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Purification of Endopolygalacturonase from Submerged Culture of *Aspergillus awamori* L1 Using a Two-step Procedure: Enzyme Precipitation and Gel Filtration

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Abstract: The purification of extracellular endopolygalacturonase (endo-PG) from the filtrate of *Aspergillus awamori* L1 submerged culture was carried out using a two-step procedure: enzyme precipitation by organic solvents, polymers or salts; and gel filtration using Sephadex G-75 column. The results show that ammonium sulfate was the suitable precipitant in the two-step procedure for endo-PG purification. Firstly, ammonium sulfate was added to the crude enzyme solution with the concentration of 47.2% (w/v) for endo-PG precipitation. The precipitates were then isolated and redissolved in a buffer solution to prepare for the gel filtration step. Consequently, the purification factor achieved 30.4-fold and the endo-PG recovery yield obtained 68.60% in comparison to the crude enzyme solution.

Keywords: ***Aspergillus awamori*** **L1, precipitation, gel filtration, submerged fermentation, endopolygalacturonase**

INTRODUCTION

Endopolygalacturonase (EC 3.2.1.15) is the most substantial member of pectolytic enzymes that are utilized to degrade various pectic substances present in plant tissues. It catalyses the reaction hydrolyzing α-1, 4-D-galacturonosidic linkage randomly on polygalacturonate substrates (Whitaker *et al.*, 1990).

Endo-PG has potential applications in various fields of the food industry, e.g., fruit juice, wine and oil production (Kashyap *et al.*, 2001; Whitaker *et al.*, 2003). In recent years, vigorous researches on biosynthesis and purification of endo-PG have been carried out (Naidu *et al.*, 1998; Jayani *et al.*, 2005; Tewari *et al.*, 2005). Significant progress has been made in purifying endo-PG to homogeneity by using different purification methods (Manachini *et al.*, 1987; Nagai *et al.*, 2000; Guo *et al.*, 2001; Gummadi, 2003). In Vietnam, however, due to high cost and lack of pectinase production, the utilization of this enzyme in the food industry has still been limited (Pham, 2007a).

This paper reports the purification of extracellular endo-PG biosynthesized by a strain of *Aspergillus awamori* in order to produce and utilize this enzyme in an industrial scale. Medium composition and fermentation conditions for endo-PG biosynthesis were previously optimized (Pham *et al.*, 2007a,b). From this submerged culture, the endo-PG was purified by the two-step procedure: enzyme precipitation by different precipitants and gel filtration using Sephadex G-75 column. Beside the endo-PG recovery yield and purification factor, the economic efficiency of the purification procedure was also evaluated.

MATERIALS AND METHODS

Materials

The strain of *Aspergillus awamori* (L1) used in this study was supplied by the Department of Biotechnology, Ho Chi Minh City University of Technology, Vietnam. This strain was maintained on Czapek agar medium, stored at 4°C and transferred onto new Czapek agar medium every three months.

Grapefruit peels, *Citrus maxima* (Nam roi, Tien Giang, Vietnam), were utilized as carbon source for endo-PG production. The peels were dried to 7% moisture and then grinded to powder. The pectin content in this raw material was 10.77% of dried weight. Commercial citrus pectin, used for endo-PG activity determination, was supplied by Sigma Corporation, US. Sephadex G-75 was taken from Pharmacia, Uppsala, Sweden. Other analytical chemicals used in this study originated from several chemical suppliers in China.

Medium and Fermentation Conditions for Endo-PG Production

The medium for endo-PG production, previously reported by Pham *et al.* (2007), contained (in g per L of distilled water): grapefruit pectin 0.788; NH_4NO_3: 0.402; KH_2PO_4: 4.0; Na_2HPO_4, $FeSO_4.7H_2O$: 0.2; $CaCl_2$, H_3BO_3: 0.01; $MnSO_4$.

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$7H_2O$: 0.07. Prior to fermentation, the medium was sterilized at 121°C for 20 min.

The initial inoculum size for endo-PG production was approximately 34mg spores (dried weight) L^{-1} medium. Fermentation was carried out at 30°C in a 4L fermentor (BioStat® B, Braun, Germany). During fermentation, the pH of the culture was automatically adjusted to 4.5. The agitation and aeration rate (using sterilized air) were 150 rpm and 0.8 vvm, respectively. The fermentation time was 24h (Pham *et al.*, 2007).

