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Purification, cDNA cloning, and characterization of ghrelin in channel catfish, *Ictalurus punctatus*

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Abstract

The ghrelin peptide and cDNA encoding precursor protein were isolated from the stomach of a channel catfish, *Ictalurus punctatus*. Catfish ghrelin is a 22-amino acid peptide with a sequence of GSSFLSPTQKPQNRGDRKPPRV. The third serine residue has been modified by *n*-decanoic acid and unsaturated fatty acids; however, an octanoylated form could not be identified. The carboxyl end of the peptide possessed an amide structure. A Gly-extended, non-amidated 23-amino acid ghrelin (ghrelin-Gly) was also isolated. Real-time quantitative PCR analysis revealed high levels of gene expression in the stomach and moderate levels in the pancreas and gall bladder. Intraperitoneal (IP) injection of ghrelin increased plasma GH levels in the catfish, but the effect of ghrelin-Gly was more potent than that of amidated ghrelin. Furthermore, IP injection with both amidated ghrelin and ghrelin-Gly caused a significant increase in pituitary GH mRNA expression over a 3-h period. These results indicate that ghrelin is present in catfish and stimulates GH gene expression and GH release in channel catfish.
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1. Introduction

Ghrelin was first identified as a 28-amino acid peptide in rat and human stomach as an endogenous ligand for the growth hormone secretagogue (GHS) receptor (Kojima et al., 1999). Unique to this peptide is the occurrence of octanoylation of the third serine residue, and acylation of the peptide, being essential for receptor binding and biological activity (Kojima et al., 1999; Muccioli et al., 2001). In rat, peripheral and central administration of ghrelin results in the release of growth hormone (GH) from the pituitary gland (Date et al., 2000; Kojima et al., 1999; Seoane et al., 2000; Tolle et al., 2001; Wren et al., 2000). Intracerebroventricular (ICV) or intraperitoneal (IP) administration of ghrelin also increases food intake and body weight gain in rodents (Nakazato et al., 2001; Tschöp et al., 2000; Wren et al., 2000), supporting a central role for ghrelin in the regulation of feeding.

In non-mammals, ghrelin has been isolated and somewhat characterized in an amphibian (Kaiya et al., 2001), a bird (Kaiya et al., 2002), four species of fish (Kaiya et al., 2003a,b,c; Unniappan et al., 2002), and a reptile (Kaiya et al., 2004). Fish ghrelin peptides are composed of 19–23 amino acids, exhibit GH-, prolactin (PRL)-, or luteinizing hormone (LH)-releasing activity (Kaiya et al., 2003a,b,c; Riley et al., 2002; Unniappan and Peter, 2004), and in goldfish, stimulate appetite (Unniappan et al., 2002, 2004). Contrary to goldfish and rodents, ICV injection of

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ghrelin in neonatal chicks inhibits food intake (Furuse et al., 2001; Saito et al., 2002). Together, these results support roles for ghrelin in pituitary function and feeding behavior in non-mammals.

The actions of ghrelin on GH release and feeding are of particular importance to researchers working to enhance channel catfish, *Ictalurus punctatus*, growth and feed efficiency. Channel catfish are the most extensively cultured food-fish species in North America, and improvements in growth and feed efficiency have, to date, been limited to the use of traditional selective breeding methods. Studies with NWAC103 and Norris strains of channel catfish demonstrate a strong correlation between increased body weight gain and increased appetite (Silverstein et al., 1999, 2001); however, there is a lack of basic information regarding the endocrine regulation of growth and feeding in this economically important species. Thus, the roles of ghrelin in mediating GH release and feeding make ghrelin a potential candidate gene for catfish stock selection. Defining the function of ghrelin in mediating catfish GH release and feeding, and whether that function is altered by differences in expression might lead to the identification and selection of individuals with desirable phenotypes. The present research describes the isolation of ghrelin peptide and the cDNA encoding precursor protein from the stomach of channel catfish, and the effects of ghrelin administration on in vivo pituitary GH mRNA expression and GH release into circulation.

2. Materials and methods

2.1. Purification of ghrelin from the stomach

During the purification process, ghrelin activity was followed by measuring changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in a cell line stably expressing rat GHS-R (CHO-GHSR62) as described previously (Kojima et al., 1999).

Frozen catfish stomachs, approximately 20 g, were used as the starting material. Detailed purification procedures have been previously described (Kaiya et al., 2003a). Briefly, boiled tissues were acidified, homogenized, and the supernatant was obtained. The crude acid extracts after a Sep-pak purification (Waters, Milford, MA) were subjected to SP-Sephadex C-25 cation-exchange chromatography. The basic peptide-enriched fraction was subjected to a carboxymethyl (CM)-ion exchange HPLC column and followed by an anti-rat ghrelin[1–11] immunoglobulin G (IgG) immuno-affinity column to purify ghrelin-immunoreactive substances. Adsorbed substances were separated by two different reverse-phase (RP)-HPLC using a μBondasphare C18 column (3.9 × 150 mm, Waters, Milford, MA) and a diphenyl column (2.1 × 150 mm, 219TP5125, Vydac, Hesperia, CA). To analyze the purified peptide sequence, 10 pmol of the purified peptide was subjected to a protein sequencer (Model 494, PE Applied Biosystems, Foster City, CA). The molecular weight of the purified peptides was determined using MALDI-TOF mass spectrometry (Voyager-DE PRO, PE Applied Biosystems).

