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Cell Bound and Extracellular Glucose Oxidases from *Aspergillus niger* BTL: Evidence for a Secondary Glycosylation Mechanism

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Abstract Two glucose oxidase (GOX) isoforms where purified to electrophoretic homogeneity from the mycelium extract (GOX_I) and the extracellular medium (GOX_{II}) of *Aspergillus niger* BTL cultures. Both enzymes were found to be homodimers with nonreduced molecular masses of 148 and 159 kDa and pI values of 3.7 and 3.6 for GOX_I and GOX_{II}, respectively. The substrate specificity and the kinetic characteristics of the two GOX forms, as expressed through their apparent K_m values on glucose, as well as pH and T activity optima, were almost identical. The only structural difference between the two enzymes was in their degrees of glycosylation, which were determined equal to 14.1 and 20.8% (w/w) of their molecular masses for GOX_I and GOX_{II}, respectively. The above difference in the carbohydrate content between the two enzymes seems to influence their pH and thermal stabilities. GOX_{II} proved to be more stable than GOX_I at pH values 2.5, 3.0, 8.0, and 9.0. Half-lives of GOX_I at pH 3.0 and 8.0 were 8.9 and 17.5 h, respectively, whereas the corresponding values for GOX_{II} were 13.5 and 28.1 h. As far as the thermal stability is concerned, GOX_{II} was also more thermostable than GOX_I as judged by the deactivation constants determined at various temperatures. More specifically, the half-lives of GOX_I and GOX_{II}, at 45°C, were 12 and 49 h, respectively. These results suggest *A. niger* BTL probably possesses a secondary glycosylation mechanism that increases the stability of the excreted GOX.

Keywords Glucose oxidase isoform · *Aspergillus niger* BTL · GOX_I · GOX_{II}

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Introduction

Glucose oxidase (GOX; β-D-glucose:oxygen 1-oxidoreductase, E.C. 1.1.3.4), is a flavoprotein that catalyzes the molecular-oxygen-mediated oxidation of β-D-glucose to β-D-glucono-1,5-lactone and hydrogen peroxide. Glucose oxidase is an enzyme of considerable commercial importance. It is produced on an industrial scale from *Aspergillus niger* and *Penicillium amagasakiense* strains and used as an additive for the removal of oxygen and/or D-glucose from various foods and beverages to improve their shelf life and to maintain flavor and color stability [1, 2]. In addition, it is used as an analytical reagent in test kits and biosensors for the determination of D-glucose in food and clinical chemistry and in industrial process monitoring [3]. Furthermore, GOX has attracted considerable interest in the recent literature as an improver for wheat flour doughs [4], in wine production with reduced alcohol content after fermentation [1] and as antimicrobial additive in toothpastes against dental plaque bacteria [5].

Although the enzyme is produced from a variety of fungal sources [6–8] including several *Penicillium* sp. [9–12], the *A. niger* enzyme is by far the most well studied. The subcellular localization of *A. niger* GOX has been a point of discussion. Early studies on *A. niger* GOX production and purification treated the enzyme as intracellular [13–15], a fact supported by the claim of its localization in peroxisomes [16]. While there also appeared reports dealing with the optimization of GOX production from *A. niger* measuring only the extracellular levels of the enzyme [17, 18], the prevalent viewpoint on the matter is that the enzyme is mostly cell associated and it appears in the extracellular medium as a result of an active secretion process, a situation described for both wild-type [19–22] and recombinant *A. niger* strains [23, 24]. The above fact is also supported by immunocytochemical studies that revealed that the bulk of the enzyme is localized in the cell wall [25]. In addition, the relatively high carbohydrate content of GOX [26, 27] also supports the existence of an active secretion system.

The role of glycosylation on GOX function and stability has been the subject of several reports were the purified enzyme was chemically or enzymatically deglycosylated and compared with its native form. Structural studies on the GOX from *A. niger* revealed no change in size and shape between native and deglycosylated enzymes [9, 28–30]. The catalytic properties, immunological reactivity, and secondary and quaternary structures of the native enzyme were also reported not to be significantly altered upon deglycosylation [29, 31]. However, strong interactions between the sugar and amino acids residues of the glycoprotein were predicted during studies of the crystal structure of GOX from *A. niger* [32]. Such interactions suggest a strong possibility of a significant effect of the carbohydrate moiety on the conformation and stability of the enzyme. The first direct experimental demonstration on the influence of carbohydrate moiety on the structure and stability of GOX was only recently presented, revealing contrasting effects between native and deglycosylated GOX, with the latter form demonstrating compaction of native conformation and enhanced stability during alkaline treatment [33]. Differences in the degree of glycosylation (ranging from 7.9 to 11.8%, w/w), also seem to be the reason for multiple isoforms of the enzyme that were reported for a commercial preparation of *A. niger* GOX [34], whereas all kinetic properties, as well as the aminoacid sequences of the isoforms, were found identical. A similar heterogeneity with respect to pI was reported while studying the kinetics of GOX excretion into the extracellular medium by a recombinant *A. niger* strain carrying multiple copies of the corresponding gene [23]. Any structural differences between the recombinant cell-bound and excreted enzymes could not been identified.

