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Purification and characterization of human brain prolyl endopeptidase

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Prolyl endopeptidase (EC 3.4.21.26) was purified from human brain by a series of column-chromatographic steps using DEAE-cellulose DE-52, hydroxyapatite, phenyl-Sepharose, Sephacryl S-200 and f.p.l.c. (Mono Q). The enzyme was purified by a factor of 943 and was homogeneous in a SDS/polyacrylamide gel as judged by Coomassie Blue staining. The M_r estimated by SDS/PAGE is 79600, and under native conditions on Sephacryl S-200 it is 85600. Therefore the enzyme exists as a monomer. With benzyloxycarbonylglycylproline *p*-nitroanilide as substrate, the optimum pH of the enzyme is 6.8, and with the substrate concentration between 0.059 mM and 0.37 mM the K_m is 9.0×10^{-4} M. The pI of the enzyme is 4.75. The enzyme is classified as a serine proteinase, as it is strongly inhibited by di-isopropyl fluorophosphate. However, other serine proteinase inhibitors do not inhibit the enzyme significantly, suggesting that the active site of prolyl endopeptidase differs from that of classical serine proteinases such as trypsin. Polyclonal antibodies were raised against purified human brain prolyl endopeptidase in rabbits. Western-blot analysis, enzyme-inhibition assays, antibody binding and immunoprecipitation experiments indicated that the polyclonal antibodies are both specific and inhibitory to the enzyme activity.

INTRODUCTION

Many biologically active peptides contain proline at their *N*-terminus or *C*-terminus or within their amino acid sequence (Turner, 1986). Proline is important both in determining molecular conformation and in conferring resistance to the action of proteinases. An enzyme activity that hydrolyses peptide bonds on the carboxyl side of L-proline residues was first found as an oxytocin-inactivating enzyme in human uterus (Walter *et al.*, 1971). Koida & Walter (1976) partially purified this enzyme using an affinity column and named it post-proline-cleaving enzyme. This enzyme has since been renamed prolyl endopeptidase (EC 3.4.21.26) and has been isolated from various tissues such as rabbit brain (Orlowski *et al.*, 1979; Carvalho & Camargo, 1981), rat brain (Rupnow *et al.*, 1979), lamb brain (Yoshimoto *et al.*, 1981), bovine brain (Yoshimoto *et al.*, 1983), pig liver (Moriyama & Sasaki, 1983), pig muscle (Moriyama *et al.*, 1988) and human placenta (Mizutani *et al.*, 1984). An enzyme with the same specificity as prolyl endopeptidase has been purified from micro-organisms, plants and mushrooms (Yoshimoto *et al.*, 1980, 1987, 1988).

Mammalian prolyl endopeptidase has been proposed to play a role in neuropeptide metabolism (Wilk, 1983), since the enzyme isolated from various tissues has been shown *in vitro* to degrade substance P, neurotensin, luteinizing-hormone-releasing hormone, thyrotropin-releasing hormone, bradykinin and angiotension II (Knisatschek & Bauer, 1979; Orlowski *et al.*, 1979; Taylor & Dixon, 1980; Camargo *et al.*, 1984). Substance P is cleaved into two biologically active fragments (Blumberg *et al.*, 1980). In the central nervous system, angiotensin (1–7), which is a potent stimulator of [arginine]vasopressin release from the hypothalamus, is most likely formed from angiotensin II by the action of prolyl endopeptidase (Schiavone *et al.*, 1988).

Interestingly, prolyl endopeptidase purified from pig muscle did not hydrolyse proteins of high M_r such as albumin, IgG, elastin, collagen and soluble and insoluble muscle proteins (Moriyama *et al.*, 1988). However, a few peptides of less than 3 kDa isolated from muscle extract was efficiently degraded by prolyl endopeptidase, suggesting that these peptides could be endogenous substrates for the enzyme (Moriyama *et al.*, 1988). Pratt *et al.* (1989) showed that loss of prolyl endopeptidase activity in intact HeLa cells after heat-shock treatment at 45 °C correlated with the decreased degradation of injected ubiquitin and BSA. This could suggest a possible role for this enzyme during heat-shock response in cells. Here we report the purification to homogeneity and extensive characterization of human brain prolyl endopeptidase as a first step in elucidating the structure and role of this enzyme.

EXPERIMENTAL

Materials

Z-Gly-Pro-pNA and Succ-Gly-Pro-pNA were purchased from BACHEM Fine Chemical Co. All chromatography materials were from Pharmacia, except DEAE-cellulose DE-52, which was from Whatman, and hydroxyapatite, which was from Bio-Rad. The f.p.l.c. Mono Q column and isoelectrofocusing standard protein markers were from Pharmacia. ZPCK, TLCK, TPCK, 3,4-dichloroisocoumarin, pAPMSF, actinonin, amastatin, pepstatin, antipain, phosphoramidon, E-64, bestatin and Thr(tBu)-Phe-Pro were from Protogen A.G. PMSF, iodoacetamide, BSA and 4-chloronaphthol were from Sigma. Aprotinin, trypsin inhibitor (from chicken-egg white), leupeptin and α_2-macroglobulin were from Boehringer Mannheim. Iodo-Beads were bought from Pierce. ^{125}I and DFP were bought from Amersham. Protein A–Sepharose 4B was from BRL and 2,6-lutidine from Aldrich.

