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Rapid process for purification of an extracellular β-xylosidase by aqueous two-phase extraction

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Abstract

A rapid process for purification of an extracellular β-xylosidase with high purity was developed. The manipulation involved the precipitation of protein from culture medium and the extraction of enzyme from the resuspended crude protein solution by an aqueous-two phase separation. A linear random copolymer, PE62, with 20% ethylene oxide and 80% propylene oxide was employed in both stages of the purification. The enzyme was precipitated effectively by using 10% (w/v) PE62 and 5% (w/v) Na_2HPO_4. The aqueous two-phase extraction was performed with PE62 (10%)–NaH_2PO_4 (15%) as phase-forming reagent. SDS–PAGE analysis revealed that the purified enzyme is near homogeneity. The yield is about 100% with a purification factor of 8.8-fold. The whole process can be completed within an hour without any column chromatography.

Keywords: Aqueous two-phase extraction; Enzymes; β-Xylosidase

1. Introduction

β-Xylosidase (EC 3.2.1.37) is an exoglycosidease that catalyzes the hydrolysis of short xylooligosaccharides to liberate xylose. Recently, the enzyme from *Trichoderma koningii* G-39 was reported to possess a strong transxylosylation activity [1,2]. This enzyme is potential useful for preparation of β-D-xylosides and even α-L-arabinosides [1]. β-Xylosidases have been found in bacteria [3,4] and fungi [5–8]. However, the purifications of the enzymes frequently involve many time-consuming column chromatographies [9–15]. For promoting the application potential in industry, developing a simple and efficient method to improve enzyme preparation becomes an important issue.

Aqueous two-phase extraction methods have shown to be useful for the purification of biomolecules [16–19]. In the presence of appropriate amounts of a polymer and a salt, two aqueous phases can be formed. By changing the percentage of polymer and salt, it is likely to tune the hydrophobicity of the phases. Consequently, the selective partitioning of biomolecules in two phases is possible. the proteins, however, obtained by this method frequently contain impurity. A developing technique, namely affinity precipitation, was considered as a useful technique for protein purification. Affinity precipitation involves the binding of a protein with the soluble affinity ligand, which is covalently attached to polymers [20–24]. This method is similar to antigen–antibody precipitation. Although affinity

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precipitation is an attractive method for biomolecular isolation, it requires extensive studies on the target protein and the ligand immobilization techniques. In order to overcome the weakness of the above methods, we developed a two-stage process, which combines the precipitation of proteins by an artificial polymer and an aqueous two-phase extraction. By using a Ucon polymer, PE62, and phosphate salts, we were able to purify the β-xylosidase to near homogeneity from the fermentation medium with a yield of 99% and high purity.

2. Materials and methods

Trichoderma koningii G-39, a gift generously supplied by Professor 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.

2.1. Chemicals

PE62, obtained from Sino-Japan, Taiwan, is a random copolymer composed of 20% ethylene oxide and 80% propylene oxide. The average molecular mass is 2475 and the cloud point is 32±2°C. 2.4-Dinitrophenyl-β-D-xylopyranoside was synthesized according to the literature [25]. The salts and other chemicals used were analytical grade and purchased from Merck.

2.2. Crude extract

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% PDA (potato dextrose agar). 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 15 min to obtain a yellowish supernatant.

2.3. 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. Enzyme unit is defined as the amount of enzyme required for releasing 1 μmol of 2,4-dinitrophenol from the substrate in 1 min. For activity assay, a suitable amount of protein was added to 0.5 ml acetate buffer (50 m*M*, pH 4.2) containing 1 m*M* 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.

2.4. Protein determination

Protein was determined by bicinchonic acid assay (BCA), using bovine serum albumin as standard and expressed in mg/ml [26].

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to a standard protocol (Bio-Rad). The molecular mass standard was the broad range protein marker from New England Biolab.

2.6. Purification

2.6.1. Protein precipitation

After the addition of suitable amounts of PE62 and Na_2HPO_4 (specified in each table) to the culture filtrate of fermentation, the mixture was vortex-mixed to homogeneity and kept at room temperature for a few minutes. The solution was centrifuged at 10 000 *g* for 5 min to collect the crude precipitant.

Alternatively, for comparison, a final concentration of 50% (v/v) ethanol was used for precipitation of the crude enzyme.

2.6.2. Aqueous two-phase extraction

The precipitants derived from the last step (by PE62–Na_2HPO_4 and by ethanol) were resuspended in 1.0 ml of NaOAc buffer (20 m*M*, pH 4.5). Appropriate amounts of PE62 and NaH_2PO_4 (specified in each table) were added to the solution and vortex-mixed for 5 min. The mixture was kept at 34°C for 30 min to form phases. The enzyme interested was present in the bottom phase, which was nicely separated by centrifugation at 10 000 *g* for 5 min. The bottom phase was then withdrawn and desalted for activity assay and SDS–PAGE analysis.

