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Recombinant fructosyl peptide oxidase preparation and its immobilization on polydopamine coating for colorimetric determination of HbA1c

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ABSTRACT

Recombinant fructosyl peptide oxidase (FPO) from *Phaeosphaeria nodorum* SN15 was functionally expressed by *Escherichia coli* cells and one-step purified from crude cells extract using immobilized metal affinity chromatography (IMAC) to achieve a specific activity of 26 U/mg. A ready-use colorimetric detection of HbA1c level in blood sample was developed based on FPO immobilized membrane. Facile bio-inspired polydopamine coating on the surface of a microporous membrane was employed for effective FPO immobilization. Glutaraldehyde activation of the polydopamine coating significantly enhanced FPO immobilization yield that at least 5-fold higher activity could be achieved. The stability of FPO membrane was also enhanced by glutaraldehyde activation that 85% activity could be maintained after 7 repeated uses. Highly correlated optical densities at 727 nm (OD_{727}) against fructosylvaline (FV) in the range of 0.02 mM to 0.7 mM ($R^2 = 0.988$) could be achieved using FPO membrane. At least 80% of the initial activity was maintained for FPO membrane stored at 4 °C for 7 days. Rather low OD_{727} but good correlation still could be obtained by using FPO membrane for the detection HbA1c levels (6–14%) in blood samples.

1. Introduction

Glycated hemoglobin A1c (HbA1c) is one of well-known glycated proteins generated via a non-enzymatic Schiff-base reaction between blood glucose and N-terminal valine residue of the β-chains in hemoglobin A molecules. Its measurement has important implications for diagnosis of diabetes and assessment of treatment effectiveness because its level reflects an average blood glucose concentration of the past 2–-3 months [1–4]. The level of HbA1c in blood is usually presented as percentage (%) of hemoglobin molecules grafted with glucose. For example, HbA1c level for normal (non-diabetic) adults is below 6.0% which means less than 6% of their hemoglobin molecules are grafted with glucose. For diabetes, the HbA1c levels are well above 6.5%. Many different methods are available for the measurement of HbA1c level in blood such as colorimetric wet chemistry [5,6], immunoassay, boronate affinity and ion exchange high performance liquid chromatography (HPLC) [7–13]. However, these methods, generally need tedious procedures and expensive equipments, are commonly employed in clinic laboratories.

Therefore, there is a strong demand in the market for a cost-effective and simple blood sugar level detection system for routine home-monitoring diabetes patients. In recent years, several facile HbA1c detection systems have been developed for point-of-care testing such as enzymatic and nonenzymatic electrochemical sensors [14–18], phenylbonoic acid based binding methods [19,20], and enzymatic colorimetric methods [21,22]. The enzymatic methods based on the fructosyl amino acid oxidase (FAO) or fructosyl peptide oxidase (FPO) to catalyze the oxidation of fructosyl group on N-terminal valine residue (Fru-Val) derived from HbA1c to generate hydrogen peroxide (H_2O_2) are more convenient and reproducible because the H_2O_2 sensing systems are well- established in many bioassays [23]. However, FAO or FPO enzymes found so far cannot directly oxidize the fructosyl group attached on HbA1c structure. As shown in Scheme 1, HbA1c must be hydrolyzed first by a protease to release fructosyl-amino acid or/and fructosyl-peptide. Then, enzymatic oxidation is employed for oxidative deglycation of the produced fructosyl amino acid or/and peptide to generate H_2O_2 that then can be easily detected by colorimetric or electrochemical methods.

Due to its commercial importance, active researches on isolation and characterization of fructosyl group oxidation enzymes (FAO and FPO) from various microorganisms have been carried out over the past decade [24–34]. The cloning and recombinant productions of these enzymes in *Escherichia coli* have also been achieved [35,36]. Many researches to improve the properties of these enzymes, such as substrate specification [37–41] and thermal stability [42] have been reported. Besides, many FAO-based electrochemical [15,17,43–46] and colorimetric detection systems [21,22,47] have recently been developed. Companies like Diazyme Lab and DiaSys have recently marketed

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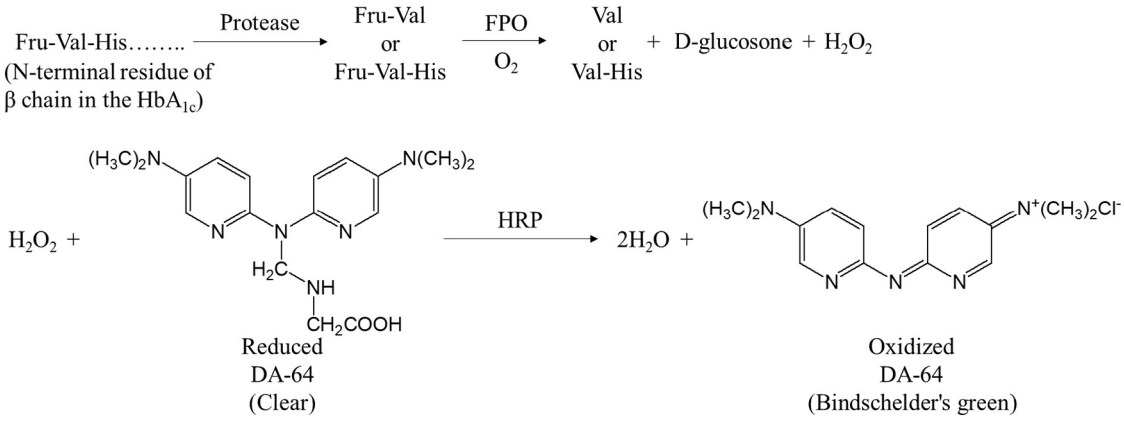

Scheme 1. Reaction steps for enzymatic detection of HbA1c in blood sample based on colorimetric method.

