CROSS-LINKING OF REVERSIBLY IMMOBILIZED ENZYMES

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1. Introduction

There are at least two reasons for the introduction of covalent, intramolecular cross-links into proteins. Valuable information concerning the solution structure of the protein may be obtained [1]. In addition stabilization of the protein may be achieved. One problem inherent in the reaction of proteins with bifunctional reagents is the production of aggregates as a result of the formation of intermolecular cross-links. We report here a new method of cross-linking which yields monomeric protein exclusively (fig.1). First, the protein is attached to a solid support via a cleavable linkage. Cross-linking is the second step. The last step is the release of the cross-linked monomer. Since the protein molecules are fixed and widely spaced on the matrix, intermolecular cross-links are prevented.

Trypsin was attached to CL-Sepharose-$NH(CH_2)_6$ $NHCO(CH_2)_2$ $S\text{-}S(CH_2)_2$ CO-(*N*-hydroxy-succinimide) (I), cross-linked with diimidates, and released by reduction of the disulfide linkage between the enzyme and support. In a second approach the protein was attached to the support through free thiols rather than through amino groups. To illustrate this method papain was reacted with CL-Sepharose-(glutathione-2,2′-dipyridyl disulfide) (II). The bound enzyme was cross-linked with glutaraldehyde and released by treatment with DTT*.

**Abbreviations*: AA-bislysine, $N^{\epsilon},N^{\epsilon}$-adipamidinobis-L-lysine; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; DTSP dithiobis (succinimidyl propionate); DTT, dithiothreitol; HMD, hexamethylene-diamine; SA-bislysine, $N^{\epsilon},N^{\epsilon}$-suberamidinobis-L-lysine.

Monomeric protein was produced exclusively in the two cases described above. The cross-linked trypsin was more stable than the control (enzyme which had been immobilized and released without cross-linking), but less stable than the native enzyme. However, the cross-linked papain was significantly more stable than the native enzyme in autolysis and thermal denaturation studies.

2. Experimental

2.1. *Materials*

Trypsin and papain were obtained from Worthington (lot nos. TRL 350888 and 36E670 respectively). C1-Sepharose-4B was the product of Pharmacia. Imidoesters and DTSP were from Pierce Chemical Co. Standards for amidino lysine adducts were kindly provided by Drs F. Wold, F. Hartman, and W. Neihaus. 2,2′-Dipyridyl disulfide was supplied by Aldrich.

2.2. *Preparation of bound enzymes*

HMD-CL-Sepharose was prepared by the method of Liberatore et al. [2] with modifications to reduce the amount of bound HMD. For this study the washed gel was oxidized for 10 min at room temperature with 0.2 mM $NaIO_4$. The amine content was determined to be 0.25 μmol/ml gel by the manual ninhydrin method of Moore [3]. Prior to the preparation of (I) the HMD-CL-Sepharose (10 ml) was washed with 100 ml of dioxane in a sintered glass filter. The gel was then equilibrated with a 10% solution of triethylamine in dioxane (20 ml) and washed with 30 ml of dry dioxane. The gel was added to 20 ml of dry dioxane containing 20 mg of DTSP. After stirring for 20 min at room

temperature, the gel was washed with at least 5 volumes of dioxane and used immediately. The damp gel (I) was added directly to 10 ml of a trypsin solution (5 mg/ml in 0.1 M borate buffer, pH 8.5 containing 1 mM benzamidine, 4°C). After 1 h the gel was washed with 100 ml of distilled water, 1 liter of 2 M KCl over a period of several hours, and finally 100 ml of distilled water. Washing was done at 4°C. The bound enzyme contained 0.48 mg protein/ml gel as determined by amino acid analysis; it was stored in the cold in 1 mM HCl.

CL-Sepharose-glutathione was prepared by the method of Liberatore et al. [2]; it was reacted with 2,2′-dipyridyl disulfide and papain according to Brocklehurst, et al. [4]. The bound enzyme derivative contained 1 mg protein ml/gel.

