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Stabilized enzymatic reagents for measuring glucose, creatine kinase and γ-glutamyltransferase with thermostable enzymes from a thermophile, *Bacillus stearothermophilus**

KOSUKE TOMITA,†‡ KUMIKO NOMURA,‡ HITOSHI KONDO,§ KAZUHIKO NAGATA§ and HIROYUKI TSUBOTA‖

‡*College of Engineering, Kanto Gakuin University, 4834 Mutsuura, Kanazawa, Yokohama 236, Japan*
§*Research and Development Center, Unitika Ltd, 23 Kozakura, Uji, Kyoto 611, Japan*
‖*Technical Section, Iatron Laboratories Inc., 1144 Oowadashinden, Yachiyo, Chiba 276, Japan*

Abstract: Stabilized enzymatic reagents for measuring some components in biological fluids have been successfully developed based on two kinds of thermostable enzymes derived from *Bacillus stearothermophilus* with separation of the reagent into two complementary solutions. The thermostable glucokinase produced was applied to the measurement of glucose and creatine kinase activity, while the alanine dehydrogenase produced was used for the measurement of γ-glutamyltransferase activity. The enzymatic reagents were also stabilized by developing two separate reagents with an optimum pH for the main reagent components. The stability of the reagents in liquid form was examined at 10°C. It was clearly shown that the reagents for measuring glucose and creatine kinase activity were stable and retained their full capability for accurate measurement in biological fluids for over one year. The alanine dehydrogenase product was stable for at least 40 days.

Keywords: *Bacillus stearothermophilus; glucokinase; alanine dehydrogenase; glucose measurement; creatine kinase measurement; γ-glutamyltransferase measurement.*

Introduction

Enzymes have become popular in clinical chemical analysis, because of their excellent characteristics of substrate and reaction specificity and stereospecificity. Many of these enzymes are obtained from mesophiles and tend to lose their activity on storage in liquid form, even at rather low temperatures. On the other hand, enzymes derived from thermophiles are superior to those from mesophiles as regards their thermal and long-term storage stability. For the practical application of enzymes in clinical analysis, the stability of enzymatic reagents in liquid form is an important factor. Several enzymatic methods using thermostable enzymes have been reported to have good stability in the liquid state [1–6]. Although this property was shown to be useful for routine analysis of some components in biological fluids over a limited time period, it is necessary to stabilize the enzymatic reagents to enable their practical and convenient use in clinical laboratories over a longer period of time.

To fulfil these requirements, the authors have developed enzymatic reagents with extremely good stability in the liquid state, based on using two kinds of thermostable enzymes and on optimizing the reagent composition. These enzymes are glucokinase (GlcK, EC 2.7.1.2) and alanine dehydrogenase (AlaDH, EC 1.4.1.1), both of which were obtained from a thermophile, *Bacillus stearothermophilus*. The long-term stability in using GlcK for measurement of glucose and creatine kinase (CK) activity, and in using AlaDH for γ-glutamyltransferase (γ-GT) activity is examined in this report.

Basic principles

The principle of the assay for glucose is

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†Author to whom correspondence should be addressed.

based on the enzymatic coupling of GlcK and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) [2]:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{GlcK}} \text{Glucose-6-phosphate (G6P)} + \text{ADP}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate (6PG)} + \text{NADPH} + \text{H}^+$$

Glucose is stoichiometrically phosphorylated to G6P by GlcK, and then, G6P is converted to 6PG by G6PDH, transforming $NADP^+$ to NADPH, whose absorbance measured at 340 nm is therefore proportional to the amount of glucose present.

The CK assay can be represented as follows [3]:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{GlcK}} \text{G6P} + \text{ADP}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6PG} + \text{NADPH} + \text{H}^+$$

The CK activity is, therefore, measured by monitoring NADPH at 340 nm.

The γ-GT assay follows this reaction scheme [6]:

$$\gamma\text{-Glutamylalanine (}\gamma\text{-GluAla)} + \text{Glycylglycine (GlyGly)} \xrightarrow{\gamma\text{-GT}} \gamma\text{-Glutamylglycylglycine} + \text{L-alanine}$$

$$\text{L-alanine} + \text{H}_2\text{O} + \text{NAD}^+ \xrightarrow{\text{AlaDH}} \text{Pyruvate} + \text{NH}_4^+ + \text{NADH}$$

The L-alanine formed by the γ-GT action on the peptide substrate γ-GluAla is stoichiometrically deaminated to pyruvate by AlaDH, transforming NAD^+ to NADH, which is continuously monitored at 340 nm.

