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Neutral Proteinase Inhibitors in PMN Leukocytes

I. Purification and Characterization of a Neutral Proteinase Inhibitor from Bovine Neutrophils

Petra HINTZ[a], Marianne JOCHUM[b] and Horst MOSSMANN[a]

[a] Max-Planck-Institut für Immunbiologie, Freiburg.
[b] Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München

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Summary: An inhibitor of neutral proteinases was isolated from the cytosol of bovine leukocytes by anion exchange chromatography on Mono Q and gel filtration on a HPLC TSK column. The gel filtration resulted in two fractions with inhibitory activity which could be identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions as dimer and monomer of the inhibitor. The latter was shown to be homogeneous in SDS-PAGE with an apparent molecular mass of 40 kDa, with calibrated HPLC a molecular mass of 36.5 kDa has been determined. Isoelectric focusing followed by Western blot analysis revealed four bands in the pH range of 5.0 to 5.9. The inhibitor was found in bovine polymorphonuclear neutrophils (PMN), whereas lymphocytes and monocytes lacked this protein. No immunological cross-reactivity between the described cell-derived PMN-inhibitor (PMN-I) and α_1-proteinase inhibitor was detectable. The mechanism of inhibition for the serine proteinases chymotrypsin, trypsin, pancreatic elastase and leukocyte elastase was studied. PMN-I could not bind to PMS-chymotrypsin. The reaction of the serine proteinases with the PMN-I was characterized by the determination of the association rate constant k_{on}.

Inhibitoren neutraler Proteinasen aus PMN-Leukozyten.
I. Reinigung und Charakterisierung eines Inhibitors neutraler Proteinasen aus polymorphkernigen Neutrophilen des Rindes

Zusammenfassung: Aus dem Cytoplasma von Rinder-Leukozyten wurde ein Inhibitor neutraler Proteinasen mit Hilfe einer Kombination von Anionenaustausch-Chromatographie an einer Mono-Q-Säule und Gelfiltration an einer HPLC-TSK-Säule gereinigt. Die Gelfiltration resultierte in zwei Fraktionen mit inhibitorischer Aktivität, die in der SDS-Polyacrylamid-Gelelektrophorese (SDS-PAGE) unter nichtreduzierenden Bedingungen als die dimere und die monomere Form des Inhibitors identifiziert werden konnten. Letztere erwies sich in der SDS-PAGE als homogen mit einer Molekularmasse von 40 kDa. Dagegen führte die Bestimmung der Molekularmasse an einer kalibrierten HPLC-Säule zu einer etwas geringeren Molekularmasse von 36.5 kDa. Bei der isoelektrischen Fokussierung mit anschließendem Western-Blot traten vier Banden

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Enzymes:
Bovine chymotrypsin (EC 3.4.21.1), human leukocyte elastase (EC 3.4.21.37, formerly EC 3.4.21.11), porcine pancreatic elastase (EC 3.4.21.36, formerly EC 3.4.21.11), porcine pancreatic trypsin (EC 3.4.21.4).

Abbreviations:
ACD, acid citrate dextrose; AMC, 7-amino-4-methylcoumarin; DMSO, dimethylsulfoxide; FPLC, fast-protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HPLC, high-pressure liquid chromatography; IEF, isoelectric focusing; MeOSuc, 4-methoxycarbonylpropionyl; Nan, 4-nitroanilide; $PO_4^{3\ominus}$/NaCl, phosphate-buffered saline; PMN, polymorphonuclear neutrophils; PMN-I, PMN-inhibitor; PMSF, phenylmethylsulfonyl fluorid; SBzl, thiobenzylester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

im pH-Bereich von 5.0 bis 5.9 auf. Weiterhin konnte gezeigt werden, daß der Inhibitor ein zellspezifisches Protein der polymorphkernigen Neutrophilen (PMN) ist; Lymphozyten und Monozyten besitzen dieses Protein nicht. Der PMN-Inhibitor und α_1-Proteinase Inhibitor haben keine gemeinsame immunologische Spezifität. Der Hemmungsmechanismus wurde für die Serin-Proteinasen Chymotrypsin, Trypsin, Pankreas-Elastase und Leukozyten-Elastase untersucht. Der PMN-I reagierte nicht mit PMS-Chymotrypsin. Die Reaktion der Serin-Proteinasen mit PMN-I wurde durch die Bestimmung der Geschwindigkeitskonstanten der Assoziation k_{on} charakterisiert.

Key words: Leukocyte elastase inhibitor, granulocytes, serine proteinases, cytosolic inhibitor.

Acute inflammatory processes are characterized by an accumulation of PMN[1], which release a series of enzymes by extrusion of their granules during phagocytosis or cell disintegration[2]. Besides acid hydrolases, mainly neutral serine proteinases such as leukocyte elastase and cathepsin G as well as the metallo proteinase collagenase[3] are released. Furthermore, toxic oxygen radicals are produced in connection with the respiratory burst of the cells[4]. The extracellularly released enzyme activity in the serum is controlled by plasma proteinase inhibitors like α_1-PI[5] and α_2-macroglobulin[6]. In addition, inhibitors of neutral proteinases were found in the cytosol of leukocytes and macrophages in man[7–9], pig[10], rabbit[11], horse[12] and cattle[13,14].

The relevance of inhibitors, found in the cytosol of leukocytes, is still under discussion. Protection of cytoplasmic proteins against proteinases released from the granules during phagocytosis seems to be a major function[9,12,15]. Cytosol inhibitors may also be responsible for the blocking of enzymes when cell degradation takes place during inflammation[12], while the concentration of plasma inhibitors is possibly not efficient in tissue for the control of proteinase activity[1].

This report describes the purification and biochemical characterization of a cytosolic neutral serine proteinase inhibitor from bovine PMN.

