# Metal-Dependent Proteinase of the Lens

## ASSAY, PURIFICATION AND PROPERTIES OF THE BOVINE ENZYME

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1. Two new assay methods were developed for the lens proteinase. In both, the substrate was $\alpha_2$-crystallin (a major lens protein); in the first method, the products were detected by reaction with trinitrobenzenesulphonate in the presence of $SO_3^{2-}$, whereas in the second method, $^3H$-labelled substrate was used, and the products were detected as radioactivity soluble in trichloroacetic acid. 2. The neutral proteinase from bovine lens was partially purified by extraction of the lens at pH 5.0 and column chromatography on hydroxyapatite and Sepharose 6B gel. 3. The purified enzyme had no detectable activity against haemoglobin, azo-casein or $\gamma$-crystallin under optimum conditions for $\alpha_2$-crystallin. 4. The enzyme showed greatest activity and stability at pH 7.5. It was reversibly inhibited by EDTA and 1,10-phenanthroline, and activated by $Ca^{2+}$ and $Mg^{2+}$. 5. Molecular weights obtained for the enzyme by chromatography on Sepharose 6B were approx. 500000 in buffer of $I = 0.02$, and 250000 at $I = 1.02$. 6. The properties of the purified lens proteinase are such as to suggest that this enzyme could account for the entire endopeptidase activity of the lens.

The protein content of the normal human lens increases progressively during life, but many cataractous lenses contain less protein than normal (van Heyningen, 1969). One explanation for this aspect of the pathology of cataract would be that protein degradation in the diseased lens is abnormally rapid, and for this reason it was decided to examine the proteolytic enzymes of the lens. Studies *in vivo* have indicated that there is little protein turnover in the normal rat lens (Young & Fulhorst, 1966; Furst *et al.*, 1973). Extracts of lenses of various species have an autolytic capacity at neutral pH values of the same order as other tissues (Waley & van Heyningen, 1962) but seem to lack the predominance of activity at acid pH values seen in most tissues (Hanson, 1962, 1968; van Heyningen & Waley, 1963; Bohley *et al.*, 1971). The proteolytic activity of bovine lens extracts can only be demonstrated, with certainty, with a lens protein ($\alpha$-crystallin) as substrate.

The object of the present study was a fuller characterization of the proteinase of the bovine lens.

## Experimental

### Materials

Lenses were removed from bovine eyes within 2h of death and stored frozen. No difference was

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noted in enzyme content or specific activity between fresh lenses and those stored frozen for several months.

Bovine serum albumin, trypsin (EC 3.4.21.4), toluene-*p*-sulphonyl-L-arginine methyl ester and 2,4,6-trinitrobenzenesulphonic acid were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Sephadex G-25, G-75 and Sepharose 4B and 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel HT hydroxyapatite was from Bio-Rad Laboratories Ltd., St. Albans, Herts., U.K. DEAE-cellulose (Whatman DE 52) and Cellogel strips (modified cellulose acetate) were from Reeve Angel Scientific Ltd., London EC4V 6AY, U.K. 4-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg was from Fluka, Buchs, Switzerland. Mes [2-(*N*-morpholino)ethanesulphonic acid], Tes [*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulphonic acid] and Hepes [2-(*N*-2-hydroxy-ethylpiperazin-*N'*-yl)ethanesulphonic acid] buffers were from Hopkin and Williams, Romford RM1 1HA, U.K. Lyphogel for small-scale protein concentration was from Hawksley and Sons, Lancing, Sussex, U.K. [³H]Acetic anhydride (2500mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were AnalaR grade wherever possible, and were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Bovine haemoglobin and azo-casein were prepared as described by Barrett (1970) and Charney & Tomarelli (1947) respectively.

### Preparation of $\alpha_1$- and $\alpha_2$-crystallins

The protein that precipitates from a lens extract at pH 5 is called $\alpha_1$-crystallin; after its removal, $\alpha_2$-crystallin is precipitated from the supernatant by the addition of ethanol (Francois et al., 1955). The $\alpha_1$ fraction contains the proteinase; the $\alpha_2$ fraction is free of proteinase activity and can be used as a substrate (Waley & van Heyningen, 1962). Both $\alpha_1$- and $\alpha_2$-crystallin are contaminated with leucine aminopeptidase (EC 3.4.11.1). The alkaline urea treatment of $\alpha_2$-crystallin described below destroys the activity of this peptidase, and in this form $\alpha_2$-crystallin is used as substrate in the present work.

