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Partition of Glucose Oxidase from *Aspergillus niger* in Aqueous Two-Phase Systems Based on Salt and Polyethylene Glycol

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ABSTRACT

The aim of this work was to study the isolation of glucose oxidase (GOx) from Aspergillus niger in aqueous two phase system consisting of PEG 7500 (150g l^{-1}), potassium phosphate (175 g l^{-1}, K_2HPO_4 +KH_2PO_4) and glucose (10 gl^{-1}), the enzyme was partitioned in polymer phase. By sequential extraction GOx (69.2%) was recovered in polymer phase by 11.8 fold purification, giving a yield of 129U mg protein-^{1}.

Key words: *Aspergillus niger*, glucose oxidase, aqueous two phase

INTRODUCTION

Glucose oxidase (EC 1.1.3.4, β-D- glucose oxygen 1- oxidoreductase) is a flavoprotein which catalyses the oxidation of β-D- glucose by molecular oxygen to D- glucolactone and H_2O_2. It removes hydrogen from glucose and gets reduced. The reduced form of the GOx is then reoxidised by molecular oxygen and the produced hydrogen peroxide is decomposed by catalase to water and oxygen. The D-glucolactone hydrolyses spontaneously to gluconic acid (Gibson et al., 1964; Duke et al., 1969., Barker and Shirley, 1980; Doppner and Hartmeir, 1984; Crueger and Crueger, 1990).

GOx is an intracellular and extracellular and is produced on industrial scale from *Aspergillus* and *Penicillium* genus. (Pazur et al., 1965; Pazur, 1966; Muller, 1977; Van Dijken and Veenheus, 1980; Mischak et al., 1985; Visser et al., 1995;). It is widely used for the determination of glucose and commercial applications have been found in the desugaring the egg products and removing oxygen from certain food and beverages (Ward, 1967; Barker and Shirley, 1980; Pitcher, 1980 and Richter et al., 1983).

Aqueous two-phase systems (ATPS) composed of salt and soluble polymers have found widespread use in biochemical research for separation and purification of macromolecules, cells and cell particles (Albertsson et al., 1981; Walter et al., 1985). ATPS can easily be scaled- up without an appreciable change in the nature or efficiency of the process. In addition, since there is no solid phase, mixing of the two phases is possible, and hence interface transport is rapid. Very little time is required to bring most two-phase systems into equilibrium. Another benefit is that the phases are compatible with almost all the known proteins. They are an attractive alternative procedure for the separation and purification of proteins on a large - scale. The question of selectivity in protein partitioning still needs to be better understood. An

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increased knowledge of protein behaviour in aqueous-two phase systems will also lead to the ability to predict the partitioning of specific materials (Silva and Franco, 2000). ATPS has very low tension (0.001-0.1 dyne cm^{-1}) at the interpahse of polymer and salt phase which promotes mass transfer, and as a result molecules can diffuse easily from one phase to another phase. Further partition is influenced by the number of factors such as molecular weight, concentration of polymer, pH value and ionic strength of salt used. Today, industry demands fast and economic downsteam processes for the partitioning and purification of biomolecules with maximum recovery and purification fold. Therefore, in light of the above demands, aqueous two phase system is an ideal technology where clarification, concentration, and partial purification can be integrated in one step. Moreover, this method can be made highly selective and can be easily scaled-up, thus allowing wider biotechnological applications. The present study investigated the possibility of using ATPS for the purification of GOx.

MATERIALS AND METHODS

Microorganism

A. niger (MTCC 281) was used in this study. Culture was maintained on potato dextrose agar at 4-6^{0}C and sub-cultured after every 20 days.

Pre-culture

Spores of fungus *A. niger* (7.5 x 10^5/ml) were grown in 250 ml Erlenmeyer flask containing 50 ml of the medium contains (g/l): $(NH_4)_2HPO_4$, 0.4; KH_2PO_4, 0.2; $MgSO_4$, 0.2; Peptone, 10; Sucrose, 70 and pH 5.5. This medium was incubated for 24 h in rotary shaker at 200 rpm at 30 ^{0}C.

Composition of fermentation medium

Fifty mille liter of the medium containing (g/l) sucrose 75, peptone 15, $(NH_4)_2HPO_4$ 2, $MgSO_4$ 2, $NaNO_3$ 2.0, KCl 0.5, $CaCO_3$ 20.0 and pH 5.5-6.0 was inoculated with the pre-germinated spores (15%) of 24 h age and culture was incubated in orbital shaker (250 rpm) at 30 ^{0}C for 48h.

