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PARTITIONING OF EXTRACELLULAR PROTEASE FROM *BACILLUS SUBTILIS* IN PEG/POTASSIUM PHOSPHATE AQUEOUS TWO-PHASE SYSTEMS

Nguyen Hoang Loc[1], Luu Thi Nguyet Minh[1], Do Thi Bich Thuy[2]

[1]*Institute of Resources, Environment and Biotechnology, Hue University*
[2]*College of Agriculture and Forestry, Hue University*

SUMMARY

The aqueous-two phase system (ATPS) has a great potential for use in the downstream processing of fermentation products. The partitioning of extracellular protease from *Bacillus subtilis* C10 culture was carried out in ATPS formed by polyethylene glycol (PEG)/potassium phosphate. Factors that influenced the partition of the extracellular protease in this system, including the concentration and molecular weight of the PEG, and the potassium phosphate concentration were investigated. The optimal ATPS was 20% (w/w) PEG 6000 and 15% (w/w) potassium phosphate, pH 7.0. The partition coefficient for extracellular protease ($K_{protease}$) was 4.99 with a top phase yield (Y) of 90.25% at room temperature. The extracellular protease specific activity of the top phase was 0.33 unit/mg in the same system. This process, therefore, is suggested to be a rapid and convenient method for protease purification.

Keywords: *Aqueous two-phase system, Bacillus subtilis, partition, polyethylene glycol, potassium phosphate, protease*

INTRODUCTION

The aqueous two-phase system (ATPS) is widely used in biochemistry and biotechnology for purification of proteins/enzymes and other labile biomolecules from crude cell or free-cell extracts, or other mixtures (Xu *et al.*, 2005). The ATPS is typically created by mixing solutions of PEG and dextran or PEG and salts such as potassium phosphate, sodium phosphate or ammonium sulphate to form two immiscible phases. Proteins and cellular debris show differential solubility between the two phases, so that the technique can be used both for the separation of proteins from cellular debris and for the partitioning of enzymes during protein purification (Scawen, Hammond, 2002). Most often, this technique is employed in industrial or laboratory production of enzymes. The ATPS has, therefore, great potential for use in the downstream processing of fermentation products. Generally, the optimal condition of the ATPS required for a particular protein is found empirically. Although the conditions required to achieve satisfactory separation can often be precisely defined, the mechanism of partitioning is not fully understood (Xu *et al.*, 2005).

The proteases (proteinases) are large group of enzymes that conduct proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases occur naturally in all organisms and constitute 1-5% of the gene content. They are the most important industrial enzymes and account for roughly 60% of the total enzyme market (Ng, Wenealy, 1986).

The aim of this study was to investigate the partition of protease from cell free culture broth of *Bacillus subtilis* C10 in different PEG/potassium phosphate aqueous two-phase systems.

MATERIALS AND METHODS

Chemicals

Polyethylene glycol 2000 and 10000 were purchased from Fluka Chemical Corp. (Ronkonkoma, USA). Polyethylene glycol 6000, K_2HPO_4, and KH_2PO_4 were supplied by Merck & Co., Inc. (Whitehouse Station, USA). All the other chemicals used were of analytical grade.

Bacillus subtilis C10 culture and protease production

Bacillus subtilis C10 was a wild strain isolated

from shrimp shell (Do *et al.*, 2004). The seed culture was grown in the medium containing 1% peptone, 0.5% NaCl, and 0.3% beef extract; on a shaker with rotation speed of 200 rpm, at 35°C for 20 h. The protease production was carried out under the same conditions using the medium of pH 7.0 with 1% peptone, 0.5% NaCl, 0.1% yeast extract, 0.3% beef extract, and 1.75% dissolved starch. The supernatant of the culture broth was harvested after 24 h by centrifugation at 14000 rpm for 5 min at 4°C and stored at 4°C for further use.

