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Traditional Food Processing Methods to Increase Mineral Bioavailability from Cereal and Legume Based Weaning Foods

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Summary

Background: Cereal grains and legume seeds usually contain high amounts of phytic acid. Phytic acid binds strongly to minerals and trace elements, such as iron, zinc, copper, calcium and can thereby reduce their bioavailability. Minerals and trace elements are of special importance in infant nutrition for the rapidly growing child. Therefore weaning foods based on cereals and legumes should preferably be low in phytic acid. Phytic acid can be degraded by the enzyme phytase, which naturally occurs in grains and seeds.

Objectives: The aim of this study was to develop a food processing method to produce cereal and legume based weaning foods with low phytic acid content using phytase naturally occurring in cereals and legumes. In order to identify potential sources of phytase, cereal grains and legume seeds were screened for phytase activity. The influence of traditional food processing methods, such as soaking and germination of grains and seeds on phytase activity and phytic acid content was investigated. The impact of phytic acid degradation on zinc and copper absorption from a weaning food was evaluated in adults.

Design: Grains and seeds of cereals, pseudocereals, legumes and oilseeds were screened for phytase activity under standardized conditions. The conditions for phytic acid degradation were optimized for the grains with the highest phytase activity. The influence of traditional food processing methods, such as soaking and germination of grains and seeds on phytase activity and phytic acid content was investigated. Weaning food mixtures based on cereals and legumes were combined with a phytase source selected from the screening study, and incubated as a slurry for phytic acid degradation. Conditions were optimized in laboratory experiments. The application of the method to commercial weaning food production was evaluated in a pilot plant (Nestlé Product Technology Centre, Orbe, Switzerland). For comparison, a weaning food was produced without the phytic acid degradation step and the composition of the weaning foods was evaluated. The influence of phytic acid on zinc and copper absorption from the weaning

foods was investigated in adults. Zinc and copper in the weaning foods were labeled extrinsically with stable isotopes (^{70}Zn and ^{65}Cu). Apparent zinc and copper absorption was measured, based on the quantification of non-absorbed isotopes excreted in feces during a period equivalent to six days dietary intake. The isotopic analysis of copper and zinc was performed by thermal ionization mass spectrometry.

Results: The phytase activity of grains and seeds varied over a wide range (0.1 to 7.0 phytase units per gram dry matter) and the most potent sources of phytase were rye, wheat and buckwheat. The optimal conditions for the phytase activity of rye, wheat and buckwheat were between 50 and 60°C and at pH values between 5.0 and 5.5. Soaking and germination showed a negative influence on phytase activity in most cereals and pseudocereals. Soaking and germination of legumes and oilseeds increased the relatively low initial phytase activity 2 to 3 fold. The phytic acid content of all grains and seeds tested (mean 1.1 g/100 g dry matter) was reduced after soaking and germination to an average of circa 80% of the initial content. Weaning food mixtures based on cereals and legumes were combined with 10% rye, wheat or buckwheat as the phytase source and incubated for complete phytic acid degradation. Time required for complete degradation was in the range of 80 to 280 minutes depending on ingredients and conditions. The results from the laboratory experiments were confirmed in the pilot plant and production of a phytic acid free weaning food based on wheat and soybeans was successfully completed. A patent for the developed processing method has been submitted. For comparison, a weaning food with the same composition was produced without phytic acid degradation. The overall composition of the two weaning foods was comparable, except for the phytic acid content; the dephytinized weaning food contained no phytic acid (< 3 mg/100 g) while the other product contained about 0.4 g/100 g. The mean apparent fractional zinc absorption was significantly higher (p = 0.005) from the weaning food containing no phytic acid (34.6 ± 8.0%), than from the weaning food with the native phytic acid content (22.8 ± 8.8%). No significant difference (p = 0.167) was found for the mean apparent fractional copper absorption between the weaning food with no phytic acid (19.7 ± 5.1%) and the weaning food with the native phytic acid content (23.7 ± 8.1%).

Conclusions: Results from this systematic study on phytase activity of cereals, pseudocereals, legumes and oilseeds under standardized conditions provides new data enabling comparisons of absolute values. The results showed that rye, wheat and buckwheat have highest values whereas legumes generally have low phytase activity (circa 10 fold lower). Soaking and germination of grains and seeds did not lead to high phytase activity, nor did these food processing techniques degrade phytic acid substantially under the experimental conditions. Cereals with high phytase activity can be used as a phytase source to degrade phytic acid in weaning foods based on cereals and legumes. Results from small scale laboratory experiments of phytic acid degradation can be used to predict phytic acid degradation during commercial weaning food production. Phytic acid can be degraded completely during commercial production by including a relatively short incubation step (80 to 140 minutes for wheat/soybean mixture) to the usual production steps. The beneficial effect of dephytinization of a weaning food on the absorption of zinc was demonstrated in an absorption study with adults while no effect was observed for copper absorption in the same study. Further studies would be desirable to adapt the new processing method to the household preparation of phytic acid free weaning foods, especially in developing countries.

IV

Kurzfassung

Hintergrund: Zerealien und Leguminosen enthalten Phytinsäure, welche Mineralstoffe wie zum Beispiel Eisen, Zink, Kupfer oder Kalzium bindet und dabei deren Bioverfügbarkeit vermindern kann. Mineralstoffe sind besonders wichtig in der Ernährung von Kleinkindern und deshalb sollten die Mineralstoffe der Beikost eine hohe Bioverfügbarkeit aufweisen. Beikost auf der Basis von Zerealien und Leguminosen sollte daher möglichst wenig Phytinsäure enthalten. Phytinsäure kann durch das Enzym Phytase, welches natürlicherweise in Zerealien und Leguminosen vorhanden ist abgebaut werden und verliert dabei seine Fähigkeit Mineralstoffe zu binden.

Zielsetzungen: Das Ziel dieser Studie war die Entwicklung einer Methode zur Herstellung von phytinsäurearmer Beikost auf der Basis von Zerealien und Leguminosen, unter Verwendung der in den Zutaten natürlicherweise vorkommenden Phytase. Zur Ermittlung potentieller Phytasequellen wurde die Phytaseaktivität einer Vielzahl von Zerealien und Leguminosen gemessen. Die Auswirkungen traditioneller Lebensmittelzubereitungsmethoden, wie das Einweichen und Keimen von Zerealien- und Leguminosensamen auf die Phytaseaktivität und den Phytinsäuregehalt wurden untersucht. Der Einfluss der Phytinsäure in Beikost auf die Absorption von Zink und Kupfer wurde in einer Absorptionsstudie mit Erwachsenen bestimmt.

Studienaufbau: Die Phytaseaktivitäten verschiedener Zerealien, Pseudozerealien, Leguminosen und Ölsamen wurden unter standardisierten Bedingungen gemessen. Für die Proben mit den höchsten Phytaseaktivitäten wurden die Bedingungen für den Phytinsäureabbau optimiert. Der Einfluss traditioneller Lebensmittelzubereitungsmethoden, wie das Einweichen und Keimen von Zerealien- und Leguminosensamen auf die Phytaseaktivität und den Phytinsäuregehalt wurde ermittelt. Mischungen aus Zerealien und Leguminosen wurden mit einer geeigneten Phytasequelle kombiniert und in Form eines Breis inkubiert um die Phytinsäure abzubauen. Die Inkubationsbedingungen wurden in Laborexperimenten optimiert und die Übertragbarkeit der

entwickelten Methode auf die industrielle Beikostherstellung wurde in einer Pilotanlage (Nestlé Product Technology Centre in Orbe) getestet. Als Vergleich wurde ein zweites Produkt hergestellt; basierend auf denselben Zutaten, jedoch ohne den Inkubations-schritt für den Phytinsäureabbau. Die Zusammensetzung beider Produkte wurde evaluiert. Der Einfluss der Phytinsäure auf die Absorption von Zink und Kupfer aus den Beikostprodukten wurde an Erwachsenen untersucht. Das in den Produkten natürlicher-weise vorhandene Zink und Kupfer wurde extrinsisch mit stabilen Isotopen markiert (^{70}Zn and ^{65}Cu). Die Zink- und Kupferabsorption wurde über die Quantifizierung der nichtabsorbierten Isotope im Stuhl bestimmt, entsprechend einer quantitativen Stuhl-sammlung während sechs Tagen. Die Analyse der Isotopen-verhältnisse von Zink und Kupfer erfolgte mittels Thermionen-Massenspektrometrie.

Resultate: Die Phytaseaktivitäten variierten über einen weiten Bereich (0.1 to 7.0 Phytaseeinheiten pro Gramm Trockenmasse), wobei die höchsten Werte für Roggen, Weizen und Buchweizen gemessen wurden. Die optimalen Bedingungen für die Phytaseaktivitäten von Roggen, Weizen und Buchweizen lagen zwischen 50 and 60°C und pH Werten von 5.0 bis 5.5. Das Einweichen und Keimen der Samen beeinflusste die Phytaseaktivität der meisten Zerealien und Pseudozerealien negativ, während die relativ tiefen Phytaseaktivitäten der Leguminosen und Ölsamen durch das Einweichen und Keimen um das 2- bis 3-fache anstiegen. Die Phytinsäuregehalte aller untersuchten Proben (Mittelwert 1.1 g/100 g Trockenmasse) wurden durch das Einweichen und Keimen auf durchschnittlich circa 80% der Ausgangswerte gesenkt. Als Beikost geeignete Mischungen aus Zerealien und Leguminosen wurden mit 10% Roggen, Weizen oder Buchweizen als Phytasequelle kombiniert. Die Phytinsäure der Mischungen konnte durch Inkubation, je nach Bedingungen und Zusammensetzung, in 80 bis 280 Minuten vollständig abgebaut werden. Die Resultate der Laborversuche konnten in der Pilotanlage bestätigt werden und phytinsäurefreie Beikost basierend auf Weizen und Sojabohnen wurde hergestellt. Ein Patent für die neuentwickelte Herstellungsmethode wurde angemeldet. Zum Vergleich wurde ein zweites Beikost-produkt hergestellt, basierend auf denselben Zutaten, jedoch ohne den Inkubations-schritt zum Phytinsäureabbau. Beide Produkte wiesen eine vergleichbare Zusammen-setzung auf, mit Ausnahme des Phytinsäuregehaltes. Während im einen Produkt keine

Phytinsäure nachweisbar war (< 3 mg/100 g), enthielt das andere den ursprünglichen Gehalt von circa 0.4 g/100 g. Die durchschnittliche Zinkabsorption vom Produkt ohne Phytinsäure (34.6 ± 8.0%) war signifikant höher (p = 0.005) als vom Produkt mit Phytinsäure (22.8 ± 8.8%). Für die Kupferabsorption wurde kein signifikanter Unterschied (p = 0.167) zwischen dem Produkt ohne Phytinsäure (19.7 ± 5.1%) und demjenigen mit Phytinsäure (23.7 ± 8.1%) gefunden.

Schlussfolgerungen: Die systematische Bestimmung der Phytaseaktivitäten verschiedener Zerealien, Pseudozerealien, Leguminosen und Ölsamen unter standardisierten Bedingungen erlaubte den Vergleich der absoluten Werte. Die Messungen zeigten, dass Roggen, Weizen und Buchweizen circa 10 mal höhere Phytaseaktivitäten aufweisen als Leguminosen und sie führten zur Kenntnis der Phytaseaktivitäten von bis dahin nicht untersuchten Lebensmitteln. Das Einweichen und Keimen der Samen erzielte keine hohen Phytaseaktivitäten und die Phytinsäure wurde nicht erheblich reduziert. Zerealien mit hohen Phytaseaktivitäten können als Phytasequelle zu Beikostmischungen zugesetzt werden um die Phytinsäure abzubauen. Die Resultate des Phytinsäureabbaus in Laborversuchen können auf die industrielle Beikostherstellung übertragen werden. Durch die Einführung eines relativ kurzen Inkubationsschrittes (80 bis 140 Minuten für Weizen/Sojabohnen-Mischung) in den herkömmlichen Prozess der Beikostherstellung kann die Phytinsäure vollständig abgebaut werden. Der positive Effekt des Phytinsäureabbaus auf die Zinkabsorption konnte an Erwachsenen gezeigt werden, während in derselben Studie kein Einfluss der Phytinsäure auf die Kupfer-absorption beobachtet werden konnte. Weiterführende Studien wären wünschenswert, um die neuentwicklete Methode auf die Herstellung phytinsäurefreier Beikost im Haushalt, speziell in Entwicklungsländern, zu übertragen.

Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakis (dihydrogen phosphate)) consists of *myo*-inositol esterified with six phosphoric acid groups. Phytic acid is present in cereal grains and legume seeds (Reddy et al. 1989) as the main phosphorus storage form. At pH values normally found in foods, and under physiological conditions, phytic acid is negatively charged and has the potential to bind cations or other positively charged functional groups of molecules (Cosgrove 1980a). Phytic acid can form complexes with minerals and trace elements, proteins and starch and can thereby influence their bioavailability. Nutritionally most important are the interactions of phytic acid with minerals and trace elements, such as iron, zinc, calcium, copper, resulting in complexes as has been shown in *in vitro* studies (Vohra et al. 1965, Persson et al. 1998). However, extrapolations from *in vitro* studies to the human digestive tract are difficult to make and the influence of phytic acid on the bioavailability of minerals and trace elements needs to be investigated in human absorption studies.

Infancy is one of the most vulnerable periods of life as the infant has a restricted number of foods. Weaning foods are the first solid foods which are often based on cereals, combined with milk or with legumes in countries were milk is not readily available, to improve the quantity and the nutritional quality of the protein component. Weaning foods based on cereals and legumes often contain considerable amounts of phytic acid, potentially impairing the mineral and trace element bioavailability. Bioavailability of minerals and trace elements is of particular concern in infant nutrition. Iron deficiency is a major problem mainly affecting infants and children as well as women of childbearing age and is often due to diets high in phytic acid. Likewise zinc is of special importance as zinc deficiency during early life can reduce growth and immune response (Prasad et al. 1963, Rimbach et al. 1996).

Phytic acid can be degraded by the enzyme phytase (*myo*-inositol hexakisphosphate phosphohydrolase), which occurs in cereal grains and legume seeds as well as in some microorganisms. Microbial phytase is commonly used for phytic acid degradation of animal feed (Simell et al. 1991, Cromwell et al. 1993), but is not used for foods for

human consumption. Traditional food processing methods such as soaking and germination of grains and seeds have been reported to increase phytase activity and to be efficient for reduction of phytic acid (Tabekhia and Luh 1980, Ologhobo and Fetuga 1984a, Beal and Mehta 1985, Laboure et al. 1993). The usefulness of the traditional methods soaking and germination of various grains and seeds to produce phytic acid free weaning foods have not yet been investigated systematically. The potential to produce phytic acid free weaning foods using phytase naturally occurring in grains and seeds needs to be evaluated and conditions optimized for such methods.

The negative influence of phytic acid on iron absorption in adults and infants is well established in the literature (Hallberg et al. 1989a, Hurrell et al. 1992, Davidsson et al. 1994a, Sandberg et al. 1999). Phytic acid has to be reduced completely as even small amounts of phytic acid inhibit iron absorption (Hurrell et al. 1992). The influence of phytic acid on zinc absorption remains contradictory as not all studies have shown increased zinc absorption from products with low phytic acid content (Nävert and Sandström 1985, Kivistö et al. 1989, Fairweather-Tait et al. 1992, Davidsson et al. 1996a, Larsson et al. 1996). Only a few animal and human studies report on the influence of phytic acid on copper absorption and results are contradictory (Turnlund et al. 1985, Lee et al. 1988, Morris et al. 1988, Lönnderdal et al. 1999). Therefore, the influence of phytic acid on zinc and copper absorption from weaning foods needs to be investigated.

This thesis is structured as a literature review followed by three experimental sections, each including introduction, materials and methods, results and discussion as well as a summary. The literature review covers various aspects of phytic acid, phytase, grains and seeds and a discussion about weaning foods. In the second part, the screening of grains and seeds for phytase activity and the influence of soaking and germination on phytase activity and phytic acid content is described. In the third part, the production of weaning foods with low phytic acid content by using phytase naturally occurring in cereal grains is reported. The impact of dephytinization of weaning foods on zinc and copper absorption in adults is described in part four.

1. Literature review

1.1. Phytic acid

1.1.1. Discovery

The discovery of phytic acid goes back to 1855 (Reddy et al. 1989), when Hartig isolated small, non-starch particles from various plant seeds and considered to be essential storage material. In 1872, Pfeffer characterized these small particles and suggested that the substance was phosphate combined with a carbohydrate. He showed that wheat contained a calcium/magnesium salt of organic phosphate, which was later found in other grains and shown to be phytic acid.

1.1.2. Structure and nomenclature

Two different structures of phytic acid have been proposed by Neuberg (1908) and Anderson (1914). The structure suggested by Neuberg had three P-O-P linkages between pairs of adjacent phosphate groups (tripyrophosphate), while in the structure proposed by Anderson the phosphate groups were not linked. Using titration methods some studies supported Neuberg, while others supported Anderson. Finally, the use of ^{31}P nuclear magnetic resonance technique confirmed Anderson's structure to be correct (Cosgrove 1966a, Johnson and Tate1969a).

Phytic acid consists of inositol esterified with six phosphoric acid groups. There are nine possible stereoisomers of inositol, *cis-*, *epi-*, *allo-*, *neo-*, *myo-*, *muco-*, D-*chiro-*, L-*chiro-* and *scyllo-*inositol depending whether the phosphate groups are in axial or equatorial position. From plants, only *myo*-inositol phosphates have been isolated, while *neo-*, *chiro-* and *scyllo*-inositol phosphates have been identified in soil (Cosgrove 1966a). There has been some controversy about the conformational structure of phytic acid. Johnson and Tate (1969a) suggested that the phosphate at C-2 is in the axial position, while the other phosphate groups are equatorial (*myo*-form). Later Isbrandt and Oertel (1980) showed that in aqueous solution under acidic conditions the 1-axial/5-equatorial conformation predominates, while under strong alkaline conditions the inverted conformation, 5-axial/1-equatorial prevails. Numbering of *myo*-inositol

phosphates may be either clockwise (L) or counter-clockwise (D) when viewed from above the ring. By convention the numbering is clockwise (Parthasarathy and Eisenberg 1991), while for biological purposes counter-clockwise numbering is proposed by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB 1989). Since 1968, the IUPAC (International Union of Pure and Applied Chemistry) name of phytic acid is *myo*-inositol 1,2,3,4,5,6 hexakis (dihydrogen phosphate) (IUPAC-IUB 1968). The chemical structure with counter-clockwise numbering is shown in Figure 1.1. (adapted from Plaami 1997).

Figure 1.1. Structure of *myo*-inositol 1,2,3,4,5,6 hexakis (dihydrogen phosphate) adapted from Plaami (1997)
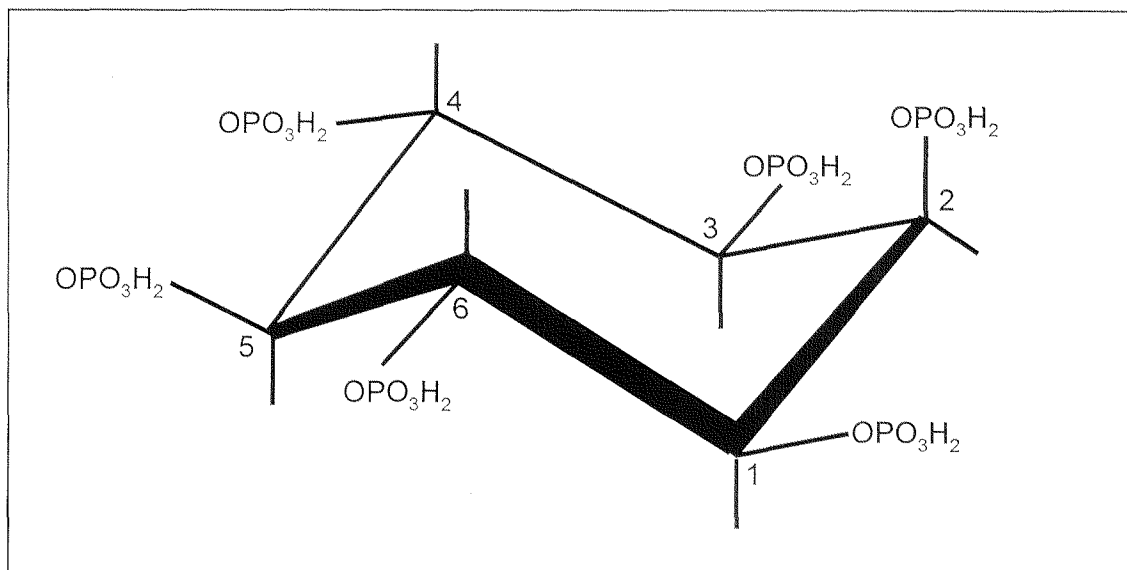
In the following text *myo*-inositol 1,2,3,4,5,6 hexakis (dihydrogen phosphate) will be termed phytic acid or abbreviated IP6. The abbreviation IP5 will be used for *myo*-inositol pentakis-, IP4 for *myo*-inositol tetrakis-, IP3 for *myo*-inositol trikis-, IP2 for *myo*-inositol bikis- and IP1 for *myo*-inositol monokis (dihydrogen phosphate). IP1, IP2, IP3, IP4 and IP5 will also be referred to as lower inositol phosphates.

1.1.3. Occurrence and content of phytic acid in plants

In plants, phytic acid mostly occurs in the grains and seeds of cereals, pseudocereals, legumes and oilseeds. It also occurs in pollen (Jackson et al. 1982), tubers, fruits and vegetables (McCance and Widdowsen 1935, Alaoui and Essatara 1985). Table 1.1.

summarizes the phytic acid content of cereal and pseudocereal grains, legume seeds, nuts, oilseeds, vegetables, tubers, coffee and tea from different studies. The contents are generally expressed in g/100 g dry matter (dm), for rapeseed in g/100 g defatted dry matter and for brewed coffee and tea in mg per cup. If the values were given on weight as is basis they were converted to dry matter basis using values from Souci et al. (1994) and for alfalfa from Hamilton and Vanderstoep (1979). When contents were expressed as phytic acid phosphorus, the values were converted to phytic acid, assuming a phosphorus content of 28.2% of the phytic acid molecule (de Boland et al. 1975, Fretzdorff 1992). If no specification is indicated the value represents the content of untreated whole grains. In studies where samples from different varieties, cultivars or locations had been analyzed, the number of samples is indicated and the mean content given. If the number of samples is not indicated, the phytic acid content represents the value of one sample. While for some grains and seeds the reported values are similar, they differ widely for others. This could be due to the method of analysis used, the varieties and species analyzed or the environmental conditions during growth.

Table 1.1. Phytic acid content

Sample	Number of samples	Phytic acid content [g/100 g dm]	Reference
Cereal grains			
Barley	2	1.3	Averill and King 1926
		0.8	McCance and Widdowson 1935
	18	1.0	Lolas et al. 1976
		1.0	Bartnik and Szafranska 1987
Maize	2	1.0	O'Dell et al. 1972
	2	1.1	de Boland et al. 1975
White maize		0.8	Marfo et al. 1990
Yellow maize		0.7	Marfo et al. 1990
Millet		1.3	Averill and King 1926
		0.8	McCance and Widdowson 1935
	2	0.8	Torelm and Bruce 1982
	8	1.0	Simwemba et al. 1984
		0.8	Khokhar et al. 1994
Oat		0.7	McCance and Widdowson 1935
	19	0.9	Lolas et al. 1976
		1.1	Bartnik and Szafranska 1987
Rice		1.0	McCance and Widdowson 1935
		0.9	O'Dell et al. 1972
		0.5	Marfo et al. 1990
		0.3	Khokhar et al. 1994
Rice polished		0.2	McCance and Widdowson 1935
Rye	3	1.5	Averill and King 1926
	16	0.9	Fretzdorff and Weipert 1986
	2	0.9	Bartnik and Szafranska 1987

Sorghum	7	1.1	Cheng Wang et al. 1959
Red sorghum		0.9	Marfo et al. 1990
Wheat	3	1.4	Averill and King 1926
		0.7	McCance and Widdowson 1935
		1.3	O'Dell et al. 1972
		1.3	de Boland et al. 1975
	38	1.0	Lolas et al. 1976
		0.9	Khokhar et al. 1994
Wheat bran	4	4.2	Graf and Dintzis 1982
Pseudocereal grains			
Amaranth	9	1.6	Becker et al. 1981
Buckwheat	2	2.1	Averill and King 1926
	3	0.8	Torelm and Bruce 1982
Quinoa	2	0.7	Valencia et al. 1999
Legume seeds			
Alfalfa, lucerne		0.8	Torelm and Bruce 1982
Bean	2	0.6	Burbano et al. 1995
Blackeyed bean		1.1	Tabekhia and Luh 1980
		1.2	Davies and Warrington 1986
Faba bean	2	1.0	Eskin and Wiebe 1983
	2	0.7	Burbano et al. 1995
Great northern bean	6	0.8	Lolas and Markakis 1975
Kidney bean		0.9	Davies and Warrington 1986
Red kidney bean		1.3	Lolas and Markakis 1975
		1.2	Tabekhia and Luh 1980
Navy bean	29	1.3	Lolas and Markakis 1975
Pink bean		0.5	Tabekhia and Luh 1980
Red Mexican bean	4	0.7	Lolas and Markakis 1975
Small white bean	2	0.6	Lolas and Markakis 1975
White bean		1.1	Davis 1981
		1.0	Torelm and Bruce 1982
Chickpea	2	0.6	Gad et al. 1982
		0.6	Davies and Warrington 1986
		0.4	Harland et al. 1988
		1.0	Khokhar et al. 1994
	2	0.5	Burbano et al. 1995
		0.7	Sharma and Khetarpaul 1995
White chickpea		1.2	Khan et al. 1988
Brown chickpea		0.8	Khan et al. 1988
Cowpea	10	0.3	Ologhobo and Fetuga 1983
	3	1.4	Ologhobo and Fetuga 1984a
		0.9	Marfo et al. 1990
Lentil		0.8	Davis 1981
	2	0.5	Gad et al. 1982
		0.5	Davies and Warrington 1986
		0.8	Harland et al. 1988
		1.0	Khokhar et al. 1994
		0.3	Burbano et al. 1995
Lupin	2	0.3	Gad et al. 1982
	2	1.1	Kirby and Nelson 1988
	2	0.6	Burbano et al. 1995
Mungbean		0.2	Tabekhia and Luh 1980
		0.6	Torelm and Bruce 1982
		0.2	Sattar et al. 1989
Pea		0.8	Gad et al. 1982
		0.8	Beal and Mehta 1985
		1.0	Harland and Oberleas 1987

Soybean	7	2.4	Averill and King 1926
	15	1.1	Lolas et al. 1976
		1.3	Torelm and Bruce 1982
	2	1.5	Ologhobo and Fetuga 1984a
		1.1	Sutardi and Buckle 1985
		0.8	Marfo et al. 1990
		2.2	Khokhar et al. 1994
Nuts and oilseeds			
Almond		1.3	Harland and Oberleas 1987
Cashew nut		2.0	Harland and Oberleas 1987
Peacan nut (shelled)		1.5	Averell and King 1926
Sesameseed		1.7	Harland and Oberleas 1987
Rapeseed		4.0-4.8	Uppström and Svensson 1980
Sunflower seed		1.7	Harland and Oberleas 1987
Vegetables			
Broccoli		0.16	Harland and Oberleas 1987
Cucumber		0.06	Harland and Oberleas 1987
Okra		0.29	Harland and Oberleas 1987
Olive (green)		0.01	Harland and Oberleas 1987
Tubers			
Cassava		0.1	Harland et al. 1988
Yam		0.3	Harland et al. 1988
Sweet potato		0.3	Harland et al. 1988
Coffee and tea			
Coffee instant		0.6	McKenzie 1984
Coffee green bean		0.3	McKenzie 1984
Coffee roasted bean		0.2	McKenzie 1984
Coffee brewed		1-18 mg/cup	Harland and Oberleas 1985
Tea brewed		0.2 mg/cup	Harland and Oberleas 1985

1.1.3.1. Phytic acid location in grains and seeds

The phytic acid content (on weight as is basis) in different morphological components of cereals and legumes is presented in Table 1.2. In many cereals, most of the phytic acid is located in the pericarp or aleurone, for example 87% in wheat and 80% in rice. Using fluorescence microscopy, the occurrence of the crystalline form of phytic in the aleurone layer could be shown in wheat (Fulcher 1982). Maize, millet and sorghum differ from most other cereals since phytic acid is mainly found in the germ. In legumes, phytic acid is distributed throughout the cotyledons associated with the protein bodies. The seed coat of legumes contains very little phytic acid and its removal can lead to an even higher phytic acid content in the dehulled seed. This was shown for two varieties of beans, where the phytic acid content increased from 0.79% and 0.81% of the whole seeds to 0.99% and 0.87% respectively after removal of the seed coat (Davies and Warrington 1986). Dehulling of lentils also led to an increase in phytic acid content, while the same process decreased the phytic acid content in lupin (Gad et al. 1982).

Table 1.2.   Phytic acid content and distribution in different morphological
             components

Sample	Morphological component	Phytic acid content [g/100 g]	Distribution [%]	Reference
Corn	germ endosperm hull	6.38 0.03 0.07	88.0 3.2 0.4	O'Dell et al. 1972
Millet	germ endosperm bran	2.67 0.31 0.99	no percentage indicated	Simwemba et al. 1984
Rice	germ endosperm pericarp	3.47 0.01 3.36	7.6 1.2 80.0	O'Dell et al. 1972
Sorghum	germ endosperm bran	4.99 0.23 1.15	no percentage indicated	Cheng Wang et al. 1959
Wheat (soft)	germ endosperm aleurone	3.90 0.003 4.11	12.9 2.2 87.1	O'Dell et al. 1972
Pea	germ cotyledon hull	1.23 0.78 0.01	2.5 88.7 0.1	Beal and Mehta 1985

1.1.3.2. Phytic acid phosphorus in relation to total phosphorus

In most cereals and legumes, phytic acid phosphorus constitutes the major part of the total phosphorus. Lolas and Markakis (1975) analyzed 50 different varieties or lines of beans and reported 54% to 82% (average 69%) of the phosphorus as phytic acid. In soybean 53%, oat 61%, barley 68% and wheat 72% of the total phosphorus was found to be in form of phytic acid (Lolas et al. 1976), whereas in cowpea the value was 39% (Ologhobo and Fetuga 1983), lupin 55% (Kirby and Nelson 1988), oat 62% (Ashton and Williams 1958), blackeyed bean 60% and red kidney bean 60% (Tabekhia and Luh 1980), mungbean 65 to 80% (Reddy et al. 1978, Feil and Fossati 1997).

Phytic acid content was found to be highly correlated with total phosphorus content in soybean, oat, barley and wheat and Lolas et al. (1976) proposed that phytic acid content could be estimated by the determination of total phosphorus. A high correlation was found for oat (Ashton and Williams 1958, Miller et al. 1980), while Singh and Reddy (1977) could not find a clear relationship between total phosphorus and phytic acid phosphorus in triticale. To our knowledge the determination of total phosphorus is not used to estimate the phytic acid content at the present time.

1.1.3.3. Influence of environmental and other factors on phytic acid content of grains and seeds

Variations in phytic acid content due to cultivation conditions, such as location and year, application of fertilizer and to cultivar or variety have been reported for different cereals and legumes. Feil and Fossati (1997) found significant differences between cultivars of triticale in the content of total phosphorus and phytic acid phosphorus. Phytic acid content was shown to depend on cultivar, location and year for oat (Ashton and Williams 1958, Miller et al. 1980), pearl millet (Simwemba et al. 1984) and wheat (Raboy et al. 1991). Several studies (reviewed by Reddy et al. 1989) reported that the application of fertilizers (nitrogen and phosphorus) increased the phytic acid content of grains or seeds.

1.1.3.4. Phytic acid biosynthesis

The enzymes and metabolic steps involved in the biosynthesis, accumulation, and degradation of phytic acid in plants have been the subject of several reviews (Cosgrove 1966a, Loewus and Loewus 1980, Majerus et al. 1988, Loewus 1990, Loewus et al. 1990, Raboy 1990, Murthy 1996, Loewus and Murthy 2000). In this chapter, the different pathways of phytic acid biosynthesis will be discussed, while the phytic acid degradation will be reviewed in chapter 1.2.3.5. which describes the functions of phytase. A schematic overview of the *myo*-inositol metabolism in plants is given by Loewus and Murthy (2000), for details of the different pathways see the cited references.

An early review (Cosgrove 1966a) suggested three possible pathways for the biosynthesis of phytic acid: (1) phosphorylation of phosphoinositide intermediates and subsequent hydrolysis to corresponding inositol phosphates; (2) successive phosphorylation in the absence of free intermediates and (3) direct stepwise phosphorylation of free *myo*-inositol and/or *myo*-inositol monophosphate by a kinase type of reaction. The first two mechanisms received little support, while most authors favored the third pathway.

All studies considered free *myo*-inositol and D-glucose 6-phosphate to play important roles as precusors in the biosynthesis of phytic acid. D-glucose is phosphorylated by the enzyme hexokinase to D-glucose 6-phosphate, which is subsequently transformed to 1L-*myo*-inositol 1-phosphate by the enzyme 1L-*myo*-inositol 1-phosphate synthase (Cosgrove 1980b, Loewus 1983, 1990). 1L-*myo*-inositol 1-phosphate plays a key role and can either be degraded to free *myo*-inositol or phosphorylated to higher inositol phosphates.

The enzyme *myo*-inositol monophosphatase links the phytic acid biosynthesis to the *myo*-inositol oxidation pathway, which finally results in the formation of cell wall polysaccharides via uridine diphosphate D-glucuronate (Loewus 1990, Loewus et al. 1990). *Myo*-inositol can be transformed back to 1L-*myo*-inositol 1-phosphate either by the enzyme *myo*-inositol kinase or by 1L-*myo*-inositol 1-phosphate synthase (Reddy et al. 1989, Loewus et al. 1990).

Phytic acid biosynthesis can either begin with *myo*-inositol 1-phosphate or *myo*-inositol 2-phosphate as the initial substrate (Reddy et al. 1989). De and Biswas (1979) proposed a direct stepwise phosphorylation of 1L-*myo*-inositol 1-phosphate to *myo*-inositol pentaphosphate by phosphoinositol kinases. Phosphorylation to *myo*-inositol hexaphosphate (phytic acid) is possible if *myo*-inositol 2-phosphate is used as substrate. For complete phosphorylation (to phytic acid), starting from *myo*-inositol 1-phosphate, the action of a reversible *myo*-inositol hexaphosphate-adenosine diphosphate phosphotransferase has been proposed.

More recent studies of second-messenger molecules have led to a hypothetical pathway proceeding via polyphosphoinositide intermediates (Majerus et al. 1986, 1988, Loewus et al. 1990). A pathway starting with *myo*-inositol via several polyphosphoinositides to *myo*-inositol 3-phosphate and successive phosphorylation to phytic acid has been proposed.

1.1.4. Occurrence of phytic acid in animals

Rapoport (1941) first observed the occurrence of an inositol polyphosphate in chicken blood and found it to have the properties and composition of phytic acid. It was later shown by Johnson and Tate (1969b) that this blood constituent was *myo*-inositol pentakisphosphate. Now it appears that phytic acid not only occurs in plants, but also in the amoebae *Dictyostelium discoideum* (Martin et al. 1987) and *myo*-inositol penta- and hexakisphosphates are synthesized in the brain of mammals (Vallejo et al. 1987). These findings suggest, that phytic acid is a common cellular constituent not only of plants, but also of unicellular species and certain animals (Loewus et al. 1990).

1.1.5. Manufacture and applications of phytic acid

Corn, rice, wheat bran and cottonseed are the main sources of commercial phytic acid. The manufacture generally involves extraction with dilute acid from plant material, neutralization to precipitate the calcium-magnesium salt and purification with ion exchange chromatography. Phytic acid is commercially available as salts, such as phytic acid dodecasodium salt, or as aqueous solutions (40-50%) (Graf 1983, Sands et al. 1986).

The ability of phytic acid to chelate metal ions over a broad pH range makes it useful in industrial and food applications. However, in industrial applications other chelating substances, such as ethylene diaminetetraacetic acid (EDTA) or citric acid, are cheaper and more readily available. In food, phytic acid can serve as a preservative or antioxidant. Phytic acid can prevent lipid containing foods against rancidity and can stabilize natural and artificial coloring agents. It is used in liquid food products, such as wine or other beverages to chelate metals, especially iron (Graf 1983). In Japan, phytic acid is used in different food products, such as meat, fish or noodles, while in the USA phytic acid can not be used, as it does not have GRAS (generally recognized as safe) status (Graf 1983, Sands et al. 1986). Further industrial, food and medical applications of phytic acid have been reviewed by Graf (1983, 1986), Sands et al. (1986) and Weiner and Franco (1986).

In the USA, phytic acid is at the present time sold as a nutrition supplement for athletes. Distributors claim that daily intakes of 0.75 to 1.50 g help to improve the building of bone tissue, increase the oxygen transport capacity of the red blood cells and accelerate the transportation of creatine and other nutrients to the muscle cells. However, these effects have not been demonstrated in humans and orally administered phytic acid is not expected to be absorbed.

1.1.6. Methods for phytic acid determination

1.1.6.1. Introduction

Since phytic acid does not have a characteristic absorption spectrum and can not be easily identified using specific colorimetric reagents, the determination has remained difficult. The analytical methods for the determination of phytic acid and inositol phosphate have been reviewed by several authors (Oberleas and Harland 1986, Reddy et al. 1989, Xu et al. 1992, Sandberg 1995).

Qualitative methods for the phytic acid determination were among the first techniques for phytic acid determination and some are still used, for example microscopy for the localization of phytic acid in grains. The first methods used for the quantitative determination of phytic acid were based on the precipitation of an insoluble salt of phytic acid. They are still used today, especially the methods of Makower (1970), Wheeler and Ferrel (1971), Davies and Reid (1979), Latta and Eskin (1980). Precipitation methods do not usually require special equipment and can easily be installed in most laboratories. Methods based on precipitation may result in falsely elevated phytic acid concentrations compared with HPLC-methods (Sandberg and Ahderinne 1986) or NMR spectroscopy (Frolich et al. 1986) due to co-precipitation of lower inositol phosphates with phytic acid.

Several methods, mainly based on chromatography, have been developed to separate and quantify the different inositol phosphates. The discovery of physiological functions of inositol phosphates, such as inositol 1,4,5-triphosphate as a second messenger in mobilizing intracellular calcium (Berridge and Irvine 1989, Berridge 1993) led to a

widespread interest in the structures of the inositol phosphates. The position of the phosphate groups in the molecule is of great importance for the physiological functions and new methods for the separation of structural isomers of inositol phosphates became necessary. A number of isomer specific ion exchange chromatography methods with gradient elution for the separation of inositol phosphates have been developed. Methods based on NIR (Near-Infrared reflectance) spectroscopy, ^{31}P NMR (phosphorus-31 nuclear magnetic resonance) spectroscopy or isotachophoresis have also been applied to determine phytic acid.

Methods for phytic acid determination must be chosen carefully, depending on raw material (cereal grains or legume seeds, processed food, intestinal contents, biological tissues), information required (qualitative or quantitative determination, total phytic acid content, different inositol phosphates, different isomeric forms of inositol phosphates), available equipment and cost.

1.1.6.2. Qualitative methods

Oberleas and Harland (1986) reviewed the qualitative methods for the separation and identification of inositol phosphates. Paper chromatography was often used in the sixties and the selection of chromatographic paper, eluent mixture and detection of inositol phosphates after separation were optimized. Paper electrophoresis was first introduced by Wade and Morgan (1955) for the separation of inositol phosphates and they also combined electrophoresis with chromatography to provide two-dimensional separations. Fulcher et al. (1981) and Fulcher (1982) applied a microscopic detection of phytic acid in cereal grains using polarizing optics or different staining agents.

1.1.6.3 Quantitative methods

1.1.6.3.1. Precipitation

The precipitation methods have evolved from the original titration method of Heubner and Stadler (1914). These methods are based on the precipitation of phytic acid as an insoluble salt, mostly as ferric phytate, using an excess of ferric chloride. The precipitation methods can be divided into direct and indirect methods. In the direct

methods, the phosphorus content of the precipitate is determined after hydrolysis, while in the indirect methods, the quantity of iron is determined in the precipitate after hydrolysis or the remaining ferric iron in the solution is measured. The estimation of phytic acid content using the indirect methods is based on the assumption that the a molar ratio Fe:P is 4:6 (four Fe atoms per phytic acid molecule). Fe:P ratios of 3.5:6 to 4.6:6 have been obtained by Makower (1970) and Wheeler and Ferrel (1971), who investigated factors influencing this ratio. Thompson and Erdman (1982) reported that the methods based on the determination of iron in the precipitate can not be recommended as they observed variations in the Fe:P ratio of ferric phytate from soybean extracts. However, the ratio 4:6 is generally used for calculations (Reddy et al. 1989).

The direct method was first introduced by McCance and Widdowson (1935). The precipitated ferric phytate was converted to sodium phytate and the phosphate content determined colorimetrically after wet-ashing. This method has been used and modified by various authors. Early and DeTurk (1944) dried the precipitate after washing with dilute acid, followed by dry-ashing before colorimetric phosphate determination. Ellis et al. (1977) reported that high concentrations of phosphate in the extract may lead to contamination of the precipitate and result in elevated phytic acid values. Mohamed et al. (1986) proposed a conversion of ferric phytate to sodium phytate after the precipitation, which can be measured without hydrolysis by the formation of a blue molybdenum complex.

Young (1936) first introduced the indirect method, measuring the unprecipitated ferric iron colorimetrically as ferric thiocyanate. This method was also used by Holt (1955) and was later modified by Davies and Reid (1979), improving the precision and using less sample and reagents. Latta and Eskin (1980) developed a rapid colorimetric method, based on the formation of a pink complex of ferric iron with sulfosalicylic acid. In the presence of phytate, color intensity decreases due to formation of ferric phytate. This method had been applied for the measurement of phytic acid in rapeseed (Thies 1991). Haug and Lantzsch (1983) described another rapid method, measuring the decrease of iron in the solution colorimetrically with 2,2'-bipyridine.

Makower (1970) tested four different methods, direct and indirect, following ferric phytate precipitation. A colorimetric determination (with o-phenanthroline) of iron after conversion of ferric phytate to ferric hydroxide; the determination of phosphorus in ashed ferric phytate; the determination of iron in ashed ferric phytate and the determination of ferric iron remaining in solution. The last method was unsatisfactory for low amounts of phytic acid concentrations, while for higher concentrations results of all four methods were comparable.

While most methods use ferric chloride for the phytic acid precipitation, Anderson (1963) proposed the use of ferric ammonium sulfate. A precipitation with cerium sulfate has been used by Hurrell et al. (1992) and Davidsson et al. (1994a, 1995, 1997).

1.1.6.3.2. Chromatographic methods

1.1.6.3.2.1. HPLC

Tangendjaja et al. (1980) introduced a high performance liquid chromatography (HPLC) method with refractive index detection for the determination of phytic acid, analyzing sample extracts without previous purification. This method has been modified by several authors, introducing sample purification steps and improving separation and sensitivity. Graf and Dintzis (1982) introduced a sample purification step using an anion exchange resin to remove inorganic phosphate and other impurities. Lee and Abendroth (1983) introduced the concept of ion-pair to avoid problems of co-elution with the solvent. While the previous methods detect only the inositol hexaphosphate, Sandberg and Ahderinne (1986) and Sandberg et al. (1989) achieved a separation of the inositol tri- to hexaphosphates by modifying pH of the mobile phase. Lehrfeld (1989, 1994) further modified the method to shorten the extraction time and concentration procedures. Burbano et al. (1995) modified the composition of the mobile phase and the column temperature to optimize the separation. The method of Sandberg and Ahderinne (1986), with modifications, is the official Swiss method for phytic acid determination (Schweizerisches Lebensmittelbuch: Kapitel 14: Methode 3.9). Camire and Clydesdale (1982) combined the precipitation method with HPLC analysis, using precipitation of phytic acid (as ferric salt) followed by a conversion to sodium phytate and HPLC analysis according to Tangendjaja et al. (1980). The HPLC methods using refractive

index detection do not differentiate between different isomers of inositol phosphates as gradient elution can not be performed with refractive index detection. Other chromatographic methods were therefore developed.

1.1.6.3.2.2. Ion exchange chromatography

Harland and Oberleas (1977) first introduced the use of an anion exchange resin to separate phytic acid from the crude extract, followed by hydrolysis and colorimetric determination of phosphate. Ellis and Morris (1982) compared this method with a precipitation method, the later resulting in higher phytic acid concentrations. The ion exchange method was improved by addition of EDTA and pH adjustment of the sample extract, thus avoiding binding of phytic acid to proteins and minerals and leading to values comparable to those of the precipitation method. The selection of an appropriate anion exchange resin for phytic acid separation was also investigated by Ellis and Morris (1986). After a collaborative study, proving accuracy and reproducibility, the modified ion exchange method was adopted as an AOAC (Association of Official Analytical Chemists) method (AOAC Official method 986.11 phytate in foods; Harland and Oberleas 1986). However, Phillippy and Johnston (1985) suggested that the AOAC method might overestimate the phytic acid content due to co-elution of lower inositol phosphates with inositol hexaphosphate. They proposed an ion chromatography method using isocratic elution with post-column derivatization and spectrophotometric detection. Bos et al. (1991) combined the method of Phillippy and Johnston (1985) with that of Cilliers and van Niekerk (1986) and included a treatment of the sample extract with EDTA. Frühbeck et al. (1995) combined the methods of Harland and Oberleas (1986) and Latta and Eskin (1980) using an anion exchange column for the separation followed by the measurement of residual iron after precipitation. Talamond et al. (1998) proposed a rapid and sensitive procedure for phytic acid detection based on a separation using an anion exchange column with a conductivity detector.

The application of a step-gradient ion exchange method followed by precipitation allowed the separation of different inositol phosphates and demonstrated the overestimation of the phytic acid content detected by precipitation methods (Phillippy et al. 1988). Based on the method of Phillippy and Johnston (1985), Phillipy and Bland

(1988) developed a ion chromatography procedure with gradient elution, postcolumn derivatization and UV detection to separate the different isomers. Skoglund et al. (1997) used a combination of two high-performance ion chromatography systems for the separation and determination of isomers of inositol mono- to hexaphosphates, using postcolumn reaction and UV detection or conductivity detection.

