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Abbreviations used: SMP, starfish myorelaxant peptide; ACh, acetylcholine; TFA, trifluoroacetic acid; RP, reversed-phase; RT-qPCR, real-time quantitative polymerase chain reaction.

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Abstract

Neuropeptides that act as muscle relaxants have been identified in chordates and protostomian invertebrates but little is known about the molecular identity of neuropeptides that act as muscle relaxants in deuterostomian invertebrates (e.g. echinoderms) that are "evolutionary intermediates" of chordates and protostomes. Here we have used the apical muscle of the starfish *Patiria pectinifera* to assay for myorelaxants in extracts of this species. A hexadecapeptide with the amino acid sequence Phe-Gly-Lys-Gly-Gly-Ala-Tyr-Asp-Pro-Leu-Ser-Ala-Gly-Phe-Thr-Asp was identified and designated starfish myorelaxant peptide (SMP). Cloning and sequencing of a cDNA encoding the SMP precursor protein revealed that it comprises 12 copies of SMP as well as 3 peptides (7 copies in total) that are structurally related to SMP. Analysis of the expression of SMP precursor transcripts in *P. pectinifera* using qPCR revealed the highest expression in the radial nerve cords and lower expression levels in a range of neuromuscular tissues, including the apical muscle, tube feet and cardiac stomach. Consistent with these findings, SMP also caused relaxation of tube foot and cardiac stomach preparations. Furthermore, SMP caused relaxation of apical muscle preparations from another starfish species - *Asterias amurensis*. Collectively, these data indicate that SMP has a general physiological role as a muscle relaxant in starfish. Interestingly, comparison of the sequence of the SMP precursor with known neuropeptide precursors revealed that SMP belongs to a bilaterian family of neuropeptides that include molluscan pedal peptides (PP) and arthropodan orcokinins (OK). This is the first study to determine the function of a PP/OK-type peptide in a deuterostome.

Introduction

A variety of neuropeptides that act as smooth muscle relaxants in vertebrates have been identified, including calcitonin-gene related peptide (CGRP), adrenomedullin, corticotropin-releasing hormone (CRH), urocortin, vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase-activating peptide (PACAP) and peptide histidine isoleucine (PHI) (Kitamura *et al.* 1993; Brain *et al.* 1985; Williams *et al.* 1987; Schilling *et al.* 1998; Grider & Makhlouf 1986; Miyata *et al.* 1989; Robberecht *et al.* 1982). Likewise, studies on protostomian invertebrates such as insects have identified a number of myoinhibitory neuropeptides; for example, type-A allatostatins, type-B allatostatins or myoinhibitory peptides (MIPs) and myosupressins (Bendena *et al.* 1997; Blackburn *et al.* 1995; Holman *et al.* 1986). In the context of our understanding of the evolutionary history and relationships of neuropeptides in the animal kingdom (Mirabeau & Joly 2013), it appears that neuropeptides belonging to different families have been recruited to act as muscle relaxants in vertebrates and protostomes. It is of interest, therefore, to identify neuropeptides that act as muscle relaxants in animals that occupy an "intermediate" position with respect to the vertebrates and protostomes in animal phylogeny – the deuterostomian invertebrates, which include two chordate sub-phyla that are closely related to vertebrates (Urochordata and Cephalochordata) and the Ambulacraria (Hemichordata and Echinodermata) (Adoutte *et al.* 2000).

Nothing is known about the molecular identity of neuropeptides that act as muscle relaxants in hemichordates but neuropeptides that act as muscle relaxants have been identified in echinoderms – the SALMFamides. The prototypes for this family of neuropeptides were both identified in the starfish *Asterias rubens* and *Asterias forbesi* – S1 (GFNSALMF-NH_2) and S2 (SGPYSFNSGLTF-NH_2) (Elphick *et al.* 1991a; Elphick *et al.* 1991b). *In vitro* pharmacological tests with S1 and S2 revealed that both peptides cause relaxation of neuromuscular preparations from *A. rubens* – the cardiac stomach, tube feet and apical muscle – but with S2 more potent/effective than S1 (Elphick & Melarange 2001; Elphick *et al.* 1995; Melarange & Elphick 2003; Melarange *et al.* 1999). Subsequently, other members of the SALMFamide neuropeptide family were identified in other echinoderms (e.g. sea cucumbers) and these peptides were also found to act as muscle relaxants (Diaz-Miranda & Garcia-Arraras 1995; Ohtani *et al.* 2002).

It is unlikely that SALMFamides are the only family of neuropeptides that act as muscle relaxants in echinoderms, given the multitude of neuropeptides that have been found to act as muscle relaxants in vertebrates and protostomian invertebrates (see above). Therefore, here we set out to employ use of an *in vitro* muscle bioassay to screen for muscle relaxants in an echinoderm. The starfish species *Patiria pectinifera* was selected as a model system because it is widely distributed in the northern Pacific Ocean, and can be easily collected and transported as it found in shallow coastal waters. This species adapts well to artificial conditions in the laboratory and as a non-specialized predator and/or scavenger it can be fed on algae, detritus and small invertebrates.

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For these reasons, this species has been used in many scientific studies as a model organism for studying starfish physiology, and it is also of interest from both economic and environmental perspectives (Dan-Sohkawa *et al.* 1986; Davydov *et al.* 1990; Ikegami *et al.* 1967; Jo *et al.* 2013; Mita *et al.* 2009; Haraguchi *et al.* 2015). The apical muscle of *P. pectinifera* was selected as a bioassay because it can be easily dissected from the aboral body wall of the arms in this species. Furthermore, as highlighted above, previous studies have revealed that SALMFamides cause relaxation of the apical muscle from the starfish *A. rubens* (Melarange & Elphick 2003).

Here we report the isolation of a novel neuropeptide from *P. pectinifera* that causes relaxation of the apical muscle from this species - starfish myorelaxant peptide (SMP). A cDNA encoding the SMP precursor protein was cloned and sequenced, enabling investigation of its expression pattern in *P. pectinifera* and investigation of relationships with neuropeptides that have been identified in other echinoderms and other phyla.

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Methods

Animals

Live specimens of the starfish species *Patiria pectinifera* (Fig. 1A and B) and *Asterias amurensis* were collected at Cheongsapo of Busan, Korea, and maintained in a recirculating seawater system at 15 °C until use. The animals were fed once every three days with live manila clam, *Ruditapes philippinarum*. Live specimens of the starfish species *Asterias rubens* were collected at low tide from the Thanet coast of Kent in the UK, and maintained in a recirculating seawater system at 12 °C until use. The animals were fed weekly with live mussels (*Mytilus edulis*).

Peptide extraction

Starfish (*P. pectinifera*) were cut into pieces using scissors, soaked in 70% methanol and then heated in a double boiler for 5 min to denature proteins and inhibit proteolytic enzyme activity. The boiled sample was cooled on ice and then homogenized (PT10-35, Kinematica, Inc., Switzerland), followed by addition of glacial acetic acid to yield a final concentration of 5% acetic acid. The homogenate was then centrifuged (10,000 × g, 40 min, 4 °C). The pellet was re-extracted in 5% acetic acid with same extraction method. The supernatant was pooled and concentrated using a rotary evaporator. The concentrated solution was diluted with 10 volumes of ethanol and then the suspension was centrifuged (10,000 × g, 40 min, 4 °C) to remove the precipitate. The supernatant was evaporated to 100 mL, and then 100 mL of ethanol with 1.1 g sodium chloride was added to it. After centrifugation to remove precipitate, the supernatant was concentrated by evaporation, and 0.1 volume of 1 N hydrochloride was added. The precipitate was removed again by centrifugation (20,000 × g, 50 min, 4 °C) and the supernatant was applied to a C18 cartridge (Sep-pak C18; Waters Corp). The column was washed with 10% methanol/0.1% trifluoroacetic acid (TFA) and retained materials were then eluted with 60% methanol/0.1% TFA. The eluate was evaporated and its biological activity on the apical muscle of *P. pectinifera* was investigated, as described below in the methods section for *in vitro* bioassay and pharmacology.

Peptide purification

The 60% methanol elutate was applied to a cation-exchange column (CM-52, 2.5 cm × 30 cm, Whatman, Oregon, USA), and eluted with a linear gradient of 0.02 to 1.5 M ammonium acetate (pH 5.0) for 6 hours at a flow rate of 2.75 ml/min. Absorbance peaks were monitored at 254 nm (ISCO Model UA-6 detector, Nebraska, USA) and fractions were collected every 4 min. The bioactive fractions, which eluted between fraction numbers 40 to 45, were pooled and then subjected to reversed phase (RP)-HPLC (Vydac 218TP510 Protein & Peptide C18, 9.2 mm x

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250mm, USA). Elution was performed with a linear gradient of 0 to 60% acetonitrile/0.1% TFA at a flow rate of 3.0 ml/min for 120 min, and fractions were collected every 2 min.

Bioactive fractions eluted between 50 and 54 min with RP-HPLC and these were subjected to further purification steps using an anion-exchange column (TSKgel DEAE-5PW, 7.5 × 75 mm, Tosho) with a linear gradient of 0 to 0.5 M sodium chloride in 10 mM Tris-HCl (pH 9.2) at a flow rate of 0.5 ml/min for 100 min. A fraction that eluted with a concentration of about 0.1 M sodium chloride from the anion-exchange column caused relaxation of the apical muscle from *P. pectinifera*. This eluate was subjected to further RP-HPLC (Capcellpak C18, 4.6 × 250 mm, Shisheido). The absorbance peaks were recovered with a linear gradient of 15 to 30% acetonitrile/0.1% TFA at flow rate of 1 ml/min for 60 min. The bioactive peak was then subjected again to RP-HPLC using the same solvent gradient as in the previous RP-HPLC step but with a different column (Hypersil-BDS C18, 2 × 125 mm, HP). Finally, the active peak was applied to the same column as in the previous step but with an isocratic elution of 20% acetonitrile/0.1% TFA at a flow rate of 0.5 ml/min.

Structure determination and synthesis of peptides

To determine the molecular mass and amino acid sequence of the purified starfish myorelaxant peptide (SMP), it was analyzed using an automated N-terminal amino acid gas-phase sequencer (PSQ-1, Shimadzu) and a MALDI-TOF mass spectrometer (Voyager-DE^{TM} PRO spectrometer, Perseptive Biosystem). On the basis of the structural determination results, two peptides, with or without the carboxyl-terminus amidated, were automatically synthesized by a conventional solid-phase method with Fmoc-protected amino acids and coupling reagents, 1-hydroxybenzotriazole (HOBT) and *N*,*N*-diisopropylcarbodimide (DIPCI), using a peptide synthesizer (PSSM-8, Shimadzu) as described previously (Kim *et al.* 2015). Other neuropeptides, S1 (GFNSALMFamide), S2 (SGPYSFNSGLTFamide), FMRFamide, and FLRFamide were synthesized to enable comparison of their activities with that of the identified peptide.

In vitro bioassay and pharmacology

Three neuromuscular preparations, apical muscle, cardiac stomach and tube feet, were dissected from *P. pectinifera* for *in vitro* bioassay and pharmacology according to a slightly modified version of previously reported methods (Elphick et al. 1995; Melarange & Elphick 2003). Synthetic neuropeptides were also tested for bioactivity on apical muscle preparations from a different starfish species - *A. amurensis*. Briefly, the apical muscle was cut from the aboral body wall of an arm, where the apical muscle forms a thickening of longitudinally orientated muscle that runs along the mid-line of the inner side (Fig. 1C). A piece of cardiac stomach between the oral opening and extrinsic retractor strand was obtained by removing the aboral body wall from the

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central disk and the proximal region. An individual whole tube foot was dissected from the arm ambulacra but without the ampulla. All muscle preparations were cut to approximately 10 mm, and both ends of the muscle preparations were tied with cotton threads. The preparations were then suspended vertically in a 2 ml polypropylene chamber containing artificial seawater (ASW) with aeration, one end being connected to silver hook on the bottom of the chamber and the other to a force displacement transducer (Type 45196A, NEC-Sanei Instrument Ltd., Tokyo, Japan). Output from the force displacement transducer was monitored by a recorder (WR7300, GRAPHTEC CORP., Yokohama, Japan) via an amplifier (AS1302, NEC-Sanei, Tokyo, Japan), which recorded the mechanical responses of the device. Prior to testing, the muscle preparations were allowed to stabilize for about 90 min. The resting tension was then adjusted to 1.0 g for apical muscle and 0.5 g for cardiac stomach and tube foot. Muscles in the chamber were allowed to equilibrate for about 30 min in ASW, during which time the ASW in the chamber was freshly replaced every 15 min. Pre-contraction of apical muscle, cardiac stomach or tube foot preparations was induced by applying 1 μM acetylcholine (ACh), 10 μM carbachol or 30 mM high-potassium ASW, respectively. Then immediately after equilibration, the muscles were treated with test samples to measure relaxation responses.

