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# MODIFICATION OF AMINO GROUPS IN PORCINE PANCREATIC ELASTASE WITH POLYETHYLENE GLYCOL IN RELATION TO BINDING ABILITY TOWARDS ANTI-SERUM AND TO ENZYMIC ACTIVITY

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Summary: Porcine pancreatic elastase was modified by activated polyethylene glycol (2-O-methoxy-polyethyleneglycol-4, 6-dichloro-s-triazine) with molecular weight of 5000. The modification of elastase in which three amino groups out of the total four amino groups in the molecule gave rise to a complete loss of the binding ability towards anti-elastase serum from rabbit. The modified enzyme showed 35% of the original enzymic activity towards succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide and 17% towards casein. The heat-denatured collagen was also digested by the modified elastase, but the enzymic activity towards the elastin substrate was completely lost. The inhibition of the modified elastase activity by $\alpha_2$-macroglobulin was found to be lesser than that of non-modified elastase.

Elastase (EC 3.4.21.11) is a unique enzyme among proteolytic enzymes of animal tissues because it has an ability to hydrolyse elastin, a fibrous insoluble protein of connective tissue (1). Because of its unique substrate specificity, many studies on the participation of elastase in arterial connective tissue metabolism have been reported with a view to atherosclerosis (2), aging (3) and its possible involvement in connective tissue diseases (4). Recently several reports (5, 6, 7) have been presented on the effects of

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porcine pancreatic elastase on rabbits with atherosclerosis experimentally induced by cholesterol feeding, showing a possible medicinal use of elastase.

Abuchowski et al. (8, 9) and Inada et al. (10) have shown that conjugation of polyethyleneglycol, non-immunogenic polymer, to catalase or L-asparaginase leads to a complete loss of its antigenicity with retaining its enzymic activity towards the low molecular weights of substrates. Recently we have also reported the modification of streptokinase with polyethyleneglycol (11).

In this report, we present that the modification of porcine pancreatic elastase with polyethyleneglycol of molecular weight of 5000 completely diminished the antigenic reactivity of enzyme towards the anti-serum with retaining its enzymic activity towards not only the low molecular weights of substrates, but also the high molecular weights of substrates, indicating a possibility of non-oral administration of the modified enzyme for a medicinal purpose. The results also provide some interesting information for the structure-activity relationsip of elastase.

## MATERIALS AND METHODS

Porcine pancreatic elastase (EC 3.4.21.11) was prepared by the method of Smillie and Hartley (12). $\alpha_2$-macroglobulin was prepared according to the combination of the methods previously reported by Kobayashi and Nagasawa (13) and by Virca et al. (14). Fresh human plasma was fractionated by differential precipitation with polyethyleneglycol #4000 (average molecular weight: 3000-3700). The fraction precipitated at 8-19%, where $\alpha_2$-macroglobulin was concentrated, was directly subjected to Blue Sepharose column chromatography. Homogeneity of $\alpha_2$-macroglobulin was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of Laemmli (15) and by immunoelectrophoresis of Scheidegger (16). Heat-denatured collagen was prepared according to Kobayashi and Nagai (17).

Monomethoxypolyethylene glycol (mw; 5000) was purchased from Polyscience Inc.. Anti-elastase serum was obtained from rabbit immunized four times by subcutaneous injection of elastase

(3.0 mg × 4) and stored at -20°C. Anti-human serum from horse and anti-human $\alpha_2$-macroglobulin from rabbit were purchased from Behringewerke.

The modification of elastase was carried out by the method of Abuchowski et al (8) as follows; monomethoxypolyethylene glycol (0.009 moles) was coupled with cyanic chlorides (0.0027 moles) to form 2-0-methoxypolyethylene glycol-4, 6-dichloro s-triazine, activated polyethyleneglycol. To an elastase solution (20 mg) in 0.1 M borate buffer (pH 9.2) was added activated polyethyleneglycol with molecular weight of 5000. The mixture was incubated for 1 hr at 4°C and then filtered with an ultrafiltration apparatus with XM-50 membrance to remove unreacted activated polyethyleneglycol. Several differently modified elastase preparations were prepared by changing the molar ratio of activated polyethyleneglycol (PEG) to amino groups in elastase molecule (PEG/-$NH_2$: 3.0, 5.0, 10.0 and 20.0). The total amino groups in the elastase molecule are 4, including $\varepsilon$-amino groups of lysine residues and 1 $\alpha$-amino group (18).

Degree of the reaction was determined by measuring the amount of free amino groups with trinitrobenzene sulfonate (19). Protein concentrations were determined using an extinction coefficient, $E^{1\%}_{cm}$ at 280 nm of 20.2 (18).

