PURIFICATION, AMINO ACID COMPOSITION AND N-TERMINUS OF THE HYPOTHALAMIC LUTEINIZING HORMONE RELEASING FACTOR (LRF) OF OVINE ORIGIN.

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Received May 21, 1971

SUMMARY: A purification sequence for the ovine hypothalamic LH-releasing factor is reported. From 300,000 sheep brains, ca. 200 μg have been obtained of a material containing ≤ 56% peptide, of which 94% can be accounted for by the amino acids His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1, Tyr 1, the N-terminal being pyroGlu (2-pyrrolidone-5-carboxylic acid, PCA). This material stimulates release of LH in vivo and in vitro (≥0.5 ng/ml); it also stimulates release of FSH concomitantly with LH.

Following the successful isolation and characterization of the thyrotropin releasing factor (TRF) present in the extract from ca. 300,000 sheep hypothalamic fragments(1,2), aliquots taken from the entire effluent of the large (15 cm x 2.5 m) Sephadex G-25 filtration stage (see 3, p.229) were assayed for LRF-activity, using a highly specific assay for LRF based on measurements of plasma LH by a solid phase radioimmunoassay(4). This study revealed that LRF-activity was fairly well localized (ca. 0.5 V_o- see Fig. 1), but that it overlapped the zone containing TRF. LRF-activity was thus followed by the bioassay in the side-fractions from the (previously performed) stages that had led to the isolation of TRF(1,2,3). It was found that LRF- and TRF-activities completely separated at the level of the first partition chromatography (see 3, p.229). This LRF-fraction was subsequently used for pilot studies from which, after 4 more steps of purification(5), 992 μg of an LRF-preparation were obtained which moved as a single component (ninhydrin -, Pauly +) in 4 TLC systems(2) as well as thin layer electrophoresis. This preparation of LRF was active at ≥ 25 ng/dose in the bioassay system as above; it contained ≥ 20% amino acids by weight.

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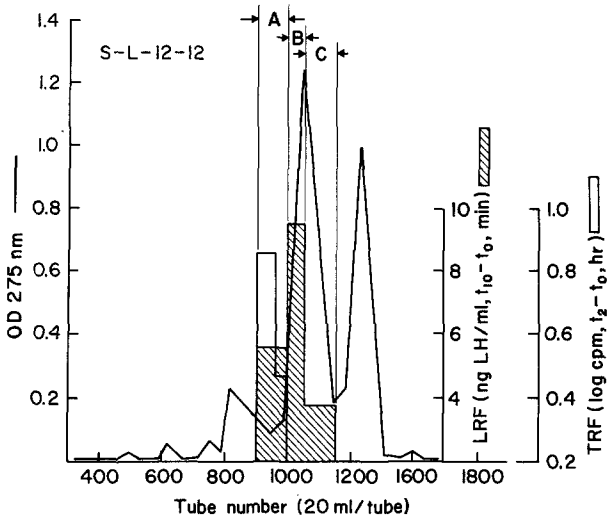

Fig. 1. Gel filtration chromatography of fraction 2, stage 3 (see Table I) on Sephadex G-25 in 0.5 M acetic acid. Column dimensions = 15 cm x 2.5 m.

In view of the negative reaction with ninhydrin, aliquots of this purified LRF-preparation were incubated with the pyrrolidonyl-carboxylyl-peptidase purified by Fellows and Mudge from B.subtilis, which we had used previously to confirm the existence of a pyroGlu N-terminal in TRF(2). In all experiments, biological activity was destroyed. These results were considered compatible with the hypothesis(5) that (ovine) LRF-activity is associated with a polypeptidic structure in which the N-terminus is pyrrolidonyl-carboxylyl (PCA), as in the case of TRF (PCA-His-Pro-NH_2). This hypothesis was confirmed by partial acid hydrolysis followed by dansylation and also by mass spectrometry on an aliquot of LRF which revealed peaks at m/e = 84 and 129, characteristic of PCA-amides (courtesy H. Fales, NIH, Bethesda).

