Human plasma kallikrein

A rapid purification method with high yield

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A simple method for isolation of kallikrein from human plasma is described. Before activation of the enzyme with acetone, the plasma was treated with 0.2 M-methylamine at pH 8.2 to inactivate α_2 -macroglobulin and thus prevent the irreversible binding of the active enzyme to the inhibitor. The enzyme was adsorbed on soya-bean trypsin inhibitor–Sephacrose 4B and eluted with 5 mM-NaOH, pH 11.3. It was further purified by immunoabsorption of contaminating proteins, and gel chromatography on Ultrogel AcA 44. About 3 mg of kallikrein was obtained from 400 ml of plasma (35% yield). The purified enzyme was shown to be homogeneous by electrophoretic and immunological criteria. The specific activities against benzyloxycarbonylphenylalanylarginine methylcoumarylamide, prolylphenylalanylarginine methylcoumarylamide and tosylarginine methyl ester were higher than any previously reported. The purified enzyme was resolved into two forms of mol.wts. 88 000 and 86 000 in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis without reduction. Each consisted of three chains linked by disulphide bonds, one containing the reactive serine residue (mol.wt. 36 000 or 34 000), and two additional chains (mol.wt. 28 000 and 22 000).

Plasma kallikrein (EC 3.4.21.8) is a serine proteinase that is activated from its precursor, prokallikrein, by the proteolytic action of activated Hageman factor (Webster, 1968).

The first recognized physiological function of kallikrein was the liberation of the nonapeptide bradykinin from high-molecular-weight plasma kininogen (Webster & Pierce, 1963). Later it was implicated in fibrinolysis (Laake & Venneröd, 1974; Mandle & Kaplan, 1977), and in the surface-mediated intrinsic pathway of coagulation (Wuepper, 1973; Saito *et al.*, 1974; Weiss *et al.*, 1974; Colman *et al.*, 1975; Griffin & Cochrane, 1976), in which kallikrein completes a positive-feedback loop by activating more Hageman factor. More recently the activation of prorenin by the intrinsic Hageman factor–prokallikrein pathway has been suggested (Yokasawa *et al.*, 1979; Sealey *et al.*, 1979; Derkx *et al.*, 1979).

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with the IUPAC–IUB Recommendations (1972). Additional abbreviations used are: -NMec, 4-methyl-7-coumarylamide; -NPhNO₂, *p*-nitroanilide; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; Dip-F, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate; Glt, glutaryl; Suc, succinyl.

Many methods have been described for the purification of human plasma kallikrein and prokallikrein (see the Discussion section for references). Generally, these start with a Cohn fraction of plasma and give a low yield of the enzyme. In the present paper we describe a simple method for purification of kallikrein that gives better results and is well suited to use with small or large quantities of plasma.

Materials and methods

Materials

Fresh human blood containing acid/citrate/dextrose anticoagulant, but unsuitable for transfusion, was provided by a local blood-transfusion service [see Barrett *et al.* (1979) for further details]. The plasma was separated by centrifugation at 10 000 *g* for 20 min in plastic bottles.

Brij 35 and methylammonium chloride were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Albumin (bovine serum, crystallized), carbonic anhydrase (bovine erythrocyte), phosphorylase *a* (rabbit muscle, twice-crystallized), trypsin inhibitor (soya bean) type I-S, trypsin inhibitor (lima bean) type II-L, transferrin (human), Tos-Phe-CH₂Cl and Tos-Arg-OMe were from Sigma (London) Chemical

Co., Poole, Dorset BN17 17NH, U.K. Tos-Lys-CH₂Cl was from Bachem, CH-4416 Bubendorf, Switzerland. Pro-Phe-Arg-CH₂Cl was generously given by Dr. C. Kettner, Department of Biology, Brookhaven National Laboratory, Upton, NY, U.S.A. Turkey ovomucoid and chicken ovomucoid were prepared as described by Lineweaver & Murray (1947) and Barrett (1974) respectively. Aprotin was from Bayer A. G., Wuppertal-Elbfeld, Germany. Pro-Phe-Arg-NMec, Z-Phe-Arg-NMec, Boc-Val-Pro-Arg-NMec, Boc-Ile-Glu-Gly-Arg-NMec, Glt-Gly-Arg-NMec and leupeptin were from Protein Research Foundation, 476 Ina, Minoh, Osaka 562, Japan. D-Val-Leu-Lys-NPhNO₂ was from Kabi Diagnostica, S-11287 Stockholm, Sweden. Suc-Ala-Ala-Pro-Val-NMec was the gift of Dr. Trevor Payne, Wander Ltd., Berne, Switzerland. Monospecific antisera against human plasma kallikrein were generously given by Dr. J. V. Pierce, National Heart and Lung Institute, Bethesda, MD, U.S.A. (sheep), and Dr. N. Heimburger, Behringwerke A. G., 3550 Marburg/Lahn, Germany (rabbit). Immunoglobulin preparations (rabbit) against human immunoglobulins IgA, IgG and IgM were from Mercia Brocades, Weybridge, Surrey KT14 6RA, U.K. Horse anti-(human serum) serum was from Wellcome Reagents, Beckenham, Kent BR3 3BS, U.K. Sepharose 4B was from Pharmacia (G.B.), Middx. TW3 1NE, U.K. Ultrogel AcA 44 was from LKB Instruments, South Croydon, Surrey CR2 8YD, U.K. [³H]Dip-F (sp. radioactivity 3 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.

