Available online at www.sciencedirect.com
SCIENCE DIRECT
Food Chemistry 100 (2007) 964–971
Food Chemistry
www.elsevier.com/locate/foodchem

Immobilization of pepsin on chitosan beads

Gamze Durgun Altun, Senay Akkus Cetinus *

Department of Chemistry, Faculty of Science and Literature, Cumhuriyet University, Sivas 58140, Turkey

Received 2 September 2005; received in revised form 1 November 2005; accepted 10 November 2005

Abstract

In this study, chitosan beads were prepared by using a cross-linking agent and the resulting beads were employed in immobilization process. Studies on free and immobilized pepsin systems for determination of optimum temperature, optimum pH, thermal stability, pH stability, operational stability, storage stability and kinetic parameters were carried out. The optimum temperature interval for free pepsin and immobilized pepsin were 30–40 and 40–50 °C, respectively. Free and immobilized pepsin showed higher activity at pH 2.0–4.0. Apparent $K_m = 12.0$ g L^{-1} haemoglobin (1.56 mM tyrosine) and $V_{max} = 5220$ μmol (mg protein min)$^{-1}$ values were obtained for free pepsin, while apparent $K_m = 20.0$ g L^{-1} haemoglobin (2.16 mM tyrosine) and $V_{max} = 2780$ μmol (mg protein min)$^{-1}$ values were obtained for immobilized pepsin. Thermal stability and storage stability of immobilized pepsin were higher than that of free pepsin. Milk clotting activity was used for evaluation of the applicability of pepsin immobilization to industrial process. Optimum milk clotting temperature was found as 40 °C for free pepsin and 50 °C for immobilized pepsin.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Chitosan; Pepsin; Enzyme immobilization

1. Introduction

Enzymes have a wide variety of biotechnological, biomedical, and pharmaceutical applications. They are used as biosensors in bioengineering, clinically as therapeutic agents, in modern diagnostic tool, and as catalyst for chemical or biochemical reactions (Betigeri & Neau, 2002). In chemical and biochemical reactions, purified enzymes can be rather costly and have to be discarded after each use which is not economical. A large research of work has been devoted to the polymeric carriers, especially to immobilization of the proteins onto carriers (Kennedy & Melo, 1990). Since the recovery yield and reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization which may offer advantages over free enzymes; for example, possibility of continuous process, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs (Cao, Langen, & Sheldon, 2003). Enzymes are often immobilized onto solid supports to increase their thermal and operational stability, and recovery. Methods available for enzyme immobilization can be divided into two general classes: chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weak interactions between support and enzyme exist. Protocols for covalent enzyme immobilization often begin with a surface modification or activation step. The surface can be bound to aldehyde groups using glutaraldehyde (Krajewska, Leszko, & Zaborska, 1990). A great number of synthetic or natural carriers with different shapes/sizes, porous/non-porous structures, different hydrophilicities and binding functionalities, have been specially designed for various bioimmobilization and bioseparation procedures (Gemeiner, 1992). Chitosan, a poly-*N*-acetylglucosamine, is a transformed oligosaccharide obtained by deacetylation of chitin, and it is the second most abundant biopolymer after cellulose. Since it is highly biocompatible and easily biodegradable, chitosan has been used as a raw material for medical applications such as surgical sutures,

* Corresponding author. Tel.: +90 346 2191010/1569; fax: +90 346 2191186.
E-mail address: scetinus@cumhuriyet.edu.tr (S.A. Cetinus).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.11.005

artificial skin and immunosuppressant (Chandy & Scharma, 1990; Chiou & Wu, 2004; Khor, 2002; Krajewska et al., 1990). Chitosan, chitin and their derivatives have been considered as versatile biopolymers in food applications. In that sense, these biopolymers offer a wide range of unique applications including bioconversion for the production of value- added food products (Shahidi & Synowiecki, 1991), preservation of food from microbial deterioration (Papineau, Hoover, Knorr, & Farkas, 1991), formation biodegradable films (Kittur, Kumar, & Tharanathan, 1998), recovery of waste material from food processing discards (Pinotti, Bevilacqua, & Zaritzky, 1997), purification of water (Deans & Dixon, 1992), and clarification and deacidification of fruit juices (Soto-Perlata, Muller, & Knorr, 1989; Spagna et al., 1996). The solubility of chitosan in organic acids allows for gel, membrane and bead fabrication (Cetinus & Oztop, 2003; Krajewska et al., 1990). Gel, membrane and beads forms of chitosan have been used for enzyme (protein, cell) immobilization as support material (Cetinus & Oztop, 2003; Chiou & Wu, 2004; Freeman & Dror, 1994; Krajewska et al., 1990).

Pepsin is an endopeptidase with a broad specify. Pepsin is produced in the mucosal lining of the stomach and degrades proteins. Pepsin is one of three principal protein-degrading, or proteolytic enzymes in the digestive system, the other two being chymotrypsin and trypsin. The three enzymes were among the first to be isolated in crystalline form. During the process of digestion, these enzymes, each of which is particularly effective in severing links between particular types of amino acids, collaborate to break down dietary proteins to their components, i.e., peptides and amino acids, which can be readily absorbed by the intestinal lining. In the laboratory studies pepsin is most efficient in cleaving bonds involving aromatic amino acids, phenylalanine, tryptophan, and tyrosine. Pepsin is synthesized in an inactive form by the stomach lining; hydrochloric acid, also produced by the gastric mucosa, is necessary to convert the inactive enzyme and to maintain the optimum acidity (pH 1–3) for pepsin function. Pepsin and other proteolytic enzymes are used in the laboratory analysis of various proteins; pepsin is also used in the preparation of cheese and other protein-containing foods (Cooper, Khan, Taylor, Tickle, & Blundeli, 1990; Hasem, 2000; Kamatari, Dobson, & Konno, 2003).

