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Human Senile Cataractous Lens Protease

ISOLATION AND SOME CHEMICAL CHARACTERISTICS

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A proteolytic enzyme was isolated from human senile cataractous lens by anion-exchange and gel-filtration chromatography. Sedimentation and zone-electrophoretic experiments indicated a high degree of homogeneity for the enzyme. A molecular weight of 27000 was calculated from measurements of sedimentation velocity and diffusion coefficient. Chelating agents decreased activity which could be restored by addition of certain bivalent metal ions. Diisopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride inhibit the proteolytic activities. Optimum rates of hydrolysis were observed at pH 5.2.

Early evidence to suggest the existence of enzymes that catalyse the intracellular hydrolysis of lens proteins was reported by Krause (1933). This investigator maintained that the bovine lens possessed a proteolytic enzyme that hydrolysed β-crystallin and albumin under acid conditions. The presence of polypeptidase in mammalian lens tissue was subsequently investigated by Abderhalden & Hanson (1938). They employed a partially purified ox lens extract which rapidly hydrolysed L-leucylglycine at pH 8.0 and appeared to be more active on the addition of Mg^{2+}. Dailey, Zeller, Wakin, Herrick & Benedict (1951) showed that certain L-peptidases in undialysed rabbit lens homogenates will hydrolyse L-glycyl-leucine, L-leucylglycine and L-leucylglycylglycine. Hanson & Methfessel (1958) found that the soluble fractions of bovine lens hydrolysed L-leucylglycylglycine, L-leucylglycine and L-leucinamide on activation with Mn^{2+} ions. Zeller, Banerjee & Shoch (1960) and Zeller & Devi (1957) studied the esteratic properties of a lens peptidase that catalysed the hydrolysis of L-glycyl-leucine, L-leucylglycylglycine and phenylalanine ethyl ester. Esterase activity was investigated on cattle lens homogenates by Devi (1959). From differences in hydrolysis of various synthetic amino acid esters, and specifically by using phenylalanine ethyl ester, he concluded that the lens enzyme is identical with chymotrypsin. Spector (1961) reports that calf and rabbit lenses appear to have only an amino acid esterase, specific for L-amino acid esters having a free NH_2 group. A variation in activity was noted in the enzyme leucinamidase of lens extracts from different species of mammals (Kleine & Hanson, 1961). Waley & van Heyningen (1962) were able to demonstrate separation of a neutral lens proteinase from leucine aminopeptidase. They concluded this enzyme might be an endopeptidase. It was found to be inhibited by thiol inactivators. Their purified enzyme preparation attacked α_2-crystallin, serum albumin and haemoglobin. Wolfe & Resnik (1963*a*,*b*) have reported results on leucine aminopeptidase from lens preparations of several mammalian species. By comparing a purified aminopeptidase from hog kidney with the proteolytic activity of bovine, porcine and ovine lens preparations (crude homogenates with purified lens preparation) with a series of amino acid β-naphthylamides, dipeptides and metal-ion requirements, they found certain similarities between the rate of hydrolysis for the two different tissue enzymes. Swanson (1966) and Swanson, Raley & Jeter (1967) described the isolation of lysosomes identified by the activities of their enzymes in bovine lens epithelium cells. Their results clearly indicate the presence of hydrolytic enzymes in mammalian lens tissue.

However, in contrast with the well-characterized proteolytic enzymes of digestive secretions (namely the pancreatic and gastric juices of various mammals) mammalian lens proteases have been investigated only to a limited extent; few have been extensively purified, and of these, much remains to be learned about either physical or catalytic properties. An interest in the loss of protein in advanced cataractous human lenses, possibly due to proteolysis, prompted us to undertake the purification of a proteolytic enzyme from human senile cataractous lenses. We describe herein a procedure for the isolation of a lens protease in apparently homogeneous form and report some of its physicochemical characteristics.

MATERIALS AND METHODS

Materials. The human senile cataract lenses were acquired from cataractomies performed at the Medical University of South Carolina. The lenses were stored at −85°C until used. Substrates employed in the enzymic assays were obtained as follows: haemoglobin substrate powder from Worthington Biochemical Corp., Freehold, N.J., U.S.A., benzyl-L-arginine ethyl ester, L-leucine β-naphthylamide, ovalbumin, casein and lactalbumin from Sigma Chemical Co., St Louis, Mo., U.S.A. and crystalline bovine albumin from Armour Laboratories, Chicago, Ill., U.S.A. Sephadex G-75 and DEAE-Sephadex A-50 (spherical resin, particle size 40–120 μm) were obtained from Pharmacia, Uppsala, Sweden and the reagents for polyacrylamide-gel electrophoresis were purchased from Canalco (Rockville, Md., U.S.A.) and Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Enzyme assays. One unit of protease, was defined as the amount of the enzyme that yields a colour equivalent of 1.0 μg of tyrosine/min with Folin's reagent by using bovine plasma albumin as substrate (Burgum, Prescott & Hervey, 1964).

