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An enzymatic colorimetric assay for glucose-6-phosphate

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ABSTRACT

A specific colorimetric assay for the determination of glucose-6-phosphate (G6P) was developed. This assay is based on the oxidation of G6P in the presence of glucose-6-phosphate dehydrogenase (G6PD) and nicotinamide adenine dinucleotide phosphate ($NADP^+$); the NADPH thereby generated reduces the tetrazolium salt WST-1 [2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] to water-soluble yellow-colored formazan with 1-methoxy-5-methylphenazium methylsulfate (1-mPMS) as an electron carrier. The assay is optimized for reaction buffer pH, enzyme/dye concentration, and reaction time course. The limit of detection of the assay is 0.15 μM (15 pmol/well). The usefulness of the assay is demonstrated by the accurate measurement of the G6P concentration in fetal bovine serum (FBS).

Hexokinase (EC 2.7.1.1) catalyzes the formation of glucose-6-phosphate (G6P)[1] from glucose. G6P is the first substrate in glycolysis, the hexose monophosphate shunt, and both the glycogen and starch biosynthesis pathways [1]. G6P concentrations may reflect ongoing biological processes such as catabolite derepression, metabolic oscillations, and metabolic regulation in diabetes, to name just a few [2–4].

Several methods have been developed for the quantitative analysis of G6P concentrations in cells and tissues. These methods include chromatographic approaches [5–9], enzyme-based amperometric biosensors [10–12], and nicotinamide adenine dinucleotide phosphate ($NADP^+$)-coupled enzymatic assays [13–15]. Because G6P has no chromophore, chromatographic methods require an expensive apparatus to separate and detect G6P. On the other hand, enzymatic methods are specific, rapid, and convenient to perform. In these methods, G6P is oxidized to gluconolactone 6-phosphate by glucose-6-phosphate dehydrogenase (G6PD) while $NADP^+$ is reduced to NADPH. Hence, G6P can be determined by measuring NADPH absorbance at 339 nm or fluorescence at 470 nm [13–15]. However, due to its low extinction coefficient and weak fluorescence, the sensitivity of NADPH-coupled tools is not high [13]. Amperometric methods are based on enzyme reactions with electronic signal detection. Because enzyme immobilization on the electrodes is required, long-term storage of functional enzyme-labeled electrodes is difficult.

Recently, we reported a sensitive enzymatic fluorimetric assay for the determination of intracellular G6P based on the stoichiometric reduction of weakly fluorescent resazurin to highly fluorescent resorufin [16]. We have found this fluorimetric assay to be particularly useful in the analysis of intracellular metabolites [17]. However, under certain conditions, a colorimetric methodology may be preferred. For example, colorimetric assays are not sensitive to autofluorescence background. Fluorescence methods are also more sensitive to impurities. We have found that fluorimetric measurements are not sufficiently reliable to quantify metabolite levels in human serum. Although colorimetric assays typically exhibit moderate sensitivities, recently developed tools have improved the sensitivities of these assays. A new generation of sulfonated tetrazolium salts (WST-1 [2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] and WST-8) have been used in clinical and cellular assays [18–21]. Ishiyama and coworkers [19] demonstrated that NADH reduced WST-1 to WST-1 formazan at pH values greater than 8.0. The absorbance change of WST-1 formazan is much more sensitive than the spectrophotometric measurement of NADH (or NADPH) at 339 nm.

In this article, we report a WST-1-coupled enzymatic assay for measurement of G6P in a 96-well microplate using redox amplifi-

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[1] *Abbreviations used:* G6P, glucose-6-phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; G6PD, glucose-6-phosphate dehydrogenase; 1-mPMS, 1-methoxy-5-methylphenazium methylsulfate; FBS, fetal bovine serum; %CV, percentage coefficient of variation; ANOVA, analysis of variance; ULOQ, upper limit of quantitation; LOD, limit of detection; LLOQ, lower limit of quantitation; %RE, percentage relative error.

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cation to increase sensitivity [22]. The mechanism of the assay is shown in Scheme 1. G6P is converted to gluconate-6-phosphate by G6PD; the simultaneously generated NADPH participates in a cycling reaction that employs the tetrazolium salt WST-1 to produce water-soluble yellow-colored formazan with 1-methoxy-5-methylphenazium methylsulfate (1-mPMS) as an electron carrier. The concentration of WST-1 formazan is measured by the change in absorbance at 440 nm, which is proportional to the G6P concentration. Colorimetric read-out is convenient, and the dynamic range is much higher than the fluorimetric method [16]. We also tested the assay's performance by measuring G6P concentrations in fetal bovine serum (FBS) samples.

