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Partitioning and purification of extracellular β-1,3-1,4-glucanase in aqueous two-phase systems*

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Abstract: The partition behaviors of β-1,3-1,4-glucanase, α-amylase and neutral proteases from clarified and whole fermentation broths of *Bacillus subtilis* ZJF-1A5 were investigated. An aqueous two-phase system (polyethylene glycol (PEG)/$MgSO_4$) was examined with regard to the effects of PEG molecular weight (MW) and concentration, $MgSO_4$ concentration, pH and NaCl concentration on enzyme partition and extraction. The MW and concentration of PEG were found to have significant effects on enzyme partition and extraction with low MW PEG showing the greatest benefit in the partition and extraction of β-glucanase with the PEG/$MgSO_4$ system. $MgSO_4$ concentration influenced the partition and extraction of β-glucanase significantly. pH had little effect on β-glucanase or proteases partition but affected α-amylase partition when pH was over 7.0. The addition of NaCl had little effect on the partition behavior of β-glucanase but had very significant effects on the partitioning of α-amylase and on the neutral proteases. The partition behaviors of β-glucanase, α-amylase and proteases in whole broth were also investigated and results were similar to those obtained with clarified fermentation broth. A two-step process for purifying β-glucanase was developed, which achieved β-glucanase recovery of 65.3% and specific activity of 14027 U/mg, 6.6 times improvement over the whole broth.

Key words: Aqueous two-phase system, Partition, β-1,3-1,4-glucanase
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INTRODUCTION

β-1,3-1,4-glucan is the major cell wall component of barley and other cereal endosperms. It consists of a polymer of glucose monomers joined by β-1,3- and β-1,4-glycosidic bonds in an irregular fashion (Woodward *et al*., 1983; Edney *et al*., 1991). Due to its unique structure, barley β-glucan can be dissolved in water in high molecular mass and resulting in a high viscosity solution. The high-molecular-mass β-glucan released during mashing causes severe filtration problems and may lead to gelatinous precipitates in the resulting beer. The use of barley varieties of high β-glucan content, the addition of adjuncts or the use of under modified malt can result in beer with troublesome levels of high molecular weight β-glucan. Barley β-glucan can also cause high intestinal viscosity that affects the digestibility of feed and absorption of nutrients by chicks and piglets fed barley (Planas, 2000). The addition of exogenous enzymes during mashing or feed preparation can reduce the negative effects of barley β-glucan (Stone and Clarke, 1992; Godfrey and Reichely, 1983; White *et al*., 1983). Some bacteria and fungi can produce β-glucanase that has the same substrate specificity as malt β-glucanase and, thus, the ability to hydrolyze barley β-glucan specifically. And β-1,3-1,4-glucanase produced by *Bacillus* has great industrial potential because of its higher thermal stability.

Generally, industry uses crude raw materials to produce β-glucanase from *Bacillus*. The high viscos-

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ity and solid mass content of the resulting broth, and the simultaneous production of α-amylase and neutral proteases, cause difficulties in selectively purifying enzymes using conventional techniques, such as filtration and centrifugation. It is also difficult to scale-up these techniques because of particulate materials in particular bacteria, compressible filter cakes and viscous slurries (Schmidt *et al*., 1994).

Partitioning in aqueous two-phase systems (ATPS) has been shown to provide a powerful method for separating and purifying mixtures of proteins. These systems are composed of aqueous solutions either of two water-soluble polymers, usually polyethylene glycol (PEG) and dextrin, or of a polymer and a salt, usually PEG and phosphate or sulfate. Some new systems have recently been developed which, for example, rely on a system of surfactant and surfactant or polymer and surfactant (Xiao *et al*., 2001). Compared with other commonly used separation and purification techniques, ATPS has a number of advantages, such as ease of scale-up, the ability to handle particulate materials and the ability to process streams continuously. ATPS can remove undesirable by-products in crude supernatants, for example, undefined polysaccharides and pigments and interfering proteins that lower the activity of the enzyme. This makes ATPS a potentially attractive technique for separation and purification of industrial enzymes, extracellular as well as intracellular. There are many factors influencing the partitioning in ATPS. Differences in partitioning result from the interaction of factors inherent in the system itself, such as choice of system components, polymer molecular weight, concentration of polymers and salts, ionic strength, and pH, and those of the target protein, such as hydrophobicity, charge and molecular weight. The causative mechanisms of uneven biomolecules distribution are poorly understood. Fundamental theories of protein partition derived from classical polymer solution thermodynamics are being developed. Although the present models provide some information, no comprehensive theory currently exists to guide the design of systems for separation of specific mixtures of proteins and particles. Therefore application of the technique requires experimentation to design an adequate phase system for optimal partitioning of a particular protein. The success of a technique can be monitored with the use of partition coefficient K, the ratio of biomolecule concentration in the top phase to that in the bottom phase.

