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Affinity chromatography of serine proteinases from the bovine intervertebral disc on BPTI–Separon HEMA 1000

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ABSTRACT

A method for the purification of serine proteinases from the bovine intervertebral disc using affinity chromatography on basic pancreatic trypsin inhibitor (BPTI) immobilized to the hydroxyalkyl methacrylate copolymer Separon HEMA 1000 E is reported. Its advantage is the possibility of obtaining serine proteinases without an artificial alteration in relative molecular mass.

INTRODUCTION

In cartilage, natural substrates of serine proteinases are mainly high-molecular-mass components such as proteoglycans and gelatin. Serine proteinases also activate metalloproteinases, the most important proteinase system in the degradation of extracellular matrix [1]. Serine proteinases enter the extracellular matrix mainly as either high-molecular-mass zymogens or complexes with inhibitors. *In vitro* they can be activated proteolytically or by the action of chaotropic extractants [2,3]. Apart from the natural substrates, they also split Arg- and Lys-peptide substrates [4]. Melrose and Ghosh [3] found a serine proteinase system in the human intervertebral disc and separated it by affinity chromatography on immobilized trypsin and by gel permeation chromatography on Sephadex G-75 into three fractions with relative molecular masses (M_r) of 70 000, 45 000–55 000 and 12 000. However, in the course of affinity chromatography on immobilized trypsin, artificial alterations in M_r took place. Cole *et al.* [4] described a similar serine proteinase system in the canine intervertebral disc and separated it by ion-exchange chromatography and gel permeation chromatography into two fractions, the major one having an M_r of 94 000 and the minor one 57 000. Another serine proteinase system was described [5] in the human hip joint cartilage. It was characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as consisting of two species, one of them having an M_r of 70 000 and the other 28 000.

In our laboratory, serine proteinases from the human and bovine intervertebral discs were studied. A method of purification was developed and tested using affinity chromatography on immobilized basic pancreatic trypsin inhibitor (BPTI), a polyvalent serine proteinase inhibitor of M_r 6500. It is known that it forms biospecific complexes with trypsin in both the active molecule and the zymogen forms. To the zymogen, however, it shows a lower affinity [6]. As the neutral serine proteinases from the intervertebral disc are of trypsin-like character, an attempt was made to separate them by affinity chromatography on immobilized BPTI, so that no alterations in molecular mass could take place.

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EXPERIMENTAL

Materials

Acrylamide, recrystallized twice (research grade) from Serva (Heidelberg, Germany), N,N'-methylenebisacrylamide, recrystallized twice (research grade) (Serva), N,N,N',N'-tetramethylenediamine (Serva), N-α-benzoyl-D,L-arginine-4-nitroanilide hydrochloride (BAPNA) (analytical-reagent grade) from Fluka (Buchs, Switzerland), *p*-aminophenylmercuric acetate (APMA) from Sigma (St. Louis, MO, USA), *p*-aminophenylmethylsulphonyl fluoride (PMSF) (Sigma), 1,10-phenanthroline from Lachema (Brno, Czechoslovakia), ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) (Lachema), caesium chloride (analytical-reagent grade) (Serva), guanidine hydrochloride (analytical-reagent grade) (Serva), SDS, recrystallized twice (analytical-reagent grade) (Serva), dimethyl sulphoxide (DMSO) (Serva), Triton X-100 (scintillation grade) from Merck (Darmstadt, Germany) and Separon HEMA 1000 E from Tessek (Prague, Czechoslovakia) were used. BPTI–Separon HEMA 1000 was a gift from Dr. J. Turková, trypsin (crystallized) from Léčiva (Prague, Czechoslovakia), plasmin from IMUNA (Šarišské Michalany, Czechoslovakia) and papain from Fluka. All the other chemicals were of analytical-reagent grade.

