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Isolation of two differentially glycosylated forms of peptidyl-dipeptidase A (angiotensin converting enzyme) from pig brain: a re-evaluation of their role in neuropeptide metabolism

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Peptidyl-dipeptidase A (angiotensin converting enzyme; ACE, EC 3.4.15.1), has been purified from pig kidney and striatum by affinity chromatography employing the selective inhibitor lisinopril as ligand. The inclusion of a 2.8 nm spacer arm improved the yield of the enzyme compared with the 1.4 nm spacer arm described in previous work. Two forms of striatal ACE (M_r 180000 and 170000), but only a single form of kidney ACE (M_r 180000), were isolated by this procedure. Both forms of striatal ACE were recognized by a polyclonal antibody to kidney ACE. No significant differences in substrate specificity or inhibitor sensitivity between kidney and striatal ACE could be detected. In particular, the amidated neuropeptide, substance P, was hydrolysed identically by both preparations and no significant hydrolysis of the related tachykinin peptides neurokinin A and neurokinin B could be detected. After chemical or enzymic deglycosylation, kidney and both forms of striatal ACE migrated identically on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 150000. We suggest that the two detectable forms of ACE in pig brain are not isoenzymes but are the result of differential glycosylation in different cell types in the brain. It appears that ACE, unlike endopeptidase-24.11, does not have the general capacity to hydrolyse and inactivate the tachykinin peptides at a significant rate in brain.

INTRODUCTION

The primary specificity of angiotensin I converting enzyme (ACE; peptidyl-dipeptidase A, EC 3.4.15.1) is to act as an exopeptidase releasing dipeptide fragments from the *C*-terminus of an oligopeptide (Soffer, 1976). Its classical physiological substrates are angiotensin I, which is converted to the vasopressor angiotensin II, and bradykinin, which is inactivated. Inhibitors of ACE (e.g. captopril, enalapril, lisinopril) have therefore a valuable role to play in the treatment of hypertension (Cushman & Ondetti, 1980; Abrams *et al.*, 1984). Apart from its peptidyl-dipeptidase activity, ACE, purified from lung and kidney, has been shown to act as an endopeptidase with certain *C*-terminally amidated peptides, for example substance P and luteinizing hormone releasing hormone (LH-RH) (Yokosawa *et al.*, 1983; Skidgel *et al.*, 1984; Skidgel & Erdös, 1985). The identification of ACE in the mammalian brain (Yang & Neff, 1972), and particularly its localization to a striatonigral pathway (Strittmatter *et al.*, 1984), has led to speculation that the brain enzyme may serve to inactivate substance P and perhaps other neuropeptide transmitters. This hypothesis has been supported, in part, by the reported isolation of an isoenzyme of ACE from rat striatum, apparently different from lung ACE in molecular size and specificity for certain amidated peptides (Thiele *et al.*, 1985; Strittmatter *et al.*, 1985). The striatal form, believed to be of neuronal origin, apparently hydrolysed not only substance P but also the structurally related neuropeptide, neurokinin A (substance K).

Our previous enzymic and immunocytochemical studies (Matsas *et al.*, 1983, 1986; Hooper *et al.*, 1985) have implicated another cell surface peptidase (endopeptidase-24.11, EC 3.4.24.11) in the inactivation of substance P and neurokinin A. Among a range of neuropeptides, substance P and neurokinin A were the most efficiently hydrolysed *in vitro* by purified endopeptidase-24.11. The ability of pig striatal synaptic membranes to hydrolyse these peptides was abolished by the selective endopeptidase inhibitor phosphoramidon, and by an antiserum to the endopeptidase (Matsas *et al.*, 1983; Hooper *et al.*, 1985). The ACE inhibitor captopril had no significant effect on substance P hydrolysis by pig striatal synaptic membranes. Finally, we were able to demonstrate immunohistochemically the co-localization of endopeptidase and substance P in certain brain regions (Matsas *et al.*, 1986). In order to explore further the relative roles of endopeptidase-24.11 and ACE in the metabolism of neuropeptides in the brain, we have now isolated ACE from pig striatum by affinity chromatography. Two forms of striatal ACE were obtained by this method, which appeared to differ in the extent of glycosylation. Both forms were recognized by an antibody to pig kidney ACE. No marked differences in the substrate specificity and inhibitor sensitivity were detectable and, in particular, neurokinins A and B were not hydrolysed at a significant rate by the pig striatal enzyme.

EXPERIMENTAL

Materials

Substance P, neurokinins A and B and other peptide substrates were obtained from Cambridge Research

Abbreviations used: ACE, angiotensin converting enzyme (peptidyl-dipeptidase A, EC 3.4.15.1); PAGE, polyacrylamide-gel electrophoresis; TFMS, trifluoromethanesulphonic acid; Dip-F, di-isopropyl phosphorofluoridate.

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Biochemicals (Harston, Cambridge, U.K.). Captopril (SQ 14 225, D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from Squibb Institute for Medical Research (Princeton, NJ, U.S.A.). Lisinopril {MK521, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline}, enalaprilat {MK422, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl-L-proline}, L155212 (Compound 11a; Wyvratt *et al.*, 1983) and L155360 (Compound 3b; Parsons *et al.*, 1983) were gifts from Dr. A. A. Patchett, Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Bestatin was purchased from Sigma Chemical Co. *N*-Glycanase (glycopeptide *N*-glycosidase, EC 3.2.2.18) was purchased from Genzyme Fine Chemicals (Suffolk, U.K.) and trifluoromethanesulphonic acid (TFMS) was from Aldrich. Other materials were from sources previously noted.

