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Comparison in effect of different metal ions, pH and reducing agent on the protease activity in human hyper mature and mature cataract

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Abstract: This study was undertaken to isolate and characterize the protease activity of human eye lens sample of mature and hyper mature cataract. Samples were collected just after surgery of the cataract lens and were stored at −20 °C. The total protein extract was isolated from 5 samples in each case (mature and hyper mature cataract) and clear supernatant obtained after centrifugation was used as an enzyme source. The optimum pH for the proteases of mature cataract was 7.5 while the proteases of hyper mature cataract were recorded for maximum activity at pH 5.5 and 7.5. The optimum temperature for both enzyme sources was 50 °C. Effect of different metal ions such as potassium, lead, silver, zinc and borate was studied. In each case protease activity was increased. Reducing agent e.g. β mercaptoethanol also caused an increase in activity indicating the involvement of sulfhydryl groups. Protease activity was also located on agar plates.

Key words: Mature cataract, Hyper mature cataract, Eye lens, Protease activity, β mercaptoehanol
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INTRODUCTION

Cataract is one of the major causes of blindness throughout the world. The hyper mature cataract can only be witnessed in developing countries, as in developed countries patients used to undergo surgery before that stage. As Pakistan is an agrarian country with hot weather (temperature range 40~48 °C most of the months), the work force faces the direct sunlight, UV light during routine working, which may also be a main causative reason of cataract in Pakistan. Although modern techniques for cataract surgery are available in Pakistan now, because of less availability, the classical surgical procedures for removal of eye lenses are used, and the sample collection of mature and hyper mature eye lenses in intact form was possible. Different biochemical changes occur during cataract surgery e.g., an increase in the amount of insoluble protein, cross-linked protein, oxidised cysteine in the protein, oxidised methionine in the protein (Truscott and Augusteyn, 1977a; 1977b; 1977c), protein amino acids which have been modified by hydroxyl radical, and a decrease in the amount of the antioxidant glutathione in the centre of the lens (Truscott and Augusteyn, 1977a; 1977b; 1977c). Proteases are reported to be involved in the development of cataract (Sulochana *et al*., 1996; Sharma and Ortwerth, 1986). A new protease enzyme in the human lens, dipeptidase, has been purified to homogeneity and characterized (Sulochana *et al*., 1996). This enzyme is specific for dipeptides in its protease function while two other lens proteases, leucin amino peptidase and amino peptidase III (Sharma and Ortwerth, 1986) can act on di-, tri- and oligopeptides. It is reported that the activity decreases in human cataract lenses (Ortwerth *et al*., 1993). There are two viewpoints on the role of such proteases: (1) a decline in proteolytic enzymes with age leading to an accumulation of degraded proteins (Taylor *et al*., 1984), and (2) excessive proteolysis destroying essential proteases (Swanson *et al*., 1984). Taylor and Davies (1987) reported that protein oxidation and loss of protease

activity may lead to cataract formation in the aged lens. Previously we have reported the detection of two proteases in human eye lens (Sami *et al*., 2006). The work described here is a comparison of protease activity from human eye lens diagnosed as mature and hyper mature cataract.

MATERIALS AND METHODS

Samples of human eye lenses from subjects (age 60~70 years) were collected immediately after their surgical removal, for the implantation of artificial human eye lens, from the Institute of Ophthalmology, King Edward Medical University, Mayo Hospital, Lahore, Pakistan. Samples were divided into two classes: hyper mature cataract lens (dark brown coloration) and mature cataract (yellow coloration). Samples were stored at −20 °C for further use. Human eye lenses (5 lenses) were homogenized in a blender taken in 50 ml of buffer (1 mol/L Tris-HCl, pH 7.5) and the homogenate was centrifuged at 13000×g for about 10 min. The supernatant was used as a source of enzyme protein. Total protein contents were estimated by Bradford (1976) method. Protease activity for mature and hyper mature cataract of human eye lens was determined. Assay method was based on the method previously reported by Sami *et al*.(1988) with some modification. Five grams of liver tissue of freshly slaughtered chicken homogenized in 100 ml of Tris-HCl buffer (pH 8.0) was used as substrate. The method was as follows.