This work describes, for the first time, the purification and biochemical comparison of two electrophoretically distinct forms of GOX from the same culture of a wild-type strain of *A. niger*, namely, *A. niger* BTL. The first form was isolated from the mycelium extract and the second from the extracellular liquid. This particular strain of *A. niger* carries only one single copy of the corresponding gene [35] and produces elevated levels of GOX upon induction by $CaCO_3$, whereas a significant amount of the enzyme starts to appear in the extracellular medium already from the initial stages of growth [20]. The molecular, kinetic, and stability properties of the two enzyme forms were determined and their differences and similarities are discussed in relation to the possibility of the existence of a secondary glycosylation mechanism in this particular *A. niger* strain.

Materials and Methods

Microorganism and Growth Conditions

A wild-type strain of *A. niger*, denoted as BTL, that was isolated from corn cobs was used [20]. The microorganism was grown in 3-l Erlenmeyer flasks, using a basal salt medium [17] supplemented with sucrose and peptone as carbon and nitrogen sources, respectively, and $CaCO_3$ as inducer for GOX expression [35].

Crude Enzyme Preparations

Liberation of cell-bound enzyme Mycelium, from a 48-h culture, was separated from the culture broth by filtration (Whatman no. 4 filter, Millipore, Billerica, MA, USA) and subjected to extensive washing with 100 mM citrate phosphate buffer pH 5.0. Liberation of intracellular glucose oxidase (GOX_I) was carried out in a Rannie laboratory homogenizer (model Mini-Lab, type 8.30H, APV Rannie, AS, Albertslund, Denmark), as described previously [35].

Extracellular enzyme The culture filtrate, from the above 48-h culture, was further clarified by centrifugation (10,000×*g*) (Sorvall RC-28S *SUPRA*speed®, Kendro, Newtown, CT, USA) and was concentrated by ultrafiltration on a Minitan® system (Millipore), using Millipore membrane plates (Millipore), with a 30,000-Da molecular weight cut-off. The retentate, containing the extracellular glucose oxidase activity (GOX_{II}), was used for further studies.

Purification of Cell-bound (GOX_I) and Extracellular (GOX_{II}) GOX

All chromatographic steps were performed at room temperature using a fast-protein liquid chromatography system (Waters, Milford, MA, USA). Purification of GOX_I was conducted using a series of steps including ammonium sulfate precipitation, anion exchange chromatography, and gel filtration, at conditions described elsewhere [35]. GOX_{II} was purified as described in the following sections.

Ammonium sulfate fractionation The culture broth was concentrated with ammonium sulfate precipitation (40–95% saturation levels) prior to chromatographic separations. The precipitate was collected by centrifugation at 12,000×*g* (4°C) for 20 min, dissolved in a minimum volume of 20 mM piperazine–HCl buffer at pH 5.0 and desalted using an Amicon apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 KDa; Millipore).

Anion-exchange chromatography The concentrated sample was loaded onto a Q-Sepharose column (Sigma Chemical, St Louis, MO, USA) (1.0 cm i.d., 18 cm length),

equilibrated with 20 mM piperazine–HCl buffer at pH 5.0. The column was first washed with 17.5 ml of 20 mM piperazine–HCl buffer at pH 5.0 and then a linear gradient from 0 to 400 mM NaCl in 182.5 ml of 100 mM piperazine–HCl buffer at pH 5.0 was applied at a flow rate of 60 ml/h. Fractions (3.5 ml) containing GOX activity were pooled, desalted, and concentrated using an Amicon apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 KDa; Millipore).

Gel filtration chromatography The concentrated fractions from the previous step were rebuffered into 10 mM citrate–phosphate buffer pH 5.0 and loaded onto a SW-300 gel filtration column (0.8 cm i.d., 30 cm length) (Waters) previously equilibrated with the same buffer at a flow rate of 48 ml/h. Fractions containing GOX activity were pooled, desalted, and concentrated using an Amicon apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 KDa; Millipore) and used for further studies.

Enzyme Assay

Glucose oxidase activity was determined using the o-dianiside/peroxidase method at pH 5.0 and 30°C. The assay conditions were described previously [20]. One unit of GOX activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose per minute at pH 5.0 and 30°C.

Substrate Specificity

Carbohydrates such as maltose, galactose, sucrose, fructose, lactose, and mannose replaced glucose in the standard assay reaction mixture to determine the substrate specificity of GOX_I and GOX_{II}.

