Sequence of two gonadotropin releasing hormones from tunicate suggest an important role of conformation in receptor activation

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Abstract The primary structure of two forms of gonadotropin releasing hormone (GnRH) from tunicate (*Chelyosoma productum*) have been determined based on mass spectrometric and chemical sequence analyses. The peptides, tunicate GnRH-I and -II, contain features unprecedented in vertebrate GnRH. Tunicate GnRH-I contains a putative salt bridge between Asp[5] and Lys[8]. A GnRH analog containing a lactam bridge between Asp[5] and Lys[8] was found to increase release of estradiol compared with that of the native tunicate GnRH-I and -II. Tunicate GnRH-II contains a cysteine residue and was isolated as a dimeric peptide. These motifs suggest that the conformation plays an important role in receptor activation.

Key words: Gonadotropin releasing hormone; Saltbridge; Disulfide bridge; Lactam bridge; Mass spectrometry; Chemical sequencing

1. Introduction

The neuropeptide gonadotropin releasing hormone (GnRH) is secreted in the median eminence of vertebrates to act on the anterior pituitary gland resulting in the release of luteinizing hormone and follicle stimulating hormone. The release of these hormones sets off a cascade of events leading to steroidogenesis. Because of the pivotal role of GnRH in vertebrate reproduction, the primary structures of GnRH peptides in a wide variety of species have been determined. In most vertebrates more than one form of GnRH is encountered, e.g. chicken GnRH-I and -II are both decapeptides with considerable identity to GnRH peptides from other species (see Table 1). In fact, the primary structures of these different forms of GnRH show considerable conservation particularly at residues 1–4 and 9–10 (e.g. chicken GnRH-I and -II differ only at residues 5, 7 and 8). Also, in mammals, two forms of the GnRH decapeptide have been found. This raises the question from which forms of GnRH, conserved throughout evolution, did these peptides arise?

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Abbreviations: DTT, dithiothreitol; GnRH, gonadotropin releasing hormone; tunGnRH, tunicate GnRH; HPLC, high performance liquid chromatography; MALD, matrix assisted laser desorption; MS, mass spectrometry; PSD, post source decay; TCEP, tris(2-carboxyethyl)-phosphine; TOF, time of flight

Evolutionary studies have attempted to interpret the variation in primary structure of GnRH peptides in terms of the hormone structure and its function. These phylogenetic arguments have in turn stimulated systematic synthetic investigations aimed at identifying antagonists or super agonists [1,2]. These agonists and antagonists have potential pharmacological value in management of sex steroid-dependent pathophysiologies.

Previously we and others have traced the structural changes in the evolution of this neuropeptide within vertebrates from the single form of mammalian GnRH through to primitive species such as Russian sturgeon (*Acipenser gueldenstaedti*) [3], spiny dogfish (*Squalus acanthias*) [4], and lamprey (*Petromyzon marinus*) [5]. In phylogenetic terms, these species are located in three different lineage bifurcations, the first leading to bony fishes, the second leading to cartilaginous fishes, and the third to jawless fishes. We are interested in continuing to trace the phylogeny of this neuropeptide and to establish whether a homolog exists in invertebrates. To address this issue we have investigated whether a GnRH-like molecule was present in tunicate (*Chelyosoma productum*). Protochordates such as the tunicate or sea squirt are at an evolutionary junction because they possess a notochord during the larval stage of development, which they loose when they become sedentary.

The introduction of electrospray [6] and matrix assisted laser desorption (MALD) [7–9] ionization techniques to mass spectrometry (MS) has resulted in a dramatic increase in sensitivity, allowing the analysis of fmol amounts of small peptides [9,10]. Although MALD has been successfully coupled with a number of types of mass spectrometers including magnetic deflection [6,11], Fourier transform ion cyclotron resonance [12] and quadrupole ion trap [13] instruments, it remains most commonly used with time of flight (TOF) mass analyzers [9]. A major advantage of TOF is the essentially unlimited mass range as demonstrated by the observation of ions in excess of 1 MDa [14]. In MALD, the high energy transfer used to effect ionization results in a small variation in the initial kinetic energy and position of the ions that are formed. As a result the mass accuracy of MALD-TOF is significantly reduced compared to that typically associated with conventional MS techniques. In order to correct for these effects with a TOF instrument, a reflector or energy analyzer can be incorporated into the design of the instrument. A more efficient TOF method of increasing the mass accuracy is the introduction of a time delay after ion formation but before the ions are accelerated out of the ion-

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ization source [15]. Alternatively, the analysis can be carried out with an instrument that can compensate and select for the spread in energies of the ions such as a double focusing magnetic sector type instrument.

The initial attempts to improve the mass accuracy by incorporating a reflector, ultimately led to an extremely useful method of fragment mass analysis [16]. The reflector can be used for analyzing the energy of fragment ions. Fragment ions formed from parents that have exited from the ionization source (and thereby experienced full acceleration) are referred to as post source decay (PSD) ions and have energies proportional to their mass. By reducing the energy applied to the reflector it is possible to bring these PSD ions to focus on the detector. The generation of PSD sequence information has been demonstrated for intact ions formed with MALD [17]. PSD with MALD has been used to investigate the structure of peptides [18–21], glycopeptides [22], phosphopeptides [23] and oligosaccharides [24].

GnRH peptides have historically been difficult to sequence because of the presence of blocked N- and C-termini (pGlu and amide). Whereas the N-terminal pyroglutaminyl residue can be cleaved by the enzyme pyroglutamate aminopeptidase [25], the C-terminal amide functionality is resistant to carboxy peptidase treatment [26]. In our hands pyroglutamate aminopeptidase has worked reliably at the cost of additional material and the additional purification step [27]. Therefore, GnRH is representative of that class of samples where the sensitivity of an MS based sequencing strategy has additional complementary advantages over the Edman based chemical approach.

Part of this work has been reported previously at the 44th American Society for Mass Spectrometry and Allied Topics in Portland, May 1996.

2. Materials and methods

The tissue collection, peptide extraction and purification of native tunicate GnRH peptides from *Chelyosoma productum* have been detailed previously [28].

2.1. Mass spectrometry

All MALD measurements were made using a nitrogen UV laser (Laserscience) and α-cyano-4-hydroxycinnamic acid, (Aldrich) which was dissolved (without further purification) in acetone (Sigma). An aliquot (0.5 μL) of the matrix solution was applied to stainless steel targets [29]. Samples were subsequently applied to the thin matrix film and left to dry. Repetitive applications (2–20 times) of the sample on or about the same spot were used to increase the amount of sample applied. Care was taken to avoid spreading of solutions and thereby lowering the concentrations of the sample. However, no special precautions were made to reduce or contain the sample area since the TOF instrument does not incorporate any means for visually locating the sample and directing the laser onto a small sample area. Reduction of disulfide bridges was carried out with tris(2-carboxyethyl) phosphine prepared as described elsewhere [30].

