A neutral endopeptidase in the microvillar membrane of pig intestine

Partial purification and properties

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An enzyme hydrolysing [¹²⁵I]iodo-insulin B chain was enriched in preparations of intestinal microvilli. The activity could be solubilized by Triton X-100 and was partially (76-fold) purified. It was very sensitive to inhibition by phosphoramidon and was also inhibited by chelating agents. In its enzymic, molecular and immunological properties the intestinal enzyme closely resembled kidney microvillar neutral endopeptidase (kidney-brush-border neutral proteinase, EC 3.4.24.11).

Previous work in this laboratory (Kerr & Kenny, 1974*a,b*) had purified and characterized an enzyme from rabbit kidneys, referred to as neutral endopeptidase and now listed as kidney-brush-border neutral proteinase (EC 3.4.24.11). It was enriched in kidney-brush-border and kidney-microvillar fractions to the same extent as other marker enzymes (George & Kenny, 1973; Booth & Kenny, 1974). The enzyme is a metalloproteinase, containing one atom of zinc/molecule (93 000 mol.wt.), and, in cleaving peptide bonds involving the amino group of hydrophobic residues, possesses a specificity similar to that of the microbial metalloproteinases such as thermolysin. Phosphoramidon, a specific inhibitor of thermolysin and others of this class of proteinase (Suda *et al.*, 1973) is also a potent inhibitor of the kidney enzyme (Kenny, 1977). This inhibitor has proved to be a valuable experimental tool in defining the activity of the enzyme in crude preparations of tissues, such as intestine, that may contain endopeptidases. So far, the kidney enzyme is the only mammalian example of an endopeptidase of this class. An enzyme with essentially identical properties has been studied in rat kidneys (Varandani & Shroyer, 1977) and is also present in kidneys from pig and man (J. P. Vyas, M. Abbs & A. J. Kenny, unpublished work).

Although microvilli from kidney and intestine have many enzymes in common, some seem to be unique to one or other cell type (for review, see Kenny & Booth, 1978). Among the peptidases in the intestinal brush border, only one endopeptidase is

known, namely the serine enzyme, enteropeptidase (EC 3.4.21.9). In the present paper we provide evidence that a second endopeptidase is present in the pig intestinal microvillar membrane and that this enzyme has similar properties to that found in kidney.

Experimental

Enzyme purification

Pig intestines were obtained from the slaughterhouse and mucosal scrapings were collected and stored at -70°C . A microvillus fraction was prepared from 150 g of mucosa by the Ca^{2+} -precipitation method (Sjöström *et al.*, 1978). The microvillus fraction was suspended (approx. 2 mg of protein/ml) in 0.1 M-Hepes/NaOH, pH 6.8, and incubated at 37°C for 1 h with papain (100 $\mu\text{g}/\text{mg}$ of microvillar protein), activated as described by Booth *et al.* (1979). Iodoacetamide (1 mg/ml) was added and, after centrifugation at 30 000 *g* for 1 h, the pellet was resuspended in 100 ml of 10 mM-Tris/HCl, pH 7.5, and re-centrifuged. The washed pellet was resuspended in 200 ml of 10 mM-Tris/HCl, pH 7.5, and Triton X-100 was added to a concentration of 1% (w/v). After being left at room temperature for 1 h, the suspension was re-centrifuged as above. The supernatant fraction (volume 200 ml) was concentrated 10-fold in a dialysis bag with solid poly(ethylene glycol) and loaded on to a column (50 mm $\times$ 800 mm) of Sepharose 6B and eluted with 10 mM-Tris/HCl (pH 7.5)/0.1% (w/v) Triton X-100. The active fractions were passed through an immunoabsorbent column containing the IgG fraction of a serum raised to kidney microvillar membrane (Danielsen *et al.*, 1980) in order to

Abbreviations used: IgG, immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

remove traces of contaminating enzymes. The preparation was stored, without significant loss of activity, at -20°C .

Trypsin treatment

The preparation (1 mg of protein/ml) was incubated with trypsin (0.1 mg/ml) at 37°C for 1 h.

Enzyme assays

Neutral endopeptidase was assayed with [^{125}I]-iodo-insulin B chain as substrate as described by Kerr & Kenny (1974a). The assays for other microvillar peptidases were fluorimetric (Kenny, 1977). The unit is defined as the hydrolysis of $1\mu\text{mol}$ of substrate/min at 37°C .

Determination of molecular weight by gel filtration

A column (25 mm $\times$ 900 mm) of Ultrogel AcA 22 was equilibrated with 10 mM-Tris/HCl/150 mM-NaCl/0.1% (w/v) Triton X-100. Detergent was omitted when the trypsin-treated samples were run. The presence or absence of detergent did not influence the elution volume of the calibration proteins. The latter were (molecular weights in parentheses): urease (483 000); ferritin (480 000); aminopeptidase M (detergent form, 400 000); dipeptidyl peptidase IV (266 000); glucose oxidase (150 000); liver alcohol dehydrogenase (80 000); and bovine serum albumin (68 000). Blue Dextran was used to determine the exclusion volume. The line relating V_e/V_0 to $\log$ (molecular weight) was based on 23 values for the calibration proteins. The detergent and trypsin forms of the endopeptidase were each run in three experiments, and the molecular weights computed by a linear regression using a programmable calculator.

