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Purification of endopeptidase-24.11 ('enkephalinase') from pig brain by immunoadsorbent chromatography

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Membrane preparations from striatum of pig brain contain endopeptidase activity towards iodoinsulin B-chain. Only 50% of the hydrolysis of insulin B-chain is inhibitable by phosphoramidon, and DEAE-cellulose chromatography can resolve the phosphoramidon-sensitive and -insensitive activities. The former activity (now designated 'endopeptidase-24.11') is responsible for hydrolysis of [D-Ala^2,Leu^5]enkephalin and is identical with an enzyme in brain that has previously been referred to as 'enkephalinase'. Pig striatal endopeptidase-24.11 has now been purified to homogeneity in a single step by immunoadsorbent chromatography using a monoclonal antibody. The overall purification was 23000-fold, with a yield of 30%. The brain enzyme appears to be identical with kidney endopeptidase-24.11 in amino acid composition as well as by immunological and kinetic criteria. However, it differs slightly in apparent subunit size ($M_r = 87000$), attributable to differences in glycosylation.

Endopeptidase-24.11 (EC 3.4.24.11) is an integral glycoprotein located in the plasma membrane of many tissues (Kenny & Fulcher, 1983). The endopeptidase resembles thermolysin (EC 3.4.24.4) in its specificity and its sensitivity to inhibition by phosphoramidon and thiorphan (Fulcher *et al.*, 1982). Kidney microvilli are a particularly rich source of the endopeptidase, where it constitutes 4% of the membrane protein (Fulcher & Kenny, 1983). An enzyme with similar characteristics is also present in mammalian brain, although in much lower concentration, and has been shown to inactivate the neuropeptide enkephalin by hydrolysis of the Gly^3–Phe^4 bond (Fulcher *et al.*, 1982; Roques *et al.*, 1980; Gorenstein & Snyder, 1979). The brain enzyme has therefore been referred to in the literature as 'enkephalinase'. This is a misnomer, since the brain enzyme appears to be immunologically identical with kidney endopeptidase-24.11 and can hydrolyse a range of neuropeptide substrates including Substance P (Matsas *et al.*, 1983), cholecystokinin (Deschodt-Lanckman & Strosberg, 1983), luliberin ('luteinizing hormone-releasing hormone', 'LH-RH') and neurotensin (S. H. Ibbotson, A. J. Kenny & A. J. Turner, unpublished work). We have postulated that endopeptidase-24.11 may play a key role in the inactivation of peptide neurotransmitters at the synapse (Fulcher *et al.*, 1982; Matsas *et al.*, 1983).

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

Previous attempts to purify the endopeptidase from brain have met with limited success (Gorenstein & Snyder, 1979; Almenoff *et al.*, 1981). In the present paper we report the purification of the endopeptidase from pig brain by immunoadsorbent chromatography employing a monoclonal antibody (GK7C2) to the pig kidney enzyme (Gee *et al.*, 1983). The brain and kidney enzymes are identical in many respects, although there are small differences in the nature and extent of their glycosylation. Evidence is also presented for the occurrence in brain of a membrane endopeptidase insensitive to phosphoramidon.

Experimental procedures

Materials

Enkephalin analogues were obtained from Cambridge Research Biochemicals (Cambridge, U.K.). Phosphoramidon was from Protein Research Foundation (Osaka, Japan). Ultrogel AcA 34 was from LKB Instruments Ltd. Other materials were from sources previously noted (Fulcher *et al.*, 1982; Fulcher & Kenny, 1983; Matsas *et al.*, 1983). Kidney and intestinal endopeptidase-24.11 were purified to homogeneity by immunoadsorbent chromatography as described previously (Fulcher *et al.*, 1983). The immunoadsorbent column, containing monoclonal antibody (GK7C2), was prepared as described elsewhere (Gee *et al.*, 1983). The particular column used for preparation of the

brain enzyme had not previously been used for purification of kidney enzyme, in order to exclude the possibility of cross-contamination. Pig brains were kindly provided by Asda Farm Products, Lofthousegate, West Yorkshire, U.K.

Assay of endopeptidase-24.11

Two procedures were routinely used for the assay of the endopeptidase. The first method employed [^{125}I]iodoinsulin B-chain (5.8 μM) as substrate (Fulcher & Kenny, 1983). The second method used [D-Ala^2,Leu^5]enkephalin (0.5 mM) as substrate (Matsas *et al.*, 1983). The sensitivity of the enzyme activity to phosphoramidon was checked at all stages of purification.

