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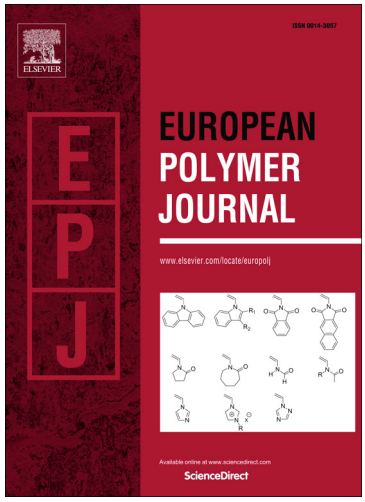

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Implications for biological function of lobe dependence of the molecular structure of liver glycogen

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Abstract

Liver glycogen, a complex branched polymer of glucose, plays a major role in controlling blood-sugar levels. Understanding its molecular structure is important for diabetes, especially since it has been found that this structure is more fragile in diabetic than in healthy mouse liver. However, there are differences in metabolic processes between liver lobes, which would be expected to be reflected in differing glycogen molecular structures. This structure was examined for separated lobe regions in rat livers, using size-exclusion chromatography (SEC) and fluorophore-assisted carbohydrate electrophoresis. The results show that the SEC weight distribution of glycogen, and the molecular weight distribution of individual branches (chains), from different lobes are similar. This shows that (a) molecular structural characterization of glycogen from whole-liver biopsy is representative (which is convenient because the commonest animal model for diabetes is the mouse, whose livers are very small), and (b) the fact that molecular structure is conserved (regulated) in different lobes suggests that this structure plays an important role in blood-sugar regulation.

Key words: glycogen; molecular structure; liver; diabetes; GPC

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1. Introduction

Glycogen is a complex, highly branched (~9% degree of branching) glucose polymer (containing a small, but functionally important, amount of bound protein [1-3]). Glycogen plays an important role in regulating blood sugar in human and other animals [4]. Liver glycogen has three levels of structure: 1) glucosyl monomer units are attached through α-(1→4) linkages to form linear chains; 2) these chains are joined together via α-(1→6)-linked branch points to form highly branched β particles (~20 nm in diameter); and 3) β particles join into α particles (up to 300 nm in diameter) [2, 5], which have a composite raspberry- or cauliflower-like appearance under transmission electron microscopy [6]. It has been found [7] that the binding between β particles to form α particles is fragile in diabetic liver glycogen, using *db/db* mice as a model for type 2 diabetes. Given that liver glycogen has similar molecular structure in both mice and humans [8], a better understanding of this molecular structure is of significance in the study of human type 2 diabetes.

The liver has multiple distinguishable lobes, and it is possible that the molecular structure of glycogen could be different in different lobes, because there are some differences in the metabolic processes between lobes. Matsubara et al. [9] examined enzyme distributions and reported that several enzymes from the cytochrome P-450 monooxygenase system, such as cytochrome P-450, cytochrome b5, cytochrome a(+a3), 7-Methoxycoumarin O-demethylase, were distributed heterogeneously in Wistar rat liver lobes, and that the median lobe showed a slightly higher glycogen content. However, this study only used 3 rats and the differences in glycogen content distributions are not statistically significant, and no enzyme related to glycogen metabolism was studied. Garcia-Moreno et al. [10] found that there are differences in area and number of silver-nucleolar organizer regions (Ag-NORs) in different lobes.

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As the Ag-NORs can indicate hepatocytic protein synthesis, the results show the existence of a lobular functional heterogeneity in liver.

The question investigated here is to see if there is a difference in the molecular structure of glycogen in these different lobes, given that there is some metabolic difference between these lobes; this metabolic difference could affect glycogen synthesis and degradation. If there were to be a significant difference, this would give data which could be used to better understand the lobe dependence of this synthesis and degradation, but would also mean that molecular structural data extracted from whole liver is not truly representative. If there were to be no significant difference, this would suggest that this molecular structure is sufficiently important for the well-being of the animal that it is controlled (regulated) in a way that is robust to some metabolic changes (as is seen, for example, in another complex branched polymer with similar bonding to glycogen, amylopectin in plant starch, for which the theoretical reasons for structural robustness are understood [11]). The structural characteristics examined here are the size distribution of the whole molecule, and the molecular weight distribution of the individual chains (branches), obtained from the whole molecule by enzymatic debranching. Overall glycogen particle morphology is also examined.

Most previous studies on the molecular structure of glycogen have used mice as an animal model. Because mouse liver is small, the glycogen in these studies have perforce been extracted from the whole liver so as to obtain sufficient sample for structural characterization. In order to ascertain the relevance of these mouse studies to humans, it is important to see if there is a difference in glycogen molecular structure in different lobes, which requires having livers which are sufficiently large that enough glycogen for structural characterization can be extracted from different lobes.

