Immobilization of Pepsin on an Acrylamide/2-Hydroxyethyl Methacrylate Copolymer and Its Use in Casein Hydrolysis

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ABSTRACT: Pepsin was immobilized through covalent bonding on a copolymer of acrylamide and 2-hydroxyethyl methacrylate via the individual and simultaneous activation of both groups. The extent of enzyme coupling upon the activation of both the amino and hydroxyl groups of the copolymer resulted in a synergistic effect. However, the order of activation of the support was critical. The covalently bound enzyme retained more than 50% of its activity even after six cycles. The storage stability of the covalently bound enzyme was 60% after storage for 1 month, whereas the free enzyme lost all of its activity within 10 days of storage at 35°C. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were 1.1×10^{-6} and 0.87 for the free enzyme and 1.2×10^{-6} and 0.98 for the covalently bound enzyme when the enzyme concentration was kept constant and the substrate concentration was varied. Similarly, K_m and V_{max} were 6.73×10^{-11} and 0.47 for the free enzyme and 7.59×10^{-11} and 0.545 for the covalently bound enzyme when the substrate concentration was kept constant and the enzyme concentration was varied; this indicated no conformational change during coupling, but the reaction was concentration-dependent. The hydrolysis of casein was carried out with a fixed-bed reactor (17 cm × 1 cm). Maximum hydrolysis (90%) was obtained at a 2 cm^3/min flow rate at 35°C with a 1 m*M* casein solution. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 1544–1549, 2005

Key words: pepsin; immobilization; hydrolysis

INTRODUCTION

Enzymes are biocatalysts involved in various chemical reactions in living systems. They mainly consist of globular proteins and are produced in living cells. Because of their wide acceptance in industry, chemical laboratories, dairy farms, and other areas, they are gaining increasing importance. Enzymes are important catalysts in a wide range of reactions because of their catalytic rates, specificity, and functioning under mild conditions.

Bergman[1] classified proteolytic enzymes on the basis of the substrates used by the proteases. Pepsin is an acidic protease found in the stomachs of almost all vertebrates. The active enzyme is released from its zymogen, that is, pepsinogen, by autocatalysis, in the presence of hydrochloric acid (HCl). Pepsin is an endopeptidase with broad specificity. Pepsin has been immobilized on many natural and synthetic supports. Pepsin was covalently bound to glass by Line et al.[2] They observed that the insoluble enzyme retained activity over 30 days during continuous operations. Hirano and Miura[3] used chitosan for the immobilization of pepsin through glutaraldehyde coupling. They reported 95% enzyme activity retention after four cycles. Gustovo and Jacqueline[4] immobilized pepsin on succenilated chitosan through the covalent binding of the enzyme with a carbodiimide coupling agent. They observed that the system retained 80% of its activity and had greater storage stability than the free enzyme. *N*-(4-Carboxy phenyl carbamoyl methyl)cellulose was used as a support for pepsin immobilization by Dumitriu et al.[5] The Michaelis constant (K_m) for the immobilized enzyme was reported to be lower than that for the free form. The immobilized system lost 25% of its activity after three cycles. Shah et al.[6] checked the clotting and stabilization of milk with pepsin covalently bound onto resinous materials. The effects of the concentration of the enzyme and crosslinking agent and the pH of the coupling medium on the immobilization of the enzyme were studied. Salicylic acid/resorcinol formaldehyde resin bound pepsin had better thermal and chemical stability, and the maximum retention of activity was observed between 30 and 40°C. Pepsin was covalently coupled to the surface of a new type of porous zirconia and to a conventional porous silica activated with 3-isothiocyanatopropyl triethoxysilane by Marion et al.[7] Pepsin exhibited a higher specific activity when it was immobilized onto silica than when it was immobilized onto zirco-

