Direct Analysis of Chains on Outer Layer of Amylopectin through Partial Hydrolysis of Normal Starch by Isoamylase

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Normal starches generally consist of 75–85% amylopectin and 15–25% amylose. It is expected that the fine structure of amylopectin plays an important role on physical property of starch because of major component, but it is not easy to elucidate the fine structures because the large molecules are composed of plenty of branches of short-amylose chains. Some cluster models of amylopectin have been proposed and the Hizukuri model pointed out different length of B chains is the most reliable.

From the study on partial hydrolysates of waxy corn starch (amylopectin) with immobilized isoamylase, it has been reported that the chains existing in the outer portion of amylopectin molecules are debranched preferentially at the early stage of the enzyme reaction. The analysis method was applied to the comparative study of two rice amylopectins isolated from Reiho and Koshihikari starches, and it has been found that their outer portions are composed of longish and shortish chains, respectively. RVA viscosograms showed that the starch of Reiho easily retrograded to Koshihikari compared. So, it is inferred that the physical property of rice starch is related to not only amylose but also amylopectin. On the other hand, the immobilized isoamylase has been known to be lacking in stabilization in addition to the costly and complicated method, and it was reported that controlled debranching reaction with free isoamylase could be used in the similar way with the immobilized enzyme.

The separation of amylopectin and amylose has been considered as a matter of course for the study on both structures. The most popular method preparing amylose with 1-butanol has problems concerning the complicated operation, the difficult certification of pure amylose and amylopectin, and the occurrence of amylopectin with long-chains capable of making a complex with 1-butanol. On the other hand, it has been known the occurrence of branched amylose in addition to linear molecule and an influence of the branched amylose should be investigated on the short-amylose chains from normal starch.

In the present study, the characteristics of short-amylose chains released in various debranching re-
actions of normal corn starch were investigated for developing a method of getting general information of outer portion of amylopectin molecule without separation of amylose and amylopectin. Then, the short-amylose chains obtained from typical rice starches were investigated with the objective for evaluating the advantage of the direct analysis of amylopectin.

**MATERIALS AND METHODS**

**Materials.** Normal corn starch was kindly donated from Nihon Shokuhin Co., Ltd. (Japan). Rice starches of four cultivars of *Oryza sativa L. japonica* (Koshihikari, Sasanishiki, Yamadaneishiki and Kirara 397) were obtained from Mie Prefectural Research Institute. *Pseudomonas* isoamylase was obtained from Hayashibara Biochemical Laboratories (Japan) and partially purified as described on previous report.5 The specific activity of the isoamylase was 50 U/mg. The gels of Toyopearl HW-50S and HW-65F were obtained from Tosoh Co., Ltd. (Japan). Maltohexaose kindly supplied from Nihon Shokuhin Kako Co., Ltd. was used as internal standard in the distribution of short-amylose chains with a high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD).

**Preparation of defatted starches.** Starch (2 g) dissolved in dimethyl sulfoxide (40 mL) was poured gently in 100 mL methanol and stood for overnight. The precipitate collected on a 3G-3 glass-filter was rinsed successively with methanol and ether, and then dried in air.

**Preparation of 0.2% starch solution and partial hydrolysates by isoamylase.** Defatted starch (30 mg) suspended in distilled water (0.5 mL) was gelatinized with 1 M NaOH (0.75 mL) in ice bath and then diluted gradually by distilled water (5.35 mL). After neutralization with 1 M HCl (0.75 mL), the solution was mixed with 3% NaN₃ (0.15 mL) and 7.5 mL of 0.1 M acetate buffer (pH 5.5).

The 0.2% starch solution (15 mL) was mixed by 0.3 μg (15 mU) of isoamylase and shaked (100 rpm) at 30 °C. In order to make a time course of the debranching reaction, partial hydrolysate (0.4 mL) was taken out continuously from the test tube at appropriate reaction time. The reducing strength was analyzed by modified Park-Johnson method and degrees of hydrolysis (%) was calculated by dividing the value of reducing strength of partial hydrolysate with that of the complete hydrolysis. A hydrolysate with 3 μg (150 mU) of isoamylase was conducted for complete debranching reaction. Various kinds of partial hydrolysates were prepared by making an estimate of reaction times from the time course.

**Gel permeation chromatography (GPC) of partial hydrolysate.** Ten milliliter of the partial hydrolysate was applied on a column (2.6 cm I.D. × 100 cm) of Toyopearl HW-50S and eluted with 50 mM NaCl containing 0.01% of NaN₃ by using peristaltic pump on the flow rate of 40 mL/h. Carbohydrate in each fraction (5 mL) was estimated by the phenol-H₂SO₄ method. Fractionated samples containing salts were concentrated by a rotary evaporator and lyophylized.