Materials and Methods

Chemicals

The enzyme substrates Suc[Ala]$_2$ValNan, Suc[Ala]$_3$Nan, Suc[Ala]$_2$ProPheNan, Suc[Ala]$_2$ProPheSBzl and ZLysSBzl were purchased from Bachem AG, Bubendorf, Switzerland, NBenzoylArgNanHCl was obtained from Sigma, St. Louis, MO, U.S.A., and Ellmanns reagent (5,5′-dithiobis-2-nitrobenzoic acid) was a product of Serva, D-6900 Heidelberg. Bovine chymotrypsin was from DeNovo, Bagsvaerd, Denmark. Porcine trypsin and porcine pancreatic elastase were from Sigma, and human leukocyte elastase was a generous gift of Dr. S. Neumann (Merck, D-6100 Darmstadt). Human α_1-PI was obtained from Sigma. Bovine α_1-PI was a product of Boehringer, D-6800 Mannheim. Trasylol (Aprotinin) was purchased from Bayer, D-5090 Leverkusen. Marker proteins for the calibration of the HPLC column and the SDS-PAGE were purchased from Sigma. Chemicals for SDS-PAGE were obtained from Bio-Rad, Richmond, CA, U.S.A. Chemicals and materials for IEF were purchased from LKB, Bromma, Sweden. Percoll was a product of Pharmacia, D-7800 Freiburg. All other chemicals of highest purity available were from Roth (D-7500 Karlsruhe) and Merck.

Protein determination

Protein was determined with the Ninhydrin reagent[16] using bovine serum albumin as standard.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels was carried out by the method of Laemmli[17] under nonreducing conditions. The samples were applied in 0.5-μg to 2.0-μg aliquots in a volume of 20 μl, respectively. Proteins were stained with Coomassie Brillant Blue R 250 (Serva, D-6900 Heidelberg).

Isoelectric focusing (IEF) was performed with 5% polyacrylamide gels containing 2.4% carrier ampholytes in the pH range of 3.5 to 9.5 according to Görg et al.[18]. Protein samples were applied in 20-μg to 60-μg aliquots in a volume of 20 μl, respectively. Coomassie Brillant Blue R250 was used for protein staining. Prior to staining the pH-gradient was determined with a pH surface electrode.

Western blot in connection with staining by ^{125}I-labeled leukocyte elastase

Iodination of human leukocyte elastase was performed according to Greenwood and Hunter[19]. Subsequent to the radiolabeling of the enzyme non-radioactive iodide was added to the mixture to give a total concentration of 0.35% (w/v). Unbound iodine was separated from ^{125}I-elastase by the use of a Sephadex G-25 column which was run with 0.1 mol/l Tris, 0.5 mol/l NaCl, 1% ovalbumin (w/v), adjusted to pH 7.5 with HCl.

The procedure used for Western blot has been described by Towbin et al.[20]. After blotting the electrophoretically separated proteins the nitrocellulose filters were

incubated in phosphate-buffered saline ($PO_4^{3\ominus}$/NaCl; 140 mmol/*l* NaCl, 3 mmol/*l* KCl, 8 mmol/*l* $Na_2HPO_4 \times 2\,H_2O$, 1.5 mmol/*l* KH_2PO_4, pH 7.2) containing additionally 0.05% Tween 20 and 2 mol/*l* NaCl to prevent unspecific binding of the ^{125}I-labeled leukocyte elastase[21]. Subsequently the nitrocellulose filters were incubated with 3 μCi ^{125}I-labeled enzyme in 10 m*l* buffer for 3 h at room temperature with constant rocking. Finally the filters were washed twice with buffer, air-dried and exposed to a Kodak X-Omat AR film for 1–3 days at – 80 °C.

Preparation of bovine leukocytes

Blood of normal cattle was collected at the slaughter house in bottles containing one fifth of the final volume of the stabilizer ACD (acid citrate dextrose: 45 mmol/*l* sodium citrate, 21 mmol/*l* citric acid, 74 mmol/*l* glucose monohydrate). After washing the cells with $PO_4^{3\ominus}$/NaCl (15 min/1000 x *g*) erythrocytes were lysed by the addition of 0.017 mol/*l* Tris, 0.14 mol/*l* ammonium chloride, pH 7.2. The remaining leukocytes were washed with $PO_4^{3\ominus}$/NaCl and adjusted to the required concentration.

Separation of bovine leukocytes

Bovine leukocytes (1.6×10^8 cells/m*l*) were separated using a discontinuous gradient of Percoll according to Hjorth et al.[22]. The interphase, containing lymphocytes as well as monocytes, and the phase between the two Percoll solutions, containing PMN, were removed and washed twice with $PO_4^{3\ominus}$/NaCl. Both cell fractions were adjusted to an amount of 5×10^7 cells/m*l*. Different cell types were identified using Giemsas and May-Grünwald's staining solutions according to Pappenheim[23].

Purification of the PMN-I

Leukocytes (1×10^8 cells/m*l*) transfered to $PO_4^{3\ominus}$/NaCl containing 8% saccharose were homogenized by nitrogen cavitation (20 atü, 13.5 min)[24]. The cytosol was separated from the membranous particles by ultracentrifugation for 60 min at 220000 x *g*. Subsequently, protein was precipitated by the addition of 2 mol/*l* ammonium sulfate to the supernatant, i.e. the cytosol. After 1 h of stirring at 2 °C the precipitate was separated by centrifugation at 4000 x *g* for 45 min. The supernatant containing the inhibitor activity was dialysed against 50 mmol/*l* Tris/phosphate, pH 7.5, and concentrated by ultrafiltration with negative pressure using a Visking dialysis tube to a concentration of 40 mg to 45 mg protein/m*l*. Two m*l* of the sample was applied to a prepacked FPLC-anion exchange chromatography column (Mono Q HR 10/10, Pharmacia). The elution was performed at 20 °C by a linear concentration gradient ranging from 0.02 mol/*l* to 0.56 mol/*l* Tris/phosphate, pH 7.5, with a flow rate of 0.5 m*l*/min using a chromatography unit of LKB. Five-m*l* fractions were collected at 4 °C and aliquots of each fraction were tested for inhibitory activity against leukocyte elastase. Fractions with inhibitory activity were pooled, concentrated by ultrafiltration and dialysed against 0.1 mol/*l* phosphate, 0.1 mol/*l* NaCl, pH 6.8. The dialysed material (10–30 mg protein) was applied in 2 m*l* to a calibrated preparative HPLC-gel permeation chromatography column (Ultro Pac TSK G 2000, LKB) for further purification. The calibration of the column was obtained with marker proteins as indicated (Fig. 2) according to Laurent and Killander[25]. Protein was eluted with 0.1 mol/*l* phosphate, 0.1 mol/*l* NaCl, pH 6.8, and the flow rate was 0.5 m*l*/min. Five-m*l* fractions were collected, analysed by SDS-PAGE (not shown) and tested for inhibitory activity against leukocyte elastase.

