コメ, トウモロコシ, コムギ, サツマイモおよびジャガイモデンプン粒表層タンパク質の抽出と同定

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Extraction and Identification of Rice, Maize, Wheat, Sweet Potato and Potato Starch Granule Surface Protein

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Starches are a major commercial food source, and different starches have distinct properties that result in part from their associated proteins. We examined the starch surface proteins (SSPs) of rice, maize, and wheat, sweet potatoes and potatoes. We identified a strong correlation between the color (b*) of various starches stained with Coomassie Brilliant Blue (CBB) and their protein content (r = 0.965). Protein was extracted from various starches using 0.1 % sodium hydroxide. The percentage of the decreased protein using this procedure differed depending on the source: Rice, maize, wheat, sweet potato, and potato yielded 71.6, 29.8, 21.6, 60.0, and 40.0 % of their SSP, respectively. The SSPs were separated to 19 protein bands by gel electrophoresis, and we successfully identified 16 of the 19 proteins. A granule-bound starch synthase or precursor was identified from each starch. The wheat SSPs included an alpha-amylase inhibitor. The potato SSPs included aspartic proteinase inhibitor homologue and cysteine protease inhibitor 7. Furthermore, several storage proteins were identified among the SSPs, including glutelin and prolamin from rice, gamma zein from maize, globulin from wheat, and sporamin from sweet potato. Since storage proteins are commonly hydrophobic, we suggest that these proteins affect the pasting characteristics of various starches.

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Key words: starch granule, starch granule surface protein LC/MS/MS デンプン粒, デンプン粒表層タンパク質

Starches are a major commercial food source. Starch granules are synthesized and stored within amyloplasts, and differ depending on their plant of origin. For example, the granule size, amylose content, and crystallinity of starch differs between plants[8-10]. These factors affect the pasting properties and digestion of the raw starch by amylase; however, the relationship is not well understood[14]. Starch granules contain amylose and amylopectin, along with small quantities of other components such as proteins, lipids, and minerals[14-16]. Cereal starches are approximately 0~0.25 % protein, and up to 1.0 % lipid, while root and tuber starches (e.g. from sweet potato and potato) are 0~0.05 % protein and 0.05~0.1 % lipid[17-19]. Even though the protein and lipid quantities are minor, they affect both the pasting properties (viscosity, swelling capacity, and gel formation) and the granule as a whole[10, 19].

Wheat starch contains starch granule bound proteins[9]. Soft wheat possesses more starch surface protein (SSP) than hard wheat, and this protein is associated with a softer endosperm texture[15]. In addition, Nierle et al., reported that removal of wheat starch SSPs by treatment with sodium dodecyl sulfate (SDS) increased viscosity[20]. This result led the authors to suggest that SSP might affect the properties of the wheat starch granules. However, it has since been suggested that SDS might interact with amylose and amylopectin, and that these interactions might explain the effects of SDS on the properties of starch[20]. Accordingly, the effects that SSPs have on the properties of a given starch are completely unknown. Therefore, it is

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important to understand the relationship between SSPs and the properties of commercially important starches.

We previously elucidated the relationship between SSP and raw maize starch digestion by alpha-amylase\(^{17}\). We discovered that maize SSPs interacted with alpha-amylase and affected the digestion of the starch; we also proposed that SSPs affect the starch granule characteristics\(^{17}\). To date, there have been relatively few studies examining SSPs. In this study, in order to better understand the relationship between SSPs and starch properties, we extracted the SSPs from rice, maize, wheat, sweet potatoes, and potatoes using a 0.1 % sodium hydroxide solution. We then determined the composition of the SSPs from these starches using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (Q-TOF) (LC-MS/MS).

Materials and Methods

1. Materials
   Rice starch was purchased from Sigma-Aldrich Co. (Japan), and maize, wheat, and sweet potato starches were purchased from WAKO Co. (Japan). Potato starch was purchased from Nacalai Tesque Co. (Japan).

