Purification and Properties of Two Membrane Alkaline Phosphatases from Bacillus subtilis 168

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Two alkaline phosphatases were extracted from the membranes of Bacillus subtilis 168 stationary-phase cells and purified as homogeneous proteins by hydroxyapatite column chromatography. Alkaline phosphatases I and II differed in several properties such as subunit molecular weight, substrate specificity, thermostability, K_m, pH stability, and peptide stability.

Bacillus subtilis makes vegetative and sporulation-specific alkaline phosphatases (APases) (2), although it is uncertain whether or not both enzymes are the product of a single gene. Preliminary data indicated differences in subunit molecular weights (5a). Also, Hulett and coworkers (5) purified two distinct species of APase from a B. subtilis mutant, JH646MS, which hyperproduces the vegetative enzyme. The failure to uncover phosphatase-negative mutants lends support to the notion that B. subtilis encodes multiple APases (1-3, 6).

To determine whether there are one or more enzymes, we undertook to purify the vegetative APases from the membrane of B. subtilis 168 and succeeded in separating two homogeneous APases. An analysis of their properties indicated that there are significant differences between these APases.

B. subtilis produced APase when the cells entered the stationary phase (data not shown). All the APase was in the cells; none was detected in the culture medium. To obtain a large quantity of APase, we grew B. subtilis 168 cells at 37°C in 6,000 ml of modified MOPS medium (pH 6.8) (8) containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 4 mM Tricine, 0.05 mM FeSO_4, 9.5 mM NH_4Cl, 0.28 mM K_2SO_4, 1 mM CaCl_2, 0.53 mM MgCl_2, 0.1 mM MnCl_2, 5 mM (NH_4)_2Mo_7O_24, 400 mM H_2BO_3, 30 mM CoCl_2, 10 mM CuSO_4, 10 mM ZnSO_4, 20 mM glucose, 0.3 mM K_2HPO_4, and tryptophan (50 μg/ml). At 2.5 h after reaching the stationary phase, the cells were harvested and suspended in 20 ml of ST buffer (20% sucrose, 50 mM Tris-HCl [pH 7.5], 1 mM phenylmethylsulfonyl fluoride [PMSF]) containing 2 ml of lysozyme solution (12.5 mg/ml in ST buffer). The mixture was incubated at 30°C for 1 h to prepare protoplasts. The envelope fraction was obtained by lysing the protoplasts in 25 ml of cold 50 mM Tris-HCl (pH 7.5) containing 0.1 mM CaCl_2 and 0.01 mM ZnCl_2 at 4°C for 60 min and centrifuging at 203,000 × g for 30 min at 4°C. The APase activity was recovered in the envelope fraction. To solubilize the APase, we incubated the envelope preparation in TM buffer (1 M Tris-HCl [pH 7.5], 1 M MgSO_4, 0.5 mM PMSF). The supernatant (Mg extract fraction) was obtained by centrifugation at 203,000 × g for 60 min at 4°C and dialyzed against 100 volumes of TKO_3 (10 mM Tris-HCl [pH 7.5], 0.3 M KCl, 0.5 mM PMSF). After dialysis, a precipitate was formed. The APase activity was recovered in the pellet fractions. The pellet thus formed was solubilized in TKO_3 buffer (10 mM Tris-HCl [pH 7.5], 0.5 M KCl, 0.5 mM PMSF). The solubilized APase was applied to a hydroxyapatite column for chromatography. Two activity peaks were resolved by the chromatography (Fig. 1A). Peak I showed one band with a molecular weight of 47,000; peak II showed one band with a molecular weight of 50,000 (Fig. 1B). We denoted the activity in peak I as APase II and the activity in peak II as APase I. APases I and II had a native molecular weight of 115,000 by gel filtration. APases I and II had molecular weights of 110,000 and 95,000, respectively, by native polyacrylamide gel electrophoresis (PAGE) by the method of Hedrick and Smith (4) (data not shown). Therefore, APases I and II are probably both homodimers. Hulett et al. (5) reported that two distinct species of APase from B. subtilis mutant were purified from the supernatant and that one APase was probably a monomer. Yamane and Maruo (10) also reported that B. subtilis 6160-BC6 produces a membrane-bound APase. Although we have no clear explanation for this discrepancy at present, strain differences may be the cause of apparent differences in localization and molecular weight of the native enzyme.

APases I and II were purified about 30-fold with the same specific activities (Table 1). Even though the amount of protein in peak II was the same as that in peak I, the lower activity of peak II relative to that of the peak I (Fig. 1A) was obviously due to the high salt concentration in the fractions in peak II. The purified APase I and II preparations were used for kinetic studies. APases I and II showed maximum activities at pH 10.2 and 10.5, respectively. APase I had a very narrow pH stability range and was most stable at pH 7.3; about 50% of its activity was lost at pH 6 and 8. APase II was stable at a broad range from pH 6.7 to 10.5. Hulett et al. (5) reported a difference in pH stability for two APases from B. subtilis JH646MS. APases I and II had optimal temperatures at 55 and 60°C, respectively. APases I and II were stable until 55°C, and they were completely inactivated at 85 and 75°C, respectively. APase I was more heat stable than APase II. The K_m values of APases I and II for p-nitrophenyl phosphate were estimated to be 5.1 × 10^{-5} and 2.7 × 10^{-4} M, respectively. APase I had stronger affinity than APase II for p-nitrophenyl phosphate. APase I hydrolyzed the following substrates in addition to p-nitrophenyl phosphate to the same extent: adenosine 2'(3')-monophosphate, adenosine 5'-monophosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, adenosine 3',5'-cyclic monophosphate, uridine 2'(3')-monophosphate, uridine 5'-

