ISOLATION AND PROPERTIES OF THE FSH AND LH-RELEASING HORMONE*

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Summary. LH-releasing hormone (LH-RH) was obtained in apparently a homogeneous state from extracts of pig hypothalami. The LH-RH preparation isolated has FSH-releasing hormone (FSH-RH) activity, which appears to be intrinsic to LH-RH. The amino acid composition of LH-RH/FSH-RH as determined on acid hydrolysates is: His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1 and Tyr 1. The hormone isolated stimulates the release of FSH and LH *in vivo* and *in vitro* in doses of a few nanograms. This polypeptide appears to represent the hypothalamic hormone which controls the secretion of both LH and FSH from the pituitary.

Hypothalamus excercises control over the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (1). This control is mediated by neurohumoral substance (s) designated LH-releasing hormone (LH-RH) and FSH-releasing hormone (FSH-RH) (2). We have previously described partial purification of porcine LH-RH (3). Recently, evidence was presented for the peptide nature of the LH-RH and FSH-RH (4). This report describes the isolation from porcine hypothalamic extracts of a polypeptide which has both LH-RH and FSH-RH activity, its amino acid composition and chemical and biological properties.

The isolation of LH-RH was accomplished essentially in 12 steps. Lyophilized fragments of 165,000 pig hypothalami (Oscar Mayer Co., Madison, Wis.), weighing 2500 grams were first pulverized, defatted and then extracted with 2 N acetic acid (3, 5). The lyophilized extract (yield 1075 g) was re-extracted with glacial acetic acid (3, 5) and lyophilized. Glacial acetic acid extracts (665 g) were subjected to gel filtration on Sephadex G-25 column (15.5 X 180 cm) in batches of 45 to 70 g as described previously (Fig. 1, ref. 5) using 1 M acetic acid as eluant. The LH-RH activity in effluents was measured by following elevation of plasma LH in ovariectomized rats pretreated with estrogen and progesterone (2-4). Plasma LH levels were determined by bioassay (6), or by radioimmunoassay for rat LH (7). FSH-RH activity was detected *in vitro* by measuring the stimulation of FSH release from rat pituitaries (8). FSH released

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into incubation medium was determined by the Steelman-Pohley assay (9) and by a radioimmunoassay for rat FSH (10). The separation pattern was followed by the Folin-Lowry reaction (11) or by optical density readings at 278 mμ. Fractions 913-1082 from Sephadex containing LH-RH and FSH-RH activity emerged between α-MSH and lysine vasopressin (See Fig. 1, ref. 5). Lyophilization of combined active areas yielded 380 g material which stimulated the release of LH in vivo in doses of 125-500 μg. The same material had FSH-RH activity in vitro in 500-600 μg amounts. The LH-RH and FSH-RH activity in this material was desalted and concentrated by extraction with 2400 ml phenol (3, 5). After recovery of the phenol soluble materials, 83 g of extract were obtained which stimulated the release of LH and FSH in doses of 30-150 μg and 100 μg respectively. This material in 5-8 g batches was subjected to ion exchange chromatography on carboxymethyl

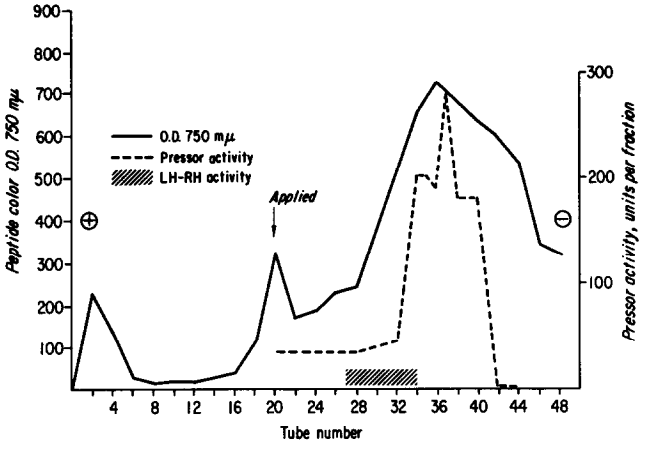

Figure 1:
Free flow electrophoresis (FFE) of 277 mg of porcine LH-RH from CMC in 0.37 M pyridine acetate buffer, pH 6.3. Conditions: 1900 volts, 160 ma, 6° C, 9 hours. Peptide analyses (11) were carried out on 25 μl aliquots.