2.1.1. TOF measurements. Intact and PSD MALD measurements were carried out on a Reflex (Bruker Instruments Inc. Billerica, MA) reflectron TOF mass spectrometer equipped with a 100 MHz digitizer. The instrument was operated with direct extraction and without precursor selection. The accelerating voltage was +31 kV and a varying potential (0–30 kV) was applied to the reflector. The mass accuracy in the reflector mode with external calibration of the instrument was typically better than 1000 p.p.m. The spectra represent the accumulation of data from approximately 20–50 laser shots.

2.1.2. Magnetic sector measurements. Accurate mass measurements were carried out on a JMS HX110 (Jeol, Japan) double focusing magnetic sector instrument equipped with a JUS MALD ionization source (Jeol USA, Peabody, MA). The instrument was operated at 10 kV accelerating voltage and with −20 kV post acceleration on to a point detector (Jeol, Japan). Based on the observed TOF *m/z*, a narrow mass range scan (< 50 Da) was generated at constant magnet field strength by rapidly (< 0.2 s) varying the accelerating voltage and electric field strength. The detector signal was dampened by selecting a 300 Hz filter and the sampling rate was increased by setting the desired resolution acquisition parameter to 10 000. The instrument slit width settings were set to give a nominal resolution of 3000. The spectra represent the accumulation of 15 000–18 000 scans where the laser was operated independently of the scan rate at 5 Hz. These measurements resulted in resolved isotope distributions albeit with a low level of statistical significance. In order to increase the statistical significance we used the sum of the observed masses for major isotopomers in each distribution. Because the separation between the isotopomers is not exactly 1 Da, we subtracted the mass corresponding with the difference between the mass of the $^{12}C_x$ and $^{12}C_{x-1}^{13}C$ or $^{12}C_{x-1}^{13}C$ and $^{12}C_{x-2}^{13}C_2$ etc., isotopomers of similar mass GnRH molecules. This results in an observed mean monoisotopic mass which can be compared with the calculated monoisotopic mass for the sequence proposed. Both internal calibration (Fig. 6) where the calibrant was mixed with the sample on the probe or external calibration (Fig. 11) where the probe was withdrawn and replaced with a sample containing the calibrant were used to calibrate the mass scans. The mass accuracy of this instrument when operated as described with external calibration is better than 20 p.p.m.

2.2. Sequence analysis

Chemical sequence analysis was carried out using an Applied Biosystems 470A Protein Sequencer equipped with an on-line PTH detector. Pyroglutamate aminopeptidase treatment was performed as described elsewhere [27].

2.3. Synthesis

The synthesis and purification of the synthetic GnRH peptides has previously been described [28].

2.4. GnRH injection and steroid assay

Peptides were separately injected (1 μl of 10^{-3} M) into the heart (visceral hemocoel) near the gonads in 15 tunicates/group at a stage of reproduction in which sperm was present in the sperm duct, but eggs were not yet in the oviduct. Samples were pooled from each group and extracted for steroids. After treatment the gonads were removed, frozen, pooled, powdered and extracted in ammonium sulfate (15 mL, pH 1) with diethyl ether/ethanol (60 mL, 4:1, v:v). The ether/ethanol phase was evaporated under nitrogen. The fluoroimmunoassay (Autodelfia) of the extract used europium-labeled steroids and specific antisera for estradiol 17β, progesterone and testosterone. Serial dilutions were necessary for two estradiol samples and for all progesterone samples. In Fig. 11, the gonad was dissected at time 0 for the control (Cont.) animals that were not injected (no inj.) or at 6 or 24 h from injected individuals from each group.

3. Results and discussion

Tunicates were dissected and the dorsal portion of the animal including the cerebral ganglion, the neural gland and the dorsal strand (see Fig. 1) was removed and collected. Two compounds (**tunI** and **tunII**) cross-reacting with antisera raised against lamprey GnRH were isolated from extracts of this material. The spectrum shown in Fig. 2, was obtained from the HPLC purified first immunoreactive fraction (**tunI**) using MALD-TOF-MS. In this spectrum (Fig. 2) a single species was observed at *m/z* 1245.8. This mass was not consistent with any of the known forms of vertebrate GnRH's (see Table 1). Based on the purification protocol and this mass measurement we could deduce that **tunI** was not a known GnRH.

The post-source decay (PSD) spectrum of the intact protonated molecule ion observed from **tunI** is shown in Fig. 3. A number of intense fragment ions observed were indicative of

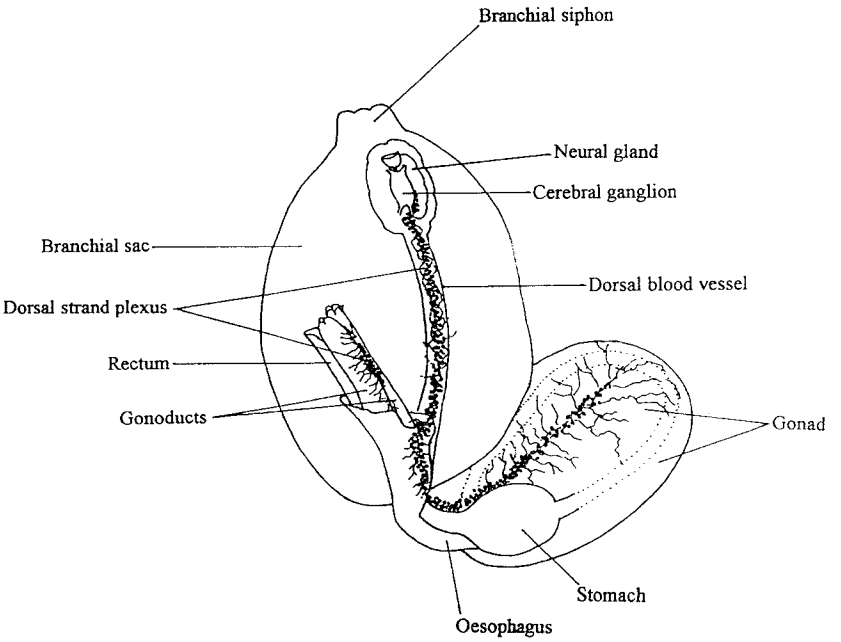

Fig. 1. Schematic drawing of the adult tunicate *Chelyosoma productum* indicating the location of the dorsal strand plexus containing GnRH cells and its proximity to the gonaducts and the gonad. To illustrate the inside of the tunicate, the drawing bisects the animal with a flap of tissue drawn to the right.

