

Identification and characterization of thermostable glucose dehydrogenases from thermophilic filamentous fungi

Kazumichi Ozawa, Hisanori Iwasa, Noriko Sasaki, Nao Kinoshita, Atsunori Hiratsuka & Kenji Yokoyama

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Identification and characterization of thermostable glucose dehydrogenases from thermophilic filamentous fungi

Kazumichi Ozawa¹ · Hisanori Iwasa¹ · Noriko Sasaki¹ · Nao Kinoshita¹ ·
Atsunori Hiratsuka¹ · Kenji Yokoyama^{1,2}

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Abstract FAD-dependent glucose dehydrogenase (FAD-GDH), which contains FAD as a cofactor, catalyzes the oxidation of D-glucose to D-glucono-1,5-lactone, and plays an important role in biosensors measuring blood glucose levels. In order to obtain a novel FAD-GDH gene homolog, we performed degenerate PCR screening of genomic DNAs from 17 species of thermophilic filamentous fungi. Two FAD-GDH gene homologs were identified and cloned from *Talaromyces emersonii* NBRC 31232 and *Thermoascus crustaceus* NBRC 9129. We then prepared the recombinant enzymes produced by *Escherichia coli* and *Pichia pastoris*. Absorption spectra and enzymatic assays revealed that the resulting enzymes contained oxidized FAD as a cofactor and exhibited glucose dehydrogenase activity. The transition midpoint temperatures (T_m) were 66.4 and 62.5 °C for glycosylated FAD-GDHs of *T. emersonii* and *T. crustaceus* prepared by using *P. pastoris* as a host, respectively. Therefore, both FAD-GDHs exhibited high thermostability. In conclusion, we propose that these thermostable FAD-GDHs could be ideal enzymes for use as thermotolerant glucose sensors with high accuracy.

Keywords FAD-dependent glucose dehydrogenase · Gene cloning · Recombinant protein production · Thermostability

Introduction

Self-monitoring of blood glucose (SMBG) is a key clinical test for diabetes patients who need to know their blood glucose levels. Traditional glucose sensors used for the clinical test are equipped with glucose oxidase (GOD), NAD(P)-dependent glucose dehydrogenase (NAD(P)-GDH), or pyrroloquinoline quinone (PQQ)-dependent GDH (PQQ-GDH).

GOD (EC 1.1.3.4), NAD(P)-GDH (EC 1.1.1.47), and PQQ-GDH (EC 1.1.5.2) are oxidoreductases that catalyze the oxidation of D-glucose to D-glucono-1,5-lactone. Use of these enzymes for glucose sensors has the following limitations: (1) GOD requires oxygen as an electron acceptor, and dissolved oxygen in the blood influences the accuracy of measurements for glucose concentration; (2) NAD(P)-GDH requires continuous coenzyme addition during measurements; and (3) PQQ-GDH exhibits broad substrate specificity (Ferri et al. 2011; Heller and Feldman 2008; Yoo and Lee 2010).

Recently, FAD-dependent GDH (FAD-GDH; EC 1.1.5.9), which contains FAD as a cofactor, has become a focus of research related to glucose sensing. FAD-GDH exhibits strict substrate specificity, and glucose sensors employing the enzyme are insensitive to dissolved oxygen and require no cofactor addition (Ferri et al. 2011; Tsujimura et al. 2006). Several studies on FAD-GDHs from filamentous fungi, including *Aspergillus oryzae* (Bak 1967a, b; Ogura 1951), *Aspergillus terreus* (Tsujimura et al. 2006; Yang et al. 2014, 2015), *Aspergillus flavus* (Mori et al. 2011; Yoshida et al. 2015), *Aspergillus niger* (Mori et al. 2011), *Glomerella cingulata* (Sygmund et al. 2011a, 2011b), *Mucor prainii*

Kazumichi Ozawa and Hisanori Iwasa contributed equally to this work.

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✉ Kenji Yokoyama
yokoyamakj@stf.teu.ac.jp

¹ Nanomaterials Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan

² School of Bioscience and Biotechnology, Tokyo University of Technology, 1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan

(Satake et al. 2015), and *Pycnoporus cinnabarinus* (Piumi et al. 2014) have been published. These FAD-GDHs have suitable enzymatic properties for the biosensors of SMBG. Preferably, more thermostable FAD-GDH is required for industrial application. Thermophilic filamentous fungi are capable of growing at temperatures between 20 and 50 °C, or above 50 °C (Maheshwari et al. 2000), and many thermostable enzymes from these fungi were characterized (Berka et al. 2011; Maheshwari et al. 2000). All previous FAD-GDHs have originated from filamentous fungi that favor and grow at ordinary temperatures, while thermophilic filamentous fungal FAD-GDHs remain unidentified.

In order to obtain a novel FAD-GDH gene homolog, we performed degenerate PCR screening of genomic DNAs from thermophilic filamentous fungi. As a result, novel FAD-GDH gene homologs were identified from the genomic DNAs of *Talaromyces emersonii* NBRC 31232, *Thermoascus crustaceus* NBRC 9129, and *T. crustaceus* NBRC 9816. Furthermore, we successfully cloned and expressed the FAD-GDH gene homologs from *T. emersonii* NBRC 31232 and *T. crustaceus* NBRC 9129 to prepare recombinant enzymes by using *Escherichia coli* and *Pichia pastoris* as hosts, and then characterized the properties of the resulting enzymes. The results indicated that the novel FAD-GDHs from the thermophilic filamentous fungi exhibited high thermostability.

Materials and methods

Strains and culture media

Thermophilic filamentous fungi used in this study are listed in Table S1. The fungal strains were purchased from the Biological Resource Center at the National Institute of Technology and Evaluation (NBRC; Chiba, Japan) and cultured in malt extract medium or potato dextrose medium as directed by NBRC. *E. coli* strains DH5 α (Takara Bio, Shiga, Japan) and BLR(DE3) (Merck Millipore, Darmstadt, Germany) were cultured in Luria-Bertani (LB) medium (Sambrook and Russell 2001). Methylotrophic yeast *P. pastoris* strain GS115 (Life Technologies, Carlsbad, CA, USA) was cultured in media (yeast extract peptone dextrose medium, minimal dextrose medium, buffered minimal glycerol medium, and buffered minimal methanol medium) with a *Pichia* expression kit (Life Technologies) according to manufacturer instructions.