Enzyme assays. Routine assays of ACE were performed at pH 8.3 in the presence of 0.3 M-NaCl and 10 μM-$ZnCl_2$ with BzGly-His-Leu (5 or 1 mM) as substrate. The reaction products (hippurate and His-Leu) were resolved and quantified by h.p.l.c. or by a fluorimetric method, modified from Yang & Neff (1972). Assays of endopeptidase-24.11 and aminopeptidase activities were performed as described elsewhere (Matsas *et al.*, 1985*a*).

Hydrolysis of peptides. Samples of ACE (100–200 ng of protein) were incubated with peptide substrates (0.5 mM) for various intervals of time. The reaction was stopped by boiling and the products were analysed by h.p.l.c. as described for substance P hydrolysis (Matsas *et al.*, 1983) except for neurokinin A, when a linear gradient of 4.5–30% (v/v) acetonitrile was used.

Preparation of lisinopril affinity resin. The preparation was modified from Bull *et al.* (1985) by including a 2.8 nm spacer arm between ligand and resin in place of the 1.4 nm spacer arm. In brief, Sepharose CL-4B was reacted with 1,4-butanediol diglycidyl ether in 0.6 M-NaOH and $NaBH_4$ (2 mg/ml) for 8 h at room temperature to form epoxy-activated Sepharose. 6-[*N*-(*p*-aminobenzoyl)amino]caproic acid (90 mM) was reacted with the epoxy-activated Sepharose in 0.1 M-NaOH for 16 h at room temperature. Residual epoxy groups were blocked with 1 M-glycine, pH 10.0, and the modified Sepharose was washed extensively. The matrix was activated to the *N*-hydroxysuccinimide ester (Cuatrecasas & Parikh, 1972) and then reacted with lisinopril (2.2 mM) in 0.3 M-K_2CO_3, pH 11.0, for 18 h at 4 °C. Residual reactive groups were blocked with 1 M-glycine, pH 10.0, and the resulting lisinopril–2.8 nm–Sepharose was washed extensively with water, 0.5 M-NaCl and equilibration buffer before use.

The coupling of 6-[*N*-(*p*-aminobenzoyl)amino]caproic acid was monitored by u.v. absorption. When caproic acid is coupled to the epoxy-activated Sepharose an absorption peak is observed at approx. 292 nm. The coupling of lisinopril can be monitored by a decrease in the lisinopril content of the coupling solution as measured by reverse-phase h.p.l.c., or by amino acid analysis of the resulting lisinopril–2.8 nm–Sepharose. The concentration of covalently bound lisinopril in the affinity gel was 1–2 μmol/ml of gel as determined by reverse-phase h.p.l.c. analysis of the coupling solution.

Affinity isolation of pig kidney and striatal ACE

The purification procedure was modified from that described by Bull *et al.* (1985) for the purification of rabbit lung ACE. The notable difference in procedure is that an increase of 1 pH unit (from pH 7.5 to pH 8.5) in the elution buffer containing free lisinopril was required in order to release bound enzyme.

Details of purification. Approx. 200 g of pig striata were homogenized in 2 litres of 0.33 M-sucrose/50 mM-Hepes, pH 7.5. The resultant homogenate was centrifuged at 26000 *g* for 2 h. The pellet was resuspended in 10 mM-Hepes/0.3 M-KCl/100 μM-$ZnCl_2$, pH 7.5 (buffer A) and ACE was solubilized from the membranes by addition of 20% (w/v) Triton X-100 to give a detergent/protein ratio of 7:1, and stirred at room temperature for 1 h. The suspension was then centrifuged at 31000 *g* for 1.5 h and the supernatant then dialysed against 8 vol. of 10 mM-Hepes/0.3 M-KCl/100 μM-$ZnCl_2$/0.1% (w/v) Triton X-100, pH 7.5 (buffer B). The dialysed extract was applied to a lisinopril–2.8 nm–Sepharose affinity column (10 ml bed volume), with a pre-column of unmodified Sepharose CL-4B (10–20 ml bed volume), both equilibrated in buffer B, at a flow rate of 10–15 ml/h. Once the sample had been applied, the pre-column was removed and the affinity column washed, first with buffer B (400 ml), then with buffer A (400 ml), and finally with 10 mM-Tris/0.3 M-KCl/100 μM-$ZnCl_2$, pH 8.5 (500 ml), at a flow rate of 15–20 ml/h. Bound enzyme was eluted from the affinity column with 10 mM-Tris/0.3 M-KCl/100 μM-$ZnCl_2$/10 μM-lisinopril, pH 8.5, and monitored by its absorbance at 280 nm. Fractions containing protein were pooled and dialysed extensively against 2 mM-Tris/0.1 M-NaCl/10 μM-EDTA, pH 8.0, to remove the lisinopril (Bull *et al.*, 1985) and finally against 5 mM-Tris/10 μM-$ZnCl_2$, pH 8.0, to re-activate the enzyme. The enzyme was concentrated by adsorption on and elution from a small column of DEAE-cellulose (0.5–1 ml bed volume) equilibrated in 5 mM-Tris, pH 8.0. Elution was effected with the same buffer containing 0.3 M-NaCl. All operations were carried out at 4 °C.

