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Role of the GlgX protein in glycogen metabolism of the cyanobacterium, *Synechococcus elongatus* PCC 7942

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Abstract

The putative *glgX* gene encoding isoamylase-type debranching enzyme was isolated from the cyanobacterium, *Synechococcus elongatus* PCC 7942. The deduced amino acid sequence indicated that the residues essential to the catalytic activity and substrate binding in bacterial and plant isoamylases and GlgX proteins were all conserved in the GlgX protein of *S. elongatus* PCC 7942. The role of GlgX in the cyanobacterium was examined by insertional inactivation of the gene. Disruption of the *glgX* gene resulted in the enhanced fluctuation of glycogen content in the cells during light–dark cycles of the culture, although the effect was marginal. The glycogen of the *glgX* mutant was enriched with very short chains with degree of polymerization 2 to 4. When the mutant was transformed with putative *glgX* genes of *Synechocystis* sp. PCC 6803, the short chains were decreased as compared to the parental mutant strain. The result indicated that GlgX protein contributes to form the branching pattern of polysaccharide in *S. elongatus* PCC 7942.
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1. Introduction

Starch or glycogen debranching enzymes (DBEs) are hydrolases catalyzing cleavage of α-1,6-glucosidic linkage of branched α-polyglucans. Depending on the substrate preferences, the DBEs are classified into pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) [1–3]. Pullulanase hydrolyzes amylopectin and yeast polysaccharide pullulan (an α-polyglucan composed of maltotriose units connected by α-1,6 linkages) but has little or no activity on glycogen. In contrast, isoamylase shows high activity on amylopectin and glycogen and does not hydrolyze pullulan. The DBE encoded by *glgX* gene from *Escherichia coli* is closely related to isoamylase in terms of the primary structure, but shows distinct substrate specificity toward limit dextrins that are produced after exhaustive digestion of branched polysaccharides with β-amylase or phosphorylase [4,5].

As hydrolases, DBEs were considered to be involved simply in the degradation of polysaccharides [6]. However, analyses of starch metabolism in a number of plant species have shown that DBEs play essential roles in the formation of distinct molecular architecture of amylopectin [7,8]. The α-1,6-branch points in amylopectin molecules are not homogeneously distributed as compared with those in glycogen, but densely branched regions and scarcely branched ones are located alternately along glucan chains with regular intervals of 9–10 nm, corresponding to the amorphous and crystalline lamellae, respectively [9,10]. This tandem-cluster structure of amylopectin contributes to the semicrystalline organization and water-insoluble nature of starch granules. In *sugary-1* mutants defective in isoamylase of maize [7,11], rice [12–14], barley [15], Arabidopsis [16], and *Chlamydomonas* [17], starch synthesis is diminished or totally lost, and replaced by the accumulation of randomly branched

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soluble polysaccharides frequently referred to as phytoglycogen. Several models or ideas have been presented to interpret the precise role of DBEs in amylopectin biosynthesis [8,15–20]. One model, termed the glucan-trimming model, proposed that DBEs act to remove excessively branched chains in pre-amylopectin molecules [17] or inappropriately positioned branches in the regions destined for crystalline lamellae during synthesis of amylopectin glucan chains [19]. Specific isozymes of starch synthases and starch branching enzymes play distinct roles to form the ordered cluster structure of amylopectin in concert with DBEs [19]. An alternative to this model, referred to as water-soluble polysaccharide (WSP)-clearing model, hypothesized that DBEs eliminate polysaccharides in the soluble phase of stroma, thereby preventing starch synthases and starch branching enzymes from being sequestered onto the improper substrate WSP to form phytoglycogen [16]. The involvement of DBE, especially isoamylase in the amylopectin biosynthesis was further confirmed by antisense inhibition of isoamylase in rice [21] and heterologous expression of the isoamylase gene of *Aegilops tauschii* (the diploid wheat) in mutants of rice [14].

Hussain et al. [22] reported that three isoforms of isoamylases (Stisa1, Stisa2 and Stisa3) were present in potato and probably in most, if not all, angiosperms. The isoform of isoamylase defective in *sugary-1* mutants of cereal plants corresponds to Stisa1 of potato and is now referred to as ISA1, whereas that encoded by Arabidopsis *DBE1* gene [16] corresponds to Stisa2 and called ISA2. Although ISA2 lacks amino acid residues essential to the catalytic activity, ISA1 and ISA2 polypeptides constitute hetero-oligomer to form fully functional enzyme [22]. The idea of complex formation between ISA1 and ISA2 was supported by the observation that the targeted mutants of Arabidopsis with defects of either one or both of ISA1 and ISA2 exhibited identical phenotypes [23,24]. In contrast to ISA1 and ISA2 that are involved in the biosynthetic role, studies with Arabidopsis mutants suggested that ISA3 is responsible for starch breakdown and mobilization in leaf tissue [24,25].

