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Purification of Frog Muscle Glycogen Synthetase *

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A detailed method for purification of frog muscle glycogen synthetase (GS) is presented in this paper. In this tissue GS is present apparently in a unique form whose activity is dependent on the presence of glucose-6-P (D-form).

The first step in the purification procedure consisted in the isolation of the glycogen-bound GS by centrifugation at 25,000 × g. At this gravitacional force maximum specific activity was obtained in the sediment. The relatively low value of this figure indicated an easy sedimentability of the glycogen-enzyme complex from frog muscle.

The enzyme could not be separated from glycogen by gel filtration through Sepharose. Treatment of the particulate glycogen fractions with α-amylase libereted the enzyme protein. The interference caused by α-amylase on glycogen synthetase activity determination could be suppersed by increasing the concentration of glycogen in the assay mixture to 4 %. Chromatography on DEAE Sephadex of the α-amylase-treated preparations yielded a considerable purification of glycogen synthetase. The eluted enzyme was unstable at 4° C but Mg^{2+} stabilized the enzymatic activity. However sediments obtained by centrifugation at 100,000 × g after addition of glycogen were stable at —20° C for months.

The specific activity of the final preparation was 4.6 μmoles of glucose incorporated per minute and mg of protein. A purification of 740 fold was achieved with a yield of 21 %.

The enzyme glycogen synthetase (UDP glucose: glycogen 4-α-glucosyltransferase E.C. 2.4.1.11) exists in the majority of species in at least two forms (23), a glucose-6-P independent (I) and a glucose-6-P dependent (D) form, which are interconverted by phosphorylation and dephosphorylation reactions catalized by a kinase and a phosphatase (6). It is believed that only the I form is active under most conditions *in vivo*, and, as a result, speculation as to the regulation of glyco-

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gen biosynthesis *in vivo* has been based on the interconversion between these forms and its control (19, 20). Both forms have been found in various mammalian organs, including muscle liver (11), heart (16), brain (7), adipose tissue (14), spleen (12), kidney (10) and blood cells (9). Recently two forms of the enzyme have been demonstrated in yeast (24) and in the mould *Neurospora crassa* (27). The amphibia (2, 22) and the toadfish (22) are interesting because in the muscle of these animal species the native enzyme is dependent on glucose-6-P and, up to now, the existence of an I form has not been demonstrated. By preincubation of crude extracts a greater activity of the D form is found, rather than a conversion to the I form, suggesting an interconversion between an inactive form and the D form (2, 22).

These special characteristics raise very interesting questions about the regulation of the biosynthesis of glycogen in these tissues. As a first step, trying to answer some of these questions, we have purified this active form of the enzyme.

Materials and Methods

Chemicals. UDP-glucose and rabbit liver glycogen were obtained from Sigma; glucose-6-P from Boehringer; Tris (hidroximetil) aminometane (Tris), EDTA and anthrone from Merck; DEAE-Sephadex A25 and Sepharose 2B from Pharmacia and UDP-(^{14}C) glucose from New England Nuclear.

Animals. The frogs were collected in the Delta of the Ebro from November to February. These animals were received on the day following their capture and were put in a tank at room temperature. Such frogs were not used until they had been in their new surroundings for 24 h. and were not fed in the laboratory.

Methods. Glycogen synthetase activity was determined by the filter paper method of THOMAS *et al.* (29) measuring the transfer of (^{14}C) glucose from UDP-(^{14}C) glucose into glycogen in the presence of 6.6 mM glucose-6-P.

Protein concentration was determined by the FOLIN-LOWRY procedure (18). Glycogen was determined by the anthrone method (5) or by the iodine method of KRISMANN (15).

Human salivary α-amylase was purified by Sephadex G-25 chromatography according to SHAINKIN and BIRK (26). α-amylase activity was determined using soluble starch dissolved (1 %) in 50 mM Tris HCl 1 mM EDTA buffer (*p*H 7.8) as substrate. The amount of reducing activity released was measured as described by BERNFELD (3).

One unit of α-amylase is defined as the amount of enzyme that released a quantity of reducing activity equivalent to 1 mg of maltose per minute in our conditions of assay.

