Biochimica et Biophysica Acta, 445 (1976) 683—693

BBA 67913

NEUTRAL ELASTOLYTIC PROTEINASE FROM CANINE LEUCOCYTES

PURIFICATION AND CHARACTERISATION

WOJCIECH ARDELT, ZDZISŁAWA TOMCZAK, STEFAN KSIEŻNY and GRAŻYNA DUDEK-WOJCIECHOWSKA *

*Department of Biochemistry, Institute of Rheumatology, 02-637 Warsaw, and * Department of Radiobiology and Health Protection, Institute of Nuclear Research, 03-195 Warsaw (Poland)*

(Received March 26th, 1976)

Summary

1. A neutral proteinase (EC 3.4.—.—) with elastolytic activity was isolated from canine bloodstream leucocytes, and purified to apparent homogeneity by a two-step procedure consisting of DEAE-Sephadex chromatography and molecular sieving on Sephadex G-75.

2. The molecular weight of the enzyme was 23 500, and the absorbance ($A^{1\%}_{1cm}$) at 282 nm was 6.1. Amino acid analysis showed high content of glycine, aspartic acid, and valine, and low proportion of methionine, lysine and histidine as well as the absence of tyrosine in the enzyme molecule.

3. The proteinase was active against several protein substrates as well as towards *N*-*t*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester, and (to a lesser extent) against *N*-acetyl-L-tyrosine ethyl ester.

4. The enzyme was inactivated by diisopropylfluorophosphate, *N*-acetyl-L-alanyl-L-alanyl-L-alanine chloromethyl ketone, and *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone. Inhibition by some natural proteinase inhibitors was also noted.

Introduction

Human granulocyte elastase, the enzyme currently thought to be involved in a variety of physiological and pathological processes in connective tissue

Abbreviations: Boc-Ala-ONp, *N*-*t*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester; Ac-Ala_3-OMe, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; iPr_2P-F, diisopropylfluorophosphate; Ac-Ala_3CH_2Cl, *N*-acetyl-L-alanyl-L-alanyl-L-alanine chloromethyl ketone; Tos-$PheCH_2Cl$, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulphate; Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; Tos-$LysCH_2Cl$, *N*-p-tosyl-L-lysine chloromethyl ketone.

[1—3] was recently purified and well characterised [4—9].

This paper presents the purification and characterisation of a canine leucocytic proteinase (EC 3.4.—.—) that is probably analogous [10] to the human enzyme.

Materials and Methods

Elastin was obtained from ox nuchal ligament by the method of Partridge et al. [11]. Porcine pancreatic elastases I and II were prepared from a commercial pancreas powder (Polfa, Warsaw), according to the method described previously [12].

Isolation of leucocytes. Dogs weighing 10—15 kg were anaesthetised with Thiopental (sodium-5-ethyl-5-methybuthyl thiobarbiturate, Spofa) 30—50 mg/kg, and blood was drawn from cannulated femoral veins. Citrate was used as anticoagulant. To each 100-ml portion of the blood, 20 ml of 6% Dextran T 500 (Pharmacia) in saline were added, and leucocytes were collected by sedimentation. Contaminating erythrocytes were lysed by a hypotonic shock in 0.2% sodium chloride [13].

Crude enzyme preparation. The disruption of leucocytes was performed in 0.2 M sucrose containing 150 units of heparin (Polfa, Warsaw) per ml [13,14]. Cell debris and intact cells were centrifuged off at 180 × *g* for 30 min at 12° C. The fraction containing mainly granules and nuclei, was then sedimented at 22 000 × *g* for 60 min at 12° C. It was suspended in saline and homogenized by freezing and thawing (5 times). The homogenate was dehydrated with acetone (—15° C) and air-dried. At this stage the crude enzyme preparation could be kept dessicated at 4° C for several months without loss of activity. This was saved until 1.2 g was accumulated and then submitted to the purification procedure as described under Results.

