Comparative studies on lens neutral endopeptidase and pituitary neutral proteinase: two closely similar enzymes

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A neutral endopeptidase (EC 3.4.24.5) previously thought to be unique to the eye lens has been found to be closely similar if not identical in native molecular size, component polypeptides and antigenic structure to a neutral proteinase from pituitary. Here we investigated some subtle differences in properties of the two enzymes, such as the effects of temperature, divalent cations and SDS on their activities with respect to different substrates. We conclude that the pituitary enzyme may have a relatively more compact structure requiring relaxation by low SDS concentration or higher temperature for maximum activity.

(Bovine lens, Pituitary) Neutral endopeptidase Neutral proteinase

1. INTRODUCTION

A neutral endopeptidase (EC 3.4.24.5) occurs in the eye lens of various mammalian species and is thought to play a role in lens cataract formation in humans. This enzyme has recently been purified from bovine lens [1]. It has been found to have a high molecular mass (~ 700 kDa) and on SDS-gel electrophoresis to show a characteristic pattern of at least 8 polypeptide subunits of molecular mass ranging from 24 to 32 kDa. This lens enzyme [1] has been shown to be indistinguishable in these respects from a previously described enzyme purified from bovine pituitary and originally referred to as a neutral proteinase [2]. Furthermore, antiserum raised in rabbit against the purified lens endopeptidase cross-reacted with the pituitary neutral proteinase and in Ouchterlony double-diffusion experiments gave a single continuous precipitin band with no spurring when the 2 enzymes were examined side by side [1]. Thus the 2 enzymes appear to be structurally very similar, if not identical. Previous studies [3] on the pituitary enzyme led to the suggestion that it represents a multicatalytic protease complex with distinct proteolytic activities associated with separate components of this high molecular mass protein. If this is so, the same is probably true for the lens enzyme.

The purpose of the experiments reported here was to examine certain properties, which it was thought from reports in literature and from some of our own earlier results might differ between the 2 enzymes. These are: (i) activities with α_2-crystallin and 2 synthetic substrates, Z-Gly-Gly-Leu-pNA and Z-Leu-Leu-Glu-2NA at different temperatures; (ii) activities using the same 3 substrates in the presence of low concentrations of SDS; (iii) effect of divalent cations on activities; (iv) ability to cleave casein and azocasein. In general, the 2 enzymes are very similar in their properties. However, certain consistent differences were identified. It is possible that the polypeptides in the 2 enzymes are coded by the same set of genes and that the subtle differences in their properties are attributable to

This paper is dedicated to Satya Prakash Datta

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Abbreviations: HA, hydroxyapatite; pNA, *p*-nitroanilide; 2NA, 2-naphthylamide; Z, benzyloxycarbonyl

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post-translational differences in the 2 tissues, lens and pituitary.

2. MATERIALS AND METHODS

2.1. *Materials*

Frozen whole bovine pituitaries and lenses were purchased from Pel-freeze. [^{3}H]Acetic anhydride (50.0 mCi/mmol) was purchased from New England Nuclear. The synthetic substrates Z-Gly-Gly-Leu-pNA and Z-Leu-Leu-Glu-2NA were kind gifts from Dr S. Wilk of the Department of Pharmacology, Mount Sinai School of Medicine, New York. All other chemicals were reagent grade or the best commercially available.

2.2. *Purification of the lens neutral endopeptidase and the pituitary neutral proteinase*

The purified enzymes were prepared as described [1].

2.3. *Labelling of the α_2-crystallin with [^{3}H]acetic anhydride*

α_2-Crystallin fraction was prepared and was freed from any contaminating protease activity including the enzyme leucine aminopeptidase by alkaline-urea treatment and then the alkali-urea treated α_2-crystallin fraction was labelled with [^{3}H]acetic anhydride as described [4].

2.4. *Protease assay using different substrates*

2.4.1. Using synthetic substrates

Enzyme activity against the synthetic substrates was measured by determining the release of 2-naphthylamine or *p*-nitroaniline from the synthetic substrates, viz. Z-Leu-Leu-Glu-2NA and Z-Gly-Gly-Leu-pNA, respectively as reported by Wilk and Orlowski [2]. The incubation mixture contained 50 mM Tris-HCl (pH 7.5), the enzyme (5 μg) and 10 μl of 0.01 M substrate (dissolved in dimethyl sulfoxide) in a total volume of 250 μl. Samples were incubated at the indicated temperature for 60 min. *p*-Nitroaniline or 2-naphthylamine released was measured by a diazotization procedure [5] and its concentration determined from a standard curve prepared under the conditions of the assay.