Enzyme assay

One millilitre (1 mg protein) of total protein extract of lens was added to 1 ml of liver homogenate/casein (10 mg protein). The reaction mixture was constantly shaken for 1 h at 50 °C. Reaction was stopped by adding 1 ml of chilled acetone. Protein pellet was separated by centrifugation at 10000×g and the pellet was dissolved in 1 ml 0.1 mol/L NaOH. The dissolved pellet (named sample A) was used for protein estimation by dye binding method as described earlier. Blank was prepared by estimating total protein contents in the reaction mixture before acetone precipitation (named sample B). Hydrolyzed proteins were estimated by subtracting amount of protein present in sample A and present in sample B. Enzyme assay was done with both the substrates.

Effect of pH, temperature, reducing agent, and ions (zinc, lead, silver, potassium and borate) were studied. All the reactions were done in triplicate. K_m value for the protease activity was also calculated. All the results were repeated twice (mean error of 2% was allowed).

Location of protease activity on agar substrate plate

Protease activity was located on the agar gel plates containing 1% casein and 2% agar. One hundred microlitres of total protein extract was loaded on the substrate agar plate after punching a hole in the centre and incubated at 50 °C overnight. Enzyme activity was visible as area hydrolyzed by the protease enzyme was lightened against blue background when stained with 1% bromophenolblue. Change in the coloration of the indicator was due to liberation of amino acids.

RESULTS

Effect of different pH values on the enzyme activity of eye lens sample of mature and hyper mature cataract was studied. Fig.1a shows optimum pH of both types (mature and hyper mature cataract) of lens proteases. Mature cataract lens protease showed optimum pH of 7.5, while the hyper mature cataract lens proteases showed two pH optima viz. 5.5 and 7.5.

For optimum temperature 50 °C was recorded for protease activity in mature and hyper mature cataract proteases (Fig.1b).

Generally boric acid is present in the eye washing solution up to concentration of 0.1% in water, and its effect was studied up to a concentration of 60 mmol/L in the reaction mixture. As shown in Fig.1c, a slight increase in enzyme activity was observed when borate ions were added upto a concentration of 60 mmol/L.

Effect of different metal ions such as lead (Fig.1d), silver, potassium (Fig.1e) and zinc (Fig.1f) on protease activity was studied. In case of lead there was more than two-fold increase in the protease activity in each case at concentration up to 30 mmol/L (Fig.1d).

Zinc caused a two-fold increase in activity of

hyper mature protease compared to the mature cataract protease (Fig.1f). It was reported by Bray and Bettger (1990) that zinc may also serve as antioxidant in retina, and that it may also be required for the activation of collagenases (a type of proteases). Effect of different routinely prescribed eye washing solutions e.g. boric acid and silver nitrate on the proteases activity of human eye lens was determined. Both of these caused an increase of protease activity. Increase in protease activity of hyper mature lens was 2 fold, while in mature cataract it was 1.5 fold.

Antioxidants/reducing agent e.g. β mercaptoethanol caused an increase in activity in both cases (mature and hyper mature cataract) indicating the involvement of sulfhydryl groups. Reducing agents caused a 10 times increase in activity of hyper mature proteases, while in mature cataract the increase was 5 times (Fig.1g).