In recent years, pepsin has been immobilized on various supports. For example, pepsin vas immobilized on modified alumina complex, designing a continuous stirred tank reactor for producing bioactive hydrolysate (Ticu et al., 2005). In another work, it was immobilized on chemically modified poly methyl methacrylate (PMMA) microspheres to investigate K_m and V_{max} values for free and immobilized enzymes (Li, Hu, & Liu, 2004). It was also reported that pepsin was immobilized on activated Sepharose 4B for affinity chromatography and pepsin was completely adsorbed to affinity columns (Frydlova, Kucerova, & Ticha, 2004). Kurimoto, Harada, Akiyama, Sakai, and Kato (2001), reported that immobilization on agarose beads for in vitro refolding, and renaturation of pepsin was extremely slow. The present study, aimed to investigate immobilization of pepsin used specially for food industry on chitosan beads and to find kinetic parameters, thermal stability, storage stability, pH stability for immobilized and free pepsin.

2. Materials and methods

Porcine pepsin (E.C. 3.4.23.1), glutaraldehyde, glyoxal (trimer dihydrate), and bovine haemoglobin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chitosan (High molecular weight $M_W \sim 600{,}000$) was obtained from Fluka Chemie AG (Buchs, Germany). Tetrasodium pyrophosphate, TCA (trichloroacetic acid), and all other chemicals were obtained from Merck AG (Darmstadt, Germany).

2.1. Preparation of chitosan solution

Chitosan flakes (2.5 g) were added into 99 ml of distilled water and suspended by magnetic stirring for 10 min. One millilitre of glacial acetic acid was then added and mixing continued for 3 h at room temperature, the solution was then filtered through a gauze pad and was stored at room temperature and used within one week.

2.2. Preparation of cross-linking solution

To prepare the cross-linking solution, glyoxal hydrate (10 g) was added to 250 ml of distilled water, heated at 80 °C for 10 min and then cooled to room temperature. The solution was mixed with an equal volume of 3% (w/v) tetrasodium pyrophosphate solution (pH 8.0). The solution was used on the same day of preparation.

2.3. Preparation of chitosan beads

Chitosan solution was added dropwise into the cross-linking solution (volume ratio 1:5) at room temperature by pumping the chitosan solution (1.5 ml/min) through the internal tube of an injector. The beads were allowed to cure for 30 min and washed three times by incubation in the same volume of 0.05 M phosphate buffer, pH 7.0.

2.4. Reinforcement of chitosan beads by glutaraldehyde treatment

Freshly prepared beads were incubated in cold 0.05% (w/v) glutaraldehyde solution in 50 mM cold phosphate buffer (pH 7.0) for one hour. The brownish reinforced beads were washed several times with 50 mM cold phosphate buffer (pH 7.0).

2.5. Immobilization of pepsin on chitosan beads

Chitosan beads were mixed with 0.5 mg ml^{-1} pepsin solution (prepared in 0.01 M HCl) at 20 °C and left for

5 h with slight stirring and finally washed until free pepsin disappeared. Then the beads dried in a vacuum incubator at room temperature and stored at 4 °C.

2.6. Determination of immobilized protein amount

The amount of immobilized enzyme was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the amount of protein used for immobilization. The protein content in solutions was determined by the method of Bradford (1976).

2.7. IR Spectra of the chitosan beads

The FTIR spectra were recorded by using a UNICAM Mattson 1000 FTIR spectrometer, and the sample and KBr were pressed to form a tablet.

2.8. Pepsin activity assays

Proteolytic activity of pepsin was estimated according to the method of Bergkvist (1963) as well as Hasem (1999, 2000), by determining the amount of hydrolysis of denatured haemoglobin solution by enzyme after incubation for 15 min at 40 °C. One unit (U) enzyme activity was taken as the amount of enzyme which liberated 1 μmole of tyrosine $(\text{min ml})^{-1}$. The reaction was started by the addition of 0.4 ml enzyme solution (or 10 mg dry chitosan beads containing immobilized enzyme after incubation in 0.01 M 2 ml of HCl at 40 °C for swelling equilibrium) to 2.0 ml of acid denatured haemoglobin solution (10 g L^{-1}) prepared in 0.01 M HCl and the mixture was gently stirred in a test tube at 40 °C. At time intervals after mixing the reaction was stopped by the addition of 4.0 ml of 5.0% (w/v) TCA solution. Samples were subjected to centrifugation and filtration. The pepsin activities were estimated from the increase in UV absorbance of the supernatant at 280 nm.

Milk clotting activity was measured by the method of Berridge (1952) as well as Hasem (1999, 2000). Free and immobilized pepsin samples were incubated with reconstituted skimmed milk at 40 °C and clotting time recorded. One unit of enzyme activity (U) was taken to be that clotted 10 ml of milk in 10 min.

