African Journal of Microbiology Research Vol. 6(24), pp. 5222-5228, 28 June, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.528
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Cloning and prokaryotic expression of *ghrelin* gene in crucian carp (*Carassius auratus*)

Chaowei Zhou, Xindong Zhang, Tao Liu, Rongbing Wei, Dengyue Yuan and Zhiqiong Li*

Department of Aquaculture, College of Animal Science and Technology, Sichuan Agricultural University, Ya'an, 625014, China.

Accepted 25 April, 2012

To make up the flaw that there is no available information about *ghrelin* gene in crucian carp. The *ghrelin* gene was amplified by reverse transcription-PCR (RT-PCR) using total RNA extracted from intestine of crucian carp. PCR product was cloned into the pMD®19-T vector to construct pMD®19-T-*ghrlein* for sequencing. Then the cDNA was subcloned into the prokaryotic expressing plasmid vector pET32a and was transformed into host *Escherichia coli* strain Rosetta for expression. In this study, 490 bp fragment of *ghrelin* was obtained by RT-PCR. In comparison with other fishes, the amino acid sequences of *ghrelin* in crucian carp showed a high similarity to that of goldfish (99%). The high expression of *ghrelin* gene was detected in the intestine and liver by real-time PCR. IPTG at concentrations of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mmol/L could efficiently induce the expression of pGh-32. The result showed that the optimal concentration of IPTG was 0.3 mmol/L by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The *ghrelin* gene expressed as early as 1 h after IPTG induction, and reached peak levels after 3 h. Successful expression of *ghrelin* fusion protein in prokaryotic cell could lay a basis for further study of industrial production.

Key words: Crucian carp, *ghrelin*, cloning, prokaryotic expression.

INTRODUCTION

The discovery of *ghrelin* was reported by Kojima et al. (1999) who were searching for a ligand for an orphan G protein coupled receptor (GHS-R1a) that stimulates the secretion of growth hormone in the pituitary gland. The *ghrelin* possesses two forms in gastrointestinal tissue, designed n-octanoyl ghrelin and des- n-octanoyl *ghrelin*. The n- octanoyl *ghrelin* plays important roles in regulation of GH release in rat (Szczepankiewicz et al., 2010), While *ghrelin* activates growth-hormone secretagogue (GHS) receptor-expressing cells, the nonmodified des-n-octanyl form of ghrelin, designated as des-acyl *ghrelin*, does not (Hosoda et al., 2000).

In mammalian, *ghrelin* is involved in various physiological functions other than GH release in mammals (Kojima and Kangawa, 2005; Korbonits et al., 2004; Van Der Lely et al., 2004). *Ghrelin* plays critical role in the body, such as appetite, adjusting of energy metabolism and immune system (Hattori, 2009). In human, octanoylation of the gastric peptide *ghrelin* could produce active forms that regulate appetite and other metabolic functions (Goodyear et al., 2010).

In recent years, in teleosts, the spot of research was focus on the *ghrelin*. To our knowledge, the cDNA cloning and sequence analysis and appraisal of all amino acid of the *ghrelin* have been reported in non-mammalian vertebrates, such as goldfish (Unniappan et al., 2002), Nile tilapia (Parhar et al., 2003), Channel catfish (Kaiya et al., 2005), Sea bream (Yeung et al., 2006), Atlantic cod (Xu and Volkoff, 2009), and it demonstrated that there were invariably homology of molecular weight, amino acids and sequence in fish species, but there were no report about *ghrelin* in the crucian carp neither at home nor abroad.

The purpose of this paper was to identify the structure of *ghrelin* cDNA in crucian carp, to detect *ghrelin* expression in the tissues and *Escherichia coli* (*E. coli*). The current study would provide useful experimental

*Corresponding author. E-mail: lizhiqiong454@163.com. Tel: 86-835-2885654.

Table 1. Primer oligonucleotide sequences and their applications.

