Nonchemical Approach for Reducing Antinutritional Factors in Rapeseed (*Brassica campestris* Var. Toria) and Characterization of Enzyme Phytase

Anupama Mahajan and Saroj Dua*

Department of Botany, Panjab University, Chandigarh 160 014, India

Antinutritional factors, viz. glucosinolates, phenols, and phytic acid, decreased with germination of rapeseed. Protein content declined during the first 24 h after imbibition and thereafter increased. Phytase activity increased with germination with a parallel decrease in phytic acid content. Two phytase fractions were separated during elution on DEAE-cellulose column, showing purifications of 26.75- and 19.96-fold, respectively. Optimum pH and temperature for both fractions were 5.2 and 50 °C, respectively. Enzyme stability decreased considerably after 50 °C, and phytase 2 was stable for a longer time. Mn, Co, Ca, and EDTA promoted the enzyme activity, whereas ions of Zn, Fe, Cu, fluoride, molybdate, and arsenate were inhibitory.

Keywords: *Antinutritional factors; characterization; germination; phytase*

INTRODUCTION

The attractiveness of oilseeds lies in the fact that they are reservoirs of protein along with oil. However, their utilization in foods is handicapped due to the presence of antinutritional constituents such as glucosinolates, phenols, phytic acid, trypsin inhibitors, and high nonprotein nitrogen content. A variety of chemical processes have been reported for the removal of these factors from oilseeds (Zhou *et al.*, 1990; Tzeng *et al.*, 1990; Kroll *et al.*, 1991; Xu and Diosady, 1994). Germination of seeds, however, appears to be a relatively simple nonchemical approach for decreasing antinutritional contents. The process of germination does not require intensive energy output and also yields natural products. Germination of rapeseed is accompanied by an increase in phytase level to effect a decrease in phytic acid, which is an important antinutritional factor present in many seeds. The phytate−phytase system seems to be linked to a given metabolic period of the seed. As the seedling grows, phytin disappears from the tissues, and this has been attributed to the increased phytase activity.

Phytase is a phosphatase that can hydrolyze phytic acid to inositol and free orthophosphate. Enzyme phytase (myoinositol-hexaphosphate phosphohydrolase, EC 3.1.3.8) has been reported in a number of seeds including soybean, dwarf beans, lettuce seeds, triticale, wheat, and fababeans (Peers, 1953; Gibbins and Norris, 1963; Mandal *et al.*, 1972; Singh and Sedeh, 1979; Eskin and Wiebe, 1983), and their kinetic properties have been described. Analysis of reports reveals species and cultivar differences for phytase characteristics.

Till now, the reduction in antinutritional factors has been carried out mainly by employing chemical and enzymatic treatments, and in each treatment a different variety has been taken to reduce a specific toxic factor. The purpose of the present work was to study the reduction of all the antinutritional factors simultaneously in rapeseed using a simple, cheap, and nonchemical method of germination.

Phytase is the predominant enzyme to reduce phytate during germination and, thereby, enhance the food value of rapeseed. Therefore, the changes in phytase activity were also monitored during germination of rapeseed, along with its purification and kinetic behavior.

MATERIALS AND METHODS

Purline seeds of *Brassica campestris* var. Toria were procured from Punjab Agricultural University, Ludhiana, India. Chemicals used were of analytical grade.

Seeds were surface sterilized with 0.1% mercuric chloride for 2 min followed by repeated washings with water. They were then allowed to germinate (after 24 h of imbibition) at 22 ± 2 °C in the dark on Petri dishes lined with moistened filter paper. Seeds were taken at intervals of 0, 2, 4, 6, 8, and 10 days of germination for estimating total protein content and antinutritional constituents.

Total protein content was measured according to Lowry's method (Lowry *et al.*, 1951) after extraction with 0.1 N NaOH. Phenols were extracted with 1% HCl in methanol and estimated according to the procedure of Swain and Hillis (1959). Glucosinolates were extracted according to the method of Carig and Draper (1979), and the liberated glucose was measured according to the Glucose−oxidase method of Colowick and Kaplan (1966). Phytic acid was extracted and estimated according to the method of Thompson and Erdman (1982), using the difference method. Phytase was extracted from seeds using the procedure of Goel and Sharma (1979), and its activity was monitored using the procedure of Lolas and Markakis (1977), by measuring the rate of increase in inorganic phosphorus using ascorbic acid method (Chen *et al.*, 1956).