ATPS has been successfully used to purify extracellular enzymes, for example, xylanase (Jain and Johri, 1999), β-xylosidase (Pan and Li, 2001), β-glucosidase (Johansson and Reczey, 1998), α-amylase (Planas, 2000; Jiao *et al*., 1998). There is no report for purification of β-glucanase of *Bacillus* with ATPS. Schmidt *et al*.(1994) studied the partition behavior of pure α-amylase and α-amylase from supernatant and whole broth of *Bacillus subtilis* fermentation in PEG/dextrin, PEG/phosphate, and PEG/sulfate systems, but did not investigate the partition behavior of the β-glucanase and proteases. Jiao *et al*.(1998) studied the purification of α-amylase and proteases from supernatant of *Bacillus* fermentation in PEG/sulfate system but did not mention β-glucanase activity.

The main purpose of this paper was to study the partition behaviors of β-glucanase, α-amylase and neutral proteases in a PEG and $MgSO_4$ aqueous two-phase system and to investigate the effects of PEG molecular weight and concentration, pH, $MgSO_4$ concentration and NaCl concentration on extraction of enzymes from clarified or whole fermentation broth.

MATERIALS AND METHODS

Bacillus subtilis ZJF-1A5 was a wild strain isolated from soil that was mutated in our Lab with UV and diethyl sulfate.

Chemicals

PEG with average MW of 2000, 4000 or 6000 was obtained from Pudong Gaonan Chemicals Ltd. Shanghai, China. Lichenin was purchased from Sigma Corporation of America and used as substrate for the β-glucanase assay. All other salts and chemicals used were of analytical grade.

Crude enzyme preparation

The medium used for seed culture contained: dextrin, 20 g/L; yeast extract, 20 g/L; KH_2PO_4, 1.0 g/L; $MgSO_4\cdot 7H_2O$, 0.1 g/L; $CaCl_2$, 0.1 g/L. The medium used for enzyme production contained: barley flour, 63.5 g/L; corn flour, 44.8 g/L; KH_2PO_4, 1.0 g/L;

$MgSO_4·7H_2O$, 0.1 g/L; $CaCl_2$, 0.1 g/L. *Bacillus subtilis* ZJF-1A5 was activated at the first preculture with 10 ml medium in a 100 ml culture flask in a 200 r/min rotary shaker at 37 °C. After 18 h cultivation, the first culture broth was divided and inoculated (4%) into 30 ml seed culture medium in 250 ml culture flask. The flasks were shaken at 200 r/min for 18 h at 37 °C. The seed culture was inoculated (3.82%) into 5 L fermentor (B. Braun) containing 3 L medium at 500 r/min, 37 °C, and 1 L/(L·min) for 40 h. After fermentation, the culture broth was centrifuged at 1500 g for 15 min. Cells and insoluble components were discarded. The supernatant was used as a crude enzyme solution.

Enzyme assays

β-glucanase: Enzyme preparation (100 μl), diluted with phosphate buffer (0.2 mol/L, pH 6.0), was incubated at 50 °C for 10 min with 0.9 ml 0.2% (*w*/*V*) lichenin in the same buffer. Reducing sugars were estimated as described by Miller (1959). One unit of β-glucanase activity (U) was defined as the amount of enzyme required to form 1 μmol reducing sugar (glucose as standard) per minute.

α-amylase (Syu and Chen, 1997, but modified): 1.2% buffered starch solution was used as the substrate for the assay of α-amylase activity. The reagent was I_2-KI solution (0.5%:5%). One millilitre phosphate buffer (0.2 mol/L, pH 6.0) was added into 2 ml of 1.2% (*w*/*V*) starch solution and the mixture was incubated in 40 °C water bath for 5 min. One millilitre sampled enzyme solution was added into the starch solution, and then incubated at 40 °C for 30 min. After that, 10 ml of 0.5 mol/L acetic acid was added to stop the reaction. One millilitre of such solution was mixed with 10 ml I_2-KI reagent and the absorbance of the solution was measured at wavelength of 660 nm. The α-amylase activity was measured by its dextrinizing power (D.P.). α-amylase unit (1 D.P.) is defined as the amount of amylase which will produce 10 percent decrease in the intensity of amylase's blue color under the conditions mentioned above.

