

THE INFLUENCE OF pH AND TEMPERATURE ON ENDOGENOUS PHYTASE ACTIVITY AND ON HYDROLYSIS OF INOSITOL HEXAPHOSPHATE IN LENTIL, FABA BEAN AND PEA SEEDS

Joanna Honke, Ann-Sofie Sandberg*, Halina Kozłowska

Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn; *Chalmers University of Technology, Department of Food Science, Gothenburg, Sweden

Key words: inositol phosphates, legume seeds, phytase, soaking

The study was conducted to determine the influence of pH and temperature on endogenous phytases activity and to investigate the influence of pH and temperature of soaking on hydrolysis of inositol hexaphosphate in lentil, pea, and faba bean seeds. In the first experiment, the sodium phytate was hydrolyzed by phytase extracted from the germinated seeds and the released inorganic phosphorus was measured. The second experiment relied on measuring the changes in seed contents of inositol phosphates as affected by soaking-activated phytases at 37°C, 45°C and 55°C and pH 4.0, 5.0, 6.4, 7.0 and 8.0. In both experiments, the highest activities of endogenous phytase were found in pH 5.0 for lentil, pH 4.0 for faba bean and pH 7.0 for pea, and the temperature of 45°C appeared to be the most suitable for enzyme activity, for all examined species. The obtained results, proved by two experiments, can indicate that above mentioned pHs and temperatures are convenient for optimal activity of phytase enzyme in examined seeds, what can be used in the technology of fodder and food production.

INTRODUCTION

Cereal grains and legume seeds constitute an enormous source of foods, thus, may satisfy the needs of increasing world population. They are properly balanced in terms of calories, and content and quality of protein [Cosgrove, 1980]. The plant-derived foods contain generally great amounts of inositol phosphates (mainly inositol hexaphosphate), the major storage medium for phosphorus with 60–90% in the organically-bound form [Scheuermann, 1984; Billington, 1993]. These compounds are of great importance to human nutrition because of their well documented negative properties and recent reports on their beneficial influence on the human organism. Myo-inositol hexaphosphate (IP-6), is considered to be the major factor impairing the absorption of several essential minerals, such as Ca^{2+} , Zn^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , and Mn^{2+} by turning them into insoluble forms, which are not readily absorbed

Author's address for correspondence: Joanna Honke, Instytut Rozrodu Zwierząt i Badań Żywności PAN, ul. Tuwima 10, PO. Box. 55, 10-747 Olsztyn, tel. (48 80) 5234647; fax (48 89) 5240124, e-mail: joanna@food.irzbz.pan.olsztyn.pl

from the intestine [Lönnerdal *et al.*, 1989; Lantzsch, 1990; Sandström & Sandberg, 1992; Saha, 1994]. IP-6 also forms the complexes with proteins [Scheuermann, 1984; Carnovale *et al.*, 1988] and starch [Houseman & de Bruyne, 1989] which lower their digestibilities and interfere with proteolytic [Vaintraub & Bulmaga, 1991] and amylolytic [Knuckles & Betschart, 1987] enzymes. The positive properties of IP-6 include its ability to inhibit cell proliferation, therefore, it is suggested that IP-6 may reduce the risk of colon cancer [Graf & Eaton, 1985]. IP-6 binds the excess of iron, thus, arrests oxidation processes [Empson *et al.*, 1991] and heavy metals which contaminate food [Rose & Quatermann, 1984].

Phosphorus deposited as inositol phosphates in the generative parts of plant is released during enzymic and non-enzymic processes. The phosphate groups are released from IP-6 in the presence of water when the dormant stage of seeds and grains is finished or interrupted. This is accompanied by an increase in activity of enzymes, including phytase, which liberates phosphate groups from IP-6.

IP-6 is hydrolyzed to myo-inositol and inorganic phosphorus via intermediate myo-inositol phosphates (penta- to monophosphates), having lowered chelating properties [Sandström & Sandberg, 1992] and potential physiological activities. The thermal processes used in the processing and home preparation of seeds involve the IP-6 degradation, but the efficiency of these processes is lower than that of enzymic reactions [Honke *et al.*, 1994; Kozłowska *et al.*, 1996].

Before consumption, legume seeds are subjected to different technological treatments, including soaking and germination, which activate an endogenous phytase, *i.e.* enzyme which hydrolyzes IP-6 [Lolas & Markakis, 1977; Tabekhia & Luh, 1980; Eskin & Wiebe, 1983; Beal & Mehta, 1985]. The optimum activity of plant phytases is generally considered to be in the temperature range of 47–57°C and pH 4.0 to 6.0, depending on the seed species [Scheuermann, 1984]. Among the cereal phytases, the highest activity was found in wheat, rye, and barley [Houseman & de Bruyne, 1989]. The range of phytase activities in legume seeds is more broad and some authors have reported the optimum at pH 7.0 or higher at the range of temperatures mentioned above [Mandal *et al.*, 1972; Scott, 1991; Gustafsson & Sandberg, 1995]. Only few works suggest that some differences occur in phytase activity and optimal conditions of its activity, also between varieties of the same legume seeds species [Eskin & Wiebe, 1983; Scott, 1991].

Because of the lack of univocal results connected with the influence of temperature and pH on phytase activity in seeds commonly used in nutrition, such as lentil, faba bean, and pea it was decided to estimate the enzyme activity by measuring: (a) the activity of the enzyme isolated from germinated seeds and the amount of inorganic phosphorus released from sodium phytate; and (b) the content of inositol phosphates as affected by soaking-activated phytases.

