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A rapid extraction method for glycogen from formalin-fixed liver

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ABSTRACT

Liver glycogen, a highly branched polymer, acts as our blood-glucose buffer. While past structural studies have extracted glycogen from fresh or frozen tissue using a cold-water, sucrose-gradient centrifugation technique, a method for the extraction of glycogen from formalin-fixed liver would allow the analysis of glycogen from human tissues that are routinely collected in pathology laboratories. In this study, both sucrose-gradient and formalin-fixed extraction techniques were carried out on piglet livers, with the yields, purities and size distributions (using size exclusion chromatography) compared. The formalin extraction technique, when combined with a protease treatment, resulted in higher yields (but lower purities) of glycogen with size distributions similar to the sucrose-gradient centrifugation technique. This formalin extraction procedure was also significantly faster, allowing glycogen extraction throughput to increase by an order of magnitude. Both extraction techniques were compatible with mass spectrometry proteomics, with analysis showing the two techniques were highly complementary.

1. Introduction

Glycogen is a highly branched glucose polymer (~9% degree of branching) which functionally stores energy in a state which can be rapidly mobilized in response to hypoglycaemia. The highest concentration of glycogen is present in the liver; glycogen is also found in skeletal muscle (Calder & Geddes, 1985), heart (Besford et al., 2012), adipose (Jurczak et al., 2007) and brain tissues (Brown, 2004). Liver glycogen consists of glucose units that are attached to form linear chains via α-(1→4) linkages. These chains are connected via α-(1→6)-linked branch points to form highly branched glycogen "β" particles (~20 nm in diameter) that can further join to form much larger "α" particles (~100–200 nm) (Sullivan, Aroney, et al., 2014).

Glycogen was first isolated by Claude Bernard in 1857 from dog liver, employing a method of heating liver tissue in an alkaline solution (Bernard, 1857). This method was shown to degrade the glycogen, making the exploration of milder techniques advantageous (Bueding & Orrell, 1964). Later methods employing cold trichloroacetic acid (TCA) (Stetten, Katzen, & Stetten, 1956) isolated glycogen with less degradation. Since then extraction methods have become progressively milder, with a cold water extraction method coupled with ultracentrifugation being shown to extract much larger, intact glycogen α particles (Lazarow, 1942; Orrell & Bueding, 1964).

More recent cold-water extraction techniques have used a Tris buffer (Parker, Koay, Gilbert-Wilson, Waddington, & Stapleton, 2007; Ryu et al., 2009; Sullivan et al., 2010a), which is a potent inhibitor of glucosidase activity (De Apodaca, Fernandez, & Delafuente, 1992). These techniques have also used sucrose-density gradient centrifugation to aid in the separation of the

Abbreviations: SEC, size exclusion chromatography; MS, mass spectroscopy; NBF, neutral buffered formalin; TCA, trichloroacetic acid; GOPOD, glucose oxidase/peroxidase; SEM, standard error of the mean; R_h, hydrodynamic radius.

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glycogen particles from the contaminating microsomal layer (Parker et al., 2007; Ryu et al., 2009; Sullivan et al., 2010a).

Liver glycogen undergoes rapid enzymatic degradation post-mortem under ambient conditions (Geddes & Rapson, 1973). Therefore unless glycogen can be immediately extracted from fresh liver tissue, which is usually an unfeasible arrangement for human samples, characterization requires a method for preserving the tissue. Two common ways to do this are by rapidly freezing the samples or by chemically fixing them in a solution such as formalin. However, it is important to ensure that it is possible to extract glycogen from samples that have been so preserved without significant loss or degradation of the glycogen (and any glycogen-bound proteins), compared to the parent glycogen from the liver extracted immediately after sacrifice.

A method employing formalin (which can dissolve glycogen and precipitate protein) to extract liver glycogen, while initially promising (Devor & Canowitz, 1962), was shown to be inferior to the cold-water extraction techniques, with a product of lower purity being obtained (Devor, Barichie, & Siddiqui, 1966). It was however noted that this method may be useful for recovering glycogen from tissues already stored in formalin. It was shown that the formalin method extracts glycogen with larger particle sizes than the alkali and TCA methods (as inferred from having higher sedimentation coefficients – note molecular conformation also affects this coefficient (Gidley et al., 2010)), indicating less degradation; however, a comparison with the cold-water extraction technique has not yet been performed. One potential problem with the formalin technique is the acidity of formaldehyde (Devor et al., 1966); however the use of neutral-buffered formalin (NBF), a common reagent used today for fixing tissue samples, can avoid potential acid degradation.

