Preparation of polyethylene glycol-modified streptokinase with disappearance of binding ability towards anti-serum and retention of activity

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1. INTRODUCTION

Streptokinase is an extracellular protein produced during the growth of certain strains of haemolytic streptococci. It has the property of promoting clot lysis by activation of the fibrinolytic system of the human blood [1] and its clinical effect has been tried in the treatment of thrombic states in man [2].

Streptokinase is a strongly antigenic bacterial protein. So, freedom from antigenicity is particularly important in the event where retreatment with the streptokinase is required at > 2 weeks after the initial medication. The failure to meet this criterion is perhaps the greatest disadvantage associated with streptokinase.

In [3–5] conjugation of polyethylene glycol, a non-immunogenic polymer, to albumin, catalase or L-asparaginase leads to a complete loss of its antigenicity with retention of its enzymic activity.

We modified the streptokinase with polyethylene glycol (M_r 5000) and tried to see the effect of the modification on the reduction of the antigenic reactivity of the streptokinase towards anti-streptokinase serum. We aimed to obtain preparations that could be administered intravenously as a safety anti-thrombic drug.

Here, we describe the modified streptokinase with a complete loss of its antigenicity and with retention of its activity.

2. MATERIALS AND METHODS

A purified streptokinase (staphylokinase, EC 3.4.99.22) (Kabikinase) was kindly provided by AB KABI (Stockholm); spec. act. 628 IU/μg nitrogen. Monomethoxypolyethylene glycol (M_r 5000) was purchased from Polyscience Inc.

Streptokinase was modified as in [3]; monomethoxypolyethylene glycol (9 mmol) was coupled with cyanic chloride (2.7 mmol) to form 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*S*-triazine, activated polyethylene glycol. To a streptokinase solution (50 mg) in 0.1 M borate buffer (pH 9.2) activated 5000 M_r polyethylene glycol was added. The mixture was incubated for 1 h at 4°C, then filtered with an ultrafiltration apparatus with XM-50 membrane to remove free activated polyethylene glycol. The modified streptokinase were synthesized by changing the molar ratio of activated polyethylene glycol (PEG) to amino groups in streptokinase molecule (PEG/$-NH_2$: 0.5, 0.8, 1.0 and 3.0). The total amino groups in the streptokinase molecule are 34, including ε-amino group in lysine residues and 1 terminal amino group [6].

The degree of modification of amino groups in the molecule were determined by measuring the amount of free amino groups with trinitrobenzene sulfonate [7]. Protein concentrations were determined using an extinction coefficient, $E^{1\%}_{cm}$ at 278 nm of 19.4 [8].

The streptokinase activity on the low M_r substrates was determined spectrophotometrically using a synthetic substrate, H–D-Val–Leu–Lys –*p*-nitroanilide, by measuring the liberated *p*-nitroaniline by the action of plasminogen–streptokinase complex [9]. The fibrinolytic activity of the streptokinase was qualitatively determined by placing it directly in a bovine fibrin–agar plate using the kit for the determination of the fibrin-

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olytic assay, modified as in [10] and purchased from Kowa Co.

The quantitative precipitin reaction curve was obtained as in [11].

Anti-streptokinase serum was obtained from rabbit immunized 4 times by subcutaneous injection of streptokinase (3.0 mg × 4) and stored at − 20°C.

Modified and non-modified streptokinase were digested with trypsin (Sigma) at 1:500 (w/w) for a given time at pH 7.6 and 37°C: To 400 μg native streptokinase and 570 μg modified streptokinase in 2 ml 0.05 M Tris–HCl, (pH 7.6) containing 0.11 M NaCl, either 0.8 μg trypsin and 1.14 μg trypsin was added. The digestion was stopped by the addition of soybean trypsin inhibitor (Sigma) and the residual streptokinase activity determined.

Chromatography of modified and non-modified streptokinase was carried out using Sephacryl S-200 column (2.5 × 100 cm) using 0.2 M Tris–HCl buffer (pH 8.0) at 20 ml/h in the cold room.

3. RESULTS AND DISCUSSIONS

Fig.1 shows the precipitin reaction curves obtained for native streptokinase (curve A) and modified streptokinases. Curves B–D represent the precipitin reaction curves for modified streptokinases in which, 0, 0 and 8 amino groups out of the total 34 amino groups [6] have been modified with activated PEG, respectively. The streptokinase, in which 8 amino groups had been modified with activated polyethylene glycol, had no binding ability.

The activity of the modified streptokinase was measured by two separate methods. The residual streptokinase activity with H–D-Val–Leu–Lys–*p*-nitroanilide as a synthetic substrate, the binding ability and the degree of modification of each modified streptokinase are summarized in table 1. The modified streptokinase, in which 8 amino groups had been substituted, showed no binding ability against its antibody and retained the activity of 33% of non-modified streptokinase. However, the fibrinolytic activity of the modified streptokinase, in which 8 amino groups had been substituted, retained 20% of non-modified streptokinase activity (not shown). The modified streptokinase, in which 12 amino groups had been substituted, showed almost a complete loss of the activity with no binding ability against its antibody. Furthermore, a consider-

Table 1

Immunogenic properties (binding ability towards anti-streptokinase serum), streptokinase activity and degrees of substitution of amino groups by polyethylene glycol (M_r 5000) of modified streptokinase

Molar ratio REG/−NH_2	n[a]	Residual streptokinase activity[b] (%)	Binding[c] ability (%)
0	0	100	100
0.5	0	92	71
0.8	0	87	56
1.0	8	33	0
3.0	12	0.8	0

[a] Number of amino groups substituted by polyethylene glycol in the streptokinase molecule; total no. amino groups in the molecule is 34