The bioassay system adopted for monitoring purification of the bioactive peptide was a system that measures relaxation of apical muscle from *P. pectinifera* pre-contracted for 2 min at 20 min intervals with 1 μM ACh. An aliquot of each test fraction was evaporated to dryness, dissolved with 50 μl of phosphate buffer saline (PBS), and added into the chamber.

At least 4 separate experiments to test the pharmacological activities of synthetic SMP, C-terminally-amidated SMP (SMPamide) and other neuropeptides were performed, using a concentration range of 10^{-10} M to 10^{-5} or 10^{-4} M at room temperature. EC_{50} values represent the concentration of peptide required to cause a response 50% of the maximum. The maximal response (E_{max}) was expressed as the percentage of the maximal relaxation induced by 10^{-4} or 10^{-5} M of each peptide compared to the maximal contraction of apical muscle by 1 μM ACh, of cardiac stomach by 10 μM carbacol or of tube foot by 30 mM high-potassium ASW. The relative activity was calculated as the ratio of the concentration of SMP or other peptides required to produce responses equivalent to a half-maximal response.

cDNA cloning and sequence analysis

Total RNA was extracted using RNeasy Mini kit (Qiagen, USA) from total tissues (except body wall) of *P. pectinifera*, and then mRNA was purified using Oligotex mRNA mini kit (Qiagen, USA) following the manufacturer's instructions. The synthesis rapid amplification cDNA end (RACE)-ready cDNA template was performed with SMARTer™ RACE cDNA amplification Kit (Clontech, UK) according to manufacturer's instructions.

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Based on the amino acid sequence of the purified peptide, two degenerate primers were designed for 3' RACE PCR, and then 5' RACE PCRs were conducted with sequence-specific primers designed from the sequencing result of the 3' RACE product. The sequences of primers used in RACE are listed in Table S1. The first PCR conditions for 3' RACE included initial denaturation at 94 °C for 3 min followed by: 5 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; 5 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min; 20 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Nested PCR for 3'RACE was performed with the same conditions as the first PCR. The first PCR product of 5' RACE was obtained by the following thermal cycle profile: 5 cycles of 94 °C for 30 sec, 67 °C for 30 sec, and 72 °C for 1 min; 5 cycles of 94 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 1 min; 25 cycles of 94 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 1 min. Nested PCR for 5' RACE was as follows: 5 cycles of 94 °C for 30 sec, 69 °C for 30 sec, and 72 °C for 1 min; 5 cycles of 94 °C for 30 sec, 67 °C for 30 sec, and 72 °C for 1 min; 25 cycles of 94 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 1 min.

PCR products in the last step of 3' and 5' RACE were introduced into the pGEM-Teasy vector system (Promega Corporation, USA) and sequenced. The full-length translated sequence of the SMP precursor, based on the cloned cDNA nucleotide sequence, was aligned by BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) and the sequence was submitted to the GenBank database (Accession number: KT870152). Multiple sequence alignment of the full-length *P. pectinifera* SMP precursor and related proteins in other species was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Asterias rubens radial nerve cord transcriptome sequence data obtained by Illumina HiSeq sequencing, as reported previously (Semmens *et al.* 2013), was analysed using BLAST to identify a homolog of the *P. pectinifera* SMP precursor. Using the sequence of the *P. pectinifera* SMP precursor as a query, a 444 bp *A. rubens* contig (1025452) comprising a partial sequence corresponding to the 3' region of the *P. pectinifera* SMP precursor cDNA was identified. Then ovarian transcriptome sequence data obtained from multiple echinoderm species ((Reich *et al.* 2015); http://www.echinobase.org/Echinobase/Blasts) was analysed and non-overlapping contigs encoding the 5' region (GAUS01027726.1) and the 3' region (GAUS01027727.1) of a SMP-type precursor transcript was identified from the starfish species *Asterias forbesi.* Combining these partial sequence data from *A. rubens* and *A. forbesi*, primers were designed to enable PCR amplification of the full-length SMP precursor coding sequence from *A. rubens*, as described below.

Total RNA was extracted from radial nerve cords of *A. rubens* using the SV Total RNA Isolation System according to the manufacturer's instructions (Promega). Then cDNA was synthesized using the QuantiTect Rev. Transcription Kit in accordance with the manufacturer's instructions (QIAgen). A cDNA containing the coding sequence of the *A. rubens* SMP-type precursor was amplified by PCR using Phusion high-fidelity PCR master mix (NEB) and the oligo

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primers 5'-ATGCGGCTCATCATGCAC-3' and 5'-TACACACCAAGCAGTGACA-3'. The conditions for PCR included initial denaturation at 98 °C for 2 min followed by: 30 cycles of 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 1 min, 72 °C for 8 min and hold at 4 °C. 1% gel electrophoresis was performed to analyse the PCR products and then the PCR product was gel-extracted and purified using a QIAquick gel extraction kit (QIAgen). Zero Blunt TOPO PCR cloning kit (Invitrogen) was used to ligate the PCR product into the pCR-Blunt II with TOPO vector for sequencing. The sequence obtained (GenBank accession number KT870153) was translated into protein sequence using ExPASy (http://web.expasy.org/translate/) and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide of the translated protein sequence.

Real time-quantitative PCR (RT-qPCR) analysis

To quantitatively analyse expression of SMP precursor transcripts in different starfish tissues/organs, RT-qPCR was employed using a LightCycler 480 Real-Time PCR System (Roche, Germany) with LightCycler 480 SYBR green master I (Roche, Germany). Total RNA extracted from the apical muscle, radial nerve cord, cardiac stomach, pyloric stomach, coelomic lining containing transverse muscles, tube feet, pyloric caecae, testis, and ovary were obtained from five specimens of *P. pectinifera*. cDNA was synthesized using the TOPscript cDNA synthesis Kit with oligo dT (dT18) (Enzynomics, Korea) according to the manufacturer's instructions. The primer pairs used for amplifying SMP precursor cDNA and elongation factor 1α (EF1α) cDNA as a control for normalization were SMP RT-F and SMP RT-R, and EF1α RT-F and EF1α RT-R, respectively (see Table S1 for sequences). Based on the standard curves for both SMP and EF1α, the relative expression levels of SMP transcripts in each tissue were normalized against the level of the EF1α control using the following formula: relative expression = $[(1 + E_{SMP})^{CP\_SMP}]^{-1}/[(1 + E_{EF1\alpha})^{CP\_EF1\alpha}]^{-1}$, in which E is PCR efficiency ($E = 10^{-1/slope} - 1$) and CP is the threshold cycle number. Triplicate amplifications were carried out independently, and the relative quantification results were expressed as the fold levels of SMP precursor transcripts. Statistical analysis of the data for comparison between tissues was carried out by one-way ANOVA, followed by Duncan's Multiple Range test, using the SPSS 21 program. P values with $p < 0.05$ were considered statistically significant.

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Results

Purification of a novel hexadecapeptide that relaxes the apical muscle of *P. pectinifera*

A whole-body extract of *P. pectinifera* induced relaxation of apical muscle pre-contracted with ACh (Fig. 1D), indicating that it was an appropriate source to isolate myorelaxants. A single absorbance peak (peak A) containing a myorelaxant was successfully purified from the whole-body extract through six steps of column purification which sequentially were cation, repeated RP, and anion HPLC. Finally, peak A was isocratically eluted with 20% acetonitrile/0.1% TFA at 16.1 min of the retention time (Fig. 2A). An aliquot of peak A relaxed the apical muscle of *P. pectinifera* that was pre-contracted by ACh (Fig. 2B). Purified peak A was identified as a sixteen-residue peptide with the sequence Phe-Gly-Lys-Gly-Gly-Ala-Tyr-Asp-Pro-Leu-Ser-Ala-Gly-Phe-Thr-Asp with a free carboxy terminus based on N-terminal amino acid sequencing and molecular mass analysis (Fig. 2C and Figure S1A). The purified hexadecapeptide was designated starfish myorelaxant peptide (SMP). To confirm the primary structure and chemical properties of SMP under RP-HPLC, SMP with a free carboxy terminus (SMP) and SMP with an amidated carboxy terminus (SMPamide) were synthesized. The synthetic SMP and native SMP eluted with an identical retention time, and a mixture of the two peptides eluted as a single peak under RP-HPLC (Fig. 2D). Moreover, SMP and SMPamide did not have an identical retention time on RP-HPLC (Figure S1B). Collectively, the results demonstrate that the purified SMP is the hexdecapeptide Phe-Gly-Lys-Gly-Gly-Ala-Tyr-Asp-Pro-Leu-Ser-Ala-Gly-Phe-Thr-Asp-OH, with a free carboxy terminus and without any post-translational modifications.

SMP is a potent relaxant of *P. pectinifera* apical muscle *in vitro*

SMP and a C-terminally amidated analog of SMP (SMPamide) both caused dose-dependent relaxation of *in vitro* apical muscle preparations from *P. pectinifera* (Fig. 2E). The threshold response, ED_{50} and E_{max} for SMP were 10^{-10} M, 6.0×10^{-8} M and 120.02±7.00 % and for SMPamide were 10^{-10} M and 4.0×10^{-8} M and 134.69±9.57 %, respectively (Fig. 2E). These results corroborate the structural determination that SMP is not C-terminally amidated and does not required C-terminal modification for its bioactivity. Comparison of the bioactivity of SMP with the starfish SALMFamide neuropeptides S1 and S2 revealed that SMP was more potent/efficacious than these peptides as a relaxant of the apical muscle from *P. pectinifera* (Fig. 2E). Thus, the E_{max} for S1 and S2 at a concentration of 10^{-5} M were only 44.15±2.41 and 29.72±8.29, respectively. Furthermore, the molluscan neuropeptides FLRFamide and FMRFamide, which share some sequence similarity with SALMFamides, exhibited little or no bioactivity as relaxants of apical muscle preparations, even at concentrations as high as 10^{-5} M (Fig. 2E).

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The *P. pectinifera* SMP precursor protein comprises twelve copies of SMP and seven copies of other SMP-like peptides

A cDNA encoding the *P. pectinifera* SMP precursor was cloned and sequenced (GenBank accession number: KT870152) and the nucleotide sequence and the deduced protein sequence are shown in Fig. 3. The cDNA sequence comprised 1682 bp, starting with a 5' untranslated region (UTR) of 148 bp, followed by an open reading frame (ORF) of 1281 bp, a 3' UTR of 253 bp including a poly-A tail. The ORF of the SMP precursor encodes a 426 amino acid residue protein. that contains four regions: a signal peptide (Met^{1}-Ala^{19}), as predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), a N-terminal spacer peptide (Ser^{20}-Arg^{56}) containing several acidic amino acids, a region containing twelve copies of SMP and seven copies of SMP-like peptides with each peptide copy bounded by dibasic cleavage sites (Phe^{57}-Arg^{402}) and a C-terminal region (Thr^{403}-Arg^{426}).

SMP transcripts are widely expressed in *P. pectinifera* and SMP causes in vitro relaxation of other muscle preparations from *P. pectinifera*

The relative expression levels of the SMP precursor mRNA in different tissues (apical muscle, radial nerve cord, cardiac stomach, pyloric stomach, coelomic lining, tube feet, pyloric caecae, testis, and ovary) were determined by RT-qPCR (Fig. 4A). The highest expression of SMP transcripts was detected in radial nerve cords, which are the major components of the nervous system in starfish. In addition, relatively high expression levels of SMP transcripts were observed, in descending order, in apical muscle, tube feet, coelomic lining, cardiac stomach, pyloric stomach and pyloric caecae. However, expression of SMP transcripts in reproductive organs (ovary and testis) was barely detectable. These findings indicate that SMP is a neuropeptide and suggest that SMP may have widespread roles as a regulator of muscle activity in *P. pectinifera*. To address this issue, SMP was tested *in vitro* on two other neuromuscular preparations in which SMP transcripts are detected –cardiac stomach and tube feet. SMP caused dose-dependent relaxation of both preparations and, as with apical muscle preparations, SMP was more potent/effective as a muscle relaxant than the SALMFamides S1 and S2 (Fig. 4B, C).