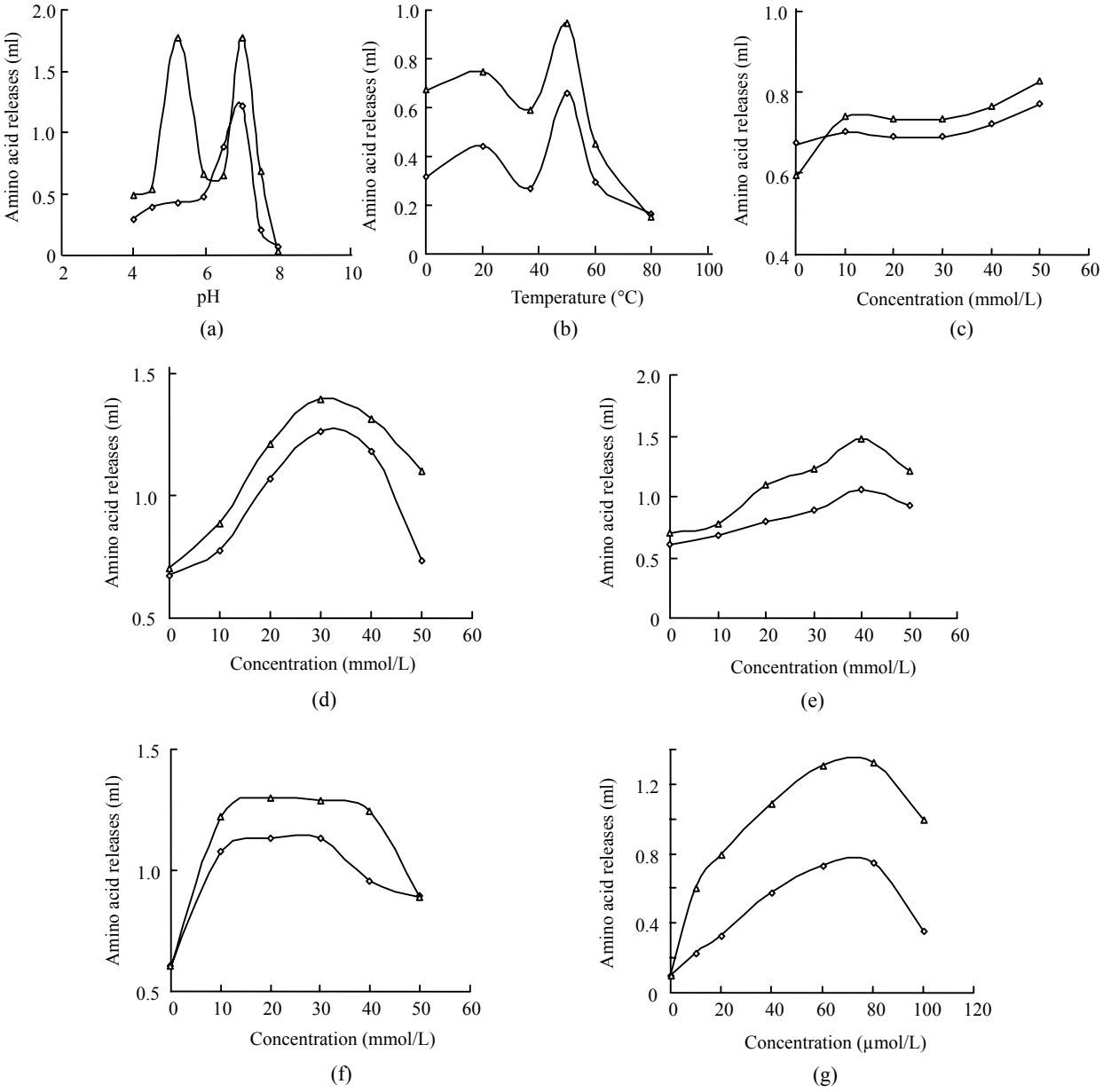

Fig.1 Effect of (a) pH, (b) temperature, (c) borate ion, (d) lead, (e) potassium ion, (f) zinc ion, and (g) reducing agent on mature and hyper mature cataract lenses

—◇—: Mature cataract; —△—: Hyper mature cataract

The K_m values for both the proteases were calculated and it was noticed that there was a difference in K_m values of both mature and hyper mature cataract lenses. The mature cataract lens protease had K_m value of 2.0 g/L while hyper mature cataract protease had K_m value of 0.75 g/L (Fig.2).

