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# Structure and solution properties of enzymatically synthesized glycogen

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ABSTRACT

Recently, a new enzymatic process for glycogen production was developed. In this process, short-chain amylose is used as a substrate for branching enzymes (BE, EC 2.4.1.18). The molecular weight of the enzymatically synthesized glycogen (ESG) depends on the size and concentration of the substrate. Structural and physicochemical properties of ESG were compared to those of natural source glycogen (NSG). The average chain length, interior chain length, and exterior chain length of ESG were 8.2–11.6, 2.0–3.3, and 4.2–7.6, respectively. These values were within the range of variation of NSG. The appearances of both ESG and NSG in solution were opalescent (milky white and slightly bluish). Furthermore, transmission electron microscopy and atomic force microscopy showed that ESG molecules formed spherical particles, and that there were no differences between ESG and NSG. Viscometric analyses also showed the spherical nature of both glycogens. When ESG and NSG were treated with pullulanase, a glucan-hydrolyzing enzyme known to degrade glycogen only on its surface portion, both glycogens were similarly degraded. These analyses revealed that ESG shares similar molecular shapes and surface properties with NSG.

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## 1. Introduction

Glycogen is the major storage polysaccharide in animals, fungi, yeast, bacteria, and archaea. Glycogen is a highly branched (1→4)(1→6)-linked α-D-glucan with a high molecular weight ($10^6$–$10^9$).[1,2] Based on the results of electron microscopy, it has been shown that glycogen consists of spherical particles with diameters of 20–40 nm (β-particles), and that the β-particles often associate into much larger α-particles (60–200 nm).[2–4] The molecular weight of an individual β-particle has been shown to be approximately $10^7$.[1,2] Glycogen is opalescent (milky white and slightly bluish) in aqueous solution, and turns a reddish brown color with the addition of iodine.[2] Amylopectin, a major component of starch, is also a highly branched (1→4)(1→6)-linked α-D-glucan. However, the degree of branching, namely, the number of (1→6)-α-D-glucosidic linkages, in glycogen is about twice that in amylopectin. A glycogen-type polymer, which is referred to as phytoglycogen, has also been isolated from several higher plants. In animal tissues, glycogen is synthesized from UDP-glucose via the cooperative action of glycogenin (EC 2.4.1.186), glycogen synthase (EC 2.4.1.11), and branching enzyme (BE, 1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-glucano)-transferase, EC 2.4.1.18).

Although isolation and purification of glycogen from natural sources are laborious and costly processes, some applications of this polymer have been developed. For example, glycogen isolated from shellfish or animal tissues has been used to stimulate the exudation of phagocytic cells into the peritoneal cavities of experimental animals in immunological studies.[5,6] It has also been reported that glycogen has an antitumor effect, probably through its immunomodulating activity.[7,8] Such activity may suggest a health benefit from the consumption of glycogen as a food ingredient. Glycogen has also been used as a raw material in the cosmetic industry, and as a carrier to enhance the yield of DNA during precipitation with organic solvent.[9]

The in vitro synthesis of glycogen has been successfully achieved by the combined action of α-glucan phosphorylase (GP, EC 2.4.1.1) and BE using glucose-1-phosphate (G-1-P) as a substrate (GP-BE method).[10–12] The enzymatically synthesized glycogen (ESG) produced by this method has properties similar to those of natural source glycogen (NSG).[13] Although G-1-P is expensive for industrial applications, it could be obtained from the inexpensive substrate sucrose using sucrose phosphorylase (EC 2.4.1.7).[14] Furthermore, we have demonstrated that the ESG produced from sucrose has immunomodulating activity.[15] However, the yield of glycogen from sucrose was less than 40%, and a more efficient method was required to meet the demand for glycogen for industrial applications.

Recently, we developed a new enzymatic process for glycogen production.[16] In this process, short-chain amylose derived from starch is used as a substrate for BE. The BE from the hyperthermophilic bacteria, *Aquifex aeolicus*, was the most suitable for the

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production of ESG of a similar molecular weight to NSG (approximately $10^7$) among the eight types of BE tested. We also found that the addition of amylomaltase (AM, EC 2.4.1.25) significantly enhanced the efficiency of this process, and the yield of glycogen reached approximately 65%. ESG produced by the new 'IAM-BE-AM' method also has similar structural properties to NSG.[16,17] In other words, both glycogens were constructed from similar-sized α-1,4 chains (9–12 glucosyl units on average), which were connected via α-1,6 linkages at their reducing ends to other chains. In spite of having the same architecture, we found slight structural differences between ESG and NSG.[17] First, the unit-chain distribution (distribution of degrees of polymerization (DP) of α-1,4 linked chains) of ESG was slightly narrower than that of NSG. Second, ESG and NSG were degraded differently by α-amylase: the final products of hydrolysis of NSG by α-amylase were glucose, maltose, maltotriose, branched oligosaccharides with DP of 4–7, and highly branched macrodextrin molecules with molecular weights of up to 10,000, whereas those of ESG were glucose, maltose, maltotriose, and much larger macrodextrins (molecular weight >$10^6$). These results suggested that the α-1,6 linkages in ESG molecules were arranged at more regular intervals than those in NSG molecules, preventing the α-amylase from acting in the core region of the ESG molecule. However, it has been still unclear whether there are any differences between physicochemical properties of ESG and NSG or not. Physicochemical properties are considered to be much more important than internal structures for developing applications of ESG. In this paper, we first confirmed the differences in internal structures between ESG and NSG[17] by using a series of ESG samples which were produced from different conditions for productions. Then, we compared certain properties of ESG with those of NSG, which are considered to reflect the surface and subsurface characteristics of polysaccharides mainly solution properties, granular shapes, viscosity, profile following pullulanase treatment, and bound water content.