2.2. Determination of cDNA encoding catfish ghrelin precursor

Nucleotide sequence of catfish ghrelin cDNA was determined by using rapid amplification of cDNA ends (RACE) PCR. Detailed procedures have been previously described (Kaiya et al., 2003a). Total RNA extracted from a portion of catfish stomach was used for cDNA preparation. For 3′-RACE PCR, four degenerate primers, based on the N-terminal seven amino acid sequence of mammalian ghrelin (GSSFLSP), were used. The amplification reaction was 94 °C for 1 min, and 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension for 3 min at 72 °C. Second-round nested PCR was performed with three degenerate sense primers, based on the amino acid sequence of eel and trout ghrelins (LSPSQ(R/K)P) under the following amplification conditions: 94 °C for 1 min, and 30 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The amplicon, approximately 380 bp, was subcloned using the TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen).

For 5′-RACE PCR, two gene-specific primers (GSP) were designed, based on the partial sequence of the catfish ghrelin cDNA as determined by 3′-RACE PCR: GSP-1, 5′-TTGAGCGGTTATGAATCAGG-3′; GSP-2, 5′-CAGGAATCCAGTAACATCC-3′. Primary PCR was performed with first-strand cDNAs transcribed from 100 ng of stomach poly$(A)^+$ RNA with oligo$(dT)_{12-18}$ primer, using GSP-1, an abridged anchor primer supplied with the 5′-RACE kit (Invitrogen) under the following reaction conditions: 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The second-round nested PCR was performed with GSP-2 and an abridged universal amplification primer (AUAP) supplied with the 5′-RACE kit under the same conditions described above. The approximately 430 bp amplified fragment was subcloned into pCRII-TOPO vector for sequencing. The nucleotide sequence was determined by automated sequencing (DNA sequencer: Model 373, PE Applied Biosystems), according to the Thermosequence II dye terminator cycle sequencing kit protocol using M13 forward or reverse primers (Amersham Pharmacia Biotech.).

2.3. Amplification of the full-length catfish ghrelin cDNA

To confirm the precise nucleotide sequence of the full-length cDNA, we performed PCR with a proofreading,

Pyrobest Taq DNA polymerase (Takara Bio, Shiga, Japan). First-strand cDNAs were synthesized from total RNA (5 μg) with SuperScript II and an adaptor primer in 3′-RACE kit (Invitrogen). PCR was conducted with a sense primer from the 5′ end of the catfish ghrelin cDNA (5′-ATAACGCCGTCCTGACATTACC-3′) and AUAP under the following conditions: 98 °C for 30 s, 30 cycles at 98 °C for 15 s, 51 °C for 30 s, and 72 °C for 1 min. An additional cycle with Ex Taq for 10 min at 72 °C was performed for subsequent TA cloning. The amplified product was subcloned into the pCRII-TOPO vector and the nucleotide sequence of the insert was determined (Model 3100, PE Applied Biosystems), according to the BigDye terminator cycle sequencing kit protocol using M13 forward or reverse primers (Applied Biosystems).

2.4. Gene expression analysis by real-time quantitative PCR

Total RNA of the hypothalamus, pituitary, gill, muscle, heart, liver, spleen, trunk kidney, head kidney, pancreas, stomach, gall bladder, and intestinal tracts was extracted separately from four individuals weighing approximately 100 g using TRI-reagent (Molecular Research Center, Cincinatti, OH). Total RNA was quantified on a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Rockland, DE), and 1 μg of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), according to manufacturer's instructions. The synthesized cDNA was then quantified and diluted to 200 ng/μL for all samples. A total of 400 ng of cDNA was used in expression analyses.

Gene expression was determined by real-time quantitative PCR using an iCycler iQ (Bio-Rad). Standards were developed by generating PCR fragments using the primers listed in Table 1, and then cloning them into the pCR4-TOPO vector (Invitrogen). Cloned inserts were sequenced to confirm sequence identity. Concentration of each resulting plasmid was measured spectrophotometrically, and serial dilutions of each plasmid were used to make the standard curves for quantification. Primer and probe sequences for the target genes are listed in Table 1. The standard curve showed a linear relationship between cycle threshold values and the logarithm of input gene copy number. All real-time PCR amplifications were performed in triplicate. All specific quantities were normalized as copy number against total RNA (Bustin, 2002).

Each PCR mixture (12.5 μL) contained 400 ng of cDNA; 1× iQ Supermix (Bio-Rad) which consisted of: 10 mM KCl, 4 mM Tris–HCl, pH 8.4, 0.16 mM dNTPs, 5 U/mL iTaq polymerase, 0.6 mM $MgCl_2$, and stabilizers; 10 nM of each primer; and 20 nM dual-labeled probe. The amplification profile was as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min. Amplification products were quantified by comparison of experimental C_t (threshold cycle—defined as the PCR cycle where an increase in fluorescence surpasses background levels) levels with those of the standard curve. The standard curve for each gene was generated from two replicates of serial dilutions of recombinant plasmid.