In this paper, the development of stabilized enzymatic reagents for measuring glucose, CK activity and γ-GT activity is described.

Experimental

Materials

GlcK was purified from *B. stearothermophilus* according to the method of Kamei *et al.* [7]. AlaDH from *Escherichia coli* C600 carrying the plasmid-encoding *B. stearothermophilus* AlaDH gene was prepared by the method of Sakamoto *et al.* [8]. G6PDH from *Leuconostoc mesenteroides*, N-acetyl-L-cysteine (NAC), P^1, P^5-di(adenosine-5′-)pentaphosphate trilithium salt (Ap5A), disodium salt of AMP, ATP and $NADP^+$, free salt of NAD^+, potassium salt of ADP and creatine phosphate disodium salt were purchased from Boehringer Mannheim GmbH, Mannheim, Germany. γ-GluAla was purchased from Kokusan Chemical Works, Ltd, Tokyo, Japan and GlyGly was obtained from Nakalai Tesque, Inc., Kyoto, Japan. The other chemicals used throughout this work were of analytical grade.

Sera were obtained from patients and healthy adults. "Ortho normal control serum" was purchased from Ortho Diagnostic Systems Ltd, Tokyo, Japan. Fixed amounts of glucose and CK from rabbit muscle were mixed with serum samples and the resultant mixtures used in the studies on linearity of the systems.

Determination of the activities of GlcK, G6PDH and AlaDH

The GlcK, G6PDH and AlaDH assays were carried out as previously described [3, 6]. The unit of GlcK, G6PDH or AlaDH activity was defined as the amount of enzyme that forms one μmol of the product at 30°C in 1 min.

Reagent composition for glucose, CK and γ-GT assays

The glucose, CK and γ-GT assays were based on mixing two separate reagents. For glucose the components in the first reagent (R1) (pH 9.0) comprised: 62 mM Tris-HCl buffer; 2.5 mM ATP; 0.9 U ml^{-1} GlcK; 1.5 U ml^{-1} G6PDH; 18 mM magnesium chloride; and 25 mM potassium chloride. The second glucose reagent (R2) (pH 4.0) comprised: 120 mM acetate buffer; 8 mM $NADP^+$; and 25 mM NAC. These reagents were mixed so that the ratio of R1:R2 was 4:1 and the final pH was 8.0 after mixing.

For CK the components in reagent R1 (pH 8.5) were: 50 mM HEPES buffer; 37.5 mM creatine phosphate; 6.25 mM AMP; 0.0125 mM Ap5A; 25 mM glucose; 25 mM potassium acetate; 4.0 U ml^{-1} GlcK; and 1.3 U ml^{-1} G6PDH. Reagent R2 (pH 3.5) comprised: 50 mM acetate buffer; 2.0 mM EDTA; 10 mM $NADP^+$; 100 mM NAC; and 50 mM magnesium acetate. These reagents were mixed to give a R1:R2 ratio of 4:1 and a final pH of 6.7.

In the γ-GT assay, the components in reagent R1 (pH 9.0) were: 100 mM diethanolamine-HCl buffer; 80 mM GlyGly; 20 mM

hydrazine sulphate; and 7.5 U ml^{-1} AlaDH. Reagent R2 (pH 5.5) comprised: 180 mM 3,3-dimethylglutaric acid–NaOH buffer; 60 mM γ-GluAla; 20 mM NAD^+; and 10 mM magnesium chloride. In this assay, hydrazine sulphate was shown to improve the linearity of the reaction. The ratio of R1:R2 was 4:1 and the final pH was 8.5 after mixing R1 and R2.

Reagent stability

Each reagent was stored in the refrigerator at 10°C. At appropriate intervals, the quantity of glucose, CK activity or γ-GT activity in a control sample was measured by the respective assays to confirm the linearity of the measurement. The concentrations of the main components, including the three coupling enzymes, were also monitored in each reagent.