The purification procedure produced approx. 80 μg of ACE from 150 g of striatum or 2.7 mg of ACE from 200 g of kidney cortex. Separate affinity columns were used for the purification of kidney and striatal ACE. Protein concentrations were determined by the method of Lowry *et al.* (1951) or by the modified method of Bensadoun & Weinstein (1976). For some purifications, a 'cocktail' of protease inhibitors [EDTA (10 mM), Dip-F (0.1 mM), *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (10 μM), antipain (10 mg/l), leupeptin (10 mg/l), pepstatin A (1 mg/l), trypsin inhibitor types II_S and II_O (each of 10 mg/ml) and bacitracin (100 mg/l)] was included up to the stage of application of enzyme preparation to the affinity column. The same two polypeptides of M_r 170000 and M_r 180000 were obtained after purification in the presence of protease inhibitors.

SDS/polyacrylamide-gel electrophoresis. This was performed by using the system of Laemmli (1970) with a 7–17% (w/v) polyacrylamide gradient as described previously (Relton *et al.*, 1983). Immunoelectrophoretic-blot analysis (Western blot) was carried out as described by Towbin *et al.* (1979). Proteins were separated by

Table 1. Purification of ACE from pig brain

ACE was purified from 156 g of pig striatum (50 brains) as described in the Experimental section. Activity was assayed with BzGly-His-Leu (5 mM) as substrate.

Stage	Protein (mg)	Activity (μmol·min^{-1})	Sp. activity (nmol·min^{-1}·mg^{-1})	Purification factor	Yield (%)
1. Homogenate	25600	0.85	0.033	1	(100)
2. Membrane pellet	12120	0.43	0.035	1.1	51
3. Triton-solubilized	5590	0.57	0.101	3.1	67
4. Affinity chromatography	0.062	0.11	1766	53500	13

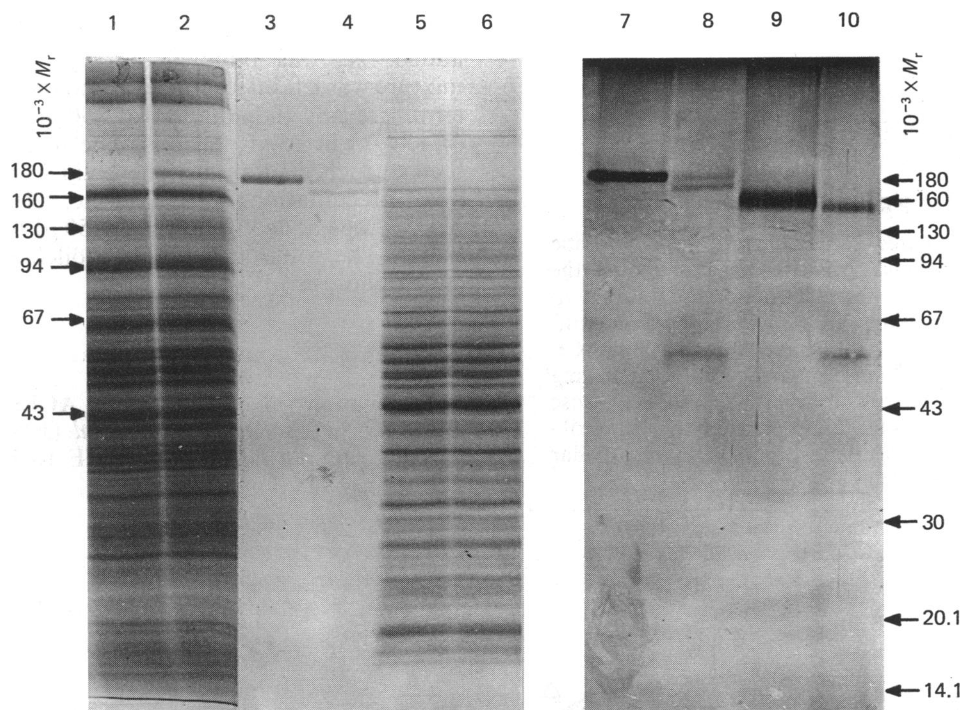

Fig. 1. SDS/polyacrylamide-gel electrophoresis of pig kidney and striatal ACE

Samples were prepared and analysed as described in the Experimental section. Lane 1, pig kidney membrane fraction solubilized with Triton X-100 after chromatography on lisinopril–2.8 nm–Sepharose, i.e. run-through fraction (200 μg); lane 2, pig kidney membrane fraction solubilized with Triton X-100 before chromatography on lisinopril–2.8 nm–Sepharose (200 μg); lane 3, pig kidney ACE purified by lisinopril–2.8 nm–Sepharose chromatography (2 μg); lane 4, pig striatal ACE purified by lisinopril–2.8 nm–Sepharose chromatography (6 μg); lane 5, pig striatal membrane fraction solubilized with Triton X-100 before chromatography on lisinopril–2.8 nm–Sepharose (100 μg); lane 6, pig striatal membrane fraction solubilized with Triton X-100 after chromatography on lisinopril–2.8 nm–Sepharose, i.e. run-through fraction (100 μg); lane 7, pig kidney ACE untreated (0.75 μg); lane 8, pig striatal ACE untreated (6 μg); lane 9, pig kidney ACE treated with *N*-glycanase (1.0 μg); lane 10; pig striatal ACE treated with *N*-glycanase (6 μg). Lanes 1–6 were stained with Coomassie Brilliant Blue; lanes 7–10 were silver stained. The contaminant at $M_r \sim 55000$ in lanes 8 and 10 appeared in one preparation of striatal ACE and was due to inadequate washing of the affinity column before elution.