Homogeneity and molecular weight. Homogeneity of the final preparation was checked by polyacrylamide gel electrophoresis without [15] as well as in the presence [16] of SDS (BDH). The latter method was also employed for molecular weight determinations. Prior to electrophoresis the enzyme samples were converted into diisopropoxy derivatives [12] and reduced with 2-mercaptoethanol in the presence of 1% SDS (2 h, 37° C). Cytochrome *C*, (Biomed, Kraków), α-chymotrypsinogen A (Sigma), egg albumin and serum albumin (Calbiochem) were used as standards assuming molecular weights of 12 600, 25 000, 45 000 and 69 000, respectively.

Amino acid analysis. Samples of the proteinase were hydrolysed in 6 M HCl at 110° C for 24 h in sealed, evacuated hydrolysis tubes. Analyses were carried out using a Beckman model Unichrom automatic amino acid analyser. Half-cystine was determined as cysteic acid [17]. Tryptophan content was determined spectrophotometrically [18].

Enzymatic assays. Activity measurements towards the protein substrates tested, were carried out (unless otherwise stated) at 37° C in 0.05 M Tris · HCl buffer (pH 8.6) during 30 min.

Elastolytic activity was determined by the direct spectrophotometric method [19] as modified previously [12], or colorimetrically, using resorcin fuchsin-elastin as a substrate. Staining of elastin was performed as follows: 10 g of elas-

tin were suspended in 100 ml of the Weigert stain [20] and left for 24 h with occasional shaking. Stained elastin was then successively washed in a Buchner funnel with ethanol, 0.05 M Tris · HCl buffer, pH 9.0, and water, to colourless filtrates. It was then dehydrated with acetone and air-dried. A standard curve was prepared using the substrate solubilized completely by pancreatic elastase I. Specific activity towards each elastin substrate was expressed as units [12] per mg of the enzyme.

Activity towards casein (Merck) was determined and expressed as described previously [21].

Activity against gelatin was determined according to the method of Sopata and Dancewicz [22]. One unit of the activity is defined as the amount of enzyme that releases 1 μg of hydroxyproline soluble in 15% trichloroacetic acid during 1 min.

Determination of the activity towards Azocoll (Calbiochem) was performed with continuous shaking using 5 mg of the substrate and 10—20 μg of the enzyme per ml. Absorbance at 520 nm was recorded. One unit of the activity is defined as the amount of enzyme that solubilizes 2 mg of the substrate in 1 min.

Digestion of calf thymus lysine-rich histone (POCH, Gliwice) was followed by the determination (Lowry method [37]) of the degradation products soluble in 10% trichloroacetic acid split off from the substrate (5 mg/ml) by the protease (20—40 μg/ml) action.

Degradation of bovine nasal cartilage proteoglycans (prepared according to the method described in ref. 23, kindly provided by Dr. J. Kaczanowska of this laboratory) was performed at 25°C for 10 min with 4 mg of the substrate and 1 μg of the enzyme per ml. Diisopropylfluorophosphate (*i*Pr_2*P*-F, Fluka) was added (up to 2 mM) in order to stop the reaction, and viscosity of the incubation mixture was measured in an Ostwald-type viscometer. The control sample was treated with *i*Pr_2*P*-F at zero time.

Esterase activity towards Boc-Ala-ONp (Serva) was determined at 30°C according to the method of Visser and Blout [24] using 0.2 mM substrate, unless otherwise stated. One unit of the activity is defined as the amount of enzyme that releases 1 μmol of p-nitrophenol during 1 min under the conditions employed. Specific activity was expressed as units per A_{280nm} unit.

Activity against Ac-Ala_3-OMe (Sigma) was determined at 30°C according to the method of Bieth and Mayer [25] except that phosphate buffer of pH 6.5 was used.

Activities towards Ac-Tyr-OEt (Sigma) and benzoyl-L-arginine ethyl ester (Bz-Arg-OEt, Koch-Light) were determined at 30°C according to the methods of Schwert and Takeneka [26] and Rich [27], respectively.

One unit of Ac-Tyr-OEt activity is defined as the amount of enzyme that hydrolyses 1 μmol of the substrate in 1 min.

