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Purification of a hybrid plasminogen activator protein

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ABSTRACT

Recombinant DNA technology has been employed to produce a hybrid gene in which the kringle and serine protease domains of tissue plasminogen activator are linked to the heavy-chain Fd region of a fibrin-specific antibody. The hybrid gene is co-expressed with antibody light chains. This communication describes a purification procedure for the hybrid protein, involving affinity and ion-exchange chromatography. The purified hybrid protein has been used *in vivo* and *in vitro* clot lysis experiments and has been shown to be effective at clot dissolution.

INTRODUCTION

It is well known that the presence of blood clots in blood vessels can be life threatening and can lead to acute cardiovascular diseases. A particular class of agents which could be used to treat such diseases are plasminogen activators. These agents act by converting plasminogen to plasmin, which then acts on the firbrin in blood clots, causing it to break down and be dissolved (see Fig. 1). There are three well known plasminogen activators, these being streptokinase, urokinase and tissue plasminogen activator (t-PA); only t-PA will be discussed in this communication. Recombinant t-PA has been marketed by Genentech under the name of "Activase" as a thrombolytic for clinical use. The unique property of t-PA is its ability to selectively activate plasminogen bound to fibrin which minimises systemic fibrinolysis. However, it is impossible to differentiate between fibrin in the thrombus or the heamostatic plug and hence its use may cause undesired bleeding. t-PA has a relatively short half life of approximately 6 min [1], and therefore treatment requires large and lengthy doses to maintain biological effect.

Although t-PA is being used as a thrombolytic it would be desirable to improve its half-life and its specificity or to produce a novel molecule having thrombolytic properties, but having an improved half-life and specificity as compared to t-PA. In order to achieve this end, recombinant DNA technology has been employed to produce novel plasminogen activators. Various approaches include: domain shuffling, *i.e.*, deleting domains from existing plasminogen activators or combining different

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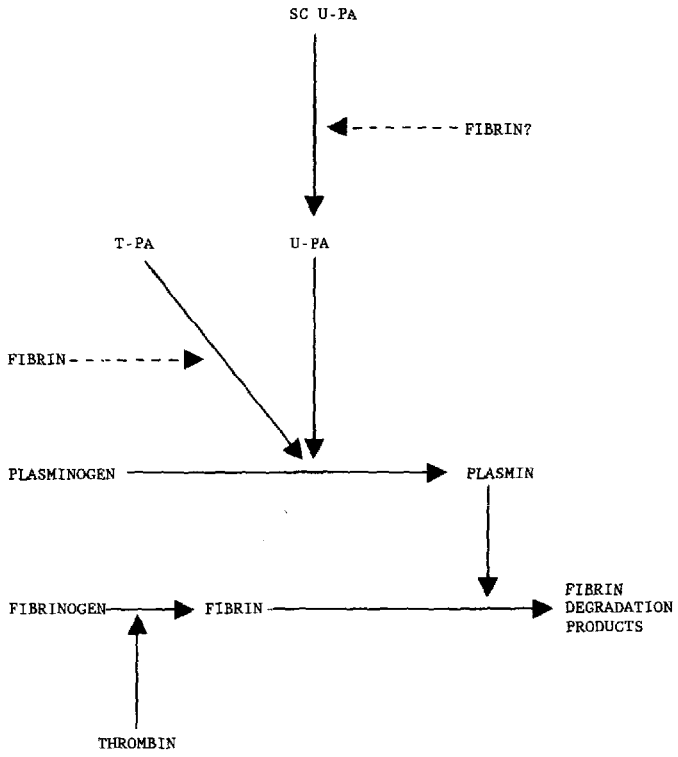

Fig. 1. A schematic representation of the fibrinolytic system. ——— = Major fibrinolytic pathways; - - - = site of cofactor action; SC U-PA = single chain urokinase; U-PA = urokinase; T-PA = tissue plasminogen activator.

domains from different activators [2]; linking a plasminogen activator to a molecule with high fibrin binding activity [3,4] or producing an antibody/plasminogen activator molecule using a monoclonal antibody having a specificity for fibrin [5]. Our approach has involved the use of recombinant DNA technology to combine the clot dissolution properties of a plasminogen activator with the fibrin specificity of an antibody in the form of a hybrid molecule (see Fig. 2). This communication is primarily concerned with the development of a purification procedure for such a hybrid protein.

