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PURIFICATION OF MICROBIAL β-GALACTOSIDASE FROM *KLUYVEROMYCES FRAGILIS* BY BIOAFFINITY PARTITIONING

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ABSTRACT

This work investigated the partitioning of β-galactosidase from *Kluyveromyces fragilis* in aqueous two-phase systems (ATPS) by bioaffinity. PEG 4000 was chemically activated with thresyl chloride, and the biospecific ligand p-aminophenyl 1-thio-β-D-galactopyranoside (APGP) was attached to the activated PEG 4000. A new two-step method for extraction and purification of the enzyme β-galactosidase from *Kluyveromyces fragilis* was developed. In the first step, a system composed of 6% PEG 4000-APGP and 8% dextran 505 was used, where β-galactosidase was strongly partitioned to the top phase (K = 2,330). In the second step, a system formed of 13% PEG-APGP and 9% phosphate salt was used to revert the value of the partition coefficient of β-galactosidase ($K = 2 \times 10^{-5}$) in order to provide the purification and recovery of 39% of the enzyme in the bottom salt-rich phase.

Key words: β-galactosidase, aqueous two-phase systems, protein purification, downstream-processing, affinity

INTRODUCTION

Partitioning of biomaterials in aqueous two-phase systems (ATPS) is a selective method for purification and for analytical studies of cellular components of several sizes, including proteins, nucleic acids, membranes and cellular organelles. An ATPS is formed by the addition of aqueous solutions of two polymers, such as PEG and dextran, or a polymer and a lyotropic salt, such as PEG and potassium phosphate. The extraction and separation process in ATPS can be used as a substitute for the initial steps of purification and of preparative chromatography of biomaterials, can be scaled-up without a significant loss of efficiency and can be accomplished in the absence of sophisticated equipment (2, 16).

The choice of a non-aggressive method such as ATPS partitioning maintains the biological properties of biomolecules. An ATPS contains a large amount of water in both phases, constituting an excellent mild biological method to recover cells, organelles or active proteins (2). Methods described in the literature, such as liquid-liquid extraction, are gaining prominence in meeting the basic demand due to the viability of their industrial applications (9).

Dextran and polyethylene glycol are not toxic chemicals, they are included in the pharmacopoeias of many countries and have applications in the food

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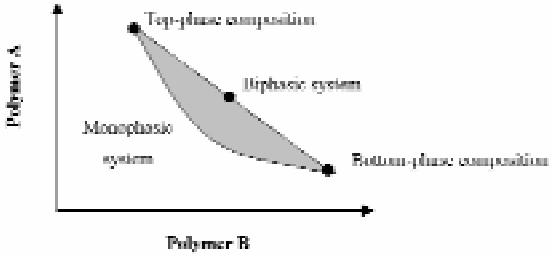
industry (17). Utilization of these polymers can be considered an advantage in the development of new technologies because a lot of applications of enzymes and biologically active proteins in the food and pharmaceutical industries (11).

In order to achieve a high recovery and the concentration of a target protein in one phase of the ATPS, it is necessary to increase the difference between the value of the partition coefficient (K = concentration of the protein in the top phase/bottom phase) of the target protein and the K value of the contaminant material (K_p). Fig. 1 shows a diagram of an ATPS.

Figure 1. Binodial curve of an aqueous two-phase system.



Parameters such as polymer molecular mass and concentration, type and concentration of salt, pH and temperature can affect K (4).

The process of purification in ATPS by bioaffinity combines the property of biological recognition and partitioning in a liquid environment. The ligand is coupled by covalent bonds to the polymer phase; therefore it will have a high partition coefficient towards the phase enriched with the polymer. If the target protein to be isolated has a specific affinity for the ligand, the formation of a ligand-biomolecule complex will induce an increase in the partition coefficient of the protein towards the phase enriched in the ligand (5, 15), while the presence of contaminant molecules will be predominant in the opposite phase (9).