Hydrolysis of esters was determined by an assay system of Schwert & Takenaka (1955) by using 1 mM-benzyl-L-arginine ethyl ester in 5 mM-tris–HCl buffer, pH 7.6. One esterase unit was arbitrarily defined as the amount of enzyme that produces an increase in E_{253} of 1.0 (1 cm light-path) in 10 min measuring the bond cleavage at 25°C.

It has already been established that lens tissue possesses the ability to hydrolyse L-leucine β-naphthylamide, which is widely used as substrate for aminopeptidase (Goldbarg & Rutenburg, 1958). The senile cataractous lens enzymes isolated, however, failed to hydrolyse L-leucine amide or L-leucylglycine and probably should be regarded as arylamidases (Patterson, Hsaio & Keppel, 1963) rather than aminopeptidase. Nonetheless, it appeared important to monitor the hydrolysis of L-leucine β-naphthylamide during the purification of the proteolytic enzyme. One unit of arylamidase activity was defined in this study as the amount of enzyme catalysing an increase in E_{560} of 1.0 (1 cm light-path) in 30 min. Protease activity employing synthetic substrates was measured by the quantitative ninhydrin procedure of Moore & Stein (1954).

Protein concentrations were determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951): crystalline bovine plasma albumin was used as the standard. Specific activities were expressed as enzyme units/mg of protein.

Electrophoretic analyses. Polyacrylamide-disc electrophoresis (Canalco Company) was performed by the procedure developed by Ornstein (1964) and Davis (1964), the only modification being that the gels were stacked at pH 6.8 and run at pH 7.6, 8.0 and 8.4.

Concentration of protein samples. Protein samples were concentrated at 4°C either by using an Amicon Diaflo ultrafiltration apparatus or by pervaporation from a dialysis bag.

Chromatographic procedures. DEAE-Sephadex A-50 and Sephadex G-75 were dispersed in water and allowed to swell. After the fine particles had been decanted several times, the gel was poured into a chromatographic column. The gel was then equilibrated in the first buffer to be used in the elution procedure. The effluents were collected as described in the legends to the figures. The E_{280} was measured, with a Gilford model 2400 automatic recording spectrophotometer.

Amino acid analysis. Dried and ash-free enzyme samples (0.42–0.5 mg/ml) were dissolved in 3.0 ml of glass-distilled, constant-boiling HCl and the solution was divided into three tubes and sealed under vacuum. After 24, 48 and 72 h hydrolysis at 110°C, the hydrolysates were filtered and evaporated to dryness *in vacuo*. The samples were dissolved in 1% HCl for application to a column (130 cm) of Chrombeads B in a Technicon amino acid analyser. Cysteic acid was determined on a sample oxidized with performic acid as described by Moore (1963). The tyrosine/tryptophan ratio was determined by the procedure of Bencze & Schmid (1957).

Ultracentrifugal analysis. Approximate molecular weights were determined by approach-to-equilibrium studies, which were done in a Spinco model E analytical ultracentrifuge by the procedure described by Archibald (1947). The sedimentation velocity experiments were performed at 4°C and 60000 rev./min by using a double-sector cell with an aluminium-filled Epon centrepiece.

With human senile cataractous lens in various stages of opacification (a partial cloudiness to a complete opaqueness for the ocular lens tissue) there were pronounced differences in the gross proteolytic activity, protein content, esterase and arylamidase activities from group to group (12 per group), and these variations affected the patterns obtained in chromatographic isolation steps. However, lens groups did not materially affect the efficiency or reproducibility of the overall enzyme purification procedure, which has been conducted with samples of crude lens homogenates ranging in size from 2 to 4 g. The studies presented below were obtained in an isolation experiment starting with 3.5 g of crude lens material.

EXPERIMENTAL AND RESULTS

Enzyme purification procedures

Phase I: preparation of the human senile cataractous lenses for column chromatography. To prepare a homogenate, lenses were stirred at 4°C with 50 ml of 5 mM-tris–HCl buffer, pH 8.5, to give a suspension of cortical and nuclear fibres. After 20–30 min the suspension of cortical fibres was decanted and homogenized in a motor-driven tissue grinder with a Teflon pestle. The suspension was centrifuged for 30 min at 4°C in a Sorvall RC2-B automatic refrigerated centrifuge (approx. 5400*g*) and the supernatant fluid decanted from the sedimented albuminoid and other insoluble proteins. The supernatant solution was dialysed for about 12 h against the 5 mM-tris–HCl buffer, pH 8.5, and centrifuged. This fluid served as the starting material.