For purification and characterization of phytase enzyme, seeds germinated for up to 8 days were taken and crude enzyme extract in water was partially purified by ammonium sulfate. The fraction precipitated between 35% and 80% saturation was dissolved in 0.5 M sodium acetate buffer (pH 5.0), dialyzed against the same buffer for 18 h, and centrifuged at 20000*g* for 30 min to remove any inactive protein precipitates. Further purification of this enzyme fraction was done on a DEAE-cellulose column (1.5 × 50 cm) equilibrated with 25 mM Tris-HCl buffer (pH 8.5). Five milliliter fractions were collected at the flow rate of 30 mL/h with gradient elution of 0.05, 0.1, 0.15, and 0.2 M NaCl in Tris-HCl buffer (25 mM, pH 8.5); 25 tubes were collected in each case.

Fractions eluted from the DEAE-cellulose column were used for kinetic studies, viz., effect of pH, substrate and product (P_i) concentration, incubation temperature, and heat stability. The effect of various ions in changing the enzyme activity was also monitored.

S0021-8561(96)00913-2 CCC: $14.00

Table 1. Changes in Protein Content and Antinutritional Factors during Germination of Rapeseed[a]

germination period (days)	protein (%)	glucosinolates (mg/g)	phenol (%)	phytic acid (%)	phytase (μmol mL^{-1} 30 min^{-1})
dry seed	25.1	4.417	0.756	3.503	0.0
imbibition (24 h)	22.0	4.047	0.510[a]	2.733	0.590
2	26.0	3.725	0.490[a]	2.250	1.050
4	28.3	2.863	0.420	1.800	1.420
6	29.1[a]	2.335	0.350	1.420	1.910
8	32.6	1.848[a]	0.190[b]	1.200	2.740
10	29.4[a]	1.800[a]	0.170[b]	1.090	2.820

[a] Results are significant at $P = 0.05$. Means with the same superscripts, within a column, are insignificantly different.

Table 2. Yields and Specific Activities of Phytase during the Course of Purification

	vol (mL)	total phytase act. (μmol mL^{-1} 30 min^{-1})	total protein (mg)	spec act. (μmol mL^{-1} 30 min^{-1})	enzyme purifn (fold)	enzyme recovery (%)
crude extract	50	180.9	71.95	2.51	1.0	100
ammonium sulfate ppt (35−80%)	10	178.75	10.90	16.40	6.53	98
1st peak	60	43.975 (15−26)[a]	0.655 (11−22)	67.14	26.75	24.3
2nd peak	80	30.865 (63−78)	0.616 (70−82)	50.10	19.96	17
remaining tubes		18.240 (27−62, 79−100)	0.769 (1−10, 23−69, 83.100)	23.72		
total		100.80	2.040	49.06	19.54	55.3

[a] Number in parentheses denote tube numbers.

RESULTS AND DISCUSSION

Germination Studies. Protein content first reduced after imbibition of 24 h and then increased up to 8 days of germination (Table 1). These studies indicate that storage proteins in rapeseed are hydrolyzed during 24 h, and new proteins are formed during subsequent periods of germination. It is a known fact that active protein metabolism takes place in germinating seeds. Due to turnover of proteins, the protein of seedlings differs in amino acid composition from that of the seed protein. The amino acids formed by degradation of storage proteins during imbibition are probably utilized for the synthesis of protein during germination (Brooker *et al.*, 1977).

Glucosinolate content was maximum (4.417 mg/g) in dry seeds and decreased with germination. The decrease was significant (at $P = 0.05$) up to the eighth day of germination; thereafter, the decrease was nonsignificant. This suggests rapid metabolism of glucosinolates for sustaining plant growth and germination. They apparently constitute a storage form for nitrogen, carbon, and especially sulfur, which became available by degradation during germination (Clossais-Besnard and Larher, 1991).

A decrease in phenol content was noted after the 2nd day of germination up to the 10th day. However, the decrease after the 8th day was insignificant. Our results are corroborated by work of Bishnoi *et al.* (1994), who also observed a marked lowering effect on antinutritional factors, polyphenols and phytate, during germination for 48 h in pea. Studies of Blumenthal-Goldschmidt (1960) showed that phenolics were present in dry seeds, but disappeared during germination. Enzymes capable of detoxicating phenolics by glycosylation have been found in number of seeds.