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This study uses rat livers. Rats have a similar liver structure to mice but the liver is much larger. Rat liver has four lobes: left, right, middle and caudate [12]. The median lobe can further be grouped into left and right portions; the right lobe can also be grouped into right superior lobe and right inferior lobe; the caudate lobe can be divided into the paracaval portion and the Spiegel lobe, which splits into two sub-lobes.

Size-exclusion chromatography (SEC, a type of gel-permeation chromatography, GPC) and fluorophore-assisted carbohydrate electrophoresis (FACE) are used here to analyze the molecular structure of extracted glycogen. SEC measures the size distributions of molecularly dispersed molecules as a function of their hydrodynamic radius, R_h. With a differential refractive index (DRI) detector, it gives the weight distribution of these molecules as a function of log R_h, $w(\log R_h)$. SEC is used here to measure the size distributions of the whole glycogen molecules. The molecular weight distribution of the individual branches (chains – the chain-length distribution, CLD) is measured here by enzymatically debranching the whole molecule, then using FACE to measure the CLD. Although FACE currently cannot go above a degree of polymerization (DP, symbol X) ~180 [13], this is sufficient for the size range of debranched glycogen. FACE directly gives the number distribution of DP following debranching, denoted $N_{de}(X)$, and gives baseline resolution between DPs in the range of interest. The morphology of the glycogen molecules is examined in this study using transmission electron microscopy (TEM).

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2. Experimental

2.1. Animals

Male Wistar rats (13 weeks) were purchased from the Hubei Provincial Centre for Food and Drug Safety. Rats were housed in a standard specific pathogen-free (SPF) animal room with standard cages. The temperature was controlled at 22 ± 1 ℃ and 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 water and standard chow (6% kcal from fat, 14.3 MJ kg^{-1}, Hubei Provincial Centre for Disease Control and Prevention). All animal experiments were approved by the Huazhong University of Science and Technology Tongji Medical College Animal Care and Ethics Committee.

2.2. Excision of liver

Rats were divided into two groups. Those in Group 1 were sacrificed in the morning, between 9 and 10 am, and those in Group 2 were sacrificed in the afternoon between 2 to 3 pm. These two times are respectively at the beginning and end of the degradation phase in glycogen synthesis [14]. Rats were anaesthetized with sodium pentobarbitone (150 mg/kg intraperitoneal) and their livers were rapidly excised and divided into six parts according to the lobe sections given in Fig. 1. Each lobe was snap-frozen in liquid nitrogen and stored at –80 °C.

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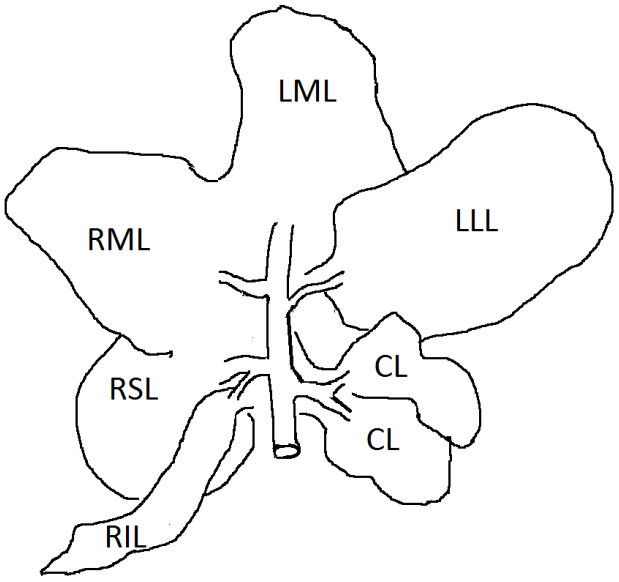

Figure 1. Six lobes of rat liver: left portion of middle lobe (LML), right portion of middle lobe (RML), right superior lobe (RSL), right inferior lobe (RIL), caudate lobe (CL) and left lateral lobe (LLL).

2.3. Purification and extraction of glycogen

Glycogen was extracted following a previous procedure [15]. Approximately 1.5 g of rat 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). 400 µL of this homogenate was reserved for glycogen content assay. The remaining samples were centrifuged at 6000 g for 10 min at 4 °C. The supernatants were further 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 % sucrose 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

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mixed with four times volume of 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).