MATERIAL AND METHODS

SEEDS: The seeds of lentil (*Lens culinaris* var. Magda 20), pea (*Pisum sativum* L. var. Ergo), and faba bean (*Vicia faba minor* var. Dino) were obtained from the experimental plots at the University of Agriculture and Technology, Olsztyn, Poland. Dried seeds were stored at room temperature ($\approx 20^{\circ}\text{C}$) until used.

111

DETERMINATION OF PHYTASE ACTIVITY: Activation of phytase by germinating the seeds: Duplicate samples of whole seeds (50 g) were pre-soaked in distilled water (500 mL) for 16 h at room temperature. The seeds were then placed on a filter paper, covered with a perforated layer of filter papers, put into an air oven (Mettmert Model BKE 30) set at 25°C, and left for germination for 72 h, being moistened by sprinkling with distilled water every 4–5 h. After germination, the seeds were dried at 30°C for 24 h. Ungerminated seeds were the control; Extraction of phytase according to Lolos & Markakis [1977]: Finely ground germinated seeds (size less than 0.5 mm) were extracted with 2 g/100 mL CaCl_2 solution at the seeds/solvent ratio of 1:6 w/v by mechanical shaking at room temperature for 30 min, and then centrifuged at 10 000xG (Sorvall Superspeed RC2-B, Automatic Refrigerated Centrifuge) for 60 min at 2°C. The crude enzyme extract obtained was used for phytase assays without further purification; Determination of phytase activity: The phytase activity was assayed by measuring the rate of release of inorganic phosphorus from sodium phytate. The reaction mixture of a total volume 2.0 mL contained 1.0 mL of either 0.2 mol/L citrate buffer, pH 4.0 or pH 5.0, or 0.2 mol/L TRIS buffer, pH 7.0 or pH 8.0, 0.3 mL 13 mmol/L of sodium phytate (BDH Chemical) pre-adjusted to pH 4.0, 5.0, 7.0, or 8.0 with 2 mol/L HCl, 0.3 mL of crude enzyme extract solution, and 0.4 mL of deionized water. The final concentrations of buffer and phytate were 0.1 mol/L and 2 mmol/L, respectively. The mixture without the enzyme extract was the blank. The mixture without sodium phytate was used as the control and the amount of inorganic phosphorus found in the control was corrected for the tested samples. The reaction mixture was incubated at 21°C, 37°C, 45°C, or 55°C in a water bath (1024 Shaking Water Bath, Tecator) for 60 min followed by the addition of 2 mL 3g/100 mL TCA to stop the reaction and precipitate protein. The precipitated protein was removed by centrifugation and the supernatant was analyzed colorimetrically at 720 nm for inorganic phosphorus by the procedure described by Fiske & Subbarow [1925]. Phytase activity was expressed in nanokatals (nKat). One katal is defined as the amount of enzyme, which liberates 1 mol of inorganic P per second under the assay condition.

DETERMINATION OF HYDROLYSIS OF INOSITOL HEXAPHOSPHATE: Activation of phytase by soaking the seeds: Duplicate samples (1g) of ground seeds (size less than 0.5 mm) were soaked in 10 mL of either 0.02 mol/L citrate buffer of pH 4.0 or pH 5.0, or deionized water of pH 6.4, or 0.5 mol/L TRIS buffer of pH 7.0 or pH 8.0. The samples were incubated in a water bath (1024 Shaking Water Bath, Tecator) at 37°C, 45°C, or 55°C for 4, 8, or 20 h. The pH of samples was measured before and after incubation (pH-meter HI 8418 Hanna Instruments). Ground unsoaked seeds were used as the control. PH remained constant except that of the samples soaked in deionized water (an decrease from 6.4 to 6.2 (at 37°C) or 6.0 (at 45°C) or 5.9 (at 55°C) was found in this case); Determination of inositol phosphates (from IP-3 to IP-6) (from inositol triphosphate to inositol hexaphosphate) by HPLC according to the Sandberg & Ahderinne [1986] and Sandberg *et al.* [1989] methods: The unsoaked (0.5g) and soaked seeds were extracted with 20 mL of 0.5 mol/L HCl and 30 mL of 0.67 mol/L HCl, respectively, for 3 h by magnetic agitation. The extract

was centrifuged at 2000xG (Labofuge M, Heraeus Sepatech) and the supernatant was decanted, frozen overnight (-18°C), thawed at room temperature, and re-centrifuged at 2000xG for 20 min. The supernatant (15 mL) was evaporated to dryness with a stream of air at 40°C , and dissolved in 15 mL 0.025 mol/L HCl. The samples were then transferred to the mini-columns filled with resin Dowex AG 1-X8, from which the inositol phosphates were eluted using 2 mol/L HCl (5x4 mL). After the solvent was evaporated the dry residue was dissolved in a mixture of methanol/0.05 mol/L formic acid and 1.5 mL/100 mL TBA-OH (tetrabutylammonium hydroxide) and analyzed by HPLC using a HPLC pump (Waters Model 510, Waters Ass. Inc.),

C-18 Chromasil column ($5\ \mu\text{m}$, 2 mm i.d.) and refractometric detector (ERC-7510 RI-detector, Erma Optical Works Ltd.). A mobile phase was methanol/0.05 mol/L formic acid (51/49 v/v) and 1.5 mL/100 mL TBA-OH. The flow rate was 0.4 mL/min. Retention times and peak areas were measured by a laboratory data system HP 3350 (Hewlett Packard Ltd.). Sodium phytate (BDH Chemical) was the external standard and the injections were made with a 20 μL loop.