Primer name	Sequence (5`→3')	Size of the product (bp)	Applications
Ghrelin-F1	CTGTGCATTCTGCATACATATTTGAG	490	Cloning of ORF
Ghrelin-R1	GTTTTGGAAGATTATTACATC		Cloning of ORF
Ghrelin-F2	CGGATCCGGCACCAGCTTCCTCAGT	248	Prokaryotic expression
Ghrelin-R2	GCTCGAGTGAATTCAAGTGGCGA		Prokaryotic expression
Ghrelin-F3	GAAGAGATGTTGCAGAGCCAGAG	152	Real time PCR
Ghrelin-R3	GCCAAGAAGATTGACCAGAACC		Real time PCR
β-Actin-F4	TTTGAGCAGGAGATGGGAACC	134	Real time PCR
β-Actin-R4	AGGAAGGATGGCTGGAAAAGAG		Real time PCR

materials for further functional analysis of *ghrelin* in crucian carp.

MATERIALS AND METHODS

Fish for cloning crucian carp *ghrelin*

Healthy crucian carp were purchased from a local fish nursery in Ya'an City, China and kept for survival when transported to the laboratory. After the fish were sacrificed, the tissues were frozen at liquid nitrogen and then removed and stored at -80°C.

Cloning of ORF sequences of crucian carp *ghrelin*

Total RNA was extracted from tissues of intestine of crucian carp by the TRIzol reagent (TaKaRa, Dalian, China) following the manufacturer's protocol. The purified RNA concentration was quantified using a photometer (Bio-Rad) and the ratio of optical densities was between 1.8 and 2.0 (at 260 and 280 nm). Subsequently, RT-PCR was performed using a commercially available RT-PCR kit (TaKaRa, Dalian, China). For PCR, 100 ng of sense primer and antisense primer (Table 1) was used. The parameters for PCR were 94°C for 5 min, ×1; 94°C for 30 s; 47°C for 30 s, 72°C for 45 s, ×38; and then 72°C for 8 min. After visualizing by 1%(w/v) agarose gels electrophoresis, the PCR product was purified and cloned into plasmid vector pMD®19-T by T-A Cloing Kit (TaKaRa, Dalian, China). The recombinant plasmid of pMD®19-T-*ghrlein* was transformed into DH5a, cultured in LB medium at 37°C and then extracted by Sambrook′s method (Sambrook et al., 1989). The recombinant plasmid was subjected to DNA sequencing by automated sequence analysis (TaKaRa).

Deducing amino acid sequence of ORF and comparison with other teleosts

To compare the sequence of the *ghrelin* with that from other teleosts, we downloaded the ghrelin sequence of other teleosts in the NCBI database (http://www.ncbi.nlm.nih.gov/). Multiple alignments of the proteins of *ghrelin* ORF were constructed using ClustalX.

Real time PCR

Tissues (that is, intestine, liver, mesonephron, head, kidney, spleen, skin, heart, muscle, gill and pituitary gland) were pooled separately according to different tissue types from ten crucian carp and total RNA was extracted by TRIzol reagent (TaKaRa, Dalian, China). The housekeeping gene β-actin was used as the endogenous control. Primers F3/R3 and F4/R4 were employed to obtain partial fragment of *ghrelin* and β-actin cDNA respectively. All the primers used in the real-time PCR were listed in Table 1.

Subcloning and Construction of pGh-32 recombinant plasmid

pMD®19-T-ghrlein was used as a template to amplify a truncated gene encoding a signal peptide-deleted *ghrelin*. The gene amplification using primer F2/R2 (Table 1) resulted in the deletion of the first 78 nucleotides in the N-terminal of the *ghrelin* gene, and then the PCR product was purified with a commercially available kit (Keygen Biotech, Nanjing, Jiangsu, China); meanwhile, pET-32a was transformed into *E. coil* DH5a, and cultured in LB medium at 37°C and then extracted by Sambrook′s method (Sambrook et al., 1989). The plasmid pET-32a was digested with *Bam*HIand XhoIenzymes, and then purified with a commercially available kit. The truncated gene was cloned into the multiple cloning sites *Bam*HIand *Xho*I of prokaryotic expression vector pET-32a and the authenticity of insert was confirmed by automated sequence analysis (TaKaRa).

Expression of ghrelin gene

The recombinant plasmid of pGh-32 was relatively cultured in LB medium at 37°C and induced by IPTG at different concentrations of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mmol/L and different times of 0, 1, 2, 3, 4, 5 and 6 h. The supernatant and precipitate were separated through centrifugation after the bacterial pellet was ultrasonically broken (300V, 3×5s). The molecular mass and output of the target recombinant protein were measured by SDS-PAGE.

