Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 287-291

REDUCTION OF IMMUNOREACTIVITY OF BOVINE SERUM ALBUMIN CONJUGATED WITH COMB-SHAPED POLYETHYLENE GLYCOL DERIVATIVES

Hideyuki Sasaki*, Yuko Ohtake, Ayako Matsushima, Misao Hiroto, Yoh Kodera and Yuji Inada

Human Science and Technology Center, Department of Materials Science and Technology, Toin University of Yokohama, 1614 Kurogane-cho, Midori-ku, Yokohama 225, JAPAN

*Yokohama Oils and Fats Industry Co., Ltd., 1-1 Minamisengen-cho, Nishi-ku, Yokohama 220, JAPAN

Received October 20, 1993

SUMMARY: Bovine serum albumin(BSA) was chemically modified with two types of comb-shaped copolymers of polyethylene glycol derivative and maleic anhydride, one with the molecular weight of 13,000(activated PM_{13}) and the other with 100,000(activated PM_{100}), to form PM_{13}- and PM_{100}-BSA. The immunoreactivity of BSA was markedly reduced by coupling with each modifier and was completely lost when 30% or 20% of amino groups in BSA were modified with activated PM_{13} or PM_{100}, respectively. The esterase activity of PM_{13}- and PM_{100}-BSA without immunoreactivity were retained 63% and 93% of non-modified one, respectively. These results were discussed with those of modified-asparaginases(1).

Chemical modification of proteins with synthetic or natural macromolecules induces the reduction of immunoreactivity and the prolongation of their blood circulation life-time(2, 3, 4). Polyethylene glycol with non-toxic, non-immunogenic and amphipathic properties has been extensively used as chemical modifying reagents, 2-[*O*-methoxypoly(ethylene glycol)]-4,6-dichloro-*s*-triazine(activated PEG_1) (5) and 2,4-bis[*O*-methoxypoly(ethylene glycol)]-6-chloro-*s*-triazine(activated PEG_2) (6). In a previous study, it was reported that BSA modified with activated PEG_2 exhibits the reduction of immunoreactivity towards anti-BSA serum(7).

Recently, the authors have explored a new type of modifier with a comb-shaped form, copolymer of polyethylene glycol and maleic anhydride, abbreviated as "activated PM".

The present paper deals with the chemical modification of BSA with two kinds of activated PM with molecular weight of 13,000, copolymer of polyoxyethylene allyl methyl diether and maleic anhydride(activated PM_{13}), and with molecular weight of 100,000, copolymer of polyoxyethylene (2-methyl-2-propenyl) methyl diether and maleic anhydride(activated PM_{100}).

0006-291X/93 $4.00

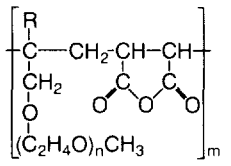

Fig. 1. Chemical structure of comb-shaped copolymers. Activated PM_{13}: copolymer of polyoxyethylene allyl methyl diether and maleic anhydride, n = 33, m = 8, R = H. Activated PM_{100}: copolymer of polyoxyethylene (2-methyl-2-propenyl) methyl diether and maleic anhydride, n = 40, m = 50 and R = CH_3.

Materials and Methods

Crystallized and lyophilized bovine serum albumin(BSA) was purchased from Sigma Chemical Co.(St. Louis, MO, USA) Two types of comb-shaped copolymers(activated PM_{13} and activated PM_{100}) were kindly gifted from Nippon Fat and Oil Co., Ltd.(Tokyo, Japan) Fig. 1 shows the chemical structure of activated PM_{13}(mw: 13,000, m=8, n=33, R=H) and activated PM_{100}(mw: 100,000, m=50, n=40, R=CH_3). These reagents react with mainly amino groups in the BSA molecule. Total amino groups in the BSA molecule are 60 including an *N*-terminal amino group. Activated PM_{13} was purified by gel permeation chromatography in chloroform with Bio-Beads S-X1(Bio Rad Laboratories Co. Ltd., Richmond, CA, USA) to remove a trace amount of monomeric form of polyethylene glycol derivatives. Anti-albumin serum from rabbits was donated from Dr. T. Koyama, University of Tsukuba. Other reagents used in this study were of analytical grade.

