Purification of neutral lens endopeptidase: Close similarity to a neutral proteinase in pituitary
(cry stallin/bovine)

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ABSTRACT A neutral endopeptidase (EC 3.4.24.5) that degrades α- and β-crystallins occurs in mammalian lens. A procedure for purification of this enzyme from bovine lens is described. The enzyme appears to have a high molecular weight (Mr ~ 700,000) and under denaturing conditions dissociates into at least eight polypeptide subunits with Mr ranging from 24,000 to 32,000. A neutral proteinase in bovine pituitary has been reported previously to have similar structural characteristics. We have found that this enzyme purified from bovine pituitary is indistinguishable in molecular weight and in subunit composition from bovine lens endopeptidase. In addition, antiserum raised in rabbit against the purified lens enzyme crossreacts with bovine pituitary enzyme. When examined side by side in Ouchterlony double-diffusion tests, the two enzymes give a continuous precipitin line with no spurring. It is concluded that lens neutral endopeptidase and pituitary neutral proteinase are structurally closely similar, if not identical. This is a surprising result because it had been thought previously that the lens endopeptidase was unique to lens, where its crystallin substrates comprise a large proportion of the total tissue protein. In other tissues, crystallin is either absent or occurs, at most, in trace amounts.

The occurrence of a neutral endopeptidase (EC 3.4.24.5) in the eye lens of various mammalian species has been known for some years (1–3) and is thought to play a role in cataract formation in humans (4). It is reported to have a high molecular weight, but it has only been partially purified and its subunit composition has not been determined (1). We describe here a method for the purification of the enzyme from bovine lens. The purified preparation has a Mr of about 700,000 in the native state and on NaDodSO4/PAGE shows a characteristic pattern of at least eight polypeptides with Mr's ranging from 24,000 to 32,000. An antiserum has been raised in rabbit against the purified enzyme. This gives on Ouchterlony double-diffusion, single precipitin bands against both the purified enzyme and a crude lens enzyme preparation, which, when examined side by side, show a continuous precipitin line with no spurring.

During the course of this work we noticed when reviewing the literature a photograph of a NaDodSO4/PAGE gel prepared from a purified preparation of a neutral proteinase obtained from bovine pituitary (5). The pattern appeared to be very similar to the NaDodSO4/PAGE pattern we were obtaining with the neutral lens endopeptidase, though the methods of purification of the two enzymes were very different. We therefore purified the pituitary enzyme using essentially the method that had been described (6) and compared the pituitary and the lens enzymes directly. The NaDodSO4/PAGE subunit patterns and also the molecular weight determinations under non-denaturing conditions for the two enzymes were indistinguishable. Furthermore, the pituitary enzyme gave a single precipitin line with the antiserum raised against the purified lens enzyme and there was no spurring when the two enzyme proteins were tested side by side.

The pituitary enzyme had been shown previously to hydrolyze certain synthetic protease substrates such as benzylxoycarbonylglucylglucyl-l-leucyl-p-nitroanilide (Z-Gly-Gly-Leu-pNA), which had been used to follow its purification. However, no naturally occurring protein substrates had been identified. We have found that the pituitary enzyme, like the lens enzyme, degrades a crystallin preparation and that the lens enzyme, like the pituitary enzyme (6), hydrolyzes Z-Gly-Gly-Leu-pNA.

We conclude that the lens and pituitary enzymes are closely similar, if not identical, in their structures. This finding was unexpected because it had been thought previously that the lens endopeptidase was unique to lens (7), where its substrates α- and β-crystallins account for a high proportion of the total tissue protein. Crystallins are absent or occur, at most, in trace amounts in other tissues.

MATERIALS AND METHODS

Materials. Frozen whole bovine pituitaries and bovine lenses were purchased from Pel-Freez. [3H]Acetic anhydride (50.0 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Hydroxylapati te (HA) (Bio-Gel HT) was obtained from Bio-Rad. Mono Q column, DEAE-Sepha cel, and Sephacryl S-300 were purchased from Pharmacia. Z-Gly-Gly-Leu-pNA was the kind gift of S. Wilk (Department of Pharmacology, Mount Sinai School of Medicine, New York).

Isoelectric Precipitation of Lens Proteins. The purification of lens endopeptidase was started by the separation of lens proteins into two fractions (α1-crystallin and α2-crystallin) as described (1). The α1-crystallin fraction contains the enzyme. The α2-crystallin fraction was used to prepare the substrate.