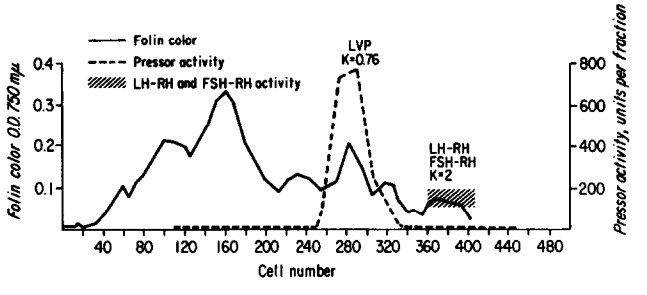

Figure 2:
Countercurrent distribution (CCD) of 94.8 mg of LH-RH from FFE in a system of 0.1% acetic acid: 1-butanol:pyridine = 11:5:3 (v/v). Lower phase was 3 ml and upper phase 5 ml. The number of transfers was 500. Peptide (11) analyses were carried out on 50 μl aliquots lower phase.

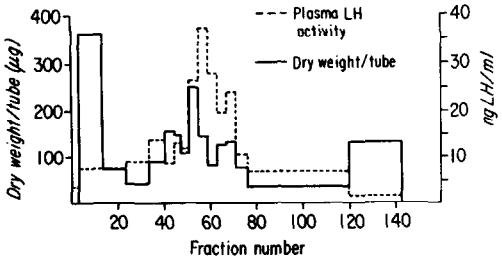

Figure 3:
Partition chromatography I of 8.9 mg of LH-RH from CCD on a column of Sephadex G-25, 0.9 X 150 cm. The solvent system consisted of upper phase of 1-butanol:acetic acid:water:benzene=4:1:5:0.33. Fraction size 5 ml; hold-up volume 24.5 ml. Flow rate 3 ml per hour; T = 68 ± 2° F.

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cellulose (CMC) columns, as described previously (3, 5). Tests on aliquots showed that LH-RH and FSH-RH activity was eluted by application of a gradient to pH 7, 0.1 M ammonium acetate and emerged in fractions No. 150-190 with conductivity of 6-8 mMHOS (see Fig. 2, ref. 5). The active fractions from thirteen CMC columns were combined and lyophilized. The material (948 mg) was divided into 4 batches, which were re-chromatographed on an analytical column of CMC (1 X 60 cm) as in (3). When fractions No. 210-270 (see Fig. 3 in ref. 3) were combined and lyophilized, 277 mg of material was obtained, active in releasing LH and FSH in doses of 0.1 µg and 0.5 µg respectively.

This material was further purified by free flow electrophoresis (FFE) in Brinkman continuous electrophoretic separator. The pattern of separation is seen in Figure 1. The results of assays indicated that the activity migrated toward the cathode and was found in tubes No. 27-34. The material obtained (94.8 mg) stimulated LH release at levels of 50 ng and FSH release in doses of 0.3 µg. This material was now subjected to 500 transfers by countercurrent distribution (CCD) as indicated in Figure 2. This procedure separated LH-RH (K=2) from traces of lysine vasopressin. After the recovery of the active area, 14.2 mg were obtained which had detectable LH-RH activity in doses of 2-25 ng and FSH-RH activity in 10-30 ng amounts. Of this material 5.3 mg, equivalent to 65,000 hypothalami, were used for various physiological (12) clinical (13) and veterinary (14) studies. The purification was continued with 8.9 mg, equivalent to about 100,000 hypothalami. This material was subjected to partition chromatography on Sephadex G-25, as shown in Figure 3. Lyophilization of fractions No. 45-71 yielded 3.6 mg material and assays showed that its LH-RH and FSH-RH activity had increased. It showed only one spot on thin layer chromatography on cellulose coated plates in the system of 1-butanol:acetic acid:water = 4:1:5 (12). This spot revealed with chlorine/o-tolidine corresponded to the LH-RH active area (12). However, the amino acid ratios indicated that the material

