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TWO GONADOTROPIN-RELEASING HORMONES FROM AFRICAN CATFISH (*CLARIAS GARIEPINUS*)

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SUMMARY: Two forms of gonadotropin-releasing hormone (GnRH) have been purified from brain extracts of the African catfish, *Clarias gariepinus*, using reverse-phase high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The amino acid sequences of both forms of African catfish GnRH were determined using Edman degradation after digestion with pyroglutamyl aminopeptidase. In addition, both GnRHs were studied by mass spectrometry. The primary structure of African catfish GnRH I is identical to Thai catfish GnRH I, pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2, and the primary structure of African catfish GnRH II is identical to the widely distributed and highly conserved chicken GnRH II, pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2.

It is well established that in vertebrates gonadotropin secretion is under control of hypothalamic peptides, the gonadotropin-releasing hormones (GnRHs). The structure of six GnRHs has been identified: all are decapeptides. The mammalian GnRH, commonly referred to as luteinizing hormone releasing hormone (LHRH), was isolated and chemically characterized for the first time in porcine and ovine hypothalamus tissue (1). Two more GnRHs were identified from chicken hypothalamus (chicken GnRH I and chicken GnRH II)(2,3), one from salmon brain (4), one from lamprey brain (5) and one from the brain of the Thai catfish (*Clarias macrocephalus*)(6).

In mammals, to date only one form of GnRH has been isolated. In contrast, in teleost fish, multiple forms of GnRH are present within brain tissue of a single species. Salmon GnRH has been found in salmon brain tissue in combination with a GnRH identical to chicken GnRH II, and in other species also a number of unidentified GnRH-like peptides have been detected (7-10). In the European eel, GnRHs identical to chicken GnRH II and mammalian GnRH are present (11).

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In mammals it has been shown that GnRH is synthesized as part of a precursor molecule. This precursor comprises a signal peptide, GnRH, and a 56 amino acid long peptide termed GAP (*G*nRH *a*ssociated *p*eptide), in this order (12). GAP has been shown to be a potent inhibitor of prolactin release in rat and human. Planas *et al.* (13) demonstrated an inhibitory effect of human GAP on total prolactin release from *Tilapia* pituitaries *in vitro*, but a stimulatory action on the release of newly synthesized prolactin.

All known GnRHs have their N- and C-termini in common (pGlu-His and Pro-Gly-NH_2, respectively). Both termini are important for receptor binding and receptor activation. Indeed, all GnRHs and their analogues with identical N- and C-termini have gonadotropin releasing activity in teleosts. However, receptor affinity, and accordingly biological activity, might be different (14,15).

In the African catfish, *Clarias gariepinus*, two GnRH-like peptides were tentatively described (10). They were identified on their chromatographic behaviour, and their binding characteristics to four different anti-GnRH antisera. One of the peptides coeluted with chicken GnRH II and showed a similar immunological binding. The chromatographic behaviour and immunological binding of the other peptide were different from any GnRH known at that time. It was assumed that this peptide was a new form of GnRH, referred to as catfish GnRH I.

The aim of the present study was to purify and chemically characterize both forms of GnRH from brains of the African catfish.

MATERIAL AND METHODS

Collection of brains. The brains of 700 specimens of adult African catfish were removed, frozen in liquid nitrogen, and stored at -80°C.

Extraction. The frozen brains (520 gr) were powdered in a Micro-Dismembrator II (Braun, Melsungen, Germany). The powdered tissue was extracted according to Sherwood *et al.* (4).

Concentration. To each 100 ml of the final aqueous phase (total volume: 1300 ml) after the petroleum ether extractions 900 ml water and 10 gr C8 (Eurosil Bioselect; Knauer, Berlin, Germany) were added. Material was allowed to bind at 4°C for 2h, after which a column of the C8 material was cast. Bound material was eluted with 80% acetonitrile in 10 mM trifluoroacetic acid.

