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Human Cathepsin B1

INHIBITION BY α_2-MACROGLOBULIN AND OTHER SERUM PROTEINS

By PHYLLIS M. STARKEY and ALAN J. BARRETT
Tissue Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

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1. Normal human serum was found to inhibit human cathepsin B1. 2. The major inhibitor present in serum was purified and identified as α_2-macroglobulin. 3. α_2-Macroglobulin was found to bind cathepsin B1 in an approximately 1:1 molar ratio. When bound, the enzyme retained about 50% of its proteolytic activity, and up to 80% of its activity against α-*N*-benzoyl-DL-arginine 2-naphthylamide. 4. Pretreatment of α_2-macroglobulin with cathepsin B1 inactivated by exposure to pH 8.5 or iodoacetic acid, in large molar excess, did not prevent the subsequent binding of active enzyme. Active enzyme, once bound, was not protected from inhibition by 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one. 5. Cathepsin B1 was also inhibited by human immunoglobulin G, at high concentration. 6. Because it had been suggested that haptoglobin is responsible for the inhibition of 'cathepsin B' by serum, a method was devised for the selective removal of haptoglobin from mixtures of serum proteins by adsorption on haemoglobin covalently linked to Sepharose. No evidence was obtained that haptoglobin has any inhibitory activity against the enzyme.

Cathepsin B1 (EC 3.4.4.–) is a thiol-dependent proteolytic enzyme, of molecular weight about 25000, present in many animal tissues [see Barrett (1973) and review by Barrett (1972*a*)].

We have undertaken a programme of work on cathepsin B1 because of its possible importance in the catabolism of cartilage matrix (Morrison *et al.*, 1973). In the course of this work we became aware of the existence in human serum and tissue extracts of inhibitors of the enzyme. Because of the possible importance of such factors in the physiological functions of cathepsin B1, we undertook the isolation and identification of the factors in human serum.

Materials

Cathepsin B1 prepared from human liver by the method of Barrett (1973) had a specific activity of approximately 5.0 units/mg when assayed with α-*N*-benzoyl-DL-arginine 2-naphthylamide as substrate (Barrett, 1972*b*). Cathepsin B1 denatured by exposure to alkaline pH was prepared by adjusting a solution of 120μg of cathepsin B1/ml to pH 8.5 with 2M-Tris–HCl, pH 9.0. The solution was left overnight and then adjusted to pH 6.5 with 1M-HCl. Cathepsin B1 inactivated by carboxymethylation was prepared by first incubating 1.22mg of cathepsin B1 in a total volume of 2ml of 2mM-cysteine, pH 6.5, for 10min to ensure that all the thiol groups on the enzyme were in a reduced form, and then the solution was made 20mM with iodoacetic acid, left for 1h, and dialysed overnight against 2 litres of 20mM-sodium–potassium phosphate buffer, pH 6.5.

Tos-PheCH$_2$Cl (TPCK, 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one) was obtained from Calbiochem Ltd., London W1H 1AS, U.K. A 200μM stock solution was made freshly in propan-2-ol. Sepharose 4B and 6B and Sephadex G-75 (superfine) were obtained from Pharmacia (G.B.) Ltd., London W.13, U.K.; DEAE-cellulose was Whatman DE-52 from W. and R. Balston Ltd., Maidstone, Kent, U.K. BRIJ-35 was obtained from British Drug Houses, Poole, Dorset, U.K.

Ultrafiltration was carried out in a Diaflo 50 apparatus fitted with an XM-50 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.).

Pooled human serum from 100 normal donors was generously supplied by Dr. C. Entwhistle, Immunohaematology Unit, Addenbrooke's Hospital, Cambridge. Before use in inhibition experiments, the serum was adjusted to pH 6.9 with 5M-sodium formate buffer, pH 2.9.

Sera and synovial fluids of known haptoglobin concentration from arthritic patients were kindly supplied by Dr. A. Howard, Medical Research Council Rheumatology Unit, Canadian Red Cross Memorial Hospital, Taplow, Bucks., U.K. The determinations of haptoglobin content had been made by a modification of the gel-filtration method of Ratcliffe & Hardwicke (1964).

Immunoglobulins from rabbit antisera against

human serum protein, human serum α_2-macroglobulin and human serum haptoglobin produced by Dakopatts, Copenhagen, Denmark, were obtained from Mercia Diagnostics Ltd., Watford, Herts., U.K.; their specificities were confirmed before they were used, by immunoelectrophoresis. α-*N*-Benzoyl-DL-arginine 2-naphthylamide and benzyloxycarbonyl-L-arginine 4-methoxynaphthylamide were obtained from Bachem Ltd., Liestal, Switzerland.

Methods

Preparation of azo-haemoglobin

A solution of bovine haemoglobin was prepared as described by Barrett (1970); 5M-NaOH (24ml) was mixed with 200ml of water, and 10g of sulphanilic acid (AnalaR grade) was dissolved in the dilute alkali, followed by 4.4g of $NaNO_2$. HCl (5M, 36ml) was introduced and the solution was stirred for 2min, then 5M-NaOH (36ml) was added and the complete mixture was immediately poured into 1 litre of bovine haemoglobin solution (5%, w/v) in $NaHCO_3$ (1%, w/v), with stirring.