Abbreviations used: Succ, 7-(succinyl-); pNA, *p*-nitroaniline; ZPCK, Z-phenylalanylchloromethane; TLCK, tosyl-lysylchloromethane; TPCK, tosyl-phenylalanylchloromethane; pAPMSF (4-amidinophenyl)methanesulphonyl fluoride; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; DFP, di-isopropyl fluorophosphate; IC_{50}, concentration of inhibitor which decreases the activity of the enzyme by a factor of 2.

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Assay for enzyme activity, kinetic studies and effect of various metal ions and proteinase inhibitors

Enzyme activity was measured by reading the absorbance of pNA released from substrate Z-Gly-Pro-pNA at 410 nm. A sample of a fraction after chromatographic separation was added to 500 μl of 0.05 M-sodium phosphate, pH 7.0, containing 1 mM-DTT and 1 mM-EDTA. Then 125 μl of 2 mM-Z-Gly-Pro-pNA in 40% (v/v) dioxan was added. The reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by addition of 1.0 ml of Triton X-100 solution (10% Triton/1 M-sodium acetate buffer, pH 4.0). The A_{410} of the sample was then read against a blank. One unit of enzyme is defined as the amount of enzyme at 37 °C which produces 1 μmol of pNA per min from Z-Gly-Pro-pNA at pH 7.0. The affinity (K_m) of the enzyme was determined by using substrate concentrations ranging from 0.059 mM to 0.37 mM and from 0.25 mM to 0.5 mM. The K_m was obtained by using a Lineweaver–Burk plot. The effect of inhibitors and metal ions on prolyl endopeptidase activity was determined by incubating the enzyme with various concentrations and types of inhibitors at 37 °C for 10 min in 0.05 M-sodium phosphate, pH 6.8, 1 mM-EDTA and 1 mM-DTT. DTT was not added to the buffer when the inhibitors E-64 and iodoacetamide were used, and EDTA were excluded from the buffer when metal ions were assessed. The assays were done in duplicate for each inhibitor and the mean value was recorded.

PAGE, isoelectric focusing and Western-blot analysis

SDS/PAGE was performed as outlined by Laemmli (1970). Native PAGE was carried out by the method of Taber & Sherman (1964). A 7.5% polyacrylamide gel was electrophoresed in 0.1 M-glycine/lutidine buffer, pH 6.7, at 5 mA constant current. Following electrophoresis, the polyacrylamide gel was sliced into 1.5 mm rectangular slices and assayed for activity as described above. Western-blot analysis was carried out as described in Towbin *et al.* (1979). Non-denaturing isoelectric focusing was performed by the procedure of Robertson *et al.* (1987), in a dual vertical minigel apparatus (Bio-Rad).

M_r determination

Native M_r was determined by gel filtration on a Sephacryl S-200 column (1.5 cm × 120 cm) as described by Andrews (1965). Subunit M_r was determined by SDS/PAGE by the method of Weber & Osborn (1969), by using high-M_r protein markers as standard.

Purification of prolyl endopeptidase

All purification steps are described here and, unless stated, they were carried out at 4 °C. All buffers contained 1 mM-DTT and 1 mM-EDTA unless otherwise stated. After each chromatographic step, fractions were assayed for prolyl endopeptidase activity, and positive fractions were pooled.

A human brain weighing 1.4 kg was obtained from the mortuary. All blood vessels were removed and the brain was washed in sodium phosphate buffer, pH 7.0, before homogenization. The homogenization was carried out in a Waring blender in 3576 ml of 0.25 M-sucrose, pH 7.0, for 1 min. The supernatant was retained. The pH of the supernatant was adjusted to pH 5.0 by addition of 0.5 M-acetic acid. The supernatant was then left for 90 min at 4 °C. The supernatant was centrifuged at 8000 *g* for 30 min. The precipitate was discarded. The pH of the supernatant was adjusted to 7.5 by addition of 0.5 M-NaOH. $(NH_4)_2SO_4$ (679.5 g) was added slowly to 2945 ml of the supernatant to give 40% saturation. After standing at 4 °C for 60 min, the supernatant was collected by centrifugation. The supernatant was adjusted to 80% $(NH_4)_2SO_4$ saturation by addition of 887.7 g of $(NH_4)_2SO_4$ to 3115 ml of supernatant. The saturated supernatant was left for 2 h at 4 °C without stirring. It was then centrifuged at 8000 *g* for 20 min and the supernatant discarded. The precipitate was resuspended in 400 ml of 0.05 M-Tris/HCl, pH 7.0. The resuspended precipitate was dialysed with 4 × 4 litres of the same buffer.

Dialysed enzyme from $(NH_4)_2SO_4$ fractionation (200 ml) was loaded on to a DEAE-cellulose DE-52 column (1.5 cm × 40 cm) which had been equilibrated with 0.05 M-Tris/HCl, pH 7.0. Equilibration buffer was passed through until no protein could be detected in the unbound fraction. The bound fraction was eluted with a linear gradient of increasing NaCl concentration to 0.3 M. Fractions were assayed for activity, and those showing prolyl endopeptidase activity were pooled and dialysed against 5 mM-sodium phosphate, pH 7.0.

The dialysed protein from the ion-exchange-chromatographic step was applied to a hydroxyapatite column (1.5 cm × 40 cm) which had been equilibrated in 5 mM-sodium phosphate, pH 7.0. Equilibration buffer was passed until no unbound fraction could be detected. The bound protein was eluted with a linear gradient of increasing phosphate concentration to 150 mM. The fractions containing enzyme were dialysed in 0.01 M-sodium phosphate, pH 7.0, containing 25% (w/v) $(NH_4)_2SO_4$.