Table 1
Nucleotide sequences of PCR primers and probes used to assay gene expression by real-time quantitative PCR

Gene	Primer	Sequence
Ghrelin	Sense	GATGCTCACCATGCTGGGTC
	Anti-sense	GAGCCACACATCACAGTTTCAG
	Probe[a]	TGTCGGTCACATGATGTTGCTCCTCTGC
Growth hormone	Sense	CATATCTCAGAGAAGCTGGC
	Anti-sense	GGTCTGGTAGAAATCCTCGA
	Probe[b]	CCACACATCCCTCGATAAGTACGCC

[a] The probe for ghrelin was dual labeled with a reporter dye (FAM, 6-carboxyfluorescein) at the 5′ end and a quencher dye (BHQ-1, Black Hole quencher-1) at the 3′ end (Integrated DNA Technologies, Coralville, IA).

[b] The probe for growth hormone (GenBank Accession No. S69215) was dual labeled with a reporter dye (FAM) at the 5′ end and a quencher dye (BHQ-1) at the 3′ end (Biosearch Technologies, Novato CA).

2.5. In vivo effect of catfish ghrelin on the secretion of pituitary GH

Immature channel catfish of the NWAC103-strain, weighing approximately 150 g, were obtained from the USDA-ARS Catfish Genetics Research Unit, Stoneville, MS aquaculture facility. Catfish were reared in 76-L aquaria with flowing 26 °C well water under a 14:10 h light/dark photoperiod and maintained following accepted standards of animal care, approved by the Institutional Animal Care and Use Committee (IACUC) according to USDA-ARS policies and procedures. Food was withheld 3 days prior to injection. Fish were anesthetized in a solution of tricaine methanesulfonate (0.1 g/L; Finquil; Argent Chemical Laboratories, Richmond, WA), and intraperitoneally injected with either 25 or 250 ng/g body weight (BW) of either amidated catfish ghrelin (ghrelin-amide) or Gly-extended catfish ghrelin (ghrelin-Gly) with decanoic acid modification (see Section 3). Control fish received 0.9% NaCl solution only (1 μL/g body weight). Blood samples were collected in syringes coated with heparin from the caudal vessels at 1, 2, 4, and 6 h after injection ($n = 5$ at each time point). Plasma was separated by centrifugation and stored at −80 °C until assayed for GH. Plasma GH concentrations were determined using a homologous ELISA validated for quantifying GH in channel catfish plasma (Drennon et al., 2003).

2.6. In vivo effects of catfish ghrelin on pituitary GH mRNA expression

Immature channel catfish of the NWAC103-strain, weighing approximately 110 g, were reared and maintained as previously described. Fish were anesthetized and injected as previously described with either 25 or 250 ng/g BW ghrelin-amide and ghrelin-Gly with decanoic acid modification. Control fish again received 0.9% NaCl solution only (1 μL/g body weight). Five fish per treatment were euthanized in a solution of tricaine methanesulfonate (0.2 g/L) at 0.5, 1, and 3 h after injection, and pituitary tissue was rapidly excised from each fish, flash-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation, as previously described. Growth hormone mRNA expression was determined by real-time quantitative PCR using the iCycler iQ (Bio-Rad) as previously described for ghrelin, except for primers and probe, which were GH-specific (Table 1).

2.7. Statistical analyses

The experimental data were analyzed using SAS software system version 8.00 (SAS Institute, Cary, NC). Assumptions for homogeneity of variance and normality of the data were tested by examination of correlation between absolute residuals and predicted values, and the Shapiro–Wilks test for normality. Tissue mRNA expression data were log transformed prior to analysis to meet the assumptions. Tissue ghrelin mRNA expression was subjected to one-way analysis of variance (ANOVA) mixed-model procedures. Plasma growth hormone concentrations and pituitary mRNA expression were subjected to two-way analysis of variance (ANOVA) mixed-model procedures with treatment and time as the fixed effects and fish within treatment as the random effect. When significant differences were found using ANOVA, pairwise contrasts were made using Fisher's LSD test to identify differences at the 5% level. Results are presented as means ± standard error (SE).

3. Results

3.1. Purification and sequence determination of catfish ghrelin

Ghrelin activity was divided into five groups in CM-HPLC (pH 4.8) of the SP-III fraction (Fig. 1A). Each active fraction was purified by two different RP-HPLC runs following anti-ghrelin IgG immuno-affinity column chromatography. Fig. 1B shows a representative HPLC profile of final purification from group "e." The yield of peptide estimated from each peak height is summarized in Table 2. Amino acid sequence of the fraction 4 peptide was analyzed, and consequently a 21-amino acid

