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Fine structure characterization of amylopectins from grain amaranth starch

Xiangli Kong [a], Harold Corke [a], Eric Bertoft [b,*]

[a] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China
[b] Department of Food Science, Swedish University of Agricultural Sciences, PO Box 7051, S-750 07 Uppsala, Sweden

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ABSTRACT

The aim of this study was to determine the fine structure of amylopectin from grain amaranth. *Amaranthus* amylopectin was hydrolyzed with α-amylase, and single clusters and a group of clusters (domain) were isolated by methanol precipitation. The domain and the clusters were treated with phosphorylase *a* and then β-amylase to remove all external chains, whereby the internal structure was obtained. The ϕ,β-limit dextrins were analyzed on Sepharose CL 6B. The average DP (degree of polymerization) and peak-DP values of fractions of clusters were 57 and 82, respectively; the values of the domain were 137 and 309, respectively. The unit chain length profiles were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC–PAD). The results showed that the domain fraction contained ~2.2 clusters, and single clusters were composed of ~13 chains. The ϕ,β-limit dextrins of the clusters were further hydrolyzed with α-amylase to characterize their building block composition. The average DP of the branched blocks was ~11 and they contained on average ~2.5 chains. Their average chain length, internal chain length, and degree of branching were approximately 4.3, 2.8, and 14, respectively. A cluster consisted of ~6 branched blocks, and the internal chain length between the blocks was ~6.8.

1. Introduction

Starch is one of the most important biopolymers in nature, which provides the main dietary energy for human. Starch contains three types of biomacromolecules: amylopectin (which is highly branched), amylose (which is almost linear with few branches), and possibly an intermediate material, which is found in some kinds of starch (such as oat and some mutant maize genotypes[1,2]) and has a structure intermediate to those of amylose and amylopectin. There are large variations in the contents of the three components in starches from different sources, but amylopectin is commonly considered the major component in storage starch and accounts for about 65–85% by weight.[3] To describe the structural properties of amylopectin, the chain length profile is usually determined, but the cluster model of amylopectin, which can provide a useful conceptual basis for understanding of the structure of the biopolymer and guide current thinking related to amylopectin biosynthesis and physical behavior, has been given less attention.[4]

Grain amaranth is a pseudo-cereal with a long cultivation history in Central and South America, which produces very small seeds with high nutritional benefits. Starch is the main component in amaranth grain and has granules between 0.5 and 2.0 μm,[5–9] smaller than found in starches from most other sources. The starch granules possess A-type crystallinity, which is more compact than B-type crystallinity. Amylopectin is the major component in grain amaranth starch and has major effects on the properties. Little work has been done on the structure of amaranth amylopectin,[10–12] and this mainly focused on the molecular size of amylopectin. The internal and fine structure of amylopectin from amaranth is largely unknown. Recently, groups of clusters (structural domains) and clusters were isolated and characterized in potato amylopectin,[13,14] which provided a new approach to characterize the cluster structure of amylopectin. In these works, domains and clusters were isolated from amylopectin by an initial, short α-amylolysis, and a continued enzymatic hydrolysis, respectively; and building blocks were obtained by an extensive hydrolysis into near-limit dextrins with the liquefying α-amylase of *Bacillus subtilis*.[13,14] A cluster was defined as a group of chains in which the branches are found closer than ~9 residues from each other; domains are composed of more than one cluster interconnected by long chains (B2- and B3-chains); and building blocks were characterized as very densely branched areas in which the average internal chain length between branches was only ~2 glucosyl residues. Building blocks build up clusters by inter-block chains with an apparent length of 7–8 glucosyl residues.[13,14]

The objectives of this study were to: (i) produce and isolate a domain and clusters from amaranth amylopectins; (ii) characterize the structure of the isolated domain and clusters; (iii) characterize the building blocks in clusters.

* Corresponding author. Tel.: +46 18 672 048; fax: +46 18 672 995.
E-mail address: eric.bertoft@lmv.slu.se (E. Bertoft).

