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Short communication

A gel filtration assay to determine glycogen synthase activity

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Abstract

We developed a gel filtration assay for the determination of glycogen synthase activity in cultured cells or tissue homogenates. Compared to the commonly used filter paper assay, the gel filtration assay resulted in a more than 5-fold reduction of background levels leading to an – at least – twofold increase in precision. These benefits allow the gel filtration method to detect differences of ±5% in enzyme activity out of 300 μg total cell protein. In addition to high precision and sensitivity, the method's additional salient advantages include lesser expenditure of time and labour and reduced exposure time of the personnel to radioactivity.
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1. Introduction

Glycogen synthase (EC 2.4.1.11) is a key enzyme in the regulation of glycogen synthesis. Its activity is regulated by phosphorylation and dephosphorylation at least at nine different sites [1,2]. Enzyme activity is also regulated by the allosteric activator glucose-6-phosphate and other phosphorylated monosaccharides [3]. Insulin stimulates glycogen synthase activity by increasing intracellular glucose-6-phosphate concentrations and, moreover, by activating a signalling cascade resulting in dephosphorylation of glycogen synthase. Glycogen synthase activity is most commonly determined to study the effects of changes in the phosphorylation pattern of glycogen synthase and of mutations in the glycogen synthase gene on enzyme activity [4,5]. Reduced glycogen synthase activity has been observed in a number of different metabolic diseases including type-2 diabetes [6–8].

To our knowledge, two different approaches have been published to determine glycogen synthase activity. The first method is based on UDP release from UDP-glucose upon incorporation into glycogen [3]. Free UDP is subsequently measured photometrically by an indicator reaction. The large amount of required sample material limits the application of this method.

The second method, originally published by Thomas et al., exhibits an increased sensitivity allowing its use with smaller samples [9]. For this assay, cell- or tissue homogenates are mixed with glycogen and radiolabeled UDP-glucose in the presence or absence of glucose-6-phosphate. The separation of incorporated from free UDP-glucose is achieved by spotting the reaction mix onto a filter paper followed by extensive washing with ethanol. Several modifications of the original protocol based on filter paper precipitation for bound/free separation have been published resulting, however, in no significant improvement of the assay's precision [10–12].

Here we report a glycogen synthase activity assay which affords a higher precision and accuracy by using gel filtration instead of filter paper precipitation to separate incorporated from free UDP-glucose.

2. Experimental

2.1. Chemicals and reagents

L6 Skeletal muscle cells were purchased from ATCC (Manassas, Virginia, USA), foetal calf serum (FCS, Cat. No.

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C-3736) was from PromoCell (Heidelberg, Germany), UDP-[U-^{14}C]-glucose (25 μCi/ml, Cat. No. CFB102) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). PMSF and the Quick Spin Columns Sephadex G50 were supplied by Roche (Hertfordshire, England). Sephacryl S100 HR was from Amersham Biosciences (Uppsala, Sweden) and insulin-free bovine serum albumin (BSA, Clinical Reagent Grade, RIA Grade, Cat. No 105033) from Valeant Pharmaceuticals (Bryan, Ohio, USA). Insulin (human recombinant, cell culture tested), α-MEM, glycogen, glucose-6-phosphate, UDP-glucose and all others reagents were from Sigma (St. Louis, Missouri, USA). Whatman filter paper No. 3 (Madiston, England) was used for the filter paper assay.

2.2. Cell culture

The L6 skeletal muscle cells were seeded at 5–6 × 10^3 cells/cm^2. Cells were cultured in α-MEM containing 10% FCS to confluency and then switched to the same media containing 2% FCS. Experiments were performed with fully differentiated L6 skeletal muscle cells grown in 60 mm cell culture dishes. Each plate contained 300 μg total protein estimated by the method of Bradford using a BSA standard. Following overnight starvation, cells were incubated without or with 100 nM insulin for 30 min at 37 °C.

2.3. Sample preparation

Cells were washed twice with ice-cold washing buffer (50 mM Tris–HCl, pH 7.6, 100 mM potassium fluoride) and subsequently scraped in 100 μl collecting buffer (50 mM Tris–HCl, pH 7.6, 30% glycerol, 1 mM EDTA, 100 mM potassium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Cells were lysed by sonification and after centrifugation at 1000 × *g* for 10 min at 4 °C, the supernatant of each sample was split in two fractions of 30 μl each. To one of these fractions, 60 μl of reaction mix (50 mM Tris–HCl, pH 7.6, 20 mM EDTA, 25 mM potassium fluoride, 10 mg/ml glycogen, 7.2 mM UDP-glucose, 25 μCi/ml UDP-[U-^{14}C]-glucose) including 0.3 mM glucose-6-phosphate concentration (low G6P) and to the other fraction, 60 μl of the above reaction mix including 6.7 mM glucose-6-phosphate concentration (high G6P) were added [13]. These concentrations of glucose-6-phosphate were chosen since after dilution of the reaction mix with 30 μl of collection buffer the resulting glucose-6-phosphate concentrations of 0.2 and 4.5 mM, respectively, correspond to physiological intracellular glucose-6-phosphate concentrations in the unstimulated (0.2 mM) [14] and in the maximally stimulated state (4.5 mM)[15]. Samples were incubated for 120 min (unless otherwise stated) in a water bath at 30 °C under constant agitation.

2.3.1. Filter paper method

For the filter paper method [9], the reaction was terminated by spotting 85 μl of the sample onto 2 cm × 2 cm squares of Whatman filter paper that were subsequently washed extensively with ice-cold 70% ethanol. Radioactivity was then counted.

