Introduction of γ-Glutamyl Residue into Chymotrypsin and Agarose Gel: Application to Cross-Linking and Immobilization of Protein

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In our previous work a new method for the cross-linking of protein was proposed. The method is based on the spontaneous chelate formation process between salicylaldehyde and α-amino acid residues. Thus, the facile procedure for the introduction of these residues into protein is required. In this paper, a modification reagnent which affords γ-glutamylation products, i.e., introducing an α-amino acid functional group to the protein was proposed. Versatility of the reagent for the preparation of a cross-linked enzyme was examined.

Keywords cross-linking; immobilization; chelate; γ-glutamylation; salicylideneglutamate; chymotrypsin; carboxypeptidase

The intermolecular cross-linking reaction of protein has gained increasing importance in biochemical research fields. Development of an immobilized enzyme and enzyme immunoassay, for example, are indebted to the reaction. In the search for new methodology for intermolecular cross-linking we are interested in the spontaneous chelate formation process. It is known that Schiff base formed from α-amino acid and salicylaldehyde is dissociated into its original components in an aqueous media,[1] but it affords a highly stable copper (II) chelate of which the dissociation constant is as small as 10^{-15} M.[2] Therefore, two macromolecules which are labelled with either salicylaldehyde or α-amino acid residue will be present independently in a copper free media, and they could be spontaneously cross-linked to one another by the addition of copper (II) ion through a carbon-nitrogen double bond (Schiff base formation). Furthermore, the cross-linking will be cancelled by the subsequent addition of ethylenediamine-tetraacetate (EDTA). This strategy was proved successful in our previous works.[3] Thus, the efficient method for the introduction of salicylaldehyde or α-amino acid residue into protein was required, and in our previous paper the introduction of the salicylaldehyde group was carried out using *N*-succinimidyl 3-formyl-4-hydroxybenzoate.[3] In the present work, design of a modification reagent introducing an α-amino acid functional group into protein was carried out.

Result and Discussion

γ-Succinimidyl *N*-Salicylideneglutamate (2) as γ-Glutamylation Reagent Introduction of an α-amino acid functional group into protein was carried out using acidic α-amino acid in which the α-functional group was protected. It is known that various α-amino acids afford crystalline salicylidene copper complexes.[4] They are well characterized compounds and are soluble in aprotic organic solvents. On this basis we synthesized salicylideneglutamate (**1**) following the reported procedure.[4] The resulting greenish crystals were dissolved in dioxane (2% solution) and were reacted with *N*-hydroxysuccinimide and dicyclohexyl-carbodiimide. Crystalline γ-succinimidyl *N*-salicylideneglutamate (**2**) was obtained in an excellent yield (93%).

The reagent (**2**) is expected to be most reactive toward amino groups of chymotrypsin. It is known that the N-terminus residue of chymotrypsin, Ile-16, is important for its catalytic activity.[5] Therefore, the *N*-terminus residue must be protected from the modification reaction. Chymotrypsinogen was subjected to the modification reaction and subsequently activated by trypsin since the *N*-terminus of chymotrypsin is produced by the tryptic cleavage of chymotrypsinogen. Chymotrypsinogen was modified with γ-succinimidyl *N*-salicylideneglutamate at pH 6.9 for 3 h. The solution was adjusted to pH 10 and incubated with hydroxylamine. Possible modification of the tyrosine residues was cancelled by the procedure since the *O*-acyl groups on the tyrosine residue would be easily removed under the conditions. After the addition of EDTA, the solution was gel-filtered and lyophilized. The resulting modified chymotrypsinogen was converted to the active enzyme by the trypsin action. The catalytic activity of the resulting modified chymotrypsin was identical to that of the original enzyme as shown in Table I. The amount of glutamic acid introduced into the chymotrypsin was determined by the amino acid analysis of the hydrolysate of the modified chymotrypsin as listed in Table I. Modification was carried out using 3.0 and 5.6 mol eq of the reagent to chymotrypsinogen which has 14 lysine residues. The mean numbers of the residue introduced, 0.6 and 3.8, reflect that very effective modification reactions were accomplished, since the reaction yields were 20 and 68% on the basis of the reagent.

