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Article

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# Thermostable FAD-dependent Glucose Dehydrogenases from Thermophilic Filamentous Fungus *Thermoascus aurantiacus*

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**ABSTRACT**

We newly identified FAD-dependent glucose dehydrogenase (FADGDH) gene homologs from thermophilic filamentous fungus *Thermoascus aurantiacus*. The gene homologs were cloned from two strains of *Th. aurantiacus*, NBRC 6766 and NBRC 9748. Recombinant FADGDHs of the two strains were prepared by using *Escherichia coli* and *Pichia pastoris* as hosts. Absorption spectra and enzymatic characterization clearly showed that these enzymes contained oxidized FAD as a coenzyme and exhibited glucose dehydrogenase activity. Analysis of thermal stability revealed that the denaturation midpoints ($T_m$) of these FADGDHs were at least above 71.4°C. The results showed that these FADGDHs exhibited remarkably high thermal stability. We also performed bioelectrochemical experiments using unglycosylated and glycosylated FADGDHs of *Th. aurantiacus* NBRC 6766, which exhibited higher affinities for glucose than those of *Th. aurantiacus* NBRC 9748 FADGDHs. *Th. aurantiacus* NBRC 6766 FADGDH-immobilized electrodes effectively showed current responses to glucose. Therefore, these thermostable FADGDHs could be applied to a bioelectro-catalyst for glucose oxidation with long-term storage and continuous use.



Keywords : FAD-dependent Glucose Dehydrogenase, Gene Cloning, Thermostable Enzyme

## 1. Introduction

Glucose oxidase and glucose dehydrogenase, which catalyze the oxidation of D-glucose at its 1-hydroxyl group to D-glucono-1,5-lactone, are core components of amperometric glucose sensors. For clinical test of diabetes, glucose oxidase (GOD; EC 1.1.3.4), NAD(P)-dependent glucose dehydrogenase (GDH) (EC 1.1.1.47) or pyrroloquinoline quinone (PQQ)-dependent GDH (EC 1.1.5.2) has been generally equipped on the biosensors to determine blood glucose concentration. However, usage of these enzymes for blood glucose sensors has the following limitations: 1) GOD requires molecular oxygen as a natural electron acceptor, and dissolved oxygen in the blood influences the accuracy of measurements for glucose concentration even if in the presence of an electron transfer mediator; 2) NAD(P)GDH needs NAD addition to the measurement system; 3) PQQGDH exhibits broad substrate specificity.[1–3]

Recently, FAD-dependent GDH (EC 1.1.5.9) containing FAD as a coenzyme has become a focus of research related to an expedient component of a mediated enzyme sensor.[2] In this type of glucose sensor, electron is transferred from FAD as the primary electron acceptor to an external electron acceptor except for molecular oxygen during the enzymatic oxidation of D-glucose. Several studies on FADGDHs from filamentous fungi, including *Aspergillus oryzae*,[4–6] *Aspergillus terreus*,[7–9] *Aspergillus flavus*,[10,11] *Aspergillus niger*,[10] *Glomerella cingulata*,[12,13] and *Mucor prainii*,[14] have been reported. These FADGDHs have suitable enzymatic properties for glucose sensors. Preferably, more thermostable FADGDH is required for industrial application.

We previously reported degenerate PCR screening of 17 species of thermophilic filamentous fungi to identify a novel FADGDH gene homolog. Two thermostable FADGDH gene homologs were identified and cloned from *Talaromyces emersonii* NBRC 31232 and *Thermoascus crustaceus* NBRC 9129.[15] In this study, we re-tried the degenerate PCR screening of the thermophilic filamentous fungi to discover a novel FADGDH gene homolog. We successfully identified and cloned novel FADGDH gene homologs from *Thermoascus aurantiacus* NBRC 6766 and *Th. aurantiacus* NBRC 9748. Furthermore, we also characterized the enzymatic properties of the recombinant FADGDHs produced by *E. coli* and *P. pastoris*.

## 2. Experimental

### 2.1 Strains, vectors, and primers

*Th. aurantiacus* NBRC 6766 and *Th. aurantiacus* NBRC 9748 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 general gene cloning. The expression vector pET-21b(+) (Merck Millipore) and *E. coli* BLR(DE3) (Merck Millipore) were used to produce unglycosylated recombinant enzymes. The expression vector pPIC9 (Life Technologies) and *P. pastoris* GS115 (Life Technologies) were used to produce glycosylated recombinant enzymes. Primers used in this study are listed in Table 1.

### 2.2 Degenerate PCR screening for FADGDH gene homologs

Degenerate PCR was performed by using KOD FX (Toyobo), degenerate primers, and genomic DNA extracted from *Th. aurantiacus* as a template. The nucleotide sequences of the degenerate primers were designed based on the primary structures of *A. oryzae* FADGDH (accession number DJ347964) and *A. terreus* FADGDH (accession number DD412265), and shown in our previous study.[15] The amplified DNA fragments in the PCR products were separated by agarose gel electrophoresis, and then extracted

[#] These authors contributed equally to this work.

