Purification and Properties of Bakers’ Yeast Trehalase*

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(Received for publication, August 19, 1963)

Since recent surveys of insect hemolymph (1, 2) have shown that trehalose is the major blood sugar, the hydrolytic enzyme trehalase has also awakened the interest of investigators. A purified preparation of the enzyme from Galleria mellonella (3) and from Phormia regina (4) was recently obtained and some of its properties described. The biosynthesis of trehalose with an enzyme preparation from brewers’ yeast (5) and from bakers’ yeast (6) has been accomplished. One of us (7) has also determined the function of trehalase in brewers’ yeast as an energy source for cell division, compared to that of trehalase of insects, in which it serves as an energy source for flight. Yeast trehalase, however, has never been purified because the bulk of it remains strongly attached to the cell debris, and only a small part is soluble (8, 9). Autolysis, therefore, does not liberate enough enzyme to permit more than a 4- to 5-fold purification. The aim of this report is to present a purification procedure for bakers’ yeast trehalase and to describe some of its properties.

EXPERIMENTAL PROCEDURE

Materials—All reagents were of analytical grade. Trehalose-14C, uniformly labeled, was prepared according to Panek (6). Cakes of bakers’ yeast, Saccharomyces cerevisiae (Fleischmann), were used as a source of enzyme.

Methods—Glucose was determined by the Nelson procedure (10), and protein was estimated by the biuret method according to Starchland (11). Trehalose was determined according to Trevelyan (12) by the anthrone method and demonstrated to be free of glucose by paper chromatography (7).

Enzyme Assay—Trehalase activity was measured by determining the rate of the enzymic release of glucose from trehalose. The reaction mixture contained 10 μmoles of trehalose, 5 to 20 units of the enzyme preparation, and 0.1 M acetate buffer, pH 5.6, in a final volume of 0.5 ml. After 15 minutes at 45°C, the reaction was stopped by immersing the tubes in a boiling water bath for 3 minutes. After cooling, the insoluble material was removed by centrifugation, and glucose was determined in the supernatant fluid. A blank was routinely run to correct for any glucose, or other reducing sugar, present in the trehalose solution and in the enzyme preparation.

One unit of enzyme was defined as that amount which will liberate 0.1 μ mole of glucose from trehalose in 15 minutes under the conditions of the assay. The specific activity is the number of units of enzyme per mg of protein. Under the conditions of the assay, the rate of glucose released from trehalose was proportional to the amount of enzyme added, as well as to the time of incubation (Fig. 1).

RESULTS

Purification of Trehalase—S. cerevisiae, 250 g, was added to 100 ml of ice-cold 0.1 M acetate buffer, pH 5.6, and to 440 g of Ballotini beads in a Waring Blender and homogenized in an ice bath for 90 minutes. The homogenate was centrifuged at 4000 rpm for 20 minutes to remove cellular debris and beads (crude extract).

Precipitation with Ethanol—To the crude extract at pH 5.6, ice-cold ethanol was added dropwise with mechanical stirring to bring the solution to a final concentration of 40% in ethanol. The mixture was allowed to stand for 15 minutes at -10°C, and the precipitate which was formed was removed by centrifugation and dissolved in 0.1 M acetate buffer, pH 5.6 (ethanol fraction).

First Ammonium Sulfate Precipitation—To the ethanol fraction, solid ammonium sulfate was added with stirring to bring the solution to 30% saturation. After standing for 15 minutes in an ice bath, the precipitate was removed by centrifugation and discarded. To the supernatant solution, solid ammonium sulfate was added to raise the concentration to 40%. After 15 minutes the mixture was centrifuged and the precipitate was again discarded. The concentration of the supernatant fluid was raised to 70% saturation, and the solution was then dialyzed overnight at 5°C against several changes of distilled water. The precipitate which formed on dialysis was removed by centrifugation (first ammonium sulfate fraction).

