THE TRYPSIN AND CHYMOTRYPSIN INHIBITORY CAPACITY OF HUMAN AND BOVINE α-CRYSTALLIN

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1. Introduction

The existence of proteolytic enzymes in mammalian lens tissue is fairly well documented and proteinases from bovine [1,2] and also from human senile cataractous lenses [3] have been purified and partially characterized. At present the exact role of these enzymes is not well understood; it has been suggested, but not proven, that the loss of protein in advanced cataractous human lenses might be due to increased proteolysis [3,4].

During our own attempts at isolating proteases from bovine and also from human cataractous lenses we discovered that crude lens homogenates were able to inhibit trypsin and to a lesser extent chymotrypsin. The present paper describes this finding and also shows that the inhibitory activity is associated with the α-crystallin fraction of the lens. Aggregates of urea denatured α-crystallin or of its acidic (αA_1, αA_2) or basic (αB_1, αB_2) components [5,6] inhibit trypsin at least five times as strongly as does native α-crystallin. The chymotrypsin inhibitory activity of α-crystallin is much weaker than its trypsin inhibitory activity and can not be increased by urea treatment or separation into the component acidic and basic polypeptide chains. To our knowledge this is the first report of a protease inhibitory activity associated with eye lens proteins. The inhibition of chymotrypsin by aqueous humor has been previously reported by other workers [7,8].

During the course of this investigation we successfully applied purification methods developed for the purification of bovine lens proteins to the purification of human senile cataractous lens proteins.

2. Materials and methods

2.1. *Materials*

Bovine trypsin (TRL 2DA) and chymotrypsin (CDI 1IC) were obtained from Worthington (Freehold, N.J.). Calf lenses were purchased from Pel-Freeze Biochemicals (Rogers, Ark.) and human cataractous lenses were obtained from the Winnipeg General and St. Boniface hospitals. Bovine albumin (Cohn fraction V) was obtained from Sigma Chemical Co. (St. Louis, Mo.); all other chemicals were reagent grade or better.

2.2. *Assays for inhibitory activity*

Assays for protease inhibitory activity were based on the method of Anson [9] for determining proteolytic activity. Trypsin or chymotrypsin (15–20 μg) and a sample eye lens protein (0–2000 μg) were preincubated for 30 min at 37°C in 200 μl of 0.006 M Tris–HCl, pH 7.6. The substrate (1 ml of 2% bovine albumin, pH 7.6) was then added and the mixture incubated for a further 20 min at 37°C. After precipitation of the proteins with trichloracetic acid (2 ml of a 5% aqueous solution) and centrifugation, the unprecipitated split products were estimated with phenol reagent [9]. For each sample a blank was carried out in an identical fashion except that the trichloracetic acid was added immediately after the addition of substrate. Protein concentrations were determined by the method of Lowry [10].

2.3. *Extraction of lens proteins*

Lenses were kept frozen at −80°C until used. The lenses were then washed with ice water, trimmed if needed and suspended in 5 to 6 times their weight of

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cold water (4°C) for 1 hr. After removal of the nuclei the suspension was homogenized in a motor driven tissue grinder and then stirred for an additional hour at 4°C. The material was then centrifuged at 12 000 *g* for 30 min and the precipitate discarded. The supernatant is referred to a crude lens extract.

2.4. *Fractionation of crude extract into α, β and γ crystallin groups*

The extract obtained in 2.3 was fractionated into its α, β and γ crystallin components by stepwise elution from a column of DEAE–cellulose with 0.002 M, 0.050 M and 0.40 M potassium phosphate pH 7.2 as described by Spector [11].

2.5. *Preparation of α-crystallin*

In separate experiments α-crystallin was also prepared by isoelectric precipitation at pH 5.0 as described by Waley and van Heyningen (1). The material so obtained was further purified by gel filtration on Sephadex G-200 as described by Van Dam and Ten Cate (12).

2.6. *Preparation of the acidic and basic polypeptide chains of α-crystallin*

α-Crystallin prepared as described under 2.5 was dissociated into its subunits with 7M urea and separated into its acidic and basic polypeptide components by chromatography on SP-Sephadex C50 as described by Schoenmakers et al. [13].