A comparison of glycogen extracted from modern cold-water extraction techniques that utilize Tris buffers, ultracentrifugation and sucrose density gradients with a formalin method that uses NBF would determine the potential of extracting glycogen from formalin-fixed tissues, allowing for the analysis of glycogen from the vast source of human tissues currently fixed with NBF in pathology laboratories (Thavarajah, Mudimbaimannar, Elizabeth, Rao, & Ranganathan, 2012). The extension of this work into human samples would allow for a more detailed study of liver glycogen and its role in type 2 diabetes. This is especially relevant given the discovery (Sullivan et al., 2011) that liver glycogen from healthy and diabetic mouse livers shows significant molecular structural differences.

The efficacy of different glycogen extraction techniques, with and without formalin, is explored here, using liver from healthy piglets. Efficacy is judged by comparing the molecular size distributions from the various extraction techniques using size-exclusion chromatography, which can show if there is a systematic loss of particles of different sizes. Mass spectroscopy proteomics was also performed on mouse-liver glycogen, confirming the ability to identify glycogen-associating proteins from glycogen extracted via both cold-water sucrose-gradient centrifugation and formalin techniques.

2. Materials and methods

2.1. Animals

Glycogen was extracted from two piglet livers following a procedure similar to that used previously (Sullivan et al., 2012) (The University of Queensland animal ethics approval certificate CNFS/217/11/PORK CRC). Two male, 34 day-old piglets (Large White breed), reared at the University of Queensland Gatton piggery, were sedated and euthanized prior to sample extraction. The piglets were fed a standard nursery diet consisting of wheat (68.6%), fishmeal (6.8%), whey powder (5.0%), soybean meal (4.0%) and soy protein concentrate (4.0%). A sample of liver from each (~10 g) was obtained from the central lobe of the liver and immediately frozen in liquid nitrogen and stored at −80 °C. Each following procedure was first performed with one liver sample and then repeated 2 days later with the other, acting as an experimental replicate.

For the proteomics analysis, one male 24-week old, non-fasted C57BL6/J mouse was euthanized via CO_2 inhalation. Following this, the liver was divided into two and either immediately snap frozen for the sucrose method or placed in 10% NBF for ~48 h. Small animal studies were performed in accordance with guidelines from the University of Queensland Ethics Committee and the National Health and Medical Research Council of Australia.

2.2. Cold-water extraction using sucrose density ultracentrifugation ("sucrose method")

The procedure for liver-glycogen extraction and purification using sucrose density ultracentrifugation was similar to that used previously (Sullivan, Aroney, et al., 2014). Approximately 1.2 g of frozen liver was homogenized in 18.2 mL of glycogen isolation buffer, an inhibitor of glucosidase activity (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, and protease-inhibiting cocktail (Roche)). Then 200 μL of the homogenate was removed and frozen at −20 °C for glycogen content analysis. The remaining homogenate was divided into six equal portions and was centrifuged at 6000 × *g* for 10 min at 4 °C with the resulting supernatants centrifuged further at 488 300 × *g* for 1 h at 4 °C. The pellets were resuspended in 400 μL of glycogen isolation buffer and layered over a 3-mL stepwise sucrose gradient (37.5% and 75% in glycogen isolation buffer). The samples were then centrifuged at 488 300 × *g* for 2 h at 4 °C. The supernatants were discarded and the resulting pellets were resuspended in 200 μL of deionized water. 1 mL of absolute ethanol was added to the samples and centrifuged at 4000 × *g* for 10 min, with the supernatants being discarded. The pellets were resuspended in 500 μL of deionized water and then lyophilized (freeze-dried; VirTis, Benchtop K).

2.3. Preparation of 10% neutral-buffered formalin

While technically 3.7% formaldehyde, historically the preparation of this fixative chemical has been achieved by diluting commercial-grade stock formaldehyde (37–40% formaldehyde, generally referred to as formalin when in solution) 10-fold in a phosphate buffer; hence the name 10% neutral-buffered formalin (NBF). A 10% NBF solution (adjusted to pH 7) was prepared by diluting 37% formaldehyde (formalin) 10-fold and adding 4% sodium dihydrogenphosphate monohydrate and 6.5% anhydrous sodium hydrogenphosphate.

2.4. Extraction of glycogen from formalin-fixed tissue ("formalin method")

The method used was modified from that employed previously (Devor & Canowitz, 1962). Approximately 1.2 g of frozen liver was divided into 6 portions (~200 mg each). These samples were taken from the same piglets as for Section 2.2. To these samples, 2 mL of 10% NBF was added, with the liver tissues being fully immersed. These samples were left at room temperature for ~48 h, which has been shown to be an adequate time to form protein crosslinks when using NBF at ~25 °C (Helander, 1994), and then homogenized. The homogenate was subsequently centrifuged at 4000 × *g* for 10 min. The supernatant of each sample was added to 10 mL of absolute ethanol and the samples were centrifuged at 4000 × *g* for 10 min.

