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Purification of glucose oxidase from complex fermentation medium using tandem chromatography

Maxim Zakhartsev [a,b,*], Carmen Momeu [a]

[a] *Biochemical Engineering, Jacobs University Bremen, Germany*
[b] *Marine Animal Physiology, Alfred Wegener Institute for Polar and Marine Research (AWI), Bremerhaven, Germany*

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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

* Corresponding author at: Institute of Biochemical Engineering (IBVT), University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.
Tel.: +49 711 685 69930.
E-mail address: maksim.zakhartsev@ibvt.uni-stuttgart.de (M. Zakhartsev).

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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.

2.3. Sample conditioning

After 12 h of induction of GOx secretory expression the yeast cells were spun down at 4×10^3 rpm for 10 min and supernatant collected. The conductivity of the collected medium was adjusted up to 183 mS cm^{-1} by $(NH_4)_2SO_4$, which corresponds to 1.5 M solution of ammonium sulphate. The pH of the supernatant (medium) was adjusted up to 5.5 by 20% aqueous ammonium solution. Finally, the conditioned medium was filtered through 0.45 μm filter under vacuum (GH PolyPro 50 mm hydrophobic polypropelene membrane filters; Cat. No. 66625, Pall Life Science).

2.4. Purification of GOx

2.4.1. Instrumentation

A column with strong hydrophobic interaction stationary phase (HIC; TOSOH Toyopearl® Butyl 650s, 35 μm, 15/125 mm) was tandemed with size exclusion column (SEC; Amersham Pharmacia Sephadex® 200, 20–50 μm, 16/600 mm) to form HIC/SEC-tandem chromatographic system on the base of ÄKTA*explorer* (Amersham Biosciences). The columns were tandemed by means of three 4-port small high pressure rotary T-valves (Cat. No. 1121 Bio-Chem Valve/Omnifit, Cambridge, UK) which were additionally installed in the system (V2-V4; Fig. 1). One of the inlets of V2 and V4 were capped (Fig. 1). The combination of flow directing positions of V2–V4 valves allowed employing either one of any columns or both together, according to the embedded scheme in the Fig. 1. Accordingly, the flow directing positions of valves V2–V4 were denoted as: "Adsorption" (or flow *Position 1*) where flow goes only through HIC column but around SEC column, "Elution" (or flow *Position 2*) where flow goes through HIC column into SEC column and through it, and "Polishing" (or flow *Position 3*) where flow goes around HIC column but through SEC column. Being at *Position 1* the system had 17 mL dead volume, measured by injection of 5 μL of acetone. All switches between flow positions were carried out through pausing of the system. The injection valve (V1) was not involved during the purification, whereas the outlet valve (V5) was used for switching between waste and fraction collector. Buffer A was pumped through inlet A1, correspondingly buffer B through inlet B1, whereas loading of the conditioned sample was implemented through the inlet A2, i.e. direct on-column sample application (Fig. 1). Monitoring of the purification was carried out at 280, 382 and 452 nm (mAU) and conductivity (mS cm^{-1}). The buffer compositions and other system's specifications are presented in Table 1.

