Effects of Degree of Branching on Dispersion Stability of Phytoglycogen in Aqueous Solution

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It was supposed that the solubility of (1→4)(1→6)-linked α-D-glucans, e.g., glycogen, phytoglycogen, and amyllopectin, in water was related to the colloidal dispersion stability of such molecules and depended on the degree of branching. Since phytoglycogen has various degrees of branching according to the maturation of plant seeds, it was extracted from sweet corn kernels at several days after pollination (DAP), and we have investigated the effects of the degree of branching on the dispersion stability of phytoglycogen (DSP) by salting out, using ammonium sulfate (\((\text{NH}_4)_2\text{SO}_4\)).

As the sweet corn kernels matured, the concentration of \((\text{NH}_4)_2\text{SO}_4\) needed for salting out of phytoglycogen increased. According to the degree of branching measured by periodate oxidation analysis and β-amylolysis, the fraction of phytoglycogen precipitated at the high concentration with \((\text{NH}_4)_2\text{SO}_4\) has a highly branched structure. The turbidity of phytoglycogen aqueous solution was also measured to discuss the relationship between the dispersion stability of colloidal particles and the degree of branching. We found that the variation of the degree of branching is closely related to DSP in an aqueous solution.

Key words: phytoglycogen; salting out; colloidal particle; dispersion stability

Glycogen is a collective name given to a group of reserve polysaccharides included in most animal cells, and it has the structure of \((1→4)(1→6)-\text{linked α-D-glucans}\). It is well known that glycogen has a similar molecular structure to amyllopectin, which exists as a reserve polysaccharide in most plants, but the degree of branching, viz. amounts of \((1→6)-\text{α-D-glucoside linkages}\), in glycogen is about twice that in amyllopectin\(^{1−4}\). The former dispersions well homogeneously in cold water, while the latter is distributed inhomogeneously in it. Furthermore, there are water-soluble polysaccharides isolated from some plants such as sweet corn, and other sources\(^1−7\). The polysaccharides like glycogen from plants are called phytoglycogens.

The exact structures of such polysaccharides were investigated by many workers. Haworth\(^8\) and Staudinger\(^9\) first explained them as ‘laminated’ and ‘comb’ form, respectively. Meyer\(^10\) proposed a different molecular model for \((1→4)(1→6)-\text{linked α-D-glucans}\). The model of a multiply branched tree-like structure by Meyer is now believed to be correct because enzymic studies by workers in other laboratories supported it\(^11\).

Since glycogen and phytoglycogen have a larger number of branching points than amyllopectin, their molecular structures are almost ‘spherical’. Moreover, solutions containing glycogen or phytoglycogen are opalescent because of colloidal dispersion. In a recent paper, glycogen or phytoglycogen was fractionated by using gel filtration and ultracentrifugation to evaluate molecular weight and average chain length (CL)\(^12\). These methods are based on colloidal dispersion, but nobody has focused on the dispersion stability of glycogen or phytoglycogen in the form of colloidal particles. It was supposed that the solubility of \((1→4)(1→6)-\text{linked α-D-glucans}\) in water was related to the colloidal dispersion stability of such molecules and which depended on the degree of branching. It seemed that the dispersion stability of colloidal particles of glycogen or phytoglycogen molecule mainly depends on particle size and hydrogen bonding between molecules and water.

Phytoglycogen has various degrees of branching during maturation of plant seeds. In this study, the effects of degrees of branching on the dispersion stability of phytoglycogen were investigated using the phytoglycogen that was extracted from sweet corn kernels at several DAP and fractionated with \((\text{NH}_4)_2\text{SO}_4\). Then, the structural change of phytoglycogen is discussed as the degree of ripeness.

Materials and Methods

Plant materials. Zea mays (cv. Golden Cross Bantam) was grown on an experimental farm at Shinshu University (Minamininoawa, Nagano, Japan). Kernels at 20, 25, 30, and 40 DAP were harvested and immediately stored at −20°C.

Extraction of phytoglycogen. Sweet corn kernels were homogenized with three volumes of a 5% \(\text{HClO}_4\) aqueous solution at 10°C for 10 min. After centrifugation at 5000 rpm for 30 min, precipitates were discarded and supernatant solution was precipitated with two volumes of ethanol. Sediment was dispersed again with distilled water, and then the supernatant solution was centrifuged at 5000 rpm for 20 min. After the centrifugation, phytoglycogen was obtained from the supernatant solution.