**Table 1.** Primers used in this study.

| Primers | Sequence (5′ to 3′) | Purpose of use |
|---|---|---|
| GSP2 | GGATTGTACGAGGCACCTGCCGCCATCTGC | Cloning of FADGDH gene homologs |
| GSP3 | TGAACGCGACAGTCCTACAGCGCCTGTTCC | |
| GSP7 | AGAGATCTCTGTCAATTTCTTACGGAAGCT | |
| GSP8 | TAGATATCTTTTTCTTTCTTTAATGACGTG | |
| E1S | ACCATGTTATTTTGTGTGAGGATTCTCAGT | Synthesis of CDSs |
| E1A | GCCATCCCATTGATCGTGCTCGACCCACCG | |
| E2S | GATCAATGGGATGGCTTATGCTCGCGCGGA | |
| E2A | TAGAATCCTTGGATTTCCAACTCCAGAAAG | |
| E3S | AATCCAAGGATTCTAAACCAGTACAACATC | |
| E3A | GAGCGGTAGCTTGCTTTGATCCAGTTCTCC | |
| E4S | AGCAAGCTACCGCTCAAATTTCCACCCTGT | |
| E4A | AGGTTACAGAGCGGCAGCATCCTCCTTGAC | |
| Esc1 | AAACATATGGCCACTTCGCTATCATCACGT | Construction of expression plasmids |
| Esc2 | TTTAAGCTTTTAGTGGTGGTGGTGGTGGTG | |
| | CAGAGCGGCAGCATCCTCCTTGACCAT | |
| Pic1 | AAAGAATTCGCCACTTCGCTATCATCACGT | |
| Pic2 | TTTGCGGCCGCTTAGTGGTGGTGGTGGTGG | |
| | TGCAGAGCGGCAGCATCCTCCTTGACC | |

from preparative agarose gels. Each extracted DNA fragment was subcloned into the cloning vector, followed by determining the nucleotide sequence. The predicted amino acid sequences were compared to those of *A. oryzae* and *A. terreus* FADGDHs.

### 2.3 Cloning of FADGDH gene homologs

The genomic DNA from *Th. aurantiacus* was digested by various restriction enzymes, and analyzed by Southern hybridization. The DNA fragment amplified by degenerate PCR, representing a portion of the FADGDH gene homolog, was used as a probe. As a result, a single band hybridized with the probe was detected at approximately 4.0 kb in *Sac*I lane. Inverse PCR was performed to clone the upstream region of the start codon and the downstream region of the stop codon of the FADGDH gene homolog. The *Sac*I-digested genomic DNA was subjected to preparative agarose gel electrophoresis, and the DNA fragments around 4.0 kb were extracted from the gel. These DNA fragments were self-ligated with T4 DNA ligase and used as the template for inverse PCR. The primers for inverse PCR, GSP2 and GSP3, were designed based on the nucleotide sequence of the internal FADGDH gene homolog from *Th. aurantiacus*. The DNA fragment containing 5′- and 3′-flanking regions of the FADGDH gene homolog was amplified by inverse PCR with KOD -Plus- Neo (Toyobo). The genomic DNA fragment containing the FADGDH gene homolog was amplified by PCR using KOD -Plus- Neo, sense primer GSP7, antisense primer GSP8, and *Th. aurantiacus* genomic DNA. Primers GSP7 and GSP8 were designed based on the nucleotide sequence of the inverse PCR-amplified DNA fragment.

### 2.4 Synthesis of the coding sequence (CDS)

The CDS of the FADGDH gene homolog from *Th. aurantiacus* was artificially synthesized by overlap extension PCR.[16] Four exons were individually amplified and connected to synthesize the complete structural gene. The primers used were as follows: E1S and E1A for exon 1, E2S and E2A for exon 2, E3S and E3A for exon 3, and E4S and E4A for exon 4.

### 2.5 Preparation of recombinant enzymes

For construction of an *E. coli* expression plasmid, the gene encoding mature FADGDH was generated by PCR using primers Esc1 and Esc2. Primers Esc1 and Esc2 contained *Nde*I site, and *Hin*dIII site and six CAC codons encoding a histidine tag located at the C-terminus, respectively. The amplified DNA fragment was inserted between *Nde*I and *Hin*dIII sites in the pET-21b(+) vector. The recombinant enzyme produced by *E. coli* cells harboring the expression plasmid was purified by using a Ni-NTA agarose column (Qiagen) and a Q Sepharose Fast Flow column (GE Healthcare).

For construction of a *P. pastoris* expression plasmid, the gene encoding mature FADGDH was generated by PCR using primers Pic1 and Pic2. 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 and *Eco*RI sites. The amplified DNA fragment was inserted between *Eco*RI and *Not*I sites in the pPIC9 vector. By inserting the DNA fragment into the vector, the yeast secretion signal peptide was fused to the mature FADGDH. The resulting plasmid cleaved with *Sal*I was introduced into *P. pastoris* GS115 genomic DNA. The recombinant enzyme produced in the culture media of the strain carrying the expression plasmid was purified by using a Ni-NTA agarose column.