Extraction

Bovine discs were removed from the spinal column on the day of slaughter, cut into pieces of *ca.* 1 × 1 × 1 mm and extracted for 48 h in a five-fold excess of 2 mol/l guanidine hydrochloride in 50 mmol/l Tris buffer (pH 7.3) at 4°C. The residue was then removed by centrifugation. To the supernatant CsCl was added at a concentration of 0.5 g/g to give a density of 1.5 g/ml. The mixture was then centrifuged at 100 000 *g* and 8°C for 48 h in a Beckman L8M ultracentrifuge using a Model 50.2Ti angular rotor. The upper third of the centrifugal tube volume was withdrawn and dialysed against 50 mmol/l acetate buffer (pH 6.0). Residual proteoglycans and other high-molecular-mass substances were removed by ultrafiltration through an XM-100 Diaflo membrane. The filtrate was then concentrated by ultrafiltration through a UM-2 Diaflo membrane.

Gel permeation chromatography on Sephadex G-75

A 6-ml volume of the concentrated sample (absorbance at 280 nm = 6.2) was applied to a Sephadex G-75 column (100 cm × 2.1 cm I.D.) in 20 mmol/l ammonium carbonate buffer (pH 8.0) and eluted at a rate of 10 ml/h. Fractions of 2.5 ml were collected and the absorbance at 280 nm and both enzymatic and inhibitory activities after activation with trypsin were determined.

Affinity chromatography on immobilized BPTI

A 1-ml volume of sample was diluted with 4 ml of 50 mmol/l formate buffer (pH 8.0) containing 10 mmol/l $CaCl_2$. The mixture was applied to the affinity column of volume 10 ml with the capacity to bind 1.2 mg of trypsin per g of wet carrier.

Elution was carried out with the same buffer at a rate of 2.8 ml in 15 min until a constant absorbance was obtained. The column was then washed with 50 mmol/l formate buffer (pH 4.0) containing 10 mmol/l $CaCl_2$. After the absorbance had stabilized again, 50 mmol/l formate buffer (pH 2.3) containing 10 mmol/l $CaCl_2$ and 1 mol/l NaCl was applied. Fractions of 2.8 ml were collected and their absorbance at 280 nm and enzymatic activity after activation with trypsin were determined. Fractions possessing enzymatic activity were combined, dialysed and concentrated on a UM-2 Diaflo membrane. Concentrated fractions were analysed by SDS-PAGE [7] followed by zymography using copolymerized gelatin (0.05%) as a substrate.

Determination of enzymatic activity

To 0, 0.1, 0.2 and 0.3 ml of the sample were added 5 μl of a solution of 0.5 mg of trypsin dissolved in 1 ml of 50 mmol/l Tris buffer (pH 7.6) containing 10 mmol/l $CaCl_2$. After preincubation at room temperature for 10 min, the volume was made up to 2 ml with the same buffer. The mixture was warmed to 37°C, 50 μl of BAPNA solution (40 mg in 1 ml of DMSO) were added and the mixture was incubated for 30 min at 37°C.

The reaction was terminated by the addition of 0.1 ml of glacial acetic acid. Activity was measured as the change in absorbance at 405 nm compared with the absorbance of the incubation mixture with trypsin, but without the sample. As the blank the buffer with BAPNA and acetic acid was used.

SDS-PAGE zymography

Electrophoresis was performed in 11% polyacrylamide gel copolymerized with 0.05% gelatin. In all instances 35 μl of the sample containing 1% (w/v) SDS and 15% (w/v) sucrose were used. The separation proceeded in rods (5 mm in diameter) at a current density of 3 mA per tube. The molecular mass standards consisted of phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carboanhydrase (30 000), soybean trypsin inhibitor (20 100) and ribonuclease A (13 700). After electrophoresis the gels were washed overnight at 6°C with 2.5% (v/v) Triton X-100 followed by distilled water. The gels were then incubated for 24 h at 37°C in 100 mmol/l glycine buffer (pH 8.0) containing 10 mmol/l $CaCl_2$ or in the same buffer containing APMA (5 mmol/l) or trypsin (2 mg per 100 ml) or plasmin (400 mg per 100 ml). After incubation the gels were stained with either Coomassie Brilliant Blue or silver. The zones of proteolytic activity were visible as transparant stripes on a dark background and those of inhibitory activity as dark stripes on light background. Relative molecular masses of the activated proteinases were calculated from the positions of their stripes relative to the position of bromphenol blue stripes and by comparison of these values with those of molecular mass markers, rather than by direct comparison of the positions of stripes, because the length of polyacrylamide rods with and without gelatin responded in different ways to the staining technique (see Fig. 4).

