*Journal of Chromatography*, 510 (1990) 83–93
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 450

# Affinity purification of tissue plasminogen activator using transition-state analogues

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ABSTRACT

The search for a simple affinity ligand to purify tissue plasminogen activator (tPA) was facilitated by a solid-phase synthesis approach. A large variety of tripeptide ligands containing argininal were synthesized on agarose gels containing a spacer with carboxy terminal. The immobilized ligands were easy to test with urokinase, and tPA. While a number of sequence combinations showed initial binding by tPA, only a few resulted in tight binding corresponding to a hemiacetal linkage with the active site serine. Hydrophobic residues, especially aromatics, flanking the N-side of argininal gave rise to ligands which were bound strongly by tPA. A gel containing D-Phe–D-Phe–Argal (an aldehyde derivative of arginine) was very effective in purifying tPA derived from cell culture media at small scale (milligrams) and at large (multi-grams).

## INTRODUCTION

Recent clinical studies have shown that thrombolytic rescue of patients undergoing an acute myocardial infarction (AMI) has definite benefit resulting in lowering patient mortality[1]. Among the newer agents showing promise in this regard is tissue plasminogen activator (tPA). Because of the large dosage requirement (100 mg), it is of interest to find more efficient ways to produce and purify this drug. We describe here a novel affinity purification process for tPA using tripeptide transition state analogues as ligands.

Since tPA is a trypsin-like serine protease, we initially surveyed inhibitors well characterized for trypsin. Simple, readily attainable ligands, such as arginine or benzamidine, which had been long shown as inhibitors of trypsin, proved to be weak in binding tPA. The macromolecular (20 000 dalton) ligand, Erythrina trypsin inhibitor (ETI), had been shown to bind tPA with great avidity, and this was verified in our own laboratory. However, its relative scarcity and high cost made it unattractive for consideration in any affinity purification system that was to be directed to large-scale (kilograms) enzyme isolation.

Thus, the challenge was to develop a novel affinity ligand of moderately low

0021-9673/90/$03.50 

molecular weight but possessing strong binding properties like that of ETI. This led us to evaluating tripeptide structures which could be generally described as follows:

P3–P2–P1–P1′–P2′–
↓
X–Y–Arg–Z–Z′–

where the "P" residues define parts of the substrate specificity and include the critical arginine the carbamide linkage of which is the scissile bond (indicated by "↓"). Other studies had indicated that lysine in the P1 position was not as good as arginine for tPA, while trypsin seems to bind both residues with more similar avidity.

To further enhance binding potency, we looked to structures that could form transition-state analogues. This could be attained by converting the C-terminal arginine into argininal, which could form a hemi-acetal structure with the active-site serine when the tripeptide ligand was bound to the enzyme. Such structures have been shown by others to bind trypsin-like proteases with great avidity[2].

Given the abundance of trypsin inhibitors from which new tPA inhibitors might be modeled, it was instructive to compare differences (in addition to those already noted above) in substrate specificity between the two enzymes.

Trypsin is capable of attacking a wide range of peptide sequences and even undergoes autolysis. By contrast tPA seems uniquely poised to hydrolyze a specific peptide loop in plasminogen and cause its conversion to plasmin. Owing to this specificity tPA does not autolyze. With its more complex structure comprised of finger, growth factor, and two kringle domains attached to the protease domain, tPA does not function through a zymogen stage, but normally does its task by first binding to a freshly formed fibrin clot which also binds plasminogen. It has been demonstrated that the catalytic activity of tPA, while intrinsically well below that of trypsin[3], is significantly stimulated by fibrin[4]. The generation of plasmin by this process results in efficient dissolution of the fibrin clot. Thus, tPA has gathered much attention as a thrombolytic of great clinical promise.

In our quest for a novel inhibitor selective for tPA binding, we studied the example of ETI[5], which in addition to binding trypsin has unusual avidity towards tPA. TPA binding to ETI may not involve a reversible scissile bond. Indeed, tPA inactivated with diisopropylfluorophosphate (DFP) is still capable of binding to ETI[6]. This suggested that inhibitor–enzyme recognition may involve structural elements some what removed in space from the catalytic triad.

