MODIFICATION OF CYSTINE OR TRYPTOPHAN RESIDUES IN THE ASPARAGINASE MOLECULE AND THEIR ROLE IN CONFORMATION AND ANTIGENICITY

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1. Introduction

Finding that asparaginase(L-asparagine amidohydrolase, EC 3.5.1.1.) is effective for remission of children with lymphoblastic leukemia led to an extensive study on the physicochemical and immunochemical characteristics of asparaginase. Asparaginase from *Escherichia coli* has the mol. wt. of 136 000 and consists of four identical subunits [1,2]. The primary structure of the enzyme was recently determined by Maita et al. [3]. In our laboratory, states of amino acid residues in the asparaginase molecule and amino acid residues in participation with the enzymic activity and also with antigenicity towards anti-asparaginase serum have been studied in relation to tertiary and quarternary structures [4–9]. And it was found that amino group, histidine and tyrosine residues in the asparaginase molecule are closely associated with the enzymic activity, and that the reduction of the antigenicity of asparaginase is accompanied by the decrease of α-helix content and the destruction of tyrosine residues.

In the present study, four cystine or four tryptophan residues in the asparaginase molecule were modified by chemicals and the physicochemical properties of these modified asparaginases were clarified and discussed in relation to their conformation and antigenicity.

Abbreviations: SM-asparaginase; *S*-methylated asparaginase, SCM-asparaginase; *S*-carboxymethylated asparaginase and HNB-asparaginase; 2-hydroxy-5-nitrobenzyl asparaginase.

2. Materials and methods

Asparaginase obtained from *E. coli* was kindly donated from Kyowa Hakko Kogyo Co.. The specific activity of the asparaginase is 220 IU/mg of protein and its molar extinction coefficient is 8.53×10^4 M^{-1} cm^{-1}. *S*-Carboxymethylated asparaginase (SCM-asparaginase) was prepared as follows; disulfide bonds in asparaginase (10 mg) were reduced with 10 μl of β-mercaptoethanol in 0.5 M Tris-glycine buffer (pH 8.6) containing 8 M urea and 0.1% EDTA and kept standing for 2 h at room temperature. And then iodoacetic acid (27.7 mg) was added to the reaction mixture and placed for 2 h at room temperature [10]. The reaction mixture was passed through a column of Sephadex G-15 and eluted with 8 M urea containing 0.5 M Tris-glycine buffer (pH 8.0). The protein thus eluted was dialyzed completely against 15 mM phosphate buffered saline. Modification of tryptophan residues with 2-hydroxy-5-nitrobenzylbromide was carried out by the method of Barman et al. [11,12]. To 4.0 ml of asparaginase (20 mg) in 0.2 M acetate buffer (pH 3.0) was added 50 μl of 2-hydroxy-5-nitrobenzylbromide (10 mg) in acetone. During the reaction, the sample solution was maintained at a constant pH (3.0) with the addition of sodium hydroxide by using a pH-stat. The reaction mixture was passed through a Sephadex G-15 column and eluted by 0.2 M acetate buffer (pH 3.0). To the eluted protein solution was added 8 M urea containing 0.1 M phosphate buffer (pH 8.0) to make a concentration of urea 6 M and pH 8.0, and then the protein

solution was dialyzed against 50 mM phosphate buffer (pH 8.0). 2-Hydroxy-5-nitrobenzyl asparaginase(HNB-asparaginase) was thus obtained. *S*-Methylated asparaginase (SM-asparaginase) was prepared by the method described previously [9]. Hybridized asparaginase formed by the association of SM- and SCM-asparaginase subunits was prepared; SM- and SCM-asparaginases were mixed in equal amount and dissolved with 8 M urea, and the mixture of the two modified asparaginases containing urea was dialyzed against 50 mM phosphate buffer (pH 8.0). A similar hybridization experiment was performed for asparaginase and SCM-asparaginase. Hybridization of these subunits was confirmed by disc electrophoresis. Amino acid compositions of the modified asparaginases were analyzed with a JEOL amino acid analyzer model JLC-5AH. α-Helix content was measured with a Jasco optical rotatory dispersion recorder model ORD/UV-5. The enzymic activity of asparaginase was determined by direct Nesslerization. Anti-asparaginase serum was obtained from rabbits and anti-asparaginase immunoglobulin G was isolated by the method previously described [9]. Disc electrophoresis was performed by using 7.5% polyacrylamide gel and thin-layer chromatography was carried out with Sephadex G-200 (Super Fine). Isotachophoresis was performed with a Tachophor LKB 2127.