Protease activity assay

Protease activity was determined spectrophotometrically by the Anson's method with a slight modification (Kalisz, 1988). The substrate was 0.6% (w/w) casein in Tris.HCl (0.05 M pH 7.0). Enzymatic hydrolysis was initiated by addition of 1 ml properly diluted enzyme solution in 5 ml of substrate. After 5 min of incubation at 40°C, 5 ml of 0.4 M trichloacetic acid was pipetted into the solution to terminate the reaction. The solution was incubated at 40°C for 20 min to precipitate the residue substrate, and filtrated. The tyrosine concentration of the filtrate was determined spectrophotometrically at $\lambda_{750\ nm}$. One unit of protease activity is defined as the amount of enzyme required to liberate 1 mg of tyrosine per 1 ml per min under the standard assay conditions.

Total protein determination

Total protein concentration was determined by the method of Bradford using BSA as a standard (Bradford, 1976). The samples were read at $\lambda_{595\ nm}$ against the blanks with the same compositions as the samples, but without any protein, to avoid the interference of PEG and phosphate. The protease specific activity is obtained by dividing units of enzyme by total protein concentration in the sample.

Preparation of aqueous two-phase systems

The aqueous two-phase system was prepared from stock solutions of PEG (60%, w/w) and potassium phosphate (30%, w/w). Stock solutions were stored at 4°C. Before use, the temperature of all stock solutions was equilibrated to room temperature. The ATPS was created by using the required amounts of PEG and potassium phosphate to achieve various concentrations with final volumes of 10 ml. Four milliliters of the supernatant from *B. subtilis* C10 culture was added to the ATPS. The pH of the ATPS was adjusted by using the appropriate ratio of K_2HPO_4 and KH_2PO_4. Low-speed centrifugation (2000 rpm) for 10 min at room temperature was used to speed up phase separation after a gentle mixing of the system components and incubation of 6 h at 20°C. The phase volume ratios were determined in graduated centrifuge tubes (15 ml Falcon tube). Samples from the top and bottom phases were then assayed for protease activity and total protein concentration.

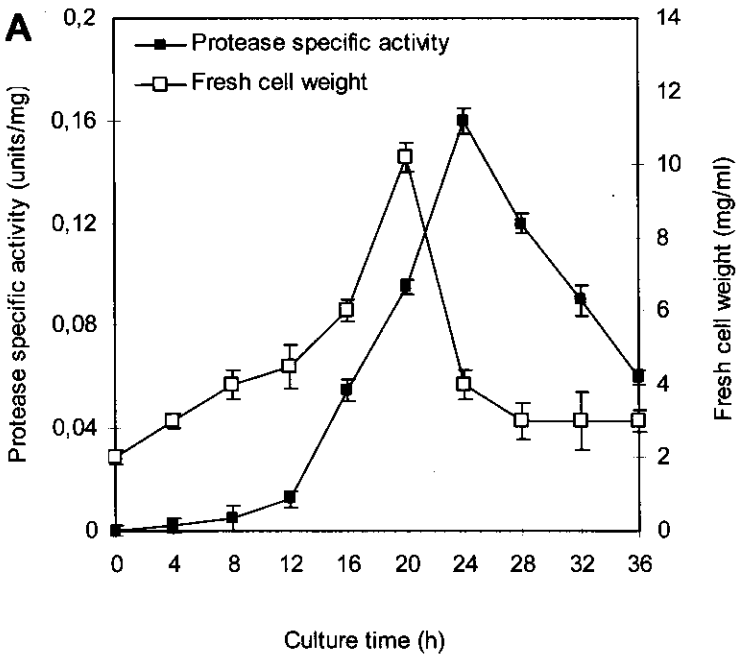
The protease partitioning coefficient ($K_{protease}$) and total protein partitioning coefficient ($K_{protein}$) were defined as the ratio of protease specific activity and total protein concentration in the top and bottom phases, respectively. The volume ratio (V) was the ratio of the volume in the top (V_T) phase to the volume in the bottom (V_B) phase. The partitioning yield of protease (%) in the top phase, Y, is given by the following equation (Albertson, 1958):

$$Y = \frac{100}{1 + \left(V_B / V_T\right) \cdot \left(1 / K_{protease}\right)}$$

