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Immobilization of phytase on epoxy-activated Sepabead EC-EP for the hydrolysis of soymilk phytate

Evran Bıçak Çelem, Seçil Önal*

Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova-İzmir, Turkey

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ABSTRACT

In this work, an active phytase concentrated extract from soybean sprout was immobilized on a polymethacrylate-based polymer Sepabead EC-EP which is activated with epoxy groups. The immobilized enzyme exhibited an activity of 0.1 U/g of carrier and activity yield of 64.7%. The optimum temperature and pH for the activity of both free and immobilized enzymes were found as 60 °C and pH 5.0, respectively. The immobilized enzyme was more stable than free enzyme in the range of pH 3.0–8.0 and more than 70% of the original activity was recovered. Both the enzymes completely retained nearly about 84% of their original activity at 65 °C. The K_m and V_{max} values were measured as 5 mM and 0.63 U/mg for free enzyme and 12.5 mM and 0.71 U/mg for immobilized enzyme, respectively. Free and immobilized soybean sprout phytase enzymes were also used in the biodegradation of soymilk phytate. The immobilized enzyme hydrolysed 92.5% of soymilk phytate in 7 h at 60 °C, as compared with 98% hydrolysis observed for the native enzyme over the same period of time. The immobilization procedure on Sepabead EC-EP is very cheap and also easy to carry out, and the features of the immobilized enzyme are very attractive that the potential for practical application is considerable.

1. Introduction

Phytate (*myo*-inositol hexakisphosphate) is the major form of phosphorus stored in cereals, pollens, legumes and oil seeds, representing 18–88% of the total phosphorus content. It is a naturally occurring component formed during the maturation of seeds and cereal grains. In seeds and grains, it functions as: a phosphorus store, a source of *myo*-inositol (a cell wall precursor), a source of cations, an energy store. Beside of this, phytate is very important for several reasons: (a) it is often considered to be an antinutritional substance in human diets, but it may have a positive nutritional role as an anti-oxidant and anti-cancer agent. (b) It has preventive effects against heart disease, diabetes and kidney stones. (c) It has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas. (d) It has a role in decreasing cholesterol and triglycerides and has a positive effect in the treatment of Parkinson's disease, Alzheimer's disease and MS [1–3]. Phytate is considered as an antinutritional factor due to its ability to chelate metal cations such as zinc, magnesium, calcium and iron rendering them nutritionally unavailable. In addition, phytate has poor availability for monogastrics including pigs, poultry and humans due to the lack of phytases. Phytate is not degraded during digestion in the small intestine and the phosphate is not available. It passes through the intestinal track and is excreted in the feces. This causes environmental problems in areas of intensive livestock production. The infant soy formula and other soy protein products contain high levels of phytate. Therefore supplementation of soy-based foods with plant phytases will increase the mineral bioavailability and the nutritional value of foods [4,5].

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) catalyse the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, *myo*-inositol and inorganic phosphate, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction [3]. In order for an enzyme to be a phytase it must display phosphatase activity. Three structurally distinct classes of enzymes have been described as phytases. These are: histidine acid phosphatases (HAP), β-propoller phytase (BPP) and purple acid phosphatases (PAP). Soybean phytase = GmPhy (*Glycine max*) has the active site motif of a purple acid phosphatase [6]. This class of enzymes are dinuclear metallohydrolases and Fe–Fe or Fe–Zn metals are located in their active sites [7]. Phytases are present in plants, certain animal tissues and microorganisms. But they have been studied most intensively in seeds of plants such as wheat, bean, rice, corn, etc. Adding phytases in food for human consumption may diminish antinutritional

* Corresponding author. Tel.: +90 232 3438624; fax: +90 232 3888264.
E-mail addresses: elvi28@hotmail.com (E.B. Çelem), secil.onal@ege.edu.tr (S. Önal).

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effects of phytate and makes phytases very important for biotechnological applications, especially for the reduction of phytate in food and feedstuffs [5]. In spite of numerous attempts to purify plant phytases [8,9] there are only a few immobilization studies [10–19].