2. Preparation of starch surface protein (SSP)
   Starch surface protein solutions were prepared from various starches following the methods described by SHUJUN et al. with minor modifications\(^{40}\). Various starches (10 g dry weight) were added to 150 mL of pure water, and the mixtures were left at room temperature for 20 min. The supernatants were discarded after a 5 min centrifugation (9,000 \( \times \) g) at 20\(^\circ\)C, and the above steps were repeated. The precipitates were then resuspended in 0.1 % NaOH solutions (100 mL) by stirring at 4\(^\circ\)C for 12 h. The supernatants were collected after a 5 min centrifugation (9,000 \( \times \) g) at 20\(^\circ\)C. The supernatants were then each separately dialyzed against pure water using Spectra/Por 3 Dialysis Membrane Standard Grade RC Tubing (molecular weight cut-off 3,500 Da). The resulting SSP solutions were freeze dried. The precipitates from the centrifugation were washed using pure water until they reached pH 7. These washed starches were then freeze dried. Control starch samples were prepared using the same procedure, with the exception that they were resuspended in pure water rather than 0.1 % NaOH before centrifugation.

3. Measurement of protein content in starch
   The protein content was determined by based on nitrogen and carbon content using CN coder (Yanaco Technical Science Co., Japan). The amounts of nitrogen from 500 mg (dry weight) of the 0.1 % NaOH treated and control starches were measured, and the amount of protein in each was calculated using the appropriate conversion factor (rice, 5.95; maize, sweet potato, and potato, 6.25; wheat, 5.70).

4. Staining the starch surface
   Coomassie Brilliant Blue (CBB) staining solution was prepared by mixing 0.25 % (w/v) Coomassie Brilliant Blue R-250 (WAKO Co., Japan) dye, 50 % (v/v) methanol (Kanto Chemical Co., Japan) and 10 % acetic acid (Kanto Chemical Co., Japan). The CBB staining solution (1 mL) was added to 0.1 % NaOH treated and control starch samples (100 mg dry weight), and the mixtures were incubated for 30 min at room temperature. The solutions were centrifuged at 16,000 \( \times \) g for 1 min at 4\(^\circ\)C, and the resulting supernatants were discarded. Subsequently, 1 mL of destaining solution (50 % (v/v) methanol, 10 % (v/v) acetic acid) was added to each of the precipitates, and they were resuspended with mixing. The centrifugation process was repeated as described, and the precipitates were again obtained. The centrifugation and resuspension cycle was repeated five times before the pellets were finally resuspended in 1 mL of water. The color of each resulting slurry was analyzed using a CM-5 spectrophotometer (Konica Minolta Co., Japan), and the \( b^* \) values were determined for each slurry.

5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
   The extracted SSPs were dissolved in 10 mM MES buffer (pH 6.0), and the supernatants were collected after a centrifugation at 16,000 \( \times \) g for 1 min at 4\(^\circ\)C. Laemmli sample buffer was added to the supernatant SSP solution, and the SSPs were resolved by SDS-PAGE in a 5~20 % polyacrylamide gradient gel (ATTO Co., Japan) according to the Laemmli method\(^{19}\). The gels were then stained with CBB staining solution, and subsequently destained using destaining solution.

6. Statistical analysis
   Analysis of variance was conducted using Statcel software (OMS Publishing Inc., Saitama, Japan). Data were subjected to analysis of variance (ANOVA), and if the means were significantly different (\( p < 0.05 \), they were then compared using
Fisher’s protected least significant difference (PLSD) test. Pearson’s correlation coefficient was also calculated.

7. Protein identification by LC-MS/MS

The protein bands separated by SDS-PAGE were digested in-gel with trypsin. The gel fragments were excised from the gel, and were then decolored with a solution of 30 % (v/v) acetonitrile and 25 mM ammonium hydrogen carbonate. The fragments were then reduced with a solution of 10 mM dithiothreitol and 25 mM ammonium hydrogen carbonate, and alkylated with a solution of 55 mM iodoacetamide and 25 mM ammonium hydrogen carbonate. The treated gels were then digested with trypsin (Promega Co., Japan).

All of the mass spectrometry experiments were performed on an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS/MS coupled to an Agilent 1,260 Infinity LC high-pressure liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA). The analytical column was a 100 mm length InertSustain C18 column with a 1.0 mm internal diameter and 3 μm particle size (GL Sciences Co., Japan). The mobile phase was 0.1 % formic acid (WAKO Co., Japan) with varying amounts of acetonitrile (Kanto Chemical Co., Japan). The injection volume was 5.0 μL. Peptide separation was carried out at 70 μL/min with a mobile phase linear gradient of 2–90 % acetonitrile for 30 min, followed by 90 % acetonitrile for 5 min. The mass spectra were compared to the subset in the NCBInr database using an in-house MASCOT search engine.