Neutral proteases (Han and Lee, 1997, but modified): the substrate was 0.6% (*w*/*V*) casein in Tris-HCl (0.05 mol/L, pH 7.5). Enzymatic hydrolysis was initiated by addition of 1 ml properly diluted enzyme solution in 5 ml substrate. After 5 min incubation at 40 °C, 5 ml of 0.4 mol/L trichloroacetic acid (TCA) 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 275 nm. One unit of proteases activity is based on the tyrosine (per μg tyrosine enzyme per min).

Protein determination

Protein content was determined by the Bradford method using bovine serum albumin (BSA) as a standard (Zhang *et al*., 1997). Blank systems, without protein, were used as reference, and no interference from phase components was observed.

Aqueous two-phase extraction

Phase systems were prepared from stock solutions of PEG (50%, *w*/*w*) and $MgSO_4$ (20%, *w*/*w*). The pH was set using NaOH or HCl. Stock solutions were stored at 4 °C. Before use, the temperature of all stock solutions was equilibrated to room temperature. Low-speed centrifugation (1000 g) was used to speed up phase separation after thorough but gentle mixing of the system components. The phase volume ratios were determined in graduated centrifuge tubes. Samples of the top and bottom phases were then assayed for activities of enzymes and total protein. All partition experiments were done at room temperature.

RESULTS AND DISCUSSION

Effect of PEG molecular weight on enzymes partitioning

The phase volume ratio (*R*=volume of top phase/volume of bottom phase) decreased with increasing PEG MW. The partitioning of total protein and enzymes depended on the PEG MW. Partition coefficient (*K*=concentration in top phase/concentration in bottom phase) and extraction rate (*E*=the total enzyme activity in top phase/the total enzyme activity in system) of total protein increased as PEG molecular weight increased from 2000 to 6000 (Table 1). The partitioning and extraction of β-glucanase in PEG and $MgSO_4$ systems was strongly depended on the PEG MW. Lower PEG MW led to higher partition coefficients and extraction rates, the difference being especially significant when the PEG concentration increased (results not shown). While PEG MW had

less effect on the partitioning and extraction of the α-amylase and neutral proteases, most of the α-amylase and proteases was partitioned to the bottom phase. High MW PEG would not be a reasonable choice for further investigation, as all enzymes had low K values and extraction rates at high PEG MW, which would lead to very poor separation and purification of enzymes. Therefore, low MW PEG (PEG 2000) was chosen as phase component for further studies.

Effects of PEG 2000 concentration on enzymes partitioning

Results (Fig.1) showed that increasing PEG 2000 concentration caused an increase (2.30-fold) of the partition coefficient for β-glucanase (from K=0.86 to K=2.84), and the extraction rate from 26.84% to 75.66%. Increasing PEG 2000 concentration had no significant effects on partition coefficients or extraction rates for α-amylase or neutral proteases, and so PEG 2000 offers a convenient method for separating β-glucanase from α-amylase and neutral proteases. Increasing the PEG concentration to 20% (w/w) caused a 2.30-fold increase in the partition coefficient for β-glucanase, although extraction rate and partition coefficient reached a relatively constant level at a PEG concentration of 16% (w/w). Therefore, the PEG 2000 concentration should be kept at 16%.

Effect of $MgSO_4$ concentration on enzymes partitioning

The effects of $MgSO_4$ concentration on enzyme partitioning and extraction are shown in Table 2. In the PEG 2000/$MgSO_4$ system, although volume ratios decreased as $MgSO_4$ concentration increased, K value and extraction rate of β-glucanase, α-amylase and neutral proteases all increased. But the α-amylase transferred to the bottom phase and extraction rate was only 15.47% when the $MgSO_4$ concentration was 16%. Additionally, when the $MgSO_4$ concentration was lower than 14%, the E value and K value of neutral proteases had no significant effect (E_p varies

Table 1 The effects of PEG molecular weight on phase ratio, extraction and partitioning of β-glucanase, α-amylase and neutral protease with 16% PEG and 10% $MgSO_4$ at pH 7.0 and (22±2) °C

PEG MW	R	K_e	E_e (%)	K_g	E_g (%)	K_a	E_a (%)	K_p	E_p (%)
2000	1.160	0.550	38.79	1.44	73.94	0.157	15.36	0.27	24.02
4000	0.975	0.660	40.49	0.44	27.70	0.525	31.48	0.22	14.36
6000	0.915	0.802	42.32	0.26	19.40	0.219	16.66	0.27	20.00

