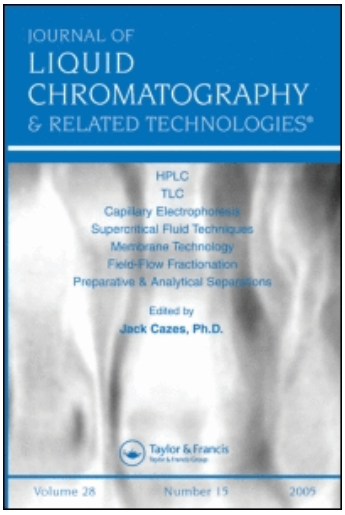

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CHROMATOGRAPHIC ANALYSIS OF SUBERIMIDATE-CROSSLINKED LYSINE*

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ABSTRACT

Quantitative analysis of bislysylsuberamidine and monolysylsuberamidinic acid, which are obtained by an acid hydrolysis of protein cross-linked with dimethyl suberimidate, on an amino acid analyzer are described. Both of ninhydrin and fluorometric detection with o-phthalaldehyde were applied and less than 50 pmol of cross-linked lysine was analyzed in the latter case. The first-order rate constant for hydrolysis of amidine bond under standard conditions of acid hydrolysis of protein was found to be $3.4 \times 10^{-3} h^{-1}$.

INTRODUCTION

During the last decade, an application of cross-linking reactions to protein has become one of the most popular tools in the field of protein biochemistry. The application is not only restricted to topological analysis of protein assembly: recent studies show further possibilities of the reaction, such as the fixation of a conformation or a functional state of protein by an artificially introduced cross-links(1,2).

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Nevertheless, against so many increasing number of the reports on the use of the reaction, there has been no method for quantitative analysis of cross-linked amino acid reported, except those of the reaction products of lysine and dimethyladipimidate(3) and radioisotopic analysis of suberimidate-treated lysine(4). In the present paper, we will describe the chromatographic analysis of the lysine derivatives of dimethylsuberimidate, which is generally selected as the best choice among the bifunctional imidoesters, together with the behaviour of the compounds under the conditions of total acid hydrolysis of the modified protein.

MATERIALS AND METHODS

Preparation of Bislysylsuberamidine (I) and Monolysylsuberamidinic acid (II). Dimethylsuberimidate dihydrochloride was prepared from suberonitrile by the method of McElvain and Schroeder(5). N^{α}-benzyloxycarbonyllysine (α-Z-Lys) was prepared according to the method of Bezas and Zervas(6). α-Z-Lys (2 g/100 ml of 0.4 M Na_2CO_3, pH 10.0) was treated with dimethylsuberimidate dihydrochloride (1.8 g), which was added as a solid in three times with 30 min-intervals. After the last addition of the reagent, the reaction mixture was gently stirred for 2 h at room temperature. The reaction was quenched by the addition of concentrated HCl to 6 N, and then the reaction mixture was refluxed overnight for the removal of the benzyloxycarbonyl group from lysyl moiety. After the reflux, the reaction mixture was extracted with ether and the aqueous layer was evaporated to leave a residue containing suberimidate-modified lysines and salts. The lysine derivatives were extracted from the residue with methanol and

chromatographed on Dowex 50X2 (1.3 x 50 cm) with pyridine-acetate buffer, pH 5.5, under a linear gradient of pyridine concentration from 1 M to 2 M (total, 2,000 ml). Pure bislysylsuberamidine (I) was obtained by the chromatography. The ninhydrin-positive material which was eluted between lysine and (I) on the chromatography was treated with 6 N HCl for 3 h at room temperature to give the monolysyl derivative (II).

The desired products, (I) and (II), were obtained as an oil and were found to be pure in terms of NMR spectra and amino acid analysis, except for the presence of a small amount of ammonia. Proton NMR spectra were obtained with JEOL FT100 in D_2O and the proton chemical shifts for (I) and (II), which were represented in ppm from the methyl signal of 3-(trimethylsilyl)-propanesulfonic acid sodium salt, are as follows: for (I); 3.70 (C_α-H of lysine), 3.26 (C_ε-H of lysine), 2.44 (C_α-H of suberamidine), 1.2-1.8 (20 protons). For (II); 4.08 (C_α-H of lysine), 3.28 (C_ε-H of lysine), 2.47 (C_α-H of suberamidinic acid), 2.38 (C_ζ-H of suberamidinic acid), 1.2-1.8 (14 protons).