RESULTS AND DISCUSSION

***Bacillus subtilis* C10 culture and protease production**

A batch culture of *B. subtilis* C10 was performed in 250 ml Erlenmeyer flasks for the extracellular protease production. Figure 1 displayed a typical growth curve based on the fresh cell weight of *B. subtilis* C10. The cell biomass reached maximum value of 10.2 mg/ml at 20 h of cultivation, and then decreased during 24-36 h. The dynamics of protease and total protein production are also shown in figure 1. Generally, the concentration of total protein and protease specific activity increased continuously from the start to 24 h of cultivation with final respective values of 3.51 mg/ml and 0.16 unit/mg, and they rapidly decreased after 28 h. For the partitioning experiments, we used the culture broth of 24 h, which contained the highest activity of protease.



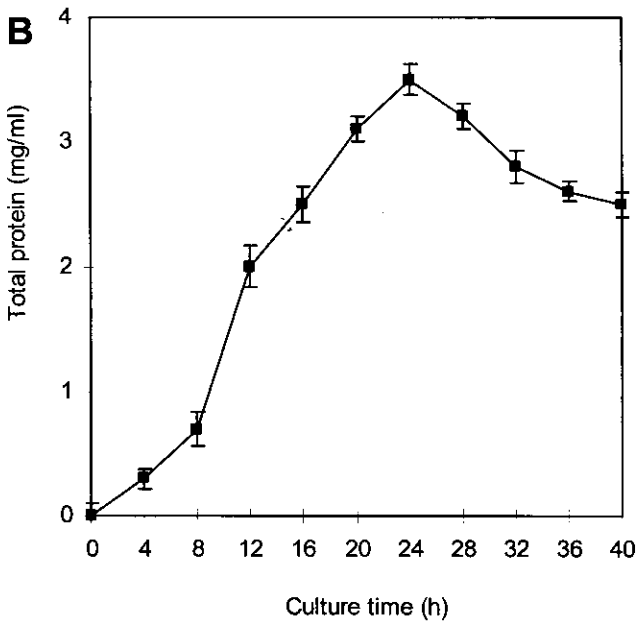


Figure 1. Dynamics of growth protease specific activity (A), and total protein concentration (B) during batch culture of *B. subtilis* C10. The values represent the means of triplicate results, and the error bars represent standard errors.

Partitioning of protease and total protein

The partitioning of a protein depends on parameters such as its molecular weight and charge, the concentration and molecular weight of the polymers, the temperature, pH and ionic strength of the mixture, and the presence of polyvalent salts such as phosphate or sulphate (Walter *et al.*, 1985).

In our study, the influences of polymer concentration and molecular weight, salt concentration were investigated. The partition was assayed at pH 7.0 in the ATPS. Both total protein and protease showed high affinity for the PEG-rich phase.

Effect of PEG concentration and molecular weight

The effect of PEG on the partition of protease and total protein were analysed in the various ATPSs (Table 1). Generally, the partitioning coefficients and volume ratio depended on the degree of polymerization. The appropriate concentration and molecular weight of PEG favoured the proteins transfer to the top phase, suggesting an important protein-polymer interaction. The PEG proved to have a stabilizing effect on the protein, increasing its secondary structure (Spelzini *et al.*, 2005).

The data in Table 1 show that the protease concentrates in the top phase with the $K_{protease}$ ranging from 0.96 to 4.06, and the other proteins tend to concentrate in the bottom phase with the $K_{total\ protein}$ ranging from 0.25 to 1.09. Both $K_{total\ protein}$ and $K_{protease}$ increase with increasing PEG molecular weight from 2000 to 10000 (PEG in 15%) or from 2000 to 6000 (PEG in 20-25%). The polymerization increase (PEG 10000 in 20-25%) results in less space for proteins to partition into the top phase. The hydroxyl groups, which lead to an increase in hydrophobicity in the PEG-rich top phase, and the size-exclusion effect are suggested to be the main reason for the decrease in the partition coefficients (Zhi *et al.*, 2004).