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral protease respectively; R represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficients of total protein, β-glucanase, α-amylase and neutral protease respectively

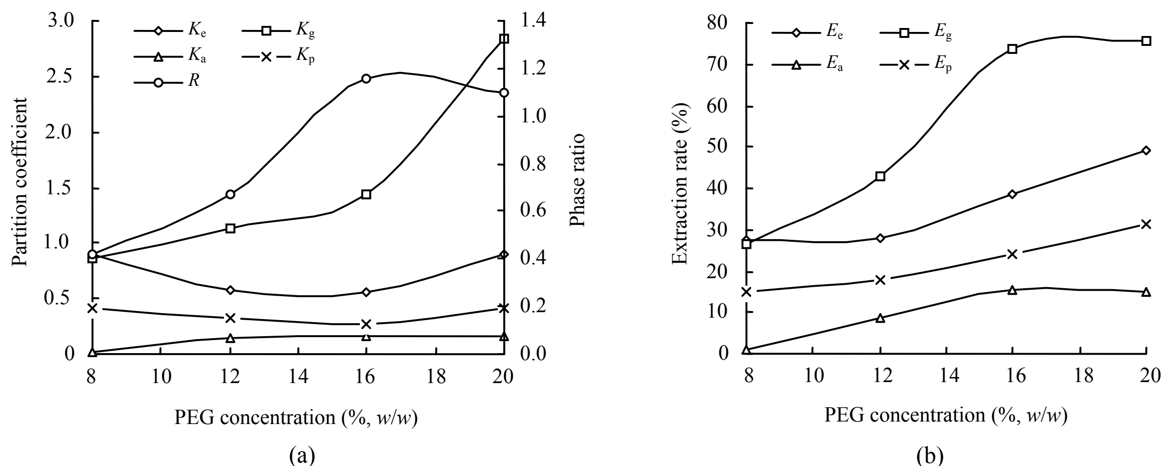

Fig.1 The effects of PEG 2000 concentration on (a) phase ratio, partitioning and (b) extraction of β-glucanase, α-amylase and neutral proteases with 10% $MgSO_4$ at pH 7.0 and temperature of (22±2) °C

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral proteases respectively; R represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficient of total protein, β-glucanase, α-amylase and neutral proteases respectively

from 22.56 % to 29.55 %, K_p varies from 0.29 to 0.64), but an increase of $MgSO_4$ concentration from 14% to 16% significantly increased the partition coefficient of neutral proteases from 0.64 to 3.35, and the extraction rate from 29.55% to 72.40%. These results indicated that increasing $MgSO_4$ concentration could increase the extraction rate of β-glucanase, and that the α-amylase extraction rate increased a little. But when the $MgSO_4$ concentration was more than 14%, a lot of neutral proteases were extracted into the top phase, which was detrimental to the purification of β-glucanase. Therefore, an $MgSO_4$ concentration of 14% was chosen for all further investigations.

Effect of pH on the enzymes partitioning

Results shown in Table 3 show that *R* was independent of pH in the PEG 2000/$MgSO_4$ system. As pH increased from 4.3 to 9.0, the partition coefficients and the extraction rates of β-glucanase and neutral proteases changed little. However, *K* values and extraction rates for α-amylase increased as pH increased. The differences could be explained by the fact that the isoelectric points (pI) of β-glucanase and neutral proteases of *Bacillus* are basic, whereas, pI of α-amylase is acidic (Han and Lee, 1997; Huddleston and Veide, 1991; Schmidt *et al*., 1994). As a general rule, negatively charged proteins partition to the top phase and positively charged proteins to the bottom phase. At higher pH, the protein was more negatively charged than at low pH, and therefore, interactions between α-amylase and PEG units became stronger. The result indicated that the pH 7.0 PEG 2000/$MgSO_4$ systems should be chosen to separate the β-glucanase from the other two enzymes.