The pellet was resuspended in 500 μL of deionized water and then lyophilized (freeze-dried; VirTis, Benchtop K).

2.5. Measuring the liver-glycogen content

The glycogen content of the liver was determined using a glucose oxidase/peroxidase (GOPOD) assay procedure, similar to that used previously (Roehrig & Allred, 1974; Sullivan, Aroney, et al., 2014). Firstly, six 20 μL aliquots of liver-glycogen homogenate (from Section 2.2) were separated, allowing for a more accurate determination of the liver-glycogen content and determination of the statistical error in the analysis. To each of these 6 samples was added 5 μL of amyloglucosidase (3260 U mL^{-1}, Megazyme) and 100 μL of sodium acetate buffer (pH 6), with the solution being made up to 500 μL with deionized water and incubated on a thermomixer (50 °C) for 30 min. A control for each of the samples, containing everything except amyloglucosidase, and a blank containing everything except the glycogen homogenate, were also analyzed. A 300 μL aliquot of each sample was added to 1 mL of GOPOD reagent (Megazyme) and incubated at 50 °C for a further 30 min on the thermomixer. The absorbance (510 nm) of each sample was analyzed on a UV-1700 PharmaSpec UV-vis spectrophotometer (Shimadzu). The glycogen content was determined by constructing a calibration curve that analyzed the absorbance of various concentrations of D-glucose that had been reacted with the same GOPOD reagent. All samples including controls were run in duplicate. Various concentrations of sucrose (up to a concentration of 1 mg mL^{-1}) were also tested, showing no reaction with GOPOD, confirming that there is no additional absorbance resulting from sucrose contamination.

The liver glycogen content, given in Table 1, is presented as the mean ± standard error of the mean (SEM) of the 6 samples.

2.6. Measuring crude glycogen yield

The crude yield from both of the glycogen extraction methods, given in Table 1, was determined by weighing the amount of sample remaining after being freeze-dried. There were 6 samples from each method, allowing the yield to be presented as the mean ± standard error of the mean (SEM).

2.7. Measuring glycogen purity

The purity of glycogen can also be determined using the same assay used to measure the glycogen content of the liver. Briefly, 100 μL of extracted-glycogen solution (~0.006 mg mL^{-1}) was added to 5 μL of amyloglucosidase (3260 U mL^{-1}, Megazyme) and 100 μL of sodium acetate buffer (pH 6), with the solution being made up to 500 μL with deionized water. The rest of the procedure is identical to that in Section 2.5, with the glycogen purity being calculated as a percentage of the determined glycogen content to that of the initial amount of sample used in the assay. Because there were 6 samples for each extraction procedure, the glycogen purity is given as the mean ± standard error of the mean (SEM); see Table 1.

2.8. Protease treatment of formalin-extracted glycogen ("formalin/protease method")

Approximately 3 mg of the glycogen extracted using the formalin-extraction method was subjected to protease treatment as follows. Glycogen was dissolved in 0.5 mL of protease solution (2.5 U mL^{-1}; bacterial type XIV, Sigma–Aldrich) in tricine buffer (pH 7.5, 250 mM) and incubated at 37 °C for 4 h. Samples were then lyophilized (freeze-dried; VirTis, BTP-9EL).

2.9. Size-exclusion chromatography (SEC) of glycogen

An aqueous SEC setup similar to that recently employed for glycogen characterization was used here (Sullivan, Powell, et al., 2014). Glycogen samples were dissolved in a thermomixer overnight at 25 °C in 50 mM ammonium nitrate/0.02% sodium azide at ~2 g L^{-1}. The effect of heating the samples overnight at 80 °C in a thermomixer was also tested. As previously stated (Sullivan, Powell, et al., 2014), the ammonium nitrate is used to minimize any potential interactions between the glycogen and the column by increasing the solution's ionic strength. Sodium azide acts as an antimicrobial agent.

Dissolved glycogen samples were injected into an Agilent 1260 infinity SEC system (Aglient, Santa Clara, CA, USA) using a column setup of SUPREMA pre-column, 1000 and 10 000 columns (Polymer Standard Service, Mainz, Germany). The columns were kept at 80 °C using a column oven and the flow rate was set to 0.3 mL min^{-1}. A refractive index detector (RID) (Optilab UT-rEX, WYATT, Santa Barbara, CA, USA) was used to determine the SEC weight distributions.