Amino Acid Analysis. Amino acid analysis was carried out with a 15-cm (i.d. = 0.5 cm, packed with JEOL LCR-1 resin) or a 5-cm(i.d. = 0.3 cm, Shodex HC095 resin) column. A 30-cm column (i.d. = 0.3 cm, Shodex HC095 resin) was used for the analysis of (II) under the conditions of single column methodology. A $NaBH_4$-ninhydrin system(7) or o-phthalaldehyde solution(8) was used to detect amino acids.

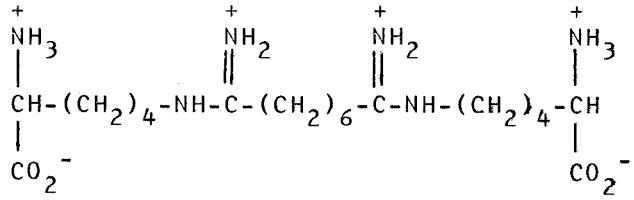
Other Procedures. Acid hydrolysis was carried out with constant-boiling HCl at 110°C in a sealed and evacuated tube. Deamidination was performed with methylamine-formate buffer (3.3 M, pH 11.5) as described by Dubois et al.(9).

RESULTS AND DISCUSSION

Analysis of (I) and (II). In this study, we prepared the authentic samples of (I) and (II) by the reaction of α-Z-Lys with dimethylsuberimidate. After the removal of the blocking group, the reaction products were chromatographed on Dowex 50 with a volatile buffer system. In the present preparation, the compound (II) was isolated as its methylester (NMR, $COOCH_3$ at 3.70 ppm), which was obtained as the result of a methanol treatment of deblocked products (see "MATERIALS AND METHODS"), and it was necessary to treat with an acid to convert it into free acid (II). The esterification of (II), which was not expected initially, facilitated the purification on Dowex 50 due to the loss of a negative charge on a carboxyl group: (II) itself was co-eluted with lysine remaining in the reaction mixture, but (II)-methylester was eluted between lysine and (I) on Dowex 50 chromatography.

Chromatographic data of (I) and (II) on an amino acid analyzer are summarized in Table 1. (I) was analyzed on a 5-cm column with citrate buffer (Na^+=1.4 M, pH 6.0, for ninhydrin analysis) or borate buffer (Na^+= 0.35M, pH 9.5, for fluorescence detection) to appear as a sharp peak with a tolerable retention time for a routine work. A 5-cm column analysis could be also applied to the analysis of bislysyladipamidine, which was eluted as a sharper peak than that observed on a 15-cm column analysis(3). Furthermore, a trislysyl derivatives of adipamidine, which was tentatively assigned and appeared with a retention time of 210 min on a 15-cm column analysis using the citrate buffer, was eluted at 40 min. The analysis of (II) was performed on a 30-cm column under the single column

(I) Bislysylsuberamidine (N,N'-bis(5-amino-5-carboxy-pentyl)suberamidine).



(II) Monolysylsuberamidinic acid (N-(5-amino-5-carboxy-pentyl)suberamidinic acid).

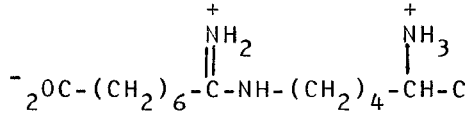


TABLE 1

Analytical conditions[a]	Retention Time (min) (I)	(II)	KWK[b]	Lys	NH_3	Arg
A	43	--[c]	21	--[c]	--[c]	--[c]
B	25	--[c]	--	--[c]	--[c]	--[c]
C	300	29	--	37	51	--
D	--	100	--	66	69	86

a) A: 5-cm column; 65°C; Na^+=1.4 M, 0.35 M citrate, pH 6.0. (For ninhydrin analysis)
B: 5-cm column; 65°C; Na^+=0.35 M, 0.1 M borate, pH 9.5. (For fluorescence detection)
C: 15-cm column; 65°C; Na^+=0.35 M, 0.12 M citrate, pH 5.6.
D: 30-cm column; first buffer, Na^+=0.2 M, 0.067 M citrate, pH 3.22 (0-12 min); second buffer, Na^+= 0.2 M, 0.067 M citrate, pH 4.25 (12-32 min); third buffer, Na^+=1.6 M, 0.2 M citrate, pH 5.00; 45°C (0-57 min), 55°C (afterward).
Buffer flow rate: 30 ml/h for A, B, and C; 12 ml/h for D.
b) KWK = bislysyladipamidine.
c) appears at the void.

