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Molecular characterization and expression pattern of an α-amylase gene (*HcAmy*) from the freshwater pearl mussel, *Hyriopsis cumingii*

G. Ren[1,2], J.Y. Tang[1] and Y. Wang[1]

[1]College of Animal Sciences, Zhejiang University, Hangzhou, China
[2]College of Life Science, Shaoxing University, Shaoxing, China

Corresponding author: Y. Wang
E-mail: ywang@zju.edu.cn

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ABSTRACT. The freshwater pearl mussel *Hyriopsis cumingii* is of commercial importance because it produces the freshwater pearl; however, knowledge about the molecular characterization and regulation mechanisms of α-amylase remains unknown for this species. In this study, the full-length cDNA of the α-amylase gene (*HcAmy*) was isolated from *H. cumingii* by the rapid amplification of cDNA ends. Tissue-specific expression analysis showed that *HcAmy* mRNA was mainly expressed in the hepatopancreas; although, the gene was also expressed in the adductor muscle, intestine, gill, and crystalline style. After 2 weeks starvation, the expression of *HcAmy* mRNA in the hepatopancreas was upregulated at 24 h after re-feeding or when exposed to algal concentration of 32 µg/L chlorophyll-*a*, indicating that the *HcAmy* mRNA expression in *H. cumingii* is regulated by algal availability. The results of this study confirm that the *HcAmy* gene is an important component of the carbohydrate metabolism of *H. cumingii* fed phytoplankton. In addition, this study demonstrates that the modulation of this gene is dependent on environmental food availability, including starvation, re-feeding time following a period of starvation, and algal

concentrations during re-feeding.

Key words: *Hyriopsis cumingii*; α-amylase; Gene expression; Food availability

INTRODUCTION

Most bivalves are filter feeders that ingest particulates suspended in the water column, such as phytoplankton, bacteria, zooplankton, and detritus (Shumway, 2011; Ezgeta-Balić et al., 2012). Carbohydrases, such as α-amylase, cellulose, and chitinase, play an important role in the utilization of phytoplankton by bivalves (Gosling, 2003). Among the carbohydrases identified from bivalves, α-amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) catalyzes the endohydrolysis of 1,4-α-D-glucosidic linkages of polysaccharides, and participates in the glycogen utilization of bivalves (Matsui et al., 1996, Areekijseree et al., 2004). In marine bivalves, α-amylase is a limiting enzyme of carbohydrate assimilation (Sellos et al., 2003; Prudence et al., 2006); consequently, the modulation of α-amylase activity affects the assimilation rate and growth of bivalves (Huvet et al., 2003).

The characteristics of α-amylase have been reported for various marine mollusks, such as *Pecten maximus* (Le Moine et al., 1997), *Crassostrea gigas* (Huvet et al., 2003), and *Haliotis discus discus* (Nikapitiya et al., 2009). However, knowledge about the molecular characteristics of the α-amylase gene in freshwater mollusks remains limited (Areekijseree et al., 2004). α-Amylase expression is influenced by intrinsic factors, such as hormones and biogenic amines (Giard et al., 1998; Nachman et al., 1999), and extraneous factors, such as food availability (Le Pennec and Le Pennec, 2002; Fernandez-Reiriz et al., 2005; Huvet et al., 2012). However, there is a paucity of information about the effect of the re-feeding regime on the α-amylase expression of bivalves after a period of starvation (Ibarrola et al., 1998; Supannapong et al., 2008).

The freshwater mussel *Hyriopsis cumingii* is of commercial importance because it produces the freshwater pearl (Wang et al., 2009). Under natural and commercial farming conditions, the mussel ingests phytoplankton as food (Fei et al., 2006), and exhibits high amylase activity in the digestive organs (Xu et al., 2008). To the best of our knowledge, the molecular characterization of the α-amylase gene in *H. cumingii* has not been previously reported. In the present study, we isolated an α-amylase gene designated *HcAmy* from the hepatopancreas of *H. cumingii*, and examined its tissue-specific expression pattern. We also evaluated the effect of re-feeding regimes on the expression of *HcAmy* mRNA in the mussel following a period of starvation. This study aimed to evaluate the function of α-amylase as a digestive enzyme in *H. cumingii* that are fed phytoplankton.

