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Expression of recombinant pro-neuropeptide Y, proopiomelanocortin, and proenkephalin: relative processing by 'prohormone thiol protease' (PTP)

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Abstract **The preference of the 'prohormone thiol protease' (PTP), a candidate prohormone processing enzyme, for different peptide precursors was assessed in vitro with recombinant prohormones near estimated in vivo levels. Pro-neuropeptide Y (pro-NPY), proopiomelanocortin (POMC), and proenkephalin (PE) were expressed at high levels in *E. coli*. Purification of prohormones utilized a combination of DEAE-Sepharose, Mono Q, and preparative electrophoresis. PTP cleaved PE most readily, and also cleaved pro-NPY. The processing of POMC by PTP was minimal. These results demonstrate PTP's preference for certain prohormone substrates.**

Key words: Recombinant prohormone; Proprotein processing; Cysteine protease

1. Introduction

Peptide hormones and neurotransmitters are synthesized as protein precursors that require proteolytic processing at pairs of basic amino acid residues to generate active neuropeptides [1–3]. In biochemical [4–7] and cell biological [8] studies of proenkephalin processing in adrenal medulla, the primary enkephalin precursor cleaving activity is represented by a novel cysteine protease termed 'prohormone thiol protease' (PTP). The role of PTP in enkephalin production in chromaffin cells was clearly demonstrated by complete blockade of cAMP-stimulated enkephalin levels by a potent cysteine protease inhibitor of PTP [8]. Proenkephalin processing also involves the subtilisin-like PC1/3 and PC2 proteases (PC = prohormone convertase) [9], and a 70 kDa aspartic proteinase [10].

The neuroendocrine-specific prohormone convertases (PC) PC1/3 and PC2 [1–3], that were cloned based on active site homology to the yeast Kex2 gene required in pro-α-mating factor processing [11], have been demonstrated to process proinsulin, POMC, and many other prohormones. Characterization of recombinant PC1/3 and PC2 through several approaches–(a) co-expression of PC enzymes with POMC, and other prohormone cDNAs, in cell lines [1–3,12,13], (b) characterization of purified recombinant PC enzymes [1–3,14–16], and (c) characterization of purified native PC enzymes from endogenous tissue sources [9,17,18]–demonstrate that these proteases possess appropriate cleavage specificity, pH optimum, and kinetic properties for prohormone processing. Evidence also indicates participation of a 70 kDa aspartic proteinase in proenkephalin processing in adrenal medulla [10], and in POMC processing in pituitary by a protease known as 'POMC converting enzyme' (PCE) [19]. Overall, these studies indicate that proteases of different mechanistic classes are involved in prohormone processing.

The different primary sequences of prohormones lead to the prediction that processing proteases may recognize and cleave one prohormone more readily than another. This hypothesis was addressed in this study by comparing the ability of the prohormone thiol protease (PTP) to process recombinant pro-neuropeptide Y, proopiomelanocortin, and proenkephalin. Pro-neuropeptide Y, proopiomelanocortin, and proenkephalin, as well as their peptide products, are co-localized with PTP in adrenal medulla and pituitary ([4,20,21]; Hook et al., manuscript in preparation). High level expression of these prohormones in *E. coli* has made it possible to achieve in vitro prohormone concentrations near estimated in vivo levels. Results demonstrate that PTP clearly possesses preference for certain prohormone substrates.

