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# Improving size-exclusion chromatography separation for glycogen

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ABSTRACT

Glycogen is a hyperbranched glucose polymer comprised of glycogen β particles, which can also form much larger composite α particles. The recent discovery using size-exclusion chromatography (SEC) that fewer, smaller, α particles are found in diabetic-mouse liver compared to healthy mice highlights the need to achieve greater accuracy in the size separation methods used to analyze α and β particles. While past studies have used dimethyl sulfoxide as the SEC eluent to analyze the molecular size and structure of native glycogen, an aqueous eluent has not been rigorously tested and compared with dimethyl sulfoxide. The conditions for SEC of pig-liver glycogen, phytoglycogen and oyster glycogen were optimized by comparing two different eluents, aqueous 50 mM $NH_4NO_3$/0.02% $NaN_3$ and dimethyl sulfoxide/0.5% LiBr, run through different column materials and pore sizes at various flow rates. The aqueous system gave distinct size separation of α- and β-particle peaks, allowing for a more detailed and quantitative analysis and comparison between liver glycogen samples. This greater resolution has also revealed key differences between the structure of liver glycogen and phytoglycogen.



## 1. Introduction

Glycogen functions as a glucose storage molecule in a wide range of organisms, ranging from bacteria to animals, while some plant varieties have a structurally similar glucan termed phytoglycogen (which may also play a role in starch biosynthesis).

Both glycogen and phytoglycogen consist of linear chains of α-(1 → 4)-linked D-glucose residues, with branching points being connected via α-(1 → 6) glycosidic linkages. Glycogen comprises smaller molecules, termed β particles (~20 nm in diameter with molecular weights ~$10^6$–$10^7$) [1,2] that can also form much larger molecules, termed α particles (anywhere between 40 and 300 nm in diameter with molecular weights reaching over $10^8$) [3,4]. In animals, glycogen is found in a number of organs, performing various functions. Liver glycogen is essential in maintaining blood-glucose homeostasis [5], whereas muscle glycogen provides rapid energy during muscular activity [6]. While muscle glycogen consists of β particles, liver [7] and cardiac [8] glycogen has been shown to contain α particles. These larger molecules are also seen in phytoglycogen [9]. It is also noted that glycogen is not simply a polysaccharide, as there is extensive evidence that all glycogens contain small but significant amounts of protein [10,11].

Insight can be gained into the biosynthesis and degradation of glycogen by analyzing glycogen's macromolecular structure. Size-exclusion chromatography (SEC) has been successfully used to determine size distributions of starch and glycogen, as recently reviewed [12–15], which has resulted in the discovery that db/db mice (a model for type 2 diabetes) have impaired α particle formation [16]. Given the greater ratio of surface area to volume of smaller molecules, it has been hypothesized that impaired α-particle formation, all other things being equal, may impact on blood–glucose homeostasis [8,16].

While dimethyl sulfoxide (DMSO)/LiBr has been used as the SEC solvent in these past studies to characterize glycogen structure, to date aqueous-SEC has not been employed for native liver glycogen, although some encouraging analysis has been performed on commercial oyster and rabbit-liver glycogen [17]. The DMSO/LiBr system has been employed in the past because it has been shown that this dissolves amylose and amylopectin (the two types of glucans in starch, with the same glycosidic linkages as in glycogen) molecularly and without aggregation [18]. However, there are a number of potential benefits of using an aqueous system: the lower

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viscosity of water should lead to better resolution and reduction of shear scission; the characterization is more physiologically relevant as glycogen is in an aqueous solvent *in vivo*; and water is a much cheaper and safer solvent than DMSO. Aqueous SEC has recently been successfully used for synthetic branched polysaccharides (with similar size ranges to glycogen), where better separation was found compared to a DMSO setup [19]. Additionally, the presence of small amounts of proteins in glycogen will affect the solubility of this molecule in water- and DMSO-based systems; if, as is usually the case, the proteins are predominantly hydrophilic on the surface, water solubilization will be increased. In this present study, the size separation of glycogen from pig liver, *sugary-1* (*su-1*) mutant maize grain (termed phytoglycogen) and oyster glycogen were analyzed using both aqueous (50 mM $NH_4NO_3$/0.02% $NaN_3$) and DMSO/LiBr SEC. Differential refractive index detection was used alone, as the objective of improved separation is not aided by further knowledge (which would be useful for mechanistic interpretation) that would result from having additional detectors.

## 2. Method

### 2.1. Glycogen extraction and purification

#### 2.1.1. Pig-liver glycogen

Pig-liver glycogen was extracted as previously described [20]. A sample from the central lobe of the liver (~25 g) from a 106-day old male pig (Large White breed), reared at the University of Queensland Centre for Advanced Animal Science, was immediately frozen in dry ice and kept at −80 °C for 6 weeks prior to glycogen extraction. Liver (~4 g) was homogenized with 5 volumes of glycogen isolation buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, and phenylmethanesulfonylfluoride (PMSF)). The homogenate was centrifuged at 6000 × *g* for 10 min at 4 °C with the resulting supernatant then being centrifuged at 50,000 × *g* for 30 min at 4 °C. The pellet was resuspended in glycogen isolation buffer (3 mL) and layered over an 18 mL, stepwise sucrose gradient (25%, 50%, and 75% in glycogen isolation buffer). The gradient was then centrifuged at 300,000 × *g* for 2 h at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 mL of 80% ethanol. The sample was then centrifuged at 4000 × *g* for 10 min at 4 °C and the supernatant was discarded. This ethanol precipitation step was repeated once more and the pellet was dissolved in 1 mL of deionized water and then lyophilized (freeze-dried; VirTis, Benchtop K).

