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IMMOBILIZED CHYMOTRYPSIN BY MEANS OF SCHIFF BASE COPPER(II) CHELATE

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SUMMARY: A new method for cross-linking of protein was proposed in our previous work. The method is based on the spontaneous chelate formation process involving three components, salicylaldehyde moiety, α-amino acid residue and copper(II). In this paper versatility of the method as a purpose of immobilization of enzyme was described. Chymotrypsin-salicylaldehyde conjugate was immobilized to the agarose gel attached α-amino acid residue in the presence of copper(II) ion. The enzyme was not eluted from the gel by washing with a copper free buffer though it was exclusively eluted by a medium containing EDTA. Catalytic activity of the chymotrypsin salicylaldehyde conjugate was not changed upon the immobilization. The method was proposed as a new tool for reversible immobilization of enzyme.

Immobilization and intermolecular cross-linking reactions of protein are applied in a wide range of biochemical research. However, it is generally difficult to attain site-specific and efficient cross-linking reaction of proteins. In the search of a new methodology for intermolecular cross-linking and immobilization reactions we are interested in a spontaneous chelate formation process. It is known that the Schiff base formed from α-amino acid and salicylaldehyde is dissociated into its original components in aqueous media (1) but it affords highly stable copper(II) chelate of which dissociation constant is as small as 10^{-15} M (2). Therefore it is expected that two macromolecules which are labelled with either salicylaldehyde or α-amino acid residue separately could be cross-linked one another *in situ* by the addition of copper(II) ion though they are present independently in a copper free media. Furthermore, the cross-linking will be cancelled by subsequent addition of ethylenediaminetetraacetate (EDTA). In our previous report (3), the validity of this strategy was proved by an analysis of chromatographic behavior of one ligand to the other ligand attached to agarose gel. In the present study the method was further extended for preparation of immobilized enzyme.

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MATERIALS AND METHODS

Materials : Crystalline bovine chymotrypsinogen A (Code CG) and crystalline bovine trypsin (Code TRL) were purchased from Worthington Biochemical Corp. N^{α}-Benzoyl-L-tyrosine-*p*-nitroanilide(BTNA) was purchased from Peptide Institute Inc.(Osaka, Japan). Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chem. Co. Sepharose lysine conjugate (N^{ε}-Lys-Sepharose) which contains 3.7 μmol of lysine per ml of wet gel was prepared as reported previously(3). N-Succinimidyl 3-formyl-4-hydroxybenzoate was prepared as reported(3).

Preparation of chymotrypsin-salicylaldehyde conjugate: Chymotrypsinogen A 20 mg (0.8 μmol) was dissolved in 3 ml of 0.1 M sodium bicarbonate (pH 8.3), and a solution of N-succinimidyl 3-formyl-4-hydroxybenzoate (6.3 mg) in 0.2 ml of dioxane was added and kept at 0°C for 5 hr. Resulted precipitation was removed by centrifugation and the supernatant was applied to Sephadex G-25 column (2.2 x 20 cm) using 10 mM ammonium bicarbonate (pH 8.0) as an eluent. Protein fraction was dialyzed against 10 mM ammonium bicarbonate (pH 8.0), subsequently 1 mM HCl, and lyophilized. Introduction of 2.0 mol of 3-formyl-4-hydroxybenzoyl group per mol of enzyme was analyzed from the colorimetric method using 2,4-dinitrophenylhydrazine(4). Enzyme concentration was determined by BCA protein assay reagent using native chymotrypsinogen as a standard(5).

Activationof the modified chymotrypsinogen was carried out using trypsin (1/50 equivalent of chymotrypsinogen). Both the proteins were dissolved in a medium containing 20 mM $CaCl_2$ and kept at pH 7.5, 0°C for 90 min. After adjusting the pH to 4 by addition of 0.1 M HCl , the reaction mixture was subjected to CM-cellulose chromatography following the method of Wilcox(6). Obtained trypsin-free chymotrypsin preparation was dialyzed against 1 mM HCl and lyophilized. Catalytic activity of the modified enzyme was measured spectrophotometrically using N^{α}-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) as a substrate in 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$ and 10 % dimethylsulfoxide at 25°C. Absorbance change at 385 nm due to the liberation of *p*-nitroaniline was monitored. Enzyme concentration was determined by the titration with N-trans-cinnamoylimidazole(7).

Immobilization of chymotrypsin: N^{ε}-Lys-Sepharose (10 ml, containing 37 μmol of lysine) was equilibrated with 50 mM BES buffer (pH 7.0) in a vial tube. Copper(II) acetate (0.2 mg, 1 μmol) and modified chymotrypsin (0.8 mg, 32 nmol) were added to the gel and the vessel was rotated gently for 24 hr at 4°C. The gel was transferred into a column (1.0 x 12.7 cm) and washed with copper free 50 mM BES buffer (pH 7.0). Amountof chymotrypsin immobilized to the gel was estimated by amino acid analysis of hydrolysate of the gel. Hydrolysis of the gel was performed in 6 M HCl at 110°C for 24 hr, and the hydrolysate was subjected to Hitachi-835 amino acid analyzer using norleucine as a standard. It was determined that the wet gel 1 ml contains 2.7 nmol of chymotrypsin.

