

## Partitioning of $\beta$ -galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus* in Polyethylene Glycol/ Potassium Phosphate Aqueous Two-Phase Systems

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### Abstract

The partitioning behaviour of  $\beta$ -galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus* was investigated in aqueous two-phase systems (ATPS) composed of polyethylene glycol and potassium phosphate buffer. Different molecular weights of PEG (3350 and 8000) with various concentrations of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) at pH 6.5 were employed. The enzyme preferably partitioned to the phosphate rich phase when high concentrations of  $\text{KH}_2\text{PO}_4$  were used. Moreover, an increase in NaCl concentrations induced the enzyme favoured in the bottom phase. At near neutral pH some part of the enzyme moved to the top phase but after changing the pH values above this point, most of the enzyme preferred to be in the bottom phase.

**Keywords:**  $\beta$ -galactosidase, *L. delbrueckii* ssp. *bulgaricus*, aqueous two-phase systems, partitioning.

### 1. Introduction

The  $\beta$ -galactosidase usually hydrolyses non reducing terminal galactosyl moiety from oligosaccharide such as lactose into glucose and galactose. It normally disrupts substrates at  $\beta$ -1, 4

bond however, some is able to break and form other covalent bonds, as  $\beta$ -1, 6 more common and  $\beta$ -1, 2 rarely [1]. The  $\beta$ -galactosidase is also capable of catalysing transferase reactions which can result in both internal rearrangement of the lactose molecule and the formation of different disaccharides, known as transgalactosylation [2]. The transferase reaction can produce a range of di-, tri- and higher oligosaccharides. However, the hydrolysis reaction of oligosaccharides can simultaneously occur during the synthesis.

The  $\beta$ -galactosidase is a very important enzyme involved in dairy industry. Many studies on the enzyme have been attracted due to its important application in solving the problem of lactose intolerance, food-technology and environmental field from milk product.

An aqueous two phase system (ATPS) is an attractive method for purification of biological materials since it constitutes mild environmental condition containing high water content in each of the liquid phase up to 70-90% and surface tension between the two phases is low [3], resulting in high mass transfer and decreasing the possibility of denaturation of labile biomolecules [4]. Many polymers used in the system have protein-stabilizing

properties. In continuous extraction with ATPS is also straightforward and requires relatively simple equipments which are easy to operate [5]. Moreover, the conditions for separation on a large scale do not considerably change from small scale, thus easy in scale-up. However, the limitation of the technique is the lack of knowledge of the molecular mechanism involved in the partitioning process.

In general, ATPS process has been widely used for the recovery of macromolecule from fermentation broth and biological products. The biological materials such as cell organelles, protein, enzyme and whole cells are distributed between the two phases based on surface properties of the particles and molecules including size, shape, surface net charge, hydrophobicity and the existence of specific binding sites [6, 7]. Normally, after phase partitioning the target product biomolecules have to be preferentially partitioned in favor of one phase, whereas the interfering substances and contaminants, such as cells, cell debris, RNA, carbohydrate and lipid, should partition into the other phase. According to phenomenon, the effective separation can be achieved. The partition of molecules between the two phases is described by the partition coefficient ( $K$ ) which is defined as the ratio of the molecules concentration in the top phase ( $C_{top}$ ) to that in the bottom phase ( $C_{bottom}$ ). If the coefficient is higher than 1, the molecules prefer the top phase and if lower than 1 is in the bottom phase.

The enzyme partitioning is also related to its surface properties. The surface of a protein is made of different types of amino acid which generally contains mostly polar amino acid residues and charged side group and probably contains significant hydrophobic regions [8, 9]. The various side groups on the protein normally have different pH values, so when the pH of solution changes from acidic to

basic values, the protein becomes less positive and more negative charge. Thus portioning behaviour in a two phase system relate to the net protein charge which is function of the solution. In addition to the effect of interaction between enzyme phase forming and surface properties of the enzyme, the physicochemical properties of the two phases also influence partitioning behaviour such as type and concentration of the polymer (and salt), molecular weight of polymer, viscosity, temperature, interfacial tension and ionic composition.

