Nano-Structures of DeBranched Potato Starch Obtained by Isoamylolysis

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Abstract: Starch debranching is fundamental for understanding the structure-function relationships of starch. In this paper, atomic force microscopy (AFM) was used to investigate potato starch by isoamylase [EC 3.2.1.68] debranching at nanometer scale. The hydrolysates were separated by gel-permeation chromatography and the fractions were imaged. In addition to linear structures and branches, coiled structures were revealed in the intermediate hydrolysates. It is concluded that isoamylolysis is very useful for a better understanding of starch structure-property relationships.

Keywords: atomic force microscopy (AFM), isoamylase, potato starch, structure

Introduction

Characterization of starch molecular structure is vital to comprehending starch structure-function relationships. Starch often applies to a mixture of 2 components: amylose and amylopectin. The molecular structure of amylose is essentially linear with a few relatively long branches. In contrast, the molecular structure of amylopectin is highly branched via 1, 6-α linkages (Zobel 1988; Wang and others 1998). Since natural starch generally has a granular structure, it needs to be destructed or modified before it can be used as food or industrial materials. Melting or disordering usually leads to partial or complete destruction of starch granule. Chemical or enzymatic agents may also be used to destruct, oxidize, or derivatize the starch. During the metabolism, degradation, and food process, the structure of starch granules ultimately affects the digestion of starch-based food, for example, as food for livestock without processing, and relate to users' health. Amylolytic enzymes, such as α-amylase, and debranching enzymes, such as pullulanase and isoamylase, are most widely used to analyze the chemical structure of starch (Dang and others 2006; Copeland and others 2009). Through the use of debranching enzymes, followed by size exclusion chromatography, chain length distribution of amylose and amylopectin can be obtained. A number of reviews have covered the 2 models of the structure of starch, the blocklet model (Gallant and others 1997; Buleon and others 1998), and the side-chain liquid-crystalline model (Waigh and others 1998, 2000). Supportive evidence of the blocklet model has come from both electron microscopy (Helbert and Chanzy 1996; Gallant and others 1997) and atomic force microscopy (AFM) (Baldwin and others 1998; Szymonska and Krok 2003; An and others 2008; Copeland and others 2009). The side-chain liquid-crystalline model was put forward based on enzymatic hydrolysis. Although starch has been studied for many years, and the chain and branch structures are commonly held by scientists, those structures have been rarely visualized.

In order to obtain the molecular details regarding the biochemical mechanisms that accomplish and control starch anabolism and catabolism, isoamylase (EC 3.2.1.68) was used in this study. Potato starch was chosen here because it is not only used for food, but also for other, nonfood purposes. Because cereal starches contain higher levels of lipids and proteins, which result in lower paste transparency and a strong persistent raw cereal flavor, than potato starch, the functional properties of starch from potato are also generally regarded to be superior to those of cereal starches (Wang and others 1998; Tester and others 2004). More than 80% of starches from potato are used in the sector of industry in Europe. Therefore, degradation of potato starches is essential for understanding the processing-structure-property relationships.

Materials and Methods

Materials

Isoamylase from *Pseudomonas amyloderamosa* [EC 3.2.1.68], activity 350 U/mL, according to supplier, was purchased from Megazyme Intl. Co., Ltd. Ireland. Potato starch (amylose content 24.2 mass%; phosphorus content 0.1%; moisture 12.5%; ash 0.25%; N 0.10%; lipid 0.12%.) was from Dingxin Starch Plant (Tianjin, China). Analyses were performed as described before (Gunaratne and Hoover 2002).

Enzymatic debranching

Starch was gelatinized before enzymatic debranching. Potato starch suspension (20 mg/mL) was gelatinized in boiling water bath for 10 min. Before adding enzymes, the sample was cooled down to 40 °C. One milliliter gelatinized starch was thoroughly mixed with 10 units of isoamylase and buffered to pH 5.5 at an acetate concentration of 30 mM in 1 vial. After stirring, the vial was incubated in water bath 40 °C for 4 h.

