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Catabolism of the octadecaneuropeptide ODN by prolyl endopeptidase: Identification of an unusual cleavage site

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DBI, diazepam-binding inhibitor
*h*UII, human urotensin II
ODN, octadecaneuropeptide
PEP, prolyl endopeptidase

ABSTRACT

The octadecaneuropeptide ODN (QATVGDVNTDRPGLLDLK), a biologically active fragment of diazepam-binding inhibitor, exerts a number of behavioral and neurophysiological activities. The presence of a proline residue in the sequence of ODN led us to investigate the role of proline endopeptidase (PEP) in the catabolism of this neuropeptide. The effect of PEP on the breakdown of ODN and related analogs was studied by combining RP-HPLC analysis and MALDI-TOF MS characterization. Incubation of ODN with PEP generated two products, i.e. ODN_{3-18} and ODN_{5-18} which resulted from cleavage of the Ala-Thr and Val-Gly peptide bonds. S 17092, a specific PEP inhibitor, significantly reduced the PEP-induced cleavages of ODN. Similarly, [Ala^2]OP showed S 17092-sensitive post-alanine cleavage, while [*p*Glu^1]ODN and OP (ODN_{11-18}) were not catabolized by the enzyme. For all these peptides, cleavage of the Pro-Gly peptide bond by PEP was never observed, even after prolonged incubation times. In contrast, PEP hydrolyzed human urotensin II at the canonical post-proline site. Collectively, these data suggest that the Ala^2 residue is the preferential cleavage site of ODN and that the Pro-Gly bond of ODN is not hydrolyzed by PEP. In addition, this study reveals for the first time that the endoproteolytic activity of PEP can specifically take place after a valine moiety.

1. Introduction

The octadecaneuropeptide (ODN) has been originally characterized as a selective ligand for central-type benzodiazepine receptors [16]. ODN is generated by proteolytic cleavage of an 86-aminoacid precursor called diazepam-binding inhibitor (DBI; Fig. 1) [21]. The primary structure of DBI has now been characterized in several mammalian species including man [34], ox [34], pig [7], dog [25], armadillo [6] and rat [24]. The sequence of ODN has been highly conserved during evolution indicating that this peptide may play important biological functions. Indeed, behavioral studies have shown that ODN

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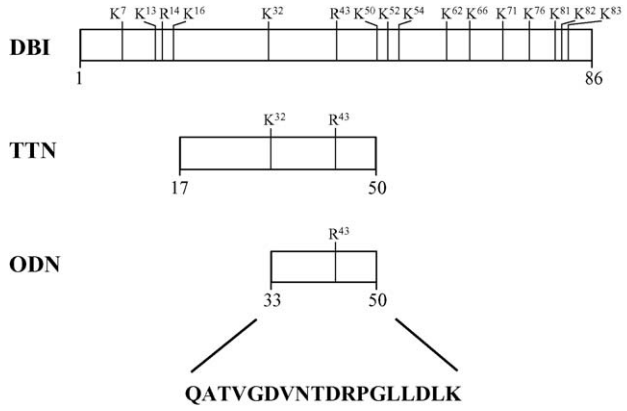

Fig. 1 – Schematic representation of the primary structure of rat diazepam-binding inhibitor (DBI) and two of its processing fragments, the triakontatetraneuropeptide TTN ($DBI_{17–50}$) and the octadecaneuropeptide ODN ($DBI_{33–50}$). Vertical bars indicate the basic amino acids which represent potential cleavage sites for prohormone convertases. The sequence of rat ODN is shown.

induces anxiety [11,23], attenuates pentylenetetrazol-evoked convulsions [20], suppresses apomorphine-induced yawning [12], and inhibits food intake [13]. At the cellular level, ODN increases intracellular calcium concentrations in cultured rat astrocytes [18], activates glial cell proliferation [19], stimulates neurosteroid biosynthesis [14], and regulates the expression of the proopiomelanocortin, neuropeptide Y, corticotropin-releasing hormone and gonadotropin-releasing hormone (GnRH) genes in the hypothalamus [8,9]. Despite the multiple behavioral and neurobiological activities of ODN, the mechanisms of inactivation of this neuropeptide have never been investigated.

Prolyl endopeptidase (PEP) is a serine protease that preferentially hydrolyzes proline-containing peptides at the carboxyl terminus of proline residues (for review, see Ref. [10]). In particular, it has been shown that PEP can efficiently cleave the post-proline bond in various neuropeptides including arginine-vasopressin (AVP), oxytocin, substance P, thyrotropin-releasing hormone (TRH) and α-melanocyte-stimulating hormone (α-MSH) [2,4,41,47]. In addition, PEP can hydrolyze peptides at the carboxyl side of alanine residues [37,48,50] and may thus contribute to the formation of β-amyloid (Aβ) peptides from the amyloid precursor protein [40].