SDS/polyacrylamide-gel electrophoresis and then transferred to a nitrocellulose membrane at 250 mA for 3 h in 20 mM-Tris/150 mM-glycine/20% (v/v) methanol, pH 8–9. The membranes were then washed with 20 mM-Tris/50 mM-NaCl, pH 7.4, before blocking with 50 mM-Tris/500 mM-NaCl/0.5% Tween 20, pH 8.0 ('blocking buffer'). The membrane was incubated for 2.5 h at room temperature with a polyclonal antibody raised to pig kidney ACE (affinity purified on lisinopril–Sepharose), which was diluted in blocking buffer. The antibody was previously purified on a protein A–Sepharose column. The membrane was then washed extensively with 20 mM-Tris/150 mM-NaCl, pH 7.4, 20 mM-Tris/150 mM-NaCl/0.1% Tween 20, pH 7.4, and 20 mM-Tris/500 mM-NaCl/0.1% Tween 20, pH 7.4, before incubation for 30 min at room temperature with an anti-rabbit IgG alkaline phosphatase-conjugated antibody diluted in blocking buffer. Before staining, the membrane was washed with blocking buffer, and then developed with nitro blue tetrazolium/5-bromo-4-

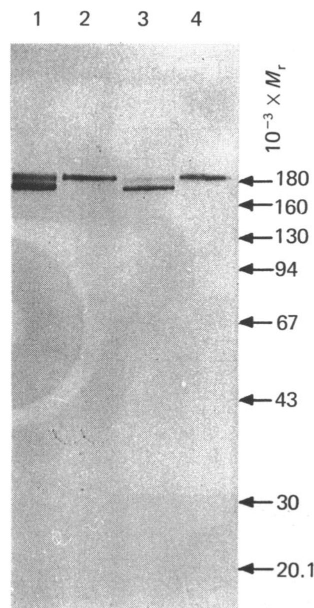

Fig. 2. 'Western' blot of pig kidney and striatal ACE

Samples were prepared and immunoelectrophoretic (Western) blot analysis was performed as described in the Experimental section. After electrophoretic transfer to nitrocellulose sheets, the tracks were blotted with antiserum to pig kidney ACE. Lane 1, pig striatal ACE purified by lisinopril–2.8 nm–Sepharose (3 μg); lane 2, pig kidney ACE purified by lisinopril–2.8 nm–Sepharose (1 μg); lane 3, pig striatal ACE purified by lisinopril–2.8 nm–Sepharose (1.5 μg); lane 4, pig kidney microvillar membrane preparation (40 μg).

chloro-3-indolyl phosphate substrate solution. The staining was stopped by adding 20 mM-Tris/5 mM-EDTA, pH 8.0.

Deglycosylation of kidney and striatal ACE. Samples of kidney and striatal ACE were deglycosylated both by enzymic and chemical methods. Enzymic deglycosylation employed *N*-glycanase (10–20 units/ml), which was incubated for 24 h at 37 °C with ACE (1–10 μg) in 0.25 M-sodium phosphate buffer, pH 8.6, containing 1,10-phenanthroline (10 mM) in a final volume of 30–50 μl. The reaction was terminated by the addition of 50 μl of electrophoresis buffer and boiled for 4 min.

For chemical deglycosylation (Stewart *et al.*, 1984), ACE (10–50 μg) was precipitated twice with ice-cold acetone and then dried under N_2. The pellet was resuspended in anisole (25 μl) and TFMS (50 μl) was added. N_2 was bubbled through the solution. The mixture was left on ice for 2.5 h and the reaction was then terminated by the addition of 250 μl of pyridine/water (4:1, v/v) in 25 μl portions. The reaction mixture was then quenched in an acetone/solid CO_2 slurry. After a final precipitation with acetone, 30 μl of 3 M-Tris, pH 7.5, was added, followed by 50 μl of electrophoresis buffer. The solution was then boiled for 4 min before electrophoresis.

RESULTS

Purification of pig striatal ACE. Although the affinity resin described by Bull *et al.* (1985), with a 1.4 nm spacer arm, purified kidney ACE to homogeneity the