The effect of substrate concentration on the activity was tested by using increasing concentrations of haemoglobin, V_{max} and K_m values of immobilized and free pepsin were determined.

2.9. The pH profiles and pH stability of free and immobilized pepsin

Effect of pH value on the activities of free and immobilized pepsin were carried out at pH range 2.2–7.0 (by using appropriate buffer solutions). Activity of pH profiles was determined at indicated pH values using a 10 g L^{-1} haemoglobin solution at 40 °C.

The pH stability of free and immobilized pepsin was ascertained by measuring the residual activity of enzyme exposed at various pH (2.2–7.0) in buffer solution for 5 h. Activities of samples were performed at optimum conditions. (10 g L^{-1} haemoglobin in 0.01 M HCl, 40 °C)

2.10. The temperature profiles and thermal stability of free and immobilized pepsin

Activity of temperature profiles was determined at indicated temperatures (20–70 °C) using a 10 g L^{-1} haemoglobin solution in 0.01 M HCl.

The thermal stability of free and immobilized pepsin was ascertained by measuring the activity of the residual enzyme exposed at various temperatures (20–70 °C) in 0.01 M HCl solution for 5 h. Activities of samples were performed at optimum conditions (10 g L^{-1} haemoglobin in 0.01 M HCl, 40 °C).

2.11. The operational stability of free and immobilized pepsin

The retention of the immobilized enzyme activity was tested as described in the activity assays of pepsin. After each reaction run, the enzyme immobilized chitosan beads were removed and washed with 0.05 M HCl solution to remove any residual substrate within the chitosan beads. They were then reintroduced into fresh reaction medium and enzyme activities were detected at optimum conditions.

2.12. The storage stability of free and immobilized pepsin

The activity of free and immobilized pepsin after storage in 0.01 M HCl at 5 °C was measured in a batch operation mode with the experimental conditions given above.

The results of pH, temperature, reusability and storage stability of free and immobilized pepsin were presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

3. Results and discussion

Enzymes have been used in several industrial processes for a long time. Detergent, textiles, food (starch, baking, drinks), animal feed, pulp and paper, leather are among the main industries that use enzymes. Pepsin has been used especially in the food industry as a milk coagulant to make cheese (Cooper et al., 1990; Hasem, 2000; Kamatari et al., 2003). Many studies on immobilization of pepsin have been reported (Frydlova et al., 2004; Li et al., 2004; Ticu et al., 2005). In this study, we investigated immobilization of pepsin onto chitosan beads.

The potential use of enzyme and cell immobilized chitosan beads carried out in acidic medium raises a general problem. As chitosan is soluble in acidic solutions, the continuous prolonged exposure of chitosan beads, made via counter-ion precipitation, may result in gel softening and bead disintegration. We used a procedure for the prepara-

tion of chemically cross-linked chitosan beads (Freeman & Dror, 1994). This procedure is based on the addition of the acidic chitosan solution into a mixture diphosphate and nontoxic dialdehyde glyoxal, resulting in chemically cross-linked chitosan beads. Glyoxal cross-linked chitosan beads were exposed to glutaraldehyde solution as a complementary stability curing treatment. As Schiff's base formed between chitosan and glyoxal is essentially reversible, continuous prolonged operation under acidic conditions may result in gradual leakage of glyoxal. Glutaraldehyde irreversible cross-linking via Schiff's base may lead to chitosan beads exhibiting high operational stability.

The FTIR spectra of the chitosan pretreated glutaraldehyde (Fig. 1A) show peaks at 910 and 1153 cm^{-1} and these peaks of the assigned to saccharide structure. There has been a significant peak at 1574 cm^{-1}, which can be attributed to the characteristic peak of C–N forming from the cross-linking reaction between chitosan and glutaraldehyde. In Fig. 1B a peak appeared at 1600 cm^{-1}, which can be attributed to the formation of C=N because of the imine reaction between amino groups from enzyme and aldehyde groups in glutaraldehyde. In our previous study (cell immobilization on chitosan film), we found a similar results (Oztop, Saraydın, & Cetinus, 2002). Glutaraldehyde activation of supports is a method for irreversible immobilization of proteins through a single amino-support link. The proposed method of immobilization of pepsin on chitosan beads using glutaraldehyde is summarized in Scheme 1.

3.1. Kinetic studies

Kinetics of the proteolytic activity of free and immobilized pepsin was investigated at various concentrations (5.0–30.0 g L^{-1}) of denatured haemoglobin as a substrate. These data were plotted according to the method of Lineweaver–Burk (Figs. 2a and 2b) and kinetic parameters, apparent K_m and V_{max} were calculated from the graphs. The apparent K_m value of free pepsin (12.0 g L^{-1} haemoglobin or 1.56 mM tyrosine) was found to be lower than that of (20.0 g L^{-1} haemoglobin or 2.16 mM tyrosine) immobilized pepsin. The pepsin immobilized chitosan beads exhibited apparent K_m value which was about 1.88-fold higher than that of free pepsin. This increase in apparent K_m value might be either due to structural changes in the enzyme induced by the applied immobilization procedure or due to the lower accessibility of the substrate to the active site of the immobilized enzyme (Kennedy & Melo, 1990). The V_{max} value of 2780 μmol (mg protein min)$^{-1}$ exhibited by pepsin immobilized chitosan beads was found to be lower than that of free pepsin [5220 μmol (mg protein min)$^{-1}$]. In another work, pepsin was immobilized on chemically modified poly methyl methacrylate (PMMA) microspheres and was found that Km value was larger and V_{max} was smaller in immobilized form than those in the free form (Li et al., 2004). A similar result involving change in Km and V_{max} values of enzyme after immobilization has been reported in the literature (Cetinus & Oztop, 2003; Kennedy & Melo, 1990; Krajewska et al., 1990). Our results are also in accordance with the literature report.

