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Affinity purification of proteinases by a combination of immobilized peptidyl aldehyde and semicarbazone

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ABSTRACT

D-Phe-Phe-argininal semicarbazone and Tyr-Gly-Gly-Phe-Leu-Arg-argininal semicarbazone were prepared using the solution phase synthesis method and characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. The tripeptide and heptapeptide semicarbazones were individually immobilized on affi-Gel 15 resulting in two affinity columns called S_3 and S_7, respectively. A third affinity column was obtained by hydrolysing the semicarbazone moiety in column S_3 to aldehyde (column A_3). Serine proteinases such as trypsin or rat plasma kallikrein almost quantitatively bind to either S_3 or A_3 affinity columns. Under optimized conditions, more than 97% of trypsin bound to both columns S_3 and A_3. At a lower ionic strength and higher pH, 80–85% of rat plasma kallikrein bound to the same columns. Elution of both enzymes was achieved using mild conditions at near neutral pH and in the presence of a small amount of denaturant. Both proteinases were identified and characterized by high-performance liquid chromatography, sodium dodecylsulphate polyacrylamide gel electrophoresis and by their substrate specificity and inhibition profiles. A single purification (six- to seven-fold) step using either column S_3 or A_3 allowed the preparation of pure trypsin from commercial sources. Starting from rat plasma partially purified by a phenyl boronate column, fractionation on the S_3 column allowed approximately an 87-fold purification of rat plasma kallikrein. However, serial purification of rat plasma kallikrein on column S_7 followed by column A_3 resulted in a purification factor of about 455.

INTRODUCTION

During the past few years, there has been an increasing number of published reports on the applications of peptidyl aldehydes, especially the argininals, as affinity ligands in the purification of many serine and other proteinases [1–4]. This is mainly because these peptide derivatives are potent inhibitors of the proteinases in question. However, it was not until 1984 that the corresponding semicarbazone derivatives were also shown to possess similar proteinase inhibition

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properties [5,6]. The use of peptidyl semicarbazones as affinity ligands for the purification of proteinases was not fully explored until Rich *et al.* [7] successfully used a dipeptide glycinal semicarbazone-bound column for the purification of the cysteine proteinase cathepsin B.

The reversible inhibition of rat plasma kallikrein (rPK) by a number of peptidyl argininal semicarbazone (SC) derivatives has been reported [8] and one of them, Tyr-Gly-Gly-Phe-Leu-argininal-SC, has been used in the purification of rPK. In an effort to improve the purification of trypsin-like enzymes from crude mixtures using affinity procedures, more selective methodologies have been developed for the separation of proteinase mixtures. This paper describes an affinity procedure which allows the purification of trypsin from commercial sources and of rPK from crude rat plasma extracts. The method relies on the judicious use of peptide argininal-SC and argininal columns either alone or in combination, giving as much as a 455-fold purification factor in just two chromatographic steps.

EXPERIMENTAL

Materials

Unless stated otherwise, all amino acid derivatives (Institute Armand Frappier, Laval, Canada or Chemical Dynamics, South Plainfield, NJ, USA) are of the L-form. Commercial trypsin and soybean trypsin inhibitor (SBTI) (Kunitz type, molecular mass 21 700) were from Sigma (St. Louis, MO, USA) and rPK was from rat plasma isolated in our laboratory. Thin-layer chromatographic (TLC) analyses were performed on silica gel plates (silica gel G, 250 μm, E. Merck 5554) precoated on aluminium sheets and the peptides were revealed with ammonium molybdate–sulphuric acid, ninhydrin or modified Sakaguchi [9] spraying reagents. The solvents used for ascending TLC were chloroform–methanol (5:1, v/v; system A); butan-1-ol—acetic acid–water (4:1:1, v/v; system B) and butan-1-ol–acetic acid–water–ethyl acetate (1:1:1:1, v/v; system C). Optical rotations were measured with a Perkin-Elmer 141 polarimeter in a 1-cm cell. Melting points were uncorrected and recorded in a digital electrothermal instrument.

^{1}H NMR spectra were recorded on a Varian Assoc. Bruker 400-MHz spectrometer in [^{2}H]dimethyl sulphoxide (Me_2SO) unless stated otherwise. ^{13}C NMR spectra, fully proton noise-decoupled, were also run in the same spectrometer at 100 MHz at ambient temperature in Fourier transform mode at a peptide concentration of 0.02 m*M* in [^{2}H]Me_2SO.

The enzymatic activities of rPK and trypsin were assayed by monitoring the fluorescence of released 7-amino-4-methylcoumarin (AMC) from the substrates, D-Phe-Phe-Arg-4-methylcoumarylamide (MCA) or *Z*-Ala-Lys-Arg-MCA as reported elsewhere [10]. The protein determination was performed using Pierce bicinchoninic acid (BCA) and a Bio-Rad kit according to the manufacturer's instructions. IC_{50} values of the inhibitors (*i.e.* concentrations causing 50% inhibition) were calculated as described previously [5]. The following buffer systems were used: buffer A, 10 m*M* N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 1 m*M* EDTA, pH 8.0; buffer B, 100 m*M* 2-(N-morpholino)ethanesulfonic acid (MES), 1 m*M* EDTA, pH 6.0; buffer C, 25 m*M* NaH_2PO_4, 1 m*M* EDTA, pH 6.0; and buffer D, 100 m*M* BES, 1 m*M* EDTA, pH 8.5.

