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Efficient Expression, Purification, and Characterization of a Novel FAD-Dependent Glucose Dehydrogenase from *Aspergillus terreus* in *Pichia pastoris*

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Flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) can utilize a variety of external electron acceptors and also has stricter substrate specificity than any other glucose oxidoreductases, which makes it the ideal diagnostic enzyme in the field of glucose biosensors. A gene coding for a hypothetical protein, similar to glucose oxidase and derived from *Aspergillus terreus* NIH2624, was overexpressed in *Pichia pastoris* GS115 under the control of an AOX1 promoter with a level of 260,000 U/l in the culture supernatant after fed-batch cultivation for 84 h. After a three-step purification protocol that included isopropanol precipitation, affinity chromatography, and a second isopropanol precipitation, recombinant FAD-GDH was purified with a recovery of 65%. This is the first time that isopropanol precipitation has been used to concentrate a fermentation supernatant and exchange buffers after affinity chromatography purification. The purified FAD-GDH exhibited a broad and diffuse band between 83 and 150 kDa. The recombinant FAD-GDH was stable across a wide pH range (3.5 to 9.0) with maximum activity at pH 7.5 and 55°C. In addition, it displayed very high thermal stability, with a half-life of 82 min at 60°C. These characteristics indicate that FAD-GDH will be useful in the field of glucose biosensors.

Keywords: Characterization, FAD-GDH, isopropanol precipitation, *Pichia pastoris*, thermal stability

Introduction

Glucose detection is of great importance in both biomedical applications and industrial processes [3]. A systematic investigation showed that, globally, diabetes is a rising chronic disease: the number of adults suffering from diabetes has doubled in nearly three decades and is now approximately 347 million people worldwide [4]. The World Health Organization (WHO) has forecasted that diabetes will be the seventh leading cause of death in 2030 [2]. For patients, a device that conveniently monitors blood glucose is very important for their therapy and daily life. The currently available glucose sensors are mostly derived from enzymatic reactions of glucose oxidases (GOX) [6, 14, 35] or water-soluble pyrroloquinoline quinine-dependent glucose dehydrogenases (PQQ-GDHs) [10, 22, 24]. However, dissolved oxygen may easily interfere with GOX detection of glucose, and the substrate spectrum of water-soluble PQQ-GDH is too broad; these drawbacks inevitably impair the accuracy of blood glucose measurement by such methods.

To solve these problems, much attention has been drawn to flavin adenine dinucleotide-dependent glucose dehydrogenases (FAD-GDHs) [20]. FAD-GDHs can utilize a variety of external electron acceptors, with the exception of oxygen, and have stricter substrate specificity than PQQ-GDHs. Several FAD-GDHs have been identified and characterized in the past several years, and most of them are derived from fungi such as *Aspergillus oryzae* [13, 29], *A. flavus* [20],

A. niger [20], *A. carbonarius* [20], *A. terreus* [20, 21], *Penicillium* [1], and *Glomerella cingulata* [25, 26]. Only one FAD-GDH, from *Burkholderia cepacia*, was prokaryote-derived [30]. It is well known that fungi-derived FAD-GDHs cannot be effectively expressed in *E. coli*, even when the codon bias of *E. coli* is carefully considered. The prokaryote-derived FAD-GDH is membrane bound and also cannot be easily produced in *E. coli* [30]. Five putative FAD-GDHs from several *Aspergillus* species were expressed in *E. coli* by Mori *et al*. [20]; however, only one showed a clear band in SDS-PAGE analysis. Most FAD-GDHs failed to be expressed, or were expressed as insoluble products. The reported yields of FAD-GDHs expressed in *E. coli* in shaking flasks were only about 8,000 U/l [29] to 13,000 U/l [20], which are relatively low. By using a 10 L jar fermentor, the production of FAD-GDH from *A. oryzae* could be improved to 72,000~73,000 U/l [13], but this yield still needs to be improved for industrial production when compared with the yield of PQQ-GDH, which is as high as 1,530,000 U/l [32]. Recently, an acceptable yield of FAD-GDH was obtained in *P. pastoris*, with up to 48,000 U/l after fermentation for 50.5 h, which was about 4,800-fold higher than that in *E. coli* [26]. Usually, FAD-GDH is extracted and purified from fungi [1, 21, 25], but the procedure is time-consuming, labor-intensive, and inefficient. Therefore, an efficient and simple expression protocol is still imperative to allow large-scale production of FAD-GDH.

A *P. pastoris* protein expression system has beneficial properties, such as rapid growth rate, ease of high cell-density fermentation, and high-level productivity, which are all desirable for protein expression [9, 11, 15]. In our previous work, an alcohol oxidase that is an important diagnostic enzyme for alcohol detection was effectively expressed in *P. pastoris* [17]. In this study, we have successfully overexpressed the synthesized gene encoding *A. terreus* FAD-GDH in *P. pastoris*, a protein that failed in an *E. coli* expression system even though the gene was synthesized with optimized codons [20]. A simple purification procedure involving two steps of isopropanol precipitation and one step of affinity chromatography was established for obtaining highly purified FAD-GDH. Isopropanol was chosen as the precipitant in this study after comparison with other commonly used precipitants. Enzymatic characterization of the purified protein was also performed and is summarized here.

