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SHORT COMMUNICATION

Molecular structure of glycogen in diabetic liver

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Abstract Liver glycogen (involved in maintaining blood-sugar levels) is a hyperbranched glucose polymer containing β particles (diameter ~20 nm), which can form composite α particles (diameter ~50–300 nm), and includes a small but significant amount of bound protein. Size distributions of glycogen from livers of healthy and diabetic mice were examined using size-exclusion chromatography with two separate eluents: aqueous eluent and dimethylsulfoxide (DMSO) eluent. Morphologies were examined with transmission electron microscopy. Diabetic glycogen (DG) exhibited many α particles in the mild water-based solvent, but in DMSO, which breaks H bonds, these degraded to β particles; α particles however were always present in healthy glycogen (HG). This DG fragility shows the binding of β into α particles is different in HG and DG. The diabetic α particle fragility may be involved with the uncontrolled blood-sugar release symptomatic of diabetes: small β particles degrade more easily to glucose than α particles. This has implications for diabetes management.

Keywords Glycogen · *db/db* mice · Size-exclusion chromatography · Type 2 diabetes

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Introduction

Liver glycogen, a complex highly-branched glucose polymer containing a small but significant amount of bound protein [1–3], acts as a blood-sugar storage molecule in humans and other animals [1]. In the liver, glycogen β particles (~20 nm in diameter) are able to form much larger composite structures termed α particles (up to ~300 nm in diameter), which show in transmission electron microscopy (TEM) a cauliflower-like appearance [2]. Size-exclusion chromatography (SEC) with dimethyl sulfoxide/LiBr as eluent revealed that glycogen extracted from the livers of diabetic (*db/db*) mice have very few α particles [4]. This discovery has important potential implications for diabetes management, because there is evidence that the rate of degradation per monomer unit of glycogen to glucose will be faster in smaller β particles [5], and a major characteristic of diabetes is uncontrolled glucose release. Subsequent to the observation that *db/db* mice have fewer α particles in DMSO eluent, it was found that the use of an aqueous-SEC system results in significantly better separation/resolution of the α-particle and β-particle components of glycogen [6]. Our previous work had also shown that α particles are strongly resistant to many chemical processes which would break certain types of non-covalent and covalent bonds [7]. We also showed that the bonding between the component β particles was quite different in α particles in glycogen from healthy (non-diabetic) liver and in phytoglycogen,

the composite particles strongly resembling liver α particle formed in certain mutant plants, where the inter-β particle bonding can only be glycosidic [8]. The objective of the present work is to compare healthy glycogen (HG) and diabetic glycogen (DG) structure using the improved aqueous-SEC method.

Materials and methods

Animal

Genetically diabetic C57BL/6J-*db/db* female mice, a model for type 2 diabetes, were used in this study. Such mice are resistant to the hormone leptin, a potent agent for satiety. This makes them susceptible to obesity, insulin resistance, hyperinsulinemia and transient hyperglycemia [9]. Wild-type (+/+) female mice (also on the C57BL/6J background) were chosen as the control group. The mice 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). When the mice were 12 weeks of age, they were anaesthetized with sodium pentobarbitone (150 mg/kg intraperitoneal) and their livers were rapidly excised, snap-frozen in liquid nitrogen and stored at –80 °C. All animal experiments were approved by the Huazhong University of Science and Technology Tongji Medical College Animal Care and Ethics Committee.

Purification and extraction of glycogen

Glycogen was extracted similarly to the method given in a previous study [10]. Approximately 1500 mg of mouse liver was homogenized in 25 mL of glycogen isolation buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF and 5 mM sodium pyrophosphate). Samples were centrifuged at 6000*g* for 10 min at 4 °C. The supernatants were then centrifuged at 260,000*g* for 2 h at 4 °C. The pellets were then resuspended in glycogen isolation buffer and layered over a 20 mL, stepwise sucrose gradient (37.5 and 75 % in deionized water). These samples were then centrifuged at 370,000*g* for 2.5 h at 4 °C. The pellets of glycogen at the bottom of the tube were resuspended in 0.5 mL of deionized water. Samples were mixed with four parts absolute ethanol to precipitate the glycogen. The samples were centrifuged at 4000*g* for 10 min and the pellets were re-dissolved in 1 mL of deionized water and lyophilized (freeze-dryer; VirTis, BTP-9EL).