Pullulan standards (PSS), with a molar mass range of 342 Da to 2.35×10^6 Da, were dissolved into the 50 mM ammonium nitrate/0.02% sodium azide solution and run through the SEC system, allowing the construction of a universal calibration curve. While this assumes that the SEC is separating solely on hydrodynamic size, a valid assumption shown for molecules with widely varied shapes (Hamielec & Ouano, 1978; Kuge, Kobayashi, Tanahashi, Igushi, & Kitamura, 1984), the purpose of this study was to compare the relative structure of glycogen obtained from different extraction methods, with any inaccuracies in calibration being equal for all of the samples as they were run consecutively.

2.10. Mass spectrometry

Mouse-liver glycogen was extracted via both the sucrose and formalin methods, as was performed with the piglet livers (see Sections 2.2 and 2.4). Mouse-liver glycogen was used instead of pig-liver glycogen based on tissue availability; this is equally suitable to test the efficacy of this method with the ultimate goal being to study human tissues in the future. For glycogen extracted from the formalin method, two treatments that have been previously employed for formalin-fixed tissue were trialed (Jiang et al., 2007). 2 mg mL^{-1} of glycogen was treated with either 6 M guanidine–HCl or 2% SDS, then heated at 100 °C for 1 h. Samples without either treatment were also tested as a control. Glycogen extracted from the sucrose method also did not undergo these additional treatments.

Extracted glycogen samples containing ~50 μg protein were resuspended in 50 mM Tris HCl buffer (pH 7.5) and 10 mM DTT with 1 μg trypsin (proteomics grade, Sigma–Aldrich) and incubated at 37 °C with constant mixing for 16 h. Insoluble material was removed by centrifugation at 18 000 *g* for 10 min, and peptides were desalted with C18 ZipTips (Millipore). Peptides were analyzed as described previously (Bailey, Jamaluddin, & Schulz, 2012) by LC-ESI–MS/MS using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometry with a Nanospray III interface (AB SCIEX). Identical LC conditions were used for SWATH-MS (Sequential Window Acquisition of all Theoretical Mass Spectra), with an MS-TOF scan from an m/z of 350–1800 for 0.05 s followed by high sensitivity information-independent acquisition with 26 m/z isolation windows with 1 m/z window overlap each for 0.1 s across an m/z range of 400–1250. Collision energy was automatically assigned by Analyst software (AB SCIEX) based on m/z window ranges.

Peptides were identified essentially as described (Bailey, Punyadeera, Cooper-White, & Schulz, 2012) using ProteinPilot (AB SCIEX), searching the UniProt database (downloaded from www.uniprot.org/ as at 4th March 2014) with standard settings:

Table 1
Glycogen content, purity and yield.

	Liver-glycogen content (%)	Crude yield (%)	Purity (%)	Glycogen yield (%)
Sucrose	4.31 ± 0.022	3.6 ± 0.15	55 ± 5.30	46 ± 4.65
Formalin		11.8 ± 0.45	31 ± 5.42	85 ± 16.94

Samples are given as the mean ± standard error of the mean (SEM), $n = 6$.

Sample type, identification; Instrument, TripleTof 5600; Species, Mouse with common contaminants; ID focus, biological modifications; Enzyme, Trypsin; Search effort, thorough ID. False discovery rate analysis using ProteinPilot was performed on all searches, and peptides identified with greater than 99% confidence and with a local false discovery rate of less than 1% were included for further analysis. ProteinPilot search results were used as ion libraries for SWATH analyses. The abundance of proteins were measured automatically using PeakView (AB SCIEX) with standard settings. Comparison of protein relative abundance was performed with the MSstats package in R (Choi et al., 2014). Gene ontology analysis was performed using the DAVID bioinformatics resource (Huang, Sherman, & Lempicki, 2009).

3. Results

The extraction of liver-glycogen using different techniques was performed on the same liver samples, allowing the direct comparison of the methods. Because the trend was the same for both pigs, the main text contains results from a single pig, with those for the corresponding size-exclusion chromatography data for the other in the SI.

The yields and purities of glycogen extracted with the sucrose and formalin methods are given in Table 1.

While the purity of the glycogen extracted using the formalin method is lower than that of the sucrose method (~31% compared to ~55%), the amount of glycogen extracted (the glycogen yield) is significantly higher with the formalin method, which extracted ~85% of the glycogen present in the liver (as calculated in Section 2.5), compared to the ~46% from the sucrose method. Because the formalin/protease method consists of taking formalin-extracted glycogen and adding protease, the glycogen yield is the same as for the formalin method. The crude yield and purity will change as a direct result of how much protease is added to the samples.