Purification of endopeptidase-24.11 from pig brain

All procedures were carried out at 4°C unless stated otherwise. Striata (130g) were dissected out from fresh pig brains and homogenized in 5 vol. of 10 mM-Tris/HCl, pH 7.5. A membrane fraction was prepared by centrifugation of the homogenate at 17000 ***g*** for 60 min. The pellet was washed in half the original volume of buffer, re-centrifuged and then resuspended in 150 ml of 50 mM-Tris/HCl, pH 7.5, to which 25 ml of 20% (w/v) Triton X-100 was added. After being stirred for 1 h at room temperature, the mixture was centrifuged at 31000 ***g*** for 2 h. The supernatant. containing the membrane-solubilized endopeptidase, was retained and NaCl added to a final concentration of 0.5 M. The enzyme preparation was applied at a rate of 10 ml/h to the immunoadsorbent column (400 μl bed volume), equilibrated in 50 mM-Tris/HCl buffer (pH 7.5)/0.5 M-NaCl/0.1% Triton X-100. The column was washed with 200 vol. of equilibration buffer and activity eluted at room temperature with 0.1 M-$NaHCO_3$/0.5 M-NaCl/0.1% Triton X-100, adjusted to pH 10.6 with 2 M-NaOH. The active fractions were immediately adjusted to pH 7.5, pooled, and dialysed against 5 mM-Tris/HCl (pH 7.5)/0.1% Triton X-100. The enzyme sample was then concentrated by adsorption to a small column (100 μl bed vol.) of DEAE-cellulose equilibrated in dialysis buffer, followed by elution in the same buffer containing 0.5 M-NaCl. The enzyme was routinely stored frozen at −70°C. After each use, the immunoadsorbent column was washed with 20 ml of 0.2 M-glycine/HCl, pH 2.3, containing 0.5 M-NaCl and 0.1% (w/v) Triton X-100 and then re-equilibrated with the starting buffer solution. Between uses the column was stored with 0.02% NaN_3 in this buffer.

Other methods

For some studies, the Triton-solubilized membrane preparation was initially subjected to ion-exchange chromatography. The sample (0.84g of protein) was applied to a column (60 ml bed vol.) of DEAE-cellulose equilibrated with 5 mM-Tris/HCl (pH 7.5)/0.1% Triton X-100. After the column had been washed extensively with equilibration buffer, enzyme activity was eluted by application of a linear gradient (0–300 mM-NaCl) in 1 litre of the same buffer. Fractions were collected and assayed, in the presence or absence of 1 μM-phosphoramidon, for endopeptidase activity using [^{125}I]iodoinsulin B-chain or [D-Ala^2,Leu^5]enkephalin as substrate. Gel filtration of the purified endopeptidase was performed on a column (530 mm × 22 mm) of Ultrogel AcA 34. Samples (20 μg in 0.5 ml) of either brain or kidney endopeptidase were applied to the column, which was equilibrated with 10 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/0.1% (w/v) Triton X-100. The column was calibrated with markers as described previously (Fulcher & Kenny, 1983). SDS/polyacrylamide-gel electrophoresis was performed by using the method of Laemmli (1970), with a 7–17% gradient of polyacrylamide.

Double immunodiffusion was carried out by the technique of Ouchterlony (1968), with agarose (1%) containing 0.1% (w/v) Triton X-100 in 0.15 M-NaCl/0.01 M-NaH_2PO_4/0.02% NaN_3, adjusted with 2 M-NaOH to pH 7.4, as the diffusion medium. The central well contained polyclonal antiserum (36 μg) to the pig kidney endopeptidase. Samples (2 μg) of purified endopeptidase from kidney, intestine and brain in 5 mM-Tris/HCl/500 mM-NaCl/0.1% Triton X-100, pH 7.5, were loaded into the peripheral wells. Immunodiffusion was allowed to occur at 4°C for 48 h in a damp atmosphere. When precipitation appeared complete, unprecipitated antibodies were removed from the gel by soaking in 0.5 M-NaCl and the precipitin arcs were stained for protein with 0.5% (w/v) Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water (9:2:9, by vol.).