Materials and Methods

Gene, Bacterial Strains, Plasmids, Chemicals, and Media

The FAD-GDH gene (XM_001216916) from *Aspergillus terreus* NIH2624 was synthesized by Qinglan Biotech (Shanghai, China) according to the codon bias of *P. pastoris* and integrated into plasmid pUC19. *E. coli* strain DH5α, *P. pastoris* GS115, and pPIC9k were preserved in our laboratory. PrimeSTAR GXL DNA polymerase, restriction enzymes, and ligase were purchased from Takara (Dalian, China). Yeast nitrogen base (YNB) without amino acids was purchased from Sangon Biotech (Shanghai, China). Minimal dextrase (MD) medium, buffered complex glycerol medium (BMGY), yeast extract peptone dextrose (YPD) medium, buffered complex methanol medium (BMMY), and fermentation basal salts medium (BSM) supplemented with trace elements solution were prepared according to the manufacturer's instructions. All chemicals used were of analytical grade. A 1 M glucose stock solution was prepared with Milli-Q water and stored at least 24 h to achieve mutarotation equilibrium.

Construction and Screening of *P. pastoris* GS115/FAD-GDH

The FAD-GDH gene was amplified from pUC19-FAD-GDH using two designed primers: FAD-GDH F (5′-CGGAATTCACCATGG ATTCTAATTCTAC-3′) and FAD-GDH R (5′-TAAAGCGGCCGC TTAatggtgatggtgatggtgACGTCTGCCAGCATCGGCTTTG-3′), in which the *Eco*RI and *Not*I restriction sites (underlined) were introduced into the gene fragment for cloning convenience, and the 6×histidine tag was fused into the C-terminus of the gene (lower case letters). The obtained PCR fragment was inserted into pPIC9K, in which the FAD-GDH gene was under the control of the AOX1 promoter and the α-mating factor signal sequence from *Saccharomyces cerevisiae*. The resulting plasmid pPIC9K-FAD-GDH was linearized with *Sac*I and transformed into electrocompetent *P. pastoris* GS115 cells prepared according to the instructions of the GenePulserXcell electroporation apparatus (Bio-Rad). The screening of transformants was carried out on MD plates. Only *P. pastoris* cells whose genome was integrated with the linearized plasmid and contained the *his4* gene could grow on the histidine auxotrophic MD plates. The positive clones were confirmed by PCR using FAD-GDH-specific primers and the genomic DNA of transformant as the template. Six positive colonies were selected for protein expression in shake flasks. Each colony was inoculated into 40 ml of BMGY medium and grown at 30°C overnight. Then pre-cultures were transferred into 250 ml shake flasks containing 40 ml of BMMY medium and incubated at 30°C and 220 rpm. Methanol (0.5% (v/v) final concentration) was added to the culture at 24 h intervals, and samples were taken every 24 h for FAD-GDH activity analysis.

Expression and Purification of Recombinant FAD-GDH

A 15 L stainless steel fermentor with 7 L of BSM containing 4% glycerol was used for recombinant FAD-GDH production. After autoclaving, 700 ml of pre-cultures was inoculated into the fermentor and cultivated according to the *Pichia* Fermentation Guideline of Invitrogen with slight modification. The pH of the medium was kept at about 5.0 by the addition of 28% ammonium hydroxide. After about 21 h, the glycerol was exhausted and

glycerol feeding was initiated to promote cell biomass increase. After feeding for about 4.5 h, glycerol was replaced by methanol for protein induction. The dissolved oxygen was kept above 15%. Samples were taken regularly from the fermentor for monitoring.

At the end of the fermentation, the broth was centrifuged at 8,000 ×*g* and 4°C for 30 min. Ammonium sulfate at different saturations (40%, 60%, 80%, or 100%) was used to precipitate FAD-GDH at 4°C. As little precipitate was obtained by ammonium sulfate precipitation, residual activity was detected in the supernatant. Then, addition of cold methanol, ethanol, acetone, or isopropanol at different concentrations (30%, 40%, or 50% (v/v)) and different processing times (0, 10, 30, or 60 min) were evaluated to optimize protein precipitation. After centrifugation at 13,000 ×*g* and 4°C for 15 min, the resulting precipitate was resuspended in buffer A (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0). Finally, the supernatant was loaded into the pre-equilibrated Ni^{2+}-affinity column. Active fractions were eluted by buffer B (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 8.0) and precipitated again by organic solvent to remove imidazole and sodium chloride. The purified protein was re-dissolved in 20 mM 3-morpholinopropanesulfonic acid buffer (MOPS; pH 7.5) and stored at 4°C.

Enzymatic Activity Assay of Recombinant FAD-GDH

The GDH activity of recombinant FAD-GDH was determined according to the method reported by Mori *et al*. [20], with slight modification. The reaction buffer (total 1.99 ml) contained 20 mM MOPS buffer (pH 7.5), 0.06 mM 2,6-dichlorophenol indophenol (DCIP), 0.6 mM phenazinemethosulfate (PMS) and 100 mM D-glucose. First, 10 µl of enzyme solution at an appropriate dilution (the concentration of enzyme was about 7 µg/ml) was added to initiate the reaction. The enzymatic reaction was then monitored by measuring the absorbance decrease of DCIP at 600 nm. One unit of FAD-GDH activity was defined as the amount of enzyme required for the reduction of 1 µmol glucose or electron acceptor per minute under the assay conditions. The recombinant protein was also assayed for oxidase activity in 100 mM 2-(*N*-morpholino) ethansulfonic acid buffer, pH 5.7, 0.3 mM EHSPT, 4 U horseradish peroxidase/ml, 1.5 mM 4-aminoantipyrine, and 100 mM glucose, at room temperature by measuring the formation of quinoneimine dye at 555 nm. The protein concentration was determined by the Bradford method using a commercial kit (Sangon, China).

