Lens neutral endopeptidase occurs in other bovine and human tissues

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Lens neutral endopeptidase (EC 3.4.24.5) was previously thought to be unique to the eye lens. We report here the finding of a neutral endopeptidase, in a variety of bovine and human tissues, which is very similar both biochemically and immunologically to the lens endopeptidase. SDS/polyacrylamide-gel electrophoresis of partially purified enzyme fractions from various bovine tissues shows the characteristic pattern of at least eight bands with M_r values ranging from 24000 to 32000 which was described for the bovine-lens neutral endopeptidase. The relative activity of the enzyme varies from tissue to tissue with lung having the highest activity. Partially purified enzyme fractions from these tissues cross-react with antiserum raised in rabbit against bovine lens endopeptidase showing apparent identity when examined side by side in Ouchterlony double-diffusion tests. The human enzyme also cross-reacts with the antiserum but when tested by double-diffusion against the bovine enzyme the precipitin lines show spurring at the joining edges indicating a structural difference between the human and the bovine enzymes. It was also found by Western blot experiments, after denaturing polyacrylamide-gel electrophoresis of the enzyme, that the polypeptide components of the human and bovine enzymes show somewhat different banding patterns.

INTRODUCTION

A neutral endopeptidase (EC 3.4.24.5) occurs in the eye lens of various mammalian species [1–3]. Previously this enzyme was thought to be unique to the eye lens [4]. Recently, however, we have reported biochemical and immunological evidence suggesting a close similarity between the bovine-lens neutral endopeptidase and a bovine-pituitary neutral endopeptidase [5]. Unlike most other endopeptidases, both the lens and the pituitary neutral endopeptidases have very high M_r values (700000) and show a characteristic pattern of at least eight bands with M_r values ranging from 24000 to 32000 on SDS/polyacrylamide-gel electrophoresis (SDS/PAGE). The pituitary enzyme gives a single precipitin line with antiserum raised against the purified bovine lens enzyme and there is no spurring when the two enzymes are tested side by side in Ouchterlony double-diffusion tests. Recently, high-molecular-mass proteinases from rat skeletal muscle [6], rat and mouse liver [7,8], human lung [9] and human erythrocyte [10] have been described. These enzymes show multiple bands of M_r between 21000 and 34000 on SDS/PAGE and the band pattern is very similar to that obtained from bovine lens and bovine-pituitary neutral endopeptidases. This prompted us to make a systematic study of the distribution of the neutral endopeptidase in bovine and human tissues.

We present here biochemical and immunological evidence which suggests that the putative high-molecular-mass endopeptidase is present in a variety of bovine and human tissues but that the relative activity of the enzyme varies from tissue to tissue. We observed by Ouchterlony double-diffusion experiments, using enzyme fractions

from different tissues and antiserum raised against purified bovine-lens neutral endopeptidase, that the neutral proteinase present in different bovine tissues are closely similar. However, the human enzyme is antigenically distinct from the bovine enzyme.

MATERIALS AND METHODS

Materials

Frozen bovine pituitaries and bovine lenses were purchased from Pel-Freez. Other bovine tissues were collected fresh from a single animal from the local slaughterhouse. Human tissue samples from a variety of subjects were obtained at autopsy. The specimens were stored at -20°C until use. Benzyloxycarbonyl-Gly-Leu-*p*-nitroanilide was purchased from Sigma Chemical Co. Mono Q column was obtained from Pharmacia. ^{125}I -Protein A (30 mCi/mg; 1 Ci = 37 GBq) was purchased from Amersham. All other chemicals were reagent grade or the best commercially available.

