STRUCTURE OF THE PORCINE LH- AND FSH-RELEASING HORMONE. II. CONFIRMATION OF THE PROPOSED STRUCTURE BY CONVENTIONAL SEQUENTIAL ANALYSES.*

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Summary. The proposed amino acid sequence of porcine LH- and FSH-releasing hormone (LH-RH/FSH-RH) was reinvestigated by Edman-dansyl degradation after the cleavage of N-terminal pyroglutamyl residue by pyrrolidonecarboxylyl (PCA) peptidase. A C-terminal fragment from chymotryptic digest of LH-RH/FSH-RH was found to be identical to synthetic Gly-Leu-Arg-Pro-Gly-NH_2. The results indicate that the structure initially proposed is correct. The amino acid sequence of porcine LH-RH/FSH-RH is thus (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.

In a previous communication (1) we proposed (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 as the amino acid sequence of LH and FSH releasing hormone (LH-RH/FSH-RH) isolated from porcine hypothalami. This structure was based mainly on the results of Edman-dansyl degradation and selective tritiation of the C-terminus which were applied directly to the chymotryptic and thermolytic digests of this hormonal polypeptide. A limited amount of the pure material (about 200 nmoles) and the blocked N-terminus as well as the C-terminus forced us to use this unconventional approach. After using less than half of the material, we were able nevertheless, to narrow down the possibilities to only one amino acid sequence. We tried to confirm this proposed amino acid sequence in two ways: (1) by conventional degradation methods for sequential analyses on the remainder of the pure natural material, (2) by the synthesis of the proposed structure. This report describes the results obtained following our first approach.

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MATERIALS AND METHODS

LH-RH/FSH-RH used in this study was isolated from pig hypothalami as described by Schally et al. (2).

Edman-Dansyl Degradation: Edman-dansyl degradation was performed as described in our previous communication (1). Polyamide sheets were used for the identification of DNS-amino acids. For DNS-histidine and DNS-arginine silica gel plates were also used and the developing solvent system consisted of benzene-2-chloroethanol-28% ammonia (8:10:4). This modification of the solvent II described by Gross and Labouesse (3) was found to separate better these two basic amino acids.

PCA peptidase Digestion: Pyrrolidone carboxylyl (PCA) peptidase (4) (G-200 and A-25 preparations) was supplied by Dr. R.F. Doolittle. Generally, the enzyme preparations, precipitated by ammonium sulfate containing pyrrolidone as a stabilizer, were dialyzed twice against 0.05 M phosphate buffer pH 7.3, in presence of 0.01 M mercaptoethanol and 0.001 M EDTA just before use. The concentration of the enzyme was estimated by absorption at 280 mμ (4). Three digestions were made using this enzyme.

Digestion I. LH-RH/FSH-RH (3 nmoles) was digested by G-200 preparation of the enzyme at 30° C for 17 hours in 5 μl of the same buffer as used for the dialysis. The optical density (O.D.) of the enzyme in the reaction medium was 0.29 at 280 mμ. A control without LH-RH/FSH-RH was run simultaneously. After the digestion, the solution was evaporated and the residue was dansylated directly.

Digestion II. LH-RH/FSH-RH (6 nmoles) was digested in 10 μl buffer by A-25 preparation as in digestion I. The O.D. of the enzyme was 0.28 at 280 mμ.

Digestion III. LH-RH/FSH-RH (30 nmoles) was digested in 10 μl buffer by A-25 preparation (the same lot No. as used in Digestion II). The initial O.D. of the enzyme was 0.20 at 280 mμ. Further additions of 1 μl of the enzyme solution (OD_{280} = 1.00) were made after two and after four hours. The final volume of the reaction mixture was 12 μl, and the O.D. of the enzyme was found to be 0.33 at 280 mμ.

Synthesis of Gly-Leu-Arg-Pro-Gly-NH_2: Solid phase synthesis of the C-terminal pentapeptide amide of the proposed sequence was carried out by the procedure described by Stewart and Young (5). t-BOC-amino acids, and t-BOC- glycine resin ester were obtained from Mann Research Lab. For the removal of the solid support and the protecting groups, ammonolysis by methanol saturated with ammonia, followed by the catalytic reduction over palladium on charcoal and the treatment with trifluoroacetic acid in dichloromethane were employed. The resulting peptide was repurified by preparative TLC on cellulose. After hydrolysis, the amino acid ratios of this preparation were found to be: Pro 0.82, Gly 2.00, Leu 1.14, Arg 1.01.

