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Immobilization of trypsin on an enteric polymer Eudragit S-100 for the biocatalysis of macromolecular substrate

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Abstract

Trypsin was covalently linked to Eudragit S-100 (an enteric coating polymer) by carbodiimide coupling method. Nearly 90% of enzyme activity was conjugated to the polymer whereas actual observed (expressed) activity was 64%. The enzyme bound to the polymer by simple adsorption as well but leached off the polymer in the presence of high ionic strength (1.0 M NaCl). The immobilized enzyme showed stability to autolysis at 45°C and had a marginal shift in pH optimum. The K_m value of the enzyme decreased from 1.0×10^{-3} M to 0.7×10^{-3} M on immobilization. The soluble Eudragit-trypsin conjugate was used to hydrolyze casein at 45°C. Eudragit being a reversibly soluble–insoluble polymer, the biocatalyst could be recovered and reused.

Keywords: Autolysis; Carbodiimide coupling; Casein hydrolysis; Eudragit; Eudragit-trypsin conjugate; Trypsin; Water-soluble polymer

1. Introduction

Immobilization of enzymes has become an established approach for enhancing their usefulness in biotechnology [1,2]. The rate limiting step in such approaches is when dealing with macromolecular or insoluble substrate material. However, in such cases, better enzymatic activity is observed if immobilization is carried out on a soluble matrix [3,4]. In recent years, Eudragit, a methacrylic-methacrylate polymer has emerged as a useful immobilizing support for hydrolysis of such substrates like starch [5], cellulose [6] and yeast cells [7]. The polymer precipitates at low pH around 4.5 and is completely soluble at higher pH (≥ 5.5). This makes it the best choice for carrying out biocatalytic reactions in soluble phase in the most favourable pH range.

Hydrolysis of food proteins is carried out for many reasons: improving nutritional characteristics, changing solubility, improving texture, removal of flavours and antinutritional factors [8]. The hydrolysis using proteases is preferred over chemical methods especially if the intended application is for nutritional purposes.

The present study describes the immobilization of trypsin on Eudragit S-100 by both covalent and non-covalent methods. The results fo-

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cus on the reusability of the covalently immobilized trypsin conjugate for casein hydrolysis.

2. Materials and methods

2.1. Materials

Eudragit S-100 was a product of Rohm Pharma, Weiterstadt, Germany. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), trypsin (Type III, from bovine pancreas) and *N*-benzoyl-*p*-nitroanilide (BAPNA) were purchased from Sigma, St. Louis, USA. Casein was obtained from Sisco Research Laboratory, India. All other chemicals used were of analytical grade.

2.2. Immobilization of trypsin on Eudragit S-100

Eudragit S-100 solution (2% w/v, 100 ml) was prepared in distilled water [9]. 30 ml of this solution was precipitated by decreasing the pH to 4.5 with 2 M acetic acid. The precipitate was centrifuged at $10{,}000 \times g$ for 15 min and dissolved in 60 ml of 0.05 M Tris–HCl (pH 8.0) containing 0.02 M $CaCl_2$ and 0.15 M NaCl. 30 mg trypsin (10 mg/ml in 0.05 M Tris–HCl containing 0.02 M $CaCl_2$, pH 8.0) was added to the polymer solution and chemically coupled by using 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) [10]. The coupling mixture was stirred at room temperature (25°C) for 3 h. To this mixture 2 ml ethanolamine (0.35 g/ml, pH 8.6) was added and stirred for 1 h. The conjugate was precipitated by decreasing the pH to 4.5 and the precipitate was centrifuged at $10{,}000 \times g$ for 15 min. The precipitated conjugate was dissolved in 0.05 M Tris–HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ to a total volume of 60 ml. This soluble conjugate was divided in three parts and precipitated in presence of (a) 0.5 M NaCl and (b) 1.0 M NaCl by lowering the pH to 4.5 with 2 M acetic acid. Also, the precipitation was done at pH 6.5 by adding 10% (v/v) acetonitrile and 0.02 M $CaCl_2$ to the conjugate (c). Finally the total volume of the each conjugate part was made up to 20 ml in 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.0. Unbound trypsin was determined in the supernatant.

The non-covalent adsorption of the enzyme to the polymer was carried out by adding 1 ml of trypsin (5 mg/ml in 0.05 M Tris–HCl containing 0.02 M $CaCl_2$, pH 8.0) to 10 ml of Eudragit S-100 solution (1% w/v). After incubation for 1 h at room temperature, polymer was precipitated by adjusting pH to 4.5 by adding 2 M acetic acid and centrifuged at $10{,}000 \times g$ for 15 min. Unbound trypsin activity was determined in the supernatant. To determine the leaching of the adsorbed enzyme, the trypsin bound polymer was precipitated in presence of 1.0 M NaCl. The precipitate was centrifuged at $10{,}000 \times g$ for 15 min and released enzyme activity was measured in the supernatant. This step was repeated until no enzyme activity could be detected in the supernatant.

