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Are the proteinase inhibitory activities in lenticular tissues real?

S. SWAMINATHAN and T. N. PATTABIRAMAN
Department of Biochemistry, Kasturba Medical College, Manipal 576 119, India

Abstract. The possibility of proteinase inhibitory activities in lenses measured with synthetic substrates being spurious, due to the effective competition of lens proteins as substrates for the target enzymes, was investigated. Goat, sheep and human cataractous lens proteins were found to be poor substrates for trypsin, elastase and papain compared to casein or bovine serum albumin. Further, the inhibition of elastase catalyzed hydrolysis of succinyl trialanyl *p*-nitroanilide by casein (500 μg, 53%) and albumin (500 μg, 49%) and of trypsin-catalyzed hydrolysis of benzoyl arginine *p* -nitroanilide by albumin (1 mg, 24%) were significant only at high protein concentrations. These data indicated that the relatively high antielastase and antitryptic activities observed in human cataractous lenses were real. On the other hand, coincident lens protein hydrolysis elevating the true antitryptic and anti-elastase activities in goat and sheep lenses (that have low activities) could not be ruled out The lesser papain inhibitory activities observed in lenses when albumin was used as substrate compared to activities with benzoyl arginine *p*-nitroanilide as substrate, appeared to be partly due to lens protein hydrolysis masking the actual inhibition in the former method. Preincubation of goat, sheep and human lens extracts with trypsin for 1 h resulted in complete loss of antitryptic and antielastase activity except in the case of human lens antielastase activity which underwent 50% loss. Papain inhibitory activity was fully stable. Similar papain treatment caused loss of 80–100% of antielastase activity and 45–55% loss of antitryptic activity.

Keywords. Proteinase inhibitor, lenticular tissue; antielastase activity; antitryptic activity.

Introduction

Tse and Ortwerth (1980) demonstrated antitryptic activity in mammalian lenses and suggested that alteration in the activity could be a determinant factor for cataracto-genesis (Tse and Ortwerth, 1980 a,b). Recently, the presence of factors capable of inhibiting trypsin, elastase and chymotrypsin in several mammalian lenses was reported from this laboratory (Swaminathan *et al.,* 1986). While the activities were manifested with synthetic substrates in the assay systems, with protein substrates like casein and albumin, the inhibitory activities could not be detected in the lenticular tissues (Swaminathan and Pattabiraman, 1986a). It was also observed that papain inhibitory activity was much higher in lens when the assay was performed with benzoyl arginine *p*-nitroanilide as substrate rather than with bovine serum albumin (BSA) or casein (Swaminathan and Pattabiraman, 1986b).

The inhibitory activities in the lenses were generally low requiring deployment of lens proteins several folds higher than the target enzymes to measure inhibition. Heat treatment of the normal lens extracts enhanced the proteinase inhibitory activities whereas preincubation with target enzymes decreased or abolished the activities (Swaminathan and Pattabiraman, 1986a,b). Further, magnitude of inhibition was linear with respect to lens protein concentration only over a limited range. These

Abbreviations used: BSA, Bovine serum albumin; BAPNA, α-N-benzoyl DL-arginine *p*-nitroanilide; STANA, succinyl tri-L-alanyl *p*-nitroanilide; TCA, trichloroacetic acid.

data taken together, suggested that the inhibitory activities observed could be artifactual, in that the lens proteins by serving as substrates could competitively diminish the hydrolysis of synthetic substrates. This will cause an apparent inhibition and the increase in inhibitory activity observed with heat treated lens extracts could merely reflect better binding and hydrolysis of lens proteins.

The present study was undertaken to evaluate this possibility. We approached the problem by determining (i) the effect of protein substrates on the hydrolysis of amides by target enzymes, (ii) the correlation of the efficacy of lens proteins (native and denatured) as substrates for proteinases and their inhibitory potencies and (iii) the alteration of inhibitory activities in lens extract on digestion with different proteinases. The results are presented and evaluated in this communication.

Materials and methods

The sources of human cataractous lenses, sheep and goat lenses were mentioned earlier (Swaminathan *et al.,* 1986). Bovine trypsin (twice crystallized) and porcine elastase (twice crystallized) were purchased from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Pepsin (porcine, crystallized), papain (twice crystallized), α-N-benzoyl DL-arginine *p*-nitroanilide (BAPNA), succinyl tri-L-alanyl *p*-nitroanilide (STANA), benzamidine and BSA were from Sigma Chemical Company, St. Louis, Missouri, USA. Casein was from British Drug House Chemical Limited, Poole, England. All other reagents were analytical grade commercial chemicals.