Dialysed fractions with prolyl endopeptidase activity from hydroxyapatite chromatography were loaded on to a phenyl-Sepharose column (1.5 cm × 40 cm) which had been equilibrated with 0.01 M-sodium phosphate, pH 7.0, containing 25% $(NH_4)_2SO_4$. The starting buffer was passed through until no unbound fraction could be detected. The bound protein was eluted with a linear gradient of increasing ethylene glycol concentration to 50% (v/v) and a decreasing $(NH_4)_2SO_4$ concentration to zero. The pooled fractions from the phenyl-Sepharose column were passed through a gel-filtration column (1.5 cm × 120 cm) of Sephacryl S-200 with 0.05 M-Tris/HCl, pH 7.0.

Fractions containing prolyl endopeptidase activity after gel filtration were concentrated, dialysed and loaded on to an f.p.l.c. Mono Q column, which had been equilibrated with 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.0, buffer. Equilibration buffer was passed through for 10 min after loading the column to remove the unbound fraction, and the bound protein was eluted with a linear gradient of increasing NaCl concentration to 0.25 M. Positive fractions were pooled and re-fractionated on an f.p.l.c. Mono Q column under similar conditions.

Raising of polyclonal antibodies, iodination and immunoprecipitation of human brain prolyl endopeptidase

Polyclonal antibodies to human brain prolyl endopeptidase were raised in rabbits. Purified enzyme (50 μg) was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously. Every 2 weeks, 50 μg of purified human brain prolyl endopeptidase was mixed with an equal volume of incomplete Freund's adjuvant and injected again. After 3 months, the rabbits were bled and the IgG fraction was purified by using Protein A–Sepharose CL-4B. Purified human brain prolyl endopeptidase was labelled by using Iodo-Beads. The iodination was carried out as described by Markwell (1982). For immunoprecipitation studies, ^{125}I-labelled human brain prolyl endopeptidase (about 50000 c.p.m.) was incubated overnight with an equal volume of purified IgG polyclonal antibodies at 4 °C with constant shaking. Protein A–Sepharose CL-4B (0.1 mg/ml) was swelled overnight in immunoprecipitation buffer (50 mM-Tris, pH 7.5, 150 mM-NaCl, 2% Triton X-100). Protein A–Sepharose CL-4B (100 μl) was added to each tube and left for

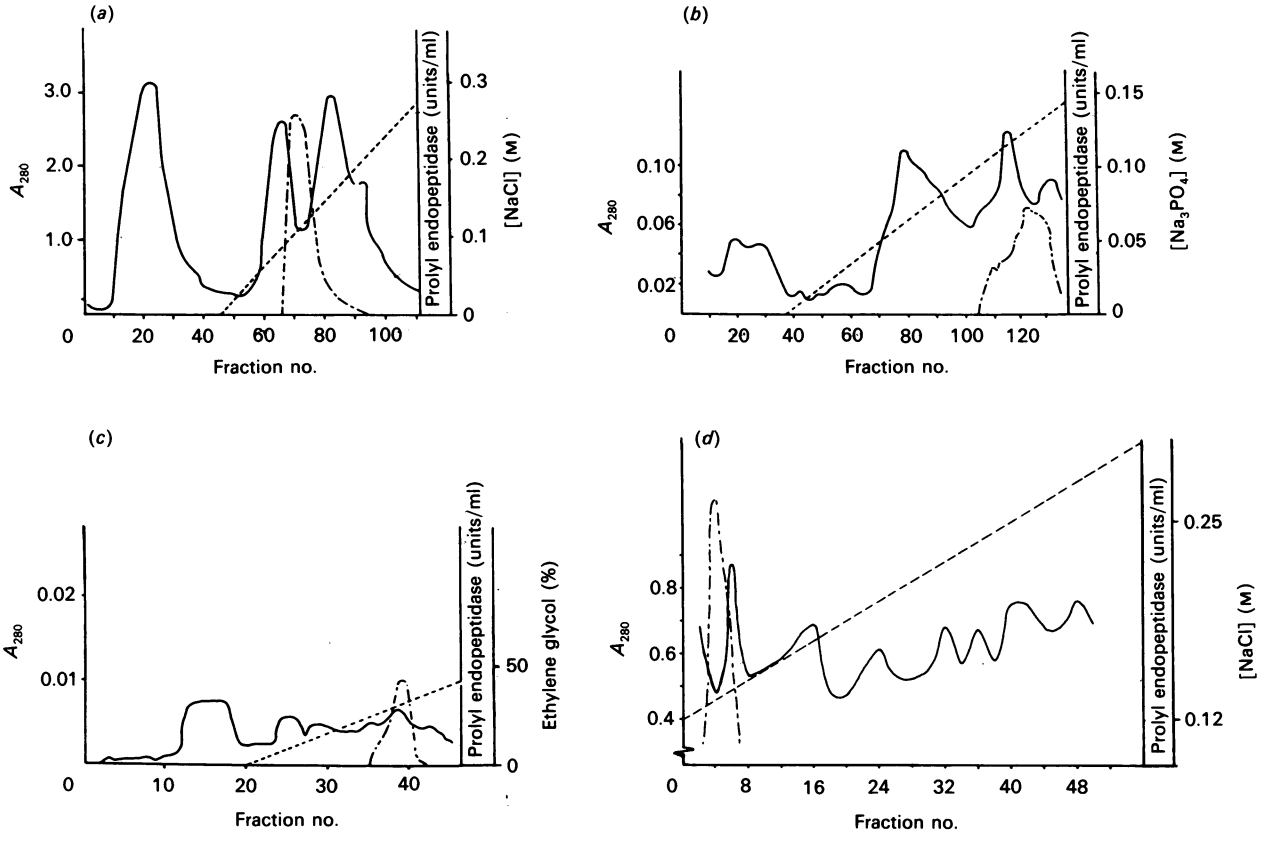

Fig. 1. Purification of prolyl endopeptidase activity by various chromatographic steps

(*a*) DEAE-cellulose DE-52 ion-exchange chromatography; (*b*) hydroxyapatite chromatography; (*c*) phenyl-Sepharose chromatography; (*d*) f.p.l.c. Mono Q chromatography. Prolyl endopeptidase activity is represented by —·—, protein concentration by —— and gradient by ----. A unit of activity represents 1 μmol of pNA released per min at 37 °C from Z-Gly-Pro-pNA.