Frozen bovine eye lens (300 g) were thawed at room temperature, an incision was made in each lens, and the tissues were stirred for 20 min at 4°C in 1000 ml of 100 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 5.0/5.0 mM 2-mercaptoethanol, 0.1 mM EDTA. The pH of the extract was monitored, any upward drift in pH being readjusted to pH 5.0 with slow addition of 1 M HCl. The extract was further stirred for 20 min. This resulted in dense white precipitation of the α1-crystallin containing the putative neutral endopeptidase. The bulk of α2-crystallin used to prepare the substrate remained soluble under these conditions. The two fractions were separated by centrifugation at 30,000 X g for 20 min at 4°C.

Abbreviations: FPLC, fast protein liquid chromatography; HA, hydroxylapatite; Mes, 2-(N-morpholino)ethanesulfonic acid; pNA, p-nitroanilide; Z, benzylxoycarbonyl.

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Preparation of the Substrate (α2-crystallin). The soluble supernatant from the total extract was taken and α2-crystallin fraction was prepared and freed from any contaminating protease activity, including the enzyme leucine aminopeptidase, by alkaline urea treatment as described (1).

Preparation of the Enzyme: Lens Neutral Endopeptidase. Solubilization of the enzyme. The α2-crystallin fraction was suspended in 100 ml of 75 mM KH2PO4/K2HPO4 buffer, pH 6.8, containing 5 mM 2-mercaptoethanol and stirred at 40°C for 60 min. The suspension was then centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was collected and the pellet was washed with 50 ml of the same buffer and centrifuged. The supernatants were pooled. Ammonium sulfate fractionation. The soluble supernatant containing the enzyme activity was subjected to 0–33% and 33–80% ammonium sulfate fractionation. The 33–80% fraction, which contained most of the enzyme activity, was suspended in a small volume and dialyzed in 1 liter of buffer A (75 mM potassium phosphate, pH 6.8/5 mM 2-mercaptoethanol/0.02% sodium azide).

HA chromatography. The dialyzed crude enzyme preparation was loaded onto a HA column (16.5 × 2.6 cm) preequilibrated in buffer A. The column was then washed with 2 column volumes of the same buffer. The contaminating proteins were eluted with buffer B (150 mM potassium phosphate, pH 6.8/5 mM 2-mercaptoethanol/0.02% sodium azide) until the column was almost free from 280-nm absorbing material. Finally, an enzyme-enriched protein fraction was eluted from the column by using buffer C (400 mM potassium phosphate, pH 6.8/5 mM 2-mercaptoethanol/0.02% sodium azide). The proteins eluted from the HA column with buffer C were fractionated into 0–33% and 33–80% ammonium sulfate fractions. The 33–80% ammonium sulfate fraction, which contained most of the enzyme activity, was dialyzed against 1 liter of buffer D (50 mM Tris-HCl, pH 7.5/100 mM NaCl/2-mercaptoethanol/0.02% sodium azide) overnight.

Sephacryl S-300 column chromatography. The dialyzed 33–80% ammonium sulfate fraction from the previous step was centrifuged at 15,000 × g for 15 min to remove any aggregated protein prior to loading onto a Sephacryl S-300 column (78 × 2.6 cm). The Sephacryl S-300 column was preequilibrated with the eluting buffer D. Following chromatography the enzyme-enriched fractions were combined, concentrated to a small volume, and rechromatographed on the same Sephacryl S-300 column.

Fast protein liquid chromatography (FPLC) of the Sephacryl S-300 column-purified enzyme by using an anion-exchange column (Mono Q). The pooled Sephacryl S-300 column-eluted fractions containing enzyme activity were concentrated to a small volume by using a Schleicher & Schuell collodion bag (M1 cutoff, 75,000) and dialyzed in buffer E (25 mM Tris-HCl, pH 7.5/50 mM NaCl/5 mM 2-mercaptoethanol). The concentrated fraction was loaded onto a Mono Q column (1 ml) attached to a FPLC system (Pharmacia). The proteins absorbed to the column were eluted as 1-ml fractions by using a salt gradient between 50 and 500 mM NaCl in buffer E. No additional protein was eluted when the salt gradient was extended to 1.0 M. The fractions were assayed for enzyme activity and active fractions were pooled, concentrated, and dialyzed in 10 mM Hepes buffer (pH 7.5).

Labeling of α2-Crystallin with [3H]Acetic Anhydride. This was done essentially as described (1). The only modification that was that the labeled protein was stored in 50 mM NaOAc/50 mM NaCl to keep the concentration of NaCl low during the enzyme assay.