Two observations prompted us to study the possible involvement of PEP in the breakdown of the neuropeptide ODN: (i) the sequence of ODN encompasses both proline and alanine residues (Pro^{12} and Ala^{2}; Fig. 1) and (ii) the regions of the rat brain that exhibit the highest concentrations of ODN, i.e. the olfactory bulb, the hypothalamus and the hippocampus [44], also contain high levels of PEP mRNA [3]. In the present report, we have investigated the in vitro effect of PEP on the catabolism of rat ODN and related peptides, and we have identified the breakdown products by combining reversed phase high-performance liquid chromatography (RP-HPLC) analysis and MALDI-TOF mass spectrometry (MS) characterization.

2. Materials and methods

2.1. Chemicals

Rat ODN, the C-terminal octapeptide fragment $ODN_{11–18}$ (OP), $ODN_{3–18}$, $ODN_{5–18}$, $ODN_{13–18}$, [$pGlu^{1}$]ODN, [Ala^{2}]OP, human urotensin II (*h*UII), and $hUII_{4–11}$ were synthesized by the solid-phase methodology on a 433A Applied Biosystems peptide synthesizer (Applera France, Courtaboeuf, France) using the standard Fmoc procedure as previously described [27,28]. The synthetic peptides were purified by RP-HPLC and characterized by MALDI-TOF MS. The purity of all peptides was higher than 99.9% except for ODN which initially contained 6% of the pyroglutamic form. The selective PEP inhibitor (2*S*, 3a*S*, 7a*S*)-1-{[(*R*,*R*)-2-phenylcyclopropyl]carbonyl}-2-[(thiazolidin-3-yl)carbonyl]octahydro-1*H*-indole (S 17092) was kindly provided by Dr. P. Morain (Institut de Recherches Internationales Servier, Courbevoie, France). Recombinant Flavobacterium meningosepticum PEP was purchased from ICN Biomedicals (Aurora, OH, USA). Benzyloxycarbonyl-glycyl-prolyl-paranitroanilide (Z-Gly-Pro-pNA) was from Bachem (Weil am Rhein, Germany). Acetonitrile was from Carlo Erba (Val-de-Reuil, France) and trifluoroacetic acid (TFA) was from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.2. Peptide hydrolysis by PEP

The activity of PEP was initially checked with the synthetic substrate Z-Gly-Pro-pNA by measuring the absorbance at 410 nm as previously described [2]. Seven microgram of each peptide was dissolved in 1 ml of 25 mM phosphate buffer (PB) (pH 7.5) and incubated with 3 μg of Flavobacterium PEP, at 37 °C for 2 to 15 h, in the absence or presence of the PEP inhibitor S 17092 (1–4 μM). Z-Gly-Pro-pNA and S 17092 were extemporaneously solubilized in dimethylsulfoxide (DMSO) and final solutions were made up in PB so that the concentration of DMSO never exceeded 1%. The reaction was stopped by chilling the samples on ice before RP-HPLC analysis.

2.3. RP-HPLC analysis

After incubation, peptide samples were submitted to RP-HPLC analysis on a Nucleosil 300-5 C_{18} column (0.8 cm × 12.5 cm) using a linear gradient of acetonitrile/TFA (99.9/0.1, v/v) as shown on the chromatograms, at a flow rate of 2 ml/min. Fractions corresponding to each discrete peak were manually collected and subjected to MALDI-TOF MS analysis.

2.4. MALDI-TOF MS analysis

Mass spectra were acquired using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applera France). The matrix, α-cyano-4-hydroxycinnamic acid (LaserBio Lab, Sophia-Antipolis, France), was prepared at a concentration of 10 mg/ml in acetonitrile. One microlitre samples of RP-HPLC fractions were deposited on the sample plate immediately followed by 1 μl matrix, and dried at room temperature according to the dried-droplet method. Peptide standards (des-Arg^{1}-bradykinin, angiotensin I, [Glu^{1}]-fibrinopeptide B and neurotensin) (Applera France) were spotted next to the samples. The

spectra represent the resolved monoisotopic MH^+ ions in positive and reflectron modes.

3. Results

3.1. Incubation of ODN with Flavobacterium PEP

The time-course of ODN breakdown by recombinant PEP was investigated by combining RP-HPLC analysis with MALDI-TOF MS characterization. In control conditions, synthetic intact ODN eluted as a major peak (peak 1) with a retention time (rt) of 38.67 min (Fig. 2A). A minor contaminant, slightly more hydrophobic than ODN (rt = 39.98 min), corresponding to the pyroglutamic form of ODN ([*p*Glu^1]ODN; peak 2) was spontaneously generated in solution (Fig. 2A) and the relative importance of peak 2 increased in a time-dependent manner during incubation of ODN at 37 °C (Fig. 2B,C). Incubation of ODN with PEP (6 and 15 h) led to the appearance of a third peak (peak 3) with a rt of 36.92 min (Fig. 2B,C) that was more hydrophobic than synthetic $ODN_{13–18}$ (rt = 35.77 min).

