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A new protocol for separation of acid soluble and insoluble fractions from total glycogen and simultaneous measurements

M. RASOULI, H. SHOKRI-AFRA, A. OSTOVAR-RAVARI

Department of Clinical Biochemistry, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran

Abstract. – OBJECTIVE: The glycogen is extracted routinely from animal tissues with cold perchloric acid (PCA). Acid soluble glycogen (ASG) is extracted, while the insoluble fraction (AIG) is liberated using hot alkaline. The current study was performed to separate and measure ASG, AIG and total glycogen in the same sample simultaneously.

MATERIALS AND METHODS: The protocol has the four phases of tissue digestion, extraction, separation of fractions and measurement. The liver tissue was weighed and digested with four volumes of 30% KOH and heated in boiling water bath for 10 min. Total glycogen was extracted with ethanol at a final concentration of 55%. The suspension of total glycogen was separated into the two fractions of acid soluble and insoluble by adding of 30 µL PCA (70%) followed by a short and mild centrifugation. Total glycogen, ASG and AIG have derived from the same sample and analyzed for glucose.

RESULTS: Analysis of different weights of the liver tissue using the current procedure shows that the fractions of glycogen are measured accurately. The CV% was less than 5% for inter- and intra-assays of total glycogen and ASG. The CV% was more than 5% for inter-assays of AIG, but it lessened in intra-assays. During 24 h starvation, total glycogen depleted completely (71.4 ± 8.3 mg/g wet vs. 4.4 ± 1.2, $p \leq 0.004$) and the change occurred entirely in ASG (66.9 ± 7.8 vs. 1.9 ± 1.1, $p \leq 0.004$), while AIG did not change significantly (4.4 ± 1.3 vs. 2.2 ± 0.9, $p \leq 0.08$).

CONCLUSIONS: The values of ASG, AIG and total glycogen obtained by the current protocol are the same as the classical homogenization method but the procedure is more easy and precise. ASG is the main and metabolically active portion of glycogen in rat liver.

Key Words:
Glycogen, Proglycogen, Macroglycogen, Liver.

Introduction

Several procedures have been described to measure glycogen in animal tissues. The tissue is digested by hot alkaline[1,2], hot acid[3] or cold acid-grinding[4,5]. Then, glycogen is extracted from the (supernatant of) tissue homogenate with ethanol[6]. Glycogen is labile in hot acid and undergoes hydrolysis, so ethanol extraction could not be used in hot acid digestion[3]. Chemical or enzymatic methods are used to hydrolyze the glycogen to glucose and subsequent assay of glucose[7,8]. In the previous study, the method of phenol-sulfuric acid was optimized for microassay of glucose-based glycogen in small tube or microplate[9]. We also re-evaluated and optimized the classical method for assays of glycogen fractions[9].

The tissue is ground by cold perchloric acid in the classical method[9,10]. The extraction must be done several times to recover any acid soluble glycogen (ASG)[11-14]. The last pellet is digested with hot alkaline to release acid insoluble fraction (AIG). The level of AIG is low in the liver tissue and the CV% is high for intra- and inter-assays[10]. Total glycogen could be calculated by summing the values of ASG and AIG or measured directly[1]. Another sample must be weighed, digested by hot alkaline, extracted with ethanol to measure total glycogen. Therefore in the classical method, one sample is used to measure ASG and AIG and another sample for total glycogen with several protracted steps. In the current study, total glycogen was extracted from the liver and separated into ASG and AIG *in vitro* and analyzed simultaneously.

Materials and Methods

Liver Sampling

The liver was isolated from male rats (200-220 g) anesthetized by diethyl ether and washed rapidly three times with ice cold isotonic saline. The lobs incised longitudinally into several parts on a filter paper and preserved at −70°C immediately.

Corresponding Author: Mehdi Rasouli, PhD; e-mail: mehdi.rasouli@yahoo.com

Tissue Digestion and Ethanol Extraction

Fifty mg of liver tissue was weighed at precision of ± 0.0001 g by an analytical balance (Sartorius, Bagno a Ripoli, FI, Italy), and transferred quantitatively to 200 μL 30% KOH and heated in boiling water bath for 10 min with regular mixing. After cooling, ethanol was added at a final concentration of 55%, vortexed and centrifuged 10 min at 1700 ×g. The supernatant was decanted off and the pellet re-suspended in 2 mL of distilled water and 10 μL was analyzed for total glycogen in triplicate (Figure 1).