2. Experimental

2.1. Prohormone expression constructs

The cDNAs for rat pro-neuropeptide Y (pro-NPY) [22], porcine proopiomelanocortin (POMC) [23], and rat proenkephalin [7] were subcloned into the pET3c expression vector [24] (Fig. 1A). Pro-NPY and POMC cDNAs were amplified by PCR, with deletion of the signal sequences and incorporation of *Nde*I and *Bam*HI restriction sites for subcloning into the pET3c vector [24]. Primers for PCR of pro-NPY were 5'-AAACATATGTACCCCTCCAAGCCG-3' and 5'-AAAGGATCCCCATCACCACATGGAAG-3'; primers for PCR of POMC were 5'-AAACATATGTGGTGCTTGGAGAGCAGCCAGTGTCAG-3' and 5'-AAAGGATCCCCCTCACTGGCCCTTCTTGTGGGCGTTCTT-3'. PCR reactions (according to the protocol of the Perkin-Elmer PCR kit) contained kit 1 ng cDNA as template, and primers at 0.2 or 0.1 μM, with thermocycling consisting of 30 cycles of 1 min each at 94, 50, and 72°C. The PCR reaction for POMC also included 10% glycerol. The amplified pro-NPY (227 bp) and POMC (748 bp) DNAs were digested with *Nde*I and *Bam*HI, ligated with T4 ligase to the pET3c plasmid vector digested with *Nde*I and *Bam*HI, and transformed into DH5α *E. coli* cells. Pro-NPY/pET3c and POMC/pET3cplasmid constructs were subjected to DNA sequencing, as previously described [25], to confirm that PCR reactions generated authentic pro-NPY and POMC nucleotide

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Abbreviations: PTP, prohormone thiol protease; Pro-NPY, pro-neuropeptide Y; POMC, proopiomelanocortin; PE, proenkephalin; PC, prohormone convertase; ACTH, adrenocorticotropin hormone; IPTG, isopropyl-β-thiogalactopyranoside

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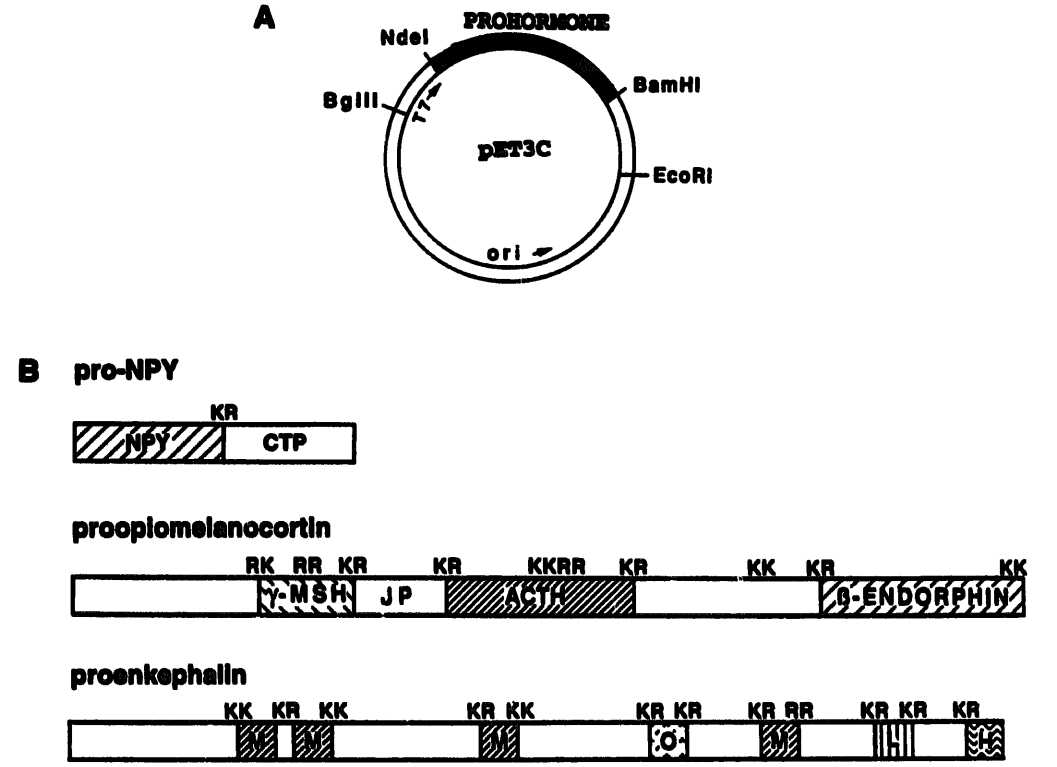

