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Research Article

Measurement of glycogen synthase activity in crude extracts by CE

Glycogen synthase catalyzes the incorporation of UDP-glucose into glycogen. The activity of the enzyme is usually measured either by a spectrophotometric method or by a radioassay. The first one is not suitable because of the difficulties regarding the use of coupled enzymes in crude extracts, while the second is a time-consuming method involving glycogen isolation and manipulation of radioactivity. We have used a CZE technique as a novel approach to measure glycogen synthase activity. The separations were performed at 22 kV (36 μA) in uncoated capillaries (53 cm × 50 μm). Sample injection time was 30 s and nucleotides were monitored at 254 nm. Best resolution was achieved in 20 mM tetraborate buffer, pH 9.2. Curves of absorbance as a function of UDP and UDP-glucose concentration were linear. Enzyme activity in oocyte extracts was linear with respect to time (up to15 min) and enzyme concentration. The $K_{m\ app.}$ for UDP-glucose was 0.87 mM, a value identical to the one reported using the radioassay. CZE enables easy quantitation of compounds, high sensitivity, and automation of the process. Small sample sizes are required, interferences by auxiliary enzymes and manipulation of radioactivity are avoided, and analysis time is significantly diminished.

Keywords:
CZE / Frog oocytes / Glycogen synthase

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1 Introduction

Glycogen synthase (EC 2.4.1.11) catalyzes the incorporation of activated glucose from uridine 5′-diphosphate (UDP)-glucose into glycogen. The enzyme is regulated both by covalent phosphorylation–dephosphorylation and by allosteric activation by glucose-6-P. The properties of the enzyme may significantly differ under *in vitro* or *in vivo* conditions [1]. Our interest in this enzyme stems from the widely accepted notion that glycogen synthase controls the rate of glycogen formation. However, this role has been challenged by the application of metabolic control analysis. Shulman and co-workers [2] have reported that, in skeletal muscle, the rate-limiting step for glycogen synthesis is at the glucose transport/hexokinase step. Also, and in agreement with these results, it has been shown that hexokinase controls the flux through the glycogen synthesis pathway in *Xenopus laevis* oocytes [3].

Two methods, based on photometric and radioactive assays were early described to measure the activity of glycogen synthase [4, 5]. Although several modifications of these techniques have been proposed later [6–10], many of the initial disadvantages still remain. During our ongoing investigation into glycogen synthesis and its regulation *in vivo*, the need for a more rapid and convenient method than the ones available at present for the determination of glycogen synthase activity became imperative. Since the enzymatic reaction uses UDP-glucose as subtrate rendering UDP as one of the products, we have taken advantage of the CE technique which has been successfully applied to the separation of nucleotides and UDP-sugars [11, 12]. This methodology enables easy quantitation of separated compounds, improves sensitivity, and automation of the process. In addition, small sample sizes are required, interferences developed by coupling several auxiliary enzymes to the spectrophotometric assay in crude extracts and manipulation of radioactivity are avoided, and analysis time is significantly diminished.

2 Materials and methods

2.1 Materials

Nucleotides, glucose-6-P, and UDP-glucose were obtained from Sigma–Aldrich. All other reagents were purchased from Merck.

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Abbreviation: UDP, uridine 5′-diphosphate

2.2 Animals and cells

Chilean female frogs *Caudiverbera caudiverbera* were obtained from a local dealer and maintained in the laboratory until used. The animals were kept in tap water at 20° and fed once a week with chicken or bovine liver. On the day of the experiments an ovary fragment was obtained from the frogs by a small costal incision and further placed in a beaker containing the widely used amphibian Barth saline [13]. Individual same size stage VI oocytes (applying to *C. caudiverbera* the classification criteria of Dumont [14]) were manually freed from surrounding tissue from the ovaries by blunt dissection with the aid of jewler's forceps and kept in saline until used [15].