Assay conditions for measurement of glucose, CK activity and γ-GT activity

All assays were carried out at 37°C with a Shimadzu UV-2200 Spectrophotometer. In the glucose assay, 6 μl of sample was added to 640 μl of R1 and incubated for 5 min; then 160 μl of R2 was added to start the reaction. The change in absorbance at 340 nm was measured after equilibrating for 5 min. The quantity of glucose (mg l^{-1}) was calculated by reference to the absorbance change at 340 nm of a standard glucose solution (2000 mg l^{-1}).

In the CK assay, 20 μl of sample was added to 800 μl of R1 and incubated for 5 min, followed by the addition of 200 μl of R2 to start the CK reaction. The change in absorbance at 340 nm was continuously monitored and the CK activity was calculated from the linear portion of the time curve appearing after the lag phase (this was typically less than about 2 min).

In the γ-GT assay, 40 μl of sample was added to 800 μl of R1 and incubated for 5 min; then 200 μl of R2 was added to start the γ-GT reaction. The change in absorbance at 340 nm was continuously monitored and the γ-GT activity was calculated from the linear portion of the time curve appearing after the lag phase (typically less than about 3 min). The CK or γ-GT activity was defined as the amount of enzyme that forms one μmol of NADPH or NADH at 37°C in 1 min.

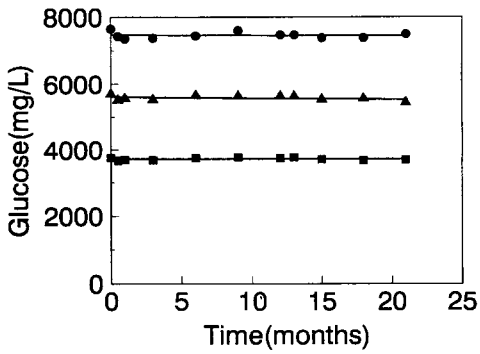

Figure 1
Glucose reagent stability in the liquid state at 10°C. ●, ▲ and ■ represent measured glucose quantities (mg l^{-1}) in the control serum samples containing higher (ca 8000 mg l^{-1}), medium (ca 6000 mg l^{-1}) and lower glucose levels (ca 4000 mg l^{-1}), respectively.

Results and Discussion

Glucose determination

The determination of glucose in clinical chemistry is a very important factor in the diagnosis of diabetes mellitus. Although the method using hexokinase (HK, EC 2.7.1.1) as the coupling enzyme is recommended as the reference method [9], the reagent is only stable for several days in the liquid state at room temperature. The thermostable GlcK has been reported for the successful determination of glucose. The reagent in liquid form was stable for at least one month at room temperature [2].

In the GlcK method, the stability of GlcK, G6PDH and $NADP^+$ in the reagent was insufficient to give a suitably stable reagent. It was found that these unstable components could be stabilized by dividing them into two reagents at an appropriate pH for each component. The stabilization of the reagent was achieved both by putting GlcK and G6PDH into R1 (at pH 9.0), and by putting $NADP^+$ into R2 (pH 4.0). The stability of the two separate reagents was examined under storage at 10°C shown in Fig. 1. The measured glucose levels in the control serum samples were found to be unchanged for about 21 months at each of three control glucose levels.

A linear relationship between the glucose concentration and the change in absorbance at 340 nm was also observed up to about 10 000 mg l^{-1} and no detectable increase in the reagent blank at 340 nm was observed over a 21-month period.

After 21 months the GlcK and G6PDH activities in solution R1 were more than 90% of their initial value; the $NADP^+$ quantity in

solution R2 was more than 85% of its initial value. This very high stability of the reagent solutions was considered to be attributable to the thermostable GlcK and the separation of the two reagents.

CK determination

The determination of serum CK activity is one of the important tests usually employed for the diagnosis of myocardial infarction or progressive muscular dystrophy. Although the HK-G6PDH method has been recognized as the most reliable method for measuring CK activity since the first report by Oliver [10], this method still has the problem that the enzymatic reagent is poorly stable in the liquid state, presumably due to the instability of HK. A stabilized reagent for measuring serum CK activity has been recently developed, based on thermostable GlcK instead of HK, and by adjusting the pH of the creatine phosphate solution to weakly alkaline [3].

