ISOLATION AND PURIFICATION OF HcPI, A NATURAL INHIBITOR OF PYROGLUTAMIL AMINOPEPTIDASE II (TRH-degrading ectoenzyme) FROM EXTRACTS OF *Hermodice carunculata* (Annelide:Polychaeta)

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ABSTRACT

An inhibitor of the metallo-ectoenzyme, pyroglutamyl aminopeptidase II (PPII), a thyrotropin releasing hormone (TRH) specific peptidase, was identified during the screening of extracts from marine species of the Havana City coast-line belonging to the *Phylla: Coelenterata, Mollusca, Annelida, Echinodermata, Porifera, Chlorophyta, Magnoliohyta* and *Chordata.* Isolation of the inhibitor (HcPI), from the marine annelide *Hermodice carunculata*, was achieved by trichloroacetic acid treatment of the aqueous extract to precipitale acid labels proteins. We designed, as first strategy, a procedure in HPLC systems, consisting in anionic exchange chromatography on Quaternary Amines Partisil SAX followed by molecular exclusion on Biosep-SEC-S2000. This procedure in HPLC systems does not allow the purification of the PPII inhibitor till homogeneity. A second strategy of purification involved anionic exchange chromatography on DEAE Sephacel followed by molecular exclusion on Sephadex G-25. RP-HPLC demonstrated inhibitor homogeneity. This procedure allowed the purification with yields up to 106 % and purification grades of 313 times regarding to the crude extract. The purified molecule was able to inhibit rat PPII (a membrane preparation with 7.5 mg protein /mL) with an IC_{50} of 4.8 μg/mL. HcPI represents the first natural inhibitor described against the metallo neuropeptidase PPII.

Key words: pyroglutamyl aminopeptidase II, inhibitor, screening, purification

RESUMEN

Un tamizaje realizado con extractos de invertebrados marinos (anélidos, moluscos, celenterados, poríferos y equinodermos), algas y algunos cordados de la costa norte de La Habana, permitió la identificación de un inhibidor de la metaloenzima piroglutamil aminopeptidasa II (PPII), una peptidasa específica para la degradación de la hormona liberadora de tirotropina. En el presente trabajo, se describe el aislamiento y purificación de este inhibidor (HcPI) a partir del anélido marino *Hermodice carunculata*. El extracto crudo se trató con ácido tricloroacético, lo cual permitió la obtención de un preparado clarificado más enriquecido en la actividad inhibidora de PPII que el extracto de partida. Se procedió primeramente a la purificación en sistemas de alta presión, para lo cual se desarrolló una cromatografía de intercambio aniónico en Aminas Cuaternarias Partisil-SAX, seguido de una cromatografía de exclusión molecular en Biosep-SEC-S2000. Este procedimiento no permitió la purificación hasta homogeneidad de la molécula responsable de la actividad inhibidora, por lo cual se procedió a diseñar un protocolo en sistemas de baja presión. Este consistió en una cromatografía de intercambio aniónico en DEAE-Sephacel, seguido de una cromatografía de exclusión molecular en Sephadex G-25. La pureza de la fracción obtenida se comprobó por RP-HPLC. Este protocolo permitió la purificación hasta homogeneidad del inhibidor de PPII, con un grado de purificación de 313 veces respecto al extracto de partida y rendimientos superiores al 100%. La inhibición de PPII mostrada por la molécula purificada fue dependiente de la dosis, con un valor de IC_{50} de 4.8 μg/mL frente a una preparación de membranas de cerebro de rata con 7.5 mg/mL de concentración de proteínas. Esta molécula representa el primer inhibidor natural descrito para la metalopeptidasa PPII.

Palabras clave: piroglutamil aminopeptidasa II, inhibidor, tamizaje, purificación.

INTRODUCTION

Marine invertebrates (such as coelenterates, molluscs, annelids, echinoderms, sponges), algae and some chordates are an interesting source of protease inhibitors (Fritz **et al.,** 1972; Wunderer **et al.,** 1976; Mebs and Gebauer, 1980; Kolkenbrock and Tschesche, 1987; Scheitz **et al.,** 1995; Shishikura **et al.,** 1996; Nagle **et al.,** 2001). These

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are mainly serine inhibitors, although there are some reports about inhibitors isolated from marine coelenterates active against proteinases of different mechanistic classes (Delfín **et al.,** 1996; Lenarcic **et al.,** 1997; Strukelj **et al.,** 2000) except metalloproteases.

