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Glycogen

Determination with Amyloglucosidase

Dietrich Keppler and Karl Decker

Glycogen occurs as a storage form of glucose in animal organs and in some micro-organisms. The structure of glycogen is well-defined, but the molecular weight is not. Native glycogen from liver has a particle weight of ca. 300 million[1,2], muscle glycogen has one of ca. 10 million[2]. For specific and sensitive assays of glycogen enzymatic hydrolysis is preferable to acid hydrolysis.

Amyloglucosidase from *Aspergillus niger* (1,4-α-D-Glucan glucohydrolase, EC 3.2.1.3)[3] is an exoglucosidase which hydrolyses the α-D-(1→4) and α-D-(1→6)-linkages[3,4] of glycogen. The specificity of the enzyme allows hydrolysis[5,6] in organ and blood cell homogenates, so that the inconvenience of isolating the glycogen is avoided. The glucose formed is specifically determined with hexokinase, HK (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) and glucose-6-phosphate dehydrogenase, G6P-DH (D-Glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49)[7] (see also p. 1196).

Application of Method: In biochemistry, clinical biochemistry and food chemistry.

Principle

(1) $$\text{Glycogen} + (H_2O)_{n-1} \xrightarrow[\text{pH 4.8}]{\text{amyloglucosidase}} (\text{Glucose})_n$$

(2) $$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{2+},\ \text{pH 7.5}]{\text{hexokinase}} \text{ADP} + \text{Glucose-6-phosphate}$$

(3) $$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-Phosphogluconolactone} + NADPH + H^+$$

The glucose liberated after hydrolysis of glycogen is proportional to the increase of NADPH measured by the extinction change at 340 (334, 365) nm.

Optimum Conditions for Measurements

To obtain quantitative hydrolysis of glycogen with amyloglucosidase the pH must be maintained between 4 and 5. The enzyme is completely active between 30° and 50 °C, and at 65 °C two-thirds of the maximal activity still remains. The optimum conditions are pH 4.8, 40 °C and a two hour incubation with shaking. The rate of hydrolysis decreases with decreasing molecular weight of the substrate[8].

Equipment

Analytical balance up to 100 g.; high-speed homogenizer; constant temperature bath with shaker; bench centrifuge; spectrophotometer or spectrum-line photometer for measurements at 334, 340 or 365 nm.

Reagents

1. Perchloric acid, A. R., 70% (w/w), sp. gr. 1.67
2. Potassium hydrogen carbonate, A. R.
3. Potassium hydroxide, A. R., 1 N
4. Acetic acid, A. R., 96% (w/w), sp. gr. 1.06
5. Sodium acetate, A. R.

6. Triethanolamine hydrochloride, A. R.
7. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
8. Adenosine-5'-triphosphate, ATP
   crystalline disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$ commercial preparation, see p. 527.
9. Nicotinamide-adenine dinucleotide phosphate, NADP
   disodium salt, NADP-Na_2H, commercial preparation, see p. 546.
10. Hexokinase, HK
    from yeast; crystalline suspension in 3.2 M ammonium sulphate; 2 mg./ml., ca. 140 U/mg. (25 °C). Commercial preparation, see p. 473.
11. Glucose-6-phosphate dehydrogenase, G6P-DH
    from yeast, for analytical purposes. Suspension in 3.2 M ammonium sulphate solution; 1mg./ml. ca. 140 U/mg. (25 °C). Commercial preparation, see p. 458.
12. Amyloglucosidase, AGS
    from *Aspergillus niger*, suspension in 3.2 M ammonium sulphate solution; ca. 14 U/mg. (25 °C). Commercial preparation, see p. 434.

Preparation of Solutions

Prepare all solutions with fresh distilled water.

I. Perchloric acid (0.6 N):
   Dilute 5.2 ml. 70% $HClO_4$ with distilled water to 100 ml.

II. Potassium hydrogen carbonate (1 M):
   Dissolve 2 g. potassium hydrogen carbonate in distilled water and make up to 20 ml.