MALDI-TOF MS analysis of peak 1 (which originally contained synthetic intact ODN) isolated after a 15-h incubation with PEP (Fig. 2C) revealed the presence of two compounds with main ions at *m/z* values of 1911.7, corresponding to intact ODN (MH^+ calcd = 1912.0), and 1712.4, corresponding to $ODN_{3–18}$ (MH^+ calcd = 1712.9) (Fig. 3A). In fact, MS analysis of peak 1 isolated after a 2-h incubation with PEP showed that $ODN_{3–18}$ had already been formed from ODN (data not shown). MS analysis of purified peak 3, isolated after a 15-h incubation with PEP (Fig. 2C), revealed the presence of a main ion with a *m/z* value of 1512.9 (Fig. 3B), indicating that this peak corresponds to $ODN_{5–18}$ (MH^+ calcd = 1512.8). Finally, MS analysis of peak 2, isolated after a 15-h incubation with PEP (Fig. 2C) showed a main ion with a *m/z* value of 1895.3 (Fig. 3C) confirming that this peak actually corresponds to [*p*Glu^1]ODN (MH^+ calcd = 1895.0).

Addition of the PEP inhibitor S 17092 (4 μM) to the ODN/PEP mixture inhibited by 85% the formation of $ODN_{5–18}$ (peak 3 in Fig. 4A versus peak 3 in Fig. 2C) and the reduction in the amplitude of peak 3 was accompanied by a concomitant increase in the magnitude of both peaks 1 and 2. Application of a shallower gradient made it possible to resolve ODN and $ODN_{3–18}$ (Fig. 4B) that initially co-eluted as a single peak (peak 1 in Fig. 2C). Addition of S 17092 (4 μM) to the ODN/PEP mixture inhibited by 50% the formation of $ODN_{3–18}$ (Fig. 4B versus Fig. 4C).

3.2. Incubation of [*p*Glu^1]ODN with Flavobacterium PEP

Incubation of synthetic [*p*Glu^1]ODN (7 μg) with recombinant PEP at 37 °C for 15 h did not provoke the formation of any peptide fragment co-eluting with peak 1 ($ODN_{3–18}$) or peak 3 ($ODN_{5–18}$) (Fig. 5) indicating that, in contrast to what has been seen with ODN (Fig. 2C), PEP could not cleave [*p*Glu^1]ODN at the Ala^2-Thr^3 nor at the Val^4-Gly^5 peptide bonds. The chromatogram also revealed the absence of $ODN_{13–18}$ (Fig. 5) confirming that PEP was unable to cleave the post-proline bond of [*p*Glu^1]ODN in very much the same as for ODN (Fig. 2C).

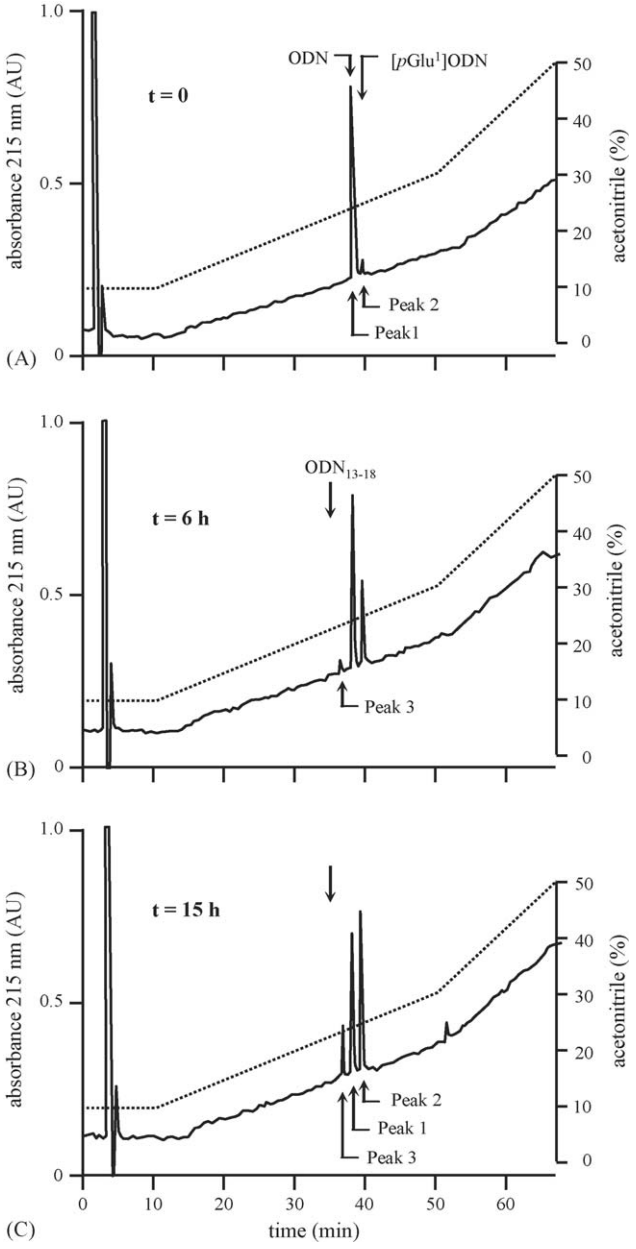

Fig. 2 – RP-HPLC analysis showing the time-course of ODN breakdown induced by recombinant PEP. Synthetic ODN (7 μg) was incubated at 37 °C in the absence (A) or presence of 3 μg PEP for 6 h (B) and 15 h (C). The incubation mixtures were then analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic intact ODN, [*p*Glu^1]ODN and $ODN_{13–18}$. The dotted lines show the percentage of acetonitrile in the eluting solvent.

