A simple method for the simultaneous decoloration and deproteinization of crude levan extract from *Paenibacillus polymyxa* EJS-3 by macroporous resin

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**A B S T R A C T**

A simple method for the simultaneous decoloration and deproteinization of crude levan extract from the endophytic bacterium *Paenibacillus polymyxa* EJS-3 was developed through static and dynamic adsorption tests of macroporous resins. S-8 resin demonstrated the highest decoloration and deproteinization ratios among various resins tested. Under optimized static adsorption conditions (pH 6.0, 35 °C and adsorption time of 70 min), the ratios of decoloration, deproteinization and polysaccharide recovery for S-8 resin were 76.8%, 78.9% and 69.0%, respectively. Under optimized dynamic adsorption condition (flow rate of 2 BV/h, 160 ml of 2.5 mg/ml crude levan extract), higher ratios of decoloration, deproteinization and polysaccharide recovery for S-8 resin (84.6%, 91.7% and 81.3%, respectively) were observed. The method developed will provide a potential approach for large-scale production of levan from *P. polymyxa* EJS-3.

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1. Introduction

Levan, a β-2,6-linked fructose polymer with β-2,1-linked side chains, is an important bioactive polysaccharide with a wide variety of applications. It can be used in medicine as hypo-cholesterol, anti-tumor and immunostimulating agents, and in food as emulsifier, stabilizer, thickener and encapsulating materials (Bae et al., 2008; Bekers et al., 2005; Oliveira et al., 2007). Levan is primarily produced by microbial fermentation from sucrose-based media (Bae et al., 2008; Yoon et al., 2004). The conventional method used to extract levan from the fermentation broth is performed by centrifugation. Notably, many impurities (especially pigments and proteins) are co-precipitated with levan by ethanol, making the subsequent purification process very difficult. Till now, several methods including H₂O₂ and Sevag reagent (Staub, 1965) have been used for the decoloration and deproteinization of crude polysaccharides (Yang and Zhang, 2009); however, these chemical methods probably cause partial hydrolysis of polysaccharides, resulting in variable bioactivities. In addition, the chemical reagents used are environmentally disadvantageous and may cause adverse impacts on the human body when the polysaccharides are added to food and medicine (Wang et al., 2007). Therefore, it is necessary to develop novel methods for the decoloration and deproteinization of crude levan extract.

Macroporous resins are durable polar, non-polar or slightly hydrophilic polymers with high adsorption capacity (Fu et al., 2006). They can selectively adsorb the targeted constituents from aqueous as well as non-aqueous system through electrostatic force, hydrogen bonding interaction, complexation and size sieving action (Gao et al., 2007). Therefore, macroporous resins have been widely used in the separation of targeted component from other impurities in crude biological samples (Crini, 2006; Fu et al., 2005; Hatano et al., 2009; Jia and Lu, 2008; Silva et al., 2007; Wan et al., 2008; Ye et al., 2008; Zhang et al., 2008); however, there is little information available on employing macroporous resins for the decoloration or deproteinization of crude polysaccharide extracts (Li et al., 2006; Wang et al., 2005). Compared to the chemical methods, the application of macroporous resins as the decoloration or deproteinization reagents for crude polysaccharide extracts should have some advantages, such as low operating costs, less solvent consumption and easy regeneration (Gao et al., 2007). Moreover, macroporous resins can simultaneously remove pigments and proteins from crude polysaccharide extracts without destroying the structures and bioactivities of polysaccharides.

Recently, we have found that the endophytic bacterium *Paenibacillus polymyxa* EJS-3, isolated from the root tissue of *Stemona japonica* (Blume) Miquel, produced high levels of levan (a yield of 35.26 g/L). In addition, we have demonstrated that both crude and purified levens showed strong superoxide and hydroxyl radical scavenging activities in vitro (Liu et al., 2009, 2010). Due to the fact that the crude levan extract contains a lot of pigments and proteins, the aim of this study was to develop a simple method for the simultaneous decoloration and deproteinization of crude levan extract.