DNA manipulation

All DNA experiments followed standard protocols (Sambrook and Russell 2001). Genomic DNA was extracted with a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Thermostable DNA polymerases and oligonucleotide primers

(Table S2) were purchased from Toyobo (Osaka, Japan) and Life Technologies, respectively. GeneAmp PCR System 9700 (Life Technologies) was used for PCR. Each DNA fragment amplified by PCR was subcloned into a pCR-Blunt II-TOPO vector (Life Technologies), and its nucleotide sequence was determined using an Applied Biosystems 3130xl genetic analyzer (Life Technologies). Restriction and modification enzymes were purchased from New England BioLabs (Ipswich, MA, USA) or Takara Bio. Southern hybridization analysis was performed according to a standard protocol (Sambrook and Russell 2001) with a DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland).

Degenerate PCR screening for FAD-GDH gene homologs

Seventeen species of thermophilic filamentous fungi were screened for FAD-GDH gene homologs by degenerate PCR using the individual fungal genomic DNA as a template. Three sense degenerate primers (HCA, HCB, and HCC) and two antisense degenerate primers (HCD and HCE) were designed based on the primary structures of *A. oryzae* and *A. terreus* FAD-GDHs (28-YDYIVVGGG-36, 100-QVLRAGKALG-109, 120-TRAEDVQI-127, 525-NFHPVGTAAAMM-535, and 565-FQVCGHLVST-574, where the numbers correspond to the amino acid sequence of *A. oryzae* FAD-GDH). The PCR mixture (50 μ l) contained 1 U KOD FX (Toyobo), PCR buffer for KOD FX, 0.3 μ M sense primer, 0.3 μ M antisense primer, dNTP mixture (0.4 mM each), and the individual fungal genomic DNA as the template. Each reaction consisted of 30 cycles of the following steps: denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 2 min. The amplified DNA fragments in the PCR products were separated by agarose gel electrophoresis and then extracted from preparative agarose gels. Each extracted DNA fragment was subcloned and sequenced. The predicted amino acid sequences were compared to those of *A. oryzae* and *A. terreus* FAD-GDHs.

Cloning of FAD-GDH gene homologs

Prior to gene cloning, the genomic DNA from *T. emersonii* or *T. crustaceus* was digested by various restriction enzymes, and analyzed by Southern hybridization. The DNA fragment amplified by degenerate PCR, representing a portion of the FAD-GDH gene homolog, was used as a probe. As a result, a single band hybridized with the probe was detected at ~4.0 kb in the lane of *Eco*RI-digested *T. emersonii* genomic DNA, or at ~5.0 kb in the lane of *Hind*III-digested *Th. crustaceus* genomic DNA.

Inverse PCR was performed to clone the upstream region of the start codon and the downstream region of the stop codon of the FAD-GDH gene homolog. The *Eco*RI-digested

T. emersonii genomic DNA or the *Hind*III-digested *T. crustaceus* genomic DNA was subjected to preparative agarose gel electrophoresis, and the DNA fragments around 4.0 or 5.0 kb were extracted from the gels. These DNA fragments were self-ligated with T4 DNA ligase and used as the template for inverse PCR. The primers for inverse PCR, GSP3, and GSP5, were designed based on the nucleotide sequence of the internal FAD-GDH gene homolog from *T. emersonii* or *T. crustaceus*. The PCR mixture (50 μ l) contained 1 U KOD-Plus-Neo (Toyobo), PCR buffer for KOD-Plus-Neo, 0.2 μ M sense primer, 0.2 μ M antisense primer, dNTP mixture (0.2 mM each), 1.5 mM magnesium sulfate, and the circularized DNA as the template. Each reaction consisted of 30 cycles of the following steps: denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 3 min.

The genomic DNA fragment containing the FAD-GDH gene homolog was amplified by PCR using KOD-Plus-Neo, sense primer GSP9, antisense primer GSP10, and either *T. emersonii* or *T. crustaceus* genomic DNA. Primers GSP9 and GSP10 were designed based on the nucleotide sequence of the inverse PCR-amplified DNA fragment.

Genetic analysis

Exon-intron splicing sites were predicted by the GT-AG rule (Breathnach et al. 1978; Catterall et al. 1978). Secretion signal sequence was predicted by SignalP 4.0 (Petersen et al. 2011), and *N*-glycosylation sites were predicted by NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>). The amino acid sequences of the FAD-GDHs were aligned using ClustalW (<http://clustalw.ddbj.nig.ac.jp/>) (Thompson et al. 1994).

Synthesis of the coding sequence

The coding sequence (CDS) of the FAD-GDH gene homolog from *T. emersonii* or *T. crustaceus* was artificially synthesized by overlap extension PCR (Horton et al. 1989). Four exons were individually amplified and connected to synthesize the complete structural gene. The primers used were as follows: GSP11 and E1A for exon 1, E2S and E2A for exon 2, E3S and E3A for exon 3, and E4S and GSP12 for exon 4.

Preparation of recombinant enzymes produced by *E. coli*

The gene encoding the mature FAD-GDH was generated by PCR using primers Esc2 and Esc4 to construct an *E. coli* expression plasmid. Primers Esc2 and Esc4 contained *Nde*I site, and *Hind*III site and six CAC codons encoding a histidine tag located at the C-terminus, respectively. The amplified DNA fragment was inserted between the *Nde*I and *Hind*III sites in the *E. coli* pET-21b(+) expression vector (Merck Millipore).

E. coli BLR(DE3) cells harboring the expression plasmid were cultured in LB medium containing 100 μ g/ml ampicillin at 37 °C for approximately 4 h, and isopropyl- β -D-thiogalactopyranoside was added into the culture medium (1 mM final concentration) to induce the gene expression. The cells were further cultured at 25 °C overnight.