Peptide synthesis

Two peptidyl argininal semicarbazones known to be potent inhibitors of rPK [8], namely D-Phe-Phe-argininal-SC and Tyr-Gly-Gly-Phe-Leu-Arg-argininal-SC, were synthesized by the solution phase method through coupling of *tert.*-butyloxycarbonyl (Boc)-D-Phe-Phe-OH or Boc-Tyr [benzyloxy(OBzl)]-Gly-Gly-Phe-Leu-Arg (ωNO_2)-OH to H-Arg(ωNO_2)-SC, followed by complete deprotection.

N-Boc-D-Phe-Phe-ωNO_2-argininal-SC (II). Boc-D-Phe-Phe-OH (I, 6.0 mmol), obtained by coupling Boc-D-Phe-OH with Phe-OMe and alkaline hydrolysis, was allowed to react with an equimolar amount of (ωNO_2)-argininal-SC · HBr in dimethylformamide (DMF)–tetrahydrofuran (THF) (10:1, v/v; 22 ml) and Et_3N. The purified material (II, 2.6 g, 66%) exhibited

the following characteristics: melting point (m.p.) 138–140°C, R_F(A) 0.33, $[\alpha]_D^{25}$ −9.21° (c, 6.30, MeOH), fast atom bombardment mass spectrometry (FAB-MS), *m/z* 677 $(M + Na)^+$ and 655 $(M + H)^+$.

N-Boc-D-Phe-Phe-argininal-SC · HOAc (III). Boc-D-Phe-Phe-ωNO_2-argininal-SC (II, 654 mg, 1 mmol) on hydrogenation in 5% acetic acid–methanol (v/v, 15 ml) with Pd black (200 mg) for 16 h at ambient temperature followed by silica gel column chromatography (30 cm × 2.5 cm I.D.) yielded on elution with chloroform–methanol (5:1, v/v) a fraction (III, 395 mg, 56%) with the following characteristics: m.p. 193–195°C, R_F(B) 0.36, $[\alpha]_D^{25}$ −8.5° (c, 2.85, MeOH), FAB-MS, *m/z* 632 $(M + Na)^+$ and 610 $(M + H)^+$.

D-Phe-Phe-argininal-SC · 2HOAc (IVa). Boc-D-Phe-Phe-argininal-SC · HOAc (III, 0.52 mmol), following deprotection with DCM–TFA (1:1, v/v, 15 ml, 30 min, room temperature), yielded after silica gel column chromatography (28 cm × 2.5 cm) a fraction eluting at methanol–chloroform–acetic acid (1:1:0.02, v/v) (IVa, 172 mg, 54%): m.p. 208–210°C, R_F(B) 0.11, $[\alpha]_D^{25}$ −6.2° (c, 3.8, H_2O); FAB-MS, *m/z* 510 $(M + H)^+$. Following similar procedure, L-Phe-Phe-argininal-SC (IVb) was also prepared and characterized.

Tyr-Gly-Gly-Phe-Leu-Arg-argininal-SC · 3HOAc (V). This peptide was prepared by coupling Boc-Tyr(OBzl)-Gly-Gly-Phe-Leu-(ωNO_2)-Arg-OH with H-(ωNO_2)-argininal-SC followed by complete deprotection. Following purification by silica gel column chromatography, it showed m.p. 240–245°C, R_F(C) 0.43; FAB-MS, *m/z* 922 $(M + Na)^+$ and 910 $(M + H)^+$.

Peptide purification

The peptide products were purified by column chromatography over silica gel (230–400 mesh, 60 Å, Aldrich) and/or by reversed-phase high-performance liquid chromatography (RP-HPLC). The purification runs were performed with a Gilson chromatograph (Model 302) consisting of pumps (6000A and M45), a programmer (720) and a semi-preparative Vydac 218TP510-C_{18} reversed-phase column (25 cm × 0.46 cm I.D.). The buffer system consisted of 0.025% trifluoroacetic acid (TFA) (v/v) in water and acetonitrile. Elution was with a linear gradient of 5–80% acetonitrile in 75 min at a flow-rate of 3.0 ml/min for preparative runs and 1.0 ml/min for analytical runs (25 cm × 0.2 cm I.D., C_{18} Vydac column) unless stated otherwise. The purification was followed by monitoring the UV absorbance at 225 nm.

Immobilization of D-Phe-Phe-argininal-SC and Tyr-Gly-Gly-Phe-Leu-Arg-argininal-SC (S_3 and S_7 columns)

D-Phe-Phe-argininal-SC (IVa, 10 mg, 0.02 mmol) and Tyr-Gly-Gly-Phe-Leu-Arg-argininal-SC (V, 30 mg, 0.03 mmol) were coupled separately to affi-Gel 15 (10 ml packed gel) following the suggested manufacturer's procedure to yield the bound peptide-SC, S_3 and S_7 columns having a concentration of around 2.0 and 3.0 μmol/ml of gel, respectively. The progress of the coupling was monitored by RP-HPLC and was almost complete after 3 h. When not in use the gels were stored at 4°C in 0.01% aqueous NaN_3 solution.

Preparation of immobilized SBTI column

Following a similar method, SBTI (10 mg, 0.46 μmol) was also coupled to affi-Gel 15 (10 ml packed gel) to yield the bound SBTI affi-Gel column with a concentration of 0.046 μmol SBTI per ml of gel.

Immobilization of D-Phe-Phe-argininal (A_3 column)

D-Phe-Phe-argininal-SC bound gel (S_3) (10 ml) was treated with methanol–formaldehyde–acetic acid (3:1:1, v/v, 10 ml) for 18 h at 4°C to hydrolyse the SC group to the aldehyde function. The total free aldehyde content in the gel was determined using the *p*-phenylazoaniline–salicylaldehyde reagent [3] and never exceeded 10%. The rest of the functional group is presumably present in the hydrated form [11].

