Isolation of Plasma Kallikrein by High Efficiency Affinity Chromatography and Its Characterization¹⁾

Mayako TADA,^{a,c} Yuko TSUDA,^{a,b} Keiko WANAKA,^c Akiko HUIKATA-OKUNOMIYA,^d Noboru HORIE,^e Utako OKAMOTO,^c Shosuke OKAMOTO,^c and Yoshio OKADA^{*,a,b}

Faculty of Pharmaceutical Sciences^a and High Technology Research Center,^b Kobe Gakuin University, Nishi-ku, Kobe 651–2180, Japan, Kobe Research Projects on Thrombosis and Haemostasis,^c Tarumi-ku, Kobe 655–0033, Japan, Faculty of Health Sciences, Kobe University School of Medicine,^d Suma-ku, Kobe 654–0142, Japan, and School of Human Environmental Science, Mukogawa Women's University,^e Nishinomiya 633–8558, Japan.

Received September 21, 2000; accepted January 22, 2001

We established a simple method for the purification of human plasma kallikrein (PK) by affinity chromatography and characterized it by analytical reverse phase-HPLC and Time of Flight Mass Spectroscopy (TOF-MS). The affinity resin (PKSI-Toyopearl) was synthesized using a selective synthetic inhibitor of plasma kallikrein (PKSI-527) as a ligand. The resin was found to have the highest efficiency in PK purification when the coupling ratio of PKSI-527 per resin was 9–14 $\mu\text{mol/g}$. PK was purified 466-fold with a yield of 83% from acetone-activated human plasma by affinity chromatography. The purity of PK thus obtained was confirmed by reverse phase-HPLC with a linear gradient of acetonitrile. The molecular weight of the purified PK was determined to be 86,151 by TOF-MS.

Key words plasma kallikrein; affinity chromatography; plasma kallikrein selective inhibitor; PKSI-527; reverse phase-HPLC profile; molecular weight

Plasma kallikrein (PK) (EC. 3.4.21.34) is a serine protease that releases bradykinin (BK) from high molecular weight kininogen.²⁾ PK is also known to activate factor XII,³⁾ prourokinase,⁴⁾ plasminogen,⁵⁾ and pro-matrix metalloproteinase 3.⁶⁾ The PK-kinin system was reported to be associated with various diseases such as disseminated intravascular coagulation,⁷⁾ septic shock,⁸⁾ allergy,⁹⁾ postgastrectomy dumping syndrome,¹⁰⁾ arthritis,¹¹⁾ and adult respiratory distress syndrome.¹²⁾ These facts mean that PK has a broad spectrum of activity. But as yet, detailed studies of its physiologic and pathologic roles remain to be solved. In order to study the roles of PK, it is essential to develop effective PK inhibitors and to prepare pure PK in sufficient yield. There have been reports on an affinity chromatography for PK purification using serine proteinase inhibitors such as soybean trypsin inhibitor (SBTI),^{13,14)} L-homoarginine benzyl ester,¹⁵⁾ and peptidyl argininal-semicarbazone.¹⁶⁾ These methods required several steps which reduced the yield of the final products.

We succeeded in finding a highly selective PK inhibitor, *N*-(*trans*-4-aminomethylcyclohexanecarbonyl)-L-phenylalanine-4-carboxymethylanilide (PKSI-527) (Fig. 1),^{17–20)} and employed it as a ligand of affinity chromatography for efficient PK purification. In the previous paper, we demonstrated that PK was purified in a yield as high as 63% from kaolin-activated human plasma by affinity chromatography in combination with DEAE-Toyopearl 650M chromatography.²¹⁾ Continuing our previous investigation, we studied a PK-inhibitor/resin coupling ratio to improve the efficiency of PK purification, then characterized the purified PK.