#### 2.1.2. Phytoglycogen

Extraction of phytoglycogen was performed following a technique developed in our laboratories, as done previously [21]. Kernels of *su-1* mutant maize, obtained from Prof. Ian D. Godwin (The University of Queensland, Brisbane, Australia), were ground into a fine powder using a cryo-mill (Freezer/Mill 6870, SPEC CertiPrep, Metuchen, NJ, USA) that used a 1 min precooling step followed by 5 min grinding. This technique is used to minimize mechanical and thermal damage and has been shown to be effective for starch extraction [22]. After grinding, 100 mg of kernel flour was incubated in 2.5 mL of tricine buffer for 30 min at 37 °C with protease (2.5 units $mL^{-1}$; bacterial type XIV, Sigma-Aldrich). An additional 2.5 mL of ice-cold tricine buffer was added to the sample, followed by centrifugation at 4000 × *g* for 10 min. The supernatant was precipitated with 4 volumes of absolute ethanol and centrifuged for an additional 10 min at 4000 × *g*. The pellet was dissolved in 1 mL of deionized water and then lyophilized (freeze-dried; VirTis, Benchtop K).

**Table 1**
Column information.

| Solvent | Column | Particle size (μm) |
|---|---|---|
| Aqueous | Suprema 30 | 5 |
| | Suprema 1000 | 5 |
| | Suprema 3000 | 5 |
| | Suprema 10,000 | 10 |
| DMSO | GRAM 30 | 10 |
| | GRAM 1000 | 10 |
| | GRAM 3000 | 10 |
| | GRAM 10,000 | 10 |

#### 2.1.3. Oyster glycogen

Oyster type II glycogen was purchased from Sigma-Aldrich. This was used as a comparative tool as it consists only of β particles [20].

### 2.2. Size-exclusion chromatography using dimethyl sulfoxide (DMSO)/LiBr as an eluent

Pig-liver glycogen, phytoglycogen and oyster glycogen were dissolved (2 g $L^{-1}$) in DMSO with 0.5 wt% LiBr on a thermomixer at 80 °C and 350 rpm overnight.

Samples were injected into an Agilent 110 Series SEC system (PSS, Mainz, Germany) using two different column setups: GRAM preColumn, 30 and 3000 columns (PSS); and GRAM preColumn, 1000 and 10,000 (PSS) (see Table 1 for column information). The columns were kept at 80 °C using a column oven and 3 different flow rates were tested (0.3, 0.6 and 0.9 mL $min^{-1}$). A refractive index detector (RID) (Shimadzu RID-10A, Shimadzu, Japan) was used to determine the SEC weight distributions. The detector temperature was 45 °C. Because SEC weight distributions are based on the relative amount of DRI signal, any small difference in the refractive index between the eluent in the column and in the detector due to a temperature difference will remain constant.

Universal calibration curves were obtained for each column setup and flow rate using pullulan standards (PSS), with a molar mass range of 1080 Da to $2.35 \times 10^6$ Da, which were directly dissolved into eluent. This allowed elution volumes to be converted into hydrodynamic volumes ($V_h$), or equivalently the hydrodynamic radius ($R_h$), where $V_h = 4/3\pi R_h^3$ [23], using the Mark-Houwink relationship (see Eq. (1)).

$$V_h = \frac{2}{5}\frac{KM^{1+\alpha}}{N_A} \quad (1)$$

The hydrodynamic radius here is defined by IUPAC as the volume of a hydrodynamically equivalent sphere [24], and thus the meaning is dependent on the particular technique used: for example, hydrodynamic radius in dynamic light scattering is a different quantity to that for SEC.

The use of universal calibration in this study is based on the assumption that SEC separates solely on hydrodynamic size, an assumption which has been shown to be valid for molecules with widely varied shapes [25–27]. As this study is aimed at improving the separation of α and β particles and very accurate values of size are not necessary for this goal, the universal calibration assumption is used here with the caveat that calibration is not absolute and may not be completely reliable for glycogen.

The Mark-Houwink parameters for pullulan in DMSO/LiBr (0.5 wt%) at 80 °C are $K = 2.427 \times 10^{-4}$ dL $g^{-1}$ and $\alpha = 0.6804$ (Kramer and Kilz, PSS, private communication; the number of significant figures is that provided by Kramer and Kilz, and are given in full to avoid the possibility of sensitivity of the data processing to these values). No uncertainty (or rather, joint confidence interval) is known for the two Mark-Houwink parameters. These values give an $R_h$ upper limit of accurate calibration of ~58 nm for this solvent.