2.4.2. Using radioactive α_2-crystallin as substrate

The enzyme (8 μg) was mixed with 10 μl α_2-[^{3}H]-crystallin (2–10 μCi) diluted to a final volume of 125 μl with 10 mM Hepes (pH 7.5). Where necessary 5 μl of 5 mM $CaCl_2$ or $MgCl_2$ was added to it. The assay mixture was incubated at the indicated temperature for 60 min. The assay tubes were then transferred to an ice bath. After 5 min, 25 μl carrier BSA (20 mg/ml) and 500 μl of 7.5% trichloroacetic acid were added. After keeping the tubes in ice bath for 10 min, they were centrifuged in a table-top Microfuge for 6 min. 65 μl of trichloroacetic acid-soluble supernatant was taken on Whatman paper disc (3 mm), dried and counted for radioactivity using toluene-based scintillation fluor.

2.5. *Polyacrylamide gel electrophoresis (PAGE) under denaturing condition*

The protein samples were diluted in sample buffer (125 mM Tris-HCl, pH 6.8, 2% 2-ME, 0.5% SDS, 10% glycerol and 0.01% bromophenol blue) and boiled in a water bath for 2–3 min. Electrophoresis was done using 25 mM Tris-glycine, pH 8.8, containing 0.1% SDS as described by Laemmli [9] with 5% polyacrylamide in the upper gel and 12.5% polyacrylamide in the lower gel. Gels were stained for protein with Coomassie brilliant blue.

3. RESULTS AND DISCUSSION

3.1. *Activity at different temperatures*

Fig.1 shows the relative activities of the 2 enzymes with 3 different substrates, α_2-crystallin, Z-Gly-Gly-Leu-pNA and Z-Leu-Leu-Glu-2NA. The optimal temperature for cleavage of α_2-crystallin is 60°C for both enzymes (fig.1a). However, the lens enzyme shows relatively higher activities than the pituitary enzyme at 50 and 55°C. For Z-Gly-Gly-Leu-pNA the optimal temperature is between 40 and 50°C for the 2 enzymes (fig.1b). However, the lens enzyme shows relatively higher activity than the pituitary enzyme at 30°C. Z-Leu-Leu-Glu-2NA shows a striking difference in optimal temperature with the 2 enzymes. For the lens enzyme the optimal temperature is 37°C whereas for the pituitary enzyme it is 50°C (fig.1c). This last finding implies that some structural difference between the 2 enzymes exists.

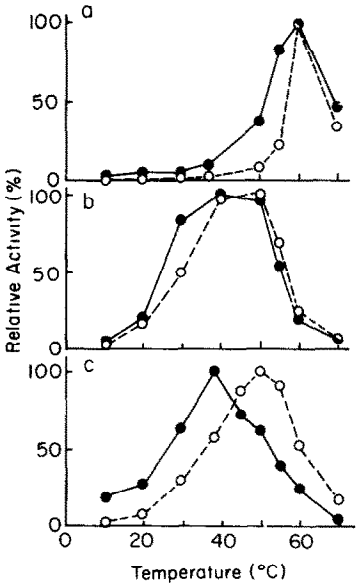

Fig.1. Temperature optima for cleavage of different substrates by lens neutral endopeptidase (●) and pituitary neutral proteinase (○) using (a) α_2-crystallin, (b) Z-Gly-Gly-Leu-pNA and (c) Z-Leu-Leu-Glu-2NA.

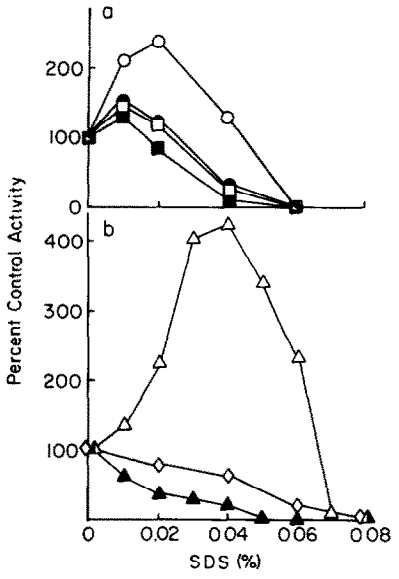

Fig.2. Effect of SDS on (a) Z-Leu-Leu-Glu-2NA and (b) α_2-crystallin cleavage activity by lens neutral endopeptidase [at 37°C (●) and at 50°C (■) or at 55°C (▲)] and pituitary proteinase [at 37°C (○) and at 50°C (□) or at 55°C (△) and at 60°C (◇)].