Pyroglutamyl aminopeptidase II (PPII; E.C. 3.4.19.6) is a high specificity zinc metallo-ectopeptidase which hydrolyses the pyroglutamyl-histidyl peptide bond (O'Connor and O'Cuinn, 1984; Garat **et al.,** 1985; Friedman and Wilk, 1986; Charli **et al.,** 1998; Heuer **et al.,** 1998) of thyrotropin releasing hormone (TRH), a tripeptide (pGlu-His-ProNH_2) enriched in the central nervous system with neural and endocrine functions (O'Leary and O'Connor, 1995). A TRH degrading enzyme present in serum, named thyroliberinase, is a soluble form of PPII, with very similar properties to those of PPII originating in liver (Schmitmeier **et al.,** 2002). PPII primary sequence, deduced from the cDNA cloning, corresponds to a type II integral membrane protein containing 1025 aminoacids (aa) including a large extracellular region with the consensus sequence His-Glu-X-X-His and a second Glu separated by 18 aa, aminoacids characteristic of the M1 family of zinc peptidases (Schauder **et al.,** 1994). Analysis of the purified enzyme indicated it is a glycoprotein composed of two identical subunits (Bauer, 1994). Studies by O'Connor and O'Cuinn, 1984, Garat **et al.,**1985 and Friedman and Wilk, 1986 demonstrated that PPII presents stringent substrate requirements. It can only hydrolyze peptides no longer than four aa long with the general structure pGlu-His-X or -Y, X being Pro, Ala or Trp, Y being Pro-NH_2 or Pro-Gly or Pro-βnaphtylamide (βNA). Most modifications of pGlu or His abolish hydrolysis (Wilk and Wilk, 1989; Elmore **et al.,** 1990). However, a more recent study with TRH-like peptides in which the His residue was replaced by several aa demonstrates that the enzyme binds preferentially moderately bulky, uncharged residues in the P_1' position. (Kelly **et al.,** 2000)

Inhibitors of ectopeptidases have been useful both to elucidate physiological role and its therapeutic properties (Roques, 2000). Studies performed *in vitro* showed that specific inhibition of PPII with N-1-carboxy-2-phenylethyl (N^{im} benzyl)-histidyl-βNA enhances the recovery of TRH released from brain slices (Charli **et al.,** 1989). Kelly **et al.,** 2000 identified pGlu-Asn-Pro-7-amido-4-methyl coumarin as a more potent inhibitor of purified PPII (Ki = 0.97 umol/L). Since the potency of the synthetic inhibitors of PPII is limited and their specificity minimally determined, natural inhibitors were looked for as an alternative.

A screening of an inhibitory activity in crude extracts from marine species of the Havana City coast-line performed in our laboratories indicated its occurrence in the species *Hermodice carunculata* (fire worm), belonging to the *Phylum Annelida, Polychaeta.* In the present paper we describe the purification of this inhibitor named HcPI and a preliminary characterization of this new molecule.

MATERIAL AND METHODS

Reagents

Except where indicated, the enzymes and substrates were purchased from Sigma (St Louis, Mo, USA) and Bachem Bioscience Inc (King of Prusia, PA, USA) respectively. All other chemicals were purchased from Sigma and solvents from J.T. Baker (DF, Mexico).

The matrixes for low pressure purification procedure were bougth from *Pharmacia Fine Chemicals*. Chromatographic supports for HPLC purification procedure were purchased from *Bio Rad*.

Biological Material

The screening of PPII inhibitory activity included 24 marine species belonging to the *Phylla Coelenterata, Mollusca, Annelida, Echinodermata, Porifera, Chlorophyta, Magnoliohyta* and *Chordata.* (see Table I)

Preparation of crude extracts

Species were collected on the northern coast of Havana (Cuba), identified by a trained biologist, transported alive on ice and homogenized up to 2h after collection in a warring blender in destillated water and centrifuged at 10 000 g for 30 min. Supernatants were liophylizated and kept at -20 ^{0}C until assayed against PPII activity (see below).

Inhibitor Purification

The purification was performed at 4 ^{0}C. 200mg of *Hermodice carunculata* liophylizated were resuspended in 5ml of 50 mmol/L sodium phosphate buffer, pH 7.5 (Buffer A). The crude extract was treated with 2.5 % final concentration of trichloroacetic acid (TCA) followed by centrifugation with the aim to precipitated acid unstable proteins and to obtain a clarified extract. The supernatants were adjusted to pH 8. The dialyzed supernatant (cut off 500 Da) containing inhibitory activity was first applied to ion exchange on Partisil SAX quaternary amine (HPLC) column equilibrated with buffer A. The elution was carried out with a linear gradient up to 0.3 mol/L NaCl in buffer A. Active fractions were pooled, liophilyzed and applied to a gel filtration column, Biosep-SEC-S2000, equilibrated with buffer A. A procedure in low presure sistems was designed. The crude extract treated with TCA 2.5 % and dialyzed (cut off 500 Da) was applied to ion exchange on DEAE-Sephacel column equilibrated with 50 mmol/L sodium phosphate buffer, pH 7.5. The elution was carried out with a linear gradient up to 1 mol/L NaCl in the same buffer. Fractions containing inhibitory activity were pooled, liophilyzed

Table I. Screening of inhibitory activities against PPII among marine species.

Species	Phyllum	Inhibition of PPII	Inhibition of DAPIV
Caulerpa racemosa	*Algae*	-	-
Dictyosphaeria cavernosa	*Algae*	-	-
Galaxaura marginata	*Algae*	-	-
Opuntia sp.	*Algae*	-	-
Halimeda incresata	*Algae*	-	-
Dictyosphaeria cavernosa	*Algae*	-	-
Ascidia sidneyense	*Chordata*	-	-
Molgula occidentalis	*Chordata*	-	-
Pyura vitata	*Chordata*	-	-
Tectitethya cripta	*Porifera*	-	-
Mycale microsigmatosa	*Porifera*	-	+
Lima scabra	*Mollusca*	-	-
Aplisia dactilomela	*Mollusca*	-	-
Zoanthus pullchelus	*Coelenterata*	-	-
Plexaura homomaya	*Coelenterata*	-	-
Condylactis gigantea	*Coelenterata*	-	+
Stichodactyla helianthus	*Coelenterata*	-	+
Cassiopea xamachana	*Coelenterata*	-	-
Physalia physalis	*Coelenterata*	-	-
Hermodice carunculata	*Annelida*	+	-
Sabellastarte magnifica	*Annelida*	-	-
Holothuria floridiana	*Echinodermata*	-	-
Holothuria mexicana	*Echinodermata*	-	-