Methods

Enzyme assays. Activity against peptidyl-4-methyl-7-coumarylamides was measured by a micro-modification of the method of Morita *et al.* (1977). Stock solutions of the substrates (10 mM in dimethyl sulphoxide, stored at 4°C) were diluted 20-fold with 20 mM-Tris/HCl buffer, pH 7.8, containing 0.2 M-NaCl and 0.02% NaN₃ before use. For Z-Phe-Arg-NMec, 0.1% Brij 35 was added to the buffer.

The dilute substrate solutions were stable for at least 3 weeks at 4°C. A 10 µl portion of enzyme solution in 20 mM-Tris/HCl, pH 7.8, was placed in a 1.5 ml conical plastic Microfuge (Alpha Laboratories, Greenford, Middx. UB6 8PW, U.K.) and mixed with 10 µl of 0.5 mM-substrate solution. The mixture was incubated for 3 min at 37°C. The reaction was stopped by adding 1.5 ml of 5% acetic acid, and the fluorescence read with a Locarte fluorimeter (Locarte Co., London S.W.7, U.K.) by using excitation filter LF/2 (340–380 nm) with emission wavelength at 460 nm. The fluorimeter was adjusted to read 500 arbitrary units with 1 µM-7-amino-4-methylcoumarin in the same solvent. The

assay gave a linear response with 5–50 ng of kallikrein/tube.

Routine assays during purification of kallikrein were made with Pro-Phe-Arg-NMec as substrate; 1 unit of activity hydrolysed 1 µmol of substrate/min under the conditions described. Activity against Tos-Arg-OME at 25°C, pH 7.8, was measured by reaction of the liberated methanol with chromotropic acid (Siegelman *et al.*, 1962). Assays with D-Val-Leu-Lys-NPhNO₂ were made as recommended by the manufacturers (Kabi Diagnostica).

Coupling of soya-bean trypsin inhibitor and antibodies to Sepharose. Sepharose 4B was activated with CNBr as described by Cuatrecasas & Anfinsen (1971). Activated Sepharose 4B (50 ml) was made to react with 100 mg of soya-bean trypsin inhibitor in 0.1 M-NaHCO₃, pH 9, overnight at 20°C. The gel was then treated with 50 mM-glycine in the same buffer for 2 h, washed thoroughly with 0.1 M-sodium formate buffer, pH 3.0, and then 5 mM-NaOH. Soya-bean trypsin inhibitor-Sepharose was stored in 10 mM-Tris/HCl, pH 7.8, containing 0.02% NaN₃.

The immunoabsorbent for human immunoglobulins was prepared by coupling rabbit anti-(human immunoglobulins) immunoglobulin to the activated Sepharose 4B as described by Barrett *et al.* (1979).

SDS/polyacrylamide-gel electrophoresis. This was done as described by Barrett *et al.* (1979) with gels of 7% total acrylamide concentration.

Fluorography. Purified kallikrein was reacted with 0.1 mM-[³H]Dip-F in 10 mM-Tris/HCl, pH 7.8, for 15 min at 20°C and subjected to SDS/polyacrylamide gel electrophoresis. The gel was stained, photographed, impregnated with 2,5-diphenyloxazole and dried according to the method of Laskey & Mills (1975) before exposure to Kodak X-OMAT H film for 48 h. The film was developed with a Kodak RPX-OMAT Processor.

Protein determination. Protein concentrations were determined either by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard, or by the measurement of $A_{280} \cdot A_{280,1\text{cm}}^{-1\%}$ for purified plasma kallikrein was found to be 10.6 (on the basis of the Lowry assay).

