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Structure and physical properties of gel prepared from α-amylase-treated cassava starch

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Takashi Ichihara
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ABBREVIATIONS

α-AMA  \(\alpha\)-amylose from *Aspergillus niger*

α-AMB  \(\alpha\)-amylose from *Bacillus amyloliquefaciens*

β-AMB  \(\beta\)-amylose from barley

\(\phi\)  Strain amplitude

\(\lambda\)  Wavelength of the incident beam

\(\lambda_{\text{max}}\)  Maximum absorption wavelength

\(\theta\)  Scattering angle

\(a\)  Measure of the extent of inhomogeneity

\(\degree\)  degree Celsius

CNP  2-chloro-4-nitrophenol

d\(_{jk}\)  Distance between the \(j\)th and \(k\)th atom

DP  degree of polymerization

\(f_i\)  Atomic scattering weight of atom \(j\)

\(G'\)  Storage modulus

\(G''\)  Loss modulus

\(g_j(q)\)  Form factor for a single atom

GPC  Gel permeation chromatography

HPAEC-PAD  High-performance anion-exchange chromatography with pulsed amperometric detection

\(I(q)\)  Scattering intensity

\(k_1, k_2, \text{ and } k_3\)  Adjustable parameters

\(Md\)  Molecular model of amylose double helix

\(Ms\)  Molecular model of amylose single chain
N3-G5-β-CNP  2-chloro-4-nitrophenyl
6⁵-azido-6⁵-deoxy-β-malopentaoside
PAS  Phosphate cross-linked cassava starch
       treated with α-amylase from Aspergillus niger
PS  Phosphate cross-linked cassava starch
q  Magnitude of the scattering vector
R_j  Van der Waals radius of the jth atom
SAXS  Small-angle X-ray scattering
SEM  Scanning electron microscope
tan δ  Dynamic mechanical loss tangent
(w/w)  weight:weight ratio
GENERAL INTRODUCTION

Starch is a major storage polysaccharide in plants where it occurs as insoluble and semi-crystalline granules made of two types of glucose polymer, amylose and amylopectin. Amylose is a linear molecule, consisting of α-(1,4)-linked D-glucopyranosyl units with a degree of polymerization (DP) in the range of 700–5,000 glucose units [1]. A fraction of the amylose molecules are slightly branched by α-(1,6)-linkages [2, 3]. Amylopectin is a highly branched polysaccharide with a DP in the range of 10^4 to 10^5 glucose units [1]. It is composed of chains of α-(1,4)-linked D-glucopyranosyl units which are interlinked by α-(1,6)-bonds. The amylose/amylopectin ratio differs between starches. For example, the amylose percentage of starch is in the range 20-36% for maize [4, 5], 18-23% for potatoes [6], 17-29% for wheat [4, 7] and 8-37% for rice [8-11]. Native starch is 20-40% crystalline [1], which is predominantly attributable to structural elements of amylopectin.

Starch granules consist of alternating layers of amorphous and crystalline domains [12]. The organization of amylose and amylopectin in the starch granule is not completely understood, but amylopectin side chains make up the framework of the crystalline lamellae, with branching points located in the amorphous domains [13-15], where amylose is also mainly localized [16]. When starch granules are heated in water to above a certain temperature (the gelatinization temperature), the granules will irreversibly swell and some of the amylose fraction will leach out. Upon cooling, the amylose in solution undergoes
a process called retrogradation. If the concentration is high enough, this process results in the formation of a network which turns the solution into a gel. Starch gel conforms to the filler-in-matrix model, where the filler is swelled starch granules and the matrix is the leached starch component from starch granules [17-19]. The matrix is mainly composed of amylose [20-22]. Starch retrogradation may be divided into two steps, short- and long-term. Short-term retrogradation seems to occur with gelation and crystallization of amylose [23, 24], whereas long-term retrogradation is attributed to amyllopectin [25].

Starch is an important renewable raw material used in various industries including foods, textiles, cosmetics, adhesives, paper, and pharmaceuticals. It is used either as extracted from plants (native starch), or after chemical or enzymatic modification. Chemical modification of starch is widespread in both the food and non-food sectors since it can alter the characteristics of starch for diverse applications. The most common chemical modification processes are acid treatment, cross-linking, oxidation and substitution, including esterification and etherification [26, 27]. In general, these chemical modifications are carried out on insoluble starch granules. Enzymatic treatment of starch is also common, usually not to alter its properties but to convert it into low molecular weight derivatives, eg glucose, fructose, maltose, maltodextrins and cyclodextrins. Enzymes used in starch processing are either hydrolytic enzymes (α-amylase, β-amylase, glucoamylase, α-glucosidase, isoamylase, pululanase) or transferases
(transglucosidase, cyclodextrin glucanotransferase, branching enzyme, amylomaltase) [28]. Enzymatic treatment is usually carried out on the gelatinized form of starch since these enzymes hardly affect insoluble starch granules.

Enzymatic treatment of insoluble starch granules at sub-gelatinization temperatures is now receiving increased attention. It is mainly used to convert starch into fermentable sugars. The process requires less energy than the conventional process requiring complete gelatinization, so it is especially important for low cost production of ethanol [29, 30].

Another application of the enzymatic treatment of insoluble starch granules is to produce granules with altered surface structure, especially microporous granules. The porosity and surface area of starch granules are potentially important characteristics for food texture [31, 32].

Enzymatic treatment of insoluble starch granules to alter the functionality of starch is an attractive area of research but there are only a limited number of studies available. The objectives of this would be to investigate the effect of enzyme treatment on insoluble starch granules and to evaluate the feasibility of this method to alter functionality of starch.

In chapter 1 we treat insoluble cassava starch granules with three enzymes, α-amylase from *Aspergillus niger* (α-AMA), α-amylase from *Bacillus amyloliquefaciens* (α-AMB) and β-amylase from barley (β-AMB), and examine the gelling properties of the residual starch granules.

In chapter 2 we study the mechanism underlying the enhanced gelling properties of Chapter 1 using small-angle X-ray
scattering (SAXS) and dynamic viscoelastic measurement, to understand the mechanism.

In chapter 3 the new enzymatic method developed in chapter 1 is combined with a conventional phosphate cross-linking method widely used to improve the gelling properties of starch.
CHAPTER 1

Limited hydrolysis of insoluble cassava starch granules results in enhanced gelling properties

1-1 Introduction

Enzymatic treatment of insoluble starch granules to alter the functionality of starch is an attractive area of research but there are only a limited number of studies available. In one relatively comprehensive study, four kinds of starch granules (corn, cassava, mung bean and sago) were treated with a commercial enzyme preparation containing α-amylase from Aspergillus kawachi and glucoamylase from A. niger. Their amylose content, X-ray diffraction pattern and pasting properties were then analyzed [33]. The amylose content of corn, cassava and mung bean starch decreased significantly but that of sago starch was unchanged. None of the four starches changed their X-ray diffraction type, but corn, mung bean and sago increased in relative crystallinity value (cassava remained the same). The peak viscosity (measured with a Rapid Visco Analyzer) of corn and cassava starch decreased but that of sago and mung bean starch increased. A similar study was carried out on cassava, sweet potato, Peruvian carrot and potato starch granules treated with α-amylase from Bacillus sp [34]. The amylose content of
the first three decreased but that of potato starch was unchanged. The intrinsic and peak viscosities of the pasting pattern (measured with a Rapid Visco Analyzer) decreased in all four hydrolyzed starches. As observed in these two studies, the effects of enzyme treatment on starch granules seem to depend on the botanical source of the starch, extent of hydrolysis and enzyme used. Retrogradation and gel formation are the most important properties of starch in the food sector, but the gelling properties of enzyme-treated starch granules have not been studied.