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extract by macroporous resin. Firstly, various macroporous resins were investigated to select one with the highest decoloration and deproteinization efficiencies. Then, both static and dynamic adsorption experiments were carried out to investigate the effects of different parameters (pH, temperature, adsorption time, flow rate, and sample concentration) on decoloration and deproteinization efficiencies of the selected resin. Finally, the levan solutions before and after adsorption by the resin were measured by UV–vis spectroscopy, digital camera and HPLC to validate the decoloration and deproteinization efficiencies. To the best of our knowledge, this is the first report on the simultaneous decoloration and deproteinization of crude levan extract by macroporous resin.

2. Methods

2.1. Preparation of crude levan extract

Levan was produced by using *P. polymyxa* EJS-3 according to our reported method (Liu et al., 2010). Briefly, the fermentation broth was properly diluted and centrifuged at 10,500g for 15 min to remove bacterial cells after incubation on a rotary shaker incubator at 24 °C for 60 h. The resultant supernatant was mixed with four volumes of anhydrous ethanol, stirred vigorously and kept overnight at 4 °C. The precipitate from the ethanol dispersion was collected by centrifugation at 10,500g for 15 min, dissolved in distilled water and dialyzed against distilled water to afford the crude levan extract for further use.

2.2. Adsorbents

Macroporous resins including D3520, D4020, AB-8, D101, NAK-II and S-8 were provided by Chemical plant of Nankai University (Tianjing, China) and their physical properties are listed in Table 1. These resins were pretreated with 1 M HCl and NaOH solutions sequentially to remove the monomers and porogenic agents trapped inside the pores during the synthesis process, and then dried at 60 °C under vacuum. Prior to adsorption experiments, pre-weighed amounts of resins were soaked in 95% ethanol and washed thoroughly with deionized water.

2.3. Static adsorption tests

Static adsorption tests were performed as follows: pre-weighed amounts of hydrated adsorbent (equal to 1 g dry resin) and 50 ml of 5 mg/ml crude levan solution were added to an air-tight Erlenmeyer flask. The flask was shaken at 150 rpm in a constant temperature (25 °C) water-bath shaker for 12 h. After the resin was separated from the sample solution by filtration, the decoloration, deproteinization and polysaccharide recovery ratios of the resin were measured as described in Section 2.5. The preliminary selection of the resins was evaluated by their decoloration, deproteinization and polysaccharide recovery ratios towards crude levan solution. Then, static adsorption tests were done at different pH values (4, 5, 6, 7, 8 and 9), different temperatures (25, 30, 35 and 40 °C) and adsorption time (60, 120, 180 and 240 min) on the decoloration, deproteinization and polysaccharide recovery ratios of the selected resin were investigated with a shaking of 150 rpm.

2.4. Dynamic adsorption tests

Dynamic adsorption tests were carried out as follows: 180 ml of crude levan solution (5 mg/ml) was loaded continuously at a constant flow rate to a glass column (2.0 × 30 cm) wet-packed with the selected resin. The bed volume (BV) of the resin was 10 ml. The decoloration and deproteinization ratios of the effluents (10 ml/tube collected) were monitored as described in Section 2.5. In this way, the effects of flow rate and sample concentration on decoloration and deproteinization efficiencies of the selected resin were investigated.

2.5. Analytical methods

2.5.1. Determination of decoloration ratio

The decoloration ratio was determined by the method of Wang et al. (2005) with some modifications. Briefly, sample solution was adjusted to pH 7.0 with 1 M HCl and NaOH, and then centrifuged at 5000 rpm for 20 min. Subsequently, the absorbance of the resultant supernatant was measured at 420 nm on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). The following equation was used to quantify the decoloration ratio of the resin:

\[
\text{Decoloration ratio} \% = \left( \frac{A_o - A_e}{A_o} \right) \times 100
\]

where \(A_o\) and \(A_e\) were the absorbance of the samples at 420 nm before and after adsorption by resin, respectively.

2.5.2. Determination of protein concentration and deproteinization ratio

The concentration of protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard. The following equation was used to quantify the deproteinization ratio of the resin:

\[
\text{Deproteinization ratio} \% = \left( \frac{C_o - C_e}{C_o} \right) \times 100
\]

where \(C_o\) and \(C_e\) were the concentrations of protein (μg/ml) in the solutions before and after adsorption by resin, respectively.