Enzyme Assay

The catalytic activity of endo-PG was measured based on viscosity decrease (Roboz *et al.*, 1952):

$$\text{Reduction in viscosity (\%)} = \frac{T_0 - T_t}{T_0 - T_w}.100\% \quad (1)$$

where T_o, T_t and T_w represent the flow rate (in a capillary viscometer) in seconds for the experimental sample, control sample and water, respectively.

The experimental sample consisted of 7.5mL of 1.0% (w/v) polygalacturonic acid (PGA) dissolved in 0.1N sodium acetate buffer (pH 4.5) and 2.5mL enzyme solution. In the control sample, 2.5mL of water was replaced for enzyme solution. One endo-PG unit (U) was defined as the amount of enzyme which reduces the viscosity of 1% PGA solution by 50% in 30 min at 45°C (Blandino, 2001).

Soluble Protein Determination

Protein concentration was determined by spectrophotometric method using Folin reagent (Lowry *et al.*, 1951). Bovine serum albumin (Sigma) was used as the standard.

Enzyme Purification

At the end of the fermentation, mycelia were promptly removed by microfiltration and the filtrate (or crude enzyme solution) was then purified by the two-step procedure:

- Step 1: Endo-PG precipitation by the following precipitants:

Organic solvents: Ethanol, isopropanol and acetone were alternatively used. Precooled solvent (-15°C) was slowly added to the crude enzyme solution until the volume ratio between enzyme solution and solvent reached 10:90; 20:80; 30:70; 40:60; 50:50; 60:40 (v/v).

Polymers - Polyethylenglycol (PEG): PEG-4000 and PEG-6000 in powder form were slowly added to the crude enzyme solutions until the concentration of these agents in the solutions reached 5%; 10%; 15%; 20% and 25% (w/v).

Salts: Ammonium sulfate was slowly added to the crude enzyme solution using various concentrations: 76.1%; 65.7%; 56.1%; 47.2%; 39.0%; 31.4% and 25.1% (w/v). Sodium chloride was also used at five concentrations: 35%; 30%; 25%; 20%; 15% (w/v).

After standing the mixtures for 30 min at -4°C, the precipitates were collected by centrifugation at 10000 g for 15 min and further redissolved in 0.05N sodium acetate buffer solution (pH 4.5) to a determined volume. These samples were used for determining the purification factor and enzyme recovery yield (Roe, 2001). Finally, the suitable precipitants were chosen for the next step.

-Step 2: Endo-PG purification by gel filtration. The enzyme solution obtained from the first step was eluted through a Sephadex G-75 column in 1.5 cm diameter and 30 cm height. 20 fractions were then collected for determining optical density at 280 nm (OD_{280nm}) and endo-PG activity. The volume of each fraction was 4mL. This step was examined at 18°C.

Endo-PG Recovery Yield (Y)

$$Y\ (\%) = \frac{TA_1}{TA_0}.100 \quad (2)$$

where TA_0: total activity in crude enzyme solution (U); TA_1: total activity in sample after purification (U).

Endo-PG Specific Activity (S)

$$S\ (U / mg\ protein) = \frac{A}{C_p} \quad (3)$$

where A: activity of enzyme solution (U/mL); C_p: protein concentration in enzyme solution (mg/mL).

Purification Factor (P)

$$P\ (fold) = \frac{S_1}{S_0} \quad (4)$$

where S_1: specific activity of endo-PG after purification; S_0: specific activity of endo-PG before purification.

Statistical Treatment

All experiments were carried out in triplicate. The data were subjected to analysis of variance (ANOVA) with a p value <0.05 using STATGRAPHICS© Plus for windows 3.0 (Copyright 1994-1997 by Statistical Graphics Corporation).

Table 1: Recovery yield and purification factor of endo-PG by ethanol precipitation

Ratio $V_{crude\ enzyme}$: $V_{ethanol}$	Protein concentration* (mg/mL)	Endo-PG activity* (U/mL)	Specific activity (U/mg protein)	Recovery yield (%)	Purification factor (fold)
10:90	0.078	1.144	14.609	92.21[b]	5.77[b]
20:80	**0.074**	**1.204**	**16.294**	**97.02[a]**	**6.43[a]**
30:70	0.068	1.005	14.678	80.96[c]	5.79[b]
40:60	0.052	0.287	5.493	23.15[d]	2.17[e]
50:50	0.009	0.123	13.673	7.95[e]	4.32[c]
60:40	0.004	0.036	9.321	2.90[f]	3.69[d]
Culture filtrate	0.489	1.244	2.544	100.00	1.00

Each value represents the mean of three independent samples. Different letters in each column mean significant difference ($P<0.05$).
* The concentration of protein and endo-PG activity in 1mL enzyme solution after redissolving the precipitates in the acetate buffer.