Purification of the neutral proteinase from bovine and human tissues

Bovine-lens neutral endopeptidase was purified to apparent homogeneity as described [5]. From other tissues the enzyme was partially purified as follows: tissue (10 g) was thawed, cleaned and minced into small pieces with scissors. The tissue was then homogenized in a Polytron (3×30 s) in 5 vol. of 20 mM-Tris/HCl, pH 7.5, with 5 mM-2-mercaptoethanol. The homogenate was centrifuged at 20000 *g* for 20 min. The supernatant was subjected to ammonium sulphate fractionation. The 40–65% ammonium sulphate fraction contained most

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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of the enzyme activity. The pelleted protein was resuspended in 25 mM-Tris/HCl, pH 7.5/5 mM-2-mercaptoethanol/50 mM-NaCl and dialysed overnight against 10 vol. of the same buffer. The dialysed protein was centrifuged at 20000 *g* for 20 min and then loaded onto a Mono Q column (1 ml) attached to a fast protein liquid chromatography system (Pharmacia). The proteins adsorbed onto the column were eluted as 1 ml fractions using a 50 mM–500 mM-NaCl linear gradient in 25 mM-Tris/HCl, pH 7.5/5 mM-2-mercaptoethanol. The fractions having endopeptidase activity were pooled, concentrated and dialysed against 20 mM-Tris/HCl, pH 7.5/5 mM-2-mercaptoethanol. Henceforth, this enzyme fraction will be referred to as the partially purified enzyme fraction.

Assay of neutral endopeptidase

Enzyme activity was measured using the synthetic substrate benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide as reported by Wilk & Orlowski [11]. The incubation mixture contained 5 mM-Tris/HCl, pH 7.5, the enzyme fraction and 10 μ l of 0.01 M-substrate (dissolved in dimethyl sulphoxide) in a total vol. of 250 μ l. Samples were incubated at 40 °C for 60 min. *p*-Nitroaniline released was measured by a diazotization procedure [12].

PAGE under non-denaturing conditions

The protein samples were diluted in sample buffer (125 mM-Tris/HCl, pH 6.8/10 % glycerol/0.01 % Bromophenol Blue). Discontinuous electrophoresis was carried out according to Davis [13] with 2.5 % polyacrylamide in the upper gel and 5 % polyacrylamide in the lower gel. The proteins from the gel were then transferred to nitrocellulose paper by Western blot.

PAGE under denaturing conditions

The protein samples were diluted in sample buffer (125 mM-Tris/HCl, pH 6.8/2 % 2-mercaptoethanol/0.5 % SDS/10 % glycerol/0.01 % Bromophenol Blue) and boiled in a water bath for 3 min. Electrophoresis was carried out as described by Laemmli [14] with 5 % polyacrylamide in the upper gel and 12.5 % polyacrylamide in the lower gel. Gels were stained for protein with Coomassie Brilliant Blue or the protein was transferred to nitrocellulose paper by Western blot as described below.

Western blot

This was carried out essentially as described by Towbin *et al.* [15] and Burnette [16] with some modifications. After electrophoresis the proteins were transferred from polyacrylamide gel to nitrocellulose paper using a Bio-Rad trans-blot cell at 4 °C with constant voltage (30 V) for 16 h. Transfer buffer for the non-denaturing gel was buffer A (25 mM-Tris, pH 8.3/192 mM-glycine) and for the denaturing gel buffer B was used [buffer A with 20 % (v/v) methanol]. Prior to transblotting, SDS was removed from the denaturing gels by washing for 30 min in 0.5 litre of buffer B. To prevent non-specific binding of proteins to nitrocellulose during subsequent treatments, the paper was soaked for 1 h at room temperature in buffer C [phosphate-buffered saline (20 mM-sodium phosphate/50 mM-NaCl, pH 7.4) containing 3 % bovine serum albumin/1 % fetal calf serum (FCS) and 0.02 % NaN₃]. Next the nitrocellulose

paper was incubated at 4 °C overnight with rabbit anti-(bovine-lens endopeptidase) serum diluted 100 times in buffer D (phosphate-buffered saline containing 1 % bovine serum albumin/0.3 % fetal calf serum/0.05 % Tween 20/0.02 % NaN₃). The paper was then washed 5 times (5 $\times$ 5 min) with buffer D, incubated with a 100-fold dilution of goat anti-rabbit IgG in buffer D at room temperature for 2 h and washed again as above. Finally, it was incubated with ¹²⁵I-Protein A (0.6 μ Ci) in buffer D for 2 h at room temperature, washed 5 times in buffer D and 2 times in phosphate-buffered saline. The nitrocellulose paper was dried at room temperature and autoradiographed.