Reduction of the PMN-I fractions with mercaptoethanol

The PMN-I samples (each 0.6 mg protein in 3.8 m*l* 0.5 mol/*l* Tris/HCl pH 8.1) were reduced by the addition of mercaptoethanol to a final concentration of 0.1 mol/*l*. After incubation of the reaction mixtures for 60 min at 20 °C iodoacetamide was added to a final concentration of 0.1 mol/*l* and the mixtures were incubated further 60 min at 2 °C. The samples were dialysed overnight against $PO_4^{3\ominus}$/NaCl and analysed for their inhibitory activity against leukocyte elastase and by SDS-PAGE.

Activity and inhibition measurements

The enzyme assays were performed in a final volume of 1 m*l* and at a temperature of 25 °C. The velocity of reaction of the enzymes with the substrates (ΔA_{405}/min) was determined using the Eppendorf calculator (CKE 6455) for the first 2 min subsequent to the addition of substrate. For the determinations of the type of inhibition, the serine proteinases were first incubated with PMN-I for 5 min at 25 °C before adding the appropriate substrate. Inhibition was studied using the methods of Lineweaver and Burk[26] and Henderson[27]. The association rate constant k_{on} was measured and calculated according to Bieth[28].

Human leukocyte elastase was assayed according to Wenzel et al.[29] using a buffer system of 0.1 mol/*l* Hepes, 2 mol/*l* NaCl and 0.05% Triton X-100 (v/v) adjusted to pH 8.0 with ammonia. The photometrical determination of the type of inhibition was accomplished with 3.9 nmol/*l* enzyme and PMN-I in a range of 1.0 nmol/*l* to 3.15 nmol/*l*. Suc[Ala]$_2$ValNan dissolved in dimethylsulfoxide (DMSO) was used as substrate in a range of 0.2 mmol to 2 mmol (DMSO < 10% of the assay volume). The association rate constant k_{on} was fluorimetrically measured with 0.5 mmol/*l* MeOSuc-[Ala]$_2$ProValAMC as substrate[30], 0.5 nmol/*l* leukocyte elastase and 0.5 nmol/*l* PMN-I using the same assay buffer as described. For the calculation of the specific inhibitory activity of PMN-I (IU/mg protein) according to Bergmeyer[31] an extinction coefficient of $\epsilon = 1.3\ l\ mmol^{-1}\ mm^{-1}$ was determined.

For the photometrical determination of the type of inhibition 7.8 nmol/*l porcine pancreatic elastase* was tested in 0.2 mol/*l* Tris, adjusted to pH 8.0 with HCl with the substrate Suc[Ala]$_3$Nan (first dissolved in DMSO) in the range of 0.2 mmol/*l* to 0.8 mmol/*l* and PMN-I in the range of 5.3 nmol/*l* to 13.1 nmol/*l*. k_{on} was fluorimetrically determined with 0.5 mmol/*l* MeOSuc[Ala]$_2$ProValAMC[30], 5 nmol/*l* pancreatic elastase and 5 nmol/*l* PMN-I in 0.2 mol/*l* Tris/HCl, pH 8.0.

Bovine chymotrypsin was assayed according to Del Mar et al.[32] with 50 mmol/l Tris, 0.4 mol/l NaCl and 0.05% Triton X-100 (v/v) adjusted to pH 7.8 with HCl as buffer. 1.2 nmol/l enzyme and PMN-I in the range of 0.6 nmol/l to 2.0 nmol/l were used for the determination of the type of inhibition. The association rate constant k_{on} was photometrically measured with 1 nmol/l enzyme, 1 nmol/l PMN-I, 0.1 mmol/l Suc[Ala]$_2$ProPheSBzl and 0.24 mmol/l Ellmanns reagent [5,5'-dithiobis-(2-nitrobenzoic acid)][33]. The latter two substances were first dissolved in DMSO. The final concentration of DMSO in the assay was 4%.

Porcine trypsin was dissolved in 2.5 mmol/l HCl to a concentration of 0.7 µmol/l and was further diluted with buffer (0.2 mol/l Tris, 0.05% Triton X-100 adjusted to pH 8.0 with HCl). The concentration of enzyme for the determinations of the type of inhibition was 14.2 mmol/l per assay, using NBenzoylArgNan · HCl (dissolved in bidistilled water) as substrate in a range of 0.2 mmol/l to 1.7 mmol/l. PMN-I was added in the range of 4.2 nmol/l to 8.4 nmol/l. For the photometrical determination of k_{on} 0.5 nmol/l trypsin, 0.5 nmol/l PMN-I and the substrate combination of 0.5 mmol/l ZLysSBzl and 0.24 mmol/l Ellmanns reagent were used[34] (4% DMSO per assay), in the above-mentioned assay buffer.

