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Heterogeneity of lotus rhizome starch granules as revealed by α-amylase degradation

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Abstract

Lotus (*Nelumbo nucifera* Gaertn.) rhizome starch granules have an elongated oval shape with the hilum located at one end. The morphologic characteristics were used as a direction anchor to study the heterogeneity of molecular organization of starch granules using microscopy before and after partial digestion by bacterial α-amylase (*Bacillus* sp.) The partially digested granule showed a single, big eroded hole at the end distant from the hilum. The enzyme-attacked end was revealed to be the loosely packed end and to be the weak point for enzyme hydrolysis. The α-amylase hydrolyzed the loosely packed central part of the granule faster than the densely packed periphery, and left an empty shell with a fish-bone-like tunnel inside. The periphery was more resistant to amylase hydrolysis and had strong birefringence. For the whole starch granule, the selectivity of α-amylase hydrolysis was low for the crystalline and amorphous regions and for amylose and amylopectin molecules. This study elucidated that the molecular organization of lotus rhizome starch granules was heterogeneous.

Keywords: Lotus rhizome; *Nelumbo nucifera*; Starch granule; α-Amylase; Microscopy

1. Introduction

Native starch is composed of water-insoluble semi-crystalline granules, which are made up of amylopectin, a highly branched component, and amylose, an essentially linear component. Some of the molecular chains in starch are folded into double helices and aggregated to form crystallites. The degree crystallinity of native starch granules varies from 15% to 45% (Zobel, 1988). As a consequence of their crystallinity, most starch granules show a Maltese Cross when viewed with the polarized light microscope. Large hydrated starch granules show as concentric growth rings, which are layers of alternating high and low refractive index (French, 1984). At the center of the granule is the original growing point, the hilum, which is usually less structurally organized than the rest of the granule. Based on X-ray diffraction patterns, cereal starches and small granules of some tuber starches were classified as A-type; most tuber starches were classified as B-type; while other tuber starches and seed starches were designated as C-type.

Microscopic observation under polarized light provides more detailed information about the molecular organization of starch granules. The birefringent nature of starch indicates the high degree of molecular orientation within the granules. It was also suggested that the high refractive index is caused by double helix arrangement of molecular chains (Banks & Greenwood, 1975). At the dark cross, the molecular chains of granules are either parallel or perpendicular to the plane of polarization. It is accepted that the optic axis under polarized light coincides with the chain

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direction of the starch molecules, and is aligned perpendicularly to the growth ring (French, 1972). Observation of crushed potato starch granules also found a radial arrangement of crystallites (Hall & Sayre, 1970; Whelan, 1975).

Amylose, the linear component of starch, is heterogeneously distributed within the starch granule. The surface gelatinization technique has demonstrated that amylose molecules are concentrated more at the periphery than at the core of potato and maize starch granules (Jane & Shen, 1993; Pan & Jane, 2000). Atkin and colleagues also observed alternating layers of densely packed amylopectin and amylose in several starch granules having different amylose contents (Atkin, Cheng, Abeysekera, & Robards, 1999). The size of starch molecules is also not uniformly distributed in starch granules. The molecular size of amylose was found to be larger at the core (around the hilum) than that located at the periphery, and amylopectin had longer B-chains at the core than at the periphery (Jane & Shen, 1993; Pan & Jane, 2000). These studies have concluded that the starch molecules are not homogeneously distributed in the individual starch granule.

The presence of pores and channels attests to the anatomical heterogeneity of starch granules. The number and type of pores and channels are characteristic of starch from different sources (Fannon, Hauber, & BeMiller, 1992; Fannon, Shull, & BeMiller, 1993; Huber & BeMiller, 1997, 2000). The surface pores of starch are connected to the central cavity of granules through narrow channels (Gray & BeMiller, 2004, 2005; Huber & BeMiller, 2000, 2001). Water-soluble dyes and chemical reagents can penetrate to the central cavity by passing through the channels, and then quickly diffuse to the starch matrix from the hilum, which is the less organized area (Gallant & Guibot, 1969; Gallant & Guilbot, 1969). For starches where no such channels have been found, such as potato starch, chemical reactions mostly occur on the granular surface (Gray & BeMiller, 2004). For different reagents, chemical reactions take place in different regions of the starch granule (Gray & BeMiller, 2004; Huber & BeMiller, 2001). Fast reacting chemicals only interact with the surface of granule and inner cavity. By contrast, slow reacting chemicals can diffuse into the starch matrix and cause reactions to occur there. These studies have demonstrated the non-uniform nature of chemical reactivity in different regions of the starch granule.