### 2.6 GDH assay

GDH activity was measured by the 2,6-dichloroindophenol (DCIP) method.[15] One unit was defined as the amount of FADGDH producing 1 µmol of reduced DCIP per minute at pH7.0 and 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 ($11.3\,mM^{-1}\,cm^{-1}$) was used to calculate the concentration of FAD in the enzyme. Activity was shown as unit per 1 µmol of FAD, which is equal to 1 µmol of holo-enzyme.

### 2.7 Current response of FADGDH-immobilized electrode

Electrochemical measurements were performed with a two-electrode system using an ALS model 1202 electrochemical analyzer (BAS). FADGDH was mixed with a hydrophilic polymer BIOSURFINE-AWP (Toyo Gosei) for UV cross-linking. The

mixture was applied onto the surface of a platinum electrode (9 $mm^2$) and immobilized by UV irradiation. The resulting electrode and a counter electrode (9 $mm^2$ platinum) were immersed in 50 mM potassium phosphate buffer (pH7.4) containing 200 mM potassium ferricyanide. Voltage between two electrodes was fixed at 300 mV. After the current value reached steady state, glucose in the same buffer was successively dropped into the solution, and the current value was measured.

### 2.8 Accession numbers

The nucleotide sequences of the FADGDH genes have been deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC096957 and LC096958 for *Th. aurantiacus* NBRC 6766, and LC096959 and LC096960 for *Th. aurantiacus* NBRC 9748.

## 3. Results and Discussion

### 3.1 Degenerate PCR screening for FADGDH gene homologs

We retried degenerate PCR screening of 17 species of thermophilic filamentous fungi to discover a novel FADGDH gene homolog. In this second trial, partial FADGDH gene homologs were successfully amplified when using each genomic DNA extracted from *Th. aurantiacus* NBRC 6766 and NBRC 9748 as a template. The results of agarose gel electrophoresis of the amplified DNA fragments by the degenerate PCR are shown in Fig. 1(a) and (b). DNA bands of approximately 1.3–2.0 kb were detected from the PCR products derived from the genomic DNAs of *Th. aurantiacus* NBRC 6766 and NBRC 9748. Nucleotide sequencing of these DNA fragments revealed that the predicted amino acid sequences were homologous to those of *A. oryzae* and *A. terreus* FADGDHs. These results indicated that the genomic DNAs of *Th. aurantiacus* NBRC 6766 and NBRC 9748 contained novel FADGDH gene homologs.

### 3.2 Cloning of FADGDH gene homologs

The genomic DNA fragments containing the FADGDH gene homologs were cloned from *Th. aurantiacus* NBRC 6766 and NBRC 9748. Nucleotide sequencing revealed that these fragments contained open reading frames of the FADGDH gene homologs, and the structural genes were divided into four exons by three introns. Both putative FADGDH gene homologs contained 1812 bp encoding 603 amino acids.

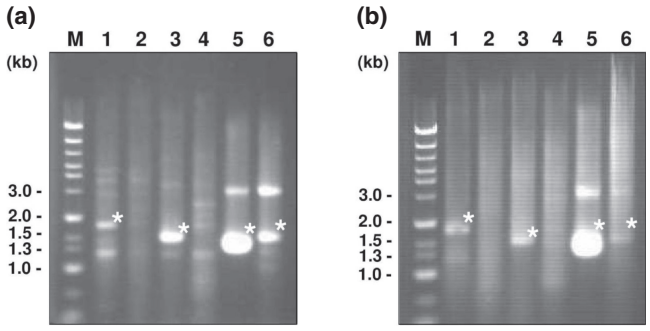


**Figure 1.** Agarose gel electrophoresis of DNA fragments amplified by degenerate PCR. The DNA fragments in the gels (1.2% agarose gel) were stained with ethidium bromide. The agarose gels of the PCR products derived from (a) *Th. aurantiacus* NBRC 6766 and (b) *Th. aurantiacus* NBRC 9748 genomic DNA as templates. Lane M indicates DNA size markers; lanes 1–6 represent the PCR products using the following primers: lane 1, HCA and HCD; lane 2, HCA and HCE; lane 3, HCB and HCD; lane 4, HCB and HCE; lane 5, HCC and HCD; lane 6, HCC and HCE. The DNA bands of partial FADGDH gene homologs are indicated by asterisks.