3.3. Incubation of OP with Flavobacterium PEP

Incubation of OP (RPGLLDLK, 7 μg) with recombinant PEP at 37 °C for 15 h did not provoke the appearance of novel compounds as evidenced by RP-HPLC analysis (Fig. 6A). In particular, no peak exhibiting the rt of $ODN_{13–18}$ was observed. MS analysis of the OP/PEP mixture confirmed the presence,

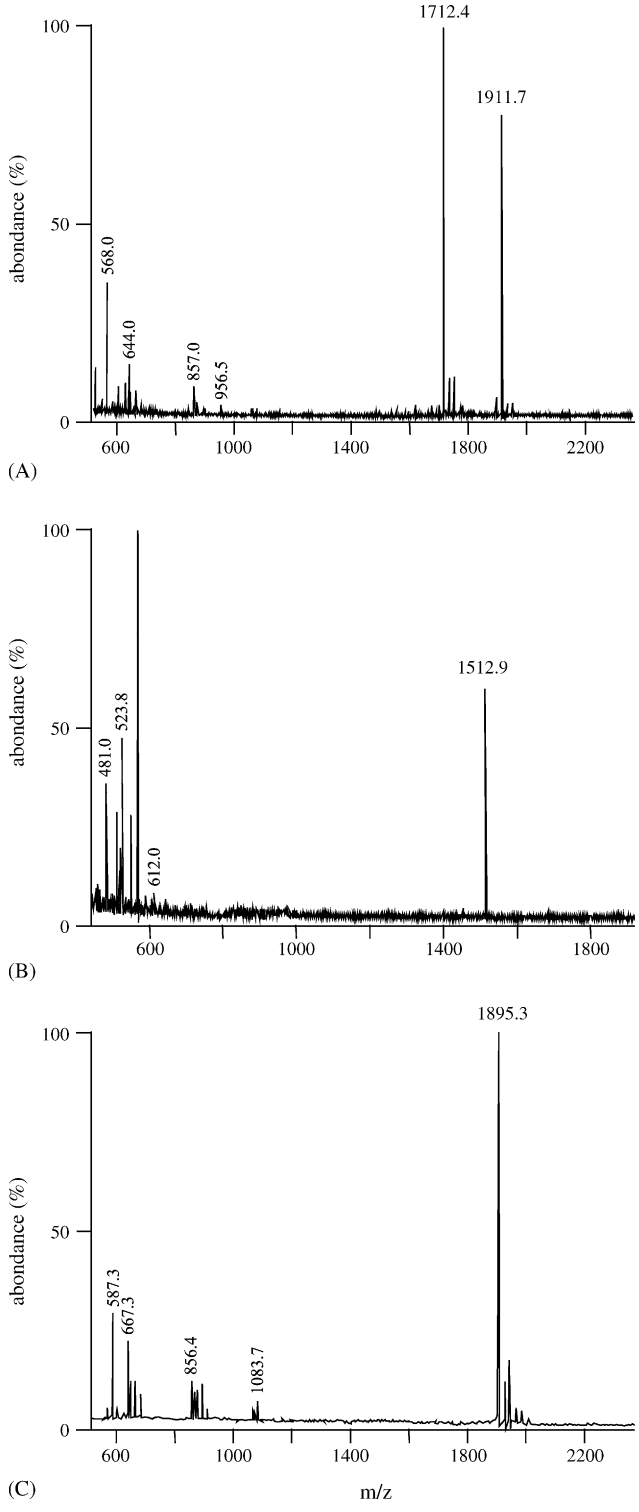

Fig. 3 – MALDI-TOF MS spectra of the RP-HPLC peaks isolated in Fig. 2C. The *m/z* ions revealed that peak 1 (A) contained both intact ODN (MH^+ exptl = 1911.7 vs. MH^+ calcd = 1912.0) and $ODN_{3–18}$ (MH^+ exptl = 1712.4 vs. MH^+ calcd = 1712.9), peak 3 (B) contained $ODN_{5–18}$ (MH^+ exptl = 1512.9 vs. MH^+ calcd = 1512.8), and peak 2 (C) contained [$pGlu^1$]ODN (MH^+ exptl = 1895.3 vs. MH^+ calcd = 1895.0).

