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Effective extraction and purification of β-xylosidase from *Trichoderma koningii* fermentation culture by aqueous two-phase partitioning

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Abstract

Effective extraction of protein from bulk medium is an important technique in bioresearch. In the present study, we describe an extracellular β-xylosidase from the fermentation supernatant of *Trichoderma koningii* G-39 that was successfully extracted and purified simultaneously in a single step by using an aqueous two-phase partitioning method. This two-phase system was prepared by dissolving suitable amount of poly(ethylene glycol) (PEG) and sodium dihydrogenphosphate (NaH_2PO_4) in aqueous solution. β-Xylosidase was recovered with high yield and high concentration in the bottom salt-rich phase when 25% (w/v) PEG 1500 and 20–25% (w/v) NaH_2PO_4 were applied. Based on a 1-liter scale extraction, the purity of the enzyme was enhanced at least 33-fold. The total activity increased 422% in comparison with that in the untreated filtrate. The effectiveness and simplicity may make this technique potentially useful in various applications. The transxylosylation activity of the enzyme purified by this technique was also investigated.

Keywords: β-xylosidase, aqueous two-phase system, purification, partitioning

1. Introduction

β-Xylosidases (EC 3.2.1.37) catalyzes the hydrolysis of xylooligosaccharides and xylobiose from the non-reducing end to release xylose. It is considered to relieve the product inhibition of xylanase. Many β-xylosidase have been purified from fungi [1–7] and bacteria [8–11]. One feature of the fungal β-xylosidase is their transxylosylation activity, which is particularly useful for preparation of β-xylosides. Owing to the biological importance, β-xylosides have received a great deal of attention. For instance, alkyl β-xylosides have been shown to act as primers for chondroitin sulfate synthesis [12]. They can be prepared by organic synthesis [13] or, more efficiently, by the application of the transxylosylation of β-xylosidase [14–19]. However, the purification of β-xylosidases from various sources frequently involves tedious chromatographic steps so that large scale preparation of the enzyme with high purity is often not commercially available. For the classical protein purification, a large amount of ammonium sulfate is added in the culture filtrate to precipitate crude protein. Alternatively, organic solvents such as alcohols are often used to reduce the polarity of the aqueous media, which leads to the precipitation of proteins. Chromatography will then be applied to enhance the purity of the enzyme of interest. Besides that, differential extraction between aqueous and organic phases is another method reported for protein purification [20,21]. Yet, the organic systems are occasionally incompatible with the conformational stability of proteins. Aqueous two-phase systems have been designed to improve this weakness in terms of the purification of biomolecules [22–26]. Two phases can be formed when appropriate amounts of a polymer (eg., PEG) and a salt are mixed in aqueous solution. Selective partitioning of protein in two phases is the key point of this technique. The feasibility of this technique in the purification of β-galactosidase has been evaluated [27–31].

Recently, we reported that a β-xylosidase was effectively induced from *Trichoderma koningii* [32]. The enzyme is somewhat heat resistant (stable at 60°C for several hours) and possesses a powerful transxylosylation characteristic. It is potentially useful in industry. In this paper, we provide a successful example on the purification of β-xylosidase from *Trichoderma koningii* fermentation culture by a rapid liq-

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Table 1
Effects of phase composition on the recovery of β-xylosidase

Phase composition		Partition Coefficient, K	Partition ratio of β-xylosidase in bottom phase, Y (%)	Specific activity of β-xylosidase in bottom phase, SA_b (U/mg)	Purification factor of β-xylosidase in bottom phase, PF
Salts [20%(w/v)]	PEG 1500 [%(w/v)]				
$MgSO_4$	5	—	—	—	—
	10	0.278	94.7	117.5	7.4
	15	0.070	97.3	129.9	8.2
	20	0.045	97.4	144.4	9.1
	25	0.049	96.6	201.9	12.8
	30	0.046	94.9	237.0	15.0
$(NH_4)_2SO_4$	5	—	—	—	—
	10	1.515	76.8	14.1	0.89
	15	0.957	67.9	14.6	0.92
	20	0.629	72.5	16.4	1.04
	25	0.850	62.4	13.3	0.84
	30	0.524	62.2	15.5	0.98
NaH_2PO_4	5	—	—	—	—
	10	—	—	—	—
	15	—	—	—	—
	20	—	—	—	—
	25	0.011	96.3	254	16.1
	30	0.014	96.7	211	13.4
Na_2HPO_4	5	—	—	—	—
	10	—	—	—	—
	15	—	—	—	—
	20	—	—	—	—
	25	0.037	90.8	83.2	5.3
	30	0.050	85.8	73.2	4.6
KH_2PO_4	5	—	—	—	—
	10	—	—	—	—
	15	—	—	—	—
	20	—	—	—	—
	25	—	—	—	—
	30	—	—	—	—