Purification of commercial trypsin by affinity chromatography on the S_3 column

Commercial trypsin (6 mg, Sigma), dissolved

in buffer C (10 ml), was loaded on the S_3 column (8 ml, packed volume) previously equilibrated with buffer C at a flow-rate of 7 ml/h at 4°C. Following successive washings with buffer C and buffer C containing 1 *M* NaCl (40 ml each), the column was filled up with buffer B containing 1 *M* guanidine hydrochloride (Gn · HCl) and 1 *M* NaCl at pH 6.0 (8 ml) and allowed to stand at 25°C for 1 h. The flow-rate was then resumed and the column was further eluted with the same buffer (32 ml). The pH of the eluate was immediately raised to 8.0 by collection in tubes containing an equal volume of buffer B, pH 9.5. The combined fractions were dialysed against buffer B (2 × 3 l) and finally concentrated in a Minitan concentrator. The enzymatic activity and the protein content were measured at various stages of purification. The identity and purity of trypsin thus eluted was confirmed by RP-HPLC, inhibition profiles and by autoradiography after labeling with [^{125}I]-D-Tyr-Glu-Phe-Lys-Arg-chloromethylketon (CK) as described previously [10,12,13].

Purification of commercial trypsin by affinity chromatography through immobilized SBTI

Commercial trypsin (1 mg) in buffer C (10 ml) was shaken with immobilized SBTI (10 ml packed gel) at 4°C until almost all the activity was bound. Following extensive washings as described earlier, the pure enzyme was eluted with buffer C containing 1 *M* Gn · HCl and 1 *M* NaCl at pH 6.0 (6 × 15 ml).

Purification of rPK by affinity chromatography through S_3 column

The rPK contained in the 1 *M* Gn · HCl eluate resulting from purification of rat plasma on a phenyl boronate–agarose column [10] was dialysed at 4°C against buffer A (3 × 3 l). The course of dialysis was monitored by conductance measurements of the dialysis buffer until this value became identical with that of buffer A (1.65 mS relative to water as 3.75 μS). The resulting mixture (55 ml) was then slowly pumped on the S_3 column (10 ml), previously equilibrated with buffer A (pumping rate 7–8 ml/h) at 4°C. Following washings, rPK was released from the column and concentrated as described for trypsin.

Purification of rPK by affinity chromatography through S_7 and A_3 columns

A similar procedure is also used for the affinity purification of rPK through a serial combination of S_7 and A_3 columns where the release of bound rPK from the final (A_3) column was achieved with buffer B containing 1 *M* NaCl, 1 *M* Gn · HCl and 1 *M* semicarbazide · HCl at pH 6.0 instead of the conventional use of leupeptin followed by treatment with $NaBH_4$.

Separation of a trypsin and rPK mixture using S_3 affinity column

A mixture of rPK and trypsin was obtained by combining the 1 *M* Gn · HCl eluate from a phenyl boronate–agarose column (143 ml, total rPK enzymatic activity 25 250 nmol/h) with a solution of commercial trypsin in water (60 μg, 5 ml, total trypsin enzymatic activity 29 550 nmol/h). After dialysis against buffer C this mixture was applied to the S_3 column (10 ml). After washing with buffer C and 1 *M* NaCl-containing buffer C, the bound trypsin was released in a pure form. The combined flow through containing almost exclusively rPK was dialysed against buffer A (3 × 3 l), diluted with 10 m*M* Bes, 2 m*M* EDTA, pH 8.0 (total volume 150 ml) and pumped on to a freshly prepared S_3 column (10 ml). The rPK activity bound was released as described for trypsin purification.

RESULTS

Chemical characterization by FAB-MS, ^{1}H NMR and ^{13}C NMR of the peptidyl SCs

Both free and Boc-protected D-Phe-Phe-arginal-SC showed molecular ion peaks at *m/z* 510 and 610, respectively. Both exhibited fragmentation patterns resulting from the losses of NH_3 [17 mass units (mu)], $CH_2=NNHCONH_2$ (87 mu), HNCO (43 mu), H_3NO (33 mu), NHCONH (58 mu) and CH_3N (29 mu), all indicative of the presence of an SC moiety. Similar patterns were also observed with the heptapeptide-SC derivative (V) (data not shown).

The ^{1}H NMR spectra of the tripeptide-SC (IVa) (Fig. 1) and of the heptapeptide-SC display

