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Isolation, purification and characterization of a novel glucose oxidase from *Penicillium* sp. CBS 120262 optimally active at neutral pH

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Abstract

A novel glucose oxidase (GOX), a flavoenzyme, from *Penicillium* sp. was isolated, purified and partially characterised. Maximum activities of 1.08 U mg^{-1}dry weight intracellular and 6.9 U ml^{-1} extracellular GOX were obtained. Isoelectric focussing revealed two isoenzymes present in both intra- and extracellular fractions, having pI's of 4.30 and 4.67. GOX from *Penicillium* sp. was shown to be dimeric with a molecular weight of 148 kDa, consisting of two equal subunits with molecular weight of 70 kDa. The enzyme displayed a temperature optimum between 25 and 30 °C, and an optimum pH range of 6–8 for the oxidation of β-D-glucose. The enzyme was stable at 25 °C for a minimum of 10 h, with a half-life of approximately 30 min at 37 °C without any prior stabilisation. The lyophilized enzyme was stable at −20 °C for a minimum of 6 months. GOX from *Penicillium* sp. Tt42 displayed the following kinetic characteristics: V_{max}, 240.5 U mg^{-1}; K_m, 18.4 mM; k_{cat}, 741 s^{-1} and k_{cat}/K_m, 40 s^{-1} mM^{-1}. Stability at room temperature, good shelf-life without stabilisation and the neutral range for the pH optimum of this GOX contribute to its usefulness in current GOX-based biosensor applications.
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Keywords: Glucose oxidase; Penicillium; Purification; Characterisation

Glucose oxidase (GOX[1]—β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) has been purified from a range of different fungal sources, primarily from the genera *Aspergillus* [1–3] and *Penicillium* [4–8].

GOX from *Penicillium* sp. are generally more advantageous than those that have been isolated from *Aspergillus* sp. since they have enhanced kinetic parameters for glucose oxidation [4,9]. GOX is a flavoprotein which catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor [3,10–12]. The reaction can be divided into a reductive and an oxidative step (Scheme 1). In the reductive-half reaction, GOX catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone, which can in certain fungi such as *Aspergillus* sp. [13], be enzymatically (lactonase; EC 3.1.1.17) or spontaneously hydrolyzed to gluconic acid. Subsequently the flavine adenine dinucleotide (FAD) ring of GOX is reduced to $FADH_2$ [11]. In the oxidative half reaction the reduced GOX is re-oxidised by oxygen to yield hydrogen peroxide.

GOX has enjoyed large-scale technological application since the 1950's [14], which includes the enzymatic determination of glucose with biosensor technology [15,16], for the production of gluconic acid and as a food preservative [10]. Implantable glucose sensors may find significant application for the monitoring of glucose in diabetics [17]. Enhancement of the properties of GOX is still receiving attention [8], presumably due to the current and extensive applications base of this enzyme. This article describes the production, purification and characterisation of a novel GOX from a *Penicillium* species.

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[1] *Abbreviations used*: GOX, glucose oxidase; MEA, malt extract agar; CAT, catalase.

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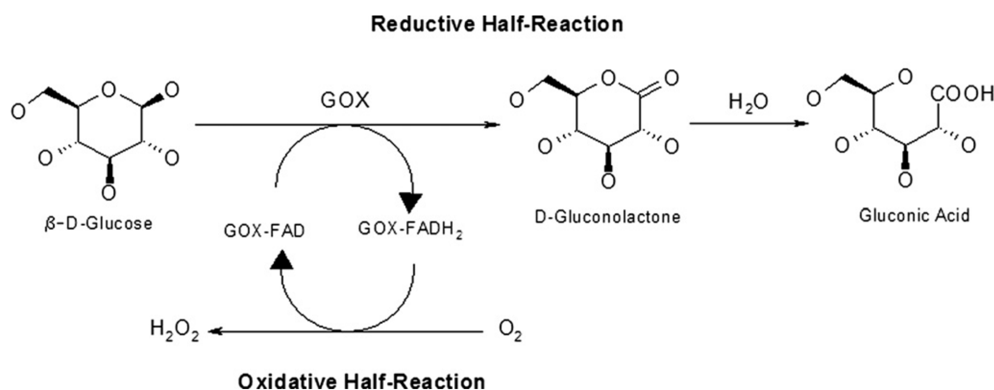

Scheme 1. Representation of the GOX reaction (adapted from [11]).

