Isolation and cloning of exendin precursor cDNAs from single samples of venom from the Mexican beaded lizard (*Heloderma horridum*) and the Gila monster (*Heloderma suspectum*)

Tianbao Chen [a], HangFai Kwok [a], Craig Ivanyi [b], Chris Shaw [a,*]

[a] *School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast BT9 7BL, UK*
[b] *The Arizona-Sonora Desert Museum, Kinney Road, Tucson, AZ 85743-8918, USA*

Abstract

Reptile venoms are complex cocktails of bioactive molecules, including peptides. While the drug discovery potential of most species remains unrealized, many are endangered and afforded protection under international treaties. In this study, we describe how potential clinically important bioactive peptides and their corresponding mRNAs can be structurally characterized from single, small samples of reptile venom. The potential type-2 diabetes therapeutics, exendin-3 and exendin-4, from the Mexican beaded lizard (*Heloderma horridum*) and the Gila monster (*Heloderma suspectum*), respectively, have been characterized at both protein and nucleic acid levels to illustrate the efficacy of the technique and its contribution to biodiversity conservation.
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1. Introduction

Biologically active molecules, including peptides, found in tissues of non-mammalian vertebrates and invertebrates, have been the subject of intensive investigation and subsequent report in the scientific literature for almost 40 years (Bertaccini, 1976; Erspamer, 1994; Raufman, 1996). The complex cocktail of bioactives contained in the venom of helodermatid lizards, secreted from glands located at the bases of their specialized grooved teeth, appears to have evolved primarily for subduing prey but also plays an effective role in defence (Datta and Tu, 1997; Mebs, 1968; Nobile et al., 1996; Russell and Bogert, 1981). The individual components are representative of many classes of biochemical, including enzymes, peptides, nucleotides, proteins and other, as yet uncharacterized molecules (Bertaccini, 1976).

The Mexican beaded lizard (*Heloderma horridum*) and the closely related Gila monster (*Heloderma suspectum*) are the only known extant venomous lizards. They are large lizards (Family Helodermatidae), related to the monitors, and are denizens of the Sonoran desert of the South Western United States and Mexico. Envenomation by these lizards produces a rapid drop in blood pressure that can lead to hypotensive shock (Tu and Murdock, 1967). Their venoms have been the subject of study since 1891, but only in recent times has their biochemistry been partially elucidated; particularly directed towards potential novel drug lead discovery. However, in 1997, due to accumulating evidence of their threatened status, the US and Mexican administrations put a commercial ban on sale or export of both helodermatid lizards and their venoms (Levell, 1997) and they became CITES (Convention on International Trade in Endangered Species) Appendix 2 designates. Early biochemical studies

* Corresponding author. Tel.: +44 2890 972129; fax: +44 2890 247794.

E-mail address: chris.shaw@qub.ac.uk (C. Shaw).

(Russell, 1983) reported that some 90–95% of freeze–dried venom consisted of proteinaceous material comprising non-toxic proteins, protein toxins, enzymes and peptides. Thus, we believe that the potential for novel bioactive peptide discovery from these venoms remains significant. Perhaps, the most significant bioactive peptides present in these venoms are the helodermins/helospectins and the exendins. These are all structurally related to the glucagon/secretin/vasoactive intestinal peptide family of peptides present in the neuroendocrine systems of vertebrates. The most interesting group from the drug lead discovery perspective is the exendins. These peptides are agonists of the mammalian glucagon-like peptide 1 (GLP-1) receptor—a receptor expressed in high levels by the pancreatic beta cell. This peptide/receptor system has received much attention as a novel target for insulinotropic drug development directed towards the treatment of Type 2 diabetes. The exendins have apparently superior pharmacokinetics when compared with the endogenous ligand, GLP-1. Thus, the molecular dissection of reptile venoms can lead to discoveries with potential for addressing chemotherapeutic utility in human disease which are not apparent at the initiation of the research programme.

In the present study, we have employed exendins from the venom of both species of helodermatid lizard to illustrate that structural characterization of peptide and cloning of autologous precursor cDNAs can be achieved from small (ca. 5–10 mg) samples of venom without recourse to sacrifice of donor specimens. This is completely in keeping with the need for conservation of endangered species without sacrificing the robustness of scientific data generated from studies on their gene products (peptides and proteins) that may have either clinical relevance or additional biotechnological applications. The structure of cloned cDNA encoding the precursor of exendin-3 from Mexican beaded lizard (*H. horridum*) venom is additionally reported for the first time.