Results

Preparation of the extract. Female frogs (30-50 g) were demedulated with a needle and the leg muscles removed, chilled on ice, and trimmed of fat and connective tissue. The muscle was weighed and homogenization was carried out in a home blender for 3 min. at 4° C with 7.5 volumes of 50 mH Tris HCl-5 mM EDTA buffer (*p*H 7.8). This buffer was chosen because the enzyme shows maximum stability and activity in it (2). The homogenate was then centrifuged in a MSE refrigerated centrifuge at 2,000 × g for 20 min. The 2,000 × g supernatant was then filtered through glasswool. This crude extract contained about 90 % of the total activity.

Obtention of the glycogen particulate fraction. The glycogen synthetase activity is usually bound to the glycogen particulate fraction (21), and can be separated by centrifugation. A particulate fraction

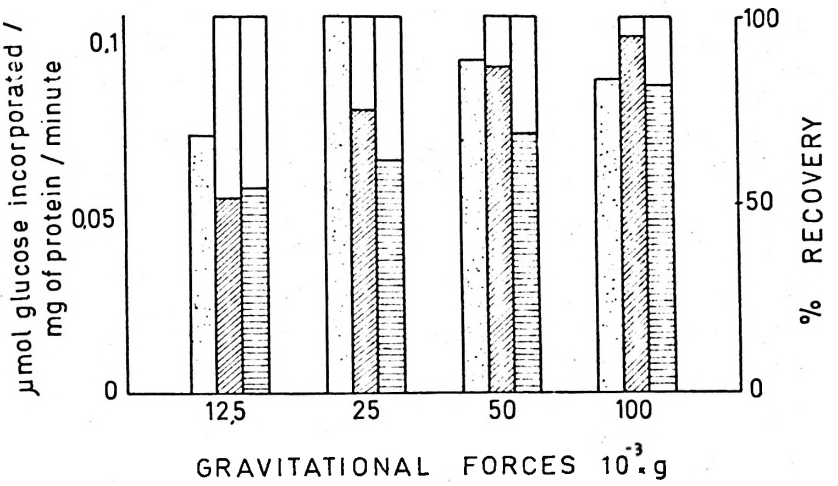

Fig. 1. *Sedimentability of the glycogen-bound enzyme.*
A crude extract was divided in four parts. Each one was centrifuged at a different centrifugal force, showed in the graph. The data presented are those of each pellet the simbols are: ⬚ specific activity; ▨ recovery of activity; ▤ recovery of glycogen.

with high glycogen synthetase specific activity was obtained by centrifuging the crude extracts at 25,000 × g (Fig. 1). In these conditions the recuperation was 76 %. Using higher gravitational forces the recuperation of activity in the particulate fractions rises, however the specific activity decreases. At lower gravitational forces both the recuperation and the specific activity in the particulate fractions were lower than in the 25,000 × g particulate fraction.

The 25,000 × g particulate fractions could be stored at —20° C for months without loss of activity. The freezing step was used as a convenient stopping point in the purification.

The particulate fractions were dispersed in 50 mM Tris HCl-1 mM EDTA buffer (*p*H 7.8) with a glass manual homogenizer to give a total volume of aproximately 1/3 the original crude extract.

Sepharose step. As one of the aims of this work was to obtain the enzyme with a considerable degree of purification and free of glycogen, the resuspended particulate fraction was applied to a Sepharose 2B column which had been equilibrated with the same buffer, trying to separate the glycogen from the protein enzyme. However, both, glycogen and glycogen synthetase activity, appeared together after the excluded protein peak (Fig. 2).

Salivary α-amylase treatment. Since gel filtration experiments with Sepharose demonstrated that the enzyme could not be separated from glycogen by gel filtration, it was found necessary to remove glycogen by α-amylase digestion.

Human salivary α-amylase was applied to the resuspended particulate fractions (0.2 U of amylase/mg glycogen) and the digestion was allowed to progress for 30-40 min. at 30° C. The digestion of the glycogen was very fast in the first half hour and at the end of this period practically 90 % of the glycogen was hydrolyzed (Fig. 3). With longer periods of digestion the percentage of glycogen hydrolyzed hardly varied but the glycogen synthetase activity went down slowly.

