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Isolation of ‹Glu-Gly-Leu-Arg-Trp-NH_2 (Antho-RWamide II), a novel neuropeptide from sea anemones

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Using a radioimmunoassay for the peptide sequence Arg-Phe-NH_2 (RFamide), a novel peptide has been purified from acetic acid extracts of the sea anemone *Anthopleura elegantissima*. This peptide has the structure ‹Glu-Gly-Leu-Arg-Trp-NH_2, and was named Antho-RWamide II. Antho-RWamide II is a neuropeptide. Its structure is closely related to an earlier characterized neuropeptide from *Anthopleura* ‹Glu-Ser-Leu-Arg-Trp-NH_2 (Antho-RWamide I).

Peptide isolation; Neuropeptide; Neurotransmitter; Coelenterate

1. INTRODUCTION

Coelenterates have the simplest nervous systems of the animal kingdom. In these primitive nervous systems peptides play an important role. In earlier papers we reported on the sequence of two novel neuropeptides from the sea anemone *Anthopleura elegantissima*, <Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide) and <Glu-Ser-Leu-Arg-Trp-NH_2 [1,2]. These peptides have excitatory actions on muscles and neuronal systems of sea anemones, suggesting that they might be neurotransmitters ([3]; McFarlane, Graff and Grimmelikhuijzen, unpublished). In the present paper we describe the isolation and sequencing of a third neuropeptide from sea anemones.

2. MATERIALS AND METHODS

A. elegantissima were obtained from Biomarine Laboratories (Venice, USA). The following procedures and techniques have been described before: acetic acid extraction of sea anemones, the desalting procedure using Sep-pak C-18 cartridges and the cation-exchange chromatography using CM-Sephadex C-25 [1,2]. HPLC was carried out using a Shimadzu LC-6A system. The HPLC columns were described earlier [1]. The amino acid composition of the peptide was determined by hydrolysis (6 N HCl, overnight, 115°C), subsequent dansylation and separation of the dansylation products on 2.5 × 2.5 cm polyamide sheets [1,4,5]. Sequencing was carried out with the 4-*N,N'*-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) method [6]. Enzymatic removal of pyroglutamate from a blocked peptide was performed by digestion with pyroglutamate aminopeptidase following a protocol described in [1]. Trypsin digestion was carried out as in [2]. The details of the Arg-Phe-NH_2 (RFamide) radioimmunoassay are given in [1]. In short, this radioimmunoassay only recognizes peptides which contain a carboxy-terminal amidation as well as an uncharged aromatic or aliphatic amino acid in the carboxy-terminal position, preceded by Arg or Lys [1]. Synthetic <Glu-Gly-Leu-Arg-Trp-NH_2 was prepared as a customer synthesis by Bachem (Bubendorf, Switzerland).

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3. RESULTS

In a former article we prepared an acetic acid extract of 150 g (wet weight) *A. elegantissima* [2]. This extract was desalted using Sep-pak and purified by cation-exchange chromatography [2]. Subsequent HPLC using a Spherisorb ODS-2 column and a 20 min gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) revealed three fractions which were immunoreactive in a radioimmunoassay for the sequence RFamide [2]. This is

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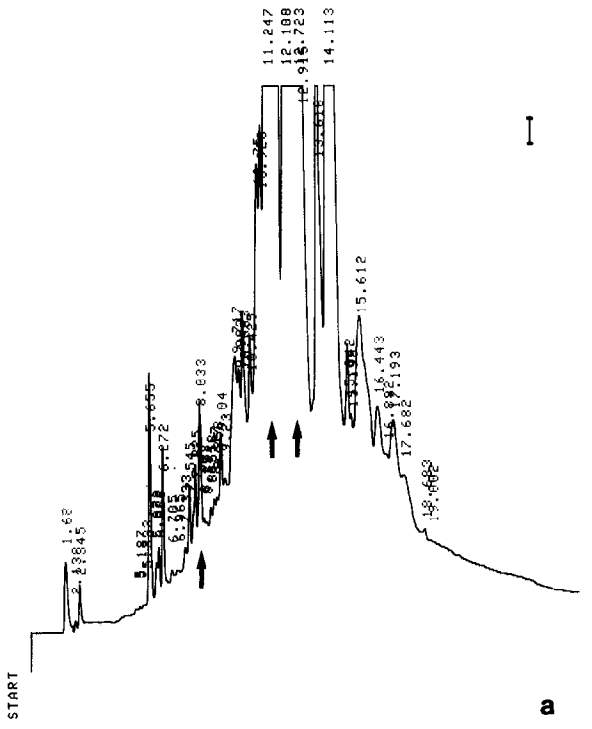