methodology. (II) was eluted later than arginine and the retention time of (II) was similar to that of tryptophan under the chromatographic conditions employed. Since tryptophan is almost completely destroyed under the standard conditions of the total acid hydrolysis, the quantitative analysis of (II) was not interfered by the presence of tryptophan residue(s) in protein. When the analysis of (II) was carried out on a 15-cm column at 60°C, (II) and lysine were eluted separately but the peak of histidine overlapped with that of (II). However, when the column temperature was 45°C, the peak of (II) was very broad and overlapped with those of lysine and histidine. We could not find the appropriate conditions under which (II) was analyzed on a 15-cm column without a peak overlapping with lysine and histidine. Ninhydrin color values for (I) and (II) were determined as 1.8 and 1.0 relative to lysine, respectively, from the alkaline hydrolysis technique(3). Our most work on cross-linked protein hydrolyzates has been accomplished with fluorescence detection system employing o-phthalaldehyde(8). Approximately, 0.5-1.0 nmol of (I) was routinely analyzed. The lowest limit of detection may be below 50 pmol of (I).

Decomposition of (I) and (II) under the Standard Conditions of Acid Hydrolysis of Protein. (I) and (II) were subjected to acid hydrolysis (6 N HCl, at 110°C) for various periods up to 340 h. The acid hydrolyzate of (I) showed the presence of (I), (II), lysine, and ammonia, and no other ninhydrin-positive material was detected. Similarly, acid hydrolysis of (II) did not produce any ninhydrin-positive material except lysine and ammonia in hydrolyzates. A semi-logarithmic plot of the hydrolysis of (II) yielded a straight line,

which gave a first order rate constant $K_1 = 3.4 \times 10^{-3}$ h^{-1} for the hydrolysis of the amidine bond. The result that (I) degraded to (II) with a rate constant $6.8 \times 10^{-3} h^{-1}$, exactly twice the above k_1, indicates that two amidines of (I) were hydrolyzed independently at the same rate, as previously observed for bislysyl-adipamidine(3).

Deamidination of Suberimidate-crosslinked Lysine. Recently Dubois et al. reported that acetamidine was readily deamidinated by the treatment with methylamine buffer and the side reactions such as non-specific cleavage of peptide bonds were not observed(9). We examined the deamidination of (I) with methylamine-formate buffer. Methylamine buffer was found to induce the rapid cleavage of the suberamidine bonds; (I) completely disappeared after the incubation of (I) in the methylamine-formate buffer for 1 h at 37°C and lysine was regenerated quantitatively.

Application to the Analysis of the Suberimidate-treated Actin. Actin, one of major muscle proteins, was treated with dimethylsuberimidate(2). Amino acid analysis revealed that the acid hydrolyzate of the suberimidate-treated actin contained (I) and (II) and that the sum of lysine, (I) and (II) gave the original number of lysine in intact actin. These results indicate the validity of the analytical method described here. A removal of suberimidate-crosslink in the modified actin was accomplished by using the methylamine buffer. After the incubation of the suberimidate-treated actin in methylamine buffer for 2 h at 37°C, (I) and (II) completely disappeared on an amino acid analysis of the acid hydrolyzate of the modified protein, and the polyacrylamide gel electrophoresis in the presence of

sodium dodecylsulfate indicated that the treatment with methylamine buffer did not induce the non-specific cleavage of the peptide bonds in the protein.

Therefore, as demonstrated, the present study has established the basis to analyze the suberimidate-induced cross-links quantitatively, thus enabling us to investigate the location of the cross-links, in a strict sense of a primary structure.

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