1.1.6.3.3. Other methods

1.1.6.3.3.1. ^{31}P NMR spectroscopy

Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P NMR) can be used for phytic acid determination as the phytic acid molecule has a plane of symmetry through the carbon atoms C-2 and C-5 and produces four major resonance peaks. The NMR procedure requires extraction and pH adjustment before recording the NMR spectrum. The C-2 phosphate peak is well resoluted and phytic acid is quantified on the basis of the area of this peak (O'Neill et al. 1980).

Frolich et al. (1986) measured phytic acid degradation products with ^{31}P NMR spectroscopy and found lower values compared to the precipitation methods, indicating that lower inositol phosphates co-precipitate with iron. Szwergold et al. (1987) used ^{31}P NMR technique to determine inositol penta- and hexakisphophates in mammalian tissues. Crans et al. (1993) investigated the influence of extraction on phosphorus metabolites determined with ^{31}P NMR spectroscopy. No signal could be detected for phytic acid, although considerable amounts were found using a precipitation method. This was attributed to the formation of phytic acid-protein or phytic acid-cation-protein complexes. Johnson et al. (1995) used a two-dimensional total correlation spectroscopy technique (2D TOSCY) for the determination of the structure of individual inositol phosphates in a mixture.

1.1.6.3.3.2. Isotachophoresis

Kikunaga et al. (1985) measured the phytic acid content of cereal grains quantitatively by isotachophoresis, an electrophoretic separation method. Phytic acid has to be extracted, precipitated as ferric salt and transformed to soluble sodium phytate before

determination. Blatny et al. (1995) developed a capillary isotachophoretic method for the determination of phytic acid in plant extracts without previous separation procedures.

1.1.6.3.3.3. NIR spectroscopy

Near infrared reflectance (NIR) spectroscopy is a simple and direct method for phytic acid measurement as it does not require laborious sample preparation procedures. The calibration is based on the relation of the phytic acid concentrations determined with other methods, such as precipitation or anion exchange methods and the absorbances at several wavelengths measured by NIR spectroscopy. This calibration must be restricted to samples which have similar matrix effects and the usefulness of the method is therefore limited (Xu et al. 1992). Parrish et al. (1990) proposed the method as rapid and routine semi-quantitative determination of phytic acid in cottonseed.

1.1.6.3.3.4. Enzymatic hydrolysis

Uppström and Svensson (1980) developed a method where the phytic acid is enzymatically hydrolyzed with wheat phytase and the liberated phosphate determined. The phytic acid content is calculated from the difference of phosphate before and after enzymatic hydrolysis.

1.1.7. Influence of food processing on phytic acid

Cereals and legumes have to be processed before human consumption. The most common food processing methods include heat treatments, such as cooking, autoclaving or extrusion cooking and soaking, germination and fermentation. These processing methods can change the phytic acid content by enzymatic and/or non-enzymatic degradation.

1.1.7.1. Heat treatment

1.1.7.1.1. Household cooking

The influence of cooking, i.e. boiling in water on the phytic acid content of legumes and cereals has been investigated by many authors and a brief review is given here. In general the loss of phytic acid during cooking is rather small. In green gram, cowpea and chickpea the acid extractable phytic acid content was decreased by 4-27% of the initial content during cooking (Kumar et al. 1978). Reddy et al. (1978) reported that the decrease in phytic acid during cooking of black gram was not due to phytic acid breakdown but to leaching into the cooking water. Tabekhia and Luh (1980) found phytic acid reduction of 6-36% during cooking of blackeyed bean, red kidney bean, mungbean and pink bean. In cowpea, lima bean and soybean phytic acid reduction of 8-11% during cooking was reported (Ologhobo and Fetuga 1984a). Cooking of maize, rice, sorghum, cowpea and soybean lowered the phytic acid content by 16-31% (Marfo et al. 1990). In faba bean a phytic acid reduction of 30% was reported by Khalil and Mansour (1995) after cooking, while Ziena et al. (1991) found maximal 16% degradation depending on cooking temperature and duration. Cooking reduced the phytic acid content by about 10% in cowpea (Ologhobo and Fetuga 1984b) and by 4-5% in quinoa (Valencia et al. 1999).

1.1.7.1.2. Autoclaving and canning

Compared to cooking the effect of autoclaving and canning at circa 120°C is more variable. Tabekhia and Luh (1980) found a phytic acid reduction of 67-91% after canning of blackeyed bean, red kidney bean, mungbean and pink bean, which was considerably higher than the effect of cooking. However, in cowpea, lima bean and soybean phytic acid reduction of only about 6% during autoclaving was reported, which was slightly lower than the effect of cooking (Ologhobo and Fetuga 1984a). Different varieties of cowpea lost an average of 6% phytic acid during autoclaving (Ologhobo et al. 1984b), while a reduction of 40% in faba bean was reported (Khalil and Mansour 1995). De Boland et al. (1975) compared the impact of autoclaving on phytic acid in grains or seeds as well as on free phytic acid in solution. After two hours of autoclaving, circa 80% of the free phytic acid was degraded, while the phytic acid in rice and wheat

was degraded by less than 10%, suggesting that the phytic acid degradation depends on the protein and cation environment in the grains and seeds. The study of Khan et al. (1991) showed that the phytic acid degradation in fresh and dried maize depends on the thermal processing conditions used as well as on the moisture content and the physical form of the raw material.

1.1.7.1.3. Extrusion cooking

The influence of extrusion cooking on phytic acid content in rye flour was shown to depend on the temperature; a maximal degradation of 23% was found at 170°C (Fretzdorff and Weipert 1986). Extrusion cooking of legume flours did not decrease the total inositol phosphate content, but IP6 was partly degraded to lower inositol phosphates (Ummadi et al. 1995). The phytic acid content of blends of rice, millet and soy flour was reduced by 11-29% during extrusion cooking (Dublish et al. 1988).

1.1.7.2. Soaking and germination

Phytic acid is used as a source of phosphate for the growing plant during germination. Thus, germination can lower the phytic acid content at a variable degree, depending on the type of grains or seeds and on the germination conditions. Germination is often combined with a previous soaking step, which refers to the soaking of whole grains or seeds and not to the incubation of flours (Reddy et al. 1989, Gustafsson and Sandberg 1995).

Soaking of peas decreased the phytic acid content by 10-12% (Bishnoi et al. 1994), and by circa 20% in mungbeans depending on the temperature (Sattar et al. 1989). Tabekhia and Luh (1980) showed a decrease of 8-20% during soaking of different legumes. Deshpande and Cheryan (1983) found less than 9% phytic acid reduction by soaking of beans in different soaking media. In cereals, phytic acid reduction after seven days of germination (after soaking for two days) ranged from 33% in oat to 67% in corn, while a complete reduction was found after five days in rye (results of Mellanby 1950, summarized by Reddy et al. 1989). Fretzdorff and Weipert (1986) reported a phytic acid reduction of circa 30% in rye after three days of germination. The phytic acid reduction

in legumes after five days of germination, reported by several authors, varied from 22% in pea (Chen and Pan 1977) to 77% in blackeyed beans (Tabekhia and Luh 1980) most of the legumes showing reductions of 30-60% (Reddy et al. 1989). Ologhobo and Fetuga (1984a) found a decrease of circa 30% in cowpea, lima bean and soybean after three days of germination. Typically, during the first two days of germination very little phytic acid is degraded (Walker 1974, Gad et al. 1982, Khokar and Chauhan 1986) and complete degradation could not be observed even after five days of germination. After ten days, circa 70% reduction was found in pea (Beal and Mehta 1985) and faba bean (Eskin and Wiebe 1983).

When the impact on phytic acid degradation of different heat treatments was compared to germination, germination was generally found to be more efficient, as shown for faba bean (Khalil and Mansour 1995), cowpea, lima bean and soybean (Ologhobo and Fetuga 1984a), different varieties of cowpea (Ologhobo and Fetuga 1984b) and mungbean (Kataria et al. 1989) as well as several legumes investigated by Chitra et al. (1996). Cooking of peas decreased the phytic acid content by 6-7%, while germination during two days led to 67-83% reduction (Bishnoi et al. 1994). Cooking of fengugreek seed, a legume commonly consumed in Egypt, reduced the phytic acid content by 26%, while four days of germination decreased the phytic acid content by 68% (El-Mahdy and El-Sebaiy 1982).

1.1.7.3. Fermentation

During fermentation, raw materials undergo overall changes in composition, flavor and textural properties. Fermentation of cereals and legumes, spontaneous or with selected microorganisms, is a common food preparation method in many African and Asian countries. Many studies have investigated the influence of fermentation on phytic acid content. Loss of phytic acid can be considerable due to the phytase of the microorganisms and/or the phytase in the grains or seeds.

During the production of tempeh, based on sorghum-bean or maize-soybean and fermented with *Rhizopus* ssp., the phytic acid content was decreased by 44% and 66% respectively (Mugula 1992a, 1992b). In bean tempeh phytic acid was reduced by 33%

(Parades-Lopez and Harry 1989). The phytic acid content of soybeans was reduced by 90% during tempeh production followed by subsequent storage and frying (Sutardi and Buckle 1985), while 94% reduction was reported directly after fermentation by van der Riet et al. (1987). Phytic acid reduction of almost 80% could be achieved by lactic acid fermentation of wheat or barley flour, leading to products typically prepared in India (Gupta et al. 1992, Gupta and Khetarpaul 1993). Lactic acid fermentation of corn flour decreased the phytic acid content by 65% (Lopez et al. 1983) while complete reduction was found in millet flour (Mahajan and Chauhan 1987). Subsequent fermentation of millet flour with yeast and lactic acid bacteria led to a complete phytic acid degradation (Khetarpaul and Chauhan 1991).

1.1.7.4 Breadmaking

Breadmaking is a special type of fermentation and phytic acid degradation can be considerable, depending on the ingredients and the fermentation conditions. During sour-dough rye bread preparation, phytic acid was reduced by 80% while in wheat bread 25-60% of the initial content was degraded (Bartnik and Florysiak 1988). Phytic acid reduction of 31-46% in bread based on whole wheat flour and 88-99% in white wheat flour was reported by Daniels and Fisher (1981). Fretzdorff and Brümmer (1992) showed that the phytic acid degradation during bread preparation depended mostly on pH and the phytase naturally occurring in the flours. Türk et al. (1996) showed that phytic acid reduction during breadmaking was due to the phytase of the wheat flour and the phytase of the yeast. A decreasing phytic acid content could be observed with increasing fermentation time in bread based on whole wheat flour and 70% extraction wheat flour (Nayini and Markakis 1983). A study of Swiss bread varieties (Blumenthal and Scheffeldt 1983), reported phytic acid contents of less than 10 mg/100 g in white bread and up to 800 mg/100 g in bread containing bran.

1.1.8. Interactions of phytic acid with other food components

In foods such as cereals and legumes, phytic acid can interact with several different food components. The chemical aspects of these interactions are discussed in this chapter, while the nutritional aspects are presented in chapter 1.6.4. Phytic acid has six

strongly dissociated protons (pK_a 1.1-2.9) and six weaker dissociated protons (pK_a 4.6-12) (Cosgrove 1980a). Therefore, at pH values normally occurring in foods and under physiological conditions (pH 1 to 10), phytic acid is negatively charged and has the potential to bind cations or positively charged functional groups of molecules. Phytic acid can bind cations within a phosphate group, between two phosphate groups of the same molecule or between phosphate groups of different phytic acid molecules (Cheryan 1980). Figure 1.2. shows the possible interactions of phytic acid with minerals and trace elements, proteins and starch (adapted from Thompson 1988 and Barclay et al. 1995).

Figure 1.2. Possible interactions of phytic acid with minerals and trace elements, proteins and starch (adapted from Thompson 1988 and Barclay et al. 1995)
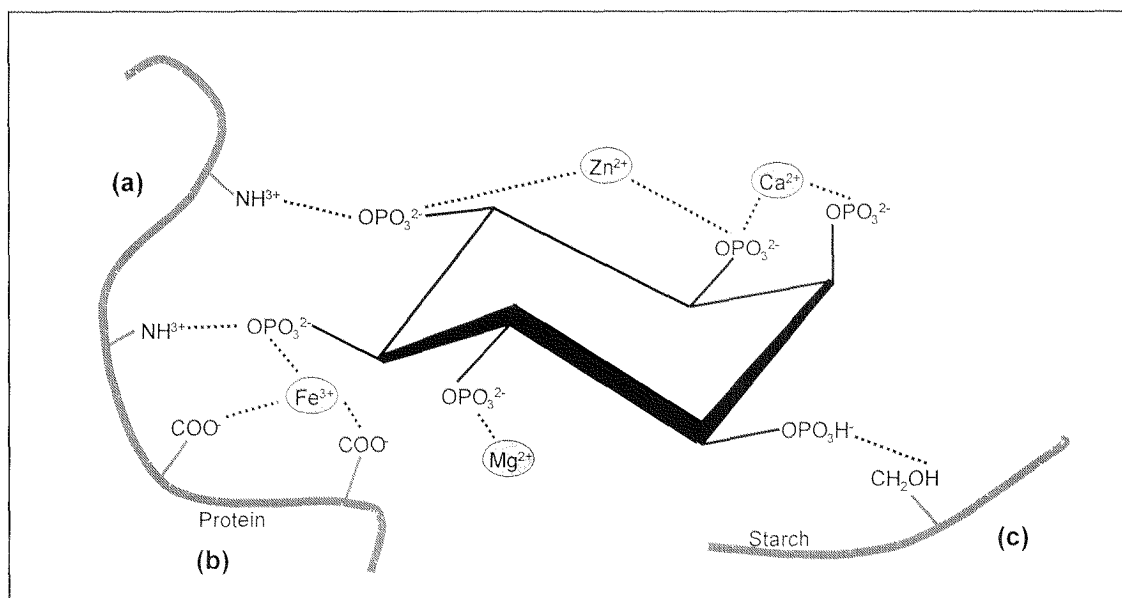
1.1.8.1. Interactions of phytic acid with minerals and trace elements

The following studies on the stability and solubility of complexes of phytic acid with minerals or trace elements were performed *in vitro* under specific conditions. Ratios of phytic acid to minerals or trace elements investigated do not necessarily represent conditions that could be encountered in complex systems, such as in the digestive tract of animals or humans. Extrapolations from *in vitro* to *in vivo* are therefore difficult to make.

The stability of phytic acid complexes with minerals and trace elements is pH dependent. Maddaiah et al. (1964) investigated the relative stability of various phytic acid-cation complexes by potentiometric titration and reported the following decreasing order: $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$. Vohra et al. (1965) described a slightly different decreasing succession of the stability of phytic acid-cation complexes: $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$. Persson et al. (1998) investigated the interaction of Cu^{2+}, Zn^{2+} and Cd^{2+} with inositol tri- (IP3), tetra- (IP4), penta- (IP5) and hexaphosphate (IP6). The study was performed at pH ranging from 3 to 7 and all inositol phosphates showed a pronounced binding capacity between pH 5 and 7. The binding capacity was lower for IP3 and IP4 than for IP5 and IP6 for all cations and was found to be in the decreasing order of $Cu^{2+} > Zn^{2+} > Cd^{2+}$ for all inositol phosphates. The difference between Cu and Zn was small for IP6.

The solubility of phytic acid complexes with minerals and trace elements depends on the pH value, the molar ratio of phytic acid to the mineral and the mineral itself. Jackman and Black (1951) investigated the solubility of complexes of phytic acid with Fe^{2+}, Al^{3+}, Ca^{2+} and Mg^{2+} at different pH values. At an excess of mineral ions, iron phytate was insoluble at pH in the range 2.5 to 8, aluminum phytate at pH 3 to 9, calcium phytate above pH 6 and magnesium phytate above pH 9.7. Graf and Eaton (1984) reported that complexes of phytic acid with mineral ions at equimolar ratios were soluble at any pH. Martin and Evans (1986a, 1986b) investigated the binding of zinc and copper to phytic acid. Each phytic acid molecule was found to bind 3.5 Zn^{2+} ions, resulting in insoluble complexes. Six Cu^{2+} ions were bound per phytic acid molecule at pH 6. Binding occurred in the pH range 2-6 and precipitation started at pH > 3.4. Soluble complexes were formed when only some of the binding sites were occupied. Nolan et al. (1987) investigated the influence of phytic acid concentrations on the solubility of Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} under physiological conditions (37°C, pH 2-7). At all phytic acid concentrations, complexes of phytic acid with copper were soluble below pH 3.5, with zinc below pH 4, with calcium below pH 5.5 and with magnesium below pH 6.0. At low iron concentration no precipitation of phytic acid-iron was observed at any pH. The solubility of phytic acid-copper and -zinc complexes was lowest at pH 6. The solubility of Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+} was investigated in cereal

products with varying phytic acid content. The extent of precipitation was in the following order for all cereal products tested at pH 7: $Zn^{2+} > Fe^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+}$. The addition of ethylene diamine tetraacetic acid (EDTA) and citric acid prevented precipitation (Lyon 1984).

Generally, complexes with three to six mineral ions per phytic acid molecule are insoluble, while mono- and di-complexes are soluble. However, mono- and di-complexes are only present at high phytic acid to mineral molar ratios. At equimolar ratios, phytic acid binds more than one mineral ion per phytic acid molecule. Champagne and Fisher (1990) reported that binding of Zn^{2+} and Cu^{2+} to phytic acid is cooperative at pH 7, i.e., binding of Zn^{2+} or Cu^{2+} to one phosphate group facilitates the binding of subsequent Zn^{2+} and Cu^{2+} to other phosphate groups of the phytic acid molecule. Thus even at high phytic acid to mineral molar ratios, insoluble complexes are formed.

1.1.8.2. Interactions of phytic acid with proteins and starch

The interactions between phytic acid and proteins are pH dependent. At low pH (below the isoelectric point) the amino terminal group of the protein is positively charged and can bind directly to the negatively charged phosphate group (Figure 1.2.a). At pH > 6 (above the isoelectric point) proteins and phytic acid are negatively charged and the interactions are somewhat uncertain. It has been proposed that multivalent cations are bound between the negatively charged phosphate group of the phytic acid molecule and the negatively charged carboxyl group of the protein, resulting in a ternary phytic acid-cation-protein complex (Figure 1.2.b). At even higher pH (> 10) it was proposed that the ternary complex dissociates and phytic acid becomes insoluble, while the unbound protein remains in solution (Cheryan 1980). Thompson (1986, 1988) suggested that other ternary complexes, for example phytic acid-protein-carbohydrate (starch) complexes may form through proteins associated with starch. Starch can also bind to phytic acid by the formation of hydrogen bonds (Figure 1.2.c).

1.2. Phytase

1.2.1. Introduction and nomenclature

Phytase was first noted in the literature in 1907 by Suzuki et al., reporting on the enzyme in rice. Due to its importance in human and animal nutrition, the reaction mechanism and the occurrence of the enzyme have been investigated extensively.

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of *myo*-inositol hexakisphosphate to inorganic phosphate (P_i) and lower phosphoric esters of *myo*-inositol and/or free *myo*-inositol. The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) distinguish between two phytases (IUPAC-IUB 1975): 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The EC (Enzyme Commission) classifications are based on the reaction the enzyme catalyzes. The first number refers to one of six general categories of reactions, the second number defines the substrate on which the enzyme acts and a third and fourth number define the individual enzyme. In the case of phytase, the first number '3' stands for hydrolase, the second number '1' means it acts on ester bonds, the third number '3' stands for phosphoric monoesters and the fourth number '8' or '26' is individual for the enzyme, based on the position of the phosphate group in the phytic acid molecule which is hydrolyzed first. 3-phytase is typically found in microorganisms, while 6-phytase is usually present in the seeds of higher plants. Phytase is a special type of acid phosphatase, capable of hydrolyzing P_i from IP6 as well as from other phosphate esters, whereas acid phosphatases can hydrolyze P_i from a wide range of phosphate esters but not from IP6.

1.2.2. Sources of phytase

1.2.2.1. Plants

Phytase has mostly been found in grains, seeds and pollen of higher plants, such as cereals, legumes, oilseeds and nuts, but also in roots of pea, gram, wheat and barley (Saxena 1964).

1.2.2.1.1. Location of phytase in grains and seeds

Circa 40% of the phytase activity of the whole wheat grain was found in the aleurone, circa 35% in the endosperm and 15% in the scutellum (Peers 1953). In rice (Yoshida et al. 1975), barley (Tronier et al. 1971) and sorghum (Adams and Novellie 1975) phytase activity was found to be mainly associated with the aleurone layer. Investigations of different triticale varieties showed a higher phytase activity in the bran as compared to the whole grain (Singh and Sedeh 1979). Little information is available on the location of phytase in legume seeds. Mandal and Biswas (1970) reported that phytase was found in the cotyledons of mungbeans.

1.2.2.2. Microorganisms

Microorganisms are an important source of phytase, especially for commercial phytase. Dvoráková (1998) summarized the sources of microbial phytase with pH and temperature optima. Recently, a novel screening method for extracellular phytase-producing microorganisms has been developed (Chen 1998).

1.2.2.2.1. Fungi

Microbial phytase has been most frequently detected in fungi. Shieh and Ware (1968) screened over 2000 microorganisms from soil for phytase activity. Only 30 of the 2000 microorganisms showed extracellular phytase activity, 28 of them belonging to the genus *Aspergillus*. *Aspergillus niger* was found to be the most active group, *Aspergillus ficuum* NRRL 3135 produced the highest phytase activity. Howson and Davies (1983) and Volfová et al. (1994) confirmed these results. Most of the microorganisms, such as *Mucor, Aspergillus* and *Rhizopus,* commonly used in oriental food fermentation, showed an ability to produce phytase, the activity depending on strains, species and growth media (Wang et al. 1980). Phytase activity was found in *Rhizopus oligosporus,* used for production of soybean tempeh, a fermented Asian food (Sudarmadji and Markakis 1977). In all fungal phytase producers, the enzyme production was induced by limiting concentrations of inorganic phosphate in the growth medium, while higher phosphate concentrations inhibited phytase synthesis (Shieh and Ware 1968).

1.2.2.2.2. Bacteria

Compared to fungi, bacteria are in general not a good source of phytase. Some strains of lactic acid bacteria have been screened for their ability to degrade phytic acid in the growth medium (Shirai et al. 1994). Phytic acid reduction was observed, however this might not only be due to phytase activity, but also to co-precipitation of phytic acid and protein with decreasing pH during fermentation, which rendered the phytic acid undetectable for the method used. Two phytases were purified and characterized from *Escherichia coli*, one of them being a 6-phytase, although phytase from microorganisms are usually considered as 3-phytase (Greiner et al. 1993). In a subsequent study this phytase has been immobilized and used in a bioreactor to produce special phytic acid breakdown products, for example certain isomers of IP5, IP4, IP3 and IP2 (Greiner and Konietzny 1996).

1.2.2.2.3. Yeast

The phytase production of yeast has been screened using sodium phytate as the sole source of inorganic phosphate in the growth medium. Nine of 21 yeast strains showed phytase activity, the highest was found in *Schwannimyces castellii* CBS 2863 (Lambrechts et al. 1992). Nayini and Markakis (1984) found *Saccharomyces cerevisiae* (Baker's yeast) to produce phytase, which contributes to phytic acid degradation during breadmaking (Türk at al. 1996).

1.2.2.3. Animals and humans

The first report of animal phytase, found in liver and blood of calves, was published by McCollum and Hart (1908). Further search for phytase in blood of mammals was unsuccessful; it was detected only in blood of lower vertebrates, such as birds, reptiles and fishes (Rapoport et al. 1941). Occurrence of phytase in the rat intestine was first noted by Patwardham (1937). Subsequently, several other researchers detected phytase activity in the intestine of rats, pigs and cows (Spitzer and Phillips 1945) and rats, rabbits, guinea pigs and hamsters (Cooper and Gowing 1983). Phytase activtiy could be demonstrated in the mucosa extracts of the small intestine of rats, chicken, calves and humans (Bitar and Reinhold 1972). In the rat, phytase was found to be localized in the

brush border of the small intestine (Cooper and Gowing 1983). The first quantification of human intestinal phytase activity was done by Iqbal et al. (1994). In comparison with rat intestinal phytase or human alkaline phosphatase the phytase activity was found to be very low and its ability to degrade phytic acid was considered to be very limited.

1.2.2.3.1. Impact of intestinal phytase on phytic acid degradation

There are three possible sources of phytase in the gastrointestinal tract of animals or humans: the diet, the intestinal mucosa and the bacterial flora. The diet is in general the most important source of phytase, while the finding on intestinal and bacterial phytase are contradictory.

To determine the main source of phytase, Wise and Gilburt (1982) studied phytic acid degradation in intestines of germfree and conventional rats fed identical diets. More phytic acid reduction was observed in conventional rats and the authors concluded, that this was due to the bacteria in the large intestine and that intestinal phytase activity was negligible. Williams and Taylor (1985) confirmed these results and showed that in rats, phytic acid was degraded in the stomach by phytase present in the diet and further degradation occurred in the large intestine, presumably due to the presence of bacterial phytase. Jany (1993) found that phytic acid was only degraded in the intestinal tract of the rat if the diets contained active phytase and concluded that rats do not possess intestinal phytase, but that phytic acid can be degraded by phytase from the diet and intestinal alkaline phosphatase. Cooper and Gowing (1983) demonstrated contradictory data i.e., that phytic acid in the lumen of the intestine of rats was degraded by the phytase from the brush border membrane. The phytase activity was found to be higher in rats than in rabbits. They concluded that phytase is present in a variety of mammalian species and that the enzyme may play an important role in the bioavailability of minerals and trace elements.

The digestion of phytic acid in the stomach and small intestine of ileostomy patients was investigated by Sandberg and Andersson (1988). When phytase-deactivated wheat bran was fed, only 5% of the phytic acid was found to be degraded. Feeding wheat bran with active phytase led to phytic acid degradation of over 50% of the initial content.

These results showed that mucosal intestinal phytase, if present, does not play an important role in phytic acid degradation in the human digestive tract.

1.2.3. Properties of phytase

1.2.3.1. Molecular properties

Molecular weight and numbers of subunits of phytase from various sources have been summarized by Dvoráková (1998). Only a few phytases were purified to such homogeneity that their molecular weight could be estimated. For example phytase from microorganisms had molecular weights ranging from 36.5 kDa for *Bacillus subtilis* to 700 kDa for *Klebsiella aerogenes*. Commonly used phytases from *Aspergillus niger* have molecular weights between 80 and 200 kDa. Soybean phytase was found to have a molecular weight of 119 kDa, mungbean phytase 158 kDa and maize phytase 76 kDa (Gibson and Ullah 1988, Laboure et al. 1993). Assuming a mean molecular weight of 100 Da for amino acids, these phytases comprise circa 360 to 7000 amino acids.

1.2.3.2. Temperature and pH stability and optima

Generally, phytase is a relatively thermostable enzyme with temperature optima in the range of 37 to 77°C (Dvoráková 1998). The heat resistance of wheat phytase was first investigated in 1944 by McCance and Widdowson. After five hours at 90°C dry heat the activity was only decreased by about 10%, whereas in water at 90°C the enzyme was destroyed instantaneously. Extremely thermostable phytase from *Aspergillus fumigatus* was shown to withstand 100°C for 20 minutes in buffer solution with a loss of only 10% of the initial activity (Pasamontes et al. 1997). Temperature optima of phytase originating from animals or humans have not been described, but they are generally assayed under physiological conditions at 37°C (Bitar and Reinhold 1972).

Plant phytases have been described to have pH optima between 4.0 and 5.6, although phytases extracted from certain legume seeds showed an alkaline pH optimum of 8.0 (Scott 1991). Microbial phytases, especially those from *Aspergillus* have two pH optima, one at 4.5 to 5.5 and a second at pH 2 to 2.5 (Howson and Davis 1983, Ullah and Gibson 1987, Simons et al. 1990, Volfová et al. 1994, Sandberg et al. 1996,

Pasamontes et al. 1997). Bacterial phytase from *Escherichia coli* showed a pH optimum at 4.5. Phytases from the intestinal mucosa of rats, chicken, calves and humans were found to have pH optima between 7.0 and 8.5 (Bitar and Reinhold 1972, Yang et al. 1991). In Table 1.3. temperature and pH optima of phytase from various cereals grains and legumes seeds are summarized. If no specification is indicated the values are representative for untreated whole grains and seeds.

Table 1.3.   Temperature and pH optima of phytase from cereal grains and legume seeds

Phytase source	Optimal temperature [°C]	Optimal pH	Reference
Cereal grains			
Barley		5.2	Preece 1962
Maize (germinated)	50	5.6	Chang 1967
	55	4.8	Laboure et al. 1993
Rice (aleurone particles)	45	4.0-4.5	Yoshida et al. 1975
Rye	48	5.5	Greiner et al. 1997
Spelt	45	6.0	Konietzny et al. 1995a
Triticale	45	5.4	Singh and Sedeh 1979
Wheat	55	5.15	Peers 1953
Wheat bran		5.0	Nagai and Funahashi 1962
		5.6; 7.2	Lim and Tate 1973
Legume seeds			
Bean (California small white bean)	35-45	5.2	Becker et al. 1974
	60	5.2	Chang and Schwimmer 1977
Dwarf bean		5.2	Gibbins and Norris 1963
Faba bean (germinated)	50	5.0	Eskin and Johnson 1987
Garden pea (germinated)	35-40	5.1	Guardiola and Sutcliffe 1971
Lupin (germinated)	50	5.0	Silva and Trugo 1996
Mungbean (germinated)	57	7.5	Mandal et al. 1972
Navy bean		5.2	Gibbins and Norris 1963
	50	5.3	Lolas and Markakis 1977
Soybean (germinated)	55	4.5-4.8	Gibson and Ullah 1988
Soybean	60	4.8	Sutardi and Buckle 1986

1.2.3.3. Substrate specificity

Phytases usually have a broad substrate specificity and only few phytases have been described as being highly specific for IP6, such as phytase from *Bacillus subtilis* (Powar and Jagannathan 1982) and from lily pollen (Barrientos et al. 1994). Relative substrate specificity, as compared to IP6, of phytase from navy bean, mungbean, rice and wheat were summarized by Reddy et al. (1989), relative substrate specificity of microbial phytase and phytase from spelt by Liu et al. (1998). None of the phytase preparations showed an absolute specificity for IP6, some having higher specificity for other

phosphate esters, such as lower inositol phosphates (IP3, IP4, IP5), glycerophosphate, phenylphosphate, pyrophosphate, adenosine triphosphate, adenosine diphosphate and fructose-phosphate. Phytase from wheat bran has been shown to be able to degrade not only *myo*-inositol hexaphosphate, but also isomers, such as *neo*-, D-*chiro*- and *scyllo*-inositol hexaphosphate (Cosgrove 1966b).

1.2.3.4. Activators and inhibitors of phytase

Certain substances can have an inhibiting or enhancing effect on phytase activity, depending on concentration and nature of the substance. Different concentrations of bivalent cations, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+} have been investigated on their influence on phytase activity in barley (Singh and Sedeh 1979), germinating maize (Laboure et al. 1993), rice bran (Hayakawa et al. 1989), spelt (Konietzny et al. 1995a), wheat (Peers 1953), dwarf bean (Gibbins and Norris 1963), pea (Guardiola and Sutcliffe 1971) and germinating soybean (Gibson and Ullah 1988). The effect of the cations on the phytase activity of these grains and seeds varied from a complete reduction to a 2.5 fold increase of phytase activity. The influence of cations on microbial phytase has been summarized by Liu et al. (1998).

Phytase is inhibited by high concentrations of phytic acid (substrate inhibition). Substrate inhibition was found in navy beans at phytic acid concentrations above 2 mM (Lolas and Markakis 1977), in faba bean above 1.5 mM (Eskin and Johnson 1987) and above 0.9 mM or 0.8 mM in crude extract or partially purified phytase of triticale (Singh and Sedeh 1979). Phytase from spelt was inhibited at substrate concentrations above 5 mM (Konietzny et al. 1995a), rye phytase above 4.0 mM (Greiner et al. 1997). In two varieties of pea no inhibition was found up to phytic acid concentrations of 4.5 mM and 10 mM respectively (Beal and Mehta 1985).

Inorganic phosphate liberated during the enzymatic reaction can inhibit the phytase (product inhibition). Inorganic phosphate was found to be a strong inhibitor of phytase purified from germinating soybeans (Gibson and Ullah 1988) and of *Aspergillus ficuum* NRRL 3135 phytase (Howson and Davis 1983).

1.2.3.5. Dephosphorylation of phytic acid by phytase

The phosphate group at C-6 of the phytic acid molecule is liberated first by the 6-phytase, usually found in higher plants. The phosphate liberation at C-6 was followed by those at C-5 and C-4, or C-1 and C-3, or C-1 and C-4, while the phosphate at C-2 was stable in mungbean (Maiti et al. 1974). The stepwise phytic acid degradation by wheat bran phytase, starting at C-2, C-6 or C-5 has been described by Lim and Tate (1973). Hayakawa et al. (1990) found that phytase from rice bran started the degradation at C-6 and described the intermediates formed during degradation to the final *myo*-inositol. The alkaline phytase from lily pollen started the degradation at C-5 resulting in the final product IP3, which could not be degraded any further (Barrientos et al. 1994). The 3-phytase from microorganisms starts the phytic acid degradation at C-3, as shown for phytase from *Pseudomonas ssp.* (Cosgrove 1969). However, phytase from *Escherichia coli* starts at C-6 (Greiner et al. 1993).

1.2.3.6. Phytase purification

Phytase is usually isolated and purified by extraction (with water, buffer or salt solutions), precipitation of the crude extract with ammonium sulfate, followed by dialysis or gel filtration and ion exchange chromatography (Reddy et al. 1989). Depending on the source of the enzyme, the purification steps need to be adapted and modified. Partial purification and characterization of phytase from different sources has been described, but complete purification to homogeneity was often difficult to achieve. Phytase from several microorganisms have been purified, for example from *Aspergillus ficuum* NRRL 3135 (Ullah and Gibson 1987) and *Aspergillus fumigatus*, after being overexpressed in *Aspergillus niger* (Pasamontes et al. 1997). Plant phytases isolated from cotyledons of mungbean (Maiti et al. 1974), from soybean (Gibson and Ullah 1988) and from germinating maize (Laboure et al. 1993) were purified to homogeneity, while phytase isolated from wheat bran (Nagai and Funahashi 1962), spelt (Konietzny et al. 1995a), rye (Greiner et al. 1997) and faba bean (Eskin and Johnson 1987) could not be completely purified.

1.2.4. Phytase activity

1.2.4.1. Definition of phytase activity

The International Union of Biochemistry (IUB) has adopted the international unit (IU) as the standard measure of enzyme activity: one IU being the amount of enzyme (or crude enzyme extract) required to catalyze the transformation of one micromole of substrate per minute or, if more than one bond of each substrate molecule is attacked, one microequivalent of the group concerned, under a specific set of defined solution conditions (Copeland 1996). The activity of phytase is expressed in phytase units (PU); one PU being the activity that liberates one micromole P_i from phytic acid per minute under specified conditions. This definition has been used by many authors (Lim and Tate 1973, Lolas and Markakis 1977, Kikunaga et al. 1991, Ranfft 1991, Greiner et al. 1993, Most et al. 1993, Pen et al. 1993, Biehl et al. 1995, Konietzny et al. 1995a, Rimbach et al. 1995, Yoon et al. 1996). Others defined the PU as nanomoles P_i per minute (Laboure et al. 1993) or micromole P_i per hour (Nagai and Funahashi 1962, Mandal et al. 1972, Chang and Schwimmer 1977, Wang et al. 1980, Hayakawa et al. 1989) or milligram or microgram per hour (Peers 1953, Shieh and Ware 1968). Picomole per second (picokatals) were used by Gibson and Ullah (1988), nanokatals (nkat) by Scott (1991) and Volfová et al. (1994). Some authors defined the phytase activity as liberated phosphorus, and not phosphate, and therefore care has to be taken when values, expressed in weight, are compared with values expressed in moles.

1.2.4.2. Determination of phytase activity

Phytase activity is usually determined by measuring the liberation of P_i from phytic acid, although some authors followed phytic acid degradation by measuring the different inositol phosphates generated, using HPLC technique (Sandberg and Andersson 1988) or ^{31}P NMR spectroscopy (Frolich et al. 1988). The conditions of the phytase assay depend on the source of the phytase and vary widely in the concentration of enzyme and substrate, pH and buffers, temperature and the use of potential activators. The enzyme is mostly used in the form of crude extracts or partly purified enzyme extracts and sometimes as purified enzyme.

For the determination of the liberated P_i colorimetric methods have mostly been used. For example, Fiske and Subbarow (1925) applied a method where phosphomolybdate is reduced to molybdenum blue and measured colorimetrically. This method was used by Shieh and Ware (1968), Guardiola and Sutcliffe (1971), Bitar and Reinhold (1972), Singh and Sedeh (1979) and Hayakawa et al. (1989 and 1990). A colorimetric method, based on the reduction of phosphomolybdate by ascorbic acid (Watanabe and Olsen 1965) was applied by Lim and Tate (1973), Lolas and Markakis (1977), Nayini and Markakis (1984) and Volfová et al. (1994). A colorimetric method which measures phosphomolybdate directly, without reduction, by dissolving the whole assay mixture in acetone (Heinonen and Lahti 1981) was used with modifications by Ullah and Gibson (1987), Gibson and Ullah (1988), Laboure et al. (1993), Greiner et al. (1993, 1997) and Konietzny et al. (1995a). The method by Allen (1940), a modification of the method of Fiske and Subbarow (1925), was used by Peers (1953), Gibbins and Norris (1963), Chang and Schwimmer (1977) and Bartnik and Szafranska (1987).

1.2.5. Influence of soaking and germination of grains and seeds on phytase activity

During germination of grains and seeds, phosphorus is required for growth and is mobilized from the main storage form, phytic acid. Phytase activity is therefore often increased during germination. It is not clear if the increase in phytase activity is due to an activation of the existing enzyme or as a result of a *de novo* synthesis. Bianchetti and Sartirana (1967) found that new phytase was synthesized in wheat during germination and that it was regulated by the concentration of inorganic phosphate, while Eastwood and Laidman (1971) blocked the protein synthesis in germinating wheat and found that the increase in phytase activity was due to an activation of the existing enzyme. Mandal and Biswas (1970), found that the increase in phytase activity in germinating mungbean was due to a *de novo* synthesis of the enzyme. The mechanism of the activation has not yet been elucidated.

Soaking is often combined with germination and sparse information is available on the influence of separate soaking on phytase activity. Soaking of faba beans at 20°C was found to increase the phytase activity constantly up to 24 hours, whereas soaking at

35°C increased the phytase activity during eight hours, followed by a decrease and seeds soaked at 50 and 65°C did not show any phytase activity (Henderson and Ankrah 1985). A rise in phytase activity during germination has been found in many legume seeds, such as mungbean (Mandal and Biswas 1970, Lolas and Markakis 1977), faba bean (Eskin and Wiebe 1983), garden pea (Guardiola and Sutcliffe 1971, Chen and Pan 1977) and bush bean (Walker 1974). For lentil, chickpea, and bean a maximum phytase activity was found after six to eight days of germination (Kyriakidis et al. 1998). A significant increase in phytase activity occured after four and more days of germination with the first two days representing a latent period (Lolas and Markakis 1977). In cereals, such as wheat, rye, barley and oat (Bartnik and Szafranska 1987), maize (Chang 1967, Laboure et al. 1993) and rice (Mukherji et al. 1971) an increase in phytase activity during germination has been reported. Konietzny et al. (1995b) found a maximal increase in phytase activity in spelt, maize and oat after four to five days of germination. Fretzdorff and Weipert (1986) could not detect an increase in phytase activity in rye during three days of germination.

1.2.6. Applications of phytase

Phytase is mainly used for phytic acid degradation of animal feeds and for the preparation of special phytic acid breakdown products, such as particular isomers of lower inositol phosphates, for biochemical investigations. In foods for human consumption, phytase has been used to degrade phytic acid in meals for absorption studies, but to our knowledge not in commercial products.

In animal feeds, based on cereals and legumes, the addition of phytase is beneficial for both animal nutrition and the environment. The P_i liberated from phytic acid becomes absorbable by the animal and in addition the degradation of phytic acid can increase the bioavailability of minerals and trace elements. As a result of increased phosphate absorption, the phosphate content of the feces, which causes environmental problems can be decreased. Phosphorus excretion by pigs and poultry was reduced by circa 50% using a phytase preparation from *Aspergillus niger* (FinaseR) to degrade phytic acid in the feed (Simell et al. 1991). FinaseR added to a corn-soy based pig diet converted circa one third of the non-absorbable phosphorus to an absorbable form (Cromwell et al.

1993). Addition of phytase from Alltech USA (Allzyme Phytase), BASF USA (Natuphos), or crude microbial phytase from *Apergillus ficuum* improved the bioavailability of phytate phosphorus in pigs and broilers (Simons et al. 1990, Cromwell et al. 1995). Several authors have confirmed the possibility of replacing inorganic phosphate supplementation in a phytic acid rich diet for monogastric animals by the use of phytase (Kornegay and Qian 1996, van Dijck 1999). In addition to the enhanced utilization of phosphorus, also calcium, magnesium, zinc and protein availability was improved in feed for piglets (Pallauf et al. 1994), zinc availability in a rat diet (Rimbach and Pallauf 1992, 1993) and the availability of several minerals and trace elements in a barley-corn based diet for pigs by the addition of phytase (Gebert 1998).

The growing interest in inositol phosphates in transmembrane signaling and calcium mobilization resulted in the request for various inositol phosphate preparations (Billington 1993). Mild reaction conditions and stereospecificity are the advantages of enzymatic preparation of inositol phosphates as compared to chemical synthesis. Several patents for the preparation of different inositol phosphates are mentioned by Dvoráková (1998) and immobilized phytase from *Escherichia coli* has been used (Greiner and Konietzny 1996).

In human nutrition, phytase has been used in studies to investigate the influence of phytic acid on the bioavailability of minerals and trace elements. Addition of phytase derived from *Aspergillus niger* to soy-protein isolate, leading to almost complete phytic acid degradation, increased iron absorption in adults (Hurrell et al. 1992). A significant increase in iron absorption was observed in infants, when the phytic acid in formulas based on soy-protein isolate was degraded by phytase derived from *Aspergillus niger* (Davidsson et al. 1994a). When phytase from *Aspergillus niger* was given with a meal, iron absorption in adults was increased, while wheat bran with active phytase added to the meal did not influence iron absorption (Sandberg et al. 1996). Zinc absorption from bread was increased, when bran with active phytase was added during dough fermentation (Nävert et al. 1985).

1.3. Cereals, pseudocereals, legumes and oilseeds

The botanical classification, the origin, uses and preparations, and the composition of cereals, pseudocereals, legumes and oilseeds are reported in this chapter. The selection is based on the following study (chapter 2), including the most important as well as less frequently consumed grains and seeds.

1.3.1. Cereals

Cereals belong to the grass family (*Gramineae*). They have been collected and cultivated for the size and quality of their grains for thousands of years and are grown as major crops throughout the world (FAO 1989a). According to worldwide production data the most important cereal grains in 1993 were wheat, rice, maize, barley, sorghum, oat, millet and rye (Rehm and Espig 1996).

The basic structure is similar for most cereal grains, consisting of bran (including pericarp, testa and aleurone layer), endosperm, scutellum and germ or embryo. As an example Figure 1.3. shows the structure of a rice grain. Starch is mainly present in the endosperm, while most fibrous tissues are found in the pericarp, testa and aleurone layer. Protein occurs throughout the grain, fat and minerals are mostly found in the germ and in the bran (FAO 1989a). The whole grains of most cereals have similar chemical composition and nutritive value. Depending on species, variety and cultivation, protein content varies from 5-15%, carbohydrate content from 60-90% and fat content from 1-5%. Most cereals have a low content of the essential amino acids lysine and tryptophan.

Figure 1.3. Structure of a rice grain (FAO 1989a)
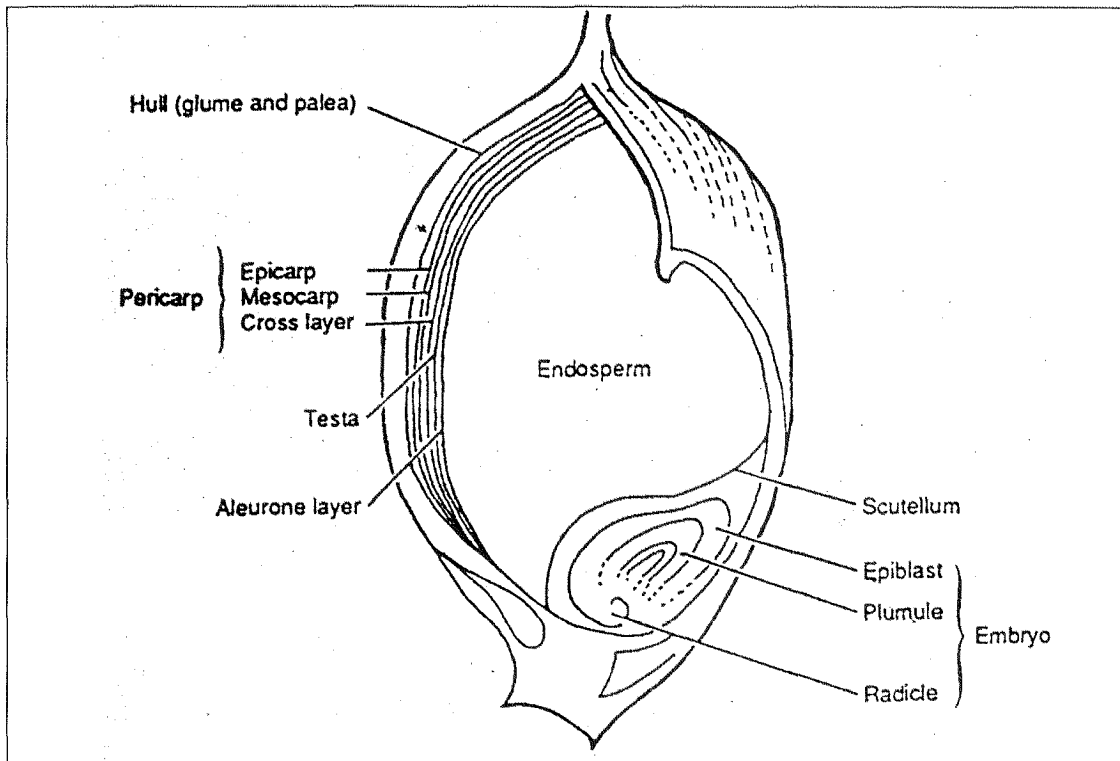
1.3.1.1. Wheat

Ninety % of the wheat production is *Triticum aestivum* (common wheat), followed by *Triticum durum* (durum wheat). Spelt (*Triticum spelta*) also belongs to the wheat family (Franke 1989). Wheat is produced in greater quantities than any other cereal and is cultivated almost everywhere, except for the tropical regions. The highest yields are achieved in Europe, China, USA and Canada (Franke 1989). Although there is only very limited wheat production in the tropics, consumption is realtively high. Wheat products are often preferred to those prepared from locally grown cereals, such as sorghum and millet, and lead to increased dependence on imported supplies (FAO 1989a).