Protein concentration. The protein solutions were concentrated in an Amicon ultrafiltration cell with Diaflo PM-10 membrane (Amicon Corp., Lexington, MA, U.S.A.) or by dialysis against Carbowax 20m [poly(ethylene glycol)] (Raymond A. Lamb, North Acton, London NW10 6JL, U.K.).

Results

Purification of plasma kallikrein

All purification procedures were carried out at 4°C unless otherwise mentioned. Plastic containers, tubes and columns were used throughout the

Table 1. *Purification of human plasma kallikrein*
See text for experimental details.

Step	Volume (ml)	Protein (g)	Activity (units)	Specific activity (unit/mg)	Purification factor	Yield (%)
Plasma	400	20.8	—	—	—	—
Acetone activation and pH 5.8 treatment	650	14.0	76.1	0.0054	1	100
Soya-bean trypsin inhibitor–Sephacrose	68	0.029	71.4	2.5	462	94
Immunoadsorbent	22	0.0077	43.6	5.6	1037	57
Ultrogel AcA 44	21	0.0028	26.8	9.73	1800	35

purification procedure, and all buffers for column chromatography contained 0.1% Brij 35. The results are summarized in Table 1.

Methylamine treatment of plasma and subsequent acetone activation of prokallikrein. α_2 -Macroglobulin was inactivated with methylamine (Barrett *et al.*, 1979), and prokallikrein was then converted into kallikrein by use of 20% (v/v) acetone.

Human plasma (400 ml) was adjusted to pH 8.2 with 2 M-Tris/HCl, pH 8.6, was made 0.2 M with respect to methylamine by the addition of solid methylamine hydrochloride, and was stirred gently for 1 h at 20°C. At this stage, plasma contained little or no activity against Pro-Phe-Arg-NMec. Prokallikrein was activated by adding acetone to 20% (v/v) to the plasma (Webster, 1968); it was left for 3 h at 20°C. (A preliminary experiment showed that maximal activity was obtained after 2.5–5 h.) The preparation was then dialysed against 20 vol. of water at 4°C for 16 h, and adjusted to pH 5.8 with 1 M-HCl. After 30 min, the precipitate was removed by centrifugation, the supernatant was adjusted to pH 7.5–7.8 by adding 1 M-Tris/HCl buffer, pH 7.8, and made 1 M-NaCl and 0.1% Brij 35.

Affinity chromatography on soya-bean trypsin inhibitor–Sephacrose. The plasma activated by the above procedure was passed through a soya-bean trypsin inhibitor–Sephacrose column (2.5 cm × 10 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.8, containing 1 M-NaCl, at a flow rate of 180–200 ml/h. More than 99% of the kallikrein was adsorbed. The column was washed with at least 4 bed vol. of the same buffer, and kallikrein was then eluted with 5 mM-NaOH (pH 11.2–11.4) at a flow rate of 60 ml/h, and 5 ml fractions were collected in tubes containing 0.5 ml of 1 M-Tris/HCl buffer, pH 7.8, for the immediate neutralization of the alkaline solution.

The yield from this step (in the combined active fractions) was about 90%, with 400–500-fold purification. Kallikrein was by far the major protein present (Fig. 1b).

Immunoadsorption of immunoglobulins. The major contaminants after soya-bean trypsin inhibitor–Sephacrose affinity chromatography were

found to be immunoglobulins. These were removed by immunoadsorption.

The crude kallikrein solution from step 2 (about 70 ml) was concentrated by ultrafiltration to about 10 ml and passed through a column (20 ml) of Sepharose 4B coupled with IgG (rabbit) against human immunoglobulins, in 10 mM-Tris/HCl buffer, pH 7.8, containing 0.4 M-NaCl. Almost all contaminants were removed by this step (Fig. 1c). The recovery was about 70%, with about 2-fold increase in specific activity (Table 1).

Further purification by gel chromatography. The preparation from step 3 (about 20 ml) was concentrated to 1.5 ml by dialysing against Carbowax, and applied to a column (1.5 cm × 95 cm) of Ultrogel AcA 44 equilibrated with 10 mM-Tris/HCl buffer, pH 7.8, containing 0.2 M-NaCl. This step removed minor contaminants of lower molecular weight.

About 3 mg of kallikrein was obtained from 400 ml of human plasma. The overall yield of enzymic activity was 35% (Table 1).

Purity and molecular weight. Samples taken during the purification procedure, and the final product, were subjected to SDS/polyacrylamide-gel electrophoresis without reduction (Figs. 1b–1d). The final product showed two bands of 88 000 and 86 000 apparent mol.wt. These were taken to represent kallikreins I and II described by Mandle & Kaplan (1977). Run in SDS/polyacrylamide gels with reduction, the purified kallikrein showed bands corresponding to mol.wts. of 36 000, 34 000, 28 000 and 22 000 (Fig. 1e).