In this chapter, I treated insoluble cassava starch granules with 3 enzymes, \( \alpha \)-amylase from \( A. \ niger \) (\( \alpha \)-AMA), \( \alpha \)-amylase from \( B. \ amyloliquefaciens \) (\( \alpha \)-AMB) and \( \beta \)-amylase from barley (\( \beta \)-AMB), and examined the gelling properties of the residual starch granules. Surprisingly, starch granules treated with \( \alpha \)-AMA produced starch gel with significantly enhanced hardness and elasticity. The mechanism behind this observation is investigated and discussed based on the filler in matrix model [17-19] of starch gel.

1-2 Materials and Methods

1-2-1 Materials

Native cassava starch was purchased from Vedan Vietnam Enterprise Co., Ltd. (Dong Nai Province, Vietnam). \( \alpha \)-amylase
(4,940 unit/g) from *A. niger* was provided by Shinnihon Chemicals Co., Ltd. (Aichi, Japan). α-amylase (10 unit/g) from *B. amyloliquefaciens* was provided by Novozymes Japan (Chiba, Japan). α-amylase activity was measured with an α-amylase Measuring Kit (Kikkoman Biochemifa Co., Ltd., Tokyo, Japan) using N3-G5-β-CNP as substrate. One unit of α-amylase activity was defined as the amount of enzyme producing 1 μmol of CNP for one minute under the assay conditions [35]. β-amylase from barley (OPTIMALT BBA, 1230 DP°/g) was purchased from Danisco Japan Ltd. Genencor Division (Tokyo, Japan) and used without any further treatment.

**1-2-2 Production of enzyme-treated starch granules**

Native cassava starch granules (300 g) were suspended in distilled water (700 g), regulated pH (pH 5.0) with 1 N HCl and incubated with or without enzyme (3 g) for 23 h at 50 °C with continuous agitation. To halt enzyme activity, 5 g of sodium hypochlorite solution (effective chlorine concentration: 10% (w/w)) was added to the starch suspension and the solution was stirred. After 10 min, disodium pyrosulfite (0.25 g) was added for neutralization and the solution was stirred for 10 min. The enzyme-treated starch granules were precipitated by centrifugation (3,000 rpm for 5 min). The amount of solubilized carbohydrates from the starch granules was determined by measuring the sugar content of the supernatant fraction by the phenol-sulfuric acid method [36]. The precipitate was re-suspended with distilled water then precipitated again by centrifugation. This washing process was repeated three times.
and the enzyme-treated starch granules were obtained by drying the washed pellets with a blow dryer.

1-2-3 Rheometric analysis of starch gel

The physical properties of the starch gel were analyzed using a rheometer (RT-2010J-CW, Rheotech, Tokyo, Japan). A starch suspension containing 20% (w/w) starch granules in distilled water was prepared, incubated at 65 °C for 10 min and placed in a polyvinylidene chloride tube. The tube was heated to 90 °C at 1 °C/min, held at 90 °C for 30 min, and kept for 1 h at 25 °C followed by 19 h at 5 °C. The starch gel formed in the casing tube was incubated at 25 °C for 4 h, sliced into gel disks 25 mm thick and loaded onto the stage of the rheometer. The rupture stress, rupture strain and Young's modulus were measured by the rheometer with an adapter (diameter 5 mm, area 19.635 mm²) using a movement speed of 6 cm/min.

1-2-4 Scanning electron microscope (SEM)

α-AMA-treated cassava starch granules were coated with Pt-Au using a Model E-1010 ion sputter coater (Hitachi Co., Ltd., Tokyo, Japan). The coated samples were then analyzed using a SU1510 SEM (Hitachi Co., Ltd., Tokyo, Japan) at an operating voltage of 10 kV.

1-2-5 Amylose content

The amylose content of the starch was determined by the methods of Hovenkamp-Hermelink et al [37]. 100 μl of starch solution in 90% (v/v) DMSO were mixed with 900 μl of distilled
water and vortexed, then mixed with 20 mL of iodine reagent. The absorbances at 618 and 550 nm were measured using a spectrophotometer (U-3900H, Hitachi Co., Ltd., Tokyo, Japan). Amylose content was calculated using the following equation.

Amylose (%) = (3.5 – 5.1 x R) / (10.4 x R – 19.9)
where R = A618 nm / A550 nm

Iodine reagent was made daily from 0.5 mL of iodine stock solution (0.26 g of I₂ and 2.6 g of KI in 10 mL of water) mixed with 0.5 mL of 1 N HCl and diluted to 130 mL with distilled water.

1-2-6 Analysis of the unit chain length distribution of starch

A starch sample (10 mg) was dissolved in 1,250 μl of 1 N NaOH and cooled to 5 ℃ for 20 h. After cooling, 5 μl of 5 N HCl, 5 μl of 1 M sodium acetate (pH 5.5) and 65 μl of distilled water were added to 25 μl of sample solution, and 2 μl of 0.11 mg/mL Pseudomonas isoamylase in 50 mM sodium acetate (pH 5.5) was added. The samples were incubated at 37 ℃ for 20 h, filtered through a 0.45 μm nylon filter and subjected to high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS-3000 (Dionex Corp., Sunnyvale, Calif.). A sample was eluted with a gradient of sodium acetate (0 – 2 min, 50 mM; 2 – 37 min, increasing from 50 mM to 350 mM with the installed gradient program 3; 37 – 45 min, increasing from 350 mM to 850 mM with the installed gradient program 7; 45 – 47 min, 850 mM) in 150 mM NaOH with a flow
rate of 1 mL min\(^{-1}\).

**1-2-7 Analysis of hot water extracts**

Each starch sample (200 mg) was dispersed in 20 mL of 0.1% AgNO\(_3\) and then incubated at 80 °C for 30 min. AgNO\(_3\) was used to inhibit \(\alpha\)-amylase activity [38]. The starch suspension was then centrifuged at 85 \(\times\) g for 3 min and the supernatant recovered for further analysis. A 2 mL aliquot of supernatant was loaded onto a Sepharose CL-2B (GE Healthcare, Buckinghamshire, U.K.) column (diameter 2.5 cm, length 45 cm) that had been equilibrated with 50 mmol/L sodium hydroxide containing 0.02% sodium azide and eluted with the same eluent at a flow rate of about 120 mL h\(^{-1}\) at room temperature. Fractions were taken at 100 drop intervals and the total carbohydrate content in each fraction was measured by the phenolic sulfuric method [36]. To learn the structure of the leached starch, the eluate in each fraction was mixed with iodine solution (6.0 mM KI, 0.4 mM I\(_2\)) and subjected to absorption spectral analysis.

