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Novel glucose dehydrogenase from *Mucor prainii*: Purification, characterization, molecular cloning and gene expression in *Aspergillus sojae*

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Glucose dehydrogenase (GDH) is of interest for its potential applications in the field of glucose sensors. To improve the performance of glucose sensors, GDH is required to have strict substrate specificity. A novel flavin adenine dinucleotide (FAD)-dependent GDH was isolated from *Mucor prainii* NISL0103 and its enzymatic properties were characterized. This FAD-dependent GDH (MpGDH) exhibited high specificity toward glucose. High specificity for glucose was also observed even in the presence of saccharides such as maltose, galactose and xylose. The molecular masses of the glycoforms of GDH ranged from 90 to 130 kDa. After deglycosylation, a single 80 kDa band was observed. The gene encoding MpGDH was cloned and expressed in *Aspergillus sojae*. The apparent k_{cat} and K_m values of recombinant enzyme for glucose were found to be 749.7 s^{-1} and 28.3 mM, respectively. The results indicated that the characteristics of MpGDH were suitable for assaying blood glucose levels.

[**Key words:** Diabetes; Glucose dehydrogenase; Glucose sensor; Flavin adenine dinucleotide; Substrate specificity]

The self-monitoring of blood glucose at home is important for management of diabetes. There has been a striking evolution in glucose monitoring technology since the first blood glucose tests for self-monitoring were introduced around 1980 (1). The currently available glucose monitoring sensors are mainly based on electron-mediator-dependent glucose oxidoreductases.

Glucose oxidase (GOD) (EC 1.1.3.4) has been widely used as a mediated amperometric glucose sensor based on its high thermostability and high glucose selectivity (2). However, errors in glucose measurement often occur because of variations in the concentration of dioxygen (O_2) in the blood samples. To avoid this problem, glucose dehydrogenases have been used in glucose sensors: NAD-dependent glucose dehydrogenase (GDH), NADP-dependent GDH [NAD(P)-dependent GDH, EC 1.1.1.47] and pyrroloquinoline quinine (PQQ)-dependent GDH (EC 1.1.99.17) (3–11). NAD(P)-dependent GDH has strict substrate specificity, though NAD(P) cofactor must be supplied exogenously together with specific artificial electron mediators to carry out the electrochemical measurement because NAD(P) cofactor is not bound to the enzyme. PQQ-GDH has high catalytic activities for glucose and can use a variety of electron acceptors as redox mediators except for O_2 (12). However, PQQ-GDH has broad substrate specificity and thus, when using PQQ-GDH, blood glucose levels higher than the actual value are obtained when the specimen contains high maltose, icodextrin, galactose, or xylose (13). In a patient whose blood glucose level was measured using a simplified self-monitoring blood glucose sensor employing PQQ-GDH during administration of a maltose-containing infusion, hypoglycemia accompanied by a disruption in consciousness was experienced when the insulin dose was adjusted based on the measured value. The strict accuracy guidelines for blood glucose meters were published by the International Organization for Standardization (ISO) (14). Recently, Fraeyman et al. (15) have reported the successful production of a mutant strain of PQQ-GDH which shows no cross-reactivity with maltose.

Flavin adenine dinucleotide (FAD)-GDH was first discovered in 1951 in *Aspergillus oryzae* (16); however, a detailed characterization of GDH has not been completed. Since the use of FAD-GDH as an electrode catalyst in glucose sensors was published, GDHs from *Burkholderia cepacia* (17), *Aspergillus terreus* (18,19), *A. oryzae* (20) and *Glomerella cingulata* (21) have been studied. The advantage of employing FAD-GDH in glucose sensors is its strict substrate specificity toward glucose. Therefore, we tried screening for fungi to get novel FAD-GDH which had substrate specificity suited to glucose sensors.

In this study, we discovered a novel FAD-GDH from *Mucor prainii* NISL0103 (MpGDH). MpGDH showed high substrate specificity toward glucose. Notably, its reactivity for xylose was much lower than any other FAD-GDH. We also describe the cloning of the gene encoding the MpGDH and its recombinant expression in *Aspergillus sojae*.

MATERIALS AND METHODS

Strains and culture media *Escherichia coli* JM109 was used for the recombinant plasmid construction. The pUTE300K′ plasmid, which was derived from pBR322 DNA (Takara, Shiga, Japan), was used for the gene cloning (22). *A. sojae* BM-7

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(*pyrG*-, *niaD*-, *Δku70::niaD*) (23) was used as the host strain for the recombinant expression of MpGDH. Malt extract medium consisting of 2.0% malt extract, 2.0% D-glucose and 0.1% polypeptone (pH 6.0) was used for screening. Yeast glucose (YD) medium containing 2.0% yeast extract and 4.0% D-glucose (pH 6.0) was used for the cultivation of *M. prainii*. The YDS medium for the cultivation of recombinant *A. sojae* was comprised 0.2% yeast extract, 2% D-glucose, 0.5% soypeptone, 0.1% KH_2PO_4 and 0.050% $MgSO_4$ $7H_2O$ (pH 6.0).