Fractionation with ammonium sulfate. Phytoglycogen (2 g) was fractionated with distilled water (100 ml) and fractionated with pulverized \((\text{NH}_4)_2\text{SO}_4\) at 60, 65, 70, 75, and 80% saturation at 25°C for 10 min in a flask containing a magnetic stirrer bar under stirring conditions. After centrifugation at 10,000 rpm for 15 min, the precipitates were dispersed again with distilled water and dialyzed against flowing water. After 72 hours, phytoglycogen was precipitated using ethanol and acetone and dried in a water bath at 90°C. The dry phytoglycogen was weighed by a chemical balance.

Measurement of the average chain length (CL). The average chain length was measured for periodate oxidation analysis. Phytoglycogen (200 mg) was dispersed with distilled water (20 ml) and added sodium periodate aqueous solution (0.2 M, 20 ml). The solution was reacted at 2°C for
24 hours in the dark, treated with ethylene glycol, and titrated in a CO₂-free atmosphere against 0.01 M NaOH. A blank experiment was done at the same time under the same conditions.

Measurement of the β-amylolysis limit. Phytoglycogen (20 mg) was dispersed with sodium acetate-acetic acid buffer (0.05 M, pH 4.8, 5 ml) and added same buffer (5 ml) containing with β-amylase (20 U). This solution was incubated at 37°C for 8 h in a water bath. Reducing power (as maltose) was measured by the Somogyi-Nelson method.15

Turbidity of phytoglycogen aqueous solution. Phytoglycogen (25 mg) was dispersed with distilled water. This solution (0.5 wt%) was heated at 50°C for 10 min, then kept at 25°C for 8 h in a water bath. Turbidity was measured every hour by the optical density at 400 nm.

Results
Fractionation with ammonium sulfate

Protein can be separated and purified by salting out using (NH₄)₂SO₄.3 Various structures in proteins are linear polyelectrolytes, so several factors seem to exist in terms of the solubility of proteins in water. In contrast, DSP with salt in an aqueous solution may be dominated by the degree of branching and the size of particles because phytoglycogen is a multiply branched nonionic molecule, thus the structure is limited. Since the hydrogen bonding between the surface of phytoglycogen particles and water becomes weaker with an increase of salt concentration, it seemed that phytoglycogen precipitated with the low (NH₄)₂SO₄ concentration dispersed more inhomogeneously than that precipitated with a high concentration. Therefore, we have investigated DSP at several DAP in an aqueous solution.

Figure 1 shows fractionation of phytoglycogen as a function of (NH₄)₂SO₄ concentration. Phytoglycogen did not precipitate below the (NH₄)₂SO₄ 60% saturated solution, regardless of the development of maturation. The precipitates were first found for all samples in the 65% saturation. The opalescence of the supernatant solution was finally extinguished with the 75% (20 and 25 DAP) and 80% (30 and 40 DAP) saturation. In Fig. 1, I, II, III, and IV indicate the fraction of phytoglycogen precipitated with 65, 70, 75, and 80% (NH₄)₂SO₄ saturation, respectively. It seemed that phytoglycogen at the same fraction number indicates almost the same dispersion stability in aqueous solution, because the concentration of (NH₄)₂SO₄ is same. As the ripening proceeded, the amount of phytoglycogen precipitated with low concentrations of (NH₄)₂SO₄ decreased and that with high concentrations increased. Since the concentration of salts is closely related to the dispersion stability of particles as described above, DSP increases with the development of maturation.

Relation between degree of branching and DSP

The structural features of glycogen and phytoglycogen have been investigated by several methods for many years. Particularly, CL values and β-amylolysis limit were measured by a lot of workers in terms of focusing on the degree of branching in glycogen and phytoglycogen.1-7 Accordingly, we have investigated the relationship between the degree of branching and DSP.