The solution was adjusted to pH4 with acetic acid, and the precipitated protein was removed by centrifugation, redissolved in dilute NaOH, and reprecipitated as before. The final pellet was redissolved in 1M-$NaHCO_3$ and dialysed thoroughly against glass-distilled water. The solution was freeze-dried and stored at −20°C. The $E_{366}^{1\%}$ of the azo-haemoglobin was 75.9. A sample run on a column of Sephadex G-75 (superfine grade) was partially separated into three components; the elution volumes were consistent with these being monomers, dimers and tetramers of the haemoglobin subunits.

Preparation of haemoglobin–Sepharose

Haemoglobin–Sepharose was made by coupling 100g of Sepharose 4B, activated by a method based on those of Cuatrecasas & Anfinsen (1971) and Axén & Ernback (1971), with 800mg of bovine haemoglobin prepared as described by Barrett (1970).

Enzyme assays

The activity of cathepsin B1 against α-*N*-benzoyl-DL-arginine 2-naphthylamide ('naphthylamidase activity') was assayed as described by Barrett (1972*b*). In this procedure, the enzymic reaction is stopped by the introduction of a 'coupling reagent' containing 4-chloromercuribenzoate and a diazonium salt, Fast Garnet GBC, that converts the free 2-naphthylamine resulting from hydrolysis of the substrate into a red azo-dye, the concentration of which is determined spectrophotometrically. One unit of activity was defined as that amount hydrolysing 1μmol of substrate per min under the assay conditions. This unit is 1000 times that used by Barrett (1972*b*). The concentration of protein in cathepsin B1 preparations was calculated from the E_{280}, on the basis that the $E_{280}^{1\%} = 20$ (Barrett, 1973).

The naphthylamidase activity of plasmin and thrombin was measured in the same way, except that 0.1M-Tris–HCl, pH8.0, without cysteine or EDTA, was used as assay buffer. The coupling reagent was 0.25mg of Fast Garnet GBC/ml in 0.5M-sodium acetate buffer, pH4.3, containing 2% (w/v) BRIJ-35.

Proteolytic activity was measured at pH6.0 in incubation mixtures (1.0ml) containing enzyme sample (0.1ml, approx. 0.3 unit), inhibitor solution or diluent (0.4ml), 40mM-cysteine, 20mM-disodium EDTA made freshly (0.05ml), 0.5M-sodium–potassium phosphate buffer, pH6.0 (0.2ml), and 5% (w/v) azo-haemoglobin (0.25ml). After 30min at 40°C, the reaction was stopped by addition of 5ml of 3.0% (w/v) trichloroacetic acid, and the mixtures were filtered. The E_{366} of the trichloroacetic acid-soluble reaction products was determined. Blanks were prepared by addition of the enzyme sample only after the reaction had been stopped with trichloroacetic acid.

Determination of inhibitory activity

Activity in the presence of inhibitor was expressed as a percentage of the activity of the enzyme in the absence of inhibitor. Unless otherwise stated, the naphthylamidase activity of the enzyme was used.

The concentration of purified serum protein preparations was determined from $E_{277}^{1\%} = 9.12$ for α_2-macroglobulin (Dunn & Spiro, 1967) and $E_{280}^{1\%} = 14.0$ for immunoglobulin G* (Little & Donahue, 1968). The mean concentrations of α_2-macroglobulin and IgG in healthy adult human serum were taken to be 265mg/100ml and 1250mg/100ml respectively (Becker *et al.*, 1969). In calculations of molar ratios, the molecular weight of human cathepsin B1 was taken to be 27000 (Barrett, 1973), that of human α_2-macroglobulin 820000 (Dunn & Spiro, 1967; Frénoy *et al.*, 1972), and of IgG 160000 (Fudenberg, 1967).

Immunoelectrophoresis and gel diffusion analysis

Immunoelectrophoresis plates (8cm×8cm) were coated with 1% (w/v) Meath agarose (from Medical and Biological Instrumentation Ltd., Ashford, Kent, U.K.) in buffer containing 21.6mM-barbitone sodium, 21.6mM-sodium acetate and 0.01% thimerosal, pH8.2. The capacity of the antigen well was 5μl, and of the antiserum trough 50μl. Electrophoresis was for 1.5h at 25mA/plate.

* Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A.

For gel diffusion analysis by the method of Ouchterlony (1967) plates were coated with 1% agarose in sodium–potassium phosphate buffer (20mM, pH 6.0) including 0.15M-NaCl. The developed plates were photographed with dark-ground illumination by use of Agfa Scientia 39 C 56 film.

To stain for lipoprotein, the plates were washed for 48h in phosphate-buffered saline (0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.12% Na_2HPO_4) containing 1% (v/v) butan-1-ol, washed for 4h in water, and then dried. The dried plate was immersed in 60% ethanol saturated with Sudan Black for approximately 10min, rinsed in ethanol, then water and counterstained for protein in Ponceau S (0.1% in 0.55M-acetic acid, 0.05M-NaOH) for 5min, and finally rinsed in water and dried.

To stain for cathepsin B1 activity, a method based on the assay method of Barrett (1972*b*) was used. The gels were incubated at 40°C in 0.1M-sodium–potassium phosphate buffer, pH 6.0 containing 1.33mM-disodium EDTA, 2.67mM-cysteine, and 0.5mg of benzyloxycarbonyl-L-arginine 4-methoxynaphthylamide/ml for 30min. The buffer was then replaced by the coupling reagent containing 1mg of Fast Garnet GBC/ml, 5mM-4-chloromercuribenzoic acid and 25mM-disodium EDTA, pH 6.0. During about 15min the coupling reagent reacted with the 4-methoxynaphthylamine produced by cathepsin B1 activity to give a red precipitate.