Data indicate a reduction in phytic acid (about 64.2% decrease) with a concomitant increase in phytase activity in rapeseed. It depicts the degradation of phytate to inositol and inorganic phosphorus. This inorganic phosphorus is probably utilized for ATP formation to meet the energy requirement of germinating seeds. This speculation is based on the fact that if this was not utilized, it may prove inhibitory for phytase activity, as is shown by further studies regarding the effect of P_i (product inhibition, on phytase activity) (Figure 3).

Table 3. Effect of pH and Incubation Temperature on Phytase Activity[a]

	act.			act.	
pH	phytase 1	phytase 2	temp (°C)	phytase 1	phytase 2
3.6	0.144	0.098	30	0.344	0.160[a,b]
4.0	0.210	0.137	35	0.386[a]	0.19l[c]
4.5	0.292	0.203	40	0.443	0.271[d]
4.8	0.508	0.311	45	0.503[b]	0.299[e]
5.0	0.708	0.486	50	0.779	0.472
5.2	0.737	0.513	55	0.662	0.299[e]
5.3	0.589	0.451	60	0.497[b]	0.225[d]
5.4	0.480	0.392	65	0.406[a]	0.179[ac]
5.5	0.443	0.374	70	0.202	0.142[b]

[a] Results are significant at $P = 0.05$. Means with the same superscripts, within a column, are insignificantly different.

Since germination is mainly a catabolic process, it supplies important nutrients to growing plant through hydrolysis of various nutrients. Their reduction in antinutritional factors may enhance the nutritional quality of rapeseed.

Characterization of Enzyme. Purification of enzyme phytase on the DEAE-cellulose column separated the enzyme into two distinct fractions with 0.05 and 0.15 M NaCl, referred to as phytases 1 and 2, which showed purifications of 26.75- and 19.96-fold, respectively (Table 2; Figure 1). These results are in general agreement with the reported results. Canola phytase fractions were separated into two isoenzymes by Kim and Eskin (1987), which showed 9.8- and 4.9-fold purifications. Purification of pea phytase after 35−80% $(NH_4)_2SO_4$ purification yielded 2.5-fold purification (Beal and Mehta, 1985).

Phytases 1 and 2 eluted from the DEAE-cellulose column exhibited the following properties.

Optimum pH of phytases 1 and 2 was found to be 5.2 (Table 3), which characterizes the enzyme as an acid phosphohydrolase. Various optimum pH values have been reported in the literature—4.5−5.0 for canola (Kim and Eskin, 1987) and 7.5 for mung bean phytase