[b] Determined by using H–D-Val–Leu–Lys–*p*-nitroanilide as a synthetic substrate

[c] Relative value of the amount of the maximum precipitate on each precipitin reaction curve in fig.1

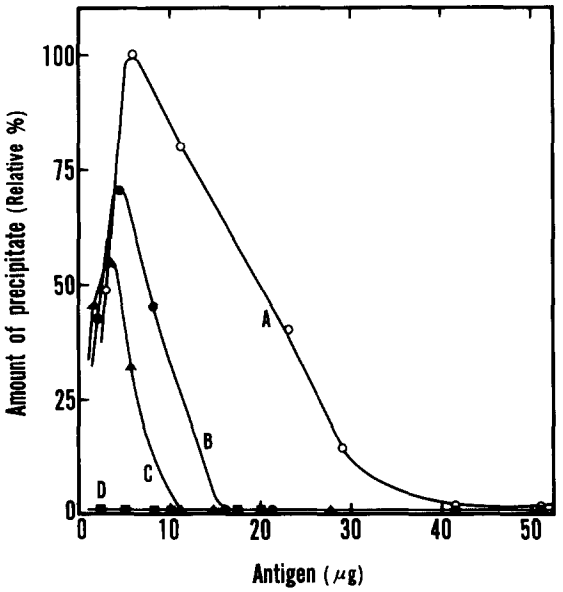

Fig.1. Precipitin reaction of native streptokinase and streptokinase modified with polyethylene glycol (M_r 5000) towards anti-streptokinase serum: (A) precipitin reaction curve of native streptokinase; (B–D) precipitin reaction curves of the modified streptokinases in which 0 (PEG/−NH_2, 0.5), 0 (PEG/−NH_2, 0.8) and 8 amino groups (PEG/−NH_2, 1.0) out of the total 34 amino groups in streptokinase molecule have been substituted by polyethylene glycol, respectively.

able amount of the reduction of the binding ability was observed for the preprarations obtained from the treatment with the molar ratio of PEG/$-NH_2$ of 0.5 and 0.8, though these preparations showed no modification of the amino groups in the molecule. This result suggests that the partially denatured streptokinases, in which 8% and 13% of their original activities have been reduced, may respond to their antibodies at a lower rate than the native streptokinase.

Fig.2 shows the chromatography of the modified streptokinase, in which 8 amino groups have been substituted, using Sephacryl S-200 column. Its elution pattern is shown in fig.2A. A single sharp band appeared with a peak at tube no. 46. Fig.2B shows the elution pattern of native streptokinase obtained under the same condition as above and its peak position was completely different from that obtained for the modified streptokinase. The streptokinase activity measured for each protein fraction of the modified streptokinase eluted from Sephacryl S-200 column using a synthetic substrate (fig.2C). Its profile was in good agreement with the elution pattern of the protein obtained for the modified streptokinase (fig.2A). The above result indicates that the modified streptokinase, in which 8 amino groups have been substituted, does not contain native streptokinase as a contaminant and must be a

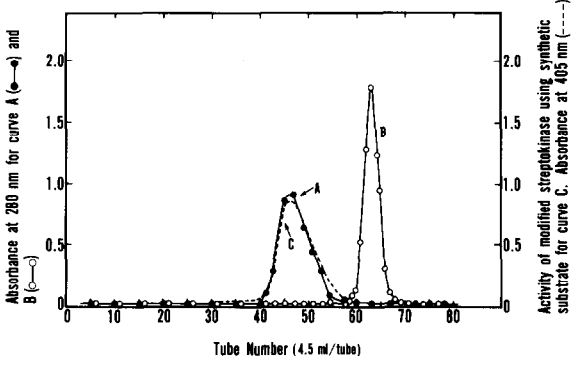

Fig.2. Chromatogram of the modified streptokinase, in which 8 amino groups have been substituted by polyethylene glycol, using Sephacryl S-200 column (2.5 × 100 cm): (A) elution pattern of the modified streptokinase (50 mg); (B) elution pattern of native streptokinase (50 mg); (C) streptokinase activity measured for each protein fraction of the modified streptokinase eluted from Sephacryl S-200. Elution was carried out with 0.2 M Tris–HCl buffer (pH 8.0).

homogeneous preparation with streptokinase activity. Sodium dodecyl sulfate gel electrophoresis and cellulose acetate membrane electrophoresis (0.05 M phosphate buffer, pH 7.0) also indicated that this modified streptokinase did not contain the native streptokinase as a contaminant.

Modification of amino groups in catalase [4] and L-asparaginase molecules [5] with polyethylene glycol give rise to the high resistance against trypsin. Thus, the native streptokinase and modified streptokinase, in which 8 amino groups had been substituted by 5000 M_r polyethylene glycol, were digested with trypsin. The two preparations incubated with trypsin showed rapid decreases in the activities at the same rate in certain time. Both the native streptokinase and modified streptokinase lost 50% of their original activities after 10 min digestion; 82% of activity was lost after 20 min digestion; a complete loss of activity was observed after 30 min digestion, in the two preparations. The low resistance of the modified streptokinase against trypsin compared with the modified preparations by polyethylene glycol in [3–5] is probably due to the lower degrees of the modification of amino groups in lysine residue, which are sites of cleavage with trypsin, in streptokinase (23.5%) than those of the modification of amino groups in lysine residues in catalase (43%) [4] and L-asparaginase (79%) [5].

The preparation of a modified streptokinase obtained here, which has no antigenicity against its antibody and retains activity, may be effective for treating patients who have antibodies formed by injection of streptokinase. Furthermore, the study indicates that research on modified streptokinase as an anti-thrombic drug will yield information of scientific and clinical value.

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