RESULTS

Bovine disc extract was characterized by SDS-PAGE followed by zymography. The gels were incubated either with buffers alone or with buffers complemented with some activators (APMA, trypsin, plasmin or papain). Fig. 1 shows that the sharpest activation was provided by plasmin, which is a natural activator of latent cartilage proteinases [8]. Activation by plasmin induced proteolytic acitvity corresponding to $M_r \approx$ 94 000 and 80 000 (rod 5). The plasmin inhibitory activity of the extract was not distinguishable by plasmin, because gelatin, used as a substrate, was not digested sufficiently to reveal the inhibitory stripes. Moreover, staining with silver is too strong in the case of plasmin activation, so that

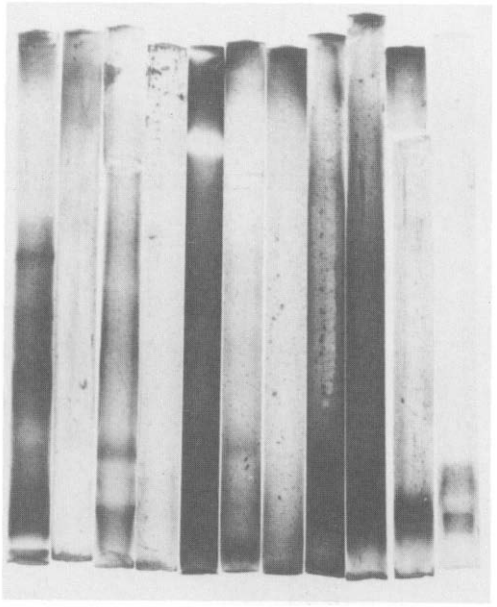

Fig. 1. Zymographic patterns of the extracts from the bovine intervertebral disc. Aliquots of 35 μl were made 1% in SDS and 15% in sucrose. Staining with silver. From the left: (1) gel stained immediately after electrophoresis; (2) gel stained after preincubation at 37°C for 24 h in 100 mmol/l glycine buffer (pH 8.0) containing 10 mmol/l $CaCl_2$ and trypsin (2 mg per 100 ml of buffer); (3) gel preincubation as in 2 but only for 3 h at room temperature; (4) gel preincubation with papain [20 mg per 100 ml of 50 mmol/l phosphate buffer (pH 6.0) supplemented with 1 mg/ml cysteine]; (5) gel preincubation with plasmin (400 mg per 100 ml of glycine buffer); (6) gel preincubation with glycine buffer alone; (7) gel preincubation with glycine buffer containing APMA (2 mmol/l); (8) gel preincubation with phosphate buffer alone; (9) gel preincubation with 100 mmol/l acetate buffer (pH 3.0); (10) BPTI processed under reducing conditions (sample buffer made 2% in β-mercaptoethanol), followed by preincubation with trypsin at 37°C for 24 h; (11) BPTI under non-reducing conditions, preincubation with trypsin at 37°C for 24 h.

co-existing stripes of inhibitory activity could be covered.

There was also weak activation by trypsin when the exposure to the enzyme was only 3 h at room temperature. One stripe with very weak activity was at a position corresponding to $M_r \approx 80\,000$ and two slightly stronger stripes were at $M_r \approx 40\,000$ and 20 000. Activation by trypsin also revealed at least two stripes of inhibitory activity, one of them corresponding to the molecular mass of BPTI monomer (6500) and the second to a molecular mass slightly higher than that of BPTI dimer (the upper zone on rod 11).

Activation by glycine buffer complemented with $CaCl_2$ alone revealed one sharp active stripe at a position corresponding to $M_r \approx 90\,000$ and a wide region of proteinase activity ranging from $M_r \approx 20\,000$ up to 90 000. The same result was obtained with glycine buffer complemented with APMA, a metalloproteinase activator, but there was no activation of the M_r 90 000 species. Acidic buffers [phosphate (pH 6.0) and acetate (pH 3.0)] did not provide any activation by themselves.