SMP causes relaxation of apical muscle preparations from the starfish *Asterias amurensis* and identification of an SMP-type precursor in *Asterias rubens*

Having identified SMP as a muscle relaxant in *P. pectinifera*, we then investigated if this peptide also acts as a muscle relaxant in other starfish species. To address this issue we tested synthetic SMP on apical muscle preparations from *A. amurensis*. SMP caused dose-dependent relaxation and the E_{max} was 82.1±1.94 % at a concentration of 10^{-5} M (Fig. 5A). Previous studies have shown that the SALMFamide neuropeptides S1 and S2 cause relaxation of apical muscle

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preparations from *Asterias rubens*, which is closely related to *A. amurensis* (Melarange & Elphick 2003). Therefore, we compared the bioactivity of SMP with S1 and S2 and found that the E_{max} for S1 and S2 at a concentration of 10^{-5} M were less than for SMP, 62.3±4.4 and 38.7±1.21, respectively (Fig. 5A). However, by comparison with S1 and S2, SMP was less effective as a relaxant of the apical muscle from *A. amurensis* (Fig. 5A) than from *P. pectinifera* (Fig. 2E). These findings indicate that SMP or a related peptide(s) exists in *A. amurensis* and that SMP-type peptides act as muscle relaxants throughout the Asteroidea. Accordingly, a cDNA encoding an SMP-type precursor was identified in *A. rubens*, comprising a 224-residue protein with a predicted 21-residue signal peptide and eight copies of putative SMP-like peptides: four copies of the peptide FGGKGAFDPLSAGFTD, two copies of the peptide FGGSRGAFDPLSAGFTD and one copy each of GFGMGAYDPLSAGFTD and SFVHGDFDPLSTGFVDGD (Fig. 5B and Figure S2, GenBank Accession number: KT870153). It is noteworthy that the C-terminal region of three of these peptides (DPLSAGFTD) is identical to the corresponding region of *P. pectinifera* SMP.

Starfish SMP precursors are homologs of neuropeptide precursors that have been identified in other echinoderms

To investigate relationships with neuropeptide precursors that have been in other animals, the *P. pectinifera* and *A. rubens* SMP precursor proteins were submitted as queries against the GenBank nr database using BLAST. The top two hits (XP_785647.1 and XP_003727926) were identified neuropeptide precursor proteins that have been described previously from the sea urchin *Strongylocentrotus purpuratus* and designated as Spnp6 and Spnp7, respectively (Rowe & Elphick 2012). In Fig. 6, we show a multiple sequence alignment of the *P. pectinifera* SMP precursor, the *A. rubens* SMP-type precursor, Spnp6, Spnp7 and a homolog of Spnp7 that has been identified in the sea cucumber *A. japonicus* (Ajnp7, (Rowe *et al.* 2014)). Furthermore, alignment of the putative neuropeptides derived from the *P. pectinifera* SMP precursor, the *A. rubens* SMP-type precursor, Spnp6, Spnp7 and Ajnp7 (Fig. 7) reveals that the peptides have a number of features in common. These include two phenylalanine residues located at or near the N- and C-termini of the peptides as well as a conserved core region with the motif (D/E)-(P)-(L/M), structural characteristics that may be important for the bioactivity of these peptides.

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Discussion

Here we have isolated a novel hexadecapeptide (FGKGGAYDPLSAGFTD) from starfish that acts as a muscle relaxant and which we have designated as starfish myorelaxant peptide or SMP. Previous studies have identified the SALMFamide neuropeptides S1 and S2 as muscle relaxants in starfish (Melarange & Elphick 2003; Melarange et al. 1999) and here the bioactivity of SMP, S1 and S2 as muscle relaxants were compared. When tested on three preparations from *P. pectinifera* (apical muscle, cardiac stomach and tube feet), SMP was more effective/potent than S1 or S2. This finding is likely to be physiologically relevant with respect to S1 because we know that S1 occurs in the closely related species *Patiria miniata*. However, *P. miniata* does not contain S2 and this species has instead an S2-like peptide (Elphick *et al.* 2013; Elphick *et al.* 2015). Therefore, the inferior bioactivity of S2 as a myorelaxant in *P. pectinifera* may in part be attributable to differences in peptide structure. Furthermore, analysis of the sequences of the two SALMFamide precursor proteins in *P. miniata* reveals that they comprise S1, the S2-like peptide and fourteen other SALMFamide-type peptides (Elphick et al. 2015; Elphick et al. 2013). So comparison of the effects of SMP with S1 or S2 tested in isolation does not reflect the physiological occurrence of "cocktails" of SALMFamides. Nevertheless, the superior bioactivity of SMP as a myorelaxant, compared to S1 and S2, in tests on muscle preparations from both *P. pectinifera* and *Asterias amurensis* clearly indicates that SMP is a physiologically important regulator of muscle relaxation in starfish.

Analysis of the distribution of the expression of the SMP precursor in *P. pectinifera* using qPCR revealed a widespread pattern of expression, including all three neuromuscular preparations that SMP causes relaxation of *in vitro* – the apical muscle, cardiac stomach and tube feet. Likewise, immunocytochemical- and radioimmunoassay-based analysis of the distribution of S1 and S2 in *A. rubens* reveals a widespread pattern of expression (Elphick et al. 1995; Moore & Thorndyke 1993; Newman *et al.* 1995b; Newman *et al.* 1995a). Therefore, it is likely that SMP and SALMFamide neuropeptides act in concert as muscle relaxants to regulate a variety of physiological processes in starfish. For example, relaxing effects on the apical muscle *in vivo* may be associated with neural mechanisms that control changes in body posture, whereas relaxing effects on tube feet *in vivo* may be associated with locomotor activity. The relaxing action of SALMFamides on the cardiac stomach is thought be relevant to neural mechanisms controlling stomach eversion during feeding in starfish (Melarange et al. 1999) and this role may equally apply to the novel SMP neuropeptide identified here. Further insights into the physiological roles of SMP and other SMP-like peptides derived from the same precursor protein may be obtained by analysis of the distribution of these peptides at the cellular level. As highlighted above, detailed immunocytochemical analyses of the distribution of S1 and S2 in *A. rubens* have been reported previously (Elphick et al. 1995; Moore &

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Thorndyke 1993; Newman et al. 1995b; Newman et al. 1995a) and it would be interesting to compare the distribution of SMP and SALMFamides using this approach.

Comparative analysis of the sequence of SMP and the SMP precursor with neuropeptides and neuropeptide precursors that have been identified in other animals has revealed that SMP belongs to a bilaterian family of neuropeptides that includes molluscan pedal peptides (PP) and arthropodan orcokinins (OK). The occurrence of PP/OK-type peptides in echinoderms has been reported previously based on analysis of genome/transcriptome sequence data (Rowe & Elphick 2012; Rowe et al. 2014) and in Fig. 6 and Fig. 7, respectively, we show alignments of SMP and the SMP precursor with related PP/OK-type neuropeptides and precursor proteins that have been identified in the sea urchin *S. purpuratus* and the sea cucumber *A. japonicus*. These alignments reveal conserved residues that may be important for the bioactivity of PP/OK-type peptides in echinoderms. In Fig. 8 we show an alignment of SMP and other representative echinoderm PP/OK-type peptides with molluscan pedal peptides and arthropodan orcokinins. What this reveals is the conservation of hydrophobic residues, which are typically phenylalanine, proximal to or at the N- and C-termini of the peptides. This suggests that these evolutionarily conserved structural features are important for the bioactivity of PP/OK-type peptides in the Bilateria. However, the motif (D/E)-(P)-(L/M) that is a conserved feature of the core of echinoderm PP/OK-type peptides, including SMP (Fig. 7), is not seen in molluscan and arthropodan PP/OK-type peptides and therefore this may be a unique characteristic of echinoderm representatives of this neuropeptide family.

With the identification of SMP as a member of the PP/OK-type family of neuropeptides, it is of interest to consider what is known about the physiological roles of these neuropeptides in other phyla. PP was originally discovered in the mollusc *A. californica* as a peptide that causes contraction of pedal muscles (Lloyd & Connolly 1989; Hall & Lloyd 1990); it also stimulates beating of cilia associated with the foot (Longley & Peterman 2013). OK was first isolated from neural extracts of the crayfish *Orconectus limosus* on account of its stimulatory effect on hindgut activity (Stangier *et al.* 1992). Subsequently, OK-type peptides have been identified in several arthropod species and found to have a variety of effects, including stimulation of the prothoracic gland and regulation of ecdysteroidogenesis in the silk moth *Bombyx mori* (Yamanaka *et al.* 2011) and regulation of circadian activity in the cockroach *Leucophaea maderae* (Hofer & Homberg 2006; Soehler *et al.* 2011; Wei & Stengl 2011). Thus, in both molluscs and arthropods, PP/OK-type neuropeptides have stimulatory effects on the activity of muscle and other tissues. This contrasts with the inhibitory effect that SMP has in causing relaxation of muscle in starfish, as reported here. It will be interesting, therefore, to investigate in future studies if PP/OK-type peptides also act as muscle relaxants in other echinoderms or if this is a unique characteristic of PP/OK-type peptides in starfish.

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Thus far, PP/OK-type peptides have not been identified in other deuterostomian phyla such as hemichordates, which are a sister clade to the echinoderms, or chordates. One possibility is that PP/OK-type peptides have been lost in hemichordates and chordates and the echinoderms are unique amongst the deuterostomes in retaining peptides belonging to this bilaterian neuropeptide family. Alternatively, the possibility remains that members of this neuropeptide family exist in hemichordates and chordates but their relationship with PP/OK-type peptides has not been observed due to sequence divergence. Addressing this issue would be facilitated if the receptors that mediate the effects of PP/OK-type peptides in echinoderms or in protostomes were identified, and therefore this represents an important objective for future research on PP/OK-type peptides. At the outset of this paper, we highlighted the variety of types of neuropeptides that have been identified as muscle relaxants in mammals and other vertebrates. Our discovery of SMP as a novel muscle relaxant in a deuterostomian invertebrate may provide a basis for discovery of evolutionarily related neuropeptides in vertebrates, with potential biomedical applications in humans.

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Figure legends

Fig. 1 The starfish *Patiria pectinifera*. The aboral side (A) and oral side (B) of an intact animal are illustrated. The position of the apical muscles on the inner surface of the aboral body wall of a dissected animal is shown in (C). An extract of *P. pectinifera* containing peptidic materials relaxed apical muscle that was pre-contracted with 1 μM acetylcholine (ACh); up and down arrows represent application of ACh and the extract, respectively (D).

Fig. 2 Isolation, structure determination, and pharmacology of purified peptide. Peak A was isocratically eluted with 20 % acetonitrile/0.1 % TFA on RP-HPLC (A), and an aliquot of purified peak A caused relaxation of the apical muscle (B). Purified peak A was identified as a peptide comprised of sixteen amino acid residues with a molecular mass of 1601.72 Da, which we have named starfish myorelaxant peptide or SMP (C). Comparison of chromatographic properties of native SMP (N) and synthetic SMP (S) on RP-HPLC showed that native SMP and synthetic SMP with a free carboxy terminal have identical retention times on RP-HPLC (D). The concentration-dependent relaxing activity of SMP on the apical muscle of *P. pectinifera*. SMP with free carboxyl terminus and amidated carboxy terminus is SMP (●) and SMPamide (○), respectively. The effects of S1 (▲) and S2 (△) from the starfish *A. rubens* and the molluscan neuropeptides FLRFamide (■) and FMRFamide (□) are shown to compare their activity with SMP. Each point represents the mean ± standard deviation determined from four separate experiments. The percentage relaxing activity was calculated by comparing each relaxation effect to the maximal contraction of the apical muscle by 1 μM ACh (D).

Fig. 3 Precursor of starfish myorelaxant peptide (SMP) in *Patiria pectinifera*. The DNA sequence of a transcript (lowercase, 1682 bases) encoding the *P. pectinifera* SMP precursor (uppercase, 426 amino acid residues) is shown. The predicted signal peptide, the purified mature SMP (SMP_a), and three other variants (SMP_b, [Met^3]-SMP_a; SMP_c, [Met^3, Glu^{16}]-SMP_a; SMP_d, SMP_a-related octadecapeptide) are shown in blue, red, pink, orange and purple, respectively, and putative dibasic cleavage sites (KR) are shown in green. The asterisk shows the position of the stop codon. The SMP precursor protein comprises twelve copies of SMP and seven copies of SMP-like peptides.

