PURIFICATION OF PLATELET PROTEASES: ACTIVATION OF PROELASTASE BY A TRYPSIN-LIKE ENZYME

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1. Introduction

The presence of proteases (cathepsins [1], elastase, trypsin and chymotrypsin-like enzymes [2,3], collagenase [4]) has already been demonstrated in human blood platelets. Among these enzymes, we have described [3] a procedure for the purification of elastase: the method was based upon the affinity chromatography of elastase rich protein fractions on a mixed bed cellulose–elastin column. Platelet elastase arises from the activation of a precursor (platelet proelastase) which has also been purified [3]: the mechanism of activation is similar to that demonstrated by Grant and Robbins [5] and involves a limited proteolysis, which can be induced in vitro by pancreatic trypsin. The purpose of the present study was to look for a direct role of platelet trypsin (and eventually of platelet chymotrypsin) like enzyme in this activation process. This has required the purification of these enzymes. We therefore devised a three step affinity chromatography method in order to purify proteolytic enzymes (trypsin, chymotrypsin, elastase-like enzymes) from a platelet lysate. Their Michaelis constant has been determined using specific substrates and the effect of the two first-mentioned enzymes on platelet proelastase has been verified.

2. Experimental

2.1. *Determination of enzyme activities*

Elastolytic activity of elastase was measured by the method of Bielefeld et al. [6] using [^{14}C]formaldehyde-labelled elastin. The titrimetric method used for the determination of the esterolytic activity of trypsin, chymotrypsin and elastase has already been described [2]: substrates used were, respectively, *N*-benzoyl-DL-arginine-ethyl ester (BzArgOEt), *N*-acetyl-L-tyrosine-ethyl ester (AcTyrOEt) and *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester ($AcAla_3OMe$) [7] from Sigma Chem. Co (St Louis, Mo.). Trypsin-like enzyme has also been measured by the esterolysis of Benzoyl-DL-arginine *p*-nitroanilide (BAPNA) [8], 6 min at 37°C, in a spectrophotometer at 420 nm. Results were expressed as equiv. (μg) of the corresponding pancreatic enzymes as read on standard curves obtained with crystallized bovine trypsin (EC 3.4.4.4) bovine chymotrypsin (EC 3.4.4.5) and porcine elastase (EC 3.4.4.7) from Worthington (Freehold, NJ) as described elsewhere [3].

2.2. *Purification of platelet trypsin, chymotrypsin and elastase by affinity chromatography*

The starting material was represented by proteins precipitated from a platelet lysate (obtained with Triton X-100 at 0.5% final concentration) by ammonium sulfate at 70% saturation as previously described [2]. The purification procedure is summarized in fig.1. The fraction was first charged on a column of agarose–trypsin inhibitor [9] (prepared from soya bean according to Birk et al. [10] Miles Research Laboratories, Slough, England. Capacity of gel 10–14 μmol/ml gel suspension. Size of column 30 × 1.5 cm). The column had been packed in a 0.1 M, pH 8.4, Tris–HCl buffer at 4°C. The same buffer was used for the elution of a single protein peak

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(peak A_1) elution speed was 2 ml/h. A second peak (peak B_1) was afterwards eluted with a 0.2 M, pH 2.0, KCl buffer. Peak A_1 was then charged on a second column packed with agarose-ε-amino caproyl D-tryptophane-methyl ester [11] purchased from the same firm (Miles Research Laboratories, Slough, England) (capacity of gel 2–5 mg/ml). The conditions of the chromatography were strictly identical: the peak eluted with Tris–HCl buffer was called peak A_2, the peak eluted with KCl buffer, peak B_2. Peak A_2 was then dialysed against a 0.05 M, pH 4.5 acetate buffer and charged on the top of a mixed bed elastin–cellulose column (2 g elastin/98 g cellulose, size of the column 40 × 2.5 cm) and a third chromatography was run as described [3]. Two fractions were thus separated: peak A_3 with the above mentioned buffer, then peak B_3 with a 1 M, pH 4.5 acetate buffer. The different fractions (peaks A_3, B_1, B_2, B_3) were then dialyzed overnight against distilled water at +4°C and lyophilized. Aliquots were dissolved in a 0.1 M, pH 8.1 Tris–HCl buffer (pH 8.6 for B_3). The protein content (method Lowry et al. [12] and the trypsin, chymotrypsin and elastase activities were determined. Their homogeneity was assessed by analytical polyacrylamide disc-gel electrophoresis in the absence or in the presence of SDS (1‰).