RESULTS

Cloning of crucian carp *ghrelin* and nucleotide sequence

As shown in Figure 1, the fragment of ghrelin gene was 490 bp in length and confirmed the target size. The nucleotide sequence and its deduced amino acid sequence were shown in Figure 2. The cDNA sequence included a whole Open Reading Frame (ORF), which

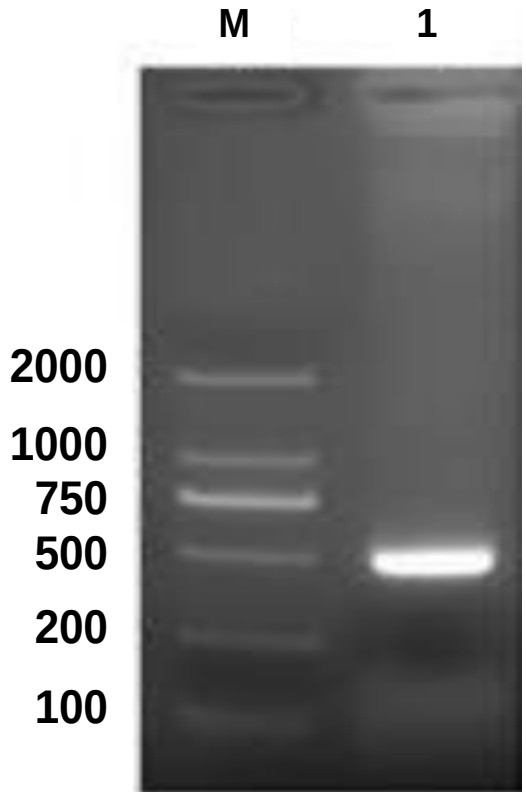

Figure 1. Amplification of *ghrelin* gene by RT-PCR. M, DL2, 000 DNA marker; Lane 1, PCR product of *ghrelin* amplification.

encoded 103 amino acids. The signal peptide region included 26 amino acids in length. (GeneBank accession number: HM567312).

Homology of the *ghrelin* gene

Figure 3 revealed the amino acid sequences of *ghrelin* of crucian carp and other fishes. The ORF region of *ghrelin* gene in crucian carp presented a high similarity with those of goldfish (99%), common carp (89.4%) and zebra fish (78.8%), as all four species derived from the Cyprinidae family. However, it only showed 53.8% similarity with channel fish. Therefore, it can be concluded that this *ghrelin* gene was rather conserved among different fish species.

mRNA expression

The expression levels of the *ghrelin* gene were shown in Figure 4. High expression levels were detected in the intestine and liver, followed by mesonephron, head kidney and the spleen, and the skin, heart, muscle, gill and pituitary gland showed relatively weak expression levels.

Construction and identification of the recombinant plasmid

The mature peptide gene fragment was amplified from the recombinant plasmid of pMD-*ghrelin* by PCR with the size of 248 bp and inserted into bacterial expression vector of pET-32a. As a result, the prokaryotic expression plasmid pGh-32 was obtained. pGh-32 was amplified and purified to recycle, digested with *Bam*HIand XhoIenzymes, and tested by 1% (w/v) agarose gels electrophoresis. The flow chart of the vector construction was shown in Figure 5.

Expression of the target recombinant protein

The expressed products detected with 15% SDS-PAGE and a 27.0 KDa protein band could be seen after staining with Coomassie brilliant blue R250 (Figures 6 and 7). IPTG at concentrations of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mmol/L could efficiently induce the expression of pGh-32. SDS-PAGE indicated that the optimal concentration of IPTG was 0.3 mmol/L. The *ghrelin* gene expressed as early as 1 h after IPTG induction, attaining peak levels around 3 h (Figure 7), the *ghrelin* fusion protein was mainly soluble protein and appeared in the precipitate only in a small amount (Figure 8).