The starch granules of lotus (*Nelumbo nucifera* Gaerth) rhizome are mostly elongated and oval in shape, being 20–40 μm across and 20–90 μm long (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994; Sung, Chang, Yang, & Lii, 1978; Suzuki et al., 1992). The hilum is not central, which is also the case in potato and Shoti starch (Banks & Greenwood, 1975), but is located near one end of the granule (Lii & Lee, 1993; Sung et al., 1978). The surface heterogeneity of starch granules – that is, dents, hollows, surface striations or irregular shape at one end of the elongated granules – has been reported (Jane et al., 1994; Sung et al., 1978; Suzuki et al., 1992). Some small, round and irregularly shaped granules have also been observed in other studies. The growth ring stripes are more obvious in granules that were partially degraded with amylase (Fuwa, Sugimoto, & Takaya, 1979). Lotus rhizome starch granules have a characteristic morphology after enzyme hydrolysis that can be applied to orient the granule. In this study, we investigated the structure of lotus starch granules that had been partially digested with bacterial α-amylase. The heterogeneity of enzyme susceptibility in the starch granule was revealed.

2. Materials and methods

2.1. Starch preparation

Lotus (*N. nucifera* Gaertn. Var. Dahou) rhizomes were harvested in Taiwan. The starches were isolated using an alkaline wet-milling process (Chang, Lii, & Yang, 1991). The chopped rhizomes were blended with cold diluted alkali solution (0.1% sodium hydroxide) in a blender. The ground slurry was filtered through a nylon screen (100 μm mesh). The pulp was ground twice more to facilitate the release of starch granules from the fibers. The starch slurry was repeatedly washed with water and recovered using centrifugation at 3000*g*. The yellow, gummy material from the top of the starch cake was scraped off and discarded. The resulting white starch was washed once with ethanol and recovered by filtration with filter paper (Whatman No. 4, Whatman, UK) and dried at 40 °C.

2.2. Preparation of partially hydrolyzed starch granules

Partially degraded starch granules were prepared by α-amylase digestion according the method described by Leach and Schoch (1961). Starch (0.1 g) was suspended in 5 ml of 0.2 M phosphate buffer (pH 7.0) containing *Bacillus* sp. α-amylase (4 U/ml starch) (Sigma–Aldrich, MO, USA). Sodium azide was added to a concentration of 0.02% as preservative. The amylolysis was carried out in centrifuge tubes and incubated in a water bath with shaking at 37 °C. The hydrolysis was stopped by immersing the tubes in an ice-bath for 3 min. The residues were collected by centrifugation at 3000*g* for 10 min. The granular residue was washed three times with distilled water, and then washed with ethanol once before oven-drying at 40 °C. The hydrolysate collected following centrifugation was filtered through Whatman No. 4 filter paper, and then heated in boiling water for 10 min to de-activate the α-amylase. The concentration of carbohydrates released by α-amylase hydrolysis was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Light and polarizing light microscopy

A starch suspension (1.25% w/v) was prepared with 50% glycerol and a drop of 0.01 N iodine–potassium iodide

solution. A small drop of starch suspension was spotted on to a glass slide and covered with a cover-slip, the sides of which were sealed with glue. The starch granules were observed under the light microscope (Microflex FX- IIA, Nikon, Tokyo, Japan) equipped with the Diorama Color CCD camera (Diorama, Taipei, Taiwan).

2.4. Scanning electron microscopy

The starch granules were placed on an adhesive tape attached to a circular aluminum specimen stub. After coating vertically with gold–palladium, the samples were photographed at an accelerator potential of 15 kV using a scanning electron microscope (SEM, Hitachi S-2400, Japan).

2.5. Confocal laser scanning microscopy

Starch suspensions (1% w/v) were stained with Rhodamine B (0.01% w/v) in sodium azide solution (0.02%). Samples were stored at 28 °C for 24 h, then removed the dye solution by using centrifugation at 7000 rpm for 20 min. The starch granules were re-suspended in distilled water and centrifuged again immediately. The starch precipitate was re-suspended to 1% (w/v) in 50% glycerol. Samples were observed under a Leica TCS SP2 Confocal Spectral Microscope Imaging System (Leica Microsystems AG, Wetzlar, Germany) using the single-photon mode with He-Ne laser. The excitation wavelength was 543 nm, and emitted light between 550 and 700 nm was collected. The images were taken using Leica Confocal Software (Leica Microsystems AG, Wetzlar, Germany).

2.6. X-ray diffraction pattern

Starch samples were moistened by equilibrating them in a saturated humidity chamber for 1 day at room temperature. Starch X-ray diffraction was performed on a Siemens D-500 X-ray diffractometer (Siemens, Madison, WI) with Cu K_α radiation. The signal of reflection angle, 2θ, from 4 to 40 deg, was recorded. Other operations followed procedures described by Zobel (1964).