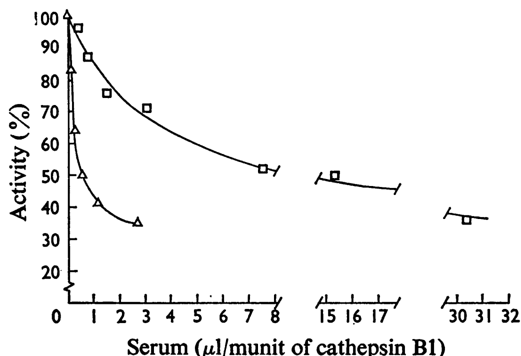


Fig. 1. *Inhibition of cathepsin B1 by whole human serum*

Mixtures of cathepsin B1 with human serum were made up to 0.50ml with 50mM-sodium acetate buffer, pH 5.5, and left at 20°C for 5min. Assays were made for naphthylamidase activity (with 13.2munits of cathepsin B1, and 5–400μl of serum/tube) (□), and for proteolytic activity (with 165munits of cathepsin B1, and 20–450μl of serum/tube) (△). Enzymic activity is expressed as a percentage of that in the absence of serum. An equimolar ratio of cathepsin B1 and α_2-macroglobulin was obtained at a serum concentration of 2.3μl/munit of cathepsin B1.

Results

Inhibition and binding of cathepsin B1 by human serum

Inhibition. Human serum was shown to inhibit to different degrees the proteolytic and naphthylamidase activities of human cathepsin B1 (Fig. 1). An amount of serum (2.7μl/munit of cathepsin B1) that inhibited the proteolytic activity by 65%, gave only 30% inhibition of the naphthylamidase activity; at this point the relative molar proportions of cathepsin B1, α_2-macroglobulin and IgG were 1, 1.18 and 28.5.

Binding of cathepsin B1 to a serum component. Human serum (5ml) was mixed with 4.75ml of a buffer containing 50mM-sodium–potassium phosphate, pH 6.0, 100mM-NaCl, 1mM-disodium EDTA and 1% (v/v) butan-1-ol. Cathepsin B1 (1.5 units in 0.25ml) was introduced, and the complete mixture was incubated at 4°C for 1h. The mixture was applied to a column (2.5cm × 88cm, 430cm³) of Sephadex G-75 (superfine grade) equilibrated with the pH 6 buffer. The sample was eluted with the same buffer at 4°C and a flow rate of 19ml/h; 3.9ml fractions were collected and assayed for naphthylamidase activity.

Two peaks of activity were eluted from the column (Fig. 2); the first appeared at the void volume of the column, and analysis of the fractions by immunoelectrophoresis showed the naphthylamidase activity to be closely correlated with the distribution of α_2-macroglobulin. The second peak of activity was eluted at the volume characteristic of free cathepsin B1 (determined in a control experiment without serum).

The fractions representing the first peak (34.5–36% bed vol.) were pooled and concentrated by ultrafiltration. The pool was run in double-immunodiffusion against rabbit anti-(human serum protein) IgG, rabbit anti-(human haptoglobin)IgG and rabbit anti-(human α_2-macroglobulin) IgG (Plate 1*a*). The gel-diffusion plate was washed for 48h at 4°C in 0.1M-sodium–potassium phosphate buffer, pH 6.0, containing 1.33mM-disodium EDTA, and stained for cathepsin B1 activity (see the Methods section). The coloured reaction product appeared over precipitin lines formed against the anti-(α_2-macroglobulin) IgG, but not anti-(haptoglobin) IgG; against the anti-(human serum protein) IgG there was a weak reaction only over a line showing identity with the specific anti-(α_2-macroglobulin) line (Plate 1*b*).

It was concluded that human α_2-macroglobulin has the capacity to bind cathepsin B1; there was no evidence for binding by any other serum protein under these conditions.

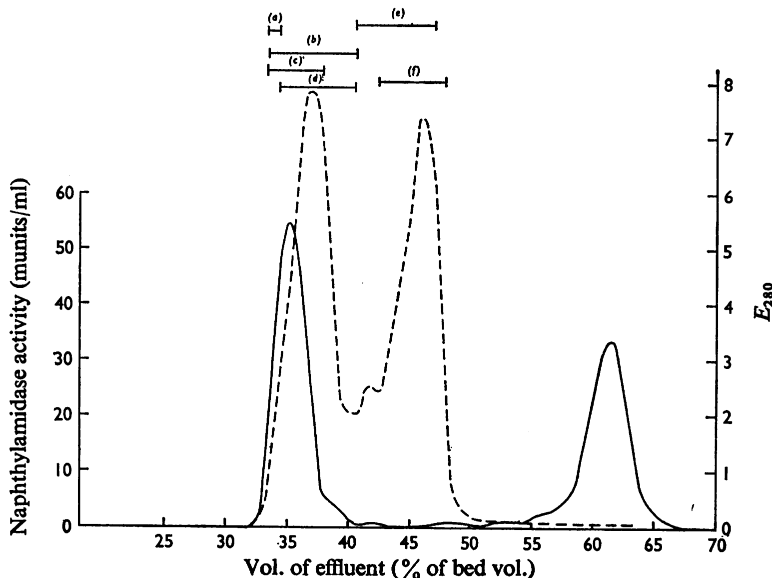


Fig. 2. *Gel chromatography of a mixture of human serum and cathepsin B*1

Fractions eluted from a column of Sephadex G-75 (superfine grade) were analysed for protein as E_{280} (---) and naphthylamidase activity (——). The distribution of various serum proteins, determined by immunoelectrophoresis, is indicated by the horizontal bars. (See the text for details.) (*a*) IgM; (*b*) haptoglobin; (*c*) α_2-macroglobulin; (*d*) IgG; (*e*) α_1-antitrypsin; (*f*) albumin.