CL values were measured by periodate oxidation, as shown in Table I. In general, the degree of branching in phytoglycogen increases with decreasing the CL value. According to the whole value, as the ripening increased, the degree of branching in phytoglycogen increased (CL values decreased). At the same degree of maturation, highly branched phytoglycogen dispersed more homogeneously in an aqueous solution than that of low branching. The degree of branching of phytoglycogen, fractionated with the equal concentration of (NH₄)₂SO₄ (each fraction I, II, III, and IV), tended to increase as the maturation developed except 40 DAP, and the CL value of each fraction was mostly large compared with the whole value. It suggested the existence of highly branched or small particles, not precipitated till 75% (20 and 25 DAP) and 80% (30 and 40 DAP) saturation of (NH₄)₂SO₄. β-Amylolyis of phytoglycogen is shown in Table II. In general, the degree of branching in phytoglycogen increases with the decreasing β-amylolysis limit. The results of β-amylolyis also indicated that highly branched molecules

| Table I. Average Chain Length (CL) for Periodate Oxidation Analysis |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
|                             | I               | II              | III             | IV              | Whole           |
| 20 DAP                      | 15.3            | 14.3            | 13.8            | 13.7            |
| 25 DAP                      | 14.5            | 13.8            | 13.3            | 13.3            |
| 30 DAP                      | 15.0            | 13.1            | 12.6            | 11.8            |
| 40 DAP                      | 16.0            | 13.8            | 13.0            | 12.4            | 11.5            |

DAP means days after pollination. I, II, III, and IV indicate the fraction of phytoglycogen precipitated with 65, 70, 75, and 80% (NH₄)₂SO₄ saturation, respectively. Whole shows a CL value of phytoglycogen before it was fractionated at several DAP.

| Table II. β-Amylolyis of Phytoglycogen (wt%) |
|--------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| I                                          | II              | III             | IV              | Whole           |
| 20 DAP                                     | 52              | 53              | 51              | 53              |
| 25 DAP                                     | 51              | 47              | 47              | 49              |
| 30 DAP                                     | 45              | 43              | 42              | 35              |
| 40 DAP                                     | 42              | 47              | 41              | 38              |

DAP means days after pollination. I, II, III, and IV indicate the fraction of phytoglycogen precipitated with 65, 70, 75, and 80% (NH₄)₂SO₄ saturation, respectively. Whole shows the β-amylolyis limit of phytoglycogen before it was fractionated at several DAP.

Fig. 1. Fractionation Profiles with Ammonium Sulfate of Phytoglycogen from Sweet Corn Kernels at (○) 20 DAP; (+) 25 DAP; (△) 30 DAP; and (□) 40 DAP.

The amount of fractionated precipitates in total precipitates (wt%) is plotted vertically, and the concentration of ammonium sulfate (% saturation) is plotted horizontally. I, II, III, and IV indicate the fraction of phytoglycogen precipitated at 65, 70, 75, and 80% (NH₄)₂SO₄ saturation, respectively.
Disperse homogeneously in an aqueous solution. However, Β-amylyolysis of fraction I was smaller than that of fraction II at 40 DAP. It seemed that Β-amylyolysis of low branched phytylgycogen was not completed under these conditions. 4)

Measurement of turbidity of phytylgycogen aqueous solution

Iodine staining has been widely used in studies of polysaccharides,4,6 but this method is unsuitable for quantitative analysis because values of λ_max depends on substances. The aqueous solution of phytylgycogen is opalescent due to colloidal dispersion. Therefore, turbidity may reveal the size and the degree of agglomeration of phytylgycogen particles in an aqueous solution.

The turbidity of the phytylgycogen aqueous solution at each DAP is shown in Fig. 2. It seemed that phytylgycogen particles had negligible absorption of visible rays (data not shown), thus, we measured the optical density at 400 nm as light scattering, and measured turbidity. In Fig. 2, turbidity is plotted as a function of time. As the development of the maturation, turbidity tended to decrease. It suggested that the whole phytylgycogen particles in an aqueous solution became smaller as the maturation developed. And turbidity also decreased with the passage of time except for 40 DAP. At the early stage, phytylgycogen particles aggregated or agglomerated but they were separated with time. In contrast, little variation of turbidity was observed in terms of phytylgycogen aqueous solution at 40 DAP. It seemed that whole phytylgycogen particles at this stage may be difficult to aggregate and immediately disperse homogeneously in water because they have a more highly branched structure than particles at other maturation stages.

Table III shows the turbidity in the phytylgycogen aqueous solution of each fraction, measured by the same procedure as in Fig. 2. Compared with the fraction of I, II, III, and IV at the same DAP, the turbidity of the solution containing short CL particles tended to be smaller. For the particles of same fraction number, as the CL value decreased the turbidity tended to increase.