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nia. Although the immobilized pepsin–zirconia matrix was more stable than the enzyme in a free solution at room temperature, a loss of activity of up to 50% over a 2-month period occurred when the matrix was stored in a 50 m*M* sodium acetate buffer at pH 4.0. Arnostova et al.[8] prepared affinity sorbents containing immobilized iodinated derivatives of L-tyrosine for the affinity chromatography of porcine pepsin. The ligand was coupled to either sepharose 4B or cellulose beads after activation with 2,4,6-trichloro-1,3,5 triazine and divinyl sulfone, respectively. Pepsin was immobilized onto agarose beads by Kurimoto et al.[9] to determine the refolding conditions. The renaturation of immobilized pepsin was reported to be extremely slow and reached equilibrium after 300 h. The use of a 60% salt concentration was observed to raise the recovery of the enzyme to 80% but had no significant effect on the refolding rate. Pepsin was immobilized onto *A*-acetyl phenyl alanine and an iodinated derivative of tyrosine, divinyl sulfone activated sepharose, by Jana et al.[10] The ligand with blocked amino groups and free carboxyl groups was linked to sepharose via an ethylenediamine spacer with a carbodiimide reagent. They further checked the effects of the pH and ionic strength.

A copolymer of acrylamide (AAm) and 2-hydroxyethyl methacrylate (HEMA) was used by us for the entrapment of α-chymotrypsin[11] and the covalent coupling of horseradish peroxidase (HRP).[12] In our recent work on HRP immobilization, we reported some interesting observations regarding the order of activation of coupling groups. Therefore, in this communication, we report our observations for pepsin coupling to the AAm–HEMA copolymer. Upon the optimization of the conditions, immobilized pepsin was further used for the hydrolysis of casein with a fixed-bed reactor.

EXPERIMENTAL

Materials

Pepsin (E.C.3.4.23.1) from porcine stomach mucosa (strength = 3380 U/mg) and casein were purchased from Sigma Chemicals Co. (St. Louis, MO). Trichloroacetic acid and AAm were purchased from Qualigens (Mumbai, India), and hemoglobin and ascorbic acid were purchased from Loba Chemie (Mumbai, India). HEMA was acquired from Fluka Chemie AG-CH-9470 (Buchs, Switzerland).

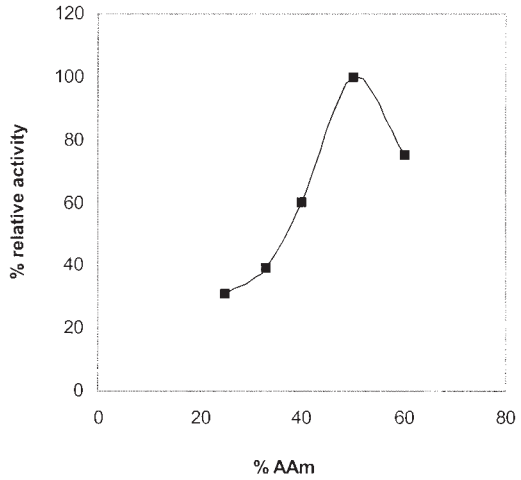

Figure 1 Effect of the AAm percentage in the copolymer on the retention of pepsin activity.

Assay method of pepsin

The activity of the free and covalently bound enzyme was determined with 4 mL of a 2% (w/v) hemoglobin solution prepared in 0.06*N* HCl as a substrate, as described by Anson.[13] The enzyme and substrate were incubated for 30 min at 37°C. The reaction was terminated with 5 mL (5% w/v) of trichloroacetic acid. The product being soluble in trichloroacetic acid, 5 cm^3 of the filtrate was taken, and 10 cm^3 of 0.5*N* sodium hydroxide and 3 cm^3 of a diluted phenol reagent were added to it; the liberated tyrosin was measured at 750 nm.

Synthesis and activation of the AAm–HEMA copolymer

The AAm–HEMA copolymer was prepared by bulk polymerization, as described elsewhere.[12] AAm (1.0 g)

TABLE I
Effect of Different Activating Agents on Different Coupling Parameters

Coupling condition	Coupling agent			
	Glu	PBQ	Glu + PBQ	PBQ + Glu
Activating agent concentration	10 cm^3 1% (w/v)	25 mg/g	10 cm^3 1% (w/v) + 25 mg/g	25 mg/g + 10 mL 1% (w/v)
Activation time	5	4	5 + 8	8 + 5
Enzyme used for coupling	7000	4000	7500	5000
Coupled enzyme concentration (μg/g)	147	52.9	559.85	151.24
Enzyme coupling time (h)	8	16	8	8
Coupling pH	2	2	2	2
Coupling yield	2.1	1.2	7.4	3

The concentrations were fixed after the optimization of the coupling agent concentration and coupling time to obtain the maximum extent of coupling. Glu = glutaraldehyde; PBQ = *p*-benzoquinone.