Frozen bovine lenses (100 g; approx. 50 lenses) were thawed at room temperature, and an incision was made in each capsule with a scalpel. The tissue was stirred in 1 litre of 0.1 M-Mes adjusted to pH 5.0 with 1.0 M-NaOH, at 4°C. After 20 min, about 7.5 ml of 1 M-HCl was slowly added to return the extract to pH 5.0 (producing a dense white precipitate of $\alpha_1$-crystallin), and stirring was continued for a further 20 min. The suspension was decanted from the lens nuclei and centrifuged at 15000 g for 20 min at 10°C. The precipitate was used as starting material in the purification of the lens proteinase (see the Results section). Crude $\alpha_2$-crystallin was precipitated from the supernatant by the addition of ethanol to 13% (v/v), and collected by centrifugation. It was dissolved in a little saturated aq. NaHCO$_3$, and the ethanol precipitation at pH 5 repeated.

The $\alpha_2$-crystallin was redissolved in a minimum volume of saturated NaHCO$_3$ and dialysed against three changes of 200 vol. of water. The protein concentration was then determined by measuring the dry weight of a portion of the solution after heating to constant weight in an oven and the remainder was diluted to 5% (w/v). Then 0.72 g of urea and 0.16 ml of 1 M-NaOH were added per ml of 5% (w/v) $\alpha_2$-crystallin, and the volume was made up to 1.60 ml with water. After 30 min at 20°C, the pH was re-adjusted to 7.5 with about 0.16 ml of 1 M-HCl and the volume made up to 2.0 ml with water. The solution was dialysed against three changes of water (500 vol.) and freeze-dried.

The leucine aminopeptidase activity of $\alpha_2$-crystallin (originally 70 munits/mg) was thus lowered to 0.16 munit/mg by the alkaline urea treatment, and to undetectable values (<0.08 munit/mg) after freeze-drying.

### Assay of proteinase

Two methods were used to measure the trichloroacetic acid-soluble degradation products of the $\alpha_2$-crystallin substrate: a modification of the Tnb-sulphonate (trinitrobenzenesulphonate) method of Fields (1972), and a radiochemical assay. Because of the inhibition of the enzyme by phosphate (see below), it was necessary to dialyse samples containing this ion before assay by either method.

*Trinitrobenzenesulphonate method.* The enzyme sample was mixed with buffered substrate solution to produce a final reaction mixture (0.5 ml) containing 10 mM-Hepes adjusted to pH 7.5 with NaOH, 5 mM-MgCl$_2$, and 0.5% substrate. The mixtures were incubated in stoppered tubes at 55°C for 1 h, and cooled in an ice bath for 2 min. Then 2 ml of 7.5% (w/v) trichloroacetic acid was added, and after a further 10 min in the ice bath the mixture was filtered through 7 cm Whatman no. 1 papers. To 0.5 ml of the filtrate was added 0.5 ml of borate buffer solution containing 3.81 g of Na$_2$B$_4$O$_7$,10H$_2$O, 46.7 ml of 1 M-NaOH and 0.372 g of EDTA (disodium salt) in 100 ml; i.e. 0.1 M-Na$_2$B$_4$O$_7$ in 0.1 M-NaOH, as used by Fields (1972), with extra NaOH to neutralize the trichloroacetic acid, and EDTA to remove Mg$^{2+}$ ions, which otherwise were precipitated by the borate. Tnb-sulphonic acid solution (20%, w/v; 0.1 ml) was added to the tubes at 15 s intervals, with immediate mixing. After 5 min, 2 ml of freshly prepared sulphite solution (1.5 mM-Na$_2$SO$_3$ in 0.1 M-NaH$_2$PO$_4$) was added, and the extinction at 420 nm measured within 10 min. Blanks were prepared by incubating substrate alone, and introducing the enzyme immediately before the trichloroacetic acid. The reaction was standardized with leucine [10–100 nmol in 0.5 ml of 6% (w/v) trichloroacetic acid]. One unit of proteinase activity was defined as the amount of enzyme that produced 1 $\mu$mol of amino groups/min under these conditions. Activity was proportional to enzyme concentration up to a $\Delta E_{420}$ of 1.0.

*Radiochemical method.* $\alpha_2$-crystallin treated with alkaline urea was labelled with [³H]acetic anhydride as follows.

The protein (50 mg) in 1 ml of 1.0% NaCl containing 0.042 M-Na$_2$HPO$_4$–0.008 M-NaH$_2$PO$_4$, pH 7.5, was treated with 2 mCi of [³H]acetic anhydride at 0°C for 1 h and dialysed against 1.0% NaCl in 0.05 M-sodium acetate until less than 1% of the radioactivity was soluble in 10% (w/v) trichloroacetic acid. The material was stored at −20°C.

Incubations were performed as in the Tnb-sulphonate method, except that the substrate contained 0.01–0.10 $\mu$Ci of ³H/tube. Just before the addition of trichloroacetic acid, 0.1 ml of 1% (w/v) bovine serum albumin was added to each tube to ensure complete precipitation of protein. Samples (1 ml) of the filtrates were taken for radioactivity counting, with 9 ml of scintillant solution (Hall & Cocking, 1965). Blanks were prepared by incubating substrate alone. Activity was expressed as percentage release of the radioactivity present.