HPLC. An HPLC system from Waters Associates (Milford, U.S.A.), consisting of an M720 System Control, an M730 Data Module, an M6000 Solvent Delivery System and an M441 UV/Vis Absorbance Detector was used for HPLC runs 1-5. An HPLC system from BioRad (Richmond, U.S.A.) consisting of an HRLC 8221T System Control/Data Module/Solvent Delivery System and an Model 1706 UV/Vis Monitor was used for HPLC runs 6 and 7. Details of the consecutive HPLC runs are given in Table I. Aliquots of the fractions from HPLC runs 1-5 were taken for RIA. Immunoreactive fractions of HPLC run 1 were combined as either early- (peak 1) or late-eluting (peak 2) fractions and reapplied together for HPLC run 2. Peaks 1 and 2 were run separately in all subsequent HPLC runs. The fractions from peak 1 (HPLC run 5) and from peak 2 (HPLC run 4), respectively, were taken for mass spectrometry, pyroglutamate aminopeptidase digestion and subsequent amino acid sequencing.

RIA. GnRH was measured as described (10). An antiserum prepared against salmon GnRH (GF-4; gift of Dr. N.M. Sherwood), shown to bind to different forms of GnRH, was used for screenings of all fractions.

Mass spectrometry. The mass spectra of 10% of peaks 1 (HPLC run 5) and 2 (HPLC run 4) were recorded in a Bio-Ion 20 plasma desorption mass spectrometer

Table I. Protocol for purification of African catfish GnRH I and II by HPLC

HPLC	Material applied to column	Column	Mobile phase[a]	Program Time (min)	%CH_3CN
0	Extract	C8 [b]	10 mM TFA/CH_3CN	0-5 5-12	0 80
1	Fractions from HPLC-0	C18 [c]	10 mM TFA/CH_3CN	0-10 10-35 35-60	0-12 12-27 27-60
2	Peaks 1 and 2 from HPLC-1 combined	C18 [d]	10 mM HFBA/CH_3CN	0-5 5-55 55-60	0-18 18-33 33-60
3	Peak 1 from HPLC-2	C18 [d]	250 mM TEAA (pH 6.4)/CH_3CN	0-5 5-45 45-50	0-12 12-24 24-60
4	Peak 2 from HPLC-2	C18 [d]	250 mM TEAA (pH 6.4)/CH_3CN	0-5 5-65 65-70	0-12 12-30 30-60
5	Peak 1 from HPLC-3	C18 [e]	7 mM TFA/CH_3CN	0-5 5-80 80-90 90-95	0-12 12-24 24-32 32-80
6	Peak 1 from HPLC-5[f]	C18 [e]	7 mM TFA/CH_3CN	0-5 5-45 45-55 55-60	0-12 12-28 28-36 36-80
7	Peak 2 from HPLC-4[f]	C18 [e]	7 mM TFA/CH_3CN	0-5 5-45 45-55 55-60	0-12 12-28 28-36 36-80

[a] Mobile phase components: TFA, trifluoroacetic acid; CH_3CN, acetonitrile; HFBA, heptafluorobutyric acid; TEAA, triethylammonium acetate.
[b] Eurosil Bioselect (Knauer, Berlin, Germany)
[c] preparative column (10 µm wide-pore; Baker Inc., Phillipsburg, U.S.A.).
[d] analytical column (5 µm wide-pore Nucleosil; Macherey-Nagel, Düren, Germany).
[e] analytical column (3 µm wide-pore Nucleosil; Macherey-Nagel, Düren, Germany).
[f] digested with pyroglutamate aminopeptidase.

(PDMS) (Applied Biosystems, Foster City, U.S.A.). The acceleration voltage was 18 kV. The mass accuracy of the instrument is 0.1-0.2%. The peptides were dissolved in 7.0 mM TFA, applied to nitrocellulose matrix and allowed to absorb for 5 min. The remaining liquid was evaporated by a stream of nitrogen. Spectra were accumulated for 4 h (peak 1) and 15 min (peak 2).