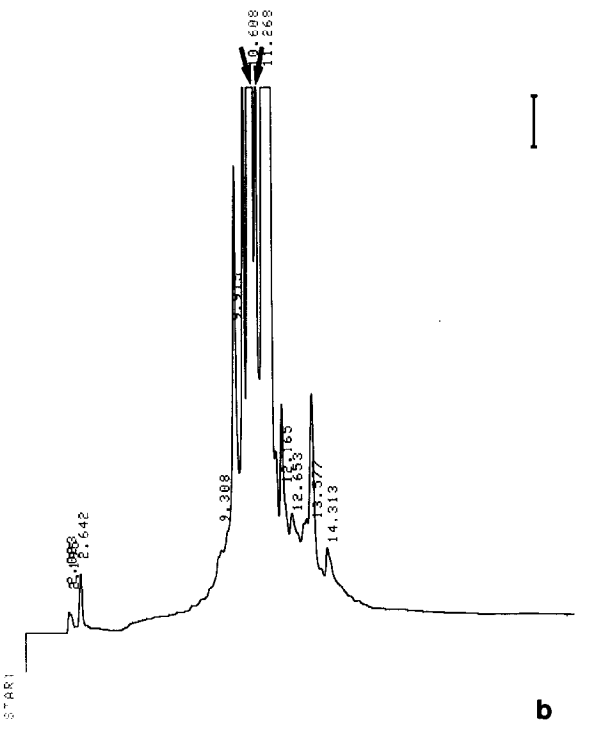

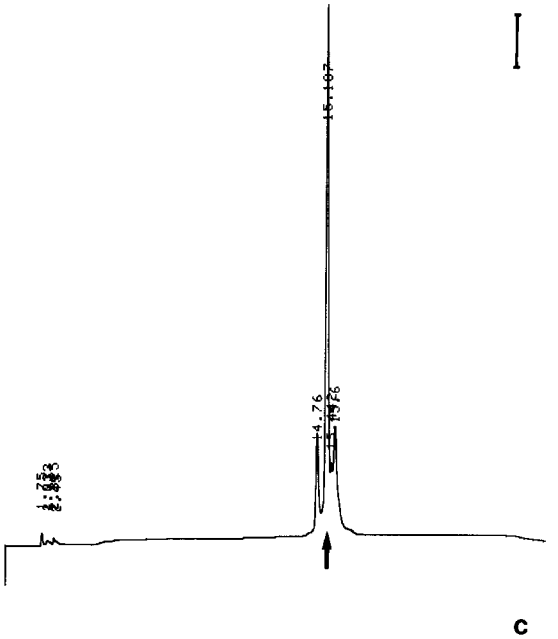

Fig.1. HPLC of an acetic acid extract of 150 g (wet weight) *Anthopleura elegantissima*. This extract is the same as the one which was prepared, purified and desalted in [2]. The vertical bar is 1.0 ΔA at 215 nm. (a) This is the same figure as fig.2a from [2]. Of a total amount of 200 nmol RFamide equivalents (400 μl), 50 nmol (100 μl) was injected. The column (Spherisorb ODS-2, 5 μm particle size, 80 Å pore diameter; dimensions: 4 × 250 mm) was equilibrated with 10% acetonitrile in 0.1% TFA. After injection of the sample, a linear gradient of 10–60% acetonitrile in 0.1% TFA was started (20 min; 1 ml/min). Immunoreactive components (arrows) were eluted at 7.9–9.0 min (30 nmol RFamide equivalents), at 10.3–11.9 min (13 nmol) and at 11.9–13.3 min (1 nmol). (b) HPLC of the combined 11.9–13.3 min fractions of a. Of a total amount of 4 nmol RFamide equivalents (300 μl), 1.3 nmol (100 μl) was injected. The column was Partisil ODS-3, 5 μm, 60 Å, 4 × 250 mm. After equilibration with 10% acetonitrile in 0.1% TFA, the sample was injected and a gradient of 10–60% acetonitrile in 0.1% TFA was started (20 min; 1 ml/min). Immunoreactive peaks (arrows) appeared at 10.500–10.890 min (800 pmol RFamide equivalents) and at 10.890–11.160 min (200 pmol). (c) HPLC of the combined 10.890–11.160 min fractions of b. Of a total amount of 600 pmol RFamide equivalents (40 μl), 300 pmol (20 μl) was injected. The column was Nucleosil C-18 (5 μm, 120 Å, 4 × 250 mm). After equilibration with 10% acetonitrile in 0.1% TFA, the sample was injected and a gradient of 10–35% acetonitrile in 0.1% TFA was started (20 min; 1 ml/min). Several peaks appeared of which the major one (15.107 min; arrow) was immunoreactive (200 pmol). Realize that 1 pmol of RFamide equivalents, as measured in our radioimmunoassay, might be more than 1 pmol of pure peptide [1].

shown in fig.1a. The first immunoreactive fraction (7.9–9.0 min) was eluted at the position of Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2). From the second fraction (10.3–11.9 min), <Glu-Ser-Leu-Arg-Trp-NH_2 was isolated [2]. The third component (11.9–13.3 min) was not characterized in our former paper [2]. This material was now further purified by HPLC using a column containing Partisil ODS-3 and a 20 min gradient of 10–60% acetonitrile in 0.1% TFA (fig.1b). Two immunoreactive peaks were eluted, one appearing