Phase II: chromatography on DEAE-Sephadex A-50. All phases or steps of the purification procedure were carried out at 4°C unless otherwise indicated. The above supernatant fluid was placed on a column of DEAE-Sephadex A-50 previously

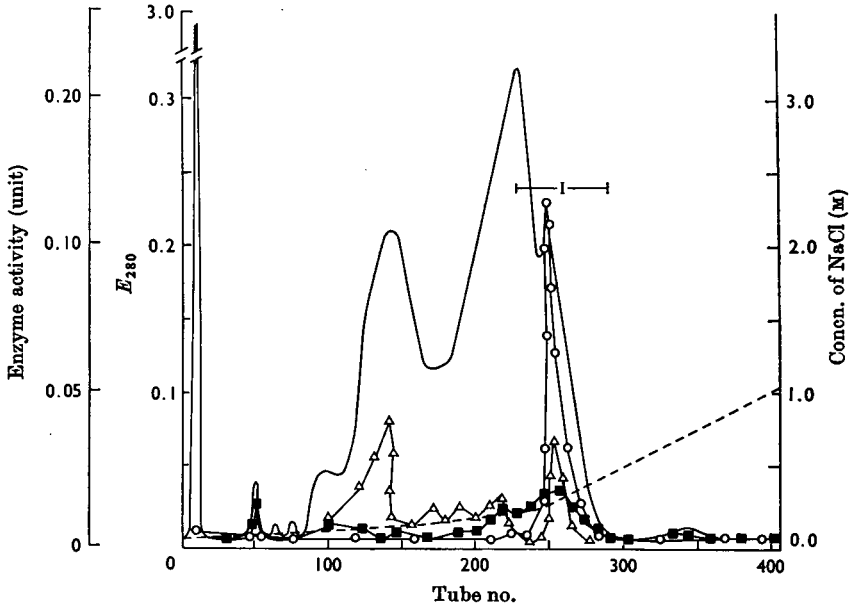

Fig. 1. Chromatogram of the crude, dialysed human senile cataractous lens protease on DEAE-Sephadex A-50 in 5mM-tris–HCl buffer, pH8.5. A 65ml sample of supernatant fluid was applied to a column (2.5cm × 50cm), and eluted at approx. 50ml/h into fractions containing 10ml, with a gradient elution from 0 to 1.0M-NaCl (-----). ——, E_{280}; ○, protease activity toward bovine plasma albumin; △, esterase activity toward benzyl-L-arginine ethyl ester; ■, arylamidase activity toward L-leucine β-naphthylamide. Peak I (tubes 240–280) was selected for further purification.

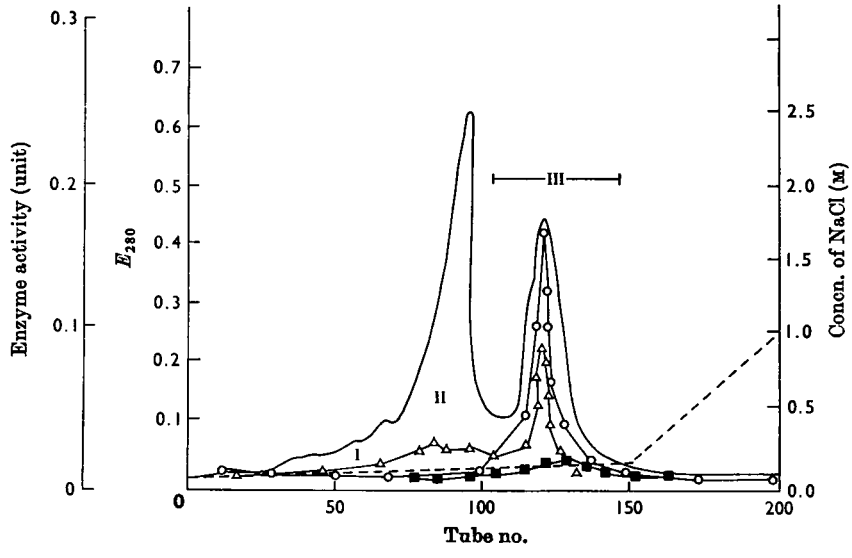

Fig. 2. Rechromatography on DEAE-Sephadex A-50 in 5mM-borate–NaOH buffer, pH9.2, of fraction I. Phase II was applied to a column (2.5cm × 50cm) and was eluted at a rate of 60ml/h. Fractions (10ml each) were collected in tubes containing 2ml of 0.1M-Na_2HPO_4 (adjusted to pH8.0) to decrease the alkalinity of the effluent and thus to minimize the possibility of denaturation. ——, E_{280}; ○, proteolytic activity; △, esterase activity; ■, arylamidase activity; -----, NaCl gradient (0–1.0M). Peak III was found to contain the highest proteolytic activity and was selected for further purification. This peak included tube numbers 112–138.