Other methods

Protein determination, preparation of IgG fractions and other methods, as well as the sources of chemicals and tissues, were those previously described (Booth *et al.*, 1979; Danielsen *et al.*, 1980).

Results and discussion

Localization in the microvillar fraction

In the three preparations shown in Table 1 the enrichment of the phosphoramidon-sensitive activity was 9.0-fold (range 8.7–9.3), whereas that for aminopeptidase M [aminopeptidase (microsomal); EC 3.4.11.2] was 10.6-fold. In three other experiments, performed on a smaller scale (20 g of pig mucosa), the enrichments (mean $\pm$ S.E.M.) of four enzymes in the microvillar fraction were: phosphoramidon-sensitive neutral endopeptidase, 13.5 ± 4.4 ; aminopeptidase M, 14.8 ± 2.6 ; dipeptidyl peptidase IV (EC 3.4.14.-), 12.5 ± 1.3 ; alkaline phosphatase (EC 3.1.3.1), 10.5 ± 1.4 . In the intestine, as in the kidney, the endopeptidase appears to be localized in the microvillar membrane. The phosphoramidon-insensitive component was mainly cytosolic and was not inhibited by 1 mM-di-isopropyl phosphorofluoridate, soya-bean trypsin inhibitor (100 $\mu\text{g}/\text{ml}$), or Trasylol [100 kallikrein-inhibitory units (k.i.u.)/ml] and is therefore unlikely to include pancreatic endopeptidases.

Partial purification of the endopeptidase

The kidney enzyme is resistant to release from the microvillar membrane by papain. Indeed one of the problems in its purification has been that of solubilization. In the present work we treated a crude microvillar fraction with papain in order to release substantial amounts of other peptidases (Kenny, 1977) and then solubilized the endopeptidase-enriched membrane with Triton X-100. Gel filtration on Sepharose 6B yielded a partially pure preparation enriched 76-fold. Passage through an immunoabsorbent column removed minor contamination by other microvillar peptidases without measurably affecting the specific activity. The results based on the preparation of three batches are shown in Table 1. The starting homogenate contained other insulin B chain-hydrolysing activity that could be distinguished by its insensitivity to phos-

Table 1. Purification of the endopeptidase from pig intestinal mucosa

Values are means for three preparations, each using 150 g of mucosal scrapings. See the Experimental section for details.

Fraction	Protein (mg)	Endopeptidase recovered (%)	Specific activity (munits/mg of protein)	Purification factor	Inhibition by $1\mu\text{M}$ -phosphoramidon (%)
Homogenate	7030	100	0.32	1	55
Microvilli	378	29	1.73	5.5	91
Triton X-100 extract	97	26	5.89	18.6	89
Sepharose 6B and immunoabsorbent column	11.4	12	28.5	75.8	98.4

phoramidon. The proportion of this contaminating activity fell from 45 to less than 2% in the product. The preparation was free of aminopeptidase M and dipeptidylpeptidase IV activities and contained less than 0.03% contamination with aminopeptidase A and γ -glutamyltransferase. These peptidases cannot initiate attack on the assay substrate, but they (particularly aminopeptidase M) may exaggerate the apparent endopeptidase activity by further degrading peptide fragments (Kerr & Kenny, 1974a). Thus, although the preparation stopped short of full purification, we conclude that the activity studied can be attributed to a phosphoramidon-sensitive endopeptidase that, as is shown below, possessed properties similar to those reported for the rabbit kidney enzyme.

Catalytic properties of the endopeptidase

pH optimum. The pH-activity curve was determined at the usual substrate concentration (6 μ M) with 0.2 M-sodium acetate/acetic acid buffers (pH 4.0–6.5) and 0.2 M-Tris/HCl buffer (pH 7–10), and was symmetrical, with a sharp maximum at pH 7 and about 25% activity at pH 5.5 and 8.5.

Metal ions. Ca^{2+} and Mg^{2+} (over a concentration range 0.01–10 mM) had no effect. Zn^{2+} and Ni^{2+} were inhibitory (I_{50} values, approx. 1 mM). In each case the enzyme was preincubated with the effector for 1 h at 37°C.

Chelating agents. Four reagents, each known to inhibit the rabbit kidney microvillar endopeptidase, also inhibited the intestinal enzyme (Fig. 1). EDTA was the strongest inhibitor (I_{50} 30 μ M); 1,10-phenanthroline was a weaker inhibitor (I_{50} 0.6 mM). The I_{50} values for dithiothreitol and 2-mercaptoethanol were 2 and 30 mM respectively.

Fig. 1. Effect of phosphoramidon, chelators and thiols on the endopeptidase

The enzyme preparation (0.5 munits/ml) was preincubated for 1 h at 37°C with each effector at the concentrations shown. In the experiments with EDTA and 1,10-phenanthroline the preparation had been exhaustively dialysed against water. Effectors: ●, Phosphoramidon; ○, EDTA; ■, 1,10-phenanthroline; □, dithiothreitol; ▲, 2-mercaptoethanol.