Fig. 4 The expression levels for the SMP precursor transcript in various organs/tissues from *P. pectinifera* and the pharmacological effects of SMP on cardiac stomach and tube foot from *P. pectinifera*. Relative expression levels of SMP transcripts in each organ/tissue were normalized against the level of the EF1α gene as an internal control. Means and standard deviations (n=3) are shown. Bars with different letters indicate statistically significant differences between tissues

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($p < 0.05$) determined by one-way ANOVA followed by Duncan's Multiple Range test (A). SMP caused concentration-dependent relaxation of the cardiac stomach (B) and tube foot (C) from *P. pectinifera*. The relaxing activity of SMP (●) was compared with S1 (▲) and S2 (△). Each point represents the mean ± standard deviation determined from four separate experiments. The percentage relaxing activity was calculated by comparing each relaxation effect to the maximal contraction of cardiac stomach caused by 10 μM carbacol and of tube foot caused by 30 mM high-potassium ASW, respectively.

Fig. 5 Pharmacological effect of SMP on apical muscle from *Asterias amunensis* and identification of an SMP-type precursor in *Asterias rubens*. (A). The concentration-dependent relaxing activity of SMP (●) compared with S1 (▲) and S2 (△) on the apical muscle of *A. amurensis*. Each point represents the mean ± standard deviation determined from four separate experiments. The percentage relaxing activity was calculated by comparing each relaxation effect to the maximal contraction of apical muscle caused by 1 μM ACh. (B) Amino acid sequence of a 224-residue SMP-type precursor protein identified in *A. rubens*, which comprises a predicted 21-residue signal peptide (blue) and eight copies of putative SMP-like peptides (red) and putative dibasic cleavage sites (KR, green). The sequence of the cDNA encoding this protein is shown in Supplementary Figure 2.

Fig. 6 Multiple sequence alignment of the *P. pectinifera* SMP precursor with related neuropeptide precursors in other echinoderms. Highlighted red, green, blue, yellow and purple boxes represent multiple copies of neuropeptides separated by putative cleavage sites (KR or KK). All of the precursors contain multiple copies of related peptides: *P. pectinifera* SMP precursor contains twelve copies of SMP and seven copies of SMP-like peptides; *A. rubens* SMP precursor contains eight copies of SMP-like peptides; *S. purpuratus* neuropeptide precursor 6 (Spnp6) contains twenty one copies of nine SMP-like peptides; Spnp7 precursor contains ten copies of nine SMP-like peptides; *A. japonicus* neuropeptide precursor 7 (Ajnp7) contains six copies of five SMP-like peptides. The sequences of Spnp6, Spnp7 and Ajnp7 are from (Rowe & Elphick 2012; Rowe et al. 2014).

Fig. 7 Alignment of SMP (SMP_a) with putative SMP-like neuropeptides derived from echinoderm SMP-type precursors: the starfish *P. pectinifera* and *A. rubens*; sea urchin *S. purpuratus*; sea cucumber *A. japonicus*. Conserved residues are highlighted in black and grey.

Fig. 8 Alignment of echinoderm SMP-type peptides with protostomian pedal peptide (PP)/orcokinin(OK)-type peptides. The basic amino acids Lys, Arg, and His are shown in the black

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with light grey highlighting, and the acidic residues Glu and Asp are shown in black with dark grey highlighting. All other amino acids are classified as hydrophobic (white with light grey highlighting) or hydrophilic (white with dark grey highlighting). Lower case "a" denotes a C-terminal amide group. Species abbreviations and references: Pp, *P. pectinifera;* Ar, *A. rubens*; Sp, *S. purpuratus* (Reich *et al.* 2015); Aj, *A. japonicas* (Rowe & Elphick 2012; Du *et al.* 2012); Ac, *Aplysia californica* (Moroz *et al.* 2006); Pd, *Platynereis dumerilii* (Conzlmann *et al.* 2011); Ce, *Caenorhabditis elegans*; Pc, *Procambrus clarkia* (Yasuda-Kamatani & Yasuda 2000); Nv, *Nasonia vitripennis*.

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Supporting information

Supplementary Table legend

Table S1 Primers used for RACE and RT-qPCR analysis of SMP precursor expression in *P. pectinifera*

Supplementary Figure legends

Figure S1 MALDI-TOF mass spectrum of purified peak A (A). Comparison of the chromatographic properties of synthetic SMP with a free carboxyl terminus (SMP) and SMP with an amidated carboxyl terminus (SMPamide) on RP-HPLC reveals that the two peptides elute at different retention times with isocratic 20% acetonitrile/0.1 % TFA (B).

Figure S2 Precursor of starfish myoactive peptide (SMP)-type neuropeptides in *Asterias rubens*. The DNA sequence of a transcript (lowercase, 948 bases) encoding the *A. rubens* SMP-type precursor (uppercase, 224 amino acid residues) is shown. The sequences that were used to design primers for PCR amplification of the cDNA are underlined. The predicted signal peptide of the precursor protein is shown in blue and the eight putative mature neuropeptides are shown in red. Putative dibasic cleavage sites (KR) are shown in green and the asterisk shows the position of the stop codon.

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Table S1 Primers used for RACE and RT-qPCR analysis of SMP precursor expression in *P. pectinifera*

Primers	Sequence (5'-3')	Amplification
SMP-3F	TTYGGNAARGGNGGNGCNTAYGA	3'RACE
SMP-3nF	GNGCNTAYGAYCCNYTNWSNGCNG	3'nested RACE
SMP-5R	AGAATCGTCAATGGAAGTGTTCATATAGTCAGTGG	5'RACE
SMP-5nR	GGAGTTGGTATTGTCTGGATCTTCTTATCGG	5'nested RACE
SMP RT-F	GGT TTC CTA CAC GGA CCA GAC	RT-qPCR
SMP RT-R	CCA CAA GGT GAC GGA AAG GG	
EF-1α RT-F	TCA ACG ACT ACC AGC CCC TA	RT-qPCR
EF-1α RT-R	TTC TTG CTA GCC TTC TGG GC	

For Peer Review

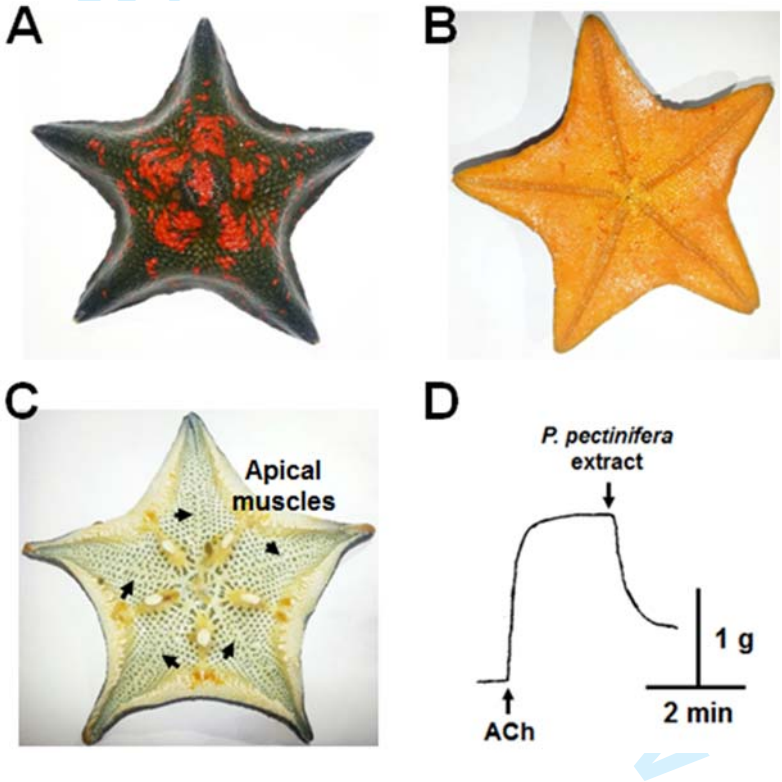
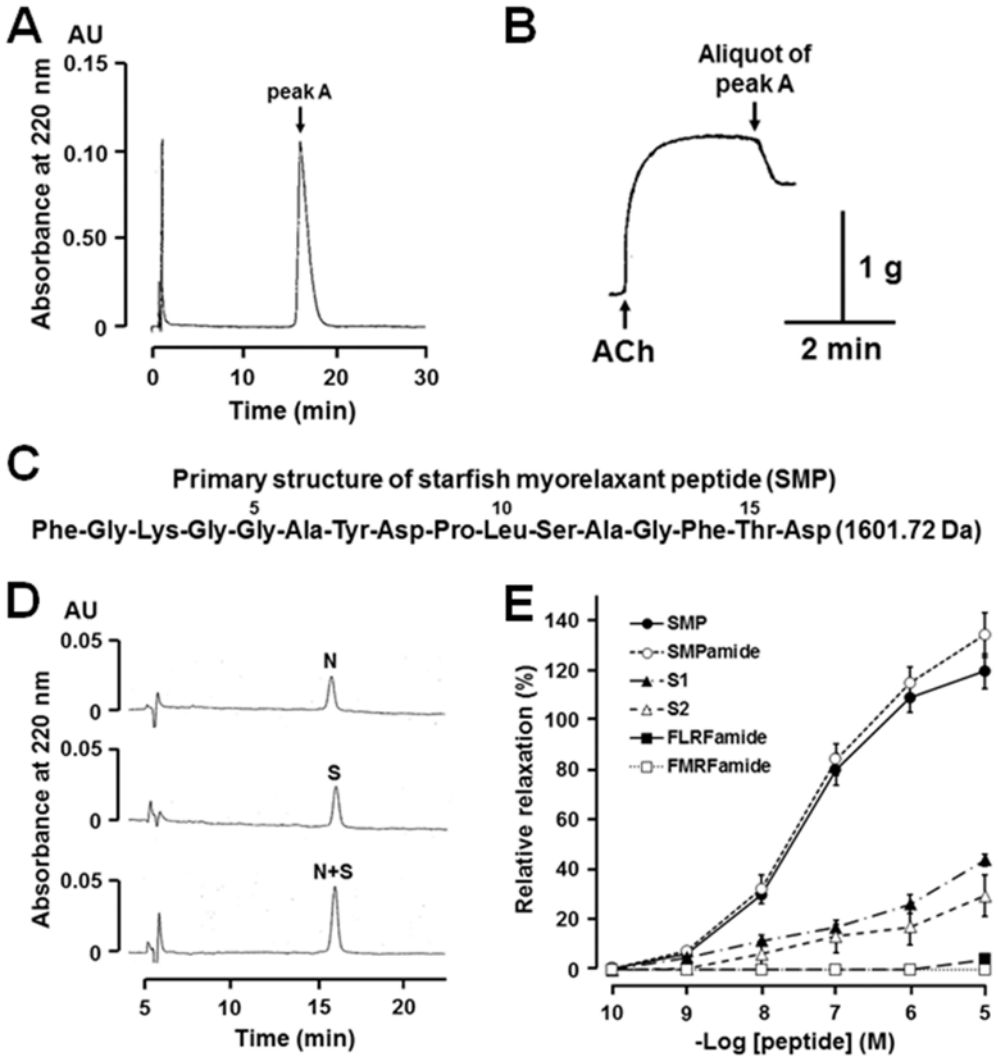


