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Secretory and continuous expression of *Aspergillus niger* glucose oxidase gene in *Pichia pastoris*

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Abstract

We proposed a yeast transformant cell incorporating the *Aspergillus niger* glucose oxidase gene (GOX gene), which is capable of constitutively as well as secretory expression. The GOX gene has been cloned in this study. This conclusion is based on the following: first, the ligated DNA determined by electrophoresis, was a 1489–1882 bp fragment, close to the size of glucose oxidase (GOD), which is 1818 bp. Secondly, the single open reading frame encoded a protein of 605 amino acids. Thirdly, secreted GOD recombinant proteins in the culture supernatants of the GOX gene transformant migrated as a single band in SDS–PAGE with an apparent molecular mass of between 75,000 and 100,000 Da, which is glycosylated GOD by the *Pichia pastoris* X-33 host machinery during the secretion process. Finally, the clones were cultured and secreted a protein, which possessed the GOD activity of catalyzing β-D-glucose oxidation. With regard to the pH characteristics, the activity was more than 80% of the maximum activity in the range between pH 5 and pH 7. As for the temperature characteristics, the activity was not less than 92% of the maximum in the temperature range between 10 and 45 °C. The GOX gene transformant was able to maintain the GOD enzyme activity and produce recombinant GOD continuously for at least 2 weeks.

Keywords: Glucose oxidase; *Aspergillus niger*; Secretory expression; Continuously purification; Biosensor

The most commonly used enzymes in biosensors contain redox groups that change state during the biochemical reaction. Enzymes of this type are glucose oxidase (GOD)[1] and glucose dehydrogenase. GOD (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as an electron acceptor [1]. Subcutaneously or totally implantable type glucose biosensors have been investigated for the continuous measurement of the blood glucose levels in real-time. However, implantable type blood glucose monitors in commercial use have a disadvantage in that they cannot be used for more than 3 days [2]. Implantable type enzyme-based glucose biosensors have the following problems: (i) difficulty to verify the bio-safety of the enzyme immobilising materials, (ii) deactivation of the enzyme with time, (iii) decrease in sensitivity due to the adhesion of proteins onto the surface of the electrode [3,4]. To address these problems the authors have been proposed the concept of a novel transformant-based biosensor using an enzyme which is produced by a transformant cell incorporating an enzyme gene, as the molecular recognition material [5]. The deactivated enzyme is replaced with newly produced recombinant enzyme from the transformant cell.

In order to realize this totally implantable glucose biosensor, a glucose oxidase gene transformant needs to be obtained so that the biosensor can continuously produce GOD. Previously, *Aspergillus niger* glucose oxidase gene

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[1] *Abbreviations used*: GOX gene, *Aspergillus niger* glucose oxidase gene; GOD, Glucose oxidase; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gelelectrophoresis; HPLC, high performance liquid chromatography; PVDF, polvinylidene difluoride; MCS, multiple cloning site.

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(GOX gene; GenBank Accession No. X16061) for GOD was determined by Marion Kriechbam et al. [6]. The GOX gene has been cloned from both cDNA and genomic libraries using oligonucleotide probes derived from the amino acid sequences of peptide fragments of the enzyme (1818 bp) [7–10]. Previously, the glucose oxidase gene has also been cloned into *Saccharomyces cerevisiae* [11,12]. Furthermore, the GOX gene linked to a secretory expression sequence has also been identified [13].

In this work, we present a transformant cell incorporating the GOX gene (GOX gene transformant), which is capable of constitutive as well as secretory expression, using the yeast strain *Pichia pastoris* X-33. The GOD activity as a function time is compared over a period of 2 weeks for recombinant GOD produced by the GOX gene transformant and commercially available GODs. Consequently, the culture solution of the GOX gene transformant is able to produce a sufficient amount of GOD for a glucose biosensor without shaking in conditions approximating those of a glucose biosensor implanted into the body.

Materials and methods

Strain and plasmid

Aspergillus niger was used as a source of the GOX gene. *Aspergillus niger* was obtained from the American Type Culture Collection (ATCC; strain No. 9029). It was grown in ATCC medium (No. 325; 2% malt extract, 2% peptone; Becton, Dickinson and Company, France) containing 2% glucose at 24 °C.