Preparation of PM-BSA: To 1.0 ml of BSA(4 mg/ml) dissolved in 0.2 M borate buffer (pH 9.5) was added a given amount(0-200 mg) of activated PM and was stirred at 4°C for 1 hr. After the reaction was completed, the mixture was diluted with 100 ml of phosphate-buffered saline, pH 7.4. The solution was ultrafiltrated through Amicon Diaflo XM-50 membrane(Dambers, MA, USA) for PM_{13} and through Advantec Ultra Filter UK-200(Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for PM_{100}.

The degree of modification was determined by measuring free amino groups in BSA molecule with 2,4,6-trinitrobenzenesulfonic acid(8). Protein concentration was determined by the biuret method(9). It is well-known that serum albumin exhibits the esterase-like activity although the activity is poor in comparison with esterolytic enzymes(10, 11). The esterase activity of BSA was measured with *p*-nitrophenyl acetate as a substrate(12). The immunoreactivity of PM-BSA towards anti-BSA serum was determined by the quantitative precipitin reaction by the method of Kabat and Mayer(13).

Results and Discussion

PM_{13}-BSA and PM_{100}-BSA were prepared by coupling with activated PM_{13} and PM_{100}, respectively. The immunoreactivity of BSA modified with each modifier towards anti-BSA serum was tested by the quantitative precipitin reaction. Fig. 2 shows these quantitative precipitin curves with PM_{13}-BSA(Fig. 2a) and PM_{100}-BSA(Fig. 2b). Curve A in Fig. 2 shows the quantitative precipitin curves obtained for non-modified BSA towards anti-BSA serum. The precipitin curves of PM_{13}-BSA in which 15.4, 22.8 and 29.5% of amino groups were modified with activated PM_{13} are shown by curves B, C and D in Fig.2a, respectively. The immunoreactivity of BSA is markedly reduced by increasing the degree of modification of amino groups with activated PM_{13}, and is completely lost at 29.5% modification of amino groups. Its enzymic activity was 63% of non-modified one. A similar line of study was conducted with activated PM_{100} in place

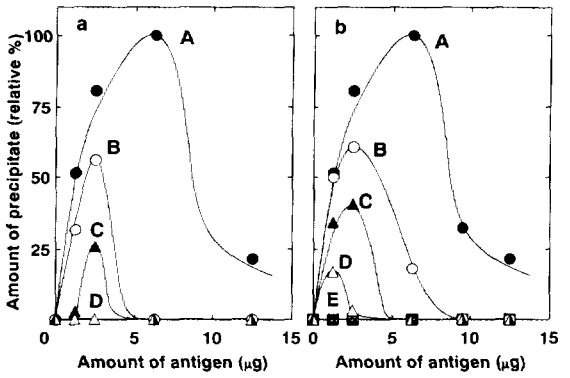

Fig. 2. Quantitative precipitin curves of PM_{13}- and PM_{100}-BSAs towards anti-BSA serum. In left panel(**a**); **curve A**: non-modified BSA, **curves B, C** and **D**: PM_{13}-BSA in which 15.4, 22.8 and 29.5% of amino groups in the molecule were modified with activated PM_{13}. In right panel(**b**); **curve A**: non-modified BSA, **curves B, C, D**, and **E**: PM_{100}-BSA in which 5.6, 10.9, 11.4 and 20.1% of amino groups were modified with activated PM_{100}.