DISCUSSION

Ghrelin plays an important role in appetite, adjusting of energy metabolism and immune system. More recently, it has been reported to be related to human diseases (Vila et al., 2007). The cDNA cloning and sequence analysis as well as appraisal of all amino acid of the *ghrelin* have been reported in non-mammalian vertebrates. However, no information is available on the role of *ghrelin* in teleost diseases. In this study, we obtained 490 bp of *ghrelin* which encoded 103 amino acids of ORF from the intestine of crucian carp, and the *ghrelin* involved 26 amino acids of the signal peptide region. The mature peptide started immediately after the signal peptide and this indicated that protein will be secreted out of the cell after its synthesis (Von Heijne, 1992) which demonstrated it was the secreted protein. The the high similarity between crucian carp and other fishes was consistent with previous studies (Kaiya et al., 2003c). Many species like *rainbow trout* (Kaiya et al., 2003a), *Janpanese eel* (Kaiya et al., 2003b), and *channel catfish* (Kaiya et al., 2005) showed highest expression levels in the stomach. Because the crucian carp lacks a stomach, our result showed the highest expression of *ghrelin* mRNA was found in intestine which had been proved in Cyprinidae family (Unniappan et al., 2002). This finding indicated that *ghrelin* could play important roles in gastrointestinal hormone in the crucian carp.

```
                                                    CATCTGTGCATTCTGCATACATATTTGAGACTTT
TAAAGTGCAGCCATTCAGAGTGTTGTCGTAAAACAGAACTAAACCAGGTGACTTCCCAGG
ATGCCTCTGCGTCGTCGTGCCAGCCACATGTTTGTGCTCTTATGTGCTCTTTCCTTGTGT
 M  P  L  R  R  R  A  S  H  M  F  V  L  L  C  A  L  S  L  C
GTTGAGTCTGTGAAAGGTGGCACCAGCTTCCTCAGTCCTGCTCAGAAACCACAGGGTCGA
 V  E  S  V  K  G  G  T  S  F  L  S  P  A  Q  K  P  Q  G  R
AGGCCACCCCGGATGGGCAGAAGAGATGTTGCAGAGCCAGAGATCCCAGTGATTAAAGAG
 R  P  P  R  M  G  R  R  D  V  A  E  P  E  I  P  V  I  K  E
GATGACCAGTTCATGATGAGTGCTCCGTTCGAACTGTCTGTGTCTCTGAGCGAAGCAGAG
 D  D  Q  F  M  M  S  A  P  F  E  L  S  V  S  L  S  E  A  E
TATGAGAAATATGGTCCTGTTCTGCAGAAGGTTCTGGTCAATCTTCTTGGCGATTCGCCA
 Y  E  K  Y  G  P  V  L  Q  K  V  L  V  N  L  L  G  D  S  P
CTTGAATTCTGACAAGAGCTAAAAGTTCTACAAGATTCAGCTCCTTATAAACCATTAAAA
 L  E  F
TGTTTGTAAGGTAGAAATGATGTAATAATCTTCCAAAAC
```

Figure 2. Nucleotide and putative amino acids sequences of crucian carp *ghrelin*. Beneath the nucleotide sequence is the puttied amino acids sequence. The signal peptide region is the black box.

```
crucian carp      1 MPLRRRASHMFVLLCA-LSLCVESVKGGTSFLSPAQKPQGR---RPPRMGRRDVAEPEIP 56
goldfish          1 MPLRRRASHMFVLLCA-LSLCVESVKGGTSFLSPAQKPQGR---RPPRMGRRDVAEPEIP 56
common carp       1 MPLHFRASHMFLLLCA-LSLCVESVRGGTSFLSPAQKPQGR---RPPRVGRRDVAEPEIP 56
zebrafish         1 MPLRCRASSMFLLLCVSLSLCLESVSGGTSFLSPTQKPQGR---RPPRVGRREAADPEIP 57
channel fish      1 MLGHGRVGHMMLLLCA-FSLWAETVMCGSSFLSPTQKPQNRGDRKPPRVGRRTAAELEAP 59
                    *  : *.. *::***. :**  *:*  *:*****:****.*   :***:*** .*: * *

crucian carp     57 VIKEDDQFMMSAPFELSVSLSEAEYEKYGPVLQKVLVNLLGDSP-LEF-- 103
goldfish         57 VIKEDDQFMMSAPFELSVSLSEAEYEKYGPVLQKVLVNLLGDSP-LEF-- 103 99.0%
common carp      57 VIKENDQFMMSAPFELSVSLSEAEYEKYGPVLQNVLGNLLSDPP-LEF-- 103 89.4%
zebrafish        58 VIKEDDRFMMSAPFELSMSLSEAEYEKYGPVLQNLLEDLLRDSS-FEF-- 104 78.8%
channel fish     60 LPSE-EKIMVSAPFQLAVSLSDAEYEDYGPVLQRMLLDVLGDPPTLDGAN 108 53.8%
                    : .* :::*:****:*::***:****.******.:* ::* *.. ::
```

Figure 3. Multiple alignment of the deduced amino acid sequences of *ghrelin* in crucian carp and other vertebrates. The multiple alignments were produced using ClustalX. '*' indicates positions that have a single, fully conserved residue. ':' and '.' indicate positions that have strong and weak similarity, respectively. Amino acid identities (%) between crucian carp and other vertebrates are show at end of each aligned sequence.