**Distribution of short-amylose chains.** HPAEC was conducted using a Dionex BioLC System (Dionex Co., USA) connected with a pulsed amperometric detector system (Model PAD II). The column used was a CarboPac PA1 Column (250 mm×4 mm I.D.) connected with a CarboPac guard column. Appropriate amount of sample was dissolved in 150 mM NaOH to prepare 0.1–0.2% carbohydrate. In HPAEC analysis, 50 μL of sample solution was injected and eluted at 1 mL/min using a mixture of eluent A (150 mM NaOH) and eluent B (500 mM sodium acetate in 150 mM NaOH). The gradient pump program was as follow: 10% eluent B at 0 min, 80% eluent B at 80 min, and 100% eluent B at 87 min. An LC 8020 software (Tosoh Co., Japan) was used to record and calculate the peak areas.

**Preparation of amylopectin and amylose from normal corn starch by GPC.** After preparation of 0.4% starch solution (22.5 mL) containing 0.03% NaN₃, the starch solution was applied on a column (2.6 cm I.D. × 100 cm) of GPC Toyopearl HW-65F and eluted by distilled water containing 0.01% NaN₃ at a flow rate of 100 mL/h. Carbohydrate in each fraction (10 mL) was estimated by the phenol-H₂SO₄ method. The fractions of amylose and amylopectin were concentrated appropri-
ately and their sugar concentrations were adjusted for analyzing sugar amounts with the colorimetric method. The fractions eluting amylose were confirmed with the commercial corn amylose B (Nacalai Tesque, Japan). In order to prepare enough amount of amylose, the procedure was repeated two times and only the amylose fractions were rechromatographed.

**Digestion of amylopectin and amylose by isoamylase.** The solution (7.5 mL) containing 30 mg of amylose or amylopectin was mixed with 7.5 mL of 0.1 M acetate buffer (pH 5.5) containing 0.02% of 3% NaN₃. The debranching reaction with 0.3 μg (15 mU) or 3 μg (150 mU) of isoamylase was conducted by shaking at 100 rpm at 30 °C, respectively.

**RESULTS AND DISCUSSION**

**Preparation of amylopectin and amylose by GPC.**

Semipreparative GPC with Toyopearl HW-65F of normal corn starch was conducted for preparing amylopectin and amylose of normal corn starch and the amount of Fr. 2 (FN 30-45) corresponding to amylose was about 30.6% (Fig. 1A). Refined amylose was obtained by splitting non-amylose fraction (10.2%) on the re-chromatography (Fig. 1B).

**Debranching actions of isoamylase on starch and amylopectin of normal corn.**

When starch and amylopectin of normal corn (30 mg each) were digested by isoamylase (15 mU and 150 mU, respectively), the increases of reducing strength were analyzed by modified Park-Johnson method⁹ and monitored by the increases of optical density (OD) at 715 nm (Fig. 2). As the partial hydrolysates obtained were suggested to contain a few branching structures from the elution

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**Fig. 1.** Gel chromatography of normal corn starch (A) on a column of Toyopearl HW-65F and rechromatography of amylose fraction (B).

A starch solution (22.5 mL) dissolving defatted normal corn starch (90 mg) was applied on the column for semipreparative chromatography. Fractions 16–29 (Fr. 1) and fractions 30–45 (Fr. 2) were collected as fractions of amylopectin and amylose, respectively.

**Fig. 2.** Time courses of formations of short-chains released from normal corn starch (□, ■), amylopectin (Δ, ▲) and amylose (○, ●) isolated from normal corn starch. Each 0.2% starch solutions was digested by 15 mU (open symboles) and 150 mU (closed symboles) of isoamylase, respectively.
The partial hydrolysates (A, B, C, D and E) were prepared by digestion with 15 mU of isoamylase and their degrees of hydrolysis (%) were calculated to be 11, 19, 30, 41 and 67, respectively. The complete hydrolysate (F) was obtained by digesting with 150 mU of isoamylase. The reaction times for the preparation of these hydrolysates were referred in Table 1. The elution curves were drawn with total saccharide ratio which were calculated by dividing the amount of carbohydrate in each fraction by the total amounts eluted.

patterns of fr. 2 shown in Fig. 3 (A–E), changes in the reducing strength were shown with direct measurements (OD). A very fast reaction with 150 mU of isoamylase was necessary to understand the complete debranching reaction and the final OD value (10.20) of normal corn starch was 29% smaller than that (14.37) of amylopectin. Considered amylose content of normal corn starch (about 25%), the difference could be acceptable. When the enzyme activity of isoamylase was reduced to 15 mU, the velocity of debranching reaction decreased remarkably and the substrates were not hydrolyzed completely. From the time course, it could be estimated the hydrolysis times for preparing 10–20% hydrolysates from normal starch (see Table 1).

