Application of Poly-L-lysine to Purification of Leukocyte Cathepsin G by Affinity Chromatography[1)]

Yoko NAGAMATSU,*,[a] Junichiro YAMAMOTO,[a] Akira KANAMOTO,[a] Yuko TSUDA,[b] and Yoshio OKADA[b]

Faculty of Nutrition[a] and Faculty of Pharmaceutical Sciences,[b] Kobe-Gakuin University, Nishi-ku, Kobe 651-21, Japan. *Received November 29, 1991*

Poly-L-lysine with molecular masses of 3.3—290 kDa increased the amidolytic activities of leukocyte elastase and cathepsin G at low concentration, but had little effect on the activities of pancreatic elastase, α-chymotrypsin, plasmin and thrombin. Highly purified cathepsin G was obtained from column of EAH Sepharose 4B or Suc-L-Tyr-D-Leu-D-Val-*p*NA-Sepharose (affinity chromatography) by elution with poly-L-lysine solution (0.4 mg/ml, molecular weight (MW.) 290000 or 2.2 mg/ml, MW. 3300). Leukocyte elastase, adsorbed to Suc-L-Tyr-D-Leu-D-Val-*p*NA-Sepharose, was not eluted with poly-L-lysine solution. The amino acid composition of purified cathepsin G has been determined.

Keywords polycation; poly-L-lysine; leukocyte cathepsin G; purification; affinity chromatography

Recent reports have demonstrated that leukocyte elastase activity (ELP) can be detected in blood in spite of an excess of plasma inhibitors.[2)] In addition, plasma levels of ELP have been measured by ELISA and the role of the enzyme in various pathological states has been investigated.[3)] In contrast, studies of cathepsin G (CLP) which is of interest as a strong platelet agonist,[4)] are relatively scarce.

Investigations of the physiological role of these enzymes are hampered by difficult multi-stage purification processes.[4)] We have previously fractionated ELP and CLP using Suc-L-Tyr-D-Leu-D-Val-*p*NA-Sepharose.[5)] CLP was bound less strongly than ELP to the Sepharose and was eluted with 2 M NaCl. ELP was eluted with 8 M urea. The purification procedures were incomplete, however, and, in particular, the specific activity of CLP was low compared to that of ELP eluted with 8 M urea.

Several studies have shown that polycations influenced endothelial functions and activated the caseinolytic activity of the erythrocyte multicatalytic proteinase.[6)] We have found that poly-L-lysine and other polycations effect the amidolytic activities of ELP and CLP. The present paper reports the purification of CLP by a simple one-step method using poly-L-lysine.

Materials and Methods

Preliminary extraction of ELP and CLP from human leukocytes was carried out using Tris–HCl buffer (0.1 M, pH 8.0) containing 2 M NaCl, as described previously.[5a)] Amidolytic activities of ELP and CLP were determined by the method described previously[5b)] using Suc-Ala-Tyr-Leu-Val-*p*NA and Suc-Ile-Pro-Phe-*p*NA, respectively. In the same manner, the amidolytic activities of pancreatic elastase (PE), α-chymotrypsin, plasmin and thrombin were determined using Suc-Ala_3-*p*NA, Suc-Ile-Pro-Phe-*p*NA, H-D-Val-Leu-Lys-*p*NA (S-2251) and H-D-Phe-Pip-Arg-*p*NA (S-2238), rspectively. Synthetic substrates for ELP and CLP and Suc-L-Tyr-D-Leu-D-Val-*p*NA-Sepharose were prepared in our laboratory,[7)] and Suc-Ala_3-*p*NA was purchased from Peptide Institute INC (Osaka). Polycations, PE (porcine pancreatic elastase, type IV) and α-chymotrypsin were purchased from Sigma Chemical Co. (St. Louis). S-2251, S-2238 and plasmin were purchased from KABI AB (Stockholm). EAH Sepharose 4B and thrombin were purchased from Pharmacia LKB (Uppsala) and Mochida Pharmaceutical Co. (Tokyo), respectively. Synthetic substrates and polycations were dissolved in 0.1 M Tris–HCl buffer (pH 7.5 for CLP and α-chymotrypsin or pH 8.0 for other proteinases). Amino acid composition of an acid hydrolysate of CLP (6 N HCl, 110 °C, 18 h) was determined using an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co.).