MATERIALS AND METHODS

Materials AF-Amino Toyopearl 650M was purchased from Tosoh Co., Tokyo. H-D-Pro-Phe-Arg-*p*NA (S-2302) was from Chromogenix AB, Mölndal, Sweden. PKSI-527 and *N*-(4-aminomethylbenzoyl)-*O*-(3'-picolyl)-L-tyrosine *n*-hexylamide dihydrochloride (PASI-535)²²⁾ (Fig. 2) were synthe-

Fig. 1. Structure of PKSI-527 and PKSI-Toyopearl

* To whom correspondence should be addressed. e-mail: okada@pharm.kobegakuin.ac.jp

Enzyme	Substrate	Ki (M)
Plasmin	S-2251	2.9×10^{-6}
Urokinase	S-2444	1.7×10^{-4}
Tissue plasminogen activator	S-2288	$>2.0 \times 10^{-4}$
Thrombin	S-2238	$>2.0 \times 10^{-4}$
Factor Xa	S-2222	$>2.0 \times 10^{-4}$
PK	S-2302	7.7×10^{-5}
Glandular kallikrein	S-2266	$>2.0 \times 10^{-4}$

Fig. 2. Chemical Structure and Enzyme Inhibitions of PASI-535

S-2251; H-D-Val-Leu-Lys-pNA. S-2444; <Glu-Gly-Arg-pNA. S-2288; H-D-Ile-Phe-Arg-pNA. S-2238; H-D-Phe-Pip-Arg-pNA. S-2222; Bz-Ile-Glu-Gly-Arg-pNA. S-2266; H-D-Val-Leu-Arg-pNA.

sized by the authors. Kaolin was purchased from Kishida Chemical Co., Osaka. Methylamine-HCl and Brij 35 from nacalai tesque Inc., Kyoto. SBTI was purchased from Sigma Chemical Co., St. Louis, U.S.A. and lima bean trypsin inhibitor (LBTI) was from Worthington, New Jersey, U.S.A.

Preparation of Human Plasma Fresh human blood was obtained from healthy donors using plastic packs containing sodium citrate as an anticoagulant, and plasma was separated by centrifugation at 1500g for 10 min. Two types of pre-treated plasma were examined. One was kaolin-activated plasma and the other was acetone-activated. Preparation of kaolin-activated plasma was described previously.²¹⁾ Human plasma (18 ml) was slowly acidified with 1 N HCl (3 ml) to pH 2–3 and incubated for 15 min at 37 °C. The pH was subsequently raised to 7.4 by the addition of 1 N NaOH (2.8 ml). A final concentration of 0.2 mg/ml kaolin was added to the acid-treated plasma and the mixture was incubated for 15 min at 37 °C. Plasma was then separated by centrifugation at 1500g for 10 min and the supernatant was used as crude PK.

Acetone-activated plasma was obtained according to the method proposed by Nagase and Barrett.¹³⁾ Methylamine-HCl (1.1 g) was added to human plasma (81 ml) until the concentration of methylamine reached 0.2 M, then the solution was stirred gently for 1 h at room temperature. Acetone (20.3 ml) was added to plasma at the concentration of 20% (v/v) and kept at room temperature for 2–3 h. The plasma thus prepared was dialyzed against distilled water (5 l × 3) at 4 °C for 19 h, and left for 30 min after adjusting the pH to 5.8 with 1 N HCl (1.3 ml). Plasma (107 ml) was then separated by centrifugation at 1500g for 10 min, and the pH of the supernatant was adjusted to 7.8 by adding 1 M Tris-HCl buffer (pH 7.8, 3.7 ml). Brij 35 and NaCl were added to the supernatant until the concentration reached 0.1% and 1 M, respectively.