GnRH peptides as explained below. Fig. 4A shows the low-mass region of PSD spectrum of 1 pmol of mammalian GnRH where several intense fragment ions are indicated y_2+2, a_2, b_2, b_3, a_4, b_4 [31,32]. The PSD spectrum of 10 pmol of mammalian GnRH contained similar fragment ions as observed in Fig. 4A (with a higher signal to noise ratio) while no distinct fragment ions were observed from 0.1 pmol of mammalian GnRH (data not shown). Based on the intensity of the MALD spectra and the UV absorbance we estimate that 1–2 pmol of peptide was applied to the probe for the **tunI** PSD analysis shown in Fig. 3. The fragment ions indicated in Fig. 4A arise as a result of peptide chain cleavage with charge retention in the conserved region of GnRH (i.e. residues 1–4 and 9–10). The low-mass region of the **tunI** PSD spectrum is shown in Fig. 4B. The appearance of the spectra shown in Fig. 4A and B are very similar. In particular, the low mass fragment ions labeled in Fig. 4B are observed at the same *m/z* in the PSD spectra of most other GnRH peptides (see Table 2). Although the cross reactivity with antisera and the intact mass suggested that **tunI** was a novel GnRH, the similarity between the spectra in Fig. 4 (and the data in Table 2) was the first direct evidence that the peptide was a novel GnRH.

The similar *m/z* and intensity of many of the low mass fragment ions observed for **tunI** in Fig. 4B and other GnRH peptides in Table 2, were taken to indicate that tunI also contained the same conserved regions as mammalian GnRH. The *m/z* 172.2, 221.7, 250.2, 435.0, 494.2 and 521.8 fragment ions were therefore assigned as y_2+2, a_2, b_2, b_3, a_4, b_4. These assignments were used as a template for interpretation of the remainder of the spectrum. Those fragment ions observed in the high-mass region of the **tunI** PSD spectrum (Fig. 5) whose *m/z* did not coincide with the fragment ions observed in mammalian GnRH were assumed to arise as a result of peptide chain cleavage with charge retention on a portion of the molecule not corresponding to the conserved region of GnRH. In addition, we assumed that as was the case with most GnRH peptides, the high mass fragment ions were of N-terminal origin (compare Tables 1 and 2). Further, in Fig. 5 fragment ions observed at *m/z* 772.2, 800.1 and *m/z* 919.1, 947.2 appeared to correspond with 'a' and 'b' type N-terminal fragment ions (since the mass differ-

Table 1
C-terminal fragment ions observed from different GnRH peptides

Species	Sequence	Mass[a]	y_2+2	y_3+2	y_4+2	$*z_5$[b] y_5+2	y_6+2	y_7+2	y_8+2
mammalian	ZHWS**YGLR**PG	1182.58	s	s	w	– w	w	w	w
dogfish III	ZHWS**HGWL**PG	1186.55	m	–	–	– –	m	–	–
salmon	ZHWS**YGWL**PG	1212.56	s	–	–	– –	–	m	m
chicken II	ZHWS**HGWY**PG	1236.53	s	–	s	m –	b6	–	–
catfish	ZHWS**HGLN**PG	1114.52	m	w	m	s –	s	w	w
chicken I	ZHWS**YGLQ**PG	1154.54	m	m	–	– –	–	–	–
herring II	ZHWS**HGLS**PG	1087.51	–	w	–	s –	s	s	–
sea bream	ZHWS**YGLS**PG	1113.51	m	m	m	– –	w	m	–
lamprey III	ZHWS**HDWK**PG	1259.57	w	w	–	– –	–	–	–
tunicate I	ZHWS**DYFK**PG	1246.56	s	s	–	– –	–	–	–
tunicate II	ZHWS**LCHA**PG	2233.51	s	s	s	– –	–	–	–

[a]Monoisotopic mass of $[M+H]^+$; [b] $*z_5 = z_5 - 18$.

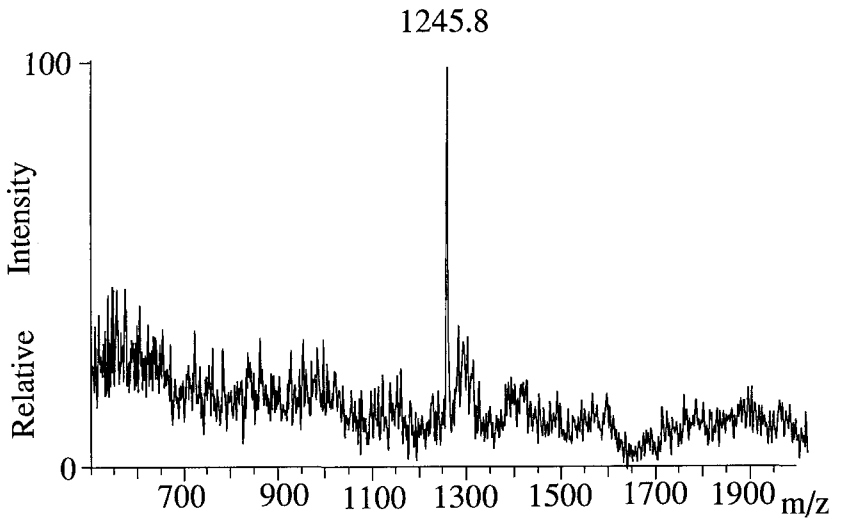

Fig. 2. MALD-TOF mass spectrum of tunGnRH-I.

ence between these fragment ions was 27.9 and 28.2 Da). We therefore assumed that *m/z* 800.1 and 947.3 were 'b' type fragment ions. The mass difference between *m/z* 521.8 and 800.1 (278.3 Da) corresponds with the sum of either tyrosine and aspartic acid (278.26 Da) or methionine and phenylalanine (278.38 Da). Inspection of the spectrum allows us to exclude the Met-Phe possibility and determine the order of the Tyr-Asp interpretation. Based on the intensity of, and mass difference between, the *m/z* 521.8 and 637.2 fragment ions (115.4 Da) we assigned the *m/z* 637.2 fragment ion as b_5 and the fifth residue as Asp. The mass difference between *m/z* 637.2 and *m/z* 800.1 fragment ions (162.9 Da) confirms our suggestion that the *m/z* 800.1 was derived by 'b' type cleavage and indicates the sixth residue is Tyr. The difference between *m/z* 800.1 and 947.2 (147.1 Da) is in accordance with our suggestion that the *m/z* 947.3 fragment ion was a 'b' type ion and indicates that the seventh residue was phenylalanine. The mass difference between the *m/z* 947.3 and 1074.1 (126.9 Da) corresponds most closely with either glutamine (128.06 Da) or lysine (128.10 Da). We assigned the eighth residue as lysine on the basis of the intense *m/z* 300.1 fragment ion assigned as y_3+2 and also observed for mammalian GnRH which contains a basic amino acid (arginine) in position 8. Finally, the mass difference between *m/z* 1074.1 and the intact $[M+H]^+$ *m/z* 1245.8 (171.7 Da) corresponds with Pro-Gly (170.19 Da).