2. Materials and methods

2.1. Acquisition of lizard venom

Venoms of the Gila monster (*H.suspectum*) and the Mexican beaded lizard (*H. horridum*), were obtained from captive specimens residing in the herpetarium of the Arizona-Sonora Desert Museum in Tucson, AZ, USA. Lizards were handled by experienced herpetologists and were permitted to repeatedly bite a sponge placed between their jaws. Venom released from the submandibular venom glands by the biting action was frequently harvested from the sponge and collected directly into chilled beakers, immediately snap-frozen and lyophilized. As these species are CITES Appendix II designates, all necessary permits for export and import of venom samples were obtained from

both the United States Department of Agriculture and the British Customs and Excise, in accordance with the international treaty.

2.2. Gel permeation chromatography

About 35 mg of lyophilized venom from each species of lizard were reconstituted in 3 ml of 2 M acetic acid, clarified by centrifugation, and chromatographed separately on a 90×1.6 cm column of Sephadex G-50 (fine), equilibrated in 2 M acetic acid and eluted at a flow rate of 10 ml/h. Fractions (2.5 ml) were collected at 15 min intervals and the column had previously been calibrated with Blue Dextran (V_o) and potassium dichromate (V_t).

2.3. Reverse-phase HLPC

Pooled aliquots (500 µl) of gel permeation chromatography fraction nos 55–65 from the venom of each species were subjected to reverse-phase HPLC fractionation using a Thermoquest gradient HPLC system fitted with a Jupiter semi-preparative C-5 column (30×1 cm). This was eluted with a linear gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals and the effluent absorbance was continuously monitored at λ 214 nm.

2.4. Structural analyses