**1-3 Results**

**1-3-1 Enzyme treatment of cassava starch granules**

Native cassava starch granules suspended in distilled water were incubated with three enzymes (\(\alpha\)-AMA, \(\alpha\)-AMB and \(\beta\)-AMB), and the amount of carbohydrates solubilized into the
water fraction was monitored along with the reaction time (Fig. 1). α-AMB most efficiently hydrolyzed insoluble starch granules, liberating 8.2% (w/w) of soluble sugars in the initial 15 min and more than 30% (w/w) in the subsequent 23 h. α-AMA and β-AMB liberated only 2.8% (w/w) and 0.5% (w/w) of soluble sugars, respectively, in 23 h. This result clearly indicates that the extent of hydrolysis of insoluble cassava starch granules depends on the enzyme used. Insoluble starch granules treated with α-AMA or β-AMB for 23 h or with α-AMB for 15 min were prepared as described in Materials and Methods, and were subjected to further studies. The extent of hydrolysis of α-AMA-, α-AMB- and β-AMB-treated cassava starch granules thus produced was 2.8% (w/w), 8.2% (w/w) and 0.5% (w/w), respectively.

1-3-2 Rheometric analysis of starch gel

When gelatinized starch paste is left at a low temperature, retrogradation occurs and the starch paste forms a gel. Gel formation is an important property of starch, especially in the food industry since the strength and hardness of starch gel affect the texture of foods. The three types of enzyme-treated starch granules described above were gelatinized with water and converted into gel disks as described in Materials and Methods. These gel disks were subjected to rheometric analysis to measure rupture stress, rupture strain and Young’s modulus. As shown in Table 1, the Young’s modulus of starch gels prepared from α-AMB- or β-AMB-treated starch granules was slightly smaller than that prepared from native cassava starch granules. These
results suggest that $\alpha$-AMB and $\beta$-AMB treatments slightly weaken the gelling properties of cassava starch granules. This is not surprising because hydrolysis of starch should have a negative effect on its gelling properties. On the other hand, the Young's modulus of the gel produced from $\alpha$-AMA-treated cassava starch granules was higher than that of the gel produced from native cassava starch granules. $\alpha$-AMA treatment appears to significantly affect the gelling properties of cassava starch granules, producing a hard and elastic starch gel. This is surprising since it contradicts the generally held view that partially hydrolyzed starch shows decreased gel-forming ability. These rheometric experiments indicate that limited hydrolysis of cassava starch granules to release only 2.8% (w/w) of solid matter into solution has a big impact on their gelling properties. It should also be noted that this is not true for all enzymes tested but only for one specific enzyme, $\alpha$-AMA.
Fig. 1. Time course of hydrolysis for different enzymes. Cassava starch granules were treated with $\alpha$-AMA (●), $\alpha$-AMB (▲) and $\beta$-AMB (□). The extent of hydrolysis is defined as the percentage of total carbohydrate in the reaction mixture that is solubilized.
Table 1. Rheometric properties of starch gel prepared from native and enzyme-treated cassava starches.

<table>
<thead>
<tr>
<th>Enzyme used</th>
<th>Rupture stress (g/cm²)</th>
<th>Rupture strain (%)</th>
<th>Young’s modulus (×10³ dyn/cm²)</th>
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<td>none</td>
<td>262.8 ± 21.9</td>
<td>49.6 ± 2.8</td>
<td>521 ± 44</td>
</tr>
<tr>
<td>α-AMA</td>
<td>404.4 ± 27.5</td>
<td>19.6 ± 1.2</td>
<td>2,040 ± 212</td>
</tr>
<tr>
<td>α-AMB</td>
<td>206.3 ± 3.6</td>
<td>42.8 ± 0.4</td>
<td>473 ± 11</td>
</tr>
<tr>
<td>β-AMB</td>
<td>173.7 ± 10.2</td>
<td>43.6 ± 6.0</td>
<td>399 ± 57</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of at least three measurements.

1-3-3 Analysis of α-AMA-treated cassava starch granules

In order to understand the mechanism underlying the unexpected gelling properties of α-AMA-treated cassava starch granules, several structural analyses of α-AMA-treated and native starch granules were carried out. However, I could not observe any difference between the two starches in scanning electron microscopy analysis (Fig. 2A), amylose content (Table 2), unit chain distribution of amyllopectin by HPAEC-PAD analysis (Fig. 2B) or amylograph analysis (data not shown). All these results indicate that α-AMA-treated and native cassava starch granules are very similar in granule morphology,
composition and structure of starch components and pasting properties, but different in gelling properties.

Table 2. Properties of native and α-AMA treated cassava starches.

<table>
<thead>
<tr>
<th>Enzyme used</th>
<th>Absorbance</th>
<th>Amylose content (%)</th>
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<tr>
<td></td>
<td>618 nm</td>
<td>550 nm</td>
</tr>
<tr>
<td>None</td>
<td>0.515 ± 0.005</td>
<td>0.507 ± 0.004</td>
</tr>
<tr>
<td>α-AMA</td>
<td>0.543 ± 0.007</td>
<td>0.531 ± 0.007</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (n = 3).
Fig. 2. Analysis of native and α-AMA-treated cassava starch granules.

(A) Morphology of native (a, b) and α-AMA-treated (c, d) cassava starch granules as observed by scanning electron microscopy. Left panels (a, c) are × 1,000 and right panels (b, d) are × 4,500. (B) Debranched unit chains from native (white) and α-AMA-treated (black) cassava starch are compared by displaying their respective percentages of total peak area.
1-3-4 Analysis of hot water extracts

The mechanism underlying the gelling properties of α-AMA-treated cassava starch granules can be understood in the context of the filler in the matrix model of starch gel [17-19], which tells us that matrix (leached material from starch granules) is an important factor determining the properties of the gel. I suspended α-AMA-treated and native starch granules in hot water and collected the leached starch component for analysis. Starch granules were suspended in water containing 0.1% (w/v) AgNO₃ as an anti-microbial agent, and incubated at 80 °C for 30 min. The leached starch component was collected as described in Materials and Methods and subjected to GPC analysis. The amount of carbohydrate and the λ_max of the starch-iodine complex of each fraction were measured and are summarized in Fig. 3.

The leached starch components collected from native and α-AMA-treated cassava starch granules showed wide molecular weight distribution as shown in Fig. 3A, and their elution profiles were similar. However, differences were found in the λ_max values of fractions 25 to 28, where very high molecular weight starch components are eluted (Fig. 3B). The λ_max values of fractions 25 to 28 for α-AMA-treated cassava starch granules were all higher than 600 nm but those for native cassava starch granules were around 600 nm or below.

The λ_max value of the amylose-iodine complex is known to be around 650 nm, whereas that of the amylopectin-iodine complex is much lower (around 550 nm). From these studies, I conclude that the leached starch components (matrix) collected
from native cassava starch granules contain high molecular weight amylopectin which is absent or much less in α-AMA-treated cassava starch granules, and that the matrix of native cassava starch granules and α-AMA-treated cassava starch granules are different, with the former composed of amylose with a wide molecular weight distribution and high molecular weight amylopectin, and the latter composed of amylose with much less amylopectin, or amylopectin with long side chains.
Fig. 3. Gel permeation chromatography of starch components leached from starch granules.