**Gel chromatographies of partial hydrolysates on Toyopearl HW-50S.**

GPC profiles of partial hydrolysates of normal corn starch on Toyopearl HW-50S were shown in Fig. 3. Toyopearl HW-65F was used in our previous experiment, but the column was not chosen in this study because amylose and short-amylose chains were eluted in similar fractions. The fractions eluted were classified into three groups (fr. 1, fr. 2 and fr. 3) by referring elution patterns of completely debranched normal corn starch (Fig. 3 F). Amylose, amylopectin and half-way hydrolysate of amylopectin were eluted in the fr. 1, and longish and shortish chains released from amylopectin were eluted in fr. 2 and fr. 3, respectively. Amounts of carbohydrate in fractions 33–47 (fr. 1), fractions 48–55 (fr. 2) and fractions 56–75 (fr. 3) were shown in Table 1. The amounts of fr. 1 and fr. 3 of 11% and 19% hydrolysates were cal-

<table>
<thead>
<tr>
<th>Isoamylase activity (mU)</th>
<th>Reaction time (h)</th>
<th>Degree of hydrolysis (%)</th>
<th>Total saccharide ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>15</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>15</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>15</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>15</td>
<td>11</td>
<td>41</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>15</td>
<td>24</td>
<td>67</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>150</td>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>
culated to be 89.5% and 85.7%, and 6.1% and 7.5%, respectively. The short-amylose chains accumulated in the fr. 3 could be speculated as chains released mainly from outer layer of amylopectin.

**Chain distribution of fr. 3 of the partial hydrolysates.**

Chain distributions of fr. 3 were analyzed by HPAEC-PAD. Chains longer than DP 36 were cut off in this analysis because of unnecessary chain. As shown in Fig. 4-A, the fr. 3 of 11% hydrolysis was composed of large amount of short chains of DP 7–12. The chain distribution expanded mainly to longer chains as the degree of hydrolysis became bigger. These distribution patterns were similar to those of waxy corn starch. However, chains of DP 4 and DP 5 were slightly detected and it was thought that a part of them came from branched amylose because amylopectins are generally composed of chains larger than DP 6. The difference of each chain was calculated between fr. 3 of each partial hydrolysate and fr. 3 of complete hydrolysate (Fig. 5). Although the patterns shown in Fig. 5 were similar, fr. 3 of 11% and 19% hydrolysates contained lots of shortish chains (Fig. 5-A and -B).

**Effect of short-amylose chains from branched amylose.**

Since it has been known that normal corn starch contains about 25% of amylose and that linear and branched molecules exist in amylose, the debranching reaction of branched amylose should be investigated. So, the isolated amylose (30 mg) was digested by 15 mU and 150 mU of isoamylase. Their increases of optical density were followed

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**Fig. 4.** Chain-length distribution of fr. 3 fractionated partial hydrolysates of normal corn starch by GPC of Toyopearl HW-50S.

Symbols A–F were referred to Fig. 3 and Table 1.

**Fig. 5.** Differences of short-amylose chains between fr. 3 of partial hydrolysate and fr. 3 of 100% hydrolysate of normal corn starch.

Symbols A–E referred to Figs. 3, 4 and Table 1.
**Table 2.** Increasing of optical density/mL reaction mixture during the hydrolysis of normal corn starch and amylose by 15 mU isoamylase.

<table>
<thead>
<tr>
<th>Reaction time (h)</th>
<th>Optical density (at 715 nm)/mL</th>
<th>Amylose in normal corn(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal corn starch [A]</td>
<td>Normal corn amylose [B]</td>
</tr>
<tr>
<td>2</td>
<td>1.85</td>
<td>0.48</td>
</tr>
<tr>
<td>4</td>
<td>3.62</td>
<td>1.00</td>
</tr>
<tr>
<td>8</td>
<td>5.06</td>
<td>1.61</td>
</tr>
<tr>
<td>11</td>
<td>5.38</td>
<td>1.77</td>
</tr>
<tr>
<td>24</td>
<td>7.59</td>
<td>2.30</td>
</tr>
</tbody>
</table>

\(^a\) Calculated the optical density of amylose in normal corn starch. \(^a\) The rate of optical density of amylose in that of normal corn starch.

and compared with normal corn starch (Fig. 2). The isolated amylose was digested by isoamylase, however, the increasing gradient was obviously slower than normal corn starch. The property of short-amylose chains in branched amylose is generally interested and HPAEC-PAD analysis was conducted. However, it was found that the elution pattern of isoamylase was slightly different from that of pullulanase and a detail study will be done in future. Considering the content of amylose in normal corn starch (about 25%), the amounts of short-amylose chains released from branched amylose were calculated to be 6.5–8.2% (Table 2). So, it could be thought that chain distributions shown in Fig. 4-A and -B (or Fig. 5-A and -B) were reflecting a general characteristics of the outer layer of amylopectin.\(^3\) These results led us to expect that this method is applicable to normal starch.