Phosphoramidon. Fig. 1 also shows the very high sensitivity of the endopeptidase to inhibition by phosphoramidon (I_{50} 10 nM). The response is comparable with that of the kidney enzyme (K_i = 2 nM) (Kenny, 1977).

Other inhibitors. No effect was seen with 1 mM-di-isopropyl phosphorofluoridate (preincubated 1 h), Trasylol (100 k.i.u./ml) or soya-bean trypsin inhibitor (100 μ g/ml).

Molecular properties of the endopeptidase

All the previous estimates of molecular weight of the rabbit kidney endopeptidase had been made on a form released from the membrane by trypsin after exposure to toluene (Kerr & Kenny, 1974a). Values obtained by gel filtration, sedimentation equilibrium and polyacrylamide gel electrophoresis under reducing conditions in the presence of SDS were in the range 87 000–96 000. Varandani & Shroyer (1977) estimated the rat kidney enzyme, solubilized in the same way, to have a mol.wt. of 210 000, a result that may possibly indicate a dimer in this species. Although trypsin successfully solubilized the enzyme after toluene treatment, it was no more successful than papain in releasing the endopeptidase when incubated with kidney microvilli. However, in the present work we observed that trypsin converted the detergent-solubilized enzyme from pig intestine into a form with distinctly different molecular properties (without any effect on enzymic activity). In repeated experiments with a carefully calibrated column of Ultragel AcA 22 the detergent forms of the enzyme were eluted in a volume consistent with a mol.wt. of 310 000 (mean of three experiments). On treatment with trypsin and on chromatography in the absence of detergent, two peaks of activity were observed. One was consistent with an aggregated form (mol.wt. approx. 800 000) resulting from imperfect conversion into the proteinase form. The other was presumed to be the true proteinase form, which on rechromatography was eluted in the same volume. The mol.wt. was 90 700 (mean of three experiments). The simplest, though somewhat speculative, interpretation of these results is that the detergent form is dimeric, the molecule comprising a micelle of detergent (approx. mol.wt. 100 000; Helenius & Simons, 1975) and two identical subunits, each larger than the proteinase form by the presence of an additional length of polypeptide including the anchoring region. If this is so, an unusual feature is the apparently ready dissociation of the dimer on trypsin treatment. Other microvillar peptidases retain their dimeric structure in the proteinase forms (see, e.g., Kenny & Booth, 1978).

Immunological properties of the endopeptidase

Several microvillar-membrane enzymes present in kidney and intestine are known to be immuno-

Fig. 2. Immunoprecipitation of the intestinal and renal microvillar endopeptidases by antiserum to kidney microvillar membrane

Samples (volume 1 ml) of the intestinal and kidney endopeptidases (each 1.2 munits/ml) were incubated at 20°C for 20 h with various amounts of an IgG fraction (17 mg/ml) in 50 mM-Tris/HCl (pH 7.5)/0.1% (w/v) Triton X-100. Tubes were gently shaken and a sample (0.1 ml) taken for enzyme assay to determine the extent of inhibition (open symbols). After centrifugation at 15 000 *g* for 15 min a sample of the supernatant fraction was assayed to determine the extent of precipitation (closed symbols). ●, ○, Intestinal endopeptidase. ■, □, Renal endopeptidase. In a control experiment the IgG fraction was absorbed with the renal endopeptidase (100 μl of IgG fraction with 5 munits of enzyme) for 18 h at 20°C. After centrifugation the supernatant fraction was tested as above with the intestinal enzyme (volumes corrected for dilution during absorption). △, Endopeptidase activity in the supernatant fraction.

logically similar, if not identical, e.g. aminopeptidase M (Vannier *et al.*, 1976) and aminopeptidase A (Danielsen *et al.*, 1980). Fig. 2 shows the titration curves with an IgG fraction from a rabbit immunized with microvillar membrane from pig kidney. The responses of the partially pure endopeptidase from intestine and that of a comparable preparation from pig kidney are generally similar. The IgG fraction inhibited the enzymic activity of both and also precipitated the enzymes, although about twice as much IgG was needed to give 50% inhibition or 95% precipitation of the intestinal form compared with the kidney enzyme. A control experiment, in which the IgG fraction had been absorbed by the kidney enzyme, did not exhibit or precipitate the intestinal form.

General conclusions

The intestine contains an enzyme closely resembling the kidney microvillar neutral endopeptidase

(EC 3.4.24.11). The similarities include (a) localization in the microvillar fraction. (b) sensitivity to chelating agents and, more importantly, to phosphoramidon, (c) molecular weight of the proteinase-released form and (d) immunoprecipitation by a serum raised to pig kidney microvilli.

The physiological role of the endopeptidase in kidney microvilli is unclear, though it may play a part in initiating the degradation of some peptide hormones. The specific activity of neutral endopeptidase in intestinal microvilli is only 10% of that in the kidney. Possibly it plays a minor role in the final stages of peptide hydrolysis at the membrane surface, but it seems unlikely that its presence would be essential to normal digestion and absorption.

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