SYNTHESIS OF THE PORCINE LH- AND FSH-RELEASING HORMONE BY THE SOLID-PHASE METHOD[1]

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Summary. The decapeptide (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, corresponding to the amino acid sequence of the porcine LH- and FSH-releasing hormone (LH-RH/FSH-RH) has been synthesized by the solid phase method. After repurification, the synthetic product showed the same physico-chemical and biological properties as natural porcine LH-RH/FSH-RH.

Recently, we reported the isolation from porcine hypothalami of the peptide hormone which controls the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland (1). The amino acid sequence of this hypothalamic hormone, designated LH-releasing hormone/FSH-releasing hormone (LH-RH/FSH-RH) was established to be (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (2, 3). In order to confirm that this decapeptide actually represents LH-RH/FSH-RH, we carried out its synthesis by the Merrifield method (4). The present paper reports the details of the synthesis and the proof of identity of the synthetic decapeptide with natural porcine LH-RH/FSH-RH.

EXPERIMENTAL

The protected decapeptide resin ester (Boc-Gln-His-Trp-(O-Bzl-Ser)-(O-Bzl-Tyr)-Gly-Leu-(NO_2-Arg)-Pro-Gly-resin ester) corresponding to the amino acid sequence of LH-RH/FSH-RH was synthesized essentially by the method described by Stewart and Young (4), starting with Boc-Gly-resin ester (1.0 g: 0.35 mmole). The tert-butyloxycarbonyl (Boc) group was used for protecting the α-amino group of all amino acids including the

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following derivatives; Gln(ONp), O-Bzl-Ser, O-Bzl-Tyr, and NO_2-Arg. (All these amino acid derivatives were obtained from Schwartz-Mann, New York).

Coupling was achieved with dicyclohexylcarbodiimide (DCCI) with the single exception that glutamine was coupled by means of its p-nitrophenyl ester. Stepwise synthesis was carried out in the methylene chloride and/or dimethylformamide, using a glass shaker (Schwartz-Mann, New York) at room temperature. Removal of the Boc-group in the first seven steps was performed by treatment with 50% (v/v) trifluoroacetic acid in methylene chloride for 20 min, as suggested by Li and Yamashiro (5). After the incorporation of the tryptophan residue, 1N HCl in acetic acid containing 1% of 2-mercaptoethanol was used for removal of the Boc-group as described by Marshall (6). The neutralization was carried out by shaking with 10% (v/v) triethylamine in chloroform. In every DCCI-coupling step, 4 equivalents of Boc-amino acid (4 X 0.35 mmole) for every equivalent of the starting glycine resin ester were shaken in the presence of 4 equivalents of DCCI for 5 hr. An additional reaction with the same reagents was performed for another 5 hr. For the incorporation of histidine residue, 5 equivalents of Boc-histidine were used and the same reaction was carried out twice as above (7). Nitrophenyl ester coupling of Boc-Gln was performed with 10 equivalents of the active ester for 5 hr, followed by an additional treatment for 5 hr in the presence of 5 equivalents of imidazole (5, 8). The yield of the protected decapeptide resin ester was 1.38 g (about 78% based on dry weight and amino acid analysis).

In order to achieve cyclization of N-terminal glutaminyl group on the resin to the pyroglutamyl-ring after removal of Boc-group, the Boc-glutaminyl-peptide resin ester was treated with 1N HCl in acetic acid containing 1% of 2-mercaptoethanol for 1 hr at room temperature (9). For the cleavage of the solid support, the peptide resin ester was submitted to the ammonolysis by stirring in 20 ml of absolute methanol saturated with ammonia for 3 days at room temperature. After filtration, evaporation of the methanolic filtrate yielded 368 mg of the corresponding amide as a yellow semi-solid material. Re-