TEM

TEM images of glycogen were obtained by a method similar to that used elsewhere [11]. Glycogen was resuspended in 50 mM Tris-HCl pH 7.0 at a concentration of 1 mg/mL. The suspension was then diluted 10-fold and applied onto a glow discharged copper grid (400 mesh). After 2 min, excess sample was drawn off with filter paper, and the grids stained with two or three drops of 1 % uranyl acetate. The preparations were examined by using a Hitachi H-7000 transmission electron microscope operating at 75 kV using AnalySiS image management software.

DMSO SEC

DMSO SEC of glycogen was obtained using a similar method to that used previously [6]. Glycogen was dissolved in a thermomixer for 8 h at 80 °C in DMSO with 0.5 wt% LiBr at 2 mg/mL. Samples were injected into an Agilent 1260 Infinity SEC system (Agilent, Santa Clara, CA, USA) using a SUPREMA pre-column, 30 and 3000 columns (Polymer Standards Service [PSS], Mainz, Germany). The columns were kept at 80 °C using a column oven with flow rate (0.3 mL/min). A refractive index detector (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 342–2.35×10^6 Da were dissolved in DMSO with 0.5 wt% LiBr and run through the SEC system, allowing the construction of a universal calibration curve. It is noted that SEC separates by molecular size (specifically the hydrodynamic volume or the corresponding hydrodynamic radius), not molecular weight. Because pullulan is a linear polymer, its molecular weight and hydrodynamic radius are uniquely related, and both can be measured independently. Thus the use of pullulan as a standard enable the volume at which a particular molecular size is eluted in the SEC set-up at hand to be converted to the corresponding hydrodynamic radius.

Water SEC

An aqueous SEC setup similar to that recently employed for obtaining SEC distributions of glycogen with a water-based eluent was also used here [6]. Glycogen was dissolved in a thermomixer for 8 h at 80 °C in 50 mM ammonium nitrate/0.02 % sodium azide at 2 mg/mL. The effect of DMSO on glycogen structure was also tested. Glycogen was first dissolved in DMSO for 8 h at 80 °C followed by ethanol precipitation and lyophilization. The glycogen with DMSO treatment was prepared in the same way as described above. Samples were injected into an Agilent 1260 infinity SEC system (Agilent, Santa Clara, CA, USA) using a 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. A refractive index detector (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 342–2.35×10^6 Da were dissolved in 50 mM ammonium nitrate/0.02 % sodium azide

and run through the SEC system, allowing the construction of a universal calibration curve.

Results

Figure 1 gives the size distributions of liver glycogen from healthy (+/+) and diabetic (*db/db*) mice from aqueous SEC. This shows the SEC weight distributions $w(\log R_h)$: the weight of molecules as a function of the SEC separation parameter, the hydrodynamic radius R_h. In contrast to a past study using DMSO-SEC, glycogen from healthy and diabetic mice have similar wide size distributions, with both having a significant population of α particles.

As can be seen in the TEM images (see Fig. 2a and b), composite α particles exist in both HG and DG; this confirms the inferences from the SEC data. While it is tempting to use a large number of TEM images to attempt to obtain quantitative

