Isolation and Structural Determination of a Novel TRH-like Tripeptide, pyroGlu-Tyr-Pro Amide, from Alfalfa*

David B. Lackey
From the Laboratory of Peptide Chemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

The tripeptide pyroGlu-Tyr-Pro amide was isolated from an aqueous extract prepared from dried alfalfa pellets. The tripeptide was quantitated using a competitive radioimmunoassay in which \(^{125}\text{I}\)-labeled thyrotropin-releasing hormone (TRH), is displaced from antibody specific to TRH (pyroGlu-His-Pro amide). The tripeptide pyroGlu-Tyr-Pro amide was purified by passing the filtered extract through QAE-Sephadex A25 at pH 5, followed by open bed chromatography on C\(_18\) silica using an H\(_2\)O/methanol gradient, then preparative high performance liquid chromatography (HPLC) on microbondapak C\(_18\) using a 10 mM HCl/methanol gradient, followed by G-10-Sephadex chromatography, SP-C25-Sephadex chromatography, QAE-Sephadex chromatography at pH 10.1, analytical HPLC on a microbondapak C\(_8\) column eluted with 10 mM HCl/acetonitrile, and analytical HPLC reverse phase chromatography on an APEX phenyl column eluted with H\(_2\)O/acetonitrile. The tripeptide was essentially homogeneous after the final chromatography step, as judged by correspondence of immunoreactivity with \(A_{280}\).

The sequence of the alfalfa tripeptide was determined to be Glu-Tyr-Pro by gas phase sequencing, after hydrolysis of pyroglutamic acid by mild acid hydrolysis. The mass of the alfalfa tripeptide was 389.1, as determined by fast ion bombardment mass spectrometry, and was found to be identical to the mass of synthetic pyroGlu-Tyr-Pro amide. The sequence of the alfalfa tripeptide was also confirmed using B/E-linked scanning. I conclude that the tripeptide isolated from alfalfa differs from human thyrotropin-releasing hormone only by the substitution of tyrosine for histidine at position 2.

The role of pyroGlu-Tyr-Pro amide in alfalfa is not known, but the existence of a family of thyrotropin-related peptides occurring in both the animal and the plant kingdoms indicates that the thyrotropin related peptides have a wide phylogenetic distribution.

Large amounts (157 ± 6 ng/g) of material with the immunoreactive characteristics of thyrotropin-releasing hormone can be extracted from dried alfalfa, and it has been suggested that this immunoreactivity is due to the presence of a tripeptide differing from thyrotropin-releasing hormone by a point change in the histidine residue (1). TRH\(^{-}\)-related peptides, identical to thyrotropin-releasing hormone except for the substitution of the histidine residue by another amino acid, have also been identified in various animal tissues and fluids. For example, the acidic tripeptide pyroGlu-Glu-Pro amide has been isolated from rabbit prostate (2) and human semen (3), and recently, two TRH-related neutral tripeptides, pyroGlu-Phe-Pro amide and pyroGlu-Glu-Pro amide, have been identified in human semen (4).

In this paper, I describe the isolation and structural determination of a novel TRH-related peptide, pyroGlu-Pro amide, which is present in high concentrations in dried alfalfa.

MATERIALS AND METHODS

Analytical grade chemicals were obtained from BDH Chemicals Ltd (Dorset, England), AE54, and Fmoc-His(2-aminoethyl)-derivatives, stabilizers, or binders. Antiserum specific for TRH was a gift from Dr. H. M. Fraser (MRC Reproductive Biology Unit, Edinburgh University, Scotland, U.K.).

Preparation of Synthetic TRH-like Peptides—pyroGlu-Tyr-Pro amide was synthesized on the polystyrene resin, Fmoc-Ultrosyn C, by a solid phase procedure using a Cambridge Research Biochemicals peptide synthesizer (Pepsynthesizer II, Norwich, Cheshire, U.K.). Coupling was performed with the pentafluorophenyl esters of the amino acids in the presence of 1-hydroxybenzotriazole. The peptides were cleaved from the support by trifluoroacetic acid. The crude products were isolated by reverse-phase HPLC on a C\(_18\) Microbondapak column (0.39 \(\times\) 30 cm) after precipitation by addition of ether.

