Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 52-58

SCHIFF BASE COPPER(II) CHELATE AS A TOOL FOR INTERMOLECULAR CROSS-LINKING AND IMMOBILIZATION OF PROTEIN

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Received March 10, 1989

SUMMARY: A new method for intermolecular cross-linking or bridging of protein has been proposed. The method is based on the spontaneous chelate formation process involving three components, salicylaldehyde, α-amino acid residue and copper(II). Reliability of the process as a tool for protein cross-linking was evaluated by chromatographic procedures. Behavior of salicylaldehyde in a column packed with Sepharose attached α-amino acid residue showed that salicylaldehyde was bound tightly to the gel in the presence of copper(II) ion and was eluted by the addition of EDTA. The association was shown strong enough to be applied for the purpose of cross-linking of proteins. It was also proved that BSA salicylaldehyde conjugate was immobilized specifically to the column, and the process was reversed by the addition of EDTA as well. The method is proposed to be useful not only for immobilization of enzyme but also for cross-linking of proteins since the method is free from unexpected random coupling products which are unavoidable with bifunctional cross-linking reagents.

Chemical modification reaction of protein such as intermolecular cross-linking and conjugation of small ligand has gained increasing importance in the current biochemical research field. The reaction is indispensable for the preparation of antigenic protein-hapten conjugate, for example. Development of enzyme immunoassay and immobilized enzyme is also indebted to the reaction. The reaction is generally attained by divalent reagents, which will be classified into two types, one step coupling and consecutive two steps modification. In either cases it is difficult to attain site-specific and efficient modification of proteins. Especially difficulty is anticipated in the cross-linking of proteins since it unavoidably involves the process attacking of a polymeric reactant to a polymeric substrate.

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Abbreviations : BSA, bovine serum albumin. EDTA, ethylenediaminetetraacetic acid di sodium salt. EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. DCC, N,N-dicyclohexylcarbodiimide. BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

0006-291X/89 $1.50

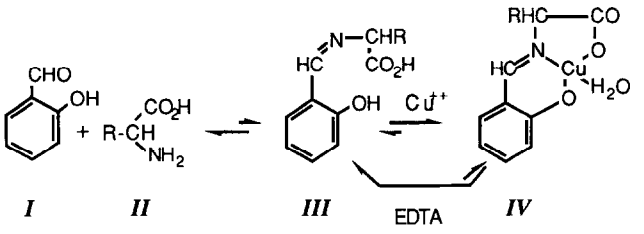

Figure 1. Equilibrium of Schiff base copper(II) chelete.

We are interested in very stable Schiff base copper(II) chelate shown in Figure 1. Salicylaldehyde(*I*) reacts with α-amino acid(*II*) to give Schiff base (*III*), but in aqueous media the dissociation constant of the resulting complex is so large that it dissociates into *I* and *II* (1). It was reported that the Schiff base (*III*) can form highly stable chelete (*IV*) of which dissociation constant is as small as 10^{-15} M (2). Therefore it is expected that two components *I* and *II* which are separated in aqueous media will be connected spontaneously by the addition of copper(II) ion. Furthermore the addition of ethylenediaminetetraacetate (EDTA) is expected to cause the disruption of chelate(*IV*) because of the extraction of copper (II) ion from *IV*. Our strategy is such that one protein modified with salicylaldehyde residue and another protein carrying α-amino acid residue can be used as a reversible cross-linking system. It is noted that protein molecules themselves contain neither of salicylaldehyde nor α-amino acid residue. Thus non specific cross-linking will not be taking place during the course of modification. Two protein components separated will be cross-linked *in situ* in aqueous media by the addition of copper(II) ion and will be separated again by the addition of EDTA.

Materials and Methods

Materials : BSA was obtained from Nacalai Tesque Inc.(lot M7T9542). All the chemicals were products of Nacalai Tesque Inc.(Kyoto, Japan).

Preparation of gels attached lysine residue : Gel ***a*** ; Sepharose 4B (20 ml) was activated by cyanogen bromide (7 g) following the reported procedure (3). Activated gel was added to a solution of copper lysinate (4)(40 mmol) in 40 ml of 0.1 M carbonate buffer (pH 10.0). After keeping at 25°C for 24 hr the gel was washed with water, 0.1 M EDTA(pH 7.0), 1M ethanolamine (pH 7.0), 0.1 M sodium bicarbonate (pH 8.3) containing 0.5 M NaCl, 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and finally water. Amount of lysine incorporated in the gel was estimated by amino acid analysis of the hydrolysate of the gel. Hydrolysis of gel was performed in 6 N HCl at 110°C for 24 hr, and the hydrolysate was subjected to Hitachi-835 amino acid analyzer using L-leucine as a standard. Gel ***b*** ; Activated gel 20 ml was added to a solution of ε-aminocaproic acid (40 mmol) in 40 ml of 0.5 M carbonate buffer (pH 10.0). After keeping at 25°C for 24 hr, the gel was washed with water, 1 M ethanolamine (pH 8.0), 0.1 M sodium bicarbonate (pH 8.3) containing 0.5 M NaCl, 0.1 M acetate (pH 4.0) containing 0.5 M NaCl and

