Deglycosylation by trifluoromethanesulphonic acid of endopeptidase-24.11 purified from pig kidney and intestine

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Endopeptidase-24.11, an integral microvillar membrane enzyme, exists in differently glycosylated forms when purified from pig kidney and intestine [Fulcher, Chaplin & Kenny (1983) *Biochem. J.* **215**, 317–323]. When these glycoproteins, and another form of the kidney enzyme prepared from the Yucatan dwarf strain of piglet, were treated, under controlled conditions, with trifluoromethanesulphonic acid, the proteins were freed of carbohydrate and all had the same apparent subunit M_r (77000) even though the untreated forms varied from M_r 89000 to M_r 95000.

Endopeptidase-24.11 (EC 3.4.24.11) is a glycoprotein that is present in plasma membranes of various cell types, but is especially abundant in the brush borders of kidney and intestine (see, e.g., Kenny & Fulcher, 1983). We have reported that the forms of this enzyme purified from pig kidney and intestine differ in apparent subunit M_r and in the extent and pattern of glycosylation (Fulcher *et al.*, 1983). The enzyme purified from pig brain also exhibits differences of this kind (Relton *et al.*, 1983). Recently, in the course of some studies on the biosynthesis of endopeptidase-24.11 by pulse-labelling with [35 S]methionine, we observed that the labelled polypeptide that was assembled in kidney microvilli had a subunit M_r of 93000, a value closer to that of the intestinal enzyme (95000) than the value (89000) previously found for the kidney enzyme (Stewart & Kenny, 1984). These biosynthesis experiments were performed on slices of renal cortex from piglets of the dwarf Yucatan strain. The data on the purified enzymes were obtained from adult pig tissues of unspecified strain. The reported difference in glycosylation of the purified enzymes seemed an adequate explanation for their structural variation. However, the higher apparent M_r of the immunoprecipitated 35 S-labelled polypeptide was unexpected. Hence we have now investigated the carbohydrate content of the enzyme purified from Yucatan-pig kidneys and have compared the polypeptide chains of both renal forms and the intestinal form after deglycosylation by the reagent trifluoromethanesulphonic acid, previously described by Edge *et al.* (1981). In

the present paper we report that all three proteins, when deglycosylated, have the same apparent subunit M_r .

Experimental

Materials

A Yucatan piglet (4 weeks old) and a fully grown Yucatan pig were obtained from the University of Leeds Field Station. Other pig tissues were obtained as previously described (Fulcher & Kenny, 1983). Anisole and trifluoromethanesulphonic acid were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Concanavalin A-Sepharose-4B was obtained from Pharmacia Fine Chemicals.

Methods

Purification of endopeptidase-24.11. This was achieved by using an immunoabsorbent column with a monoclonal antibody, GK7C2, as described previously (Gee *et al.*, 1983).

Chemical deglycosylation of endopeptidase-24.11. The method of Edge *et al.* (1981) was used, with the following modifications. The protein sample (500 μ g) in a 1 ml Reacti-vial [Pierce and Warriner (U.K.)] was precipitated at 5°C by 10 vol. of acetone, washed with acetone (5°C) and dried in a stream of N_2 . The pellet was suspended in 25 μ l of anisole, and 50 μ l of trifluoromethanesulphonic acid was added. The vial was capped after bubbling N_2 through the solution and left for 2.5 h at 0°C. The reaction was terminated by the addition of 250 μ l of pyridine/water (4:1, v/v) in

Abbreviation used: SDS, sodium dodecyl sulphate.

ten 25 μ l portions. During this treatment, the vial was surrounded by an acetone/solid- CO_2 slurry, since considerable heat is generated at this stage. The solution was dialysed against water for 18 h at 4°C and then for 24 h against two changes of 1% SDS. The protein was precipitated by cold acetone as before and taken up in 1 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl and 0.1% Triton X-100.

Removal of carbohydrate and residual glycoproteins by concanavalin A-Sepharose 4B. The column (200 μ l) was equilibrated with the same buffer in which the trifluoromethanesulphonic acid-treated protein had been dissolved (see above), the protein sample was added slowly over 10 min and the column was washed with the same buffer. The effluent (2 ml) was collected and concentrated by acetone precipitation in order to prepare the sample for analysis by SDS/polyacrylamide-gel electrophoresis and determination of carbohydrate and protein, the latter by amino acid analysis.