Cells were collected and disrupted by sonication in PBS [137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, and 2 mM potassium dihydrogen phosphate (pH 7.4)]. The extract was centrifuged at 8000g for 10 min at 4 °C to remove unbroken cells, and further centrifuged at 12,000g for 20 min at 4 °C to obtain the soluble fraction. The soluble fraction was applied to a Ni-NTA agarose column (QIAGEN, Hilden, Germany) equilibrated with PBS. The adsorbed proteins were eluted using 20 mM HEPES buffer (pH 7.5) containing 100 mM imidazole. The eluate was applied to a Q Sepharose Fast Flow column (GE Healthcare, Little Chalfont, UK) equilibrated with 20 mM HEPES buffer (pH 7.5). The adsorbed proteins were eluted using 20 mM HEPES buffer (pH 7.5) containing 100 mM sodium chloride. The eluate was desalted with an Amicon Ultra-15 10 K centrifugal filter device (Merck Millipore) and applied to a RESOURCE Q (1 ml) column (GE Healthcare) equilibrated with 20 mM HEPES buffer (pH 7.5). The adsorbed proteins were eluted by a linear gradient of 0–1.0 M sodium chloride in 20 mM HEPES buffer (pH 7.5). The fractions containing recombinant FAD-GDH were desalted and stored at 4 °C until use.

Preparation of recombinant enzymes produced by *P. pastoris*

The gene encoding mature FAD-GDH was generated by PCR using primers Pic1 and Pic2 to construct a *P. pastoris* expression plasmid. Primers Pic1 and Pic2 contained *Eco*RI site, and *Not*I site and six CAC codons encoding a histidine tag located at the C-terminus, respectively. Before PCR amplification, the structural gene was modified by silent mutagenesis to synthesize the artificial gene lacking *Sal*I sites. The amplified DNA fragment was inserted between the *Eco*RI and *Not*I sites in the *P. pastoris* pPIC9 expression vector (Life Technologies). By inserting the DNA fragment into the vector, the yeast secretion signal peptide was fused to the mature FAD-GDH. The resulting plasmid was cleaved with *Sal*I, and the linearized DNA was introduced into *P. pastoris* GS115 genomic DNA at the *his4* locus by electroporation.

The recombinant strain was cultured in buffered minimal methanol medium at 30 °C for 5 days. Methanol was added into the culture medium every 24 h (0.5 % final concentration).

Culture supernatant was dialyzed against PBS, and applied to a Ni-NTA agarose column equilibrated with PBS. The adsorbed proteins were eluted using 20 mM HEPES buffer

(pH 7.5) containing 200 mM imidazole. The eluate was desalted and stored at 4 °C until use.

Electrophoresis

SDS-PAGE was performed by the method of Laemmli (Laemmli 1970). Proteins were stained with Coomassie brilliant blue (CBB) G-250 (CBB Stain One; Nacalai Tesque, Kyoto, Japan), and glycosylated proteins were detected by periodic acid-Schiff (PAS) staining with a Pierce glycoprotein staining kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was estimated with a Pierce BCA protein assay kit (Thermo Fisher Scientific) (Smith et al. 1985). Bovine serum albumin was used as a standard. Purity of the purified proteins was evaluated with ImageJ (<http://imagej.nih.gov/ij/>) (Abramoff et al. 2004).

Absorption spectrum

The absorption spectrum of purified recombinant enzyme (2 mg/ml) was measured with a DU-800 UV/visible spectrophotometer (Beckman Coulter, Brea, CA, USA) using a micro cell (1-cm path length). The molar absorption coefficient of FAD at 450 nm ($11.3 \text{ mM}^{-1} \text{ cm}^{-1}$) (Macheroux 1999) was used to calculate the concentration of FAD in the enzyme.

GDH assay

GDH activity was measured by the 2,6-dichloroindophenol (DCIP) method. The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.14 mM 1-methoxy-5-methylphenazinium methylsulfate (1-mPMS; Dojindo, Kumamoto, Japan), 0.07 mM DCIP (2,6-dichlorophenol-indophenol sodium salt dihydrate; Merck Millipore), 0.2 % Triton X-100, and 300 mM glucose. The enzymatic reaction was initiated by the addition of purified recombinant enzyme (0.1 ml). The absorbance of oxidized DCIP at 600 nm was monitored with a UV-1700 Pharma Spec UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). One unit was defined as the amount of FAD-GDH producing 1 μmol of reduced DCIP per minute at pH 7.0 and 37 °C. The molar absorption coefficient of oxidized DCIP at 600 nm ($16.3 \text{ mM}^{-1} \text{ cm}^{-1}$) was used to determine the enzyme activity. Specific activity was shown as units per 1 μmol of holoenzyme (U/ μmol).

Assay of oxygen-reducing activity

Oxygen-reducing activity was measured by the method of Swoboda and Massey (1965). *A. niger* GOD (Wako Pure Chemical Industries, Osaka, Japan) was used as a positive control.

Accession numbers

The nucleotide sequences of the FAD-GDH genes have been deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC069047 and HW503402 for *T. emersonii* NBRC 31232 and LC069048 and HW503403 for *T. crustaceus* NBRC 9129.

Results

Screening for FAD-GDH gene homologs

We performed degenerate PCR screening of genomic DNAs from 17 species of thermophilic filamentous fungi to identify novel FAD-GDH gene homologs. Genomic DNA extracted from each thermophilic filamentous fungus was used as the template for the degenerate PCR, and results of agarose gel electrophoresis of the amplified DNA fragments are shown in Fig. S1. DNA bands of ~1.3–2.0 kb were detected from the PCR products derived from the genomic DNAs of *T. emersonii* NBRC 31232, *T. crustaceus* NBRC 9129, and *T. crustaceus* NBRC 9816. These DNA fragments were successfully amplified by all primer pairs for *T. emersonii* NBRC 31232 (Fig. S1a), in contrast to only two primer pairs successfully amplified for both *T. crustaceus* strains (Fig. S1b, c). Nucleotide sequencing of these DNA fragments revealed that the predicted amino acid sequences were homologous to those of *A. oryzae* and *A. terreus* FAD-GDHs. These results indicated that the genomic DNA of *T. emersonii* NBRC 31232 and the two *T. crustaceus* strains contained FAD-GDH gene homologs. Furthermore, the nucleotide sequences of the two *T. crustaceus* FAD-GDH gene homologs were identical each other.