+ presence of inhibitory activity of PPII
- absence of inhibitory activity of PPII

and applied to a gel filtration column, Sephadex G-25, equilibrated with buffer A. The inhibitory component was concentrated by ultrafiltration (cut off 500 Da). Homogeneity of the inhibitor was confirmed by reversed phase high performance liquid chromatography (RP-HPLC) on C-18 column (7.5 x 200 mm; Altex) at a flow rate of 0.8 mL/min using a 60 min linear gradient (B: 0 - 60 %; B = acetonitrile containing 0.1 % trifluoroacetic acid).

Protein concentration

Protein concentration was determined by the method of Lowry modified by Peterson in 1977.

Inhibitory assay against PPII

PPII activity was measured in rat brain synaptosomal membranes prepared as described by Vargas **et al.** 1987. Membrane PPII activity was determined with the substrate TRH-ßNA in a coupled assay in the presence of excess dipeptidyl aminopeptidase IV (DAPIV) according to Friedman and Wilk, 1986, except for a few modifications. The incubation mixture contained 100-200 µg membrane protein, DAPIV (4 nmoles Gly-Pro-ßNA hydrolyzed per min), bacitracin and N-ethylmaleimide (200 µ mol/L each) in 50 mmol/L sodium phosphate buffer, pH 7.5 (total volume of 290 µL). This mixture was pre-incubated at 37 ^{o}C for 10 min before addition of 10 µL TRH-ßNA (400 µ mol/L final) in buffer A. Reaction was run at 37 ^{o}C. Aliquots (50 µL) were taken at time 15, 30, 45 and 60 min. Reaction was stopped by addition of one vol. of methanol. Liberated ßNA was detected fluorometrically. Fluorescence measurements were made using a Perkin-Elmer luminescence spectrometer. Wavelengths for excitation and emission were set at 335 nm and 410 nm, respectively, with slit widths of 15 and 20 nm, respectively. Activity was linear with time. Inhibition was assayed by measuring the decrease of activity

in a sample pre-incubated with HcPI (or other extracts) for 10 min at 37 ^{0}C.

Inhibition of other proteases

Serine proteases: bovine trypsin (E.C. 3.4.21.4) was evaluated by monitoring the esterase activity against BAEE as substrate (Trautschold and Werle, 1961). Bovine chymotrypsin (E.C. 3.4.21.1) was tested using Suc-Phe-p-nitroanilide as substrate (Geiger, 1984) and human leucocyte elastase (E.C. 3.4.21.37) was determined using methasuccinyl-(Ala)2-Pro-Val-p-nitroanilide (Nakajima *et al.,* 1979). Porcine kidney DAPIV (E.C. 3.4.14.1; purified according to Yoshimoto and Walter, 1977) was assayed by a fluorimetric method with Gly-Pro-ßNA as substrate. (Yoshimoto and Walter, 1977)

Cysteine proteases: bacterial pyroglutamyl peptidase I (E.C. 3.4.19.6) was evaluated using pGlu-ßNA as substrate (Szewczuk and Mulczyk, 1969). Papain from papaya (E.C. 3.4.22.2) and bromelain from pineapple (E.C. 3.4.22.5) were assayed using Benzoil-arginyl-p-nitro-anilida (Barrett and Kirschke, 1981) as substrate.

Aspartic proteases: porcine pepsin (E.C. 3.4.23.1) was determined with Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMet as substrate (Martin **et al.,** 1980). Human immunodeficiency virus 1 protease (PR-HIV 1) (E.C. 3.4.23.16) activity was measured as described by Gutiérrez **et al.,** 2002 using a novel solid-phase procedure with the specific peptide SQNYPIVβARQSTPIGLGQALYTT as substrate.

Metalloproteinases: *Clostridium histolyticum* collagenase (E.C. 3.4.24.3) and gelatinase (E.C. 3.4.24.24) were determined according the method described by Masui *et al.,* 1977, using dinitrophenyl-Pro-Gln-Ile-Ala-Gly-Gln-D-Arg-OH as substrate. Angiotensin converting enzyme from procine kidney (ACE; E.C. 3.4.15.1; Sigma) was monitored by the hydrolysis of furacryloil-Phe-Phe-Gly, as described by Buttery and Stuart, 1993. Aminopeptidase N (E.C. 3.4.11.2), prepared from intestinal cells of *Manduca sexta* as described Wolfersberger *et al.,* 1987, was determined using Leu-p-nitroanilide as substrate (Sjöström *et al*., 1978) and porcine carboxypeptidase A (E.C. 3.4.17.1) was evaluated using N-(4-methoxyphenylazoformyl)-L-Phe as substrate. (Mock **et al.,** 1996)

Inhibition was assayed by hydrolysis of the corresponding substrate for the indicated enzyme after pre-incubation regarding a sample without inhibitor. Enzyme and inhibitor, HcPI (10 ng to 200 ug) were pre-incubated either for 5, 10 or 30 min at 25 ^{o}C. Assays were performed out in triplicate.