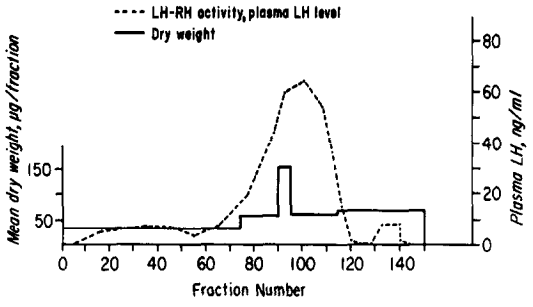

Figure 4:
Partition chromatography of 3.6 mg of LH-RH from partition chromatography I on a column of Sephadex G-25, 0.9 X 150 cm. The solvent system consisted of upper phase of 1-butanol:ethanol:water:acetic acid: Pyridine:Benzene=25:7:30:2:1:10. Fraction size 5 ml; hold-up volume 24.5 ml. Flow rate 5 ml per hour; T = 70 ± 2° F.

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was still not homogeneous. The material was further repurified by partition chromatography in another solvent system as illustrated in Figure 4. The weight of active fractions was reduced to 2.3 mg.

The final purification step consisted of zone electrophoresis (15) as shown in Figure 5. Assays on aliquots of fractions showed that LH-RH activity migrated strongly toward the cathode under these conditions. After lyophilization 830 µg of material was obtained which contained both LH-RH and FSH-RH activity. Since the recovery at each step was essentially complete, the purification was over 2 million fold. The product appeared to be homogeneous, but due to the very limited amounts of material available many conventional methods for proof of purity were inapplicable. The amino acid composition, determined after hydrolysis with 6 N HCl (22 hrs, 110° C) was: His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1 and Tyr 1 (Table I).

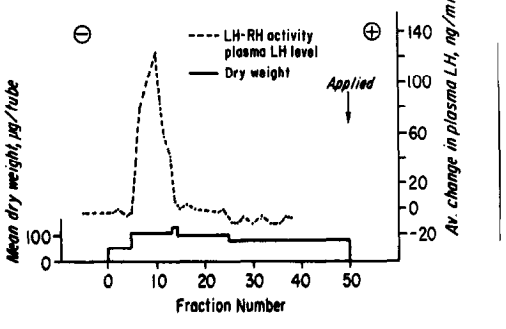

Figure 5:
High voltage electrophoresis of 2.3 mg LH-RH in 0.18 M pyridine acetate buffer, pH 6.3. Vertical column 0.9 X 97.6 cm, with external cooling at 5° C, packed with cellulose powder (15). After the electrophoretic separation at 2570 V; 20 mA for 18 hrs, the column was eluted with buffer and 1.3 ml fractions were collected.

Table I

AMINO ACID COMPOSITION OF PORCINE LH-RH/FSH-RH

	mumoles/10 μg material*	Amino Acid Ratio**	Nearest Integral
Histidine	2.351	1.10	1
Arginine	2.459	1.15	1
Serine	1.773	0.83	1
Glutamic Acid	1.987	0.93	1
Proline	2.708	1.27	1
Glycine	4.271	2.00	2
Leucine	2.234	1.05	1
Tyrosine	1.563	0.73	1
* Total Amino Acid Content = 30.5%.	** Gly = 2.0.		9 residues

The view that the polypeptide moiety, which probably consists of 9 amino acids, is essential for biological activity of LH-RH and FSH-RH is supported by experiments with proteolytic enzymes (4). Both LH-RH and FSH-RH activities were simultaneously abolished by incubation with endopeptidases (chymotrypsin, papain, subtilisin, thermolysin) but not by exopeptidases (leucine aminopeptidase, aminopeptidase M, carboxypeptidase A and B). Lack of inactivation by Edman procedure and failure to detect any N-terminal amino acid by Dansyl method indicate a blocked N-terminus. Inactivation by pyrrolidone carboxylyl peptidase (16) (G-200 preparation, supplied by Dr. R. Doolittle) suggests that the N-terminus may be occuppied by pyroglu-