Preparation of the PKSI-Toyopearl The affinity resin called PKSI-Toyopearl (Fig. 1) was prepared as follows. *tert*-Butyloxycarbonyl (Boc)-PKSI-527 (0.25 g, 0.46 mmol) and AF-Amino Toyopearl 650M (5.6 ml) were coupled using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide · HCl (0.485 g, 2.43 mmol) in dimethylformamide (DMF)/water (20 ml/0.2 ml) with gentle shaking at room temperature. After being

washed with the solvents, the remaining amino groups were capped with acetic anhydride (0.11 ml, 1.11 mmol) and diisopropylethylamine (0.19 ml, 1.11 mmol) at room temperature for 1 h. Boc-PKSI-Toyopearl was treated with trifluoroacetic acid (TFA) to give PKSI-Toyopearl. The peptide content was determined by amino acid analysis of the acid hydrolysate (12 M HCl, 0.5 ml-propionic acid, 0.5 ml, 110 °C for 24 h) using an amino acid analyzer, K-202 SN (Kyowa Seimitsu Co., Ltd.).

Effect of Reaction Time for the Coupling of Boc-PKSI-527 with AF-Amino Toyopearl 650M In the procedure described above, the reaction times for the coupling of Boc-PKSI-527 (0.50 g, 0.92 mmol) with AF-Amino Toyopearl 650M (5.6 ml) were 2, 6, 24, and 72 h. PKSI-527 content was determined by measuring the concentration of phenylalanine in PKSI-Toyopearl by amino acid analysis as a function of time.

Effect of PKSI-527 Content on the Efficiency of PKSI-Toyopearl PKSI-Toyopearl resins were prepared as mentioned above using various amounts of PKSI-527. The purification efficiency of PKSI-Toyopearl was evaluated by measuring the specific activity and yield of PK purified by affinity chromatography. Kaolin-activated plasma (8 ml) was applied to an affinity column (19 × 25 mm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. After being washed with the same buffer, purified PK was eluted with 0.05 M glycine-HCl buffer (pH 3.0) at a flow rate of 1 ml/min at room temperature.

Affinity Chromatography on PKSI-Toyopearl A PKSI-Toyopearl affinity column (19 × 25 mm) was washed with 0.02 M sodium phosphate buffer solution containing 1 M NaCl and 0.1% Brij 35 (pH 7.8). The activated plasma was applied to the column, then the column was washed with running buffer until the absorbance of the eluate at 280 nm dropped below 0.005. The elution of PK was carried out with 0.05 M glycine-HCl buffer (pH 3.0) at a flow rate of 1 ml/min using plastic tubes and columns at room temperature. Tris-HCl buffer (1 M) at pH 7.8 was added to collection tubes during the elution of PK.

Assay of PK Activity The activity of PK was assayed by measuring amidolytic activity using S-2302 as described previously.²¹⁾ Twenty-five microliters of the enzyme solution was added to a mixture of 50 μl of 2 mM S-2302 and 425 μl of 0.01 M sodium phosphate buffer (pH 7.4, 0.15 mM NaCl) at 37 °C. The *p*-nitroaniline released was then determined by measuring OD at 405 nm. One unit of activity indicates that the PK hydrolyzes 1 μmol of S-2302 per minute.

Protein Determination The protein concentration was determined by measuring OD at 280 nm.

Assay of BK Release The proteolytic activity of PK was measured by its ability to release BK from heated plasma. The human heated plasma (7.5 ml) was prepared by its incubation at 60 °C for 1 h. The PK solution (10 μl) was added to a mixture of heated plasma (50 μl) and borate saline buffer, pH 7.4, or inhibitor solution (40 μl) with incubation at 37 °C. The reaction was stopped by adding 20% TFA (20 μl) after the incubation for 2 min. Then the mixture was centrifuged at 1500g for 10 min at 4 °C. The BK in the supernatant was quantitated by the enzyme immunoassay method (Markit-M-Bradykinin Kit, Dainippon Pharmaceutical Co., Osaka).

