〈Original Article〉

Establishment of a rapid and simple assay for measuring serum trehalase activity

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Summary Trehalase (TREH, trehalose-1-glucohydrolase, EC 3.2.1.28) catalyzes the hydrolysis of α,α-trehalose (1-α-$_D$-glucopyranosyl-α-$_D$-glucopyranoside) to form two molecules of glucose. TREH has been identified in the brush border membranes of the kidney, liver, and small intestine of mammals, but its physiological role has not yet been clarified. Some methods for measuring TREH activity have been reported, but they have been time-intensive, and its suitability for quantitative analysis could not be ascertained. Therefore, we developed a simple and rapid enzymatic assay for measuring serum TREH activity using a Hitachi 7600 type automated analyzer and evaluated the assay performance. The precision, linearity, detection limit, and recovery test were good. This new method effectively measures the serum TREH activity and can be easily integrated with an automated analyzer for rapid, high performance assays. This method can also support further research on understanding TREH activity.

Key words: Trehalase (TREH), Trehalose, Enzymatic assay, Automated analyzer

1. Introduction

Trehalase (TREH, trehalose-1-glucohydrolase, EC 3.2.1.28) catalyzes the hydrolysis of α,α-trehalose (1-α-$_D$-glucopyranosyl-α-$_D$-glucopyranoside) to form two molecules of glucose. This enzyme, first indentified by Bourquelet (1893) in *Aspergillus niger*, has been subsequently found in bacteria, fungi, yeast, insects, fish, higher plants, and

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mammals[1]. In insects, the high TREH activity has been observed in many organs, because they rely on the glucose derived from trehalose for energy[2]. However, considering that trehalose is not required for energy metabolism in mammals, what could be the reasons for the prevalence of TREH activity in the brush border membranes of their kidney, liver, and small intestine[2,3]? The physiological role of TREH in mammalian bodies has not yet been clarified.

Several relationships between blood TREH activity and diabetes have been reported so far. Eze *et al*.[4] found that TREH activity in plasma was higher in diabetic subjects than that in non-diabetic subjects. Baumann *et al*.[3] observed elevated serum TREH activity in both alloxan-induced and genetically (Ob/Ob, Db/Db) diabetic mice. Further, Muller *et al*.[5] reported that one of the genetic variants that determines human TREH activity is reproducibly associated with type 2 diabetes.

Some methods for measuring TREH activity have been reported[4,6]. For example, Eze *et al*.[4] reported a method that involves adding trehalose, buffer, *etc*. to 200 μL of plasma, reacting them at 37°C for 6 h, and calculating the TREH activity based on isolated glucose concentration. However, this method is sample and time-intensive, and its suitability for quantitative analysis could not be ascertained due to insufficient documentation about precision, linearity, and influence of co-existing substances. These drawbacks make it inappropriate for clinical use in the laboratory, and highlight the necessity of developing simpler, rapid, automated, and quantitative methods.

In this paper, we present a simple, rapid method for quantitatively analyzing serum TREH activity, which deploys an automated analyzer and is well-suited for routine clinical laboratory use.

2. Materials and Methods

2.1. Method Principle

As seen in Fig. 1, the reaction sequence of the present method consists of two steps: first, endogenous glucose is removed by mutarotase, glucose oxidase (GOD), and catalase. Second, glucose liberated from trehalose by TREH is detected as follows: GOD acts on glucose to produce H_2O_2, which then reacts with 4-aminoantipyrine (4-AA) and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline

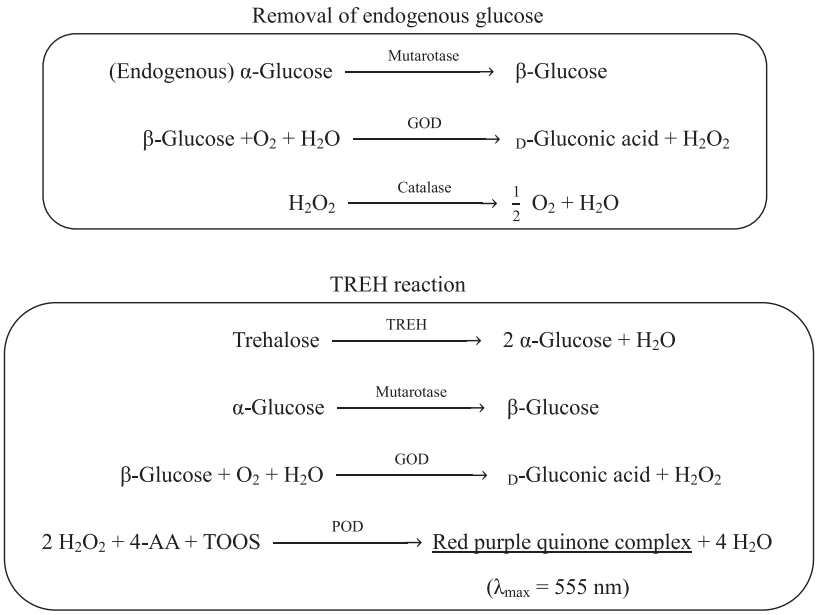

Fig. 1 Principles of the method for measuring serum TREH activity. First, endogenous glucose is removed by mutarotase, GOD, and catalase. Second, substrate trehalose is added and glucose liberated by TREH is detected by POD-catalyzed reaction.

(TOOS) in a peroxidase (POD)-catalyzed reaction to generate a quinoneimine derivative. The absorbance of this colored product is measured at 555 nm and is proportional to the serum TREH activity.

2.2. Instrumentation and reagents

Instruments. A Hitachi 7600 type automated analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) was used for the assay.

Reagent. TREH (EC 3.2.1.28, from porcine kidney) and glucose were obtained from Sigma-Aldrich Japan Co. (Tokyo, Japan). Mutarotase (EC 5.1.3.3, from pig kidney), trehalose, 4-AA, and NaN_3 were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). GOD (EC 1.1.3.4, from *Aspergillus* sp.), ascorbate oxidase (ASOD, EC 1.10.3.3, from *Cucurbita* sp.), POD (EC 1.11.1.7, from horseradish) were obtained from Toyobo Co., Ltd. (Osaka, Japan). Catalase (EC 1.11.1.6, from bovine liver) and NaOH were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TOOS, ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K), and 2-morpholinoethanesulfonic acid (MES) were obtained from Doujin Laboratories (Kumamoto, Japan). A two-reagent system was used in the assay. Reagent-1 (R-1) consisted of a 0.1 mol/L MES buffer (pH 5.7 at 25°C), 2.2 kU/L mutarotase, 200 kU/L GOD, 833 kU/L catalase, 1.67 kU/L ASOD, 12.5 kU/L POD, 16.7 μmol/L TOOS, and 1% (w/v) EDTA-2K. Reagent-2 (R-2) consisted of a 0.1 mol/L MES buffer (pH 5.7 at 25°C), 85.7 mmol/L pretreated trehalose, 714 μmol/L 4-AA, 714 μmol/L TOOS, 11.4 mmol/L NaN_3, amd 1% (w/v) EDTA-2K. The pretreated trehalose solution was obtained by mixing 171 mmol/L trehalose, 0.55 kU/L mutarotase, 50 kU/L GOD, 416 kU/L catalase and 0.1 mol/L MES buffer (pH 5.7 at 25°C), and reacting them at 37°C for 1 h, to remove glucose from the trehalose reagent. The TREH standard solution for the calibrator (25 U/L) was diluted with deionized water.

2.3. Procedure

Analytical conditions. The analytical conditions for the automated analyzer were as follows: 15 μL of the sample was mixed with 180 μL of R-1. After incubation for 1.5 min at 37°C, the reaction solution was stirred to supply oxygen. After incubation for 3.5 min at 37°C, 105 μL of R-2 was added. After another 5 min, the mixture was measured by a rate assay (23–30 points, 6.6–8.7 min) at 37°C, using wavelengths of 800/546 nm (sub/main wavelengths).

2.4. Statistical analysis

Statistical analyses were performed using the StatFlex Ver.6.0 software (Artech Co., Ltd.). The reference interval was determined by the non-parametric probability paper method. The gender difference in the healthy group was assessed with the Mann-Whitney U-test.

3. Results

3.1. Reaction time course

Figure 2 shows the reaction time course for some of the samples. After the addition of R-2, the absorbance in each case increased linearly.

3.2. Precision

Table 1 shows the within-run (n = 20) and between-day (n = 10) variations of data for the three different solutions. The within-run CVs were 0.60–2.36%, and the between-day CVs were 1.25–3.41%.

3.3 Linearity

Linearity was determined using the working solution of the 100 U/L TREH solution (10 different concentrations: 0–100 U/L TREH solution) as sample. As a result, the linearity was obtained in the range of 0–100 U/L and correlation between the measured activity (y) and the theoretical activity (x) was good ($y = 0.929x + 0.707$, $r = 0.999$) (Fig. 3).

3.4. Detection limit

We examined the detection limit by repeated assay of the zero concentration as sample: mean = 0.007 U/L, SD = 0.082 U/L (n = 10). The detection limit, defined as the mean concentration of the TREH zero sample + 3SD, was 2 U/L.