MATERIALS AND METHODS

Recombinant DNA techniques and cell culture

Methods used for the production of expression vectors and to transfect cell lines were as described by Maniatis *et al.* [6] and Bebbington and Hentschel [7]. A Chinese hamster ovary cell line was established and cultured at 100-l scale using Airlift Fermenter (ALF) technology as described by Rhodes and Birch [8]. Fermenter harvests containing hybrid protein were clarified by continuous centrifugation and concentrated 10-fold by ultrafiltration.

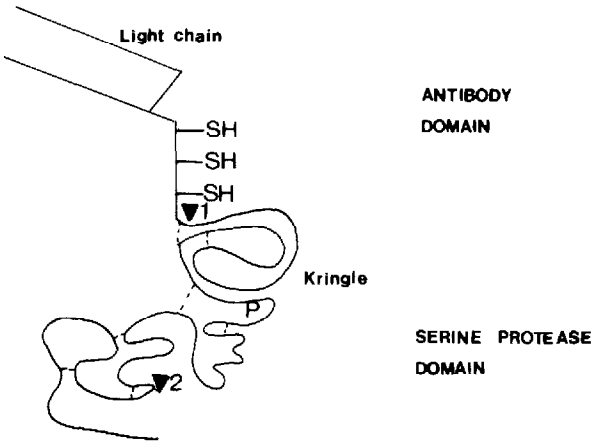

Fig. 2. Diagrammatic representation of the hybrid molecule. The molecule has two domains —the antibody domain and the serine protease domain of t-PA. The molecule has two potential glycosylation sites (▼) which are situated in the serine protease domain. These correspond to Asn 184(1) and Asn 448(2) from the t-PA molecule, the former site is only optionally glycosylated. P indicates the site of plasmin cleavage whereupon the molecule can be cleaved to the two chain form. – – – = Disulphide bridges.

Purification of the protein

Initial purification of the hybrid protein was achieved by the use of a Fibrinogen–Reacti-Gel adduct. Fibrinogen–Reacti-Gel was prepared by coupling fibrinogen (human, Kabi Vitrum) to carbonyldiimidazole (CDI) Reacti-Gel (6 ×) from Pierce according to manufacturers instructions.

Concentrated culture supernatant (2.5 l) was loaded onto the Fibrinogen–Reacti-Gel matrix (100 ml) pre-equilibrated in 50 m*M* sodium phosphate buffer pH 7.4 containing 0.9% (w/v) NaCl; 0.05% (v/v) Tween 80. After loading the matrix was washed with the same buffer containing 50 m*M* ε-aminocaproic acid. Bound hybrid protein was then eluted from the matrix using 0.3 *M* glycine hydrochloride buffer pH 2.5, containing 0.9% (w/v) NaCl and 0.05% (v/v) Tween 80, and immediately adjusted to neutrality using 1 *M* Tris.

The eluate from Fibrinogen–Reacti-Gel™ was diluted two-fold with 50 m*M* sodium phosphate buffer pH 5.5 containing 0.01% Tween 80 and the pH adjusted to 5.5 by the addition of hydrochloric acid and then loaded onto an sulfopropyl Sepharose (S-Sepharose) column (40 ml) pre-equilibrated with 50 m*M* sodium phosphate buffer (pH 5.5) containing 0.01% (v/v) Tween 80. Bound protein was eluted with 50 m*M* sodium phosphate buffer (pH 7.4) containing 0.1 *M* NaCl and 0.01% (v/v) Tween 80. Fractions containing enzymatically active hybrid protein were combined, concentrated as necessary by ultrafiltration and dialysed into final buffer.

Active site titrations

The activity of the protein was measured (using the S2288 chromogenic substrate assay, see Dodd *et al.* [12]) in the presence of an inhibitor, diisopropyl fluorophosphate (DFP), which binds irreversibly to the protein in a 1:1 ratio. A plot of activity against DFP concentration thus yields the activity of the enzyme as the intercept on the

ordinate and the concentration of the enzyme as the intercept on the abscissa. The titration was calibrated using commercially available t-PA (American Diagnostics) of known concentration. Both the standard and hybrid protein were converted to their two chain forms by incubation with plasmin before titration. This ensures that mixtures of single and two chain species are not present in the titration.