For the industrial development of biocatalytic process an effective immobilization method is commonly required to allow the reuse of enzymes or continuous processing. Covalent immobilization has the advantage of forming strong and stable linkages between the enzyme and the carrier that result in robust biocatalysts. Immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols. For example, multipoint covalent immobilization improves the stability of the enzyme. It should be considered that any other technique employed to get a stable enzyme may be compatible with the stabilization of the enzyme by multipoint covalent attachment [20]. Epoxy-activated supports have been proposed as very efficient materials for the immobilization of proteins at the industrial scale for different reasons: e.g. high stability of the groups at neutral pH values even in wet conditions, commercial supports can be stored for long periods of time, high stability of the enzyme-support bonds, possibility of performing a final blocking of the remaining groups, and possibility of achieving stabilization of the enzymes via multipoint covalent attachment [20]. Epoxy-activated carriers were usually used for the immobilization of different industrial enzymes such as; β-galactosidase [20–22], lipase [23], glutarylacylase [24] and penicillin G acylase [25]. Immobilization of proteins on commercial epoxy supports follows a two-step mechanism: first, the enzyme is hydrophobically adsorbed on a fairly hydrophobic support (e.g., Eupergit, Sepabeads) at very high ionic strength, and then, the covalent reaction between the enzyme and the support proceeds [20,21]. Sepabeads EC are polymethacrylate-based carriers and can be successfully used for the immobilization of enzymes [26–30]. The series Sepabead EC-EP are epoxy activated, with high reactive group density. The chemistry for attachment of the enzyme to the support is straight forward. Compared with other epoxy acrylic polymers, Sepabeads EC-EP posses a high mechanical stability, high resistance to microbial attack and do not swell in water. They are particularly suitable for covalent immobilization of enzymes for industrial applications because of their excellent mechanical properties when used in bioreactors. Furthermore, the raw materials applied for the production of these supports are included in the EU list of resins for the processing of foodstuffs [31]. Mateo et al. [20] have immobilized various industrial enzymes on epoxy-Sepabeads and amino-epoxy-Sepabeads. Amino-epoxy immobilized enzyme was more stable than the epoxy-immobilized enzyme. This stabilization was explained by the differences between both kind of supports; orientation of enzyme molecules onto the support surface and different possibilities of multipoint attachment [20]. Soybean, a concentrated source of isoflavones has received a considerable attention for its potential role in preventing and treating cancer and osteoporosis. Moreover, soymilk is considered as a low cost substitute for cows milk in developing countries as a nutritive supplement for lactose intolerance infants and children [32,33].

In the present investigation, we report the application of Sepabead EC-EP for the immobilization of phytase. The first aim of the study was to investigate the effects of initial enzyme concentration on activity yields. Having obtained the highly efficient immobilized phytase, it was then characterized. Some parameters effecting to the enzyme activity and stability were analyzed. Moreover, a kinetic study of phytate hydrolysis in soymilk by both forms of enzyme was carried out. To our knowledge, this is the first report of the usage of Sepabead EC-EP as a support for the immobilization of phytases.

2. Materials and methods

2.1. Materials

Soybean sprout and soy flour were obtained from local markets. Sepabead EC-EP was kindly provided by Resindion S.R.L (Mitsubishi Chemical Co., Milan, Italy). All other chemicals and reagents were of the highest available purity and used as purchased.

All the experiments were performed at least by triplicate and experimental error was always under 10%.

2.2. Assay of phytase activity

Phytase activity was determined by measuring the amount of inorganic phosphate liberated from sodium phytate [34]. The assay mixture consisted of 0.4 ml of 2 mM Na-phytate (in 0.1 M pH 5.0 acetate buffer) and 0.1 ml free enzyme solution. For the immobilized enzyme, assay mixture consisted of 0.4 ml of Na-phytate, 100 mg immobilized enzyme and 0.1 ml of 0.1 M acetate buffer (pH 5.0). After incubation for 30 min at 37 °C, the reaction was stopped by adding 0.5 ml of 15% (w/v) trichloroacetic acid. Reaction mixture centrifugated at 10 000 rpm for 10 min. The liberated phosphate was determined spectrophotometrically at 660 nm using the method as reported previously [35].

One unit (1 U) of phytase activity was defined as 1 μmol inorganic phosphate released per min under conditions explained above.

2.3. Protein determination

Protein concentrations were determined according to the Coomassie blue G-250 dye-binding assay using bovine serum albumin as a standard [36]. The amount of bound protein was determined indirectly from the difference between the amount of protein in the filtrates and washing solutions after immobilization.

2.4. Phytate analysis

Phytate content of soymilk was measured by modified Latta and Eskin method [37]. 0.1 ml of sample was taken out from reactor and then the reaction was stopped by adding 0.2 ml of 3% (w/v) trichloroacetic acid. After dilution with bidistilled water in a ratio 1:5 (depending on phytate content), 0.5 ml Wade reactive (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalisilic acid in bidistilled water) was added. The sample centrifugated at 4000 rpm for 2 min and then phytate content measured spectrophotometrically at 500 nm.