The secretion signal peptides were identified as 16 residues from Met1 to Ala16 in *Th. aurantiacus* FADGDHs by SignalP 4.1.[17] The amino acid sequences implicated in FAD-binding (Gly-X-Gly-X-X-Gly, where X represents any amino acid residue)[18] were conserved in the FADGDHs. Eleven and ten asparagine residues for potential *N*-glycosylation (Asn-X-Ser/Thr, where X represents any residue, except for proline)[19] were found in *Th. aurantiacus* NBRC 6766 FADGDH and *Th. aurantiacus* NBRC 9748 FADGDH, respectively. Six amino acid residues were different between these *Th. aurantiacus* FADGDHs (99% identity). *Th. aurantiacus* NBRC 6766 FADGDH showed 57% and 58% identity with *A. oryzae* and *A. terreus* FADGDHs, respectively. *Th. aurantiacus* NBRC 9748 FADGDH showed 57% and 57% identity with *A. oryzae* and *A. terreus* FADGDHs, respectively.

### 3.3 Preparation of recombinant enzymes

*Th. aurantiacus* NBRC 6766 mature FADGDH (6766FADGDH) and *Th. aurantiacus* NBRC 9748 mature FADGDH (9748FADGDH) were produced by *E. coli*. Although the recombinant FADGDHs formed inclusion bodies in *E. coli* cells, the slightly produced soluble FADGDHs were purified from the soluble fractions of the cells. Protein bands denoting the purified FADGDHs were detected by SDS-PAGE at 65 kDa for 6766FADGDH and 9748FADGDH, which were consistent with the theoretical molecular masses of both FADGDHs [Fig. 2(a)]. The theoretical molecular masses of these enzymes with their histidine tags were calculated at 65 kDa for both FADGDHs. When produced by *P. pastoris*, both FADGDHs were secreted into the culture media, and then purified. Protein bands denoting the purified FADGDHs were detected at 80–150 kDa for both FADGDHs by Coomassie brilliant blue (CBB) staining [Fig. 2(b), *left*]. These enzymes were also stained with periodic acid-Schiff (PAS), indicating that they had undergone glycosylation during post-translational modification in *P. pastoris* [Fig. 2(b), *right*].

Absorption spectra of the FADGDHs produced by *E. coli* (unglycosylated FADGDH) [Fig. 3(a)] and *P. pastoris* (glycosylated FADGDH) [Fig. 3(b)] were similar to the typical spectrum observed for oxidized flavin with two major peaks at 380 nm and 450 nm. The peaks corresponding to oxidized flavin disappeared after addition of glucose, and also after addition of sodium dithionite as a reductant. These results suggested that 6766FADGDHs and 9748FADGDHs produced by *E. coli* and *P. pastoris* were purified as holo forms containing oxidized FAD as a coenzyme.

### 3.4 GDH activity

GDH activity at pH7.0 and 37°C was measured by the DCIP method. The specific activities of unglycosylated and glycosylated 6766FADGDHs in the presence of 300 mM glucose were $3.1 \times 10^2$ U/μmol-FAD and $3.2 \times 10^2$ U/μmol-FAD, respectively, whereas those of unglycosylated and glycosylated 9748FADGDHs were $2.1 \times 10^2$ U/μmol-FAD (see Table 2 as a summary of enzymatic properties). The specific activities of 6766FADGDHs were slightly higher than those of 9748FADGDHs. In addition, the GOD activities of 6766FADGDHs and 9748FADGDHs were investigated. None of the FADGDHs prepared in this study exhibited GOD activity. Therefore, our results revealed that these enzymes were FAD-dependent GDHs.

The activities of 6766FADGDHs and 9748FADGDHs depended on glucose concentration [Fig. 4(a) and (b)]. The Michaelis-Menten constant ($K_m$) and the maximum velocity ($V_{max}$) were calculated by a Lineweaver-Burk plot. The $K_m$ values were 1.7 M for unglycosylated 6766FADGDH and glycosylated 6766FADGDH, 3.7 M for unglycosylated 9748FADGDH, and 3.5 M for glycosylated 9748FADGDH. The $V_{max}$ values were $2.1 \times 10^3$ U/μmol-FAD for unglycosylated 6766FADGDH, $2.2 \times 10^3$ U/μmol-FAD for glycosylated 6766FADGDH, whereas those of unglycosylated

