

## Enzymatic Elimination of Phytate in Soybean Milk

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The phytic acid content of commercial soybean milk in Japan was from 0.52 to 1.11 mg/g. Phytate in soybean milk was hydrolyzed by wheat phytase. The enzyme was immobilized on Dowex MWA-1, MSC-1, Duolite ES-762 with glutaraldehyde. The activity yield was in the range of 4.5~21% depending upon the immobilization method. The optimum pH and  $K_m$  values of the immobilized enzyme on Duolite ES-762 were similar to those of the free enzyme. On the other hand, the optimum pH of the immobilized enzyme on Dowex MSC-1 shifted by one pH unit toward the alkaline region, and its  $K_m$  value was considerably increased. The optimum temperature did not change by immobilization. Immobilized enzymes on Duolite ES-762 and Dowex MSC-1 were used for the hydrolysis of phytate in soybean milk.

Phytic acid, *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, is a unique stock compound for phosphorus in cereal grains and legumes such as rice, wheat, barley, and soybeans accounting for 18 to 88% of the total phosphorus<sup>1,2)</sup>. The interaction of phytic acid with minerals or proteins is considered to be one of the primary factors which limit the nutritional values of cereal grains and legume proteins. Phytic acid forms insoluble products with various metals and is believed to interfere with assimilation of calcium, iron, and zinc in human and animal bodies. High levels of phytate may also cause magnesium deficiency<sup>1,2)</sup>. Although less attention has been paid to phytate-protein interaction, it has been found that phytic acid inhibits pepsin<sup>3)</sup>, trypsin<sup>4)</sup>, and  $\alpha$ -amylase<sup>5)</sup> *in vitro*. Therefore, many attempts have been made to eliminate phytate from soybeans and its products for nutritional improvement by cooking<sup>6)</sup>, soaking<sup>2)</sup>, germination<sup>7)</sup>, autolysis<sup>2)</sup>, fermentative processes<sup>8)</sup>, ultrafiltration<sup>9)</sup>, and special treatments<sup>10,11)</sup>.

Phytase, *myo*-inositol hexakisphosphate phosphohydrolase is an enzyme which catalyzes the hydrolysis of phytate, and has been demonstrated to be present in various kinds of seeds<sup>1,2)</sup>. If the phytate in soybean products could be

eliminated before consumption, their nutritional value would be improved and the phytate would be utilized as a source of inositol and inorganic phosphate.

Soybean milk has drawn considerable attention as an economical high-protein beverage. Applications of  $\alpha$ -galactosidase to remove oligosaccharides from soybean milk were reported<sup>12)</sup><sup>13)</sup>. However, little information is available on the hydrolysis of phytate in soybean milk by soluble and immobilized enzymes.

This paper demonstrates that the phytate in soybean milk can be hydrolyzed not only by free wheat phytase but also by the immobilized phytase. The properties of immobilized phytase are also described.

### Materials and Methods

#### Materials

Commercially available soybean milk was used. Phytase from wheat (Sigma Co., Ltd) was used as a source of enzyme. Ion exchange resins (20~50 mesh), Dowex MSC-1, MWA-1, and Duolite ES-762, used as supports for immobilization, were the products of the Dow Chemical Co., Ltd, and the Diamond Shamrock Chemical Co., Ltd. Sodium phytate and *p*-nitrophenylphosphate as substrates were purchased

from the Nakarai Chemical Co. Pepsin was from the Wako Chemical Co., Ltd. All the other reagents used were of analytical grade.

#### *Determination of phytic acid*

Extraction and precipitation of phytate from soybean milk were performed according to WHEELER and FERREL<sup>14</sup>). A 6% trichloroacetic acid (TCA) solution was added to an equal volume of soybean milk to remove protein. Then the slurry obtained was centrifuged at 3 000 rpm for 10 min and 25 ml of supernatant was used for the assay of phytic acid. Phytate in the supernatant was precipitated as ferric phytate. The conversion of ferric phytate to ferric hydroxide was carried out by MAKOWER's procedure<sup>15</sup>). The precipitate produced was dissolved in HCl and the ferric iron content was measured colorimetrically with *o*-phenanthroline. The contents of iron and phosphorus were assumed to be in a 4 : 6 ratio in order to calculate the phytate phosphorus content. The weight of phytate was determined by multiplying the iron content by 2.98.

