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Purification of recombinant neutral protease (NPRC10) by partitioning in aqueous two-phase systems

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ABSTRACT

A study was made of the partition and purification of recombinant neutral protease (NPRC10) from a culture supernatant of E. coli BL21 (DE3) in the polyethylene glycol (PEG)/potassium phosphate aqueous two-phase systems. Factors that influenced the partition of the enzyme in these systems, including the PEG molecular weight and concentration, potassium phosphate concentration, incubation time and temperature, and pH were investigated. The optimum condition of the primary aqueous two-phase extraction was 35% PEG 6000/30% potassium phosphate (pH 6.5) at 30°C for 25 min. The partitioning coefficient for protease ($K_{protease}$) was 4.69 with a partitioning yield (Y) of 94.93%, a purification fold (PF) of 1.7, and protease specific activity ($SA_{protease\ T}$) of 694.61 unit/mg protein in top phase. The protease, which was concentrated in the top phase, was further mixed with 35% potassium phosphate in combination with 3% potassium chloride at room temperature to elute the bottom phase (salt-rich phase). Using this step, the PF of enzyme achieved a higher value of 2.38 with a recovery yield of 76.17% and $SA_{protease\ B}$ of 983.04 unit/mg protein.

Keywords: Aqueous two-phase system, neutral protease, NPRC10, polyethylene glycol, potassium phosphate, purification

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INTRODUCTION

An aqueous two-phase system (ATPS) or aqueous biphasic system is obtained either by mixture of aqueous solution of two polymers, or a polymer and a salt. The former is comprised of polyethylene glycol (PEG) and polymers like dextran, the latter is composed of PEG and phosphate or sulfate salts. The polymer-salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in the first extraction step. Aqueous two-phase systems have been successfully used for the separation and purification of proteins because of their advantages compare with the traditional methods. These systems have good resolution, low material cost, high yield, less energy consumption, and less process time [1, 2].

Several results on partitioning of protease in the different ATPSs were reported such as PEG/ammonium sulfate [3]; PEG/potassium phosphate [4]; PEG/dextran, PEG/potassium phosphate, PEG/sodium citrate, PEG/magnesium sulfate, and PEG/sodium dihydrogen phosphate [5]; PEG/monosodium phosphate and sodium chloride [6]; PEG/ammonium sulfate [7]; PEG/sodium citrate [8]; PEG/potassium phosphate [9]…

In this work, we describe the partitioning features of neutral protease (NPRC10) in PEG/potassium phosphate ATPS in order to improve purification yield of enzyme from recombinant *E. coli* strain BL21 (DE3).

MATERIALS AND METHODS

Chemicals

Polyethylene glycol 2000, 6000 and 10000 were purchased from Sigma (USA). Mono- and dibasic potassium phosphate (K_2HPO_4 and KH_2PO_4) were supplied by Nacalai (Japan). All the other chemicals used were of analytical grade.

Bacterial strain and plasmid

E. coli strain BL21 (DE3) (Invitrogen) harboring expression vector pET200/D-TOPO was used in this study. The plasmid pET200/D-TOPO contains *npr*C10 gene (accession No. FJ822054) encoding extracellular neutral protease (NPRC10) of 42 kDa under the control of T7 promoter. The *npr*C10 gene contains the signal peptide-like sequence from nucleotides 161 to 250 and the mature peptide sequence from nucleotides 824 to 1723 [10].

Cultures and enzyme production

The initial culture was grown in 250-mL Erlenmayer flasks containing 50 mL of LB medium supplemented with 50 mg/L kanamycin. The flasks were incubated at 37^{o}C on rotation shaker at a speed of 200 rpm for overnight to obtain an OD_{600} value of 2.2.

The biomass production was done in 250-mL Erlenmeyer flasks with 50 mL medium. The culture was grown in the light modified HSG medium (3% glycerol, 0.7% yeast extract, 1.35% tryptone, 0.014% $MgSO_4.H_2O$, 0.15% KH_2PO_4, 0.23% K_2HPO_4, 0.5% glycine and 1.5% soluble starch) at 37^{o}C, 200 rpm rotation speed, and 0.5% (v/v) inoculum size of initial culture for 24 hours.