## Leucine aminopeptidase

Leucine aminopeptidase was assayed by its action on L-leucine amide, which is converted into leucine and $NH_3$. A new procedure was devised in which the Berthelot reaction (Weatherburn, 1967) slightly modified from that used to assay urease (Kaplan, 1969), was used to follow the release of $NH_3$. Incubation mixtures (0.5 ml) contained 50 mM-leucine amide in 0.03 M-$NaHCO_3$-0.07 M-$Na_2CO_3$, pH 9.5, and 5 mM-$MgCl_2$. Incubation was at 40°C for 10 min. The reaction was stopped with 1 ml of phenol–nitroprusside reagent (31 g of phenol and 0.125 g of sodium nitroprusside, in 500 ml of water) and the colour was developed with 1 ml of alkaline hypochlorite reagent (10.75 ml of NaOCl solution, containing 10–14% available chlorine, and 125 ml of 1 M-NaOH in 250 ml of water) by incubation at 40°C for 20 min. The solutions were diluted with water to give an $E_{625}$ of less than 1.0, and samples were filtered if the final solutions were turbid. The reaction was standardized with $NH_4Cl$ containing up to 6 μg of ammonia N per tube. One unit of leucine aminopeptidase hydrolyses 1 μmol of leucine amide/min under these conditions. Interference in the colour reaction was encountered with Tris, Tes and Hepes buffers, which prevented the development of colour by standard $NH_3$ solution.

## Cellogel electrophoresis

Cellogel strips were soaked in Tris–glycine buffer (3.0 g of Tris and 14.4 g of glycine made up to 1 litre with water, pH 8.3). The buffer was used at double strength in the buffer compartments of the electrophoresis apparatus. The sample was applied as a relatively concentrated solution (20–30 mg/ml) in a small volume (normally 1.5 μl) by using the proprietary applicators. Small-scale sample concentration was done with Lyphogel. Electrophoresis was at 200 V for 30–60 min, and the strips were stained in Coomassie Brilliant Blue [1.25 g in methanol–acetic acid–water (5:1:5, by vol.)] for 15–20 min. Rapid destaining was done in acetic acid–methanol (1:9, v/v). After 5 min the strips were clear and were stored in 7.5% (v/v) acetic acid.

## Protein determination

Protein in chromatographic column effluents was measured by the $E_{280}$ (assuming $E_{280}^{1\%} = 10$). The specific activity of enzyme preparations was based on measurement of protein by a modification of the Folin–Ciocalteu method of Lowry et al. (1951) (Leggett-Bailey, 1967), standardized with bovine plasma albumin.

## Results

### Neutral proteinase assays

The two assay methods were compared with that based on the $E_{280}$ of trichloroacetic acid filtrates and

with the ninhydrin determination of new end groups, described by Waley & van Heyningen (1962) (Table 1).

The Tnb-sulphonate method was equal in sensitivity to that using ninhydrin, but was more convenient in practice, and had the advantage of relative insensitivity to $NH_3$. The radiochemical assay was still more sensitive, and more economical of sample because no enzyme was required for use in blank reaction mixtures.

The dose–responses of the assay methods were compared (Fig. 1). The rate of enzymic activity was constant for at least 1 h in both methods of assay. The Tnb-sulphonate method gave a good linear relationship between $\Delta E_{420}$ and amount of enzyme, whereas the standard curve for the radiochemical assay was convex. End-group methods for the measurement of proteolytic activity, such as the Tnb-sulphonate procedure, are highly sensitive to exopeptidase activity, unlike methods based on the detection of total trichloroacetic acid-soluble material (e.g. the radiochemical procedure). The difference was reflected, in the present study, in a changing ratio of units measured by the two methods as exopeptidases were removed during the purification procedure.

## Purification of the enzyme

The results of the purification procedure are summarized in Table 2. $\alpha_1$-Crystallin (see the Experimental section) prepared from 100 g of lenses was resuspended in 100 ml of 0.075 M-$KH_2PO_4$-0.075 M-$K_2HPO_4$ buffer, pH 6.8, containing 5 mM-$MgCl_2$, adjusted to pH 6.8 with saturated $NaHCO_3$, and stirred at 40°C for 1 h, after which it was centrifuged at 15000 g for 20 min at 10°C. If the crude enzyme

Table 1. *Comparison of assay methods for the lens neutral proteinase*

The sample required was defined empirically as that activity producing an increase in extinction or radioactivity equal to the blank value. The amount of enzyme required per assay included that required for the blank assay (not needed in the radiochemical assay). The methods based on $E_{280}$ and ninhydrin were as described by Waley & van Heyningen (1962), whereas the Tnb-sulphonate and radiochemical methods were those described in the Experimental section.