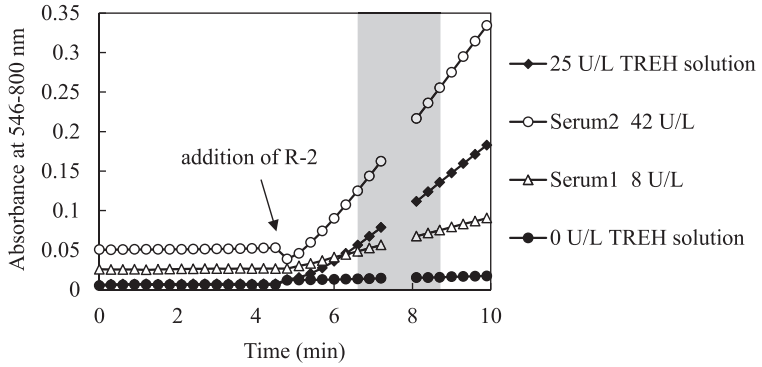

Fig. 2 Reaction time course with the TREH solutions and the serum samples.
Five minutes after adding R-2, the absorbance in each case increased linearly. The increase in absorbance per minute 23–30 point, 6.6–8.7 min (we showed it with a gray color) is obtained as the serum TREH activity. ◆, 25 U/L TREH solution; ●, 0 U/L TREH solution; ○, 42 U/L serum sample; △, 8 U/L serum sample. Note: As for 26, 27 point (7.5, 7.8 min), the absorbance is not measured in the performance of a Hitachi 7600 type automated analyzer.

Table 1 Precision of the present method

	Within-run (n = 20)			Between-day (n = 10)		
Sample	S1	S2	S3	S1	S2	S3
Mean (U/L)	5.32	20.68	48.82	4.57	25.26	44.55
SD (U/L)	0.13	0.23	0.29	0.16	0.32	0.69
CV (%)	2.36	1.12	0.60	3.41	1.25	1.54

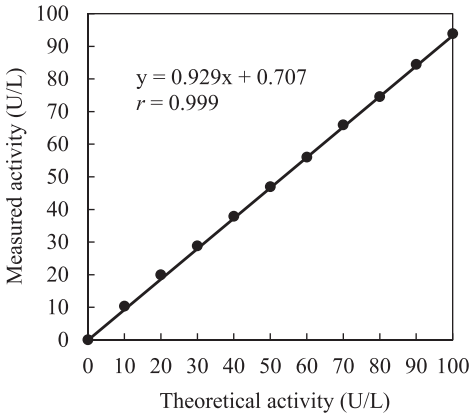

Fig. 3 Linearity.
The linearity was obtained in the range of 0–100 U/L and correlation between the measured activity (y) and the theoretical activity (x) was good (y = 0.929x + 0.707, r = 0.999).

3.5. Effect of endogenous glucose

The effect of endogenous glucose was examined using the 18 U/L TREH solutions containing 0, 5.6, 8.3, 11.1, and 13.9 mmol/L glucose as sample. We observed no effect up to a concentration of 11.1 mmol/L endogenous glucose.

3.6. Interferences

Various materials in serum, such as conjugated and unconjugated bilirubin, hemoglobin, and chyle might interfere with the assay. To study their interference, we used “The Interference Check-A Plus kit” (Sysmex Co., Kobe, Japan). These substances were added to the pooled serum (1:9 by volume), and the grade of interference was estimated as a

percentage of interference rate: relative activity (%) = TREH activity of the added interference substances/TREH activity in the reference sample (without interference materials) × 100. Figure 4 shows the results of the interferences. The interference rates for the samples containing 182 μmol/L conjugated bilirubin, 162 μmol/L unconjugated bilirubin, and 0.28 g/L hemoglobin were –9.42%, –9.78%, and –5.92% respectively. No effect was observed in the samples containing 1,410 formazin turbidity units (FTU).

3.7. Recovery test

Nine volumes of the pooled serum were mixed with one volume of 50, 100, and 200 U/L TREH solutions, in order to prepare samples at three different levels (approximately 5, 10, and 20 U/L) for the analytical recovery tests. The average analytical recoveries of triplicate assays were 103%, 96%, and 99% respectively.

3.8. Stability of TREH activity in serum

The stability of TREH activity in serum was examined using sera obtained from five healthy volunteers and thier pooled sera. Each serum was stored at 25°C, 4°C, and –80°C. The averages of the relative activity (%) of all sera are summarized in Fig. 5. We found the serum TREH activity to remain stable for up to 1 month at 25°C, 3 months at 4°C and more than 1 year at –80°C.

