Schiff Base Copper(II) Chelate as a Tool for Immobilization of Protein

Katsuhiro MORIYA, Kazutaka TANIZAWA,* and Yuichi KANAOKA

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan. *Received March 27, 1989*

In our previous report a new methodology for intermolecular cross-linking or bridging of protein utilizing a spontaneous chelate formation process was proposed. In this paper the reliability of the process as a tool for protein immobilization has been further evaluated. The chromatographic behavior of tryptophan in a column packed with Sepharose coupled with salicylaldehyde residue showed that the α-amino acid was bound tightly to the gel in the presence of copper(II) ion and was eluted by the addition of ethylenediaminetetraacetate (EDTA). It was also proved that subtilisin modified with an α-amino acid residue was immobilized on the column, and this binding was reversed by the addition of EDTA as well.

Keywords chelate; α-amino acid; salicylaldehyde; copper ion; ligand attached gel; chromatography; elution; modified subtilisin; protein immobilization

Immobilization and cross-linking reactions of proteins have gained increasing importance in biochemical research. These reactions are generally carried out by the use of divalent reagents which carry out in one step or two steps. In either case it is difficult to attain site-specific and efficient modification of proteins since the reaction unavoidably involves attack of a polymeric reactant on a polymeric substrate.

It is known that in aqueous media the Schiff base (III) is present predominantly in the hydrolyzed form, *i.e.*, salicylaldehyde (I) and α-amino acid (II).[1] It is also known that the Schiff base (III) and copper(II) ion can form a highly stable chelate (IV), of which the dissociation constant is as small as 10^{-15} M.[2] Therefore it is expected that two components I and II in aqueous media will be spontaneously bound by chelation by the addition of copper(II) ion. Furthermore, the addition of ethylenediaminetetraacetate (EDTA) is expected to reverse the process by extracting the copper(II) ion from IV. Consequently, a reversible intermolecular cross-linking system could be produced by using proteins modified with salicylaldehyde residues and with residues having an α-amino-α-carboxyl group.

In our previous work,[3] direct evidence for the intermolecular interaction of such a system was observed. The stability of IV was concluded to be very high based on the chromatographic behavior of salicylaldehyde with lysine residues covalently connected to Sepharose gel through the ε-amino group.

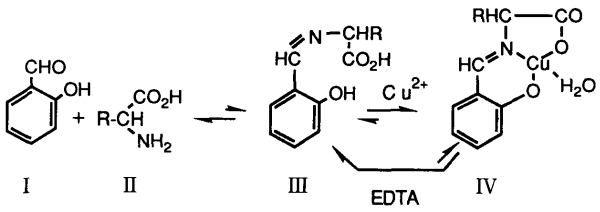

Fig. 1. Equilibrium of Schiff Base Copper(II) Chelate

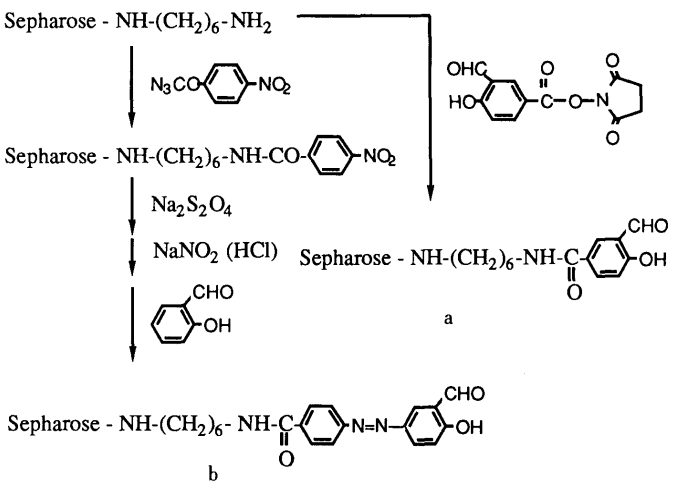

Fig. 2. Preparation of Sepharose Linked with Salicylaldehyde Residue

Results and Discussion

In order to verify the applicability of the process in Fig. 1 as a tool for the immobilization of protein, we analyzed the chromatographic behavior of one component when the other was attached to an immobilizing phase. For this purpose, agarose gels coupled with salicylaldehyde residues (a and b) were prepared. The synthetic routes are summarized in Fig. 2. Preparation of gel a was carried out by the reaction of activated ester of 3-formyl-4-hydroxybenzoic acid with AH-Sepharose. The procedure was successful for the introduction of a sufficient amount of salicylaldehyde residues onto the gel; the content was determined to be 12 μmol per ml of wet gel. Gel b was prepared by coupling of diazotized gel to salicylaldehyde following the reported procedure.[4] The content of salicylaldehyde residue in gel b was analyzed as 6.8 μmol per ml of wet gel.

