Purification Method for Human Plasma Kallikrein by a New Affinity Chromatography

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We synthesized a new affinity gel (PKSI-Toyopearl) using a selective synthetic inhibitor of plasma kallikrein (PKSI-527) as an affinity ligand, and employed it for the rapid purification of plasma kallikrein from human plasma. Human plasma activated with kaolin after acid treatment was applied to a PKSI-Toyopearl column. Adsorbed protein was eluted with 50 mM glycine–hydrochloric acid buffer (pH 3.0). Plasma kallikrein was purified 181-fold with a yield of 85% from the kaolin-activated plasma. Further purification was performed by chromatography on a DEAE-Toyopearl 650M column. Plasma kallikrein was finally purified 1720-fold with a 63% yield by these procedures. On sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, a band was observed at approximately 88 kDa. These findings indicate that PKSI-Toyopearl is a valuable tool for the purification of plasma kallikrein from human plasma.

Key words plasma kallikrein; affinity chromatography; synthetic inhibitor; simple purification

Plasma prekallikrein circulates in the blood as the zymogen of plasma kallikrein, and is activated by factor XIIa to form plasma kallikrein.[1] Plasma kallikrein releases bradykinin from high molecular weight kininogen.[2] Furthermore, it has been reported that plasma kallikrein activates factor XII,[3] pro-urokinase[4] and plasminogen.[5] Plasma kallikrein is also known to cause neutrophil aggregation[6] and elastase release.[7] These observations suggest that plasma kallikrein has many functions, although its precise role remains to be determined. Study of this role and the relationship between plasma kallikrein and certain diseases has required development of a simple and convenient purification procedure.

Various techniques have been described for the purification of plasma kallikrein.[8—14] Affinity chromatography is a valuable tool for the purification of enzymes. The inhibitor of an enzyme can be employed as an affinity ligand. Soybean trypsin inhibitor (SBTI) is a protein inhibitor (MW=21600) isolated from soybeans,[15] and has a potent inhibitory effect on plasma kallikrein in addition to trypsin.[16] SBTI-Sepharose affinity chromatography was also applied for purification or isolation of proteinases such as trypsin, plasma kallikrein and factor Xa.[8—11,17,18] However, a synthetic selective inhibitor with small molecular weight has not yet been successfully employed as an affinity ligand for purification of plasma kallikrein.

Many different attempts were made in our group to design and synthesize new compounds in order to develop selective inhibitors for proteinases.[19—23] Finally, we succeeded in finding a novel, highly selective plasma kallikrein inhibitor, *N*-(*trans*-4-aminomethylcyclohexanecarbonyl)-L-phenylalanine 4-carboxymethylanilide (PKSI-527, MW=474).[24,25] PKSI-527 exhibits a reversible and competitive inhibitory activity against plasma kallikrein with a K_i value of 0.81 μM. The K_i values for urokinase, plasmin, thrombin, glandular kallikrein and factor Xa, in contrast, are more than 200-fold greater, revealing an extremely high selectivity of PKSI-527 for plasma kallikrein.[24]

We developed a new affinity gel, PKSI-Toyopearl (Fig. 1), by coupling PKSI-527 with AF-Amino Toyopearl 650M. This paper describes a simple and rapid method for the purification of plasma kallikrein from human plasma employing this PKSI-Toyopearl.

MATERIALS AND METHODS

Materials AF-Amino Toyopearl 650M and DEAE-Toyopearl 650M were purchased from Tosoh Co., Tokyo. H–D-Phe–Pro–Arg–pNA (S-2302), H–D-Val–Leu–Lys–pNA (S-2251), H–D-Ile–Phe–Arg–pNA (S-2288), <Glu–Gly–Arg–pNA (S-2444), H–D-Val–Leu–Lys–Arg–pNA (S-2266), H–D-Phe–Pip–Arg–pNA (S-2238), and Bz–Ile–Glu–Gly–Arg–pNA (S-2222) were purchased from Chromogenix AB, Mölndal. PKSI-527 was synthesized by the authors.[25] SBTI was purchased from Sigma Chemical Co., St. Louis, and lima bean trypsin inhibitor (LBTI), from Worthington, New Jersey. Argatroban was provided by Mitsubishi Chemical Co., Tokyo. Kaolin was purchased from Kishida Chemical Co., Osaka and Briji 35 from Nakarai Tesque Inc., Kyoto. Fresh human blood was obtained from healthy donors using plastic packs containing sodium citrate as an anticoagulant, and plasma was separated by centrifugation at 1500 ***g*** for 10 min.