Size distributions of the glycogen extracted by the sucrose method, the formalin method and the formalin/protease method are given in Fig. 1.

As shown in Fig. 1A, each of the three extraction methods have a similar and relatively good level of repeatability, with little variation between the six distributions within each extraction method. There is however some variation so care must be taken when drawing conclusions from very similar distributions. As expected there are α- and β-particle peaks for each distribution, as has been seen in recent SEC distributions of pig-liver glycogen (Sullivan, Powell, et al., 2014).

The effect of dissolving samples at 80 °C overnight (compared to the much milder 25 °C) was also tested, as this method has been employed previously (Sullivan, Powell, et al., 2014). As can be seen in Fig. 2, glycogen from all extraction methods showed some level of degradation when dissolved at 80 °C compared to 25 °C.

Understanding the biosynthesis and regulation of the structure of glycogen requires identification and quantification of glycogen-associated proteins. We therefore compared mass spectroscopy (MS) proteomic analysis of mouse-liver glycogen extracted via the sucrose and formalin methods. Proteins were digested with trypsin and detected by LC-ESI–MS/MS. This identified 147 proteins with guanidine–HCl treatment, 40 with SDS treatment and 137 with no additional treatment (Supplementary Tables 1–4). Glycogenin and glycogen phosphorylase, proteins associated with glycogen biosynthesis, were confidently identified in formalin-extracted glycogen after guanidine–HCl treatment (Table 2).

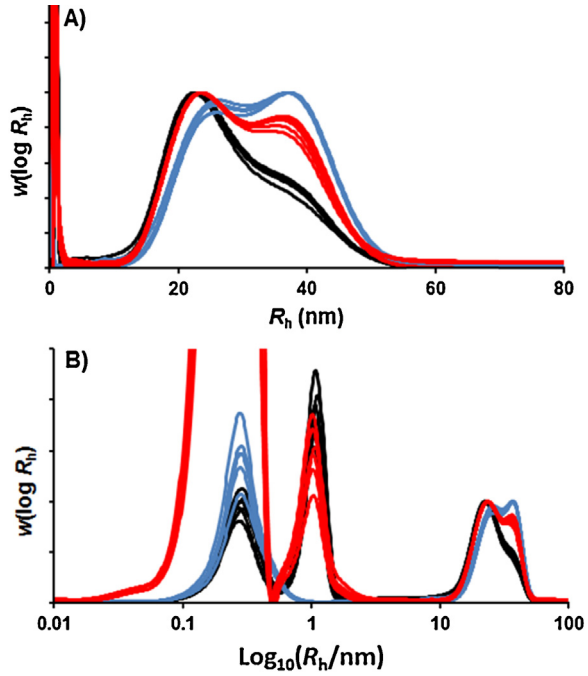

Fig. 1. Comparing extraction techniques. SEC weight distributions, $w(\log R_h)$, normalized to have equal heights for the maximum glycogen peak as a function of molecular size (the hydrodynamic radius R_h) for pig-liver glycogen extracted via the sucrose method (blue), formalin method (black) and formalin/protease method (red). The same data are provided with a linear X-axis in R_h (A) and a logarithmic X-axis (B), aiding in the visual observation of this large range of molecular sizes. While there are 6 replicates for each extraction technique, there is significant overlap between distributions of the same method. The SEC samples were also run at a concentration 5 times more dilute, with no changes occurring in the distribution, indicating no aggregation (see SI). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Peptides identified from glycogen-associated proteins enriched from formalin fixed tissue.

Sequence	ΔMass	m/z	z	Score
PYGL_MOUSE				
ARPEFMLPVHFYGR	−0.0043	430.7241	4	7
DIWNMEPSDLK	0.0025	674.3174	2	8
GIVGVENVAELK	0.0006	614.3511	2	8
HLEIIYEINQK	−0.0032	467.2564	3	8
ISLSNESSNGVSANGK	0.0027	783.3694	2	10
IVALFPK	−0.0026	394.2562	2	5
LHSFVSDDIFLR	0.0017	483.5898	3	9
TFAYTNHTVLPEALER	−0.0050	621.3179	3	9
VFADYEAYVK	0.0009	602.7983	2	8
VLYPNDNFFEGK	−0.0005	721.8510	2	10
YEYGIFNQK	0.0556	581.3102	2	9
GLYG_MOUSE				
MVVLTSPQVSDSMR	0.0034	775.3909	2	13

However, as expected by the measured purity of the samples, many non-glycogen associated proteins were also identified