Wheat is used for human consumption and animal feeds. The efficiency in feed utilization is higher when wheat is processed before being fed to cattle, poultry or pigs. For human consumption wheat flour is most commonly used for bread preparation, due to its high gluten content. Durum wheat is mostly used for the production of pasta. Wheat is rarely eaten as whole grains; most of the wheat is consumed milled, crushed or

cracked (FAO 1989a). Milling removes the bran and the germ from the endosperm and reduces the content of fiber, fat, mineral and vitamin of the flour, but increases storage stability. Although flour of every extraction rate can be used to prepare leavened bread, low extraction flour is generally preferred in most countries (Pedersen et al. 1989).

1.3.1.2. Rice

There are two species of cultivated rice: *Oryza sativa* of Asian origin and *Oryza glaberrima* of African origin. However, nearly all cultivated rice is *Oryza sativa*. Rice domestication occurred independently in China, India and Indonesia and was thereby leading to different varieties. *Oryza sativa japonica* (synonym *sinica*) is short-grained and sticky or glutinous after cooking. *Oryza sativa indica* is long-grained (FAO 1989a, 1993).

About 95% of all rice is produced in developing countries, 92% is grown in Asia (FAO 1993). Rice is mostly eaten boiled or steamed but it can also be processed to rice flakes or flour, which can be fermented or used for noodle preparation. It is also the raw material for brewing of rice beer and for rice wine production (FAO 1989a). The rice bran fraction has high contents of energy and protein, riboflavin, niacin and minerals. The germ has high fat and α-tocopherol content. Rice is mostly consumed as white rice, which means that the pericarp, testa, aleurone layer and the germ are removed resulting in losses of fat, protein, fiber, vitamins and minerals. Compared with other cereals, rice is low in protein and fiber, but has a rather high lysine content (FAO 1993).

1.3.1.3. Maize

All maize is classified as *Zea mays* although there are many different varieties, such as *Zea mays saccharata* (sweet corn), which contains more sugar than starch in the upper endosperm, *Zea mays mays* (flint corn) or *Zea mays dentiformis* (dent corn) and special breeding such as high-oil or high-protein maize (Franke 1989, FAO 1992). Maize most probably originates from Central America, from where it spread northwards and southwards, and after the discovery of the American continent also to Europe. Maize is grown in almost every agricultural region of the world, the biggest part of the world production is cultivated in North and Central America (FAO 1992).

Maize is used for human consumption, animal feeding and it serves as raw material for the production of starch, oil, protein, alcoholic beverages and food sweeteners (FAO 1992). Many different dishes can be prepared for human consumption, such as gruels, polenta, corn flakes, corn bread or popcorn, depending on the region. Fermented maize is mostly consumed in Africa, where it is also used for brewing beer. Maize flour after alkaline treatment is widely used for the preparation of tortillas in Central and South America (FAO 1992). The maize germ has a high fat content (circa 33%) and can be used for oil production. Maize protein is especially low in the essential amino acid tryptophan.

1.3.1.4. Barley

Barley (*Hordeum vulgare*) descends from the wild type *Hordeum spontaneum* which originated in Asia (Franke 1989). The two main types are two-rowed barley (*Hordeum vulgare* convar. *distichon*) and six-rowed barley (*Hordeum vulgare* convar. *hexastichon*) (Aufhammer 1998). Barley is mostly cultivated in Europe and the ex-USSR with minor amounts grown in North America, in the Middle East, India and Africa (Franke 1989, Pedersen et al. 1989).

Barley is used for human consumption, for feeding of monogastric animals (in particular pigs) and for beer brewing and whisky manufacturing (Franke 1989, Pedersen et al. 1989). The use of barley for human consumption is rather limited in industrialized countries, while barley is consumed as soup, porridge or unleavened bread in developing countries (Kent and Evers 1994). Barley contains more fiber than wheat, irrespective of the extraction rate (Pedersen et al. 1989). The ß-glucans in the endosperm and aleurone cells of barley have implications on glucose and lipid metabolism (Pedersen et al. 1989, McIntosh et al. 1995). The average protein content of barley is 13%, which is about equal to wheat and generally higher than in other cereal grains (McIntosh et al. 1995). High-lysine cultivars have been developed for an optimal utilization of barley proteins for humans and animals nutrition (Pedersen et al. 1989, McIntosh et al. 1995).

1.3.1.5. Sorghum

Sorghum belongs together with millet to the small-grain cereals. *Sorghum vulgare*, correctly termed *Sorghum bicolor* is cultivated in South Africa, *Sorghum guineense* in West Africa, *Sorghum dura* in North Africa, India and China, *Sorghum halepense* in the Philippines (FAO 1989a). *Sorghum saccharatum* (sweet sorghum) was mainly cultivated for the sugar content of the stem, as a substitute to sugar cane, and it is now used to produce molasses 'sorgho syrup' (FAO 1989a).

Sorghum probably originated in North Africa and spread throughout Africa and the Middle East and later on to China and America. Sorghum is grown in latitudes below 45° in all continents, with the USA, India and China as the major producers (Kent and Evers 1994). In the USA, Australia, Japan and Europe, sorghum is almost entirely used for animal feeding, while in Africa and India it is mostly used for human consumption (Kent and Evers 1994). Sorghum grains are hard, and the outer bran layer is bitter and astringent and has to be removed by milling. In traditional home processing, sorghum is soaked prior to removal of the bran. Sorghum is used, similar to millet, for the preparation of gruels, weaning foods, porridge or unleavened bread. Porridge and gruels are also prepared with fermented sorghum. The color of sorghum grains may be white, yellow, brown or red. The white varieties are the most palatable, while the darker varieties contain more tannins in the husk and protein availability and digestibility are reduced. For beer brewing the dark-colored grains are preferred (FAO 1989a, Kent and Evers 1994).

1.3.1.6. Oat

The oat species most cultivated are *Avena sativa* (white or yellow oat) *and Avena byzantina* (red oat) and crossings thereof. Rarely cultivated species are *Avena strigosa, Avena abyssinica, Avena fatua* and *Avena nuda* (Schrickel 1986). Oat originates from the Middle East. A large proportion of the oat production is found in the Northern Hemisphere, mostly in the ex-USSR, USA and Europe. The highest yields are achieved in Northern Europe (UK, Sweden, Germany) (Schrickel 1986).

Oat is mainly used for animal feeding, especially for horses and poultry. Oat is mostly prepared into oatmeal or rolled oat for porridge, infant food or breakfast cereals for humans (Kent and Evers 1994). Oat products are generally made of whole grain oat, after removal of the hulls, and therefore only minor losses of vitamins or minerals concentrated in the bran and germ occur. Oat has high content of fat (ca. 7%) compared to other cereals (Kent and Evers 1994). Fiber and ß-glucans in oat have been found to have beneficial physiological effects on lipid and glucose metabolism (Pedersen et al. 1989).

1.3.1.7. Millet

Millet belongs to the small-grain cereals and includes a large number of different botanical species. *Pennisetum americanum* (synonym *Pennisetum typhoides*) is the most important species and is called pearl or bulrush millet, cattail, bajra, cumbo, sajja or candle millet and is mostly cultivated in Africa (especially in West Africa), India and South Asia. The second most important species (*Setaria italica)* is called Italian or foxtail millet, and is cultivated in Japan, India and China. *Panicum miliaceum* (common, hog or golden millet, broom corn or proso) is cultivated in East Asia, India, Iran, China and Japan. *Eleusine coracana* is called finger or African millet, wimbe or ragi and is cultivated in East Africa, India, China and Ethiopia. Less important species are *Setaria verticillata, Panicum obtusum* and *sumatrense, Echinocola* and *Digitaria* (FAO 1989a, Rehm 1989, Kent and Evers 1994). Millet is assumed to have originated from wild African grasses which were subsequently taken to China and India for cultivation. Millet is mostly grown in areas too dry for the cultivation of wheat and rice, especially in Africa and India. Statistics on millet consumption is lacking since millet is mostly consumed locally and therefore does not appear in cereal trade figures. Millet is often mentioned together with sorghum (Hulse et al. 1980, FAO 1989a).

The pericarp of millet grains is hard and has to be removed before consumption. Millet is used for the preparation of gruels, weaning foods, unleavened bread, couscous, for beer brewing or animal feeding. In Africa and Asia, millet is often germinated and fermented to improve the flavor and digestibility of cereal gruels (FAO 1989a). Wide diversity in the chemical composition of different millet varieties has been reported.

Protein content ranges from 4-20%, fat content from 1-7% and carbohydrate content from 60-90% (Hulse et al. 1980).

1.3.1.8. Rye

Rye (*Secale cereale*) was domesticated in Southern Europe. It is mainly cultivated in the ex-USSR, in Eastern and Northern Europe, in Canada and in the North Central States of the USA were emigrants from Eastern Europe have settled (Kent and Evers 1994).

Rye is mostly used for animal feeding in Europe and the ex-USSR. Although rye is high in energy, growth of animals fed rye is slower than with other cereals, possibly because its unpalatability restricts intake (Kent and Evers 1994). For human consumption rye is mainly eaten in the form of sour-dough bread. It is a major bread grain of Scandinavian and Eastern European countries, while elsewhere bread is mainly produced with wheat flour (Kent and Evers 1994). Compared to wheat, rye contains less starch and crude protein (especially low in gluten), but rye contains more free sugars and fiber (Franke 1989, Åman et al. 1997).

1.3.1.9. Triticale

Triticale (*Triticosecale*) is a cross breeding of wheat and rye (*Triticum* x *Secale)* to combine the grain quality, productivity and disease resistance of wheat with the vigor and hardiness of rye (Franke 1989, Kent and Evers 1994). Triticale (variety 'Rosner'), a cross between durum wheat and rye was first grown in the USA in 1970. In 1977 a cross between bread wheat and rye was grown in China. Production greatly increased over the following years and in 1989 the largest contribution to the world production came from Poland, China, France, Spain and Australia (Kent and Evers 1994).

As animal feed, triticale is especially suitable for pigs and poultry. Triticale is mainly consumed in the form of bread, either as sour-dough bread, or mixed with wheat flour as leavened, or as unleavened bread (Rehm 1989). Triticale has a higher protein content

(13-15%) than wheat or rye and also a higher proportion of lysine (several references compiled by Rehm 1989).

1.3.2. Pseudocereals

The composition of pseudocereal grains is similar to cereal grains, although botanically they do not belong to the grass family (*Gramineae*). Pseudocereals are also called non-grass food grains (FAO 1989a).

1.3.2.1. Amaranth

Amaranth is an ancient plant belonging to the family *Amaranthaceae*. The main varieties used for grain production are *Amaranthus cruentus*, *Amaranthus hypochondriacus* and *Amaranthus caudatus*. From other varieties, such as *Amranthus tricolor* and *Amaranthus lividus*, the leaves are consumed as vegetables (FAO 1989a). Amaranth had been cultivated for over 4000 years in Central America and was later transported to Asia and Africa (Rehm 1989). Amaranth grows very well in tropical and subtropical regions and is resistant to heat and dryness. Amaranth is mostly grown in South America and Mexico, but also in India and Africa.

Amaranth grains can be consumed cooked as gruels or soups, popped or as flour made into unleavened bread (FAO 1989a, Rehm 1989). The grains are very small, 1-1.5 mm, the color varies from gray-white, beige, brown, red to black. Amaranth grains contain 60-70% starch, a rather high fat content (6-7%) and 16-18% protein. The protein is high in lysine i.e. circa 3 fold the content of wheat (Franke 1989, Rehm 1989, Dodok et al. 1994, 1997).

1.3.2.2. Buckwheat

There are three different varieties of buckwheat: *Fagopyrum esculentum*, *Fagopyrum tataricum* and *Fagopyrum cymosum*, the first being the most common variety in Europe (Aufhammer, 1998). Buckwheat originates from Central Asia from where it spread to India, China, Japan and Europe. It is now mostly cultivated in Russia and Eastern Europe (Rehm 1989, Aufhammer 1998).

Buckwheat is consumed in the form of gruels or soups, buckwheat flour can be made into pancakes ('blinis') or mixed with wheat flour for bread preparation. Buckwheat is also used as animal feed, especially for poultry, or buckwheat flour can be added to cattle feed (Rehm 1989). The composition of buckwheat grains is similar to cereals, containing circa 10% protein and 2% fat, and 70% carbohydrates (Rehm 1989).

1.3.2.3. Quinoa

Quinoa belongs to the species *Chenopodium* which comprises circa one hundred different varieties, the most common being *Chenopodium quinoa*. Quinoa originates from the Andes in South America where it is still grown. Quinoa is cultivated from sealevel up to 4000 m altitude with different varieties appropriate for each climate (Aufhammer 1998).

The grains are roasted and consumed as soups or gruels or quinoa flour can be mixed with wheat flour for bread preparation. The leaves are consumed as vegetables or used for animal feeding (Rehm 1989). Quinoa has high protein content (12-18%), often high in methionine, and a fat content of 4-5%. Quinoa contains up to 5% saponins in the outer layer of the grain, which cause a bitter taste and have a hemolytic effect. They have to be removed before consumption, either by soaking in alkaline solution or milling (FAO 1989a, Rehm 1989). 'Sweet' quinoa varieties contain less saponins, circa one tenth that of normal varieties (Gee et al. 1993).

1.3.3. Legumes

Legumes belong to the family of *Leguminosae*. The family comprises about 18000 species divided into three subfamilies: *Papilionideae* (or *Faboideae*), *Caesalpinioideae* and *Mimosoideae*, the first being the most important group. The nomenclature of legumes has been revised and the former *Phaseolus* species have been reclassified as *Vigna*. Some species have many common names depending on regions and population group. An alternative term, often used for the edible seeds of leguminous plants is 'pulse' (Aykroyd and Doughty 1982, Deshpande and Damodaran 1990). Legumes are second to cereals as staple foods for humans and animals, the most commonly produced

legumes worldwide are soybean, bean (including *Phaseolus* ssp. and *Vigna* ssp.), pea, chickpea and lentils (Rehm and Espig 1996).

Legume seeds consist of three major components: the seed-coat (testa or hull), the cotyledons which comprise about 80-90% of the total seed weight and the embryo axis. As an example the structure of a soybean seed is shown in Figure 1.4. Legumes can be divided in two main types, those which store energy mostly as fat (e.g. soybean, lupin) and those which store energy mostly in form of starch (e.g. chickpea). The fat content of soybean is about 15-20% while most other legumes have lower fat contents, 1-5%. All legumes have high protein content ranging from 16 to 44%, most of them between 20 and 30%, generally about double the protein content of cereals. This is due to the ability of legumes to bind nitrogen from the air with the help of a symbiotic system with N_2-fixing microorganisms (Franke 1989, Marschner 1995). The sulfur-containing amino acids, methionine and cysteine, as well as tryptophan tend to be rather low, while legumes are generally rich in lysine (Aykroyd and Doughty 1982). Legumes are therefore a good supplement to cereal-based diets to improve protein quality (FAO 1989b, Deshpande and Damodaran 1990). A detailed overview of the chemical composition of legumes is given by Deshpande and Damodaran (1990). Legumes contain a range of antinutritional factors, such as trypsin inhibitors, hemagglutinins, goitrogens, cyanides, saponins, polyphenols, phytic acid and flatulence producing oligosaccharides depending on species and variety. As some of the antinutritional factors can be removed, degraded or inactivated, appropriate preparation of legumes is important.

Figure 1.4. Structure of a soybean seed (Liu 1997)
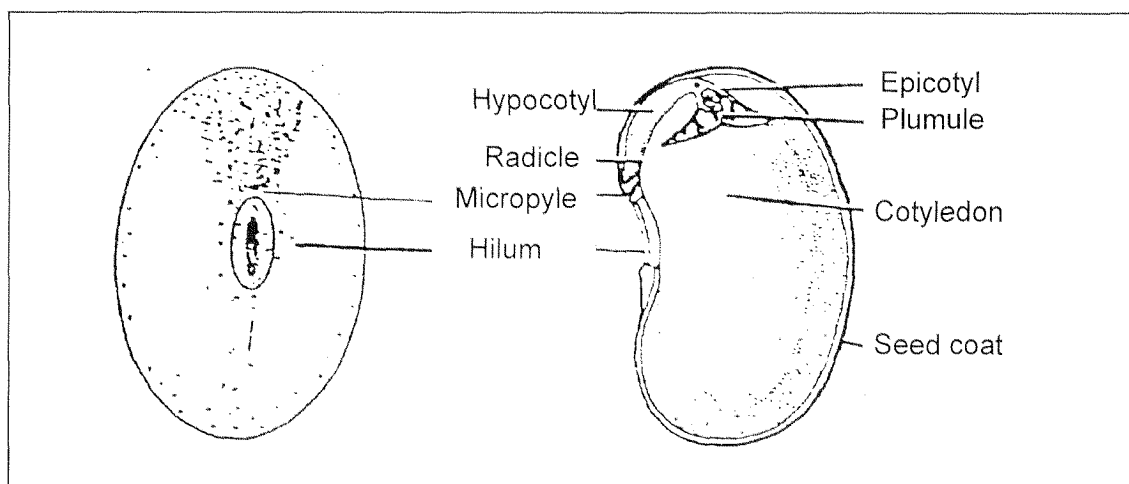
1.3.3.1. Soybean

Soybean (*Glycine max*) was originally domesticated in China and is grown in the USA, Brazil and to a limited extent in Africa and the Near East (Aykroyd and Doughty 1982). In 1995, half of the world's production was cultivated in the USA. In the Western countries, the main use of soybeans is as a raw material for cooking oil (Franke 1989). The residue after oilextraction is used for the production of flour, protein concentrates and isolates or for animal feed. In Eastern countries such as China, Japan, Indonesia, where whole soybeans are an important constituent of the human diet, many traditional food preparation methods are used. Fermentation is of great importance resulting in products such as soy sauce, soy paste (miso) and natto, especially in Japan, and tempeh in Indonesia (Aykroyd and Doughty 1982). Soybeans have high protein content, ranging from 30 to 50% as well as high fat content, ranging from 12 to almost 30% (Liu 1997).

1.3.3.2. Bean

Many common names are used for bean (*Phaseolus vulgaris*), such as dry bean, haricot bean, kidney bean, French bean, snap bean and others. There are many types which can be classified according to shape and color (Aykroyd and Doughty 1982). Beans originate from Mexico and Central America, where they are still important in the diet. Beans are the most often consumed legumes in Mexico, Central and South America and it is also important in Asia and parts of Africa (Aykroyd and Doughty 1982).

Beans are often consumed in form of bean stew or bean paste. The seeds are soaked overnight, cooked, occasionally mixed with other ingredients and often served with maize tortillas or rice (FAO 1989b). A detailed overview of the composition of different varieties and strains of *Phaseolus vulgaris* is given by Sgarbieri (1989), showing a mean protein content of 24% and an average fat content of 1.5%. Protein digestibility of beans is low, due to a globulin fraction of the protein which shows protease inhibition even after cooking (FAO 1989b).

1.3.3.3. Pea

Pea (*Pisum sativum*) comprises two main subgroups, garden pea (*Pisum sativum* var. *sativum*) and the smaller field pea (*Pisum sativum* var. *arvense*). Peas originate from somewhere between India and the Eastern Mediterranean and are grown in temperate climates throughout the world (Aykroyd and Doughty 1982). Garden peas are often consumed as immature seeds (green peas), the leading processed vegetable in the world (canned, frozen or dried). In India, mature seeds are used dehulled as dhal and flour. In the ex-USSR and Europe, mature field peas are used as animal feed (Aykroyd and Doughty 1982).

1.3.3.4. Chickpea

Another common name for chickpea (*Cicer arietinum*) is Bengal gram. Chickpea originates from the East Mediterranean and Near East and is most important in India (Aykroyd and Doughty 1982). In India, seeds are usually consumed dehulled as dhal (a rather liquid dish of cooked legumes) and sometimes pounded to flour which can be used for the preparation of unleavened bread. The seeds may also be soaked, boiled, fermented or sprouted. Chickpea have a slightly higher fat content (about 6%) as compared to other legumes (Aykroyd and Doughty 1982).

1.3.3.5. Lentil

Lentils (*Lens culinaris*) are also called split pea or red dhal. They originate from the Eastern Mediterranean and Asia (Aykroyd and Doughty 1982). The largest producer is India, followed by Turkey, Canada, Syria, Ethiopia and Morocco. In India and Africa the lentils are consumed locally, while most of the lentils produced in Canada and the USA are exported (Muehlbauer et al. 1995). The seeds are usually dehulled and split and used as dhal in soups or stews, but they may also be ground to flour and mixed with cereal flour (Aykroyd and Doughty 1982). Lentils are often used in dishes combined with rice or other cereals, to increase protein quality (Muehlbauer et al. 1995). Residues from threshing, the lentil crop are used as animal feed in India, the Middle East, North Africa and Ethiopia. In Canada, lentils are used as green manure to a limited extent (Muehlbauer et al. 1995).

1.3.3.6. Mungbean

Mungbean (*Vigna radiata, Phaeolus aureus*) is also called green gram or mung dhal (India). The seeds resemble closely *Vigna mungo* or *Phaseouls mungo*, called black gram or urd bean. Both originate from Asia, where they are still important in the diet, especially in India and Pakistan. The beans have successfully been introduced in East and Southern Africa and the USA (Aykroyd and Doughty 1982). Mungbeans have low contents of raffinose and stachyose, thus not causing flatulence (FAO 1989b). Mungbeans are often eaten as whole seeds after soaking and boiling or dehulled and split as dhal. Mungbeans are used to produce sprouts, which are eaten fresh or cooked in many Asian dishes (FAO 1989b).

1.3.3.7. Cowpea

Cowpea (*Vigna unguiculata, Vigna sinensis*) is also called blackeye pea or blackeyed bean or niebe. The main subspecies are ssp. *unguiculata*, the common cowpea, ssp. *cylindrica* often used as forage and ssp. *sesquipedalis* cultivated in Asia (Aykroyd and Doughty 1982). Cowpea probably originated in India, but wild forms were also found in Africa. Cowpeas are widely cultivated in Africa, the tropical regions of Asia and in the USA. Cowpeas are especially important in Africa, as almost 90% of the world's production is cultivated in Nigeria, Niger, Burkina Faso and Uganda (Aykroyd and Doughty 1982). In Africa, cowpeas are used for the preparation of a wide variety of dishes such as, cowpea paste, soups, snacks, flour and weaning foods, mostly after soaking and dehulling. In India the seeds are used whole or dehulled and split as dhal.

1.3.3.8. Lucerne

Lucerne (*Medicago sativa*), also called alfalfa, originates from Western Asia and is now grown in temperate and sub-tropical regions throughout the world (Aykroyd and Doughty 1982). The entire plant is mostly used for animal feeding and green manure (Aykroyd and Doughty 1982). Sprouts of lucerne seeds are used for human consumption. Lucerne seeds have a high protein content of 33-35% and can have a rather high fat content of 4-13% (Kylen and McCready 1975, Hamilton and Vanderstoep 1979).

1.3.3.9. Lupin

The three main species of lupins (*Lupinus* ssp.) are *Lupinus albus*, white or field lupin, *Lupinus luteus*, yellow lupin and *Lupinus augustifolius* blue or narrow-leaved lupin. White lupin is of Mediterranean origin and is still used partly for human consumption in Southern Europe and North Africa. The use of yellow lupin, originating from the Mediterranean, has been limited for human consumption by high alkaloid content. Today alkaloid-free cultivars are the dominant lupins in a number of European countries. Blue lupin originates from Northern Europe and is grown in the USA, Australia and New Zealand for animal feeding (Aykroyd and Doughty 1982).

Lupin seeds have a high fat content, but their use for human nutrition is very limited because the thick seat coat and the high alkaloid content require special processing. Lupin seeds have high protein content, but are very low in methionine (Aykroyd and Doughty 1982, FAO 1989b). Composite flours of lupin and quinoa for weaning food preparation have been proposed, as quinoa protein is rich in methionine, which would lead to a well-balanced amino acid profile (FAO 1989b).

1.3.4. Oilseeds

Oilseeds are characterized by a high fat content in the seeds and are mainly used for production of cooking oil. Botanically, oilseeds are a heterogeneous group and several legumes, such as soybean and peanut, are often classified as oilseeds due to their high fat content (Aufhammer 1998). The most important oilseeds in 1993 were soybean, coconut, cotton seed, rapeseed, peanut, sunflower, olives, sesame and castor bean (Rehm and Espig 1996).

1.3.4.1. Rapeseed

Rapeseed (*Brassica napus*) belongs to the family *Cruciferae* and comprises the two subspecies *Brassica napus oleifera* and *Brassica napus napus*. Rapeseed originates most probably from Southern Europe and is now grown in China, Canada, India and Europe (Franke 1989). Rapeseed is mostly used for oil production, but only partly used for human consumption due to the content of erucic acid and glucosinolates. Special

cultivars, called '00', are almost free of erucic acid and glucosinolates and are mainly used for margarine production (Franke 1989).

1.3.4.2. Sunflowerseed

Sunflower (*Helianthus annuus*) belongs to the family *Compositae*. Sunflower was first used for oilproduction by the American Indians. The main producers are ex-USSR, Argentina, France, China and other European countries (Franke 1989). The sunflower seeds contain 24-28% protein and 36-57% fat (Franke 1989). The oil is mainly used for human consumption and margarine production. The residue of the oilproduction is high in protein and is used for animal feeding (Franke 1989, Rehm and Espig 1996).

1.4. Weaning food

The weaning period represents the gradual transition from human milk or infant formula to the usual family diet. Weaning foods are also called complementary foods or beikost. The quantitative and qualitative nutritional requirements of rapidly growing infants are different from those of older children and adults and therefore foods with high energy and nutrient density are required. For example, the requirements of an infant for energy and protein per kilogram body weight are about three times those of an adult (Akre 1989). Weaning foods are recommended to be introduced to infants after four to six months (WHO 1998). Earlier introduction is not advisable, because of the immature physiological maturation of the digestive tract and as it can make the infant more vulnerable to diarrhoeal and other diseases, including allergies. After six month of age, exclusive breast feeding can not satisfy the energy requirement of the infant (Akre 1989).

1.4.1. Weaning food composition

The type of weaning foods introduced to the infant depends on tradition and availability and thus varies considerably among countries. Many infants receive cereals as the first solid food, in industrialized as well as in developing countries (Ahmad 1987, Anderson and Ziegler 1987, Ballabriga and Schmidt 1987). Gluten should not be introduced

before the age of six months to prevent allergies (European Society for Pediatric Gastroenterology and Nutrition (ESPAGAN) 1982) and therefore infant cereals are often based on rice or maize. As mentioned in the previous chapter, cereals are generally low in the essential amino acids lysine and tryptophan. To improve the protein quality, cereals are combined with other foods. In industrialized countries, cow milk is often added to cereal based weaning foods, either during food preparation or during manufacturing of industrially produced infant cereals. In developing countries, where milk is not part of the usual diet and is not readily available, cereal based weaning foods are often combined with legumes. Legumes are generally low in the essential amino acid methionine, but high in lysine. The combination of cereals and legumes can lead to a well-balanced protein composition.

For commercial manufacture of weaning foods in the European Union, the Commission Directive 96/5/EC (1996) of the European Communities on processed cereal-based foods and baby foods for infants and young children, regulates the essential composition of these products as well as the addition of vitamins, minerals and trace elements. Upper limits for the content of protein and fat (relative to energy content) and lower limits for the protein quality are included in this directive. The Codex Alimentarius for processed cereal-based foods for infants and children defines the ingredients, the lower limit of the protein content and quality, allowed additives, as well as packaging and labeling of the products (FAO/WHO 1994).

1.4.1.1. Viscosity and energy density

Weaning foods are often fed to infants as semi-solid gruels. Viscosity and energy density of weaning foods are important issues to assure appropriate energy intake for the infant. The required energy density of weaning foods depends on the age of the child, the feeding frequency and the amount of human milk consumed. Cereal based weaning foods have high contents of starch, resulting in high viscosity. In developing countries, weaning foods are often based on foods prepared for adults, i.e., thick cereal and/or legume gruels, diluted with water to reduce the viscosity, resulting in low energy and nutrient density. The most efficient way to reduce viscosity, without decreasing energy and nutrient density, was found to be by the addition of the starch degrading

enzyme amylase (WHO 1998). Amylase rich flours ('power flour') can be prepared at the household level by germinating local cereal grains, such as millet, maize or sorghum, which are subsequently dried, milled and added to the weaning foods (Gopaldas and Deshapande 1992). However, the viscosity reduction by addition of amylase is still being discussed, as some studies showed contradictory results (WHO 1998).

1.4.1.2. Protein and fat

Requirements for protein are about three times higher (per kilogram body weight) during the first months of life than during adulthood. The requirements for essential amino acids are also elevated and would therefore be difficult to satisfy with proteins of low quality (Akre 1989). Fats provide essential fatty acids and fat soluble vitamins and are an important source of energy. Dietary fat intake should range from 30-45% of the total dietary intake for children less than two years of age (WHO 1998).

1.4.1.3. Dietary fiber

A maximum level of 5% crude fiber in weaning foods was suggested by the Food and Agricultural Organization (FAO 1985). This would correspond to > 10% dietary fiber, since during sample preparation for crude fiber analysis a large part is removed (Davidsson et al. 1996a). Commercial weaning foods are generally low in dietary fiber, since they are often based on low extraction rate cereal flours. In developing countries, weaning foods often contain whole grain cereals and legumes, thus resulting in high dietary fiber contents. No difference in energy and nutrient intake was found, when healthy formula fed infants received weaning foods with either low or high dietary fiber content (Davidsson et al. 1996a). The optimal dietary fiber content of weaning foods has often been discussed, however further studies are still needed before an optimal level can be defined.

1.4.1.4. Micronutrients

The most problematic micronutrients in weaning foods are iron, zinc, calcium and vitamin A (WHO 1998). Infants use their iron stores present at birth in addition to the

very small amounts of iron provided by human milk for growth and red blood cell synthesis. Therefore the iron stores are decreasing, leading to risk of iron deficiency after the age of six months, if weaning foods do not provide enough iron with adequate bioavailability (Dallmann 1992). While in industrialized countries commercial weaning foods are often fortified with iron, in developing countries the weaning foods with low iron content and/or poor bioavailability represent a major problem. The situation for zinc and calcium is similar. While animal products have high zinc (meat) and high calcium contents (milk and milk products), plant foods contain less zinc and calcium and the bioavailability is poor. Vitamin A requirements are also difficult to meet, as retinol is not present in plant foods and β–carotine is absent or at low level in most cereals and legumes.

1.5. Germination of grains and seeds

Water uptake of dry quiescent grains and seeds causes a rise in general metabolic activity and the formation of a seedling from the embryo. Germination is identified by the protrusion of some part of the embryo from the seed coat; in many seeds the root protrudes first, in some seeds it is the shoot (Mayer and Poljakoff-Mayber 1989). The protrusion is a result of cell division or cell growth.

1.5.1. Factors affecting germination

Several parameters, such as viability and life span of the seeds, and environmental factors i.e., water, temperature, gas atmosphere and light influence germination. Viability is best retained under conditions of storage favoring low metabolic activity of the seeds, such as low temperature, low moisture and high carbon dioxide concentration. Mathematical models to predict viability and life span of seeds under various storage conditions have been developed by Roberts (1960) and were later modified (Mayer and Poljakoff-Mayber 1989). Viability is retained for long periods of time under dry conditions and it was shown that 70-100% of the seeds still germinated after seven years of storage for rye, eight years for barley, nine years for wheat and 11 years for oat (various sources compiled by Mayer and Poljakoff-Mayber 1989). An adequate water supply is the most important factor for germination. The water uptake is

due to imbibition and depends on the seed coat, the seed composition and the availability of water. It is mainly protein which imbibes water. After 24 hours, imbibition was found to be 150% (% of the original seed weight) for wheat and around 140% for corn (Mayer and Poljakoff-Mayber 1989). Optimal germination temperatures vary with different seeds, usually between 20 and 30°C, but optimal temperatures above 30°C have been reported for corn (32-35°C) and rice (30-37°C) (various sources compiled by Mayer and Poljakoff-Mayber 1989). Most seeds germinate in a gas atmosphere of 20% oxygen and 0.03% carbon dioxide, i.e. normal composition of air. Certain seeds show increased germination at higher oxygen concentrations, while most seeds fail to germinate at greatly increased carbon dioxide concentrations. There is little evidence that light is a factor influencing germination, as most seeds germinate equally well in the dark and in the light (Mayer and Poljakoff-Mayber 1989). However, Finney (1983) reported on studies where light increased the ascorbic acid formation during germination up to two fold in certain grains and seeds.

1.5.2. Germination procedure

Several methods have been described for the home preparation of germinated seeds. For more controlled germination of small quantities of seeds, specials screen-lidded jars, called sprouters, sprouting pails or trays have been described (Lorenz 1980) as have untreated cellulose sponges, regulating the moisture content (Fordham et al. 1975). For production of large quantities of sprouts on a commercial scale, rotating jars on a device developed by Miller (1978) are suggested.

1.5.3. Effect of germination on chemical composition

During germination the metabolic rate and enzyme activities increase and changes in composition in various parts of the seed occur. The following compositional changes have been reported: breakdown of certain components, transport from one part of the seed to another and synthesis of new components from breakdown products (Lorenz 1980). The influence of germination on phytic acid has been discussed in chapter 1.1.7.2. and on phytase activity in chapter 1.2.5., while the influence on dry matter, vitamins, especially ascorbic acid will be discussed in this section. Changes in other

components, often discussed, but less relevant in the context of this study are mentioned. A detailed overview of compositional changes is given by Mayer and Poljakoff-Mayber (1989).

1.5.3.1. Dry weight

Germination leads to a loss of dry weight due to oxidation and leaching. A loss of carbon (17%) due to respiration was reported after five to seven days germination using radioactive labeled (^{14}C) wheat (McConnell 1957). Weight loss was found to be higher with increased temperature and duration. In wheat, a loss of dry weight of 5-6% after four days of germination at 20°C was reported (Nielsen et al. 1978). In millet, dry weight loss increased from 4% after one day of germination to 16.5% after seven days (Parvathy Parameswaran and Sadasivam 1994).

1.5.3.2. Minerals and trace elements

Contradictory results have been reported for the changes in mineral and trace element content of grains and seeds during germination. Some authors have described an increase during germination, for example iron and zinc in lentil and mungbean (Kavas and El 1992), while others found no difference in wheat (Lemar and Swanson 1976), or a decrease in millet (Malleshi and Desikachar 1986), in wheat, mungbean and chickpea (Harmuth-Hoene et al. 1987). An increase in total mineral and trace element content is considered to be an apparent but not a true increase, due to the losses of dry matter during germination (Lorenz 1980, Chavan and Kadam 1989). A decrease can be attributed to losses due to leaching during soaking and rinsing (Harmuth-Hoene et al. 1987, Chavan and Kadam 1989). Changes in mineral contents during malting should be interpreted carefully, as minerals can be translocated to the sprouts or rootlets, which are often removed after completed germination (Malleshi and Desikachar 1986).

1.5.3.3. Vitamins

There are several publications, and a review by Finney (1983), regarding the influence of germination on the content of different vitamins, especially ascorbic acid. It has been reported that ascorbic acid increases during germination of legumes (Fordham et al.

1975, Farhangi and Valadon 1981, Harmuth-Hoene and Bognar 1988, Sattar et al. 1989, Kavas and El 1992, Riddoch et al. 1998), cereals (Danisová et al. 1994, Malleshi and Klopfenstein 1998) and pseudocereals (Lintschinger et al. 1997). The biosynthetic pathway of ascorbic acid in plants has not been firmly established, although ascorbic acid seems to be involved in a wide range of important functions during growth, for example antioxidant defense, photosynthesis, regulation of cell division and growth (Smirnoff 1996).

Lemar and Swanson (1976) reported an 1.2 fold increase of thiamin and an 1.4 fold increase of riboflavin content in wheat during germination. An increase in riboflavin during germination was found in lentil (1.5 fold) and mungbean (3.1 fold) (Kavas and El 1992). Miller (1978) reported an increase of carotenoids up to 6 times the initial value in germinated wheat. The results of Miller (1978) were questioned by Lorenz (1980) as the increase might be a result of dry weight loss. An increase in riboflavin (1.8-4.3 fold) and niacin (1.2-1.4 fold), but slightly reduced thiamin (0.6-0.8 fold) content was found in millet and sorghum (Malleshi and Klopfenstein 1998). Harmuth-Hoene et al. (1987) found an increase in riboflavin and pyridoxine in germinated wheat (4.6 and 1.9 fold respectively) and an increase in thiamin and riboflavin content (2.1 and 17 fold respectively) in germinated mungbean.

1.5.3.4. Other components

The influence of germination on carbohydrates (starch, sugars), nitrogenous compounds (total protein, amino acids, nucleic acids, enzymes), lipids (fatty acids) and polyphenols has been investigated. Increase of enzymatic activity of amylase and maltase during germination causes a decrease in starch and a concomitant increase in reducing and non-reducing sugars (Lemar and Swanson 1976, Mayer and Poljakoff-Mayber 1989, Gopaldas and Deshpande 1992). A slight increase in fiber content after germination has been found in millet, but was considered to be only apparent and mainly due to the degradation of starch (Malleshi and Desikachar 1986). No significant changes in fiber content have been detected in wheat (Lemar and Swanson 1976).

Reports on changes in total protein content during germination show either an increase, a decrease or indifference. Total protein content was found to increase significantly during germination in wheat, triticale, barley, rice, rye and oat (Dalby and Tsai 1976, Lemar and Swanson 1976, Miller 1978, Nielsen et al. 1978). For wheat an increase of total protein from 13 to 15% of dry weight after five days of germination was reported (Dalby and Tsai 1976). The increase in protein content has been attributed to loss in dry weight, particularly loss of carbohydrates (Chavan and Kadam 1989). Although the changes in total protein content are contradictory, complex qualitative changes are reported to occur during germination. Proteins are partially hydrolyzed by proteolytic enzymes, resulting in an increase in free amino acids (Nielsen et al. 1978). An increase in lysine and tryptophan was reported for wheat, barley, triticale, rye and oat (Dalby and Tsai 1976) and millet (Malleshi and Desikachar 1986). Nielsen et al. (1978) found an increase in lysine, but not in other essential amino acids in germinated wheat.

The reports on the influence of germination on fat content of seeds are also contradictory. While Lemar and Swanson (1976) found an increase in the lipid content in wheat during germination, which was attributed to a transformation from starch, reduced fat content was observed in sorghum (Nielsen et al. 1978) and millet (Malleshi and Desikachar 1986).

Polyphenols present in cereals like sorghum and millet are known to inhibit several hydrolytic enzymes, such as trypsin, chymotrypsin, amylase and cellulase (Salunkhe et al. 1982), to bind with proteins, making them unavailable and to have detrimental effect on the availability minerals (Chavan et al. 1981, Brune et al. 1989, Brown et al. 1990). Germination has been reported to considerably decrease tannin content in high-tannin sorghum (Chavan et al. 1981, Mukuru et al. 1992) and in peas (Bishnoi et al. 1994).

1.6. Nutritional aspects of phytic acid

In this chapter the nutritional aspects of phytic acid, including intake, absorption, suggested preventive effects and the influence on the bioavailability of other food components will be discussed. The focus is on the most important nutritional aspect of phytic acid, i.e., the influence on mineral and trace element bioavailability.

1.6.1. Phytic acid intake

Cereals and legumes are the major source of phytic acid in omnivorous and vegetarian diets. Ellis et al. (1987) found that vegetarians consumed higher amounts of phytic acid than non-vegetarians and men consumed more phytic acid than women, which was attributed to higher energy intake. Harland and Peterson (1978) estimated the daily phytic acid intake of North American men (weighing 75 kg) to be 750 mg, while phytic acid intake of ovo-lacto-vegetarian Trappist monks ranged from 615 to 5770 mg. Wise et al. (1987) reported a daily phytic acid intake of 500 mg for female and 840 mg for male students and staff in an institution of higher education in Aberdeen, Scotland. In this study breakfast cereals were the most important contributors to phytic acid at breakfast, while bread was more important at lunch and evening meals. Phytic acid intake of rural Indian vegetarians ranged from 1300 mg for women to 2500 mg for men per day (Khokar et al. 1994). Phytic acid intake from bread was estimated to be 107 mg in Switzerland (Blumenthal and Scheffeldt 1983) and 110 mg from bread and 70 mg per day from other foods in Sweden (Torelm and Bruce 1982). Estimated phytic acid intake in different countries was summarized by Plaami (1997).

1.6.2. Absorption of phytic acid

In humans there are to our knowledge no studies on the absorption of phytic acid. However, phytic acid would not be expected to be absorbed from the gastrointestinal tract as such (Weiner and Franco 1986). There are only a few studies in animals and *in vitro* using radioactively labeled phytic acid. (*Myo*-(inositol-2-^{3}H(N))hexaphosphate was administered to rats and the absorption and distribution in various tissues measured (Sakamoto et al. 1993). About 80% of the radioactivity was rapidly absorbed in the upper gastrointestinal tract and distributed in the tissues, mostly in the form of *myo*-inositol and *myo*-inositol monophosphate. The authors proposed that *myo*-inositol hexaphosphate was absorbed as such in the upper intestine and dephosphorylated during its passage through the mucosal cells, although earlier degradation could not be excluded. In a later study, using the same radioactive labeled phytic acid, a rapid uptake in murine and human malignant cells *in vitro* was reported (Vucenik and Shamsuddin 1994). However, there seemed to be a difference in the magnitude and/or speed at which

normal versus malignant cells take up inositol hexaphosphate (Vucenik and Shamsuddin 1994).

1.6.3. Various effects of phytic acid

1.6.3.1. Anticarcinogenic activity

The incidence of colon cancer differs widely among populations and has partly been attributed to dietary habits. Several authors (Graf and Eaton 1985, 1993, Harland and Morris 1995) proposed that diets rich in phytic acid might suppress colon carcinogenesis by the inhibition of free radical generation via chelation of reactive iron by phytic acid. The proposed mechanisms were reported by Plaami (1997).

In rats, oral treatment with phytic acid (2% phytic acid in drinking water) reduced colon cancer, which was induced earlier by exposure to the carcinogen azoxymethane (Shamsuddin and Ullah 1989). It was proposed that the antineoplastic effect was exerted by regulating cell proliferation. Using a different carcinogen (1,2-di-methylhxdrazine) the antineoplastic effect of phytic acid could also be shown in mice (Shamsuddin et al. 1989). Using human erythroleukemia cells, a reduction in cell population and increased differentiation could be observed *in vitro* after treatment with phytic acid (Shamsuddin et al. 1992). The results of these studies suggest that phytic acid reduces the generation of free radicals and thereby prevents the formation of malignant cell and/or decreases the cell proliferation after carcinogenic induction (Shamsuddin 1995). Phytic acid has an anticarcinogenic effect not only in the colon but also on induced mammary carcinoma in rats (Vucenik et al. 1993). This effect was also shown *in vitro* with human mammary cancer cells (Shamsuddin 1996). However, in rats oral administration of phytic (2% phytic acid in the diet) has not shown an anticarcinogenic effect in all tissues in which cancer was initiated (Hirose et al. 1991). Zhou and Erdman (1995) reviewed the anticarcinogenic role of phytic acid and concluded that human studies are indispensable before making any recommendations for intake of phytic acid.

1.6.3.2. Heart disease

It has been proposed that dietary fiber may influence the etiology of heart disease due to its cholesterol lowering effect (Trowell 1976). Klevay (1975) hypothesized that an imbalance of zinc and copper in the diet (high ratio zinc:copper) results in hypercholesterolemia. Therefore, a phytic acid rich diet might reduce serum cholesterol levels by decreasing zinc absorption. It has been demonstrated that dietary phytic acid lowered serum cholesterol in rats (Jariwalla et al. 1990). This effect was accompanied by decreased serum zinc concentration and decreased ratio zinc:copper in the serum, probably by lowering zinc absorption without affecting copper absorption (Turnlund et al. 1984, 1985). Another heart protective mechanism of phytic acid, i.e., by chelating iron and suppressing radical formation, was reported in rats (Rao et al. 1991). However, it should be stressed, that the indications for a preventive role of phytic acid in heart disease are only speculative as they are based only on a few animal and *in vitro* studies and that human studies are still lacking (Zhou and Erdman 1995).

1.6.3.3. Renal stones

Epidemiological studies have shown increased renal stone incidence with diets low in fiber and phytic acid (reviewed by Zhou and Erdman 1995). *In vitro* experiments indicated that inositol diphosphate and inositol triphosphate are effective in preventing the formation of hydroxyapatite crystals (Thomas and Tilden 1972), which can function as nuclei for renal stone formation (Pak 1972, Malek and Boyce 1973). Thus, it was hypothesized that high urinary content of phosphorylated inositol from increased dietary phytic acid intake can inhibit renal stone formation (Modlin 1980).

1.6.4. Influence of phytic acid on the bioavailability of other food components

The interactions of phytic acid with proteins may influence the digestibility and bioavailability of proteins. The effect depends on the character of the phytic acid-protein complex which varies with the protein source, pH and temperature. Contradictory results of *in vivo* studies regarding the digestibility and bioavailability of proteins have been summarized by Lásztity and Lásztity (1990). The influence of phytic

acid-starch interactions on starch digestibility and blood glucose response was reviewed by Thompson (1986, 1988). A lowered starch digestibility and slower increase in blood glucose from phytic acid containing foods was attributed to direct binding of phytic acid to starch, the binding of starch degrading enzymes (amylase) and/or chelation of calcium required for the activity of amylase. Nutritionally most important is the influence of phytic acid on the bioavailability of minerals and trace elements. Some of the numerous studies, *in vitro*, as well as *in vivo* in animals and humans that have been conducted will be discussed, with special focus on iron, zinc and copper bioavailability. The metabolism and requirements of zinc and copper in humans will be reviewed briefly as they are important in the context of the study. Methods to determine the bioavailability of minerals and trace elements in humans will be discussed in the following chapter.

