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Fluorimetric assay of the neurotensin-degrading metalloendopeptidase, endopeptidase 24.16

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Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (Mcc = 3-carboxy-7-methoxycoumarin; Dnp = dinitrophenyl), a quenched fluorimetric substrate originally designed as a probe to measure Pz-peptidase (also called endopeptidase 24.15), was examined as a putative substrate of the neurotensin-degrading neutral metalloendopeptidase, endopeptidase 24.16. During the purification of endopeptidase 24.16 the neurotensin(1–10) and neurotensin(11–13) formation due to this enzyme was co-eluted with Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity. By both fluorimetric and h.p.l.c. analyses, we observed that the latter activity was dose-dependently and completely abolished by neurotensin with an IC_{50} value (2.6 μM) that closely corresponds to the affinity of purified endopeptidase 24.16 for neurotensin (K_m = 2.5 μM). Furthermore, Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis was inhibited by a series of dipeptides with a rank of order of potencies that parallels that observed in competition experiments of tritiated neurotensin hydrolysis by brain and intestinal endopeptidase 24.16. Altogether, these data clearly demonstrate that, in addition to Pz-peptidase, Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp also behaves as a substrate of endopeptidase 24.16, with a K_m of about 26 μM. In addition, we show that, even in crude membrane preparations, Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp behaves as a useful tool to monitor and accurately quantify endopeptidase 24.16.

INTRODUCTION

Previous studies have established that neurotensin(1–10) and neurotensin(11–13) were the only two biologically inactive neurotensin-degradation products that were ubiquitously generated by various membrane preparations and cell lines from central and peripheral origins (Checler *et al.*, 1988) bearing the neurotensin receptors that probably mediate the physiological message. Pharmacological approaches by means of specific peptidase inhibitors developed against various proteolytic activities demonstrated that the formation of these metabolites remained insensitive to all these agents (Checler *et al.*, 1984, 1988). These observations led us to suggest that the peptidase responsible for the formation of neurotensin(1–10) and neurotensin(11–13) could correspond to endopeptidase 24.16, an enzyme that was first detected in (Checler *et al.*, 1984) and purified from rat brain synaptic membranes (Checler *et al.*, 1986) and later from intestinal tissue (Barelli *et al.*, 1988*b*). Such a possibility was verified by an immunological approach by means of a monospecific polyclonal antiserum directed towards rat brain endopeptidase 24.16 that revealed the presence of such activity in these biological models (Checler *et al.*, 1989). Such a tool also proved useful to establish the qualitative distribution of endopeptidase 24.16 in rat peripheral organs (Checler *et al.*, 1989) and to demonstrate the co-localization of the enzyme with neurotensin receptors in pure cultured neurons from mouse embryos (Chabry *et al.*, 1990). However, the IgG-purified fraction of this polyclonal antiserum did not allow quantitative estimation of endopeptidase 24.16 and was restricted to the detection of the enzyme in the mouse, since the antiserum displayed an exclusive specificity towards this species.

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp displayed a Gly-Pro-Xaa feature corresponding to the repeated sequence of the collagen helical sequence. However, this peptide did not behave as a substrate of clostridial collagenase but was readily hydrolysed by a thiol-dependent metalloendopeptidase widely distributed within mammalian tissues, Pz-peptidase (Tisljar *et al.*, 1990). This substrate was reported to be poorly susceptible to cleavage by other proteolytic activities such as endopeptidase 24.11 and proline endopeptidase (Tisljar *et al.*, 1990). The only peptidase that efficiently hydrolysed the substrate was the rat brain soluble metalloendopeptidase, endopeptidase 24.15. However, since Pz-peptidase was co-purified with endopeptidase 24.15 by high-resolution gel chromatography (Barrett & Tisljar, 1989) and was dose-dependently blocked by the endopeptidase 24.15 inhibitors *N*-[(1*R*,1*S*)carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (CPP-A-A-Y-pAB) and *N*-[(1*R*,1*S*)carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate with K_i values (9.9 nM and 16.6 nM respectively) virtually identical with those reported for endopeptidase 24.15 (16 nM and 27 nM), it was concluded that Pz-peptidase and endopeptidase 24.15 activities were due to a single enzyme (Barrett & Tisljar, 1989).

