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Kinetic properties of glycerophosphate oxidase isolated from dry baker's yeast

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Abstract

The glycerophosphate oxidase is a flavoprotein responsible for the catalysis of the oxidation of the glycerophosphate to dihydroxyacetone phosphate, through the reduction of the oxygen to hydrogen peroxide. The glycerophosphate oxidase from baker's yeast was specific for L-α-glycerol phosphate. It was estimated by monitoring the consumption of oxygen with an oxygraph. An increase of 32% in consumption of oxygen was obtained when the enzyme was concentrated 16-fold. The assay of enzyme was determined by the peroxidase chromogen method followed at 500 nm. The procedure for the standardization of the activity of the glycerophosphate oxidase from baker's yeast was accomplished, and the pH and temperature stability showed that the enzyme presented a high stability at pH 8.0, and the thermal stability was maintained up to 60 °C during 1 h. Such method allowed quantifying in the range 92–230 mM of glycerol phosphate, an important intermediate metabolite from lipid biosynthesis and glycolytic routes.

Keywords: Glycerophosphate oxidase; Kinetic properties; Baker's yeast

1. Introduction

Glycerophosphate oxidase (*sn*-glycerol-3-phosphate: oxygen 2-oxidoreductase, EC 1.1.3.21, GPO) is an oxido-reductase isolated from different microorganisms such as lactic acid bacteria [1–5] and yeast [6]. Evidence for the involvement of this enzyme in lactic acid bacteria, was shown through the lack of the membrane-associated electron transport chain found in *E. coli* and in eukaryotic mitochondria [7,8]. The enzyme differs markedly from the L-α-glycerophosphate dehydrogenases isolated from both mitochondria [9] and from *E. coli* [10,11], in fact that two of these membrane-bound enzymes contain non-heme iron as well as FAD; furthermore, these enzymes do not catalyze the reduction of oxygen. The animal and yeast oxidases are mitochondrial origin and catalyze transfer of electrons from L-α-glycerol phosphate via the respiratory chain, and the bacterial enzymes (*E. coli*, for example) are bound to the membrane and do not utilize molecular oxygen as an electron acceptor. Whereas the enzymes isolated from the lactic acid bacteria are soluble enzymes, which do utilize molecular oxygen [2].

This enzyme catalyzes the oxidation of α-glycerol phosphate to dihydroxyacetone phosphate with the concomitant reduction of oxygen to hydrogen peroxide. Glycerophosphate oxidase from the cells of the mutant strain *A. viridans* was isolated and purified and its properties were established [5]. GPO production is dependent on the time and on the composition of culture medium [5]. Commercial dry yeast can be used as a source of glycerophosphate oxidase for enzymatic assay. Most studies on these oxidative enzymes have utilized crude extracts, however, the assays that use partially purified preparations have a quite low cost and are viable for different samples.

GPO has practical application in several coupled systems for the quantitative determination of magnesium [12], glycerol phosphate [4], triacylglycerol [13–16], glycerol [17,18], phosphatidic acid [19] and other phospholipids. It can also be used for the measurement of enzymatic activities (glycerol kinase [20] and similar enzymes coupled to other enzymes and chromogen reagent) in blood serum or other biological materials [3,5,21].

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This paper describes the kinetics parameters of GPO from dry baker's yeast, and the effect of various stabilizing compounds, pH and temperature on the stability of enzyme and the measure of the oxygen consumed by the reaction in oxygraph.

2. Experimental

2.1. Microorganism

Dry baker's yeast from Mauri Brazil Ind. Com. e Imp. Ltda.

2.2. Cell disruption and crude cell extract preparation for enzymatic assay

2.2.1. GPO from dry baker's yeast

The cells (10 g, dry weight) of baker's yeast were suspended in 100 mL of 2 mmol/L sodium citrate buffer, at pH 6.2 containing 2 mmol/L of β-mercaptoethanol, and 100 g of glass beads (425–600 μm) was added to the cells suspension and the disruption was carried out for 15 min in a Bead Beater (Biospec Products Inc., USA), cooled by ice and operated under full speed. The cells debris was separated by centrifugation for 10 min at $8130 \times g$.