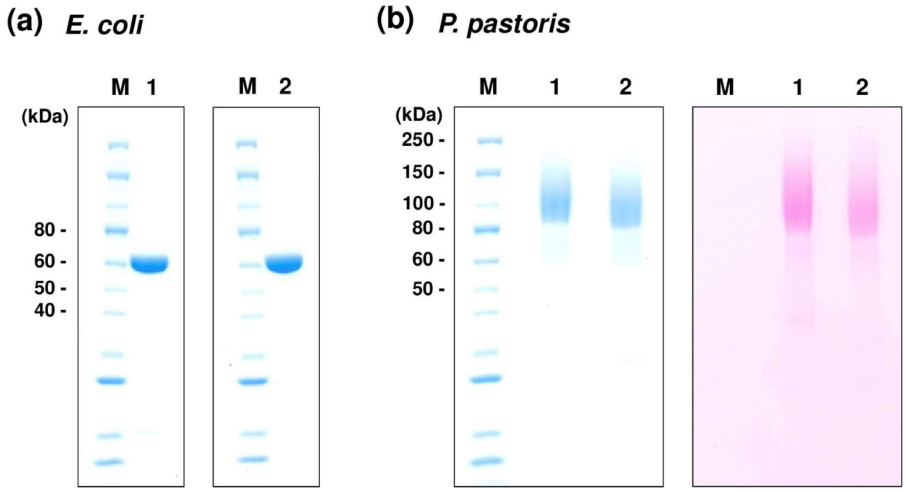


**Figure 2.** (Color online) SDS-PAGE of FADGDHs. Purified FADGDHs were subjected to SDS-PAGE (5 µg/lane). (a) FADGDHs produced by *E. coli*. Proteins were stained with CBB. (b) FADGDHs produced by *P. pastoris*. Proteins in the left gel were stained with CBB, and glycosylated proteins in the right gel were stained with PAS. M indicates molecular mass markers. In both panels, lane 1 represents 6766FADGDH, and lane 2 represents 9748FADGDH.

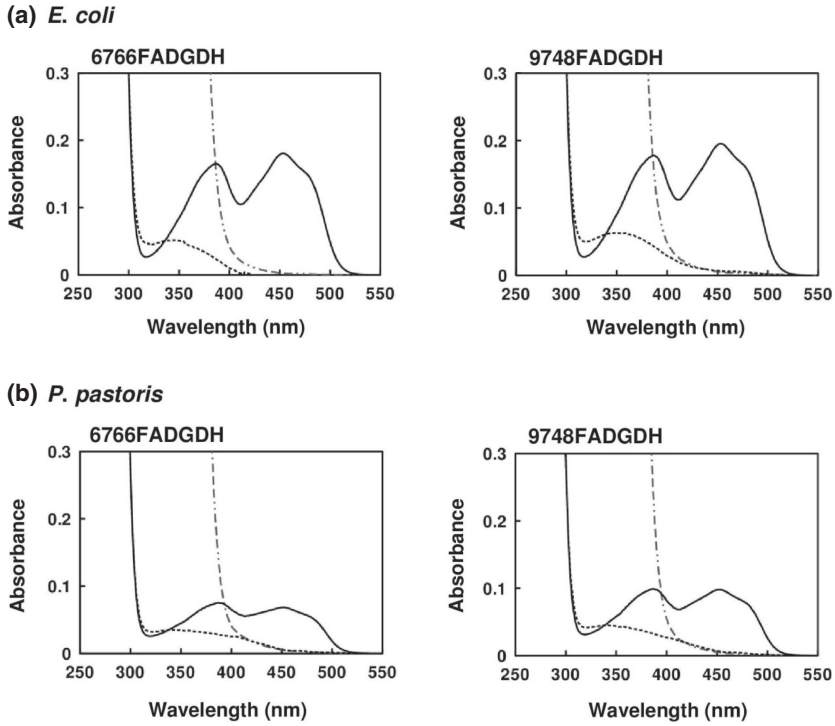


**Figure 3.** Absorption spectra of FADGDHs. (a) Unglycosylated FADGDHs produced by *E. coli*. (b) Glycosylated FADGDHs produced by *P. pastoris*. The spectra of 6766FADGDHs and 9748FADGDHs are on the left and right sides, respectively. The spectra of purified FADGDHs (2 mg/mL) are drawn as solid lines, the spectra of FADGDHs in the presence of 150 mM glucose are drawn as dashed lines, and the spectra of FADGDHs in the presence of sodium dithionite are drawn as dotted and dashed lines.

**Table 2.** Summary of enzymatic properties.

| Enzymatic properties | 6766FADGDH | | 9748FADGDH | |
|---|---|---|---|---|
| | Unglycosylated | Glycosylated | Unglycosylated | Glycosylated |
| Specific activity (U/µmol-FAD) | $3.1 \times 10^2$ | $3.2 \times 10^2$ | $2.1 \times 10^2$ | $2.1 \times 10^2$ |
| $V_{max}$ (U/µmol-FAD) | $2.1 \times 10^3$ | $2.2 \times 10^3$ | $2.8 \times 10^3$ | $2.8 \times 10^3$ |
| $K_m$ (M) | 1.7 | 1.7 | 3.7 | 3.5 |
| pH stability | 4.0–11.0 | 3.0–11.0 | 3.0–11.0 | 3.0–11.0 |
| Optimum pH | 6.0 | 6.0–7.0 | 5.0 | 5.0 |
| $T_m$ (°C) | 71.4 | 76.8 | 72.6 | 77.8 |
| Optimum temperature (°C) | 70 | 70 | 70≤ | 70≤ |