2.4. Glycogen content

The liver-glycogen content was analyzed using a method reported elsewhere [14, 16]. Liver homogenates from Group 1 (sacrificed in the morning) were used for glycogen content assay. Amyloglucosidase was used to degrade glycogen to glucose units and then glucose oxidase/peroxidase (GOPOD, Megazyme, Ireland) reagent to quantify the amount of glucose. 20 μL of homogenate (from the glycogen extraction) was put in a 1.5 mL tube, 5 μL of amyloglucosidase (Megazyme), 100 μL of sodium acetate buffer (pH 6) added, then made up to 0.5 mL with deionized water and incubated in a thermomixer at 50 °C for 30 min. A control, with everything except amyloglucosidase, was also analyzed. A 300 μL aliquot of each sample was then added to 1 mL of GOPOD and incubated for a further 30 min at 50 °C on a thermomixer. The absorbance (510 nm) of each sample was then analyzed on a UV-61000s UV−vis spectrophotometer (Mapada). The glycogen content was calculated based on a calibration curve (constructed by reacting D-glucose of various concentrations with the same GOPOD reagent). All samples and controls were run in triplicate with the absorbance values averaged.

2.5. Size-Exclusion Chromatography

Glycogen from two groups was analyzed using an aqueous SEC system, following a previous method [17], to obtain the size distribution of the whole (undebranched) molecules. Ammonium nitrate solution (50 mM) containing 0.02 % sodium azide (w/w) was used as the mobile phase. Glycogen was dissolved for 4 h

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at 80 °C in 50 mM ammonium nitrate/0.02 % sodium azide at 1 mg/mL. 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; the flow rate was 0.3 mL/min. A refractive index detector (Optilab UT-rEX, Wyatt, Santa Barbara, CA, USA) was used to determine weight distributions, and multiple-angle laser light scattering detector (DAWN HELEOS-II, Wyatt, Santa Barbara, CA, USA) were used to measure weight-average molecular weight ($\bar{M}_w$). Pullulan standards (Polymer Standard Service, Mainz, Germany), with molar masses ranging 342–2.35×10^6 Da, were used for elution volume calibration.

2.6. Fluorophore-assisted carbohydrate electrophoresis

Glycogen from Group 1 (rats sacrificed in the morning) was analyzed using FACE to give the chain length distribution (CLD) of debranched glycogen from various lobes of livers, following a procedure described elsewhere [13]. Glycogen was firstly debranched and freeze-dried, and then separated with a carbohydrate separation buffer (Beckman-Coulter) in an N-CHO coated capillary at 25 ˚C using a voltage of 30 kV. Each sample was analyzed in duplicate.

2.7. TEM

TEM images of glycogen from different lobes of rat 7 were obtained using a method similar to that reported elsewhere [18]. Glycogen (from Group 1) was dissolved in 50 mM Tris-HCl buffer (pH 7.0) at 1mg/mL. Copper grids (230 mesh) with carbon film were discharged using a Harrick plasma cleaner (PDC-32G-2) before use. The solution was diluted 10 times and applied to the grid for 1 min, and excess sample was drawn off with filter paper. Then the grid was stained with one

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drop of 2% uranyl acetate. The grids were examined with a Hitachi H-7000 TEM operating at 75 kV using AnalySiS image management software.

2.8. Statistical analysis

Analysis of variance (ANOVA) with the general linear model and Tukey's pairwise comparisons were used for statistical analysis in Minitab 16 (Minitab Inc., State College, PA, USA). Significant differences of the mean values were determined at $p < 0.05$.

3. Results and Discussion

3.1. Glycogen content in liver lobes

The glycogen contents of homogenate from different lobes in each rat, shown in Table 1, show some apparent differences. However, comparing this content in the same lobe but from different rats as biological repeats, there is no statistically significant difference ($p < 0.05$, n=24).

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Table 1 The glycogen content of glycogen (% wet liver weight) extracted from various lobes of rat livers (sacrificed in the morning) (means ± SD)[a]

Glycogen content (% wet liver weight)	LML	LLL	RML	CL	RSL	RIL
HR1	1.9±0.2	2.1±0.1	1.8±0.2	1.7±0.1	2.0±0.1	1.8±0.2
HR2	2.0±0.4	2.3±0.2	2.2±0.2	2.2±0.1	2.2±0.1	1.9±0.2
HR3	1.2±0.1	2.1±0.1	1.8±0.2	3.5±0.0	2.2±0.1	1.9±0.1
HR4	2.8±0.3	2.2±0.2	2.6±0.5	3.6±0.2	1.0±0.0	2.3±0.2
HR5	2.6±0.1	1.9±0.3	2.6±0.2	2.3±0.2	2.7±0.3	2.4±0.1
HR6	2.4±0.1	2.2±0.1	2.3±0.2	2.0±0.1	2.5±0.1	2.2±0.1
HR7	2.8±0.1	2.4±0.3	2.5±0.3	1.8±0.1	2.7±0.2	2.3±0.1
HR8	3.0±0.1	2.8±0.1	2.8±0.1	2.7±0.1	2.9±0.1	2.6±0.0
mean	2.3±0.6[a]	2.2±0.3[a]	2.3±0.4[a]	2.5±0.7[a]	2.3±0.6[a]	2.2±0.3[a]

[a] Mean ± standard deviation is calculated from duplicate measurements. Values with different letters in the same row are significantly different at $p < 0.05$.