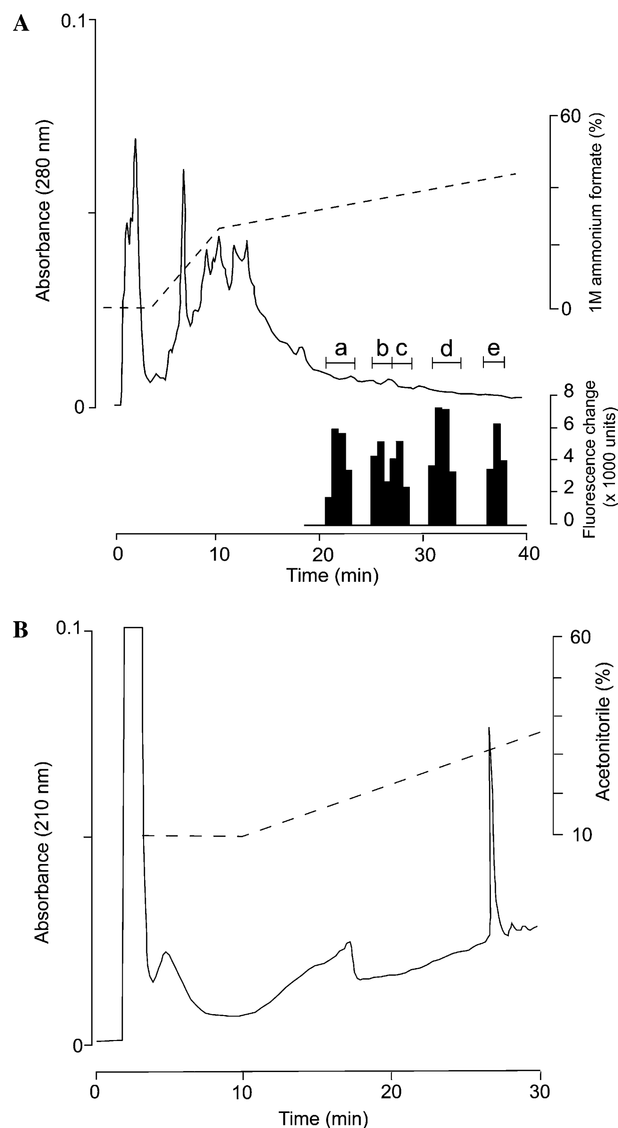

Fig. 1. Purification of catfish ghrelin from stomach extracts. Black bars indicate the measured fluorescence changes corresponding to intracellular calcium concentrations in CHO-GHSR62 cells. (A) Carboxymethyl (CM)-cation ion exchange HPLC (pH 4.8) of SP-III fraction of stomach extract. Each active fraction (a–e) was separately purified by an anti-rat ghrelin[1–11] IgG immuno-affinity chromatography. (B) Final purification of fraction "e" by reverse-phase (RP)-HPLC.

sequence was determined: GSXFLSPTQKPQN RGDRKPPR (X, unidentified). An amino acid of the position 3 was not detected, probably due to acyl modification as previously observed (Kaiya et al., 2004).

To determine the complete amino acid sequence of the peptide, we isolated cDNA encoding the preproghrelin from catfish stomach mRNA. We isolated several cDNAs. Two reasons for the different lengths of cDNA may relate to: (i) an AG-repeated sequence ranging from 15 to 19 bases was present in the 5′ non-coding region; and (ii) two different nucleotide sequences encoding the last three amino acids (Gly-Ala-Asn) of the prohormone were present. Taking these into consideration, two

Table 2
Summary of purified ghrelin and expected acyl modification

CM-HPLC	RP-HPLC fraction	Yield (pmol)	Theoretical [M + H]	Detected [M + H]	Difference[a]	Expected molecular form
a	1	19	2663.3[b]	2661.7	1.6	(C10:1) ghrelin-Gly
b	2	25	2663.3	2663.8	−0.5	(C10:0) ghrelin-Gly
c	3	23	2605.3[c]	2601.2	4.1	(C10:2) ghrelin
d	4	44	2605.3	2603.7	1.6	(C10:1) ghrelin
e	5	48	2605.3	2605.8	−0.5	(C10:0) ghrelin

(C10:0) represents Ser[3] acyl modification with saturated decanoic acid.
(C10:1) and (C10:2) represent decanoyl-modified Ser[3] with the number of unsaturated fatty acid.
[a] The value is difference between theoretical mass and detected mass.
[b] The value is the molecular weight of (C10:0) ghrelin-Gly.
[c] The value is the molecular weight from (C10:0) ghrelin.

representative nucleotide sequences of catfish ghrelin cDNA were deposited to GenBank as catfish ghrelin-1 and catfish ghrelin-2 (Fig. 2; AB196449 and AB196450). The nucleotide sequence of catfish ghrelin-1 was 502 bp in length, containing an 87 bp 5′-untranslated region (UTR), a 327 bp coding region, and an 88 bp 3′-UTR. This cDNA clone had two polyadenylation signals in the 3′-UTR. Another catfish ghrelin-2 cDNA was 522 bp long, containing a 95 bp 5′-UTR, a 327 bp coding region, and a 100 bp 3′-UTR. In this cDNA, one polyadenylation signal was identified in the 3′-UTR. The deduced amino acid sequences of the 327 bp coding region, present in the two cDNAs, are identical and showed that the catfish ghrelin precursor consists of 108 amino acids (Fig. 3). However, two nucleotide alleles are present at amino acid position 65, where Lys (AAG) changed to Asn (AAC), and at amino acid position 81, where GCG changed to GCC but the amino acid (Ala) does not change. As a result, two amino acid sequences of prohormone are present in catfish ghrelin. From the sequence determination of the mature ghrelin, the unidentified third amino acid was identified as serine, as seen in other species, except bullfrog (Kaiya et al., 2001). Furthermore, a typical dibasic processing and amidation signal (Gly-Arg-Arg), which was seen in other ghrelin precursors of fish, followed the carboxyl-terminal end of the mature peptide. Thus, the primary sequence of catfish ghrelin was predicted to be a 22-amino acid peptide, GSSFLSPTQKPQNRGDRKPPRV.