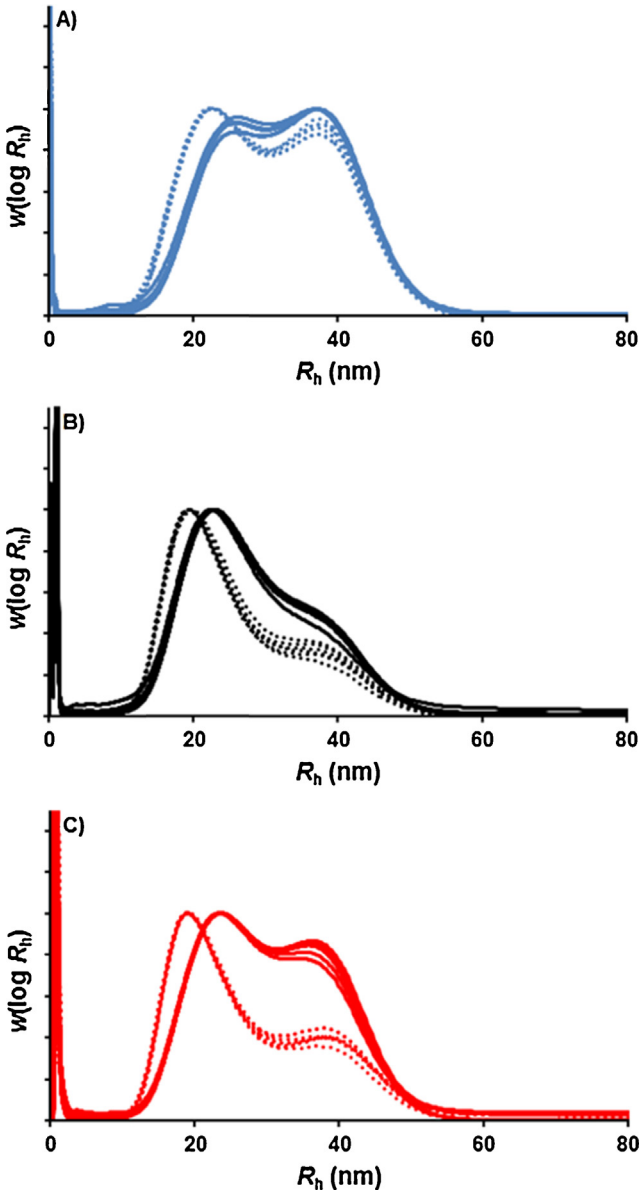

Fig. 2. The effect of heating. SEC weight distributions, $w(\log R_h)$, normalized to have equal heights for the maximum glycogen peak, as a function of molecular size (the hydrodynamic radius R_h) for pig-liver glycogen dissolved overnight at 25 °C (full line) and 80 °C (broken line) extracted via the sucrose method (A, blue), formalin method (B, black) and formalin/protease method (C, red). While there are 6 replicates for each extraction technique, there is significant overlap between distributions of the same method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in these preparations. To compare formalin-extracted glycogen with standard sucrose enriched glycogen, we performed semi-quantitative proteomics with SWATH-MS. Proteomic analysis of glycogen enriched using the sucrose method identified 290 proteins. SWATH-MS comparison of sucrose and formalin extracted glycogen found 72% of proteins had significantly different relative abundances (adjusted $P < 0.05$), suggesting that these extraction methods are qualitatively complementary (Fig. 3 – volcano plot).

Gene ontology analysis was performed to compare the proteins enriched by the sucrose and formalin extraction methods (see Table 3). The main contaminants after the sucrose method were ribosome-associated proteins, consistent with co-sedimentation of ribosomes with ultracentrifuge purified glycogen. On the other hand, the formalin method was contaminated primarily with

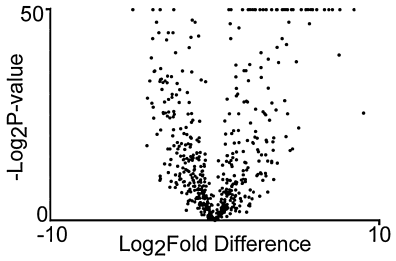

Fig. 3. Comparison of relative protein abundance of all proteins present in glycogen enriched via the sucrose and formalin methods. Volcano plot comparing differences in relative protein abundances in glycogen samples enriched via the sucrose method and the formalin method. Technical triplicates of each enrichment method were compared with SWATH-MS. Each data point represents an independent protein. Proteins with $-\text{Log}_2(P\text{-value})$ greater than 4.32 ($P = 0.05$) were considered significant. Proteins with a positive or negative Log_2(Fold Difference) were enriched in the sucrose or formalin method, respectively.

Table 3
Gene ontology analysis of proteins enriched by sucrose versus formalin methods.