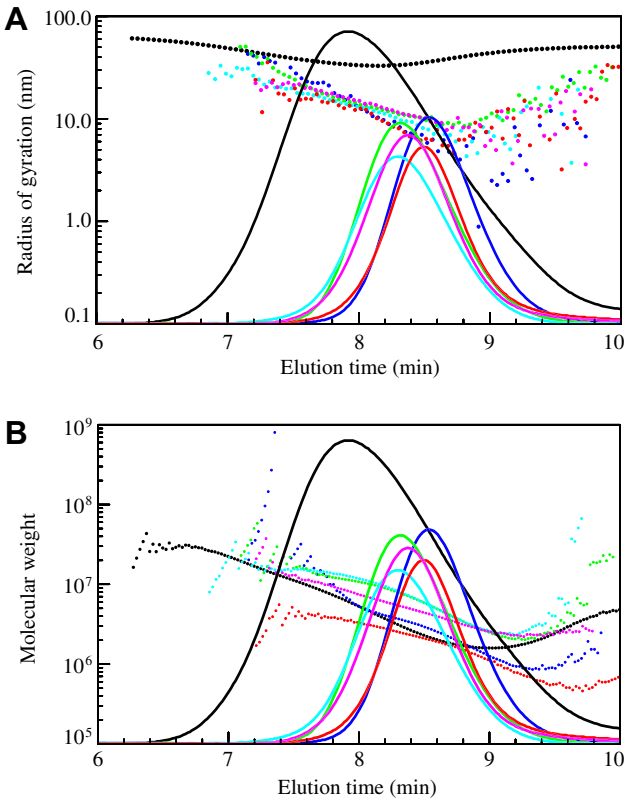


**Figure 1.** Typical HPLC chromatograms of ESG and NSG. Solid lines are refractive index detector response of partially hydrolyzed amylopectin (black), ESG A (blue), ESG C (yellow green), ESG D (light blue), NSG from mussel (red), and NSG from bovine liver (pink). Dots show radii of gyration (A) and molecular weights (B) calculated from the RI and MALLS responses at the respective elution points.

## 2. Results

### 2.1. Structural parameters of ESG and NSG

We prepared nine types of ESGs (A–I) with narrow molecular weight distributions as described in Section 4. Firstly, ESGs (A–I) and NSGs were subjected to HPSEC-MALLS-RI analysis. The radius of gyration (Fig. 1A) and molecular weight (Fig. 1B) at each elution point were calculated and plotted on the chromatograms. The plots of radii for ESG and NSG roughly formed into a line, whereas those for amylopectin were separate from the line. This result implied that ESG and NSG molecules similarly behaved in a solution but amylopectin did not. The $M_w$ of ESGs ranged from $3.0 \times 10^6$ to $3.0 \times 10^7$ (Table 1). Secondly, we compared the structural parameters of ESGs with those of NSGs. Three parameters, that is, the average chain length (CL), the exterior chain length (ECL), and the interior chain length (ICL), have often been used to compare the structure of glucose polymers.[2,18] The CLs, ECLs, and ICLs of ESGs (A–I) tended to be slightly shorter than those of NSGs (Table 1). However, the values were considered to be within the variation of the reported values for NSG, since the structures of NSGs depend on the source and extraction method and have structural heterogeneity.[2,19,20] Thirdly, we confirmed the wave-length with maximum absorption ($\lambda_{max}$) of ESGs (A–I) and NSGs. Amylopectin and amylose give a $\lambda_{max}$ at 520–540 nm and 690 nm, respectively.[21] Unlike amylopectin and amylose, ESGs (A–I) and NSGs gave a $\lambda_{max}$ ranged from 398 to 467 nm (Table 1). Fourthly, we performed an α-amylase degradation test. Huge macrodextrin molecules of around 20% of the original size were detected in all ESG preparations after extensive treatment with α-amylase (data not shown). The results were consistent with the previous results for four other types of ESG.[17] Thus, we concluded that these nine types of ESG had the same architecture as NSG but with more regularly arranged α-1,6 linkages in their internal structure compared with NSG, as described in our previous paper.[17]

### 2.2. Appearance of aqueous solution

Figure 2 shows the comparison of the solutions of amylopectin, dextrin, NSG from rabbit liver, and ESG F. The solution of amylopectin was cloudy, whereas the solution of dextrin was clear/ colorless. In contrast, the solutions of NSG from rabbit liver and ESG F were opalescent (milky white and slightly bluish), as described by Manners.[2] All ESGs listed in Table 1 were readily soluble in water and the aqueous solutions were also opalescent, resembling NSGs (data not shown).

The larger the molecular size, the deeper were the opalescent colors of the aqueous solutions of ESGs and NSGs (data not shown). The concentrations of ESG and NSG also affected the depth of color (data not shown). In the relatively low concentration range (1–10% (w/v)), the higher the concentration, the deeper was the color. However, in the high range (10–20% (w/v)), the color of the solution became lighter. Similar phenomena were also observed for the NSG solutions (data not shown). The bluish white color of glycogen is considered to be resulted from irregular scattering of visible light by particles with appropriate sizes. Therefore, the lighter color could be obtained from particle solutions with higher uniformity. In a concentrated solution of glycogen, the uniformity may increase by steric hindrance of particles making the color of the solution lighter. Further investigations are needed for better understanding of this phenomenon.

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