Gel-permeation chromatography (GPC)

Isoamylase-treated starch samples (0.2 mL) were loaded on a column (1.5 × 90 cm) of Sepharose CL-6B (Pharmacia) and then eluted with 0.5M KOH at 0.5 mL/min. Fractions were analyzed for carohydrates with the phenol sulfuric acid reagent (Dubois and

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others 1956). The column was calibrated with dextrins of known degree of polymerization (DP) (Bertoft and Spoof 1989).

Atomic force microscopy

The solution (5 μL) of the hydrolysates, including starch molecules, intermediate product and isoamylase molecules, or from fractions was dropped onto a newly cleaved mica surface by a pipette. After 3 min of deposition, the samples were immediately mounted onto AFM sample stage and covered with butanol. AFM experiments were performed using a Multimode AFM (Nanoscope IIIa, Veeco/Digital Instruments, Santa Barbara, Calif., U.S.A.) equipped with a J scanner. The scanner XYZ was calibrated by imaging standard gratings with a step height of 180 nm. Images were collected using Tapping Mode AFM. Silicon cantilevers (CSC-11, MikroMasch) with a typical spring constant of 0.35 N/m were used. All operations were carried out in butanol with a fluid cell using O-ring at room temperature. AFM imaging was conducted in triplicate. All the AFM images are presented without any image processing. The height, length, and particle distribution were measured by AFM offline software. The apparent height is equal to the vertical distance in a section analysis; while the apparent width may be broadened because of tip-broadening effect. The diameter of particles was obtained through particle analysis. Length statistical analysis was performed with Image J software.

Results

Amylopectin and amylose have very different properties probably due to their different structures. Under conditions that provide sufficient freedom of molecular movements, primarily by dilution with suitable solvents, and in some instances, dilution coupled with heating, the linear amylose chains can be oriented into preferentially parallel alignments and easy form strong aggregates. That is why it is not easy to visualize the chain structures. In contrast, the amylopectin molecules are highly branched so that they could not move as freely and do not align and associate as readily. Because the branches are quite short, normally 25 monomer units, the branch structures of amylopectin molecules are invisible at granule level. In this study, starches were gelatinized before adding isoamylase, which enable the branches and chains at molecular level to release completely from starch granule. After debranching, enzymes were not inactivated so that they were still active and were expected to be viewed in a binding state, starch-enzymes complex. Both the separated and nonseparated hydrolysates samples were used for AFM imaging. Also because the completely debranched starches were studied elsewhere in the literatures, we do not conduct a completed isoamylolysis in current work.

Topography of debranched potato starch by isoamylase

Figure 1 showed the typical topography of nonseparated hydrolysates. The hydrolysates were quite different from the gelatinized potato starches before isoamylase added. In Figure 1A, linear long and short chains, branches, coiled structures, and prominent particles co-existed in the hydrolysates. The detailed structures were shown in a small scan size image (Figure 1B). The linear chains were 1.02 $\pm$ 0.17 nm (n = 35) in height. The length of the chains ranged from 20 nm to approximately 2 μm. The apparent height of coiled feature is 1.75 $\pm$ 0.21 nm (n = 30). Some starch chains branched with angles about 60 degrees, which were clear in AFM images (Figure 1A). The prominent structures were 6.02 $\pm$ 0.31 nm (n = 36). In a 3-D image, a prominent structure was observed circling around a linear chain