Preparation of PKSI-Toyopearl *tert*-Butoxycarbonyl (Boc)-*trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanine 4-carboxymethylanilide (Boc-PKSI-527) was prepared as described.[25] Boc-PKSI-527 (0.6 g, 1.1 mmol) and AF-Amino Toyopearl 650M (15 ml) were coupled using water-soluble carbodiimide (1.5 g, 7.5 mmol) in dimethylformamide (DMF)/water (50 ml/25 ml) at room temperature overnight with gentle shaking. After washing with the same solvent, the product (Boc-PKSI-Toyopearl) was further washed successively with DMF, water and methanol. The remaining amino groups were blocked by treatment with acetic

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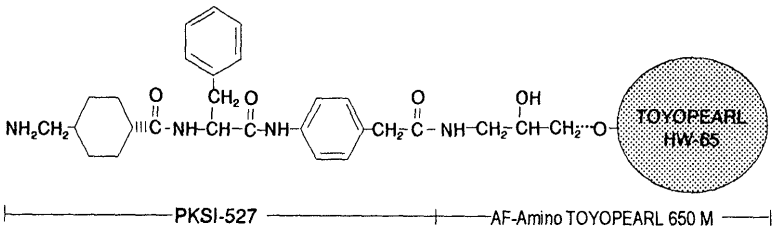

Fig. 1. Chemical Structure of PKSI-Toyopearl

Table 1. Isolation and Purification of Human Plasma Kallikrein

Step	Total volume (ml)	Total protein (mg)	Total activity[a] (units)	Specific activity (unit/mg)	Purification factor (fold)	Yield (%)
Kaolin-activated plasma	16	1024	6.5	0.0064	1	100
Affinity chromatography on PKSI-Toyopearl	10	5.0	5.5	1.16	181	85
Chromatography on DEAE-Toyopearl 650M	11	0.37	4.1	11	1720	63

a) S-2302 amidolytic activity.

anhydride (0.31 ml, 3.0 mmol) and diisopropylethylamine (0.52 ml, 3.0 mmol) at room temperature for 60 min. A Boc-PKSI-Toyopearl was treated with trifluoroacetic acid to give a PKSI-Toyopearl. A concentration of the ligand on PKSI-Toyopearl was determined by amino acid analysis of hydrolysate (6 N HCl, 110 °C for 24 h). The concentration of the ligand was 14 μmol/g resin in this experiment.

Kaolin-Activated Plasma The plasma was slowly acidified with 1 N hydrochloric acid to pH 2—3, and incubated for 15 min at 37 °C. The pH was subsequently raised to 7.4 with 1 N sodium hydroxide. 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 to activate prekallikrein to kallikrein. After centrifugation at 1500 ***g*** for 10 min at 4 °C, the reaction mixture was used as kaolin-activated plasma.

Assay of Plasma Kallikrein Activity The amidolytic activity of the enzyme was estimated as follows. Fifty μl of the enzyme solution was added to a mixture of 50 μl of 2 mM S-2302 and 400 μl of 10 mM sodium phosphate buffer (pH 7.4, 150 mM sodium chloride) at 37 °C. The *p*-nitroaniline released was then measured at OD 405 nm. One unit of activity hydrolyzed 1 μmol of substrate per min.

Assay of Kinin Release One hundred μl of enzyme solution was added to a mixture of 500 μl of human heated plasma (for 1 h at 60 °C) and 80 μl of borate saline buffer (BSB, pH 7.4) or inhibitor solution. Following incubation for 0, 2, 5, 10 min at 37 °C, the reaction was stopped by adding 40 μl trichloroacetic acid (20%) to 200 μl of the reaction mixture, and then the mixture was centrifuged at 1500 ***g*** for 10 min at 4 °C. The amount of kinin in the supernatant was measured by the enzyme immunoassay method (Markit-A-Bradykinin Kit, Dainippon Pharmaceutical Co., Osaka).

