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Characterization of Glucose Oxidase from *Penicillium notatum*

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Summary

In the present study glucose oxidase (GOD) has been isolated from a culture filtrate of *Penicillium notatum*. The enzyme was purified by ammonium sulphate precipitation, diethylaminoethyl (DEAE) cellulose ion-exchange chromatography and Sephadex gel filtration. This protocol gave 16.47-fold purification and 25 % recovery of the enzyme. The optimum pH and temperature for the activity were 5.4 and 45 °C, respectively. The K_m and v_{max} values for the enzyme were 10.5 mM and 456 U/mg, respectively. A detailed kinetic study of thermal inactivation was carried out. Both enthalpy of activation (ΔH^*) and entropy of activation (ΔS^*) decreased at higher temperatures. Moreover, free energy of denaturation (ΔG^*) increased at higher temperature, making the enzyme thermally stable. A possible explanation for the thermal inactivation of GOD at higher temperatures is also discussed.

Key words: *Penicillium notatum*, glucose oxidase, thermostability, purification

Introduction

Glucose oxidase (GOD, β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as the electron acceptor. D-glucono-δ--lactone, a weak competitive inhibitor of GOD, is hydrolyzed non-enzymatically to gluconic acid and the reduced $FADH_2$-enzyme is reoxidized by molecular oxygen (*1*). GOD is a commercially important enzyme which has application in pharmaceutical industry as a biosensor for the enzymatic determination of glucose in the fermentation of liquor, beverages and body fluids (*2–4*) and in the food industry for the removal of glucose and/or oxygen to improve colour, flavour, texture and shelf life of various products (*5,6*). Recently, GOD has also been used in biofuel cells (*7*).

The mycelial fungi *Aspergillus* and *Penicillium* serve as industrial producers of glucose oxidase. It is generally accepted that the suitability of an enzyme for practical purposes depends on its thermal stability and stability in various media (*8*). Thermodynamic parameters are also important for inactivation studies of enzymes (*9,10*). Enhancement of the properties of GOD is still receiving attention, presumably due to current and extensive applications of this enzyme.

In order to exploit new industrial potentials of glucose oxidase, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this important enzyme. In a previous report, the enhanced production of GOD by *Penicillium notatum* using rice polish was described (*11*). In this paper, the purification, kinetics and thermodynamics of irreversible thermal inactivation of glucose oxidase from *Penicillium notatum* grown on rice polish are described. The results are expected to add new information on the nature of inactivation of this industrially important enzyme.

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Materials and Methods

All the chemicals used were of analytical grade and mainly purchased from Sigma-Aldrich Chemical Company, USA.

Microorganism

Penicillium notatum was obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad. The culture was maintained on potato dextrose agar (PDA) slants, subcultured once a month and stored at 4 °C.

Inoculum development and enzyme production

Inoculum development was carried out by transferring spores from 5- to 6-day-old slant culture into a 500-mL Erlenmeyer flask containing 150 mL of sterile basal culture medium. Composition of the basal culture medium was (in g/L): glucose 20, trisodium citrate 2.5, KH_2PO_4 5, NH_4NO_3 2, $(NH_4)_2SO_4$ 4, $MgSO_4{\cdot}7H_2O$ 0.2, peptone 2, microelement solution 10 mL and vitamin solution 5 mL. The pH of the medium was adjusted to 5.0 using 1 M HCl/1 M NaOH. The flasks were incubated on a rotary shaker at 150 rpm at (30±1) °C for 48 h to get mycelia and spore suspension (*12*).

Shaken cultures were grown in 100-mL Erlenmeyer flask containing 50 mL of sterile basal culture medium (pH=6), 5 g of rice polish, inoculated with 3 mL of the spore suspension and incubated on a rotary shaker (150 rpm at 30 °C) for 72 h as described earlier (*11*). After fermentation the enzyme was isolated by filtering the culture fluid through a Whatman no. 4 filter paper. Finally the cell debris was removed by centrifugation (39 200×*g* for 10 min at 4 °C) and the clear supernatant was used as enzyme source to determine its activity.

Determination of enzyme activity and protein

GOD activity was determined with the help of a coupled *o*-dianisidine-peroxidase reaction (*13*). Appropriately diluted enzyme (100 μL) was added to dianisidine buffer mixture (pH=5.5) containing glucose and peroxidase after proper mixing. The increase in absorbance at 460 nm was monitored with spectrophotometer (Hitachi, U-2001). One unit (U) of GOD activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose per mL per min under the above assay conditions. Total proteins were estimated by the method of Bradford (*14*) using bovine serum albumin as standard.