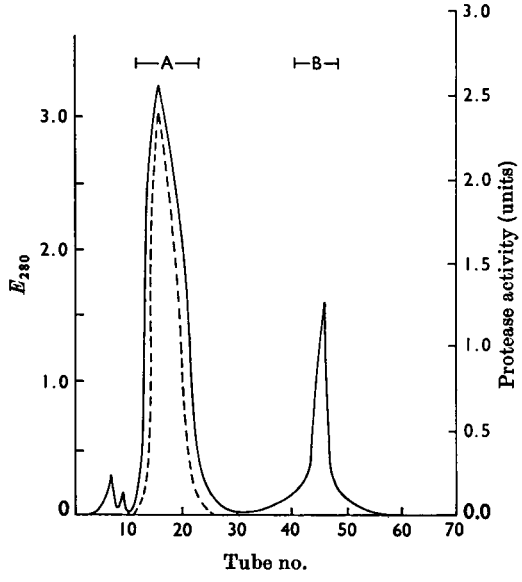

Fig. 3. Purification of the human senile cataractous lens protease by gel filtration on Sephadex G-75 of peak III, phase III (tubes 112–138). The sample was applied to a column (4.25 cm × 60 cm) and eluted at a rate of 160 ml/h in 5 mM-tris–HCl, pH 8.5, which was 0.1 M with respect to NaCl. Peak A represents the proteolytic activity toward the bovine plasma albumin substrate; it was devoid of arylamidase and esterase activity. Peak B revealed most of the esterase activity.

equilibrated with 5 mM-tris–HCl buffer, pH 8.5, and the unadsorbed material was washed through by the addition of this buffer until the breakthrough peak had completely emerged. The column was developed by the addition of sodium chloride to the 5 mM-tris–HCl buffer, pH 8.5, as follows. First, 2 l of a linear gradient (0–0.1 M-sodium chloride) was passed through, then the sodium chloride concentration was successively increased to 0.2 M and 1.0 M. Every third tube was assayed for protease, arylamidase and esterase activities. Each peak that emerged from the column was pooled separately and concentrated to 10 ml for further purification. The chromatography on DEAE-Sephadex A-50 is shown in Fig. 1, which is a representative chromatogram of crude senile cataractous lenses indicating a sharp separation of a major proteolytic peak (I) emerging at 0.05 M-sodium chloride. This peak always possessed the strongest activity toward bovine plasma albumin substrate and was selected as the enzyme of primary interest in this study.

Phase III: rechromatography on DEAE-Sephadex A-50. Peak I from the previous treatment was

Table 1. *Purification of human senile cataractous lens protease*

See the text for experimental details and definitions of enzyme units. The recovery of protease represents the accumulated recovery during isolation (this varies with each group of lenses).

Phase and treatment	Total protein (mg)	Total enzyme units			Specific activity			Recovery of protease (%)
		Protease	Esterase	Arylamidase	Protease	Esterase	Arylamidase	
I. Preparation of senile cataractous lens for purification	1200	1185	1465	1214	0.988	1.221	1.012	—
II. Chromatography on DEAE-Sephadex A-50 at pH 8.5	210	350	207	216	1.67	0.986	1.029	30
III. Rechromatography on DEAE-Sephadex A-50 at pH 9.4	97	208	104	180	2.144	1.072	1.86	18
VI. Chromatography on Sephadex G-75	28	197	22	10	7.04	0.786	0.357	17
V. Rechromatography on DEAE-Sephadex A-50 at pH 9.6	17	164	0	0.09	9.65	0	0	14