We note that the PSD fragmentation could not be used to distinguish between the order of the first two amino acids (i.e. pGlu-His versus His-pGlu). Although, His-pGlu is non-sensical, the absence of significant fragmentation between these two residues permits alternative assignments (e.g. Thr-Phe (248.28 Da) cannot be distinguished from pGlu-His (248.24 Da)). Similarly, the PSD fragmentation cannot distinguish between the order Pro-Gly or Gly-Pro at the C-terminus of the peptide. In each case the sequence was deduced based on the presumed identity with all other known GnRH peptides. In addition, an aliquot (corresponding to 10× the amount of sample applied for MALD PSD analysis) of the sample was

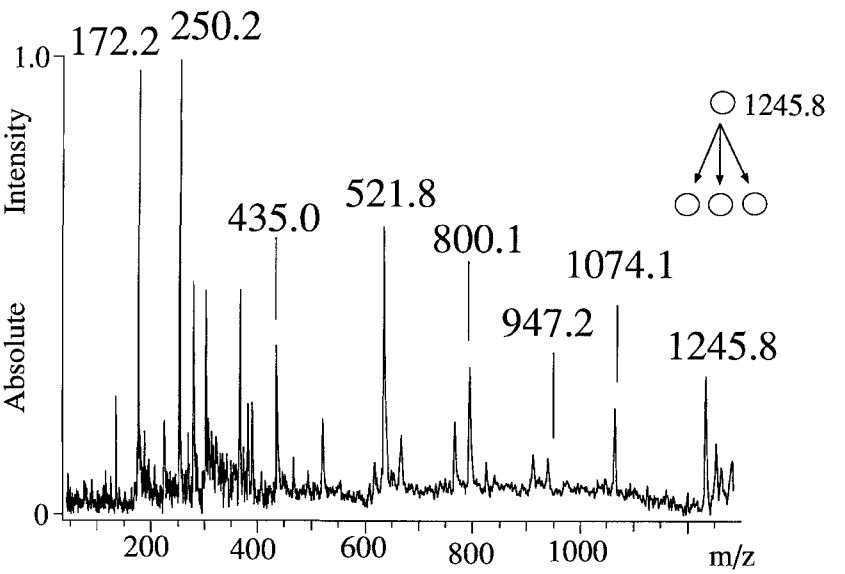

Fig. 3. PSD spectrum of tunGnRH-I.

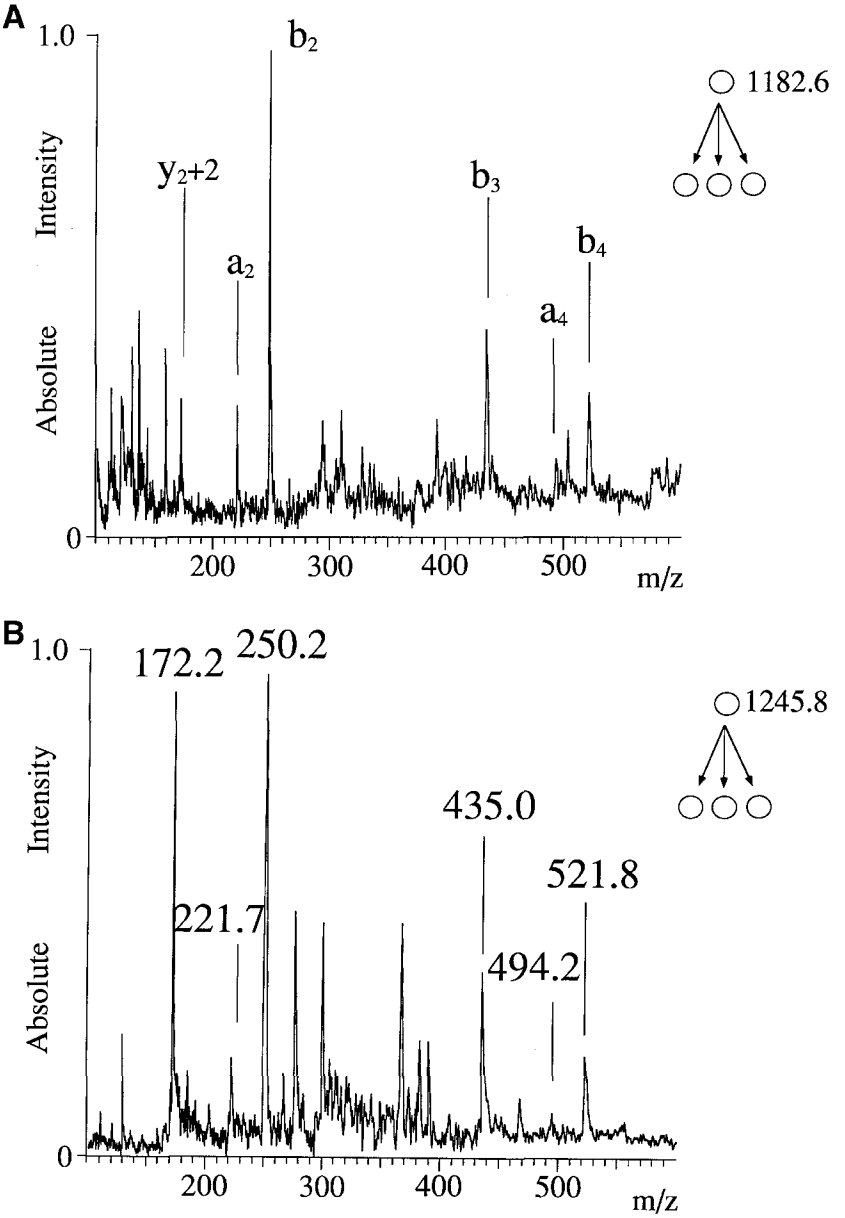

Fig. 4. Low-mass region (100–600 Da) of PSD spectrum of (A) mammalian GnRH and (B) tunGnRH-I.

treated with pyroglutamate aminopeptidase. In this way we were able to exclude either Thr-Phe or Phe-Thr on the basis of the shift in retention time and observed mass after pyroglutamate aminopeptidase treatment (data not shown). Chemical sequence analysis after HPLC purification yielded the sequence assignment (H)(W)SDYF(K)PG, where residues in pa-