For enzyme production, a 14-L fermenter (BioFlo 110, New Brunswick Scientific, Edison, USA) with 10 L of the biomass production medium was used. Lactose at the final concentration of 0.5% was added into the culture when the OD_{600} reached at value of 2 to induce enzyme production at 20^{o}C. The pH was controlled to 7 by addition of 3 N NaOH solution. 0.1% antifoam solution (Sigma-Aldrich, St. Louis, MO) was added as needed. The fermentation was done at 4 L/min aeration rate, 500 rpm agitation speed, and 1% (v/v) inoculum size.

Protease activity assay

Protease activity was determined by modified procedure based on the method of Anson [11] using casein as the substrate. One-milliliter of the supernatant was mixed with 2 mL of 2% (w/v) casein in 50 mM Tris.HCl buffer (pH 7). The reaction was incubated at 50^{o}C for 15 min, subsequent to 5 mL of 5% (w/v) trichloroacetic acid was added into the solution to terminate the reaction. The solution was incubated at room temperature for 20 min to precipitate the residue substrate. The tyrosine concentration of the supernatant was determined at the 750 nm wavelength by the spectrophotometer (SmartSpecTM Plus, Bio-Rad, USA). One unit of protease activity is defined as the amount of enzyme required to release 1 µg tyrosine per 1 mL per min under the standard assay condition.

Total protein concentration was determined by the method of Bradford [12] using bovine serum albumin as a standard. The sample was read at wave length of 595 nm against the blank. The protease specific activity is expressed as units of enzyme per milligrams of total proteins.

Preparation of aqueous two-phase systems

The ATPS was created by using the required amounts of PEG and potassium phosphate to achieve different concentrations with final volumes of 10 mL (Table 1). Four milliliters of the supernatant from *E. coli* culture was added to the ATPS. The pH of the ATPS was adjusted by using the appropriate ratio of mono- and dibasic potassium phosphate. Centrifugation at 2000 rpm for 10 min at room temperature was done to speed up phase separation. 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 ($K_{protease}$) was defined as the ratio of protease specific activity in the top and bottom phases. The volume ratio (V) was the ratio of the volume in the top phase (V_T) to the volume in the bottom phase (V_B). The partitioning yield of protease (%) in the top phase, Y, is given by the following equation [13]:

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

Purification by aqueous two-phase extraction and back extraction

The purification process of protease by the ATPS is done by the method of Cho et al [14] with light modification. In the primary extraction, protease was selectively partitioned to the PEG-rich top phase. In the back extraction, fresh potassium phosphate (30-40%, w/w) and potassium chloride (3-9%, w/w) were added to the PEG-rich top phase of the primary extraction. Centrifugation at 2000 rpm for 10 min at room temperature was performed to speed up phase separation. Sample from the bottom phase was dialyzed at 4^{o}C for 20 h and then freeze-dried to obtain purified protease.

SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run at 4^{o}C by using a Mini-PROTEAN III system (Bio-Rad), 12% polyacrylamide gel, and 0.1% casein as a substrate. The gel was then shaken gently at room temperature for 30 min in 2.5% Triton X-100 to remove SDS. The gel slab was transferred to a bath containing 0.05 M Tris.HCl buffer (pH 6.7) and incubated at 37°C for 3 h to hydrolyze casein. The gels were then fixed and stained in Coomassie Brillant Blue R250, and the image was analyzed by Quality One software (ver 4.1, Bio-Rad).

Statistical analysis

All experiments were repeated thrice. The data were analyzed in terms of means followed by Duncan's test with $p<0.05$.

RESULTS AND DISCUSSION

Culture and protease production

A batch culture of *E. coli* BL21 (DE3) was done in 14-L fermenter for the extracellular protease production. Figure 1 displayed a typical growth curve based on the OD_{600} of the cell density. The cell biomass increased continuously from the start to 40 h of culture, with final OD_{600} value of about 6.5. It was found that the maximum production of protease was also obtained after 40 h of batch fermentation at aeration rate of 4 L/min, agitation speed of 500 rpm and inoculum size of 1%. The highest total activity of protease during the course of present studies was approximately 103 unit/mL. For the partitioning experiments, we used the culture broth of 40 h for investigation.

