Journal of Chromatography, 376 (1986) 385—393
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2960

AFFINITY PURIFICATION OF KALLIKREIN AND ELASTASE FROM HOG PANCREAS POWDER

TOSHIYUKI HONDA, AKIRA FUJITA, YASUO TSUBAKIHARA and KAZUYUKI MORIHARA*

Toho Pharmaceutical Ind. Co., Ltd., Kyoto Research Laboratories, Chikatake 7-4, Shohryuji, Nagaokakyo-shi, Kyoto 617 (Japan)

SUMMARY

The present report describes a method that is efficient for simultaneous isolation of kallikrein and elastase from hog pancreas powder. Both enzymes were separated by successive column chromatography on CM-cellulofine and *p*-aminobenzamidine-Sepharose 4B. Kallikrein was further purified by column chromatography on DEAE-Sephadex and elastase was purified by repeated gel chromatography on Sephadex G-75. The kallikrein obtained was composed of two components, which were separable by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the elastase had one component. The activity yields of kallikrein and elastase were 49 and 38%, respectively.

INTRODUCTION

Many reports have appeared concerning the purification of kallikrein [1—4] or elastase [5] using hog pancreas powder as the starting material. These reports are mainly concerned with the purification of one of them and not the simultaneous purification of both, indicating that the conditions for purification of one enzyme are not always suitable for purification of the other from the same starting material. The present study was thus undertaken to try to prepare kallikrein and elastase simultaneously for industrial production from the same starting material, hog pancreas powder, by adapting affinity chromatography using *p*-aminobenzamidine-Sepharose (PABA-Sepharose). The application of PABA-Sepharose had previously been used [6] for purification of rat stomach kallikrein but not for purification of hog pancreas kallikrein.

EXPERIMENTAL

Materials

Hog pancreas powder (lot 225-00837) was obtained from V.G.F. (New

0378-4347/86/$03.50

York, NY, U.S.A.). Benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), L-prolyl-L-phenylalanyl-L-arginine-4-methylcoumarine amide (Pro-Phe-Arg-MCA), succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (Suc-Ala-Ala-Ala-PNA), elastinal and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (water-soluble carbodiimide) were obtained from Protein Research Foundation (Minoh, Osaka, Japan). Soy bean trypsin inhibitor (SBTI) and beef pancreatic trypsin inhibitor (BPTI) were obtained from Sigma (St. Louis, MO, U.S.A.). Sephadex G-75 and CM-cellulofine were obtained from Seikagaku Kogyo (Tokyo, Japan) and DEAE-Sepharose and Sepharose 4B were from Pharmacia (Uppsala, Sweden). *p*-Aminobenzamidine hydrochloride was obtained from Aldrich (Milwaukee, MI, U.S.A.) and elastin (50–100 mesh) was from ICN NBC Labs. (Cleveland, OH, U.S.A.). ϵ-Aminocaproic acid, tosyl-L-phenylalanine chloromethyl ketone (TPCK), tosyl-L-lysine chloromethyl ketone (TLCK), *p*-chloromercuric benzoic acid (PCMB) and ethylenediaminetetraacetic acid (EDTA) were obtained from Nakarai (Kyoto, Japan). Cyanogen bromide and diisopropylphosphofluoridate (DFP) were supplied by Kishida (Osaka, Japan).

PABA-Sepharose 4B was prepared by first coupling ϵ-aminocaproic acid to Sepharose 4B by the cyanogen bromide method [7], then attaching *p*-aminobenzamidine to the Sepharose 4B using water-soluble carbodiimide according to the method of Holleman et al. [8].

Enzyme assay of kallikrein

Amidase activity was determined using Pro-Phe-Arg-MCA as a substrate according to the method of Morita et al. [9]. Before the reaction, the enzyme solution (0.1 ml) was pretreated with 0.1 ml of SBTI solution (4 mg/ml in water) for 5 min at 37°C. The pretreated enzyme solution was suitably diluted with 50 m*M* phosphate buffer (pH 7.4). Reaction was started by adding 5 μl of 18 m*M* substrate (in dimethylformamide) to 1 ml of the enzyme solution. After 15 min at 37°C, 1 ml of 20% acetic acid was added to the reaction mixture to stop further reaction. The amounts of 7-amino-4-methylcoumarin liberated were measured using a Hitachi fluorescence spectrophotometer (Model 650-10 S) with excitation at 380 nm and emission at 460 nm, and the μmol/min values of hydrolysed substrate were calculated.

