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Neuropeptide Specificity and Inhibition of Recombinant Isoforms of the Endopeptidase 3.4.24.16 Family: Comparison with the Related Recombinant Endopeptidase 3.4.24.15

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Endopeptidase EC 3.4.24.16 (EP24.16c, neurolysin) and thimet oligopeptidase EC 3.4.24.15 are close related members of a large family of metalloproteases. Besides their cytosolic and membrane bound form, endopeptidase EC 3.4.24.16 appears to be present in the inner membrane of the mitochondria (EP24.16m). We have overexpressed two porcine EP24.16 isoforms in *E. coli* and purified the recombinant proteins to homogeneity. We show here that these peptidases hydrolyse a series of neuropeptides with similar rates and at sites reminiscent of those elicited by classically purified human brain EP24.16c. All neuropeptides, except neurotensin, were similarly cleaved by recombinant endopeptidase 3.4.24.15 (EP24.15, thimet oligopeptidase), another zinc-containing metalloenzyme structurally related to EP24.16. These two EP24.16 isoforms were drastically inhibited by Pro-Ile and dithiothreitol and remained unaffected by a specific carboalkyl inhibitor (CFP-AAY-pAb) directed toward the related EP24.15. The present purification procedure of EP24.16 should allow to establish, by mutagenesis analysis, the mechanistic properties of the enzyme.

Key Words: **endopeptidase; peptide processing; pGEX; recombinant enzyme.**

Endopeptidase 3.4.24.16 (also referred to as neurolysin) was first detected (1) and later purified from rat brain synaptic membranes on the basis of its ability to inactivate the gut-brain tridecapeptide neurotensin (2). This activity appeared to display a dual cytosolic and membrane-associated subcellular localization (1), a feature that appeared clearly to be cell specific as the membrane-bound counterpart was evidenced at the electron microscopic level in primary cultured neurons but not in astrocytes (3). The molecular cloning of the rat brain enzyme did not elucidate the structural features accounting for such membrane anchor (4). More recently, Serizawa et al. (5) reported on the isolation of a rat liver neurolysin counterpart that appeared, after subcellular fractionation, to be associated with the inner mitochondrial membrane. Interestingly, these authors demonstrated that the N-terminal sequence of purified liver neurolysin matched that of oligopeptidase M, a rabbit liver microsomal endopeptidase (6), the specificity of which did not support a direct link with endopeptidase 3.4.24.16 since this enzyme was described as a pro-protein processing enzyme. The above data suggested that various related isoforms of endopeptidase 3.4.24.16 could contribute to a wider neurolysin family. This hypothesis was recently supported by a study showing that the alternative splicing of the endopeptidase 3.4.24.16 gene could result in the production of species differing in their N-terminus (7). Most interesting was the observation that such N-terminal truncation could trigger distinct subcellular localization, thereby reconciliating most of the above biochemical observations (7). However, it was not clear

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whether such structural differences could in fact influence the catalytic, specificity or inhibitor sensitivity of the various isoforms.

Several lines of evidence indicate that (4, 8), endopeptidase 3.4.24.16 displays a very similar specificity towards a series of neuropeptides than that exhibited by another, structurally homologous, metal-dependent endopeptidase called endopeptidase 3.4.24.15 or thimet-oligopeptidase (8, 9, 10, 11). However, it appeared that the rat brain endopeptidases 3.4.24.15 and 3.4.24.16 could be distinguished with respect to the cleavage they trigger on neurotensin, their sensitivity to carboalkyls, phosphinic and Pro-Ile inhibitors (12, 13, 14) and their drastic distinct sensitivity to thiol compounds (15, 16).

Unambiguous characterization of proteolytic activities necessits an homogenous source of enzyme and the obtention of such an activity, devoid of contaminating enzyme, is often difficult to achieve, especially when peptidase isoforms display mostly identical physical properties. We have therefore expressed in and purified the mitochondrial and cytosolic recombinant endopeptidase 3.4.24.16 isoforms and compared their substrate specificity and inhibitor sensitivity with those of the purified human brain counterpart. These properties were further compared with those exhibited by recombinant endopeptidase 3.4.24.15.