Materials and methods

Organism and maintenance

The fungus used in this study was isolated from a soil sample obtained in the Northern Province of South Africa. The microorganism was identified as *Penicillium* sp. according to Pitt [18] and subsequently filed with Centraalbureau voor Schimmelcultures (CBS) in The Netherlands and designated as a *Penicillium* sp. CBS 120262. The organism was identified as a glucose oxidase producer by screening using the method of Sharif and Alaeddinoglu [19]. The microorganism was maintained on malt extract agar (MEA) and subcultured once a month with storage at 4 °C.

Culture conditions

Medium optimised for the production of GOX from *Aspergillus niger* by Rogalski et al. [20] was used to culture the microorganism. This medium contained: 3 mM $(NH_4)_2HPO_4$; 1.38 mM KH_2PO_4; 0.63 mM $MgSO_4 \cdot 7H_2O$; 3% (w/v) peptone; 8% (w/v) glucose and 3.5% (w/v) $CaCO_3$. Glucose and $CaCO_3$ stock solutions (80% and 35%, respectively) were autoclaved separately. A conidia suspension was prepared by washing the organism, grown on MEA plates for 3 days at 28 °C, with sterile Tween 80 solution (0.1% w/v) to yield a stock spore suspension of $\pm 1.2 \times 10^8$ conidia/ml (hemocytometer count). Five Erlenmeyer flasks, each containing 200 ml of the medium, were inoculated with 6 ml of the conidia suspension. The flasks were cultured for 72 h on a rotary shaker (200 rpm, 28 °C). The mycelial mass was harvested by centrifugation at 2135*g* for 20 min in a Sorvall RT Du Pont benchtop centrifuge fitted with a swinging bucket rotor. The supernatant constituted the extracellular GOX fraction while the resuspended mycelial mass (20% wet w/v in 0.1 M potassium phosphate buffer, pH 7) was disrupted with a Constant Systems (Z-Plus series) cell disrupter at 38 kpsi (one pass). The supernatant of the broken cell suspension (centrifuged as above) constituted the intracellular GOX fraction.

Enzyme assays

GOX activity was measured using the indirect oxidation of *o*-dianisidine by horseradish peroxidase (HRP). The assays were performed according to Bergmeyer [21]. The following reagents were prepared: reagent A, 0.1 M potassium phosphate buffer, pH 7, containing *o*-dianisidine·2HCl (0.006%) (sparged with oxygen for 5 min prior to addition of reagents B and C); reagent B, 10% aqueous solution of β-D-glucose (allowed to mutarotate for 1 h before use); reagent C, 60 U ml^{-1} HRP aqueous solution. Reagents A, B and C were mixed immediately prior to assaying for GOX in the ratio 24:5:1, respectively. The reaction contained 3 ml of the final reagent and was initiated by the addition of 0.1 ml of sample. GOX samples were diluted where necessary to fall within the range of 0.15–0.2 U ml^{-1}. The reaction was measured kinetically at 436 nm ($\varepsilon = 8300\ M^{-1}\ cm^{-1}$) and 25 °C using a Beckman Coulter DU800 spectrophotometer. Reagent solutions (A, B and C) were stored at 4 °C and prepared fresh weekly. One unit of GOX activity is defined as the amount of enzyme that catalyses the conversion of 1 μmole β-D-glucose to D-glucono-δ-lactone and H_2O_2 per minute at 25 °C and pH 7.

Catalase (CAT) activity was determined in the final purified preparation of GOX according to the method described by Bergmeyer [21]. The decomposition of hydrogen peroxide by CAT was measured kinetically at 240 nm ($\varepsilon = 41\ M^{-1}\ cm^{-1}$) and 25 °C in potassium phosphate buffer (0.1 M, pH 7), using a Beckman Coulter DU800 spectrophotometer. One unit of CAT activity is defined as the amount of enzyme catalysing the decomposition of 1 μmole of hydrogen peroxide per minute at 25 °C and pH 7. Total protein assays were performed using the Bio-Rad Total Protein Assay kit with bovine serum albumin as the standard. All assays were performed in triplicate.