—, No phase separation

uid-liquid (aqueous two-phase) extraction system. For 1-liter scale manipulation, the enzyme can be obtained within 1 h.

2. Materials and methods

2.1. Materials

PEG 200, 300, 400, 1500, 6000, 8000, 10000 were obtained from Merck. *Trichoderma koningii* G-39, a gift generously supplied by prof. T-H. Hseu from the National Tsing Hua University, Hsinchu, Taiwan, is a mutated strain derived from the wild strain W-10 by UV mutagenesis. 2,4-Dinitrophenyl-β-D-xylopyranoside was synthesized according to the method of Sharma et al. [33]. The salts and other chemicals used were of analytical grade.

2.2. Cultivation and cell harvest

The basal medium used for growth and enzyme induction was MRE medium containing (per liter) KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1.4 g; $CaCl_2$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnCl_2 \cdot 4H_2O$, 1.6 mg; $ZnSO_4 \cdot 7H_2O$, 4 mg; $CoCl_2 \cdot 6H_2O$, 3.7 mg; peptone, 1 g; urea, 0.3 g; Tween 80, 1 ml. Spores for inoculation were obtained by culturing at 28°C in Petri dishes, each containing 15 ml MRE with 3.9% potato dextrose agar (PDA). After a week of incubation, the spores from four PDA plates were suspended in the basal MRE medium with 2% glucose and inoculated into a liter culture flask containing 800 ml of medium. The flask was shaken at 180 rpm for 24 h at 28°C. The seed culture was then filtered. The mycelia were washed twice with sterile water. Enzymes were then induced by adding the clean mycelia in 400 ml MRE medium containing 1% xylan and 0.1% xylose for 24 h at 28°C. After fermentation, the culture was centrifuged at 10,000 × g for 30–50 min to obtain a yellowish supernatant.

2.3. Aqueous two-phase extraction

Phase systems were prepared in a 1.5-ml microcentrifuge tube by adding appropriate amounts of PEG and NaH_2PO_4 in 1 ml of fermentation culture. The mixtures were vortexed

Table 2
Effects of PEGs on the partition of β-xylosidase in a PEG-NaH_2PO_4 system

Phase composition		Partition Coefficient, K	Partition ratio of β-xylosidase in bottom phase, Y (%)	Purification factor of β-xylosidase in bottom phase, PF
Salt	Polymer [25%(w/v)]			
	PEG 200	—	—	—
	PEG 300	—	—	—
	PEG 400	—	—	—
NaH_2PO_4 [20%(w/v)]	PEG 1500	0.011	96.3	16.1
	PEG 6000	0.069	90.7	10.1
	PEG 8000	0.064	91.2	9.1
	PEG10000	0.053	92.8	8.3

for 5–10 min and the phase was separated by centrifugation at 10,000 × g for 5 min. Phase volumes were measured. The partitioned cell debris was visually estimated from the turbidity of the two phases. β-Xylosidase was found in the bottom salt-rich phase, which was then withdrawn and desalted for activity assay and SDS-PAGE analysis.

Results were evaluated by a few parameters including: the partition coefficient, K; activity distribution ratio, G; partition ratio in the bottom phase, Y; and purification factor, PF. The parameters are defined as follows:

$K = C_t/C_b$, where C_t and C_b denote the measured activity units per ml (units/ml) in the top and the bottom phases.

