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Large-scale purification and characterization of recombinant tick anticoagulant peptide

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ABSTRACT

Recombinant tick anticoagulant peptide (r-TAP), a potent and specific inhibitor of blood coagulation factor Xa, was purified to >99% homogeneity at the multi-gram scale. Genetically engineered yeast secreted 200–250 mg/l of the heterologous protein into the medium. Cells were separated from broth by diafiltration and purification was done by two chromatographic steps, both conducive to operation on a large scale. Analysis of the purified protein by several methods indicated that it was >99% homogeneous and no incompletely processed or truncated proteins were detected. Physico-chemical characterization data of r-TAP show that it exists as a monomer in solution and no evidence of post-translational modification was observed. The purified protein was fully active in inhibiting human coagulation factor Xa.

INTRODUCTION

Tick anticoagulant peptide (TAP) is a 60 amino acid peptide that was originally isolated from the soft tick *Ornithodorous moubata* [1] and shown to be an effective anticoagulant in several *in vitro* blood clotting assays. It is a Kunitz-type protease inhibitor that is highly specific for blood coagulation factor Xa (fXa) and does not inhibit several other serine proteases (for examples see ref. 1). These data indicated that it might be therapeutically useful in restoring and maintaining hemostasis but further investigations to compare TAP with other inhibitors of coagulation would require a large amount of protein, and only 200–250 μg of peptide can be isolated from 500 ticks. In order to obtain sufficient peptide for a thorough *in vitro* and *in vivo* evaluation, a recombinant *Saccharomyces cerevisiae* secretion system was developed in which a synthetic gene for TAP

was fused to the secretory pre-pro leader of the yeast α-factor mating pheromone under control of a galactose-inducible promoter and the cloning, secretion and purification of recombinant TAP (r-TAP) were described by Neeper *et al.* [2]. A thorough kinetic analysis of r-TAP demonstrated that it is a reversible, slow, tight-binding competitive inhibitor of fXa with stoichiometric binding [3]. Whole animal studies with the recombinant protein showed efficacy in suppressing disseminated intravascular coagulation by preventing the production of fibrinopeptide peptide A through inhibition of the prothrombinase complex [2]. This paper describes the development of a process to purify multi-gram amounts of r-TAP from a single 250-l fermentation of recombinant yeast cells by two chromatographic steps and a decolorization step. Physico-chemical characterization of r-TAP by circular dichroism spectroscopy, light scattering, mass spectrometry, capillary zone electrophoresis, isoelectric focusing, reversed-phase high-performance liquid chromatography (RP-HPLC) and N-terminal sequencing is also presented.

EXPERIMENTAL

Construction of the yeast plasmid vector used for r-TAP expression

The synthetic gene segment encoding r-TAP was constructed and cloned into a yeast expression vector which directs secretion of TAP under the control of the galactose-inducible *GAL*10 promotor. This vector was transformed into a diploid strain of *S. cerevisiae* DMY6 (*Mat*a/α, *ade*1,*ura*3-52,*his3Δ*::*GAL*10*p-GAL*4-*ura*3,*leu2-2,112/leu2-04, cir*^{0}/*cir*^{0}) as described [2]. A clonal isolate designated 281-3 was selected to prepare a master seed which was stored frozen at −70°C in the presence of 20% (v/v) glycerol.

Fermentation

Cells were grown by inoculating frozen stock culture of 281-3 on leu$^-$ agar medium [4] for 3 days at 28°C. Cells were scraped from the agar plate and inoculated into 2-l flasks, each containing 600 ml of selective medium (5X leu$^-$ medium with 4% glucose) [5]. After incubating for 19 h at 28°C in a rotary shaker incubator at 300 rpm (Model G26, New Brunswick, Scientific), a 6% (v/v) inoculation was made into either two 16-l fermenters (New Brunswick Scientific) or one 250-l fermenter (New Brunswick 250-l Magnaferm) and fed-batch fermentation was performed in the following way.

Initially, the 16-l fermenters contained 6 l of 5X leu$^-$ medium with 4% galactose and were operated at 28°C, 500 rpm and 5 l/min of air. Dissolved oxygen was maintained at ⩾30% saturation by automatic adjustments to the air flow and agitation. At 30–31 h post-inoculation, feeding was initiated with leu$^-$–galactose concentrate (all concentrations are %, w/v: yeast nitrogen base without amino acids and ammonium sulfate, 1.7; adenine, 0.08; tyrosine, 0.05; uracil, 0.04; succinic acid, 2.0; $FeCl_3$, 0.008; $ZnSO_4$, 0.008; NaOH, 0.8; arginine, 0.02; histidine, 0.01; isoleucine, 0.06; lysine, 0.04; methionine, 0.01; phenylalanine, 0.06; tryptophan, 0.04; galactose, 25.0) at a constant rate of 1.6 ml/min and continued for 52 h.