TABLE II
Effect of Temperature and Time on the Deactivation of the Enzyme

	Time (min)									
	15		30		45		60		120	
Temperature	Free	Covalalently bound	Free	Covalalently bound	Free	Covalalently bound	Free	Covalalently bound	Free	Covalalently bound
35	0.07	0.048	0.83	0.65	1.20	0.84	1.42	1.02	1.45	1.02
45	1.66	1.12	1.93	1.63	2.01	1.61	2.21	1.87	2.24	2.18
55	2.30	2.10	2.50	2.42	2.55	2.48	2.62	2.43	3.19	2.88
65	3.45	3.38	3.97	3.44	4.16	3.89	4.82	4.72	—	—

and 2.0 g of HEMA were stirred for 10 min, and 0.3 cm^3 of 30% (w/v) hydrogen peroxide and 0.1 g of ascorbic acid dissolved in 0.5 cm^3 of water were added. The reaction mixture, upon stirring at 35°C for 10–15 min, turned into a gel. Copolymers with different monomer ratios were prepared and were characterized by Fourier transform infrared (FTIR).

The hydroxyl and amine groups in the copolymer were activated with *p*-tolyl sulfonyl chloride or *p*-benzoquinone and glutaraldehyde, respectively. The coupling of the enzyme to the activated support was carried out through the simultaneous and individual activation of the amine and hydroxyl groups, which was followed by enzyme coupling. Various conditions such as the concentration of the activating agent, the activation time, the coupling time, and the coupling pH were optimized.

pH

The pH activity profile of the free and covalently bound pepsin was studied through the incubation of the enzyme at pH 1–7 at 37°C for 30 min with hemoglobin as the substrate.

Thermal stability

The thermal stability of the free and covalently bound enzyme was studied by the heating of the enzyme at 35–65°C for various times and by the measurement of its activity. The thermodeactivation constant (K_d) was calculated as follows:[14]

$$\ln A_t = \ln A_0 - K_d(t)$$

where A_0 is the initial activity of the enzyme and A_t is the activity after the heat treatment for t min.

Storage stability

The storage stability of free and covalently bound pepsin was checked at 35°C, and the retention of the activity was determined at regular times.

Reusability of the covalently bound pepsin

The reusability of the covalently bound pepsin was examined with the same aliquot of the enzyme with a fresh aliquot of the substrate until 50% of the enzyme activity was lost.

Kinetic parameters

We calculated the kinetic parameters K_m and V_{max} from a Lineweaver–Burk plot by varying the substrate concentration from 0.075×10^{-3} to 0.375×10^{-3} M and keeping the enzyme concentration at 13.15×10^{-11} M and by varying the enzyme concentration from 2.63×10^{-11} to 13.15×10^{-11} M and keeping the substrate concentration at 0.225×10^{-3} M.

Hydrolysis of casein

Casein is the principal protein of cow's milk. It is the most commonly used milk protein in industry and contains 21 amino acids. Acid casein, a granular milk protein, is available in two types: edible and technical. Hydrolyzed proteins are added to food to serve vari-

TABLE III
Kinetic Parameters of Free and Covalently Bound Enzyme

Conditions	Free enzyme		Covalently bound Enzyme	
	K_m	V_{max}	K_m	V_{max}
Substrate concentration = 0.075×10^{-3} to 0.375×10^{-3} M and enzyme concentration = 13.15×10^{-11} M	1.1×10^{-6}	0.87	1.2×10^{-6}	0.98
Enzyme concentration = 2.63×10^{-11} to 13.15×10^{-11} M and substrate concentration = 0.225×10^{-3} M	6.73×10^{-11}	0.47	7.59×10^{-11}	0.545