Purification of GOX

The extracellular fraction (600 ml) was used to purify the GOX. The sample was mixed with 150 ml of GOX buffer and centrifuged at 9632*g* for 10 min, in a Beckman

J2-21 centrifuge using a JA14 rotor, for clarification. The protein was subsequently precipitated with ammonium sulphate (60–70% fractionation) and centrifuged at 3000*g* for 1 h in a Beckman J2-21, centrifuge using a JA14 rotor, to pellet the resultant precipitate. The precipitate was resuspended in 200 ml ddH_2O (MilliQ) and concentrated to 10 ml in an Amicon (2800) stirred cell ultrafiltration unit using a 30 kDa PES ultrafiltration membrane. The sample was further washed with 3×200 ml volumes ddH_2O followed by concentration in the ultrafiltration unit. The final washed sample (10 ml) was diluted to 50 ml with 20 mM Tris buffer containing 50 mM NaCl (pH 7.2) and applied to a Super Q 650M (TosohBiosep) anion exchange resin column (XK 26—Amersham Biosciences) pre-equilibrated with the same buffer. Bound protein was eluted with a 500 ml linear gradient from 50 to 500 mM NaCl in 20 mM Tris buffer (pH 7.2) at a flow rate of 4 ml min^{-1}, with collection of 10 ml fractions. Fractions were assayed for protein by monitoring ultraviolet absorption at 280 nm, and GOX activity determined for each fraction with the abovementioned assay. Fractions containing high GOX activity were pooled and washed with 10 volumes ddH_2O in the Amicon ultrafiltration unit. This sample was then lyophilized and stored at −20 °C. The enzyme sample was later dissolved in the minimum amount of ddH_2O (±1 ml) and applied to a size exclusion column (XK16—Amersham Biosciences) packed with Sephacryl S200 HR (Amersham Biosciences) for approximation of native GOX size and as a final purification step. The sample was eluted from the column at a flow rate of 0.7 ml min^{-1} with 20 mM potassium phosphate buffer (pH 7.4). The approximate molecular weight of the GOX was determined by calibration against a set of Bio-Rad Gel Filtration Standards: thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.35 kDa). Peaks with GOX activity were pooled, concentrated and lyophilized. The pooled sample containing GOX was shown to be CAT free and was therefore subsequently used for kinetic characterization of GOX.

Characterization of GOX

Molecular weight determination

Native GOX molecular weight was determined by size-exclusion chromatography and subunit molecular weight by SDS–PAGE with 12% acrylamide gels using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). GOX purity after purification was determined by analysis of SDS–PAGE with quantification of bands using Quantity-one software (Bio-Rad). A total of 1.3 µg of purified GOX was loaded per lane and the proteins were visualized with Coomassie® Brilliant Blue R-250. The approximate subunit molecular weight was calculated by calibration against PageRuler™. Protein Ladder standards containing 14 proteins with sizes of 10, 15, 20, 25, 30, 40, 50, 60, 70, 85, 100, 120, 150 and 200 kDa.

pI determination

The pI’s of intra- and extracellular GOX were determined by isoelectric focusing (IEF) using an Amersham IPGPhor™ 2 on 13 cm (pH 4–7) linear Immobiline Strips (Amersham Biosciences). Rehydration buffer was modified from the manufacturer’s protocol to contain glycerol (20% v/v), CHAPS (2% w/v) and IPG buffer (0.4%). Samples (200 µl of 1 mg ml^{-1} protein) were loaded onto the Immobiline strips using the rehydration loading protocol [22]. The loaded samples were rehydrated at 20 °C for 12 h and focused stepwise: 500 V for 2 h, 1000 V for 2 h and 5000 V for 5 h.

