Plant Science 233 (2015) 95–106

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Protein–protein interactions among enzymes of starch biosynthesis in high-amylose barley genotypes reveal differential roles of heteromeric enzyme complexes in the synthesis of A and B granules

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ARTICLE INFO

Article history:
Received 11 September 2014
Received in revised form
10 November 2014
Accepted 20 December 2014
Available online 7 January 2015

Keywords:
Starch biosynthesis
Barley
Enzyme complexes
A and B granules

ABSTRACT

The present study investigated the role of protein phosphorylation, and protein complex formation between key enzymes of amylopectin synthesis, in barley genotypes exhibiting "high amylose" phenotypes. Starch branching enzyme (SBE) down-regulated lines (ΔSBEIIa and ΔSBEIIb), starch synthase (SS)IIa (*ssiia*$^{-}$, *sex6*) and SSIII (*ssiii*$^{-}$, *amo1*) mutants were compared to a reference genotype, OAC Baxter. Down-regulation of either SBEIIa or IIb caused pleiotropic effects on SSI and starch phosphorylase (SP) and resulted in formation of novel protein complexes in which the missing SBEII isoform was substituted by SBEI and SP. In the ΔSBEIIb down-regulated line, soluble SP activity was undetectable. Nonetheless, SP was incorporated into a heteromeric protein complex with SBEI and SBEIIa and was readily detected in starch granules. In *amo1*, unlike other mutants, the data suggest that both SBEIIa and SBEIIb are in a protein complex with SSI and SSIIa. In the *sex6* mutant no protein complexes involving SBEIIa or SBEIIb were detected in amyloplasts. Studies with Pro-Q Diamond revealed that GBSS, SSI, SSIIa, SBEIIb and SP are phosphorylated in their granule bound state. Alteration in the granule proteome in ΔSBEIIa and ΔSBEIIb lines, suggests that different protein complexes are involved in the synthesis of A and B granules.

1. Introduction

Starch accumulates in cereal endosperm as an energy reserve for the next generation and is used globally as a human food, livestock feed as well as numerous important industrial applications including biofuels. Starch is found in higher plants, mosses, ferns and some microorganisms [1] and is an insoluble polyglucan composed of two polymers of glucose, amylose and amylopectin. Amylose is, essentially, a linear molecule with a molecular weight varying between (10^5–10^6 Da), in which glucose residues are joined via α-1,4 linkages with very few α-1,6 linkages, and typically constitutes up to 20–30% of total starch. Amylopectin has a molecular weight of (10^7–10^9 Da) constitutes 70–80% of total starch, containing linear chains of various degree of polymerization. Almost 5% of the glucose units in amylopectin are joined by α-1,6 linkages, which introduce branches in the amylopectin in a non-random fashion. Starch exists in the form of structurally well-organized granules in which amylopectin exhibits non-random distribution of linear chains and a clustered arrangement of branch linkages which gives rise to a high degree of structural organization. This conserved architecture of amylopectin is responsible for the semi-crystalline water insoluble starch granule [2]. Amylopectin is required for normal size and shape of the granules, whereas granules with varying low, or no, amylose retain the same shape and size as granules with normal amylose content [1]. In Festucoides, such as wheat, oats and barley, granules can be divided into B (1–15 μm, round) and A-granules (>15 μm, lenticular) [3–6], synthesis of which is developmentally

Abbreviations: *Ae*, *amylose extender*; AGPase, adenosine diphosphate glucose pyrophosphorylase; AMG, amyloglucosidase; APase, alkaline phosphatase; ATP, adenosine triphosphate; Da, Dalton; DBE, starch debranching enzyme; GBSS, granule bound starch synthase; GPC, gel permeation chromatography; HAG, high amylose glacier; ISA, isoamylases; PHO1, plastidic isoform of starch phosphorylase; PU, pullulanase; SBE, starch branching enzyme; SDS, sodium dodecyl sulphate; SP, starch phosphorylase; SS, starch synthase; *Su2*, *sugary2*.

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http://dx.doi.org/10.1016/j.plantsci.2014.12.016
0168-9452/

regulated [4,7]. The amylose:amylopectin ratio, and the size and shape of granules, are important parameters which impact the end use of starch [8].

The organization of the starch granule is a complex process involving several classes of enzyme, each with isoforms, which include adenosine diphosphate glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSSI and GBSSII), soluble starch synthases (SSI, SSIIa, SSIII, SSIV), starch branching enzymes (SBEI, SBEIIa and SBEIIb), starch debranching enzymes (DBEs) such as isoamylases (ISAI, ISAII and ISAIII), pullulanase (PU), and starch phosphorylase (SP) [9–13]. These different enzymes and their isoforms are differentially distributed in the soluble and starch granule fractions of plastids [14].

Recent evidence has demonstrated that many enzymes involved in starch biosynthesis are subjected to post translational modification by protein phosphorylation, and also interact to form heteromeric protein complexes [15–18]. In wheat GBSS, SSI and SSII are phosphorylated in their starch granule bound state [18,19]. In wheat amyloplasts phosphorylation of SBEIIb, SSIIa and SP has also been reported by radioactive labelling of amyloplasts with γ ^{32}P-ATP [20].

