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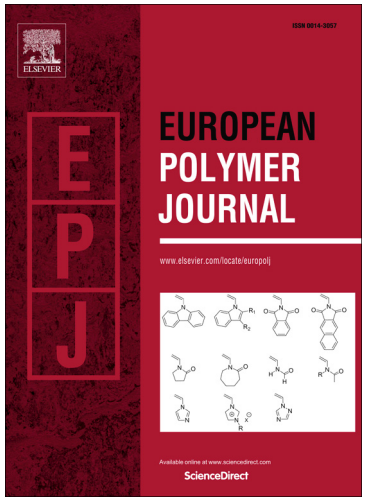

Molecular-size dependence of glycogen enzymatic degradation and its importance for diabetes

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Molecular-size dependence of glycogen enzymatic degradation and its importance for diabetes

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Abstract

Glycogen, a hyperbranched glucose polymer, is the blood-sugar reservoir in animals. Liver glycogen comprises small β particles, which can join together as large composite α particles. It had been shown that the binding between β in α particles in the liver of diabetic mice is more fragile than in healthy mice. This could be linked to the loss of blood-sugar control characteristic of diabetes if the rate per monomer unit of the enzymatic degradation to glucose of α particles were significantly slower than that of β particles. This is tested here by examining the *in vitro* time evolution of the molecular size distribution of glycogen from the livers of healthy rats and diabetic mice, containing distinct components of both α and β

particles; this treatment is analogous to the "competitive growth" method used to explore mechanisms in emulsion polymerization. Simulations for the time evolution of the molecular size distribution were also performed. It is found that the degradation rate per monomer unit is indeed faster for the smaller particles, supporting the hypothesis of a causal link between chemical fragility of glycogen from diabetic liver with poor control of blood-sugar release. Comparison between simulations and experiment indicate that α and β particles have significant structural differences.

Key words: glycogen, branched polymer, particle size, kinetics, diabetes.

1. Introduction

Glycogen is a highly branched polymer of glucose that comprises two types of particles: small β particles (~20 nm in diameter), which can form large α particles (up to 300 nm in diameter) which have a cauliflower-like appearance under transmission electron microscopy [1-3]. Glycogen predominantly exists in brain, muscle [4], heart [5] and liver, functioning as a buffer to release or store glucose as appropriate.

We have shown previously that liver glycogen in db/db mice (an animal model of type 2 diabetes) has similar amounts of α particles to healthy liver glycogen. However, the α particles from diabetic mice are much more fragile [6], e.g. to solvents which break hydrogen bonds, than those from healthy mice. They would thus be more readily broken down into β particles [7] in the liver. This phenomenon could be related to the hyperglycemia (high blood glucose, loss of blood-sugar control) that is characteristic of diabetes if the degradation process of β particles to glucose were to be at a faster rate than that of α particles.

This study investigates the relative rates of the degradation processes of α and β particles from the livers of healthy rats, healthy mice and diabetic db/db mice, by examining the time evolution of glycogen molecular size distribution during *in vitro* enzymatic degradation.

Glycogen phosphorylase, which catalyzes the breakdown of glycogen by cleaving (1⟶4)-α glycosidic bonds [8, 9], was used to imitate glycogenolysis *in vitro*. Size-exclusion

chromatography (SEC, also termed gel-permeation chromatography, GPC), which separates fully dissolved and dispersed polymer molecules by size (the hydrodynamic radius R_h), was used to examine the molecular size distributions of glycogen at different degradation times [10]. Studying the time evolution of bimodal size distributions to make mechanistic inferences is analogous to the "competitive growth" experiments used to develop mechanistic understanding of growth processes in emulsion polymerization [11].

2. Experimental

2.1. Animals

Male Wistar rats (8 weeks), male C57 mice (12 weeks) and male C57BL/6j-db/db mice (12 weeks) were used. Rats and C57 mice were purchased from Hubei Provincial Food and Drug Safety Center. C57BL/6j db/db mice were purchased from the Model Animal Research Center of Nanjing University. Animals were housed in standard cages, with the temperature controlled at 22 ± 1°C. A 12 h dark/light cycle was used, with lights on at 7 am and off at 7 pm. Animals had *ad libitum* access to standard chow (6% kcal from fat, 14.3 MJ kg^{-1}, Hubei Provincial Center for Disease Control and Prevention) and water. Animals were anaesthetized with sodium pentobarbitone (150 mg kg^{-1}, i.p.), with their livers rapidly excised and snap-frozen in liquid nitrogen. Samples were 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.