Our preliminary data suggested that there existed a CPP-A-A-Y-pAB-insensitive fraction of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity in a whole rat brain homogenate indicating that, besides endopeptidase 24.15, an additional enzyme appeared to be able to hydrolyse this substrate. Since such an activity was detected during the course of endopeptidase 24.16 purification in fractions devoid of endopeptidase 24.15 activity, we examined Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp as a putative substrate of endopeptidase 24.16. In the absence of a chromogenic or fluorimetric substrate to use for quantitative studies on endopeptidase 24.16, such a tool would be very convenient for monitoring and accurately quantifying endopeptidase 24.16.

MATERIALS

CPP-A-A-Y-pAB and Z-Pro-prolinal were kindly provided by Dr. M. Orlowski and Dr. S. Wilk (Mount Sinaï School of Medicine, New York, NY, U.S.A.). Mcc-Pro-Leu-Gly-Pro-D-

Abbreviations used: Mcc, 3-carboxy-7-methoxycoumarin; Dnp, dinitrophenyl; CPP-A-A-Y-pAB, *N*-[(1*R*,1*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate; IC_{50}, concentration causing 50% inhibition.

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Lys-Dnp was generously given by Dr. A. J. Barrett or purchased from Novabiochem. Dipeptides were from Sigma Chemicals. Neurotensin was from Neosystem (Strasbourg, France).

METHODS

Purification of endopeptidase 24.16

Endopeptidase 24.16 was purified as previously described (Checler *et al.*, 1986; Barelli *et al.*, 1988*a*). By this procedure, the post-DEAE-cellulose chromatography pool of endopeptidase 24.16 appeared to be slightly contaminated by endopeptidase 24.15. These two peptidases were totally separated by hydroxyapatite chromatography (Checler *et al.*, 1986; Barelli *et al.*, 1991). This is illustrated in Fig. 1 by the h.p.l.c. analysis of neurotensin hydrolysis by the post-hydroxyapatite pools of endopeptidase 24.16 (H_1) and endopeptidase 24.15 (H_2). As expected from homogeneous preparations, in the absence of inhibitor, neurotensin(1–10) and neurotensin(11–13) were the only degradation products generated by endopeptidase 24.16 activity (Fig. 1*a*, inset) while the two metabolites derived from hydrolysis by endopeptidase 24.15 activity (Fig. 1*b*, inset) were neurotensin(1–8) and neurotensin(9–13).

H.p.l.c. analysis of neurotensin hydrolysis

Neurotensin (2 nmol, 20 μM) was incubated at 37 °C with a portion of each fraction (20–50 μl) in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5. After acidification with 10 μl of 1.25 M-HCl, incubation mixtures were submitted to reversed-phase column chromatography and analysed by h.p.l.c. by means of a 42 min linear gradient of 0.1% trifluoroacetic acid, 0.05% triethylamine/0.1% trifluoroacetic acid and 0.05% triethylamine in acetonitrile from 9:1 (v/v) to 3:2 (v/v) as previously described (Checler *et al.*, 1988).

H.p.l.c. analysis of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (4 nmol, 20 μM) was incubated at 37 °C with 25 μl of purified enzyme in a final volume of 200 μl of 20 mM-Tris/HCl, pH 7.5. Incubation mixtures were acidified and analysed by h.p.l.c. according to the following biphasic gradient: 15 min from 9:1 to 3:1 then 40 min from 3:1 to 1:4 in the trifluoroacetic acid/triethylamine system described above.

Fluorimetric analysis of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (10 μM) was incubated at 37 °C with a portion of post-hydroxyapatite fractions or H_1 pooled proteins in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5, in the absence (control) or presence of various agents. When activity was measured in whole rat brain homogenate (final concentration of 0.2/0.3 mg of protein/ml), 0.5 μM-CPP-A-A-Y-pAB was added in order to block endopeptidase 24.15. Incubations were stopped with 2.5 ml of 80 mM-sodium formate, pH 3.7, and monitored at $\lambda_{ex.}$ 345 nm and $\lambda_{em.}$ 405 nm as described (Tisljar *et al.*, 1990). Quantification of Mcc-Pro-Leu released was by use of standard curve established with known amounts of Mcc-Pro-Leu.