EXPERIMENTAL PROCEDURES

Plasmid constructions. The cDNA sequence coding for EP24.15 was subcloned into a pGEX4T-1 vector as previously described (17).To generate the cytosolic/membrane bound (EP24.16c) and the mitochondrial (EP24.16m) recombinant porcine EP24.16, a glutathione S-transferase fusion-protein system expressed in Escherichia coli was utilized (18). The EP24.16c or EP24.16m were constructed and named pGEX-EP2416c and pGEX-EP2416m, respectively (Fig. 1A). A 2.7-kb Bam HI-Xho I fragment of pPAB-L1 (19), which encodes the entire open reading frame of EP24.16c, was inserted into the Bam HI-Xho I cloning sites of pGEX-4T vector (Pharmacia), and the 5′ non-coding region was removed using Bam HI and Bsp EI. The 5′-end of EP24.16c was amplified by PCR using primers 5′-TAAGAGGATCCACTTTAGGGAGAGAAGCAATG-3′ (sense oligonucleotide) and 5′-GTCAATACATAGCTCACTCATTCT-3′ (antisense oligonucleotide). The 5′-end of EP24.16m was amplified by PCR using primers 5′-TAAGAGGATCCTCCTATACTGTGGATGGCAGA-3′ (sense oligonucleotide) and 5′-GTCAATACATAGCTCACTCATTCT-3′ (antisense oligonucleotide). The PCR fragments were digested with Bam HI and Bsp EI, and inserted into Bam HI-Bsp EI sites of pGEX-4TL1. To confirm correct amplifications and ligations, DNA sequencing was performed by the dideoxynucleotidechain-termination method (20).

Expression of recombinant enzymes. The expression of the recombinant EP24.16c and EP24.16m proteins were conducted as originally described for the rat testis recombinant endopeptidase EP24.15 (17). Briefly, DH5- *E. coli* cells were transformed with the correspondent pGEX plasmid containing the cDNA sequence for either EP24.15, EP24.16c or EP24.16m. Cells were grown with antibiotic selection to a OD600 nm of 0.6, when the expression of the recombinant fusion protein gene was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), proceeding for 4 h. Bacterial culture (1 l) was centrifuged at 5,000 g for 5 min, at 4C, ressuspended in 50 ml of PBS and then lysed by sonication at 40 Hz (3 cycles of 40 sec.). After removal of the bacterial debris by centrifugation at 10,000 g for 15 min the supernatants were incubated with gluthatione-Sepharose beads (Pharmacia) for 18 h at 4C. After extensive washing with 0.05 M tris-HCl, pH 8.0, containing 0.150 M NaCl (TBS+), the beads were equilibrated in 0.05 M tris-HCl, pH 7.5, containing 2.5 mM CaCl2 and incubated in the presence of thrombin (30 Units/ml of beads; Calbiochem) for 3 h at room temperature. Recombinant proteins were eluted from the column with TBS+ and submitted to filtration in a Centricon 50 (Amicon) to eliminate contamination with thrombin. Purified recombinant enzymes were aliquotted and stored at −20 C. Protein concentration was determined as previously described (21) using bovine serum albumin as a standard. Homogeneity of the recombinant enzyme preparations were analyzed by polyacrylamide gel electrophoresis (22) after staining with comassie blue R250 (BioRad).

