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STUDIES ON A HUMAN BLOOD PLATELET PROTEASE WITH ELASTOLYTIC ACTIVITY

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SUMMARY

The fractionation by ammonium sulfate precipitation and gel filtration on Sephadex G-150 of Triton X-100 extracts of human blood platelets is described, as well as the determination of the enzymatic (proteolytic and esterolytic) activities of the fractions obtained with ^{125}I-labeled elastin, hemoglobin and synthetic substrates $AcAla_3OMe$, BzArgOEt and AcTyrOEt.

Activity towards $AcAla_3OMe$ was significantly higher in all fractions than that towards AcTyrOEt and BzArgOEt.

The highest specific activity towards $AcAla_3OMe$ and ^{125}I-labeled elastin was obtained in the 2nd Sephadex peak of the 70% ammonium sulfate precipitate. The increase in specific activity towards $AcAla_3OMe$ was of the order of 1200, with respect to the Triton extract.

The 2nd Sephadex peak of the 70% ammonium sulfate precipitate gives three bands on acrylamide electrophoresis, the 2nd band containing all elastolytic activity.

Preincubation with trypsin significantly increases the ^{125}I-labeled elastin splitting activity of the Triton extract and of the 40% ammonium sulfate precipitate. No or only a very slight activation was observed in the 70% ammonium sulfate precipitate.

These results suggest the presence of an inactive precursor in the 40% ammonium sulfate precipitate and of an activated protease in the 70% precipitate.

The high specific activity of the purified fraction (2nd peak of 70% ammonium sulfate precipitate) with $AcAla_3OMe$ and ^{125}I-labeled elastin as substrate, as well as the absence of activity on other substrates (hemoglobin, BzArgOEt and AcTyrOEt) justified the denomination suggested of "platelet elastase" for the protease in this fraction.

Abbreviations: $AcAla_3OMe$, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester.

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INTRODUCTION

Since the discovery of pancreatic elastase (pancreatopeptidase, EC 3.4.4.7) by Banga and Balo[1], elastolytic enzymes have been described in serum[2], vessel wall[3] and in leucocyte granules[4] from human and other animal species. We have shown that human blood platelets contain proteases acting on elastin. This proteolytic activity could be released from blood platelets by collagen, adrenalin and ADP, as well as by sonication and incubation with Triton X-100 (ref. 5). The extent of release of these proteases depends on the method of treatment, the total activity being distributed between the released supernatant and the particulate fractions. Investigations on the mechanism of this release and activation suggested a double mechanism consisting of the release of a particulate-bound enzyme as well as the activation of a precursor of this enzyme[6].

We report here the results obtained on the fractionation of platelet extracts, the purification of fully activated platelet elastase, its separation from other proteolytic activities and from its inactive precursor or zymogen, as well as the activation of this precursor by trypsin.

MATERIALS

^{125}I-labeled elastin was prepared as described elsewhere[7]. Twice-crystallized porcine pancreatic elastase (EC 3.4.4.7) was purchased from Whatman Chemical Co. (England).

Three times crystallized trypsin and chymotrypsin were obtained from Worthington Biochemical Co. (Freehold, N.J., U.S.A.). The synthetic substrates used for enzymatic assays were *N*-acetyl-L-analyl-L-alanyl-L-alanine methyl ester from Sigma Chemical Co., St. Louis, Mo., U.S.A.; benzoyl-L-arginine ethyl ester (HCl) and acetyl-L-tyrosine ethyl ester from Koch and Light Laboratories, Colnbrook, Bucks, England. Sephadex G-150 was the product of Pharmacia (Uppsala, Sweden). All the reagents used for acrylamide gel electrophoresis were the products of Eastmann Organic Chemicals, Rochester, U.S.A.

METHODS

Fractionation procedure

Human blood platelets were obtained from voluntary donors by an IBM cell separator as described[5,8]. This method allows a good separation of platelets from white and red blood cells. Platelets were washed twice in saline, recentrifuged, the pellet resuspended in 0.1 M Tris–HCl buffer, pH 7.4, and incubated with a Triton X-100 solution at a final concentration of 0.1 or 0.2% for 60 min, at 4 °C. After centrifugation at 3500 × *g* in the cold for 30 min, the supernatant (Triton extract) was fractionated with ammonium sulfate, by first adding crystalline ammonium sulfate to 40% saturation. After centrifugation, more ammonium sulfate was added to the supernatant to 70% saturation, as described[8]. Both the 40% and 70% precipitates were dissolved in 0.2 M KCl, centrifuged to discard a small insoluble fraction and dialysed against the same solvent. Both precipitates were then fractionated by

gel filtration on a Sephadex G-150 column (100 cm × 2.5 cm) and eluted by 0.05 M KCl, adjusted to pH 7 with KOH.