Protein Estimation

For quantitative protein estimation, both Lowry [36] and Bradford [37] methods were employed, using bovine serum albumin as standard. A_{280} was used to monitor protein in column effluents.

Determination of Optimum pH and Temperature

The combined effect of pH and temperature to the activity of the purified enzymes was determined applying a full 6×6 experimental design under standard assay conditions. Ranges of pH values from 3 to 8 (step 1 pH unit) and temperature values from 10 to 60°C (step 10°C), were employed. All experiments were performed in triplicate. The behavior of the system was explained by the following second-degree polynomial equation:

$$y = a_0 + a_1(\mathrm{pH}) + a_2(T) + a_{11}(\mathrm{pH})^2 + a_{22}(T)^2 + a_{12}(\mathrm{pH})(T) \quad (1)$$

where, y is the relative enzyme activity, a_0 is the offset term, α_1 and α_2 are linear effect terms, α_{11} and α_{22} are squared effect terms, and α_{12} is the interaction effect term.

The constants in Eq. 1 were determined by fitting the experimental results using the linear regression routines of SigmaPlot Program (SigmaPlot 2001 for Windows version 7.0, SPSS, Chicago, IL, USA). The optimum values of the independent variables were determined by solving the linear system of equations resulting from zeroing the partial derivatives, with respect to temperature and pH, of Eq. 1.

pH and Thermal Stability of the Purified GOX_I and GOX_{II}

For the pH-stability experiments, both enzyme preparations were diluted to the same initial activity in appropriate buffers, at pH values from 2.5 to 9.0, and placed in a water bath at 25°C. Samples were withdrawn at specific time intervals and assayed for residual enzyme activity. Thermostability of the two enzymes was determined similarly. Purified enzyme preparations were diluted to the same initial activity in 50 mM citrate–phosphate buffer pH 5.0 and incubated at temperatures ranging from room temperature to 60°C. At different time intervals, samples were withdrawn and assayed for residual GOX activity.

Determination of Kinetic Constants

The Michaelis–Menten constant (K_m) and the maximum velocity of substrate hydrolysis (V_{max}) were measured. The apparent K_m values of GOX_I and GOX_{II} towards glucose were determined by measuring the activity at varying concentrations of glucose (5.5×10^{-3}–1.66 M). Because GOX is a two-substrate enzyme, special care was taken to ensure that all measured velocities were independent of oxygen concentration. Reactions were carried out in a 50-ml vessel, thermostated at the reaction temperature (water circulation through double walls). Aeration level was monitored by an oxygen electrode and controlled through agitation speed (magnetic stirrer) and air sparging flowrate. The reaction mixture was oxygen-saturated (100±1.5% saturation) prior to the addition of the enzyme and this aeration level was manually maintained until the end of the reaction for all glucose concentrations tested. Reaction velocity was determined from the rate of absorbance increase in the reaction mixture at 435 nm [20].

Determination of Molecular Mass and Isoelectric Point

The apparent molecular weight of the native enzyme forms was determined by gel filtration chromatography on a SW-300 column (0.8 cm i.d., 30 cm length) (Waters) using the standards α-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). A minimum concentrated sample volume (30 μl loop) was introduced into the column, which was run at an elution flowrate of 0.8 ml/min with distilled water. Nine independent runs were conducted for the standards, purified GOX_I and GOX_{II} (three of each). Elution volumes were determined from the corresponding A_{280} recordings.

Molecular weight under denaturing conditions was determined by SDS-PAGE in a PhastSystem electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) using appropriate PhastGels (Pharmacia™). Electrophoresis conditions and Coomasie Brilliant Blue R staining of the gels were according to the manufacturer's recommendations.

The pI values of GOX_I and GOX_{II} were determined by chromatofocusing using a PBE94 resin (Pharmacia Biotech). The column (1.0 cm i.d., 24 cm length) was equilibrated with 25 mM piperazine–HCl buffer pH 5.0 and eluted with Polybuffer®–HCl to a final pH of 3.3 at a flow rate of 1 ml/min in 2-ml fractions.

Carbohydrate Content

The carbohydrate content of the purified enzymes (GOX_I and GOX_{II}) was determined by the phenol-sulfuric acid method [38], using mannose as a standard [30, 34].

