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TITLE:

The Extraction of Liver Glycogen Molecules for Glycogen Structure Determination

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SUMMARY:

An optimal sucrose concentration was determined for the extraction of liver glycogen using sucrose density gradient centrifugation. The addition of a 10 min boiling step to inhibit glycogen-degrading enzymes proved beneficial.

ABSTRACT:

Liver glycogen is a hyperbranched glucose polymer that is involved in the maintenance of blood sugar levels in animals. The properties of glycogen are influenced by its structure. Hence, a suitable extraction method that isolates representative samples of glycogen is crucial to the study of this macromolecule. Compared to other extraction methods, a method that employs a sucrose density gradient centrifugation step can minimize molecular damage. Based on this method, a recent publication describes how the density of the sucrose solution used during centrifugation

was varied (30%, 50%, 72.5%) to find the most suitable concentration to extract glycogen particles of a wide variety of sizes, limiting the loss of smaller particles. A 10 min boiling step was introduced to test its ability to denature glycogen degrading enzymes, thus preserving glycogen. The lowest sucrose concentration (30%) and the addition of the boiling step were shown to extract the most representative samples of glycogen.

INTRODUCTION:

Glycogen is a complex, hyperbranched polymer of glucose found in animals, fungi, and bacteria[1]. In mammals, liver glycogen functions as a blood glucose buffer, preserving homeostasis, while muscle glycogen acts as a short-term glucose reservoir to provide energy directly[2]. The structure of glycogen is often described by three levels (shown in **Figure 1**): 1. Linear chains are formed by glucose monomers via (1→4)-α glycosidic bonds, with branch points being connected via (1→6)-α glycosidic bonds; 2. highly branched β particles (~20 nm in diameter) that, especially in tissues such as skeletal muscle, act as independent glycogen molecules[3,4]; 3. larger α glycogen particles (up to 300 nm in diameter) that consist of smaller β glycogen units, which are found in the liver[5], heart[6], and in some non-mammalian species[7]. Hepatic α particles from diabetic mice are molecularly fragile, with a propensity to degrade to β-particles when dissolved in dimethyl sulfoxide (DMSO), while α particles from non-diabetic controls generally remain unchanged. One hypothesis is that this fragility may exacerbate the poor blood glucose balance seen in diabetes, with the fragile α particles potentially resulting in higher proportions of the more rapidly degraded β particle[8-11].

Traditional glycogen extraction methods utilize the relatively harsh conditions of exposing the liver tissue to hot alkaline solution[12] or acid solutions such as trichloroacetic acid (TCA)[13] or perchloric acid (PCA)[14]. While effective at separating the glycogen from other components of the liver tissue, these methods inevitably degrade the glycogen structure to some extent[15,16]. Although these methods are suitable for quantitative measurement of the glycogen content, they are not ideal for studies focused on obtaining structural information on the glycogen due to this structural damage. Since the development of these methods, a milder extraction procedure has been developed that utilizes cold Tris buffer (shown to inhibit glucosidase degradation) with sucrose density gradient ultracentrifugation[17-19]. With the pH controlled at ~8, this method does not subject the glycogen to the acid or alkaline hydrolysis seen in previous procedures.

Sucrose density gradient ultracentrifugation of homogenized liver tissue can separate glycogen particles from the majority of cell material. If necessary, additional purification can be performed by preparative size exclusion chromatography, resulting in the collection of purified glycogen with attached glycogen-associating proteins[20]. Although this method, with milder conditions, is more likely to preserve the structure of glycogen, it is difficult to prevent some portion of the glycogen from being lost in the supernatant, especially smaller glycogen particles that are less dense[15]. Another potential cause of glycogen loss is that the milder conditions allow some enzymatic degradation, resulting in lower glycogen yields compared to harsher extraction methods. Recent research reported optimization of the liver-glycogen extraction method to preserve the structure of glycogen[21]. Here, various sucrose concentrations (30%,50%, 72.5%) were tested to determine

whether lower sucrose concentrations minimized the loss of smaller glycogen particles. The rationale was that the lower density would allow for smaller, less dense particles to penetrate the sucrose layer and aggregate in the pellet with the rest of the glycogen.

In this study, the extraction methods with and without a 10 min boiling step were compared to test whether glycogen degradation enzymes could be denatured, resulting in the extraction of more glycogen that was also free from partial degradation. Whole molecular size distributions and the glycogen chain length distributions were used to determine the structure of the extracted glycogen, similar to a starch extraction optimization published previously[22]. Size exclusion chromatography (SEC) with differential refractive index (DRI) detection was used to obtain the size distributions of glycogen, which describe the total molecular weight as a function of molecular size. Fluorophore-assisted carbohydrate electrophoresis (FACE) was used to analyze the chain-length distributions, which describe the relative number of glucoside chains of each giver size (or degree of polymerization). This paper describes the methodology of extracting glycogen from liver tissues based on the previous optimization study[21]. The data suggest that the method most suited to preserve glycogen structure is a sucrose concentration of 30% with a 10 min boiling step.

PROTOCOL:

Mouse livers used to optimize this procedure[21] were from 12 male BKS-DB/Nju background mice (7.2 weeks old, see the **Table of Materials**). Animal use was approved by Renmin Hospital of Wuhan University Animal Care and Ethics Committee, IACUC Issue No. WDRM 20181113.

1 Animal tissues

1.1. Weigh mouse liver (1–1.8 g of whole liver from each mouse).

1.2. Rapidly freeze the mouse liver in liquid nitrogen and store it at -80 °C.

2. Preparation of buffer and reagents

2.1. Prepare glycogen isolation buffer containing 5 mM Tris,150 mM NaCl, 2 mM EDTA, 50 mM NaF, and 5 mM sodium pyrophosphate with deionized water, and adjust the pH to 8.