3. Results and discussion

The amino acid compositions of asparaginase, SM-asparaginase and SCM-asparaginase are shown in table 1, in which the number of each amino acid per a subunit in the asparaginase molecule is represented. As is clear from the table, all of four cystine in the asparaginase molecule were completely modified by methyl-*p*-nitrobenzenesulfonate, and iodoacetic acid. No modification of another amino acid residues took place with these reagents. Degree of the modification of tryptophan residues in the asparaginase molecule by 2-hydroxy-5-nitrobenzylbromide was spectrophotometrically determined according to the method of Barman [11], and it was found that four tryptophan residues in the molecule are modified.

Fig.1 shows the disc electrophoretic patterns

Table 1
Amino acid composition of asparaginase, SM- and SCM-asparaginase

Amino acid	Asparaginase	SM-asparaginase	SCM-asparaginase
Lysine	21.6	21.7	22.0
Histidine	3.0	2.8	3.0
Arginine	8.1	7.6	7.5
Aspartic acid	52.8	53.3	51.0
Threonine	34.1	34.9	33.4
Serine	14.2	14.6	15.4
Glutamic acid	21.4	21.1	21.1
Proline	14.2	15.2	13.2
S-Methylcysteine[a]	0.0	1.9	0.0
Glycine	27.7	27.5	28.6
Alanine	33.0	33.0	33.0
1/2 Cysteine	2.0	0.0	0.0
Valine	27.8	28.3	30.0
Methionine	6.0	5.7	6.0
Isoleucine	12.3	12.6	11.4
Leucine	22.9	19.8	22.4
Tyrosine	12.5	11.9	11.9
Phenylalanine	8.2	7.7	7.9

The samples were hydrolyzed with 6 N HCl for 22 h at 110°C. Amino acid composition was expressed as the number of amino acid residues in one subunit (34 000) of the asparaginase molecule.

[a] The value of *S*-methylcysteine was calculated by the method of Heinrikson [13].

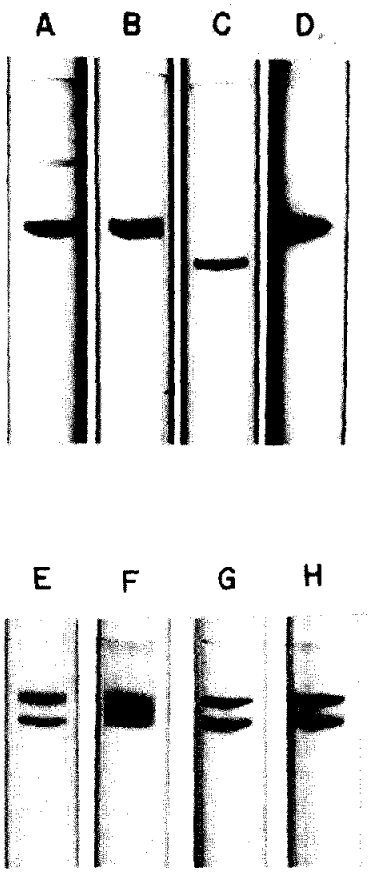

Fig.1. Disc gel electrophoretic patterns of asparaginase and modified asparaginases. Electrophoresis was carried out with 7.5% polyacrylamide gel in Tris-glycine buffer (pH 8.3). Tubes A, B, C and D; asparaginase, SM-asparaginase, SCM-asparaginase and HNB-asparaginase, respectively. Tubes E and F; the mixture of SM- and SCM-asparaginases and the same mixture treated with urea, respectively. Tubes G and H; the mixture of asparaginase and SCM-asparaginase and the same mixture treated with urea, respectively. The small band present at the upper portion of the band of asparaginase (Tube A) is a polymerized form of the asparaginase molecule [14].

obtained for asparaginase (tube A), SM-asparaginase (tube B), SCM-asparaginase (tube C) and HNB-asparaginase (tube D). A single and sharp band for each asparaginase was observed and the mobility was 0.37 for SM- or HNB-asparaginase, which is in good agreement with that of asparaginase. The mobility for SCM-asparaginase was 0.44, which is a little larger than 0.37.

From thin-layer chromatographic analyses of asparaginase and modified asparaginases, it was found that the mol. wt. was 136 000 for asparaginase, SM-asparaginase and SCM-asparaginase, and 34 000 for HNB-asparaginase which is one-fourth of the mol. wt. of native asparaginase. These results suggest that the SM- or SCM-asparaginase molecule consists of four identical subunits even after asparaginases were modified. The HNB-asparaginase molecule, however, dissociates into subunit with mol. wt. of 34 000. The enzymic activities of SM- and SCM-asparaginases are slightly lower than that of native asparaginase, 220 IU/mg of protein, while HNB-asparaginase has no enzymic activity. Optical rotatory dispersion analyses of SM- or SCM-asparaginase gave almost the same value as $b_0 = -163$ of native asparaginase, while the value of b_0 for HNB-asparaginase was $b_0 = -88$, which is a half value of that of asparaginase. These results are summarized in table 2.