Previous study (Itakura et al., 1977) reported that it was a milestone in genetic engineering to make a foreign gene successfully expressed in *E. coil* with the advantage of rapid growth rate, capacity for continuous fermentation, and relatively low cost. The purpose of the current study was to obtain the high expression levels of target gene to facilitate further functional analysis. The 26-amino acid signal peptide was identified by SingalP v3.0 software, which was often useless but influenced the protein expression in prokaryotic system; therefore, we cloned a truncated gene encoding the target protein without signal peptide into prokaryotic vector pET-32a. SDS-PAGE performed in this study confirmed that a 27 KDa protein band could be seen after staining which indicated that the recombinant prokaryotic expression system of pGh-32 was constructed successfully.

The product of crucian carp *ghrelin* gene was mainly soluble protein, which was consistent with the pig *ghrelin* protein (Yang et al., 2005). Intriguingly, the *ghrelin* gene in Black-feather chicken was expressed in *E. coli* 2566 by pTYB11 prokaryotic expression plasmids and the *ghrelin* protein was cytorrhyctes (Dai et al., 2008). These could be explained that the vector, form or condition might impact the existing form of the *ghrelin* protein in the host cell, and further study needed to be continued. The output of *ghrelin* was relatively high (approximately 33% of the total bacterial proteins) and this was beneficial to industrial production.

Conclusions

In conclusion, the *ghrelin* gene was obtained by molecular cloning techniques and was successfully

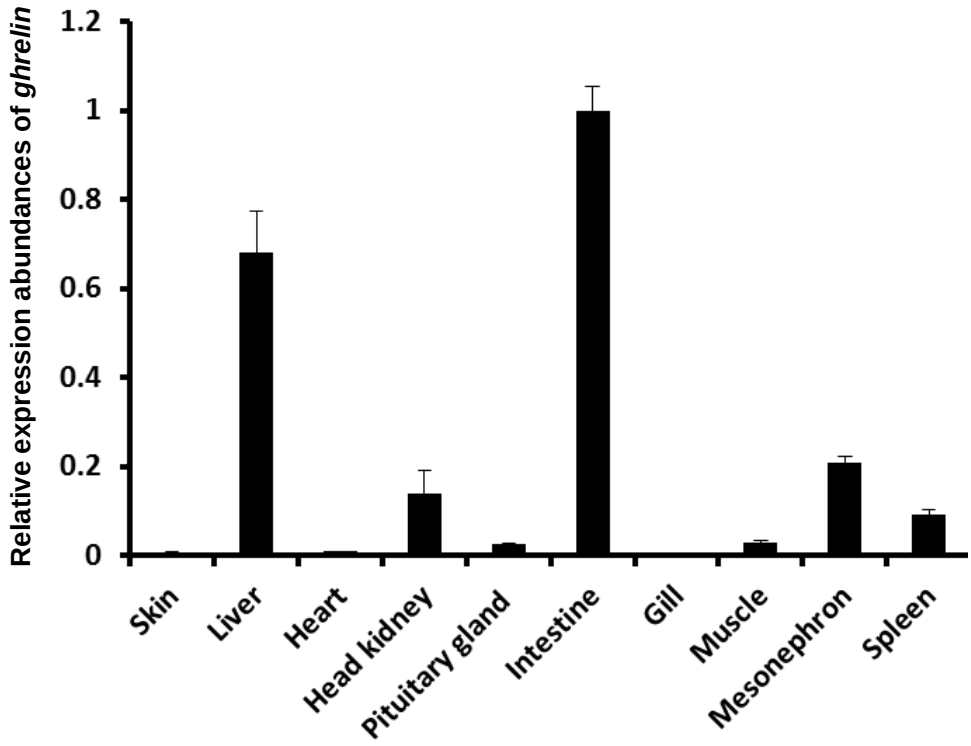

Figure 4. Expression level of *ghrelin* gene in ten tissues of the crucian carp.