2.5. Isolation and partial purification of phytase from soybean sprout

Soybean sprout was separated from green leaves and the root and then grounded in ice-cold blender with 0.1 M pH 5.0 sodium acetate buffer. Homogenate was filtered from a fine muslin. The cell debris was removed by centrifugation at 10 000 rpm, 4 °C for 10 min and then treated to 80% ammonium sulfate saturation. The precipitate was collected by centrifugation at 10 000 rpm for 10 min, suspended in 100 mM acetate buffer (pH 5.0) and then dialyzed against 20 mM acetate buffer (pH 5.0). The dialyzed enzyme was analysed for phytase activity.

2.6. Immobilization of phytase on Sepabead EC-EP

Sepabead EC-EP (10 g) was previously washed with distilled water, suspended in 60 ml of 0.1 M acetate buffer (pH 5.5) and then 10 ml of enzyme solution was added. The reaction mixture was first incubated for 18 h at room temperature with roller shaking and then

incubated at room temperature for another 24 h without shaking. After this reaction period, the immobilized enzyme conjugate was separated by filtration. In order to remove unbound proteins it was washed with 40 ml of 20 mM pH 5.5 acetate buffer. To completely block the residual epoxy groups, the enzyme derivative was incubated in 3 M glycine solution for 24 h at 4 °C and then stored at 4 °C in fresh buffer until use.

2.7. Characterization of phytase

2.7.1. Influence of temperature on the activity and stability

The temperature profile of the free and immobilized phytases were conducted from 4 to 80 °C using the standard phytase assay at the given temperature. To check the thermal stability, the enzymes which have the same amount of protein in incubation mixtures were incubated at different temperatures for 30 min with continuous shaking and after desired incubation periods enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual phytase activities.

2.7.2. Effect of pH on phytase activity and stability

The pH activity profile of all the two forms of phytase was studied by incubating samples with sodium phytate in buffers of different pH ranging from 2.5 to 9.0 at 37 °C. The buffers (0.1 M) used were Glycine/HCl (pH 2.5–3.5), Na-acetate/acetic acid (pH 4.0–6.5) and Tris/HCl (pH 7.0–9.0). For the pH stability, the free and immobilized enzymes were incubated in above buffers for 3 h at 4 °C. The remaining activity was estimated using the standard activity assay procedure.

2.7.3. Effect of substrate concentration

In order to determine maximum velocity of reaction (V_{max}) and Michaelis–Menten constant (K_m) for free and immobilized phytase, activity assay was applied for different concentrations (0.5–10 mM) of sodium phytate at 37 °C. The apparent K_m and V_{max} values were calculated from Lineweaver–Burk plot which is a plot of $1/V$ against 1/[S] for systems obeying the Michaelis–Menten equation. The graph being linear can be extrapolated at anywhere approximating to a saturating substrate concentration, even if no experiment has been performed and from the extrapolated graph, the values of K_m and V_{max} can be determined.

2.7.4. Reusability

The stability of immobilized phytase on repeated use was examined by measuring the activity for the hydrolysis of phytate at 37 °C. After each activity measurement, the immobilized enzyme was separated and washed three times with acetate buffer (0.1 M, pH 5.0) and then fresh reaction medium was introduced onto the immobilized enzyme. By this way, the next activity measurements were carried out.

2.7.5. Storage stability

Free and immobilized enzymes were stored at 4 °C in 100 mM of acetate buffer (pH 5.0). The storage stability of the free and immobilized enzyme was investigated by measuring their activities after being stored at 4 °C for a 8 month period and the remaining activity measurements were performed once a week.

2.7.6. Effect of effector concentration

The influence of various effectors ($FeCl_3{\cdot}6H_2O$, citric acid, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $CuSO_4{\cdot}5H_2O$, Tartarat, $Na_2MoO_4{\cdot}2H_2O$, NaF, $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$) in concentrations of 0.1–2.5 mM on enzyme activity was investigated by preincubating the free and immobilized phytase with different compounds for 10 min at room temperature. Residual activity was calculated against control.

2.8. Biodegradation of soymilk phytate

2.8.1. Preparation of soymilk

Dried soybeans obtained from local market were ground to flour and defatted with n-hexane in a ratio of 1:1 (w/v). The fat free soybean flour was suspended in 10 volume of distilled water and heated to boiling. Undissolved residue was separated from soymilk by centrifugation for 5 min at 5000 rpm. The supernatant containing soymilk was stored at 4 °C for a short period till further use and diluted three times before usage [38].