A characteristic of ghrelin is modification by fatty acids. To determine acyl modification as well as the amino acid sequences of purified peptides, we deter-

Catfish ghrelin-1

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  1 ATAACGCCGTCCTGACATTACCTGCCTCTAGTTTTACTCCAGATCCTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATGCTCACCATG  90
                                                                                          M
 91 CTGGGTCACGGTCGTGTCGGTCACATGATGTTGCTCCTCTGCGCTTTTTCTTTGTGGGCTGAAACTGTGATGTGTGGCTCCAGCTTCCTC 180
     L  G  H  G  R  V  G  H  M  M  L  L  L  C  A  F  S  L  W  A  E  T  V  M  C  G  S  S  F  L
181 AGTCCAACCCAAAAACCACAGAATCGTGGAGACCGAAAGCCACCTCGGGTGGGACGGAGGACTGCAGCTGAACTCGAGGCTCCTCTTCCT 270
     S  P  T  Q  K  P  Q  N  R  G  D  R  K  P  P  R  V  G  R  R  T  A  A  E  L  E  A  P  L  P
271 TCCGAGGAGAAGATCATGGTGAGCGCACCGTTCCAGCTGGCCGTGTCTCTCAGTGACGCGGAATACGAGGATTATGGTCCTGTGCTACAG 360
     S  E  E (K) I  M  V  S  A  P  F  Q  L  A  V  S  L  S  D  A  E  Y  E  D  Y  G  P  V  L  Q
361 AGGATGTTACTGGATGTCCTGGGTGATCCGCCTACTTTGGACGGCGCAAACTAACAACATTTGAACTTAAATGATTCCTGATTCATAACC 450
     R  M  L  L  D  V  L  G  D  P  P  T  L  D  G  A  N  *
451 GCTCAAATTATTAAAACAAACTGTGAAATGTG[AATAAA]ATGAA[AATAAA]ACG  502
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Catfish ghrelin-2

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  1 ATAACGCCGTCCTGACATTACCTGCCTCTAGTTTTACTCCAGATCCTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATGC  90

 91 TCACCATGCTGGGTCACGGTCGTGTCGGTCACATGATGTTGCTCCTCTGCGCTTTTTCTTTGTGGGCTGAAACTGTGATGTGTGGCTCCA 180
           M  L  G  H  G  R  V  G  H  M  M  L  L  L  C  A  F  S  L  W  A  E  T  V  M  C  G  S  S
181 GCTTCCTCAGTCCAACCCAAAAACCACAGAATCGTGGAGACCGAAAGCCACCTCGGGTGGGACGGAGGACTGCAGCTGAACTCGAGGCTC 270
       F  L  S  P  T  Q  K  P  Q  N  R  G  D  R  K  P  P  R  V  G  R  R  T  A  A  E  L  E  A  P
271 CTCTTCCTTCCGAGGAGAACATCATGGTGAGCGCACCGTTCCAGCTGGCCGTGTCTCTCAGTGACGCGGAATACGAGGATTATGGTCCTG 360
       L  P  S  E  E (N) I  M  V  S  A  P  F  Q  L  A  V  S  L  S  D  A  E  Y  E  D  Y  G  P  V
361 TGCTACAGAGGATGTTACTGGATGTCCTGGGTGATCCGCCTACTTTGGATGGAGCCAACTAACAGCAGGGATCCTGATTCATAACGCTCT 450
       L  Q  R  M  L  L  D  V  L  G  D  P  P  T  L  D  G  A  N  *
451 CTTTCAAATTGCACATTATTAAAACAACAGATCGTGAGGAAAATGAG[AATAAA]GTGACGCTATGGCGATCTC  522
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Fig. 2. Nucleotide sequences and deduced amino acid sequence of the catfish ghrelin cDNA. Two cDNAs have been deposited in the DDBJ/EMBL/GenBank databases as catfish ghrelin-1 (AB196449) and catfish ghrelin-2 (AB196450). Boxes indicate the polyadenylation signal (AATAAA). Typical amidation signal (GR) is underlined. Double underline in catfish ghrelin-2 sequence indicates a splice sequence (GA/TG) of the gene. An amino acid substitution was circled. Prepro- and mature-ghrelin (bolded letters) are composed of 108 and 22 amino acids, respectively.