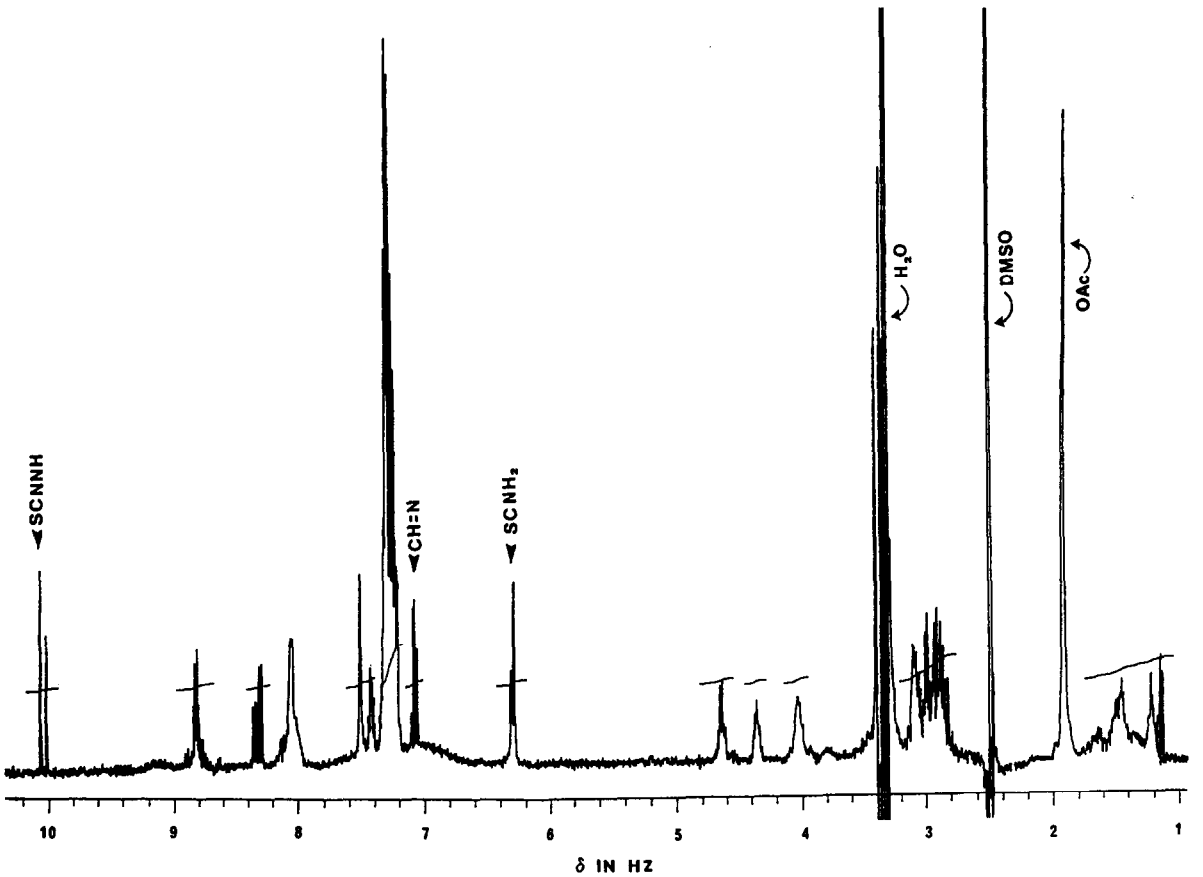

Fig. 1. 400-MHz ^{1}H NMR spectrum of D-Phe-Phe-argininal-SC in [^{2}H]Me_2SO at 25°C.

characteristic signals at δ (ppm) 10.0 (1H) and 6.30 (2H) for the three protons belonging to the SC function (NH*H*CONH$_2$ and NNHCON*H*$_2$, respectively). The proton originally belonging to the aldehydic moiety (CH–C*H*=NNHCONH$_2$) appeared as a doublet or a triplet at δ (ppm) 7.10 (J = 6.0 Hz) because of splitting with the adjacent Arg α proton. These data confirmed the presence of the SC functionality in the two derivatives IVa and V.

Both protected and free D-Phe-Phe-argininal-SC were also examined by ^{13}C NMR. Although unambiguous assignments of all the carbon atoms may not be possible in the absence of further studies, the signal positions for the two carbon atoms belonging to the SC and the Gn functions were very distinct as shown in Table I.

Chromatography through the tripeptidyl-SC column (S_3)

When applied separately under appropriate buffer conditions, both rPK (80–85%) and trypsin (more than 97%) very efficiently bound to the S_3 column. Optimum binding of trypsin required buffer C, whereas for rPK buffer A was more suitable. The effect of pH and ionic strength of the loading buffer on the binding of rPK to the S_3 column is shown in Table II, where it is seen that 80, 51 and 25% of the activity was bound after an overnight incubation in buffers A, D and C, respectively. Most of the non-enzyme protein impurities were removed during the wash steps. More than 78% rPK and 98% of trypsin could be released by buffer B containing 1 *M* Gn · HCl and 1 *M* NaCl at pH 6.0. This method allowed an

TABLE I

^{13}C NMR DATA OF D-Phe-Phe-ARGININAL-SC AND Boc-D-Phe-Phe-ARGININAL-SC IN [^2H]Me_2SO

Carbon type	Chemical shifts[a] (Hz)	
	Boc-Phe-Phe-argininal-SC	H-Phe-Phe-argininal-SC
Boc-tertiary	37.94	–
Boc-methyl	27.98	–
Boc-carbonyl	77.96	–
Phe_1-amide carbonyl	171.35	173.95
Phe_2-amide carbonyl	170.58	170.55
$-NNHCONH_2$ (SC)	157.86	157.78
$-CH=NNHCONH_2$ (SC)	141.72	141.74
All α-carbons	55.81 (Phe_1) 53.10 (Phe_2) 49.48 (Arg)	55.99 53.50 49.40
Phe_1 and Phe_2	37.50	38.18
β-Carbon	37.37	38.05
Arg β-carbon	29.29	29.17
Arg γ-carbon	27.69	24.15
Arg δ-carbon	40.72	40.58
Arg-Gn-carbon	155.86	156.72
Phe_1 and Phe_2 aromatics	138.11 (1 carbon) 137.45 (1 carbon) 129.11 (4 carbons), $C_{2,6}$ 127.95 (4 carbons), $C_{3,5}$ 126.0 (2 carbons)	138.61 (1 carbon) 137.30 (1 carbon) 129.11 (4 carbons), $C_{2,6}$ 127.95 (4 carbons), $C_{3,5}$ 126.0 (2 carbons)

[a] The strong signals at δ 175.22, 23.8 (MeCOOH) and 39.5 (Me_2SO) were attributed to solvents.