Discussion

The existence of many factors have been investigated to clarify the dispersion stability of colloidal particles. In this study, it seemed that the size and the agglomeration of particles in water mainly affect DSP. In this section, a multiply branched spherical structure was assumed for a particle of phytylgycogen, and we have discussed the effects of the degree of branching on DSP in an aqueous solution.

First, we discussed the effects of the size of the dispersoid. Small colloidal particles may disperse more homogeneously than large particles, because the size of particles have been closely related to the sedimentation velocity and brownian motion.

Secondly, we considered how hydrogen bonding between the surface of phytylgycogen molecules and water might contribute to DSP. When the hydrogen bonding is strong, the hydrophilicity of the phytylgycogen particles become strong. Since the agglomeration of those particles may be difficult, those particles may be difficult to precipitate.

The schematic representations of spherical phytylgycogen particles in an aqueous solutions are shown in Fig. 3(a), (b), and (c). As is shown in Fig. 3(a), large phytylgycogen particles tend to precipitate with the lower concentration of (NH₄)₂SO₄ compared with small phytylgycogen particles. The size of phytylgycogen particles depends on not only molecular weight but existence of entangled particles, because if the molecule has a long CL, external and internal chain length are also long. Hence, as described in Fig. 2, a molecule tends to get entangled easily with other molecules. Therefore, colloidal particles having large CL show large size and precipitate with a lower concentration of (NH₄)₂SO₄ compared with those of small CL values.

As is also shown in Fig. 3(b), phytylgycogen is a high molecular weight polymer consisting of α-D-glucose and multifunctional compounds containing a large number of hydroxyl groups. Therefore, all glucose residues are regarded as hydrophilic parts, hydrogen bonding of the surface of particles with a high degree of branching becoming stronger, and these molecules show higher hydrophilicity than those of lower branched phytylgycogen.

For the turbidity of phytylgycogen aqueous solution, there are two factors, as described above. As is shown in Tables I, II, and III, at the same fraction, the turbidity and the degree of branching were different in spite of the concentration of (NH₄)₂SO₄ in an aqueous solution being the same. Figure 3(c) shows phytylgycogen particles with long CL and small size dispersed homogeneously similar to that of short CL and large size. As is shown in Table

Fig. 2. Turbidity of the Aqueous Solution of Phytylgycogen from Sweet Corn Kernels at (C) 20 DAP; (+) 25 DAP; (△) 30 DAP; and (□) 40 DAP as a Function of Time. The concentration of phytylgycogen is fixed, 0.5% (w/w). Optical density was measured at 400 nm.

Table III. Turbidity of Various Fraction of Phytylgycogen in Aqueous Solutions

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 DAP</td>
<td>0.429</td>
<td>0.200</td>
<td>0.169</td>
<td>0.295</td>
<td></td>
</tr>
<tr>
<td>25 DAP</td>
<td>0.935</td>
<td>0.272</td>
<td>0.222</td>
<td>0.296</td>
<td></td>
</tr>
<tr>
<td>30 DAP</td>
<td>0.965</td>
<td>0.311</td>
<td>0.241</td>
<td>0.238</td>
<td>0.257</td>
</tr>
<tr>
<td>40 DAP</td>
<td>1.587</td>
<td>0.291</td>
<td>0.176</td>
<td>0.170</td>
<td>0.261</td>
</tr>
</tbody>
</table>

DAP means days after pollination. I, II, III, and IV indicate the fraction of phytylgycogen precipitated with 65, 70, 75, and 80% (NH₄)₂SO₄ saturation, respectively. Whole shows the value before it was fractionated at several DAP. These values indicate the optical density of phytylgycogen aqueous solutions measured after keeping at 25°C for 8 hours.
pectin are similar, but amyllopectin has lower degrees of branching than glycogen or phyoglycogen and it is not dissolved in water. The cluster model\(^{17}\) and other models\(^{18,19}\) of amyllopectin, glycogen, and phyoglycogen particles have been proposed to explain the different solubility, but we consider that other factors exist for the dispersion stability of such substances as mentioned above. Consequently, in this study, we concluded that the variation of the degree of branching are closely related to the dispersion stability of (1→4)(1→6)-linked α-D-glucans in an aqueous solution.

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References