Cloning of FAD-GDH gene homologs

The genomic DNA fragments containing the FAD-GDH gene homologs were cloned from *T. emersonii* NBRC 31232 and *T. crustaceus* NBRC 9129. Nucleotide sequencing revealed that these fragments contained open reading frames of the FAD-GDH gene homologs, and that the structural genes were divided into four exons by three introns. The putative FAD-GDH gene homologs from *T. emersonii* and *T. crustaceus* contained 1782-bp encoding 593 amino acids and 1755-bp encoding 584 amino acids, respectively.

The secretion signal peptides were identified as 17 residues from Met1 to Ala17 in *T. emersonii* FAD-GDH, and 16 residues from Met1 to Ala16 in *T. crustaceus* FAD-GDH. The amino acid sequences implicated in FAD-binding (Gly-X-Gly-X-X-Gly, where X represents any amino acid residue) (Dym and Eisenberg 2001) were conserved in the FAD-GDHs of both strains. The 12 and 11 asparagine residues that

undergo *N*-glycosylation (Asn-*X*-Ser/Thr, where *X* represents any residue, except for proline) (Tschopp et al. 1987) were predicted in *T. emersonii* FAD-GDH and *T. crustaceus* FAD-GDH, respectively. The alignment of the amino acid sequences of the FAD-GDHs from *T. emersonii*, *T. crustaceus*, *A. oryzae*, and *A. terreus* is shown in Fig. S2. *T. emersonii* FAD-GDH showed 63 % identity with both *A. oryzae* and *A. terreus* FAD-GDHs, whereas *T. crustaceus* FAD-GDH showed 54 and 56 % identity with *A. oryzae* and *A. terreus* FAD-GDHs, respectively. Additionally, *T. emersonii* FAD-GDH and *T. crustaceus* FAD-GDH showed 62 % identity with each other.

Preparation of recombinant FAD-GDHs

Talaromyces emersonii mature FAD-GDH (TeGDH) and *T. crustaceus* mature FAD-GDH (TcGDH) were successfully produced in the soluble fractions of *E. coli*, and then purified (>95 % purity). The yields per 1 l of *E. coli* culture media were 30 mg for TeGDH and 5 mg for TcGDH. Protein bands denoting the purified recombinant enzymes were detected by SDS-PAGE at ~60 kDa for TeGDH and ~58 kDa for TcGDH, which were consistent with the theoretical molecular masses of both enzymes (Fig. 1a). The theoretical molecular masses of these enzymes with their respective histidine tags were calculated at ~63 kDa for TeGDH and ~62 kDa for TcGDH. When produced by *P. pastoris*, both enzymes were effectively secreted into the culture media, and then purified (>95 % purity). The yields per 1 l of *P. pastoris* culture media were 46 mg for TeGDH and 39 mg for TcGDH. Protein bands denoting the purified recombinant enzymes were detected at 90–200 kDa for TeGDH, and 80–200 kDa for TcGDH by CBB staining (Fig. 1b, left). These enzymes were also stained with PAS, indicating that they had undergone glycosylation during post-translational modification in *P. pastoris* (Fig. 1b, right). Deglycosylation under the denaturing condition with PNGase F exhibited decreased molecular masses, which were

~65 kDa for TeGDH and ~63 kDa for TcGDH (Fig. S3). The results revealed that the glycosylated enzymes were modified by *N*-linked sugar chains as major components of glycosylation. The molecular masses of glycosylated enzymes would be overestimated by SDS-PAGE. Glycosylated proteins are known to have decreased charge-mass ratios when complexed to SDS, resulting in decreased migration rates and overestimated molecular masses (Werner et al. 1993).

The solutions of the purified TeGDHs and TcGDHs were yellow. Absorption spectra of the enzymes produced by *E. coli* (unglycosylated FAD-GDH) (Fig. 2a) and *P. pastoris* (glycosylated FAD-GDH) (Fig. 2b) were similar to the typical spectrum observed for oxidized flavin with major peaks at 380 and 450 nm. The peaks corresponding to oxidized flavin disappeared when glucose was added, and also when sodium dithionite was added as a reductant. These results suggested that TeGDHs and TcGDHs produced by *E. coli* and *P. pastoris* were purified as holo forms containing oxidized FAD as a cofactor.

GDH activity

GDH activity at pH 7.0 and 37 °C was measured. The specific activities of unglycosylated and glycosylated TeGDHs were $(9.21 \pm 0.24) \times 10^3$ U/μmol and $(8.79 \pm 0.12) \times 10^3$ U/μmol, respectively, whereas those of unglycosylated and glycosylated TcGDHs were $(16.0 \pm 1.2) \times 10^3$ U/μmol and $(13.5 \pm 0.9) \times 10^3$ U/μmol, respectively. The specific activities of TcGDHs were slightly higher than those of TeGDHs.

The activities of TeGDHs and TcGDHs depended on glucose concentration (Fig. 3a, b). The maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) were calculated by the Michaelis-Menten curve-fitting with the least square method. The V_{max} values of unglycosylated FAD-GDHs were higher than those observed for glycosylated FAD-GDHs. The K_m values were calculated at $(4.06 \pm 0.40) \times 10^2$ mM for unglycosylated TeGDH and $(3.37 \pm 0.31) \times 10^2$ mM for

Fig. 1 SDS-PAGE of FAD-GDHs. Purified FAD-GDHs were subjected to SDS-PAGE (5 μg/lane). **a** FAD-GDHs produced by *E. coli*. The proteins in the gels were stained with CBB. **b** FAD-GDHs produced by *P. pastoris*. The proteins in the left gel were stained with CBB, and the glycosylated proteins in the right gel were stained with PAS. *M* molecular mass markers. In both panels, lane 1 TeGDH and lane 2 TcGDH