SDS Polyacrylamide Gel Electrophoresis Electro-

phoresis was carried out according to the literature.²³⁾

Reverse Phase-HPLC Analysis Reverse phase-HPLC was performed according to the method described by Basak *et al.*¹⁶⁾ A Vydac C₄ column (4.6×250 mm) was used on Waters liquid chromatography (600E) with an elution buffer consisting of acetonitrile containing 0.05% TFA (eluent A) and H₂O containing 0.05% TFA (eluent B). The proteins were eluted with a gradient of the eluent A:B starting at 20:80 to 80:20 over a period of 50 min; flow rate of 1 ml/min; and detection at 225 nm. Twenty-five microliters of acetone-activated plasma were injected into the Vydac C₄ column. Purified PK was concentrated by freeze-drying and was then dissolved in 100 μ l of water. The prepared PK was injected into the column. The eluted solution was assayed by measuring the amidolytic activity as follows: fifty microliters of the enzyme solution was added to a mixture of 100 μ l of 2 mM S-2302 and 800 μ l of 0.01 M sodium phosphate buffer containing 0.15 mM NaCl (pH 7.4) at 37 °C over a period of 4 min. Then, 100 μ l of 50 % acetic acid was added to the mixture. The *p*-nitroaniline released was then determined by measuring OD at 405 nm. The effects of the inhibitors were examined using 100 μ l of solution, both PKSI-527 (1 mM) and PASI-535 (1 mM).

TOF-MS Analysis TOF-MS was measured on a Kratos Kompact MALDI 4 (Kratos Analysis) instrument. Sinapinic acid was used as the matrix.

RESULTS AND DISCUSSION

The Preparation of Boc-PKSI-Toyopearl In the preparation of PKSI-Toyopearl, the couplings of Boc-PKSI-527 (0.50 g, 0.92 mmol) with AF-Amino Toyopearl 650M were performed for 2, 6, 24, and 72 h. The coupling reaction of Boc-PKSI-527 with AF-Amino Toyopearl 650M was found to be completed after 24 h.

Effect of a PKSI-527 Content on the Efficiency of PKSI-Toyopearl We attempted to improve the purification efficiency of PK by controlling a PKSI-527/AF-Amino Toyopearl 650M ratio in PKSI-Toyopearl. Figure 3 shows the yield and specific activity of PK purified with various PKSI-Toyopearl columns. The highest specific activity was obtained at the coupling ratio below 7 μ mol/g, although the yield was rather low. In contrast, kaolin-activated plasma was purified in high yield at the ratio of 20 μ mol/g, but the product contained various proteins as impurities, reducing the specific activity. PKSI-Toyopearl, performed at the ratio of 9–14 μ mol/g, showed the highest efficiency in PK purification from kaolin-activated plasma. Almost the same result was obtained for PK purification from acetone-activated plasma.

PKSI-Toyopearl Affinity Chromatography The acetone-activated plasma (40 ml) was applied to a PKSI-Toyo-

pearl (11.8 μ mol/g) column (size; 19×25 mm). After washing the column with 0.02 M sodium phosphate buffer (pH 7.8) containing 1 M NaCl and 0.1% Brij 35, PK was eluted with 0.05 M glycine-HCl buffer (pH 3.0). The eluate was then neutralized with 1 M Tris-HCl buffer (pH 7.8) in order to assure retention of the activity. Acetone-activated human plasma was purified 466-fold with an overall yield of 83% (Fig. 4, Table 1). Purified PK had the specific activity of 28.9 units/mg against S-2302. This value is much higher than that obtained for PK purified from kaolin-activated plasma using PKSI-Toyopearl and DEAE-Toyopearl 650M.

BK Release from Heated Plasma by Purified PK Heated plasma was used as a source of kininogen. As shown in Fig. 5, the purified PK released BK from heated plasma in dose dependent manner. The BK release was strongly inhibited by PKSI-527 and SBTI, which inhibit plasmin and PK, but not by LBTI, which inhibits plasmin.

SDS Polyacrylamide Gel Electrophoresis The purified PK was examined by SDS polyacrylamide gel electrophoresis. As shown in line (B) of Fig. 6, the purified PK exhibited

Fig. 3. Effect of PK Purification as a Function of the Coupling Ratio of PKSI-527 to AF-Amino Toyopearl 650M

Fig. 4. Elution Pattern of Isolation by PKSI-Toyopearl Chromatography from Acetone-Activated Plasma

Buffer A, 0.02 M phosphate buffer solution containing 1 M NaCl and 0.1% Brij 35 (pH 7.8). Buffer B, 0.05 M glycine-HCl buffer (pH 3.0).