Results and Discussion

Table I shows the effect of polycations on the amidolytic activity of various serine proteinases. All polycations enhanced the activity of ELP and CLP, but, in contrast, tended to inhibit the activity of plasmin. The polycations had little effect on the other enzymes tested. The effect of poly-L-lysine on ELP and CLP was dependent on the size of the polycation molecules in the reaction mixture. The effect decreased as the molecular weight of poly-L-lysine became smaller. In addition, the effect of polycations on the activity of plasmin and thrombin, in reaction mixtures containing 0.05 M NaCl, was negligible compared to those containing 0.01 M NaCl (data not shown). Note especially that the results in Table I show the activity of plasmin and thrombin at a final concentration of 0.01 M NaCl.

TABLE I. Effect of Polycations on the Amidolytic Activity of Serine Proteinases

Polycation	% activity[a)]					
	CLP	ELP	α-Chymotrypsin	PE	Plasmin[b)]	Thrombin[b)]
Poly-L-arginine						
MW. 139200	200	320	90	110	60	100
Protamin	200	300	90	110	90	110
Polybrene	180	320	120	110	115	110
Histon	170	300	110	110	105	100
Poly-L-lysine						
MW. 290000	200	320	110	110	60	120
MW. 26600	200	310	80	110	90	110
MW. 10200	175	285	105	105	70	110
MW. 3300	160	200	90	105	115	115

The final concentration of polycations and NaCl in reaction mixture was 44 μg/ml and 0.05 M, respectively. *a*) The activities in the absence of polycation were taken as 100% (control). *b*) Final concentration of NaCl was 0.01 M.

Figure 1 illustrates the affinity chromatography of preliminary leukocyte extracts on Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose. The columns were equilibrated with Tris–HCl buffer (0.1 M, pH 7.5) containing 0.15 M NaCl and 2 ml of leukocyte extract (2 M NaCl) adjusted NaCl concentration to 0.15 M with Tris–HCl buffer (0.1 M, pH 7.5) was applied. When poly–L-lysine (0.4 mg/ml, MW. 290000) was used as an eluate (Fig. 1A), the specific activity of the fraction was 3—4 times higher than that of CLP obtained using 2 M NaCl (Fig. 1B).

Similarly, the specific activity of CLP obtained by chromatography on EAH Sepharose 4B using poly-L-lysine (0.4 mg/ml, MW. 290000, Fig. 2A) was markedly higher than that obtained using 2 M NaCl (Fig. 2B). The yield of CLP was approximately 80% using Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose and 88% using EAH Sepharose 4B.

Poly-L-lysine of MW. 3300 was also used to elute CLP from Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose and EAH Sepharose 4B columns. In these instances 2.2 mg/ml poly-L-lysine was required to obtain the enzyme at high specific activity and for high recovery. In contrast, the lower concentration of poly-L-lysine (MW. 3300) was not efficient at eluting CLP from either column (data not shown). The peak fraction of CLP which was eluted by poly-L-lysine (MW. 3300) from Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose or EAH Sepharose 4B was dialyzed against Tris–HCl buffer (0.1 M, pH 7.5) and used for the determination of

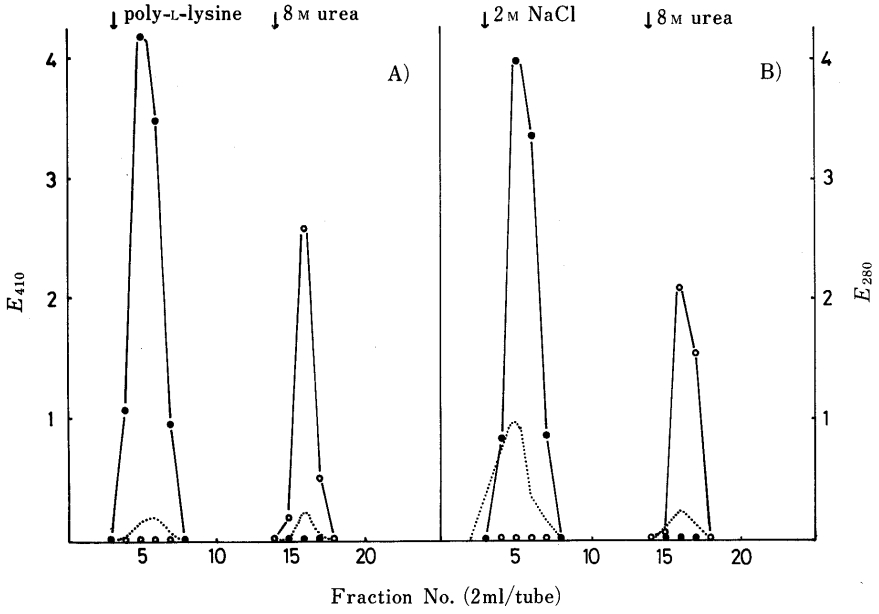

Fig. 1. The Purification of CLP on Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose

