

The TRH-related peptide pyroglutamylglutamylprolinamide is present in human semen

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We have recently identified a novel peptide in the rabbit prostate complex which cross-reacts with an antibody to thyrotrophin-releasing hormone (TRH) and has the structure pGlu-Glu-ProNH₂. In the present study, high concentrations of a TRH-related tripeptide and also a polypeptide (10–12 kDa) containing a TRH-immunoreactive peptide at its C-terminus were detected in human semen. The low molecular mass TRH-like peptide and the immunoreactive fragment from the polypeptide were isolated from human semen and shown to have identical structures. Amino acid analysis suggested compositions Glu₂, Pro₁, and after mild acid hydrolysis, the same sequence, Glu-Glu-Pro, was established for the two peptides. Fast atom bombardment (FAB) mass spectrometry yielded a pseudomolecular ion (M + H)⁺ of 355.38 which was identical to that of the synthetic peptide pGlu-Glu-ProNH₂. The data demonstrate that human semen contains the TRH-like peptide pyroglutamylglutamylprolinamide and also a polypeptide terminating in the sequence Gln-Glu-ProNH₂.

Semen; Thyrotrophin-releasing hormone; Pyroglutamylglutamylprolinamide; FAB mass spectrometry; (Human)

1. INTRODUCTION

Rat prostate [1,2] and human semen [3,4] have been reported to contain high levels of immunoreactive thyrotrophin-releasing hormone (TRH). We have recently detected a TRH-like tripeptide and a polypeptide containing a TRH immunoreactive fragment at its C-terminus in the rabbit prostate complex and semen [5]. These TRH immunoreactive peptides did not correspond with authentic TRH, and after purification the low molecular mass peptide was identified as pyroglutamylglutamylprolinamide by amino acid analysis, N-terminal sequence analysis after mild acid hydrolysis, and fast atom bombardment (FAB) mass spectrometry [6]. This peptide differs from TRH by the substitution of glutamic acid for histidine at position 2.

We report here that human semen contains the same TRH-related peptide pGlu-Glu-ProNH₂

although it could not be detected in bull or ram semen.

2. EXPERIMENTAL

2.1. Semen collection

Whole ejaculates were collected by 5 normal men aged between 28 and 37 years. After ejaculation each volunteer immediately mixed the semen with 10 ml acidified acetone (acetone/HCl/H₂O, 40:1:5) which was stored on ice for up to 16 h before analysis.

Bulls from an artificial insemination centre (Milk Marketing Board Freezing Unit, Little Horwood), which were accustomed to regular semen collection, were used as semen donors. Semen was collected using an artificial vagina from pre-stimulated ('teased') bulls [7], although on one occasion two sequential ejaculates were obtained from an unteased bull. Rams of mixed breed, which had been bred and kept at the National Institute for Medical Research, were trained to ejaculate into an artificial vagina when presented with a teaser ewe [7]. After ejaculation, bull and ram semen (pooled ejaculates from 2 animals) was immediately mixed with 15 ml acidified acetone and stored on ice for a maximum of 4 h.

2.2. Peptide synthesis

The tripeptide pGlu-Glu-ProNH₂ and the C-terminally extended TRH-related peptide (43 residues) from the rat prohormone: pGlu-His-Pro-Gly-Arg-Arg-Ala-Asn-Gln-Asp-Lys-Tyr-

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Ser-Trp-Ala-Asp-Glu-Glu-Asp-Ser-Asp-Trp-Met-Pro-Arg-Ser-Trp-Leu-Pro-Asp-Phe-Phe-Leu-Asp-Ser-Trp-Phe-Ser-Asp-Val-Pro-Gln-Val, were synthesised by conventional fluorenyl-methoxycarbonyl methodology as described [5,6].

2.3. Detection of TRH-related peptides in semen

The semen mixed with acidified acetone was centrifuged and the supernatant dried in vacuo before dissolving in 25% acetic acid [5]. Peptides in the semen were resolved by gel-exclusion chromatography on Sephadex G-50 superfine (1 × 100 cm) in 25% acetic acid. Aliquots of each fraction were dried in vacuo and immunoreactive TRH was detected by radioimmunoassay (RIA) as in [8]. The TRH-related polypeptide in human semen was detected by RIA with the TRH antibody after excision of the immunoreactive fragment with trypsin [20 µg enzyme in 50 µl of 0.05 M sodium phosphate (pH 8.2), 37°C, 6 h] followed by cyclisation of the N-terminal glutamine (50% acetic acid, 100°C, 20 min).