Cell extract is treated with 1% of streptomycin sulfate and the clarified supernatant is obtained by centrifugation ($16{,}260 \times g$/20 min) [22]. The precipitate obtained by addition of an equal volume of a solution of 30% (w/v) polyethylene glycol 3350, and after one night at 4 °C the suspension was centrifuged ($16{,}260 \times g$/20 min) and dissolved in 1 mL 10 mM Tris/HCl (pH 7.2) buffer. The fraction of enzyme was dialyzed for 24 h at 4 °C against a solution of 2 mmol/L sodium citrate at pH 5.5 containing 2 mmol/L β-mercaptoethanol, and 10 mmol/L $MnSO_4$, and this fraction, after centrifugation ($11{,}292 \times g$/10 min), was used as the source of GPO.

2.2.2. Concentration of extract from bakerĭs yeast

The extract was obtained as described previously and it was concentrated 16-fold in Speed Vacuum at 30 °C for 10 h for analyses in oxygraph.

2.2.3. Horseradish peroxidase (PO)

The crude cell extract was obtained by disruption of the 500 g of peeled horseradish in an electric liquidizer with 20 mL of 0.1 M acetate buffer at pH 5.0. The homogenate was filtered in gauze and it was clarified by centrifuged ($1807 \times g$/10 min) in refrigerated centrifuge, and used as peroxidase source.

2.3. Estimation of glycerophosphate oxidase from baker's yeast

The glycerophosphate oxidase was estimated by monitoring the consumption of oxygen with Oxygraphy (model YSI 5300A) and oxygen electrode type Clark. The method was described by Lin et al. [23]. The reaction was initiated by adding a 50 μL of enzyme to the reaction mixture containing 2.5 mL of 60 mM glycerol phosphate and 0.5 mL of 0.1 M acetate buffer (pH 6.0), and the initial velocity of oxygen consumption was measured.

2.4. GPO assay

GPO activity was determined by the peroxidase chromogen method as described by Suchová et al. [5] with modifications. The absorbance at 500 nm (molar absorption coefficient of $6.65 \times 10^3\ M^{-1}\ cm^{-1}$) was measured using a mixture of 750 μL of glycerol phosphate 0.5 M in 0.1 M Tris/HCl buffer pH 8.0 containing 0.1% Triton X-100; 150 μL of 4-aminoantipyrine 0.1%, 300 μL of phenol 0.1%; 15 μL (1.5 units) of horseradish peroxidase; 100 μL water. The reaction was started by adding 15 μL of the enzymatic preparation diluted 10-fold, and stopped with 300 μL of SDS 10%, after incubation at 60 °C for 120 min. The assay measures the production of the oxidated derivatives (quinone-imine) of 4-aminoantipyrine. One unit (U) of enzyme was defined as the amount of the enzyme producing 1 μmol of H_2O_2 per minute.

2.5. PO assay

PO activity was determined by a spectrophotometric method at 460 nm using a mixture of 0.2 mL of *o*-dianisidine 15 mM; 0.2 mL of hydrogen peroxide 30 mM; 5 μL of horseradish extract and 2.595 mL of 0.1 M citrate–phosphate buffer at pH 5.5. The assay measures the production of the oxidated derivatives of *o*-dianisidine during the first 15–90 s of the reaction rate (molar absorption coefficient of $11.3 \times 10^3\ M^{-1}\ cm^{-1}$). One unit of enzyme was defined as the unit of absorbance generated per minute in the assay conditions.

2.6. Effect of pH on GPO activity and stability

The optimum pH of GPO activity was determined by assaying the enzyme activity at various pH levels, between 5.0 and 10.0. The pH stability of the enzyme was determined by measuring the enzymatic activity under standard enzyme assay conditions after incubating the enzyme for 14 days at 40 °C, at various pH levels ranging from 6.0 to 10.0. The following 0.1 M buffer systems of varying pH, but fixed ionic strength (buffer capacity), were used: acetate buffer (NaAc–HAc) for pH 5.0; phosphate buffer (NaH_2PO_4–Na_2HPO_4) for pH 6.0–7.0; Tris–HCl buffer (Tris–HCl) for pH 7.5–9.0; and carbonate–bicarbonate (Na_2CO_3–$NaHCO_3$) for pH 9.5–10.0.