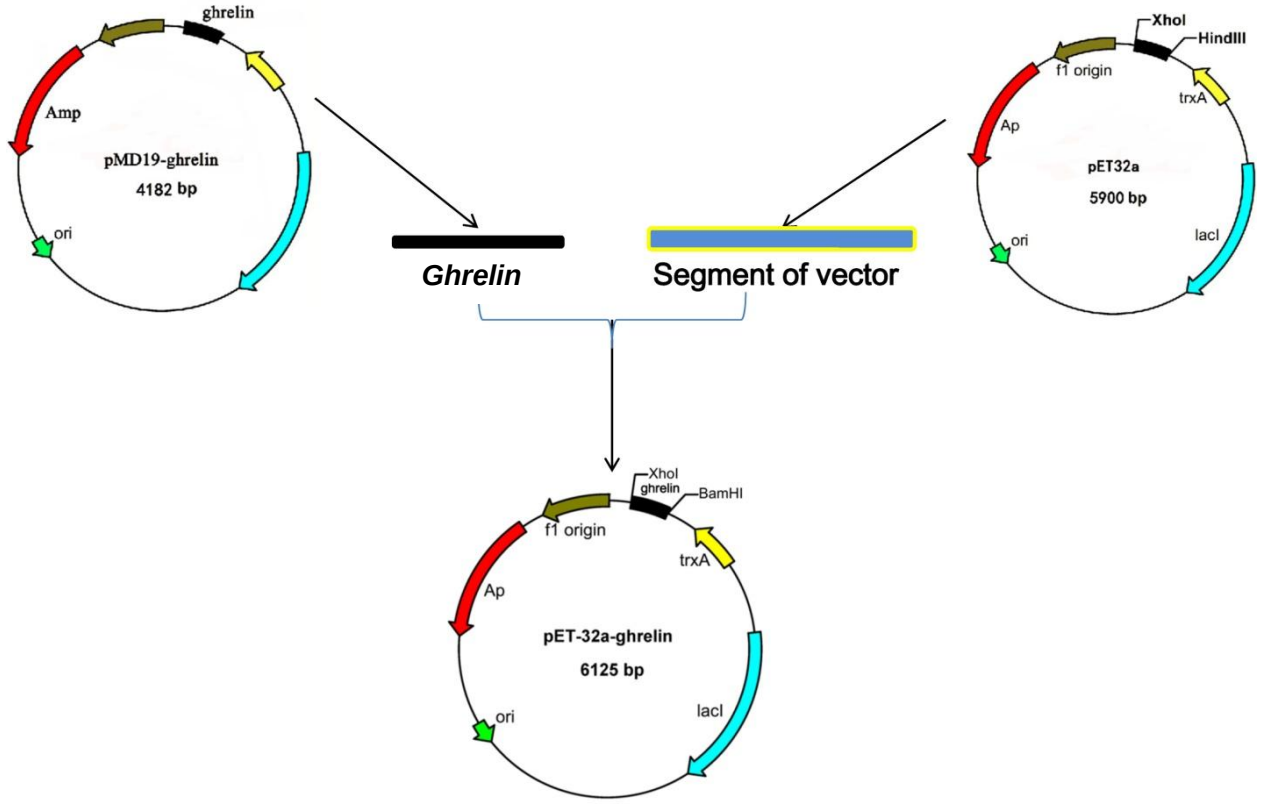

Figure 5. Flow chart of the vector construction.