Catalysis by immobilized chymotrypsin

Batchwise method: After the enzyme bound gel was washed with 50 mM Tris buffer(pH 8.0) containing 20 mM $CaCl_2$, the gel (1ml, containing 2.7 nmol of chymotrypsin) was transferred in a vessel containing 0.8 ml of Tris buffer and 0.2 ml of BTNA solution (1.7 mM in dimethylsulfoxide). The vessel was rotated (65 rpm) for 10 min at 25°C. After addition of 1 ml of acetic acid-dioxane mixture (3:7), the gel was filtered off. Optical density at 385 nm of the filtrate was measured.

Continuous method: Immobilized enzyme was packed in a column (1.3 x 2.3 cm) and 0.2 mM solution of BTNA in 50 mM Tris (pH 8.0) containing 20 mM $CaCl_2$ was passed through the column at 25°C. Optical density at 385 nm of the eluent was measured.

RESULTS AND DISCUSSION

It is known that the N-terminus residue of chymotrypsin is important for its catalytic activity(8). In order to avoid possible modification at the N-terminus of the enzyme, chymotrypsinogen was subjected to the modification reaction and subsequently activated. Introduction of single salicylaldehyde residue per mol of enzyme is sufficient for the purpose. However, the modification condition was selected to afford modified enzyme containing about 2 salicylaldehyde residues with a view to excluding the possible contamination of unmodified enzyme.

Conversion of chymotrypsinogen to chymotrypsin was performed by the action of trypsin. The introduced salicylaldehyde residues will be kept in the resulting chymotrypsin molecule, since the activation process does not accompany the liberation of peptide fragments which contain lysine residue(9).

Kinetic parameters for the hydrolyses of BTNA catalyzed by native and modified chymotrypsin were shown in Table 1. Catalytic property of the modified chymotrypsin is similar to that of native chymotrypsin at pH 8.0. However, activity-pH profile showed that optimum pH of the modified enzyme shifted about 0.5 pH unit to the higher region (Figure 1).

Immobilization of the modified enzyme was carried out using N^{ε}-Lys-Sepharose which was reported in our previous work (3). The modified chymotrypsin was incubated with the gel in the presence of copper(II) ion. The gel was transferred into a column and elution profile of the modified chymotrypsin was analyzed. As a control experiment, incubation of the gel with unmodified enzyme was carried out and the gel was subjected

Table 1. Kinetic parameters for native, modified and immobilized chymotrypsin[a]

Chymotrypsin	Km (M)	k_{cat} (min^{-1})	k_{cat} / Km ($min^{-1}M^{-1}$)
Native	8.65×10^{-5}	19.8	2.29×10^{5}
Modified	7.08×10^{-5}	16.5	2.33×10^{5}
Immobilized	11.5×10^{-5}	18.9	1.64×10^{5}

[a] Analysis was carried out in 50 mM Tris buffer (pH 8.0) containing 20 mM $CaCl_2$ and 10 % dimethylsulfoxide at 25°C using N^{α}-Benzoyl-L-tyrosine-*p*-nitroanilide(BTNA) as a substrate. [E] : 1.4 μM , [S] : 10 - 100 μM .

to the same chromatographic procedure. As shown in Figure 2, the modified enzyme was trapped in the column and was not liberated even after the exhaustive washing with copper free buffer. Addition of EDTA to the buffer caused complete elution of the trapped enzyme. In the case of native chymotrypsin, however, no adsorption was observed. No adsorption was also observed for the modified chymotrypsin with the gel in the absence of copper.

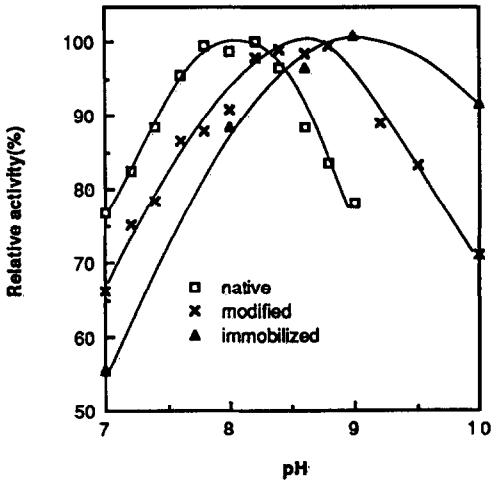

Figure 1. Activity-pH profiles of native, modified and immobilized chymotrypsin samples. Catalytic activities were determined in 50 mM Tris-20 mM $CaCl_2$ containing 10 % dimethylsulfoxide, at 25°C using BTNA(0.17 mM) as a substrate. Activities relative to that at the optimum pH were plotted for native (□), modified (×) and immobilized enzymes(▲).