Elastase activity towards low molecular weight substrate was determined spectrophotometrically by using a synthetic substrate, succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (Suc-(Aℓa)$_3$-pNA), according to Bieth et al. (20). Elastase activity toward high molecular weight substrate was determined with elastin as a substrate according to Grant and Robbins (21). Caseinolytic activity was determined according to Kunitz (22). Activity of $\alpha_2$-macroglobulin was determined spectrophotometrically by using a synthetic substrate, Nα-benzoyl-D, L-arginine-p-nitroanilide; by measuring p-nitroanilide liberated by the action of trypsin-$\alpha_2$-macroglobulin complexes after inhibition of the residual trypsin by soybean trypsin inhibitor, because trypsin-$\alpha_2$-macroglobulin complexes can act on the low molecular weight substrates (23).

Quantitative precipitin reaction curve was obtained by the method of Kabat and Mayer (24).

Digestion of heat-denatured collagen with the modified elastase was carried out as follows; a mixture of 100 μℓ of 0.2% acid soluble collagen and 2 μg of the modified elastase was incubated for 6 hrs at 37°C. Then, the sample solution was boiled for 3 min in the presence of 1% SDS and an aliquot (20 μℓ) was applied to SDS-polyacrylamide gel electrophoresis according to Laemmli (15).

Interaction of $\alpha_2$-macroglobulin with the modified and non-modified elastase was examined as follows; a mixture of 25 μℓ of $\alpha_2$-macroglobulin, which can inhibit 2.5 μg of bovine trypsin, and 1 μg of either non-modified elastase or modified elastase, in 1 mℓ of 0.05 M tris-HCℓ buffer, pH 8.0 was pre-incubated at 37°C. Then, 1 mℓ of 2 mM suc-(Aℓa)$_3$-pNA was added and the mixture was incubated for 5 min for native elastase and 10 min for modified elastase at 37°C. After 1 mℓ of 1 M acetic acid was added to stop the reaction, the absorbance at 410 nm was measured.

Chromatography of modified and non-modified elastase were carried out using Sephacryl S-200 column (1.0 × 120 cm) using 0.2 M tris-HCℓ buffer, pH 8.5 containing $5 \times 10^{-3}$ M $CaCℓ_2$ at 10 mℓ/hr in the cold room.

## RESULTS AND DISCUSSIONS

Figure 1 shows the immuno-precipitin reaction curves obtained with native elastase (curve A) and the modified elastases. The modified elastase with 3 amino groups substituted with activated PEG (the modified elastase $E_3$) (curve D), showed almost a complete loss of the binding ability against anti-elastase serum. Curve E represents the precipitin reaction curve with the purified modified elastase $E_3$ by Sephacryl S-200 column chromatography (Fig. 3). As shown by curve E, it is evident that the modified elastase (the modified elastase $EC_3$) has no binding ability.

Enzymic activity of the modified elastase was measured by two separate methods. In table 1, those activities towards suc-(Aℓa)$_3$-pNA as a synthetic substrate and towards elastin as a natural substrate, the binding ability against the anti-serum and the degree of the modification of each modified elastase are summarized. When the modified elastase $E_3$ was purified by

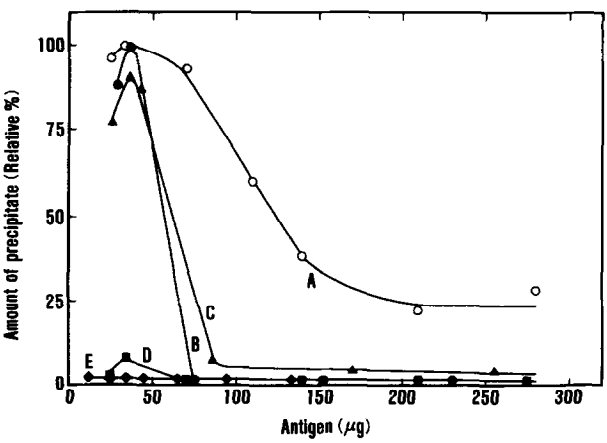


Fig. 1. Precipition reaction of native elastase and elastase modified with polyethylene glycol (mw;5000) towards anti-elastase serum.
Curve A; precipitin reaction curve of native elastase.
Curve B,C and D; precipitin reaction curves of the modified elastases with 1 (the modified elastase $E_1$), 2 (the modified elastase $E_2$) and 3 substituted amino groups (the modified elastase $E_3$), respectively. Curve E; precipitin reaction curve of the modified elastase with 3 substituted amino groups purified on a Sephacryl s-200 column (tube no. 24-30; the modified elastase $EC_3$) in Fig. 3.