2.3.2. Gel filtration method

The reaction was terminated by adding 50 μl of 0.6N perchloric acid to the sample. Then 50 μl of the sample were loaded onto Quick Spin Columns Sephadex G50 (Roche). The columns were subsequently centrifuged at 1000 × g for 4 min and eluted radioactivity was counted. In a pilot experiment we first confirmed that the addition of perchloric acid to our samples did not affect the retention and filtration properties of the columns.

2.4. Calculations

To determine total and basal activity, 30 μl collecting buffer, 60 μl reaction mix and 50 μl of 0.6N perchloric acid were mixed. For total activity (DPM_{total}), 50 μl of this mix was counted. For the basal activity (DPM_{basal}), 50 μl of the above mix was loaded onto a column, centrifuged and then radioactivity was counted in the eluate.

Glucose integration (GI) into glycogen was calculated as $GI = (DPM_{sample} - DPM_{basal})/DPM_{total}$.

Glycogen synthase activity (GSA) was then calculated as $GSA = GI_{low\ G6P}/GI_{high\ G6P}$.

3. Results and discussion

In a series of pilot experiments we analysed the filtration- and retention properties of the Quick Spin Columns using pure glucose- and glycogen solutions. We were able to demonstrate that 0.2–0.5% of glucose and over 95% of glycogen were found in the eluate indicating a strong separation power of the gel filtration method (Table 1). Comparable results were achieved using Sephacryl S100 HR instead of Quick Spin Columns (data not shown).

To determine the linear range of glycogen synthase activity as a function of time in our cell culture system, the glycogen synthase reaction was performed for various lengths of time. Cells were incubated without or with 100 nM insulin, lysed and subsequently analysed for glycogen synthase activity using gel filtration as the separation technique. As shown in Fig. 1, glucose incorporation into glycogen was found to be linear for up to 240 min of incubation time. Linearity is also

Table 1
Filtration and retention properties of Quick Spin Columns Sephadex G50

	Loaded (μg)	Eluted (μg)	% eluted	*N*
Glucose	529 ± 7	1.55 ± 0.86	0.3 ± 0.16	8
Glycogen	430 ± 12.5	412.5 ± 3	96 ± 0.71	8

Fifty microlitres of pure glucose or glycogen solutions were loaded onto columns, and filtration was performed as described. Glucose and glycogen concentrations were measured in the sample prior to loading and in the eluate. Experiments were performed twice in quadruplicates. Numbers are expressed as mean ± standard deviation.

Table 2
Comparison of gel filtration and filter paper method

	Filter paper	Gel filtration	*p*-value *t*-test	*p*-value Levene test
Background levels (DPM)	245.2 ± 60.5	52.6 ± 22.5	<0.001	0.04
Glycogen synthase activity without insulin in % of the glucose-6-phosphate stimulated condition	52.0 ± 15.1%	45.7 ± 6.0%	NS	0.003
Glycogen synthase activity with 100 nM insulin in % of the glucose-6-phosphate stimulated condition	68.2 ± 16.2%	70.5 ± 5.4%	NS	0.01

Cells were incubated without or with 100 nM insulin for 30 min, lysed and incubated with low and high glucose-6-phosphate as described in Section 2. Glycogen synthase reaction was performed for 2 h. Experiments were performed seven times in duplicates ($N = 14$). *p*-values for assessing differences of the means were calculated using the unpaired *t*-test; *p*-values for assessing differences of the standard deviation were calculated using the Levene test. Numbers are expressed as mean ± standard deviation. NS, not significant.

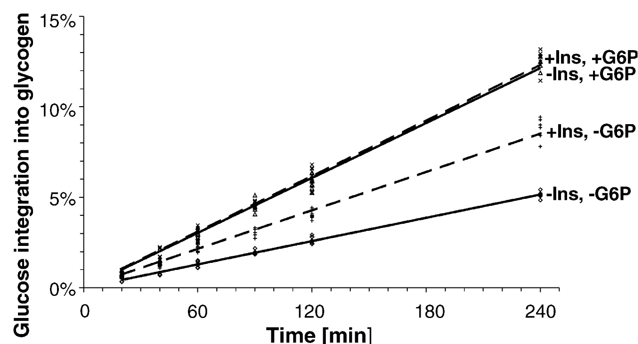

Fig. 1. Glucose integration into glycogen as a function of glycogen synthase reaction time. Cells were incubated without or with 100 nM insulin for 30 min, lysed and incubated with low and high glucose-6-phosphate concentrations as described in Section 2. The glycogen synthase reaction was terminated after 0, 20, 40, 60, 90 120 and 240 min. Glycogen was separated from unincorporated UDP-glucose by gel filtration. Glucose integration into glycogen was expressed in percent of total labelled glucose. Experiments were performed twice in triplicates for each time point. G6P, glucose-6-phosphate; Ins, insulin.

evidenced by high correlation coefficients ($r^2 > 0.97$, $N = 6$ for each of the 6 time points). For all following experiments, we performed a 120 min incubation for the glycogen synthase reaction.

Next, we compared the gel filtration method with the filter paper assay which, in our hands, showed similar degrees of variance compared to a number of published reports [5,10,11,16].

As shown in Table 2, no significant differences in glycogen synthase activity were found between both separation techniques. However, using the gel filtration method the background could be reduced 5-fold ($N = 14$) and standard deviation of glycogen synthase activity was reduced significantly by about 50% ($N = 14$). The reduced background levels of the assay allow the detection of even ±5% differences in enzyme activity among various conditions. In addition to the improved precision and sensitivity, the gel filtration's salient advantages compared to the filter paper assay include lesser expenditure of time and labour, avoidance of contaminated washing solutions and, thus, reduced exposure time of the personnel to radioactivity.

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