RESULTS

THE INFLUENCE OF pH AND TEMPERATURE ON ENDOGENOUS PHYTASE ACTIVITY IN LENTIL, FABA BEAN, AND PEA

Figure 1 presents the results concerning the phytase activity in germinated seeds of lentil, faba bean, and pea measured in different pHs (4.0, 5.0, 7.0, 8.0) and temperatures (21°C , 37°C , 45°C , 55°C). The phytase activities in non-germinated seeds were not detected, as the extracts from these seeds did not release phosphorus from sodium phytate, indicating either the lack of phytase activity or inability to extract phytase from these seeds.

The highest phytase activities in lentil, faba bean, and pea were found in the same temperature (45°C), although each species had the highest activity of this enzyme in different pH:

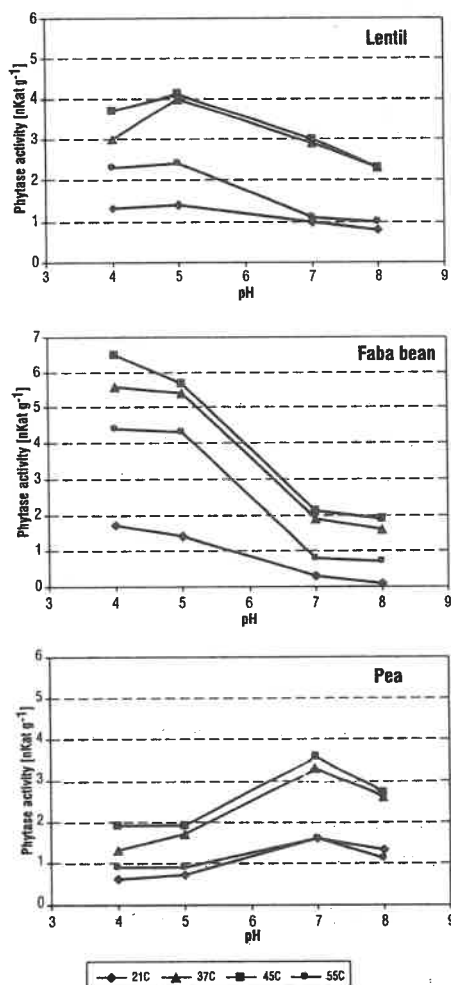


FIGURE 1. Phytase activity in germinated lentil, faba bean, and pea seeds at different pHs and temperature.

5.0 for lentil, 4.0 for faba bean, 7.0 for pea. For mentioned values of pH, the phytase also showed high activities at 37°C, being only slightly lower, *i.e.* by 4 to 14% ($P \leq 0.01$). The phytase activities at extreme temperatures of 21°C and 55°C were lower than optimal activities, but more efficient at 55°C than at 21°C ($P \leq 0.01$).

In order to estimate the influence of both examined features (temperature and pH) on phytase activity, the analysis of variation of the obtained results was conducted. It indicated that pH and temperature radically influenced the results of assays of phytase activity. It was also ascertained that the interaction of both features has essential influence on values of the enzyme activity, what indicates that activity of one feature on different levels of the second feature, is different.

THE INFLUENCE OF pH AND TEMPERATURE ON HYDROLYSIS OF IP-6 IN LENTIL, FABA BEAN, AND PEA SEEDS

Lentil seeds

The contents of IP-6 and lower forms of inositol phosphates (IP-5 to IP-3) in unsoaked and soaked lentil seeds are shown in Table 1. Unsoaked lentil seeds contained predominantly IP-6 with only small amounts of IP-4 and IP-5, and with IP-3 not being detected. The hydrolysis of IP-6 was observed for the soaked lentil seeds, being dependent on pH, temperature, and incubation time (Figure 2). IP-6 were hydrolyzed the most (in about 87%) at pH 5.0 and 45°C. At this pH, but at 37°C, less intensive (by 12%) hydrolysis of IP-6 was observed ($P \leq 0.01$). The IP-6 content also decreased at pH 4.0 and 45°C or 37°C, but to a lesser degree than at pH 5.0 and 45°C, *i.e.* by 22% ($P \leq 0.01$) and 24% ($P \leq 0.05$), respectively. Soaking the seeds at other pH values also caused distinct decreases of the IP-6 content, ranging from 55% (water, pH 6.4, 37°C and 45°C) to 29% (pH 8.0, 55°C) ($P \leq 0.01$).

The 20 h-soaked seeds, for which the highest decrease of the IP-6 content was found, *i.e.*, at pH 4.0 and pH 5.0 and 45°C and 37°C were repeated and soaked at the same pH and tempera-

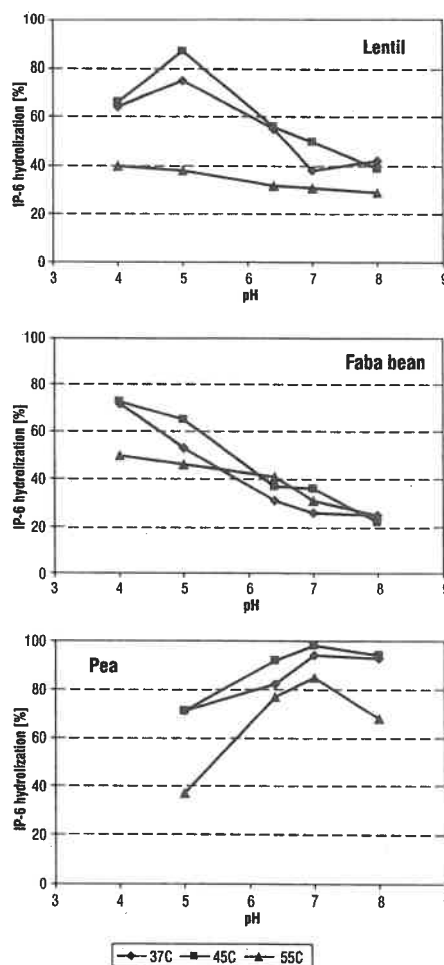


FIGURE 2. Hydrolysis of IP-6 in soaked (20 hours) lentil, faba bean, and pea seeds at different pHs and temperatures.

