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A new non-degradative method to purify glycogen

Xinle Tan[a,b], Mitchell A. Sullivan[b,c], Fei Gao[a], Shihan Li[b,d], Benjamin L. Schulz[e], Robert G. Gilbert[b,f,*]

[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] Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada
[d] Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria 3052, Australia
[e] School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia
[f] Joint International Research Laboratory of Agriculture and Agri-Product Safety, College of Agriculture, Yangzhou University, Yangzhou 225009, Jiangsu Province, PR China

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ABSTRACT

Liver glycogen, a complex branched glucose polymer containing a small amount of protein, is important for maintaining glucose homeostasis (blood-sugar control) in humans. It has recently been found that glycogen molecular structure is impaired in diabetes. Isolating the carbohydrate polymer and any intrinsically-attached protein(s) is an essential prerequisite for studying this structural impairment. This requires an effective, non-degradative and efficient purification method to exclude the many other proteins present in liver. Proteins and glycogen have different ranges of molecular sizes. Despite the plethora of proteins that might still be present in significant abundance after other isolation techniques, SEC (size exclusion chromatography, also known as GPC), which separates by molecular size, should separate those extraneous to glycogen from glycogen with any intrinsically associated protein(s). A novel purification method is developed for this, based on preparative SEC following sucrose gradient centrifugation. Proteomics is used to show that the new method compares favourably with current methods in the literature.

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1. Introduction

Glycogen, a complex glucose polysaccharide found in a number of organisms, is highly branched and works in regulation of blood-sugar homeostasis (glucose buffering). One of its main locations in mammals is the liver. Liver glycogen comprises α-(1 → 4) linear linkages and α-(1 → 6) branch points, and forms small β particles (~20 nm in diameter); these may bond together to form a composite α particle (~50–200 nm) which has a broccoli-like appearance under transmission electron microscopy. It also contains a small but significant amount of protein (Meyer, Heilmeyer, Haschke, & Fischer, 1970; Rybicka, 1996; Smythe, Villar-Palasi, & Cohen, 1989).

Recent work (Deng et al., 2015; Sullivan et al., 2011) has found that the binding of β into α particles is impaired in diabetic mouse livers. There is evidence (Powell, Sullivan, Sheehy, Schulz, Warren, & Gilbert, 2015; Sullivan et al., 2012) that this binding involves a protein which is structurally part of the glycogen molecule. However, identifying this protein is burdened with the difficulty of there being a large number of proteins in the liver, only a small number of which would be directly part of the glycogen molecule. Separation of this polymer, without its molecular degradation, and so that the intrinsic glycogen protein(s) would be preserved intact while extraneous ones are removed, is a significant problem. Identifying endogenous glycogen proteins could open up new developments in diabetes drug targets (Sullivan, Harcourt, Xu, Forbes, & Gilbert, 2015).

The glycogen molecule is directly associated with various proteins, including glycogen phosphorylase, glycogen debranching enzyme and other complex regulatory proteins (Stapleton et al., 2010); these are involved in its metabolism, control its structure, particle size and sub-cellular distribution. Previous studies focussed on the proteome associated with the surface of glycogen, using malto-oligosaccharides to compete for glycogen binding activities and release the related proteome (Stapleton, Nelson,

* Corresponding author at: The University of Queensland, Centre for Nutrition and Food Sciences, Building 83, Brisbane 4072, QLD, Australia.
E-mail address: b.gilbert@uq.edu.au (R.G. Gilbert).

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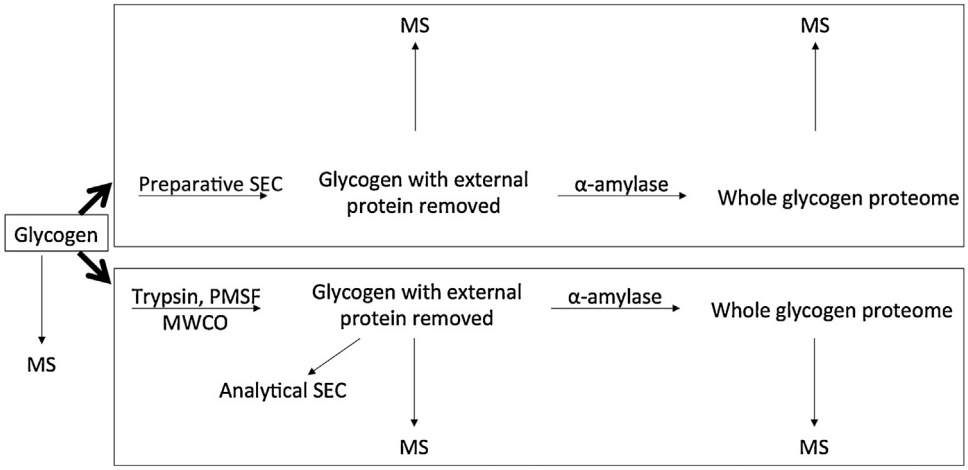

Fig. 1. Schematic of the preparative SEC and trypsin procedures for glycogen purification.