Fractionation of Total Glycogen to ASG and AIG

30 μL PCA (70%) was added to the suspension of total glycogen and mixed, the final pH was about 3. ASG was remained in the suspension while AIG was precipitated. The sample was centrifuged 5 min at 280×g. The short and low extent centrifugation is critical to prevent co-precipitation of some ASG with AIG. The supernatant contains ASG in suspension was decanted into another tube. The pellet was resolved in 2 mL of distilled water with help of 10 μL 30% of KOH, the final pH was about 9.5. Any increase in the amount of PCA and KOH causes $KClO_4$ to precipitate.

Evaluation of Contamination of AIG with ASG

To assess the extent of ASG co-precipitates with AIG during fractionation, 2 mL of the suspension of AIG was acidified with PCA, centrifuged 5 min at 280×g and glycogen was measured in the supernatant and precipitant. No any acid soluble glycogen was found in the supernatant.

Assay of Glycogen

The suspension of glycogen was mixed by vortex 1 sec just before the sampling. A short

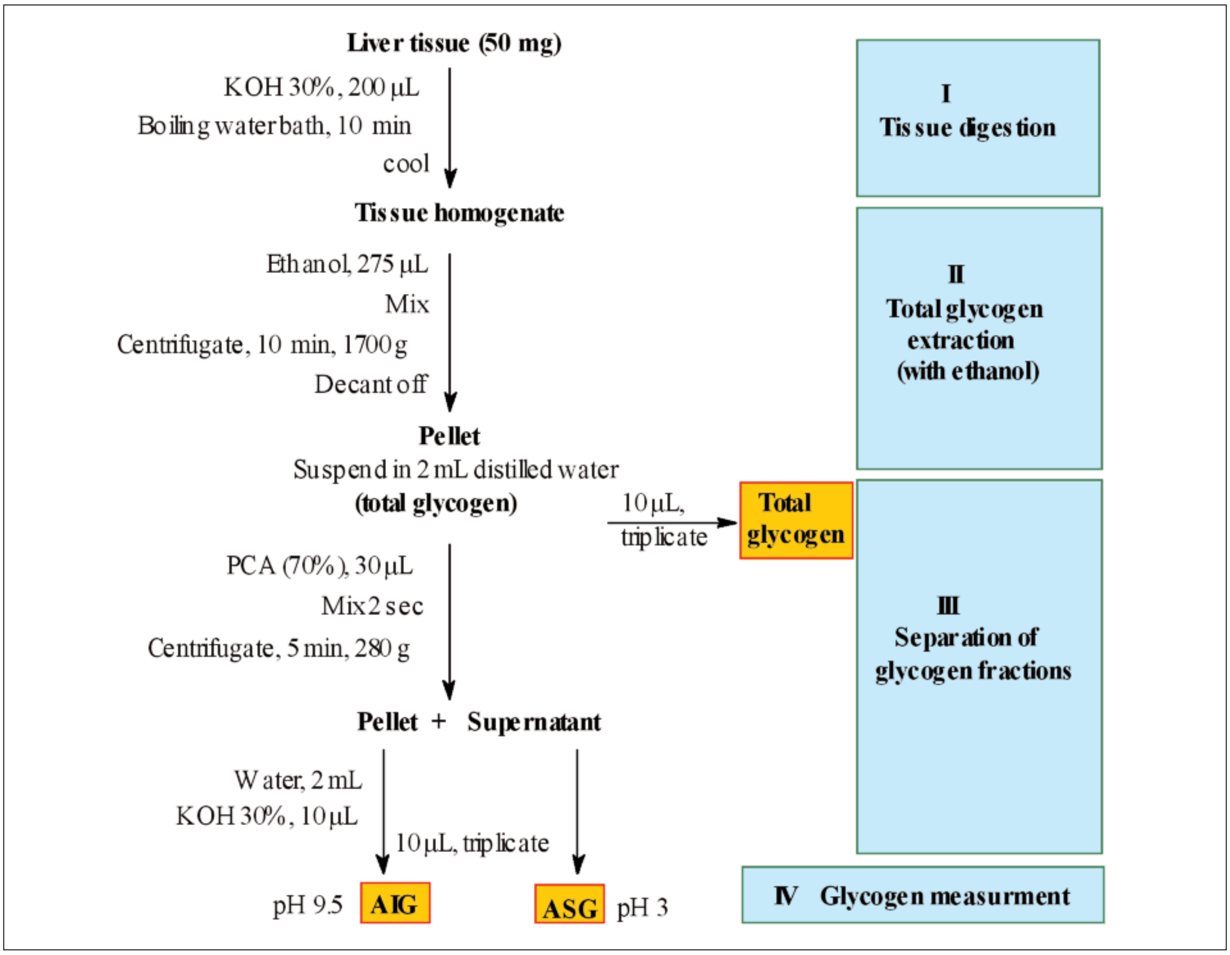

Figure 1. Procedural flow chart for tissue digestion, extraction and separation of glycogen fractions. The protocol has described in details in the method section.