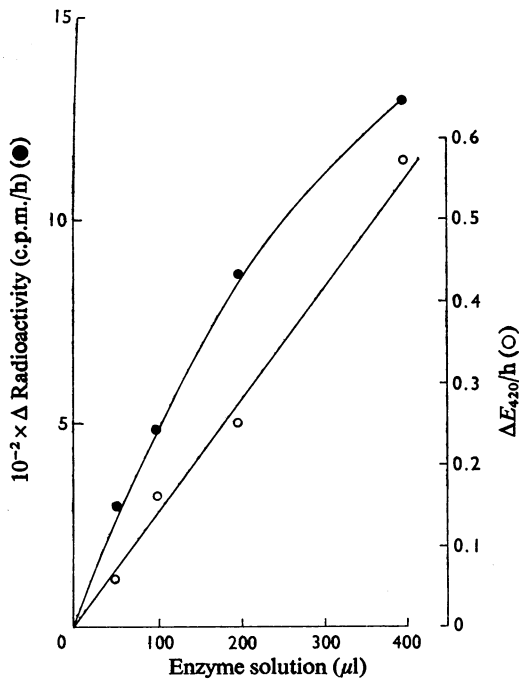| | Sample required | | |
|---|---|---|---|
| | Amount | | |
| Method | (munits/ tube) | (munits/ assay) | Concentration (munits/ml) |
| $E_{280}$ | 16.7 | 33.4 | 83.3 |
| Ninhydrin | 5.0 | 10.0 | 25.0 |
| Tnb-sulphonate | 4.7 | 9.4 | 23.3 |
| Radiochemical | 1.7 | 1.7 | 4.2 |

Fig. 1. *Standard curves for the Tnb-sulphonate* (○) *and radio-chemical* (●) *assay methods*

For details see the Experimental section.

preparation was to be stored, rather than run immediately on hydroxyapatite, a 10mM-sodium diethylbarbiturate buffer (5.8mM-diethylbarbituric acid–4.2mM-sodium diethylbarbiturate, pH7.5) or 10mM-Hepes–NaOH buffer, pH7.5, containing 5mM-MgCl$_2$ was used. The same buffers were used also for storage of the enzyme at later stages of the preparation for optimum stability.

The supernatant was applied to a column (26mm × 190mm; 100cm³) of hydroxyapatite equilibrated with the same buffer. Some 80–90% of the proteinase activity was adsorbed, whereas most of the protein passed straight through the column. The column was washed with 0.15M-KH$_2$PO$_4$–K$_2$HPO$_4$ buffer, pH6.8, containing 5mM-MgCl$_2$. The enzyme was eluted as a sharp peak with 0.40M-phosphate buffer.

The fractions containing enzymic activity were combined and the phosphate concentration was measured (Fiske & SubbaRow, 1925) to allow dilution to 0.15M-potassium phosphate buffer containing 5mM-MgCl$_2$. The solution was applied to a second column (14mm × 60mm; 10cm³) of hydroxyapatite equilibrated as above. The column was washed with 5 bed vol. of the equilibrating buffer, and the enzyme was eluted once more with the 0.40M-phosphate buffer. The enzyme was not eluted by KCl up to 1M concentration.

Table 2. *Purification of lens neutral proteinase*

Lenses (50; 104g) were used as starting material in the purification procedure described in the text. The unit of the radiochemical assay was arbitrarily made equivalent to that of the Tnb-sulphonate assay at the final stage of purification. Activities listed for the lens cortex are based on recoveries found for acid precipitation of a lens cortex homogenate, and the known protein content per wet wt. of lens cortex.

| | Proteinase | | | | | | | | Leucine aminopeptidase | |
| | Tnb-sulphonate assay | | | | Radiochemical assay | | | | | |
| | Protein (g) | Activity (munits) | Specific activity (munits/mg) | Purification (fold) | Activity (munits) | Specific activity (munits/mg) | Recovery (%) | Purification (fold) | Activity (units) | Specific activity (unit/mg) |
|---|---|---|---|---|---|---|---|---|---|---|
| Lens cortex | 15.6 | 4333 | 0.283 | (1.0) | 1042 | 0.067 | (100) | (1.0) | 317 | 0.02 |
| Extraction and acid precipitation | 2.57 | 3667 | 1.42 | 5 | 883 | 0.33 | 85 | 5 | 158 | 0.06 |
| 1st hydroxyapatite | 0.15 | 1500 | 10.0 | 35 | 667 | 4.5 | 64 | 66 | 1.3 | 0.02 |
| 2nd hydroxyapatite | 0.05 | 825 | 16.7 | 57 | 825 | 16.7 | 79 | 250 | <0.03 | <0.0007 |

The purified enzyme was stored at −20°C in 10mM-Hepes–NaOH buffer, pH7.5, and the activity fell to 50% of the initial value in about 4 months. Further purification could be obtained by gel chromatography on Sepharose 6B (see below), but this gave rise to very unstable preparations. Attempts at further purification by DEAE-cellulose chromatography at pH6.5 resulted in very poor recoveries of activity.