Other methods. Enzymic assays, gel electrophoresis and protein determinations were as described previously (Fulcher & Kenny, 1983). Carbohydrate and protein were also analysed as described previously (Fulcher *et al.*, 1983). Microvilli were prepared by the method of Booth & Kenny (1974).

Results and discussion

Purification of endopeptidase-24.11 from Yucatan-pig kidneys

The purification by immunoabsorbent chromatography is shown in Table 1. The specific activity of this batch was similar to that purified from slaughterhouse material (Fulcher & Kenny, 1983), and the activity was fully inhibited by 1 μ M-phosphoramidon. Fig. 1(a) compares the mobilities on SDS/polyacrylamide-gel electrophoresis of this preparation with that purified under identical conditions by the same monoclonal-immunoabsorbent column. The Yucatan-piglet enzyme migrated as a polypeptide of M_r 93 000, and that prepared from slaughterhouse pigs as one of M_r 89 000, as

previously reported. This anomaly seems to be both strain- and age-related. The enzyme purified from a 4-week-old Landrace $\times$ White-cross piglet had the same mobility as that from slaughterhouse kidneys, which were generally of this strain, as did that purified from a mature Yucatan pig (results not shown). Fig. 1(b) reveals some differences in the pattern of polypeptides present in Yucatan-piglet kidney microvillar membranes compared with those from slaughterhouse pigs, the most striking difference being a strong band of apparent subunit M_r 80 000 (Fig. 1b).

Carbohydrate and amino acid analyses of Yucatan-piglet endopeptidase

The amino acid composition of the form from kidney and intestine (Fulcher *et al.*, 1983) and brain (Relton *et al.*, 1983) agreed well, except for the value obtained for glycine. The analysis of the Yucatan-piglet form did not differ significantly from these values (results not shown). The carbohydrate analysis is given in Table 2. It shows some differences from those reported before: the value for *N*-acetylgalactosamine is low (cf. 10.5 μ g/mg of protein for slaughterhouse-pig kidney enzyme) and the glucose content is exceptionally high (cf. 31.6 μ g/mg). Since glucose may arise adventitiously during preparation of the sample, we have previously put more reliance on the value for carbohydrate excluding glucose. However, this total (11.3%) is lower than those reported for brain (12.9%), kidney (15%) and intestine (18.3%), and hence cannot account for the higher apparent subunit M_r . If glucose is included in the total, the Yucatan-pig kidney enzyme contains 25.2% carbohydrate, higher than that in the intestinal enzyme (22.1%). However, the significance of the high values for glucose remains uncertain. Although it is possible that some of the total represents a difference in post-translational glycosylation in this strain, it is very unlikely that all of the glucose can be so attributed. An analysis of the carbohydrate in the endopeptidase purified from a mature Yucatan pig also showed a low value for *N*-

Table 1. *Purification of endopeptidase-24.11 from Yucatan-piglet kidney cortex*

See the Experimental section for details. The results are from one purification starting with 20 g of piglet kidney cortex with the use of only a single immunoabsorbent step. The specific activity of the purified enzyme was calculated from protein estimated by amino acid analysis. The assay substrate was ^{125}I -labelled insulin B-chain, 1 unit being the hydrolysis of 1 μ mol of substrate/min.

Fraction	Protein (mg)	Total activity (munits)	Specific activity (munits/mg of protein)	Recovery (%)	Purification (fold)
Homogenate	2315	2361	1.02	100	1
Microsomal	764	1375	1.80	58	1.8
Triton X-100-solubilized extract	378	1360	3.60	57	3.5
Immunoabsorbent chromatography	1.24	198.4	160	8.4	157

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified endopeptidase-24.11 and microvillar proteins from kidneys of Yucatan piglet and those from slaughterhouse pigs.