2.7. Effect of temperature on GPO activity and thermal stability

The optimum temperature of GPO activity was determined by assaying enzyme activity at pH 8.0 in 0.1 M Tris–HCl buffer, containing 0.1% Triton X-100 at temperatures of 25, 30, 37, 45, 50, 55, 60, 65, 70 °C. The thermal stability of the enzyme was determined by measuring the enzymatic activity under standard enzyme assay conditions after incubating the enzyme

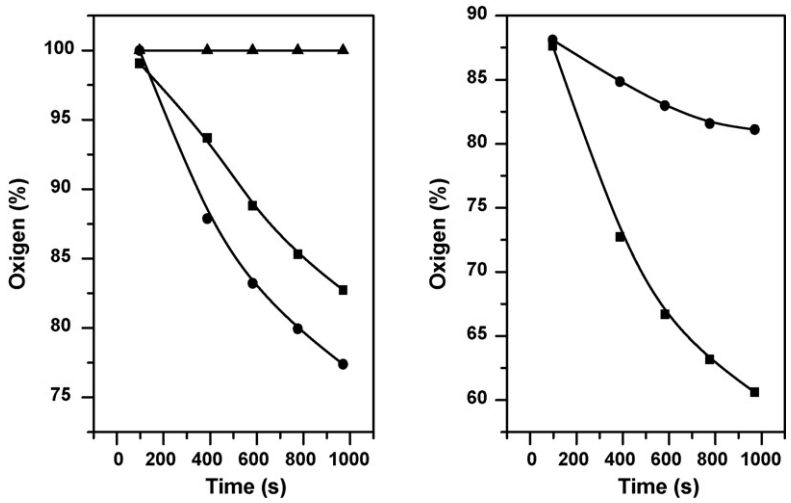

Fig. 1. Oxygen consumed by the reaction of the glycerol 3-phosphate oxidase (A) and in the concentrated crude enzymatic extract (B). The following samples were used: supernatant (■), precipitate (●), control (▲), and 16-fold concentrated crude extract (♦).

solution for 1 h, at various temperatures of 0, 30, 50, 60, 70, 80 °C.

2.8. Protein assay

Total protein was assayed according to the method of Lowry, modified by Layne [24], using bovine serum albumin as the standard protein. The levels of total protein were 2.53–3.30 mg/mL for horseradish extract, and around of 16.0–18.5 mg/mL for yeast extract.

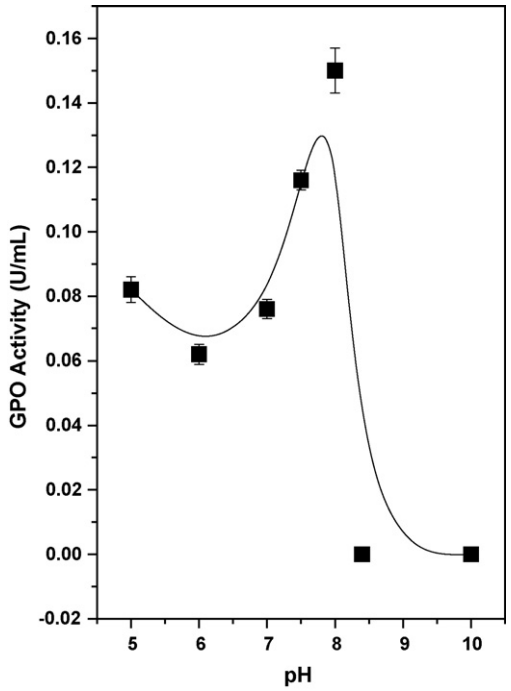

Fig. 2. Effect of pH on glycerol 3-phosphate oxidase activity. The GPO was assayed at the pH range 5.0–10.0, using different buffers (acetate buffer, 5.0; phosphate buffer, pH 6.0–7.0; Tris–HCl, pH 7.5–8.5, and carbonate–bicarbonate for pH 10.0).