Characterization of Recombinant FAD-GDH

To determine the optimal pH for FAD-GDH, different buffer solutions, including sodium phosphate buffer (pH 6.0 to 8.0), MOPS buffer (pH 6.5 to 7.5), and Tris-HCl buffer (pH 7.5 to 9.0), were employed in the enzymatic activity assay. The reaction solutions were maintained at different temperatures from 30°C to 65°C during the assays to determine the optimum temperature for FAD-GDH.

To evaluate the pH stability, FAD-GDH was dissolved in different 100 mM buffer solutions, including glycine-HCl buffer (pH 2.5 to 3.5), sodium citrate buffer (pH 3.0 to 6.5), sodium phosphate buffer (pH 6.0 to 8.0), MOPS buffer (pH 6.5 to 7.5), Tris-HCl (pH 7.5 to 7.5), and glycine-NaOH buffer (pH 8.5 to 10). After being kept at 40°C for 60 min, the enzymatic activity of each FAD-GDH solution was examined. The ratio (%) of residual enzymatic activity was calculated, and the highest enzymatic activity was defined as 100%. To determine thermal stability, FAD-GDH was dissolved in 20 mM MOPS buffer and kept at 60°C. Samples were taken every 10 or 20 min and kept at 0°C for 2 min before enzymatic activity measurement. The K_m value of FAD-GDH was evaluated from a Lineweaver-Burk plot of enzymatic assay results using different concentrations of D-glucose (10–100 mM).

To characterize the substrate specificity of FAD-GDH, various substrates at a final concentration of 100 mM were employed instead of D-glucose, and the corresponding enzymatic activity was measured. The enzymatic activity for each substrate was calculated as a relative value, while the enzymatic activity of FAD-GDH for D-glucose was defined as 100%.

In order to confirm the coenzyme of FAD-GDH, 200 µl of purified FAD-GDH was scanned between the wavelengths 300 nm and 550 nm before and after 10 µl of 1 M D-glucose was added.

Results

Construction of Recombinant *P. pastoris* GS115/FAD-GDH

As shown in Fig. 1, the 1722 bp DNA fragment encoding a 574-amino-acid FAD-DGH without the putative signal sequence, but with a 6×histidine tag at the C-terminus, was successfully amplified from pUC19-FAD-GDH and ligated into pPIC9K under the control of a methanol-inducible AOX primer. After validation by sequencing, the reconstructed plasmid was linearized and integrated into the genome of *P. pastoris*. The recombinant *P. pastoris* GS115/FAD-GDH was confirmed by PCR using FAD-GDH-specific primers. Six positive transformants were picked up for expression of FAD-GDH in flasks. The best-producing clone (19,000 U/l enzymatic activity) was selected for further studies.

Fermentation and Purification of Recombinant FAD-GDH

Production of recombinant FAD-GDH was performed in a 15 L stainless steel fermentor. As seen in Fig. 2, 120 g/l of wet biomass was obtained after 21 h of cultivation from the initial culture, and this increased to 161 g/l after glycerol feeding for 4.5 h. FAD-GDH expression was initiated by methanol induction. The maximum wet biomass reached 228 g/l, and the concentration of soluble protein in the culture supernatant rose to 0.71 g/l. The fermentation lasted for about 84 h and was stopped once the specific FAD-GDH activity in the broth began to decrease. The

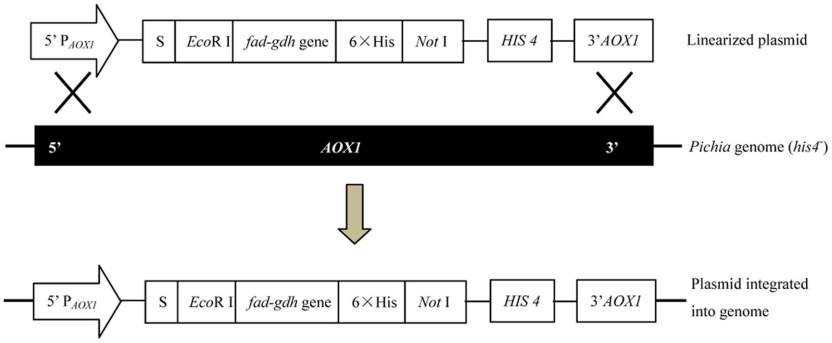

Fig. 1. Genetic components of recombinant linearized pPIC9K and its integration into the *Pichia* genome.
S: α-mating factor signal sequence.

maximum activity of FAD-GDH achieved in the supernatant was about 260,000 U/l.

Usually, ammonium sulfate is the first choice for protein precipitation because of its low cost and availability. However, ammonium sulfate was not a suitable precipitant for FAD-GDH in this study. Even when using 100% saturated ammonium sulfate solution, more than 93% of the enzymatic activity remained in the supernatant (Fig. 3). In contrast, several organic solvents can precipitate FAD-GDH to differing degrees. As shown in Fig. 4A, after precipitation for 30 min, 40% isopropanol and acetone effectively precipitated FAD-GDH with a recovery of more than 80%. When 40% ethanol was used as precipitant, the recovery was about 65%. Methanol was not the ideal precipitant among the four candidates, as recovery of FAD-GDH was only about 10% with a 40% solution, and, when the concentration was 50%, only about one-half of the FAD-GDH precipitated. Owing to its lower recovery rate, methanol was not used in further studies. The recovery was inversely proportional to the precipitation time for all three organic solvents. The "0 min treatment" was defined as the point when the organic solvent was mixed in and the fermentation broth was centrifuged. In this treatment, the recovery of FAD-GDH using 40% isopropanol reached about 94%, which was superior to ethanol and acetone (Fig. 4B). Therefore, isopropanol was selected as the precipitant for purification. After purification, FAD-GDH was obtained with a moderate recovery of 65% (Table 1), and the purity of FAD-GDH was analyzed by SDS-PAGE (Fig. 5). The final recombinant FAD-GDH solution was bright-yellow, demonstrating dye-mediated GDH activity of 541 U/mg (Table 1), but no GOX activity.

