Purification and Characterization of Maltose Phosphorylase from *Bacillus* sp. RK-1

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Received June 4, 2001; Accepted August 20, 2001

*Bacillus* sp. RK-1 was isolated as a bacterium that produced maltose phosphorylase (MPase) in the culture supernatant. Screening was done from among about 400 isolates that could grow at 55°C in a medium containing maltose as the sole carbon source. The enzyme was purified to an electrophoretically homogeneous state and some properties were investigated. The *M*<sub>e</sub> of the enzyme was estimated to be 170 kDa by gel filtration and 88.5 kDa by SDS-PAGE, suggesting that it consisted of two identical subunits. The enzyme showed optimum activity around pH 6.0-7.0 and the optimum temperature was about 65°C. The enzyme was stable in the range of pH 5.5-8.0 after keeping it at 4°C for 24 h and retained the activity up to about 55°C after keeping it for 15 min. This is the first report about an MPase that could be produced in the culture supernatant. Furthermore, these investigations showed that this MPase is one of the most thermostable ones reported so far.

**Key words:** maltose phosphorylase; *Bacillus*; thermostable; extracellular

Maltose phosphorylase (MPase; maltose: orthophosphate 1-β-D-glucosyltransferase, EC 2.4.1.18) is one of the enzymes classified as disaccharide phosphorylases. It catalyzes the following reversible reaction:

\[
\text{Maltose + phosphate} \rightarrow \beta-D-glucose-1-phosphate (\beta GIP) + D-glucose
\]

In the phosphorylization direction, it is known that MPase is highly specific for maltose, and arsenate can serve as a substitute for phosphate at the cleavage of maltose. In the maltose synthesis direction, β-D-glucosyl-fluoride can be used instead of βGIP.

In the field of applications, MPase is used for the measurement of maltose, taking advantage of its high substrate specificity. Furthermore, as one of the coupling enzymes, it is used for the measurement of α-amylase activity using maltopentaose as a substrate. Recently, MPase was used as the key enzyme in a biosensor for phosphate, consisting of MPase and glucose oxidase. MPase was also used for the synthesis of trehalose from maltose enzymatically coupled with trehalose phosphorylase.

It has been found in various microorganisms such as *Neisseria meningitidis*, *Neisseria perflava*, not specified bacterium lactobacilli, *Lactobacillus brevis*, *Plesiomonas*, *Enterococcus hirae*, and *Propionibacterium freudenreichii*. MPases produced by these bacteria were all intracellularly located, and were not sufficiently stable. From the viewpoint of industrial large-scale fermentation for supplying the enzyme, extracellular production is preferable and more stable activities are desirable.

We screened at a moderately high temperature for microorganisms producing extracellular MPase that could be suitable for industrial use, and isolated *Bacillus* sp. RK-1. In this paper, we describe the screening, purification, and some properties of this enzyme.

**Materials and Methods**

*Chemicals.* Isomaltose was purchased from Seikagaku Kogyo Co., Ltd., Japan. p-Nitrophenyl α-D-glucopyranoside, and p-nitrophenyl β-D-glucopyranoside were purchased from Sigma-Aldrich Co., St. Louis, USA. DEAE-Toyopearl 650M was a product of Tosoh Co., Japan. Superdex 200 pg 16/60, MonoP HR 5/20, and Polybuffer 74 were products of Amersham Pharmacia Biotech, Uppsala, Sweden. Other chemicals were purchased from Wako Pure Chemical Industries, Japan.

*Medium.* The test media used in screening were as follows. The selection medium was composed of 0.2% maltose, 0.05% yeast extract, 0.2% (NH₄)₂SO₄, 0.1% sodium citrate, 0.02% MgSO₄, 7H₂O, 0.35% KH₂PO₄, and 1.5% agar, pH 7.0. The standard medium was composed of 0.5% peptone (Difco Laboratories, Detroit, USA), 0.25% yeast extract, 0.1% glucose, and 1.5% agar, pH 7.0. The medium YPM contained 1% yeast extract, 2% poly-
pepton (Nihon Seiyaku Co., Japan) and 1% maltose, pH 7.0.