2.3. Determination of K_m, pH optimum and thermal stability

K_m values of native and immobilized enzyme was determined by measurement of enzyme activity with various concentrations of substrate. The same amount of free and immobilized enzyme were used in these experiments. Effect of pH on native and immobilized enzyme was studied by assaying both the preparations at different pH values. Similarly, thermal stability of the enzyme was studied by incubating at different temperatures. An appropriate aliquot of native and immobilized enzyme was drawn at various time intervals of incubation and their activities were determined.

2.4. Casein hydrolysis

The hydrolysis of casein using covalently immobilized trypsin was carried out according to the method described in Ref. [11] with some modifications. 2 ml of 1% (w/v) casein solu-

tion was incubated with 2 ml of Eudragit–trypsin complex (pH 8.0) for 30 min at 45°C. The reaction was stopped by adding 6 ml of 5% (w/v) trichloro acetic acid (TCA) solution and incubated for 15 min. The precipitated Eudragit–trypsin complex and unhydrolysed casein was centrifuged at $10{,}000 \times g$ for 15 min. TCA soluble peptides were estimated in the supernatant [12]. The precipitate of immobilized enzyme was washed free of TCA by dissolving in 5 ml of 0.05 M Tris–HCl containing 0.02 M $CaCl_2$, pH 8.0 and then decreasing the pH to 4.5 with 2 M acetic acid. The recovered Eudragit–trypsin was reused for another cycle of casein hydrolysis as above. The process was repeated till three cycles of reuse.

2.5. Enzyme assay

Trypsin activity was determined by the method of Erlanger et al. [13] using *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate.

2.6. Protein estimation

Protein was estimated using the dye-binding method of Bradford [14] using bovine serum albumin as standard protein.

3. Results and discussion

Hydrolysis of protein when the latter is present in a complex mixture requires a robust and economical technology in the context of food/feed improvement and treatment of biological waste from food processing industries. Proteases immobilized on soluble–insoluble polymer may be one such approach. This approach is evaluated here with an immobilized system in which trypsin was linked to Eudragit S-100.

The conjugation of trypsin with Eudragit S-100 was carried out with the carbodiimide coupling method [10] (see Table 1). Trypsin also shows binding to the polymer non-covalently which could be a more convenient alternate to the covalent coupling method [15,16]. The adsorbed trypsin showed initial expressed activity of 74% (Fig. 1). However, this leached off during reprecipitation cycles in presence of 1 M NaCl. The final expressed activity as shown in Table 1 was 3%, as almost all enzyme comes off the polymer. However, in our other study, it was demonstrated that enzyme leaching was minimized as the polymer concentration during immobilization was increased [9], which shows that by employing higher polymer concentration during immobilization, one could strengthen the

Table 1
Immobilization of trypsin on Eudragit S-100

Conjugation	Protein bound (%)	Activity bound (%) A	Expressed activity (%) B	Activity yield (B/A) × 100 (%)
Chemical coupling				
Eudragit–trypsin (Precipitation in presence of 0.5 M NaCl)	86	91	64	70
Eudragit–trypsin (Precipitation in presence of 1.0 M NaCl)	86	91	62	68
Eudragit–trypsin (Precipitation with organic solvent and Ca^{2+})	75	84	56	67
Adsorption				
Eudragit–trypsin (Repeated precipitation in presence of 1.0 M NaCl)	30	32	3	9

Precipitation of conjugates (in 0.05 M Tris–HCl + 0.02 M $CaCl_2$, pH 8.0) were done by lowering the pH to 4.5 in presence of desired concentration of NaCl. In case of precipitation using organic solvent at pH 6.5, 10% (v/v) acetonitrile and 0.2 M $CaCl_2$ was used in the above buffer. Repeated precipitation were carried out in case of adsorbed trypsin in presence of 1.0 M NaCl.

