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MODIFICATION OF E. COLI ASPARAGINASE WITH 2,4-BIS(O-METHOXYPOLYETHYLENE GLYCOL)-6-CHLORO-S-TRIAZINE(ACTIVATED PEG_2); DISAPPEARANCE OF BINDING ABILITY TOWARDS ANTI-SERUM AND RETENTION OF ENZYMIC ACTIVITY

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Asparaginase from E. coli was modified with activated PEG_2 which was synthesized from monomethoxypolyethylene glycol and cyanuric chloride. The PEG_2-modified asparaginase had no binding ability towards anti-asparaginase serum but retained 11% of the enzymic activity(GOT method) of the native asparaginase.

Various chemical modifiers such as amino acid polymer[1], polysaccharide[2], lipid[3] and polyalcohol[4,5] have been used on protein antigens including allergens to alter their immuno responses. However, only limited number of successful attempts has been presented as far as modifications of active enzymes are concerned. In an earlier paper dealing with E. coli asparaginase[6], which has been used as an anti-tumor agent, modified with 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine (activated PEG_1), it was shown that modified asparaginase has no binding ability towards anti-asparaginase serum but a considerable enzymic activity. However, it was found later that the remaining enzymic activity was almost negligible as measured by GOT method which should reflect most appropriately the physiological circumstances.

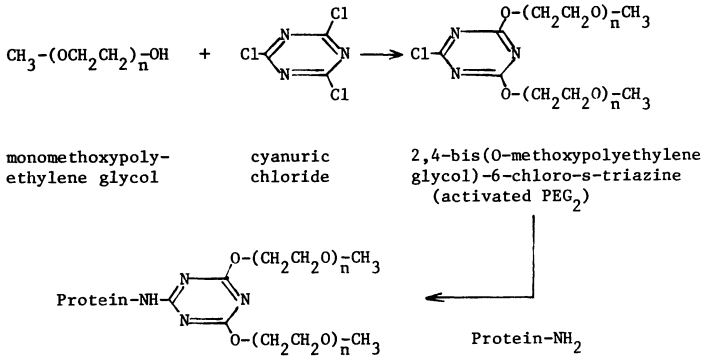
In order to obtain non-immunoreactive asparaginase but with high enzymic activity (by GOT method), 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine(activated PEG_2) was used as a new modifying reagent in the present study. Modification of amino groups(possibly also tyrosine residues) in asparaginase with activated PEG_2 having two polyethylene glycol chains is far more effective than with activated PEG_1 having only one polyethylene glycol chain in the molecule.

The present study deals with the preparation of non-immunoreactive asparaginase retaining the high enzymic activity by modification with a new reagent, activated PEG_2, and may give a clue to the development of enzyme therapy.

A sample of crystalline L-asparaginase(EC 3.5.1.1) from Escherichia coli A-1-3 was a gift from Kyowa Hakko Kogyo Co. and its specific activity was 262 IU/mg of protein. Monomethoxypolyethylene glycol(MW 5,000) was purchased from Polyscience Inc.

Activated PEG_2 was synthesized as follows(Scheme 1); 20 g of monomethoxypolyethylene glycol was dissolved in 100 ml of dried benzene containing 5 g of Molecular Sieve 3A and 10 g of anhydrous sodium carbonate, and then 365 mg of cyanuric

chloride was added. The mixture was refluxed at 80° C for 44hr under vigorous stirring and crude activated PEG_2 solution was obtained by decantation. Activated PEG_2 was precipitated by adding 200 ml of petroleum ether and was filtered. Purification procedure was repeated six times to remove unreacted cyanuric chloride and its hydrolyzates. The content of chlorine in the activated PEG_2 was determined by Mohr method[7], and it was found that two out of three chlorine atoms in the cyanuric chloride molecule were substituted with monomethoxypolyethylene glycol under the condition described above.