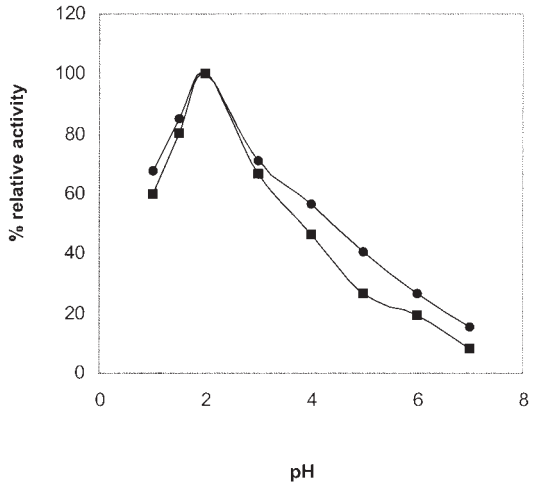

Figure 2 pH activity profile of (■) free and (●) covalently bound pepsin.

ous functions. They can be used as leavening agents, stabilizers, thickeners, flavor enhancers, and nutrients. Dharmapuri and Saiprakash[15] reported greater catalytic activity of pepsin in casein hydrolysis in an aquoalcoholic medium than in an aqueous medium. The maximum activity of pepsin was reported to range from 20 to 40% in a 0.2*M* aquoalcoholic medium. Pepsin immobilized on egg shells and synthetic zeolites was used for the hydrolysis of casein by Sunitha and Saiprakash.[16] They covalently bound pepsin onto egg shell with a glutaraldehyde crosslinking agent and also physically adsorbed it onto synthetic zeolites. They observed that the covalently bound enzyme possessed higher retention of activity and stability at higher temperatures and extreme pH conditions than the pepsin adsorbed onto zeolites. We used pepsin

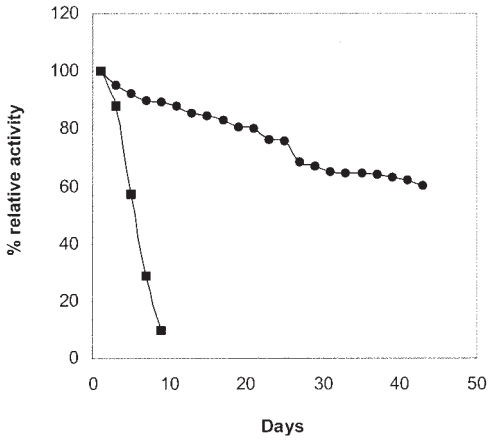

Figure 4 Storage stability of (■) free and (●) covalently bound pepsin at 35°C.

covalently coupled to the copolymer of AAm–HEMA with different activating agents for the hydrolysis of casein with a fixed-bed reactor. Reactor conditions such as the flow rate, substrate concentration, reactor temperature, and length to diameter ratio (l/d) ratio were optimized.

RESULTS AND DISCUSSION

Optimization of the monomer ratio and coupling conditions

The maximum extent of pepsin coupling to a copolymer was observed with a copolymer synthesized with a 1:1 ratio of AAm and HEMA (Fig. 1). Hence, further studies were carried out with this copolymer. The FTIR spectra of the copolymer showed bands at 3700–3200 cm^{-1} corresponding to —NH stretching and —OH stretching, at 1710 cm^{-1} due to —C═O stretch-

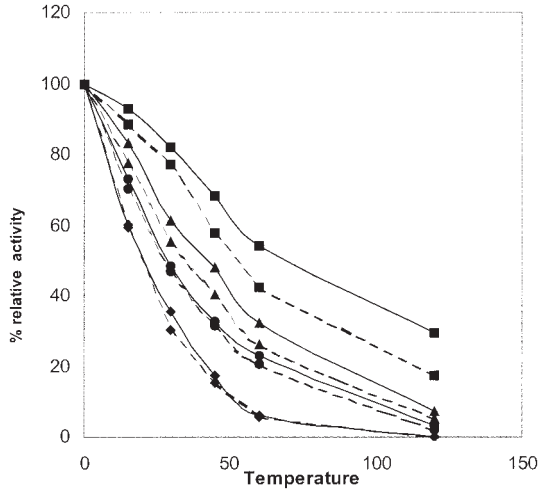

Figure 3 Thermal stability of (- - -) free pepsin and (—) covalently bound pepsin for different time intervals at (■) 35, (▲) 45, (●) 55, and (♦) 65°C.