The molecular masses of polypeptides in each chromatographic fraction were determined using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as $\pm 0.1\%$. The major polypeptides with masses of approximately 4.2 kDa, that were identified in the venom, were each subjected to primary structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode. The limit for detection of phenylthiohydantoin (PTH) amino acids was 0.1 pmol.

2.5. In vitro cDNA library construction from lyophilized venom

About 10 mg samples of lyophilized venom from each lizard species were dissolved separately in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). mRNA was eluted in 20 µl of RNase free water and first strand cDNA synthesis for subsequent

RACE reactions was performed using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the RACE reactions were amplified using a sense primer (S1, 5′-GGTGCCTGTG CAAAGGAGAA-GATG-3′) and an antisense primer (AS1-12, 5′-GGCTGTGTCGCTCACTAATTGGAAA-3′) for the exendin cDNAs by thermostable polymerase (Invitrogen). These primers were complementary to a domain of nucleotide sequences in the 5′ and 3′-untranslated regions of the exendin-4 precursor cDNA cloned previously from Gila monster (*H. suspectum*) venom gland tissue (Chen and Drucker., 1997). The PCR cycling procedure was as follows: an initial denaturation step for 1 min at 94 °C followed by 35 cycles consisting of denaturation for 30 s at 94 °C, primer annealing for 30 s at 62 °C and extension for 3 min at 72 °C. Gel electrophoresis of the PCR products from each specie's venom library was followed by further purification, cloning using a pGEM-T vector system (Promega Corporation) and subsequent sequencing using an ABI 3100 automated capillary sequencer.
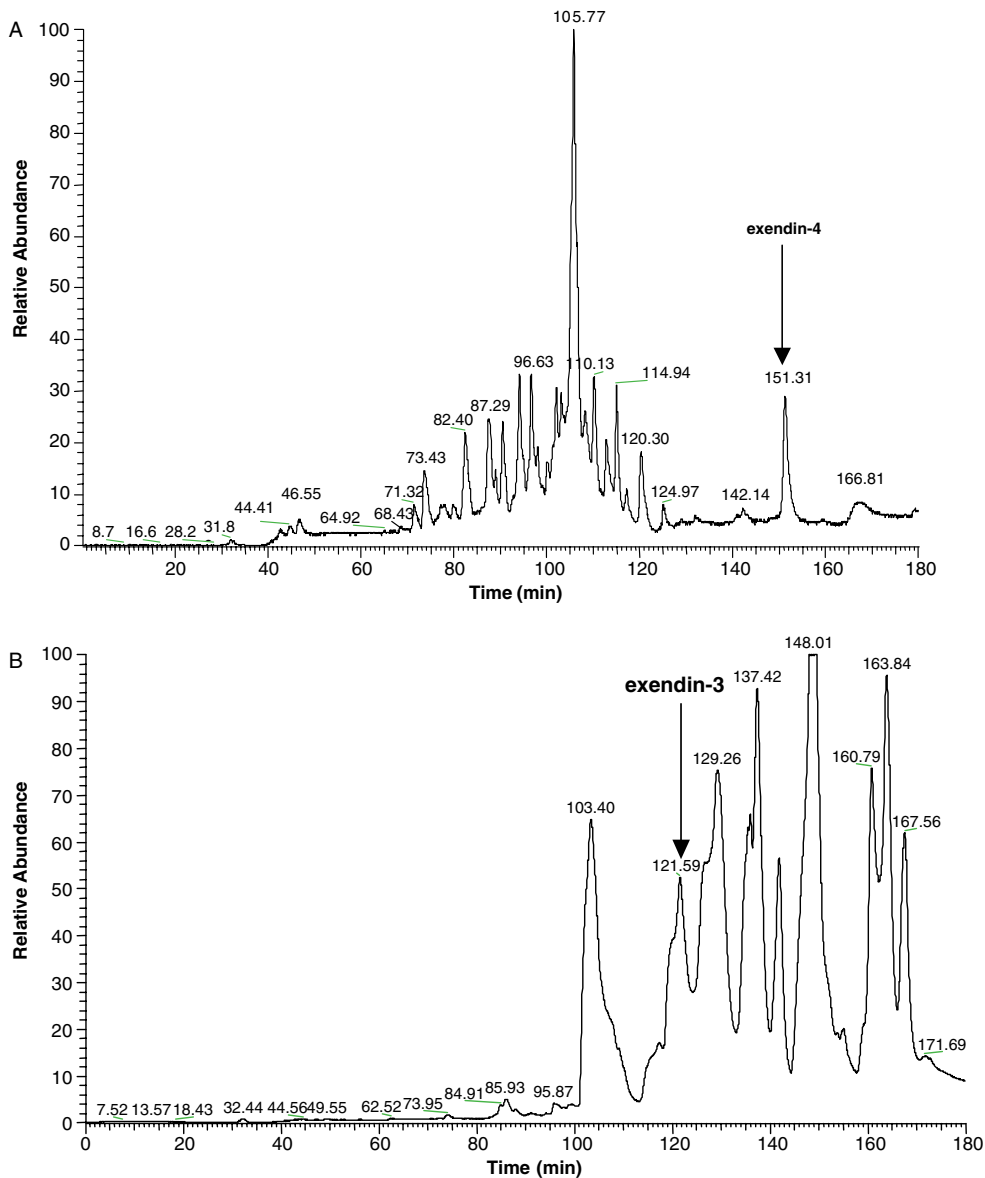

