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Review article

# Critical and speculative review of the roles of multi-protein complexes in starch biosynthesis in cereals

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ABSTRACT

Starch accounts for the majority of edible carbohydrate resources generated through photosynthesis. Amylopectin is the major component of starch and is one of highest-molecular-weight biopolymers. Rapid and systematic synthesis of frequently branched hydro-insoluble amylopectin and efficient accumulation into amyloplasts of cereal endosperm is crucial. The functions of multiple starch biosynthetic enzymes, including elongation, branching, and debranching enzymes, must be temporally and spatially coordinated. Accordingly, direct evidence of protein–protein interactions of starch biosynthetic enzymes were first discovered in developing wheat endosperm in 2004, and they have since been shown in the developing seeds of other cereals. This review article describes structural characteristics of starches as well as similarities and differences in protein complex formation among different plant species and among mutant plants that are deficient in specific starch biosynthetic enzymes. In addition, evidence for protein complexes that are involved in the initiation stages of starch biosynthesis is summarized. Finally, we discuss the significance of protein complexes and describe new methods that may elucidate the mechanisms and roles of starch biosynthetic enzyme complexes.

## 1. Introduction

The unique structure of starch has been investigated in multiple studies and was characterized by the hydrolysis of specific starch regions. These regions include the hydrolysis of α1,6 glycosidic linkages by isoamylase (ISA), α1,4 glycosidic linkages by α and β-amylases, and the actions of glucan phosphorylase (Pho) [1,2]. However, the structure of starch at the macro-level remains largely uncharacterized [3].

Starch comprises glucose monomers with α1,4 and α1,6 glycoside linkages. These bonding patterns of starch differ from cell walls, which comprise β-glycosidic bonds, and are exclusively digestible by human enzymes to convert the potential energy of this polymer into energy-rich molecules such as ATP. Starches include glucose polymers of nearly linear amylose and frequently branched amylopectin. The average degrees of glucose polymerization (DP) of amylose and amylopectin vary depending on the tissue type, genetic background, species, and growth environment. The sizes of amylose in DP range from 200 to 10,000 [4]. The sizes of amylopectin encompass a much wider range than amylose, depending on the different methods of measurement and the preparation of amylopectin. For example, the molecular weight of purified cereal amylopectin measured by high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors is approximately DP $4 \times 10^5$–$3 \times 10^7$ [5], whereas that measured by the number of reducing ends ranges from DP 500–20,000 [6]. These inconsistencies may be due to the degradation or aggregation of amylopectin molecules during the purification, dissolution and separation steps, and even now it is very difficult to accurately measure the molecular size of undecomposed whole amylopectin [3]. Adjacent chains of amylopectin branches twist to form double helices, and multiple botryoidal grape-bunch like clusters are layered at 9-nm intervals [6] in all plant species (Cluster model [7]). Although it is accepted that starch molecules are stored as granules, where and how amylose and amylopectin reside in these granules remains unknown.

Multiple models for the structure of starch have been proposed [7–10]. Among these, the cluster model by Hizukuri [7,10] proposed that clusters built from A chains and $B_1$ chains are interconnected through $B_{2\text{-}3}$ chains and that these long B chains are located in both crystalline and amorphous lamellae (Fig. 1A and B). By contrast, the two-directional backbone model by Bertoft [9] proposed that the clusters connecting the chains are positioned perpendicular to the direction of the backbone. Long B chains and amylose molecules are

*Abbreviations: ae, amylose extender*; BE, starch branching enzyme; BN-PAGE, blue-native-polyacrylamide gel electrophoresis; DP, degree of glucose polymerization; ISA, isoamylase; Pho, phosphorylase; SS, starch synthase; *su2*, *sugary2*

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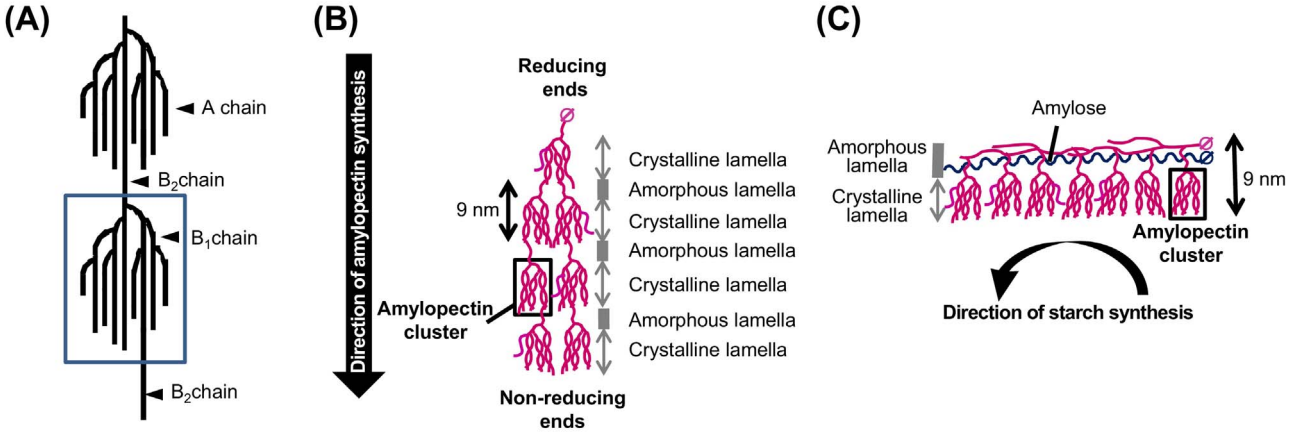