1 h at room temperature with constant shaking. The Protein A–Sepharose CL-4B was then pelleted, and the pellet was washed three times with immunoprecipitation buffer and three times with 10 mM-Tris, pH 7.4. The pellet was resuspended in loading buffer and electrophoresed in a SDS/7.5%-polyacrylamide gel. The dried gels were exposed overnight to Kodak X-Omat AR film.

RESULTS

Purification of prolyl endopeptidase

Prolyl endopeptidase was purified from human brain by a combination of f.p.l.c. and classical column-chromatographic procedures (Fig. 1). The homogenate was assayed for prolyl endopeptidase activity, and 1 μM-Z-Pro-Prolinal, a specific inhibitor of prolyl endopeptidase (Friedman *et al.*, 1983), completely inhibited all activity. Purification by selective precipitation was carried out with acetic acid and $(NH_4)_2SO_4$. The first chromatographic separation was performed with a DEAE-cellulose DE-52 ion-exchange column. Subsequent separations were performed with hydroxyapatite, phenyl-Sepharose and Sephacryl S-200. However, the enzyme was still not homogeneous as judged by SDS/PAGE. An f.p.l.c. Mono Q column was subsequently used and gave good resolution and recovery, but with a limitation of low loading capacity. The enzyme activity and protein profile were analysed after each chromatography step, and a summary is shown in Fig. 1. The purified protein was assessed for homogeneity after the f.p.l.c. Mono Q chromatographic step by SDS/PAGE (Fig. 2). Coomassie Blue staining revealed that the purified protein migrated as a single band at 80 kDa (Fig. 2, lane 4). Silver staining did not reveal any other bands apart from a minor band at 55 kDa (Fig. 2, lane 6), which

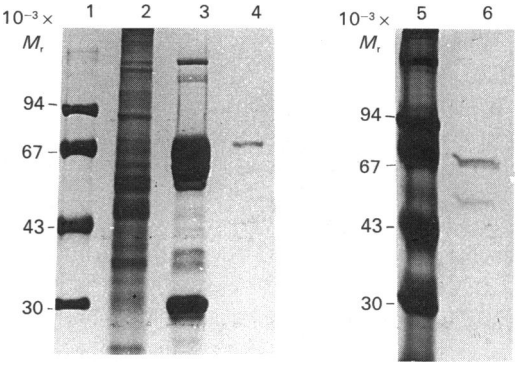

Fig. 2. SDS/PAGE of human brain prolyl endopeptidase

The enzyme purified from human brain was electrophoresed in a 0.1%-SDS/7.5%-polyacrylamide gel. Left panel, Coomassie Blue stain. Lane 1, M_r markers. Lane 2, 50 μg of human brain homogenate. Lane 3, 50 μg of protein fraction after phenyl-Sepharose chromatography. Lane 4, 5 μg of purified human brain prolyl endopeptidase after f.p.l.c. Mono Q chromatography. Lanes 5 and 6 are M_r markers and purified human brain prolyl endopeptidase respectively after silver staining of the SDS/PAGE gel.

Table 1. Purification of human brain prolyl endopeptidase

Enzyme activity was assayed with Z-Gly-Pro-pNA as substrate. One unit of activity is defined as the amount of enzyme which produces 1 μmol of pNA per min at 37 °C.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor (fold)	Recovery (%)
1. Homogenate	4565.0	228.3	66192.5	0.003		
2. Supernatant	2945.0	147.3	1222.3	0.012	4.0	64.5
3. pH 5.0 (acetic acid)	2800.0	115.1	6860.0	0.017	5.7	50.4
4. 40%-$(NH_4)_2SO_4$ ppt.	3115.0	103.0	4672.5	0.022	7.3	45.1
5. 80%-$(NH_4)_2SO_4$ ppt.	405.0	93.2	3347.0	0.027	9.0	40.8
6. DEAE -cellulose DE52	82.0	46.6	594.4	0.078	26.1	20.4
7. Hydroxyapatite	103.0	16.0	23.2	0.690	230.0	7.0
8. Phenyl-Sepharose	94.0	14.5	11.8	1.23	410.0	6.2
9. Sephacryl S-200	150.0	14.3	10.5	1.36	454.3	6.3
10. Mono Q	48.0	7.2	3.8	1.88	626.7	3.2
11. Mono Q	16.0	2.7	1.0	2.83	943.3	1.2

