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CHEMICAL MODIFICATION OF L-ASPARAGINASE WITH A COMB-SHAPED COPOLYMER OF POLYETHYLENE GLYCOL DERIVATIVE AND MALEIC ANHYDRIDE

Yoh Kodera, Hideki Tanaka, Ayako Matsushima and Yuji Inada

Department of Materials Science and Technology, Toin University of Yokohama, Kurogane 1614, Midori-ku, Yokohama 227, JAPAN

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Summary: L-Asparaginase from *Escherichia coli*, an anti-tumor enzyme, was chemically modified with two types of maleic anhydride copolymers with a comb-shaped form, the one composed of polyoxyethylene allyl methyl diether with the molecular weight of 13,000 (activated PM_{13}) and the other of polyoxyethylene 2-methyl-2-propenyl methyl diether with 100,000 (activated PM_{100}). The modified asparaginases (PM_{13}- and PM_{100}-asparaginases) exhibited the complete loss of immunoreactivity towards anti-asparaginase serum. The enzymic activity of PM_{100}-asparaginase without immunoreactivity was well retained by 85% of non-modified one, while that of PM_{13}-asparaginase was retained 46%. These results were discussed in relation to the chemical structure of modifying reagents including chain shaped-polyethylene glycol derivatives.

Chemical modification of protein drugs with polyethylene glycol derivatives leads to the prolongation of their blood circulation life-time as well as the reduction of their antigenicity and immunogenicity (1, 2). Polyethylene glycol derivatives, being non-toxic, non-immunogenic and amphipathic macromolecule, were extensively used as chemical modifying reagents. Among them, 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine, activated PEG_1, and 2,4-bis[*O*-methoxypoly(ethylene glycol)]-6-chloro-*s*-triazine, activated PEG_2, are chain-shaped modifying reagents which couple directly with amino groups on the surface of protein molecules (3, 4). Ono *et al.* synthesized activated PEG_2 without any contaminants including activated PEG_1 (5).

Quite recently, we explored a new modifying reagent with comb-shaped form, a copolymer of polyoxyethylene allyl methyl diether and maleic anhydride. Lipase from *Pseudomonas fluorescens* coupled with the modifying reagent catalyzed the ester synthesis reaction in organic solvents with high heat-stability (6). The modification of L-asparaginase with polyethylene glycol derivatives with a chain-shaped form had been extensively studied with the purpose of clinical use for the treatment of leukemia and sarcoma (7).

The present paper deals with the chemical modification of asparaginase with two types of copolymers composed of maleic anhydride and polyethylene glycol derivatives with a comb-shaped form in relation to the immunoreactivity and the enzymic activity of the modified asparaginases.

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Materials and Methods

Crystallized L-asparaginase (EC 3.5.1.1) from *Escherichia coli* was kindly gifted from Kyowa Hakko Kogyo Co. (Tokyo, Japan) It has 92 amino groups in the molecule with the molecular weight of 136,000 (8). Glutamate-oxalacetate transaminase and malate dehydrogenase were purchased from Boehringer Manheim GmbH (Manheim, Germany). Two types of comb-shaped copolymers were gifted from Nippon Fat and Oil Co., Ltd. (Tokyo, Japan) The chemical structure is shown in Figure 1. One designated as activated PM_{13} has n = 33, m = 8 and R = H with the average molecular weight of 13,000, which is the copolymer of polyoxyethylene allyl methyl diether and maleic anhydride. The other as activated PM_{100} has n = 40, m = 50 and R = CH_3 with 100,000, which is the copolymer of polyoxyethylene 2-methyl-2-propenyl methyl diether and maleic anhydride.

Preparation of PM-asparaginase: To 1.0 ml of asparaginase (4.0 mg/ml) dissolved in 0.5 M borate buffer (pH 8.5) was added a given amount (0 - 200 mg) of activated PM_{13} to form PM_{13}-asparaginase, and was stirred at 4°C for 1 hr. The reaction mixture was diluted with 100 ml of 50 mM borate buffer (pH7.0) and then the uncoupled activated PM_{13} was removed by ultrafiltration with Amicon Diaflo YM-100 membrane (Danvers, MS). The same procedure was performed with activated PM_{100}, except the ultrafiltration membrane Amicon Diaflo XM-300.

The degree of modification was determined by measuring the amount of free amino groups in the modified asparaginase with trinitrobenzene sulfonate (9). The protein concentration was determined by the biuret method (10). The enzymic activity of asparaginase was determined by measuring the absorbance decrease at 340 nm of NADH according to the GOT method (11). The quantitative precipitin reaction curve was obtained by the method of Kabat and Mayer (12). Anti-asparaginase serum was obtained from rabbits immunized three times by subcutaneous injection of the enzyme and stored at -80°C.