Co-immunoprecipitation of SBEIIb, SBEI, and SP provided direct evidence of multi enzyme complex formation in soluble extracts of wheat endosperm [20], dependent upon the phosphorylation of target proteins. Other enzyme complexes, containing SSI, SSIIa, SSIII, SBEIIa, and/or SBEIIb, in various combinations have also been reported [15,21]. Hennen-Bierwagen et al. [22] also reported a novel complex in maize endosperm containing SSIII, SSIIa, SBEIIa, SBEIIb, large and small subunits of AGPase, and pyruvate phosphate dikinase (PPDK). Liu et al. [16] reported that, in maize, a null mutant of SBEIIb (*amylose extender*, *ae*$^-$) contained a novel protein complex comprising SSI, SSIIa, SBEI, SBEIIa and SP which became entrapped in the starch granule. Another *ae*$^-$ allele was described which expressed a catalytically inactive SBEIIb protein. Although this protein was not able to bind glucan substrate, it was still found as a granule associated protein, as a result of being able to form a heteromeric protein complex leading to the entrapment of inactive protein within the starch granule [17]. In related work [23] it was reported that a point mutation in SSIIa in a *su2*$^-$ mutant of maize, led to loss of SSI and SBEIIb, as well as SSIIa from starch granules. These observations suggest that single gene mutations affect partitioning of several proteins between the soluble and granule-bound fractions of amyloplasts and impact amylopectin fine structure. They further imply that alteration in the protein fingerprint of the granule may reflect variations in protein–protein interactions in the stroma. Such changes in the granule proteome arising from allelic variations also give rise to variation in granule structure and composition [17]. The present study examines the relationship between the granule proteome and starch properties in several varieties of barley with defined mutations, and relates this to variation in protein–protein interactions in the stroma. Variation in the granule proteome of A- and B-granules was also investigated with a view to understanding the relationship between granule type, its proteome and mechanisms underpinning granule size and morphology.

2. Materials and methods

2.1. Plant material

The genotypes used in this study included, wild-type, starch branching enzyme down-regulated lines (ΔSBEIIa and ΔSBEIIb), *ssiia*$^-$ (*sex6*) mutant, and *amo1* (*ssiii*$^-$) mutant. The sources of mutant seed were, ΔSBEIIa and ΔSBEIIb [24], *sex6* [25], HAG *amo1* [26]. The seeds for OAC Baxter (reference genotype) and Neopolis (*waxy*) were obtained from Dr. Duane E. Falk, Plant Agriculture, University of Guelph and Dr. Brian Rossnagel, Crop Development Centre, University of Saskatchewan, Canada, respectively. The plant material was grown in the glasshouse at the University of Guelph under conditions previously described for growing wheat [18].

2.2. Isolation of starch granules

Mature dry barley seeds weighing ≈(55) g were completely ground to flour with a Retsch® MM301 homogenizer in liquid nitrogen. 50 g of flour was suspended in 150 ml buffer containing 100 mM Tricine-KOH, pH 7.8, 1 mM Na_2-EDTA, 1 mM DTT and 5 mM $MgCl_2$ at 4 °C. The suspension was vortexed (Eppendorf) for 5–10 min to make a uniform suspension and left for 5–10 min on ice. The well-mixed suspension was sieved through six-layers of cheese cloth to remove debris and bran. Buffer was added to wash traces of starch from the cheese cloth. The sieved milky suspension, 450–500 ml, containing starch, fine pieces of debris and bran was centrifuged at 16,000 × *g* for 15 min. The supernant was discarded and the pellet resuspended in 100 ml buffer containing 50 mM TRIS-acetate, pH 7.5, 1 mM Na_2-EDTA, and 1 mM DTT (wash buffer). The resuspended pellet was centrifuged at 6000 × *g* for 5 min. This washing step was repeated 5–7 times until a thick yellow layer of debris was left on the top of starch. The yellow layer, containing very fine pieces of debris and bran, was completely removed with a spatula whilst minimizing loss of starch. After removing debris the starch was again washed and centrifuged twice (see above) in wash buffer. The purified starch was washed three times, each with 45 ml of 99% acetone (termed acetone-washed starch) followed by three washes, each with 45 ml of 2% (w/v) sodium dodecyl sulphate (SDS) in water, to remove proteins bound to the surface of starch granules. This was followed by 3 washings with 45 ml distilled water and the starch finally dried under vacuum (Eppendorf, vacufuge™) at 25 °C for 3 h.

2.3. Separation of A- and B-type starch granules

A- and B- starch granules were separated based on a method previously described [27]. Approximately 0.5 g of the acetone-washed starch was suspended in 5 ml of dH_2O. This starch suspension was then carefully laid on top of 10 ml of 70% (v/v) Percoll in dH_2O in a 15 ml tube, followed by centrifugation at 10 × *g* for 10 min at room temperature. The larger A-granules were centrifuged through the Percoll pad and precipitated at the bottom of the tubes, whereas the smaller B-granules remain in suspension. After centrifugation, the supernatant (containing B-granules) was carefully removed. The pellet containing A-granules was washed twice with dH_2O, by resuspension and centrifugation at 4000 × *g* for 5 min. The resulting pellet was resuspended in 5 ml dH_2O and laid on top of 10 ml of 70% (v/v) Percoll. Centrifugation through Percoll with subsequent washing in dH_2O was repeated 3 times. A-granules were then centrifuged 3 times through 100% Percoll for 10 min at 10 × *g*. All supernants from each Percoll centrifugation step were pooled and centrifuged at 4000 × *g* for 5 min. The resulting pellet was washed twice with dH_2O, resuspended and centrifuged at 4000 × *g* for 5 min, and comprised B-granules.

2.4. Extraction of amyloplasts and preparation of endosperm whole cell extracts

Barley endosperm amyloplasts were isolated as described earlier [18]. Endosperm whole cell extracts were prepared according to a previously described protocol [28].