The presence of a Tyr residue in the preparation of highly purified LRF suggested the possibility of attaching a radioactive ^{125}I label on the peptide to be used as a convenient marker. A modification of the method of Greenwood et al.(6) was employed which yielded a specific radioactivity of 1.28 mCi/μg of LRF-peptide. The iodinated peptide was separated from unreacted iodine and other reagents by chromatography on a 2 mm x 24 cm column of Sephadex G-10 in

0.5 M acetic acid. The addition of the iodine reduced the biological activity to approximately 50% of untreated LRF and to 60% of LRF subjected to the iodination reagents minus the iodine. However, ^{125}I-LRF exhibited the same Rf as native LRF (γ counts, Pauly color) in the TLC systems and in TLE utilized previously(5).

Purification of the LRF from the bulk of the LRF-active fraction (fractions B + C in Fig. 1) from the original 300,000 sheep hypothalami was then undertaken, according to the sequence outlined in Table I. An aliquot of 0.2 μg of ^{125}I-labeled LRF obtained as above was added to the bulk of the LRF-concentrate prior to stage 5. At stages 1 through 7 of the sequence described below, the

TABLE I. Sequence of Purification of Ovine LRF.

Stage	Weight	LRF(Units/mg)[a]
1. Lyophilized ovine hypothalami	25 kg	
2. Alcohol-chloroform extract	294 g	
3. Ultrafiltration (Diaflo Membranes, UM-05)	71 g	1.5
4. Sephadex G-25 in 0.5 M acetic acid	51.18 g	1.6
5. Ion-exchange chromatography on CMC	61.1 mg	250
6. Column electrophoresis	11.92 mg	600
7. Partition chromatography 11:5:3, 0.1% HOAc:n-BuOH:pyridine	2.028 mg	3600
8. Partition chromatography 4:1:5, n-BuOH:HOAc:H_2O	ca. 200 μg	Not ascertained

a) One LRF unit defined as the biological activity of 1 mg of the material designated Fraction B on Fig. 1.

peak of LRF-activity was found to coincide with a peak of radioactivity (^{125}I-LRF); at the last step (stage 8), a definite shift was observed, the peak of ^{125}I-LRF preceding the peak of biological activity by as much as 0.25 V_o (see Fig. 2).

Stage 5: Ion-exchange chromatography on carboxymethyl cellulose (Whatmann CM32), 10 x 16 cm column, equilibrated with 0.01 M ammonium acetate, pH 4.5; step gradients in molarity and pH, first to 75 mM, pH 6.5 then to 250 mM,

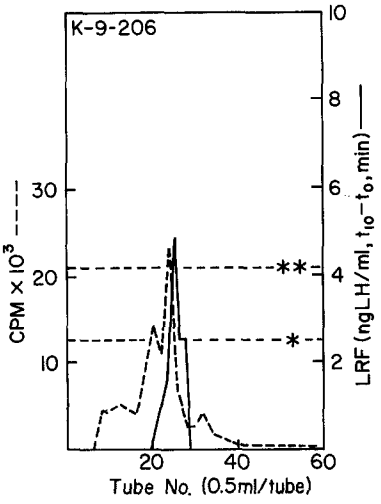

Fig. 2. Partition chromatography of the LRF fraction from stage 7 on Sephadex G-25 in the system [n-butanol:acetic acid:water, 4:1:5] . Column dimensions = 6 mm x 60 cm. LRF activity determined on 25 ng doses/rat.