TABLE 1. Content of inositol phosphates (IP-3 - IP-6) in unsoaked and soaked lentil seeds.

Incubation conditions			Inositol phosphates ($\mu\text{mol g}^{-1}$ DM)			
Temp. (°C)	pH	Time (hours)	IP-3	IP-4	IP-5	IP-6
Unsoaked seeds (control)			—	0.4±0.03	0.9±0.01	11.3±0.02
37	4.0	20	1.1±0.03	0.4±0.00	0.8±0.07	4.1±0.12
		8	1.1±0.10	1.0±0.05	1.3±0.00	7.8±0.16
		4	1.3±0.02	0.7±0.06	1.0±0.05	8.4±0.34
	5.0	20	1.3±0.08	1.0±0.01	2.1±0.09	2.8±0.09
		8	1.5±0.01	0.9±0.01	1.0±0.04	5.9±0.01
		4	1.5±0.01	0.7±0.07	0.8±0.00	7.5±0.13
	6.4	20	1.4±0.08	1.1±0.01	2.3±0.17	5.1±0.27
	7.0	20	0.2±0.04	1.1±0.18	2.4±0.12	7.0±0.06
	8.0	20	0.1±0.01	0.8±0.01	1.5±0.00	6.6±0.07
	4.0	20	1.1±0.00	0.3±0.01	0.6±0.01	3.9±0.03
		8	1.3±0.02	0.5±0.01	0.6±0.01	5.0±0.27
		4	1.0±0.08	0.6±0.04	1.3±0.03	7.5±0.20
45	5.0	20	1.3±0.07	1.1±0.06	1.6±0.12	1.5±0.09
		8	1.5±0.05	0.9±0.07	0.9±0.04	2.8±0.14
		4	1.4±0.04	0.7±0.01	0.9±0.03	6.3±0.18
	6.4	20	2.6±0.01	1.5±0.05	2.7±0.01	5.0±0.11
	7.0	20	0.2±0.01	1.3±0.02	2.7±0.01	5.7±0.05
	8.0	20	0.4±0.02	1.6±0.18	2.1±0.00	6.9±0.01
	4.0	20	0.9±0.05	0.2±0.4	1.1±0.09	6.8±0.15
		5.0	0.4±0.05	0.4±0.02	1.9±0.02	7.0±0.10
		6.4	0.5±0.06	0.5±0.05	1.8±0.26	7.7±0.12
	7.0	20	—	1.3±0.09	2.5±0.01	7.8±0.07
	8.0	20	0.1±0.02	1.0±0.06	2.3±0.15	8.0±0.14

The values are means $\pm$ SD for duplicate samples from two independent experiments.

ture conditions for 4, 8, and 20 h (Figure 3). Shorter times of soaking were applied because an influence of enzymes of microbiological origin on degradation of IP-6 can be considered when the samples were soaked for 20 h. The rate of IP-6 degradation after 4, 8, and 20 h was the greatest for samples incubated at pH 5.0 and 45°C, being 44% ($P \leq 0.02$), 75% ($P \leq 0.05$), and 87% ($P \leq 0.01$), respectively. Thus, pH 5.0 and 45°C were assumed as the most profitable, among all examined, for hydrolysis of IP-6 in lentil seeds.

A decrease of IP-6 content upon endogenous phytase was accompanied by an increase in the contents of IP-5, IP-4, and IP-3 in most analyzed samples, especially in those incubated for 20 h at the pH above 6.4. There was more IP-3 in acid- than in basic medium (Table 1).

Faba bean seeds

Unsoaked faba beans had higher content of IP-6 by about 30% compared with lentil seeds (Table 2). Other forms of inositol phosphates occurred either in mini-

mum (IP-5) or trace amounts (IP-3 and IP-4). After 20-h soaking, IP-6 was hydrolyzed the most at pH 4.0 and 45°C or 37°C. The difference in the degradation of IP-6 at both temperatures was small, and about 73% degradation was found in both cases. The efficiency of IP-6 hydrolysis decreased with an increase of pH ($P \leq 0.01$) (Figure 2).

The seeds were soaked for shorter times of 4 and 8 h to find out which temperature 45°C or 37°C at pH 4.0 degrades more efficiently IP-6 and to observe the hydrolysis rate of this compound (Table 2, Figure 3). The samples soaked at pH 5.0 and 45°C or 37°C were also included in this experiment, although, the amount of IP-6 hydrolysed in these samples was lower after 20 h, being 65% ($P \leq 0.01$) and 53% ($P \leq 0.01$), respectively. As in the case of lentil an influence of microbiological hydrolysis could not be excluded when the samples were soaked for 20 h. Following the hydrolysis rates of IP-6 after 4 and 8 h, it was found that the faba bean phytase hydrolyzed IP-6 most efficiently at pH 4.0 and 45°C. At 45°C, the hydrolysis efficiency of IP-6 was by about 6% higher than at 37°C after 4 ($P \leq 0.05$) and 8 h ($P \leq 0.05$) incubation. This allowed to assume 45°C and pH 4.0 as the most advantageous for hydrolysis of IP-6 in faba bean at the conditions of experiment.