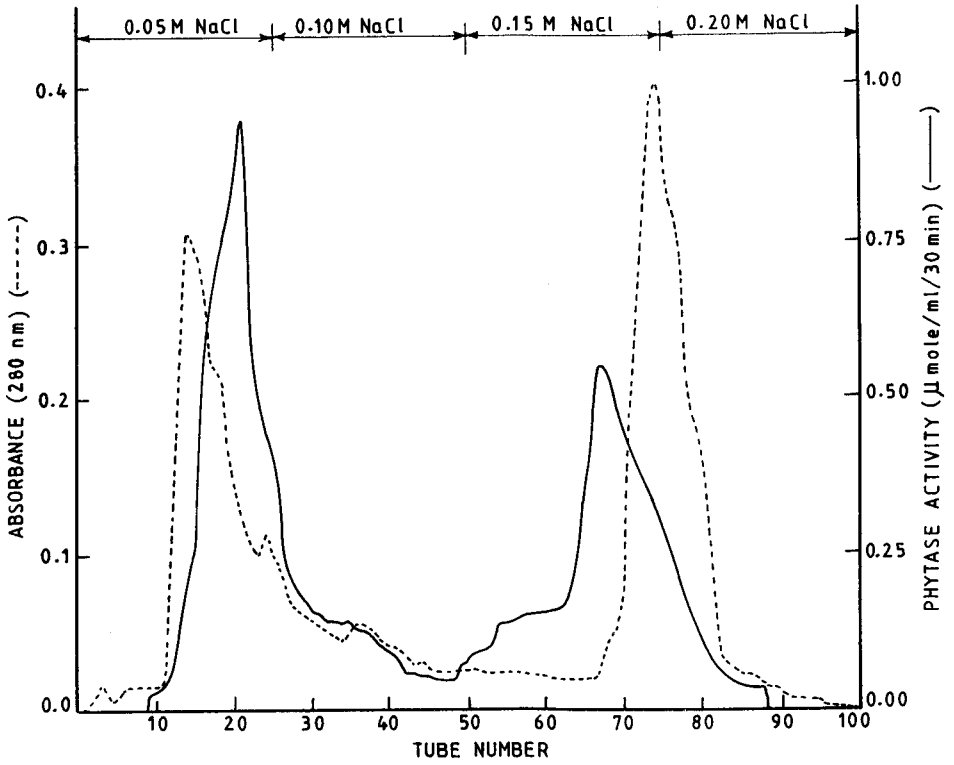

Figure 1. Elution profile of phytase purification on DEAE-cellulose column in rapeseed.

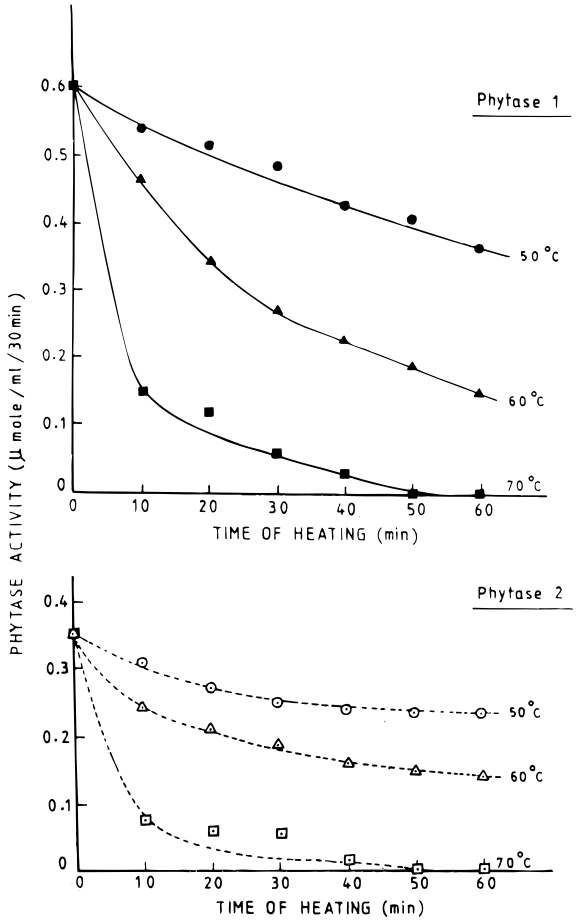

Figure 2. Effect of time of preincubation heating on the activity of phytases 1 and 2 in rapeseed.

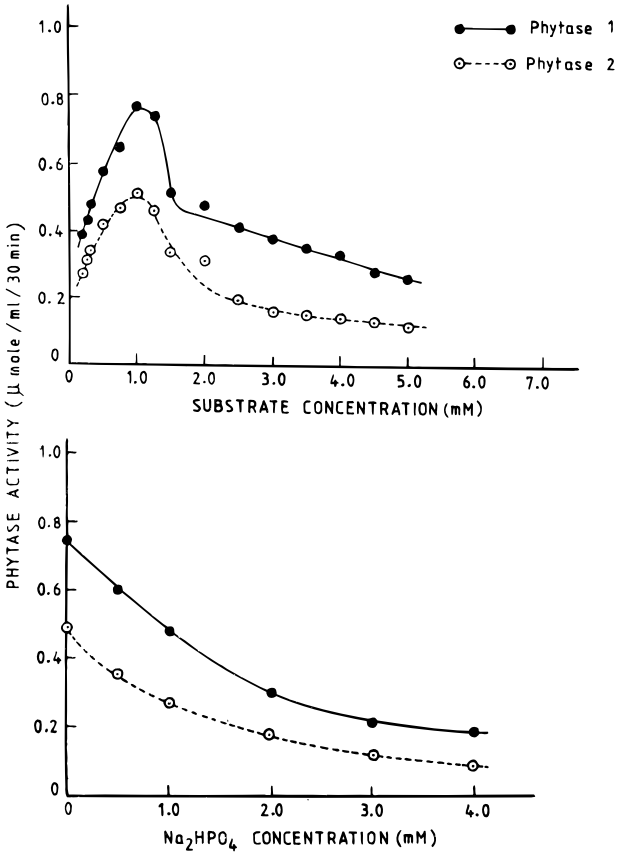

Figure 3. Effect of substrate and product (P_i) concentration on the activity of phytases 1 and 2 in rapeseed.