Table 2: Endo-PG recovery yield and purification factor by various precipitants

Precipitants	Amount of precipitants	Specific activity (U/mg protein)	Recovery yield (%)	Purification factor (fold)
Organic solvents				
Ethanol	20:80*	16.29	97.02	6.43
Isopropanol	40:60*	19.24	88.95	8.43
Acetone	40:60*	6.46	70.74	5.08
Polymers				
PEG 4000	20.0%**	3.63	9.40	1.42
PEG 6000	25.0%**	3.76	8.67	1.57
Salts				
$(NH_4)_2SO_4$	47.2%**	14.03	78.17	5.26
NaCl	35.0%**	12.29	48.87	5.30

Amount of precipitants: * Volume ratio of enzyme solution to organic solvent; ** Weight of precipitant added to enzyme solution volume (w/v).

RESULTS AND DISCUSSION

Endo-PG Purification by Precipitation

(i) Precipitation by organic solvents

The enzyme recovery yield and purification factor when using ethanol as a precipitant are shown in Table 1.

The results show that the recovery yield as well as the purification factor augmented significantly when the volume ratio of crude enzyme solution to ethanol decreased from 60:40 to 20:80. According to Rosenberg (1996), adding a solvent to an enzyme solution reduces its dielectric constant and therefore enhances interaction of protein molecules. It leads to an increase in enzyme recovery yield.

The highest endo-PG recovery yield and purification factor reached 97.02% and 6.43-fold, respectively when the volume ratio of crude enzyme solution to ethanol was 20:80. In contrast, when the volume ratio of crude enzyme solution to ethanol was 10:90, these values decreased conspicuously due to the irreversible denaturation of some enzyme molecules at high concentration of ethanol (Scopes, 1994). In this research, the same phenomenon was also observed when using other solvents such as isopropanol and acetone (data not shown).

In summary, the appropriate ratio of crude enzyme solution to ethanol for endo-PG purification from *A. awamori* L1 submerged culture was 20:80 (v/v). In comparison with a previous study of Manachini *et al.* (1987) on the purification of endo-PG from culture filtrate of *Rhizopus stolonifer*, the optimum volume ratio of crude enzyme extract to ethanol was about 30:70, the purification factor and recovery yield of this enzyme were 9.2 (fold) and 77%, respectively.

With regard to other organic solvents, our experimental results showed that the suitable volume ratio of crude endo-PG solution to isopropanol or acetone was 40:60. Under these

conditions, the purification factor and enzyme recovery yield were 8.43-fold and 88.95% for isopropanol, and 5.08-fold and 70.74% for acetone, respectively (Table 2).

(ii) Precipitation by polymers

Table 2 shows that the purification factor and recovery yield of endo-PG reached a maximum when the concentration of PEG-4000 and PEG-6000 was 20.0% and 25.0% (w/v), respectively. Nevertheless, the obtained values were quite low which is attributable to the fact that adding PEG to the enzyme solution increased the viscosity of the mixture. In addition, the difference in density between liquid and solid phases of the mixture became insignificant. The above problems made the centrifugation difficult. Hence, the precipitates could not be entirely recovered under the experimental conditions. To overcome this phenomenon, an increase in centrifugal force of more than 10000*g* should be examined. However, this leads to a higher cost of enzyme preparation. In brief, the use of PEG-4000 and PEG-6000 as endo-PG precipitants is industrially impracticable. This conclusion is in agreement with the results of Scope (1994) and Rosenberg (1996) who reported that PEG has been an ineffective precipitant for intrinsically high-solubility proteins like enzymes.

(iii) Precipitation by salts

By salting out the crude enzyme solution with ammonium sulfate and sodium chloride at concentrations of 47.2% (w/v) and 35% (w/v), the results obtained were the most efficient. The purification factor was 5.26 and 5.20 (fold), and the enzyme recovery yield was 78.17% and 48.87%, respectively (Table 2). In comparison with sodium chloride, ammonium sulfate is a better precipitant with much higher recovery yield in endo-PG activity.

Ammonium sulfate has a great advantage to precipitate protein because of its low cost and rarely causing irreversible denaturation of protein molecules (Scopes, 1994). According to previous research by Afifi *et al.* (2002); Guo *et al.* (2001); Jaffar *et al.* (1993); Miyazaki (1991), the saturation degree of ammonium sulfate appropriate for endo-PG purification varied from 60 to 80% and that depended on the enzyme sources. In this study, the suitable concentration of ammonium sulfate, which is 47.2% (w/v), is equivalent to 70% saturation degree of this salt in the enzyme solution. Thus, the result obtained is similar to that suggested by other researchers.

