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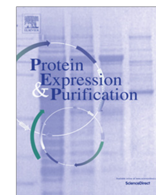

Expression, purification, and characterization of galactose oxidase of *Fusarium sambucinum* in *E. coli*

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ABSTRACT

A gene encoding a galactose oxidase (GalOx) was isolated from *Fusarium sambucinum* cultures and overexpressed in *Escherichia coli* yielding 4.4 mg enzyme per L of growth culture with a specific activity of 159 U mg^{-1}. By adding a C-terminal His-tag the enzyme could be easily purified with a single affinity chromatography step with high recovery rate (90%). The enzyme showed a single band on SDS–PAGE with an apparent molecular mass of 68.5 kDa. The pH optimum for the oxidation of galactose was in the range of pH 6–7.5. Optimum temperature for the enzyme activity was 35 °C, with a half-life of 11.2 min, 5.3 min, and 2.7 min for incubation at 40 °C, 50 °C, and 60 °C, respectively. From all tested substrates, the highest relative activity was found for 1-methyl-β-galactopyranoside (226 U mg^{-1}) and the highest catalytic efficiency (k_{cat}/K_m) for melibiose (2700 mM^{-1} s^{-1}). The enzyme was highly specific for molecular oxygen as an electron acceptor, and showed no appreciable activity with a range of alternative acceptors investigated. Different chemicals were tested for their effect on GalOx activity. The activity was significantly reduced by EDTA, NaN_3, and KCN.

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Introduction

Galactose oxidase (GalOx[1]; D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) is a monomeric 68-kDa enzyme that contains a single copper ion [1] and an amino acid-derived cofactor [2,3], formed by cross-linking of a Cys and a Tyr residue in the direct vicinity of the copper [4–6]. The thioether bond of the Tyr-Cys cross-link is post-translationally generated [4,7] and has been shown to affect the stability, the reduction potential [8] and the catalytic efficiency of the enzyme [9,10]. It has been classified as a member of the carbohydrate active-enzyme family AA5, subfamily 2 [11]. GalOx catalyzes the two-electron oxidation [3,12] of the C_6-hydroxyl group of nonreducing D-galactose residues [13] as well as a range of primary alcohols to the corresponding aldehydes with concomitant reduction of oxygen to hydrogen peroxide [14–17]. During catalysis both the metal ion and the cysteine-modified tyrosine group undergo 1-electron redox interconversions [18]. Despite a wide substrate specificity, GalOx is strictly regioselective and no secondary alcohols are oxidized [19]. However, the enzyme accepts a wide variety of primary alcohols such as benzyl alcohol [20], and glycerol [21] as reducing substrates. GalOx displays remarkable stereospecificity in its reaction with sugars [22], being highly sensitive for the orientation of the C_4-OH group, and hence it shows activity with galactose but not with glucose. Because of this specificity, various analytical techniques are based on GalOx, such as the determination of lactose in milk and dairy products [23] or the histochemical examination of mucus-secreting cells [24]. Furthermore, GalOx has been used in biosensors for the measurement of galactose and its derivatives in biological fluids [25], to label galactose residues in glycoconjugates [26], and for the induction of interferon in human lymphocyte culture [27,28]. GalOx is viewed as a competitive and cost-effective catalyst compared to chemical conversion for the manufacturing of fine chemicals for pharmaceutical purposes or in food industry, for example GalOx was used for conversion of sugars like D-galactose to food-grade cross-linking agents [29–32]. Another important application for GalOx is the modification of cell surface carbohydrates and has been used in cell labeling studies and histochemical staining [19]. GalOx is interesting for the use in industrial processes such as derivatization of guar gum and related polymers as well [33,34].

The enzyme is secreted by a number of fungal species, of which *Fusarium graminearum* (formerly classified as *Dactylium dendroides*) is the most extensively studied [35–43]. The production and

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[1] *Abbreviations used:* GalOx, galactose oxidase; IMAC, immobilized metal affinity chromatography; SDS–PAGE, one dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis; ABTS, 2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid); DCIP, 2,6-dichloro-indophenol; $FcPF_6$, ferrocenium; DMP, 2,6-dimethoxyphenol; BTA-1, 2-(4′-methylaminophenyl)benzothiazole.