Analysis of fractions obtained by chromatography of human serum alone under these conditions revealed no naphthylamidase activity at pH6.0. Assays for naphthylamidase activity at pH8.0 were also negative both with whole serum and purified α_2-macroglobulin.

Purification of the inhibitors from human serum

Removal of lipoprotein. Lipoproteins were removed from the pooled serum as described by Fischer (1969, p. 239). Immunoelectrophoresis plates developed with rabbit anti-(human serum protein) IgG showed that all lipoproteins stainable with Sudan Black had been removed by this treatment. The inhibitory capacity of the serum was unaffected.

Chromatography on Sepharose 6B. The lipid-free serum was applied to a column of Sepharose 6B (2.5cm×100cm, 490cm^3) that had been equilibrated with buffer containing 20mM-Tris–HCl, pH7.8, 200mM-NaCl, 2mM-disodium EDTA and 1% (v/v) butan-1-ol. The column was run at a flow-rate of 25ml/h at 4°C. Three peaks of inhibitory activity were eluted from the column (Fig. 3). Analysis of the fractions by immunoelectrophoresis showed that the major peak of activity, peak 1 (50–59% bed vol.) was associated with the high-molecular-weight proteins α_2-macroglobulin, IgM, IgA and haptoglobin.

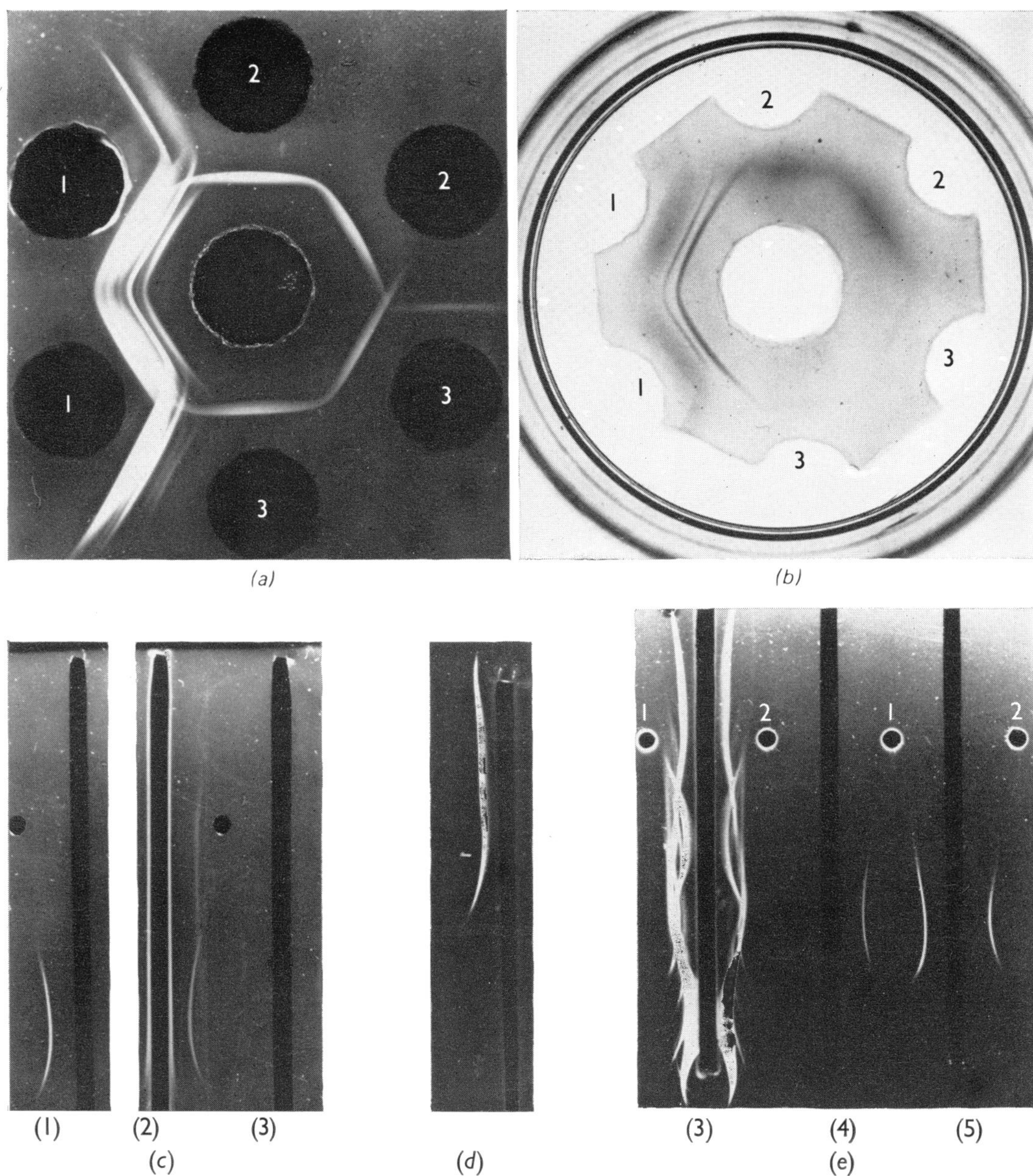
All the fractions comprising the second peak of inhibitory activity contained IgG, but other proteins were present too.