Among the early candidates for a tPA-specific ligand was the tripeptide sequence (Pro558–Gly559–Arg560) neighboring the scissile bond in plasminogen. The structure proved disappointing. In addition, several other structures suggested by known substrates and inhibitors, proved to be equally disappointing. Thus, we were prompted to empirically search for a more avid binding tripeptide, argininal-containing sequences.

Our approach was to synthesize a series of affinity gels containing tripeptide structures bearing a C-terminal argininal moiety. These gels were tested in mini-columns with partially purified tPA preparations for effectiveness in capturing and purifying the enzyme.

Upon identifying a effective ligand, it was necessary to determine the best

conditions for capturing tPA from conditioned media and for its further purificaton. Unlike chemically unreactive affinity ligands, one which contains an argininal moiety has the potential to non-selectively react with primary and secondary amines in buffers or culture media. Beyond these studies, it was of interest to study the process at large-scale with a view to developing a system for meeting the growing needs of tPA in treating acute myocardial infarctions[7].

## EXPERIMENTAL

### *Materials sources*

Amino acids and protected derivatives were purchased from Bachem Bioscience (Philadelphia, PA, U.S.A.) Affi-Gel-10 was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Chromogenic enzyme substrates were purchased from Helena Labs. (Beamont, TX, U.S.A.). Bovine thrombin was from Miles Diagnostics (Kankakee, IL, U.S.A.). Urokinase was obtained from Calbiochem (San Diego, CA, U.S.A.) tPA was obtained from in-house souces. Other specialty chemicals were procured from Sigma (St. Louis, MO, U.S.A.). Arginine semicarbazone (ArgSC) was synthesized according to a new method (patent applied for). Alternatively one could prepare the reagent according to Patel and Schultz[8].

### *Synthesis of ESEP-Cl-6B-EDA-SA gels*

This was done according to the epibromohydrin procedure described by Nishikawa and Bailon[9]. Instead of shake flasks a modified stirred resin flask (Kontes Glass, No. K-614012-9006) was used. The epoxy-derivative of Sepharose Cl-6B (obtained from Pharmacia) was reacted with ethylenediamine in water at room temperature overnight. Unreacted ethylenediamine was washed out from the gel with 0.1 *M* acetic acid followed by water. Next the gel was reacted with succinic anhydride in water at pH 6. Finally, the gel was washed with 0.1 *M* sodium carbonate followed by water until washings were neutral. The product gel was rinsed three times with isopropanol and stored as a moist powder.

### *Synthesis of ESEP-Cl-6B-EDA-SA-X-Y-ArgSCgels*

The "X" (P3), "Y" (P2) and ArgSC (P1) groups were coupled sequentially to the succinyl moiety using water-soluble carbodiimide [N-ethyl-N′-(3-dimetylaminopropyl) carbodiimide hydrochloride], triethylamine, and N-hydroxybenztriazole. The X and Y groups were added as methyl ester derivatives and followed by saponification with 0.1 *M* sodium carbonate[10].

### *Synthesis of Affi-Gel-10-X-Y-ArgSCgels*

The chemically reactive Affi-Gel-10 gels were coupled directly at pH 7 to X–Y groups already preformed as dipeptides. The ArgSC moiety was coupled to the Affi-Gel-10–X–Y using water-soluble carbodiimide (procedure as above). The semicarbazide protecting group was removed by treatment with formaldehyde in dilute acetic acid.

### *Testing of sorbents*

Usually a 5 × 1 cm column was packed and pre-equilibrated with a pH

6 imidazole buffer, except with tPA where 0.1 *M* sodium acetate pH 4.5 (plus sodium chloride) was used. After loading enzyme, the column was washed with various buffers to remove contaminants. The enzyme could be recovered with either 0.1 *M* acetic acid or 0.1 *M* semicarbazide. Occasionally 0.2 *M* ammonium hydroxide as used.