2.2. Prepare 30% (w/w) sucrose solution (found to be most optimal for liver glycogen[21]).

2.3. Prepare sodium acetate buffer (1 M, pH 4.5), acetic acid buffer (0.1 M, pH 3.5), sodium hydroxide solution (0.1 M), and sodium cyanoborohydride (1 M).

2.4. Prepare 8-aminopyrene-1,3,6-trisulfonate (APTS) solution by adding 5 mg of APTS to 50 µL of 15% glacial acetic acid.

2.5. Prepare ammonium nitrate solution containing 50 mM ammonium nitrate with 0.02% sodium azide.

3. Glycogen extraction (Figure 2)

3.1. Transfer the frozen liver tissue (~1 g) to a 15 mL tube containing 6 mL of glycogen isolation buffer.

3.2. Keeping it on ice, homogenize the liver tissue using a tissue homogenizer.

3.3. Transfer half of the suspension (3 mL) to a new tube and boil for 10 min (shown to be optimal for glycogen structural studies[21]). Keep the other half of the suspension (3 mL) on ice to extract glycogen containing associated proteins that are not denatured.

NOTE: Unboiled samples should always be kept on ice during glycogen extraction steps. If glycogen proteins are not important for study, the whole sample can undergo the 10 min boiling step.

3.4. Remove an 8 µL aliquot from each tube, keep the aliquots on ice, and use them for the glycogen content determination (see section 4).

3.5. Centrifuge the remaining suspension at 6,000 × *g* for 10 min at 4 °C.

3.6. Transfer the supernatants to ultracentrifuge tubes, and centrifuge them at 3.6×10^5 *g* for 90 min at 4 °C.

3.7. Discard the remaining supernatants and resuspend the pellets in 1.5 mL of glycogen isolation buffer.

3.8. Layer the samples over 1.5 mL of 30% sucrose solution in 4 mL ultracentrifuge tubes and centrifuge at 3.6×10^5 *g* for 2 h at 4 °C.

3.9. Discard the remaining supernatants and resuspend the pellets in 200 µL of deionized water.

3.10. Add 800 µL of absolute ethanol to the suspensions and mix well to precipitate glycogen[23,24]. Store the mixtures at –20 °C for at least 1 h to allow precipitation.

3.11. Centrifuge the samples at 6,000 × *g* for 10 min at 4 °C. Discard the supernatants and resuspend the pellets in 200 µL of deionized water.

3.12. Repeat this ethanol precipitation process 3x and resuspend the final glycogen pellet in 200 µL of deionized water.

3.13. Remove an aliquot of 8 µL from each tube for glycogen content determination (see section 4).

3.14. Freeze the remaining supernatants in liquid nitrogen and freeze-dry (lyophilize) overnight. Store the dry glycogen samples in the freezer at -20 °C.

NOTE: The dry glycogen samples should be stable at -20 °C; however, there are no data to indicate how long they last without any structural changes.

4. Glycogen content determination (Figure 3)

4.1. Add 8 µL of the glycogen supernatants, (see sections 3.13 and 3.4), 5 µL of amyloglucosidase (3269 U/mL), and 100 µL of sodium acetate buffer (1 M, pH 4.5) to a microcentrifuge tube and fill the tube to the 500 µL mark with deionized water.

4.2. Prepare controls that use deionized water instead of amyloglucosidase.

4.3. Incubate the samples at 50 °C for 30 min, while keeping the controls on ice.

4.4. Centrifuge at 6,000 × *g* at 4 °C for 10 min, and mix 300 µL of each resulting supernatant with 1 mL of glucosidase oxidase/peroxidase (GOPOD) reagent.

4.5. Construct a calibration curve by mixing 300 µL of denionized water containing 0, 10, 20, 30, 40, and 50 µg of D-glucose with 1 mL of GOPOD reagent.

4.5.1. Incubate the mixtures at 50 °C for a further 30 min.

4.5.2. Read the absorbance (510 nm) of each sample using 96-well plates (150 µL per well) using a UV-vis spectrophotometer.

4.5.3. Subtract the absorbances of control samples (with no amyloglucosidase) from the absorbances of experimental samples, then calculate the glycogen content based on the D-glucose standard curve

5. Crude yield, glycogen yield, and purity determination

5.1. For the crude yield, weigh the freeze-dried glycogen sample and calculate the yield as a percentage of the wet liver tissue.

NOTE: This yield should be adjusted to correct for the aliquots taken in each glycogen content step.

5.2. For glycogen purity, determine the glycogen content in the final pellets, as described in section 4. Calculate the purity as a percentage of the determined glycogen content relative to the crude yield (see step 5.1).

5.3. For glycogen yield, determine the glycogen content of the homogenized samples without boiling and before any centrifugation, as described in section 4. Calculate the glycogen yield as a percentage of the glycogen content in the final pellets (see step 5.2) to that of the glycogen content determined in the initial homogenate.

6. Analysis of chain-length distributions (Figure 4)

6.1. Weigh 0.5 mg of freeze-dried glycogen in 1.5 mL tubes.

6.2. Add 90 µL of deionized water and 1.5 µL of sodium azide (0.04 g/mL) to the tubes.

6.3. Add 5 µL of acetic acid buffer (0.1 M, pH 3.5) and 2 µL of isoamylase solution (180 U/mg) to the tubes to debranch the glycogen.

6.4. Incubate the samples at 37 °C for 3 h.

6.5. Add 5 µL of sodium hydroxide solution (0.1 M) to the samples to increase the pH to 7.0.

6.6. Freeze the samples in liquid nitrogen and freeze-dry (lyophilize) overnight.

6.7. Add 2 µL of APTS solution (5 mg of APTS in 50 µL of 15% glacial acetic acid) and 2 µL sodium cyanoborohydride (1 M) to the freeze-dried debranched glycogen.