Purification of GOD

The extracellular fraction (500 mL) was used to purify the GOD. The protein was subsequently precipitated with ammonium sulphate 75 % (by mass per volume). The mixture was kept at 4 °C overnight. After 24 h the resulting precipitate of protein was collected by centrifugation in a Beckman J2-21 centrifuge at 39 200×*g* for 30 min. The protein was dissolved in minimum quantity of distilled water and dialyzed extensively against three changes of distilled water to remove the salts. The dialyzed sample was concentrated by freeze drier and then applied on DEAE-cellulose column (2.4×26 cm) equilibrated with Tris/HCl buffer at pH=7.5. Bound protein was eluted with 200 mL of linear gradient from 0–0.5 M NaCl in 20 mM Tris/HCl at pH=7.5. Fractions of 3 mL were collected and assayed for protein using the Bradford method. The active fractions were pooled and dialyzed against distilled water. The sample from DEAE--cellulose column was again concentrated and then applied to Sephadex G-100 column (2.4×26 cm) previously equilibrated with acetate buffer, pH=5.0 and eluted with the same buffer (*15*). Fractions of 2 mL were collected and active fractions were pooled. The pooled sample containing GOD activity was used for kinetic and thermodynamic characterization.

GOD characterization

The effect of pH on GOD activity was determined by assaying the enzyme as mentioned before with the difference that the activity was determined using glucose in 0.1 M acetate buffer (pH=3.5–6), 0.1 M phosphate buffer (pH=6–8), or 0.1 M NaOH/glycine buffer (pH=8–10) as a substrate. The apparent value of GOD K_m for the substrate was determined by measuring the initial velocities at pH=5.5 over a range of substrate concentrations (2–25 mM). Apparent value was determined from the double reciprocal plot. Effect of temperature on GOD activity was determined as described before (*13*) in the temperature range of 30–70 °C.

Thermal stability of GOD was determined by incubating the enzyme in 50 mM sodium acetate buffer (pH=5.0) at different temperatures (45–65 °C) in the absence of substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to assay as described above. The residual activity was measured and expressed as a percentage of the initial activity. From a semi-logarithmic plot of residual activity *versus* time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half-lives were estimated. The temperature dependence of k_d was analyzed from the Arrhenius plot and the activation energy (E_a) was obtained from the slope of the plot as described earlier (*16*). The activation enthalpy (ΔH^*) was calculated according to the following equation:

$$\Delta H^* = E_a - RT \qquad /1/$$

where R is universal gas constant (8.314 J/(K·mol)) and T is the absolute temperature.

The values for free energy of inactivation (ΔG^*) at different temperatures were obtained from the equation:

$$\Delta G^* = -RT\ln(k_d\, h/kT) \qquad /2/$$

where h is the Planck constant and k is the Boltzmann constant. The activation entropy (ΔS^*) was calculated as follows:

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \qquad /3/$$

The effect of metal ions (metal nitrates) on the activity of GOD was examined by incubating the enzyme in the presence of metal ion solution (1 mM) at 30 °C for 40 min. The residual activity (%) was determined as described before (*13*). All experiments were conducted in triplicate and the results were reported as mean±SD.

Results and Discussion

An extracellular GOD isolated from mycelium extracts of *Penicillium notatum* was purified after being subjected to ammonium sulphate, anion exchange and gel filtration. The specific activity of crude extract was 17 U/mg protein. The complete precipitation of the GOD was observed at 75 % ammonium sulphate. Purification of the enzyme on anion exchange column was 8.47-fold with 69 % recovery. Purified GOD, from the anion exchange column, was further purified by application on gel filtration column, where the enzyme was purified to 16.47-fold, to a specific activity of 280 U/mg with a yield of 15 % (Table 1). In a previous study (*15*), GOD was purified from *Aspergillus niger* using a combination of ammonium sulphate, anion exchange and gel filtration to a specific activity of 123 U/mg with 28.43 % yield. Sukhacheva *et al.* (*17*) purified GOD from *Penicillium funiculosum* 433 by a combination of ammonium sulphate, acetone, DEAE cellulose and Sephadex G-150. The specific activity of the purified enzyme was 3730 U/mg with 56 % recovery. Similarly, GOD from *Penicillium* sp. CBS 120262 was purified to 11.1-fold with 14.1 % yield (*18*).

The GOD from *Penicillium notatum* was optimally active at pH=5.4 and displayed a narrow pH profile (Fig. 1). The activity was found to decrease sharply above and below pH=5.4. Thus, pH of the medium affects the state of the active site of GOD and thereby the mode of interaction with its substrate, on which the rate of enzymatic glucose oxidation depends. The results are generally consistent with those reported for GOD from other fungi. The GOD activities from *Aspergillus niger* (*15*) and *Penicillium adametzii* (*13*) exhibited a pH optimum of 5.5 and 5.0, respectively. The activation energy (E_a) and optimum temperature of GOD from *P. notatum* were found to be 26.52 kJ/mol and 45 °C, respectively. It is obvious from the Arrhenius plot (Fig. 2) that the enzyme had a single conformation up to the transition temperature. The enzyme showed a low E_a at 45 °C, which makes this GOD superior to those from various other sources (*13*,*19*). The K_m and v_{max} values as determined from Lineweaver-Burk plot were 1.8 mM and 456 U/mg, respectively (Fig. 3). Referring to properties of biotechnological relevance, this GOD exhibited a high affinity for D-glucose (K_m=10.5 mM). This value is less than the reported value (K_m=25 mM) for GOD from *A. niger* (*15*). Such high substrate affinity and specificity, in addition to its stability at 45 °C, makes the GOD from *P. notatum* a suitable biocatalyst for industrial applications.