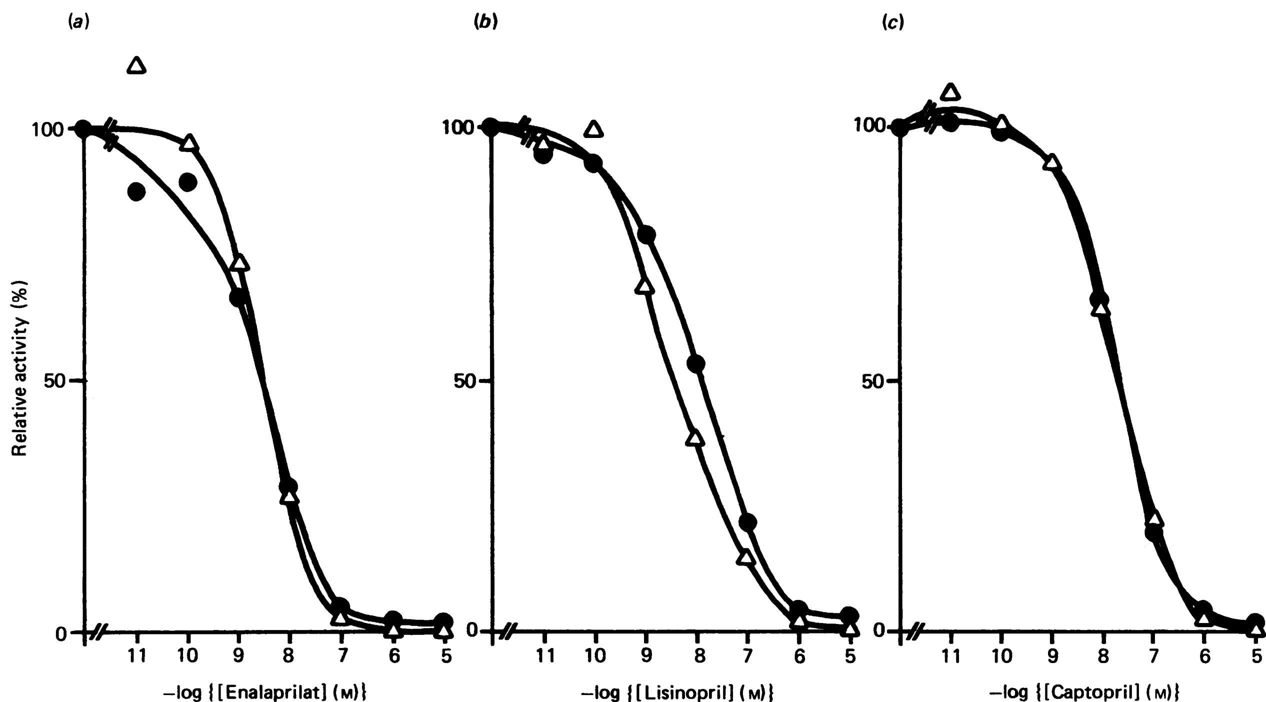

Fig. 3. Inhibition curves of pig kidney and striatal ACE

Pig kidney ACE (100 ng) or pig striatal ACE (100 ng) was incubated with substrate (BzGly-His-Leu, 10 mM). The reaction product (His-Leu) was assayed fluorimetrically. The inhibitors used were: (*a*) enalaprilat; (*b*) lisinopril; (*c*) captopril. Each point is the mean of duplicates. ●, Kidney ACE; △, striatal ACE.

Table 2. Sensitivity to selected inhibitors of pig kidney and striatal ACE

The I_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity at pH 8.3 and 37 °C with BzGly-His-Leu (1 mM) as substrate. The reaction product (His-Leu) was assayed fluorimetrically. The numbers in parentheses represent the number of separate determinations of I_{50} for each inhibitor. Where more than one determination was carried out, mean values and ranges are quoted.

	I_{50} (μM)	
Inhibitor	Kidney ACE	Striatal ACE
Captopril (3)	0.021±0.013	0.019±0.015
Lisinopril (3)	0.011±0.004	0.003±0.002
Enalaprilat (2)	0.037±0.010	0.024±0.006
L155,212 (1)	0.027	0.028
L155,360 (1)	0.056	0.026
EDTA (1)	48.0	37.0

yields were too low from pig striatum (2 μg/200 g of striatum) to obtain purification of the less abundant striatal ACE in sufficient yield for further studies. When the 2.8 nm spacer arm was employed, a much improved yield was obtained (62 μg from 156 g of striatum). This modified affinity resin was also effective for purification of the enzyme from kidney. Table 1 lists the results from a typical purification schedule of the striatal enzyme. The activity was purified 53000-fold with an overall yield of 13%. When the purified enzyme was subjected to SDS/PAGE, two polypeptide bands (M_r 180000 and 170000) were detected (Fig. 1). The larger polypeptide migrated identically with pig kidney ACE. Both polypeptides were recognized in Western blots by a polyclonal antiserum to pig kidney ACE (Fig. 2). The antibody recognized only a single polypeptide in a preparation of kidney microvillar membranes.

Further attempts to resolve the striatal 180000 and 170000 proteins have proved unsuccessful. No separation of the two proteins was obtained by f.p.l.c. gel filtration on Superose 12 in the presence of 0.3 M-NaCl and 0.2% emulphogen, nor by f.p.l.c. anion-exchange chromatography at pH 7.3 on MonoQ resin in the presence of 0.2% emulphogen. Partial separation of protein could be achieved by f.p.l.c. chromatofocusing in the range pH 4–7 on Pharmacia PBE94 but, under these conditions of separation, little activity was detectable since ACE was found to inactivate very rapidly below pH 6.0. Captopril, lisinopril and enalaprilat, all at 1 μM, virtually completely inhibited (> 98%) the hydrolysis of BzGly-His-Leu and of [D-Ala[2],Leu[5]]enkephalin by striatal ACE. The purified enzyme contained no detectable endopeptidase-24.11 nor aminopeptidase activities.

Inhibitor sensitivity of striatal and kidney ACE. The inhibitor sensitivity of striatal and kidney ACE with a variety of specific and non-specific enzyme inhibitors was compared. No significant differences were apparent. Some inhibitors were explored in more detail. Inhibition curves for the enzyme from the two different tissues were, in general, very similar (Fig. 3). However, lisinopril appeared to be somewhat more potent as an inhibitor of the striatal than of the kidney enzyme (Table 2).