Inhibition studies. Samples of the protease (final concentration 10^{-5} M) were preincubated (0.05 M Tris · HCl, pH 7.5, room temperature, 20 min) with various natural proteolytic inhibitors, and residual Boc-Ala-ONp activity was determined. The following inhibitors were used: pancreatic trypsin inhibitor (Kunitz-type), and soybean trypsin inhibitor (Worthington); basic pulmonary trypsin inhibitor, and pancreatic trypsin inhibitor (Kazal-type, POCH-Gliwice); ovo-

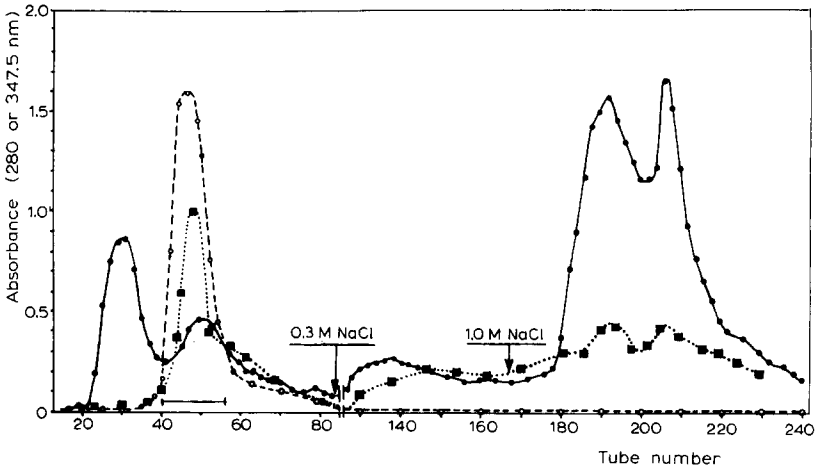

Fig. 1. Elution pattern of the leucocyte proteinase from DEAE-Sephadex A-50. The solution of the crude preparation (62 ml, total absorbance at 280 nm, 1153, total Boc-Ala-ONp-activity, 8.1 units) was applied to the column (4 × 40 cm) with the exchanger (Pharmacia), equilibrated and eluted (room temperature) with 0.05 M sodium carbonate buffer (pH 8.7) made in 3 M urea. Fractions of 5 ml were collected at the downward flow rate of 75 ml/h. The strongly adsorbed material was recovered by a step-by-step elution with 0.3 and 1.0 M NaCl in the buffer. •———•, protein (A_{280nm}); ○- - - - -○, Boc-Ala-ONp activity ($A_{347.5nm}$ units min per 0.1 ml); ■. . . . .■, activity towards casein (A_{280nm} units 30 min per 0.1 ml).

mucoid and lima bean trypsin inhibitor (Koch-Light); egg-white trypsin inhibitor (Calbiochem); garden bean isoinhibitors * SP-I and SP-IIB (kindly provided by Dr. M. Laskowski, Sr., Roswell Park Mem. Inst., Buffalo, N.Y., U.S.A.); and normal human serum.

Inactivation studies. Samples of the enzyme (final concentration 10^{-5} M) were preincubated (pH 7.5, room temperature, 1 h) with iPr_2P-F, Tos-Phe-CH_2Cl (Sigma), *N-p*-tosyl-L-lysine chloromethyl ketone (Tos-Lys CH_2Cl, Sigma) or with Ac-Ala_3 CH_2Cl (synthesised by Dr. K. Bańkowski, Inst. of Fundamental Problems of Chemistry, Warsaw, Univ.), and the residual Boc-Ala-ONp-activity was determined.

Results

Purification of the enzyme

The crude enzyme preparation (1.2 g) was solubilized (1 h, room temperature) in 0.05 M sodium carbonate buffer of pH 8.7 made in 3 M urea, containing 0.5% Tween 80. The solution was clarified by centrifugation and subjected to DEAE-Sephadex chromatography under the conditions employed previously [12] for the purification of porcine pancreatic elastases. The elution profile is presented in Fig. 1.

