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Fungal FAD-dependent glucose dehydrogenases concerning high activity, affinity, and thermostability for maltose-insensitive blood glucose sensor

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HIGHLIGHTS

- Six FAD-dependent glucose dehydrogenase genes were cloned from *Aspergillus* species.
- The recombinant enzymes produced by *Pichia pastoris* were characterized.
- All the enzymes exhibited maltose insensitivity.
- The enzyme from *A.phoenicis* exhibited D-xylose insensitivity.
- The enzymes exhibited high activity, high affinity, or high thermostability.

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ABSTRACT

Six FAD-dependent glucose dehydrogenase (FAD-GDH) genes, for blood glucose sensors, from the genomic DNAs of *Aspergillus* species were identified by degenerated PCR screening. The enzymatic properties of the gene products expressed by *Pichia pastoris* were characterized. Investigation of substrate selectivity to fourteen saccharides revealed that these enzymes were maltose-insensitive FAD-GDHs. *A.bisporus*, *A.terreus* var. *aureus*, and *A. phoenicis* FAD-GDHs showed high substrate selectivity; they exhibited reactivity to only one or two saccharides. *A. phoenicis* FAD-GDH specifically exhibited insensitivity to D-xylose. Furthermore, six FAD-GDHs exhibited superior parameter values, such as maximum velocity (V_{max}), Michaelis-Menten constant (K_m), and apparent transition midpoint temperature (T_m). *A. phoenicis* and *A. terreus* var. *aureus* FAD-GDHs exhibited high activity, with V_{max} values of $(8.66 \pm 0.17) \times 10^4$ U/μmol and $(1.14 \pm 0.03) \times 10^5$ U/μmol, respectively. *A. brunneo-uniseriatus*, *A.carneus*, and *A. malignus* FAD-GDHs exhibited very high affinity for glucose, with K_m values between 0.41 and 0.96 mM. *A. bisporus* FAD-GDH exhibited high thermostability, with a T_m value of 72.5 °C. Therefore, we propose that the FAD-GDHs will be practical in blood glucose sensors and in other bioelectrochemical devices exhibiting high substrate selectivity, high current output, high sensitivity, and/or high stability to storage and use.

1. Introduction

Self-monitoring of blood glucose is a key clinical test for patients with diabetes, a common disease worldwide. Such patients need to monitor their blood glucose levels continuously on a daily basis using a blood glucose sensor to manage the disease. Glucose oxidase (GOD) and glucose dehydrogenase (GDH) have been widely used in commercially available blood glucose sensors [1–3]. Among these enzymes, fungal FAD-dependent GDH (FAD-GDH; EC 1.1.5.9) has recently received considerable attention for its accuracy in self-monitoring of blood glucose.

FAD-GDH catalyzes the oxidation of D-glucose at its 1-hydroxyl group to D-glucono-1,5-lactone using the cofactor FAD as the electron acceptor. Recent blood glucose sensors using FAD-GDHs are mainly of the mediator type. In these sensors, an electron is transferred from FAD to an artificial electron acceptor (mediator) during the enzymatic

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oxidation of D-glucose. The reduced mediator is then oxidized at the electrode, and the released electron is detected as the current output. The following advantages are ascribed to the use of the fungal FAD-GDH in blood glucose sensors: (1) the cofactor FAD is tightly bound to the enzyme, and this enables easier fabrication processes owing to no requirement of exogenous cofactor addition to the systems; (2) FAD-GDH is insensitive to dissolved oxygen; therefore, only the artificial mediator on the sensor systems receives the electron from reduced FAD, which confers improved accuracy; and (3) some FAD-GDHs are maltose-insensitive; the presence of maltose in the blood can result in falsely elevated blood glucose levels [1,3]. In addition to blood glucose sensors, other applications that take advantage of the features of FAD-GDH include sensors used in various clinical tests and food and brewing management [4–8]. Moreover, biofuel cells utilizing FAD-GDH as an anode catalyst have also been studied [9,10]. Implantable power sources for devices such as glucose sensors, pacemakers, and small pumps have been proposed as examples of the applicability of enzymatic biofuel cells [11,12]. Fungal FAD-GDH was first found from *Aspergillus oryzae* [13–18]. The number of reports regarding the discovery and recombinant production of new fungal FAD-GDHs, including *Aspergillus terreus* [19–21], *Aspergillus flavus* [22,23], *Aspergillus niger* [22,24], *Glomerella cingulata* [25,26], *Mucor prainii* [27], and *Pycnoporus cinnabarinus* [28], has been increasing. We believe that the discovery of high-spec FAD-GDHs will lead to the development of blood glucose sensors with high performances, and enable FAD-GDH use in other bioelectrochemical devices.

We previously discovered FAD-GDH gene homologs in thermophilic fungi such as *Talaromyces emersonii* NBRC 31232, *Thermoascus aurantiacus* NBRC 6766, *Thermoascus aurantiacus* NBRC 9748, and *Thermoascus crustaceus* NBRC 9129 by using degenerate PCR to screen their genomic DNAs [29,30]. We cloned the gene homologs and characterized the enzymatic properties of the recombinant enzymes. The enzymes exhibited high thermostability; hence, the application of these enzymes to blood glucose sensors exhibiting stability for storage and use was proposed.