Table II

EFFECT OF LH-RH ON PLASMA LH LEVELS IN OVARIECTOMIZED RATS PRETREATED WITH ESTROGEN AND PROGESTERONE

Sample	dose ng/rat	Plasma LH (RIA) ng/ml* ± S.E.	P Value
Saline	----	7.6 ± 0.6	----
LH-RH	0.1	9.2 ± 0.6	NS
LH-RH	0.25	11.0 ± 0.6	0.01
LH-RH	0.625	13.8 ± 2.0	0.05
LH-RH	1.56	26.0 ± 2.2	0.001
LH-RH	3.89	60.3 ± 7.2	0.001

* In terms of NIH-LH-S-14.

tamic acid (17). The mass spectral fragmentation patterns of free and trifluoroacetylated LH-RH, exhibit similar peaks at 83, 84, 111, and 112 mass units, probably due to the presence of a pyroglutamyl N-terminus. Inactivation with N-bromosuccinimide and diazotized sulfanilic acid (4) suggests that tyrosine and/or histidine are necessary for both biological activities (4).

As suggested previously (4, 18) the FSH-RH activity appears to be intrinsic to LH-RH. Partition chromatography in 10 different solvent systems did not separate FSH-RH activity from LH-RH. The material isolated as described above stimulated the release of both LH and FSH *in vivo* as well as *in vitro*. Results illustrated in Table II indicate that intravenous administration of doses as small as 0.25 - 0.625 ng LH-RH significantly increased plasma LH levels in ovariectomized rats pretreated with estrogen and progesterone. Table III shows that doses as small as 0.5 ng LH-RH/ml augmented the release of both LH and FSH *in vitro* as ascertained by bioassays (9) and radioimmunoassays (7, 10) of media. The stimulation of FSH and LH release was linearly related to the log dose of LH-RH/FSH-RH over the range 1.5 - 13.5 ng/ml. The highest dose resulted is a 4 fold increase in FSH and LH activity in incubation medium. Table IV shows that plasma LH and FSH levels were increased 10 and 45 min after intracarotid injection of LH-RH/FSH-RH into castrated male rats pretreated with testosterone. It is of interest that in humans administration of highly purified porcine LH-RH also increased the levels of FSH as well as LH (13). Monkeys, sheep and rabbits also respond to LH-RH (12, 14). This indicates lack of species specificity to LH-RH/FSH-RH in mammals. The results reported here indicate that the nonapeptide isolated from porcine hypothalami has both LH-RH and FSH-RH activity and it represents the hypothalamic hormone which controls the secretion of both LH and FSH from the pituitary.

Table III

STIMULATION BY LH-RH/FSH-RH OF FSH AND LH RELEASE *IN VITRO* FROM PITUITARIES OF MALE RATS

Sample	Dose ng/ml*	FSH CONTENT OF MEDIUM Steelman - Pohley Assay			LH CONTENT RIA
		Ovarian Wt mg ± S.E.	P Value	FSH** μg/ml	LH ng+/ml
Control	----	71.4 ± 4.2	----	21	238
LH-RH	0.5	94.9 ± 4.9	.01	28	362
LH-RH	1.5	101.6 ± 6.4	.005	32	460
LH-RH	4.5	143.2 ± 20.0	.01	53	628
LH-RH	13.5	169.6 ± 10.0	.001	77	978

* Total volume 10 ml. ** Expressed as NIH-FSH-S-4. + As NIH-LH-S-14.

Table IV

EFFECT OF LH-RH/FSH-RH ON PLASMA LH AND FSH LEVELS IN CASTRATED MALE RATS TREATED WITH TESTOSTERONE

Sample	Dose ng/rat	PLASMA FSH ACTIVITY Steelman - Pohley Assay		PLASMA LH ACTIVITY RIA
		Ovarian wt. mg	FSH* μg/ml with 95% limits	LH ng/ml+
Saline	----	89.4 ± 6	10 (6.5 - 15.3)	0
LH-RH	1	----	----	12 ± 4.5
LH-RH	5	----	----	42 ± 4.0
LH-RH	25	134.2** ± 10.9	19 (12.1 - 28.9)	66 ± 5.9

* As NIH-FSH-S-8, 45 min after injection. ** P = 0.05 vs saline. + As NIH-LH-S-14, 10 min after injection.

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