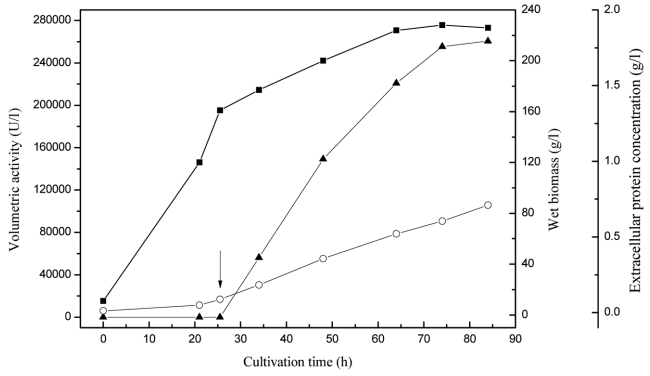

Fig. 2. Production of recombinant *A. terreus* FAD-GDH in *P. pastoris*.
The yeast was cultivated in a 15 L bioreactor. The induction was started by a methanol feed phase. Key: wet biomass (■); volumetric activity (▲); extracellular protein concentration (○); the arrow indicates induction initiation.

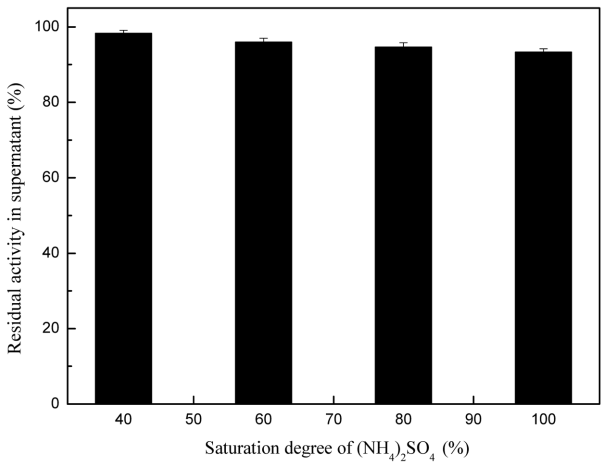

Fig. 3. Residual activity remaining in the supernatant after precipitation by ammonium sulfate solutions with different degrees of saturation.

Table 1. Purification of recombinant FAD-GDH.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Broth supernatant	52,679	177	297	100
First-round isopropanol precipitation	49,623	142	347	94
Affinity chromatography	36,880	70	526	70
Second-round isopropanol precipitation	34,242	63	541	65

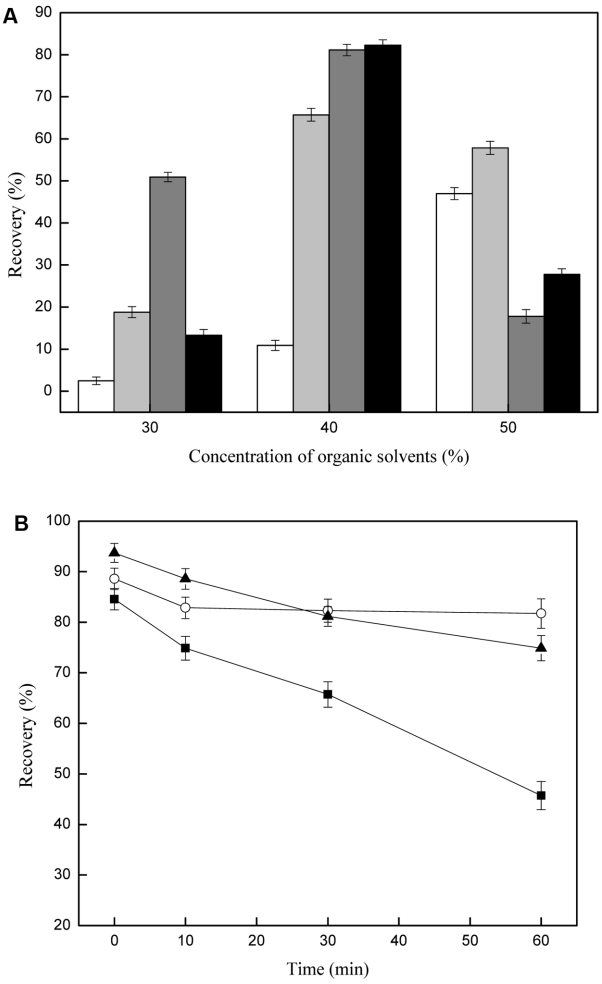

Fig. 4. Optimization of precipitant and precipitation time.
(**A**) Precipitation of FAD-GDH by different organic solvents at three concentrations for 30 min. Key: methanol (white); ethanol (light gray); isopropanol (gray); acetone (dark gray). (**B**) Processing time of FAD-GDH precipitation with the concentrations of organic solvents fixed at 40% (v/v). Key: isopropanol (▲); acetone (○); ethanol (■).

Characterization of Recombinant FAD-GDH

Recombinant FAD-GDH produced from *P. pastoris* was glycosylated, and it presented a broad and diffuse band between 83 and 150 kDa upon SDS-PAGE analysis (Fig. 5).