6.8. Centrifuge the samples at 4,000 × *g* for 2 min.

6.9. Incubate the samples at 60 °C for 3 h in the dark.

NOTE: The tube can be covered with aluminum foil to protect the contents from light.

6.10. Add 200 µL of deionized water to the samples and vortex them until all precipitate is dissolved.

6.11. Centrifuge the samples at 4,000 × *g* for 2 min.

6.12. Transfer aliquots of 50 µL to fluorophore-assisted carbohydrate electrophoresis (FACE) micro-vials for analysis.

NOTE: The data are shown as the relative abundance of (debranched) chains ($N_{de}(X)$) for each degree of polymerization (DP, symbol *X*).

7. Analysis of molecular size distributions (Figure 5)

7.1. Dissolve 0.5 mg of freeze-dried glycogen in 50 mM ammonium nitrate and 0.02% sodium azide at 1 mg/mL.

7.2. Incubate the samples in a thermomixer at 80 °C for 3 h at 300 rpm.

7.3. Inject the samples into an SEC system using a pre-column and 1000 Å and 10,000 Å columns at 80 °C with a flow rate of 0.3 mL/min (see the **Table of Materials**). Use a refractive index detector to determine the relative weight of molecules at each elution volume.

7.4. Using pullulan standards (PSS) with molar masses ranging from 342 to 1.22×10^6, plot an SEC universal calibration curve to convert the elution time to R_h (hydrodynamic radius). Express the data from the differential refractive index (DRI) detector as an SEC weight distribution w (log R_h) as a function of R_h.

REPRESENTATIVE RESULTS:

While the procedure described above is for the most optimal method (30% sucrose with the addition of a 10min boiling step), data are provided here for glycogen extracted via three sucrose concentrations (30%, 50%, 72.5%), with and without a boiling step, as previously described[21]. Following the protocol, the purity, crude yield, and glycogen yield of dry glycogen extracted by different conditions are given in **Table 1**, reproduced from[21]. There were no significant differences in crude yield and glycogen yield between the groups extracted with the various conditions. In contrast, the glycogen purity was significantly influenced by both the sucrose concentrations (**Table 1**, $P < 0.001$) and by the addition of a boiling step (**Table 1**, $P < 0.0001$). Glycogen with the highest purity was extracted using the 30% sucrose concentration along with a 10 min boiling step, which is why it was determined to be the most optimal out of the conditions tested.

Molecular size distributions were used to assess the effects of the various conditions on the size of molecules in the final extract. These were obtained using an aqueous SEC system, as described previously[25]. Normalizing each distribution to the same maximum value allowed the relative proportion of α to β particles to be compared from each method, shown in **Figure 6**, reproduced from[21]. The R_h at which the maxima occurs ($R_{h,max}$) and the average R_h ($\bar{R}_h$) are shown in **Table 2**, reproduced from[21]. Glycogen molecules with $R_h < 30$ nm were defined as β particles[11]. The β particle content was calculated as the area under the curve (AUC) of ($R_h < 30$ nm)/AUC (total R_h). The boiled samples had lower average R_h values and a higher β particle content than the unboiled samples (**Table 2**, $P < 0.05$). Lower sucrose concentrations resulted in lower $\bar{R}_h$ values and higher β particle contents (**Table 2**, $P < 0.05$). The introduction of a boiling step also led to lower $R_{h,max}$ values (**Table 2**, $P<0.05$), while the sucrose concentration had no significant effect.

Chain-length distributions (CLDs) provide the relative number of chains of each given length (number of connected glucose units, or degree of polymerization), obtained using FACE. The CLDs are shown in **Figure 7**, reproduced using data from[21]. The number-average chain length (ACL) was calculated as ($\Sigma_X X N_{de}(X)$)/ ($\Sigma_X N_{de}(X)$) (**Table 2**). The ACLs of unboiled samples were significantly smaller and more varied than those of the boiled samples **(Table** 2, $P < 0.05$). However, the sucrose concentration did not significantly affect the ACLs. This supported the hypothesis that boiling the samples for 10 min as a pre-extraction step would preserve the glycogen structure. The proposed mechanism is the denaturing of glycogen-degrading enzymes.

FIGURE AND TABLE LEGENDS:

Figure 1: The three levels of glycogen structure.

Figure 2: Glycogen extraction process. Steps to extract and purify the glycogen from mouse liver.

Figure 3: Glycogen content determination. Steps to determine the glycogen content in liver homogenate, purified dry glycogen or glycogen solution.

Figure 4: Analysis of chain-length distributions. Steps to analyze chain-length distributions by a fluorophore-assisted carbohydrate electrophoresis system.

Figure 5: Analysis of molecular size distributions. Steps to analyze the molecular size distributions by an aqueous size-exclusion chromatography system.

Figure 6: SEC weight distributions of whole (not debranched) mouse liver glycogen. Extraction was performed by different conditions, normalized to have the same maximum in $w(\log R_h)$. Each liver homogenate was divided into six equal volumes, and glycogen was extracted by either 30%, 50%, or 72.5% sucrose, boiled or unboiled. Curves represent the mean at a given R_h with the SD being provided on either side of the main line (n = 4–6 with samples having insufficient signal to noise being removed). (**A**) Glycogen extracted by 30% sucrose, boiled or unboiled; (**B**) glycogen extracted by 50% sucrose, boiled or unboiled; (**C**) glycogen extracted by 72.5% sucrose, boiled or unboiled. This figure was adapted from [21]. Abbreviations: SEC = size exclusion chromatography; SD = standard deviation; Rh = hydrodynamic radius; $w(\log R_h)$ = SEC weight distribution.