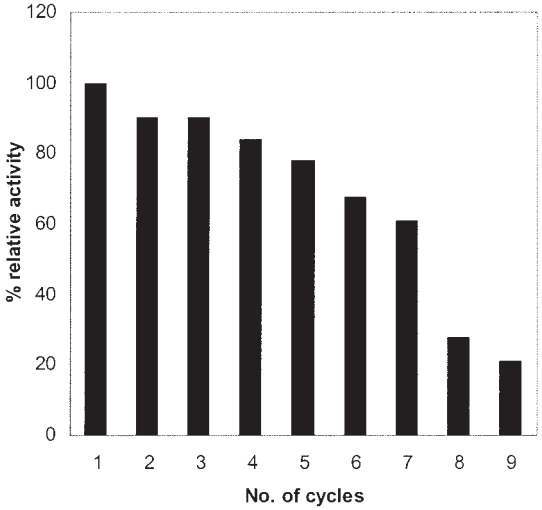

Figure 5 Reusability of covalently bound pepsin at 35°C and pH 2.

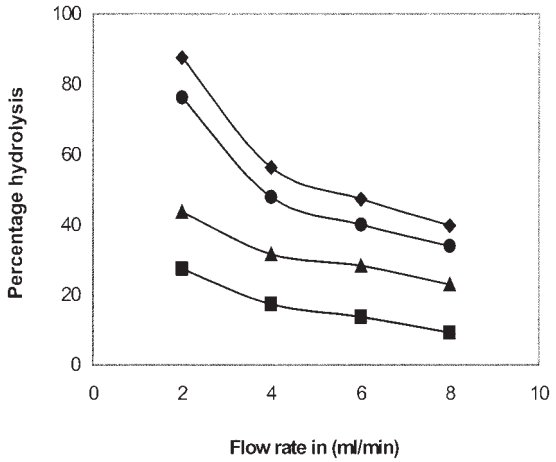

Figure 6 Effect of the flow rate on casein hydrolysis at 35°C with a 1 m*M* casein solution: (■) first cycle, (●) second cycle, (▲) third cycle, and (♦) fourth cycle.

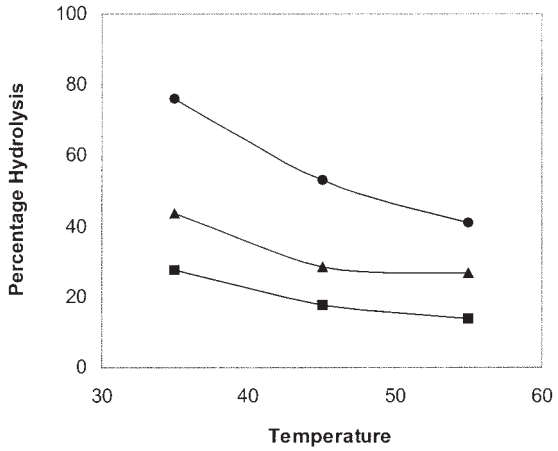

Figure 8 Effect of the temperature on hydrolysis of 1 m*M* casein at a 2 cm^3 min^{-1} flow rate: (■) first cycle, (●) second cycle, and (▲) third cycle.

ing, at 2925 cm^{-1} due to C—H stretching, and at 1155 cm^{-1} due to C—N stretching.

The activation of the functional groups of the copolymer was carried out with glutaraldehyde and *p*-benzoquinone for amino and hydroxyl groups, respectively. The conditions optimized for the maximum coupling of pepsin to the copolymer were as follows:

For the activation of NH_2 groups, 10 cm^3 of 1% (w/v) glutaraldehyde, a 5-h activation time, an enzyme concentration of 147 μg/g, a pH 2 coupling medium, and a coupling time of 8 h.

For the activation of OH groups, a *p*-benzoquinone concentration of 25 mg/g, an activation time of 4 h, an enzyme concentration of 52.8 μg/g, a pH 2 coupling medium, and a coupling time of 16 h.