3.2. Molecular size distributions of glycogen from various lobes

For healthy rats sacrificed in the morning, the SEC weight distributions of liver glycogen from different lobes, $w(\log R_h)$, are shown in Figs. 2 and 3. The data are normalized to the height of the highest peak. A main peak with a distinct shoulder peak was observed for most of the samples. The main peak, with maximum at R_h ~ 40 nm, comprises α particles (Table 2 and Fig 2), and the shoulder at R_h = 20~25 nm comprises β particles. The molecular structure of glycogen from different lobes in each individual rat show apparent differences in the size distribution, especially in the ratio of α and β particles. However, when comparing glycogen from the same lobe but from different rats as biological repeats, the molecular size distributions of α particles from different lobes of the same rat are similar (Table 2). While there are

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differences in the fraction of β particles in different lobes (quantified as ratio of the areas under the $w(\log R_h)$ curves over 1 – 25 nm for β particles and 1 – 100 nm for all particles), the differences are small and not statistically significantly different.

The $\overline{M}_w$ values of glycogen from different lobes are also not statistically significantly different (Table 2). For the samples obtained in the afternoon, the main peak comprises α particles with maxima at R_h ~37 nm (Fig. 3), where the regions separating α and β particles are slightly different from those in the morning. Some of these samples show a less obvious β-particle peak at ~20 nm, corresponding to degradation of β particles during the day. The shift of the peak position of α particles is probably because the samples were analyzed at slightly different times.

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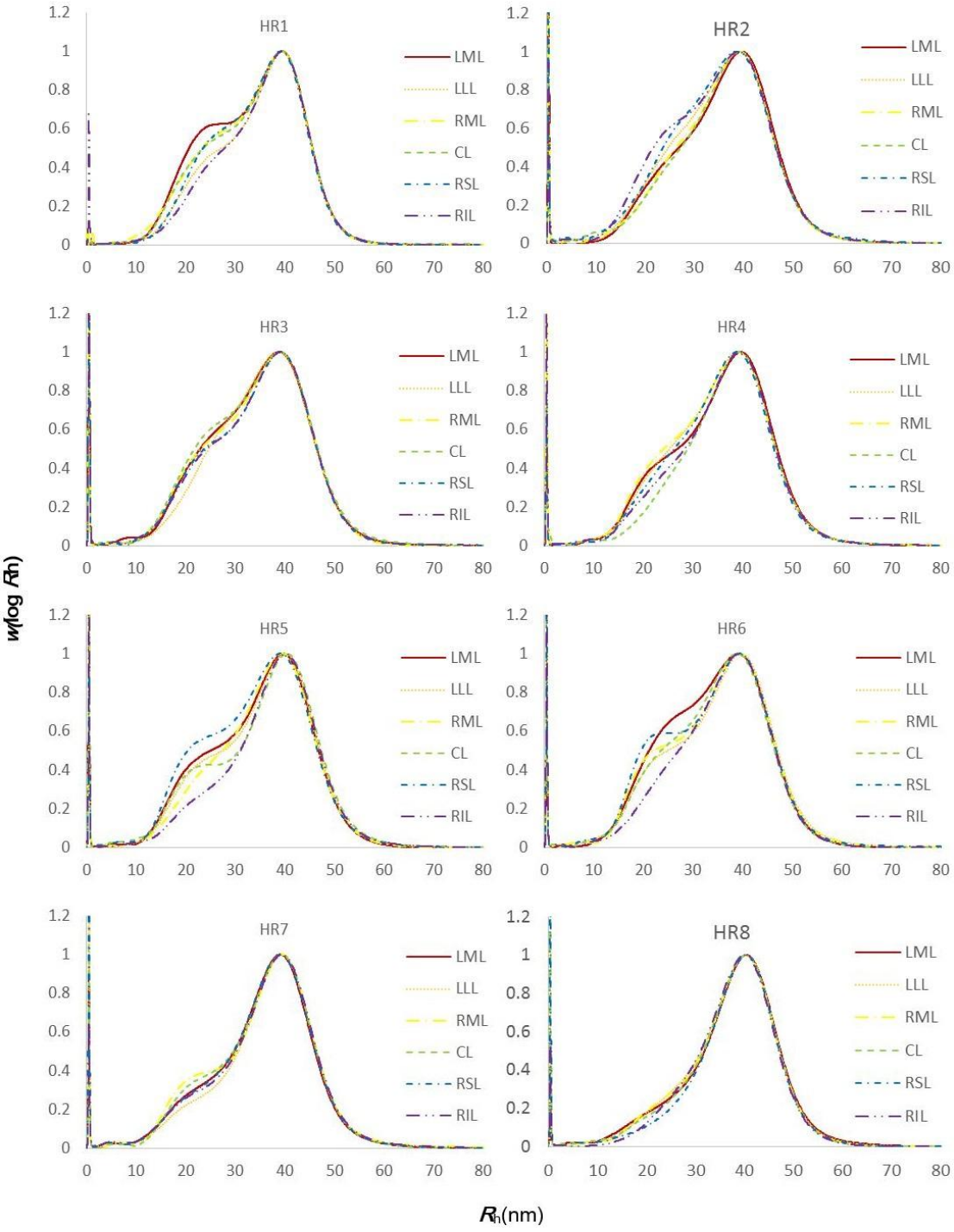