2.8.2. Phytate degradation in batch-stirred-tank reactor

Hydrolysis of soymilk phytate was carried out in batch-stirred-tank reactor at 60 °C by using free and Sepabead EC-EP immobilized phytase. Reservoir components were magnetically stirred. The initial level of phytate was determined prior to incubation and then the aliquots were withdrawn at different time intervals. By measuring the phytic acid content of samples, hydrolysis % was calculated. All treatments were again carried out in triplicate.

3. Results and discussion

In the present work, phytase was isolated from soybean sprout, immobilized on Sepabead EC-EP and then characterized. After the extraction of phytase from soybean sprout, an enzyme extract (0.18 U/ml, 1.06 mg/ml) was obtained and then used for the immobilization studies (Table 1). The basic characteristics of an immobilized enzyme include the amount of immobilized protein, its activity and specific activity, thermal and pH profiles and also kinetic properties. The coupling of enzymes to epoxy-activated carriers is commonly carried out at high ionic strength, because it has been postulated that, in a first step, a salt-induced association between the macromolecule and the support surface takes place. This interaction increases the effective concentration of nucleophilic groups on the protein close to epoxide reactive sites. However, the salt concentration needed to immobilize an enzyme is highly protein-dependent. The epoxy groups may react with different nucleophiles of the protein as a function of pH. At neutral or slightly alkaline pH, with the thiol groups; at pH >9, with the amino groups; at pH >11, with phenolic groups of tyrosines; at slightly acidic pH, with carboxyl groups.

For above reasons, the effect of pH and ionic strength on the immobilization of phytase was studied. In order to bind the enzyme to the support, the immobilization was performed at different pH values. The buffer concentration was varied over the range 0.05–1.0 M. Regarding biocatalyst activity, a low buffer concentration resulted in higher activity. The highest activity of the biocatalyst was achieved with Sepabead EC-EP, using 0.1 M sodium acetate buffer (pH 5.5) (data not shown). The immobilization time was chosen and applied according to suppliers' protocol. We observed that a further increase in time did not result in a significant increase of biocatalyst activity.

An attempt was also made to achieve binding of high levels of enzyme with a high retention of its initial activity. Thus, the effect of varying phytase concentration in the immobilization medium in the range of 0.18–1.44 U/g wet support on the total protein loading on the Sepabead EC-EP and activity coupling yields is investigated (Table 1). The expressed activity and activity yield of the enzyme were defined respectively as:

$$\text{expressed activity}\,(\%) = \frac{\text{activity of immobilized enzyme}}{\text{activity of free enzyme}} \times 100$$

$$\text{activity yield}\,(\%) = \frac{\text{specific activity of immobilized enzyme}}{\text{specific activity of free enzyme}} \times 100$$

Table 1
Immobilization of soybean sprout phytase on Sepabead EC-EP[a].

Free enzyme			Bound protein (mg)	Protein binding yield (%)	Immobilized enzyme		
Activity (unit)	Protein (mg)	Specific activity (U/mg)			Activity (unit)	Specific activity (U/mg)	Activity yield (%)
0.18	1.06	0.17	0.94	88.7	0.10	0.11	64.7
0.36	2.12	0.17	1.73	81.6	0.12	0.07	41.2
0.72	4.24	0.17	3.29	77.6	0.14	0.04	23.5
1.44	8.48	0.17	6.12	72.2	0.15	0.02	11.8

[a] The values were given per g of Sepabead EC-EP.

As is seen from Table 1, the activity yield of phytase immobilization varied significantly with the amount of loaded protein. The immobilized enzyme exhibited an activity of 0.1 U/g of support (the expressed activity was 55.6%) with an activity yield of 64.7. It is generally acknowledged that, catalytic efficiency of immobilization processes decreases when enzyme loading exceeds a certain value and an optimum activity should be selected [39]. Considering all analyzed variables (pH and ionic strength of the immobilization medium, enzyme amount per g of support, immobilization time) the optimal protocol to prepare a stable immobilized biocatalyst of phytase seems to be; incubation at pH 5.5 (0.1 M acetate buffer) for 18 h with shaking and then for 24 h without shaking at 25 °C, 1.06 mg protein/g wet support and also 0.18 U/g wet support. Detachment of immobilized enzyme from polymer was not observed.