The protein peaks were detected by measuring the absorbance at 280 nm, dialysed, concentrated by evaporation and tested for their enzyme activity. At each fractionation step, proteins were determined by the Lowry method[9] or by the microbiuret reaction described by Itzhaki and Gill[10].

Enzyme assays

Elastase-like activity was determined by two independent methods: one using ^{125}I-labeled elastin as described elsewhere[7] and the other using the synthetic specific substrate for pancreatic elastase, $AcAla_3OMe$[11]. This determination was performed on the titrigraph Radiometer (Copenhagen, Denmark), using 20 μmoles of the synthetic substrate in 2 ml of a 0.005 M Tris–HCl buffer, pH 8.0, containing 0.1 M KCl at 30 °C under a nitrogen atmosphere. Titration was performed with 0.02–0.1 M NaOH (ref. 12).

Protease activities were determined with hemoglobin as substrate according to Anson[13].

Trypsin-like and chymotrypsin-like activities were determined by using the synthetic substrates BzArgOEt and AcTyrOEt, respectively, with the titrigraph Radiometer as described for the determination of elastase-like activity. Calibration curves were prepared with all the synthetic substrates using crystalline porcine pancreatic elastase, crystalline trypsin and chymotrypsin. The activities of the various platelet extracts were expressed in equivalents of crystalline pancreatic enzymes (elastase, trypsin or chymotrypsin), as read on the standard curves[12] and are given in μg enzyme (elastase, trypsin or chymotrypsin) per mg protein.

Acrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed at pH 8.4 in a Tris–glycine buffer in the Canalco equipment (Canal Industrial Corp., Rockville, Md., U.S.A.), 2 mA were applied to each gel. The gels were stained with Amidoblack, destained with 7% acetic acid and analysed with a recording electrophoresis densitometer (Colab., Chicago, Ill., U.S.A.). In some experiments, unstained gels were cut into 2-mm sections with a gel slicer. Each fragment was eluted by mechanical stirring for 2 h with 0.1 M KCl, and the eluates tested for their elastase activity, using ^{125}I-labeled elastin as substrate.

Effect of trypsin

Activation of elastolytic activity by trypsin was measured by incubating the platelet extracts or the purified fractions in the presence of various amounts of trypsin (between 10 and 100 μg) for 10 min at 37 °C. Elastase activity was determined in the incubated extract with ^{125}I-labeled elastin[7].

RESULTS

Precipitation of the platelet Triton extract with 40% and 70% ammonium sulfate and gel filtration of the precipitates on Sephadex G-150 columns gave the following results[8]: a large excluded peak was obtained with the 40% fraction, and

two peaks with the 70% ammonium sulfate precipitate. The first smaller peak was eluted with the void volume (designated as Peak I), followed at a distance, of about twice the void volume, by a second larger peak (designated as Peak II), corresponding to proteins penetrating the gel. All three peaks contain protease activities (see Tables I and II).

The Sephadex peak of the 40% ammonium sulfate precipitate presents a single band on acrylamide gel electrophoresis (see Fig. 2/A) in the conditions indicated in Methods. Peak II of the 70% ammonium sulfate fraction gives three bands on acrylamide electrophoresis (see Fig. 3).

Table I shows the elastase-like activity of the platelet fractions as well as their trypsin-like and chymotrypsin-like activities. The first column of the table gives the quantity of protein recovered in the fractions. Most of the platelet proteins are extracted during the Triton incubation, only about 10% remained associated with the insoluble pellet. Elastase-like activity is higher in all the fractions, than trypsin-like and chymotrypsin-like activities.

TABLE I

ELASTASE-LIKE ACTIVITY (SUBSTRATE: $AcAla_3OMe$), TRYPSIN-LIKE ACTIVITY (SUBSTRATE: BzArgOEt) AND CHYMOTRYPSIN-LIKE ACTIVITY (SUBSTRATE: AcTyrOEt) OF THE PLATELET FRACTIONS

Activities determined in the pH stat, as described in Methods. Results are expressed in equivalents (μg) of crystalline enzymes per mg of platelet protein.