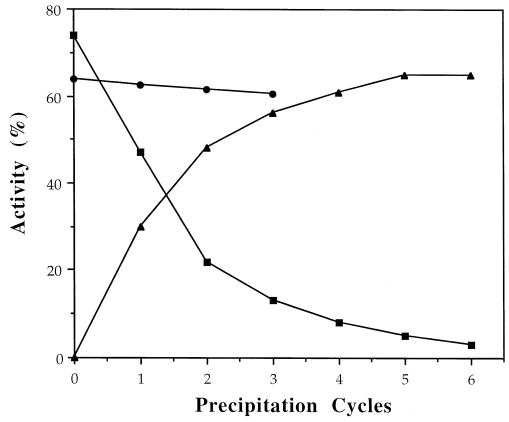

Fig. 1. Release of adsorbed trypsin from Eudragit S-100 by repeated precipitation in presence of 1.0 M NaCl. (-▲-) cumulative activity released: (-■-) expressed activity and (-●-) expressed activity of covalently linked Eudragit–trypsin conjugate after each precipitation cycle. The enzyme activities were determined by using BAPNA as substrate.

non-covalent forces between the polymer and an enzyme. The covalently coupled trypsin, on the other hand, retained 67–70% of the bound activity (see Table 1). The extent of loss upon conjugation is similar to what is observed by other workers [7]. It was pointed out recently that in such cases, some protein is invariably bound non-covalently during the covalent coupling procedure, which may leach slowly during the use of the immobilized biocatalyst [17]. The trypsin covalently coupled to Eudragit was dissolved in buffer containing 1.0 M NaCl and reprecipitated. This reduced the activity by mere 2–4%. Eudragit and Eudragit coupled proteins can also be precipitated at neutral pH by adding Ca^{2+} and organic solvents [18]. Precipitation of Eudragit–trypsin conjugate by this method reduced the expressed activity by 6%. As the intended application was in an aqueous medium, the preparation treated with 1.0 M NaCl was used in further work. However, the data reinforces the wisdom of investigating extensively the stability of the binding of the protein with the matrix before use [17].

Eudragit as well as Eudragit–trypsin conjugate precipitated below pH 4.5 and were soluble above pH 6.0 (Fig. 2). The latter, thus, could be used as a reversibly soluble–insoluble immobilized protease. A positive feature about the recovery of this conjugate (for reuse) is that precipitation is possible at pH 4.5, which is a reasonably safe pH for many enzymes. The immobilization of the enzyme also resulted in its stabilization (Fig. 3). In the absence of Ca^{2+}, at 45°C, the enzyme in free solution lost nearly all of its activity within 20 min whereas the enzyme conjugated to Eudragit S-100 showed no loss even after 90 min. The pH vs. activity curve of the enzyme (Fig. 4) altered, reflecting the way the negatively charged polymer affected the enzyme microenvironment in this regard. The K_m value of the immobilized enzyme decreased from 1.0×10^{-3} M to 0.7×10^{-3} M using BAPNA as substrate. This decrease in K_m value is likely to be due to the fact that the local concentration of the substrate (with its positive charge) increases because of the negatively charged matrix near the immobilized enzyme.

The polymer-enzyme conjugate was used to hydrolyze milk casein. The biocatalyst could be

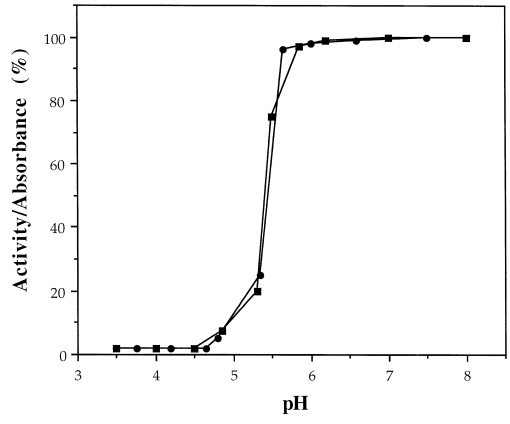

Fig. 2. Effect of pH on a soluble–insoluble form of (-■-) Eudragit S-100 and (-●-) Eudragit–trypsin conjugate. Eudragit and Eudragit–trypsin was dissolved in 0.05 M Tris–Hcl buffer (pH 8.0) and the pH of the solution was adjusted to a specified value by the addition of 2.0 M acetic acid. The solution was centrifuged and activity was determined in the supernatant using BAPNA as substrate. Activity at pH 8.0 was taken as 100%. In case of Eudragit alone, absorbance at 280 nm was measured to estimate unprecipitated Eudragit. This was expressed in percentage and 100% was taken as the absorbance of 2% (w/v) Eudragit solution at pH 8.0.

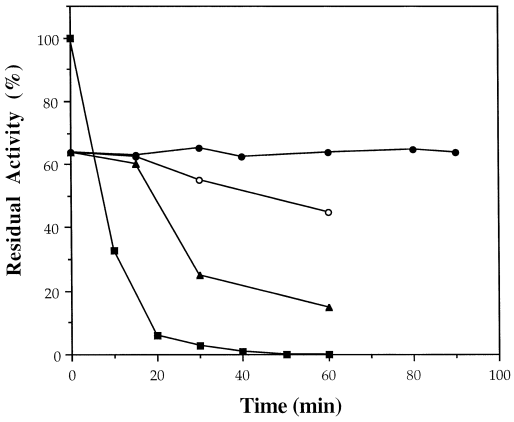

Fig. 3. Autolysis of native and immobilized trypsin. Native and immobilized trypsin in 0.05 M Tris–HCl, pH 8.0 were incubated at different temperatures; (-■-) native trypsin at 45°C; (-●-) immobilized trypsin at 45°C; (-○-) 50°C and (-▲-) 60°C. An aliquot of 0.6 ml of the enzyme was taken at regular intervals and assayed for enzyme activity.