2.3 CZE

Separations were accomplished on an Ion Analyzer Capillary Electrophoresis system (Waters, Milford, MA, USA) with a Millenium software (Waters) for data handling. CZE was performed by a modification of the procedure of Xu and co-workers [12], who successfully applied the CZE to the analysis of UDP-sugars. The separations were performed at 22 kV (constant voltage, approximate current 36 μA) using 53 cm × 50 μm uncoated fused-silica capillaries (52.5 cm to the detection window) manufactured by Polymicro Technologies (Phoenix, AZ, USA). The samples were introduced by hydrodynamic injection (10 cm height, for 30 s) and nucleotides were detected online at 254 nm. Best resolution was achieved with 20 mM tetraborate buffer, pH 9.2 at 25°. After use, the capillary column was sequentially washed by 5 min injections of 0.5 M NaOH and deionized water.

2.4 Glycogen synthase activity

One or more oocytes were homogenized by means of a glass pestle specially adapted to Eppendorf tubes containing in a final volume of 100 μL of the following: 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, 1 mM DTT, 100 mM NaF, 3 mM glucose-6-P, and 3 mM UDP-glucose. The different preparations were normalized considering that each oocyte contributes to the solutions with a volume of 3 μL. Thus, all conditions in the assay medium were kept constant except for the crude extract bearing the enzyme of interest (homogenized oocytes). After incubating at 30°C for 10 min, the reaction was stopped by heating at 100°C during 5 min. Then the homogenate was centrifuged at 14 000 rpm for 30 min. The resulting supernatant liquid was saved for CZE analysis. Control experiments were performed in order to study the stability of UDP-glucose and UDP at 100°C during different times. Both compounds proved to be stable under this condition at least for the time required for the assay.

3 Results and discussion

3.1 Nucleotide separation

The procedure of Xu and colaborators [12] for the separation of UDP-sugars includes the use of 20 mM borate buffer, pH 9.0. However, poor resolution was obtained with oocyte samples under this condition. Instead, 20 mM tetraborate buffer, with a greater conductivity than borate, allowed optimal separation of nucleotides and sugar-nucleotides of interest, although resulting in longer running times (20 min). The separation profile obtained from a mixture of different nucleotides is shown in Fig. 1A. An excellent separation between UDP-glucose and UDP was achieved under the described conditions. This is a crucial point for the