The chromatographic behavior of α-amino acid with gel a was examined using L-tryptophan, which is spectrometrically detectable. As shown in Fig. 3, L-tryptophan was not eluted from the gel which was preincubated with tryptophan in the presence of copper(II) even after exhaustive washing, though a certain amount of tryptophan leaked out at the initial stage of the elution. L-Tryptophan adsorbed in the column was quantitatively recovered when the eluent was changed to the buffer containing 5 mM EDTA. The behavior of an incubate which was prepared in copper free buffer was quite different. In this case, all L-tryptophan in the incubate passed through the column. The result with gel b (data not shown) was identical to that with gel a except that the shape of the elution peak was broader in the case of gel b. The results were consistent with those of the previous work in which interaction of salicylaldehyde with L-lysine-

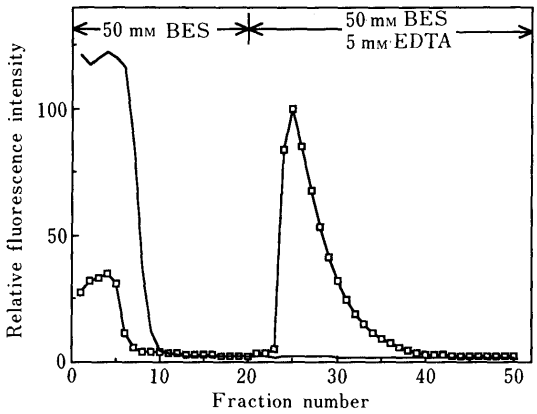

Fig. 3. Elution Profile of Tryptophan with Salicylaldehyde Sepharose Column

L-Tryptophan was preincubated with gel a at pH 7.0 in the presence of 0.4 mM copper (II) acetate. The incubate was transferred into a column, and the column was washed with the copper-free buffer. The elution profile of tryptophan from the gel preincubated in the absence of copper(II) was compared. —□—, Cu(+); ——, Cu(−).

Sepharose conjugate was examined.[3]

Applicability of the gel to large-molecular substances was next examined. Subtilisin BPN′ modified with an α-amino acid residue was prepared. The reaction process in Fig. 4 afforded modified subtilisin containing 1.5 mol of the α-amino acid moiety per mol of protein. The catalytic activity of the enzyme preparation was found to be 95% of that of the native enzyme. The chromatographic behavior of the resulting subtilisin conjugate in a column of gel a is shown in Fig. 5. More than 86% of the protein was trapped by the gel and it was completely recovered by the addition of EDTA as shown. However, the incubate which was prepared in the absence of copper(II) gave less satisfactory elution pattern. Some nonspecific adsorption was observed in this case. Such nonspecific adsorption was also found in the interaction of native subtilisin with gel a. Furthermore, other proteins such as chymotrypsin and trypsin were shown to be partially trapped by the gel in both the absence and presence of copper(II). A small ligand, L-lysine, showed no nonspecific adsorption. This observation suggests that the introduced salicylaldehyde groups may undergo nonspecific interaction such as stable Schiff base formation and hydrophobic bonding with protein. It was shown in Fig. 5 that the nonspecific adsorption was less in buffer of lower pH and higher ionic strength (100 mM acetate, pH 4.0).

It may be concluded that lysine-Sepharose is superior to gel a with respect to the specific interaction with macromolecular substances since lysine-Sepharose did not show nonspecific adsorption with salicylaldehyde–protein conjugate as reported in the previous work.[3]

In the present study, the stability of salicylidene copper(II) chelate has been evaluated using α-amino acid derivatives as a free ligand and salicylaldehyde group as an immobilized ligand. These results, together with those of the previous work, in which the roles of the two ligands were reversed, lead to the conclusion that the chelate formation process in Fig. 1 is a useful tool for cross-linking or immobilization of protein.