The molecular mass of the TRH-related polypeptide was estimated by gel-exclusion chromatography on Sephadex G-100 (1 × 100 cm) in 25% acetic acid. Aprotinin (6.5 kDa), ¹²⁵I-labelled rat β-lipotrophin (9.9 kDa) and cytochrome c (12.4 kDa) were used to calibrate the column.

2.4. Purification of the TRH immunoreactive peptides from human semen

The peptides from a total of 17 ejaculates were separated by gel-exclusion chromatography as described above. A maximum of 4 ejaculates were analysed at one time and the fractions were frozen until all samples had been processed. Fractions containing the TRH-related polypeptide were dried in vacuo, the immunoreactive fragment being excised by trypsin digestion and the N-terminal glutamine cyclised as described above. The immunoreactive fragment thus generated and the low molecular mass TRH-related peptide were purified by ion-exchange chromatography and high-performance liquid chromatography (HPLC) as detailed elsewhere [6].

2.5. Amino acid analysis

Peptides were hydrolysed for 20 h at 110°C in 6 M HCl, 2 mM phenol in the vapour phase under N₂. Analysis was performed using an Applied Biosystems 420A derivatiser-analyser fitted with an on-line 130A PTC-HPLC detection [9].

2.6. Automated sequence analysis

The peptides were incubated with 3 M HCl for 10 min at 100°C in order to achieve partial conversion of the N-terminal pyroglutamic acid to glutamic acid [10]. Sequence analysis was performed using Applied Biosystems 470A gas-phase and 477A pulsed liquid-phase peptide sequencers [11].

2.7. Mass spectrometry

The endogenous peptides were further purified and concentrated to final volumes of 50 µl by microbore reverse-phase HPLC on an Applied Biosystems microsyringe pumping system [6]. For FAB mass spectrometry the peptides were dried to 1.5 µl on the probe and mixed with 1 µl thioglycerol matrix containing 1% trifluoroacetic acid. Spectra were recorded by normal scanning (310–1200, scan time = 0.05 decade) in the positive ion mode on a VG 70-250 SE mass spectrometer at an

accelerating voltage of 8 keV using a 35 keV caesium ion gun [12].

3. RESULTS AND DISCUSSION

The TRH-related peptides present in extracts of human semen are illustrated in fig.1. Before enzymic digestion, RIA with the TRH antibody revealed only the presence of low molecular mass TRH immunoreactivity in human semen (13.2 pmol immunoreactive peptide/ejaculate). After treatment with trypsin a high concentration of a TRH-related polypeptide (31.3 pmol immunoreactive peptide/ejaculate) with a molecular mass of approx. 10–12 kDa was detected. This polypeptide must terminate with a TRH immunoreactive fragment because one of the requirements for recognition by the TRH antibody is a C-terminal prolinamide which can only occur at the end of a peptide. The pattern of TRH-like peptides in human semen is remarkably similar to those in rabbit prostate complex and semen. In rabbit, high concentrations of a polypeptide (5–6 kDa) containing a TRH-like peptide at its C-terminus were observed in addition to low molecular mass TRH immunoreactivity [5,6].

In contrast to rabbit and human, TRH immunoreactivity could not be detected in untreated fractions after gel-exclusion chromatography of extracts of bull and ram semen. This pronounced species difference in the distribution of TRH-related peptides is difficult to explain. The peptides

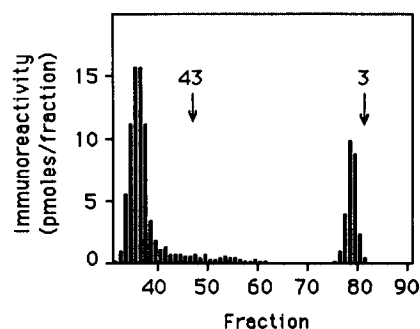


Fig.1. TRH-related peptides in human semen. Peptides extracted from 2 pooled semen samples (2 individuals) were resolved by gel-exclusion chromatography (Sephadex G-50 superfine). Iodinated TRH (3 residues) and TRH-related peptide (43 residues) were used as molecular markers. Aliquots of each fraction were assayed for TRH after enzymic digestion as described in section 2.

may be present in the semen of bull and ram but at concentrations too low to be detected by our radioimmunoassay. Another explanation could be that the peptides occur in a fraction of the ejaculate that is not collected effectively by the standard protocol (see section 2). In this context, bulls are generally pre-stimulated or teased before collection of semen [7] and the fluid, which contains secretions from the accessory reproductive glands, is discarded because it does not contain viable sperm. For this reason, an ejaculate was collected from a bull which had not been pre-stimulated; nevertheless, TRH immunoreactivity was not detected in extracts of this ejaculate.