Scheme 1

Modification of the protein with activated PEG_2 was carried out as follows; Activated PEG_2 was added to 2 ml of asparaginase solution(5 mg/ml) in 0.1 M borate buffer(pH 10.0) and the mixture was stirred at 37° C for 1hr. To the sample solution was added 80 ml of cold phosphate buffered saline(pH 7.0) to stop the reaction. The reaction mixture was filtered with an ultrafiltration apparatus with A-50T membrane to remove free activated PEG_2. The degree of modification of amino groups in the asparaginase molecule was determined by measuring the amount of free amino groups with trinitrobenzene sulfonate[8]. The protein concentration was determined by Biuret method[9].

The enzymic activity of asparaginase was determined by measuring the absorbance decrease at 340 nm of NADH due to formation of malate from L-asparagine caused by the enzymic action of glutamate-oxalacetate transaminase and malate dehydrogenase (GOT method)[10]. The quantitative precipitin reaction curve was obtained by the method of Kabat and Mayer[11]. Anti-asparaginase serum was obtained from rabbits immunized three times by subcutaneous injection of the enzyme(3.3 mg x 3) and stored at -20° C[12].

Modified and non-modified asparaginases were digested with trypsin(weight ratio, 100:1) at pH 8.0 and 37° C. The digestion was stopped by adding soybean trypsin inhibitor(Miles Lab.). Chromatography of modified asparaginase was carried out using Sepharose 4B equilibrated with 15 mM phosphate buffer(pH 7.0) conatining 150 mM sodium chloride.

Native asparaginase was modified with activated PEG_2. Figure 1 shows the precipitin reaction curves obtained for native asparaginase(curve A) and modified asparaginases. Curves B, C, D and E represent the precipitin reaction curves for asparaginases in which 33, 48, 50 and 52 amino groups out of the total 92 amino groups[13] have been modified with activated PEG_2, respectively. Increasing the degree of modification of amino groups in asparaginase resulted in the reduction of the binding ability. The asparaginase in which 52 amino groups had been modi-

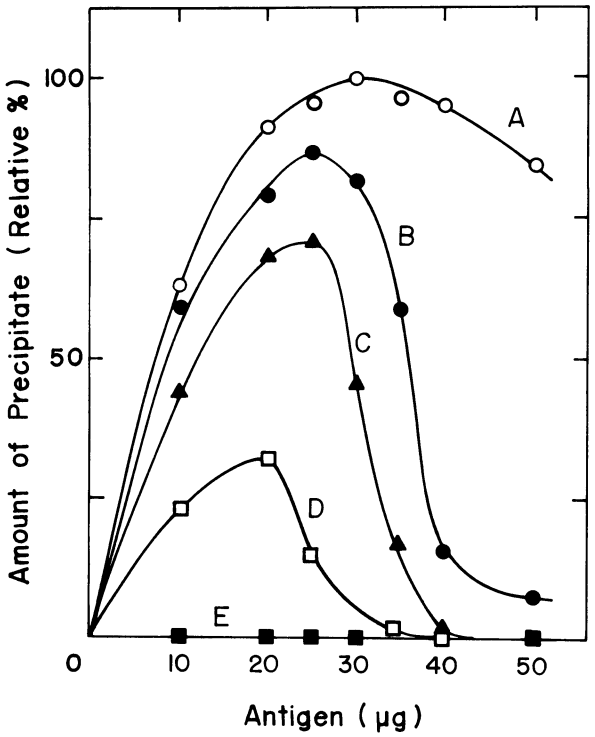


Fig. 1. Precipitin reaction curves of asparaginase(curve A) and asparaginase modified with activated PEG_2 towards anti-asparaginase serum. Curves B, C, D and E: Modified asparaginases in which 33, 48, 50 and 52 amino groups out of the total 92 amino groups in the molecule were modified.