87-fold enrichment of the partially purified rPK and a six- to seven-fold enrichment of commercial trypsin.

In addition to the purification of commercial trypsin and of partially purified rPK, the S_3 column was found to be effective in the separation of a mixture containing approximately equal amounts of each activity against D-Phe-Phe-Arg-MCA. To follow easily the purification, it was decided to rely on two properties, namely the sensitivity of either enzyme towards inhibitors and their different elution characteristic on RP-HPLC separation. Indeed, approximately 50% of the enzymatic activity can be abolished by inclusion of Nα-*p*-tosyl-lysine-CK (TLCK) or lima bean trypsin inhibitor (LBTI), both of which inhibit trypsin quantitatively but not rPK. Addition of SBTI to either TLCK or LBTI completely abolishes the enzymatic activity present in the mixture, as expected as SBTI fully inhibits rPK (Table III) [13]. When this mixture is loaded on the S_3 column in buffer C only trypsin was bound (more than 90%), as evidenced by the near disappearance of the corresponding peak on RP-

TABLE II

BINDING OF TRYPSIN AND rPK TO D-Phe-Phe-ARGININAL-SC (S_3) COLUMN AND THEIR SUBSEQUENT ELUTION

rPK was obtained from 5 g of rat plasma and trypsin from 6 mg of commercial sample.

Enzyme	Buffer[a]	Enzymatic activity[b] (nmol/h)	Protein (mg)	Specific activity (nmol/h/mg)	Flow through + buffer wash		Activity bound (%)	Gn · HCl pool[c]			Purification factor	Activity recovered (%)
					Activity (nmol/h)	Protein (mg)		Activity (nmol/h)	Protein (mg)	Specific activity (nmol/h/mg)		
Trypsin	C	$3.46 \cdot 10^6$	6.0	577 166	6129	2.90	99.8	$3.20 \cdot 10^6$	0.88	$3.64 \cdot 10^6$	6.3	93
rPK	C	53 688	4.0	13 422	40 560	3.57	25	7800	–	–	–	60
	A	60 140	4.2	14 319	12 028	3.88	80	37 527	0.03	$1.25 \cdot 10^6$	87.4	78
	D	62 328	4.2	14 804	30 508	3.92	51	23 483	–	–	–	74

[a] Buffer A, 10 m*M* BES and 1 m*M* EDTA, pH 8.0; buffer B, 100 m*M* Mes and 1 m*M* EDTA, pH 6.0; buffer C, 25 m*M* NaH_2PO_4 and 1 m*M* EDTA, pH 6.0; and buffer D, 100 m*M* BES and 1 m*M* EDTA, pH 8.5.

[b] Enzymatic activity was measured in terms of released AMC from D-Phe-Phe-Arg-MCA as fluorogenic substrate.

[c] Buffer B containing 1 *M* Gn · HCl and 1 *M* NaCl.

TABLE III

INHIBITION PROFILES OF TRYPSIN, rPK AND TRYPSIN–rPK MIXTURE

The enzymes were preincubated with the inhibitor for 30 min in buffer D before addition of the substrate. Following the preincubation period, the activity remaining relative to the control (enzyme preincubated without inhibitor) was measured using D-Phe-Phe-Arg-MCA as fluorogenic substrate.

Inhibitor	Amount (μg/ml)	Enzymatic activity (nmol/h)		
		Trypsin[a]	Trypsin/rPK mixture[b]	rPK[c]
None	–	13.02	15.91	13.26
TLCK	0.74	0	7.85	13.26
LBTI	1.0	0	7.37	13.06
TLCK + SBTI	0.74 + 1.0	0	0	0
LBTI + SBTI	1.0 + 1.0	0	0	0

[a] Trypsin was obtained from a commercial source.

[b] A mixture of trypsin and rPK was obtained as described in the Experimental section.

[c] rPK was present in the Gn pool after phenyl boronate–agarose column of rat plasma extract.

HPLC and by the minimal inhibition with TLCK and LBTI of the unretained activity on the S_3 column. The bound trypsin was later released (80–85%) and further characterized as described in the following section. The rPK activity present in the flow through was then purified using another S_3 column equilibrated with buffer A; the final product exhibited characteristics identical to purified rPK. These results demonstrated that under the optimized conditions of pH and buffer solution, the S_3 column allows the efficient separation of trypsin from rPK, two proteinases sharing similar substrate specificity.

In this study, the D-Phe-Phe-argininal-SC derivative has been preferred over L-Phe-Phe-argininal-SC because the former is much stronger towards rPK ($IC_{50} = 7.44$ μM) than the latter ($IC_{50} = 0.96$ mM) (data not shown).

Chromatography through the tripeptidyl-aldehyde column (A_3)

Almost identical results were also achieved with the A_3 column, where we observed more rapid binding kinetics. The enzymes were recovered by elution with buffer B containing 1 M semicarbazide · HCl, 1 M Gn · HCl and 1 M NaCl (data not shown). In both instances the enzymes are stable in the eluting buffer but are preferably stored after dialysis against suitable buffers and after dilution with glycerol.