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2. Experimental

2.1. Starch isolation, amylopectin fractionation, and debranching

Two amaranth cultivars, Cr049 and V69, were the same samples as in our previous report.[9] The starch isolation, amylopectin purification, ϕ,β-limit dextrin production from amylopectin, and debranching procedures were performed as previously described.[9,15]

2.2. Time course for α-amylolysis of amylopectins

Amylopectin (100 mg) was dissolved in 2 mL of 90% dimethyl sulfoxide (DMSO) with constant stirring for 2 days, then 7 mL hot MilliQ water was added. After cooling, 1 mL of 0.9 U/mL α-amylase of liquefying type (*B. subtilis*, also known as *Bacillus amyloliquefaciens*, EC 3.2.1.1, Seikagaku Corp., Tokyo, Japan) in 0.01 M sodium acetate buffer (pH 6.5) was added to start the reaction in a water bath (25 °C) with magnetic stirring. The enzyme activity was quantified on the day of analysis using Amylazyme assay (Megazyme, Wicklow, Ireland). Samples (100 μL) were taken every half hour from 0.5 h to 4 h. If analyzed immediately, 200 μL MilliQ water and 30 μL of 5 M NaOH were added and mixed; if not analyzed directly, 2 μL of 5 M NaOH was added and the sample was then stored at −20 °C. Prior to analysis, 200 μL MilliQ water and 30 μL of 5 M NaOH were added. The mixtures of the α-amylolysates were centrifuged and then analyzed by gel-permeation chromatography (GPC) on a column (1 × 90 cm) of Sepharose CL 6B (Pharmacia, Uppsala, Sweden) with 0.5 M NaOH as eluent at a rate of 0.5 mL/min.[13] The column was calibrated as described by Bertoft and Spoof.[16] The carbohydrate content of the collected fractions (0.5 mL) was determined by phenol–sulfuric acid reagent.[17]

2.3. Production of fractions of clusters and domains

The cluster and domain preparation was performed according to the method described by Bertoft[13] with some modifications and the procedure was similar to that described in Section 2.2. The amylopectin samples were incubated with α-amylase for the appropriate time (40 min for domain isolation and 3 h for cluster isolation) and the reaction was ended by adjusting the pH to ~13 with 5 M NaOH. After 1 h at room temperature, 5 volumes of methanol was added to the cluster preparations (1.2 volumes for domain preparations) and the solution was left at room temperature for another 1 h to precipitate the clusters or domains. The solution was centrifuged for 10 min at 1800*g*, the supernatant was removed, the precipitate was rinsed with methanol and centrifuged again. The precipitates were dissolved in 10 mL MilliQ water and were centrifuged to remove traces of undissolved matter, whereafter the supernatants were evaporated (Büchi Rotavapor R-3000, Flawil, Switzerland) at 60 °C to adjust the concentration to ~10 mg/mL. The concentrated samples were lyophilized (CT60e, Heto-Holten, Denmark) if not used directly for further experiments. An aliquot of 100 μL was taken for analyzing the molecular weight distribution on Sepharose CL 6B as described in Section 2.2.