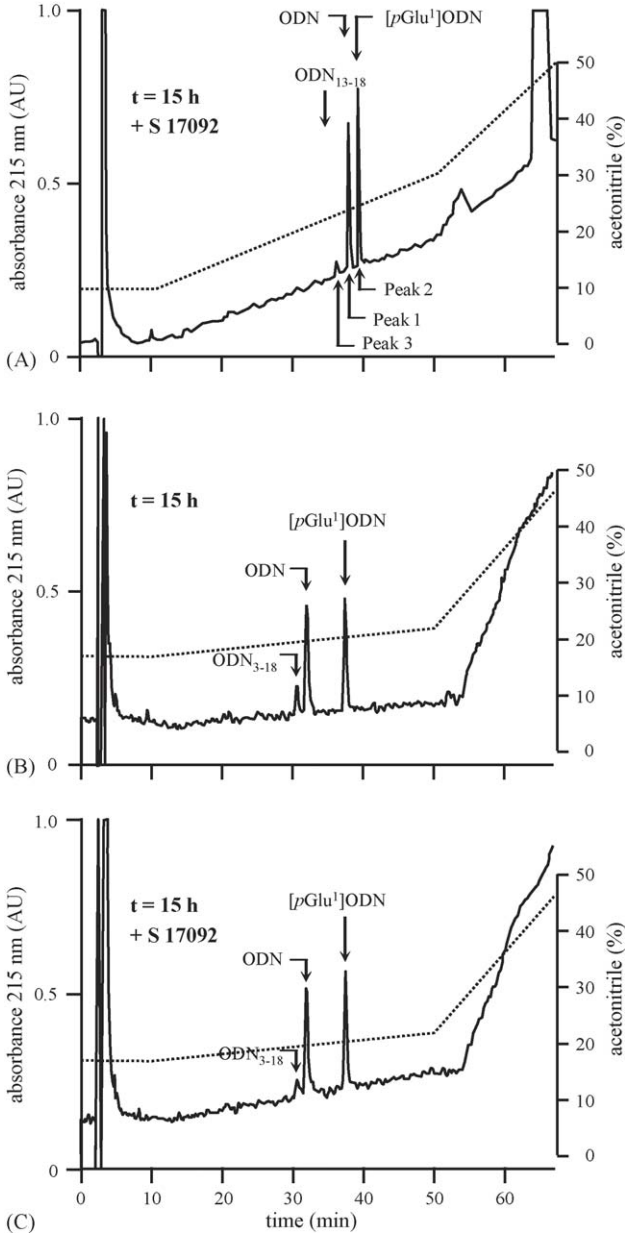

Fig. 4 – Effect of S 17092 on ODN breakdown induced by recombinant PEP. Synthetic ODN (7 μg) was incubated with 3 μg of PEP, at 37 °C for 15 h, in the presence (A and C) or absence (B) of the selective PEP inhibitor S 17092 (4 μM). The incubation mixtures were analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic intact $ODN_{13–18}$, ODN, [$pGlu^1$]ODN and $ODN_{3–18}$. The dotted lines show the percentage of acetonitrile in the eluting solvent.

after a 15-h incubation, of a compound with a *m/z* value of 911.3 corresponding to intact OP (MH^+ calcd = 911.6) and the absence of any ion with a *m/z* value of 658.4 that would have corresponded to the calculated MH^+ value for $ODN_{13–18}$ (Fig. 6B).

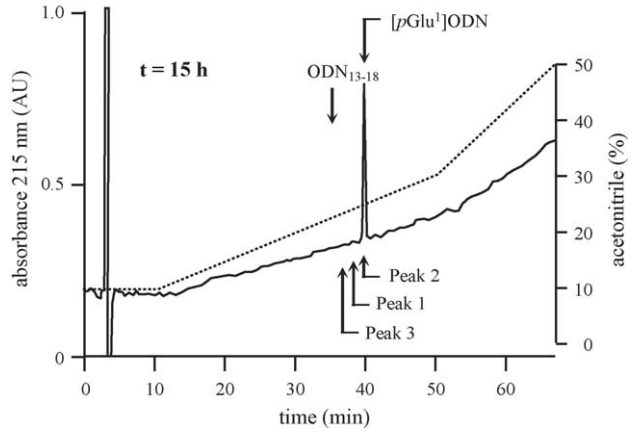

Fig. 5 – Effect of recombinant PEP on [*p*Glu^1]ODN breakdown. Synthetic [*p*Glu^1]ODN (7 μg) was incubated with 3 μg of PEP at 37 °C for 15 h. The incubation mixture was then analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic ODN$_{13–18}$, ODN$_{5–18}$ (peak 3), ODN$_{3–18}$ (peak 1) and [*p*Glu^1]ODN (peak 2). The dotted line shows the percentage of acetonitrile in the eluting solvent.