Preparation and sequencing of degradation products

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (50 nmol) was incubated overnight with 8 μg of H_1 pooled proteins. Degradation products were separated by h.p.l.c. as described above. Sequencing was performed on an Applied Biosystems Inc. 477 A protein/peptide sequencing system equipped with an on-line phenylthiohydantoin analyser, model 120 A.

RESULTS

Table 1 indicates that, among a series of specific peptidase inhibitors, CPP-A-A-Y-pAB was the most potent blocker of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by rat brain homogenate. However, saturating concentrations of this inhibitor only exerted half-inhibition of the hydrolysis. Furthermore, complete dose–response analysis of the effect of CPP-A-A-Y-pAB resulted in a shallow curve, with a half-maximal effect around 0.5 μM (not shown), a value noticeably higher than the expected K_i value (16 nM) previously reported (Orlowski *et al.*, 1988). This indicates that, as well as endopeptidase 24.15, an additional proteolytic activity contributed to the metabolism of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp in rat brain. Endopeptidase 24.16 was therefore examined as a putative candidate for such cleavage of the fluorimetric substrate.

Fig. 1 illustrates the complete chromatographic separation of endopeptidase 24.15 and endopeptidase 24.16 achieved on hydroxyapatite. The pool H_1 corresponding to endopeptidase 24.16 generates only two neurotensin-degradation products, namely neurotensin(1–10) and neurotensin(11–13). The fact that neurotensin(1–8) and neurotensin(9–13) were totally undetectable together with the observation that the H_1 pooled proteins were unable to hydrolyse the endopeptidase 24.15 substrate benzoyl-Gly-Ala-Ala-Phe-*p*-aminobenzoate (not shown) indicate that the endopeptidase 24.16 activity was totally devoid of endopeptidase 24.15 activity. In the same way the H_2 pool contains only neurotensin(1–8)- and neurotensin(9–13)-forming activity, which could be ascribed to endopeptidase 24.15 since this activity could be totally blocked by CPP-A-A-Y-pAB. It should be noted that Western blot analysis of H_2 pooled protein did not reveal any label after incubation with the IgG-purified fraction of a monospecific polyclonal antiserum directed towards endopeptidase 24.16, indicating the absence of contamination of the H_2 pool by this peptidase (not shown).

Table 1. Effect of peptidase inhibitors on Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by rat brain homogenate

Inhibition studies were carried out in the presence of the indicated saturating concentrations of specific inhibitors. Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity present in rat brain homogenate (final concentration 0.3 mg of protein/ml) was measured as described in the Methods section. Values represent the percentage inhibition of control activities obtained without inhibitors and correspond to the means ± S.E.M. ($n = 6$).

Inhibitor	Concentration (μM)	Spectrum of inhibition	Percentage inhibition
Benzyloxycarbonyl-Pro-Prolinal	1	Proline endopeptidase	0.9 ± 0.6
Captopril	1	Angiotensin-converting enzyme	3.1 ± 1.7
CPP-A-A-Y-pAB	1	Endopeptidase 24.15	52.2 ± 4.5
Phosphoramidon	1	Endopeptidase 24.11	3.2 ± 1.2
Arphamenine B	0.5	Aminopeptidase B	2 ± 0.7
Diprotin A	100	Dipeptidyl aminopeptidase IV	10.3 ± 1.3
Kelatorphan	1	Dipeptidyl aminopeptidase	2 ± 0.9