(Mandal *et al.*, 1972) and certain bacteria (Powar and Jaganathan, 1967). Optimum temperature for both fractions of phytase was 50 °C (Table 3), which was quite high. This may be a reflection of high thermostability of this enzyme in rapeseed. This optimal temperature is similar to those reported for navy bean phytase (Lolas and Markakis, 1977) and corn seed phytase (Chang, 1967), whereas it appears to be lower

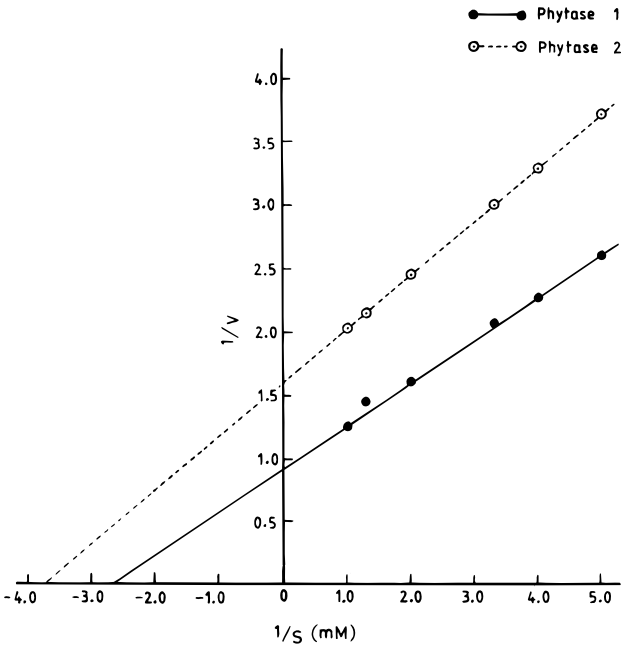

Figure 4. Lineweaver–Burk plot of 1/*V* against 1/*S* of phytases 1 and 2 in rapeseed.

than that for some legumes, mung bean, 57 °C (Mandal *et al.*, 1972), and higher than that for pea phytase, 45 °C (Beal and Mehta, 1985).

Thermal stability of enzyme showed that both the fractions were quite stable up to 50 °C. Phytase 2 is stable for a longer time at 50 °C. At 70 °C, most of the enzyme activity was diminished after 10 min of heating and got completely inactivated after 50 min of heating (Figure 2).

Maximum enzyme activity of both fractions in the present study was at the substrate concentration of 1.0 mM, showing that the enzyme system was saturated with this substrate concentration. Higher substrate concentrations proved to be inhibitory for the enzyme (Figure 3). From a plot of 1/*V* against 1/*S*, K_m values of 0.37 and 0.27 mM were observed for phytases 1 and 2, respectively (Figure 4). These enzyme fractions have V_{max} values of 1.11 and 0.625 μmol mL^{-1} 30 min^{-1}. These Michaelis constant values compared well to literature values for other phytases (Kim and Eskin, 1987; Chang and Schwimmer, 1977).

Effect of the product, P_i, on phytase activity was studied by including different concentrations of Na_2HPO_4 in assay mixtures (Figure 3). It was recorded that at smaller concentration (up to 0.5 mM) of P_i, only 20.0% inhibition was found for phytase 1 and 29.1% for phytase 2. At 4.0 mM concentration, phytases 1 and 2 showed 75.5% and 80.2% inhibition, respectively, showing that phytase 2 is more prone to the product inhibition. A literature survey of other seeds indicates that pea phytase activity was inhibited three-fourths at 2.0 mM concentrations of P_i (Beal and Mehta, 1985), while mung bean phytase activity was inhibited by 49% at a concentration of 2 μmol of P_i/mL (Mandal *et al.*, 1972).