A) and B) in Figs. 1 and 2 show elution patterns with poly-L-lysine (0.4 mg/ml, MW. 290000) and 2 M NaCl, respectively. Column size and flow rate were 1.5 × 4.5 cm and 30 ml/h, respectively. ●—● and ○—○; amidolytic activities of CLP and ELP, respectively. ------; protein. The final concentration of synthetic substrates in Fig. 1 and 2 was 0.5 mM, and amidolytic activities in each fraction were measured after incubation for 5 min at 37 °C.

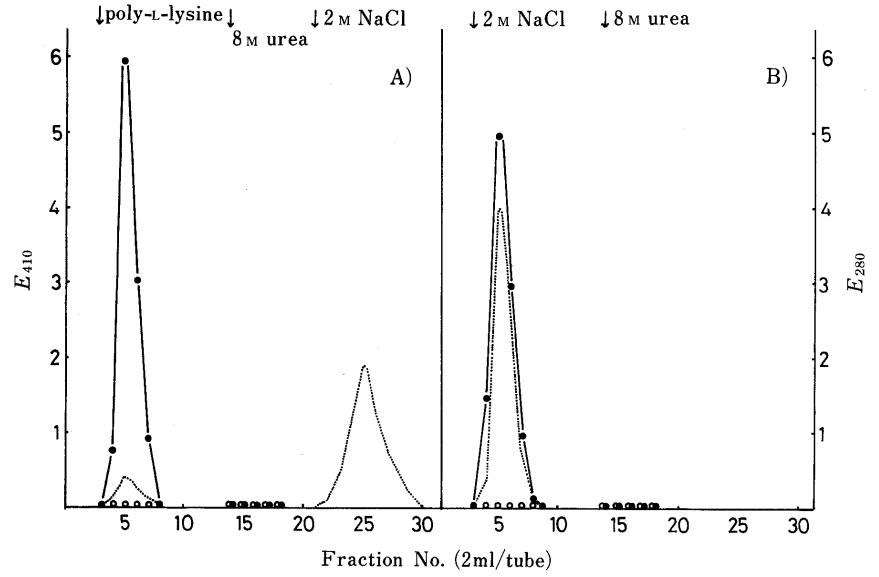

Fig. 2. The Purification of CLP with EAH Sepharose 4B

All conditions were the same as Fig. 1. Figures 1 and 2 show patterns of adsorbed protein and proteinase activities.

Table II. Amino Acid Compositions of Cathepsin G

Amino acid	mol/mol		
	Travis *et al.*	Feinstein *et al.*	Ours
Lysine	3	5	8
Histidine	6	5	5
Arginine	31	18	20
Aspartic acid	17	20	19
Threonine	12	13	9
Serine	15	18	14
Glutamic acid	23	24	26
Proline	13	8	12
Glycine	21	21	21
Alanine	13	13	10
Half-cystine	6	4	ND
Valine	14	11	13
Methionine	4	3	2
Isoleucine	11	9	10
Leucine	16	14	15
Tyrosine	5	5	3
Phenylalanine	10	6	9
Tryptophan	ND	ND	ND
Total No.	220	197	196

ND: not determined.

amino acid composition (Table II). The purity of CLP used for the determination of amino acid composition was over 90%. This differed from those of Feinstein and Janoff[8] and Travis *et al.*[9] in some amino acids (low values of Thr, Ala, Met and Tyr, and a high value of Lys), whose results were also different from each other. This variation may be due to the preparations used for purification being different because of autolysis of proteinases extracted from leukocytes. The elution of CLP by lower molecular weight poly-L-lysine is now under way to clarify whether or not the high value of lysine is due to contamination by poly-L-lysine.

Also, in the present study, polycations, which are known to affect endothelial function and permeability, and the proteolytic activity of erythrocyte multicatalytic proteinase, increased the amidolytic activities of ELP and CLP in the same manner as that observed using NaCl. While in some experiments the activities was increased 300% and 200%, respectively, at a final concentration of 0.2 M NaCl (data not shown), similar increase in activity was obtained with only low concentration of polycations. Although poly-L-lysine increased amidolytic activities of both ELP and CLP, it could elute only CLP from Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose column. From these results, it might be deduced that the active site of ELP bound strongly with the affinity Sepharose and a region apart from the active site of CLP bound only weakly. This is compatible with the fact that Suc–L-Tyr–D-Leu–D-Val–*p*NA was a competitive inhibitor of ELP, but not of CLP.[10] Further, the elution of CLP with poly-L-lysine suggested that binding mode of CLP with Suc–L-Tyr–D-Leu–D-Val–*p*NA–Sepharose was similar to that with EAH Sepharose.