Influences of some factors on partition behavior of protease in PEG/potassium phosphate ATPSs

In the present work, in order to select the ATPS in which the protease and others concentrate in opposite phases, the partition behavior was studied using systems comprising a stock solution of protease from recombinant *E. coli*. The influences of some factors on the partition were investigated and protease shown high relative affinity for the PEG-rich phase.

As shown in table 2, protease in the nearly ATPSs concentrated in top phase with the $K_{protease}$ ranging from 1.06 to 5.04. The PEG, potassium phosphate, incubation time and temperature, and pH affected on the partitioning of protease significantly. The $K_{protease}$ remained at lowest value of 0.77 in 40% PEG2000/30% potassium phosphate system at pH 7 and room temperature with incubation time of 30 min. Whereas the $K_{protease}$ reached its highest value of 5.04 in 35% PEG6000/30% potassium phosphate system at pH 6.5 and temperature of 30^{o}C with incubation time of 25 min.

The partitioning yield of protease depends on two parameters: V_B/V_T and $K_{protease}$. In most of the ATPSs investigated, protease partitioned more towards the top phase ($K_{protease}>1$), and the volume values, V_T, were usually large. 35% PEG6000/30% potassium phosphate system yields the highest protease (94.93%) with partitioning coefficient of 4.69 and volume ratio (V) of 13. The protease specific activity and purification fold of top phase increased up 694.61 unit/mg protein and 1.7 in the same system, respectively (Table 2).

Banik and Prakash [5] used 13.71% PEG4000/12.12% potassium phosphate system to purify alkaline protease from *B. cereus* MTCC 3105. The results shown that enzyme activity of top phase reached a highest value of 708.56 unit/mL with a partitioning coefficient of 4.35 and a volume ratio of 1.28. Wongmongkol and Prichanont [4] found the most suitable ATPS compositions for partition of alkaline protease from *B. subtilis* NS99 were 18% PEG1000 (w/w), 13.01% potassium phosphate (w/w) under pH 9.5, temperature of 30±2^{o}C, and without NaCl addition. Partitioning coefficient and yield obtained were 20, and 95.1%, respectively. A study was carried out by Peričin et al [6] for the partition and purification of aspergillopepsin I, an acid protease, from *Aspergillus niger*. Purification of protease was attained with a purification factor (PF) of over 5 and partitioning yield of over 99% in a 17.3% PEG4000 (w/w)/15% NaH_2PO_4 (w/w) and 8.75% NaCl (w/w) system. Sarangi et al [8] shown that for efficient separation of alkaline proteases from chicken intestine, an ATPS of 15% PEG6000 and 15% sodium citrate, initial protein concentration of 1-1.5 mg/mL in the system, a temperature of 20°C, with a pH of 8-9 was more suitable.

Aqueous two-phase back extraction

As the PEG-protease mixture in the top phase was unable to separate from each other, the utilization and further purification of the protease extracted in the top phase was difficult. After the aqueous two-phase extraction (primary extraction), different methods such as ultrafiltration or chromatography were used to remove the PEG from the top phase and to purify the enzyme. However, the back extraction by the ATPS has been known to be the most favorable method. It could facilitate further treatment of protease since protease is separated from the PEG-rich top phase [14]. In this work, protease was efficiently purified from the top phase when the top phase was mixed with 35% potassium phosphate buffer and 3% potassium chloride. The purification fold increased to 2.38 and specific activity of protease reached 983.04 unit/mg protein by the back extraction step (Table 3). Table 4 summarizes the overall purification folds and recovery yields of protease during the present aqueous two-phase separation process.

In a previous report, we investigated the aqueous two-phase back extraction of α-amylase from *B. subtilis* C10 in the PEG/potassium phosphate system, shown that the increase of the purification fold (3.56) with a recover yield of 59.37% [15]. The high purification fold of enzyme suggests that this method may be more cost-effective than the other conventional means. Furthermore, industries are strongly urged to consider application of an ATPS to purifying biological materials.