3.1. Properties of free and immobilized phytase

We attempt to examine the properties of the immobilized soybean sprout phytase and compare them with those of the free counterpart. The temperature dependence of the phytase activity was investigated in the temperature range 4–80 °C and the results are shown in Fig. 1. The maximum activity was observed at 60 °C for both forms of the enzyme. These results compare well with the previous reports. In general, plant phytases show high activity in the temperature range of 50–70 °C and usually has an optimum temperature of 60 °C [3,40]. The calculated values of activation energy (E_a) obtained from Arrhenius plots (log activity versus $1/T$) were equal to 12.8 kJ mol^{-1} and 7.1 kJ mol^{-1} for free and immobilized enzyme, respectively. This decrease in the E_a of the immobilized enzyme could be attributed to steric hindrances or diffusional limitations. Similar results were reported previously [41]. Thermal stability experiments were carried out with free and immobilized enzymes, which were incubated in the absence of substrate at various temperatures. The effect of the temperature on the stability of the enzyme is shown in Fig. 2. As shown from the Fig. 2, the immobilized enzyme was as thermostable as free enzyme. Free enzyme shows 96% activity at 60 °C while it decreases to 65% at 80 °C. Meanwhile immobilized enzyme shows 88% activity at 60 °C and the activity decreases to 63% at 80 °C. The wheat bran phytase activity was also affected by the temperature. The enzyme remains very stable at temperatures below 70 °C but when the temperature was increased to 80 °C, the activity decreased rapidly [2]. Liu et al. [14] studied the effect of immobilization on thermal stability of *A. ficuum* phytase. The optimum temperature was increased to 58 °C, which was 8 °C higher than that of the free enzyme [14]. Greiner and Konietzny [19] described the covalent immobilization of *E. coli* phytase NHS-activated Sepharose (R) high performance. The stability against heat treatment was enhanced as a consequence of immobilization [19].

The performance of free and immobilized enzyme was studied by changing the pH of the medium. The comparison of pH profile on the activity between free and immobilized enzymes is shown in Fig. 3. As can be seen from the figure, the pH optima is

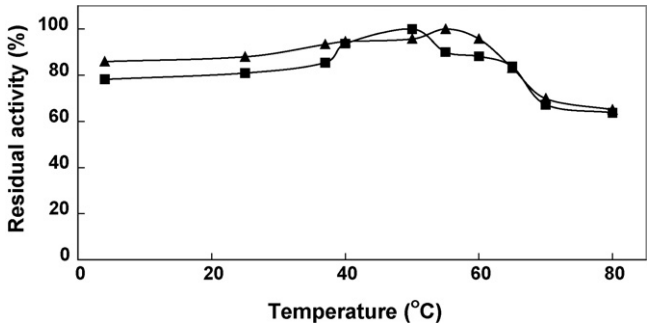

Fig. 2. Thermal stability of free (▲) and immobilized (■) phytase. (After incubation at indicated temperatures activities were assayed at 37 °C by using 2 mM sodium phytate prepared in 0.1 M acetate buffer at pH 5.0.)

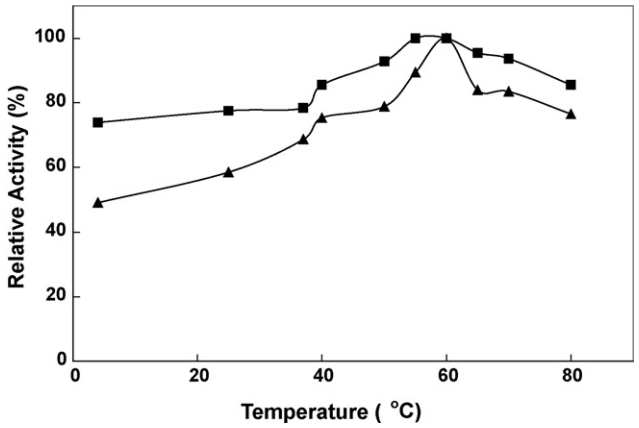

Fig. 1. The effect of temperature on the activity of free (▲) and immobilized (■) phytase activity. (Activities were assayed at indicated temperatures by using 2 mM sodium phytate prepared in 0.1 M acetate buffer at pH 5.0.)