Figure 2. SEC weight distribution, $w(\log R_h)$, of whole (undebranched) glycogen extracted from different lobes of rat livers; these rats were sacrificed in the morning. Most of the glycogen comprised α particles. Normalized to have the same maximum height.

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Table 2 Whole molecules: α-particle peak positions, weight-average molecular weight. Debranched molecules: slopes of approximately linear regions (from the ranges indicated) in log $N_{de}(X)$. For glycogen extracted from different rat-liver lobes (samples from rats sacrificed in the morning) (mean ± SD)[a]

	α-particle peak maximum positions (nm)	proportion of β particles (R_h < 25 nm) (%)	$\bar{M}_w$ /10^8 Da	slope of log $N_{de}(X)$ ($20 \leq X \leq 40$)
LML	39.5±0.5 [a]	17.0±0 [a]	7.5±0.9 [a]	0.2±0 [a]
LLL	39.4±0.5 [a]	15.4±0 [a]	7.6±0.6 [a]	0.2±0 [a]
RML	39.4±0.4 [a]	16.8±0 [a]	7.5±0.8 [a]	0.2±0 [a]
CL	39.5±0.6 [a]	15.9±0 [a]	8.2±3.5 [a]	0.2±0 [a]
RSL	39.2±0.5 [a]	16.8±0 [a]	6.5±2.1 [a]	0.2±0 [a]
RIL	39.5±0.4 [a]	14.3±0 [a]	6.7±2.2 [a]	0.2±0 [a]

[a] Mean ± standard deviation is calculated from duplicate measurements. Values with different letters in the same row are significantly different at $p < 0.05$.

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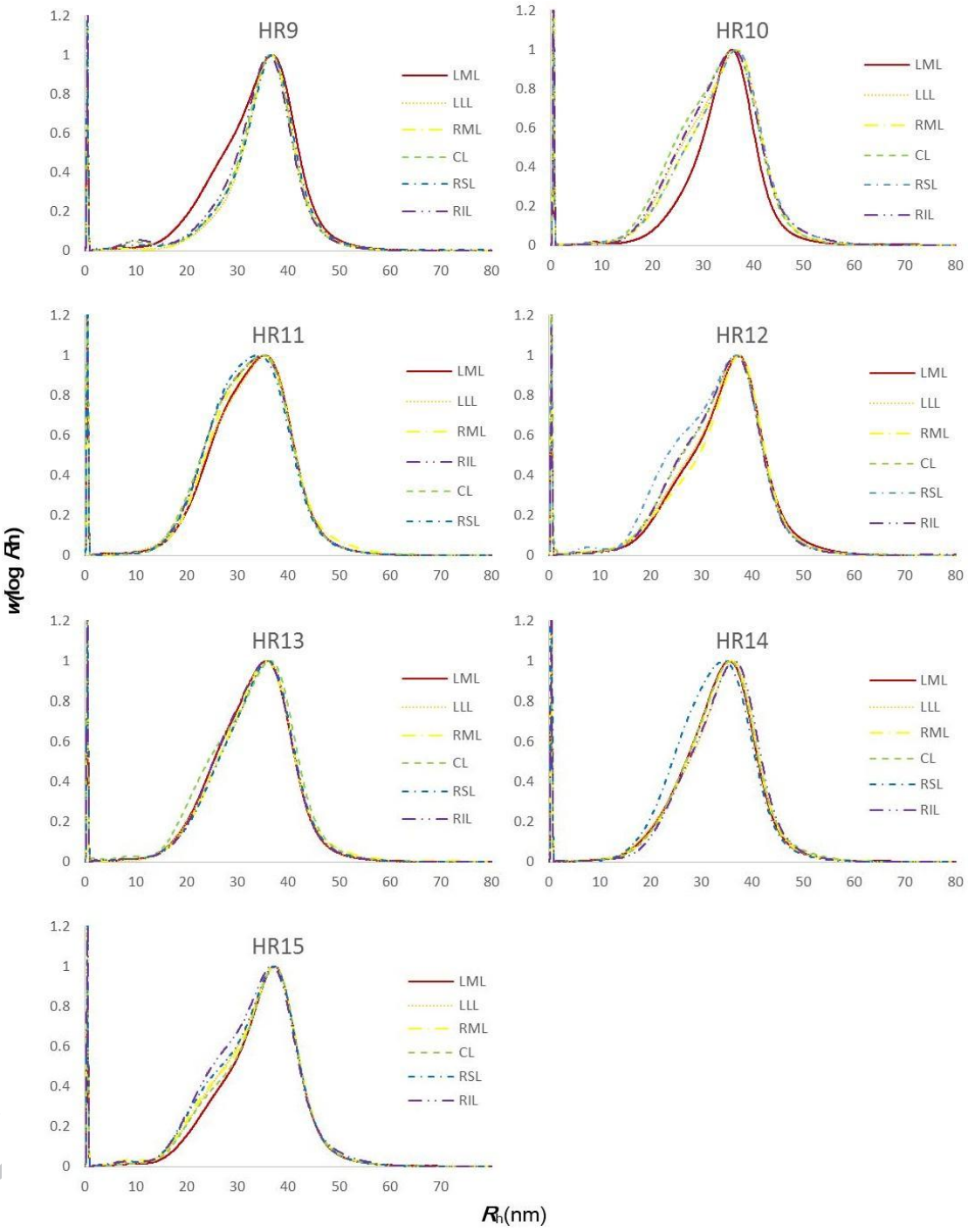