Protein Determination Protein concentrations were determined by the method of Lowry *et al.*[26] or by measurement of the OD 280 nm.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[27]

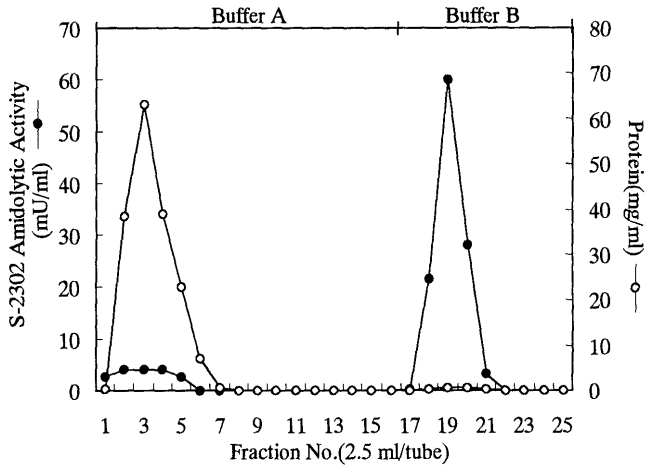

Fig. 2. Affinity Chromatography of Human Plasma Kallikrein by PKSI-Toyopearl from Kaolin Activated Human Plasma

Buffer A, 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM sodium chloride; buffer B, 50 mM glycine-hydrochloric acid buffer (pH 3.0).

RESULTS AND DISCUSSION

Affinity Chromatography on PKSI-Toyopearl PKSI-527 showed a potent and selective inhibition of plasma kallikrein, and the novel affinity gel, PKSI-Toyopearl, was successfully synthesized by coupling PKSI-527 with AF-Amino Toyopearl 650M (Fig. 1). Affinity chromatography on the PKSI-Toyopearl was carried out at room temperature employing plastic tubes and plastic columns. Plasma was activated with kaolin after acid treatment to remove inhibitors in the plasma. The kaolin-activated plasma (8 ml) was applied to a PKSI-Toyopearl column (1.9×2.5 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM sodium chloride. After washing with the same buffer, plasma kallikrein was eluted with 50 mM glycine-hydrochloric acid buffer (pH 3.0) containing 150 mM sodium chloride at a flow rate of 60 ml per hour and purified 181-fold with a yield of 85% from kaolin-activated plasma (Table 1, Fig. 2).

In the SBTI-Sepharose affinity chromatography, plasma kallikrein was eluted with benzamidine, urea, alkali (pH 11.3) and acid (pH 3.5).[8—11] We also attempted to elute

plasma kallikrein with benzamidine, urea, alkali (pH 11.3), acid (pH 3.0—5.0) and PKSI-527 from the PKSI-Toyopearl column. Among the various elutions examined, the elution with acid showed the highest purity and yield. In the present study, plasma kallikrein was eluted with 50 mM glycine-hydrochloric acid buffer (pH 3.0), and no loss of activity was observed when it stood for a week at 4 °C.

Further Purification by DEAE-Toyopearl 650M Chromatography After being concentrated by ultrafiltration on a Centricut (Biofield Co., Tokyo) and adjusted to pH 7.4, the kallikrein solution was applied to a DEAE-Toyopearl 650M column (1.9×29 cm) equilibrated at 4 °C with 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM sodium chloride. The plasma kallikrein was eluted with the same buffer as that used for equilibration, at a flow rate of 30 ml per hour. The eluate was collected in tubes containing 0.1 volume of 0.1% Briji 35. Plasma kallikrein activity was found in the unabsorbed protein and the yield was 75%, with a 9.5-fold increase in specific activity. The plasma kallikrein was finally purified 1720-fold with a 63% yield from the kaolin-activated plasma, as shown in Table 1.