The enzymes as well as the PMN-I sample used in the assays were previously active site titrated, i.e. the percentages of active molecules were determined using corresponding substrates and inhibitors respectively. First of all trypsin was titrated with the covalently binding substrate 4-nitrophenyl guanidinobenzoate by the method of Shaw et al. as described in ref.[35]. The inhibitor aprotinin, required for the standardization of chymotrypsin, was assayed by the reaction with titrated trypsin and found to be 100% active. Both the inhibitor and the enzyme were standardized by the above-mentioned photometrical enzyme assays. Subsequently, chymotrypsin was taken for the titration of human α_1-PI. This inhibitor was used for the titration of leukocyte as well as pancreatic elastase using the corresponding photometrical enzyme assays. PMN-I was active site titrated by the already titrated serine proteinases using again the corresponding photometrical enzyme assays. The percentage of active molecules was calculated from the complex-forming enzyme- and inhibitor-molecules, respectively, in relation to the total concentration of the corresponding molecules in the assay.

Preparation of PMS-chymotrypsin

PMS-chymotrypsin was prepared according to the method of Ako et al.[36]. In detail, 500 mg chymotrypsin in 50 ml 0.1 mol/l Tris buffer, pH 8.0, containing 0.1 mol/l NaCl, 0.12 mol/l $CaCl_2$ and 13% methanol were titrated with 0.04 mol/l PMSF (dissolved in acetone) by adding 0.1-ml aliquots at five-minute intervals until no enzymatic activity could be determined in the photometrical assay. The PMS-chymotrypsin preparation was dialysed overnight against 1 mol/l potassium phosphate pH 7.0 and in order to test the binding of PMN-I to PMS-chymotrypsin, the latter was first immobilized on Eupergit C[37].

Immunological analysis in agarose gels

For the preparation of antisera rabbits were immunized with α_1-PI and PMN-I according to Mossmann et al.[38] with an amount of 750 µg antigen per animal. In the latter case PMN-I was first bound to chymotrypsin, which was immobilized on Eupergit C[37], assuming that the complex would be of higher antigenicity than the isolated inhibitor. Both antisera were tested for their reaction with α_1-PI and PMN-I according to Ouchterlony[39].

Results and Discussion

Comparison of the inhibitory activity of PMN and lymphocytes-monocytes

The leukocytes, separated by discontinuous Percoll gradients, were homogenized and tested for their inhibitory activity in the leukocyte elastase assay. In contrast to the PMN-fraction only a low specific inhibitory activity was found in the lymphocyte-monocyte fraction. The latter fraction was contaminated by 13% PMN. Therefore, it could be calculated that the inhibitory activity of the lymphocyte-monocyte fraction was due to the residual PMN (Table 1). This result gives evidence for the bovine inhibitor of neutral proteinases being a PMN-specific protein.

Table 1. Comparison of the specific inhibitory activity of PMN and lymphocytes-monocytes.

Sample	Content of PMN [%]	Content of lymphocytes and monocytes [%]	Specific inhibitory activity[a] [IU/mg protein]
PMN cell fraction	98	2	0.042
Lymphocyte-monocyte cell fraction	13	80[b]	0.0053

[a] Inhibition of leukocyte elastase.
[b] The remaining 7% were undifferentiated cells.

Purification of PMN-I

The following purification procedure was established:

(i) ammonium sulphate precipitation,
(ii) anion exchange chromatography (Fig. 1) and,
(iii) gel permeation chromatography (Fig. 2).

The result of a typical purification procedure is summarized in Table 2 showing a 60-fold concentration of the PMN-I and a yield of 2.3%. In gel permeation chromatography two

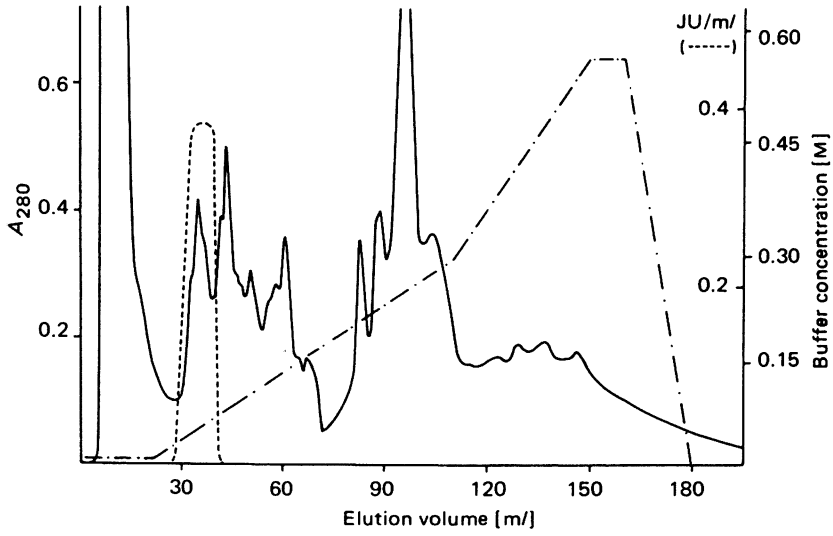

Fig. 1. Anion exchange chromatography subsequent to ammonium sulphate precipitation on a FPLC Mono Q HR 10/10 column (Pharmacia).

Trisphosphate was used as buffer in a concentration range from 0.02 mol/l to 0.56 mol/l (–·–·–, right ordinate). Protein concentration is given as absorption at 280 nm (——, left ordinate). The PMN-I enriched fraction is marked by dashes (– – –).

Table 2. Purification of the bovine PMN-inhibitor from leukocytes.
The data represent one preparation as an example of the purification procedure.

Step	Total protein [mg]	Total inhibitory activity[a] [IU]	Specific inhibitory activity[a] [IU/mg protein]	Purification factor	Purification yield [%]
1. Leukocyte homogenate	1390	44.0	0.0317	1	100
2. Leukocyte cytosol	580	44.7	0.077	2.4	101.6
3. Ammonium sulphate precipitation	104	33.8	0.325	10.2	76.8
4. Anion exchange chromatography	3.13	4.4	1.4	44.2	10.0
5. Gelpermeation chromatography					
Fraction a:	0.32	0.16	0.5	–	–
Fraction b:	0.56	1.0	1.9	60.0	2.3

[a] Inhibition of leukocyte elastase.