Cell disruption

For the breakage of cells, 5g of fungal wet weight was taken in mortar and liquid nitrogen was added. After the evaporation of nitrogen, biomass was crushed to powder form and 5ml of sodium citrate buffer (pH 5.75,50mM) was added. Biomass was centrifuged at 3000g for 20 minutes at 4^{0}C and supernatant was used for further study.

Enzyme assay and protein determination

GOx activity was determined spectrophotometrically by the method of Ciucu and Petroescu, (1984) as modified by Markwell et al,(1989) method by the reduction of benzoquinone to hydroquine. One unit of GOx activity is defined as amount of enzyme which reduces 1.0 μM of benzoquinone ml^{-1} $minute^{-1}$. Protein concentration was determined using absorption method (Kirschenbaum, 1975; Kalb and Bernlohr,1977).

Preparations of two phase system

Predetermined amount of Polyethylene glycol (PEG), potassium phosphate (KH_2PO_4 and K_2HPO_4 in different molar ration), enzyme solution and water were mixed. The contents in the tubes were mixed in centrifuge (25-50 rpm) for half an hour. The top and bottom phases were withdrawn for the analysis.

RESULTS AND DISCUSSION

Effect of PEG molecular weight and concentration on the partition coefficient of GOx

Effect of different PEG molecular weight on partition behaviors was examined employing 150 g l^{-1} PEG with different molecular weight. PEG 7500 at 30 ^{0}C and pH 5.75 gave the maximum partitioning (1.5) of GOx to PEG-rich phase. With low molecular weight PEG, there was no phase separation (Table 1).

Table 1 - Dependence of partition coefficient of GOx on the molecular weight and concentrations of polyethylene glycol (PEG).

Step 1		Step 2	
Average Molecular weight of PEG[b]	**Partition coefficient (K) [a]**	**Concentration of PEG(gl^{-1})[c]**	**Partition coefficient (K)[a]**
200	--	100	--
400	--	125	1.40
6000	1.30	150	1.59
75,00	1.59	200	1.57
10,000	1.59	250	1.50
15,000	1.55	300	1.50

(K=Specific activity in polymer phase/ Specific activity in salt phase);[b] PEG 150 gl^{-1}, Phosphate 150 gl^{-1}, pH 5.75 and Temp.30 ^{0}C.; [C] PEG 7500, Phosphate (KH_2PO_4+K_2HPO_4) 150 gl^{-1}, pH 5.75 and Temp. 30 ^{0}C

With an increase in the molecular weight of PEG, osmotic pressure of PEG phase would have decreased, which in turn increased the protein recovery to the top phase. Maximum GOx partition to polymer phase was at 15 % PEG concentration at which 1.5 Partition coefficient was observed but at higher concentration GOx yield declined. This would be because decrease in the volume of PEG would have forced the GOx protein to concentrate and eventually precipitate when the limits of protein solubility were exceeded

Effect of phosphate concentration and pH on the partition coefficient of GOx

Partition coefficient of protein depends upon the ionic strength of medium as following equation given by the Albertson(1971):

$$\mathbf{In\ Kp = lnK^0s + (ZF/RF)\Psi \text{----}(1)}$$

Where Ψ is interfacial potential, Z is net charge on the protein, R is gas content, F is faraday constant, T is absulate temperature and lnKp is partition coefficient of protein. Interfacial potential is given by:

$$\mathbf{\Psi = \{RT/Z^+ + Z^-)\ F\}\ In\ (K- / K^+) \text{---}(2)}$$

Where K^- / K^+ is partition coefficient of protein in two phase system when $Z^+ + Z^-$ are the charge strength due to salts concentration. Change in phosphate concentarion influences the ionic strength of phase and hence partition coefficient of protein. When Phosphate concentration (KH_2PO_4+ K_2HPO_4) was increased from 125-175gl^{-1}, there was increase in partition coefficient of enzyme (1.4 to 1.8), but above 200gl^{-1} concentartion there was decline in the partition coefficient (Table 2).

Different molar ratio of KH_2PO_4 and K_2HPO_4 influenced the pH of the two-phase system. There was maximum partition coefficient (3.67) at pH 6.0 and above that there was constant partition coefficient up to pH 6.5 (Fig. 1).