Results and Discussion

Production and Purification of GOX_I and GOX_{II}

A 48-h culture of *A. niger* BTL on sucrose supplied the mycelium and the culture filtrate that were used for the isolation of cell-bound (GOX_I) and extracellular (GOX_{II}) GOX forms, respectively. Although this cultivation time is not the optimum for maximum enzyme production [20], it was chosen to avoid any uncontrolled secretion of GOX due to autolysis. The amounts of cell-bound and extracellular enzymes that were produced, however, were enough for efficient purification. GOX_I (cell-bound GOX) was purified from the mycelium extract of *A. niger* BTL, using a procedure that involved ammonium sulfate fractionation, two ion-exchange chromatographic steps, and a gel filtration chromatographic step, whereas GOX_{II} (extracellular GOX) was purified from the culture broth of *A. niger* BTL applying ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration chromatography. The specific activities of GOX_I and GOX_{II}, after the final chromatographic steps, were 525.2 and 343.7 $\mu mol \cdot min^{-1}$ mg^{-1}, the total yields were 49.3 and 71.5%, and the enzymes were purified 41- and 45-fold, respectively. Summaries of the purification procedures for GOX_I and GOX_{II} are presented in Tables 1 and 2.

Physical Properties of GOXI and GOXII

Both enzymes (GOX_I and GOX_{II}), as eluted from the final chromatographic step, appeared to be electrophoretically pure. Purified GOX_I and GOX_{II} revealed native molecular masses of 148 and 159 kDa, respectively, as determined by gel filtration chromatography. However, under denaturating conditions, GOX_I and GOX_{II} showed apparent molecular masses of 76.0 and 79.0 kDa, respectively (Fig. 1), that imply homodimeric structure for both enzymes. Intracellular GOXs isolated from different *A. niger* strains [28, 29], as well as from other fungi like *Phanerochaete chrysosporium* [6] and *Penicillium pinophilum* [11], are also homodimers with reduced molecular masses of approximately 70–80 kDa. *Pleurotus ostreatus* and *Botrytis cinerea* intracellular GOXs exhibited tetrameric structure with reduced molecular mass of 70 and 35 kDa, respectively [7, 8]. Extracellular GOXs purified from *P. amagasakiense* [9], *Penicillium chrysogenum* [10], and *Penicillium funicolosum* [39] are also homodimers with reduced molecular masses of approximately 70–80 kDa. The pI values of GOX_I and GOX_{II}, determined by chromatofocusing as described under the "Materials and Methods" section, were very close (3.7 and 3.6,

Table 1 Summary of the purification procedure for the cell-bound GOX (GOX_I) of *A. niger* BTL (purification procedure according to Hatzinikolaou et al. [35]).

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)
Crude extract	7,240	570	12.7	1	100
$(NH_4)_2SO_4$	6,340	142	44.6	3.5	87.6
Anion exchange (Q-sepharose, pH=6.0)	5,376	25.3	212.5	16.7	74.3
Anion exchange (Q-sepharose, pH=4.5)	4,611	11.5	401.0	31.6	63.7
Gel filtration (SW-300)	3,570	6.8	525.2	41.4	49.3

Table 2 Summary of the purification procedure for the GOX_{II} of *A. niger* BTL.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)
Culture broth	494	64.6	7.65	1	100
$(NH_4)_2SO_4$	473	40.7	11.6	1.5	96.0
Anion exchange (Q-Sepharose, pH=5.0)	393	1.6	247.3	32.3	79.6
Gel filtration (SW-300)	353	1.0	343.7	44.9	71.5

respectively). These values are lower than those reported in literature for other purified fungal intra- or extracellular GOXs, whose pI values are in the range of 4.0 to 4.5 [8–10, 29].

Kinetic and Specificity Studies

Both GOX_I and GOX_{II} were optimally active against glucose, with only 2.5% of the corresponding activity appearing against maltose. Both enzymes were inactive against galactose, sucrose, fructose, lactose, and mannose. Other GOXs isolated from several strains of *A. niger* reported in literature also exhibited high specificity against glucose [28–30, 34, 40]. On the other hand, *P. chrysosporium* and *P. ostreatus* GOXs exhibited significant activity against other carbohydrates like maltose, xylose, and sorbose [6, 7].

The apparent K_m values on glucose were experimentally determined under oxygen saturation conditions (see corresponding "Materials and Methods" section). Their values were calculated through nonlinear regression (SigmaPlot, SPSS Science) directly on the original [initial reaction velocity] vs [substrate concentration] data, assuming Michaelis–Menten kinetics. K_m and V_{max} values obtained from the corresponding Lineweaver–Burk plots were used as initial estimates for the final nonlinear regression routines (Fig. 2a, b). Apparent K_m values on glucose for GOX_I and GOX_{II} were practically identical and equal to 19.4±1.1 and 21.3±0.8 mM, respectively. The apparent K_m values against glucose for other GOXs from different strains of *A. niger* ranged from 19 to 37 mM [28–30, 34, 40], whereas