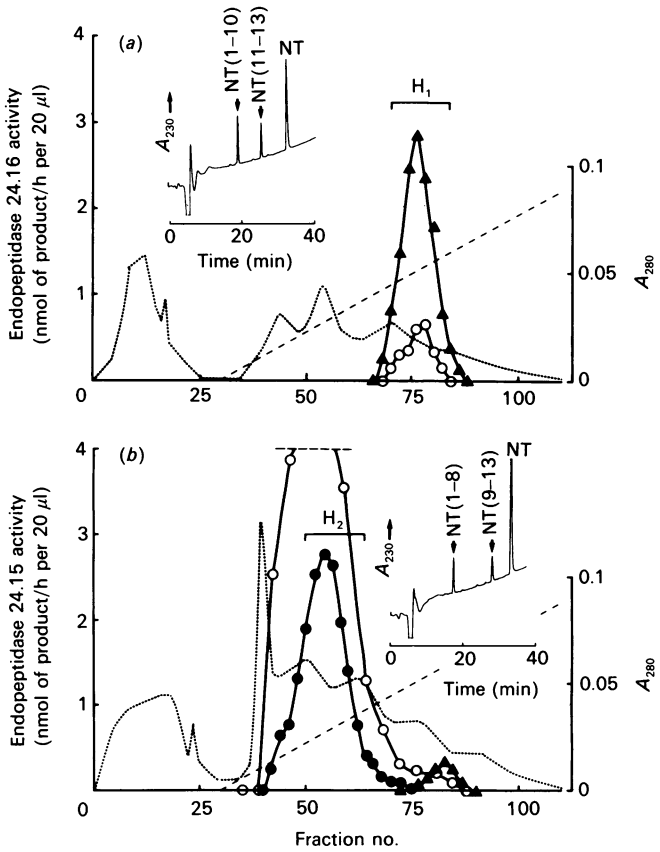

Fig. 1. Hydroxyapatite chromatography of DEAE-cellulose-enriched pools of endopeptidase 24.15 and endopeptidase 24.16

Endopeptidase 24.16 and endopeptidase 24.15 were purified as extensively described previously (Checler *et al.*, 1986; Barelli *et al.*, 1988*b*, 1991). The DEAE-cellulose-enriched pools of endopeptidases 24.16 (*a*) and 24.15 (*b*) were placed on an hydroxyapatite column previously equilibrated with 1 mM-KH_2PO_4, pH 7.5. A linear gradient of 75 ml of 1 mM-KH_2PO_4 was then applied (----). All fractions were tested for degradation of dynorphin(1–8) and neurotensin and analysed by h.p.l.c. as described in the Methods section. Insets correspond to the h.p.l.c. analysis of neurotensin (NT) degradation by H_1 (endopeptidase 24.16) or H_2 (endopeptidase 24.15) pooled proteins. ▲, Neurotensin(1–10); ●, neurotensin(1–8); ○, [leucine]enkephalin; ····, A_{280}.

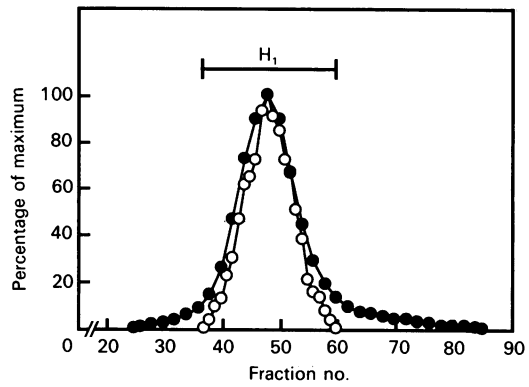

Fig. 2. Elution profiles of endopeptidase 24.16 and Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activities from hydroxyapatite

Neurotensin (2 nmol, 20 μM) was incubated at 37 °C for 3 h with 50 μl of fractions obtained during purification of endopeptidase 24.16 on hydroxyapatite. Neurotensin(1–10) formation (●) was detected and quantified by h.p.l.c. analysis as described in the Methods section. Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (10 μM) was incubated for 1 h with 30 μl of fractions and fluorescence (○) was monitored as described in the Methods section. Values are expressed as the percentage of the maximal activity recovered.