Rabbit antiserum

Antiserum against pure bovine-lens neutral endopeptidase was raised in a New Zealand White rabbit. The rabbit was injected subcutaneously with 200 μ g of pure enzyme in complete Freund's adjuvant. The injections were repeated at 4-week intervals for 3 months using incomplete Freund's adjuvant. Serum was collected at 1-week intervals following each immunization.

RESULTS

Preliminary results from Ouchterlony double-diffusion experiments, using rabbit anti-bovine lens neutral endopeptidase antiserum and crude extracts from different bovine and human tissues, showed precipitin lines indicating the presence of the enzyme. We therefore partially purified the enzyme from different tissues to study the enzyme biochemically and immunologically.

Purification and quantification of proteinase from bovine and human tissues

Partial purification of the enzyme from different tissues included ammonium sulphate fractionation and ion-exchange chromatography steps, as described in the Materials and methods section. The enzyme activity from both bovine and human tissue extracts was eluted from fast protein liquid-chromatography anion-exchange column (Mono Q) at 0.35 M-NaCl. The same salt

Table 1. Neutral endopeptidase activity in partially purified enzyme fractions from bovine tissues

Ammonium sulphate (40–65 %) fraction (18 mg) of total soluble protein from tissue extract was loaded to the ion-exchange column as described in the Materials and methods section. Total activity eluted from the column was determined. The activities given are means of three assays done on each tissue sample, all from the same animal.

Tissue	<i>p</i> -Nitroanilide released (μ mol/h)	Relative activity
Lung	255.00	(100)
Pituitary	213.11	83.6
Kidney	173.21	67.9
Liver	159.34	62.5
Heart	100.47	39.4
Muscle	87.17	34.2
Brain	29.15	11.4
Lens	15.57	6.1
Red blood cells	12.74	5.0

concentration was used to elute the bovine-lens neutral endopeptidase [5]. All the tissues examined contained enzyme activity. However, the relative amount of enzyme activity for different bovine tissues varied widely. Table 1 gives the mean activities from three determinations on each tissue from a single animal. The relative activities observed were in the following order: lung > pituitary > kidney > liver > muscle > heart > brain > lens > red blood cells. We did not measure total enzyme activities of the enzyme in crude homogenates because such a measurement may be influenced by cleavage of the synthetic substrate by other proteinases present and by variable amounts in various tissues. Although we detected the enzyme activity in all the human tissues tested (heart, lung, kidney, liver, brain, spleen and lens) we did not attempt to quantify the relative amounts of the enzyme in different human tissues because they were collected from different subjects with different pathological conditions and had been stored for variable periods of time.

PAGE of partially purified enzyme fractions under denaturing conditions

A characteristic complex pattern of bands is obtained [5], so it appeared that a rigorous test for the identity of the enzyme in other tissues would be to examine their band pattern in this system. We performed SDS/PAGE on the partially purified enzyme fractions from different tissues and compared the results with that obtained for pure bovine lens enzyme. It is evident from Fig. 1 that for bovine heart, lung, kidney, liver and muscle the typical pattern of eight bands is clearly observed. In the case of bovine brain and blood, the pattern is less clear, probably

Fig. 1. SDS/polyacrylamide-gel electrophoresis of bovine tissue proteins containing the neutral endopeptidase using a 12.5% polyacrylamide gel

Lane 1, pure bovine lens endopeptidase (14 μ g); lanes 2, 4, 6, 8, 10 and 12, 40–65% ammonium sulphate fraction of soluble proteins (50 μ g each) from heart, lung, kidney, liver, muscle and brain respectively; lanes 3, 5, 7, 9, 11, 13 and 14, anion-exchange column purified enzyme fractions from heart (22 μ g), lung (23 μ g), kidney (24 μ g), liver (20 μ g), muscle (19 μ g), brain (20 μ g) and blood (5 μ g) respectively; lane 15, molecular-mass markers: phosphorylase *b* (M_r 94000), albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20100), α -lactalbumin (M_r 14400). Abbreviation: RBC, red blood corpuscle.

owing to the smaller amounts of enzyme present in those tissues.