A starch (○, native cassava starch; ●, α-AMA-treated cassava starch) suspension in water was heated to 80 °C for 30 min and the leached starch component was fractionated using a Sepharose CL-2B column as described in “Analysis of hot water extracts” in the Experimental section. (A) The amount of carbohydrate in each fraction is displayed as a percentage of the total amount of carbohydrate eluted from the column. (B) $\lambda_{\text{max}}$ of the polysaccharide-iodine complex is shown. The value is the mean of three independent experiments.
1-4 Discussion

Enzymatic treatment of insoluble starch granules to alter their functionality is an attractive area of research. In the present study, I found that α-AMA-treated cassava starch granules can produce gel with greater hardness and elasticity. This is surprising because α-AMA treatment liberates only 2.8% (w/w) of the insoluble starch component into the soluble fraction, but has a big impact on gelling properties. It should be emphasized that this finding contradicts the generally held view that partially hydrolyzed starch produces gel with decreased viscosity and elasticity [39]. α-AMB- and β-AMB-treated starch granules obtained in this study both showed weakened gelling properties (Table 1), so the enhanced gelling properties of α-AMA-treated cassava starch granules may be specific to this particular enzyme. Further studies are necessary to address this issue.

In spite of many basic and applied studies of starch gel, our understanding of starch gel formation is still incomplete and varies depending on the type of starch and the conditions of formation. It is known that starch gel is a composite of filler (swelled starch granules) in a matrix (leached starch component) as proposed by Carnali [17] and subsequently confirmed by Goesaert [19]. When starch granules suspended in water are heated to temperatures under 100 °C, they swell and leach starch components (mainly amylose) resulting in the dispersion of swelled starch granules in water containing leached amylose. Subsequent cooling helps the leached amylose to form local
intra-molecular cross-links and a continuous macro gel (matrix) which includes swelled starch granules (filler), as schematically shown in Fig. 4A.

If starch gel is like the filler in a matrix model, the leached starch component should be an important factor determining the properties of the gel. I carefully collected the starch component leached from native and \( \alpha \)-AMA-treated cassava starch granules and analyzed its amount and composition with GPC. The component from native cassava starch granules contained amylose with a wide molecular weight distribution and high molecular weight amyllopectin; the component from \( \alpha \)-AMA-treated starch granules contained amylose with much less amyllopectin, or amyllopectin with long side chains (Fig. 3B).

This suggests that the matrix of native starch gel is composed of amylose and amyllopectin, while that of \( \alpha \)-AMA-treated starch gel is composed of amylose and less amyllopectin, as schematically summarized in Fig. 4B. I would like to conclude that the enhanced gelling properties of \( \alpha \)-AMA-treated cassava starch are produced by the strong matrix structure made from amylose.

Amylose is the minor component of starch and its content in the cassava starch granule varies between 17.9-23.6\% [40]. Amylose is the predominant starch component leached from the starch granule [41]. However, amyllopectin is also leached as I observed in the native cassava starch granule (Fig. 3B). It is thus very interesting why amyllopectin is not leached from \( \alpha \)-AMA-treated cassava starch granules. I cannot find any significant difference in amylose content or amyllopectin unit
chain distribution profiles when whole starch is subjected to analysis (Fig. 2B, Table 2). This means that the difference produced by α-AMA treatment occurs in a limited part of the starch granule. In this particular case, the limited part is the surface of the starch granule. α-AMA may preferentially hydrolyze amylopectin molecules at the surface and increase the local (surface) amylose content, or it may alter the surface structure for selective leaching of amylose. Our understanding of starch granules and the organization of amylose and amylopectin especially at the surface of the granule is not sufficient to address the mechanism of this interesting finding.

Our results suggest that enzymatic treatment of cassava starch granules can have a significant impact on the properties of the starch without changing the size or morphology of the starch granules. However, this may not be true of cereal starches. In the case of maize and rice, α-amylase treatment produces starch granules covered with holes [42]. These holes go toward the center of the granule and gradually break it [43]. Our α-AMA-treated cassava starch granules were smooth with no holes (Fig. 2A). This difference is of great interest and will be the subject of further studies that will provide a better understanding of the structure and organization of starch granules.

In conclusion, enzymatic treatment of insoluble starch granules at sub-gelatinization temperatures is potentially a novel and powerful tool to alter the properties of starch. The efficacy and feasibility of this approach should be examined for different enzymes and sources of starch.
Fig. 4. Schematic illustrations of the gelling process of (A) native and (B) α-AMA-treated cassava starch granules.
CHAPTER 2

Small-angle X-ray scattering measurements of gel produced from α-amylase-treated cassava starch granules

2-1 Introduction

In chapter 1, I reported the interesting and unexpected finding that cassava starch granules after very limited hydrolysis by α-AMA show significantly enhanced gelling properties. The α-AMA-treated cassava starch was produced by treating suspended insoluble native cassava starch granules with α-AMA to remove only 2.8% of the starch component into soluble fraction. In this chapter, α-AMA-treated cassava starch granules are subjected to dynamic viscoelastic measurement and small-angle X-ray scattering (SAXS) to understand the mechanism.

SAXS is a method for structural analysis of solutions and gels at the colloidal level and may help to understand the gelling process of starch. Starch has characteristic structures at different scales. At the nano level it has a lamella structure composed of amorphous and crystalline regions, the latter composed of double helical structures of branched amylopectin chains. It can be characterized by the position of an interference
peak appearing in SAXS. The distance between periodic alternating crystalline and and amorphous lamellae layers is around 9 nm [44]. The progress of gelatinization and retrogradation can be observed by means of time-resolved SAXS with synchrotron radiation [14, 45]. I analyzed the formation and deformation of the lamella and crystal structures. Gel formation of gelatinized starch paste is thought to occur with a complicated molecular association of starch components. A time-lapse SAXS analysis of the gelling process of starch would be interesting, but has not been carried out.

2-2 Materials and Methods

2-2-1 Materials

Native cassava starch was purchased from Vedan Vietnam Enterprise Co., Ltd. (Dong Nai Province, Vietnam). α-amylase (4,940 unit/g) from A. niger was provided by Shinnihon Chemicals Co., Ltd. (Aichi, Japan). α-amylase activity was measured with an α-amylase Measuring Kit (Kikkoman Biochemifa Co., Ltd., Tokyo, Japan) using N3-G5-β-CNP as substrate. One unit of α-amylase activity was defined as the amount of enzyme producing 1 μmol of CNP for one minute under the assay conditions [35].
2-2-2 Production of α-AMA-treated cassava starch granules

Native cassava starch granules (300 g) were suspended in distilled water (700 g), pH-regulated (pH 5.0) with 1 N HCl and incubated with α-amylase (3 g) for 23 h at 50 °C with continuous agitation. To halt enzyme activity, we added 5 g of sodium hypochlorite solution (effective chlorine concentration: 10% (w/w)) to the starch suspension and stirred. After 10 min, we added disodium pyrosulfite (0.25 g) for neutralization and stirred for 10 min. The enzyme-treated starch granules were precipitated by centrifugation (3,000 rpm for 5 min). The starch granules released 2.8% of their carbohydrate into soluble fraction (determined by measuring the sugar content of the supernatant fraction by the phenol-sulfuric acid method [36]). The precipitate was re-suspended with distilled water then precipitated again by centrifugation. This washing process was repeated three times and the α-AMA-treated cassava starch granules were obtained by drying the washed pellets with a blow dryer.