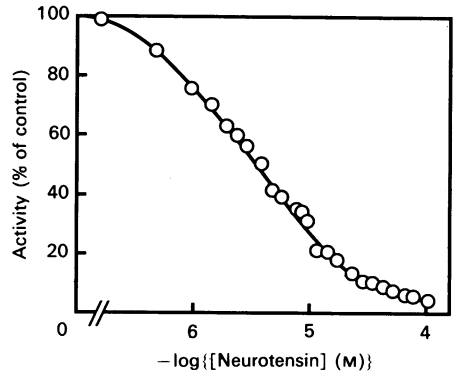

Fig. 3. Inhibition of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by neurotensin

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (10 μM) was incubated for 2 h at 37 °C with 20 μl of H_1 pooled proteins in the absence (control) or presence of increasing concentrations of neurotensin. Incubations were stopped and fluorimetrically monitored as described in the Methods section. Values are expressed as the percentage of the control and are the mean of two independent determinations.

Fig. 2 shows that during the hydroxyapatite chromatography of endopeptidase 24.16, Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity was detectable. This activity was superimposable on the neurotensin(1–10)-forming activity since the ratio between both activities remained constant all along the peak and the activities were both recovered in maximal amounts in fraction 48 (Fig. 2). Fig. 3 shows that the Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity was dose-dependently and fully abolished by neurotensin with a half-maximal effect of 4 μM. It was therefore possible to deduce a K_i value of 2.6 μM for neurotensin which closely agreed with the K_m value of purified endopeptidase 24.16 for neurotensin (Checler *et al.*, 1986; Barelli *et al.*, 1988*a*).

H.p.l.c. analysis of the hydrolysis of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp by the H_1 pool allowed the recovery of two degradation products. One of them was eluted with the retention time of Mcc-Pro-Leu and, as expected, appeared to be blocked at its *N*-terminus sequence analysis. Sequencing of the second product indicated that it corresponded to the complementary product Gly-Pro-D-Lys-Dnp. Hydrolysis of the fluorimetric substrate was fully abolished by 80 μM-neurotensin (Fig. 4). Here again, a complete competition curve indicated a K_i value of 2.5 μM, close to the K_m value (see above). On the other hand, hydrolysis of neurotensin was completely blocked by 200 μM-Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp with a half-maximal effect of about 26 μM (Fig. 4). Together, these data clearly indicate that Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp behaved as a substrate of endopeptidase 24.16. This was confirmed by additional experiments showing that Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by H_1 and H_2 pools could be dose-dependently blocked by CPP-A-A-Y-pAB, although it was with distinct potencies (Fig. 5). K_i values derived from these experiments appeared to be in agreement with theoretical values observed with neurotensin and Bz-Gly-Ala-Ala-Phe-*p*-aminobenzoate (Orlowski *et al.*, 1988) as substrates for endopeptidases 24.16 and 24.15 respectively (Table 2). Finally, a series of dipeptides that were previously shown to compete for