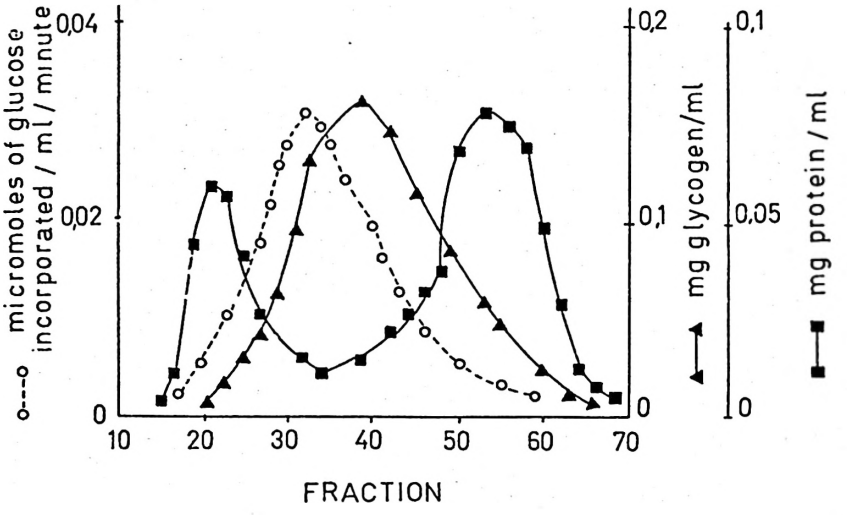

Fig. 2. *Sepharose 2B gel filtration of the resuspended 25,000 × g pellet.* The buffer used was 50 mM Tris HCl-1 mM EDTA, *p*H 7.8.

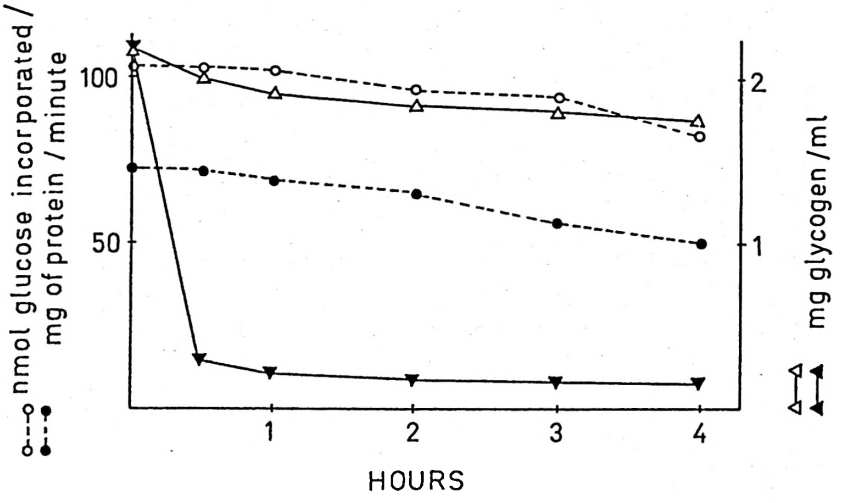

Fig. 3. *α-amylase digestion of the resuspended 25,000 × g pellet.* 0.2 units of α-amylase per mg of glycogen were added. The amount of remaining glycogen and glycogen synthetase activity were measured at the intervals indicated in the graph.

The interference of α-amylase on the glycogen synthetase determination could be avoided by increasing the glycogen concentration in the reaction mixtures to 4 %. In these conditions no interference could be detected and the apparent loss of glycogen synthetase activity observed with lesser glycogen concentrations disappeared (Fig. 4).

After α-amylase treatment the digested suspension was centrifuged at 2,000 × g for 10 minutes with practically all the