Thus we observed from the proteinase present in different bovine and human tissues that (a) they hydrolyse the synthetic substrate benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, (b) the enzyme activity is eluted from anion-exchange columns at the same salt concentration and (c) the enzyme appears to split into a multiple-band pattern similar to lens neutral endopeptidase on SDS/PAGE.

Ouchterlony double-diffusion experiments

Using both Ouchterlony double-diffusion tests and Western blotting, we compared the cross-reactivity of the putative high-molecular-mass neutral proteinases from different tissues with antiserum raised in rabbit against the purified bovine-lens neutral endopeptidase.

In Ouchterlony double-diffusion experiments the rabbit anti-(bovine lens endopeptidase) serum gave single precipitin bands against partially purified enzyme fractions obtained from the different bovine and human tissues (Figs. 2 and 3). On testing bovine lens endopeptidase and proteinase fractions from other bovine tissues side by side, it was observed that the precipitin lines obtained were continuous at the joining edges with no spurring (Fig. 2). A similar result was obtained when different bovine tissue fractions were tested side by side (Fig. 2c). This shows that the putative high-molecular-mass proteinases present in different bovine tissues are immunologically closely similar to, if not identical with, the bovine-lens neutral endopeptidase. However, when Ouchterlony double-diffusion experiments were done using bovine-lens neutral endopeptidase and the proteinase fractions from different human tissues, it was observed that the precipitin lines for the two enzymes showed spurring (Fig. 3). A similar experiment done using only human tissue enzyme fractions side by side resulted in single precipitin lines which were continuous at the joining edges with no spurring (Fig. 3c). These results indicated that this high-molecular-mass proteinase is similar antigenically in different tissues of the same species but the bovine enzyme has some differences in antigenicity from the human enzyme.

Fig. 2. Ouchterlony double-diffusion experiment using partially purified endopeptidase fractions from bovine tissues

Centre wells of (a), (b) and (c) contain rabbit antisera to pure bovine lens endopeptidase (16 μ l/well). Outer wells contain either pure endopeptidase (15 μ g) from bovine lens, Le, or partially purified endopeptidase from other bovine tissues: K, kidney (24 μ g); H, heart (23 μ g); Lu, lung (40 μ g); B, brain (28 μ g); Li, liver (16 μ g); M, muscle (15 μ g).

Fig. 3. Ouchterlony double-diffusion experiment using partially purified endopeptidase fractions from human tissues and pure bovine lens endopeptidase

Centre wells of (a), (b) and (c) contain rabbit antisera to pure bovine lens endopeptidase (8 μ l/well). Outer wells contain either pure endopeptidase (9 μ g) from bovine lens, Le, or partially purified endopeptidase from different human tissues: H, heart (20 μ g); Lu, lung (16 μ g); K, kidney (20 μ g); Li, liver (20 μ g); B, brain (30 μ g); S, spleen (15 μ g). The plate was washed in 0.9% saline and stained with Coomassie Blue.

Fig. 4. Western blot of partially purified bovine and human endopeptidases under non-denaturing conditions

(a) Bovine tissue enzyme. Lane 1 contains pure enzyme (5 μ g) and lanes 2–8 contain partially purified enzyme fractions (20–30 μ g) from: 1, lens; 2, pituitary; 3, heart; 4, lung; 5, kidney; 6, liver; 7, brain; 8, muscle. Bovine and human tissue enzymes. Lanes 1 and 2 contain pure bovine enzymes (3 μ g) from: 1, pituitary; 2, lens. Lanes 3–8 contain partially purified enzyme fractions (20–30 μ g) from human tissues of: 3, heart; 4, lung; 5, kidney; 6, liver; 7, brain; 8, spleen.