Characterization of the purified enzymes

The purified rPK and trypsin were characterized by RP-HPLC (Fig. 2) and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) following affinity labeling with [^{125}I]-D-Tyr-Glu-Phe-Lys-Arg-CK (Fig. 3) [10]. Thus affinity-purified trypsin exhibited on analysis by HPLC a single peak at 47.1 min (52% acetonitrile) containing more than 90% of the total enzymatic activity. Furthermore, the ratio of the initial rate of cleavage of D-Phe-Phe-Arg-MCA *versus* *Z*-Ala-Lys-Arg-MCA was found to be between 1.2 to 1.5, similar to that of unpurified trypsin (Table IV). The above activity was completely inhibited by LBTI, SBTI and TLCK (Table III), and migrated as a single band on SDS-PAGE with an apparent M_r of 27 kilodaltons (Fig. 3).

The affinity-purified rPK from S_3 column eluted on HPLC (Fig. 2) as a major peak at 26.3 min (51% acetonitrile) and two minor peaks around

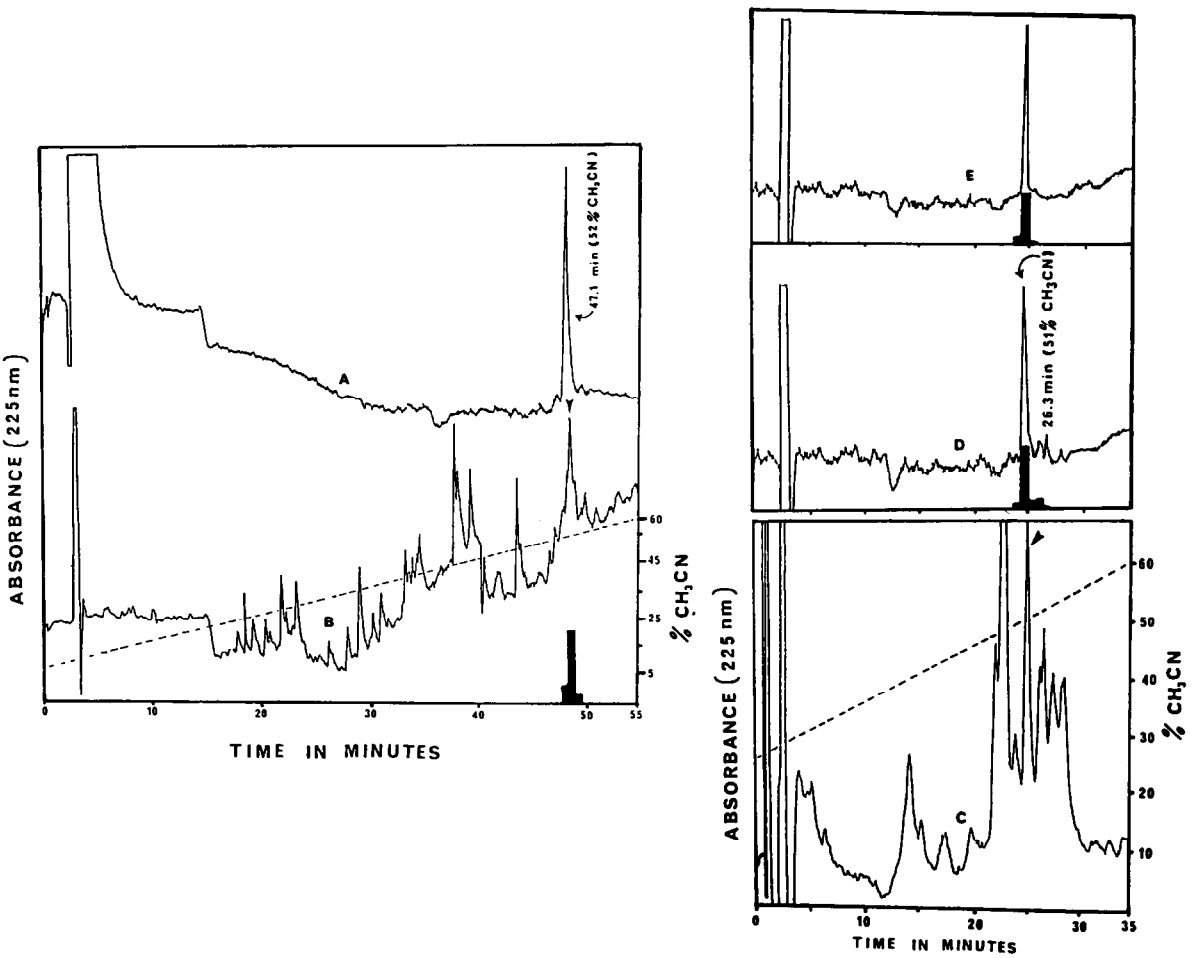

Fig. 2. Reversed-phase HPLC profiles of (A) trypsin purified *via* SBTI or S_3 column, (B) commercial sample of trypsin, (C) Gn · HCl pool containing rPK of phenyl boronate–agarose column, (D) rPK purified *via* column S_3 alone and (E) rPK after purification through a combination of the S_7 and A_3 columns. The Vydac C_{18} analytical column was eluted with the linear gradient of acetonitrile–TFA (0.025%, v/v) as depicted. The bar charts indicate the relative enzymatic activity of each fraction (1 ml) collected. The chromatogram was monitored at 225 nm, 0.05 a.u.f.s.

28 min (53% acetonitrile); these three fractions displayed enzymatic activity when assayed with fluorogenic substrates. Whereas the elution position of the first peak was identical to that observed previously [12,13], the remaining two minor peaks may possibly represent autolytic degradative products. These conclusions are further strengthened by the results of SDS-PAGE as shown in Fig. 3. The presence is noted of a major band migrating close to 90 kilodaltons together with two other minor bands of lower molecular mass which, under reducing conditions, migrate as a 44 kilodalton single band. This latter form corresponds to the molecular mass of the catalytic chain of rPK, suggesting that they represent molecules in which the regulatory chain of rPK has undergone autocatalytic cleavages [13]. The identity of rPK was further confirmed by the measured 5.6–5.9 activity ratio using the D-Phe-Phe-Arg-MCA and *Z*-Ala-Lys-Arg-MCA substrates (Table IV), a value characteristic of rPK [13].