2-2-3 Dynamic viscoelastic measurement

The physical properties of the starch gel were studied using a dynamic rheometer (Physica MCR 301, Anton Paar GmbH, Graz, Austria) equipped with a parallel plate system (2.5 cm diameter). A starch suspension containing 20% (w/w) starch granules in distilled water was prepared, incubated at 65 °C for 10 min and gelatinized at 90 °C for 30 min. We placed the hot starch paste (2 ml) in a dynamic rheometer within the gap between a parallel plate and a base plate preset to 25 °C, and
formed gels by keeping it at 25 °C for 1 h followed by cooling at 5 °C for 19 h (1,140 min). The frequency sweep measurements of the storage modulus \( G' \) and loss modulus \( G'' \) were made at 5 °C over the angular frequency range of 0.1 to 10 rad/s. The strain amplitude \( (\varphi) \) was 0.1% which was selected in the linear viscoelastic region for the samples, according to the results of strain sweep tests performed at frequencies of 0.1, 1 and 10 rad/s (data not shown).

2-2-4 Small-angle X-ray scattering

SAXS was carried out at the experimental station BL40B2 of the synchrotron radiation facility, SPring-8, located in Hyogo Prefecture, Japan. The sample was irradiated with an incident X-ray beam with a wavelength of 0.1 nm, and the scattered X-ray was detected with an imaging plate after passing through a vacuum chamber of about 1 m. The two-dimensional data obtained was transformed to one dimensional data by circular averaging. A starch suspension containing 20% (w/w) starch granules in distilled water was prepared and incubated at 60 °C for 10 min, and then at 90 °C for 30 min. The gelatinized starch paste was loaded into the cell for time-resolved SAXS measurements. Flat sample cells of 2 mm in path length were made of stainless steel with mica windows. Except for measurement time, they were kept at room temperature for the first 1 h, then at 5 °C in a refrigerator.
2-3 Results

2-3-1 Dynamic rheologic properties

In order to understand the difference between native and α-AMA-treated cassava starch granules, two starch samples were first subjected to dynamic viscoelastic analysis.

As shown in Fig. 5a, the $G'$ of native cassava starch paste measured after incubation for 19 h (1,140 min) at 5 °C increased with increasing angular frequency, while that of α-AMA-treated cassava starch paste changed little. This indicates that in this relatively short period of time α-AMA-treated cassava starch paste can form a stronger gel than native cassava starch. The $G'$ of α-AMA-treated cassava starch paste was about twice as large as that of native cassava starch, indicating that α-AMA treatment increased the elasticity of the native starch. As shown in Fig. 5b, the $G''$ of native starch increased markedly with increasing angular frequency, while that of α-AMA-treated cassava starch gently increased. The dynamic mechanical loss tangent values ($\tan \delta = G''/G'$), are shown in Fig. 5c. The $\tan \delta$ of α-AMA-treated cassava starch paste was lower (at any angular frequency tested) than that of native cassava starch paste, and did not change with increasing angular frequency. This suggests that α-AMA-treated cassava starch granules behave like solid particles in the paste. All these results support our previous finding that limited hydrolysis of native cassava starch with α-AMA increases the gel-forming ability of the cassava starch paste and gives the gel more elasticity.
Fig. 5. Angular frequency-dependent change of (A) $G'$, (B) $G''$, and (C) tan $\delta$ of 20% (w/w) starch paste prepared from native (○) and α-AMA-treated (●) cassava starch, held at 5°C for 19 h.
2-3-2 Small-angle X-ray scattering

I used SAXS to investigate the gelling and subsequent retrogradation process of native and \( \alpha \)-AMA-treated cassava starch granules. The 20% (w/w) starch paste was heated to 90 °C, and then injected into a flat sample cell of 2 mm in path length made of stainless steel with mica windows. Samples were kept at room temperature for the first hour, then at 5 °C in a refrigerator. For SAXS measurements sample cells were taken from the refrigerator and returned immediately after each measurement.

Fig. 6 shows Kratky plots of \( q^2 I(q) \) vs \( q \), where \( I(q) \) is the scattering intensity and \( q \) is the magnitude of the scattering vector defined by \( (4\pi/\lambda)\sin(\theta/2) \) with \( \lambda \) the wavelength of the incident beam and \( \theta \) the scattering angle. All profiles for both kinds of starches give broad maxima around \( q \approx 2.3 \) and upturned tails in the higher \( q \) region, reflecting the properties of amylose chain conformation [46]. A definite peak appeared in the smaller \( q \) region (\( q < 1 \)) due to aggregation. This peak for \( \alpha \)-AMA-treated cassava starch gel was different from that for native cassava starch gel. The small but sharp peak detected at \( q = 4 \), during retrogradation, was due to the formation of the crystal structure of the amylose chain corresponding to type-B [47]. In order to investigate the appearance of peak around \( q = 4 \), the SAXS profile is enlarged and shown in Fig. 7, respectively. In native cassava starch gel, peak around \( q = 4 \) appeared at 780 min. On the other hand, the peak is appeared at 120 min in \( \alpha \)-AMA-treated cassava starch gel which is much faster than native cassava starch gel. This shows that \( \alpha \)-AMA-treated cassava starch gel crystallizes faster than native cassava starch gel.
Fig. 6. Time-resolved SAXS profiles during gelation and retrogradation. (A) native cassava starch and (B) α-AMA-treated cassava starch. Scattering data are shown as Kratky plots ($q^2I(q)$ vs $q$). Gray and black bold lines represent data corresponding to 0 min and 1,140 min, respectively. Dashed lines represent data corresponding to 90, 420 and 780 min.

Fig. 7. The growth of diffraction peak around $q=4$ as Kratky plots. (A) native cassava starch and (B) α-AMA-treated cassava starch. The arrows mark the diffraction peak around $q=4$. 
Fig. 8. Molecular models of amylose. (A) Double helix and (B) single coil generated by the Monte Carlo method [49].

In order to analyze the scattering from the amylose chain in the solvated region, I used molecular models with a double helical structure [48] and a simulated amylose single chain produced by the Monte Carlo method [49] (see Fig. 8). The scattering of the molecular model can be calculated with the Debye formula [50] as follows.

\[ I(q) = \sum_{j=1}^{n} f_i^2 g_j^2(q) + 2 \sum_{j=1}^{n} \sum_{k=j+1}^{n} f_j f_k g_j(q) g_k(q) \cdot \frac{\sin d_{jk}q}{d_{jk}q} \]  

(1)

where \( f_i \) and \( d_{jk} \) are the atomic scattering weight of atom \( j \) and the distance between the \( j \)th and \( k \)th atom, respectively. The form factor \( g_j(q) \) for a single atom is represented by a rigid sphere with a radius equal to the van der Waals radius, \( R_j \), of the \( j \)th atom.

\[ g_j = \frac{3[\sin(R_jq)-(R_jq)\cos(R_jq)]}{(R_jq)^3} \]  

(2)

The experimental SAXS curve except for the low \( q \) region can be explained as a linear sum of the scattering calculated from a
single amylose chain and that of the double helix.