Western blots of native and denatured enzyme fractions

Partially purified enzyme fractions were electrophoresed on polyacrylamide gel under both denaturing and non-denaturing conditions. The bands of the gel cross-reacting with rabbit antiserum raised against bovine-lens neutral endopeptidase were detected by Western blotting as detailed in the Materials and methods section. Under non-denaturing conditions, the enzyme from the various bovine tissues had mobilities identical with the bovine lens enzyme (Fig. 4a). Similarly the enzyme from human tissue fractions had the same mobilities as the bovine lens enzyme (Fig. 4b). The polypeptide components of the enzyme cross-reacting with the antiserum were identified by Western blotting

Fig. 5. Western blot of partially purified bovine and human endopeptidases under denaturing conditions

(a) Bovine tissue enzyme. Lanes 1 and 2 contain pure enzyme (5 μ g) and lanes 3–8 contain partially purified enzyme fractions (20–30 μ g) from: 1, lens; 2, pituitary; 3, heart; 4, lung; 5, kidney; 6, liver; 7, muscle; 8, brain. (b) Human tissue enzyme (20–30 μ g) from: lanes 1, heart; 2, lung; 3, kidney; 4, liver; 5, brain.

using enzyme fractions electrophoresed under denaturing conditions. With bovine tissue fractions the band pattern was similar to that of the bovine lens and bovine pituitary enzymes (Fig. 5a) with some subtle differences. The polypeptide band with M_r 28000 which is present in the partially purified tissue fractions is lost during further purification of the enzyme. Not all the minor bands are clearly seen in the purified preparations (Fig. 5a, lanes 1 and 2) because of the small amount of protein loaded, but could be detected when larger amounts were applied. With enzyme fractions from human tissues the band pattern was different (Fig. 5b) suggesting that the enzyme in human tissues is structurally different from the bovine enzyme. Thus the components of the enzyme in human tissues cross-reacting with the antiserum appear to be a cluster of at least four polypeptides in the M_r range of 24000–26000 and a polypeptide with M_r 68000. In addition to these bands, the antisera cross-reacted with a polypeptide of low M_r (12000) from human lung and with a polypeptide of high M_r (200000) from human kidney.

DISCUSSION

The existence of the lens neutral endopeptidase has been known for some years [1–3]. It had been thought to be unique to the eye lens [4]. Historically, the only biological substrates then known for the enzyme were α - and β -crystallin which only occur in significant amounts in the lens. A possible role for the enzyme in some types of cataractogenesis, where the amount of total lens protein is reduced, has been suggested. Until recently, the enzyme had not been purified to homogeneity, thus

preventing a direct comparison of the lens neutral endopeptidase with proteinases from other tissues.