Figure 7: Chain length distributions, $N_{de}(X)$, of glycogen. Chain length analysis was performed on six livers for both boiled and unboiled using 30%, 50%, and 72.5% sucrose concentrations in the ultracentrifugation step. Values for each DP represent the mean ± SD (N = 6). (**A**) Glycogen extracted by 30% sucrose, boiled or unboiled; (**B**) glycogen extracted by 50% sucrose, boiled or unboiled; (**C**) glycogen extracted by 72.5% sucrose, boiled or unboiled. This figure was adapted from [21]. Abbreviation: $N_{de}(X)$ = chain length distribution.

Table 1: Purity, crude yield, glycogen yield. Purity, crude yield, glycogen yield for liver glycogen samples extracted by 30%, 50%, or 72.5% sucrose, boiled or unboiled. -c were samples extracted by cold buffer; -b were samples extracted by boiling for 10 min. The values are given as the mean

± standard deviation (SD), n = 6. Differences in values with different superscript letters in the same column are statistically significant ($P < 0.05$). This table was adapted from [21].

Table 2: $\bar{R}_h$ and R_h at which the maxima occurs ($R_{h,max}$) and average chain length (ACL). $\bar{R}_h$ and R_h at which the maxima occurs ($R_{h,max}$) and average chain length (ACL) of glycogen extracted by either 30%, 50%, or 72.5% sucrose, boiled or unboiled. -c unboiled; -b involved a 10 min boiling step. Differences in values with different superscript letters in the same column are statistically significant ($P < 0.05$). This table was produced using the same data from [21]. Abbreviations: R_h = hydrodynamic radius; $\bar{R}_h$ = ; ACL = average chain length; R_h,max = R_h at which maxima occurs.

DISCUSSION:

Previous studies have shown that the structure of glycogen is important for its properties; for example, the molecular size affects the degradation rate of glycogen[10], and the chain length distribution affects its solubility[26]. To properly understand these relationships, it is important to extract glycogen with a procedure that isolates, as much as possible, a representative and undamaged sample. Traditional methods of extraction utilized either hot alkaline conditions or cold acid. While effective in separating the glycogen from other tissue components, these methods are chemically harsh and have been shown to degrade the molecular structure of glycogen[27].

A relatively gentle method has since been developed that uses sucrose density gradient centrifugation[17,18], allowing glycogen to form in the pellet while most of the cell material remains in the supernatant. This method is particularly useful for liver tissue, with the glycogen α particles being sensitive to acid hydrolysis[28]. This milder method does, however, have at least two potential mechanisms for the isolation of glycogen diverging in structure from that seen *in vivo*: 1) smaller, less dense glycogen particles are more susceptible to being left in the supernatant during sucrose density gradient centrifugation [17,18], as they may be unable to reach the pellet. 2) The milder conditions may allow glycogen degradation enzymes, which would be denatured in the harsher alkaline/acid extraction conditions, to continue to degrade glycogen particles during extraction.

A recent publication[21] aimed to help resolve these potential issues by testing a series of sucrose concentrations (and therefore densities), finding that using a concentration of 30%, as opposed to the traditionally used 72.5%, helped minimize the loss of smaller glycogen particles. Future experiments could test even lower concentrations to see if some smaller particles are still preferentially lost in the supernatant during centrifugation. This publication also tested the efficacy of introducing a 10 min boiling step directly after tissue homogenization to denature the glycogen degradation enzymes, thereby preserving the structure of glycogen. It was shown that this step helped inhibit glycogen degradation, with the glycogen chain lengths being significantly preserved. Further experiments in this study provided evidence that this 10 min boiling step was unlikely to cause significant damage to the glycogen structure. However, this boiling step may influence the structure of glycogen-associated proteins, potentially resulting in the denaturation

and subsequent dissociation of proteins from the glycogen. Therefore, if proteomics is of interest, using the low sucrose concentration (30%) without boiling (samples kept on ice) might be preferable, with the caveat that the glycogen may be slightly degraded.