All Boc-Ala-ONp activity as well as the highest activity towards casein were recovered in the second peak. The fractions of this peak were pooled and dialysed overnight against 0.005 M sodium acetate buffer (pH 5.0) containing 0.1

* Isoinhibitors: SP-I and SP-IIB represented rather crude preparations corresponding to the isoinhibitors II and IIIa + IIIb, respectively, from the original paper [28].

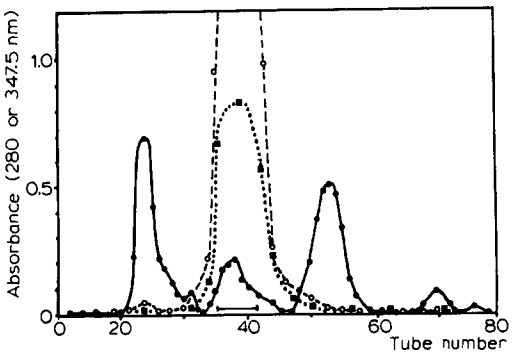

Fig. 2. Sephadex G-75-chromatography of the leucocyte proteinase recovered from DEAE-Sephadex A-50. The material from the previous step (6.7 ml, total absorbance at 280 nm, 24.1, Boc-Ala-ONp-activity 45 units) was applied to the column (2 × 75 cm) with Sephadex G-75, medium (Pharmacia) in 0.05 M sodium acetate buffer (pH 5.0) containing 1.0 M NaCl. The column was eluted (room temperature) with the buffer; 3-ml fractions were collected at the downward flow rate of 18 ml/h. Symbols as in Fig. 1.

M NaCl. Then the enzyme solution was concentrated ten times in a rotatory evaporator, and introduced on to the Sephadex G-75 column. The elution pattern is presented in Fig. 2. The proteolytic and esterase activity was recovered in the second of three peaks obtained. The enzyme was isolated, dialysed exhaustively against 0.02 M ammonium acetate buffer, pH 6.0, concentrated ten times and stored at −20° C. A summary of the purification procedure is given in Table I.

Molecular properties

The preparation obtained appeared homogenous in disc gel electrophoresis without as well as in the presence of SDS (Fig. 3, a and b).

The ultraviolet spectrum (not shown) revealed typical features of tryptophan with a minor contribution of phenylalanine. The main peaks observed were at 282 and 289 nm.

The absorbance ($A^{1\%}_{1cm}$) at 282 nm in 0.02 M sodium acetate buffer (pH 5.0) of 6.1 was also estimated. This was used in further experiments for the determination of the enzyme concentration.

The molecular weight of the leucocyte proteinase was determined by SDS polyacrylamide gel electrophoresis. The value of 23 500 ± 1200 was evaluated as the average from six separate runs.

The results of amino acid analysis are presented in Table II. The enzyme ap-

TABLE I

PURIFICATION OF THE CANINE LEUCOCYTE PROTEINASE

Steps	Total A_{280nm}	Total Boc-Ala-ONp-activity (units)	Specific activity (A_{280nm} units)
Crude enzyme preparation	1153	8.1	0.007
DEAE-Sephadex A-50	24.1	45.1	1.87
Sephadex G-75	3.9	41.0	10.5