MATERIAL AND METHODS

Study animals

The *H. cumingii* mussels used in this study were collected from a pond in a commercial mussel farm located at Fengqiao Town, Zhuji City (Zhejiang Province, China) in September 2012. The mussels were 1 and 2 years of age. The shell length of the 1-year-old mussels was 40 ± 3 mm (means ± SD, N = 15), while the shell length of the 2-year-old mussels was 95 ± 5 mm (means ± SD, N = 6).

cDNA amplification and bioinformatic and phylogenetic tree analyses

Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from the hepatopancreas of the 2-year-old mussels using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer protocols. The RNA extracted was quantified by measuring absorbance at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The 5'-RACE and 3'-RACE were carried out with the SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and the Advantage 2 cDNA Polymerase Mix (Clontech), following manufacturer protocols. Gene-specific primers (HcAmyR1 and HcAmyR2 for 5'-RACE and HcAmyF1 and HcAmyF2 for 3'-RACE) were designed based on an amylase-like *H. cumingii* EST (GenBank accession No. FE968625) (Table 1). Polymerase chain reaction (PCR) products were purified using a DNA fragment purification kit (TaKaRa, Dalian, China), and were subsequently cloned into a pMD19-T vector using a TA cloning kit (TaKaRa), and sequenced.

Table 1. Primers and their applications in this study.

Primer names	Sequence 5'→3'	Use
HcAmyF1	GTCTGGTTATGACCGTCTTTGG	1st round 3' RACE
HcAmyF2	GTGTCCAGGTATCACCGCCGAATG	2nd round 3' RACE
HcAmyR1	TGTTGTTCATTTCCACTGCGGGTCT	1st round 5' RACE
HcAmyR2	TTCATTCGGCGGTGATACCTGGAC	2nd round 5' RACE
RT-HcAmyF	TCGGCATACCATCACCCAT	Realtime PCR Forward
RT-HcAmyR	GATTGATTACCAGATCAGGGAAGAT	Realtime PCR Reverse
β-actin-F	CCCTGGAATCGCTGACCGTAT	Realtime PCR Forward
β-actin-R	GCTGGAAGGTGGAGAGAGAAG	Realtime PCR Reverse

Bioinformatic analysis

Determination of the gene, open reading frame (ORF), and protein sequence were performed using the Expert Protein Analysis System (ExPASy; http://au.expasy.org). The signal peptide was predicted using the SignalP program (http://www.cbs.dtu.dk/). Domain prediction was performed using the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/).

Phylogenetic analysis

The deduced amino acid sequence of HcAmy was aligned with the other sequences from the mollusk α-amylase family using Cluster W 1.81 (Thompson et al., 1994) (Table 2). Phylogenetic reconstruction was performed by the MEGA 5 program (Tamura et al., 2011) by the neighbor-joining method (Nei and Kumar, 2000). The evolutionary distance was measured as the proportion of difference (p-distance). The confidence in the phylogenetic tree branch topology was accessed by bootstrap, using 10,000 replicates.

Tissue-specific expression of *HcAmy* mRNA

The tissue-specific expression of *HcAmy* mRNA in 2-year-old mussels (N = 4) was analyzed by real-time quantitative PCR (qRT-PCR) using the iQ™5 apparatus (BioRad, Hercules,

Table 2. Amino acid sequences of α-amylase family from GenBank database.

Species	Accession No.	Species	Accession No.
Corbicula fluminea	AAO17927	*Haliotis discus discus*	ABO26611
Crassostrea gigas	AAL37207	*Mytilus edulis*	ACA34372
Crassostrea gigas	AAL37183	*Patella vulgata*	ACA34388
Crassostrea gigas	EKC28393	*Pecten maximus*	CAA68065
Crassostrea gigas	EKC28396	*Pinctada maxima*	AEI58897
Haliotis discus discus	ABO26610	*Pteria penguin*	AEI58894