When using sucrose density gradient centrifugation without further optimization experiments, the most suitable method is to utilize a relatively low concentration of sucrose (30%) with the introduction of a 10 min boiling step directly after tissue homogenization. There are some limitations to this technique. First, this was optimized for liver glycogen, and it is important to note that it may not be as appropriate for glycogen from other tissues. Second, as mentioned above, the lowest sucrose concentration tested was 30%, and it is possible that lower concentrations could be preferable. Third, an optimized technique that prevents the enzymatic degradation of glycogen while preserving the associated proteome is not yet available.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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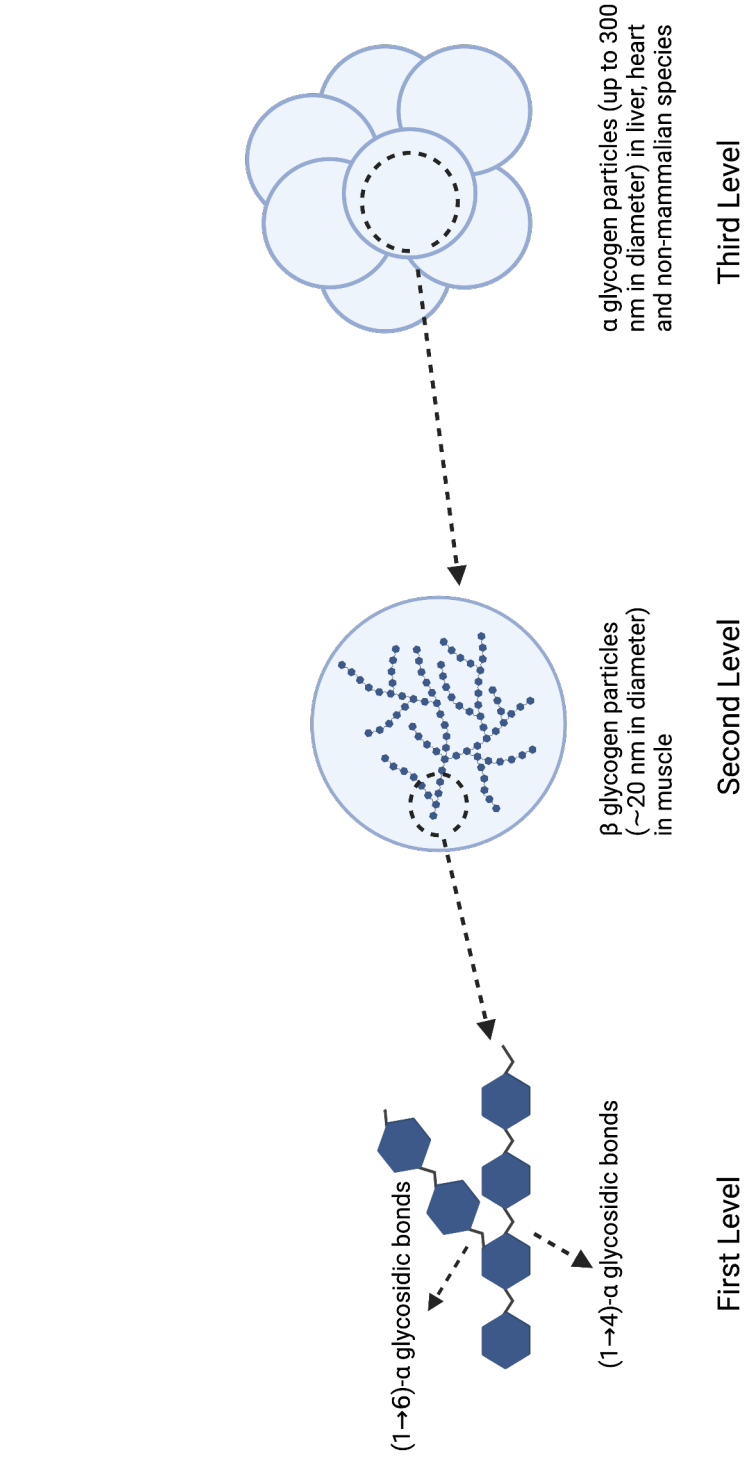