Assay of Lens Neutral Endopeptidase. The enzyme fraction was mixed with 10 μl of α2-[3H]crystallin (2–10 μCi) diluted to a final volume of 125 μl with 10 mM Hepes (pH 7.5). The assay mixture was incubated at 55°C for 60 min and then transferred to an ice bath. After 5 min, 25 μl of carrier bovine serum albumin (20 mg/ml) and 500 μl of 7.5% trichloroacetic acid were added. After keeping the tubes in ice for 10 min, the tubes were centrifuged in a table top Microfuge for 6 min. Sixty-five microliters of the trichloroacetic acid-soluble supernatant was applied to a Whatman paper disc (3 mm), dried, and assayed for radioactivity with toluene-based scintillation fluor. Protein was measured as described (8). Enzyme activity was also measured against the synthetic substrate Z-Gly-Gly-Leu-pNa, as described (6).

PAGE Under Nondenaturing Conditions and Determination of Molecular Weight of the Enzyme. Native PAGE of the protein samples was done in a Pharmacia apparatus using a Pharmacia-prepared (4–30%) gradient polyacrylamide slab gel. The gel was preelectrophoresed (without sample) for 20 min at 70 V in the electrophoresis buffer (90 mM Tris/80 mM boric acid/2.5 mM Na3EDTA, pH 8.4). The sample proteins were diluted in electrophoresis buffer, loaded onto the gel, and electrophoresed initially at 70 V for 20 min until protein moved into the gel. Then the electrophoresis was continued at 150 V for 16 hr. After electrophoresis the gel was fixed by shaking in 10% sulfosalicylic acid for 30 min. The gel was then stained overnight in 0.2% Coomassie blue in 7% acetic acid and destained in 7% acetic acid. By using molecular weight markers a standard curve was derived from which the molecular weights of the protein were determined. PAGE Under Denaturing Conditions. The protein samples were diluted in sample buffer (125 mM Tris-HCl, pH 6.8/2% 2-mercaptoethanol/0.5% NaDodSO4/10% glycerol/0.01% bromophenol blue) and boiled in a water bath for 2–3 min. Electrophoresis was done by using 25 mM Tris/glycine (pH 8.8) containing 0.1% NaDodSO4 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.

Rabbit Antiserum. Antiserum against pure lens neural endopeptidase was raised in a New Zealand White rabbit. The rabbit was injected s.c. with 200 μg of pure enzyme in complete Freund’s adjuvant. After 4 weeks, the injections were repeated at 4-week intervals for 3 months by using incomplete Freund’s adjuvant. Serum was collected at 1-week intervals following each immunization.

RESULTS

Purification of the Lens Neutral Endopeptidase. Only partial purification of the enzyme has been reported so far (1). Here we report purification of the enzyme to apparent homogeneity. The results of the purification procedure are summarized in Table 1. Relatively much lower total activity in the initial "crude protein extract" fraction compared to that found later was due to dilution of the specific activity of the substrate α2-[3H]crystallin by the presence of a large excess of unlabeled α2-crystallin. Isoelectric precipitation separated the α2-crystallin-enriched fraction containing most of the enzyme activity from α2-crystallin that remained soluble. Thus, the isoelectric-precipitated protein fraction has been arbitrarily considered to contain 100% activity (Table 1).

The large excess of α2-crystallin present at that stage was partially removed from the enzyme by ammonium sulfate fractionation. Most of the enzyme activity remained in the 33–80% ammonium sulfate fraction that was subjected to HA chromatography. Almost the entire enzyme activity was eluted from the HA column using 400 mM potassium phosphate (pH 6.8), as described in Materials and Methods. The increase in specific activity was 78-fold. However, as revealed by NaDodSO4/PAGE, the HA-purified enzyme still contained large amounts of α-crystallin (Fig. 1), the amount of which was reduced in the subsequent ammonium sulfate treatment.
fractionation. The 33–80% ammonium sulfate fraction of HA-purified enzyme preparation contained most of the enzyme activity and was further purified by gel-filtration chromatography on a Sephacryl S-300 column (Fig. 2). The enzyme activity was eluted from the column at the beginning of the first protein peak and was thus contaminated with other proteins. Therefore, the enzyme-enriched fraction was rechromatographed on the same column. The Sephacryl S-300-purified enzyme fraction had some low and high molecular weight contaminants, which were finally removed by purification of the enzyme on an ion-exchange column using FPLC (Fig. 3). The enzyme eluted from the column at 0.35 M NaCl, as shown in Fig. 3. At this stage, the purification of the enzyme compared to the original isoelectric-precipitated enzyme fraction was 2532-fold and recovery of total activity compared to isoelectric-precipitated enzyme fraction was 31.7% (Table 1). No divalent cation had been used in the enzyme assay to estimate total recovery and the specific activity at the different stages of purification (Table 1). This is because, although the enzyme has been reported as divalent metal ion dependent (1, 7), the extent of stimulation of the enzyme activity by Mg2+ or Ca2+ in different purification stages was progressively found to decrease and the final, apparently homogeneous, enzyme preparation shows very little stimulation.