In conclusion, three agents that resulted in higher enzyme recovery yield and purification factor than the others are ethanol, isopropanol and ammonium sulfate. The appropriate ratio of precipitant to crude enzyme solution for endo-PG purification was as follows: 80:20 (v/v) for ethanol; 60:40 (v/v) for isopropanol, and 47.2% (w/v) for ammonium sulfate.

Endo-PG Purification by Gel Filtration

In this experiment, three enzyme samples were previously precipitated by ethanol, isopropanol and ammonium sulfate from the crude enzyme solution. The precipitates were then isolated and redissolved in the buffer solution. Finally, each enzyme sample was alternatively passed through the Sephadex G-75 column.

As shown in Figure 1, in all cases, two peaks of OD_{280nm} value were observed, and only one endo-PG activity peak was found in the eluted fractions from Sephadex G-75 column. The highest endo-PG activity was primarily present in the 5^{th} and 6^{th} fractions. It should be mentioned that the OD_{280} value of these fractions, however, was quite low. This result indicates that the desired enzyme contributed a low amount to total protein concentration of the enzyme solution. In fact, total protein content in these fractions varied from 0.35 mg to 1.02 mg in comparison with 31.98 mg in crude enzyme solution (Table 3).

In this experiment, to examine the purification efficiency of the desired endo-PG, the fractions which was responsible for high endo-PG activities were mixed together and the obtained mixture considered as the purified enzyme solution after gel filtration was successively used to determine endo-PG activity and soluble protein concentration. Correspondingly, the gel filtration step resulted in significant increase in specific activity of endo-PG, and that obviously led to an important augmentation in purification factor.

Table 3 shows the experimental results of the 3 enzyme samples purified by the two-step procedure of precipitation and gel filtration. Obviously, sample 1 precipitated by isopropanol had a much higher purification factor (47.2-fold) than the others. Contrary to the purification factor, the enzyme recovery yield only reached 51.6%. Sample 2 precipitated by ethanol expressed the highest enzyme recovery yield which was 81.7%, but the least in enzyme purification factor which only reached 25.6-fold compared with the crude enzyme solution.

Besides, it can be noted that sample 3 precipitated by ammonium sulfate resulted in intermediate values of purification factor (30.4-fold) and enzyme recovery yield (68.6%) in comparison with samples 1 and 2. In order to select the suitable precipitant for endo-PG purification, an evaluation of the cost of chemicals used in the three enzyme samples above must be examined.