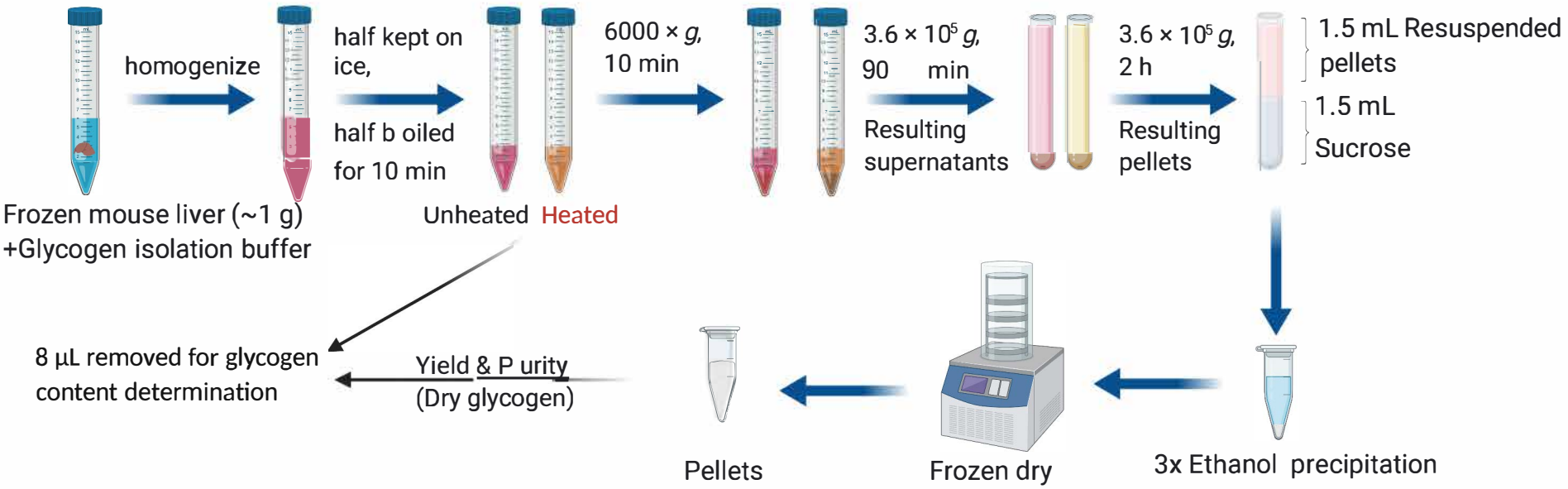


Figure 3

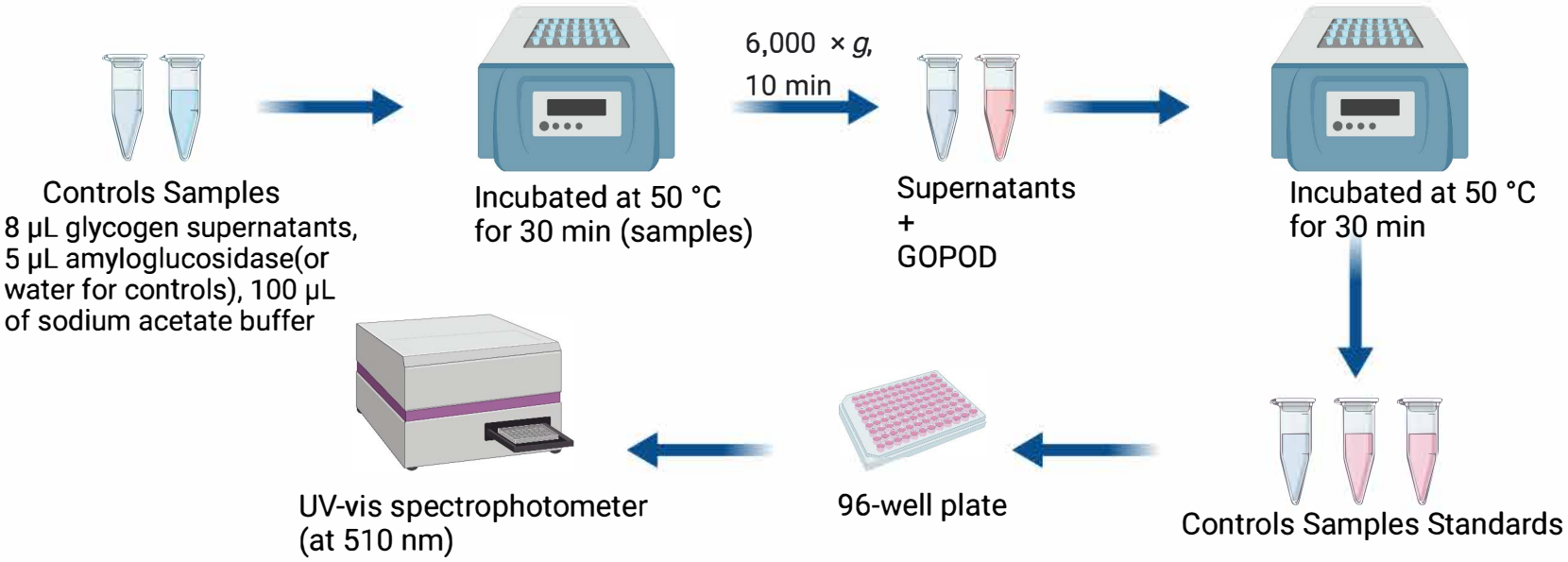
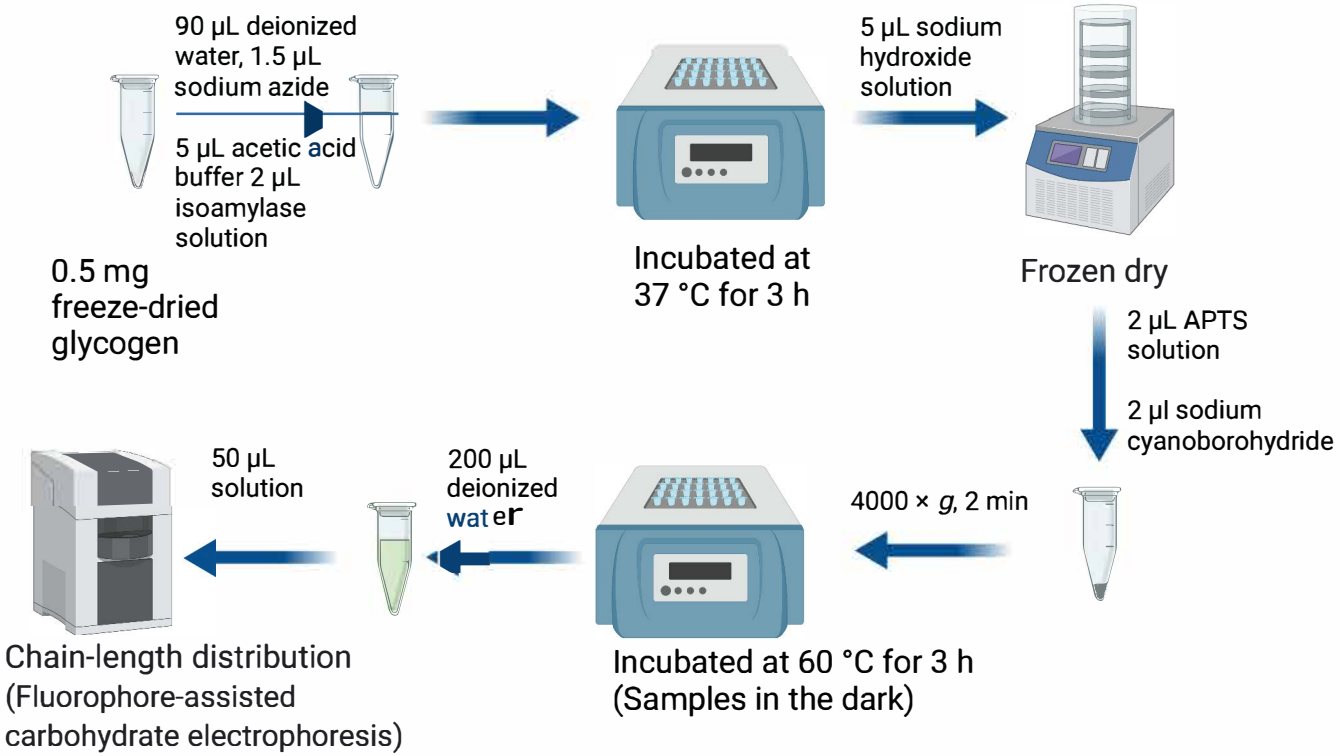