finally water. Amino acid analysis of hydrolysate of the gel revealed that 2.7 μmol of ε-aminocaproic acid was incorporated per ml of wet gel. To a solution of copper lysinate (4 mmol) in 40 ml water was added 20 ml of the resulting gel and the pH was adjusted to 5.5. Solid EDC 8 mmol was added in two portions at 12 hr intervals keeping the pH at 5.5. After keeping at 25°C for 24 hr (total period), the gel was washed in a similar manner as gel ***a***.. Lysine content was determined as above. Gel ***c*** ; Sepharose 20 ml was treated with 1,4-butanediol diglycidyl ether 15 ml following the reported procedure (5). The resulting oxirane-containing gel was added to a solution of copper lysinate (0.6 mmol) in 30 ml of water, pH was adjusted to 11.0 and kept at 40°C for 18 hr. The gel was washed in a same manner as gel ***a***.. Amount of bound lysine was estimated by determining the amount of unreacted lysine in the washing.The washing was treated with fluorescamine and the concentration of lysine was determined fluorometrically following the reported procedure (6).

Analysis of chromatographic behavior of salicylaldehyde : Behavior of salicylaldehyde with gel ***a*** was analyzed as follows: Wet gel 10 ml (37 μ mol lysine) and salicylaldehyde (3.7 μ mol) were mixed with 10 ml of 50 mM BES buffer (pH 7.0) containing 0.4 m M copper(II) acetate in a vial tube and the vessel was rotated gently for 24 hr at 25°C. The gel was transferred to a column (1.0 x 12.7 cm) and 50 mM BES buffer (pH 7.0) without copper ion was passed through the column. After eluting with 60 ml of the buffer, the eluent was changed to the buffer containing 5 mM EDTA. For the control experiment an incubate of salicylaldehyde and the gel in the absence of copper was subjected to the same chromatographic procedure. Flow rate 20 ml per hour was maintained throughout the experiment. Concentration of salicylaldehyde was determined taking $\varepsilon_{327\,nm}$: $3700M^{-1}\,cm^{-1}$.

Synthesis of N-succinimidyl 3-formyl-4-hydroxybenzoate : 3-Formyl-4-hydroxybenzoic acid (7) 300 mg and N-hydroxysuccinimide 210 mg were dissolved in 10 ml of dioxane. To this solution DCC 370 mg was added and the solution was stirred at 25°C for 24 hr. The precipitated urea was filtered off and the solvent was evaporated 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.

Modification of BSA : BSA 20 mg(0.3 mmol) was dissolved in 4 ml of 0.1 M citrate buffer (pH 6.0), and a solution of N-succinimidyl 3-formyl-4-hydroxybenzoate (9.6 mg) in 0.4 ml acetone was added and kept at 0°C for 5 hr. The reaction mixture was applied to Sephadex G-25 column (2.2 x 20.5 cm) using 10 mM ammonium bicarbonate (pH 8.0) as a eluent, dialyzed against distilled water and lyophilized. Introduction of 2.2 mol of 3-fromyl-4-hydroxybenzoyl residue per 66000g of BSA was determined from the colorimetric analysis using 2,4-dinitrophenylhydrazine (8).

Analysis of chromatographic behavior of BSA conjugate : Gel ***a*** 10 ml and copper(II) acetate (1 μmol in 1 ml water) were mixed with a solution of 1.0 mg of modified BSA (15 nmol) in 9 ml of 50 mM BES (pH 7.0) in a vial tube and the vessel was rotated gently for 24 hr at 25°C. Then the gel was transferred to a column (1.0 x 12.7 cm) and the column was subjected to the same procedure as that for salicylaldehyde. Elution of BSA was detected measuring fluorescence intensity developed by fluorescamine (6).