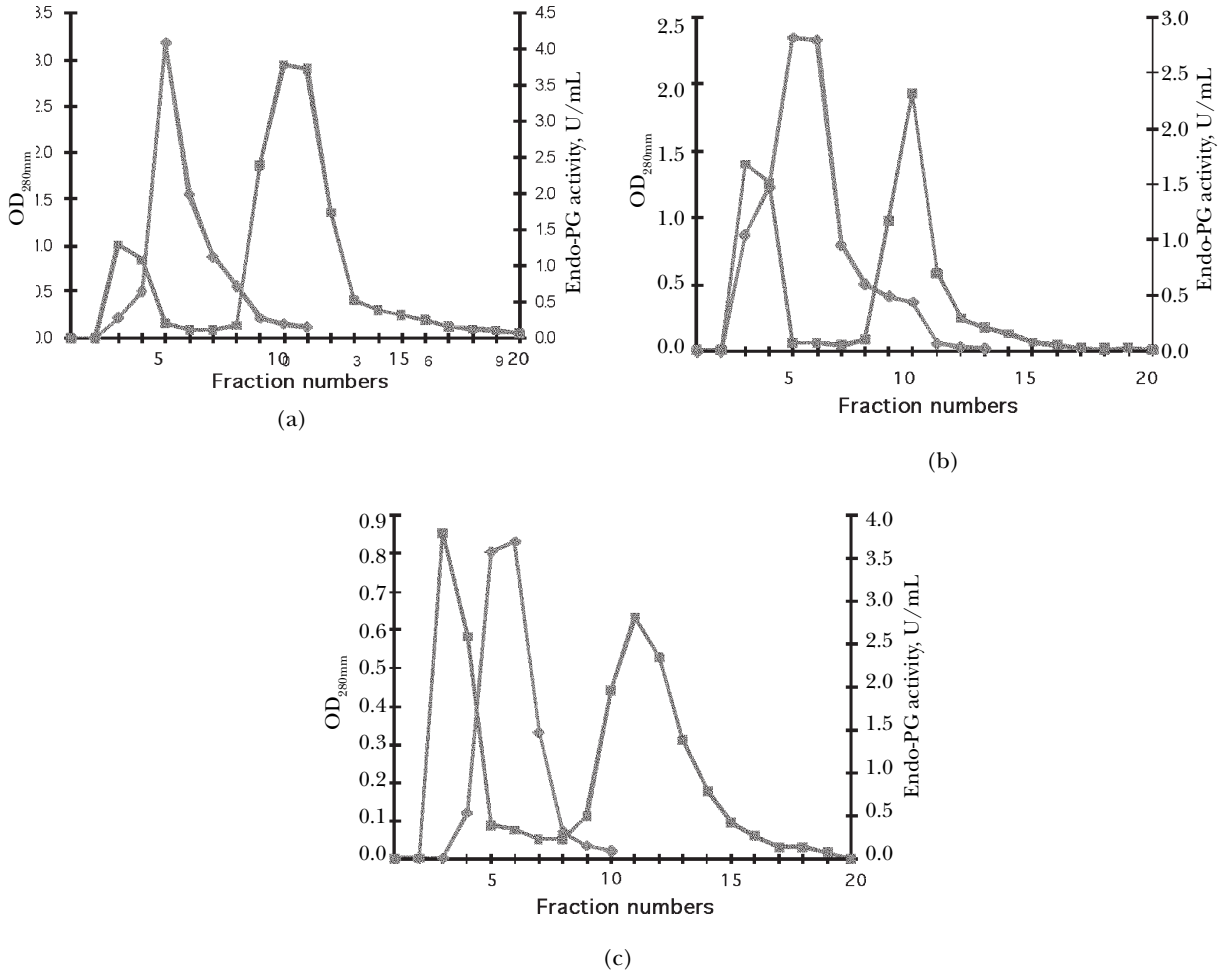

Figure 1: Gel filtration by Sephadex G-75 after endo-PG precipitation with: (a) ethanol ($V_{ethanol}$: V_{enzyme}=20:80); (b) isopropanol ($V_{isopropanol}$: V_{enzyme}=40:60); and (c) ammonium sulfate (47.2% w/v); (■)OD_{280nm} (◆) endo-PG activity

Table 3: Purification of *A. awamori* L1 endo-PG by precipitation and gel filtration

	Volume (mL)	Total protein (mg)	Recovery activity (U)	Specific activity (U/mg protein)	Recovery yield (%)	Purification factor (fold)
Culture filtrate	60.0*	31.98	76.32	2.39	100.0	1.0
Sample 1	3.0**	0.35	39.36	112.63	51.6	47.2
Sample 2	3.0**	1.02	62.39	61.26	81.7	25.6
Sample 3	3.0**	0.72	52.37	72.59	68.6	30.4

In the enzyme precipitation step, isopropanol was used in sample 1 (V_{enzyme}: $V_{isopropanol}$ = 40:60), ethanol was used in sample 2 (V_{enzyme}: $V_{ethanol}$ = 20:80), and ammonium sulfate was used in sample 3 (47.2% w/v). The conditions of the gel filtration step for all samples were similar. Volume of enzyme solution before: (*) precipitation step, (**) gel filtration step.

Table 4: Economic evaluation of endo-PG purification from *Aspergillus awamori* L1

Precipitants	Industrial price (VND)	Unit	Amount*	Total cost (VND)	Total PG recovery (U)	Cost per endo-PG unit** (VND/U)
Isopropanol	44200	1 kg	1.5 kg	66300	656.00	**101.07**
$(NH_4)_2SO_4$	48000	1 kg	472 g	22656	872.83	**25.96**
Ethanol	25000	1 L	4 L	100000	1039.80	**96.17**

* Amount of precipitant used for purifying 1L of crude enzyme solution. **Cost of precipitant used for recovering 1 endo-PG activity unit in the enzyme precipitation step.

According to Table 4, the cost of using ammonium sulfate in the enzyme precipitation step for recovering one unit of endo-PG activity was significantly lower than that of using the others. Hence, this precipitant was chosen for the first step of the purification process.

In summary, the procedure including enzyme precipitation by ammonium sulfate and gel filtration showed the most potential.

CONCLUSION

From both technological and economic points of view, the use of ammonium sulfate as a precipitant gives better results than that of other precipitants. Endo-PG from *A. awamori* culture filtrate can be purified by using the two-step procedure: enzyme precipitation and gel filtration. The purification factor and endo-PG recovery yield reached about 30.6-fold and 68.6%, respectively. The industrial application of this result for producing endo-PG has a big potential.

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