Figure 4



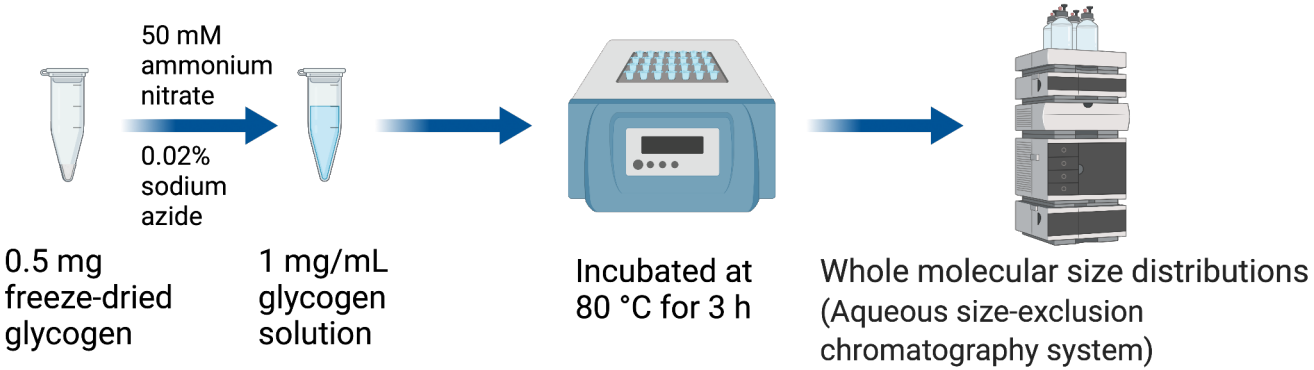


Fig 5

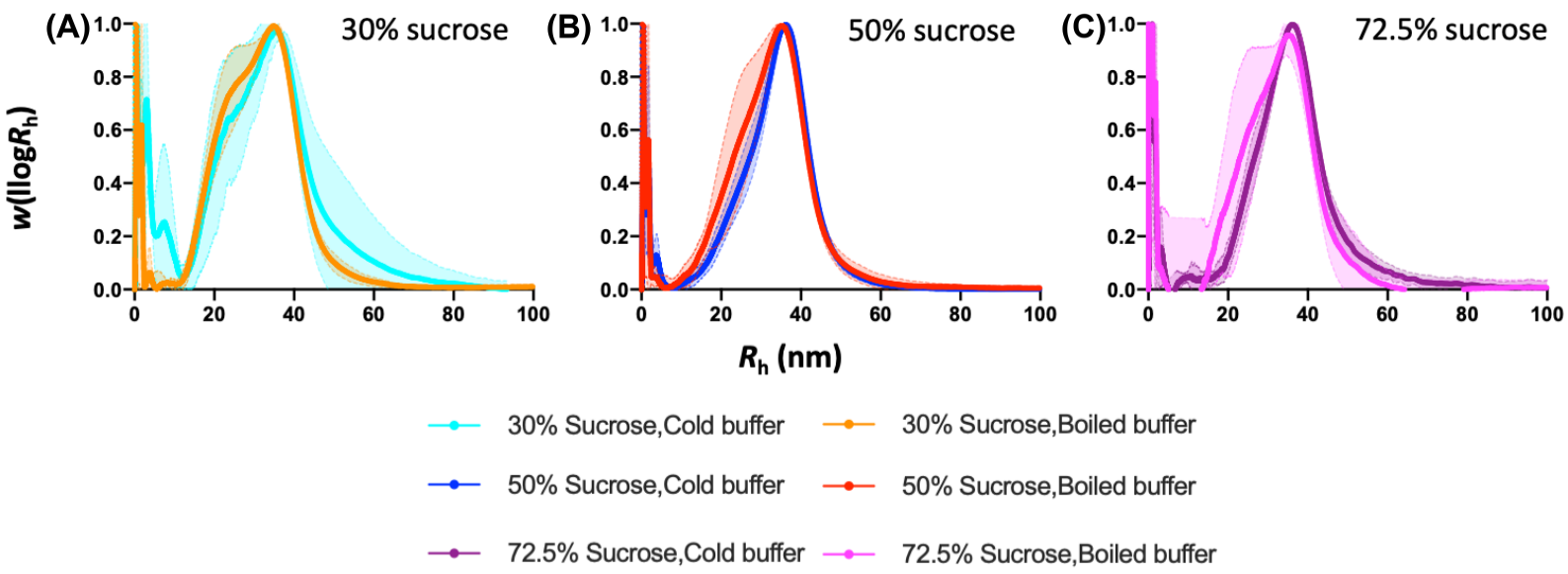


Figure 6

Figure 7

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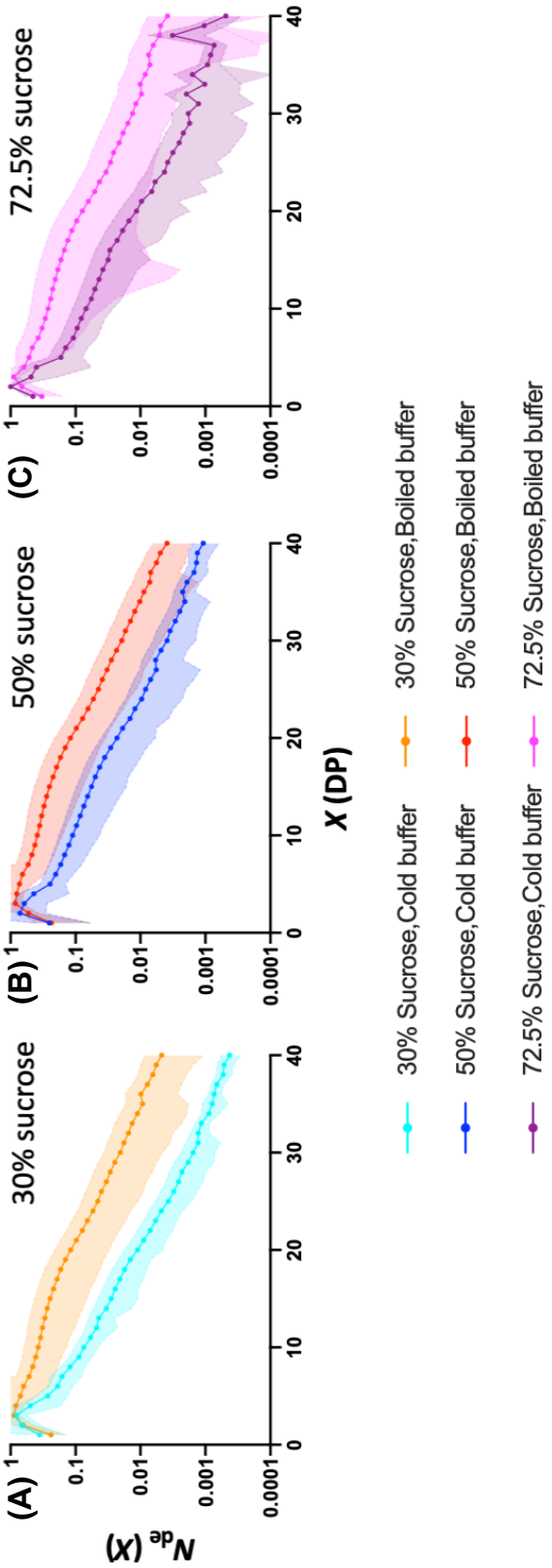