FAD-GDHs from *A. oryzae* TI (Toyobo Co., Ltd.) [31] and *A. terreus* FERM BP-08578 (Ikeda tohka Industries Co., Ltd.) [32] are patented and will be practically used in commercially available blood glucose sensors. Although some FAD-GDHs from *Aspergillus* species have been reported, the existence of FAD-GDHs from other *Aspergillus* species remain unknown. It was expected that FAD-GDHs from other *Aspergillus* species exhibited practical properties. Here, we identified novel FAD-GDH gene homologs from *Aspergillus* species. We obtained six novel FAD-GDHs as recombinant enzymes produced by *P. pastoris*, and these enzymes exhibited reactivity toward glucose and no reactivity toward maltose. Recently, in the development of glucose sensors, particularly blood glucose sensors, improvement of substrate selectivity of sensor enzymes has been deemed important. Such devices have been recommended to show insensitivity to not only maltose but also galactose and xylose [33]. Thus, reactivity and selectivity of the enzymes to various saccharides such as galactose and xylose were investigated. Furthermore, FAD-GDH is being used in sensors for various fields and applications, as well as in biofuel cells. In addition to high substrate selectivity, the bioelectrochemical devices in these fields are required to exhibit various performances, including high current output, high sensitivity, and/or high stability to different pH and heat levels. Therefore, varied enzymatic properties of the enzymes were investigated, and then compared to the *P. pastoris*-produced recombinant enzymes of *A. oryzae* TI FAD-GDH and *A. terreus* FERM BP-08578 FAD-GDH as benchmark enzymes.

2. Materials and methods

2.1. Strains, vectors, and primers

The strains of *Aspergillus* species used in this study were purchased from the Biological Resource Center at the National Institute of Technology and Evaluation (NBRC; Chiba, Japan). The cloning vector pCR-Blunt II-TOPO (Life Technologies) and *E. coli* DH5α (Takara Bio) were used for gene cloning. The expression vector pPIC9 (Life Technologies) and *P. pastoris* GS115 (Life Technologies) were used to produce recombinant enzymes. The primers used in this study are listed in Table S1.

2.2. Degenerate PCR screening for FAD-GDH gene homologs

Degenerate PCR was performed by using KOD FX (Toyobo), degenerate primers, and genomic DNA extracted from each *Aspergillus* strain, which was used as a template. The nucleotide sequences of the five degenerate primers—HCA, HCB, HCC, HCD, and HCE—were designed based on the primary structures of FAD-GDHs from *A. oryzae* TI (accession number DJ347964) and *A. terreus* FERM BP-08578 (accession number DD412265), as shown in our previous study [30]. 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 into the cloning vector and its nucleotide sequence was determined. The predicted amino acid sequences were compared to those of *A. oryzae* and *A. terreus* FAD-GDHs.

2.3. Cloning of FAD-GDH gene homologs

The genomic DNA from each *Aspergillus* strain 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. The restriction enzymes and sizes of DNA bands detected by Southern hybridization were as follows: *A. bisporus* NBRC 32017, *Bam*HI (7.0 kb); *A. brunneouniseriatus* NBRC 6993, *Bam*HI (3.5 kb); *A. carneus* NBRC 5861, *Apa*I (7.0 kb); *A. malignus* NBRC 8132, *Xba*I (3.5 kb); *A. phoenicis* NBRC 6648, *Apa*I (3.0 kb); *A. terreus* var. *aureus* NBRC 30536, *Xho*I (4.5 kb); *A. ustus* NBRC 4104, *Pst*I (8.0 kb). The digested genomic DNA was subjected to preparative agarose gel electrophoresis, and the DNA fragments that were approximately of the same size as the Southern band were extracted from the gel. These DNA fragments were self-ligated with T4 DNA ligase and used as templates for inverse PCR using KOD-Plus-Neo (Toyobo). Inverse PCR was then 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 primers for inverse PCR were designed based on the nucleotide sequence of the internal FAD-GDH gene homolog from each *Aspergillus* strain.

The genomic DNA fragment containing the entire FAD-GDH gene homolog was then amplified by PCR using KOD-Plus-Neo, genomic DNA from each *Aspergillus* strain, and primers designed based on the nucleotide sequence of the inverse PCR-amplified DNA fragment.

2.4. Genetic analysis

Exon-intron splicing sites were predicted by the GT-AG rule [34,35]. The secretion signal sequence was predicted by using SignalP 4.0 [36], and *N*-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The amino acid sequences of FAD-GDHs were aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp/) [37].

2.5. Synthesis of the coding sequence

The coding sequence (CDS) of the novel FAD-GDH gene homolog from the *Aspergillus* strain was artificially synthesized by overlap extension PCR [38]. Exons were individually amplified and connected.

2.6. Preparation of recombinant enzymes produced by P. pastoris

The gene encoding mature FAD-GDH was generated by PCR using the primers Pic1 and Pic2. Primer Pic1 contained the *Eco*RI site, and primer Pic2 contained the *Not*I site and six CAC codons encoding a histidine tag located at the C-terminus. Before PCR amplification, the structural gene was modified by silent mutagenesis to synthesize an artificial gene lacking the *Eco*RI and *Sal*I sites. The amplified DNA fragment was inserted between *Eco*RI and *Not*I sites in the pPIC9 vector. The resultant plasmid was cleaved with *Sal*I and introduced into *P. pastoris* GS115 genomic DNA at its *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). The culture supernatant was dialyzed against PBS [137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, and 2 mM potassium dihydrogen phosphate (pH 7.4)], and applied to a Ni-NTA agarose column (QIAGEN) 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 further use. Protein concentration was determined by using a Pierce BCA protein assay kit (Thermo Fisher Scientific) [39], with bovine serum albumin as a standard.