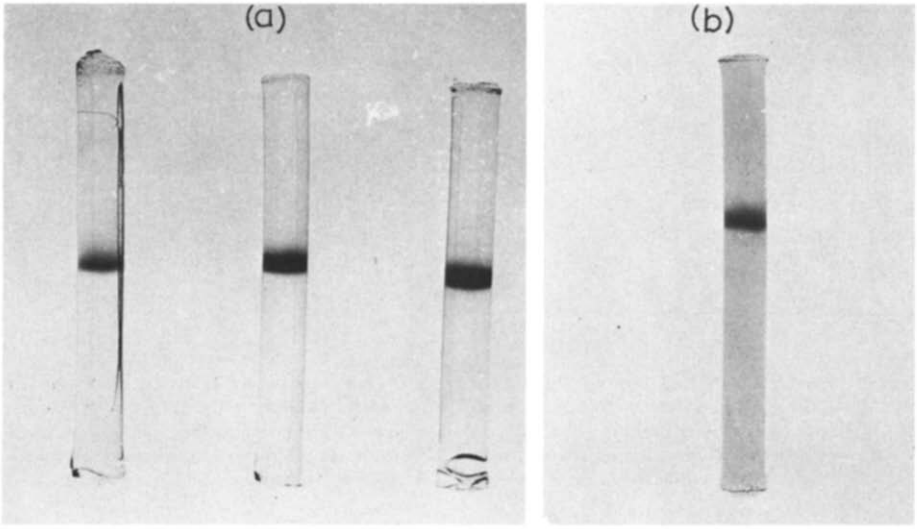

Fig. 3. Polyacrylamide disc gel electrophoresis of the leucocyte proteinase. (a) pH 4.3 [15], migration from top (anode) to bottom (cathode); 19, 39, and 78 μg of the enzyme, respectively, from left to right. Amido Black stain. (b) pH 7.0, 0.1% sodium dodecyl sulphate [16]. Migration from top (cathode) to bottom (anode), 30 μg of the enzyme. Stained with Coomassie Brilliant Blue.

TABLE II

AMINO ACID COMPOSITION OF THE CANINE LEUCOCYTIC PROTEINASE

Amino acid residue	No. of residues per 23 500 daltons [a]	Nearest integer per 23 500 daltons
Lysine	1.56	2
Histidine	3.91	4
Arginine	15.64	16
Aspartic acid [b]	24.04	24
Threonine	8.79	9
Serine	12.12	12
Glutamic acid [b]	18.76	19
Proline	14.85	15
Glycine	24.62	25
Alanine	17.59	18
Cysteine [c]	5.86	6
Valine [d]	22.47	22
Methionine	2.34	2
Isoleucine [d]	8.79	9
Leucine	17.59	18
Tyrosine	0.00	0
Phenylalanine	12.51	13
Tryptophan [e]	4.69	5
Total		219

[a] 24-h hydrolysis.
[b] Free plus amide.
[c] As cysteic acid.
[d] Uncorrected.
[e] Determined spectrophotometrically.

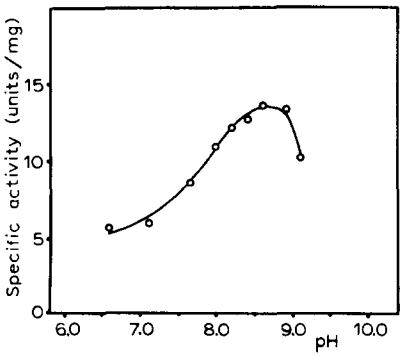

Fig. 4. Effect of pH on the enzyme activity. The buffers used were: 0.05 M Tris/acetate (pH 6.6, 7.1) and 0.05 M Tris/Cl (pH 7.6—9.1). The incubation mixture contained 50 mg of resorcin fuchsin-elastin and 39 μg of the leucocyte proteinase in a final volume of 5 ml.

parently does not contain tyrosine but is rich in phenylalanine. It is in accordance with the observed spectral features. High content of glycine, aspartic acid and valine as well as low content of methionine, lysine and histidine were also recorded.

Catalytic properties

The pH-dependence of the elastolytic activity of the leucocytic proteinase is presented in Fig. 4. No sharp profile was observed. The maximal activity was found at pH 8.5—8.7, but a considerable activity was retained below pH 7.0. Ionic strength of the buffers used in this experiment varied from 0.055 to 0.099. However, no effect of ionic strength on the enzyme activity was observed within that range.

Table III gives the results of the proteinase action on various proteins. The enzyme exhibited considerably lower activity towards each substrate tested, as compared to the activity of porcine pancreatic elastases. Unfortunately, the results for various proteins (and one enzyme) are not directly comparable, due to differences in assay systems and, therefore, in arbitrary units assumed. However, the comparison of the caseinolytic activity of the three enzymes tested,

TABLE III

ACTIVITY OF THE CANINE LEUCOCYTIC PROTEINASE TOWARDS PROTEIN SUBSTRATES

Substrate	Specific activity (units/mg)		
	Leucotycic protease	Pancreatic elastases	
		I	II
Unstained elastin	14.5	171 *	312 *
Resorcin fuchsin-elastin	13.8	110	n.d.
Casein	0.35	1.1 *	4.3 *
Gelatin	40.5	n.d.	n.d.
Azocoll	2.9	97	34

* According to the previous work [12].
n.d. not determined.