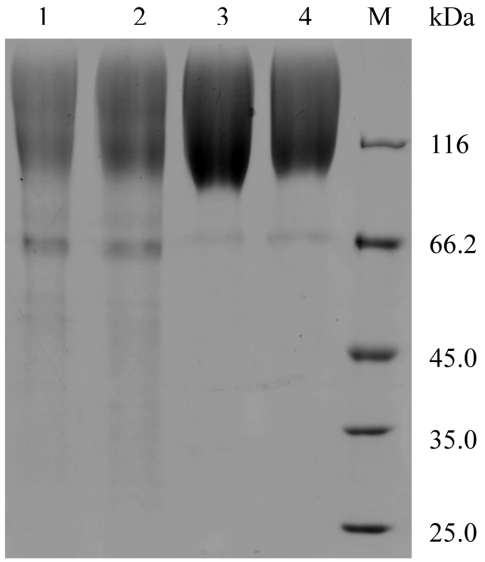

Fig. 5. SDS-PAGE analysis of recombinant FAD-GDH during purification.
Lane 1, broth supernatant; lane 2, re-dissolved solution after the first isopropanol precipitation; lane 3, elution during affinity chromatography; lane 4, re-dissolved solution after the second isopropanol precipitation; and lane M, molecular marker.

As shown in Fig. 6, the optimal pH and temperature for FAD-GDH production were pH 7.5 (Fig. 6A) and 55°C (Fig. 6B), respectively. The enzyme had activity within a broad pH range from 3.5 to 9.0 (Fig. 6C). The half-life of FAD-GDH at 60°C was about 82 min (Fig. 6D), and its K_m value was 89.7 mM (Fig. 6E). The typical absorption peaks of FAD at 378 and 454 nm were detected (Fig. 6F, solid line), and these two peaks disappeared because of the reduction of FAD-GDH upon the addition of D-glucose (Fig. 6F, dashed line). As summarized in Table 2, in addition to D-glucose, FAD-GDH displayed partial enzymatic activity towards maltose and D-xylose as substrates, and the relative enzymatic activities were 17.7 ± 1.6% and 7.4 ± 0.8% of that towards D-glucose, respectively. FAD-GDH showed negligible activity with respect to other monosaccharides and disaccharides.