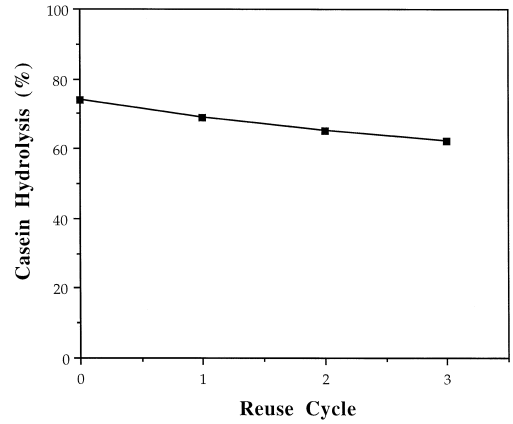

Fig. 5. Reuse of Eudragit–trypsin conjugate for the hydrolysis of 1% (w/v) casein. The hydrolysis of casein (pH 8.0) was carried out at 45°C and the recovered enzyme was reused for the three cycles.

reused successfully, although it showed 12% losses in activity upon 3 reuse cycles (Fig. 5). Similar losses in activity during recycling have been observed by others [5,7] but the cause of this loss have not been generally investigated. We believe that this is perhaps mostly due to less than 100% precipitation of the polymer in each centrifugation step. This problem has been discussed elsewhere [19]. The data shown in Fig. 2 also shows that the polymer cannot be recovered quantitatively. The loss of activity at each precipitation as shown in Fig. 1 works out to be about 1–1.5%. Taking into account two precipitation steps (one for recovering enzyme conjugate and another for TCA removal) during each reuse cycle of casein hydrolysis, the cumulative loss of 12% activity (Fig. 5) is almost well accounted for.

In the system described here, the undigested casein precipitates along with the biocatalyst and is again present in the next cycle along with the freshly added casein solution. As also pointed out by other workers [7], such a situation is in fact desirable and leads to more efficient catalysis as compared to the removal of residual substrate before each cycle.

Earlier, the conjugation of papain with Eudragit S-100 for obtaining a reusable soluble–insoluble protease have been described [20]. The immobilized papain showed only 40% of the activity of the bound enzyme. These workers also conjugated trypsin, but to another polymer, MPM-06. In that case, the immobilized enzyme showed very similar expressed activity as ours (70% of the bound enzyme). The focus of that work was on the use of such conjugates for peptide synthesis in heterogenous reaction system containing water miscible organic sol-

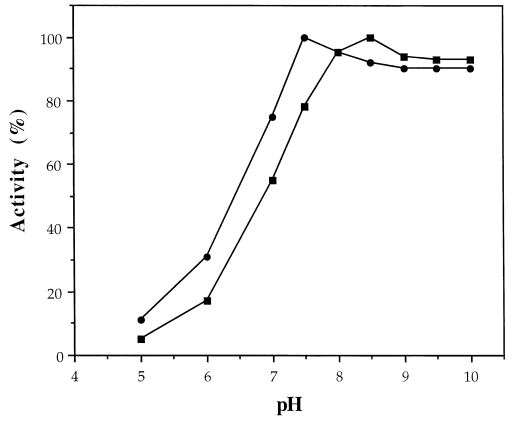

Fig. 4. Effect of pH on enzyme activity. (-■-) Native trypsin and (-●-) Eudragit–trypsin. Buffers used were 0.1 M acetate (from pH 4.0–6.0); 0.05 M Tris–maleate (from pH 6.0–8.0) and 0.05 M Tris–HCl (from pH 8.0–10). All buffers contain 0.02 M $CaCl_2$. Activity was determined at 25°C using BAPNA as substrate.

vent, although activities of immobilized papain towards casein and haemoglobin were reported. This work on the other hand focuses on evaluation of an Eudragit–trypsin preparation for hydrolysis of casein. The useful features of this approach is that the polymer component in the bioconjugate has several attractive features which favours its use as an immobilization matrix for enzymes/proteins. It is nontoxic (safe for use in food processing industries), water soluble, recoverable from solution by altering pH, economical, and commercially available [21].

While we have treated casein hydrolysis as a model system, it is felt that results with this system may be useful in the development of a general approach for protein hydrolysis for application in food and feed industry [22,23].

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