The GOD thermal stability is presented in Fig. 4. At 45 °C the enzyme half-life was 99 min and at 65 °C it was 20.38 min under similar conditions. The enzyme had ΔH^* values of 71.85 and 71.68 kJ/mol at 45 and 65 °C, respectively. The Gibbs free energy (ΔG^*) value for GOD was 102 kJ/mol at 45 °C, showing an increasing trend with an increase in temperature. Maximum ΔG^* value (104.17 kJ/mol) was observed at 65 °C. When ΔS^* was calculated at each temperature, negative values were found (Table 2). GOD from *P. notatum* showed a ΔS^* value of –96.12 J/(mol·K) at 65 °C.

Catalytic protein molecules, like all other proteins, are only marginally stable especially at elevated temperatures. Thermal inactivation of enzymes occurs in two steps as shown below:

$$E \leftrightarrow X \rightarrow D \qquad /4/$$

where E is the enzyme in its native state, X is an intermediate state which could be reversibly refolded upon

Table 1. Purification of extracellular GOD from *Penicillium notatum*

Treatment	Total activity/U	*m*(total protein)/mg	Specific activity/(U/mg)	Purification factor	Yield/%
Crude supernatant	56000	3270	17	1.00	100.0
Ammonium sulphate precipitation	49000	2145	23	1.35	88.0
DEAE-cellulose	34000	236	144	8.47	60.7
Gel filtration	8400	30	280	16.47	15.0

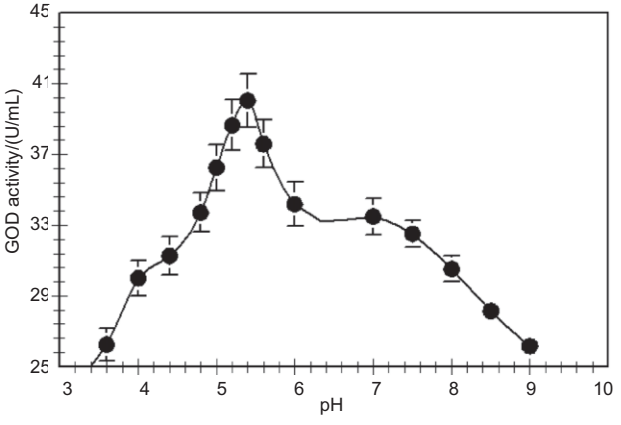

Fig. 1. Effect of pH on the activity of glucose oxidase from *Penicillium notatum*

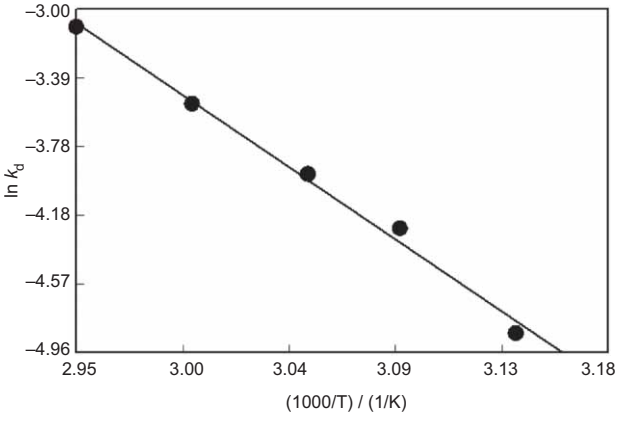

Fig. 2. Arrhenius plot for the determination of activation energy for irreversible thermal inactivation of glucose oxidase from *Penicillium notatum*

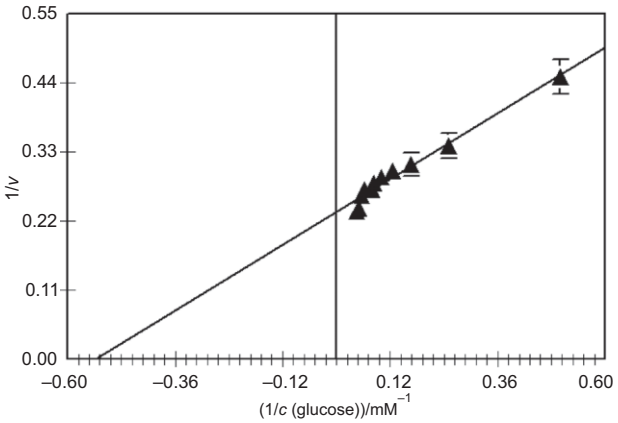

Fig. 3. Lineweaver–Burk plot to determine the kinetic constants for glucose oxidation by glucose oxidase from *Penicillium notatum*

