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Characterization of the functional interactions of plastidial starch phosphorylase and starch branching enzymes from rice endosperm during reserve starch biosynthesis

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ABSTRACT

Functional interactions of plastidial phosphorylase (Pho1) and starch branching enzymes (BEs) from the developing rice endosperm are the focus of this study. In the presence of both Pho1 and BE, the same branched primer molecule is elongated and further branched almost simultaneously even at very low glucan concentrations present in the purified enzyme preparations. By contrast, in the absence of any BE, glucans are not, to any significant extent, elongated by Pho1. Based on our *in vitro* data, in the developing rice endosperm, Pho1 appears to be weakly associated with any of the BE isozymes. By using fluorophore-labeled malto-oligosaccharides, we identified maltose as the smallest possible primer for elongation by Pho1. Linear dextrins act as carbohydrate substrates for BEs. By functionally interacting with a BE, Pho1 performs two essential functions during the initiation of starch biosynthesis in the rice endosperm: First, it elongates maltodextrins up to a degree of polymerization of at least 60. Second, by closely interacting with BEs, Pho1 is able to elongate branched glucans efficiently and thereby synthesizes branched carbohydrates essential for the initiation of amylopectin biosynthesis.

1. Introduction

During evolution, plants have developed a conserved fine structure of amylopectin, which is the major component of hydroinsoluble starch [1–3]. To synthesize starch, plants possess specialized isozymes of several α-glucan-related enzymes. In principle, isozymes mediate the same type of reaction (such as chain elongation or branching), but differ in some kinetic properties. They are often designated using a Roman numeral (e.g., starch synthase I, starch synthase II). When a further distinction is needed within a group of kinetically similar isozymes (e.g., those are preferentially expressed in distinct organs), the suffixes a, b, and so on are commonly used (e.g., BEIIa and IIb) [2,4]. During evolution, some (iso)enzymes, such as distinct starch debranching isozymes, took over novel metabolic functions [5,6]. In addition to the chloroplasts of the leaves, many higher plant species have evolved non-green plastids that reside in storage organs. These plastids are incapable of photosynthesis but synthesize and store huge amounts of reserve starch.

In contrast to the more frequently and rather randomly branching pattern of hydrosoluble glycogen (the principal carbon store of animals), amylopectin has a distinct fine structure. The structural unit of amylopectin is called a cluster and consists of areas that are deficient or enriched in branching points. In the latter, double helices of two adjacent glucan chains frequently occur, provided that they have a degree of polymerization (DP) exceeding 10 [7,8]. Double helix formation largely contributes to the hydrophobic nature of native starch.

Abbreviations: AGPase, ADPglucose pyrophosphorylase; APTS, 8-amino-1,3,6-pyrenetrisulfonic acid; BE, starch branching enzyme; BN-PAGE, Blue-native PAGE; CBB, Coomassie brilliant blue; CD, cluster dextrin; CE, capillary electrophoresis; DBE, starch debranching enzyme; DP, degree of polymerization; DPE, disproportionating enzyme; FACE, fluorophore-assisted carbohydrate electrophoresis; G1P, glucose-1-phosphate; GS, grinding solution; HPLC, high-performance liquid chromatography; ISA, isoamylase; MD, maltodextrin; PAGE, polyacrylamide gel electrophoresis; PaISA, *Pseudomonas amyloderamosa* isoamylase; Pho1, plastidial phosphorylase; Φ-LD, phosphorylase-limit dextrin; PUL, pullulanase; SS, soluble starch synthase

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Currently, the fine structure of amylopectin is described by two models designated the cluster and the backbone models [9,10], which differ in the proposed orientation of the clusters and other structural details [11].

How is the structure of amylopectin synthesized in plant tissues? Numerous studies have provided evidence that amylopectin is formed by the concerted actions of various isozymes of starch synthases (SSs), starch branching enzymes (BEs), and debranching enzymes (DBEs). In plant tissues, SS, BE, and DBE generally exist as 4 (SSI, SSII, SSIII, and SSIV), 2 (BEI and BEII), and 2 [isoamylase (ISA) and pullulanase (PUL)] isozyme types, respectively [1,2].

How do these many isozymes cooperate during the synthesis of starch granules? Several lines of evidence have shown that distinct (iso) enzymes interact closely during amylopectin biosynthesis. In rice endosperm, the activities of BEIIb, SSI, and SSIIa are coordinated based on their preferences for different chain-lengths. SSI acts on very short chains having a degree of polymerization (DP) of 6 or 7, which are almost exclusively transferred by BEIIb [12], and elongates these chains to a DP of 8 or slightly higher [13,14]. Subsequently, these glucan chains are further elongated by SSIIa to form the crystalline lamellae of the cluster [15]. Another type of interaction represents the formation of enzyme complexes that consist of two different activities, such as SSI and BE, as shown by *in vitro* studies for rice [14] and *Arabidopsis thaliana* [16]. Similarly, starch biosynthesis-related heterologous protein complexes have been isolated from the cereal endosperm of wheat [17,18], maize [19–23], rice [24], and barley [25]. In these complexes, the activities of the protein compounds are mutually favored [see also [26]].

Recent studies have clearly indicated that other enzyme activities are involved in starch biosynthesis. The plastidial disproportionating enzyme (DPE1) is known to metabolize malto-oligosaccharides (MOS). Considering that MOS would be liberated during the trimming of the amylopectin cluster structure by DBE in starch biosynthesis, it is highly possible that DPE1 plays some role in starch biosynthesis. In *Chlamydomonas reinhardtii*, DPE1 has been reported to be functional during starch biosynthesis [27,28]. In contrast, in *Arabidopsis* leaves, this enzyme appears to be mainly active during starch degradation [29].