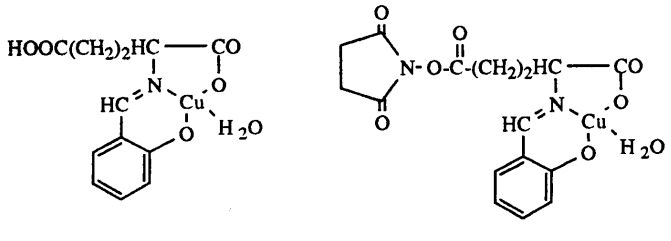

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Chart 1

TABLE I. Characterization of Modified Chymotrypsin

Reagent used[a] (mol eq)	Introduced glutamyl residues per mol enzyme[b]	Relative catalytic activity[c] (%)
3.0	0.6	101
5.6	3.8	99

a) Mol eq of reagent (**2**) to chymotrypsinogen used in the modification reaction. *b*) Determined by amino acid analysis. *c*) Catalytic activity relative to that of native chymotrypsin. Hydrolysis of benzoyl-L-tyrosine *p*-nitroanilide was analyzed at pH 8.0.

γ-Succinimidyl *N*-salicylideneglutamate was also shown to be useful for the preparation of the insoluble matrix attached glutamic acid through its γ-carboxyl group. Aminohexyl-Sepharose 4B (Pharmacia) was treated with an excess amount of the reagent at pH 8 and subsequently treated with EDTA solution. The procedure afforded the gel contained a sufficient amount of the ligand (8.15 μmol per ml wet gel). The amount of the ligand, α-amino acid residue, was determined based on the amount of salicylaldehyde released by EDTA treatment.

Chromatographic Behavior of Salicylaldehyde in a Column of Sepharose Attached α-Amino Acid Residue Stability of Schiff base copper chelate formed from salicylaldehyde and glutamic acid derivative was analyzed using the glutamic acid-gel conjugate. To a suspension of 10 ml of wet gel (containing 81.5 μmol of α-amino acid ligand) in 6 ml of 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (pH 7.0), salicylaldehyde (8.15 μmol) and copper (II) acetate (81.5 μmol) were added. After incubating at 25 °C for 24 h, the gel was transferred to a column and washed with 50 mM BES buffer (pH 7.0) and subsequently with 100 mM acetate buffer (pH 4.0). As shown in Fig. 1 no salicylaldehyde was eluted. Salicylaldehyde trapped in the column was eluted immediately after the eluent was changed to 100 mM acetate buffer containing 5 mM EDTA (pH 4.0). In Fig. 1 the elution pattern of the gel-salicylaldehyde incubated in the absence of copper ion was also shown. The elution experiment was also carried out using a Sepharose column (no glutamic acid ligand) under the presence of copper ion. No holding of salicylaldehyde on the column was observed.

Preparation of Carboxypeptidase Attached Salicylaldehyde Residue and Its Interaction with the Modified Chymotrypsin Introduction of salicylaldehyde residue into carboxypeptidase was carried out using *N*-succinimidyl 3-formyl-4-hydroxybenzoate.[3] Treatment of carboxypeptidase A with 100 eq of the reagent in a phosphate buffer (pH 7.5) resulted in the incorporation of 1.2 residue per mol enzyme. Catalytic activity of the modified enzyme was nearly identical to that of the unmodified enzyme.

Cross-linking between glutamyl-chymotrypsin and 3-formyl-4-hydroxybenzoyl-carboxypeptidase was examined next. Equimolar amounts of the modified chymotrypsin (3.8 residues of glutamic acid per mol of enzyme) and the modified carboxypeptidase (1.2 residues of salicylaldehyde per mol enzyme) were dissolved in 50 mM BES (pH 7.5). The solutions in three different concentrations (0.5, 1 and 5 μM for the respective enzyme) were prepared, and none of them afforded precipitation. Electrophoretic analysis of each sample on sodium dodecyl sulfate (SDS)-polyacrylamide gel showed two bands corresponding to chymotrypsin and carboxypeptidase. The addition of copper ion (20 molar excess) to the enzyme solution resulted in the exclusive formation of precipitation for both the 1 and 5 μM solutions. Only in the case of the 0.5 μM solution, a detectable amount of protein component remained in the medium though precipitation occurred as well. In Fig. 2 an electrophoretic pattern of the soluble fraction of the 0.5 μM solution was shown. Besides bands at 25 and 34 kDa which correspond to the modified chymotrypsin and carboxypeptidase, a weak band was detected at the region near 59 kDa. The band is assumed to correspond to the chymotrypsin-carboxypeptidase complex. The sample prepared from the modified chymotrypsin and carboxypeptidase in the absence of copper did not show any band around the region as shown in Fig. 2.