Fraction	*Protein in fraction (mg)*	*Elastase-like activity*	*Trypsin-like activity*	*Chymotrypsin-like activity*
Platelet extract (Triton 0.1%)	77.0	0.4	0.8	0.1
Residual pellet*	8.2	2.4	1.6	0
40% Ammonium sulfate precipitate	16.2	3.0	0.4	0.1
Peak of 40% precipitate	0.9	7.0	0.2	0
70% Ammonium sulfate precipitate	3.0	6.5	0.2	0.4
Peak I of 70% precipitate	0.12	50.0	3.2	1.2
Peak II of 70% precipitate	0.67	495.0	0	0
Supernatant 70% ammonium sulfate	1.8	0	0	0

* Fraction insoluble in Triton X-100, see Methods.

The 70% ammonium sulfate precipitate contains a relatively high specific activity of 6.5 μg equivalents elastase per mg protein. A significant elastase-like activity is associated with the 40% ammonium sulfate precipitate also. The highest specific activity was recovered from the second peak of the gel-filtration column obtained with the 70% ammonium sulfate precipitate. This peak material is devoid of any BzArgOEt or AcTyrOEt splitting activities.

Distribution of the enzymatic activities in the various fractions

The distribution of protease activities at three pH values and elastase-like activity in platelet extracts is represented in Table II. Significant proteolytic activities are found in the Triton extract at all three pH values studied. The distribution

TABLE II

DISTRIBUTION OF ELASTASE-LIKE AND PROTEASE ACTIVITIES IN PLATELET FRACTIONS

Elastase-like activity determined at pH 8.6 with ^{125}I-labeled elastin and protease activity at different pH values with hemoglobin. Results are expressed as mg substrate hydrolysed by mg platelet protein. Buffers used with hemoglobin: at pH values 3.5 and 5.5, 0.1 M sodium acetate; and at pH 7.5, 0.1 M sodium cacodylate.

Fraction	*Elastase-like activity*	*Protease activity*		
		pH 3.5	*pH 5.5*	*pH 7.5*
Platelet extract	0.55	1.02	0.74	0.80
40% Precipitate	1.18	0.59	0.46	0.70
Peak 40%	1.73	0.50	0.54	0.64
70% Precipitate	4.0	0.27	0.30	0.25
Peak I 70%	1.49	0.20	0.32	0.22
Peak II 70%	10.2	0.1	0.1	0.1

of elastase-like activity as determined with ^{125}I-labeled elastin as substrate, corresponds to that obtained with $AcAla_3OMe$ as substrate (see Table I), although the ratio of specific activities is different when determined with these two substrates (*cf.* Tables I and II).

In agreement with our previous results[8] the highest protease activity (as measured with hemoglobin) is recovered in the 40% ammonium sulfate precipitate. This activity is entirely recovered in the excluded peak of the Sephadex column. Some protease activity is also present in the 70% ammonium sulfate precipitate, and is recovered entirely in its first (excluded) gel filtration peak. The second peak of this fraction is the highest in elastase-like activity, but does not have any detectable acid or neutral protease activities against hemoglobin as substrate. It appears therefore that the protease activity measured with hemoglobin and the elastase-like activity measured with elastin is represented by separate enzymes.

Effect of trypsin on the elastolytic activity of the different fractions

The incubation of platelet Triton extract in the presence of trypsin significantly enhanced its activity towards ^{125}I-labeled elastin.

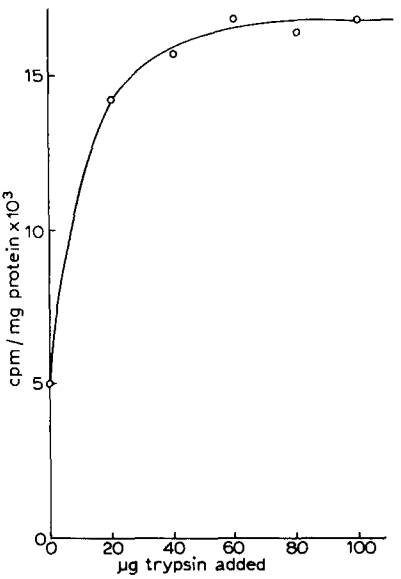
Fig. 1 shows the effect of increasing concentration of trypsin on the elastolytic activity of a Triton platelet extract. About 60 μg trypsin per mg platelet protein is sufficient to obtain a maximal activation of the elastolytic activity. This activity is not increased any further by adding more trypsin.

The effect of trypsin has been tested at the different steps of the purification procedure described. The elastase-like activity of the Triton extract is activated about three times. The elastase-like activity of the 40% precipitate as well as its Sephadex peak, was activated about four times.