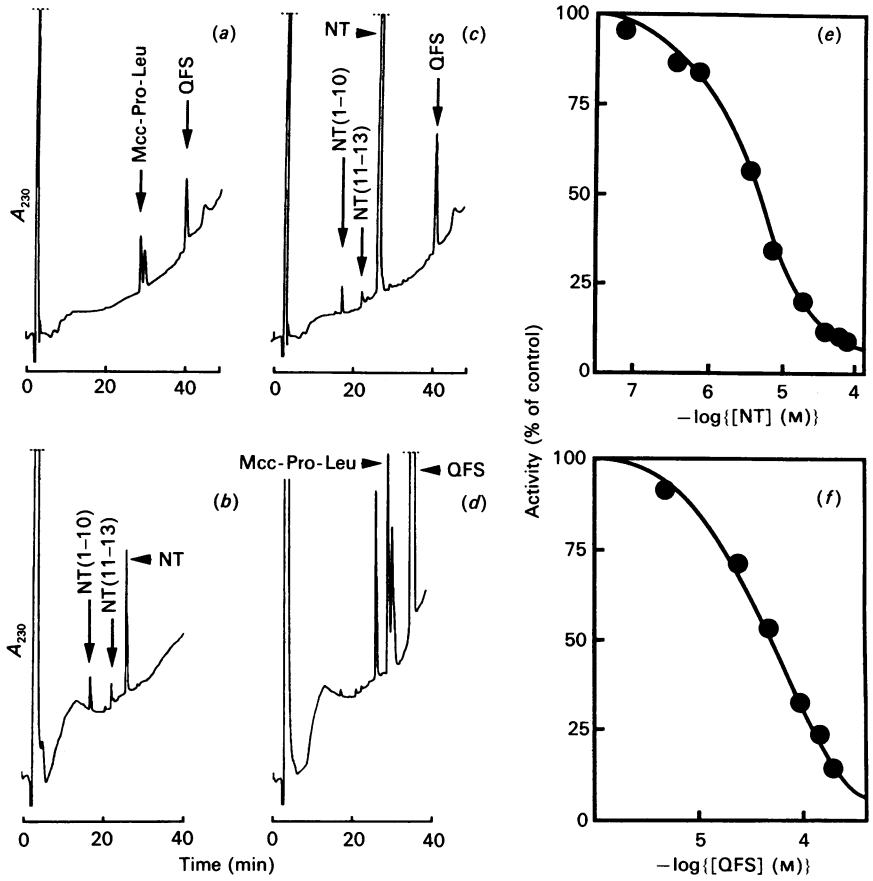

Fig. 4. H.p.l.c. analysis of neurotensin and Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by H_1 pooled proteins

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (QFS, quenched fluorimetric substrate) (4 nmol, 20 μM) (*a*, *c*) and neurotensin (NT; 1 nmol, 2 μM) (*b*, *d*) were incubated for 30–90 min with 20–25 μl of H_1 pooled proteins in the absence (*a*, *b*) or presence of the maximally active concentration (*c*, 80 μM; *d*, 200 μM) of the indicated competitor. H.p.l.c. analysis was carried out as described in the Methods section. Complete dose–response curves of the effect of neurotensin (*e*) or Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (*f*) were established by h.p.l.c. Values correspond to amounts of Mcc-Pro-Leu (*e*) or neurotensin(1–10) (*f*) as estimated by absorbance and are expressed as the percentage of the control obtained in the absence of competitor.

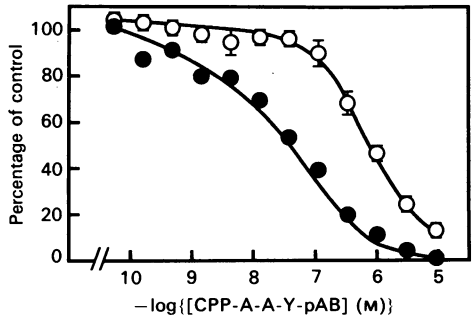

Fig. 5. Effect of CPP-A-A-Y-pAB on Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by H_1 and H_2 pooled proteins

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (10 μM) was incubated for 2 h at 37 °C with 10–20 μl of H_1 (endopeptidase 24.16; ○) or H_2 (endopeptidase 24.15; ●) pooled proteins in the absence (control) or presence of increasing concentrations of CPP-A-A-Y-pAB. Activities were monitored by fluorimetry as described in the Methods section and are expressed as the percentage of control activities recovered in the absence of inhibitor. Values are the means of two to four independent determinations.

Table 2. Comparison of the effect of CPP-A-A-Y-pAB on endopeptidase 24.15 and 24.16 activities

	Inhibition by CPP-A-A-Y-pAB (μM)		
Peptidase	IC_{50}	Calculated K_i	Theoretical K_i
Endopeptidase 24.15	0.044	0.02	0.016*
Endopeptidase 24.16	1	0.66	1†

* K_i value from Orlowski *et al.* (1988).

† K_i value was from P. Dauch, H. Barelli, J.-P. Vincent & F. Checler (unpublished work).

degradation of tritiated neurotensin by brain and ileal endopeptidase 24.16 (Barelli *et al.*, 1988*a*,*b*) also inhibited Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by endopeptidase 24.16 (Table 3). Although the IC_{50} values were not totally identical, the same order of potency was observed for the series of dipeptides whatever the substrate used.