KCl and NaCI were also used to study the effect on the partition behavior of the protein (result not shown). KCl was more effective as compared to NaCl. Different concentrations of KCl affected the partition coefficient and as the concentration of KCl was increased from 0.05-0.1%, there was increase in the partition coefficient (Fig. 2).

Glucose at varying concentration (0.05-4%) was added in the mixture and it was observed that there was maximum partition coefficient (6.44) at 0.1% and was constant upto 0.2%, but above this there was slight fall in the partition coefficient (Fig.3).

Table 2 - Effect of phosphate concentration on the partition coefficient of GOx.

Phosphate salt (gl^{-1}) (KH_2PO_4+K_2HPO_4)[a]	Recovery of Gox in Top phase (%)	Recovery of protein in top phase (%)	Partition coefficient (K)
125	59	25	1.40
150	64	24	1.59
175	69	24	1.80
200	69	26	1.70
250	58	29	1.70
300	52	29	1.70

PEG 75 00 (150 gl^{-1}), pH 5.75 and Temp. 30^0C.

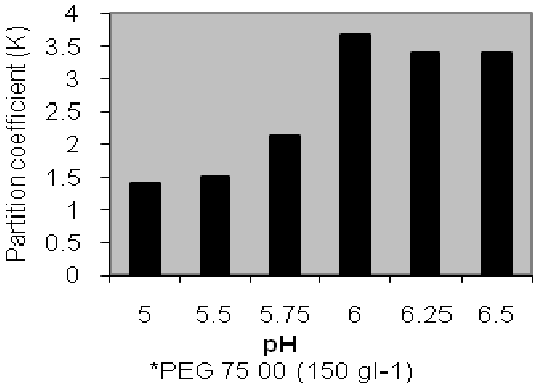

Figure 1 - Dependences of partition coefficient* of Gox on the pH of Phase system.

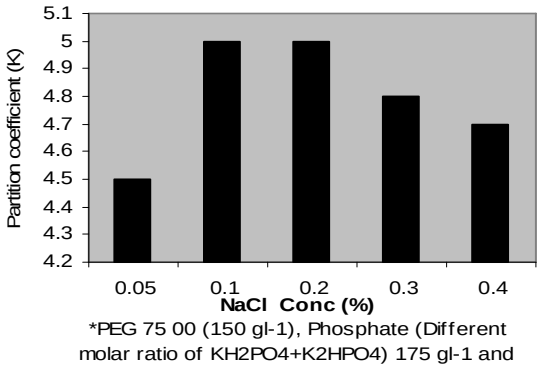

Figure 2 - Effect of NaCl concentrations on the partition behavior** of GOx in two phase system.

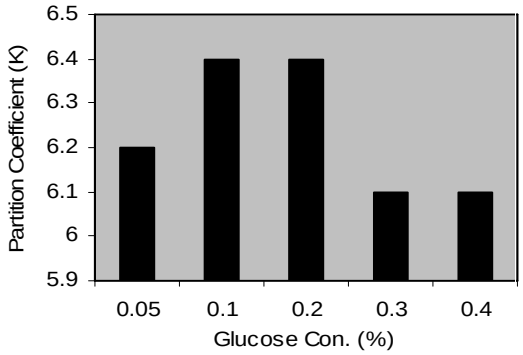

Figure 3 - Effect of Glucose concentration on the partition behaviors of GOx in two phase system.

Effect of temperature on the partition coefficient of Gox

The effect of temperature on the partition coefficient of Gox was studied (Table 3).

The results showed a increase of K with temperature and maximum partition coefficient (6.9) at temperature 35 ^{0}C. At 40^{0}C there was sharp decline. The effect of temperature is quite complex because the phase composition, electrostatic interactions and hydrophobic interactions are all coupled to the temperature. In addition, the proteins can undergo denaturation, conformational changes, and self association or dissociation when the temperature is raised. Some reports have described an increase in the partition coefficient with the temperature (Diamond and Hsu, 1992; Forciniti et al., 1991) others have found that the partition coefficient showed no temperature dependence.

Table 3 - Dependences of partition coefficient of GOx on the temperature of Phase system.

Temperature (^{0}C)	Specific activity of top phase (PEG phase)	Specific activity of Bottom phase(Salt phase)	Partition coefficient (K)
25	20	3.27	6.1
30	21	3.2	6.5
35	21.5	3.16	6.8
40	19	3.3	5.6

[a]PEG 7500 (150 **gl^{-1}**), Phosphate 175 **gl^{-1}**, pH 6.0, KCl. 0.2% and Glucose 0.1%.

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