Table 1. Purification of PK from Acetone-Activated Human Plasma by PKSI-Toyopearl Affinity Chromatography with a Coupling Ratio of 11.8 μ mol/g resin

Step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification Factor (fold)	Yield (%)
Acetone-activated plasma	40	58.0	930	0.062	1	100
PKSI-Toyopearl affinity chromatography	30	48.2	1.67	28.9	466	83

a single band at approximately 88 kDa.

Analysis on the Reverse Phase-HPLC PK purified from the acetone-activated plasma by PKSI-Toyopearl chromatography was analyzed on the reverse phase-HPLC. The reverse phase-HPLC profile of the original acetone-activated plasma and that of purified PK are shown in Figs. 7(a) and (b), respectively. The composition of peak I observed at 26.1 min (46.1% acetonitrile) in Figs. 7(a) and (b) had amidolytic activity against S-2302. Furthermore, the activity was inhibited more than 95% by the PK specific inhibitor, PKSI-527, while it was not inhibited by the plasmin specific inhibitor, PASI-535. It was concluded that we could obtain pure PK by PKSI-Toyopearl affinity chromatography from human plasma.

TOF-MS Analysis The molecular weight of human PK,

Fig. 5. BK Release from Heated Plasma by Purified PK

Fig. 7. Reverse Phase-HPLC of (a) Acetone-Activated Plasma and (b) Purified PK from Acetone-Activated Plasma by PKSI-Toyopearl Affinity Chromatography

Reverse phase-HPLC profile of the purified PK at 225 nm on a Vydac C_4 Column with a depicted linear gradient of acetonitrile (A) and water (B) (from A:B=20:80 to A:B=80:20 for 50 min).

Fig. 8. TOF-MS of Purified PK Using Sinapinic Acid as the Matrix

as assessed by gel filtration, was reported to be approximately 100 kDa.^{24,25)} SDS polyacrylamide gel electrophoresis studies showed that PK consisted of two components with molecular weights of 86 kDa and 88 kDa,¹³⁾ possibly due to the difference in the degree of glycosylation. The molecular weight of purified PK was measured with TOF-MS. Figure 8 shows the TOF-MS spectrum of the PK obtained from the component of peak I in the reverse phase-HPLC. The molecular weight of the purified PK was determined to be 86151. This result was almost the same as that measured by SDS polyacrylamide gel electrophoresis (88 kDa). It was reported that the zymogen of PK (pre-PK) consists of a nature protein

Fig. 6. Purified PK on SDS Polyacrylamide Gel Electrophoresis

(A) Acetone-activated plasma, (B) purified PK, (C) protein standards [phosphorylase (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa)].

of 619 amino acids.²⁶⁾ Therefore, it can be deduced that the PK examined in the present experiment contains some sugar by glycosylation.

In summary, we established a simple method for the purification of PK from human plasma. We prepared ultra pure PK in a high yield. The purity and molecular weight of PK was characterized by reverse phase HPLC and TOF-MS, respectively.

Acknowledgement The authors are grateful to Dr. Lawrence H. Lazarus of the National Institute of Environmental Health Sciences (NIEHS) for his kind help during preparation of this manuscript.