The activation energy (E_a) for free and immobilized pepsin was evaluated, as shown in Table 1. The activation energies for free and immobilized pepsin were found to be 8.01 and 6.95 kcal/mol, respectively. The activation energy of the immobilized pepsin was lower than that of the free pepsin, which indicates a lower sensitivity to temperature as well as significantly higher affinity for the active site of the chitosan support.

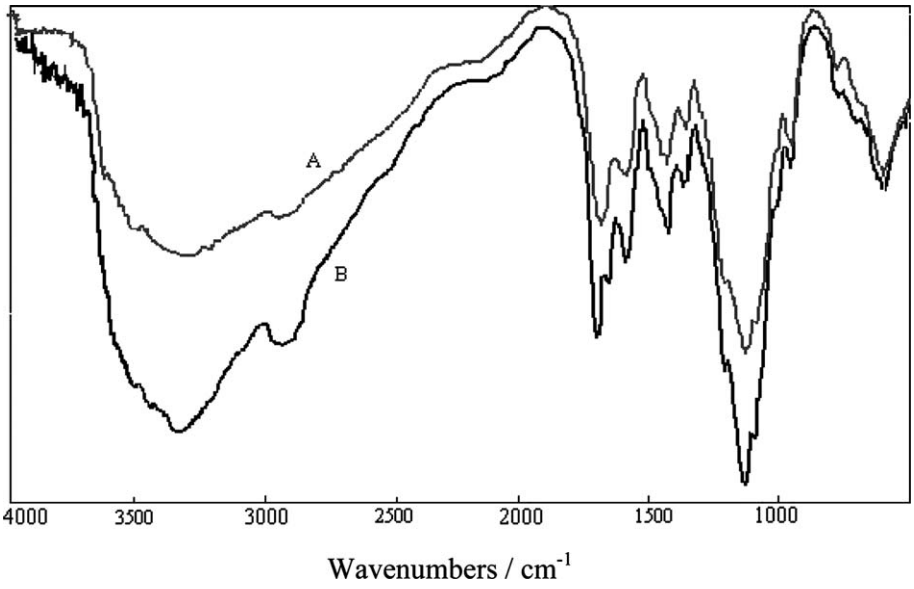
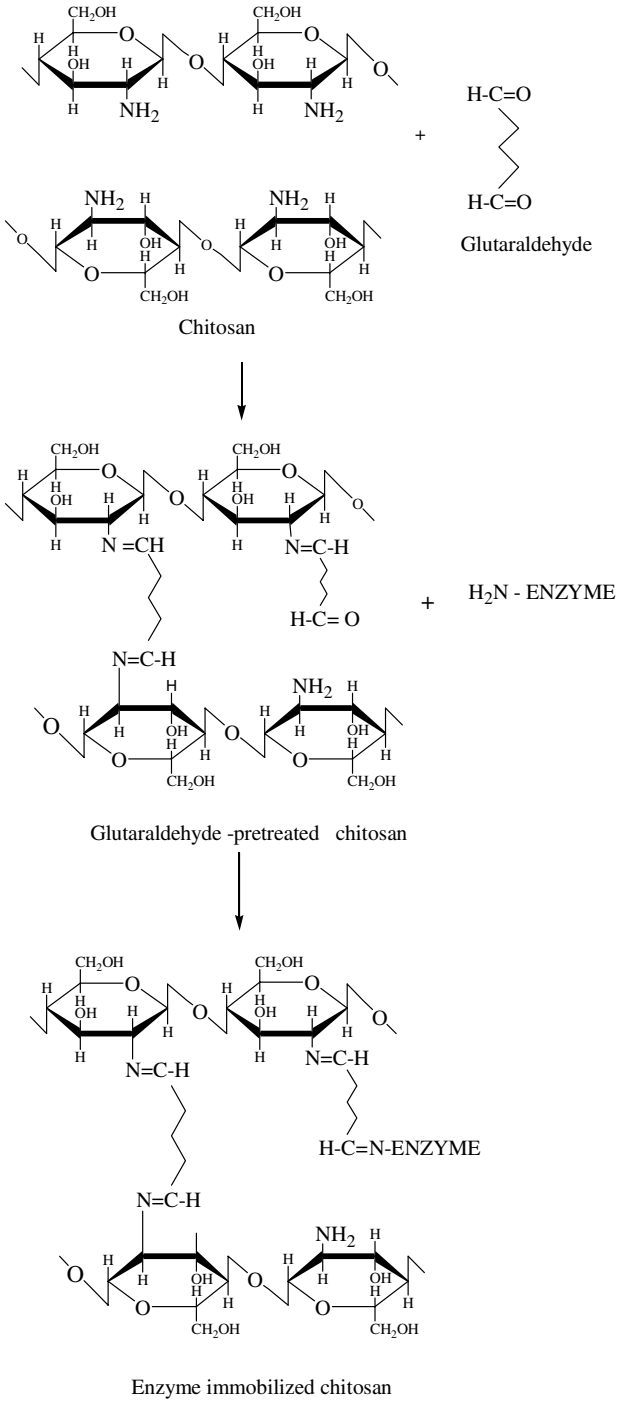


Fig. 1. The IR spectra of chitosan beads: pretreated glutaraldehyde (A) and pepsin immobilized (B).



