Partial Purification and Properties of d-Glucosamine 6-Phosphate N-Acetyltransferase from Zoospores of Blastocladiella emersonii

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A d-glucosamine 6-phosphate N-acetyltransferase from Blastocladiella emersonii zoospores was partially purified to a specific activity of 2.41 IU per mg of protein. Its pH optimum was 8.05 and its $K_m$ values were $2.4 \times 10^{-4}$ M d-glucosamine 6-phosphate and $0.38 \times 10^{-4}$ M Na$_2$S-acetyl coenzyme A.

d-Glucosamine 6-phosphate N-acetyltransferase (EC 2.3.1.4) catalyzes a step in the biosynthesis of uridine 5'-diphosphate-N-acetyl d-glucosamine, substrate for a chitin synthetase in zoospores (2,4; R. B. Myers and E. C. Cantino, The gamma particle. A study of cell-organelle interactions in the development of the water mold Blastocladiella emersonii, in press) of Blastocladiella emersonii Cantino and Hyatt. The acetyltransferase has been purified and characterized. Although it was refined 75- and 314-fold from tissues of mammals (12) and higher plants (15), the specific activities (SA) achieved were not remarkable. On the other hand, Neurospora crassa (5) and, as we report here, B. emersonii contain much higher starting levels of the acetyltransferase, hence they can be processed to yield a higher final SA. Some properties of the B. emersonii acetyltransferase are herewith compared with information presently available about it in other organisms.

The standard assay mixture contained 40 mM potassium phosphate (pH 8.1), 0.4 mM Na$_2$S-acetyl coenzyme A (AcCoA; Sigma, 92% pure), and 0.8 mM d-glucosamine 6-phosphate (Gm6-P; Sigma) in a final volume of 0.25 ml. Preparations with high activity were diluted in 25 mM potassium phosphate (pH 8.1) containing 1.0 mM ethylene-glycol bis (β-aminophenylether)-N,N'-tetraacetic acid (EGTA) and 1.0 mM KCl. Assays were initiated at 30 °C by addition of enzyme; they were terminated after 10 min by addition of 0.05 ml of 0.8 M K$_2$B$_4$O$_7$ (pH 9.0) and immersion in boiling water for 3 min. Removal of denatured protein was necessary only when whole spore lysates were assayed. N-acetylglucosamine 6-phosphate (AcGm6-P) was determined by a modified (15) Reissig et al. (14) procedure. Protein was estimated turbidimetrically (8), the Lowry et al. method (7) being used simultaneously in some instances. Routine controls without substrates, without enzyme, and with heat-inactivated enzyme were employed as needed. One unit (IU) of enzyme is the amount which catalyzed formation of 1 μmol of AcGm6-P per min; SA is expressed as IU/mg of protein.

The original strain (3) of B. emersonii was used. Zoospores were processed by procedures (11) employed for investigating their L-glutamine: Δ-fructose 6-phosphate amidotransferase. Lysates were prepared by centrifugation (1,000 × g, 8 min, 4°C) of spores (ca. 5 × 10⁷), by resuspension in 8 to 12 ml of 1 M sucrose containing 25 mM potassium phosphate (pH 6.8), 1 mM EGTA, and 1 mM KCl for 10 min, by recentrifugation, and by a second resuspension in 9 ml of 0.4 M sucrose in the same buffer for 5 min to rupture the cells. Lysates were fractionated with the procedure used to isolate gamma particles (10; R. B. Myers and E. C. Cantino, in press). The final 110,000 × g supernatants (HSS), containing ca. 0.6 M sucrose in buffer, were stored at −20°C. To 18-ml portions of ice-cold HSS, 4 ml of acetone (−20°C) was added; the precipitate was resuspended in 5 ml of pH 8.1 phosphate buffer. Diethylaminoethyl (DEAE)-cellulose (Whatman DE-11) was washed (13). Columns (1.8 by 25 cm) were equilibrated with three bed volumes of buffer (25 mM potassium phosphate, pH 8.1, 1 mM EGTA, 1 mM KCl), and 5 ml of resuspended acetone precipitate (15 to 32 mg of protein) were applied. Stepwise elution was made with above buffer and then with 10 mM, 100 mM, 500 mM, and 1 M KCl in the same buffer; 10-ml fractions were collected (75 ml/h), highly active fractions being diluted 1:4 with buffer and stored at −20°C.

