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*Short Communication*

# Methods used in the study of TRH-like peptides in rat prostate*

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## Introduction

Recent studies have demonstrated the presence of a series of thyrotropin-releasing hormone (TRH)-like peptides in the male reproductive system which differ from TRH (*p*Glu-His-Pro amide) in the replacement of histidine at position 2 of the tripeptide by an acidic or neutral amino acid [1–4]. The absence of a basic group in these 'TRH-like' peptides permits their separation from TRH by chromatography on a cation exchange resin and the distinction can be confirmed by high-performance liquid chromatography (HPLC). The major TRH-like peptide in rabbit prostate has been identified as *p*Glu-Glu-Pro amide [2], and a neutral TRH-like peptide present in human semen has been identified as *p*Glu-Phe-Pro amide [5]. In addition, at least two other neutral TRH-like peptides are present in human semen.

The sequences of the TRH-like peptides terminate in an α-amide group which is a characteristic feature of many peptides that possess biological activity. It is of interest, therefore, to examine whether the tissue concentrations of these peptides are influenced by hormone status. As a first step towards understanding their function, an investigation has been undertaken on whether the new peptides are sensitive to hormones known to influence the tissues in which they occur. In this communication the methods used to determine the concentrations of TRH-like peptides are described and a preliminary report is made on the effects produced by testosterone on the peptides in rat prostate.

## Experimental

*Extraction of prostate tissue*

Prostate tissue was obtained by dissection from male Wistar rats and the tissue was immediately frozen and stored at −80°C. In a group of four castrated rats, testosterone cyprionate (200 μg) was administered daily by a subcutaneous route over a period of 1 week, after which the animals were killed by nembutal anaesthesia. For comparison, a second group of four castrated Wistar rats was treated in parallel but without administration of the steroid. In addition, the prostates were removed from four sham castrated rats as a control.

The tissues (two in each experiment) were homogenized at 13,000 rpm for 1 min at room temperature in 1 M acetic acid containing $^{125}$I-TRH (2000 cpm; 0.2 μg synthetic TRH was labelled with 1 mCi of $^{125}$I by the chloramine T method) using an Ultra-Turrax T25 homogenizer (Janke and Kunkel gmbH and Co. KG, Staufen, Germany). The remaining two tissues

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in each series were homogenized in parallel, the same procedure being used for the control, castrated and hormone-treated animals. The suspensions were allowed to stand for 1 min and homogenization was repeated for a further 1-min period; after 10 min at room temperature, the suspensions were raised to approximately 100°C to inactivate degrading enzymes. The suspensions were centrifuged for 30 min at 20,000 rpm, 4°C, and the supernatant liquids were evaporated to dryness *in vacuo*. The residues were extracted three times with methanol (5 ml) and the solvent was removed by evaporation *in vacuo*. The peptides were taken up in 1.5 ml of 50% acetic acid (v/v), clarified by centrifugation in a microfuge tube, and added to mini-columns of SP-Sephadex C25 (KB, Uppsala, Sweden).

*Resolution of TRH-related peptides by mini-column chromatography*

Cation exchange chromatography was carried out on columns (0.9 × 6 cm) of SP Sephadex C25 prepared in the pyridinium form by washing with 1 M hydrochloric acid, water; 1 M pyridine, water; and finally with 50% acetic acid. Neutral and acidic peptides were eluted in 50% acetic acid (8 × 0.5 ml fractions), and a further 12 fractions (0.5 ml) containing basic peptides were collected with 0.4 M pyridine in 50% acetic acid as eluent. Aliquots (50 μl) were taken for radioimmunoassay (RIA) with TRH antibody after removal of the acetic acid *in vacuo*. The retention of TRH on the mini-column was confirmed by including $^{125}$I-TRH (2000 cpm) in the sample added to the column and its elution was detected by γ-counting.

*Resolution of TRH-related peptides by HPLC*

HPLC was performed on a μBondapak C18 column (0.39 × 30 cm, dp 10 μm, Millipore–Waters U.K., Watford, Herts, UK) using a linear 10 mM hydrochloric acid–methanol gradient and a flow rate of 1.5 ml min$^{-1}$. Minor changes in the gradient were employed to achieve the most satisfactory separation of TRH-like peptides, as indicated in the figures. The elution positions of the peptides were detected by RIA after the fractions had been dried *in vacuo*.