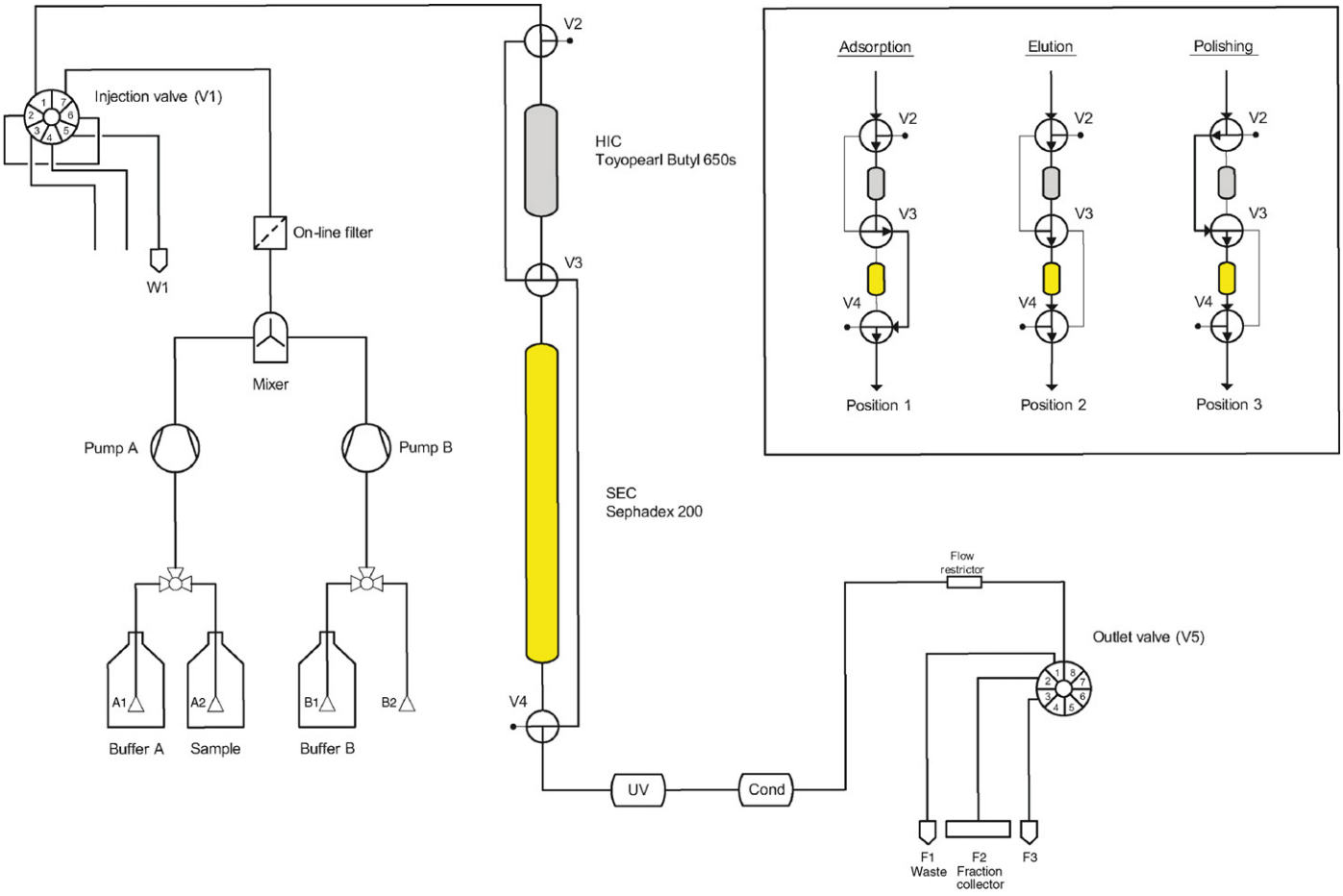

Fig. 1. Map of the HIC/SEC-tandem chromatographic system based on ÄKTA*explorer* represented in the "Adsorption" flow position. Embedded map shows all others flow directions (bold arrowed lines) through combination of three 4-port small rotary T-valves (V2, V3 and V4). One of the outlets of V2 and V4 are capped.

Table 1
Instrumentation and specification of the HIC/SEC-tandem chromatographic system

Instrument	ÄKTA*explorer* (Amersham Biosciences)
Column 1—HIC (Hydrophobic interaction chromatography)	
HIC medium	TOSOH Toyopearl® Butyl 650s
Particle size	20–50 μm
Exclusion limit	5×10^6 Da (globular proteins)
Protein adsorption capacity	40 ± 10 mg mL^{-1} (lysozyme)
Column	15/125 mm (KRONLab Cat. No. TAC15/125PE5-AB-1)
Int. volume	22 mL
Gradient	Stepwise 0 → 100% buffer B in 0 min
Flow rate	5 mL min^{-1} = 170 cm h^{-1}
Pressure	0.21 MPa
Column 2—SEC (size exclusion chromatography)	
SEC medium	Sephadex™ 200 (Amersham Biosciences)
Particle size	20–50 μm
Column	16/600 mm (custom prepacked column Amersham Biosciences)
Int. volume	120 mL
Flow rate	1 mL min^{-1} = 30 cm h^{-1}
Pressure	0.1 MPa
Buffer A	50 mM CH_3COONH_4 pH 5.5; 1.5 M $(NH_4)_2SO_4$
Buffer B	50 mM CH_3COONH_4 pH 5.5
Detection	UV–vis at 280, 382, 452 nm; conductivity
Sample	GOx in conditioned expression medium
Sample injection	On-column sample application through A2 inlet (see Fig. 1)
Sample volume	Up to 2000 mL

2.4.2. Purification

2.4.2.1. The system conditioning. Initially, the HIC/SEC-tandem system was conditioned with buffer B being set in flow *Position 2* ("elution"; Fig. 1) at 1 mL min^{-1} flow rate for three column volumes of SEC column. Then the system was switched into flow *Position 1* ("Adsorption"; Fig. 1) and conditioned with buffer A at 5 mL min^{-1} flow rate for 3–5 column volumes of HIC column.