Digestion. The fractions, containing peak 1 (HPLC run 5) and peak 2 (HPLC run 4), respectively, were lyophilized. 100 µl of reaction buffer containing 88 mM TES (N-tris-(hydroxymethyl)methyl-2-amino-ethanesulfonic acid) (pH 8), 10 mM EDTA (Ethylenediaminetetraacetic acid), 5 mM dithiothreitol (DTT), 5% glycerol and 100 µg of pyroglutamate aminopeptidase (Boehringer Mannheim, Mannheim, Germany) was added to each of them. The solutions were incubated at 20°C for 5h. Salmon GnRH and chicken GnRH II were used as controls. The digested catfish GnRHs were separated from the reaction mixture in HPLC runs 6 and 7, respectively (Table I).

Amino acid sequencing. Automated repetitive Edman degradations were performed on all material of peak 1 (HPLC run 6), and on half the material of peak 2 (HPLC run 7), respectively, using an Applied Biosystems 473 pulse liquid phase sequencer. The data were analyzed with the 610A Data Analysis software from Applied Biosystems, using the subtraction and filter mode for ultra-sensitive phenylthiohydantoin amino acid detection.

RESULTS AND DISCUSSION

This study shows that the African catfish contains two forms of GnRH. Figure 1A-E summarizes the purification results of HPLC runs 1-5. Peak 1 (Fig. 1E) and peak