Table 1.

Binding ability towards anti-serum, enzymic activities and degree of substitution of amino groups by polyethyleneglycol (PEG) (m w; 5000) of the modified elastases

| Molar Ratio $PEG/NH_2$ | n[a] | Residual Enzymic Activity (%) | | Binding[c] Ability (%) |
|---|---|---|---|---|
| | | Elastin | STANA[b] | |
| 0 | 0 | 100 | 100 | 100 |
| 3.0 | 1 | 81 | 84 | 100 |
| 5.0 | 2 | 0 | 31 | 91 |
| 10.0 | 3 | 0 | 21 | 9 |
| | 3[d] | 0 | 35 | 0 |
| 20.0 | 4 | 0 | 3 | 0 |

a) Number of amino groups substituted by polyethyleneglycol. The total number of amino groups in the molecule is 4.
b) Determined by using a synthetic substrate, suc-(Aℓa)$_3$-pNA.
c) Relative value of the amount of the maximum precipitate shown in each precipitin reaction curve (Fig. 1).
d) Purified by Sephacryl S-200 column chromatography (Fig. 3).

Sephacryl S-200 column (Fig. 3), it showed no binding ability against anti-elastase serum but retained 35% of the activity of intact enzyme elastase towards a synthetic substrate, suc-(Aℓa)$_3$-pNA. On the other hand, the elastin hydrolysing activity of the modified elastase $EC_3$ was completely lost. The modified elastase with 4 substituted amino groups (the modified elastase $E_4$) showed almost a complete loss of the activity towards suc-(Aℓa)$_3$-pNA and a complete loss of the activity towards elastin, in addition to no binding ability against the anti-serum. It should be noted that the casein hydrolysing activity of the modified elastase $EC_3$ was 17% of that of the intact enzyme (data not presented in the table).

Figure 2 shows the SDS-polyacrylamide gel electrophoresis of heat-denatured collagen treated by the modified elastase $EC_3$. The result clearly indicates that heat-denatured collagen can be hydrolysed by the modified elastase. Both α and β-chains of the collagen were apparently cleaved by the modified elastase and

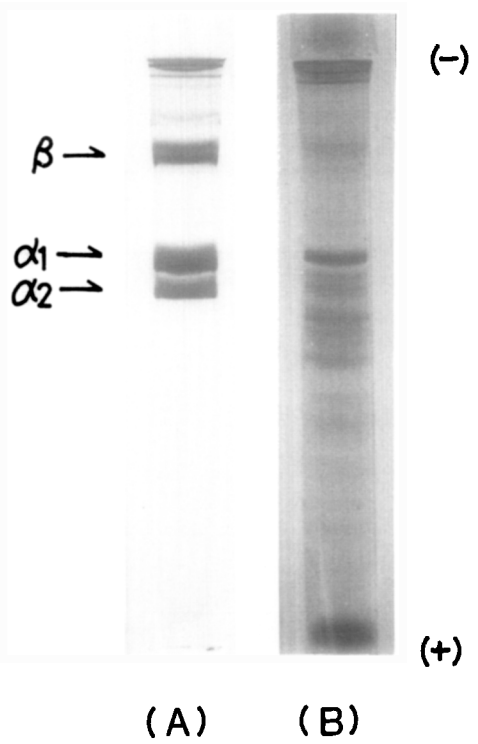


Fig. 2. Digestion of heat-denatured collagen with the modified elastase. A mixture of 100 μℓ of 0.2% acid soluble collagen and 2 μg of the modified elastase $EC_3$ was incubated for 6 hrs at 37°C. Then the sample solution was boiled for 3 min in the presence of 1% SDS and aliquot (20μℓ) was applied to SDS-polyacrylamide gel electrophoresis.

(A); heat-denatured collagen only.
(B); heat-denatured collagen incubated with the modified elastase

the increasing new bands with lower molecular weights were observed.