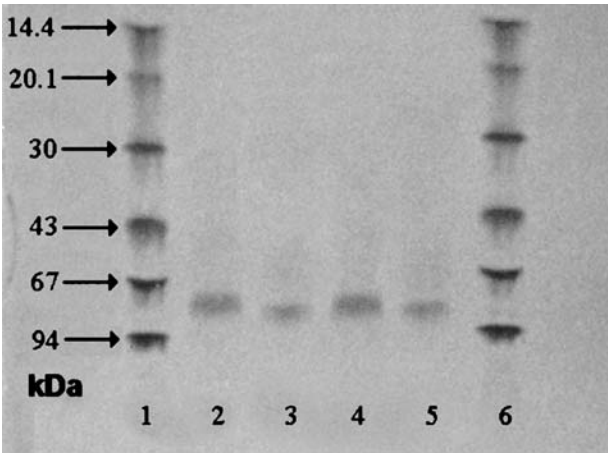

Fig. 1 SDS-PAGE of purified GOX_I and GOX_{II} from *A. niger* BTL on 10–15% gradient PhastGels. *Lanes 1* and *6*: standard protein markers in the order of increasing molecular mass are lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B. *Lanes 2* and *4*: GOX_I. *Lanes 3* and *5*: GOX_{II}

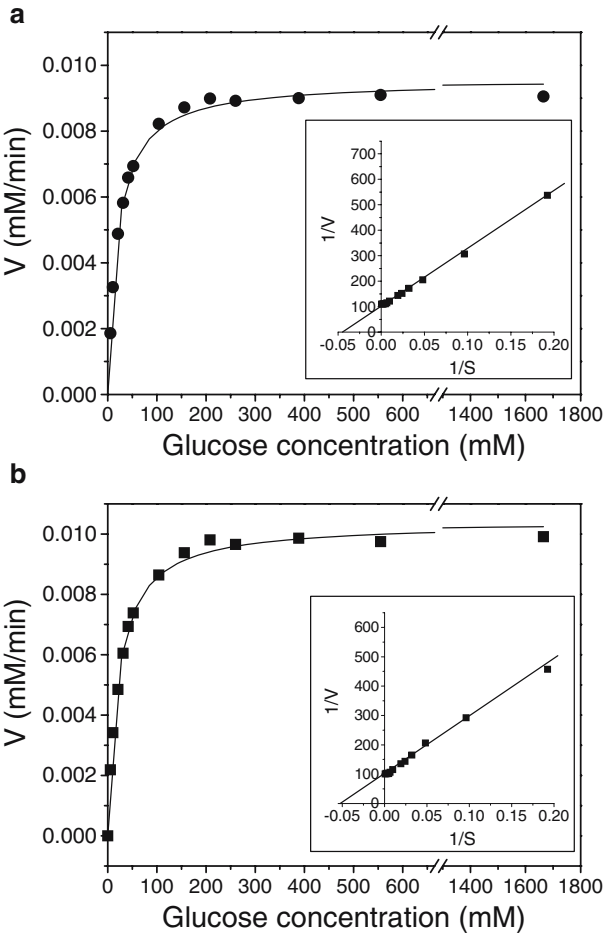

Fig. 2 Michaelis–Menten plots of **a** GOX_I and **b** GOX_{II}. *Solid symbols*: experimental data. *Solid lines*: Nonlinear best fit for the determination of K_m. *Inserts*: the corresponding Lineweaver–Burk plots used to obtain good initial estimates for K_m and V_{max} introduced into the nonlinear regression routines

the values for *P. amagasakiense*, *P. pinophilum*, *P. ostreatus*, and *P. funiculosum* were 5.2, 6.2, 1.34, and 3.3 mM, respectively [7, 9, 11, 39].

Determination of Optimum pH-Temperature

To study the combined effect of pH and temperature on enzyme activity, the combination of six values of pH (pH=3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) and six temperatures (T=10, 20, 30, 40, 50, 60°C) was examined. Using the results of the experiments, the following equations (Eqs. 2 and 3) giving the relative enzyme activities as a function of pH and temperature were obtained:

$$y_{GOX_I} = -227.38 + 103.85(\text{pH}) + 3.87(T) - 9.14(\text{pH})^2 - 0.04(T)^2 - 0.28(\text{pH})(T) \quad (2)$$

$$y_{GOX_{II}} = -230.54 + 100.98(\text{pH}) + 4.75(T) - 9.35(\text{pH})^2 - 0.06(T)^2 - 0.22(\text{pH})(T) \quad (3)$$

where, y_{GOX_I} and $y_{GOX_{II}}$ are the relative activities (%) of GOX_I and GOX_{II}, respectively.

The contour plots that explain the behavior of the system are shown in Fig. 3a and b. The values obtained after optimizing the regression equation are: for GOX_I, 5.2±0.2 and 29.3±1.6°C for pH and temperature, respectively, and for GOX_{II}, 5.0±0.3 and 32.4±2.3°C for pH and temperature, respectively.