2.7. Debranched chain-length distribution

The debranched starch was prepared from dimethyl sulfoxide (DMSO) gelatinized starch using a debranching enzyme, isoamylase, from *Pseudomonas amyloderamosa* (crystalline grade, Hayashibara Biochemical Laboratories Ltd, Okayama, Japan). The chain-length distribution was determined by size-exclusion chromatography coupled with multi-angle laser-light-scattering (MALS) detection. The starch (50 mg, dry weight) was suspended in 5 ml water before adding 45 ml DMSO and heated with stirring in boiling water for 1 h. The starch solution was continuously stirred for 12 h at room temperature. The starch was precipitated by adding 100 ml alcohol and recovered by centrifugation at 3000*g* for 10 min. The starch precipitate was re-dissolved in 90% DMSO, heated with stirring in boiling water for 10 min and precipitated with ethanol before the debranching procedure. A starch solution (25 mg/2.4 ml water) was prepared from re-dissolving the precipitate in water. Concentrated acetate buffer and isoamylase were added to make the hydrolyzing solution, which contained 10 mg/ml of starch in 25 mM acetate buffer (pH 3.5), 0.02% sodium azide and 30 U isoamylase. The mixture was shaken in a water bath at 45 °C for 1 h; then extra isoamylase (30 U) was added to the mixture and the reaction continued for another 2 h. The solution was neutralized with 25 mM sodium hydroxide and heated in boiling water for 5 min to de-activate the enzyme. The debranched starch solution was diluted with deionized water to 5 ml, and filter with 0.45 μm syringe filter (PVDF, Millipore, MA, USA). The solution was deionized using a Vivapure® D mini L spin column (Vivascience-Sartorius AG, Goettingen, Germany) and centrifuging at 3000 rpm for 5 min.

The debranched starch solution (500 μl) was injected into a HPSEC-MALS-RI system, which consisted of high-performance size-exclusion chromatography (HPSEC) with MALS photometer (MALS, DAWN EOS, Wyatt Technology Inc., Santa Barbara, USA) and refractometer (RI, Optilab DSP, Wyatt Technology Inc., Santa Barbara, USA) as detectors. A series of columns of TSK-gel G3000PW_{XL}, and two of G2500PW_{XL} (Tosoh, Tokyo, Japan) were connected and maintained at 70°C. The columns were eluted with 0.3 N sodium nitrate with 0.02% sodium azide at 0.5 ml/min. The RI detector was maintained at 35 °C. The MALS photometer was calibrated with toluene, and the laser intensity of multi-angles was normalized with pullulan (P20, Shodex Showa Denko, Kavasaki, Japan).

3. Results

3.1. Morphology

Most of the collected lotus starch granules were elongated in shape or oval with relative smaller size. The sizes of elongated starch granules are about 15–20 μm across and 50–70 μm long (Figs. 1a–f). The hilum was clearly visible owing to its different refractive index under the light microscope (Fig. 1-c), and as the center of the Maltese Cross when viewed with the polarized light microscope (Fig. 1-d). The centrical stripes of the growth rings could be also clearly observed (Fig. 1-c). A dent was observed at the one end of the granule under the scanning electron microscope (Fig. 1-e). Under the confocal laser scanning microscope (CLSM), the Rhodamine B dye stained native granule showed brilliant color (Figs. 1-a and f). The center of granule was darker than periphery. Comparing the two ends of the granule, the hilum end showed brilliant color while the other end was not as dense showed less brilliant color. (Fig. 1-f). After partial digestion with α-amylase,