Esterase activity was measured using Bz-Arg-OEt as a substrate by the formation of hydroxamic acid–ferric complex from the residual substrate [10]. The enzyme solution pretreated with SBTI as mentioned above was used. Next, 0.5 ml of 10 m*M* substrate in 50 m*M* phosphate buffer (pH 7.4) and 0.5 ml of enzyme solution suitably diluted with 50 m*M* phosphate buffer (pH 7.4) were mixed and incubated at 37°C. After 30 min of reaction, 1.5 ml of a mixture of equal volumes of 3.5 *M* sodium hydroxide and 2 *M* $NH_2OH \cdot HCl$ were added to the reaction mixture to stop further hydrolysis, and the reaction mixture was kept at 25°C for 20 min. Finally, 1 ml of 4 *M* hydrochloric acid, 1 ml of 18% trichloroacetic acid and 1 ml of 10% ferric chloride (in 0.1 *M* hydrochloric acid) were added to the reaction mixture, which was kept at 25°C for 20 min. After filtration, the ferric complex formed was determined colorimetrically at 530 nm. Esterolytic activities were expressed in μmol/min of hydrolysed substrate.

Enzyme assay of elastase

Amidase activity was determined using Suc-Ala-Ala-Ala-PNA as a substrate according to the method of Bieth et al. [11]. The reaction mixture containing 0.1 ml of enzyme solution, 0.4 ml of 20 m*M* Tris—HCl buffer (pH 8.0) and 0.5 ml of 2 m*M* substrate was kept at 37° C. After 15 min, the reaction was terminated by adding 1 ml of 0.15 *M* acetic acid to the reaction mixture. The released *p*-nitroaniline was determined at 410 nm using a Hitachi spectrophotometer (Model 200-20). The enzyme activity was expressed in μmol/min of hydrolysed substrate.

Elastin digestion was determined as follows. Elastin (12 mg) was suspended in 2 ml of 50 m*M* borate buffer (pH 8.8), which was stirred for 30 min at 37° C. Enzyme solution (0.5 ml) was added to the elastin suspension and stirred at this temperature. After 30 min of reaction, 2.5 ml of 0.6 *M* acetic acid were added to the reaction mixture to stop further reaction. After filtration, the absorbance was read at 275 nm. A control experiment without enzyme was also carried out. The activity was expressed in μmol/min of tyrosine released.

Amino acid analysis

The determination was carried out with an amino acid analyser, Hitachi Model 835-50. The protein samples had previously been hydrolysed with 6 *M* hydrochloric acid at 110° C for 24, 48 and 72 h, respectively. Data were obtained by the average of three analyses. Data for threonine and serine were extrapolated to zero time by the method of least squares.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The gel electrophoresis was performed in the presence of SDS and mercaptoethanol according to the method of Laemmli [12].

RESULTS

Isolation of kallikrein and elastase

All purification procedures were at 4° C except when otherwise specified.

Step 1: extraction. Hog pancreas powder (250 g) was suspended in 5 l of distilled water and the pH of the suspension was adjusted to 4.5. The suspension was stirred for 2 h at room temperature. After centrifugation (7500 *g* for 30 min), the supernatant was concentrated to 1.5 l using ultrafiltration module SEP 1013 (MW 3000).

Step 2: column chromatography on CM-cellulofine. The concentrate mentioned above was applied to a column of CM-cellulofine (8 × 5 cm, I.D.), which had previously been equilibrated with 0.1 *M* acetate buffer (pH 4.5). The flow-rate was 900 ml/h. Kallikrein passed through the column, which was washed with 2000 ml of 20 m*M* phosphate buffer (pH 8.0). Elastase was retained and eluted with alkaline buffer solution (0.1 *M* sodium bicarbonate, the pH adjusted to 9.0 with concentrated sodium hydroxide) containing 1 *M* sodium chloride.

Step 3: affinity chromatography on PABA-Sepharose 4B. The pH and ionic strength of the kallikrein fraction that had passed through the column of CM-cellulofine were adjusted to pH 8.0 and ionic strength (*I*) of 11 mS for affinity

chromatography. The kallikrein solution obtained (≈ 2000 ml) was applied to a column of PABA-Sepharose 4B (7 × 3 cm I.D.), which had previously been equilibrated with 10 m*M* phosphate buffer (pH 8.0) and 0.1 *M* sodium chloride. The flow-rate was 480 ml/h. The kallikrein adsorbed on the column was eluted with alkaline solution (0.1 *M* sodium bicarbonate) containing 1 *M* sodium chloride, after the column had been washed with the equilibrated buffer solution. The kallikrein fraction was concentrated by ultrafiltration using Toyo ultrafilter UK-10.