Figure 3. SEC weight distribution, w(logR_h) (normalized to maximum heights), of whole (undebranched) glycogen extracted from different lobes of rat livers; rats sacrificed in the afternoon. Glycogen mainly comprised α particles in these samples.

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3.3. CLDs of glycogen from different lobes

Glycogen from different lobes of liver have measurable CLDs up to ~ DP 50 (Fig. 4); above this DP, there is so little glycogen that the signal:noise ratio is very low. These CLDs were quantified by finding the average slopes from the approximately linear regions in log $N_{de}(X)$ in the range $20 \leq X \leq 40$, using the method of Deng et al. [19]. In some of the rats, the CLDs of glycogen from different lobes show small apparent differences, but when comparing samples from the same lobe but different rats as biological repeats, the differences are not statistically significant (Table 2).

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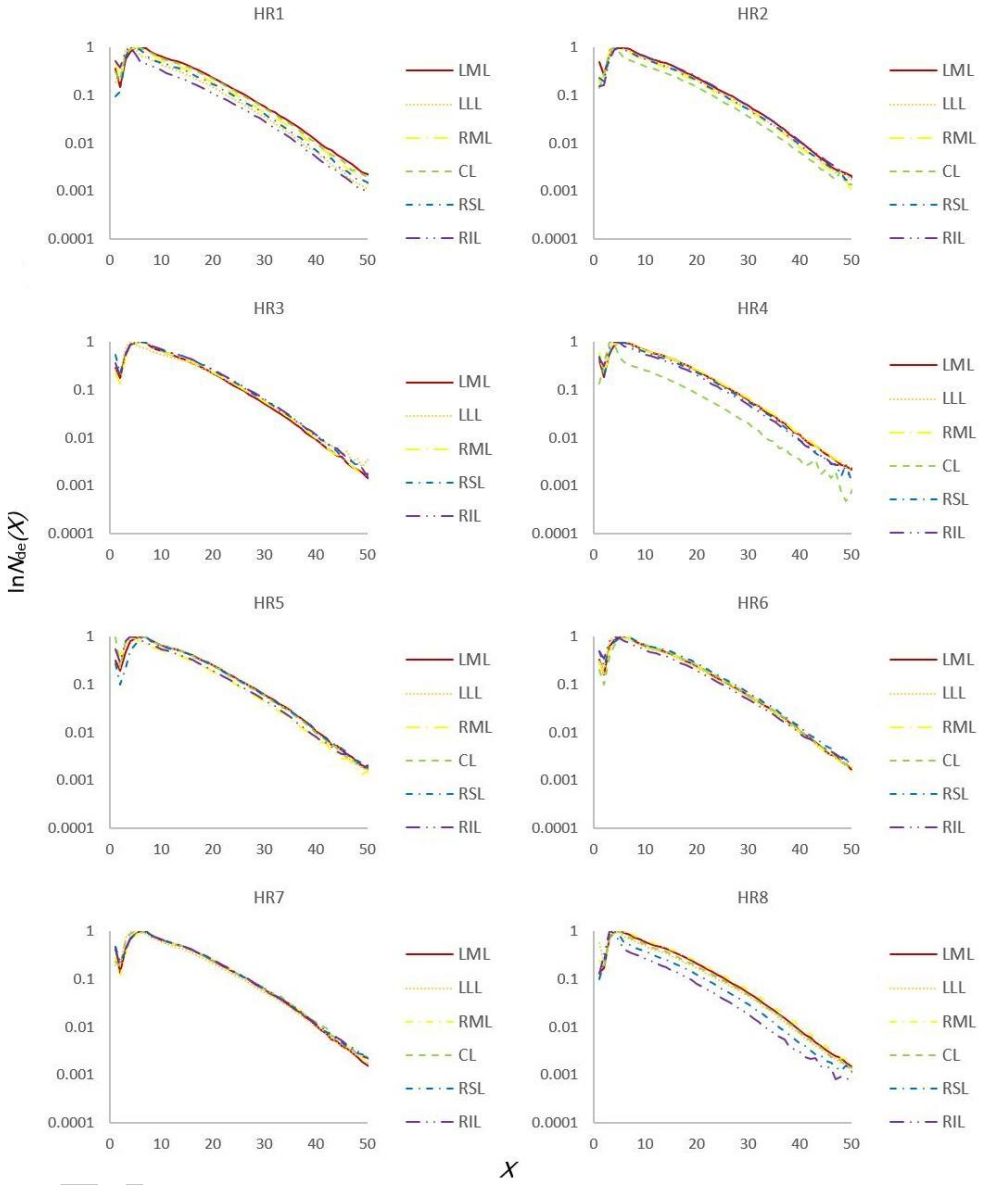