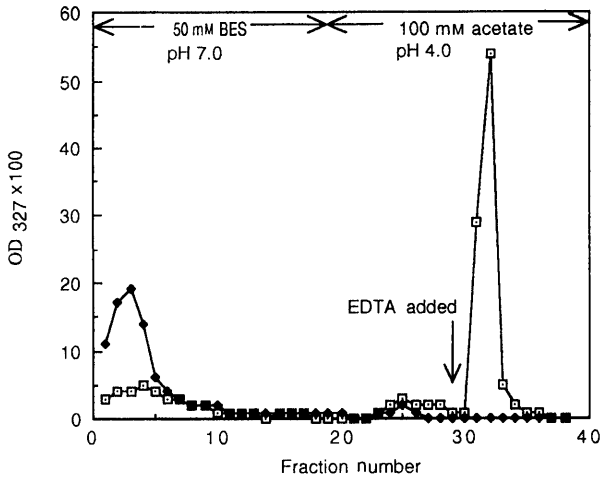

Fig. 1. Elution Diagram of Salicylaldehyde with Glutamic Acid Attached Sepharose Column

Salicylaldehyde was preincubated with the gel in the presence (□) and absence (◆) of copper acetate. Absorbance at 327 nm of each fraction (3 ml) *vs.* fraction number was plotted.

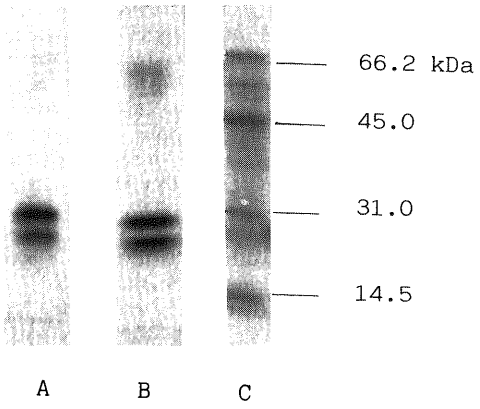

Fig. 2. SDS-Polyacrylamide Gel Electrophoretic Pattern of Modified Enzymes

A mixture of the modified chymotrypsin and the modified carboxypeptidase was applied to electrophoresis; lane A. Modified chymotrypsin (0.5 μM) and modified carboxypeptidase (0.5 μM) were mixed in the presence of 10 μM copper ion. The resulting precipitate was removed by centrifugation and the soluble fraction was applied to electrophoresis; lane B. A standard protein mixture; lane C.

The precipitation was solubilized with 0.1% SDS and subjected to SDS-polyacrylamide gel electrophoresis. It was shown that the precipitate was composed of an equimolar amount of chymotrypsin and carboxypeptidase.

Now it may be concluded that cross-linking is successful though the formation of precipitate is unexpected. It is attributed to the formation of a dimeric complex which has very low solubility. Since the number of ligands attached to the chymotrypsin is as large as 3.8, formation of a polymeric complex may be possible to some extent.

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). *N*-Carbobenzoxy-glycyl-

L-phenylalanine was synthesized as reported.[6] *N*-Succinimidyl 3-formyl-4-hydroxybenzoate was prepared as reported.[3]

Synthesis of γ-Succinimidyl *N*-Salicylideneglutamate *N*-Salicylideneglutamic acid was synthesized following the reported procedure.[4] *N*-Salicylideneglutamic acid (385 mg, 1.0 mmol) and *N*-hydroxysuccinimide (115 mg, 1.0 mmol) were dissolved in 20 ml of dioxane. To this solution, *N*,*N*-dicyclohexylcarbodiimide (DCC) (1.0 g, 5.0 mmol) was added and the reaction mixture was stirred at 10 °C for 1 h and at 25 °C for 12 h. Excess DCC was deactivated by the addition of 50% aqueous acetic acid. The precipitated dicyclohexylurea was filtered off and the solvent was evaporated under reduced pressure. Recrystallization from methanol yielded 397 mg of a green powder, mp 189—191 °C, 93% yield. *Anal.* Calcd for $C_{16}H_{16}CuN_{20}O_6$: C, 44.91; H, 3.77; N, 6.55. Found: C, 45.05; H, 3.55; N, 6.33.

Preparation of γ-Glutamyl Chymotrypsin To an ice-cold solution of chymotrypsinogen A (40 mg, 1.6 μmol) in 3 ml of water (pH 6.9), γ-succinimidyl *N*-salicylideneglutamate (3.7 mg, 8.7 μmol) was added with stirring. The pH was maintained at 6.9 by the addition of 1 mM sodium hydroxide solution and the temperature was kept at 0 °C for 3 h. The pH of the reaction mixture was readjusted to 10 and 0.5 ml of 0.1 M hydroxylamine solution was added. After remaining at 0 °C for 30 min, 0.2 ml of 0.1 M EDTA was added and the pH was adjusted to 4.0. The solution was gel-filtered (Sephadex G-25, 1 mM HCl) and lyophilized.