Focussed strips for the intra- and extracellular GOX fractions were equilibrated in 20 ml potassium phosphate buffer (0.1 M, pH 7) for 10 min and subsequently stained (30 min) for GOX activity in assay reagent containing 24 ml potassium phosphate buffer (0.1 M, pH 7) containing *o*-dianisidine·2HCl (0.006% w/v), 5 ml of aqueous glucose solution (10%w/v) and 1 ml HRP (60 U ml^{-1}).

Optimum temperature and pH

GOX assay reagents were equilibrated for 10 min at the temperatures of interest (ranging from 10 to 65 °C) in the temperature-controlled cuvette holder (Peltier) of a Beckman DU800 spectrophotometer, before initiating the reactions with the addition of the GOX at 0.15 U ml^{-1}. The pH profile for GOX was performed in universal buffer containing 50 mM potassium dihydrogen orthophosphate, 33 mM citric acid and 50.7 mM boric acid, adjusted to pH values of interest ranging from 3.5 to 9 with potassium hydroxide [23]. The assay reagent buffer in the assay was replaced with universal buffer and a GOX concentration of 0.4 U ml^{-1} was used to initiate the reaction.

Stability testing

The stability of purified GOX was determined at 25 and 37 °C, since these temperatures correlated to potential applications of glucose oxidase, namely implantable glucose biosensors for human application (operating at 37 °C and physiological pH of 7.2) and glucose determination test strips (room temperature operation ±25 °C). Purified GOX at a concentration of 0.15 U ml^{-1} was prepared by dissolution in ddH_2O water, and subsequently filter sterilised (0.2 µm cellulose acetate syringe filter) into a sterile vessel. Volumes of 10 ml were placed in water baths pre-equilibrated to 25 and 37 °C. Samples (500 µl) were removed periodically and analysed for GOX activity. The GOX activities of the samples were compared to an initial sample taken at the onset of the experiment. A lyophilized GOX preparation was stored at −20 °C and assayed monthly for 6 months to provide an indication of shelf-life.

Kinetic constants

The kinetic constants for the purified GOX were determined from a Hanes–Woolf linear plot. The Michaelis constant (K_m), the maximal limiting rate velocity (V_{max}), the

turnover number (k_{cat}) and the specificity constant (k_{cat}/K_m) were all calculated. The turnover number was determined per mole of native purified GOX.

Results and discussion

Enzyme production

Both the intra- and extracellular GOX concentrations increased until glucose depletion, after 3 days, with maximum values of 1.08 U mg^{-1} dry weight and 6.9 U ml^{-1}, respectively. Maximum GOX activity coincided with complete glucose utilisation. GOX was produced from the start of cultivation indicating constitutive production of this enzyme in *Penicillium* sp. CBS 120262.

Isoelectric focusing

The GOX from *Penicillium* sp. CBS 120262 was shown to consist of two isoenzymes with approximate pI values of 4.30 and 4.67. Both the intra- and extracellular GOX fractions had both these isoenzymes, with the enzyme activity at pI 4.30 being vastly dominant.

Purification

Since the intra- and extracellular fractions contained both isoenzymes, the extracellular fraction was used for purification as this ensured that all the GOX used for purification was correctly folded. pH adjustment of the culture supernatant to 7 with 100 mM potassium phosphate (to a final concentration of 10 mM) resulted in the formation of a precipitate (likely calcium phosphate). The precipitate was removed from the extracellular fraction by centrifugation and confirmed to be GOX free by assay (Table 1). The purification protocol followed in this study resulted in a yield of 10.3% GOX with 8.6-fold purification (Table 1). There were slight decreases in specific activity during the freeze-drying steps since no stabilizers were added which could potentially interfere with subsequent purification steps (Table 1). The purified GOX was confirmed to be catalase free and therefore suitable for kinetic characterisation. Catalase contamination could potentially interfere with characterisation of GOX by reducing the availability of H_2O_2 for the secondary reaction (*o*-dianisidine oxidation by HRP).

Approximate molecular weight and purity

The final lyophilized GOX was 92.86% pure according to SDS–PAGE, with a contaminant peak of 7.14% (Fig. 1). The molecular weight of native GOX from Tt42 was approximately 148 kDa (size exclusion) while denaturing SDS–PAGE indicated a single band with a molecular weight of approximately 70 kDa (Fig. 1), indicating that the GOX was likely a homo-dimeric protein consisting of two equal subunits.