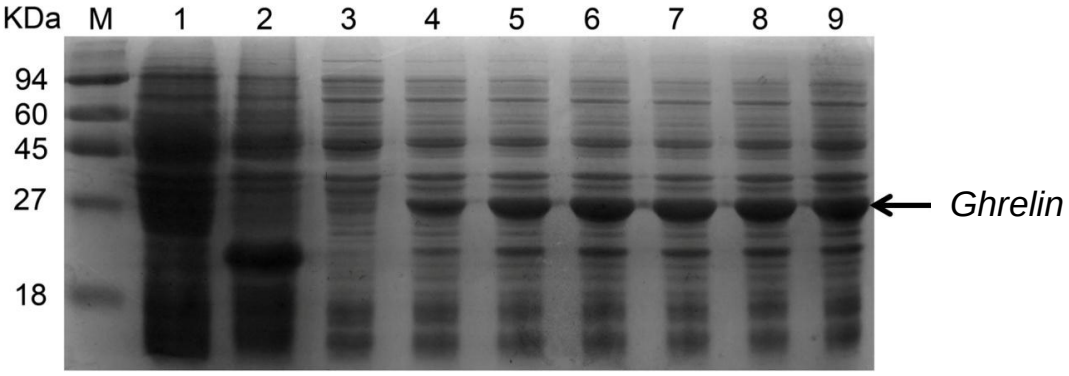

Figure 6. *Ghrelin* expression induced with different dosages of IPTG. M: Protein marker; Lane 1: Blank control; Lane 2: PET32a after induced; Lane 3: None-induced with IPTG; Lane 4-9: Induced with 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mmol/L IPTG, respectively.

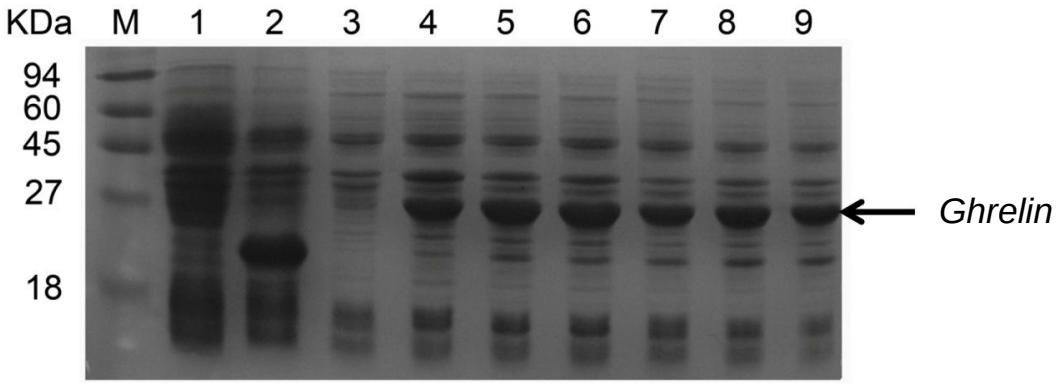

Figure 7. *Ghrelin* expression induced with IPTG at different times. M: Protein marker; Lane 1: Blank control; Lane 2: PET32a after induced; Lane 3: None-induced with IPTG; Lane 4-9: Induced with IPTG at 1, 2, 3, 4, 5 and 6 h, respectively.

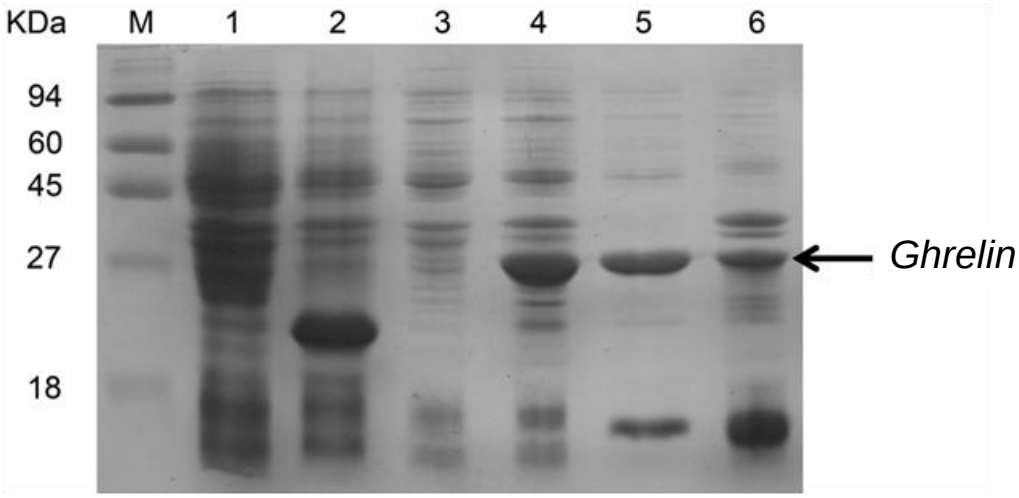

Figure 8. Identification of soluble protein of recombinant PET32a/*ghrelin*. M: Protein marker; Lane 1: Blank control; Lane 2: PET32a after induced; Lane 3: None-induced with IPTG; Lane 4: PET32a/*ghrelin* after induced; Lane 5 and 6: Bacterial supernatant and precipitate with IPTG, respectively.

expressed in *E. coli* in this study, and this will lay the foundation for the further study on the function of this protein and its mechanism.