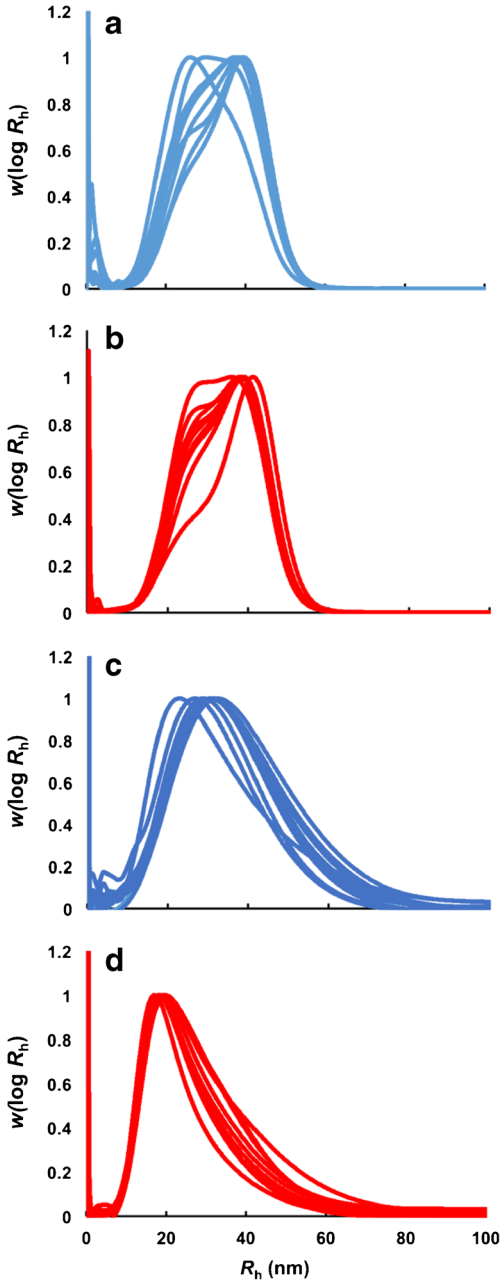

Fig. 1 SEC weight distributions, $w(\log R_h)$ (arbitrary units), for HG (**a**) and DG (**b**) using an aqueous-SEC setup, and the same HG (**c**) and DG (**d**) using a DMSO-SEC system. Curves have been normalized to equal maximum heights. Identification of individual mice is given in ESM

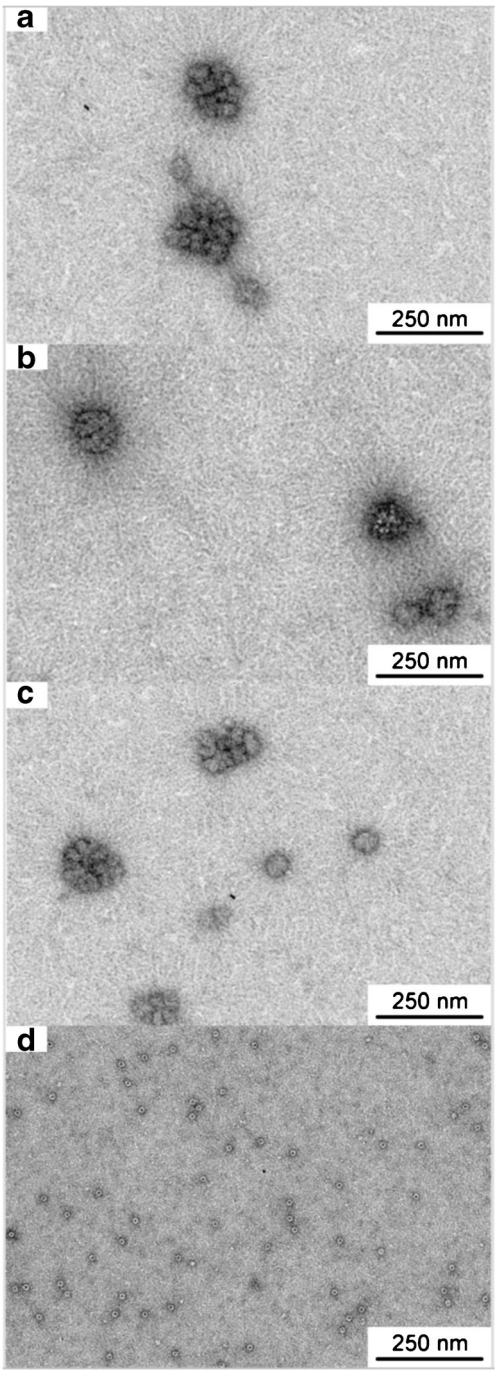

Fig. 2 Transmission electron microscope (TEM) images of glycogen from healthy (+/+) mice (**a**) and diabetic (*db/db*) mice (**b**) and glycogen after DMSO treatment from healthy (+/+) mice (**c**) and diabetic (*db/db*) mice (**d**)

