International Journal of Biological Macromolecules 91 (2016) 768–777

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

On the molecular structure of the amylopectin fraction isolated from "high-amylose" *ae* maize starches☆,☆☆

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ARTICLE INFO

Article history:
Received 6 May 2016
Received in revised form 8 June 2016
Accepted 10 June 2016
Available online 11 June 2016

Keywords:
Amylose extender starch mutant
Amylopectin clusters
Building blocks

ABSTRACT

The amylopectin fractions from starch of a series of *amylose-extender* (*ae*) maize samples (HYLON® V, VII and VIII starches) were isolated and analysed for their molecular composition and structure. The fractions from all samples contained both a high and a low molecular weight fraction (HMF and LMF), of which LMF increased with the amylose content of the starch and appeared to have substantially more of long chains than HMF. A normal amylose-containing maize starch (NMS), which served as a reference sample, contained very little LMF, which suggested that LMF was the inherent result of the effect of the loss of starch branching enzyme IIb activity in the *ae* mutants. Clusters were isolated from the amylopectin fractions using *Bacillus amyloliquefaciens* α-amylase, which effectively hydrolyses long internal chain segments between clusters. During the hydrolysis process, clearly more of small dextrins were released from the *ae* starches in comparison to NMS. It appeared that some of these small dextrins did not precipitate in methanol together with the majority of the clusters. Nevertheless, isolated clusters from the HYLON starch samples were smaller than in NMS and the clusters possessed a lower density of branches with longer chains. The composition of small, branched building blocks was also clearly different: HYLON starch samples possessed much more of single-branched blocks and less multiple-branched blocks than NMS.

1. Introduction

Starch is synthesized by a large diverse group of enzymes that gives rise to water-insoluble granules containing predominantly amylopectin with a high molecular weight and chains consisting of α-(1,4)-linkages and about 5% α-(1,6)-branches, and amylose with lower molecular weight, considerably longer chains and only a few branches [1]. The enzyme responsible for the synthesis of amylose is granule-bound starch synthase I (GBSSI), whereas amylopectin is synthesized by the concerted action of a number of enzyme classes; (soluble) starch synthases (SSs), starch branching enzymes (SBEs), and starch debranching enzymes. In addition, several isoforms of each class exist alongside a similarly large suite of starch degrading enzymes, making the biochemical pathway complex, and understanding the precise role of each of the enzymes in determining the final polymer structure very difficult [2,3]. Whereas the molecular structure of amylose is fairly simple, the structure of amylopectin is complex and apparently depends on the relative activities of the enzymes involved in the biosynthesis as well as the genetic composition of the plant [3].

Differences in the structure and composition of amylose and amylopectin in starch give rise to important differences in the properties and the functionality of starch from different plants [4,5]. Nevertheless, the principle structure of amylopectin is believed to be highly conserved throughout the plant kingdom [6]. The true nature of the principle structure remains uncertain, however, and to-date two major structural models are under discussion, namely the traditional cluster model [7–9] and the more recently suggested building block backbone model [10,11]. In the traditional cluster model, short chains with less than approximately 36 glucosyl units form clusters and longer chains interconnect the clusters [12]. In

☆ This publication is dedicated to the memory of Dr. Koushik Seetharaman (1966–2014).
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http://dx.doi.org/10.1016/j.ijbiomac.2016.06.029
0141-8130/

the backbone model, mostly the long chains (>35 glucosyl units) form collectively a backbone and branched building blocks, which are smaller than clusters, are outspread along the backbone and form the basic structural unit [13]. The building blocks in the clusters are units containing two, three, or more chains and are divided into group 2, 3, or more, respectively [14]. Larger entities, which can be considered as clusters, have been isolated using the α-amylase of *Bacillus amyloliquefaciens* (see discussion in [13]). The enzyme preferentially attacks longer internal chain segments between the apparent cluster units as well as external chains. The fine structure of the apparent clusters has been characterized with the conclusion that their structure is better compatible with the backbone model than with the traditional model [15].

Because of the simultaneous attack by the α-amylase on external chains, the length of these chains are reduced making the blend of chains that can be analysed after debranching of the clusters difficult to interpret [16,17]. The residual external chains are therefore largely removed by the successive action of phosphorylase *a* and β-amylase, which trim the chains to certain lengths. In the resulting φ,β-limit dextrin (φ,β-LD), A-chains (chains that do not carry other chains) remain as maltosyl stubs and the external segment of B-chains (carry other chains) remain as glucosyl stubs [18]. In clusters, these chains were assigned small case letters, however, because many of the chains in the clusters are not comparable to those in the original amylopectin macromolecule, as they are formed by the α-amylase during cluster isolation [15]. The b-chains of the clusters were further divided into sub-categories (b0–b2, etc.) based on the apparent number of inter-block segments they are involved in [15].

Several mutations that affect the activities of the starch synthesizing enzymes are known and they affect the structure of the starch components to varying degrees. Most of the investigations made so far have concentrated the analyses on the composition of amylose in the starch granules [19–24] and the composition of unit chains in the amylopectin component [19–28]. A few studies have also focussed on the internal unit chain composition, i.e., on the limit dextrins of the amylopectin [27–33]. However, the literature regarding the cluster and building block structure of mutant starches is sparse [34–39].