at 10.50–10.89 min and another at 10.89–11.16 min. An aliquot of the first peak (100 pmol RFamide equivalents; realize that the absolute amount of peptide might be higher, cf. fig.2 of [1]) was hydrolyzed with HCl and subsequently dansylated. This hydrolysate showed the same amino acid composition as a hydrolysate of <Glu-Ser-Leu-Arg-Trp-NH_2 (Trp is always destroyed under these conditions). The 10.50–10.89 min component is therefore most likely <Glu-Ser-Leu-Arg-Trp-NH_2 and was not further investigated. The 10.89–11.16 min component was purified by HPLC using Nucleosil C-18 and a 20 min gradient of 10–35% acetonitrile in 0.1% TFA (fig.1c). Several peaks appeared of which the major one (15.107 min) was immunoreactive.

This major component, although not totally pure, was analyzed for its amino acid composition (20 pmol RFamide equivalents). This revealed Glu, Gly, Leu and Arg. An end group determination of the intact peptide (10 pmol RFamide equivalents), using the DABITC method [6], showed that no reactive amino-terminal amino acid was present. As Glu was one of the amino acids in the hydrolysate, the amino-terminus could be <Glu. To investigate this, intact peptide (80 pmol RFamide equivalents) was incubated with 6 mU pyroglutamate aminopeptidase. After this digestion, the sample was purified by HPLC using Partisil ODS-3 and a 20 min gradient of 10–35% acetonitrile in 0.1% TFA. Two immunoreactive components appeared during this chromatography: one at 15.308 min, which is the position of intact peptide (cf. table 1) and another, new component at 13.900 min (not shown). An aliquot of this new material (20 pmol RFamide equivalents) was hydrolyzed and dansylated. Analysis showed Gly, Leu and Arg, whereas Glu was failing. Therefore, the amino-terminus of the intact peptide is most likely <Glu and the new 13.900 min component is the carboxy-terminal fragment of the intact peptide after removal of <Glu. The 13.900 min component was sequenced using the DABITC method. This yielded the sequence Gly-Leu-Arg with no amino acids following Arg using the extraction procedures of Chang et al. [6]. Because such a sequence is not recognized in our RFamide radioimmunoassay (see section 2), and because aromatic or aliphatic amino acid amides cannot be detected by the DABITC method of Chang [6], we supposed that an amino acid amide follows Arg. This was investigated by incubating intact peptide (20 pmol RFamide equivalents) with