The enzyme lactase or ß-galactosidase has many applications in dairy technology, such as for industrialized products containing lactose. ß-galactosidase can also be applied in crystal removal, production of sweeteners, solubilisers and toothpaste and research and analytic activities (12). The transglycosylation activity of ß-galactosidase, leading to the synthesis of oligosaccharides, has been reported by Prenosil *et al.* (23) and Brena *et al.* (6). Lactase-hydrolyzed products are currently being manufactured in Brazil, and the demand for such products would increase if this enzyme were produced at a lower cost and a higher quality.

This enzyme is intracellularly formed in yeasts and bacteria and secreted by fungi. The most important microorganisms which produce this enzyme are species of *Aspergillus niger, A. oryzae, Kluyveromyces fragilis* and *K. lactis* (16). Its production by *Neurospora* (19), *Escherichia coli* (29), *Saccharomyces lactis* (13), *Bacillus circulans* (14), *Scopulariopsis* sp (22) and *Erwinia aroidea* (10) has also been reported.

MATERIALS AND METHODS

PEG 4000 was purchased from Fluka (Switzerland) and tresyl chloride from Sigma (St. Louis, MO, USA). The *Kluyveromyces fragilis* strain was obtained from the American Type Culture Collection (ATCC 46537). A commercial β-galactosidase (Lactozym) was the kind gift of Novo Nordisk.

Enzyme production

For the production of β-galactosidase, *K. fragilis* was grown at 37°C for 24 h in the following culture medium: 30 ml of commercial milk, 0.15 g of $(NH_4)_2SO_4$ and 0.06 g of KH_2PO_4, with pH adjusted to 6.5. The fermented culture broth was centrifuged at 3,000 g for 20 min. The mass of cells was weighed and chloroform was added in the proportion of 1:1 (w/w). This suspension was kept under magnetic stirring for 1 hour, and then 20 ml of 0.05 M phosphate buffer, pH 7, was added and centrifuged. This procedure was repeated three times. The supernatant containing intracellular material of *K. fragilis* was used in the experiments.

Determination of β-galactosidase activity

The enzyme activity was determined by using o-nytrophenyl-β-galactopyranoside (ONPG) as the substrate and by spectrophotometric measurement at 420 nm. The enzyme was assayed at 37°C. One β-galactosidase unit corresponds to 1.0 µmol of orthophenol released per minute under the given condition.

Partitioning in the ATPS

One hundred ml of enzyme sample was mixed into the ATPS, using a Vortex for 30 s. Phase

separation was achieved by centrifugation for 5 min at 3,000 g and the interface of each system discharged. A known volume of each phase was collected and the activity was determined. The partition coefficient (K_E) of the enzyme was calculated from the ratio of β-galactosidase activity found in the top phase and activity found in the bottom phase.

Protein assay

The main contaminant protein concentration was determined by the dye-binding technique of Sedmak and Grossberg (25). Fifty to 100 µl of the top phase was transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD_{595} was measured in a spectrophotometer, versus a blank which had 50 µl of a top phase of a system which had been equally prepared without any sample. The blanks were done to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A BSA standard curve was used to calculate protein concentration. The partitioning of the main contaminant protein, K_P, was calculated as the ratio of protein in the top phase to that in the bottom phase at room temperature.

Synthesis of PEG-APGP

The PEG-APGP was synthesized according to Delgado *et al.* (8) and Nilsson and Mosbach (21).

Activation of PEG 4000 with tresyl chloride

(I) Solid PEG 4000 (20 g) was dried by azeotropic distillation in toluene and then dried in vacuum. The white solid was dissolved in 45 ml of dry dichloromethane at room temperature. The mixture was cooled to 0°C and stirred magnetically, and 1.125 ml of pyridine and 1 g of tresyl chloride at 0°C were added drop by drop. The reaction was continued at room temperature with constant stirring for 1.5 h, and the dichloromethane was removed by evaporation under reduced pressure. The white solid was dried in vacuum overnight at room temperature and formed the PEG-tresilated precursor (TPEG). TPEG was washed twice with HCl-ethanol (1:250, v:v), precipitated at 4°C and kept in a dissecator. This procedure was repeated sixtimes and the white solid was collected and dried in vacuum. In a second step, TPEG (I) reacted with Tris-HCl 0.2 M buffer, pH 8.0 for 12 hours at 4°C. It was dialyzed against water to remove the excess Tris-HCl and then concentrated by ultrafiltration (50 ml) **(II)**.