In order to locate the active-site serine residues, we treated the kallikrein preparation with [³H]Dip-F before subjecting it to SDS/polyacrylamide-gel electrophoresis with reduction. The fluorograph (Fig. 1f) of the gel showed that only the two chains, of mol.wt. 36 000 and 34 000, incorporated [³H]Dip. The difference in molecular weight between these chains agreed with that between kallikreins I and II. It is likely that each form of kallikrein contain both of the chains not reactive with Dip-F (22 000 and 28 000 mol.wt.) together with one of the reactive chains (34 000 or 36 000 mol.wt.).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of human plasma kallikrein

Samples containing 8–10 μ g of protein after (b) soya-bean trypsin inhibitor–Sephadex 4B affinity chromatography, (c) immunoadsorption of contaminants, and (d) gel chromatography on Ultrogel AcA 44, were run without reduction. (e) Purified kallikrein (16 μ g) treated with [3 H]Dip-F was run with reduction and stained for protein, and (f) the fluorograph of [3 H]Dip-kallikrein was prepared as described under 'Methods'. Protein standards [immunoglobulin G (150 000), phosphorylase *a* (94 000), transferrin (77 000), bovine serum albumin (68 000), carbonic anhydrase (29 000) and soya-bean trypsin inhibitor (21 000)] were run (a) without reduction and (g) with reduction.

The purity of the preparations was also examined by double immunodiffusion. Precipitin lines formed against two antisera to human plasma kallikrein (sheep and rabbit) fused in a reaction of complete identity (Fig. 2). There was no reaction with anti-(whole human serum) serum, or immunoglobulin preparations against human immunoglobulins (IgA, IgG and IgM). The lack of reaction with commercial anti-(whole human serum) serum implied that the antiserum contained little or no antibody against human plasma kallikrein, but also indicated that the kallikrein preparation was free from other plasma proteins (Fig. 2).

Substrate specificity and inhibition characteristics. Morita *et al.* (1977) described peptidyl-NMec substrates specific for some plasma proteinases. These were tested with the purified human plasma kallikrein (Table 2). Plasma kallikrein readily hydrolysed Z-Phe-Arg-NMec and Pro-Phe-Arg-NMec, but the other methylcoumarylamides were relatively resistant. The specific activity of our preparation with Z-Phe-Arg-NMec (11.3 μ mol/min per mg) may be compared with the $V_{\max}$ value of 8.0 reported by Iwanaga *et al.* (1979) for their preparation of bovine plasma kallikrein.

The specific activity against Tos-Arg-OMe of 74 μ mol/min per mg may be compared with the

Fig. 2. Double immunodiffusion

The plates contained: K, 20 μ g of the purified kallikrein; a, sheep anti-(human plasma kallikrein) serum; b, anti-(human IgM) immunoglobulin; c, anti-(human IgA) immunoglobulin; d, anti-(human IgG) immunoglobulin; e, anti-(human immunoglobulins) immunoglobulin; f, anti-(whole human serum) serum; and g, rabbit anti-(human plasma kallikrein) serum. The diffusion was for 42 h at 20°C, and the plate was washed, dried and stained for protein as described previously (Barrett, 1974).

highest value previously reported of 45.1 μ mol/min per mg (Sampaio *et al.*, 1974). The other substrates tested have been used for urokinase, thrombin, factor Xa, elastase and plasmin respectively, and the low activity against each of them precludes major contamination by any of these enzymes.

Inhibition studies with the purified enzyme showed that the enzyme was inhibited by soya-bean trypsin inhibitor, aprotinin, leupeptin and the specific inhibitor, Pro-Phe-Arg-CH₂Cl, but not by lima-bean trypsin inhibitor, ovomucoid, Tos-Phe-CH₂Cl or Tos-Lys-CH₂Cl. These results indicate that our preparation has the same inhibition characteristics as those previously reported for plasma kallikrein (reviewed by Barrett & McDonald, 1980). The second-order rate constant for the inactivation of our plasma kallikrein preparation by Pro-Phe-Arg-CH₂Cl calculated according to the equation described by Vincent & Lazdunski

Table 2. *Specific activity of human plasma kallikrein with synthetic substrates*

The assays were carried out as described under 'Methods' with the methylcoumarylamides or nitro-anilide at a final concentration of 0.25 mM, and Tos-Arg-OMe at 20 mM.