The system obtained from polymer and salt are usually preferred since salt is much cheaper than dextran and the phases have a lower viscosity, then it is easier to handle and a shorter time for phase partitioning is required [5]. In this study, the two phases formed then composed of polymer (PEG) and salt ( $KH_2PO_4$ ). The partition is occurred by the unvarying distribution of the salt ions in the top and bottom phase and by the different of the electric current which causes from inequality of the salt distribution [10]. In general, phosphate ion move to the bottom phase which has a higher density, then the bottom phase enriches with negative charge rather than the top phase. Therefore, the enzymes might prefer to partition to the phase which enriches the opposite their surface charge.

The aim of this work was to study the partitioning behaviour of  $\beta$ -galactosidase in two-phase system. Two types molecular weight of PEG (MW 3350 and 8000) were investigated on various concentrations of potassium phosphate. In addition the role of pH and influence of NaCl were considered.

## 2. Materials and Methods

### 2.1 Materials

*Lactobacillus delbrueckii* ssp *bulgaricus* NCTC

11778 used for the source of  $\beta$ -galactosidase was purchased from NCIMB LTD (Aberdeen, U.K.). Polyethylene glycols (MW. 3350 and 8000) and ortho-nitrophenol- $\beta$ -galactosidase (ONPG) were purchased from Sigma. Potassium dihydrogen phosphate and Bovine Serum Albumin (BSA) (98%) were obtained from Aldrich (Sigma-Aldrich Co. Ltd. UK). Potassium hydroxide and Sodium chloride were obtained from BDH Limited Poole England. All the chemical compounds were analytical grade.

## 2.2 Fermentation and cell collection

Fermentation was conducted in a 100 L fermenter placed into a 115 L steam-jacketed fermentor (ML-4100 New Brunswick Scientific Co., New Brunswick, NJ, USA) equipped with a temperature controller at  $37 \pm 1$  °C and a pump for automatic addition of a neutralizer for pH control. The culture medium consisted of 10 g soy peptone, 10 g yeast extract, 20 g lactose, 2.5 g  $\text{KH}_2\text{PO}_4$ , 5g sodium acetate, 2 g Triammonium citrate, 1 ml Tween80, 0.2 g magnesium sulphate and 0.05 g manganous sulphate in 1000 ml of distilled water. 10 M NaOH was added for maintaining pH  $5.5 \pm 0.2$  and agitation was controlled at 200 rpm. After 10 hours the fermentation was terminated and quickly harvested. The fermentation medium was removed and the cells were washed with phosphate buffer using microfiltration membrane system.

## 2.3 Cell disruption

The cell disruption was carried out by using mechanical disrupter (Constant Systems Ltd, Coventry, UK, B series, model: UPCD40). The temperature was controlled at  $20 \pm 1$  °C. The pressure used was 25 kpsi. The cell debris was removed after centrifuge and the supernatant was used in partitioning study.

## 2.4 Determination of enzyme activity and protein concentration

The enzyme activity was determined with ONPG method. The rate of change in absorbance with time was measured after mixing 2 ml of 0.1M phosphate buffer pH 6.5, 1 ml of crude enzyme and 0.5 ml of 45 mM ONPG substrate which had the final concentration available in excess of the reaction. The solution was rapidly and completely mixed in cuvette. The reaction velocities of each condition were then determined during the first few minutes of the assay in absorbance at 420 nm temperature at 20 °C. All experiments were run in triplicate and were averaged. The activity of the enzyme was expressed as enzyme unit (U).

One unit of enzyme was defined as the amount of enzyme required to liberate 1  $\mu\text{mol}$  of o-nitrophenol per minute [11].

The amount of total protein was measured according to the Coomassie Blue G-250 method described by Bradford [12] at 595 nm. Different concentrations of Bovine Serum Albumin were prepared as a standard protein.