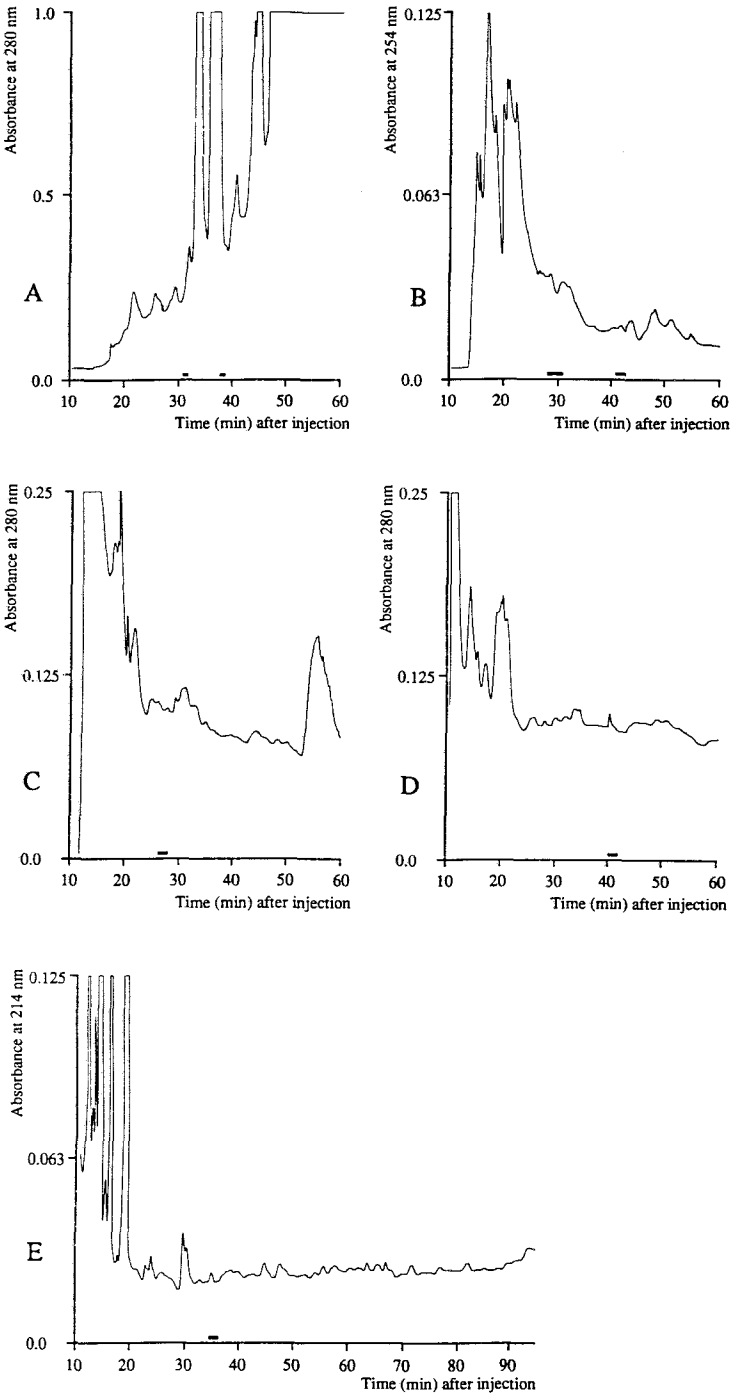

Figure 1. Purification of both African catfish GnRH molecules. HPLC fractions immunoreactive with antiserum GF-4 are indicated by bars. African catfish GnRH I: A, B, C, E. Fraction 32 in A, fractions 29, 30, and 31 in B, and fractions 27 and 28 in C were used for the next step in purification. Fractions 35 and 36 (peak 1) in E were used for structural characterization. African catfish GnRH II: A, B, D. Fraction 38 in A, and fractions 42 and 43 in B were used for the next step in purification. Fractions 41 and 42 (peak 2) in D were used for structural characterization.

2 (Fig. 1D) were resolved as single chromatographic fractions, achieved after 4 and 3 HPLC runs, respectively. In different HPLC runs, peak 2 was more hydrophobic and eluted later compared with peak 1 (Fig. 1A-D).

Mass spectrometry revealed that peak 1 (HPLC run 5) consisted of a peptide with a MH^+ of 1114.0 D (Fig. 2A; calculated mass: 1114.3 D), and that peak 2 (HPLC run 4) consisted of a peptide with a MH^+ of 1235.7 D (Fig. 2B; calculated mass: 1236.4 D).

All known GnRHs contain N-terminal 5-Pyrrolidone-2-carboxylic acid residues that are resistant to Edman degradation. Therefore, peaks 1 (Fig. 1E) and 2 (Fig. 1D) were treated with pyroglutamate aminopeptidase, and each peak was purified by HPLC (HPLC runs 6 and 7, respectively; results not shown). After digestion, both peaks

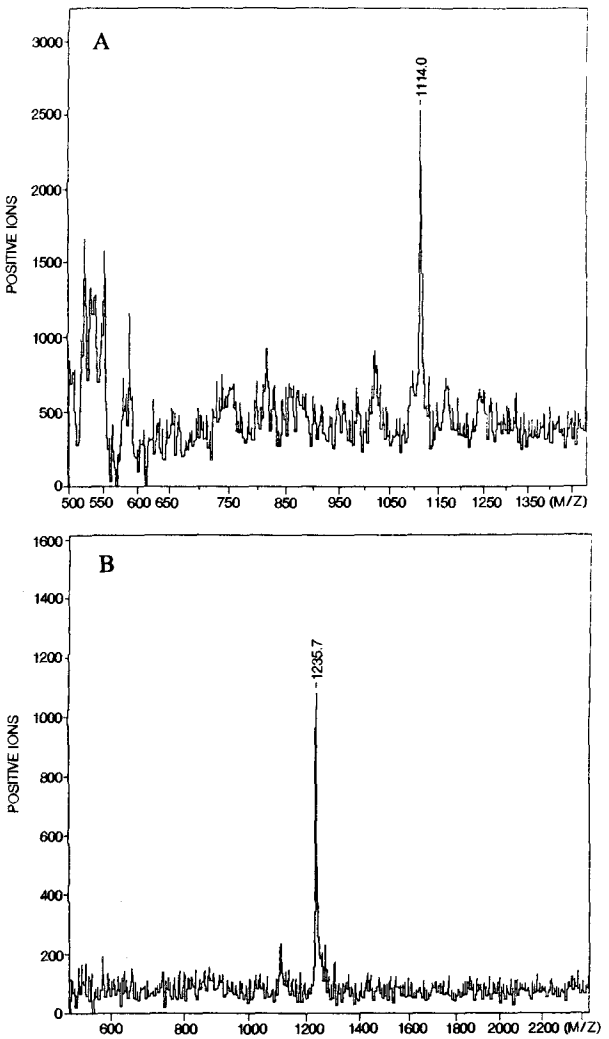

Figure 2. Mass spectra of African catfish GnRH I (A) and African catfish GnRH II (B). GnRH I has a MH^+ of 1114.0 D (calculated mass: 1114.3 D), and GnRH II has a MH^+ of 1235.7 D (calculated mass: 1236.4 D).

