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MODIFICATION OF E. COLI L-ASPARAGINASE WITH POLYETHYLENE GLYCOL: DISAPPEARANCE OF BINDING ABILITY TO ANTI-ASPARAGINASE SERUM

Yoshihiro ASHIHARA, Takashi KONO, Shojiro YAMAZAKI and Yuji INADA

Laboratory of Biological Chemistry, Tokyo Institute of Technology,

Ookayama, Meguroku, Tokyo 152, Japan

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SUMMARY

L-Asparaginase from Escherichia coli A-1-3 was modified with activated polyethylene glycols(2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine) with molecular weights of 750, 1900 and 5000. The modification of asparaginase to 73 amino groups out of the total 92 amino groups in the molecule with polyethylene glycol of 5000 daltons gave rise to a complete loss of the binding ability towards anti-asparaginase serum from rabbit. This modified asparaginase retained the enzymic activity(7%) and had a resistivity against trypsin. Asparaginases modified with polyethylene glycols of 750 and 1900 daltons did not show a substantial change of the immunogenic properties.

L-Asparaginase is effective for remission in patients with lymphoblastic leukemia, and its physicochemical properties and the clinical effects have been extensively studied by many investigators(1-3). The chemical modification of L-asparaginase has been studied in order to clarify the active site of the enzymic activity(4-7) and also to reduce the antigenic reactivity towards anti-asparaginase antibodies(8-11). Tamaura et al.(9) demonstrated that the photooxidation of asparaginase in the presence of methylene blue causes the reduction of the antigenicity(85%) and a complete loss of the enzymic activity, accompanied with photooxidation of histidine, methionine and tyrosine residues and with the reduction of α-helix content of asparaginase. Makino et al.(8) reported that acetylation of amino groups in asparaginase gives rise to the reduction of antigenicity(20%) without conformational change.

Recently, Abuchowski et al.(12,13) reported that covalent attachment of serum albumin or catalase to polyethylene glycol, nonimmunogenic polymer,

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leads to a complete loss of its antigenicity with retaining its enzymic activity. Their reports led to the present study on the reduction of the antigenic reactivity of L-asparaginase towards anti-asparaginase serum, with a hope of a possible clinical use of the modified asparaginase.

MATERIALS AND METHODS

A crystalline L-asparaginase(EC 3.5.1.1) obtained from E. coli A-1-3 was kindly gifted from Kyowa Hakko Kogyo Co. and its specific activity was 200 IU/mg of protein. Monomethoxypolyethylene glycols of 750, 1900 and 5000 daltons were purchased from Polyscience Inc. and Union Carbide Co.

The modification of asparaginase was carried out by the modified method of Abuchowski et al.(12); monomethoxypolyethylene glycol(1.9 mmoles) was coupled with cyanuric chloride(5.7 mmoles) to form 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine, activated polyethylene glycol. To an asparaginase solution(10 mg) in 0.1 M borate buffer(pH 9.2) was added activated polyethylene glycols with molecular weights of 750, 1900 and 5000. The mixture was incubated for 1 hr at 4°C and then filtrated with an ultrafiltration apparatus and an XM-50 membrane to remove free activated polyethylene glycol.
The modified asparaginases were synthesized by changing the molar ratio of activated polyethylene glycol(PEG) to amino groups in the asparaginase molecule(PEG/-NH_2; 0.5, 1.0, 3.0, 5.0 and 10.0). The total amino groups in the asparaginase molecule is 92, including 88 ε-amino groups in lysine residues and 4 terminal amino groups(14). The degree of modification of amino groups in the molecule was determined by measuring the amount of free amino groups with trinitrobenzene sulfonate(15). Protein concentrations were determined by the biuret method(16).

The enzymic activity of asparaginase was determined spectrophotometrically by using a synthetic substrate, aspartic acid-β-p-nitroanilide, ANA(17), or by measuring the amount of asparty1-β-hydroxamate formed from L-asparagine and hydroxylamine by the action of the enzyme(18). The quantitative precipitin reaction curve was obtained by the method of Kabat and Mayer(19). Anti-asparaginase serum was obtained from rabbit immunized three times by subcutaneous injection of asparaginase(3.3 mg x 3) and stored at -20°C(8). Modified and non-modified asparaginases were digested with trypsin(crystallized, Sigma)(weight ratio, 1:300) for a given time at pH 7.3 and 37°C. The digestion was stopped by the addition of trypsin inhibitor(Miles Lab.) and the enzymic activity of asparaginases was determined. Chromatography of modified asparaginase was carried out using Sephadex G-200 equilibrated with 15 mM phosphate buffer(pH 7.0) containing 150 mM sodium chloride.