## 2.5 Phase diagram

Stock solution containing 45% w/w polyethylene glycol (PEG MW.3350 and 8000) and a stock solution containing 30 % w/w potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) were prepared. The salt stock solution was adjusted pH to 6.5 using KOH. Different amounts of PEG stock solution were taken into 20 ml test tubes and diluted with demineralized water until the final total volume in each tube was 5 ml. Then a small amount of salt stock solution was gradually added to each of the tubes and completely mixed. The salt solution was added until the cloud point shown. The first appearance of turbidity indicated that the system was about to enter the two-phase area. The

experiments were carried out at temperature 25 °C and done in triplicate.

## 2.6 Partitioning behaviour in ATPS

A certain amount of suspension was weighed out 10 g and dissolved in the solution consisted of calculated amount of PEG and phosphate buffer pH at 6.5. Both PEG and phosphate concentrations in ATPS were chosen based on slightly higher than the critical concentrations obtained from binodal curve. The total mixture was set to a total mass of 30 g by adding demineralised water. The system was then left to equilibrium at temperature 25 °C for overnight. The phases between upper and bottom were carefully separated using a syringe with a long needle. The volume of phase, enzyme and protein partitioning in each phase were determined. The enzyme partition coefficient was defined as the ratio of total enzyme activity in the top phase ( $E_T$ ) to in the bottom phase ( $E_B$ ) ( $K_E = E_T / E_B$ ).

## 3. Results

### 3.1 Phase diagrams of aqueous two phase systems.

Phase diagram of the different PEG molecular weights and potassium dihydrogen phosphate at pH 6.5 indicated the concentrations of phase components at which separation occurred above binodal line. After phase separation was allowed to take place, high concentration of PEG was found at the upper phase while the lower become salt rich phase. Figure 1 shows that the amount of salt needed for phase separation decreased with increasing the concentration of PEG. As can be seen from curve, the molecular weight of polymer influenced the phase diagram. Lower concentration of polymer was needed for two phase separation when the higher molecular weight was applied.

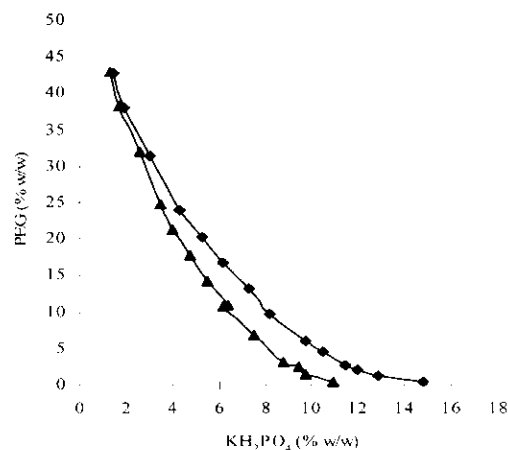


Figure 1 Phase diagram of PEG 3350 (◆) and 8000 (▲) with  $KH_2PO_4$  two phase system; pH 6.5, temperature at 25 °C.

### 3.2 Partitioning behaviours of $\beta$ -galactosidase in different concentrations of $KH_2PO_4$

The effect on the partition coefficient of  $\beta$ -galactosidase as function of  $KH_2PO_4$  concentrations varied from 7 to 9 %w/w were examined with constant PEG concentrations 13.5 %w/w for PEG 3350 and 8.5 %w/w for PEG 8000. The pH value in the system was at 6.5 and temperature was at 25 °C.

The influence of  $KH_2PO_4$  concentration on partition coefficient of  $\beta$ -galactosidase is shown in Figure 2. The tendency of result was decreased when the concentrations of  $KH_2PO_4$  were raised. It was obviously seen in PEG 3350 and 8000 the partition coefficient reduced quickly when the system composed of  $KH_2PO_4$  from 8.5 to 9 %w/w. Han and Lee [10] noted that increasing potassium phosphate concentration made the bottom phase became more negatively charge while the top PEG-rich phase became more positively charge. Thus most proteins which are positively charge will partition to the bottom phase. For example neutral protease from *Bacillus subtilis*, had the isoelectric point at pH about

8.95, partitioning to the bottom phase increased as the potassium phosphate concentration increased. The result was similar to partition behaviour of Lysozyme, whose surface charge is positive, in PEG and phosphate system.