The third peak of inhibitory activity was due to a diffusible component, perhaps the Ca^{2+} introduced into the serum during the removal of lipoprotein. In a separate experiment, 2.5mM-$CaCl_2$ inhibited the naphthylamidase activity of human cathepsin B1 by 25%.

Chromatography on DEAE-cellulose. The high-molecular-weight fraction from gel filtration was applied to a column (1.5cm×9cm, 16cm^3) of DEAE-cellulose that had been equilibrated with buffer containing 50mM-Tris–HCl, pH7.8. The sample was eluted with a linear gradient (total volume 100ml) of 0–0.3M-NaCl in the same buffer.

α_2-Macroglobulin was shown by immunoelectrophoresis to be partially separated from IgM, IgA and haptoglobin, and the peak of inhibitory activity was most closely associated with that of α_2-macroglobulin.

Removal of haptoglobin by use of haemoglobin–Sepharose. The distribution of inhibitory activity in the effluent fractions from Sepharose 6B and DEAE-



EXPLANATION OF PLATE 1

Immunological characterization of protein fractions

(*a*) Immunodiffusion of pool representing peak 1 from the chromatography (Fig. 2) of a mixture of serum and cathepsin B1 on Sephadex G-75 (superfine grade) (centre well) against (1) rabbit anti-(human serum protein) IgG, (2) rabbit anti-(human α_2-macroglobulin) IgG and (3) rabbit anti-(human haptoglobin) IgG. (*b*) The immunodiffusion pattern shown in (*a*), but stained for cathepsin B1 activity (see the Methods section). The red reaction product is seen at wells (2) and less strongly at (1), whereas the sharp lines seen elsewhere are due to non-specific yellow staining of precipitin lines by the Fast Garnet GBC. (*c*) Immunoelectrophoresis of purified α_2-macroglobulin (in both wells) developed with (1) rabbit anti-(human α_2-macroglobulin) IgG, (2) rabbit anti-(human serum protein) IgG and (3) rabbit anti-(human haptoglobin) IgG. (*d*) Immunoelectrophoresis of purified IgG developed with rabbit anti-(human serum protein) IgG. (*e*) Immunoelectrophoresis of human serum before and after treatment with haemoglobin–Sepharose. The wells contain (1) untreated serum and (2) serum treated with haemoglobin–Sepharose. The troughs contain (3) rabbit anti-(human serum protein) IgG, (4) rabbit anti-(human haptoglobin) IgG and (5) rabbit anti-(human α_2-macroglobulin) IgG.

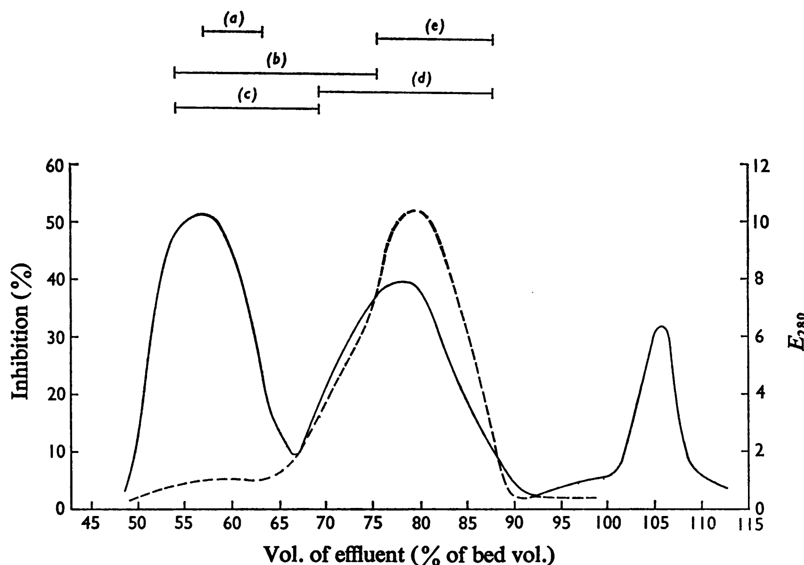


Fig. 3. *Chromatography of lipoprotein-free serum on Sepharose 6B*

The distribution of protein as E_{280} (---), and of inhibitory activity against cathepsin B1 in the naphthylamidase assay (——), is shown. Inhibition is expressed as the difference between activity in the presence and in the absence of the sample, as a percentage of activity in the absence of inhibitor. The distribution of various serum proteins, determined by immunoelectrophoresis, is indicated by the horizontal bars. (See the text for details.) (*a*) IgM or IgA; (*b*) haptoglobin; (*c*) α_2-macroglobulin; (*d*) IgG; (*e*) α_1-antitrypsin.

cellulose suggested that the inhibitor was likely to be either α_2-macroglobulin or haptoglobin. Several attempts at the complete separation of these proteins by re-chromatography on Sepharose 6B or DEAE-cellulose were unsuccessful, perhaps partly because of the genetically determined heterogeneity of haptoglobin in pooled human serum.

Because of the capacity of haptoglobin to bind haemoglobin specifically (Bocci, 1970, p. 384), it seemed likely that α_2-macroglobulin might be freed from haptoglobin by treatment with haemoglobin covalently linked to Sepharose 4B (see the Methods section).

Peak 1 from a Sepharose 6B column was concentrated by ultrafiltration and applied to a column (2.5cm × 28cm; 138cm^3) of haemoglobin–Sepharose equilibrated with buffer containing 20mM-Tris–HCl, pH7.8, 200mM-NaCl, 2mM-disodium EDTA and 1% (v/v) butan-1-ol. The sample was recycled five times, then eluted with the above buffer. Immunoelectrophoresis showed that the treatment with haemoglobin–Sepharose had resulted in very efficient removal of haptoglobin, the relative concentrations of the other proteins being unaffected.