Plasmid pGAPZαC (3152 bp, Invitrogen Corporation, CA, USA) was used as a cloning vector. The pGAPZαC is a *Pichia* expression vector for constitutive expression and purification of recombinant proteins in *Pichia pastoris* [14]. The yeast strain *Pichia pastoris* X-33 (wild type; Invitrogen Corporation, CA, USA) was used as a host for cloning and enzyme expression [15]. The cells are spheroidal to ovoidal, (2.0–4.0) × (2.2–5.8) μm, and occur singly or in pairs [16]. *Pichia pastoris* X-33 was grown in YPD medium (1% yeast extract, 2% peptone; Becton, Dickinson and Company, France) containing 2% glucose at 30 °C. The vector has Zeocin-resistance as a selectable marker of the expression vectors. Zeocin is an antibiotic that acts as a strong anti-bacterial and anti-tumor drug [17]. In addition, the vector produces secretory expression as it codes for an α-factor secretion signal.

DNA manipulation, ligation and transformation

Aspergillus chromosomal DNA was isolated using a DNA extraction kit (No. 314-02731; Nippon Gene Co., Ltd., Japan) [18]. A two-step polymerase chain reaction (PCR) was carried out using oligonucleotide primers as follows:1st PCR: 5′-CCTTTCCTCTCTCATTCCCTCA-3′ and 5′-AATGCCCTTGTTTGGTAGTAAT-3′ 2nd PCR: 5′-atccatcgatGAGCCAATGGCATTGAAGCCAGCCTCCT-3′ and 5′-attagcggccgcCTACATGGAAGCATAATCTTCCAAGATAG-3′where, atccatcgatg: Bsu15I restriction enzyme site; attagcggccgc: NotI restriction enzyme site.

Electrophoresis was done using λ-EcoI14I as a molecular weight marker following Sambrock et al. [19]. The nucleotide sequence was analyzed using a sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, CA, USA). The identified GOX gene (1818 bp) was ligated to the plasmid vector pGAZαC using a DNA ligation kit (Code: 6023, Takara Bio Inc., Japan) [20].

The ligated DNA was used to transform *Pichia pastoris* X-33 to obtain the GOX gene transformant. Yeast transformations were done following Neumann et al. [21] using electroporation (Gene Pulser II system, Bio-Rad Laboratories, CA, USA). Ten randomly transformants were picked and grown in a selective medium (YPD medium containing 300 mg/l Zeocin (0.03%)) in order to acquire the GOX gene transformant.

Culture of GOX gene transforment

Preparation of the strain solution: the GOX gene transformant and wild type *Pichia pastoris* X-33 (wild type strain) were grown in YPD agar medium containing 300 mg/l Zeocin (0.03%) for 3 days in the dark. Then, 10 ml of the YPD liquid medium was inoculated with a single colony from the YPD agar medium and grown overnight with shaking (500 rpm) at 30 °C to create a starter culture. Next, 1 ml of the culture solution was added to 150 ml of YPD liquid medium and cultured with shaking (500 rpm) for few days at 30 °C in order to reach enzyme activity 1 U/ml.

Finally, the culture was incubated with shaking (500 rpm) at 30 °C. During incubation the GOX gene transformant and wild type strain were monitored the density of each strain solution at 660 nm using a spectrophotometer (OD_{660}, Hitachi Ltd., Japan) until the OD_{660} reached 20. This solution is hereafter called the stationary growth phase strain solution. The culture of positive clones was directly screened for GOD activity, and the secreted enzymes were monitored by SDS–PAGE and N-terminal amino acid sequence analysis.

SDS–PAGE and N-terminal amino acid sequence analysis

The stationary growth phase strain solution was centrifuged at 140 rpm for 3 min. The filtrated solution was centrifuged once more using an Amicon Ultra filter (Millipore Co., MA) at 140 rpm for 15 min. To investigate the secretion of GOD, cell precipitates were suspended in 15 μl of 2 × sample buffer consisting of 56% Tris–HCl (0.25 M, pH 6.8), 4% SDS, 11% sucrose, 0.4% bromophenol blue and distilled water. This was subjected to 10% SDS–PAGE (Ready Gels J, 161-J320, Bio-Rad Laboratories, Inc., CA) at 200 V for 60 min. The separated proteins were stained with a Silver Stain kit (161-0443, Bio-Rad Laboratories, Inc., CA).