information about the size distribution, this is impossible in practice. Even for perfectly spherical, compositionally homogeneous, polystyrene particles, which are resistant to any deformation of the electron beam, many hundreds of particles from many different randomly-chosen sets of micrographs need to be counted for quantitative accuracy [12]. The situation is far worse with glycogen, since the particles are easily deformed, not perfectly spherical, and readily degraded under the electron beam.

Figure 1 also shows HG and DG SEC distributions that have been run through a DMSO-LiBr SEC setup. As expected due to the much poorer separation/resolution of the DMSO setup [6], only one peak was present for each distribution. It can be clearly seen, however, that when comparing HG and DG using DMSO/LiBr, the HG has on average more large particles, similar to results shown previously.

In the light of this observation, the susceptibility of DG to degradation when dissolved in DMSO was tested. The liver glycogen samples from eight healthy and eight diabetic mice were both left in DMSO (where they completely dissolved) for 8 h at 80 °C, followed by ethanol precipitation and lyophilization. The SEC distributions of these DMSO-treated samples were compared with those of untreated glycogen in the aqueous-SEC system.

Figure 3 gives the size distribution of HG and DG, both DMSO-treated and untreated. The size distributions of the HG

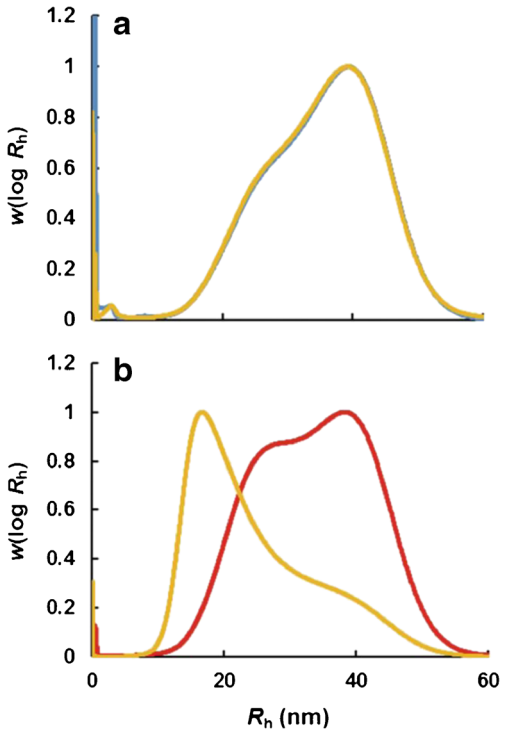

Fig. 3 SEC weight distribution $w(\log R_h)$, arbitrary units, of **a** HG samples (untreated, *blue*; treated with DMSO, *yellow*) and **b** DG samples (untreated glycogen, *red*; treated with DMSO, *yellow*). All distributions are normalized to equal maxima. Only one sample of each kind of glycogen is displayed here. The data for all 16 samples is given in ESM, and all show the same trend as those given here

sample do not change significantly after DMSO treatment. However, the DG samples show a significant decrease in the relative amount of α particles after DMSO treatment. This is also seen qualitatively in the TEM images (Fig. 2). Indeed, the TEM of DG after DMSO treatment (Fig. 2d) apparently shows tiny particles with a core-shell morphology; this morphology may or may not be a TEM artifact, but what is apparent is that there are no remaining α particles. This confirms that while diabetic (*db/db*) mice are capable of synthesizing α particles. These α particles are significantly more susceptible to degradation in DMSO, compared to the healthy particles. This explains why a previous report using DMSO-SEC found much fewer α particles in diabetic mice than the healthy controls [4].