Substrate	Specific activity ($\mu\text{mol}/\text{min}$ per mg)
Pro-Phe-Arg-NMec	9.73
Z-Phe-Arg-NMec	11.3
Glt-Gly-Arg-NMec	0.09
Boc-Val-Pro-Arg-NMec	0.46
Boc-Ile-Glu-Gly-Arg-NMec	0.46
Suc-Ala-Ala-Pro-Val-NMec	<0.01
D-Val-Leu-Lys-NPhNO ₂	0.02
Tos-Arg-OMe	74.2

(1972) was $1520 \pm 55 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$ ($n=3$); this was in good agreement with the value of $1500 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$ obtained by Kettner & Shaw (1978).

Discussion

Kallikrein and prokallikrein have previously been purified from plasma of several species: man (Colman *et al.*, 1969; Fritz *et al.*, 1972; Bagdasarian *et al.*, 1974; Sampaio *et al.*, 1974; Tamura & Fujii, 1976; Mandle & Kaplan, 1977; Heber *et al.*, 1978; Gallimore *et al.*, 1978; Scott *et al.*, 1979; Yarovaya *et al.*, 1979), cattle (Takahashi *et al.*, 1972; Heimark & Davie, 1979), pig (Habermann & Klett, 1966; Fritz *et al.*, 1972) and rabbit (Wuepper & Cochrane, 1972).

Prokallikrein has proved difficult to purify; its pI and molecular weight are inconveniently close to those of other proteins, including IgG. Purification as the active enzyme has the major advantage that affinity chromatography can be employed, but kallikrein is rapidly complexed by α_2 -macroglobulin in plasma. This has led to poor yields in our early experiments, and in the hands of others. We have found (results not shown) that even activation by shaking of plasma with chloroform, or treatment with 20% acetone, does not inactivate the α_2 -macroglobulin, and acid treatment tends to inactivate the prokallikrein. The inactivation of the inhibitor with methylamine (Barrett *et al.*, 1979) was highly effective, however, and allowed the purification of the enzyme on soya-bean trypsin inhibitor-Sepharose in good yield.

Immobilized soya-bean trypsin inhibitor has been used previously in the purification of kallikrein, but with a high concentration of benzamidine to elute the enzyme efficiently (Fritz *et al.*, 1972; Sampaio *et al.*, 1974). We found that the elution of kallikrein from soya-bean trypsin inhibitor-Sepharose could be easily done with 5 mM-NaOH, pH 11.3, with a

recovery of more than 90% of the activity. This allowed the direct monitoring of A_{280} and the enzymic activity in the eluate, which is not possible with benzamidine. The problem of non-specific binding of immunoglobulins, particularly IgG, to soya-bean trypsin inhibitor-Sepharose has previously been recognized by Heber *et al.* (1978). By adding 1 M-NaCl and 0.1% Brij 35 to the sample, we could minimize, but not eliminate, this non-specific interaction.

Although Scott *et al.* (1979) have reported only one prokallikrein, with mol.wt. 88 000, Mandle & Kaplan (1977), and, more recently, Y. Hojima (personal communication) have described two forms of prokallikrein with mol.wts. of 88 000 and 85 000. A doublet of bovine prokallikrein with mol.wt. of 89 000 and 86 000 was also reported by Heimark & Davie (1979). Our SDS/polyacrylamide-gel electrophoresis of non-reduced samples gave results in agreement with the latter authors. Heber *et al.* (1978) obtained the slightly higher mol.wt. of $98 000 \pm 5000$ by SDS/polyacrylamide-gel electrophoresis.

Reduction of kallikrein showed four protein bands on SDS/polyacrylamide-gel electrophoresis. Two bands labelled with [³H]Dip-F corresponded to light chains I and II from prokallikrein I and II respectively (Mandle & Kaplan, 1977). Two other unlabelled bands, corresponding to mol.wts. 22 000 and 28 000, were seen in place of the expected 52 000-mol.wt. heavy chains (Mandle & Kaplan, 1977). We suggest that these two chains arise by cleavage of the heavy chain. Y. Hojima (personal communication) has obtained evidence that the heavy chain is cleaved by the autolytic action of plasma kallikrein itself. Probably this further step of limited proteolysis is unavoidable in kallikrein purified as the active enzyme, whereas the enzyme can be obtained with the heavy chain intact by controlled activation of purified prokallikrein (Mandle & Kaplan, 1977; Scott *et al.*, 1979). It remains to be established whether or not the two-chain and three-chain forms of the kallikreins have minor differences in catalytic activity, but there are no indications of major differences.

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