Results and Discussion

The reported value (2) on the stability of chelate ***IV*** will rationalize the strong intermolecular interaction between salicylaldehyde and α-amino acid group in the presence of copper(II) ion. The value, however, is that determined spectrometrically, and no direct evidence for the binding interaction has been, so far, reported. For the application of the principle to the present purpose it seemed necessary to observe direct evidence for the complexation and to evaluate its potency. One of the best way for this will be the analysis using an immobilizing phase, i.e., the adsorptive ability of one

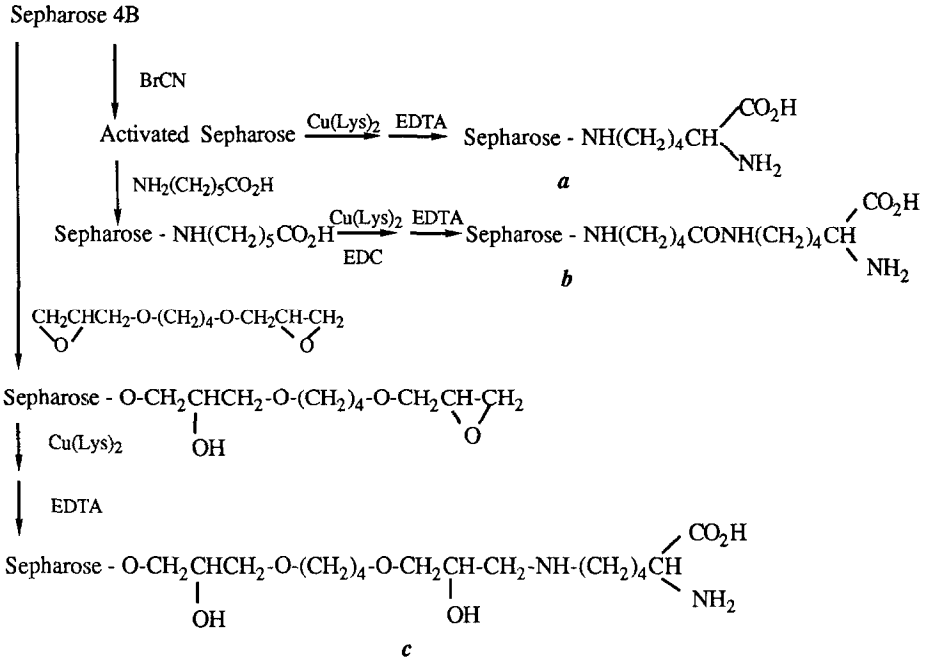

Figure 2. Synthetic routes for Sepharose gels attached lysine residue.

component to another component attached on a matrix which is analyzable through the chromatographic behavior. In this respect Sepharose gels attached α-amino acid residue were prepared and their synthetic routes are summarized in Figure 2. Preparation of ***a*** was carried out by the coupling of copper lysinate with BrCN-activated Sepharose. Gel ***b*** was prepared by coupling of the activated gel with ε-aminocaproic acid and subsequent reaction with copper lysinate and water soluble carbodiimide. The contents of the ligand molecule were determined 3.7 and 1.9 μ mol per ml of wet gel for ***a*** and ***b***, respectively. The highest content, 15 μ mol, was obtained for the gel ***c*** which was prepared from the reaction of Sepharose 4B with 1,4-butanediol diglycidyl ether and subsequent coupling with copper lysinate and water soluble carbodiimide.

Preincubated solution of salicylaldehyde with gel ***a*** in 0.1 M phosphate buffer (pH 7.0) in the presence of 0.4 mM copper(II) acetate was transferred into a column, and the column was washed with the copper-free buffer. As shown in Figure 3, the emergence of salicylaldehyde was not observed even after the exhaustive washing though small amount of salicylaldehyde was leaked out at the initial stage of the elution. Salicylaldehyde adsorbed in the column was quantitatively recovered when the eluent was changed to the buffer containing 5 mM EDTA. The result is in good contrast to that in