The partitions of protease and total protein in ATPSs with PEG 2000 (low molecular weight) are not significantly affected. Meanwhile, for PEGs 6000 and 10000 (high molecular weight) protease and total protein tend to partition into the bottom phase as its concentration increased to 25%, which may also be due to the size-exclusion effect caused by the relative large size of PEG molecules with high molecular weight (Table 1).

Table 1. Effect of PEG molecular weight and concentration on the partition of total protein and protease.

PEG		V	$K_{total\ protein}$	$K_{protease}$
Molecular weights	**Concentrations (%)**			
2000	15	0.23 ± 0.02	0.25 ± 0.01	0.96 ± 0.12
	20	0.35 ± 0.01	0.31 ± 0.01	1.31 ± 0.14
	25	0.43 ± 0.04	0.39 ± 0.03	1.41 ± 0.21
6000	15	0.41 ± 0.02	0.35 ± 0.03	1.35 ± 0.17
	20	0.94 ± 0.13	1.09 ± 0.21	2.14 ± 0.28
	25	0.57 ± 0.06	0.48 ± 0.03	1.61 ± 0.15
10000	15	1.41 ± 0.20	0.76 ± 0.05	4.06 ± 0.38
	20	0.51 ± 0.03	1.01 ± 0.22	1.52 ± 0.11
	25	0.43 ± 0.04	0.37 ± 0.01	1.36 ± 0.18

Note: Concentration of potassium phosphate in systems was 12% (w/w). The values represent the means of triplicate results ± the standard errors.

Effect of potassium phosphate concentration

Using the results in Table 1, the polymer concentrations (w/w) were maintained at 25% (PEG 2000), 20% (PEG 6000) and 15% (PEG 10000) with the potassium phosphate concentration was 9% or 15% (w/w). Table 2 represents the values of $K_{total\ protein}$ and $K_{protease}$ in changing potassium phosphate concentration.

Our data show that the PEG molecular weight had little influence on the partitioning of protease

and total protein, but the potassium phosphate concentrations affected the protease and total protein partition significantly. Since the $K_{\text{total protein}}$ are relatively low (0.35-1.09) in potassium phosphate concentrations of 9-12%, it is assumed that the proteins tend mainly in the bottom phase of the ATPSs. $K_{\text{total protein}}$ become progressively greater at the value of 1.75 with an increasing potassium phosphate concentration to 15%. The K_{protease} increased by the increment of potassium phosphate concentration from 9-15% and achieved the highest value of 4.99. It shows that the increment of K_{protease} is much greater than the K_{protein} increment (Table 1 and 2).

In the PEG/potassium phosphate ATPS, the top phase is the PEG-rich phase, while the bottom phase is the potassium phosphate-rich phase. Protein solubility in the top phase was determined by hydrophobic interaction between the ethylene group of PEG and the hydrophobic residue of protein, while that in the bottom phase was determined by salting-out effect with the presence of salts (Baskir *et al.*, 1989; Lee, Sandler, 1990). Decreasing the protein solubility in the bottom phase by increment of potassium phosphate concentration increased the partition coefficient of both total protein and protease. Because the partition coefficient of protease was larger than that of total protein at the same potassium phosphate concentration, it can be suggested that protease may be concentrated and separated from the rest of the proteins (Cho *et al.*, 1999).

