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INTERACTION OF BOVINE ALPHA$_2$ MACROGLOBULIN WITH PROTEINASES

INTERAÇÃO DE ALPHA$_2$ MACROGLOBULINA BOVINA COM PROTEINASES

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SUMMARY

The alpha$_2$-macroglobulin (alpha$_2$M) protease inhibitor was purified from bovine plasma. The alpha$_2$M preparations at various purification steps were identified by immunodiffusion and crossed immunoelectrophoresis with anti-human alpha$_2$ serum. Anti-bovine alpha$_2$M serum was prepared for the quantitative determinations. The purest alpha$_2$M preparation was obtained by affinity chromatography and used as primary standard in radial immunodiffusion. Alpha$_2$M preparations were submitted to binding tests with p' - NPGB (p' - nitrophenyl-p-guanidine-benzoate HCL) titrated trypsin and plasmin. Alpha$_2$M protected 35% of the esterolytic activity of trypsin and 50% of the amidolytic activity of plasmin.

UNITERMS: Cattle; Plasma; Proteinase inhibitors; Alpha$_2$M; 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). Alpha$_2$M is one of the major protease inhibitors of blood plasma.

Human alpha$_2$-macroglobulin (alpha$_2$M) 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-JENSEN[20], 1989).

Free and alpha$_2$-macroglobulin-bound proteinase molecules retain to various degrees their ability to hydrolyze small substrates (BARRET; STARKEY[4], 1973).The hydrolysis rates of chromogenic substrates by trypsin, chymotrypsin, elastase or plasmin when bound to alpha$_2$M 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, alpha$_2$-macroglobulin dramatically decreases the affinity of proteinases for macromolecular inhibitors or their catalytic power on macromolecular substrates (GANROT; NIHEHN[10], 1983; NAGASAWA et al.[16], 1970; BIETH[5], 1981), inhibitors or antibodies (CREWS et al.[7], 1987). Proteins homologous to human alpha$_2$M have been isolated and characterized from a number of mammalian plasmas (ANDERSEN; KROLE[1], 1975). In the present study, alpha$_2$M was isolated from bovine plasma.

In an attempt to contribute to a better understanding of the mechanism of action of alpha$_2$M, the objectives of the present study were: 1) to purify alpha$_2$M by existing or adapted methods; 2) to quantify alpha$_2$M by immunlogical reactions; 3) to determine the binding properties of bovine alpha$_2$M with plasmin and trypsin.

MATERIAL AND METHOD

1-Preparation of starting material containing alpha$_2$M

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^{-4}M 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 alpha$_2$M

The crude material containing alpha$_2$M was submitted to chromatography on a DEAE-Sephadex A-50 column equilibrated with 0.02 M potassium phosphate buffer, pH 8.0. Alpha$_2$M 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 alpha$_2$M 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-alpha$_2$M human serum. The eluates containing semi- purified alpha$_2$M, 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 LAEMMLI[13] (1970). The separation gel contained 7.5% acrylamide and the stackin 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 alpha$_2$M

a-Immunodiffusion by the method of OUCHTERLONY; NILSOON[17] (1986).

For alpha$_2$M quantitation, the eluates obtained by Sepharose CL-6B chromatography were submitted to immunodiffusion against anti-human alpha$_2$M 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 alpha$_2$M, anti-human IgG and IgM sera (Behring) and total anti-human serum were used in these experiments.

4- Preparation of bovine anti alpha$_2$M serum

The Sepharose CL-6B eluates containing highly purified alpha$_2$M, 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 alpha$_2$M 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 alpha$_2$M 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 el al.[2], 1974)

One hundred mg of the gamma fraction of the rabbit anti-bovine alpha$_2$M 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 alpha$_2$M were submitted to immunoaffinity chromatography on Sepharose 4B resin bound to rabbit anti-bovine alpha$_2$M antibodies. The column was equilibrated with 0.1 M borate buffer, pH 8.0 and alpha$_2$M 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 TASTALDI[22] (1965).

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

The alpha$_2$M 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 alpha$_2$M serum mixture. The results of immunodiffusion were plotted as the standard concentrations against the square diameters of the diffusion and precipitation haloes, respectively. Unknown alpha$_2$M samples were then evaluted concerning the different steps of purification in this system.

8- Binding capacity

The capacity of alpha$_2$M to bind proteolytic enzymes, protecting them from the action of their physiological inhibitors was tested according to BARRETT; STARKEY[4] (1973). Trypsin and plasmin were initially titrated with p'NPGB, by the method of CHASE; SHAW[6] (1967), and then assayed with bovine alpha$_2$M obtained by affinity chromatography. The free enzyme was inhibited by adding SBTI, while the activity of the "protected enzyme" (bound to alpha$_2$M) 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 alpha$_2$M at the concentration of 44mg/ml. This crude alpha$_2$M solution was submitted to gel filtration chromatography with a linear NaCl gradient, as determined by agarose gel electrophoresis and immunoelectrophoresis against anti-human alpha$_2$M serum.

