Purification and characterization of *Pseudomonas aeruginosa* PAO1 Asparaginase

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**Abstract**

The prevalence of bacterial infection has been gradually increased for the last decades. This phenomenon is predicted as one of serious problems to human security in the next decades. *Helicobacter pylori* has been clarified to be related to gastric inflammation and a variety of diseases including gastric cancer. Recently, it has been known that *H. pylori* asparaginase is necessary for the growth in host infection and might be involved in inhibition of lymphocyte function at gastric niche. On the other hand, *Pseudomonas aeruginosa* is known to cause a fence-sitting infectious disease. However, no studies on a physiological role of asparaginase in relation to pathogenicity of *P. aeruginosa* have been found. Also, there are no studies on its physiological role in relation to pathogenicity of *P. aeruginosa* have been found. Therefore, we focused on purification and characterization of *P. aeruginosa* PAO1 asparaginase in order to clarify its physiological roles in this study. Intracellular asparaginase was highly purified and characterized from *P. aeruginosa* PAO1. The profile of DEAE-cellulose A 500 column chromatography suggested that two types of asparaginases were produced in *P. aeruginosa* PAO1. The optimum pH and temperature of the one enzyme was 9.5 and 40°C, respectively while those of the another was 9.5 and 65°C, respectively. Those results indicated that *P. aeruginosa* PAO1 had two different types of asparaginases.

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**Keywords:** Asparaginase; *Pseudomonas aeruginosa* PAO1; purification; characterization

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1. Introduction

L-asparaginase (E. C. 3. 5. 1. 1) catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia and exists in many animal tissues, bacteria and plants. Microbial asparaginases have been particularly studied for their applications as therapeutic agents in the treatment of certain types of human cancer. Enzymes isolated from *Escherichia coli* and *Erwinia carotovora* have been used for the treatment of acute lymphoblastic leukemia. The critical factors required for a chemotherapeutically active L-asparaginase are optimal activity under physiological conditions, a low $K_m$ for L-asparagine, high substrate specificity, and a long half-life in the blood stream.

The prevalence of bacterial infection has been gradually increased for the last decades. This phenomenon is predicted as one of serious problems to human security in the next decades. *Pseudomonas aeruginosa* is a ubiquitous environmental bacterium and causes various severe opportunistic infections in patients of serious hospitalization. The following is one of the three causes of opportunistic infections. Feature of this microbe as a pathogen is of intrinsic resistance to antibiotics and disinfectants. Therefore, nosocomial infection has become a major problem. Recently it has been demonstrated that asparaginase from *Helicobacter pylori*, a common human pathogen that predisposes individuals to gastric inflammation and a variety of diseases including gastric cancer is required for utilization of asparagine and the establishment of colonization. No reports on physiological roles of asparaginase from *P. aeruginosa* have been found so far.

Characterization of asparaginase from *P. aeruginosa*, pathogenic microorganism is of interest and significance from the viewpoint of its pathophysiological roles. In this report the characterization of asparaginase from *P. aeruginosa* is described.

2. Materials and Methods

2.1. Materials

DEAE-cellufine A-500 and Butyl-cellulofine were purchased from JNC Co. and DEAE-TOYOPEARL was from TOSOH Co. All of the other chemicals were of analytical grade and purchased from Wako Pure Chemical Co.

2.2. Bacterial strain, medium and culture conditions

*Pseudomonas aeruginosa* PAO1 (NBRC 106052) used in this study was purchased from National Biological Resource Center. The strain was cultured in 802 medium (polypepton1% (w/v), yeast extract 0.2% (w/v), MgSO$_4$7H$_2$O 0.1% (w/v), pH 7.0). Culture condition was at 30°C for 24 hours at 200 rpm in shake culture in 2L Sakaguchi flasks containing 800 mL of medium.