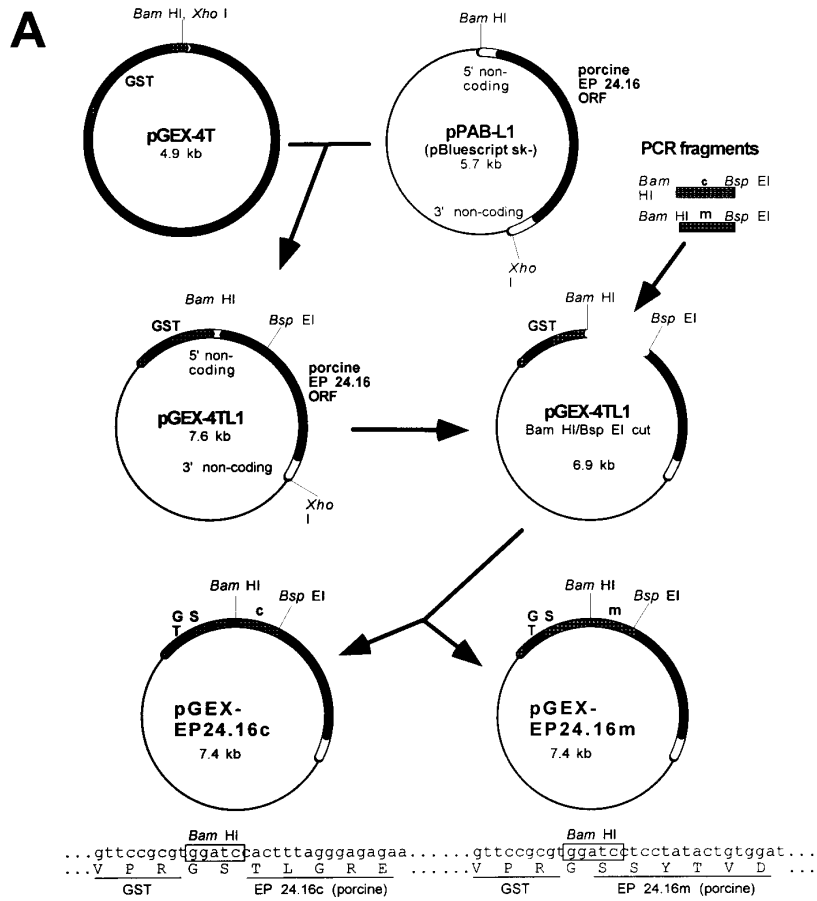
Purification of the human brain endopeptidase 24.16. The human brain endopeptidase 24.16 was purified as previously described (23).

HPLC peptide hydrolysis analysis and enzyme assay. Peptide hydrolysis were analyzed by reverse phase HPLC using a C18 μBondapak column (4.6 x 250 mm; Millipore Corp.) with a linear gradient of 5-65% acetonitrile in 0.1 % TFA for 20 min at a flow rate of 2ml/min, as previously described (24). Absorbance was monitored at a wavelength of 214 nm. Cleveage sites were assigned by using internal synthetic peptide fragments standards (24). Enzyme assays were conducted using bradykinin (30 μM) as a substrate. One milli-unit (mU) of either recombinant EP24.15, EP24.16c, EP24.16m or human brain EP24.16 was defined as the amount of enzyme able to hydrolyze 1 nmol of bradykinin/min, at 37 C, in 50 mM tris-HCl, pH 7.5, containing 100 mM NaCl. Specific enzymatic activity was expresses in mU/mg of protein.

RESULTS

IPTG induction of transformed DH5 *E. Coli* cells triggers a time-dependent overexpression of specific proteins, the apparent molecular weight of which corresponds to the calculated mass of EP24.15 (Fig.2A1), EP24.16c (Fig.2A2) and EP24.16m (Fig.2A3) fused with glutathione S-transferase. The maximal production of the fusion proteins similarly reaches a plateau by 4 hours. Proteolytic removal of glutathione S-transferase and subsequent purification of the recombinant proteins allow the recovery of apparently homogenous peptidases after SDS-PAGE analysis (Fig. 2B). The production yields (mg/l of culture) and specific activities (mU/mg of protein) of EP24.15, EP24.16c and EP24.16m are 3.6 /3,200, 2.6/2,800 and 2.6/2,500, respectively.

