Endopeptidase-24.11 purified from pig intestine is differently glycosylated from that in kidney

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Endopeptidase-24.11 (EC 3.4.24.11) was purified from pig intestinal microvilli by immunoabsorbent chromatography, using antibodies raised to kidney endopeptidase-24.11. In many respects, the kidney and intestinal enzymes were indistinguishable, but some structural differences were demonstrated. In particular, the detergent form of the intestinal enzyme had an apparent subunit M_r of 95 000, which, on treatment with trypsin, fell to a value of 89 000, identical with that of the kidney form. The intestinal enzyme contained 3–4% more carbohydrate and many more fucose residues than that from kidney. Although these results show that post-translational processing was different in the two cell types, the possibility that the primary translation products also differed cannot be excluded.

We have recently described the molecular properties of the detergent form of endopeptidase-24.11 (EC 3.4.24.11) purified from pig kidneys (Fulcher & Kenny, 1983; Kenny *et al.*, 1983). This name, using the last two numbers of the Enzyme Commission classification, has been proposed (Matsas *et al.*, 1983) in order to avoid confusion when referring to an enzyme that is now known to be widely distributed in cell membranes. Although it was first recognized as a renal brush-border enzyme (George & Kenny, 1973), it is also present in the intestinal brush border (Danielsen *et al.*, 1980) and membranes of several other tissues and organs (Kenny & Fulcher, 1983; Matsas *et al.*, 1983). The intestinal form was shown to have similar properties to that in kidney, but was not obtained in a pure form (Danielsen *et al.*, 1980). We have now made use of an immunoabsorbent column, developed from the renal enzyme, to prepare samples of the intestinal endopeptidase. In comparing the two forms, some unexpected structural differences have emerged. The detergent forms from kidney and intestine differ in molecular size and in glycosylation. It is possible that post-translational processing wholly accounts for the structural differences observed, but we cannot at this stage exclude the possibility that the primary translation products are not identical.

Abbreviations used: d-form, the detergent (Triton X-100)-solubilized form of endopeptidase-24.11; dt-form, the d-form after trypsin treatment; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

Experimental

The sources of the materials and details of methods are those previously described by Fulcher & Kenny (1983), with the following additions.

Preparation of intestinal microvilli

Pig intestines were obtained from the slaughterhouse and the lumen washed through with 0.1 mM-di-isopropyl fluorophosphate in 0.9% NaCl. The mucosa was scraped off with a microscope slide; about 400 g (wet wt.) was obtained for each preparation. Microvilli were prepared (Kessler *et al.*, 1978) and resuspended in 250 ml of 5 mM-Tris/HCl, pH 7.4.

Purification of endopeptidase-24.11 from intestine

Microvilli (as above) were solubilized by detergent. Triton X-100 was added to achieve a ratio to protein (w/w) of 10:1 and stirred for 1 h at 20°C. The supernatant after centrifugation for 2 h at 31 000 g was loaded on a column (25 ml volume) of DEAE-cellulose, equilibrated and washed with the same buffer containing 1% (w/v) Triton X-100. A gradient, 0–250 mM-NaCl (500 ml total), in the same buffer with 0.1% Triton X-100 was applied and active fractions were pooled and subsequently purified by immunoabsorbent chromatography (Fulcher & Kenny, 1983).

Treatment of samples of enzyme for carbohydrate and amino acid analysis

The d-form (200 µg) was precipitated by the addition of an equal volume of 25% (w/v) trichloroacetic acid and the pellet was analysed without further washing. The preparation of the dt-form in these experiments was modified so that any fragments produced by trypsin were removed before carbohydrate analysis. The d-form (200 µg) was incubated with 20 µg of trypsin at 37°C for 2 h in 50 mM-Tris/HCl, pH 8.0, also containing 10 µM-phosphoramidon. A further 20 µg of trypsin was then added and the incubation continued for 18 h at 4°C. Di-isopropyl fluorophosphate was added (to 0.1 mM) and the sample (0.5 ml volume) loaded on to a column (300 mm × 15 mm) of Ultragel AcA 44, equilibrated with 10 mM-Tris/HCl/100 mM-NaCl/0.1% Triton X-100, pH 7.4. Endopeptidase-24.11 was recovered in the void volume; trypsin and other components were retarded. The voided fractions were pooled, concentrated by a small (0.1 ml) column of DEAE-cellulose and batch-eluted with 500 mM-NaCl in 10 mM-Tris/HCl/0.1% Triton X-100, pH 7.4. The protein was then precipitated with trichloroacetic acid as previously.