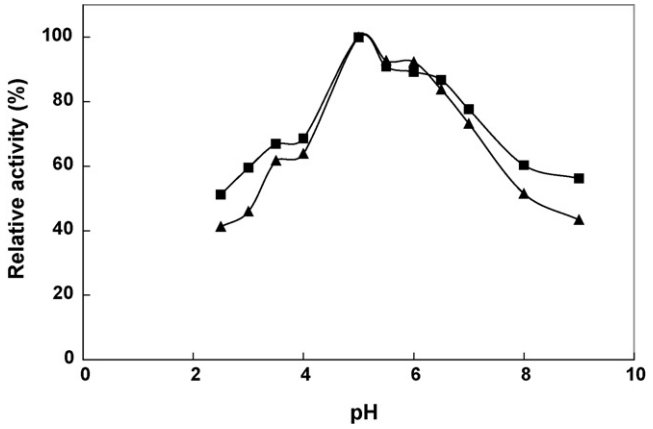

Fig. 3. The effect of pH on the activity of free (▲) and immobilized (■) phytase. (Activities were assayed at 37 °C by using 2 mM sodium phytate prepared in appropriate buffer solution.)

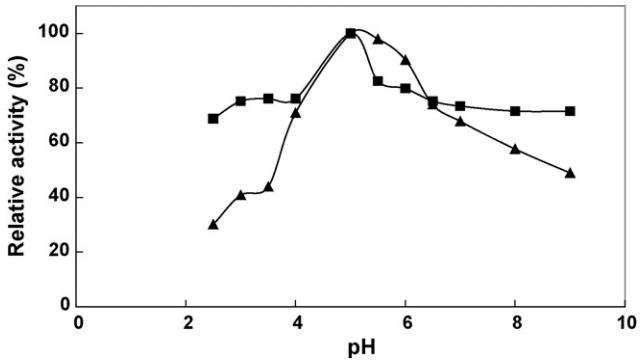

Fig. 4. pH stability of free (▲) and immobilized (■) phytase phytase. (After incubation at indicated pH's activities were assayed at 37 °C by using 2 mM sodium phytate prepared in 0.1 M acetate buffer at pH 5.0.)

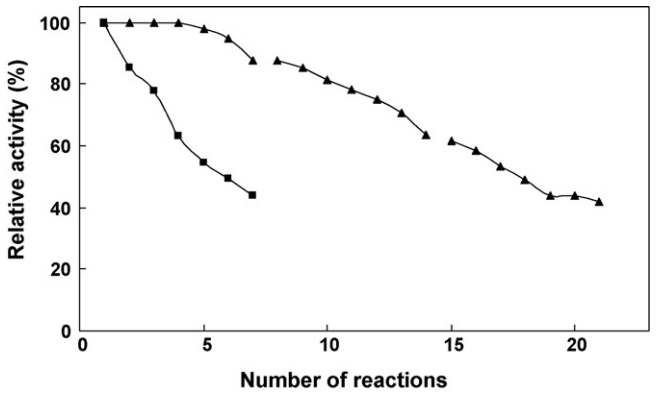

Fig. 5. Repeated use of immobilized phytase at 37 °C (▲) and 60 °C (■). (Activities were assayed at 37 °C by using 2 mM sodium phytate prepared in 0.1 M acetate buffer at pH 5.0.)

same (pH 5.0) for both enzymes. This is an expected result, because Sepabead EC-EP is not a charged carrier and also there is no any acidic or basic product as a result of enzymatic reaction. The pH profiles of the enzymes were also very broader in a pH range of 3.0–8.0. The broadened pH range can be an advantage in the application of immobilized enzyme at lower pH, which can eliminate the possibility of microbial contamination during long-term operation. Similar results have been reported in different immobilization studies [14,17]. The optimum pH for the immobilized *A. ficuum* phytase was not much different from that of the intact enzyme [17]. The pH optimum of the plant phytases changes in the range of pH 4.0–8.0 and usually between 4.0 and 5.6 [22]. A phytase from *Vicia faba* beans has an pH optimum at pH 5.0. A rapid decline of the enzyme activity was observed on both sides of the pH optimum. The enzyme is virtually inactive below pH 3.0 and above pH 8.0 [8]. The effect of pH on the stability of the enzyme was determined in various buffers. As shown in Fig. 4, the immobilized enzyme is more stable than free enzyme. The free enzyme from soybean sprout is stable over pH 4.0–7.0 whereas activity decrease observed on both sides of these pH values.