2.7. GDH assay

GDH activity was measured by using the 2,6-dichloroindophenol (DCIP) method [30]. One unit was defined as the amount of FAD-GDH producing 1 μmol of reduced DCIP per minute at a pH of 7.0 and temperature of 37 °C. The molar absorption coefficient of oxidized DCIP at 600 nm (16.3 $mM^{-1}\,cm^{-1}$) was used to determine the enzyme activity. The molar absorption coefficient of FAD at 450 nm (11.3 $mM^{-1}\,cm^{-1}$) [40] was used to calculate the concentration of FAD present in the enzyme. Activity was shown as units per μmol of the holoenzyme.

2.8. Assay of oxygen-reducing activity

Oxygen-reducing activity was measured by the method of Swoboda and Massey [41]. GOD from *A. niger* (Wako Pure Chemical Industries) was used as a positive control.

2.9. Accession numbers

The nucleotide sequences of the FAD-GDH genes have been deposited in the DNA Data Bank of Japan (DDBJ). Their accession numbers are LC307123–LC307136.

3. Results

3.1. Identification and cloning of FAD-GDH gene homologs

We identified novel FAD-GDH gene homologs from *A. bisporus* NBRC 32017, *A. brunneo-uniseriatus* NBRC 6993, *A. carneus* NBRC 5861, *A. malignus* NBRC 8132, *A. phoenicis* NBRC 6648, *A. terreus* var. *aureus* NBRC 30536, and *A. ustus* NBRC 4104 by degenerate PCR for the genomic DNA of each *Aspergillus* strain. DNA fragments with lengths of ~1.3–2.0 kb were amplified by degenerate PCR, as shown in Fig. S1. Nucleotide sequencing of these DNA fragments revealed that the genomic DNAs of the *Aspergillus* strains contained FAD-GDH gene homologs. The genomic DNA fragments containing the entire FAD-GDH gene homologs were successfully cloned from all of the strains.

The results of genetic analyses of the seven FAD-GDH gene homologs are shown in Table 1. The CDSs were divided into three or four exons by introns. The putative FAD-GDHs were 583–596 amino acids long. In all putative FAD-GDHs, the secretion signal peptides were identified at the N-terminus, and the amino acid sequences implicated in FAD-binding (Gly-*X*-Gly-*X*-*X*-Gly, where *X* represents any amino acid residue) [42] were conserved. The 7–13 *N*-glycosylation sites (Asn-*X*-Ser/Thr, where *X* represents any residue, except for proline) [43] were predicted. The alignment of the amino acid sequences of putative FAD-GDHs from the *Aspergillus* strains is shown in Fig. S2. The amino acid sequence identities among these FAD-GDHs and the thermophilic fungal FAD-GDHs found in our previous study [29,30] were 53.2–92.9% (Fig. S3).

3.2. Preparation of recombinant FAD-GDHs

To prepare recombinant enzymes, we used the production system of *P. pastoris*. The mature FAD-GDH gene was inserted into the pPIC9 vector to fuse with the yeast secretion signal peptide. The recombinant enzymes—*A. bisporus* FAD-GDH (AbGDH), *A. brunneo-uniseriatus* FAD-GDH (AbuGDH), *A. carneus* FAD-GDH (AcGDH), *A. malignus* FAD-GDH (AmGDH), *A. phoenicis* FAD-GDH (ApGDH), *A. terreus* var. *aureus* FAD-GDH (AtaGDH), and *A. ustus* FAD-GDH (AuGDH)—were purified. Recombinant enzymes *A. oryzae* FAD-GDH (AoGDH) and *A. terreus* FAD-GDH (AtGDH) were also prepared by *Pichia* expression system using the pPIC9 and the strain GS115. The yields per liter of culture media were as follows: AbGDH, 33 mg; AbuGDH, 48 mg; AcGDH, 12 mg; AmGDH, 30 mg; ApGDH, 54 mg; AtaGDH, 22 mg; AuGDH, 1 mg; AoGDH, 32 mg; and AtGDH, 75 mg.

The purified recombinant enzymes were subjected to SDS-PAGE (Fig. 1A). The protein bands of the purified enzymes were detected with Coomassie brilliant blue G-250. The apparent molecular masses of the purified enzymes in the SDS-PAGE gel were as follows: AbGDH, ~75 kDa; AbuGDH, ~80 kDa; AcGDH, ~85 kDa; AmGDH, ~80 kDa; ApGDH, 90–200 kDa; AtaGDH, 90–250 kDa; AuGDH, 100–250 kDa; AoGDH, 80–250 kDa; and AtGDH, 80–250 kDa. The theoretical molecular masses of the recombinant enzymes were as follows: AbGDH, 64 kDa; AbuGDH, 63 kDa; AcGDH, 63 kDa; AmGDH, 63 kDa; ApGDH, 64 kDa; AtaGDH, 65 kDa; AuGDH, 64 kDa; AoGDH, 64 kDa; and AtGDH, 65 kDa. The apparent molecular masses obtained by SDS-PAGE were higher than the estimated theoretical molecular masses. Decreased protein band shift was observed by Peptide-N-Glycosidase F (PNGase F; New England Biolabs) treatment of the enzymes under denaturing conditions (Fig. 1B). It was revealed that the recombinant enzymes had undergone glycosylation during post-translational modification in *P. pastoris*, and *N*-linked sugar chains were the major components of the glycosylation process. The molecular masses of the glycosylated enzymes would be overestimated by SDS-PAGE. Glycosylated proteins are known to have decreased charge-mass ratios when complexed with SDS, resulting in decreased migration rates and overestimated molecular masses [44].