Isolation of trypsin-cleaved fragments for carbohydrate analysis

The d-form of the intestinal enzyme was treated with trypsin as above, but instead of subjecting the sample (0.5 ml) to gel filtration it was allowed to bind for 18 h at 4°C to 50 µl of an immuno-adsorbent prepared by covalently coupling a monoclonal antibody, GK7C2, to Sepharose 4B (Gee *et al.*, 1983). This antibody is specific for endopeptidase in kidney and other tissues. After centrifugation, the supernatant was shown to be free of endopeptidase-24.11 activity. Acetone at -20°C was added (20 vol.) and after 1 h at -20°C the precipitate was collected for analysis.

Analysis of carbohydrate

Carbohydrate was analysed after methanolysis using a micromethod developed for glycoproteins (Chaplin, 1982).

Silver stain

Slab gels were stained by a simplified method described by Ansorge (1980).

Results

Purification of endopeptidase-24.11 from pig intestinal mucosa

Intestinal microvilli contain much less endopeptidase-24.11 than those of kidney; the specific activities at this stage of purification differed by a factor of ten. The purification of one batch of the intestinal enzyme is shown in Table 1. The mean specific activity (154 m-units/mg of protein) of three purified preparations was comparable, though slightly less than that (177 m-units/mg of protein) reported for the kidney enzyme (Fulcher & Kenny, 1983). In calculating both values, the protein had been accurately determined by amino acid analysis. The lower content of endopeptidase in intestine is reflected in the higher enrichment value for this purification. We estimate that endopeptidase-24.11 constitutes 0.4% of the intestinal microvillar protein and 0.76% of the Triton X-100-solubilized proteins. The conversion by trypsin of the d-form into the dt-form had no significant effect on specific activity (162 m-units/mg of protein; mean of three preparations).

The purity of each batch of intestinal endopeptidase-24.11 was checked by SDS/polyacrylamide-gel electrophoresis (e.g. Fig. 1) and cross-immunoelectrophoresis against antisera raised to both kidney and intestinal microvilli and was shown to be homogeneous by both criteria.

The binding by octyl- and phenyl-Sepharoses of the d- and dt-forms was comparable with that observed for the kidney enzyme (Table 2). Both forms were therefore amphipathic proteins by the criterion of hydrophobic chromatography.

Immunological comparison

The use of a polyclonal antibody, raised in a rabbit to the renal enzyme, to purify the intestinal form by immuno-adsorbent chromatography is evidence of some degree of immunological similarity,

Table 1. *Purification of endopeptidase-24.11 from pig intestinal mucosa*

The data from one purification are shown except for the final specific activity and recovery, which are mean values. The capacity of the last step (immuno-adsorbent column) was about 130 m-units. The recovery (4.2%) required the processing of four batches from the previous step. Values marked * are means ± S.E.M. for three preparations.

Fraction	Protein (mg)	Total specific activity		Recovery (%)	Purification factor (fold)	Inhibition by phosphoramidon (%)
		(m-units)	(m-units/mg of protein)			
Homogenate of mucosal scrapings	17 400	2110	0.121	100	1	5
Microvilli	912	567	0.622	27	5.1	92
Triton X-100-solubilized fraction	472	557	1.18	26	9.7	92
DEAE-cellulose chromatography	79.6	489	6.14	23	51	98
Immuno-adsorbent chromatography	0.132	20.4	154.5 ± 4.2*	4.2 ± 0.1*	1270	100