The eluates containing semi-purified alpha$_2$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 alpha$_2$M was determined by SDS-polyacrylamide gel electrophoresis. On the basis of the molecular weight standards, the alpha$_2$M dimers (MW = 360,000) were found to be still slightly contamined. Immunoelectrophoresis of these fractions against anti-human IgG and IgM sera revealed a slight contamination with IgG.

Alpha$_2$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 alpha$_2$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-alpha$_2$M antibodies in the eluates was confirmed by immunodiffusion using bovine alpha$_2$M preparations.

To further purify the chromatographic preparations containing alpha$_2$M, the material was submitted to affinity chromatography on Sepharose 4B, with the resin coupled to 4 ml anti-bovine alpha$_2$M serum at 25 mg/ml concentration. The solution containing alpha$_2$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. Alpha$_2$M was mobilized by passing 2.5 N potassium thiocyanate. The experiment yielded a highly purified alpha$_2$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 alpha$_2$M concentration during the various stages of purification by radial immunodiffusion (MANCINI et al.[15], 1965) in wich anti-bovine alpha$_2$M serum was incorporated into agarose. Using the standard alpha$_2$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-binging assays with alpha$_2$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 alpha$_2$M inhibitor maintained only 58% of its activity, a phenomenon also observed by GONIAS; PIZZO[11] (1983) in a study on a human alpha$_2$M plasmin complex. The present results show that in the alpha$_2$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 alpha$_2$M-trypsin complex in the presence and absence of SBTI: 35% of the enzimatic activity of trypsin was maintained when alpha$_2$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 (alpha$_2$M) is formed.

TABLE 1
Amidolytic activity of free and alpha$_2$M bound plasmin with and without SBTI. Ribeirão Preto, 1985.

plasmin ug	alpha$_2$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.

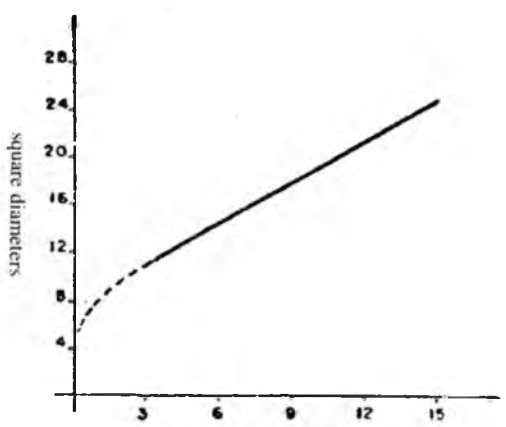

FIGURE 1
Relationship between sample concentrations and the squares of the diameters $(d)^2$ of the precipitation haloes.

TABLE 2
Esterolytic activity of free and alpha$_2$M 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 alpha$_2$M and to determine the binding ratio between bovine alpha$_2$M and trypsin and plasmin, since the results reported in the literature are controversial. In the alpha$_2$M-protease binding tests we observed that increasing amounts of alpha$_2$M 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; STARKEY[4] (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 alpha$_2$M inhibitors in the preparations. This hypothesis was not tested in this study; however, NAGASAWA et al.[16] (1970) stated that bovine alpha$_2$M 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 alpha$_2$M molecule undergoes conformational changes when it binds to proteinases (JAMES[12], 1990). The conformation acquired by the molecule of the inhibitor is essential for the proteinase to be inactivated and depends on the methods of alpha$_2$M preparation. Precipitation with ammonium sulfate is one of the methods used by several investigators for alpha$_2$M extraction from bovine or human plasma (NAGASAWA et al.[16], 1970). However, BARRET et al.[3] (1979) reported that alpha$_2$M purified in the presence of ammonium ions suffers a conformational change from the S-alpha$_2$M to the F-alpha$_2$M form, the slow (S) and fast (F) migration forms, respectively, and that the S-alpha$_2$M form, by binding to the protease, is converted to the F-alpha$_2$M form, which looses its ability to bind proteases, as also confirmed by others (SOTTRUP-JENSEN[20], 1989). However, SWENSON et al.[21] (1979) have reported data on the interaction of the alpha$_2$M-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 alpha$_2$M-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 alpha$_2$M 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 alpha$_2$M-protease complex, with a consequent partial protection of alpha$_2$M against the proteolytic enzyme.

RESUMO

Alpha$_2$ Macroglobulina, uma proteína inibidora de proteases, foi isolada do plasma bovino. O processo de purificação foi monitorado por imunodifusão e imunoeletroforese cruzada com soro anti alpha$_2$M-humana. Para as determinações quantitativas foi preparado um soro anti alpha$_2$M bovino. A preparação mais pura de alpha$_2$M foi obtida por cromatografia de afinidade e usada como padrão primário na imunodifusão radial de Mancini. Preparações de alpha$_2$M 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 alpha$_2$M. Não foi possível determinar se houve ineficiência na ligação ou se a perda de atividade ocorreu por alterações na afinidade do complexo alpha$_2$M-protease, em relação aos substratos usados.

UNITERMOS: Bovinos; Plasma; Inibidores da protease; Alpha$_2$M; Plasmina; Tripsina

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