Separation of Chymotryptic Fragments of LH-RH/FSH-RH: LH-RH/FSH-RH (80 nmoles) was digested by chymotrypsin (Worthington, 2 x recryst lot TRL 6259) in 200 µl of 0.1 M ammonium acetate buffer, pH 8.1 at 37° C for 16 hours. The E:S ratio was 1:20. After evaporation of the buffer, the resulting peptide fragments were separated by TLC on cellulose MN 300 HR, previously washed by the method of Haworth and Heathcote (6). The solvent system was 1-butanol-acetic acid-water (4:1:1). The sample was applied on half of the plate and the other half was used as a control, to subtract the amino acids contamination in cellulose. The guide regions were visualized by spraying with chlorine-tolidine reagent and the unsprayed bands were eluted by 0.2 N acetic acid. Amino acid analysis was performed as described previously (2).

RESULTS AND DISCUSSION

The N-terminal pyroglutamyl group of LH-RH/FSH-RH, proved by mass spectra (1, 2), was consistent with the failure to detect a free N-terminus by dansylation (2) as well as with the lack of inactivation by aminopeptidases (2). Except for the pyroglutamyl residue, no other glutamic acid or glutamine are present in this polypeptide. This indicated that LH-RH/FSH-RH was suitable for the application of Edman-dansyl degradation, if only the pyroglutamyl group could be removed, or if its pyrrolidone ring was opened. Attempts to use alkaline hydrolysis to open the pyrrolidone ring gave unde-

sirable results. PCA peptidase was then tried for this purpose. The G-200 preparation of the enzyme was found unsatisfactory (Digestion I), because of the probable contamination with endopeptidases. Dansylation of the enzyme digest showed at least four new N-termini: Gly, Leu, Tyr, and His and/or Ser. However, only DNS-histidine was detected when a more purified A-25 preparation was used (Digestion II). This confirmed the (pyro)Glu-His linkage, previously unproven. This result suggested that the digestion of LH-RH/FSH-RH by A-25 enzyme followed by Edman-dansyl degradation would be successful. A larger amount of LH-RH/FSH-RH (30 nmoles) was then digested (Digestion III) under slightly different conditions from that of Digestion II. The volume of the reaction medium was reduced because the presence of large amounts of inorganic salts would disturb the identification of DNS-amino acids on polyamide layer, and particularly, of DNS-serine, DNS-arginine and DNS-histidine. On N-terminal analysis of the enzyme digest, DNS-histidine appeared as the major spot together with some DNS-serine. When Edman-dansyl degradation was continued up to ninth step, the amino acid sequence His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly was confirmed clearly. At the attempted tenth step no DNS-amino acids were detected, other than a very faint spot of DNS-glycine. At the second, the third and the fourth steps, faint spots of DNS-tyrosine, DNS-glycine and DNS-leucine respectively were found in agreement with the unexpected cleavage of Trp-Ser linkage. Thus, the A-25 preparation of PCA peptidase proved to be useful for the sequential study on a micro scale of peptide whose N-terminus is pyroglutamic acid. However, more purified preparation of the enzyme to eliminate contaminating endopeptidase would have been preferable.

An attempt was also made to get further confirmation of the C-terminal amide group. After the digestion of LH-RH/FSH-RH by chymotrypsin, a fragment corresponding to synthetic Gly-Leu-Arg-Pro-Gly-NH_2 on TLC was found and eluted. The spot given by this fragment was strong and well separated and its mobility was exactly the same as that of

the synthetic peptide. The amino acid ratios of this fragment were: Pro 1.21, Gly 2.20, Leu 1.00, Arg 0.94.

These results are in good agreement with the proposed amino acid sequence of porcine LH-RH/FSH-RH (1). The amino acid sequence of this decapeptide which stimulates the release of both LH and FSH from the pituitary is (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (NH_2). The linkage Arg-Pro explains the lack of inactivation by trypsin (7). No acidic function was found in this polypeptide, nor in the other two hypothalamic hormones whose structures have been elucidated so far (thyrotropin-releasing hormone (8) and melanocyte-stimulating hormone-inhibiting hormone (9)). The synthesis of the amino acid sequence corresponding to LH-RH/FSH-RH will be reported shortly.

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