though it does not necessarily imply that the two forms have all determinants in common. Another immunoadsorbent column prepared from the monoclonal antibody, GK7C2 (Gee *et al.*, 1983), was equally efficient in purifying the enzyme from either organ (results not shown). GK7C2 was also very effective, by immunofluorescence microscopy, in demonstrating the enzyme in intestinal brush borders (Gee *et al.*, 1983). Thus the epitope recognized by GK7C2 is a common determinant. Ouchterlony diffusion against the polyclonal serum (Fig. 1) showed precipitates consistent with immunological identity. A more discriminating test of immunological identity is shown in Fig. 2. Here, the d- and dt-forms of kidney and intestinal endopeptidase have been titrated by the polyclonal antiserum raised

to the kidney enzyme. This serum contained antibodies that are partially inhibitory as well as causing precipitation. Both effects are shown in Fig. 2. The inhibition curves of the d-forms (Fig. 2a) are different for kidney and intestinal endopeptidases, the former being slightly more sensitive. The precipitation curves also show a small but consistent difference: again the kidney enzyme is very slightly more sensitive. When the dt-forms were compared (Fig. 2b), these differences were less apparent. Some difference in inhibition remains, but the precipitation curves are now indistinguishable.

Amino acid analyses

Three preparations (all d-forms) purified from both kidney and intestine were hydrolysed and subjected to amino acid analysis. The results are shown in Table 3. No significant differences in amino acid composition are apparent.

Molecular weight of the d- and dt-forms of the endopeptidase from intestine

We have reported that trypsin treatment of the detergent form altered the properties of the kidney enzyme without producing a perceptible change in the apparent subunit M_r (89 000) (Fulcher & Kenny, 1983). The only observed change in properties was the loss of any tendency for the dt-form to aggregate, the d-form invariably containing a small proportion of molecules that appeared in the void volume on gel filtration with Ultragel AcA34. The Stokes radii of the predominant species were the same before and after trypsin treatment (M_r values

Table 2. *Hydrophobic chromatography of d- and dt-forms of endopeptidase-24.11 purified from intestine*

See Fulcher & Kenny (1983) for experimental details. The results are expressed as the percentage of the applied activity (1.8 m-units) that was not bound or eluted by 2.5% (w/v) Triton X-100 in a buffer of low ionic strength.

Column	Enzyme form	Endopeptidase-24.11 activity		
		Unbound (%)	Eluted (%)	Total recovered (%)
Octyl-Sepharose	d-form	16.9	84.9	101.8
	dt-form	27.3	71.6	98.9
Phenyl-Sepharose	d-form	24.1	76.3	100.4
	dt-form	36.0	64.1	100.1

Fig. 1. *Ouchterlony immunodiffusion of renal and intestinal endopeptidase-24.11 against polyclonal antibody to the kidney enzyme*

Antibody (Ab) in the centre wells was an IgG fraction (50 μ g). Endopeptidase-24.11 from kidney (k) or intestine (i) was loaded (5 μ g of d-forms) in alternate wells. (a), Coomassie Blue stain; (b), histochemical stain using glutaryl-Gly-Gly-Phe-2-naphthylamide as substrate (Fulcher & Kenny, 1983).

Fig. 2. Immunotitration of endopeptidase-24.11 from kidney and intestine

Endopeptidase-24.11 (2 μ g) from each source was incubated (2 h at 37°C, 18 h at 4°C) with a Protein A-Sepharose-purified IgG fraction of rabbit antiserum raised to the kidney enzyme. Each mixture (volume 0.5 ml) was first resuspended and a sample removed for assay to determine inhibition by the antiserum (filled symbols). The tubes were then centrifuged at 18000 g for 15 min and the supernatant fraction assayed for enzyme activity to demonstrate precipitation. Results are expressed as activity relative to the control tubes from which IgG had been omitted. Symbols: ● and ○, kidney enzyme; ■ and □, intestinal enzyme; ● and ■, inhibition; ○ and □, precipitation. The broken line shows a control experiment using comparable amounts of IgG prepared from pre-immune serum. In (a), the d-forms of the enzymes are compared; in (b), the dt-forms are shown.

321000 and 318000) and both forms contained similar amounts of bound Triton X-100.