2.3. *Cross-linking procedures*

Bound trypsin (2 ml) was suspended in 8 ml of 0.2 M triethanolamine-HCl, pH 8.5 containing 1 mM benzamidine, 25°C. Solid diimidate (100 μmol) was added every 30 min to the suspension and the pH was adjusted to 8.5 by addition of 1 N NaOH. After stirring for 3 h, the gel was washed with 50 ml of 0.1 M borate buffer, pH 9.0 and 50 ml of 0.1 M acetate buffer, pH 4.0.

Papain was cross-linked with glutaraldehyde rather than diimidates. Bound papain (1 ml) was suspended in 12.5 ml of 0.1 M phosphate buffer, pH 7.0. Glutaraldehyde (25 μl of a 25% solution, w/v) was added and the mixture was stirred for 1 h at 25°C. The reaction was terminated by washing with this phosphate buffer (50 ml) and water (200 ml).

2.4. *Release of enzymes from the supports*

Bound trypsin (2 ml) was placed in a column (1 × 5 cm) and washed with 2 mM DTT in 0.05 M Tris, pH 8.0 containing 1 mM benzamidine at 25°C. Fractions (1 ml) were collected into tubes containing 1 ml of this Tris buffer which contained 10 mM iodoacetamide to block free thiols generated by reduction. After 30 min at 25°C the fraction were dialyzed against 2.5 mM HCl at 4°C.

Bound papain (1 ml) was washed in a column (1 × 5 cm) with 7 ml of 5 mM DTT in 0.1 M Tris, pH 8.5 containing 1 mM EDTA with and without 3 M urea. The released protein was desalted with a Sephadex G-25 column.

2.5. *Enzyme assays: thermal stability studies*

Soluble trypsin was assayed spectrophotometrically according to Schwert and Takenaka [5] (1 mM BAEE, 0.05 M Tris, pH 8.0, 0.025 M $CaCl_2$, 25°C). Bound trypsin was assayed titrimetrically: ~50 mg damp enzyme conjugate, 20 ml of 1 mM BAEE in 0.5 mM Tris, pH 8, containing 0.025 M $CaCl_2$ at 25°C. Soluble and insoluble forms of papain were assayed titrimetrically: 40 mM BAEE, 1 mM EDTA, 5 mM cysteine, 0.3 M KCl, pH 6.3 at 25°C. Proteinase activity was measured as described by Lin et al. [6].

In the thermal stability study of trypsin derivatives, enzyme solution (2 ml, 0.1 mg enzyme/ml, 0.1 M Tris, pH 8.0, 1 mM benzamidine, 25 mM $CaCl_2$) was incubated at 65°C. At timed intervals 0.1 ml samples were removed, cooled to 25°C and assayed against BAEE.

For the determination of the thermal stability of papain the active-site thiol was blocked with Ellman's reagent (0.5 mM in 0.02 M phosphate buffer pH 7.0, 1 mM EDTA and 0.1 M NaCl; excess reagent was removed by gel filtration). Samples (0.5 ml) of the blocked enzyme were placed in tubes and incubated in a constant temperature bath. At timed intervals the samples were cooled to 25°C and assayed against BAEE.

3. Results and discussion

The reaction schemes of fig.1 illustrate the reversible immobilization of proteins either through amino groups or thiol groups. In the case where the desired chemical modification is cross-linking, the capacity of the solid support should be controlled so that bound protein molecules are not sufficiently close to permit intermolecular cross-links. The levels of reactive groups on supports (I) and (II) were 0.25 μmol/ml and 3.9 μmol/ml, respectively. In the case of trypsin the number of linkages between enzyme and the support was estimated by the difference in loss of amino groups as measured by TNBS [7] and the amount of amidinated lysine as measured by amino acid analysis. About 1.5 linkages per enzyme molecule were found (table 1).

The reaction of diimidates with proteins can be quantitated in two ways: disappearance of free lysines and the appearance of amidinated amino acid deriva-

tives on the amino acid analyzer [8]. Reaction of reversibly bound trypsin with DMA and DMS resulted in substantial loss of lysine and introduction of nearly one intramolecular cross-link per molecule (table 1). Protein recovery was over 80% and the control sample retained complete specific activity. The specific activities of the amidinated derivatives are lower than that of native enzyme. Reaction of the bound enzyme with methyl acetimidate gave a fully active product with about the same extent of modified lysine. This result suggests the possibility of reduced activity brought about by the cross-link.