Goat, sheep and human lens extracts were prepared in 20 mM sodium phosphate buffer, pH 7·6 as described earlier (Swaminathan and Pattabiraman, 1986b). For experiments involving the determination of antitryptic and antielastase activities the lens extracts were prepared in 20 mM Tris-HCl buffer pH 7·6 (Swaminathan *et al.*, 1986). The lens extracts when necessary were subjected to heat treatment at 60°C for 15 min prior to use. Albumin and casein solutions were prepared in Tris-HCl buffer (0·1 M), pH 7·6 at concentrations of 10mg/ml. Heat denatured albumin was prepared by subjecting the solution to heat treatment at 100°C for 2 min. Similarly, casein was denatured by heating at 100°C for 10 min.

Assay of proteinases and inhibitors

Trypsin and elastase were assayed using BAPNA and STANA respectively as substrates (Swaminathan *et al.,* 1986). Under the assay conditions (37°C, 15 min, pH 7·6) trypsin (160 μg protein) and elastase (2·0 μg protein) released *p*-nitroaniline equivalent to an absorbance of 0·6 (λ 410). Aor routine purposes, papain was assayed using either BSA or BAPNA (Swaminathan and Pattabiraman, 1986b). Under the assay conditions with albumin (5 mg, 12·5 μg papain, 15 min, 37°C, pH 7·6) papain liberated trichloroacetic acid (TCA) soluble fragments equivalent to an absorbance of 0·6 (λ 540). With BAPNA as substrate (5 μmol) papain (125 μg) liberated under the assay conditions (30 min, pH 7·6, 37°C) *p*-nitroaniline equivalent to an absorbance of 0·5. Aor determination of inhibitory activity, aliquots of lens extract (or casein or albumin as the case may be) were included in the assay system. The difference in absorbance values between the test and control systems was a measure of inhibitory acitivity.

Hydrolysis of lens proteins by different proteinases

The assays were based on the method of Kunitz (1947). The system consisted of lens extracts (native or denatured), 40 μmol of sodium phosphate buffer pH 7·6 and the enzyme (trypsin 16·0 μg, elastase 2·0 μg or papain 12·5 μg) in a volume of 2·0 ml. After 15min incubation at 37°C, the reaction was arrested by the addition of 3·0 ml of 5% TCA. The suspension was centrifuged (2500 rpm) and the extent of lens protein degradation was estimated by determining the TCA soluble fragments in 1 ml fractions of the supernatant by the method of Lowry *et al.* (1951). To study pepsin digestion, 3·7 μg of the enzyme was incubated with the lens extract in the presence of 40 μmol of HCl-KCl buffer pH 2·0. The processing was otherwise similar·

Effect of treatment of lens proteins with proteinases on their inhibitory activities

Lens extracts were preincubated with different proteinases individually for definite time intervals at 37°C. The residual inhibitory activities were determined according to the methods described above. In the cases of preincubation with the target enzyme. itself, inactivation of the enzyme, prior to assay of inhibitory activities did not arise. The enzyme was treated with the lens extracts at pH 7·6 for definite time followed by the addition of the substrate. Control system with enzyme alone was run concurrently. In other cases controls without the digesting proteinases were run simultaneously·

Pepsin treatment: Lens extracts (5–15 mg protein) were incubated with pepsin (3·7 μg) at pH 2·0 (0·04 M HCl-KCl) in a volume of 1·0 ml· The reaction was stopped by adding 2·0 ml of 0·2 M Tris-HCl buffer pH 7·6. Aliquots were assayed for residual antitryptic, antielastase and antipapain activities·

Papain treatment: Lens extracts (0·15–2·20 mg protein) were treated with papain (12·5 μg) in the presence of 4 μmol of EDTA, 9 μmol of β-mercaptoethanol and 40 μmol of Tris-HCl buffer pH 7·6. After incubation, 10 μmol of $CaCl_2$ was added (final volume 1·8 ml) and aliquots were assayed for antitryptic and antielastase activities·

Trypsin treatment: Lens extracts (0·08–l·40mg protein) were incubated with 16 μg of trypsin in the presence of 40 μmol of buffer pH 7·6 (Tris-HCl for antielastase and sodium phosphate for antipapain determinations) in a volume of 1·8 ml· Aliquots were taken and directly assayed for residual antielastase activity· For measurement of antipapain activity with BAPNA as substrate 5 mg of benzamidine was added (final volume 1·8 ml) to completely inactivate trypsin and aliquots were then used for the estimation· Benzamidine at the concentration used did not affect papain action and also it did not alter the papain inhibitory activity in lens extracts·