Table 2
N–terminal fragment ions observed from different GnRH peptides

Species	Sequence	a2	b2	b3	a4	*4[a]	b4	a5	*5[b]	b5	a6	b6	a7	b7	a8	*8[c]	b8
mammalian	ZHWS**YGLR**PG	m	s	s	m	s	s	m	m	w	–	s	w	w	s	m	s
dogfish III	ZHWS**HGWL**PG	m	m	w	–	–	s	s	–	s	w	s	s	s	–	–	s
salmon	ZHWS**YGWL**PG	w	m	s	w	w	s	m	s	m	–	s	s	s	–	–	s
chicken II	ZHWS**HGWY**PG	w	s	m	w	w	m	s	m	s	–	s	s	s	–	–	s
catfish	ZHWS**HGLN**PG	w	m	s	w	m	s	s	w	s	–	s	s	s	–	–	s
chicken I	ZHWS**YGLQ**PG	–	–	s	w	–	s	w	s	m	–	s	m?	s	w	–	s
herring II	ZHWS**HGLS**PG	–	w	w	–	w	m	m	w	m	–	s	m	s	–	m	s
sea bream	ZHWS**YGLS**PG	–	m	s	w	w	s	–	–	m	–	s	m	m	–	s	s
lamprey III	ZHWS**HDWK**PG	w	m	s	–	w	s	m	w	w	–	s	w	m	–	–	s
tunicate I	ZHWS**DYFK**PG	m	s	s	w	–	s	w	–	s	s	s	m	m	–	–	s
tunicate II	ZHWS**LCHA**PG	–	s	m	–	–	m	–	–	m	–	s	–	s	–	–	s

[a]*4 = b_4–18; [b]*5 = b_5–18; [c]*8 = b_8–18.

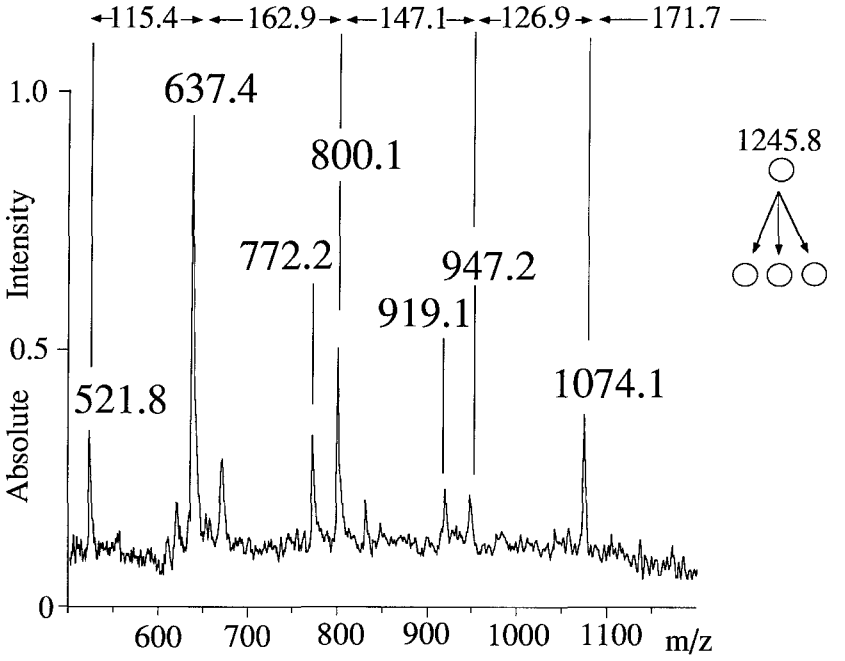

Fig. 5. High-mass region (500–1100 Da) of PSD spectrum of tunGnRH-I.

rentheses were assigned with less than 50% confidence. This analysis also resolved the assignment of the C-terminal residues. Therefore based on the PSD measurement and chemical sequence analysis we proposed the sequence pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly.

In order to verify the proposed sequence and also to identify whether the carboxy-terminus of the molecule was amidated a more accurate mass measurement was undertaken. Using MALD with the magnetic sector instrument (as described in the experimental section) the free acid and amidated forms of bombesin and a GnRH analog were analyzed (data not shown). Although the intensity of different isotopomers were observed to vary in consecutively measured spectra, the monoisotopic isotopomer could be clearly identified in each spectrum and thereby the difference between the two forms of the peptides determined. In addition, to the $[M+H]^+$ species we observed intense isotopomer distributions for the $[M+Na]^+$ and $[M+K]^+$ species which could be used to confirm the monoisotopic peak in each distribution and thereby the molecule mass (M). Fig. 6 shows the resolved isotope distributions of the $[M+H]^+$ and $[M+Na]^+$ species from **tunI** (an internal calibrant chicken GnRH-II (denoted as C) present on the probe generated $[C+Na]^+$ and $[C+K]^+$ species). The observed *m/z* of 1246.58 compares very closely with that calculated for the monoisotopic $[M+H]^+$ of 1246.564 Da for the amidated form of the proposed sequence. Based on these analyses we assign **tunI** as a GnRH, tunGnRH-I (sequence as listed in Table 3).

Purification of the second immunoreactive fraction (**tunII**) led to the identification of a major component which when analyzed with MALD-TOF-MS gave a single species at *m/z* 2233.1 (Fig. 7A). This mass was significantly greater than that of the 11 known forms of vertebrate GnRH. This component appeared to be a non-GnRH that shared some common GnRH epitope and therefore cross-reacted with the antisera used (based on the orthogonal purification protocol, we could discount the possibility that an impurity of this mass was present that was more abundant than a minor GnRH compo-

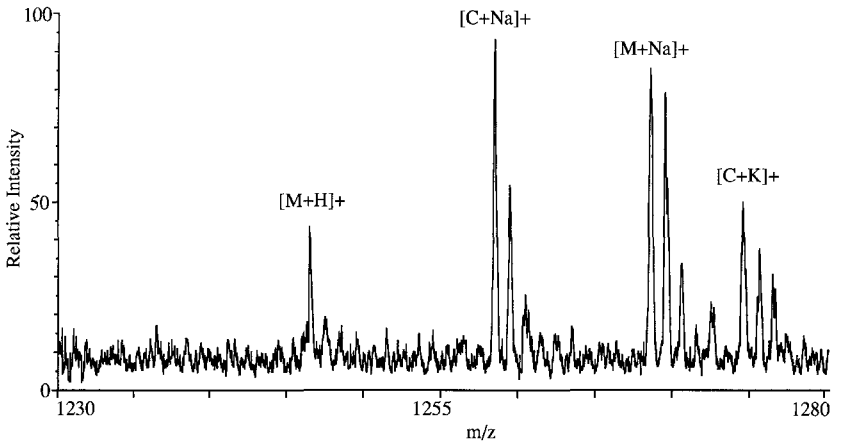

Fig. 6. Accurate mass measurement of the $[M+H]^+$ of tunGnRH-I.