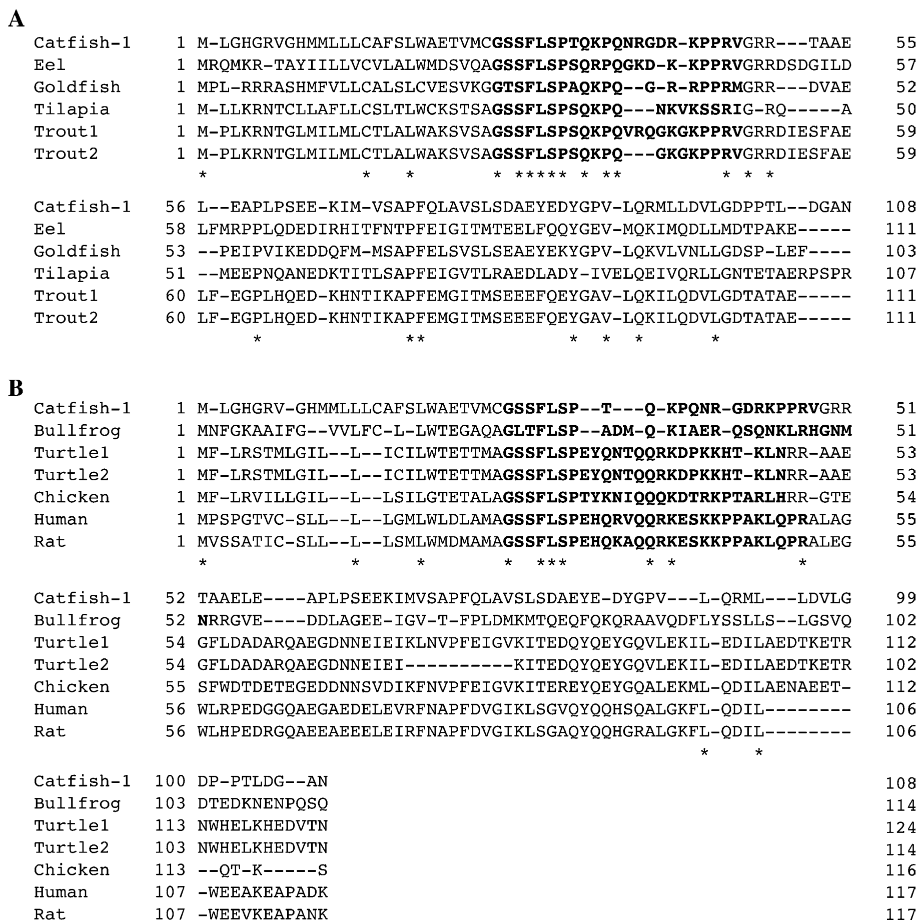

Fig. 3. Comparison of amino acid sequences of catfish preproghrelin. Alignment of preproghrelin among fish species (A) and some tetrapods (B) is shown. Bolded letters indicate the sequence of mature ghrelin.

mined the molecular mass of the purified peptides by MALDI-TOF mass spectrometry (Table 2). The theoretical mass, calculated from the 22-residue amidated peptide, is 2450.3. Furthermore, in the rainbow trout, Gly-extended, non-amidated ghrelin has been identified (Kaiya et al., 2003a), suggesting the presence of Gly-extended ghrelin in catfish. Theoretical mass of the Gly-extended, 23-amino acid peptide, is 2508.3. All of the detected masses were over 2600, indicating that octanoylated ghrelin, which has an amide structure at the C-terminus (2576.3), is not present in the purified peptides. Molecular masses of fraction 2 (2663.8) and fraction 5 (2605.8) were 154 mass units greater than the theoretical mass of the 23-residue, non-amidated, and the 22-residue amidated peptides, respectively. Thus, these Ser^3 hydroxyl groups are modified by *n*-decanoic acid. As such, the complete structure of peak 5 ghrelin is GSS(*O*-*n*-decanoyl) FLSPTQKPQNRGDRKPPRV-amide and peak 2 ghrelin is GSS(*O*-*n*-decanoyl) FLSPTQKPQNRGDRKPPRVG-OH. We designated these peptides as catfish ghrelin-amide and catfish ghrelin-Gly, respectively. It is predicted that other peptides have been modified by unsaturated decanoic acid (Table 2).

3.2. Expression of ghrelin mRNA in channel catfish tissues

Real-time quantitative PCR analysis revealed high levels of ghrelin gene expression in the stomach, moderate levels in the pancreas and gall bladder, and low levels in the remaining tissues (Fig. 4).

3.3. IP injection of catfish ghrelin

The effects of IP injection of ghrelin on circulating GH, time post-injection, and the interaction between the two were significant ($P < 0.05$). Plasma GH levels increased ($P < 0.05$) 1 h after injection of ghrelin-Gly at both doses and ghrelin-amide at 250 ng/g BW (Fig. 5). Plasma GH levels of fish injected with 25 ng/g of ghrelin-amide were not different ($P > 0.05$) from saline injected catfish at any time point. Plasma GH levels decreased to basal levels between 2 and 4 h post-injection and were similar ($P > 0.05$) among all treatments throughout the remainder of the experiment.

IP injection with ghrelin-Gly and ghrelin-amide, irrespective of concentration, caused an overall increase in the expression of pituitary GH mRNA, 1.59 ± 0.18 and

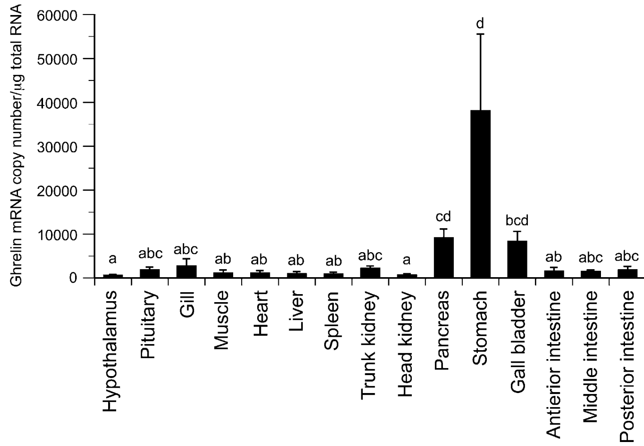

Fig. 4. Expression pattern of ghrelin mRNA in channel catfish tissues as determined by real-time quantitative PCR. Ghrelin copy number was normalized as a ratio to total RNA. Values represent means (±SE) of four individual catfish. Significant differences ($P < 0.05$) in tissue ghrelin expression are indicated by differing letters.