The purity of the final enzyme preparation was examined by nondenaturing PAGE. As evident from Fig. 4, the enzyme was apparently purified to homogeneity. The M_r of the native enzyme was found to be about 700,000 estimated from the standard curve drawn from the marker proteins used during the gel electrophoresis. A similar molecular weight for the native enzyme was also obtained from the gel-filtration chromatography of the enzyme on the Sephacryl S-300 column.

The subunit pattern of the native enzyme was determined by denaturing PAGE using 0.1% NaDodSO_4 as described in Materials and Methods. We found that the native enzyme dissociates into at least eight subunits in the M_r range of 20,000–32,000 (Fig. 1).

Purification of the Neutral Protease from Bovine Pituitary. Purification of the neutral protease from the bovine pituitaries was done essentially as described by Wilk and Orlowski (6). In short, the 40–60% ammonium sulfate fraction of the soluble supernatant was chromatographed on a DEAE-Sephacel column. The DEAE-Sephacel-purified protease was applied to a Sephacryl S-300 column (78 × 2.6 cm) equilibrated with 0.05 M Tris/EDTA, pH 7.5. The enzyme activity eluted in the first major protein peak in a position identical to the elution volume of the lens neutral endopeptidase. To get rid of small amounts of high molecular weight contaminating proteins we purified the enzyme further by FPLC using an ion-exchange column (Mono Q, Pharmacia) under the conditions identical to those used in the final stage of the lens enzyme purification. The pituitary

**Table 1. Summary of purification of lens neutral endopeptidase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein, mg</th>
<th>Total activity, units</th>
<th>Specific activity, units/mg</th>
<th>Purification, fold</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude protein extract</td>
<td>23,171</td>
<td>*</td>
<td>*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2. Isoelectric precipitate</td>
<td>10,528</td>
<td>1818</td>
<td>0.173</td>
<td>(1.0)</td>
<td>(100.0)</td>
</tr>
<tr>
<td>3. Soluble enzyme</td>
<td>3,556</td>
<td>1755</td>
<td>0.494</td>
<td>2.9</td>
<td>96.5</td>
</tr>
<tr>
<td>4. 33–80% (NH_4)_2SO_4 fraction</td>
<td>2,214</td>
<td>1830</td>
<td>0.827</td>
<td>4.8</td>
<td>100.7</td>
</tr>
<tr>
<td>5. HA chromatography</td>
<td>109</td>
<td>1470</td>
<td>13.45</td>
<td>78.1</td>
<td>80.9</td>
</tr>
<tr>
<td>6. 33–80% (NH_4)_2SO_4 fraction of HA-purified enzyme</td>
<td>60.5</td>
<td>1484</td>
<td>24.53</td>
<td>142</td>
<td>81.6</td>
</tr>
<tr>
<td>7. Sephacryl S-300 chromatography</td>
<td>11.7</td>
<td>1045</td>
<td>89.29</td>
<td>517</td>
<td>57.5</td>
</tr>
<tr>
<td>8. Rechromatography of Sephacryl S-300 fraction</td>
<td>3.86</td>
<td>715</td>
<td>185.35</td>
<td>1073</td>
<td>39.4</td>
</tr>
<tr>
<td>9. FPLC on anion-exchange column</td>
<td>1.32</td>
<td>577</td>
<td>438.05</td>
<td>2552</td>
<td>31.7</td>
</tr>
</tbody>
</table>

One unit of enzyme is described as the amount that gives 100 cpm in trichloroacetic acid-soluble supernatant. Steps 1–9 relate to lanes 1–9 of Fig. 1.

*See text.*
Biochemistry: Ray and Harris

in enzyme activity, enzyme has been found to degrade α2-crystallin as well as Z-Gly-Gly-Leu-pNA and the activity peaks coeluted. This enzyme has been reported to dissociate into three (6) to five (5) bands with Mr's between 24,000 and 28,000 on NaDodSO4/PAGE. We observed on NaDodSO4/PAGE that the enzyme dissociates into at least eight closely spaced bands (Mr's 24,000–32,000), as was found with the lens neutral endopeptidase (Fig. 5).