Effect of NaCl concentration on the enzymes partitioning

Generally the addition of neutral salts, such as NaCl, affects partitioning in ATPS by speeding up phase separation, by influencing the phase potential or by decreasing protein hydrophobicity. Results shown in Fig.2 show that NaCl concentration in the PEG 2000/$MgSO_4$ system had little effect on β-glucanase partition and extraction, although, it did change slightly the partition coefficient and extraction rate when the concentration of NaCl increased from 0 to 10%. However, the NaCl concentration did have significant effects on the partitioning and extraction of α-amylase and neutral proteases. Partition coefficient and extraction rate for the neutral proteases increased very rapidly when 1% (*w*/*w*) NaCl was added. The extraction rate reached 97.1% when NaCl

Table 2 The effect of $MgSO_4$ concentration on phase ratio, extraction and partition of β-glucanase, α-amylase and neutral proteases at pH 7.0 and temperature of (22±2) °C

$MgSO_4$ (%, *w*/*w*)	*R*	K_e	E_e (%)	K_g	E_g (%)	K_a	E_a (%)	K_p	E_p (%)
8	1.32	0.57	43.05	1.23	61.76	0.0079	1.04	0.29	27.93
10	1.16	0.77	38.26	1.68	57.32	0.0446	3.45	0.36	22.56
12	1.00	0.89	46.96	2.47	71.14	0.1050	9.54	0.41	29.26
14	0.65	1.16	43.06	4.00	72.34	0.1190	6.44	0.64	29.55
16	0.78	1.94	60.24	5.45	81.01	0.2340	15.47	3.35	72.40

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral proteases respectively; *R* represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficient of total protein, β-glucanase, α-amylase and neutral proteases respectively

Table 3 The effects of pH on phase ratio, extraction and partition of β-glucanase, α-amylase and neutral proteases at temperature of (22±2) °C

pH	*R*	K_e	E_e (%)	K_g	E_g (%)	K_a	E_a (%)	K_p	E_p (%)
4.3	0.73	1.58	53.48	3.60	72.31	0.14	14.16	0.50	34.58
5.0	0.73	1.63	56.03	4.45	71.70	0.16	15.82	0.48	26.73
6.0	0.77	0.96	42.43	5.05	79.56	0.13	15.07	0.54	27.18
7.0	0.72	1.17	45.82	5.07	77.46	0.13	13.70	0.54	27.89
8.0	0.75	0.58	30.43	5.00	78.95	0.23	14.65	0.82	28.71
9.0	0.72	1.07	55.75	5.28	79.17	0.27	24.82	0.73	36.40

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral proteases respectively; *R* represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficient of total protein, β-glucanase, α-amylase and neutral proteases respectively

concentration was increased to 2% (*w*/*w*). At these concentrations, NaCl had only slight effects on α-amylase partition coefficient and extraction rate. When the NaCl concentration reached 5% (*w*/*w*), however, partition coefficients and extraction rates of α-amylase increased significantly and when the NaCl concentration was over 8%, the extraction rate of α-amylase exceeded 80%. This could have been caused by the different partitioning of Na^+ and Cl^- ions between top and bottom phase resulting in a increase of potential difference between the two phases. As a result, adjustments of the NaCl concentration in PEG 2000/$MgSO_4$ system can be used to efficiently separate the three enzymes studied.

Partitioning of enzymes from whole broth

The PEG 2000/$MgSO_4$ system was used with whole broth to purify β-glucanase, α-amylase and neutral proteases and to separate typical *B. subtilis* contaminants. Results in Table 4 show that the partition behavior of enzymes in whole broth was superior to that obtained from clarified broth. Most of the β-glucanase was partitioned to the top phase (Extraction rate reached 79.6%) and almost all of the α-amylase (>99%) and most of the neutral proteases (>80%) was partitioned to the bottom phase of whole broth. The specific activity of β-glucanase was 4903 U/mg, 2.66 times that in unpartitioned whole broth. Cells and solid mass were absorbed to the interface but were easily removed by centrifugation.

Purify β-glucanase in two steps

The PEG 2000/$MgSO_4$ system is extremely attractive for purifying β-glucanase in two steps. In the first step, a system with 16% PEG 2000 and 14% $MgSO_4$ was used, where most of β-glucanase (72.0%) partitioned to the top phase along with some neutral proteases and other contaminants. Almost all of the α-amylase (>99%) and some of the neutral proteases (>70.00%) partitioned to the bottom phase. Cell debris and other contaminants partitioned to the bottom and absorbed to the interface. The top phase was then repartitioned (back extraction) by adding magnesium sulfate and water. When the $MgSO_4$ concentration was