Figure 4. Number CLDs, $N_{de}(X)$, plotted with a logarithmic Y axis, of glycogen from various lobes characterized using FACE. All distributions were normalized to the global maximum. Although the FACE data comprise points for individual DPs, they are presented as continuous lines for visual ease.

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3.4. Morphology of glycogen molecules

TEM images of glycogen from different lobes are similar. Representative TEM micrographs of glycogen show large amounts of α particles, and few β particles (Fig 5). The β particles have diameters around 30 nm. The α particles have a composite, cauliflower-like, appearance with diameters 80 – 250 nm. Particle sizes in TEM are much larger than those inferred using SEC, for which "size" is hydrodynamic radius. Now, R_h is proportional to cube root of the product of the weight-average intrinsic viscosity and the number-average molecular weight; it has the dimensions of length but is not an easily interpreted physical "size" in the way that the radius of gyration is. Moreover, for a nonglobular polymer like glycogen, the sizes from TEM and SEC can only be compared semiquantitatively [20]. In addition, the samples were under different conditions as the solvent was removed in TEM, which would cause some size change.

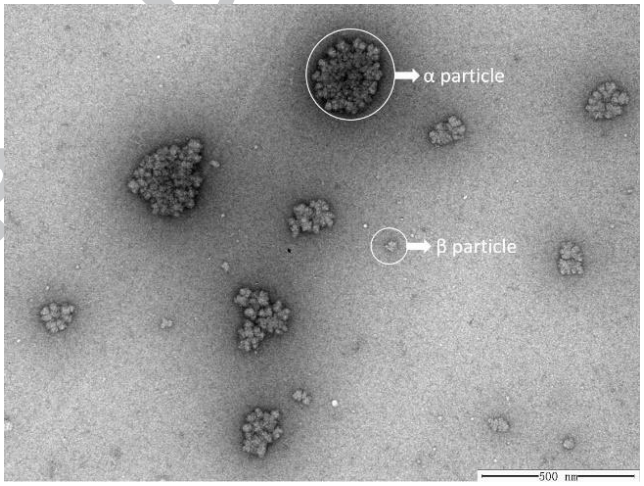

Figure 5. Typical TEM micrograph of glycogen, showing far more α than β particles. The cauliflower-like composite appearance of α particles is very apparent.

SEC and TEM results show that rat glycogen taken at the times used here mainly consisted of many α particles and smaller proportions of β particles, as has

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been seen elsewhere, e.g. [14]. The SEC and FACE results show that glycogen samples from different lobes of liver have similar molecular structures, meaning that glycogen extracted from part of liver is representative of the whole. The SEC results show small (particularly relative amounts of β and α particles) and not statistically significant differences for glycogen from different liver lobes. The same holds for the slopes from the FACE results; for most rats, glycogen from different lobes show very similar CLDs. In some rats, there are a few glycogen samples from different lobes that have small differences. However, the slopes of the linear regions in log $N_{de}(X)$ over the range $20 \leq X \leq 40$ are similar. These slopes yield the ratio of the rates of chain stoppage to growth [19, 21], and thus different lobes have similar glycogen growth kinetics.

4. Conclusions

Glycogen from different liver lobes of rats have very similar molecular structures, both for the molecular weight distribution (CLD) of individual chains (branches) and the $\overline{M}_w$'s and size distributions of the whole molecules. The same similarity is seen in rats sacrificed at different times of the day, when the molecular structures vary with time of day but not with the lobe. Since the variation is small and random within each rat, it means that structural characterization of glycogen from any part of the liver is quantitatively representative of that from the whole liver.

