Enzyme and Microbial Technology 49 (2011) 441–445

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

A biosensor based on the self-entrapment of glucose oxidase within biomimetic silica nanoparticles induced by a fusion enzyme

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ARTICLE INFO

Article history:
Received 31 January 2011
Received in revised form 11 April 2011
Accepted 13 July 2011

Keywords:
Biosilicification
Enzyme immobilization
Fused enzyme
Silaffin
Glucose oxidase
Biosensor

ABSTRACT

We constructed a fusion protein (GOx–R5) consisting of R5 (a polypeptide component of silaffin) and glucose oxidase (GOx) that was expressed in *Pichia pastoris*. Silaffin proteins are responsible for the formation of a silica-based cell matrix of diatoms, and synthetic variants of the R5 protein can perform silicification *in vitro* [1]. GOx secreted by *P. pastoris* was self-immobilized (biosilicification) in a pH 5 citric buffer using 0.1 M tetramethoxysilane as a silica source. This self-entrapment property of GOx–R5 was used to immobilize GOx on a graphite rod electrode. An electric cell designed as a biosensor was prepared to monitor the glucose concentrations. The electric cell consisted of an Ag/AgCl reference electrode, a platinum counter electrode, and a working electrode modified with poly(neutral red) (PNR)/GOx/Nafion. Glucose oxidase was immobilized by fused protein on poly(neutral red) and covered by Nafion to protect diffusion to the solution. The morphology of the resulting composite PNR/GOx/Nafion material was analyzed by scanning electron microscopy (SEM). This amperometric transducer was characterized electrochemically using cyclic voltammetry and amperometry in the presence of glucose. An image produced by scanning electron microscopy supported the formation of a PNR/GOx complex and the current was increased to 1.58 $\mu A\,cm^{-1}$ by adding 1 mM glucose at an applied potential of −0.5 V. The current was detected by way of PNR-reduced hydrogen peroxide, a product of the glucose oxidation by GOx. The detection limit was 0.67 mM (S/N = 3). The biosensor containing the graphite rod/PNR/GOx/Nafion detected glucose at various concentrations in mixed samples, which contained interfering molecules. In this study, we report the first expression of R5 fused to glucose oxidase in eukaryotic cells and demonstrate an application of self-entrapped GOx to a glucose biosensor.

1. Introduction

Immobilized biomolecules are more useful than their soluble counterparts in an industrial enzyme system owing to their stability and ease of handling. Enzymes can be immobilized by non-covalent adsorption, covalent bonds, entrapment, and cross-linking [2]. Biosilicification is an entrapment method that utilizes biological extracts (including protein and carbohydrates). It is related to the type of silicification that occurs in diatoms, sponges, and grass [3]. Biomimetic synthesis has become of interest recently because it can take place under mild conditions with greater ease [4]. Silaffin is a polycationic peptide (R_x) that is present in diatoms and induced nano-structured silica precipitates [5]. Synthetic variants of the R5 protein (a repeating unit of silaffin polypeptides) can perform silicification *in vitro* [1]. Several groups have used silaffin to induce biosilicification, which can immobilize fusion enzymes [6,7]. Our group also demonstrated that silaffin-fused proteins (Green Fluorescence Protein, GFP-R_x) were biosilicificated at lower concentrations that were 14–17 times less than those of synthetic polypeptides (R_x) [8]. In addition to the requirement of less protein to induce silicification, an enzyme fused with a silaffin protein takes advantage of the composite sol–gel electrode, leading to increased sensitivity due to voids or less dense film, biocompatibility without any formation of covalent bonds to the enzyme, and good adhesion to the supporting electrode [9,10]. Here, we present the expression of the novel fusion protein glucose oxidase–R5 (GOx–R5) in *Pichia pastoris*, and an application involving the self-immobilization of the enzyme on an electrode, which can function as a biosensor.

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doi:10.1016/j.enzmictec.2011.07.005

This process functions by taking advantage of the simple and rapid immobilization of the enzyme, which does not need an additional catalyst for silicification, similar to that of poly-L-lysine [11,12] and lysozyme [13,14], or any treatment such as electrodeposition [15].

Glucose oxidase (GOx) is one of many enzymes used in biosensors due to its high enzymatic activity. GOx catalyzes the oxidation of glucose to gluconolactone and the subsequent hydrolysis of gluconolactone to gluconic acid. It was demonstrated that the following materials can increase the conductivity of GOx: carbon nanotubes (CNT) [16–18], platinum and silica nanoparticles [19], tetrathiafulvalene (TTF) [20], and Tm_2O_3 nanoparticles [21]. Moreover, electrochemically prepared poly(neutral red) (PNR) can mediate the electron transfer from glucose to a cathode [22–25]. Neutral red (NR) has a phenazine structure, similar to that of flavins, which can mimic NADH dehydrogenase [24]. To overcome the handicap of the limited GOx production by *P. pastoris* in the laboratory, we used PNR as an electron shuttle to accelerate the electron transport between the electrodes and chemicals produced by glucose oxidase.