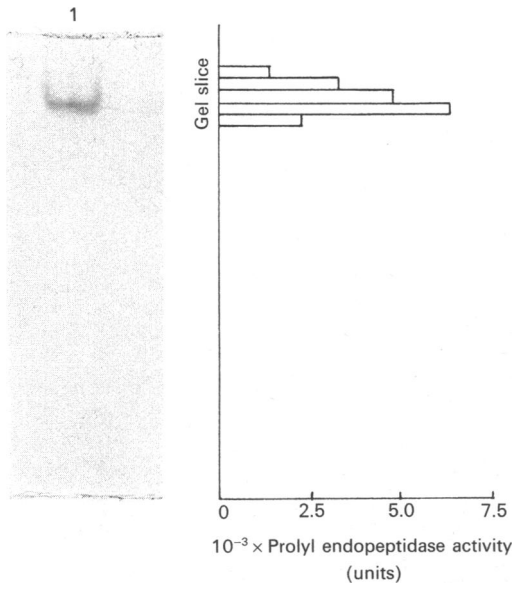

Fig. 3. Native PAGE of human brain prolyl endopeptidase

Purified human brain prolyl endopeptidase (5 μg) was electrophoresed in a 7.5%-polyacrylamide gel in 0.1 M-glycine/lutidine buffer, pH 6.7. The panel on the right shows the enzyme activity from the gel slices with Z-Gly-Pro-pNA as substrate.

was not related to prolyl endopeptidase (see below). The enzyme was purified 943-fold with a recovery of 1.2%. Full details of the purification at each chromatographic step are summarized and presented in Table 1.

Physicochemical properties of prolyl endopeptidase

The subunit M_r of prolyl endopeptidase was determined by SDS/PAGE, using standard marker proteins, to be 79600 (Fig. 2). The native M_r of the enzyme was determined by its elution volume relative to that of standard protein markers to be 85600 (results not shown). Therefore the enzyme exists as a monomer. The activity of the purified enzyme was analysed in a native polyacrylamide gel containing 0.1 M-glycine/lutidine buffer, pH 6.7. Following electrophoresis, the gel was sliced and assayed for activity, while a corresponding lane was stained using Coomassie Blue (Fig. 3). The activity coincided with the stained band confirming the identity of the protein (Fig. 3). By using the synthetic substrate Z-Gly-Pro-pNA, the optimum pH was determined to be 6.8. The enzyme was relatively stable at 37 °C, retaining 60% of its activity after 90 min. At 50 °C, the enzyme lost 50% of its activity within 4 min, and at 60 °C the enzyme was totally inactivated within 4 min. The K_m value of the enzyme for Z-Gly-Pro-pNA over a concentration range of 0.059–0.37 mM was determined by Lineweaver–Burk plot to be 0.9 mM. The K_m for this substrate over a range of 0.25–0.5 mM was found to be 0.21 mM.

The K_m for another substrate, Succ-Gly-Pro-pNA, was found to be 2.56 mM over the substrate concentration range of 0.49–12.3 mM. The pI was determined to be 4.75 by using standard protein pI markers. The amino acid composition indicated that the enzyme is rich in lysine (95 mol/mol) and has five methionine residues, which should be useful in CNBr digestion of the enzyme to obtain fragments for internal amino acid sequence analysis.

Inhibitors of prolyl endopeptidase

The effects of metal ions and proteinase inhibitors on prolyl endopeptidase activity are summarized in Table 2. Zn^{2+} at 0.1 mM partially inhibited the enzyme, whereas Ca^{2+} inhibited the enzyme only at a concentration of 10 mM. Other metal ions did not significantly affect prolyl endopeptidase activity. At 1 mM, DTT consistently caused a 5% increase in activity of the enzyme (Table 2) and was included in all the buffers used in assays and purification of the enzyme. Consistent with activation of the enzyme by DTT, iodoacetamide at 5 mM partially inhibited the enzyme indicating that one or more cysteine groups might be important for enzymic activity (Table 2). However, E-64, another thiol-proteinase inhibitor, did not have any effect on enzyme activity even at 0.1 mM. DFP, a specific serine-proteinase inhibitor, was found to be the most effective inhibitor, exerting 40% inhibition at nanomolar concentrations (Table 2). TLCK and TPCK, which inhibit trypsin and also some thiol proteinases, were found to inhibit prolyl endopeptidase partially at 0.1 mM and 0.01 mM concentration respectively, whereas other serine-proteinase inhibitors, such as 3,4-dichloroisocoumarin and pAPMSF, failed to inhibit the enzyme (Table 2). Bestatin and Thr(tBu)-Phe-Pro, which are specific inhibitors of amino-peptidases, did not inhibit the enzyme even at high concentrations.

The purification of prolyl endopeptidase was monitored using a synthetic substrate, Z-Gly-Pro-pNA. Therefore the ability of

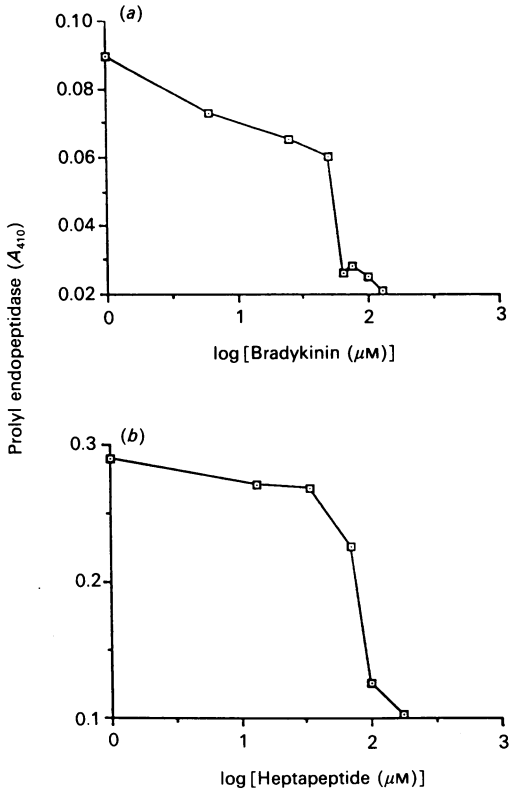

Fig. 4. Inhibition of hydrolysis of Z-Gly-Pro-pNA by human brain prolyl endopeptidase by (*a*) bradykinin and (*b*) des-Phe^8-Arg^9-bradykinin (heptapeptide)