The 250-l fermenter initially contained 100 l of 5X leu$^-$ medium containing 4% galactose. The fermenter was operated at 28°C, 260 rpm and 90 l/min of air. The dissolved oxygen was maintained at ⩾30% saturation by automatic adjustments to agitation. At 31 h post-inoculation, feeding with leu$^-$–galactose concentrate was started and continued at a constant rate of 32 ml/min for 52 h.

The absorbance at 600 nm at 120 h was 135–140, which corresponded to a dry cell weight of 39–41 g/l.

Harvesting by cross-flow filtration

After 120 h, the broth was separated from the cells. r-TAP was recovered from the fermentation fluid by a modification of the cross-flow filtration method described by Bailey *et al.* [6], utilizing microfiltration through 0.1-μm pore size hollow fibers in tandem with ultrafiltration through 100 000 molecular mass cut-off (M_rCO) hollow-fiber cartridges.

At the 16-l scale, two Amicon DC10 units were

used in tandem. Fermentation broth was pumped directly into the first unit and concentrated from 22 to 7 l with the 0.1-μm pore size cartridges (Amicon H5MP01). Cells were then washed by diafiltering with 14 l of water purified with a Milli-Q system (Millipore) (Milli-Q water). The combined diafiltrate and wash were fed directly to and diafiltered through the 100 000 M_rCO fibers (Amicon H5MP100). The retained fluid was diafiltered with 4 l of Milli-Q water and then the diafiltrate and wash (37 l) were collected in a single 50-l carboy. Half of the volume was purified as described below.

A schematic diagram of the harvesting system for the 250-l fermenter is shown in Fig. 1. Fermentation broth was piped from the fermenter directly to the primary filtration system, consisting of three Amicon DC-30 hollow-fiber cartridges of 0.1-μm porosity and 2.4 m^2 filtration surface area (Amicon H26MP01). The broth was

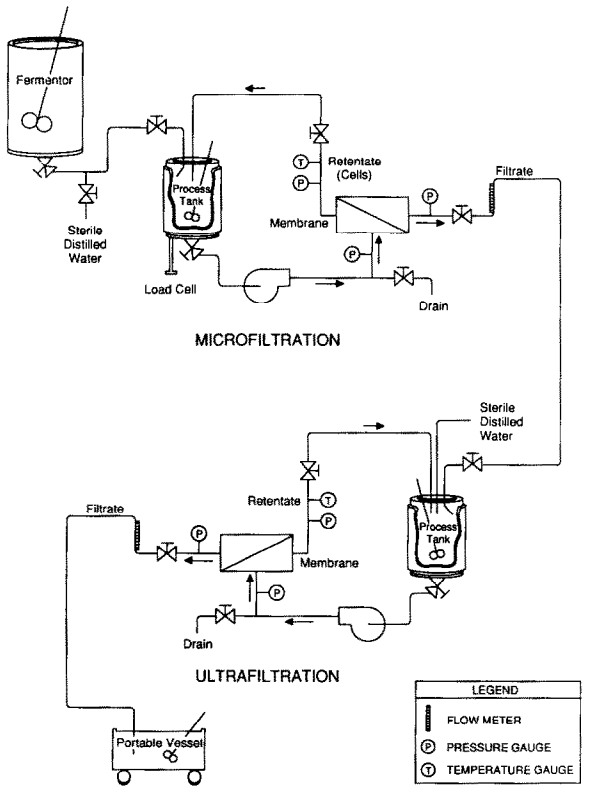

Fig. 1. Process diagram of filtration used to harvest r-TAP from fermentation broth.

concentrated to 50 l, after which the retained cells were washed with 175 l of cold, sterile distilled water. The r-TAP in the ultrafiltrate and wash was piped directly to the secondary filtration system consisting of three 100 000 M_rCO fibers of 2.4 m^2 filtration area (Amicon H26P100-43) and concentrated to 15 l. The concentrated, retained fraction was diafiltered with 25 l of distilled water. The total volume of the filtrate (385 l) was collected in a 400-l portable, stainless-steel tank.