With a decline in amount of IP-6 the intermediate products IP-3 to IP-5 of this compound were formed. Distinct amounts of IP-3 (Table 2) in the samples soaked in acid media (pH 4.0 and 5.0) deserve attention. The amount of these compounds was greater after 4 ($P \leq 0.05$) and 8 h ($P \leq 0.01$) soaking at 45°C than after 20-h soaking, whereas the amounts of IP-4 and IP-5 were greater after 4-h soaking than after 8 ($P \leq 0.02$) or 20 h ($P \leq 0.02$) soaking. A decrease of the amount of IP-6 in seeds soaked at pH 6.4, 7.0, and 8.0 was lower ($P \leq 0.01$), but the IP-5 accumulation was higher compared to seeds soaked at pH 4.0 ($P \leq 0.05$) and 5.0 ($P \leq 0.05$).

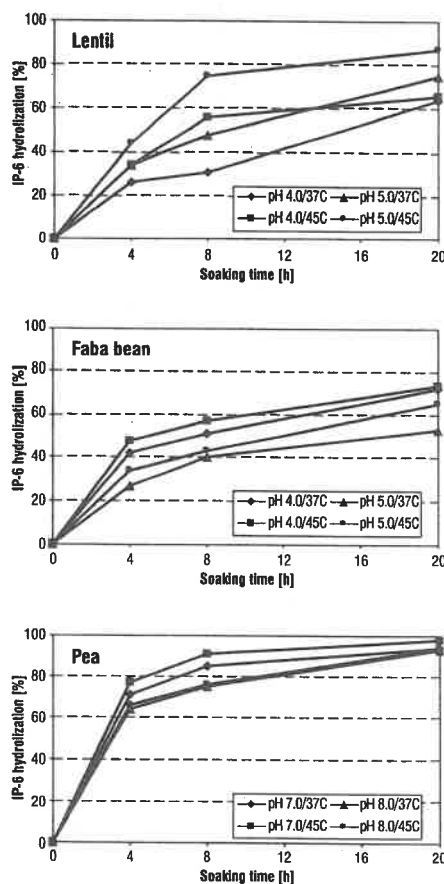


FIGURE 3. Percentage hydrolysis of IP-6 during soaking of ground lentil, faba bean, and pea seeds.

TABLE 2. Content of inositol phosphates (IP-3 - IP-6) in unsoaked and soaked faba bean seeds.

Incubation conditions			Inositol phosphates ($\mu\text{mol g}^{-1}$ DM)			
Temp. (°C)	pH	Time (hours)	IP-3	IP-4	IP-5	IP-6
Unsoaked seeds (control)			tr	Tr	0.8±0.02	17.9±0.01
37	4.0	20	4.6±0.09	0.6±0.01	0.2±0.01	5.0±0.08
		8	6.0±0.13	1.7±0.01	0.6±0.02	8.7±0.17
		4	4.0±0.10	2.3±0.05	0.7±0.06	10.4±0.14
	5.0	20	2.8±0.04	1.3±0.05	0.6±0.05	8.4±0.10
		8	2.6±0.10	1.4±0.09	0.8±0.06	10.8±0.01
		4	2.6±0.05	1.8±0.01	0.9±0.04	13.0±0.00
	6.4	20	1.7±0.02	1.4±0.02	1.1±0.01	12.3±0.20
	7.0	20	0.5±0.01	0.5±0.01	2.9±0.02	13.3±0.17
	8.0	20	0.4±0.01	0.7±0.06	2.4±0.06	13.5±0.14
45	4.0	20	2.9±0.01	1.1±0.02	0.7±0.05	4.8±0.12
		8	5.2±0.24	1.9±0.02	1.2±0.02	7.7±0.06
		4	3.4±0.14	2.1±0.01	1.2±0.03	9.3±0.10
	5.0	20	2.1±0.05	1.3±0.08	1.3±0.06	6.2±0.08
		8	3.4±0.07	1.6±0.03	1.2±0.01	10.2±0.10
		4	2.5±0.04	1.9±0.01	1.2±0.11	11.8±0.24
	6.4	20	1.2±0.02	1.4±0.01	3.1±0.07	11.3±0.10
	7.0	20	0.1±0.01	0.8±0.03	3.7±0.02	11.4±0.18
	8.0	20	0.3±0.03	0.9±0.09	2.2±0.20	13.9±0.13
55	4.0	20	1.2±0.01	0.9±0.04	2.6±0.05	8.9±0.13
	5.0	20	0.4±0.02	1.1±0.04	3.0±0.04	9.6±0.10
	6.4	20	0.6±0.02	1.4±0.02	4.0±0.17	10.6±0.15
	7.0	20	0.4±0.05	1.1±0.12	3.5±0.07	12.3±0.22
	8.0	20	0.3±0.04	0.4±0.01	2.0±0.05	13.4±0.15

The values are means $\pm$ SD for duplicate samples from two independent experiments.

Pea seeds

Like lentil and faba bean seeds, unsoaked peas contained only small or trace amounts of lower inositol phosphate forms IP-3 to IP-5, apart from a dominant IP-6. Soaking the seeds for 20 h resulted in substantial hydrolysis of IP-6, except for seeds soaked at 55°C and pH 5.0 ($P \leq 0.001$) (Table 3). IP-6 was, however, hydrolyzed most efficiently at pH 7.0 and 8.0 and 37°C or 45°C. At these conditions, the phytase hydrolyzed IP-6 in 94 to 99% (pH 7.0, 45°C) after 20 h soaking (Figure 2). The same possible influence of microbiological hydrolysis of IP-6 when the samples were soaked 20 h should be considered also in this case. To determine the rate of IP-6 hydrolysis, the seeds which had been soaked at pH 7.0 and 8.0, and at 37°C and 45°C were analyzed after 4 and 8 h soaking (Figure 3). It was found that phytase degraded IP-6 most efficiently at pH 7.0 and 45°C ($P \leq 0.01$), allowing these conditions to be assumed as the most profitable, among all conditions of the experiment, for hydrolysis of this compound in pea. Although, at other conditions of soaking, the rate of IP-6 hydrolysis and the degree of hydrolysis were lower compared to the most profitable conditions, the latter was, however, higher than in faba bean and lentil seeds, proving that the pea phytase had the highest activity.