Results and Discussion

PM_{13}-asparaginases were prepared by coupling with activated PM_{13}. Using these PM_{13}-asparaginases, the quantitative precipitin reaction was conducted with anti-asparaginase serum. Figure 2a shows the quantitative precipitin curves of non-modified asparaginase (curve A) and of PM_{13}-asparaginases with 36, 43, 51 and 50% modification of amino groups in the asparaginase molecule (curves B, C, D and E, respectively). The immunoreactivity of asparaginase is markedly reduced by increasing the degree of modification of amino groups with activated PM_{13}, and is completely lost at approximately 50% modification of amino groups. Its enzymic activity is 45.5% of non-modified one. A similar line of study was conducted with activated PM_{100} in place of activated PM_{13}. PM_{100}-asparaginases, in which 20, 33 and 34% amino groups in the molecule were modified with activated PM_{100}, were subjected

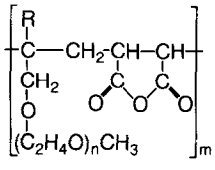

Fig. 1. Chemical structure of comb-shaped copolymer. Activated PM_{13}, copolymer of polyoxyethylene allyl methyl diether and maleic anhydride, n = 33, m = 8 and R = H. Activated PM_{100}, copolymer of polyoxyethylene 2-methyl-2-propenyl methyl diether and maleic anhydride, n = 40, m = 50 and R = CH_3.

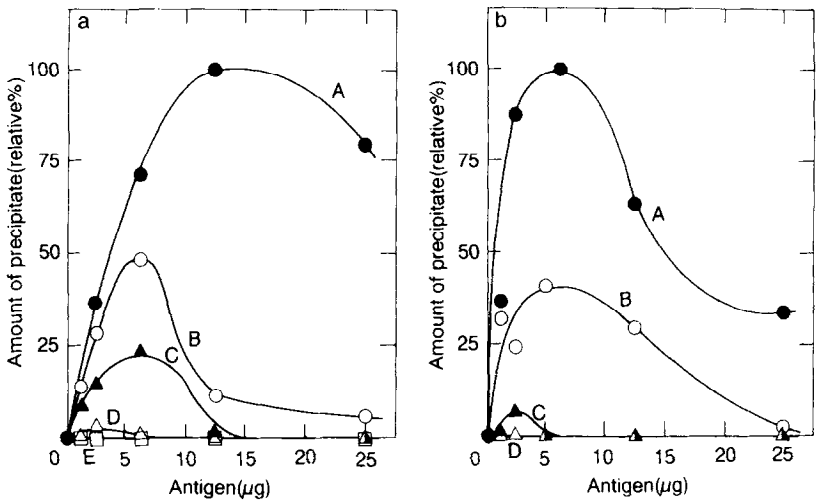

Fig. 2. Quantitative precipitin curves of PM_{13}- and PM_{100}-asparaginases towards anti-asparaginase serum. In the left side panel (**a**); **curve A**: non-modified asparaginase, **curves B, C, D,** and **E**: PM_{13}-asparaginases in which 36, 43, 51 and 50% amino groups in the molecule were modified. In the right side panel (**b**); **curve A**: non-modified asparaginase, **curves B, C** and **D**: PM_{100}-asparaginases in which 20, 33 and 34% amino groups were modified.

to the precipitin reaction. The immunoreactivity is completely lost at 34% modification as seen by curve D in Figure 2b. Its enzymic activity is well retained by 85.3%.

Figure 3 represents the degree of modification, the immunoreactivity and the enzymic activity of PM-asparaginases modified with activated PM_{13} (Figure 3a) or activated PM_{100} (Figure 3b). Various kinds of the modified asparaginase were synthesized by changing the molar ratio (PM_{13}/NH_2) of activated PM_{13} to an amino group in the