Studies with porcine pancreatic elastase have shown that the enzyme must be adsorbed on to insoluble elastin before hydrolysis can occur (25). This phenomenon takes place by the basicity of the enzyme and several basic proteins are also adsorbed on to elastin in the same way as elastase (25). Thus disappearance of the activity of the modified elastase towards elastin substrate, unlike to those activities towards suc-$(Ala)_3$-pNA, casein and heat-denatured collagen as substrates, probably is

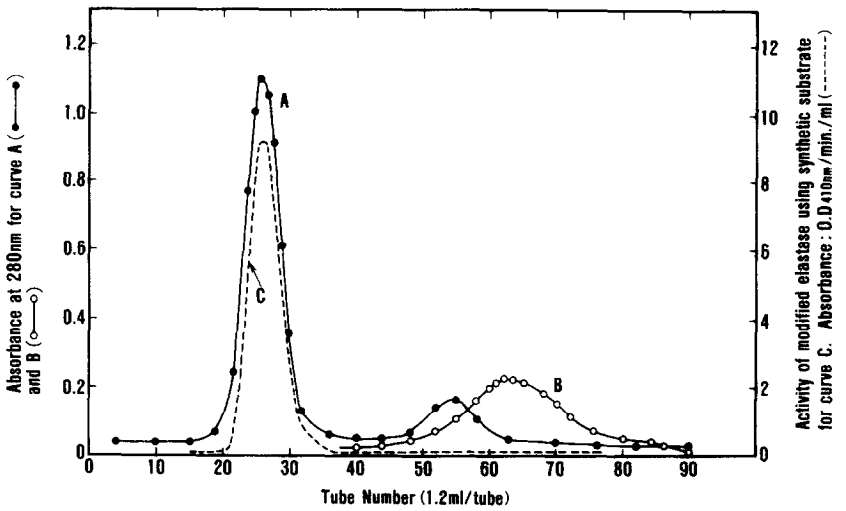


Fig. 3. Chromatogram of the modified elastase $E_3$ on a Sephacryl S-200 column (1.0 × 120 cm). Curve A; elution profile of the modified enzyme (27 mg). Curve B; elution profile of native enzyme (2.4 mg). Curve C; enzymic activity measured with the modified enzyme eluted from Sephacryl S-200. The column was equilibrated and eluted with 0.2 M tris-HCℓ buffer, pH 8.5, containing $5 \times 10^{-3}$M $CaCℓ_2$.

due to a loss of the adsorptive properties on to elastin molecule by the modification of basic groups in the molecule.

Figure 3 shows chromatography of the modified elastase $E_3$ on a Sephacryl S-200 column. Its elution profile is shown by curve A in Fig. 3. Two peaks were observed in the profile. The first peak around the tube numbers 24-30 showed enzymic activity towards suc-$(Aℓa)_3$-pNA but no activity was observed with the second peak, as shown in curve C. Curve B shows the elution profile of native elastase obtained under the same chromatographic conditions as above. Its elution profile was entirely different from that obtained with the modified elastase. The above results indicate that the modified elastase with 3 substituted amino groups is eluted at a unique position from Sephacryl S-200 column (tube no.24-30), excluding contamination of the native elastase and, therefore, must be a homogeneous preparation possessing suc-$(Aℓa)_3$-pNA, casein and heat-denatured collagen hydrolysing activities.

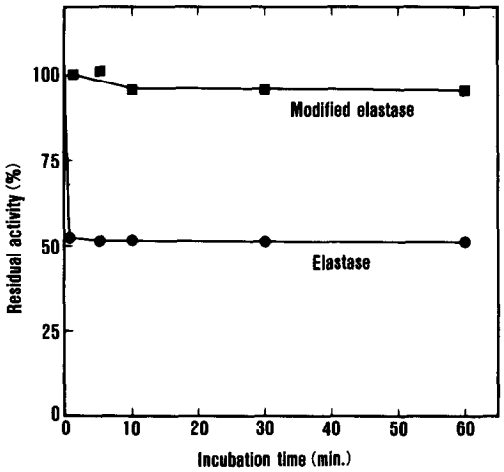


Fig. 4. Interaction of elastase and the modified enzyme with $\alpha_2$-macroglobulin.
(■——■); the modified elastase $EC_3$
(●——●); non-modified elastase

Figure 4 shows interaction of $\alpha_2$-macroglobulin with the modified and the non-modified elastase using suc-(Aℓa)$_3$-pNA as a substrate. The enzymic activity of the non-modified elastase was reduced to about 50% of its original activity by addition of $\alpha_2$-macroglobulin, while that of the modified elastase $EC_3$ was more than 95% of its original activity in the presence of the inhibitor. This modified elastase, showing a high resistance against the plasma proteinase inhibitor, probably can not readily form the complex with $\alpha_2$-macroglobulin, unlike to the non-modified elastase.

Unfortunately the modified elastase prepared in this study lost completely its elastin hydrolysing activity, but the preparation of the modified elastase, which has no binding ability against the anti-serum but retains the enzymic activities towards both the synthetic and the natural substrates such as suc-(Aℓa)$_3$-pNA, casein and heat-denatured collagen, may provide interesting informations with respect to possible medicinal applications and also the structure-activity relationship of the enzyme.

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