For amino acid and carbohydrate analysis, samples (5–20 μg) of purified brain endopeptidase were precipitated with an equal volume of 25% (w/v) trichloroacetic acid. The precipitates were washed twice with acetone and then dried over P_2O_5 *in vacuo*. For amino acid analysis, the samples were then hydrolysed in 6 M-HCl containing 0.3% (w/v) phenol for 24 h at 110°C, and the hydrolysate was analysed by using a Rank–Hilger Chromaspek J180 analyser. Carbohydrate analysis of the protein samples employed the g.l.c. method of Chaplin (1982).

Results and discussion

Phosphoramidon-sensitivity of brain membrane endopeptidase activity

In pig striatum, 50% of membrane endopeptidase activity towards insulin B-chain is inhibitable by phosphoramidon. Only the phosphoramidon-sen-

sitive component hydrolysed [D-Ala^2,Leu^5]-enkephalin and hence is the same as the enzyme referred to as 'enkephalinase'. Since the specificity of this enzyme is by no means restricted to enkephalin, we have proposed (Matsas *et al.*, 1983) the more general name *'endopeptidase-24.11'*, on the basis of its Enzyme Commission number. This enzyme is the principal subject of the present report.

The nature and specificity of the phosphoramidon-insensitive activity are at present unclear. On DEAE-cellulose chromatography, this activity was eluted as a broad peak (120–180mM-NaCl), whereas the peak of endopeptidase-24.11 appeared at 80mM-NaCl. The phosphoramidon-insensitive peak was strongly inhibited by 1,10-phenanthroline and EDTA in the range 0.1–1mM, but was not inhibited by phenylmethanesulphonyl fluoride, di-isopropyl fluorophosphate (each 1mM), leupeptin or pepstatin (each 25μg/ml). Although it failed to hydrolyse [D-Ala^2,Leu^5]enkephalin, it must be considered as a potential candidate for the metabolism of other brain peptides. It is possible that it may prove to be similar to the phosphoramidon-insensitive membrane endopeptidases present in mouse (Beynon *et al.*, 1981) and rat (Kenny *et al.*, 1981) kidneys and other tissues (Kenny & Fulcher, 1983), but further speculation on its role must await its purification and determination of its specificity.

Purification of endopeptidase-24.11

The striatum is one of the richest sources of endopeptidase-24.11 in pig brain (Matsas *et al.*, 1983) and this region was therefore selected for purification purposes. After solubilization of enzyme activity with Triton X-100, it proved possible to purify the endopeptidase to homogeneity in a single immunoadsorbent step using our monoclonal antibody, GK7C2. An overall purification of 23000-fold was achieved, with a yield of 30% (Table 1, mean result for two preparations). The yield of pure endopeptidase was about 1μg/g (wet weight) of pig striatum by this procedure. The specific activity of the final product (160nmol/min per mg) was the same as that of the pig kidney endopeptidase (Fulcher & Kenny, 1983). The overall purification factor for endopeptidase-24.11 was not influenced by the use of either insulin B-chain or [D-Ala^2,Leu^5]-enkephalin as substrate (Table 1).

Immunological cross-reactivity

We had previously shown that a polyclonal antiserum to the pig kidney endopeptidase was able to inhibit kidney and brain enzymes identically (Matsas *et al.*, 1983); indeed, the similarity was closer for these two than for the renal and intestinal enzymes (Fulcher *et al.*, 1983). The ability of GK7C2 to recognize the brain endopeptidase provides additional evidence for their immunological similarity. By Ouchterlony immunodiffusion the endopeptidase purified from pig brain, intestine and kidney cross-reacted identically with the polyclonal antiserum rasied to the kidney enzyme (Fig. 1).

Kinetic and inhibitor properties

The purification brain endopeptidase was inhibited by phosphoramidon in a dose-dependent manner with an I_{50} (concentration causing half-maximal inhibition) (9nM) comparable with that reported for the kidney enzyme or intact synaptic membranes (Fulcher *et al.*, 1982). The chelating agent 1,10-phenanthroline (I_{50} 145μM) also inhibited the brain endopeptidase. Captopril (1mM) and di-isopropyl fluorophosphate (1mM) had no effect on the purified enzyme, and 1mM-puromycin gave 10% inhibition of brain endopeptidase activity. Again, these values are comparable with those obtained with kidney enzyme or intact synaptic membranes (Fulcher *et al.*, 1982; Matsas *et al.*, 1983). The K_m for hydrolysis of [D-Ala^2,Leu^5]-enkephalin was 104μM (mean for three experiments) (cf. K_m for kidney enzyme, 87μM). The peptides produced by hydrolysis of Substance P by the purified brain enzyme had identical retention times

Table 1. *Purification of endopeptidase-24.11 from pig brain*

Endopeptidase-24.11 was purified from 130g of pig striatum (25 brains) as described in the Experimental procedures section. Activity was assayed by using either iodoinsulin B-chain (5.8μM) or [D-Ala^2,Leu^5]enkephalin (0.5mM) as substrate.