Fig. 1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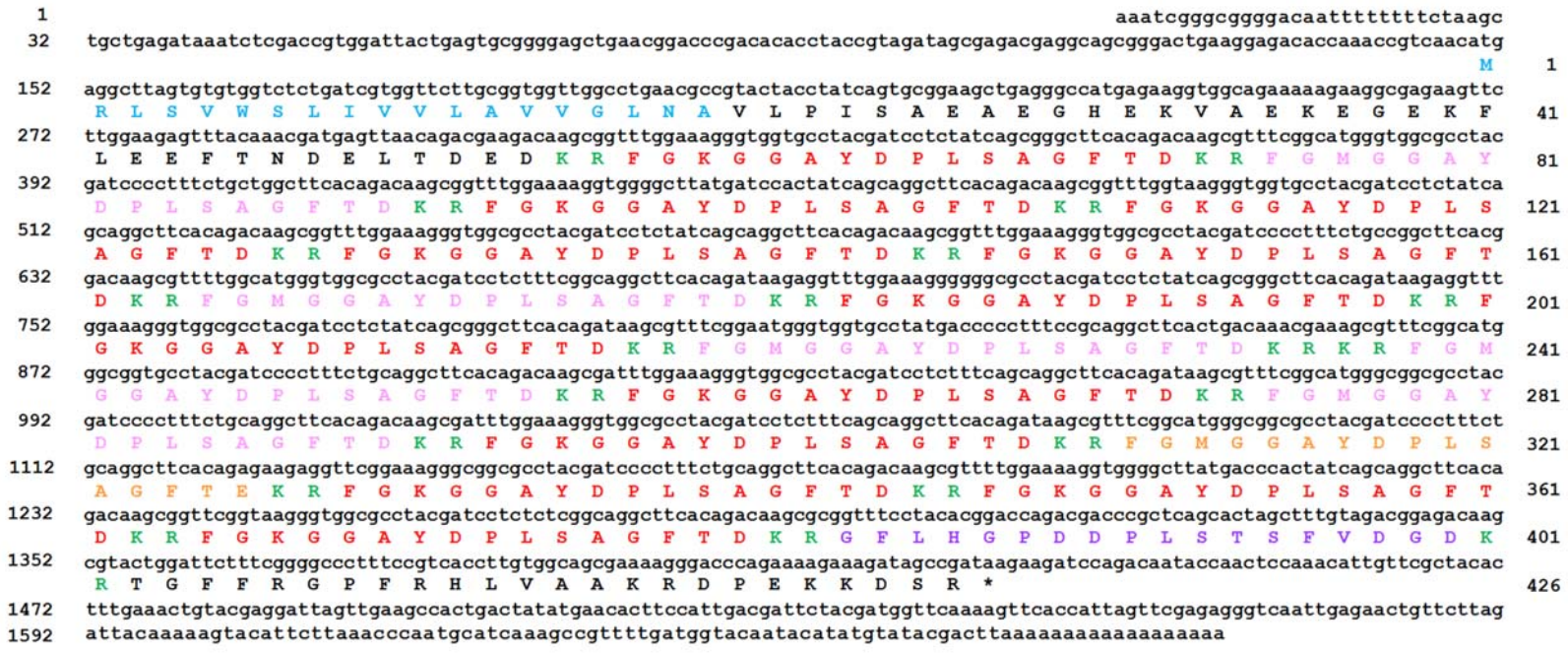
AU
0.15
0.10
0.50
0
Absorbance at 220 nm
peak A
0
10
20
30
Time (min)
B
Aliquot of peak A
1 g
ACh
2 min
C
Primary structure of starfish myorelaxant peptide (SMP)
Phe-Gly-Lys-Gly-Gly-Ala-Tyr-Asp-Pro-Leu-Ser-Ala-Gly-Phe-Thr-Asp (1601.72 Da)
5
10
15
D
AU
0.05
0
N
S
N+S
Absorbance at 220 nm
5
10
15
20
Time (min)
E
140
120
100
80
60
40
20
0
Relative relaxation (%)
SMP
SMPamide
S1
S2
FLRFamide
FMRFamide
10
9
8
7
6
5
-Log [peptide] (M)

Fig. 2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M 1
152 aggcttagtgtgtggtctctgatcgtggttcttgcggtggttggcctgaacgccgtactacctatcagtgcggaagctgagggccatgagaaggtggcagaaaaagaaggcgagaagttc
R L S V W S L I V V L A V V G L N A V L P I S A E A E G H E K V A E K E G E K F 41
272 ttggaagagtttacaaacgatgagttaacagacgaagacaagcggtttggaaagggtggtgcctacgatcctctatcagcgggcttcacagacaagcgtttcggcatgggtggcgcctac
L E E F T N D E L T D E D K R F G K G G A Y D P L S A G F T D K R F G M G G A Y 81
392 gatccccttttctgctggcttcacagacaagcggtttggaaaaggtggggcttatgatccactatcagcaggcttcacagacaagcggtttggtaagggtggtgcctacgatcctctatca
D P L S A G F T D K R F G K G G A Y D P L S A G F T D K R F G K G G A Y D P L S 121
512 gcaggcttcacagacaagcggtttggaaagggtggcgcctacgatcctctatcagcaggcttcacagacaagcggtttggaaagggtggcgcctacgatccccttttctgccggcttcacg
A G F T D K R F G K G G A Y D P L S A G F T D K R F G K G G A Y D P L S A G F T 161
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D K R F G M G G A Y D P L S A G F T D K R F G K G G A Y D P L S A G F T D K R F 201
752 ggaaagggtggcgcctacgatcctctatcagcgggcttcacagataagcgtttcggaatgggtggtgcctatgacccccttccgcaggcttcactgacaaacgaaagcgtttcggcatg
G K G G A Y D P L S A G F T D K R F G M G G A Y D P L S A G F T D K R K R F G M 241
872 ggcggtgcctacgatcccctttctgcaggcttcacagacaagcgatttggaaagggtggcgcctacgatcctctttcagcaggcttcacagataagcgtttcggcatgggcggcgcctac
G G A Y D P L S A G F T D K R F G K G G A Y D P L S A G F T D K R F G M G G A Y 281
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D P L S A G F T D K R F G K G G A Y D P L S A G F T D K R F G M G G A Y D P L S 321
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A G F T E K R F G K G G A Y D P L S A G F T D K R F G K G G A Y D P L S A G F T 361
1232 gacaagcggttcggtaagggtggcgcctacgatcctctctcggcaggcttcacagacaagcgcggtttcctacacggaccagacgacccgctcagcactagctttgtagacggagacaag
D K R F G K G G A Y D P L S A G F T D K R G F L H G P D D P L S T S F V D G D K 401
1352 cgtactggattctttcggggccctttccgtcaccttgtggcagcgaaaagggacccagaaaagaaagatagccgataagaagatccagacaataccaactccaaacattgttcgctacac
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1472 tttgaaactgtacgaggattagttgaagccactgactatatgaacacttccattgacgattctacgatggttcaaaagttcaccattagttcgagagggtcaattgagaactgttcttag
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Fig. 3

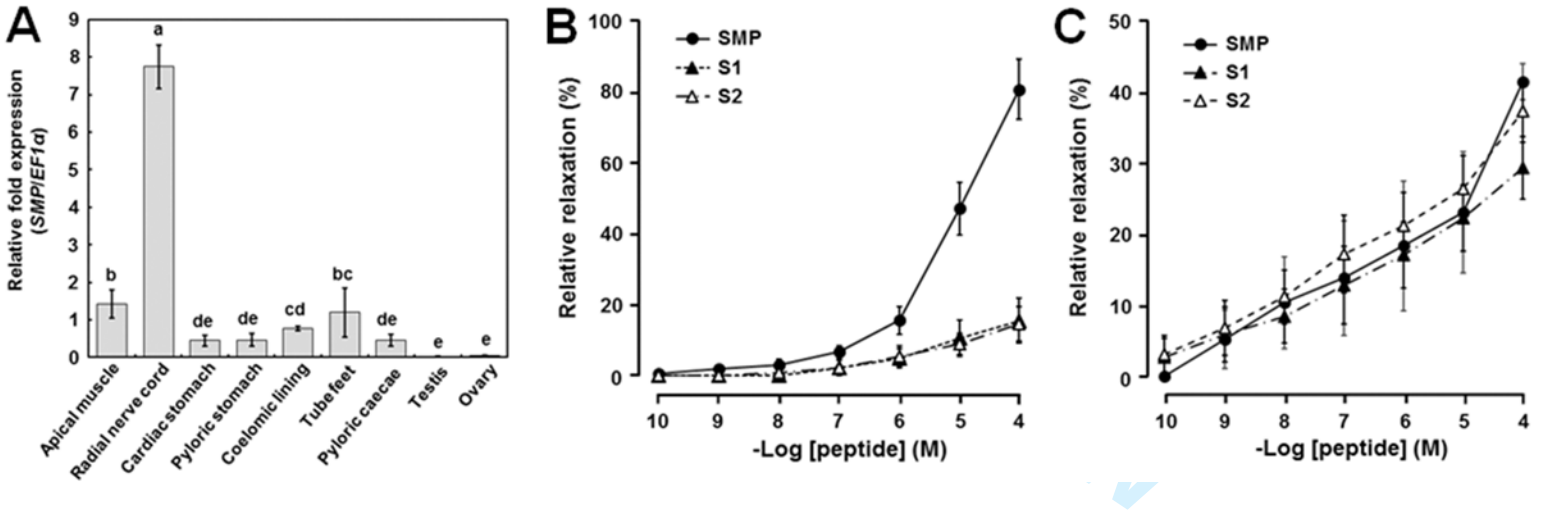
A
Relative fold expression (SMP/EF1α)
a
b
de
de
cd
bc
de
e
e
Apical muscle
Radial nerve cord
Cardiac stomach
Pyloric stomach
Coelomic lining
Tube feet
Pyloric caecae
Testis
Ovary
B
Relative relaxation (%)
SMP
S1
S2
-Log [peptide] (M)
C
Relative relaxation (%)
SMP
S1
S2
-Log [peptide] (M)

Fig. 4

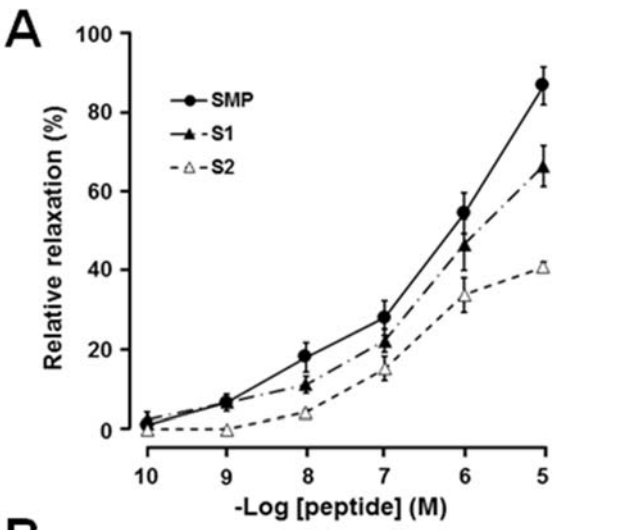
A
100
80
60
40
20
0
Relative relaxation (%)
SMP
S1
S2
10
9
8
7
6
5
-Log [peptide] (M)
B
MRLIMHSVVLLLAVIGLSMALPASKDSEDKEKLTEKEKEE
IFEEFGEEDDGKRGFGMGAYDPLSAGFTDKRFGGKGAFDP
LSAGFTDKRFGGKGAFDPLSAGFTDKRFGGSRGAFDPLSA
GFTDKRFGGKGAFDPLSAGFTDKRFGGSRGAFDPLSAGFT
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Fig. 5