moval of all protecting groups from the decapeptide amide was carried out by treatment with liquid hydrogen fluoride in the presence of 2 ml of anisole for 1 hr at 0° (4, 10). After evaporation, the residual material was dissolved in 5 ml of acetic acid containing 1% of 2-mercaptoethanol and heated in an evacuated sealed tube at 100° for 10 min for the completion of cyclization to the pyroglutamyl-group (11, 12). After addition of 50 ml of water, followed by lyophilization, the residual solid was dissolved in 0.2 N acetic acid and lyophilized. The resulting slightly yellow solid was purified by the counter-current distribution (CCD) in the solvent system of 0.1% acetic acid:butanol: pyridine (11:5:3) for 400 transfers (1). Lyophilization of fractions No. 282-343 (K = 2.0) (1) after CCD afforded 153 mg of purified LH-RH as a white fluffy solid, which showed about 25% of biological activity of natural porcine LH-RH. On paper-chromatography (Whatman No. 1) in 1-butanol:pyridine:acetic acid:water = 15:10:3:12, this material showed two spots, positive to Pauly's reagent and negative to ninhydrin, with R_f = 0.67 and 0.79 respectively. Paper-electrophoresis (pH 6.4 pyridine acetate, 2,500 volts, 1 hr) also exhibited two spots with R_{LVP}[2] 0.95 and 0.76, respectively. The main spot with R_f 0.67 and R_{LVP}[2] 0.95 was found to be identical with natural porcine LH-RH. Further purification of 9 mg portions of the CCD-purified material was carried out on a column of carboxymethylcellulose (1 X 60 cm), using 0.1 M ammonium acetate, pH 7.0 for elution (1). This yielded 2 mg of essentially pure synthetic LH-RH, which was identical chromatographically and electrophoretically with natural porcine LH-RH. Comparisons of chromatographic mobilities of synthetic and natural LH-RH were carried out by paper-chromatography in 1-butanol:pyridine:acetic acid:water (15:10:3:12) (R_f = 0.67) and in 1-butanol:acetic acid:water = 4:1:5 (R_f = 0.5), as well as by thin layer chromatography (TLC) in 1-butanol:pyridine:acetic acid:water (15:10:3:12) (R_f = 0.59 on microcrystalline cellulose) and in 1-butanol:acetic acid:water (4:1:5) (R_f = 0.71 on cellulose

2. R_{LVP}: relative mobility toward cathode compared to that of Lys-vasopressin.

MN 300 HR, Brinkman). Electrophoretic mobilities were compared on Whatman No. 1 filter paper (pH 6.4 pyridine-acetate, 2,500 volts, 1 hr) and by thin layer electrophoresis (TLE) on cellulose (pH 4.5 pyridine-acetate, 310 volts, 3 hr). In every case, synthetic LH-RH exhibited one spot with identical mobilities with those of natural LH-RH. Amino acid analyses of this synthetic LH-RH after acid hydrolysis by the Matsubara method (13) showed: $Trp_{0.95}$ $His_{0.89}$ $Arg_{1.10}$ $Ser_{0.94}$ $Glu_{0.86}$ $Pro_{0.88}$ $Gly_{1.90}$ $Leu_{1.00}$ $Tyr_{0.82}$. Because of the shortage of natural LH-RH (1), the $[\alpha]_D$ values of synthetic LH-RH could not be compared.

LH-RH activity of the purified decapeptide was compared at 2 dose levels with that of natural LH-RH (AVS 77-33 #215-269) in ovariectomized, estrogen-progesterone treated rats. Serum LH levels 15 min after intravenous injection were used as the index of LH-RH

Table 1

LH-RH ACTIVITY OF NATURAL AND SYNTHETIC LH-RH AS TESTED IN OVARIECTOMIZED, ESTROGEN PROGESTERONE TREATED RATS.

Sample	Dose ng	Mean serum LH ng/ml ± SE	Relative potency vs,natural LH-RH %
Saline	----	2.3 ± 0.9 (4)*	
Natural LH-RH	0.5	5.5 ± 0.9 (4)	
	2.5	25.2 ± 3.0 (4)	
Synthetic LH-RH	1	18.3 ± 1.6 (5)	122(63 - 339)%**
	5	42.3 ± 7.3 (5)	

* Number in the parentheses indicates numbers of rats per group.

** 95% confidence limits.

Non-parallelism for dose response regression lines of natural LH-RH vs.synthetic LH-RH = not significant $\lambda = 0.280$.

activity. LH was measured by radioimmunoassay as described by Niswender et al. (14) and expressed in terms of NIH-LH-S 17. As shown in Table 1, the dose response regression lines for natural and synthetic LH-RH preparations were parallel. Using four point factorial assay (15), the LH-RH activity of this synthetic preparation calculated as 122% of the potency of natural LH-RH preparation with 95% of fiducial limits of 63-339% i.e., LH-RH activity of this synthetic decapeptide was a little higher than that of the preparation of natural porcine LH-RH. This was not unexpected since the amino acid content of natural LH-RH was 67.5% (16). Its FSH-RH activity, measured as in (1), was essentially the same as that of the natural LH-RH/FSH-RH. Thus little if any racemization is thought to have occurred during the synthesis.

On the basis of behavior on CCD, paper chromatography and electrophoresis, TLC and TLE as well as biological results, it was concluded that the synthetic product was similar to the natural porcine LH-RH/FSH-RH.

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