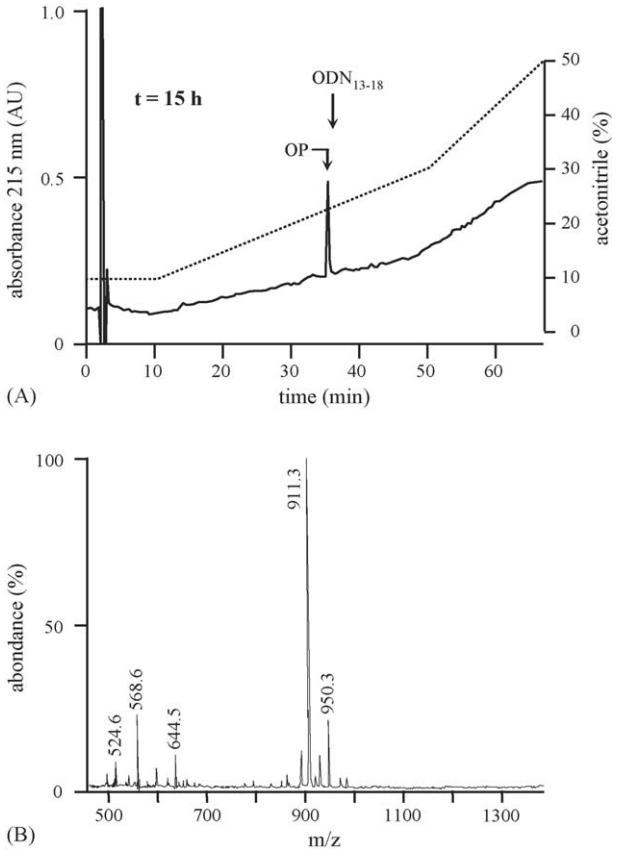

Fig. 6 – Effect of recombinant PEP on OP breakdown. Synthetic OP (7 μg) was incubated with 3 μg of PEP at 37 °C for 15 h. (A) The incubation mixture was then analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic OP and ODN$_{13–18}$. The dotted line shows the percentage of acetonitrile in the eluting solvent. (B) MALDI-TOF MS spectrum of the OP/PEP mixture after a 15-h incubation time. The *m/z* ion revealed the presence of intact OP (MH$^+$ exptl = 911.3 vs. MH$^+$ calcd = 911.6), and the absence of ODN$_{13–18}$ (MH$^+$ calcd = 658.4).

3.4. *Incubation of [Ala^2]OP with Flavobacterium PEP*

Incubation of [Ala^2]OP (7 μg) with PEP at 37 °C for 15 h provoked the formation of a more hydrophobic peptide (peak 4) that co-eluted with synthetic ODN$_{13–18}$ (Fig. 7A). Addition of S 17092 (1 μM) to the incubation medium totally blocked the cleavage of [Ala^2]OP induced by PEP (Fig. 7B). MS analysis of the resolved peak 4 (Fig. 7A) showed the presence of a main ion with a *m/z* value of 658.3 corresponding to ODN$_{13–18}$ (MH$^+$ calcd = 658.4) (data not shown).

3.5. *Incubation of hUII with Flavobacterium PEP*

Incubation of *h*UII (ETPDCFWKYCV, 7 μg) with PEP at 37 °C for 6 h provoked the formation of a more hydrophilic peptide (peak 5) that exhibited the same rt as that of synthetic *h*UII$_{4–11}$ (Fig. 8A). Addition of S 17092 (1 μM) to the incubation medium totally blocked the cleavage of *h*UII induced by recombinant PEP (Fig. 8B). MS analysis of the resolved peak 5 (Fig. 8A) showed the presence of a main ion with a *m/z* value of 1061.3 corresponding effectively to *h*UII$_{4–11}$ (MH$^+$ calcd = 1061.4) (data not shown).

4. Discussion

The present study has shown that recombinant PEP can hydrolyze the endozepine ODN in vitro. The cleavage of ODN induced by PEP occurs at the Ala^2-Thr^3 and to a certain extent at the Val^4-Gly^5 bonds but not at the expected Pro^{12}-Gly^{13} bond. Endoproteolysis of ODN by PEP was markedly reduced by the selective PEP inhibitor S 17092. These data provide the first evidence that PEP can hydrolyze peptides at the carboxyl side of other residues than proline and alanine.

RP-HPLC analysis combined with MALDI-TOF MS revealed that incubation of ODN with recombinant PEP for 2 h generated ODN$_{3–18}$ while prolonged hydrolysis produced ODN$_{5–18}$. Paradoxically, we did not observe any formation of the expected ODN$_{1–12}$ and ODN$_{13–18}$ fragments that would have resulted from cleavage of the neuropeptide at the C-terminus of the proline residue. The absence of effect of PEP on the ODN Pro^{12}-Gly^{13} peptide bond could not be ascribed to a lack of activity of the enzyme inasmuch as the same batch of recombinant PEP was able to specifically cleave *h*UII at the expected Pro^3-Asp^4 peptide bond. The fact that only 15% of *h*UII was hydrolyzed during a 6-h incubation with PEP is attributable to the occurrence of an Asp residue flanking the Pro moiety on its carboxyl side since it has been shown that the presence of an acidic residue (Asp or Glu) adjacent to Pro impairs post-proline cleavage by PEP [26,42]. At any rate, the Pro-Gly bond is certainly a favorable cleavage site for PEP as GnRH (<QHWSYGLRPG-NH$_2$) is efficiently cleaved by PEP at