**Screening method.** Soils from various areas in Japan were used. A small amount (about 2 g) of soil was suspended in 5 ml of sterilized saline. Then the suspension (0.2 ml) was spread on agar plates of selection medium. The plates were incubated at 55°C for 1 d. Colonies that appeared on the plates were streaked on agar plates of standard medium to isolate a single colony. The plates were incubated at 55°C for 1 d. Single colonies that appeared on the plates were inoculated into 3 ml of the YPM medium in test tubes, followed by shaking cultivation at 55°C for 3 d. The cells in 1 ml of the culture broth were removed by centrifugation at 10,000 × g for 10 min, and the precipitate that formed at 80% saturation with ammonium sulfate was collected by centrifugation at 15,000 × g for 20 min. The precipitate was washed with 1 ml of 10 mM acetate buffer, pH 6.0, containing 80% of ammonium sulfate, and dissolved in 0.2 ml of the same buffer without ammonium sulfate. The maltose phosphorylase activity of this enzyme solution was tested under the standard assay conditions. To distinguish MPase activity from α-D-glucosidase activity, acetate buffer was also used as a substitute for potassium phosphate-citrate buffer in the standard assay conditions. The maltose synthetic activity was also tested by the following conditions. The enzyme solution was dialyzed against the acetate buffer. The reaction mixture contained 0.03 ml of the enzyme solution, 0.02 ml of 0.2 M glucose, 0.02 ml of 0.2 M βG1P, 0.008 ml of 0.5 M acetate buffer, pH 6.0, and water, in a total volume of 0.1 ml. The mixture was incubated at 55°C for 1 d and then boiled for 10 min to stop the reaction. The detection of maltose in this reaction mixture was done by HPLC. A strain capable of producing MPase was selected as the enzyme producer. Identification of the isolated bacterium was done by Japan Food Research Laboratories (Tokyo, Japan) according to the methods described in "Bergey's Manual of Systematic Bacteriology" and "The Genus Bacillus".

**Assay of MPase activity.** MPase activity was assayed by the following method. The reaction mixture for maltose phosphorylase contained 0.6 ml of 2% maltose, 0.06 ml of 0.5 M potassium phosphate-citrate buffer (pH 6.0), enzyme solution, and water in a total volume of 1.2 ml. The mixture was incubated for 10 min at 60°C and then boiled for 10 min to stop the reaction. The amount of glucose liberated was measured by the mutarotase-glucose oxidase method using the Glucose CII-Test Kit (Wako Pure Chemical Ind., Japan). One unit of the enzyme activity was defined as the amount of the enzyme that liberates 1 jumole of glucose per min under these conditions. The specific activity was expressed by the enzyme activity per mg of protein.

**Assay of protein.** In the purification steps by column chromatography, the protein elution pattern was monitored spectrophotometrically as the absorbance at 280 nm. Protein concentration was also measured by the method of Lowry et al. using bovine serum albumin as the standard.

**Purification of MPase.** The purification of MPase was monitored by assaying MPase activity and all procedures were done at 10°C.

