The activities of 'Pz-peptidase' and 'endopeptidase 24.15' are due to a single enzyme

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It was found that Pz-peptidase (assayed with 2,4-dinitrophenyl-Pro-Leu-Gly-Pro-Trp-D-Lys) and endopeptidase 24.15 (assayed with benzoyl-Gly-Ala-Ala-Phe-*p*-aminobenzoate) were co-purified from rat skeletal muscle, were co-eluted in high-resolution gel chromatography and co-existed in a homogeneous preparation of rat testis endopeptidase 24.15. The action of partially purified Pz-peptidase from rat testis on 4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg was blocked by an inhibitor of endopeptidase 24.15, and also by a substrate of this enzyme. The partially purified enzyme hydrolysed two substrates of endopeptidase 24.15 with K_m values similar to those published previously, and its action on 2,4-dinitrophenyl-Pro-Leu-Gly-Pro-Trp-D-Lys was inhibited by compounds that are considered specific for endopeptidase 24.15. We conclude that the activities previously attributed to two distinct enzymes are due to only one, and that the merging of the two literatures may lead to new lines of research.

INTRODUCTION

'Pz-peptidase' is an enzymic activity of mammalian tissues that cleaves a synthetic substrate, Pz-Pro-Leu-Gly-Pro-D-Arg, which was designed for the collagenase of *Clostridium histolyticum* (Morales & Woessner, 1977; Barrett *et al.*, 1989); it is described as 'tissue-endopeptidase degrading collagenase-synthetic-substrate' (EC 3.4.99.31) in the recommendations of the Nomenclature Committee of I.U.B. (1984). The enzyme is more conveniently assayed with a quenched fluorescence substrate, Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys (Barrett *et al.*, 1989), and using this assay, we have purified Pz-peptidase from rabbit skeletal muscle (Tisljar & Barrett, 1989).

Recently, we have used a similar procedure to isolate Pz-peptidase from rat skeletal muscle. In the course of characterization of the enzyme, we encountered a number of resemblances to an enzyme that has previously been reported from other rat tissues, and named endopeptidase 24.15. Endopeptidase 24.15 is found mainly in the soluble fraction of homogenates of various tissues, although a minor component is membrane-associated. It cleaves a number of biologically active peptides, including bradykinin, neurotensin and substance P, and generates enkephalins from their precursors (Chu & Orlowski, 1985; Acker *et al.*, 1987). In the assay for endopeptidase 24.15, cleavage of a synthetic substrate such as Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate yields Ala-Ala-Phe-*p*-aminobenzoate, from which free *p*-aminobenzoate is released during a second incubation with alanyl aminopeptidase, and finally quantified in a colorimetric procedure (Orlowski *et al.*, 1983). No continuous assay has been described for the enzyme. A series of highly selective inhibitors is available for endopeptidase 24.15, of which *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate is a good example (Chu & Orlowski, 1984; Orlowski *et al.*, 1988).

We now report the results of comparisons of Pz-peptidase with endopeptidase 24.15, and conclude that both activities are due to a single enzyme.

EXPERIMENTAL

Materials

Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys (Barrett *et al.*, 1989) was provided by Dr. C. G. Knight of this Laboratory. Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoic acid, *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoic acid and *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoic acid and rat testis endopeptidase 24.15 (Orlowski *et al.*, 1989) were provided by Dr. M. Orlowski, Mount Sinai School of Medicine, New York, NY, U.S.A. Microsomal alanyl aminopeptidase was purchased from Boehringer Mannheim (catalogue no. 102768) and further purified as described by Almenoff & Orlowski (1983).

Enzyme assays

Continuous assays for Pz-peptidase activity were made with the quenched fluorescence substrate Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys as described by Barrett *et al.* (1989). One unit of activity was defined as hydrolysing 1 μ mol of substrate/min, at 40 °C, or 23 °C on one occasion, as stated in the text. The hydrolysis of Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate and Boc-Phe-Ala-Ala-Phe-*p*-aminobenzoate was assayed as described by Orlowski *et al.* (1983), but with the addition of 5 μ M-phosphoramidon to eliminate interference by endopeptidase 24.11, as described in the text. With these substrates, 1 unit of activity was defined as hydrolysing 1 μ mol of substrate/h at 37 °C. K_m and K_i values were determined as described by Barrett *et al.* (1989) and Nicklin & Barrett (1984) respectively. Assays with Pz-Pro-Leu-Gly-Pro-D-Arg were made as described by Tisljar & Barrett (1989).