Fig. 1. Expression vector pET3c and illustration of prohormone structures for pro-NPY, POMC, and proenkephalin. (A) Expression with the pET3c T7 expression vector. Prohormone cDNAs were subcloned at *Nde*I and *Bam*HI restriction sites of the pET3c vector. (B) Prohormone structures. Pro-NPY contains NPY and COOH-terminal peptide (CTP) sequences separated by a Lys-Arg pair. Proopiomelanocortin (POMC) contains the peptides ACTH, β-endorphin, γ-MSH, joining peptide (JP). Proenkephalin contains 4 copies of (Met)enkephalin (M) (YGGFM), one copy of (Leu)enkephalin (L) (YGGFL), and the enkephalin-related peptides known as the octapeptide (O) (YGGFMRGL) and heptapeptide (H) (YGGFMRF).

sequences. The proenkephalin (PE) expression construct, PE/pET3c, was prepared as described previously [7].

2.2. Recombinant prohormone expression and purification

Recombinant pro-NPY, POMC, and proenkephalin were expressed by IPTG (isopropyl-β-thiogalactopyranoside) induction of BL21(DE3) *E. coli* cells containing the prohormone/pET3c constructs, with expression conducted as described previously [7]. Expression was assessed by Western blots utilizing anti-NPY serum (1:200 final dilution, from Accurate Chemicals, New York) to detect pro-NPY, and anti-β-LPH serum (1:1000 final dilution, from the National Hormone and Pituitary Program, Gaithersburg, MD) to detect POMC. Expression of recombinant proenkephalin in *E. coli* has been confirmed by Western blots with anti-PE monoclonal antibody [7]. For large-scale expression and purification, pro-NPY and POMC were expressed as 10 and 5 l cell cultures, respectively; proenkephalin was expressed from 5 l of culture.

For purification of pro-NPY, cells were pelleted (2600×*g* for 20 min), resuspended in 55 ml of 50 mM Tris-HCl, pH 7.5 (buffer A), and lysed by sonication. After centrifugation of the lysate (27000×*g* for 30 min at 4°C), the supernatant was chromatographed on a DEAE-Sepharose ion exchange column (1.5×40 cm) equilibrated in buffer A, with elution of pro-NPY by a 0–500 mM NaCl gradient (500 ml gradient) in buffer A (25 ml/h flow rate). Pro-NPY in column fractions (4.5 ml/fraction) was monitored by anti-NPY Western blots and Coomassie blue staining of SDS-PAGE gels. Pro-NPY fractions were pooled, concentrated by the Speed-Vac, and chromatographed two times on a molecular sieving Superose-12 FPLC column equilibrated in buffer A. Purified pro-NPY was subjected to peptide microsequencing and amino acid composition analyses that confirmed the purified prohormone as pro-NPY.

For purification of POMC, cells from 5 l of culture were pelleted (2600×*g* for 20 min), resuspended in 20 ml of 50 mM Tris-HCl, pH 7.5, pelleted again by centrifugation, and resuspended in 20 ml 50 mM MOPS-HCl, pH 6.5, 6.0 M urea (buffer B). Cells were lysed by sonication, and solubilized POMC was obtained as the supernatant resulting from centrifugation at 27000×*g* for 30 min. The supernatant was applied to DEAE column (1.5×36 cm) equilibrated with buffer B (flow rate of 16 ml/h), and POMC was eluted with a 0-500 mM NaCl gradient (400 ml gradient) in buffer B. Fractions (3.7 ml) were assayed for POMC by anti-β-LPH Western blots. POMC positive fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.0, and concentrated to 200–500 μl by the Speed-Vac. POMC was then subjected to preparative SDS-PAGE gel electrophoresis using the Biorad model 491 preparative cell, performed as previously described [7]. Purified POMC obtained from this step was dialyzed against 0.2 mM Tris-HCl pH 7.5, lyophilized, and resuspended in 250 μl H_2O. It is noted that any residual SDS has no effect on PTP enzyme activity, since proenkephalin, whose purification includes the SDS-PAGE step, was appropriately cleaved by PTP at paired basic residues to generate products known to be present in vivo [7].