Enzyme activity and protein assays Glucose dehydrogenase activity was assayed in 90 mM phosphate buffer (pH 7.0), containing 0.1 mM 2,6-dichlorophenol indophenol dehydrate (DCIP) and 0.2 mM phenazine methosulfate (PMS) at 37°C in the presence of 50 mM glucose or various concentrations of substrates. The activity was calculated by monitoring the decrease in absorbance of DCIP at 600 nm using a Hitachi U-3010 spectrophotometer (Hitachi High-Tech Fielding, Tokyo, Japan). One unit of enzymatic activity was defined as the amount of enzyme that caused the reduction of 1 μmol of DCIP per minute under the assay conditions. The extinction coefficient of 20.4 $cm^2/\mu mol$ and 18.3 $cm^2/\mu mol$ were used for the calculation in phosphate buffer (pH 7.0) and phosphate buffer (pH 6.5), respectively. The glucose oxidase activity assay in 80 mM phosphate buffer (pH 7.0), 135 mM glucose, 0.2 mM 4-aminoantipyrine (4-AA), 0.3 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) and 4 U/ml peroxidase (Kikkoman Biochemifa, Chiba, Japan) at 37°C. The activity was calculated by monitoring the increase in absorbance at 555 nm using a Hitachi U-3010 spectrophotometer (Hitachi High-Tech Fielding). One unit of enzymatic activity was defined as the amount of enzyme that caused a reduction of 1 μmol of H_2O_2 per minute under the above assay conditions. An extinction coefficient of 39.2 $cm^2/\mu mol$ was used for the calculation. The protein concentration was determined using the absorbance at 280 nm. The absorbance of a protein at 280 nm depends on the content of tryptophan, tyrosine and cystine (24). After cloning of MpGDH, it was revealed MpGDH contained 9 of tryptophan and 31 of tyrosine. The molar absorption coefficient, ε, of MpGDH was calculated and the protein concentration was recalculated using the absorbance at 280 nm and ε.

Screening for fungi producing glucose dehydrogenase Fungi isolated from soils or stock cultures collections were grown at 30°C in 3 ml of malt extract medium with shaking for several days. The mycelia were harvested and washed with 10 mM acetate buffer (pH 5.0). Subsequently, the washed mycelia were resuspended in 1 ml of 10 mM acetate buffer (pH 5.0) and disrupted using glass beads. Then insoluble extracts were removed by centrifugation (2000 ×*g*). The cell extracts were assayed for glucose dehydrogenase activity.

Preparation of purified MpGDH *M. prainii* NISL0103 was cultured in 40 L of YD medium with shaking at 30°C for 4 days. The mycelia were harvested and washed with 10 mM acetate buffer (pH 5.0), and disrupted using glass beads (diameter 0.75 mm) with a Dyno-Mill (Shinmaru Enterprises, Osaka, Japan). Then the insoluble portions were removed by centrifugation (6000 ×*g*, 30 min). The resulting extract was concentrated and filtered in a hollow fiber crossflow module (AIP2013, 6 kDa cut-off, Asahi Kasei Chemicals) to remove low-molecular-mass components. Solid ammonium sulfate was added to the enzyme solution to a final concentration of 70%. The solution was stored at 4°C overnight and the precipitate was removed by centrifugation at 20,000 ×*g*. The resulting supernatant was applied to a hydrophobic interaction column, Toyopearl-Butyl 650C (Tosoh, Tokyo, Japan), which was equilibrated with 10 mM acetate buffer (pH 5.0) containing 2 M ammonium sulfate. Proteins were eluted within a liner gradient from 2 M to 0 M ammonium sulfate in 10 column volumes. Fractions containing GDH activity were pooled and concentrated using a Centricon Plus-70 (Merck Millipore, Billerica, MA, USA). The concentrated enzyme solution was loaded onto a cation exchange column, SP-Sepharose FF (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), previously equilibrated with 10 mM acetate buffer (pH 4.5). Proteins were eluted within a linear salt gradient from 0 mM to 200 mM of KCl in 20 column volumes. The elution was dialyzed against 10 mM acetate buffer (pH 5.0) using a Centricon Plus-70.