The effect of trypsin on the d-form from intestine was to lower the molecular weight. The apparent subunit M_r determined by SDS/polyacrylamide-gel electrophoresis of the intestinal d-form was 95000. The dt-form was smaller (apparent M_r 89000), indistinguishable in mobility from the d- and dt-forms of the kidney enzyme. Fig. 3 shows that the d-form and dt-form are clearly resolvable when

Table 3. Amino acid analyses of endopeptidase-24.11 purified from kidney and intestine

Three different preparations (d-form) of each enzyme (25 μ g) were hydrolysed with 6M-HCl/0.3% (w/v) phenol for 36 h at 110°C. The amino acid analyses are means $\pm$ S.E.M. expressed as mol %. Methionine, tryptophan and cysteine were not determined.

Amino acid	Endopeptidase-24.11 ...	Amino acid content (mol %)	
		Kidney	Intestine
Aspartic acid		10.2 $\pm$ 0.7	10.1 $\pm$ 1.0
Glutamic acid		12.4 $\pm$ 1.2	10.8 $\pm$ 1.0
Histidine		3.7 $\pm$ 0.8	3.7 $\pm$ 0.4
Lysine		6.7 $\pm$ 0.5	4.9 $\pm$ 0.1
Arginine		5.5 $\pm$ 0.4	5.3 $\pm$ 0.3
Threonine		5.4 $\pm$ 1.1	5.5 $\pm$ 1.0
Serine		7.0 $\pm$ 1.2	8.2 $\pm$ 0.2
Glycine		14.6 $\pm$ 0.8	11.7 $\pm$ 2.0
Tyrosine		3.3 $\pm$ 0.6	3.9 $\pm$ 0.1
Alanine		7.8 $\pm$ 0.5	7.2 $\pm$ 0.1
Valine		6.1 $\pm$ 0.6	6.7 $\pm$ 0.1
Isoleucine		5.2 $\pm$ 0.2	4.7 $\pm$ 0.1
Leucine		8.9 $\pm$ 0.5	9.0 $\pm$ 0.3
Phenylalanine		2.9 $\pm$ 1.0	4.5 $\pm$ 0.2
Proline		3.6 $\pm$ 0.1	3.0 $\pm$ 0.2

electrophoresed in the same track. Gel filtration by a column of Ultragel AcA34 also demonstrated a clear-cut difference in molecular weight. The peak fraction of the d-form from intestine eluted in a volume corresponding to M_r 339000 and 342000 (two samples). The dt-form was slightly retarded, the peak indicating an M_r of 325000 and 326000 (two samples). These values for the dt-form agree well with that (310000) we reported for the partially purified enzyme chromatographed on Ultragel AcA22 (Danielsen *et al.*, 1980). In these previous experiments, the intestinal microvilli had been treated with papain before solubilization with detergent, and this form might be considered analogous to the dt-form. The observation that trypsin treatment generated a hydrophilic monomeric form has not been confirmed in the present work.

Thus trypsin caused the loss of a fragment of apparent M_r 6000 from each subunit, a value in agreement with the difference of 13000 Da between the two dimeric forms. A peptide of M_r 6000 might be expected to be seen in the SDS/polyacrylamide gels and such a fragment was sought in 10–28% acrylamide gradient gels and in 10% acrylamide gels containing 8M-urea. Loss of small peptides was minimized by fixation with formaldehyde. Two staining methods were used: Coomassie Brilliant Blue and the more sensitive silver method. No peptide fragment was visually detected in any experiment. Possible degradation of the fragment by endo-

Fig. 3. SDS/polyacrylamide-gel electrophoresis of d- and dt-forms of endopeptidase-24.11 purified from intestine and kidney

See Fulcher & Kenny (1983) for details of methods. The gels (7–17% acrylamide) were stained with Coomassie Blue. Tracks: (1), microvilli (intestine); (2), d-form (intestine); (3), d-form + dt-form (intestine); (4), dt-form (intestine); (5), microvilli (kidney); (6), d-form (intestine) + d-form (kidney); (7), d-form (kidney); (8), dt-form (intestine); (9), markers (Pharmacia) of M_r ($\times 10^{-3}$) 94, 67, 43, 30, 20 and 14; (10), d-form (kidney); (11), dt-form (kidney). Loadings: microvilli, 100 μ g of protein; enzyme samples, approx. 10 μ g.