A portion of enzyme (0.01 unit) was incubated with increasing concentrations of bradykinin or des-Phe^8-Arg^9-bradykinin. The remaining activity was assayed with Z-Gly-Pro-pNA. A graph of log concentration of substrate against absorbance is shown.

the purified enzyme to degrade the natural peptide bradykinin was investigated. Bradykinin was specifically cleaved into Arg-Pro-Pro-Gly-Phe-Ser-Pro and Phe-Arg as judged by comparison

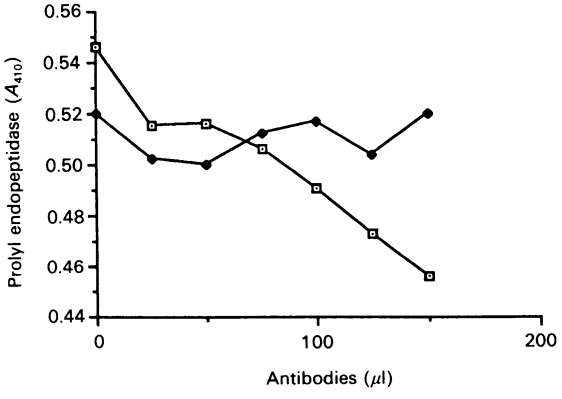

Fig. 5. Inhibition of prolyl endopeptidase activity by polyclonal antibodies

A portion of enzyme (0.05 unit) was incubated with increasing concentrations of the IgG-purified fraction of the polyclonal antibodies, which were diluted 4-fold before use. The quantity of antibodies was plotted against remaining activity. ◆, Control antibodies; ⊡, polyclonal antibodies against prolyl endopeptidase.

Table 2. Inhibition of human brain prolyl endopeptidase by various metal ions and proteinase inhibitors

Enzyme (0.01 unit) was incubated with each inhibitor for 10 min at 37 °C. The remaining activity was assayed with Z-Gly-Pro-pNA as substrate. No DTT was included when iodoacetamide and E-64 were assessed, and no EDTA was present when metal ions were assessed. These assays were carried out in duplicate and the mean values recorded. The inhibition did not vary by more than 5% for each duplicate.

Inhibitor	Concn. (mM)	Remaining activity (%)
ZPCK	10^{-1}	100
TPCK	10^{-4}	100
	10^{-3}	85
	10^{-2}	68
	10^{-1}	42
TLCK	10^{-2}	98
	10^{-1}	76
3,4-Dichloroisocoumarin	10^{-1}	100
pAPMSF	10^{-1}	100
PMSF	1.0	95
	10	78
Trypsin inhibitor (egg white)	1.0	100
	10	117
DFP	1.4×10^{-6}	70
	2.8×10^{-6}	40
α_2-Macroglobulin	10	92
DTT	1.0	105
	10	91
Iodoacetamide	1.0	96
	5.0	60
	10	51
E-64	10^{-1}	97
Actinonin	10^{-1}	100
Pepstatin	10^{-1}	100
Antipain	10^{-1}	98
Phosphoramidon	10^{-1}	100
Bestatin	10^{-1}	97
Thr(tBu)-Phe-Pro	10^{-1}	100
Amastatin	10^{-3}	88
	10^{-2}	85
Aprotinin	10	111
Leupeptin	1.0	98
	10	108
Ca^{2+}	1.0	91
	10	66
Zn^{2+}	10^{-1}	71
	1.0	41
	10	26

with authentic peptides by using h.p.l.c. (results not shown). Bradykinin strongly inhibited the hydrolysis of Z-Gly-Pro-pNA with an IC_{50} value of 53.1 μM (Fig. 4*a*). A heptapeptide derived from bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro, which has an internal cleavage site at Pro^3–Gly^4, also inhibited hydrolysis of Z-Gly-Pro-pNA with an IC_{50} value of 83.2 μM (Fig. 4*b*) (Orlowski *et al.*, 1979). Finally, Z-Pro-prolinal, a potent and specific inhibitor of prolyl endopeptidase (Friedman *et al.*, 1983), inhibited the human enzyme with an IC_{50} value of 0.6 nM, which is similar to the value obtained for the enzyme from bovine brain (0.74 nM) and also the enzymes isolated from other species (Tsuru *et al.*, 1988; Wilk, 1983).