In the GlcK method, the reagent was stable for only one week at room temperature. The stabilization of GlcK, G6PDH and $NADP^+$ was found to be very important for prolonging the reagent stability. Reagent R1 containing GlcK, G6PDH and creatine phosphate could be stabilized by adjusting its pH to 8.5; while R2 containing $NADP^+$ and NAC (an essential compound to activate serum CK activity) was stabilized by adjusting its pH to 3.5. As shown in Fig. 2, the measured CK activities in the serum sample could be fully recovered after about 13 months at 10°C, for each of three CK levels. A CK activity of about 4000 U l^{-1} was confirmed as the upper limit of linearity for CK

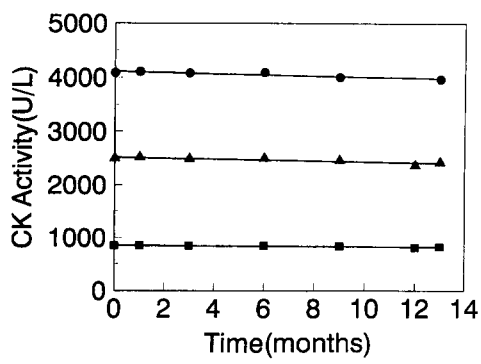

Figure 2
CK reagent stability in the liquid state at 10°C. ●, ▲ and ■ represent measured CK activities (U l^{-1}) in the control serum samples containing higher (ca 4000 U l^{-1}), medium (ca 2500 U l^{-1}) and lower CK activity levels (ca 1000 U l^{-1}), respectively.

measurement. In solution R1 the main components (GlcK, G6PDH and creatine phosphate) were sufficiently stable even after 13-months' storage: the quantities of GlcK, G6PDH and creatine phosphate recovered were more than 90, 75 and 95%, respectively. The quantities of NADP and NAC in solution R2 were also recovered to more than 85%.

Good correlation was confirmed between the present GlcK method and the established GlcK method: for 50 sera the linear regression equation was found to be: Y (the present GlcK method) = 0.987 X (the previous GlcK method) + 7.56 (correlation coefficient r = 0.9997). This high stability of these reagent solutions may be useful for the emergent analysis of CK activity in patients with acute myocardial infarction.

γ-GT determination

The assay of γ-GT is routinely employed in the diagnosis of hepatic diseases. A colorimetric method using the chromogenic substrate γ-glutamyl-3-carboxy-4-nitroanilide as the γ-glutamyl donor has been proposed for the routine assay of γ-GT by the International Federation of Clinical Chemistry [11], but there are still problems such as the interference by coloured substances [12], the relatively poor solubility and the poor stability of γ-glutamyl substrate. As an alternative, an ultraviolet spectrophotometric method for measuring γ-GT activity was reported using the thermostable AlaDH and the γ-glutamyl dipeptide, γ-Glu-Ala, which is more soluble than the chromogenic synthetic γ-glutamyl substrates [6]. The ultraviolet method has the advantage of being a stoichiometric assay and being little affected by interfering substances present in serum.

In this paper, the authors have further examined the stability of reagents used in the ultraviolet method by optimizing the reagent composition. Both the NAD^+ and γ-GluAla could be stabilized by separating these components in solution R2 and adjusting the pH to around 5.5. Under these conditions, it was more difficult for γ-GluAla to spontaneously hydrolyse than the synthetic γ-glutamyl substrates. The storage of the two separate reagents over a 40 day-period at 10°C did not affect the measured γ-GT activity or the reagent blank at 340 nm. The thermostable AlaDH activity in the R1 solution was recovered to more than 99% and the reagent

blank at 340 nm was not unchanged over the storage. However, further work will be necessary to enhance the stability of γ-GluAla to give a stable reagent for periods longer than 40 days.

Conclusions

The stabilized enzymatic reagents have been successfully developed based on thermostable enzymes derived from *B. stearothermophilus* and by splitting the reagents into two complementary solutions. The reagents for measuring glucose quantity and CK activity, especially, gave excellent stability for about 21- and 13-month periods, respectively, at 10°C. These stability dates are attributable to the use of thermostable GlcK and two separate complementary reagents. Although the reagent for measuring γ-GT activity was stable for only 40-days, further work continues in this laboratory to enhance the stability of this reagent.

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