RK-1 was cultured aerobically for 16 h by shaking at 55°C in 3 l of YPM medium. Solid ammonium sulfate was added to the culture supernatant after the cells had been removed by centrifugation at 10,000 × g for 10 min, and precipitate that formed at between 40 and 60% saturation with ammonium sulfate was collected by centrifugation at 15,000 × g for 20 min. The precipitate was dissolved in a small volume of 10 mM potassium phosphate-citrate buffer, pH 6.0 (buffer A), and then dialyzed against the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation (crude enzyme). This crude enzyme sample was put on a column (2.5 × 20 cm) of DEAE-Toyopearl 650M previously equilibrated with buffer A. After the column was washed with buffer A, the enzyme was eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer A at a flow rate of 4 ml/min. The eluate was fractionated into 8-ml portions and the active fractions were pooled. The enzyme solution was concentrated to a small volume by ultrafiltration using Centriprep-10 (Millipore Co., Bedford, USA) and put on a Superdex 200 pg 16/60 column previously equilibrated with 10 mM acetate buffer, pH 6.0, containing 0.2 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min and the active fractions were pooled. The enzyme solution was concentrated and the buffer was exchanged for 25 mM bis-Tris-HCl, pH 6.3 using Centriprep-10. The concentrated enzyme solution was put on a MonoP HR 5/20 column previously equilibrated with 25 mM bis-Tris-HCl, pH 6.3. After the column was washed with the same buffer, the enzyme was eluted with Polybuffer 74 adjusted to pH 4.0 with HCl at a flow rate of 1 ml/min. The eluate was fractionated into 1-ml portions and the active fractions were pooled as the purified MPase.

**Electrophoresis.** SDS-PAGE of the purified MPase was done by the method of Laemmli using a Phast System (Pharmacia, Uppsala, Sweden) and a PhastGel Gradient 10-15 (10-15% polyacrylamide gel; Pharmacia). Protein bands were stained with Coomassie Brilliant Blue R-250.

**Molecular weight (M.) measurement.** A Superdex
200 pg 16/60 column was used to measure the $M_v$ of the purified MPase. The analysis was done in 10 mm acetate buffer, pH 6.0, containing 0.2 M NaCl at 4°C at a flow rate of 0.5 ml/min. A $M_v$ marker kit (Boehringer Mannheim, Mannheim, Germany) was used for standard proteins.

Isoelectric point (pl) measurement. The pl of purified MPase was measured by chromatofocusing using a MonoP HR 5/20 column described above.

HPLC conditions for measuring of sugars and phosphate. HPLC conditions for measurement of maltose and glucose were as follows: TSK gel Amido 80 (4.6 × 250 mm, Tosoh Co., Japan); mobile phase, acetonitrile: H$_2$O = 76: 24 (v/v); flow rate, 0.8 ml/min; column temp., 80°C and detector, refractive index indicator (RI). HPLC conditions for measurement of βG1P and phosphate were as follows: TSK gel SAX (6.0 × 150 mm, Tosoh Co., Japan); mobile phase, 0.1 M potassium acetate (pH 5.0, adjusted by HCl); flow rate, 1.0 ml/min; column temp., 30°C, and detector, RI.

Kinetic parameters measurement. The Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) for maltose were obtained from Lineweaver-Burk plot by varying the concentration of maltose at the fixed phosphate concentration of 50 mM. The $K_m$ and $V_{max}$ for phosphate were obtained at the fixed maltose concentration of 50 mM in a similar way.

Analysis of the N-terminal amino acid sequence. The amino-terminal amino acid sequence of purified MPase was analyzed with a gas-phase protein sequencer (ABI model 477A). The sample for sequence analysis was prepared by the method of Matsudaira.¹⁸

Results

Screening for MPase-producing bacteria
From among about 400 isolates that could grow at 55°C, 8 isolates that had maltose phosphorolytic activity were obtained. One isolate (RK-1) was chosen for further experiments because of its high productivity of MPase (data not shown). The isolate was identified as one species belonging to the genus Bacillus. Although the taxonomical characteristics of the isolate are similar to those of B. circulans and B. coagulans, the species could not be identified (data not shown).

Purification of MPase
The purification procedure resulted in 12% recovery of the enzyme activity and a 125-fold enrichment of MPase compared to the culture supernatant, as shown in Table 1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate (40-60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200 pg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MonoP</td>
<td></td>
<td></td>
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</tbody>
</table>

¹ Corresponding to 3 liters of the culture broth.