The peaks in the smaller angle region ($q<1$) seem to correspond to the growth of aggregation during gelation and retrogradation. This can be simulated with a Debye-Bueche type scattering function [51] from a two-phase random aggregation model as

$$I_{DB}(q) \approx \frac{1}{(1+a^2q^2)^2}$$  \hspace{1cm} (3)

where $a$ is the extent of inhomogeneity. The experimental scattering profile was fitted with the sum of a scattering from molecular models and a Debye-Bueche type scattering function as follows.

$$I(q) \approx \{k_1I_{Ms}(q)+k_2I_{Md}(q)\}+k_3I_{DB}(q)$$  \hspace{1cm} (4)

where the term in curly braces is the scattering from molecular models. Here $I_{Ms}(q)$ and $I_{Md}(q)$ are from the amylose single chain and double helix, respectively, and $k_1$, $k_2$, and $k_3$ are the adjustable parameters. The fitted curves are shown as solid lines in Fig. 9. In addition, $a$ is plotted as a function of time in Fig. 10. For native cassava starch gel, $a$ increased from 1.8 to 2.6 nm. For $\alpha$-AMA-treated cassava starch gel, it increased from 2.2 to 3.5 nm, indicating a larger size of aggregation for $\alpha$-AMA-treated cassava starch paste.
**Fig. 9.** Kratky plots for SAXS of α-AMA-treated cassava starch left for 15 min (A) and 1,140 min (B) after putting gelatinized starch paste in the cell. The simulated scattering curves are also shown.
Fig. 10. Time course of the $a$ value, the measure of the extent of inhomogeneity, for native cassava starch (white dot) and $\alpha$-AMA-treated cassava starch (black dot). The arrows mark the appearance of the diffraction peak around $q=4$ as Kratky plots.

2-4 Discussion

I reported in chapter 1 that cassava starch granules show significantly enhanced gelling properties after very limited hydrolysis by $\alpha$-AMA. Starch granules before and after $\alpha$-AMA treatment were carefully compared but no significant difference in surface morphology, amylose content or unit chain distribution of amylpectin was observed. However, GPC analysis showed that leached starch components from native
cassava granules contained amylose and high molecular weight amylopectin, while components from α-AMA-treated cassava starch granules contained amylose but little or no amylopectin. I proposed a hypothesis in chapter 1 that α-AMA-treated cassava starch granules form a stronger gel because the gel matrix is composed of amylose with decreased levels of amylopectin. I examined α-AMA-treated cassava starch granules using two experimental methods in order to understand the mechanism of these enhanced gelling properties.

I subjected native and α-AMA-treated cassava starch granules to dynamic viscoelastic analysis. $G'$ was higher than $G''$ for both native and α-AMA-treated cassava starch paste, regardless of angular frequency, indicating that both starches formed gel. The $G'$ of α-AMA-treated cassava starch paste was greater than that of native cassava starch paste at any angular frequency. The dynamic mechanical loss tangent values ($\tan \delta = G''/G'$) of α-AMA-treated cassava starch paste were almost zero for the angular frequencies tested and were much lower than those of native cassava starch paste. This suggests that α-AMA treatment doesn't affect viscosity but has a significant effect on elasticity. The resulting starch paste appeared to behave like an elastic body. These results demonstrate that α-AMA treatment can enhance elasticity, probably by forming a strong three dimensional network of amylose [52] during the gelling process.

Understanding the starch gelling process is scientifically and practically important. However, methods for analyzing the structural characteristics of starch gel formation are limited, and time-resolved small-angle X-ray scattering method is a
promising tool to observe the gelling process. I used SAXS to study the gelling processes of native and α-AMA-treated cassava starch. Solubilized amylose chains with partial helical conformation were detected in the gel of both native and α-AMA-treated cassava starch granules. Characteristic peaks appearing over time in the smaller angle region ($q<1$) are due to the inhomogeneous structure, accompanied by the formation of cross-linked zones and aggregation of amylose chains [53, 54]. These characteristic peaks appeared more quickly and predominantly in α-AMA-treated cassava starch, indicating that the aggregation of amylose chains proceeds quickly in α-AMA-treated cassava starch gel to produce larger aggregates. In other words, amylose molecules can make cross-links with other amylose molecules more quickly in α-AMA-treated cassava starch gel than they can in native starch gel. The increased number of cross-links should contribute to the formation of a highly cross-linked continuous gel matrix produced by leached amylose, and result in a highly elastic starch gel. Leached amylose from native cassava starch granules produces cross-links less efficiently, probably because of the presence of high molecular weight amylopectin, which is known to inhibit the cross-link formation of amylose. SAXS is a useful technique to characterize and understand the gelling process of starch. I found that microscopic observation from SAXS data was clearly correlated with macroscopic gelling properties.

I would like to conclude this section by describing our updated hypothesis on the α-AMA-treated cassava starch granule. Treatment of insoluble cassava starch granules with α-amylase
from *A. niger* produces changes in the composition of the material that leaches out of the starch granule. The leached material from native cassava starch granules contains amylase as well as amylopectin, while that of α-AMA-treated cassava starch granules is predominantly amylase and little or no amylopectin. Amylose is known to take single coil conformations in aqueous solution. However, solubilized amylase in a single coil conformation is not stable, and easily forms double helical conformations with other amylase chains to produce cross-links between pairs of amylase molecules [55-57]. It is well known that amylase and its double helical formation are responsible for the gelling properties of starch. Amylopectin, on the other hand, is not responsible for gel formation but inhibits cross-linking and gel formation of amylase [23]. Considering these distinct natures of amylase and amylopectin, the elastic gel produced by α-AMA-treated cassava starch granules is comprehensible. α-AMA-treated cassava starch granules should produce a strong and elastic composite gel with swelled starch granules embedded in an amylase-rich matrix with many cross-links. The mechanism of reduced amylopectin content in the matrix is not fully understood, but is due to the substrate specificity of enzymes, since the gelling properties of enzyme-treated starch greatly depend on the source and type of enzymes (unpublished observation).

The results presented in this study demonstrate the mechanism and impact of limited enzymatic hydrolysis of insoluble starch granules. I think it is a promising tool to alter the properties and functionality of starch.
CHAPTER 3

Development of a new phosphate cross-linked cassava starch by enzymatic treatment

3-1 Introduction

Due to its low cost, availability, and ability to impart a broad range of functional properties to food and nonfood products [58], starch is used in several industrial applications [26]. It is also one of the most promising candidates for future materials [59]. Starch represents more than 85% of all hydrocolloids used in food systems [60]. It is used as starch paste and starch gel.

When starch granules are heated in water to above a certain temperature (the gelationization temperature), the starch granules are gelatinized to form paste. When gelatinized starch paste is left at a low temperature, retrogradation occurs and the starch paste forms a gel. Gel formation is the most important properties of starch. For example, it is used to improve the physical properties of boiled rice, noodles, bread and fishery paste products [61]. The properties of starch gel are predominantly determined by plant sources but they can be changed to a significant extent by chemical modification, which greatly contributes to the exploitation of starch in an increasing
number of applications [62].

There are a lot of chemical modification technologies of starch. Phosphate cross-linking is one. This is a reaction to form intramolecular or intermolecular cross-linked points in starch.

