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ScienceDirect
Journal of Chromatography B, 858 (2007) 151–158
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of glucose oxidase from complex fermentation medium using tandem chromatography

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Received 6 October 2006; accepted 13 August 2007
Available online 22 August 2007

Abstract

A fast and efficient purification method for recombinant glucose oxidase (rGOx) for flask fermentation scale (up to 2 L) was designed for the purposes of characterization of rGOx mutants during directed protein evolution. The *Aspergillus niger* GOx was cloned into a pYES2-αMF-GOx construct and expressed extracellularly in yeast *Saccharomyces cerevisiae*. Hydrophobic interaction (HIC)/size exclusion (SEC)-tandem chromatographic system was designed for direct purification of rGOx from a conditioned complex expression medium with minimum preceding sample preparation (only adjustments to conductivity, pH and coarse filtering). HIC on Butyl 650s (50 mM ammonium acetate pH 5.5 and 1.5 M ammonium sulphate) absorbs GOx from the medium and later it is eluted by 100% stepwise gradient with salt free buffer directly into SEC column (Sephadex 200) for desalting and final polishing separation. The electrophoretic and UV–vis spectrophotometric analyses have proven enzyme purity after purification.

Keywords: Recombinant glucose oxidase; Purification; Hydrophobic interaction chromatography; Size exclusion chromatography

1. Introduction

Glucose oxidase (GOx, β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is an important biotechnological object for industrial applications. GOx catalyses the oxidation of β-D-glucose to D-glucono-1,5-δ-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor and releasing hydrogen peroxide. In aquatic phase D-glucono-1,5-δ-lactone spontaneously hydrolyzes to gluconic acid, therefore GOx-producing moulds such as *Aspergillus* and *Penicillum* species are used for the biological production of gluconic acid. GOx is highly specific to β-D-glucose, although 2-deoxy-D-glucose, D-mannose and D-fructose are also oxidised, albeit at a much reduced rate [1]. GOx is used for removing residual glucose and oxygen from beverages, wine [2], foodstuffs [3], and also in bleaching cellulose fibers [4]. However, one of the most exciting GOx applications is in biosensors for the monitoring of glucose levels during fermentation of beverages and also in body fluids, such as blood and urine [5], or as converter of chemical energy into electrical energy in a miniaturised implantable biofuel cell [6]. However, GOx from natural sources (mainly fungi) is not stable for long terms under the conditions used in such applications. Therefore there is an increasing need for a stable GOx, suitable for the biosensor application.

Directed protein evolution (DE) provides a methodological platform [7] for the improvement of properties of a wild type GOx, such as: increased glucose affinity, higher turnover number, increased thermostability, increased electron transfer rate, increased chloride resistance and activity at pH 7.0 [6]. According to the DE methodology, first, the variety of GOx random mutants (i.e. a library) is generated (for example by error prone PCR method), which is then screened to find improved mutants. Selected mutants later will be separately cultivated to yield enough enzymes for the further characterization. Therefore, a

Abbreviations: GOx, glucose oxidase; cGOx, commercially available GOx from *A. niger*; rGOx, recombinant *A. niger* GOx derived from yeast *S. cerevisiae*; IEC, ion exchange chromatography; HIC, hydrophobic interaction chromatography; SEC, size exclusion chromatography

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doi:10.1016/j.jchromb.2007.08.015

fast and simple purification procedure for mutants of recombinant GOx from batch flask fermentation (1–2 L) is required.

The natural GOx source is fungi (such as *Aspergillus niger*, *Penicillium chrysogenum*, *P. amagasakiense* and *Botrytis cinerea*), consequently, the major body of information about GOx properties has been derived for this source. The mature GOx is a homodimer with a number of different non-bonded interactions between monomers. Mature GOx from *A. niger* is N- or O-glycosylated at Asn, Thr and Ser (MW = 160–180 kDa) with a carbohydrate moiety of 16% (w/w), which has high mannose content (80%, w/w) [8]. GOx is flavoportein and each monomer contains one tightly, but not covalently bound flavin adenine dinucleotide (FAD), which is an important part in the oxidative reaction chain. FAD undergoes independent reversible half-reactions through two stable redox states (oxidized and reduced) following a ping-pong kinetic mechanism. Therefore, the apo-enzyme is not active. Under conditions of oxygen excess and glucose deficiency the FAD of GOx is very quickly oxidizes. The oxidized FAD has unique spectral characteristics therefore pure GOx is yellow with unique adsorption maxima at 280, 382 and 452 nm (the last two bands belong to oxidized FAD). The pH optimum of GOx is 5.5, but GOx is able to operate within relatively broad pH range from 4 to 7 and has pI = 4.2.