Purification of r-TAP

All operations were done at 4–8°C unless stated otherwise. The diafiltered broth was adjusted to pH 4.0 with glacial acetic acid and diluted with distilled water (10 ± 2°C) to a conductivity of 5.0 mS before being pumped on to an S-Sepharose FF column that had been equilibrated with 50 mM sodium acetate, pH 4 (buffer A). For chromatography at the 16-l fermentation scale, a 1.2-l column (15 × 10 cm I.D.) was charged with 38 l of diafiltered broth at 2 l/h (25 cm/h); for chromatography at the 250-l fermentation scale, a 10-l column (20 × 25.2 cm I.D.) was charged with 770 l at 40 l/h (80 cm/h). Each column was washed with buffer A to remove unadsorbed materials after all the diluted broth had been pumped through and then the adsorbed material was eluted with a linear gradient of NaCl. A 16-l gradient of 0–1.0 M NaCl in buffer A was used to elute the 1.2-l column at 2 l/h with mixing of the gradient being done in a Kontes 16-l gradient mixer sitting on a magnetic stirring base. For the large column, a 160-l gradient of 0.1–0.6 M NaCl in buffer A was generated by placing equal volumes of low and high salt concentrations in 200-l carboys (Nalgene, 11150-0055) and attaching a three-channel pump (Masterflex L/S Digi-Staltic pump with three L-07015-21 pump heads). Buffer was pumped from the mixing chamber on to the column at 20 l/h with two channels while high salt buffer was fed to the mixing chamber with the third channel. Mixing in the mixing chamber was done by recirculating the buffer with a second peristaltic pump fixed with a single pump head. The column effluents were monitored by absorbance at 280 nm and by HPLC. Those frac-

tions which contained r-TAP and were >95% homogeneous by HPLC were combined and filtered through a low-protein-binding membrane (0.22-μm pore size, Millipak 60, Millipore) and stored at 4°C. The product from S-Sepharose FF chromatography was then processed by preparative HPLC on a Waters Delta-Pak C_{18} PrepPak cartridge, 300 × 47 mm I.D., containing 15-μm, 300-Å pore size particles at a flow-rate of 100 ml/min. The filtered S-Sepharose FF pool was pumped on to the column, the column was washed with four column volumes of 10% aqueous acetonitrile–0.1% trifluoroacetic acid (TFA), and then r-TAP was eluted with a linear gradient of 10–30% aqueous acetonitrile–0.1% TFA over 80 min. Fractions that were >95% homogeneous by analytical HPLC were combined and concentrated by removing organic solvent under vacuum in a Speed-Vac centrifuge and subsequently shell frozen and lypophilized to dryness. The dry protein was dissolved in Milli-Q water to a protein concentration of 20 mg/ml, and decolorizing carbon (Norit-A, Fisher Scientific) was added to a concentration of 4 mg/ml. After 90 min the suspension was filtered through Whatman No. 1 filter-paper followed by filtration through a 0.2-μm pore size membrane (Millipak 20 filter unit, Millipore) and the filtered solution was re-lyophilized, weighed and stored at −70°C.

Analytical

Determination of r-TAP in fermentation broth and fractions generated during purification was done by analytical HPLC on a Vydac C_{18} column (150 × 4.6 mm I.D.) operated on a Spectra-Physics chromatography system consisting of a Model 8800 gradient pump, Model 8490 detector and Model 4270 integrator. The column was initially equilibrated with 10% acetonitrile–0.1% TFA. After sample injection it was operated isocratically with the same solvent for 8 min, after which a linear gradient to 45% acetonitrile–0.1% TFA was run over 35 min. The flow-rate was 1.0 ml/min.

Capillary zone electrophoresis (CZE) analysis of purifed r-TAP was performed in an Applied Biosystems Model 270A CZE system using an uncoated open capillary 72 cm long (50 cm working length) with a 50-μm bore. All samples were analyzed at 30°C using 20 m*M* sodium citrate running buffer (pH 2.5). A 27-kV electric field was applied across the capillary, with the detector end of the capillary being at negative potential with respect to the inlet of the capillary. The typical running current was *ca.* 30 μA. The sample injection time was 1.5 s using vacuum, which resulted in a *ca.* 15-nl sample. A 2-min wash with 0.1 *M* NaOH followed by a 3-min wash with running buffer preceded each sample injection. Sample detection was by measuring the absorbance at 200 nm.

Electrophoretic analysis in 10–20% gradient polyacrylamide gels, 1.0 mm thick, with 0.1% sodium dodecyl sulfate (SDS-PAGE) (Novex, San Diego, CA, USA), was done on non-reduced samples using the Tris–tricine buffer system [7] and proteins were detected by silver staining [8].

Free solution isoelectric focusing (IEF) was done in a Protein Technologies Model RF3 cell in the electrodialysis mode, 1% pH 2.5–5 ampholytes (Pharmacia), in 10% glycerol at 15°C. Ampholytes were prefocused for 35 min at 1500 V and the sample was injected. Focusing was continued at 1500 V for 1 h, 1000 V for 30 min and 500 V for 2 h. The pH of the fractions was determined and r-TAP was detected by analytical HPLC. The calculated p*I* was obtained from the amino acid composition using the Sequence Analysis Software Package for VAX/VMS computers developed by the University of Wisconsin Department of Genetics [9].