The absorption spectra of the recombinant enzymes were similar to the typical spectrum observed for oxidized flavin, with major peaks at 380 nm and 450 nm, except for AuGDH (Fig. 2). After the addition of glucose, the two peaks corresponding to oxidized flavin disappeared. These results indicated that the holoenzyme forms containing oxidized FAD as a cofactor were purified. AuGDH showed an unclear broad spectrum in the absence of glucose, and exhibited no change in absorbance at 450 nm. It was indicated that AuGDH contained no FAD and exhibited no dehydrogenase activity for glucose. We thus performed the characterization of the enzymatic properties of all the recombinant enzymes except for AuGDH.

3.3. Enzymatic properties

The characterization of the enzymatic properties was performed by the DCIP method. The specific activities, shown as units per μmol of the holoenzyme, were measured in the presence of 300 mM glucose at a pH of 7.0 and a temperature of 37 °C (Table 2). The six recombinant enzymes exhibited GDH activity. In addition, the oxygen-reducing activity

Table 1
Genetic analysis of the FAD-GDH gene homologs from *Aspergillus* strains. Number of nucleotides includes stop codon.

	A.bisporus	*A.brunneo-uniseriatus*	*A.carneus*	*A.malignus*	*A.phoenicis*	*A.terreus* var.*aureus*	*A.ustus*
Number of exons	3	3	3	3	3	3	4
Number of nucleotides (bp)	1779	1752	1758	1764	1791	1779	1770
Number of amino acid residues	592	583	585	587	596	592	589
Signal peptide	Met1-Ala16	Met1-Ser17	Met1-Ala18	Met1-Ala19	Met1-Ala20	Met1-Ala16	Met1-Ala15
Number of *N*-glycosylation sites	9	11	8	9	13	9	7

was examined for these enzymes using *A. niger* GOD as a positive control; none of the recombinant enzymes exhibited oxygen-reducing activity. Therefore, our results revealed that these enzymes were FAD-dependent GDHs.

Table 3 shows dehydrogenase activity to fourteen saccharides. Importantly, all the enzymes showed no reactivity to maltose. In detail, high substrate selectivity, with reactivity to only two saccharides, was observed in the following enzymes: AtaGDH, AoGDH, and AtGDH, 2-deoxy-D-glucose (23.0–37.5%) and D-xylose (10.1–10.5%); AbGDH, D-raffinose (3.0 ± 1.8%) and D-xylose (36.1 ± 8.3%). These enzymes exhibited insensitivity to D-galactose. Furthermore, ApGDH had the strictest substrate selectivity among the eight enzymes in this study; this enzyme exhibited reactivity to only 2-deoxy-D-glucose (17.4 ± 3.9%), and was the only enzyme that was insensitive to D-xylose. On the other hand, the following enzymes exhibited reactivity to several saccharides such as D-galactose (4.6–93.9%) and D-xylose (28.1–83.1%): AcGDH, which was reactive to five saccharides, and AbuGDH and AmGDH, which were reactive to six saccharides. The results indicated that AcGDH, AbuGDH, and AmGDH had broad substrate selectivity.

We investigated enzymatic properties related to the performance of blood glucose sensors and other electrochemical devices: glucose concentration dependence (Fig. S4), effect of pH (Fig. S5) and temperature (Fig. 3) on stability, and effect of pH (Fig. S6) and temperature (Fig. S7) on activity. These results are summarized in Table 2, and the properties of the enzymes were compared to those of AoGDH and AtGDH, as practical benchmark enzymes. In addition to the common property of maltose insensitivity, superior parameter values were exhibited.

ApGDH and AtaGDH exhibited high activity. These enzymes exhibited high V_{max} values of (8.66 ± 0.17) × 10^4 U/μmol and (1.14 ± 0.03) × 10^5 U/μmol, respectively. These V_{max} values were similar to or higher than the values for AoGDH [(6.61 ± 0.22) × 10^4 U/μmol] and AtGDH [(9.23 ± 0.10) × 10^4 U/μmol]. The K_m values of ApGDH [(1.76 ± 0.07) × 10^2 mM] and AtaGDH [(5.38 ± 0.36) × 10 mM] were relatively higher, indicating their relatively lower affinity for glucose. ApGDH was more stable at acidic pHs, exhibiting > 90% residual activity after incubating at pH 3.0–5.0 for 20 h.

AbuGDH, AcGDH, and AmGDH exhibited very high affinity for glucose. The K_m values were between 0.41 and 0.96 mM, indicating that the GDH activity of these enzymes reaches V_{max} at lower glucose concentrations. These enzymes also exhibited high stability at acidic to neutral pH between 2.0 and 7.0. Moreover, their optimum pH values were also acidic: AbuGDH, pH 5.0–6.0; AcGDH and AmGDH, pH 5.0. AmGDH exhibited relatively high thermostability. The apparent T_m value, determined from the data in Fig. 3, where the GDH activities were measured after heat treatment at 30–80 °C for 15 min, was 62.0 °C; the apparent T_m values of AbuGDH and AcGDH were 56.2 °C and 55.8 °C, respectively.