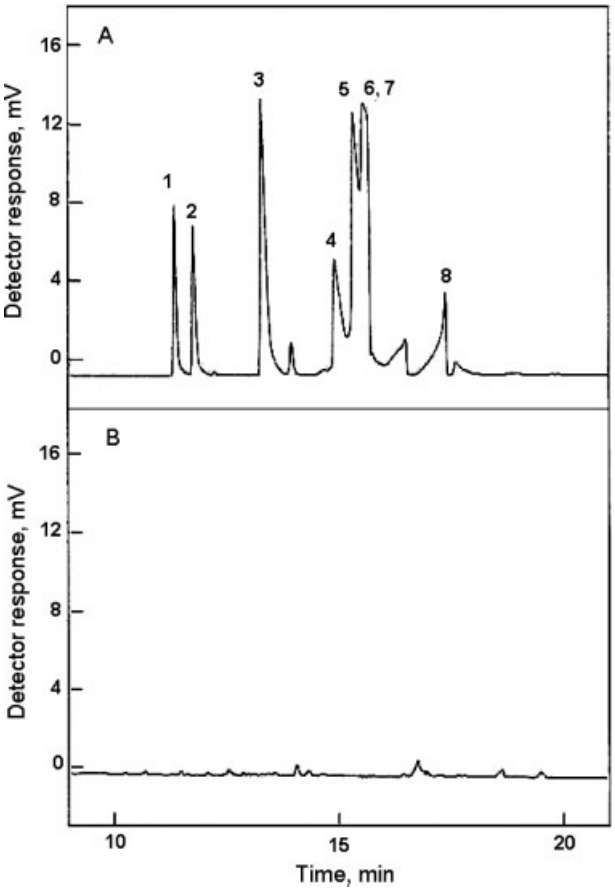

Figure 1. Nucleotide elution profile. (A) A mixture of different nucleotides dissolved in the enzyme assay mix was subjected to CZE as described in Section 2. Numbers correspond to the elution time of the following nucleotides: 1: unknown; 2: UDP-glucose; 3: adenosine 5′-monophosphate (AMP); 4: ADP; 5: cytidine 5′-triphosphate (CTP); 6: uridine 5′-monophosphate (UMP); 7: adenosine 5′-triphosphate (ATP); 8: UDP. (B) A homogenate obtained by resuspending two oocytes in the enzyme assay mix lacking the substrate was centrifuged as described in Section 2 and 50 μL from the supernatant liquid were subjected to CZE under the same conditions as in (A).

estimation of the glycogen synthase-catalyzed reaction, where the conversion of the substrate UDP-glucose to the product UDP is being monitored. Moreover, the oocytes seem to be devoid of measurable amounts of both nucleotides since a control experiment showed that no detectable nucleotides that could interfere with enzymatic activity estimations were apparent in oocyte crude extracts (Fig. 1B).

3.2 UDP-glucose and UDP calibration curves

Several UDP-glucose and UDP (substrate and product of the glycogen synthase reaction, respectively) dilutions were prepared in the assay medium used for enzyme activity determination and subjected to CZE under the conditions described above. As seen from Figs. 2A and B, the increase in absorbance exhibits a linear relationship with the increase in nucleotide concentration. The relation is linear at least up to 3 mM, which is the highest concentration used in further assays. It should be stressed that the latter is the concentration of UDP-glucose present as saturating substrate in the assay medium, therefore this is also the highest product concentration to be expected. Detection limit (considered as the concentration of nucleotide corresponding to a signal around three times the noise level of the background that gave reproducible results) was around 8 μM for both compounds.

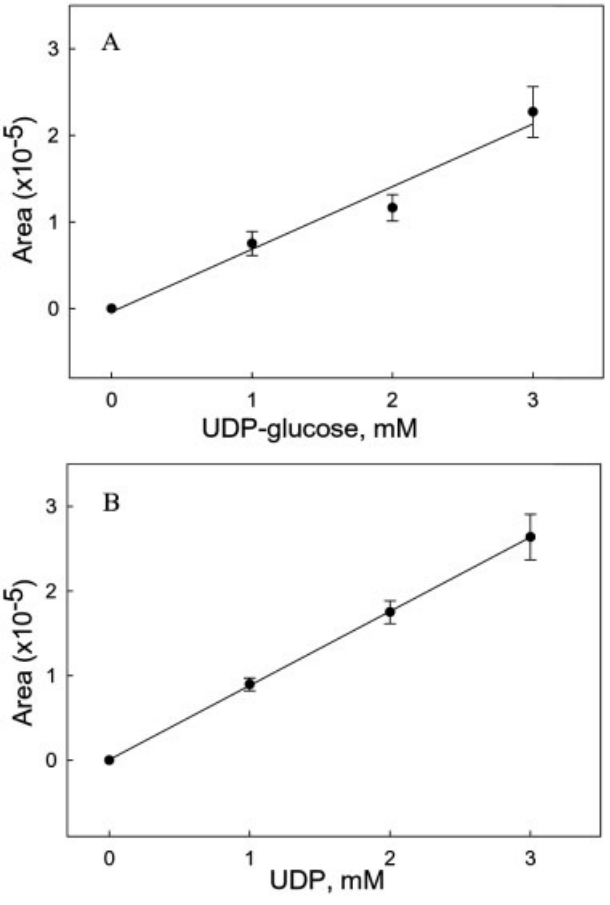

Figure 2. UDP-glucose and UDP calibration curves. Increasing amounts of UDP-glucose (A) or UDP (B) dissolved in the assay medium were subjected to CZE under the conditions described. The results shown are the means of three determinations ± SD.