3. Results and discussion

The enzyme L-α-glycerophosphate oxidase was isolated from bacteria [2] and does not utilize molecular oxygen as an electron acceptor. On the other hand, this article presents the activity of GPO from baker's yeast estimated by oxygen consumed in oxygraph and this consumption is shown in Fig. 1. An increase of about 32% was obtained with 1000 s of reaction time when the extract was concentrated 16-fold in Speed Vacuum (Fig. 1B). The monitoring of the consumption of oxygen in oxygraph is also described by Lin et al. [23] for the activity of glycerol oxidase from *Penicillium* sp.

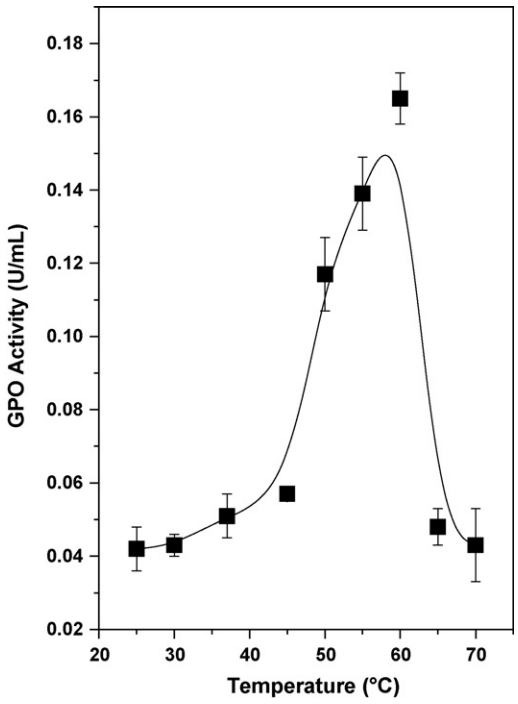

Fig. 3. Effect of temperature on glycerol 3-phosphate oxidase activity. GPO activity was assayed in the Tris/HCl buffer at pH 8.0.

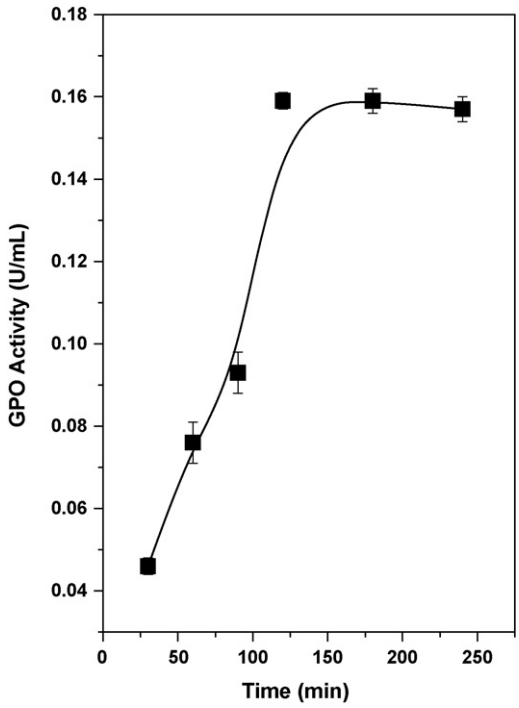

Fig. 4. Effect of reaction time on glycerol 3-phosphate oxidase activity. The activity was assayed at pH 8.0 and at 60 °C.

The GPO was partially purified by precipitation with 15% (w/v) polyethyleneglycol (PEG) as described by Ince et al. [1]. The use of PEG 8000 purified 1.4-fold the GPO from *Propionibscterium freudenreichii*, while the use of a solution 30% (w/v) PEG 3350, in the present work, in the yeast extract caused a significant increase of fourfold in the purification of GPO (data not shown), and this extract was used as source of glycerophosphate oxidase.