Fig. 6 illustrates the determination of the main experimental parameters that concerned the assay of endopeptidase 24.16 with Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp. Initial-velocity measurements at various concentrations of substrate are illustrated in Fig. 6(*a*). The Lineweaver–Burk plot gave a K_m value of about 30 μM. This value was in close agreement with the K_i (26 μM) observed by h.p.l.c. for the inhibition of neurotensin hydrolysis by Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (Fig. 4). The initial velocity was directly

Table 3. Effect of dipeptides on endopeptidase 24.16

Dipeptide	Half-maximal inhibitions of endopeptidase 24.16 (μM)		
	Rat ileum	Rat brain	Rat brain
Pro-Ile	50*	46*	89†
Pro-Leu	190	130	296
Pro-Tyr	200	100	469
Pro-Xaa (Xaa = Ala, Ser, Gly)	$\gg$ 1000	$\gg$ 1000	$\gg$ 1000
Xaa-Pro (Xaa = Ser, Pro, Val, His, Met)	$\gg$ 1000	$\gg$ 1000	$\gg$ 1000
Val-Tyr	380	200	734
Trp-Tyr	460	290	847
Xaa-Tyr (Xaa = Ala, Ser, Lys, Gly, His)	$\gg$ 1000	$\gg$ 1000	$\gg$ 1000

* IC_{50} values were from Barelli *et al.* (1988*a*,*b*) and were established with tritiated neurotensin as substrate.

† K_i values were from the present study performed with Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (see the Methods section).

proportional to the amount of endopeptidase 24.16 (Fig. 6*b*). Kinetic analysis carried out at maximal velocity (i.e. at virtual saturating concentration of substrate) indicated that initial velocity could be observed during the first 60 min of incubation (Fig. 6*c*). The specific activity of endopeptidase 24.16 towards the fluorimetric substrate was 215 nmol/h per mg of protein.

Finally, the usefulness of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp for assaying endopeptidase 24.16 in crude tissue was established. Data in Fig. 7 were obtained in the presence of 0.5 μM-CPP-A-A-Y-pAB in order to block the contribution of endopeptidase 24.15. Under these conditions, an important Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity was observed in rat brain homogenate. This hydrolysis could be dose-dependently and completely abolished by neurotensin (Fig. 7). Here again, the IC_{50} value derived from these experiments was in agreement with the K_m value of purified endopeptidase 24.16 for neurotensin. Therefore the hydrolytic activity observed in Fig. 7 can only be ascribed to endopeptidase 24.16 in rat brain homogenate.

DISCUSSION

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp was first designed as a quenched fluorimetric substrate of Pz-peptidase, a peptidase widely distributed within mammalian tissues (Tisljar *et al.*, 1990). Since Pz-peptidase was co-eluted with endopeptidase 24.15 and was completely blocked by its inhibitor CPP-A-A-Y-pAB, it was concluded that the two proteolytic activities were identical (Barrett & Tisljar, 1989). Our data first demonstrate that Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp could not be considered as an exclusive substrate of Pz-peptidase since the endopeptidase 24.15 inhibitors only exerted a partial inhibition when the enzyme source was a crude tissue or membrane preparation. Therefore, in brain, there seemed to exist additional peptidase(s) responsible for the degradation of the fluorimetric substrate. The present work clearly establishes that such a peptidase could be the metalloproteinase endopeptidase 24.16. First, purified endopeptidase 24.16 (totally devoid of endopeptidase 24.15 activity) was co-eluted with Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-hydrolysing activity from a hydroxyapatite column; secondly, the hydrolysis of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp, determined by either fluorimetry or h.p.l.c. analysis, was dose-dependently and completely inhibited by neurotensin with a K_i value that