2.4.2.2. Sample loading to HIC. To begin on-column sample application the pump A was switched over to A2 inlet while the tandem system was kept at the same flow *Position 1* and flow rate (5 mL min^{-1}). This approach allowed application of relatively large volumes onto HIC column.

2.4.2.3. Washing. The pump A was switched back to A1 to begin the wash of HIC column from unbounded compounds while keeping the same flow *Position 1* and flow rate (5 mL min^{-1}). The wash was continued until a stable baseline almost at zero was reached.

2.4.2.4. Elution. The elution of adsorbed compounds began with a 100% stepwise gradient with Buffer B from the B1 inlet, while the tandem system at the same flow *Position 1* and flow rate (5 mL min^{-1}) was kept constant. Exactly after system's dead volume (17 mL) the flow rate decreased to 1 mL min^{-1} and flow direction switched to flow *Position 2* ("elution"; Fig. 1) to expel all desorbed compounds that were eluted from the front into SEC column.

2.4.2.5. SEC. After 6 mL had been eluted in flow *Position 2* the flow direction of the tandem system switched to flow *Position 3* ("SEC"; Fig. 1) for the further size exclusion separation, while the same flow rate 1 mL min^{-1} with buffer B from B1 inlet was maintained. GOx was eluted as single desalted peak that was fractioned (2 mL fractions).

2.4.2.6. GOx concentration and lyophilization. Collected fractions with GOx activity were pooled and either concentrated on centrifugal filter devices Centricon-50 with 50 kDA cut-off threshold (Cat No. 4224, Millipore Ireland BV, Tullagreen, Carrigtwohill, County Cork, Ireland) or frozen at −80 °C and dried to powder in Lyophilizer Alpha 1–2 LD (Crist GmbH, Osterode, Germany).

2.5. GOx kinetic activity

GOx activity of collected fractions was assayed in F-bottom PS 96-well microplate with 300 μL volume (Greiner Bio-One GmbH, Frickenhausen, Germany), always in triplicate at room temperature. ABTS/HRP coupled assay that detects production of hydrogen peroxide was used to detect GOx activity [6,23]. The final content of the reaction mixture was: 200 mM phosphate/citrate buffer pH 5.0, 100 μM ABTS (2.2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; λ_{ex} = 414 nm; $\xi^{1\,M}_{1\,cm} = 36800$), 1.25 U mL^{-1} of horseradish peroxidase (Fluka) and different β-D-glucose concentrations (0–300 mM) in 100% air saturated solution (∼300 μM of O_2 at 25 °C). The reaction was initiated by adding of 50 μL sample and monitored at 414 nm during 5 min in FLASHScan S12 microplate reader (Analytik Jena, Jena, Germany) every 12 s. GOx performs two-substrate reaction with glucose (glc) and oxygen (O_2):

$$\upsilon = \frac{V_{max}[glc]}{K_M^{glc} + [glc](K_M^{O_2}/[O_2])} \quad (1)$$

The total GOx activity in collected chromatographic fractions was always measured at both β-D-glucose (300 mM) and oxygen (∼300 μM at 25 °C) saturating concentrations, whereas kinetic parameters were evaluated at different β-D-glucose concentrations (0–300 mM) in 100% oxygen saturated aquatic phase (∼300 μM at 25 °C) or at different oxygen concentrations (0–1000 μM) under saturation with β-D-glucose (500 mM) [6]. The Michaelis constants were calculated by non-linear regression analysis using Eq. (2) (GraphPad Prism v4.0, GraphPad Inc., USA):

$$\upsilon = \frac{V_{max}[glc]}{K_M^{glc} + [glc]} \quad \upsilon = \frac{V_{max}[O_2]}{K_M^{glc} + [O_2]} \quad (2)$$

The GOx activity was expressed in units (1 unit = 1 μmol glucose min^{-1}).

2.6. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was conducted according to the method of Laemmli et al. [24] using 6% acrylamide separating gel and 5% stacking gel containing 1% SDS. Purified rGOx from *S. cerevisiae* and cGOx from *A. niger* samples were heated at 95 °C for 10 min in Tris–glycine buffer containing 2% SDS, 0.1% bromophenol blue and 100 mM dithiothreitol in buffer pH 2.2. Electrophoresis was carried out at a constant current of 200 V for 35 min at +4 °C using a running buffer of Tris–glycine containing 0.1% SDS. After electrophoresis, the gel was stained with 0.025% Coomassie Brilliant Blue R-250 solution (1 h, room temperature), and was finally destined in methanol: acetic acid 1:1 solution (2 h, room temperature) with changing the destining solution one or two times. PageRuler^{TM} prestained protein ladder (Fermentas, Cat. No. #SM0671) was used as the molecular weight standard for gel analysis (Fig. 4) (ImageQuant TL v2005, Amersham Biosciences).