The enzyme kinetics obeyed simple Michaelis–Menten kinetics. Lineweaver–Burk plots for free and immobilized enzymes were drawn and the kinetic parameters (K_m and V_{max}) were calculated from graphs as 5 mM and 0.63 U/mg for free and 12.5 mM and 0.71 U/mg for immobilized enzyme, respectively. The values for free enzyme obtained in this work are in agreement with those presented in the literatures [3]. The K_m value of immobilized phytase was approximately 2.5-fold higher than that of free enzyme. Similar results were obtained from the immobilization of *E. coli* phytase. The immobilized *E. coli* phytase showed an increased K_m value compared to the free phytase [19]. The increase in K_m value was either due to the conformational change of the enzyme resulting in a lower possibility of forming a substrate–enzyme complex, or to the immobilized enzyme caused by the increased diffusional limitations. The variations in both K_m and V_{max} values of the enzyme upon immobilization are also attributed to several factors such as; the noncovalent interactions of the immobilized enzyme molecule with the carrier surface might have induced an inactive conformation to the enzyme molecules. It should be noted that the immobilization process does not also control the proper orientation of the immobilized enzyme on the support.

Reusability and storage stability are of considerable importance for various applications of biocatalysts in a commercial point of view. An increased stability can make the immobilized enzyme more advantageous than its free counterpart. Enzymes can easily lose their catalytic activity and denatured, thus, careful storage and handling are essential. The stability of immobilized phytase on repeated use was examined by measuring the activity for the hydrolysis of Na-phytate at 37 and 60 °C (Fig. 5). As is seen from the figure, the immobilized enzyme retained 42% of its initial activity after 21 cycles of reuse at 37 °C and 44% of its initial activity after 7 cycles of reuse at 60 °C. This study shows that, the phytase immobilized on Sepabead EC-EP can be easily recovered and used repeatedly although significant loss of its activity at 60 °C is an avoidable under the conditions of our experiment. As no detachment of immobilized enzymes from support was observed. Mckelvie et al. [15] utilized immobilized phytase for the determination of phytase hydrolysable phosphorus present in natural waters. The immobilized enzyme exhibited good operational and storage stability over a period of time [15]. Such reusability is advantageous for the continuous use of the enzyme in industrial applications and also could significantly reduce the operation costs in practical applications.

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually decreased overtime. Hence, both free and immobilized enzyme were stored at 4 °C under the same conditions mentioned before. Under the same storage conditions, the free enzyme lost about 33% of its activity over a period of 8 months, whereas the immobilized enzyme lost about only 17% of its original activity over the same period of time (Fig. 6). The experiment revealed that storage stability of the immobilized enzyme was improved compared to free form, and 4 °C was very suitable for storage. On the basis of this observation, modified support should provide a stabilization effect, minimizing possible distortion effects imposed from aqueous medium on the active site of the immobilized enzyme. Various reports can confirm that the storage stability

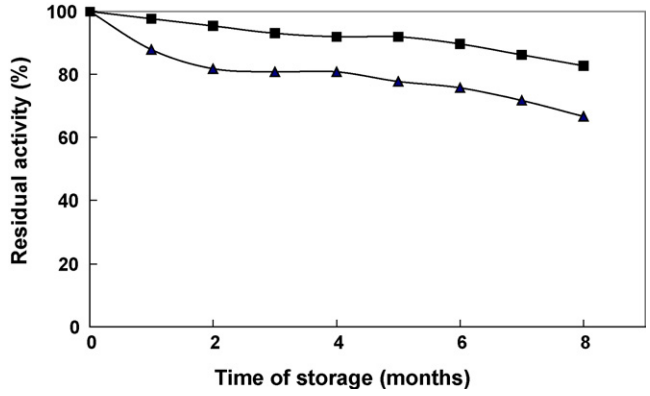

Fig. 6. Storage stability of free (▲) and immobilized (■) phytase. (Activities were assayed at 37 °C by using 2 mM sodium phytate prepared in 0.1 M acetate buffer at pH 5.0.)

Table 2
The effects of some minerals and ions on free (a) and immobilized (b) enzyme activities.

Effector	0.1 mM	1.0 mM	2.5 mM
(a)			
Control	100	100	100
$FeCl_3 \cdot 6H_2O$	94.2	76.5	53.7
$CaCl_2$	92.2	100	101.2
$MgCl_2 \cdot 6H_2O$	102.9	92.2	91.3
$CuSO_4 \cdot 5H_2O$	61.2	52.5	42.6
Tartarat	99.2	98.2	97.5
Citric acid	121.9	116.1	109.9
$Na_2MoO_4 \cdot 2H_20$	52.1	44.2	32.2
NaF	93.4	85.5	78.1
$ZnSO_4 \cdot 7H_2O$	90.1	78.9	59.1
$MnCl_2 \cdot 4H_2O$	100.8	95.2	92.6
(b)			
Control	100	100	100
$FeCl_3 \cdot 6H_2O$	99	98.3	97.9
$CaCl_2$	103.2	104.1	105.3
$MgCl_2 \cdot 6H_2O$	104.2	93.7	90.5
$CuSO_4 \cdot 5H_2O$	85.3	70.5	63.2
Tartarat	102.1	97.9	97.1
Citric acid	131.6	127.4	111.6
$Na_2MoO_4 \cdot 2H_20$	75.8	67.4	60
NaF	95.8	89.5	85.3
$ZnSO_4 \cdot 7H_2O$	95.8	93.7	89.5
$MnCl_2 \cdot 4H_2O$	101.1	98.7	97.9