Experimental

Materials Subtilisin BPN′ was a gift from Prof. K. Hiromi of Kyoto

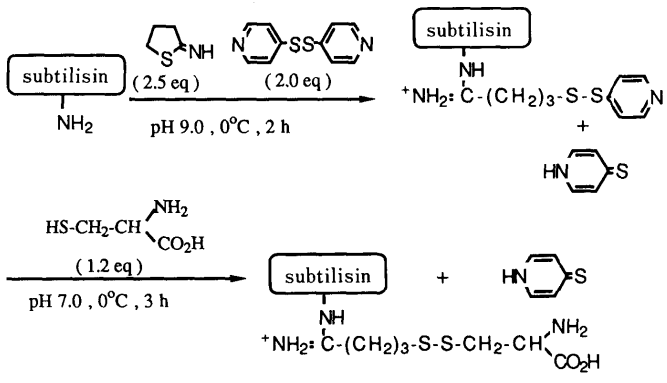

Fig. 4. Preparation of Modified Subtilisin BPN′

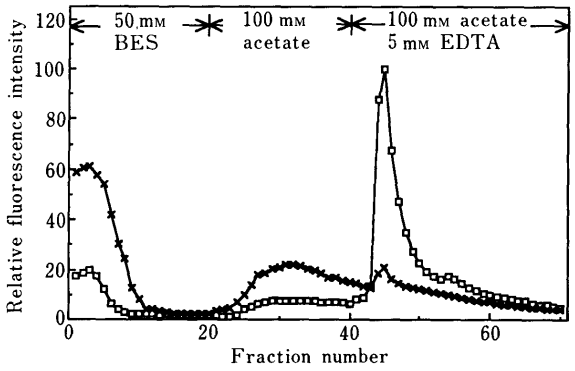

Fig. 5. Elution Profile of Modified Subtilisin BPN′ from a Salicylaldehyde-Sepharose Column

Chromatographic behavior was analyzed following the procedure used for the elution of tryptophan. —□—, Cu(+); —×—, Cu(−).

University. The concentration of the active enzyme was determined to be 80% by initial burst titration.[5] All the chemicals used were products of Nacalai Tesque Inc. (Kyoto, Japan).

Synthesis of *N*-Succinimidyl 3-Formyl-4-hydroxybenzoate 3-Formyl-4-hydroxybenzoic acid[6] (300 mg) and *N*-hydroxysuccinimide (210 mg) were dissolved in 10 ml of dioxane. To this solution, *N*,*N*-dicyclohexylcarbodiimide (DCC) (370 mg) was added and the solution was stirred at 25 °C for 24 h. The precipitated urea was filtered off and the solvent was evaporated off under reduced pressure. Recrystallization from ethanol gave 410 mg of pale yellow needles (87% yield), mp 167—168 °C. *Anal.* Calcd for $C_{12}H_9NO_6$: C, 54.76; H, 3.45; N, 5.32. Found: C, 54.67; H, 3.40; N, 5.25.

Preparation of Gels Coupled with Salicylaldehyde Residue Gel a: Wet AH-Sepharose 4B (Pharmacia) (10 ml) was washed with 0.5 M NaCl and water, and suspended in 0.1 M sodium bicarbonate (pH 8.3). To this suspension, a solution of 79 mg of *N*-succinimidyl 3-formyl-4-hydroxybenzoate in 3 ml of dioxane was added. The suspension was shaken gently at room temperature for 3 h, then the gel was washed with water, 0.1 M Tris (pH 8.0) containing 10% dioxane, 0.1 M acetate (pH 4.0) and water. The amount of the salicylaldehyde residue introduced into the gel was determined by analyzing salicylaldehyde in the washing with 2,4-dinitrophenylhydrazine according to the reported procedure.[7]

Gel b: AH-Sepharose 4B was reacted with *p*-nitrobenzoylazide. The resulting *p*-nitrobenzoylated gel was treated with sodium hydrosulfite and subsequently with sodium nitrite, and then coupled with salicylaldehyde according to the reported procedure.[4] The amount of salicylaldehyde residue in the gel was determined in the same manner as for gel a.

Preparation of Subtilisin BPN′ Modified with an α-Amino Acid Residue Subtilisin was modified according to the reported procedure.[8] To a solution of subtilisin (20 mg) in 2.5 ml of 50 mM borate buffer (pH 9.0), solutions of 2-iminothiolane hydrochloride (3.4 mg) in 0.2 ml of 50 mM borate (pH 9.0) and 4,4′-dithiodipyridine (4.4 mg) in 0.3 ml of acetone were added. After standing at 0 °C for 2 h, the reaction mixture was gel-filtered (Sephadex G-25, 10 mM ammonium acetate, pH 6.5) and lyophilized. The amount of dithiopyridine groups introduced was determined as follows. Lyophilized enzyme sample (0.16 mg) was dissolved in

3 ml of 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA. To this solution, 2-mercaptoethanol (5.3 μl) was added at room temperature. The increase of optical density at 324 nm due to the liberation of 4-thiopyridone was analyzed based on $\varepsilon 324 = 19800$. It was found that 2.1 mol of dithiopyridyl groups had been introduced per mol of enzyme. Enzyme concentration was determined with the BIO-RAD protein assay reagent using native subtilisin as a standard.