The protein eluted from the haemoglobin–Sepharose was concentrated by ultrafiltration and applied to a column of Sepharose 6B as before. The fractions were analysed by immunoelectrophoresis and those rich in α_2-macroglobulin were pooled, concentrated by ultrafiltration to 7.6mg/ml and stored at 4°C. Immunoelectrophoresis of this final product showed it to consist of α_2-macroglobulin contaminated only by traces of IgM and haptoglobin (Plate 1*c*).

Purification of immunoglobulin G. IgG was precipitated from human serum by making the serum 1.6M with ammonium sulphate (Stelos, 1967). The precipitate was twice re-dissolved in water and re-precipitated. The supernatant solutions were pooled and shown by immunoelectrophoresis to contain no IgG. The final precipitate was resuspended in water and dialysed against buffer containing 50mM-Tris–HCl, pH7.8. The non-diffusible fraction was applied to a column (1.5cm × 28cm; 50cm^3) of DEAE-cellulose equilibrated and subsequently eluted with the same buffer. The protein that passed through the column under the starting conditions was shown to be pure IgG in immunoelectrophoresis against rabbit anti-(human serum protein) IgG (Plate 1*d*).

Inhibition by α_2-macroglobulin

Stoicheiometry and importance of substrate. The inhibition of the naphthylamidase activity of human

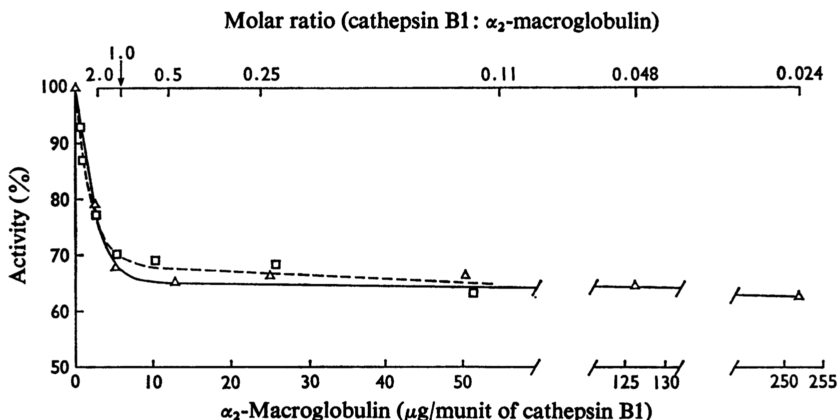


Fig. 4. *Inhibition of the naphthylamidase activity of cathepsin B1 by purified α_2-macroglobulin*

The experiment was conducted in the same way as that described in Fig. 1, except that 7.6–760 μg of purified α_2-macroglobulin was used with 3 munits of cathepsin B1/tube (△) or 15 munits of cathepsin B1/tube (□).

cathepsin B1 by purified α_2-macroglobulin increased very little as the molar ratio cathepsin B1 : α_2-macroglobulin fell below 1.0 (Fig. 4). Closely similar results were obtained with 3 munits or 15 munits of cathepsin B1/assay.

The proteolytic activity of human cathepsin B1 was more strongly inhibited by α_2-macroglobulin than was the naphthylamidase activity (50% and 20% respectively in one experiment). Again, the inhibition did not increase appreciably when the amount of α_2-macroglobulin was increased above an equimolar ratio to cathepsin B1.

Effect of inactive forms of cathepsin B1 on the inhibition. Cathepsin B1 undergoes irreversible denaturation at pH values of 7 and above (Otto, 1971; Barrett, 1973), and is irreversibly inhibited by iodoacetic acid, presumably through carboxymethylation of a thiol group at the active site.

α_2-Macroglobulin (137 μg) and pH-inactivated cathepsin B1 (originally 65 munits) in 0.45 ml of 20 mM-sodium–potassium phosphate buffer, pH 6.0, were incubated at 20°C for 15 min before 5.4 munits of active enzyme was introduced in 0.05 ml. The molar proportion α_2-macroglobulin : inactive cathepsin B1 : active cathepsin B1 was 1 : 3 : 0.25. Controls were set up with bovine serum albumin (13 μg) in place of the inactivated enzyme.

In a second experiment, α_2-macroglobulin (106 μg) and iodoacetic acid-inactivated cathepsin B1 (originally 153 munits) in 0.45 ml of buffer, as above, were incubated at 20°C for 15 min before 6.1 munits of active enzyme was introduced in 0.05 ml. The molar proportion of α_2-macroglobulin : inactive cathepsin B1 : active cathepsin B1 was 1 : 9.65 : 0.35. Controls were set up with bovine serum albumin (30.5 μg) in place of the inactivated enzyme.

In each experiment the final mixtures were incubated for 5 min at 20°C and their naphthylamidase activities were measured as usual. It was found that neither pH-denatured nor iodoacetic acid-inactivated cathepsin B1 interfered with the inhibition of active enzyme by α_2-macroglobulin. It thus appeared that the catalytic site of cathepsin B1 plays some part in the interaction with α_2-macroglobulin.