We also found that low concentrations of poly-L-lysine stimulated the release of ELP and CLP from leukocytes *in vitro*, and that poly-L-lysine was an especially useful reagent for the fractionation of CLP in a simple one-step chromatographic procedure.

References and Notes

1) The customary L indication for amino acid residues of synthetic substrates is omitted. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used: Suc, succinyl; *p*NA, *p*-nitroanilide, Pip, pipecolyl; ELISA, enzyme-linked immunosorbent assay.
2) *a*) J. I. Weitz, A. J. Huang, S. L. Landman, S. C. Nicholson, and S. C. Silverstein, *J. Exp. Med.*, **166**, 1836 (1987); *b*) S. J. Weiss, J. T. Curnutte, and S. Regiani, *J. Immunol.*, **136**, 636 (1986).
3) *a*) L. A. Smedly, M. G. Tonnesen, R. A. Sandhaus, C. Haslett, L. A. Guthrie, and R. B. Johnston Jr., *J. Clin. Invest.*, **77**, 1233 (1986); *b*) C. Von Ritter, R. Be, and D. N. Granger, *Gastroenterology*, **97**, 605 (1989); *c*) E. O. Adeyemi, S. Neumann, V. S. Chadwick, H. J. F. Hodgson, and M. B. Pepys, *Gut*, **26**, 1306 (1985); *d*) C. P. Speer, M. Rethwilm, and M. Gahr, *J. Pediatr.*, **111**, 667 (1987); *e*) M. Saito, H. Asakura, C. Uotani, H. Jokaji, I. Kumabashiri, and T. Matsuda, *Thromb. Res.*, **53**, 163 (1989).
4) M. A. Selak, M. Chignard, and J. B. Smith, *Biochem. J.*, **251**, 293 (1988).
5) *a*) Y. Nagamatsu, S. Tsuboi, K. Nakabayashi, Y. Tsuda, Y. Okada, and J. Yamamoto, *Jpn. J. Thromb. Haemost.*, **1**, 203 (1990); *b*) Y. Nagamatsu, U. Okamoto, Y. Tsuda, and Y. Okada, *Thromb. Haemostas.*, **51**, 243 (1984).
6) *a*) V. M. Vehaskari, C. T. C. Chang, J. K. Stevens, and A. M. Robson, *J. Clin. Invest.*, **73**, 1053 (1984); *b*) R. L. Mellgren, *Biochim. Biophys. Acta*, **1040**, 28 (1990); *c*) L. Needham, P. G. Hellewell, T. J. Williams, and J. L. Gordon, *Lab. Invest.*, **59**, 538 (1988).
7) *a*) Y. Okada, Y. Tsuda, A. Hirata, Y. Nagamatsu, and U. Okamoto, *Chem. Pharm. Bull.*, **30**, 4060 (1982); *b*) Y. Okada, Y. Tsuda, N. Teno, Y. Nagamatsu, and U. Okamoto, "Peptide Chemistry 1986," ed. by T. Miyazawa, Protein Research Foundation, Osaka, 1987, p. 261; *c*) Y. Okada, Y. Tsuda, Y. Nagamatsu, and U. Okamoto, *Chem. Pharm. Bull.*, **30**, 1528 (1982).
8) G. Feinstein and A. Janoff, *Biochim. Biophys. Acta*, **403**, 477 (1975).
9) J. Travis, R. Baugh, P. J. Giles, D. Johnson, J. Bowen, and C. F. Reilly, "The Neutral Proteases of Human Polymorphonuclear Leukocytes," ed. by K. Havemann and A. Janoff, Urban & Schwarzenberg, Baltimore-Munich, U.S.A., 1978, p. 118.
10) *a*) Y. Okada, Y. Tsuda, Y. Nagamatsu, and U. Okamoto, *J. Peptide Protein Res.*, **24**, 347 (1984); *b*) Y. Tsuda, N. Teno, Y. Okada, Y. Nagamatsu, and U. Okamoto, *Chem. Pharm. Bull.*, **36**, 3119 (1988).