1.6.4.1. Iron

Iron occurs in two forms in foods, as heme and as non-heme iron. Heme iron, which is present as hemoglobin and myoglobin, is absorbed directly as the intact iron porphyrin complex. Heme iron is well absorbed (15-35%) and little influenced by physiological or dietary factors (Monsen et al. 1978). The absorption of non-heme iron varies widely and is influenced by dietary components and iron status of the individual. Iron deficiency anemia (defined as low hemoglobin and two iron status parameters outside the normal range) occurs mostly in infants, children and women of childbearing age, particularly in developing countries, where iron is mostly consumed as non-heme iron.

1.6.4.1.1. Influence of phytic acid and ascorbic acid on iron absorption

Several dietary factors, including phytic acid, polyphenols, calcium, protein, muscle tissue and ascorbic acid influence the absorption non-heme iron either negatively or positively (Hurrell 1997). Many studies, both *in vitro* and *in vivo*, in animals or humans have been performed to investigate the influence of phytic acid on iron absorption, started in 1943 by McCance et al. In this chapter, some human studies on the influence of phytic acid or ascorbic acid on iron absorption will be reviewed. While in studies

with adults radioisotope techniques were used, studies with infants were performed using the stable isotope technique.

Different methods to reduce phytic acid during food preparation had a positive effect on iron absorption. The degradation of phytic acid in wheat bran by endogenous phytase almost completely overcame the inhibiting effect on iron absorption in adults, thus showing that phytic acid is the main inhibitory factor in bran (Hallberg et al. 1987). Fermentation of bread, and malting and soaking of oat, increased the iron absorption in adults (Brune et al. 1992, Larsson et al. 1996). Phytic acid degradation by microbial phytase in soy-protein isolate increased iron absorption in adults fed a liquid formula meals (Hurrell et al. 1992). A marked increase in iron absorption was also observed in infants, when infant formula with phytic acid free soy-protein isolate was fed (Davidsson et al. 1994a).

A dose dependent inhibiting effect of phytic acid on iron absorption in adults was shown by Hallberg et al. (1989a), when phytic acid was added in various amounts (7-890 mg) to phytic acid free wheat rolls. The dose dependent inhibitory effect of phytic acid was also shown in women, fed bread with added maize bran, containing different amounts of phytic acid (35-205 mg) (Siegenberg et al. 1991). To achieve a meaningful increase in iron absorption, phytic acid had to be reduced to 0.03% in soy-protein isolate (corresponding to 10 mg phytic acid per meal) (Hurrell et al. 1992). The molar ratio of phytic acid:iron had to be reduced from 2.2:1 to 0.1:1 to achieve an almost 5 fold increase in iron absorption.

The above mentioned studies evaluated the effect of phytic acid (IP6) or the sum of different inositol phosphates on iron absorption. Sandberg et al. (1999) investigated the influence of IP3, IP4, IP5 and IP6 and mixtures thereof on iron absorption in humans. IP6 and IP5 showed an inhibiting effect, whereas IP3 and IP4 in isolated form had no such effect. However, IP3 and IP4 contributed to the negative effect of IP5 and IP6 in processed foods.

Ascorbic acid has been shown to increase iron absorption and to at least partly overcome the inhibiting effect of phytic acid (Hallberg et al. 1986, 1989a, Siegenberg et al. 1991, Davidsson et al. 1994a, 1998). The mechanism of this effect is suggested to be due to the prevention of the formation of insoluble iron compounds not available for absorption, and due to the reduction of ferric to ferrous iron (Hallberg et al. 1989b). Iron absorption from cereal based weaning foods, measured in women, was significantly improved by the addition of ascorbic acid at molar ratios of ascorbic acid to iron above 0.9:1 (Derman et al. 1980). Addition of 50 mg ascorbic acid to meals based on different cereals led to a 2.7 fold increase in iron absorption in adults (Cook et al. 1997). Addition of 50 mg ascorbic acid to weaning foods based on cereals or legumes improved iron absorption in infants 2 fold (Fairweather-Tait et al. 1995). Iron absorption in children was increased 3 fold by addition of 25 mg ascorbic acid to a chocolate-flavored milk drink. The addition of 50 mg ascorbic acid as compared to 25 mg further increased the iron absorption (Davidsson et al. 1998). No difference on iron absorption in infants was found when they were fed infant cereals with 0.01% or 0.08% phytic acid. Both infant cereals contained ascorbic acid (17 mg per meal), which was proposed to be sufficient to overcome the negative effect of phytic acid (Davidsson et al. 1997).

1.6.4.2. Zinc

1.6.4.2.1. Zinc metabolism

In 1934 zinc was reported to be essential for rats by Todd et al. (cited by Sauberlich et al. 1999). The essentiality of zinc for humans was demonstrated by Prasad et al. (1963), based on observations of zinc deficiency in male adolescents manifested as dwarfism and hypogonadism. Zinc is present in various organs, tissues and fluids of the body; the total body content is between 2 and 3 g (Rimbach et al. 1996). The major part, 80-90% is found in the skeletal muscles and bones. Zinc is part of numerous enzymes such as superoxide dismutase, alcohol dehydrogenase, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) polymerase. Zinc is required in the metabolism of nucleic acids, thus zinc deficiency decreases cell replication resulting in reduced growth. Zinc deficiency is also associated with reduced immune response, loss of appetite, impaired

taste acuity, skin lesions and a variety of disorders of cell metabolism (Rimbach et al. 1996).

Zinc is absorbed in the small intestine via a carrier mediated transport process or at higher concentrations by passive diffusion (Rimbach et al. 1996). The non-absorbed zinc is excreted in feces. Daily endogenous zinc losses of 1.5-3 mg occur mainly via urine, skin and the gastrointestinal tract. Urinary excretion is relatively constant, circa 0.5 mg per day (Sandström 1997). Body zinc homeostasis is maintained by changes in absorption and endogenous excretion. Changes in excretion were proposed to be the rapidly responding mechanism to small daily variations of zinc intake, while changes in absorption are responding more slowly but to larger fluctuations in dietary zinc (Jackson et al. 1984). Infants, fed formulas with low zinc concentrations, showed increased fractional zinc absorption and decreased excretion of endogenous zinc, demonstrating their ability to maintain body zinc homeostasis (Ziegler et al. 1989).

1.6.4.2.2. Zinc requirements

The recommended dietary allowances (RDA) were set at 15 mg/day for adult men, 12 mg/day for adult women, 10 mg/day for children and 5 mg/day for infants (National Research Council, Food and Nutrition Board 1989). These values are based on estimated zinc losses in adult men of circa 2.5 mg/day and assuming a fractional zinc absorption of 20%. The German recommendations are similar with additional differentiation for children of different age groups (Deutsche Gesellschaft für Ernährung 1992).

1.6.4.2.3. Zinc status

Only a small part of the body zinc, circa 0.1%, circulates in serum, bound to albumin or α-macroglobulin. Plasma or serum zinc concentrations are commonly used to evaluate zinc status, although plasma or serum zinc are influenced by various factors, such as infections, stress, injury, exercise, pregnancy and other physiological factors (Rimbach et al. 1996, Brown 1998, Sauberlich 1999). Other methods to assess zinc status have been proposed to be more sensitive and specific, but the alternative techniques are

tedious (Fairwaether-Tait 1988, Sauberlich 1999). Therefore, plasma or serum zinc concentrations are most commonly used to evaluate zinc status. Plasma or serum zinc concentrations of 10.7 μmol/L and more were considered as acceptable levels by Sauberlich (1999). Smith et al. (1985) summarized extensive data on normal plasma and serum zinc levels. For the determination of plasma or serum zinc concentrations, special care has to be taken during sample collection and analysis. To avoid falsely high zinc concentrations, trace element free collecting tubes are required, the sampling procedure has to be standardized and blood has to be drawn from fasting subjects (Sauberlich 1999).

1.6.4.2.4. Influence of phytic acid on zinc absorption

Good sources of dietary zinc are oysters, meat and meat products and whole grain cereals (Heseker 1998). The bioavailability of zinc is generally higher from animal foods than from plant products. Fractional zinc absorption in humans can vary considerably from circa 5 to 50%, depending on the composition of the diet (Fairweather-Tait and Hurrell 1996). For the establishment of zinc requirements, mean fractional absorption of 20% was assumed (National Research Council, Food and Nutrition Board 1989). However higher fractional absorption of (circa 30%) could be expected if zinc intake mainly derives from animal products (Rimbach et al. 1996). Fractional zinc absorption varied from 8 to 38% from composite meals and from 8 to 27% from different cereals (Sandström et al. 1980, 1987). Zinc absorption was found to be < 15% from meals with high contents of phytic acid (Rossander et al. 1992).

Based on data from animal studies, molar ratios of phytic acid:zinc (Oberleas and Harland 1981) and molar ratios of calcium x phytic acid:zinc (Davies et al. 1985, Fordyce et al. 1987) have been proposed to estimate the inhibiting effect of phytic acid on zinc absorption. Ellis et al. (1987) suggested ratios phytic acid:zinc of > 10:1 and calcium x phytic acid:zinc > 200:1 [mM] to negatively influence zinc absorption. Most omnivorous diets were found to be below these critical ratios, while vegetarian diets were above. The molar ratios have been used to estimate zinc bioavailability from different food sources in humans (Fitzgerald et al. 1993, Paul et al. 1998). However,

Wise (1995) doubted the predictive use of these ratios, derived from animal studies, for humans.

Different methods have been used to measure zinc absorption in humans, such as the metabolic balance technique, radioisotope or stable isotope techniques and plasma response curve technique. The methods were summarized and discussed by Fairweather-Tait (1988) and Sandström (1997). The results of several human studies investigating the influence of phytic acid on zinc absorption are reported chronologically in this review.

Turnlund et al. (1984) reported that phytic acid reduced zinc absorption by 50% from a liquid formula diet, using stable isotope technique. Lönnderdal et al. (1984) found a negative effect of phytic acid in soy formula on zinc absorption in adults, reducing zinc absorption from 31% to 16%, using radioisotope technique. The negative effect of calcium on zinc absorption from phytic acid rich-diets reported in animals (Davies et al. 1985, Fordyce et al. 1987) was not shown in humans (Lönnerdal et al. 1984). Nävert and Sandström (1985) demonstrated doubled zinc absorption in adults from leavened bread with phytic acid partly degraded, using a radioisotope technique. Sandström et al. (1987) found increased zinc absorption from different cereals when the phytic acid content was reduced, using a radioisotope technique. Morris et al. (1988) showed that the dephytinization of wheat bran increased zinc absorption in a balance study with adult men. Kivistö et al. (1989) reported an increase in zinc absorption from 6 to 18% from extruded bran products after phytic acid reduction, using radioisotope technique. Zinc absorption in adult men, determined by stable isotope technique, from white bread sandwiches (low phytic acid content) was slightly higher than from wholemeal bread sandwich (high phytic acid content), but not significantly different (Fairweather-Tait et al. 1992). The higher zinc intake in the wholemeal bread more than compensated the slightly lower fractional absorption. A significant increase in zinc absorption was found in adults when dephytinized soy formula was compared with formula containing its native phytic acid level (Davidsson et al. unpublished observations). In infants, using stable isotope techniques, the inhibitory effect of phytic acid could not be demonstrated after feeding two wheat/soy cereals, presumably because the phytic acid content of the

low phytic acid product was still relatively high (Davidsson et al. 1996a). Zinc absorption determined by radioisotope technique from oat products was improved from 12 to 18% when the oat was malted to reduce phytic acid content (Larsson et al. 1996). Hunt et al. (1998) reported increased zinc absorption in women consuming an omnivorous diet as compared to a lactoovo-vegetarian diet containing more phytic acid, determined by radioisotope techniques.

While the above mentioned studies evaluated the effect of IP6 or the sum of different inositol phosphates (IP3 to IP6) on zinc absorption, Sandström and Sandberg (1992) demonstrated that only IP6 and IP5 had an inhibitory effect on zinc absorption in humans, whereas the lower inositol-phosphates (IP3, IP4) had less or no effect, when added to white wheat bread.

1.6.4.3. Copper

1.6.4.3.1. Copper metabolism

The essentiality of copper in nutrition was first established in animals and later in humans (Cartwright and Wintrobe 1964). Copper deficiency is rare in humans and mostly associated with genetic disorders (Menkes disease) and malnourished children (Danks 1988, Olivares and Uauy 1996). Copper is part of many enzymes, including the antioxidant enzymes superoxide dismutase, ceruloplasmin and cytochrome oxidase. Copper is present in lysyl oxidase, required for the formation and function of connective tissue and tyrosinase, responsible for the synthesis of melanin (Danks 1988, Shaw 1992, Olivares and Uauy 1996). Some of the features of copper deficiency, including anemia, neutropenia, bone abnormalities, depigmentation of hair and skin and neurological disturbances can be explained by the function of these enzymes (Olivares and Uauy 1996, Sauberlich 1999).

Copper is absorbed in the duodenum and jejunum. Copper uptake probably occurs by non-energy-dependent diffusion into the mucosal cells, from where it is transferred across the basolateral membrane by a rate-limiting energy-dependent mechanism. In plasma, copper is bound to albumin and transcuprein and rapidly transported to the

liver, where it is incorporated into ceruloplasmin. Daily copper losses (circa 0.5-1 mg) are mainly excreted via bile, and hence in the feces with only a minor fraction excreted in the urine (Linder and Hazegh-Azam 1996). Copper homeostasis is regulated by changes in absorption and endogenous excretion. When young men were fed low copper diets, fractional absorption increased and endogenous excretion decreased. However, this regulatory mechanism was not sufficient to maintain copper status at the lowest intake of dietary copper (Turnlund et al. 1998).

1.6.4.3.2. Copper requirements

Dietary copper intakes of approximately 1.6 mg/day are required to replace endogenous losses in adult men, assuming 36% absorption (National Research Council, Food and Nutrition Board 1989). However, many diets provide less than 1.6 mg/day copper without resulting in symptoms of copper deficiency. This suggests either an adaptation to low copper diets or incorrect estimation of copper intake. Due to these uncertainties, no recommended dietary allowances (RDA) have been set for copper, but an estimated safe and adequate daily dietary intake (ESADDI) of 1.5-3 mg/day was established. The German recommendations are similar (Deutsche Gesellschaft für Ernährung 1992). Recommendations for copper intake of pre-term and full-term infants vary from 10 to 120 µg/100 kcal (Shaw 1992).

1.6.4.3.3. Copper status

Ceruloplasmin binds the main part of the copper present in plasma. Copper status is often evaluated by measurements of plasma or serum copper concentrations, ceruloplasmin or erythrocyte Cu/Zn superoxide dismutase levels (Sauberlich 1999). Plasma or serum copper concentrations are influenced by a number of factors, such as infection, estrogen, age, sex, and pregnancy, which must be considered in the evaluation of copper status. Women have generally higher plasma or serum copper levels than men, which may be further increased by the use of oral contraceptives (Johnson et al. 1992). Serum copper concentrations > 0.75 µg/ml (> 11.8 µmol/L) were considered acceptable by Sauberlich (1999), while Cartwright and Wintrobe (1964) previously defined hypocupremia as serum copper concentrations of 12.6 µmol/L or less.

1.6.4.3.4. Influence of dietary factors on copper absorption

The richest dietary sources of copper are liver, shellfish, nuts, seeds and cereals. Water can also contribute to copper intake, depending on the pH of the water and the plumbing system (Pennington and Calloway 1973). Copper absorption in humans is reported to range from 25 to 70% (Fairweather-Tait and Hurrell 1996). The average absorption from diets typically consumed in developed countries is 30 to 40% (Wapnir 1998). Different dietary factors reported to influence copper absorption were summarized by Turnlund (1988), Lönnerdal (1996), Fairweather-Tait (1997) and Wapnir (1998). In this review, the influence of phytic acid and some other dietary factors on copper absorption will be discussed.

Results from *in vitro* studies suggest phytic acid to be an inhibitor of copper absorption, as the binding is even stronger for copper than for zinc (Vohra et al. 1965, Persson et al. 1998). In animals and humans however there are only a few studies reported and they have given contradictory results. In rats the addition of phytic acid to a copper deficient diet increased the copper bioavailability. These results were explained as due to the binding of phytic acid to other dietary components, such as zinc, resulting in competition with copper at the site of intestinal absorption (Lee et al. 1988). A study in rats and monkeys, using radioisotope technique, evaluated the influence of dephytinizing soy formula on copper absorption (Lönnerdal et al. 1999). There was no difference in copper absorption in monkeys and rats from regular and dephytinized (80% reduction of phytic acid) soy formula. A balance study showed negative effect of phytic acid in wheat bran on copper absorption in adult men, as compared to dephytinized wheat bran (Morris et al. 1988). However, the balance was positive, even during the period of consumption of wheat bran with phytic acid. Turnlund et al. (1985) used stable isotope technique to study copper absorption in humans and found no influence of phytic acid, which was added to the diet, although zinc absorption from the same diet was markedly reduced (Turnlund et al. 1984).

Results from animal studies suggested that high dietary levels of ascorbic acid can have negative effects on copper absorption (van Campen and Gross 1968). However, this effect was not confirmed in humans. High intakes of ascorbic acid (600 mg per day)

resulted in a reduction of serum ceruloplasmin, but copper absorption was not decreased in young men (Jacob et al. 1987). Reduced copper absorption was reported in the presence of excessive zinc intake (150 mg/day) (Turnlund 1988). However, zinc intake of 16.5 mg/day as compared to 5.5 mg/day did not influence copper absorption in young men (Turnlund et al. 1988). The influence of zinc on copper metabolism in human intestinal cell models (Caco-2) has recently been investigated (Reeves et al. 1998). High concentrations of zinc were shown to increase copper uptake and to reduce copper transport in the cells. Copper deficiency results in microcytic anemia similar to iron deficiency anemia, but not responsive to iron supplementation. The interactions between iron and copper do not appear to be related to absorption, but to impaired utilization of one trace element in the absence of the other (Turnlund 1988). Influence of other factors on copper absorption, such as carbohydrates, in particular fructose, amino acids, molybdenum, cadmium and drugs have been discussed by Turnlund (1988), Lönnerdal (1996) and Wapnir (1998).

1.6.4.4. Other minerals and trace elements

Phytic acid may interact with calcium and cause a reduction in calcium absorption. Animal and human studies have been reviewed by Reddy et al. (1989), Torre et al. (1991) and Zhou and Erdman (1995). Reports on nutritional rickets and osteomalacia in populations consuming unleavened bread had been attributed to the high levels of phytic acid in the diet. However, other authors suggested that vitamin D deficiency or interference of phytic acid with vitamin D metabolism could be responsible for these deficiency symptoms (Reddy et al. 1989). Morris and Ellis (1985) suggested that diets with a molar ratio of phytic acid:calcium > 0.2:1 might cause calcium deficiency in humans. Calcium absorption from soybeans with high phytic acid content was significantly lower (31%) than from soybeans with low phytic acid content (41%) when measured in women, using intrinsic labeling with ^{45}Ca (Heaney et al. 1991). Weaver et al. (1991) demonstrated that leavening of products based on wheat flour improved calcium absorption. However, wheat products, except bran cereal, did not have a negative effect on calcium absorption, which was even better than from milk with a similar calcium content.

Phytic acid was also suggested as a possible cause of magnesium deficiency symptoms in rats (Roberts and Yudkin 1960). Later studies showed that the addition of phytic acid to the diets resulted in a decreased magnesium absorption in rats (Brink et al. 1991, Pallauf et al. 1998, Rimbach and Pallauf 1999). Human studies on magnesium bioavailability have been mainly performed using chemical balance techniques and the individual effect of enhancers or inhibitors on magnesium absorption is often not clear (Fairweather-Tait and Hurrell 1996).

Manganese absorption in adults was increased 2.3 fold, when dephytinized soy formula was fed as compared to regular soy formula (Davidsson et al. 1995). In the same study manganese absorption was not influenced by increased ascorbic acid content in the test meal. In an earlier study (Davidsson et al. 1991) the addition of phytic acid to cow milk based formula showed no effect on manganese absorption in adults.

1.6.5. Mineral and trace element absorption studies in humans

Bioavailability is that proportion of a mineral or trace element in a food, meal or diet that is absorbed and utilized by the body for normal functions. It is not possible to measure the utilization of minerals and trace elements directly, except for iron which is incorporated in hemoglobin. Therefore, bioavailability is assessed by different techniques such as absorption or retention measurements (Mellon and Sandström 1996). Absorption is defined as the difference between intake and fecal excretion of an element. When no allowance is made for the endogenous losses of the element in feces and urine, the term 'apparent absorption' is used. Recent reviews on the methods for the assessment of the bioavailability of iron were given by Hurrell (1997) and Wienk et al. (1999) and for zinc by Sandström (1997). In this chapter, the use of stable isotopes to label native minerals and trace elements in foods in order to follow their absorption and metabolism in humans will be discussed.

1.6.5.1. Stable isotopes versus radioisotopes

The advantages of the use of stable isotopes in human absorption studies, as compared to radioisotopes, are the safety and the possibility to simultaneously study several

isotopes of one or different elements (Sandström 1997). Stable isotope techniques are especially useful for investigating mineral and trace element absorption and metabolism in infants (Davidsson 1994), pregnant or lactating women. Another benefit of stable isotopes is that samples can be stored indefinitely before analysis (Sandström 1996a). A major drawback of stable isotopes is the higher amount that has to be added to test meals, as compared to radioisotopes, which are used as 'tracers'. A significant amount of a stable isotope label has to be added to a meal before an increase above the natural abundance of the isotope can be determined in biological samples. The stable isotope label added influences the total content of the mineral or trace element investigated in a meal. In addition, the high costs of enriched stable isotopes and the access to instrumentation used for analysis are limiting factors. Using radioisotopes, retention can be measured directly by whole-body measurements. Absorption and retention of stable isotopes are most often measured indirectly, by analysis of excreted isotopes in feces or urine.

Among the nutritionally important minerals and trace elements, the absorption and metabolism of calcium, copper, iron, magnesium, selenium and zinc can be studied with stable isotope techniques as these elements have several stable isotopes in nature (Hurrell et al. 1993). The mass and natural abundance of stable isotopes used in nutrition research were reviewed by Sandström (1996b). The dosage of the stable isotopes depends on the expected absorption or retention, the distribution between different body tissues and fluids, the natural abundance and the required degree of isotope enrichment in biological samples (Sandström et al. 1993).

1.6.5.2. Extrinsic versus intrinsic labeling

The food from which the mineral or trace element absorption will be measured, can be labeled intrinsically (biological incorporation of the isotope) or extrinsically (mixing the isotope with the food). The use of extrinsically labeled foods is simpler and less expensive than intrinsically labeled foods (Fairweather-Tait and Fox 1996). However, extrinsically labeled foods can only be used on the condition that the isotope exchanges completely with the native mineral or trace element and that they are absorbed and metabolized identically (Hurrell et al. 1993). Good agreement in absorption from

intrinsically and extrinsically added tracers were found for zinc, calcium, magnesium and copper (Fairweather-Tait and Fox 1996). Care should be taken to ensure that the extrinsic label has enough time to equilibrate fully with the native mineral or trace element before administration (Fairweather-Tait and Fox 1996).

1.6.5.3. Study techniques

Different study techniques can be used with stable isotopes, including fecal monitoring, tissue retention, simultaneous oral and intravenous isotope administration and plasma appearance (Sandström et al. 1993). Fecal monitoring is most widely used and the principle is the same as for the conventional balance technique. Fecal monitoring allows the measure of apparent absorption, but not true absorption, as it does not account for the re-excretion of the absorbed mineral or trace element. It has been applied to several minerals and trace elements with relatively high fractional absorption. The design of zinc, copper and iron absorption studies using fecal monitoring was summarized by Sandström et al. (1993). The length of the fecal collection period depends on the age of the subjects and on the diet. In adults, different duration of fecal collections have been used (Sandström et al. 1993), while in weanling infants 72 hour periods were found to be sufficiently long (Davidsson et al. 1996b). A non-absorbable fecal marker is usually administered to determine the beginning and the end of the collection period (Hurrell et al. 1993). The major drawbacks of fecal monitoring studies are the cumbersome fecal collection and the errors associated with incomplete collections and homogenization of the samples.

Tissue retention technique can be used only if the mineral or trace element is incorporated to a high extent in a specific tissue or body compartment, as for example iron. Simultaneous oral and intravenous isotope administration has been used to measure calcium (reviewed by Sandström et al. 1993), but has also been proposed for zinc, magnesium and selenium absorption. The advantage of this technique as it is used for calcium is that true absorption can be measured and only spot urine samples are required. The use of stable isotopes in plasma appearance techniques requires lower doses than with unlabeled minerals or trace elements, as the degree of isotope

enrichment can be measured in the plasma as compared to the increase in plasma concentration (van Dokkum et al. 1996).

1.6.5.4. Stable isotope quantification

Measurements of stable isotopes are based on changes in isotopic ratios determined by neutron activation analysis or mass spectrometry (Sandstöm et al. 1993). The neutron activation technique has been mostly used for total element analysis, but also to quantify isotopic enrichment in early studies. Different mass spectrometry methods are currently used, such as thermal ionization mass spectrometry (TIMS), fast atom bombardment mass spectrometry (FABMS), inductively coupled plasma mass spectrometry (ICPMS), electron impact mass spectrometry (EIMS) and gas chromatography-mass spectrometry (GC-MS). Each method has its advantages and disadvantages, but best analytical precision and accuracy can be achieved by TIMS. For reviews see Turnlund (1987, 1989), Janghorbani and Ting (1990) and Turnlund and Keyes (1990).

1.6.5.5. Rare earth elements in fecal monitoring studies

The main source of errors in fecal monitoring studies are incomplete fecal collections. Rare earth elements can be used to verify the completeness of the fecal collection in these studies. Rare earth elements are a group of 15 elements (lanthanons), which are non-toxic, occur only in very low concentrations in food (< 10 ppb) and the absorption after oral administration is very low, less than 0.05% (Arvela 1979). It has been shown that the rare earth elements, samarium, ytterbium and dysprosium have similar excretory patterns to iron in humans (Fairweather-Tait et al. 1997). Dysprosium also showed a similar excretory pattern to zinc and magnesium but not to copper (Schuette et al. 1993).

2. Screening of grains and seeds for phytase activity and the effect of soaking and germination

2.1. Introduction

Grains and seeds of cereals, pseudocereals, legumes and oilseeds usually contain high amounts of phytic acid (Reddy et al. 1989). Phytic acid strongly binds to minerals and trace elements, such as iron, zinc, copper, calcium and can thereby reduce their bioavailability. A high bioavailability of minerals and trace elements is especially important in infant nutrition and therefore weaning foods based on cereals and legumes should be low in phytic acid.

Phytic acid can be degraded by the enzyme phytase, which naturally occurs in grains and seeds. The aim of this screening study was to compare the activity of naturally occurring phytase in grains and seeds of cereals, pseudocereals, legumes and oilseeds under standardized conditions. Those grains and seeds with the highest phytase activities were further studied so as to define the optimum conditions for phytic acid degradation with a view to producing phytic acid free weaning foods.

Soaking and germination of grains and seeds have been reported to increase enzyme activities and to be among the most efficient of the food processing methods to reduce phytic acid (Tabekhia and Luh 1980, Ologhobo and Fetuga 1984a, Beal and Mehta 1985, Laboure et al. 1993). Germination of grains and seeds is also known to increase the content of certain vitamins, such as ascorbic acid (Finney 1983, Harmuth-Hoene and Bognar 1988, Riddoch et al. 1998). Ascorbic acid has an enhancing effect on iron absorption (Hallberg et al. 1986, 1989a, Davidsson et al. 1994a, 1998). The addition of ascorbic acid containing germinated grains or seeds to weaning foods could increase iron absorption and partly overcome the negative effect of phytic acid. The influence of soaking and germination on the phytase activity and the phytic acid content of cereals, pseudocereals, legumes and oilseeds was investigated, as was the effect of germination on the ascorbic acid level.

2.2. Materials and methods

All chemicals and reagents were of analytical grade (Fluka Chemie AG, Buchs, Switzerland; Merck, Darmstadt, Germany) if not mentioned otherwise and water was purified with ion exchange and reverse osmosis (18MΩ) (RD 2000, Renggli, Rotkreuz, Switzerland; Nanopure Cartridge System, Skan AG, Basel, Switzerland).

2.2.1. Grains and seeds

The common and botanical name of all cereals, pseudocereals, legumes and oilseeds used in the study are listed in Table 2.1. Most of the grains and seeds were purchased from commercial seed suppliers (Fenaco, Winterthur, Switzerland; UFA, Bussingy, Switzerland; Delley Samen und Pflanzen, Delley, Switzerland), some from supermarkets and health stores in Zurich, Switzerland or local markets in the Ivory Coast. All grains and seeds were of whole grain quality, not dehusked nor dehulled, and without any thermal or chemical treatment. The grains and seeds were purchased between spring 1997 and autumn 1998 and stored at $4 \pm 2°C$.

Table 2.1. Grains and seeds

	Common name	Botanical name, variety
Cereals	Barley	*Hordeum vulgare*
	Maize	*Zea mays* (variety not specified)
	Millet	*Pennisetum typhoides*
	Oat	*Avena sativa*
	Rice	*Oryza sativa*
	Rye	*Secale cereale*
	Sorghum	*Sorghum sudanensis*
	Sweet corn	*Zea mays saccharata*
	Triticale	*Triticosecale*
	Wheat	*Triticum aestivum*
Pseudocereals	Amaranth	*Amaranthaceae* (variety not specified)
	Buckwheat	*Fagopyrum esculentum*
	Quinoa	*Chenopodium quinoa*
Legumes	Blackeyed bean	*Vigna unguiculata*
	Chickpea	*Cicer arietinum*
	Cowpea	*Vigna niebe*
	Dwarf bean	*Phaseolus vulgaris nana*
	Lentil	*Lens culinaris*
	Lucerne or alfalfa	*Medicago sativa*
	Lupin	*Lupinus albus*
	Mungbean	*Phaseolus aureus or Vigna radiata*
	Pea	*Pisum sativum* (variety not specified)
	Soybean	*Glycine max*
	White bean	*Phaseolus vulgaris*
Oilseeds	Rapeseed	*Brassica napus oleifera*
	Sunflowerseed	*Helianthus annuus*

2.2.2. Treatment of grains and seeds

2.2.2.1. Soaking

Circa 100 g grains or seeds were rinsed with water and soaked in 500 ml water in covered beakers for 16 hours in the dark at 25°C in an incubator (WTB Binder, Tuttlingen, Germany). The water was poured off and the soaked samples were stored frozen (-25°C).

2.2.2.2. Germination

The grains or seeds were rinsed with water and germinated for 24, 48 and 72 hours using a household germination system with 3 trays per unit (BioSnacky, Biokosma GmbH, Konstanz, Germany). Depending on the size of the grains or seeds, 20 to 30 g were germinated per tray for each time period. The germination system was kept in the dark at 25°C in an incubator (WTB Binder) and the samples were rinsed with water twice daily. The germinated grains or seeds were frozen (-25°C) immediately after germination. The weight of the grains or seeds after 0, 24, 48 and 72 hours of germination was recorded.

Grains or seeds for ascorbic acid determination were soaked (see above) prior to germination. After 72 hours of germination, the samples were homogenized with 10% *meta*-phosphoric acid and stored frozen (-25°C).

2.2.2.3. Cooking

Circa 100 g germinated mungbean were cooked in 1 L boiling water in a covered beaker at > 95°C for 1, 5, 10 and 20 minutes, drained, cooled in water (circa 20°C) and dried for 15 minutes at room temperature. Samples were prepared in duplicate and the weight of the seeds before and after cooking was recorded.

2.2.2.4. Freeze-drying

The frozen grains or seeds (soaked or germinated) were spread in a thin layer on glass dishes and freeze-dried (Freeze-dryer Lyolab B, LSL Secfroid SA, Aclens-Lausanne, Switzerland). The weight of the samples before and after freeze-drying was recorded.

2.2.2.5. Milling

All grains or seeds (untreated, soaked or germinated) were frozen in liquid nitrogen and milled with a laboratory mill (0.5 mm mesh, centrifugal mill, Retsch ZM1, Retsch GmbH, Haan, Germany). The milled samples were stored frozen (-25°C).

Wheat was milled additionally with a pilot plant mill (Bühler MLU 202, Bühler AG, Uzwil, Switzerland) and three of the different milling fractions obtained were further analyzed.

2.2.3. Analytical methods

2.2.3.1. Dry matter

Circa 1 g of milled (untreated or freeze-dried) or circa 3 g of fresh germinated grains or seeds were weighed in pre-dried weighing flasks and the dry matter (dm) was determined gravimetrically after drying for 24 hours at 105°C (oven KVTS 11/22, Salvis AG, Reussbühl-Luzern, Switzerland) and cooling in a desiccator. Analyses were repeated if the coefficient of variation based on triplicate determination was > 0.2%.

2.2.3.2. Total ash

Circa 5 g milled sample were weighed in pre-dried crucibles and ashed overnight at 630°C. Total ash content was determined gravimetrically after cooling in a desiccator. Analyses were repeated if the difference between duplicate determination was > 2 mg/100 g. The results were expressed in g/100 g dm.

2.2.3.3. Phytase activity

The determination of phytase activity was based on the measurement of liberated inorganic phosphate (P_i) from phytic acid in a certain time interval. The technique is a modification of the method of Peers (1953), based on direct incubation of the milled grains and seeds without purification. The liberated P_i was quantified with a colorimetric method (van Veldhoven and Mannaerts 1987) adapted to microtiterplates.

2.2.3.3.1. Phytase assay

Circa 1 g milled sample was weighed into Erlenmeyer flasks and dissolved in 20 ml buffer containing 5 mg/ml IP6 (phytic acid dodecasodium salt, P-8810, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as substrate. For the determination of phytase activity at pH 5.0, 0.2 M acetate buffer (sodium acetate/acetic acid), for pH 8.0, 0.2 M Tris buffer (tris(hydroxymethyl)-aminomethane/hydrochloric acid) preheated to 45°C were used. The Erlenmeyer flasks were covered and the sample solution stirred (600 rpm, Variomag multipoint HP15, H+P Labortechnik GmbH, Oberschleissheim, Germany) for 1 hour in an incubator (WTB Binder) set at 45°C. Aliquots of 0.5 ml were taken at the start of the experiment and every 20 minutes and the reaction terminated by adding 0.5 ml 15% trichloroacetic acid. The samples were centrifuged at 14000 rpm (centrifuge 5415C, Eppendorf, Hamburg, Germany) during 10 minutes and the P_i in the supernatant was quantified as described below. Together with each series of samples a blank was analyzed.

2.2.3.3.2. Internal control material

Whole grain wheat was analyzed together with each series of samples and was used as an internal control material to monitor reproducibility.

2.2.3.3.3. Optimization of conditions

Phytase activity of wheat, rye and buckwheat was measured under various pH and temperature conditions. For all measurements, 0.2 M acetate buffer was adjusted to the required pH. The liberation of P_i was measured during 30 minutes and aliquots were

taken at the start of the experiment and every 10 minutes, if not mentioned otherwise. All other conditions were as described in the phytase assay.

The phytase activity of rye was measured at 35, 40, 45, 48, 50, 55, 60 and 63°C at pH 5.5. At 55°C, the phytase activity was measured at pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.25. The phytase activity of wheat was measured at 10, 25, 35, 45, 50, 55, 60, 65 and 70°C at pH 5.0. At 60°C, the phytase activity was measured at pH 4.0, 4.5, 5.0, 5.15, 5.5 and 6.0. Additional measurements over two hours with time intervals of 20 minutes were performed at 60 and 65°C and pH 5.15. The phytase activity of buckwheat was measured at 25, 35, 45, 55 and 65°C at pH 5.0. At 55°C, the phytase activity was measured at pH 4.0, 4.5, 5.0, 5.5 and 6.0.

2.2.3.3.4. Determination of inorganic phosphate

For phosphate quantification, a standard solution was prepared by dissolving potassium dihydrogenphosphate anhydrous (suprapur) in water. Sulfuric acid, 150 µl 0.5 M, was added to each well of a microtiterplate (96 well polystyrene microtiterplate, Dynatech No. M29A, Dynatech Laboratories, Billingshurst, West Sussex, UK). Sample or standard solution (150 µl) were added to one column of wells and the samples diluted by transferring each time 150 µl to the following column (resulting in 12 dilutions per sample). Ammonium molybdate solution (30 µl, 1.75%) was added to each well, mixed gently on a shaker (Titramax 100, Heidolph, Kelheim, Germany) and, after 10 minutes, 30 µl of 0.035% malachite green in 0.35% polyvinyl alcohol were added to each well. The microtiterplates were gently shaken and after 45 minutes the absorbance was measured at 610 nm with a microtiterplate reader (Microtech MRX 1.2, Dynatech Laboratories, Billingshurst, West Sussex, UK). On each microtiterplate, a blank and a standard was included and diluted as described for the sample solution. For each sample, the dilution with an absorbance value between 0.4 to 0.8 (in the middle of the linear range) was chosen, corrected for the value of the blank and the phosphate content calculated according to the absorbance value of the standard.

2.2.3.3.5. Calculation of phytase activity

Phytase activity was calculated by linear regression of the determined liberated P_i during one hour and expressed in phytase units (PU) per g dm of grains or seeds; 1 PU equivalent to the enzymatic activity which liberates 1 μmol P_i from phytic acid per minute. Analyses were repeated if the coefficients of variation based on triplicate analysis was > 15%.

2.2.3.4. Inositol phosphates

The procedure included extraction of inositol phosphates from the milled grains and seeds, separation from the crude extract by anion exchange chromatography, and ion-pair C18 reverse phase HPLC with refractive index detection. The technique is based on the methods of Sandberg and Ahderinne (1986) and Sandberg et al. (1989) with modifications.

2.2.3.4.1. Extraction and purification

Circa 0.5-1.0 g milled sample were weighed into a centrifugation tube, 20 ml 0.5 M hydrochloric acid added and the extraction performed during three hours under vigorous agitation (Unimax 2010, Heidolph). After centrifugation at 3500 rpm (Omnifuge 2.0 RS, Heraeus GmbH, Hanau, Germany) for 10 minutes, 15 ml of the supernatant were transferred to a glass tube (circa 50 ml) and evaporated overnight under a stream of air in a water bath set at 40°C. Plastic columns (Poly-Prep, Bio-Rad Laboratories, Hercules, California, USA) with polyethylene frits (20 μm pore size, Sorbent AB, Frölunda, Sweden) were filled with circa 2 ml anion exchange resin (AG 1-X8, 200-400 mesh, chloride form, Bio-Rad Laboratories) and preconditioned with 0.025 M hydrochloric acid. The residue after evaporation was dissolved in 15 ml 0.025 M hydrochloric acid and loaded on the anion exchange column. The column was washed with 15 ml 0.025 M hydrochloric acid and the inositol phosphates eluted with 5 times 4 ml 2 M hydrochloric acid. The sample was evaporated to complete dryness under a stream of air in a water bath set at 40°C during circa 24 hours. The residue was dissolved in 2 ml water, using an ultrasonic bath (5210E-MT, Branson Ultrasonics Corporation, Danbury, Connecticut, USA) and diluted with water according to the

expected inositol phosphate content. The sample solution was filtered through a 0.45 μm filter (Macherey-Nagel AG, Oensingen, Switzerland) before HPLC analysis.

2.2.3.4.2. HPLC

The analysis was performed with an HPLC system (L-6200A Intelligent Pump, D-6000 Interface, AS-4000 Intelligent Autosampler, Merck Hitachi, Hitachi Ltd., Tokyo, Japan) with a C18 Chromasil PEEK (polyetheretherketone) analytical column (pore size 5 μm, 150 mm x 2 mm, GROM Analytik + HPLC GmbH, Herrenberg-Kayh, Germany) with a refraction index detector (Differential Refractometer RI-71, Merck Hitachi, Hitachi Ltd.). The injector was made of titanium and capillaries were made of PEEK. The flow rate was 0.4 ml/min and the column temperature was set at 40°C. The mobile phase was prepared freshly for each analysis by mixing 600 ml methanol, 480 ml 0.05 M formic acid, 17.4 ml tetrabutylammonium hydroxide solution (~40%) and 1 ml 0.1% phytic acid in water. pH was adjusted to 4.3 with 50% sulfuric acid and the mobile phase filtered (0.45 μm, Alltech Ass. Inc. Deerfield, Illinois, USA) under vacuum and degassed with helium. Sample or standard solution 20 μl were injected for HPLC analysis.

2.2.3.4.3. Standards

Standard solutions in the concentration range 0.07 to 0.7 mg/ml IP6 were prepared by dissolving phytic acid dodecasodium salt (P-8810, Sigma-Aldrich Chemie GmbH) in water. The IP6 content of phytic acid dodecasodium salt was calculated for each lot, based on the certificate of analysis provided by the supplier. To achieve a linear standard curve, several injections of a standard containing 3.3 mg/ml IP6 were made before starting the analysis in order to saturate the system. The standard solutions were prepared freshly and analyzed with each batch of samples to quantify the IP6 content. For IP5, IP4 and IP3, which have a different detector response, correction factors were used according to A.-S. Sandberg and colleagues (Chalmers University of Technology, Göteborg, Sweden, personal communication). Correction factors were 1.1 for IP5, 1.5 for IP4 and 2.0 for IP3. The correction factor for IP3 had been verified on the above

mentioned HPLC system using IP3 in form of Na$_5$H(IP3) (Perstorp Pharma, Perstorp, Sweden).

2.2.3.4.4. Retention times

The retention times of the different inositol phosphates were determined by analyzing a mixture of inositol phosphates, prepared by an acid hydrolysis of IP6. Circa 1 g IP6 (phytic acid dodecasodium salt, P-8810, Sigma-Aldrich Chemie GmbH) was dissolved in 100 ml 0.5 M hydrochloric acid and boiled for 15 hours at 150°C (Heating block, Liebisch, Bielefeld, Germany). The acid was evaporated (Rotavapor RE 111, Büchi Labortechnik AG, Flawil, Switzerland) and the residue dissolved in 100 ml water. The sample was filtered (0.45 µm, Alltech Ass. Inc.) and after appropriate dilution analyzed by HPLC.

2.2.3.4.5. Calculation

Samples were prepared in duplicate and injected twice for HPLC analysis. The mean peak area of the duplicate HPLC analysis was used to calculate the inositol phosphate content of the samples with the standard curve for IP6 and the correction factors for IP5, IP4 and IP3. Results were given as the sum of IP6 and IP5, expressed in g/100 g dm. Analyses were repeated if the difference of duplicate samples relative to the mean value, was > 10%.

2.2.3.4.6. Detection and determination limit

For the determination of the detection limit, a low concentration of the standard solution (0.03 mg/ml IP6) was analyzed ten times. The detection limit corresponds to the IP6 concentration of three times the standard deviation of the peaks area. The detection limit was expressed in mg/ml. The determination limit corresponds to the IP6 concentration of six times the standard deviation of the peak area. It was expressed in g/100 g sample under consideration of the sample preparation.

2.2.3.4.7. Internal control material

Wheat bran (American Association of Cereal Chemists, St. Paul, Minnesota, USA: Certified Hard Red Wheat Bran, Lot 195, 1995) was analyzed together with each series of samples and was used as an internal control material to monitor reproducibility.

2.2.3.5. Ascorbic acid

The method used included stabilization of ascorbic acid in the grains or seeds, extraction, reduction of dehydroascorbic acid and C18 reverse phase HPLC with UV detection. The technique is based on a protocol of an AOAC (Association of Official Analytical Chemists) collaborative study (AOAC 1994).

2.2.3.5.1. Stabilization of ascorbic acid

After milling or completed germination the samples were homogenized with 10% *meta*-phosphoric acid (Polytron PT 6000, Kinematica AG, Littau/Luzern, Switzerland) for 5 minutes at 8000 rpm and stored frozen (-25°C).

2.2.3.5.2. Extraction and reduction

Samples of homogenate corresponding to 10 g grains or seeds were weighed in 100 ml volumetric flasks and 4 ml 10% *meta*-phosphoric acid plus 40 ml 50°C water were added. The flasks were shaken for five minutes, the solution cooled to room temperature and water added up to the final volume. Circa 30 ml of the extract were centrifuged at 3500 rpm for 10 minutes (Omnifuge 2.0 RS, Heraeus GmbH). Ten ml of the supernatant were transferred to a 30 ml polypropylene container and pH was adjusted to 5.0-5.2 with 10 M sodium hydroxide. Dithiotreitol 10 mg was added, carefully mixed, and the covered sample left in the dark for one hour. The sample solution was transferred to a 20 ml volumetric flask and 2% *meta*-phosphoric acid was added to the final volume. The solution was filtered through a 0.45 µm filter (Macherey-Nagel AG) before HPLC analysis. Sample preparation was performed in triplicate.

2.2.3.5.3. HPLC

Analysis was performed with an HPLC system (L-6200A Intelligent Pump, D-6000 Interface, AS-4000 Intelligent Autosampler, Merck Hitachi, Hitachi Ltd.) with a analytical column (C18, Hypersil ODS, pore size 3 μm, 200 mm x 4.6 mm, GROM Analytik + HPLC GmbH) and a pre-column (C18, Hypersil ODS, pore size 3 μm, 20 mm x 4.6 mm, GROM Analytik + HPLC GmbH) with an UV detector (L-4500 Diode Array Detector, Merck Hitachi, Hitachi Ltd.). The mobile phase consisted of 0.5% potassium dihydrogenphosphate with the pH adjusted to 2.5 with 85% *ortho*-phosphoric acid. The mobile phase was filtered (0.45 μm, Alltech Ass. Inc.) under vacuum and degassed with helium. The flow rate was 0.8 ml/min and the column temperature was set at 25°C. Sample or standard solution 20 μl were injected in duplicate for HPLC analysis.

2.2.3.5.4. Standard

Standard solutions containing 1 to 10 mg/l L(+)-ascorbic acid in 2% *meta*-phosphoric acid were prepared freshly and analyzed with each batch of samples to quantify the ascorbic acid content.

2.2.3.5.5. Calculation

The ascorbic acid content was calculated with the mean peak area of duplicate HPLC analysis and the standard curve and expressed in mg/100 g dm and in mg/100 g fresh weight.

2.2.3.5.6. Detection and determination limit

For the determination of the detection limit, a low concentration of the standard solution (0.5 μg/ml) was analyzed ten times. The detection limit corresponds to the ascorbic acid concentration of three times the standard deviation of the peaks area. It was expressed in μg/ml. The determination limit corresponds to the ascorbic acid concentration of six times the standard deviation of the peak area. It was expressed in mg/100 g sample under consideration of the sample preparation.

2.2.4. Statistical analysis

Paired t-test was used to evaluate statistically significant differences between phytase activity and inositol phosphate content before and after soaking or germination of grains and seeds.