Unmodified silica is highly hydrophilic [26,27]. The entrapment of GOx by biogenic silica production creates a favorable environment for accessing diffused glucose and H_2O_2. In situations in which enzymes are lipophilic, such as the case with lipases, siloxane can be applied to enhance the level of hydrophobicity using alkylsilanes [28]. Additionally, a sol–gel matrix can improve the chemical and thermal stability of enzymes [6,7,13,27].

The primary goal of this study was to design and express a glucose oxidase–R5 fusion protein in *P. pastoris*. To demonstrate a possible application of the GOx–R5 fusion protein, we used self-entrapment characteristics of GOx–R5 to immobilize GOx on an electrode, which could function as a biosensor.

2. Materials and methods

2.1. Strains, plasmid construction, and transformation

The GOx gene was cloned from *Aspergillus niger* KCTC 6278, which was obtained from the Korean Collection for Type Cultures (KCTC). *A. niger* KCTC 6278 was cultured on potato dextrose agar (PDA, BD, USA). The GOx gene was cloned into yeast expression plasmid pGAPZαC (Invitrogen, USA) and expressed in wild-type *P. pastoris* X-33 yeast (Invitrogen).

Chromosomal DNA from *A. niger* KCTC 6278 was extracted with the PowerSoil® DNA Isolation Kit (Mo Bio, USA). The GOx gene from *A. niger* KCTC 6278 was cloned into pGAPZαC using the methods reported previously [29]. Briefly, the GOx gene was amplified by polymerase chain reaction (PCR) with primers pGO1f (5′-CCT TTC CTC TCT CAT TCC CTC A-3′) and pGO1r (5′-AAT GCC CTT GTT TGG TAG TAA T-3′). The resulting PCR product was re-amplified with PCR primers pGOef (5′-ATC CAT CGA TGA GCA ATG GCA TTG AAG CCA GCC TCC T-3′) and pGOer (5′-ATT AGC GGC CGC CTG CAT GGA AGC ATA ATC TTC CAA GAT AG-3′), where pGOef and PGOer contained restriction enzyme sites for Cla I (ATCGAT) and Not I (GCGGCCGC), respectively. The second PCR product and pGAPZαC were digested with Cla I and Not I, and ligated using the LigaFastTM Rapid DNA Ligation System (Promega, USA). pGAPZαC containing the GOx gene was named pGAP-GOx.

The R5 peptide, Ser-Ser-Lys-Lys-Ser-Gly-Ser-Tyr-Ser-Gly-Ser-Lys-Gly-Ser-Lys-Arg-Arg-Ile-Leu, was attached to the C-terminus of GOx. For the construction of the fusion enzyme, the GOx gene in pGAP-GOx was modified with a polyhistidine (HHHHHH) sequence (5′-CAT CAT CAT CAT CAT CAT-3′) and R5 (5′-TCT TCT AAG AAG TCT GGT TCT TAC TCT GGT TCT AAG GGT TCT AAG AGA AGA ATC TTG-3′), at the N-terminus and C-terminus of GOx gene, respectively. Gly-Gly-Gly-Ser (GGGS; 5′-GGT GGT GGT TCT-3′) was used as a linker peptide in an attempt to reduce steric interference and was inserted between the polyhistidine, R5 and the GOx genes. Insertion of the polyhistidine-GGGS sequence into the N-terminal region of the GOx gene was accomplished using a 64 bp DNA fragment which was amplified with primers HGf (5′-GAG GCT GAA GCA TCG CAT CAT CAT CAT CAT CAT GGT GGT GGT TCT G-3′) and HGr (5′-TGC CAT TGC TCA TCG ATC CAG AAC CAC CAC CAT GAT GAT G-3′). After digestion of the 64 bp PCR product and pGAP-GOx with Cla I (ATCGAT), the two DNA fragments were ligated and the resulting plasmid was named pGAP-HGOx. Insertion of GGGS-R5 into the C-terminus of the GOx gene contained in pGAP-HGOx was accomplished using a 107 bp fragment of DNA, which was amplified with the primers LR5f (5′-TCC ATG CAG GCG GCC GCC GGT GGT GGT TCT TCT TCT AAG AAG TCT GGT TCT TAC TCT GGT TCT AAG G-3′) and LR5r (5′-AGA AAG CTG GCG GCC CCT CAC AAG ATT CTT CTC TTA GAA CCC TTA GAA CCA GAG TAA GAA CCA GAC-3′). The 107 bp PCR product and pGAP-HGOx were digested with Not I and ligated. The ligation product was named pGAP-HGOxR5 (Fig. 1). Ligations of the digested PCR products and pGAP-GOx or pGAP-HGOx were accomplished using an In-FusionTM 2.0 Dry-Down PCR Cloning Kit (Clontech, USA). The plasmid pGAP-HGOxR5 was expected to express a polypeptide having the following sequence, HHHHHH-GGGS-GOx-GGGS-SSKKSGSYSGSKGSKRRIL.