The elastase fraction eluted from the column of CM-cellulofine was applied to a column of PABA-Sepharose 4B (7 × 3 cm I.D.), which had previously been equilibrated with alkaline solution (pH 9.0, 0.1 *M* sodium bicarbonate and concentrated sodium hydroxide) containing 1 *M* sodium chloride. The elastase fraction passed through the column, but trypsin, which had contaminated the fraction, was retained. The elastase fraction obtained was concentrated by ultrafiltration using Toyo ultrafilter UK-10.

Step 4A: column chromatography on DEAE-Sephadex. The kallikrein fraction was applied to a column of DEAE-Sephadex (15 × 8 cm I.D.), which had previously been equilibrated with 0.3 *M* ammonium acetate buffer (pH 6.0). Elution was carried out in a linear gradient system with 5 l of 0.3—0.7 *M* ammonium acetate buffer (pH 6.0), as shown in Fig. 1. The main part was collected and dialysed against distilled water, which was then concentrated using Toyo ultrafilter UK-10.

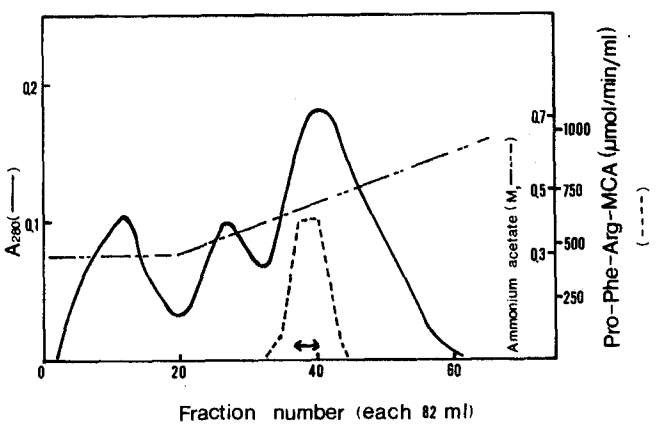

Fig. 1. DEAE-Sephadex A-50 chromatography of hog pancreas kallikrein. The kallikrein fraction (500 ml) from step 3 was applied to the column (15 × 8 cm I.D.), which had been equilibrated with 0.3 *M* ammonium acetate buffer (pH 6.0). Elution was carried out in a linear-gradient system with 5 l of 0.3—0.7 *M* ammonium acetate buffer (pH 6.0).

Step 4B: gel chromatography on Sephadex G-75. The elastase fraction concentrated to 1/100 (step 3) was applied to a column of Sephadex G-75 (90 × 3 cm I.D.), which had previously been equilibrated with 50 m*M* phosphate buffer (pH 7.4) containing 0.15 *M* sodium chloride (Fig. 2A). Gel chromatography was repeated with part a of Fig. 2A, as shown in Fig. 2B. Part b of Fig. 2A and part c of Fig. 2B were combined and then concentrated by ultrafiltration.

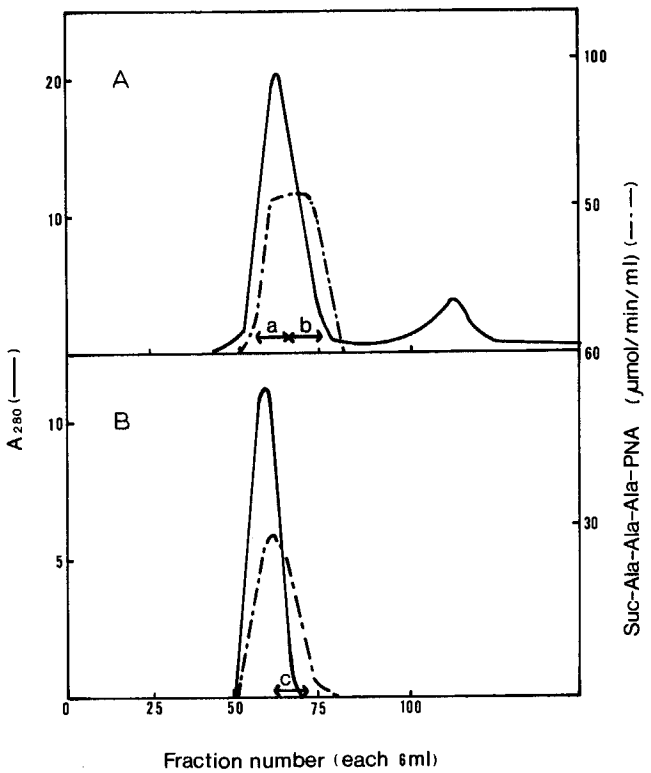

Fig. 2. Gel chromatography of hog pancreas elastase. The elastase fraction (1500 ml) from step 3 was concentrated to 15 ml by ultrafiltration, which was then applied to a column (90 × 3 cm I.D.) of Sephadex G-75 that had been equilibrated with 50 m*M* phosphate buffer (pH 7.4) containing 0.15 *M* potassium chloride. By first flow (A), fraction b was obtained and fraction a was rechromatographed in a similar way as mentioned above. Fraction c was obtained by the second flow (B).