Attachment of APGP to TPEG

(III) - APGP (0.60 g) was added to the TPEG (II) and it reacted for 12 hours, ultrafiltered with water and then dried in vacuum.

Aqueous two-phase system preparation

Aqueous two-phase systems were prepared according to Franco *et al.* (11). They were prepared from stock solutions of PEG 4000 (50%, w/w), dextran T505,000 (30%, w/w) and potassium phosphate solution with K_2HPO_4 to KH_2PO_4 (40%, w/w) with a molar ratio of 0.6, pH 6.5. In the first step, the compounds were mixed to form 8.0 g of a system having a final concentration of 6% PEG 4000-APGP (compound III) and 8% dextran T505, pH 6.5. In the second step, the bottom phase of the PEG 4000-APGP/dextran system was discarded and replaced with a fresh phosphate phase, pH 6.5. The composition of the system (6.0 g total weight) was 13% PEG 4000-APGP and 9% K_2HPO_4/KH_2PO_4, pH 6.5 The system was mixed and centrifuged.

Electrophoresis

SDS electrophoresis (SDS-PAGE) was carried out in 12% homogeneous gel (18). The gels were stained with Bio-Rad silver. The molecular mass markers consisted of thyroglobulin (330 KDa), ferritin (220 KDa – half unit), albumin (67 KDa), catalase (60 KDa), lactate dehydrogenase (36 KDa) and ferritin (18.5 KDa), available as a standard kit (Pharmacia Biotech).

Specific β-galactosidase activity ($SA_{\beta\text{-gal}}$)

It is defined as the ratio of enzyme activity (U/ml) to the total protein concentration (mg/ml) and is expressed in U/mg of protein (equation 1).

$$SA_{\hat{a}-gal} = \frac{\text{Enzime activity}}{\text{Protein concentration}} \qquad (1)$$

Purification factor (PF)

The PF concept has been used in this work as a measurement to follow the purification operations (24, 27) and is defined as the ratio of the specific β-galactosidase activity after a purification step to the initial specific β-galactosidase activity (from the aqueous enzyme extract or from a previous purification step) (equation 2).

$$PF = \frac{SA_{\hat{a}\text{-gal}} \text{ in the collected phase}}{\text{Initial } SA_{\hat{a}\text{-gal}}} \qquad (2)$$

Recovery (R)

It is defined as the ratio of the enzyme activity collected from an aqueous phase after partitioning to the total enzyme activity added to the system (equation 3).

$$R(\%) = \frac{\text{enzyme activity of the phase}}{\text{total enzime activity added to the system}} \qquad (3)$$

Selectivity (S)

It is defined as the ratio of the partition coefficient of the enzyme, $K_{E,}$ to the partition coefficient of the protein, K_p.

$$S = \frac{K_E}{K_p} \qquad (4)$$

RESULTS AND DISCUSSION

Our earlier experiments showed that β-galactosidase from *Kluyveromyces lactis* was not separated from the main contaminant proteins of the broth (27) in conventional aqueous two-phase systems. The pool of total proteins was mainly partitioned towards the bottom salt-rich phase of PEG/phosphate systems independently of PEG molecular mass. In order to achieve a good and efficient separation of the β-galactosidase and its main contaminants, it would be desirable to find an ATPS composition where they are mostly extracted in opposite phases. Therefore an affinity system was developed in which the APGP biospecific ligand chemically attached to PEG 4000, which was accomplished in two chemical reactions. In the first reaction PEG hydroxyls were activated with chloride to become more reactive. In the second reaction the APGP ligand was finally bound to PEG. In this present work, the amount of activated and reacted PEG was not measured because our earlier results had shown that approximately 74% of the total number of hydroxyl groups in PEG had been transformed into tresyl esters in the first activation reaction. Also, the amount of APGP bound to PEG in the second reaction had been indirectly observed to be higher than 80% of the activated hydroxyls after five hours of reaction (27). Therefore in order to assure at least the same amount of APGP bound to PEG in this work, the second reaction lasted 12 hours. Another modification of the synthesis of the PEG-APGP was the removal of the excess of free APGP molecules and of the excess of Tris-HCl buffer, which was done by extensively washing by ultrafiltration.