We have recently reported the purification of the lens neutral endopeptidase and its striking similarity to a bovine pituitary endopeptidase [5]. The bovine pituitary endopeptidase has been described by Wilk & Orlowski [17] as a multicatalytic protease complex because it degrades different synthetic substrates. It shows trypsin-, chymotrypsin- and peptidyl glutamyl-like activities, each of which could be inhibited by a specific inhibitor while the other activities remained unaffected. This suggested that multiple active sites for proteolysis are present on a single enzyme complex. We have made similar observations using the same synthetic substrates and the lens enzyme [18]. We have also found that both the lens and the pituitary endopeptidase have similar molecular sizes and polypeptide components and each enzyme cross-reacts with antisera raised against the other [5]. Dahlmann *et al.* [6] have reported the presence of such a multicatalytic proteinase complex (M_r 650 000) from rat skeletal muscle which has polypeptide components of similar size. It has been suggested that the muscle enzyme [6] is very similar to the pituitary enzyme though with some subtle differences. Recently, Rivett [7] has purified an alkaline protease from rat liver, which preferentially degrades oxidatively-modified glutamine synthetase. The rat liver protease has a high M_r (650 000) and contains polypeptide components (M_r between 22 000 and 34 000) similar to bovine pituitary and bovine lens endopeptidase. It has also been suggested that the liver protease has several properties in common with multicatalytic muscle proteinase [7]. Tanaka *et al.* [8] also have purified the high-molecular-mass protease from rat liver cytosol and have demonstrated the presence of the enzyme in other rat tissues by Ouchterlony double-diffusion analyses. The presence of similar high-molecular-mass proteinase has also been reported from human tissues. Thus, a 'high-molecular-mass neutral endopeptidase-24/5' (M_r 650 000) has been reported to be present in human lung [9]. A similar proteinase termed 'macropain' from human erythrocytes (M_r 650 000) has been described [10]. The proteinase from both the human tissues on SDS/PAGE dissociates into several lower- M_r components (M_r 21 000–32 000) similar to bovine pituitary and lens endopeptidase. All these observations taken together indicated the presence of a high-molecular-mass multicatalytic proteinase in various tissues similar to the enzyme classified in the protease glossary [4] as lens neutral proteinase (EC 3.4.24.5).

An examination of different bovine and human tissues revealed the presence of the high-molecular-mass proteinase similar to the bovine lens neutral endopeptidase. Partially purified enzyme fractions were observed to have the following similarities: (a) cleavage of the synthetic substrate benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, (b) elution of the enzyme activity from an ion-exchange column at the same ionic concentration of NaCl, (c) a similar cluster of bands on denaturing PAGE, (d) cross-reactions with rabbit antiserum raised against bovine lens neutral endopeptidase, and (e) similar mobility of the enzyme in non-denaturing PAGE as detected by Western blots. These observations strongly suggested the presence of a similar proteinase in the different tissues. This protease is different from most other known proteases. However, the putative proteinase from bovine tissues has some difference in antigenicity

from the human tissue enzyme as revealed by Ouchterlony double-diffusion experiments. Also, the polypeptide components within the proteinase from bovine and human tissues cross-reacting with anti-(bovine lens endopeptidase) serum were different. The reason for the variation in polypeptide components within human tissue fractions (e.g. liver, lung and kidney) cross-reacting with the antiserum is not clear but non-specific interaction of human tissue proteinase fraction with rabbit serum was not observed by using rabbit pre-immune serum under similar experimental conditions. A better understanding about such variation could be achieved by purification of the proteinase to homogeneity from different tissues and analysing their polypeptide components.

The physiological significance of the enzyme is not yet well understood. However, different functional roles have been suggested for the same, or very similar, enzymes occurring in different tissues. Thus Wilk & Orlowski [11] have observed that the pituitary endopeptidase digests biologically active peptides (e.g. luteinizing-hormone-releasing hormone, angiotensin, neurotensin etc.). They have suggested that the enzyme may be involved in the sequestering of peptide hormones within the pituitary. Dahlmann *et al.* [19] have shown activation of the rat skeletal muscle proteinase by fatty acids and suggest that during diabetes and starvation when the concentration of fatty acids is known to increase within the muscle tissues [20], fatty acids could dramatically increase the activity of the multicatalytic proteinase. This is consistent with the observed increased protein breakdown under these metabolic conditions. Rat liver proteinase has been reported to degrade oxidatively-modified glutamine synthetase more readily than the native enzyme [7] suggesting that the proteinase might be involved in preferential breakdown of oxidized proteins *in vivo* [7]. So, it appears probable that the enzyme participates in overall intra-cellular, non-lysosomal, protein breakdown. Purification and characterization of the enzyme from yet other tissues is needed for an understanding of the significance of the enzyme in cellular processes. It remains to be seen how many different genes code for the multiple polypeptides present in the enzyme and how far the multiplicity of components can be attributed to pre- and post-translational modifications. Different processing methods could be responsible for some differences observed between the enzymes from different tissues.

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