pH 7.0, were utilized; the last step exchanges both LRF and Arg-8-vasopressin present in the starting material. Stage 6: High voltage column electrophoresis on Sephadex G-25 (LKB Model 3540), in 0.05 M pyridine acetate, pH 5.0, 5 hrs, 100 mAmps, 1000 volts. Stage 7: Partition chromatography with the system 0.1% acetic acid:n-BuOH:pyridine (11:5:3) on a 4 mm x 60 cm column of Sephadex G-25 as matrix; this step achieved total separation of LRF from Arg-8-vasopressin. Stage 8: Partition chromatography in the system n-BuOH:acetic acid: H_2O (4:1:5) on a 6 mm x 60 cm column of Sephadex G-25 (Fig. 2). Hydrolysis (6 N HCl) of a 31.5 μg aliquot of the material in tube 25 yielded the following composition(normalized to Leu): Lys 0.03, His 1.06, Arg 1.24, Asp 0.17, Ser 0.85, Glu 1.20, Pro 0.78, Gly 2.02,Ala 0.02, Leu 1.00, Tyr 1.09; these amino acids accounting for 56% of the weight of the aliquot and for 94% of the total peptide present, the overall yield in the LRF peak being ca. 200 μg. It is thus reasonable to propose that the preparation of LRF obtained here has the composition His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1, Tyr 1, and represents highly purified LRF peptide, the additional material, non-

peptidic in nature, likely being constituted by water soluble substances eluted from the last columns' matrices.

Nikitovitch-Winer et al.(7) had reported that a purified preparation of ovine LRF was ninhydrin-negative; our results are in agreement with their early observation. Recently, Currie et al.(8), using a PCA-peptidase from P. fluorescens and crude preparations of bovine LRF, have concluded that bovine LRF is associated with a peptide moiety with an N-terminal PCA, confirming our earlier observations with ovine LRF(5). It is of considerable interest that, working independently and with a different purification sequence, Schally et al.(9) have recently obtained for porcine LRF, the same amino acid composition reported here for ovine LRF.

Besides the results obtained in vivo in the assays reported here, the biological activity of LRF was also studied in vitro: In several experiments, hemi-pituitaries from rats of different age and sex were incubated for 1 hr with increasing doses (0.5 - 10 ng/ml) of LRF (material from tube 25, see Fig. 2). Four-point bioassays of both LH and FSH released in the incubation

TABLE II. Highly Purified LRF on LH and FSH Release In Vitro

Dose of LRF	Hormone determined	Potency Ratio[a] Pituitary Donors		
		Immature females (30 days)	Immature males (30 days)	Mature males 150 grams
0.5 ng/ml	LH	1.77	0.95(b)	1.81
	FSH	1.21(b)	1.12(b)	1.13(b)
2.0 ng/ml	LH	4.22	8.95	2.12
	FSH	2.83	5.28	1.23(b)
10.0 ng/ml	LH	51.47	44.55	53.23
	FSH	7.57	20.40	13.90

a) Obtained in classical four-point bioassays.

b) Not significantly different from a potency of 1.0; all other potency ratios, statistically different from 1, at 95% or 99% significance by factorial analysis.

fluids, as determined by specific murine radioimmunoassays, indicated that LRF elicits concomitant release of LH and FSH (Table II); the functions relating amounts of FSH and LH released, to the doses of LRF, appear to be of different slopes and different intercepts. These results, obtained with a highly purified preparation of LRF, are at variance with previous claims(10, 11) that preparations of LRF free of FSH-releasing activity had been obtained in the very early stages of purification of LRF. That highly purified LRF stimulates concomitantly the secretion of LH and FSH as seen here, does not exclude the possible existence of a specific FRF distinct from LRF, both releasing factors perhaps sharing the activity to release LH and FSH though with different ratios of specific activity. It is also possible that the molecule of LRF could account for all the LH- and FSH-releasing activities seen in hypothalamic extracts.

ACKNOWLEDGEMENTS: Research supported by AID (Contract No. AID/csd 2785), NIH (AM 08290; AM 14894), Ford Foundation and Rockefeller Foundation. We gratefully acknowledge the devoted collaboration of M. Butcher, D. Cedergren, A. Erenea, R. Givens, E. Raines, R. Smith, K. Wendler and P. Wilson.

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