Fig. 1. SDS-PAGE of the Purified MPase and $M_v$ Measurement. (A) SDS-PAGE. Lane 1, purified MPase; Lane 2, standard protein mixture containing myosin ($M_v$ 200 kDa), β-galactosidase (116.2 kDa), bovine serum albumin (66.3 kDa), and aldolase (42.4 kDa). (B) Gel filtration (Superdex 200 pg). 1, purified MPase; 2, 3, and 4, respectively, correspond to catalase (240 kDa), bovine serum albumin (68 kDa), hen egg albumin (45 kDa), and chymotrypsin (25 kDa).

$M_v$ and pl
The apparent $M_v$ of the purified enzyme was estimated to be 170 kDa by gel filtration using a Superdex 200 pg column (Fig. 1(B)). SDS-PAGE of the purified enzyme gave a single protein band with an apparent $M_v$ of 88.5 kDa (Fig. 1(A)). These results suggested that the enzyme consisted of two identical subunits. The pl of the purified enzyme was estimated to be 4.9 by chromatofocusing using MonoP HR 5/20 column (data not shown).

Analysis of maltose phosphorolysis
In order to confirm the purified enzyme to be a phosphorylase and calculate the equilibrium constant, a maltose phosphorolysis reaction was done until no change was observed in the concentrations of reaction components (Table 2). From this result, the equilibrium constant ([βG1P][glucose]/[maltose] [Pi]) was estimated to be approximately 0.08.
Table 2. Analysis of Maltose Phosphorylase

<table>
<thead>
<tr>
<th>Analysis of reaction components (mM)</th>
<th>Maltose</th>
<th>Pi</th>
<th>Glucose</th>
<th>βG1P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>25.0</td>
<td>25.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>After 1 h</td>
<td>20.1</td>
<td>19.3</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>After 2 h</td>
<td>19.7</td>
<td>17.5</td>
<td>5.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

The reaction mixture containing 25 mM maltose, 25 mM potassium phosphate-citrate buffer (pH 6.0), and 20 u/ml purified MPase was incubated at 60°C. At the intervals indicated, the concentrations of reaction components were measured by HPLC as described in Materials and Methods.

Table 3. Substrate Specificity of Phosphorylation of MPase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>100</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>2</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>2</td>
</tr>
<tr>
<td>p-Nitrophenyl</td>
<td>0</td>
</tr>
<tr>
<td>α-D-glucopyranoside</td>
<td>0</td>
</tr>
<tr>
<td>β-D-glucopyranoside</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme activity was assayed as described in the text in the presence of 1% various substrates except for the 60-min incubation. The activity with maltose is taken as 100%.

**Substrate specificity**

The specificity of phosphorylation of various disaccharides and related compounds by the purified enzyme was examined. The results are shown in Table 3. Trehalose, sucrose, lactose, and cellobiose were completely non-reactive for the MPase. Slight action was observed with isomaltose and gentiobiose. These results suggested that this enzyme had high substrate specificity for the α, 1\-4 glycosidic linkage between D-glucose and D-glucose.

**K_m and V_max for substrates**

The reaction of MPase with various concentrations of substrate proceeded according to a Michaelis-Menten type reaction. Double reciprocal plots for the reaction velocities against substrate concentrations gave K_m of 12.3 mM and 2.9 mM for maltose and phosphate, respectively. V_max for maltose and phosphate were 24.1 μmol/min/mg and 20.3 μmol/min/mg, respectively.

**Effects of pH and temperature**

The activities were measured in the range of pH 4.0 to 9.0 using 25 mM potassium phosphate-citrate buffer (pH 4.0–9.0) and 25 mM Tris-HCl buffer (pH 7.5–9.0). When using Tris-HCl buffer, 25 mM K_2HPO_4 was added to the reaction mixture, and pH after mixing with the later compound are indicated.