In vitro and in vivo clot lysis

In vitro clot lysis by the hybrid protein was tested using fresh human blood labelled with ^{125}I according to the method described by Zamarron *et al.* [9]. The *in vivo* clot lysis was tested using the rabbit jugular vein model using the method described by Collen *et al.* [10].

Miscellaneous

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially according to Laemmli [11]. Fibrin zymography and amidolytic activity assays using S2288 chromogenic substrate were carried out as described by Dodd *et al.* [12].

RESULTS AND DISCUSSIONS

Purification of the hybrid protein

The hybrid protein was purified as described in the Materials and Methods section and monitored throughout using the assay systems described previously. Table I shows that 32 mg of purified protein (calculated from S2288 activity assay) was obtained from the process, corresponding to a 39% recovery of activity units. The major loss in recovery for the process occurred upon chromatography using Fibrinogen–Reacti-Gel. This reflects the removal of degraded protein which contributes to the S2288 chromogenic substrate activity of culture supernatants. Analysis of eluates throughout the purification was carried out using SDS-PAGE (reducing and non-reducing) and zymography.

Analysis by zymography is shown in Fig. 3. Strong clearance of the fibrin agar gel was observed at a single spot indicating that most of the enzymic activity was associated with a single species, identified as the hybrid protein by N-terminal sequence analysis. The relative molecular mass (M_r) of this species was determined by

TABLE I

YIELD TABLE FOR THE PURIFICATION OF THE HYBRID PROTEIN

Total yield (mg) obtained from each step in the process was calculated from S2288 activity assay data using a conversion factor of 444 000 IU = 1 mg.

	Total recovery (mg)	Cumulative yield (%)
Culture supernatant (10 × concentrate)	92	100 (assumed)
Fibrinogen–Reacti-Gel	39.5	43
S-Sepharose	34.5	37
Dialylsis	32	35

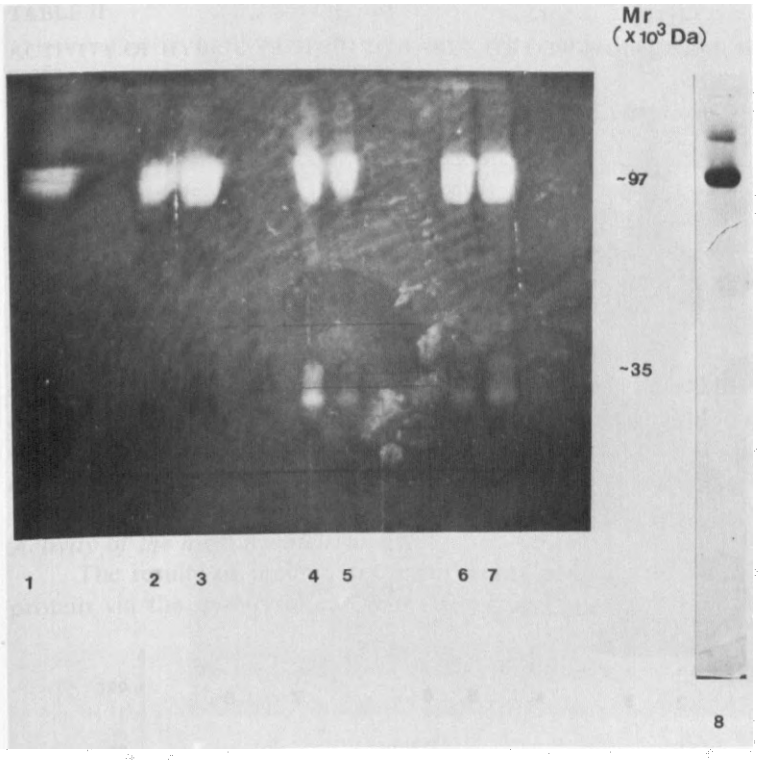

Fig. 3. Analysis of eluates from the purification of the hybrid protein using zymography and SDS-PAGE. Lanes: 1 = culture supernatant (10 × concentrate); 2,3 = eluate from Fibrinogen–Reacti-Gel; 4,5 = eluate from S-Sepharose; 6,7 = final product after dialysis; 8 = final product after analysis by Coomassie blue staining of SDS-PAGE (non-reducing).

Coomassie blue-stained SDS-PAGE (non-reduced) to be approximately 97 000 dalton (Da) and constitutes $\geqslant 90\%$ of total protein as determined by scanning densitometry. Slight clearing of the zymogram was also observed at approximately 35 000 dalton, which is consistent with some cleavage of the serine protease domain of the molecule.