2.5.3. Determination of levan concentration and its recovery ratio

The concentration of levan was measured by the phenol–sulfuric acid method using fructose as standard (Dubois et al., 1956). The recovery ratio of levan was calculated by the following equation:

\[
\text{Polysaccharide recovery ratio} \% = \left( \frac{M_e}{M_o} \right) \times 100
\]

where \(M_o\) and \(M_e\) were the concentrations of levan (μg/ml) in the solutions before and after adsorption by resin, respectively.

2.5.4. Characterization of levan before and after adsorption by resin

The UV–vis spectra of the levan solutions before and after adsorption by resin were determined by a Shimadzu UV-2450 spectrophotometer. In addition, the levan solutions before and after adsorption by resin were photographed by a Sony T700 digital camera (Sony Corp., Park Ridge, NJ).

### Table 1

<table>
<thead>
<tr>
<th>Resin</th>
<th>Particle diameter (mm)</th>
<th>Surface area (m²/g)</th>
<th>Average pore diameter (nm)</th>
<th>Appearance</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3520</td>
<td>0.3–1.25</td>
<td>480–520</td>
<td>8.5–9.0</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>D4020</td>
<td>0.3–1.25</td>
<td>540–520</td>
<td>10–10.5</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>D101</td>
<td>0.2–0.6</td>
<td>400–650</td>
<td>10–12</td>
<td>Milk white</td>
<td>Non-polar</td>
</tr>
<tr>
<td>AB-8</td>
<td>0.3–1.25</td>
<td>480–520</td>
<td>13–14</td>
<td>Milk white</td>
<td>Weak-polar</td>
</tr>
<tr>
<td>NAK-II</td>
<td>0.3–1.25</td>
<td>160–200</td>
<td>14.5–15.5</td>
<td>Reddish brown</td>
<td>Polar</td>
</tr>
<tr>
<td>S-8</td>
<td>0.3–1.25</td>
<td>100–120</td>
<td>28–30</td>
<td>Slight yellow</td>
<td>Polar</td>
</tr>
</tbody>
</table>
The molecular weights of levan before and after adsorption by resin were determined on an Agilent 1100 HPLC system equipped with a refractive index detector (RID) and a TSK-GEL G3000SWxl column (7.5 × 300 mm, Tosoh Corp., Tokyo, Japan). The column was eluted with 0.1 M Na₂SO₄ solutions at a flow rate of 0.85 ml/min. Pullulan P-800, P-400, P-200, P-100, P-20, P-10 and P-5 (Showa denko, Japan) were used as standards for molecular weight measurement.

2.6. Statistical analysis

The data were presented as mean ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests. Difference was considered to be statistically significant if \( P < 0.05 \). All statistical analyses were carried out by SPSS for Windows, Version11.5 (SPSS, Chicago, IL).

3. Results and discussion

3.1. Comparison of the decoloration and deproteinization ratios of different resins

In order to compare the decoloration and deproteinization ratios of different resins, pre-weighed amount of hydrated adsorbent and crude levan solution (5 mg/ml, pH 7.0) were shaken at 25 °C for 12 h. As shown in Fig. 1, the polysaccharide recovery ratios for the resins tested were all more than 65%, indicating that macroporous resins had weak adsorption capacities to levan. Moreover, the decoloration and deproteinization ratios of S-8 resin were considerably higher than those of other resins (\( P < 0.05 \)), which correlated with the physical properties of the resins (such as polarity, average pore diameter and surface area) and the chemical features of the adsorbed substance. As shown in Table 1, S-8 resin had much bigger average pore diameter than other resins. This feature allowed the solute molecules to more easily reach and adsorb to the inner of the resin and resulted in better decoloration and deproteinization efficiencies of the crude levan solution. Therefore, S-8 resin was chosen for further study.

3.2. Effect of pH on the decoloration and deproteinization ratios of S-8 resin

The pH value of sample solution is very important for the adsorption properties of resins, since the pH value determines the extent of ionization of adsorbed molecules, thereby affecting their adsorption affinity (Fu et al., 2005, 2006). Therefore, the effects of pH on the decoloration and deproteinization ratios of S-8 resin were investigated. As shown in Fig. 2a, the decoloration and deproteinization ratios of S-8 resin decreased with the increase of pH value. With the increase in pH, however, the polysaccharide recovery ratio increased. These results indicated that pigments, proteins and levan were adsorbed more easily at a relatively low pH. It has been reported that that hydrogen bonding plays an important role in the adsorption process of macroporous resins. At higher pH value, the hydrogen bonding interactions are reduced, which result in the decrease of the adsorption capacity of resins (Fu et al., 2005, 2006).