Results were evaluated by purification factor (PF), which 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.

3. Results and discussion

Regular protein purification always involves several steps of manipulation such as salting out proteins and various column chromatographies. The yield of the enzyme activity is unavoidably decreased in each step of the purification. For precipitating protein from culture filtrate, a large amount of ammonium sulfate is used. Yet, ammonium sulfate sometimes deactivates the activity of the enzyme interested. Alternatively, organic solvent such as ethanol or acetone, which reduces the polarity of the aqueous medium and results in the precipitation of protein, is employed. However, the incompatibility of organic solvents with proteins limits their applications. Aqueous two-phase extraction is developed for obtaining intact protein from a bulk fermentation medium. Owing to the simplicity and the effectiveness of this technique, it is potential useful in enzyme preparation in industry scale. In our previous study (Li, unpublished data), the application of 25% (w/v) PEG1500 and 20~25% (w/v) NaH_2PO_4 was found to be effective in concentrating and purifying the β-xylosidase. However, the final product contained 10~15% of impurity. This study was performed to directly improve the purity of the β-xylosidase by using PE62 polymer. Table 1 summarizes the effect of the PE62–Na_2HPO_4 system under various conditions. In either 10% of PE62 or 10% of Na_2HPO_4 alone, no phase separation and precipitation can be observed indicating the combination of both is necessary. Higher percentage of PE62 enhances the phase separation (data not shown). Yet, interestingly, protein precipitation was somewhat prohibited in higher concentrations of salt. Fig. 1 clearly shows that increasing Na_2HPO_4 or PE62 concentration resulted in a less activity recovery as well as a poorer specific activity. The best condition was shown to be the system of 5% Na_2HPO_4 and 10% PE62. By carrying out this precipitation, a 5.7-fold purification factor can be easily achieved.

In order to enhance the purity of the enzyme interested, an aqueous two-phase system was employed after the precipitation. The suitable conditions for this stage of purification were evaluated and summarized in Table 2. The experiments were performed by applying the PE62–NaH_2PO_4 system in the culture filtrate directly. The system composed of 10% (w/v) PE62 and 15% (w/v) NaH_2PO_4 not only provided a nice phase separation but also enhanced the enzyme purity by four fold. More interestingly, the total β-xylosidase activity was increased by 340%, presumably due to the removing of the potential inhibitors, such as xylose, during the manipulation of extraction.

For evaluation of the effectiveness of protein precipitation of this study, the crude protein were obtained by ethanol precipitation and a PE62 (10%)–Na_2HPO_4 (5%) system. The resulting proteins were then extracted by the same aqueous two-phase conditions. Table 3 and Table 4 show the corresponding results derived from the two precipitation methods. For the case of ethanol precipitation, the purification factor is 6.5-fold with the recovery yield of 84.2%, whereas, for the PE62 precipitation system, the purification factor and yield are 8.8-fold and 99%, respectively. The purity of the enzyme was analyzed by SDS–PAGE. As can be seen in Fig. 2, the enzyme obtained by this process was near homogeneity with estimated molecular mass of

Table 1
Effect of phase composition on the precipitation of β-xylosidase in PE62–Na_2HPO_4 affinity precipitation system[a]

Phase composition		Specific activity of precipitate (U/mg)	Recovery (%)
PE62 (%, w/v)	Na_2PO_4 (%, w/v)		
0	5	–[b]	–
	10	–	–
10	0	–	–
	5	114.5	97.8
	10	90.2	89.4
	20	16.2	14.7
	30	14.1	9.8
20	5	66.0	61.6
	10	31.0	28.3
	20	20.2	19.4
	30	–	–
30	5	59.6	56.2
	10	17.2	18
	20	–	–
	30	–	–

[a] A 1-ml volume of culture filtrate was applied for the experiment. The total protein, total activity, and specific activity were determined as 1.12 mg, 22.4 unit, and 20 unit/mg, respectively.
[b] – indicates that no precipitation occurred and therefore data not available for recovery (%).

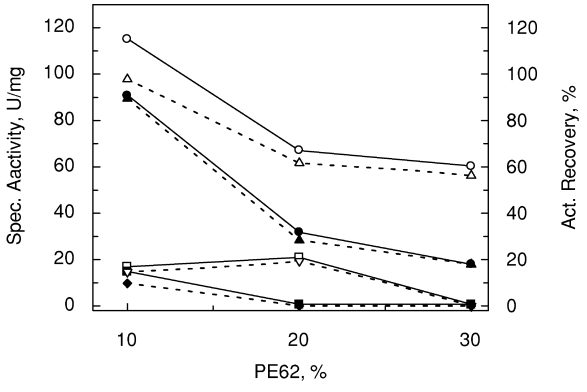

Fig. 1. The specific activity and the activity recovery of the enzyme obtained from various phase compositions. The precipitates obtained from 1 ml culture filtrate by the PE62–Na_2HPO_4 affinity precipitation system were resuspended in 0.2 ml sodium acetate buffer (pH 4.5, 50 m*M*). For specific activity: – ○ –, 5% Na_2HPO_4; – ● –, 10%; – □ –, 20%; – ■ –, 30%. For activity recovery: – △ –, 5% Na_2HPO_4; – ▲ –, 10%; – ▽ –, 20%; – ♦ –, 30%.