A representative partial purification of the transferase is summarized in Table 1. The B. emersonii spore lysate is the most active source
lized to avoid complications (9) while establishing kinetic data. Chromatography resolved the material into four peaks (Fig. 1), most of the enzyme activity being eluted on the front shoulder of the third peak. Although purification was only 13-fold, the SA (2.41 IU/mg of protein) of the column purified enzyme (CPE) was much greater than the best values reported for sheep brain and mung bean seeds (Table 2). CPE did not change significantly at -20°C but lost activity with successive freezings and thawings.

Production of AcGm6-P increased linearly with increasing volumes of CPE (28 μg of protein per ml) up to 0.05 ml; hence, the following data were based on assays using 0.04 ml (1.1 μg of protein) of CPE. Since reaction velocity in the standard assay was linear for 16 min, all of the data below were based on 10-min incubations. Enzyme activities in acetate buffers (pH 4.0 to 5.7) were very low; representative activities in phosphate buffers are illustrated in Fig. 2. Studies of reaction velocities with varying concentrations of Gm6-P showed that the enzyme was nearly saturated at 0.8 mM Gm6-P. Double-reciprocal plots yielded straight lines by linear regression analysis (r = 0.99), from which a $K_m$ of $2.4 \times 10^{-4}$ M was calculated (Table 2). Similarly, the effects of AcCoA concentration showed that the enzyme was saturated at 0.4 mM. The $K_m$ calculated as above (again, r = 0.99) was $0.38 \times 10^{-4}$ M (Table 2). Additions of up to 20 mM ethylene diaminetetraacetic acid or Ca and Mg salts had no effect on enzyme activity.

**Table 1. Representative partial purification of B. emersonii acetyltransferase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (IU)</th>
<th>SA</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole spore lysate</td>
<td>74.0</td>
<td>13.85</td>
<td>0.19</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>HSS</td>
<td>32.5</td>
<td>8.94</td>
<td>0.28</td>
<td>1.5</td>
<td>65</td>
</tr>
<tr>
<td>Acetone precipitates</td>
<td>17.3</td>
<td>7.63</td>
<td>0.44</td>
<td>2.3</td>
<td>55</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>10.5*</td>
<td>4.32*</td>
<td>2.41*</td>
<td>12.7*</td>
<td>31*</td>
</tr>
</tbody>
</table>

Procedures which were ineffective in improving the SA of this acetyltransferase included: precipitation by acidification (± heat) of the HSS to pH 4.5 (activity lost); (NH$_4$)$_2$SO$_4$ fractionation of the HSS and of DEAE-cellulose column fractions (activity lost unless sucrose present); sephadex chromatography of the HSS and of acetone precipitates (poor separations with some losses in activity); and recycling of acetone-precipitated fractions from DEAE columns through a second DEAE-cellulose column (activity lost unless sucrose present).