Statistical analysis showed that the decoloration and deproteinization ratios of S-8 resin at acidic solutions were higher than those of neutral and alkaline solutions (\( P < 0.05 \)). However, there was no significant difference among pH 4.0, 5.0 and 6.0 for the decoloration and deproteinization ratios of S-8 resin (\( P > 0.05 \)). In addition, the polysaccharide recovery ratio of S-8 resin at pH 6.0 was higher than that of pH 4.0 or pH 5.0 (\( P < 0.05 \)). These results indicated that pH value of 6.0 was favorable for the decoloration and deproteinization of crude levan solution. Thus, the pH value of the solution was adjusted to 6.0 for all the following experiments.

3.3. Effect of temperature on the decoloration and deproteinization ratios of S-8 resin

Temperature is also a very important factor affecting the adsorption properties of resins. To investigate the effect of temperature on the decoloration and deproteinization ratios of S-8 resin, pre-weighed amount of hydrated adsorbent (equal to 1 g dry S-8...
and 50 ml of 5 mg/ml crude levan solution (pH 6.0) were shaken in a constant temperature water-bath shaker at 25, 30, 35 and 40 °C for 12 h. As shown in Fig. 2b, the highest decoloration and deproteinization ratios were both achieved at 35 °C ($P < 0.05$). However, statistic analysis showed that there was no significant difference among all the temperatures tested for the polysaccharide recovery ratio ($P > 0.05$). It has been reported that two kinds of counteraction coexist in the adsorption process of the resin (Fu et al., 2006). On one hand, the thermal motion of the solute molecules increases with the increase of temperature, which benefits for the molecules to reach and adsorb on the surface and inner of the resin, thereby the adsorption capacity of the resin increases. On the other hand, the increased thermal motion of the solute molecules could also cause the adsorbed substances to desorb from the resin, which results in the decrease of the adsorption capacity of the resin. Our results indicated that S-8 resin had the highest adsorption capacity to pigments and proteins at 35 °C. Thus, the following experiments were carried out at 35 °C.

3.4. Effect of adsorption time on the decoloration and deproteinization ratios of S-8 resin

To investigate the effect of adsorption time on the decoloration and deproteinization ratios of S-8 resin, pre-weighed amount of hydrated adsorbent and crude levan solution (5 mg/ml, pH 6.0) were shaken at 35 °C. As shown in Fig. 3, the decoloration and deproteinization ratios increased rapidly in the first 30 min, and reached a plateau at 70 min. In addition, the polysaccharide recovery ratio decreased with the increase of adsorption time and also reached the plateau at 70 min. These results indicated that the adsorption property of S-8 resin reached the equilibrium at around 70 min. It has been suggested that the initial fast adsorption process of the resin in 30 min was due to the high diffusivity of solute molecules into micropores of the resin, and the slow adsorption process of the resin after 30 min was due to the high intraparticle mass transfer resistance within the resin (Fu et al., 2005; Gao et al., 2007). Under the optimized static adsorption conditions, the decoloration, deproteinization and polysaccharide recovery ratios of S-8 resin were 76.8%, 78.9% and 69.0%, respectively.

3.5. Effect of flow rate on the decoloration and deproteinization ratios of S-8 resin

It has been reported that the adsorption capacity of the resin was achieved via surface adsorption, size sieving action, surface electrical property, hydrogen bonding interactions, etc. When the
adsorption process reaches the break point, the adsorption capacity of the resin will decrease or even disappear and the solutes will leak from the resin (Fu et al., 2006). Thus, it is essential to investigate into the processing volume and the flow rate of sample solution. To investigate the effect of flow rate on the decoloration and deproteinization ratios, 180 ml of 5 mg/ml crude levan solution (pH 6.0) was loaded at different flow rate (2, 4, 6 and 8 BV/h) to a glass column (2.0 × 30 cm) wet-packed with 10 ml of S-8 resin. As shown in Fig. 4, both of the decoloration and deproteinization ratios decreased gradually with the increase of the volume of sample solution. In addition, the highest decoloration and deproteinization ratios were achieved at the flow rate of 2 BV/h ($P < 0.05$),