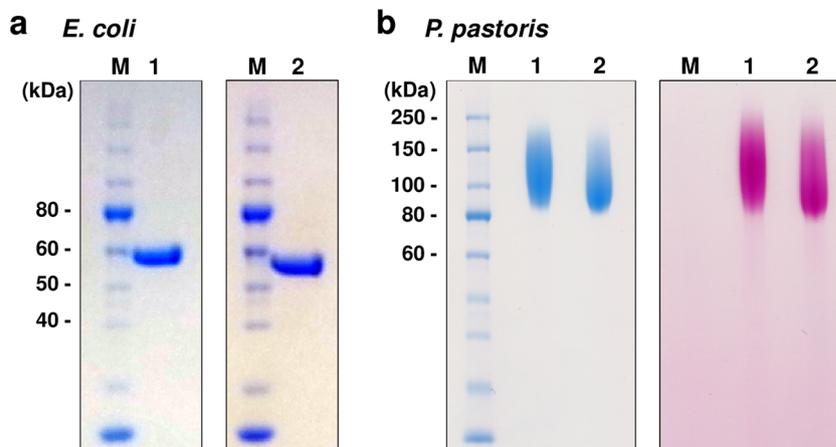
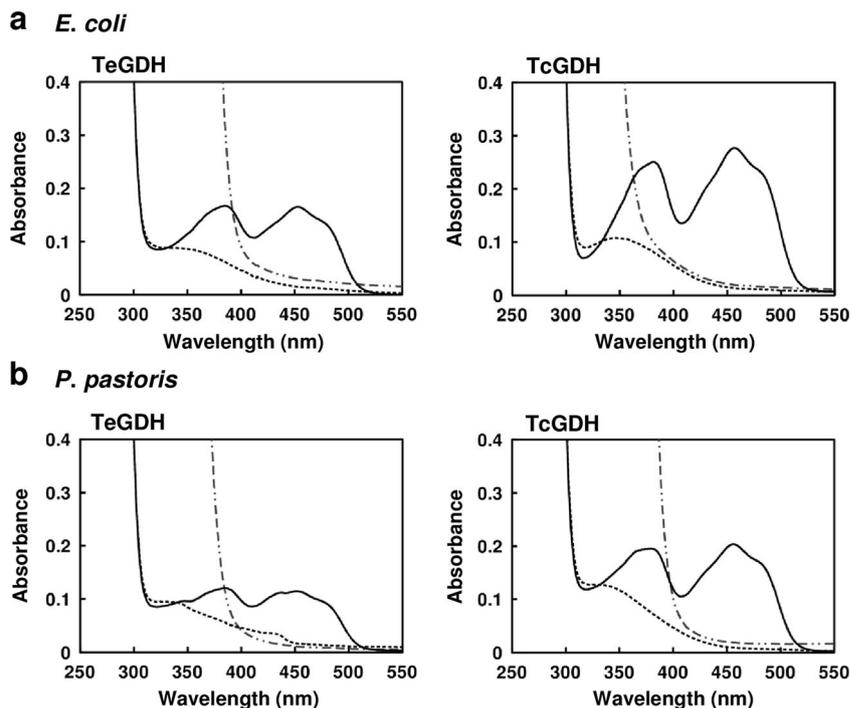


Fig. 2 Absorption spectra of FAD-GDHs. **a** Unglycosylated FAD-GDHs produced by *E. coli*. **b** Glycosylated FAD-GDHs produced by *P. pastoris*. The spectra of TeGDHs and TcGDHs are on the *left* and *right sides*, respectively. The spectra of purified FAD-GDHs are drawn as *solid lines*, the spectra of FAD-GDHs in the presence of 100 mM glucose are drawn as *dashed lines*, and the spectra of FAD-GDHs in the presence of sodium dithionite are drawn as *dotted and dashed lines*



glycosylated TeGDH. Unglycosylated and glycosylated TcGDHs exhibited higher substrate affinities with the K_m values of 4.63 ± 0.19 and 2.82 ± 0.17 mM, respectively.

The oxygen-reducing activities of TeGDHs and TcGDHs were also investigated, using *A. niger* GOD as a positive control. However, none of the enzymes prepared in this study exhibited oxygen reducing activity. Therefore, our results revealed that these enzymes were FAD-dependent GDHs.

Effect of pH on FAD-GDH stability

The stability of the FAD-GDHs at various pH values was examined (Fig. 4). Unglycosylated TeGDH was stable at a range of pH values between 3.0 and 8.0 (Fig. 4a). Additionally, glycosylated TeGDH showed high stability at a wide range of pH values between 2.0 and 9.0. Also, the stability of unglycosylated and glycosylated TcGDHs appeared similar to that of unglycosylated and glycosylated TeGDHs (Fig. 4b). These results indicated that TeGDHs and TcGDHs exhibited stability

over a broad pH range, and that glycosylated FAD-GDHs were more stable than unglycosylated FAD-GDHs.

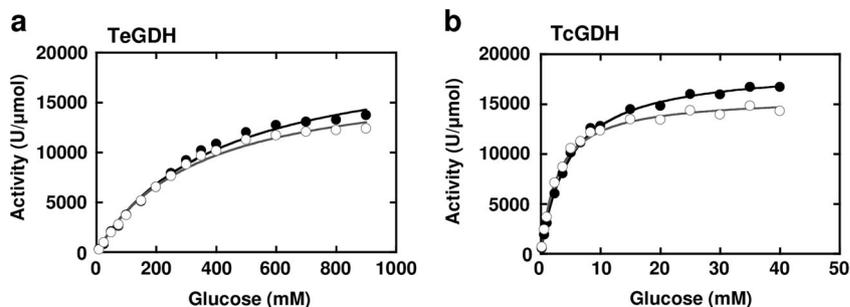
Effect of reaction pH on FAD-GDH activity

The pH profile of FAD-GDH activity was examined (Fig. 5). The relative activities of TeGDHs were over 50 % at a wide range of pH values between 3.0 and 10.0. The optimum reaction pH values were 6.0 for unglycosylated TeGDH and 5.0 for glycosylated TeGDH (Fig. 5a). The optimum reaction pH values for unglycosylated and glycosylated TcGDHs were 5.0, although the activities of TcGDHs were relatively low at a range of pH values between 8.0 and 10.0 (Fig. 5b).