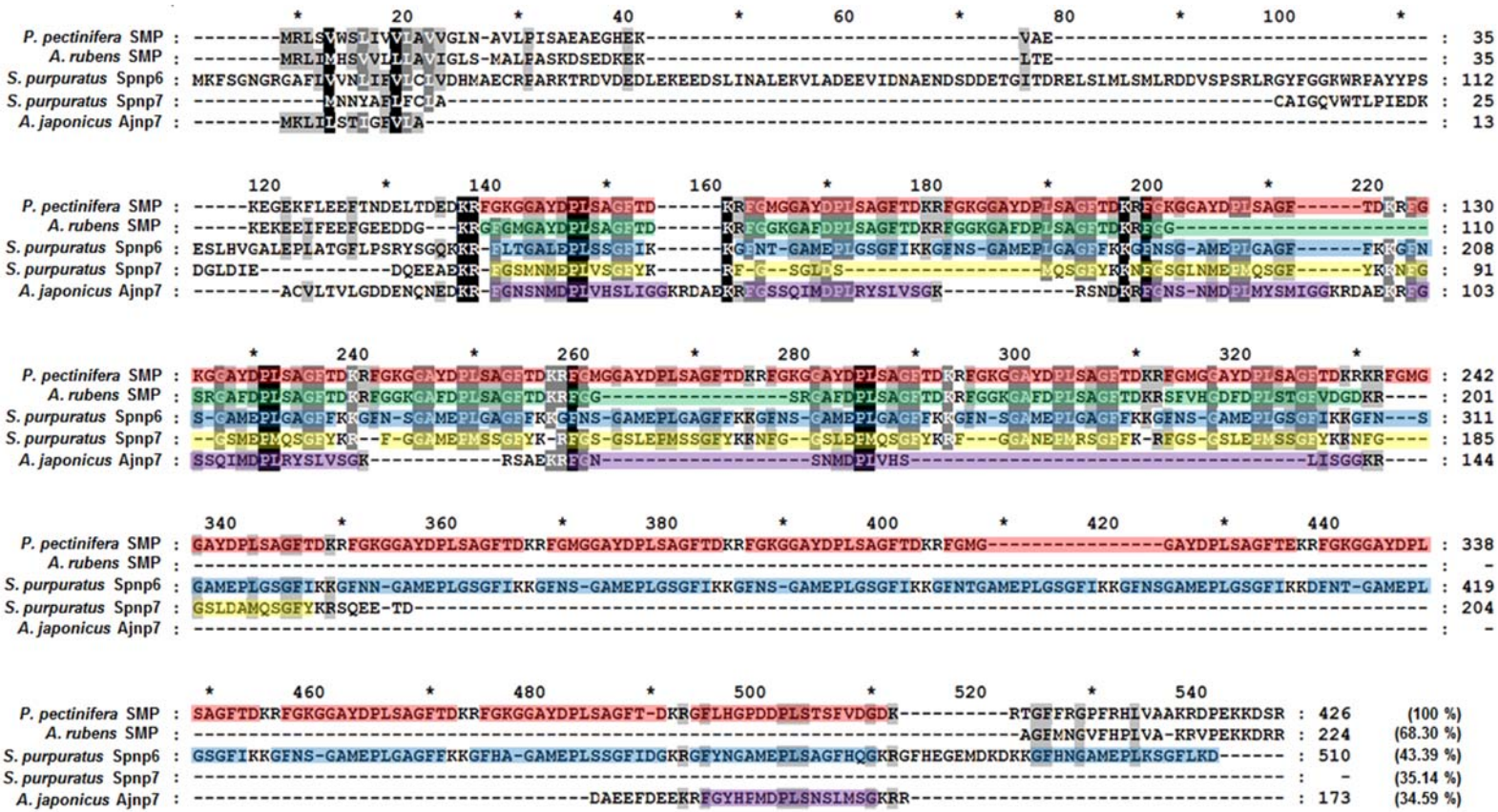
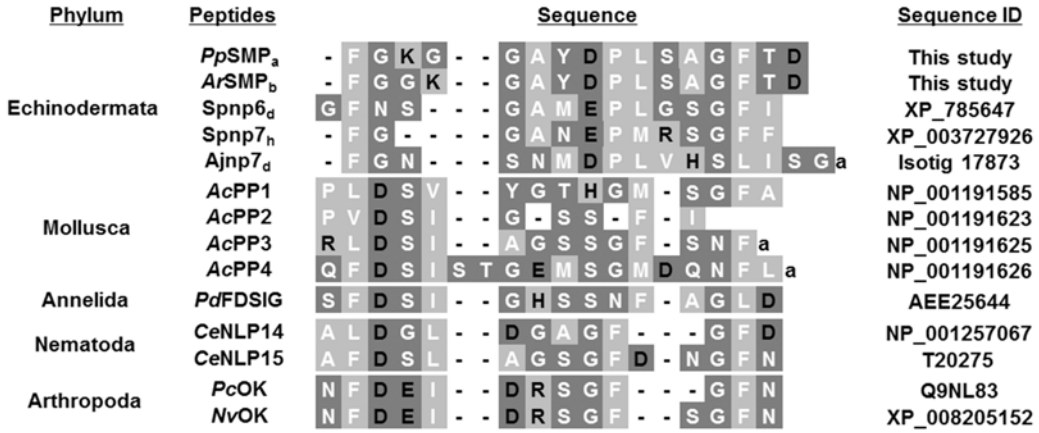

P. pectinifera SMP
A. rubens SMP
S. purpuratus Spnp6
S. purpuratus Spnp7
A. japonicus Ajnp7

Fig. 6

Origin	Peptides	Sequence	No. residues	Identity	References
P. pectinifera	SMP_a	-EGK-GGAYDPLSAGFTD--	16		This study
	SMP_b	-EGM-GGAYDPLSAGFTD--	16	93.75	
	SMP_c	-EGM-GGAYDPLSAGFTE--	16	87.50	
	SMP_d	GFL--HGPDDPLSTSFVDGD	18	43.75	
A. rubens	$ArSMP_a$	GFGM--GAYDPLSAGFTD--	16	75.00	This study
	$ArSMP_b$	-FGG-KGAFDPLSAGFTD--	16	81.25	
	$ArSMP_c$	-FGGSRGAFDPLSAGFTD--	17	75.00	
	$ArSMP_d$	SFV--HGDFDPLSTGFVDGD	18	50.00	
S. purpuratus	$Spnp6_a$	RFLT-G-ALEPLSSGFI---	15	46.67	Reich *et al.* 2015
	$Spnp6_b$	GFNT-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_c$	GFNS-G-AMEPLGAGFF---	15	46.67	
	$Spnp6_d$	GFNS-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_e$	GFNN-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_f$	DFNT-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_g$	GFHA-G-AMEPLSSGFIDG-	17	50.00	
	$Spnp6_h$	GFYN-G-AMEPLSAGFHQG-	17	50.00	
	$Spnp6_i$	GFHN-G-AMEPLKSGFLKD-	17	37.50	
	$Spnp7_a$	-FGS-MN-MEPLVSGFY---	14	28.57	
	$Spnp7_b$	-FGS-G--LDSMQSGFY---	13	38.46	
	$Spnp7_c$	NFGS-GLNMEPMQSGFY---	16	40.00	
	$Spnp7_d$	NFGG-S--MEPMQSGFY---	14	46.15	
	$Spnp7_e$	-FGG-A--MEPMSSGFY---	13	61.54	
	$Spnp7_f$	-FGS-G-SLEPMSSGFY---	14	50.00	
	$Spnp7_g$	NFGG-S--LEPMQSGFY---	14	46.15	
	$Spnp7_h$	-FGG-A--NEPMRSGFF---	13	53.85	
	$Spnp7_i$	NFGG-S--LDAMQSGFY---	14	46.15	
A. japonicus	$Ajpnp7_a$	-FG--NSNMDPLVHSLIGG-	16	33.33	Rowe & Elphick 2012
	$Ajpnp7_b$	-FGS-SQIMDPLRYSLVSG-	17	31.25	
	$Ajpnp7_c$	-FG--NSNMDPLMYSMIGG-	16	33.33	
	$Ajpnp7_d$	-FG--NSNMDPLVHSLISGG	17	33.33	
	$Ajpnp7_e$	-FG--YHPMDPLSNSLMSG-	16	40.00	

Fig. 7


Phylum
Peptides
Sequence
Sequence ID
Echinodermata
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ArSMP$_b$ - F G G K - - G A Y D P L S A G F T D This study
Spnp6$_d$ G F N S - - - G A M E P L G S G F I XP_785647
Spnp7$_h$ - F G - - - - G A N E P M R S G F F XP_003727926
Ajnp7$_d$ - F G N - - - S N M D P L V H S L I S G a Isotig 17873
Mollusca
AcPP1 P L D S V - - Y G T H G M - S G F A NP_001191585
AcPP2 P V D S I - - G - S S - F - I NP_001191623
AcPP3 R L D S I - - A G S S G F - S N F a NP_001191625
AcPP4 Q F D S I S T G E M S G M D Q N F L a NP_001191626
Annelida
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Nematoda
CeNLP14 A L D G L - - D G A G F - - - G F D NP_001257067
CeNLP15 A F D S L - - A G S G F D - N G F N T20275
Arthropoda
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NvOK N F D E I - - D R S G F - - S G F N XP_008205152

Fig. 8

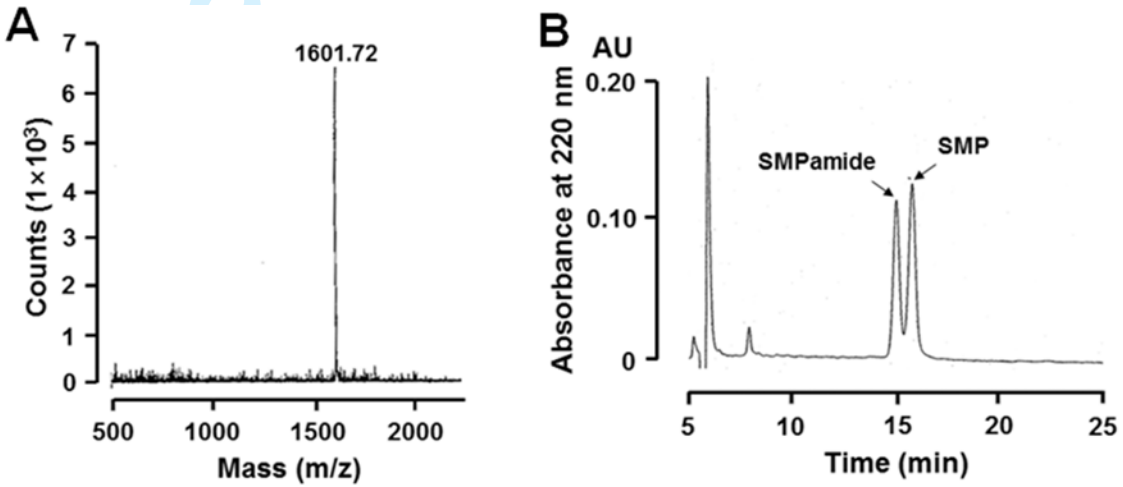
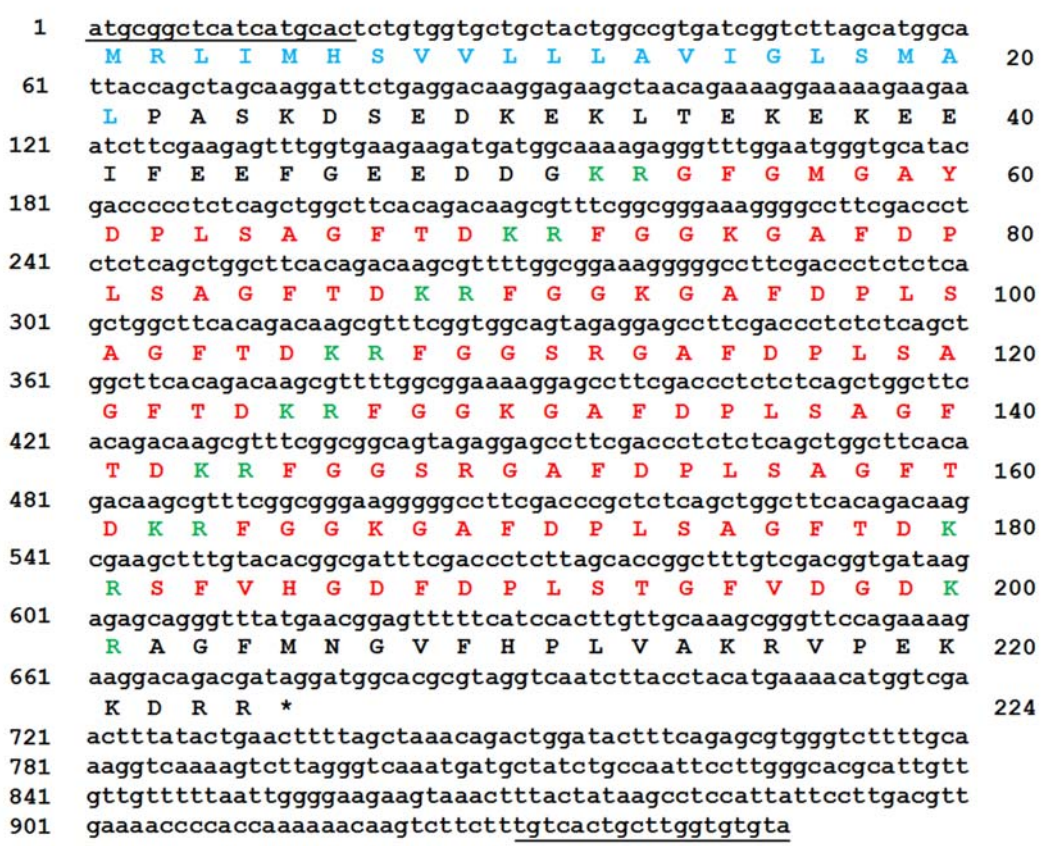

A
1601.72
Counts (1×10³)
7
6
5
4
3
2
1
0
500
1000
1500
2000
Mass (m/z)
B
AU
Absorbance at 220 nm
0.20
0.10
0
SMPamide
SMP
5
10
15
20
25
Time (min)