2.3. Prohormone thiol protease (PTP) incubation with prohormones

The prohormone thiol protease (PTP) was purified from bovine chromaffin granules of adrenal medulla as previously described [4]. Pro-NPY (4 μg, equivalent to 6 μM pro-NPY) and PTP (1 ng) were incubated in 100 mM citrate-NaOH, pH 5.0, 1 mM EDTA, 1 mM DTT, and 10 mM CHAPS (3-[3-chloramidopropyldimethylammonio]-1-propanesulfonate) in a total volume of 80 μl for 10 min to 8 h at 37°C. Enzyme reactions were subjected to 15% polyacrylamide Tricine-buffered SDS-PAGE gels by the method of Schaegger and Von Jagow [26], transblotted to PVDF membranes, and stained with amido black. POMC (4 μg) and PTP (1 ng) were incubated for 6 h at 37°C; POMC reactions were analyzed by western blots with anti-β-LPH serum. PE and PTP were incubated for 1–24 h at 37°C, and PE reactions were analyzed by SDS-PAGE gels blotted to PVDF

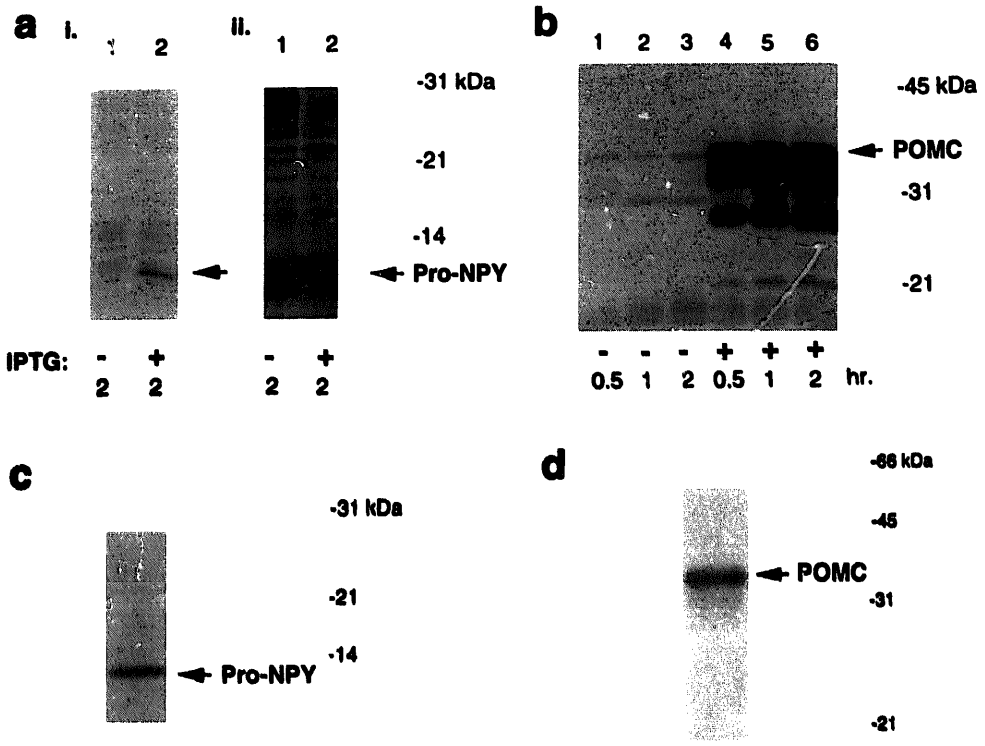

Fig. 2. Expression of pro-NPY and POMC, and purified recombinant prohormones. (a) Pro-NPY expression. (i) Pro-NPY expression detected on SDS-PAGE gels. Expression of pro-NPY is indicated by IPTG induction (2 h incubation of cells with IPTG) of an 8 kDa band detected by Coomassie blue staining of a SDS-PAGE gel. (ii) Pro-NPY expression detected by Western blot. Anti-NPY immunoblots indicated IPTG induction of the pro-NPY immunoreactive 8 kDa band. (b) POMC expression. Expression of POMC was induced when cells were incubated with IPTG for 0.5, 1, or 2 h. Anti-β-LPH immunoblots demonstrate expression of intact 35 kDa POMC. (c) Purified pro-NPY. Pro-NPY (5 μg) was purified to apparent homogeneity, as illustrated by the single band of 8 kDa pro-NPY. (d) Purified POMC. POMC (2 μg) was purified to apparent homogeneity, as illustrated by the single band of 35 kDa POMC.

membranes and stained with amido black. Densitometry of gels (performed as previously described [7]) was used to quantitate the amount of prohormones cleaved.

3. Results and discussion

3.1. Expression and purification of recombinant pro-NPY and POMC