Fig. 1. Reverse phase HPLC chromatograms of Gila monsters (*H. suspectum*) (A) and Mexican beaded lizard (*H. horridum*) (B) venom. Retention times of exendins are indicated.

3. Results

3.1. Isolation and structural characterization of exendin-4 and exendin-3 from H. suspectum *and* H. horridum *venom, respectively*

Polypeptides with non-protonated molecular masses corresponding to exendin-4 (4185.0 Da) and exendin-3 (4202.6 Da) were detected using MALDI-TOF MS in reverse phase HPLC fractions of *H. suspectum* and *H. horridum* venom gel permeation chromatographic fractions (Fig. 1). Each fraction was rechromatographed producing single-peak pure polypeptides in each case as revealed by mass spectroscopy (Fig. 2). The primary structures of each purified peptide were established unequivocally by automated Edman degradation as: (1)

exendin-4 (*H. suspectum*) HGEGTFTSDLSKQ-MEEEAVRLFIEWLKNGGPSSGAPPPS and (2) exendin-3 (*H. horridum*) HSDGTFTSDLSKQMEEEAVRL-FIEWLKNGGPSSGAPPPS (data not shown)

3.2. Cloning of exendin precursor cDNAs from venom-derived libraries

Single discrete bands of approximately 400 bp were resolved in PCR reactions from the venom-derived cDNA libraries from both species of lizard (Fig. 3). Following cloning of sequencing of these products and sequencing, cDNAs encoding the entire reading frames of exendin-4 (*H. suspectum*) and exendin-3 (*H. horridum*) were represented in at least 40 clones from each species library, respectively. The sequences of exendin precursor cDNAs
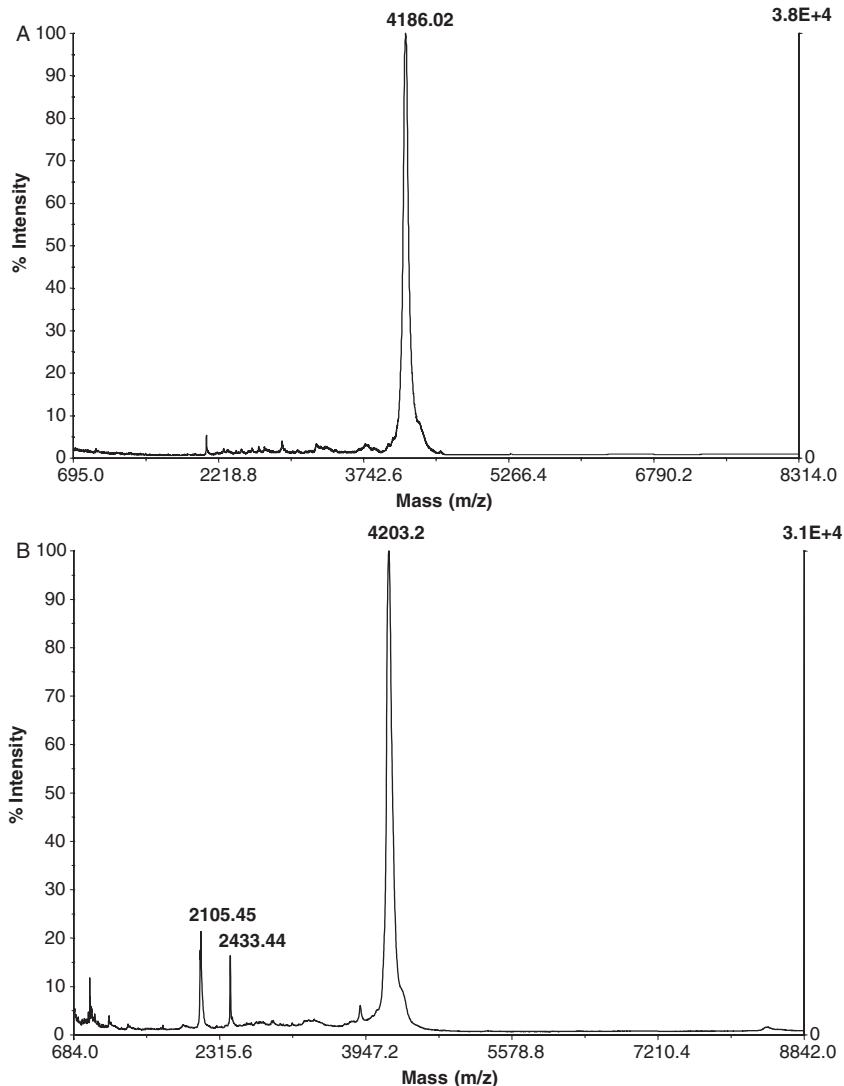

Fig. 2. MALDI-TOF mass spectrograms of fraction 151 containing exendin-4 (A) and fraction 122 containing exendin-3 (B).
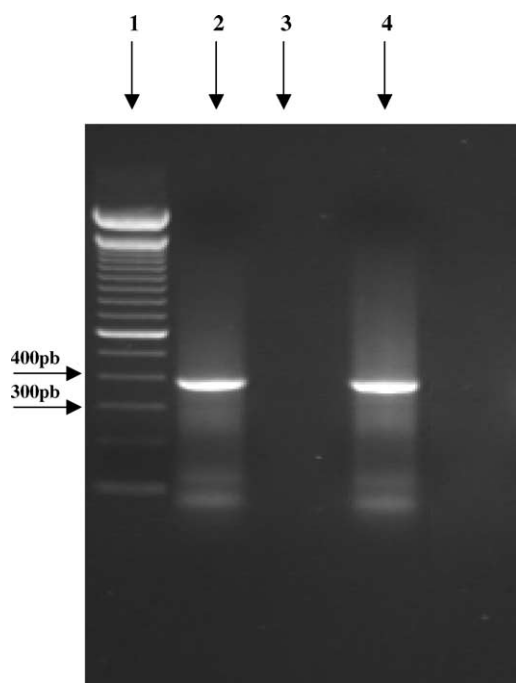
Fig. 3. Gel electropherogram of RT-PCR products generated from the gene-specific primer. Lane 1 contains a standard DNA ladder, each band representing 100 bp increments. Lane 2: RT-PCR product from venom libraries of Gila monsters (*H. suspectum*). Lane 3: Non-template control. Lane 4: RT-PCR product from venom libraries of Mexican beaded lizards (*H. horridum*).