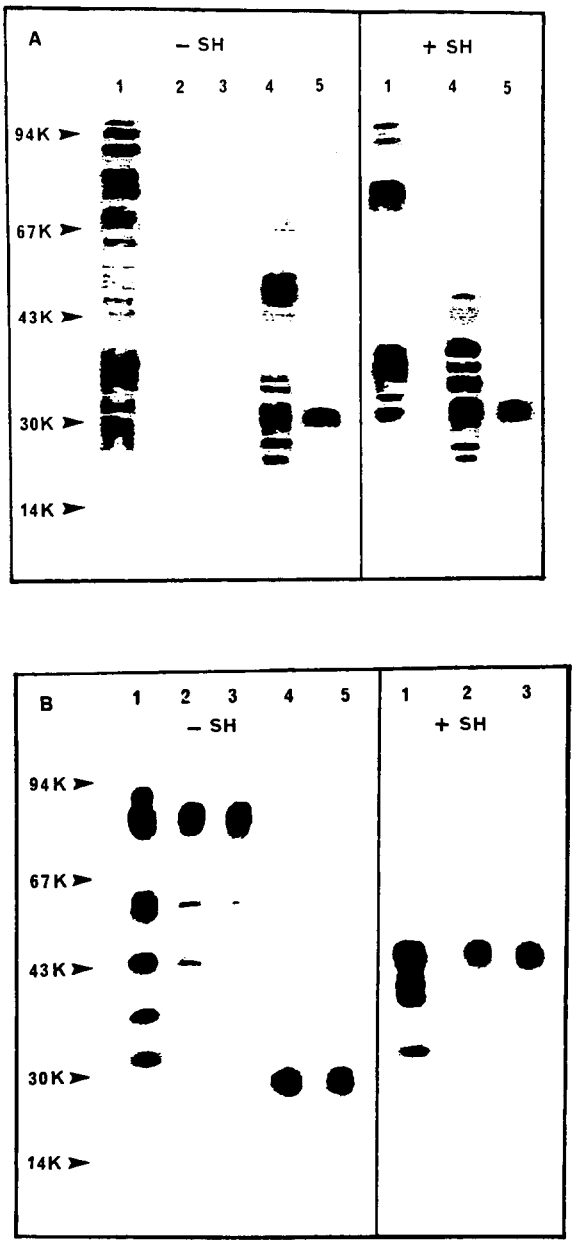

Fig. 3. (A) SDS-PAGE of rPK and trypsin after labelling with [^{125}I]-D-Tyr-Glu-Phe-Lys-Arg-CK and staining with Coomassie brilliant blue and run under non-reducing conditions (– SH) or reducing conditions (+ SH) on 8% acrylamide gel as described previously [17]. Lane 1, crude rPK after phenyl boronate–agarose column; lane 2, rPK after purification by S_3 column; lane 3, rPK after purification by S_7 and A_3 column; lane 4, commercial trypsin; and lane 5, trypsin purified by S_3 column. (B) Autoradiogram of the SDS-PAGE in part A. Identification of the material analysed corresponds to lane assignment described above.

Comparison of trypsin purification using immobilized SBTI and the tripeptidyl-SC columns

To evaluate and analyse the efficiency of S_3 column with respect to a typical trypsin purification protocol, a comparative study was conducted with immobilized S_3 and immobilized SBTI using identical support material freshly prepared in our laboratory. Both columns bound trypsin activity almost quantitatively (in excess of 98%) in 2 h under identical experimental conditions. Purified enzyme could be eluted from either of these columns with the same eluting buffer system at an 81 and 69% yield from immobilized S_3 and immobilized SBTI, respectively (Table V). The HPLC profiles and SDS-PAGE analysis also confirm the high degree of purity achieved with both methods. As trypsin samples are often contaminated with chymotrypsin activity, the amount of such an activity was determined in the original commercial as well as in the purified samples, using the fluorogenic substrates Succ-Ala-Ala-Pro-Phe-MCA and Succ-Leu-Leu-Val-Tyr-MCA, known to be specific for chymotrypsin [14,15]. Although the chymotrypsin contamination in the original commercial sample was very low (around 0.2% only), the purified trypsin samples obtained from either method failed to show any detectable amount of chymotrypsin activity (Table V). These results thus demonstrated that the SC-based affinity column is equally efficient as the SBTI column.

Chromatography through a combination of heptapeptidyl-SC (S_7) and tripeptidyl-aldehyde (A_3) columns

The serial application of the two affinity columns S_7 and A_3 resulted in a much improved degree of purification (455-fold) for rPK as shown in Table VI. The use of 1 *M* semicarbazide · HCl in the eluting buffer was found to be necessary for the effective elution of bound rPK from the A_3 column.