Quantitative amino acid analysis, except cysteine/cystine, was done after 20 h of hydrolysis in 6 *M* HCl, *in vacuo*, with 0.1% phenol at 110°C, using a Beckman Model 6300 amino acid analyzer with ninhydrin post-column detection as specified by the manufacturer. Cysteine/cystine analysis as cysteic acid was conducted by subjecting protein samples to performic acid oxidation [10]. Samples were hydrolyzed and analyzed as described above.

Automated N-terminal sequence analysis was done with an Applied Biosystems Model 470A sequenator.

All circular dichroism (CD) spectra were recorded on an Aviv 62DS spectrometer calibrated with camphorsulfonic acid. A cuvette with a path length of 1.0 mm was used. Spectra were collected at 10°C with a bandwidth of 1.5 nm. The ellipticity is reported as the mean residue ellipticity, $[\Theta]$, in units of deg cm^2/dmol. The peptide concentration used for the CD spectra was 2 μM. The program VARSELEC [11], provided by Dr. W. Curtis Johnson of Oregon State University, was used for secondary structure prediction. For comparative purposes, predictions were also calculated from a map of the Chou and Fasman algorithm [12] for secondary structure analysis using Intelligenetics software for the VAX computer.

Estimation of protein size from light-scattering experiments was done after filtering the solutions through a 0.1-μm filter with a Malvern 9700 scattering spectrometer (Malvern Instruments, Malvern, UK) equipped with a 5-W argon ion laser (Spectra-Physics, Piscataway, NJ, USA). The intensity autocorrelation function of the scattering light was analyzed using the method of cumulants [13] to derive the apparent hydrodynamic radii and the normalized variance reflecting the size polydispersity. All measurements were recorded at 25°C with the incident light (488 nm wavelength) at 90° scattering angle. The data accumulation time was typically 5 min.

Peptide concentrations for determining yields during purification were determined by measuring the absorbance at 280 nm and using a molar absorptivity of 18 140 l mol^{-1} cm^{-1}, calculated by the method of Gill and Von Hippel [14]. The value which was used for CD and light-scattering calculations, 18 859 l mol^{-1} cm^{-1}, was determined experimentally.

Molecular mass (M_r) determination by mass spectrometry was performed on a SCIEX API III quadrupole mass spectrometer, employing the ion spray technique [15]. About 10 pmol/μl of r-TAP in methanol–acetonitrile containing 0.1% TFA was introduced to the ion spray interface at a rate of 20 μl/min using a syringe pump. Data acquisition, retrieval and hypermass calculations were accomplished on an on-line Macintosh computer.

HFXa inhibition assay. Inhibition of human coagulation factor Xa (HfXa) activity was determined with the chromogenic substrate Spectrozyme fXa [16], using purified r-TAP as standard. Kinetic analysis was done as described by Jordan *et al.* [3].

RESULTS

A fermentation process was developed using a recombinant *S. cerevisiae* strain transformed with a synthetic TAP gene fused to the pre-pro leader sequence of yeast α-mating factor and transcription was under control of a galactose inducible promoter. Infusing nutrients into the fermenter by batch feeding resulted in the secretion of 200–250 mg/l of r-TAP into the culture fluid 120 h after induction with galactose. A 16-l fermentation was done for method development and the process was scaled up to the 250-l fermentation scale, with a corresponding increase in the amount of r-TAP secreted.

Tick anticoagulant peptide was readily detected by HPLC in the fermentation medium several hours after induction (Fig. 2A). Therefore, an analytical HPLC procedure was developed to quantify r-TAP in fermentation broth and fractions generated during purification. The results of the HfXa assay on fermentation fluids were compared with those obtained by HPLC and the values were within 20% of each other (data not shown). The HPLC assay was used for quantification during purification because, in addition to determining the amount of r-TAP, it also gave a measure of the degree of homogeneity, which was helpful in selecting and pooling the best fractions.

A knowledge of the amino acid sequence permitted the calculation of the molar absorptivity (18 140 l mol^{-1} cm^{-1}) and so peptide concentration was determined from the absorbance at 280 nm. A retrospective experimental determination on purified r-TAP whose concentration was quantified by amino acid analysis gave a value of 18 859 l mol^{-1} cm^{-1}.