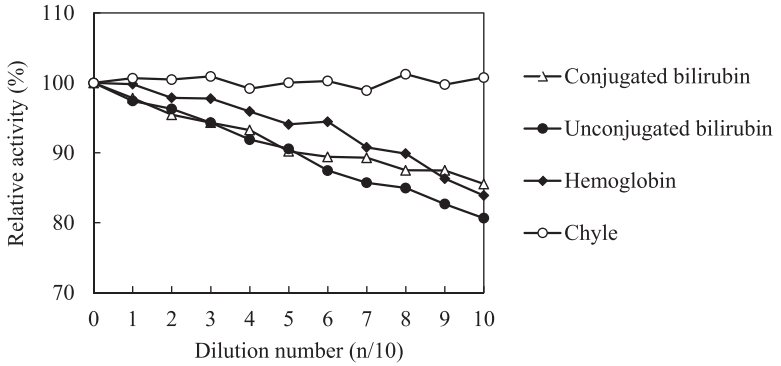

Fig. 4 Interferences.
Each level of maximum dilution number (10/10): Conjugated bilirubin (△); 364 μmol/L, Unconjugated bilirubin (●); 324 μmol/L, Hemoglobin (◆); 0.55 g/L, and Chyle (○); 1,410 FTU.

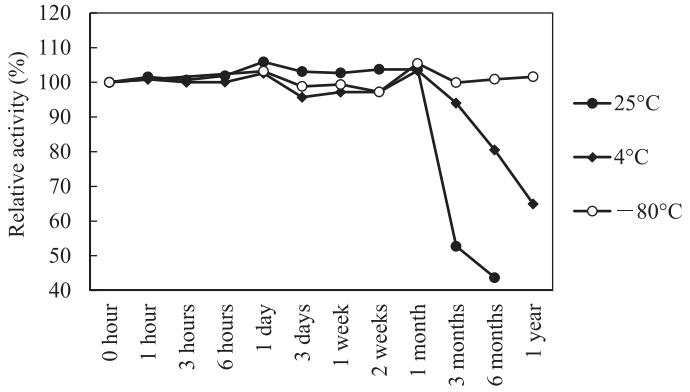

Fig. 5 Stability of serum TREH activity.
The sera obtained from five healthy volunteers and thier pooled sera were stored for 1 year at 25°C (●), 4°C (◆), and –80°C (○).

3.9. Reference interval

The reference interval was examined using 105 serum specimens (male:female = 48:57, aged 23.2 ± 4.2 (SD) years ranging from 20–45) collected from healthy volunteers. The reference interval for serum TREH activity was 2.8–26.1 U/L; the gender difference was not confirmed (P= 0.78).

4. Discussion

TREH catalyzes the hydrolysis of α,α-trehalose to form two molecules of glucose. In mammals, it has been identified in the brush border membranes of the kidney, liver, and small intestine[2,3]. TREH from the human kidney has an optimum pH of 5.7, *Km* value 3.0 mmol/L, isoelectric point of 4.8, and molecular weight of 97,000 by electrophoresis[7]. Methods for evaluating TREH activity have previously been reported[4,6], but are known to be sample and time-intensive; they are also unsuitable to develop simpler, automated, rapid, and quantitative methods for routine clinical use in the laboratory.

As described in the results, the reaction time course showed a linear increase and the photometric point was properly set. Within-run and between-day as indices of precision were good. In the recovery test, satisfactory recoveries of 96–103% were obtained. The limit of detection was 2 U/L. The linearity was shown up to 100 U/L. Moreover, our method has sufficient accuracy because correlation between the measured activity and the theoretical activity was good. These results confirm that our method has sufficient quantitative characteristics for specimen measurements. Our method for using trehalose as a substrate for measuring TREH activity mandates the removal endogenous glucose; the deployed procedure has a glucose removal ability of up to 11.1 mmol/L. We note that higher blood glucose concentrations will require measures such as specimen dilution. In the interference studies, we observed the presence of bilirubin and hemoglobin to result in slight negative errors, and recommend that specimens should not be measured at high concentrations of bilirubin and hemoglobin. TREH activity in serum remained quite stable, and was observed to be stable for 1 month even at 25°C. The reference interval was 2.8–26.1 U/L. However, we should examine it again because there was deflection in the age group.

In this paper, we have presented a simple, rapid method for accurately quantifying the serum TREH activity, which is suitable for routine clinical used in the laboratory. This method can also support further research on understanding TREH activity.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

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