#### *Preparation of immobilized enzyme*

The ion exchange resins were equilibrated with 0.02 M citrate buffer (pH 6.0). These resins were separated from the buffer solution through a glass filter and 10 g of each resin was added to 100 ml of the enzyme solution (13.6 mg protein/ml) and shaken gently for 24 h at 5°C. After discarding 70 ml of solution, 3 ml of glutaraldehyde solution (25%) was added to the mixture to make crosslinkage among the enzyme proteins, by shaking for 2 h at 5°C. The enzyme-bound resin was filtered through a glass filter and was washed thoroughly with chilled citrate buffer (0.02 M, pH 6.0) to remove the enzyme protein loosely adsorbed or attached to the resin beads. The immobilized enzyme was equilibrated with 0.2 M acetate buffer (pH 5.0), and the activity was measured. The activity yield was evaluated according to the following equation: activity yield (%) = (activity of enzyme beads) × 100 / [(activity of the free enzyme used for immobilization) - (activity of the free enzyme in the solution after removal of the enzyme beads)].

#### *Assay of enzyme activity*

Phytase activity was assayed in terms of the inorganic phosphorus by the method of ALLEN<sup>16</sup>).

The optimum pH was determined by using 0.2 M acetate buffer (pH 3.5~5.5) and 0.2 M Tris-malate buffer (pH 5.25~7.0). The reaction mixture consisted of 1 ml of 1% phytate in 0.2 M buffer, and 1 ml of the enzyme solution. The reaction was allowed to proceed at 40°C for 30 min and terminated by the addition of 2 ml of 10% TCA. The effect of temperature was studied in the range of 20 to 60°C. For pH stability studies, the enzyme was incubated in buffer solutions of lower molarity (0.02 M) with different pH's at 40°C for 30 min. Then residual enzymatic activity was measured at 40°C, pH 5.0. Similarly, the crude enzyme was preincubated at different temperatures for 30 min at pH 5.0 and residual enzymatic activity was determined. The activity of immobilized phytase was measured by the addition of 100 mg of beads to 10 ml of 0.5% phytate in 0.1 M acetate buffer, pH 5.0. The reaction mixture was incubated at 40°C for 2 h with stirring. Then 2 ml aliquot of the mixture was taken into 2 ml of TCA. One unit of enzyme activity was defined as the activity which liberated 1  $\mu$  mol of phosphorus per min at 40°C, pH 5.0.

#### *Enzymatic elimination of phytate in soybean milk*

Soybean milk was treated enzymatically by the following procedures. Two ml of enzyme solution (0.65~26 units) was added to 50 ml of soybean milk and the mixture incubated at 50°C up to 6~8 h. In the case of the immobilized phytase, an appropriate amount (wet weight: 0.5~5 g) of the beads was added to 50 ml of the soybean milk in a 100 ml flask. The reaction mixture was stirred with a magnetic stirrer. At proper time intervals, 1 ml of sample was taken into 2 ml of 10% TCA and the protein precipitated was separated by centrifugation. The supernatant was used for the determination of inorganic phosphorus.

#### *Determination of non-protein nitrogen released by pepsin*

The effect of phytase treatment on the proteolysis of soybean milk by pepsin was evaluated in terms of non-protein nitrogen released. Forty-five ml of soybean milk was added to 5 ml of 1% enzyme solution and the mixture was incubated at 40°C for 3 h. Then the mixture

was kept in boiling water bath for 10 min, cooled, and finally diluted with glycine-HCl buffer (pH 1.8). Fifteen ml of 0.5% pepsin solution was added to 25 ml of phytase-treated or untreated soybean milk and the mixture was incubated at 40°C. At proper time intervals, 2 ml aliquot was removed and mixed with 2 ml of 10% TCA. Then the samples were filtered and nitrogen in the filtrate was determined. Non-protein nitrogen content was determined according to LOWRY *et al.* as modified by HAGIHARA<sup>17)</sup> with tyrosine as the standard. The emulsifying property of protein of soybean milk treated by phytase was determined in terms of the turbidity at 550 nm of the supernatant obtained after the pH was adjusted with buffer<sup>18)</sup>.