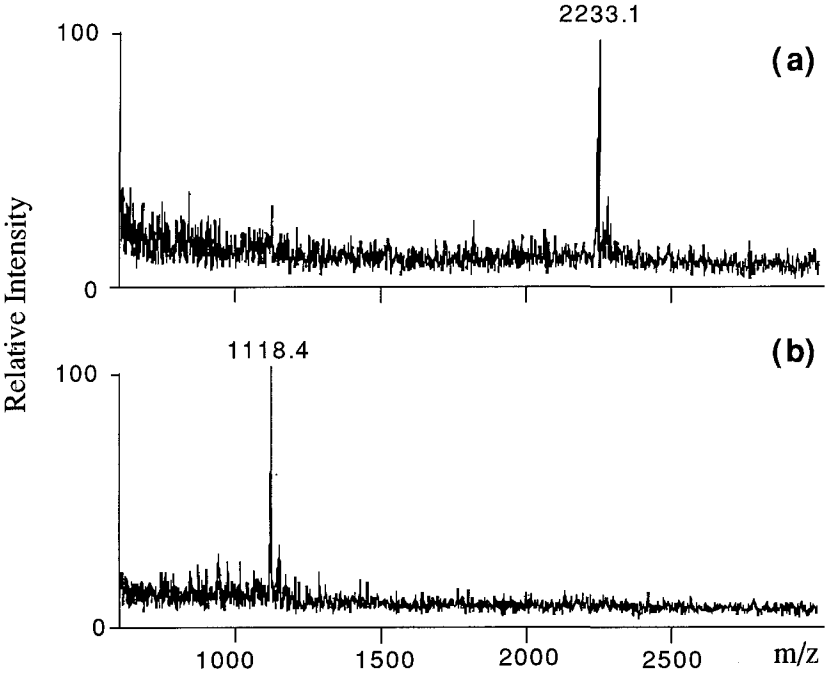

Fig. 7. MALD-TOF mass spectra of tunGnRH-II (a) before and (b) after reduction.

nent). However, the PSD spectrum of the protonated molecule ion (shown in Fig. 8) contained fragment ions at *m/z* 110.7, 172.3, 209.7, 248.7, 436.1, 505.8, 522.8 and 583.4. The observed masses of several of these fragment ions (*m/z* 110.7, 172.3, 248.7, 436.1, 505.8 and 522.8) are consistent with those observed in the low-mass region of known GnRH peptides (see Fig. 4 and Table 2). This suggested that **tunII** also contained regions with sequence identity to GnRH peptides. The situation was further clarified after treatment of the fraction with pyroglutamate aminopeptidase. Re-purification and measurement with MALD-TOF-MS revealed a species at *m/z* 1006.0 (data not shown). Because the enzyme treatment was carried out in a buffer containing the reducing agent DTT, we investigated the effect of reduction alone on the second immunoreactive fraction. After addition of the reducing reagent (TCEP) [33] we observed a shift in the retention time of the **tunII** fraction. MALD-TOF-MS analysis of this fraction gave a species at *m/z* 1118.4 (see Fig. 7B), indicating that the peptide present in this fraction was a cystine-containing homodimer and explaining the species observed at *m/z* 1006.0 after pyroglutamate aminopeptidase treatment. Both the intact mass (see Fig. 7B and Table 1) and the presence of a cysteine residue indicated that the peptide monomer was not a known GnRH. The low-mass region of the PSD spectrum of the protonated molecule ion of the reduced monomer is shown in Fig. 9. An uncharacteristic pattern was observed as a result of superimposition of the typical GnRH fragments with other intense fragment ions. Extending from the familiar *m/z* 172.3

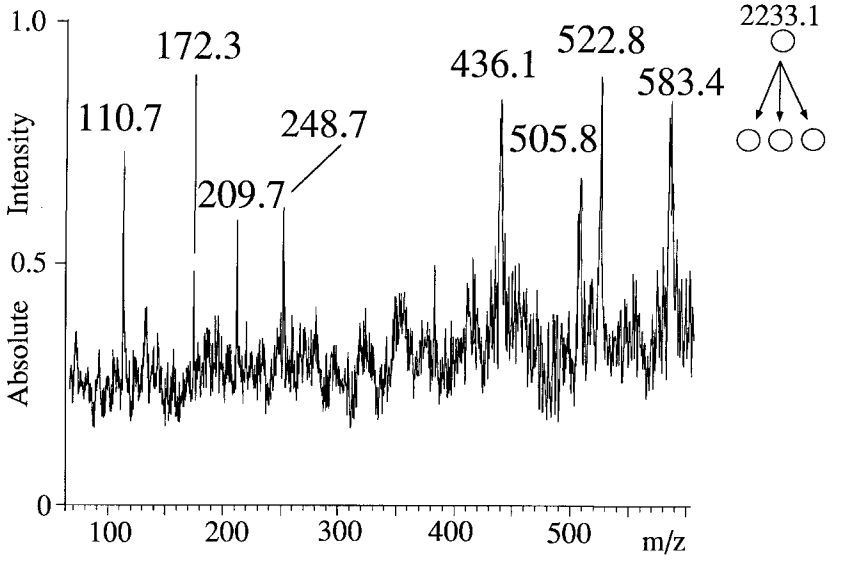

Fig. 8. Low-mass region (50–600 Da) of PSD spectrum of dimeric tunGnRH-II.

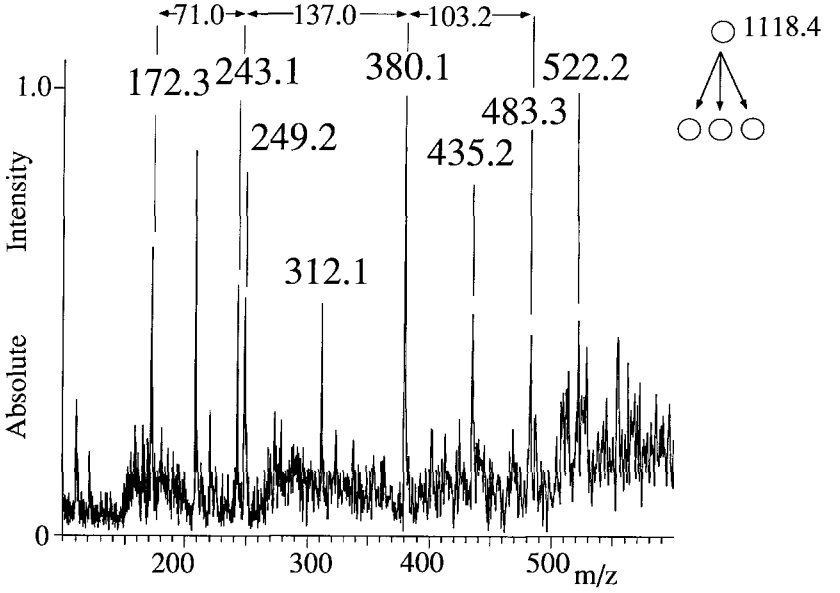

Fig. 9. Low-mass region (100–600 Da) of PSD spectrum of monomeric tunGnRH-II.