2.7. Protein quantification

The protein concentration was assayed using NanoOrange® protein quantification kit using BSA for calibration (Cat. No. N6666, Molecular Probes, Invitrogen).

3. Results

To work out the purification procedure we made 100 mL of SC minimal medium after expressing pYES2 without GOx insert in yeast and 2.8 mg of commercial GOx (cGOx) [Fluka, Cat. No. 49181]. The medium was then conditioned according to Section 2 (conductivity up to 183 mS cm^{-1} with $(NH_4)_2SO_4$, pH 5.5 with 20% NH_3 aqueous solution, filtering through 0.45 μm) and cGOx was purified from there. Prior sample application the HIC/SEC-tandem chromatographic system was equilibrated initially with buffer B (Table 1) being in flow *Position 2* (Fig. 1) for three column volumes of SEC column at 1 mL min^{-1}, then switched into flow *Position 1* (Fig. 1) and additionally equilibrated with buffer A (Table 1) for 3-5 HIC-column volumes at 5 mL min^{-1}. Hundred milliliters sample was applied directly on-column through A2 inlet at 5 mL min^{-1}, correspondingly during ~20 min (Fig. 2). System pressure during "Adsorption" and washing phases was $P = 0.21$ MPa. Unbound compounds were washed out during approximately the same volume/time (Fig. 2) through A1 inlet. The 100% stepwise gradient was started with buffer B from B1 after the baseline was approached zero and was stable. Such acute type gradient elution allows condensing of all eluting high molecular weight proteins into a relatively narrow front, which improves the efficiency of the following SEC separation. Then exactly after 17 mL (this was equal to the dead volume of the system in flow *Position 1*) of the gradient elution the system was switched into flow *Position 2* through pausing of the flow and concomitant reducing flow rate to 1 mL min^{-1} (Fig. 2). After 6 mL in flow *Position 2* the system was switched to flow *Position 3* (Fig. 2). Six milliliter is a sufficient volume for passing the complete front of the gradient from HIC column into SEC column and at the same time this is the limit of the injection volume for this SEC column. The SEC separation (flow *Position 3*) was then completed at 1 mL min^{-1} with buffer B from B1 (Fig. 2). System pressure during "SEC" phase was $P = 0.1$ MPa. The GOx peak was monitored at three unique wave-

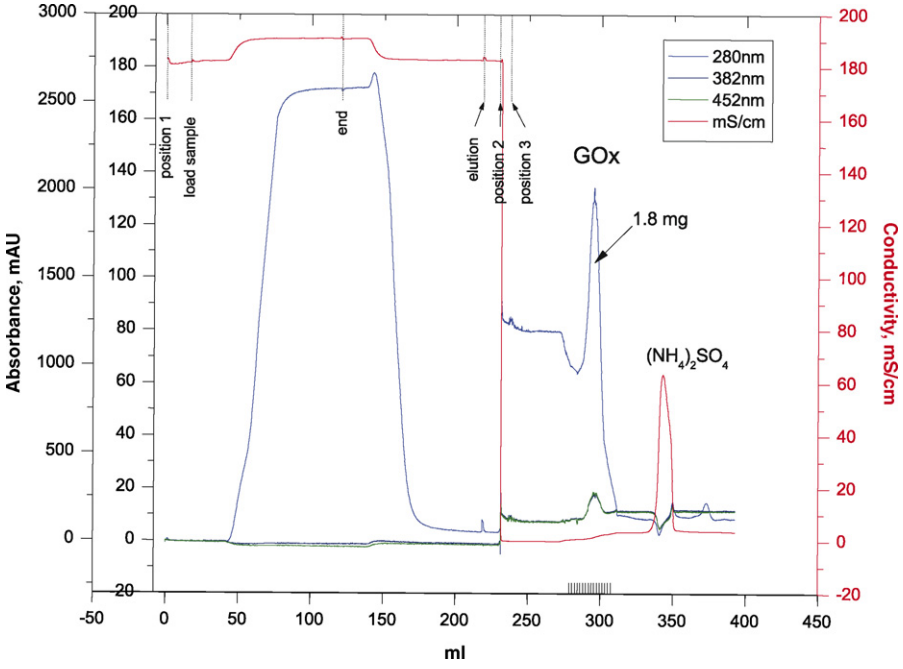

Fig. 2. Purification of commercial glucose oxidase (cGOx) by HIC/SEC-tandem chromatographic system from 100 mL of complex expression medium. Corresponding purification phases are indicated according to Fig. 1. The time/volume course of the purification is documented in Table 2. From 0 to 230 mL (switch to flow *Position 2*) the absorbance scale is up to 3000 mAU, whereas afterwards it is up to 200 mAU.