and translated open-reading frames are illustrated in Fig. 4. Alignment of both nucleotide sequences (Fig. 5A) and open-reading frame amino acid sequences (Fig. 5B), using the AlignX programme of the Vector NTI Bioinformatics suite (Informax), revealed a high degree of structural similarity between the exendin-3 and exendin-4 transcripts and precursors. The exendin-4 nucleotide sequence and translated open-reading frame amino acid sequence, displayed 100% identity with that previously cloned from the venom glands of *H. suspectum* and deposited in the EMBL database (Accession no. U77613) (Chen and Drucker., 1997). The mature exendin-3 peptide displayed 100% identity with that previously deposited in SwissProt (P20394) and as the nucleotide sequence of its precursor cDNA and translated open-reading frame deduced here had not been previously reported, it was deposited in the EMBL database under the accession number, AJ580309. The entire open-reading frames of exendin-3 and exendin-4 precursors exhibit only three sites of amino acid substitution. Two of these occur at the near N-terminus of both peptides (Gly^2-Glu^3 in exendin-4 for Ser^2-Asp^3 in exendin-3) accounted for by single base changes in codons (GGT/AGT–Gly/Ser and GAA/GAT–Glu/Asp. The additional site of amino acid substitution (occurs at position 20) in the open-reading frame at the end of the putative signal peptide (Ile/Val) accounted for by

a single base change in the specifying codon (ATC/GTC). All of these substitutions are chemically conservative. A second site of base substitution occurs in the codon specifying the Phe residue at position 10 in the open-reading frame putative signal peptide domain but this change (TTT/TTC) is accommodated within the codon degeneracy for this amino acid residue. Three other sites of single base substitutions occur in the 3′-untranslated region.

4. Discussion

Tissues from non-mammalian vertebrates remain a largely untapped resource for the peptide biochemist with an interest in the identification, structural characterization and cloning of precursor cDNAs of novel bioactive peptides. While some of these peptides may represent novel analogs of known peptide families and provide much insight into molecular evolutionary pathways related to phylogenesis, others will exhibit such dramatic structural alterations that very different pharmacological properties may be imparted providing important information for analogue design or receptor sub-type targeting. However, perhaps the most interesting group will always remain the smaller number that represent prototype peptides not encountered before in nature (Clarke, 1997; Ramoutsaki et al., 2000; Raufman, 1996).

Molecular cloning of bioactive polypeptide mRNAs from the tissues of various species can provide much useful information on species relatedness, post-translational or signature motifs, endogenous propolypeptide convertase specificities, co-encoded peptides and can be a vital step in the initiation of studies designed to map genomic organization of respective genes (Chen and Drucker, 1997; Chen et al., 2002; Junqueira et al., 2001; Zhu et al., 2002). Until now, whilst isolation and structural characterization of proteins/peptides could be achieved using lyophilized venom, construction of transcriptome cDNA libraries for the purpose of molecular cloning of precursors required the use of tissue samples that necessitated sacrifice of living specimens (Chen and Drucker, 1997; Chen et al., 2002; Junqueira et al., 2001;; Zhu et al., 2002). The only two known extant venomous lizards of the family Helodermatidae, are representative of many groups of vertebrates that are suffering unexplained declines due to a combination of factors, including habitat destruction and pollution on one hand, and pathogenic viral and fungal diseases, on the other (Ast, 2001). Thus, conservation of even individual specimens of threatened species and the acquisition of potentially useful gene products from these in a non-invasive and non-lethal fashion, would be highly desirable for scientists interested in novel drug lead discovery from disappearing biodiversity. We thus extended our studies on cloning venom gland peptide precursors from amphibian venom cDNA libraries (Chen et al., 2003) to determine if novel transcripts could be cloned from the venom of helodermatid

A

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                                     M   K   I    I   L   W    L   C   V   F
      1  GGTGCCTGTG CAAAGGAGAA GATGAAAATC ATCCTGTGGC TGTGTGTTTT

           G   L   F    L   A   T    L   F   P   I    S   W   Q    M   P   V
     51  TGGGCTGTTC CTTGCAACTT TATTCCCTAT CAGCTGGCAA ATGCCTGTTG

           E   S   G   L    S   S   E    D   S   A    S   S   E    F   A   S
    101  AATCTGGGTT GTCTTCTGAG GATTCTGCAA GCTCAGAAAG CTTTGCTTCG

           K   I   K    R   H   G   E    G   T   F    T   S   D    L   S   K   Q
    151  AAGATTAAGC GACATGGTGA AGGAACATTT ACCAGTGACT TGTCAAAACA

             M   E   E    E   A   V   R    L   F   I    E   W   L    K   N   G
    201  GATGGAAGAG GAGGCAGTGC GGTTATTTAT TGAGTGGCTT AAGAACGGAG

           G   P   S   S    G   A   P    P   P   S   G   *
    251  GACCAAGTAG CGGGGCACCT CCGCCATCGG GTTAAGGTCT TTCAATTGTG

    301  GAACAAGACA CACACCTGAT GTTTGATGAC CATTTTAAAG AAATGTTTCC

    351  AGAAATACGT CACATGTCTT TGTTTCCAAT TAGTGAGCGA CACAGCC
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B

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                                     M   K   I    I   L   W    L   C   V   F
      1  GGTGCCTGTG CAAAGGAGAA GATGAAAATC ATCCTGTGGC TGTGTGTTTT