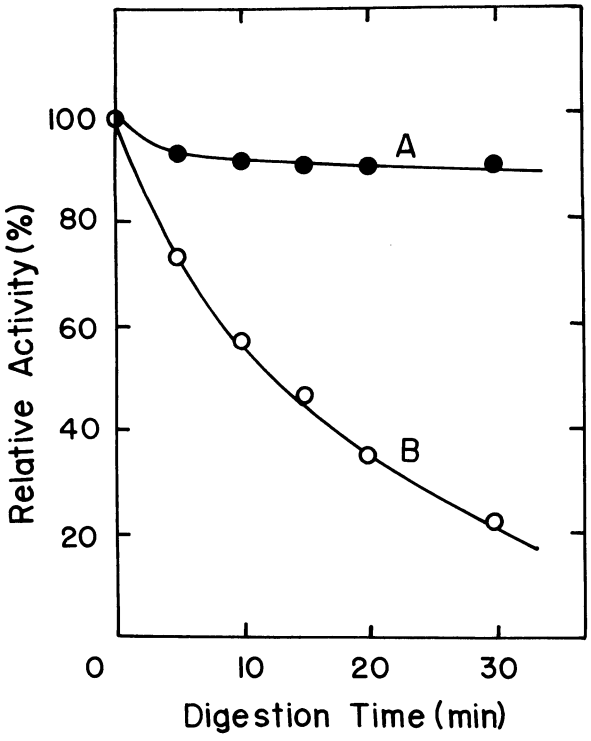


Fig. 2. Resistance to trypsin of the modified asparaginase in which 52 amino groups were modified with activated PEG_2. Curve A; modified asparaginase. Curve B; native asparaginase. Digestion with trypsin was carried out at pH 8.0 and 37° C.

Table 1. Binding ability of modified asparaginase with activated PEG_2(MW: 5,000 x 2) towards anti-asparaginase serum and its enzymic activity together with degree of modification of amino groups in the molecule.

Molar ratio, PEG_2/NH_2	n[a]	Enzymic activity[b] (%)	Binding ability[c] (%)
0	0	100	100
4	33	30	86
10	48	20	72
12.5	50	14	32
15	52	11	0

a] Number of amino groups modified with activated PEG_2 in the asparaginase molecule.
b] Determined by measuring NADH with glutamate-oxalacetate transaminase(GOT method).
c] Relative value of the amount of the maximum precipitate on each precipitin reaction curve(see Fig. 1).

fied with activated PEG_2, had no binding ability.

The enzymic activity of each modified asparaginase is shown in Table 1, in which the binding ability and the degree of modification are also summarized. The loss of the enzymic activity of asparaginase by the modification proceeded parallel to the increment of the degree of modification. The asparaginase in which 52 amino groups had been modified with activated PEG_2 was more than 10 times as active as that modified with activated PEG_1 when the enzymic activity was determined by GOT

method.

This modified asparaginase solution was passed through a column of Sepharose 4B. A sharp band appeared on the chromatogram and its elution position corresponded to the molecular weight of approximate 600,000, which agrees with the sum of molecular weight of asparaginase(136,000) and bound polyethylene glycol(5,000 x 2 x 52).

Native asparaginase and modified asparaginase with no binding ability were digested with trypsin and their enzymic activities were measured(Fig. 2). The modified asparaginase(52 amino groups were modified) retained 90% of the original activity after 30 min-digestion with trypsin(curve A). Digestion of non-modified asparaginase for 30 min with trypsin caused significant loss of the enzymic activity(curve B). The high resistance of the modified asparaginase against trypsin may be due to the modification of amino groups in lysine residues in asparaginase which are sites of cleavage by trypsin. The modified asparaginase should be much less vulnerable than the native enzyme to the digestion by trypsin-like enzymes present in plasma, which makes this procedure highly suitable for clinical application.

The data presented in this work support the notion that activated PEG_2, as a modifier, is superior to activated PEG_1 for preparing non-immunoreactive asparaginase with high enzymic activity. This may be due to the high effectiveness for masking or covering the antigenic site(s) with activated PEG_2 having two polyethylene glycol chains. The modified asparaginase thus prepared may be effective for treating patients with lymphoblastic leukemia, who have antibodies formed by injection of asparaginase.

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