Phosphate cross-linking reactions are used to strengthen the structure of swollen granules upon gelatinization, enhancing the resistance to viscosity breakdown as a result of mechanical shear, acid conditions, or high temperature. Very low levels of phosphate cross-linking usually stabilize the granule structure to allow the modified starch to attain a higher degree of granule swelling during heating than would be achieved by native starch. In such cases, higher swelling powers and paste peak viscosities can be observed [63-67].

In contrast, higher levels of phosphate cross-linking generally lead to reduced granule swelling [68-76], solubility [66, 67, 69, 77], amylose leaching [68, 76], paste clarity [66, 67, 74, 75, 78], and paste peak viscosity [73, 79, 80].

Furthermore, phosphate cross-linked starches exhibit higher pasting temperatures [68, 71, 80], stability to shear [80-83], and tolerance to acid pH conditions [76]. Phosphate cross-linking is the most important technique for increasing the elasticity of starch, and is widely used in many commercial food starches [26].

In this chapter, we combined the new enzymatic method developed in chapter 1 with the conventional phosphate cross-linking method, widely used to improve the gelling properties of starch. We produced phosphate cross-linked cassava starch (PS) and PS treated with α-AMA (PAS) from
native cassava starch, and studied their gelatinization and gelling properties.

3-2 Materials and Methods

3-2-1 Materials

Native cassava starch was purchased from Vedan Vietnam Enterprise Co., Ltd. (Dong Nai Province, Vietnam). α-amylase (565 unit/g) from *A. niger* was provided by Danisco Japan Ltd. (Tokyo, Japan). α-amylase activity was measured with an α-amylase Measuring Kit (Kikkoman Biochemifa Co., Ltd., Tokyo, Japan) using N3-G5-β-CNP as substrate. One unit of α-amylase activity was defined as the amount of enzyme producing 1 μmol of CNP for one minute under the assay conditions [35].

3-2-2 Production of starch granules

Native cassava starch (500 g) was suspended in an aqueous 6.7% (w/w) sodium sulfate solution (780 g), pH adjusted to 11.0, mixed with 10 μl of phosphorus oxychloride and incubated for 1 h at 30 °C with continuous agitation. After 1 h, the pH of the suspension was adjusted to 6.0 and the reaction was terminated. After completion of the reaction, the starch granules were precipitated by centrifugation (3,000 rpm for 5 min). The precipitate was re-suspended with distilled water then
precipitated again by centrifugation. This washing process was repeated three times and PS granules were obtained by drying the washed pellets with a blow dryer.

PS granules (400 g) were suspended in distilled water (900 g), pH adjusted to 5.0 with 1 N HCl and incubated with \( \alpha \)-amylase (4 g) for 18 h at 50 °C with continuous agitation. To halt enzyme activity we added 5 g of sodium hypochlorite solution (effective chlorine concentration: 10% (w/w)) to the starch suspension and stirred. After 10 min, we added disodium pyrosulfite (0.25 g) for neutralization and stirred for 10 min. The enzyme-treated starch granules were precipitated by centrifugation (3,000 rpm for 5 min). The starch granules released 14.7% of their carbohydrate into the soluble fraction (determined by measuring the sugar content of the supernatant fraction by the phenol-sulfuric acid method [36]). The precipitate was re-suspended with distilled water then precipitated again by centrifugation. This washing process was repeated three times and PAS granules were obtained by drying the washed pellets with a blow dryer.

3-2-3 Analysis of pasting properties

The pasting properties of starch were analyzed using a Rapid Visco Analyzer, TECMASTER (FOSS JAPAN, Tokyo, Japan). A starch suspension containing 6% (w/v) of starch granules in distilled water was prepared and loaded to a sample container of the apparatus, then incubated at 50 °C for 10 sec with continuous agitation (910 rpm). The starch suspension was incubated at 50 °C for 1 min with continuous agitation (120 rpm),
heated to 95 °C at 4.5 °C/min, held at 95 °C for 10 min, and cooled to 72.5 °C at 2.25 °C/min. The peak viscosity reached was regarded as the maximum viscosity.

3-2-4 Rheometric analysis of starch gel

The physical properties of the starch gel were analyzed using a rheometer (RT-2010J-CW, Rheotech, Tokyo, Japan). A starch suspension containing 20% (w/w) starch granules in distilled water was prepared, incubated at 65 °C for 10 min and placed in a polyvinylidene chloride tube. The tube was heated to 90 °C at 1 °C/min, held at 90 °C for 30 min, and kept for 1 h at 25 °C followed by 19 h or 21 days at 5 °C. The starch gel formed in the casing tube was incubated at 25 °C for 4 h, sliced into gel disks 25 mm thick and loaded onto the stage of the rheometer. The rupture stress, rupture strain and Young’s modulus were measured by the rheometer with an adapter (diameter 5 mm, area 19.635 mm²) using a movement speed of 6 cm/min.

3-3 Results and discussion

I reported in chapter 1 that cassava starch granules after very limited hydrolysis by α-AMA show significantly enhanced gelling properties. Now I aimed to combine α-AMA treatment with the conventional phosphate cross-linking method which has been widely used to improve the gelling properties of starch.
I analyzed the pasting properties of starch granules using a Rapid Visco Analyzer. As shown in Fig. 11, the peak viscosity temperature of PS was about 13 °C higher than that of native cassava starch, and the viscosity of PS was slightly lower than that of native cassava starch. However, when it was held at 95 °C for 10 min, the viscosity of PS was about twice as high as that of native cassava starch. In general, phosphate cross-linking is used to prevent starch from losing too much viscosity during cooking, and PS shares this property.

Next, the starch paste of PAS was compared with that of PS. Their peak viscosity temperatures and viscosity profiles were the same. When it was held at 95 °C for 10 min the peak viscosity of PAS was 1,511 cp, which is higher than that of native cassava starch. But the difference between PAS and PS was unobservable. So α-AMA treatment does not change the gelatinization and pasting characteristics of PS. A similar result also obtained in chapter 1 where α-AMA treatment of native cassava starch does not change its gelatinization and pasting characteristics.
Next we looked at the gel properties of the three starch samples. Native cassava starch, PS and PAS were gelatinized with water and converted into gel disks as described in Materials and Methods and subjected to rheometric analysis to measure rupture stress, rupture strain and Young’s modulus. In general, rupture stress and Young’s modulus increase when the gel becomes more elastic, and rupture strain decreases when the gel becomes more fragile.

Gels kept for 19 h at 5 °C were analyzed, and are shown in Table 3a. The rupture stress and Young’s modulus of PS were about 1.2 times and about 1.8 times greater, respectively, than those of native cassava starch. These results suggest that the PS gel is more elastic than native cassava starch gel. The rupture strain of PS was lower than that of native cassava starch,
indicating that PS gel is more fragile than native cassava starch gel. The rupture stress and Young’s modulus of PAS gel were about 3.4 times and about 4 times greater, respectively, than those of native cassava starch gel, and were also higher than those of PS. The rupture strain of PAS was also higher than that of PS, almost as high as that of native cassava starch. Similar results were obtained with starch gels kept for 21 days at 5 °C as shown in Table 3b. The rupture stress and Young’s modulus of PS were about twice as large as those of native cassava starch, while those of PAS were about 4 times greater. There was no significant difference in rupture strain between PS and PAS.
Table 3. Rheometric properties of starch gel prepared from native cassava starch, PS, and PAS, held at 5 °C for 19 h (A) and 21 days (B).