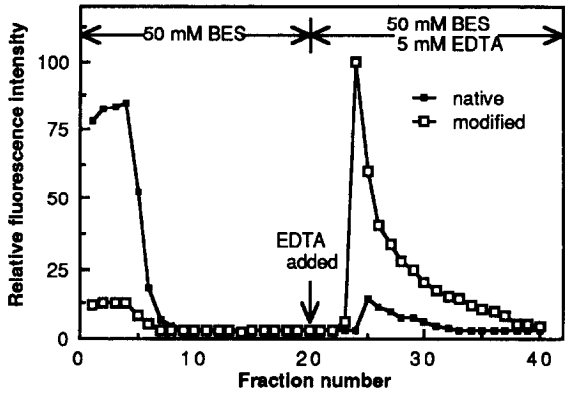

Figure 2. Elution diagrams of salicylaldehyde-chymotrypsin conjugate (□)and native chymotrypsin(■) from the lysine attached Sepharose column (1.0 x 12.7 cm) with 50 mM BES buffer (pH 7.0) at 25°C. The conjugate was preincubated with the gel in the presence (0.1mM) of copper(II) acetate. Aliquot of each fraction (3 ml) was reacted with fluorescamine and the fluorescence intensity at 475 nm (excitation at 390 nm) was plotted. After the elution with 60 ml of BES buffer (6 times of the column volume) EDTA was added to the buffer (final concentration, 5 mM).

Achievement of efficient contact of reactants is a prerequisite for the kinetic analysis of reaction in a heterogeneous solution. Under the batchwise reaction condition used, catalytic rate which was not influenced by the mixing efficiency was determined as shown in Table 1. The observed catalytic rate was not changed when the reaction vessel was rotated with higher speed. The immobilization did not cause a major change of the catalytic activity. It is noted that the optimum pH was shifted to the higher region and activity at the region around pH 10 was considerably enhanced(Figure 1). The activity at the higher pH region is exceedingly high if compared to that of native chymotrypsin. Possibly this will be explained to be due to the anionic nature of the modified gel at the alkaline pH region(10).

Continuous reaction of hydrolysis of the substrate was studied. Into a column of immobilized chymotrypsin, BTNA solution was passed and the effluent was monitored spectrophotometrically. Extents of the hydrolysis are dependent on the flow rate as shown in Figure 3. The extents correspond to 43 and 14 % of the substrate with flow rates 18 ml per hr and 58 ml per hr, respectively. Complete hydrolysis of the substrate will be readily attained by using longer column or slower flow rate. Stability of

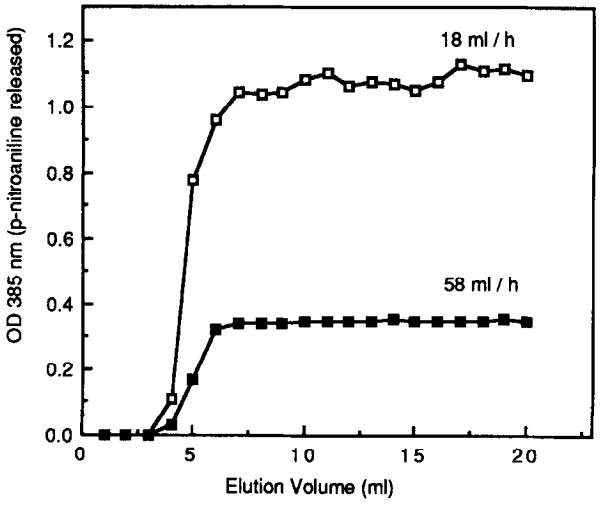

Figure 3. Hydrolysis of BTNA in the continuous flow system at 25°C. Solution of BTNA (0.2 mM) was continuously introduced into a column packed with immobilized chymotrypsin (1.3 x 2.3 cm, 8.2 nmol of chymotrypsin) and optical density of eluent was monitored. Buffer used was 50 mM Tris-20 mM $CaCl_2$ (pH 8.0) containing 10 % dimethylsulfoxide. Flow rate: 18 ml per hr (□) and 58 ml per hr (■).

the immobilized enzyme was also studied. It was analyzed that the enzyme column remained 72 % of that of original activity after keeping in Tris buffer (pH 8.0) at room temperature for 10 days.

It will be concluded that the proposed procedure will provide a new method for immobilization. It should be noted that protein molecules themselves contain neither of salicylaldehyde nor α-amino acid residue. Thus specific cross-linking will be taking place only between introduced ligand residues. Substitution of copper to zinc, nickel and cobalt afforded less stabilized chelate.

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