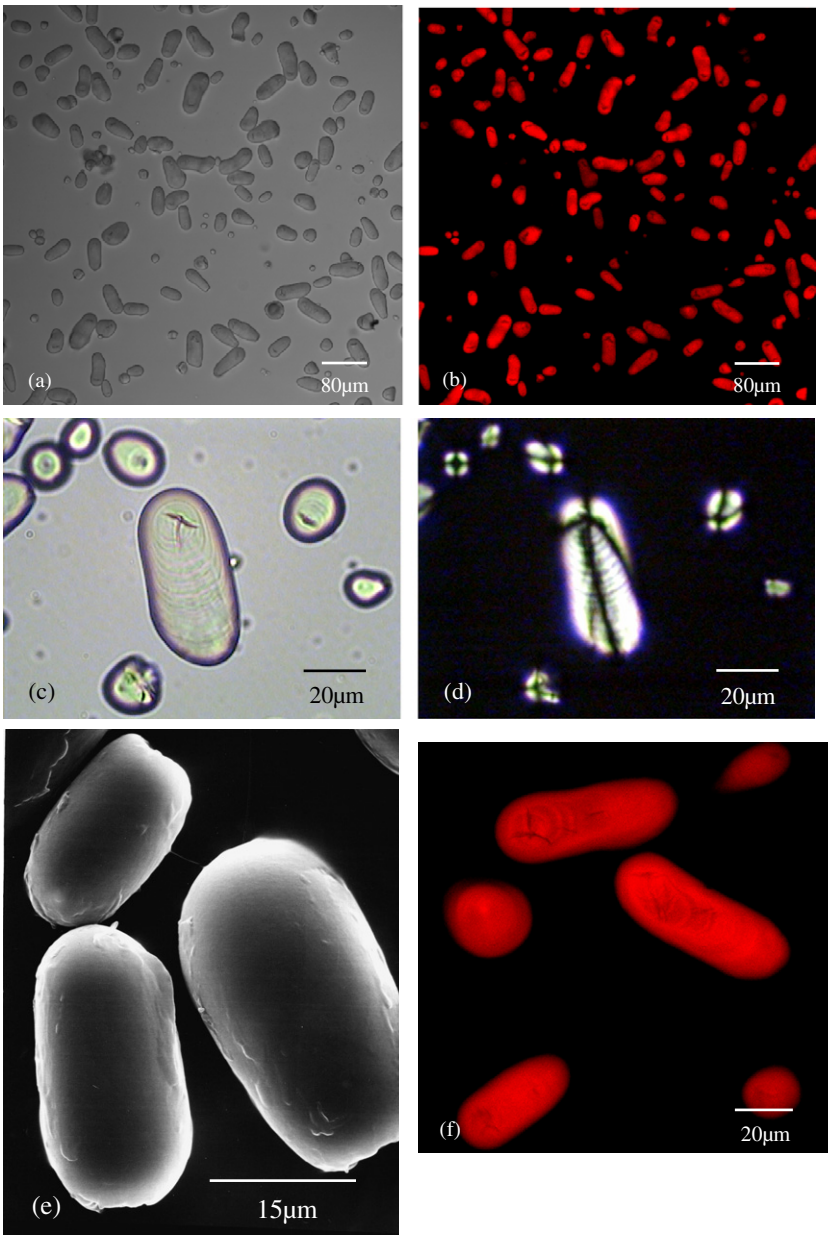

Fig. 1. Native lotus rhizome starch granules visualized by the light microscope (a, c), confocal laser scanning microscope (b, f), polarized light microscope (d) and scanning electron microscope (e).

only one end of the starch granule was attacked by α-amylase and formed a big hole. However, the other end that was near the hilum remained intact, even when the average hydrolysis ratio reached 50% of the weight of the starch granules. Thus the two ends of the starch granules showed different susceptibility to the α-amylase (Fig. 2).

The partially digested granule could be categorized into three parts under the light microscope (Figs. 2-a and b). The first part was where the α-amylase attacked the granule and formed a big hole. It was observed that this end lost its dye-binding capacity under the CLSM (Fig. 2-e), and also lost birefringence under the polarized light microscope (Fig. 2-b). The second part was the central tunnel area, which had a fish-bone-like shape in the core of the starch granule. The third part was the intact regions, which included the hilum end and the periphery. The remaining granules still showed strong birefringence in this region (Fig. 2-b), even when the granule was 50% hydrolyzed (picture not shown), which implied that a high degree of molecular organization still existed.

After partial digestion by α-amylase, only one end of the lotus starch granule was significantly degraded and the other end remained intact (Fig. 2). From the evidence provided by polarized light microscopy and light microscopy, the α-amylase degraded the granule from the end opposite to the hilum location; however, the hilum end was intact (at 18% degraded sample) (Figs. 2-b and e). At the degraded end, penetration was apparent and formed a single opening (Fig. 2-c) at the dented end. In addition to degrading from specific end, the α-amylase burrowed along a unique route