2.2. Glycogen extraction and purification

Glycogen extraction was carried out following our published method with some modifications [12]. Liver glycogen was homogenized, and a stepwise sucrose gradient (37.5% and 75% in deionized water) method was used to remove non-glycogen components. Glycogen was further purified by ethanol precipitation, and then dissolved in 1 mL of deionized water and lyophilized (VirTis BTP-9EL freeze-dryer).

2.3. Glycogen degradation

Glycogen degradation was analyzed as described elsewhere [12]. This used a buffer solution obtained by mixing 500 mM potassium phosphate (100 mL), 300 mM magnesium chloride (5 mL) and 100 mM EDTA (1 mL) (adjusted to pH 6.8). The digestion buffer was obtained by mixing this solution (1.5 mL) with deionized water (10 mL), NADP (6.5 mM) and 0.1% (w/v) α-D glucose 1,6-disphosphate. 2 mg of liver glycogen was dissolved in 1.2 mL digestion buffer and then incubated at 80 °C with agitation at 350 rpm for 4 h. The following reagents (Sigma) were added in this mixture: glycogen phosphorylase (40 μL, 1 mg mL^{-1}), phosphoglucomutase (24 μL, 10 U mL^{-1}), glucose-6-phosphate dehydrogenase (24 μL, 10 U mL^{-1}) and 5′AMP (2 μL, 100 mM). Samples were collected after 0, 20 and 60 min, and absolute ethanol added (4 times) to terminate the reaction. These times were chosen so as to cover the range from the time the degradative enzyme was added to when the glycogen was almost completely degraded. Digested glycogen was collected after centrifugation at 4000 g for 10 min, and was re-dissolved in 0.3 mL of deionized water and lyophilized (freeze-dryer; VirTis, BTP-9EL) for SEC analysis.

2.4. Size-chromatography (SEC) of glycogen

The SEC weight distribution of glycogen, $w(\log R_h)$, which gives the distribution of the weight of particles as a function of $\log R_h$, was analyzed using an Agilent 1260 Infinity SEC system (Agilent, Santa Clara, CA, USA) with a refractive index detector (RID, Optilab UT-rEX, Wyatt, Santa Barbara, CA, USA) following a previously reported method [6]. Water containing 50 mM ammonium nitrate/0.02% sodium azide was used as eluent, and separation of molecules by size used a SUPREMA pre-column, 1000 and 10,000 columns (Polymer Standard Service, Mainz, Germany).

3. Simulations

Two approaches were used to simulate the time evolution of the molecular size distribution under enzymatic degradation. Both were based on analogous methods used in emulsion polymerization. The first approach was through the partial differential equation involving a

single model describing this evolution, analogous to the full description of the particle size distribution in emulsion polymerization during particle formation and growth [13], and is an extension of that used previously for glycogen degradation under acidic conditions [14]. The second approach treated the α- and β-particle populations as separate entitles, analogous to the description used for competitive growth in emulsion polymerization [11].

The partial differential equation describing the time evolution of the molecular size evolution [14] is:

$$\frac{\partial N(R,t)}{\partial t} = \frac{\partial}{\partial V}\left(K\,N\right) \tag{1}$$

where $N(R,t)$ is the number distribution of molecules of radius $R = R_h$, $V = {}^4/_3\ \pi\ R^3$ is the corresponding volume, and K is the rate coefficient for enzymatic degradation. In the extension of the form of this equation implemented here, it is assumed that

$$K = kR^n \tag{2}$$

where $n = 3$ corresponds to uniform degradation throughout the particle (as implemented previously [14]), $n = 2$ to degradation on the surface, and $n = 1$ to diffusion-controlled degradation (e.g. eq 6 in [11]). The number distribution is converted to the SEC weight distribution $w(\log R_h)$, and specific inclusion of the radius dependence of K gives:

$$\frac{\partial w(\log R,t)}{\partial t} = \frac{1}{4\,\pi}\left[k(n-6)\,R^{n-3}w(\log R,t) \;+ kR^{n-2}\,\frac{\partial w(\log R,t)}{\partial R}\right] \tag{3}$$

This equation is solved numerically by finite difference, as set out in the Supporting Information.