Data presented in Figure 5 indicate that salts of zinc, ferrous, ferric, copper, silver, and mercuric ions at concentrations of 10^{-2} M showed a significant inhibitory effect on activity of phytases 1 and 2. Most of these inhibitory metal ions have strong affinity for phytase itself, competing with the enzyme for the substrate. Ions of molybdate, arsenate, fluoride, and iodide also proved to be inhibitory for phytases 1 and 2. Of the above inhibitory ions, zinc acted as an uncompetitive inhibitor, whereas mercuric ions acted as noncompetitive inhibitor in the present study. Molybdate and arsenate ions showed competitive inhibition for phytase 1 and mixed type of inhibition for phytase 2. Ferric and fluoride ions also showed mixed type of inhibition for both fractions.

Ions of magnesium, manganese, ammonium, cobalt, calcium, and EDTA promoted the enzyme activity in both fractions. Usually, EDTA decreases or has no effect on enzyme activity. Activation of the enzyme with EDTA in the present study seems to be due to the removal of contaminating inhibitory metals. Enzyme phytase seemed to have no demonstrable requirement for divalent and monovalent ions, it being fully active in the presence of EDTA.

To conclude, the present data indicate that germination appears to be a simple and nonchemical approach for reducing antinutrutional factors and to improve the

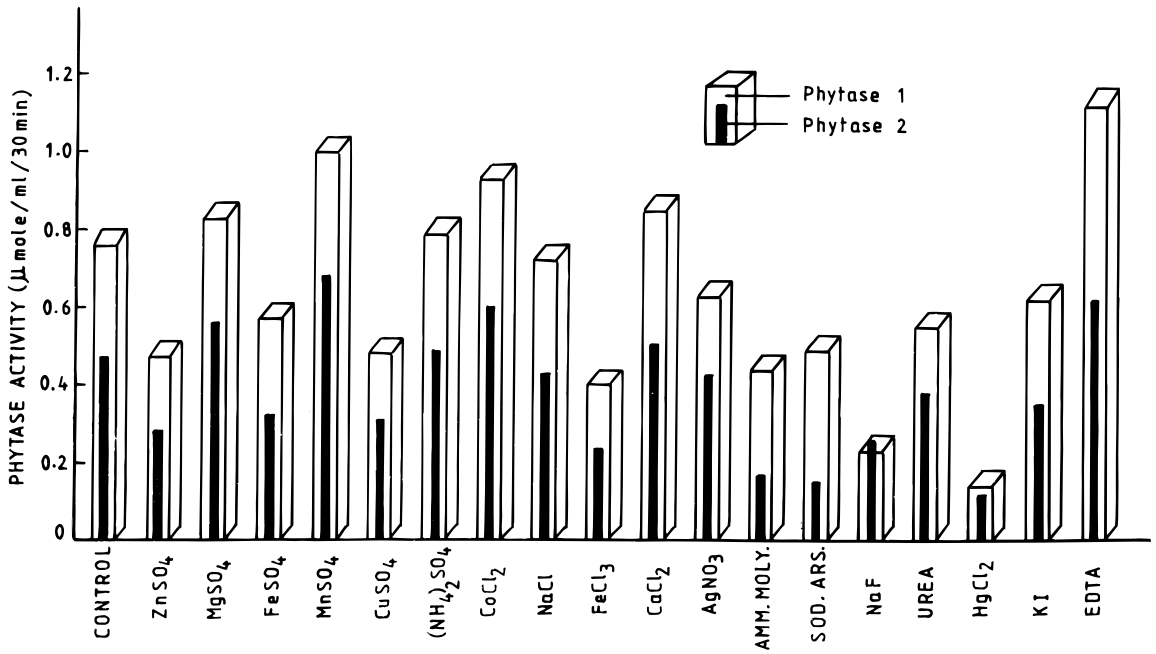

Figure 5. Effect of various metal ions on the activity of phytases 1 and 2 in rapeseed.

nutritional quality of rapeseed. Phytase fractions purified from the germinated seeds can be used for the reduction of phytic acid in different rapeseed products, as has been reported by Nair *et al.* (1991).

LITERATURE CITED

Beal, L.; Mehta, T. Zinc and phytase distribution in peas. Influence of heat treatment, germination, pH, substrate and phosphorus on pea phytate and phytase. *J. Food Sci.* **1985**, *50*, 96−100.