Substrate Specificity and Inhibition Characteristics The final preparation was tested for its substrate specificity using the following synthetic substrates: S-2302 (plasma kallikrein), S-2288 (tissue plasminogen activator), S-2238 (thrombin), S-2266 (glandular kallikrein), S-2222 (factor Xa), S-2251 (plasmin) and S-2444 (urokinase). The purified enzyme displayed a high amidolytic activity for S-2302, and low amidolytic activities for S-2288, S-2238 and S-2266 (Table 2). We examined these amidolytic activities for the inhibitory effects of various inhibitors. The amidolysis of S-2302 was not inhibited by LBTI which can inhibit plasmin, but was strongly inhibited by PKSI-527 and SBTI which inhibits plasmin and plasma kallikrein. The amidolysis of S-2238 was not inhibited by the thrombin selective inhibitor, argatroban,[28] but was strongly inhibited by PKSI-527 and SBTI. PKSI-527 and SBTI also inhibited the amidolysis of S-2288 and S-2266 by the enzyme (Table 3).

Table 2. Amidolytic Activity of Purified Plasma Kallikrein on Various Substrates

Substrate	Amidolytic activity (nmol/min)
S-2302	39.0
S-2288	11.0
S-2238	5.6
S-2266	4.8
S-2222	1.0
S-2251	0.8
S-2444	0.7

Fifty μl of enzyme solution was added to a mixture of 50 μl of 2 mM substrate and 400 μl of 0.05 M Tris HCl buffer at 37 °C. The pH of buffer was 7.8 for S-2302, 8.4 for S-2288, 8.3 for S-2238, 9.0 for S-2266, 8.3 for S-2222, 7.4 for S-2251 and 8.8 for S-2444.

Table 3. Effects of Inhibitors on Amidolytic Activity of Purified Human Plasma Kallikrein

Substrate	Inhibitor	Inhibition (%)
S-2302	PKSI-527	>98
	SBTI	>98
	LBTI	0
S-2288	PKSI-527	>98
	SBTI	>98
S-2238	PKSI-527	>98
	SBTI	>98
	Argatroban	0
S-2266	PKSI-527	>98
	SBTI	>98

Concentrations of inhibitors: PKSI-527, 100 μM; Argatroban, 20 μM; LBTI and SBTI, 100 mg/ml.

The properties of the final purified enzyme were also investigated by measuring release of kinin in heated human plasma. Kinin was measured by enzyme immunoassay. The data in Fig. 3 show that the purified enzyme was able to release kinin in heated human plasma; this kinin release was clearly inhibited by the plasma kallikrein inhibitor, PKSI-527 dose dependently. SBTI also inhibited the release, but LBTI did not. The plasma kallikrein purified by PKSI-Toyopearl showed the same results on the substrate specificity, indicating that PKSI-Toyopearl was able to purify plasma kallikrein successfully.

SDS Polyacrylamide Gel Electrophoresis of the Purified Plasma Kallikrein The purity of our final preparation was examined by SDS polyacrylamide gel electrophoresis.

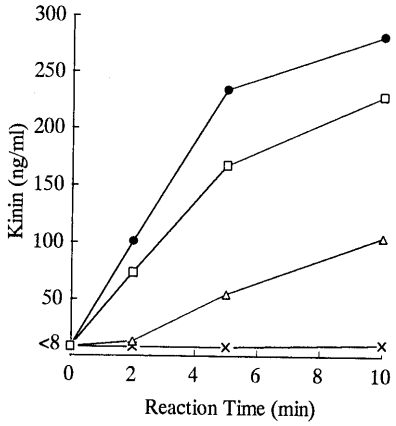

Fig. 3. Kinin Release by Purified Human Plasma Kallikrein and the Inhibitory Effect of PKSI-527

Purified plasma kallikrein was added to a mixture containing heated plasma and incubated at 37 °C in the absence (●) or presence of PKSI-527; 1 μM (□), 10 μM (△), 100 μM (×). The kinin in the reaction mixture was determined by the enzyme immunoassay method.

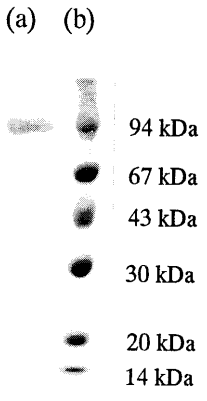

Fig. 4. SDS Polyacrylamide Gel Electrophoresis of Human Plasma Kallikrein

(a) Purified human plasma kallikrein. (b) Protein standards [phosphorylase (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14 kDa)].

Figire 4(a) shows a band at approximately 88 kDa.

These findings indicate that PKSI-Toyopearl is a valuable tool for the rapid purification of plasma kallikrein. Moreover, since PKSI-Toyopearl was fairly durable and its ability was not reduced after repeated use at room temperature, it is considered very useful for purification of the plasma kallikrein enzyme as affinity chromatography.