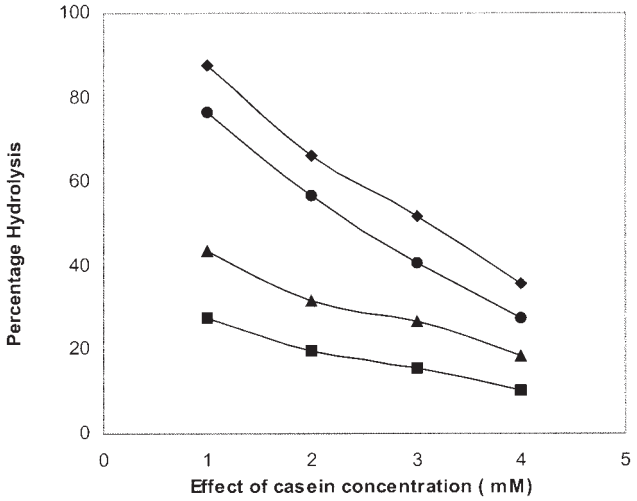

Figure 7 Effect of the casein concentration on hydrolysis at a 2 cm^3 min^{-1} flow rate and 35°C: (■) first cycle, (●) second cycle, (▲) third cycle, and (♦) fourth cycle.

A synergistic effect was observed when the activation of amino groups followed by enzyme loading and then the activation of hydroxyl groups followed by enzyme coupling were carried out under the optimized conditions. When the order of activation was reversed, the enzyme activity decreased. The results are given in Table I. The extent of enzyme coupling was almost 3 times higher when the coupling order was as follows: the activation of amino groups by glutaraldehyde followed by enzyme coupling and then the activation of hydroxyl groups by *p*-benzoquinone followed by enzyme coupling. When the order was reversed, a drastic decrease in the total enzyme coupling was observed. We observed a similar trend for the covalent coupling of HRP on the AAm–HEMA copolymer.[12] From our study, we have found that two different classes of enzymes show similar trends, although the extents of coupling are different.

pH

Enzymatic reactions are sensitive to pH and operate within a very narrow pH range. There was no change in the optimum pH for the free and covalently bound enzyme, and the maximum activity was exhibited at pH 2.This indicated no change in the catalytic sites of the enzyme during enzyme immobilization (Fig. 2).

Thermal stability

Enzymes are susceptible to temperature and lose their activity at higher temperatures (Table II). At high temperatures, the free and covalently bound enzyme showed similar reductions. However, the covalently coupled pepsin showed better thermal stability than the free enzyme (Fig. 3).

Storage stability and reusability

The storage stability is an important parameter for the commercial applications of enzymes. The free enzyme lost all its activity within 10 days at 35°C, whereas the covalently bound enzyme retained 60% of its activity even after 1 month at 35°C (Fig. 4). This shows the advantage of the immobilization of the enzymes in comparison with free ones. The covalently coupled pepsin retained 55% of its activity after sic cycles (Fig. 5). The turnover number was 650.

Kinetic parameters

K_m and V_{max} for the free enzyme were 1.1×10^{-6} and 0.88, whereas for the covalently bound enzyme, K_m was 1.2×10^{-6}, and V_{max} was 0.98 when the enzyme concentration was kept constant and the substrate concentration was varied (Table III). The correlation coefficients for the linear plots were 0.97 and 0.96 respectively. When the enzyme concentration was varied and the substrate concentration was kept constant K_m and V_{max} were 6.73×10^{-11} and 0.47 for the free enzyme and 7.59×10^{-11} and 0.604 for the covalently bound enzyme with correlation coefficients of 0.98 and 0.98. This indicated no conformational changes in the enzyme during immobilization.

Casein hydrolysis

The hydrolysis of casein was carried out with a fixed-bed reactor (17 cm × 1 cm). The casein solution was prepared in 0.05*M* lactic acid, as described by Ohtsuru et al.[17] The effect of the flow rate on the hydrolysis of a 1 m*M* casein solution was checked through the variation of the flow rate from 2 to 8 cm^3/min. With an increase in the flow rate, the hydrolysis decreased as the time of contact of the covalently bound support and casein solution decreased. Moreover, 90% hydrolysis was achieved after four cycles at a 2 cm^3/min flow rate (Fig. 6). The hydrolysis of casein decreased when the casein concentration was increased from 1 to 4 m*M* and the flow rate was maintained at 2 cm^3/min at 35°C (Fig. 7). A similar trend was observed with an increase in the reaction temperature. The hydrolysis decreased with increasing temperature (Fig. 8). This may be due to the fact that some enzyme was deactivated at a higher temperature. With an increase in the l/d ratio up to 4, the casein hydrolysis increased, but with a further increase in the l/d ratio, it did not show much change. A maximum of 90% hydrolysis was achieved after four cycles at the l/d ratio of 4 (Fig. 9). All the experiments were repeated three times, and the presented results are with the maximum standard deviation not exceeding ±0.0025.