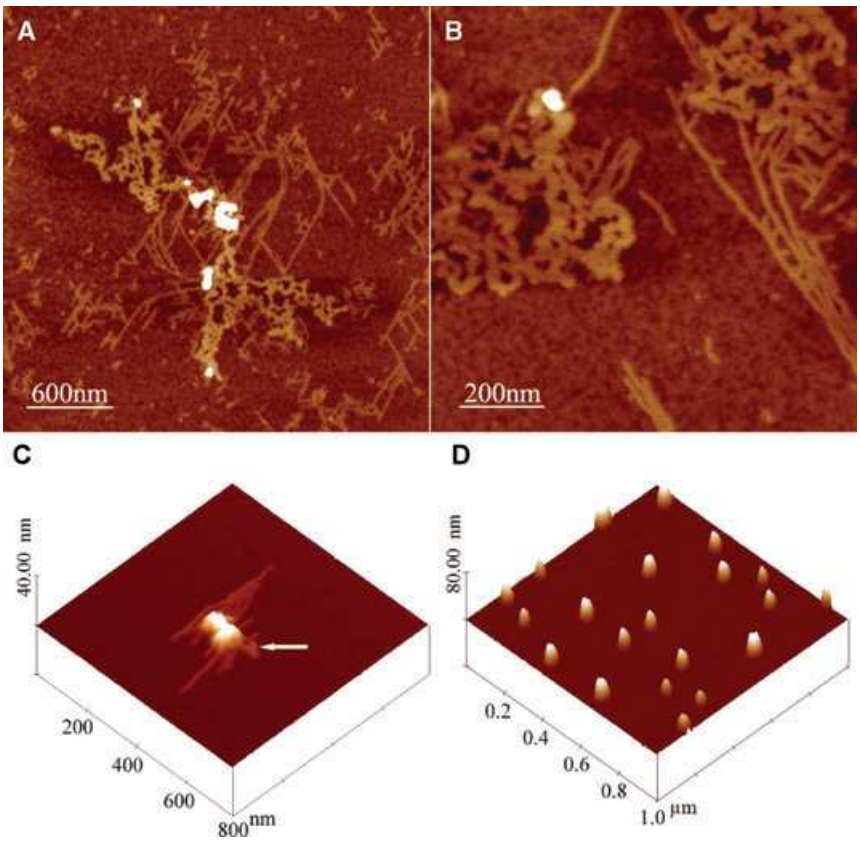

Figure 1–Nano-structures of product of isoamylolysis of gelatinized potato starch. (A) and (B). Linear chains, branches, and coiled structures were clear. (C) Three-dimensional structural information of an isoamylase-starch complex (as indicated by arrow). (D) Control of isoamylase. (A) scan size: 3 μm x 3 μm, height range 5 nm; (B) scan size: 1 μm x 1 μm, height range 5 nm; (C) scan size: 800 nm x 800 nm, height range 40 nm; (D) scan size: 1 μm x 1 μm, height range 80 nm.

(as arrow pointed, in 3-D image [Figure 1C]). Most prominent structures bound with coiled structures adjacent to linear structures. According to previous study (Rindlav-Westling and Gatenholm 2003), these prominent features were probably enzymes. The control of isoamylase without interaction with starches was also imaged (Figure 1D). The apparent height of isoamylase control was 13.30 ± 0.39 nm (n = 153). Compared to the pure isoamylase, the structures shown in Figure 1C indicated a binding state of isoamylase-starch complex.

Characterization of fractions obtained from the isoamylase debranched potato starch

Size distribution of debranched starches was analyzed by GPC on Sepharose CL-6B and were separated into 3 fractions, I (DP greater than about 1000), II (DP 60 to approximately1000), and III (DP lower than about 60) (Figure 2). After isoamylolysis, weight% of hydrolysates with DP greater than 1000 accounted up to 30%. A double peak appeared below DP 1000. The fractions I at 50 mL, II at 90 mL, and III at 100 mL were used for topographical characterization. AFM height images of the 3 fractions were shown in Figure 3. Coiled structures dominated in fraction I with an apparent height of 1.68 ± 0.35 nm (Figure 3A), while some of them showed linear chains or branches. Fraction II showed short linear chains with branches (Figure 3B). Length of the chains was measured from the branch points. The length distribution of the hydrolysate was analyzed by software Image J. Gaussian distribution was showed in Figure 4. Length of branches typically showed in Figure 3B was 98 nm (Gaussian width 29.7 nm, n = 1000). Fraction III showed very short chains or dots (Figure 3C), and most molecules sized around 10 nm.

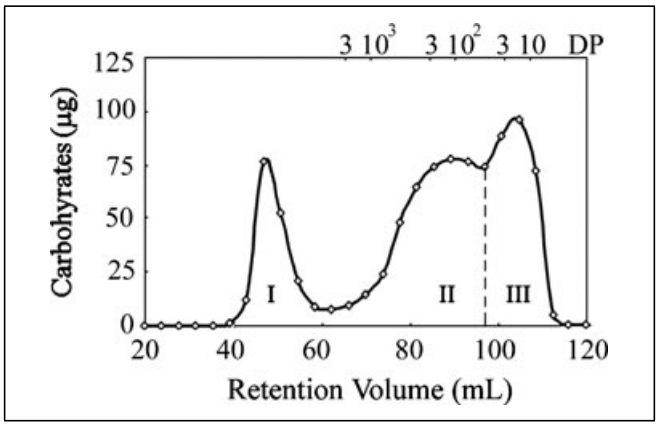

Figure 2–Elution of isoamylolysis of gelatinized potato starch on Sepharose CL-6B.