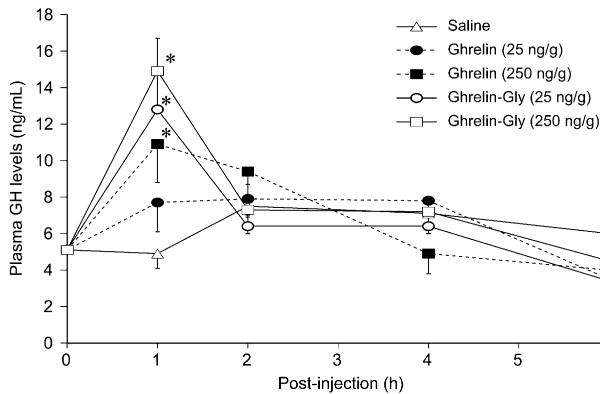

Fig. 5. Changes in plasma growth hormone (GH) levels after intraperitoneal injection of amidated ghrelin and ghrelin-Gly at two concentrations. Values represent means (±SE) of five individual catfish. Significant differences ($P < 0.05$) are expressed by an asterisk (*), compared with the values from saline injected catfish at each time point.

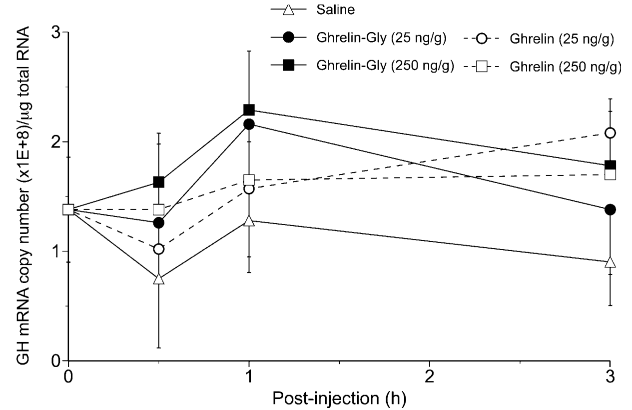

Fig. 6. Changes in pituitary growth hormone (GH) mRNA expression levels after intraperitoneal injection of amidated ghrelin and ghrelin-Gly at two concentrations. Values represent means (±SE) of five individual catfish. Two-way ANOVA revealed significant effects of treatment ($P < 0.05$) and time on pituitary GH mRNA levels, but no significant ($P > 0.05$) treatment × time interaction.

1.44 ± 0.18 (means ± SE) copy number (x1E + 8)/μg total RNA, respectively, relative to levels (0.82 ± 0.23 copy number (x1E + 8)/μg total RNA) in saline injected fish (Fig. 6). A significant effect ($P < 0.05$) of sampling time was also observed, with mean levels increasing over time. The interaction between IP injection of ghrelin and sampling time was not significant ($P > 0.05$); however, pituitary GH mRNA expression tended to be higher ($P < 0.1$) in catfish injected with ghrelin-Gly (250 ng/g) at 0.5 h post-injection, ghrelin-Gly (25 and 250 ng/g) at 1 h post-injection, and amidated ghrelin (25 ng/g) at 3 h post-injection.

4. Discussion

The present study demonstrated that ghrelin was present in a channel catfish and stimulated GH expression and the release of GH into circulation. Here, we discuss several new findings that have not been described in teleosts.

The hydroxyl group of Ser^3 or Thr^3, in all the known mammalian and non-mammalian ghrelins, is modified by *n*-octanoic acid except tilapia where only decanoylated ghrelin was identified (Kaiya et al., 2003c). The acylation is essential for receptor binding (Muccioli et al., 2001) and for eliciting ghrelin activity (Kojima et al., 1999; Matsumoto et al., 2001). In the present study, ghrelins modified by decanoic acid or unsaturated fatty acid forms at Ser^3 were also identified. The mechanisms governing acylation of ghrelin are still unknown, even in mammals. Recently, it has been reported that ingested medium-chain fatty acids influence the nature of the attached fatty acid (Nishi et al., 2005).

Two cDNAs comprising channel catfish preproghrelin were identified. Alternative splicing of the gene would be involved in the conformation of the cDNA. Interestingly, the last three amino acids of preproghrelin, Gly-Ala-Asn, consisted of different nucleotide fragments, which may be derived from the exon–intron junctions. Since there is a GA-TG sequence at the boundary between the amino acid Asp and Gly, catfish ghrelin-2 cDNA may consist of a fragment derived from an intron. However, splice variants that have been seen in the region of the mature peptide thus far, wherein des-Gln^{14} ghrelin (Hosoda et al., 2000, 2003) or des-VRQ ghrelin in rainbow trout (Kaiya et al., 2003a) occurs, have not been found in this study. Consequently, further study is needed to clarify genomic organization of the catfish ghrelin gene.

Catfish ghrelin has an amide structure at the carboxyl end of the peptide, which is a structure specific to teleost ghrelins and is not found in any tetrapod ghrelins, including mammalian, avian (Kaiya et al., 2002), amphibian (Kaiya et al., 2001) or reptilian (Kaiya et al., 2004). When the potency between eel and rat ghrelins, on

GH release in vivo in rats, was compared, there was no difference (Kaiya et al., 2002). Therefore, it is likely that the amide structure does not affect the receptor binding and activity at least in the rat GHS receptor; however, the effect of catfish ghrelin-Gly on GH release was more potent than that of ghrelin-amide, suggesting C-terminal modification alters the biological activity of ghrelin in the channel catfish. Such an effect of C-terminal modification has not been previously described for any vertebrate ghrelin. Further studies concerning the determination of catfish ghrelin receptor(s) followed by functional analysis may provide novel information such as a different receptor binding.