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activities were (203 evelope Cell Hydroxyapatite chro-

extraction Mg2+ p-nitrophenyl phosphate, as the proteins. at incubated 3, APase milliliters was column eluted 37°C for 15 min. The released inorganic phosphate was measured by the method of Nakamura (7). One unit of activity was defined as the amount of enzyme releasing 1 nmol of p-nitrophenol per min. (B) Sodium dodecyl sulfate-PAGE of APases I and II. Lanes: 1, 2, and 3, APase II preparation; 4, 5, and 6, APase I preparation; 7, mixture of APase I and II preparations; M, molecular weight (M.W.) marker proteins. A total of 2 μg (lanes 1 and 4), 1 μg (lanes 2 and 5), and 0.3 μg (lanes 3, 6, and 7) of protein were applied to the gel.

![Graph](image)

**FIG. 1.** (A) Hydroxyapatite chromatography of alkaline phosphatase. The solubilized APase in TKo.4 buffer was applied to a hydroxyapatite column (9.5 by 2.3 cm) equilibrated with TKo.3 buffer. The column was washed with TKo.3 buffer, and APase activity was eluted by a linear gradient of KCl from 0.5 to 1.0 M and then from 1.0 to 3.0 M in Tris-HCl buffer (pH 7.5) containing 0.5 mM PMSF. Four milliliters was collected in each tube. For determination of APase activity, we prepared a reaction mixture which contained enzyme, 10 mM p-nitrophenyl phosphate, 0.2 M MgSO4, and 0.2 M ethanalamine buffer (pH 10.2) in a total volume of 0.5 ml. The reaction mixture was incubated at 37°C for 15 min. The released inorganic phosphate was measured by the method of Nakamura (7). One unit of activity was defined as the amount of enzyme releasing 1 nmol of p-nitrophenol per min. (B) Sodium dodecyl sulfate-PAGE of APases I and II. Lanes: 1, 2, and 3, APase II preparation; 4, 5, and 6, APase I preparation; 7, mixture of APase I and II preparations; M, molecular weight (M.W.) marker proteins. A total of 2 μg (lanes 1 and 4), 1 μg (lanes 2 and 5), and 0.3 μg (lanes 3, 6, and 7) of protein were applied to the gel.

**TABLE 1.** Purification of APases I and II from *B. subtilis* 168

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U) (10^4)</th>
<th>Total protein (mg)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell envelope</td>
<td>923.6</td>
<td>823.4</td>
<td>1,100</td>
<td>1.0</td>
</tr>
<tr>
<td>Mg2+ extraction</td>
<td>856.2</td>
<td>210.3</td>
<td>4,100</td>
<td>3.7</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;APase I fraction&quot;</td>
<td>158.2</td>
<td>4.7</td>
<td>33,700</td>
<td>30.6</td>
</tr>
<tr>
<td>&quot;APase II fraction&quot;</td>
<td>118.6</td>
<td>3.5</td>
<td>33,900</td>
<td>30.8</td>
</tr>
</tbody>
</table>

a The fractions containing the two peaks, APase I (275 to 330) and APase II (205 to 245) in Fig. 1, were separately dialyzed against TKo.3 buffer, and their activities were measured.

monophosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, 6-phosphogluconate, phosphoenolpyruvate, β-nicotinamide adenine dinucleotide phosphate, pyridoxal 5-phosphate, α-naphthyl phosphate. On the other hand, APase II showed a relatively higher activity on synthetic substrates containing a benzene ring such as p-nitrophenyl phosphate and α-naphthylphosphate. APase II generally had lower activity than APase I on various substrates except adenosine 2′(3′)-monophosphate as described above. APases I and II had no detectable phosphodiesterase activity if we used bis-p-nitrophenyl phosphate as the substrate. Hulet et al. (5) reported that APase IV has low phosphodiesterase activity.

Finally, we did the peptide mapping of APases I and II. To prepare the peptide map of APases I and II by using trypsin, we radiiodinated APases I and II with carrier-free Na125I (obtained from Dupont, NEN Research Products) by the
1826 NOTES

ammonium analyzed by electrophoresis incubated for (trypsin-TPCK) (15:3:12:10, acetone of V. For phy. Fifteen preparations respectively (Fig. 2C). No peptide overlapped any spots from APase I and II (Fig. 2C). Therefore, APases I and II almost certainly differ in their primary sequence.

We concluded, on the basis of differences in subunit molecular weight, substrate specificity, thermostability, $K_m$, pH stability, and peptide maps between APases I and II, that the two distinct species of APase from B. subtilis 168 were the products of separate structural genes of this strain.

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REFERENCES