of activated PM_{13}. PM_{100}-BSAs, in which 5.6, 10.9, 11.4 and 20.1% amino groups in the molecule were modified with activated PM_{100}, were subjected to the precipitin reaction. The immunoreactivity is completely lost at 20.1% modification as seen by curve E in Fig. 2b. Its enzymic activity is well retained by 93%. Fig. 3 represents the enzymic activity(curve A), the immunoreactivity(curve B) and the degree of modification(curve C) of PM-BSA modified with activated PM_{13}(Fig. 3a) or with activated PM_{100}(Fig. 3b). Various kinds of the modified BSAs were synthesized by changing the molar ratio($PM/-NH_2$) of activated PM to an amino group in the BSA molecule. In the case of PM_{13}-BSA, the degree of modification of amino groups(curve C) is enhanced by increasing the molar ratio(Fig. 3a). At the molar ratio, $PM_{13}/-NH_2$ = 1.6, 29.5% of amino groups were modified with activated PM_{13}. The immunoreactivity is sharply decreased by increasing the degree of modification and approaches

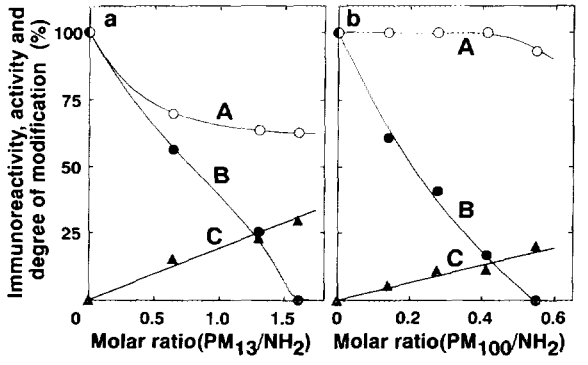

Fig. 3. Enzymic activity, immunoreactivity and degree of modification of modified BSAs. Left panel(**a**); PM_{13}-BSA. Right panel(**b**); PM_{100}-BSA. **Curves A, B** and **C**: enzymic activity, immunoreactivity and degree of modification of amino groups in BSA, respectively.

Table 1. Relation between enzymic activity, immunoreactivity and degree of modification obtained by BSA and asparaginase modified with comb-shaped modifiers and chain-shaped modifiers

Modifying reagent (molecular weight)	Degree of modification (%)	Enzymic activity (%)	Immuno-reactivity (%)	Ref.
Bovine Serum Albumin(BSA)				
non-modified BSA	0	100[a]	100	-
activated PEG_1 (5,000)	42	-[b]	0	(5)
activated PEG_2 (10,000)	25	63	0	(7)
activated PM_{13} (13,000)	30	63	0	-
activated PM_{100} (100,000)	20	93	0	-
Asparaginase				
non-modified asparaginase	0	100	100	-
activated PEG_1 (5,000)	79	0.9	0	(14)
activated PEG_2 (10,000)	57	11.0	0	(6)
activated PM_{13} (13,000)	50	45.5	0	(1)
activated PM_{100} (100,000)	34	85.3	0	(1)

[a] Activity of the non-modified BSA is 32μmol/min/g protein using *p*-nitrophenyl acetate as substrate.
[b] Hydrolytic activity of BSA was not measured.

zero(curve B). The enzymic activity is retained by 63% at the molar ratio of 1.6(curve A). In case of PM_{100}-BSA(Fig. 3b), 20.1% of amino groups in the BSA molecule are modified with activated PM_{100}, at the molar ratio, $PM_{100}/-NH_2$ = 0.55. Its immunoreactivity is completely lost(curve B), and the enzymic activity of PM_{100}-BSA is well retained by 93%(curve A) which is approximately 1.5 times higher enzymic activity than 63% obtained for PM_{13}-BSA. Furthermore, the degree of modification of amino groups, 20.1%, with activated PM_{100} is less than 29.5% with activated PM_{13} when the immunoreactivity is completely lost.

Table 1 shows the degree of modification and the enzymic activity of the modified BSA when their immunoreactivities were completely lost by coupling with various kinds of the modifying reagents(5, 7). It can be clearly concluded that activated PM_{100} with comb-shaped form is the superior modifying reagent for BSA to reduce its immunoreactivity and to retain its enzymic activity, comparing with activated PEGs with chain-shaped form.