Purification of intracellular GOx from fungi is based on adsorption chromatographic techniques (IEC and HIC) with gradient elution, therefore laborious and time consuming sample preparation is required due to the relatively low GOx intracellular content and relatively high content of bulk proteins, lipids, carbohydrates, nucleic acids and intrinsic salts in a cell homogenate. For example, a combination of dialysis [8,9], ammonium or copper sulphate fractionating [8,10,11], ultrafiltration [11], consecutive precipitation in potassium haxacyanoferrate(II) and isopropanol [11], and desalting and coarse fractionating on SEC [9,12] are main sample preparation methods for following purification on IEC [8–10,12] or HIC [11] with finalizing polishing step on SEC [8] or chromatofocusing [12].

Extracellular expression of recombinant GOx (rGOx) in fungi (*Saccharomyces cerevisiae*, *Pichia pastoris*, *Penicillium funiculosum*) using a signal sequence (like α-mating factor) is more attractive strategy, because yeast can successfully secrete large amount of active rGOx into the surrounding medium, e.g. up to 1.5 g L^{-1} [13]. However, the expression level depends on chosen promoter/terminator pair (constitutive, inducible, etc) [2]. Unlike bacteria [14], yeast also performs post-translational protein modification, particularly *N*-linked glycosylation which is essential for GOx stable activity. However, yeast usually hyperglycosylate recombinant proteins with a variety of carbohydrate moieties with lengths that result in broad range of molecular weights of recombinant enzyme [15,16]. Regardless, the secretory strategy somewhat simplifies a medium preparation for the adsorption chromatography (e.g. IEC), since rGOx source (expression medium) possesses less bulky and more diluted contents than cell extracts. Therefore medium dilution to reduce salt concentrations [13] or ultrafiltration with following dialysis [17] or adsorption on quartz sand or Al_2O_3 [18] is sufficient to condition a sample for the IEC. There is a method that solely uses filtration techniques (cross-flow filtration, microfiltration, ultrafiltration and diafiltration) without resorting to chromatographic methods to recover the rGOx from large volume batch fermentation [19,20]. Thus, this approach is time consuming although is compromisingly efficient and capable of treating large amounts of fermentation medium. Alternatively, the *gox* gene can be fused with His- [21] or Lys-tags [17] to use affinity chromatography (e.g. HisTrap). However, unfortunately tagging significantly affects rGOx kinetic properties [17,21], which conflicts with the purposes of protein engineering. In conclusion, such approaches of preparation of extracellular rGOx sources for chromatographic purification are not practical for characterisation of a number of mutants in the course of the protein engineering due to either the length of time (dialysis, ultrafiltration) or inflation of the sample volume (dilution) or low yielding (adsorption on quartz sand) or affecting kinetic properties (tagging).

Thus, the aim of the paper is to provide a fast and simple method to purify milligrams of extracellular rGOx from flask-scale batch fermentations (up to 2 L) for the purposes of further mutant validation in course of the protein engineering by directed protein evolution.

2. Experimental

2.1. GOx cloning

The *gox* gene from *A. niger* has been obtained from Institute of Wine Biotechnology, Stellenbosch, South Africa [2]. The *gox* gene and yeast mating pheromone α-factor secretion signal (*MFα1s*) were cloned into pYES2 (Invitrogen) via KpnI–XhoI restriction sites [pYES2-MFα1s-GOx] under GAL1 promoter and CYC1 terminator. The DNA manipulations were performed according to Sambrook et al. [22]. The restriction enzymes were purchased from (MBI Fermentas GmbH, St. Leon-Rot, Germany) and used according to supplier's recommendations. Initially pYES2-MFα1s-GOx was transformed in *E. coli* (DHα5 strain) which were grown on ampicillin selective LB medium plates, and then the construct was transformed in yeast *Sacharomyces cerevisiae* (INVSc1 diploid strain, Invitrogen) using S.c. EasyComp transformation kit (Invitrogen) and selected on SC-minimal medium (URA 3) selective plates.

2.2. Medium and yeast culture conditions

Successfully transformed yeast were cultured (250 rpm, 30 °C) in liquid phase of growth SC minimal medium (1.7 g L^{-1} yeast nitrogen base, 5 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} amino acids (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan), 0.05 g L^{-1} amino acids (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine) and 20 g L^{-1} glucose; pH 5.0; 20–25 mS cm^{-1}). The GOx expression was induced by exchange of growth medium (with glucose) onto expression medium (with galactose). The induction was performed at 0.4 OD of the culture density and lasted for 12 h, where maximum of rGOx induction was observed.