In the experiment with testosterone, four laboratory synthesized $^{3}$H-labelled marker peptides [6] were added to the prostate extracts before HPLC analysis to indicate the elution positions of TRH, *p*Glu-Glu-Pro amide, *p*Glu-Val-Pro amide, and *p*Glu-Phe-Pro amide. Their elution positions were detected by scintillation counting.

*Radioimmunoassay*

The concentrations of TRH-like peptides were determined by RIA using a sheep antiserum [7] raised against TRH. The details of the procedure have been described previously [4]. It may be noted that the immunoreactivities of *p*Glu-Glu-Pro amide and *p*Glu-Phe-Pro amide are approximately 50% of the immunoreactivity of TRH; thus the concentrations of these peptides shown in the figures represent minimum values. The TRH-antibody was used at a final dilution of 1:96,000 and the separation of bound from free ligand was by the use of 20% (w/v) polyethylene glycol and heat inactivated horse serum.

## Results

*TRH-like peptides in the prostates of castrated rats: analysis by mini-column chromatography*

In all experiments, the peptides obtained from two prostates in each experimental group were resolved by chromatography on SP-Sephadex C25 to give an unretained fraction when eluted with 50% acetic acid (neutral and acidic TRH-like peptides) and a retained fraction when eluted with 0.4 M pyridine in acetic acid (basic TRH-like peptides). A typical elution pattern is shown in Fig. 1. The amounts of non-retained TRH-like peptides obtained were 1.6 and 3.9 pmoles immunoreactive TRH (ir-TRH) per gram of tissue; the concentrations of non-retained TRH-like peptides obtained from the castrated rats were 5.4 and 9.0 pmoles ir-TRH per gram of tissue; the concentrations in the castrated, testosterone-treated castrate rats were 1.9 and 2.6 pmoles ir-TRH per gram of tissue. These results suggest that the rat prostate concentration of TRH-like peptide is elevated by castration and that testosterone treatment of castrated rats restores the normal levels of TRH.

*Fractionation of TRH-like peptides from rat prostates by HPLC*

Experiments were carried out with different gradient profiles with a view to obtaining optimal resolution of four synthetic TRH-like peptides. The best separation was obtained by

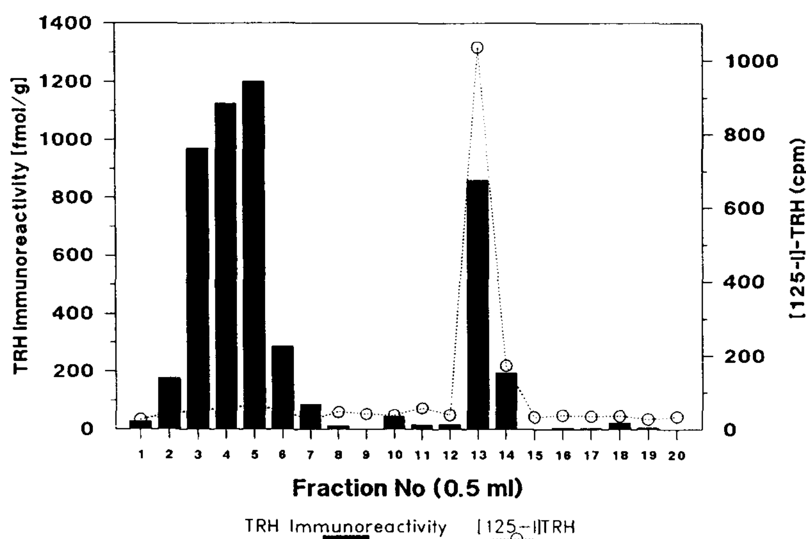


**Figure 1**
Mini-column cation exchange chromatography of TRH-like peptides from rat prostate. The chromatography was carried out as described in the Methods section. Note that the majority of the TRH-immunoreactivity was not retained on the column and can thus be distinguished from TRH.