RESULTS

Inhibitor screening

A screening of PPII inhibitory activity among 24 different marine species extracts revealed an inhibitory activity from the whole body extracts of the worm *Hermodice carunculata*, in the anemones *Condylactis gigantea* and *Stichodactyla helianthus* and in the sponge *Mycale microsigmatosa.* The other species were devoid of any activity. Since the assay of PPII is coupled to DAPIV, it was necessary to separate the effects due to DAPIV inhibition to those due only to PPII inhibition. Among the active species, only the *H. carunculata* extract did not inhibit DAPIV, suggesting that this extract contains an inhibitor of PPII activity (Table I). This was confirmed by an independent assay of PPII activity using a radiometric method with [L-2,3,4,5-^{3}H-Pro]-TRH as substrate according to Vargas **et al.,** 1987. The other extracts, that inhibited DAPIV, did not inhibit PPII activity in the radiometric assay (not shown). The following data are the results of multiple purifications of the *H. carunculata* inhibitor (named HcPI) from distinct collections; we did not detect any significant difference in the properties of the inhibitor from purification to purification or collection to collection.

Purification

Isolation of the inhibitor was achieved in three steps. The first step involved TCA treatment of the crude extract and centrifugation with the aim to precipitated acid unstable proteins and to obtain a clarified extract. The best result in terms of activity recovered was obtained using a final concentration of TCA of 2.5 % (not shown). Total inhibitory activity increased after this treatment (Table II) suggesting the dissociation of endogenous inhibitor complexes.

Recovery of HcPI through dialysis of the TCA extract was only possible using membranes with a very low cut off (500 Da), according to the inhibitor molecular weight, confirmed later by chromatography. The TCA extract was applied to a Partisil SAX quaternary amine column. The chromatographic profile indicated that fractions containing PPII inhibitory activity elute just before the beginning of the gradient (Fig. 1) with yields of 83 %. (Table II)

Gel filtration on Biosep-SEC-S2000 (Fig. 2) does not allow the final purification of the inhibitor as shown in RP-HPLC profile obtained for this fraction (Fig. 3); PPII inhibitory activity was obtained with yields of 66 % and a 83 fold purification (Table II).

This procedure in HPLC systems does not allow the purification of the PPII inhibitor to homogeneity. For this reason a second strategy in low pressure systems was designed.

The TCA extract was applied to a DEAE Sephacel column. The chromatographic profile indicated that fractions containing PPII inhibitory activity elute just before the beginning of the gradient (Fig. 4) with yields of 122 %. (Table III)

Table II. Summary of the purification procedure of HcPI in HPLC systems.

Step	*Protein (mg/mL)*	*Inhibitory activity (U/mL)*	*Specific activity (U/mg))*	*Purification (n fold)*	*Yield (%)*
Crude extract	124.82 ± 4.25	3000 ± 11	24	1	100
TCA treatment	48.09 ± 3.05	3420 ± 10	71	2.95	114
Ion-exchange	1.45 ± 0.05	2500 ± 8	1715	71.00	83
Gel filtration	1.00 ± 0.02	2000 ± 15	2000	83.22	66

The data shown are an average of 10 purification processes.
* U:1 unit was defined as the amount of inhibitor needed to inhibit 1 unit of PPII activity. One unit of PPII activity was defined as the enzyme activity that hydrolysed 1 pmol of TRH-βNA per min under the specified conditions.

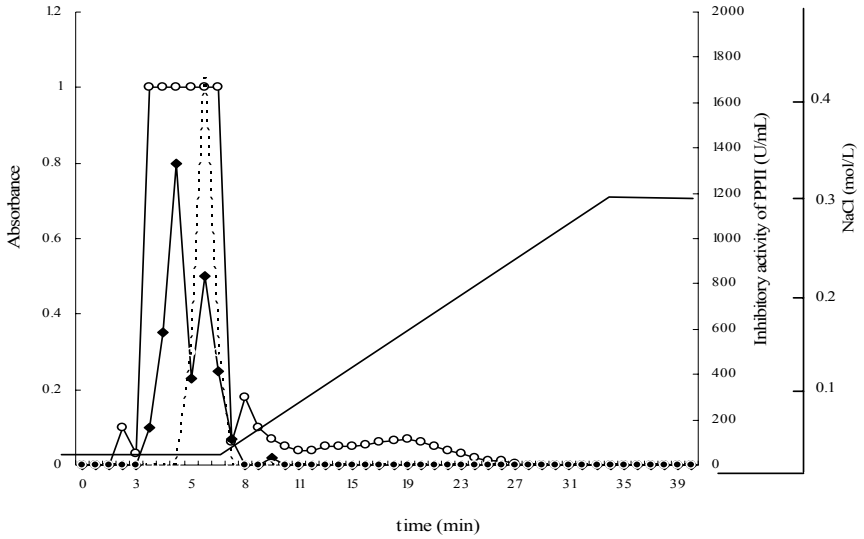

Fig.1. Ion-exchange chromatography of the *H. carunculata* 2.5 % TCA extract in HPLC systems. Chromatography was carried out on 20,5 x 0.46 cm Partisil SAX quaternary amine column equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The elution was performed using a linear salt gradient (0.3 mol/L NaCl in the same buffer). Flow rate, 226 cm h^{-1}; white circle: absorption at 215 nm; black rhomboid: absorption at 280 nm; dashed line: inhibitory activity of PPII; solid line: NaCl gradient. This profile is typical of various independent chromatographies.