Partitioning of the *Kluyveromyces fragilis* β-galactosidase

In order to extract and separate β-galactosidase from the contaminant proteins, a strain of *K. fragilis* was fermented and the cells were disrupted with chloroform. The enzyme extract was partitioned in the aqueous two-phase systems described in Table 1. The observed results show a 3,280-fold increase in the K_E value, when PEG-APGP replaced the plain PEG in the 6% PEG 4000 and 8% dextran system, indicating the strong affinity of β-galactosidase for the phase containing the APGP ligand. The purification factor rose from 2.8 to 9.7 and the selectivity factor of the system rose from 1.6 to 1,650. The high selectivity value indicates the potentiality of the system for a selective extraction of β-galactosidase by liquid extraction. Enzyme yield in the first-step partitioning in the affinity system was 55%, and the 45% β-galactosidase loss at the interface would possibly be due to the strong interaction between the APGP and the enzyme, which was not totally disrupted by the condition of the β-galactosidase assay with the ONPG substrate. STEERS *et al.* (26) observed that in affinity chromatography the interaction between APGP and β-galactosidase was stronger than the interaction between β-galactosidase and other substrates such as lactose, o-nitrophenyl-β-D-galactopyranoside and isopropyl-β-D-galactopyranoside. They found that the APGP-enzyme binding could only be disrupted when a low degree of ligand substitution was used or when an alkaline buffer of borate, pH 10, was employed to elute the enzyme. A second-step purification was developed by separating the top PEG-APGP-rich phase where the enzyme was collected and mixing it with a new fresh phosphate phase.

Albertsson (2) reports on the binodial curve of the PEG 4000/dextran system, where the top phase contained 8% PEG 4000 and 3% dextran and the bottom phase contained 5% PEG 4000 and 9%

Table 1. Liquid-liquid extraction process of β-galactosidase from *Kluyveromyces fragilis,* pH 6.5.

System	K_E	K_P	R (%)	SA (U/mg)	PF	S
6% PEG 4000 8% Dextran	0.71	1.2	57 (bottom phase)	104	2.8	1.6
6% PEG 4000-APGP 8% Dextran	2,330	1.4	55 (top phase)	361	9.7	1,650
13% PEG 4000-APGP 9% phosphate	2.2 x 10-5	0.8	39 (bottom phase)	708	19	2.7 x 10-5

β-galactosidase activity = 295 U/ml
Protein concentration = 7.92 mg/ml
Specific activity = 37 U/mg

dextran. As a new PEG 4000/phosphate system, pH 6.5, had to be used in order to invert the partition coefficient of the β-galactosidase by disrupting the interaction between the APGP and the enzyme, the top PEG-APGP phase was mixed with a stock phosphate solution to give a final composition of 13% PEG-APGP and 9% phosphate system. It was assumed that the binodial curve of the PEG-APGP/dextran system was similar to the PEG 4000/dextran system.

The results from Table 1 show that the partition coefficient of β-galactosidase decreased from 2,330 to 2.2 x 10^{-5} in the second partitioning procedure in the PEG 4000/phosphate system. The purification factor was 19, the specific activity was 708 U/mg and the selectivity was 2.7 x 10^{-5}, respectively. Due to the extremely low enzyme concentration in the salt-rich phase from the PEG-APGP systems (first step) and in the PEG-APGP-rich phase (second step), the partition coefficients given are lower limit estimates based on the limit of detection of the spectrophotometer.

A commercial enzyme (Lactozym) produced by the same specie of microrganism was also extracted and purified by the procedure developed in this work (Table 2).

Table 2. Liquid-liquid extraction process of β-galactosidase from Lactozym.