Effect of temperature on FAD-GDH stability

In order to investigate whether thermophilic filamentous fungal FAD-GDHs are highly thermostable, the enzymatic activities of TeGDHs and TcGDHs were measured after heat treatment at 40–70 °C for 15 min (Fig. 6). Unglycosylated TeGDH

Fig. 3 Glucose concentration dependence of FAD-GDH activity. **a** TeGDHs. **b** TcGDHs. In both panels, *closed circles* unglycosylated FAD-GDHs and *open circles* glycosylated FAD-GDHs



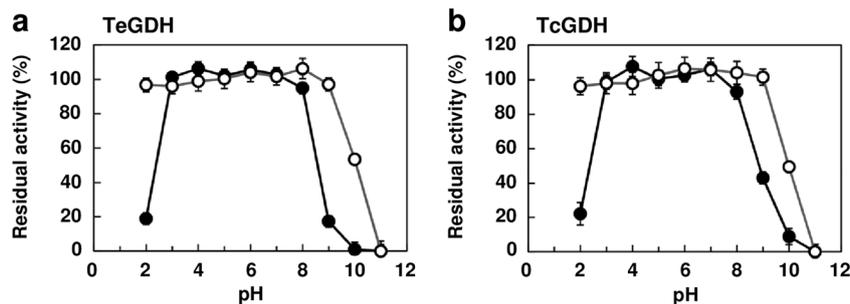


Fig. 4 Effect of pH on FAD-GDH stability. FAD-GDH (7 U/ml) was incubated in 50 mM Britton-Robinson universal buffer at indicated pH at 25 °C for 20 h, and then diluted with 50 mM potassium phosphate buffer

(pH 7.0) containing 0.1 % Triton X-100. This diluted solution was used for assay. **a** TeGDHs. **b** TcGDHs. In both panels, *closed circles* unglycosylated FAD-GDHs and *open circles* glycosylated FAD-GDHs

maintained nearly 100 % activity at temperatures up to 45 °C, but completely lost activity at 60 °C (Fig. 6a). Glycosylated TeGDH displayed high thermostability, maintaining 89 % activity even at 60 °C. Unglycosylated TcGDH was stable at temperatures up to 45 °C (Fig. 6b), while glycosylated TcGDH maintained 75 % activity even at 60 °C. The apparent T_m values were 52.7 °C for unglycosylated TeGDH, 66.4 °C for glycosylated TeGDH, 51.6 °C for unglycosylated TcGDH, and 62.5 °C for glycosylated TcGDH. These results revealed that glycosylated TeGDH and glycosylated TcGDH exhibited high thermostability.

Effect of reaction temperature on FAD-GDH activity

The temperature profile of FAD-GDH activity was examined (Fig. 7). The optimum reaction temperatures of unglycosylated and glycosylated TeGDHs were observed at 55–60 and 65–70 °C, respectively (Fig. 7a). The optimum reaction temperatures of unglycosylated and glycosylated TcGDHs were observed at 45–60 and 60–65 °C, respectively (Fig. 7b). Our results revealed that the optimum reaction temperatures of unglycosylated TeGDH and unglycosylated TcGDH were ~60 °C and that glycosylated TeGDH and glycosylated TcGDH were ~65 °C. The activities of glycosylated TeGDH and glycosylated TcGDH at their optimum reaction

temperatures were ~4.2 and ~3.3 times higher relative to those observed at 37 °C, respectively.

Substrate specificity of FAD-GDHs

The substrate specificity of TeGDHs and TcGDHs was examined (Table 1). TeGDHs and TcGDHs exhibited the highest specificity for D-glucose, while they exhibited no activity in the presence of maltose, to which PQQ-GDH exhibited cross-reactivity. TeGDHs and TcGDHs exhibited low reactivity to maltotriose and D-mannose, respectively, although TeGDHs and TcGDHs exhibited cross-reactivity toward 2-deoxy-D-glucose and D-xylose. These results revealed that TeGDHs and TcGDHs exhibited high substrate specificity for D-glucose.

Discussion

Here, we identified novel FAD-GDH gene homologs from *T. emersonii* NBRC 31232, *T. crustaceus* NBRC 9129, and *T. crustaceus* NBRC 9816. Since the partial nucleotide sequences of the *T. crustaceus* FAD-GDH gene homologs were identical with each other, we cloned two FAD-GDH gene homologs from *T. emersonii* NBRC 31232 and *T. crustaceus*

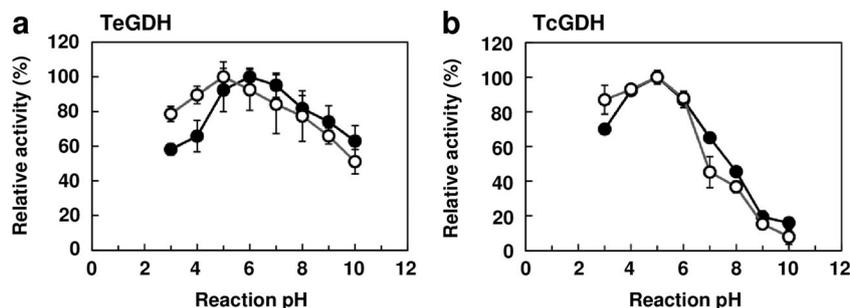


Fig. 5 Effect of reaction pH on FAD-GDH activity. Reaction mixture containing FAD-GDH was incubated in 50 mM Britton-Robinson universal buffer at the indicated pH at 37 °C for 2 min. The oxidized DCIP was quantified after adding 0.2 M Tris-HCl buffer (pH 8.0)

containing 8 M urea into the mixture to stop the reaction. The reaction mixture was finally diluted fivefold with the stopping buffer. **a** TeGDHs. **b** TcGDHs. In both panels, *closed circles* unglycosylated FAD-GDHs and *open circles* glycosylated FAD-GDHs