3.2. *Activity in the presence of different concentrations of SDS*

Wilk and Orlowski found that in the presence of a low concentration of SDS (0.02%), cleavage of Z-Leu-Leu-Glu-2NA at 37°C by the pituitary enzyme was greatly enhanced. Fig.2a shows our results with Z-Leu-Leu-Glu-2NA, using both pituitary and lens enzymes over a range of SDS concentrations at 37 and 50°C. The pituitary enzyme at 37°C shows enhancement of activity reaching a maximum at 0.02% SDS similar to that of Wilks and Orlowski [3] except that the degree of enhancement (about 2.5-fold) is less marked than in their experiments (about 9-fold). At 50°C, which is the optimal temperature for the pituitary enzyme with this substrate (fig.1c), enhancement of activity was less marked (about 1.5-fold at 0.01% SDS). With higher SDS concentrations inhibition of activity was observed. The lens enzyme with Z-Leu-Leu-Glu-2NA showed modest activation with 0.01% SDS, and inhibition at higher concentrations. The effect was somewhat more marked at 37°C than at 50°C. Wilk and Orlowski [3] had previously shown that there is no activation of the pituitary enzyme with SDS using Z-Gly-Gly-Leu-pNA as substrate. Instead there was progressive inhibition with increasing SDS concentration. Our observation using the lens enzyme was similar under identical conditions.

Fig.2b shows the effect of SDS on the cleavage of α_2-crystallin by the lens enzyme at 55°C and the pituitary enzyme at 55 and 60°C. There is marked enhancement of pituitary enzyme activity at 55°C with a maximum of more than 4-fold activation at 0.04% SDS. In contrast, at 60°C no activation is seen with the pituitary enzyme and there is increasing inhibition with increasing SDS concentration. At 55°C, the lens enzyme is inhibited by SDS.

Thus, low concentrations of SDS result in marked activation of the pituitary enzyme with either Z-Leu-Leu-Glu-2NA or α_2-crystallin at temperatures below the optima for these substrates. At higher temperatures closer to the optima less activation with Z-Leu-Leu-Glu-2NA and no activation with α_2-crystallin are observed. A possible explanation is that low concentrations of SDS open up the tertiary or quaternary structure of the pituitary enzyme and allow easier access and binding of the substrates to the active site. Higher

temperatures in the absence of SDS may produce a similar effect. Consequently, at these higher temperatures SDS per se would produce much less enhancement of activity. The lens enzyme shows only a modest SDS activation with Z-Leu-Leu-Glu-2NA and inhibition with α_2-crystallin. It is therefore possible that the lens enzyme has a more open structure than the pituitary enzyme.

3.3. *Effect of divalent cations on enzyme activity*

The activity of lens neutral endopeptidase has always been described in the literature as being strongly stimulated by Ca^{2+} or Mg^{2+} [4,7]. In contrast, the pituitary neutral proteinase has been shown not to be so stimulated [2].

The demonstration of Ca^{2+} and Mg^{2+} stimulation of α_2-crystallin cleavage by the lens endopeptidase has in the past been based on experiments using relatively impure enzyme preparations [4]. During the course of our purification of the lens enzyme we noticed that the degree of stimulation produced by 5 mM Ca^{2+} or Mg^{2+} was progressively diminished as the purification proceeded, from more than 5-fold in the earlier stages to only 1.5-fold in the final stages (table 1). Furthermore, the relatively small amount of Ca^{2+} or Mg^{2+} stimulation of the final product of the purification was found to disappear altogether on storage of the purified enzyme for a few days. It seems probable that a protein activator of the enzyme occurs in crude lens preparations and is progressively eliminated during the purification. Trace amounts of such a postulated activator may be present in the final enzyme product though it could not be detected by SDS-PAGE. The loss of all activation after storage suggests that the postulated activation may be relatively unstable.