Figure S1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L P A S K D S E D K E K L T E K E K E E 40
121 atcttcgaagagtttggtgaagaagatgatggcaaaagagggtttggaatgggtgcatac
I F E E F G E E D D G K R G F G M G A Y 60
181 gacccccctctcagctggcttcacagacaagcgtttcggcgggaaaggggccttcgaccct
D P L S A G F T D K R F G G K G A F D P 80
241 ctctcagctggcttcacagacaagcgttttggcggaaaggggccttcgaccctctctca
L S A G F T D K R F G G K G A F D P L S 100
301 gctggcttcacagacaagcgtttcggtggcagtagaggagccttcgaccctctctcagct
A G F T D K R F G G S R G A F D P L S A 120
361 ggcttcacagacaagcgttttggcggaaaaggagccttcgaccctctctcagctggcttc
G F T D K R F G G K G A F D P L S A G F 140
421 acagacaagcgtttcggcggcagtagaggagccttcgaccctctctcagctggcttcaca
T D K R F G G S R G A F D P L S A G F T 160
481 gacaagcgtttcggcgggaaggggccttcgacccgctctcagctggcttcacagacaag
D K R F G G K G A F D P L S A G F T D K 180
541 cgaagctttgtacacggcgatttcgaccctcttagcaccggctttgtcgacggtgataag
R S F V H G D F D P L S T G F V D G D K 200
601 agagcagggtttatgaacggagtttttcatccacttgttgcaaagcgggttccagaaaag
R A G F M N G V F H P L V A K R V P E K 220
661 aaggacagacgataggatggcacgcgtaggtcaatcttacctacatgaaaacatggtcga
K D R R * 224
721 actttatactgaacttttagctaaacagactggatactttcagagcgtgggtcttttgca
781 aaggtcaaaagtcttagggtcaaatgatgctatctgccaattccttgggcacgcattgtt
841 gttgtttttaattggggaagaagtaaactttactataagcctccattattccttgacgtt
901 gaaaaccccaccaaaaaacaagtcttctttgtcactgcttggtgtgta

Figure S2

Graphical abstract

Little is known about the molecular identity of neuropeptides that act as muscle relaxants in deuterostomian invertebrates (e.g. echinoderms) that are “evolutionary intermediates” of chordates and protostomes. In this study, a hexadecapeptide was identified from a starfish, *Patiria pectinifera*, and designated starfish myorelaxant peptide (SMP). The SMP precursor comprises 19 copies of SMP and related peptides and is widely expressed in *P. pectinifera*, including several neuromuscular organs. SMP causes relaxation of several muscle preparations from *P. pectinifera* and another starfish species, *Asterias amurensis*, indicating that SMP has a general physiological role as a muscle relaxant in starfish. Interestingly, comparison of the sequence of the SMP precursor with known neuropeptide precursors revealed that SMP belongs to a bilaterian family of neuropeptides that include molluscan pedal peptides (PP) and arthropodan orcokinins (OK). This is the first study to determine the function of a PP/OK-type peptide in a deuterostome.

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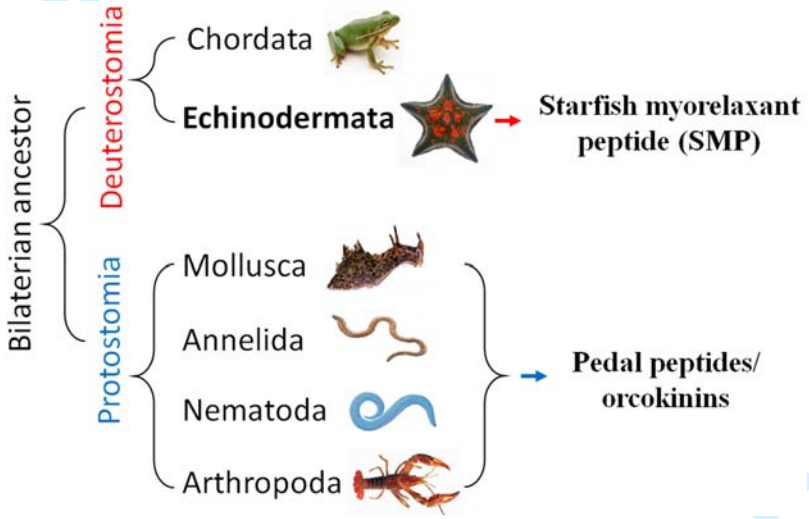

Graphical abstract

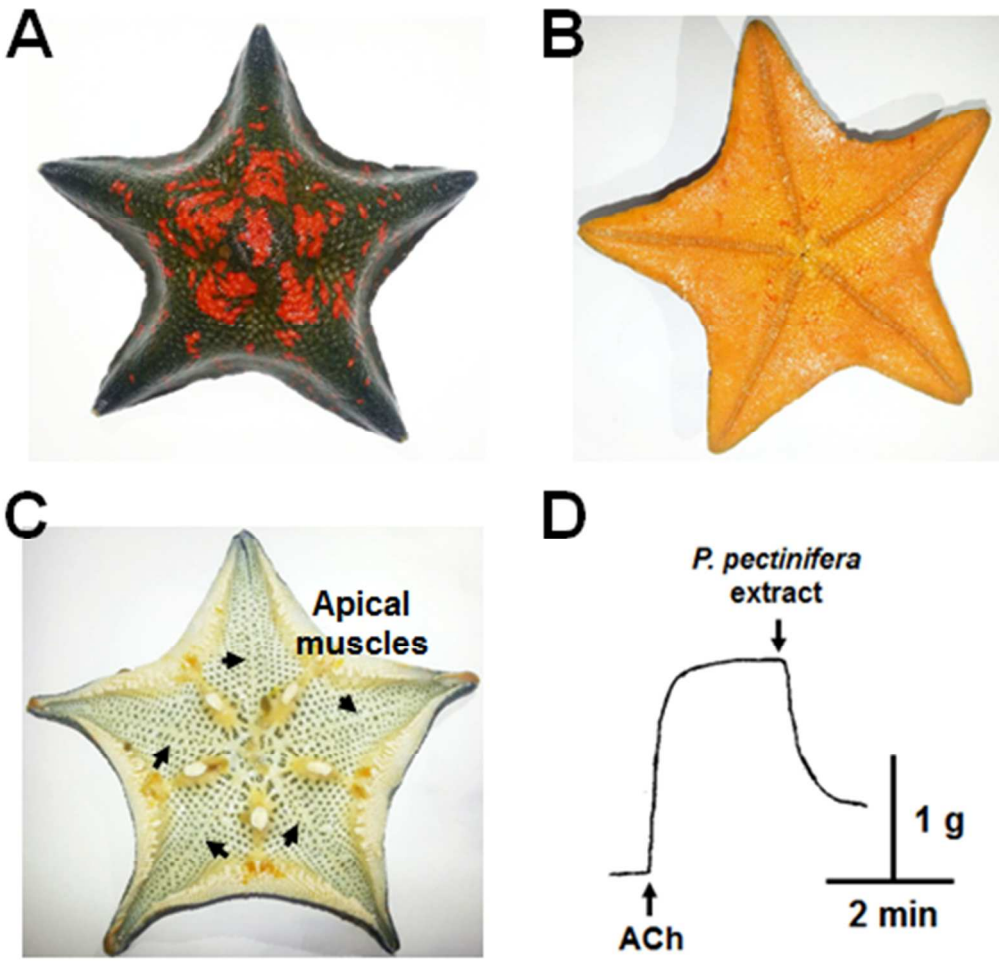

Fig. 1
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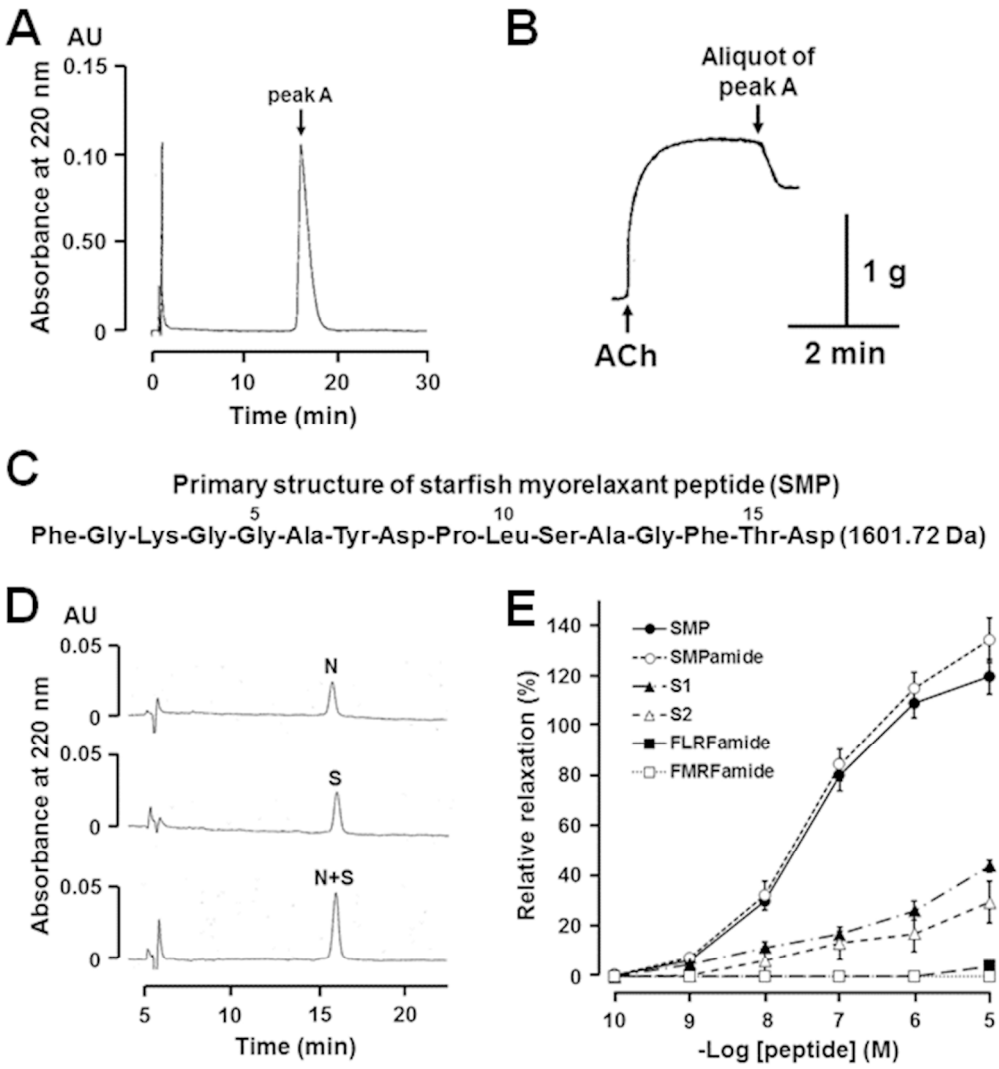

Fig. 2
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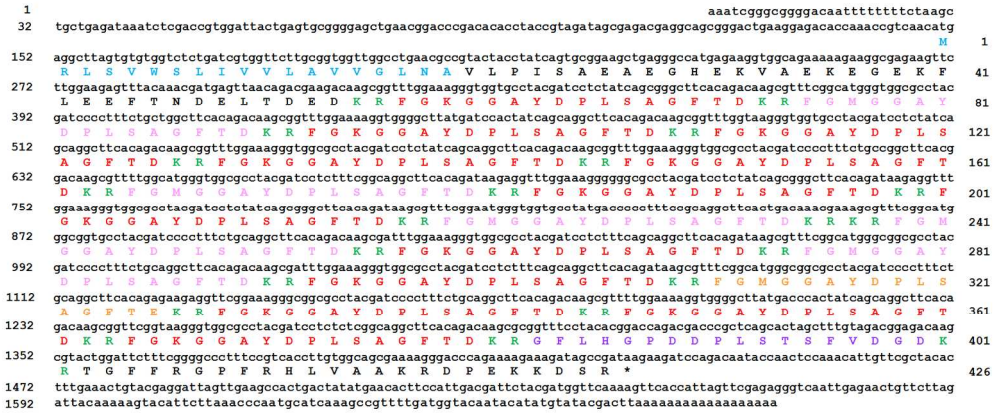