           G   L   F    L   A   T    L   F   P   V    S   W   Q    M   P   V
     51  CGGGCTGTTC CTTGCAACTT TATTCCCTGT CAGCTGGCAA ATGCCTGTTG

           E   S   G   L    S   S   E    D   S   A    S   S   E    F   A   S
    101  AATCTGGGTT GTCTTCTGAG GATTCTGCAA GCTCAGAAAG CTTTGCTTCG

           K   I   K    R   H   S   D    G   T   F    T   S   D    L   S   K   Q
    151  AAGATTAAGC GACATAGTGA TGGAACATTT ACCAGTGACT TGTCAAAACA

             M   E   E    E   A   V   R    L   F   I    E   W   L    K   N   G
    201  GATGGAAGAG GAGGCAGTGC GGTTATTTAT TGAGTGGCTT AAGAACGGAG

           G   P   S   S    G   A   P    P   P   S   G   *
    251  GACCAAGTAG CGGGGCACCT CCGCCATCGG GTTAAGGTCT TTCAATTGTG

    301  GAACAAGACA CACACCTGAT GTTTGACGAC CATTTTAAAG AAATGTTTCC

    351  AGCAATAAGT CACATGTCTT TGTTTCCAAT TAGTGAGCGA CACAGCC
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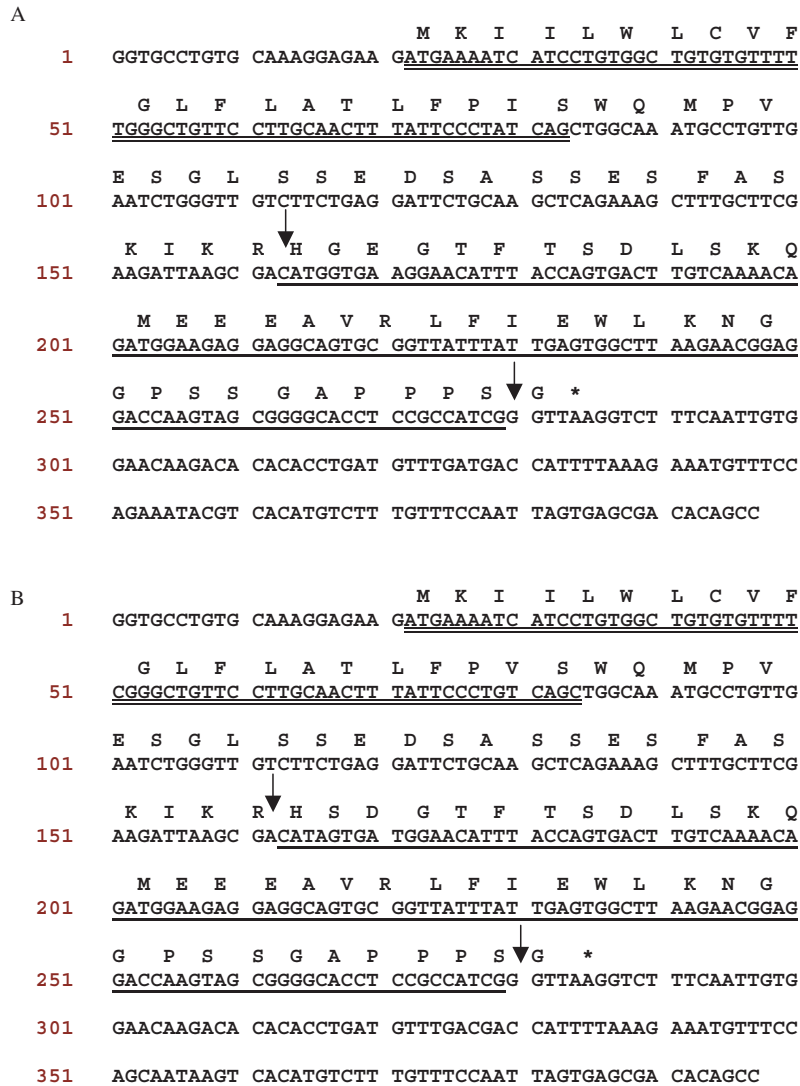
Fig. 4. Nucleotide sequences of cDNAs encoding exendins from Gila monsters (*H. suspectum*) (A) and Mexican beaded lizards (*H. horridum*) (B) venom. The putative signal peptides (double-underlined), mature peptides (single-underlined) and stop codons (asterisks) are indicated. The cleavage site of the precursor protein is indicated by an arrow.

lizards. Why are these species of interest to the scientist studying natural peptide/protein-based secretions for novel chemical entities?