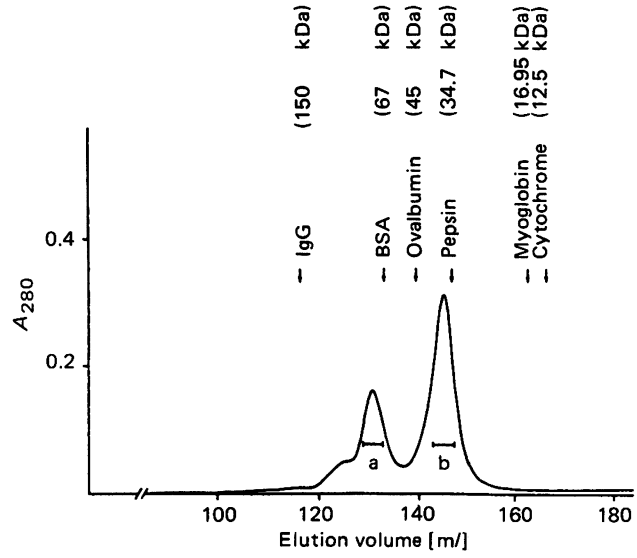

Fig. 2. Gel permeation chromatography of the PMN-I enriched fraction from FPLC on a calibrated HPLC Ultro Pac TSK G 2000 column (LKB).

0.1 mol/l phosphate, 0.1 mol/l NaCl, pH 6.8, was used as buffer. Protein concentration is given as absorption at 280 nm. The inhibitory active fractions are indicated by a and b.

Table 3. Effect of the reduction by mercaptoethanol on the specific inhibitory activity of the PMN-I fractions a and b.

Sample	Specific inhibitory activity [IU/mg protein][a]	
	prior to reduction	subsequent to reduction
PMN-I, fraction a	0.5	1.3
PMN-I, fraction b	1.95	2.1

[a] Protein concentration was determined by Ninhydrin reaction.

peaks – (a) and (b) – with a molecular mass of 73 kDa and 36.5 kDa, respectively, were obtained which differed in their specific inhibitory activity (Table 3). Whereas PMN-I fraction (a) appears to contain a minor contamination, the single band obtained with fraction (b) indicate the purity of this preparation (Fig. 3).

The PMN-I fractions were stored frozen at – 80 °C. At this temperature the PMN-I is stable for 5 to 6 months.

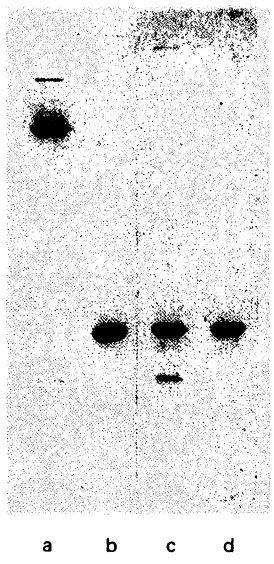

Fig. 3. SDS-PAGE of the PMN-I monomer and dimer under nonreducing conditions in 10% acrylamide.

The samples were preincubated with sample buffer for 5 min at 100 °C. a) PMN-I, fraction a; b) PMN-I, fraction b; c) PMN-I fraction a, preincubated with mercaptoethanol; d) PMN-I fraction b, preincubated with mercaptoethanol.

Chemical and physical characteristics

The molecular mass of the monomeric PMN-I could be determined as 36.5 kDa using a calibrated HPLC column (Fig. 2) and 40 kDa under nonreducing conditions in the SDS-PAGE (not shown).

The granulocyte-derived inhibitor mentioned by Marossy et al.[13,40] has a molecular mass of 50 kDa and is therefore not identical with the PMN-I described in the paper.

On IEF, PMN-I fraction (b) showed five protein bands with isoelectric points of 5.0, 5.5, 5.7, 5.9 and 6.3, whereas fraction (a) displayed only the band of pI 5.0 (Fig. 4, A). The bands of pI 5.0, 5.5, 5.7 and 5.9 formed complexes with ^{125}I-labeled leukocyte elastase (Fig. 4, B), whereas the band of pI 6.3 did not bind the radiolabeled enzyme.

As shown in Table 3, treatment of PMN-I fraction (a) with mercaptoethanol followed by alkylation increased the specific inhibitory activity (IU/mg protein) from 25% to 62% of that of fraction (b). The activity of the latter was only marginally increased. When, however, the sam-