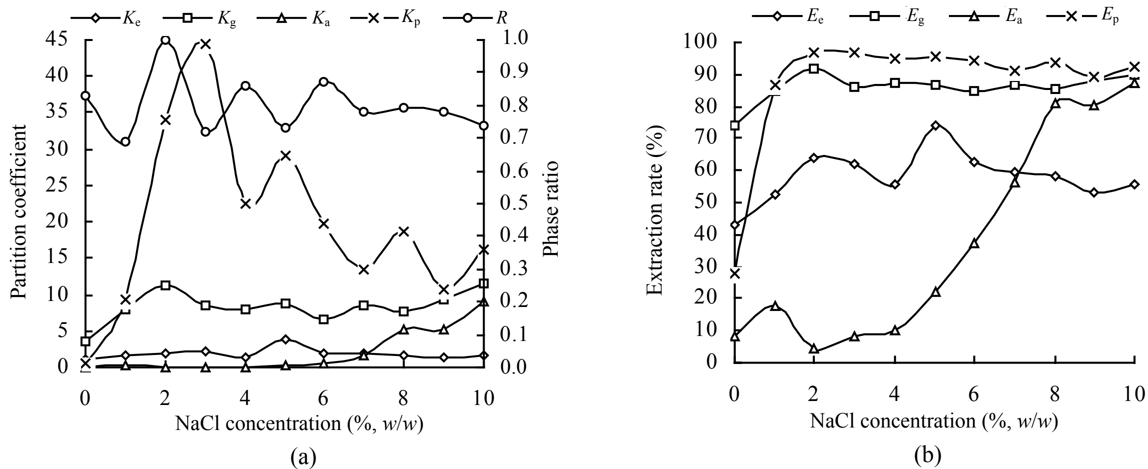

Fig.2 The effect of NaCl concentration on (a) phase ratio, partitioning and (b) extraction of β-glucanase, α-amylase and neutral proteases at pH 7.0 and temperature of (22±2) °C

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral proteases respectively; R represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficient of total protein, β-glucanase, α-amylase and neutral proteases respectively

Table 4 Comparison of partition behaviors of β-glucanase, α-amylase and neutral proteases in clarified and whole broth at pH 7.0 and temperature of (22±2) °C

	R	K_e	E_e (%)	K_g	E_g (%)	K_a	E_a (%)	K_p	E_p (%)
Clarified broth	0.83	0.84	41.03	3.22	72.76	0.196	14.01	0.72	37.28
Whole broth	0.72	0.39	22.08	2.80	79.55	0.047	3.25	0.33	18.95

E_e, E_g, E_a and E_p represent the extraction rate of total protein, β-glucanase, α-amylase and neutral proteases respectively; R represents phase ratio; K_e, K_g, K_a and K_p represent the partition coefficients of total protein, β-glucanase, α-amylase and neutral proteases respectively

increased to 14.67%, most of the β-glucanase (82.20%) in the top phase was salted-out and precipitated in the bottom phase. β-glucanase recovery rate and specific activity was 65.34% and 14027 U/mg respectively, 6.6 times higher than that in whole broth.

CONCLUSION

Based on the experimental results above, it can be concluded that: in PEG/$MgSO_4$ system, low MW PEG (PEG 2000) is beneficial for purification of β-glucanase; 16% PEG can be used to achieve a high extraction rate of β-glucanase and significantly decrease the proportion of α-amylase and neutral proteases in the upper phase. $MgSO_4$ concentration should be maintained at 14% to increase the extraction rate of β-glucanase and decrease the contamination by neutral proteases. In order to separate the β-glucanase from the other two enzymes, the pH 7.0, PEG 2000/$MgSO_4$ system should be used. It was considered that the hydrophobicity difference of the three enzymes caused the differences of NaCl concentration required for the extraction in the upper phase. So adjustment of NaCl concentration in PEG 2000/$MgSO_4$ system is an efficient way to separate the three enzymes. Partition behaviors of β-glucanase, α-amylase and protease in whole broth are similar to those in supernatant based on the experimental results. Most of the β-glucanase is partitioned to the top phase (Extraction rate reached 79.6%) and almost all of the α-amylase (>99%) and most of the neutral proteases (>80%) was partitioned to the bottom phase. The two-step process for purifying β-glucanase was established, and β-glucanase recovery was 65.34% and specific activity of β-glucanase was 14027 U/mg. These results indicated that this ATPS provided mild aqueous environments for biological molecules separation. In industrial process crude raw materials are generally adopted to produce β-glucanase from *Bacillus*. The high viscosity, solid mass content and different enzymes produced at the same time cause difficulties in selectively purifying enzymes using conventional techniques. ATPS is a potentially attractive and efficient technique for resolving the difficulties in separating and purifying β-glucanase enzymes.

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