2.3. *Purification of proelastase by preparative polyacrylamide gel electrophoresis*

Proteins precipitated from a platelet lysate by ammonium sulfate at 40% saturation were fractionated by three successive affinity chromatography as described above. Peak A_3 (18.7 mg protein associated with 7.5 equiv. (μg) of proelastase) was used as starting material for the purification of proelastase by polyacrylamide gel electrophoresis in preparative conditions described elsewhere [3].

3. Results

3.1. *Affinity chromatography of the proteins precipitated by ammonium sulfate at 70% saturation*

3.1.1. Protein recovery in each fraction (see the legend of figure 1)

Column 1 had been charged with 14 mg protein,

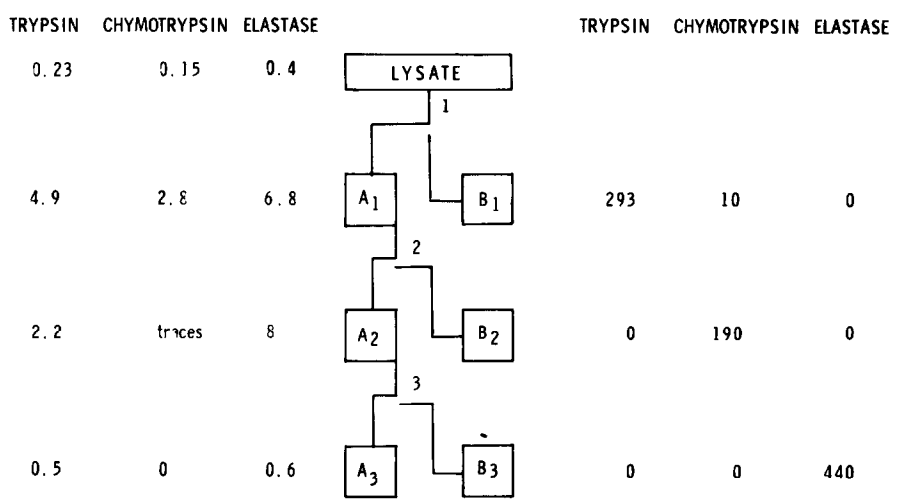

Fig.1. Purification of platelet proteases by affinity chromatography. A protein fraction (14 mg) obtained by addition of ammonium sulfate (70% saturation) to a platelet lysate (460 mg protein) was charged on column 1 (agarose–trypsin inhibitor). Peak A_1 (11 mg protein) was eluted by 0.1 M, pH 8.4, Tris–HCl, peak B_1 (1.13 mg) by 0.2 M, pH 2.0, KCl. Peak A_1 was then charged on column 2 (agarose–amino caproyl D-tryptophane methyl ester). Peak A_2 (7.2 mg proteins) and peak B_2 (0.8 mg) were eluted as above. Peak A_2 was charged on column 3 (mixed bed elastin–cellulose). Peak A_3 (2.8 mg protein) was eluted by 0.05 M, pH 4.5, sodium acetate. Peak B_3 (0.45 mg) by 1 M, pH 4.5 sodium acetate. The activity of the trypsin, chymotrypsin and elastase-like enzymes were measured using respectively BzArgOEt, AcTyrOEt and $AcAla_3OMe$ as substrate. The results, expressed in equivalents (μg) of crystalline pancreatic trypsin (EC 3.4.4.4) chymotrypsin (EC 3.4.4.5) and elastase (EC 3.4.4.7) [3] are indicated on the left for the platelet lysate and for peaks A_1, A_2, A_3 and on the right for peaks B_1, B_2 and B_3.

86.5% were recovered. 78.5% in peak A_1, 8% in peak B_1. Peak A_2 represents 68% and peak B_2 7.6% of the material charged on column 2 (10.5 mg). 43% of the protein deposited on column 3 (6.5 mg) was associated with peak A_3 and 7% with peak B_3.