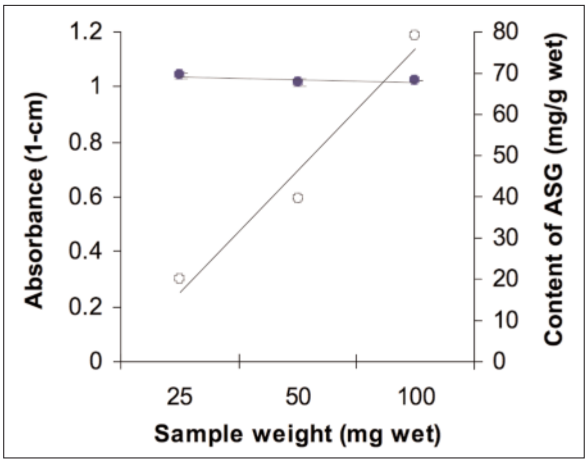

Figure 2. The effect of sample weight on the accuracy of measurement. Different weights of the liver of fed rat were analyzed for ASG, AIG and total glycogen. The opened circles (°) indicate the absorbance of 10 µL of the final suspension of ASG with the phenol-sulfuric reagent. The filled circles (•) show the glycogen content of the samples calculated as mg/g wet weights of liver. All measurements were done on three samples in triplicate.

vortex step is essential to have uniform suspension of glycogen and reproducible results. 10 µL of sample was used for the measurement of glycogen fractions by chemical method of phenol-sulfuric acid optimized previously[9]. The curve of glucose standard was used to calculate glycogen concentration[9]. The factor is multiplied by 0.927 to convert the results to glycogen that corresponds to 97% hydrolysis of glycogen[6].

Statistical Analysis

The results are presented as the means ± SD of three inter-assays performed at least in three samples. The significant differences between samples and corresponding control were accessed by student's *t*-test. All *p*-values are two-tailed and differences were considered significant if *p*-values were ≤ 0.05.

Results

The effect of Sample Weight on Assay Accuracy

Different weights of the liver tissue were used to address the effect of sample size on the accuracy of the method. The weights of 25, 50 and 100 mg of fed rat liver were weighed and analyzed for ASG, AIG and total glycogen. Figure 2 shows that, the absorbance of equal volumes (10 µL) of the final ASG suspension with phenol-sulfuric acid reagent increases with the sample size linearly. In addition, the glycogen content of the samples calculated as mg/g wet of liver was the same for all sample preparations. A similar pattern was seen for total glycogen (results not shown). The weight of 50 mg of liver was adopted for analysis in subsequent experiments.

The inter- and Intra-Assay Precision of Glycogen Assay

Three 50 mg pieces of the same lob of liver of a single rat were weighed separately and analyzed for ASG, AIG and total glycogen. The final assay for glycogen on any sample was also done in triplicate. Table I shows the mean, SD and CV% for the assays of ASG, AIG and total glycogen. The results indicate that CV% was less than 5% for the inter- and intra-assays of total glycogen and ASG. The CV% was more than 5% for inter-assays of AIG, but it lessened in intra-assays.

The Changes of Glycogen Fractions During 24 h Starvation

To test and compare the classical and new procedures, the levels of ASG, AIG and total glycogen were measured by both methods in fed and 24 h starved rat liver (Table II). The data shows that during 24 h fasting, total glycogen depleted completely (71.4 ± 8.3 vs. 4.4 ± 1.2, $p \leq 0.004$)

Table I. The inter- and intra-assay precision of glycogen measurement.

(Mean ± SD, % CV) mg/g wet	N	ASG	AIG	Total glycogen
Inter-assay	3	67.7 ± 1.3, 1.9%	4.8 ± 1.8, 37.5%	71.7 ± 4.1, 5.7%
	3	68.6 ± 1.8, 2.7%	4.2 ± 1.2, 27.2%	72.1 ± 0.8, 1.1%
	3	69.2 ± 3.8, 5.4%	4.4 ± 0.4, 9.8%	74.1 ± 1.6, 2.2%
Intra-assay	3	68.5 ± 0.8, 1.2%	4.5 ± 0.3, 6.0%	72.6 ± 1.3, 1.8%

Three 50 mg pieces of liver were digested with hot alkali, extracted with ethanol, separated to ASG and AIG and analyzed for glucose. The final assay for any fraction of glycogen has also done in triplicate.