(y_2+2) to the *m/z* 243.1 fragment ions we observe a mass separation of 71 Da consistent with alanine. The separation between the *m/z* 243.1 and 380.1 (137 Da) corresponds with histidine, while the separation to *m/z* 483.3 (103.2 Da) indicates cysteine. The presence of cysteine is confirmed by experiments detailed above. This information suggests the sequence Cys-His-Ala-Pro-Gly for the C-terminus. The high-mass region of the **tunII** PSD spectrum is shown in Fig. 10. The mass separation between the *m/z* 522.2 (b_4) and the intense *m/z* 738.4 (216.2 Da) can correspond with either threonine-aspartic acid, serine-glutamic acid or leucine-cysteine. The presence of a fragment at *m/z* 635.6 favors the leucine-cysteine sequence assignment. The separation between the *m/z* 875.9, 738.4 and 945.6 verified the presence of the His-Ala residues. Chemical sequence analysis carried out on an aliquot after pyroglutamate aminopeptidase treatment led to the sequence assignment X(W)SL(C)HAPG, where X designates that no residue could be assigned confidently and residues in parentheses were assigned with less than 50% certainty. On the basis of this PSD measurement and chemical sequence analysis we proposed the sequence pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly for the monomeric form. Accurate mass measurement were obtained using external calibration with MALD on the magnetic sector instrument. Fig. 11A shows the resolved isotope distribution of the $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ species generated from **tunII**, before reduction where the monoisotopic $[M_d+H]^+$ species was observed at *m/z* 2232.08 (cf. 2231.977 for the calculated monoisotopic $[M_d+H]^+$ where M_d denotes the cystine-containing dimer). Fig. 11B shows the resolved isotope distribution of the

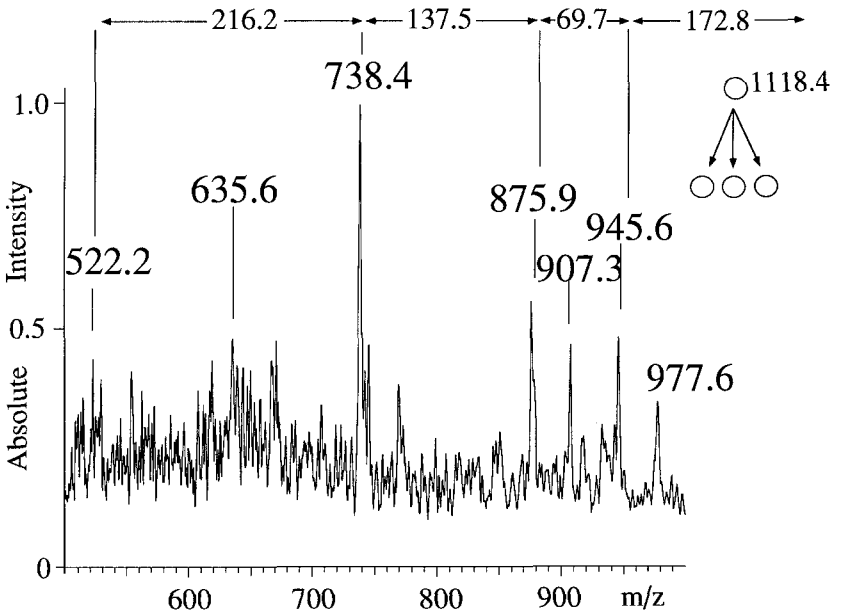

Fig. 10. High-mass region (500–1000 Da) of PSD spectrum of monomeric tunGnRH-II.

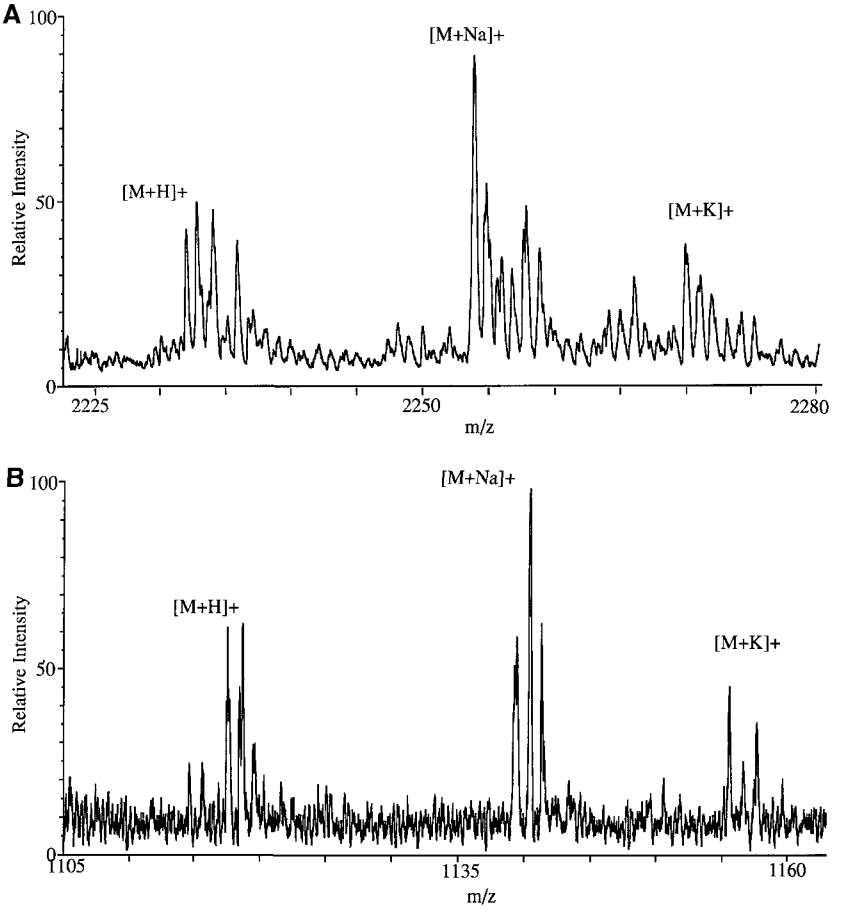

Fig. 11. Accurate mass measurement of the $[M+H]^+$ of tunGnRH-II (A) before reduction and (B) after reduction.

$[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ after reduction (with TCEP) where the monoisotopic $[M_m+H]^+$ species was observed at *m/z* 1117.52 (cf. 1117.500 for the calculated monoisotopic $[M_m+H]^+$, where M_m denotes the cysteine-containing monomer). On the basis of these analyses we assign **tunII** as tunGnRH-II (sequence as listed in Table 3).