Cells were separated from the supernatant liquid in 24–200 l of broth within 2 h by microfiltration with 0.1 μm and diafiltration with

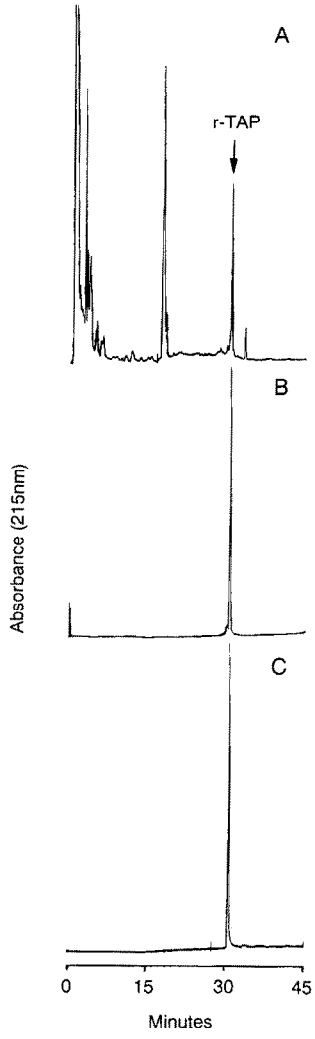

Fig. 2. HPLC analysis of (A) 20 μl of final harvested fermentation broth, (B) S-Sepharose FF product (20 μg) and (C) final product (24 μg) during the purification of r-TAP. Experimental conditions: 150 × 4.6 mm I.D. Vydac C_{18} protein and peptide column, 5-μm particles, 300-Å pore size; flow-rate, 1.0 ml/min; gradient, 10% acetonitrile–0.1% TFA to 45% acetonitrile–0.1% TFA after an 8-min wash with 10% acetonitrile–0.1% TFA; detection, UV absorbance at 215 nm.

100 000 M_rCO hollow-fiber units. The r-TAP permeated both types of fibers and the recovery was about 80% at both the 16- and 250-l fermentation scales (Table I). The r-TAP was adsorbed and concentrated from the clarified fermentation broth by cation-exchange chromatography on S-Sepharose FF after being adjusted to pH 4 and diluted to the appropriated ionic strength ($\leqslant 5$ mS). The unadsorbed column fraction contained UV-absorbing material and pigments from the fermentation broth but no r-TAP was detected. An NaCl gradient was used to elute the bound proteins from the cation-exchange columns (Fig. 3). Each fraction was analyzed by HPLC and only those that were >95% homogeneous by integration of peak areas were pooled. Recovery of r-TAP in the final product from S-Sepharose FF chromatography from the 16-l fermentation was 70%, although 86% was recovered when impure fractions were accounted for. The recovery of r-TAP from the 10-l S-Sepharose FF column was 56% and reflects the poorer resolution obtained with the larger column. This was caused, in part, by the higher loading of the column (3.4 g/l) compared with the smaller column (1.2 g/l) and, in part, by the higher linear flow-rate of 80 cm/h *vs.* 25 cm/h.

Peptide purified by cation-exchange chromatography was >95% homogeneous as estimated by the determination of percentage peak areas on the chromatogram (Fig. 2B). However, it contained pigmented materials from the fermentation broth that were not detected on the chromatograms but which did contribute to the absorbance at 280 nm of the solution. Table I shows that protein determination by absorbance indicated that the product of chromatography at the 16-l scale was only 84% homogeneous, whereas the HPLC profile indicated a purity of >95%. Additionally, desalting of the solution was needed before the protein could be lyophilized. Preparative HPLC removed the salts, and also most of the pigments, but a pink pigment co-purified with the protein and was evident after lyophilization. Advantage was taken of the ability of carbon to absorb conjugated organic molecules by mixing it with a 2% (w/v) solution of the protein, removing the carbon by filtration and relyophilizing. The resulting protein was white. Recovery of r-TAP from HPLC and decolorization was 80% at the 16-l scale and 60% at the 250-l scale. The lower recovery at the 250-l scale was the result of dividing the S-Sepharose product into six aliquots before doing HPLC, which multiplied the losses at this step.

TABLE I

PURIFICATION

Step and scale	Volume (l)	Protein (g)	r-TAP (g)	Yield (%)	Purification (-fold)
Fermentation broth					
16 l	11	88[a]	2.55[c]	100	1 ×
250 l	200	1466[a]	41.4[c]	100	1 ×
Diafiltered broth					
16 l	18.8	73[a]	1.93[c]	76	1 ×
250 l	385	1255[a]	34.0[c]	82	1 ×
S-Sepharose chromatography					
16 l	1.94	1.59[a]	1.37[c]	54	30 ×
250 l	24.4	19.2[a]	19.1[c]	46	37 ×
C_{18} RP-HPLC					
16 l	Dry	1.60[b]	1.28[d]	50	
250 l	Dry	17.3[b]	13.3[d]	32	
Decolorization					
16 l	Dry	1.33[b]	1.10[d]	43	
250 l	Dry	16.3[b]	13.2[d]	32	

[a] Absorbance at 280 nm.
[b] Dry weight.
[c] HPLC.
[d] Amino acid analysis.

Total recoveries of purified protein were 43% at the 16-l scale and 32% at the 250-l scale.