Detection of TRH-like Tripeptides by Radioimmunoassay—TRH immunoreactivity was assayed essentially as described previously (5, 6). Aliquots of samples to be assayed were dried under vacuum to remove acetic acid or HCl, if necessary. 200 \(\mu\)l of 50 mM NaPO\(_4\), pH 7.5, containing 250 \(\mu\)g/ml bovine serum albumin, 50 \(\mu\)l of \(^{125}\text{I}\)-TRH, and 50 \(\mu\)l of TRH antiserum (1:48,000 dilution) (7) was added to each unknown, and to a series of 12 doubling dilutions of a TRH standard, beginning with 16 pmol of TRH in the first tube. The unknowns and standards were incubated at room temperature for 2 h, or overnight at 4 °C. After this incubation, 200 \(\mu\)l of a 3% (w/v) solution of activated charcoal in 0.05 M NaPO\(_4\) containing 66% horse serum and 0.74% dextran was added. Samples were then centrifuged at 2,000 \(\times\) g for 15 min in a Beckman GS ultracentrifuge at 4 °C.

Isolation of TRH-IR from Alfalfa—2 kg of alfalfa pellets were reduced to powder in a Waring Blender, then allowed to steep in distilled water for 60 min at room temperature. Solid material was removed by vacuum filtration through a cotton tea-towel cut to fit a Buchner funnel. The vacuum filtrate was clarified by two rounds of
Isolation of pyroGlu-Tyr-Pro Amide

FIG. 1. Phenyl reverse-phase chromatography of purified alfalfa TRH-immunoreactive material. Alfalfa peptide was purified as described under “Materials and Methods” and applied to a Jones Apex phenyl RPC column as the final purification step. Material was eluted with a gradient of distilled water/acetonitrile. Column fractions were assayed for TRH immunoreactivity as described under “Materials and Methods.”

FIG. 2. Gas phase sequencing of purified alfalfa tripeptide. The alfalfa tripeptide was analyzed after hydrolysis of the pyroGlu ring in 3 M HCl for 10 min at 100 °C.

FIG. 3. Mass spectroscopy of purified alfalfa tripeptide and synthetic pyroGlu-His-Pro amide. 6 nmol of each peptide was analyzed by fast atom bombardment mass spectroscopy in the positive ion mode on a VG70-250 SE mass spectrometer.

filtration through Whatman No. 1 filter paper, and the clarified filtrate was passed through an Amicon YM2 ultrafiltration membrane at 70 psi nitrogen in an Amicon 8400 filtration apparatus.

The ultrafiltrate was passed through a 250-ml column of QAE-Sephadex A25 in distilled water, and fractions containing TRH immunoreactivity were applied directly to an 80-ml reverse phase column (2 × 30 cm) consisting of Bio-Sil C-18 HL Silica (fully end-capped) 40-63 mm. The Bio-Sil column was washed with 10 column volumes of distilled water, then eluted with a methanol gradient (500 ml of distilled water, 500 ml of 60% methanol). Fractions (10 ml) were assayed for TRH immunoreactivity, and peak fractions were pooled and concentrated by rotary evaporation. The concentrated material was further purified by preparative HPLC on a Waters Microbondapak C18 column (3.9 × 300 mm). TRH immunoreactivity was eluted with a 0.25% gradient of 10 mM HCl/methanol at a flow rate of 3 ml/min. Fractions were collected at 0.3-min intervals, and peak fractions were pooled and evaporated to dryness in 10 × 75-mm polystyrene tubes using a Uniscience vacuum centrifuge. TRH immunoreactivity was redissolved in 25 mM NH₂OH, pH 10.1, and applied to a 2 ml column of QAE A25-Sephadex equilibrated with the same buffer. After washing the column with several volumes of distilled water, the TRH immunoreactivity was eluted with 25 mM Tris, pH 7.5. Fractions containing immunoreactivity were applied to a Jones Apex phenyl RPC column (4.6 × 250 mm) and eluted with a 0.25% gradient of distilled water/acetonitrile at a flow rate of 1.5 ml/min. Fractions were collected at 0.3-min intervals.

Automated Sequence Analysis—NH₂-terminal pyroglutamic acid residues were converted to glutamic acid by incubation for 10 min in 3 M HCl at 100 °C. (8). The Applied Biosystems 470A gas-phase sequenator was used to determine amino acid sequence; an Applied Biosystems 120A analyzer was used to detect phenylthiohydantoin amino acids (9). An Applied Biosystems 900A module calibrated with 25 pmol of phenylthiohydantoin amino acid standard was used for data collection and analysis.

