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A rapid extraction method for glycogen from formalin-fixed liver

Mitchell A. Sullivan[a,b], Shihan Li[a,b], Samuel T.N. Aroney[b], Bin Deng[a], Cheng Li[a,b], Eugeni Roura[b], Benjamin L. Schulz[c], Brooke E. Harcourt[d], Josephine M. Forbes[d,e], Robert G. Gilbert[a,b,*]

[a] Tongji School of Pharmacy, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China
[b] The University of Queensland, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, Brisbane, QLD 4072, Australia
[c] School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia
[d] Glycation and Diabetes Complications, Mater Research-UQ, Translational Research Institute, Woolloongabba, QLD 4102, Australia
[e] Mater Clinical School, University of Queensland, Brisbane, QLD 4072, Australia

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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.

* Corresponding author at: The University of Queensland, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, Brisbane, QLD 4072, Australia. Tel.: +61 7 3365 4809; fax: +61 7 3365 1188.
E-mail addresses: m.sullivan7@uq.edu.au (M.A. Sullivan), shihan.li@uqconnect.edu.au (S. Li), samuel.aroney@uqconnect.edu.au (S.T.N. Aroney), bindeng@hust.edu.cn (B. Deng), cheng.li1@uqconnect.edu.au (C. Li), e.roura@uq.edu.au (E. Roura), b.schulz@uq.edu.au (B.L. Schulz), brooke.harcourt@mater.uq.edu.au (B.E. Harcourt), josephine.forbes@mater.uq.edu.au (J.M. Forbes), b.gilbert@uq.edu.au (R.G. Gilbert).

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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.