Scheme 1. Possible immobilization mechanism of pepsin on chitosan beads.

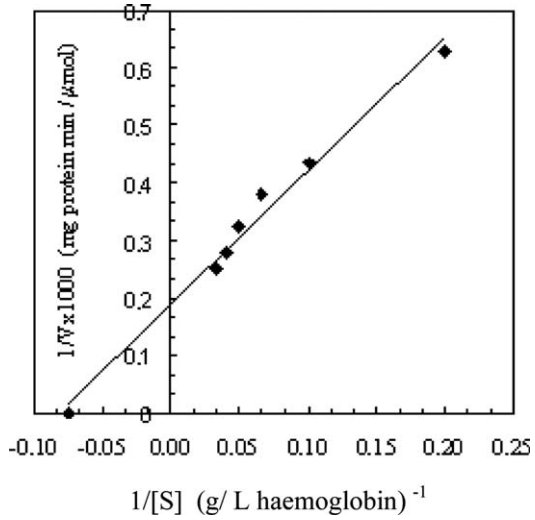


Fig. 2a. Lineweaver–Burk plot for free pepsin at 40 °C.

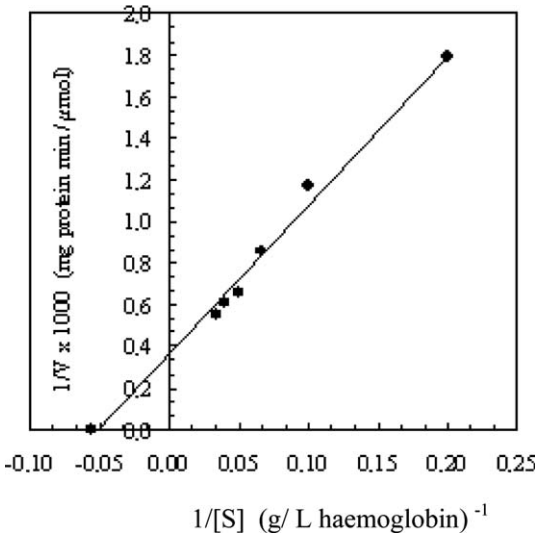


Fig. 2b. Lineweaver–Burk plot for immobilized pepsin at 40 °C.

Table 1
Activation energy (E_a) and kinetic parameters for free and immobilized pepsin

Types	E_a (kcal/mol)	V_{max} (U/g chitosan or mL)	K_m (g L^{-1} haemoglobin)
Free pepsin	8.01	5220	12.0
Immobilized pepsin	6.95	2780	20.0

3.2. The pH profiles and pH stability of free and immobilized pepsin

The pH activity profiles of free and immobilized pepsin were compared. The results are given in Fig. 3. The optimum pH values for free and immobilized pepsin were the same. The optimum pH range was obtained as 2.2–3.0 for free and immobilized pepsin.

The pH stabilities of immobilized and free pepsin were compared by immersing in buffer solutions pH range of 2.2–7.0 for 5 h at 40 °C and then determining the activity at its optimum pH. The results are shown in Fig. 4. The free and immobilized pepsin remained more stable in the pH range at 2.2–4.0.

The stability of various immobilized enzymes can frequently be improved when inorganic supports are used instead of organic polymers because of greater dimensional stabilities of inorganic supports. Shifts in pH optimum with immobilization have been found for many enzymes. Anionic supports tend to shift the pH optimum toward the alka-

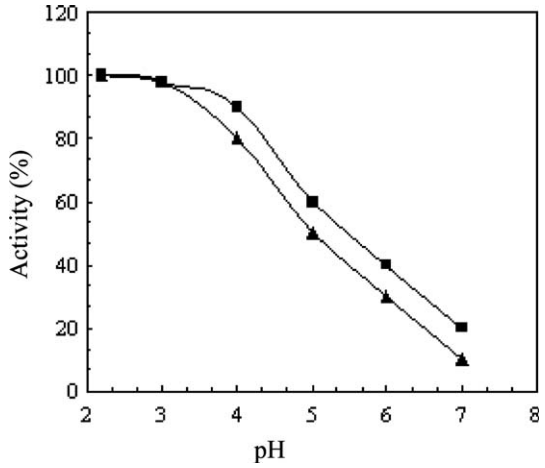


Fig. 3. Effect of pH value on the activities of free and immobilized pepsin at 40 °C: (▲) free enzyme and (■) immobilized enzyme.