The enzyme had an optimum pH range from 6.0–7.0 as shown in Fig. 2(A). The pH stability was also assessed by treatment at 4°C for 24 h from pH 4.0 to 9.0 using 25 mM potassium phosphate-citrate buffer (pH 4.0–7.5) and 25 mM Tris-HCl buffer (pH 7.5–9.0). The MPase was stable at pHs between 5.5 and 8.0 under these conditions as shown in Fig. 2(B).

The effects of temperature on activity were also examined. The enzyme activities were measured at various temperatures under standard assay conditions. The maximum activity was found at 65°C, as shown in Fig. 3(A). The thermal stabilities of both of the purified and crude enzyme (see Purification of MPase in Materials and Methods) were also assessed in 25 mM potassium phosphate-citrate buffer, pH 6.0, after incubation at various temperatures for 15 min. As shown in Fig. 3(B), the purified and crude MPase were completely stable up to 55°C and 60°C, respectively.
Amino-terminal amino acid sequence

The amino-terminal amino acid sequence of MPase, analyzed chemically, was MYYNRLFVDEWTLKAQLD. No proteins similar to this amino acid sequence were found in the databases.

Discussion

We have isolated Bacillus sp. RK-1 producing extracellular MPase. We have also purified this enzyme and investigated some of its properties.

The extracellular production is one of the characteristic points that are distinct from other MPases. This is of great advantage for supplying the enzyme for commercial use. To our knowledge, this is the first report about bacterial extracellular production of disaccharide phosphorylase. We also found that the MPase from Bacillus sp. RK-1 is a maltose-dependent inducible enzyme because no or only a slight activity was detected when glucose, sucrose, or soluble starch was used in place of maltose as a carbon source (data not shown). It is interesting to investigate the mechanisms of extracellular and inducible production of MPase although we have not studied them in full yet. Detailed examination will clarify them in the near future.

The study of some properties of this MPase show that it is one of the most thermostable MPases reported so far. A crude enzyme preparation retained 94% of its activity after it was kept at 65°C for 15 minutes. But the purified enzyme showed a bit lower thermostability than this crude preparation. The cause of this observation remains unknown. One possibility is that some stabilizers of MPase existed in the culture supernatant and were eliminated by the purification procedures. Another possibility is dissociation of the enzyme, consisting of two subunits, into its monomeric, inactive form during purification procedures. The existence of di- or tetrameric active forms is a common feature for various types of phosphorylases, however, it was reported that the monomeric forms of different phosphorylases have no or only a greatly reduced activity.9,20

From the viewpoint of industrial large-scale fermentation for supplying the enzyme for commercial use, MPase from Bacillus sp. RK-1 has some great advantages. Besides extracellular production by an aerobic bacterium and thermostability, it has a potential for the highest productivity among all MPases reported so far (Table 4). We are considering that it is worthwhile to clone this enzyme gene. We are also interested in the mechanisms of extracellular and maltose-dependent inducible production and molecular comparison with other disaccharide phosphorylases such as sucrose phosphorylase and cellobiose phosphorylase that have been cloned and sequenced. Therefore we are now cloning and characterizing of this enzyme gene.

Table 4. Comparison of Productivities and Stabilities of Various Microbial MPases

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Enzyme activity</th>
<th>Stability</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus brevis</td>
<td>72</td>
<td>&gt;90% (35°C for 10 min)</td>
<td>5</td>
</tr>
<tr>
<td>ATCC 8287</td>
<td>40</td>
<td>94% (50°C for 15 min)</td>
<td>8</td>
</tr>
<tr>
<td>Propionibacterium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>freudenreichii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus hirae</td>
<td>107</td>
<td>&gt;90% (55°C for 10 min)</td>
<td>7</td>
</tr>
<tr>
<td>IFO 3181</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus sp. RK-1</td>
<td>165</td>
<td>&gt;95% (55°C for 15 min)</td>
<td>this study</td>
</tr>
</tbody>
</table>

* Phosphorylolytic activities in the cell extracts were reported in each reference, and are indicated as u/l broth.

References

Maltose Phosphorylase from Bacillus sp. RK-1