TABLE 3. Content of inositol phosphates (IP-3 – IP-6) in unsoaked and soaked pea seeds.

Incubation conditions			Inositol phosphates ($\mu\text{mol g}^{-1}$ DM)			
Temp. (°C)	pH	Time (hours)	IP-3	IP-4	IP-5	IP-6
Unsoaked seeds (control)			tr	Tr	0.9±0.03	11.9±0.08
37	5.0	20	2.8±0.02	3.6±0.01	2.0±0.02	3.4±0.09
		20	3.3±0.03	3.9±0.10	2.7±0.05	2.2±0.01
	6.4	20	1.2±0.05	0.1±0.00	0.1±0.01	0.7±0.01
		8	1.5±0.03	0.5±0.01	0.2±0.01	1.8±0.05
	8.0	4	0.7±0.04	2.2±0.15	0.9±0.08	3.4±0.03
		20	2.4±0.08	0.5±0.02	0.1±0.01	0.8±0.00
		8	1.3±0.07	2.4±0.01	0.4±0.01	3.0±0.06
		4	0.6±0.03	1.9±0.13	0.7±0.01	4.3±0.04
	5.0	20	2.2±0.02	3.9±0.02	2.5±0.01	3.4±0.09
		20	5.5±0.06	4.2±0.11	1.7±0.00	0.9±0.06
45	6.4	20	1.7±0.01	0.2±0.00	0.1±0.00	0.2±0.02
		8	3.5±0.07	2.5±0.07	0.4±0.05	1.1±0.08
	8.0	4	1.2±0.05	3.4±0.06	1.4±0.04	2.7±0.07
		20	2.0±0.06	0.7±0.07	0.1±0.01	0.7±0.03
		8	2.1±0.04	2.4±0.08	0.4±0.02	2.9±0.09
		4	1.9±0.02	4.8±0.06	0.9±0.03	4.1±0.08
	5.0	20	0.4±0.01	1.2±0.01	2.9±0.01	7.5±0.09
		20	2.4±0.02	4.3±0.02	2.9±0.01	2.7±0.03
	7.0	20	1.1±0.06	1.5±0.23	1.2±0.00	1.8±0.01
		20	0.9±0.01	0.9±0.02	0.6±0.01	3.8±0.02

The values are means $\pm$ SD for duplicate samples from two independent experiments.

Lower forms of inositol phosphates (Table 3) were formed as the amount of IP-6 decreased, like for lentil and faba bean seeds. A substantial accumulation of IP-6 hydrolysis products in peas soaked in water (pH 6.4) and each temperature examined is worth noticing.

DISCUSSION

The activities of phytases of all examined species as well as the level of hydrolysis of IP-6 were the most efficient at 45°C, which temperature is within the range of 45–57°C, reported by many authors as optimal for phytases of plant origin [Mandal *et al.*, 1972; Lolas & Markakis, 1977; Chang & Schwimmer, 1977]. There are, however, deviations from these optimal temperatures for legumes and cereals. Ranhotra & Loewe [1975] found the optimum temperature of 37°C for the activity of wheat phytase, Larsson & Sandberg [1992] found the optimum temperature of 38°C for oat phytase, and Scott [1991] found that pea and bean phytases had the highest activities at 37°C. Our study also showed high phytase activities of lentil, pea, and faba bean seeds at 37°C. However, the results of both methods used showed the highest IP-6 hydrolysis at 45°C, which allows to assume this temperature as the most advantageous for the three examined seed species of legumes.

The most profitable pH, among all tested during both experiments, for the activity of phytase and the level of hydrolysis of IP-6 differed, however, between the species, being 5.0 for lentils, 7.0 for peas, and 4.0 for faba bean. According to the literature, the pH optima of phytase activities range from 4.0 to 6.0 [Scheuermann, 1984]. Similarly to our finding for pea seeds, some authors also found neutral or even slightly alkaline pH as optimal for legume phytases. Mandal *et al.* [1972] reported that the optimum pH for phytase from germinating mung bean was 7.5 and Gustafsson & Sandberg [1995] showed that the brown bean IP-6 was hydrolyzed the most at pH 7.0. Scott [1991] examined the phytase activities of nine bean varieties and one pea variety at two pH 5.0 and 8.0, finding that in six cases the activities were higher at pH 8.0. It should, however, be added that, apart from the optimum conditions for phytase activity, also the enzyme/substrate availability is important for the hydrolysis of IP-6 by phytase.

The possibility of bacterial microflora increase, during soaking (especially for 20 h) of lentil, faba bean, and pea, should be also taken into consideration. In conducted experiment microflora have not been controlled, however, the previous researches concerning the process of natural fermentation in lentil [Frias *et al.*, 1996], showed an explicit increase of microorganisms, where the bacteria of lactic acid were dominant. Therefore, it can be assumed that in the process of soaking the enzymes of microbiological origin have the share in the hydrolysis of IP-6.

The attempts to determine the phytase activities in native seeds of lentil, pea, and faba bean were unsuccessful, probably because of difficulties in extracting the enzyme. Mandal & Biswas [1970] also failed to determine the activity of phytase in native mung beans, and other authors reported very low activities in native seeds of California small white beans [Chang & Schwimmer, 1977], navy beans [Lolas & Markakis, 1977], and faba beans [Eskin & Wiebe, 1983]. Native legume seeds seem not to contain phytases of high activities. According to Housemann & de Bruyne [1989] only cereal phytases, particularly of wheat, rye, and barley are highly active.