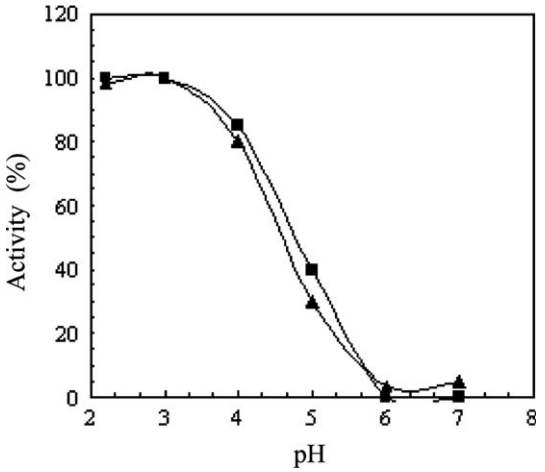


Fig. 4. pH stability of free and immobilized pepsin at 40 °C: (▲) free enzyme and (■) immobilized enzyme.

line side; cationic supports tend to shift the pH optimum toward the acidic side. Pepsin is a strongly acidic protein and shows high enzymatic activity at lower pH values (Cooper et al., 1990; Hasem, 2000; Kamatari et al., 2003). In this study, chemically cross-linked chitosan beads were used as a support material. Chitosan beads do not carry a charge at neutral pH. If the pH values decrease in solution, chitosan beads can be charged positively at lower pH because hydrogen ions can bind to free amino groups. However, limited number of available amino groups on cross-linked chitosan beads reduces number of bound hydrogen ions. Therefore, solution pH was not expected to shift to acidic side significantly. For that reason acidic microenvironment did not change the pH profile and pH stability of pepsin.

3.3. The temperature profiles and thermal stability of free and immobilized pepsin

The temperature dependence of the activities of the free and immobilized pepsin were studied in 0.05 M HCl solution at temperature range of 20–70 °C and temperature profiles of free and immobilized pepsin are shown in Fig. 5. The optimum temperature range for free and immobilized pepsin was found to be about 30–40 and 40–50 °C, respectively. The conformational flexibility of the enzyme was affected by immobilization. Immobilization of pepsin on chitosan beads caused an increase in enzyme rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature (Abdel-Naby, 1993; Jiang & Zhang, 1993).

Thermal stability was investigated by incubating free and immobilized pepsin on chitosan beads at temperatures ranging from 20 to 70 °C for 5 h and then determining the activity at optimum reaction temperature. The effect of temperature on stability of free and immobilized pepsin is illustrated in Fig. 6. Free pepsin has shown high stability at 40 °C, and immobilized pepsin has high stability at 40–50 °C. There was no activity loss for the immobilized pepsin at 50 °C. However, the free pepsin activity decreased at

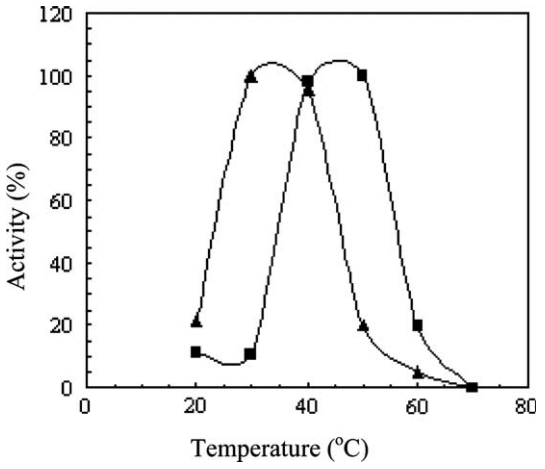


Fig. 5. Effect of temperature on the activities of free and immobilized pepsin: (▲) free enzyme and (■) immobilized enzyme.

the same temperature. At 50 °C, immobilized pepsin on chitosan beads retained an activity of about 100%, whereas the activity retained by the free pepsin was only 60%. The authors demonstrated that the thermal stability of enzymes may be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint (Martinek, Kilbanov, Goldmacher, & Berezin, 1977).

3.4. Reusability and storage stability

When comparing performance of immobilized biocatalysts, intended for preparative or industrial use, characterization of their operational stabilities is very important. The operational stability of immobilized pepsin in the current study was evaluated in repeated batch process. Fig. 7 shows the effect of repeated use on activity of immobilized pepsin. The pepsin immobilized chitosan beads retained a specific activity of 95% after three reuses. After that activity decreased gradually. This brings an advantage over using free pepsin.

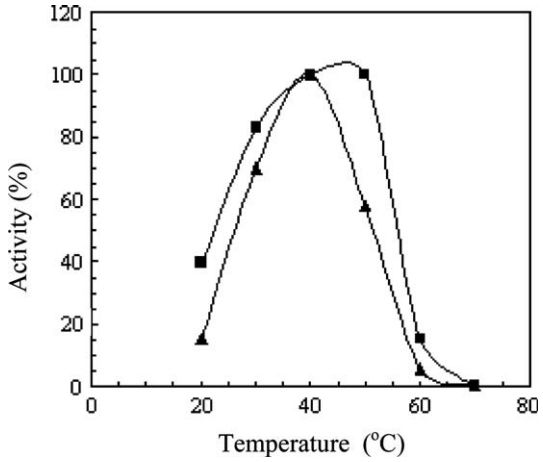


Fig. 6. Thermal stability of free and immobilized pepsin: (▲) free enzyme and (■) immobilized enzyme.