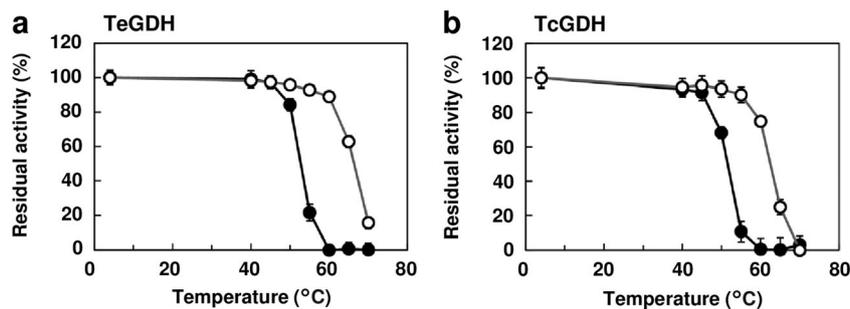


Fig. 6 Effect of temperature on FAD-GDH stability. FAD-GDH (7 U/ml) was incubated at indicated temperatures at pH 5.0 for 15 min, and then diluted with 50 mM potassium phosphate buffer (pH 7.0) containing

0.1 % Triton X-100. This diluted solution was used for assay. **a** TeGDHs. **b** TcGDHs. In both panels, *closed circles* unglycosylated FAD-GDHs and *open circles* glycosylated FAD-GDHs

NBRC 9129, and characterized the enzymatic properties of the gene products.

The culture media of *A. oryzae* and *A. terreus* exhibited GDH activity, and then FAD-GDHs were purified and their enzymatic properties were examined (Bak 1967a, b; Omura et al. 2010). In order to obtain thermostable FAD-GDHs from thermophilic filamentous fungi, we first attempted to detect GDH activity in the culture media of the fungi. However, no GDH activity was detected from the culture media, indicating that the production level of FAD-GDH depended on the culture conditions. Thus, the purification of native FAD-GDHs from any fungal culture media was resigned, and thermophilic filamentous fungi with FAD-GDH gene homologs on their genomic DNAs were screened by degenerate PCR. Successfully, novel FAD-GDH gene homologs were identified from the genomic DNAs of *T. emersonii* NBRC 31232, *T. crustaceus* NBRC 9129, and *T. crustaceus* NBRC 9816 by degenerate PCR using primers designed based on the primary structures of *A. oryzae* and *A. terreus* FAD-GDHs. The genomic DNA fragments containing the FAD-GDH gene homologs were subsequently cloned from *T. emersonii* NBRC 31232 and *T. crustaceus* NBRC 9129.

The enzymatic properties of the recombinant FAD-GDHs are summarized in Table 2. In the experiment of the effect of temperature on stability, the apparent T_m values of glycosylated TeGDH and glycosylated TcGDH were 66.4 and 62.5 °C,

respectively (Fig. 6). Studies on the thermostability of native *A. oryzae* and *A. terreus* FAD-GDHs purified from their culture media were previously reported (Bak 1967a; Omura et al. 2010). The apparent T_m value of *A. oryzae* FAD-GDH was ~53 °C after heat treatment for 15 min. *A. terreus* FAD-GDH retained ~60 % of the activity after heat treatment at 55 °C for 15 min. These results revealed that glycosylated TeGDH and glycosylated TcGDH were highly thermostable. Additionally, glycosylated TeGDH and glycosylated TcGDH were stable at a broad pH range. The thermostability and pH stability of TeGDH and TcGDH were improved when the enzymes were produced by *P. pastoris*, likely a result of glycosylation. Several studies on glycosylated proteins produced by *P. pastoris* support our results (Daly and Hearn 2005). The numbers of predicted *N*-glycosylation sites were 12 for TeGDH and 11 for TcGDH. The evidence supporting the glycosylation of the FAD-GDHs produced by *P. pastoris* was confirmed by PAS staining (Fig. 1b) and PNGase F treatments (Fig. S3).

Generally, the structures of *N*-glycans produced by filamentous fungi and *P. pastoris* are high-mannose type. *N*-glycans produced by filamentous fungi are $\text{Man}_{6-9}\text{GlcNAc}_2$ or $\text{Man}_{5-12}\text{GlcNAc}_2$ sizes (Maras et al. 1999). The majority of *N*-glycans produced by *P. pastoris* are $\text{Man}_{8-14}\text{GlcNAc}_2$ sizes, with the remainder being much larger $\text{Man}_{30-50}\text{GlcNAc}_2$ sizes (Brethauer and Castellino 1999). These suggest that the

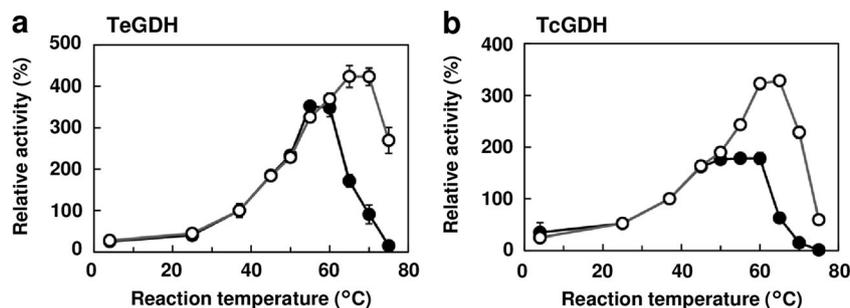


Fig. 7 Effect of reaction temperature on FAD-GDH activity. Reaction mixture containing FAD-GDH was incubated at the indicated temperatures at pH 5.0 for 2 min. The oxidized DCIP was quantified after adding 0.2 M Tris-HCl buffer (pH 8.0) containing 8 M urea into

the mixture to stop the reaction. The reaction mixture was finally diluted fivefold with the stopping buffer. **a** TeGDHs. **b** TcGDHs. In both panels, *closed circles* unglycosylated FAD-GDHs and *open circles* glycosylated FAD-GDHs

Table 1 Substrate specificity of FAD-GDH activity. Dehydrogenase activity to various saccharides (40 mM) was examined at pH 7.0 and 37 °C. Activity to D-glucose was set as 100 %