The 72-h germination of seeds of the examined species increased the phytase activities enough to measure them. It should, however, be noticed that phytase and phosphatase could have been co-extracted. Eskin & Wiebe [1983] reported about sixfold increase of faba bean phytase activity and Beal & Mehta [1985] reported about fivefold increase of pea phytase activity after the same germinating period (3 days) of these seeds. These authors found that the phytase activities were both species- and variety-dependent. Studying two faba bean varieties, Eskin & Wiebe [1983] obtained threefold higher phytase activity in one of them. Compared to other authors [Mandal *et al.*, 1972; Lolas & Markakis, 1977; Eskin & Wiebe, 1983; Beal & Mehta, 1985], we found for the same germinating time (3 days) lower phytase activities in lentil, pea, and faba bean seeds. Lentils and peas had similar phytase activities in the most advantageous conditions (pH 5.0/45°C for lentil and pH 7.0/45°C for pea), whereas the activity of faba bean phytase was comparatively slightly higher in the most advantageous conditions (pH 4.0/45°C), but these seeds required shorter time to germinate.

The contents of lower forms of inositol phosphates (IP-5 to IP-3) in the soaking-activated seeds of lentil, pea, and faba bean were higher than in native seeds. These compounds were also hydrolyzed during the examined time of soaking.

When seeds were soaked at the same conditions at different times, IP-3 and IP-4 were first accumulated, but later their amount decreased (Tables 1–3). This may prove that not only IP-6, but also IP-5, IP-4, and IP-3 are the substrates for endogenous phytase or phosphatase. Our studies showed that the phytases hydrolyzed the lower forms of inositol phosphates independently on pH. The IP-5 and IP-6 hydrolysis is very important from the nutritive point of view because they substantially decrease the bioavailabilities of bulk- and trace-elements, and other nutrients [Knuckles & Betschart 1987; Carnovale *et al.*, 1988; Lantzsch, 1990; Saha *et al.*, 1994], in contrast to lower inositol phosphates, such as IP-3 and IP-4. The studies on humans conducted by Sandström & Sandberg [1992] and Sandberg *et al.* [1993], and those on suckling rats made by Lönnerdal *et al.* [1989] showed that the bioavailabilities of iron, zinc, and calcium were not decreased upon the additions of IP-3 and IP-4 to a diet. It seems, however, that IP-3 and IP-4 can adversely affect the absorption of minerals from the processed foods containing a mixture of inositol phosphates [Brune *et al.*, 1992; Sandström & Sandberg, 1992]. It is assumed that lower forms of inositol phosphates may play an important role in human organism as cell stimulators, *i.e.* they can stimulate the entry of calcium ions into cells [according to Billington, 1993], provided that they are absorbed in the intestine.

The processes (soaking and germination) used in this study to induce phytase activity are commonly used for preparing meals from legume seeds. Thus, the obtained results provide an important information about the influence of these processes on IP-6 hydrolysis, thereby, on potentially increased digestibility and bioavailability of valuable nutrients such as proteins, starch, or bulk- and trace-elements. It is worth noticing that the hydrolysis of IP-6 during the used bioprocesses was always accompanied by the formation of lower forms of inositol phosphates. These compounds, the importance of which has not yet been fully recognized, may have a beneficial influence on the activity of human and animal organisms [according to Reddy *et al.*, 1989].

CONCLUSIONS

Under conditions of the conducted experiment the highest activities of phytase were found in pH 5.0 and temperature 45°C for lentil, in pH 4.0 and temperature 45°C for faba bean, and in pH 7.0 and temperature 45°C for pea.

Mentioned above, the most profitable conditions of inositol hexaphosphate hydrolysis were proved by two independent experiments.

ACKNOWLEDGEMENTS

The financial support of the Swedish Institute in Stockholm and helpful contribution of the staff of the Department of Food Science at The Chalmers University of Technology in Gothenburg are gratefully acknowledged.