3. Results and discussion

3.1. *Inhibitory activities of crude bovine lens extract and its α, β and γ crystallin fractions*

In initial experiments we found that crude eye lens extracts had the capacity to inhibit trypsin and to a lesser extent chymotrypsin. In an attempt to see if this inhibitory activity was associated with any particular eye lens protein the extract was fractionated into its α, β and γ crystallin groups. The inhibitory activities of crude lens homogenate and of the three partially purified components are shown in fig. 1. It can be seen from the figure that the inhibitory activity is associated with the α-crystallin fraction. The inhibition is not

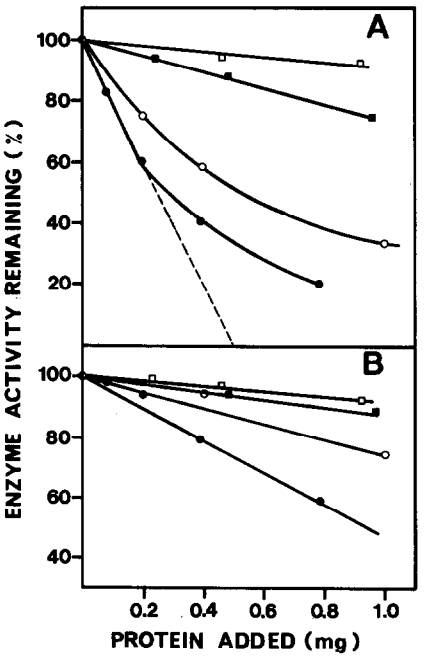

Fig. 1. Protease inhibitory activities of bovine lens proteins. The assays are described in section 2.2. and the results are expressed in terms of percent enzyme activity remaining as a function of the amount of protein added to a constant amount of enzyme. Panel A: 20 μg trypsin. Panel B: 15 μg chymotrypsin. (○–○–○) crude extract; (■–■–■) γ-crystallin fraction; (□–□–□) β-crystallin fraction; (●–●–●) α-crystallin fraction.

linear with the amount of protein added but by extrapolation of the linear part of the curve one can estimate that it takes about 250 μg of α-crystallin to inhibit 10 μg of trypsin and about three to four times as much to inhibit the same amount of chymotrypsin. Assuming a mol. wt. of 800 000 for α-crystallin [14] it takes a little less than one mole of α-crystallin to inhibit one mole of trypsin. The β and γ crystallin fractions of the bovine lens are essentially devoid of trypsin and chymotrypsin inhibitory activity.

3.2. *Inhibitory Activity of Bovine α-Crystallin and its acidic and basic polypeptide components*

α-Crystallin is believed to be built up mainly from four distinct polypeptide chains of mol. wt. 20 000 – 25 000; two are acidic (αA_1 and αA_2) and two are basic (αB_1 and αB_2) [5,15]. α-Crystallin purified as

described under 2.5. was dissociated in 7M urea and the resulting mixture fractionated into its acidic and basic polypeptide components as described under 2.6. It can be seen from fig. 2 and table 1 that purified α-crystallin and its acidic and basic components are all about equally active against chymotrypsin. On the other hand the trypsin inhibitory activity of the components is about six fold that of native α crystallin, at least at low inhibitor to enzyme ratio. The curves showing the amount of residual enzyme activity with increasing amounts of protein level off at about 30% in the case of the acidic polypeptides and at about 15% in the case of the basic polypeptides. This is probably an indication that the K_{ass} for binding between enzyme and inhibitor to form an inactive complex is not very large [16].

The marked increased trypsin inhibitory activity of the acidic and basic polypeptide components over that of native α-crystallin is surprising and led us to do a control experiment. A sample of purified α-crystallin was treated with 7M urea under conditions of concentration and pH identical to those used for the chromatographic separation into acidic and basic components; the urea was then removed by dialysis and the sample lyophilized and assayed for trypsin inhibitory activity. The results are included in table 1 and show that this material has an inhibitory activity very similar to that of the isolated acidic and basic components. It therefore appears that mere treatment of α-crystallin with urea and subsequent removal of the urea results in a new molecular species with increased trypsin inhibitory activity. Li and Spector [17] have previously shown that urea treated α-crystallin and also its isolated subunits are capable of reaggregating to form large macromolecules (mol. wt. 400 000 – 600 000) which are nonetheless significantly smaller than native α-crystallin. Our own preliminary ultracentrifuge studies show that whereas our α-crystallin preparation has a sedimentation constant of 19.43 S the reaggregated acidic component of α-crystallin has a sedimentation constant of 14.08; these values are in very good agreement with those of Li and Spector [17]. Assuming a mol. wt. of 400 000 for the reaggregated subunits it can be calculated by extrapolation of the data in fig. 2 that 1 mole of this macromolecule can inhibit about 5 moles of trypsin. At the present time we believe that this observed inhibition is non-specific and is not of physiological importance.