Characterization of r-TAP

The r-TAP gave a single symmetrical peak that was >99% homogeneous when analyzed by HPLC on a C_{18} column (Fig. 2C) and CZE (Fig. 4A). Electrophoretic analysis in non-reduced tris–tricine SDS polyacrylamide gels (Fig. 4B) demonstrated a homogeneous protein migrating with a mass equivalent to M_r = 6000. This is consistent with the value of 6977 predicted from the cDNA. N-Terminal sequencing of 20 nmol of protein through fifteen cycles yielded only the expected sequence of r-TAP (H_2N–Y–N–R–L–C–I–K–P–R–D–W–I–D–E–C–) [2]. Less than 10 pmol of a contaminating peptide sequence could been detected by the method. Quantitative amino acid analysis (Table II) gave results that indicated a homogeneous preparation with composition consistent with that predicted from the cloned gene.

Light-scattering experiments were used to estimate the molecular size of r-TAP also. Assuming r-TAP is a globular protein, the *Z*-average hydrodynamic diameter of 1.6 nm estimates the molecular mass as 7200 g/mol. The data indicate that r-TAP exists as a monomer in solution.

The mass spectrum (Fig. 5), derived from ion spray mass spectrometric analysis of r-TAP, is a derivative of hypermass calculations for the observed multiply charged mass peaks. Accordingly, the final mass estimate was 6977.24 dalton, with a standard deviation of 0.69 dalton. This observed mass is consistent with the theoretical mass of 6977.02 dalton, as evident from the amino acid content (Table II). Other mass peaks present in the spectrum were attributable to M + Na (6999), M + K (7016) and M + Na + K (7038).

The far-UV circular dichroism spectrum of r-TAP is shown in Fig. 6 and is characterized by a positive peak at 192 nm, a strong negative ellipticity at 201 nm and a broad shoulder centered

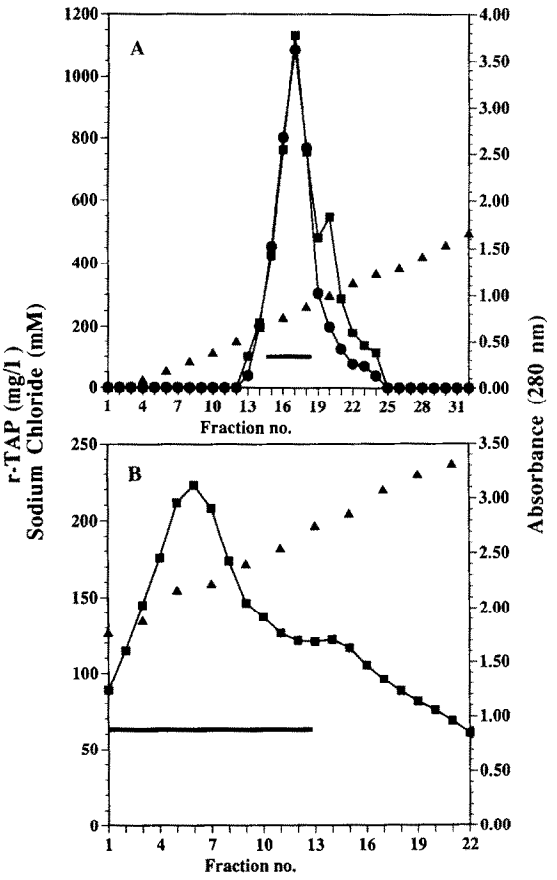

Fig. 3. S-Sepharose FF cation-exchange chromatography elution profiles. (A) 1.2-l column, used to purify 1.93 g of r-TAP at the 16-l fermentation scale; fractions 0.40 l each. (B) 10-l column, used to purify 34 g of peptide at the 250-l fermentation scale; fractions 1.85 l each. ■ = Absorbance at 280 nm; ● = r-TAP by HPLC; ▲ = NaCl. The unadsorbed fractions for these columns are not shown because, although a significant amount of UV-absorbing material and pigment passed through unadsorbed no r-TAP was detected. The fractions marked by the bars were combined to make the pool of purified r-TAP.