DISCUSSION

Although a number of affinity-based procedures using natural or synthetic inhibitors are

TABLE IV

COMPARISON OF ENZYMATIC ACTIVITIES DUE TO TRYPSIN AND rPK TOWARDS DIFFERENT FLUOROGENIC SUBSTRATES

Enzyme	Enzymatic activity (nmol/h)			Ratio of activities	
	Z-Ala-Arg-Arg-MCA (*A*)	*Z*-Ala-Lys-Arg-MCA (*B*)	D-Phe-Phe-Arg-MCA (*C*)	*C*/*A*	*C*/*B*
Trypsin	43.4	96.2	120.9	2.8	1.3
rPK	2.2	26.7	153.0	69.5	5.7

TABLE V

PURIFICATION TABLE FOR TRYPSIN USING IMMOBILIZED SBTI AND IMMOBILIZED D-Phe-Phe-ARGININAL-SC

Enzyme (source)	Amount (mg)	Total enzyme activity[b] (μmol/h)			Specific activity[c] (μmol/h/mg)	Purification factor
		A	B	C		
Trypsin (commercial)	1.00	163.0	0.22	0.26	163.0	–
Chymotrypsin (commercial)	1.00	5.10	105.9	40.0	105.9	–
Trypsin[a] (SBTI column)	0.13	118.7	N.D.[d]	N.D.	913.1	5.60
Trypsin[a] (SC column)	0.12	130.6	N.D.	N.D.	1088.3	6.68

[a] The trypsin used for the purification on either column was of the commercial type and corresponded to 1 mg.

[b] Total enzymatic acitivity was determined using the following peptidyl substrates: (A) *Z*-Ala-Lys-Arg-MCA; (B) Succ-Ala-Ala-Pro-Phe-MCA; and (C) Succ-Leu-Leu-Val-Tyr-MCA.

[c] Specific activity as determined using *Z*-Ala-Lys-Arg-MCA for trypsin and Succ-Ala-Ala-Pro-Phe-MCA for chymotrypsin.

[d] N.D. = not detectable.

TABLE VI

PURIFICATION OF RAT PLASMA KALLIKREIN USING A COMBINATION OF IMMOBILIZED Tyr-Gly-Gly-Phe-Leu-Arg-ARGININAL-SC (S_7) AND D-Phe-Phe-ARGININAL (A_3) COLUMNS

Fraction	Protein (mg)	Activity (μmol/h)	Specific activity[b] (μmol/h/mg)	Purification factor
1 *M* Gn · HCl pool[a]	95.0	0.21	0.002	–
S_7 eluate	4.20	0.18	0.043	19
A_3 eluate	0.16	0.16	1.001	455

[a] Rat plasma kallikrein used was obtained from 70 g of rat plasma as previously described [13]. The fraction used corresponds to the Gn · HCl eluate from the phenyl boronate–agarose column.

[b] Specific activity as determined using D-Phe-Phe-Arg-MCA.

currently available for the purification of trypsin-like enzymes, some of these methods are time-consuming, give a poor recovery of active enzymes sometimes contaminated with large amounts of inactive proteins, or use very drastic eluting conditions such as a reducing agent, high or low pH, high salt and/or high amount of denaturant to retrieve the active enzyme [8,16–20]. In this respect the proposed method has some specific advantages namely: (i) it uses only chaotropic agens and a salt buffer system for elution of the bound enzymes; (ii) it can be extended to other classes of enzymes such as thiol-proteinase [5,7,8]; and (iii) the immobilized ligand is extremely stable, even more so because it is not submitted to extreme pH values or to harsh conditions during use. Indeed, for example, the elution of bound proteinase from aldehyde-based columns is practically impossible by increasing the ionic strength, lowering the pH or the addition of denaturants, whereas elution was easily accomplished here with excellent enzymatic activity recovery from the corresponding SC column.

Until now the purification of plasma kallikrein was a very cumbersome and tedious procedure because of the need for three or more chromatography purification steps [21–23]. Previous attempts to purify rPK using a SBTI–agarose column yielded an extremely low amount of active proteinase because of the non-availability of any mild eluting buffer systems [13,22]. Thus the peptidyl-SC immobilized column introduced in this study could be extremely useful for the affinity purification of plasma kallikrein as well as other serine proteases. The substrate specificity of the enzyme to be purified will therefore dictate the peptide sequence within the peptidyl-SC derivatives.

Although the mechanism by which peptidyl-SC derivatives associate with serine proteinases is not presently understood, it is likely to involve ionic interactions, hydrogen bonding or reversible covalent binding, as noted for the inhibition of papain (a cysteine proteinase) by peptidyl nitriles [24,25]. The nature and geometry of the catalytic pocket of the enzyme where the C-terminal SC group ($-CH=N-NH-CO-NH_2$) attaches, may also play an important role in the binding phenomena. Further studies, particularly using ^1H and ^{13}C NMR, may be useful to shed more light on this mechanism. Indeed, it has been shown that peptidyl-SC derivatives exhibit characteristic signals for protons and carbon atoms belonging to the SC function. The unique positions of these peaks in the ^{13}C NMR spectrum may find useful application in monitoring their involvement in the binding mechanism of these ligands with a serine proteinase.

These studies revealed the potential application of peptidyl argininal-SC immobilized columns in the separation and purification of serine proteinases such as rPK and trypsin. Furthermore, a high degree of enzyme purification can be achieved by exploiting the powerful combination of two affinity-based procedures utilizing ligands displaying non-covalent (SC-containing peptides) and covalent (peptidyl aldehydes) reversible binding. In the latter case, the covalent bond between the proteinase and the immobilized inhibitor could be reversed by a displacement reaction with a more potent inhibitor. This finding is of particular importance as numerous serine proteinases are involved in the regulation of many biological functions. One example is shown by the number of proteinases found in the glandular kallikrein family. This procedure may find applications in this field as presently no technique is available for the selective purification of each member of this family in which the overall sequence similarity between the different tissue kallikreins is close to 80–85% [26].

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