CA, USA). Total RNA was isolated from the tissues of the adductor muscle (AM), gill (GL), hepatopancreas (HP), intestine (IN), labial palps (LP), crystalline style (CS), and mantle center (MC) using TRIzol Reagent (Invitrogen). cDNA from 2.0 μg total RNA was synthesized by a PrimeScript® Reverse Transcriptase kit (TaKaRa), using oligo $(dT)_{18}$ as primer. The gene-specific primers for qRT-PCR, RT-HcAmyF and RT-HcAmyR, were designed based on the *HcAmy* cDNA sequence. *β-actin* (GenBank accession No. HM045420) was selected as the endogenous reference gene for calibration, and amplified with 2 primers, actin-F and actin-R (Table 1). qRT-PCR was performed with the SYBR® Premix Ex Taq™ PCR kit (TaKaRa). A volume of 1.0 μL of the template cDNA was used for PCR amplification in a 25-μL reaction volume for 40 cycles (95°C for 30 s, 72°C for 30 s, and 56°C for 30 s). Melting curves were performed with 0.5°C increments from 50 to 95°C, to ensure that a single PCR product was amplified using each pair of primers. The relative mRNA level was determined by the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001), using *β-actin* as a reference gene and the adductor muscle as the calibration tissue.

Regulation of *HcAmy* mRNA expression by the re-feeding regime after a period of starvation

Algal concentrations during re-feeding

A feeding trial (I) was conducted in the mussel farm at Fengqiao Town (Zhuji, China) in September 2012. One-year-old mussels were fed an algal mixture at 6 chlorophyll-a (Chl-a) concentrations (0, 16, 32, 48, 64, and 96 μg/L) following 2 weeks starvation. The dominant species in the algal mixture included *Merismopedia* sp, *Scenedesmus* sp, *Crucigenia* sp, *Pediastrum* sp, *Schroederia* sp, *Melosira* sp, *Coelosphaerium* sp, *Cyclotella* sp, *Synedra* sp, and *Chlorella* sp (the ratio of the algal cells was 224:37:16:15:5.5:4.5:2:2:1.5:1, respectively). The algae were collected from a mussel pond, and cultured in polyethylene (PE) tanks, in which gibel carp *Carassius auratus gibelio* and grass carp *Ctenopharyngodon idellus* were cultured and fed a commercial formulated fish feed (Kesheng Feed Co. Ltd., Shaoxing, China) twice daily. The algal concentration was determined using a 10-005 field fluorometer (Turner Designs, Inc., Sunnyvale, CA, USA). The tested algal concentrations were established by diluting the algae collected from the tanks with well water (Chl-a concentration was 0 μg/L). The algae were stored in 6 PE tanks (volume 300 L) before use.

During trial I, mussels were cultured in glass aquariums (volume 30 L) at a density of 15 individuals per aquarium. Each algal concentration was replicated 3 times. Therefore, a total of 18 aquariums were used. The mussels were starved for 2 weeks, and then re-fed for 3 weeks, during which the different algal mixture concentrations continuously flowed from the store tanks to each aquarium at 3 L/min. The aquariums were placed in a pool to avoid dramatic changes in water temperature (the water temperature was 24° ± 2°C) during the experiment. The algal concentra-

tion in each aquarium was monitored 3 times daily. At the end of the trial, the hepatopancreas of the mussels was collected rapidly, frozen in liquid nitrogen, and transported to the Aquatic Ecosystem and Aquaculture Laboratory (AEA) at Zhejiang University (Hangzhou, China) and stored at -80°C until analysis. The expression profile of *HcAmy* mRNA in the hepatopancreas was tested by the qRT-PCR method, as described in the tissue-specific expression analysis.

Re-feeding duration

A feeding trial (II) was conducted in the mussel farm at Fengqiao Town (Zhuji City, China) in September 2012. Three PE tanks (volume 1000 L) were located near a mussel pond, and filled with aerated well water (Chl-a 0 μg/L). A total of sixty 2-year-old mussels were hung in the tanks at a density of 20 individuals per tank. The mussels were deprived of food for 2 weeks, and then re-fed with the green algae *Chlorella pyrenoidosa* (algal concentration: 1.0 x 10^6 cells/mL). During re-feeding, 2 mussels were sampled randomly from each tank at 0, 6, 12, 24, 48, and 96 h. The hepatopancreas of the 6 mussels collected was collected and frozen in liquid nitrogen. The samples were transported to AEA and stored at -80°C until analysis. The expression profile of *HcAmy* mRNA in the hepatopancreas was tested by the qRT-PCR method, as described in the tissue-specific expression analysis.