The purified native MpGDH was subjected to SDS-PAGE (SuperSep Ace 10–20%, Wako Pure Chemical Industries, Osaka, Japan). A broad band of MpGDH ranging between 90 and 130 kDa was excised and then internal sequencing was performed by APRO Life Science Institute Inc. MpGDH was deglycosylated using the Enzymatic Deglycosylation Kit (Prozyme, Hayward, CA, USA) according to the manufacturer's instructions.

Measurement of enzyme stability Purified MpGDH was diluted to 80 U/ml using the proper buffer, and enzyme stability was determined by measuring the residual activity after incubation under the conditions described below. For measurement of pH stability, MpGDH was diluted in various buffers having different pH values between 2.5 and 9.0, and incubated at 25°C for 16 h. For measurement of thermal stability, MpGDH was diluted with 100 mM potassium acetate buffer (pH 5.0) and incubated for 15 min between 25°C and 60°C. To investigate the effect of some compounds on MpGDH stability, MpGDH was diluted in 100 mM potassium phosphate buffer (pH 7.0) with each compound at a final concentration of 50 mM and incubated at 40°C for 15 min. Prior to this test, some compounds were previously adjusted to pH 7.0 with NaOH as appropriate.

Cross-reactivity test To investigate the effect of other saccharides on MpGDH activity, GDH activity was assayed in the presence of 100 mg/dl glucose and other saccharides: maltose, galactose, xylose or all of them. The final concentrations of maltose were between 120 mg/dl and 600 mg/dl. The final concentrations of galactose were between 60 mg/dl and 300 mg/dl. The final concentrations of xylose were between 40 mg/dl and 200 mg/dl.

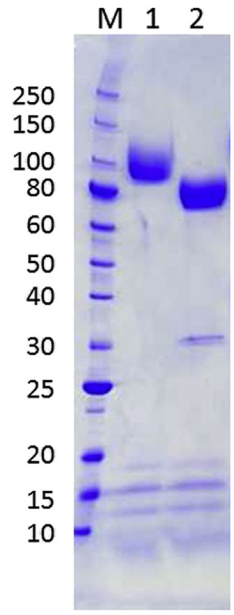

FIG. 1. SDS-PAGE of native MpGDH. Lane 1, MpGDH; lane 2, MpGDH treated by the Enzymatic Deglycosylation Kit; lane M, molecular mass markers (Molecular Weight Standards Low Range, Bio-Rad).

Cloning and sequencing of the GDH gene from *M. prainii* NISL0103 Total RNA was extracted from disrupted mycelia using the Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Degenerate RT-PCR was performed using the Prime Script RT-PCR Kit (Takara) according to the manufacturer's instructions. The Oligo dT primer from the kit was used for the synthesis of the 1st strand DNA, and the degenerate primers based on the partial amino acids sequences were used for PCR. The PCR products (approximately 800 bp) were sequenced with a CEQ200XL DNA Analysis System (Beckman Coulter, Brea, CA, USA). To obtain the complete open reading frame (ORF), 5′ and 3′ rapid amplification of the cDNA ends (RACE) was performed using the 3′-Full Race Core Set (Takara) and 5′-Full RACE Core Set (Takara), respectively, according to the manufacturer's instructions. Primers for the 3′-RACE and 5′-RACE were designed from the DNA sequences obtained from degenerate RT-PCR products. The full-length *mpgdh* was amplified using Prime Script RT-PCR Kit with the total RNA as a template and the MpGdh-full_F and MpGdh-full_R primers with a *NdeI* site. The PCR products were digested with *NdeI* and inserted into the pUTE300K′ vector to yield p300K′-Mpgdh.

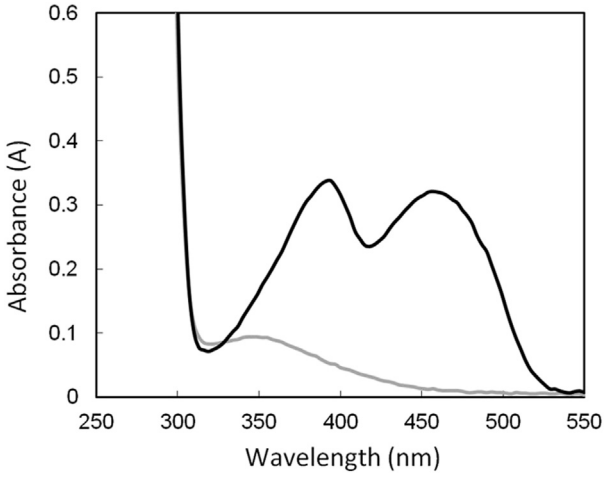

FIG. 2. Characterization of spectra MpGDH showing both the oxidized (black) and reduced (gray) forms of the enzyme. To reduce the enzyme, glucose was used.