Table 1. Concentrations of the phase-forming PEG/potassium phosphate in the aqueous two-phase systems

System designation	PEG (%)			Potassium phosphate (%)
	MW 2000	MW 6000	MW 10000	
P1	45	-	-	25
P2	50	-	-	25
P3	45	-	-	30
P4	50	-	-	30
P5	-	35	-	25
P6	-	40	-	25
P7	-	35	-	30
P8	-	40	-	30
P9	-	-	25	25
P10	-	-	30	25
P11	-	-	25	30
P12	-	-	30	30

MW: molecular weight

Table 2. Effect of some factors on *V*, $K_{protease}$, $Y_{protease}$, $SA_{protease\ T}$ and purification fold in primary extraction

Factors	*V*	$K_{protease}$	$Y_{protease}$ (%)	$SA_{protease\ T}$ (unit/mg)	Purification fold
PEG (MW)					
2000	0.83	0.77^{c}	24.79^{c}	153.93^{c}	0.37^{c}
6000	1.44	1.24^{a}	45.83^{a}	251.95^{a}	0.61^{a}
10000	0.56	1.06^{b}	36.69^{b}	205.47^{b}	0.50^{b}
Incubation time (min)					
20	1.15	0.97^{b}	37.34^{c}	204.51^{c}	0.50^{c}
25	1.12	1.69^{a}	55.17^{a}	332.85^{a}	0.81^{a}
30	1.15	1.57^{a}	50.86^{b}	299.51^{b}	0.73^{b}
Temperature (°C)					
20	2.26	1.98^{cd}	64.75^{b}	405.02^{c}	0.98^{c}
25	2.11	2.09^{c}	65.05^{b}	409.47^{c}	0.99^{c}
30	2.50	2.48^{a}	68.85^{ab}	494.53^{a}	1.20^{a}
35	2.68	2.30^{b}	70.79^{a}	450.34^{b}	1.09^{b}
PEG (%)					
30	-	-	-	-	-
35	3.67	4.15^{a}	84.69^{a}	517.94^{a}	1.26^{a}
40	3.00	3.40^{ab}	79.39^{bc}	430.66^{bc}	1.04^{dc}
45	3.24	2.43^{b}	80.58^{b}	454.98^{b}	1.01^{b}
Potassium phosphate (%)					
20	3.67	3.33^{c}	77.79^{c}	490.36^{c}	1.19^{c}
25	4.60	3.77^{b}	81.10^{b}	537.94^{b}	1.30^{b}
30	5.09	4.01^{a}	87.22^{a}	613.61^{a}	1.49^{a}
pH					
6.0	3.67	5.04^{a}	93.51^{b}	674.66^{b}	1.64^{a}
6.5	13.00	4.69^{b}	94.93^{a}	694.61^{a}	1.70^{a}
7.0	3.38	3.89^{c}	83.84^{c}	571.29^{dc}	1.39^{bc}

Different letters in the column for each factor indicate significantly different means using Duncan's test ($p<0.05$). $SA_{protease\ T}$: specific activity of protease in top phase.

Substrate-SDS-PAGE of protease

The protease activity was also confirmed by SDS-PAGE with casein as a substrate. A protease band clearly occurred in the dark colored gel after staining. The molecular weight of purified enzyme was found to be approximately 42 kDa (Fig 2).

Table 3. Effect of some factors on 1/*V*, $K_{protease}$, $Y_{protease}$, $SA_{protease\ B}$ and purification fold in back extraction

Factors	1/*V*	$K_{protease}$	$Y_{protease}$ (%)	$SA_{protease\ B}$ (unit/mg)	Purification fold
Potassium phosphate (%)					
30	2.03	6.97^{a}	69.11^{b}	764.85^{c}	1.86^{c}
35	2.57	6.04^{b}	76.29^{a}	974.32^{a}	2.36^{a}
40	3.00	5.61^{b}	64.36^{c}	838.87^{b}	2.03^{b}
Potassium chloride (%)					
3	3.00	7.64^{a}	71.72^{a}	983.04^{a}	2.38^{a}
6	3.00	5.17^{b}	63.13^{b}	863.66^{b}	2.04^{b}
9	1.86	4.67^{bc}	60.83^{b}	772.12^{c}	1.87^{c}

$SA_{protease\ B}$: specific activity of protease in bottom phase.