employing a 0.5% $min^{-1}$ linear gradient of methanol for the first 20 fractions (0.3 min each) and then a 2% $min^{-1}$ gradient for the following 80 fractions (Fig. 2). By using this procedure, it was shown that the principal peptide in the unretained fractions from ion-exchange mini-column chromatography of the prostate extracts corresponded chromatographically to *p*Glu-Glu-Pro amide (Fig. 3). Other peptides were also present but in much lower concentrations. The identification of *p*Glu-Glu-Pro amide was confirmed by inclusion of four $^{3}$H-labelled marker peptides in the mixture resolved by HPLC. These were: $^{3}$H-TRH (fraction 33), $^{3}$H-*p*Glu-Glu-Pro amide (fraction 48), $^{3}$H-*p*Glu-Val-Pro amide (fraction 63), and $^{3}$H-*p*Glu-Phe-Pro amide (fraction 84). The major immunoreactive component corresponded to $^{3}$H-*p*Glu-Glu-Pro amide. The amounts of *p*Glu-Glu-Pro amide observed in the mixtures resolved by HPLC were as follows: the control rats contained 0.8 pmoles $g^{-1}$, the castrated rats contained 3.1 pmoles $g^{-1}$, and the castrated, testosterone-treated rats contained 0.6 pmoles $g^{-1}$,

Purification of the retained immunoreactive components from the mini-columns was carried out by HPLC under the same conditions that were employed with the non-retained peptides. Minor immunoreactive components were observed but negligible concentrations of immunoreactive components chromatographed in the position of $^{3}$H-TRH.

## Discussion

The procedure described for the resolution of TRH-like peptides has been applied in a study of the effects of castration and testosterone replacement on the levels of these peptides in rat prostate. Within the limits of the number of experiments carried out, it is apparent that castration leads to a substantial increase in the concentrations of TRH-like peptides and that testosterone treatment restores the levels of these peptides to the normal values. The experiments also provide good evidence that the principal TRH-like peptide in the prostate is either *p*Glu-Glu-Pro amide or another TRH-like peptide with similar HPLC mobility.

The methods employed in this study involve mini-column chromatography on columns of SP-Sephadex C25 in a dissociating solvent (50% acetic acid), followed by HPLC in 10 mM hydrochloric acid, using a refinement of our previous procedure [8] which involved a linear methanol gradient. The retention of $^{125}$I-TRH during the ion-exchange step confirms that the columns would retain endogenous TRH if it were present and consequently the peptides in the non-retained fractions do not include this hormone. This was further demonstrated by the second stage of purification which involved HPLC with methanol gradients. With the non-retained fractions from the ion-exchange step, no immuno-