Table 2
Time/volume protocol of commercial GOx purification at HIC/SEC-tandem chromatographic system (Fig. 2) from 100 mL of complex fermentation medium

Min	mL	Event	Purification phase
0	0.00	Wavelength 280 nm, 382 nm, 452 nm	Adsorption
0	0.00	Flow Position 1 (see Fig. 1)	
0	0.00	Start: flow = 5.0 mL min^{-1} from A1	
3.4	17.12	Sample loading Start from A2	
23.9	119.73	Washing HIC column from A1	
43.8	219.05	Gradient 100.0% B, length 0.00 min from B1	Elution
46.1	230.27	Pause: flow Position 2 (see Fig. 1)	
46.1	230.27	Flow = 1.0 mL/min	
46.1	230.27	Continue	
52.1	236.31	Pause: flow Position 3 (see)	Polishing
52.1	236.31	Continue	
94.2	278.43	Fractionation start; 2.00 mL	
124.2	308.42	Fractionation stop	
208.5	392.76	Stop	

lengths (280, 382 and 452 nm) by UV–vis detector, whereas salt was monitored by conductometer. The desalted GOx peak was fractioned (2 mL) and fractions exhibiting GOx activity were pooled ($\sum V = 16$ mL) (Fig. 2). Nevertheless, the GOx activity was checked in all combined fractions along the purification procedure such as during adsorption, washing, etc. steps, and none of them exhibited any enzyme activity. The pooled GOx fraction was either concentrated immediately with a centrifugal filter device, Centricon-50 with 50 kDA cut-off threshold, or frozen at −80 °C and then lyophilized. None of additional dialysis treatment was required since ammonium acetate is volatile buffer. The collected cGOx (1.72 mg) gave a 61.4% yield efficiency, due to some impurities in the commercial preparation (Fig. 3)

Cloning of the wild type GOx from *A. niger* (cGOx) and expressing of it in yeast *S. cerevisiae* (rGOx) did not affect significantly the affinity of the enzyme to β-D-glucose: K_M^{glc}(cGOx) = 18.01 ± 1.70 mM versus K_M^{glc}(rGOx) = 18.66 ± 0.96 mM (*F*-test; $F(df_n, df_d) = 5.15 \times 10^{-5}$ (2.72)) at pH 5.5 and 25 °C.

Spectral ratio (280:382:452 nm) of commercial GOx (cGOx) from *Aspergilus niger* [Cat. No. 49181, Fluka] was quantified in 100 μg cGOx per mL of 50 mM ammonium acetate pH 5.5 (buffer B) and it was 9.83:0.89:1.0, whereas after purification it had become 6.90:0.89:1.0 (the spectral values were taken from the chromatogram; Fig. 2). Additionally, size exclusion separation of cGOx dissolved in buffer A shows that commercial preparation possesses a significant amount of impurities (f_1, f_3, and f_4; Fig. 3).

SDS-PAGE analysis of cGOx dissolved in conditioned fermentation medium expressing pYES2 without the GOx insert showed that the strongest band in the gel has a size of 74.8 kDa. A number of small heavier minor bands are obscured by its tail (line 2; Fig. 4). Additionally, a number of lightweight minor compounds (<70 kDa; line 2; Fig. 4) are contained in the fermentation medium itself. Moreover, none of compounds of ∼74 kDa were observed in such abundance in the fermentation medium after expressing empty pYES2 (without GOx) (data are not shown). Nevertheless, purified cGOx, also showed a smeared