Table 4. Summary of purification of protease from *E. coli*

Steps	Total activity (unit/mL)	Specific activity (unit/mg)	Purification fold	Recovery yield (%)
Culture filtrate	103.25	440.44	1	100
Primary extraction	83.35	694.61	1.70	80.73
Back extraction	78.64	983.04	2.38	76.17

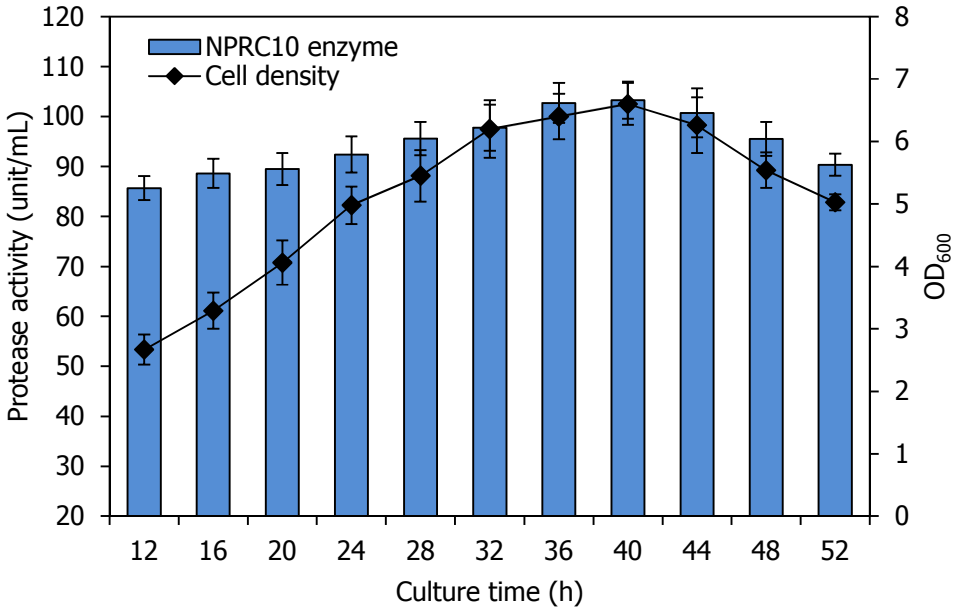

Figure 1. Profile on the growth and protease production of recombinant *E. coli* BL21 (DE3) in batch culture

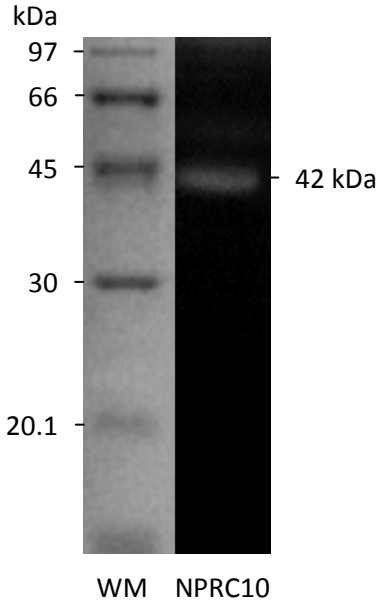

Figure 2. SDS-PAGE (12%) with 0.1% casein. Lanes: WM-protein weight markers (97-14.4 kDa), NPRC10-recombinant neutral protease sample purified by back extraction.

CONCLUSION

In the present study, we have found an optimal ATPS (35% PEG6000/30% potassium phosphate) and an appropriate back-extraction procedure for purification process of protease NPRC10 from recombinant *E. coli* strain BL21 (DE3).

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