The T7 expression system utilizing the pET3c expression vector (Fig. 1A) generates high levels of recombinant prohormones. Expression of pro-NPY, POMC, and proenkephalin (PE) prohormones (Fig. 1B) was successfully achieved in this study with the pET3c vector. Expression and purification of recombinant pro-NPY and POMC is described in this report; expression and purification of proenkephalin has been described [7].

Induction by IPTG of pro-NPY expression in *E. coli* was apparent after 2 h incubation at 37°C (Fig. 2a). The expression of pro-NPY as a 8 kDa band was detected on SDS-PAGE gels (Fig. 2ai); western blot with anti-NPY (Fig. 2aii) indicated this 8 kDa band to be immunoreactive for NPY. Also, the observed apparent M_r of 8 kDa is consistent with the theoretical size of 8059 M_r of pro-NPY, based on the deduced primary sequence from the pro-NPY cDNA [22]. POMC expression was detected by western blots with anti-β-LPH serum (Fig. 2b) which recognizes the COOH-terminal segment of POMC that includes β-endorphin [23]. Intact POMC of 35 kDa is consistent with the theoretical M_r of POMC [23]. The time-dependent appearance of two lower molecular weight bands of POMC immunoreactivity indicated some degradation of POMC within the cells. In addition, it was noted that lower levels of POMC were expressed compared to pro-NPY, since POMC could only be detected by western blots, and not by Coomassie blue staining of proteins on SDS-PAGE gels.

Pro-NPY was purified by chromatography of solubilized pro-NPY by DEAE-Sepharose and Superose-12 FPLC columns. The DEAE-Sepharose step provided a 20-fold purification of pro-NPY with a 30% yield. The Superose-12 FPLC step resulted in purified pro-NPY, as indicated by a single 8 kDa pro-NPY band on SDS-PAGE (Fig. 2c). Pro-NPY was purified with a yield of 20% and a 170-fold purification.

Table 1
Rates of PTP cleavage of proenkephalin, pro-NPY, and POMC

Prohormone	Rate of processing (μmol/min per mg)
Proenkephalin	3.13
Pro-NPY	1.75
POMC	4.7×10^{-3}

The rates of PTP cleavage of proenkephalin, pro-NPY, and POMC were calculated. Prohormones (at micromolar concentrations, see section 2) were incubated in vitro with PTP under optimum, standard PTP assay conditions [4,7].