Parsawar, Flores-Opazo, McClain, & Parker, 2013; Stapleton et al., 2010). This work identified both expected and unexpected proteins with metabolic roles involving glycogen. Many other proteins unrelated to glycogen metabolism have also identified (Stapleton et al., 2010), such as cytochrome P450, which indicates that not all the extraneous proteins were being excluded. The majority of the proteins associated with glycogen will be extraneous contaminants in the liver which are not actually attached (either covalently or non-covalently) to the glucan component.

In the present paper, two new methods are developed and tested to remove these extraneous contaminants. The first method (which has some similarities to those used by some other researchers) uses trypsin to digest the external proteins affiliated with liver glycogen, followed by deactivation with PMSF (phenylmethylsulfonide fluoride), an inhibitor of serine proteases. The result is tested using size-exclusion chromatography (SEC, also denoted GPC) to see if it retains its intact molecular structure. PMSF is used to inactivate the added trypsin, to avoid the latter cleaving the α-amylase that is added in the following step and to avoid the trypsin from degrading the interior proteins released after amylase treatment. The second method, preparative SEC, is used to purify glycogen from its associated proteins by size separation, as the size of these proteins is significantly smaller than the size range of molecular glycogen, which is 10 nm in diameter, e.g. (Deng et al., 2015). SEC separates by hydrodynamic volume, a parameter based on molecular size. Thus despite the plethora of proteins that might still be present in significant abundance after initial separation, this should neatly separate proteins extraneous to glycogen from glycogen with any intrinsically associated protein(s).

A combination of analytical and preparative SEC is used here to provide an efficient purification and testing. The preparative SEC is used first to purify glycogen according to elution volume, using the signal from the DRI (deferential reflective index) detector to measure concentration, followed by running a small aliquot of the collected samples through an analytical SEC to validate the purification of the glycogen. Although analytical SEC gives better size separation than preparative SEC, using just analytical SEC in both purification and validation is not practical with the goal of obtaining samples for subsequent further analysis, because this technique has only a small load capacity.

It is important to qualitatively and quantitatively identify the proteins involved in the various steps in this procedure. This is done here using proteomics, particularly an LC–MS/MS technique, with time-of-flight (TOF) MS. An IDA (information-dependent acquisition) identification method was used to explore the glycogen proteome. Efficacy is judged by which methods has the fewest remaining proteins, and whether these proteins are likely be directly associated with glycogen, and also by the similarities in the glycogen size distributions before and after purification.

2. Materials and methods

2.1. Glycogen

Mouse-, rat- and pig- liver glycogen were obtained as follows. Healthy C57BL/6J mice and Wistar rats were raised in a standard specific pathogen-free (SPF) animal room with the temperature controlled at 22 ± 1 °C and a 12-h dark/light cycle (lights on at 7 am). The animals were anaesthetized with sodium pentobarbitone (150 mg/kg intraperitoneal) when they reached the age of 12 weeks. Livers were then excised, dropped into liquid nitrogen and stored at −80 °C. All procedures on these animals were approved by the Huazhong University of Science and Technology Tongji Medical College Animal Care and Ethics Committee. Pig-liver glycogen was obtained as detailed elsewhere (Sullivan et al., 2012).

2.2. Initial glycogen extraction

Initial glycogen extraction followed procedures detailed elsewhere (Sullivan, Harcourt et al., 2015; Sullivan, Li et al., 2015). Approximately 1500 mg of animal liver was removed and homogenized with 25 mL glycogen extraction buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, protease-inhibiting cocktail (Roche)). Samples were then centrifuged at 6000 *g* for 10 min at 4 °C. Separate supernatants were obtained from further centrifugation at 260,000 *g* for 2 h at 4 °C. The resulting pellets were resuspended in glycogen isolation buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, and PMSF) and run through a 20 mL stepwise sucrose gradient (37.5% and 75%) at 370,000 *g* for 2.5 h at 4 °C. Finally, pellets were dissolved in 0.5 mL deionized water, then 2 mL absolute ethanol added, followed by centrifugation at 4000 *g* for 10 min. The resulting pellet was dissolved in 1 mL deionized water and lyophilized overnight (freeze-dryer; VirTis, BTP-9EL).

2.3. Analytical SEC

An aqueous SEC set up was used as described elsewhere (Deng et al., 2015). All glycogen samples were dissolved in SEC eluent (50 mM NH_4NO_3 with 0.02% sodium azide) in a thermomixer at 80 °C overnight.

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