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purification of GalOx has been reported from its natural fungal source [26,39,44–49], furthermore, various GalOx genes were cloned and successfully expressed in the filamentous fungi *Aspergillus nidulans* [50,51], *Aspergillus oryzae* and *Fusarium venenatum* [52], which have no endogenous GalOx, in the methylotrophic yeast *Pichia pastoris* [4,10,33,36,53–56] and in the bacterium *Escherichia coli* [55,57–59]. Typically, wild-type fungal GalOx is produced as a preproform carrying an N-terminal signal sequence, which is removed upon secretion, yielding the immature proform. The prosequence in this form was suggested to function as an intramolecular chaperone supporting copper binding and cofactor formation [42,50]. The maturation of GalOx requires several successive steps including cleavage of the signal sequence, which directs translocation, metal binding and cofactor processing [12,43]. Subsequently, the prosequence is removed and the Tyr-Cys cofactor is formed by self-processing reactions [2,7].

In the present paper we describe cloning and recombinant expression of a new *gao* gene without its prepro sequence from *Fusarium sambucinum* in *E. coli*. Furthermore, the purification and biochemical characterization of the enzyme are reported. Alternative electron acceptors, and possible activators as well as inhibitors were tested for their effect on GalOx activity.

Materials and methods

Chemicals, strains and vectors

Chemicals for enzyme assays, buffers and media were purchased from Sigma–Aldrich (Steinheim, Germany) and were of the highest purity available. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Amresco (Solon, OH, USA). Restriction enzymes, dNTP mix, Rapid DNA Ligation Kit and standard for Agarose gelelectrophoresis (GeneRuler DNA Ladder Mix) were from Fermentas (Vilnius, Lithuania) and the Phusion polymerase was from New England BioLabs (Ipswich, UK). Synthetic oligonucleotides were synthesized by VBC-Biotech (Vienna, Austria). *E. coli* strain BL21 (DE3) was purchased from Invitrogen (Carlsbad, CA, USA), the cloning vector pJET 1.2 was from Fermentas and the expression vector pET21a was from Novagen (Madison, WI, USA). The HisPrep FF 16/10 column was from GE Healthcare Bioscience AB (Uppsala, Sweden). SDS–PAGE protein standard (Precision Plus Protein prestained standard) was from BioRad (Herts, UK). The electron acceptors ferrocenium ($FcPF_6$), guaiacol, 2,6-dimethoxyphenol, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, Thioflavin T, 2-(4′-methylaminophenyl)benzothiazole, 1,1′-diethyl-2,2′-carbocyanine iodide, 1,4-benzoquinone, 2,6-dichloro-indophenol, and ferricyanide were purchased from Sigma–Aldrich. *F. sambucinum* (synonym *Gibberella pulicaris*) strain MA1886 was kindly provided by Gerhard Adam (Department of Applied Genetics and Cell Biology, BOKU Vienna, Austria).