A series of natural peptides have been examined as substrates of EP24.16c and EP24.16m. The two recombinant enzymes display the same relative rates of cleavage (Table1) and appear particularly efficient in degrading neurotensin as well as on the blood pressure modulators bradykinin and angiotensin I. HPLC analysis of the cleavage products (Fig.3) indicate a single cleaveage event giving rise to catabolites, the identity of which agrees well with previous studies on EP24.16c classically purified from various sources (16). Accordingly, the same qualitative pattern is ob-



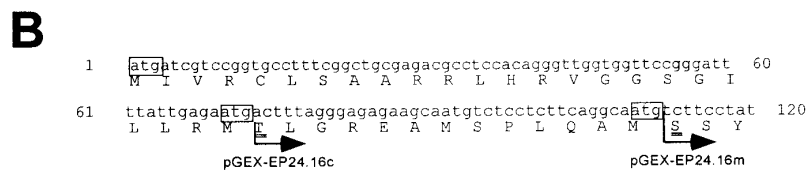


FIG. 1. Schematic representation of the EP24.16 plasmid constructions -**A,** schematic representation of the plasmid constructions. The structure of pPAB-L1 is described elsewhere (1). The coding regions of EP24.16 are indicated by filled bars. The non-coding regions are indicated by open bars. The hatched bars indicate coding regions for the glutathione S-transferase in the pGEX vectors. The PCR fragments encoding amino-termini of cytosolic and mitochondrial EP24.16 are indicated by gray bars and marked by c and m, respectively. Nucleotide and deduced amino-acid sequences of the flanking region of Bam HI site of the plasmids are shown at the bottom. **B,** nucleotide and deduced amino-acid sequence of 5′ region of cDNA for porcine EP24.16. Positions of 5′ end of the cDNA inserted into pGEX vector are indicated by arrows. Two translational initiation codons (ATG) are boxed. The residues for amino-termini of EP24.16, which were determined by Serizawa et al. (1995), are double underlined.

served with purified human brain EP24.16 (not shown). EP24.16c and EP24.16m also hydrolyse the C-terminally blocked metorphinamide peptide, thereby confirming the endopeptidase nature of both isoforms (Table 1 and Fig.3). By contrast, GnRH behaves as a poor substrate of EP24.16c/m (Table1). Recombinant EP24.15 cleaves most of the neuropeptides at similar rates and identical peptide sites than EP24.16c/m, with the noticeable exception of neurotensin that is hydrolysed at the Arg8-Arg9 bond by EP24.15 while EP24.16c/m attack occurs between Pro10 and Tyr11 (Table 1 and Fig. 3).

Table 2 documents the spectrum of inhibition/activation of recombinant EP24.15, EP24.16c and EP24.16m. The EP24.16c is substantially inhibited by Pro-Ile and dithiothreitol (Table2) as was previously reported for