Effect of pH. Inhibition of the naphthylamidase activity of cathepsin B1 by α_2-macroglobulin was measured over the pH range 5.0 to 7.5. The buffers employed, at 0.1 M concentration, were sodium acetate (pH 5.0 to 5.5), sodium–potassium phosphate (pH 6.0 to 6.5) and Tris–HCl (pH 7.0 to 7.5); at all pH values the assay mixtures contained 1 mM-disodium EDTA, 2 mM-cysteine, 10 munits of cathepsin B1 and 150 μg of α_2-macroglobulin, in a total volume of 2.0 ml.

The extent of inhibition was independent of pH below pH 6.5, but increased slightly with pH above this value.

Ability of α_2-macroglobulin to protect cathepsin B1 from the action of other inhibitors

Tos-PheCH$_2$Cl is an inhibitor of papain (Bender & Brubacher, 1966) and cathepsin B1 (Barrett, 1973). Cathepsin B1 (5.7 munits) was preincubated at pH 6.0 in a total volume of 0.40 ml, with 345 μg of α_2-macroglobulin (an 11-fold molar excess). The naphthylamidase activity of the enzyme was then assayed with and without 10 μM (final concentration) Tos-PheCH$_2$Cl. Inhibition of 87% was produced by Tos-PheCH$_2$Cl under these conditions, whereas enzyme preincubated without α_2-macroglobulin was 79% inhibited. We conclude that the cathepsin B1

complexed by α_2-macroglobulin was not protected from inhibition by the low-molecular-weight inhibitor.

On the other hand, preincubation of human cathepsin B1 with α_2-macroglobulin partially protected the enzyme from further inhibition by either whole human serum or purified human IgG (Fig. 5).

Inhibition by IgG

The inhibition of the naphthylamidase activity of cathepsin B1 by purified human IgG increased with the concentration of the immunoglobulin up to a molar ratio of 650:1 (Fig. 5). IgG was clearly a much less effective inhibitor than α_2-macroglobulin, but it may well account for the progressive increase in inhibition of the naphthylamidase activity with serum concentrations beyond an equimolar ratio of the enzyme to α_2-macroglobulin (Fig. 1).

When added to cathepsin B1 that had been preincubated with an excess of α_2-macroglobulin, an amount of whole serum which alone gave 32% inhibition, caused an additional 7% inhibition of enzyme activity. An amount of purified human IgG equivalent to this amount of serum, introduced under the same conditions, caused some 6–10% additional inhibition. These results therefore suggest that α_2-macroglobulin and IgG are the major serum inhibitors of human cathepsin B1.

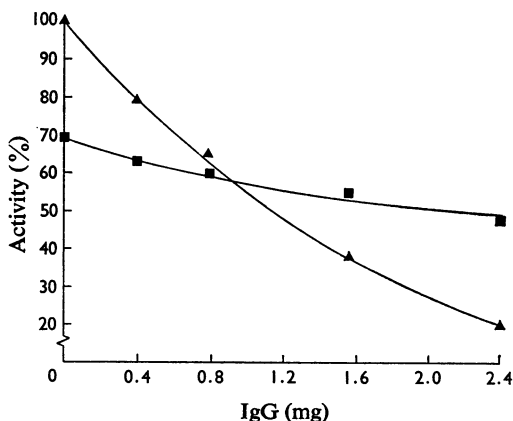


Fig. 5. *Effect of IgG on the naphthylamidase activity of cathepsin B1 and of cathepsin B1–α_2-macroglobulin complex*

A solution of IgG (0.4–2.4mg) was mixed with 3munits of cathepsin B1 alone (▲) or with 3munits of cathepsin B1 that had been preincubated with an 8.5-fold molar excess (0.15mg) of α_2-macroglobulin (■). Naphthylamidase activity was measured as usual.

Effect of human haptoglobin on the activity of cathepsin B1

A sample of pooled human serum was treated with haemoglobin–Sepharose to remove the haptoglobin. Immunoelectrophoresis of the serum before and after treatment with the adsorbent (Plate 1*e*) showed that the haemoglobin–Sepharose almost completely removed the haptoglobin, without appreciably altering the concentrations of the other serum proteins.

Measurements of the inhibitory capacity of the untreated serum and the haptoglobin-depleted serum showed that the removal of haptoglobin had not decreased the inhibitory capacity of the serum (Fig. 6).

Inhibitory capacity of sera of known haptoglobin content. Human sera and synovial fluids, of known haptoglobin content, from patients with various forms of arthritis were used to determine whether the inhibitory activity of a sample of serum is correlated with its content of haptoglobin.

Equal volumes of the sera had approximately equal inhibitory capacities against human cathepsin B1 (Fig. 7) despite their very different haptoglobin contents; 20μl of serum gave approximately 44% inhibition, 10μl of serum gave approximately 32% inhibition. Thus there appeared to be no correlation between the haptoglobin content and the inhibitory capacity of a serum.

Serum inhibitors of cathepsin B1 from other species. The proteolytic activity of rabbit liver cathepsin B1 (prepared by a method closely similar to that used for

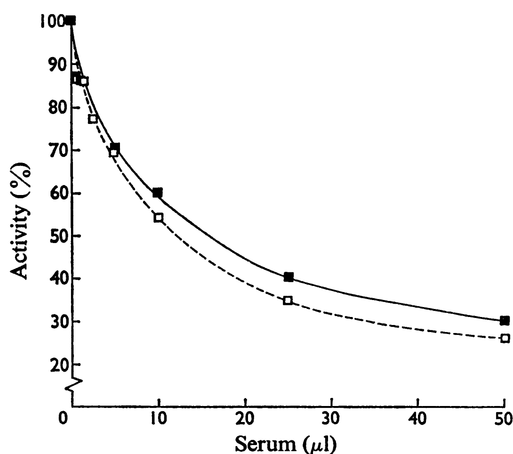


Fig. 6. *Inhibition of cathepsin B1 by untreated human serum and haptoglobin-depleted serum*

Cathepsin B1 (11munits) was incubated with 0.5–50μl of serum (■) or haptoglobin-depleted serum (□) and the naphthylamidase activity was determined as usual.