The GOXs of different *A. niger* strains, as well as from *P. amagasakiense*, *P. chrysogenum*, *P. chrysosporium*, *P. pinophilum*, *P. ostreatus*, and *Talaromyces flavus* exhibited pH optima between 5.0 and 6.5 [6, 7, 9–11, 29, 41, 42]. An exception to this pattern is the GOX isolated from *B. cinerea*, which exhibited higher pH optimum (7.5) [8]. The optimum temperatures for GOX_I and GOX_{II} (29.3 and 32.5°C, respectively) were lower than those reported in literature for other cell-bound or extracellular GOXs of fungal origin [7, 9, 11, 29, 43].

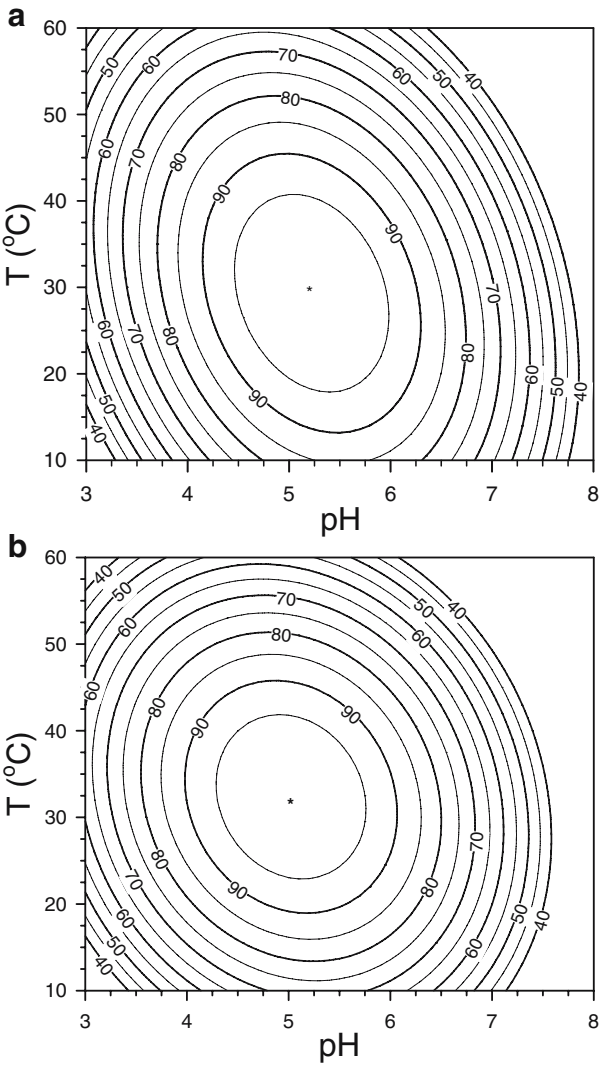

Fig. 3 Contour plot of relative activity of **a** GOX_I and **b** GOX_{II}. Effect of pH and temperature

pH and Thermal Stability of GOX_I and GOX_{II}

pH stability of GOX_I and GOX_{II} was studied over a range of pH values from 2.5 to 9.0. At pH values from 4.0 to 6.0, no loss of their activity was observed after 24 h incubation at 25°C. At pH 7.0 and for 24 h at 25°C, both enzymes exhibited approximately 15% loss of their initial activity. At pH 2.5 and 3.0, as well as 8.0 and 9.0, deactivation of GOX_I and GOX_{II} (Fig. 4a,b) follows first-order kinetics (Eq. 4):

$$\frac{dE}{dt} = -k_d \cdot E \Rightarrow \ln\left(\frac{E}{E_0}\right) = -k_d \cdot t \Rightarrow \frac{E}{E_0} = e^{-k_d \cdot t} \tag{4}$$

where, k_d is the first-order deactivation constant (h^{-1}), E_0 is the initial enzyme activity, and E is the enzyme activity at time t (h).

GOX_{II} was more stable than GOX_I and exhibited lower k_d values at all extreme pH values tested (Table 3). Especially, half-lives of GOX_I at pH 3.0 and 8.0 were 8.9 and 17.5 h, respectively, whereas the values for GOX_{II} were 13.5 and 28.1 h, respectively, under the same experimental conditions. In general, these levels of pH stability for both forms of *A. niger* BTL GOX are within the ranges reported in literature for the same enzyme from different *A. niger* strains as well as other fungi [7, 11, 30, 41].

Thermal stability of purified GOX_I and GOX_{II} was studied as described under the "Materials and Methods" section. The thermal deactivation of both enzyme forms followed first-order kinetics (Eq. 4). The k_d values for GOX_I and GOX_{II} are presented in Table 4. It should be noted that, at room temperature (~25°C), both enzymes were stable for 48 h.