2.3. Results and Discussion

2.3.1. Development of the phytase assay

A phytase assay for the screening of grains and seeds for phytase activity was developed in order to obtain maximal phytase activities. The incubation of the milled grains and seeds with or without previous extraction was investigated in barley, rye and wheat in collaboration with Dr. R. Greiner, The Federal Research Center for Nutrition, Karlsruhe, Germany). The impact of freezing and freeze-drying of grains and seeds, as well as the influence of the following parameters on the phytase activity was investigated: substrate concentration, buffer, pH stability, temperature and duration of the phytase assay. The development of the method is discussed in this section, while the final protocol of the phytase assay was described in the materials and methods section (chapter 2.2.3.3.1.)

2.3.1.1. Sample preparation

While in most phytase assays, the enzyme is isolated from the grains and seeds by extraction or dialysis, in this study, the milled grains and seeds were incubated directly. A separation step generally leads to lower values, as phytase can partly be associated with the cell walls and/or as phytase can be degraded during extraction by protease activity. When phytase was extracted from barley, rye and wheat only 35-50% of the phytase activity found by direct incubation was determined (unpublished results in collaboration with Dr. R. Greiner, The Federal Research Center for Nutrition, Karlsruhe, Germany). Therefore, direct incubation of the milled grains and seeds was chosen for this study.

2.3.1.2. Freezing and freeze-drying of grains and seeds

The grains or seeds were frozen in liquid nitrogen before milling, since the milling process would have heated the grains or seed and this might have influenced phytase activity. No changes in phytase activity due to the freezing or due to storage at −25°C were observed as tested with wheat and maize. No change in phytase activity in milled wheat, stored at −25°C, was observed by occasional analysis over two years. Freeze-drying of the soaked and germinated grains or seeds was necessary in order to measure the phytase activity under standardized conditions. No influence of freeze-drying on phytase activity was observed.

2.3.1.3. Substrate concentration

Substrate concentration has a strong influence on the velocity of enzymatic reactions (Copeland 1996). The substrate concentration of 5 mg/ml (7.6 mM) IP6 led to a maximum reaction velocity, when tested for wheat and maize. This substrate concentration did not include the phytic acid present in the grains or seeds. Therefore, the final substrate concentrations varied from 5.25 mg/ml IP6 for grains or seeds containing 0.5% phytic acid to 5.5 mg/ml IP6 for grains and seeds containing 1% phytic acid. Substrate inhibition was not observed up to concentrations of 10 mg/ml, while other authors found inhibitions at lower concentrations (Lolas and Markakis 1977, Eskin and Johnson 1987, Konietzny et al. 1995a, Greiner et al. 1997). The reason for the contradictory results is not known.

2.3.1.4. Buffers and pH-stability

The pH value has an important influence on the enzyme activity. The tertiary structure of enzymes is sensitive to pH changes and denaturation occurs at very low and high pH values. While the enzyme conformation can be maintained over a relatively broad range; circa 4-5 units; the velocity of the enzymatic reaction varies considerably with pH (Copeland 1996). The pH optima of phytase from cereals and legumes is around 5 (Table 1.3., literature review) and for certain legumes around 8 (Scott 1991). The phytase activity was therefore measured at pH 5.0 for all grains and seeds and additionally at pH 8.0 for legumes. For the measurement at pH 5.0, acetate buffer

0.2 M, at pH 8.0 Tris buffer 0.2 M was chosen. These buffers, typically used in phytase assays, were appropriate for the pH chosen (acetate buffer pK_a 4.75 at 25°C; Tris buffer pK_a 8.3 at 25°C) and the pH remained stable (maximum shift 0.1 pH units) during the measurement, as tested with wheat and maize at pH 5.0 and pea and soybean at pH 8.0.

2.3.1.5. Temperature

The optimal temperature for phytase from most cereals and legumes is between 50 and 60°C, although lower optima (35 to 48°C) have been reported (Table 1.3., literature review). Generally, the rate of chemical reactions increases with temperature and enzymatic reactions are no exception. However, enzymes undergo thermal inactivation at elevated temperature and therefore a relatively low temperature (45°C) was chosen.

2.3.1.6. Duration

The phytase activity of all untreated grains or seeds was measured during two hours, with samples taken every 20 minutes. For all samples, P_i liberation was at a constant rate during the first hour, for some samples the reaction slowed down slightly during the second hour. The phytase activity calculated over the first hour, in relation to the phytase activity over two hours, resulted in a mean ratio of 1.1. Higher ratios were not related to higher values of phytase activity and therefore it could not be attributed to a decrease in substrate concentration. In the final protocol, phytase activity measurements were terminated after one hour and all results were calculated as enzyme activity during one hour.

2.3.1.7. Internal control material and blank

Whole grain wheat was analyzed together with each series of samples and was used as an internal control material to monitor reproducibility. The mean phytase activity of wheat (measured at the screening conditions of pH 5.0 and 45°C) of 45 independent determinations performed over nine months was 3.078 ± 0.207 PU/g dm resulting in a coefficient of variation of 7%. A blank (substrate in buffer) analyzed together with each series of samples, demonstrated that the substrate was not degraded by any chemical or thermal reaction.

2.3.1.8. Determination of inorganic phosphate

The different buffers, acetate and Tris buffer, used in the phytase assay had no influence on the determination of liberated P_i, measured by a very sensitive colorimetric method reported by van Veldhoven and Mannaerts (1987). The values obtained were comparable to those determined with a modification of the method of Heinonen and Lahti (1981) when the same samples (barley, wheat, rye) were analyzed (unpublished results in collaboration with Dr. R. Greiner, The Federal Research Center for Nutrition, Karlsruhe, Germany).

2.3.1.9. Phytase unit

The most common definition of phytase activity was used; one phytase unit (PU) being the activity that liberates one μmol P_i from phytic acid per minute under specified conditions.

2.3.1.10. Phytase and phosphatase activity

Phytase is a special type of phosphatase, able to liberate P_i from phytic acid and lower inositol phosphates. The phytase assay developed did not allow the differentiation between phytase and unspecific phosphatase activity. It is not possible to selectively inhibit phytase, as thermal or chemical treatments also inhibit other enzymes, such as phosphatase. The liberation of P_i from other phosphate esters could not be measured as all grains and seeds investigated contained native phytic acid. We assumed however, that the measured P_i was mainly liberated from phytic acid. Our assumption is based on the following argument: 1 g cereal or legume containing 1% phytic acid (80% of the total phosphorus being bound in phytic acid) would contain circa 20 μmol of phosphate bound to phosphate esters other than phytic acid. Their degradation would take circa three minutes by the phytase activity measured in rye (7 PU/g dm) or circa one hour by the phytase activity measured in soy (0.3 PU/g dm). As P_i was liberated at a constant rate over two hours for most samples, it can be assumed that P_i was mainly liberated from the excess of phytic acid and not from other phosphate esters which would have quickly become limiting. The total phosphatase activity, measured by the liberation of P_i, was therefore called 'phytase activity'.

2.3.2. Phytase activity

2.3.2.1. Screening of grains and seeds for phytase activity

2.3.2.1.1. Selection of grains and seeds

The most important cereals, legumes and oilseeds were included in the screening study as well as less frequently consumed pseudocereals, cereals and legumes. The botanical classification, origin, uses and preparations and the composition of these grains and seeds had been discussed in the literature review (chapter 1.3).

2.3.2.1.2. Phytase activity of cereals, pseudocereals, legumes and oilseeds at pH 5.0

The phytase activity ($\pm$ standard deviation) of all grains and seeds is shown in Figure 2.1. Phytase activity of cereals varied over a wide range, from 0.1 PU/g dm for sorghum to 6.9 PU/g dm for rye. The pseudocereals showed relatively high values, 0.6 PU/g dm for quinoa, 1.3 PU/g dm for amaranth and 2.9 PU/g dm for buckwheat. All legumes had rather low phytase activity, 0.2 to 0.4 PU/g dm, with the exception of lucerne (0.8 PU/g dm). Phytase activity of sunflowerseed and rapeseed were low, 0.1 PU/g dm and 0.3 PU/g dm respectively. The highest phytase activity was found in rye, triticale, wheat and buckwheat.

The phytase activity found in this screening study is difficult to compare with earlier published data. There are no standardized conditions for the determination and many authors express the results in relative values, comparing different conditions of the phytase assay or different grains or seeds. Some results are discussed, converted to the phytase units (μmol P_i per minute) as used in this study, although it was not always obvious if the results were expressed in liberated phosphate or phosphorus. Singh and Sedeh (1979) measured phytase activity in crude extracts of different triticale varieties and found values between 2.4 to 4.1 PU/g dm at 45°C. Peers (1953) found circa 0.6 PU/g dm in whole grain wheat at 55°C. Most et al. (1993) found 4.6 PU/g for rye, 1.1 for wheat, 1.3 for barley, 0.1 for oat and 0.2 for beans and peas at 37°C. Bartnik and Szafranska (1987) found 0.6 PU/g dm for wheat, 2.0 for rye, 0.3 for barley and 0.03 for oat at 55°C.

None of these authors investigated phytase activity of a wide range of grains or seeds under standardized conditions. The results of this screening study are thus very useful as they allow the comparison of absolute values of phytase activity of a wide range of grains and seeds, including many that had not been investigated before.

From a practical consideration, the phytase activity in rye (7 PU/g dm) is high enough, that the phytic acid content in rye (circa 1%) could theoretically be degraded to *myo*-inositol in about 15 minutes by incubating the whole grain flour at pH 5, 45°C as slurry. Alternatively, the addition of 10% rye to a cereal/legume mixture containing 1% phytic acid (assuming no phytase activity in the mixture) would be sufficient to degrade all phytic acid to *myo*-inositol in about two hours.

Figure 2.1. Phytase activity of cereals, pseudocereals, legumes and oilseeds at pH 5.0
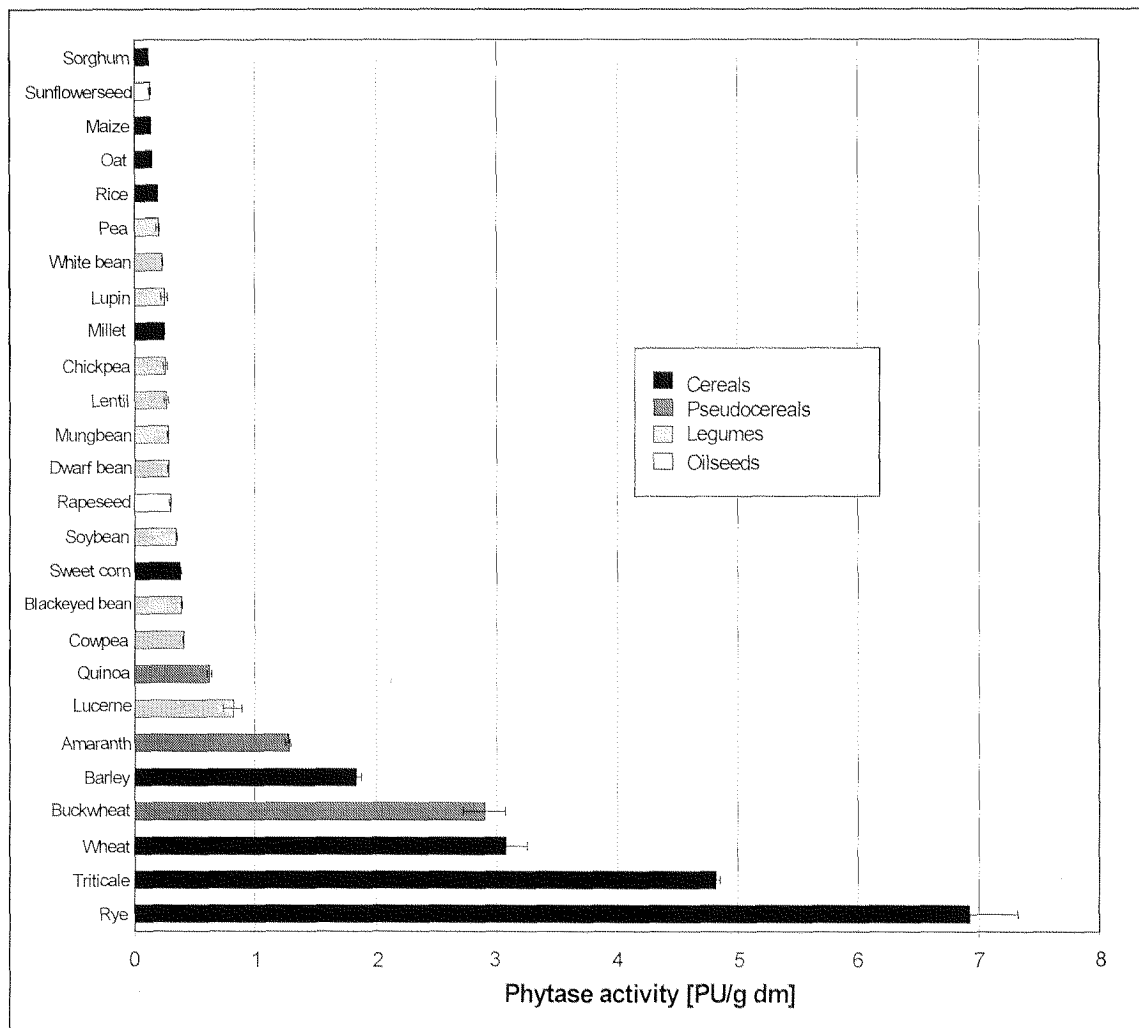
2.3.2.1.3. Phytase activity of legumes at pH 8.0

Figure 2.2. shows the phytase activity (± standard deviation) of legumes measured at pH 5.0 and pH 8.0. For all legumes tested, the values at pH 5.0 were higher than at pH 8.0 except for lupin, which showed a 2.4 times higher value at pH 8.0. No phytase activity could be detected in lentils at pII 8.0. These results show that the screening for phytase activity at pH 5.0 did not underestimate the activity of the legumes, as compared to the cereals due to the pH.

These results are in contradiction to other studies reporting pH optima for legumes between 7 and 8. Mandal et al. (1972) found an optimum pH at 7.5 for phytase from germinated mungbean and Gustafsson and Sandberg (1995) an optimum pH at 7 for phytase from brown beans. Scott (1991) extracted an alkaline phytase with an optimum pH at 8 from seeds of different varieties of beans, pea and lucerne and in six of nine bean varieties, phytase activity at pH 8 was higher than at pH 5, while pea and alfalfa showed a higher activity at pH 5. The data for the beans could not be confirmed in this study, but it is not likely that phytase activity would be lost due to the extraction procedure as proposed by Scott (1991), as direct incubation was used.

Figure 2.2. Phytase activity of legumes at pH 5.0 and 8.0
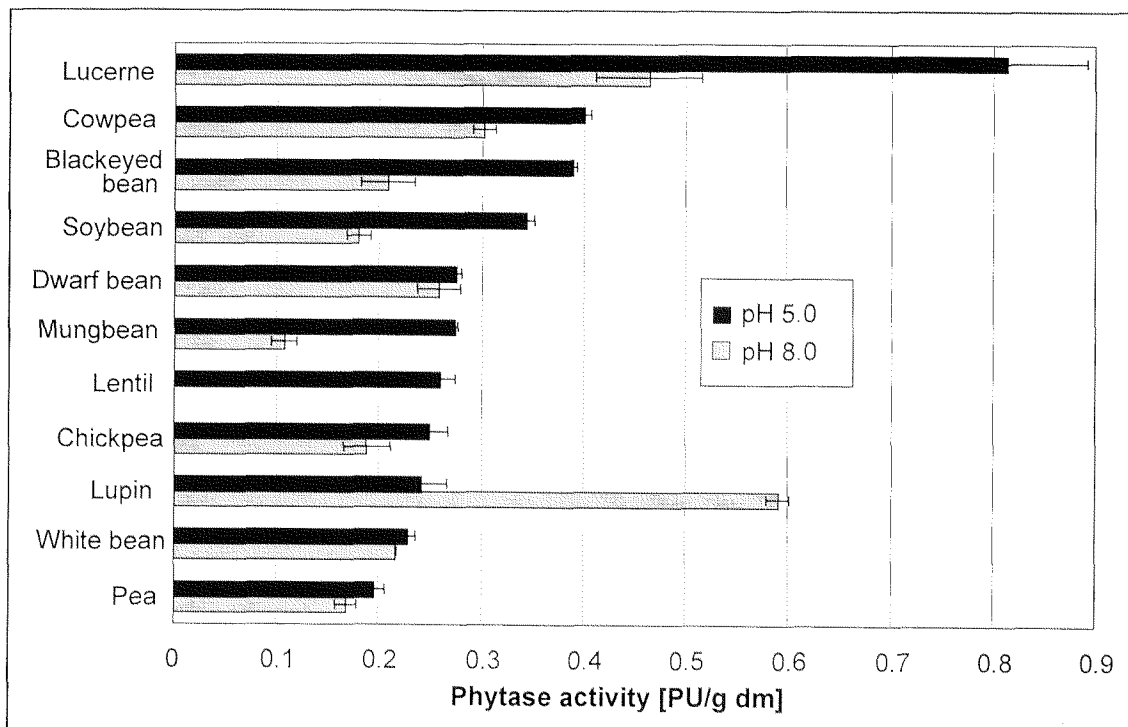
2.3.2.1.4. Influence of variety of the grains or seeds on phytase activity

Different varieties of maize, millet, sorghum and soybean were screened for phytase activity and within species showed comparable phytase activities. Two different millet varieties showed similar phytase activity, 0.18 and 0.24 PU/g dm. Four varieties of sorghum, purchased in the Ivory Coast and from a commercial seed supplier in Switzerland, had phytase activity from 0.11 to 0.17 PU/g dm. The phytase activity of three soybean varieties was comparable, ranging from 0.34 to 0.40 PU/g dm at pH 5.0 and from 0.18 to 0.20 PU/g dm at pH 8.0. As phytase activity did not vary considerably between varieties, only one sample of millet, sorghum and soybean was chosen for further investigations (soaking and germination). Phytase activity was found to vary in maize (0.13 PU/g dm) and sweet corn (0.38 PU/g dm), although they both belong to the same botanical family. Therefore, further investigations were conducted with both varieties.

2.3.2.2. Optimizing conditions for phytase from rye, wheat and buckwheat

Rye, wheat and buckwheat were chosen for further investigations as they showed high phytase activity in the screening study. Rye had the highest phytase activity, wheat is the most important cereal worldwide and buckwheat is a gluten-free source of phytase. Although optimum conditions for phytase activity in rye (Greiner et al. 1997) and wheat (Peers 1953) had been described earlier, the experiments were performed to investigate the effect of the optimization on absolute values. No information was available previously about optimum conditions for buckwheat phytase. For the determination of pH optima, acetate buffer with pH ranging from 4.0 to 6.25 was used. Although pH 4.0 and 6.25 were at the limit of the buffering capacity, pH remained stable during the phytase assay.

2.3.2.2.1. Rye

The influence of pH and temperature on phytase activity in rye is shown in Figure 2.3. and Figure 2.4. The optimum pH of 5.5 for rye phytase described by Greiner et al. (1997) was confirmed, whereas the temperature optimum was found to be slightly higher, 55°C, as compared to 48°C (Greiner et al. 1997). The high phytase activity of

almost 7 PU/g dm (screening conditions) could be increased 1.9 fold (13 PU/g dm) by optimizing the conditions.

Figure 2.3. Phytase activity of rye at different pH (temperature 55°C)

Figure 2.4. Phytase activity of rye at different temperatures (pH 5.5)
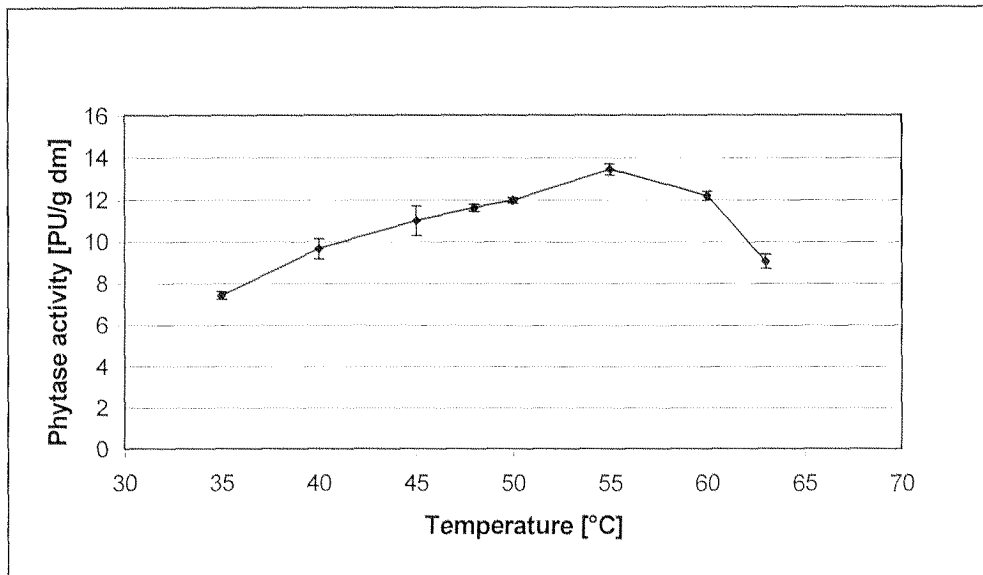
2.3.2.2.2. Wheat

The influence of pH and temperature on phytase activity in wheat is shown in Figure 2.5. and Figure 2.6. The pH optimum of 5.15 described by Peers (1953) for wheat phytase was confirmed. Very little variation was detected between pH 5.0 and 5.5. The

temperature optimum was found to be slightly higher, 65°C, as compared to 55°C (Peers 1953). At 60°C, the liberation of P_i was at a constant rate during one hour and slowed down slightly afterwards. The total amount of liberated P_i was higher after one hour at 65°C as compared to 60°C, but the reaction slowed down after only 20 minutes. Thermal denaturation of the enzyme started at 60°C and therefore, temperatures below 60°C should be used, when the phytic acid degradation requires more than one hour of incubation time. By optimizing the conditions for wheat phytase, to pH 5.15 and 60°C, the phytase activity was increased 1.6 fold (from 3.1 to 4.9 PU/g dm).

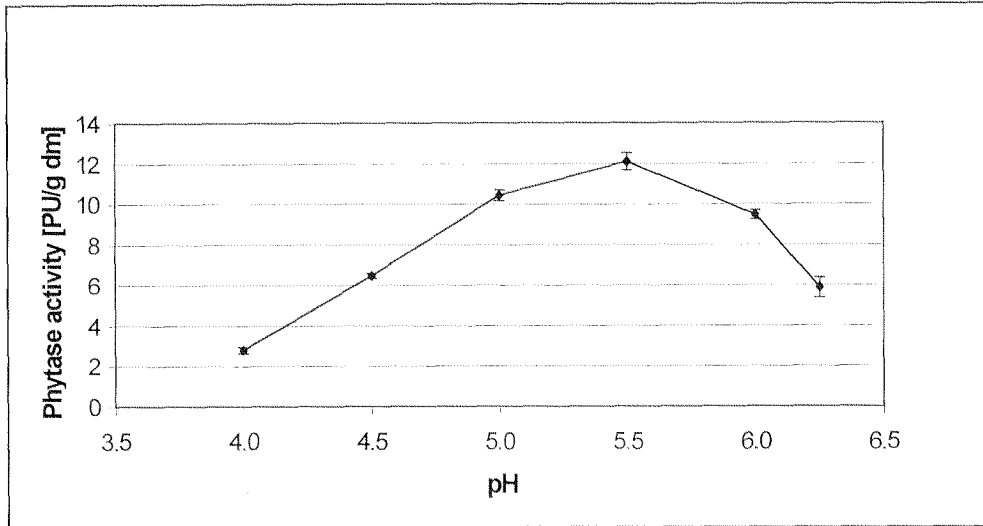Figure 2.5. Phytase activity of wheat at different pH (temperature 60°C)
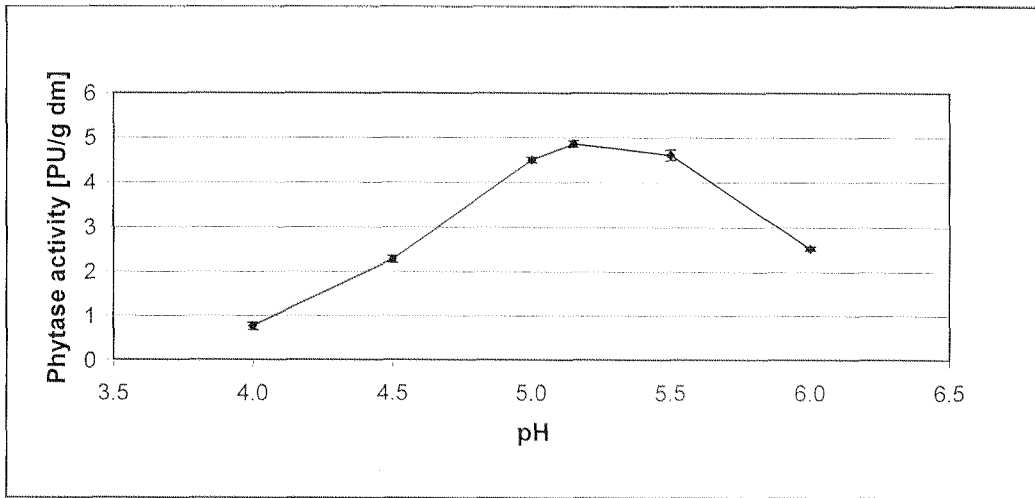
Figure 2.6. Phytase activity of wheat at different temperatures (pH 5.0)
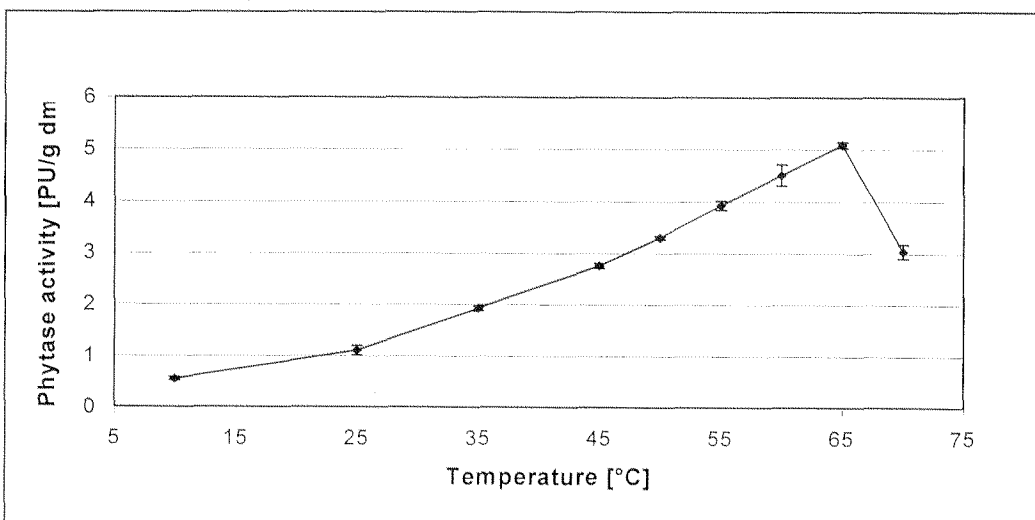
2.3.2.2.3. Buckwheat

The influence of pH and temperature on phytase activity in buckwheat is shown in Figure 2.7. and Figure 2.8. The pH optimum of 5.0 and the temperature optimum of 55°C of buckwheat phytase are in the same range as for other cereals. The phytase activity could hardly be improved, as the pH was already at the optimum under the screening conditions and as the curve of the temperature dependence was rather flat.

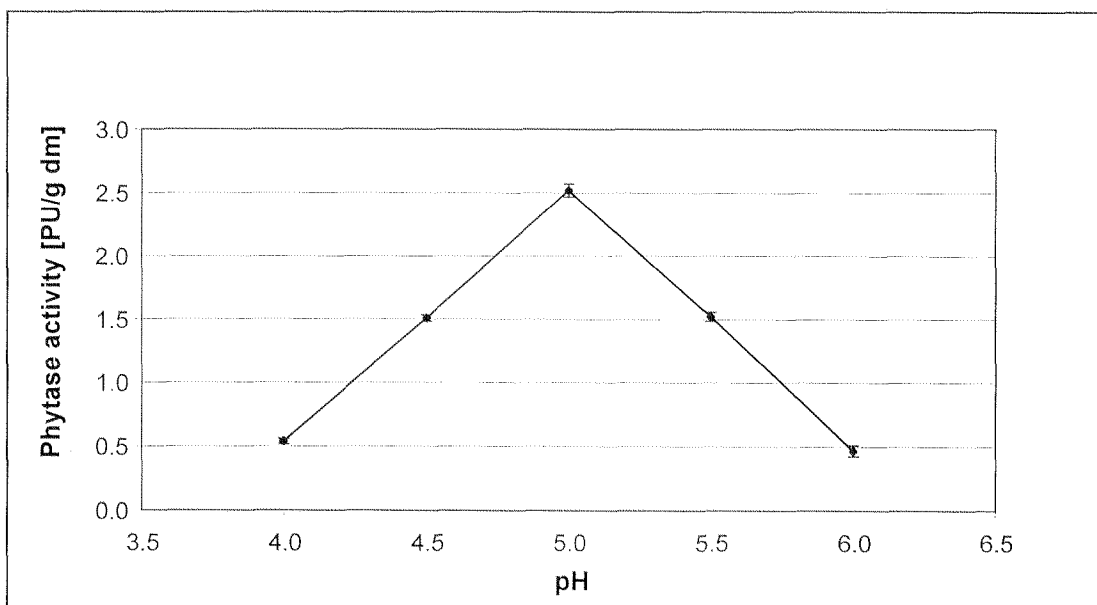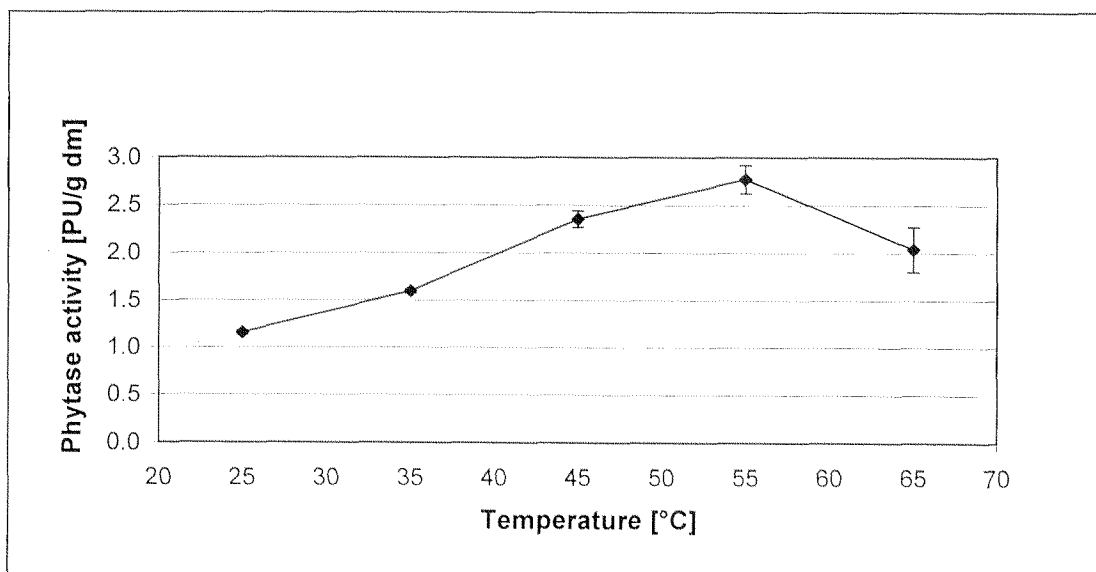Figure 2.7. Phytase activity of buckwheat at different pH (temperature 55°C)

Figure 2.8. Phytase activity of buckwheat at different temperatures (pH 5.0)

2.3.2.3. Phytase activity of wheat flour of different extraction rates

The wheat flours were obtained by milling whole grain wheat with a pilot plant mill and they were classified as flours 405, 550 and 812 according to their ash content (Arens 1992). Ash content, inositol phosphate content and phytase activity of the different wheat flours, as well as of whole grain wheat flour are shown in Table 2.2. and Figure 2.9. Phytase activity and inositol phosphate content were increasing with the ash content, the highest values were found for whole grain flour. Phytase activity and inositol phosphate content of the different flours were expressed relative to the values for whole grain flour. In the flours of different extraction rates, phytase activity and inositol phosphate content were found in approximately the same proportions as in the whole grain flour. The rather low phytase activity found in the flours 405, 550 and 812 would be sufficient to degrade the inositol phosphate content in these flours at the same velocity as in whole grain flour. However, only whole grain wheat flour is a good source of phytase activity, which could be used to degrade high inositol phosphate levels in mixtures of cereals and legumes.

Table 2.2.   Ash content, inositol phosphate content and phytase activity of
wheat flour of different extraction rates

Wheat flour	Ash [g/100 g dm]	Phytase activity [PU/g dm]	Phytase activity relative to whole-grain [%]	IP5+IP6 [g/100 g dm]	IP5+IP6 relative to whole grain [%]
Whole-grain	1.90	3.119	100	1.033	100
812	0.85	0.875	28	0.429	42
550	0.51	0.485	16	0.175	17
405	0.46	0.466	15	0.146	14

Figure 2.9.   Phytase activity and inositol phosphate content of wheat flour of
different extraction rates

The columns represent the phytase activity (left axis) and the connected dots the inositol phosphate content (right axis).
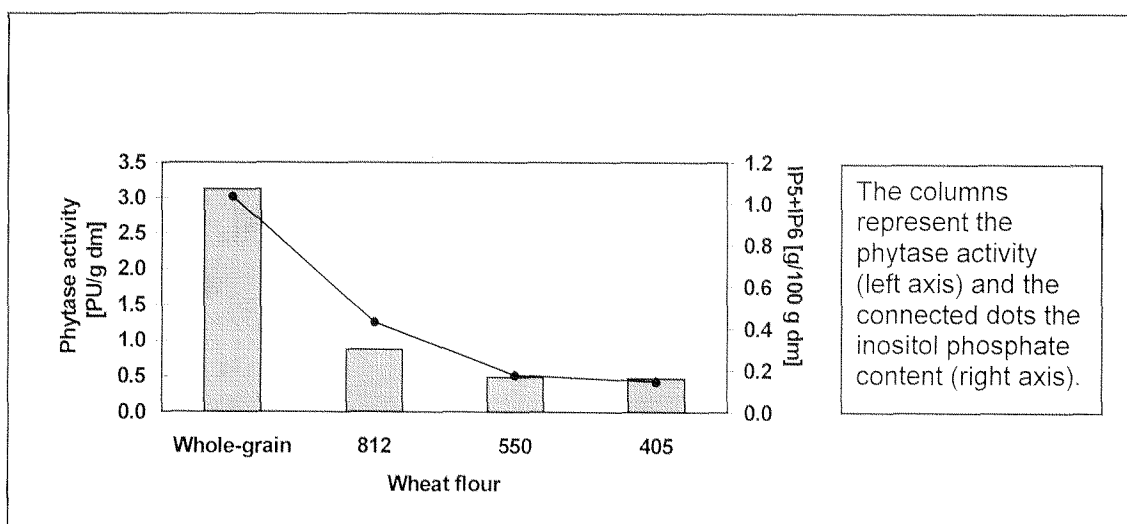
2.3.3. Soaking and germination of grains and seeds

2.3.3.1. Conditions for soaking and germination

Although germination is often combined with soaking (Lorenz 1980), in this study the effects of soaking and germination were investigated separately and the grains and seeds were germinated without previous soaking. All grains and seeds were soaked for 16 hours, and they were germinated for 72 hours at 25°C in the dark. Samples were taken every 24 hours during germination. The effect of soaking and germination, using standardized protocols, on phytase activity and inositol phosphates was investigated without optimizing the conditions for the different grains and seeds.

Soaking for 16 hours resulted in maximal water uptake. Generally, legumes imbibed more water than cereals, presumably due to the higher protein content. Germination started during the first 24 hours for all grains and seeds tested. Either roots or shoots were formed first, reaching lengths from 1 to 5 cm after 72 hours of germination. For most legumes, the formation of leaves had begun at 72 hours of germination. The limiting factor for the duration of germination was the formation of moulds on the surface of the germinated seeds or grain, especially on small grains, such as amaranth and quinoa. The germination system used, which had a grooved surface, was primarily designed for larger seeds, keeping the small grains too wet and therefore susceptible to mould growth. As the grains would not have been edible after 72 hours, results for amaranth and quinoa are reported only for 24 and 48 hours of germination.

To avoid rapid mould formation, a treatment with hypochlorite solution prior to germination, was tested with wheat, rye and soy. The germination was slowed down as compared to untreated seeds and as such pre-treatment would not be feasible at the household level, untreated grains and seeds were germinated. The microbial contamination of germinated mungbean and wheat, investigated by Bomar (1987) and Harmuth-Hoene and Bognar (1988), was found to be significant and was considered as a potential health risk, as pathogenic microorganisms can not be excluded.

2.3.3.2. Water uptake and dry matter loss during germination

The wet weight of all grains and seeds increased considerably during germination due to water imbibition. The water uptake after 72 hours germination was about twice the weight of dry cereal grains and about 3 times the weight of dry legume seeds. The dry grains and seeds had a mean water content of 12% which increased to 75% during germination, ranging from circa 65% for barley and maize to almost 90% for mungbean and lucerne. At the same time, the dry matter content of the grains and seeds decreased due to oxidation and leaching. The mean dry matter loss of all grains and seeds after 72 hours of germination, calculated by comparing the dry matter before and after germination; was about 9%, ranging from circa 5% for barley, buckwheat, oat and rye, up to almost 15% for lucerne, mungbean and cowpea. The values are in agreement with other studies, summarized by Lorenz (1980). The dry matter loss was not taken into consideration for calculations of phytase activity and inositol phosphate content after soaking and germination, as it had not been considered in most previous publications and as it would not change the values considerably.

2.3.3.3. Influence of soaking and germination on phytase activity

Absolute and relative values of phytase activity before and after soaking and germination are summarized in Table 2.3. The relative values after soaking and germination were calculated in relation to the values of the untreated grains or seeds, set at 100%. The relative values of cereals and pseudocereals are shown in Figure 2.10. and the relative values of legumes and oilseeds in Figure 2.11.

2.3.3.3.1. Cereals and pseudocereals

During soaking, the decrease in phytase activity of all cereals and pseudocereals ranged from circa 10% for millet and rye to circa 60% for barley. In cereal grains, phytase is mostly located in the aleurone layer (Peers 1953, Tronier et al. 1971, Adams and Novellie 1975, Yoshida et al. 1975) and the losses might therefore be explained by leaching of the phytase into the soaking water. To our knowledge no information is available on the effect of soaking on phytase activity in cereals.

Table 2.3. Phytase activity of grains and seeds before and after soaking and germination

Grains and seeds		Phytase activity [PU/g dm]								
		untreated	soaked 16 hours		germinated 24 hours		germinated 48 hours		germinated 72 hours	
		mean	mean	% of untreated	mean	% of untreated	mean	% of untreated	mean	% of untreated
Cereals	Barley	1.830	0.791	43	0.815	45	0.822	45	1.092	60
	Maize	0.132	0.091	69	0.167	127	0.594	450	0.765	580
	Millet	0.242	0.217	90	0.276	114	0.645	267	0.792	327
	Oat	0.141	0.122	87	0.090	64	0.067	48	0.083	59
	Rice	0.189	0.119	63	0.141	75	0.128	68	0.129	68
	Rye	6.920	6.425	93	5.781	84	5.568	80	6.255	90
	Sorghum	0.113	0.066	58	0.146	129	0.326	288	0.434	384
	Sweet corn	0.377	0.295	78	0.360	95	0.695	184	1.094	290
	Triticale	4.821	3.009	62	3.083	64	2.479	51	2.318	48
	Wheat	3.084	1.917	62	3.055	99	1.218	39	1.316	43
Pseudocereals	Amaranth	1.272	1.005	79	1.214	95	1.572	124	n.a.*	
	Buckwheat	2.896	1.490	51	2.181	75	2.009	69	1.268	44
	Quinoa	0.621	0.395	64	0.738	119	0.540	87	n.a.	
Legumes	Blackeyed bean	0.390	0.404	104	0.468	120	0.543	139	0.608	156
	Chickpea	0.249	0.250	100	0.265	106	0.611	245	0.627	252
	Cowpea	0.400	0.346	87	0.454	114	0.414	104	0.641	160
	Dwarf bean	0.275	0.254	92	0.280	102	0.412	150	0.524	191
	Lentil	0.260	0.153	59	0.380	146	0.577	222	0.917	353
	Lucerne	0.814	0.840	103	1.007	124	1.526	187	2.042	251
	Lupin	0.242	0.241	100	0.282	117	0.425	176	0.533	220
	Mungbean	0.274	0.325	119	0.466	170	0.817	298	1.001	365
	Pea	0.195	0.143	73	0.220	113	0.522	268	0.625	321
	Soybean	0.344	0.270	78	0.393	114	0.573	167	0.766	223
	White bean	0.228	0.260	114	0.324	142	0.506	222	0.473	207
Oilseeds	Rapeseed	0.295	0.242	82	0.265	90	0.413	140	0.823	279
	Sunflowerseed	0.126	0.115	91	0.127	101	0.174	138	0.195	155

* not analyzed

Figure 2.10. Relative values of phytase activity of cereals and pseudocereals before and after soaking and germination
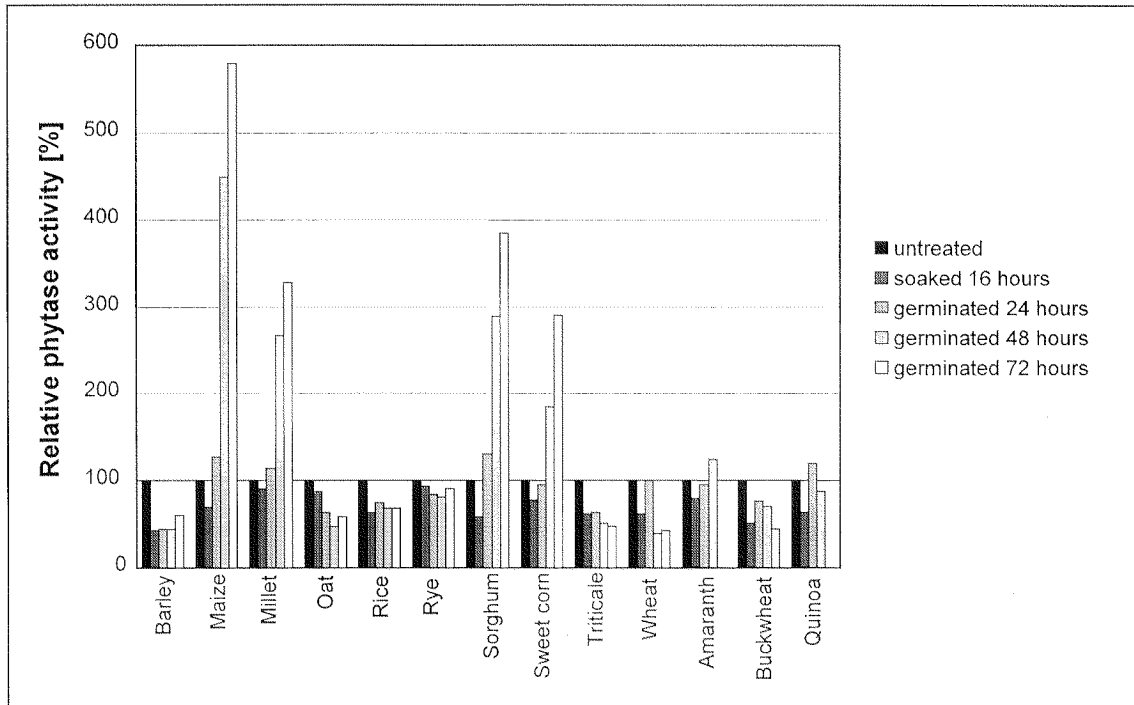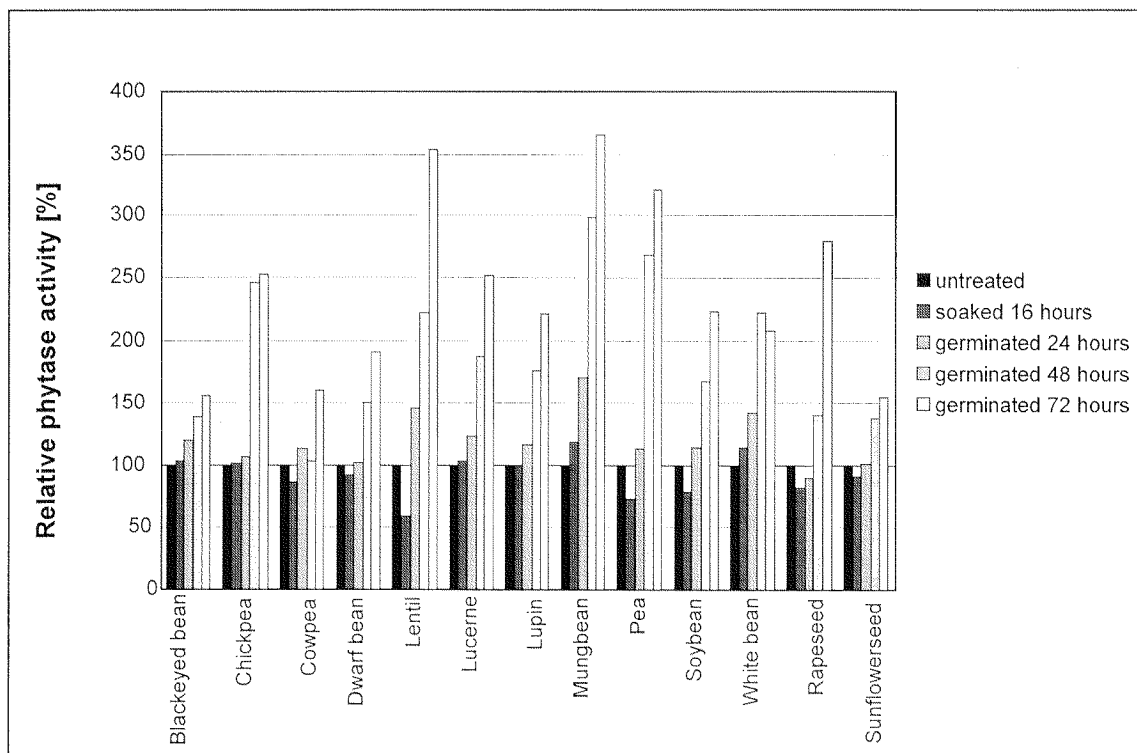

Figure 2.11. Relative values of phytase activity of legumes and oilseeds before and after soaking and germination

The effect of germination was different from the effect of soaking. While the phytase activity decreased for most of the cereals and pseudocereals, it increased substantially for maize, millet, sorghum and sweet corn, relative to the initial values (Figure 2.10). After 72 hours, maize, millet, sorghum and sweet corn had increased their phytase activity between 300% and almost 600% of the initial values, although this increase was observed between 24 and 72 hours of germination and not before. In maize, millet and sorghum, phytic acid is mostly localized in the germ, while in other cereals the major part of phytic acid is found in the aleurone layer (Table 1.2., literature review), however, it is not clear if this difference would have an influence on phytase activity during germination. Information about the distribution of phytase in maize and millet is lacking; phytase activity in sorghum was found in the protein bodies from the aleurone layer (Adams and Novellie 1975).