## Results and Discussion

### Phytic acid content of soybean milk

Phytic acid contents of soybean milk are given in Table 1. The phytic acid content in the soybean milk ranged from 0.52 to 1.11 mg/g LOLAS *et al.*<sup>19)</sup> reported that the phytic acid content of 15 soybean varieties ranged from 1.00 to 1.47% (dry weight basis) which represented between 51.4 and 57.1% of the total phosphorus. The solid content in soybean milk ranged from 6 to 9%. Thus, the phytic acid content of soybean milk obtained in this study is in reasonable agreement with the result by LOLAS *et al.*

### Characteristics of crude phytase preparation

Fig. 1. shows pH vs. enzyme activity curves for the crude enzyme preparation. The free phytase had a relatively sharp pH optimum around 5.0 with a rapid diminution in activity on either side of this optimum. The optimum pH reported so far ranged from 5.0 to 5.7<sup>2)</sup>. Optimum temperature of enzymatic activity was around 50°C. An optimum of 45°C has been reported for triticale phytase by SINGH and SEDEH<sup>20)</sup>, 50°C for navy bean phytase by LOLAS and MARKAKIS<sup>21)</sup>, 57°C for germinating mung bean by MANDAL and BISAWAS<sup>22)</sup> and 55°C for wheat phytase by PEERS<sup>23)</sup>. Heating for 30 min up to 45°C did not depress the enzymatic activity, but at 60°C the activity was decreased to about 40% of the control and at 70°C the enzyme was completely inactivated. The en-

Table 1 Phytic acid content of soybean milk

No.	Phytic acid (mg/g)	pH
1	1.11	7.05
2	1.11	7.03
3	1.08	6.96
4	1.00	6.66
5	0.78	6.32
6	0.52	6.67

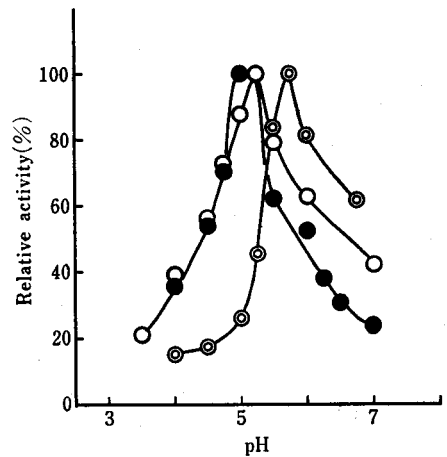


Fig. 1 pH-activity profile of phytase

- : free state
- : immobilized on Duolite ES-762
- ◐ : immobilized on Dowex MSG-1

zyme was stable at pH 3.5 to 7. In the substrate concentration from 0.2 to 9 mM,  $K_m$  value was 0.83 mM, although the enzymatic activity was inhibited at the concentration higher than 2 mM. LOLAS *et al.*<sup>19)</sup> and SINGH *et al.*<sup>20)</sup> observed a similar relationship between substrate and enzyme activity for navy beans and triticale. This might be due to the electrostatic attraction between substrate and enzyme. GIBBINS and NORRIS<sup>24)</sup> suggested that the inhibition of phytase under high substrate concentrations was indicative of two-point attachment of phytate to phytase. The activation energy for hydrolysis of phytate was about 8500 cal/mol. This value of wheat phytase was within the range found for other plants. A number of studies have been done on phytases

from various kinds of plant materials. Though highly purified phytase has rarely been available<sup>25,26</sup>). These phytases show broad substrate specificity and are classified as a kind of acid phosphatase.

#### *Characteristics of immobilized enzyme*

The activity yields of the immobilized phytase are listed in Table 2. The recovery of enzymatic activity of phytase immobilized on Duolite ES-762 was the highest among the three resins. Phytase immobilized on Dowex MWA-1 was so low that its enzymatic properties were not determined.

The pH profiles of immobilized phytase are shown in Fig. 1. The optimum pHs for Duolite ES-762 and Dowex MSC-1 were 5.25 and 5.75, respectively. In soybean milk, the incubation with the enzyme at pH lower than 6.0 caused isoelectric precipitation of soybean proteins. The incubation with the enzyme at neutral pH would be more desirable. Therefore, the immobilized enzyme is suitable for treatment of soybean milk. The other enzymatic properties of immobilized phytase are summarized in Table 3. Immobilization did not shift the optimum temperature of these enzymes. Optimum temperature for hydrolysis of phytate was around 50°C. The activation energies were evaluated as 12 000 cal/mol for

Duolite ES-762 and 10 000 cal/mol for Dowex MSC-1. Slight increase in activation energy by immobilization might be due to the modification of protein conformation.