**Comparison of short-amylose chains released from rice starches.**

As amylose contents of rice starches are commonly less than 20%, it was thought that the fine structure of amylopectin was more influence on the textural and physical properties of rice and/or rice starch. Yamadanishiki is the most famous rice as raw material of “sake” because of an unique resistance in the process of saccharification, Kirara 397 is a quality rice improved in Hokkaido and Koshihikari has been evaluated as the best taste in Japan. These typical rice starches were tested as an

**Table 3.** Composition of each fraction of complete hydrolysate of rice starches by isoamylase eluted on GPC Toyopearl HW-50S.

<table>
<thead>
<tr>
<th>Rice</th>
<th>Total saccharide ratio (%)</th>
<th>fr. 1 / fr. 2</th>
<th>fr. 3 / fr. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koshihikari</td>
<td>13.9</td>
<td>25.6</td>
<td>60.6</td>
</tr>
<tr>
<td>Sasanishiki</td>
<td>12.9</td>
<td>24.1</td>
<td>63.0</td>
</tr>
<tr>
<td>Kirara 397</td>
<td>16.7</td>
<td>23.8</td>
<td>59.5</td>
</tr>
<tr>
<td>Yamadanishiki</td>
<td>15.8</td>
<td>33.8</td>
<td>50.5</td>
</tr>
</tbody>
</table>

![Fig. 6. Differences of short-amylose chains between fr. 3 of Sasanishiki (A), Kirara 397 (B), or Yamadanishiki (C) and fr. 3 of Koshihikari.](image-url)
appropriate example in order to know whether the direct analysis method was useful for getting an essential structural information of amylopectin or not.

Two kinds of hydrolysates were prepared with isoamylase; one was a complete hydrolysate and another one was a partial hydrolysate with degree of hydrolysis of about 15%. The GPC data of complete hydrolysates on Toyopearl HW-50S were shown in Table 3. An approximate amount of amylose could be estimated from fr. 1 in the figure. It was known that the amylose contents of Koshihikari and Sasanishiki were similar, and that it was recognized that the amylose contents of Kirara and Yamadanishiki were also similar. The value dividing fr. 3 by fr. 2 was expected to offer information of amylopectin and the value of Yamadanishiki impressed that the amylopectin was composed of lots of longish short-amylose chains. The partial hydrolysates were also conducted on a column of Toyopearl HW-50S in order to prepare the fr. 3 for the analysis by HPAEC-PAD. The difference of each chain was calculated between fr. 3 of Sasanishiki (Fig. 6-A), Kirara 397 (Fig. 6-B) or Yamadanishiki (Fig. 6-C), and fr. 3 of Koshihikari. As short-amylose chains released from rice starches were focused on outer-chains of amylopectin and the chains around DP 30 was very few in the case of rice, chains of DP 6-23 were calculated. Compared with Koshihikari, it was suggested that the outer layers of amylopectins of Yamadanishiki and Kirara 397 were composed of longish and shortish chains, respectively. On the other hand, it was found that outer layers of amylopectin of Sasanishiki was similar to that of Koshihikari.

Thus, it was confirmed that the analytical method putting together the debranching reaction of starch and GPC on Toyopearl HW-50S were convenient for studying on structures of normal starches without a separation of amylose and amylopectin. As an outside structure of amylopectin could be regarded as an important factor in the physical property of starch because of high possibility on the interaction with chemical stuffs, the direct analysis without purification of amylopectin will be worth using as the first screening.

REFERENCES


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イソアミラーゼによるウルチ澱粉の
部分加水分解物よりアミロベクチン外部層に
存在する鎖長構造の直接分析

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アミロベクチンの構造情報を直接取得する簡便な分
析方法を確立する目的で，イソアミラーゼによるウル
チトウモコシ澱粉から切り出される短鎖アミロース
鎖を調べた。アミロースとアミロベクチンを分離せず
に得られる部分加水分解物を，Toyopearl HW-50S の
ゲルクロマトグラフィーにより3区分(fr.1, fr.2, fr.
3)に分けた。短い鎖長を含むfr.3の鎖長分布を
HPAEC-PADで分析した。fr.3に含まれる短鎖アミ
ロースはアミロベクチンのほかに分岐アミロース由来
のものも含まれるが，後者由来のものはわずかである
と推定した。このアミロベクチンの一般的な構造情報
を直接得る分析法は，日本で栽培された代表的な米澱
粉で応用性を調べた。