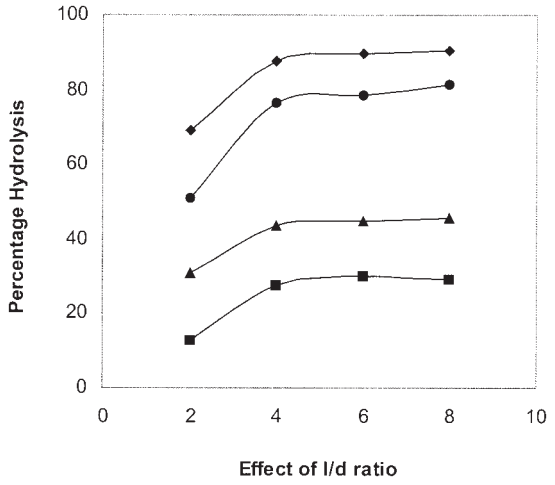

Figure 9 Effect of the l/d ratio on casein hydrolysis with a 1 m*M* casein solution at a 2 cm^3 min^{-1} flow rate and 35°C: (■) first cycle, (●) second cycle, (▲) third cycle, and (♦) fourth cycle.

CONCLUSIONS

Pepsin was immobilized onto an AAm–HEMA copolymer by covalent coupling. A synergistic effect was observed when both amino and hydroxyl groups were activated in a particular sequence, and the activity increased almost 3 times, but when the order of activation was reversed, a reverse effect was observed. The covalently bound enzyme showed better thermal and storage stability than the free enzyme. The turnover number was 650. Under optimized conditions, 90% casein hydrolysis was achieved with immobilized pepsin in a fixed-bed reactor (17 cm × 1 cm).

References

1. Bergman, M. Adv Enzymol 1942, 2, 49.
2. Line, W. F.; Kwong, A.; Weetall, H. H. Biochem Biophys Acta 1971, 242, 194.
3. Hirano, S.; Miura, O. Biotechnol Bioeng 1979, 21, 711.
4. Gustavo, G.; Jacqueline, A. J Chem Technol Biotechnol 1885, 63, 247.
5. Dumitriu, S.; Popa, M.; Duritriu, C. J Macromol Sci Chem 1987, 24, 1135.
6. Shah, B.; Kumar, S. R.; Devi, S. Proc Biochem 1995, 30, 63.
7. Marion, H.; Hans, J. W.; Milton, H. T. W. J Biochem Biophys Methods 1996, 31, 165.
8. Arnostova, H.; Kucerova, Z.; Tislerura, J.; Trnka, T.; Ticha, M. J Chromatogr A 2001, 911, 211.
9. Kurimoto, E.; Harada, T.; Akiyama, A.; Sakai, T. J Biochem 2001, 30, 295.
10. Jana, F.; Zdenka, K.; Marie, T. J Chromatogr B 2004, 800, 109.
11. Soni, S.; Desai, J. D.; Devi, S. J Appl Polym Sci 2000, 77, 2996.
12. Shukla, S. P.; Devi, S. Proc Biochem 2005, 40, 147.
13. Anson, M. L. J Gen Physiol 1939, 22, 77.
14. Shukla, S. P.; Modi, K.; Ghosh, P. K.; Devi, S. J Appl Polym Sci 2004, 91, 2063.
15. Dharmapuri, A.; Saiprakash, P. Indian J Chem Sect A 1999, 38, 1181.
16. Sunitha, J.; Saiprakash, P. K. J Indian Chem Soc 1996, 73, 335.
17. Ohtsuru, M.; Tang, J.; Delaney, R. Int J Biochem 1982, 14, 925.