The affinity of the enzyme for the substrate phytate was evaluated as the  $K_m$  value. The  $K_m$  values of the enzyme immobilized on Duolite ES-762 and Dowex MSC-1 were 2.94 mM and 33.3 mM, respectively. The  $K_m$  values were considerably increased as a result of immobilization. Especially, in the case of Dowex MSC-1, the  $K_m$  value was increased 40 times by immobilization. This might result from electrostatic repulsion between substrate and support, since phytate is considered to have a negative charge at pH 5.0 and the resin Dowex MSC-1 is a type of polysulfonic acid (negative charge).

#### *Enzymatic elimination of phytate in soybean milk*

The optimum temperature for the hydrolysis of phytate in soybean milk was 50°C both in the immobilized and free enzyme. From the dependency of the reaction rate on temperature, the activation energies were evaluated: they were 12 800 cal/mol for Duolite ES-762 and 12 900 cal/mol for Dowex MSC-1 in the immobilized state; whereas they were 7 500 cal/mol for phytase in the free state. The increase

Table 2 Activity yields of immobilized phytase

Support	Amount of enzyme adsorbed (units/g-wet resin)	Activity of immobilized enzyme (units/g-wet resin)	Yield (%)
Duolite ES-762	6.82	1.28	18.8
Dowex MSC-1	2.62	0.55	20.9
Dowex MWA-1	4.75	0.22	4.6

Table 3 Characteristics of immobilized wheat phytase

Property	Free	Immobilized	
		Duolite ES-762	Dowex MSC-1
Optimum pH (50°C)	5.0	5.25	5.75
Optimum temperature	50°C	50°C	50°C
$K_m$ at (pH 5.0) (mM)	0.83	2.94	33.3
Activation energy (cal/mol)	8 500	12 000	10 000

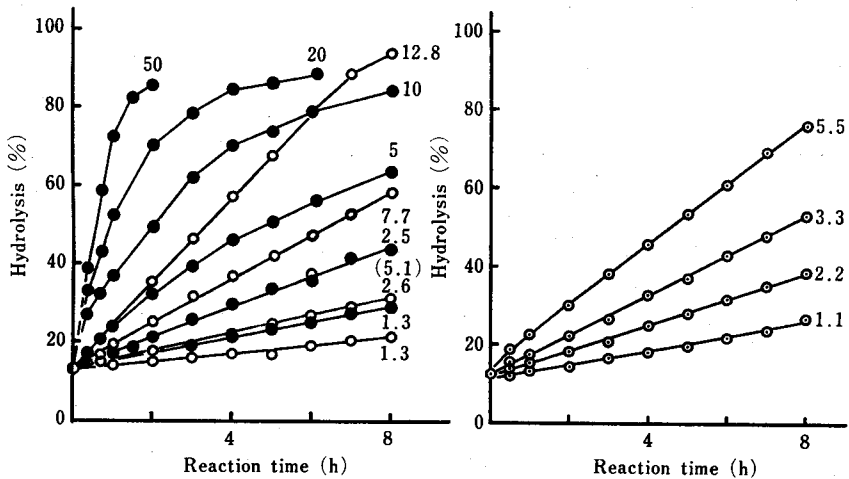


Fig. 2 Course of hydrolysis of phytate in soybean milk

All the values in the Fig. indicate units of phytase activity per 100 ml of soybean milk.

- : free state
- : immobilized on Duolite ES-762
- : immobilized on Dowex MSC-1

in activation energy by immobilization found in soybean milk is slightly higher than that found in phytate solution. However, the reason is not clear.

The course of the hydrolysis of phytate in soybean milk is shown in Fig. 2. In soybean milk (pH 7.0), the enzyme was quite stable. The amount of phosphorus liberated from phytate by the action of phytase increased linearly with the incubation time. The rate of hydrolysis was approximately proportional to the amounts of phytase in the soluble or immobilized form in the mixtures. The activity of phytase immobilized on Dowex MSC-1 was higher than the activity of enzyme immobilized on Duolite ES-762. This result suggests that the properties of the support have a great influence on the activities of enzymes. Duolite ES-762 is a type of polyphenol resin being able to adsorb hydrophobic substances such as proteins and lipids. Thus the adsorption of soybean protein on Duolite ES-762 might have interfered with the enzymatic hydrolysis of phytate in soybean milk. On the other hand, Dowex MSC-1 does not adsorb the protein because of electrostatic repulsion between pro-

tein and this support.