TABLE IV

HYDROLYSIS OF *N*-tert-BUTYLOXYCARBONYL-L-ALANINE-*p*-NITROPHENYL ESTER AND *N*-ACETYL-L-ALANYL-L-ALANYL-L-ALANINE METHYL ESTER BY THE LEUCOCYTE PROTEINASE

The Michaelis constant was evaluated from the Lineweaver-Burk plot. K_{cat} was calculated from the maximal velocity and the initial enzyme concentration.

Substrate	K_{cat}/K_m (app) ($M^{-1} \cdot s^{-1}$)	K_{cat} (s^{-1})	K_m (app) (mM)	[S] (Range of substrate concns., mM)
Boc-Ala-ONp	46 050	3.5	0.076	0.02—0.4
Ac-Ala$_3$-OMe	6 150	20.5	3.33	1.8 —6.0

revealed that casein is a relatively good substrate for the leucocytic proteinase. The enzyme was also active against proteoglycans from nasal cartilage and towards lysine-rich histone. However, numerical values are not given because of nonlinearity of the time curves as well as enzyme curves.

The apparent K_m and k_{cat} values for esterase activity of the enzyme are presented in Table IV. Boc-Ala-ONp appeared to be a better substrate than Ac-Ala$_3$-OMe, as judged from the 7.5 times higher k_{cat}/K_m(app) value for the hy-

TABLE V

THE EFFECT OF SOME NATURAL PROTEOLYTIC INHIBITORS AND SYNTHETIC INACTIVATORS ON THE ACTIVITY OF THE LEUCOCYTE PROTEINASE

Compound tested	Concentration	Concn. units	Residual Boc-Ala-ONp activity (%)
		%	
Normal human serum	5		55
	10		20
		weight ratio, inhibitor: enzyme	
Pancreatic trypsin inhibitor/ Kunitz-type/	5		100
Pancreatic trypsin inhibitor/ Kazal-type/	5		100
Basic pulmonary trypsin inhibitor	5		100
Ovomucoid	2		100
Egg white trypsin inhibitor	2		80
Soybean trypsin inhibitor	2		17
Lima bean trypsin inhibitor	2		50
Garden bean trypsin inhibitor SP-I	3		10
Garden bean trypsin inhibitor SP-IIB	3		5
		molar ratio inactivator: enzyme	
Ac-Ala$_3$CH$_2$Cl	25		18
Tos-LysCH$_2$Cl	25		100
Tos-PheCH$_2$Cl	20		48
*i*Pr$_2$*P*-F	100		0

drolysis of the former compound. The difference is first of all due to the nearly 45 times lower K_m for Boc-Ala-ONp; k_{cat} value was distinctly higher for the hydrolysis of Ac-Ala_3-OMe.

The leucocyte proteinase was inactive towards Bz-Arg-OEt, the specific trypsin substrate, but exhibited some chymotryptic activity against Ac-Tyr-OEt. The specific activity against this substrate was 12.7 units/mg as compared to 133 units/mg of the BDH α-chymotrypsin.

To characterise further the canine leucocytic proteinase, preliminary inhibition studies were performed using various proteinase inhibitors and inactivators. The results are gathered in Table V. Among the natural inhibitors used, the most effective appeared the isoinhibitors from garden bean, soybean trypsin inhibitor, and human serum. Ac-Ala_3CH_2Cl the specific active site reagent for elastase, as well as iPr_2P-F were powerful inactivators. Tos-PheCH_2Cl, the specific inactivator of chymotrypsin was less active while Tos-LysCH_2Cl had no effect on the enzyme activity.