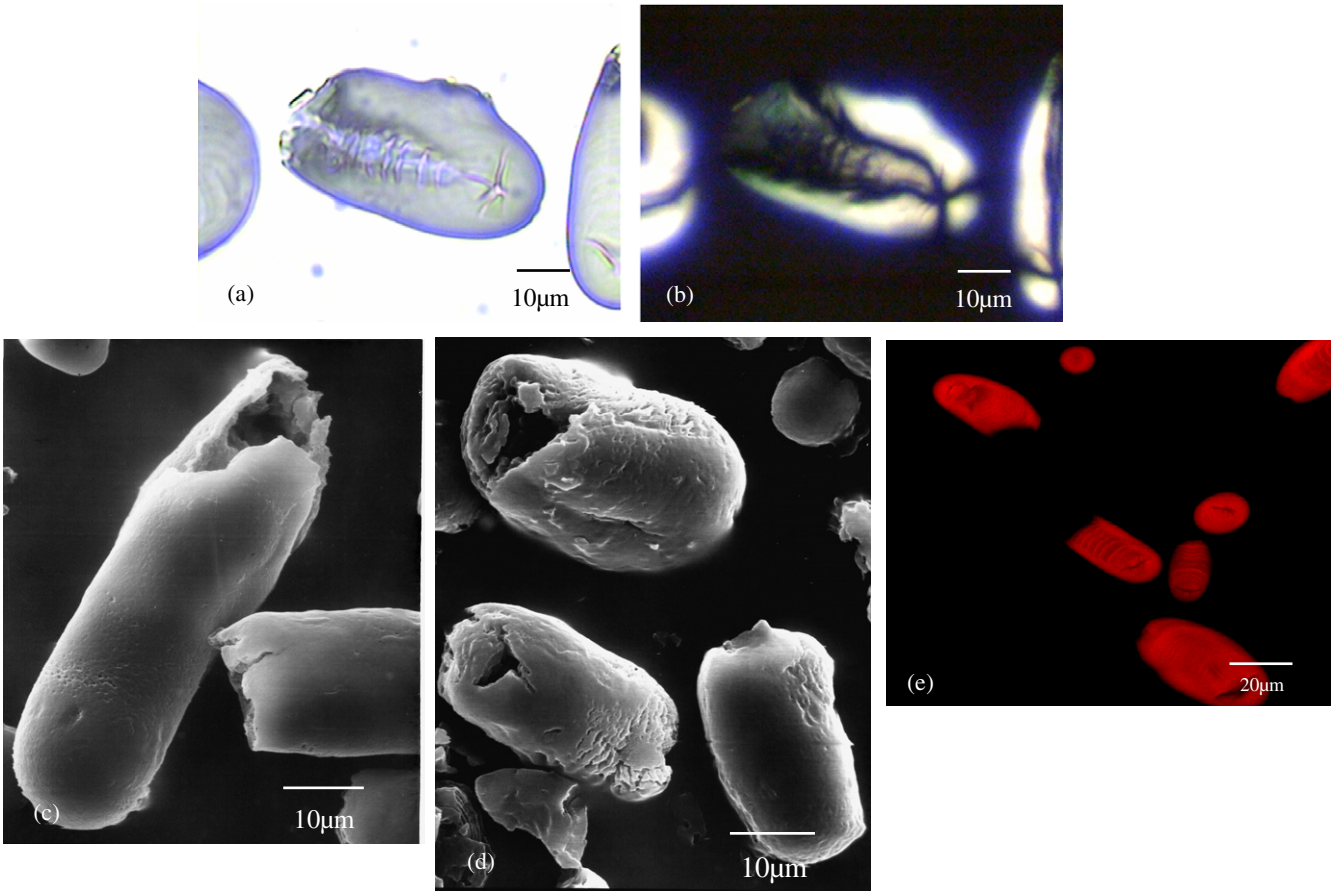

Fig. 2. Partially digested lotus rhizome starch granules visualized by the common light (a), polarized light (b), scanning electron (c, d) and confocal laser scanning microscopes (e). The granules were collected from α-amylase digested samples in which the average degree of digestion was 18% (a, b, c, e) or 50% (d).

into the granule. The α-amylase dug a fish-bone-like channel with a big opening (Figs. 2-a and b). Shells were observed along the channel under the polarized light microscope. Near the opening at the degraded end, the regions around the channel were darker under the light microscope. The birefringence was disturbed in this region when viewed with the polarized light microscope (Figs. 2-a and b).

3.2. X-ray diffraction pattern

The X-ray diffraction pattern of native lotus rhizome starch was of the C-type (Fig. 3). After partial digestion with α-amylase, the intensity decreased. The X-ray pattern remained in similar pattern and only slightly changed towards C_b-type characteristics by showing decrease of the shoulder at 18°, and separation of the peaks at 22–24° of 2θ degree.

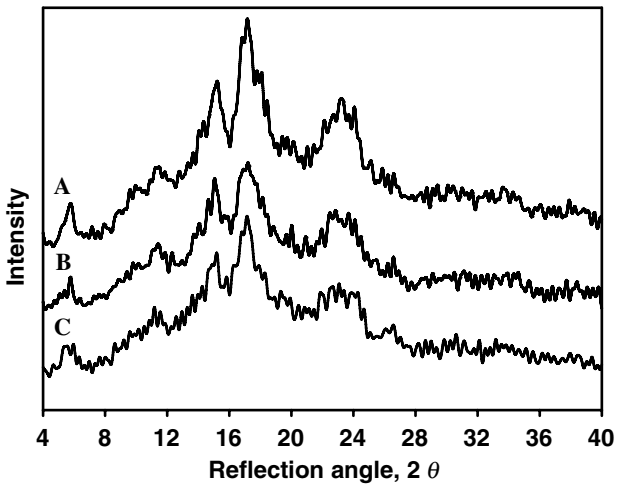

Fig. 3. X-ray diffractograms of native (A), 24% (B) and 69% (C) α-amylase digested starch granules.