Protein was determined by the method of Lowry *et al.* (1951) using BSA as standard·

Results

Figure 1 depicts the action of BSA and casein on the amidolysis of BAPNA by trypsin and of STANA by elastase· The concentrations of the proteins used were in the same range in which lens proteins were employed for routine inhibitory assays. Hydrolysis of BAPNA was virtually unaffected by casein (figure 1A). Heat denatured casein also behaved similarly (data not shown)· While BSA did not reduce the action of trypsin on BAPNA, heat treated BSA showed moderate reduction of *p*-nitroaniline release· Elastase catalyzed hydrolysis of STANA was more sensitive to the inclusion of proteins in the assay medium. Casein was effective in diminishing STANA hydrolysis (figure 1B). Heat treated casein however did not exhibit any enhanced action (data not shown)· While BSA did not reduce elastase action, heat denatured BSA was moderately effective in reducing STANA hydrolysis. Comparison of the data with that of lens proteins showed that 1 mg of casein, heat treated BSA, goat lens protein, sheep lens protein and human cataract lens protein diminished elastase activity on STANA to the extents of 2·33 μg, 2·50 μg, 0·5–1·1 μg (n= 10), 0·5–2·35 μg (n = 10) and 8·3–25·1 μg (n = 10) equivalents of elastase, respectively. Similarly, 1 mg of heat treated BSA, goat, sheep and human lens proteins inhibited 3·73 μg, 1·87–4·0 μg (n = 10), 1·06–6·6 μg (n = 10) and 12·0–42·9 μg, (n = 10) of trypsin in terms of suppression of BAPNA hydrolysis. Similar studies on papain catalyzed hydrolysis of BAPNA showed that 1 mg of BSA, goat, sheep and human lens proteins (all heat denatured) will suppress papain action to the tune of 8·4 μg, 24·5 μg (n = 5), 25–42·0 μg (n = 5) and 45–90 μg (n = 5) of the enzyme, respectively· The values are based on the linear ranges of inhibition·

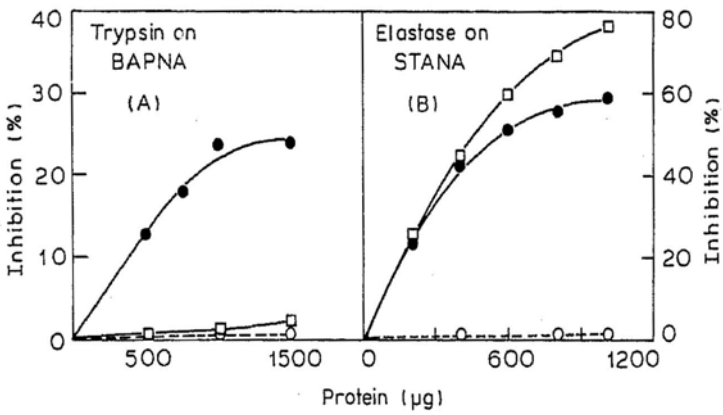

Figure 1. Diminition of tryptic and elastase activity on synthetic substrates by casein and albumin· (❑), Casein; (O), albumin; (●), heat treated albumin. Other details are given under 'materials and methods'·

If observed 'inhibitions' are due to coincident hydrolysis of protein substrates by target enzymes, it should be expected that human cataractous lens proteins will be efficient substrates for the proteinases· The extents of hydrolysis of lens proteins by different proteinases in the range of concentrations used for inhibition assays are presented in figure 2. For comparison, the action of pepsin is also shown. Under the assay conditions, tryptic digestion was far more effective than elastase action with all the 3 lens extracts. Papain hydrolysis occupied an intermediate position. In the case

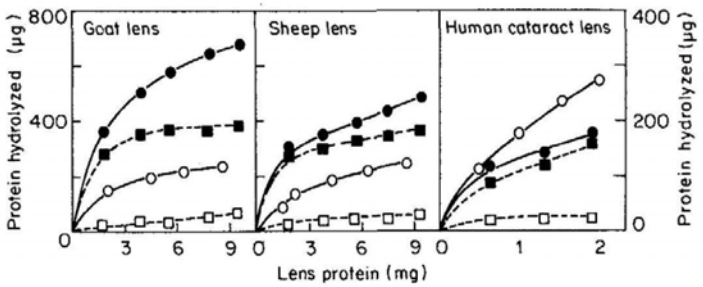

Figure 2. Hydrolysis of lens proteins by proteinases.·(●), Trypsin; (■), papain; (O), pepsin; (❑), elastase. Other details are given under 'materials and methods'.