*Assay of enzyme activity*

Samples were assayed with appropriate chromogenic substrates at room temperature. Increases in absorbance (at appropriate wavelength) were recorded in a spectrophotometer for 300 s. Samples were diluted so that linear absorbance tracings were obtained during the enzyme reactions.

*Trypsin. A* 247 nm, 900 µl 0.1 *M* Tris–0.01% PEG-3400 pH 8.1 buffer, 100 µl 0.01 *M* tosylarginine methyl ester in water, 100 µl of sample.

*tPA. A* 405 nm, 900 µl Tris buffer (see trypsin), 50 µl 0.01 *M* D-Ile–Pro–Arg–*p*-nitroanilide (pNA) · HCl (S-2288), 50 µl of sample; also assayed by S-2251 (ref. 11).

*Urokinase. A* 405 nm, 800 µl Tris buffer (see trypsin), 50 µl 0.003 *M* pyro-glutamate (Pyr–Glu)–Gly–Arg–pNA · HCl (S-2444), 200 µl of sample.

*Thrombin. A* 405 nm, 900 µl 0.1 *M* Tris–0.025 *M* $CaCl_2$–0.01% PEG-3400 pH 8.1 buffer, 50 µl 0.002 *M* D-Phe–pipecolic acid (Pip)–Arg–pNA (S-2238), 50 µl of sample.

*Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis*

General method was according to Laemmli[12]. Stacking gel was 4% acrylamide and 5% cross-linker. Separating gel was 10% acrylamide and 5% cross-linker. Dithiothreitol was used to reduce proteins.

## RESULTS AND DISCUSSION

*Inhibitor studies*

*ETI.* Among the serine protease inhibitors (serpins) described in the literature, ETI[5] stands out as being uniquely selective for tPA (in addition to reacting with trypsin). Heussen *et al.*[6] had shown that it was capable of avidly binding even active-site blocked inhibited tPA. This suggested that a considerable part of the binding of tPA to ETI migh involve macroscopic structural domains in the two proteins (as depicted in Fig. 1). But since trypsin (which is similar in size to the protease domain of tPA), appears to avidly recognize and bind the sequence: Arg61–Leu62–Arg63 in ETI, we decided to test an oligopeptide containing this sequence as an affinity ligand for tPA.

Affi-Gel-10–Arg–Leu–Argal was prepared as described above and tested with tPA. The results are shown in Table I. While tPA appears to adsorb initially to the gel,

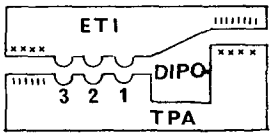


Fig. 1. Model depicting ETI binding to tPA as diisopropylphosphoryl (DIPO) derivative.

TABLE I

BINDING STUDY OF tPA WITH AFFINITY MATRIX (Affi-Gel-10–Arg–Leu–Argal) CONTAINING HOMOLOGOUS SEQUENCE TO ETI ACTIVE SITE SEQUENCE

| *Test sample* | *Enzyme activity (%)* *t-PA* |
|---|---|
| Load | 100 |
| Flow through | 0 |
| 0.2 *M* sodium chloride/buffer wash | 49 |
| 1.0 *M* sodium chloride/buffer wash | 53 |
| 0.2 *M* Ammonium hydroxide elution | 8 |

it is readily desorbed by buffer containing salts to simply disrupt ionic interactions between enzyme and ligand. The results point to the importance of macro-surface recognition features (even though not yet known) in ETI which cause tight binding to tPA.

*The plasminogen loop.* Owing to the efficiency with which tPA can cleave the scissile Arg560 bond in plasminogen, we decided to explore an oligopeptide containing the sequence: Pro558–Gly559–Arg560 as a possible affinity ligand.