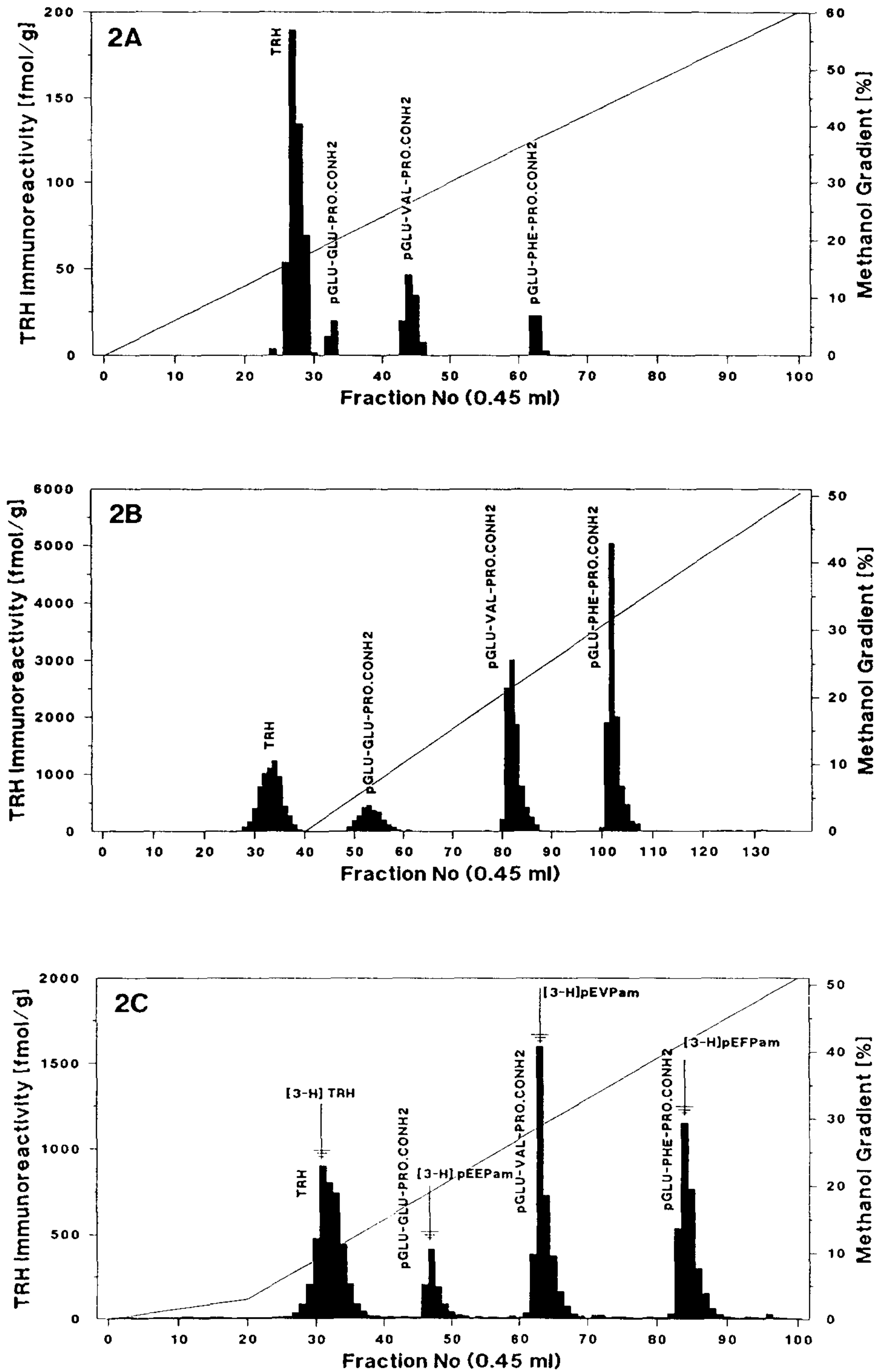


**Figure 2**
Resolution of synthetic TRH-like peptides by HPLC. In (A), there is insufficient resolution of TRH from *p*Glu-Glu-Pro amide; in (B), the TRH peak is relatively broad due to the isocratic elution; in (C) the four TRH marker peptides are effectively resolved.

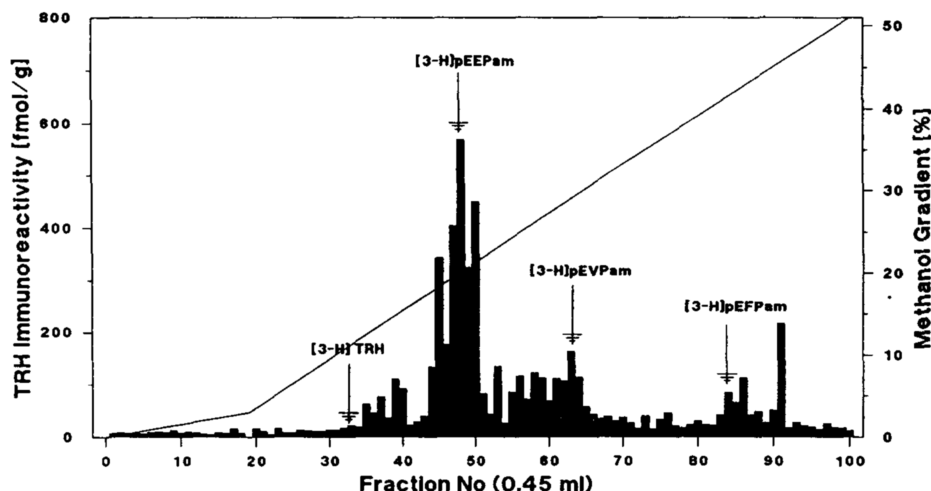


**Figure 3**
Resolution of TRH-like peptides from rate prostate by HPLC. The arrows (left to right) indicate the elution positions of $^3$H-TRH, $^3$H-*p*Glu-Glu-Pro amide, $^3$H-*p*Glu-Val-Pro amide, and $^3$H-*p*Glu-Phe-Pro amide, respectively. Note the principal TRH-like peptide corresponds chromatographically to *p*Glu-Glu-Pro amide.

reactive components co-chromatographed with $^3$H-TRH; a similar result was obtained by HPLC of the retained fractions. Thus, it is clear that the TRH-immunoreactive peptides in the prostate do not include TRH itself.

The methodology described has been used to demonstrate the absence of TRH in rat prostate and the results indicate that *p*Glu-Glu-Pro amide is a prominent TRH-like peptide in this tissue. The concentrations of this peptide are sensitive to a hormone that has a profound effect on the male reproductive system.

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