This model implicitly assumes that all glycogen particle sizes have the same type of structure: there is only a single population of particles. However, as stated, α particles are agglomerates of β particles, and so one expects qualitative structural differences between isolated β particles and their aggregated form as α particles; this is distinct from *phytoglycogen*, where there is evidence [14] that these particles have a uniform type of structure at all sizes. If one

treats α and β particles as structurally different entities, then the system would be described by two dependent variables, the distributions for α and β particles as separate $w(\log R_h)$. A much simpler approach is to treat the populations as kinetically separate, analogous to what has been done for competitive growth in emulsion polymerization [11], as set out in the Supporting Information.

4. Results and Discussion

The time evolutions of the molecular size distributions of liver glycogen from healthy rats, diabetic and healthy mice are shown in Figure 1; the reproducibility of the data is given in the Supporting Information. Two peaks are observed, at R_h ~ 40 and 20 nm, which are the glycogen α and β particles, respectively. The healthy rat-liver glycogen used in this study comprises predominantly β particles, the healthy mice-liver glycogen involved in this study contains more α than β particles, and the diabetic mouse-liver glycogen has commensurate components of both α and β particles. The relative amounts of α and β particles depends on the time of day in a given animal [12]; the different relative amounts of particles in the different animals arises because the three glycogen samples are from rats sacrificed at 8 am (Figure 1A), and from healthy mice sacrificed at 10 am (Figure 1C) and diabetic mice sacrificed at 4 pm (Figure 1B).

Figure 1 shows that the amount of β particles decreased rapidly over time for healthy rat-liver glycogen (Figure 1A), whereas the decrease in α particles only becomes obvious after 20 min, and a significant amount of α particles remains after 60 min degradation. The same effect was seen for diabetic mice-liver glycogen (Figure 1B) and healthy mice-liver glycogen (Figure 1C). This shows that the enzymatic degradation rate of β particles is much quicker than that of α particles for both healthy and diabetic glycogen derived from rats and mice.

The enzymatic degradation rate of glycogen may be affected by many factors. The nature of the link between β into α particles is not yet known (although it may involve a protein) [15]. The ratio of surface area to volume could play a major role in the enzymatic degradation rate.

β particles have a greater surface/volume ratio (per glucose monomer unit), which would increase the availability of glucose monomer units (per monomer unit) to degradation enzymes, compared to α particles [10]. These data indicate that the size of glycogen molecules has a major influence on their enzymatic degradation rate: it is clear from Fig. 1A and Fig. 1C that β particles degrade much more quickly than α particles.

Rats and mice follow a diurnal eating cycle, with the first meal in a 24-h period taken early in the evening, and the last meal early in the morning. During the fasting stage (i.e. well after the last meal), glycogen phosphorylase is released and/or activated, and this enzyme will quickly degrade β particles. The fragility of α particles in diabetes means that the α particles would be easily degraded to β particles, which then would degrade much more rapidly (per glucose monomer unit) than would be the case for a healthy animal, where the α particles are not fragile.

More information about this was obtained using the simulation of eqs 1 and 2 to see how well the data could be fitted. Some results are shown in Fig. 2, using as the zero-time distribution the observed $w(\log R_h)$ for healthy rats and diabetic mice of Fig. 1.

An apparent inconsistency in the simulation results is that one expects the distribution to move towards smaller R_h with degradation, but this is not apparent from the data in terms of $w(\log R_h)$. In actuality, it is the *number* distribution that should show this behaviour, and on presenting the same simulation data as $\log N(\log R_h)$, as shown in the Supporting Information, this expected behaviour is indeed seen.

The best fit was obtained with $n = 1$, and $k = 0.75$ and 0.45 nm^{-2} min^{-1} for healthy rats and diabetic mice, respectively. Superficially, it appears that the model is able to reproduce the data quite well. However, this is not quite the case, because closer inspection shows some a subtle feature which is not reproduced by the simulation. This is the fact that the data for healthy rats at $t = 20$ min shows two distinct peaks (corresponding to β and α particles) of essentially the same height. The simulations however for $t = 20$ min show that the smaller size (β) peak is significantly higher than that for the larger (α) particles, and at a later time (the

black lines in the simulation) when the two simulated peaks are the same height, they are merging into each other and are not as distinct as in experiment. Bearing in mind that experimental SEC band broadening means that the true experimental peaks are even more distinct, these apparently small differences are in fact significant. There is no such qualitative difference between the simulated and observed distributions for the diabetic livers.