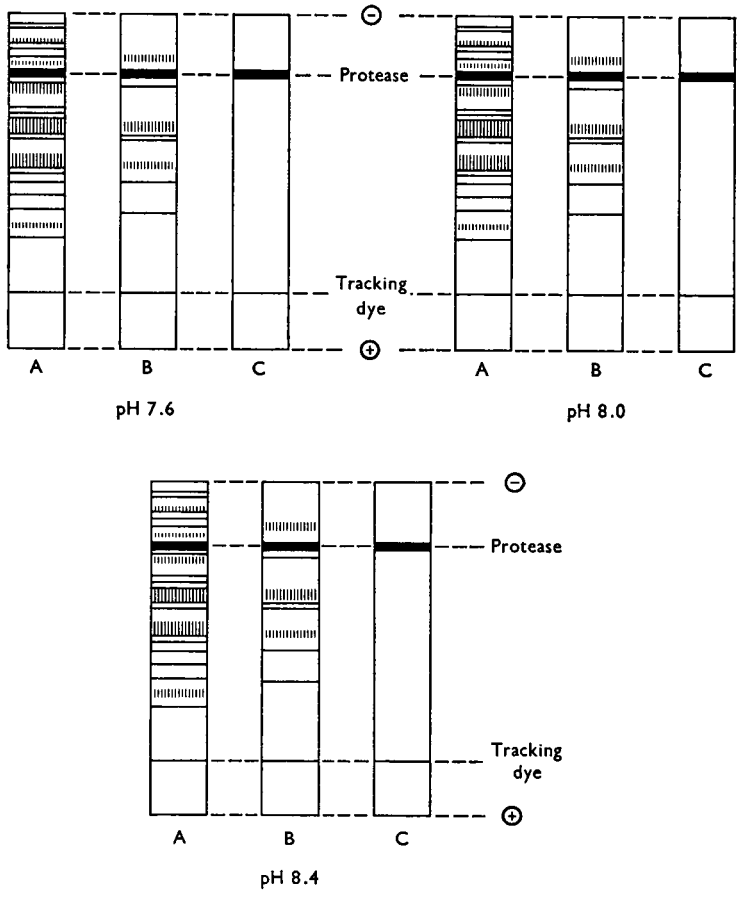

Fig. 4. Electrophoretic patterns on polyacrylamide gels of fractions obtained during the purification of the human senile cataractous lens protease. Gels were run at pH 7.6, 8.0 and 8.4 with samples containing 100 μg of enzyme. Shown are major bands in the crude dialysed lens supernatant (A), the active fraction from phase III (B) and the final isolated protease after phase V (C).

dialysed against 5 mM-borate–NaOH buffer, pH 9.2, then was placed on a column of DEAE-Sephadex A-50 equilibrated with this buffer. The sample was eluted with a linear gradient of increasing sodium chloride concentration (700 ml of 0–0.1 M; 500 ml of 0.1–0.5 M), in the borate buffer, and the collected peaks were pooled separately, concentrated and assayed as previously described. The major proteolytic fraction designated as peak III contained esterase together with a very small amount of arylamidase activity. The shapes of the peaks in Fig. 2 indicate that more than one enzyme was present in peak I of phase II as their elution patterns were not identical. Arylamidase activity began to emerge from the column as the ionic strength reached 0.1 M-sodium chloride.

Phase IV: chromatography on Sephadex G-75. Peak III was dialysed for about 10 h against 4 l of 5 mM-tris–HCl buffer, pH 8.5, and applied to a column of Sephadex G-75. The protease was eluted in 5 mM-tris–HCl buffer, pH 8.5. The first peak of extinction contained opalescent material; a second peak (peak A), possessing all the protease activity, emerged in tubes 11–24. The elution was continued until the extinction at 280 nm dropped sharply. This filtration step facilitated the separation of all the esterase activity from the protease, thus revealing two major components, as shown in Fig. 3.

In some preparations it was found necessary to fractionate the enzyme preparation further to eliminate the esterolytic activity present in phase IV.

Table 2. *Amino acid composition of human senile cataractous lens protease*

Values for recovery are means of duplicate analysis on each of two 24, 48 and 72 h hydrolysates as outlined in the text. Minimum molecular weight = $\frac{\text{mol. wt. of amino acid residue}}{\%\text{ amino acid residue in protein}} \times 100$.

Amino acid	Recovery (%)	Minimum molecular weight	Calculated number of residues	Nearest integer	Integral no. × mol. wt. of residue
Serine*	8.81	988	17.6	18	1567
Threonine*	5.70	1773	14.8	15	1516
Glutamic acid	10.85	1190	18.1	18	2324
Aspartic acid	9.58	1201	27.7	28	3222
Glycine	4.56	1251	12.2	12	684
Proline	6.23	1559	6.1	6	582
Alanine	5.96	1193	10.4	10	711
Valine	3.43	2891	14.6	15	1487
Isoleucine	5.38	2104	10.3	10	1132
Leucine	9.36	1209	18.4	18	2037
Half cystine†	1.27	8043	4.3	4	605
Methionine	2.60	5046	6.1	6	787
Phenylalanine	3.82	3853	10.2	10	1472
Tyrosine	4.08	3999	9.1	9	1469
Histidine	5.13	2674	10.8	11	1508
Arginine	5.14	3039	6.8	7	1093
Tryptophan‡	2.65	7027	12.4	12	2235
Lysine	4.58	2799	5.2	5	640
Totals	99.13			214	25071§

* Extrapolated to zero hydrolysis time on the basis of 24 and 72 h hydrolysis.

† Performic acid oxidation of separate sample before hydrolysis, expressed as cysteic acid.

‡ Spectrophotometric determination on intact enzyme sample.

§ Corrections have been made for the mol of water, but no corrections to account for the amides.