100 000, which is consistent with our previous study [1,2]. The affinity precipitation combined with aqueous extraction presents a persuasive result that this process enhances both the purity and the yield in comparison with that from classical purification. It also offers a useful method for a large-scale preparation of enzyme for industrial applications.

4. Conclusions

Many methods have been reported for the purification of β-xylosidase, most of them used ammonium sulfate or organic solvents to precipitate proteins in the culture medium and then followed by few chromatographies. These tedious and time-consuming processes can be overcome by our method. By combining the PE62 precipitation system and aqueous two-phase extraction, an extracellular β-xylosid-

Table 2
Effect of phase composition on partitioning of β-xylosidase in PE62–NaH_2PO_4 aqueous two-phase extraction[a]

Phase composition		Total protein (mg)	Total activity (U)	Purification factor (PF)	Specific activity (U/mg)
NaH_2PO_4 (%, w/v)	PE62 (%, w/v)				
5	10	–[b]	–	–	–
10	10	3.3	168	2.5	51
15	10	2.8	228	4.1	81
20	10	2.6	200	3.9	77
15	15	2.3	103	2.3	45
15	20	2.2	167	3.8	76
15	30	2.3	120	2.6	52

[a] A 3-ml volume of culture filtrate was applied for the experiment. The total protein, total activity, and specific activity were determined as 3.4 mg, 67 unit, and 20 unit/mg, respectively.
[b] – indicates that no precipitation occurred and therefore data not available.

Table 3
Purification of β-xylosidase by combining ethanol precipitation and aqueous two-phase extraction (ATPE)[a]

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (PF)	Recovery (%)
Crude extract	2936	40	73.4	1	100
Precipitate[b]	3060	7.5	408	5.6	100
Bottom phase of ATPE[c]	2472	5.2	477	6.5	84.2

[a] The experiments were triplicate and carried out with 40 ml culture filtrate. All results derived from different experiment were within 10% standard deviations. The results shown were the average of all data.
[b] Precipitation condition: 50% (w/w) ethanol.
[c] ATPE condition: 10% PE62 and 15% NaH_2PO_4.

ase was obtained with high purity and high recovery yield. Most importantly, this process is simple, effective, and industrially applicable. The best conditions applied in this study were PE62 (10%)–Na_2HPO_4 (5%) and PE62 (10%)–NaH_2PO_4 (15%) for precipitation and extraction, respectively.

Table 4
Purification of β-xylosidase by combining of affinity precipitation and aqueous two-phase extraction (ATPE)[a]

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (PF)	Recovery (%)
Crude extract	2936	40.0	73.4	1	100
Precipitate[b]	2884	6.7	430.4	5.8	98
Bottom phase of ATPE[c]	2907	4.5	645.9	8.8	99

[a] The experiments were done in triplicate and carried out with 40 ml culture filtrate. All results derived from different experiment were within 10% standard deviations. The results shown were the average of all data.
[b] Precipitation condition: 10% PE62, 5% Na_2HPO_4.
[c] ATPE condition: 10% PE62, 15% NaH_2PO_4.

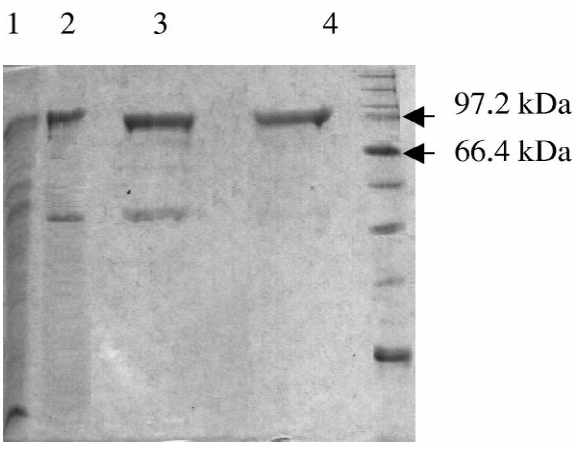

Fig. 2. SDS–PAGE analysis of of β-xylosidase. Lanes: 1=culture filtrate; 2=sample obtained from the extraction by using 25% (w/v) PEG1500 and 20% (w/v) NaH_2PO_4; 3=sample obtained from the ethanol precipitation followed by PE62 (10%)–NaH_2PO_4 (15%) extraction; 4=sample obtained from the affinity precipitation followed by PE62 (10%)–NaH_2PO_4 (15%) extraction; 5=protein marker.

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