There is only limited information available on the effect of germination on phytase activity of cereals. Maximum phytase activity in maize was found after five days of germination (Laboure et al. 1993). In wheat, Eastwood and Laidman (1971) found an increase in phytase activity during germination in the bran, while there was only little increase in the other tissues. Bartnik and Szafranska (1987) reported an increased phytase activity during germination of wheat (4.5 fold), rye (2.5 fold), barley (6 fold) and oat (9 fold). Fretzdorff and Weipert (1986) found no increase in phytase activity in rye after three days of germination. The reason for the contradictory results is not known.

The absolute values of phytase activity after germination were 1.1 PU/g dm in sweet corn, 0.8 PU/g dm in maize and millet and 0.4 PU/g dm in sorghum. Thus, phytase activity after germination was still much lower than in untreated rye (6.9 PU/g dm) or wheat (3.1 PU/g dm).

2.3.3.3.2. Legumes and oilseeds

During soaking of legumes and oilseeds, the phytase activity per gram dry matter remained rather stable as compared to the untreated seeds, with the exception of a 40% reduction in lentil. Assuming that the phytase distribution in the legume seeds is similar

to the distribution of phytic acid i.e., in the cotyledons, this might explain the different response of cereal and legume phytase to soaking. Leaching of phytase into the soaking water might be lower in legumes as the enzyme is not concentrated in the surface of the seeds. Henderson and Ankrah (1985) have reported an increase in phytase activity in faba bean during soaking which was not observed in the present study.

Germination enhanced the phytase activity in all legumes tested. While during the first 24 hours, the activity remained rather stable, an increase was observed after 48 hours and this increase continued until 72 hours. The most pronounced increase was found in lentil, mungbean and pea exceeding 300% of the initial value. Others legumes increased phytase activity between 150% and 250%. Often a latent period of several days has been described before the increase in phytase activity, reaching a maximum after six to seven days germination for peas (Guardiola and Sutcliffe 1971), six days for beans (Eskin and Wiebe 1983) and six to eight days germination for lentils, chickpeas and beans (Kyriakidis et al. 1998). An important increase of phytase activity after two days of germination was described for beans (Walker 1974, Lolas and Markakis 1977). These findings suggest, that the increase observed in this study could be expected to continue after 72 hours germination. However, in the germination system used, 72 hours were maximum duration before microbial contamination.

The absolute values of phytase activity after 72 hours germination of legumes and oilseeds investigated were statistically significant different (p = 0.0002) from the values of the untreated seeds. The mean of all values was 0.75 PU/g dm after germination as compared to 0.31 PU/g dm before germination. The highest value was found for lucerne (2.0 PU/g dm), which also had the highest initial value. Relatively high values were detected for mungbean (1.0 PU/g dm) and lentil (0.9 PU/g dm). However, the increased values of the legumes and oilseeds did not reach the much higher values in untreated rye and wheat.

2.3.3.4. Influence of soaking and germination on inositol phosphates

2.3.3.4.1. Determination of inositol phosphates

In order to measure phytic acid content of grains and seeds and to monitor the degradation during soaking and germination, a method which allowed the quantification of the different inositol phosphates was chosen. The most appropriate method was found to be an ion-pair high performance liquid chromatography (HPLC) technique with refractive index detection, based on considerations of sample throughput and available equipment. The technique chosen was based on the methods of Sandberg and Ahderinne (1986) and Sandberg et al. (1989) with modifications according to the current protocol for the analysis as used by A.-S. Sandberg and colleagues (Chalmers University of Technology Göteborg, Sweden; personal communication). The protocol was adopted with minor modifications, including the use of a different HPLC system with autosampler, dissolving samples in water instead of mobile phase before HPLC analysis to avoid changes in sample concentration by evaporation of methanol on the autosampler and the addition of phytic acid to the mobile phase to saturate the system. The relative difference (sum of IP5 and IP6) between five samples analyzed in both laboratories did not exceed 5%.

Figure 2.12. shows the chromatogram of partly hydrolyzed phytic acid. To determine the retention times of the different inositol phosphates (IP3, IP4, IP5, IP6), IP6 was hydrolyzed with hydrochloric acid until an equilibrated mixture of the inositol phosphates was achieved. The peaks were well separated and they were all eluted within seven minutes.

Figure 2.12. Chromatogram of partly hydrolyzed phytic acid
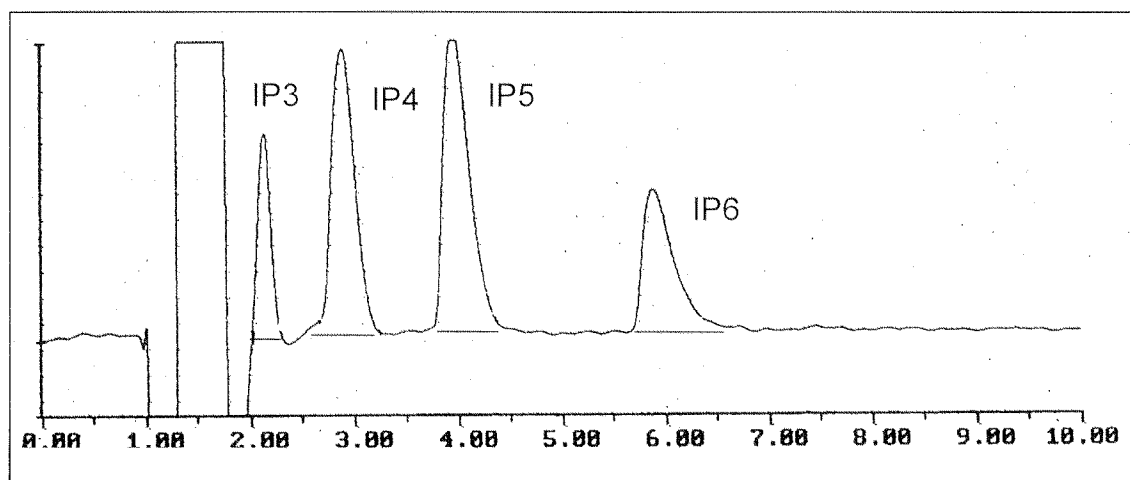
2.3.3.4.1.1. Detection and determination limit

The detection limit was 0.01 mgl/ml, the determination limit 3 mg/100 g sample (0.05 µmol/g sample).

2.3.3.4.1.2. Internal control material

Wheat bran was used as internal control material to monitor reproducibility. The mean value of 20 independent determinations over two years (sum of IP5 and IP6) was 3.82 ± 0.15 g/100 g, resulting in a coefficient of variation of 4.3%.

2.3.3.4.2. Inositol phosphate content of untreated, soaked and germinated grains and seeds

In all samples analyzed for their phytase activity (untreated, soaked and germinated), the inositol phosphate content was also determined. The results of all grains and seeds in absolute values, and in relation to the inositol phosphate content of the untreated grains and seeds, set at 100%, are given in Table 2.4. The inositol phosphate content of cereals and pseudocereals are shown in Figure 2.13., the inositol phosphate content of legumes and oilseeds in Figure 2.14.

The inositol phosphate content represents the sum of IP6 and IP5, the lower inositol phosphates (IP4 and IP3) were not included, as they can not form strong complexes with minerals and trace elements and thus have less negative influence on iron and zinc bioavailability (Sandström and Sandberg 1992, Sandberg et al. 1999). Most of the untreated grains and seeds contained only IP6 and IP5, with IP5 only contributing circa 5 to 10% of the sum of IP5 and IP6. It is not known if IP5 naturally occurs in the grains and seeds or if it is formed during sample preparation. After soaking and germination, IP4 was detected in most of the grains and seeds, contributing circa 1 to 5% of the sum of IP5 and IP6. In only two samples, were small amounts of IP3 also detected. As IP5, IP4 and IP3 did not accumulate significantly during soaking and germination, IP6 was either degraded directly to IP2, IP1 or *myo*-inositol, which could not be detected with the method used, and/or the losses were due to leaching into the soaking or rinsing water.

Table 2.4. Inositol phosphate content of grains and seeds before and after soaking and germination

Grains and seeds		Inositol phosphate content IP5+IP6 [g/100 g dm]								
		untreated	soaked 16 hours		germinated 24 hours		germinated 48 hours		germinated 72 hours	
		mean	mean	% of untreated	mean	% of untreated	mean	% of untreated	mean	% of untreated
Cereals	Barley	1.013	0.951	94	0.858	85	0.804	79	0.815	80
	Maize	1.147	1.139	99	1.159	101	1.047	91	0.751	65
	Millet	0.827	0.719	87	0.710	86	0.493	60	0.365	44
	Oat	0.882	0.884	100	0.918	104	0.915	104	0.941	107
	Rice	0.878	0.622	71	0.530	60	0.390	44	0.299	34
	Rye	0.785	0.620	79	0.713	91	0.436	56	0.466	59
	Sorghum	1.083	1.091	101	1.078	100	1.105	102	0.764	71
	Sweet corn	1.628	1.716	105	1.704	105	1.627	100	1.587	97
	Triticale	1.288	1.043	81	0.923	72	0.718	56	0.750	58
	Wheat	1.033	1.043	101	1.120	108	0.785	76	0.694	67
Pseudocereals	Amaranth	1.388	1.430	103	1.435	103	1.303	94	n.a.*	
	Buckwheat	1.418	1.303	92	1.443	102	1.456	103	1.444	102
	Quinoa	0.965	1.026	106	0.929	96	0.852	88	n.a.	
Legumes	Blackeyed bean	0.860	0.939	109	0.938	109	0.926	108	0.805	94
	Chickpea	0.484	0.414	86	0.509	105	0.474	98	0.405	84
	Cowpea	0.657	0.614	93	0.661	101	0.681	104	0.684	104
	Dwarf bean	1.127	1.068	95	1.114	99	1.063	94	0.953	85
	Lentil	1.149	1.122	98	1.115	97	0.985	86	0.866	75
	Lucerne	1.361	1.350	99	1.262	93	1.008	74	0.942	69
	Lupin	0.674	0.739	110	0.675	100	0.676	100	0.627	93
	Mungbean	0.827	0.887	107	0.790	96	0.614	74	0.411	50
	Pea	0.629	0.618	98	0.570	91	0.607	97	0.595	95
	Soybean	1.400	1.357	97	1.444	103	1.319	94	1.027	73
	White bean	1.083	1.213	112	1.218	112	1.100	102	1.063	98
Oilseeds	Rapeseed	1.521	1.676	110	1.614	106	1.655	109	1.468	97
	Sunflowerseed	1.515	1.655	109	1.512	100	1.521	100	1.333	88

* not analyzed

Figure 2.13. Inositol phosphate content of cereals and pseudocereals before
             and after soaking and germination
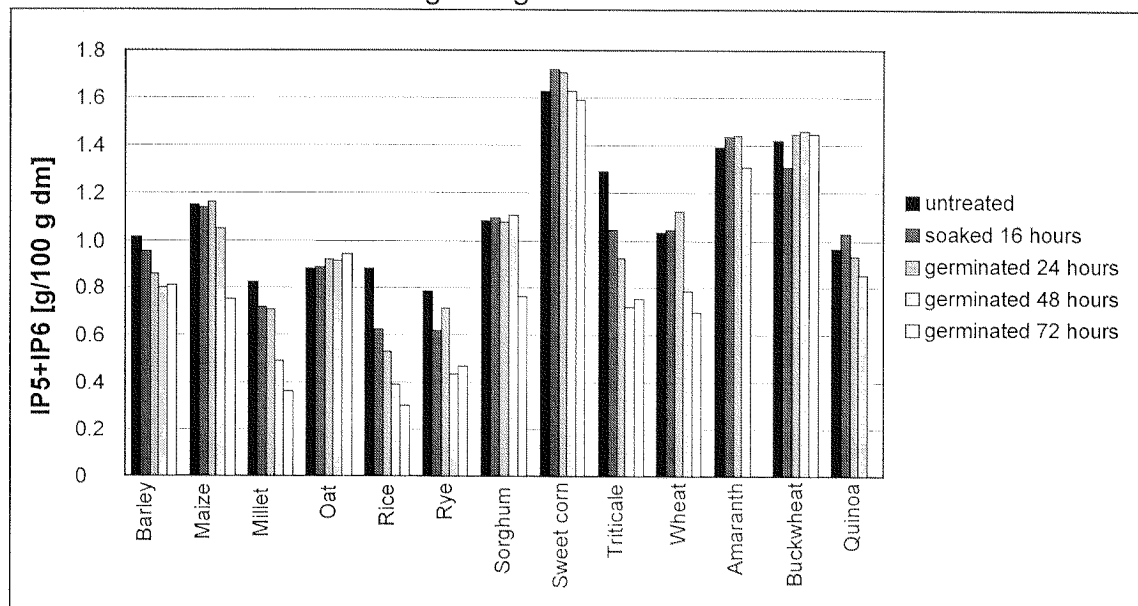
Figure 2.14. Inositol phosphate content of legumes and oilseeds before and
             after soaking and germination
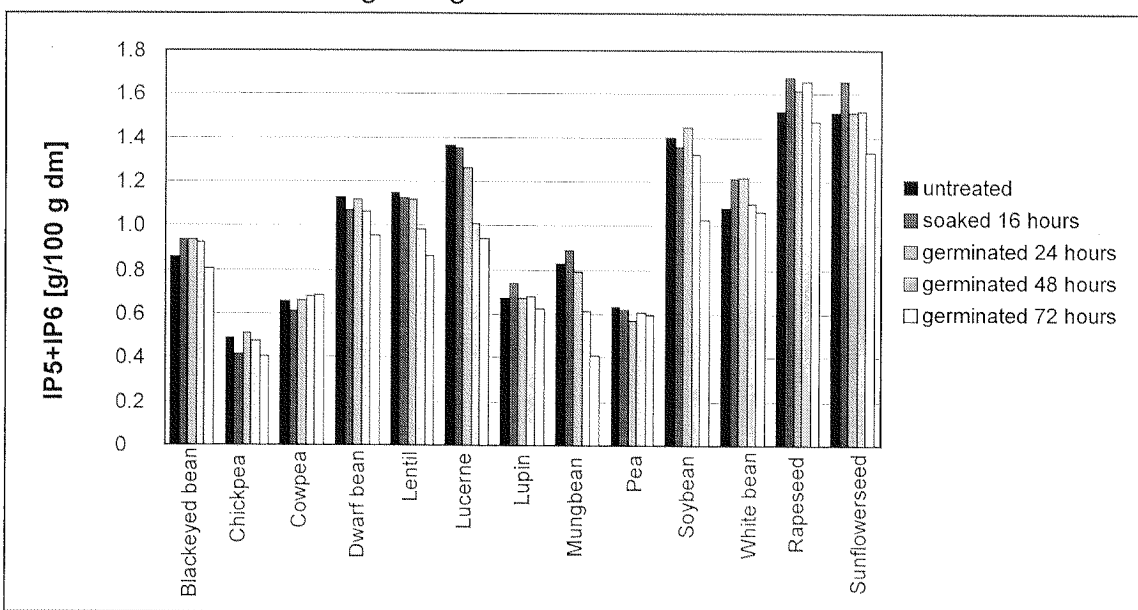
The mean value of the inositol phosphate content of all untreated grains and seeds tested
was 1.1 g/100 g dm. The lowest value of 0.5 g/100 g dm was found in chickpea, the
highest in sweet corn, 1.6 g/100 g dm. The results are in good agreement with the phytic
acid content of grains and seeds as summarized in the literature review (Table 1.1.).

Soaking of grains and seeds had very little influence on the inositol phosphate content and there was no statistically significant difference (p = 0.4) between the inositol phosphate content before and after soaking. The most pronounced effects were found for rice, rye and triticale, in which the inositol phosphate content was decreased to 71, 79 and 81% of the initial values. For the other grains and seeds, the inositol phosphate content remained between 86 and 100% of the initial values. Some samples showed a slight increase compared to the content before soaking, probably due to inaccuracy of the determination and/or to dry matter loss. No difference between cereals and legumes could be observed, although phytic acid in the cereal grains (except maize, millet, sorghum) is mostly located in the aleurone layer and could therefore be expected to be more easily leached into the soaking water than the phytic acid of legume seeds.

The inositol phosphate content of all grains and seeds tested decreased slightly during germination, except for oat, buckwheat and cowpea. Oat, buckwheat and cowpea apparently contained more inositol phosphate after germination (102 to 107% of the initial values), which might be explained by inaccuracy of the determination and/or dry matter loss. The greatest decrease was found for millet, rice and mungbean, where only 44, 34 and 50% of the initial content remained after germination for 72 hours. Comparing the inositol phosphate content of all samples before and after germination for 72 hours, a statistically significant difference (p = 0.00001) was observed.

The inositol phosphate decrease found during germination was in the same range as described by other authors (Chen and Pan 1977, Ologhobo and Fetuga 1984a, Fretzdorff and Weipert 1986, Reddy et al. 1989), but they were not substantial enough to expect an influence on mineral and trace element bioavailability. For example, phytic acid had to be reduced to levels below 0.03% (or below 10 mg per meal) before a meaningful increase in iron absorption could be observed (Hurrell et al. 1992).

No relationship was found between the phytase activity and the inositol phosphate content of the untreated grains and seeds, indicating that high phytase activity of cereals, such as rye or wheat is not linked with high inositol phosphate contents. There was no relationship between the phytase activity and the decrease in inositol phosphate

content during germination of the grains and seeds. Thus, demonstrating that the phytase can not act well on its substrate phytic acid in the whole grains or seeds under the conditions tested. In rye for example, which has a high phytase activity, the initial inositol phosphate content was only decreased by 40%, after germination for 72 hours. If water was added to milled rye to form a slurry, the inositol phosphate could be expected to be completely degraded in a few hours at the conditions used for the germination (25°C). For products based on flours of grains and seeds, as for example weaning foods, phytic acid degradation is therefore preferentially done in the flours than in the whole grains or seeds.

2.3.3.5. Influence of soaking and germination on ascorbic acid content

2.3.3.5.1. Determination of ascorbic acid

For the determination of ascorbic acid in grains and seeds a high performance liquid chromatography (HPLC) technique with UV detection was used. This method allowed the determination of ascorbic acid and dehydroascorbic acid simultaneously by reduction of dehydroascorbic acid to ascorbic acid prior to HPLC analysis. The results were therefore expressed as total ascorbic acid.

2.4.2.5.1.1. Detection and determination limit

The detection limit was 0.23 µg/ml, the determination limit 0.5 mg/100 g sample.

2.3.3.5.2. Total ascorbic acid content

2.3.3.5.2.1. Reproducibility of ascorbic acid formation

The reproducibility of the ascorbic acid formation was investigated in soybean. Three batches of soybean were germinated under standardized conditions and analyzed independently, leading to a coefficient of variation of the three mean values of 6.4%.

2.3.3.5.2.2. Total ascorbic acid content of grains and seeds after 72 hours germination

A selection of grains and seeds used for the analysis of phytase and inositol phosphate content was investigated for the formation of ascorbic acid during germination. The procedure included a soaking step (16 hours) prior to germination.

No ascorbic acid was detected before soaking and germination in any grains and seeds tested. Table 2.5. shows the total ascorbic acid content after combined soaking and germination for 72 hours. The values are expressed as mg/100 g wet weight with standard deviations (sd) and as mg/100 g dm to compensate for the differences in water uptake. All grains and seeds investigated showed formation of ascorbic acid during germination, resulting in values from 3.0 mg/100 g wet weight in buckwheat to 13.5 mg/100 g wet weight in pea. Expressed as mg/100 g dm the values ranged from 10.2 for buckwheat to 93.8 mg/100 g dm, for lucerne.

Table 2.5.   Total ascorbic acid content of raw materials after 72 hours germination

Grains and seeds		Total ascorbic acid content		
		[mg/100 g wet weight]		[mg/100 g dm]
		mean	sd	mean
Cereals	Barley	3.2	0.5	13.0
	Maize	10.0	0.6	27.8
	Rye	5.6	0.2	23.9
	Sorghum	7.7	0.1	29.0
	Sweet corn	7.1	0.2	25.6
	Triticale	6.3	0.1	24.1
	Wheat	4.8	1.2	18.1
Pseudocereal	Buckwheat	3.0	0.2	10.2
Legumes	Blackeyed bean	12.9	0.3	61.1
	Chickpea	6.8	0.4	20.7
	Lentil	11.9	0.2	50.7
	Lucerne	8.9	0.3	93.8
	Mungbean	11.2	0.5	79.9
	Pea	13.5	0.2	49.2
	Soybean	10.4	0.8	45.5

The results are difficult to compare with values reported by other authors due to different germination procedures and analytical methods. Generally, the values found were in the same range, or slightly lower, than those reported by other authors (Harmuth-Hoene and Bognar 1988, Kavas and El 1992, Danisová et al. 1994, Lintschinger et al. 1997, Riddoch et al. 1998). For some of the grains and seeds, such as maize, rye, sorghum and triticale no literature values are available.

The influence of cooking on the total ascorbic acid content after germination was investigated in mungbean. Cooking for one minute (> 95°C) decreased the initial content by 65%, cooking for five minutes by 78%, cooking for 10 minutes by 90% and after 20 minutes no ascorbic acid could be detected. Similar results were obtained by other authors. A treatment of germinated mungbeans of one minute in boiling water resulted in approximately 50% loss of ascorbic acid (Farhangi and Valadon 1981) and cooking for five minutes in circa 60% loss (Harmuth-Hoene and Bognar 1988, Riddoch et al. 1998). In amaranth and cowpea the ascorbic acid content was reduced to 3% and 5% of the initial content after five minutes at 95°C (Mosha et al. 1995). The losses were attributed to leaching during the heat treatment and oxidation during the consecutive drying. Lane et al. (1985) reported that a high surface to mass ratio of vegetables resulted in excessive loss of ascorbic acid during processing.

2.3.3.5.2.3. Influence of ascorbic acid on iron absorption

Germinated cereals and legumes might be a useful source of ascorbic acid in developing countries where fruits and vegetables rich in ascorbic acid are often unavailable and/or too expensive to be added to the diet. Riddoch et al. (1998) evaluated germinated legumes as a source of ascorbic acid to protect against scurvy and reported that the daily consumption of 50 g germinated beans would provide sufficient ascorbic acid. In the present study, the focus was on the potential enhancing effect of ascorbic acid from germinated cereals or legumes on iron absorption from weaning foods. Ascorbic acid has been shown to increase iron absorption and to overcome the inhibiting effect of phytic acid (Hallberg et al. 1986, 1989a, Davidsson et al. 1994a, 1998). Iron absorption from weaning foods in adults was significantly improved by the addition of ascorbic acid at molar ratio of ascorbic acid to iron above 0.9:1 (Derman et al. 1980).

The addition of 50 g germinated mungbean (corresponding to 7 g dry matter), with an ascorbic acid content of 11 mg/100 g wet weight to 100 g of weaning food, based on wheat and soy with 2 mg iron/100 g would lead to a molar ratio of ascorbic acid to iron of 0.9:1. This addition should theoretically improve iron absorption from the weaning food (Derman et al. 1980). A heat treatment of the germinated mungbeans would be necessary before being fed to infants and even a short heat treatment was shown to

decrease the ascorbic acid content to such extent that no positive effect on iron absorption could be expected. It remains to be investigated if the addition of germinated grains or seeds during the weaning food preparation would result in a higher retention of the ascorbic acid, as leaching into the cooking water could be avoided. However, the ascorbic acid content even before heat treatment might not be sufficient to overcome the negative influence of the relatively high phytic acid content (0.4 g/100 g) that could be expected in a wheat/soy based weaning food (Davidsson et al. 1997, Davidsson 1998).

2.4. Summary

A range of cereals, pseudocereals, legumes and oilseeds, consumed in developed and developing countries have been screened for phytase activity under standardized conditions at pH 5.0. All legumes had a relatively low phytase activity, between 0.2 to 0.8 PU/g dm, while the phytase activity of cereals and pseudocereals was in the range of 0.1 to 6.9 PU/g dm. Phytase activity in the legumes was also measured at pH 8.0 and found to be lower than at pH 5.0. The highest phytase activity was found in rye (6.9 PU/g dm), triticale (4.8 PU/g dm), wheat (3.1 PU/g dm) and in the pseudocereal buckwheat (2.9 PU/g dm). The optimum conditions for phytase in rye, wheat and buckwheat were found to be at pH values between 5.0 and 5.5 and at temperatures between 55 and 60°C. Phytase activity and inositol phosphate content of wheat flour of different extraction rates were found to be correlated. Only whole grain wheat flour had a high phytase activity, sufficient to degrade high levels of phytic acid.

Soaking decreased the phytase activity of most cereals and pseudocereals. Germination for 72 hours of cereals and pseudocereals also decreased the phytase activity, except for maize, millet and sorghum, where the activity was increased 3 to 6 fold. In legumes and oilseeds, the phytase activity remained rather stable during soaking with the exception of lentil (60% of the initial activity). Germination of legumes and oilseeds generally increased the phytase activity 2 to 3 fold. However, the high phytase activity in untreated rye and wheat could not be reached by germinating legumes or the cereals maize, millet and sorghum. The inositol phosphate content of most grains and seeds was stable during soaking and decreased only slightly during germination for 72 hours,

although rather high losses of 50-65% were found for mungbean, millet and rice. However, the losses are probably too small to expect a positive influence on mineral and trace element bioavailability. During germination, ascorbic acid was formed in the grains and seeds tested. The ascorbic acid content (3-13 mg/100 g wet weight) of germinated grains and seeds might have an enhancing effect on iron absorption, but after a heat treatment of mungbeans the values were too low to expect any positive influence. More experiments would be necessary to investigate the influence of different heat treatments on ascorbic acid content in other grains and seeds.

In further investigations, untreated whole grain rye, wheat and buckwheat were used as a phytase source to degrade phytic acid in weaning food mixtures of cereals and legumes.

3. Phytic acid degradation in weaning foods using naturally occurring phytase

3.1. Introduction

Weaning foods are often based on cereals, which are combined with milk or with legumes in countries were milk is not readily available, so as to improve the quantity and the nutritional quality of the protein component. In this study the feasibility of reducing the phytic acid content during weaning food production by using the naturally occurring phytase, present in whole grain cereals was investigated.

First, on a laboratory scale, cereals with high phytase activity, selected from the screening study, were added to cereals and legumes and the milled mixtures incubated in the form of a slurry. Incubation conditions were optimized so as to achieve complete phytic acid degradation. The mixtures were selected because of their potential use as weaning foods. Once the laboratory conditions for complete phytic acid degradation were defined, the procedure was scaled up to the pilot plant. Potential weaning food mixtures were selected and phytic acid degradation was monitored during weaning food production.

Finally weaning foods based on wheat and soybeans were produced, one with no phytic acid and one with the native phytic acid content. Phytic acid degradation was achieved by using the phytase naturally occurring in whole grain wheat. The chemical composition and the microbiological safety of the weaning foods were evaluated.

3.2. Materials and methods

All chemicals and reagents were of analytical grade (Fluka Chemie AG, Buchs, Switzerland; Merck, Darmstadt, Germany) except for mineral and trace element analysis, where suprapur quality of chemicals and reagents was used (Fluka Chemie AG; Merck), and where nitric acid and hydrochloric acid were further purified by sub-

boiling. For mineral and trace element analysis, all tubes, containers and pipette-tips were acid-washed. Water was purified with ion exchange and reverse osmosis (18MΩ) (RD 2000, Renggli, Rotkreuz, Switzerland; Nanopure Cartridge System, Skan AG, Basel, Switzerland), except for pilot plant trials where tap water was used.

3.2.1. Grains and seeds

The grains and seeds listed in Table 3.1. were selected based on data from the screening study as the phytase source and/or as the ingredients for cereal/legume based weaning food mixtures. Whole grain rye, wheat and buckwheat were selected as the phytase sources, due to their high enzymatic activity. The cereals maize, millet, rice and wheat and the legumes chickpea, cowpea and soybean were chosen as ingredients for weaning food mixtures. While in the screening study grains and seeds were used only as whole grain quality, in this study the weaning food ingredients were used as they are commonly consumed, i.e., whole grain maize, dehulled millet, polished rice and wheat with low extraction rate (classified as wheat flour 550 according to the ash content (Arens 1992)). Chickpea was used with hulls, while cowpea was dehulled and soybean was dehulled and toasted. In contrast to the screening study, where experiments were carried out with whole grains and seeds, in this study milled grains and seeds were used. For laboratory experiments, whole grain rye and wheat were purchased from a commercial seed supplier (Fenaco, Winterthur, Switzerland). All other cereals and buckwheat came from supermarkets and health stores in Zurich, Switzerland. Chickpea was provided by Nestlé, Singapore, cowpea by Nestlé, Abidjan, Ivory Coast, and soybean was purchased from Zwicky AG, Müllheim-Wigoltingen, Switzerland. The grains and seeds were obtained between spring 1997 and spring 1999 and stored at 4 ± 2°C. Grains and seeds for pilot plant trials were purchased from Zwicky AG, Müllheim-Wigoltingen, Switzerland (rye, soybean) and from Moulins Cossonay SA, Cossonay-Ville, Switzerland (wheat) and were stored at ambient temperature until used.

Table 3.1.   Grains and seeds

	Common name	Botanical name, variety
Cereals	Maize	*Zea mays* (variety not specified)
	Millet	*Pennisetum typhoides*
	Rice	*Oryza sativa*
	Rye	*Secale cereale*
	Wheat	*Triticum aestivum*
Pseudocereal	Buckwheat	*Fagopyrum esculentum*
Legumes	Chickpea	*Cicer arietinum*
	Cowpea	*Vigna niebe*
	Soybean	*Glycine max*

3.2.1.1. Milling

All grains and seeds for laboratory experiments were frozen in liquid nitrogen and milled (0.5 mm mesh, centrifugal mill, Retsch ZM1, Retsch GmbH, Haan, Germany). Grains and seeds for pilot plant trials were milled by Nestlé Product Technology Centre, Orbe, Switzerland or by the suppliers (Zwicky AG, Moulins Cossonay SA).

3.2.1.2. Inositol phosphates

The inositol phosphate content of the milled grains and seeds was determined as described in chapter 2.2.3.4. The results were given as the sum of IP5 and IP6, expressed in g/100 g as is.

3.2.1.3. Phytase activity

The phytase activity was measured under the conditions of the screening study (pH 5.0, 45°C) as described in chapter 2.2.3.3.1. The results were expressed in phytase units (PU)/g as is. One PU being the activity that liberates one μmol inorganic phosphate (P_i) from phytic acid per minute.

3.2.2. Laboratory scale trials of inositol phosphate degradation

The inositol phosphate degradation was investigated in the mixtures shown in Table 3.2. Milled grains and seeds were weighed individually into 500 ml polypropylene containers, the total weight being 50-100 g and mixed under vigorous shaking of the containers by hand during circa five minutes. Samples (2-4 g) of the mixtures were weighed into Erlenmeyer flasks and preheated to the selected incubation temperature

(45-60°C). Ten ml water, 10 ml 0.2 M acetate buffer (pH 5.0) or 10 ml 2-11 mM citric acid, preheated to the incubation temperature were added to form a slurry. The Erlenmeyer flasks were covered and the inositol phosphate degradation was performed under constant stirring of the slurry (600 rpm, Variomag multipoint HP15, H+P Labortechnik GmbH, Oberschleissheim, Germany) in an incubator (WTB Binder, Tuttlingen, Germany) at the selected incubation temperature (45-60°C). Depending on the ingredients of the mixtures and the conditions, incubation times varied from 20 to 280 minutes. For each time point separate samples were incubated for inositol phosphate degradation and determination. To determine the inositol phosphate content of the mixtures after incubation, the enzymatic reaction was terminated by addition of 70 ml 0.57 M hydrochloric acid to the slurries. For the determination of the initial inositol phosphate content of the mixtures (incubation time zero) 70 ml 0.57 M hydrochloric acid was added before incubation. The inositol phosphates were extracted from the slurries under stirring (600 rpm, Variomag multipoint HP15, H+P Labortechnik GmbH) during three hours at room temperature. Circa 30 ml of the sample solution were centrifuged at 3500 rpm (Omnifuge 2.0 RS, Heraeus GmbH, Hanau, Germany) for 10 minutes and 15 ml of the supernatant were used for the inositol phosphate determination as described in chapter 2.2.3.4.

Table 3.2.   Composition (weight%) of mixtures for inositol phosphate degradation

Ingredients:	Phytase source (whole grain quality):
90% soybean	10% wheat
90% soybean	10% rye
90% maize	10% wheat
90% maize	10% rye
90% rice	10% buckwheat
30% rice, 60% chickpea	10% buckwheat
33% rice, 66% chickpea	-
50% millet, 40% cowpea	10% wheat
55% millet, 45% cowpea	-
70% wheat (low extraction rate), 20% soybean	10% wheat
70% wheat (low extraction rate), 20% soybean	10% rye

For each time point of the inositol phosphate degradation, the experiment was performed in duplicate for the mixtures based on 90% soybean and 10% whole grain wheat or 10% whole grain rye and in triplicate for all other mixtures shown in Table 3.2. The inositol phosphate content (IP3, IP4, IP5, IP6) at the different time points of the degradation was expressed in g/100 g as is. Mean values were calculated as the

sum of IP3, IP4, IP5 and IP6 and experiments were repeated if the difference of duplicate analysis relative to the mean value, or the coefficient of variation based on triplicate analysis, was > 15%.

3.2.3. Weaning food production in the pilot plant

Pilot plant trials were carried out at Nestlé Product Technology Centre, Orbe.

3.2.3.1. Weaning food with no phytic acid

A weaning food mixture with a total weight of 40-60 kg was prepared by mixing the milled ingredients in the following proportions: 70% wheat with low extraction rate, 20% soybean and 10% whole grain wheat or 10% whole grain rye. Water (90-100 kg) was heated to circa 55°C in a tank and the weaning food mixture was added under constant stirring, decreasing the temperature to circa 50°C. pH was adjusted by addition of 1 M citric acid to pH 5.1 for the mixture containing whole grain wheat and to pH 5.5 for the mixture containing whole grain rye. Once the slurry was homogeneously mixed and the pH adjusted, i.e. after 10-20 minutes, duplicate samples (circa 10 g of the slurry) for inositol phosphate determination were weighed in 100 ml polyethylene bottles containing 70 ml 0.57 M hydrochloric acid to terminate the enzymatic reaction. Further samples for inositol phosphate determination were taken every twenty minutes. pH was measured at the beginning of the incubation and every 60 minutes thereafter. The temperature was monitored continuously. After 180 minutes, the slurry was heated by steam injection (circa 135°C) and roller dried. The weaning food was packed in sealed paper bags and stored at ambient temperature.

The inositol phosphates in the samples taken during the incubation were extracted under stirring (600 rpm, Variomag multipoint HP15, H+P Labortechnik GmbH) for three hours at room temperature. Circa 30 ml of the solution were centrifuged at 3500 rpm (Omnifuge 2.0 RS, Heraeus GmbH) for 10 minutes and 15 ml of the supernatant were used for the inositol phosphate determination as described in chapter 2.2.3.4.

The inositol phosphate content (IP3, IP4, IP5, IP6) at the different time points of the degradation was expressed in g/100 g as is. Mean values were calculated as the sum of IP3, IP4, IP5 and IP6 and determinations were repeated if the difference of duplicate analysis relative to the mean value was > 15%.

3.2.3.2. Weaning food with native phytic acid content

A weaning food based on 70% wheat with low extraction rate, 20% soybean and 10% whole grain wheat was produced without the inositol phosphate degradation step so as to compare the overall composition and the absorption of zinc and copper (chapter 4) with the dephytinized weaning food. The weaning food mixture was continuously mixed with water of about 10°C and 1 M citric acid, resulting in a slurry with a dry matter content of circa 35% and pH 5.2. The slurry was heated by steam injection (135°C) and roller dried. The weaning food was packed in sealed paper bags and stored at ambient temperature.

3.2.4. Weaning food analysis

The weaning foods were analyzed by Nestlé Product Technology Centre, Orbe, except for inositol phosphate, mineral and trace element analysis.

3.2.4.1. Inositol phosphates

The inositol phosphate content was determined as described in chapter 2.2.3.4. The content of IP3, IP4, IP5 and IP6 was expressed in g/100 g as is.

3.2.4.2. Dry matter

Dry matter content was determined as described in chapter 2.2.3.1.

3.2.4.3. Total ash

The total ash content was determined gravimetrically after combustion of the organic matter at 550°C, according to the official Swiss method for the determination of ash (Schweizerisches Lebensmittelbuch; Kapitel 22; Methode 3) and to the method of

AOAC (Association of Official Analytical Chemists) (Official method 923.03) with modifications.

Samples of 2-5 g weaning food were weighed into pre-dried quartz crucibles. The samples were pre-ashed using an electric heating mantle until they were well carbonized and than ashed in a muffle furnace (M 104, Heraeus GmbH) at 550°C for 8-12 hours. The crucibles were cooled in a desiccator and the total ash content determined gravi-metrically. Samples were analyzed in duplicate and repeated if the difference relative to the mean value was > 2%. The total ash content was expressed in g/100 g as is.

3.2.4.4. Total nitrogen

The total nitrogen content was determined according to Kjeldahl and the official Swiss method for the determination of nitrogen (Schweizerisches Lebensmittelbuch; Kapitel 22; Methode 4.1) with modifications.

Samples of 0.5-1.0 g weaning food were weighed into mineralization tubes, two Kjedahl tablets (Missouri catalyst: 49.8% Na_2SO_4 + 49.8% K_2SO_4 + 0.3% $CuSO_4$) and 15 ml of 95-97% sulfuric acid were added. A blank was analyzed with each series of samples. The samples were mineralized during one hour at about 370°C in a preheated mineralization unit (Büchi 435, Büchi Labortechnik AG, Flawil, Switzerland). After cooling for 10 minutes the samples were diluted with 50-100 ml water. The mineralization tubes were placed in a distillation device (Büchi 339, Büchi Labortechnik AG), 40 ml water and 60 ml 32% sodium hydroxide were added and the distillation started. The distillate was collected in 0.6% boric acid containing an indicator (methyl red and bromocresol green) and the subsequent titration was performed with 0.1 M hydrochloric acid using a titration stand (Methrom 719, Methrom AG, Herisau, Switzerland) until pH 4.65 was reached, indicated by a color change. The volume of the 0.1 M hydrochloric acid was recorded, corrected for the value of the blank and the total nitrogen content calculated based on 1 ml of 0.1 M hydrochloric acid corresponding to 1.4007 mg nitrogen. Samples were analyzed in duplicate and repeated if the difference was > 0.03 g/100 g.

3.2.4.5. Protein

The protein content was calculated from the total nitrogen content, using the conversion factors 5.83 for wheat (wholemeal flour), 5.7 for wheat flour and 5.7 for soybean according to Holland et al. (1991) referring to FAO/WHO (1973). The composition of the weaning foods, i.e., 70% wheat with low extraction rate, 20% soybean and 10% whole grain wheat resulted in a conversion factor of 5.71. The results were expressed in g/100 g as is.

3.2.4.6. Fat

The fat content was determined gravimetrically after acid hydrolysis and extraction according to Weibull-Stoldt and the official Swiss method for the determination of total fat (Schweizerisches Lebensmittelbuch; Kapitel 22; Methode 5.1) with modifications.

Samples of 10-20 g weaning food were weighed in hydrolysis tubes, 5 g celite (545, Macherey-Nagel AG, Oensingen, Switzerland) and 100 ml 4 M hydrochloric acid were added and the hydrolysis performed in a hydrolyzation unit (Büchi B-428, Büchi Labortechnik AG). The hydrolyzed fat was collected quantitatively in a filter containing 20 g purified sand (Zimmerli Mineralwerk AG, Zurich, Switzerland) and 5 g celite (545, Macherey-Nagel AG). The filters were dried in a microwave oven (HMG 610, Siemens AG, München, Germany) for circa 30 minutes (depending on sample weight and amount of samples). The beakers for the extraction were dried in a microwave oven during 12 minutes (power 600 W), cooled in a desiccator and the weight recorded before they were placed in a Soxhlet unit (Büchi B-810, Büchi Labortechnik AG). The fat in the dried filters was extracted during two hours with petroleum ether (boiling range 40-60°C). The beakers with the extracted fat were dried in the microwave oven for 12 minutes (power 600 W) and the fat content determined gravimetrically. Samples were analyzed in duplicate and repeated if the difference was > 0.2 g/100 g. The fat content was expressed in g/100 g as is.

3.2.4.7. Dietary fiber

The total dietary fiber content was determined gravimetrically after enzymatic hydrolysis and correction for protein and ash content, according to AOAC (Official method 985.29) and the official Swiss method for the determination of dietary fiber (Schweizerisches Lebensmittelbuch; Kapitel 22; Methode 8) with modifications.

Circa 1 g weaning food was weighed in duplicate in beakers, the difference in weight of the duplicate samples not exceeding 20 mg. A blank was analyzed with each series of samples. The sample was dissolved in 50 ml 0.08 M phosphate buffer (pH 6.0) and 100 µl α-amylase suspension (Termamyl 120L, Novo Nordisk SA, Nanterre, France) was added. The covered beakers were placed in a boiling water bath during 15 minutes, shaken every five minutes and cooled to room temperature. The pH was adjusted to 7.5 ± 0.2 with 10 ml 0.275 M sodium hydroxide before 5 mg protease (P-3910, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added. The covered beakers were placed in a shaking water bath (60°C) for 30 minutes. After cooling to room temperature, pH was adjusted to 4.5 ± 0.2 with 10 ml 0.35 M hydrochloric acid. Amyloglucosidase 300 µl (A-9913, Sigma-Aldrich Chemie GmbH) were added and the covered beakers placed for 30 minutes in a water bath (60°C). Ethanol 280 ml 95% preheated to 60°C were added and the covered beakers left for one hour at room temperature. Pre-weighed glass filter crucibles dried overnight (102°C) with 0.9 g celite (545, Fluka Chemie AG) were used to filter the solution and the residue was transferred quantitatively to the filters using a Fibertec System E (Tecator, Höganäs, Sweden). The residue was washed 3 times with 20 ml 78% ethanol, once with 10 ml 95% ethanol, 3 times with 10 ml acetone and twice with 10 ml petroleum ether. The filters with the residue were dried overnight at 102°C and weighed. One of the two residues of the same sample was used to determine the protein content according to the method described for the total nitrogen determination (see above) using the factor 6.25 for the conversion to protein. The ash content of the second residue was determined gravimetrically by ashing in a muffle furnace (Heraeus M 104, Heraeus GmbH) during five hours at 525°C. The fiber content was calculated by dividing the mean weight of the two residues, corrected for the blank, the ash and the protein content by the mean

weight of the two samples. A single analysis was performed and the total fiber content was expressed in g/100 g as is.

3.2.4.8. Carbohydrates

The available carbohydrates were calculated according to Souci et al. (1994) using the following formula: available carbohydrates = 100 – (water + protein + fat + total ash + dietary fiber).

3.2.4.9. Reducing sugars

The reducing sugars were determined according to Fehling and the method of Lane-Eynon (Journal Officiel des Communautés Européennes du 22.9.79/No L239/46 à 50) with modifications. The content of reducing sugars in the sample solution was calculated by comparison with a solution containing a defined reducing sugar concentration after titration with Fehling reagent.

Samples of 0.6-2.5 g weaning food were extracted in 70 ml water at 70°C during 15 minutes under frequent agitation. After cooling to room temperature, 50 ml Carrez I solution (3.6% $K_4[Fe(CN)_6]x3\ H_2O$), 5 ml Carrez II solution (7.2 % $ZnSO4x7\ H_2O$) and 10 ml 0.1 M sodium hydroxide were added. Water was added to 100 ml. The solution was used for titration after filtration. The standard solution containing glucose was prepared by dissolving 0.1 g D-glucose (anhydrous) in 100 ml water. The titration was first performed with the standard solution. Fehling solution 25 ml (1:1 mixture of 6.93% $CuSO_4x5\ H_2O$ in water and 34.6% $KNaC_4H_4O_6x4H_2O$ and 100 g sodium hydroxide in water) and 18 ml standard solution were heated to boiling point using an infra red heater and maintained for two minutes at this temperature before adding 1 ml 0.1 % methylene blue in water. Standard solution was added until the disappearance of the blue color and the volume was recorded. The sample solution was titrated in the same way, but starting with 10 ml instead of 18 ml used for the standard solution. Depending on the results, the concentration of the sample solution had to be adjusted. Each sample solution was titrated twice. The content of reducing sugars in the sample was calculated by comparing the mean volumes of sample and standard solution required to titrate 25 ml

Fehling solution to the end point of the titration. A single analysis was performed and the of content reducing sugars was expressed in g/100 g as is.

3.2.4.10. Minerals and trace elements

Calcium, copper, iron and zinc were analyzed using atomic absorption spectrometry (AAS) (atomic absorption spectrometer SpectrAA-400 and GTA-96 graphite furnace atomizer, Varian, Techtron Pty. Limited, Mulgrave, Victoria, Australia) after mineralization of the samples. The parameters of the atomic absorption spectrometer were chosen according to the instructions of Varian (Beach et al. 1988).

3.2.4.10.1. Mineralization

Circa 500 mg weaning food were weighed in high pressure Teflon tubes of a microwave digestion unit (MLS 1200 MEGA, MLS GmbH, Leutkirch, Germany). Five ml nitric acid 65% and 2 ml 30% hydrogen peroxide were added and the covered tubes left for 30 minutes at room temperature, before mineralization in the microwave digestion unit. After mineralization and cooling to room temperature the solutions were transferred to 10 ml polypropylene tubes and water added up to 10 ml. A blank was analyzed with each series of samples. Accuracy of the determinations was verified by analyzing a standard reference material (wheat flour 1567a, National Bureau of Standards, Gaithersburg, USA) in parallel.