Phase V: rechromatography on DEAE-Sephadex A-50 at pH 9.6. It was apparent that both the esterase and arylamidase contamination, when they appeared, could be removed by rechromatography on DEAE-Sephadex A-50 at pH 9.6. When this treatment was used, peak A from phase IV was dialysed for 24 h against 5 mM-borate–NaOH buffer, pH 9.6, concentrated to about 0.7 mg/ml, and then placed on a column of DEAE-Sephadex A-50 equilibrated with the dialysing buffer. After the sample had entered the gel bed, the protease was eluted with a linear gradient of sodium chloride (20 mM–0.5 M) in the same buffer. This procedure produced two sharp peaks; the first peak was active toward benzyl-L-arginine ethyl ester and inactive toward bovine plasma albumin, the second peak was the protease free from esterase and arylamidase. If a third peak was eluted it was active toward leucine β-naphthylamide substrate and inactive toward the other two substrates. Representative separations on DEAE-Sephadex A-50 and Sephadex G-75 depict steps of phases in the isolation experiment summarized in Table 1, which reveals that the specific proteolytic activity was increased about tenfold.

Disc electrophoresis. In Fig. 4 disc-gel-electrophoretic patterns for the crude senile cataractous lens supernatant, for one intermediate step and for the final product purified through phase V, are compared. Zone electrophoresis was used at three different pH values to evaluate the effectiveness of the various isolation phases. The effectiveness of the purification protocol is evident from the decrease in the number of components giving only a single band in the final product.

Properties of the protease

Amino acid composition. Phase V purified protease was used for amino acid analyses that are shown in Table 2. The values for all residues except tryptophan were determined from results obtained by using enzyme samples hydrolysed for 24, 48 and 72 h with constant-boiling HCl.

Substrate specificity. The cataractous lens protease readily hydrolysed denatured haemoglobin,

lactalbumin and both the soluble and insoluble bovine lens proteins, but purified protease preparations were devoid of activity towards casein and ovalbumin substrates (Table 3). It is noteworthy that the cataractous lens protease exhibited activity toward both the soluble and insoluble bovine lens proteins.

The behaviour of the protease on some synthetic substrates is also shown in Table 3. The human senile cataractous lens protease possessed peptidase activity, as evidenced by its action on *N*-Z-L-glycylphenylalanine. Hydrolysis was noted with *N*-acetyl-L-phenylalanine ethyl ester and *N*-benzoyl-L-arginine amide hydrochloride substrates. Weak activity was observed toward the trypsin substrate *p*-tosy-L-arginine methyl ester during the 2h period of incubation. However, the enzyme was devoid of activity toward benzylarginine ethyl ester, leucine β-naphthylamide, *N*-acetyl-L-tyrosine amide, glycyl-L-phenylalanine amide atceate, glycyl-L-tyrosine amide acetate, DL-leucine amide hydrochloride and L-tyrosine ethyl ester.

pH *optimum*. The effects of pH on enzymic activity and stability were investigated with a buffer composed of equimolar (0.1M) citric acid, sodium citrate and veronal, adjusted with hydrochloric acid to the pH values desired (Peterson, Johnson & Price, 1948). The bovine plasma albumin substrate was dissolved in the buffer at the pH values indicated in Fig. 5, and activity was assayed in the usual manner. Maximum activity occurred near pH5.2.

Effect of bivalent cations. The need of metal ions for enzymic activity was tested by dialysing the purified enzyme against 0.02M-EDTA for 18h at pH5.2 (sodium veronal buffer) and the excess of EDTA was removed by dialysis against deionized water.

As can be seen from Table 4, the cations Co^{2+}, Ni^{2+} and Mn^{2+} approximately doubled and tripled the rate of hydrolysis of bovine plasma albumin by the purified enzyme. There was no activation

Table 3. *Hydrolysis of substrates by purified human senile cataractous lens protease*

Enzyme activity with natural substrates (2% concentration) was determined as E_{660} units/ml. Protease activities with synthetic substrates were measured by the quantitative ninhydrin procedure of Moore & Stein (1954). Substrate concentrations were 0.1mM in 0.05M-veronal buffer, pH6.5; 0.5ml of substrate was incubated with 50μl of enzyme at 40°C. Activity is expressed as μmol of amino acid released/ml of reaction mixture per 2h. The extent of hydrolysis was determined by referring to previously prepared tables of amino acid equivalents, and of colour yields for individual amino acids (Moore & Stein, 1954).