Immunocharacterization of human brain prolyl endopeptidase

Polyclonal antibodies were raised against purified human brain prolyl endopeptidase in rabbits, and the IgG fraction was purified by using Protein A–Sepharose CL-4B. The polyclonal

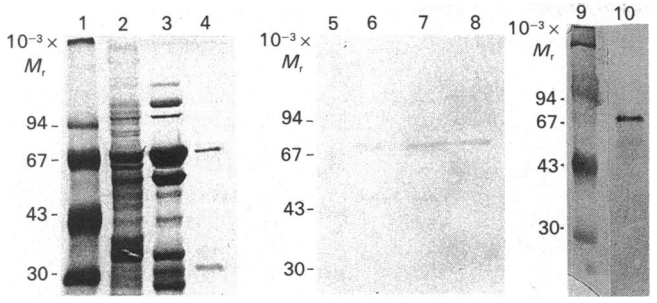

Fig. 6. Western-blot analysis of human brain prolyl endopeptidase

Lanes 1, 2, 3 and 4 show SDS/PAGE analysis, with corresponding Western-blot analysis of the blotted gel in lanes 5, 6, 7 and 8. Lane 1, M_r markers. Lane 2, protein fraction after DEAE-cellulose DE-52 ion exchange. Lane 3, protein fraction after phenyl-Sepharose chromatography. Lane 4, protein fraction after one f.p.l.c. Mono Q separation. Lane 5, M_r markers (rainbow markers). Lanes 6, 7 and 8 correspond to lanes 2, 3 and 4 after Western-blot analysis. Lanes 9 and 10, Western-blot analysis of the final purified enzyme. Lane 9, M_r markers (rainbow markers). Lane 10, purified human brain prolyl endopeptidase.

antibodies were found to inhibit the enzyme activity in a dose-dependent manner (Fig. 5). A Western-blot analysis was carried out with the DEAE-cellulose DE-52, phenyl-Sepharose and first Mono Q fractions, demonstrating that the antibodies are specific, recognizing the 79.6 kDa prolyl endopeptidase (Fig. 6, lanes 2, 3, 4, and corresponding Western blot in lanes 6, 7 and 8). A Western blot of purified prolyl endopeptidase is also shown (Fig. 6, lane 10). Immunoprecipitation analysis was carried out with a sample of ^{125}I-labelled human brain prolyl endopeptidase, and results indicated that prolyl endopeptidase was recognized by the polyclonal antibodies (Fig. 7, lane 2). To confirm specificity, it was shown in immunoprecipitation experiments that unlabelled purified human brain prolyl endopeptidase competed with the ^{125}I-labelled enzyme (Fig. 7, lanes 3 and 4). The polyclonal antibodies also cross-reacted with the placental enzyme (Fig. 7, lane 5), and likewise, unlabelled purified brain enzyme competed with the ^{125}I-labelled placental enzyme (Fig. 7, lane 7).

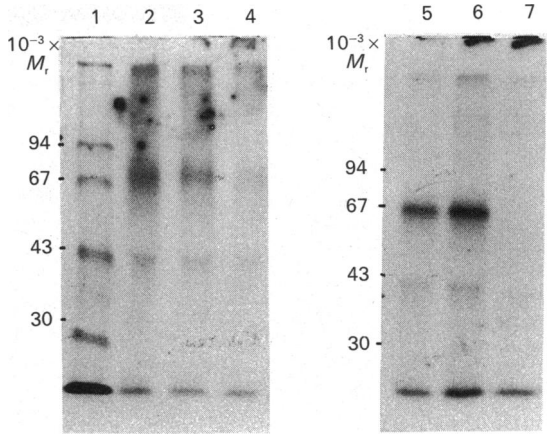

Fig. 7. Immunoprecipitation of human brain prolyl endopeptidase

Lanes 1, 2, 3 and 4 show immunoprecipitation with ^{125}I-labelled human brain prolyl endopeptidase. Lane 1, ^{14}C-labelled M_r markers. Lane 2, ^{125}I-labelled human brain prolyl endopeptidase. Lane 3, ^{125}I-labelled human brain prolyl endopeptidase preincubated with 0.1 μg of unlabelled enzyme. Lane 4, ^{125}I-labelled human brain prolyl endopeptidase preincubated with 1.0 μg of unlabelled enzyme. Lanes 5, 6 and 7, immunoprecipitation with ^{125}I-labelled human placental prolyl endopeptidase. Lane 5, ^{125}I-labelled human placental enzyme. Lane 6, ^{125}I-labelled human placental enzyme preincubated with 0.1 μg of unlabelled brain enzyme. Lane 7, ^{125}I-labelled human placental enzyme preincubated with 1.0 μg of unlabelled brain enzyme.

DISCUSSION

Prolyl endopeptidase was purified to homogeneity from human brain by a combination of both classical chromatography and f.p.l.c. The purified enzyme was homogeneous, as a single protein band of 80 kDa was seen after denaturing PAGE. Silver staining revealed another band of 55 kDa. Separation on a native polyacrylamide gel and assaying gel slices for ability to cleave Z-Gly-Pro-pNA confirmed that the purified enzyme is prolyl endopeptidase.

The molecular mass of the enzyme from lamb kidney was reported to be 115 kDa and the enzyme was suggested to be a

Table 3. Summary of various physicochemical properties of prolyl endopeptidases

Source	Molecular mass (kDa) (SDS/PAGE/gel filtration)	DFP Inhibition	pI	Reference
Rabbit brain	66	Yes	–	Orlowski *et al.* (1979)
	68	–	4.9	Oliveira *et al.* (1976)
	68–69	–	4.9	Carvalho & Carmargo (1981)
Bovine brain	76	Yes	4.8	Yoshimoto *et al.* (1983)
	62–65	–	–	Tate (1981)
	75	–	–	
Lamb kidney	115 (2 subunits of 57)	–	–	Koida & Walter (1976)
	74–77	Yes	4.9	Yoshimoto *et al.* (1981)
Lamb brain	74–77	Yes	4.8	Yoshimoto *et al.* (1981)
Rat brain	70–73.5	–	4.5	Rupnow *et al.* (1979)
Rat kidney	75	–	–	Hersh (1981)
Bovine anterior pituitary	76	Yes	–	Knisatchek & Bauer (1979)
Pig liver	72–74	Yes	4.9	Moriyama & Sasaki (1983)
Human placenta	67–140 (2 subunits)	Yes	4.75	Mizutani *et al.* (1984)
Human blood	78.5±2	Yes	4.7–4.8	Pratt *et al.* (1989)
Flavobacterium	74–76	Yes	9.6	Yoshimoto *et al.* (1980)
Lyophyllum cinerascens (mushroom)	76	Yes	5.2	Yoshimoto *et al.* (1988)
Agaricus bisporus (carrot)	78±2	Yes	4.8	Sattar *et al.* (1990)
	75	Yes	4.8	Yoshimoto *et al.* (1987)