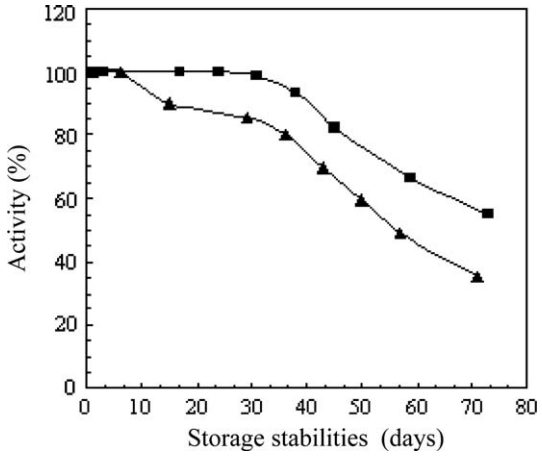


Fig. 8. Storage stability of free and immobilized pepsin at 5 °C: (▲) free enzyme and (■) immobilized enzyme.

The storage stability of free and immobilized pepsin is shown in Fig. 8. The pepsin immobilized chitosan beads did not exhibit any activity loss up to 30 days at 5 °C, but the activity decreased after 30 days as opposed to 5 days for that of free pepsin. The decrease in activity is explained as time dependent natural loss, and this can be prevented to a significant degree by immobilization. Also this immobilization brings another advantage by increasing enzyme stability for another 25 days at 5 °C as judged by storage stability experiments.

3.5. Temperature effects of free and immobilized pepsin on milk clotting activity

The temperature dependence of milk clotting activities of the free and immobilized pepsin were studied in 10 ml milk in the temperature range 20–70 °C and temperature profiles of free and immobilized pepsin are shown Table 2. As can be seen from Table 2 optimum milk-clotting temperatures for free and immobilized pepsin were 40 and 50 °C, respectively.

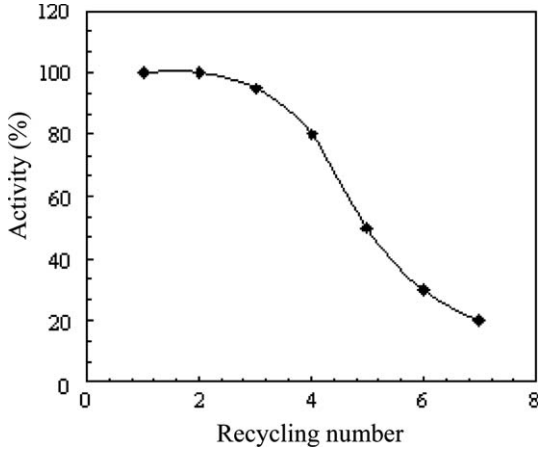


Fig. 7. Reusability of immobilized pepsin at 40 °C.

Thermal stability was investigated by incubating the free pepsin and immobilized pepsin on chitosan beads at temperatures ranging from 20 to 70 °C for 5 h, the milk clotting activity at is optimum reaction temperature was then determined. The effect of temperature stability on milk clotting activity of free and immobilized pepsin is illustrated in Table 3. It was found that thermal stability temperature of immobilized pepsin and free pepsin were 40 °C, and milk clotting activity and thermal stability of immobilized pepsin at 50 °C was higher than that of free

Table 2
Optimum milk clotting temperature for free and immobilized pepsin[a]

Temperature (°C)	Milk clotting activity (%)	
	Free pepsin	Immobilized pepsin
20	2.52	4.2
30	7.50	8.2
40	100.0	83.0
50	90.0	100.0
60	21.0	5.0
70	0.63	1.0

[a] Each experiment performed at least three times and average values were reported. Activities were presented normalized form, with the highest value of each set being assigned the value of 100% activity.

Table 3
The effect of thermal stability on the milk clotting activity of free and immobilized pepsin[a]

Temperature (°C)	Milk clotting activity (%)	
	Free pepsin	Immobilized pepsin
20	2.80	2.5
30	40.0	30.0
40	100.0	100.0
50	45.0	88.0
60	NC[b]	NC[b]
70	NC[b]	NC[b]

[a] Each experiment performed at least three times and average values were reported. Activities were presented normalized form, with the highest value of each set being assigned the value of 100% activity.

[b] NC = no clotting.

pepsin. Because of thermal denaturation, free and immobilized pepsin did not show milk clotting activity at 60–70 °C.

4. Conclusions

Immobilization of porcine pepsin on chitosan beads has been successfully carried out. Our experiments have shown that the pepsin immobilized on chitosan beads exhibits an improved resistance against thermal denaturation. It was found that thermal stability and storage stability of immobilized pepsin were greater than that of free pepsin. As shown by milk clotting experiments, immobilizations of pepsin can have several applications in industrial processes.

Acknowledgement

The authors gratefully acknowledge the financial support provided by the Cumhuriyet University Research Found through Project F-86.

References

Abdel-Naby, M. A. (1993). Immobilization of *Aspergillus niger* NRC 107 xylanase and β-xylosidase, and properties of the immobilized enzymes. *Applied Biochemistry Biotechnology, 38*, 69–81.

Bergkvist, R. (1963). The proteolytic enzyme of *Aspergillus oryzae* 1. Methods for the estimation and isolation of the proteolytic enzymes. *Acta Chemica Scandinavica, 17*, 1521–1540.

Berridge, N. J. (1952). Some observations on the determination of the activity of rennet. *Analyst London, 17*, 57–62.

Betigeri, S. S., & Neau, S. H. (2002). Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads. *Biomaterials, 23*, 3627–3636.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248.

Cao, L., Langen, L. V., & Sheldon, R. A. (2003). Immobilised enzymes: carrier-bound or carrier free? *Current Opinion in Biotechnology, 14*, 387–394.