Discussion

In current work, potato starches were gelatinized and hydrolyzed by isoamylase. In contrast to the structure of the gelatinized starches, the isoamylolysis of potato starch revealed chains, branches, and coiled structures (Figure 1 and 3). The branched linear structures suggest that isoamylase could perform as an endoenzyme as it is generally termed, endo-α-1, 6-glucan hydrolase (Harada and others 1972). However, size distribution obtained by GPC also showed a peak around DP 25 (Figure 2), and images from fraction III showed very short linear chains, suggesting that isoamylase could also perform like an exoenzyme.

The prominent features were observed in the nonseparated samples, and missing in the separated fractions. If we assume these prominent structures were isoamylase, the conformational changes were huge during the interactions, from 13 nm in height (Figure 1A) to 6 nm in height, when compared to the control (Figure 1C). As the 3-D image showed, a rod-like chain was circled by a special prominent feature. It is more likely occurred when the enzyme reached its chains to wrap or to fix the substrates at the active center. In this way, a binding complex could

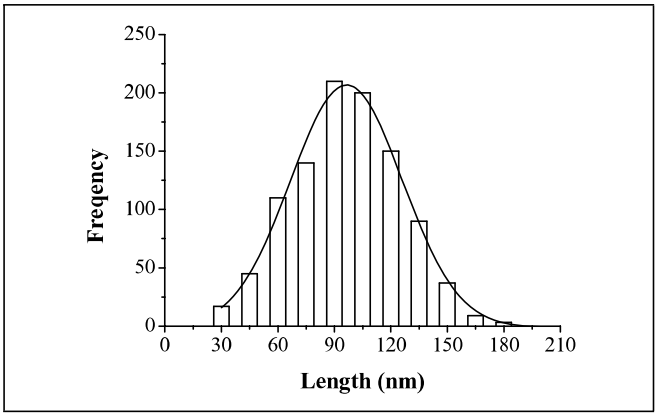

Figure 4–Length of linear chains from fraction at 90 mL was determined by Image J software. The length was measured from the end of a branch to the branch point (Gaussian width 29.7 nm, n = 1000).