The elastolytic activity present in the 70% ammonium sulfate precipitate as well as in its second gel filtration peak was not enhanced by incubation with trypsin: these fractions contain only "a trypsin-independent" elastolytic activity.

We concluded that an inactive proenzyme was present in the 40% ammonium sulfate precipitate, activated by proteolysis: this conclusion is further strengthened by the acrylamide gel electrophoresis patterns of these fractions.

The gel electrophoresis pattern of the Sephadex peak of the 40% ammonium



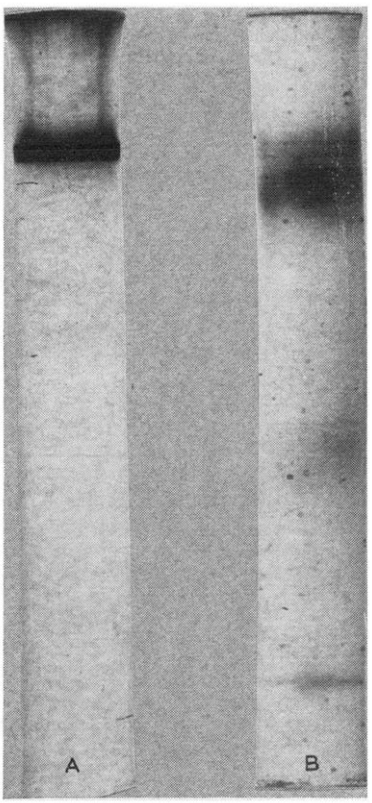


Fig. 1. Effect of increasing concentration of trypsin (on the abcissa, μg of trypsin added), on the activation of elastolytic activity of a platelet Triton extract (ordinates cpm/mg protein $\times$ 10^{-3}).

Fig. 2. Acrylamide gel electrophoresis of the Sephadex peak of the 40% ammonium sulfate precipitate. A, before and B, after activation by trypsin.

sulfate fraction (Fig. 2A) shows a single band very slightly penetrating the gel. After incubation with trypsin (100 μg for 10 min) this fraction migrates somewhat faster and another fast moving band also appears (see Fig. 2B). This modification accompanies the appearance of the activated elastase-like enzyme (see above) and confirms the suggestion concerning the proteolytic transformation of an inactive precursor into an active elastase-like protease.

Fig. 3 shows the densitometric recording of an acrylamide gel electrophoresis of the second peak eluted from the 70% ammonium sulfate precipitate. The diagram shows the presence of three major protein fractions. These fractions have been eluted from unstained gels and tested separately for their elastase activity as described in Methods. All the elastase-like activity was recovered in the second fraction and none was present in the other 2 fractions. The recovery of the elastolytic activity in this second fraction was about 63% of the total activity charged on the gel as determined by its action on ^{125}I-labeled elastin. As this active fraction gave one band on acrylamide electrophoresis it may represent a homogeneous protein. This point, however, necessitates further confirmation.

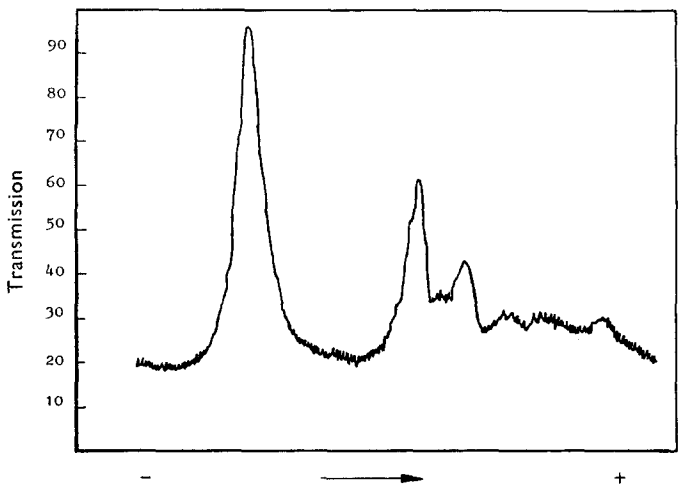


Fig. 3. Densitometric recording of an acrylamide gel electrophoresis of the second elution peak of the 70% ammonium sulfate precipitate.