AbGDH exhibited the highest thermostability among the examined enzymes, and the apparent T_m value was 72.5 °C. Moreover, AbGDH exhibited the highest optimum temperature of 65 °C. The activity of AbGDH at the optimum temperature reached 279 ± 10% of its activity at 37 °C; the activities of AoGDH and AtGDH at their optimum temperatures were 170 ± 4% and 145 ± 2% of their activities at 37 °C, respectively. AbGDH also exhibited the highest pH stability, exhibiting stability at a wide pH range of 3.0–10.0. However, its specific activity was comparatively lower [(3.21 ± 0.10) × 10^3 U/μmol]. This was due to its K_m value, which was 1.66 ± 0.35 M, indicating a very low affinity toward glucose.

4. Discussion

FAD-GDH is a key enzyme for blood glucose sensors. This enzyme can also be used in other bioelectrochemical devices. Although FAD-

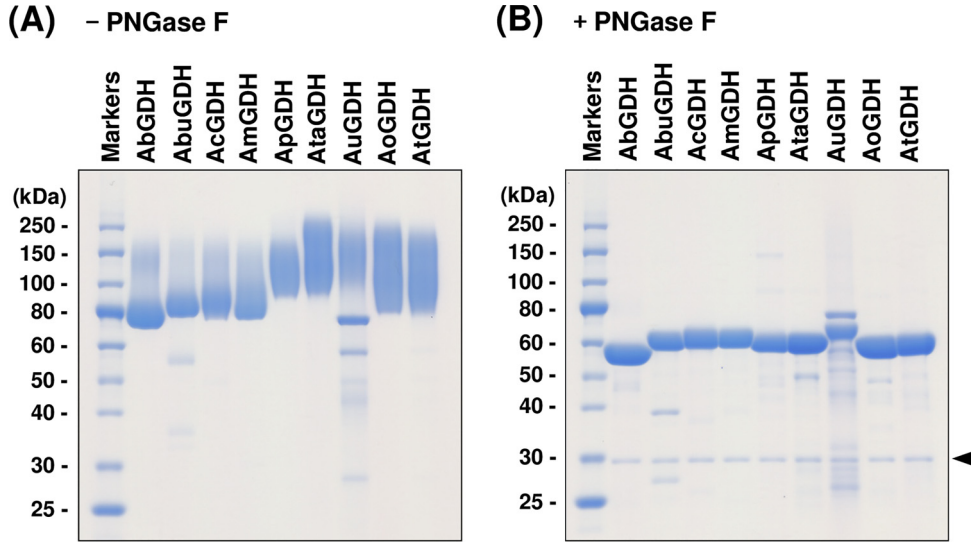

Fig. 1. SDS-PAGE of the recombinant enzymes purified from the *P. pastoris* culture supernatant. The enzymes in the (A) absence and (B) presence of PNGase F were subjected to SDS-PAGE (5 μg/lane). The proteins in the gels were stained with CBB. *Arrowhead* indicates PNGase F.