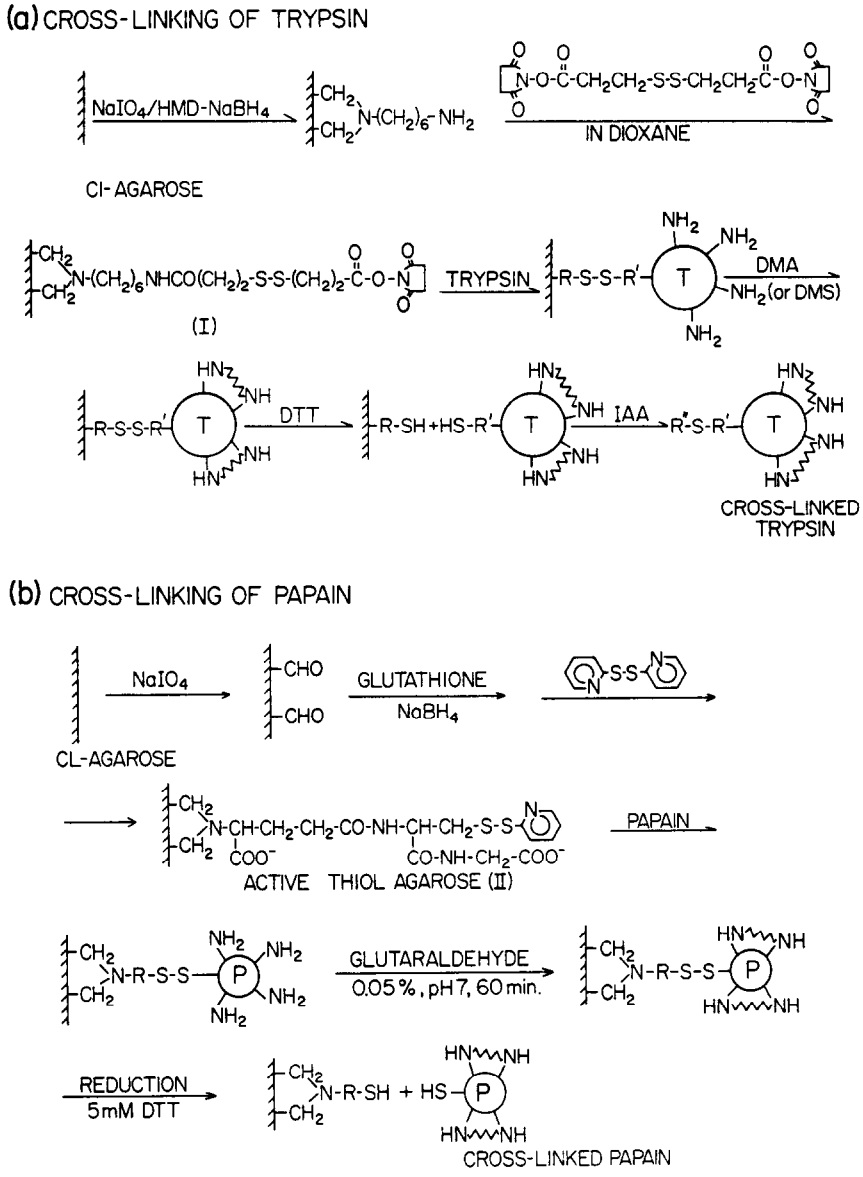

Fig.1. Preparation of intramolecularly cross-linked enzymes. (a) Trypsin: R, $-CH_2\ NH(CH_2)_6\ NHCO\ (CH_2)_2^-$; R′, $-(CH_2)_2\ CO-$: R″, $-CH_2\ CONH_2$. (b) Papain; R, $-CH\ CH_2CH_2CONHCHCH_2-$ (COO⁻ COO⁻)

Table 1
Analytical data for cross-linked trypsin derivatives

Cross-linking reagent used	Total protein recovered (%)	Specific activity (%)[b]	Loss of amino[c] groups (by TNBS)	Lys modified by cross-linking reaction[c]	AA-bislysine or SA-bislysine[c]
DMA	93	69	10.4	8.6	0.72
DMS	85	57	9.8	8.7	0.92
none[a]	87	102	1.6	–	–