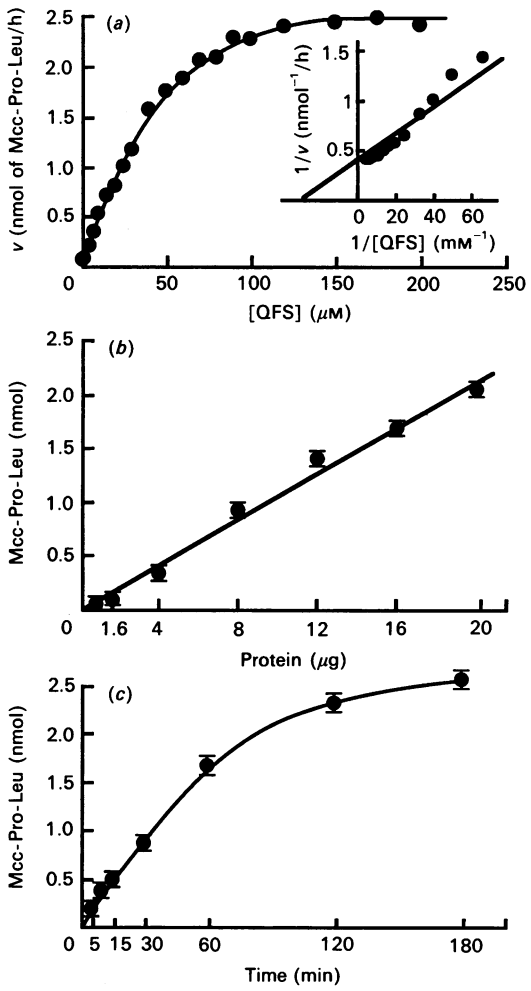

Fig. 6. Kinetic parameters of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by purified endopeptidase 24.16

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (QFS) was incubated at 37 °C with purified endopeptidase 24.16 (H_1 pool). Substrate hydrolysis was monitored by fluorimetry as described in the Methods section. (*a*) Reaction rate as a function of substrate concentration. Inset: Lineweaver–Burk plot of the data. (*b*) Proportionality between substrate hydrolysis and endopeptidase 24.16 concentration. (*c*) variation of product formed as a function of time under nearly saturating conditions of substrate concentration (100 μM-Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp).

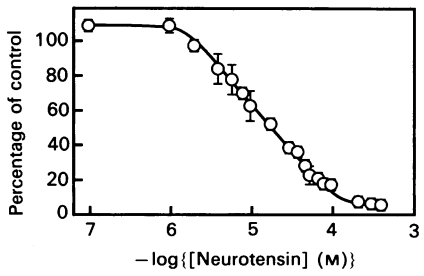

Fig. 7. Effect of neurotensin on Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by rat brain homogenate

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (10 μM) was incubated for $2\frac{1}{2}$ h at 37 °C with 10 μl of brain homogenate (final concentration 0.2–0.3 mg of protein/ml) in the absence (control) or presence of increasing concentrations of neurotensin. All incubations were carried out in the presence of 0.5 μM-CPP-A-A-Y-pAB in order to block the putative contribution of endopeptidase 24.15. Values are expressed as a percentage of the control and represent the mean ± S.E.M. of six independent determinations.

closely corresponded to the K_m value of endopeptidase 24.16 for neurotensin; thirdly, hydrolysis of neurotensin was fully blocked by Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp with a K_i value of 26 μM, which corresponded to the K_m value deduced from direct fluorimetric measurements; fourthly, hydrolysis of the fluorimetric substrate was inhibited by several dipeptides that were previously shown to protect selectively tritiated neurotensin from cleavage by purified rat brain and ileal endopeptidase 24.16. Together, these observations indicate that Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp could be used to monitor and quantify endopeptidase 24.16.

Experiments carried out with whole brain homogenate indicated that, even if endopeptidase 24.15 is blocked, the substrate is still cleaved by the homogenate and hydrolysis could be competitively inhibited by neurotensin. Therefore Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis seems to be due to two enzyme components. The first one is inhibited by 0.5 μM-CPP-A-A-Y-pAB and must be ascribed to endopeptidase 24.15. The other one is due to endopeptidase 24.16 since it is blocked by dipeptides that selectively inhibit this enzyme. The corollary of these statements is that both endopeptidase 24.15 and 24.16 activities can be measured in a complex enzyme mixture by using Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp. Endopeptidase 24.15 should be considered as responsible only for the CPP-A-A-Y-pAB-sensitive fraction of activity whereas the CPP-A-A-Y-pAB-resistant fraction of substrate hydrolysis should be attributed to endopeptidase 24.16. The possibility of measuring endopeptidase 24.16 activity by a fluorimetric assay will allow its complete biochemical characterization and aid studies on its subcellular localization, distribution in brain and ontogeny in the central nervous system.

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