RESULTS AND DISCUSSION

Native asparaginase was modified with polyethylene glycol of 5000 daltons in molar amounts equal to 0.5, 1.0, 3.0, 5.0 and 10.0 equivalents of the available amino groups in the asparaginase molecule. These modifications resulted in products with 18, 23, 38, 48 and 73 amino groups substituted by polyethylene glycol.

Native asparaginase and modified asparaginases prepared as above were

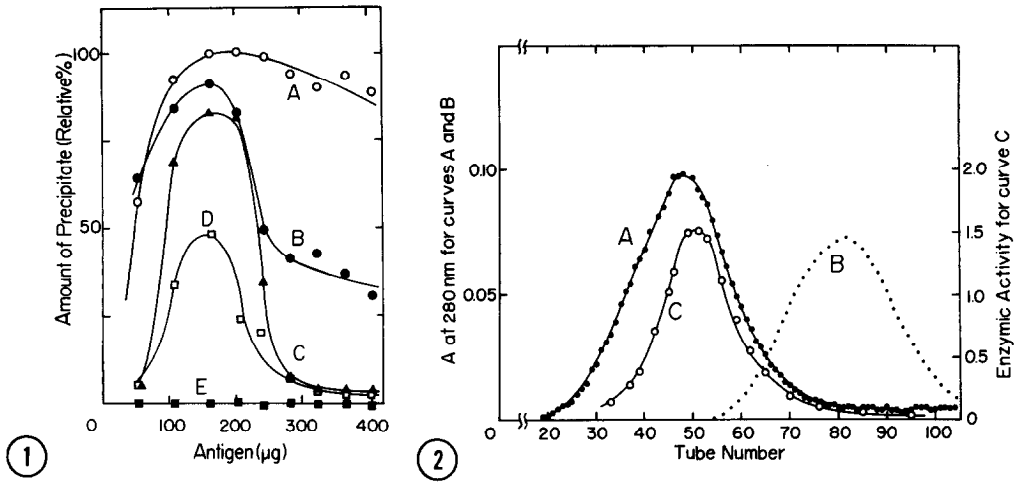

Fig. 1. Precipitin reaction of native asparaginase and the asparaginases modified with polyethylene glycol(mw; 5000) towards anti-asparaginase serum. Curve A; precipitin reaction curve of native asparaginase. Curves B, C, D and E; precipitin reaction curves of the modified asparaginases in which 18, 38, 48 and 73 amino groups out of the total 92 amino groups in the asparaginase molecule have been substituted by polyethylene glycol, respectively.

Fig. 2. Chromatogram of the modified asparaginase, in which 73 amino groups have been substituted by polyethylene glycol, using Sephadex G-200 column(2 x 40 cm). Curve A; elution pattern of the modified asparaginase(1.6 mg). Curve B; elution pattern of native asparaginase(1.5 mg). Curve C; enzymic activity measured for each protein fraction of the modified asparaginase eluted from Sephadex G-200 column. Elution was carried out with 15 mM phosphate buffer(pH 7.0) containing 150 mM NaCl.

subjected to studies on the binding ability of them with anti-asparaginase serum. The result is shown in Fig. 1. Curve A shows the precipitin reaction curve obtained for native asparaginase towards anti-asparaginase serum. The amount of precipitate, antigen-antibody complex, was increased markedly with raising asparaginase concentration and then gradually decreased. The maximum amount of precipitate was taken as 100%. The precipitin reaction curves obtained for modified asparaginases, in which 18, 38, 48 and 73 amino groups in the molecule have been substituted by polyethylene glycol of 5000 daltons, are shown by curves B, C, D and E, respectively. Increasing the degree of substitution of amino groups by polyethylene glycol resulted in the reduction of the binding ability of asparaginase against its antibodies.

Table 1. Immunogenic properties(binding ability towards anti-asparaginase serum), enzymic activities and degrees of substitution of amino groups by polyethylene glycol(PEG)(mw; 5000) of modified asparaginases

Molar Ratio PEG/-NH_2	n[a]	Enzymic Activity(%)		Binding Ability[d](%)
		Asn[b]	ANA[c]	
0	0	100	100	100
0.5	18	40	50	90
1.0	23	25	37	94
3.0	38	6	22	83
5.0	48	7	17	39
10.0	73	7	15	0

a] Number of amino groups substituted by polyethylene glycol in the asparaginase molecule. The total number of amino groups in the molecule is 92.
b] Determined by measuring the formation of asparatyl hydroxamate.
c] Determined by using aspartic acid β-p-nitroanilide as a synthetic substrate.
d] Relative value of the amount of the maximum precipitate on each precipitin reaction curve in Fig. 1.