[a]Trypsin was immobilized and released but not cross-linked
[b]Specific activity relative to native trypsin (38.0 μmol/min · mg)
[c]moles/mole of enzyme

Reaction of reversibly immobilized papain with diimidates yielded products with very low numbers of cross-links (< 0.1/mol). The stability and the activities of these amidinated derivatives were identical to those of the native enzyme. Glutaraldehyde treatment (table 2) yielded a highly substituted enzyme with improved stability. The loss of histidine and lysine suggests about nine points of reaction per molecule which almost certainly means one or more intramolecular cross-links. Glutaraldehyde-treated papain retains

Table 2
Analytical data for glutaraldehyde-treated papain

A. Activity

Preparation	Total activity recovered (%)	Specific activity[a] (%)
1. Immobilized and released without glutaraldehyde treatment	68.8	100
2. Glutaraldehyde-treated. Released with urea-DTT	49.0	65.0
3. Same treatment as 2. Released without urea	28.0	54.2

B. Amino acid analysis

	Residues per mole		
Amino acid	Literature[b]	Untreated	Treated
Lys	10.0	10.3	2.4
His	2.0	2.1	1.2
Arg	12.0	11.4	10.6

[a]Specific activity relative to untreated papain (36.9 μmol/min · mg)
[b]Reference [12]

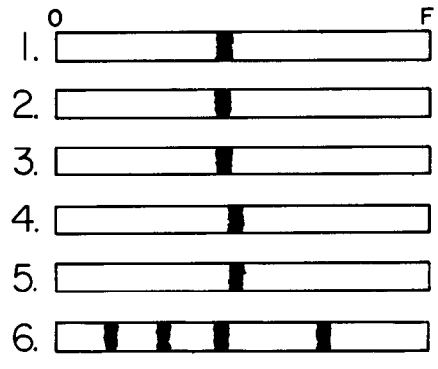

Fig.2. SDS-polyacrylamide gel electrophoresis of cross-linked trypsin and papain. (1) Native trypsin; (2) DMA-treated trypsin; (3) DMS-treated trypsin; (4) native papain; (5) glutaraldehyde-treated papain; (6) marker proteins (from origin, protein bands correspond to albumin (mol. wt. 68 000), pepsinogen (35 000), trypsinogen (23 900) and lysozyme (14 300). The procedure of Weber and Osborn [13] was used with the modification that mercaptoethanol was not added to protein solutions (1), (2) and (3) to prevent chain separation of α-trypsin.

substantial activity against BAEE and essentially complete activity against *N,N'*-dimethyl bovine serum albumin.

Figure 2 clearly shows that the reaction procedures described here yield intramolecular cross-links exclusively. Cross-linking soluble enzymes, especially with glutaraldehyde, produces large amounts of polymer unless the proteins are very dilute. Another advantage of our system is that enzymes could be cross-linked in the presence of specific substrates or effectors [9–11]. These compounds can be maintained at high levels in a flow system since the enzyme is fixed.

Glutaraldehyde-treated papain is considerably more stable than the native enzyme (table 3). This finding would be of practical value in applications which involve the use of a soluble enzyme to transform an insoluble substrate. In such applications enzyme stabilization without increase in size would be an advantage.

The cross-linked trypsin derivatives are more stable than the control (enzyme immobilized and released but not cross-linked), but less stable than native enzyme. This observation may be explained by the fact the disulfide bridges in trypsin are ruptured during the release step. Although the external cross-link provides some stabilization, the disruption of disulfides is the dominant factor. However (I) would be perfectly acceptable for enzymes which contain no essential disulfides.

Acknowledgements

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Table 3
Stability of glutaraldehyde-treated papain

Treatment	Time	Remaining activity (%)[a]	
		Untreated	Treated
(1) At 60°C	10 min	70.0	78.2
	20	45.5	69.4
	40	32.7	60.0
(2) At 80°C	10 min	1.2	6.5
(3) With 5 mM DTT at 37°C	3 h	100	100
	16	70.5	81.8

[a]In each experiment, the activity of both treated and untreated preparations before incubation was taken as 100%

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