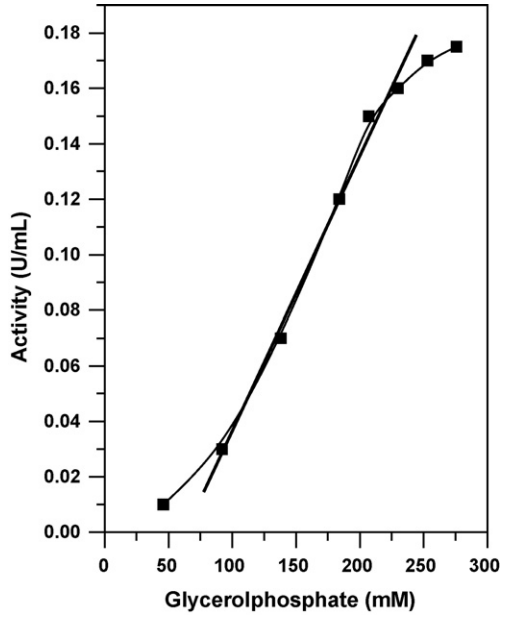

Fig. 5. Analytical curve of the glycerol phosphate (linear correlation coefficient equal to 0.995).

Table 1
Stability of glycerophosphate oxidase at different temperatures

Temperature (°C)	Glycerophosphate oxidase activity (U/mL)[a]
0	0.222 ± 0.020
30	0.244 ± 0.010
50	0.257 ± 0.028
60	0.262 ± 0.016
70	0.260 ± 0.020
80	0.238 ± 0.011

[a] Each point represents the average of two determinations.

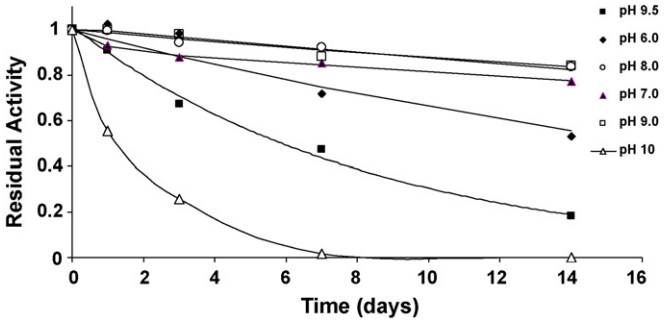

Fig. 6. Stability of glycerophosphate oxidase activity at different pH. The GPO was assayed in the pH range 6.0–10.0, using different buffers 0.1 M (phosphate buffer, pH 6.0–7.0; Tris–HCl, pH 8.0, 9.0; carbonate–bicarbonate pH 9.5, 10.0).

The optimum pH and temperature curves for the assay of the activity of the glycerophosphate oxidase can be observed in Figs. 2 and 3. Temperature and pH are usually the most important factors that can affect the enzyme activity [3]. The highest activity was found at pH 8.0 (pH optimum) and optimum temperature was 60 °C. Values higher or lower of pH and temperature showed a negative influence in the activity of GPO from baker's yeast. It is worth mentioning that the optimum pH obtained coincides with the data in literature [21], while the optimum temperature presented values that disagree with the literature for *Aerococcus* [3,21].

The catalytic activities vary in response to the concentrations of different substances, because of that, we varied the concentration of Triton X-100 as described by Suchová et al. [5] and a wide range of reduced horseradish peroxidases substrates as described by Vojinovic et al. [25]. GPO activity was positively affected by the presence of 0.018% phenol (ranging from 0.006 to 0.123%) and 0.009% 4-aminoantipirine (ranging from 0.006 to 0.074%) and the presence 0.1% of Triton X-100 stimulated slightly the enzyme activity (data not shown). The linear range of the assay was adjusted by shorting the reaction time and determining the maximum velocity of reaction (Figs. 4 and 5). A 120 min reaction time, and the 0.5 M the concentration of glyc-

Table 2
Parameters of stability assays adjusted to Sadana model, at different pH

pH	C_1	K_1 (day^{-1})	K_2 (day^{-1})	$t_{1/2}$ (days)
6.0	0.120	0.042	0.042	16.4
7.0	0.086	1.337	0.012	53.8
8.0	0.130	0.062	0.007	73.9
9.0	1.008	0.014	2.962	50.9