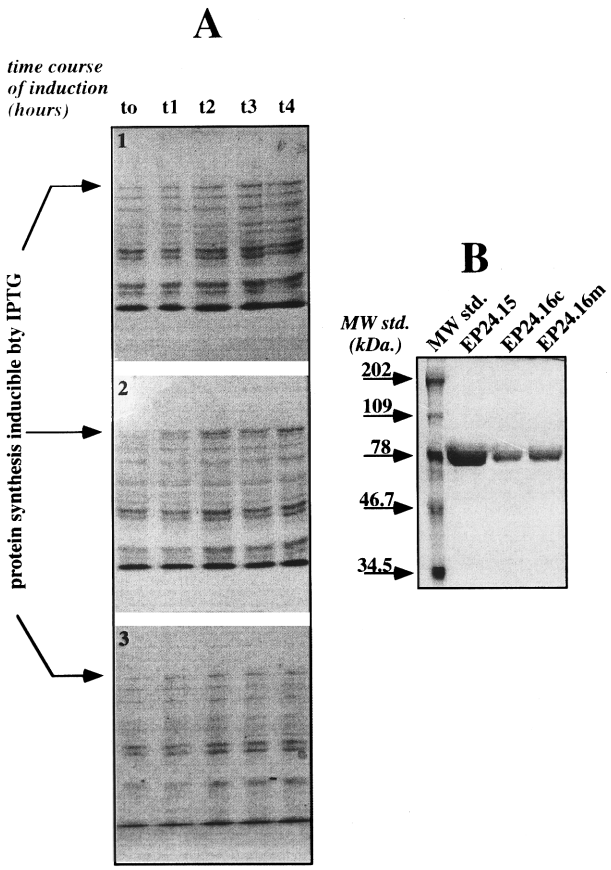


FIG. 2. Expression and purification of recombinant EP24.16c, EP24.16m, and EP24.15. **Panel A.** Time-course expression of the endopeptidases by DH5- *E. Coli* analyzed by SDS-PAGE electrophoresis after Commassie blue staining. Arrows indicated a specific protein band that increases in intensity after addition of IPTG. A1, EP24.15; A2, EP24.16c; A3, EP24.16m. **Panel B.** Homogeneous endopeptidase samples obtained after a single step affinity chromatogrhaphy using a Sepharose-GST column (Pharmacia). EP24.15, 20 g, EP24.16c, 10 g, and EP24.16m, 10g.

the purified enzyme (16). Interestingly, our study indicates that the mitochondrial isoform displays similar sensitivity to these agents. Both EP24.16c/m are not affected by the carboalkyl inhibitor CFP-AAY-pAB (Table2). By contrast, this agent strongly inhibits EP24.15, the activity of which appears highly stimulated by dithiothreitol (Table2).

DISCUSSION

The endopeptidase 3.4.24.16 (EP24.16) belongs to the large family of zinc-containing metallopeptidases and is thought to participate to the termination of peptidergic signals and particularly to contribute to the physiological inactivation of neurotensin in the central nervous system and in the periphery (24, 3). The subcellular localization of this peptidase indicated a main cytosolic distribution, but also clearly revealed a cell-specific neuronal membrane-bound counterpart (23), a cell compartment thought to contribute to the catabolic inactivation of neuropeptides in the synaptic cleft (26). Recently, an EP24.16-related activity was shown to be associated with a mitochondrial compartment (6, 5). The recent elucidation of the gene structure encoding EP24.16 indicated an alternative splicing could explain the specific subcellular targeting of the peptidase, and particularly evidenced the importance of a N-terminal sequence responsible for the targeting of one of the two isoforms to the mitochondrial compartment (7). As the function of the mitochondrial EP24.16 (EP24.16m) remained unknown, a question arises as to whether EP24.16m displays the same neuropeptide substrate specificity and inhibitor sensitivity than its non mitochondrial counterpart (refered to as EP24.16c). We took advantage of a bacterial expression system previously designed to overproduce endopeptidase 3.4.24.15 (EP24.15; 17) to produce and purify in

TABLE 1

Relative Rate of Hydrolysis and Sites of Cleavage on Naturally Occuring Peptides by Recombinant EP24.15, EP24.16c and EP24.16m

Peptides	Cleavage site	Hydrolysis (%)*		
		EP24.15	EP24.16c	EP24.16m
Bradykinin	RPPGF ⇓ ↓↑SPFR	100	108	105
Neurotensin	⟨ELYENKPR ⇓ RP↑↓YIL	80	95	95
Metorphinamide	YGGFM ⇓ ↓↑RRV-(NH_2)	130	85	82
GnRH	⟨EHWSY ⇓ ↓↑GLRPG-(NH_2)	15	10	11
Dynorphin A 1–8	YGGFL ⇓ ↓↑RRI	110	60	58
Angiotensin I	VYIHP ⇓ ↓↑FHL	80	95	98

* 100% of hydrolysis represents the EP24.15 cleavage of 16.6 nmol of bradykinin in 10 min at 37°C, in 50 mM Tris-HCl, pH 7.5, containing 100mM NaCl. To determine the relative rate of hydrolysis the reactions were conducted in the presence of 35 μM of peptide substrate and 2 mU of either EP24.15, EP24.16c or EP24.16m. Reactions were stopped with 10 μl TFA and the remaining peptide concentration was analysed by HPLC as described in Materials and Methods. The data represents the mean of three independent determinations. ⇓ hydrolyzed by EP24.15; ↓ hydrolyzed by EP24.16c and ↑ hydrolyzed by EP24.16m.