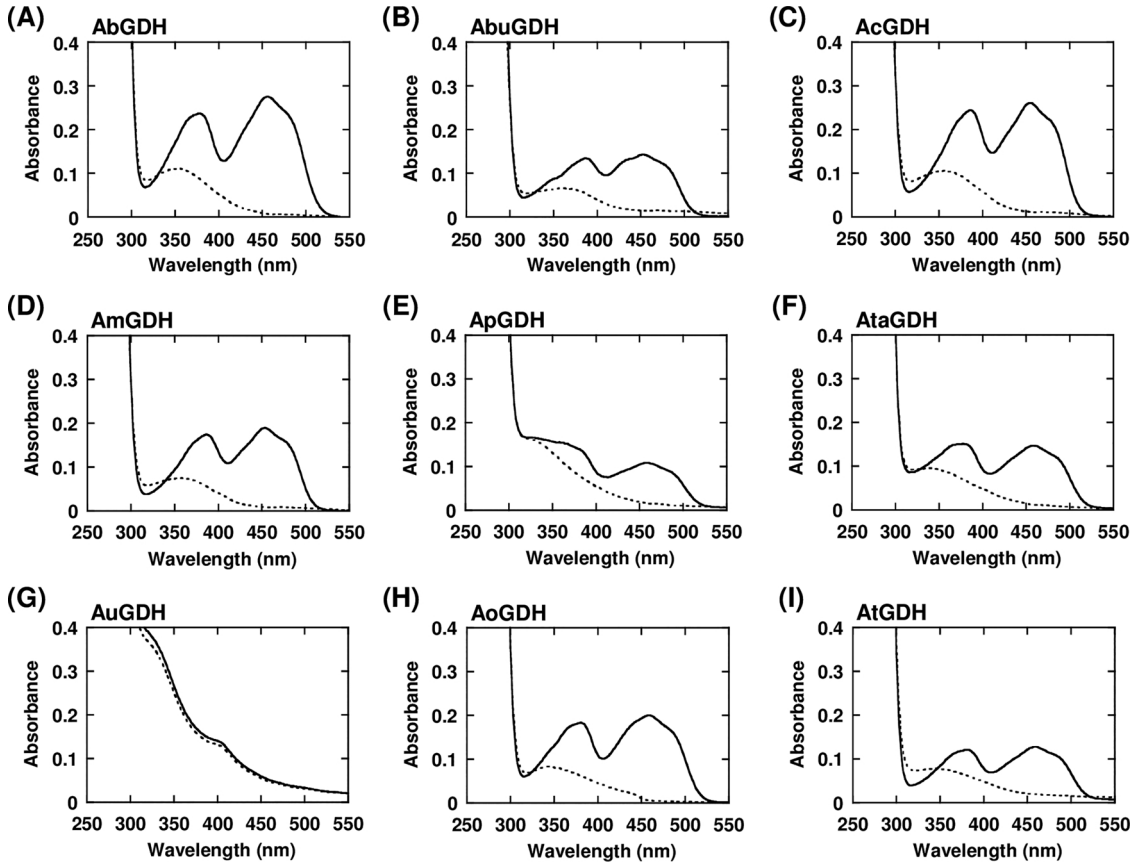

Fig. 2. The absorption spectra of the recombinant enzymes. (A) AbGDH. (B) AbuGDH. (C) AcGDH. (D) AmGDH. (E) ApGDH. (F) AtaGDH. (G) AuGDH. (H) AoGDH. (I) AtGDH. The spectra of the enzymes (2 mg/mL) in the absence and presence of 100 mM glucose are indicated as *solid lines* and *dashed lines*, respectively.

GDHs from *A. oryzae* and *A. terreus* were found as extracellular enzymes [13,32], the activity of FAD-GDH in fungal culture media often depends on the culture conditions and the production level. In previous study, we identified four FAD-GDH gene homologs from thermophilic fungi by the degenerate PCR screening method [29,30]. Degenerate PCR screening is an effective method to directly examine the existence of FAD-GDH gene homologs contained in fungal genomic DNAs even if the complete genomic DNA sequences are unknown. In the present study, we identified seven FAD-GDH gene homologs in the genomic DNAs of *Aspergillus* strains, and successfully cloned them. We subsequently prepared the recombinant enzymes by using the secreted production system of *P. pastoris*, and characterized their enzymatic properties.

Investigation of dehydrogenase activity to various saccharides revealed the substrate selectivity of six FAD-GDHs (Table 3). Maltose insensitivity was the common property. This property is important because of past fatal accidents, in which blood glucose sensors using pyrroloquinoline quinone (PQQ)-dependent GDH (EC 1.1.5.2) exhibited falsely elevated blood glucose levels by reacting to maltose contained in medical products [33]. This can result in the administration of insulin, which may lead to hypoglycemia [45]. In Table 3, AoGDH and AtGDH, as practical benchmark enzymes, exhibited high substrate selectivity, although these enzymes exhibited reactivity to D-xylose (10.5 ± 6.7% and 10.1 ± 1.1%, respectively). AbGDH and AtaGDH with high substrate selectivity also exhibited reactivity toD-xylose (36.1 ± 8.3% and 10.1 ± 6.6%, respectively). Notably, it was revealed that ApGDH had the strictest substrate selectivity and exhibited no reactivity toward not only maltose and D-galactose but also D-xylose. On the other hand, broad substrate selectivity was observed in AbuGDH, AcGDH, and AmGDH, which exhibited reactivity to five or six saccharides, including D-galactose (4.6–93.9%) and D-xylose (28.1–83.1%). PQQ-GDH, utilized in blood glucose sensors, exhibited reactivity to not only maltose but also galactose and xylose [1,33,46].

Table 2
Summary of enzymatic properties. Specific activity indicates the GDH activity in the presence of 300 mM glucose. V_{max} and K_m were calculated by Michaelis-Menten curve-fitting using the least square method. pH stability indicates the pH values of enzymes with > 90% of the residual activity in Fig. S5. The apparent T_m value was determined from the data in Fig. 3.

FAD-GDH	Specific activity (U/μmol)	V_{max} (U/μmol)	K_m (mM)	pH stability	Optimum pH	T_m (°C)	Optimum temperature (°C)
AbGDH	(3.21 ± 0.10) × 10^3	(2.41 ± 0.28) × 10^4	(1.66 ± 0.35) × 10^3	3.0–10.0	5.0–6.0	72.5	65
AbuGDH	(4.84 ± 0.20) × 10^3	(1.19 ± 0.02) × 10^4	0.41 ± 0.04	2.0–7.0	5.0–6.0	56.2	50–55
AcGDH	(9.99 ± 0.49) × 10^3	(2.62 ± 0.06) × 10^4	0.96 ± 0.12	2.0–7.0	5.0	55.8	50–55
AmGDH	(7.35 ± 0.29) × 10^3	(1.95 ± 0.04) × 10^4	0.75 ± 0.09	2.0–7.0	5.0	62.0	60
ApGDH	(5.38 ± 0.34) × 10^4	(8.66 ± 0.17) × 10^4	(1.76 ± 0.07) × 10^2	3.0–5.0	7.0	49.9	45–50
AtaGDH	(9.57 ± 0.18) × 10^4	(1.14 ± 0.03) × 10^5	(5.38 ± 0.36) × 10	5.0–6.0	6.0–8.0	53.9	50
AoGDH	(6.12 ± 0.21) × 10^4	(6.61 ± 0.22) × 10^4	(1.29 ± 0.18) × 10	4.0–6.0	6.0–7.0	52.2	50–60
AtGDH	(7.73 ± 0.03) × 10^4	(9.23 ± 0.10) × 10^4	(5.97 ± 0.20) × 10	4.0–7.0	6.0–8.0	56.8	50–65

Table 3
Substrate specificity of FAD-GDHs. Dehydrogenase activity toward various saccharides (40 mM) were examined at pH 7.0 and 37 °C. Activity toward D-glucose was set as 100%. ND indicates that activity was not detected.