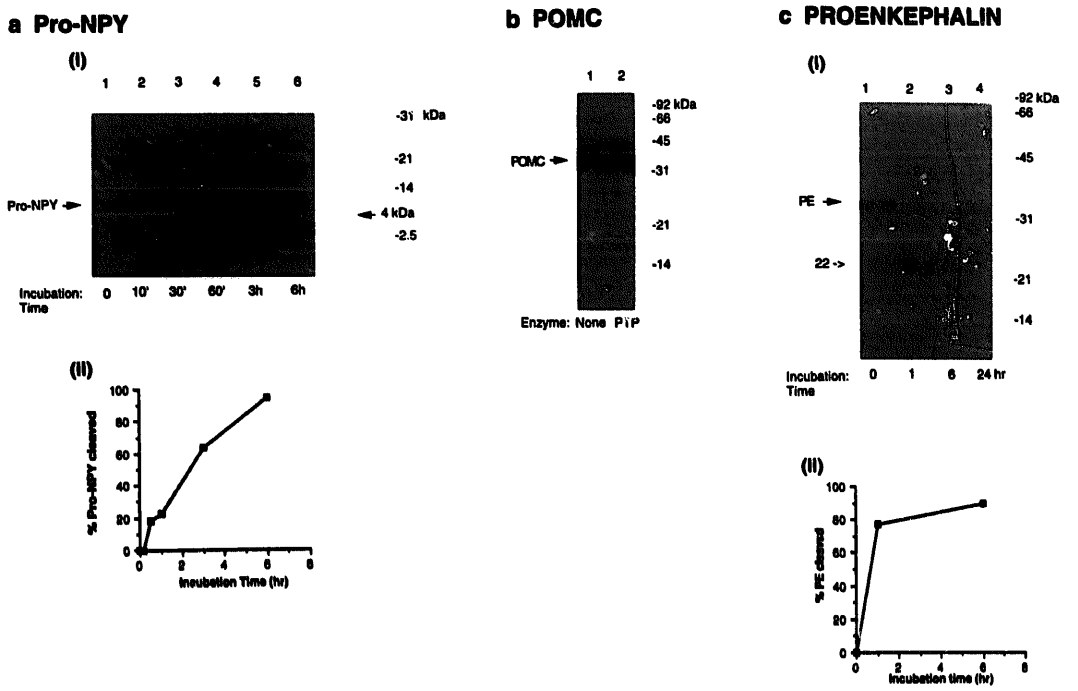

Fig. 3. PTP processing of pro-NPY, POMC, and PE. (a) Pro-NPY. (i) SDS-PAGE of pro-NPY incubated with PTP in a time course study. Pro-NPY (4 μg) was incubated with PTP (1 ng) for 0–8 h. Reaction samples were subjected to SDS-PAGE, electrophoretically blotted to PVDF membranes, and stained with amido black. The arrow indicates a product of 4 kDa. (ii) Time course of pro-NPY processing. Densitometry of pro-NPY on SDS-PAGE gels (from panel (ai)) quantitated the degree of pro-NPY processing by PTP. (b) POMC. POMC (4 μg) was incubated without (lane 1) and with purified PTP (1 ng) (lane 2), and anti-β-LPH western blotting was utilized to assess the degree of POMC remaining after incubation with PTP. (c) Proenkephalin. (i) Proenkephalin and PTP, SDS-PAGE gels. Proenkephalin (8 μg) was incubated with PTP (1 ng) for 0, 1, 6, and 24 h (lanes 1-4, respectively) at 37°C. Reactions were subjected to SDS-PAGE, electrophoretically transferred to PVDF membranes, and stained with amido black. (ii) Percent proenkephalin cleaved. Densitometry assessed the relative amounts of proenkephalin cleaved.

POMC was purified by DEAE-Sepharose and preparative SDS-PAGE gel electrophoresis. The DEAE-Sepharose column provided a 60% recovery and a 30-fold purification. Subsequent preparative gel electrophoresis isolated the intact 35 kDa POMC. Purification of POMC to apparent homogeneity was indicated by the single band of 35 kDa POMC on SDS-PAGE (Fig. 2d).

3.2 *Prohormone thiol protease preference for prohormone substrates*

The ability of the prohormone thiol protease (PTP) to cleave pro-NPY, POMC, and proenkephalin (PE) was assessed under identical PTP reaction conditions, with in vitro prohormones at micromolar concentrations that represent in vivo prohormone levels [27]. Pro-NPY was readily cleaved by PTP, as demonstrated in a time course study showing cleavage of 50% of pro-NPY within 2.5 h (Fig. 3a). PTP generated a 4 kDa product that is consistent with the production of NPY since the NH_2-terminus (YPSKXDNXGE, determined by peptide microsequencing) of the 4 kDa band corresponds to NPY, and its apparent M_r is consistent with NPY. These results are compatible with PTP cleavage at a Lys-Arg paired basic residue site [4–7] that would result in NPY.