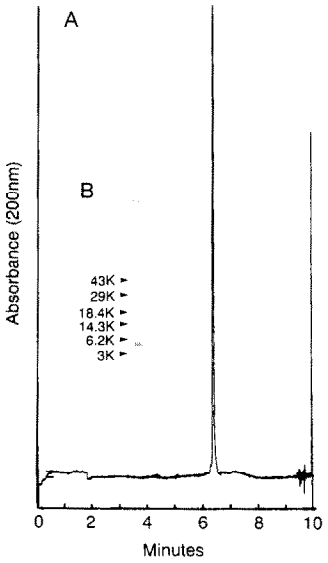

Fig. 4. (A) CZE analysis of r-TAP. Carrier, 20 m*M* sodium citrate (pH 2.5); applied potential, 27 kV; detection, UV absorption at 200 nm; sample, 1.5 ng of protein. (B) SDS-PAGE analysis of non-reduced r-TAP in tris–tricine 10–20% gradient polyacrylamide gels [6], 1.0 mm thick, with detection by silver staining [7]. K = kilodalton.

around 215 nm. Analysis of the spectrum by the VARSELEC program [11] predicted a secondary structure content of *ca.* 10% α-helix, 41% β-sheet, 25% β-turns and 26% disordered structure. For comparative purposes, calculations from a map of the Chou and Fasman algorithm for secondary structure analysis [12] predicted a secondary structure of 10% α-helix, 34% β-sheet, 27% β-turns and 29% disordered structure.

Free solution IEF in the electrodialysis mode gave a p*I* of 4.83–4.93 (Fig. 7). This is slightly higher than the value predicted by calculation with the Genetics Computer Group Sequence Analysis Software [9], p*I* = 4.49, and represents an "apparent" p*I* that reflects the proportion of charged amino acids that are in contact with the aqueous environment of the protein, rather than a p*I* due entirely to difference in the number of negatively and positively charged residues.

The *in vitro* inhibitory activity of the purified protein was assessed by titration of HfXa, 0.500 n*M*, with r-TAP and 50% inhibition of the activity (IC_{50}) was achieved with 0.286 n*M* r-TAP, a value that is reasonably close to the theoretical value of 0.250 n*M* predicted for stoichiometric binding. A thorough kinetic analysis showed that r-TAP purified by this process was a slow-tight binding competitive inhibitor with a K_i of 0.18 n*M* [3].

DISCUSSION

The strategy of expressing recombinant proteins in yeast by fusing a synthetic gene to the

TABLE II

AMINO ACID CONTENTS OF r-TAP

Amino acid	Residues/mol	
	Theoretical[a]	Experimental[b]
Asp	7	11.0[c]
Asn	4	
Thr[d]	1	1.1
Ser[d]	4	3.7
Glu	4	4.0[e]
Gln	0	
Pro	2	2.0
Gly	6	6.0
Ala	3	3.1
Cys[f]	6	5.2
Val	0	N.D.[g]
Met	0	N.D.[g]
Ileu	4	3.7
Leu	1	1.0
Tyr	5	5.0
Phe	3	3.0
His	1	1.1
Lys	2	2.0
Trp	2	–[h]
Arg	5	4.9

[a] Predicted from cDNA.
[b] Average of six determinations.
[c] Asp + Asn.
[d] Corrected for degradation.
[e] Glu + Gln.
[f] Determined as cysteic acid.
[g] N.D. = none detected.
[h] Destroyed by acid hydrolysis.

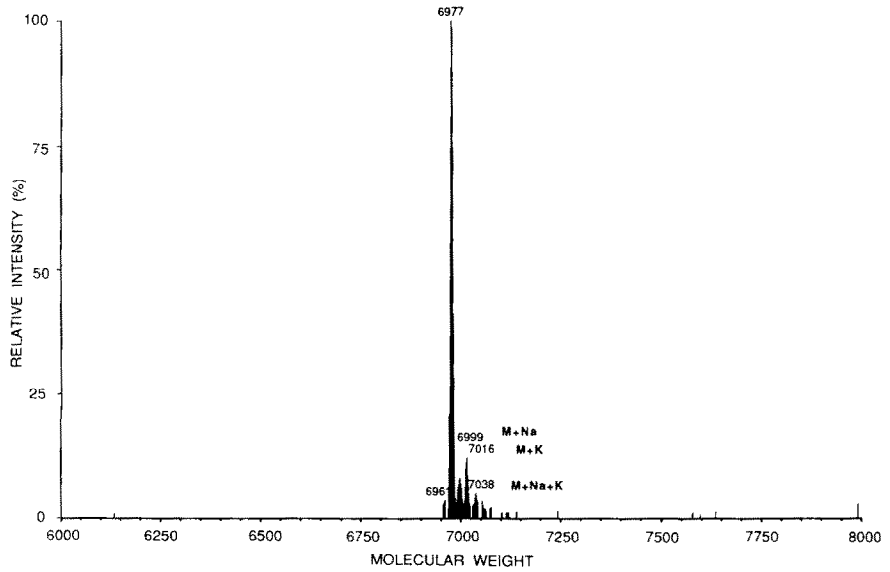

Fig. 5. Determination of the mass estimate for r-TAP by ion spray mass spectrometric analysis.