FPO-based colorimetric HbA1c detection systems. In the colorimetric detection method, pre-prepared free FPO enzyme solution is employed to react with the protease digested HbA1c sample to generate H_2O_2, then peroxidase along with reduced dye are added to develop a color with intensity in proportional to H_2O_2 generated. For the point-of-care testing, how to maintain the diagnostic enzyme activity in solution for ready-use is always a great challenge because enzymes are known to easily lose their activity in solution. In comparison with free enzyme, the immobilized enzyme has been believed to be more stable due to its less flexible structure when fixed on a solid surface. In addition to improved stability, the immobilized enzyme also has a ready-use advantage that benefits the point-of-care testing significantly. To the best of our knowledge, the immobilized FPO for colorimetric detection of HbA1c has never been reported yet.

Since reported by Lee et al. [48] that dopamine (DA) can be oxidatively self-polymerized in an alkaline condition and form an adherent coating on various organic or inorganic materials surfaces, this facile polydopamine coating has drawn a significant increased researches on its formation mechanisms and surface modification applications [49–54]. Polydopamine surface coating layer has a heterogeneous chemical structure with composition not precisely known. However, the presence of the oxidized catechol products in the polydopamine coating has been confirmed are capable of covalent coupling to biological molecules through their —SH and —NH2 functional groups via Michael addition or Schiff-base reaction [51–53,55]. Previously, we believed that there are remaining amino groups in polydopamine coating can be further activated by glutaraldehyde to enhance the enzyme immobilization on the coating surface via Schiff-base reaction [56]. Based on this line, in this work the synergetic effect of glutaraldehyde activation on enzyme immobilization on the surface of polydopamine coating was studied. The facile immobilization of FPO on a polydopamine coated polymeric membrane surface was employed for colorimetric HbA1c detection. The novel FPO from *Phaeosphaeria nodorum* SN15 reported by Kim et al. [36] was first produced using recombinant DNA technique in *Escherichia coli* cells and purified by immobilized metal affinity chromatography (IMAC) for immobilization on polydopamine coating surface. The free and immobilized FPOs were characterized for their thermal stability and reaction kinetics using a synthetic substrate fructosyl-valine. The detection of HbA1c concentration in blood samples based on free FPO and the ready-use FPO immobilized membrane were also investigated.

2. Materials and methods

2.1. Materials

E. coli BL21(DE3), *E. coli* BL21(DH5α) and plasmid pET23a vector were obtained from Novagen (USA). FPO expression plasmid pET23a-FPO was constructed using the FPO gene fragment synthesized by Yao-Hong Biotech Co. (Taipei, Taiwan) according to the published sequence [36]. Horseradish peroxidase (HRP) was obtained from Toyobo (Japan). Fructosyl valine (FV) was synthesized according to a previous published method [57]. *N* (carboxymethylaminocarbonyl) 4,4′ bis (dimethylamino) diphenylamine sodium salt (DA-64) was obtained from WAKO Chemicals. IMAC Sepharose™ 6 Fast Flow was purchased from Amersham Pharmacia Biotech. Dopamine hydrochloride (99%), isopropyl β D(−) thiogalactopyranoside (IPTG) and other related chemicals were obtained from ACROS Chemicals. Microporous polyethylene membrane was obtained from Coin Nanotech (Taipei, Taiwan).

2.2. Preparation of recombinant FPO

The gene encoding FPO from *Phaeosphaeria nodorum* SN15 was synthesized according to published sequence [36] and inserted into plasmid pET23a as an expression plasmid pET23-FPO. The expression plasmid was then transformed into *E. coli* BL21(DE3) for FPO production using 0.1 mM IPTG as inducer. The transformant was grown at 26 °C in Luria-Bertani (LB) medium (NaCl 10 g/L, tryptone 10 g/L and yeast extract 5 g/L). When OD_{600} reached 0.6–0.8, IPTG was then added to induce FPO expression. The cells were harvested at 4 h after IPTG induction. After disruption of the collected cell pellets by ultrasonication, FPO in the crude extract was purified by IMAC Sepharose™ 6 Fast Flow gel via the 6xHis tag on its structure based on the protocol provided by the manufacturer.

2.3. FPO activity assay

All the reaction reagents except DA-64 dye solution used for FPO activity measurement were prepared in pH 7.4, 10 mM phosphate-

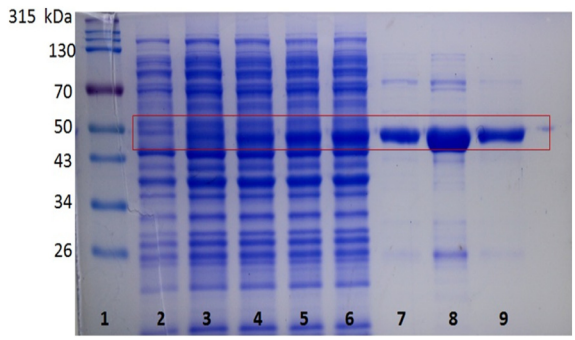

Fig. 1. SDS-PAGE analysis of FPO expressed by *E. coli* and its purification. Lane 1: marker protein. Lane 2: before IPTG induction; Lane 3–6: 1, 2, 3 and 4 h after IPTG induction; Lane 7–9: 1st, 2nd, and 3rd 1 mL fraction of FPO eluted from IMAC column.