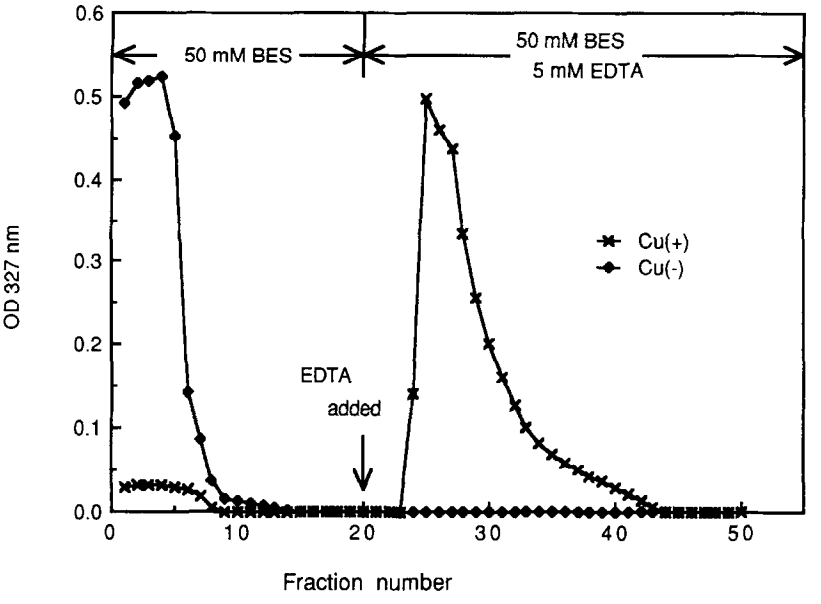

Figure 3. Elution diagram of salicylaldehyde (3.7 μmol) with lysine attached Sepharose column (1.0 x 12.7 cm). Salicylaldehyde was preincubated with gel *a* in the presence (0.4 mM) (×–×) and absence (♦–♦) of copper(II) acetate. Absorbance at 327 nm of each fraction (3 ml) vs. fraction number was plotted. After the absorbance of the eluent reached to zero (at 60 ml of eluent), EDTA was added to the buffer (final concentration, 5 mM).

the absence of copper(II) ion. Behavior of salicylaldehyde with gel *b* and gel *c* was found essentially identical to that with gel *a* , though the amount of the leakage at the initial stage of the elution is increased. Under the condition shown in Figure 3, amount of salicylaldehyde was determined to be 81 %. As shown in Table 1, the highest extent of

Table 1. Interaction of salicylaldehyde with lysine attached gel

Gel	Eluant[a]	Salicylaldehyde eluted by EDTA addition (%)[b]
	water	94
a	50 mM phosphate	81
	50 mM BES	64
	water	86
b	50 mM phosphate	61
	50 mM BES	55
	water	77
c	50 mM phosphate	63
	50 mM BES	50

[a] pH was adjust to 7.0. [b] amount of salicylaldehyde : recovered / (leaked + recovered).

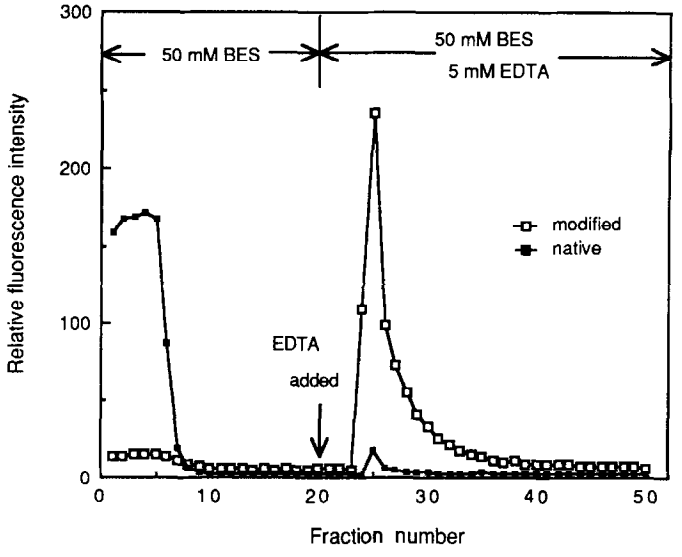

Figure 4. Elution diagrams of native BSA (■—■) and BSA-salicylaldehyde conjugate (□—□) with lysine attached Sepharose Column (1.0 x 12.7 cm). BSA preparations were preincubated with gel ***a*** and copper(II) acetate (100 μM). Aliquot of each fraction (3 ml) was reacted with fluorescamine and the fluorescence intensity at 475 nm (excitation at 390 nm) was plotted.

adsorption was observed when water is used as a medium. Comparison of gels ***a***, ***b*** and ***c*** suggests that the more specific adsorption is attained by a gel with the shorter or less polar spacer group.

In order to verify the applicability of gel ***a*** to large molecule substances, bovine serum albumin (BSA) salicylaldehyde conjugate was prepared. The reaction of BSA with excess N-succinimidyl 3-formyl-4-hydroxybenzoate at pH 6 , 0°C for 5 hr afforded modified BSA which contains 2.2 mol of the ligand per mol of protein. Chromatographic behavior of the resulting BSA conjugate in a column of gel ***a*** was analyzed following the procedure used for salicylaldehyde. As shown in Figure 4 specific adsorption of protein was analyzed. More than 90 % of protein was recovered by the elution with EDTA. It is noted that behavior of native BSA in the presence of copper(II) ion is almost identical to that of BSA conjugate in the absence of copper(II) ion.

A useful method for cross-linking or immobilization based on chelete formation reaction was proposed, although the method will not be applicable for certain proteins such as pyridoxal enzymes and metalloproteins. Other ions such as zinc, nickel and cobalt were found also applicable for the purpose, though cheletes with these metals

were less stabilized. Application of the principle to immobilization of enzyme and cross-linking between protein molecules are now in progress.

Acknowledgment

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

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