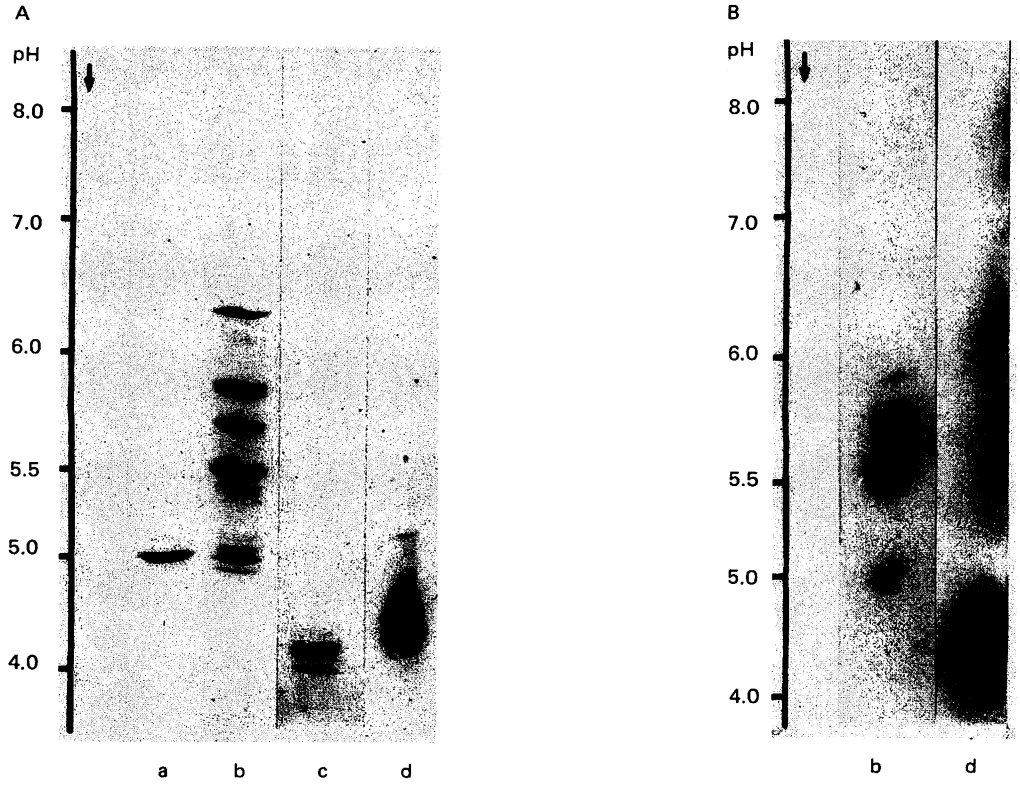

Fig. 4. IEF of the PMN-I with subsequent Western blot in 5% acrylamide with a pH range of 3.5 to 9.5.

A) Protein staining with Coomassie Brillant Blue R250. B) Western blot using ^{125}I-labeled leukocyte elastase. a) PMN-I, fraction a; b) PMN-I, fraction b; c) Bovine α_1-PI; d) Human α_1-PI.

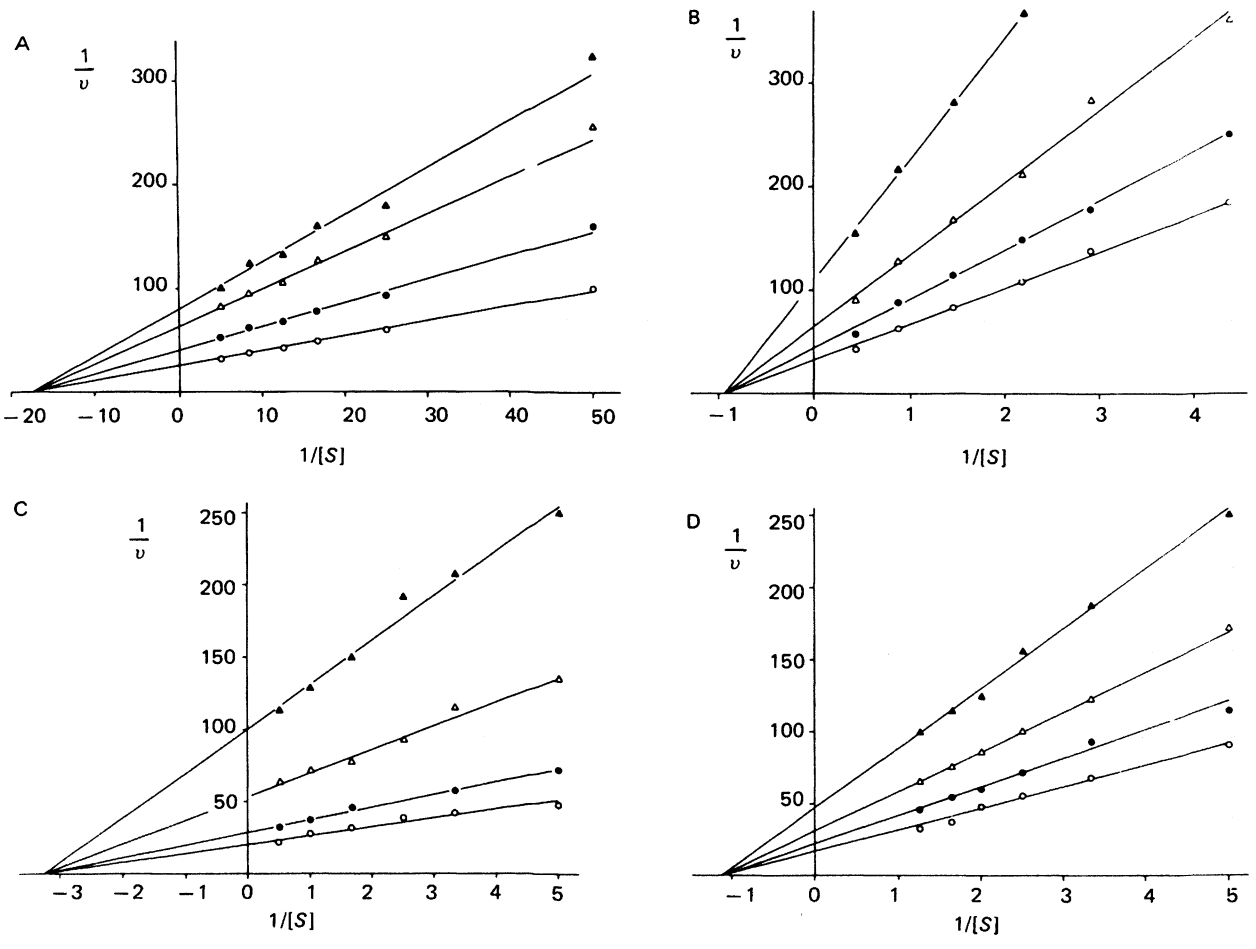

Fig. 5. Lineweaver-Burk plots of the inhibition of serine proteinases by PMN-I.