Table I summarizes the purification procedures for kallikrein and elastase from hog pancreas powder. From 250 g of the starting material, the activity yields of kallikrein and elastase were 49 and 38%, respectively.

Properties of purified kallikrein and elastase

Kallikrein. PAGE was carried out in the presence of SDS, as shown in Fig. 3. This indicates that the purified kallikrein in step 4A is mainly composed of two components, while that in step 3 is composed of several. It has been shown [4] that hog pancreas kallikrein contains at least two components, which are separable by SDS-PAGE, the molecular masses being 25 000 and 30 300, respectively. The present result with purified kallikrein agrees well with the previous observation.

Table II gives the results of amino acid analysis of purified kallikrein together with the comparative literature data. Good agreement was found between both sets of data. The effects of various inhibitors on the activity of purified kallikrein were determined (Table III), indicating that beef pancreas trypsin inhibitor and DFP inhibit kallikrein activity, but the other

TABLE I

SUMMARY OF SIMULTANEOUS PURIFICATION OF KALLIKREIN AND ELASTASE

Step	Treatment	Volume (ml)	Protein content ($A_{280\ nm}$/ml)	Kallikrein			Elastase		
				Specific activity (μmol/min/$A_{280\ nm}$)	Total activity (μmol/min)	Yield (%)	Specific activity (μmol/min/$A_{280\ nm}$)	Total activity (μmol/min)	Yield (%)
1	Extracted from 250 g of hog pancreas powder at pH 4.5	1500	198.1	2.1	$624 \cdot 10^3$	100	0.029	8870	100
2	CM-cellulofine equilibrated with 0.1 *M* acetate buffer (pH 4.5) (8 × 5 cm I.D.)								
	Passed through	2000	34.3	8.76	$601 \cdot 10^3$	96			
	Washed (20 m*M* PO_4, pH 8.0)								
	Eluted with 0.1 *M* $NaHCO_3$ (pH 9.0), 1 *M* NaCl	1250	3.4				1.30	5526	62
3	PABA-Sepharose 4B equilibrated with 10 m*M* PO_4 (pH 8.0), 0.1 *M* NaCl (7 × 3 cm I.D.)								
	Passed through	1500	1.96				1.82	5349	60
	Eluted with 0.1 *M* $NaHCO_3$, 1 *M* NaCl	500	0.89	1278	$568 \cdot 10^3$	91			
4A	DEAE-Sephadex equilibrated with 0.3 *M* ammonium acetate (pH 6.0) (Fig. 1)								
	Passed through								
	Linear gradient, 0.3—0.7 *M* ammonium acetate (pH 6.0)	246	0.186	6058	$278 \cdot 10^3$	49			
4B	Sephadex G-75 equilibrated with 50 m*M* PO_4 (pH 7.4), 0.15 *M* KCl (Fig. 2)								
	First filtration	54	10.6				4.19	2407	27
	Second filtration	60	4.06				4.00	962	11

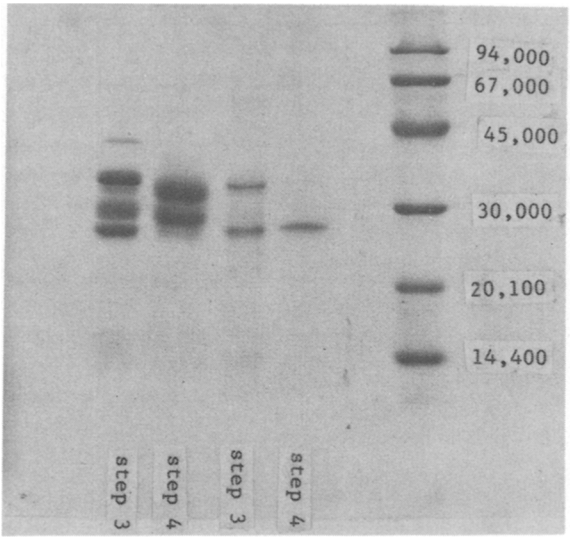

Fig. 3. SDS-PAGE of kallikreins and elastases in steps 3 and 4. Markers of various molecular weights are shown for reference.