In the published literatures, there were some studies reported on the partitioning of enzyme from the various ATPSs, especially protease from *B. subtilis* cultures. For examples, Han and Lee (1997) obtained $K_{\text{neutral protease}}$ value of 1.5 in 20% PEG 5500/17% potassium phosphate system from *B. subtilis*. Cho *et al.* (1999) obtained K_{protease} value of 5.2 in 16% PEG 4000/14% potassium phosphate system from *Bacillus* sp. PSE-68. Chouyyok *et al.* (2005) obtained $K_{\text{alkaline protease}}$ value of 4.12 in 25% PEG1000/18% potassium phosphate system from *B. subtilis* TISTR 25 *etc.* Our results also show an effective partition of protease from *B. subtilis* C10 in 20% PEG 6000/15% potassium phosphate system.

Table 2. Effect of potassium phosphate concentration on the partition of total protein and protease.

PEG molecular weights and concentrations	Potassium phosphate concentrations (%)	*V*	$K_{\text{total protein}}$	K_{protease}
25% PEG 2000	9	0.21 ± 0.02	0.35 ± 0.01	0.83 ± 0.08
	15	0.57 ± 0.04	0.44 ± 0.03	1.79 ± 0.13
20% PEG 6000	9	0.49 ± 0.03	0.84 ± 0.09	0.62 ± 0.04
	15	2.26 ± 0.32	1.75 ± 0.13	4.99 ± 0.32
15% PEG 10000	9	0.56 ± 0.03	0.99 ± 0.12	0.54 ± 0.04
	15	1.41 ± 0.11	0.79 ± 0.06	4.20 ± 0.37

Note: The values represent the means of triplicate results ± the standard errors.

Specific activity and partitioning yield of protease in the top phase

The partitioning yield of protease (Y) depends on two parameters: V_B/V_T and K_{protease}. In most of the ATPSs investigated, protease partitions more towards the top phase, and the volume values, V_T, are relative large. Therefore, the partitioning yield has high values. The highest protease yield is 90.25% (respective specific activity of protease is 0.33 unit/mg) with K_{protease} coefficients of 4.99, and V_B/V_T value of 0.44 (with V_T/V_B ratio of 2.26) in the phase compositions of 20% PEG 6000/15% potassium phosphate (Figure 2).

CONCLUSION

In conclusion, our experiments show that the 20% PEG 6000/15% potassium phosphate system was highly effective in the partitioning of bacillus protease. In this system, the protease was concentrated

in the top phase and high K-values of the enzyme guaranteed high yield of partitioning. This process is relatively convenient and inexpensive, therefore, its application can be further exploited.