To quantify the degree of degradation caused by DMSO on both HG and DG, the average R_h [13], $\overline{R}_h$, of each sample before and after DMSO treatment is given in Table 1, which also shows the ratios of the initial $\overline{R}_h$ without treatment to that after DMSO treatment. This ratio will be referred to as the degradation quotient. The larger this quotient, the greater is the extent of degradation due to the DMSO treatment (a quotient of one meaning no degradation). The average degradation quotients for the eight healthy and eight diabetic glycogen samples were analyzed using SPSS 17.0, giving 1.02±0.01 and 1.36±0.02, respectively. The difference between the HG and DG degradation quotients was statistically significant ($p<0.01$). The data demonstrate that DMSO causes little, if any, degradation of liver glycogen extracted from healthy mice, but significant degradation of DG.

Table 1 Average R_h ($\overline{R}_h$) before and after DMSO treatment and the degradation quotient of each mouse

Sample	Phenotype (healthy/diabetic)	$\overline{R}_h$ (nm)	$\overline{R}_h$ (nm) after DMSO treatment	Degradation quotient
H0932	Healthy	28.2	27.5	1.03
H0941	Healthy	26.6	25	1.07
H0950	Healthy	30.5	30.4	1.00
H1001	Healthy	29.3	29.2	1.00
H1005	Healthy	30.4	30.3	1.00
H1025	Healthy	28.5	28.2	1.01
H1029	Healthy	28.5	27.9	1.02
H1009	Healthy	27.7	27.2	1.01
Db0901	Diabetic	28.7	19.3	1.48
Db0903	Diabetic	27.9	20.5	1.36
Db0904	Diabetic	29.9	20.9	1.43
Db0912	Diabetic	29.4	21.9	1.34
Db1007	Diabetic	28.3	22.3	1.27
Db1002	Diabetic	29.2	22.5	1.3
Db1003	Diabetic	28.4	20.7	1.37
Db1004	Diabetic	28.2	20.9	1.34

Discussion

Since liver glycogen from diabetic (*db/db*) mice is significantly more vulnerable to degradation in DMSO than non-diabetic glycogen, the question remains as to why there is this difference and whether this is physiologically important, given liver glycogen's role in maintaining blood-glucose control. For example, do these more fragile α particles in *db/db* mice behave differently *in vivo*? There is increasing evidence that smaller glycogen particles are more vulnerable to enzymatic degradation, presumably due to their higher ratio of surface area to volume [5]. Thus if the fragile bonding of α particles in diabetic mice leads to increased degradation into β particles, this may contribute to their characteristically high blood-glucose levels.

Another question arising from the results given here involves determining what is causing this difference in vulnerability to DMSO degradation. What holds α particles together is still unknown, with a number of different studies coming to conflicting conclusions [14]. Recent acid hydrolysis experiments are consistent with the inference that there is a non-glycosidic bond holding α particles together [7]. This is reinforced by the present data: DMSO, while being an effective solvent, has no conceivable chemical reactivity under these conditions capable of cleaving a glycosidic linkage. The present results also provide evidence that any protein acting as a "glue" most likely interacts via non-covalent interactions. If there is a protein "glue" holding α particles together, the question remains as to why α particles in *db/db* mice are more susceptible to degradation in DMSO. One possibility is that there is a difference in either the confirmation or quantity of this protein "glue", resulting in more loosely bound particles. Another possibility is that there is a difference in the structure of DG that makes the protein "glue" less effective. There is evidence that DG has a slightly lower level of long chains than healthy glycogen [4]. Perhaps this or another physical property of the glycogen decreases the ability of a protein "glue" to hold α particles together.