Reciprocal plots of the velocity v of the enzyme reaction versus the concentration of the substrate S [mmol/l] in the presence of different fixed concentrations of PMN-I. A) 1.2 nmol/l chymotrypsin, incubated with 1.97 nmol/l PMN-I (▲), 1.3 nmol/l PMN-I (△), 0.66 nmol/l PMN-I (●), respectively, without PMN-I (○). B) 14.2 nmol/l trypsin, incubated with 8.4 nmol/l PMN-I (▲), 6.3 nmol/l PMN-I (△), 4.2 nmol/l PMN-I (●), respectively, without PMN-I (○). C) 3.9 nmol/l leukocyte elastase, incubated with 3.15 nmol/l PMN-I (▲), 2.1 nmol/l PMN-I (△), 1.0 nmol/l PMN-I (●), respectively, without PMN-I (○). D) 7.8 nmol/l pancreatic elastase, incubated with 13.1 nmol/l PMN-I (▲), 9.2 nmol/l PMN-I (△), 5.3 nmol/l PMN-I (●), respectively, without PMN-I (○).

ples were applied to SDS-PAGE the reduced fraction (a) migrated at the same rate as fraction (b) which in turn did not differ from the non-reduced fractions (Fig. 3).

The behaviour of PMN-I fraction (a) on reduction by mercaptoethanol and its molecular mass suggest that this material is a dimer of PMN-I contained in fraction (b). In the following studies, only the properties of PMN-I monomer (fraction b) were further investigated and the name PMN-I was used for this preparation.

Inhibitory properties

A prerequisite for the calculation of the kinetic data is the determination of the proportion of active molecules of the enzyme or the inhibitor solutions. Therefore, active-site titrations were performed. The percentages of catalytically active enzyme molecules were in a range of 40% to 70% for the tested serine proteinases, whereas in the two inhibitor solutions – α_1-PI and PMN-I – 10% of the molecules were active.

The reciprocal plotting of the kinetic data apparently demonstrate that the interaction of PMN-I with serine proteinases is a non-competitive one (Fig. 5). But, if the inhibitor is tightly bound to the enzyme a competitive inhibition mechanisms is virtually indistinguishable from a non-competitive mechanism under these circumstances as shown by Henderson[27]. Thus a clear definition on the inhibition mechanism cannot be given. However, the fact that PMN-I does not bind to PMS-chymotrypsin may indicate that the reactive OH-group of the

serine residue in the active site of the enzyme is involved in the reaction mechanism of inhibitor and serine proteinase (Table 4) as postulated by Valentine et al.[14].

Table 4. Binding assay of PMN-I to chymotrypsin and PMS-chymotrypsin, both coupled to Eupergit C.

PMN-I sample	Specific inhibitory activity [IU/mg protein][a]	
	PMS-chymotrypsin	Chymotrypsin
Original material	0.29	0.2
Supernatant of the binding assay	0.3	0.06[b]

[a] The specific inhibitory activity was tested with human leukocyte elastase.
[b] The measurement is below the linear correlation.

Additionally, it should be mentioned that the PMN-I binds very tightly to immobilized chymotrypsin and only treatment with high urea concentration (6 mol/l) could dissociate the enzyme-inhibitor complexes resulting in inactive inhibitor molecules (data not shown). Therefore, according to the definition given by Bieth[28] the association of chymotrypsin and PMN-I should be characterized to irreversible.

This is comparable to the inhibitory properties of α_1-PI, the most important inhibitor of serine proteinases in plasma[5]. α_1-PI reacts with proteinases in a noncompetitive or irreversible way resulting in a hydrolytic cleavage of the inhibitor molecule and a strong complex forming of the main part of the inhibitor with the enzyme[41].

The most significant constant for the classification of inhibitors is the association rate constant