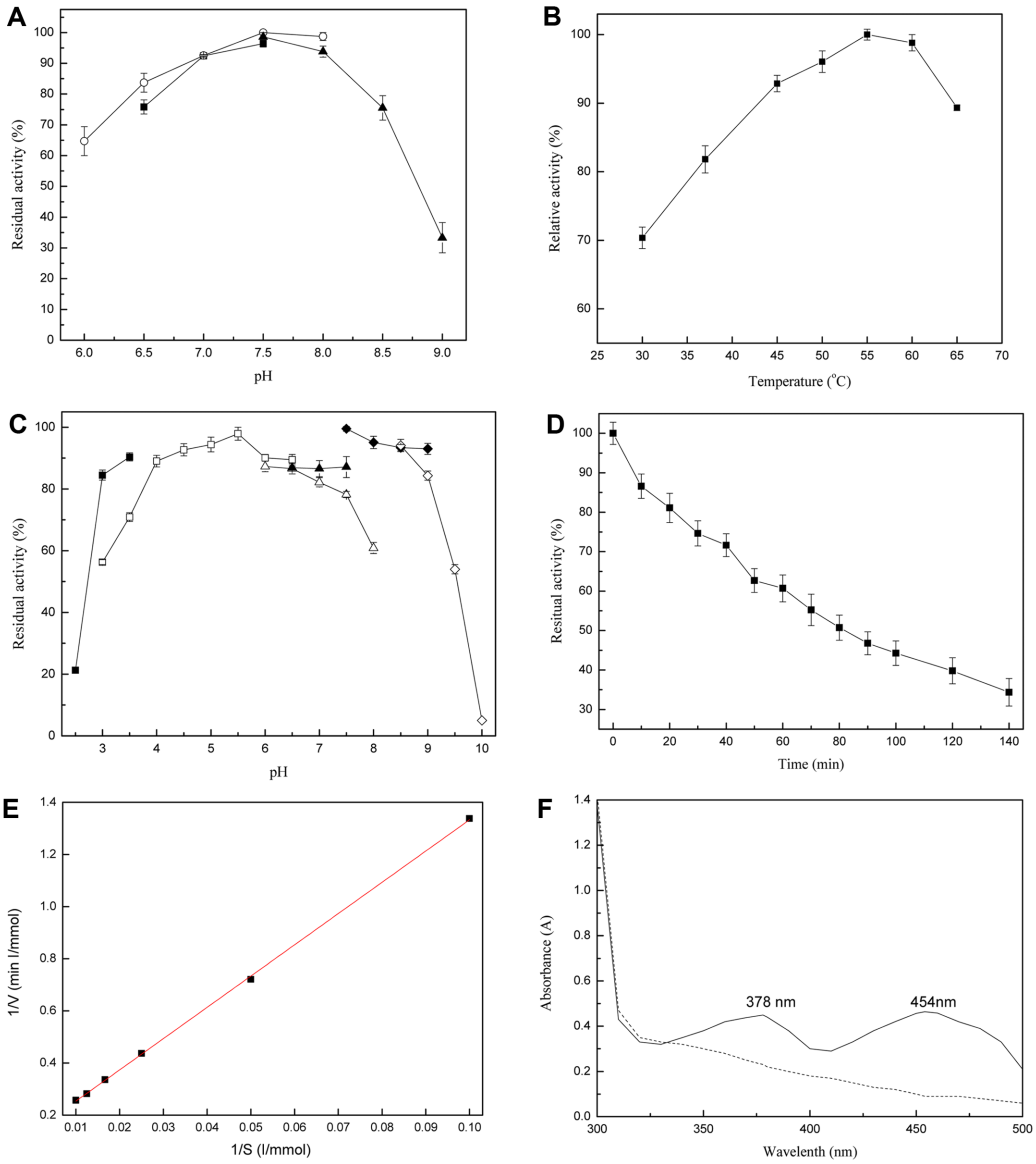

Fig. 6. Characteristics of recombinant FAD-GDH.
(**A**) Optimal pH. Key: sodium phosphate (○); MOPS (■); Tris-HCl (▲). (**B**) Optimum temperature. (**C**) pH stability. Key: glycine-HCl (■); sodium citrate (□); MOPS (▲); sodium phosphate (△); Tris-HCl (◆); Glycine-NaOH (◇). (**D**) Thermal stability under 60°C. (**E**) Determination of the K_m value. (**F**) UV spectra of the oxidized (solid line) and reduced (dashed line) FAD-GDH.

Table 2. Substrate specificities of FAD-GDH.

Substrates	Relative activity (%)[a]	Substrates	Relative activity (%)
D-Glucose	100	D-Lactose	0.6 ± 0.6
Maltose	17.7 ± 1.6	α-Trehalose	0.5 ± 0.6
D-Xylose	7.4 ± 0.8	D-Fructose	ND[b]
D-Galactose	2.2 ± 1.0	D-Sorbitol	ND
L-Arabinose	1.5 ± 0.8	D-Sucrose	ND

[a]The enzymatic activities are expressed as relative activity to that for β-D-glucose.
[b]ND, not detectable.

Discussion

FAD-GDH is a priority selection in the fields of glucose biosensors and biofuel cells owing to its high turnover rate and good stability. Several types of FAD-GDH have been used as glucose biosensors [19, 33, 34]. However, the productivity of FAD-GDH previously did not meet the requirements for industrial-scale production. As summarized in Table 3, only FAD-GDH from *A. oryzae* was expressed at an acceptable level in *E. coli*, in which the productivity reached 72,000~73,000 U/l after fermentation for 25 h [29]. The productivities of other FAD-GDHs from *E. coli* were obviously not good enough to justify commercial production for potential applications. Other than *P. pastoris*, several eukaryotic hosts have been tried for producing FAD-GDH, but none gave a satisfactory yield [1, 21, 25]. It is worth mentioning that the recently reported FAD-GDH from *G. cingulate* can be expressed effectively in *P. pastoris* (48,000 U/l) [26]. This may indicate that *P. pastoris* is an effective host for FAD-GDH expression. In this study, high-level expression of FAD-GDH from *A. terreus* was achieved in *P. pastoris*, and the productivity of 260,000 U/l is 5.4-fold higher than that of FAD-GDH from *G. cingulate* [26]. Such a high productivity may facilitate biochemical and structural studies as well as the engineering of FAD-GDH.

The recombinant FAD-GDH from *P. pastoris* displayed a broad band on SDS-PAGE (Fig. 5) because of glycosylation to different degrees. This is a common phenomenon; similar results were obtained for another FAD-GDH and other proteins expressed in *P. pastoris* [7, 16, 26]. In general, glycosylation may not affect the enzymatic activity [18], but the degree of glycosylation did affect the charge and redox hydrogel catalytic efficiency of GOX [31]. In other reports, glycosylation seems to play an important role in peptide folding into the correct conformation [26, 31]. Our results suggest that the glycosylation of FAD-GDH did enhance its tolerance towards general organic solvents when compared with unglycosylated FAD-GDH from the *E. coli* expression system. As shown in Fig. 4B, more than 80% of *P. pastoris*-expressed FAD-GDH was recovered after organic solvent precipitation, but for FAD-GDH expressed in *E. coli*, all enzymatic activity was lost after precipitation. This characteristic facilitated the subsequent purification of FAD-GDH. Our results indicate that precipitation of recombinant FAD-GDH from the broth supernatant by ammonium sulfate was not applicable; the enzyme did not precipitate even when 100% saturated ammonium sulfate solution was used, and more than 93% of the enzymatic activity remained in the supernatant (Fig. 3). Hence, four other precipitants were chosen for FAD-GDH purification. Ethanol and acetone are generally used to precipitate proteins [23, 28], whereas methanol and isopropanol have seldom been used. In our study, FAD-GDH could be precipitated from the supernatant using all organic solvents except for methanol, and isopropanol was the most efficient candidate (about 94%). Moreover, the pellets obtained by isopropanol precipitation could be resuspended much more easily than those from ethanol or acetone precipitation. In addition, 40% isopropanol did not precipitate imidazole and sodium chloride, even when

Table 3. Comparison of FAD-GDHs produced *via* different expression systems.