dimer (Koida and Walter, 1976). However, the molecular mass of the same enzyme from many other mammalian tissues indicated that the enzyme is a monomer ranging from 66 to 79 kDa (Table 3). Later work showed that the lamb kidney enzyme has a molecular mass ranging from 74 to 77 kDa, which is similar to the value obtained for the lamb brain enzyme (Yoshimoto *et al.*, 1981). Therefore it is clear that the enzyme is a monomer with a molecular mass around 72.5 ± 7.0 kDa, depending on the source.

Prolyl endopeptidase has also been purified from human placenta (Mizutani *et al.*, 1984). The K_m value for placental prolyl endopeptidase using the synthetic substrate 7-(succinyl-Gly-Pro-4-methylcoumarinamide) was quoted as 10^{-3} M, similar to the value we obtained for the human brain enzyme with Z-Gly-Pro-pNA as substrate. The K_m value for another substrate, Succ-Gly-Pro-pNA, was found to be 2.56 mM, which also correlates well with that of the placental enzyme. However, the placental enzyme was suggested to be a dimer of 67 kDa and a thiol proteinase, even though it was strongly inhibited by DFP (Mizutani *et al.*, 1984). We have also purified the human placental enzyme, and the molecular mass was found to be 79 kDa on SDS/PAGE (Fig. 7, lane 5). Purified prolyl endopeptidase from human blood also has a molecular mass of 78.5 ± 2 kDa (Pratt *et al.*, 1989).

Bradykinin and des-Phe^8-Arg^9-bradykinin inhibited cleavage of Z-Gly-Pro-pNA by the brain enzyme, with IC_{50} values of 53.1 μM and 83.2 μM respectively. H.p.l.c. analysis confirmed that bradykinin and des-Phe^8-Arg^9-bradykinin were cleaved at the expected sites (results not shown) (Wilk & Orlowski, 1979). Z-Pro-prolinal, a specific inhibitor of the enzyme, was shown to be a potent inhibitor (K_m 0.6 nM).

Table 3 summarizes the properties of various prolyl endopeptidases from different sources. The human brain prolyl endopeptidase is strongly inhibited by DFP, but not by other classes of inhibitor, indicating it is a serine proteinase. This is in agreement with the results from various tissues (Table 3). Other serine-proteinase inhibitors, such as trypsin inhibitor, pAPMSF and 3,4-dichloroisocoumarin, which inhibit trypsin did not inhibit prolyl endopeptidase. This may indicate that the active site of prolyl endopeptidase is different from that of the trypsin-like serine proteinases. Some earlier studies had indicated that mammalian brain prolyl endopeptidases are highly sensitive to thiol-blocking reagents and DFP (Yoshimoto *et al.*, 1977; Knisatschek & Bauer, 1979; Orlowski *et al.*, 1979; Rupnow *et al.*, 1979; Tate, 1981). On the other hand, the kidney prolyl endopeptidase was reported to be a serine proteinase, and was only slightly inhibited by thiol-blocking agents (Koida & Walter, 1976; Yoshimoto *et al.*, 1978). Like the kidney enzyme, we have found the human brain enzyme to be only slightly sensitive to thiol reagents.

The pI of purified human brain prolyl endopeptidase was found to be 4.75. This is similar to the values for the corresponding enzyme from other mammalian tissues, such as lamb brain, lamb kidney, rat brain and rabbit brain (pI values of 4.8, 4.9, 4.5 and 4.9 respectively; Table 3). However, the bacterial enzyme seems distinctly different, as it has a pI of 9.6. The enzyme from carrot seems to be similar to the mammalian enzyme, as it has a pI of 4.8. Ultimately, cDNA cloning and elucidation of the amino acid sequence, together with site-directed mutagenesis studies, will clarify the relatedness, mechanism of catalysis and also the role of cysteines in prolyl endopeptidases.

Polyclonal antibodies were found to be specific and reacted with human brain prolyl endopeptidase as shown by Western-blot analysis and immunoprecipitation. The antibodies were found to be inhibitory to prolyl endopeptidase activity. The placental enzyme was also recognized by the antibodies as shown by immunoprecipitation.

By using immunoprecipitation, it has been shown that some prolyl endopeptidase activity is associated with the membrane fraction of nervous tissue (Camargo *et al.*, 1984). The enzyme activity was only loosely associated with the membrane, as it could be solubilized with 1% Triton X-100 or 1 mM-EGTA. This implies a role for the peptidase in neuropeptide inactivation at the membrane. The cytosolic enzyme might be involved in the processing of neuropeptides such as β-neoendorphin and is likely to be involved in general protein metabolism, as proline-containing peptides are often resistant to many proteinases (Wilk, 1983).

In conclusion, we have purified and extensively characterized human brain prolyl endopeptidase. Apart from sensitivity to thiol reagents, the physicochemical properties of the human brain enzyme and the corresponding enzymes from other mammalian species are very similar.

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