The major SBE isoform expressed in maize endosperm is SBEIIb, and its loss in the *amylose-extender* (*ae*) mutant causes well-characterized alterations in amylopectin structure [30]. The *ae* mutation is known to give elevated amylose content in the starch [40–43], but it also affects the structure of the amylopectin component, which is expected as the mutation affects SBEIIb, responsible for most of the branching activity in this tissue [3]. The amylopectin fraction has elevated chain lengths compared to amylopectin in normal starch [27,28,41,43,44]. Boyer et al. [45] analysed the structure of *aewx* maize (devoid of amylose) and Klucinec and Thompson [46] isolated the non-complexing fraction (the amylopectin fraction) from *ae* maize in a mixture of isoamyl alcohol and 1-butanol. Both groups reported that the amylopectin consisted of two components based on their size, of which the iodine complex with the low molecular weight fraction gave rise to slightly higher wavelength maximum (λ_{max}) values than the large component, suggesting longer chains in the former component. Klucinec and Thompson [30] also analysed the internal chain distribution of the isolated amylopectin fraction and concluded that not only the chain length, but also the pattern of branching was affected by the *ae* mutation. In addition, *ae* starches have also been shown to demonstrate B-type crystallinity, indicating alterations in amylopectin cluster and blocklet architecture [19,40,47]. The higher proportion of relatively long-chain branches in *ae* starches leads to them being termed "high-amylose" starches, although this term is misleading, since studies with *aewx* double mutants (i.e., *ae* in an amylose-free background) demonstrate these starches have modified amylopectin [19,27], and recent studies with SBEIIb-deficient rice demonstrate no alteration in the amylose content [48]. In maize, SBEIIb is part of a larger enzyme complex with SSI and SSIIa, however, in its absence in the *ae* mutant a new complex is formed in which SBEIIb is substituted with SBEI, SBEIIa and starch phosphorylase [49].

The purpose of the present investigation was to study the branching pattern of a range of *ae* maize starches further by isolating the clusters from the amylopectin fraction and analyse their constitution of chains and building blocks.

2. Experimental

2.1. Materials

Normal maize starch (MELOJEL® starch) and three *ae* genotype maize starches (HYLON V, VII and VIII starches) were supplied by Ingredion Incorporated (Bridgewater, NJ, USA). The following enzymes were supplied by Megazyme (Wicklow, Ireland): α-amylase from *Bacillus amyloliquefaciens* [(1,4)-α-D-glucan glucanohydrolase, EC 3.2.1.1; 555 U/mg], β-amylase from barley [(1,4)-α-D-glucan maltohydrolase, EC 3.2.1.2; 705 U/mg], isoamylase from *Pseudomonas* sp. (glycogen 6-glucanohydrolase, EC 3.2.1.68; 260 U/mg), and pullulanase from *Klebsiella planticola* (amylopectin 6-glucanohydrolase, EC 3.2.1.41; 34 U/mg). Phosphorylase *a* from rabbit muscle [(1,4)-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1; 25 U/mg] was purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.2. Isolation of amylopectin

The amylopectin was isolated as the non-complexing fraction in isoamyl alcohol–1-butanol following the method of Klucinec and Thompson [46], with minor modifications [17]. The soluble polysaccharides in the isoamyl alcohol–1-butanol mixture were precipitated by three volumes of methanol, re-dissolved in warm water and freeze-dried. The isolation was made three times and all subsequent analyses were performed separately on these three preparations.

2.3. Molecular size-distribution of amylopectin

Amylopectin (5 mg) was dissolved in 400 μL 90% (v/v) dimethyl sulfoxide (DMSO) and heated in a warm water bath (80 °C) for 5 min and then stirred at room temperature (22 °C) overnight. The samples were diluted with 1100 μL warm water. An aliquot (800 μL) of the sample was eluted through a Sepharose CL 2B column (1.6 × 32 cm) (GE Healthcare, Uppsala, Sweden) with 0.01 M NaOH at a rate of 0.5 mL/min. Fractions (1 mL) were collected, and the carbohydrate content was determined in every second tube using the phenol-sulphuric acid reagent [50]. The other tubes were used for analysis of the spectrum and λ_{max} of the iodine-glucan complex as described elsewhere [51]. The values given are averages of duplicates.

Fractions number 15–33, containing the high-molecular weight component of the amylopectin from HYLON VIII starch, were collected and pooled separately. The pooled fractions were filtered by tangential flow filtration (Minimate™ TFF System, Canton, MA) using a membrane filter with MWC 10,000 Da to remove NaOH and excess water from the carbohydrates. The sample was used for unit chain length analysis as described below.

2.4. Isolation of clusters

Clusters of amylopectin were prepared following the description by Zhu et al. [36]. 100 mg amylopectin was dissolved in 2.0 mL of 90% (v/v) DMSO by heating in a boiling water bath for 5 min and then stirred overnight at room temperature. 7.0 mL hot water was