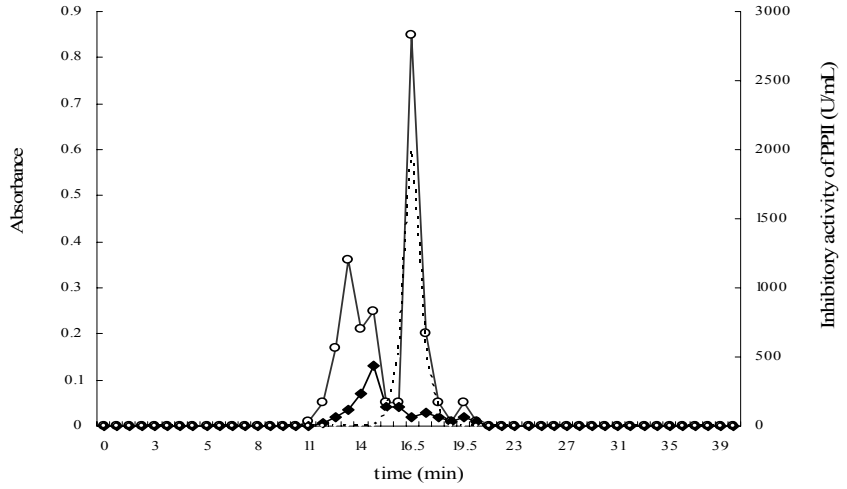

Fig. 2. Gel filtration of the inhibitory fractions purified by ion-exchange chromatography. Chromatography was carried out on a Biosep-SEC-S2000 column (1 x 100 cm) equilibrated with 50 mmol/L sodium phosphate buffer, pH 7.5. Flow rate, 78 cm h^{-1}; white circle: absorption at 215 nm; black rhomboid: absorption at 280 nm, dashed line: inhibitory activity of PPII; Vo: Exclusion volume. This profile is typical of various independent chromatographies.

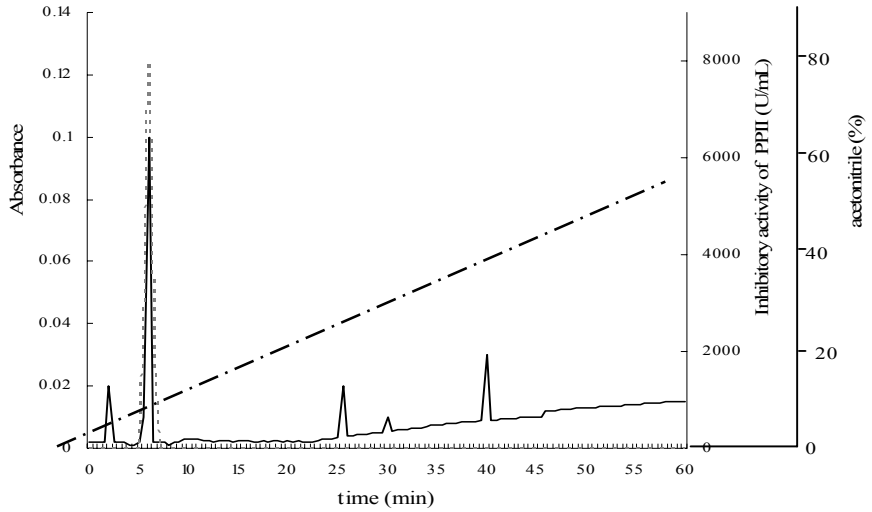

Fig.3. Reversed phase HPLC of the high pressure systems purified inhibitor. RP-HPLC was performed on a C-18 (7.5 x 200 mm) column with a linear gradient from 0 to 60 % of acetonitrile in 0.1 % of trifluoroacetic acid for 60 min. Flow rate, 75 cm h^{-1}; solid line: absorption at 215 nm; dashed line: inhibitory activity of PPII; dashed and pointed line: acetonitrile gradient (%). This profile is typical of various independent chromatographies.