DISCUSSION

Proteolytic activities have been demonstrated in platelet extracts using various substrates[14]. We have shown previously that platelet extracts hydrolyse ^{125}I-labeled elastin[5] and $AcAla_3OMe$[12], both being considered as relatively specific substrates of pancreatic elastase[1,11]. The present results confirm the presence of activities to these substrates in platelet extracts, the highest specific activity to both of them being recovered in the second gel filtration peak of the 70% ammonium sulfate precipitate (see Tables I and II). The apparent purification achieved of this elastase-like protease according to its action on $AcAla_3OMe$, was about 1200-fold[12] (see Table I). This factor may reflect partially a purification and partially the activation of an inactive precursor during the extraction and ammonium sulfate fractionation procedure.

The exact contribution of the activation process cannot yet be evaluated with certainty. According to the activation by trypsin of the platelet Triton-extract (about 3-fold, see Fig. 1), the degree of purification of the activated elastase-like enzyme may be of the order of $1200/3 = 400$. It should be noted also that the purification factor calculated from the determinations using ^{125}I-labeled elastin is only about 20 (see Table II). The reason for this discrepancy between the two estimates is not clear, although it could be attributed to the further splitting of released peptides from elastin (competitive substrate), or to the inhibition of the enzyme by some breakdown products.

The material recovered from Peak II of the 70% ammonium sulfate fraction represents about 1% of the total protein recovered from the Triton extract. This peak can be separated into three fractions by acrylamide gel electrophoresis (Fig. 3) the second band recovered from the gel contains all the elastolytic activity. About 60% of the elastase-like activity of Peak II of the 70% ammonium sulfate precipitate could be recovered from this second band. Peak II had no activity on BzArgOEt or on AcTyrOEt and did not act on hemoglobin at any of the pH values studied.

Therefore it can be concluded that this fraction corresponds to the purified elastase-like protease of platelets.

The strong activation of the ^{125}I-labeled elastin splitting activity in the Triton extract obtained by preincubation with trypsin suggests the presence of an inactive precursor of platelet elastase. Trypsin alone had no measurable activity on the substrate used[15]. The 40% ammonium sulfate precipitate contains this inactive precursor form of the elastase-like protease. This is shown by the pronounced activation of the elastase-like activity of this fraction after incubation with trypsin and by the modification of the acrylamide gel electrophoresis pattern after trypsin treatment (Figs 2A and 2B).

The existence of activating platelet proteases is indicated by the relatively strong hemoglobin-splitting activity found in the platelet Triton-extract, as well as in the 40% and 70% ammonium sulfate precipitates. We propose therefore that the hemoglobin-splitting activity should be attributed to a separate endopeptidase. This endopeptidase could accompany the pro-elastase-like enzyme in the 40% precipitate and in its Sephadex peak. It could be responsible for the gradual activation of the pro-elastase during the extraction and fractionation procedure. A similar situation was found by Baumstark[16] for pancreatic extracts which also contained an endopeptidase associated with elastase in partially purified preparations.

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REFERENCES

1 Banga, I. and Balo, J. (1953) *Nature* 171, 44–45
2 Hall, D. A. and Wilkinson, J. E. (1963) *Nature* 197, 454–455
3 Loeven, W. A. (1969) *J. Atheroscler, Res.* 9, 35–45
4 Janoff, A. and Scherrer, J. (1968) *J. Exp. Med.* 128, 1137–1155
5 Robert, B., Legrand, Y., Pignaud, G., Caen, J. and Robert, L. (1969) *Pathol. Biol.* 17, 615–622
6 Robert, B., Szigeti, M., Robert, L., Legrand, Y., Pignaud, G. and Caen, J. (1970) *Nature* 227, 1248–1249
7 Robert, B. and Robert, L. (1969) *Eur. J. Biochem.* 11, 62–67
8 Legrand, Y., Robert, B., Szigeti, M., Pignaud, G., Caen, J. and Robert, L. (1970) *Atherosclerosis* 12, 451–465
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
10 Itzhaki, R. F. and Gill, D. M. (1964) *Anal. Biochem.* 9, 401–410
11 Gertler, A. and Hofmann, T. (1970) *Can. J. Biochem.* 48, 384–386
12 Robert, B., Legrand, Y., Soria, C., Caen, J. and Robert, L. (1972) *C.R. Acad. Sci. Paris* 274, 1749–1752
13 Anson, M. L. (1939) *J. Gen. Physiol.* 22, 79
14 Nachman, R. L. and Ferris, B. (1968) *J. Clin. Invest.* 47, 2530–2540
15 Robert, B., Robert, L., Legrand, Y., Pignaud, G. and Caen, J. (1971) *Ser. Haematol.* 4, 175–185
16 Baumstark, J. S. (1970) *Biochim. Biophys. Acta* 220, 534–551