The expression of the catfish ghrelin gene was highest in the stomach followed by the pancreas and gall bladder. Thus, it is also likely that, in catfish, the stomach is the main expression and production site of ghrelin, as demonstrated in human (Ariyasu et al., 2001). Intestinal expression of the ghrelin gene is relatively low compared with the other fish such as eel and rainbow trout (Kaiya et al., 2003a,b), but it is similar to reported findings in tilapia (Kaiya et al., 2003c). In goldfish, which lacks a stomach, the intestinal tract is the main site for ghrelin gene expression (Unniappan et al., 2002). The role of intestinal ghrelin is, as yet, unclear but it has been reported that ghrelin stimulates gut motility in the rat (Edholm et al., 2004). Moderate ghrelin gene expression was also found in pancreas and gall bladder of the channel catfish, and this is the first report of such a finding in a teleost. In this regard, ghrelin mRNA expression has been found in pancreas of rat and bullfrog (Date et al., 2002; Kaiya et al., 2001) and in gall bladder of bullfrog (Kaiya et al., 2001). Recently, pancreatic ghrelin has been shown to be involved in endocrine function or cell development in the rodent pancreas (Adeghate and Parvez, 2002; Broglio et al., 2003; Date et al., 2002; Egido et al., 2002; Prado et al., 2004; Reimer et al., 2003), and similar functions may exist in the teleost pancreas. Expression of the ghrelin gene was observed in the catfish gill and kidney. While ghrelin mRNA and its peptide have been identified in mouse kidney (Mori et al., 2000), the effects of ghrelin on these tissues in catfish are unclear. However, Kozaka et al. (2003) reported a potent, antidipsogenic effect of ghrelin in seawater acclimated eels, suggesting that ghrelin may be involved in osmoregulation in fish. Thus, ghrelin may regulate water and electrolyte balances in gill and kidney. Hypothalamic expression of the ghrelin gene was not significant in catfish or in previous reports in fish. However, the effect of ghrelin on the central nervous system, such as stimulation of feeding and pituitary hormone secretion in fish, is clear (Unniappan et al., 2002, 2004; Unniappan and Peter, 2004). Furthermore, ghrelin receptor (GHS-R) is certainly present in seabream brain (Chan and Cheng, 2004). Consequently, detailed examination of ghrelin gene expression in brain regions, other than the hypothalamus, of catfish, which is a stenohaline teleost (Eckert et al., 2001), will undoubtedly provide valuable insight into the central functions of ghrelin as well as the possible osmoregulatory actions of this peptide in euryhaline and stenohaline teleosts.

Plasma GH levels increased after IP injection of ghrelin. In fish, similar in vivo effects have been demonstrated in rainbow trout (Kaiya et al., 2003a) and goldfish (Unniappan and Peter, 2004). However, the potency of ghrelin-Gly was greater in catfish than ghrelin-amide in this study. Although the injected molar concentration is somewhat higher in the case of ghrelin-Gly than ghrelin-amide, the difference of the effect is remarkable. Even though the Gly-extended ghrelin was isolated in rainbow trout, the differences in bioactivity of the ghrelin-Gly and ghrelin-amide were not examined (Kaiya et al., 2003a). Consequently, the bioactivity of the Gly-extended and amidated ghrelins, in channel catfish, appears to be the first such report of both ghrelins affecting GH release in a teleost system. However, it is evident that further studies are needed to determine whether there is a difference in the action between the effects of ghrelin-Gly and ghrelin-amide on GH release in other teleosts.

Catfish ghrelins, irrespective of the C-terminal modifications (ghrelin-Gly and amidated ghrelin), stimulated expression of pituitary GH mRNA. It has been shown, in rodent monolayer pituitary cell cultures, that the effects of ghrelin, or GHSs, on GH mRNA transcription are dependent upon the transcription factor, Pit-1 (Garcia et al., 2001; Soto et al., 1995), which is also present in teleosts (Higa et al., 2001). Interestingly, ICV injection of ghrelin stimulated GH release, but had no effect on GH mRNA expression in rat pituitary (Date et al., 2000); however, this lack of an effect on GH mRNA may be related to the sampling time points used to examine the effects of ICV ghrelin administration. There, Date and co-workers (2000) examined GH gene expression at time points where circulating GH levels were not different from controls (2 h after icv injection and 12 days after minipump implantation) and may have missed the point at which GH mRNA levels were elevated. In contrast, it has been reported that, in goldfish, ghrelin stimulates the GH mRNA expression in dispersed pituitary cells (Unniappan and Peter, 2004). It is, therefore, likely that ghrelin has an effect on stimulation of GH gene expression as well as that of GH release in fish. Whether this represents a key difference in the regulation of the GH gene, by ghrelin, between mammals and teleosts, will require further study and intense focus.

In summary, ghrelin was present in the catfish stomach and stimulated pituitary GH expression and the release of GH in vivo. However, different effects of ghrelin-Gly and ghrelin-amide on GH release were found and may suggest a difference in receptor binding of these two forms of ghrelin in teleosts.

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