A solution of L-cysteine (1.1 μmol) in 110 μl of 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA was added to a solution of 12 mg of dithiopyridyl subtilisin in 3.4 ml of the same buffer. The reaction mixture was kept at 0 °C for 3 h, gel-filtered (Sephadex G-25, 10 mM ammonium acetate, pH 6.5) and lyophilized. During the course of reaction with L-cysteine, 2.0 mol of 4-thiopyridone per mol of enzyme was liberated. This reflects incorporation of 2.0 mol of cysteine per mol of enzyme. The value is in good accordance with that of cysteinic acid determined from amino acid analysis of the preparation after performic acid oxidation[9] and hydrolysis.

Analysis of Catalytic Activity of Modified Subtilisin BPN' Hydrolysis of a fluorogenic substrate, carbobenzyloxy-diglycyl-L-leucine methylcoumarylamide, was monitored according to the reported procedure.[10]

Analysis of Chromatographic Behavior of L-Tryptophan with Gel a Gel a (10 ml; 120 μmol of salicylaldehyde residue) was suspended in a solution of L-tryptophan (12 μmol) and copper acetate (12 μmol) in 10 ml of 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.0. The reaction mixture was incubated at 25 °C for 24 h. The gel and the medium were transferred to a column (1.0 × 12.7 cm) and the column was eluted with the buffer shown in Fig. 3. A flow rate of 20 ml per h was maintained throughout the experiment. For the control experiment, the incubate of L-tryptophan and the gel in the absence of copper was subjected to the same chromatographic procedure. Concentration of L-tryptophan in the eluate was determined from the absorption at 280 nm ($\varepsilon = 3700$) or fluorometrically ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 475$ nm).[11]

Analysis of Chromatographic Behavior of L-Tryptophan with Gel b Gel b (8.5 ml; 57.8 μmol of salicylaldehyde residue), L-tryptophan (2 μmol) and copper acetate (2 μmol) were used. The analysis was carried out following the same procedure as used for gel a.

Analysis of Chromatographic Behavior of Modified Subtilisin with Gel a Gel a 2.5 ml was diluted with 7.5 ml of Sepharose 4B prior to use. The diluted gel (10 ml; 30 μmol of salicylaldehyde residue) was suspended in a solution of 1.0 mg of the modified subtilisin (36 nmol) and copper acetate(1 μmol) in 10 ml of 50 mM BES, pH 7.0. The column was eluted with 50 mM BES, pH 7.0, and subsequently with 100 mM acetate, pH 4.0. The concentration of the protein in the eluate was determined based on the optical density at 280 nm or from the fluorescence intensity developed with fluorescamine.[11]

Acknowledgement This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture.

References

1) R. W. Green and E. L. Le Mesurier, *Aust. J. Chem.*, **19**, 229 (1966).
2) D. L. Leussing and K. S. Bai, *Anal. Chem.*, **40**, 575 (1968).
3) K. Moriya, K. Tanizawa, and Y. Kanaoka, *Biochem. Biophys. Res. Commun.*, **161**, 52 (1989).
4) P. Cuatrecasas, *J. Biol. Chem.*, **245**, 3059 (1970).
5) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
6) K. Reimer and F. Tiemann, *Ber.*, **9**, 1268 (1876).
7) N. Williams, Jr. and R. M. Jacobs, *Biochem. Biophys. Res. Commun.*, **22**, 695 (1966).
8) T. P. King, Y. Li, and L. Kochoumian, *Biochemistry*, **17**, 1499 (1978).
9) S. Moore, *J. Biol. Chem.*, **238**, 257 (1963).
10) Y. Kanaoka, T. Takahashi, H. Nakayama, and K. Tanizawa, *Chem. Pharm. Bull.*, **33**, 1721 (1985).
11) P. Böhlen, S. Stein, W. Dairman, and S. Udenfriend, *Arch. Biochem. Biophys.*, **155**, 213 (1973).