α-mating factor pre-pro leader gene has been described several times [17–25] and, in the case of r-TAP, the expression of the recombinant protein was regulated by a galactose-inducible promoter. Purification of the heterologous proteins so produced is greatly facilitated in that large amounts can be secreted, 200–250 mg/l in the case of r-TAP, and significant enrichment of the heterologous protein is obtained in the fermentation broth.

The fermentation process described produced 41.4 g of r-TAP in a 250-l fermenter. A process capable of purifying multigram amounts of r-TAP was developed at the 16-l fermentation scale and then scaled-up to the 250-l scale. Cells and broth were rapidly separated by diafiltration. Two additional chromatographic steps, both capable of being operated at a large scale, produced 13.2 g (32% yield) of protein that was >99% homogeneous by CZE, HPLC, SDS-PAGE, N-terminal sequencing and IEF. Mass spectrometry gave an M_r of 6977.24, a value well within the standard deviation and consistent with the M_r of 6977.02 calculated for the peptide with three disulfide bonds, and therefore no post-translational modification was evident. The molecular mass obtained by light scattering, 7200 g/mol, indicates that r-TAP is a monomer in aqueous solution. The secondary structure determined experimentally by circular dichroism spectroscopy is in agreement with that calculated by the semi-em-

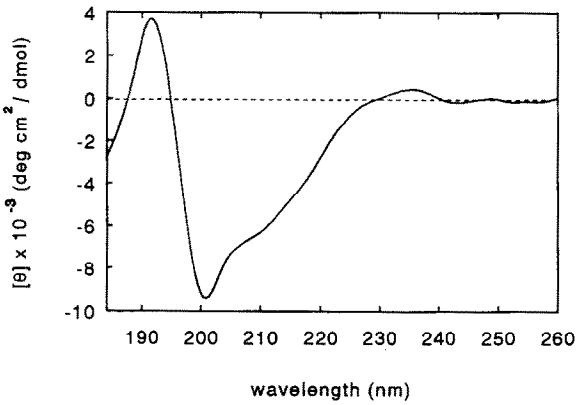

Fig. 6. Far-UV circular dichroism of r-TAP. Spectrum is shown at a peptide concentration of 2 μM in Milli-Q water at 10°C with a 1.0-mm path length and 1.5-nm bandwidth.

pirical method of Chou and Fasman [12]. Biological activity, as determined by inhibition of HfXa, exhibited stoichiometric binding [2] with a dissociation constant of 0.18 nM [3]. Purified r-TAP did not contain any detectable amounts of contaminants shown to purify closely with other heterologous proteins secreted by yeast, *i.e.*, incompletely processed fusion protein [2] and C-terminal cleavage products [5,20,21,26].

Of course non-peptide contaminants, such as yeast pigment, might not be detected by these analytical methods and the colorimetric analysis

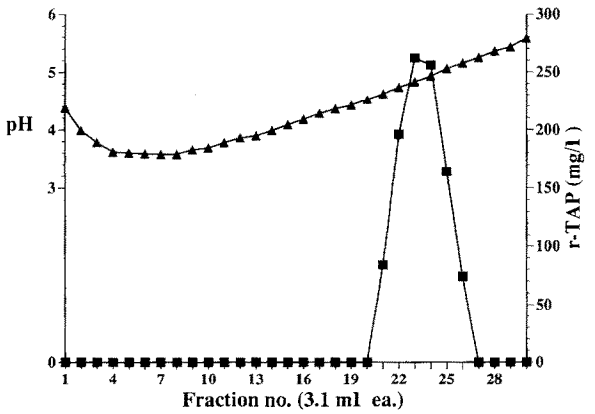

Fig. 7. Isoelectric focusing of r-TAP. The experiment was done in the electrodialysis mode using 1% 2.5–5 ampholytes (Pharmacia), 10% glycerol, 15°C. Ampholytes were prefocused for 35 min at 1500 V before 5 mg of protein were injected, then they were focused at 1500 V for 1 h, 1000 V for 30 min and 500 V for 2 h. r-TAP was determined by HPLC. ▲ = pH; ■ = r-TAP.

in the visible range of the spectrum is not sensitive enough to assure the analyst convincingly that they are absent from the protein. However, treatment with decolorizing carbon removed the residual pigment to the point that it was undetectable to the eye. An alternative method for removing pigments originating in yeast fermentations was described by Bischoff *et al.* [26] during the purification of yeast-derived recombinant hirudin, a small protein inhibitor of thrombin isolated from the medicinal leech. Hirudin was precipitated with organic solvent at 4°C and centrifuged; the pigment remained soluble and was decanted in the supernatant solvent while the protein remained in the pellet. Batch-wise decolorization with an adsorbent such as charcoal might offer advantages when working at a large scale because it does not cause a significant increase in the volume, handling and disposal of organic solvents is avoided and centrifugation, a method often difficult to scale up, is eliminated.

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