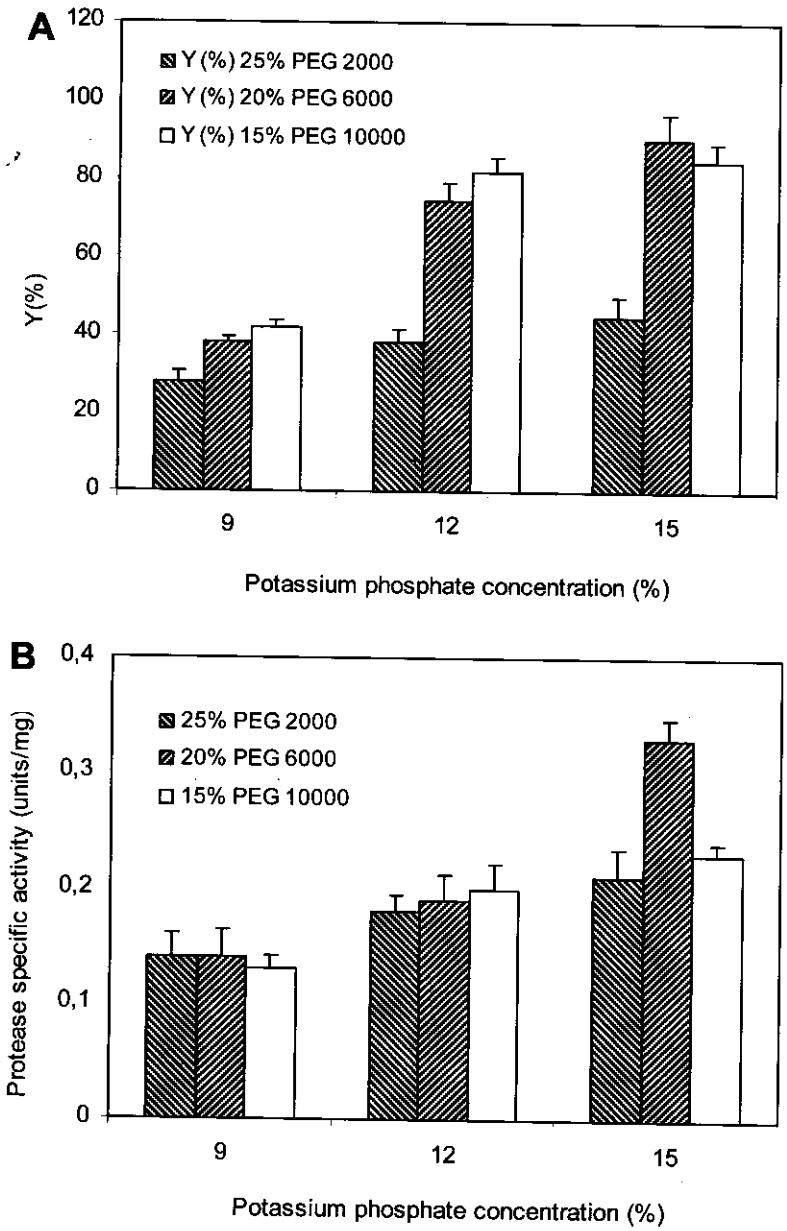


Figure 2. Protease partitioning yield and specific activity in the top phase of various ATPSs. Protease partitioning yield (A), protease specific activity (B). The values represent the means of triplicate results, and the error bars represent standard errors.

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PHÂN TÁCH PROTEASE NGOẠI BÀO CỦA *BACILLUS SUBTILIS* TRONG HỆ HAI PHA NƯỚC PEG/POTASSIUM PHOSPHATE

Nguyễn Hoàng Lộc[1,*], Lưu Thị Nguyệt Minh[1], Đỗ Thị Bích Thủy[2]

[1]Viện Tài nguyên, Môi trường và Công nghệ sinh học, Đại học Huế
[2]Trường Đại học Nông Lâm, Đại học Huế

TÓM TẮT

Hệ hai pha nước có một tiềm năng ứng dụng quan trọng trong quá trình tinh sạch các sản phẩm lên men. Trong nghiên cứu này, để phân tách protease ngoại bào từ dịch nuôi cấy của chủng *Bacillus subtilis* C10 chúng tôi đã sử dụng hệ hai pha nước được tạo thành nhờ polyethylene glycol (PEG) và potassium phosphate. Các nhân tố ảnh hưởng đến sự phân tách protease ngoại bào trong hệ thống này, bao gồm nồng độ và khối lượng phân tử của PEG, và nồng độ của potassium phosphate đã được khảo sát. Hệ hai pha nước tối ưu là 20% (w/w) PEG 6000 và 15% (w/w) potassium phosphate, pH 7,0. Hệ số phân tách của protease ngoại bào ($K_{protease}$) là 4,99 với hiệu suất pha đỉnh (Y) là 90,25% ở nhiệt độ

*Author for correspondence. Tel: *84-54-3830208;* Fax: *84-54-3820438;* E-mail: nhloc@hueuni.edu.vn

phòng. Hoạt độ riêng của protease ngoại bào ở pha đỉnh là 0,33 unit/mg trong cùng một hệ thống. Từ những kết quả trên, quá trình này được xem là một phương pháp nhanh và thuận lợi cho việc tinh sạch protease.

Từ khóa: *Bacillus subtilis, hệ hai pha nước, polyethylene glycol, potassium phosphate, protease, sự phân tách*