III. Acetate buffer (0.2 M; pH 4.8):
   Dissolve 4.8 ml. 96% acetic acid and 9.75 g. sodium acetate in distilled water and make up to 1000 ml. Check the pH with a glass electrode.

IV. Triethanolamine buffer (0.3 M TRA; pH 7.5; 4.05 mM $MgSO_4$):
   Dissolve 5.6 g. triethanolamine hydrochloride and 100 mg. $MgSO_4 \cdot 7H_2O$ in ca. 50 ml. distilled water and add 12 ml. 1 N KOH. Check the pH with a glass electrode and make up to 100 ml. with distilled water.

V. Adenosine triphosphate/nicotinamide-adenine dinucleotide phosphate/glucose-6-phosphate dehydrogenase (1 mM ATP; 0.9 mM β-NADP; 5 μg. G6P-DH/ml.):
   Dissolve 6 mg. ATP-$Na_2H_2 \cdot 3H_2O$ and 8 mg. NADP-Na_2H in 10 ml. triethanolamine buffer (solution IV) and add 0.05 ml. G6P-DH suspension.

VI. Hexokinase, HK (2 mg./ml.):
   Use the suspension undiluted.

VII. Amyloglucosidase, AGS (10 mg./ml.):
   Dissolve 20 mg. of enzyme protein in 20 ml. acetate buffer (solution III).

Stability of Solutions

Store all solutions, stoppered, at 0–4 °C. The amyloglucosidase solution can be stored deep-frozen without any appreciable loss of activity. Solution V is stable for a week at 0–4 °C.

Procedure

Collection, Treatment and Stability of Sample

Collection:

Obtain tissue by the freeze-clamping technique (see p. 400). Isolated blood cells at below 4 °C. Various anaesthetics (diethylether, halothane) bring about a rapid glycogenolysis in liver and are therefore to be avoided.

Deproteinization:

Weigh sample or deep-frozen piece of tissue and thoroughly homogenize at high speed with about 5 parts by weight ice-cold perchloric acid (I). Immediately after the homogenization 0.2 ml. of the homogenate is pipetted into a centrifuge glass and kept in an ice bath. This aliquot is later used for the glycogen hydrolysis.
Alternatively, a thermal deproteinization of the sample may be employed and also leads to rapid inactivation of glycogen decomposing enzymes[9].

Stability of sample:

The homogenate can be stored in the cold for several days.

Assay System

Glycogen hydrolysis:

Incubate 0.2 ml. homogenate in perchloric acid (0.01–3 mg. glycogen) with 0.1 ml. potassium hydrogen carbonate solution (II) and 2.0 ml. amyloglucosidase solution (VII) in a stoppered centrifuge tube with shaking at 40 °C for 2 hr.
Stop incubation by addition of 1.0 ml. perchloric acid (I). After centrifugation take 0.05 ml. of the acid supernatant fluid for the determination of glucose. If the glycogen content of the sample is very low neutralize with solid $KHCO_3$, so that 0.5–1.0 ml. of the supernatant fluid can be taken for the glucose assay. This must be allowed for in the calculations.

Homogenate glucose blank:

Centrifuge homogenate in perchloric acid for 15 min. and take 0.05 ml. of the supernatant, after neutralisation with solid $KHCO_3$, for the determination of glucose.

Spectrophotometric measurements:

Wavelength: 340 (Hg 334, Hg 365) nm; light path: 1 cm.; final volume: 1.055 ml.; room temperature: read against air.
Prepare a cuvette for the glucose blank and the glycogen hydrolysate.