Discussion

Preliminary experiments showed that the majority of Boc-Ala-ONp activity (that mimicked elastolytic activity: ref. 6) was present in the leucocyte granule fraction but a considerable amount of the activity was also associated with the fraction sedimented between 180 and 800 $\times g$, containing mainly leucocyte nuclei. It might have been due to a contamination of this fraction with granules. Therefore, in the procedure here described, the fraction sedimenting between 180 and 22 000 $\times g$ and containing nuclei and granules, was used as a starting material for the purification of the enzyme.

A great increase of total activity was observed in the first purification step making yield calculations impossible. The increase may be due to the elimination of an unknown inhibitor or inhibitors on DEAE-Sephadex. The enzyme activity might be blocked in the starting extract by the specific inhibitor(s) [29,30] and/or by nonspecific interactions, for instance with polyanions (heparin and/or nucleic acids).

The amino acid analysis of the purified enzyme preparation revealed a high proportion of arginine to lysine. The same was found for human granulocyte elastase [7,9] and porcine pancreatic elastase I [31], whereas in pancreatic elastase II these residues are present in almost equimolar proportion [32].

The absence of tyrosine is rather an unusual property that differentiates the canine leucocyte proteinase from human granulocyte elastase and from pancreatic elastases. It also offers an explanation for the relatively low absorbance value found in this paper. The cysteine content implies the presence of three disulphide bridges while four can be predicted for human granulocyte elastase [9], five for pancreatic elastase II [32], and four were found for pancreatic elastase I [31]. A large amount of ammonia appeared during the acid hydrolysis of the proteinase, indicating the presence of dicarboxylic amino acids in their amide form. This, together with rather high proportion of basic residues explains the high basicity of the enzyme molecule as indicated by its chromatographic behaviour.

Electrophoretic experiments suggest rather one polypeptide chain in the molecule having a molecular weight (23 500) of the same order as those of pancreatic serine proteases. Close to this result are the values established for human granulocyte elastase by Taylor and Crawford [33] and Baugh and Travis [34] of 22 000 and 26 000 respectively; other authors found higher values: 33 000—36 000 [7] and 34 400 [9].

The leucocyte proteinase can hydrolyse protein substrates at physiological pH values with rather broad specificity (Table III). Therefore, the enzyme may be involved in pathological processes associated with connective tissue damage, as was postulated for human granulocyte elastase [1—3]. Its potential substrates in vivo might be elastin, macromolecular degradation products of collagen by collagenase, soluble proteins and proteoglycans of connective tissue ground substance.

Like known elastases, the leucocytic proteinase exhibits esterase activity towards esters of N-substituted alanine, and peptides with esterified C-terminal alanine (Table IV).

Unexpectedly, a more effective binding of Boc-Ala-ONp (the shorter of the two substrates) than of Ac-Ala_3-OMe was noted as judged from the comparison of the K_m values. It may indicate a considerable contribution of hydrophobic interactions between largely hydrophobic Boc group and the binding site in the enzyme molecule. On the other hand, the catalytic rate constant is distinctly higher for hydrolysis of the longer substrate. Thus, the concept of the influence of S_4P_4 on S_1P_1 interactions [35] postulated for pancreatic elastase I may be also valid for the leucocyte enzyme.

Like human granulocyte elastase [9], the canine enzyme was found to have activity against a chymotrypsin substrate. However, its Ac-Tyr-OEt-activity was considerably lower than that of α-chymotrypsin as well as of pancreatic elastase II [12].

Inactivation of the leucocyte proteinase by iPr_2P-F as well as by Ac-Ala_3-CH_2Cl and Tos-PheCH_2Cl proved the presence in the active of the essential serine and histidine residues.

The lower efficiency of Tos-PheCH_2Cl, compared to that of Ac-Ala_3CH_2Cl and of iPr_2P-F is in accordance with rather weak chymotryptic activity of the enzyme.

Soybean trypsin inhibitor appeared to be also highly active. However, the question arises whether it is a property of pure Kunitz inhibitor or of accompanying elastase inhibitors found recently in commercial preparations of the inhibitor [36]. The interaction of the enzyme with inhibitors and inactivators needs further, kinetic studies.