Specific α-amylase activity

Specific α-amylase activity was assayed by 3,5-dinitrosalicylic acid (DNS) method according to Areekijseree et al. (2004) based on Bernfeld (1951), using 1% soluble starch as substrate. The incubation period lasted 30 min at 37°C. Then, reaction was stopped by adding 250 μL of DNS (dissolved in 2 M NaOH and 0.6% sodium potassium tartrate) and heating in boiling water bath for 5 min. The reaction solution was thereby read at 540 nm against a control blank. Protein concentration of enzyme extract was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard protein. The unit (U) of α-amylase activity was defined as mM of maltose produced per hour (at the specified reaction condition). Maltose was prepared similarly for the calibration curve.

Statistical analysis

Differences in the expression level of *HcAmy* mRNA among treatments in trials I and II were examined by one-way ANOVA, and further comparison between the treatments was examined by the Tukey HSD test. The significance level was set at $P < 0.05$. The statistical analysis was performed with the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Molecular characterization of *HcAmy*

Full-length cDNA of HcAmy

The full-length *HcAmy* cDNA was 1701 bp, including a 24-bp 5'-untranslated region (UTR), a 1572-bp ORF, and a 101-bp 3'-UTR (Figure 1). The sequence was deposited in the

GenBank database under accession No. KC342529.

