

INTERACTION OF BOVINE ALPHA₂ MACROGLOBULIN WITH PROTEINASES

INTERAÇÃO DE ALPHA₂ MACROGLOBULINA BOVINA COM PROTEINASES

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SUMMARY

The alpha₂-macroglobulin (alpha₂M) protease inhibitor was purified from bovine plasma. The alpha₂M preparations at various purification steps were identified by immunodiffusion and crossed immunoelectrophoresis with anti-human alpha₂ serum. Anti-bovine alpha₂M serum was prepared for the quantitative determinations. The purest alpha₂M preparation was obtained by affinity chromatography and used as primary standard in radial immunodiffusion. Alpha₂M preparations were submitted to binding tests with p' - NPGB (p' - nitrophenyl-p-guanidine-benzoate HCL.) titrated trypsin and plasmin. Alpha₂M protected 35% of the esterolytic activity of trypsin and 50% of the amidolytic activity of plasmin.

UNITERMS: Cattle; Plasma; Proteinase inhibitors; Alpha₂M; Plasmin; Trypsin

INTRODUCTION

Inhibitors are important in the study of proteases by providing the clearest evidence on the type of catalytic site, information that forms the basis for the classification of the biological functions of proteases (SALVESEN et al.¹⁸, 1981). Alpha₂M is one of the major protease inhibitors of blood plasma.

Human alpha₂-macroglobulin (alpha₂M) is a plasma glycoprotein composed of two non-covalently bound subunits, each formed from two identical 180 KDa peptide chains linked by disulfide bridges. This protein produces enzymatically active complexes with nearly all endoproteases. The bindings occur in several stages. After complex formation, the protease catalyzes the cleavage of the peptide bonds at a locus called "bait region" which induces the exposure and then the hydrolysis of a labile thiol in each monomer (FELDMAN et al.⁹, 1985; SOTTRUP-JENSEN²⁰, 1989).

Free and alpha₂-macroglobulin-bound proteinase molecules retain to various degrees their ability to hydrolyze small substrates (BARRET; STARKEY⁴, 1973). The hydrolysis rates of chromogenic substrates by trypsin, chymotrypsin, elastase or plasmin when bound to alpha₂M are about 2-fold smaller than those measured for the free protease (TOURBEZ et al.²³, 1984; DEXPERT et al.⁸, 1987).

The low level of enzymatic activity is generally related to a decreased accessibility of the bound enzyme. On the other hand, alpha₂-macroglobulin dramatically decreases the affin-

ity of proteinases for macromolecular inhibitors or their catalytic power on macromolecular substrates (GANROT; NIHEHN¹⁰, 1983; NAGASAWA et al.¹⁶, 1970; BIETH⁵, 1981), inhibitors or antibodies (CREWS et al.⁷, 1987). Proteins homologous to human alpha₂M have been isolated and characterized from a number of mammalian plasmas (ANDERSEN; KROLE¹, 1975). In the present study, alpha₂M was isolated from bovine plasma.

In an attempt to contribute to a better understanding of the mechanism of action of alpha₂M, the objectives of the present study were: 1) to purify alpha₂M by existing or adapted methods; 2) to quantify alpha₂M by immunological reactions; 3) to determine the binding properties of bovine alpha₂M with plasmin and trypsin.

MATERIAL AND METHOD

1-Preparation of starting material containing alpha₂M

Oxalated bovine plasma containing 0.1M/ml SBTI (Soya Bean Trypsin Inhibitor) was treated with 50% saturated ammonium sulfate and left overnight at 4° C. After centrifugation, the precipitate was dissolved in distilled water and exhaustively dialyzed. The euglobulins were precipitated with 1M acetic acid containing 10⁻⁴M EDTA to a final pH value of 5.5. After centrifugation, the supernatant containing pseudoeuglobulins was harvested and dialyzed with 0.02M potassium phosphate buffer, pH 8.0.

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2- Chromatographic purification of the material containing alpha₂M

The crude material containing alpha₂M was submitted to chromatography on a DEAE-Sephadex A-50 column equilibrated with 0.02 M potassium phosphate buffer, pH 8.0. Alpha₂M was eluted by passing a 0.3 M NaCl linear gradient through the chromatography column. The flow rate was 4.5ml/h and 3.8 ml fractions were collected.