We anticipate that purified plasma kallikrein preparations and the highly selective plasma kallikrein inhibitor, PKSI-527, can be very useful tools in investigations of the physiological and pathological roles of plasma kallikrein. Further studies are continuing in our laboratory employing these tools *in vivo* and *in vitro*.

REFERENCES

1) DeLa Cadena R. A., Wachtfogel Y. T., Colman R. W., "Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 3rd ed.," ed. by Colman R. W., Hirsh J., Marder V. J., Salzman E. W., J. B. Lippincott Company, Philadelphia, 1994, pp.219—240.
2) Yano M., Nagasawa 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) Schapira M., Despland E., Scott C. F., Boxer L. A., Colman R. W., *J. Clin. Invest.*, **69**, 1199—1202 (1982).
7) Wachtfogel Y. T., Kucich U., James H. L., Scott C. F., Schapira M., Zimmerman M., Cohen A. B., Colman R. W., *J. Clin. Invest.*, **72**, 1672—1677 (1983).
8) Nagase H., Barrett A. J., *Biochem. J.*, **193**, 187—192 (1981).
9) Fritz H., Wunderer G., Dittmann B., *Hoppe-Seyler's Z. Physiol. Chem.*, **353**, 893—900 (1972).
10) Yarovaya G. A., Dotsenko V. L., Orekhovich V. N., Kawiak J., Kawalec M., *Clin. Chim. Acta.*, **93**, 321—327 (1979).
11) Sampaio C., Wong S-C., Shaw E., *Arch. Biochem. Biophys.*, **165**, 133—139 (1974).
12) Gallimore M. J., Fareid E., Stormorken H., *Thromb. Res.*, **12**, 409—420 (1978).
13) Colman R.W., Mattler L., Sherry S., *J. Clin. Invest.*, **48**, 11—21 (1969).
14) Tamura Y., Fujii S., *J. Biochem.* (Tokyo), **80**, 507—511 (1976).
15) Rackis J. J., Sasame H. A., Mann R. K., Anderson R. L., Smith A. K., *Arch. Biochem. Biophys.*, **98**, 471—478 (1962).
16) Wingender W., Bayer-Symposium V., "Proteinase Inhibitors," ed. by Fritz H., Tschesche H., Greene L. J., Truscheit E., Springer-Verlag, Berlin, Heidelberg, New York, 1974, pp.548—559.
17) Yung B. Y., Trowbridge C. G., *Biochem. Biophys. Res. Commun.*, **65**, 927—930 (1975).
18) Bock P. E., Craig P. A., Olson S. T., Singh P., *Arch. Biochem. Biophys.*, **273**, 375—388 (1989).
19) Okamoto S., Hijikata A., "Drug Design VI," ed. by Ariëns E. J., Academic Press, New York, 1975, pp. 143—169.
20) Wanaka K., Okamoto S., Horie N., Hijikata-Okunomiya A., Okamoto U., Naito T., Ohno N., Bohgaki M., Tsuda Y., Okada Y., *Thromb. Res.*, **82**, 79—86 (1996).
21) Hijikata-Okunomiya A., Okamoto S., *Semin. Thromb. Hemostas.*, **18**, 135—149 (1992).
22) Hijikata-Okunomiya A., Okamoto S., Tamao Y., Kikumoto R., *J. Biol. Chem.*, **263**, 11269—11273 (1988).
23) Okada Y., Tsuda Y., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **29**, 1483—1486 (1981).
24) Wanaka K., Okamoto S., Bohgaki M., Hijikata-Okunomiya A., Naito T., Okada Y., *Thromb. Res.*, **57**, 889—895 (1990).
25) Wanaka K., Okada Y., Tsuda Y., Okamoto U., Hijikata-Okunomiya A., Okamoto S., *Chem. Pharm. Bull.*, **40**, 1814—1817 (1992).
26) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
27) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
28) Okamoto S., Hijikata A., Kikumoto R., Tonomura S., Hara H., Ninomiya K., Maruyama A., Sugano M., Tamao Y., *Biochem. Biophys. Res. Commun.*, **101**, 440—446 (1981).