3.3. Chain-length distribution

The high-performance size-exclusion chromatogram of debranched starch showed three different chain-length fractions (peaks) designated as F1, F2, and F3, respectively, according to the eluting order (Fig. 4). F1 contained debranched amylose and possible existed extra-long-chains; F2 and F3 contained long- and short-chains of amylopectin molecules. The debranched chain-length distribution of native starch granules and partially digested remaining

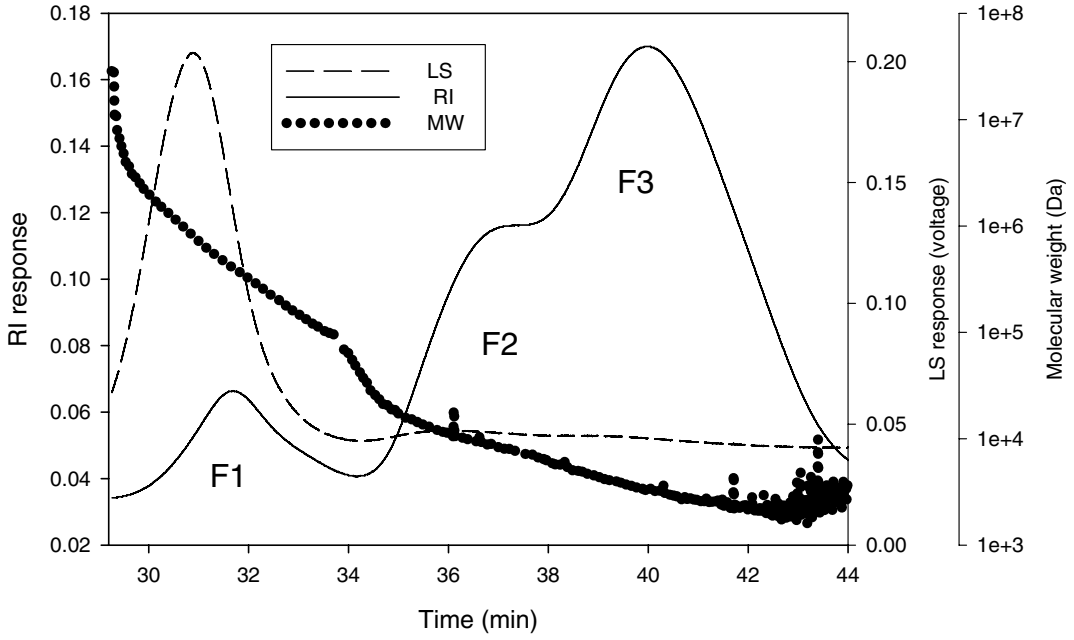

Fig. 4. High-performance size-exclusion chromatogram of debranched lotus rhizome starch detected by both multi-angle laser light scattering (LS) and refractive index (RI).

showed that the mass ratio of amylose (F1), long-chains (F2), and short-chains (F3) of amylopectin molecules were not obviously changed as the digestion degree increasing (Fig. 5). It demonstrated that both amylose and amylopectin were digested by α-amylase at the similar ratio. The amylose molecules had an obvious decrease of molecular weight (Fig. 6), the long-chains of amylopectin also showed slight decrease as the digestion degree increasing. The results demonstrated that both amylose and amylopectin were digested by α-amylase at similar ratios. The amylose molecules had obviously decreased in molecular weight (Fig. 6); the long-chains amylopectin also showed a slight decrease as the degree of hydrolysis increased. Amylose was suggested to distribute among the clusters of amylopectin branches. As the hydrolysis took place on the cluster, a portion of amylose molecules was digested at the same time as the cluster lamella. Amylopectin long-chains connecting clusters were also gradually digested with cluster lamella. Amylopectin short-chains were protected with-

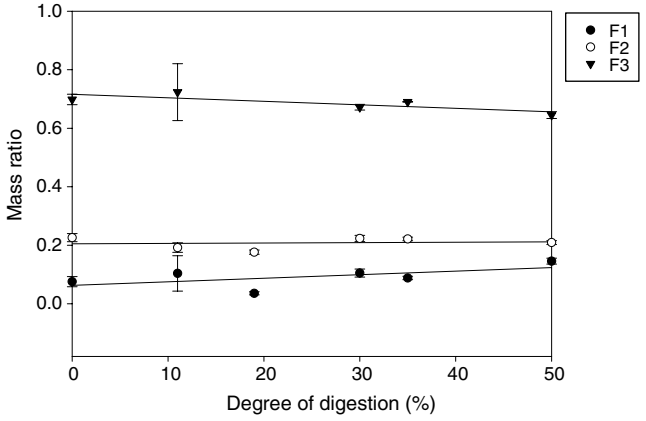

Fig. 5. Changes of mass ratio of three fractions of debranched lotus rhizome starch with different degrees of α-amylase digestion.