In contrast to pro-NPY, POMC was cleaved more slowly by PTP (Fig. 3b). Even after 24 h incubation of POMC with PTP, only 2-3% of the POMC was cleaved. Pro-NPY, on the other hand, was completely cleaved by 6 h incubation (Fig. 3a). In addition, PTP did not cleave β-lipotropin hormone (β-LPH), the COOH-terminal segment of POMC that contains β-endorphin flanked by a paired basic residue cleavage site (data not shown). These results suggest that POMC and its intermediates may be poor substrates for PTP.

Comparison of proenkephalin (PE) with pro-NPY and POMC processing by PTP indicated that PTP prefers proenkephalin among the prohormones tested. PE was readily cleaved by PTP, as shown by cleavage of the majority of PE within 1 h (Fig. 3c). Comparison of the extent of prohormones cleaved after 1 h incubation showed that 80% of PE was processed, 20% of pro-NPY was processed, and almost no POMC was processed (Fig. 4). The observed rates of prohormone cleavage (Table 1) indicate that proenkephalin was cleaved at a rate that was nearly 2-fold greater than that for pro-NPY. However, both proenkephalin and pro-NPY were cleaved at approximately 1000 times (3 orders of magnitude) greater rates than that observed for POMC. These results suggest proenkephalin or pro-NPY as preferred PTP prohormone substrates.

3.3. *Conclusions*

Results from this study demonstrate that the prohormone thiol protease (PTP), a candidate prohormone processing enzyme, shows preference for prohormone substrates. In particular, proenkephalin was the most efficiently cleaved precursor among the prohormones examined in this study. Pro-NPY was also processed quite efficiently, with processing of pro-NPY occurring at approximately half the rate observed for

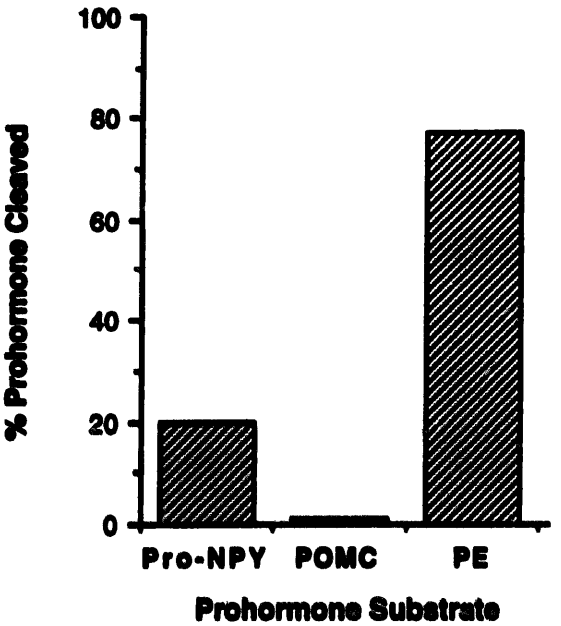

Fig. 4. Relative processing of proenkephalin, pro-NPY, and POMC by PTP. Comparison of the extent of processing of prohormones (at micromolar concentrations) after 1 h incubation with PTP from time course studies is illustrated.

proenkephalin. However, PTP processing of POMC was slower by 3 orders of magnitude.

These findings suggest that elucidation of the primary processing protease(s) involved in processing a particular prohormone may depend on the selectivity of processing enzymes for prohormone substrates. The preference of a processing enzyme for a proprotein substrate may involve selective recognition of particular prohormone structures, as defined by the unique primary sequence and three-dimensional conformation of each prohormone. It is clear that further studies of prohormone selectivity of processing enzymes will be important in the elucidation of the major processing proteases required for converting different prohormones into bioactive neuropeptides.

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