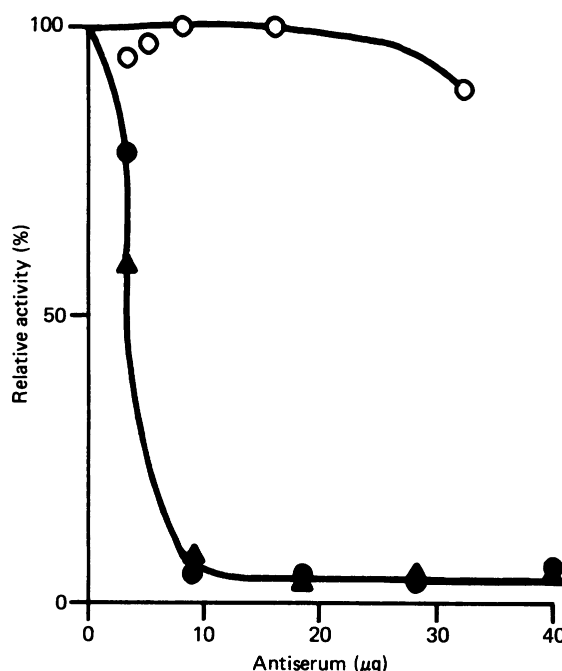

Fig. 4. Immunoprecipitation of pig kidney and striatal ACE

Pig kidney or pig striatal ACE were incubated at 4 °C for 18 h with increasing amounts of a polyclonal antibody raised to pig kidney ACE. After precipitation, enzyme was incubated with substrate (BzGly-His-Leu, 50 mM) and the reaction product (Hip) quantified by h.p.l.c. Each point is the mean of duplicates. ●, Kidney ACE with immune serum; ▲, striatal ACE with immune serum; ○, kidney ACE with pre-immune serum.

Immunoprecipitation of affinity-purified kidney and striatal ACE. Fig. 4 compares the ability of a polyclonal antibody to pig kidney ACE to precipitate purified preparations of ACE from pig kidney and striatum. The immunoprecipitation curves were essentially identical. No precipitation occurred with pre-immune serum.

Hydrolysis of tachykinins by kidney and striatal ACE. Both kidney and striatal ACE were able to hydrolyse substance P and the peptide products are compared in Fig. 5. A similar pattern of metabolism was seen in both cases, consistent with a primary cleavage at the Phe[8]-Gly[9] bond followed by successive dipeptide cleavages from the newly formed *C*-terminus. Cleavage of the Gly[9]-Leu[10] bond was relatively minor. The identity of the peptide products is listed in Table 3. Negligible hydrolysis of neurokinins A and B occurred after 18 h incubation at 37 °C with either kidney ACE (100 ng) or striatal ACE (200 ng).

Hydrolysis of other peptides by kidney and striatal ACE. No differences in the pattern of hydrolysis, or the relative rates of hydrolysis, of several amidated and non-amidated peptides could be detected between kidney and striatal ACE. The identified sites of cleavage are shown in Table 4.

Deglycosylation of kidney and striatal ACE. Samples of kidney and striatal ACE were deglycosylated both by

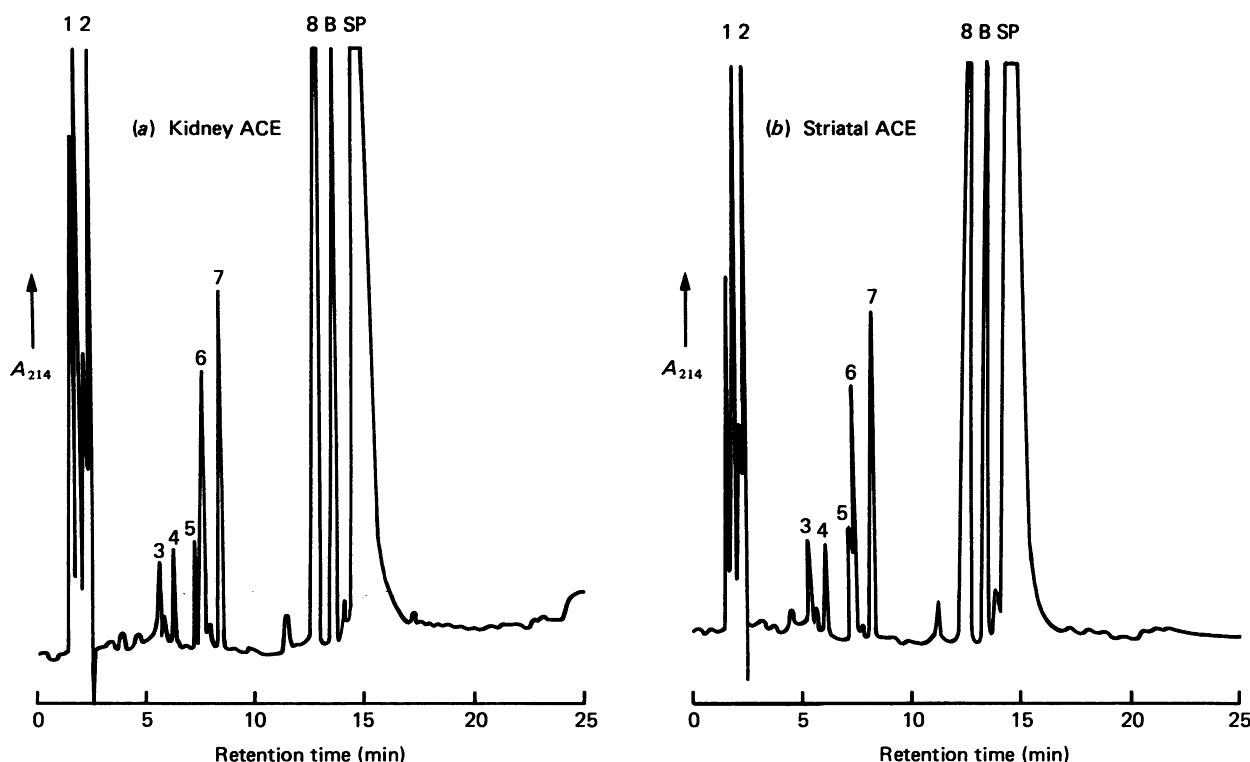

Fig. 5. Hydrolysis of substance P by (a) pig kidney ACE and (b) striatal ACE

Pig kidney ACE (100 ng) or pig striatal ACE (200 ng) was incubated with substance P (5 mM) for 18 h at 37 °C. Phosphoramidon (10 μM) and bestatin (100 μM) were present. Products were resolved by h.p.l.c. and monitored at 214 nm. SP, substance P; B, bestatin. Numbers correspond to the peptide fragments in Table 3.