Table II. Sequence results of peak 1 (GnRH I) and peak 2 (GnRH II)

peak 1			peak 2		
residue	amino acid	pmol	residue	amino acid	pmol
1	His	2.8	1	His	7.2
2	Trp	3.2	2	Trp	3.5
3	Ser	0.4	3	Ser	1.1
4	His	0.9	4	His	3.0
5	Gly	2.4	5	Gly	8.4
6	Leu	2.7	6	Trp	2.4
7	Asn	3.0	7	Tyr	6.4
8	Pro	0.8	8	Pro	1.0
9	Gly	*	9	Gly	*
10	-		10	-	

* assumed to be present, since R42 antibody recognition of the undigested peptide (see text).

were no longer immunoreactive with the GF-4 antibody, and eluted at different positions. Therefore it was concluded that peaks 1 and 2 contained $pGlu^1$-residues. Edman degradation of peak 1 yielded a sequence of: His-Trp-Ser-His-Gly-Leu-Asn-Pro (Table II). The sequence of peak 2 was determined to be: His-Trp-Ser-His-Gly-Trp-Tyr-Pro (Table II). The Gly^{10}-residues were not detectable but assumed to be present. This is based on recognition of peaks 1 and 2 by antiserum R42 (10), which is raised against mammalian GnRH, and which does not detect extended forms, fragments of GnRH, or the free acid forms lacking Gly^{10}-NH_2-residues (16). Moreover, African catfish GnRH II coelutes with chicken GnRH II. In addition, the C-terminal Gly^{10}-NH_2-residue is highly conserved in all known GnRHs. These results are in accordance with mass determination of the peptides.

We conclude that the primary structure of African catfish GnRH I is: pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2, whereas the primary structure of African catfish GnRH II is: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2. Thus, the African catfish GnRHs I and II are identical to Thai catfish GnRH I (6) and chicken GnRH II (3), respectively.

The biological significance of multiple forms of GnRH in the brain is not clear. Studies in chicken (17) revealed that chicken GnRH I, in contrast to chicken GnRH II, is involved in reproductive functions. Thus, it seems that in this species, as in mammals, the two gonadotropins are under control of a single GnRH. In fish the situation might be different. Different GnRHs are found in distinct brain areas, suggesting more functions than the control of gonadotropin secretion only. In goldfish, GnRHs identical to salmon GnRH and chicken GnRH II are present. In this species the two GnRHs have been shown to have differential distribution over the brain and pituitary, suggesting some differences in neuroendocrine and neuromodulator functions (18-20). In rainbow trout, GnRHs identical to salmon GnRH and chicken GnRH II also have a differential distribution. The GnRH identical to salmon GnRH is predominant in all brain regions except the cerebellum, and is the only GnRH found in the pituitary (21). African catfish GnRHs I and II were found in the same neurosecretory cells in the anterior hypothalamic region of this species. In the proximal

pars distalis of the pituitary, they are even colocalized within the same secretory granules in neurosecretory fibres, which are in close contact with the gonadotropic cells (22). It is, however, not known what this means for the control of the secretion of the gonadotropic hormone. In goldfish, it was shown that GnRH not only regulates gonadotropin secretion, but also the secretion of growth hormone (23). Moreover, as suggested by Chang and Jobin (24) the GnRHs in goldfish may compete for the same receptors on the gonadotropic cells, stimulating hormone release from the same cells, but via different post-receptor signal transduction mechanisms.

The presence of immunoreactive GnRHs in different brain areas suggests a neurotransmitter function of the peptides. In this respect, it has been postulated that GnRH in the olfactory tract may serve the transmission of sex pheromonal stimuli (25).

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