Substrate	Relative activity (%)							
	AbGDH	AbuGDH	AcGDH	AmGDH	ApGDH	AtaGDH	AoGDH	AtGDH
D-Glucose	100	100	100	100	100	100	100	100
D-Arabinose	ND	ND	ND	ND	ND	ND	ND	ND
2-Deoxy-D-glucose	ND	106.1 ± 7.9	108.1 ± 4.8	110.5 ± 4.8	17.4 ± 3.9	23.0 ± 2.7	37.5 ± 3.8	30.6 ± 1.3
D-Fructose	ND	ND	ND	ND	ND	ND	ND	ND
D-Galactose	ND	93.9 ± 7.2	4.6 ± 3.8	7.6 ± 1.6	ND	ND	ND	ND
Lactitol	ND	ND	ND	ND	ND	ND	ND	ND
Maltitol	ND	ND	ND	ND	ND	ND	ND	ND
Maltose	ND	ND	ND	ND	ND	ND	ND	ND
D-Mannitol	ND	ND	ND	ND	ND	ND	ND	ND
D-Mannose	ND	89.8 ± 3.3	25.3 ± 3.7	38.7 ± 3.6	ND	ND	ND	ND
D-Raffinose	3.0 ± 1.8	17.2 ± 2.2	5.4 ± 2.7	11.2 ± 1.9	ND	ND	ND	ND
D-Sorbitol	ND	ND	ND	ND	ND	ND	ND	ND
Sucrose	ND	15.9 ± 3.4	ND	10.4 ± 6.0	ND	ND	ND	ND
D-Trehalose	ND	ND	ND	ND	ND	ND	ND	ND
D-Xylose	36.1 ± 8.3	28.1 ± 6.5	66.8 ± 4.1	83.1 ± 5.1	ND	10.1 ± 6.6	10.5 ± 6.7	10.1 ± 1.1

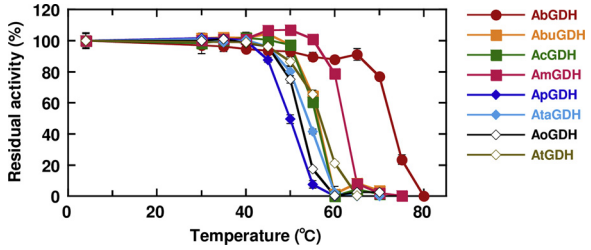

Fig. 3. Effect of temperature on FAD-GDH stability. FAD-GDH (7 U/ml) was incubated at indicated temperatures at pH 5.0 for 15 min, and then diluted with 50 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. This diluted solution was used for the assay.

Thus, insensitivity to galactose and xylose in blood glucose sensors is recommended [33]. On the other hand, in current situation, commercially available blood glucose sensors using FAD-GDH can exhibit reactivity to xylose [47–49], and those using PQQ-GDH can exhibit reactivity to galactose [50,51]. Therefore, in the aspect of substrate selectivity, we suggest that six FAD-GDHs identified in this study will be used in blood glucose sensors. Recently, several studies of PQQ-GDH have achieved an improvement in its substrate selectivity by amino acid substitution approach [52–54]. Thus, improvement of the substrate selectivity of the six FAD-GDHswould be preferable to minimalize the reactivity to saccharides for blood glucose sensors, as necessary.

In addition to the common property of maltose insensitivity, enzymatic parameters related to the performance of blood glucose sensors and other electrochemical devices were revealed (Table 2). The properties of six FAD-GDHs were compared to those of AoGDH and AtGDH. The properties of AoGDH and AtGDH were similar to those of previously reported FAD-GDHs from *A. oryzae* and *A. terreus* [13,14,31,32]. ApGDH and AtaGDH exhibited high activity, and the V_{max} values were similar to or higher than those of AoGDH and AtGDH. AbuGDH, AcGDH, and AmGDH exhibited very high affinity for glucose, and the K_m values were 0.41–0.96 mM. These K_m values indicate that their glucose affinity is higher than that of AoGDH, AtGDH, and other fungal FAD-GDHs such as *A. niger* FAD-GDH (3.8 mM) [24], *T. crustaceus* FAD-GDH (2.82 mM) [30], and *T. emersonii* FAD-GDH (337 mM) [30]. Among the examined enzymes, AbGDH exhibited the highest thermostability with an apparent T_m value of 72.5 °C. Moreover, AbGDH exhibited the highest optimum temperature (65 °C) and pH stability, exhibiting stability at a wide pH range of 3.0–10.0. It is surprising that the T_m value of AbGDH was higher than the T_m values of *T. crustaceus* and *T. emersonii* FAD-GDHs (apparent T_m, 62.5 and 66.4 °C, respectively) produced by *P. pastoris* [30]. High thermostability was also observed in AmGDH, whose apparent T_m value was 62.0 °C. Another study on FAD-GDH from *A. terreus* NIH2624 reported that the recombinant enzyme exhibited high thermostability and approximately 84% residual activity after heat treatment at 60 °C for 15 min [20]. These data suggest that the thermostable FAD-GDH gene homologs are contained in genomic DNAs of some *Aspergillus* species or other mesophilic fungi. A comparison of the amino acid identities of FAD-GDHs showed 60.4% of the average value (Fig. S3). The sequence identities among GDHs with high V_{max} values (ApGDH, AtaGDH, AoGDH, and AtGDH) were 62.6–92.9%. The sequence identities among GDHs with high affinity for glucose (AbuGDH, AcGDH, AmGDH, and *T. crustaceus* FAD-GDH) were 69.2–79.9%. The sequence identities among GDHs with high thermostability (AbGDH and thermophilic fungal FAD-GDHs) were 54.7–66.7%. These data indicated that FAD-GDHs with high activity and high affinity for glucose have relatively higher amino acid identities, respectively.