3.3 Glycogen synthase activity

The procedure was next applied to oocyte crude extracts in order to measure the activity of glycogen synthase present in the cells, *i.e.*, quantitate UDP, the product of the glycogen synthase reaction. In order to express the enzyme activity in a quantitative manner, we must make sure that the new method is measuring the reaction rate. A time curve for the enzyme was constructed by homogenizing groups of two oocytes in the assay mix and incubating during different times. After the reaction was stopped and the mixtures centrifuged, aliquots from the supernatant fractions were subjected to CZE as described. The time curve obtained under these conditions shows that the enzyme reaction is linear at least up to 15 min (Fig. 3A). In further experiments related with enzyme activity estimations, incubation times were 10 or 5 min, thus, working under conditions of initial velocity was assured. After, the effect of enzyme concentration over the reaction rate was tested. Measurements were performed in samples obtained from homogenates of two to eight oocytes as above, incubation time being 10 min. As shown in Fig. 3B, increase in enzyme concentration (number of oocytes used) increments glycogen synthase reaction rate in a linear way. From these results, glycogen synthase activity was estimated to be around 1.3 mU *per* oocyte. This value is in agreement with the one reported in the literature for the same enzyme by means of the radioactive assay [1].

Since the properties of the frog oocyte enzyme under *in vivo* and *in vitro* conditions are well known [1], we decided to test and apply the CZE method to the study of a kinetic parameter of the enzyme. Hence, the K_m value for the substrate UDP-glucose was determined, and its value compared with the ones available for the same enzyme obtained by other techniques. Oocyte glycogen synthase activity as a function of UDP-glucose is depicted in Fig. 4. The results show that the enzyme exhibits normal Michaelis–Menten kinetics towards the substrate UDP-glucose. An apparent K_m value of 0.87 mM for this substrate was obtained. This value is identical to the one reported by Báez and co-workers [1] for the frog oocyte enzyme measured by the radioactive technique. This example application together with the above results illustrates that the CZE procedure proved to be reliable for glycogen synthase determinations.

4 Concluding remarks

The results presented in this work demonstrate that the CZE technique can be successfully used for the detection and

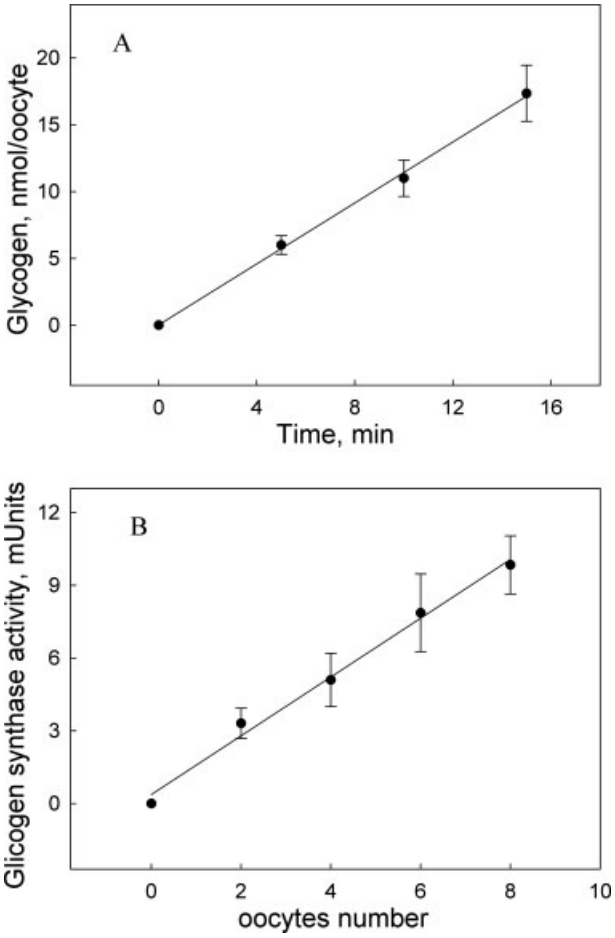

Figure 3. Glycogen synthase activity. (A) Two oocytes were homogenized and incubated in the assay medium during the indicated times. Then the reaction was stopped and the mixture was centrifuged as described. Fifty microliters from the supernatant liquid was subjected to CZE. In (B), an increasing number of oocytes were treated in the same conditions as in A, except for a fixed incubation time of 10 min. The results shown are the means of three determinations ± SEM.