Protease activity was located on substrate-agar plate; it appeared lighter against the blue background as shown in Fig.3.

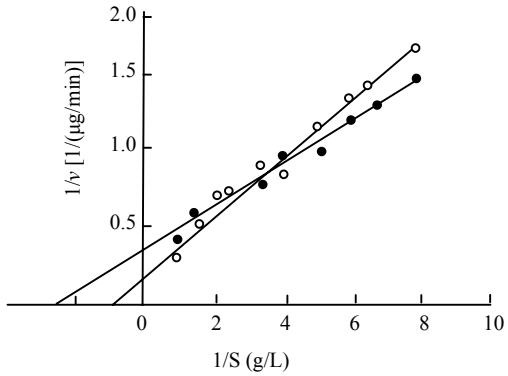

Fig.2 Line weaver burk plot for protease activity of mature and hyper mature cataract lenses
Black circles for hyper mature cataract lens (optimum pH 5.5) and white circles for mature cataract lens (optimum pH 7.5) protease activity

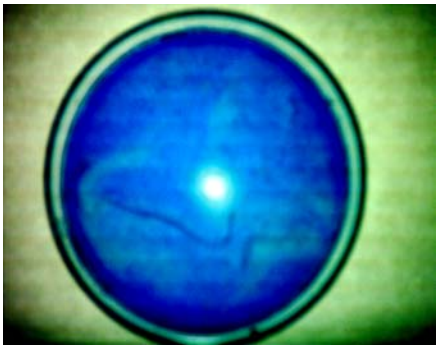

Fig.3 Location of eye lens proteases on substrate-agar plate. The hydrolyzed area is shown lighter against the blue background

DISCUSSION

Cataract is very common in Pakistan where classical treatment of cataract such as removal of affected eye lens is still in fashion. People are not used to routine checkup and vision impairment is considered as normal, that is why cataract lenses are available at latter stages of the disease. Srivastava and Srivastava (1999) reported a healthy eye lens proteinase showed a pH optimum of 7.8 and pI between 4.5 and 5.0. Our studies of mature cataract were closer to results reported by Srivastava and Srivastava (1999) e.g., 7.5 and in case of hyper mature cataract, presence of two proteases, with different optimum pH values (5.5 and 7.5) was recorded (Fig.1a). Our results for the mature cataract are comparable to the results reported by Srivastava and Srivastava (1999) for optimum pH. There is a possibility that the acidic protease may be of lysosomal origin and released from the lysosome during the advance stages of hyper mature cataract, while the protease with pH optima is the one which is already present in the healthy eye lens. So far, there is no study on proteases of mature and hyper mature cataract lens proteases. The optimum temperature reported for healthy eye lens protease was 45 °C (Srivastava and Srivastava, 1999) and we have reported the optimum temperature for mature and hyper mature cataract proteases was 50 °C, which is comparable to the results reported before. Reducing agents caused a 10 times increase in activity of hyper mature proteases, while in mature cataract the increase was 5 times (Fig.1g). This showed the involvement of sulfur containing amino acid residues. A higher level zinc caused an increase in activity of hyper mature protease in case of mature and hyper mature cataract. It was reported that Zn ions may act as activator by serving as an antioxidant for collagenases in the retina (Bray and Bettger, 1990). The other metal ions such as K ions, Ag ions and Pb ions also activated the enzyme activity. There may be a binding cleft for the metal ion in the enzyme. Proteases present in the human eye lens could be considered as metallo enzymes. The K_m values of both the enzymes were calculated and compared (Fig.2). There was a difference in the K_m value for the acidic protease found in hyper mature cataract lens and in mature cataract lens (0.75 g/L and 2.0 g/L). It could be speculated that acidic protease could be released from the lysosome during lifetime of the hyper mature cataract, as the lysosomal hydrolases has optimum pH of nearly 5.5. Another possibility may be two different genes or may be a mutation/change in gene with age/cataract being a causative reason of different protease.

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