Results and Discussion

1. Protein content and b* values of various starches

The protein content (mg protein per g of starch) and color (b*) of 0.1 % sodium hydroxide treated starches and untreated starches were analyzed, and the results are shown in Table 1. In untreated starch, the protein content was highest for rice, followed by maize, wheat, sweet potato, and lastly potato. These results are similar to the findings of previous reports (9-10). The b* values were highly correlated with the protein content (r = 0.965), and this result suggests that the b* value of CBB-stained starch is an accurate indicator of starch protein content.

The protein content of various starches was significantly decreased by treatment with 0.1 % sodium hydroxide (Table 1). The amount of protein removed during sodium hydroxide treatment was calculated for each starch type as a percentage of the total protein in the starch, based on the protein content of the appropriate untreated control. Rice starch had the highest protein loss (71.6 %), followed by sweet potato (60.0 %), potato (40.0 %), maize (29.8 %), and lastly wheat (21.6 %). Overall, the amount of SSP that dissolved in 0.1 % sodium hydroxide differed depending on the starch source. These results suggest that maize and wheat SSPs may be more hydrophobic and therefore less soluble in the sodium hydroxide solution. Additionally, the b* values were also highly correlated with the protein content, with the exception of rice starch. This correlation was expected, and the small amount of rice SSP that remained after sodium hydroxide treatment may explain the difference in the degree of CBB staining.

2. Identification of individual SSPs

The SSPs were separated and visualized by SDS-PAGE (Fig. 1). Several proteins migrating at distinct molecular weights were evident from each SSP sample. We analyzed 19 bands by MS (numbered 1-19 in Fig. 1), and successfully identified 16 proteins (Table 2). A granule-bound starch synthase or precursor thereof was present in each SSP sample. An alpha-amylase inhibitor was also identified among the wheat SSPs. Both aspartic proteinase inhibitor homologue and cysteine protease inhibitor 7 were identified among the potato SSPs (Table 2). Furthermore, we identified several storage proteins including glutelin and prolamin from rice, gamma

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amount of protein (mg per g starch)</th>
<th>b* value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.1% NaOH treated</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.1% NaOH treated</td>
</tr>
<tr>
<td>Rice</td>
<td>0.67a</td>
<td>0.19b</td>
</tr>
<tr>
<td>Maize</td>
<td>0.57a</td>
<td>0.40b</td>
</tr>
<tr>
<td>Wheat</td>
<td>0.37a</td>
<td>0.29b</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>0.15c</td>
<td>0.06d</td>
</tr>
<tr>
<td>Potato</td>
<td>0.10e</td>
<td>0.06d</td>
</tr>
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</table>

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<td>Sweet potato</td>
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</tr>
<tr>
<td>Potato</td>
<td>0.10e</td>
<td>0.06d</td>
</tr>
</tbody>
</table>
Fig. 1 Analysis of SSPs by SDS-PAGE

The SSPs from rice (R), maize (M), wheat (W), sweet potato (SP), and potato (P) were resolved on a 5–20% gradient polyacrylamide gel and visualized with coomassie brilliant blue dye. Molecular weight markers were loaded for reference (Mw), and their sizes are indicated along the left edge of the gel (Mass). Numbers indicate individual protein bands that were excised for further analysis.

zein (glutelin-2) from maize, globulin 3 from wheat, and sporamin A from sweet potato. Collectively, these results indicate that SSPs are primarily made up of storage proteins. Hamaker et al. reported that adding dithiothreitol to reduce disulfide bonds in rice powder slurry decreased the viscosity, and Derycke et al. showed that SSPs covering starch granules interfered with water absorption. We suggest that the hydrophobicity of storage proteins likely affects the pasting properties of the starch granules. In addition, some of the potato surface protein that matches on the database was not (15, 17 and 19 bands: Fig.1). It will be necessary to analyze these proteins.

We examined rice, maize, wheat, sweet potato, and potato starch surface proteins. The protein contents of their starch granules were different, and there was a strong correlation between $b^*$ values in colorimetric analysis following Coomassie staining and protein content. Treatment with 0.1% sodium hydroxide decreased the protein content, and accordingly $b^*$ value, for each of the starch types. However, the amount of protein extracted by sodium hydroxide was different for each starch type, and we hypothesize that this difference reflects the variation in hydrophobicity among the SSPs of each starch. Further, we identified proteins including starch synthases and storage proteins among the SSPs. Storage proteins are commonly hydrophobic, which may explain why SSP content affects the pasting properties of starch granules.