Source	Expression system	Fermentation mode	Culture time	Productivity (U/l)	References
A. oryzae	*E. coli*	-	16 h	8,000	[29]
A. oryzae	*E. coli*	Batch in 10 L jar fermentor	25 h	72,000–73,000	[13]
A. flavus	*E. coli*	Batch in 500 ml flask	24 h	13,000	[20]
A. flavus	*E. coli*	Batch in 500 ml flask	24 h	200	
A. niger	*E. coli*	Batch in 500 ml flask	24 h	990	
A. niger	*E. coli*	Batch in 500 ml flask	24 h	93	
A. carbonarius	*E. coli*	Batch in 500 ml flask	24 h	130	
A. terreus	*A. terreus*	Batch in 5 L jar fermentor	41 h	-	[21]
	E. coli	-	24 h	90	
A. terreus	*P. pastoris*	Batch in 15 L jar fermentor	84 h	260,000	This paper
Penicillium	*Penicillium*	Batch in 10 L jar fermentor	64 h	-	[1]
G. cingulate	*G. cingulate*	Batch in 300 ml flask	6 days	3,400	[25]
	P. pastoris	Batch in 7 L glass vessel fermentor	50.5 h	48,000	[26]
	E. coli	Batch in 125 ml flask	17 h	10	[26]
B. cepacia	*E. coli*	-	10 h	-	[30]

their concentrations in buffer B increased to as much as 500 mM. This showed that isopropanol is an ideal precipitant for FAD-GDH purification. Compared with time-consuming dialysis, our simple purification protocol with three steps resulted in a preparation of highly pure protein (as checked by SDS-PAGE) with a specific activity of 541 U/mg. Since the enzymatic activity of FAD-GDH is acceptable with the 6×histidine tag, there is no need to cut the tag off after purification. Moreover, it is preferable to target the purity of FAD-GDH for its application as a biosensor; thus Ni^{2+}-affinity chromatography could be integrated into one whole process for the industrial production of FAD-GDH for special applications. Meanwhile, isopropanol is a commonly used organic solvent in the pharmaceutical industry, and several efficient methods have been developed for isopropanol recovery [8, 12, 27]. Therefore, the simple purification protocol set up in our study is feasible for the industrial-scale production of FAD-GDH.

Characterization of the recombinant FAD-GDH had also revealed its good thermal stability and stability across a wide pH range. The half-life of FAD-GDH at 60°C was 82 min, and it was stable at pH values ranging from 3.5 to 9.0, which was broader than the previously reported pH range (4.5 to 8.5) [21]. All of these advantages suggest our recombinant FAD-GDH would be suitable for industrial processes with little enzymatic activity lost. The K_m value of FAD-GDH was 89.7 mM, which was higher than the previously reported value from *A. terreus* (49.7 mM) [21]. Further research is necessary to understand the significance of the relatively high K_m value for its industrial applications. The recombinant FAD-GDH showed better substrate specificity compared with PQQ-GDH, whose relative activities towards maltose and D-xylose were up to 90% and 20%, respectively [5]. However, the relative activities of FAD-GDH towards maltose and D-xylose need to be reduced to guarantee accuracy when it is used in blood sugar testing.

The high-level expression of *A. terreus* FAD-GDH in *P. pastoris* and the highly efficient purification procedure provide a suitable method that allows us to readily prepare sufficient FAD-GDH for structural and functional studies, or other practical applications. To improve the substrate specificity, the engineering and evolution of FAD-GDH should be explored in future studies.

Acknowledgments

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References

1. Aiba H. 2007. Novel glucose dehydrogenase. US Patent No. 2007/0105174.
2. Alwan A. 2011. *Global Status Report on Noncommunicable Diseases 2010*. World Health Organization.
3. Chen C, Xie Q, Yang D, Xiao H, Fu Y, Tan Y, Yao S. 2013. Recent advances in electrochemical glucose biosensors: a review. *RSC Advances.* **3:** 4473-4479.
4. Danaei G, Finucane M, Lu Y, Singh G, Cowan M, Paciorek C, *et al.* 2011. Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* **378:** 31-40.
5. Dokter P, Frank J, Duine J. 1986. Purification and characterization of quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* LMD 79.41. *Biochem. J.* **239:** 163-167.
6. Dong YP, Huang L, Chu XF, Pei LZ. 2013. An amperometric glucose biosensor based on the immobilization of glucose oxidase on the $CuGeO_3$ nanowire modified electrode. *Russ. J. Electrochem.* **49:** 571-576.
7. Goossens KV, De Greve H, Willaert RG. 2013. Cloning, expression, and purification of the N-terminal domain of the Flo1 flocculation protein from *Saccharomyces cerevisiae* in *Pichia pastoris*. *Protein Expr. Purif.* **88:** 114-119.
8. Han Y-J, Wang K-H, Lai J-Y, Liu Y-L. 2014. Hydrophilic chitosan-modified polybenzoimidazole membranes for pervaporation dehydration of isopropanol aqueous solutions. *J. Memb. Sci.* **463:** 17-23.
9. Hang TT, Quyen DT, Dao TT, Nguyen SLT. 2012. Cloning, high-level expression, purification, and properties of a novel endo-beta-1,4-mannanase from *Bacillus subtilis* G1 in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **22:** 331-338.
10. Heller A, Feldman B. 2008. Electrochemical glucose sensors and their applications in diabetes management. *Chem. Rev.* **108:** 2482-2505.
11. Hoa T, Quyen DT, Nghiem NM, Vu TD. 2011. Cloning, expression, purification, and properties of an endoglucanase gene (Glycosyl Hydrolase Family 12) from *Aspergillus niger* VTCC-F021 in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **21:** 1012-1020.
12. Hua D, Ong YK, Wang Y, Yang T, Chung T-S. 2014. ZIF-90/P84 mixed matrix membranes for pervaporation dehydration of isopropanol. *J. Memb. Sci.* **453:** 155-167.