of human cataract lenses alone pepsin was more effective than trypsin· Nevertheless, the lens proteins were found to be poor substrates for proteinases compared to casein or albumin· The relative rates of hydrolysis based on initial rates, at 1 mg protein level for tryptic action, was calculated to be 20·2% for goat lens, 15·4% for sheep lens, 17·3% for human cataract lens and 40·4% for BSA compared to casein. Similarly, the relative values for goat, sheep and human lens for elastase were respectively 20·1, 24·2 and 30·3% and for papain, 50·0, 44·4 and 38·9%. The relative values for pepsin hydrolysis (with hemoglobin as 100%) for the 3 lens proteins were 13·0, 11·1 and 37·0%, respectively·

The lens extracts were subjected to heat treatment (60°C, 15 min) and were then used as substrates for the proteinases. The magnitudes of increase in proteolytic activities are shown in table 1. The changes in proteinase inhibitory activities on heat treatment are also shown· Heat treatment enhanced hydrolysis by trypsin, elastase and papain in all cases excepting the action of elastase on human lens. Heat denaturation of lens proteins had no effect on peptic action. No correlation between increase in proteolysis and enhancement of inhibitory activity could be found in individual cases except that heat treatment of human cataract lens extracts neither accentuated its digestion by elastase nor affected its antielastase activity. The data suggest that increased susceptibility of lens proteins to proteinase digestion need not reflect the observed increase in inhibitory activity, on heat treatment·

The lens extracts were treated with different proteinases and the residual proteinase inhibitory activities were estimated in goat, sheep and human lenses·

Table 1. Effect of heat treatment of lens extracts on their proteolysis and proteinase inhibitory activities·

	Increase in proteolysis (%)			Increase in proteinase inhibitory activity (%)		
Proteinase	Goat	Sheep	Human	Goat	Sheep	Human
Trypsin	49	123	71	180	140	0
Elastase	233	166	0	74	90	0
Papain	76	106	83	301	307	295
Pepsin	0	0	0	—	—	—

Lens extracts were subjected to heat treatment at 60°C for 15 min· Values are mean of triplicate· Papain inhibitory activity was estimated with BAPNA as substrate as described under 'materials and methods'·

Table 2. Proteinase inhibitory activities in lens extracts after prior treatment with proteinases·

Digestion system	Residual activity (%)		
	Goat lens	Sheep lens	Human lens
	Elastase inhibition		
Trypsin, 30 min	0·0	0·0	48·6
Papain, 30 min	28·0	26·5	7·5
Papain, 60 min	11·5	19·5	0·0
Pepsin, 60 min	78·0	81·0	87·0
Pepsin, 120 min	69·0	65·0	81·0
Elastase, 60 min	0·0	0·0	75·0
	Trypsin inhibition		
Papain, 30 min	75·0	72·5	62·0
Papain, 60 min	56·0	44·5	52·0
Pepsin, 60 min	80·0	89·0	91·0
Pepsin, 120 min	80·0	83·0	91·0
Trypsin, 60 min	0·0	0·0	0·0
	Papain inhibition		
Trypsin, 60 min	104·0	100·0	85·4
Trypsin, 120 min	104·0	95·0	63·3
Pepsin, 60 min	100·0	104·0	100·0
Pepsin, 120 min	104·0	100·0	90·0
Papain[a], 60 min	0·0	0·0	0·0
Papain[b], 60 min	100·0	104·0	100·0

[a]Inhibitory activity was measured with BAPNA as substrate.
[b]Inhibitory activity was measured with BSA as substrate.
Other details are given under 'materials and methods'.