Systems	K_E	R (%)
6% PEG 4000 8% Dextran	0.40	87 (bottom phase)
6% PEG 4000-APGP 8% Dextran	2,380	50 (top phase)
13% PEG 4000-APGP 9% phosphate	5.3 x 10^{-4}	26 (bottom phase)

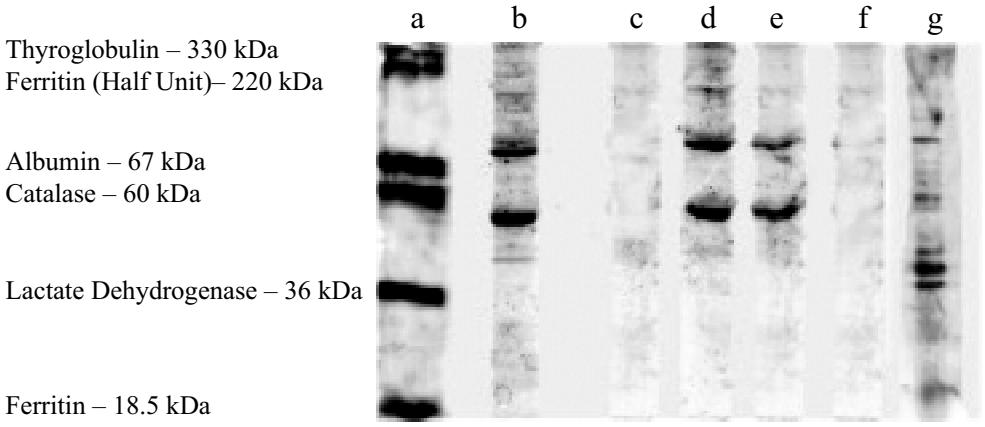
The K_E increased 5,950-fold and recovery was only 26% of the enzyme activity. The K_E observed for the Lactozym enzyme was 2,330, similar to the K_E observed for the β-galactosidase from fermentation, which was equal to 2,380 for *K. fragilis*. The system composed of 13% PEG 4000-APGP and 9% phosphate favoured the disruption of the complex enzyme ligand, and in the same way, the commercial β-galactosidase was concentrated in the bottom phosphate-rich phase.

In systems without a ligand, contaminant proteins were almost evenly distributed in both phases (K = 1.2). When the ligand was added, an insignificant increase in the partition coefficient of the contaminant proteins was found. Fig. 2 shows an electrophoretic gel of the β-galactosidase purification.

The gel shows two major bands of corresponding molecular mass, 117 KDa and 70 KDa, on the purified material. According to the literature, the molecular mass of β-galactosidase from *Kluyveromyces fragilis* is 201 KDa (20), calculated by size exclusion chromatography. They also found by SDS-PAGE that this enzyme was composed of two protein chains of MM of 120 KDa and 90 KDa, which in native conditions, would behave as a dimmer of approximately 200 KDa. If the enzyme purified in our work is assumed to be globular, a molecular mass of 187 KDa is calculated, which is only 5% below the β-galactosidase described by Mahoney and Whitaker (20).

The K/K_0 value determines the efficiency of an affinity partitioning procedure. It is described by the ratio of K, the partition coefficient in system with ligands and K_0, the partition coefficient of the enzyme in a system without ligands under otherwise identical conditions. When a ligand is coupled to the top

Figure 2. SDS-PAGE gel electrophoresis of β-galactosidase from Lactozym: a) marker proteins; b) Lactozym β-galactosidase; c) top phase of ATPS, without ligand; d) β-galactosidase extracted without ligand, bottom phase of ATPS; e) β-galactosidase extracted with ligand, top phase of ATPS; f) bottom phase of ATPS, with ligand, g) β-galactosidase (fermented).

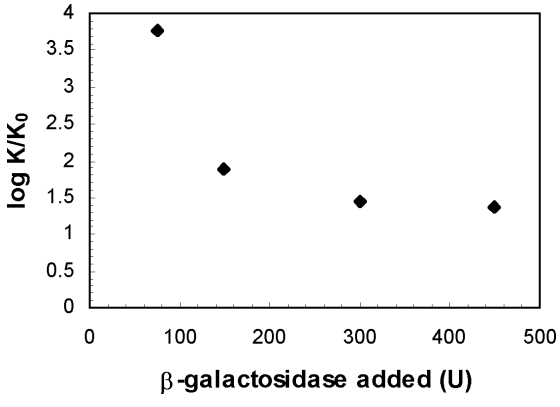


polymeric phase, K_E increases until some saturating value is reached. The value K/K_0 is proportional to the number of available ligands in the polymeric phase (1, 7).