3.1.2. Distribution of the enzymes

The values quoted on fig.1 were respectively obtained using BzArgOEt, AcTyrOEt and $AcAla_3OMe$ as substrates. After elution of proteins adsorbed on each column, purification factors were calculated equal to 1275 for trypsin-like enzyme (peak B_1), 1260 for chymotrypsin like enzyme (peak B_2), 1100 for elastase (peak B_3). No, or only a very little, contamination by each of the other enzymes measured was found associated with these values. In fact, peak B_1, peak B_2 and peak B_3 represent highly purified form of platelet trypsin, chymotrypsin and elastase since SDS–polyacrylamide disc-gel electrophoresis revealed for each of them one single-band corresponding to an approximate mol. wt (calculated from standard proteins) of 40 000 for the trypsin-like enzyme, 32 000 for the chymotrypsin-like enzyme and 26 000 for elastase. The aspect of the gel is presented on fig.2.

3.2. *Determination of the Michaelis constant* K_m *of each enzyme using synthetic substrates*

The Michaelis constant K_m of purified platelet trypsin, chymotrypsin and elastase was calculated from Linewaever-Burk plots obtained with 0.10–5 mM BzArgOEt (trypsin), AcTyrOEt (Chymotrypsin), $AcAla_3OMe$ (elastase) and 100 μg of each enzyme. K_m for the substrates was found equal to 0.625 mM for trypsin, 1.26 mM for chymotrypsin and 0.5 mM for elastase. This last value is quite comparable to the K_m of pancreatic elastase which has been found by Feinstein et al. [13] equal to 0.43 mM using the same substrate.

3.3. *Activation of platelet proelastase by the platelet trypsin like enzyme*

The effect of purified platelet trypsin and chymotrypsin on platelet proelastase has been checked on a platelet lysate, on a protein fraction enriched in proelastase (proteins precipitated by ammonium sulphate at 40% saturation) and directly on a pure preparation of proelastase. Table 1 presents the activation obtained when 1 mg of these fractions has been incubated, during 20 min, 37°C, in 1 ml of 0.1 M, pH 8.1, Tris–HCl buffer, with 10 μg platelet trypsin or with the same amount of a commercial preparation of pancreatic trypsin. The activating effect observed with platelet trypsin is quite equivalent to that given by the pancreatic enzyme. The differences observed comparing the values obtained after incubation with both kinds of trypsin and those given by the control

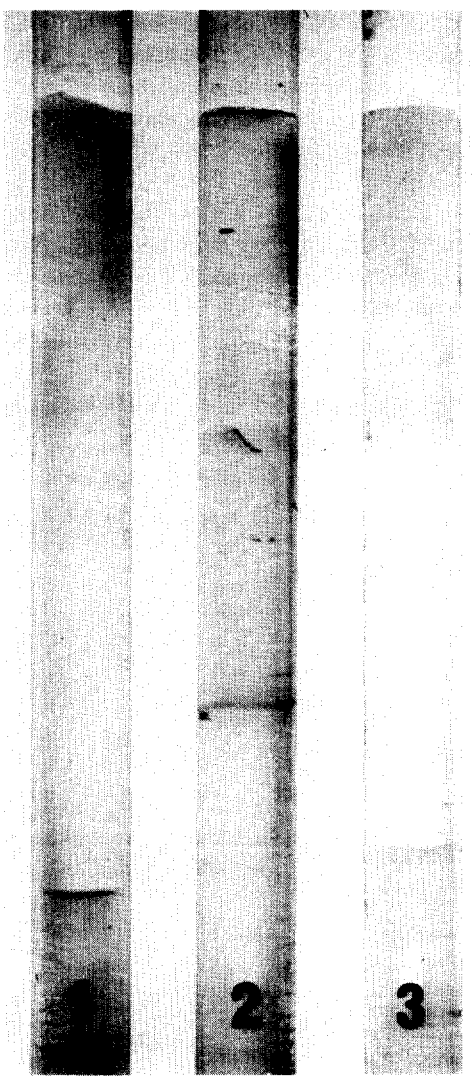

Fig.2. SDS–Acrylamide disc-gel electrophoresis of purified platelet elastase (gel 1), trypsin (gel 2) and chymotrypsin-like enzymes: 7% acrylamide containing 1‰ SDS, stained with amido black and destained with 7% acetic acid. 1.5 m/gel.