No binding ability was observed for one of the modified asparaginases, in which 73 amino groups had been substituted(curve E).

The enzymic activity of the modified asparaginase was measured by two separate methods. Although the enzymic activity was decreased by increasing the degree of modification, the modified asparaginase in which 73 amino groups had been substituted retained the enzymic activity above 7% of non-modified asparaginase. These results are summarized in Table 1.

The effects of the molecular weight of polyethylene glycol on the binding ability of modified asparaginase towards anti-asparaginase serum was tested. The result is shown in Table 2. Polyethylene glycol with the molecular weight of 750 or 1900 was not effective for preparing the modified asparaginase with a complete loss of the binding ability, although the degree of modification of amino groups was almost the same as that obtained for polyethylene glycol of 5000 daltons.

In order to check a homogenity of the asparaginase modified with poly-

Table 2. Effect of molecular weight of polyethylene glycol(PEG) on the binding ability and the enzymic activity of the modified asparaginases.

PEG(mw)	Molar Ratio PEG/-NH_2	n[a]	Enzymic Activity(%) Asn[b]	Enzymic Activity(%) ANA[c]	Binding Ability(%)[d]
750	10	77	12	20	83
1900	10	70	14	25	40
5000	10	73	7	15	0

a], b], c] and d]; See Table 1.

ethylene glycol of 5000 daltons, in which 73 amino groups had been substituted, the modified asparaginase was subjected to chromatography with Sephadex G-200 column. Its elution pattern is shown by curve A in Fig. 2, in which a single sharp band appeared with a peak at the tube number of 50. Curve B shows the elution pattern of native asparaginase obtained under the same experimental condition as above and its peak position was quite different from that obtained for the modified asparaginase. From the position of the elution pattern, the molecular weight of the modified asparaginase was assumed to be approximately 500,000, which is far greater than that of native asparaginase, 136,000. The enzymic activities measured for each protein fraction of the modified asparaginase eluted from Sephadex G-200 column are shown by curve C. Its profile was in good agreement with the elution pattern obtained for the modified asparaginase(curve A). The above result indicates that the modified asparaginase, in which 73 amino groups have been substituted, is a homogeneous preparation with the enzymic activity and does not contain native asparaginase as a contaminant.

Modification of amino groups in the asparaginase molecule with polyethylene glycol give rise to the high resistivity against trypsin. The result is demonstrated in Fig. 3. Native asparaginase and modified asparaginase in which 73 amino groups were substituted by polyethylene glycol of 5000 daltons were digested with trypsin. The enzymic activity of native asparaginase

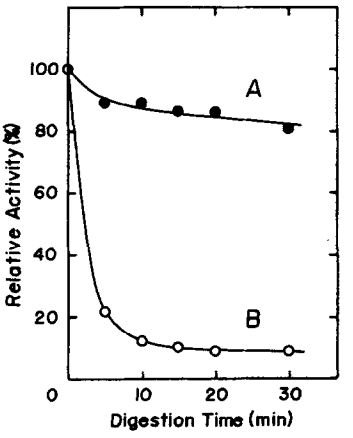

Fig. 3. Resistivity of the modified asparaginase in which 73 amino groups have been substituted by polyethylene glycol(mw; 5000) against trypsin. Curve A; the modified asparaginase. Curve B; non-modified asparaginase. Digestion with trypsin was carried out at pH 7.3 and 37°C.

was rapidly reduced with time(curve B), while that of the modified asparaginase was retained more than 80% of the original activity(curve A).

With the purpose of clinical use of the modified asparaginase without antigenicity and with enzymic activity, various modified asparaginases have been prepared in our laboratory for past five years, applying chemical modification reagents such as acetylimidazole, acetic anhydride, glyoxal, hydrogen peroxide, methyl-p-nitrobenzene sulfonate and 2-hydroxy-5-nitrobenzylbromide(8-11). A partial reduction of the antigenicity was observed for each modified asparaginase, accompanied with a complete loss of the enzymic activity. The preparation of a modified asparaginase, which has no antigenicity, retains the enzymic activity and has resistivity against trypsin, may be useful in patients with lymphoblastic leukemia and the modified asparaginase obtained in the present study may be currently available in a medical field.

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