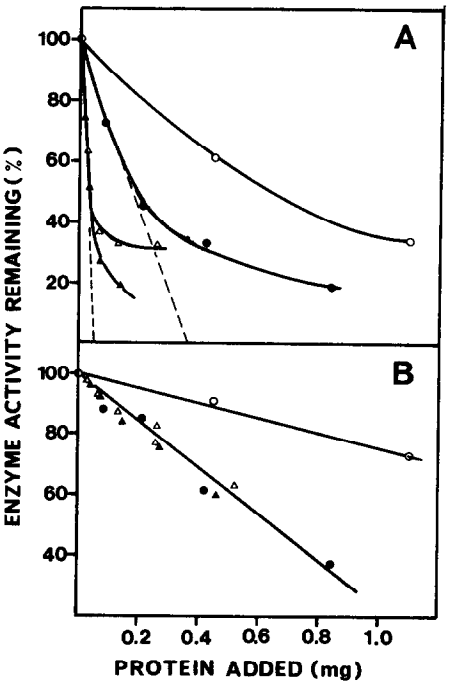

Fig. 2. Protease inhibitory activities of bovine α-crystallin and its acidic and basic components. The assays were performed as described in fig. 1. Panel A: 15 μg trypsin. Panel B: 15 μg chymotrypsin. (○–○–○) crude extract; (●–●–●) α-crystallin, (△–△–△) acidic α-crystallin components; (▲–▲–▲) basic α-crystallin components.

Table 1
Protease inhibitory activities* of lens protein preparations

Sample	μg needed to inhibit	
	10μg Trypsin	10μg Chymotrypsin
Bovine lens		
α-crystallin	250	930
α-crystallin urea treated	60	–
acidic components	40	930
basic components	40	930
Human cataractous lens		
α-crystallin	210	570
acidic components	65	570
basic components	40	570

* The values in this table were obtained by extrapolating the linear sections of the inhibition curves in figs. 1–3 to zero enzyme activity.

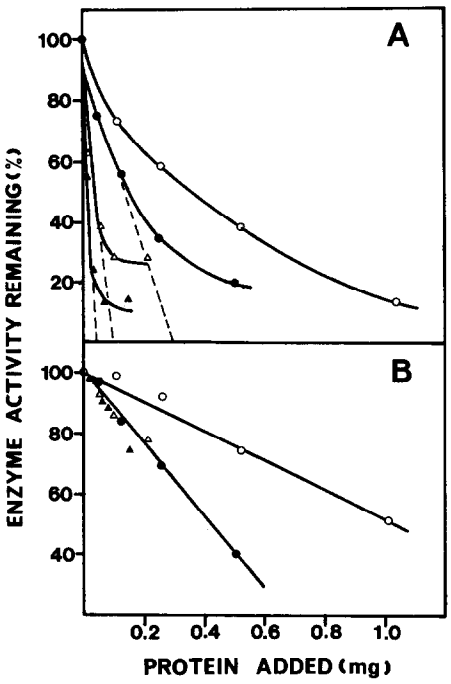

Fig. 3. Protease inhibitory activities of preparations from human cataractous lenses. The assays were performed as described in fig. 1. Panel A: 15 μg trypsin. Panel B: 15 μg chymotrypsin. (○–○–○) crude extract; (●–●–●) α-crystallin; (△–△–△) acidic α-crystallin components; (▲–▲–▲) basic α-crystallin components.

3.3. *Inhibitory activity of protein fractions prepared from human senile cataractous lenses*

The fractions used were prepared as described previously for the preparation of bovine lens proteins. The inhibitory activities of the various preparations as shown in fig. 3 and summarized in table 1. Qualitatively and quantitatively the results are essentially similar to those obtained for the bovine lens.

Acknowledgement

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