Table 1

Comparison by HPLC of the chromatographic behaviour of the natural *Anthopleura* peptide and synthetic <Glu-Gly-Leu-Arg-Trp-NH_2

Column material	Gradient of acetonitrile in 0.1% TFA	Retention time (min) of the natural peptide	Retention time (min) of <Glu-Gly-Leu-Arg-Trp-NH_2
Spherisorb ODS-2, 5 μm, 80 Å	10–35% (20 min)	17.532	17.547
Nucleosil C-18, 5 μm, 120 Å	10–35% (20 min)	15.042	15.032
Nucleosil C-18, 5 μm, 300 Å	10–35% (20 min)	13.488	13.497
Partisil ODS-3, 5 μm, 60 Å	10–35% (20 min)	15.457	15.450
Spherisorb C-8, 5 μm, 80 Å	10–35% (20 min)	17.028	17.038
Nucleosil propyl-nitrile, 7 μm, 300 Å	10–10% (15 min)	6.417	6.408

Column materials with different ligands and pore diameters were used. The dimensions of the columns were 4 × 250 mm. The retention-time variability of the HPLC system was ± 1%

Sepharose-bound trypsin. Dansylation of the digestion products and analysis on polyamide sheets showed a prominent spot of Trp-NH_2.

From all these data, the complete sequence of the intact peptide has to be <Glu-Gly-Leu-Arg-Trp-NH_2. This peptide was synthesized by Bachem (Bubendorf, Switzerland). The chromatographic behaviour of the natural *Anthopleura* peptide was subsequently compared with that of synthetic <Glu-Gly-Leu-Arg-Trp-NH_2 using HPLC and six different HPLC columns. Table 1 shows that the natural and synthetic peptide were always eluted at the same retention times. This confirmed the structure of the *Anthopleura* peptide.

4. DISCUSSION

Two neuropeptides have recently been isolated from the sea anemone *Anthopleura elegantissima*: <Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide) and <Glu-Ser-Leu-Arg-Trp-NH_2 [1,2]. The novel peptide that we describe in the present paper is closely related to <Glu-Ser-Leu-Arg-Trp-NH_2 and only differs from this by a Gly in position 2. This suggests that the DNA sequences coding for the two peptides have a common evolutionary origin.

In analogy to the name Antho-RFamide (originating from *Anthopleura* RFamide peptide), we would like to call <Glu-Ser-Leu-Arg-Trp-NH_2 Antho-RWamide I, and <Glu-Gly-Leu-Arg-Trp-NH_2 Antho-RWamide II. Using antisera to the sequence Arg-Trp-NH_2, we have found that Arg-Trp-NH_2 immunoreactivity was located in neurones of sea anemones and that non-neuronal cells were not stained [2]. This shows that both Antho-RWamide I and II are neuropeptides.

Recent experiments have shown that Antho-RWamide I is biologically active: low concentrations (10^{-8}–10^{-7} M) of neuropeptide induce contractions in endodermal muscles of sea anemones (McFarlane, Graff and Grimmelikhuijzen, unpublished). The action of Antho-RWamide II has not been investigated so far. One can assume, however, that also this neuropeptide plays a role in neurotransmission.

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