Typical data are shown in table 2. Digestion with trypsin for 30 min or with elastase for 60 min completely abolished the antielastase activity in goat and sheep lenses whereas the human lens retained antielastase activity to the extent of 50% following trypsin treatment and 75% following elastase treatment. Papain treatment also caused considerable loss of antielastase activity. Pepsin was not very effective in this respect. While preincubation with trypsin resulted in rapid loss of antitryptic activity, papain and pepsin were less effective in this regard· Papain inhibitory activity was highly resistant to the action of trypsin and pepsin. Preincubation studies with papain with reference to papain inhibitory activity differed depending on the assay procedure· In the assay with BAPNA as substrate, papain inhibitory activity was completely abolished on pretreatment with the target enzyme whereas by the BSA assay, no loss of inhibitory activity could be observed. This appears to be more of a reflection of the enzyme concentration used. In the BAPNA assay method the amount of enzyme used is nearly an order of magnitude higher compared to the quantity used in the BSA method· Since elastase showed relatively weak proteinase activity under the assay conditions (9–16 times lower than tryptic activity on different lens proteins), preincubation effect on inhibitory activities other than the target enzyme was not studied. Correlation of the data in table 2 with magnitudes of hydrolysis of lens proteins by different enzymes (figure 1) suggests that proteolytic activities do not parallel the loss of inhibitory activities. If competitive substrate effect were the

only factor responsible for apparent inhibition observed, it should be expected that trypsin treatment should abolish all the 3 proteinase inhibitory activities.

It has been shown earlier (Swaminathan and Pattabiraman, 1986b), the papain inhibitory activity in heat treated lens extracts was much higher when BAPNA was used as substrate rather than BSA. Papain inhibitory activities were compared in this study using both the substrates, but with identical concentration of the enzyme (17 μg). In this modified BAPNA assay system, the time of incubation was increased to 5 h so that the control enzymatic activity (λ 410, 0·5) was comparable to the value obtained in the earlier described assay (Papain 125 μg; assay time 30 min). The papain inhibitory activities estimated under the different assay conditions are shown in table 3. The data indicate that the wide differences observed between the values by the BSA method and BAPNA method were narrowed down when the concentration of the enzyme used in the two methods were the same. To evaluate the real magnitude of papain inhibition with BSA as substrate, a sequential experiment with goat lens extract was performed and the results are shown in table 4. Difference between system A and B will be an index of the measured inhibitory activity. Preincubation of the lens extract for 10 min with papain followed by addition of substrate and incubation for further 15 min did not alter the value of inhibition (A–C). Incubation of the lens extracts with papain for 10 min and 25 min represented by D and E indicate the hydrolysis of lens proteins by papain. The data suggest that while the apparent inhibition is 0·10, the actual inhibition could be a maximum of 0·20 (A – B + D) for

Table 3. Papain inhibitory activities in lens extracts measured with BSA and BAPNA as substrates·

	Papain (μg) inhibited per mg lens protein		
Lens	**BSA**	**BAPNA[a]**	**BAPNA[b]**
Human-1	4·85	17·20	54·0
Human-2	3·76	9·15	39·0
Human-3	3·04	5·11	35·0
Goat-1	2·74	8·90	25·0
Goat-2	2·06	5·02	22·0
Goat-3	1·78	2·81	18·0

[a]Assay conditions were, 17 μg papain, 5h assay time at 37°C·

[b]Assay conditions were, 125 μg papain, 30 min assay time at 37°C·

Table 4. Sequential studies on papain inhibition with goat lens extract·

Assay condition	λ 540
A. Papain (12·5 μg) + BSA (5 mg): 15 min	0·58
B. Papain + BSA + Lens extract (0·54 mg): 15 min	0·48
C. Papain + Lens extract: 10 min followed by BSA: 15 min	0·48
D. Papain + Lens extract: 10 min	0·10
E. Papain + Lens extract: 25 min	0·12

10 min and 0·22 (A–B + E) for 25 min, accounting for interfering hydrolysis of lens proteins·

Papain inhibitory activity was found to be still lower when casein instead of BSA was used as substrate in the assay system (Swaminathan and Pattabiraman, 1986b). The effect of heat treatment of the lens extracts on inhibitory activity against papain was studied using BSA and casein as substrates· While the heat treatment increased the papain inhibitory activity with BSA as substrate, in goat lens (1·92-3·05 fold, n = 6) and sheep lens (2·18–3·53 fold, n = 6), in human cataractous lens the changes were marginal (0·95–1·06 fold, n = 6). In contrast, with casein as substrate the papain inhibitory activity in goat lens and sheep lens were completely abolished whereas in human lens the activity remained virtually unchanged·