Materials and methods

Materials

WST-1 was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). FBS was purchased from Invitrogen (Carlsbad, CA, USA). G6P, G6PD from *Leuconostoc mesenteroides,* 1-mPMS, and $NADP^+$ were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of FBS samples

Heat-inactivated FBS samples were deproteinized by the addition of an equal volume of ice-cold 0.5 M $HClO_4$. After incubation for 5 min on ice, the mixture was centrifuged at 10,000*g* for 5 min to remove proteins and then the supernatant was collected. Next, 200 μl of supernatant was neutralized with 10 μl of 2.5 M K_2CO_3 at 4 °C. Using an Integrated SpeedVac System (model ISS110; Thermo Savant, Holbrook, NY, USA), samples were simultaneously degassed and centrifuged while set to a low drying rate for 5 min. The clear supernatant was pooled for the G6P assay, as described below.

Microplate assay procedure

A total assay volume of 100 μl was used for each reaction. The assay solution was freshly prepared by mixing the following: 250 μl of 20 mM $MgCl_2$, 250 μl of 0.5 mM $NADP^+$, 250 μl of 10 mM WST-1, 250 μl of 1-mPMS, 250 μl of 4 U/ml G6PD, and 2.25 ml of 50 mM Tris buffer (pH 8.5). A total assay solution volume of 3.5 ml is sufficient for approximately 50 assays.

The assay procedure was as follows. A 30-μl volume of serially diluted G6P standards was pipetted into individual wells of a 96-well plate (Costar 3626, Corning, Corning, NY, USA), followed by the addition of 70 μl of the assay solution. These mixtures were incubated for 30 min at room temperature in the dark. The absorbance at 440 nm was measured with a plate reader (Molecular Devices, Sunnyvale, CA, USA). Background absorbance was corrected by subtracting the value of the no-G6P control from all sample readings. For FBS samples, we used 0.2 M Tris buffer (pH 8.5) for the assay to stabilize the reaction pH. G6P standards were treated in the same way as FBS to prepare the calibration curves.

Statistical analyses

Unless otherwise noted, all experiments were performed in triplicate, and the results are presented as means ± standard deviations.

The repeatability (within-run) and intermediate precision of the assay were determined by the percentage coefficient of variation (%CV), as shown in Eqs. (1) and (2) [23]:

$$\text{Repeatability (within-run) (\%CV)} = 100 \times s_w/\text{mean} = 100 \times (MS_w)^{0.5}/\text{mean} \quad (1)$$

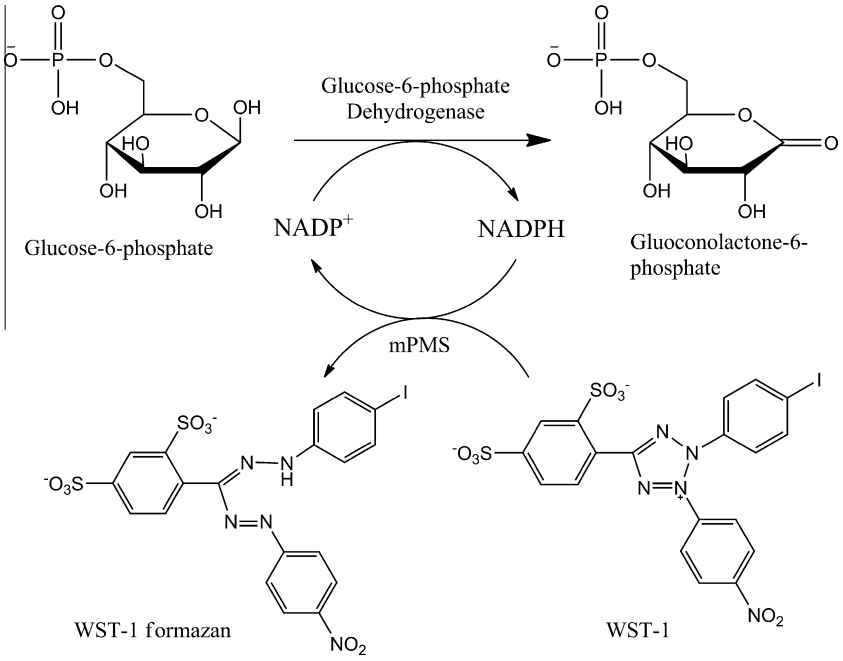
$$\text{Intermediate precision (\%CV)} = 100 \times (s_w^2 + s_b^2)^{0.5}/\text{mean} \quad (2)$$

where $s_b^2 = (MS_b - MS_w)/n$, MS_b and MS_w are between- and within-run analysis of variance (ANOVA) mean square errors, respectively, and s_w and s_b are within- and between-run variance components, respectively. The mean square variances were calculated with Excel analysis ToolPak.

Results and discussion

Optimization of assay

The G6P assay is expected to exhibit sensitivity to the concentrations of WST-l, the electron mediator, $NADP^+$, and G6PD as well



Scheme 1. Mechanism of the chemical reactions for detection of G6P.