of immobilized enzymes varies depending on the immobilization method applied and storage conditions [15,41].

The influence of various effectors on the activity of free and immobilized enzymes were also studied by using sodium phytate as a substrate. The results were shown in Table 2. Citric acid shows an activator effect especially at 0.1 mM concentration for both enzyme. The enzyme activity was not greatly inhibited by the other compounds. The addition of chelating agents together with phytase enzyme increased the solubility of mineral elements, except for potassium. The combined effect of phytase and citric acid increased the solubilities of Ca, Mg, Zn and Mn in oat bran sample significantly. With the addition of 3% citric acid the total solubility of Mg and Mn increased from 21 to 70% and from 6 to 54%, respectively. The increase in the solubility of Ca was also significant with a citric acid concentration of 1.0% [42].

3.2. Biodegradation of soymilk phytate by free and immobilized phytases

Hydrolysis of soymilk phytate by free and immobilized phytase was carried out in batch-stirred-tank reactor at 60 °C as mentioned in Section 2. The initial level of phytate was determined prior to incubation and then the aliquots were withdrawn at different time intervals. By measuring the phytic acid content of samples, % hydrolysis was calculated. A time course of hydrolysis of phytate present in soymilk is shown in Fig. 7. As is seen from the figure, both free and immobilized enzyme hydrolysed the soymilk phytate very quickly within 5 h (free enzyme hydrolysed 90% and immobilized enzyme hydrolysed 76% of phytate). This is the highest reduction compared to other reports. But interestingly, the prolonged incubation times (after 5 h) does not significantly increase the percent hydrolysis and hydrolysis after 5 h was very slow probably due to substrate depletion. Commercial immobilized wheat phytase (Sigma P 1259) hydrolysed 78% of the phytate present in the soymilk in 8 h, whereas only 42% hydrolysis was observed with native enzyme under similar conditions [43].

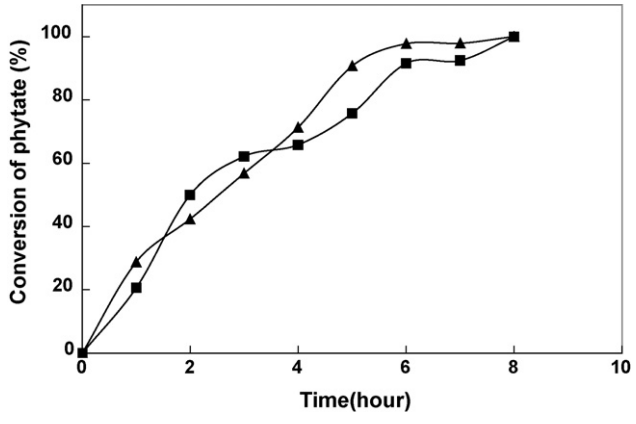

Fig. 7. Hydrolysis of phytate in batch-stirred-tank reactor with free (▲) and immobilized (■) phytase.

4. Conclusion

Potentials of phytase in human nutrition and health have increasingly been gained attention. Biotechnology has been and will continue to be an exceptionally effective tool for developing and improving phytase enzymes and their delivery systems. This study shows that, soybean sprout phytase immobilized on epoxy-activated Sepabead EC-EP has good stability and has a potential for practical industrial applications. The system has various advantages; these supports are very stable, allowing a long storage and a prolonged transport from manufacturers to consumers, they permit large enzyme-support reaction periods. In addition they are very reactive with different moieties of proteins (amine, thiol, hydroxyl groups) yielding very stable protein-support bonds. Moreover the remaining epoxy groups may be easily blocked after enzyme immobilization with different compounds yielding an inert surface. The immobilization procedure on Sepabead EC-EP is very cheap and easy to carry out. The features of the immobilized enzyme is very attractive that the potential for practical application is considerable. Moreover, the use of the above biocatalyst for the continuous hydrolysis of phytate in soymilk is only one example of their biotechnological applications.

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