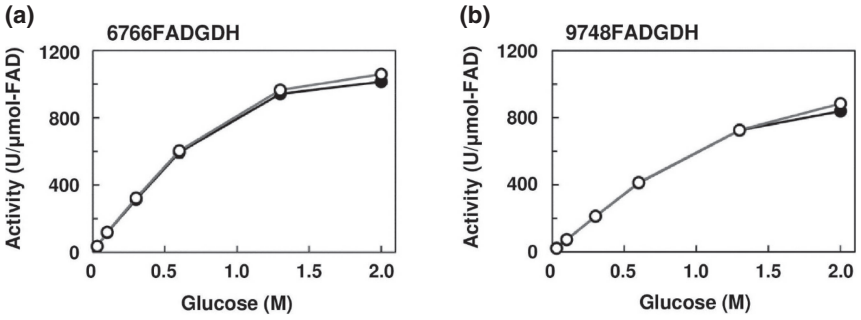


**Figure 4.** Glucose concentration dependence of FADGDH activity. Michaelis-Menten saturation curves for (a) 6766FADGDHs and (b) 9748FADGDHs. In both panels, closed circles represent unglycosylated FADGDHs, and open circles represent glycosylated FADGDHs.

**Table 3.** Substrate specificity of FADGDH activity. Dehydrogenase activity to various saccharides (40 mM) was examined at pH7.0 and 37°C. Activity to D-glucose was set as 100%. N.D. indicates that activity was not detected.

| Substrate | Relative activity (%) | | | |
|---|---|---|---|---|
| | Unglycosylated 6766FADGDH | Glycosylated 6766FADGDH | Unglycosylated 9748FADGDH | Glycosylated 9748FADGDH |
| D-Glucose | 100 | 100 | 100 | 100 |
| D-Arabinose | N.D. | N.D. | N.D. | N.D. |
| D-Cellobiose | N.D. | N.D. | N.D. | N.D. |
| 2-Deoxy-D-Glucose | 8.4 | 8.4 | 11.0 | 11.0 |
| D-Fructose | N.D. | N.D. | N.D. | N.D. |
| D-Galactose | N.D. | N.D. | N.D. | N.D. |
| Lactitol | N.D. | N.D. | N.D. | N.D. |
| Maltitol | N.D. | N.D. | N.D. | N.D. |
| Maltose | 22.3 | 19.8 | 12.2 | 11.5 |
| D-Mannitol | N.D. | N.D. | N.D. | N.D. |
| D-Mannose | N.D. | N.D. | N.D. | N.D. |
| D-Raffinose | 4.6 | 4.5 | 3.2 | 3.3 |
| D-Sorbitol | N.D. | N.D. | N.D. | N.D. |
| Sucrose | N.D. | N.D. | N.D. | N.D. |
| D-Trehalose | N.D. | N.D. | N.D. | N.D. |
| D-Xylose | 39.6 | 42.0 | 49.8 | 52.5 |

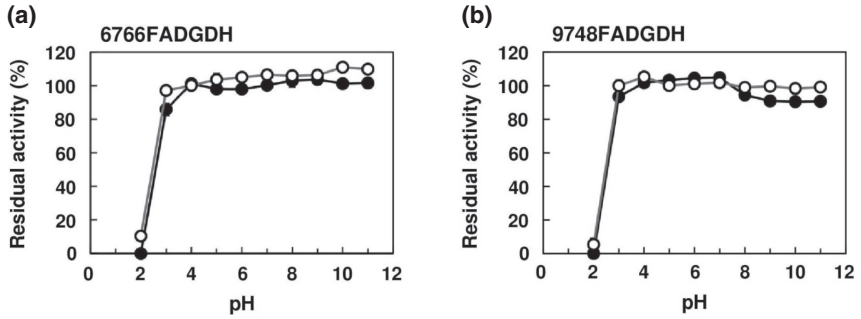


**Figure 5.** Effect of pH on FADGDH stability. FADGDH (7 U/mL) was incubated in 50 mM Britton-Robinson universal buffer[21] at indicated pH at 25°C for 20 h, and then diluted with 50 mM potassium phosphate buffer (pH7.0) containing 0.1% Triton X-100. This diluted solution was used for assay. (a) 6766FADGDHs. (b) 9748FADGDHs. In both panels, closed circles represent unglycosylated FADGDHs, and open circles represent glycosylated FADGDHs.

9748FADGDH and glycosylated 9748FADGDH were $2.8 \times 10^3$ U/μmol-FAD.

The substrate specificity of FADGDH activity was examined (Table 3). 6766FADGDHs and 9748FADGDHs exhibited the highest activity to D-glucose, although they exhibited cross-reactivity to 2-deoxy-D-glucose, maltose, D-raffinose, and D-xylose. Glucose sensors employing enzymes with broad substrate specificity would lead to potentially fatal errors for blood glucose monitoring.[20] Therefore, improvement of the substrate specificity by protein engineering of *Th. aurantiacus* FADGDHs should be required.