The interaction of soybean protein with phytate is considered to be one of the primary factors limiting the hydrolysis of phytate. The incubation of the enzyme at any pH lower than 6 would cause an isoelectric precipitation of soybean proteins. Published reports<sup>9)11)</sup> indicate that phytic acid interacts with protein to form complexes at acidic pH. At higher pHs than the isoelectric point of glycinin, e.g. pH 4.9, no binding between phytic acid and protein is detected by gel filtration on Sephadex G-75. The degree of binding is found to increase with lower pH, from a value of zero at the isoelectric point to a maximum value of 424 equivalents of phytate per mol glycinin dimer (360 000 daltons)<sup>9)</sup>. Therefore, the incubation of soybean milk with the enzyme at the natural pH would be desirable.

#### *Characteristics of soybean milk treated by phytase*

To examine the nutritive value of soybean milk treated by phytase, the digestibility by pepsin at pH 1.8 was evaluated in terms of non-protein nitrogen released. The courses of nitrogen production from treated and non-

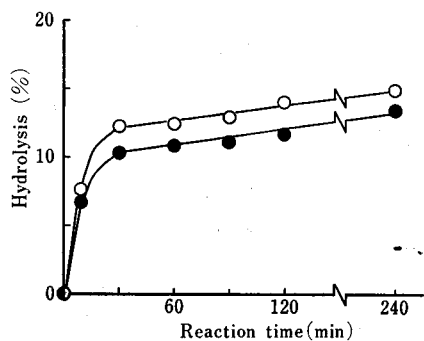


Fig. 3 Effect of enzymatic pre-treatment of phytate on the hydrolysis of soybean milk by pepsin

○ : treated ● : untreated

treated soybean milk are shown in Fig. 3. Proteolysis with pepsin was somewhat enhanced by the elimination of phytate. REDDY *et al.*<sup>3)</sup> reviewed that the phytate-protein complex was less subjected to proteolytic attack than the same protein alone.

PEARCE and KINSELLA proposed a method to evaluate the emulsifying properties of proteins by turbidimetric technique<sup>19)</sup>. The influence of pH on the turbidity of soybean milk pretreated by phytase is shown in Fig. 4. The emulsifying property of protein was improved by the treatment.

Phytic acid is known to form complexes with protein at both acidic and alkaline pH. The interaction between phytic acid and protein is thought to be of the ionic binding. The phytate is strongly negatively charged at pH 1.8, while proteins are strongly positively charged. A phytate-protein complex could be formed under such circumstances. On the other hand, at high pHs, multivalent cations such as  $Ca^{2+}$  are thought to mediate the formation of phytate-protein complexes<sup>11)</sup>. The interaction between phytate and proteins leads to decrease the solubility of the proteins. It has also been shown that calcium ion interacts with protein and phytate to decrease the solubility of proteins further. The reduced solubility of proteins as a result of formation of protein-phytate complexes can negatively affect certain functional properties of proteins which have close influence upon their hydra-

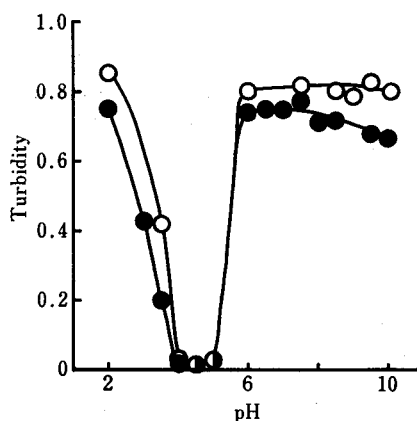


Fig. 4 Effect of pH on the turbidity of soybean milk pre-treated by phytase

○ : treated ● : untreated

tion, solubility, emulsifying property, and dispersibility in aqueous medium.

In this study, the phytate in soybean milk was proved to be hydrolyzed by the wheat phytase. Wheat phytase was immobilized by adsorption on rigid supports such as Dowex MSC-1, MWA-1, and Duolite ES-762 with glutaraldehyde treatment. The phytate present in soybean milk was extensively hydrolyzed by enzymatic treatment. The procedure to immobilize phytase reported here can be used for practical application because of its simplicity and the high mechanical strength of resin beads.