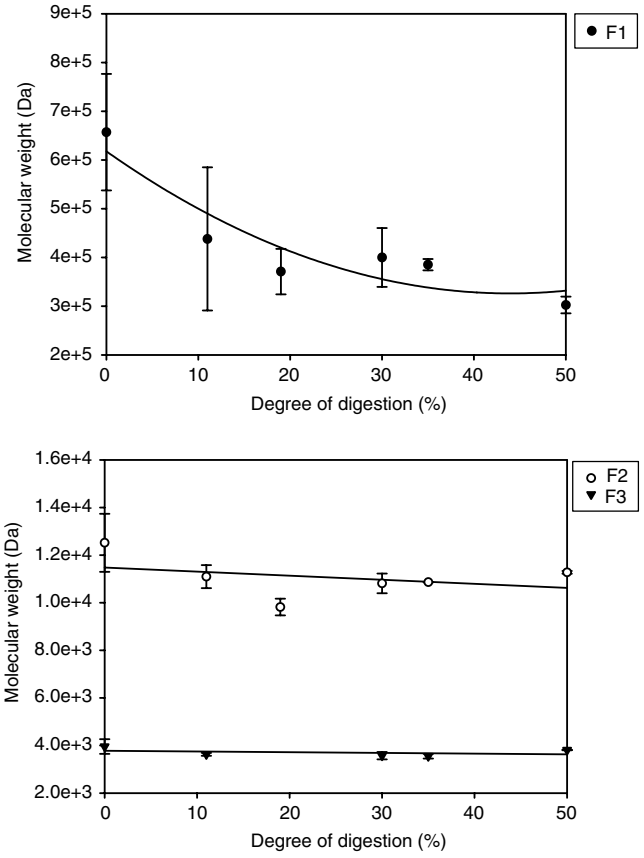

Fig. 6. Average molecular weight of three fractions of debranched lotus rhizome starch with different degrees of α-amylase digestion.

in a cluster and consequently changed little in molecular weight.

4. Discussion

Using the hilum location on the granule as an orientation-identifying anchor, it was revealed in lotus rhizome starch that α-amylase selectively attacked the starch granule at one end, which was distant from hilum. This enzyme-attacked end was the vulnerable point for enzyme digestion, and had loosely packed molecular organization, as shown by less dye-binding capacity under the CLSM. The central portion of the starch granule also showed a loose molecular organization, which had greater susceptibility to α-amylase and left a fish-bone-shaped tunnel when viewed under the light microscope. The amylase susceptible and resistant regions did not have composition and crystalline type difference. The α-amylase digestion did not significantly change the chemical composition of starch granules remaining, which showed similar mass ratio of amylopectin and amylose. The X-ray diffraction pattern was not obviously changed either after high degree of digestion. These results indicated that the molecular organization in an individual lotus rhizome starch granule is heterogeneous.

This heterogeneity of lotus rhizome starch granules was also observed by thermal transition research published by Lii and Lee (1993), who showed that lotus starch had two endotherm peaks in salt solution. One end of lotus starch granule was swollen first when the temperature achieved the first endotherm. At the same time, the other end was still intact as shown by its birefringence. In our study, it was observed that the α-amylase attacked the lotus starch granule at a specific point and degraded a specific region. Only one end of the lotus starch granule was attacked by amylase, and the other end was still intact when the starch granule was partially digested. The CLSM result provided direct evidence of the relationship between molecular organization and amylase digestion. From the outcome of CLSM study of native lotus starch, we found that the hilum end showed brilliant red color; however, the α-amylase-attacked end was not as dense as hilum end and periphery. The α-amylase-attacked end had weaker binding capacity towards dye compared with the intact regions, which means that the α-amylase-attacked end had a loosely packed organization. This explains the relationship between the amylase digesting pattern and granule organization. Koch and Jane (2000) demonstrated that the salt gelatinization of starch granules with calcium chloride seemed to take place at sites similar to those for amylase attack on starch granules (Koch & Jane, 2000). It is plausible that the loosely packed organization was a weak point for both enzymatic attack and chemical gelatinization.

Lii and Lee (1993) suggested that the lotus starch granule, which has C-type X-ray diffraction pattern, consists of A-type and B-type polymorphism within the granule. This hypothesis was also proved by a differential scanning calorimetric experiment on pea starch, another C-type starch, which had a mixture of A- and B-type polymorphs (Bogracheva, Morris, Ring, & Hedley, 1998). The synchrotron microfocus mapping of pea starch also showed two phases of crystallites existing in an individual granule (Buleon, Gerard, Riekel, Vuong, & Chanzy, 1998). Lii and Lee (1993) proposed that the double helices of B-type crystallites are parallel-stranded and arranged in a hexagonal array with more water in the center; and A-type crystalline was more compact and more resistant to the gelatinization effect of the chloride ion (Imberty & Perez, 1988; Lii & Lee, 1993). However, our X-ray results demonstrated that α-amylase did not have obviously selective digestion between A- and B-type polymorphs in the lotus starch granule, and maintained the C-type pattern after partial digestion. These results indicated that the crystallite polymorphs in loosely and densely packed regions did not have significantly different.

