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Molecular cloning and characterization of glycogen synthase in *Eriocheir sinensis*

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ABSTRACT

Glycogen plays an important role in glucose and energy homeostasis at cellular and organismal levels. In glycogen synthesis, glycogen synthase (GS) is a rate-limiting enzyme catalysing the addition of α-1,4-linked glucose units from $(UDP)^3$-glucose to a nascent glycogen chain using glycogenin (GN) as a primer. While studies on mammalian liver GS (GYS2) are numerous, enzymes from crustaceans, which also use glycogen and glucose as their main energy source, have received less attention. In the present study, we amplified full-length GS cDNA from *Eriocheir sinensis*. Tissue expression profiling revealed the highest expression of GS in the hepatopancreas. During moulting, GS expression and activity declined, and glycogen levels in the hepatopancreas were reduced. Recombinant GS was expressed in *Escherichia coli* Rosetta (DE3), and induction at 37 °C or 16 °C yielded EsGS in insoluble inclusion bodies (EsGS-I) or in soluble form (EsGS-S), respectively. Enzyme activity was measured in a cell-free system containing glucose-6-phosphate (G6P), and both forms possessed glycosyltransferase activity, but refolded EsGS-I was more active. Enzyme activity of both GS and EsGS-I in the hepatopancreas was optimum at 25 °C, which is coincident with the optimum growth temperature of Chinese mitten crab, and higher (37 °C) or lower (16 °C) temperatures resulted in lower enzyme activity. Taken together, the results suggest that GS may be important for maintaining normal physiological functions such as growth and reproduction.

1. Introduction

Glucose is stored as glycogen in many cell types when intracellular carbon is abundant. Glycogen therefore plays an important role in glucose and energy homeostasis at both cellular and organismal levels. Glycogen is a branched polysaccharide of $(UDP)^3$-glucose joined through α-1,4-glycosidic linkages with intersecting α-1,6-linked glucose residues that serve as branch points (Roach et al., 2012). Glycogen is synthesized through the cooperative action of glycogen synthase (GS), glycogenin (GN), and glycogen-branching enzyme (GBE) (Roach, 2002). The rate-limiting enzyme for glycogen synthesis is GS, which catalyzes the addition of α-1,4-linked glucose units from $(UDP)^3$-glucose to a nascent glycogen chain (Ferrer et al., 2003). In mammals, there are two GS isoforms: muscle GS (GYS1), which is abundantly expressed in skeletal and cardiac muscle and universally expressed in other tissues (Browner et al., 1989), and liver GS (GYS2), which is expressed only in the liver (Irimia et al., 2010). Although the two isoforms share 70% sequence identity, the similarity in amino and carboxyl terminal regions is only 50% (Bai et al., 1990), and the C-terminal domain of GYS2 is shorter (Hanashiro and Roach, 2002).

GS is controlled by a complex interaction between the allosteric activator glucose-6-phosphate (G6P) and reversible phosphorylation through glycogen synthase kinase-3 (GSK-3) and protein phosphatase 1 (PP1) (Von Wilamowitz-Moellendorff et al., 2013). Reversible phosphorylation of regulatory sites in GYS1 occurs at Ser8 and Ser11 in the N-terminal region, and at Ser641, Ser645, Ser649, Ser653, Ser657, Ser697 and Ser710 in the C-terminal region. Unlike GYS1, the shorter GYS2 lacks Ser697 and Ser710 (Ros et al., 2009). Furthermore, these phosphorylation sites on GS can be phosphorylated by several kinases including cAMP-dependent protein kinase (cAMP-PK), protein kinase C (PKC), phosphorylase kinase (PK), and GSK-3 (Roach, 1990). When hepatocytes are stimulated by hormones such as adrenaline and glucagon, GYS2 is inhibited by phosphorylation at several sites (Ros et al., 2009). Moreover, the GBE adds short stretches of glucose residues via α-1,6-glycosidic links to the glycogen chain to yield a branched polymer with increased water solubility (Lee et al., 2011).

Abbreviations: GS, glycogen synthase; GN, glycogenin; G6P, glucose-6-phosphate; GSK-3, glycogen synthase kinase-3; PP1, protein phosphatase-1; UDP, uridine diphosphate; EsGS, *E. sinensis* hepatopancreas GS; EsGS-I, EsGS in inclusion bodies; EsGS-S, EsGS in soluble form; GBE, glycogen branching enzyme; RACE, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; GCS, enzymatic activity of GS; GT-A, glycosyltransferase superfamily-A; IMAC, immobilized metal affinity chromatography

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Table 1
Primers used for the GS cloning. Underlined characters indicate the restriction sites.

Primer name	Length (bp)	Direction	Sequences	Restriction enzyme	Description
GS 5′ R	24	R	GGTCAGAGGCGAGTGAGATGGAAA		Targeting the 5′-untranslated region of the EsGS
GS 3′ F	23	F	CTGCTGGACTGGAGGATACTTGG		Targeting the 3′-untranslated region of the EsGS
GS F	33	F	GGAATTCCATATGATGTCCCGGGTAGCTAAACG	Nde I	Forward primer used for EsGS ORF cloning
GS R	29	R	CCCAAGCTTCCTGCTGCTGACGGTACTGC	Hind III	Reverse primer used for EsGS ORF cloning
Q-GS-F	22	F	CTGTCACCAAGTCTTTACGGGA		Forward primer used for quantitative PCR of GS
Q-GS-R	22	R	CAACAGTTCTTCAGACTCGGGA		Reverse primer used for quantitative PCR of GS
β-Actin-F	22	F	GGTTGCCGCCCTGGTTGTGGAC		forward primer used for quantitative PCR of β-Actin
β-Actin-R	19	R	TTCTCCATGTCGTCCCAGT		reverse primer used for quantitative PCR of β-Actin