Cetinus, S. A., & Oztop, H. N. (2003). Immobilization of catalase into chemically crosslinked chitosan beads. *Enzyme and Microbial Technology, 32*, 889–894.

Chandy, T., & Scharma, C. P. (1990). Chitosan as a biomaterial. *Biomaterials Artificial Cell and Artificial Organs, 18*, 1–24.

Chiou, S. H., & Wu, W. T. (2004). Immobilization of Candida rugosa lipase on chitosan with activation of the hydroxyl groups. *Biomaterials, 25*, 197–204.

Cooper, J. B., Khan, G., Taylor, G., Tickle, I. J., & Blundeli, T. L. (1990). X-ray analyses of aspartic proteinases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 A° resolution. *Journal of Molecular Biology, 214*, 199–222.

Deans, J. R., & Dixon, B. G. (1992). Bioabsorbents for waste water Treatment. In C. J. Birine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 648–656). Oxford, UK: Elsevier Applied Science.

Freeman, A., & Dror, Y. (1994). Immobilization of disguised yeast in chemically crosslinked chitosan bead. *Biotechnology and Bioengineering, 44*, 1083–1088.

Frydlova, J., Kucerova, Z., & Ticha, M. (2004). Affinity chromatography of porcine pepsin and pepsinogen using immobilized ligands derived from the specific substrate for this enzyme. *Journal of Chromatography B, 800*, 109–114.

Gemeiner, P. (1992). Materials for enzyme engineering. In *Enzyme engineering-immobilised biosystems* (pp. 13–119). England: Ellis Horwood Limited.

Hasem, A. M. (1999). Optimization of milk-clotting enzyme productivity by *Penicillium oxalicum*. *Bioresource Technology, 70*, 203–207.

Hasem, A. M. (2000). Purification and properties of a milk-clotting enzyme produced by *Penicillium oxalicum*. *Bioresource Technology, 75*, 219–222.

Jiang, B., & Zhang, Y. (1993). Immobilization of catalase on crosslinked polymeric hydrogels effect of anion on the activity of immobilized. *European Polymer Journal, 29*, 1251–1254.

Kamatari, Y. O., Dobson, C. M., & Konno, T. (2003). Structural dissection of alkaline-denatured pepsin. *Protein Science, 12*, 717–724.

Kennedy, J. F., & Melo, E. H. M. (1990). Immobilized enzymes and cells. *Chemical Engineering Progress*, 81–89.

Khor, E. (2002). Chitin: a biomaterial in waiting. *Current Opinion in Solid state and Materials Science, 6*, 313–317.

Kittur, F. S., Kumar, K. R, & Tharanathan, R. N. (1998). Functional packaging properties of chitosan films. *Zeitschrift Fur Lebensmittel Untersuchung und Forschung A-Food Research and Technology, 206*, 44–47.

Krajewska, B., Leszko, M., & Zaborska, W. (1990). Urease immobilized on chitosan membrane, preparation and properties. *Journal of Chemical Technology and Biotechnology, 48*, 337–350.

Kurimoto, E., Harada, T., Akiyama, A., Sakai, T., & Kato, K. (2001). In vitro refolding porcine pepsin immobilized on agarose beads. *Journal of Biochemistry, 130*, 295–297.

Li, S., Hu, J., & Liu, B. (2004). Use of chemically modified PMMA microspheres for enzyme immobilization. *Biosystems, 77*, 25–32.

Martinek, K., Kilbanov, A. M., Goldmacher, V. S., & Berezin, I. V. (1977). The principles of enzyme stabilization. *Biochimica et Biophysica Acta, 485*, 1–12.

Oztop, H. N., Saraydın, D., & Cetinus, S. (2002). pH – Sensitive chitosan films for baker's yeast immobilization. *Applied Biochemistry and Biotechnology, 101*, 239–249.

Papineau, A. M., Hoover, D. G., Knorr, D., & Farkas, D. F. (1991). Antimicrobial Effect of water-soluble chitosans with high hydrostatic Pressure. *Food Biotechnology, 5*, 45–57.

Pinotti, A., Bevilacqua, A., & Zaritzky, N. (1997). Optimization of the Flocculation stage in a model system of a food emulsion waste using chitosan as polyelectrolyte. *Journal of Food Engineering, 32*, 69–81.

Shahidi, F., & Synowiecki, J. (1991). Isolation and characterization of nutrients and value-added products from Snow Crab (*Chinoecetes opilio*) and Shrimp (*Pandalus borealis*) processing discards. *Journal of Agriculture and Food Chemistry, 39*, 1527–1532.

Soto-Perlata, N. V., Muller, H., & Knorr, D. (1989). Effect of chitosan treatments on the clarity and color of apple juice. *Journal of Food Science, 54*, 495–496.

Spagna, G., Pifferi, P. G., Rangoni, C., Mattivi, F., Nicolini, G., & Palmonari, R. (1996). The stabilization of white wines by adsorption of phenolic compounds on chitin and chitosan. *Food Research International, 29*, 241–248.

Ticu, E. L., Marko, D. V., Froidevaux, R., Huma, A., Artenie, V., & Guillochon, D. (2005). Use of protease-modified-alumina complex to design a continuous stirred tank reactor for producing bioactive hydrolysates. *Process Biochemistry, 40*(8), 2841–2848.