### 3.5 Effect of pH and temperature on FADGDH stability

The pH stability of the FADGDHs was examined (Fig. 5). All the FADGDHs were stable in a wide pH range between 3.0 and 11.0

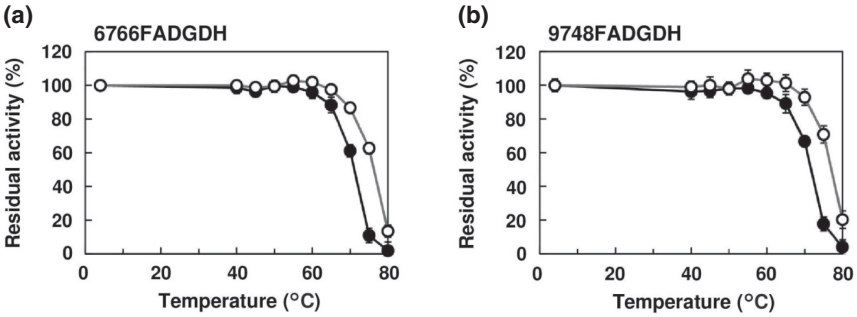


**Figure 6.** Effect of temperature on FADGDH stability. FADGDH (7 U/mL) was incubated at indicated temperatures at pH5.0 for 15 min, and then diluted with 50 mM potassium phosphate buffer (pH7.0) containing 0.1% Triton X-100. This diluted solution was used for assay. (a) 6766FADGDHs. (b) 9748FADGDHs. In both panels, closed circles represent unglycosylated FADGDHs, and open circles represent glycosylated FADGDHs.

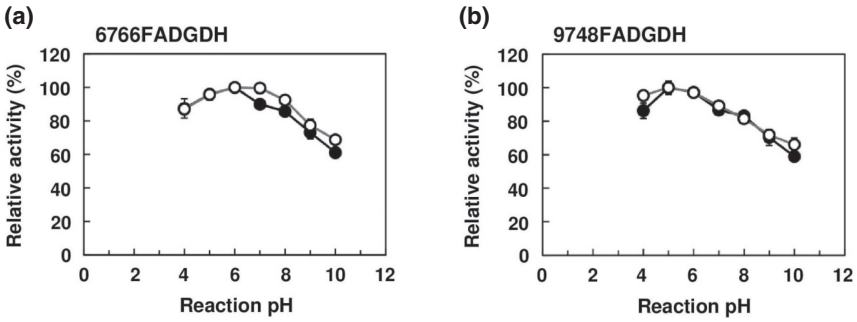


**Figure 7.** Effect of reaction pH on FADGDH activity. Reaction mixture containing FADGDH was incubated in 50 mM Britton-Robinson universal buffer at indicated pH at 37°C for 2 min. Oxidized DCIP was quantified after adding 0.2 M Tris-HCl buffer (pH8.0) containing 8 M urea into the mixture to stop the reaction. The reaction mixture was finally diluted 5-fold with the stopping buffer. (a) 6766FADGDHs. (b) 9748FADGDHs. In both panels, closed circles represent unglycosylated FADGDHs, and open circles represent glycosylated FADGDHs.

[Fig. 5(a) and (b)]. In spite of the presence or absence of sugar chains, they were unstable at pH2.0, losing their activities absolutely.

In order to investigate thermal stability of the FADGDHs, activities of 6766FADGDHs and 9748FADGDHs were measured after heat treatment at 40–80°C for 15 min (Fig. 6). Interestingly, unglycosylated 6766FADGDH and glycosylated 6766FADGDH maintained their activities close to 100% at temperatures up to 60°C and 65°C, respectively [Fig. 6(a)]. Unglycosylated 9748FADGDH and glycosylated 9748FADGDH were also stable up to 60°C and 65°C, respectively [Fig. 6(b)]. The thermal stability of native *A. oryzae* and *A. terreus* FADGDHs purified from their culture media was studied.[5,22] *A. oryzae* FADGDH retained approximately 60% of the activity after heat treatment at 50°C for 15 min. *A. terreus* FADGDH retained approximately 60% of the activity after heat treatment at 55°C for 15 min. These results revealed that 6766FADGDHs and 9748FADGDHs were highly thermostable. The $T_m$ values were calculated at 71.4°C for unglycosylated 6766FADGDH and 72.6°C for unglycosylated 9748FADGDH, whereas those of glycosylated 6766FADGDH and glycosylated 9748FADGDH were 76.8°C and 77.8°C, respectively. The improved thermal stability of the enzymes produced by *P. pastoris* is due to glycosylation. Several studies on glycosylated proteins produced by *P. pastoris* support our results.[23] Evidence of the glycosylation of the FADGDHs produced by *P. pastoris* was confirmed by PAS staining [Fig. 2(b)].

### 3.6 Effect of pH and temperature on FADGDH activity

The pH profile of FADGDH activity was examined (Fig. 7). The optimum reaction pH values were 6.0 for unglycosylated 6766FADGDH and 6.0–7.0 for glycosylated 6766FADGDH [Fig. 7(a)], whereas those were 5.0 for unglycosylated 9748FADGDH and glycosylated 9748FADGDH [Fig. 7(b)]. The relative activities of 6766FADGDHs and 9748FADGDHs remained over 50% in a wide pH range between 4.0 and 10.0.