The recovery of activity during the purification, as judged by the Tnb-sulphonate method, was less than that indicated by the radiochemical method (Table 2), and thus the final degree of purification also seemed less. This effect was attributable to two factors. In the Tnb-sulphonate method, the removal of exopeptidases, including leucine aminopeptidase, will have decreased the apparent recoveries. In the radiochemical assay, on the other hand, some apparent increase in activity could have resulted from the removal of unlabelled α-crystallin during purification, so that the effective specific radioactivity of the substrate was greater during the later assays. Leucine aminopeptidase activity was decreased to an undetectable concentration by the second run on hydroxyapatite, so that the total decrease in contamination by leucine aminopeptidase by use of hydroxyapatite was at least 5000-fold.

### Some properties of the enzyme

*Electrophoresis.* When run on cellulose acetate strips at pH8.3, the enzyme preparation showed one major band with relatively high mobility, similar to that of α-crystallin, together with traces of slower moving components. It is considered quite possible that most of the protein present was, indeed, α-crystallin. Electrophoresis in polyacrylamide gel by the method of Davis (1964) was not informative since none of the proteins in the purified enzyme preparation entered the gel, presumably because of their high molecular weights.

*Gel chromatography.* A column (26mm × 900mm; 500cm³) of Sepharose 6B in 10mM-Hepes–NaOH buffer, pH7.5, containing 5mM-MgCl₂, was calibrated with Blue Dextran (for the void volume), α₂-crystallin (mol.wt. 800000), leucine aminopeptidase (mol.wt. 326000), bovine serum albumin (mol.wt. 68000) and cytochrome c (mol.wt. 11700) (Andrews, 1965).

The results showed a good linear relationship between $K_{av}$. (Fischer, 1969) and log(molecular weight). Partially purified lens proteinase (after a single run on hydroxyapatite) was applied to the column, and in two identical runs activity was eluted at an effluent volume indicative of a molecular weight of about 550000. The column was re-equilibrated with 1M-KCl in the same buffer, and another enzyme sample was run. The bed volume and void volume of the column remained the same at the higher ionic

strength, but the elution volume of the enzyme increased to indicate a molecular weight of about 250000 (by reference to the original calibration run).

*Effect of pH on activity.* An equimolar mixture of Mes and Hepes buffers (to give a final concentration of 25mM each, with 5mM-MgCl₂) was adjusted to pH values in the range 4.5–9.5 with 1M-NaOH (the pH values were measured at room temperature although the incubations were at 55°C). The buffers were used in place of the normal assay buffer in determinations of enzymic activity by the Tnb-sulphonate method, and the $\Delta E_{420}$ values were used to plot percentage activity, relative to the maximum value, against pH. The pH of every reaction mixture was checked both before and after incubation, and the mean value was used in the graph (Fig. 2). Maximum activity was at pH7.8.

*Effect of pH on stability.* Samples of the pH7.0 buffer used in the pH-dependence experiment (see above) were adjusted to pH values in the range pH2–10 with 4M-HCl or 4M-NaOH. To the resulting solutions were added portions of purified lens proteinase, and the individual pH values were measured before the mixtures were incubated at 37°C for 1h. The pH values were readjusted to 7.0 and samples were removed for assay by both Tnb-sulphonate and radiochemical methods. The results (Fig. 3) were expressed as percentages of the activity of a control sample kept at 0°C and pH7.0. The activity was reasonably stable between pH5.5 and 7.5, but fell sharply at about pH8.

Comparison of the data in Figs. 2 and 3 suggests that the stability of the enzyme persisted to a slightly higher pH value in the presence of substrate.

*Confirmation of endopeptidase activity.* In earlier work (van Heyningen & Waley, 1963) leucine aminopeptidase was an important contaminant of substrate and enzyme, and this contamination probably contributed to the finding that only amino acids
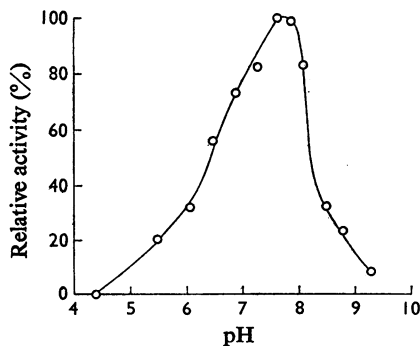