Table 1
Activation of platelet proelastase

	Elastase activity		
	Control	After platelet trypsin	After pancreatic trypsin
Platelet extract	0.35	0.58	0.60
Protein fraction	2.6	5	4.6
Proelastase	0.55	230	215

10 μg Trypsin-like enzyme purified from a platelet lysate or of a commercial preparation of pancreatic trypsin (EC 3.4.4.4) were incubated with 1 mg (method of Lowry et al. [12]) platelet lysate, with the same amount of a protein fraction precipitated by ammonium sulfate (40% saturation) and also with 1 mg purified platelet proelastase, during 20 min 37°C in a 0.1 M, pH 8.1, Tris–HCl buffer. A control was done with buffer alone. Elastase activity was then directly measured in a pH stat. using $AcAla_3OMe$ as substrate. Results are expressed in equiv. (μg) pancreatic elastase (EC 3.4.4.7).

indicate that the platelet lysate contains 0.25 μg of proelastase, the semi-purified protein fraction between 2 μg and 2.5 μg, whereas the purified proelastase is associated with a slight amount of elastase.

The effect of platelet chymotrypsin like protease has been checked in the same conditions: with this enzyme, no activation was observed.

The in vitro activation of proelastase by platelet trypsin has been visualized by SDS–polyacrylamide gel electrophoresis. As already shown with pancreatic trypsin, the activation involves a change in the mobility due to the limited proteolysis of an inactive molecule (approx. mol. wt 30 000) into the active enzyme (approx. mol. wt 26 000). The aspect of the gels is shown on fig.3.

4. Conclusions

The use of affinity chromatography has permitted a good separation of three proteolytic enzymes (trypsin-, chymotrypsin-, elastase-like proteases) present in blood platelets. Their degree of purification was quite high. This type of technique has now been frequently applied to the purification of different proteases [14–18] from distinct origins. Various authors [3,19,20] have pointed out the difficulty to discard a contamination by other proteolytic enzymes from highly purified preparations of proteases. This problem has been solved by the method described. Our results confirm that the trypsin-like enzyme has a

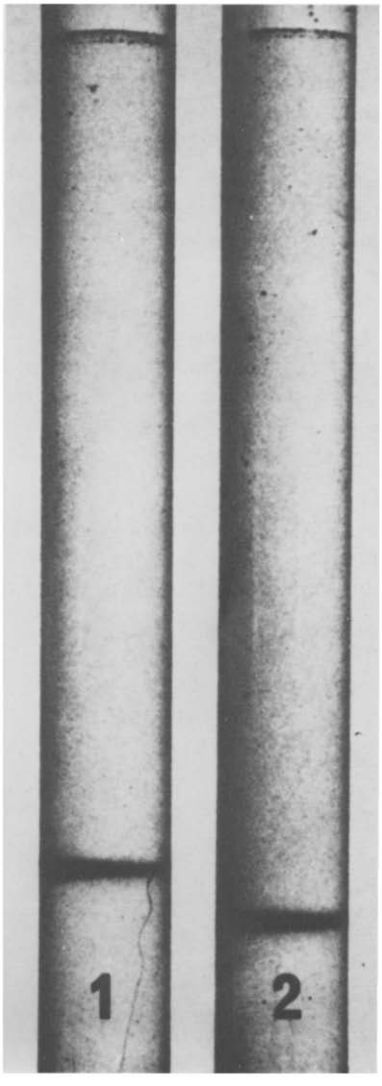

Fig.3. SDS–Acrylamide disc-gel electrophoresis of purified platelet proelastase incubated with buffer (gel 1) and with platelet-trypsin-like enzyme, 10 μg/mg of proelastase, (gel 2) as described. The conditions of electrophoresis are the same as in fig.2.

potential role in arterial elastolysis since it represents an activator of platelet proelastase. The activation of the precursor of platelet elastase can thus be paralleled to that of pancreatic proelastase [5]. Both of them involve a limited proteolysis of an inactive molecule by proteolytic enzymes (pancreatic- and platelet-trypsin-like activity) of similar specificity.

Acknowledgements

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