Based on PSD sequencing information and the identity observed within this class of peptides we have determined the amino acid sequence using 1–2 pmol of purified peptide. We note that at the sensitivity level that this investigation was undertaken the ability to obtain sequence information with Edman techniques was essentially unaltered by treating only 80% of the material with the enzyme pyroglutamate aminopeptidase (compared with consuming 100% of the sample). Of the remaining material (20%) the use of ca. half to obtain PSD and half to obtain accurate mass information gave important additional information. The low-mass region of the PSD spectra proved to be a useful diagnostic tool for identifying GnRH peptides. We note that there was considerable synergism between the sequence information obtained from the Edman approach and the PSD technique. Finally, we found that the localization of basic residues and the conservation of the amino terminus in vertebrate GnRH molecules does give rise to a common motif in the PSD spectrum which can be used diagnostically when scanning for putative GnRH-like molecules.

The sequence of these novel forms of invertebrate GnRH have significant substitutions compared with that of previously known vertebrate GnRHs. Tunicate GnRH-I differs from all vertebrate GnRHs at positions 5, 6 and 7. The presence of Lys in position 8 of tunGnRH-I, has previously been observed in lamprey GnRH-I and lamprey GnRH-III, but not in GnRH's from higher vertebrate species. The tunGnRH-II differs from all vertebrate GnRHs at positions 6, 7 and 8. The presence of Leu in position 5 of tunGnRH-II, has previously been observed only in lamprey GnRH-I.

The tunGnRH-I contains a putative salt bridge between residues Asp^5 and Lys^8. In mammalian GnRH a number of

Table 3

tunI	tunGnRH-I	pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2
tunII	tunGnRH-II	pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2 | pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2

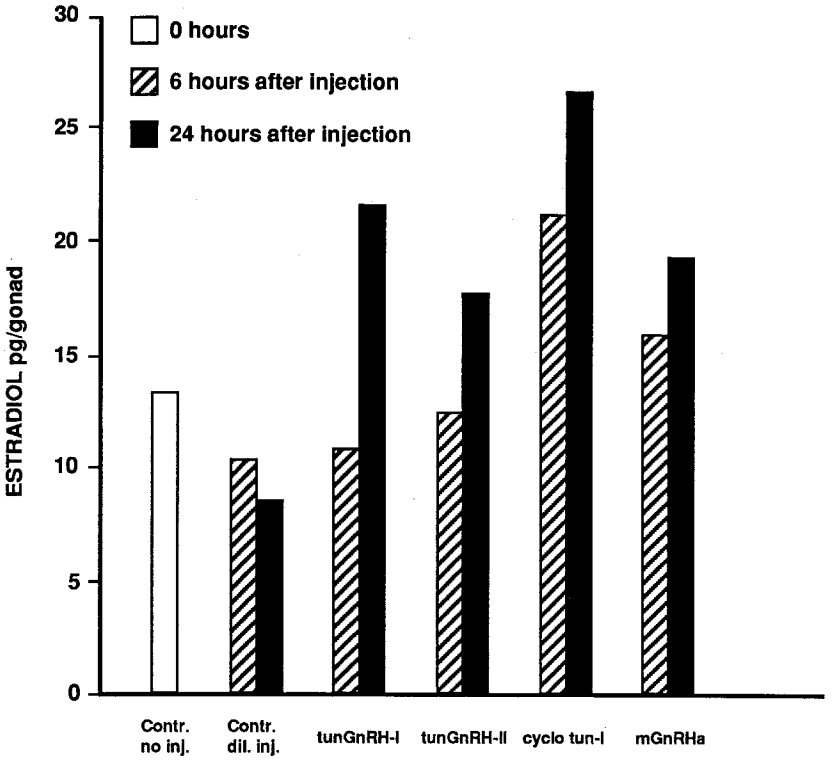

Fig. 12. Content of estradiol (pg/gonad) after injection of GnRH peptides into the tunicate, *Chelyosoma productum*. Control (Contr.); injection (inj.); diluent (dil.); tunicate (tun); gonadotropin-releasing hormone (GnRH); cyclo(5–8)tunicate GnRH-I (cyclo tun-I); [D-Ala^6, Pro^9-NEt] mammalian GnRH agonist (mGnRHa).

investigations support a proposed beta turn in this general location. The presence of a type II beta turn between these residues in tunGnRH-I would facilitate the formation of a salt bridge between Asp^5 and Lys^8 (or conversely the attraction between the charged groups of these two residues which constitute a salt bridge may result in the beta turn formation) and thereby serve to stabilize a bioactive conformation. The validity of these proposals has been investigated by synthesizing the lactam bridge stabilized analog of tunGnRH-I [cyclo(5–8)tunGnRH-I]. Although sex steroids have not been reported for Tunicata, we used highly specific antisera to detect estradiol, progesterone and testosterone. Both synthetic tunGnRH-I and -II resulted in a doubling of the gonadal content of estradiol after 24 h. The most effective peptide was the lactam bridge stabilized cyclo(5–8)tunGnRH-I which was found to double and triple estradiol content after 6 and 24 h as compared to the control group injected with diluent. As shown in Fig. 12 this analog resulted in higher estradiol release from whole tunicates than a potent linear mammalian GnRH agonist, [D-Ala^6, Pro^9-NEt] mammalian GnRH. This result also extends the observation from amphioxus to tunicates that a mammalian GnRH analog can induce an increase in estradiol [34]. The same gonadal extracts contained progesterone, but the treatment had little effect compared with the control groups; the average values in all groups ranged from 120 to 210 pg/gonad (Fig. 12). Testosterone was either below (< 0.3 nmol/L) or close (0.5–2.1 nmol/L) to the lower level of detection. Synthetic tunGnRH-I was also tested on 5 *Corella* by placing them in sea water that had a concentration of GnRH of 10^{-7} M. Gamete release was not observed although the sperm ducts were full of sperm. The synthetic tunGnRH-I and -II were also separately injected into the visceral hemocoel in the vicinity of the gonads in 13 *Corella*. A dose of 1 μl of 10^{-3} M peptide did not release the sperm observed to be in the sperm duct. Some of the injections with the picospritzer contained coloring and showed that the correct space was injected and that the color circulated through the heart followed by dispersal in the circulatory system. The animals continued to live for 3–4 weeks.

Tunicate GnRH-II is the first 'GnRH-like' peptide to have a cystine residue in its sequence. Clearly, tunGnRH-II is more than a 'GnRH-like' peptide since each monomer is a decapeptide where residues 1–4 and 9–10 are conserved compared with all other known forms of GnRH peptides. The dimeric structure has no precedent in this peptide hormone family and we speculate that this ligand may represent a means to effect receptor dimerization. Dimerization of occupied GnRH receptors is sufficient without further aggregation to activate the release of luteinizing hormone from porcine pituitary cells [35]. Structure function studies of GnRH emphasize the role both the N- and C-terminus of the molecule plays in receptor binding. The presence of cysteine in position 5 of the monomer would presumably allow the N- and C-terminus of the molecule to bind the receptor whereas the cysteine remains free to form the disulfide bridge (cystine). The tunGnRH-II may illustrate an ancestral mechanism to ensure receptor dimerization.

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