Dauvillée et al. [30] provided evidence that, in *Chlamydomonas*, Pho1 participates in starch granule formation. The same appears to be true in potato tubers [31]. Biochemical analysis of rice mutants that lack Pho1 strongly suggests that, in the endosperm, Pho1 is involved in the initiation of starch biosynthesis [32]. These results are consistent with the finding that rice Pho1 is capable of elongating MOS (DP < 10) and linear malto-dextrins (MD, with a DP from 10 up to approximately 100) even under high orthophosphate (Pi)/glucose 1-phosphate (G1P) ratios [33]. Furthermore, Pho1 seems to be essential for starch biosynthesis in rice endosperm especially at temperatures below 20 °C because, at this range of temperatures, the enzyme activities of SSs are considerably lower than that of Pho1 [34].

How is Pho1 involved in the initiation of starch biosynthesis in rice endosperm? Our previous *in vitro* studies [35] established a clear functional interaction between Pho1 and BE from rice; for example, the effect of BE is not merely to supply Pho1 with the non-reducing ends in the acceleration of glucan synthesis by Pho1. It was also shown that Pho1 can interact with any of the BE isozymes, BEI, BEIIa, or BEIIb, whereas the chain-length distribution of the branched glucan product reflects the chain-length specificity for each BE. However, the mechanism of the synergistic action has remained unclear. In the present investigation, we aimed to characterize in detail the interactions between the Pho1 and BE isozymes from rice with emphasis on the Pho1-BEI interaction. We analyzed the phenomenon of the lag period before the maximum interacting reaction and the features of fine structures of glucans synthesized by the enzymatic reactions. In addition, we also attempted to detect the physical association between Pho1 and BEs by using the co-immunoprecipitation method, blue-native PAGE (BN-PAGE), and gel filtration HPLC chromatography. We also discussed the physiological roles of the Pho1-BEI interaction in starch biosynthesis, especially the possible role in the glucan initiation process. Finally, we propose possible roles of Pho1 and other enzymes in glucan initiation in cereal endosperm.

During the review process of this paper, a report was published proposing the involvement of Pho1 in the glucan initiation of glucan synthesis through *de novo* synthesis in barley endosperm [36].

2. Materials and methods

2.1. Reagents

G1P was obtained from Wako Pure Chemical Industries, Ltd. Cluster dextrin (CD) and potato amylose were purchased from Ezaki Glico Co., Ltd. (Osaka, Japan), and Sigma, respectively. Glucose and maltose were obtained from Wako Pure Chemical Industries, Ltd., and maltotriose, maltotetraose, and maltoheptaose were obtained from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). The fluorophore 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) was obtained from Beckman Coulter/AB SCINEX. Glucoamylase from *Rhizopus* sp., α-amylase from *Bacillus subtilis*, and isoamylase from *Pseudomonas amyloderamosa* (PaISA) were obtained from Wako Pure Chemical Industries, Ltd., Seikagaku Biobusiness Corp. (Tokyo), and Hayashibara Biochemical Laboratories Inc., respectively.

2.2. Plant materials

The wild-type japonica-type rice cultivar Nipponbare was grown under natural environmental conditions in the experimental paddy fields of Akita Prefectural University. To prepare the crude enzyme extract, developing seeds were harvested at the mid-milking stage and stored at −80 °C until use. For the analysis of the chain-length distribution of amylopectin, starch was isolated from dry mature seeds that had been harvested and stored at approximately 8 °C until use.

2.3. Purification of Pho1 from developing rice endosperm

Approximately 30 g of developing seeds from Nipponbare were homogenized using a chilled mortar and pestle on ice in 150 ml of a grinding solution (GS), which consisted of 50 mM imidazole-HCl (pH 7.4), 8 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 12.5% (v/v) glycerol. The homogenate was filtered through four layers of gauze and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was collected and centrifuged again. The resulting supernatant was applied to a HitrapQ HP column (10 ml, Amersham Biosciences), which had been equilibrated with solution A (50 mM imidazole-HCl, pH 7.4, 8 mM $MgCl_2$, 1 mM dithiothreitol) at 4 °C. The proteins were eluted with a linear gradient of 0–1.0 M NaCl (dissolved in solution A), at a flow rate of 2.0 ml/min for 60 min at 4 °C. The fractions were collected into tubes at 1.5-min intervals and stored on ice. The fractions containing the peak of Pho1 activity were concentrated to a final volume of approximately 1.3 ml of GS using an Ultrafree column (Millipore) and stored at −80 °C. The concentrated Pho1 fraction was slowly combined with an equivalent volume of 3.0 M ammonium sulfate. The mixture was applied to an Ether-5PW hydrophobic chromatography column (7.5 mm in diameter × 7.5 cm in length; Tosoh Corporation, Tokyo, Japan), which had been equilibrated with solution A including 1.6 M ammonium sulfate at room temperature. The proteins were eluted with a linear decreasing gradient of 1.6–0 M $(NH_4)_2SO_4$ in solution A at a flow rate of 1.0 ml/min for 60 min at room temperature. Fractions were collected at 1 min intervals and stored on ice. The fractions that contained peak Pho1 activity were concentrated to approximately 150 μl of GS at 4 °C using an Ultrafree column (Millipore) and stored at −80 °C before use. By these procedures, Pho1 was purified to near-homogeneity, although one minor protein band with a molecular size of