This similarity has biological implications. The different lobes have significant differences in metabolic processes, but the liver glycogen molecular structure, which is controlled by metabolic processes, does not show a lobe dependence. The biological function of liver glycogen is essentially controlling blood-sugar storage

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and release (processes which are compromised in diabetes). This "negative" result of lobe structural similarity implies that this molecular structure is biologically regulated, and thus must have a significant role in this biological function, which is to maintain blood-sugar level. A homogeneous distribution of the enzymes involved in glycogen metabolism could be the mechanism for this regulation; however this hypothesis needs further exploration. The present study is confined to the time evolution of the first two levels of glycogen molecular structure in liver. Some areas meriting future investigation arising from the observations in the present paper are as follows. The degree of glycogen phosphorylation is one of the determinants of glycogen structure and functionality. Also of interest are the time evolution of the expression level of different proteins (e.g. glycogenin, glycogen synthase and glycogen branching enzyme) related to glycogen metabolism and structure. These future studies will be of value to diabetes prevention and management.

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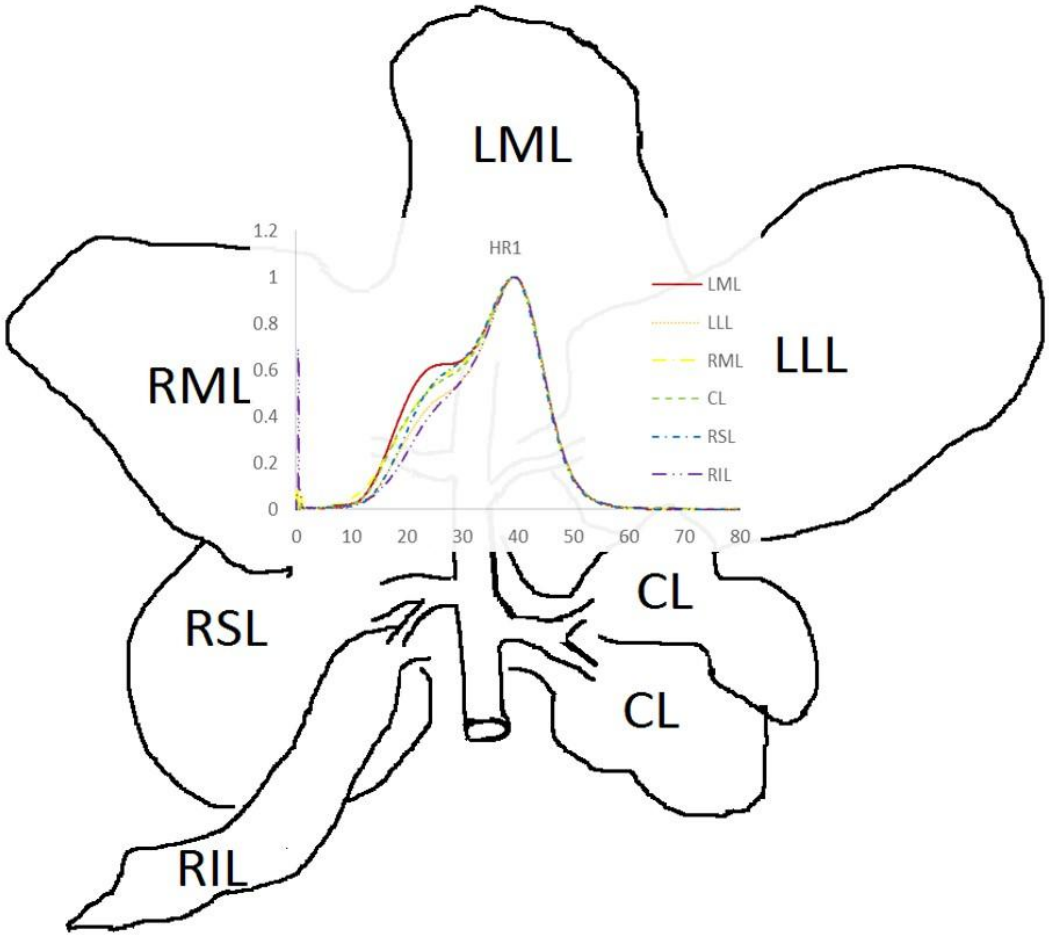
Graphical abstract



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Highlights

- Glycogen, a complex branched glucose polymer, helps maintain blood-sugar level.
- Molecular structure of glycogen from different liver lobes is examined.
- Liver lobes (main glycogen storage organ) have differences in metabolic processes
- However, molecular structure is the same in different lobes: structure is regulated
    - Implies that structural details are important in blood-sugar regulation

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