Table 4. Carbohydrate analyses of renal and intestinal endopeptidase-24.11

See the experimental section for details of methods. The results are expressed as μ g/mg of protein and are means $\pm$ s.d. Each determination was made in triplicate, the number of samples is shown in parentheses (significant differences: *, $P > 0.01$).

Carbohydrate	Content (μ g/mg of protein)			
	Kidney		Intestine	
	d-form (4)	dt-form (1)	d-form (3)	dt-form (3)
Fucose	$3.26 \pm 0.49^*$	2.39	$22.4 \pm 5.2^*$	$25.9 \pm 4.6^*$
Mannose	37.1 ± 4.4	35.1	42.2 ± 3.0	36.4 ± 4.4
Galactose	45.2 ± 4.0	45.2	40.2 ± 1.2	37.7 ± 4.5
N-Acetylgalactosamine	10.5 ± 3.1	17.7	20.7 ± 8.4	9.15 ± 3.02
N-Acetylglucosamine	46.0 ± 3.7	44.7	52.6 ± 12.9	65.3 ± 4.1
N-Acetylneuraminate	8.13 ± 2.51	8.99	4.89 ± 2.92	2.40 ± 1.15
Glucose	31.6 ± 31.7	12.5	38.2 ± 2.8	24.9 ± 27.6
Total	181.9 ± 29.4	166.6	221.2 ± 2.5	201.8 ± 17.5
Total (excluding glucose)	$150.2 \pm 7.1^*$	154.1	$183.0 \pm 4.8^*$	176.9 ± 11.8

peptidase-24.11 itself was excluded in these experiments by the presence of 1 μ M-phosphoramidon during the incubation. Nevertheless we cannot rule out the possibility that trypsin itself may have further degraded the peptide into very small fragments that migrated at the dye front.

Another attempt was made to isolate the tryptic fragments in order to establish if they contained glycosylated residues. In this experiment, the dt-form was removed from the incubation mixture by

adsorption to GK7C2 monoclonal antibody column. The unbound fraction, which was free of endopeptidase-24.11 activity, contained no significant amount of carbohydrate.

Carbohydrate analyses of endopeptidase-24.11 from kidney and intestine

The analyses are shown in Table 4. The intestinal enzyme contained more carbohydrate than the renal form. For the d-forms, the values were 183 and

150 µg/mg of protein ($P < 0.01$), excluding glucose, which may be partly derived from exogenous sources during preparation of the samples for analysis. The inclusion of the values for glucose actually increased the difference, but obscured the statistical significance. Among the individual components, the most striking result is that for fucose, which is seven times more abundant in the intestinal form. This difference is also statistically significant ($P < 0.01$). The observed differences for *N*-acetylgalactosamine and *N*-acetylneuraminic acid were not shown to be significant. For the intestinal enzyme, these two sugars were depleted when the d-form was converted into the dt-form, but again the differences did not achieve significance and we cannot be sure that trypsin released a glycosylated fragment. Only one sample of the kidney dt-form was analysed. Apart from glucose, which may be discounted, no obvious differences were apparent.

Elution of the endopeptidase from the immuno-adsorbent column involved brief exposure to a buffer of pH 10.6 (Fulcher & Kenny, 1983). The possibility that this treatment caused significant losses of carbohydrate from *O*-linked chains was considered. A batch of the kidney enzyme was prepared by eluting the monoclonal antibody column at pH 8.0 with a buffer of low ionic strength (1 mM-Tris/HCl). Portions of this preparation were then exposed for 18 h at 4°C to either 0.2 M-NaHCO₃/NaOH, pH 10.6, or 0.2 M-Tris/HCl, pH 7.0, both containing 0.1% Triton X-100. Triplicate samples of each were precipitated with trichloroacetic acid and analysed for carbohydrate. The analyses were in close agreement, revealing 0–8% differences for fucose, mannose, galactose and *N*-acetylgalactosamine and an 18% difference for *N*-acetylglucosamine, but in every case the higher value was for the sample treated at pH 10.6. Elution at the higher pH did not therefore change the carbohydrate composition of the protein.