The conclusion to be drawn from this is that the small but qualitatively significant difference suggests that there are significant structural differences between α and β particles. This is indeed consistent with our previous inferences from a different type of study of glycogen degradation [14].

As stated, this model implicitly assumes that all particles have the same type of structure, and an alternative model is to treat α and β particles as separate entities. A simple two-state treatment, as detailed in the Supporting information, and assuming each population obeys first-order kinetics, (concentration) $\propto \exp[-k(R_h)]$ with $t\ k = AR_h^n$, gives $n = 1.4 \pm 0.2$. Although simplistic, this two-population treatment gives the value of the exponent n which is consistent with that of $n = 1$ found to give the best agreement of the full single-population simulation.

5. Conclusions

Our data imply a more rapid release from β particles under enzymatic degradation following the breakup of fragile diabetic α particles, an effect that may well be a significant contribution to the high postprandial blood glucose (uncontrolled glucose release) characteristic of diabetes. Simulation of the data support the inference that there are some structural differences between α and β particles, as expected for liver glycogen where the α particles are composites of many β particles.

The relation of the present results to *in vivo* systems is an important one, and to investigate this requires an examination of the dependence of time after last meal prior to sacrifice of the molecular size distribution of glycogen in healthy and diabetic animals. This work is currently

underway, analogous to our earlier study for healthy animals [12], extended to *db/db* mice and with the use of improved SEC techniques [16].

This new understanding of a probable link between diabetes and glycogen macromolecular structure has the potential to provide guidance to develop new drug targets to prevent, and/or to alleviate the symptoms of, diabetes.

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Figure captions

Figure 1. SEC weight distributions, $w(\log R_h)$, of liver glycogen from healthy rats (A), diabetic mice (B) and healthy mice (C) as a function of hydrodynamic radius, R_h, at different times as indicated. Three repeats were performed (see Supporting Inofrmation) and the data in this figure are representative.

Figure 2. Simulated time evolution of SEC weight distributions for healthy rats (A) and diabetic mice (B), from the model of eqs 1 and 2, at $t = 0$ (blue dots), 20 (red dashes) and 60 (green unbroken line) min. The $t = 0$ values are the experimental initial distributions of Fig. 1, and the best-fit simulation parameter values are given in the text. Black lines are for intermediate and later times.