**Similarity Between the Lens Neutral Endopeptidase and the Pituitary Neutral Proteinase.** Although the lens neutral endopeptidase has been reported to cleave α- and β-crystallin as well as the synthetic substrate Z-Gly-Gly-Leu-pNA (10), the pituitary enzyme has been shown to degrade Z-Gly-Gly-Leu-pNA and other synthetic substrates as well as some biologically active small peptides, but no protein substrate has been identified (6). We report here that the pituitary enzyme is capable of degrading α2-crystallin.

Both enzymes have very high molecular weights (about 700,000), elute from a Sephacryl S-300 column at the same elution volume, and elute from an anion-exchange column (Mono Q) at the same salt concentration. All this suggests close similarity between the two enzymes. We found that after mixing the two enzymes in equal amounts and running native PAGE, they comigrate and are nonseparable (Fig. 4).

A more rigorous test of their identity was a similar experiment under denaturing conditions, where, due to the multiple number of bands for each enzyme, any difference between them would be more conspicuous. When we mixed the two enzymes in equal proportion and performed NaDodSO4/PAGE, the band pattern was indistinguishable from either the lens or the pituitary enzyme alone (Fig. 5).

Finally, antibodies were raised in rabbit against lens neutral endopeptidase. In one experiment (Fig. 6A) it was observed that on Ouchterlony double-diffusion the antibodies gave single precipitin bands against both the purified and a crude lens enzyme preparation, which, when examined side by side, showed continuity with no spurring. This suggests that the antibody is directed against the neutral endopeptidase only and no other protein of the eye lens. In another similar experiment using the pituitary neutral proteinase and the lens endopeptidase it was observed that both of the enzymes gave single precipitin lines with the antiserum (Fig. 6B) and that the lines were continuous at the joining edges with no spurring, indicating identity of the antigenic sites on the two enzymes.

**DISCUSSION**

The main difficulty in purifying lens endopeptidase is the removal of contaminating crystallins that constitute about 90% of the total lens protein. In the partially purified preparations of the enzyme reported in the literature, crystallins still seem to have been a major impurity (1). Our purification method, though it follows the earlier report (1) in some of its initial steps, differs by the addition of several new steps designed to eliminate crystallin contamination. These include ammonium sulfate fractionations both before and after the HA chromatography, Sephacryl S-300 gel filtration, and, finally, FPLC using an anion-exchange column. The final product appears to be free of crystallin, but it is difficult to exclude the presence of trace amounts of this contaminant.

Since pituitary contains either no crystallin or, at most, very minute amounts, crystallin contamination was not to be expected here. We therefore followed the method of purifi-
cation reported previously (6). In our hands, the final enzyme product still appeared to contain minor protein impurities. These were eliminated by using the same final purification step as used with the lens enzyme—namely, FPLC with an anion-exchange column.

The close similarity of the two enzymes is most dramatically demonstrated by NaDodSO₄/PAGE of the polypeptide subunits. The patterns obtained for the two enzymes were indistinguishable either when run separately or as a 50:50 mixture. We found at least eight polypeptides with Mr's in the 24,000–32,000 range. Previous studies on the pituitary enzyme (5) showed only five polypeptide subunits, but the difference is probably attributable to a somewhat increased resolving power of the system used here. It remains possible that there are minor differences in the relative amounts of the different polypeptides seen that cannot be determined by this method. It is also possible that other polypeptides may be resolved by other techniques (e.g., reverse-phase HPLC). However, the close similarity, if not structural identity, between the two enzymes is not in doubt.

The estimate of molecular weight (about 700,000) was obtained by gradient PAGE of the nondenatured proteins and also by gel filtration. The proteins were indistinguishable in this respect. This high molecular weight had also been noted by Wilk and Orlowski for the pituitary enzyme (6). They have suggested from other studies that the enzyme represents a multicatalytic protease complex with distinct proteolytic activities associated with separate components of this high molecular weight protein (5).

A detailed study of various properties of the two enzymes, including substrate specificities, temperature activity curves, and cation effects on activity, will be reported elsewhere.

Preliminary data indicate that so far the enzymes appear to be closely similar in these various characteristics. Such minor differences as have been detected, if confirmed, may prove to be the consequence of differences in processing at the RNA or protein level in the two very different tissues, lens and pituitary.

The finding of close similarity of the two enzymes is unexpected and surprising because it had been thought previously that lens neutral endopeptidase was peculiar to lens, where its crystallin substrates, in contrast to all other tissues, represent a considerable proportion of the total protein present. The results raise the questions as to whether the enzyme also occurs in other tissues besides pituitary and what the nature of the natural substrates in these locations may be.

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