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Novel fungal FAD glucose dehydrogenase derived from *Aspergillus niger* for glucose enzyme sensor strips

Koji Sode [a,b,c,*], Noya Loew [b], Yosuke Ohnishi [a], Hayato Tsuruta [a], Kazushige Mori [c], Katsuhiro Kojima [c], Wakako Tsugawa [a,b], Jeffrey T. LaBelle [d], David C. Klonoff [b,e]

[a] *Department of Biotechnology, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan*
[b] *Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu, Tokyo 183-8538, Japan*
[c] *Ultizyme International Ltd., 1-13-16, Minami, Meguro, Tokyo 152-0013, Japan*
[d] *School of Biological and Health System Engineering, Ira A. Fulton Schools of Engineering, Arizona State University, P.O. Box 879709, Tempe, AZ 85287-9709, USA*
[e] *Diabetes Research Institute, Mills-Peninsula Health Services, 100 South San Mateo Drive, San Mateo, CA 94401, USA*

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ABSTRACT

In this study, a novel fungus FAD dependent glucose dehydrogenase, derived from *Aspergillus niger* (AnGDH), was characterized. This enzyme's potential for the use as the enzyme for blood glucose monitor enzyme sensor strips was evaluated, especially by investigating the effect of the presence of xylose during glucose measurements. The substrate specificity of AnGDH towards glucose was investigated, and only xylose was found as a competing substrate. The specific catalytic efficiency for xylose compared to glucose was 1.8%. The specific activity of AnGDH for xylose at 5 mM concentration compared to glucose was 3.5%. No other sugars were used as substrate by this enzyme. The superior substrate specificity of AnGDH was also demonstrated in the performance of enzyme sensor strips. The impact of spiking xylose in a sample with physiological glucose concentrations on the sensor signals was investigated, and it was found that enzyme sensor strips using AnGDH were not affected at all by 5 mM (75 mg/dL) xylose. This is the first report of an enzyme sensor strip using a fungus derived FADGDH, which did not show any positive bias at a therapeutic level xylose concentration on the signal for a glucose sample. This clearly indicates the superiority of AnGDH over other conventionally used fungi derived FADGDHs in the application for SMBG sensor strips. The negligible activity of AnGDH towards xylose was also explained on the basis of a 3D structural model, which was compared to the 3D structures of *A. flavus* derived FADGDH and of two glucose oxidases.

1. Introduction

Glucose monitoring is essential for proper metabolic control of diabetes mellitus, a common worldwide illness. Many patients regularly perform self-monitoring of blood glucose (BG) to know which treatments and amounts of treatments to self-administer and to learn how activities affect their blood glucose levels. Patients want simpler and more accurate sensors for self-monitoring of blood glucose (SMBG). Among current commercially available BG monitoring systems, glucose oxidase (GOx) and glucose dehydrogenase (GDH) are the most widely utilized (Ferri et al., 2011). Among the two enzymes, GDH is the most widely used in outpatient BG Monitor systems. GDH is bound to one of several cofactors, such as FAD (Flavin adenine dinucleotide). Fungi derived FAD-dependent GDHs (FADGDHs) have received much attention for their accuracy in BG monitoring systems. This is because FADGDHs are insensitive to oxygen and do not have any activity towards maltose. Xylose is another saccharide which patients may receive as part of a clinical test. If the enzyme in a SMBG test strip uses these saccharides as substrate, then falsely elevated glucose levels will be reported. A false elevation of blood glucose can result in a decision to administer too much insulin, which in turn may lead to hypoglycemia (Frias, 2010; Schleis, 2007).

Historically, fungi FADGDHs have been reported since 1937 (Bak, 1967a, 1967b; Bak and Sato, 1967a, 1967b; Müller, 1977; Ogura, 1951; Ogura and Nagahisa, 1937). Recently, the number of reports on the discovery and recombinant production of new fungi derived FADGDHs increased (Mori et al., 2011; Piumi et al., 2014; Satake et al., 2015; Sygmund et al., 2011a, 2011b; Tsujimura et al.,

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan.
E-mail address: sode@cc.tuat.ac.jp (K. Sode).

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2006; Yang et al., 2014), enzyme electrodes using fungi derived FADGDHs have been studied (Monošík et al., 2012; Murata et al., 2014; Pinyou et al., 2016; Zafar et al., 2012). Many glucose enzyme sensor strips using FADGDH have recently been introduced into the BG Monitor system market (Dunne et al., 2015; Freckmann et al., 2014; Huang et al., 2014).

FADGDHs do not use oxygen as electron acceptor and are thus suitable for electrochemical applications using artificial electron acceptors. Lan et al. (2011) reported that sensors using FADGDH reacted only with xylose to the same degree as to glucose, and that FADGDH does not use any other examined mono- or disaccharide as substrate. In the presence of xylose, therefore, sensors using FADGDH show an obvious positive bias of the signal towards glucose, whereas sensors using GOx do not have this effect, because GOx does not react with xylose. By definition, this property of xylose makes it an interfering substance for FADGDH Monitor systems. There have been several attempts to engineer or screen for FADGDHs that are less active towards xylose (Satake et al., 2015; Yang et al., 2014). However, the challenges to overcome the interferent effect of xylose on sensor signals have yet to be overcome using any FADGDH.