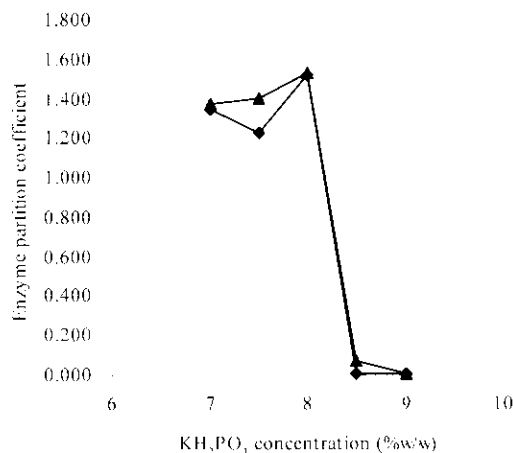


Figure 2 Influence of  $\text{KH}_2\text{PO}_4$  concentrations on partition coefficient of  $\beta$ -galactosidase in 13.5 %w/w for PEG 3350 (♦) and 8.5 %w/w for PEG 8000 (▲) systems.

The influence of  $\text{KH}_2\text{PO}_4$  concentrations on yield of  $\beta$ -galactosidase in the top phase with different molecular weights of PEG provided similar tendency with the enzyme partitioning coefficient (data not shown).

### 3.3 Effect of NaCl concentration on partitioning

The aqueous two phase system was set up for measuring the influence of NaCl on enzyme partitioning in different concentration from 0 to 5 %w/w. The fixed condition used in the system for PEG 3350 was 13.5% w/w PEG and 8% w/w  $\text{KH}_2\text{PO}_4$  and for PEG 8000 was 8.5 %w/w with 8% w/w  $\text{KH}_2\text{PO}_4$ . The pH value through this experiment was set at 6.5 and temperature was at 25 °C.

Figure 3 shows the partition coefficient of  $\beta$ -galactosidase in different molecular weight of PEG with various concentration of NaCl (%w/w).

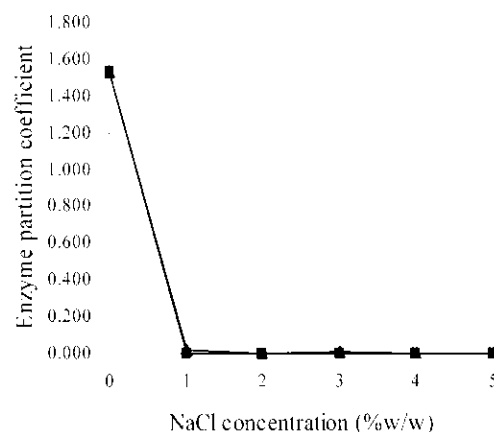


Figure 3 Influence of NaCl concentrations on  $\beta$ -galactosidase partition coefficient; phase composition 13.5% w/w PEG 3350 with 8% w/w  $\text{KH}_2\text{PO}_4$  (♦) and 8.5 %w/w PEG 8000 with 8% w/w  $\text{KH}_2\text{PO}_4$  (▲) systems

The addition of NaCl contributed to the increase of the  $\beta$ -galactosidase recovery in the bottom phase rather than top phase because salts were able to change the electrostatic charge of the system and cause the molecules with positive charge to prefer the salt-rich phase. As can be seen, the partition coefficient in the PEG 3350 and 8000 decreased from 1.530 in the system without the salt to almost 0% when the system containing NaCl from 1 to 5 %w/w. Then the addition of NaCl in PEG 3350 and 8000 systems assisted to enhance the  $\beta$ -galactosidase recovery in the bottom phase. Generally, an increase in salt concentration induces the partitioning of proteins between two phases differently. Gündüz and Korkmaz [13] found BSA favourably partitioned to the bottom phase with increasing NaCl concentration up to 0.2 M as has been observed with Ipomoea peroxidase [14] while increasing the recovery of amylase from the top phase was succeeded by using NaCl at 1-10% w/w concentration to induce the enzyme partition in the

PEG-rich phase [15]. The ion of NaCl distributed unequally between the phases causes an electrostatic potential difference between the phases and leads to the change of partition coefficient of specific proteins according their charge [6, 16-18].