The mixture of SM- and SCM-asparaginases or the mixture of asparaginase and SCM-asparaginase was subjected to disc electrophoretic analysis. The results are shown by tubes E and G in fig.1, in which two distinct sharp bands were observed. These two mixtures were treated with 8 M urea and then dialyzed, and they were also subjected to the analysis. Disc electrophoretic pattern of the mixture of SM- and SCM-asparaginases treated with urea shows a broad band (tube F), indicating that the hybridized asparaginase was formed by association of SM- and SCM-asparaginase subunits. Analyzing the hybridized asparaginase with a Tachophor revealed that there exist at least a few peaks on the isotachophoretic pattern. Tube H represents the disc electrophoretic pattern of the mixture of asparaginase and SCM-asparaginase treated with urea. The existence of two sharp bands indicates that the hybridization of asparaginase and SCM-asparaginase subunits does not take place.

Fig.2 shows the plotting of the amount of precipitates formed by binding of asparaginase or modified asparaginase to anti-asparaginase antibody against antigen concentration. The precipitin reaction curve of SM-(curve B) and SCM-asparaginases (curve C) or the asparaginase hybridized with SCM- and SM-asparaginase subunits (curve D) agrees in the equivalent point and in the amount of precipitate with the precipitin reaction curve of asparaginase (curve A).

Table 2
Characteristics of asparaginase, SM-, SCM- and HNB-asparaginase

	Asparaginase	SM-asparaginase	SCM-asparaginase	HNB-asparaginase
Number of cystine residues in the molecule	4	0	0	4
Number of tryptophan residues in the molecule	4	4	4	0
Number of subunits in the molecule	4	4	4	1
Optical rotatory dispersion parameter, b_0 (α-helix content)	−163 (26.1%)	−166 (26.5%)	−159 (25.4%)	−88 (14.8%)
Disc electrophoretic mobility	0.37	0.37	0.44	0.37
Enzymic activity (%)	220 IU/mg (100%)	187 IU/mg (85%)	190 IU/mg (86%)	0.0 IU/mg (0%)
Antigenicity[a] towards anti-asparaginase serum (%)	100%	97%	97%	86%
Hybridization of subunits between	−	+	+	−

[a] Relative amount of antigen–antibody complex at the equivalent point of precipitin reaction.

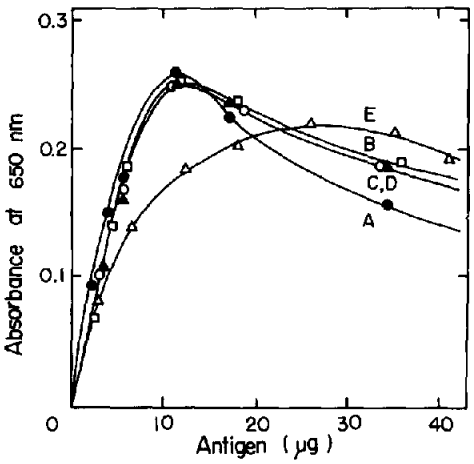

Fig.2. Quantitative precipitin reaction curves obtained for plotting the amount of precipitates of the complex of asparaginases and anti-asparaginase immunoglobulin G against antigen concentration. Curve A; asparaginase, curves B, C and D; SM-asparaginase, SCM-asparaginase and the asparaginase hybridized with SM- and SCM-asparaginase subunits, respectively. Curve E; HNB-asparaginase.

The precipitin reaction curve of HNB-asparaginase which has mol. wt. of 34 000 (curve E) is quite difference in the equivalent point with that of asparaginase. The maximum amount of precipitates for HNB-asparaginase are smaller by 14% than that for asparaginase.

It is a noteworthy fact that the physicochemical and the enzymic properties of SM- and SCM-asparaginases are the same as those of asparaginase even after four disulfide bonds in the molecule are reduced and modified with each reagent, and that the hybridized asparaginase having mol. wt. of 136 000 was formed by association of the SM- and SCM-asparaginase subunits. Another interesting finding is that the modification of four tryptophan residues in the asparaginase molecule gives rise to a complete reduction of the enzymic activity and also to the destruction of quaternary structure of the asparaginase molecule.

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