2.3. Purification of asparaginase

The cells grown in the total 14.4 L of 802 medium were harvested, washed with 10mM Tris buffer (pH 8.0), and suspended in the same buffer. The cells were disrupted by sonication. Cell debris was then removed by centrifuging at 15,650×g for 15 min at 4°C. The supernatant was collected, and ammonium sulfate added to final concentration of 70 % saturation. After centrifugation at 15,650 × g for 15min, the supernatant was removed, and the precipitated protein re-suspended in 10mM Tris buffer (pH 8.0) and dialyzed against the same buffer. The sample was loaded a DEAE-cellufine A500 column and eluted with a linear gradient of 0-300 mM NaCl. Asparaginase activity was detected in the two fractions eluted at the different NaCl concentration. The result suggested that two types of asparaginases were produced (ASNase I and ASNase II). These fractions containing asparaginase activity was collected each and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). ASNase I was loaded a DEAE-TOYOPEARL column and the fractions containing asparaginase activity found in non-absorbed fractions were collected and dialyzed against 10 mM Tris buffer (pH 8.0). ASNase I containing 25% ammonium sulfate was loaded in a Butyl-cellulofine column and eluted with a linear gradient of 25-0% ammonium sulfate in 10 mM Tris buffer (pH 8.0). After the fractions containing asparaginase activity were dialyzed against 10 mM Tris buffer (pH 8.0),
ASNase II was loaded a DEAE-cellulofine A500 column and eluted with a linear gradient of 0-250 mM NaCl. The fractions containing asparaginase activity were collected and dialyzed against 10 mM Tris buffer (pH 8.0). ASNase II containing 25% ammonium sulfate was loaded in a Butyl-cellulofine column and eluted with a linear gradient of 25-0% ammonium sulfate in 10 mM Tris pH 8.0. The fractions containing asparaginase activity were then dialyzed against 10 mM Tris buffer (pH 8.0). ASNase II containing 15% ammonium sulfate was loaded in a Butyl-cellulofine column and eluted with a stepwise of 15-10% ammonium sulfate in 10 mM Tris buffer (pH 8.0). The fractions containing asparaginase activity were then dialyzed against 10 mM Tris buffer (pH 8.0).

2.4. Enzyme assay

Asparaginase activity was measured by determining the amount of ammonia formed with glutamate dehydrogenase (GLDH). The reaction mixture contained 100 mM Tris buffer (pH 8.0), 30 mM L-asparagine, 100 μL enzyme, and water in a final volume of 1 mL. After the reaction was set for 30 min at 30°C, the reaction was then terminated by boiling it for another 3 minutes. The 20 μL of reaction mixture was transferred to a GLDH reaction mixture containing 100 mM Tris buffer pH 8.0, 2.4 mM NADH, 100 mM 2-oxoglutarate, and 5 units of GLDH in a total volume of 1 mL. The absorbance change at 340 nm was measured after incubating the mixture at 30°C for 90 minutes. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol ammonia per min5. Protein concentration was measured using the Lowry method with ovalbumin as the standard6.

3. Results and Discussions

3.1. Purification of Asparaginase from Pseudomonas aeruginosa PAO1

Two asparaginases were found and highly purified by ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography as described in Materials and Methods. Through these purification steps, the specific activities of ASNase I and ASNase II were 16.9 and 0.714 U/mg, respectively (Table 1,2), and the purity was evaluated at each step by SDS-PAGE.

Table 1. Purification profile of ASNase I

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein (mg)</th>
<th>Total activity (U)</th>
<th>S.A. (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4239</td>
<td>731</td>
<td>0.172</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>3994</td>
<td>774</td>
<td>0.194</td>
<td>1.13</td>
<td>106</td>
</tr>
<tr>
<td>DEAE-cellufine (pH 8.0)</td>
<td>19</td>
<td>80</td>
<td>4.19</td>
<td>2404</td>
<td>10.9</td>
</tr>
<tr>
<td>DEAE-Toyopearl (pH 7.0)</td>
<td>7.5</td>
<td>71</td>
<td>9.46</td>
<td>55</td>
<td>9.71</td>
</tr>
<tr>
<td>Butyl-cellulofine</td>
<td>3.7</td>
<td>62</td>
<td>16.9</td>
<td>98.3</td>
<td>8.48</td>
</tr>
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</table>