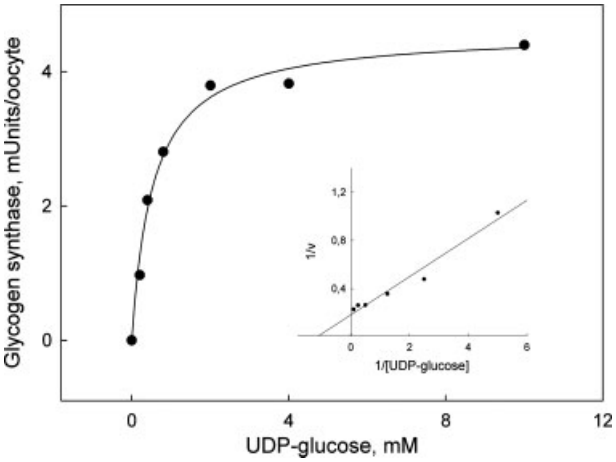

Figure 4. Glycogen synthase activity as a function of UDP-glucose concentration. The assays were performed in homogenates obtained by resuspending two oocytes in the enzyme assay mix containing increasing amounts of UDP-glucose, and incubated during 5 min. After stopping the reaction, 50 μL from the supernatant liquid obtained after centrifugation of the samples were subjected to CZE as described. The apparent K_m value for the substrate was calculated from the data shown in the inset. Results correspond to the mean of two determinations.

quantitation of UDP-glucose and UDP in frog oocyte extracts.

Thus, the easy quantitation and separation of the substrate and product of the glycogen synthase-catalyzed reaction allows enzyme activity to be routinely measured by this method. The procedure was applied to measure the glycogen synthase activity present in oocytes and to the determination of the K_m value of the enzyme for its substrate UDP-glucose. In both the cases, although the values were obtained by different methods, the results were similar to the ones reported in the literature.

Glycogen degradation and synthesis are central to the metabolism of most living organisms, especially vertebrates. The process is essential for survival mainly because of its role in maintaining blood-glucose levels, and therefore, several complex regulatory mechanisms have evolved. Important medical implications arise from the above statement, such as diabetes origin and treatment, among others.

Glycogen synthase is one of the enzymes involved in the anabolic part of glycogen metabolism, and because it is under multiple regulatory mechanisms, it has been extensively studied and characterized. It has generally been considered that glycogen synthase controls the rate of polysaccharide synthesis, although this role has been challenged in recent years.

Since the enzyme continuous to be under active research, the addition of a new procedure for assaying the activity of the enzyme should expand the experimental resources available for different conditions which may be needed. When working with biological crude extracts, serious interferences appear with the spectrophotometric assay. These are related to the use of several auxiliary enzymes which cause high backgrounds and also because of the very high K_m of pyruvate kinase for UDP, which in the assay replaces adenosine 5′-diphosphate (ADP), the natural substrate of the enzyme. On the other hand, the radioactive technique, besides the inconvenience intrinsic to radioactivity manipulation, is laborious, time-consuming, and expensive.

Compared with the classic methods available at present for glycogen synthase estimations, the CZE technique offers considerable advantages inherent to CE, such as the possibility of automation and small sample sizes. Most of all, it is a simple, less expensive, and fast method, in which analysis time is significantly diminished (from days to hours). With appropriate modifications, the described procedure could be expanded to monitor the activity of other enzymes having nucleotides as substrates.

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