Optimum temperature and pH determination

The GOX from *Penicillium* sp. CBS 120262 was optimally active at 25 °C and exhibited more than 70% of the maximum activity between 10 and 45 °C. Above 45 °C the activity decreased rapidly (Fig. 2). The GOX was optimally active at pH 7 and displayed a broad pH profile with more than 70% of the maximum activity between pH 4.9 and 8.9. Below pH 4.9 the activity decreased sharply, maintaining only 25% activity at pH 3.4 (Fig. 2). The GOX maintained high activity of 90% at 37 °C, when compared to optimal activity of this enzyme between 25 and 30 °C. This is indicative that this enzyme is suitable for operation at this temperature after stabilisation.

Stability

The residual activity of purified GOX remained relatively unchanged over 10 h at 25 °C, whilst exhibiting a half-life of approximately 30 min at 37 °C (Fig. 2A). The GOX would thus not be effective for applications at 37 °C without prior stabilisation through immobilisation or use of additives such as polyhydric alcohols (e.g., polyethylene glycol) which have been shown to have a stabilising effect on the GOX from *A. niger* [24]. The lyophilized GOX preparation remained stable for a minimum of 6 months stored at −20 °C without the addition of stabilisation agents. The shelf-life of the purified GOX without the addition of potentially costly stabilisers is thus an attractive feature of this enzyme for commercial applications.

Table 1
Purification table for the extracellular GOX from *Penicillium* sp. CBS 120262

Sample description	Protein (mg)	GOX (U)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Initial sample	153.9	4308.0	28.0	100.0	1.0
Calcium precipitation	146.0	4082.0	28.0	94.7	1.0
Ammonium sulphate precipitation	19.3	2748.0	137.7	63.8	4.9
Ultrafiltration with washing	12.7	1947.0	153.0	45.2	5.5
Anion exchange chromatography	4.1	1096.0	268.1	25.4	9.6
Ultrafiltration with washing	2.9	880.0	300.0	20.4	10.7
Lyophilization	2.4	677.0	288.0	15.7	10.3
Size-exclusion chromatography	2.0	609.0	312.0	14.1	11.1
Lyophilization	1.9	445.0	240.5	10.3	8.6

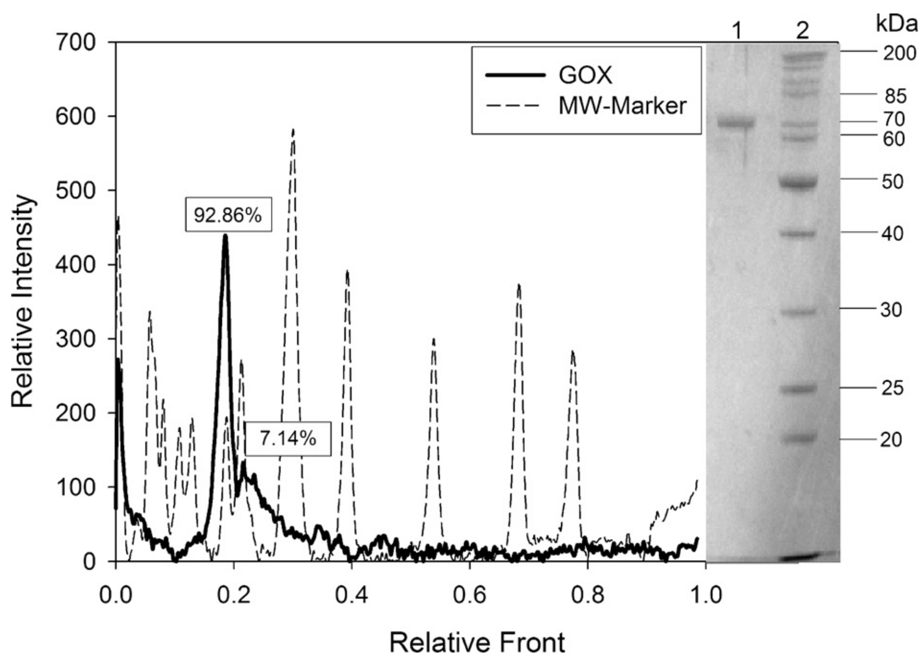

Fig. 1. Purity profile and SDS–PAGE of final purified GOX from Penicillium sp. (CBS 120262) (at a protein load of 1.3 μg). Lane 1, final purified GOX from Tt42; lane 2, PageRuler® Protein ladder molecular weight standards.