Natural substrates	Activity (units/6h per ml)
Bovine plasma albumin	68.5
Denatured haemoglobin	55.3
Casein	0
Lactalbumin	31.4
Ovalbumin	0
Soluble bovine lens protein	97.9
Insoluble bovine lens protein	86.3
Synthetic substrates	**(μmol/2h per ml)**
N-Z-L-glycylphenylalanine	4.22
N-Acetyl-L-phenylalanine ethyl ester	2.12
N-Benzoyl-L-arginine amide hydrochloride	1.10
p-Tosyl-L-arginine methyl ester	0.49
Benzyl-L-arginine ethyl ester	0
N-Acetyl-L-tyrosine amide	0
L-Leucine β-naphthylamide	0
Glycyl-L-phenylalanine amide acetate	0
Glycyl-L-tyrosine amide acetate	0
DL-Leucine amide hydrochloride	0
L-Tyrosine ethyl ester	0

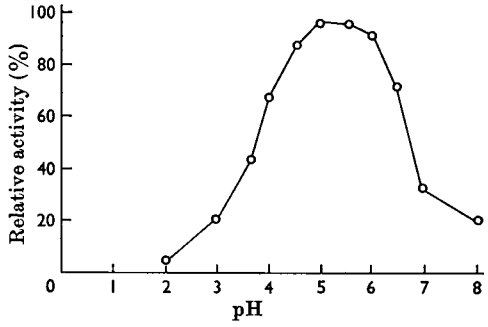

Fig. 5. Effects of pH on the hydrolysis of bovine plasma albumin by the purified human senile cataractous lens protease. The relative activity values are expressed as a percentage of the activity at pH5.0–5.2. Conditions of the experiment are described in the text.

Table 4. *Effect of bivalent cations on hydrolysis by human senile cataractous lens protease*

The lens protease preparations were incubated for 1.5h at 40°C with 10mM solutions of the respective bivalent cations (as their chloride salts). Assay for proteolytic activity was with bovine plasma albumin as described in the text.

Cation	Proteolytic activity (%)
None	100
Co^{2+}	213
Ni^{2+}	185
Mg^{2+}	0
Mn^{2+}	300
Ca^{2+}	0
Zn^{2+}	2
Cu^{2+}	0
Cd^{2+}	0

Table 5. *Effect of inhibitors on hydrolysis of human senile cataractous lens protease and restoration of activity by the addition of* Co^{2+}, Ni^{2+} *and* Mn^{2+} *to EDTA and phenylmethanesulphonyl fluoride solutions*

Assays for enzymic activity were performed as described in the text. The enzyme was incubated with inhibitor for 1 h at 37°C before assay.

Inhibitor	Concentrations (mM)	Remaining proteolytic activity (%)
None		100
o-Phenthroline	1	90
o-Phenanthroline	10	85
EDTA	1	0
p-Chloromercuribenzoate	1	65
Cysteine	5	100
NaCN	5	100
Di-isopropyl phosphorofluoridate	5	5
Phenylmethanesulphonyl fluoride	5	5
EDTA+Co^{2+}	1 and 10	180
EDTA+Ni^{2+}	1 and 10	120
EDTA+Mn^{2+}	1 and 10	250
Phenylmethanesulphonyl fluoride +Co^{2+}	5 and 10	110
Phenylmethanesulphonyl fluoride +Ni^{2+}	5 and 10	129
Phenylmethanesulphonyl fluoride +Mn^{2+}	5 and 10	314

Table 6. *Effect of temperature on proteolysis*

The enzyme was incubated with bovine plasma albumin substrate (pH 5.2) for 15 min at the temperatures shown. The extent of hydrolysis at 37°C was assigned a value of 100, and activities at other temperatures are expressed as relative values.

Temperature (°C)	Activity (%)
10	0
25	0
30	34
35	80
37	100
40	160
45	100
50	52
55	20
60	5
65	0
70	0

observed in the presence of Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Cd^{2+}. On the contrary complete inhibition results with 10 mM cation on treatment of the enzyme for 1.5 h before assay.

Effect of inhibitors. The effect of a number of group-specific inhibitors is shown in Table 5 together with restoration of activity accomplished by the addition of several cations. Di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride, and EDTA all strongly inhibited the enzyme, whereas *o*-phenanthroline at two different concentrations, *p*-chloromercuribenzoate, cysteine and sodium cyanide showed partial or no inhibition at all. The activity of the enzyme apparently does not bear a unique relationship to a single metal, inasmuch as Co^{2+}, Ni^{2+} and Mn^{2+} ions all were capable of restoring activity to the EDTA- and phenylmethanesulphonyl fluoride-inhibited protease, albeit with different effectiveness.

Temperature stability of the enzymes. The enzyme was more stable in tris and veronal buffers than in phosphate or borate–NaOH buffers. Substantial losses in activity were encountered during purification in freezing or freeze-drying (0.104 g/ml) the enzyme; storage at 4.6°C proved to be the most satisfactory. When the protease was exposed to room temperature for 2 h or longer, 80–95% of the activity was lost. Table 6 shows the effects of the temperature of incubation and indicates that maximum proteolytic activity for a 15 min reaction occurs at approx. 40°C. At 50°C, the half-life of the enzyme was 0.5 min, as determined by heating 0.1 ml samples of the enzyme at that temperature for various periods of time, then quickly cooling in an ice–water bath, and determining the remaining activity by the bovine plasma albumin substrate assay.