3.2.4.10.2. Calcium

Flame AAS (using N_2O as oxidizing gas and acetylene as fuel gas) with standard addition was used to measure the calcium concentration in the mineralized samples. Standard additions were made to each sample after appropriate dilution. Calcium concentrations of the final solutions were in the range 0.2-1.4 µg/ml. Potassium nitrate was added as a matrix modifier. The absorbance of each solution was measured four times and corrected for the value of the blank. The calcium content was calculated with the mean value of absorbance and expressed in mg/100 g as is. Analyses were repeated if the coefficient of variation based on triplicate determination was > 7%.

3.2.4.10.3. Copper

The graphite furnace technique (using argon as purge gas) with external calibration was used to measure the copper concentration in the mineralized samples. Standard solutions with copper concentrations in the range 5-20 ng/ml were prepared for calibration and appropriate dilution of the samples was chosen to be within this range. The absorbance of each sample and standard solution was measured in triplicate. The copper content was calculated with the mean value of absorbance and expressed in mg/100 g as is. Analyses were repeated if the coefficient of variation based on triplicate determination was > 7%.

3.2.4.10.4. Iron

The graphite furnace technique (using argon as purge gas) with external calibration was used to measure the iron concentration in the mineralized samples. Standard solutions with iron concentrations in the range 4-10 ng/ml were prepared for calibration and appropriate dilution of the samples was chosen to be within this range. The absorbance of each sample and standard solution was measured in triplicate. The iron content was calculated with the mean value of absorbance and expressed in mg/100 g as is. Analyses were repeated if the coefficient of variation based on triplicate determination was > 7%.

3.2.4.10.5. Zinc

Flame AAS (using air as oxidizing gas and acetylene as fuel gas) with standard addition was used to measure the zinc concentration in the mineralized samples. Standard additions were made to each sample after appropriate dilution. Zinc concentrations of the final solutions were in the range 0.2-0.7 µg/ml. The absorbance of each solution was measured four times. The zinc content was calculated with the mean value of absorbance and expressed in mg/100 g as is. Analyses were repeated if the coefficient of variation based on triplicate determination was > 7%.

3.2.4.11. Viscosity

The dynamic viscosity was measured with a rheometer Mettler RM260 (Mettler-Toledo GmbH, Greifensee, Switzerland). Samples of 18 g weaning food were mixed with 182 g

water preheated to 40°C. The measurement was conducted under a constant temperature of 40°C, applying a constant shear rate of 100 s^{-1} during 2 minutes. The final viscosity was measured after 1.98 minutes and expressed in mPas. Samples were analyzed in duplicate and repeated if the difference was > 50 mPas.

3.2.4.12. Microbiology

Microbiological analyses of the weaning foods were carried out according to the International Standards for coliforms (ISO 4831:1991), *Escherichia coli* (ISO 7251:1993), yeast and molds (ISO 7954:1987), *Staphylococcus aureus* (ISO 6888:1983). Enumeration of aerobic mesophilic microorganisms was performed according to the FDA Bacteriological analytical manual (1992) and validated according to AOAC (Method 990.12 Petrifilm validation).

3.3. Results and Discussion

3.3.1. Inositol phosphate content and phytase activity of grains and seeds

All milled grains and seeds used for laboratory experiments and pilot plant trials were analyzed for their inositol phosphate content. Milled grains used as the phytase source, i.e., whole grain rye, wheat and buckwheat, as well as wheat with low extraction rate and soybean were analyzed for phytase activity. In this study, all results were expressed on weight as is basis. Table 3.3. shows the inositol phosphate content in g/100 g and the phytase activity in PU/g of the milled grains and seeds used in laboratory experiments and pilot plant trials.

Table 3.3. Inositol phosphate content and phytase activity of grains and seeds used in laboratory experiments and pilot plant trials

Grains and seeds		Inositol phosphate content IP5+IP6 [g/100 g]	Phytase activity [PU/g]
Laboratory experiments			
Cereals	Maize whole grain	0.90	n.a.*
	Millet dehulled	0.55	n.a.
	Rice polished	0.13	n.a.
	Rye whole grain	0.68	6.0
	Wheat whole grain	0.89	2.7
	Wheat low extraction rate (550)	0.19	0.6
Pseudocereal	Buckwheat whole grain	1.00	2.2
Legumes	Chickpea whole grain	0.44	n.a.
	Cowpea dehulled	0.74	n.a.
	Soybean dehulled toasted	0.90	0.2
Pilot plant trials			
Cereals	Rye whole grain	0.81	4.8
	Wheat whole grain	0.92	3.4
	Wheat low extraction rate (550)	0.15	0.5
Legume	Soybean dehulled toasted	1.07	0.2

*n.a.: not analyzed

The inositol phosphate content (sum of IP5 and IP6) of whole grain cereals and buckwheat was between 0.7 and 1.0 g/100 g, while polished rice and wheat with low extraction rate contained circa 0.1 and 0.2 g/100 g inositol phosphate. The inositol phosphate content of the legumes varied from 0.4 for chickpea to 1.1 g/100 g for soybean. The phytase activity in whole grain rye was higher in the sample used in the laboratory experiments than in the sample used in the pilot plant trials, 6.0 and 4.8 PU/g respectively. Phytase activity in whole grain wheat was slightly higher in the sample used in the pilot plant trials than in the sample for the laboratory experiments, 3.4 and 2.7 PU/g respectively. Phytase activity in wheat with low extraction rate was 0.5-0.6 PU/g and 0.2 PU/g in soybean. These differences were not expected to substantially influence inositol phosphate degradation.

3.3.2. Inositol phosphate degradation in laboratory experiments

The influence of the addition of a phytase source, i.e., whole grain rye, wheat or buckwheat on inositol phosphate degradation in cereals or legumes and weaning food mixtures based on cereals and legumes was investigated. All grains and seeds were milled to flours and made into a slurry in which the conditions (pH, temperature, dry matter) were optimized for inositol phosphate degradation.

3.3.2.1. Conditions for inositol phosphate degradation

Conditions for the inositol phosphate degradation were optimized by investigating stability of temperature and pH, dry matter content of the slurry and reproducibility. Temperature of the samples was kept constant ($\pm 2°C$) by using an incubator and preheated samples. A maximum pH shift of 0.2 units was found during incubation of the samples. Maximum dry matter content was 25% to make homogeneous slurries. For each time point, separate samples were incubated in duplicate or triplicate, and the reaction was stopped by addition of hydrochloric acid, which was directly used for the extraction of the inositol phosphates. Depending on the initial inositol phosphate content and the phytase source, incubation times from 20 to 280 minutes were chosen to monitor the inositol phosphate degradation.

3.3.2.2. Determination of inositol phosphate degradation

Inositol phosphate (IP3, IP4, IP5, IP6) concentrations obtained using an HPLC technique with refractive index detection were similar to the concentrations calculated from liberated inorganic phosphate (P_i) using a colorimetric method (described in chapter 2.2.3.3.4.), indicating that P_i was mainly liberated from inositol phosphates. The more time consuming HPLC technique was used because the individual inositol phosphate concentrations were needed in order to evaluate mineral and trace element bioavailability.
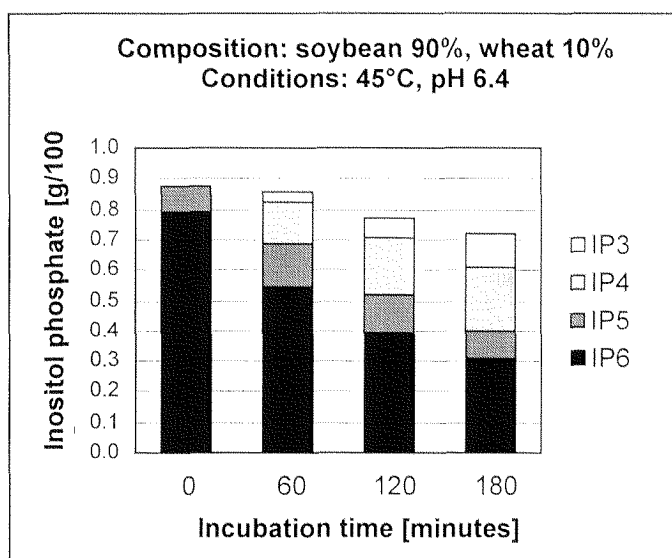
3.3.2.3. Inositol phosphate degradation in cereals and legumes

The influence of the addition of whole grain wheat, rye or buckwheat as a phytase source on the inositol phosphate degradation was first investigated in soybean, maize and rice without and with pH adjustment. In the following figures all detectable inositol phosphate (IP3, IP4, IP5, IP6) concentrations are reported in g/100 g for each time point of the incubation. The evaluation mostly focuses on the content of IP5 and IP6, as they have the strongest influence on mineral and trace element bioavailability (Sandström and Sandberg 1992, Sandberg et al. 1999).

3.3.2.3.1. Influence of whole grain wheat or rye and pH adjustment on inositol phosphate degradation in soybean and maize

Inositol phosphate degradation in soybean with 10% whole grain wheat is shown in Figure 3.1. Slurries with an approximate dry matter content of 15% were incubated at 45°C for 0-180 minutes at pH 6.4 (no pH adjustment). After 180 minutes of incubation, the sum of IP5 and IP6 was decreased to 45% of the initial content. The total inositol phosphate content (IP3, IP4, IP5, IP6) was still rather high at 0.72 g/100 g.

Figure 3.1. Inositol phosphate degradation in soybean with 10% whole grain wheat

Inositol phosphate degradation in soybean with 10% whole grain rye is shown in Figure 3.2. Dry matter content of the slurries, pH, temperature and incubation time were identical to the soybean/wheat mixture. The inositol phosphate degradation was faster as compared to wheat. After 180 minutes, IP6 was degraded to 11% of its initial content, IP5 was no longer detectable and the total inositol phosphate content was 0.32 g/100 g.

While in the first two experiments the inositol phosphate degradation was performed in water, the influence of pH adjustment with acetate buffer was investigated in the third experiment. Figure 3.3. shows the inositol phosphate degradation in soybean with 10% whole grain rye with pH adjustment to circa 5.4 (using 0.2 M acetate buffer). Dry matter content of the slurries, temperature and incubation time as previously described. After

60 minutes, the sum of IP5 and IP6 was decreased to 20% of the initial content and after 120 minutes IP5 and IP6 were not detectable. After 180 minutes, only 0.1 g/100 g IP3 and 0.06 g/100 g IP4 were left.

Figure 3.2. Inositol phosphate degradation in soybean with 10% whole grain rye
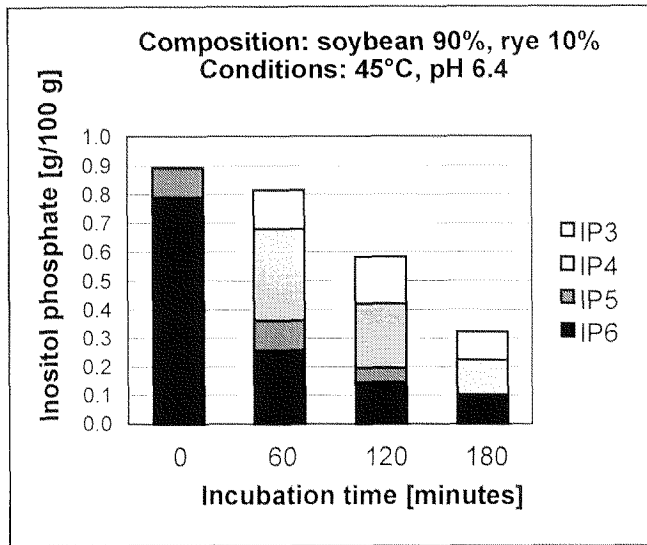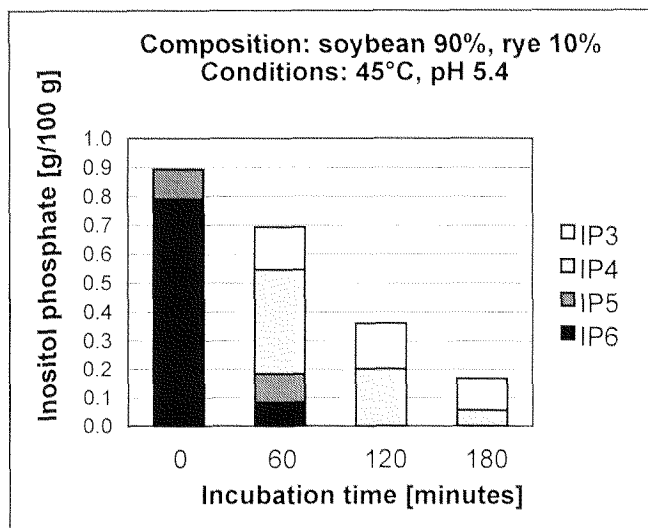

Figure 3.3. Inositol phosphate degradation in soybean with 10% whole grain rye with pH adjustment

Compared to the incubation in water, the inositol phosphate degradation was considerably accelerated when pH was adjusted to the optimal conditions for rye phytase. Similar results were obtained by using citric acid instead of acetate buffer for pH adjustment (results not shown). In all following experiments, pH was adjusted with

citric acid as it is a permitted additive for processed cereal-based foods for infants and children according to Codex Alimentarius (FAO/WHO 1994) and to the Swiss food legislation (Zusatzstoffverordnung 1995). pH was adjusted to the optimum for each phytase source, i.e., 5.1 for wheat, 5.5 for rye and 5.0 for buckwheat, according to data of the screening study.

Experimental conditions used for the inositol phosphate degradation in soybean with 10% whole grain rye (45°C, pH 5.4) were similar to the phytase assay in the screening study (45°C, pH 5.0). The liberation of P_i, calculated from the inositol phosphate degradation was at a constant rate during 120 minutes and slowed down slightly afterwards. After 120 minutes circa 53 µmol P_i were liberated per gram, thus resulting in a phytase activity of approximately 4.5 PU/g for rye, not considering the phytase activity of soybean. This value was somewhat lower than 6 PU/g determined for rye in the phytase assay, possibly due to the lower substrate concentration, which generally slows down enzymatic reactions and/or to the availability of the substrate, which is mostly associated with proteins in soybean, as in all legumes. However, it shows that the inositol phosphate degradation in cereals or legumes can be estimated from the enzyme activity of the phytase source determined in the phytase assay.

Figure 3.4. and Figure 3.5. show the inositol phosphate degradation in maize with 10 % whole grain wheat or rye respectively. pH was adjusted with citric acid to 5.2 in the maize/wheat mixture and to 5.4 in the maize/rye mixture. Slurries with a dry matter content of circa 15% were incubated at 50°C. The phytic acid degradation with wheat as the phytase source was slower as compared to rye: after 180 minutes of incubation 0.02 g/100 g IP5 and 0.02 g/100 g IP6 were left, as well as IP3 (0.28 g/100 g) and IP4 (0.21 g/100 g). Complete inositol phosphate degradation required six hours of incubation. When rye was used as the phytase source, IP5 and IP6 were not detectable after 120 minutes. After 180 minutes only 0.01 g/100 g IP3 was left.

In both soybean and maize, whole grain rye was shown to be the more efficient phytase source than whole grain wheat, thus confirming the results of enzyme activity determined in the phytase assay since phytase activity in rye was more than double that

of wheat. Addition of 10% whole grain rye to soybean or maize was shown to degrade the high initial inositol phosphate content (IP5+IP6) of 0.89/100 g or 0.97 g/100 g completely in 240 minutes, after adjustment of pH.

Figure 3.4.   Inositol phosphate degradation in maize with 10% whole grain wheat
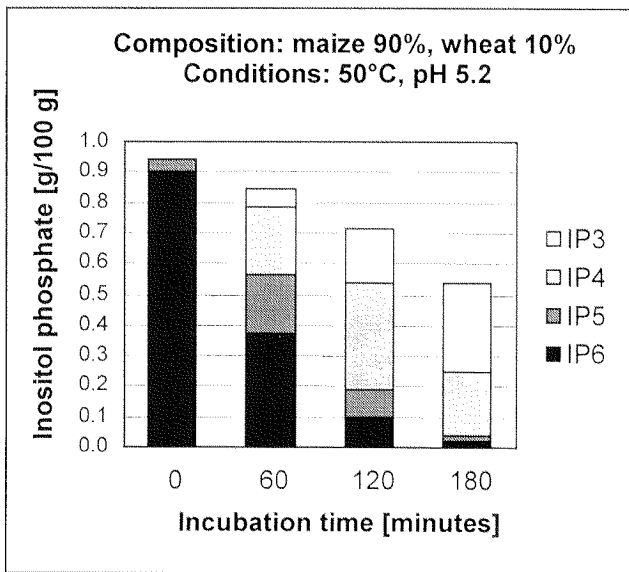

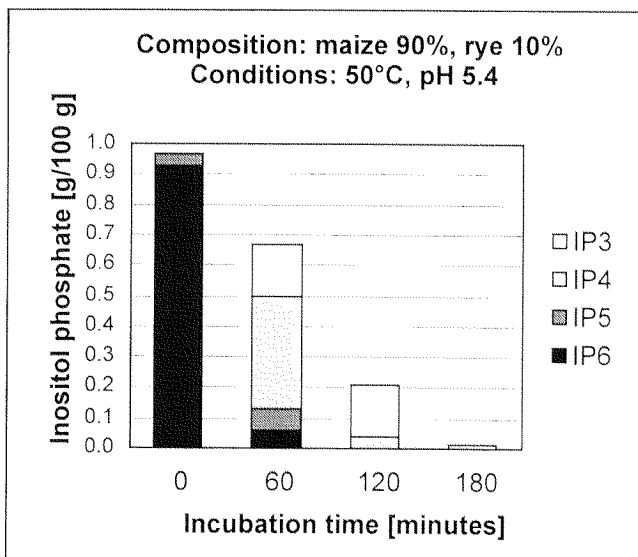Figure 3.5.   Inositol phosphate degradation in maize with 10% whole grain rye


Addition of 10% whole grain rye, wheat or buckwheat as the phytase source was based on the results from the screening study and on the impact on the overall composition of the mixtures. Addition of 10% of the phytase sources was expected to sufficiently increase phytase activity to degrade even high initial inositol phosphate contents

completely within a reasonable incubation time. Addition of higher amounts of whole grain cereals would not only increase the phytase activity, but also the initial inositol phosphate content as well as the fiber content.

3.3.2.3.2. Influence of whole grain buckwheat on inositol phosphate degradation in rice

Buckwheat has the advantage over wheat and rye, that it is gluten free. Figure 3.6. shows the inositol phosphate degradation in polished rice with 10% whole grain buckwheat. Slurries with a dry matter content of circa 20% and pH adjusted to 5.0 were incubated at 55°C. After 100 minutes the rather low initial inositol phosphate content of 0.21 g/100 g was completely degraded. The liberation of P_i, calculated from the inositol phosphate degradation was at a constant rate during 60 minutes, but slowed down afterwards. After 60 minutes circa 17 μmol P_i were liberated per g, thus resulting in a phytase activity of approximately 2.8 PU/g for buckwheat, not considering the phytase activity of rice. This corresponds to the enzyme activity determined in the phytase assay in the screening study under the same conditions (2.4 PU/g).

Figure 3.6. Inositol phosphate degradation in rice with 10% whole grain buckwheat
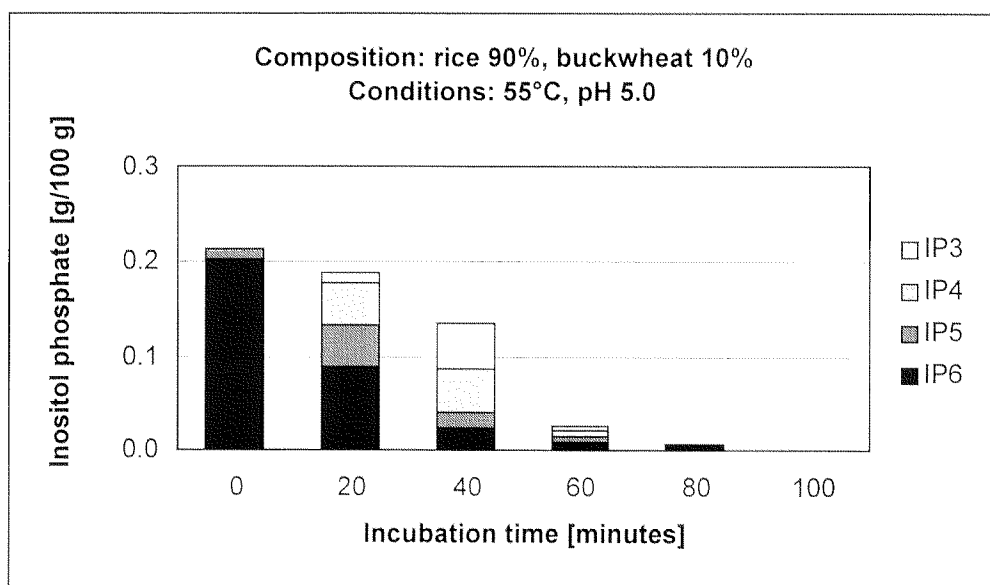
3.3.2.4. Inositol phosphate degradation in weaning food mixtures based on cereals and legumes

Cereals and legumes were mixed to produce weaning food mixtures with a protein content of circa 15%. The proportions of cereals and legumes were calculated, based on the analyzed protein content of the grains and seeds or from food composition tables (Souci et al. 1994). As weaning foods are often based on cereal/legume mixtures, in this study they will be called weaning food mixtures. The phytase source, i.e. 10% whole grain rye, wheat or buckwheat was added to the weaning food mixtures and conditions (pH and temperature) were optimized for each phytase source. The inositol phosphate degradation was performed as described previously. The aim was to reach complete degradation, i.e. no more detectable inositol phosphates. The determination limit of the method was 3 mg/100 g (chapter 2.3.3.4.1.1.) and 'no phytic acid' means that the inositol phosphate content was below the determination limit.
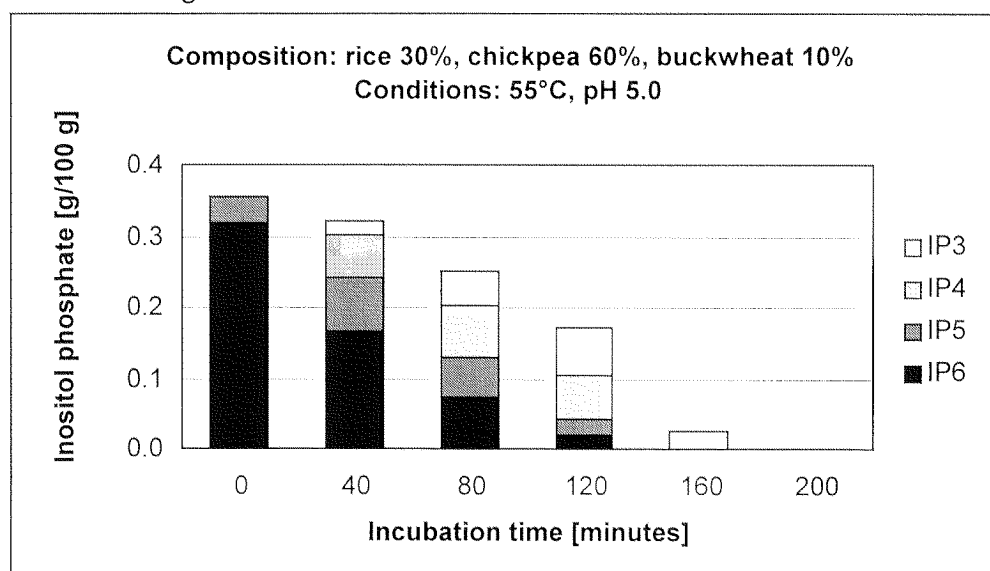
Inositol phosphate degradation during incubation was monitored in the following weaning food mixtures: (a) a gluten free mixture based on rice and chickpea with 10% whole grain buckwheat as the phytase source; (b) a mixture based on millet and cowpea with 10% whole grain wheat, and (c) a mixture based on the most commonly consumed cereal and legume, i.e. wheat and soybean with either 10% whole grain wheat or 10% whole grain rye.

Figure 3.7. shows the inositol phosphate degradation in the gluten free mixture based on 30% rice and 60% chickpea with 10% whole grain buckwheat. The relatively high proportion of the legume in the mixture was due to the low protein content of the rice. Slurries with circa 20% dry matter content and pH 5.0 were incubated at 55°C. The rather low initial inositol phosphate content of 0.36 g/100 g was completely degraded after 200 minutes.

The liberation of P_i, calculated from the inositol phosphate degradation was at a constant rate during 160 minutes. After 160 minutes, circa 30 μmol P_i were liberated per gram, resulting in a phytase activity of approximately 1.9 PU/g for buckwheat, not considering the phytase activity of rice and chickpea. This value was lower than the

phytase activity of buckwheat (2.8 PU/g) in the rice/buckwheat mixture incubated under the same conditions. This shows that not only the conditions, but also the components of the mixtures and probably the binding of the inositol phosphate to other food components in the grains and seeds influence the degradation. As in all legumes, inositol phosphates in chickpea are mostly associated with proteins.

Figure 3.7. Inositol phosphate degradation in rice and chickpea with 10% whole grain buckwheat
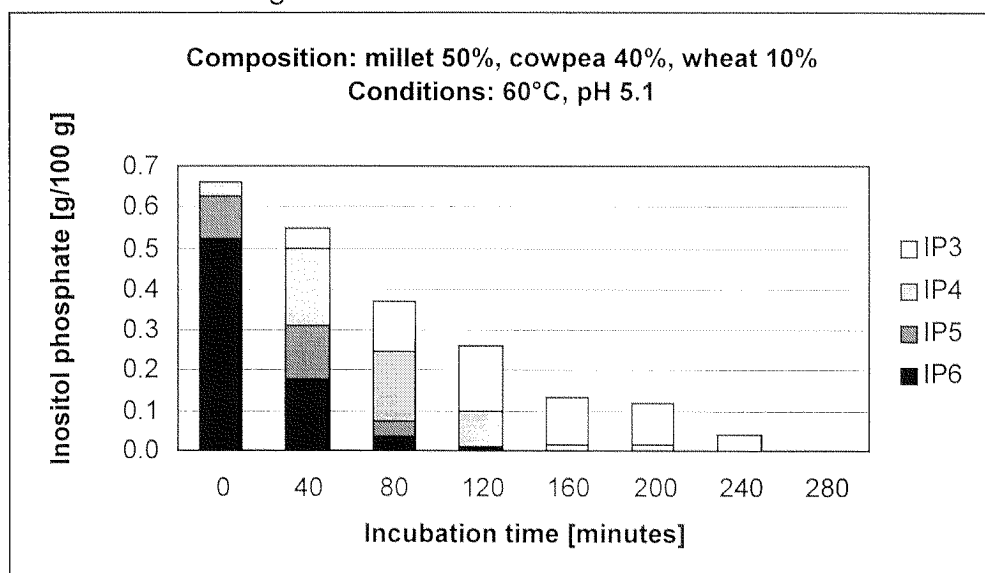

Inositol phosphate degradation was also measured under the same conditions as in the rice/chickpea mixture without addition of whole grain buckwheat (results not shown). After 200 minutes the initial inositol phosphate content of 0.3 g/100 g was degraded to 0.07 g/100 g IP3, 0.07 g/100 g IP4, 0.02 g/100 g IP5 and 0.01 g/100 g IP6, comparable to the inositol phosphate content after 120 minutes in the mixture containing buckwheat. Thus, the phytase activity of chickpea and rice also contributed to the inositol phosphate degradation. However, addition of buckwheat considerably shortened the incubation time for complete inositol phosphate degradation.

Figure 3.8. shows the inositol phosphate degradation in a weaning food mixture based on 50% millet, 40% cowpea and 10% whole grain wheat. Slurries with circa 25% dry matter content and pH 5.1 were incubated at 60°C. Complete inositol phosphate degradation required 280 minutes. IP5 and IP6 were completely degraded after 160 minutes. The rather long incubation time to achieve complete degradation of all inositol phosphates was due to the relatively high initial inositol phosphate content

(0.66 g/100 g) and to the slow enzymatic reaction after 160 minutes. However, an incubation time of 160 minutes might be sufficient as IP3 and IP4 are not expected to have a negative influence on mineral and trace element bioavailability.

Figure 3.8. Inositol phosphate degradation in millet and cowpea with 10% whole grain wheat

Inositol phosphate degradation was also measured under the same conditions in a millet/cowpea mixture without addition of whole grain wheat (results not shown). After 240 minutes 70% of the initial IP5 and IP6 content were still left and the total inositol phosphate content was 0.59 g/100 g, showing that phytase in millet and cowpea did not contribute significantly to the inositol phosphate degradation. Inositol phosphate degradation without addition of a phytase source would not be feasible in this mixture.

Figure 3.9. shows the inositol phosphate degradation in a weaning food mixture based on 70% wheat with low extraction rate and 20% soybean with 10% whole grain wheat and Figure 3.10. shows the inositol phosphate degradation in the same mixture with 10% whole grain rye. Slurries of the mixture with whole grain wheat with a dry matter content of circa 25% and pH 5.1 were incubated at 50°C. The initial inositol phosphate content of 0.40 g/100 g was decreased to 0.01 IP6 and 0.01 g/100 g IP3 after 60 minutes. After 80 minutes only IP3 was detected. The degradation slowed down after 60 minutes and complete degradation required 120 minutes. Slurries of the mixture with whole grain rye with a dry matter content of circa 25% and pH 5.4 were also incubated at 50°C. The initial inositol phosphate content of 0.38 g/100 g was degraded to 0.01 IP6

and 0.01 g/100 g IP3 after 40 minutes. The rapid initial inositol phosphate was reduced after 40 minutes and complete degradation required 100 minutes. Although the enzymatic reaction in the mixture with whole grain wheat was slower during the first 40 minutes of incubation, complete inositol phosphate degradation required only 20 minutes longer as compared to the mixture with whole grain rye. Compared to the other mixtures, complete inositol phosphate degradation was achieved in a relatively short time (100 to 120 minutes), probably due to the contribution of phytase activity in the wheat with low extraction rate (0.6 PU/g under phytase screening conditions).

Figure 3.9.   Inositol phosphate degradation in wheat with low extraction rate and soybean with 10% whole grain wheat
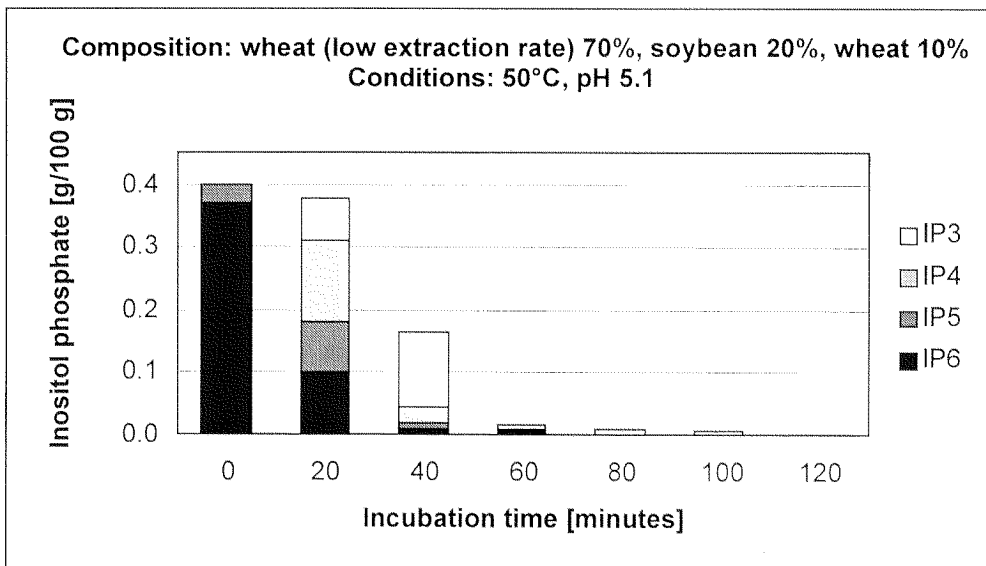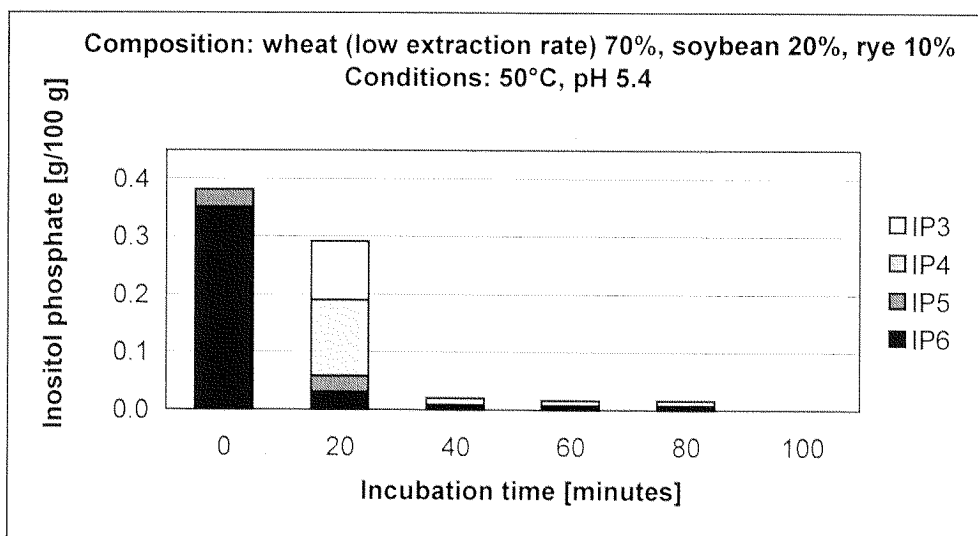

Figure 3.10. Inositol phosphate degradation in wheat with low extraction rate and soybean with 10% whole grain rye

3.3.3. Weaning food production in the pilot plant

3.3.3.1. Weaning foods with no phytic acid

The feasibility of applying the laboratory scale method for inositol phosphate degradation to large scale weaning food production was tested in a pilot plant. The two weaning food mixtures based on 70% wheat with low extraction rate and 20% soybean, with 10% whole grain wheat or 10% whole grain rye were chosen for the pilot plant trials, as the degradation could be completed in a relatively short time and because these ingredients are widely used for commercial weaning foods. The conditions (dry matter content of the slurry, pH, temperature), remained as in the laboratory experiments, but the weight of weaning food components was increased from 4 g to 40 kg. A maximum shift of 0.1 pH unit was observed and the temperature ranged from 48 to 52°C during incubation. The incubation of the slurry was followed by heating and drying to produce a precooked, dry weaning food, to be reconstituted with water. Samples of the slurries were taken every 20 minutes to monitor the inositol phosphate degradation during incubation.

Figure 3.11. shows the inositol phosphate degradation during weaning food production in a mixture based on 70% wheat with low extraction rate and 20% soybean with 10% whole grain wheat and Figure 3.12. shows the inositol phosphate degradation in the same mixture with 10% whole grain rye. The first sample for the inositol phosphate determination was taken when the slurry was homogeneously mixed and pH adjusted, i.e., after circa 10 minutes. This time point is indicated as incubation time zero in Figure 3.11. and 3.12. Therefore, the initial inositol phosphate content was calculated based on the inositol phosphate content of the weaning food components. The corresponding columns in the Figures 3.11. and 3.12. are labeled 'calc.' as they are calculated. In the weaning food containing whole grain wheat, IP5 and IP6 were decreased to 48% of the initial calculated value (0.41 g/100 g) in the first sample (incubation time zero). Complete degradation of all inositol phosphates required 80 minutes. In the weaning food containing whole grain rye, IP5 and IP6 were decreased to 35% of the initial content in the first sample (incubation time zero). Complete degradation of all inositol phosphates was reached after 100 minutes.

Figure 3.11. Inositol phosphate degradation during weaning food production in wheat with low extraction rate and soybean with 10% whole grain wheat

Composition: wheat (low extraction rate) 70%, soy 20%, wheat 10%
Conditions: 50°C, pH 5.1
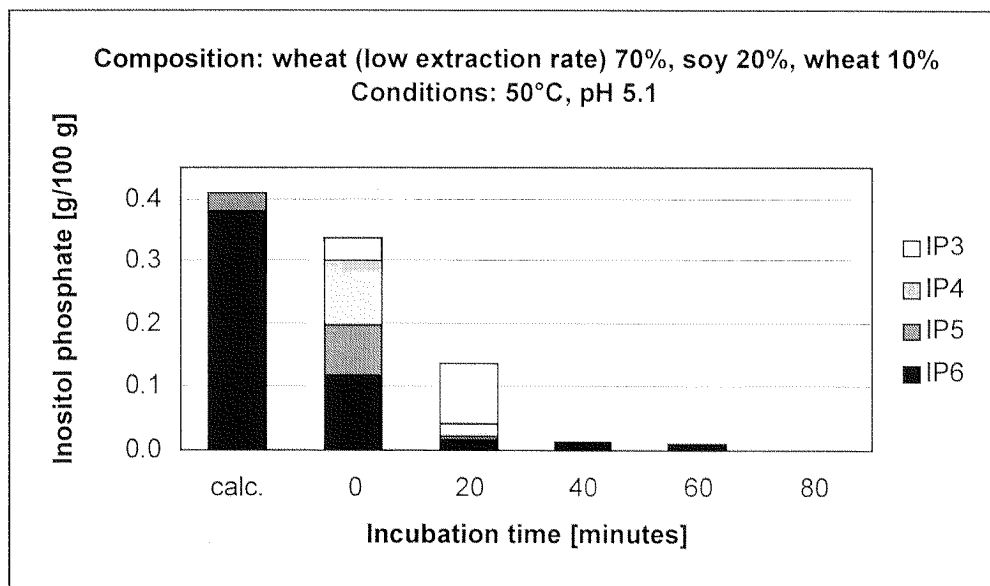
Figure 3.12. Inositol phosphate degradation during weaning food production in wheat with low extraction rate and soybean with 10% whole grain rye

Composition: wheat (low extraction rate) 70%, soy 20%, rye 10%
Conditions: 50°C, pH 5.5
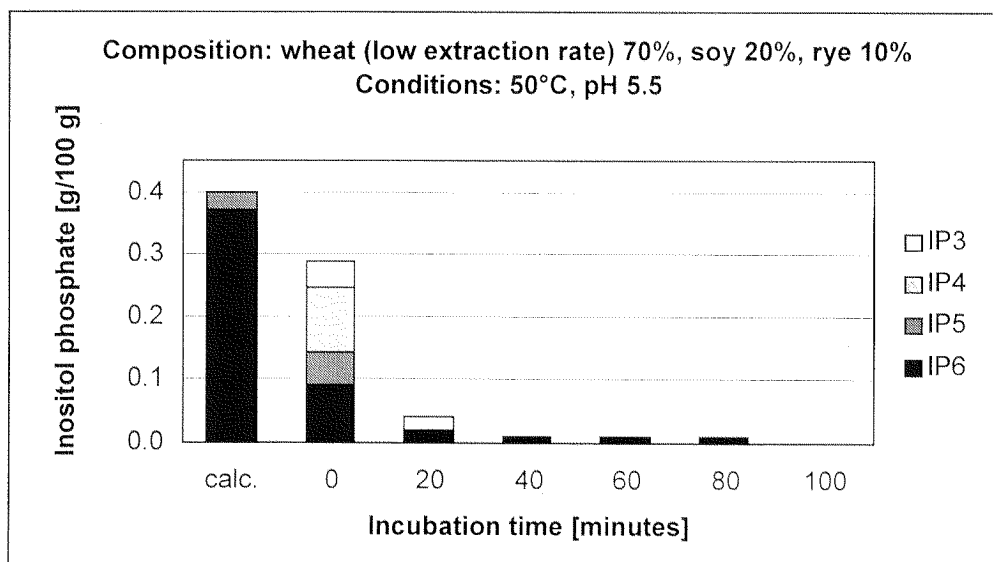
Results of the inositol phosphate degradation during the pilot plant trials correspond well to results of the laboratory experiments, thus showing that upscaling from 4 g to 40 kg had little influence on the enzymatic reaction. Laboratory experiments are therefore a useful tool to predict inositol phosphate degradation in large scale production.

3.3.3.1.1. Increase of dry matter of the slurry

In the laboratory experiments, the dry matter content was limited to circa 25% due to practical considerations. This is a low dry matter content for large scale weaning food production, especially for roller drying. Therefore a pilot plant trial with increased dry matter content of the slurry, circa 35% was carried out. The weaning food mixture, based on 70% wheat with low extraction rate, 20% soybean and 10% whole grain wheat was used, 60 kg added to 90 kg water (40 kg to 100 kg water in the previous trials), while all other conditions remained unchanged.

The addition of the weaning food mixture to the water and pH adjustment required circa 20 minutes and IP5 and IP6 were degraded to 47% of the initial content when the first sample was taken (results not shown). After 60 minutes, 0.008 g/100 g IP6 were left and another 80 minutes were required for complete inositol phosphate degradation. The inositol phosphate degradation during the first 40 minutes of the incubation was comparable to results from the slurry with 25% dry matter. Complete degradation required a slightly longer incubation time, 140 minutes.

3.3.3.2. Weaning food with native phytic acid content

A weaning food with identical composition, i.e., 70% wheat with low extraction rate, 20% soybean and 10% whole grain wheat, but without inositol phosphate degradation was produced for comparison. The weaning food production included continuos mixing of the components with water (circa 10°C) and citric acid for pH adjustment, followed by immediate heating and roller drying. An sample taken before heating and drying of the slurry had an inositol phosphate content of 0.38 g/100 g as compared to the calculated initial content of 0.41 g/100 g. Thus, inositol phosphate was not degraded during weaning food production.

3.3.4. Weaning food characteristics

The two weaning foods based on wheat with low extraction rate, soybean and whole grain wheat, one with the native phytic acid content and one with no phytic acid were

analyzed for their chemical composition and viscosity, shown in Table 3.4. and for microbiological safety.

Table 3.4. Chemical composition and viscosity of the weaning foods

			Weaning food with native phytic acid	Weaning food with no phytic acid
Inositol phosphates	IP3	[g/100 g]	0.001	n.d.*
	IP4	[g/100 g]	0.018	n.d.
	IP5	[g/100 g]	0.062	n.d.
	IP6	[g/100 g]	0.338	n.d.
	IP5+IP6	[g/100 g]	0.400	
Dry matter		[g/100 g]	98.1	98.2
Total ash		[g/100 g]	1.6	1.6
Protein		[g/100 g]	18.3	18.6
Fat		[g/100 g]	6.2	6.4
Dietary fiber		[g/100 g]	5.6	6.0
Available carbohydrates		[g/100 g]	66.4	65.7
Reducing sugars		[g/100 g]	0.8	6.6
Minerals, trace elements	Zinc	[mg/100 g]	2.0	2.0
	Copper	[mg/100 g]	0.4	0.4
	Iron	[mg/100 g]	2.1	2.0
	Calcium	[mg/100 g]	71.7	74.1
Viscosity		[mPas]	353	180

n.d.*: not detectable

3.3.4.1. Chemical composition

The major difference between the two weaning foods was the phytic acid content. In the weaning food which had been produced according to the newly developed method, no phytic acid was detectable (< 3 mg/100 g). The other weaning food, which had been heated and dried immediately after mixing of the components, contained 0.001 g/100 g IP3, 0.02 g/100 g IP4, 0.06 g/100 g IP5 and 0.34 g/100 g IP6. The phytic acid content of circa 0.4 g/100 g corresponded well to inositol phosphate contents of commercial weaning foods. Thirty weaning foods, available in Switzerland, based on cereals and/or legumes were analyzed and a mean content of 0.3 g/100 g (sum IP5 and IP6) was found, with values ranging from 0.02 to 1.02 g/100g (results not shown).

Another important difference between the two weaning foods was the content of reducing sugars. In the weaning food with no phytic acid, starch was partly degraded during incubation due to activation of carbohydrate degrading enzymes, such as

amylase, which have pH and temperature optima in the same range as phytase (Hamer 1995).

The Codex Alimentarius for processed cereal-based foods for infants and children defines a minimum protein content of 15% for dry cereals, which are intended to be mixed with water before consumption (FAO/WHO 1994). The protein content (circa 18%) of the weaning foods in this study is in agreement with the Codex Alimentarius and the combination of cereals and legumes is known to results in a high protein quality. The optimal content of dietary fiber in weaning foods is not known, but a maximum level of 5% crude fiber has been suggested (FAO 1985), which would correspond to circa 10% dietary fiber (Davidsson et al. 1996a). The dietary fiber content (circa 6%) of the weaning foods in this study are within the recommendations and are not expected to have a negative influence on the absorption of energy and nutrients (Davidsson et al. 1996a). The mineral and trace element contents of the weaning foods were as would be expected from the ingredients.

3.3.4.2. Viscosity

The viscosity was measured after reconstitution of the weaning foods with water. The viscosity of the weaning food with no inositol phosphates was lower (180 mPas), than in the weaning food with the native inositol phosphate content (350 mPas). The lower viscosity can be explained by activation of the carbohydrate degrading enzymes during weaning food production. The viscosity decreased due to the degradation of polysaccharides, i.e., starch.

3.3.4.3. Microbiology

The microbiological quality of the weaning food was in agreement with the Swiss food legislation (Hygieneverordnung 1995).

3.3.5. Advantages of weaning food production with naturally occurring phytase

The method developed to degrade phytic acid during weaning food production using naturally occurring phytase has not been described before and has several advantages. The phytic acid in mixtures of cereals and legumes can be degraded during the normal weaning food production by including an incubation step. The processing conditions (temperature, pH) have to be only slightly adapted. The phytase used for the degradation naturally occurs in the ingredients of the weaning foods, and therefore no phytase, such as that extracted from *Apergillus niger* or genetically engineered phytase has to be added. In foods for human consumption microbial phytase has only been used to degrade phytic acid in meals for absorption studies and there might be objections to the use of microbial phytase in weaning foods. The overall composition of the weaning foods does not change considerably as the addition of 10% whole grain rye, wheat or buckwheat is sufficient to increase the phytase activity. Wheat is the most commonly consumed cereal and thus readily available worldwide.

Other processing techniques to degrade phytic acid in weaning foods, such as soaking, germination and fermentation have been described by several authors (Marero et al. 1988, 1991, Gupta and Sehgal 1991, Lorri and Svanberg 1995, Mensah et al. 1995, Gimbi et al. 1997, Sanni et al. 1999). These techniques are much more complicated and change the composition, viscosity and taste of the weaning foods considerably which can result in problems of acceptance by the infants. Complete phytic acid degradation generally requires prolonged fermentation which may cause problems of microbiological safety.