ACKNOWLEDGEMENTS

The researchers would like to thank the staff of the Department of aquaculture, Sichuan Agricultural University, Ya' an, Sichuan, China.

REFERENCES

Dai X, Ran X, Wang J (2008). cDNA and Genomie DNA Cloning and Expression of Ghrelin Gene in Blaek-feather Chicken of Guizhou. China Poult., 30(7): 25-28.

Goodyear S, Arasaradnam RP, Quraishi N, Mottershead M, Nwokolo CU (2010). Acylated and des acyl ghrelin in human portal and systemic circulations. Mol. Biol. Rep., 37(8): 3697-3701.

Hattori N (2009). Expression, regulation and biological actions of growth hormone (GH) and ghrelin in the immune system. Growth Horm. IGF Res., 19(3): 187-197.

Hosoda H, Kojima M, Matsuo H, Kangawa K (2000). Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem. Biophys. Res. Commun., 279(3): 909-913.

Itakura K, Hirose T, Crea R, Riggs AD, Heyneker HL, Bolivar F (1977). Expression in Escherichia coli of a chemically synthesized gene for the hormone somatostatin. Science, 198(4321): 1056-1063.

Kaiya H, Kojima M, Hosoda H, Moriyama S, Takahashi A, Kawauchi H (2003a). Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. Endocrinology, 144(12): 5215-5226.

Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG (2003b). Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. J. Endocrinol., 176(3): 415-423.

Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG (2003c). Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, Oreochromis mossambicus. Comp. Biochem. Physiol. B., 135(3): 421-429.

Kaiya H, Small BC, Bilodeau AL, Shepherd BS, Kojima M, Hosoda H (2005). Purification, cDNA cloning, and characterization of ghrelin in channel catfish, Ictalurus punctatus. Gen. Comp. Endocrinol., 143(3): 201-210.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature, 402(6762): 656-660.

Kojima M, Kangawa K (2005). Ghrelin: structure and function. Physiol. Rev., 85(2): 495-522.

Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB (2004). Ghrelin--a hormone with multiple functions. Front. Neuroendocrin., 25(1): 27-68.

Parhar IS, Sato H, Sakuma Y (2003). Ghrelin gene in cichlid fish is modulated by sex and development. Biochem. Bioph. Res. Co., 305(1): 169-175.

Sambrook J, Fritsch E, Maniatis T (1989). Molecular cloning: a laboratory manual. 2nd. New York: Cold Spring Harbor Laboratory, 18: 58.

Szczepankiewicz D, Skrzypski M, Pruszynska-Oszmalek E, Zimmermann D, Andralojc K, Kaczmarek P (2010). Importance of ghrelin in hypothalamus - pituitary axis on growth hormone release during normal pregnancy in the rat. J. Physiol. Pharmacol., 61(4): 443-449.

Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K (2002). Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology, 143(10): 4143-4146.

Van Der Lely AJ, Tschop M, Heiman ML, Ghigo E (2004). Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr. Rev., 25(3): 426.

Vila G, Maier C, Riedl M, Nowotny P, Ludvik B, Luger A (2007). Bacterial endotoxin induces biphasic changes in plasma ghrelin in healthy humans. J. Clin. Endocr. Metab., 92(10): 3930.

Von Heijne G (1992). Membrane protein structure prediction: Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol., 225(2): 487-494.

Xu M, Volkoff H (2009). Molecular characterization of ghrelin and gastrin-releasing peptide in Atlantic cod (Gadus morhua): cloning, localization, developmental profile and role in food intake regulation. Gen. Comp. Endocr., 160(3): 250-258.

Yang L, Yang W, ZHAO Y, QIAN J, Wang Z (2005). Chemical Synthesis and Prokaryotic Expression of Ghrelin of Pig. Chin. J. Vet. Sci., 25(6): 614-616.

Yeung CM, Chan CB, Woo NY, Cheng CH (2006). Seabream ghrelin: cDNA cloning, genomic organization and promoter studies. J. Endocrinol., 189(2): 365-379.