A gel bearing Pro–Gly–Argal was tested with tPA and urokinase (UK) another well established plasminogen activator. The results seen in Table II indicate that both enzymes initially bind to the affinity sorbent. tPA is readily desorbed with buffer containing salts to disrupt ionic interactions. While a large portion of UK desorbs readily with salt-containing buffers, a smaller but significant amount requires low pH to disrupt the hemiacetal linkage and allow release of the enzyme. This agrees with other studies involving amidolysis of oligopeptide substrates bearing the Pro–Gly–Arg sequence[3]. Perhaps the low avidity of tPA to this linear sequence is not surprising. The Pro–Gly sequence is typically found in $\beta$-turns and this tripeptide sequence is part of a small disulfide enclosed loop in plasminogen[13]. Thus one might expect that the scissile bond in the zymogen might be present in a highly accesible conformation to the tPA which has been activated by the attachment of fibrin.

TABLE II

BINDING STUDY OF tPA AND UROKINASE WITH AFFINITY MATRIX [Sepharose–ETHYLENEDIAMINE (EDA)–SUCCINIC ACID (SA)–Pro–Gly–Argal] CONTAINING PLASMINOGEN LOOP SEQUENCE HOMOLOGY

| *Test sample* | *Enzyme activity (%)* | |
|---|---|---|
| | *tPA* | *Urokinase* |
| Load | 100 | 100 |
| Flow through | 1 | 0 |
| 0.1 *M* Sodium chloride/buffer wash | 81 | 48 |
| 1.0 *M* Sodium chloride/buffer wash | 7 | 9 |
| 0.1*M* Acetic acid | 6 | 21 |

TABLE III

BINDING OF tPA-1 (ONE CHAIN) TO KNOWN PEPTIDE INHIBITOR SEQUENCES

Affi–E–G–Argal = Affi–Glu–Gly–Argal; Affi–Q–G–Argal = Affi–Gln–Gly–Argal.

| *Test sample* | *Enzyme activity (%)* | |
|---|---|---|
| | *Affi–E–G–Argal* | *Affi–Q–G–Argal* |
| Load | 100 | 100 |
| Flow through | 0 | 3 |
| Low salt wash | 84 | 74 |
| High salt wash | 6 | 13 |

*Dansyl-Glu–Gly–Arg–chloromethyl ketone.* As a serine protease, tPA was studied sometime ago with active-site directed alkylating agents bearing chloromethyl ketones. One such reagent comprised of dansylated Glu–Gly–Arg was found to have particular avidity for tPA[14]. Without the dansyl group, the reagent was about 100-fold less potent but still appreciable.

Hence, we prepared an agarose gel containing Glu–Gly–Argal for testing. In addition, a Gln–Gly–Argal gel was prepared to assess the charged group effect of the γ-carboxyl of glutamate. The results seen in Table III indicate that while tPA binds to the gel but no apparent hemiacetal is formed. To rule out interference by low-molecular-weight amine compounds in the sample (conditioned media), the binding study was repeated with diafiltered enzyme (data not shown), which also bound but then eluted with a salt wash. The results with a gel containing Gln instead of Glu (Table III) indicate that loss of a negative charge at the P3 position of the ligand has little consequence and the tPA still desorbs easily with low salt buffer.

*PPACK.* D-Phe–Pro–Arg–chloromethyl ketone (PPACK) was originally developed as a thrombin inhibitor[15]. It was found that this compound has to have good activity against tPA bu a weak effect on plasmin[16]. This has prompted its use as an agent for preserving blood samples following fibrinolytic therapy[16].

Owing to this effectiveness we decided to explore an affinity gel containing

TABLE IV

BINDING STUDY OF tPA WITH AFFINITY MATRIX (Sepharose–EDA–SA–Phe–Pro–Argal) CONTAINING PPACK ANALOGUE

| *Test sample* | *Enzyme activity (%)* | |
|---|---|---|
| | *tPA-1*[a] | *t-PA-2*[b] |
| Load | 100 | 100 |
| Flow through | 1 | 0 |
| Low salt wash | 33 | 4 |
| High salt wash | 2 | 1 |
| 0.1 *M* Acetic acid | 74 | 84 |

[a] One chain.
[b] Two chains.