REFERENCES


Table 2 Identification of various SSPs with LC/MS/MS

<table>
<thead>
<tr>
<th>Samples</th>
<th>Band*</th>
<th>Identified protein</th>
<th>Data base accession number</th>
<th>Matched peptides</th>
<th>MASCOT score</th>
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<tr>
<td>Rice</td>
<td>1</td>
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<td>2</td>
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<td>119395188</td>
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<td>79</td>
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<tr>
<td></td>
<td>4</td>
<td>prolamin [Oryza sativa japonica Group]</td>
<td>gi</td>
<td>1842176</td>
<td>20</td>
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<td>Maize</td>
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<td>granule-bound starch synthase precursor [Zea mays]</td>
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<td>33321047</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>gamma zein [Zea mays]</td>
<td>gi</td>
<td>168695</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>legumin 1 [Zea mays]</td>
<td>gi</td>
<td>16305144</td>
<td>13</td>
</tr>
<tr>
<td>Wheat</td>
<td>8</td>
<td>granule-bound starch synthase precursor [Triticum aestivum]</td>
<td>gi</td>
<td>4760582</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>globulin 3 [Triticum aestivum]</td>
<td>gi</td>
<td>215398470</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>alpha-amylose inhibitor [Triticum aestivum]</td>
<td>gi</td>
<td>123963</td>
<td>8</td>
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<tr>
<td>Sweet</td>
<td>11</td>
<td>granule-bound starch synthase precursor [Ipomoea batatas]</td>
<td>gi</td>
<td>291461608</td>
<td>5</td>
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<td>potato</td>
<td>12</td>
<td>sporamin A [Ipomoea batatas]</td>
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<td>215398470</td>
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<tr>
<td></td>
<td>13</td>
<td>sporamin A precursor [Ipomoea batatas]</td>
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<td>168278</td>
<td>6</td>
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<tr>
<td>Potato</td>
<td>14</td>
<td>granule-bound starch synthase isoform X2 [Solanum tuberosum]</td>
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<td>565353704</td>
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<tr>
<td></td>
<td>16</td>
<td>aspartic proteinase inhibitor homologue [Solanum tuberosum]</td>
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<td>cysteine protease inhibitor 7 [Solanum tuberosum]</td>
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<td>20137684</td>
<td>4</td>
</tr>
</tbody>
</table>

*15, 17 and 19 bands were no significant hits.
(7) Identification of Various SSPs 241


7) IMBERTY, A., BULÉON, A., TRAN, V. and PEREZ, S.: Recent advances in knowledge of starch structure, Starch/Stärke, 43 (10), 375~384 (1991)


コメ, ツウモロコシ, コムギ, サツマイモおよびジャガイモデンプン粒表層タンパク質の抽出と同定
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佐藤広顕**・高野克己**

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デンプン粒は、起源によって粒子径, 形状, アミロース含量およびデンプン分解酵素の作用性が異なることで性状の違いがある。さらにデンプン粒の性状にデンプン粒表層のタンパク質（SSP）の関与が推察されており, SSPを減少させたデンプンは糊化特性に変化が生じるこ
とが報告されている。しかし、SSPを構成するタンパク質に関する研究例は少ない。そこで私たちは、コメ、トウモロコシ、コムギ、サツマイモおよびジャガイモのSSPを同定することを試みた。デンプン粒のタンパク質含有量は起源によって異なり、その値はデンプン粒のクマシープリリアントブルー（CCB）による染色度（b*値）と高い相関がみられた。0.1%水酸化ナトリウムによるデンプン粒中のタンパク質の減少率はデンプンの起源によって異なり、コメ、サツマイモ、ジャガイモ、トウモロコシ、コムギの順で高かった。SDS-PAGEにより、各起源のSSPが19のタンパク質バンドに分離され、そのうち16のタンパク質を同定した。その結果、各起源のSSPからデンプン合成酵素が同定されたほか、コムギSSPからはαアミラーゼ、ジャガイモからはアスパラギン酸およびシステインプロテアーゼが同定された。また、コメからはグルテリンおよびプロラミン、トウモロコシからはゼイン、コムギからはジロシン、サツマイモからはスポラミンといった貯蔵タンパク質が同定された。一般的に貯蔵タンパク質は熟水性の高いタンパク質であり、これら貯蔵タンパク質が各起源のデンプン粒の性状に影響を与えていいる可能性が示唆された。

（平成28年8月18日受付、平成28年9月21日受理）