3.4. Summary

The whole grain cereals rye and wheat and the pseudocereal buckwheat were selected from the screening study as sources of phytase. They were milled, added to milled cereals and legumes and the mixtures incubated as slurries for inositol phosphate degradation. Rye was found to be the most efficient phytase source, thus confirming the results of the screening study. Incubation conditions were optimized on a laboratory

scale and applied to mixtures of cereals and legumes, suitable for weaning food production. Weaning food mixtures based on the cereals maize, millet, rice or wheat and the legumes chickpea, cowpea or soybean were prepared and inositol phosphate degradation monitored. Depending on the weaning food mixtures and the phytase source, complete inositol phosphate degradation required 80 to 280 minutes. Generally, the enzymatic reaction was rapid at the beginning and slowed down during incubation, probably due to decreasing substrate concentration as well as to the availability of the substrate.

The application of the method for phytic acid degradation, developed in the laboratory, to commercial weaning food production was evaluated in a pilot plant. Weaning food mixtures based on wheat and soybean were selected for the pilot plant trials. Inositol phosphate degradation during weaning food production in the pilot plant was found to be comparable to the laboratory experiments. Depending on the phytase source and dry matter content of the incubated slurries, complete inositol phosphate degradation was achieved in 80 to 140 minutes.

Two weaning foods were produced in the pilot plant, one according to the method for inositol phosphate degradation and one without the degradation step. The chemical composition and microbiological safety of the weaning foods were evaluated. The major difference between the two weaning foods was the phytic acid content, one containing no phytic acid (< 3 mg/100 g) one with the native content of circa 0.4 g/100 g. Other differences were found in the content of reducing sugars and in the viscosity measured after reconstitution of the weaning foods. The overall composition of the two products was comparable and similar to commercial products and the microbiological quality was within the Swiss regulations. The two weaning foods were used in a human study to evaluate the impact of phytic acid on zinc and copper absorption.

4. The influence of phytic acid on zinc and copper apparent absorption in adults. An evaluation of a weaning food based on wheat and soybeans.

4.1. Introduction

Weaning foods based on cereals and legumes often contain relatively high amounts of phytic acid. The negative influence of phytic acid on iron absorption in adults and infants is well established in the literature (Hallberg et al. 1989a, Hurrell et al. 1992, Davidsson et al. 1994a, Sandberg et al. 1999). Even small amounts of phytic acid inhibit iron absorption and phytic acid has to be reduced completely to overcome this effect (Hurrell et al. 1992). The influence of phytic acid on zinc absorption has been investigated in numerous studies and molar ratios of phytic acid to zinc have been derived from animal studies to estimate the effect of phytic acid on zinc absorption (Oberleas and Harland 1981, Ellis et al. 1987). However, the predictive use of these ratios for humans was doubted (Wise 1995). The results of human studies remain contradictory as not all showed an increased in zinc absorption from products with low phytic acid content (Nävert and Sandström 1985, Kivistö et al. 1989, Fairweather-Tait et al. 1992, Davidsson et al. 1996a, Larsson et al. 1996). There is only very limited information on the influence of phytic acid on copper absorption. *In vitro* studies suggest phytic acid to be an inhibitor of copper absorption, as the binding of phytic acid to copper is stronger than to zinc (Vohra et al. 1965, Persson et al. 1998). However, there are only few animal and human studies which showed contradictory results (Turnlund et al. 1985, Lee et al. 1988, Morris et al. 1988, Lönnderdal et al. 1999).

The aim of this study was to measure the influence of complete phytic acid degradation in a weaning food based on wheat and soybeans on zinc and copper apparent absorption in adults. A dephytinized weaning food, produced using a newly developed method which utilizes naturally occurring phytase, was compared with a weaning food containing the native phytic acid content. The weaning foods were labeled extrinsically with stable isotopes of zinc and copper and six day fecal monitoring technique was used

to determine the apparent zinc and copper absorption. The study was conducted with ten adults, each subject acting as her/his own control.

4.2. Subjects and methods

All chemicals and reagents were of suprapur quality (Fluka Chemie AG, Buchs, Switzerland; Merck, Darmstadt, Germany) and nitric acid and hydrochloric acid were further purified by sub-boiling. For analytical procedures, all tubes, containers and pipette-tips were acid-washed. Water, purified with ion exchange and reverse osmosis (18MΩ) (RD 2000, Renggli, Rotkreuz, Switzerland; Nanopure Cartridge System, Skan AG, Basel, Switzerland) was used for analytical procedures, preparation of test meals and as drinking water.

4.2.1. Subjects

Eight healthy premenopausal women and two adult men were recruited among students and employees at the University and the Swiss Federal Institute of Technology in Zurich, Switzerland. Exclusion criteria included pregnancy, lactation, gastrointestinal disorders, metabolic or chronic diseases. No medication (except for oral contraceptives) nor any vitamin or mineral supplements were allowed two weeks before the start of the study and during the entire study. The subjects were asked not to change dietary habits or life style during the study. The aims and procedures of the study were explained orally and in writing and written informed consent was obtained from each subject. All data obtained was coded and treated confidentially and was exclusively used for scientific purposes. The study protocol was approved by the Ethical Committee of the Swiss Federal Institute of Technology Zurich.

4.2.2. Stable isotopes

Elemental zinc highly enriched in ^{70}Zn (73.7%) was purchased from Chemgas (Paris, France). ^{70}Zn was dissolved in concentrated hydrochloric acid and diluted with water to a final concentration of circa 0.8 mg/g in 0.1 M hydrochloric acid. Elemental copper highly enriched in ^{65}Cu (99.2%) was purchased from Chemgas. ^{65}Cu was dissolved in

concentrated nitric acid and transformed into its chloride form by fuming with concentrated hydrochloric acid. The solution was diluted with water to a final concentration of circa 1 mg/g in 0.1 M hydrochloric acid.

The isotopic composition of the isotopic labels was determined by thermal ionization mass spectrometry (TIMS, see below). The zinc and copper concentration of the isotopic labels were determined by reversed isotope dilution mass spectrometry. Elemental concentrations of zinc were determined against a commercial standard (Titrisol, Merck). For the characterization of the copper isotopic label, a standard was prepared gravimetrically from an isotopic reference material in elemental form (SRM 976, National Institute of Standards and Technology, Gaithersburg, USA).

4.2.3. Dysprosium

Dysprosium(III) chloride hexahydrate (Sigma-Aldrich, Steinheim, Germany) was dissolved in 1 mM hydrochloric acid to a final concentration of circa 1.5 mg/g dysprosium. The concentration was determined by inductively coupled plasma mass spectrometry (ICPMS, see below). Circa 1 g of the dysprosium solution was added to 150 g water and the exact weight of the dysprosium solution was recorded. The diluted dysprosium solution was administered to the subjects and the beaker rinsed with water to ensure complete consumption of the dysprosium dose.

4.2.4. Test meals

The test meals consisted of weaning foods based on wheat and soybeans, one with the native phytic acid content (test meal A) and one containing no phytic acid, i.e. < 3 mg/100 g (test meal B). The production of the weaning foods was described in chapter 3.2.3 and the composition is given in Table 3.4. The test meals were prepared in ceramic bowls by mixing 40 g dry weaning food with 5 g sugar (saccharose, Migros, Zurich, Switzerland) and 300 g hot (circa 80°C) water. The test meals were labeled extrinsically with 0.4 mg ^{70}Zn and 0.5 mg ^{65}Cu and the exact weight of each dose of the isotopic solutions was recorded. The test meals were well mixed and administered immediately after preparation. The bowls were cleaned with a spatula to ensure

complete consumption of the labeled test meals. Cold water (200 g) was served as a drink with the test meals.

4.2.5. Study protocol

Each subject was given test meal A and B in a cross-over design. The first test meal (A or B) was allocated randomly to each subject. The two study periods were separated by a time lapse of approximately 2-4 weeks. Women started each study period immediately after menstruation. The subjects consumed their normal self-selected diet during the study, except on days when the labeled test meals were administered.

On the day before intake of each test meal (A or B), a venous blood sample was drawn after an overnight fast. Body weight and height were measured and body mass index (BMI) was calculated by dividing the body weight in kg by the square of the body height in meters. The subjects completed a questionnaire for basic information about dietary habits. A fecal marker, 100 mg brilliant blue in a gelatin capsule (Apoteksbolaget, Göteborg, Sweden) and 1.5 mg dysprosium dissolved in water (see above) were administered. A second brilliant blue gelatin capsule was administered seven days later.

On the day following the intake of the fecal markers, test meals were administered in the morning after an overnight fast and second identical test meals were administered four hours later. No food or drink was allowed between the test meals and four hours following intake of the second test meal. In order to standardize the dietary intake during the days when labeled test meals were fed, dinner (Lasagne with mushrooms, Frisco-Findus SA, Rorschach, Switzerland), white bread rolls (Coop, Zürich, Switzerland) and water (ad libitum) were provided. No other food or drink was permitted until the next morning.

The complete collection of fecal material started immediately after intake of the first fecal markers (brilliant blue and dysprosium) on the day before intake of the test meals and continued until the excretion of the second fecal marker (brilliant blue), administered seven days later was completed, corresponding to six day fecal collection

period after intake of the labeled test meals. Feces were collected in individual portions in pre-weighed polypropylene containers (500 or 1000 ml), marked with each subject's code. The subjects labeled the containers with the date and time of each fecal sample.

4.2.6. Analytical methods

4.2.6.1. Blood

4.2.6.1.1. Sample collection and separation

Venous blood samples (about 10 ml) were drawn at the start of each study period, using acid washed glass tubes and Wasserman needles (Süddeutsche Feinmechanik GmbH, Wächtersbach, Germany). The serum was separated from blood cells by centrifugation at 3500 rpm during five minutes (Omnifuge 2.0 RS, Heraeus GmbH, Hanau, Germany). Serum was stored frozen (-25°C) until analysis.

4.2.6.1.2. Zinc and copper in serum

Zinc and copper in serum were analyzed using atomic absorption spectrometry (AAS) (atomic absorption spectrometer SpectrAA-400 and GTA-96 graphite furnace atomizer, Varian, Techtron Pty. Limited, Mulgrave, Victoria, Australia). The parameters of the atomic absorption spectrometer were chosen according to the instructions of Varian (Beach et al. 1988). Accuracy of the determinations was verified by analyzing a reference material, Seronorm trace element serum (Nycomed Pharma SA, Oslo, Norway) in paralell.

4.2.6.1.2.1. Zinc

Flame AAS (using air as oxidizing gas and acetylene as fuel gas) with standard addition technique was used to measure zinc concentration in serum samples. Standard additions were made to each sample after appropriate dilution with water. Zinc concentrations of the final solutions were in the range of 10-130 ng/ml. The absorbance of each solution was measured four times. The zinc concentration was calculated with the mean value of absorbance and expressed in μmol/L. Analyses were repeated if the coefficient of variation based on triplicate determinations was > 10%.

4.2.6.1.2.2. Copper

Graphite furnace technique (using argon as purge gas) with standard addition technique was used to measure copper concentration in the serum samples. Standard additions were made to each sample after appropriate dilution with water. Copper concentrations of the final solutions were in the range of 10-25 ng/ml. The absorbance of each sample solution was measured in triplicate. The copper concentration was calculated with the mean value of absorbance and expressed in μmol/L. Analyses were repeated if the coefficient of variation based on triplicate determinations was > 5%.

4.2.6.2. Feces

4.2.6.2.1. Drying and pooling

Feces were frozen and freeze-dried (Freeze-dryer Lyolab B, LSL Secfroid SA, Aclens-Lausanne, Switzerland) in the polypropylene containers used for fecal collection. The freeze-dried feces were homogenized with a pestle and dried for 24 hours at 65°C in an incubator (WTB Binder, Tuttlingen, Germany). The weight of the fecal samples before and after drying was recorded. Dried fecal samples (including the first fecal sample dyed by the fecal marker and including all samples before the appearance of the second fecal marker) were milled (0.75 mm mesh, centrifugal mill, Retsch ZM1, Retsch GmbH, Haan, Germany) in the order of collection and combined in 2 L polyethylene containers. The fecal pools were homogeneously mixed mechanically by overhead rotation of the containers at circa 60 rpm for one hour and dried 24 hours at 65°C before further analysis. The weight of individual and pooled fecal samples was recorded before and after each step.

4.2.6.2.2. Mineralization

Circa 1 g of the pooled fecal samples was divided and weighed into two separate high pressure Teflon tubes of a microwave digestion unit (MLS 1200 MEGA, MLS GmbH, Leutkirch, Germany). Circa 250 μl aqueous ^{67}Zn spike (circa 20 μg/g), prepared as ZnCl$_2$ in 0.1 M hydrochloric acid were added and the weight recorded. Five ml 65% nitric acid and 2 ml 30% hydrogen peroxide were added and the covered tubes were left for 30 minutes at room temperature before mineralization in the microwave digestion

unit. After mineralization and cooling to room temperature, the sample solutions were combined and quantitatively transferred to 30 ml polyethylene tubes. The solutions were centrifuged for 10 minutes at 3500 rpm (Omnifuge 2.0 RS, Heraeus GmbH) to separate insoluble silicates from the solution. Each sample was mineralized in duplicate and together with each series of samples two blanks containing 500 µl ^{65}Cu solution (circa 100 µg/g) were analyzed.

Circa 10 ml of the supernatant was used for separation of copper and zinc from the matrix for TIMS analysis and the remaining solution (circa 10 ml) for the determination of copper and dysprosium concentration.

4.2.6.2.3. Copper

Copper in mineralized fecal samples was analyzed using atomic absorption spectrometry (AAS) (atomic absorption spectrometer SpectrAA-400 and GTA-96 graphite furnace atomizer, Varian, Techtron Pty. Limited). The parameters of the atomic absorption spectrometer were chosen according to the instructions of Varian (Beach et al. 1988). Flame AAS (using air as oxidizing gas and acetylene as fuel gas) with standard addition technique was used.

Standard additions were made to each sample after appropriate dilution. Copper concentrations of the final solutions were in the range of 0.3-1.6 µg/ml. The absorbance of each sample solution was measured four times. The copper content was calculated with the mean value of absorbance and expressed in µg/g. Samples were analyzed in duplicate and repeated if the difference relative to the mean was > 5%.

4.2.6.2.4. Dysprosium

Dysprosium was determined by inductively coupled plasma mass spectrometry (ICP-MS) in mineralized fecal samples. External calibration with dysprosium concentrations of 10 to 30 ng/ml was used, prepared from a stock solution (Johnson Matthey GmbH, Karlsruhe, Germany). The mineralized samples and the orally

administered dysprosium solution were diluted with 0.5 M nitric acid to be within this concentration. Rhodium was used as an internal standard at a concentration of 20 ng/ml.

The ICP-MS instrument (Perkin-Elmer Elan 6000, Perkin-Elmer Europe, Rotkreuz, Switzerland) was equipped with a GemTip^{TM} cross-flow nebulizer and a Scott-type double-pass spray chamber. The instrument was optimized using a 10 ng/ml multi-element solution (Plasma setup solution, Perkin-Elmer Europe). The operating conditions were as follows: RF power 1000 W, nebulizer argon flow rate 0.83 L/min, mass resolution 0.8 Da at 10% of peak height. The following data collection variables for dysprosium were used: selected isotope ^{163}Dy, detector mode: pulse counting, total replicates per integration 5, dwell time 20 ms, sweeps 50, integration time per replicate 1 s, scanning mode: peak hopping, 1 point per peak, sample uptake rate 1.2 ml/min.

The results were expressed in % of the expected dysprosium concentration, calculated by assuming that all orally administered dysprosium was excreted. Samples were analyzed in duplicate and repeated if the difference relative to the mean was > 7%.

4.2.6.2.5. Separation

Separation of copper and zinc from the mineralized matrix was performed by anion exchange chromatography similar to previously described techniques (Götz and Heumann 1987, Turnlund and Keyes 1990).

Circa 10 ml of the mineralized sample solution were evaporated to dryness in Teflon vials, dissolved in 5 ml 6 M hydrochloric acid and loaded on a commercial pyrex column (Econo column, 0.7 cm x 10 cm; Bio-Rad Laboratories, Hercules, California, USA) filled with circa 2.7 ml anion exchange resin in its chloride form (AG 1-X8, 200-400 mesh, Bio-Rad Laboratories). After rinsing with 10 ml 6 M hydrochloric acid, copper was eluted with 5 ml 2.5 M hydrochloric acid. The column was rinsed with 15 ml 0.5 M hydrochloric acid and zinc was eluted with 10 ml 0.005 M hydrochloric acid.

The copper fraction was evaporated to dryness in Teflon vials, the residue dissolved in 100 µl concentrated hydrochloric acid and transferred to quartz crucibles and heated for four hours at 450°C in a muffle furnace (M110, Heraeus GmbH). The residue was dissolved in 50 µl concentrated hydrochloric acid, transferred to eppendorf tubes and evaporated to dryness at 60°C in an incubator (WTB Binder). The zinc fraction was treated as described for the copper fraction, but the residue was dissolved in 100 µl concentrated nitric acid instead of hydrochloric acid after evaporation.

4.2.6.2.6. Isotopic analysis

TIMS analysis was done with a single-focussing magnetic sector field TIMS instrument (MAT 262, Finnigan MAT, Bremen, Germany) equipped with a multi-collector system for simultaneous ion detection. At least 50 ion intensity measurements were performed per run by Faraday-Cup detection.

4.2.6.2.6.1. Zinc

Isotopic ratios of zinc were determined by TIMS based on the generation of Zn^+ ions in a rhenium double-filament ion source similar to the technique described by Turnlund and Keyes (1990). Three µl of an aqueous silicagel suspension (10 µg silicagel and 0.03 µl concentrated phosphoric acid per µl) were loaded on top of the evaporation filament and dried electrothermally at 1.0 A. Samples (5-10 µg zinc) were loaded as $ZnCl_2$ onto the silicagel layer and dried at 1.0 A. The loaded evaporation filament was heated at 1.2, 1.5 and 1.8 A, respectively, for 1 minute and finally glowed for 1-2 seconds at 2.4 A. The ionization filament remained unloaded. Isotopic ratio measurements were performed at 1450-1500°C for the evaporation and ionization filament, at ion intensities of $1-2 \times 10^{-11}$ A for the main signal.

4.2.6.2.6.2. Copper

Copper isotopic analysis was performed with a novel TIMS measurement technique using $Cu(CN)_2^-$ ions (Walczyk, submitted for publication). Samples (5-10 µg copper) were loaded as $CuCl_2$ (in aqueous solution) together with 40 µg zinc as $ZnCl_2$ and 100 µg NaCN on top of the evaporation filament. The solution was dried

electrothermally at 0.8 A while the ionization filament remained unloaded. Measurements were performed at ionization filament temperature of 930°C and evaporation filament temperature of circa 350°C, at ion intensities of $1\text{-}2\text{x}10^{-11}$ A for the main signal.

4.2.6.2.7. Calculation of apparent fractional zinc and copper absorption

Apparent absorption was calculated based on fecal excretion during six days of ^{70}Zn and ^{65}Cu isotopic labels, following previously described principles (Turnlund et al. 1982, 1989). The amount of zinc isotopic label (^{70}Zn) in the fecal pool was obtained by calculations based on the measured isotopic ratios, ^{67}Zn/^{64}Zn and ^{70}Zn/^{64}Zn. The absolute amounts of non-absorbed copper isotopic label were derived from the total copper concentration in the pooled fecal sample determined by AAS. Calculations were performed following isotope dilution principles, considering that the isotopic labels were not mono-isotopic (Walczyk et al. 1997).

Standard deviations for independent runs of the same sample were circa 0.1-0.3% for the ^{67}Zn/^{64}Zn and ^{70}Zn/^{64}Zn isotopic ratio. Zinc isotopic ratios were shifted by 35-65%, compared to their natural isotopic ratios. The relative difference of the ^{70}Zn/^{64}Zn isotopic ratio of duplicate analyses was < 1.5%. Standard deviations for independent runs of the same sample were circa 0.05% for the ^{65}Cu/^{63}Cu isotopic ratio. Copper isotopic ratios were shifted by 12-26%, compared to their natural isotopic ratios. The relative difference of the ^{63}Cu/^{65}Cu isotopic ratio of duplicate analyses was maximal 0.9%.

Results were expressed as apparent fractional absorption, i.e. the amount of the absorbed isotopic label relative to the total amount of orally administered isotopic label. The arithmetic mean and standard deviation (sd) were calculated for ech test meal and paired t-test was used to evaluate statistically significant differences in zinc and copper absorption between test meals.

4.3. Results and discussion

4.3.1. Subjects

Eight women and two men participated in the study. All subjects completed the study, but one woman was excluded due to incomplete fecal collection in one of the two study periods. The mean age of the subjects was 26 years (22-34 years) and the mean body mass index (BMI) was 20.2 kg/m² (18.1-23.3 kg/m²). The individual data in Table 4.1. represent the mean of two measurements of body weight at the beginning of each study period. Changes in body weight of individual subjects during the entire study ranged from 0 to 1 kg.

Table 4.1. Age, BMI, serum zinc and copper concentrations

Subject No.	Sex	Age [years]	Body weight [kg]	BMI [kg/m²]	Serum concentration	
					Zinc [µmol/L]	Copper [µmol/L]
1	F	26	68.0	23.3	18.1	14.8
2	M	27	63.9	21.6	13.7	15.0
3	F	23	50.5	21.3	18.1	24.6
4	F	25	49.3	18.1	13.8	14.7
5	F	22	56.8	19.9	14.8	22.1
6	F	28	61.5	20.8	12.9	14.5
7	M	34	58.8	18.3	15.8	12.3
8	F	25	59.5	19.2	11.0	24.2
9	F	23	56.5	19.1	9.9	26.7

4.3.2. Zinc and copper serum concentration

4.3.2.1. Zinc

The results presented in Table 4.1. show mean zinc concentrations based on two blood samples drawn at the beginning of each study period. The serum zinc concentration varied from 9.9 to 18.1 µmol/L with a mean concentration of 14.2 µmol/L. Changes in serum zinc concentration in individual subjects between the two study periods ranged from 0.5 to 4.5 µmol/L. The intraindividual differences during the study can partly be explained by the relatively high standard deviation of triplicate analysis (almost 2 µmol/L, corresponding to a maximal coefficient of variation of 10%). Plasma and serum zinc concentrations > 10.7 µmol/L were proposed as acceptable level by Sauberlich (1999). However, based on extensive data from the Department of Clinical Nutrition at University Göteborg, Sweden, the normal range of serum zinc

concentrations was considered to be between 9-18 µmol/L (Davidsson et al. 1996c). All subjects in this study were within this range.

4.3.2.2. Copper

The results presented in Table 4.1. show mean copper concentrations based on two blood samples drawn at the beginning of each study period. The serum copper concentration varied from 12.3 to 26.7 µmol/L, with a mean concentration of 18.8 µmol/L. Changes in serum copper concentration of individual subjects between the two study periods ranged from 0.2 to 3.1 µmol/L. All serum copper concentrations were in the normal range, according to Sauberlich. (1999) who proposed 11.8 µmol/L as the lower cut-off level.

Serum concentrations of zinc and copper are not specific and sensitive indicators of zinc and copper status. In this study, serum zinc and copper concentrations were used as indicators of trace element status, i.e., to confirm that the subjects were within the normal range and that no significant changes were observed during the study.

4.3.3. Dysprosium

Dysprosium was determined in the mineralized fecal pools by ICP-MS, which allows the measurement of very low concentrations. The results were expressed as % of the administered dose. A low recovery rate (60%) was found in one fecal pool (test meal B) for one subject, indicating that the fecal collection was incomplete. After exclusion of this subject (both study periods), the mean dysprosium recovery in 18 fecal pools was 106 ± 5% (range 95 to 114%). All these fecal pools were considered to be complete.

Similar values were obtained by Schuette et al. (1993), who reported mean dysprosium recovery of 104 ± 9%. In this study, dysprosium was administered on the day before the intake of the test meals to avoid any potential interaction between dysprosium and phytic acid in the test meals. Thus, the dysprosium data can only be used to verify the completeness of fecal collections but not to normalize the absorption values. Schuette et al. (1993) showed, that the excretory pattern of dysprosium was very similar to zinc, but

not to copper. Administration of dysprosium together with the labeled test meals would therefore only allow to normalize data on zinc absorption, but not on copper. However, in this study dysprosium was useful to verify completeness of the fecal collections, a major factor of uncertainty in studies based on fecal monitoring.

4.3.4. Apparent zinc and copper absorption

4.3.4.1. Sample preparation and isotopic analysis and calculation

Losses of fecal material during freeze-drying, subsequent drying and milling were not exceeding 1.2% (0.1 to 1.2%) of the total dry weight of fecal pools. The major part of the losses can be attributed to the residual fecal material in the mill and in the containers used for collection of individual fecal samples. As the fecal samples were milled in consecutive order of collection, the fecal material remaining in the mill was predominantly from the latter part of the collection period. Therefore, this residue can be assumed to contain negligible quantities of isotopic labels.

A second isotopic label (^{67}Zn) was added to each sample before mineralization to monitor for zinc contamination during sample preparation. Copper contamination was monitored by analysis of blanks containing known amounts of ^{65}Cu isotopic label. For copper isotopic analysis, a novel TIMS measurement technique using $Cu(CN)_2^-$ ions was developed to improve the attainable precision in isotopic analysis (Walczyk, submitted for publication). Calculation principles were according to those described for iron (Walczyk et al. 1997), while the detailed calculations for zinc and copper are not yet published (Walczyk, unpublished). The arithmetic means of the apparent zinc and copper absorption were calculated for each test meal as in previous zinc and copper absorption studies (Turnlund et al. 1985, Davidsson et al. 1996b, 1996c), although the sample size was too small to demonstrate normal distribution.

4.3.4.2. Apparent zinc absorption

The mean ($\pm$ sd) apparent fractional zinc absorption from test meal A (native phytic acid content) was 22.8 $\pm$ 8.8% and from test meal B (no phytic acid) 34.6 $\pm$ 8.0%. The difference was statistically significant (p = 0.005). The individual apparent fractional zinc absorption from test meal A and B is shown in Table 4.2. and Figure 4.1. The dots

in Figure 4.1., representing the individual data for test meal A and B are connected for each subject. The individual apparent fractional zinc absorption varied from 10.4% to 32.3% for test meal A and from 26.1% to 51.7% for test meal B. The mean absorption ratio of test meal B to test meal A was 1.74, range 0.96 to 3.66. Except for subject 4, all subjects had higher apparent fractional zinc absorption from test meal B than from test meal A.

Table 4.2. Apparent fractional zinc and copper absorption from test meal A and test meal B

	Zinc			Copper		
	Apparent fractional absorption [%]			Apparent fractional absorption [%]		
Subject No.	Test meal A native phytic acid	Test meal B no phytic acid	Ratio B:A	Test meal A native phytic acid	Test meal B no phytic acid	Ratio B:A
1	19.6	31.8	1.62	24.6	26.3	1.07
2	15.4	26.1	1.70	15.5	14.4	0.93
3	12.2	28.1	2.30	23.8	24.7	1.04
4	32.2	30.8	0.96	42.3	20.4	0.48
5	32.3	35.8	1.11	17.5	11.0	0.63
6	10.4	38.0	3.66	28.2	17.0	0.60
7	28.2	51.7	1.83	21.2	19.7	0.93
8	23.0	28.8	1.25	23.5	24.9	1.06
9	32.0	40.1	1.26	16.7	18.4	1.10
Mean	**22.8**	**34.6**	1.74	**23.7**	**19.7**	0.87
sd	**8.8**	**8.0**		**8.1**	**5.1**	

Figure 4.1. Apparent fractional zinc absorption from test meal A and test meal B
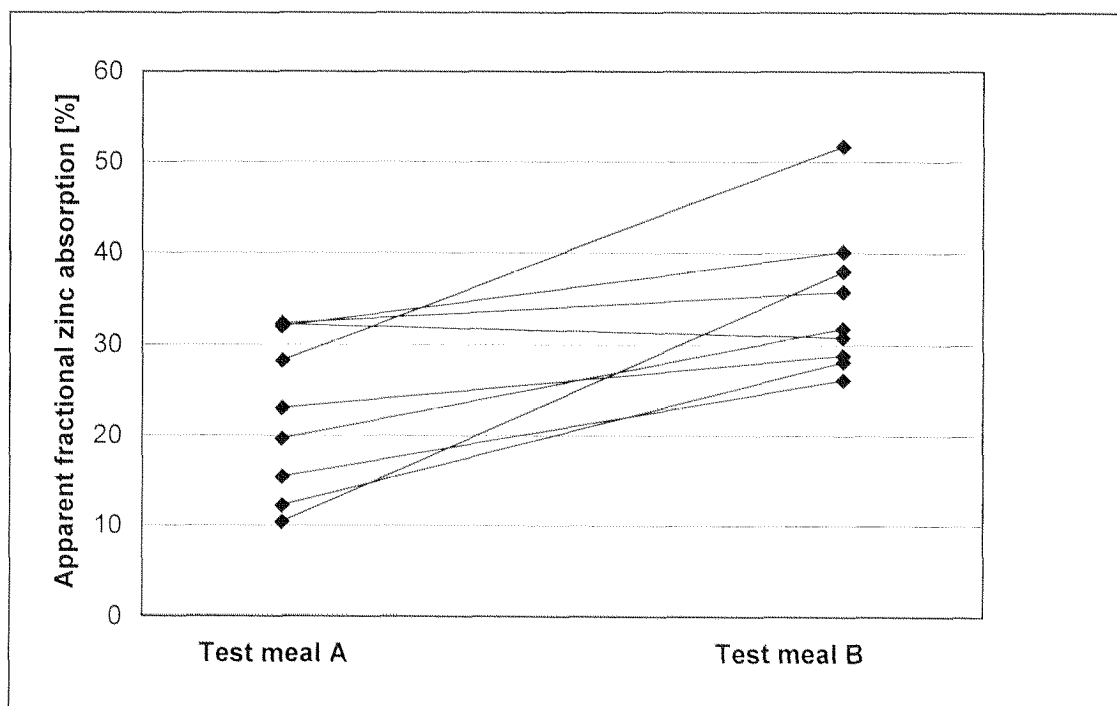
To avoid a significant increase of the zinc content in the test meals, the total dose of zinc stable isotope label (0.8 mg) was administered in two identical test meals consumed on the same day. The total zinc content of the test meals increased from 0.8 to 1.2 mg. The molar ratio of phytic acid:zinc in test meal A decreased from 16:1 to 11:1 by the addition of the stable isotope label. The molar ratio of calcium x phytic acid:zinc decreased from 290:1 to 200:1 [mM]. This is a well known disadvantage when using stable isotopes as compared with radioisotopes. However, zinc absorption was found to be inhibited in this study at a molar ratio of phytic acid:zinc of 11 and calcium x phytic acid:zinc of 200. Thus, similar or more pronounced effects can be expected in the weaning food when consumed under normal circumstances, i.e. without added ^{70}Zn. The interindividual fractional zinc absorption varied considerably for both test meals. A molar ratio of phytic acid:zinc of 11:1 can be assumed to inhibit zinc absorption moderately (Gibson and Ferguson 1998) and circa 20% apparent fractional absorption from test meal A was within the expected range.

Traditionally, apparent zinc absorption has been determined from the difference between zinc intake and fecal excretion. Absorption from single test meals can not be measured with this method and zinc isotopes are used to label zinc in the test meals, assuming that they are metabolized identically as the naturally occurring zinc. The use of stable isotopes to measure zinc absorption by fecal monitoring was shown to result in comparable values to those measured by whole-body retention of radioisotopes and by fecal monitoring of radioisotopes (Knudsen et al. 1995). A methodological study comparing intrinsic and extrinsic addition of stable isotopes (^{67}Zn and ^{70}Zn) to infant formula showed no significant difference in fractional zinc absorption in infants (Serfass et al. 1989). No difference in zinc absorption was found in women fed milk-based formulated diets comparing intrinsic and extrinsic labeling with stable isotopes (Egan et al. 1991). The methodology applied in this study, i.e. extrinsic labeling of test meals with stable isotopes and six day fecal collection had been used by us before (Davidsson et al. 1994b). The technique allows determination of apparent, but not true zinc absorption, as no correction for the re-excretion of absorbed isotopic label during the fecal collection period can be made. Fairweather-Tait et al. (1992) showed that true

zinc absorption was circa 7% higher than apparent zinc absorption in men, using a double-label stable isotope technique.

The results from this study confirmed the positive effect of dephytinization on zinc absorption in adults previously reported (Lönnderdal et al. 1984, Turnlund et al. 1984, Nävert and Sandström 1985, Sandström et al. 1987, Kivistö et al. 1989, Larsson et al. 1996).

4.3.4.3. Apparent copper absorption

The mean ($\pm$ sd) apparent fractional copper absorption from test meal A (native phytic acid content) was 23.7 $\pm$ 8.1% and from test meal B (no phytic acid) 19.7 $\pm$ 5.1%. The difference was not statistically significant (p = 0.167). The individual apparent fractional copper absorption from test meal A and B is shown in Table 4.2. and Figure 4.2. The dots in Figure 4.2., representing the individual data for test meal A and B are connected for each subject. The individual apparent fractional copper absorption varied from 15.5% to 42.3% for test meal A and from 11.0% to 26.3% for test meal B. The mean absorption ratio of test meal B to test meal A was 0.87, range 0.48 to 1.10. Three subjects (subjects 4, 5 and 6) had higher copper absorption from test meal A than from test meal B, with absorption ratio B:A 0.48, 0.63 and 0.60, respectively. The other subjects had absorption ratios close to 1 (range 0.93 to 1.10).

The addition of copper stable isotope labels increased the total copper content of the test meal from circa 0.2 to 0.7 mg, changing the molar ratio phytic acid:copper from 75:1 to 19:1. The influence of this change in molar ratio is difficult to evaluate since no conclusive data are available on the effect of phytic acid on copper absorption. To investigate whether the molar ratio phytic acid:copper normally present in cereals or legumes could influence copper absorption would need the addition of free phytic acid.

Figure 4.2. Apparent fractional copper absorption from test meal A and test meal B
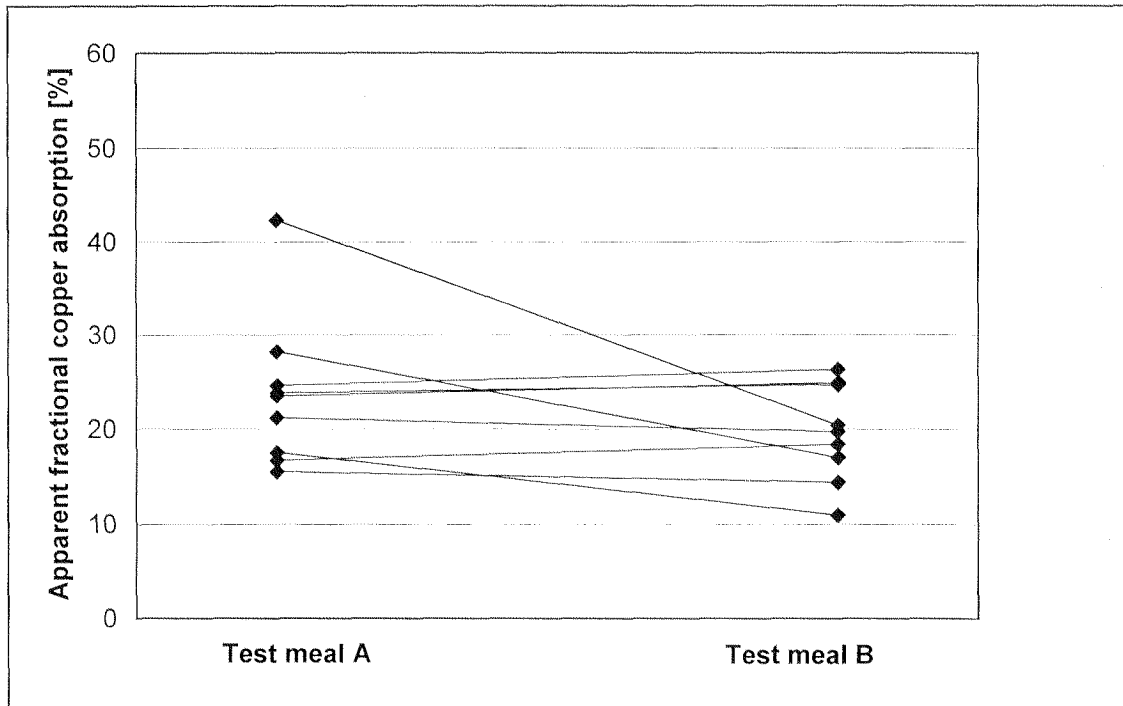
The mean apparent fractional copper absorption from test meal A (23.7%) and from test meal B (19.7%) is somewhat lower than proposed average fractional copper absorption from diets in developed countries, 30-40%, (Wapnir 1998). Fractional copper absorption has been reported to be inversely related to dietary copper intake (Turnlund et al. 1985, 1989). However, high copper intakes of 3 mg per day or more, which might result in low fractional absorption, can not be expected in the present study since most diets in Western countries provide less than 1.6 mg per day (National Research Council, Food and Nutrition Board 1989). Based on data on dietary habits of the subjects, there is no indication that the subjects consumed diets high in copper.

Results from *in vitro* studies suggested phytic acid as a potent inhibitor of copper absorption as copper binds strongly to phytic acid (Vohra et al. 1965, Persson et al. 1998). Despite the strong phytic acid-copper binding, no negative influence of phytic acid on copper absorption could be demonstrated in this study, maybe due to the solubility of the phytic acid-copper complex and to the influence of other chelating substances. An *in vitro* study (Nolan et al. 1987), using pure phytic acid mineral solutions investigated the solubility of phytic acid-copper complexes at different pH and

molar ratios phytic acid:copper. At molar ratios phytic acid:copper > 1:1, as it would be expected in meal digests, the formation of soluble Cu(phytate) and Cu_2(phytate) complexes were reported. Champagne and Fisher (1990) demonstrated that at pH 7 and molar ratio phytic acid:copper of 10:1 phytic acid-copper complexes stayed in solution. These findings are difficult to extrapolate to the situation in the digestive tract where numerous substances would influence the solubility of the phytic acid-copper complexes. However, when the solubility was tested in cereal products at pH 7, there was little precipitation of the phytic acid-copper complex (Lyon 1984). Jacobsen and Slotfeld-Ellingsen (1983) suggested that phytic acid in the soluble phytic acid-copper complex can be easily replaced by other chelators, such as amino acids and ethylene diaminetetraacetic acid (EDTA) and therefore copper absorption would not be influenced by phytic acid.

The results from this study confirmed the previous data based on stable isotope technique presented by Turnlund et al. (1985) where no influence of phytic acid on copper absorption was found in young men. It is also in agreement with the study by Lönnerdal et al. (1999) reporting no difference in copper absorption from regular and dephytinized soy formula in rats and monkeys. Extrinsic labeling of test meals with ^{65}Cu (as chloride) to measure copper absorption has been validated by Johnson et al. (1988), who observed no differences in the absorption of intrinsically and extrinsically labeled foods, such as goose meat, goose liver, peanut butter and sunflower butter in women.

In studies based on fecal monitoring, the period of fecal collection is of great importance. Short periods result in incomplete excretion of unabsorbed copper, while prolonged periods result in increased amounts of re-excreted absorbed copper. Turnlund et al. (1989) found an average of 7.7% of an intravenous dose of ^{65}Cu excreted in feces during six days after administration. True absorption would therefore be higher than apparent absorption as determined in this study. The difference between apparent and true copper absorption can be expected to be in the same order of magnitude as for zinc (Fairweather-Tait et al. 1992).

The differences between the influence of phytic acid on copper and zinc absorption might be due to the binding strength and the solubility of the respective complexes. The binding of copper to phytic acid is stronger than the binding of zinc to phytic acid (Vohra et al. 1965, Persson et al. 1998), but other chelators also bind copper stronger than zinc (Jacobsen and Slotfeld-Ellingsen 1983). Therefore the substitution of phytic acid by other chelators is more likely in complexes of phytic acid with copper than with zinc. Champagne and Fisher (1990) reported that at pH 7 and molar ratios of phytic acid:metal 10:1 phytic acid-copper complexes stayed in solution, while phytic acid:zinc complexes slowly precipitated. When the solubility was tested in cereal products at pH 7, the extent of precipitation of the phytic acid-zinc complex was higher than of the phytic acid complex-copper (Lyon 1984).

4.3.5. Benefits of phytic acid degradation in weaning foods

An advantage of the newly developed method used to degrade phytic acid during production of weaning foods is that the general composition of the weaning food remains largely unchanged. The influence of other factors than phytic acid on zinc and copper absorption can be excluded, such as differences in mineral or trace element content, as for example when comparing white bread with wholemeal bread (Fairweather-Tait et al. 1992).

Zinc is important in infant nutrition as zinc deficiency leads to reduced growth and weaning foods should therefore be good sources of highly bioavailable zinc. The beneficial effect of dephytinization on zinc absorption was clearly demonstrated in this study. The phytic acid content (0.4 g/100 g) of the non-dephytinized weaning food, represents a level which can be expected in many commercial products. Analysis of thirty cereal based weaning foods available in Switzerland resulted in a mean phytic acid content of 0.3 g/100 g (range 0.02 to 1.02 g/100g, unpublished results). Low zinc bioavailability from many of these products can be expected. The recommended dietary allowances (RDA) for zinc of 5 mg/day for infants are based on an estimated fractional zinc absorption of 20% (National Research Council, Food and Nutrition Board 1989). This value seems difficult to meet with weaning foods, assuming a zinc content of 2 mg/100 g as in the weaning food in this study. However, a weaning food with an

improved fractional zinc absorption of 35% (as for the dephytinized product), would lower the required intake to meet the RDA by circa 40% as compared to a product with 20% fractional absorption. As home-prepared weaning foods do usually not contain high levels of zinc and as very few commercial weaning foods are zinc fortified, dephytinization could help to meet the zinc requirements of infants.

The positive effect of dephytinization on iron absorption has been shown in several studies in adults and infants (Hurrell et al. 1992, Davidsson et al. 1994a, Larsson et al. 1996, Cook et al. 1997). Dephytinization can also be an alternative to ascorbic acid addition to increase iron absorption, if ascorbic acid is not available or if it might be degraded during processing (Mosha et al. 1995, Riddoch et al. 1998). As even small amounts of phytic acid were found to have an inhibiting effect on iron absorption (Hurrell et al. 1992), phytic acid was completely reduced (< 3 mg/100 g) in the weaning food used in this study and a positive effect of dephytinization of product B on iron absorption can therefore be assumed. For iron, it was shown that absorption studies in adults can be used to assess the influence of enhancers and inhibitors of iron absorption in infants, consuming identical infant formulas (Hurrell et al. 1998). This remains to be verified for zinc and copper, feeding identical test meals to adults and infants.

4.4. Summary

The influence of phytic acid degradation on zinc and copper absorption from weaning foods was investigated in adults. Two weaning foods based on wheat and soybeans, one with the native phytic acid content (0.4 g/100 g) and one with no phytic acid (< 3 mg/100 g) were compared.

Seven premenopausal women and two men completed the study. Each subject consumed the weaning food with the native phytic acid content and the weaning food with no phytic acid in a cross-over study design. Zinc and copper were labeled extrinsically with stable isotopes (^{70}Zn and ^{65}Cu) and apparent zinc and copper absorption was based on fecal monitoring technique. The completeness of fecal collections was verified by excretion of dysprosium a non-absorbable fecal marker.

Isotopic analyses were performed by thermal ionization mass spectrometry. Apparent fractional zinc and copper absorption was calculated based on fecal excretion of zinc and copper isotopic labels. The start and end of the fecal collection periods was indicated by brilliant blue.

The mean apparent fractional zinc absorption was significantly higher (p = 0.005) from the weaning food containing no phytic acid (34.6 ± 8.0%), than from the weaning food with the native phytic acid content (22.8 ± 8.8%). No significant difference (p = 0.167) was found for the mean apparent fractional copper absorption between the weaning food with no phytic acid (19.7 ± 5.1%) and the weaning food with the native phytic acid content (23.7 ± 8.1%). The results confirmed data from earlier studies and clearly demonstrated the beneficial effect of dephytinization of weaning foods on the absorption of zinc, an important nutrient in infant nutrition.

Conclusions and perspectives

A new food processing method was developed to degrade phytic acid during weaning food production by using phytase naturally occurring in the ingredients of cereal/legume based weaning foods. The application of the new processing method to commercial weaning food production was evaluated using a weaning food based on wheat and soybeans. An incubation step was included to the usual production, requiring only minor modifications of the processing conditions. The processing method had little influence on the overall composition, viscosity and taste of the weaning food. The nutritional benefit of dephytinization was demonstrated as a statistically significant increase in apparent fractional zinc absorption from the phytic acid free weaning food in adults. No effect of dephytinization was found on copper absorption. According to literature data an increased fractional iron absorption from the dephytinized weaning food can be expected.

Future development of this methodology would include the adaptation to the household level or to small scale industries producing cheap weaning foods, especially in developing countries. In developing countries commercial weaning foods are not affordable and/or not available and infants are often fed gruels based on cereals and legumes containing high amounts of phytic acid. Wheat is readily available worldwide and could be added to the gruels as a source of phytase. For example, about 10% whole grain wheat flour could be added to the gruels after cooking, the pH reduced to about 5 by adding juice from acidic fruits and the gruels kept warm during two hours or more to reduce the phytic acid content. Attention has to be paied to hygiene and the gruels would have to be heated once more before being fed to the infants. The optimal conditions for complete phytic acid degradation in various cereal/legume mixtures and the microbiological quality of the products would have to be further investigated in field studies. Rye is not commonly consumed in most countries worldwide but its usefulness as a source of phytase should be considered, due to the high phytase activity, even at low temperatures. The potential of degrading phytic acid at ambient temperature by the addition of rye to gruels needs to be investigated.

The household level processing would be similar, but even easier, as the earlier development of amylase rich flour ('power flour') to decrease the viscosity of weaning foods. The addition of amylase rich flours, produced by germination and subsequent drying and milling of cereals, has been introduced at the household level in India (Gopaladas and Deshpande 1992). The acceptability of the addition of wheat or rye flour to traditional weaning foods needs to be evaluated in mothers and infants in developing countries. The additional work for the mother is minimal and the addition of wheat or rye would not substantially change the taste of the weaning foods, indicating that acceptability would not be a major problem at the household level.

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