REFERENCES AND NOTES

- 1) The customary L-configuration for amino acid residues is omitted. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—364 (1967); **11**, 1726—1732 (1972).
- 2) Yano M., Nagawa S., Horiuchi K., Suzuki T., *J. Biochem. (Tokyo)*, **62**, 504—506 (1967).
- 3) Cochrane C. G., Revak S. D., Wuepper K. D., *J. Exp. Med.*, **138**, 1564—1583 (1973).
- 4) Ichinose A., Fujikawa K., Suyama T., *J. Biol. Chem.*, **261**, 3486—3489 (1986).
- 5) Colman R. W., *Biochem. Biophys. Res. Commun.*, **35**, 273—279 (1969).
- 6) Okada Y., Harris E. D., Jr., Nagase H., *Biochem. J.*, **254**, 731—741 (1988).
- 7) Mason J. W., Colman R. W., *Thromb. Diath. Haemorrh.*, **26**, 325—331 (1971).
- 8) Hirsch E. F., Nakajima T., Oshima G., Erdos E. G., Herman C. M., *J. Surg. Res.*, **17**, 147—153 (1974).
- 9) Smith P. L., Kagey-Sobotka A., Bleecker E. R., Traystman R., Kaplan A. P., Gralnick H., Valentine M. D., Permutt S., Lichtenstein L. M., *J. Clin. Invest.*, **66**, 1072—1080 (1980).
- 10) Wong P. Y., Talamo R. C., Babior B. M., Raymond G. G., Colman R. W., *Ann. Intern. Med.*, **80**, 577—581 (1974).
- 11) Bhoola B. K. D., Elson C. J., Dieppe P. A., *Br. J. Rheumatol.*, **31**, 509—518 (1992).
- 12) Schapira M., Gardaz J. P., Py P., Lew P. D., Perrin L. H., Suter P. M., *Bull. Eur. Physiopathol. Respir.*, **21**, 237—241 (1985).
- 13) Nagase H., Barret A. J., *J. Biochem. (Tokyo)*, **193**, 187—192 (1981).
- 14) Sampaio C., Wong S. C., Shaw E., *Arch. Biochem. Biophys.*, **165**, 133—139 (1974).
- 15) Tamura Y., Fujii S., *J. Biochem. (Tokyo)*, **80**, 507—511 (1976).
- 16) Basak A., Gong Y. T., Cromlish J. A., Paquin J. A., Jean F., Seidah N. G., Lazure C., Chretien M., *Int. J. Peptide Protein Res.*, **36**, 7—17 (1990).
- 17) Wanaka K., Okamoto S., Bohgaki M., Hijikata-Okunomiya A., Natio T., Okada Y., *Thromb. Res.*, **57**, 889—895 (1990).
- 18) Wanaka K., Okada Y., Tsuda Y., Okamoto U., Hijikata-Okunomiya A., Okamoto S., *Chem. Pharm. Bull.*, **40**, 1814—1817 (1992).
- 19) Teno N., Wanaka K., Okada Y., Taguchi H., Okamoto U., Hijikata-Okunomiya A., Okamoto S., *Chem. Pharm. Bull.*, **41**, 1079—1090 (1993).
- 20) Nakamura M., Tomoo K., Ishida T., Taguchi H., Tsuda Y., Okada Y., Okunomiya A., Wanaka K., Okamoto S., *Biochem. Biophys. Res. Commun.*, **213**, 583—587 (1995).
- 21) Tada M., Wanaka K., Okamoto S., Okamoto U., Nakaya Y., Horie N., Hijikata-Okunomiya A., Tsuda Y., Okada Y., *Biol. Pharm. Bull.*, **21**, 105—108 (1998).
- 22) Wanaka K., Okamoto S., Horie N., Hijikata-Okunomiya A., Okamoto U., Naito T., Ohno N., Bohgaki M., Tsuda Y., Okada Y., *Thromb. Res.*, **82**, 76—86 (1996).
- 23) Laemmli U. K., *Nature (London)*, **227**, 680—685 (1970).
- 24) Bagdasarian A., Lahiri B., Talamo R. C., Wong P., Colman R. W., *J. Clin. Invest.*, **54**, 1444—1454 (1974).
- 25) Gallimore M. J., Fareid E., Stormorken H., *Thromb. Res.*, **12**, 409—420 (1978).
- 26) Chung D. W., Fujikawa K., McMullen B. A., Davie E., *Biochemistry*, **25**, 2410—2417 (1986).