Fig. 2. *pH-dependence of lens proteinase activity*

See the text for experimental details. Activity was expressed as a percentage of the maximum value.
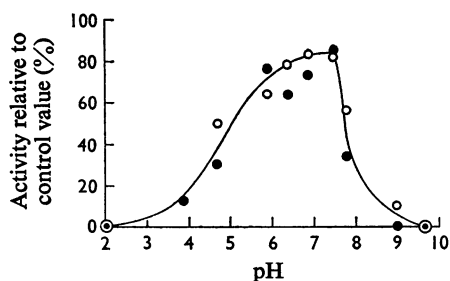
Fig. 3. *pH-dependence of lens proteinase stability*

Assays were made by the Tnb-sulphonate method ($\bigcirc$) and by the radiochemical method ($\bullet$). See the text for experimental details.

could be detected as the products of proteolysis. The substrate used in the present work was free of leucine aminopeptidase activity, and this exopeptidase was separated from the proteinase during purification. The average degree of polymerization of the peptide products, resulting from digestion of $\alpha$-crystallin by the lens proteinase, was determined by measuring the ratio of *N*-terminal groups [by the Tnb-sulphonate method of Fields (1972)] after hydrolysis with 6M-HCl (110°C, 20h, *in vacuo*) to those present before hydrolysis (Bohley *et al.*, 1971). The ratios obtained for trichloroacetic acid filtrates produced by the normal assay procedure were 2–4 for the enzyme after acid precipitation, 5–6 after one hydroxyapatite-column chromatography and 10–14 after gel chromatography on Sepharose 6B in 1M-KCl. In all cases digestion amounted to no more than 15% solubilization of radioactivity.

Several trichloroacetic acid filtrates that had resulted from radiochemical assays of the lens proteinase at various stages of purification were combined, and used in an attempt to demonstrate directly the formation of peptide products by the enzyme. The solution (40ml) was treated by the method of Hughes & Williamson (1951) to remove the trichloroacetic acid, concentrated to 1ml, and run on a column (1cm × 144cm) of Sephadex G-25 in 0.5% formic acid. Some 40% of the radioactivity, and 70% of the amino groups detectable after hydrolysis, were eluted in the region characteristic of peptides, ahead of free amino acids. Since some of the enzyme samples used in this experiment contained leucine aminopeptidase, it is clear that a still higher proportion of peptides would have been produced by the lens proteinase itself (as was indicated by the previous experiment).

*Inhibitors and activators.* Experiments with potential inhibitors and activators of the lens proteinase were made in 10mM-Hepes–NaOH buffer, pH7.5. In the first series of experiments the effect of various

Table 3. *Effect of potential inhibitors on magnesium-activated lens proteinase*

Assays were made by the radiochemical method. See the text for details.

| Compound | Final concn. (mM) | Inhibition (%) |
|---|---|---|
| EDTA | 5.0 | 100 |
|  | 1.0 | 72 |
|  | 0.1 | 32 |
|  | 0.01 | 15 |
| 1,10-Phenanthroline | 5.0 | 50 |
|  | 1.0 | 55 |
|  | 0.1 | 49 |
|  | 0.01 | 30 |
| 4-Chloromercuribenzoate | 0.5 | 94 |
|  | 0.1 | 30 |
| Di-isopropyl phosphorofluoridate | 6.0 | 66 |
|  | 0.6 | 0 |
| Urea | 2500 | 99 |
|  | 1000 | 53 |
|  | 100 | 0 |
| Dithiothreitol | 4.0 | 10 |
| ZnCl$_2$ | 10 | 100 |
|  | 1 | 100 |
|  | 0.01 | 0 |
| KCl | 1000 | 55 |
|  | 100 | 30 |
| Hepes | 300 | 83 |
|  | 100 | 36 |
|  | 10 | 0 |
| KH$_2$PO$_4$–K$_2$HPO$_4$, pH7.0 | 30 | 70 |
|  | 5 | 30 |

substances on the activity of the enzyme was investigated in the presence of 5mM-MgCl$_2$. Possible artifacts due to the effects of exopeptidases were minimized by the use of an enzyme preparation free of detectable leucine aminopeptidase, and by use of the radiochemical, rather than the Tnb-sulphonate, assay method. Generally, preincubation was for 5min at 0°C, with the effector at 1.25 times the final concentration. Preincubation with 4-chloromercuribenzoate and di-isopropyl phosphorofluoridate was for 2h and that with urea, 20min. The results (Table 3) are expressed relative to the activity of control samples preincubated alone at 0°C for the appropriate time.