In mammals, although a small amount of glycogen may be present in most tissues, liver and muscle are the main storage sites (Huang et al., 2015). In glucose homeostasis, the liver stores excess carbohydrates in the form of glycogen, and this store can be rapidly mobilised when blood sugar levels are low (Wang et al., 2008). While the mammalian enzyme is well studied, glycogen synthase (GS) from crustaceans, which also use glycogen and glucose as their main energy source, has received less attention. The Chinese mitten crab *Eriocheir sinensis* is one of the most economically important aquaculture species in China, due to its desirable taste and high nutritional value. This species is found in coastal estuaries from North Korea to the Fujian province of China in the south (Li et al., 2016). The economic and biological importance of Chinese mitten crab is attracting the attention of researchers, and the genetics of this organism are being explored (Uawisetwathana et al., 2011). Similar to the mammalian liver, the hepatopancreas of crustaceans plays a role in carbohydrate and lipid metabolism, physiological balance, and energy storage and use (Bollen et al., 1998). In addition, a large amount of energy is stored in the hepatopancreas in preparation for glucose metabolism, reproduction, limb regeneration and other physiological processes including moulting (Bélanger et al., 2011; Ghafoory et al., 2013).

In recent years, moulting in crustaceans has received increasing attention. Moulting is the process of shedding the old exoskeleton and forming a new one during growth and development. The moulting cycle can be divided into Ecdysis (E stage), Postmoult (AB stage), Intermoult (C stage) and Premoult (D stage) (McConaugha, 1982; Drach and Tchernigovtzeff, 1967). The moulting process is regulated by the ecdysone and is closely correlated with external ecological factors such as salinity, light and nutrition (Drach and Tchernigovtzeff, 1967). The Chinese mitten crab undergoes 20 ± 1 moulting cycles during its lifespan, which may be slow or fast. By passing through a number of moults, crabs increase in size, undergo essential morphological changes and regenerate limbs when required (Skinner, 1985).

In the present study, we cloned the GS enzyme of *E. sinensis* and performed tissue expression profiling, revealing that the highest expression of GS in the hepatopancreas. The relative expression and viability of GS in the hepatopancreas during different moulting stages were also assessed, as were changes in GS activity at different temperature. During moulting, GS expression and activity declined, accompanied by the glycogen levels reduced in the hepatopancreas. The recombinant GS possessed glycosyltransferase activity, but refolded EsGS-I (EsGS in insoluble inclusion bodies) was more active. Enzyme activity of both GS and EsGS-I in the hepatopancreas was optimum at 25 °C, which is coincident with the optimum growth temperature of Chinese mitten crab, and higher (37 °C) or lower (16 °C) temperatures resulted in lower enzyme activity. The results provide a theoretical basis for further study of the mechanism of glucose metabolism during the growth and development of *E. sinensis*.

2. Materials and methods

2.1. Animals

Chinese mitten crabs (*E. sinensis*) approximately 15 ± 5 g (n = 8) in weight were purchased from a local market in Tianjin, China. The crabs were acclimatised to the laboratory culture environment and maintained in aerated water at 24–26 °C for one week before the experiment. All animal studies were performed with the approval of Tianjin Normal University Animal Ethics Committee.

2.2. Cloning of glycogen synthase (EsGS) cDNA

Total RNA was extracted from *Eriocheir sinensis* hepatopancreas using TRIzol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA) and resuspended in RNase-free water. Then 2 μg of high-quality DNase I-treated (Promega, Madison, WI, USA) total RNA was reverse transcribed using M-MLV reverse transcriptase according to the manufacturer's protocol (Promega). The synthesized first-strand cDNA was used for the rapid amplification of cDNA ends (RACE), to clone full length of EsGS.

In our previous study, to identify the downstream effector molecule in glucose metabolism regulated by CHH, the transcriptome of *E. sinensis* hepatopancreas was revealed by Illumina RNA-Seq and DGE analysis. All gene-specific primers were designed based on this transcriptome, which included complete ORF fragments of EsGS. Gene-specific primers targeting the 5′- and 3′-untranslated regions of the EsGS were designed based on the transcriptome sequence (GS 5′ R, GS 3′ F, and GS F/R, Table 1). Polymerase chain reaction (PCR) was performed using the cDNAs synthesized from the hepatopancreas of *E. sinensis* as a template. The PCR products were separated by electrophoresis on a 1% agarose gel and then purified, cloned into the pMD18-T vector (TaKaRa, China). After transformation into *E.coli* DH5α, inserted nucleotide sequences of the recombinant plasmids were confirmed by Sanger DNA sequencing in Invitrogen (Invitrogen, Carlsbad, CA, USA). During the following sequence analysis, the full length of EsGS cDNA was assembled with DNA-Star 5.01.

2.3. Analysis of nucleotide and amino acid sequences

The obtained full-length EsGS cDNA and amino acid sequences were blasted against the GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Then the sequences were aligned using an online sequence alignment constructed including *Homo sapiens* and *Eriocheir sinensis* (http://www.biosoft.net/sms/index.html). Moreover, the homology analyses were carried out using ClustalW (http://www.genome.jp/tools/clustalw/). A rooted neighbor-joining (NJ) tree was constructed to determine the phylogenetic relationship using MEGA 6.0 software. Bootstrap trials were replicated 1000 times to derive confidence values for the phylogenetic analysis (Bai et al., 1990).

Protein functions were analyzed using the simple modular architecture research tool (SMART) program (http://www.smart.embl-heidelberg.de/), the molecular weight (Mw) were calculated with an online software program (http://web.expasy.org/compute_pi/).