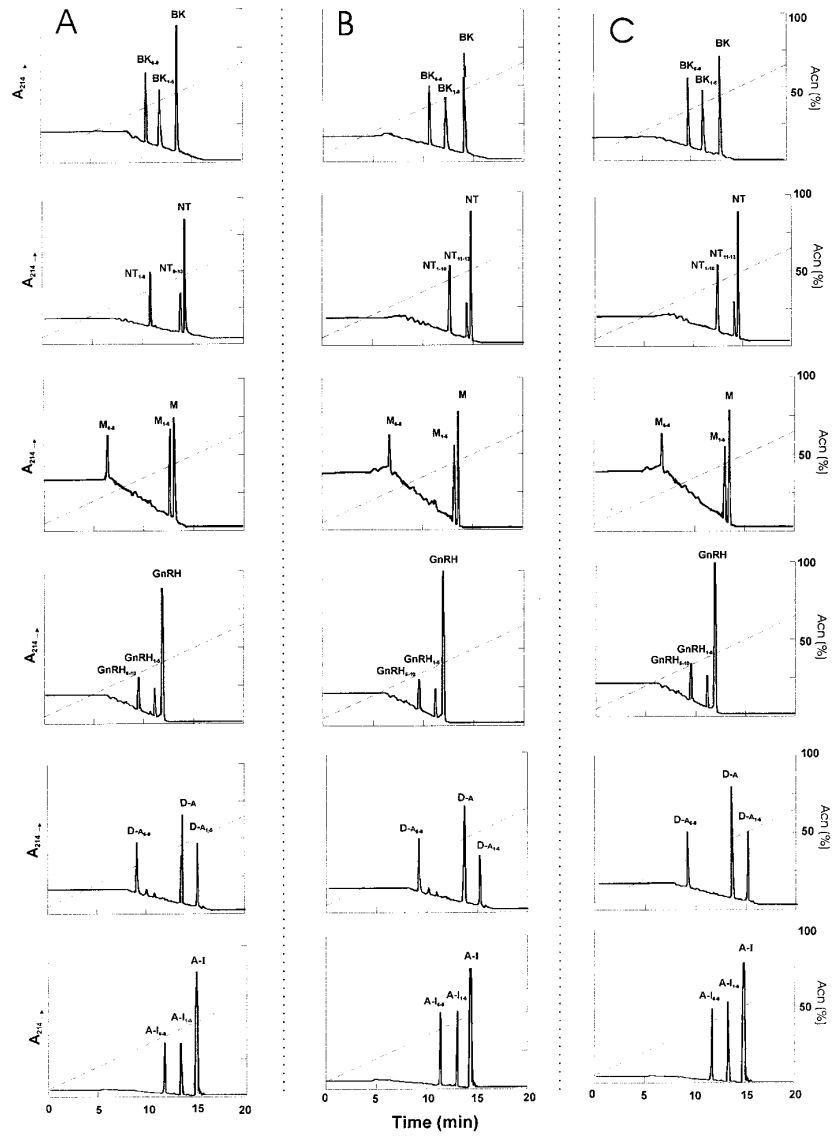


FIG. 3. HPLC profile of bradykinin (BK), neurotensin (NT), metorphinamide (M), gonadotrophin-releasing hormone (GnRH), dynorphin A1-8 (D-A) and angiotensin I (A-I) hydrolyzed by recombinant EP24.15 (Panel A), EP24.16c (Panel B), and EP24.16m (Panel C). Each peptide (30 μM) was incubated in 500 μl of 50 mM tris-HCL, pH 7.5, containing 100 mM NaCl, at 37 C with 2 mU, 2.2 mU and 2.3 mU of EP24.15, EP24.16c, and EP24.16m, respectively. Reactions were stoped by addition of TFA 10% v:v (10 μl) and subjected to HPLC. The cleveage sites were determined by using internal synthetic peptide fragments standards, as described in Experimental Procedures.

high yields the two recombinant EP24.16 isoforms. The two purified sources of enzymes elicit identical cleavages on neuropeptides involved in the blood pressure control (bradykinin and angiotensin I) and nociception (metorphinamide and dynorphin A 1-8). These products are also generated by the purified humain brain EP24.16 (data not shown; 16), indicating that classically purified and recombinant EP24.16 displayed identical specificity properties.