2.4. Production of ϕ,β-limit dextrins

The production of ϕ,β-limit dextrins was conducted following Bertoft[13] with minor modifications. Samples of clusters or domain were dissolved in hot MilliQ water and adjusted to ~3 mg/mL. For 1 volume of the solution, 0.1 volume of 1.1 M sodium phosphate buffer (pH 6.8), 0.05 volumes of 2.8 mM EDTA, and 0.25 volumes of freshly prepared phosphorylase *a* solution (from rabbit muscle, ~25 U/mg, EC 2.4.1.1, Sigma–Aldrich, Deisenhofen, Germany; 2.5 mg dissolved in 25 mL MilliQ water) were added. The solution was stirred overnight at room temperature, the reaction was terminated in a boiling water bath for 5 min, the solution was centrifuged and the collected supernatant was reduced to 20% in volume by evaporation as in Section 2.3. The purification (removal of glucose 1-phosphate) was performed on two PD-10 columns (Sephadex G-25, Pharmacia, Uppsala, Sweden) coupled in series: 2 mL sample was applied on the columns followed by 3 mL MilliQ water. The eluate was discarded and then 4.5 mL MilliQ water was added and the sample was collected. The carbohydrate content was adjusted to ~3 mg/mL and the phosphorolysis and purification procedures were repeated to produce ϕ-limit dextrins. The carbohydrate concentration of purified ϕ-limit dextrin solution was adjusted to ~3 mg/mL, for 1 volume of the purified ϕ-limit dextrin solution, 1/3 volume 0.01 M sodium acetate buffer (pH 6.0) and β-amylase (from barley, 10,000 U/mL, EC 3.2.1.2, Megazyme, Wicklow, Ireland; ~4 U/mg substrate) were added, the solution was stirred overnight at room temperature and then placed in boiling water bath for 5 min to terminate the reaction. The solution was reduced to 20% of its original volume by rotary evaporation and was purified on PD-10 columns as described above. The β-amylolysis was repeated and the produced ϕ,β-limit dextrin was finally purified two times on PD-10 columns to completely remove maltose. The purified ϕ,β-limit dextrin was lyophilized or employed directly to perform the following analyses.

2.5. Analysis of fractions of ϕ,β-limit dextrins

The solutions of ϕ,β-limit dextrins were adjusted to ~3 mg/mL. (i) To 300 μL sample was added 30 μL of 5 M NaOH, the mixed sample was applied on Sepharose CL 6B as in Section 2.2. (ii) Unit chain length profile analyses of ϕ,β-limit dextrins were conducted as follows: 50 μL of 0.1 M sodium acetate buffer (pH 5.5) was added to 450 μL volume of the sample, 1 μL isoamylase (from *Pseudomonas amyloderamosa*, 250 U/mL, EC 3.2.1.68, Hayashibara Shoji Inc., Okayama, Japan) and 1 μL pullulanase (from *Klebsiella pneumoniae*, 429 U/mL, EC 3.2.1.41, Hayashibara Shoji Inc.) were added. The debranching reaction was conducted overnight at room temperature with slow constant stirring, the debranched samples were centrifuged and analyzed by HPAEC (Program 1 in Section 2.7).

2.6. Characterization of building blocks in ϕ,β-limit dextrins of clusters

ϕ,β-Limit dextrin (2 mg) was dissolved in 360 μL hot MilliQ water and 40 μL of 60 U/mL α-amylase in sodium acetate buffer (0.01 M, pH 6.5) was added. The α-amylolysis was conducted in water bath (35 °C) for 3 h and terminated in boiling water bath for 5 min. (i) 250 μL MilliQ water was added to 250 μL sample, then applied on a calibrated[16] column (1 × 90 cm) of Superdex 30 (Pharmacia, Uppsala, Sweden) with an elution rate of 0.5 mL/min (0.5 M NaCl). The carbohydrate content of collected fractions (0.5 mL) was analyzed by phenol–sulfuric acid reagent.[17] (ii) 470 μL MilliQ water was added to 30 μL sample, the solution was analyzed on HPAEC (Program 2 in Section 2.7). (iii) To another 30 μL sample were added 470 μL MilliQ water, 50 μL of 0.01 M sodium acetate (pH 5.5), 1 μL isoamylase, and 1 μL pullulanase, the debranching reaction was performed overnight as in Section 2.5. The debranched sample was boiled to terminate the reaction and was then analyzed by HPAEC (Program 2 in Section 2.7).

2.7. High-performance anion-exchange chromatography (HPAEC)

The high-performance anion-exchange chromatography (Series 4500i, Dionex Corp., Sunnyvale, CA, USA) equipped with a BioLC