The presence of alpha₂M in the eluates was monitored by 1) 1.3% agarose gel electrophoresis in 0.19 M TRIS/0.37 M glycine/0.035M barbital buffer, pH 9.2, carried out for 45 min at 12 mA, and 2) immunoelectrophoresis against anti-alpha₂M human serum. The eluates containing semi-purified alpha₂M were pooled, concentrated and then chromatographed on Sepharose CL-6B gel equilibrated with 0.02 M potassium phosphate buffer, pH 8.0. The flow rate was 7.7 ml/h and 1.8 ml fractions were collected per tube. The eluates obtained by Sepharose CL-6B chromatography were submitted to SDS-polyacrylamide gel electrophoresis by the method of LAEMMLI¹³ (1970). The separation gel contained 7.5% acrylamide and the stacking gel, 4%. The buffer used for the run was of the following composition: 1g/l SDS, 6g/l Tris-base, and 28.6g glycine, pH 8.3. Electrophoresis was carried out at 30mA for 3-4 hours. The following molecular weight standards were used: myosin, 200,000; phosphorylase, 92,000; serum albumin, 68,000; ovalbumin, 43,000; pepsin, 33,000; chymotrypsin, 25,000; SBTI, 20,000; lysozyme, 14,300.

3- Quantification of bovine alpha₂M

a-Immunodiffusion by the method of OUCHTERLONY; NILSOON¹⁷ (1986).

For alpha₂M quantitation, the eluates obtained by Sepharose CL-6B chromatography were submitted to immunodiffusion against anti-human alpha₂M serum and total anti-human serum to detect possible contaminants.

b-The Sepharose CL-6B eluates were submitted to immunoelectrophoresis, showing more than one precipitation line (immunodiffusion) with total anti-human serum. Anti-human alpha₂M, anti-human IgG and IgM sera (Behring) and total anti-human serum were used in these experiments.

4- Preparation of bovine anti alpha₂M serum

The Sepharose CL-6B eluates containing highly purified alpha₂M, as identified by SDS-polyacrylamide gel electrophoresis, were used as antigens in female rabbits weighing 3-4 kg. Antibody titers were determined by immunodiffusion. Serum containing anti-bovine alpha₂M antibodies was submitted to chromatographic purification on DEAE-cellulose

equilibrated with 50mM potassium phosphate buffer, pH 8.0. The column was washed with the equilibrium buffer, and the gamma fraction in the bovine alpha₂M anti-serum was eluted passing 150 ml of a 0.3 M NaCl linear gradient through the chromatography column. The flow rate was 2.8 ml/h and 1.2 ml fractions were collected.

5- Affinity chromatography (ANDERSET et al.², 1974)

One hundred mg of the gamma fraction of the rabbit anti-bovine alpha₂M serum were mixed 2g of activated Sepharose 4B resin. The resin was treated accordingly to manufacturer instructions (Pharmacia). Eluates from chromatography on Sepharose CL-6B containing bovine alpha₂M were submitted to immunoaffinity chromatography on Sepharose 4B resin bound to rabbit anti-bovine alpha₂M antibodies. The column was equilibrated with 0.1 M borate buffer, pH 8.0 and alpha₂M was eluted by passing 2.5 N potassium thiocyanate through the column. The experimental conditions were 7.4 ml/h flow and 3.7 ml per tube.

6- Protein assay

All eluates were assayed by the method of LOWRY et al.¹⁴ (1951), except those from the affinity chromatography column which were assayed by the macro Kjeldahl method as described by TASTALDI¹² (1965).

7- Radial immunodiffusion (MANCINI et al.¹⁵, 1965)

The alpha₂M samples obtained by affinity chromatography were used as primary standard for radial immunodiffusion. Agarose plates were prepared with the gamma fraction of the anti-bovine alpha₂M serum mixture. The results of immunodiffusion were plotted as the standard concentrations against the square diameters of the diffusion and precipitation haloes, respectively. Unknown alpha₂M samples were then evaluated concerning the different steps of purification in this system.

8- Binding capacity

The capacity of alpha₂M to bind proteolytic enzymes, protecting them from the action of their physiological inhibitors was tested according to BARRETT; STARKEY⁴ (1973). Trypsin and plasmin were initially titrated with p'NPGb, by the method of CHASE; SHAW⁶ (1967), and then assayed with bovine alpha₂M obtained by affinity chromatography. The free enzyme was inhibited by adding SBTI, while the activity of the "protected enzyme" (bound to alpha₂M) was determined by hydrolysis of the substrates TAME (p-tosyl-arginine-methyl ester) for trypsin and the synthetic tripeptide H-D-Val-Leu-Lys-p-Na for plasmin. The trypsin and plasmin assays, and their ester activities were determined by the method of SIEGELMAN et al.¹⁹ (1962).