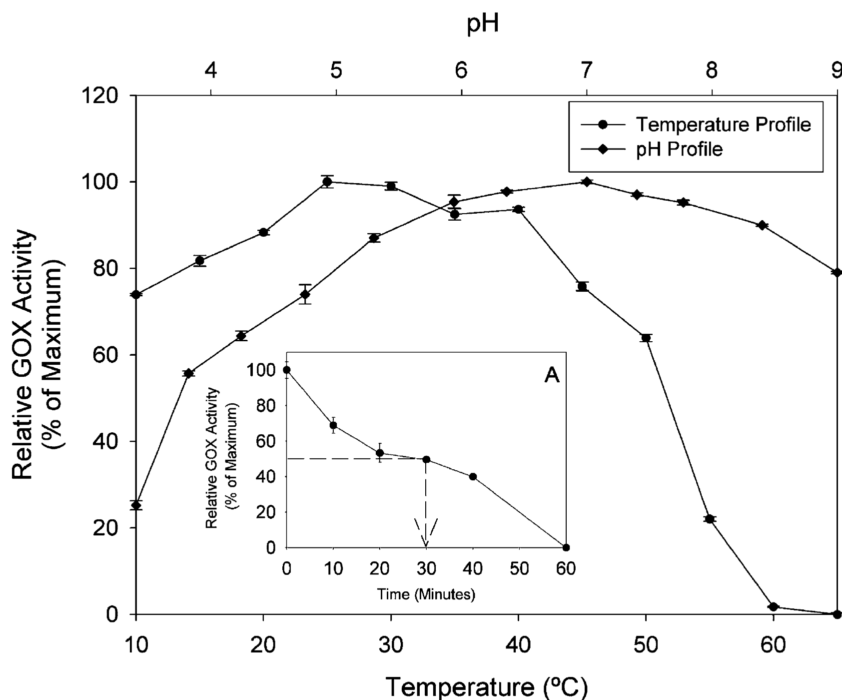

Fig. 2. Temperature and pH profile for purified GOX from Penicillium sp. (CBS 120262). A, Thermostability profile of GOX from Tt42 at 37 °C.

Kinetic constants

The Hanes–Woolf plot was used to determine the kinetic constants of the GOX from the *Penicillium* sp. The V_{max} and K_m values of the GOX reaction were determined to be 5.41×10^{-2} μmol min^{-1} and 18.43 mM β-D-glucose, respectively. The turnover number (k_{cat}) of the GOX was determined to be 741 s^{-1}. The specificity constant (k_{cat}/K_m) for GOX was determined to be 40 s^{-1} mM^{-1}.

Comparison of characteristics

The kinetic parameters calculated for the purified *Penicillium* sp. GOX were compared with those available from literature (Table 2). The comparisons were limited to characterisations of free enzyme and the k_{cat} values were calculated per mole of native GOX. The purified GOX was calculated to have a 1.63-fold higher affinity for β-D-glucose than that of *A. niger* (Sigma type VII), while

Table 2
Kinetic parameters for oxidation of β-D-glucose by GOX from various fungi

GOX	Kinetic constants				Reference
	V_{max} (U mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	
P. amagasakiense (ATCC 28686)	925	5.7	2001	351	[9]
A. niger (Sigma type VII)	458	30	920	31	[2]
Penicillium sp. (CBS 120262)	240.5	18.4	741	40	Present work

Table 3
Comparison of various characteristics for GOX from *Penicillium* sp. (CBS 120262) with other fungal sources of the enzyme