The significant role of DBEs, especially the counterparts of ISA1 and ISA2, in amylopectin biosynthesis is not exclusive to terrestrial plants, but has also been demonstrated in such a simple organism as the unicellular green alga *Chlamydomonas reinhardtii* [17,26–28]. The function of DBE in the biosynthesis of polysaccharide is thus emerged at very early stage of evolution of photosynthetic eukaryotes. As compared to the extensive studies with photosynthetic eukaryotes as described above, only a limited number of works have been carried out on the polysaccharide metabolism in more primitive phototrophs cyanobacteria [8,29–34]. It is now firmly established that an ancient phototroph related to extant cyanobacteria underwent endosymbiosis in a non-photosynthetic eukaryote to give rise to plastids as we see today in the plant cell [35]. It is therefore plausible that considerable aspects of starch biosynthesis have originated from cyanobacteria [36].

Nakamura et al. [37] reported that while many cyanobacterial species accumulated glycogen, some particular strains e.g. *Cyanobacterium* sp. MBIC 10216 synthesized polysaccharide that was intermediate between rice amylopectin and typical cyanobacterial glycogen, in terms of chain length distribution, molecular size and length of the most abundant α-1,4-chain. Based on the distinct properties, the novel polysaccharide was designated as semi-amylopectin [37]. Basic studies in glycogen metabolism of cyanobacteria are required to fully elucidate the mechanism underlying distinct polysaccharide structure in some species. Moreover, these studies will also provide clues in understanding the evolutionary relationship between glycogen in cyanobacteria and starch in plants.

In the present work, a gene homologous to *glgX* encoding isoamylase-type DBE was identified in *Synechococcus elongatus* PCC 7942 that accumulates typical cyanobacterial glycogen. The mutant specifically defective in the putative *glgX* gene was constructed and its physiological characteristics were examined. Most interestingly, we sought possibility that DBE in cyanobacteria has a role in shaping the branching pattern of storage polysaccharides when they are synthesized.

2. Materials and methods

2.1. Culture conditions and DNA manipulations

For the wild type (WT) of *Synechococcus elongatus* PCC 7942, the SPC strain that lacks the small plasmid pANS [38] was used throughout this work. *Synechocystis* sp. PCC 6803 was used for the cloning of putative *glgX* genes. The cyanobacterial cells were grown at 30 °C in BG-11 medium [39] supplemented with 10 mM HEPES-NaOH (pH 8.0) under continuous illumination by a fluorescent lamp (50 μmol of photons m^{-2} s^{-1}). The cultures were bubbled with air during growth. For the determination of glycogen content, the cells were grown under the same conditions except that the cultures were illuminated with a light–dark cycles of 12–12 h. Total DNA was extracted from the cyanobacterial cells according to the method described by Golden et al. [40]. Transformation of *S. elongatus* PCC 7942 was carried out by the standard procedure [40]. The transformants were selected on 1% agar plates of BG-11 medium containing 10 μg/ml of kanamycin or 2 μg/ml of ampicillin.

2.2. Cloning of the putative DBE gene from Synechococcus

Two genes (slr0237 and slr1857) have been annotated as *glgX* in the whole genome sequence of *Synechocystis* sp. PCC 6803 [41] at CyanoBase (http://www.kazusa.or.jp/cyano). Based on the sequences conserved in the two *glgX* genes as well as in ISA1 genes of plants [3,42], a pair of oligonucleotide primers 5′-GGCACCTACGCTGGTCTAATCGAAAAAATTCCC-3′, and 5′-TAGACATCGGGGCTACCCAGCAGGCGTTGA-3′ was designed. Using these primers, the corresponding region of the *glgX* gene was amplified from the genomic DNA of *S. elongatus* PCC 7942 by PCR. Amplification reaction was carried out 30 cycles at 94 °C for 45 s, 50 °C for 30 s and 72 °C for 60 s. The amplified DNA fragment was cloned into the plasmid pGEM-T Easy (Promega). The cloned DNA fragment was used as the probe for screening the genomic library of *S. elongatus* PCC 7942 constructed with Lambda gt11 vector (Stratagene). Plaque hybridization was carried out according to the standard protocol [43] to identify clones covering the entire region of the *glgX* gene. DNA sequence was determined using ABI 373S sequencer (Applied Biosystems).

2.3. Gene disruption and its confirmation

For the disruption of the *glgX* gene in *S. elongatus* PCC 7942, the kanamycin resistant cassette (containing *aph* that encodes aminoglycoside 3′-phosphotransferase) derived from pUC4K [44] was inserted at the *Nco*I restriction site within the coding region that had been subcloned in the pGEM-T Easy vector. The recombinant plasmid DNA was used to transform *S. elongatus* PCC 7942. The transformants were selected on 1% agar plates of BG-11

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