![Graph](image)

**Fig. 6.** The UV–vis spectra (a), photograph (b) and HPLC chromatograms (c) of levan extract before (A) and after (B) adsorption by S-8 resin.
which was probably due to that the solute molecules were easier to reach and adsorbed on the surface and inner of the resin at relatively lower flow rate. Notably, at the flow rate of 2 BV/h, the decoloration and deproteinization ratios were still over 80% when the volume of sample solution reached 8 BV. Therefore, 2 BV/h was selected as the best flow rate for further experiments.

3.6. Effect of sample concentration on the decoloration and deproteinization ratios of S-8 resin

To investigate the effect of sample concentration on the decoloration and deproteinization ratios, 180 ml of crude levan solution (pH 6.0) with different concentrations (2.5, 5, 7.5 and 10 mg/ml) was loaded at 2 BV/h to a glass column (2.0 × 30 cm) wet-packed with 10 ml of S-8 resin. As shown in Fig. 5, the highest decoloration and deproteinization ratios were achieved at the sample concentration of 2.5 mg/ml (P < 0.05). In this case, the decoloration and deproteinization ratios remained over 80% when the volume of sample solution reached 16 BV. Therefore, 2.5 mg/ml was the suitable sample concentration for dynamic adsorption tests.

Under optimized dynamic adsorption conditions (flow rate of 2 BV/h, 160 ml of 2.5 mg/ml crude levan solution), the decoloration, deproteinization and polysaccharide recovery ratios of S-8 resin were 84.6%, 91.7% and 81.3%, respectively. These results showed that dynamic adsorption process had higher decoloration, deproteinization and polysaccharide recovery ratios than those of the static adsorption process. This was probably due to that the solute molecules were prone to reach and adsorbed to the inner of the resin in dynamic adsorption process. In addition, due to the fact that macroporous resin had weak adsorption capacity to levan, some adsorbed polysaccharides further desorbed from the resin in the continuous sample loading process. In comparison, hexadecimal reagent (CHCl3-BuOH, v/v = 5:1) was used to treat the crude levan extract for seven times. As a result, the corresponding ratios of deproteinization and polysaccharide recovery were 75.5% and 54.8%, respectively. Notably, the use of S-8 resin was much more efficient and economical.

3.7. Characterization of levan before and after adsorption by the resin

In order to validate the decoloration and deproteinization efficiencies of S-8 resin, the levan solutions before and after treatment by the resin were characterized by UV–vis spectroscopy, digital photography and HPLC. As shown in Fig. 6a, the absorbance in the region of 190–900 nm disappeared after S-8 resin treatment, indicating that most of the pigments and proteins were removed by the resin. In addition, the absorbance of levan solutions before and after adsorption (Fig. 6b) also demonstrated the excellent decoloration efficiency of S-8 resin. The molecular weights of levan before and after adsorption were determined to be 1.18 × 10^5 and 1.20 × 10^6, respectively (Fig. 6c), indicating no degradation of levan during the treating process. In addition, the concentrations of polysaccharide and protein in the treated sample were 1.50 mg/ml and 5.19 μg/ml, respectively, while their corresponding concentrations in the crude levan extract were 1.85 mg/ml and 62.5 μg/ml, respectively. Therefore, our results suggested that the method developed could be a promising procedure for the simultaneous decoloration and deproteinization of crude levan extract by using S-8 resin.

4. Conclusion

In this study, a simple method for simultaneous decoloration and deproteinization of crude levan extract using macroporous resin has been successfully developed. Among various macroporous resins, tested, S-8 resin offered the best decoloration and deproteinization ratios. In addition, dynamic adsorption tests showed higher decoloration, deproteinization and polysaccharide recovery ratios than static adsorption tests. Compared to the conventional chemical methods, the method developed is superior because of its low-cost, high efficiency and procedural simplicity. Therefore, the method would provide a potential approach for large-scale production of levan for its wide applications in food and medicine.

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References


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