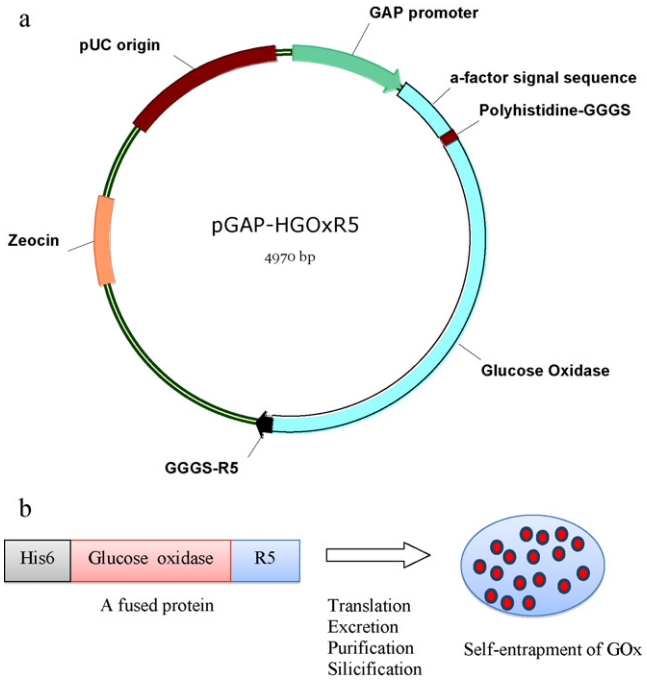

Fig. 1. (a) Structure of the yeast expression plasmid pGAP-HGOxR5, designed for the production of the GOx–R5 fusion protein in *P. pastoris*. (b) The overall scheme to design, express, and self-entrap the fusion protein.

The plasmid pGAP-HGOxR5 was transformed into *P. pastoris* X-33 to obtain a transformant expressing GOx–R5. Yeast transformations were performed using electroporation (Gene Pulser II System, Bio-Rad Laboratories, USA). Transformants were randomly selected after growth on a selective YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) containing 300 mg/l Zeocin (0.03%, w/v).

Expressed GOx–R5 was purified using an IMAC HyperCel (Pall, USA) that was the sorbent for immobilized metal affinity chromatography, according to the manufacturer's instructions, and used for the measurement of enzymatic activity and silicification.

2.2. Recombinant protein expression and purification

P. pastoris was aerobically incubated at 150 rpm in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) fructose) and sealed with a silistopper at 30 °C for improved air permeability. Fructose was used instead of glucose for GOx–R5 collection because GOx can oxidize glucose added to the media as a carbon source.

After 72 h of cultivation, the activity of GOx excreted from *P. pastoris* reach a plateau (data not shown), and the supernatant was collected from the pelleted biomass after centrifugation. GOx–R5 was harvested from the supernatant using the QIA*express*® Ni-NTA Fast Start kit (Qiagen, Valencia, CA), which can purify recombinant 6-His-tagged proteins.

2.3. Evaluation of GOx activity

The activity of glucose oxidase was kinetically analyzed by continuous spectrophotometric rate determination. Peroxidase reacts with hydrogen peroxide produced by GOx in the presence of glucose, resulting in a colorimetric change of *o*-dianisidine. After GOx (0.02 mL) was injected into a reaction solution (0.58 mL, 0.17 mM *o*-dianisidine and 1.72% (w/v) glucose), the increase in absorbance was measured by a UV–vis spectrophotometer (UV mini-1240, Shimadzu, Japan) at 500 nm, after which it was continuously monitored by an interfaced personal computer loaded with UV Probe 2.20 software (Hiroshima, Japan). The quantity of GOx produced by *P. pastoris* was measured using a NanoDrop in protein assay mode (Thermo Scientific, Wilmington, DE, USA).

2.4. Preparation of the GOx immobilized electrode

Before modification, a bare graphite rod electrode (GR, 6.0 mm diameter) was cut and polished with 0.05 μm alumina powder on a microfiber cloth. The working