On the other hand, the action of papain at pH 6.0 (rod 4) and trypsin at pH 8.0 (rod 2) caused total degradation of the gelatin substrate, so that

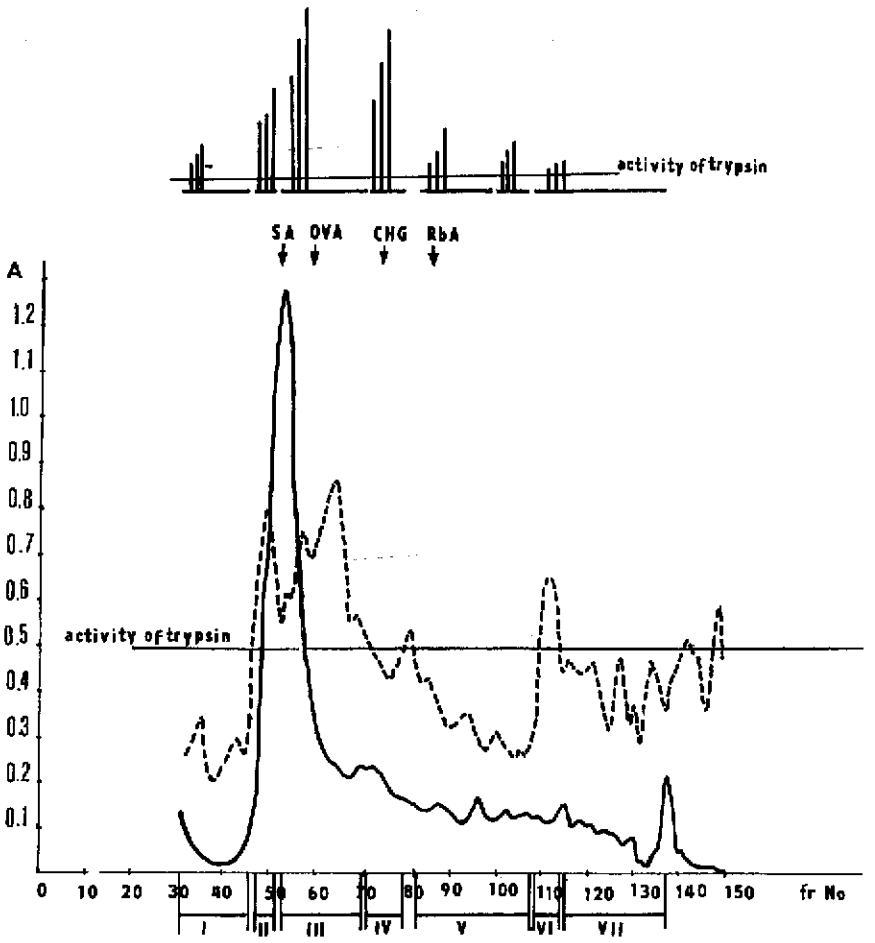

Fig. 2. Gel permation chromatography of the extract from the bovine intervertebral disc on Sephadex G-75. A 6-ml volume of the extract ($A_{280} = 6.2$) was applied to a column of Sephadex G-75 (100 cm × 2.1 cm I.D.) and eluted at a rate of 10 ml/h. Fractions of 2.5 ml were collected. Absorbance at 280 nm (full line) and amidolytic activity after trypsin activation (absorbance at 405 nm, dashed line) were determined. Amidolytically active fractions were combined and concentrated by ultrafiltration. Total amidolytic activity was then determined (shown at the top of the figure). The arrows mark the elution profiles of bovine serum albumin (SA), ovalbumin (OVA), chymotrypsinogen (CHG) and ribonuclease A (Rb A).

neither activation of proteinases nor inhibitory activity could be detected.

The extracts were also characterized by gel permeation chromatography on Sephadex G-75. The presence of three fractions was revealed: a major fraction of M_r 90 000, a smaller fraction of $M_r \approx$ 45 000–50 000 and a very small fraction of $M_r \approx$ 20 000. Proteolytic activity after activation with trypsin and papain was followed in the extracts (Fig. 2). No increase in proteolytic activity was detected in the extracts; on the contrary, inhibition of these enzymes, especially trypsin, took place (Table I).