(A)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rupture stress (g/cm²)</th>
<th>Rupture strain (%)</th>
<th>Young’s modulus (×10³ dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native starch</td>
<td>257.5 ± 7.0</td>
<td>53.5 ± 0.8</td>
<td>472 ± 6</td>
</tr>
<tr>
<td>PS</td>
<td>313.4 ± 15.1</td>
<td>36.7 ± 1.3</td>
<td>838 ± 21</td>
</tr>
<tr>
<td>PAS</td>
<td>867.6 ± 70.1</td>
<td>44.8 ± 2.0</td>
<td>1,897 ± 81</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rupture stress (g/cm²)</th>
<th>Rupture strain (%)</th>
<th>Young’s modulus (×10³ dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native starch</td>
<td>457.7 ± 10.3</td>
<td>23.9 ± 0.6</td>
<td>1,882 ± 57</td>
</tr>
<tr>
<td>PS</td>
<td>867.6 ± 38.7</td>
<td>21.6 ± 0.7</td>
<td>3,944 ± 230</td>
</tr>
<tr>
<td>PAS</td>
<td>1713.7 ± 75.8</td>
<td>22.4 ± 1.0</td>
<td>7,530 ± 626</td>
</tr>
</tbody>
</table>

The extent of hydrolysis of PAS granules was 15% (w/w). The value is the mean ± SD of at least three measurements.
This rheometric analysis of starch gels demonstrates that the gelling properties of PS can be significantly enhanced by \( \alpha \)-AMA treatment.

Phosphate cross-linking has been widely used to strengthen starch gel, but it also introduces fragility. Furthermore, high cross-linking inhibits the swelling and gel-forming of starch, which introduces an unpleasant sandy texture into food products.

Our present study clearly demonstrates that \( \alpha \)-AMA treatment is a very effective technology to improve the beneficial gelling properties of conventional phosphate cross-linked starch while diminishing its bad effect on texture.

The gelling process of starch granules is schematically illustrated in Fig. 12. When starch granules suspended in water are heated to temperatures under 100 °C, they swell and some starch component (mainly amylose) leaches from the granules, resulting in the dispersion of swelled starch granules in water containing leached amylose. Subsequent cooling helps the leached amylose to form local intra-molecular cross-links and a continuous macro gel (matrix) which includes swelled starch granules (filler) [17-19], as schematically shown in Fig. 12A.

I reported in the previous section that the matrix of native starch gel is composed of amylose and amylopectin, while that of \( \alpha \)-AMA-treated starch gel is composed of amylose with less amylopectin, as schematically summarized in Fig. 12B. I concluded that the enhanced gelling properties of \( \alpha \)-AMA-treated cassava starch are produced by the strong matrix structure made from amylose.
The distribution of phosphate cross-links within starch granules is not clearly understood, but I think they are predominantly localized in the filler (Fig. 12C), since cross-linked molecules should be difficult to leach from starch granules. I therefore think that the strong gel property observed in phosphate cross-linked starch (PS) is not due to the matrix but to the stiff cross-linked filler.

The strong and elastic gel of PAS, on the other hand, is probably produced by the combined effects of $\alpha$-AMA treatment and phosphate cross-linking. As described above, $\alpha$-AMA treatment contributes a strong matrix while phosphate cross-linking contributes a stiff filler. I believe that PAS gel is like Fig. 12D, where an amylose-rich matrix and phosphate cross-linked filler constitute a very strong filler-in-matrix structure that shows improved gelling that cannot be achieved by conventional chemical modification technologies.

Limited hydrolysis of starch granules with $\alpha$-AMA was recently discovered to be a powerful tool to enhance the gelling properties of cassava starch. The present study clearly demonstrates that this new enzymatic method can be successfully combined with conventional chemical modification technologies to further improve the properties of starch. I believe that this new enzymatic method can be used not only for cassava starch but also for starch from other botanical sources (corn, potato, wheat and rice) and can be combined with various chemical modification technologies. These technologies have already been used to produce new commercial starch products with brand names like “Chemistar E226” and “Chemistar E246”. These
starches are distinct from existing starches on the market, and find application in noodles, flower pastes and other things. I believe this new enzymatic method is a promising tool to alter the properties and functionality of starch and to expand the applications of starch in food and non-food industries.
Fig. 12. Schematic illustrations of the gelling process of (A) native cassava starch, (B) α-AMA-treated cassava starch, (C) PS, and (D) PAS granules.
CONCLUSION

Cassava starch granules were treated with three types of amylase and the resulting insoluble starch granules collected and their gelling properties investigated. Gel produced from starch granules treated with either \( \alpha \)-amylase from \textit{B. amyloliquefaciens} (\( \alpha \)-AMB) or \( \beta \)-amylase from barley (\( \beta \)-AMB) was slightly weaker than that from native cassava starch granules. On the other hand, starch granules treated with \( \alpha \)-amylase from \textit{A. niger} (\( \alpha \)-AMA) produced starch gel with significantly enhanced hardness and elasticity. This is surprising because very limited hydrolysis (2.8\%) of starch produces such a great impact on the gelling properties of starch and because it contradicts the generally held view that partially hydrolyzed starch shows decreased gel-forming ability. In order to understand the mechanism behind this, the starch component leached from starch granules into hot water was analyzed using gel permeation chromatography. The results suggest that enzymatic treatment of starch granules significantly changes the properties of starch by altering the composition of leached material (matrix) from swelled starch granules (filler). The matrix of native starch gel is composed of amylose and amylopectin, while that of \( \alpha \)-AMA-treated starch gel is composed of amylose with less amylopectin. I conclude that the enhanced gelling properties of \( \alpha \)-AMA-treated cassava starch are produced by the strong matrix structure made from amylose.
The mechanism of the enhanced gelling properties of the α-AMA-treated cassava starch was investigated by dynamic viscoelastic analysis and small-angle X-ray scattering (SAXS). The storage modulus ($G'$) of α-AMA-treated cassava starch paste was greater than that of native cassava starch paste, and the $\tan \delta$ ($G''/G'$) of the treated paste was almost zero at the tested angular frequency, which is much lower than for native cassava starch. The SAXS results suggest that the microscopic gel structure is composed of highly aggregated inhomogeneous nano-scale structures and diluted regions of single and helical amylose chains. Starch crystallization and growth of aggregates were faster, and the size of aggregates larger, for α-AMA-treated cassava starch than for native starch. These results demonstrate that α-AMA treatment improves elasticity by forming a strong three-dimensional network of amylose.

Finally, the new enzymatic method developed in chapter 1 was combined with the conventional chemical cross-linking method, widely used to improve the gelling properties of starch. Phosphate cross-linked cassava starch (PS) and PS subsequently treated with α-AMA (PAS) were produced from native cassava starch, and their gelatinization and gelling properties were investigated. Among the three starches tested, PAS produced the most elastic gel, indicating that the enzymatic method is effective on PS.

The limited enzymatic hydrolysis of insoluble starch granules at sub-gelatinization temperature is potentially a novel and powerful tool to alter the properties and functionality of
starch. The efficacy and feasibility of this approach should be examined for different enzymes and sources of starch.
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