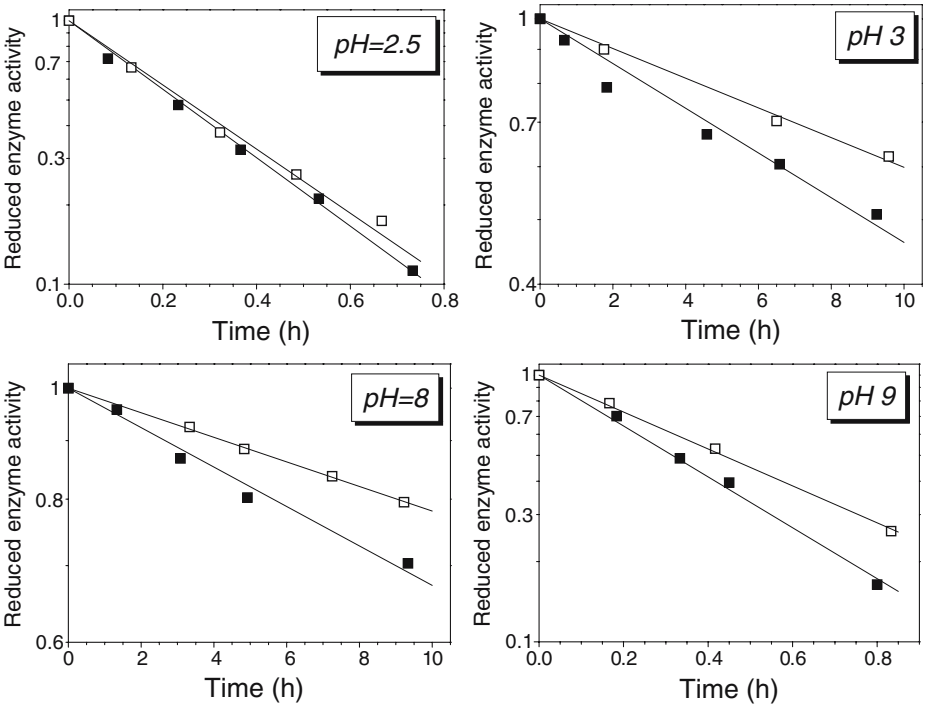

Fig. 4 Stability of GOX_I (*black squares*) and GOX_{II} (*white squares*) at pH values 2.5, 3.0, 8.0, and 9.0

Table 3 pH deactivation constants (k_d, h^{-1}) and half-lives ($t_{1/2}$, h) of GOX_I and GOX_{II} at pH 2.5, 3.0, 8.0, and 9.0 (enzymes were incubated at 25°C).

pH	GOX_I		GOX_{II}	
	k_d	$t_{1/2}$	k_d	$t_{1/2}$
2.5	2.9917	0.23	2.7283	0.25
3.0	0.0772	8.98	0.0513	13.5
8.0	0.0397	17.5	0.0247	28.1
9.0	2.1963	0.32	1.5934	0.43

Both forms of GOX were stable at 40°C, whereas a gradual loss of activity was observed at temperatures over 50°C. GOX_{II} was significantly more thermostable than GOX_I, exhibiting lower k_d at all temperatures tested (Table 4). In particular, half-lives of GOX_I and GOX_{II} at 45°C were 12 and 49 h, respectively. The results are comparable to those reported for an *A. niger* cell-bound GOX studied by O'Malley and Ulmer [44]. Enzymes from different *A. niger* strains studied by Kalisz et al. [29], and Underkofler [42] exhibited higher thermal stabilities. No loss of activity was observed for the cell-bound GOX from the fungus *P. ostreatus* when incubated for 2 h at 70°C [7].

Glycosylation

The carbohydrate content of GOX_I and GOX_{II} accounts for 14.1 and 20.8% (w/w), respectively, as determined using the phenol-sulfuric acid method and the Lowry protein assay. The corresponding values obtained using the Bradford protein assay were 15.8 and 21.5% (w/w), respectively. The carbohydrate content of GOXs from different strains of the fungus ranged from 12 to 24% [29, 34]. Glucose oxidase from *P. amagasakiense* contains 11–13% carbohydrates in its molecule [9], whereas GOXs isolated from *P. chrysosporium*, *P. ostreatus*, and *B. cinerea* are not glycosylated [6–8].

Southern blot analysis of the genomic DNA from *A. niger* BTL indicated the presence of only one GOX gene in the genome of the microorganism [35]. Thus, the molecular mass differences between GOX_I and GOX_{II} could not be attributed to differences in their amino acid sequence. The molecular mass of the nonglycosylated enzyme as calculated from the amino acid sequence derived from the open reading frame of GOX gene was found to be 65 kDa [35]. Comparing this value with the reduced molecular masses of GOX_I and GOX_{II} (75 and 79 kDa, respectively) determined in this work results in a carbohydrate contents of about 13.5 and 18%, respectively. These values are very close to those determined using the phenol-sulfuric acid method.