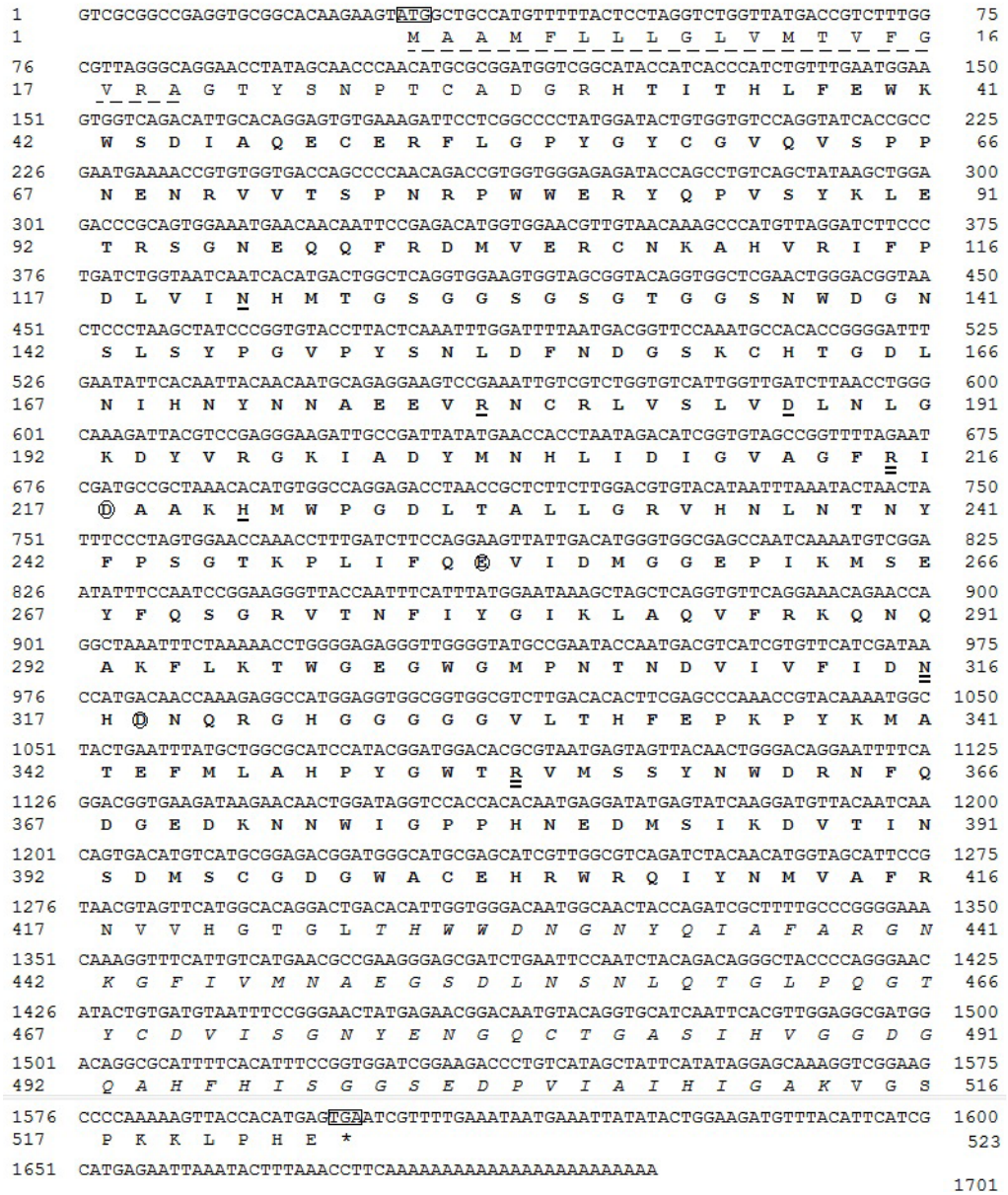

Figure 1. Nucleotide and deduced amino acid sequence of *HcAmy* gene in the mussel *Hyriopsis cumingii*. The start codon (ATG) and stop codon (TGA) were boxed. The putative signal peptide was underlined with dotted line. The predicted active sites were circled. Proposed Ca^{2+}-binding residues and chloride binding residues were underlined with single lines and double lines, respectively. Amino acids of catalytic domain and C-terminal all-beta domain were shown in bold letters and italics, respectively.

Structure of deduced HcAmy protein

The deduced HcAmy protein was composed of 523 amino acids, with a predicted molecular weight of 58.4 kDa. The protein contained a putative signal peptide, with 19 residues and a mature polypeptide with a catalytic domain (amino acids 32-416), and a C-terminal all-beta domain (amino acids 425-513). In the catalytic domain, the predicted active sites were conserved at sites Asp217, Glu253, and Asp318. Ca^{2+}-binding residues (Asn121, Arg178, Asp187, and His221) and chloride binding residues (Arg215, Asn316, and Arg354) were also found in HcAmy (Figure 1).

Phylogenetic analysis of *HcAmy*

The phylogenetic tree of the α-amylase family from mollusk species showed that the amylases were divided into 2 major groups; specifically, the α-amylase group and the maltogenic 'amylase' group (MacGregor et al., 2001) (Figure 2). In the α-amylase group, *H. cumingii* was clustered with the other bivalve species, excluding the gastropod species *H. discus discus*.

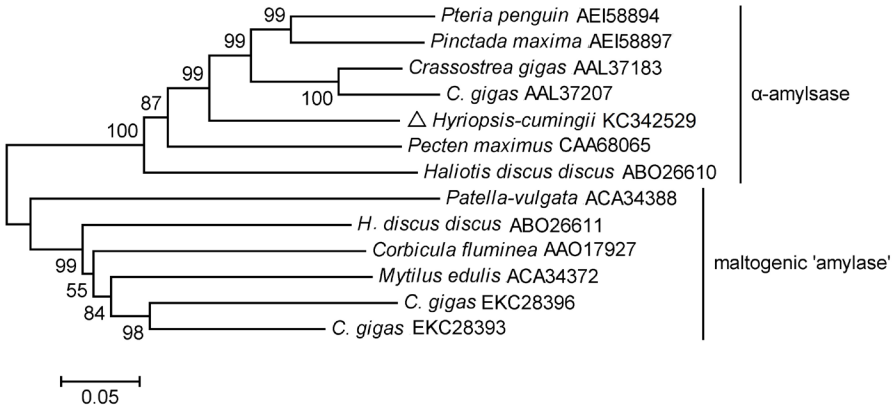

Figure 2. Neighbor-Joining tree based on protein sequences of molluscan α-amylase family. The evolutionary distances were computed using the p-distance method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The GenBank accession numbers had shown next to each species.

Tissue-specific expression pattern of *HcAmy* mRNA in 2-year-old mussels

The result of tissue expression pattern showed that *HcAmy* mRNA transcripts were detectable in AM, GL, IN, and CS, and very intensely in HP (Figure 3).