The results here presented seem to characterise the canine leucocyte enzyme as a serine-histidine proteinase, probably analogous to human granulocyte elastase with respect to the enzymatic function, mechanism, and biological properties.

Acknowledgement

We wish to thank Mrs. T. Gołaszewska for technical assistance.

References

1 Janoff, A. (1972) Am. J. Pathol. 68, 572—592
2 Janoff, A. (1972) Annu. Rev. Med. 23, 177—190
3 Galdston, M., Janoff, A. and Davis, A.L. (1973) Am. Rev. Respir. Disord. 107, 718—723
4 Janoff, A. and Scherer, J. (1968) J. Exp. Med. 128, 1137—1155
5 Folds, J.D., Welsh, J.R. and Spitznagel, J.K. (1972) Proc. Soc. Biol. Med. 139, 461—463
6 Janoff, A. (1973) Lab. Invest. 29, 458—464
7 Ohlsson, K. and Olsson, I. (1974) Eur. J. Biochem. 42, 519—527
8 Taylor, J.C. (1975) Arch. Biochem. Biophys. 169, 91—101
9 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 16, 493—505
10 Neurath, H., Walsh, K.A. and Winter, W.P. (1967) Science 158, 1638—1644
11 Partridge, S.M., Davis, H.P. and Adair, G.S. (1955) Biochem. J. 61, 11—20
12 Ardelt, W. (1974) Biochim. Biophys. Acta 341, 318—326
13 Chodirker, W.B., Bock, G.N. and Vaughan, J.H. (1968) J. Lab. Clin. Med. 71, 9—19
14 Koj, A., Chudzik, J., Pajdak, W. and Dubin, A. (1972) Biochim. Biophys. Acta 268, 199—206
15 Reisfield, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281—285
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Schram, E., Moore, S. and Bigwood, E.J. (1954) Biochem. J. 57, 33—37
18 Edelhoch, H. (1967) Biochemistry 6, 1948—1954
19 Ardelt, W. Księżny, S. and Niedźwiecka-Namysłowska, I. (1970) Anal. Biochem. 34, 180—187
20 Roulet, F. (1948) Methoden der Pathologischen Histologie, p. 304, Springer-Verlag, Vienna
21 Ardelt, W. and Księżny, S. (1970) Acta Biochim. Polon. 17, 279—289
22 Sopata, I. and Dancewicz, A.M. (1974) Biochim. Biophys. Acta 370, 510—523
23 Sajdera, S.W. and Hascall, V.C. (1969) J. Biol. Chem. 244, 77—87
24 Visser, L. and Blout, E.R. (1972) Biochim. Biophys. Acta 268, 257—260
25 Bieth, J. and Mayer, J.F. (1973) Anal. Biochem. 51, 121—126
26 Schwert, G.W. and Takenaka, Y. (1955) Biochim. Biophys. Acta 16, 570—575
27 Rick, W. (1963) in Methods of Enzymatic Analysis (Burgmeyer, H.U., ed.), pp. 815—818, Academic Press, New York
28 Wilson, K.A. and Laskowski, Sr., M. (1973) J. Biol. Chem. 248, 756—762
29 Janoff, A. and Blondin, J. (1971) Proc. Soc. Exp. Biol. Med. 136, 1050—1053
30 Janoff, A. and Blondin, J. (1971) Lab. Invest. 25, 565—571
31 Shotton, D.M. and Hartley, B.S. (1970) Nature 225, 802—806
32 Ardelt, W. (1975) Biochim. Biophys. Acta 393, 267—273
33 Taylor, J.C. and Crawford, I.P. (1975) Fed. Proc. 34, 534
34 Baugh, R. and Travis, J. (1975) Fed. Proc. 34, 484
35 Thompson, R.C. and Blout, E.R. (1973) Biochemistry 12, 57—65
36 Bieth, J. and Frenchin, J-C. (1974) Biochim. Biophys. Acta 364, 97—102
37 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275