An attempt was made to separate bovine disc serine proteinases by affinity chromatography on BPTI immobilized on Separon HEMA 1000. The material from the disc was separated into three fractions, all of them being activatable by trypsin. The first fraction was the breakthrough fraction, the second was eluted from the column with buffer of pH 4.0 and the third was released with 1 mol/l NaCl at pH 2.3 (Fig. 3). After separation, each fraction was concentrated twenty-fold by ultrafiltration and the absorbance of each fraction at 280 nm was measured (Table II). Fractions were characterized by zymography. The activation was performed by plasmin. In the first fraction, weakly active zones were detected with $M_r \approx$ 94 000, 90 000 and 80 000. The second fraction also revealed a zone of M_r 94 000 and two weak zones of $M_r \approx$ 85 000 and 80 000. Both

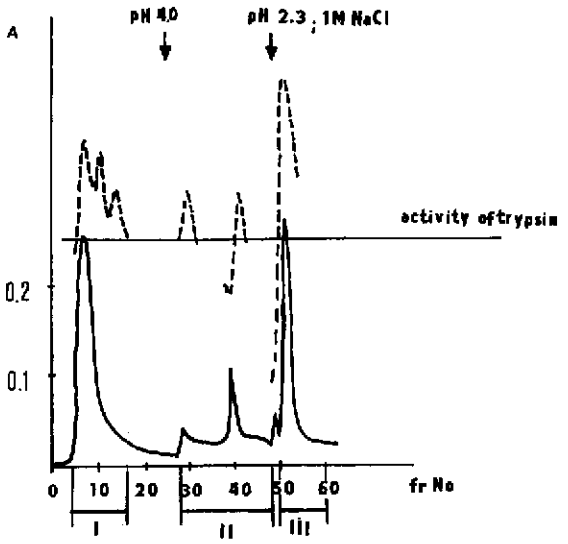

Fig. 3. Affinity chromatography of the extracts from the bovine intervertebral disc on BPTI–Separon HEMA 1000. A 1-ml volume of the extract was diluted with 4 ml of 50 mmol/l ammonium formate buffer (pH 8.0) containing 10 mmol/l $CaCl_2$ and applied to the column (10 ml). It was then developed successively at a rate of 34 ml/h with formate buffers of pH 8.0, 4.0 and 2.3, the last one containing 1 mol/l NaCl. Fraction of 2.8 ml were collected and absorbance at 280 nm (full line) and amidolytic activity after trypsin activation (dashed line) were determined.

fractions also showed a strong inhibitory activity; in the first fraction there were inhibitory activity stripes in the region of $M_r \approx$ 70 000, 28 000 and 15 000, and in the second fraction in the regions of $M_r \approx$ 28 000 and 15 000. In the third fraction a significant proteinase activity of $M_r \approx$ 80 000 was found, but no inhibitory activity (Fig. 4). All fractions were tested by the class-specific inhibitors PMSF or 1,10-phenantroline (Table III).

TABLE I

ACTIVATION OF PROTEINASES PRESENT IN THE EXTRACT FROM BOVINE INTERVERTEBRAL DISC BY TRYPSIN OR PAPAIN

Volume of extract (μl)	Volume of trypsin (μl)	Volume of papain (μl)	Amidolytic activity (%)
0	–	100	100
100	–	100	100
200	–	100	91
300	–	100	86
0	5	–	100
100	5	–	93
200	5	–	46
300	5	–	23

TABLE II

ABSORBANCE AT 280 nm OF COMBINED AND CONCENTRATED FRACTIONS OF THE BOVINE DISC EXTRACTS ISOLATED BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED BPTI

No.	Fraction	Absorbance at 280 nm
I	Uncaptured	0.940
II	Released at pH 4.0	0.400
III	Released at pH 2.3	0.880