There are many examples in diverse biological systems of carbohydrate-binding proteins, or lectins, which can cross-link large carbohydrate-rich moieties. Lectins are a diverse family of proteins found in many species that regulate protein-protein and cell-cell interactions through their specific glycan binding activities [15]. They are commonly small protein domains with binding affinity to specific carbohydrate structures, including galactosides, mannosides, sialic acids, fucose, and glucosides [16]. Lectin domains can be present as part of a larger protein, or as isolated small domains. When present as isolated domains, lectins typically form dimers or higher oligomers, and this oligomerization allows cross-linking of other bound glycoconjugates. Polymeric matrices have also been engineered, consisting of large, highly glycosylated mucin proteins, cross-linked by lectins [17]. Finally, lectin domains are often unusually stable to proteolytic, chemical or thermal denaturation [18, 19]; this would provide an alternative explanation to the resistance that liver α particles show to degradation by protease. Together, these features make it not unreasonable that a multimeric cytosolic lectin or lectin-like protein with specificity for glucosides could bind β particles to regulate formation and disassembly of glycogen α particles *in vivo*.

Another possibility is that the binding agent is glycogenin, which is the initiator for the growth of a glycogen molecule. While glycogenin can only form one bond with glycogen, it can exist as a dimer [20], which would then serve to link two β particles.

At this stage, what has just been suggested are only hypotheses about the binding; further work (especially proteomics) is needed to determine what kind of protein could bind β particles to form α particles.

In summary, both SEC size distributions and TEM images show that, in contrast to a past study [4], α particles exist in DG (*db/db*). This apparently conflicting finding is explained by these current results, revealing that diabetic α particles are significantly more fragile in DMSO (the solvent used in the past study) than HG. There is therefore a difference between the efficacy of α-particle binding in diabetic and healthy mice. This discovery provides greater understanding of the mechanism of glycogen formation and type 2 diabetes. It is not unreasonable to suppose that DG is much more easily degraded into its component β particles during glycogenolysis (degradation to glucose when the body needs blood sugar), and there is evidence that the rate of degradation is slower if the glucose is stored as large compact α particles. The fragility of α particles in DG shown here then suggests that this glycogenolysis in diabetes will result in the uncontrolled rate of release of blood sugar which is a characteristic of the disease. Further investigation into this impaired binding may have significant implications for developing new drug targets for type 2 diabetes.

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References

1. Meyer, F., Heilmeyer, L., Haschke, R.H., Fischer, E.H.: Control of phosphorylase activity in a muscle glycogen particle .1. Isolation and characterization of protein-glycogen complex. J. Biol. Chem. **245**(24), 6642–6648 (1970)
2. Rybicka, K.K.: Glycosomes—the organelles of glycogen metabolism. Tissue Cell **28**(3), 253–265 (1996)
3. Stapleton, D., Nelson, C., Parsawar, K., McClain, D., Gilbert-Wilson, R., Barker, E., Rudd, B., Brown, K., Hendrix, W., O'Donnell, P., Parker, G.: Analysis of hepatic glycogen-associated proteins.