Abbreviations and symbols used: Dnp-, *N*²-2,4-dinitrophenyl-; Bz-, *N*²-benzoyl-; Pz-, *N*²-4-phenylazobenzoyloxycarbonyl-; Boc-, *N*²-*t*-butyloxycarbonyl; †, symbol used to show the point at which a peptide substrate is cleaved.

Table 1. Co-purification of Pz-peptidase and endopeptidase 24.15 activities from rat skeletal muscle

The samples were taken from a purification procedure in which the phenyl-Sepharose step was included. The substrates used were Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys for Pz-peptidase and Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate for endopeptidase 24.15, as described in the Experimental section, except that phosphoramidon ($5\ \mu\text{M}$) was used in the assays for endopeptidase 24.15 to minimize non-specific hydrolysis of the substrate.

Stage	Pz-peptidase (munits/mg of protein)	Endopeptidase 24.15 (units/mg of protein)	$10^3 \times \text{Ratio}$
CM-cellulose (unbound)	1.86	0.421	4.4
DEAE-cellulose	8.89	1.98	4.5
Phenyl-Sepharose	20.6	4.12	5.0
Sephacryl S-200 HR	31.6	6.22	5.0

Protein determination

The Bio-Rad protein assay was used (Bradford, 1976), with bovine serum albumin as standard.

Enzyme purification

Skeletal muscle and testis were collected from black hooded rats for the partial purification of Pz-peptidase as described for rabbit muscle (Tislar & Barrett, 1989) up to step 4. Briefly, the procedure consisted of homogenization of tissue in 4 parts (w/v) or water, adjustment to pH 5.0, centrifugation at $33000\ g$ for 20 min and treatment of the supernatant with CM-cellulose. The filtrate was adjusted to pH 6.7 with Tris, and stirred with DEAE-cellulose. The ion-exchanger was packed as a column, and a gradient up to 250 mM-NaCl in 50 mM-Bistris/HCl buffer, pH 6.7, was run. As stated in the text, an additional column of phenyl-Sepharose was sometimes used at this stage, the enzyme being adsorbed from $0.8\ \text{M}-(\text{NH}_4)_2\text{SO}_4$, and eluted with a gradient from 0.8 to $0\ \text{M}-(\text{NH}_4)_2\text{SO}_4$. The final step was gel chromatography on Sephacryl S-200 HR.

Analytical gel chromatography

Partially purified Pz-peptidase was run on an LKB Ultropac TSK-G 2000 SW column in 50 mM-Tris/HCl buffer, pH 7.2, containing 200 mM-NaCl and 0.05% Brij 35 at 0.25 ml/min. Fractions (0.25 ml) were collected and assayed for activity against Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys and Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate.

RESULTS

Co-purification of Pz-peptidase and endopeptidase 24.15

The two activities were determined at four stages during the partial purification of Pz-peptidase from rat muscle, with the results shown in Table 1. The ratio of specific activities remained close to 4.7:1 during the 17-fold purification from the CM-cellulose-unbound material.

Gel chromatography

The partially purified rat muscle Pz-peptidase was run on the high-resolution TSK-G 2000 column, as described in the Experimental section. The elution profiles of Pz-peptidase and endopeptidase 24.15 activity were superimposable (Fig. 1). The peak of both activities emerged at an elution volume close to that of serum albumin, indicating an M_r greater than that of 56000

Fig. 1. Activities of Pz-peptidase (○) and endopeptidase 24.15 (●) in effluent fractions from the analytical chromatography column

See the text for details.

previously reported for Pz-peptidase from rat granuloma tissue by Aswanikumar & Radhakrishnan (1972). The M_r of rat endopeptidase 24.15 has been reported as 67000 (Orlowski *et al.*, 1983).

Action of partially purified Pz-peptidase on substrates of endopeptidase 24.15

Partially purified rat muscle Pz-peptidase hydrolysed the two substrates of endopeptidase 24.15 tested. K_m values were 0.53 mM for Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate and 0.19 mM for Boc-Phe-Ala-Ala-Phe-*p*-aminobenzoate. These values agree well with those of 0.49 mM and 0.22 mM respectively reported by Orlowski *et al.* (1983) for rat brain endopeptidase 24.15. We obtained similar values with the enzyme partially purified from rat testis.