	Substrate ... Insulin B-chain					[D-Ala^2,Leu^5]Enkephalin			
	Protein (mg)	Activity (nmol · min^{-1})	Sp. activity (nmol · min^{-1} · mg^{-1})	Yield (%)	Purification (fold)	Activity (μmol · min^{-1})	Sp. activity (nmol · min^{-1} · mg^{-1})	Yield (%)	Purification (fold)
1. Homogenate	9300	64.9	0.0069	(100)	1	10.5	1.1	(100)	1
2. Membrane pellet	4700	38.4	0.0060	60	0.9	8.4	1.7	81	1.5
3. Triton-solubilized	1000	27.9	0.028	43	4.1	5.6	5.6	54	5.1
4. Immunoadsorbent chromatography	0.118	18.7	158	29	22600	3.1	26300	30	23900

by h.p.l.c. analysis with those previously reported for the kidney enzyme (Matsas *et al.*, 1983; results not shown).

Molecular properties of brain endopeptidase-24.11

When subjected to SDS/polyacrylamide-gel electrophoresis, the brain endopeptidase migrated slightly faster than the kidney enzyme (Fig. 2). This was a consistent observation with four successive preparations. The brain enzyme revealed a single component of apparent subunit M_r 87000 (cf. kidney, 89000). In contrast, the intestinal endopeptidase migrated slower than the kidney enzyme (Fig. 2), a difference that has been attributed to differences in glycosylation (Fulcher *et al.*, 1983). The brain enzyme contained about 13% carbohydrate, if glucose is excluded (Table 2). This content is less than that of the kidney or intestinal preparations (Fulcher *et al.*, 1983), but the pattern of glycosylation is closer to that shown by the kidney enzyme, especially in the low fucose content. This difference in glycosylation provides the most likely explanation for the smaller apparent subunit

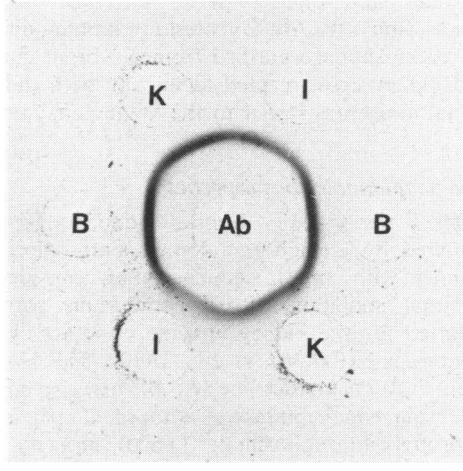

Fig. 1. *Ouchterlony double-immunodiffusion analysis of pig endopeptidase-24.11*
See the Experimental procedures section for further details. The central well contained polyclonal (Ab) antiserum to pig kidney endopeptidase-24.11. The peripheral wells contained samples (2 μg) of purified endopeptidase-24.11 from kidney (K), intestine (I) and brain (B).

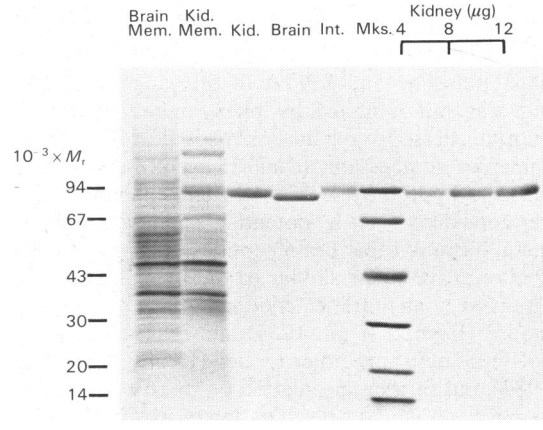

Fig. 2. *SDS/polyacrylamide-gel electrophoresis*
See the Experimental procedures section for further details. The gradient gel comprised 7–17% (w/v) polyacrylamide. Tracks (from left to right): Brain Mem., brain membrane preparation (100 μg); Kid. Mem., kidney microvillar membrane preparation (100 μg); Kid., kidney endopeptidase-24.11 (10 μg); Brain, brain endopeptidase-24.11 (10 μg); Int., intestinal endopeptidase-24.11 (10 μg); Mks, marker proteins (phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin). Kidney (μg), 4, 8 and 12 indicate these amounts of kidney endopeptidase-24.11 run.