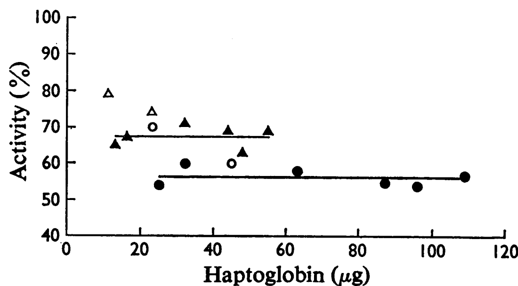


Fig. 7. *Inhibition of the naphthylamidase activity of cathepsin B1 by sera and synovial fluids of known haptoglobin content*

Cathepsin B1 (8.5munits) was incubated with 10μl of serum (▲), 10μl of synovial fluid (△), 20μl of serum (●) or 20μl of synovial fluid (○) and the naphthylamidase activity was determined as usual. The horizontal lines represent the arithmetic mean of the values obtained with 10 or 20μl of serum.

the human enzyme) was much more potently inhibited (up to 98%) by either rabbit or human serum than was the human enzyme by human serum. Gel chromatography of each of these sera indicated that the rabbit enzyme was inhibited by several proteins in addition to α_2-macroglobulin and IgG. The extent of inhibition of the naphthylamidase and proteolytic activities of the enzyme by purified human α_2-macroglobulin was similar to that of human cathepsin B1.

Discussion

We have found that human cathepsin B1 is partially inhibited by human serum, the proteolytic activity being more strongly affected than that against benzoylarginine naphthylamide. The inhibitory activity was resolved into two peaks by gel chromatography, and these correlated with the distributions of α_2-macroglobulin and IgG in the effluent fractions. Gel chromatography of a mixture of cathepsin B1 with serum showed the binding of cathepsin B1 only by the high-molecular-weight α_2-macroglobulin fraction, and naphthylamidase activity was demonstrated in a precipitin line formed against antibodies specific for α_2-macroglobulin.

The binding and partial inhibition of cathepsin B1 by purified α_2-macroglobulin had the general characteristics of the interaction of α_2-macroglobulin with other proteinases. Thus inhibition was more pronounced with the protein substrate than with the low-molecular-weight naphthylamide. Dose–response curves for the inhibition of cathepsin B1 by α_2-macroglobulin showed a progressive increase in inhibition with amount of α_2-macroglobulin, until a plateau was reached at an equimolar ratio of cathepsin B1 to α_2-macroglobulin (Fig. 4). Binding ratios previously reported for other proteolytic enzymes with α_2-macroglobulin from serum have been 1:1 for elastase (Baumstark, 1970), or 2:1 for trypsin and subtilopeptidases A and B (Dolovich & Wicher, 1971). The absence of naphthylamidase activity at pH 8 from the serum and α_2-macroglobulin used in our experiments indicates that thrombin is unlikely to have been bound to α_2-macroglobulin as we isolated it.

The failure of two inactive forms of cathepsin B1 to compete in the binding of the active enzyme by α_2-macroglobulin agrees with the results previously obtained with active and inactive thrombin (Steinbuch, 1971) and subtilopeptidase A (Dolovich & Wicher, 1971).

α_2-Macroglobulin partially protected cathepsin B1 against inhibition by IgG, but did not protect against the low-molecular-weight compound Tos-Phe-CH_2Cl.

The inhibition of cathepsin B1 by human IgG had different characteristics from that by α_2-macroglobulin. Inhibition was appreciable only at very high molar ratios of IgG to enzyme, and gel chromatography provided no evidence for binding of cathepsin B1 to IgG in a partially active state.

The failure of serum proteins of molecular weight lower than IgG (including α_1-antitrypsin, α_1-antichymotrypsin, inter-α-trypsin inhibitor and antithrombin III) to inhibit or bind cathepsin B1 underlines the relatively broad specificity of α_2-macroglobulin for the inhibition of proteolytic enzymes (A. J. Barrett & P. M. Starkey, unpublished work).

It has been reported that bovine 'cathepsin B' is inhibited by human haptoglobin (Snellman & Sylvén, 1967; Snellman, 1971). From our results it seems certain that human cathepsin B1 is not inhibited by this protein. The difference in species of origin of the enzyme may be significant, since we found that rabbit cathepsin B1 was inhibited not only by α_2-macroglobulin and IgG, but also by several other serum proteins.

We conclude that the most potent inhibitor in serum of human cathepsin B1 on a molar basis is α_2-macroglobulin, although it does not produce by any means complete inhibition. One can speculate that inhibition may not be the most important physiological consequence of interaction of a molecule of cathepsin B1 with α_2-macroglobulin. The enormously increased effective molecular size of the enzyme may limit its penetration of connective tissue matrices, and so prevent it from acting in their catabolism. It also seems that a mechanism exists for the rapid clearance from the plasma of complexes of proteolytic enzymes with α_2-macroglobulin (Ohlsson, 1971).

Note added in proof (*received* 16 *January* 1973)

It has come to our attention that weak reversible inhibition of proteolytic enzymes, similar to that described for IgG in the present paper, has previously been reported for IgA (Counitchausky *et al.*, 1970).

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