The temperature profile of FADGDH activity was examined (Fig. 8). The optimum reaction temperatures of unglycosylated 6766FADGDH and glycosylated 6766FADGDH were observed at 70°C [Fig. 8(a)]. The optimum reaction temperatures of unglycosylated 9748FADGDH and glycosylated 9748FADGDH were 70°C or higher [Fig. 8(b)]. We obtained no reliable data above 70°C, likely due to the limitation of the GDH activity measuring system. The activities of unglycosylated 6766FADGDH and glycosylated 6766FADGDH at optimum reaction temperatures were 3.6 and 4.2 times higher relative to those at 37°C, respectively. Similarly, the activities of unglycosylated 9748FADGDH and glycosylated 9748FADGDH at optimum reaction temperatures were 4.5 and 4.6 times higher relative to those at 37°C, respectively.

### 3.7 Current response of FADGDH-immobilized electrode

Electrochemical experiments were performed by using unglycosylated and glycosylated 6766FADGDHs whose affinities for glucose were higher than those of 9748FADGDHs. A linear correlation between raw current value and glucose concentration was observed up to 250 mM, as shown by least squares regression lines. The coefficients of determination, $R^2$, were 0.995 for unglycosylated 6766FADGDH and 0.992 for glycosylated 6766FADGDH [Fig. 9(a) and (b)]. These results suggested that 6766FADGDHs could be used for bioelectrochemical oxidation of glucose.

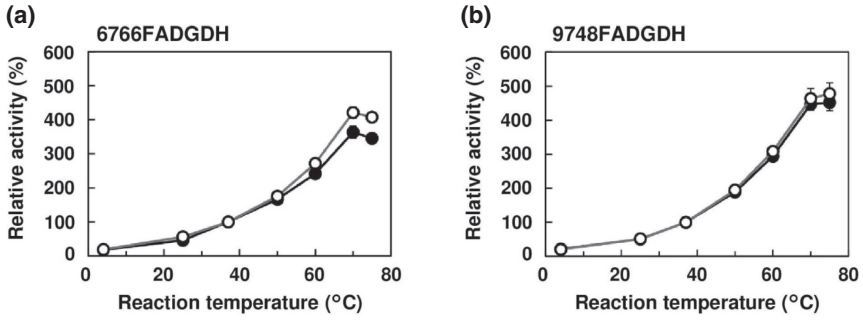


**Figure 8.** Effect of reaction temperature on FADGDH activity. Reaction mixture containing FADGDH was incubated at indicated temperatures at pH5.0 for 2 min. Oxidized DCIP was quantified after adding 0.2 M Tris-HCl buffer (pH8.0) containing 8 M urea into the mixture to stop the reaction. The reaction mixture was finally diluted 5-fold with the stopping buffer. (a) 6766FADGDH. (b) 9748FADGDH. In both panels, closed circles represent unglycosylated FADGDHs, and open circles represent glycosylated FADGDHs.

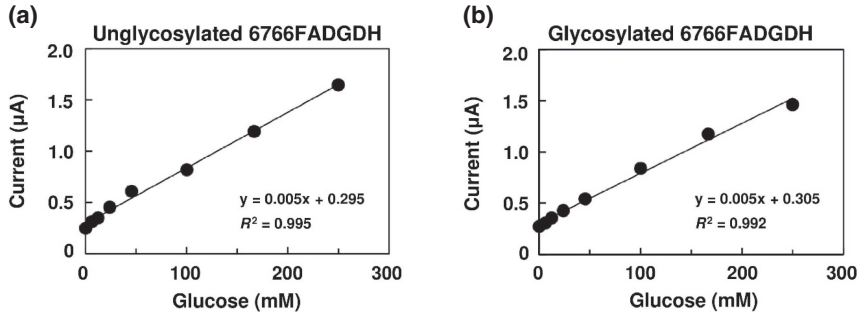


**Figure 9.** Current response of FADGDH-immobilized electrode. (a) Unglycosylated 6766FADGDH. (b) Glycosylated 6766FADGDH.

## 4. Conclusions

In this study, we newly identified and cloned the FADGDH gene homologs from thermophilic filamentous fungi *Th. aurantiacus* NBRC 6766 and *Th. aurantiacus* NBRC 9748, and then characterized the enzymatic properties of the gene products. The enzymatic characteristics showed that these FADGDHs exhibited remarkably high thermal stability. Furthermore, electrochemical experiments showed that the thermostable FADGDHs effectively worked as bioelectro-catalyst of glucose oxidation. These thermostable FADGDHs, especially from *Th. aurantiacus* NBRC 6766, could be applied to bioelectrochemical devices with long-term storage and continuous use.

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