Keyword	Count	%	Adjusted *P*-value
Enriched in sucrose preparation			
Ribosomal protein	67	31	8.20E−18
Ribonucleoprotein	71	32.9	4.80E−13
Acetylation	157	72.7	7.70E−05
Ribosome	17	7.9	1.50E−02
Enriched in formalin preparation			
Cytoplasm	95	47.5	2.60E−06
Hydrolase	40	20	3.70E−03
Secreted	23	11.5	2.30E−02
Disulfide bond	32	16	3.30E−02
Metal-binding	52	26	3.80E−02

soluble cytoplasmic and secreted proteins, consistent with the presence of these classes of proteins in the supernatant after formalin crosslinking.

4. Discussion

While a cold-water extraction method that utilizes sucrose density centrifugation has been shown to be effective in extracting glycogen with minimal degradation (Parker et al., 2007; Ryu et al., 2009; Sullivan et al., 2010b), a method that allows glycogen to be extracted from formalin-fixed tissues would permit the study of glycogen from formalin-fixed human tissues in pathology laboratories. The analysis of human glycogen would result in studies that are more physiologically relevant to human health. Given the discovery that the glycogen from diabetic (*db*/*db*) mice has a significantly different structure to that of the non-diabetic controls (Sullivan et al., 2011) and the evidence that glycogen structure may be important in its metabolism (Sullivan, Aroney, et al., 2014), the potential impacts of having a better understanding of glycogen metabolism in terms of structure for humans is considerable.

Because the normalization of these distributions is arbitrary, one cannot say, for example, that one technique or other results in more or less extraction of α particles; however, comparison of *relative* amounts is meaningful.

The distributions of glycogen extracted using the formalin method (without protease) have relatively fewer α particles then the sucrose method; however, when treated with protease, there is a substantial increase in the relative height of the α particle peak (see Fig. 1A). While there are still more α particles in relative terms from the sucrose method, it is possible that this is due to a loss of β particles from this method, as opposed to a loss of α particles in the formalin/protease method. Indeed, the preferential loss of

β particles in the sucrose method appears to be more likely, both because the total yield is lower (see Table 1) and because, given the method's reliance on the larger, denser particles forming a pellet after the sucrose-gradient centrifugation step, it is more likely that smaller particles would be lost. The shift of the β-particle peak from the sucrose method to higher sizes is consistent with the preferential loss of smaller particles.

One possible explanation for the increased amount of α particles in the formalin/protease method, compared to the formalin method (see Fig. 1A), is that a significant amount of α particles may be left insoluble after formalin fixation, most likely due to glycogen-associating proteins being linked together to form large insoluble aggregates which can be liberated when exposed to protease. This may suggest that there are significantly more proteins on the outside of α particles than β particles; however this is only speculation. Another possibility is that there is a network of proteins that are not connected to the larger glycogen α particles, but form a physical barrier that allows smaller β particles, but not α particles, to pass into solution. Here protease would be able to destroy this barrier, allowing these glycogen α particles to be analyzed using SEC.

If the predominant aim of an experiment is to analyze the size distributions of the liver glycogen, then small contaminant molecules (such as sucrose or small proteins) that do not overlap with the glycogen in the size distribution are inconsequential. As can be seen in Fig. 1B, there are a large amount of non-glycogen contaminants for all extraction methods; however these do not overlap in molecular size with the glycogen distributions. There is a large contaminant peak of small molecules in the formalin/protease extracted samples resulting from the tricine that was used in the buffer for the protease treatment.

If higher purities are required, the use of an S500 chromatography column has been shown to be effective at removing smaller particles such as free sugars and protein contaminants; however as is common with additional purification techniques, this leads to lower yields and also may affect the size distributions (Parker et al., 2007; Ryu et al., 2009).

The effect of heating samples at 80 °C was also analyzed, with the results showing that care must be taken when dissolving glycogen in an aqueous solvent, with lower temperatures being preferable (see Fig. 2). Of particular interest is that the α particles of glycogen extracted via the formalin and formalin/protease method are much more susceptible to degradation than glycogen extracted via the sucrose method. Glycogen extracted using these methods should therefore always be dissolved at mild temperatures. The pH of the samples from all of the extraction methods was ~7, ruling out acid hydrolysis as the reason for degradation.