Table 1

Stimulation of lens neutral endopeptidase by divalent cations at different steps of purification described in [1]

Purification steps	Stimulation of activity over control	
	$+Mg^{2+}$ (-fold)	$+Ca^{2+}$ (-fold)
(1) Iso-electric precipitate	5.4	5.3
(2) Soluble enzyme	5.4	5.5
(3) 33–80% ammonium sulfate fraction	5.6	5.9
(4) HA chromatography	4.9	4.7
(5) 33–80% ammonium sulfate fraction of HA-purified enzyme	3.7	3.5
(6) Sephacryl S-300 chromatography	2.4	2.7
(7) HPLC on anion-exchange column	1.6	1.4

In each step of purification, an aliquot of the enzyme fraction was assayed for α_2-[^{3}H]crystallin cleavage activity with either $MgCl_2$ or $CaCl_2$ and compared with a control ($-Mg^{2+}$ or Ca^{2+})

3.4. *Cleavage of casein but not azocasein*

It has been reported that neither the lens enzyme [4] nor the pituitary enzyme [2] digests azocasein. It was generally assumed that the same would be the case for casein, though this could not be tested using the standard spectrophotometric assay for azocasein hydrolysis. To test for casein digestion we used an electrophoretic method. After incubation of the substrate with the enzymes, the reaction mixture was examined by SDS-PAGE. Digestion of the substrate was indicated by a reduction in the intensity of staining of the substrate band compared with the appropriate control and the concomitant appearance of lower molecular mass polypeptide products. Typical results are shown in fig.3. Both the lens and the pituitary enzymes can be shown by this method to digest casein very efficiently. With the same procedure no digestion of azocasein could be detected. The finding, that casein serves as a substrate for the 2 enzymes, but azocasein does not, was unexpected since it has usually been assumed that both casein and its azo derivative behave similarly with specific proteases.

4. CONCLUDING REMARKS

Wilk and Orlowski, on the basis of studies on the pituitary neutral proteinase using a variety of synthetic substrates and inhibitors, concluded that this high molecular mass enzyme is a multicatalytic protease complex with distinct proteolytic activities associated with separate domains of the enzyme [3]. Both the lens and the pituitary enzyme have the same molecular size (M_r ~700 000) and on SDS-PAGE show the same pattern of at least 8 polypeptide subunits with M_r values ranging from 24 000 to 32 000. It remains to be seen how many

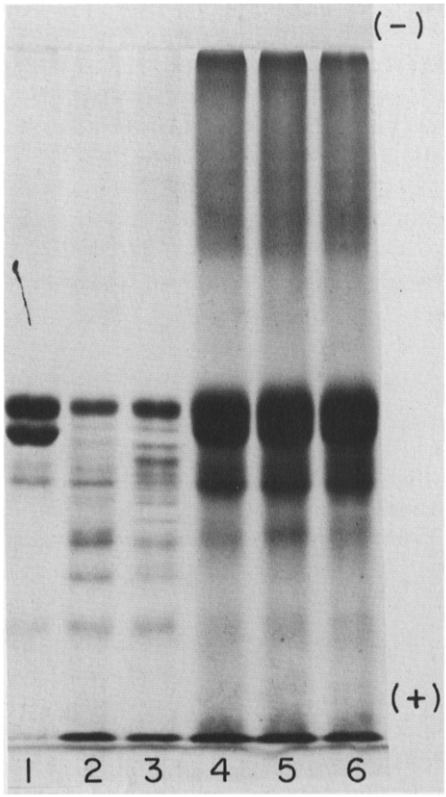

Fig.3. SDS-PAGE to detect the cleavage of casein and azo-casein by lens neutral endopeptidase and pituitary neutral proteinase. Casein (180 μg) or azo-casein (300 μg) was incubated with or without lens or pituitary enzyme (12 μg) in 60 μl of 50 mM Tris-HCl, pH 7.5, at 50°C for 60 min. Similar aliquots were taken and treated as described in section 2 and electrophoresed. Lanes: 1, casein; 2, casein and lens enzyme; 3, casein and pituitary enzyme; 4, azo-casein; 5, azo-casein and lens enzyme; 6, azo-casein and pituitary enzyme.

different genes are concerned in coding for these polypeptides, and how far the multiplicity of components can be attributed to post- or possibly pre-translational modifications in processing. Differences in processing may well occur in the 2 very different tissues, lens and pituitary. If so, such differences in processing could well explain the subtle differences in catalytic properties between the enzymes that we have identified in this report.

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