Bishoni, S.; Khetarpaul, N.; Yadav, R. K. Effect of domestic processing and cooking methods on phytic acid and polyphenol content of pea cultivars. *Plant Foods Hum. Nutr.* **1994**, *45*, 381−388.

Blumenthal-Goldschmidt, S. Thermostable substance from lettuce seeds and seedlings inhibiting 3-1AA oxidation. *Bull. Res. Counc. Isr.* **1960**, *9D*, 93−99.

Brooker, J. D.; Cheung, C. P.; Marcus, A. Protein synthesis and seed germination. In *Physiology and Biochemistry of Seed Dormancy and Germination*; Khan, A. A., Ed.; Elsevier/North-Holland Biomedical Press: Amsterdam, 1977; pp 347−360.

Chang, C. W. Study of phytase and fluoride effect in germinating corn seeds. *Cereal Chem.* **1967**, *44*, 122−142.

Chang, R.; Schwimmer, S. Characterization of phytase of beans *(Phaseolus vulgaris). J. Food Biochem.* **1977**, *1*, 45−48.

Chen, P. S.; Toshibara, T. Y.; Warner, H. Microdetermination of phosphorus. *Anal. Chem.* **1956**, *28*, 1756−1758.

Clossais-Besnard, N.; Larher, F. Physiological role of glucosinolate in *B. napus.* Concentration and distribution pattern of glucosinolates among plant organs during a complete life cycle. *J. Sci. Food Agric.* **1991**, *56*, 25−38.

Colowick, S. P.; Kaplan, N. O. *Methods Enzymol.* **1966**, *8*, 672.

Craig, E. A.; Draper, S. R. The glucosinolate content of oil seed rape varieties. *J. Natl. Inst. Agric. Biol.* **1979**, *15*, 98−103.

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.

Gibbins, L. N.; Norris, F. W. Phytase and acid phosphatase in *Phaseolus vulgaris. J. Biochem.* **1963**, *86*, 67−71.

Goel, M.; Sharma, C. B. Inhibition of plant phytases by phloroglucinol *Phytochemistry* **1979**, *18*, 969−970.

Kim, H.; Eskin, N. A. M. Canola phytase: isolation and characterization. *J. Food Sci.* **1987**, *52*, 1353−1354.

Kroll, J.; Kiyawa, M.; Schnaak, W. Preparation of rapeseed proteins by extraction, ultrafiltration and diafiltration. *Fat Sci. Technol.* **1991**, *93*, 61−64.

Lolas, G. M.; Markakis, P. The phytase of navy beans *(Phaseolus vulgaris). J. Food Sci.* **1977**, *42*, 1094−1097.

Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Mandal, N. C.; Burman, S.; Biswas, B. B. Isolation, purification and characterization of phytase from germinating mung beans. *Phytochemistry* **1972**, *11*, 495−502.

Nair, V. C.; Laflamme, J.; Duvnjak, Z. Production of phytase by *A. ficuum* and reduction of phytic acid content in Canola meal. *J. Sci. Food Agric.* **1991**, *54*, 355−365.

Peers, F. G. Phytase of wheat. *Biochem. J.* **1953**, *53*, 102−110.

Powar, V. K.; Jaganathan, V. Phytase from *Bacillus subtills. Indian J. Biochem.* **1967**, *4*, 184−186.

Singh, B.; Sedeh, H. G. Characteristics of phytase and its relationship to acid phosphatase and certain minerals in triticale. *Cereal Chem.* **1979**, *56*, 267−270.

Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica I.* The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63−68.

Thompson, D. B.; Erdman, J. W., Jr. Phytic acid determination in soyabeans. *J. Food Sci.* **1982**, *47*, 573−576.

Tzeng, Y. M.; Diosady, L. L.; Rubin, L. J. Production of Canola protein material by alkaline extraction, precipitation and membrane processing. *J. Food Sci.* **1990**, *55*, 1147−1151.

Xu, L.; Diosady, L. L. Functional properties of Chinese rapeseed protein isolates. *J. Food Sci.* **1994**, *59*, 1127−1130.

Zhou, B.; He, Z. Q.; Yu, H. M.; Mukherjee, K. D. Proteins from double zero rapeseed. *J. Agric. Food Chem.* **1990**, *38*, 690−694.

Received for review December 3, 1996. Accepted April 29, 1997.[⊗]

JF9609136

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.