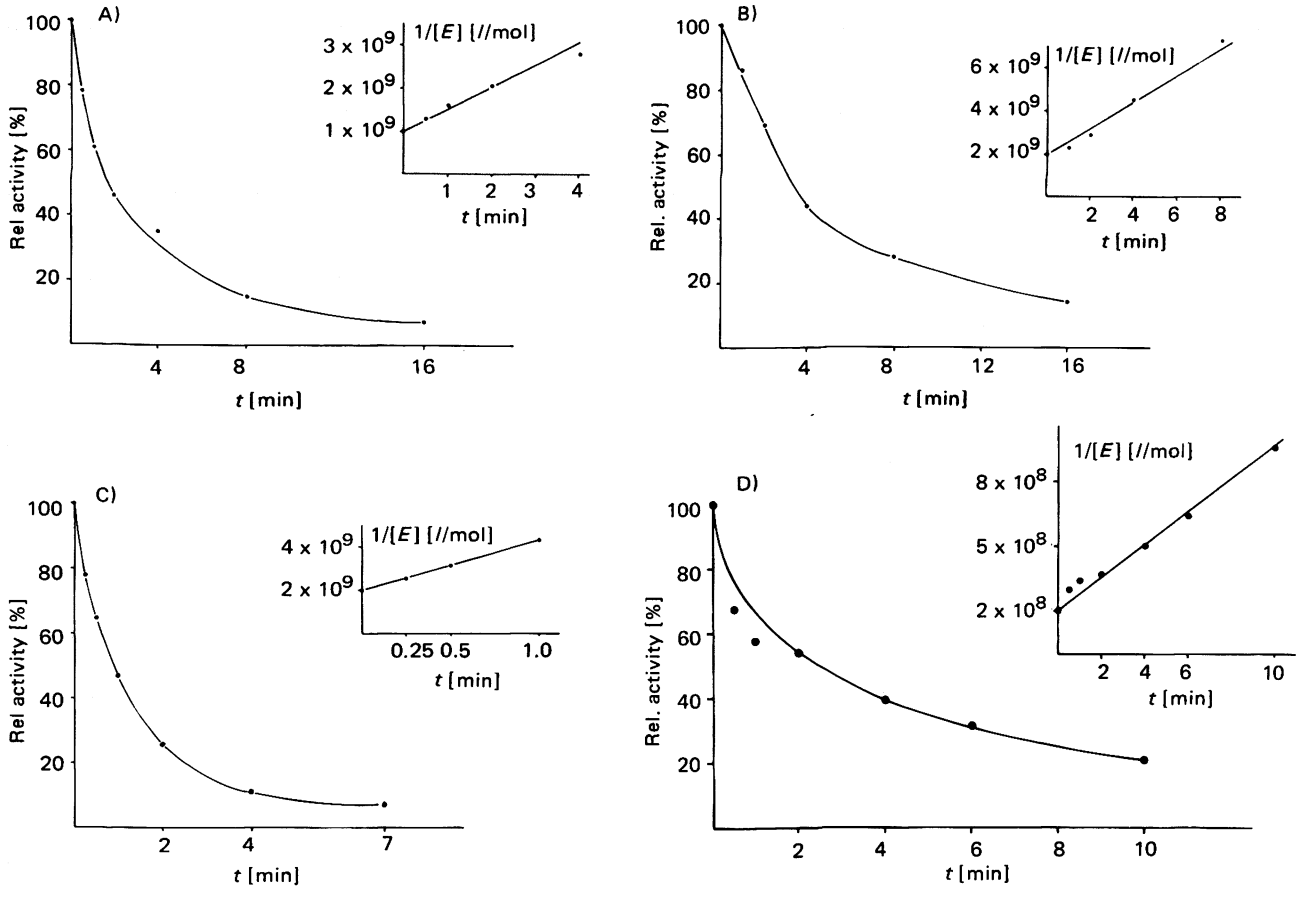

Fig. 6. Determination of the association rate constants k_{on} of the inhibition of serine proteinases by PMN-I.
The activity of the enzyme subsequent to complex forming with PMN-I (in percent) versus the incubation time [min] of the complex before starting the assay. *Inserts:* Reciprocal plots of the concentration of resting free enzyme subsequent to complex forming versus incubation time. A) 1 nmol/l chymotrypsin, incubated with 1 nmol/l PMN-I and the substrate combination of 0.1 mmol/l $Suc(Ala)_2ProPheSBzl$ and 0.24 mmol/l Ellmanns reagent. B) 0.5 nmol/l trypsin, incubated with 0.5 nmol/l PMN-I and the substrate combination of 0.5 mmol/l ZLysSBzl and 0.24 mmol/l Ellmanns reagent. C) 0.5 nmol/l leukocyte elastase, incubated with 0.5 nmol/l PMN-I and 0.5 mmol/l $MeOSuc(Ala)_2$-ProValAMC. D) 5 nmol/l pancreatic elastase, incubated with 5 nmol/l PMN-I and 0.5 mmol/l $MeOSuc(Ala)_2ProVal$-AMC.

Table 5. Association rate constants of the inhibition of serine proteinases by PMN-I.

Enzyme	Origin of the enzyme	Substrate	k_{on} [l/(mol × s)][a]
Chymotrypsin	Bovine pancreas	$Suc(Ala)_2ProPheSBzl$	8.7×10^6
Elastase	Human PMN	$MeOSuc(Ala)_2ProValAMC$	3.8×10^7
Elastase	Porcine pancreas	$MeOSuc(Ala)_2ProValAMC$	1.3×10^6
Trypsin	Porcine pancreas	ZLysSBzl	1.0×10^7

[a] The calculations of k_{on} were based on a molecular mass of 38 kDa for PMN-I.

k_{on}[28]. For PMN-I relative high values were calculated (Fig. 6, Table 5) which indicates that PMN-I reacts very fast with serine proteinases. Other physiologically active inhibitors like α_1-PI show comparable values for k_{on}[42,43].

Immunological properties

In the immunodiffusion assay according to Ouchterlony, the rabbit antiserum raised to α_1-PI showed no cross reaction with PMN-I and vice versa (Fig. 7). This immunological discrepancy indicates the different origin of both inhibitors.

The physiological relevance of PMN-I may be restricted to the protection of the cell from its own proteinases[12], but could also be relevant for the protection of tissue in infectious diseases because of the inadequate concentration of serum inhibitors[1]. Probably, they are also involved in the regulation of tumor growth[44]. However, further investigations have to clarify the precise role of PMN-I in physiological and pathological events as discussed in the accompanying paper.

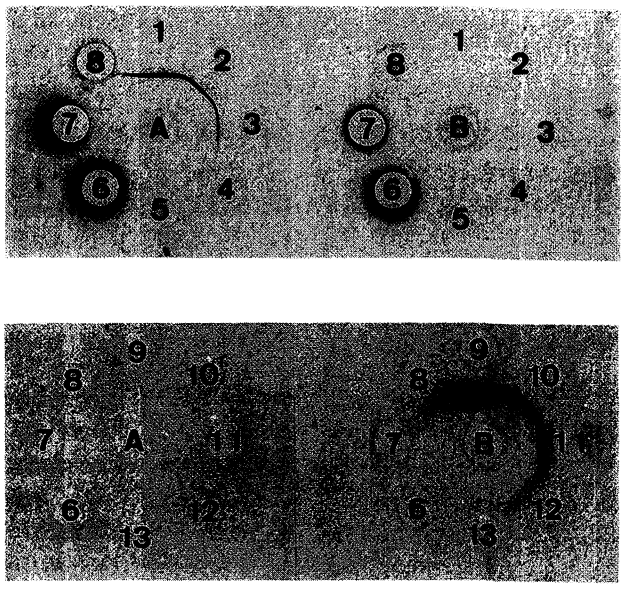

Fig. 7. Immunological specificity of α_1-PI and PMN-I in agarose gels.

A) α_1-PI; B) PMN-I. 1–5) Anti-α_1-PI antiserum in the concentration range from concentrated (No. 1) to 1:16 diluted (No. 5) antiserum. 6–8) Normal serum of rabbit in the concentration range from concentrated (No. 6) to 1:4 diluted (No. 8) serum. 9–13) Anti-PMN-I antiserum in the concentration range from concentrated (No. 9) to 1:16 diluted (No. 13) antiserum.

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Petra Hintz and Dr. Horst Mossmann*, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg.

Dr. Marianne Jochum, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nußbaumstraße 20, D-8000 München 2.

* To whom correspondence should be addressed.