Table 2. *Carbohydrate analysis of pig brain endopeptidase-24.11*
See the Experimental procedures section for methods. Each determination was made in triplicate; the number of samples is shown in parentheses. The data for kidney and intestine are from Fulcher *et al.* (1983).

Endopeptidase-24.11 ...	Composition [μg/mg of protein (± S.D.)]		
Carbohydrate	Brain (2)	Kidney (4)	Intestine (3)
Fucose	3.4 ± 1.9	3.3 ± 0.49	22.4 ± 5.2
Mannose	33.4 ± 3.9	37.1 ± 4.4	42.2 ± 3.0
Galactose	30.9 ± 2.9	45.2 ± 4.0	40.2 ± 1.2
N-Acetylgalactosamine	9.5 ± 3.2	10.5 ± 3.1	20.7 ± 8.4
N-Acetylglucosamine	46.6 ± 3.1	46.0 ± 3.7	52.6 ± 12.9
N-Acetylneuraminate	5.1 ± 1.3	8.1 ± 2.5	4.9 ± 2.9
Glucose	45.3 ± 5.6	31.6 ± 31.7	38.2 ± 2.8
Total	174.2 ± 10.1	181.9 ± 29.4	221.2 ± 2.5
Total (excluding glucose)	128.9 ± 5.1	150.2 ± 7.1	183.0 ± 4.8

M_r of the brain endopeptidase. The native brain enzyme also appeared to be slightly smaller than the kidney enzyme when subjected to gel filtration on Ultrogel AcA 34. The apparent M_r was 280000, consistent with a dimer binding a micelle of detergent (Fulcher & Kenny, 1983). The amino acid composition of the brain enzyme (results not shown) compared well with those for the enzyme from kidney and intestine (Fulcher *et al.*, 1983). Only the value for glycine showed any possible significant deviation in the three analyses, being lowest for the brain enzyme. Thus in all respects so far tested, namely specificity, kinetics, immunological and structural properties, brain endopeptidase-24.11 appears to be identical with that of kidney. It is likely that the brain and kidney forms of the endopeptidase are products of the same gene, but are subjected to different post-translational processing in their respective tissues after translation. Since the enzyme is so much more abundant in kidney, this source has obvious advantages for studying the structure and activity of endopeptidase-24.11.

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References

Almenoff, J., Wilk, S. & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun.* **102**, 206–214

Beynon, R. J., Shannon, J. D. & Bond, J. S. (1981) *Biochem. J.* **199**, 591–598

Chaplin, M. F. (1982) *Anal. Biochem.* **123**, 336–341

Deschodt-Lanckman, M. & Strosberg, A. D. (1983) *FEBS Lett.* **152**, 109–113

Fulcher, I. S. & Kenny, A. J. (1983) *Biochem. J.* **211**, 743–753

Fulcher, I. S., Matsas, R., Turner, A. J. & Kenny, A. J. (1982) *Biochem. J.* **203**, 519–522

Fulcher, I. S., Chaplin, M. F. & Kenny, A. J. (1983) *Biochem. J.* **215**, 317–323

Gee, N. S., Matsas, R. & Kenny, A. J. (1983) *Biochem. J.* **214**, 377–386

Gorenstein, C. & Snyder, S. H. (1979) *Life Sci.* **25**, 2065–2070

Kenny, A. J. & Fulcher, I. S. (1983) *Ciba Found. Symp.* **95**, 12–25

Kenny, A. J., Fulcher, I. S., Ridgwell, K. & Ingram, J. (1981) *Acta Biol. Med. Germ.* **40**, 1465–1471

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Matsas, R., Fulcher, I. S., Kenny, A. J. & Turner, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3111–3115

Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis*, Ann Arbor Science Publishers, Ann Arbor, MI

Roques, B. P., Fournié-Zaluski, M. C., Soroka, E., Lecomte, J. M., Malfroy, B., Llorens, C. & Schwartz, J. C. (1980) *Nature (London)* **288**, 286–288