REFERENCES

1. Beal L., Mehta T., Zinc and phytate distribution in peas: influence of heat treatment, germination, pH, substrate and phosphorus on pea phytate and phytase. *J. Food Sci.*, 1985, 50, 96–100.
2. Billington D. C., 1993, *The Inositol Phosphates. Chemical Synthesis and Biological Significance*. VCH Weinheim, New York, Basel, Cambridge.
3. Brune M., Rossander-Hultén L., Hallberg L., Gleeup A., Sandberg A. S., Iron absorption from bread in humans: Inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J. Nutr.*, 1992, 122, 442–449.
4. Carnovale E., Lugaro E., Marletta L., Nutrient-antinutrient interactions in legumes, 1988, *In: Food Safety and Health Protection*. (Eds Lintas C., Spadoni M. A.), CNR, IPRA, Rome, Italy Monogr., pp 13–24.
5. Chang R., Schwimmer S., Characterization of phytate of beans (*Phaseolus vulgaris*). *J. Food Biochem.*, 1977, 1, 45–56.
6. Cosgrove D. J., Biosynthesis of inositol phosphates, 1980, *In: Inositol Phosphates: Their Chemistry, Biochemistry and Physiology*. (Ed D J Cosgrove), Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York, pp 118–127.
7. Empson K. L., Labuza T. P., Graf E., Phytic acid as a food antioxidant. *J. Food Sci.*, 1991, 56, 560–563.
8. Eskin N. A. M., Wiebe S., Changes in phytase activity and phytate during germination of two faba bean cultivars. *J. Food Sci.*, 1983, 48, 270–271.
9. Fiske C. H., Subbarow Y., The colorimetric determination of phosphorus. *J. Biol. Chem.*, 1925, 66, 375–400.
10. Frias J., Vidal-Valverde C., Kozłowska H., Tabera J., Honke J., Hedley C. L., Natural fermentation of lentils. Influence of time, flour, concentration, and temperature on the kinetics of monosaccharides, disaccharide, and α -galactosides. *J. Agric. Food Chem.*, 1996, 44, 579–584.
11. Graf E., Eaton J. W., Dietary suppression of colonic cancer: fiber or phytate? *Cancer*, 1985, 56, 717–718.
12. Gustafsson E. L., Sandberg A. S., Phytate reduction in brown beans (*Phaseolus vulgaris* L.). *J. Food Sci.*, 1995, 60, 149–152.
13. Honke J., Piskula M., Troszyńska A., Kozłowska H., The content of phytic compounds in legume seeds and their behaviour during technological processes, 1994, *In: Proceedings of the International Euro Food Tox IV Conference: Bioactive Substances in Food of Plant Origin*, (ed. Kozłowska H., Fornal J., Zduńczyk Z.), Olsztyn, pp 322–326.
14. Houseman R. A., de Bruyne K., Phytin-Phosphor und Phytase – Ein Überblick. *Kraftfutter*, 1989, 4, 113–115.
15. Knuckles B. E., Betschart A. A., Effect of phytate and other myo-inositol phosphate esters on α -amylase digestion of starch. *J. Food Sci.*, 1987, 52, 719–721.
16. Kozłowska H., Honke J., Sadowska J., Frias J., Vidal-Valverde C., Natural fermentation of lentils: Influence of time, concentration and temperature on the kinetics of hydrolysis of inositol phosphates. *J. Sci. Food Agric.*, 1996, 71, 367–375.
17. Lantzsch H. J., Untersuchungen über Ernährungsphysiologische Effekte des Phytats bei Monogastriern (Ratte, Schwein). *Übers. Tierernährg.*, 1990, 18, 197–212.
18. Larsson M., Sandberg A. S., Phytate reduction in oats during malting. *J. Food Sci.*, 1992, 57, 994–997.
19. Lolas G. M., Markakis P., The phytase of navy beans (*Phaseolus vulgaris*). *J. Food Sci.*, 1977, 42, 1094–1097.

- and other inositol phosphates on zinc and calcium absorption in suckling rats. *J. Nutr.*, 1989, 119, 211–214.
21. Mandal N. C., Biswas B. B., Metabolism of inositol phosphates. 1. Phytase synthesis during germination in cotyledons of mung beans (*Phaseolus aureus*). *Plant. Physiol.*, 1970, 45, 4–7.
 22. Mandal N. C., Burman S., Biswas B. B., Isolation, purification and characterization of phytase from germinating mung beans. *Phytochemistry*, 1972, 11, 495–502.
 23. Ranhotra G. S., Loewe R. J., Effect of wheat phytase on dietary phytic acid. *J. Food Sci.*, 1975, 40, 940–942.
 24. Reddy N. R., Pierson M. D., Sathe S. K., Salunkhe D. K., Influence of processing technologies on phytate, 1989, *In: Phytates in Cereals and Legumes*. CRS Press, Boca Raton, FL., pp 111–135.
 25. Rose H. E., Quateman J., Effects of dietary phytic acid on lead and cadmium uptake and depletion in rats. *Environ. Res.*, 1984, 35, 482–489.
 26. Saha P. R., Weaver C. M., Mason A. C., Mineral bioavailability in rats from intrinsically labeled whole wheat flour of various phytate levels. *J. Agric. Food Chem.*, 1994, 42, 2531–2535.
 27. Sandberg A. S., Ahderinne R., HPLC method for determination of inositol tri-, tetra-, penta- and hexaphosphates in food and intestinal contents. *J. Food Sci.*, 1986, 51, 547–550.
 28. Sandberg A. S., Brune M., Carlsson N. G., Hallberg L., Rossander-Hulthén L., Sandström B., The effect of various inositol phosphates on iron and zinc absorption in humans, 1993, *In: Proceedings of the International Conference Bioavailability '93 – Nutritional, Chemical and Food Processing Implications of Nutrient Availability*. Ettlingen, Germany, pp 53–57.
 29. Sandberg A. S., Carlsson N. G., Svanberg U., Effects of inositol tri-, tetra-, penta-, and hexaphosphates on *in vitro* estimation of iron availability. *J. Food Sci.*, 1989, 54, 159–161.
 30. Sandström B., Sandberg A. S., Inhibitory effects of isolated inositol phosphates on zinc absorption in humans. *J. Trace Elem. Electrolytes Health Dis.*, 1992, 6, 99–103.
 31. Scheuermann S. E., 1984, Untersuchungen zur Hydrolyse von Phytat *in vitro* und *in vivo* und ihr Einfluss auf die Bioverfügbarkeit von Zink. Dissertation der Universität Hohenheim.
 32. Scott J. J., Alkaline phytase activity in nonionic detergent extracts of legume seeds. *Plant Physiol.*, 1991, 95, 1298–1301.
 33. Tabekhia M. M., Luh B. S., Effect of germination, cooking and canning on phosphorus and phytate retention in dry beans. *J. Food Sci.*, 1980, 45, 406–408.
 34. Vaintraub I. A., Bulmaga V. P., Effect of phytate on the *in vitro* activity of digestive proteinases. *J. Agric. Food Chem.*, 1991, 39, 859–861.

Received and accepted May 1999