Effect of algal concentration on *HcAmy* expression in the hepatopancreas of 1-year-old mussels during re-feeding after a period of starvation

The algal concentration significantly affected the expression of *HcAmy* in the hepato-

pancreas of 1-year-old mussels ($P < 0.05$, ANOVA). The expression level of *HcAmy* mRNA was 8.9 times higher in mussels fed 32 µg/L Chl-a compared to mussels fed 0 µg/L Chl-a (well water) ($P < 0.05$, Tukey's test). The *HcAmy* transcripts decreased with increasing Chl-a concentration from 48 to 96 µg/L (Figure 4).

Effect of re-feeding time on *HcAmy* expression in the hepatopancreas of 2-year-old mussels following a period of starvation

The expression level of *HcAmy* mRNA in the hepatopancreas of 2-year-old mussels was dependent on re-feeding time after 2 weeks starvation ($P < 0.05$, ANOVA). The expression level of *HcAmy* mRNA slightly increased with increasing re-feeding time from 0 to 24 h. The highest expression level occurred at 24 h after the onset of re-feeding ($P < 0.05$, Tukey's HSD test). The expression level of *HcAmy* mRNA tended to decline with any further increase in re-feeding time from 24 to 96 h (Figure 5).

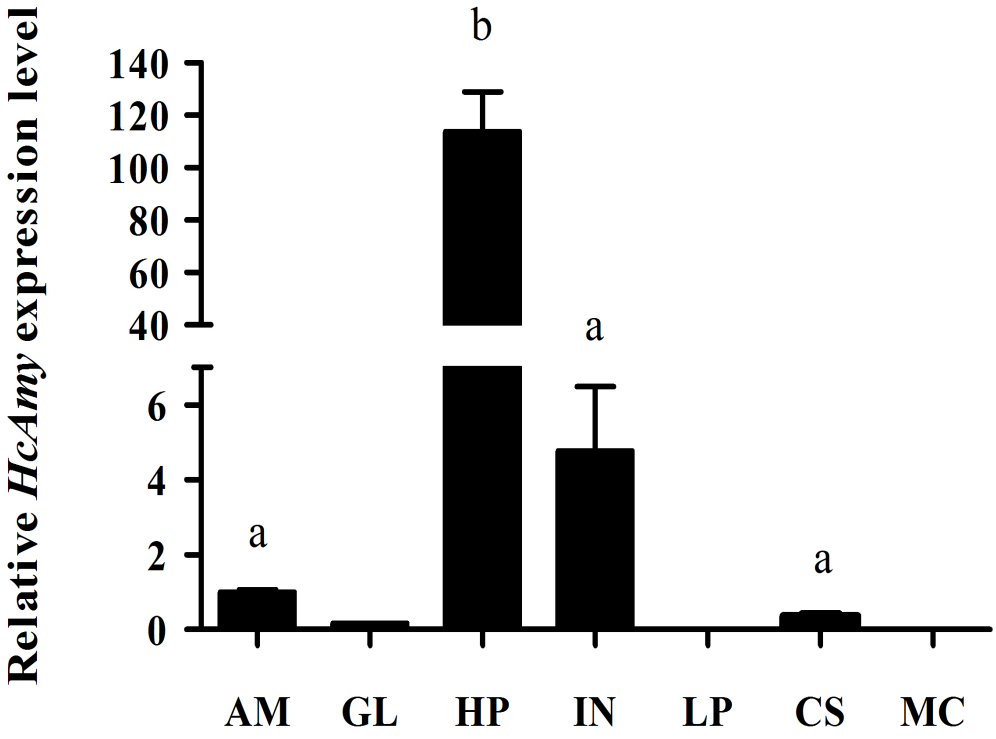

Figure 3. Expression of *HcAmy* mRNA analyzed by real-time quantitative PCR in different tissues of *Hyriopsis cumingii* with *β-actin* as a reference gene. AM was used as calibrator tissue. AM = adductor muscle; GL = gill; HP = hepatopancreas; IN = intestine; LP = labial palps; MC = mantle centre. Each bar is reported means ± SE of four pearl mussels for different tissues. Means with different superscript letters were significantly different at $P < 0.05$ by the Tukey HSD test.