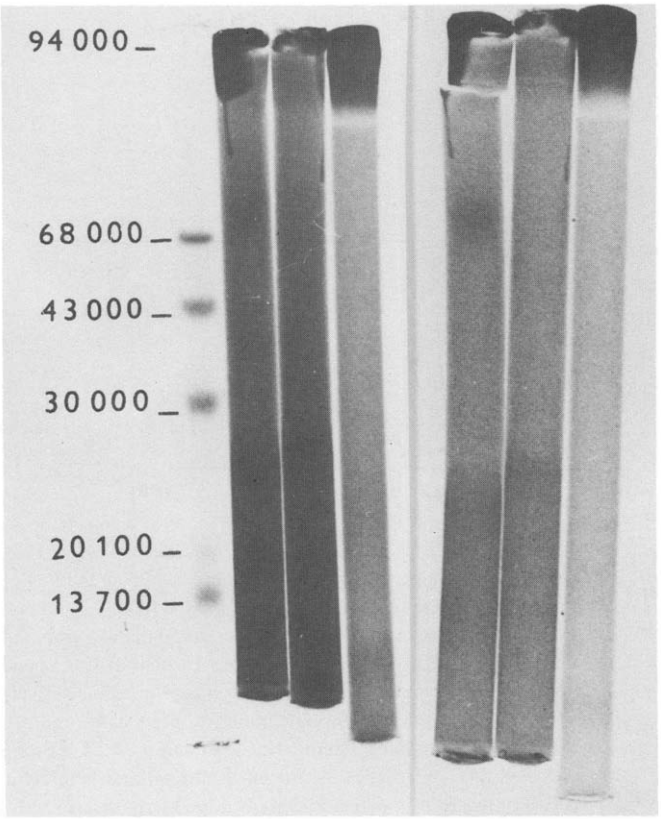

Fig. 4. Zymographic patterns of concentrated fraction isolated by affinity chromatography of the disc extract on BPTI–Separon HEMA 1000. Aliquots of 15 μl of individual fractions were made 1% in SDS and 15% in sucrose. Staining with Coomassie Brilliant Blue. From the left: (1) molecular mass markers ribonuclease A (13 700), soybean trypsin inhibitor (20 100), carboanhydrase (30 000), ovalbumin (43 000), bovine serum albumin (68 000) and phosphorylase *b* (94 000); (2) uncaptured material; (3) material released by buffer of pH 4.0; (4) material released by buffer of pH 2.3 containing 1 mol/l NaCl. The right-hand photograph shows the same zymograph, but in such a position that the zones of activated proteinases and inhibitors could be seen better.

TABLE III

COMPARISON OF THE EFFECTS OF CLASS-SPECIFIC INHIBITORS PMSF AND 1,10-PHENANTHROLINE ON THE AMIDOLYTIC ACTIVITY OF CONCENTRATED FRACTIONS FROM AFFINITY CHROMATOGRAPHY ON IMMOBILIZED BPTI

Fraction	Amidolytic acitivity (absorbance at 405 nm)[a]				
	No inhibitor present	$\frac{A_{405}}{A_{280}}$	5 mmol/l PMSF	5 mmol/l 1,10-phenanthroline	$\frac{A_{405}}{A_{280}}$
I	0.320	0.34	0.000	0.540	0.57
II	0.128	0.32	0.000	0.220	0.55
III	0.440	0.50	0.000	0.740	0.85

[a] 20 h at 37°C, 50 mmol/l Tris buffer (pH 7.5), 10 mmol/l $CaCl_2$.

Whereas 5 mmol/l PMSF completely inhibited the amidolytic activity of all fractions, 1,10-phenantroline increased it.

DISCUSSION

The results demonstrate that affinity chromatography on a carrier with bound BPTI can be used for the selective separation of serine proteinases in bovine disc extracts, even though the proteinases are present mostly in inactive forms, either as zymogens or as complexes with inhibitors. Affinity chromatography on an immobilized inhibitor has the advantage that no reduction in the molecular masses of the proteinases investigated takes place as is the case with affinity chromatography on immobilized trypsin. The component with $M_r \approx 80\,000$ was bound most strongly. It is not clear, however, if this component is present in biological materials in the form of a zymogen or in the form of a complex with inhibitor. It seems probable that the enrichment of the M_r 80 000 species in fraction III is the result of the inhibitor exchange between the naturally occurring and enzyme-bound inhibitor on the one hand, and the carrier-bound inhibitor on the other. In the course of affinity chromatography a gradual decrease in inhibitory activity was observed. In contrast to Melrose *et al.* [2], we did not observe any activation by the treatment with APMA (data not shown). The metalloproteinase inhibitor 1,10-phenanthroline not only did not inhibit these proteinases, but activated them. On the other hand, the serine proteinase inhibitor PMSF revealed a strong inhibitory effect on these proteinases, which can be seen as evidence of the appurtenance of the proteinases in question to the serine proteinase class.

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