#### References

- 1) MAGA, J. A.: *J. Agric. Food Chem.*, **30**, 1 (1982).
- 2) REDDY, N. R., SATHE, S. K. and SALUNKHE, D. K.: *Adv. Food Res.*, **28**, 1 (1982).
- 3) KANAYA, K., YASUMOTO, K. and MITSUDA, H.: *Eiyo to Shokuryo*, **29**, 341 (1976).
- 4) SINGH, M. and KRICKORIAN, A. D.: *J. Agric. Food Chem.*, **30**, 799 (1982).
- 5) SHARMA, C. B., GOEL, M. and IRSHAD, M.: *Phytochemistry*, **17**, 201 (1978).
- 6) BOLAND, A. de, GARNER, G. B. and O'DELL, B. L.: *J. Agric. Food Chem.*, **23**, 1186 (1975).
- 7) CHEN, L. H. and PAN, S. H.: *Nutr. Rep. Int.*, **46**, 125 (1977).
- 8) SUDARMADJI, S. and MARKAKIS, P.: *J. Sci. Food Agric.*, **28**, 381 (1977).
- 9) OKUBO, K., WALDRUP, A. B., IACOBUCCI, G. A.

- and MYERS, D.V. : *Cereal Chem.*, **52**, 263 (1975).
- 10) MCKINNEY, L.L., SOLLARS, W.F. and SETZKOW, E.A. : *J. Biol. Chem.*, **178**, 117 (1949).
- 11) RHAM, O. de and JOST, T. : *J. Food Sci.*, **44**, 596 (1979).
- 12) SUGIMOTO, H. and VAN BUREN, J.P. : *J. Food Sci.*, **35**, 655 (1970).
- 13) CRUZ, R. and PARK, Y.K. : *J. Food Sci.*, **47**, 1973 (1982).
- 14) WHEELER, E.L. and FERREL, R.E. : *Cereal Chem.*, **48**, 312 (1971).
- 15) MAKOWER, R.U. : *Cereal Chem.*, **47**, 288 (1970).
- 16) ALLEN, R.J.L. : *Biochem. J.*, **34**, 858 (1940).
- 17) HAGIHARA, B. : "Koso Kenkyuho Vol. 1" (Asakura Shyoten, Tokyo), p. 166 (1955).
- 18) PEARCE, K.N. and KINSELLA, J.E. : *J. Agric. Food Chem.*, **26**, 716 (1978).
- 19) LOLAS, G.M., PALAMIDIS, N. and MARKAKIS, P. : *Cereal Chem.*, **53**, 867 (1976).
- 20) SINGH, B. and SEDEH, H.G. : *Cereal Chem.*, **56**, 267 (1979).
- 21) LOLAS, G.M. and MARKAKIS, P. : *J. Food Sci.*, **42**, 1094 (1977).
- 22) MADAL, N.C. and BISWAS, B.B. : *Plant Physiol.*, **45**, 4 (1970).
- 23) PEERS, F.G. : *Biochem. J.*, **53**, 102 (1953).
- 24) GIBBINS, L.N. and NORRIS, F.W. : *Biochem. J.*, **86**, 67 (1963).
- 25) AKIYAMA, T., UCHIMIYA, H. and SUZUKI, H. : *Plant Cell Physiol.*, **22**, 1023 (1981).
- 26) YAMAGATA, H., TAKAKA, K. and KASAI, Z. : *Plant Cell Physiol.*, **21**, 1449 (1980).

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## 豆乳中のフィチン酸の酵素分解について

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市販豆乳中のフィチン酸含量について分析した結果、その含量は52~110mg%の範囲にあった。豆乳中のフィチン酸は、小麦由来のフィターゼにより加水分解されることが認められた。そこで、本酵素をDowex MWA-1, MSC-1, Duolite ES-762 にグルタルアルデヒドを用いて固定化した。その活性収率は4.5~21%であった。Duolite ES-762 に固定化した酵素の最適 pH 及び  $K_m$  値は、固定化前の酵素の値とほぼ類似した値を示した。しかし、Dowex MSC-1 に固定化した場合、最適 pH はアルカリ側で pH 1 ほど移動した。また、その  $K_m$  値も固定化により著しく増大した。固定化による酵素の最適温度には変化は認められなかった。これら Duolite ES-762, Dowex MSC-1 に固定化した酵素を用いて豆乳中のフィチン酸の加水分解を行なうことができた。