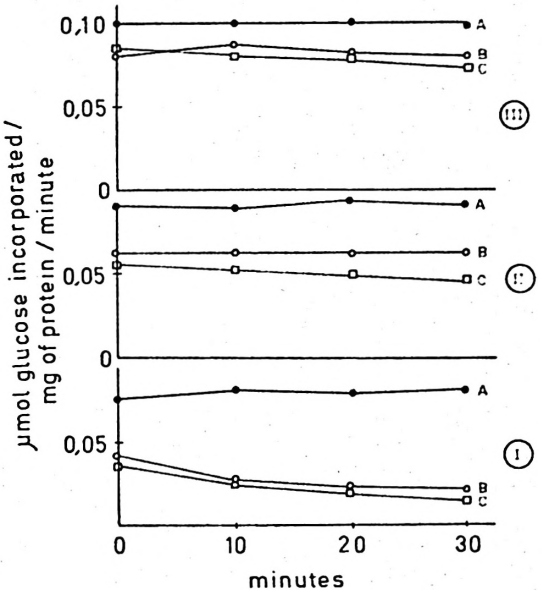

Fig. 4. *Effect of glycogen concentration in the reaction mixtures on the glycogen synthetase apparent activity measured in presence of α-amylase.*
In I the concentration of glycogen was 1 %, in II 2 % and in III 4 %. A: no α-amylase; B: with 0.12 units of α-amylase/mg glycogen; C: with 0.20 units of α-amylase/mg glycogen.

glycogen synthetase activity remaining in the supernatant. The little sediment formed was discarded.

Sepharose gel filtration of the amylase treated enzyme. When the α-amylase treated enzyme was applied to the Sepharose column in the same conditions used for the first Sepharose filtration, all the synthetase activity appeared in the last fractions.

DEAE-Sephadex column chromatography. The supernatant obtained after centrifugation of the α-amylase process was applied to a column of DEAE-Sephadex previously equilibrated with Tris-EDTA buffer (*p*H 7.8). The column was then washed with the same buffer containing 200 mM NaCl which removed α-amylase. Finally the enzyme was eluted with Tris-EDTA buffer containing 300 mM NaCl (Fig. 5).

Stability of the enzyme eluted from DEAE columns. The enzyme eluted from the column was unstable at 4° C (Fig. 6). The addition of 5 mM Mg^{2+} stabilized the enzymatic activity. The addition of glycogen hardly increased enzyme stability neither in the absence nor in the presence of Mg^{2+}.

However, if glycogen added preparations (10 mg/ml) were centrifuged at 100,000 × g for 1 hour, sediments were obtained that at —20° C maintained glycogen synthetase activity for months. An additional purification of the enzyme was achieved in this process.

General purification schedule. The data of the different steps of the process

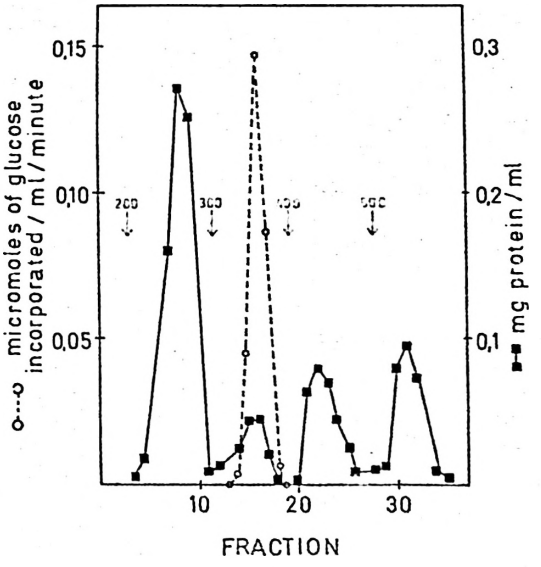

Fig. 5. *DEAE-Sephadex chromatography of α-amylase treated pellets.*
The buffer employed was 50 mM Tris HCl-1 mM EDTA (*p*H 7.8). The arrows show the application of buffers with increasing NaCl concentrations (mM).

Table I. *General schedule for purification of glycogen synthetase-D from frog muscle.*

ENZYME PREPARATION	PROTEIN mg/ml	SPECIFIC ACTIVITY μmol glucose/mg protein/min	PURIFICATION × times	RECOVERY %
Homogenate	12.66	0.006	—	100
Crude extract	6.18	0.011	1.9	89
Particulate fraction from 25,000×g	1.62	0.107	18.0	76
Digested supernatant	1.10	0.153	25.4	71
DEAE-Sephadex fraction pool	0.03	3.486	560.7	32
Sedimented fraction at 100,000×g	0.06	4.601	740.1	21

of purification described here are shown in table I. The final specific activity was of about 4.6 μmole of glucose incorporated per minute and mg of protein. A purification of about 740 fold was achieved with a yield of 21 %.