Discussion

Gaudin and Stevens (1974) showed that α-crystallin a major lens protein can inhibit trypsin and chymotrypsin. It was shown later that trypsin can degrade α-crystallin (Siezen and Hoenders, 1977). The present observations that lens proteins undergo hydrolysis, although to a limited degree, and that heat treatment of lens extract enhances digestion by proteinases in most cases would suggest that the observed antielastase, antitryptic and antipapain activities in lens extracts can be spurious. The observed inhibitions thus, would merely be due to decreased hydrolysis of the synthetic substrates used in the assays, due to effective competition of lens protein for the target enzymes as substrates. The reduction in elastase-catalyzed STANA hydrolysis by casein and of the elastase-catalyzed STANA hydrolysis and tryptic action on BAPNA by heat denatured albumin enforce this view·

However, a closer scrutiny of the available data counteracts this possibility at least partially. Even though casein and heat treated albumin reduce STANA hydrolysis by elastase, the lens proteins themselves were found to be poor substrates for elastase emphasizing that the analogy is not wholly valid. Further, with human cataract lenses which had high antielastase activity about 20–60 μg lens protein caused around 25% inhibition of the enzyme whereas at these levels casein and albumin had negligible effect. Even though the inhibitory activity in human lenses was higher by an order of magnitude compared to goat and sheep lenses, the rates of digestion of the 3 lens proteins by elastase did not differ significantly. While preincubation with elastase caused complete inactivation of antielastase activity in goat and sheep lenses, it was observed earlier that in the case of human lenses the decrease in inhibitory activity is marginal (Swaminathan and Pattabiraman, 1986a). The relative rates of digestion of lens proteins also do not parallel the magnitudes of loss of antielastase activity on proteinase treatment. The data suggest that the observed antielastase activity even in goat and sheep lenses cannot exclusively be due to competition of lens proteins as substrates·

With reference to antitryptic activity also, the available data lead to similar conclusions. Casein a good substrate for trypsin did not reduce BAPNA hydrolysis and hence it is not possible to expect that the lens proteins that are poor substrates could efficiency reduce tryptic action on BAPNA. Further, while heat treatment of lens extracts made them better substrates for trypsin in all cases, increase in inhibitory activity was observed only with goat and sheep lenses· While pepsin digested human lens proteins more effectively than trypsin, the former casused only a marginal loss of

antitryptic activity whereas, trypsin completely abolished the activity on preincubation·

In regard to thiol proteinase inhibitory activity in lens, the possibility of substrate competition resulting in apparent inhibition seems rather remote. Heat treatment of the lens extracts increased papain inhibitory activity measured with BSA as substrate, only in goat and sheep lenses but not in human lenses. However, proteolysis by papain was found to be enhanced by heat treatment in all the 3 lenses· If competitive proteolysis is responsible for inhibitory effect, it should be expected that heat treatment should actually decrease the observed inhibition in this assay system. Interestingly, papain inhibitory activity, measured with casein as substrate, which was much lower than the values observed with BSA (Swaminathan and Pattabiraman, 1986b) was abolished in goat and sheep lenses on heat treatment. The variations in papain inhibitory activities measured with different substrates and the differential behaviour of the activity on heat treatment between normal and cataractous lenses could be due to the presence of more than one papain inhibitor and their altered proportions in cataractous lenses. The importance of difference in the affinity of substrates for papain can also be a causative factor·

The earlier observation on the higher papain inhibitory activity (by an order of magnitude) observed with BAPNA as substrate compared to the values with BSA is now shown to be mainly due to differential assay conditions. At present, we do not have an explanation as to why inhibitory activity measured with BAPNA as substrate is dependant on enzyme concentration. However, the higher inhibitory activities observed with synthetic substrate compared to BSA system even at comparable concentration of enzyme can be partially explained as due to the limitation of the BSA assay method. In this procedure the digestion of lens protein coincident to inhibition, tends to mask the inhibitory activity. Failure to observe antitryptic and antielastase activities in lens extracts when casein or albumin is used as substrate (Swaminathan and Pattabiraman, 1986a) might be due to this limitation· In this context it is worthwhile to note that Tse and Ortwerth (1980b) observed antitryptic activity in lenses using azocasein as substrate. In their assay procedure it is the 'solubilized' dye that was measured as an index of proteolysis and inhibition·

Collation of the available information indicates that antielastase and antitryptic activities measured in human cataractous lenses are real and coincident lens protein hydrolysis has no effect on the observed values. With sheep and goat lenses, which have relatively low serine proteinase inhibitory activities, substrate competition effect contributing to observed inhibition cannot be completely ruled out. In regard to papain inhibition the magnitude of observed inhibition with BSA as substrate will be an underestimation due to interfering lens protein hydrolysis·

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