$G = K(V_t/V_b)$, where V_t and V_b are the volumes of the top and the bottom phases, respectively. Therefore, G gives the ratio of the amount of enzyme in the top to in the bottom phases.

Y (%) is defined as $100C_bV_b/(C_bV_b + C_tV_t)$, equal to $100/[1 + G]$. Note that the sum of the activity in the top and the bottom phase is not necessarily equal to the activity present in the original fermentation culture.

The purification factor, PF, is defined as SA_b/SA_{ori}, where SA_b and SA_{ori} are the specific activities of β-xylosidase in the bottom phase and in the original untreated culture filtrate, respectively.

2.3.1. Enzyme assays

β-Xylosidase activity was assayed with 2,4-dinitrophenyl-β-D-xylopyranoside as substrate by determining the amount of 2,4-dinitrophenol released. One enzyme unit is defined as the amount of enzyme required for releasing 1 μmole of 2,4-dinitrophenol from substrate in 1 min. For activity assay, a suitable amount of purified protein was added in 0.5 ml acetate buffer (50 mM, pH 4.2) containing 1 mM 2,4-dinitrophenyl-β-D-xylopyranoside. The extinction coefficient of 2,4-nitrophenolate/2,4-nitropenol at 400 nm was determined to be 6100 $M^{-1}cm^{-1}$ at pH 4.2.

2.3.2. Protein determination

The protein content of enzyme preparation was determined by UV absorption at 280 nm (1 OD is assumed to correspond to 1 mg/ml).

2.3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to a standard protocol. The molecular weight standard was the broad range protein marker from NEB Co.

2.3.4. Transxylosylation activity and products analyses

^{1}H-NMR spectra in D_2O solution were obtained at 300 MHz on a Varian UNITY-300FT spectrometer. The reactions were performed by adding 50 μl of enzyme (~0.2 unit) to 1 ml ammonium acetate buffer, pH 4.5, containing 20% alcohol and 10 mM *p*-nitrophenyl-β-D-xylopyranoside for 16 h at 40°C. The reaction mixture was concentrated to dryness. The crude solid was then resuspended in 0.5 ml H_2O to form a yellowish solution, which was then subjected to extraction by ethyl acetate (0.5 ml). The resulting aqueous solutions were lyophilized and exchanged with 0.5 ml D_2O for NMR analysis. Since the yields for the tested reactions were greater than 95% (estimated by the integration of the Cl anomeric protons of xylose and alkyl-xylosides), no further purification step were necessary. Ethanol, *n*-propanol, and *n*-butanol were served as xylosyl acceptors in the transxylosylation. The critical signals of the resulting products were listed below:

Ethyl-β-D-xyloside. δ: 4.25(d, J = 7.8 Hz, 1H, H_1), 3.56 (m, 2H, OCH_2), 1.06 (t, J = 7.2 Hz, 3H, CH_3).

n-Propyl-β-D-xyloside. δ: 4.24 (d, J = 7.8 Hz, 1H, H_1), 3.55 (m, 2H, OCH_2), 1.45 (m, 2H, CH_2), 0.73 (t, J = 6.6 Hz, 3H, CH_3).

n-Butyl-β-D-xyloside. δ: 4.23 (d, J = 7.8 Hz, 1H, H_1), 3.54 (m, 2H, OCH_2), 1.41 (m, 2H, CH_2), 1.12 (m, 2H, CH_2), 0.73 (t, J = 7.2 Hz, 3H, CH_3).