### 3.4 Effect of pH on partitioning

The effect of various pH values (4.5-8.5) on enzyme partitioning coefficient was investigated (Figure 4). The influence of pH value was observed to alter the  $\beta$ -galactosidase partition coefficient. At near neutral pH some part of the enzyme moved to the top phase. However, most of the enzyme preferred to be in the bottom phase when changing the pH values above this point.

The best partition coefficients in PEG 3350 and 8000 are obtained at pH value 6.5. At pH value higher than 6.5, the ionic interactions predominated the hydrophobic partitioning then the partition coefficient started to decrease.

The function of pH on the aqueous two phase system has been investigated with many proteins. Normally, negatively charged protein should prefer the PEG rich phase while the positively charged protein distributes in salt phase [15, 19]. The isoelectric point (pI) of  $\beta$ -galactosidase from microorganism is generally around 4-5 [20], so when the pH increases above the isoelectric point of the proteins, their charge become negative and will strongly interact with PEG-rich phase, then the partition coefficient increases [17]. Shang et al [18] observed distribution of four amino acids in PEG/salt system with changing pH value. When the pH of the system increase, the concentration of  $\text{OH}^-$  increases, the lysine cations were partially neutralized causing reduction of electrostatic interaction between the molecules and phase forming system while the increase of the  $\text{HPO}_4^{2-}$  caused an increase in repulsion with phenylalanine, methionine and

cysteine anions.

The pH can also change the charge of chemical groups in the side chain of protein leading to a net charge modification of overall macromolecule [21]. The results in partition coefficient at high pH (above 6.5) indicated that pH modified the net charge of the enzyme to be more positive then changing its partitioning properties to bottom phase.

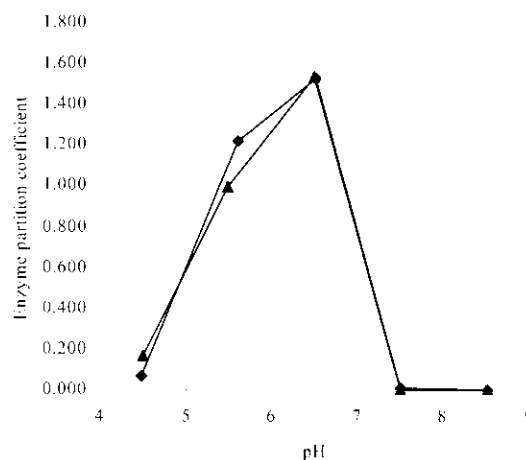


Figure 4 Influence of pH on partition coefficient of  $\beta$ -galactosidase in phase composition 13.5% w/w PEG 3350 with 8% w/w  $\text{KH}_2\text{PO}_4$  (◆) and 8.5% w/w PEG 8000 with 8% w/w  $\text{KH}_2\text{PO}_4$  (▲) systems.

### 4. Conclusions

This study indicates the partitioning behaviour of  $\beta$ -galactosidase in the PEG/phosphate ATPS. The partition coefficients of the enzyme altered with phosphate concentrations, NaCl concentrations and pH values since these parameters influenced protein surface charge. The  $\beta$ -galactosidase obviously favored to the bottom salt-rich phase when increasing the concentration of phosphate buffer above 8 % w/w and adding NaCl into the system. The enzyme preferentially partitioned to the top phase when pH was at 6.5 without the addition of

salt but above this point it preferred to bottom phase. Therefore, the environmental conditions were the importance factors for enzyme partitioning.

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