Discussion

The d- and dt-forms of pig kidney endopeptidase-24.11 were not distinguishable in size. The subunit M_r was 89 000 and the higher value for the dimeric form (320 000) was attributable to bound Triton X-100, which contributed 87 000 to this value for the dimer (Fulcher & Kenny, 1983). In contrast, the d-form of the enzyme purified from intestine was larger, with an apparent subunit M_r 95 000, which decreased to 89 000 on treatment with trypsin. There are two questions raised by the present paper. First, how do the d-forms purified from the two sources differ? Secondly, how does trypsin modify the structure of the d-form?

By many criteria, the kidney and intestinal

enzymes were identical. These included catalytic activity, amino acid composition and several immunological properties. The immuno-adsorbent column containing the polyclonal antibody raised to the kidney enzyme was effective in purifying the enzyme from both tissues. The monoclonal antibody, GK7C2, recognized the antigen in histological sections of pig intestine (Gee *et al.*, 1983) and an immuno-adsorbent column prepared with GK7C2 was equally effective in purifying both preparations (A. J. Kenny, I. S. Fulcher & N. S. Gee, unpublished work). Hence the epitope recognized by GK7C2 was common to both. Ouchterlony diffusion against the polyclonal antibody showed complete identity. The only immunological differences were those detected by titration of the enzymes by the polyclonal antibody. The intestinal d-form was slightly less sensitive to inhibition and to precipitation than was that from kidney. This difference was less apparent when the dt-forms were titrated. These experiments suggest minor differences in antigenic determinants between the two forms and raise the possibility that trypsin treatment of the intestinal d-form revealed a common determinant that had been masked in the intestinal d-form. The detailed analyses of carbohydrate of the renal and intestinal enzymes provide some explanation for the size difference. The intestinal enzyme contained 3–4% more carbohydrate and seven times as much fucose as the renal enzyme. Several other sugars, particularly *N*-acetylgalactosamine, were apparently present in higher amounts in the intestinal enzyme, but these differences could not be shown to be significant. However, we cannot be sure that the carbohydrate analyses provide a sufficient explanation of the difference in M_r values. The possibility must remain that the primary translation products are not quite identical in the two cell types.

The effect of trypsin on both d-forms was not satisfactorily resolved. A decrease in some carbohydrate components was noted in the intestinal dt-form, but these were not within significant limits. If we can assume that post-translational changes fully explain the structural differences, the simplest explanation would be that trypsin yielded the same peptide product(s) and that those from the intestine were more heavily glycosylated. The products were sought, but could not be found either as peptide bands on gel electrophoresis or as glycosylated components that could not bind to the GK7C2 monoclonal antibody column. The first failure may be due to trypsin further degrading the peptide into very small fragments. The second might be explained if the epitope for GK7C2 was present on both the fragment and the dt-form, but this point has not yet been explored. Some suggestion that the intestinal dt-form was depleted in carbohydrate was seen in the analyses, both in total and in some individual

sugars, e.g. *N*-acetylgalactosamine, but these differences were not within significant limits.

We have shown in the present paper that there are clear-cut structural differences between endopeptidase-24.11 purified from kidney and intestinal microvilli. These differences were certainly in part, and possibly wholly, explained by post-translational modifications in glycosylation of the protein synthesized by the two cell types. Although several of the major microvillar enzymes are known to be common to the brush border of both organs (for review, see Kenny & Maroux, 1982), this is the first time that the same enzyme from both sources has been compared in such detail. However, other surface membrane glycoproteins that are expressed on different cell types have been reported to exhibit different glycosylation. Differences in carbohydrate composition have been demonstrated for Thy-1 (Barclay *et al.*, 1976) and OX-2 (Barclay & Ward, 1982) antigens purified from rat thymocytes and brain. More recently, Hsieh *et al.* (1983) have shown that the Sinbis virus glycoprotein E1 is glycosylated differently by primary chick embryo fibroblasts, BHK-21 cells and CHO cells. At one glycosylation site (an asparagine residue) in the E1 protein, different oligosaccharides were demonstrated when the virus was grown in three cell cultures. Thus the amino acid sequence is not sufficient to determine the final state of glycosylation of a protein. Although in the case of endopeptidase-24.11 we cannot conclude that the renal and intestinal versions were products of identical genes, equally we do not need to postulate differences in sequence to account for the structural variation we have observed.

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