Mass Spectrometry—Peptides in a matrix consisting of thioglycerol and 1% trifluoroacetic acid were analyzed by fast atom bombardment mass spectroscopy utilizing normal scanning in the positive ion mode on a VG 70-250 SE mass spectrometer at an operating voltage of 8 keV, using a 35 keV cesium ion gun (10). A linked scan spectrum at constant B/E was used to determine the masses of the products formed by decomposition of the tripeptide MH⁺ ion (11).
Isolation of pyroGlu-Tyr-Pro Amide

RESULTS

TRH immunoreactivity was isolated from dry alfalfa by a succession of ultrafiltration, cation exchange, anion exchange, size exclusion, and reverse-phase chromatography procedures, as described under "Materials and Methods." Adsorption to QAE A25 at pH 10.1 was used as a purification step, to take advantage of the deprotonation of the tyrosine phenolic hydroxyl at alkaline pH; TRH-IR was eluted from QAE at neutral pH. Chromatography of the purified material by analytical HPLC on a reverse-phase phenyl column resulted in the co-chromatography of TRH immunoreactivity with the major peak of \( A_{280} \) (Fig. 1), indicating that the TRH-IR material was sufficiently pure for sequence determination after this step.

After mild acid hydrolysis to open the \( \text{NH}_2 \)-terminal pyroGlu ring, the sequence Glu-Tyr-Pro was obtained by analysis of the purified TRH immunoreactivity by gas-phase sequencing (Fig. 2). The repetitive yield of the alfalfa tripeptide was approximately the same as that obtained for synthetic pyroGlu-Tyr-Pro (data not shown), indicating that the efficiency of the Edman reaction was similar for both peptides.

The antibody used to detect TRH immunoreactivity is highly specific for the structure at the amino and carboxyl termini of the tripeptide, requiring an intact pyroglutamate ring and carboxyl-terminal amide group for recognition (7). Furthermore, the alfalfa tripeptide is not retained by either anion (QAE A25) or cation (SP C25) exchange Sephadex columns, indicating that the tripeptide is uncharged at neutral pH. The structure of the tripeptide termini was verified by subjecting the purified alfalfa tripeptide to mass spectroscopy in the positive ion mode. A single major mass ion corresponding to a molecular mass of 389 daltons was observed in fractions from the final reverse-phase HPLC column containing peak immunoreactivity, indicating that the structure of the alfalfa tripeptide is indeed pyroGlu-Tyr-Pro amide (Fig. 3). The mass ion corresponding to 390 daltons is probably attributable to \( ^{13} \text{C} \) pyroGlu-Tyr-Pro amide; a similar peak is observed in the mass spectrum of synthetic pyroGlu-Tyr-Pro amide. The mass ion corresponding to 389 daltons (pyroGlu-Tyr-Pro amide) was not present in fractions from the final reverse-phase HPLC column immediately preceding or following the peak of immunoreactivity, indicating that this molecular structure is indeed responsible for the observed peak of immunoreactivity (data not shown).

REFERENCES

1. Beaton, A. J., and Wurtman, R. J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2749-2753.

2. Udenfriend, S., and Michelson, A. M. (1964) Anal. Biochem. 11, 509-517.

3. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

4. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

5. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

6. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

7. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

8. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

9. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

10. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

11. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

12. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

13. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

14. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

15. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

16. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

17. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

18. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

19. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

20. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.

21. Udenfriend, S., and Michelson, A. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 516-523.
decomposition products at constant B/E (Fig. 4) shows daughter ions formed by the cleavage of specific bonds of the tripeptide pyroGlu-Tyr-Pro amide. Mass ions corresponding to 84, 115, 247, 262, 344, and 372 daltons are formed by breaking individual tripeptide bonds, as indicated in Fig. 5.

Because not all bonds are equally susceptible to collision-induced decomposition, not all possible products are observed. The pattern of bonds broken observed for the purified alfalfa tripeptide was identical with the pattern given by the synthetic tripeptide (data not shown). Typically, only one of the two daughter fragments formed by cleavage of a given bond will produce a stable mass ion. Several mass ions were observed that could not be assigned to possible daughter products of pyroGlu-Tyr-Pro amide; it is conceivable that some of these may be due to collection of product ions formed during acceleration or during passage through the electric sector of the mass spectrometer.

As an additional check on the identity of the alfalfa TRH-immunoreactive peptide, the HPLC retention time of purified alfalfa tripeptide was compared with that of synthetic pyroGlu-Tyr-Pro amide on a Microbondapak analytical C18 column. The retention times of the alfalfa peptide was found to be identical to that of synthetic pyroGlu-Tyr-Pro amide (Fig. 6).