TABLE V

BINDING STUDY OF MYELOMA tPA (ONE CHAIN) WITH VARIOUS AFFINITY MATRICES WITH X–Y–Argal AS LIGANDS

| *Ligand, X–Y–Argal* | *Enzyme activity (%)* | | |
|---|---|---|---|
| | *Unbound* | *Wash* | *Recovered* |
| D-Phe–L-Ala–Argal | 0 | 24 | 54 |
| L-Ile–L-Ala–Argal | 0 | 73 | 3 |
| L-Ile–D-Phe–Argal | 0 | 98 | 8 |
| L-Val–D-Phe–Argal | 0 | 92 | 10 |
| L-Ala–D-Phe–Argal | 0 | 64 | 34 |
| L-Tyr–L-Ala–Argal | 1 | 55 | 31 |
| L-Tyr–L-Val–Argal | 0 | 87 | 25 |

D-Phe–Pro–Argal for purifying tPA. The results shown in Table IV suggest that this ligand is fairly effective in capturing and purifying tPA. However, this depended on the chain content of tPA. Preparations where the cell line and/or culture conditions yielded primarily single-chain enzyme, showed as slightly weaker binding in that some activity was lost on low salt elution (experimental error in activity determinations is about 10%). This unexpected finding was further explored (see below) with ligands which appeared to better capture both forms of tPA.

*Search for better ligands*

After we examined ligands based on analogy to substrates and inhibitors of historical precedence and found them wanting, we concluded that an empirical screening for structure was an appropriate endeavor. The prospect was not that of a totally random screen, however. There were clues that hydrophobic residues in the P3

TABLE VI

BINDING STUDY OF MYELOMA tPA (ONE CHAIN) WITH VARIOUS AFFINITY MATRICES WITH X–Y–Argal AS LIGANDS

Hydrophobic residues in P2 and P3.

| *Ligand, X–Y–Argal* | *Enzyme activity (%)* | | |
|---|---|---|---|
| | *Unbound* | *Wash* | *Recovered* |
| D-Phe–D-Phe–Argal | 1 | 0 | 72 |
| L-Phe–D-Phe–Argal | 1 | 0 | 58 |
| L-Phe–L-Phe–Argal | 0 | 0 | 74 |
| D-Phe–L-Phe–Argal | 0 | 0 | 53 |
| D-Phe–L-Trp–Argal | 1 | 0 | 64 |
| D-Phe–L-Val–Argal | 0 | 3 | 80 |
| D-Phe–L-Ile–Argal | 0 | 0 | 59 |
| D-Phe–L-Tyr–Argal | 0 | 0 | 63 |
| L-Tyr–D-Phe–Argal | 0 | 1 | 78 |
| L-Trp–D-Phe–Argal | 0 | 0 | 50 |

and P2 positions of a ligand could enhance binding. Table V summarizes the ligand structures which were screened and found deficient. Table VI list the structures with hydrophobic moieties and branching on the beta carbon which were found to be most effective in binding tPA. The D- or L-enantiomers seemed by and large equally effective. In view of this and to take advantage of resistance to proteolytic cleavage by D-amino acid moieties, we focused attention on D-Phe–D-Phe–ArgSc sequence.

*Chain content of tPA preparations*

Two-chain tPA appears to arise from one-chain molecules by the action of proteases contaminating the culture media. Since the two forms seem to act similarly *in vivo*, it was of interest to harvest both equally in any purification scheme. With the gel containing D-Phe–D-Phe–Argal the problem of capturing both forms was solved. Although slight differences in mobility could be noted during sequential elution, the bulk of the tPA was recovered in the acetic acid eluent.

*Small-scale affinity purification*

At various times in the search for a better affinity ligand for tPA, samples of the enzyme expressed in conditioned media were tested directly, *i.e.* without prior fractionation or concentration. For reasons that are not entirely clear, the results were irregular and rather unpromising. In conjunction with an ongoing in-house project for large-scale purification of the enzyme, we examined partially purified samples of tPA. These turned out to be predictable and reproducible. Hence, most of the chromatographic tests in the preceding sections were done with partially purified samples of tPA.