Isolation and cloning of the GalOx gene

F. sambucinum MA1886 was cultivated in 50 mL Sabouraud medium (5 g L^{-1} peptone from casein, 5 g L^{-1} peptone from meat, 10 g L^{-1} glucose, 10 g L^{-1} maltose, 5 g L^{-1} yeast extract) in shaken flasks at 25 °C and 110 rpm for 3 days. Fungal mycelia were collected by centrifugation at 4 °C and 5000×g for 15 min and the pellet was washed in 50 mL saline solution (5 g L^{-1} NaCl, 0.12 g L^{-1} $MgSO_4 \cdot 7H_2O$). Genomic DNA was isolated from 100 mg of frozen mycelia ground in liquid nitrogen by the phenol–chloroform-extraction as described by Chomczynski and Sacchi [60]. The *gao* gene coding for GalOx was amplified by PCR using degenerated primers based on the published sequences from related organisms (Accession Number: FGSG_11032.3/M86819/FOXG_09956.2/FVEG_08555.3): 5′-GCCTCAGCA/TCCC/TA/CTCGG-3′ and 5′-CTGAGTAACGA/CGAAG/TA/CGT-3′, purified by agarose gel electrophoreses and subcloned into the pJET 1.2 cloning vector using the CloneJET PCR Cloning Kit (Fermentas). Restriction sites were introduced using the following forward primers: 5′-TCGCACATATGTACCTTTTGTCACTCGCTC-3′ and 5′-GCTGACATATGGCCTCAGCACCCATTGGA-3′ for *gao* with and without the prepro sequence, respectively, and 5′-GCTACGCGGCCGCCTGAGTAACGCGAAT-3′ as the reverse primer (restriction sites underlined). Subsequent, the PCR product was digested with *Nde*I and *Not*I and cloned in the equally treated expression vector pET21a in frame with the C-terminal His_6-tag by the Rapid DNA Ligation Kit. The resulting plasmid was transformed into *E. coli* BL21 (DE3) by electroporation. DNA sequencing was performed as a commercial service (LGC Genomics; Berlin, Germany). The amino acid sequence derived from the GalOx gene was used to generate a three-dimensional model based on the published structure of GalOx from *F. graminearum* [5] using SWISS-MODEL [61–63].

Heterologous expression and purification

Cultivation of *E. coli* BL21 (DE3) for production of the recombinant enzyme was performed in 30 mL of double concentrated LB medium (20 g L^{-1} peptone from casein, 10 g L^{-1} yeast extract and 10 g L^{-1} NaCl) with 50 mg L^{-1} ampicillin in 125-mL baffled flasks. Cells were grown at 37 °C and 120 rpm until reaching an OD_{600} of 0.4–0.6. Then recombinant protein expression was induced by addition of 5% lactose and cultivation was continued at 25 °C and 130 rpm overnight. The cell pellet after centrifugation was resuspended in 20 mM potassium phosphate buffer pH 7.0, and an aliquot of 500 μL was homogenized by Precellys24 (PEQLAB, Erlangen, Germany). The cell homogenate was tested for the presence of GalOx activity. Large scale cultivation was done in 1-L baffled flasks containing 300 mL medium [59].

The biomass from these cultivations was harvested by centrifugation at 4000×g for 20 min and 4 °C, and resuspended in phosphate buffer (20 mM, pH7.0). After disruption in a French Press at 100 MPa the crude cell extract was separated from cell debris by centrifugation (30,000×g, 4 °C, 30 min) and used for protein purification by Immobilized Metal Affinity Chromatography (IMAC) with a 20 mL Ni-charged Sepharose 6 Fast Flow column (HisPrep FF 16/10; GE Healthcare). Before loading the sample the column was equilibrated with 10 column volumes (CV) of buffer A (20 mM KH_2PO_4, 1 M NaCl, 10 mM imidazole, pH 8.0). After the protein sample was applied to the column, it was washed with 3 CV of the same buffer, and eluted in a linear gradient from 0.01 to 1 M imidazole in 10 CV. Fractions containing GalOx activity were pooled and the purity of the purified GalOx was checked by electrophoresis. SDS–PAGE was performed in principle as described by Laemmli [64] using the PerfectBlue vertical electrophoresis apparatus (PEQLAB) and the Precision Plus Protein Dual Color kit as mass standard. Proteins were visualized by Coomassie brilliant blue staining.

Enzyme activity assay

Prior to activity measurement GalOx was activated by incubation with 1 mM $CuSO_4$ for 30 min at 800 rpm and 25 °C. GalOx was measured with the chromogenic ABTS (2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)) assay [65]. The absorbance change at 420 nm (ε_{420} = 43.2 mM^{-1} cm^{-1}) was recorded at 30 °C for 180 s. The standard assay mixture (total volume, 1 mL) contained 1 μmol of ABTS in 20 mM potassium phosphate buffer (pH 7.0), 2 U horseradish peroxidase, 100 μmol D-galactose, and a suitable amount of GalOx sample. One Unit of GalOx activity was defined as the amount of enzyme that is necessary for the oxidation of

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