13. Kitabayashi M, Tsuji Y, Kawaminami H, Kishimoto T, Nishiya Y. 2010. Method for highly expressing recombinant glucose dehydrogenase derived from filamentous fungi. Patent No. 7,741,100.
14. Krishna R, Chandra S, Bardhan N, Salimian M, Yang Y, Titus E, *et al.* 2013. Design of an amperometric glucose biosensor based on glucose oxidase/arginated-Fe_3O_4/glassy carbon electrode. *Sci. Adv. Mater.* **5:** 333-340.
15. Li P, Anumanthan A, Gao X-G, Ilangovan K, Suzara VV, Düzgüne N, Renugopalakrishnan V. 2007. Expression of recombinant proteins in *Pichia pastoris*. *Appl. Biochem. Biotechnol.* **142:** 105-124.
16. Li R, Xie C, Zhang Y, Li B, Donelan W, Li S, *et al.* 2014. Expression of recombinant human IL-4 in *Pichia pastoris* and relationship between its glycosylation and biological activity. *Protein Expr. Purif.* **96:** 1-7.
17. Liu Y, Pan J, Wei P, Zhu J, Huang L, Cai J, Xu Z. 2012. Efficient expression and purification of recombinant alcohol oxidase in *Pichia pastoris*. *Biotechnol. Bioprocess Eng.* **17:** 693-702.
18. Monošík R, Stred'anský M, Šturdík E. 2012. Application of electrochemical biosensors in clinical diagnosis. *J. Clin. Lab. Anal.* **26:** 22-34.
19. Monošík R, Streanský M, Lušpai K, Magdolen P, Šturdík E. 2012. Amperometric glucose biosensor utilizing FAD-dependent glucose dehydrogenase immobilized on nanocomposite electrode. *Enzyme Microb. Technol.* **50:** 227-232.
20. Mori K, Nakajima M, Kojima K, Murakami K, Ferri S, Sode K. 2011. Screening of *Aspergillus*-derived FAD-glucose dehydrogenases from fungal genome database. *Biotechnol. Lett.* **33:** 2255-2263.
21. Omura H, Sanada H, Yada T, Atsumi A, Morita T, Ishimaru E. 2012. Coenzyme-linked glucose dehydrogenase and polynucleotide encoding the same. US Patent No. 8691547 B2.
22. Razumiene J, Gureviciene V, Sakinyte I, Barkauskas J, Petrauskas K, Baronas R. 2013. Modified SWCNTs for reagentless glucose biosensor: electrochemical and mathematical characterization. *Electroanalysis* **25:** 166-173.
23. Schmourlo G, Mendonça-Filho RR, Alviano CS, Costa SS. 2005. Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. *J. Ethnopharmacol.* **96:** 563-568.
24. Strack G, Babanova S, Farrington KE, Luckarift HR, Atanassov P, Johnson GR. 2013. Enzyme-modified buckypaper for bioelectrocatalysis. *J. Electrochem. Soc.* **160:** G3178-G3182.
25. Sygmund C, Klausberger M, Felice AK, Ludwig R. 2011. Reduction of quinones and phenoxy radicals by extracellular glucose dehydrogenase from *Glomerella cingulata* suggests a role in plant pathogenicity. *Microbiology* **157:** 3203-3212.
26. Sygmund C, Staudigl P, Klausberger M, Pinotsis N, Djinovi-Carugo K, Gorton L, *et al.* 2011. Heterologous overexpression of *Glomerella cingulata* FAD-dependent glucose dehydrogenase in *Escherichia coli* and *Pichia pastoris*. *Microb. Cell Fact.* **10:** 1-9.
27. Tang YP, Widjojo N, Chung TS, Weber M, Maletzko C. 2013. Nanometric thin skinned dual-layer hollow fiber membranes for dehydration of isopropanol. *AIChE J.* **59:** 2943-2956.
28. Thongboonkerd V, Mcleish KR, Arthur JM, Klein JB. 2002. Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int.* **62:** 1461-1469.
29. Tsuji Y, Kitabayashi M, Kishimoto T, Nishiya Y. 2010. Glucose dehydrogenase from *Aspergillus oryzae*. US Patent No. 7,655,130 B2.
30. Tsuya T, Ferri S, Fujikawa M, Yamaoka H, Sode K. 2006. Cloning and functional expression of glucose dehydrogenase complex of *Burkholderia cepacia* in *Escherichia coli*. *J. Biotechnol.* **123:** 127-136.
31. Witt S, Singh M, Kalisz HM. 1998. Structural and kinetic properties of nonglycosylated recombinant *Penicillium amagasakiense* glucose oxidase expressed in *Escherichia coli*. *Appl. Environ. Microbiol.* **64:** 1405-1411.
32. Yu Y, Wei P, Zhu X, Huang L, Cai J, Xu Z. 2012. High-level production of soluble pyrroloquinoline quinone-dependent glucose dehydrogenase in *Escherichia coli*. *Eng. Life Sci.* **12:** 574-582.
33. Zafar MN, Beden N, Leech D, Sygmund C, Ludwig R, Gorton L. 2012. Characterization of different FAD-dependent glucose dehydrogenases for possible use in glucose-based biosensors and biofuel cells. *Anal. Bioanal. Chem.* **402:** 2069-2077.
34. Zafar MN, Wang X, Sygmund C, Ludwig R, Leech D, Gorton L. 2011. Electron-transfer studies with a new flavin adenine dinucleotide dependent glucose dehydrogenase and osmium polymers of different redox potentials. *Anal. Chem.* **84:** 334-341.
35. Zhu L, Deng C, Chen P, You X-D, Su H-B, Yuan Y-H, Zhu M-F. 2014. Glucose oxidase biosensors based on carbon nanotube non-woven fabrics. *Carbon* **67:** 795-796.