In a previous study, we reported the chemical modification of asparaginase with two types of modifiers, activated PEGs and activated PMs. As is seen in Table 1, activated PM_{100} was also the superior modifier of asparaginase to reduce immunoreactivity and to retain high enzymic activity. BSA is a prolate ellipsoid with major and minor axes of 14.1 and 4.2 nm(15) and a single peptide chain with the molecular weight of 66,267(16). On the other hand, L-asparaginase is a globular protein(5.8 x 6.3 x 8.7 nm) (17) and consists of four identical subunits(18) with molecular weight of 34,000(19). The presumptive length of main chain of activated PM_{13} and activated PM_{100} are 4 nm (m = 8) and 26 nm (m = 50). Though BSA and asparaginase differ in molecular weight, overall dimensions and subunit structure each other, the length of PM_{100}(26nm) seems

to be enough to encircle the surfaces of asparaginase and BSA. Advantages of the copolymer of polyethylene glycol derivative and maleic anhydride with comb-shaped form as a modifying reagent are as follows; 1. Maleic anhydride in the modifier reacts directly amino groups located at the surface of protein molecule. 2. Polyethylene glycol chains in the modifier may cover effectively at the surface of the protein molecule.
3. By changing the degree of polymerization in activated PM molecule(m and n), the most suitable modifier towards a protein can be selected.

References

1. Kodera, Y., Tanaka, H., Matsushima, A., and Inada, Y. (1992) Biochem. Biophys. Res. Commun. **184**, 144-148.
2. Inada, Y., Matsushima, A., Kodera, Y., and Nishimura, H. (1990) J. Bioactive and Compatible Polym. **5**, 343-364.
3. Fuertges, F., and Abuchowski, A. (1990) J. Controlled Release **11**, 139-148.
4. Kamisaki, Y., Wada, H., Yagura, T., Matsushima, A., and Inada, Y (1981) J. Pharmacol. Exp. Ther. **216**, 410-414.
5. Abuchowski, A., Van Es, T., Palczuk, N. C., and Davis, F. F. (1977) J. Biol. Chem. **252**, 3578-3581.
6. Matsushima, A., Nishimura, H., Ashihara, Y., Yokota, Y., and Inada, Y. (1980) Chem. Lett. 773-776.
7. Matsushima, A., Sasaki, H., Kodera, Y., and Inada, Y. (1992) Biochem. Int. **26**, 485-490.
8. Habeeb, A. F. S. A. (1966) Anal. Biochem. **14**, 328-336.
9. Layne, E. (1957) Methods in Enzymology, **vol. 3**, pp. 450-451, Academic Press, New York.
10. Huggins, C., and Lapides, J. (1947) J. Biol. Chem. **170**, 467-482.
11. Nango, M., Kimura, Y., Kanda, S., Ihara, Y., Koga, J., and Kuroki, N. (1986) Chem. Lett. 229-232.
12. Heymann, E., and Mentlein, R. (1981) Methods in Enzymology, **vol. 77**, pp. 333-344, Academic Press, New York.
13. Kabat, E. A., and Mayer, M. M. (1961) Experimental Immunochemistry, 2nd edn., pp. 72-76, Thomas, Springfield.
14. Ashihara, Y., Kono, T., Yamazaki, S., and Inada, Y. (1978) Biochem. Biophys. Res. Commun. **83**, 385-391.
15. Wright, A. K., and Thompson, M. R. (1975) Biophys. J. **15**, 137-141.
16. Reed, R. G., Putnam, F. W., and Peters, T., Jr. (1980) Biochem. J. **191**, 867-868.
17. Itai, A., Yonei, M., Mitsui, Y., and Iitaka, Y. (1976) J. Mol. Biol. **105**, 321-325.
18. Greenquist, A. C., and Wriston, J. C. (1972) Arch. Biochem. Biophys. **152**, 280-286.
19. Maita, T., Morokuma, K., and Matsuda, G. (1974) J. Biochem. **76**, 1351-1354.