**Fig. 1.** Schematics of starch structures.
(A) Branch structure of amylopectin clusters. Cluster structures are established by controlled positioning of branches, and their lengths are determined by specific starch biosynthetic isozymes of starch synthases (SSs), branching enzymes (BEs), and debranching enzymes. A-type chains are branches that do not bear other branches. B-type chains bear multiple branches, and numbers in subscripts indicate numbers of amylopectin clusters spanned by the inter-connecting chains. (B) Schematic representation of the cluster structure based on the Hizukuri model [7,10]. Starch is thought to be synthesized from the reducing ends towards the non-reducing ends. Amylopectin (magenta) is synthesized as clusters and comprises highly branched crystalline lamellae, in which adjacent branches form double helices, and amorphous lamellae that connect clusters. The distance between one crystalline lamellae and the next is approximately 9 nm. (C) Schematic representation based on the two-directional backbone model [9]. The direction of the clustered chains is perpendicular to the direction of the backbone. Long B chains and amyloses (blue) are located in amorphous lamellae.

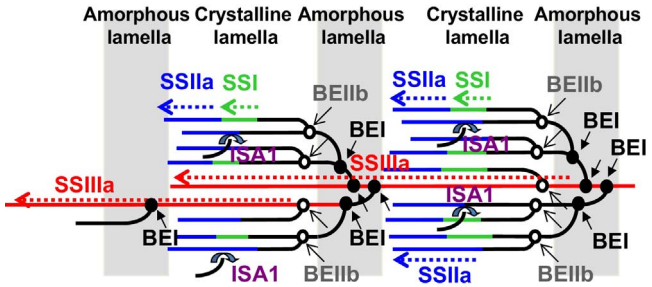


**Fig. 2.** A model of amylopectin biosynthesis in *indica* rice; SS, starch synthase; BE, branching enzyme; ISA1, isoamylase1; black circles, branch points in the amorphous lamellae; open circles, branch points in the crystalline lamellae; dashed arrows, chain elongation by SS isozymes; swing arrows, trimming of improper branch points by ISA1. Fig. 2 was reprinted with permission from [61] Kagaku to Seibutsu, Fujita, Denpunhe-nitaimai no kaiseki to riyou, 51, (©2013) 500-407, Fig. 2, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

located in amorphous lamellae (Fig. 1C). Although there is no doubt that starch biosynthesis occurs from reducing to non-reducing ends, which of these models corresponds more closely to the actual structure of starch is currently unclear (Fig. 1). As described later, protein–protein complexes composed of the starch synthase (SS) SSI, SSIIa and branching enzyme (BE) BEIIb, which contribute to the synthesis of short and intermediate amylopectin chains within the clusters, are likely to be located in crystalline lamellae. In addition, BEI and SSIII(a), which are thought to produce amylopectin long chains, possibly by forming high-molecular-weight protein complexes with SSI, SSIIa and BEIIb, are likely to be located at amorphous lamellae (see Fig. 2).

Starch biosynthesis can be divided into initiation and amplification stages with the likely involvement of different starch biosynthetic enzymes at each stage [11]. At the initiation stage, a precursor glucan with the cluster structure is synthesized from simple sugars such as maltose and malto-oligosaccharides. However, at the amplification stage, the precursor glucans and/or amylopectin are reproduced to form amylopectin molecules, and therefore the reproduced glucans can be new precursors. In this way, during the latter stage, the number of amylopectin molecules is exponentially increased. Therefore, as starch matures, starch biosynthesis must accelerate and become more efficient. The formation of double helices by adjacent amylopectin branched chains repels water molecules that confer hydro-insoluble properties. In addition, the above-mentioned unique starch structures allow large amounts of glucose monomers to be packaged with a density as high as 1.5 g/cm$^3$ [12]. This feature is important for the use of starch as a carbon source by subsequent generations of plants, providing large amounts of glucose in forms that are not easily degraded by micro-organisms during storage.

Several starch biosynthetic enzymes are known to be entrapped in starch granules [13]. Starch biosynthesis occurs in relatively aqueous environments during the early stages of seed development. However, starch synthesis continues during the late stages of seed development as desiccation progresses. Under these conditions, the diffusion rates of enzymes and substrates are likely to decelerate. Although the substrate may be more concentrated, the time required for all starch biosynthetic enzymes to randomly encounter the appropriate preferred substrates would be inefficient and, thereby, benefitting from the formation of multi protein–protein complexes.

In this review, we described protein–protein interactions in cereal endosperm between starch biosynthetic enzymes, including SSs, starch branching enzymes (BEs), starch debranching enzymes, and plastidial Pho1.

## 2. Protein–protein complex formation during starch biosynthesis

The formation of protein–protein complexes for starch biosynthesis has been suggested in biochemical studies of enzymes from developing wild-type and mutant maize kernels after chromatographic fractionation [14–16]. Moreover, direct evidence of phosphorylation-dependent multi-enzyme complex formation in starch biosynthesis was first shown in developing wheat endosperm [17].

Amyloplasts from developing wheat endosperm contained phosphorylated BE and Pho1 isozymes. Immuno-precipitation of amyloplast extract treated with ATP or alkaline phosphatase suggested that the formation of protein complexes among BEs and SSs were dependent on the phosphorylation of these enzymes. Dephosphorylation of these enzymes is associated with reduced activities and protein complex formation [17]. In addition, Q-TOF-MS, immuno-precipitation, and western blotting analyses revealed that soluble fractions from mid–late developing seeds contained a 260-kDa protein complex of SSI, BEIIa, and BEIIb, and that this protein complex was not present in seeds during the early stage of development [18]. Again, the treatment of amyloplast extract with ATP or alkaline phosphatase suggested that at least SS and BE isozymes can form phosphorylation-dependent protein