The modified chymotrypsinogen was dissolved in 20 mM of calcium chloride solution (pH 7.5) and incubated with 2% w/w of trypsin for 90 min. The solution was adjusted to pH 4.0 and subjected to carboxymethyl (CM)-cellulose chromatography following the reported method.[7] The protein fraction was dialyzed against 1 mM HCl and lyophilized. The amount of the glutamyl residue introduced was estimated from the amino acid analysis of the hydrolysate of the modified chymotrypsin. The lyophilized modified chymotrypsin was hydrolyzed in 6 M HCl at 110 °C for 24 h and subjected to amino acid analysis (Hitachi-835). Catalytic activity of the modified chymotrypsin was determined using benzoyl-L-tyrosine *p*-nitroanilide as a substrate following the method of Bundy.[8]

Preparation of Gel Attached α-Amino Acid Residue AH-Sepharose 4B (Pharmacia) 12 ml was suspended in 0.1 M bicarbonate buffer (pH 8.0). The suspension was placed in a vial tube and γ-succinimidyl *N*-salicylideneglutamate 257 mg (0.6 mmol) was added. The vessel was rotated slowly (about 60 rpm) at 25 °C for 18 h. The gel was washed with 0.05 M acetate buffer (pH 4.0) and 0.05 M Tris buffer (pH 8.0). Then 60 ml of 0.02 M EDTA solution was added and the vessel was rotated at room temperature for 1 h. The gel was washed with 0.05 M acetate buffer (pH 4.0), 0.05 M Tris (pH 8.0) and finally with water. The amount of α-amino acid residue introduced into the gel was determined based on the amount of salicylaldehyde liberated by the addition of EDTA taking $\varepsilon_{327\,nm} = 3700$.[9]

Analysis of Chromatographic Behavior of Salicylaldehyde with Gel Attached α-Amino Acid Residue Gel (wet volume 10 ml, 81.5 mmol of α-amino acid residue) was placed in a vial tube and was suspended in 6 ml of 50 mM BES buffer (pH 7.0). To this suspension, salicylaldehyde (8.15 mmol) and copper acetate (81.5 mmol) was added. The vessel was rotated for 24 h at room temperature. The gel was transferred to a column (1.2 × 9.0 cm) and 50 mM BES buffer was passed through the column. After passing BES buffer, the medium was changed to 100 mM acetate (pH 4.0). Salicylaldehyde trapped in the column was eluted by an acetate buffer containing 5 mM EDTA (pH 4.0). The flow rate was maintained at 20 ml per hour. The concentration of salicylaldehyde was determined spectrometrically taking $\varepsilon_{327\,nm} = 3700$ or the colorimetric method using 2,4-dinitrophenylhydrazine.[9]

Preparation of Carboxypeptidase A Attached Salicylaldehyde Residue Carboxypeptidase A (6.9 mg, 0.2 μmol) was dissolved in 3 ml of 5 mM phosphate buffer containing 1 M NaCl (pH 7.5). To this solution, *N*-succinimidyl 3-formyl-4-hydroxybenzoate[3] (5 mg, 20 μmol) was added and the reaction mixture was incubated for 1.5 h at 0 °C. The formed precipitate was removed by centrifugation and the supernatant was gel-filtered (Sephadex G-25, 5 mM phosphate containing 1 M NaCl, pH 7.5) at 4 °C. The enzyme concentration was determined by Bio-Rad protein assay reagent using native carboxypeptidase as a standard.[10] The amount of introduced salicylaldehyde residue was estimated based on the colorimetric method using 2,4-dinitrophenylhydrazine. The catalytic activity of the modified enzyme was measured using *N*-carbobenzoxy-glycyl-L-phenylalanine as a substrate.[11]

Cross-Linking Between Chymotrypsin and Carboxypeptidase Both the modified chymotrypsin (37.5 μg, 1.5 nmol) and carboxypeptidase A (51.0 μg, 1.5 nmol) were dissolved in 3 ml of 50 mM BES buffer, 0.5 M NaCl (pH 7.5) at 0 °C. To this solution, copper acetate (6.0 μg, 30 nmol) was added and the reaction mixture was kept at 4 °C for 24 h.

Analysis of Cross-Linked Enzyme with SDS-Polyacrylamide Gel Electrophoresis Electrophoresis in SDS was performed by the method of Laemmli.[12] The gel was stained by the silver staining method described by Merril *et al.*[13]

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