Acetone Precipitation—To the above fraction, 0.5 ml per volume of acetone previously cooled to -20°C was added with stirring during an interval of 15 minutes. The precipitate was removed and discarded. An additional 1.8 ml per initial volume were added to the supernatant solution over a period of 20 minutes. The resulting precipitate was collected by centrifugation and dissolved in 0.1 M acetate buffer, pH 5.6 (acetone fraction).

Alumina Cy Treatment—To the acetone fraction, 2 mg of alumina per mg of protein were added, and after standing for 15 minutes with occasional stirring, the suspension was centrifuged and the precipitate discarded (alumina fraction).

Second Ammonium Sulfate Precipitation—To the alumina-treated supernatant solution, solid ammonium sulfate was added to 70% saturation. After 15 minutes, the precipitate was removed and discarded. The ammonium sulfate saturation of the supernatant fluid was raised to 90%. After 15 minutes the precipitate was removed and discarded (second ammonium sulfate fraction).

At this point the total purification achieved ranged from 30- to 34-fold in different batches (Table I).

pH Effect—The effect of pH upon the hydrolysis of trehalose was determined by the use of phosphate and acetate buffers at suitable pH values. Fig. 2 indicates that the highest activity...
Fig. 1. a, proportionality of glucose formation relative to enzyme concentration. Trehalose, 10 \textmu{}moles, was incubated in 0.1 M acetate buffer, pH 5.6, at 45° for 15 minutes. b, time course of the enzymic reaction. Trehalose, 10 \textmu{}moles, was incubated with 5 units of enzyme preparation in 0.1 M acetate buffer, pH 5.6, at 45°.

**TABLE I**

**Purification of trehalase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, 40%</td>
<td>90</td>
<td>7500</td>
<td>6000</td>
<td>1.25</td>
<td>100.00</td>
</tr>
<tr>
<td>First ammonium sulfate</td>
<td>120</td>
<td>4000</td>
<td>800</td>
<td>5.00</td>
<td>53.50</td>
</tr>
<tr>
<td>Acetone, 70%</td>
<td>15</td>
<td>2100</td>
<td>188</td>
<td>11.20</td>
<td>28.00</td>
</tr>
<tr>
<td>Alumina Cr</td>
<td>15</td>
<td>2100</td>
<td>94</td>
<td>22.40</td>
<td>28.00</td>
</tr>
<tr>
<td>Second ammonium sulfate</td>
<td>40</td>
<td>1300</td>
<td>32</td>
<td>40.50</td>
<td>17.30</td>
</tr>
</tbody>
</table>

Fig. 2. pH optimum curve. The incubation mixture was set up in the appropriate buffer containing 5 units of enzyme and 10 \textmu{}moles of trehalose and incubated at 45° for 15 minutes. ●, 0.1 M acetate buffer; ▲, 0.1 M phosphate buffer.

was obtained at pH 5.7, a value in good agreement with that found by other workers (1, 3) but not with the crude preparations assayed by Lukes and Phaff (9) and by Courtois, Peter, and Kolahi-Zanouzi (13), which gave a pH optimum ranging from 4.5 to 5.35.

**TABLE II**

**Stoichiometry of trehalase reaction**

The indicated amounts of trehalose were incubated in 0.1 M acetate buffer, pH 5.6, with 12 units of enzyme in a total volume of 0.5 ml at 45°. All values have been corrected for a zero time control.

<table>
<thead>
<tr>
<th>Trehalose added</th>
<th>Glucose formed after incubation for</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textmu{}mole</td>
<td>30 min</td>
</tr>
<tr>
<td>0.07</td>
<td>0.13</td>
</tr>
<tr>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>0.11</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Temperature Effect—The maximum rate of hydrolysis occurred when the enzyme was incubated at 40–50° (Fig. 3). Inactivation
FIG. 4. Activity of trehalase as a function of the concentration of trehalose. The reaction mixture contained the indicated amount of trehalose in 0.1 M acetate buffer, pH 5.6, containing 12 units of the enzyme. Incubation was performed for 30 minutes at 45°. •, the Lineweaver-Burk plot; △, the velocity curve.