In the early 1990s, two novel 39 amino acid residue polypeptides, exendin-3 and exendin-4 [$M_r \sim 4184$], were discovered in the venoms of the Mexican beaded lizard (*Heloderma horridum*) and Gila monster (*Heloderma suspectum*), respectively (Eng, 1992). Subsequent studies showed that exendin-3 and exendin-4 are most homologous (50 and 53%, respectively) to human glucagon-like peptide-1 (GLP-1), and interact with mammalian GLP-1 receptors (Eng et al., 1992). GLP-1 has been reported can stimulate insulin secretion and inhibit glucagon secretion, gastric emptying, and feeding, suggesting it may be biologically useful adjunct for the treatment of diabetes (Radosavljevic et al., 2002). Therefore, the isolation of the exendin peptides from lizard venom, that display greatest primary structural similarities to human GLP-1, prompted proteomic and genomic studies examining the biological properties and genetic evolutionary relationship between these peptides. Over the past 20 years, the level of research interest in exendin peptides has intensified; particularly that directed towards exendin-3 and exendin-4 for the treatment of diabetes. However, the US and Mexican Governments have listed both *H. horridum* and *H. suspectum* as CITES Appendix II species (Levell, 1997). Most of the molecular biological studies on *Heloderma* species, related to cloning of cDNAs encoding venom gland peptides, have been

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                      1                                                50
Exendin-3    (1)   GGTGCCTGTGCAAAGGAGAAGATGAAAATCATCCTGTGGCTGTGTGTTTT
Exendin-4    (1)   GGTGCCTGTGCAAAGGAGAAGATGAAAATCATCCTGTGGCTGTGTGTTTT

                      51                                               100
Exendin-3   (51)   CGGGCTGTTCCTTGCAACTTTATTCCCTGTCAGCTGGCAAATGCCTGTTG
Exendin-4   (51)   TGGGCTGTTCCTTGCAACTTTATTCCCTATCAGCTGGCAAATGCCTGTTG

                      101                                              150
Exendin-3  (101)   AATCTGGGTTGTCTTCTGAGGATTCTGCAAGCTCAGAAAGCTTTGCTTCG
Exendin-4  (101)   AATCTGGGTTGTCTTCTGAGGATTCTGCAAGCTCAGAAAGCTTTGCTTCG

                      151                                              200
Exendin-3  (151)   AAGATTAAGCGACATAGTGATGGAACATTTACCAGTGACTTGTCAAAACA
Exendin-4  (151)   AAGATTAAGCGACATGGTGAAGGAACATTTACCAGTGACTTGTCAAAACA

                      201                                              250
Exendin-3  (201)   GATGGAAGAGGAGGCAGTGCGGTTATTTATTGAGTGGCTTAAGAACGGAG
Exendin-4  (201)   GATGGAAGAGGAGGCAGTGCGGTTATTTATTGAGTGGCTTAAGAACGGAG

                      251                                              300
Exendin-3  (251)   GACCAAGTAGCGGGGCACCTCCGCCATCGGGTTAAGGTCTTTCAATTGTG
Exendin-4  (251)   GACCAAGTAGCGGGGCACCTCCGCCATCGGGTTAAGGTCTTTCAATTGTG

                      301                                              350
Exendin-3  (301)   GAACAAGACACACACCTGATGTTTGACGACCATTTTAAAGAAATGTTTCC
Exendin-4  (301)   GAACAAGACACACACCTGATGTTTGATGACCATTTTAAAGAAATGTTTCC

                      351                                       397
Exendin-3  (351)   AGCAATAAGTCACATGTCTTTGTTTCCAATTAGTGAGCGACACAGCC
Exendin-4  (351)   AGAAATACGTCACATGTCTTTGTTTCCAATTAGTGAGCGACACAGCC
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B

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                      1                                                50
Exendin-3    (1)   MKIILWLCVFGLFLATLFPVSWQMPVESGLSSEDSASSESFASKIKRHSD
Exendin-4    (1)   MKIILWLCVFGLFLATLFPISWQMPVESGLSSEDSASSESFASKIKRHGE