TABLE II

AMINO ACID COMPOSITION OF HOG PANCREAS KALLIKREIN AND ELASTASE

The results were obtained from 24-, 48- and 72-h hydrolysis at 110° C; values are expressed in residues per molecule. Values of threonine and serine were obtained by extrapolation to zero time of hydrolysis.

Amino acid	Kallikrein		Elastase	
	This study	Ref. 13	This study	Ref. 5
Asp	28	27	25	24
Thr	14	14	20	19
Ser	17	14	25	22
Glu	24	23	20	19
Pro	17	16	7	7
Gly	25	22	27	25
Ala	14	13	18	17
Cys	4	5	3	4
Val	11	10	25	27
Met	4	4	2	2
Ile	10	12	9	10
Leu	18	19	18	18
Tyr	6	7	10	11
Phe	9	10	3	3
Lys	12	10	5	3
His	10	8	7	6
Arg	3	3	11	12
Molecular mass	25 200	25 200	25 900	25 900

TABLE III

EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITIES OF HOG PANCREAS KALLIKREIN AND ELASTASE

Each enzyme solution (0.14 mg/ml) containing 50 m*M* Tris–HCl buffer (pH 7.5), 0.1 *M* potassium chloride and various inhibitors (concentrations shown in the table) were kept at 37°C. After 10 or 60 min, the remaining activities of each enzyme were determined.

Inhibitor	Final concentration	Time of incubation (min)	Remaining activity (%)	
			Kallikrein	Elastase
SBTI	0.075%	10	99	102
BPTI	0.01%	10	0.02	100
Elastinal	2.5 m*M*	60	–	2
DFP	2.5 m*M*	60	31	5
TPCK	5.0 m*M*	60	98	99
TLCK	5.0 m*M*	60	101	99
EDTA	5.0 m*M*	60	99	100

inhibitors do not. These results coincide with those of previous studies [1–4]. The activity of purified kallikrein against Bz-Arg-OEt was found to be 982 μmol/min/$A_{280\ nm}$.

Elastase. The homogeneity of elastases purified in steps 3 and 4 was determined by SDS-PAGE, as shown in Fig. 3. This indicates that purified elastase of step 4 is almost of homogeneous nature, the molecular mass being 25 900. Table II lists the amino acid analysis results of the elastase, which were compared with those of a previous study. Good agreement was found. The effects of various inhibitors were also measured, as seen in Table III. Elastinal, which has been shown [14] to be a potent inhibitor of pancrease elastase, inhibits elastase activity, as well as DFP. These physicochemical and enzymatic properties of purified elastase agree with those described in the references [5, 14].

ACKNOWLEDGEMENT

The authors are grateful to Professor S. Fujii of Otsuka Pharmaceutical Co. for his advice and constant encouragement throughout this work.

REFERENCES

1 F. Fiedler, Methods Enzymol., 45 (1976) 289.
2 C. Kutzbach and G. Schmidt-Kastner, Hoppe-Seyler's Z. Physiol. Chem., 353 (1972) 1099.
3 M. Zuber and E. Sache, Biochemistry, 13 (1974) 3098.
4 H. Kira, S. Hiraku and H. Terashima, Adv. Exp. Med. Biol., 120A (1979) 273.
5 D.M. Shotton, Methods Enzymol., 19 (1970) 113.
6 K. Uchida, A. Yokoshima, M. Niinobe, H. Kato and S. Fujii, Adv. Exp. Med. Biol., 120A (1979) 291.
7 P. Cuatrecassas, J. Biol. Chem., 245 (1970) 3059.
8 W.H. Holleman, W.W. Andres and L.T. Weiss, Thromb. Res., 7 (1975) 683.

9 T. Morita, H. Kato, S. Iwanaga, K. Takada, T. Kimura and S. Sakakibara, J. Biochem. (Tokyo), 82 (1977) 1495.
10 P.S. Roberts, J. Biol. Chem., 232 (1958) 285.
11 J. Bieth, B. Spiess and C.G. Wermuth, Biochem. Med., 11 (1974) 350.
12 U.K. Laemmli, Nature, 227 (1970) 680.
13 F. Fiedler, C. Hirschaner and F. Werle, Hoppe-Seyler's Z. Physiol. Chem., 356 (1975) 1879.
14 H. Umezawa, T. Aoyagi, A. Okura, H. Morishima, T. Takeuchi and Y. Okami, J. Antibiot., 26 (1973) 787.