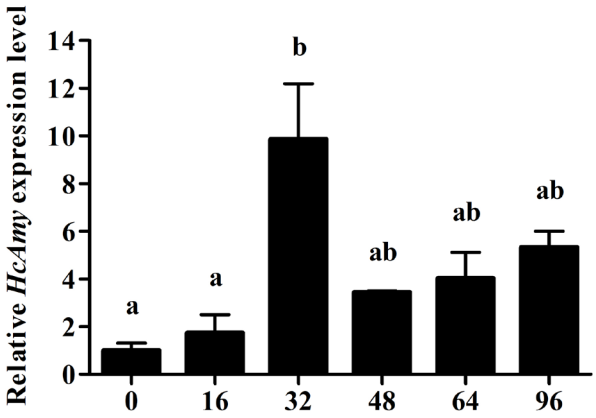

Figure 4. Expression of *HcAmy* mRNA in hepatopancreas of 1-year-old mussel under different algae concentrations. *β-actin* was used as an internal control to normalize the expression data analyzed by real-time quantitative PCR. Values are reported as means ± SE for three replicate real-time reactions from hepatopancreas (N = 15). The chlorophyll concentration represented the food density. Mean values with different superscript letters were significantly different at $P < 0.05$ by the Tukey HSD test.

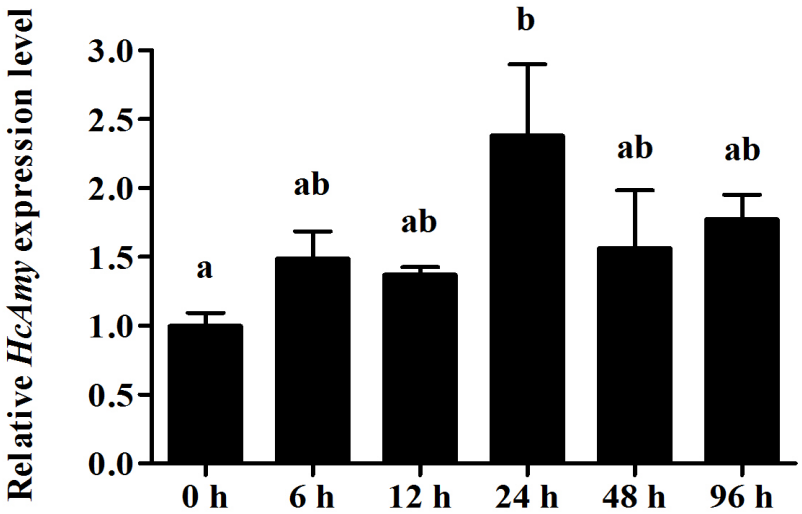

Figure 5. Expression of *HcAmy* mRNA in hepatopancreas of 2-year-old mussel after re-feeding. *β-actin* was used an internal control to normalize the expression data analyzed by real-time quantitative PCR. Values are reported as means ± SE for three replicate real-time reactions from hepatopancreas (N = 6). Means with different superscript letters were significantly different at $P < 0.05$ by the Tukey HSD test.

DISCUSSION

In this study, the full-length cDNA of the α-amylase gene (*HcAmy*) was cloned from the freshwater pearl mussel *H. cumingii*. The deduced HcAmy protein has the characteristics of the conserved catalytic domain, C-terminal all-beta domain, active site residues for catalytic function, Ca^{2+}-binding residues, and chloride binding residues in α-amylase protein (MacGregor et

al., 2001) (Figure 1). Phylogenetic analysis confirmed the classification of HcAmy as a member of the α-amylase subfamily (Figure 2), indicating that HcAmy was conserved during bivalve evolution, and might have a similar function to other bivalve α-amylase proteins.