Analysis of eluates across the purification using Coomassie blue staining of SDS-PAGE (reducing) is shown in Fig. 4. From this figure it can be seen that most of the contaminant protein elutes in the flow through from the Fibrinogen–Reacti-Gel step with remainder being eluted from the matrix in the subsequent wash steps. Chromatography using S-Sepharose serves as a final polishing step and remove trace amounts of albumin present after chromatography on Fibrinogen–Reacti-Gel.

Bands for the heavy and light chain moieties of the hybrid protein (M_r 67 000 and 30 000 dalton, respectively) can be observed as well as three additional bands (M_r 40 000, 38 000 and 35 000 dalton) which have been subjected to N-terminal sequence analysis to identify their origin. The 40 000- and 38 000-dalton bands correspond to the molecule without light chain and serine protease domains. The difference in molecular mass is a result of differential glycosylation of glycosylation

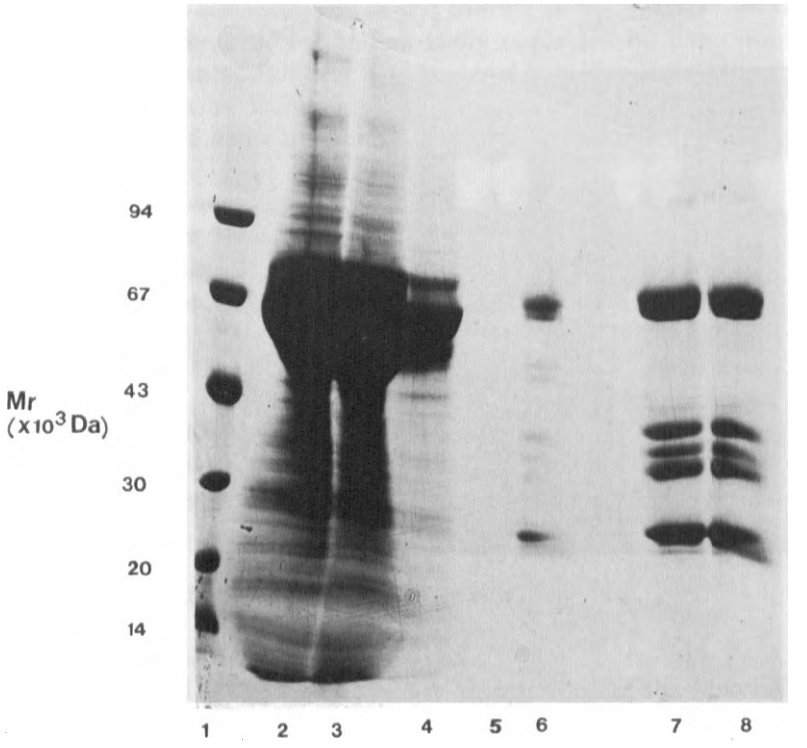

Fig. 4. Analysis of eluates from the purification of the hybrid protein using SDS-PAGE (reducing). Lanes: 1 = molecular mass markers; 2 = culture supernatant; 3 = flow through from Fibrinogen–Reacti-Gel; 4,5 = wash eluates from Fibrinogen–Reacti-Gel; 6 = eluates from Fibrinogen–Reacti-Gel; 7 = eluate from S-Sepharose; 8 = final purified hybrid protein.

site 1 (Fig. 2). The 35 000-dalton band corresponds to cleaved serine protease. The 67 000-dalton band appears as a doublet which is a result of differential glycosylation of the heavy chain moiety. This cleavage is not observed by SDS-PAGE under non-reducing conditions as internal disulphide bonds hold the molecule together (Fig. 2).

Active site titration of purified hybrid protein

Active site titrations of the purified hybrid protein were carried out as described in the Materials and Methods and compared to t-PA. The intrinsic activities of these molecules towards the low-molecular-mass substrate (S2288) were determined. The results of these measurements are shown in Table II. It can be seen from these data that the activity of the hybrid molecule towards the low-molecular-mass substrate S2288 is similar to that of t-PA and appears to be unaffected by the removal of part of the t-PA molecule and addition of the antibody variable region.