RESULTS

After precipitation of fractionat euglobulin, bovine plasma containing oxalate at the concentration of 84 mg/ml yielded a material containing α_2 M at the concentration of 44mg/ml. This crude α_2 M solution was submitted to gel filtration chromatography with a linear NaCl gradient, as determined by agarose gel electrophoresis and immunoelectrophoresis against anti-human α_2 M serum.

The eluates containing semi-purified α_2 M at 30mg/ml concentration were passed through a Sepharose CL-6B column equilibrated with 0.02 M potassium phosphate, pH 8.0.

The purity of the fractions containing α_2 M was determined by SDS-polyacrylamide gel electrophoresis. On the basis of the molecular weight standards, the α_2 M dimers (MW = 360,000) were found to be still slightly contaminated. Immunoelectrophoresis of these fractions against anti-human IgG and IgM sera revealed a slight contamination with IgG.

α_2 M fractions corresponding to the eluates from the Sepharose CL-6B column were injected into non-pregnant female rabbits at a weekly dose of 780ug antigen. The antibody produced was tested by immunodiffusion and when it reached the 1:256 titer the animals were bled. To obtain the gamma fraction of the anti-bovine α_2 M serum, immunized rabbit serum (approximately 7 ml at 160mg/ml concentration) was submitted to chromatography on DEAE-cellulose with 0.05 M potassium phosphate buffer, pH 8.0, with a yield of 100mg protein. The presence of anti- α_2 M antibodies in the eluates was confirmed by immunodiffusion using bovine α_2 M preparations.

To further purify the chromatographic preparations containing α_2 M, the material was submitted to affinity chromatography on Sepharose 4B, with the resin coupled to 4 ml anti-bovine α_2 M serum at 25 mg/ml concentration. The solution containing α_2 M used in this experiment contained 13.6 mg protein, corresponding to the Sepharose CL-6B eluates. Sepharose 4B was equilibrated with 0.1 M borate buffer, pH 8.0. α_2 M was mobilized by passing 2.5 N potassium thiocyanate. The experiment yielded a highly purified α_2 M solution at 14.24 mg% concentration, which was considered as the primary standard for subsequent tests.

The next step in the study was to determine α_2 M concentration during the various stages of purification by radial immunodiffusion (MANCINI et al.¹⁵, 1965) in wich anti-bovine α_2 M serum was incorporated into agarose. Using the standard α_2 M fraction, a curve was construted by plotting the standard sample concentrations against the squares of the diameters of the respective precipitation haloes. Fig. 1

shows the linear relationship existing between the two parameters.

Plasmin and trypsin were the enzymes used in the protease-binding assays with α_2 M.

Tab. 1 presents the results of the amidolytic activity of plasmin on its specific substrate (H-D-Val-Leu-Lys-p-NA). The enzyme bound to the α_2 M inhibitor maintained only 58% of its activity, a phenomenon also observed by GONIAS; PIZZO¹¹ (1983) in a study on a human α_2 M plasmin complex. The present results show that in the α_2 M plasmin complex, part of the plasmin was bound and its affinity for the substrate was modified, and part was not bound and was blocked in the presence of SBTI.

Tab. 2 shows the results of the esterase activity of the α_2 M-trypsin complex in the presence and absence of SBTI: 35% of the enzymatic activity of trypsin was main-

TABLE 1

Amidolytic activity of free and α_2 M bound plasmin with and without SBTI. Ribeirão Preto, 1985.

plasmin ug	α_2 M ug	SBTI ug	H-D-Val-Leu-Lys-p-Na substrate (Absorbance at 410nm)*
14.4	-	-	0.72
14.4	194	-	0.42-58%
14.4	194	-	0.20-27.7%

* Mean of 6 determinations.

tained when α_2 M and SBTI were present, corresponding to the "protected enzyme" phenomenon. However, when SBTI was omitted and total expression of the bound or free enzyme should have theoretically occurred, only 65% of the esterase activity was maintained, confirming that the affinity of the enzyme for the substrate changes when a complex with the inhibitor (α_2 M) is formed.

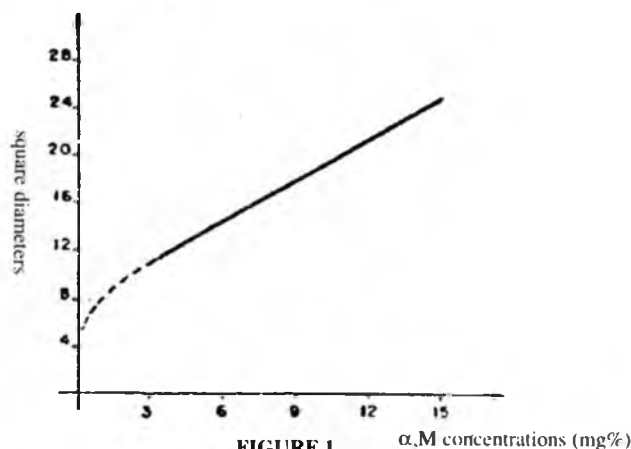


FIGURE 1
Relationship between sample concentrations and the squares of the diameters (d)² of the precipitation haloes.