Recombinant human tPA was expressed in CHO or myeloma cel lines. The enzyme was harvested from conditioned media by capture onto a zinc chelate gel[17]. The tPA recovered from this column was then used for further affinity purification studies.

tPA can bind to arginine-containing ligands over a wide pH range (usefully about 3 through 9). But owing to its marginal solubility about pH 7, we opted to work

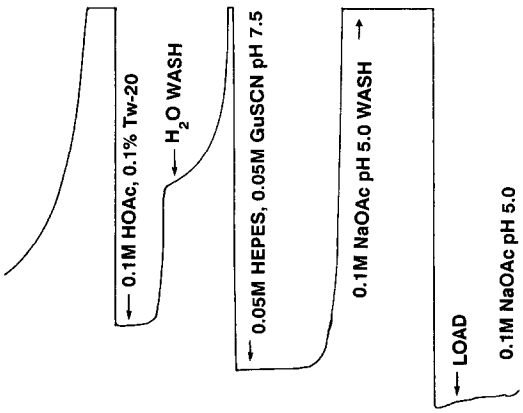


Fig. 2. Small-scale affinity purification of tPA. HOAc = Acetic acid; NaOAc = sodium acetate; TW-20 = Tween-20.

TABLE VII

SMALL-SCALE AFFINITY PURIFICATION OF tPA

| *Step* | *Enzyme activity (%)* | | |
|---|---|---|---|
| | *Volume (ml)* | *S-2288* | *S-2251* |
| Load | 50 | 100 | 100 |
| Flow through | 45 | 6.7 | — |
| Buffer wash | 40 | 1.0 | — |
| Chaotrope wash | 20 | 23.0 | — |
| Water wash | 40 | — | — |
| Recovery (%) | 30 | 44.41 | 87 |

with the enzyme in acidic pH. Partially purified tPA could be conveniently handled in 0.1 *M* sodium acetate buffer pH 4.5 containing 0.6 *M* sodium chloride for loading onto a gel of D-Phe–D-Phe–Argal. At pH 3 at least 0.3 *M* sodium chloride was required to allow enzyme binding to the affinity sorbent. Here the tPA could then be recovered by using pH 3 buffer *without* salt. We surmise that at these low pH conditions, moderate concentrations of electrolytes are necessary to overcome electrostatic repulsions between protein and ligand in order to allow binding.

A chromatographic tracing of one small-scale preparation is shown in Fig. 2.

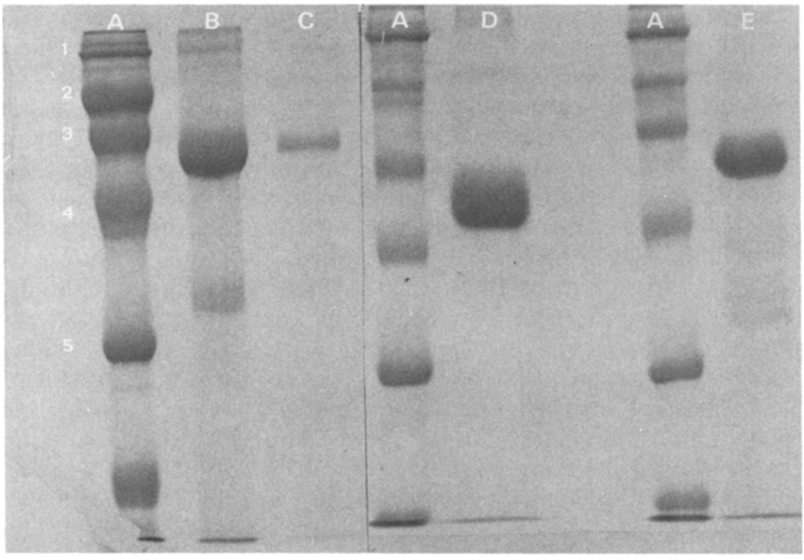


Fig. 3. SDS-PAGE analysis of affinity-purified tPA. Lane A: molecular weight markers; 1 = 200 000 dalton; 2 = 92 500 dalton; 3 = 69 000 dalton; 4 = 46 000 dalton; 5 = 30 000 dalton. Lane B: 5 $\mu$l column load (reduced). Lane C: 3.25 $\mu$l (2.5 $\mu$g) eluted band (reduced). Lane D: 20 $\mu$l eluted band (high load) (non-reduced). Lane E: 20 $\mu$g eluted band (high load) (reduced).