Complete inhibition was produced by 5mM-EDTA, and 1,10-phenanthroline was also markedly inhibitory. 4-Chloromercuribenzoate was also inhibitory, but dithiothreitol had little effect. Di-isopropyl phosphorofluoridate was ineffective below 1mM concentration, and the enzyme was only moderately sensitive to urea. Zn$^{2+}$ appeared to be very inhibitory at concentrations of 1mM or above, but this effect could be a result of the precipitation of substrate from the assay mixtures. The moderate inhibition by KCl

Table 4. *Effect of bivalent cations on the activity of the lens proteinase*

Activity is expressed as a percentage of that in the presence of 10mM-CaCl$_2$.

| Effector | Concn. (mM) | Activity |
|---|---|---|
| None | — | 16 |
| CaCl$_2$ | 10 | (100) |
| | 1.0 | 52 |
| | 0.1 | 15 |
| MgSO$_4$ | 10 | 83 |
| | 1.0 | 29 |
| | 0.1 | 17 |
| ZnSO$_4$ | 1.0 | 0 |
| | 0.01 | 24 |
| CaCl$_2$+ZnSO$_4$ | 10+0.01 | 106 |
| EDTA | 10 | 0 |
| | 1.0 | 5 |
| 1,10-Phenanthroline | 5.0 | 15 |
| | 1.0 | 12 |

and Hepes buffer was attributable to the non-specific effect of ionic strength reported by Waley & van Heyningen (1962).

Phosphate buffers caused quite marked inhibition of activity, similar to that observed for the neutral metallo-endopeptidase from rabbit kidney brush-border (Kerr & Kenny, 1974*b*).

The ability of bivalent cations to activate the enzyme was determined by use of an enzyme preparation made by the standard method, except that no Mg$^{2+}$ was included in any of the buffers. Enzyme samples were preincubated with effectors as indicated in Table 4, for 5 min at 0°C.

The activity shown without added activator was sensitive to EDTA, but not to 1,10-phenanthroline. Ca$^{2+}$ activated somewhat more efficiently than Mg$^{2+}$, and slight activation was produced by 0.01 mM-Zn$^{2+}$, in the presence or the absence of Ca$^{2+}$.

The reversibility of the effect of chelators with certain bivalent metal ions is shown in Table 5, with a partially activated enzyme sample. Removal of the chelator gave up to 30% reactivation, and this was increased to 80% for EDTA-treated enzyme and 66% for 1,10-phenanthroline-treated enzyme, by the addition of 10mM-Ca$^{2+}$. Zn$^{2+}$ at 0.01 mM again gave slight activation in the absence or the presence of Ca$^{2+}$ (10mM), but 1mM-Zn$^{2+}$ was completely inhibitory.

*Effect of cations on stability.* Proteolytic enzymes of several classes are stabilized by metal ions (Rupley, 1967; Matsubara & Feder, 1971), whereas only metallo-proteinases are activated by metal ions (Hartley, 1960). The possibility that Ca$^{2+}$ and Mg$^{2+}$ were stabilizing the lens proteinase was investigated by measuring the stability of the enzyme preparation

Table 5. *Effect of bivalent cations on chelator-treated lens proteinase activity*

Enzyme solution in 10mM-Hepes–NaOH buffer, pH 7.5, containing approximately 1 mM-MgCl$_2$ was treated with the chelator to 5mM final concn. for 5min at +5°C. The mixture was then separated on Sephadex G-25. Activity (per mg of protein) is expressed as a percentage of that obtained in the presence of 10mM-Ca$^{2+}$, before chelator treatment. —, Not assayed.

| Metal ion | Metal ion concn. (mM) | Activity after chelator treatment | |
|---|---|---|---|
| | | EDTA | 1,10-Phenanthroline |
| None | — | 30 | 8 |
| Ca$^{2+}$ | 10.0 | 83 | 66 |
| | 1.0 | 53 | 44 |
| Mg$^{2+}$ | 10.0 | 62 | 32 |
| | 1.0 | 33 | 19 |
| Zn$^{2+}$ | 10.0 | 0 | — |
| | 1.0 | 0 | 0 |
| | 0.01 | — | 16 |
| Mg$^{2+}$ or Ca$^{2+}$ +Zn$^{2+}$ | 10.0 } 1.0 } | 0 | — |
| Ca$^{2+}$ +Zn$^{2+}$ | 10.0 } 0.01 } | — | 70 |

isolated in Mg$^{2+}$- and Ca$^{2+}$-free buffers. Samples were incubated in the absence of substrate for 1 h at 37°C, with and without 10mM-Mg$^{2+}$ or -Ca$^{2+}$. Assays by the radiochemical method in the presence of 10mM-Ca$^{2+}$ or -Mg$^{2+}$ showed that the loss of activity in the absence of the metal ions was approx. 20%, and this value was not significantly altered by the metal ions.