Action of highly purified endopeptidase 24.15 on Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys

Endopeptidase 24.15 highly purified from rat testis (Orlowski *et al.*, 1989) hydrolysed both Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys and Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate. In this experiment the fluorimetric assay was made at 23 °C for practical reasons. The ratio between the two activities was $3.5 \times 10^{-3}:1$. The lower temperature of the fluorimetric assay can adequately account for the difference between this value and those close to $4.7 \times 10^{-3}:1$ in Table 1.

Inhibition of Pz-peptidase by specific inhibitors and a substrate of endopeptidase 24.15

Pz-peptidase partially purified from rat testis was assayed for activity on Pz-Pro-Leu-Gly-Pro-D-Arg alone and in the presence of $0.5 \mu\text{M}$ -*N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate or 0.5 mM -Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate. The inhibitor of endopeptidase 24.15 gave 95% inhibition of the hydrolysis of the Pz-peptide, whereas the competing substrate of that enzyme completely suppressed activity on the Pz-peptide.

The activity of the partially purified enzyme on Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys was inhibited by *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate and *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate, specific inhibitors of endopeptidase 24.15 (Orlowski *et al.*, 1988). The K_i values were 16.6 nM and 9.9 nM respectively. Orlowski *et al.* (1988) reported values of 27 nM and 16 nM for rat brain endopeptidase 24.15 with these inhibitors. We consider the results consistent with a single enzyme being responsible for both activities.

DISCUSSION

We have found that the enzymic activities hydrolysing Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys and Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate co-purify during the partial purification of Pz-peptidase from rat skeletal muscle, are co-eluted in high-resolution gel chromatography and co-exist in a homogeneous preparation of rat testis endopeptidase 24.15. The action of Pz-peptidase partially purified from rat testis on Pz-Pro-Leu-Gly-Pro-D-Arg was blocked by an inhibitor of endopeptidase 24.15, and also by a substrate of this enzyme. The partially purified enzyme hydrolysed two substrates of endopeptidase 24.15 with K_m values similar to those published previously, and its action on Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys was inhibited by compounds that are considered specific for endopeptidase 24.15.

The obvious conclusion from the above findings, namely that a single enzyme acts on both substrates, is consistent with much of the literature on Pz-peptidase and endopeptidase 24.15. Endopeptidase 24.15 shows a preference for hydrolysis of substrates with hydrophobic residues in positions P_3' and P_1 or P_2 (in the nomenclature of Berger & Schechter, 1970), and it is notable that the best synthetic substrate described by Chu & Orlowski (1985), Bz-Gly-Phe-Ala-Pro-Phe-*p*-aminobenzoate, con-

tains residues in positions P_1 to P_3' that are of similar character to those in Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys. The M_r of endopeptidase 24.15 is reported to be 67000 (Orlowski *et al.*, 1983), and that of rabbit Pz-peptidase about 74000 (Tisljar & Barrett, 1989).

An unusual property common to both Pz-peptidase and endopeptidase 24.15 is that of activation by dithiothreitol at low concentrations and inhibition at higher concentrations (Rajabi & Woessner, 1984; Orlowski *et al.*, 1983). Both activities are also inhibited by 1,10-phenanthroline and EDTA, and re-activated by metal ions after the latter (Lukac & Koren, 1977; Chu & Orlowski, 1983).

A large literature exists on Pz-peptidase, with most papers emphasizing biological correlations of activity with turnover of connective-tissue components. In contrast, the much smaller literature on endopeptidase 24.15 deals primarily with the potential of the enzyme to degrade bioactive peptides and to generate enkephalins. The merging of the two literatures with the recognition that both activities are attributable to a single enzyme may well lead to new insights into the biological functions of the enzyme. For example, the indication that the enzyme repeatedly found to be present in high activity in association with connective-tissue matrix turnover is one that degrades some bioactive peptides and generates others may be of interest. Work on the enzyme is likely to be facilitated by the availability of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys as a substrate, permitting much more powerful assays than have hitherto been possible. We suggest that the final decision on the most appropriate name for the enzyme should await further characterization, including direct determination of the metal content.

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