Table 3. Products released by incubation of substance P with ACE

The products were identified by amino acid analysis. The one-letter code for amino acids [see Biochem. J. (1984) **219**, 366–368] is used. The peak numbers refer to Fig. 5. All incubations occurred in the presence of 100 μM-bestatin (B) and 1 μM-phosphoramidon. No hydrolysis occurred in the presence of 10 μM-enalaprilat. NH_2 at the *C*-terminus indicates an amidated residue. n.d., not defined; SP, substance P.

Peak no.	Retention time (min)	Peptide identified	Fragment of substance P
1	1.66	n.d.	1–4 (?)*
2	2.24	R-P-K-P-Q-Q	1–6
3	5.40	L-MNH_2	10–11
4	6.08	F-G	8–9
5	7.22	n.d.	1–7 (?)*
6	7.42	Q-F	6–7
7	8.22	G-L-MNH_2	9–11
8	12.48	F-F	7–8
SP	14.26	R-P-K-P-Q-Q-F-F-G-L-MNH_2	

* Fragments (1–4) and (1–7) are tentatively assigned.

enzymic (*N*-glycanase) and chemical (TFMS) methods. The products were analysed, after deglycosylation, by SDS/PAGE and proteins were detected by silver staining (Fig. 1). In both cases only a single deglycosylated form of ACE was detected, with apparent M_r 150000. The M_r 180000 form of ACE was estimated to contain approx. 17% carbohydrate, consistent with the reported degree of glycosylation of ACE in other tissues.

DISCUSSION

Angiotensin converting enzyme has been purified from pig striatum, a brain region enriched in the tachykinin peptides, substance P and neurokinin A. The enzyme was purified essentially in a single step with the inhibitor lisinopril used as an affinity ligand. The construction of an affinity resin in which lisinopril was separated from

Table 4. Sites of hydrolysis of peptides by ACE

The scissile bonds were identified as described previously (Matsas *et al.*, 1984; Hooper *et al.*, 1985; Hooper & Turner, 1985). No difference in the pattern of hydrolysis, or in the relative rates of hydrolysis, was observed between kidney and striatal ACE. ↑ and ⇡ indicate primary and secondary sites of hydrolysis, respectively. dA, D-alanyl residue. No significant hydrolysis of neurokinin A, neurokinin B, SP(9–11) was detectable. Hydrolysis of each peptide was completely inhibited by enalaprilat (10 μM). The data are documented for one peptide (substance P) in Fig. 5 and Table 3.

Peptide	Sites of hydrolysis
Non-amidated	
Angiotensin I	D - R - V - Y - I - H - P - F ↓ H - L
Bradykinin	R - P - P - G - F ⇣ S - P ↓ F - R
BzGly-His-Leu	BzG ↓ H - L
[Leu⁵]Enkephalin	Y - G - G ↓ F - L
[Met⁵]Enkephalin	Y - G - G ↓ F - M
[D-Ala²,Leu⁵]Enkephalin	Y -dA - G ↓ F - L
Neurotensin	<E - L - Y - E - N - K - P - R - R - P - Y ↓ I - L
Substance P (deamidated)	R - P - K - P - Q ⇣ Q - F ⇣ F - G ↓ L - M
Amidated	
Substance P	R - P - K - P ⇣ Q ⇡ Q ⇣ F ⇡ F ↓ G ↑ L - MNH_2
SP(3–11)	K - P - Q - Q ⇣ F - F ↓ G ↑ L - MNH_2
SP(5–11)	Q - Q ⇣ F - F ↓ G ↑ L - MNH_2
SP(7–11)	F - F - G ↓ L - MNH_2
SP(8–11)	F - G ↓ L - MNH_2
[Leu⁵]Enkephalinamide	Y - G - G ↓ F - LNH_2
[D-Ala²,Leu⁵]Enkephalinamide	Y -dA - G ↓ F - LNH_2
LH-RH	<E - H - W ↓ S - Y ⇣ G - L ↓ R - P - GNH_2

the matrix by a 2.8 nm spacer arm provided a greater recovery of activity (13% as opposed to < 1%) than did the affinity resin described by Bull *et al.* (1985), which utilized a 1.4 nm spacer arm. The increased yield was critical to obtain sufficient striatal enzyme for further studies. The resin was effective for purification of ACE both from striatum and from kidney. The efficiency of purification of ACE by the affinity resin with 2.8 nm spacer arm is evident from tracks 1 and 2 of Fig. 1. After affinity chromatography, the polypeptide of M_r 180000 in kidney membrane preparations that corresponds to ACE is completely adsorbed and is not visible in the non-adsorbed ('run-through') eluate from the column. No other polypeptides appear to be removed by the affinity resin. Pantoliano *et al.* (1984) have previously shown that a 2.8 nm spacer arm appears optimal for affinity purification of ACE and have speculated that the active site may be located deep in a cleft within the enzyme.