**Table 2. Comparative data for D-glucosamine 6-phosphate N-acetyltransferase from different sources**

<table>
<thead>
<tr>
<th>Determinants</th>
<th>N. crassa (5)</th>
<th>Baker's yeast (10)</th>
<th>Sheep brain (12)</th>
<th>Mung bean seeds (15)</th>
<th>B. emersonii spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA of starting material</td>
<td>$0.47 \times 10^{-1}$</td>
<td>$0.47 \times 10^{-1}$</td>
<td>$0.72 \times 10^{-4}$</td>
<td>$0.85 \times 10^{-4}$</td>
<td>0.19</td>
</tr>
<tr>
<td>Highest SA attained</td>
<td>12.2</td>
<td>to</td>
<td>$0.54 \times 10^{-2}$</td>
<td>$0.27 \times 10^{-1}$</td>
<td>2.41</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>57</td>
<td></td>
<td>59</td>
<td>66</td>
<td>31</td>
</tr>
<tr>
<td>Purification</td>
<td>254-fold</td>
<td>75-fold</td>
<td>314-fold</td>
<td>13-fold</td>
<td></td>
</tr>
<tr>
<td>$K_m$ for Gm6-P (M; $\times 10^4$)</td>
<td>7.8</td>
<td>5</td>
<td>4.5</td>
<td>15.1</td>
<td>2.4 ± 0.1*</td>
</tr>
<tr>
<td>$K_m$ for AcCoA (M; $\times 10^4$)</td>
<td>7.8</td>
<td></td>
<td>2.5</td>
<td>7.1</td>
<td>0.38 ± 0.04*</td>
</tr>
<tr>
<td>Effect of EDTA</td>
<td>none</td>
<td>A</td>
<td>1</td>
<td>8.05*</td>
<td></td>
</tr>
<tr>
<td>pH optimum</td>
<td>6.0 to 7.1*</td>
<td>&gt;7.2 (8.2?)</td>
<td>7.4</td>
<td>5.2</td>
<td>8.05*</td>
</tr>
</tbody>
</table>

* IU, International units; EDTA, ethylenediaminetetraacetic acid; A, activation; I, inhibition.
* Standard deviation; 4 determinations.
* Standard deviation; 3 determinations.
* Vessel and Hassid (15) express misgivings about the high pH optimum reported by Davidson et al. (5), saying that apparently these investigators did not consider the buffering capacity of Gm6-P, that the pH of this substrate had not been adjusted to the desired level, and that this might have accounted for the optimum pH values reported for the enzyme. However, Davidson et al. did, in fact, specifically emphasize that because of its excellent buffering capacity Gm6-P had to be adjusted to the desired pH prior to the addition of enzyme. In any case, there should be no such question about the results obtained with the B. emersonii enzyme since the pH was measured on the complete assay mixtures before and after incubation.
activity. Neither glucosamine nor galactosamine was acetylated by either CPE or HSS.

The pH optimum (8.05) for the B. emersonii acetyltransferase is the highest reported to date; contrary to a recent opinion (footnote d, Table 2), this enzyme seems to have a fairly wide range of pH optima. The B. emersonii $K_m$ values of $2.4 \times 10^{-4}$ M and $0.38 \times 10^{-4}$ M for Gm6-P and AcCoA, respectively, are lower than the sheep brain and mung bean values. They also differ significantly from those for Neurospora. Judging from their insensitivity to ethylenediaminetetraacetic acid, Ca, and Mg, the fungal enzymes may not require divalent cations (although, since both enzymes were assayed in phosphate buffers, cation effects cannot be ruled out entirely).

The spore lysate of B. emersonii is the most active starting source of those reported thus far, its SA being four times that of Neurospora and $>2,000$ times that of other sources (Table 2). In both fungi, the enzyme is presumably an integral part of a system for chitin synthesis. Two other enzymes involved in chitinogenesis and partially purified and characterized from zoospores of B. emersonii are l-glutamine:d-fructose 6-phosphate amidotransferase (6, 11) and chitin synthetase (2, 4; Myers and Cantino, in press). Since one of the earliest studies (6) of this amidotransferase seems to have been inadvertently lost in the botanical literature and thereby ignored for 14 years in reviews on the history of this enzyme (16), it may be appropriate to emphasize here that the zoospore of B. emersonii is an exceptionally good starting source (6, 11) of the amidotransferase as well.

The zoospores of B. emersonii are not known to synthesize chitin. Our assays (unpublished) of purified gamma particles (Myers and Cantino, in press) which carry chitin synthetase, and of mitochondrial fractions, suggest that the acetyltransferase is not associated with these organelles. The uneven intracellular distribution of the amidotransferase, acetyltransferase, and synthetase may be part of a control mechanism for chitin synthesis in zoospores of B. emersonii.

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**LITERATURE CITED**