Parameter	GOX source						
	A. niger (Sigma type VII)	*A. niger* BTL	*P. funiculosum* 433	*P. chrysogenum*	*P. pinophilum* (DSM 11428)	*P. amagasakiense* ATCC 28686	*Penicillium* sp. (CBS 120262)
Molecular weight (kDa)	157	ND	140	175	155	130	148
Subunit molecular weight (kDa)	80	75	70	72	78	77	70
Isoelectric point	Five bands between 3.97 and 4.16	3.7	ND	4.2	ND	4 bands of 4.37; 4.42; 4.46 and 4.51	Two bands at 4.3 and 4.67
Optimum pH range	5.5–6	ND	6–8.6	5–6	4–6	4.5–6.5	6–8
Optimum temperature (°C)	40–60	ND	30	ND	ND	40–50	25–30
K_m for β-D-glucose (mM)	30	23.7	3.3	ND	6.2	5.2	18.4
Reference	[2]	[4]	[7]	[8]	[9]	[25]	Present work

ND, not determined by authors.

Penicillium amagasakiense (ATCC 28686) GOX displayed a 3.23-fold better affinity for β-D-glucose as the substrate. The turnover number of the purified GOX (k_{cat}) was calculated to be 2.7-fold, and 1.2-fold lower than *P. amagasakiense* (ATCC 28686) and *A. niger* (Sigma type VII) GOX, respectively. The specificity constant (k_{cat}/K_m) of purified *Penicillium* sp. GOX was 1.3-fold higher than that of *A. niger* (Sigma type VII) and 8.7-fold lower than that of *P. amagasakiense* (ATCC 28686). Overall the purified *Penicillium* sp. GOX displayed more advantageous kinetic properties than GOX from *A. niger* (Sigma type VII) due to the enzyme having a higher affinity for β-D-glucose and a higher specificity constant despite the lower turnover number.

Table 3 outlines a comparison of characteristics of the purified GOX from this study with those reported in relevant literature. Both molecular weight and subunit molecular weight of the purified GOX were comparable to those for various *Aspergillus* and other *Penicillium* species which ranged from 130 to 175 kDa (native) and 70–80 kDa (subunit). The pI values for the different GOX enzymes from the *Aspergillus* and other *Penicillium* species were shown to range between 3.7 and 4.51. Two other strains were reported to contain more than one isoenzyme of GOX, these being, *A. niger* (Sigma type VII) and *P. amagasakiense* (ATCC 28686) containing five and four isoenzymes of GOX, respectively. The optimum pH of the purified GOX (pH 6–8) was determined to be slightly acidic to alkaline, comparable to only one other GOX reported in literature, that from *P. funiculosum* 433 with an optimum pH range of 6–8.6. The other GOX enzymes all displayed optimum activity in the range between pH 4 and 6. The temperature optimum of the purified GOX from this study was comparable to that of *P. funiculosum* 433, both displaying optimal activity in the region between 25 and 30 °C. The optimum temperatures for the other GOX enzymes were relatively higher and reported to be in the range of 40–60 °C. The purified GOX was shown to have higher affinity towards β-D-glucose than those reported for other GOX enzymes from *A. niger* BTL and *A. niger* (Sigma type VII) (Table 3).

Conclusions

GOX was successfully purified to homogeneity from the extracellular culture supernatant of *Penicillium* sp. CBS 120262 and was shown to be comprised of two isoenzymes with pI values of 4.30 and 4.67. The enzyme was kinetically characterised and displayed characteristics with a $V_{max}$of 240.5 U mg^{-1}, $K_m$of 18.4 mM, k_{cat} of 741 s^{-1} and a k_{cat}/K_m of 40 s^{-1} mM^{-1}, similar to GOX from other *Penicillium* sp. reported in literature. The combination of neutral pH optimum, optimum temperature (25–30 °C), long-term storage stability at −20 °C as well as stability at 25 °C make this GOX suitable for diagnostic applications such as glucose determination in for example blood glucose test strips. Investigations into stabilisation of this enzyme may broaden the applicability to include biosensor and biofuel cell applications where a temperature stability of 37 °C is required [26].

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2006.09.013.

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