*Specificity of the enzyme.* Tests were made for activity of the purified enzyme against substrates other than α-crystallin. Haemoglobin, azo-casein and calf lens γ-crystallin were tested at final concentrations of 10, 5 and 5 mg/ml respectively, by the standard enzyme incubation methods, for various time-intervals up to 4h. The trichloroacetic acid filtrates were examined for the presence of amino groups by the Tnb-sulphonate method (haemoglobin and γ-crystallin), for the presence of material absorbing at 366 nm (azo-casein) and for ninhydrin-positive material detectable after paper electrophoresis at pH 3.6 (Bennett, 1967) (γ-crystallin). No increase in these indices over that found by incubating the enzyme without substrate (haemoglobin and γ-crystallin), and substrate without enzyme (azo-casein) could be detected. The modified peptide 4-phenylazo-benzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, which is hydrolysed by the metallo-proteinase, clostridial collagenase (Wursch & Heidrich, 1963), but not by the specific collagenase from rabbit fibroblasts (Werb & Burleigh, 1974), was tested. It was not hydrolysed by the lens proteinase.

## Discussion

Partial purification of the bovine lens neutral proteinase was achieved by van Heyningen & Waley (1963). Insofar as the different assay methods allow direct comparison, it seems that the degree of purification obtained in the earlier work was similar to that reported here, but the present method has important advantages; it can be applied to large amounts of starting material, and gives a good recovery of activity in a more stable form. The results of electrophoresis suggested that contamination by $\alpha$-crystallin persisted into the final enzyme preparations. The difficulty of separating the enzyme from $\alpha$-crystallin suggested that the enzyme was binding tightly to its substrate; this possibility was supported by the effect of salt on the apparent molecular weight of the enzyme, and the values for molecular weight should clearly be regarded as tentative.

Swanson & Nichols (1971) reported a tenfold purification of a proteinase from human cataractous lenses, with an acid pH optimum (on bovine plasma albumin), which they concluded was a serine proteinase; it appeared that the enzyme was a major component of the lens. In the present study, no evidence was obtained for the existence of such a proteinase in bovine lens.

In the earlier work of van Heyningen & Waley (1963) it was tentatively concluded that the lens neutral proteinase was an endopeptidase, but definitive evidence could not be obtained because of the high activity of leucine aminopeptidase in both enzyme and substrate preparations. In the present work, particular attention was paid to the removal of leucine aminopeptidase from both enzyme and substrate, and clear evidence has been obtained for the formation of peptide products. Since these can only be detected in the absence of leucine aminopeptidase, this suggests that leucine aminopeptidase further degrades the products of action of the endopeptidases, and also that the two enzymes might act sequentially in the physiological catabolism of lens proteins.

The effect of inhibitors and activators on the enzyme gives some indication of the nature of its catalytic site. On the basis of the work of Hartley (1960), endopeptidases can be classified as of carboxyl, thiol, serine or metallo types. The powerful inhibition of the lens proteinase by EDTA, and its activation by $Ca^{2+}$ and $Mg^{2+}$, point to its being a metallo-proteinase. Other examples of this class include the collagenases of clostridia, amphibia and mammals (Seifter & Harper, 1971; Harris & Krane, 1974*a,b,c*), the endopeptidase of rabbit kidney brush-border (Kerr & Kenny, 1974*a,b*) and thermolysin and related enzymes (Matsubara & Feder, 1971). These enzymes all seem to contain zinc, however, and thus are distinguished from the lens proteinase by their

sensitivity to inhibition by dithiothreitol. This, taken together with the partial inhibition by 6mM-diisopropyl phosphorofluoridate, could indicate that the lens enzyme is a serine proteinase, with an unusual metal requirement. The present results, together with those of van Heyningen & Waley (1963) show that the enzyme is almost completely inhibited by 4-chloromercuribenzoate and $N$-ethylmaleimide at relatively high concentrations (0.5 and 1.3mM respectively), but little or no inhibition was obtained with these reagents at 0.01–0.1mM concentration. This indicates that the enzyme has one or more thiol groups necessary for activity, but without the extreme reactivity characteristic of the catalytic thiol group of thiol proteinases such as papain. The conclusion that the enzyme is not a typical thiol proteinase is supported by the lack of stimulation by dithiothreitol.

Of the proteins tried as substrates, only $\alpha$-crystallin was hydrolysed, though an indication that $\beta$-crystallin may also be hydrolysed by the enzyme was obtained during autolysis studies (Blow, 1974). Attempts at finding a low-molecular-weight substrate for this enzyme were unsuccessful (van Heyningen & Waley, 1963). Too little is known of the mechanism of degradation of $\alpha$-crystallin by the lens proteinase to allow any conclusions about the apparent specificity of the enzyme for this substrate, but it is reminiscent of that of mammalian collagenase for collagen (Werb & Burleigh, 1974).

In respect of pH optimum, substrate specificity and inhibition characteristics the activity of a crude homogenate of lens appears identical with that of the purified enzyme. It therefore seems probable that this enzyme is the only endopeptidase in the lens that is active against the major proteins of the tissue.

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