In human, both the low molecular mass peptide and the immunoreactive fragment cleaved from the polypeptide exhibited identical chromatographic properties to the rabbit peptide: the peptides were retained on an anion exchanger (QAE-Sephadex) at pH 7.6 but not on a cation exchanger (SP-Sephadex) at pH 2.0. Unexpectedly, the immunoreactive fragment from the polypeptide in rabbit prostate and semen was not retained on the cation and anion exchangers or on a reverse-phase HPLC column.

The TRH-related peptides were purified from human semen by anion-exchange chromatography and reverse-phase HPLC yielding, as estimated by amino acid analysis, approx. 0.5 nmol low molecular mass peptide and 1.0 nmol tryptic fragment. In order to conserve the peptides for mass spectrometry, less than 20 pmol of each were subjected to amino acid analysis. The recoveries of both aspartic and glutamic acids are often poor when strong acid hydrolysis is carried out at these low levels. For this reason, a similar concentration of the synthetic peptide pGlu-Glu-ProNH₂ was hydrolysed under the same conditions as the purified peptides in order to give an internal estimate for the recovery of glutamic acid (50%). Both peptides, after correction for loss of glutamic acid, gave an approximate ratio of Glx:Pro of 2:1 by amino acid analysis: low levels of the ubiquitous amino acids serine and glycine were also present. Both of the purified peptides appeared to cross-react 50% with the TRH antibody as do the synthetic peptide pGlu-Glu-ProNH₂ and the endogenous peptide from rabbit prostate [6].

The purified peptides were resistant to Edman degradation but after mild acid hydrolysis to con-

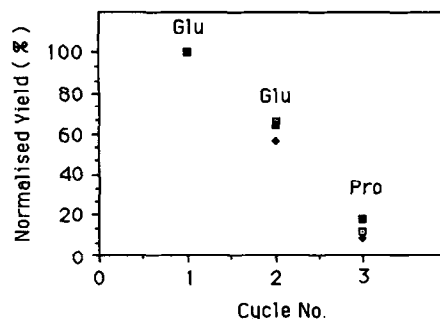


Fig.2. Sequence analysis of the purified TRH-related peptides from human semen. Automated sequencing on 100 pmol synthetic pGlu-Glu-ProNH₂ (□), 100 pmol purified TRH-immunoreactive fragment from the 10 kDa polypeptide from human semen (◆) and 60 pmol low molecular mass TRH-like peptide in human semen (■). Results are normalised with respect to the yield on the first cycle of Edman degradation for ease of comparison.

vert partially the N-terminal pyroglutamic acid to glutamic acid [10], sequence analysis of the low molecular mass TRH-like peptide and the immunoreactive fragment from the polypeptide demonstrated the sequence Glu-Glu-Pro (fig.2). The yields of the amino acids in each of the three cycles of Edman degradation were similar to those of the synthetic peptide pGlu-Glu-ProNH₂. It is interesting that proline was observed in the second round of Edman degradation of the low molecular mass peptide from rabbit prostate [6] which we had previously suggested may be 'due to slight cleavage of the Glu-Glu peptide bond during the opening of the pyroglutamyl ring'. In the present study, although both of the purified peptides and the synthetic peptide were subjected to mild acid hydrolysis under identical conditions, proline was observed in the second round of Edman degradation of only the low molecular mass TRH-like peptide. An alternative explanation of these results is that the low molecular mass peptides purified from both rabbit prostate and human semen contain a minor dipeptide contaminant. Glu-Pro diketopiperazine would not react with the TRH antibody used in this study but may have similar chromatographic properties on the ion exchangers used for purification. The diketopiperazine elutes slightly after pGlu-Glu-ProNH₂ during reverse-phase HPLC and incomplete separation of the two peptides may lead to a minor contamination with

the dipeptide. The conditions of mild acid hydrolysis used for sample treatment before gas-phase sequencing may be sufficient to open the ring structure of the diketopiperazine to reveal a free N-terminus for Edman degradation.

Synthetic pGlu-Glu-ProNH₂ and purified peptides were subjected to FAB mass spectrometry and the spectra, generated on the same day and using the same calibration file, are illustrated in fig.3. A clear pseudomolecular ion, $(M+H)^+ =$