Expression of recombinant EP24.16 derived from the porcine cDNA expression allowed us to definitely solve a controversy concerning the hydrolysis of neurotensin. Millican et al. (27) indicated that porcine EP24.16 not only inactivated neurotensin at the previously reported Pro10-Tyr11 bond but also triggered an additional cleavage at the Arg8-Arg9 bond, a cleavage that was never detected with various other sources of EP24.16 (16, 28). Here, we clearly show that porcine recombinant EP24.16 isoforms only elicit a single cleavage on neurotensin, confirming the demonstration by Vincent et al. (3) that the additional Arg8-Arg9 cleavage was indeed due to a contaminating enzyme.

Recombinant EP24.16 isoforms display identical susceptibility to peptidase inhibitors. As previously docu-

TABLE 2

Effects of Dithiothreitol, CFP-A-A-Y-pAB or Pro-Ile on the Recombinant EP24.15, EP24.16c or EP24.16m Enzymatic Activity

	Hydrolysis (%)*			
Enzyme	Control	+DTT	+CFP	+Pro-Ile
EP24.15	100*	850	8	97
EP24.16c	108	37	95	24
EP24.16m	105	35	96	21

* 100% represents EP24.15 hydrolysis of 4 nmol of bradykinin/10 min at 37°C in 500μl of 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. Purified recombinant EP24.15, EP24.16c and EP24.16m (2 mU, 2.2 mU and 2.3 mU, respectively) were incubated in the presence of either 0.5 mM DTT, 1 μM of CFP-AAY-pAb (CFP) or 1.6 mM of Pro-Ile. Reactions were stopped with 10μl TFA and the remaining bradykinin concentration was analysed by HPLC as described in Materials and Methods. The data represents the mean of three independent experiments.

mented (13, 16), EP24.16c is less sensitive than EP24.15 to the carboalkyl blocker CFP-AAY-pAb and potently inhibited by the dipeptide Pro-Ile and dithiothreitol. It is important to state that both EP24.16c/m isoforms fully behave as the purified EP24.16c. Therefore, the bacterial expression system employed here appears adequate to envision the overproduction of mutated enzyme allowing to further delineation of the residues involved in the catalytic mechanism and the possible function of the various EP24.16 isoforms.

The yield of production of recombinant EP24.15 compares well with the previous large scale purification of the rat testis enzyme in E. Coli (17, 29, 30). Recombinant EP24.15 was potently inhibited by CFP-AAY-pAB, insensitive to Pro-Ile and drastically activated by dithiothreitol (Table 2) as was reported in a previous study on recombinant EP24.15 (17, 30) and purified enzyme (13). Recombinant EP24.15 and EP24.16 display similar peptide specificities except for neurotensin (Table 1). The present study also documents the fact that recombinant EP24.15 triggers the same cleavages than does the purified activity. Particularly, it is striking that EP24.15 hydrolyses methorphinamide and angiotensin I (Table 1, and Figure 3), two peptides that did not behave as a substrate of recombinant EP24.15 in a previous study (30). The reason of such a discrepancy still remains unclear.

In conclusion, our study documents the specificity and inhibition susceptibility of the two recombinant EP24.16 isoforms and indicate that the two counterparts display identical specificities reminiscent of those exhibited by the classically purified enzymes (31). The demonstration that recombinant EP24.16c/m and also EP24.15 can be obtained in high yield with identical biochemical properties to the native enzyme should prove useful to further study the structural, mechanistic properties and function of these enzymes.

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