Table 3
The effect of different compounds on glycerophosphate oxidase stability during long-term incubation at 40 °C[a]

Time (days)	Stabilizing agents			
	Control	Azide 0.05%	Cobalt 6.7 mM	Sucrose 5%
Initial	0.242 (100%)	0.261 (100%)	0.261 (100%)	0.260 (100%)
1	0.247	0.261	0.262	0.250
3	0.242	0.268	0.257	0.248
7	0.239	0.269	0.255	0.243
14	0.223	0.261	0.233	0.218
21	0.192 (79.21%)	0.257 (98.44%)	0.219 (83.85%)	0.193 (74.34%)

[a] Enzyme stability was incubated in the buffers at the pH 8.0. After incubation at 40 °C, the residual activities were estimated at pH 8.0 by the peroxidase-chromogen method. Each point represents the average of two determinations.

erol phosphate, were considered as the best conditions for the determination of the GPO activity. The method presented linearity in the range of 92–230 mM in the dosage tube, as it is observed in Fig. 5, with a linear correlation of 0.995.

Thermal stability was investigated by incubating the enzyme in buffer Tris/HCl at various temperatures for 1 h, as shown in Table 1. The temperature stability studies showed that the enzyme was relatively stable from 30 to 80 °C, with the maximum activity at 60 °C. In relation to the stability of the pH the results obtained showed that the enzyme presented a high stability at pH 8.0 and lower its activity over pH 9.0, as shown in Fig. 6. A non-linear approach between time and residual activity is observed for all cases. The decay is single exponential [$a = a_i e^{-kt}$ (k, deactivation constant; t, time)] for the results of experiments carried out at pH 9.5 and 10, while for the other pH values (6.0, 7.0, 8.0 and 9.0) a two exponential equation of the form:

$$a = C_1 e^{(-q_1 t)} + (1 - C_1) e^{(-q_2 t)} \quad (1)$$

could be fitted, where a is the normalized residual activity related to the initial value and C_1, q_1 and q_2 are the equation parameters, which can then be evaluated by the fitting. This double exponential deactivation can be explained by a simple series type mechanism: the initial enzyme form (E) first deactivates to a still active intermediate state (E_1), which slower deactivates to the final fully inactivated state (E_d):

$$E \underset{k_2}{\longrightarrow} E_1 \underset{k_1}{\longrightarrow} E_d$$

k_1 and k_2 are the respective rate constants of the first and second deactivation reactions. Assuming that these steps are irreversible, the second rate constant k_2 is equal to the parameter q_2 from Eq. (1) [26]. When an equals 0.5 a half-life value ($t_{1/2}$) can be calculated from Eq. (1). At pH 9.5 and 10 deactivation constant of 0.12 and 0.44 day^{-1} was obtained with half-life ($t_{1/2} = \mathrm{LN}(2)/k$) of 5.8 and 1.6 days, respectively. In Table 2 is presented the rate constants of the first and second deactivation reactions at different pH and the respective half-life. The high stability at pH 8.0 is patent with the high half-life of 73.9 days.

In order to increase glycerophosphate oxidase activity the effect of different stabilizing compounds was tested, mainly, sodium azide [27], cobalt [3,23] and sucrose [3], as shown in Table 3. Results showed that the enzyme without any stabilizer lost about 20% of its original activity during storage for 21 days at 40 °C. The addition of sodium azide was much more effective on glycerophosphate oxidase activity than the other agents, after 21 days, the sodium azide in the concentration of 0.05% maintained the activity of the enzyme in 98.4%, while the addition of sucrose maintained only 74% of activity.

4. Conclusion

Assays employing glycerol 3-phosphate oxidase, which rely on hydrogen peroxide formation, form a coloured product, which has a major advantage for bioanalysis of food and biotechnological product, as biodiesel. This method also allowed quantifying of glycerol phosphate, an important intermediate metabolite of lipid biosynthesis and glycolysis, in the range 92–230 mM. GPO presented high thermal stability, pH of stability at 8.0, and a better way to prolong enzymatic stability is storage in a solution with sodium azide in the concentration of 0.05% for 21 days at 40 °C.

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