Our previous studies indicated that one of the factors on thermostability of recombinant enzymes was glycosylation [29,30], where an increased thermostability of 5.2–13.7 °C was observed in glycosylated *T. aurantiacus*, *T. crustaceus*, and *T. emersonii* FAD-GDHs. Increased thermostability would in many cases be attributable to the sugar chains contributing to the overall protein structural stability [55]. Therefore, glycosylation by *P. pastoris* in our present study would contribute to the thermostability of recombinant FAD-GDHs from the *Aspergillus* strains. Among these glycosylated enzymes, there was no correlation between the degree of glycosylation (Fig. 1) and the apparent T_m values (Table 2). The stability of glycosylated FAD-GDHs would depend on the enzymes, rather than the degree of glycosylation [30].

The SDS-PAGE of the recombinant enzymes (Fig. 1) give us perspective regarding the relationship between the number of *N*-glycosylation sites and apparent molecular masses. For instance, AbGDH had nine *N*-glycosylation-predicted asparagine residues and exhibited an apparent molecular mass of ~75 kDa. In contrast, larger apparent molecular masses of 90–250 kDa were observed in AtaGDH, which also had nine *N*-glycosylation-predicted asparagine residues. These results may be attributed to two reasons. The first is that some asparagine residues in the sequence of Asn-*X*-Ser/Thr were prevented from undergoing *N*-glycosylation. The prevention of *N*-glycosylation may be attributable to some sequences that are buried in the core of the protein after folding, and are involved in strong hydrogen-bonding, or disulfide bonding nearby [55]. The second reason is that the *N*-linked sugar chains have diverse sizes. Generally, the structures of *N*-linked sugar chains produced by *P. pastoris* are of the high-mannose-type. The majority of the *N*-linked sugar chains are of the $Man_{8–14}GlcNAc_2$ size, with the remainder being much larger, i.e., of the $Man_{30–50}GlcNAc_2$ size

[56]. The diverse sizes of the *N*-linked sugar chains may also result in diverse structural inhomogeneity of the enzyme population where two different molecules of the same enzymes in a cell may have different amounts of *N*-linked sugar chains [55]. As seen in Fig. 1A, the inhomogeneity of the enzyme population was observed as broad smeared bands in the lanes with ApGDH, AtaGDH, AuGDH, AoGDH, and AtGDH. In contrast, comparatively narrower smeared bands were observed in the lanes with the other enzymes, indicating that the structures of the *N*-linked sugar chains in the enzyme populations were comparatively homogeneous. As all the recombinant enzymes in this study were produced by *P. pastoris* under the same culture conditions, the structural homogeneity of the *N*-linked sugar chains in the enzyme populations would depend on the enzymes, but not on the protein yield per liter of culture media. Recently, we discovered mediatorless direct electron transfer (DET) between FAD-GDHs and single-walled carbon nanotubes [57,58]. The DET systems employing glycosylated FAD-GDHs produced by *P. pastoris* exhibited higher current output than those employing unglycosylated FAD-GDHs. Notably, the current output of the DET systems employing *Saccharomyces cerevisiae*-produced FAD-GDHs with a much larger amount of glycosylation was smaller than those employing *P. pastoris*-produced FAD-GDHs. Therefore, we suggest that it is important to understand the roles of glycosylation on stability, and control the amount of glycosylation and the structural homogeneity of sugar chains in the enzyme population for the effective use of FAD-GDH in bioelectrochemical systems, especially for the mediatorless DET systems using single-walled carbon nanotubes.

5. Conclusions

In this study, we obtained six maltose-insensitive FAD-GDHs. The maltose insensitivity is important for use in blood glucose sensors. Furthermore, we revealed the enzymatic properties of the FAD-GDHs, which exhibited high activity, high affinity for glucose, or high thermostability. Recently, FAD-GDH has been also used in sensors for various fields and applications, as well as in biofuel cells. The bioelectrochemical devices in these fields are required to exhibit high current output, high sensitivity, and high stability for pH and heat. Therefore, we propose that our FAD-GDHs will lead to the development of blood glucose sensors and other bioelectrochemical devices, such as those exhibiting high substrate selectivity, high current output, high sensitivity, and/or high stability for usage and storage.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.bej.2018.09.014.

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