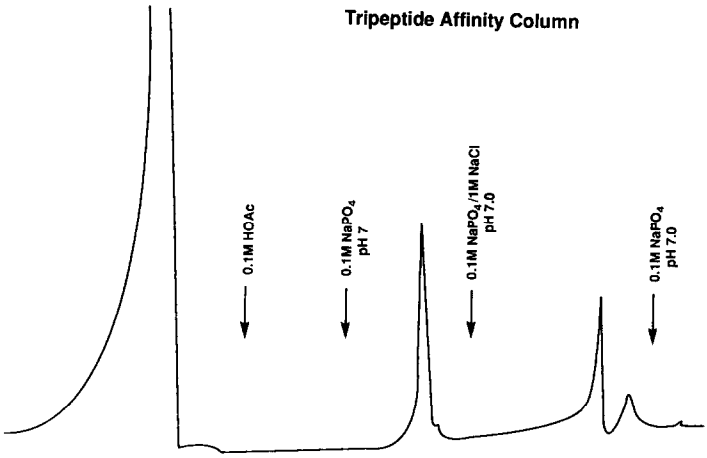


Fig. 4. Large-scale affinity purification of tPA.

The sample of partially purified tPA[17] equivalent to $11.25 \cdot 10^6$ I.U. (S-2251) was loaded on a 5 × 1 cm affinity column at pH 5. After various washes of the column, the enzyme was recovered with 0.1 *M* acetic acid–0.1% Tween-20 solution. Fractions were assayed using S-2288, a general substrate for trypsin-like proteases and S-2251, a specific tPA-Plasmin linked assay. Results summarized in Table VII indicate an 87% recovery based on assay with S-2251 substrate assay. The lower apparent recovery seen with S-2288 substrate may be due to proteases other than tPA in the starting sample.

The significant clean-up achieved in this affinity step is seen in the SDS-PAGE gel shown in Fig. 3. Akey accomplishment was the removal of serum albumin, which was a frequent contaminant carried over from the culture media.

*Large-scale affinity purification*

There was interest to develop a large-scale affinity process, which could be used to prepare tPA for clinical studies. Conditions for convenient handling and loading of partially purified tPA were chosen from the data accumulated in the bench-scale studies (noted above). The results from one large-scale purification study are shown in Fig. 4. The tPA had been captured from conditioned media on a zinc chelate column. After recovery from the column, the enzyme was concentrated by isoelectric precipitation. This protein (3.4 g) was dissolved in 0.1 *M* acetic acid–0.3 *M* sodium chloride pH 4.5 buffer and applied to a 420 ml (20 × 5 cm) pre-equilibrated with 0.1 *M* sodium acetate pH 4.5 buffer. The pH 7 buffer containing 1 *M* sodium chloride served to wash out serum albumin contaminant. This was followed by a dilute pH 7 buffer to wash out the salt, which would interfere with the recovery of tPA by 0.1 *M* acetic acid. Yields were typically 95% for this step, and protein purity routinely exceeded 95%.

## CONCLUSION

By adopting a solid-phase synthesis approach to prepare oligopeptide affinity resins, were were able to conveniently prepare a wide variety of sorbents that could be

effective in purifying tPA. Thus we were able to readily explore sequences known from the literature, as well to screen for novel ones. This enabled the discovery of Phe–Phe–Argal peptide wich have good avidity for tPA. We found that tripeptide ligands containing Argal can be readily used in large-scale affinity purification of tPA.

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