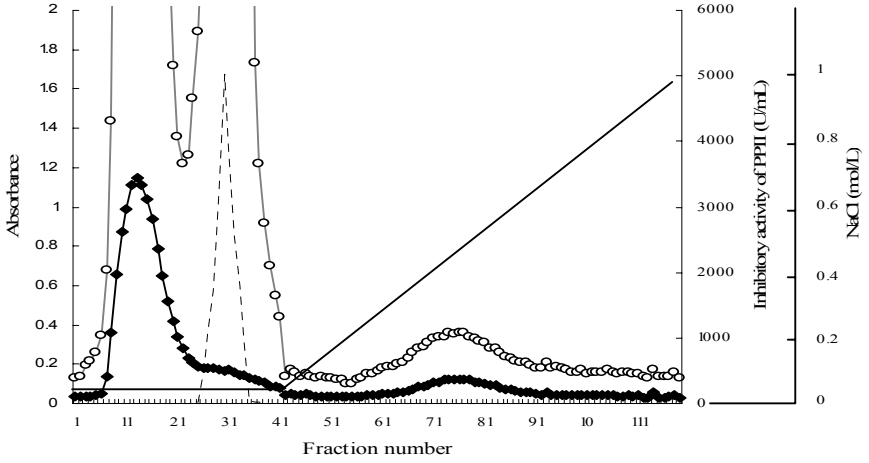

Fig.4. Ion-exchange chromatography of the *H. carunculata* 2.5 % TCA extract. Chromatography was carried out on 2 x 24 cm DEAE Sephacel column equilibrated with 50 mmol/L sodium phosphate buffer, pH 7.5. The elution was performed using a linear salt gradient (1 mol/L NaCl in the same buffer). Flow rate, 15 cm h^{-1}; white circle: absorption at 215 nm; black rhomboid: absorption at 280 nm; dashed line: inhibitory activity of PPII; solid line: NaCl gradient. This profile is typical of various independent chromatographies.

Table III. Summary of the purification procedure of HcPI in low pressure systems.

Step	*Protein (mg/mL)*	*Inhibitory activity (U/mL)*	*Specific activity (U/mg)*	*Purification (n fold)*	*Yield (%)*
Crude extract	312.00 ± 3.00	7500 ± 15	24	1	100
TCA treatment	120.00 ± 2.00	8550 ± 11	71	2.95	114
Ion-exchange	4.07 ± 1.00	9140 ± 16	2240	93.3	122
Gel filtration	1.06 ± 0.05	7980 ± 17	7530	313	106

The data shown are an average of 10 purification processes.
***** U:1 unit was defined as the amount of inhibitor needed to inhibit 1 unit of PPII activity.
One unit of PPII activity was defined as the enzyme activity that hydrolysed 1 pmol of TRH-βNA per min under the specified conditions.

Gel filtration on Sephadex G-25 allowed the final purification of the inhibitor; PPII inhibitory activity was obtained in a symmetrical peak (Fig. 5) with yields of 106 % and a 313 fold purification (Table III).

HcPI homogeneity was confirmed by RP-HPLC: a peak containing the inhibitory activity was detected at 215 nm (Fig. 6) and eluted at 6.0 min. The purified inhibitor did not absorb at 280 nm as showed in Fig. 7.

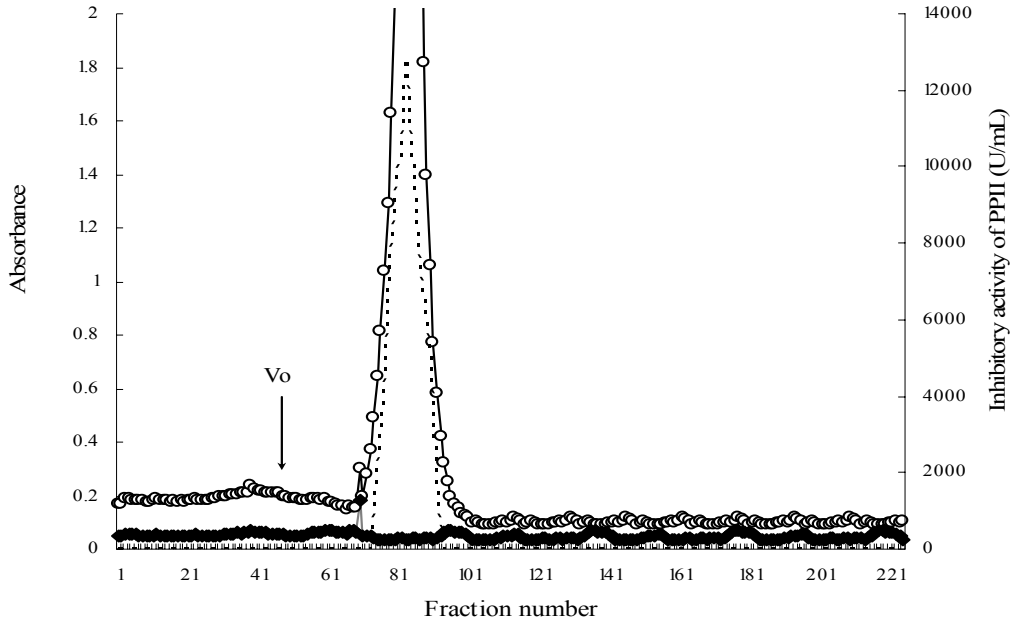

Fig. 5. Gel filtration of the inhibitory fractions purified by ion-exchange chromatography. Chromatography was carried out on a Sephadex G-25 column (1 x 100 cm) equilibrated with 50 mmol/L sodium phosphate buffer, pH 7.5. Flow rate, 5 cm h^{-1}; white circle: absorption at 215 nm; black rhomboid: absorption at 280 nm, dashed line: inhibitory activity of PPII; Vo: Exclusion volume. This profile is typical of various independent chromatographies.