TABLE 2

Esterolytic activity of free and alpha₂M bound trypsin with and without SBTI. Ribeirão Preto, 1985.

trypsin ug	alpha ₂ M ug	SBTI	TAME (Absorbance at 580 nm)*
2.16	-	-	0.40
2.16	194	-	0.26-65%
2.16	194	400	0.14-35%

* Mean of 5 determinations.

DISCUSSION

The initial objective of the present study was to purify alpha₂M and to determine the binding ratio between bovine alpha₂M and trypsin and plasmin, since the results reported in the literature are controversial. In the alpha₂M-protease binding tests we observed that increasing amounts of alpha₂M protected a maximum of 50% of the amidolytic activity when plasmin was used, and 35% of the esterolytic activity when trypsin was used. In the experiments in which SBTI was omitted, the values indicated reduced activity, when, according to the theory of BARRET; STARKEY⁴ (1973), they should have been identical to those of the protease alone (Tab. 1 and 2).

An explanation for these results may be the presence of other alpha₂M inhibitors in the preparations. This hypothesis was not tested in this study; however, NAGASAWA et al.¹⁶ (1970) stated that bovine alpha₂M binding does not affect the activity of trypsin on TAME but reduces by 35% the activity on the lysine analogue TLME (p-tosyl-lysine-methyl ester) and by 77% the activity on BAEE (benzoyl-arginine-ethyl ester). In

an attempt to clarify these discrepancies, we may propose the following line of reasoning. We know that the alpha₂M molecule undergoes conformational changes when it binds to proteinases (JAMES¹², 1990). The conformation acquired by the molecule of the inhibitor is essential for the proteinase to be inactivated and depends on the methods of alpha₂M preparation. Precipitation with ammonium sulfate is one of the methods used by several investigators for alpha₂M extraction from bovine or human plasma (NAGASAWA et al.¹⁶, 1970). However, BARRET et al.³ (1979) reported that alpha₂M purified in the presence of ammonium ions suffers a conformational change from the S-alpha₂M to the F-alpha₂M form, the slow (S) and fast (F) migration forms, respectively, and that the S-alpha₂M form, by binding to the protease, is converted to the F-alpha₂M form, which loses its ability to bind proteases, as also confirmed by others (SOTTRUP-JENSEN²⁰, 1989). However, SWENSON et al.²¹ (1979) have reported data on the interaction of the alpha₂M-protease complex with inhibitors such as SBTI which are considered to be inaccessible to the protected enzyme. These data explain in part why the "protected" enzyme treated with SBTI presented reduced activity. According to these investigators, the reaction of the alpha₂M-protease complex with SBTI is qualitatively similar to that of the enzyme-SBTI. In quantitative terms, the difference is great but depends on the SBTI/trypsin molar ratio. With a 50 ratio, 45% of the alpha₂M protease activity is inhibited within approximately 2 hours. In the present experiments, the incubations with SBTI lasted only 15 minutes before the addition of the substrate but the molar ratio was high and possibly contributed to the inhibition of the alpha₂M-protease complex, with a consequent partial protection of alpha₂M against the proteolytic enzyme.

RESUMO

Alpha₂ Macroglubulina, uma proteína inibidora de proteases, foi isolada do plasma bovino. O processo de purificação foi monitorado por imunodifusão e imunoelctroforese cruzada com soro anti alpha₂M-humana. Para as determinações quantitativas foi preparado um soro anti alpha₂M bovino. A preparação mais pura de alpha₂M foi obtida por cromatografia de afinidade e usada como padrão primário na imunodifusão radial de Mancini. Preparações de alpha₂M foram usadas em testes de ligação com tripsina e plasmina (tituladas com NPGB). Nos testes de ligação 50% de plasmina e 35% de tripsina foram "protegidas" pela alpha₂M. Não foi possível determinar se houve ineficiência na ligação ou se a perda de atividade ocorreu por alterações na afinidade do complexo alpha₂M-protease, em relação aos substratos usados.

UNITERMOS: Bovinos; Plasma; Inibidores da protease; Alpha₂M; Plasmina; Tripsina

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