Table 4 Thermal deactivation constants (k_d, h^{-1}) and half-lives ($t_{1/2}$, h) of GOX_I and GOX_{II} at temperatures ranged from 45 to 60°C (enzymes were diluted in 50 mM citrate–phosphate buffer pH 5.0).

Temperature (°C)	GOX_I		GOX_{II}	
	k_d	$t_{1/2}$	k_d	$t_{1/2}$
45	0.0576	12.0	0.0141	49.1
50	0.1500	4.62	0.0849	8.16
55	0.4981	1.39	0.4635	1.49
60	2.8944	0.24	1.9696	0.35

Hecht et al. [32], in studying the crystal structure of GOX from *A. niger*, predicted strong interactions between the sugar and amino acids residues of the glycoprotein. Such interactions suggest a strong possibility of a significant effect of carbohydrate moiety on the conformation and stability of the enzyme. GOX_I and GOX_{II} exhibited similar kinetic properties, which leads to the conclusion that the carbohydrate moiety does not affect catalytic action. The two forms of GOX from *A. niger* BTL showed differences in thermal stability and in pH stability at extreme pH values. Thus, the carbohydrate content of GOX_{II} and GOX_I seems to influence the pH and thermal stability of the enzymes. The higher carbohydrate content of GOX_{II} appears to contribute to its thermostability, as well as its stability at low and high pH values. Cleavage of the 30–40% of the protein-bound carbohydrate moiety of intracellular GOXs from different *A. niger* strains did not affect catalytic action, immunological reactivity, and secondary and tertiary structure of the enzyme, whereas the deglycosylated enzyme showed decreased thermal stability [28, 30]. A comparison of the extracellular *P. amagasakiense* GOX [9] with the *A. niger* enzyme [29] showed that cleavage of the protein-bound carbohydrate moiety only marginally affected most of the biochemical properties of both enzymes. However, deglycosylated GOX from *P. amagasakiense* exhibited decreased pH and thermal stability [9], whereas deglycosylation of *A. niger* GOX affected the stability of the enzyme at low pH values [29]. Akhtar and Bhakuni [33] observed contrasting effects on the structure and stability of deglycosylated and glycosylated forms of a commercial GOX during alkaline treatment. The glycosylated enzyme undergoes partial unfolding with decreased stability at alkaline pH; however, a compaction of native conformation and enhanced stability of the enzyme was observed for the deglycosylated enzyme under similar conditions. The nonglycosylated recombinant *P. amagasakiense* GOX was less thermostable than the native enzyme, whereas the kinetic properties of the recombinant and native enzyme were the same [45].

The present study is the first report that describes the simultaneous isolation and biochemical comparison of two GOX forms from a wild-type *A. niger* strain, with differences in their carbohydrate composition, that affect the relative enzyme stabilities. The fact that both forms are glycosylated, in combination with the finding that the carbohydrate content of the extracellular enzyme is approximately 40% higher than that of the cell-bound enzyme, suggests that *A. niger* BTL probably possesses a secondary glycosylation mechanism, which is reported for the first time for this microorganism and enzyme. Pluschkell et al. [23], in studying the kinetics of GOX excretion from a recombinant *A. niger* strain carrying multiple copies of the corresponding gene, reported a heterogeneity, with respect to the pI clearly depicted in 2D-electrophoresis gel. In that work [23], no differences between the cell-bound and extracellular enzyme could be identified. The fact, though, that the enzyme was overwhelmlingly expressed in the recombinant strain (more than 40% of the intracellular protein) could very well have overloaded the secretory pathway, masking any naturally secondary glycosylation mechanism that seems very likely to exist in the *A. niger* wild-type strain studied in this work.

Conclusions

Two distinct forms of GOX were produced by a wild-type strain of *A. niger*. GOX_I was isolated from the mycelium extract, whereas GOX_{II} was isolated from the culture broth of the fungus. Both forms were purified to homogeneity applying chromatographic techniques. GOX_I and GOX_{II} were found to be homodimers, as the majority of fungal GOXs are, with a relatively small but experimentally distinct difference in their native and

reduced molecular masses. These differences were detected in the carbohydrate content of the enzymes. GOX_I contained fewer carbohydrates in its molecule than GOX_{II}. Their kinetic properties (optimum pH and temperature, as well as apparent K_m values) were found to be practically the same, which implies that the difference in the carbohydrate moiety does not affect the catalytic properties of the enzymes. On the other hand, the carbohydrate content of GOX_{II} and GOX_I seems to influence their pH and thermal stability. GOX_{II} proved to be more stable at extreme pH values and more thermostable and than GOX_I. The higher carbohydrate content of GOX_{II} appears to contribute to its thermostability, as well as to its stability at low and high pH values.

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