4.1. Inferences for bonding between β particles in α particles

The difference in degradation rates of glycogen extracted via the different methods may shed some light on the bond that holds glycogen α particles together, the nature of which there is as yet no unambiguous evidence. The fact that the formalin extraction technique leads to a significant weakening of the bonds holding α particles together provides further evidence (in addition to that reported previously (Sullivan et al., 2012)) that glycogen α particles are not held together via glycosidic linkages. While at room temperature protein reacts relatively quickly with formaldehyde, carbohydrates are unreactive with formaldehyde at this temperature (Eltoum, Fredenburgh, Myers, & Grizzle, 2001), remaining chemically unaltered unless exposed to fixation for several weeks (Kiernan, 2000). Therefore the preferential degradation of α particles in the presence of formaldehyde is additional evidence that the bond holding them together is different to glycosidic linkages. Given the well established ability of formaldehyde to form both inter- and intra-molecular crosslinks between protein residues, it is possible that the conformation of this hypothesized protein "glue" is altered by reacting with formaldehyde. It has been shown that whether a protein maintains its native confirmation after treatment in 10% NBF depends on that protein. For example, in one study RNase A maintained a conformation almost identical to the native, untreated protein while myoglobin showed significant structural changes after treatment with formalin (Fowler, Evers, O'Leary, & Mason, 2011). However when heated, in both cases the formalin-treated proteins behaved differently to the untreated controls, having a broad and non-cooperative thermal transition as opposed to the cooperative, relatively sharp transitions of the native proteins. It is therefore entirely possible that any protein "glue" would be in a significantly different conformation at 80 °C when the glycogen was extracted with the formalin method as opposed to the sucrose method. How this difference would affect the ability of the protein to join the β particles together can only be speculated at this point and is beyond the scope of this study; however we will offer a brief description of two possibilities. Firstly, if there is a protein linked covalently to join together β particles, it is possible that denaturing this protein will make the protein backbone more susceptible to shear scission; given the relatively large molecular weights of β particles (10^6–10^7), the amount of shear scission during SEC characterization that could be subjected to a single-molecule glue holding these together may be sufficient to cleave a bond. While such shear scission is very unlikely in small molecules, it becomes increasingly likely with larger molecules, and certainly occurs with amylopectin, which is of a size commensurate with that of glycogen α particles (Cave, Seabrook, Gidley, & Gilbert, 2009). Secondly, while the possibility of a non-covalent protein linkage has been inconsistent with a number of studies that have used powerful denaturants (Orrell & Bueding, 1964), the presence of a highly resistant protein cannot be completely disregarded. If so, it is possible that this resistant protein is denatured to the extent of failing as a glue when treated with formalin and heated to 80 °C.

4.2. Identification of glycogen-associated proteins

Further investigations of the regulation of the structure of glycogen would require identification and measurement of the proteins physically associated with glycogen particles. Mass spectrometry proteomics would be a useful approach for this purpose. We therefore tested if formalin-extracted glycogen was compatible with MS proteomic analyses. Several sample preparation methods were tested, including denaturing proteins in formalin-extracted glycogen samples with guanidine–HCl or SDS, compared with no additional treatment. It has been shown here that formalin-extracted glycogen can be analyzed successfully for associated proteins when using guanidine–HCl; however due to the low purity of the samples, there is a large amount of contaminating proteins. Again this problem can be largely circumvented by employing further purification with an S500 gel chromatography column. Gene ontology analysis of the differentially abundant proteins showed that the sucrose method enriched contaminating proteins from intracellular ribosomes, whereas the formalin method enriched secreted proteins, confirming the complementarity of these methods for glycogen enrichment (see Table 3). The volcano plot (Fig. 3) illustrates these differences between the two methods. Future studies aimed at identifying bona fide glycogen-associated proteins would require additional purification steps to remove contaminating proteins.

5. Conclusions

Glycogen extracted from pig liver using a cold-water (Tris buffer) extraction method that employs sucrose gradient

centrifugation is here compared to glycogen extracted from formalin-fixed tissues. While the glycogen extracted using the formalin method was not as pure as that from the sucrose method, the overall yield of glycogen was greater. Because the impurities from both methods do not overlap with the glycogen peaks (having much smaller hydrodynamic sizes), it is not necessary to employ further purification techniques when the goal is to obtain accurate SEC size distributions.

While glycogen extracted from formalin-fixed liver had significantly fewer α particles than from the sucrose method, these "missing" particles were easily recovered by treating the glycogen with protease. The size distributions were still not identical to glycogen extracted via the sucrose method; however it is likely that this is due to a preferential loss of smaller β particles in the sucrose method. This study also highlights the importance of dissolving glycogen at mild temperatures, with glycogen from all extraction methods showing degradation when dissolved overnight at 80 °C. Interestingly, glycogen extracted from formalin-fixed tissues is significantly more susceptible to this degradation than glycogen extracted from the sucrose method. This is further evidence that glycogen α particles are not held together via glycosidic linkages, with the most reasonable hypothesis being that there is some type of protein "glue" holding them together and that this glue is weakened when treated with formalin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.005.

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