Table 1

	Crude yield (%)	Purity (%)	Glycogen yield (%)
30% Sucrose-c	2.1 ± 1.0[a]	13.1 ± 12.0[b]	10.7 ± 9.1[a]
50% Sucrose-c	1.2 ± 0.7[a]	23.3 ± 20.1[b]	10.2 ± 8.1[a]
72.5% Sucrose-c	1.9 ± 0.8[a]	9.8 ± 9.0 [b]	5.3 ± 2.4[a]
30% Sucrose-b	0.8 ± 0.4[a]	67.9 ± 16.8[a]	16.0 ± 5.1[a]
50% Sucrose-b	1.1 ± 0.6[a]	48.6 ± 16.9[a]	14.8 ± 7.6[a]
72.5% Sucrose-b	2.0 ± 0.9[a]	14.7 ± 12.6[b]	6.9 ± 3.7[a]
Two-way ANOVA	Sucrose: P = 0.053 Temperature: P = 0.108 Interaction: P = 0.11	Sucrose: P < 0.001 Temperature: P < 0.0001 Interaction: P = 0.003	Sucrose: P = 0.034 Temperature: P = 0.116 Interaction: P = 0.801

Table 2

	Mean $\bar{R}_h$	Mean $R_{h,max}$
30% Sucrose-c	29.4 ± 1.5^{b}	34.9 ± 0.6^{a}
50% Sucrose-c	$32.0 \pm 1.1^{a,b}$	36.1 ± 0.5^{a}
72.5% Sucrose-c	34.3 ± 1.8^{a}	36.2 ± 0.4^{a}
30% Sucrose-b	29.4 ± 1.2^{b}	33.7 ± 3.1^{a}
50% Sucrose-b	30.1 ± 1.2^{b}	34.9 ± 0.7^{a}
72.5% Sucrose-b	$30.9 \pm 3.5^{a,b}$	33.6 ± 3.5^{a}
Two-way ANOVA	Sucrose: P = 0.002 Temperature: P = 0.010 Interaction: P = 0.431	Sucrose: P = 0.442 Temperature: P = 0.032 Interaction: P = 0.640

β content	Mean ACL
$40.9 \pm 6.2\%^{a,b}$	4.8 ± 0.5^{c}
$28.5 \pm 3.0\%^{b,c}$	$5.6 \pm 1.1^{b,c}$
$23.7 \pm 3.5\%^{c}$	4.7 ± 0.9^{c}
$43.1 \pm 5.1\%^{a}$	8.6 ± 1.8^{a}
$36.0 \pm 7.2\%^{a,b,c}$	8.8 ± 1.7^{a}
$34.0 \pm 13.1\%^{a,b,c}$	$7.6 \pm 1.9^{a,b}$
Sucrose: $P < 0.001$	Sucrose: $P = 0.290$
Temperature: $P = 0.016$	Temperature: $P < 0.0001$
Interaction: $P = 0.441$	Interaction: $P = 0.750$

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Table of Materials

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Commented [A1]

Response: The following in red should be added: “Add 8 µL of glycogen supernatants (see section 3.4 and section 3.13), 5 µL of amyloglucosidase (3269 U/mL) and 100 µL of sodium acetate buffer (1 M, pH 4.5) to a microcentrifuge tube and fill to 500 µL with deionized water.’

Commented [A2]

Response: Should change to “to a microcentrifuge tube and fill to 500 µL with deionized water.” We do not want to have “to the 500 uL” mark because that is not quite accurate enough. The experimenter can calculate exactly how much to add (which will change if for example they want to add more of the glycogen supernatant for higher signal).

Commented [A3]

Response: The following in red should be changed: “Subtract the absorbances of control samples (with no amyloglucosidase) from the absorbances of experimental samples, then calculate the glycogen content based on the D-glucose standard curve.

Commented [A4]

Response: Apologies, it should be 21.

Commented [A5]

Response: 21

Commented [A6]

Response: Can be changed to: X (degree of polymerization, DP)

Commented [A7]

Response: All figures and tables are from reference 21. Reference 21 has already been accepted.

The reference can be updated to the following:

“Wang, Z., Liu, Q., Wang, L., Gilbert, R. G. & Sullivan, M. A. Optimization of liver glycogen extraction when considering molecular fine structure. *Carbohydrate Polymers.* **261** 117887, doi:10.1016/j.carbpol.2021.117887, (2020).”

Regarding the permission for the table data, we will forward the agreement from the journal.

Commented [A8] and [A9]

Response: Reference 21 has now been accepted. We believe we now have all references in the correct style.

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