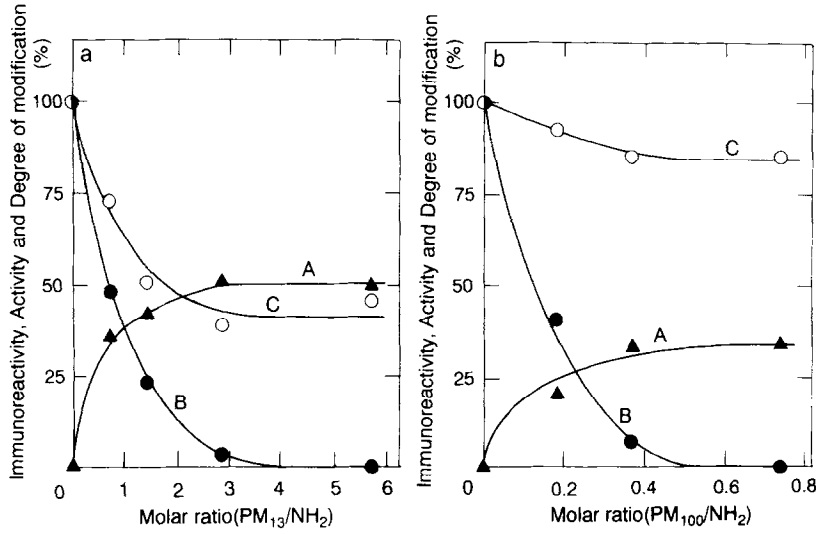

Fig. 3. Degree of modification, immunoreactivity and enzymic activity of modified asparaginases. The left side panel (**a**) and the right side panel (**b**); PM_{13}- and PM_{100}-asparaginases, respectively. **Curves A, B** and **C**: degree of modification, immunoreactivity and enzymic activity, respectively.

Table 1. Activity and immunoreactivity of *E. coli* asparaginase modified with polyethylene glycol derivatives

Modifying reagent (molecular weight)	Degree of modification (%)	Enzymic** activity (%)	Immuno-reactivity (%)	Ref.
non-modified asparaginase	0 (0)*	100	100	-
activated PEG_1 (5,000)	79 (73)	0.9	0	(3)
activated PEG_2 (10,000)	57 (52)	11.0	0	(4)
activated PM_{13} (13,000)	50 (46)	45.5	0	-
activated PM_{100} (100,000)	34 (31)	85.3	0	-

* Parentheses: number of amino groups coupled with each modifying reagent. Total number of amino groups in the asparaginase molecule is 92.
** Activity of the non-modified enzyme is 200 U/mg protein by GOT method.

asparaginase molecule. In case of PM_{13}-asparaginase, the degree of modification of amino groups (curve A) is enhanced by increasing the molar ratio. At the molar ratio, $PM_{13}/NH_2 > 3$, 50% of amino groups were modified with activated PM_{13}. The immunoreactivity is sharply decreased by increasing the degree of modification and approaches zero (curve B). The enzymic activity is retained by 45.5% although sharp decrease of activity is observed at lower ratio of PM_{13}/NH_2 (curve C). In case of PM_{100}-asparaginase (Figure 3b), the degree of modification reaches a constant level at the ratio, $PM_{100} > 0.4$, (curve A), in which approximately 34% of amino groups in the asparaginase molecule are modified with activated PM_{100}. Its immunoreactivity is completely lost (curve B), and the enzymic activity of PM_{100}-asparaginase is well held by 85.3% (curve C) which is approximately two times greater enzymic activity than 45.5% of PM_{13}-asparaginase. Furthermore, the degree of 34% modification with activated PM_{100} is less than that of 50% modification with activated PM_{13}.

Table 1 shows the degree of modification and the enzymic activity of the modified asparaginases when their immunoreactivities were completely lost by coupling with various kinds of the modifying reagents. The two comb-shaped copolymers of activated PM_{13} and PM_{100} were more effective than the chain-shaped polymers, activated PEG_1 and PEG_2 reported previously (3, 4). The former group made the modified asparaginase less number of amino groups modified and higher enzymic activity in comparison with the latter group. Comparing activated PEG_2 (mw: 10,000) with activated PM_{13} (mw:13,000) as modifying reagents with almost the same molecular weight, PM_{13}-asparaginase without immunoreactivity has higher enzymic activity (45.5%) at lower degree of modification (50%) than those (11.0% at 57%) of PEG_2-asparaginase without immunoreactivity. The comb-shaped polymers with many reactive groups of maleic anhydride (activated PM_{13} and activated PM_{100}) react directly with ε-amino groups in lysine residues and *N*-terminal amino groups in the asparaginase molecule to form internal crosslinkages of acid-amide bonds. Furthermore, they may cover the whole surface of asparaginase molecule by hydrogen bonds between side chains of amino acid residues (e. g., -COOH and -OH) and

oxygen atoms in polyethylene glycol chains. From the consideration mentioned above, it can be concluded that activated PM_{100} with a comb-shaped form is one of the superior modifying reagents for asparaginase with molecular weight of 136,000 to reduce its immunoreactivity and to retain its enzymic activity. This is supported by a report of Hershfield *et al.*, who introduced the additional amino groups to purine nucleoside phosphorylase by the site-directed mutagenesis for the attachment of polyethylene glycol on its whole surface and succeeded in the effective reduction of its immunoreactivity (13).

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