Substrate	Relative activity (%)			
	Unglycosylated TeGDH	Glycosylated TeGDH	Unglycosylated TeGDH	Glycosylated TeGDH
D-Glucose	100	100	100	100
D-Arabinose	ND	ND	ND	ND
2-Deoxy-D-glucose	17.7 ± 5.3	11.5 ± 4.8	49.8 ± 5.4	60.1 ± 2.9
D-Fructose	ND	ND	ND	ND
D-Galactose	ND	ND	ND	ND
Maltose	ND	ND	ND	ND
Maltotetraose	ND	ND	ND	ND
Maltotriose	2.7 ± 1.6	3.4 ± 1.0	ND	ND
D-Mannitol	ND	ND	ND	ND
D-Mannose	ND	ND	5.6 ± 1.7	6.8 ± 5.2
D-Raffinose	ND	ND	ND	ND
D-Sorbitol	ND	ND	ND	ND
Sucrose	ND	ND	ND	ND
D-Trehalose	ND	ND	ND	ND
D-Xylose	24.6 ± 5.1	24.8 ± 1.3	21.7 ± 3.5	27.6 ± 0.9

ND indicates that activity was not detected

degree of *N*-glycosylation of recombinant protein produced by *P. pastoris*, which can be observed by SDS-PAGE, is similar to or higher than that of *N*-glycosylation of native protein produced by filamentous fungus. In the case of *G. cingulata* FAD-GDH, native enzyme and recombinant enzyme produced by *P. pastoris* exhibited similar degree of *N*-glycosylation, as well as similar optimum temperatures (Sygmond et al. 2011a; Sygmond et al. 2011b). In other examples, *A. niger* GOD, *Penicillium variable* GOD and *Agaricus meleagris* FAD-dependent pyranose dehydrogenase (EC 1.1.99.29), which were produced by *P. pastoris*, exhibited higher degree of *N*-glycosylation than native enzymes (Crognale et al. 2006; Guo et al. 2010; Sygmond et al. 2012). In the matter of stability, while these recombinant GODs exhibited increased pH stability, decreased thermostability was unexpectedly exhibited (Crognale et al. 2006; Guo et al. 2010). Therefore, among native FAD-GDHs produced by filamentous

fungi and recombinant FAD-GDHs produced by *P. pastoris*, the stability of the glycosylated FAD-GDHs would depend on the enzymes, rather than the degree of glycosylation.

The kinetic parameters, including V_{\max} and K_m , were calculated. The V_{\max} values were 1.57×10^4 – 2.08×10^4 U/ μ mol for unglycosylated and glycosylated FAD-GDHs. The K_m values of TeGDHs were ~100-fold lower relative to those of TeGDHs. The reported K_m values of *A. oryzae* FAD-GDH and *A. terreus* FAD-GDH were 25 and 49.7 mM, respectively (Bak 1967b; Omura et al. 2010). These data indicated that TeGDHs exhibited higher affinities for glucose. The fasting blood glucose level in a healthy individual is almost 5 mM. On the other hand, in a diabetes patient, the fasting blood glucose level is over 7.0 mM, or the blood glucose level after an oral glucose tolerance test is over 11.1 mM (American Diabetes Association 2016). The blood glucose level of a severe diabetes patient sometimes reaches over 33.3 mM

Table 2 Summary of enzymatic properties

Enzymatic properties	TeGDH		TcGDH	
	Unglycosylated	Glycosylated	Unglycosylated	Glycosylated
Specific activity (10^3 U/ μ mol)	9.21 ± 0.24	8.79 ± 0.12	16.0 ± 1.2	13.5 ± 0.9
V_{\max} (10^3 U/ μ mol)	20.8 ± 0.9	17.9 ± 0.7	18.8 ± 0.21	15.7 ± 0.21
K_m (mM)	$(4.06 \pm 0.40) \times 10^2$	$(3.37 \pm 0.31) \times 10^2$	4.63 ± 0.19	2.82 ± 0.17
pH stability	3.0–8.0	2.0–9.0	3.0–8.0	2.0–9.0
Optimum pH	6.0	5.0	5.0	5.0
T_m (°C)	52.7	66.4	51.6	62.5
Optimum temperature (°C)	55–60	65–70	45–60	60–65

(American Diabetes Association 2004). Therefore, at the glucose concentrations, *T. crustaceus* FAD-GDH can exhibit high activity, but the activity of *T. emersonii* FAD-GDH becomes low. By a site-directed mutagenesis approach, improvement of *T. emersonii* FAD-GDH would be required to decrease the K_m value.

TeGDHs and TcGDHs highly recognized glucose as a substrate (Table 1). Glucose sensors employing PQQ-GDH that react with non-glucose saccharides, including maltose, galactose, and xylose, would lead to potentially fatal errors for blood glucose monitoring (US Food and Drug Administration 2009). Protein engineering studies have succeeded in improving its substrate specificity (Hamamatsu et al. 2006; Igarashi et al. 2004; Sode et al. 2002). In our present experiments, TeGDHs and TcGDHs exhibited cross-reactivity toward 2-deoxy-D-glucose and D-xylose. *A. oryzae* FAD-GDH and *A. terreus* FAD-GDH also exhibited reactivity to these saccharides (Bak 1967b; Omura et al. 2010). However, glucose sensors employing these enzymes would provide appropriate glucose concentrations for SMBG, because these saccharides are generally absent in the blood of diabetes patients. Based on the findings, we suggested that *T. emersonii* FAD-GDH and *T. crustaceus* FAD-GDH could be used for biosensors to SMBG, although improvement of the substrate specificity would be required to minimize cross-reactivity to non-glucose saccharides.

In the present study, we cloned novel FAD-GDH gene homologs from the thermophilic filamentous fungi *T. emersonii* NBRC 31232 and *T. crustaceus* NBRC 9129, and characterized the enzymatic properties of the gene products. The results revealed that these FAD-GDHs exhibited high thermostability and substrate specificity. Moreover, these thermostable FAD-GDHs have potential to be ideal enzymes for biosensors of SMBG, enabling long-term storage and high accuracy.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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