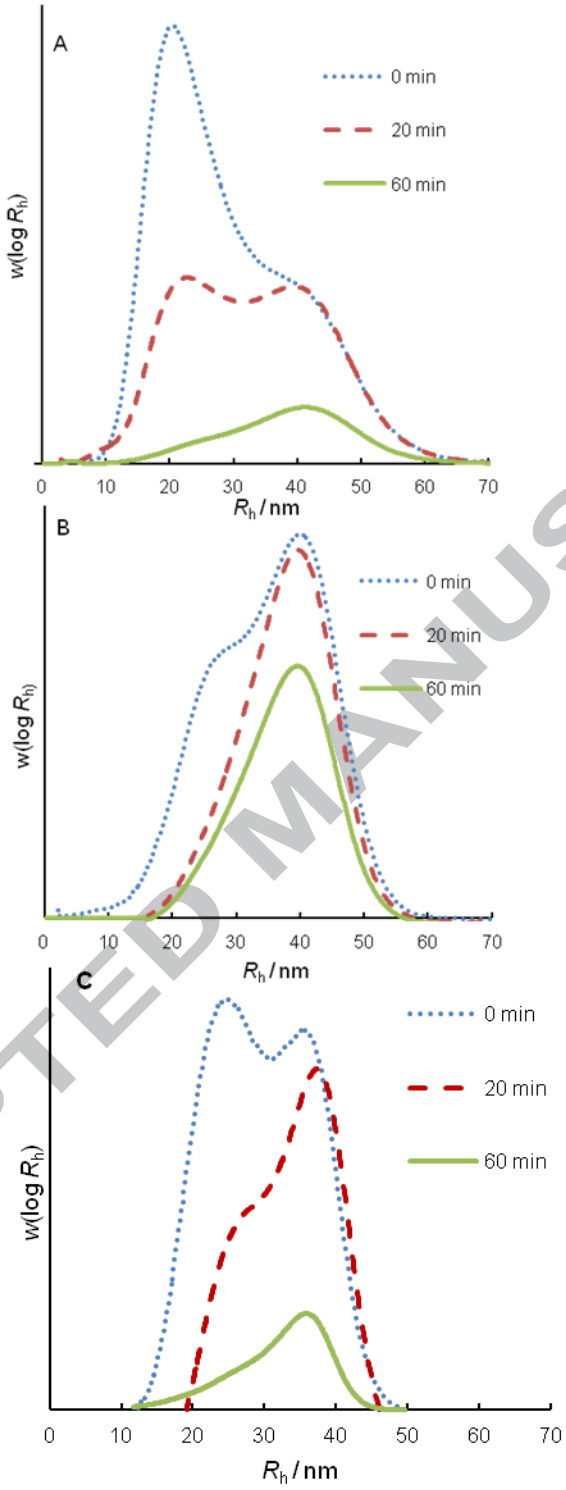


Figure 1

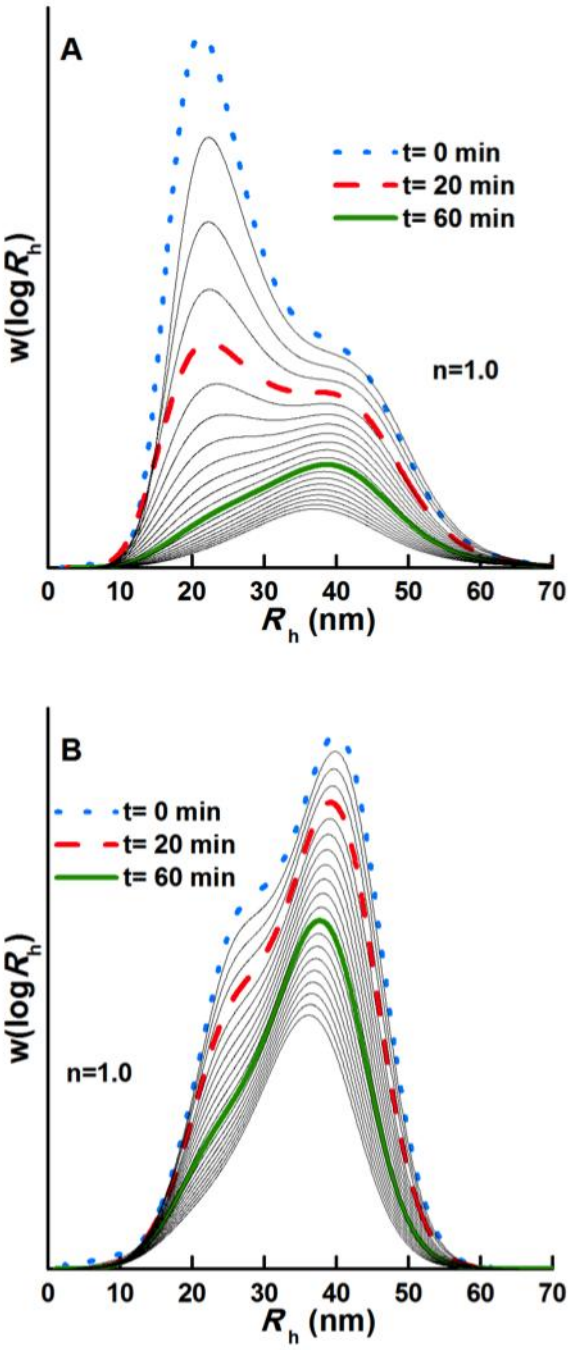
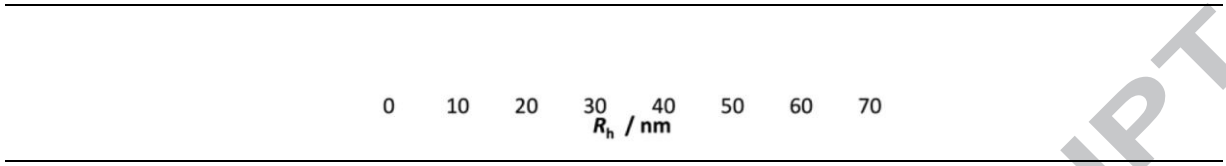


Figure 2

Graphical abstract



Highlights

- Glycogen, a complex branched glucose polymer, helps maintain blood-sugar level
- Small β particles bind into large α particles; the binding is fragile in diabetes
- We study the time evolution of molecular size distribution with degradative enzyme
- This shows small particles degrade more rapidly to glucose than large ones
- This is consistent with uncontrolled blood sugar levels in diabetes