Most A-type starch had higher susceptibility to enzyme hydrolysis compared with B-type starches (Jane et al., 2003). A-type crystallite derived from lintnerization also had greater susceptibility to enzyme hydrolysis than the B-type one (Planchot, Colonna, & Buleon, 1997). Gerard, Colonna, Buleon, and Planchot (2001) also suggested that distribution of B-type crystallites within the granules was related to enzyme resistance (Gerard et al., 2001). In 69% digested lotus starch granules, the C-type pattern only slightly lost A-polymorph characteristics. The A-polymorph and B-polymorph ratio was maintained because of the barrier presented by densely packed crystalline clusters. The reason for low selectivity of A-polymorph crystallites of α-amylase digestion might be due to the difficulty of enzyme penetration to the starch matrix. The α-amylase digested the granule layer by layer of and resulted in a decreasing X-ray intensity. From the chain-length distribution analysis, the starch molecules were maintained at a similar mass ratio after partial digestion. This also demonstrates that the α-amylase did not have obvious selectivity among starch molecules in lotus starch granule. Therefore, we concluded that amylase attacked at specific points and regions in the granule were dependent on the molecular organization in granule.

In the core of lotus starch granule, α-amylase hydrolyzed the central region and left a fish-bone-like tunnel. It demonstrated that the amylase hydrolyzed the loosely packed region faster than the densely packed one. The surrounding periphery had higher resistance to α-amylolysis. Valetudie, Colonna, Bouchet, and Gallant (1993) have shown similar results: the α-amylase enters the granules and hydrolyzes the interior of the granules and leaves the shells and internal layers (Valetudie et al., 1993). Gallant, Bouchet, and Baldwin (1997) hypothesized that the hydrolysis rate was dependent on the different layers distribution, size and interaction with non-starch constituents (Gallant et al., 1997). They found B- and C-type starch had larger blockets compared with A-type starch (Baldwin, Adler, Davies, & Melia, 1998; Gallant et al., 1997; Gallant, Bouchet, Buleon, & Perez, 1992). In potato starch, there were large blocklets arranged near the surface of the granule.

This may have contributed to the resistance of enzyme hydrolysis on the periphery.

From the viewpoint of starch synthesis, it was accepted that the starch molecules were synthesized in the amyloplast stroma and then deposited on the growing surface of the starch granule (French, 1984). French (1984) proposed that the granule grows mainly by elongation of the chains already incorporated into the granule structure from the growing point, the hilum, with simultaneous elongation and crystallization (French, 1984). French also suggested that the optic axis under the polarized light microscope coincides with the chain direction of the starch. We hypothesize that the end of molecular elongation is the loosely packed region, which results in greater susceptibility to hydrolysis and chemical gelatinization in the case of the elongated lotus starch granule. The hilum-end, begin of molecular elongation, was densely packed area, although the hilum was loosely packed. The hilum was embedded in a densely packed matrix and remained intact, even in granules that were subjected to a greater degree of digestion. The hilum-end was intact, too. This observation carries back the study of Buttrose (1960) on the large lenticular granules of wheat starch reviewed by Lineback and Rasper (1988). The equatorial surface, the edge of lenticular disc, is first attacked area by α-amylase and glucoamylase, when raw starch is digested by amylase in vitro or during germination. Starch granules normally grow by radial extension from the hilum. The development of lenticular starch granules of wheat is through a unique nucleus process (Evers, 1971). The arrangement of molecular chains is considered in perpendicular direction to the surface of both sides of the lenticular disc (French, 1972), based on the observation of that wheat lenticular granules show very weak Maltese Cross but when they are turned on edge, they become brilliantly birefrigent. At the edge of the lenticular disc (groove), the chain arrangement is loosely packed. Both amylase hydrolysis and chemical gelatinization take place from this weak point.

5. Conclusion

The molecular organization in the lotus rhizome starch granule was heterogeneous. The molecular arrangement was loosely packed at one end of the elongated and oval-shaped lotus starch granule, which was the end of molecular extension, and was the weak point to α-amylolysis and chemical gelatinization. The periphery region of the starch granule was more resistant to α-amylolysis, whereas the central part of starch granule had greater susceptibility. In the central part, the enzyme hydrolyzed the loosely packed region faster than densely packed region, and left a fish-bone-like tunnel. For the whole starch granule, the amorphous and crystalline regions were hydrolyzed at a similar ratio; amylose and amylopectin were also maintained at a similar ratio to the native starch. Amylase attacked the granule from the weak point and eroded the loosely packed regions within the granule.

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