Proteomics **10**(12), 2320–2329 (2010). doi:10.1002/pmic.200900628

4. Sullivan, M.A., Li, J., Li, C., Vilaplana, F., Zheng, L., Stapleton, D., Gray-Weale, A.A., Bowen, S., Gilbert, R.G.: Molecular structural differences between type-2-diabetic and healthy glycogen. Biomacromolecules **12**(6), 1983–1986 (2011). doi:10.1021/bm2006054
5. Sullivan, M.A., Aroney, S.T.N., Li, S., Warren, F.J., Joo, L., Mak, K.S., Stapleton, D.I., Bell-Anderson, K.S., Gilbert, R.G.: Changes in glycogen structure over feeding cycle sheds new light on blood-glucose control. Biomacromolecules **15**(2), 660–665 (2014). doi:10.1021/bm401714v
6. Sullivan, M.A., Powell, P.O., Witt, T., Vilaplana, F., Roura, E., Gilbert, R.G.: Improving size-exclusion chromatography for glycogen. J. Chromatogr. A **1332**(1), 21–29 (2014). doi:10.1016/j.chroma.2014.01.053
7. Sullivan, M.A., O'Connor, M.J., Umana, F., Roura, E., Jack, K., Stapleton, D.I., Gilbert, R.G.: Molecular insights into glycogen alpha-particle formation. Biomacromolecules **13**(11), 3805–3813 (2012). doi:10.1021/bm3012727
8. Powell, P.O., Sullivan, M.A., Sheehy, J.J., Schultz, B.L., Warren, F.J., Gilbert, R.G.: Acid hydrolysis and molecular density of phytoglycogen and liver glycogen helps with understanding the bonding in alpha (composite) glycogen molecules. PLoS One. (2015). doi:10.1371/journal.pone.0121337
9. Roesler, W.J., Khandelwal, R.L.: Age-related changes in hepatic glycogen metabolism in the genetically diabetic (db/db) mouse. Diabetes **34**(4), 395–402 (1985)
10. Sullivan, M.A., Li, S., Aroney, S.T., Deng, B., Li, C., Roura, E., Schulz, B.L., Harcourt, B.E., Forbes, J.M., Gilbert, R.G.: A rapid extraction method for glycogen from formalin-fixed liver. Carbohydr. Polym. **118**(1), 9–15 (2015). doi:10.1016/j.carbpol.2014.11.005
11. Powell, P.O., Sullivan, M.A., Sweedman, M.C., Stapleton, D.I., Hasjim, J., Gilbert, R.G.: Extraction, isolation and characterisation of phytoglycogen from su-1 maize leaves and grain. Carbohydr. Polym. **101**(1), 423–431 (2014). doi:10.1016/j.carbpol.2013.09.061
12. Lichti, G., Gilbert, R.G., Napper, D.H.: Mechanisms of latex particle formation and growth in the emulsion polymerization of styrene using the surfactant sodium dodecyl sulfate. J. Polym. Sci. Polym. Chem. Ed. **21**, 269–291 (1983)
13. Vilaplana, F., Gilbert, R.G.: Two-dimensional size/branch length distributions of a branched polymer. Macromolecules **43**(17), 7321–7329 (2010). doi:10.1021/ma101349t
14. Gilbert, R.G., Sullivan, M.A.: The molecular size distribution of glycogen and its relevance to diabetes (review). Aust. J. Chem. **67**(4), 538–543 (2014). doi:10.1071/CH13573
15. Varrot, A., Basheer, S.M., Imberty, A.: Fungal lectins: structure, function and potential applications. Curr. Opin. Struct. Biol. **23**(5), 678–685 (2013). doi:10.1016/j.sbi.2013.07.007
16. Arnaud, J., Audfray, A., Imberty, A.: Binding sugars: from natural lectins to synthetic receptors and engineered neolectins. Chem. Soc. Rev. **42**(11), 4798–4813 (2013). doi:10.1039/c2cs35435g
17. Crouzier, T., Beckwitt, C.H., Ribbeck, K.: Mucin multilayers assembled through sugar-lectin interactions. Biomacromolecules **13**(10), 3401–3408 (2012). doi:10.1021/Bm301222f
18. He, S., Simpson, B.K., Ngadi, M.O., Xue, S.J., Shi, J., Ma, Y.: pH stability study of lectin from black turtle bean (Phaseolus vulgaris) as influenced by guanidinium-HCl and thermal treatment. Protein Peptide Lett **22**(1), 45–51 (2015)
19. Pusztai, A., Grant, G.: Assessment of lectin inactivation by heat and digestion. Methods Mol. Med. **9**, 505–514 (1998). doi:10.1385/0-89603-396-1:505
20. Roach, P.J., Depaoli-Roach, A.A., Hurley, T.D., Tagliabracci, V.S.: Glycogen and its metabolism: some new developments and old themes. Biochem. J. **441**(3), 763–787 (2012). doi:10.1042/BJ20111416