Activity of the hybrid protein in vitro

A comparison of *in vitro* clot lysis activity of t-PA and the hybrid protein was

TABLE II

ACTIVITY OF HYBRID PROTEIN TOWARDS ITS LOW-MOLECULAR-WEIGHT SUBSTRATE S2288

This compares the activity of the hybrid protein with that of t-PA towards the low-molecular-mass substrate, Ile–Pro–Arg–pNA (S2288).

Enzyme	Concentration of enzyme (n*M*)	Enzyme activity (AU/min)[a]	Specific activity (AU/min/n*M* enzyme)
t-PA	4.6	8.30	1.8
Whole hybrid	4.3	8.15	1.89

[a] AU = Activity units.

carried out. The hybrid protein and t-PA were present at 500 units/ml in plasma (as determined using the S2288 chromogenic substrate assay). Fig. 5 shows the results of this experiment. From this preliminary study it is apparent that the hybrid protein has comparable clot lysing activity *in vitro* to that of t-PA.

Activity of the hybrid protein in vivo

The results of preliminary experiments giving a local infusion of the hybrid protein via the ipsilateral ear vein (10 ml over a period of 10 min) are shown in

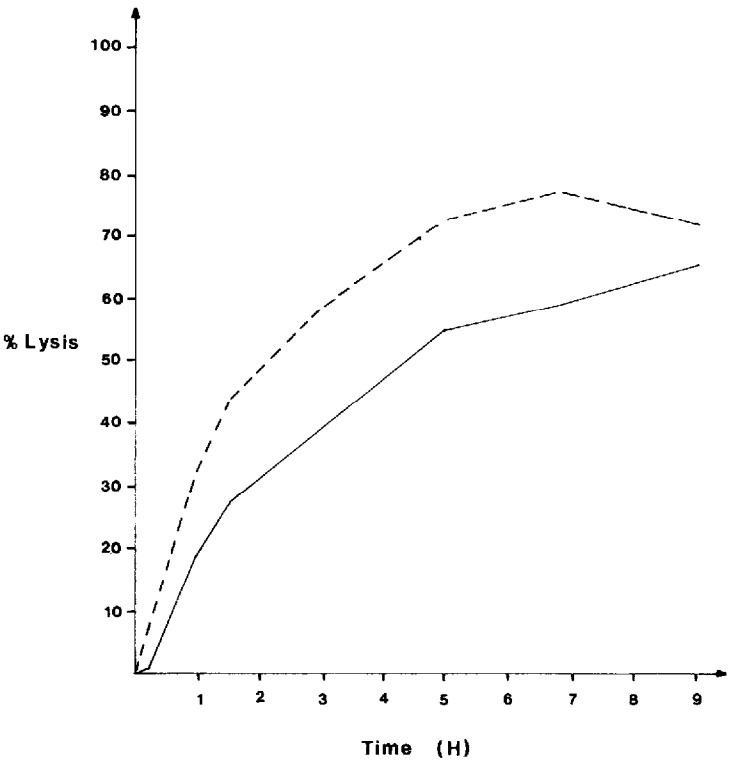

Fig. 5. Activity of the hybrid protein *in vitro*. Activity of the hybrid protein in *in vitro* clot lysis experiments and compared to that of t-PA. – – – = t-PA; ——— = hybrid protein.

TABLE III

ACTIVITY OF THE HYBRID PROTEIN *IN VIVO*

This table compares the activity of the hybrid protein and t-PA *in vivo* at two dose levels (calculated using S2288 assay). Each data point was obtained using a separate rabbit ($n = 3$).

Dose (IU/kg)	% Lysis (t-PA)	% Lysis (hybrid)
50 000	17.53 ± 5.17	20.03 ± 3.52
500 000	50.9 ± 1.85	41.7 ± 8.79

Table III. Lytic activity was compared with that of t-PA. These results indicate that the hybrid protein is thrombolytically active *in vivo* and that its activity is broadly similar to that of t-PA. However, further experiments will be required to fully evaluate the efficacy of the hybrid protein *in vivo*.

CONCLUSIONS

A purification procedure has been described for the hybrid plasminogen activator molecule from Chinese hamster ovary (CHO) culture supernatant, which includes both an affinity chromatography and an ion-exchange chromatography step. The purified material has been obtained with an overall yield of 35% with a purity $\geqslant 90\%$. The hybrid plasminogen activator molecule has been shown to be effective at clot dissolution in both *in vitro* and *in vivo* clot lysis experiments. Further experiments are necessary to fully evaluate this molecule as a potential thrombolytic agent.

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