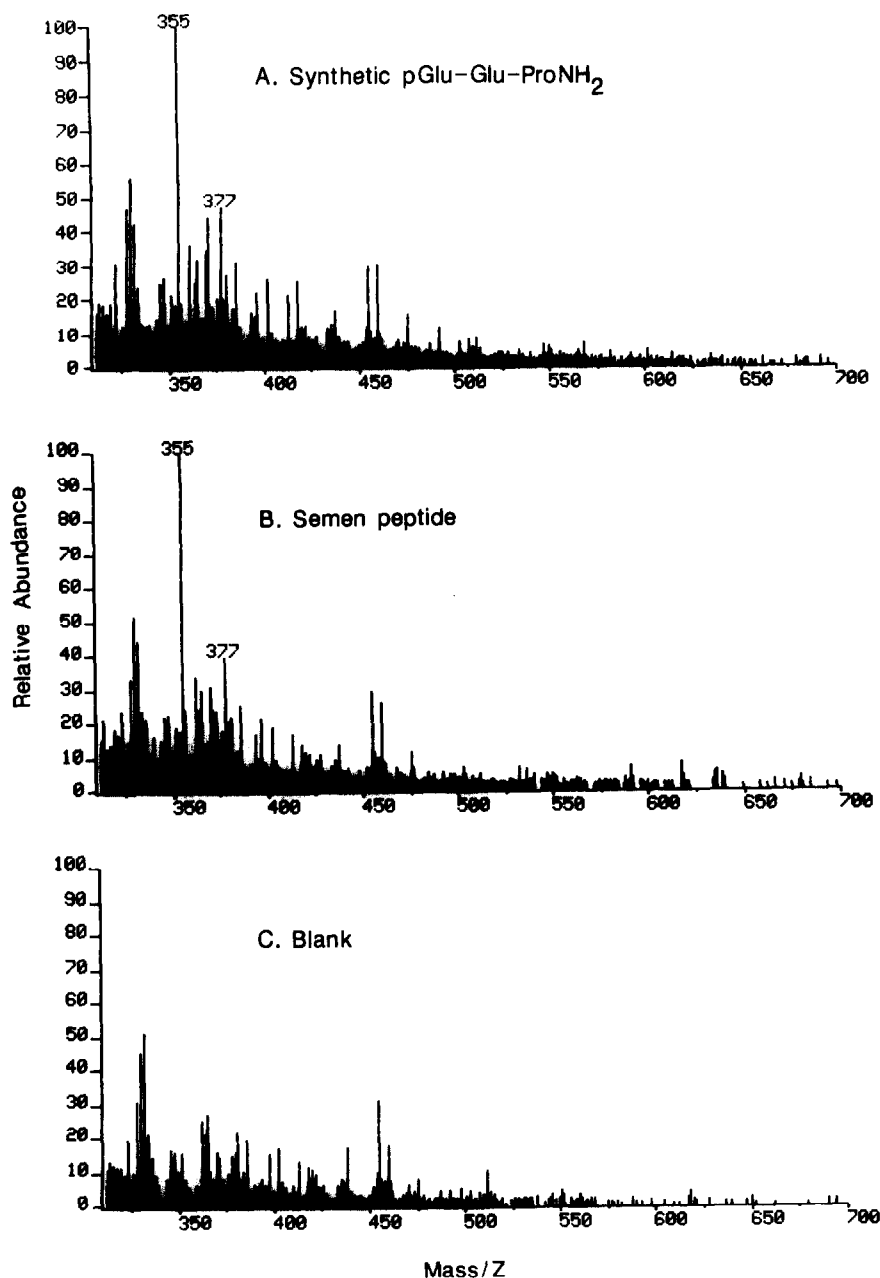


Fig.3. Structural analysis of TRH-related peptide in human semen by FAB mass spectrometry. The spectra illustrated are taken at the same time interval after introduction of the sample into the FAB source (scan 13). (A) 700 pmol synthetic peptide pGlu-Glu-ProNH₂, (B) 700 pmol purified TRH-immunoreactive fragment from the 10–12 kDa polypeptide and (C) HPLC buffer (blank).

355.38, was observed in the positive ion mode for the endogenous peptide which was identical to that for the synthetic peptide, 355.38. Additional peaks in the spectra represented matrix or buffer components (329, 331, 361, 455) and were present in the buffer blank with the exception of the ion at 377 which represented the sodium adduct of the peptide, $(M + Na)^+ = 354 + 23$. These results confirmed the structure of the purified peptide from human semen as pGlu-Glu-ProNH₂.

In conclusion, it is clear that the TRH-like tripeptide in human semen is pyroglutamylglutamylprolinamide. This peptide occurs naturally in the semen but can also be generated in vitro after tryptic cleavage of a polypeptide (10–12 kDa) and incubation under conditions which facilitate cyclisation of N-terminal glutamine. Thus, the polypeptide must terminate in the sequence Gln-Glu-ProNH₂. As the tripeptide is released by trypsin, the TRH-like sequence must be immediately preceded by at least one basic amino acid residue. It is likely that the new tripeptide is formed from the polypeptide in vivo by proteolytic processing at this site [13] followed by N-terminal cyclisation of the exposed glutamyl residue by glutamyl cyclase [14,15].

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