Fig. 3 shows the effect of the enzyme concentration added to the system on the K/K_0 values. It is observed that the highest K/K_0 value 5,950, was obtained when 75 U of commercial β-galactosidase was added to the 8.0 g system.

Pastore and Park (22) purified β-galactosidase from *Scopulariopsis sp* by precipitation with ammonium sulphate and two chromatographic steps leading to a 4% yield of the pure enzyme desired. Veide *et al.* (28) developed a process of industrial isolation and purification by PEG 4000/potassium phosphate partitioning systems, for *E. coli* β-galactosidase which is followed by an ultrafiltration step to recycle the salt-rich phase. They were able to recover 95% of the β-galactosidase. Silva *et al.* (27) observed that the partition coefficient of β-galactosidase from *Kluyveromyces lactis* increased 3.7-fold in a 6% PEG 4000-APGP and 12% dextran system. The purification factor increased 1.6-fold and the recovery of the target enzyme was 83%.

Figure 3. Effect of the enzyme concentration added to the system on the K/K_0 values.



It seems clear that partitioning in ATPS can be an effective way of purifying and concentrating enzymes and other biomolecules. It can be greatly improved by designing efficient and specific bioligands which are not expensive and can be recycled (i.e., ultrafiltrated). It is desirable that the interaction between the ligand and the target enzyme can be easily disrupted by salts or by pH changes. Research on affinity techniques, binding chemistry and the designing of specific equipments for extraction in ATPS will be of great help in the improvement of downstream enzyme processing.

CONCLUSIONS

An extractive liquid-liquid system was developed with the objective of purifying the microbial enzyme β-galactosidase. As the enzyme from *Kluyveromyces fragilis* is very hydrophilic, being partitioned with the main contaminant proteins to the saline phase in conventional PEG/phosphate systems, a new two-step process was developed for the extraction and

purification of β-galactosidase. In the first step, the system involves a biospecific ligand composed of 6% PEG4000-APGP and 8% dextran. In this system the partition coefficient of β-galactosidase increased 3,280-fold compared to the initial value in a system without ligands. In the second step, a system formed of PEG 4000 and phosphate was used to revert the value of the partition coefficient of the β-galactosidase ($K = 2.2 \times 10^{-5}$), providing the purification and recovery of 39% of the enzyme. The main benefit of this work was the development of a feasible process of chemical activation of the polymer polyethylene glycol and the subsequent binding of a ligand to PEG (a hydrosoluble polymer), capable of completely altering the value of the partition coefficient of the enzyme. In the affinity step, a great number of contaminant materials were removed to the bottom phase, as the enzyme was partitioned to the top phase. In the second step, the enzyme-ligand bond was broken in the presence of a high concentration of phosphate, providing a 19-fold purification of the β-galactosidase in the saline phase in a procedure of just two steps.

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RESUMO

Purificação de β-galactosidase de *Kluyveromyces fragilis* por partição por bioafinidade

Foi desenvolvido um método novo de extração e purificação da β-galactosidase de *Kluyveromyces fragilis* em sistema de duas fases aquosas (SDFA). PEG 4000 foi ativado quimicamente com cloreto de tresila e o ligante bioespecífico p-aminofenil-β-D-tiogalactopiranosídeo (APGP) foi acoplado ao PEG 4000 ativado. Na primeira etapa foi usado um sistema composto de 6% PEG-APGP e 8% dextrana 505, onde a partição da β-galactosidase ocorreu na fase superior (K = 2.330). Na segunda etapa foi usado um sistema composto por 13% PEG-APGP e 9% fosfato para reverter o valor do coeficiente de partição da β-galactosidase ($K = 2,2 \times 10^{-5}$), obtendo-se recuperação de 39% da enzima na fase salina.

Palavras-chave: β-galactosidase, sistema de duas fases aquosas, purificação de proteína, afinidade.

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