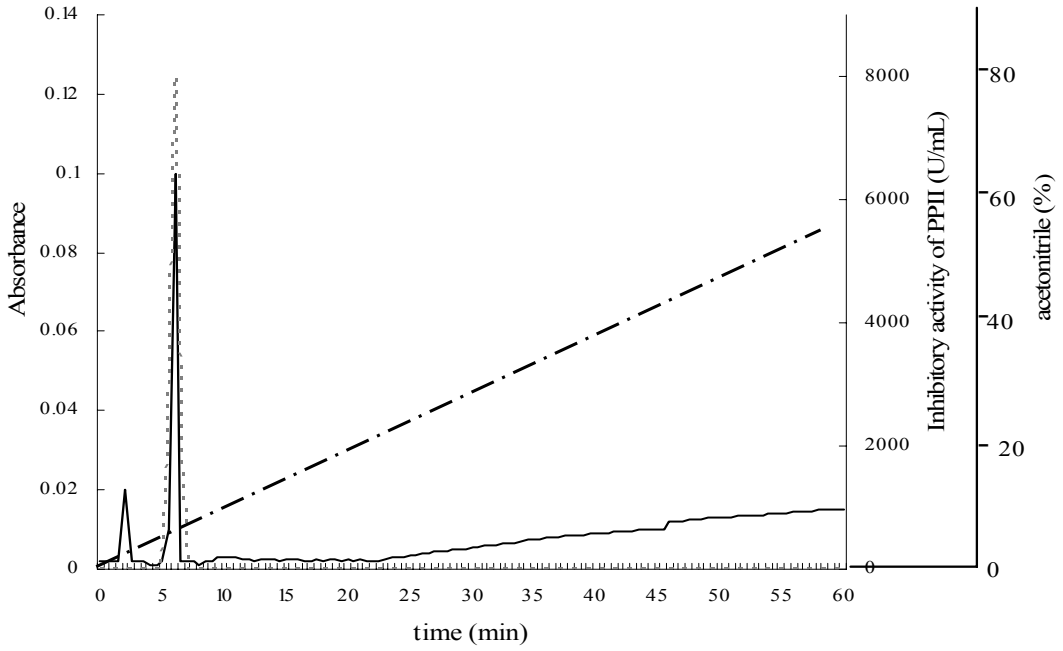

Fig. 6. Reversed phase HPLC of the purified inhibitor. RP-HPLC was performed on a C-18 (7.5 x 200 mm) column with a linear gradient from 0 to 60 % of acetonitrile in 0.1 % of trifluoroacetic acid for 60 min. Flow rate, 75 cm h^{-1}; solid line: absorption at 215 nm; dashed line: inhibitory activity of PPII; dashed and pointed line: acetonitrile gradient (%). This profile is typical of various independent chromatographies.

Purity was also confirmed by HPLC on microbore C18 coupled to a variable wavelength detector (not shown). Gel filtration chromatography on Sephadex G25 (Fig. 5) as well as HPLC gel filtration on Biosep-SEC-S2000 indicated a molecular weight below 1000 Da after calibration runs with molecular weight markers (not shown). HcPI submitted to a final purification step by RP-HPLC was used in all subsequent experiments.

Partial characterization

Gel filtration indicated a low molecular weight for HcPI. The specificity of the inhibitory activity was assayed using several enzymes belonging to four groups of proteinases : cysteine, metallo, aspartic and serine. The inhibitor, in a concentration range from 10 ng to 200 μg was not active against serine (trypsin, chymotrypsin, elastase and DAP IV), or cysteine (papain, bromelain and PPI) or aspartic (pepsin and PR-HIV) proteases. The assays against metalloproteinases (collagenase, gelatinase, ACE, aminopeptidase N and carboxypeptidase A) were also negative. (not shown)

The potency of PPII inhibitory activity was evaluated at different concentrations of HcPI. There was a dose-dependent inhibition of the membrane PPII activity (7.5 mg/mL) with an IC_{50} value of 4.8 μg/mL under the experimental conditions described in Methods. (Fig. 8)

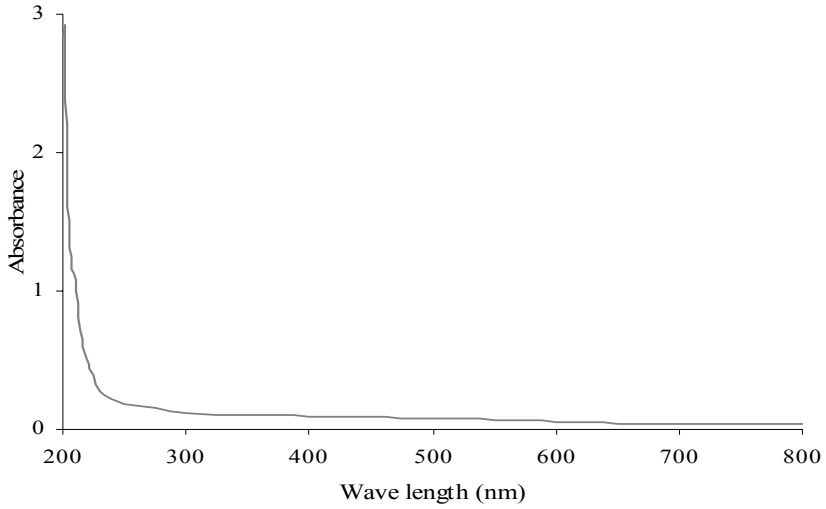

Fig. 7. Absorption spectrum of HcPI in destillated water.