                      51                                      87
Exendin-3   (51)   GTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSG
Exendin-4   (51)   GTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSG
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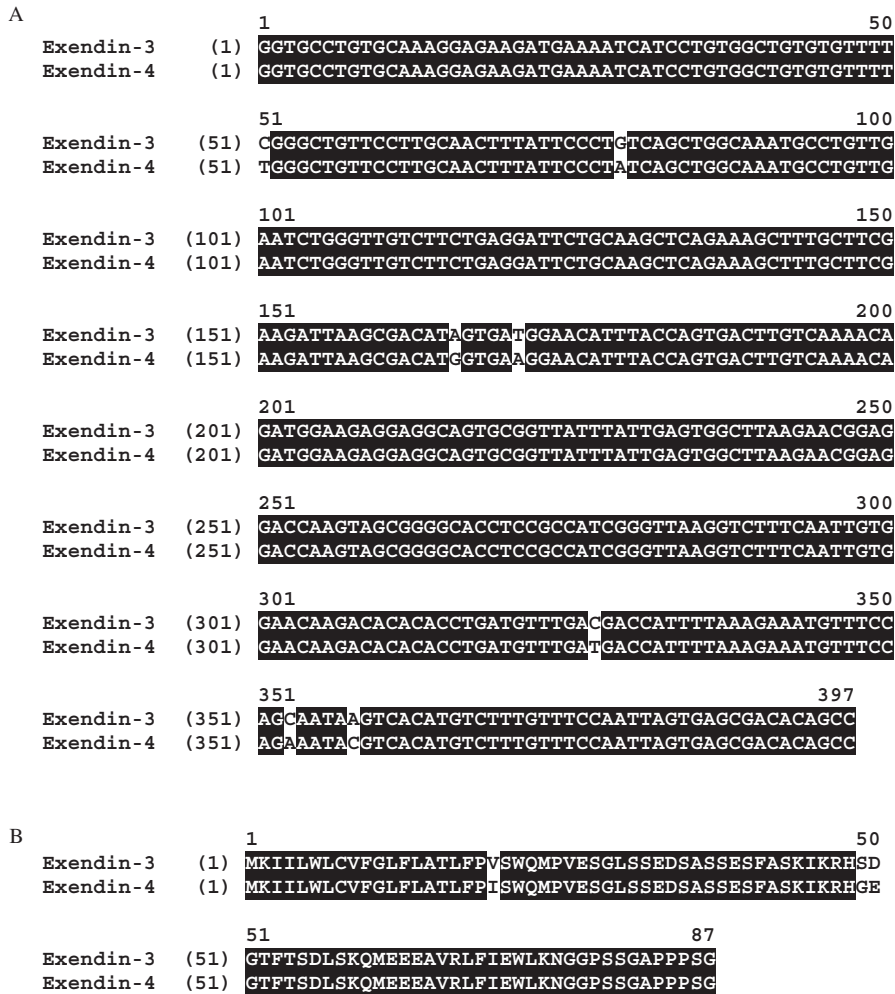
Fig. 5. Alignment of nucleotide sequences of cDNAs encoding exendin-3 (*H. horridum*) and exendin-4 (*H. suspectum*). Identical bases in all three shaded in black. Consensus bases in two sequences shaded grey. Gaps inserted to maximise alignment (A); Alignment of translated open-reading frame amino acid sequences of exendin-3 and exendin-4. Identical amino acid residues shaded in black (B).

brought to an end as these traditional methods required sacrifice of specimens and library construction from excised tissue (Raufman, 1996). Exendin-4 was chosen as a 'control' for the technique described here insofar as the nucleotide sequence of the precursor has been established (Chen and Drucker, 1997;).

Although the structure of exendin-3 has been known for some time, until now the nucleotide sequence of a precursor cDNA has not been reported. One of the major possible reasons for this is that the molecular biological studies on *Heloderma* species, related to cloning of cDNAs encoding venom gland peptides, have required the sacrifice of specimens and library construction from excised tissue (Chen and Drucker, 1997;). Using the approach described here, a novel precursor cDNA encoding exendin-3 was obtained in a rapid manner and the propeptide convertase cleavage sites utilised would have been readily predictable

from translated cDNA data (Fig. 5B). Exendin-3 was N-terminally cleaved from its precursor protein at a classical—Lys-Arg—processing site immediately upstream of the N-terminal histidyl residue. Also, the penultimate glycyl residue in the open-reading frame is appropriately positioned to act as an amide donor for generation of the C-terminal prolylamide residue present on the mature peptide. An interesting finding in the present study was the extremely high degree of primary structural conservation between two open-reading frame amino acid sequences, with only two sites of amino acid residue substitutions observed, and also the very high degree of nucleotide sequence conservation. Both species (*H. horridum* and *H. suspectum*) have overlapping distributions in North America and in fact the close relationship of these species is reflected in zones of hybridization where both species occur sympatrically (Ast, 2001).

This simple technology described here in cloning precursor cDNAs from lyophilized venom is robust can been applied readily to other species of venomous animals. The discovery of venom cloning provides an elegant means of establishing a global functional genomic repository for amphibian and reptile venom gland research and other molecular applications, without interfering with already fragile native populations and ecosystems (Gibbons et al., 2000).

Acknowledgements

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