Fig. 3
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Peer Review

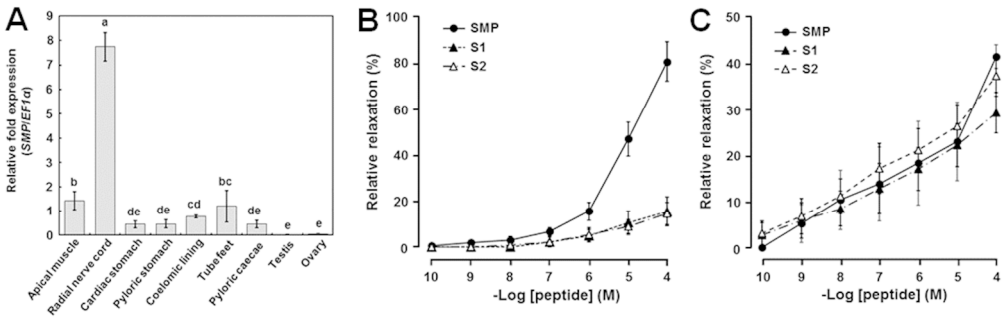
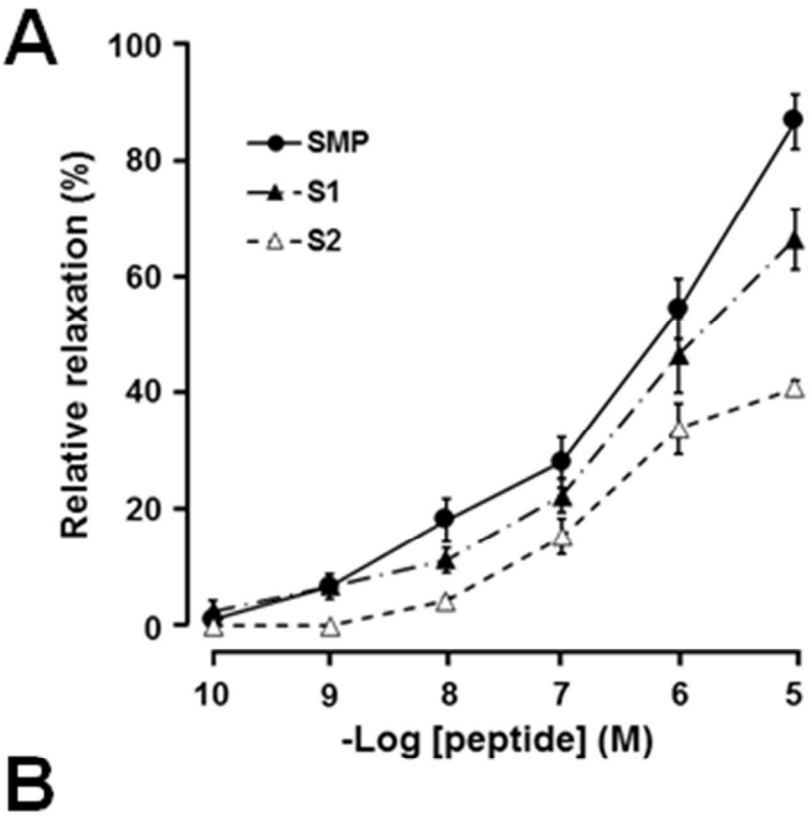


Fig. 4
447x140mm (300 x 300 DPI)

For Peer Review



B

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GFTDKRFGGKGAFDPLSAGFTDKRFGGSRGAFDPLSAGFT
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Fig. 5
179x227mm (300 x 300 DPI)

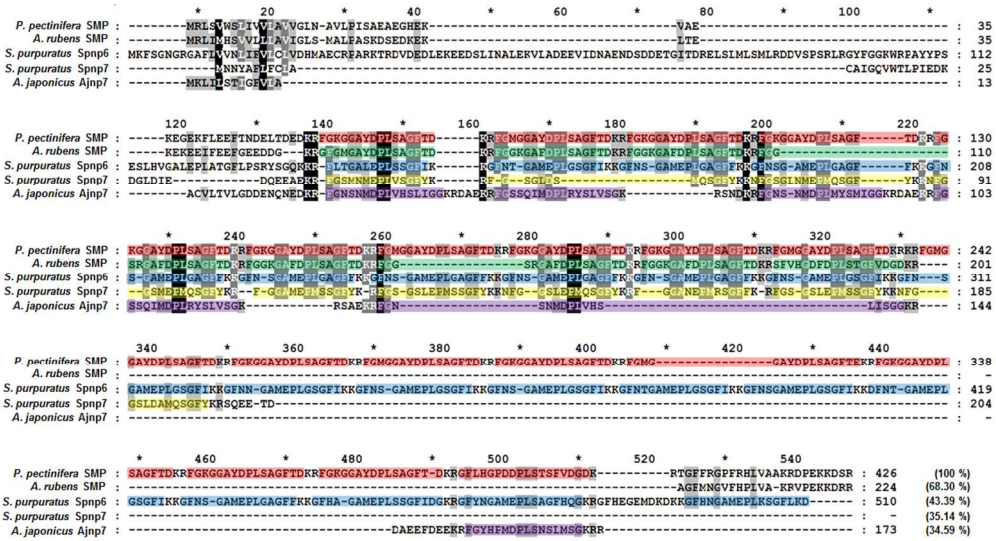

Fig. 6
422x228mm (300 x 300 DPI)

eer Review

Origin	Peptides	Sequence	No. residues	Identity	References
P. pectinifera	SMP_a	-FGK-GGAYDPLSAGFTD--	16		This study
	SMP_b	-FGM-GGAYDPLSAGFTD--	16	93.75	
	SMP_c	-FGM-GGAYDPLSAGFTE--	16	87.50	
	SMP_d	GFL--HGPDDPLSTSFVDGD	18	43.75	
A. rubens	$ArSMP_a$	GFGM--GAYDPLSAGFTD--	16	75.00	This study
	$ArSMP_b$	-FGG-KGAFDPLSAGFTD--	16	81.25	
	$ArSMP_c$	-FGGSRGAFDPLSAGFTD--	17	75.00	
	$ArSMP_d$	SFV--HGDFDPLSTGFVDGD	18	50.00	
S. purpuratus	$Spnp6_a$	RFLT-G-ALEPLSSGFI---	15	46.67	Reich *et al.* 2015
	$Spnp6_b$	GFNT-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_c$	GFNS-G-AMEPLGAGFF---	15	46.67	
	$Spnp6_d$	GFNS-G-AMEPLGSGFI---	15	40.00	
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	$Spnp6_f$	DFNT-G-AMEPLGSGFI---	15	40.00	
	$Spnp6_g$	GFHA-G-AMEPLSSGFIDG-	17	50.00	
	$Spnp6_h$	GFYN-G-AMEPLSAGFHQG-	17	50.00	
	$Spnp6_i$	GFHN-G-AMEPLKSGFLKD-	17	37.50	
	$Spnp7_a$	-FGS-MN-MEPLVSGFY---	14	28.57	
	$Spnp7_b$	-FGS-G--LDSMQSGFY---	13	38.46	
	$Spnp7_c$	NFGS-GLNMEPMQSGFY---	16	40.00	
	$Spnp7_d$	NFGG-S--MEPMQSGFY---	14	46.15	
	$Spnp7_e$	-FGG-A--MEPMSSGFY---	13	61.54	
	$Spnp7_f$	-FGS-G-SLEPMSSGFY---	14	50.00	
	$Spnp7_g$	NFGG-S--LEPMQSGFY---	14	46.15	
	$Spnp7_h$	-FGG-A--NEPMRSGFF---	13	53.85	
	$Spnp7_i$	NFGG-S--LDAMQSGFY---	14	46.15	
A. japonicus	$Ajpnp7_a$	-FG--NSNMDPLVHSLIGG-	16	33.33	Rowe & Elphick 2012
	$Ajpnp7_b$	-FGS-SQIMDPLRYSLVSG-	17	31.25	
	$Ajpnp7_c$	-FG--NSNMDPLMYSMIGG-	16	33.33	
	$Ajpnp7_d$	-FG--NSNMDPLVHSLISGG	17	33.33	
	$Ajpnp7_e$	-FG--YHPMDPLSNSLMSG-	16	40.00	

Fig. 7
364x383mm (300 x 300 DPI)

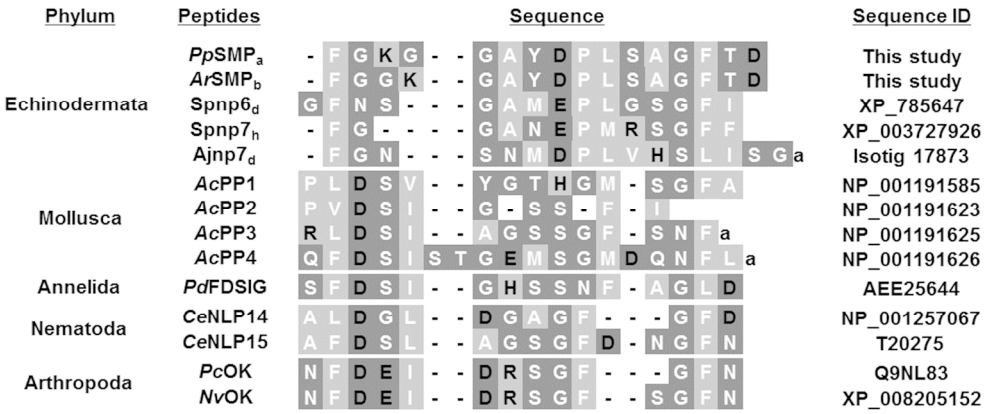

Fig. 8
480x204mm (300 x 300 DPI)

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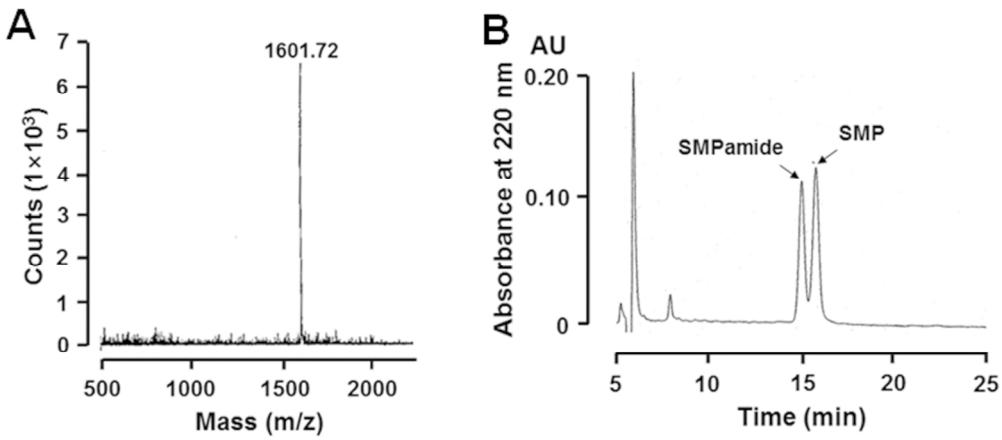

Figure S1
345x148mm (300 x 300 DPI)

Peer Review

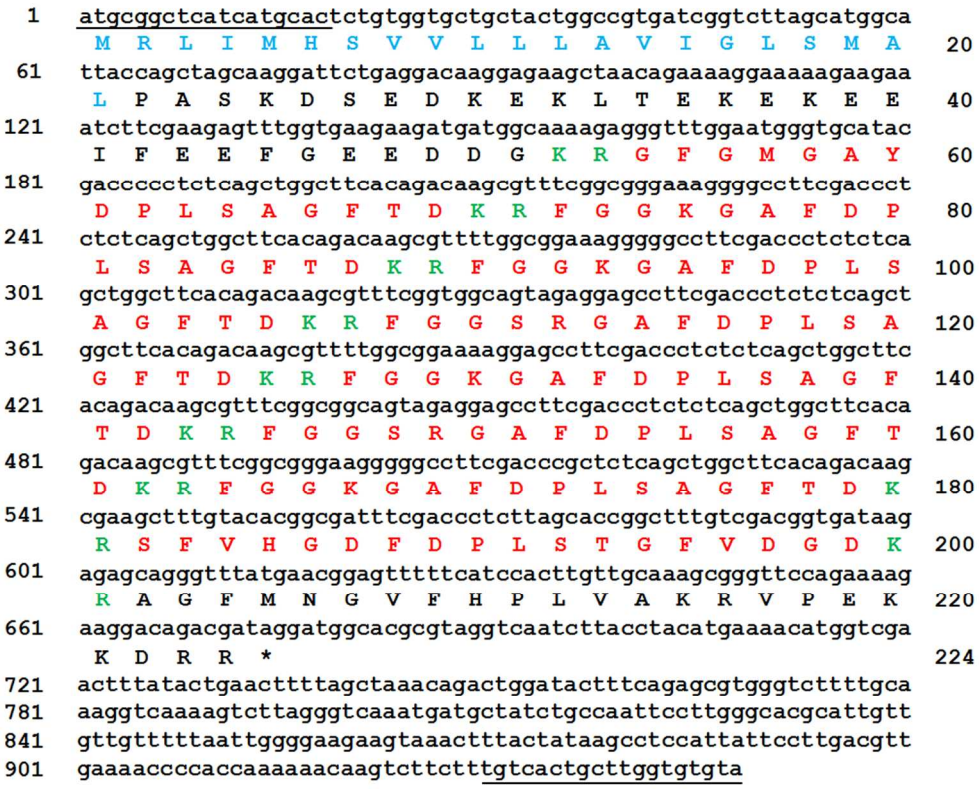

Figure S2
350x287mm (300 x 300 DPI)

For Review