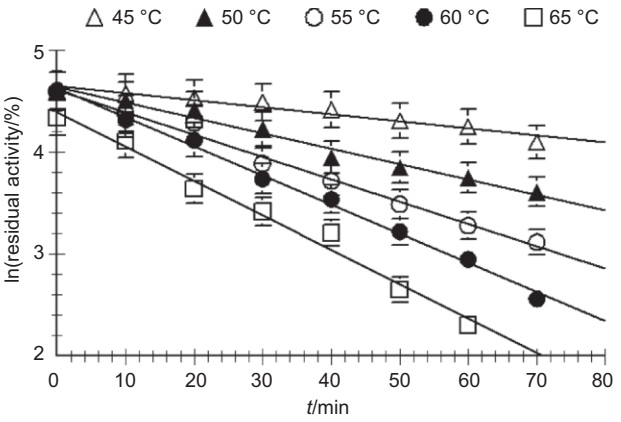

Fig. 4. Irreversible thermal inactivation of *Penicillium notatum* glucose oxidase at different temperatures

Table 2. Kinetics and thermodynamics of irreversible thermal denaturation of GOD from *Penicillium notatum*

Temperature/°C	k_d/min^{-1}	$t_{1/2}$/min	ΔH^*/(kJ/mol)	ΔG^*/(kJ/mol)	ΔS^*/(J/(mol·K))
45	0.007	99.00	71.85	102.0	–94.81
50	0.015	46.20	71.80	101.6	–92.23
55	0.022	31.50	71.76	102.2	–92.83
60	0.029	23.89	71.72	103.0	–93.96
65	0.034	20.38	71.68	104.2	–96.12

k_d (first order rate constant of denaturation) is determined from Fig. 4
$t_{1/2}$ (half-life)=0.693/k_d
$\Delta H^*=E_a$(74.49 kJ/mol)–RT
E_a (activation energy of denaturation) is calculated from Fig. 2
$\Delta G^*=-RT\ln(k_d \cdot h/k_b \cdot T)$
$\Delta S^*=(\Delta H^*-\Delta G^*)/T$

cooling and D is the denatured enzyme formed after long exposure to heat and therefore cannot be recovered after cooling. The thermal denaturation of multimeric enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, leading to subunit dissociation with a concomitant increase in the enthalpy of activation (*20,21*). The opening up of enzyme structure is accompanied by an increase in disorder, randomness or entropy of activation (*22*). On the other hand, the transition state of α-amylase from *Bacillus licheniformis* was found to be more ordered, as revealed by its negative ΔS^* at a high temperature of 80 °C (*23*). The negative entropy of inactivation (ΔS^*) observed for GOD suggested that there was negligible disorderliness, like that of invertase from *Fusarium* sp. (*10*). The values of ΔH^* and ΔS^* decreased with an increase in the temperature, indicating that the conformation of the enzyme was altered. Moreover, a high value for free energy of thermal denaturation (ΔG^*) at 65 °C indicated that the GOD exhibited resistance against thermal unfolding at higher temperatures. Values of 123.1 J/(mol·K) and 105.44 kJ/mol for ΔS^* and ΔG^*, respectively, have been reported for *A. niger* GOD, indicating that the enzyme has more unfolding configuration and that it is unstable at 60 °C (*13*). GOD is a glycoprotein that contains tightly bound FAD, which is not cleaved from the protein part at even higher temperature treatments (*24*). The activity of GOD isolated from *P. notatum* was inhibited by metal ions like K^+, Na^+, Ba^{2+}, Pb^{2+}, Hg^{2+}, Mg^{2+}, Cd^{2+} and Cu^{2+} (Table 3). The enzyme displayed a high thermostability, which allows its use for analytical and other industrial applications.

Table 3. Effects of metal ions on the activity of *P. notatum* GOD

Cations (c=1 mM)	Relative activity/%
Control (H_2O)	100
K^+	97
Na^+	95
Ba^{2+}	70
Pb^{2+}	45
Hg^{2+}	35
Mg^{2+}	85
Cd^{2+}	60
Cu^{2+}	30

Conclusion

In the present work, GOD from *P. notatum* was isolated and purified. Thermostability studies that were also carried out indicated that this enzyme is stable at high temperature. The high stability, high catalytic activity and substrate affinity open a good perspective for practical use of this enzyme.

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