The activity proceeded rapidly above this limit, so that at 55° 40% of the activity was lost. The crude extracts showed a rapid loss of activity at 3°. The purified enzyme was stable in solution at pH 5.6, and could be stored for over 3 months at -10° without loss of activity.

**Stoichiometry of Reaction**—The data in Table II show that at the concentrations tested, trehalose is hydrolyzed to 2 equivalents of glucose.

**Effects of Substrate Concentration**—The rate of trehalose reaction was studied as a function of trehalose concentration (Fig. 4). The $K_m$ calculated from the data obtained through a Lineweaver-Burk plot for this trehalase preparation was $4.1 \times 10^{-4}$ M. Activities and Inhibitors—Magnesium ions at a concentration of $2.0 \times 10^{-2}$ and $1.0 \times 10^{-2}$ M strongly inhibited trehalase activity. EDTA at $1.0 \times 10^{-3}$ M and brought to pH 6.0 did not alter the activity, whereas EDTA in the presence of magnesium ions reversed the inhibitory action. Under the conditions of the assay, 0.1 M phosphate, 0.1 M acetate, and 0.2 M NaCl neither inhibited nor stimulated hydrolysis.

**Specificity**—In its present state of purity the enzyme preparation was active only with regard to trehalose and raffinose. No hydrolysis of the following compounds could be detected: lactose, sucrose, maltose, melezitose, cellobiose, starch, glycogen, glucose 1-phosphate, glucose 6-phosphate, uridine diphosphate glucose, and trehalose 6-phosphate. Besides sugar determinations, the specificity of the reaction was followed by thin layer chromatography. Kieselguhr G plates were prepared and developed in toluene-ethyl acetate-ethanol (10:5:5) (14) for 20 minutes. The sugars were detected by spraying the plate with a mixture of anisic aldehyde, sulfuric acid, and 95% ethanol (0.5:0.5:9) to which a few drops of acetic acid had been added. The color was developed after heating at 100° for 10 minutes (15).

**Reaction Products**—Trehalase activity was assayed on uniformly labeled trehalose-14C. After incubation, samples were put on paper strips and subjected to electrophoresis on a Ketal apparatus at 200 volts for 4 hours, in 0.2 M borate buffer, pH 10.0. Autoradiograms of the strips showed the formation of only one spot, identified as glucose by fingerprint.

**DISCUSSION**

With some minor exceptions, the properties of bakers’ yeast trehalase were found to be quite similar to those described for insect trehalase. It is interesting to note that bakers’ yeast trehalase does not attack trehalose 6-phosphate, which is cleaved by a specific phosphatase that is also found in the cell. The results obtained by the specificity experiments, as well as by the stoichiometry of the reaction, preclude the existence of a phosphorolytic mechanism of trehalose cleavage, at least in vitro. The expected product of such a phosphorolysis would be glucose and glucose-1-P. Only 1 mole of glucose would be formed per mole of trehalose unless the preparation were also active on glucose 1-phosphate, which was not the case. The activity of this preparation on raffinose is probably due to the presence of another enzyme. The separation of both enzymes to achieve a further purification is under study at present.

**SUMMARY**

An enzyme, trehalase, which hydrolyzes trehalose has been purified 30- to 34-fold from bakers’ yeast. Trehalase showed maximal activity at pH 5.7 in a range between 40° and 50°, the $K_m$ value being $4.1 \times 10^{-4}$ M. The glucose produced in the reaction was isolated and identified by comparison with the authentic substance.

**Acknowledgment**—The authors are indebted to Professor Raymundo Moniz de Aragão for his many helpful suggestions during the course of this study.

**REFERENCES**

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_J. Biol. Chem. 1964, 239:1671-1673._