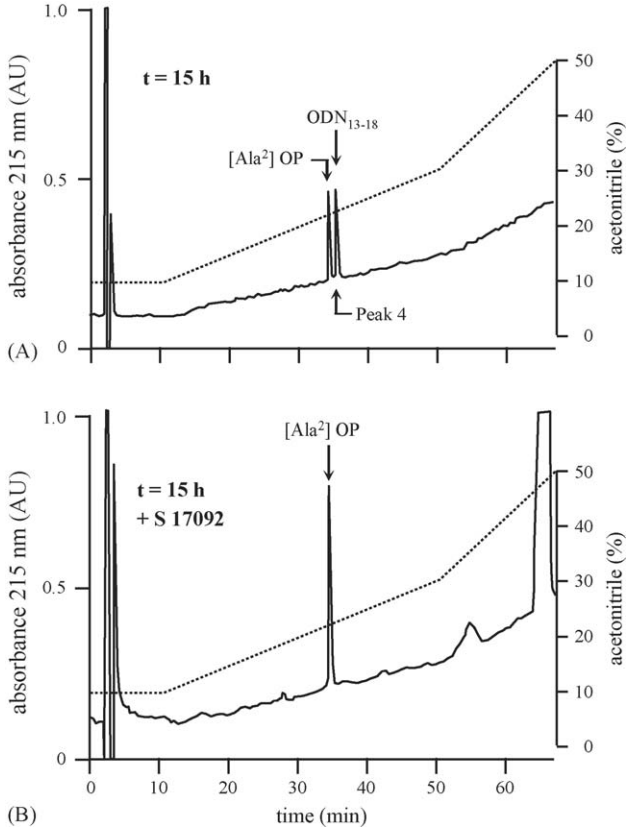

Fig. 7 – Effect of recombinant PEP on [Ala^2]OP breakdown. Synthetic [Ala^2]OP (7 μg) was incubated with 3 μg of PEP at 37 °C for 15 h, in the absence (A) or presence (B) of the selective PEP inhibitor S 17092 (1 μM). The incubation mixtures were analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic [Ala^2]OP and ODN$_{13–18}$. The dotted lines show the percentage of acetonitrile in the eluting solvent.

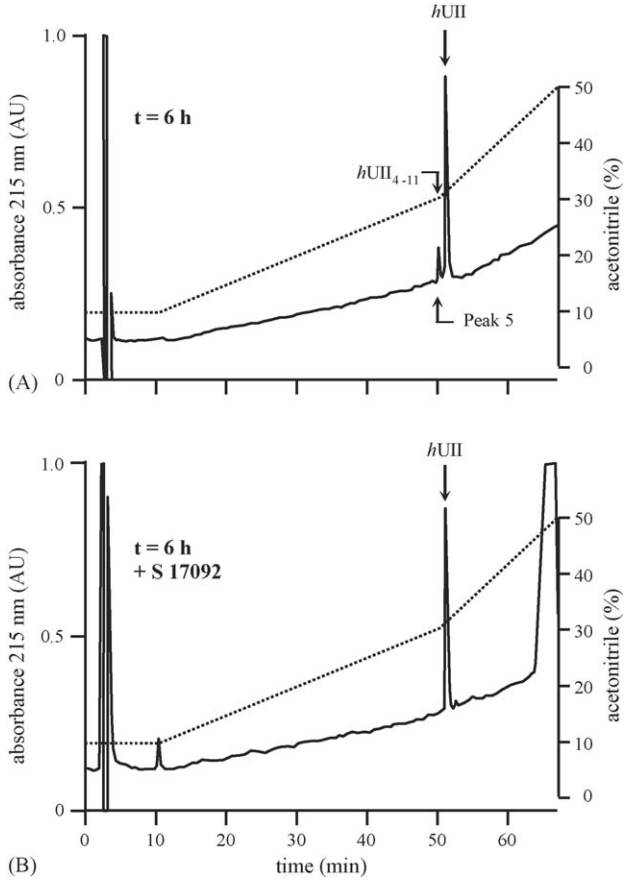

Fig. 8 – Effect of recombinant PEP on *h*UII breakdown. Synthetic *h*UII (7 μg) was incubated with 3 μg of PEP at 37 °C for 6 h, in the absence (A) or presence (B) of the selective PEP inhibitor S 17092 (1 μM). The incubation mixtures were analyzed by RP-HPLC and the resolved peptides were monitored by UV detection at 215 nm. The arrows indicate the retention times of synthetic *h*UII and *h*UII$_{4–11}$. The dotted lines show the percentage of acetonitrile in the eluting solvent.

this site [31,35,41]. Finally, it has been previously shown that the substrate specificities of Flavobacterium PEP and bovine brain PEP are indistinguishable [49] indicating that the cleavage observed with recombinant Flavobacterium PEP is actually representative of the proteolytic activity of the enzyme on ODN in the mammalian brain.

It has previously been shown that PEP is unable to cleave the Pro-Xaa bond if a free α-amino group exists in the N-terminal sequence H-Yaa-Pro-Xaa [10], hence the lack of hydrolytic activity of PEP on the post-proline bond of OP observed herein. In contrast, the absence of cleavage of the post-proline bond of ODN is more intriguing. One possible explanation may reside in the relative abundance of the *cis*/*trans* conformations at the amino side of the peptide bond of the Pro^{12} residue since PEP is known to selectively hydrolyze the peptide bonds on the C-terminal side of prolyl residues whose N-terminal bonds are in a *trans* configuration [17,32]. Thus, the present data would suggest that the Arg-Pro bond of ODN preferentially adopts a *cis* configuration. In support of this hypothesis, we have recently shown that a substantial proportion of the cyclic ODN derivative, cyclo$_{1–8}$[DLeu^5]OP, exhibits a *cis* conformation at the Arg-Pro peptide bond [29].