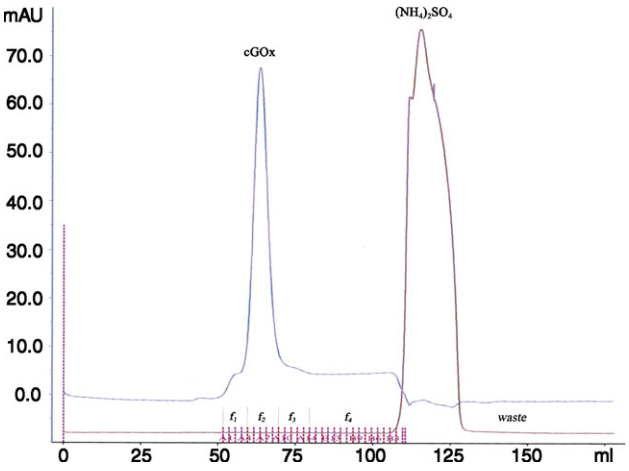

Fig. 3. Size exclusion separation of commercial GOx (cGOx) on Sephadex 200. Only fractions A5–A9 ($\sum V = 10$ mL) have shown GOx activity and unique absorption spectrum (with maxima at 280:382:452 nm), therefore they were pooled (f_2), all the rest collected fractions (f_1, f_3 and f_4) including waste did not show GOx presence.

Column	Sephadex 200 (16 mm × 600 mm)
Mobile phase	Buffer B (50 mM ammonium acetate pH 5.5)
Flow rate	1 mL min^{-1}
Sample	0.2 mg cGOx per mL of buffer A (buffer B + 1.5 M ammonium sulphate)
Inj. vol.	2 mL
Detection	280 nm (mAU), conductivity (mS cm^{-1})
Fraction vol.	2 mL

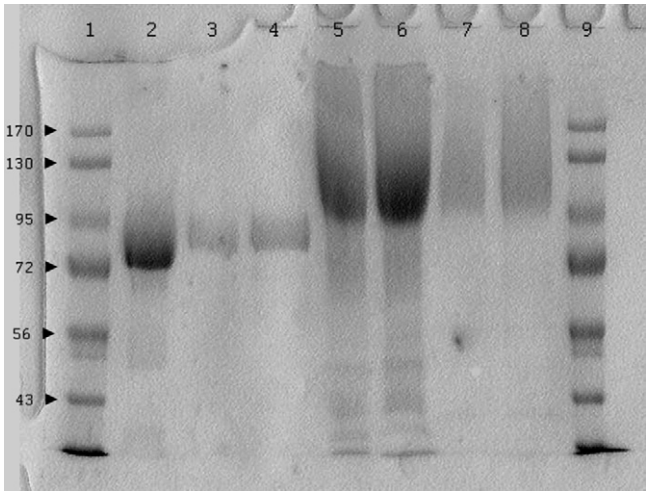

Fig. 4. SDS-PAGE of commercial (cGOx from *Aspergillus niger*) and recombinant (rGOx from *A. niger* expressed in yeast *S. cerevisiae*) GOx before and after purification by HIC/SEC-tandem chromatographic system. Lines 1 and 9—MW ladder (kDa); line 2—freshly dissolved cGOx in conditioned fermentation media after expressing pYES2 without GOx insert (74.8 kDa); lines 3 and 4—cGOx after purification (monomer is 80.8 kDa); lines 5 and 6—rGOx in conditioned fermentation media derived from *Saccharomyces cerevisiae*; lines 7 and 8—rGOx after purification (monomer is 105 kDa).

but single band with a maximum at 80.8 kDa (lines 3 and 4; Fig. 4), which proves the specificity of the purification.

Finally, rGOx was expressed (lines 5 and 6; Fig. 4), and 20.2 mg of the recombinant enzyme was purified from 546 mL of conditioned fermentation medium (lines 7 and 8; Fig. 4). Fig. 4 also shows that the purified monomer of rGOx is 105 kDa (210 kDa dimmer), which is quite different from original *A. niger* GOx. It is clearly because the purified rGOx demonstrates wider electrophoretic band with a long tail (Fig. 4) that indicates higher variety of hyperglycosylation patterns derived from yeast.

4. Discussion

Although, efficient ion exchange chromatography (IEC) analytical separation methods of isoforms of commercial GOx exist and are well characterised [25–27], mid-scale purification of GOx from expression medium by IEC is more complicated. This is because the intrinsic salt content in the expression medium ($\sim$20 mS cm^{-1}) defines the way the sample is prepared for the adsorption chromatography. Correspondingly salt content should be reduced either by dilution (volume inflation) [13] or dialysis [17] or ultrafiltration. Unfortunately, these approaches are time-consuming for a sample with a volume larger than 500 mL, therefore we have chosen an alternative route using hydrophobic interaction chromatography (HIC) as the adsorption technique, as it is easier to increase a salt content in the sample.