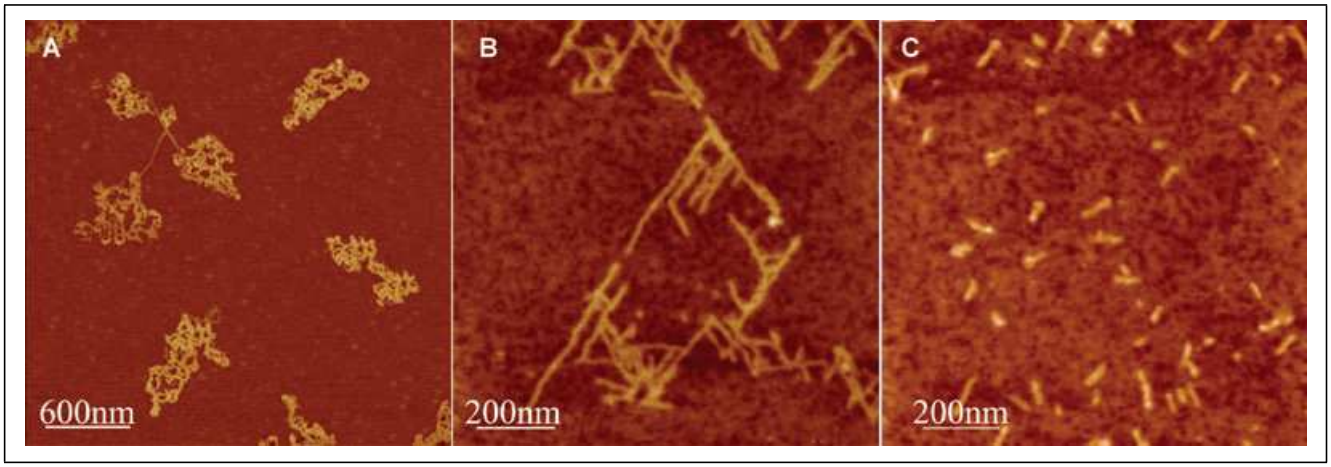

Figure 3–Topography of isoamylase treated potato starch. (A) Fraction I, scan size: 3 μm × 3 μm, height range 5 nm; (B) Fraction II, scan size: 1 μm × 1 μm, height range 5 nm; (C) Fraction III, scan size: 1 μm × 1 μm, height range 5 nm.

show the details how the enzymes attack a large starch molecule and produce some small products. AFM images also showed most prominent features bound to the connection between the coiled structure and linear chains. Therefore, we believe that isoamylase may assist to form a linear structure after a α-1, 6-glucosidic bond was cut.

AFM have shown that coiled structures and linear chains dominate the product of isoamylolysis. Images from fraction I confirmed that coiled structures were high weight component, incomplete debranched starches. Because branches (Figure 3A) were also revealed in fraction I, the component could be amylopectin component with high molecular weight. Therefore, these coiled structures are surely composed of a great number of undebranched short chains, such as A-chain and B-chain. As illustrated in Figure 1A and 1B, the coiled structures linked to linear chains, suggesting that the removal of small branched chains could transfer the coils into linear chains.

The height of linear chains (1.02 ± 0.17 nm [$n = 35$]) is comparable with duplex DNA (Watson and Crick 1953), suggesting that the linear structure might have a dia of 1 to 2 nm. Previous studies (Yamashita and Monobe 1971;Imberty and others 1988; Immel and Lichtenthaler 2000) have reported that the dia of A-amylose helix was 1.03 nm and that of V_H-amylose was 1.35 nm. Therefore, the linear chains in this work are probably helical structures of amylose composed of α-1, 4-glycosidic bond. We assume that the linear chains from one end to the branch point are B-chains, and the length was measured from the AFM images. Length distribution of the fraction at 90 mL revealed an averaged 98 nm (Gaussian width 29.7 nm, $n = 1000$). As 6-fold helical amylose has been reported repeating in 2.1 nm (Imberty and others 1988), the DP of a linear chain of 98 nm in length is about 280. This value is consistent with the size distribution obtained with GPC.

The coiled structures were not only showed in the mixture of isoamylolysis, but mainly contained in fraction I from GPC. It is noted that isoamylolysis was conducted for only 4 h and the residue contained both a considerable quantity of debranched chains and a great deal of residual starches. The results suggested that coils are more likely the amylopectin undegraded or lightly degraded. Since never reported before, the coiled structures pose a challenge to the most commonly held models, both side-chain liquid-crystalline model and blocklet model.

It is not only something interesting, but an issue worth thinking about. In addition, the debranching of starch from other botanic species, such as waxy starch, could be different and should be studied in future. Pullulanase, another starch debranching enzyme, will be investigated, which will allow researchers to compare the 2 debranching enzymes and to determine their fine catalytic specificities. The dynamic carbohydrate-protein interactions in solutions need to be conducted in the future to elucidate their precise roles that the starch debranching enzymes played in starch anabolism and catabolism.

Conclusions

The current work showed that the nano-structures of gelatinized potato starch can be revealed by AFM. Images from the mixture and/or the fraction of isoamylolysis demonstrated 3 dominant structures. The results confirmed a traditional concept of starch structure, chain and branch structures with length ranging up to 2 μm, and dia of about 1 nm. Gaussian distribution suggested a large proportion of the chains are about 100 nm long. It is clear that the amylopectin remained in enzymatic hydrolysates are in the form of coiled structures. The data also indicated that isoamylase could perform as an endoenzyme and exoenzyme as well. AFM provided vast amount of valuable information, and will play an essential role in exploring starch structures and functions. In the near future, more efforts will be committed to characterize the coiled structures, and to continue the studies of isoamylase in amylopectin degradation and synthesis the near future.

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