DISCUSSION

It is noteworthy that the level of TRH immunoreactivity in dried alfalfa is more than half the level of TRH immunoreactivity present in the rat hypothalamus (3). The presence of high levels of TRH immunoreactivity in extracts prepared from the alfalfa plant suggests that the tripeptide pyroGlu-Tyr-Pro amide may have a biological role; in mammals, peptides that terminate in the α-amide group are, as a rule, biologically active, and the α-amide group is generally required for functional activity. Although the role of peptides in plants has not been as extensively studied as the peptide hormones of mammals, secretin, an 18-amino acid peptide isolated from the tomato plant, has been shown to induce protease inhibitors in tomato at very low concentrations (14). The tripeptide pyroGlu-Tyr-Pro amide may also function in alfalfa as an endogenous signal; alternatively, pyroGlu-Tyr-Pro amide may be involved in the interaction between the plant and insects, perhaps as a deterrent to predators, or as an attractant to facilitate pollination.

The structural similarity of the alfalfa tripeptide and the hypothalamic releasing hormone TRH results from the substitution of tyrosine in the place of the histidine of TRH. In this regard, it may be recalled that TRH-related peptides have been detected in a number of mammalian tissues (6, 12, 15–18), and that the tripeptides pyroGlu-Glu-Pro amide, pyroGlu-Phe-Pro amide, and pyroGlu-Gln-Pro amide have been unambiguously identified in human semen. The biological role of these three TRH-related peptides from human semen has not yet been determined, but a role in fertility is one possibility.

From these results, it seems likely that the family of tripeptides related structurally to mammalian thyrotropin-releasing hormone may represent a diverse and widely distributed group of structurally related peptides. In this regard, I have recently identified two new TRH-immunoreactive peptides by sequencing partially purified fractions of alfalfa. These tripeptides differ from TRH only by the substitution of phenylalanine and leucine, respectively, for histidine. At present, it is not possible to state positively whether the biological role of these peptides will be similar, or whether the genes for the peptides will be found to have a common ancestor in animals and plants. However, the structural determination and synthesis of pyroGlu-Tyr-Pro amide and other TRH-related peptides is expected to facilitate this research.

Acknowledgments—I thank Dr. Derek Smyth for many insightful discussions and critical reading of this manuscript. I am also grateful to Dr. H. M. Fraser for his generous gift of antiserum directed against TRH, to Alan Harris for the gas-phase peptide sequencing, to Stephen Howell for performing the mass spectroscopy, and to Rita Findon for assistance in the preparation of this manuscript.

REFERENCES

1. Jackson, I. M. D. (1961) J. Endocrinol. 108, 344–346
2. Cockle, S. M., Aitken, A., Beg, F., and Smyth, D. G. (1989) J. Biol. Chem. 264, 7786–7791
3. Cockle, S. M., Aitken, A., Beg, F., and Smyth, D. G. (1989) FEBS Lett. 252, 113–117
4. Khan, Z., Aitken, A., de Río Garcia, J., and Smyth, D. G. (1992) J. Biol. Chem. 267, 7404–7409
5. Zakarian, S., and Smyth, D. G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5972–5976
6. de Río Garcia, J., and Smyth, D. G. (1990) J. Endocrinol. 127, 445–450
7. Fraser, H. M., and McNeilley, A. S. (1982) Endocrinology 111, 1964–1973
8. Stark, G. R., and Smyth, D. G. (1983) J. Biol. Chem. 258, 214–226
9. Norrice, N., Geary, P., Cammack, R., Harris, A., Beg, F., and Aitken, A. (1988) FEBS Lett. 231, 336–340
10. Aitken, A., and Cohen, P. (1984) Methods Enzymol. 106, 205–210
11. Jennings, K. R., and Delnake, G. G. (1990) Methods Enzymol. 193, 37–61
12. Youngblood, W. W., Lipton, M. A., and Kizer, J. S. (1979) Brain Res. 151, 99–116
13. Jackson, I. M. D., and Reichlin, S. (1974) Endocrinology 95, 854–858
14. Pearce, G., Strydom, D., Johnson, S., and Ryan, C. A. (1991) Science 253, 890–898
15. Pekary, A. E., Rosen, J. I., Geola, F., Vaillant, C., Sharp, B., Meyer, N., and Hershman, J. M. (1981) Biochem. Biophys. Res. Commun. 99, 73–80
16. Youngblood, W. W., Humm, J., and Kizer, J. S. (1979) Brain Res. 163, 101–110
17. Sheward, W. J., Harmer, A. J., Fraser, H. M., and Fink, G. (1983) Endocrinology 113, 1865–1569
18. Youngblood, W. W., Humm, J., Lipton, M. A., and Kizer, J. S. (1980) Endocrinology 106, 541–546

D. B. Lackey, unpublished results.