Pilot measurements have shown that cGOx precipitates at 90% (3.78 M) of $(NH_4)_2SO_4$ at pH 5.0 and +4 °C overnight. However, we found that minimal concentration of ammonium sulphate which results in complete GOx adsorption from expression medium on the strong HIC stationary phase (Butyl 650s) at pH 5.5 is 1.5 M, whereas a lot of other compounds from the expression medium were not adsorbed at this salt concentration (Fig. 2). This peculiarity was exploited on the first purification step of HIC/SEC-tandem chromatographic system – Adsorption (Fig. 1). Direct on-column application of the conditioned and filtered sample allows the adsorption of GOx from samples of any volume. The volume of HIC stationary phase was 22 mL, which has large protein adsorption capacity (40 ± 10 mg per mL of the HIC medium) (Table 1). Therefore, for the flask batch fermentation scale ($\leq$2000 mL), we were far below saturation of the adsorption capacity of the HIC medium. The rate of HIC on-column sample application (up to 10 mL min^{-1} or 340 cm h^{-1}) has no effect on protein adsorption capacity of Butyl 650s and upper rate limit is mainly defined by backpressure of the system due to chromatographic materials and sample condition.

We chose an ammonium acetate buffer as a medium for the purification because it is volatile and therefore facilitates lyophilization. The buffer system (A and B) was designed in a way that desorption buffer from HIC column is the same time as the separation buffer for the SEC column.

A decrease in salt content below 1.2 M of ammonium acetate causes immediate desorption of GOx from Butyl 650s. This property was used to elute GOx from HIC in a very concentrated front by applying a 100% stepwise gradient with salt free buffer B in 0 min, which resulted in very narrow and concentrated front of the eluted proteins from HIC column (Elution), which in turn placed into SEC column and further separated from impurities and salt, i.e. polished. The pilot measurements showed that the elution front with cGOx fit into 6 mL volume and could be seen by eye as a yellow front. This particular aspect matches the limit of the injection volume for the SEC column well (16 mm × 600 mm). Unlike the HIC (Adsorption) step, the SEC (Polishing) step has constrained flow rate (<1 mL min^{-1}) as well as limited injection volume (<6.0 mL), therefore it imposes a rate limitation for the entire purification procedure.

Spectral analysis of purified cGOx from expression medium showed that the ratio among spectral markers changes after purification, particularly at 280 nm. This indicates that some compounds absorbing at 280 nm were removed from the cGOx. Indeed, this is clearly seen from size exclusion chromatography of cGOx (Fig. 3) and additionally from comparison of line 2 with lines 3 and 4 at Fig. 4. The difference that has resulted in 6 kDa shift of the band maximum in the pattern of the major bands (line 2 versus lines 3 and 4) cannot be solely assigned to overloading of line 2. Therefore, SDS-PAGE indicates that probably some non-flavonoid protein impurities (follows from the spectral data) were removed from commercial GOx (Fig. 4) and proves that cGOx dissolved in expression medium was purified to a single compound (lines 3 and 4; Fig. 4). Therefore, the low yielding efficiency of the purification of cGOx (61.4%) can be solely explained by the impurities in the commercial preparation that were removed by HIC/SEC-tandem chromatographic purification.

To identify impurities in non-purified cGOx the samples were sent for analysis on Finnigan^{TM} LTQ^{TM} Orbitrap Hybrid FT mass spectrometer at Thermo Fisher Scientific (Bremen, Germany). Panoramic mass spectrum has shown very complicated overlapping mixture of various unresolved organic polymeric species with characteristic fragmentation patterns on top of the GOx, which has made spectrum uninterpreted (personal communication Dr. Alexander Makarov, Advanced Mass Spectrometry, Thermo Fisher Scientific, Bremen, Germany).

SDS-PAGE has shown that cGOx and rGOx were purified to single molecular species (Fig. 4). Purified cGOx shows a bit smeared band with MW maximum at 80.8 kDa that result in 161.6 kDa dimer. The widths of the band reflect variability of glycosylaton patterns in original enzyme source *A. niger* [28]. Whereas, rGOx derived from yeast has even higher molecular weight with much broad pattern of the glycosylation, i.e. hyperglycosylation (Fig. 4). This is the concomitant feature of yeast derived recombinant proteins using secretion strategy [15,16]. Yeast derived hyperglycosylation of the GOx affects molecular weight of the recombinant enzyme, but does not affect its affinity to β-D-glucose. This fact allows concluding that deglycosylation of rGOx is not required for the high throughput enzyme screening (ABTS/HRP) of GOx mutant libraries in course of the directed evolution. Therefore, we did not investigate an effect of deglycosylation on the chromatographic and electrophoretic behaviour of rGOx.