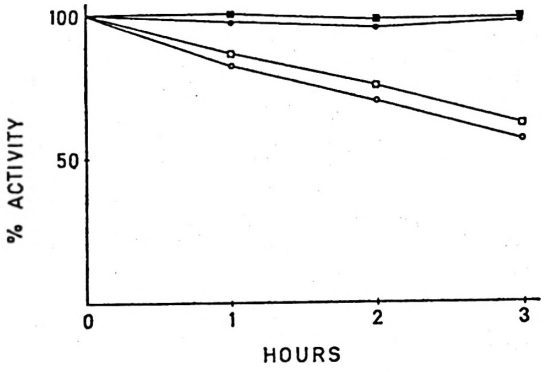

Fig. 6. *Stability of the DEAE-Sephadex eluted enzyme with different additions.*
O—O no additions; ●—● plus 5 mM Mg^{2+}; □—□ plus glycogen 10 mg/ml; ■—■ plus 5 mM Mg^{2+} and glycogen 10 mg/ml.

Discussion

The procedure developed in the present study has yielded a glycogen synthetase D preparation purified about 740 fold from extracts of frog muscle. With this procedure it is possible to obtain a glycogen free enzyme or a very stable glycogen-bound preparation.

The final specific activity of the preparation, 4.6 μmoles of glucose incorporated per minute and mg of protein is about the same as that reported for the enzyme purified from bovine heart, 4.7 (28), rabbit muscle, 12.1 (4), swine kidney, 9.1 (13), and the enzyme isolated from rat liver, 7.6 (25).

It is worthwhile noting that in frog muscle extracts, glycogen synthetase activity was sedimented at gravitational forces much lower than those usually employed to precipitate the glycogen bound enzyme from mammalian muscle (about 100,000 × g) (23). This is due to the easy sedimentability of the glycogen-enzyme complex from frog muscle. The frog liver glycogen-bound enzyme also sediments in high percentages at very low gravitacional forces (8).

The glycogen could not be separated from the enzyme by gel filtration, suggesting a rather strong bond between glycogen and the enzyme protein. After α-amylase digestion, only traces of glycogen remained and accordingly the pattern of elution from Sepharose columns was changed.

To measure glycogen synthetase activity in the presence of α-amylase presents a big difficulty, due to the contrary effects of both enzymes on the molecule of the polysaccharide, but it has been seen that the interference caused by α-amylase, could be suppresed by increasing the concentration of glycogen in the assay mix-

ture. α-amylase was later separated in the DEAE-Sephadex step, avoiding further contamination.

The glycogen free preparation is stable for hours at 4° C in the presence of Mg^{2+} and permits the study of the characteristics on the enzyme in the absence of the polysacharide. As has been pointed out in other systems (1, 17) in this case also, Mg^{2+} exerts a protective effect on the activity of glycogen synthetase. The glycogen-bound enzyme is very stable when frozen and is useful for many studies in which the absence of the polysaccharide is not a determining condition.

Resumen

Se describe un método para la obtención de preparaciones purificadas de glucógeno sintetasa (GS) de músculo de rana, que en este tejido se presenta únicamente como forma dependiente de la glucosa-6-P (forma D).

El primer paso del proceso de purificación consiste en aislar la fracción de glucógeno particulado, a la que va unido la glucógeno sintetasa, por centrifugación a 25.000 × g, condiciones en las que se obtiene la preparación con mayor actividad específica. Ello indica una fácil sedimentabilidad del complejo enzima-glucógeno en este tejido.

El enzima no puede separarse del glucógeno por filtración a través de Sepharose 2B pero sí por digestión con α-amilasa salivar. La interferencia que causa la amilasa en la determinación de la actividad de la glucógeno sintetasa puede ser evitada incrementando la concentración de glucógeno en la mezcla de determinación. Por cromatografía por DEAE-Sephadex del digesto amilásico se consigue una notable purificación de la glucógeno sintetasa. El enzima eluido de la columna de DEAE-Sephadex es inestable a 4° C pero el Mg^{2+} lo estabiliza. Se pueden obtener preparaciones que conservan la actividad durante meses a —20° C sedimentando la glucógeno sintetasa a 100.000 × g previa adición de glucógeno.

La preparación final obtenida tiene una actividad de 4,6 μmoles de glucosa incorporados por minuto y por mg de proteína, lo que representa una purificación de 740 veces y la recuperación es el del 21 % de la actividad total.

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