See the Experimental section for details. A 7–17% polyacrylamide gradient gel was used and the proteins were stained with Coomassie Blue. (a) Track 1, pig kidney microvilli (100 µg of protein); track 2, Yucatan-piglet kidney endopeptidase (3 µg); track 3, M_r markers [phosphorylase *b* (M_r , 94000); serum albumin (M_r , 67000); ovalbumin (M_r , 43000); carbonic anhydrase (M_r , 30000); trypsin inhibitor (M_r , 20000); α -lactalbumin (M_r , 14000)]; track 4, pig kidney endopeptidase (5 µg); track 5, pig kidney microvilli (100 µg). (b) Track 1, microvilli prepared from slaughterhouse-pig kidney (100 µg); track 2, microvilli prepared from Yucatan-piglet kidney (100 µg). The M_r values indicated are those for aminopeptidase N, endopeptidase-24.11 and actin (Booth & Kenny, 1976). The arrow indicates an intense band (M_r , 80000) in the preparation from the Yucatan piglet.

Table 2. Carbohydrate analyses of endopeptidase-24.11 purified from various sources after treatment with trifluoromethanesulphonic acid

See the Experimental section for details. Pig kidney and pig intestine forms were purified from slaughterhouse tissues. Triplicate samples (10 µg) were analysed. Abbreviation: N.D., not detectable.

Carbohydrate	Carbohydrate content (µg/mg of protein)			
	Untreated form Yucatan-piglet kidney	After treatment with trifluoromethanesulphonic acid		
		Pig kidney	Yucatan-piglet kidney	Pig intestine
Fucose	1.9 ± 0.2	0.16	0.16	0.16
Mannose	34.7 ± 8.2	0.03	N.D.	0.02
Galactose	40.9 ± 5.9	0.02	0.03	0.14
N-Acetylgalactosamine	1.1 ± 0.1	N.D.	N.D.	N.D.
N-Acetylglucosamine	30.7 ± 2.7	0.01	0.01	0.01
N-Acetylneuraminate	3.8 ± 0.1	N.D.	N.D.	N.D.
Glucose	139 ± 14.2	0.16	0.15	0.03
Total	252 ± 17.6	—	—	—
Total (excluding glucose)	113 ± 10.5	—	—	—

acetylgalactosamine (<2.0 µg/mg of protein), but contained very much less glucose (13.6 µg/mg) than any of the enzymes hitherto analysed.

Deglycosylation by treatment with trifluoromethanesulphonic acid

This reagent was introduced by Edge *et al.*

(1981), who showed it to be efficient in cleaving O-glycosyl bonds, while N-glycosyl bonds between asparagine and N-acetylglucosamine remained intact. Hence, apart from these residues, glycoproteins can be effectively stripped of carbohydrate. In our hands, the yield of deglycosylated endopeptidase was 10–15%. In order to avoid the hydrolysis

Fig. 2. SDS/polyacrylamide-gel electrophoresis of deglycosylated endopeptidase-24.11

See the Experimental section for details. A 7–17% polyacrylamide gradient was used, and the proteins were stained with Coomassie Blue. Tracks 1 and 7, pig kidney microvilli (100 µg of protein); tracks 2 and 6, M_r markers [phosphorylase *b* (M_r 94 000); transferrin (M_r 76 000); serum albumin (M_r 67 000); ovalbumin (M_r 43 000)]. Track 3, deglycosylated Yucatan-piglet kidney endopeptidase (7 µg); track 4, deglycosylated pig kidney endopeptidase (5 µg); track 5, deglycosylated pig intestinal endopeptidase (10 µg). The M_r values indicated on the left are those for the extrinsic glycoprotein, aminopeptidase N, dipeptidyl peptidase IV and endopeptidase-24.11 (Booth & Kenny, 1976).

of peptide bonds, it was important to limit any rise in temperature, especially when the reaction was quenched with pyridine. When the three forms of endopeptidase were treated with this reagent and subsequently analysed, no significant carbohydrate was detectable (Table 2). The low values (0.01 µg/mg of protein) for *N*-acetylglucosamine were unexpected in relation to the stated mode of attack by the reagent. It is likely that trifluoromethanesulphonic acid deacetylated this sugar, thus rendering it resistant to methanolic HCl (Chaplin, 1982). On SDS/polyacrylamide-gel electrophoresis (Fig. 2) all three forms migrated as polypeptides of M_r 77 000. Deglycosylation by this treatment destroyed enzymic activity.

Conclusions

Trifluoromethanesulphonic acid was effective in removing carbohydrate from endopeptidase-24.11. The deglycosylated forms purified from Yucatan-piglet kidney and kidney and intestine from slaughterhouse pigs had identical apparent subunit M_r values, consistent with the view that they are products of the same gene and that their structural differences arise post-translationally.

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