The hepatopancreas is one of the main digestive organs of bivalves, and has an intra- and an extracellular function in the digestion of nutrients, such as starch and glycogen (Morton, 1983). The observed prominent expression of *HcAmy* mRNA in the hepatopancreas during the current study (Figure 3) implied that this gene has an important digestive function in the mussel *H. cumingii*. Similar tissue distribution of the α-amylase gene is also found in other marine mollusks (Le Moine et al., 1997; Huvet et al., 2003; Nikapitiya et al., 2009). Previous studies reported that α-amylase plays a role in the glycogen utilization of the hepatopancreas in bivalves subject to extraneous stress, such as starvation and low temperature (Thompson et al., 1974; Bayne et al., 1976; Gosling, 2003). Nikapitiya et al. (2009) reported that over 60% of the expression level of α-amylase (*HdAmyI*) was retained in the abalone *H. discus discus* after 8 weeks of starvation. In our study, the expression level of *HcAmy* mRNA in 2-year-old mussels starved for 5 weeks was less than 30% of the level recorded for mussels that were re-fed for 3 weeks (Figure 4). Moreover, the expression level of *HcAmy* mRNA in 1-year-old mussels starved for 2 weeks was less than 42% of the level observed for mussels that had been re-fed for 24-h (Figure 5). However, starved 1 and 2-year-old mussels retained over 66% of specific α-amylase activity compared to mussels that were adequately re-fed (**Figures S1** and **S2**). These results indicate that the mussel *H. cumingii* might select the strategy of decreasing the metabolic rate and maintaining digestive enzyme activity in response to the stress of food shortage.

In our study, the *HcAmy* transcripts significantly increased in 1-year-old mussels after being re-fed for 24 h following 2 weeks starvation (Figure 4). In addition, the *HcAmy* transcripts was higher in 2-years-old mussels fed 32 μg/L Chl-a compared to mussels fed 0 or 16 μg/L Chl-a (Figure 5). These findings indicate that refeeding rapidly awakens *HcAmy* mRNA expression in starved mussel, with the maximum expression being attained at optimal algal concentrations. This relationship between α-amylase expression levels and food condition has been widely observed in bivalves. For instance, the seasonal expression pattern of the α-amylase gene in the bivalve *Pecten maximus* coincides with the development of phytoplankton blooms (Le Pennec and Le Pennec, 2002). The dietary supplementation of starch upregulated the expression level of the α-amylase gene (*AMYB*) in the digestive gland of the Pacific oyster *C. gigas* (Huvet et al., 2012).

Bivalves sort suspended particles with the gill, labial palps, mucus, and stomach before ingestion (Bayne et al., 1989; Gosling, 2003). Food quality is an important factor that regulates bivalve filtration rates (Wilson, 1983; Jorgensen, 1996) and the mRNA expression of α-amylase in bivalves (Ibarrola et al., 1996; Fernandez-Reiriz et al., 2005). This is because the composition and content of carbohydrates varies among algal species (Moal et al., 1987). In our study, the 2-year-old mussels were fed an algal mixture that was dominated by a blue-green algae, *Merismopedia* sp.; consequently, the *HcAmy* mRNA expression level slightly decreased with the further increase of Chl-a from 32 to 96 μg/L. This result is attributable to the unfavorable algal composition (Figure 4). This finding demonstrates that the occurrence of *Merismopedia* sp blooms might downregulate the expression of *HcAmy* in *H. cumingii*. In commercial *H. cumingii* farming practices, organic manure, such as duck manure, is generally used to fertilize phytoplankton, and to provide detritus as a natural food source for mussels and co-cultured filter fishes, such as silver carp and bighead carp. However, the excessive input of organic manure

causes eutrophication, which generates blue-green algal blooms. Our results demonstrate that the expression profiles of the α-amylase gene *HcAmy* decrease at a high Chl-a concentrations if *Merismopedia* sp dominates in the algal community, indicating the negative effect of this alga on *H. cumingii* feeding. This observation is consistent with the conclusion that the occurrence of *Merismopedia* sp reduces the filtration and digestive rates of *H. cumingii* (Fei et al., 2006).

In conclusion, this study identified and established the tissue-specific pattern of the α-amylase gene *HcAmy* from the freshwater pearl mussel *H. cumingii*. The expression of *HcAmy* mRNA in the hepatopancreas of 1-year-old and 2-year-old mussels is dependent on food conditions, including starvation, re-feeding time following a period of starvation, and algal concentration. We showed that re-feeding induces the upregulation of *HcAmy* after starvation, with algal concentration causing an increase in upregulation. These observations indicate that *HcAmy* expression in *H. cumingii* is regulated by the quantity and quality of the algal supplement.

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Supplementary material

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