Table II. The effect of 24 h starvation on the glycogen fractions.

	Fed		24 h starved rat	
Mean ± SD, mg/g wet	**Classical**	**New procedure**	**Classical**	**New procedure**
ASG	[≠]64.5 ± 6.4	66.9 ± 7.8	2.5 ± 1.6*	1.9 ± 1.1*
AIG	3.6 ± 1.0	4.4 ± 1.3	3.7 ± 0.3	2.2 ± 0.9
Measured total glycogen	70.5 ± 7.7	71.4 ± 8.3	5.9 ± 3.3*	4.4 ± 1.2*

The 50 mg portions of liver tissue of fed and 24 h starved rats (n=3) was analyzed for ASG, AIG and total glycogen by the classical (#) and new procedures. In the classical method, 50 mg of liver tissue was ground with 2 mL cold 10% PCA, centrifuged 10 min at 280 × g. The pellet was re-extracted for further two times with 1 mL fresh PCA. The supernatants were collected and extracted with ethanol at a final concentration of 55% and centrifuged 10 min at 1500 × g. The pellet was dissolved in 2 mL distilled water and 10 µL was analyzed for ASG. The last pellet was digested with 200 µL of 30% KOH in boiling water bath for 10 min, extracted with ethanol and analyzed for AIG. All measurements were done on three samples in triplicate. *Indicates significance at a confidence levels of $p \leq 0.004$. [≠]Sum of three extractions.

and the change occurred entirely in ASG (66.9 ± 7.8 vs. 1.9 ± 1.1, $p \leq 0.004$), while AIG did not change significantly (4.4 ± 1.3 vs. 2.2 ± 0.9, $p \leq 0.08$). The results obtained by the new procedure were also as the same as the classical method. The sum of the ASG and AIG was equal to total measured glycogen in the new procedure but was lower about 4% in classical method. This means that some of the glycogen (ASG) has lost via several extraction steps in the classical method[9].

Discussion

The early studies showed that extraction of animal tissues with cold water or tri-chloro acetic acid yielded less glycogen that was obtained with hot-alkaline[1,2]. The names lyo- and desmo-glycogens are designations that have been used for acid and alkaline extractable fractions respectively[4]. Whelan et al[14] indicated that AIG is composed mainly of low MW particles. The high protein-to-carbohydrate ratio of AIG is responsible to its poor solubility in acid. ASG is composed of large particles with low protein content. Now, lyo- and desmo-fractions are named as pro- and macro- glycogens respectively[13]. Two forms of glycogen could be separated, but the existence of two fractions with the same ratio in intact cell and the physiological importance of the fractions are questionable[2].

The accurate analysis of glycogen fractions is required to study their physiological roles. In the present study, total glycogen was extracted from the liver and fractioned to ASG and AIG *in vitro*. By this means total glycogen, ASG and AIG are separated and measured simultaneously in the same sample more easily and accurately. The results of the measurements of glycogen fractions using the new protocol were the same as the classical method (Table II). But, no any glycogen has lost via extraction step and the CV% was improved for inter- and intra-assays and the procedure became more concise[9]. Extraction of total glycogen from the tissue followed by fractionation to ASG and AIG is more logical, straight and precise. The procedure avoided several extraction-centrifugation steps, hence no any ASG is lost through successive extractions and less AIG is lost via autolysis. The time and extent of centrifugation has been chosen to be low in the fractionation step, so that no any ASG is co-precipitated with AIG[9].

The findings of the current study show that total glycogen depleted during 24 h starvation and the decrease occurred wholly in ASG, while AIG changed insignificantly. The finding is clearly in accordance with the early experiments used the classical homogenization procedure[1-3], but is in contrast to the recent homogenization free protocol of Adamo and Graham[14,15]. The method of Adamo and Graham is encountered with three main problems; high relative error in weighting, incomplete homogenization and overestimation of AIG. The high relative error could be seen as high CV% of their results[14,15] and is attributed to very small sample size taken by biopsy. In homogenization free protocol, the extraction has been done only once by a glass rod followed by unnecessarily high speed centrifugation. Therefore, ASG is not extracted completely and some extracted ASG precipitates again causing a marked overestimation of AIG. As Barnes et al mentioned[11,12], earlier studies that used a homogenization procedure have consistently reported more ASG than the recent studies without homogenization[1,2,15-19].

Conclusions

The values of ASG, AIG and total glycogen obtained by the current protocol are the same as the classical method (Table II), but the procedure is more easy and precise.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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