Table 3
The influence of the concentration of NaH_2PO_4 for β-xylosidase extraction

Phase composition		Partition Coefficient, K	Partition ratio of β-xylosidase in bottom phase, Y (%)	Purification factor of β-xylosidase in bottom phase, (PF)
Polymer	NaH_2PO_4 [%(w/v)]			
	5	—	—	—
	10	—	—	—
	20	0.011	96.3	16.1
PEG 1500 [25%(w/v)]	25	0.007	99.2	12.2
	30	0.009	98.5	11.7
	40	0.009	99.5	11.8
	50	0.014	99.6	9.0

3. Results and discussion

3.1. Effects of salt on the partitioning of β-xylosidase in PEG 1500-containing aqueous two-phase system

The selection of salt employed in the extraction system is one of the key points of this technique. To ensure the efficiency of the extraction, five different salts were applied for this purpose. The results were summarized in Table 1. With the exception of KH_2PO_4, the applications of salt led to the formation of phases. $MgSO_4$ and NaH_2PO_4 showed better partition ratios ($Y > 94\%$). Yet, based on the purification factor, NaH_2PO_4, $PF > 16$ is considered to be more applicable for the subsequent study.

3.2. Effects of PEG on the partition of β-xylosidase in PEG-NaH_2PO_4 System

According to the results shown in Table 1, we chose the PEG-NaH_2PO_4 aqueous two-phase system for further study. Another component present in the system was PEG. The addition of PEGs with different degree of polymerization affected the extraction efficiency. Table 2 shows the effects of various PEGs on the partition of β-xylosidase. As anticipated, the lower molecular weights of PEGs failed to form phases. PEG 1500 turned out to be the minimum size of polymer that was applicable for the extraction system. Note that the better choice of PEG in the two-phase system is PEG 1500, which exhibited the highest partition ratio (96.3%) and purification factor (16.1).

Table 4
The influence of the loaded mass on the partitioning and purification of β-xylosidase*

Response of the phase system on β-xylosidase partition						
Protein Conc. (mg/ml)	K	Y (%)	SA_{ori}	SA_b	PF	Y_{tot} (%)§
0.50	0.0147	98.4	11.6	205	17.7	626
1.08	0.0066	99.3	10.4	166	16.0	627
1.75	0.0036	99.6	17.1	310	18.0	498
2.24	0.0041	95.6	18.7	290	15.5	448
2.67	0.0051	94.7	19.5	209	10.7	410

* Phase Compositions contain 25% (w/v) PEG and 25% (w/v) NaH_2PO_4. Specific activity and protein concentration of the original untreated culture filtrate are 10.4 U/mg and 1.08 mg/ml, respectively. The loading samples are 6 ml regardless of their concentrations.

§ Y_{tot} is defined as the enzyme unit in bottom phase to the unit in the original untreated culture filtrate.

3.3. Influence of salt concentration on the partitioning and purification of β-xylosidase

To optimize the NaH_2PO_4 concentration, a series of solutions containing 25% PEG 1500 and 5–50% of salt concentration were investigated. The results are shown in Table 3. Among them, the system contains 25% (w/v) PEG 1500 and 20–25% (w/v) NaH_2PO_4 gave the highest enzyme recovery in the bottom phase (96.3–99.2%) and the best partition coefficient (0.011–0.007).

3.4. Influence of the mass of loaded substance in the extraction system

The effect of loaded mass on the partition and purification of a target product is important in a large-scale process. The loaded biomass can alter not only the phase ratio (by volume) [22] but also the partitioning behavior of macromolecules since some components of the biomass (especially cell-disrupted biomass) contribute to the formation of the two-phase system. In our study, the fermentation supernatant was concentrated to different concentrations by using ultracentrifugation membrane (30 kDa cut off, Millipore Co.). These samples were then subjected to performing the extraction of enzyme by the aqueous two-phase process. Several parameters describing the extraction efficiency are summarized in Table 4. In general, the partition ratio (Y) are greater than 94%, indicating the high specificity of enzyme partitioning in the bottom phase of this system. The high purification factors (10–18) suggest that the target protein was selectively extracted. More interestingly, the total activity yields in all cases were enhanced at least 410% and up to 627% when the sample concentration was decreased from 2.67 mg/ml to 0.5 mg/ml of protein. There are a variety of possible explanations for this unusual activity enhancement.