While PEP did not cleave ODN at the expected post-proline bond, it efficiently hydrolyzed both the post-alanine and post-valine bonds of ODN. Post-alanine scission of peptide bonds by PEP has been previously reported although this type of cleavage occurs at a much slower rate than that observed with post-proline bonds. For instance, incubation of the H-Ala-Ala-Ala-OH substrate with PEP produces only trace amounts of free alanine after 4 h but generates substantial quantities of alanine after 18 h [48]. Consistent with this observation, we found that post-alanine cleavage of ODN proceeded at a slow rate: trace amounts of ODN$_{3–18}$ were spectrometrically detectable after 2 h while significant amounts were formed after 15 h of incubation. As a comparison, post-proline cleavage of AVP and TRH by PEP occurs at a much higher rate, i.e. about 98% of both peptides are hydrolyzed after a 1-h incubation with PEP [4]. Incubation of ODN with PEP very unexpectedly generated the fragment ODN$_{5–18}$ that resulted

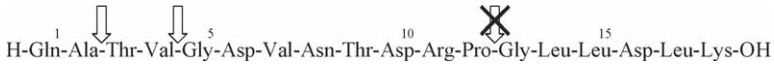

Fig. 9 – Schematic representation of PEP-induced breakdown of rat ODN. The arrows point to the observed positions of cleavage and the arrow with an × through it indicates that PEP does not cleave ODN at this canonical post-proline site.

from hydrolysis of the Val-Gly bond. Cleavage of a Val-Xaa bond by PEP has never been described before. In fact, it has been postulated that the activity of PEP was restricted to substrates with proline or, to a certain extent, with residues carrying substituents at the N and/or Cα atoms that do not exceed the size of the pyrrolidine ring of proline such as alanine [38]. Clearly, this is the first evidence that PEP can cleave post-valine peptide bonds.

The specific PEP inhibitor S 17092 [1] markedly reduced the PEP-induced post-alanine and post-valine scissions of ODN and totally blocked the effect of the enzyme on [Ala^2]OP and UII breakdowns, demonstrating that these cleavages were specifically due to PEP activity. In agreement with these observations, it has previously been shown that selective PEP inhibitors suppress in vitro post-alanine cleavage of amyloid precursor protein which yields to the formation of Aβ peptides [40] and prevent in vivo Aβ peptides deposition in the hippocampus of senescence-accelerated mice [22].

Previous studies have shown that PEP can hydrolyze various peptides possessing a N-terminal *p*Glu residue. In particular, PEP can efficiently cleave the prolinamide bond of TRH [4], the Pro^7-Arg^8 bond of neurotensin [5] and the Pro^9-Gly^{10} bond of GnRH [35]. Here, we found that PEP was totally unable to cleave the [*p*Glu^1]ODN analog, indicating that cyclization of the N-terminal Gln residue of ODN prevents hydrolysis of the Ala^2-Thr^3 and Val^4-Gly^5 bonds of ODN.

Several observations suggest that DBI and its processing products are implicated in various neurological disorders (for review, see Ref. [45]). In particular, an increased concentration of DBI-derived peptides has been measured in the cerebrospinal fluid of patients with Alzheimer's disease [15] and Aβ peptides have been shown to stimulate the expression of the DBI gene and the secretion of ODN-related peptides in cultured rat astrocytes [43]. On the other hand, there is strong evidence that PEP plays a role in the pathogenesis of Alzheimer's disease. It has notably been reported that, in vitro, PEP can hydrolyze the Ala^{42}-Ala^{43} peptide bond of the amyloid protein, and that a selective PEP inhibitor suppresses the production of Aβ peptides [40]. There is also strong evidence that PEP is involved in the catabolism of mnemocognitive-enhancing neuropeptides such as substance P, α-MSH [2], TRH and AVP [4], suggesting that specific PEP inhibitors could have beneficial effects on learning and memory impairment in Alzheimer's patients [36,46]. In support of this notion, it has been reported that PEP inhibitors improve memory processes in aged mice and elderly humans [30], alleviates chemically induced amnesia in rodents [33] and restore cognitive functions in monkeys exposed to the neurotoxin MPTP [39]. In addition, the fact that brain regions that contain the highest concentrations of ODN-related peptides [44] actively express PEP mRNA [3] is consistent with a physiological role of PEP in the breakdown of ODN.

In conclusion, we have demonstrated that PEP preferentially cleaves ODN on the carboxyl side of the Ala^2 and Val^4 residues. In contrast, PEP is totally unable to process the Pro^{12}-Gly^{13} bond of ODN (Fig. 9). To the best of our knowledge, this is the first evidence that PEP can hydrolyze peptides at a post-valine bond. The fact that, in the rat brain, the regions that contain the highest concentrations of ODN-like immunoreactivity are also enriched with PEP mRNA strongly suggests that PEP is actually involved in the breakdown of ODN.

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