Two forms of striatal ACE were obtained after affinity chromatography (Fig. 1). These forms differed in M_r by approx. 10000, as reported previously for rat brain ACE (Strittmatter *et al.*, 1985). The larger form (M_r 180000) was indistinguishable from pig kidney ACE on SDS/PAGE. The same two forms were obtained after purification in the presence of protease inhibitors (data not shown). Both forms of the pig brain enzyme were recognized by a polyclonal antiserum to pig kidney ACE (Figs. 2 and 3) but we have been unable to resolve the two forms in an enzymically active state. Strittmatter *et al.* (1985) have claimed that the rat brain isoenzyme of ACE shows a unique specificity for amidated substrates (e.g. substance P and neurokinin A) and have therefore speculated that this isoenzyme may play a role in inactivation of such peptides in the brain. We could obtain no such evidence for specificity differences between the pig brain and kidney enzymes. The pattern of hydrolysis of substance P by both enzymes was

identical, with the major cleavage occurring at the Phe[8]-Gly[9] bond followed by the successive removal of dipeptides from the new *C*-terminus (Fig. 5). Hydrolysis at the Gly[9]-Leu[10] bond was a minor cleavage (approx. 20% of initial rate of cleavage of the Phe[8]-Gly[9] bond). This pattern of hydrolysis of substance P is identical with that originally reported for ACE from rat brain and rabbit lung (Yokosawa *et al.*, 1983; Cascieri *et al.*, 1984). *C*-Terminal fragments of substance P could also be degraded by ACE (Table 4). The specificity of cleavage varied with chain length of the substance P fragments. The *C*-terminal tripeptide (Gly-Leu-MetNH$_2$) was not cleaved. SP(8-11)-fragment and SP(7-11)-fragment were hydrolysed at the Gly-Leu bond with release of Leu-MetNH$_2$. Larger fragments of substance P were predominantly cleaved at the Phe-Gly bond, releasing the tripeptide Gly-Leu-MetNH$_2$, although some release of Leu-MetNH$_2$ was also detectable. ACE also released only dipeptides from the two amidated enkephalins studied, suggesting that shorter amidated peptides (pentapeptides or tetrapeptides) are cleaved by ACE to release *C*-terminal dipeptides, whereas longer peptides are cleaved to release tripeptides, presumably due to a difference in the mode of binding of substrate at the active site. A possible mechanism for the binding and hydrolysis of substance P by ACE has been suggested by Skidgel *et al.* (1984). The closely related peptides neurokinin A and neurokinin B were not significantly hydrolysed by pig kidney or striatal ACE, even after 18 h incubation. Thus, although two forms of striatal ACE which differ in molecular size can be obtained from striatum, we are unable to support the suggestion (Strittmatter *et al.*, 1985) that the striatal enzyme shows a unique specificity for amidated substrates, at least in the pig. No other specificity differences between kidney and striatal ACE were observed with any of the other peptides studied (Table 4).

Several possibilities may account for the observed size differences seen with striatal ACE. The two forms may be isoenzymes synthesized as separate gene products. There is a precedent for this suggestion in that testicular ACE is clearly the product of a different mRNA from that produced in lung and kidney (El-Dorry *et al.*, 1982). Alternatively, the two forms may arise as a result of post-translational modifications or, artifactually, as a result of proteolysis during the purification procedure. The latter possibility was minimized by the inclusion during the purification of a 'cocktail' of protease inhibitors. The same two polypeptides of M_r 180000 and 170000 were obtained under these conditions.

In view of the failure to detect any specificity differences between pig kidney and striatal ACE, we believed that differences in post-translational processing between kidney and brain could best account for the two forms of the enzyme observed. Indeed, endopeptidase-24.11 has previously been shown to differ in size between kidney, intestine and brain as a result of differential glycosylation in these tissues (Relton *et al.*, 1983). Two forms of endopeptidase-24.11 differing in their degree of glycosylation have been observed in human placental membranes (Johnson *et al.*, 1984).

Kidney and striatal ACE were therefore deglycosylated both enzymically and chemically and then compared by SDS/PAGE. After deglycosylation, only a single band of pig striatal ACE (M_r 150000) was detectable, which migrated identically on electrophoresis with the deglycosylated kidney enzyme (Fig. 1). Similar results were obtained with *N*-glycanase and TFMS, suggesting that the bulk of the carbohydrate in the enzyme is present as *N*-linked sugars. These results imply that the differences in size between pig kidney and striatal ACE are predominantly, if not exclusively, due to differential glycosylation, as is the case with endopeptidase-24.11, in different cell types. We believe, therefore, that it is premature to describe brain and kidney ACE as isoenzymes, and to ascribe a unique specificity for amidated substrates to brain ACE.

Since the $k_{cat.}/K_m$ for the hydrolysis of substance P by ACE is several hundred-fold lower than for the reaction catalysed by endopeptidase-24.11 (Matsas *et al.*, 1985*b*), we have previously suggested that only in those brain regions or tissues where the concentration of ACE substantially exceeds that of the endopeptidase will the former enzyme contribute to any significant extent to substance P hydrolysis. In membranes prepared from pig striatum, and from human striatum and diencephalon, the major contributor to substance P hydrolysis was found to be endopeptidase-24.11 and not ACE (Matsas *et al.*, 1983, 1985*b*). Endopeptidase-24.11 also hydrolyses the other mammalian tachykinin peptides, neurokinin A and neurokinin B (Hooper *et al.*, 1985; Hooper & Turner, 1985). Our present data, therefore, still support the hypothesis that endopeptidase-24.11 is the predominant cell surface peptidase involved in tachykinin metabolism in brain. More direct physiological studies employing selective enzyme inhibitors will be required to confirm this hypothesis. The endogenous peptide substrate(s) for ACE in the brain remain to be identified.

Note added in proof (received 5 November 1986)

The *N*-Terminal sequences of pig kidney and striatal ACE were found to be identical: L-D-S-A-L-.

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