Table 2. Purification profile of the ASNaseII

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein (mg)</th>
<th>Total activity (U)</th>
<th>S.A. (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4239</td>
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<tr>
<td>Ammonium sulphate</td>
<td>3994</td>
<td>774</td>
<td>0.194</td>
<td>1.13</td>
<td>106</td>
</tr>
<tr>
<td>DEAE-cellufine (pH 8.0)</td>
<td>176</td>
<td>232</td>
<td>1.32</td>
<td>7.67</td>
<td>31.7</td>
</tr>
<tr>
<td>DEAE-Toyopearl (pH 7.0)</td>
<td>65</td>
<td>156</td>
<td>2.4</td>
<td>14</td>
<td>21.3</td>
</tr>
<tr>
<td>Butyl-cellulofine I</td>
<td>8.7</td>
<td>9</td>
<td>0.981</td>
<td>5.7</td>
<td>1.23</td>
</tr>
<tr>
<td>Butyl-cellulofine II</td>
<td>7</td>
<td>5</td>
<td>0.714</td>
<td>4.15</td>
<td>0.684</td>
</tr>
</tbody>
</table>

Both ASNase I and ASNase II were highly purified through the purification process. The yield of the purified ASNase I was 8.48 %, while the yield of ASNase II was only 0.684 %. Protein profile analysis showed that the
molecular masses of ASNase I and ASNase II were 38 kDa and 35 kDa, respectively (Fig 1). Based on genomic information which is available in KEGG, *P. aeruginosa* PAO1 has two genes encoding asparaginase. The first gene was AnsB gene encoding 362 amino acids with 38.66 kDa. The other gene was AnsA gene encoding 328 amino acids with 34.77 kDa. L-asparaginase from *Pectobacterium carotovorum* and *Thermococcus kodakaraensis* is about 35 kDa [7,8]. This result is similar to the result of ASNase I. Judging from molecular sizes of both enzymes, the ASNase I was supposed to be glutaminase-asparaginase, and the ASNase II was supposed to be L-asparaginase.

![Fig. 1. SDS-PAGE profile of ASNase I. Lane 1, molecular marker; lane 2, crude extract fraction; lane 3, ammonium sulphate-precipitated fraction; lane 4, ion-exchange column-eluted fraction; lane 5 ion-exchange column-eluted fraction, lane 6, hydrophobic interaction column-eluted fraction.](image1)

![Fig. 2. SDS-PAGE profile of T ASNase II Lane 1, molecular marker; lane 2, crude extract fraction; lane 3, ammonium sulfate-precipitated fraction; lane 4, ion-exchange column-eluted fraction; lane 5 ion-exchange column-eluted fraction; lane 6, hydrophobic interaction column-eluted fraction; lane 7, hydrophobic interaction column-eluted fraction.](image2)

### 3.2 Characterization of Asparaginases

The optimum pH and temperature of two asparaginases were further examined. The optimum pH of both ASNase I and ASNase II were 9.5 (Fig. 3). The optimum temperatures of the ASNase I and ASNase II were 65°C and 45°C, respectively (Fig. 4). The result suggested that ASNase I might be more stable than ASNase II.
Fig. 3. The optimum pH of ASNase I (A) and ASNase II (B). Activity of asparaginase under different pH buffer conditions was measured by determining the amount of ammonia formed with glutamate dehydrogenase.

Fig. 4. The optimum temperature of ASNase I (A) and ASNase II (B). Activity of asparaginase under different temperature conditions was measured by determining the amount of ammonia formed with glutamate dehydrogenase.

This indicated that this asparaginase belongs to alkaline amidohydrolase. The optimum pH was different from those of asparaginases from Yersinia pseudotuberculosis and Helicobacter pylori where optimum pH values were 8 and 10, respectively. However, this pH was exactly the same as that of asparaginase found in Thermococcus kodakaraensis.5,9,10

4. Conclusion

In conclusion, two asparaginases, namely ASNase I and ASNase II was highly purified from P. aeruginosa PA01. ASNase I was glutaminase-asparaginase which belongs to different subclass of asparaginase compared to ASNase II which is considered as L-asparaginase.

References