Thus, the HIC/SEC-tandem chromatographic system allows purifying recombinant glucose oxidase from complex expression medium in a single step procedure.

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References

[1] V. Leskovac, S. Trivic, G. Wohlfahrt, J. Kandrac, D. Pericin, Int. J. Biochem. Cell Biol. 37 (2005) 731.
[2] D.F. Malherbe, M. du Toit, R.R.C. Otero, P. van Rensburg, I.S. Pretorius, Appl. Microbiol. Biotechnol. 61 (2003) 502.
[3] J.G. Banks, R.G. Board, N.H.C. Sparks, Biotechnol. Appl. Biochem. 8 (1986) 103.
[4] T. Tzanov, S.A. Costa, G.M. Gьbitz, A. Cavaco-Paulo, J. Biotechnol. 93 (2002) 87.
[5] G. Richter, T. in, J. Godfrey, Reichelt (Eds.), Industrial Enzymology, McMillian Publishers, London, 1983, p. 428.
[6] Z. Zhu, C. Momeu, M.V. Zakhartsev, U. Schwaneberg, Biosens. Bioelectron. 21 (2006) 2046.
[7] F.H. Arnold, G. Georgiou, Directed Evolution Library Creation: Methods and Protocols, Humana Press, Totowa, New Jersey, 2003.
[8] H. Tsuge, O. Natsuaki, K. Ohashi, J. Biochem. 78 (1975) 835.
[9] B.E.P. Swoboda, V. Massey, J. Biol. Chem. 240 (1965) 2209.
[10] J.H. Pazur, K. Kleppe, Biochemistry 3 (1964) 578.
[11] K. Eriksson, I. Kourteva, K. Yao, J. Liao, F. Kilar, S. Hjerten, G. Chaga, J. Chromatogr. 397 (1987) 239.
[12] S. Liu, S. Oeljeklaus, B. Gerhardt, B. Tudzynski, Physiol. Mol. Plant Pathol. 53 (1998) 123.
[13] K.R. Frederick, J. Tung, R.S. Emerick, F.R. Masiarz, S.H. Chamberlain, A. Vasavada, S. Rosenberg, S. Chakraborty, L.M. Schopter, V. Massey, J. Biol. Chem. 265 (1990) 3793.
[14] S. Witt, M. Singh, H.M. Kalisz, Appl. Environ. Microbiol. 64 (1998) 1405.
[15] R. Conde, R. Cueva, G. Pablo, J. Poliana, G. Larriba, J. Biol. Chem. 279 (2004) 43789.
[16] T. Bulter, M. Alcalde, V. Sieber, P. Meinhold, C. Schlachtbauer, F.H. Arnold, Appl. Environ. Microbiol. 69 (2003) 987.
[17] L.Q. Chen, X.E. Zhang, W.H. Xie, Y.F. Zhou, Z.P. Zhang, A.E.G. Cass, Biosens. Bioelectron. 17 (2002) 851.
[18] A.N. Eryomin, A.P. Drozhdenyuk, G.K. Zhavnerko, T.V. Semashko, R.V. Mikhailova, Appl. Biochem. Microbiol. 40 (2004) 151.
[19] A. De Baetselier, P. Dohet, M. De Beukelaer, V. Ha-Thi, J. Hanotier, K. Frederick, S. Rosenberg, A. Vasavada, J. Biotechnol. 24 (1992) 141.
[20] A. De Baetselier, A. Vasavada, P. Dohet, V. Hathi, M. Debeukelaer, T. Erpicum, L. Declerck, J. Hanotier, S. Rosenberg, Bio-Technology 9 (1991) 559.
[21] J.H. Ko, M.S. Hahm, H.A. Kang, S.W. Nam, B.H. Chung, Protein Expr. Purif. 25 (2002) 488.
[22] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
[23] M. Fortelius, P. Mattjus, Chem. Phys. Lipids 142 (2006) 103.
[24] U.K. Laemmli, F. Beguin, G. Gujerkel, J. Mol. Biol. 47 (1970) 69.
[25] H.M. Kalisz, J. Hendle, R.D. Schmid, J. Chromatogr. 521 (1990) 245.
[26] G.L. Dai, J.R. Li, L. Jiang, Colloids Surf. B-Biointerfaces 24 (2002) 171.
[27] M. Kiess, H.J. Hecht, H.M. Kalisz, Eur. J. Biochem. 252 (1998) 90.
[28] A. Kohen, T. Jonsson, J.P. Klinman, Biochemistry 36 (1997) 2603.