Table 5
Scale up purification of β-xylosidase by using aqueous two-phase system[#]

	Volume of each phase (ml)	Activity of each phase (U/ml)	Protein Conc. (mg/ml)	Partition coefficient, K	Specific Activity (U/mg)	Purification factor of β-xylosidase in bottom phase, (PF)	Partition ratio of β-xylosidase in bottom phase, (%)	Total recovery yield, Y_{tot}* (%)
Top phase	190	0.91	1.2		0.76			
Bottom phase	180	240	0.7	0.0038	343	33.2	99.6	422

* Y_{tot} is defined as the note in table 4.
[#] 1000 ml culture filtrate was applied in this experiment.

Two of these are: 1) Metabolites or secondary metabolites, which may be removed during extraction, inhibit the enzyme activity. For example, xylose and alkyl-β-xylosides are inhibitors of the enzyme with K_i values in mM range (unpublished data). 2) High salt and/or high protein concentrations may help to maintain the protein conformation in an active form.

3.5. Scale up isolation of β-xylosidase by using aqueous two-phase system

To ensure the feasibility of this technique in large-scale preparation of β-xylosidase, a liter-scale extraction was performed. The extraction system contained 25% PEG 1500 and 25% NaH_2PO_4 at pH 4.4. The culture filtrate was concentrated to 1.75 mg/ml of protein prior to extraction. The whole process was completed in 1 h. Results (Table 5) were comparable to those of small-scale extractions. Figure 1 shows the SDS-PAGE analysis of protein samples from various extraction steps and the purified enzyme obtained by regular column chromatography [27].

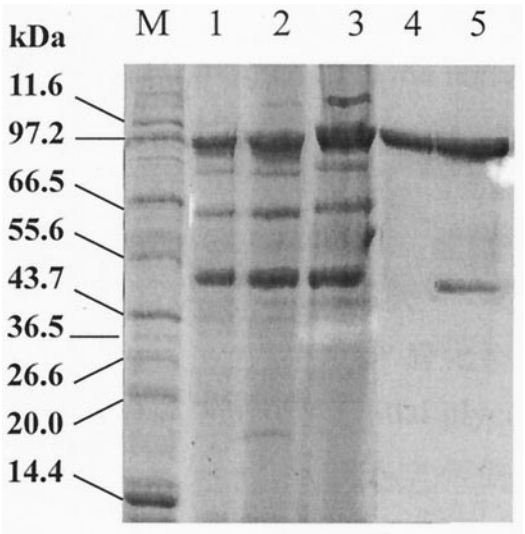

Fig. 1. SDS-PAGE analysis of the purity of β-xylosidase. Lane 1: 3× concentrated culture filtrate; Lane 2: 6× concentrated; Lane 3: 12× concentrated; Lane 4: sample from column purification [32]; Lane 5: sample from this study; M: markers.

3.6. Application to the preparation of alkyl-β-xylosides

In order to evaluate the application potential of the purified β-xylosidase. The catalytic hydrolysis of *p*-nitrophenyl-β-D-xyloside was carried out in the presence of 20% of various alcohols including ethanol, *n*-propanol and *n*-butanol. The enzyme obtained from the same strain and purified by column chromatography has been shown to be highly stable in solution containing alcohols. The powerful transxylosylation activity was comfirmed. Based on NMR analysis, the products of the enzymatic reactions are exclusively the correspondent alkyl-β-xylosides. The pretreatment of the sample was described in Methods. The products were unequivocally identified and no significant concentration of xylose (the hydrolysis product) was detected in any of the cases. Further applications of this enzyme will be developed in our subsequent studies.

4. Conclusion

A simple process for simultaneously concentrating and purifying β-xylosidase from *Trichoderma koningii* G-39 fermentation culture is developed. In the case of handling a 1-liter sample solution, the whole process can be complete in 1 h with a high recovery yield. The purity of the target protein is comparable to that from column chromatography. In combing the applications of PEG and NaH_2PO_4 in the system has successfully demonstrated the power of this aqueous two-phase extraction. This system has shown to be feasible for a liter-scale manipulation. For scale up extraction of β-xylosidase, the total activity, and the purity was enhanced by 422% and 33.2-fold, respectively. The purified enzyme was used for preparing alkyl-β-xylosides by its powerful transxylosylation activity.

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