Pipette successively into cuvettes:			Concentration in assay mixture
ATP/NADP/G6P-DH/buffer	(V)	1.00 ml.	0.3 M TRA 1 mM ATP 0.9 mM NADP 4.0 mM $MgSO_4$ 5.0 μg. G6P-DH/ml. ≙ 0.7 U/ml.
Sample (deproteinized)		0.05 ml.	up to ca. 0.4 mM glucose
Mix thoroughly and follow the increase in extinction (oxidation of glucose-6-phosphate) until constant (ca. 5 min.). Then read extinction E_1.			
HK suspension	(VI)	0.005 ml.	9 μg./ml. ≙ 1.4 U/ml.
Mix and when the extinction is constant (5–10 min.) read extinction E_2. $E_2 - E_1 = \Delta E$ is used for the calculations.			

ΔE_H represents the extinction difference of the sample from the hydrolysate.
ΔE_B represents the extinction difference of the glucose blank.

Calculations

Under the above conditions the enzymatic hydrolysis is quantitative, so that the glucose content after subtraction of the glucose blank (free glucose before hydrolysis) corresponds to the glycogen content of the sample. The glycogen content can be expressed in μmole glucosyl units per g. wet wt. of tissue. If the results are required in g. glycogen per 100 g. tissue it is necessary to include in the calculations the molecular weight of the anhydroglucose (mol. wt. 162) present in the glycogen. In the calculations the total glucose content per g. homogenate is first calculated and the free glucose content per g. homogenate is subtracted from this. If 0.05 ml. sample is taken for the glucose assay the glycogen content is calculated as follows:

Wavelength:	*334 nm*	*340 nm*	*365 nm*
Content =	$57.1 \times \Delta E_H - 3.5 \times \Delta E_B$	$56.0 \times \Delta E_H - 3.4 \times \Delta E_B$	$100.9 \times \Delta E_H - 6.1 \times \Delta E_B$ [μmole/g.]

The factors given are based on a liquid content of the tissue investigated of about 75% and a dilution of the tissue sample in the homogenate of about 6 times. Multiplying by this dilution factor gives the result in μmole glucosyl units per gram tissue wet weight. If the glycogen content is to be given in grams per 100 g. the multiplication factor is 0.0162.

Accuracy and Precision

Six replicate determinations on a purified rat liver glycogen[1] gave a mean value of 100 with a standard deviation of 2.5. The coefficient of variation is therefore 2.5%. The variation coefficient for determinations in thoroughly homogenized homogenate carefully pipetted into the hydrolysis mixture is less than 3.5%.

Normal Values

The biological variation of the glycogen content of tissues is large because it is very dependent on the nutritional state and on hormonal factors. The glycogen content of rat liver was found to be 338 ± 62 μmole glucosyl units per g. fresh wt. or 5.5 ± 1.0 g./100 g.[10]. Human liver contains 1.2–9.5 g./100 g.[6,11], skeletal muscle, 1.0–2.0 g./100 g.[12], whole blood, 6.8 mg./100 ml.[13], leucocytes 5.3–9.3 mg. per 10^9 neutrophil granulocytes[14].

Sources of Error

Effects of drugs and other therapeutic agents: None known.

Interference in the assay: The first time an amyloglucosidase preparation is used its effectiveness should be checked by comparison with the acid hydrolysis method (2N H_2SO_4, 95 °C, 4 hr.). Enzymatic hydrolysis of a solution of purified glycogen gives values which are ca. 5% higher than those obtained by acid hydrolysis. Contamination of the amyloglucosidase preparation with amylo-1→6-glucosidase and proteases can be advantageous to obtain quantitative hydrolysis of glycogen. The incomplete hydrolysis of old glycogen solutions which occurs on degradation with phosphorylase and amylo-1→6-glucosidase[16] is not detectable with amyloglucosidase.

Specificity of Method

Amyloglucosidase specifically hydrolyses oligo- and polyglucosides of the amylose, amylopectin and glycogen-type[4,11]. Maltosyloligosaccharides, which can arise from glycogen by the too slow inactivation of liver α-amylase[9] are also determined. The normal content of maltosyl-oligosaccharides in liver is below 0.4% of the glycogen content[9]. Alkaline extraction, ethanol precipitation and acid hydrolysis of glycogen can result in a partial loss of glycogen[6,15].

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