Molecular weight. On the basis of the values of sedimentation and diffusion measurements the molecular weight was calculated to be 27000 from the following Svedberg equation: $M = RTS/(1-\bar{v}\rho)D$, where T is the absolute temperature,

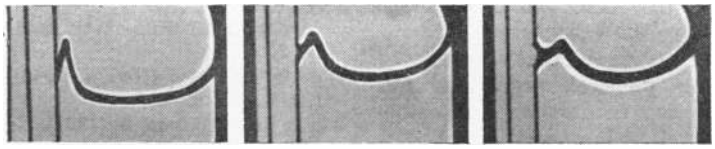

Fig. 6. Analytical ultracentrifugation of purified human senile cataractous lens protease. Shown are schlieren images obtained during the sedimentation at 22, 46 and 62 min after reaching full rotor speed of 60000 rev./min; the protein concentration was approx. 1% in 0.1 M-tris–HCl buffer and 0.1 M-NaCl, pH 7.0; the bar angle was 60° and the temperature was 4°C; sedimentation was from right to left.

Table 7. *Molecular weight of cataractous protease by approach to equilibrium*

Molecular weights were calculated from the cell meniscus at four time-intervals after the rotor attained a speed of 7137 rev./min.

Time after rotor reached speed (min)	Molecular weight
60	27550
65	27370
80	26840
95	27200
Mean	27240

R the ideal gas constant, and ρ the density of the solution. With $D_{20,w} = 8.44 \times 10^{-7}\,cm^{-2} \cdot s^{-1}$, $s_{20,w} = 2.5 \times 10^{-13}\,s$, $\bar{v} = 0.725$. Sedimentation patterns by ultracentrifugal analysis are shown in Fig. 6. The molecular weights obtained by the approach to equilibrium procedure are shown in Table 7 and agree closely with the molecular weight by sedimentation velocity and diffusion. This compares with the minimum molecular weight as calculated from amino acid composition of 25000.

DISCUSSION

The results presented above indicate that a reproducible procedure for the isolation of a protease from human senile cataractous lens has been achieved. This procedure results in the isolation of a protease in apparently homogeneous form as evidenced by several criteria including chromatography on DEAE-Sephadex A-50 at pH 9.6, electrophoresis, ultracentrifugation and enzymic activity. The modest increase in specific activity attained (9- to 9.65-fold) is probably due to the fact that this enzyme is quantitatively one of the principal components of the unfractionated cataractous lens, as indicated by the disc-gel-electrophoretic pattern in Fig. 4. Also, the cataract lens contains more insoluble protein than normal lens. Quantitative densitometric measurement of the disc-gel-electrophoretic pattern of the crude lens indicated that the protease constituted approx. 8% of the total protein.

Individual groups of cataractous lenses, however, differed somewhat from one another with respect to the total and relative amounts of protease, and other proteins present in the starting material. Variation between groups could be expected because the lenses were not uniform in size, age and degree of opacification reflected as denatured protein. The human senile cataract lenses that were removed at cataractomies ranged in age from 45 to 75 years. These variables necessitated that every isolation experiment be conducted by monitoring the results of each step by assays for all of the enzymic activities and for total protein to permit the repetition or insertion of a step whenever it was desirable.

Our evidence clearly shows that purified senile cataractous lens protease differs in properties from some of the known aminopeptidases isolated from the lens by Wolfe & Resnik (1963*a*) and Spector (1963*a*,*b*) or esterases discussed by Spector (1961). Likewise, our protease bears no resemblance to Devi's (1959) esterase, suggesting his enzyme was identical with mammalian chymotrypsin. It must be pointed out that Devi (1959) employed a non-purified bovine lens homogenate to hydrolyse various synthetic amino acid esters, specifically the phenylalanine ethyl ester. In the course of our purification of a human senile cataractous lens protease, we show esterase activity, but have not characterized it. Also, other protein preparations (denatured haemoglobin and lactalbumin) were degraded by the protease, although more slowly or less exhaustively than bovine lens protein. Casein, which is stable under these conditions of assay, was entirely unaffected by the protease. The situation with lactalbumin seems to be intermediate between these two extremes (bovine lens protein and casein). The other differences observed in the presence of metal ions and inhibitors do not concur with any known proteolytic enzyme isolated from lens tissue. The observed inhibition by di-isopropylphosphorofluoridate and phenylmethanesulphonyl fluoride implicate a serine residue in the active site

of this enzyme, as inhibition by these reagents is presumably restricted to the 'serine enzymes'.

It seems reasonable to expect that a variety of enzymes of differing physical properties and catalytic specificities may be discovered in cataractous lens tissue as well as in normal lenses.

It will be worth while to make detailed kinetic analyses of the human senile cataractous lens protease and to identify the esterase and arylamidase as well as other protease peaks found in various fractions.

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