The authors have been engaged in the screening (Mori et al., 2011), engineering (Sakai et al., 2015) and structural study (Yoshida et al., 2015) of fungi derived FADGDHs. The screening of FADGDHs, which was based on genomic information, led to the discovery of several FADGDH structural genes. Among them, the *Aspergillus flavus* FADGDH (AfGDH) has a primary structure identical to the one derived from *A. oryzae*, which has been widely used for commercial SMBG sensors. Next to glucose, the preferred substrate of AfGDH is xylose, and the activity of AfGDH towards xylose is approximately 20% of that towards glucose.

Another unique FADGDH was found from genomic information of *A. niger* (AnGDH, ANG544 in Mori et al. (2011)). While other FADGDHs, including AfGDH, show more than 20% activity towards xylose compared with that towards glucose, AnGDH showed less than 10% activity towards xylose (Mori et al., 2011), although its detailed enzymatic characteristics have not yet been reported, as its recombinant preparation as soluble enzyme was difficult.

In this paper, we introduce the novel fungi-derived GDH, AnGDH, which has a low reactivity towards xylose and the selectivity of glucose enzyme sensors using this enzyme. Recombinant AnGDH was prepared and its substrate specificity elucidated. Then, enzyme sensor strips based on screen printed carbon electrodes were constructed using AnGDH as well as AfGDH, and their activity towards xylose was investigated. Enzyme sensor strips using AnGDH showed no positive bias in the presence of 5 mM xylose while measuring a 5 mM glucose solution, whereas the sensor using AfGDH showed a positive bias of more than 23% with the same conditions. The negligible activity of AnGDH towards xylose was also discussed on the basis of a 3D structural model, compared to the 3D structures of AfGDH and of GOx.

2. Materials and methods

2.1. Materials

Disposable screen-printed carbon electrode (SPCE)-strips with two carbon-plate electrodes and an Ag/AgCl electrode were obtained from i-SENS Inc. (Seoul, Korea). Phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Methoxy PMS (mPMS) was obtained from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). All other chemicals were reagent grade.

2.2. Recombinant expression of AnGDH and AfGDH

E. coli BL21 (DE3) were transformed with expression vectors for AnGDH and AfGDH, respectively. The nucleic acid sequences of the genes for AnGDH and AfGDH are shown in supplementary Figs. S-1 and S-2, respectively, together with the corresponding amino acid sequences. The transformed *E. coli* were cultured in 100 ml ZYP-5052 medium (0.5% glycerol, 0.05% glucose, 0.2% lactose, 25 mM $(NH_4)_2SO_4$, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, and 1 mM $MgSO_4$) (Studier, 2005) at 20 °C for 30 h. After harvesting, the cells were washed twice with 0.85% NaCl and disrupted by ultrasonication in 20 mM potassium phosphate buffer (PPB), pH 6.5. After centrifugation to remove the cell debris and soluble fraction, the insoluble fraction containing AnGDH or AfGDH, respectively, was obtained as pellet.

2.3. Preparation of refolded AnGDH and AfGDH

The insoluble fraction was washed twice in 20 mM PPB containing 100 mM NaCl and 1 mM EDTA (pH 6.5), once with 1% Triton X-100 and once without. During a third washing step with 2 M urea in 20 mM PPB (pH 6.5) most of the protein contaminants were removed. AnGDH and AfGDH, respectively, were subsequently solubilized by adding in 20 mM PPB (pH 6.5) containing 8 M urea and 30 mM dithiothreitol (DTT) to the pellet and incubating at 4 °C for 4 h. The solubilized enzymes were diluted in refolding buffer (0.05 mM flavin adenine dinucleotide [FAD], 10% [vol/vol] glycerol, 20 mM PPB, pH 6.5) to a concentration of 0.1 mg/ml and incubated at 4 °C for 24 h. The sample was concentrated by ultrafiltration and dialyzed three times at 4 °C against 10 mM MOPS pH 6.5 for 12 h each. The enzyme samples obtained by this process were treated as refolded AnGDH and refolded AfGDH, respectively.

2.4. Protein and activity assay

Protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad, California, USA).

Dehydrogenase activity towards glucose and xylose was determined by: first, mixing the refolded enzyme sample with PMS (final concentration 0.6 mM) and DCIP (final concentration 0.06 mM) in 10 mM PPB (pH 6.5) and, to start the reaction, the respective substrate (final concentration 0–100 mM); and second, monitoring the decrease of DCIP absorbance at 600 nm. The reduction of 1 μmol DCIP in 1 min, corresponding to the oxidation of 1 μmol/min substrate, was defined as 1 U dehydrogenase activity.

Substrate specificity was determined by measuring the dehydrogenase activity towards 5 mM of each glucose, xylose, maltose, cellobiose, lactose, mannose, fructose, allose, galactose and sucrose.

2.5. Enzyme electrode preparation

0.1 U (for glucose) of refolded AnGDH or AfGDH was dried onto the electrode area of SPCE strips at 4 °C for 12 h. Then, a spacer and cover were attached to each electrode to form a capillary space. The thus prepared enzyme electrode was ready to use.

2.6. Electrochemical measurement

Samples containing 0–40 mM substrate and 200 mM mPMS were prepared. As substrate, glucose, xylose, or a mixture of glucose and xylose was used. 1 μL sample was injected into the capillary space of the enzyme electrodes. 5 s after loading the sample, +200 mV vs Ag/AgCl was applied and the current was recorded with a HSV-100, Hokuto Denko Co. (Tokyo, Japan).