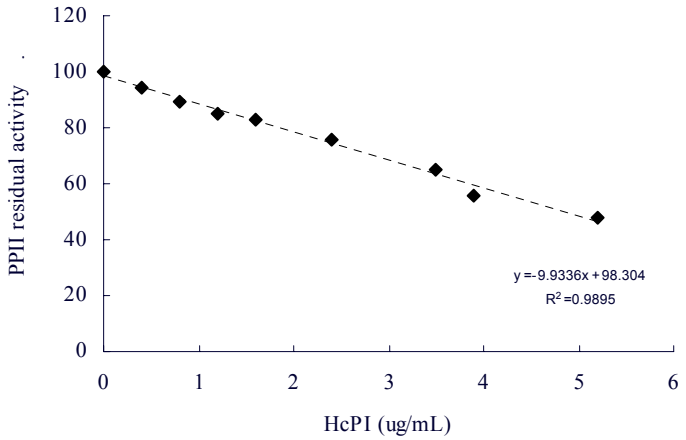

Fig. 8. Dose-response curve of HcPI effect on PPII activity in brain membranes. Membranes (7.5 mg/mL) were pre-incubated with eight different dilutions of HcPI, in 0.05 mol/L sodium phosphate buffer, pH 7.5 containing bacitracin and N-ethyl maleimide (200 μ mol/L each) for 10 minutes at 37 °C before addition of 10 μL of TRH-βNA (400 μ mol/L final). PPII assay was performed out in triplicate as described in Materials and Methods.

DISCUSSION

PPII is probably the only enzyme responsible for TRH extracellular inactivation, apart from its serum isoenzyme, thyroliberinase (Charli ***et al.*****,** 1998). The high specificity of this peptidase puts it apart from many neuropeptide-degrading peptidases that have generally multiple biological substrates. It is therefore probable that interfering with PPII activity *in vivo* will have a specific effect on TRH communication and may reveal with precision the functions of the peptide in a physiological context. Development of specific inhibitors of PPII will therefore have an important application for understanding TRH physiology. Since synthetic inhibitors of PPII have not yet been developed with sufficient potency and specificity, the finding of a specific inhibitor from a natural source is a major finding.

The screening reported in this study let us to identify the first inhibitor for a neural metalloectopeptidase isolated from marine species. The only metalloprotease inhibitor isolated from marine invertebrates, Jaspisin (from the sponge *Jaspis species*), inhibits the activities of the matrix metallo-endoproteinases members of the family M10, clan MB of metallopeptidases but not of other types of proteinases (Ikegami **et al.,** 1994; *Kato* **et al.,** 1998). The marine invertebrate *H. carunculata* (fire worm) contains a substance that is able to inhibit the metallo-ectoenzyme PPII with relatively good effectiveness and high selectivity. This is the first biological activity reported for such extract. Moreover, the screening also showed that other organisms contain inhibitory activities against DAPIV. These activities remain to be characterized except that of *Stichodactyla helianthus.* (Delfín **et al.,** 1996)

The procedure described in low pressure systems allows the purification of HcPI with a high yield. HcPI is homogeneous, as confirmed by RP-HPLC. The procedure is reproducible since we have consistently obtained the activity from purifications to purifications and collections to collections. Therefore, we have found a rich source of a PPII inhibitor.

This inhibitor is a small compound. In brain membranes 10 min pre-incubation with HcPI is sufficient to obtain the maximum inhibition of PPII activity.

The most striking property of the inhibitor is its selectivity. It does not inhibit any of the enzymes tested, whatever its mechanistic class. In particular, various metallopeptidases were not affected. The reason for this selectivity will be elucidated once its structure is solved and the mechanism of its interaction with PPII understood. This specificity is striking compared to that of proteinase inhibitors isolated from marine species or other natural sources that generally inhibit multiple enzymes and may be related to the narrow specificity of PPII. (Delfín **et al.,** 1996; Lenarcic **et al.,** 1997; Kato **et al.,** 1998; Strukelj **et al.,** 2000)

It remains to be determined what function has HcPI in this marine invertebrate. HcPI was consistently detected from collections to collections suggesting that its presence does not depend on specific physiological conditions. We could not detect PPII activity in crude extracts of this marine animal (not shown) but it is still possible that PPII was present but inhibited by HcPI. However, an analysis of the genome of invertebrates did not permit the detection of sequences homologous to PPII (unpublished). Therefore it is probable that in *H. carunculata* HcPI has a biological function unrelated to PPII inhibition.

In conclusion, we have identified and partially characterized a specific inhibitor of pyroglutamyl aminopeptidase II. This is the first specific inhibitor of a neural metallo proteinase found in marine invertebrates. This inhibitor will be an important tool to understand the role of PPII in TRH communication in brain and hypophysis.

ACKNOWLEDGMENTS

We thank the technical help of Riseth Robles and Dagmara Díaz in extracts preparation and Aida Hernández, Beatriz Guitart, Macario and Esperanza González for species collection and classification. This work was partially funded by CONACYT (31729N, proyecto integral #3 Mexico-Cuba 98), IFS grant F/3276-1, LARC-IBRO program and “Alma Mater” research project from University of Havana.

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Recibido: noviembre 2002
Aceptado: mayo 2003