Isolation and Characterization of Protoporphyrin IX from Bacterial Catalase*

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Catalase has been isolated and crystallized from the bacterium, Micrococcus lysodeikticus, but the prosthetic group of the enzyme was identified only by indirect means (1). The method described by Herbert and Pinsent (1) for the isolation of catalase from bacteria employed 78 liters of starting material. This procedure could not be applied with equal success when the starting material consisted of only 1 liter of culture medium. The method described in the present communication is based on that of Herbert and Pinsent, but includes modifications which permit the isolation of relatively pure catalase from 1 liter of culture medium. Protoporphyrin IX has been characterized as the prosthetic group by isolation of the compound from the purified enzyme.

EXPERIMENTAL PROCEDURE

Methods

M. lysodeikticus strain 2665 was grown in 1 liter of liquid medium containing 0.5% Bacto-peptone, 0.5% NaCl, 0.3% beef extract, and 0.1% yeast extract. Cultures were harvested after incubation at 37° for 48 hours on a shaker (2). The washed cells were partially disrupted in a mechanical disintegrator (3), and then lysed by treatment with crystalline lysozyme for 4 hours at 37° (4).

The early steps in purification of the enzyme were essentially those presented by Herbert (4). After the first partition in ethanol and (NH₄)₂SO₄, the lower layer was repeatedly treated with absolute ethanol until the layer was colorless indicating a nearly complete separation of the catalase. The upper layer and ethanol fractions were combined, shaken for 15 minutes with an equal volume of chloroform, and centrifuged. The supernatant was dialyzed against several changes of cold distilled water, and lyophilized.

Catalase activity was determined by iodometric titration (5). Ultracentrifugal analysis of the enzyme was done in a Beckman Spinco, model E, ultracentrifuge. Lyophilized catalase was dissolved to a concentration of 57.7 mg per ml in 0.1 M acetate buffer at pH 5.6.

Hemin was split from the enzyme by acetone-HCl treatment (6). For comparative purposes hemin also was prepared from the hemoglobin of human red blood cells (7). Crystalline hemin from the latter preparation was recrystallized from a pyridine-chloroform solution (8).

Protoporphyrin was prepared from bacterial hemin by modifying the procedure of Fisher and Orth (9). The progress of the reaction was followed by examination under an ultraviolet lamp with maximal light emission at 3,600 Å. When a pink fluorescence appeared in the solution, excess iron was removed by filtration, and the porphyrin precipitated by the addition of saturated NaCl. The precipitated porphyrin was extracted into chloroform by repeated treatment, and the combined extracts were evaporated to dryness in a vacuum. The method of Ramsey (10) was employed to prepare protoporphyrin from blood hemin. All procedures involving preparation of protoporphyrin were carried out in darkness except for ultraviolet illumination.

Further purification of the porphyrin was effected by chromatography on cellulose powder with lutidine-water (5:3) as the developing solvent. Protoporphyrin was dissolved in 2% NH₄OH, and applied to the column. During development the porphyrin zone was detected by its pink fluorescence in ultraviolet light.

The protoporphyrins were esterified by the procedure of Chu (11). The dimethylesters were chromatographed on Whatman No. 1 paper with the use of the method of Bogorad and Granick (12). Melting points were determined with a Fisher-Johns apparatus, and are reported as uncorrected values.

Spectrophotometric characterization of the compounds was determined in a Beckman model DU spectrophotometer. The concentration of bacterial protoporphyrin was determined from a calibration curve obtained by plotting optical density at 408 nm against concentration (13). To check the accuracy of the calibration curve, a similar curve was constructed with samples of protoporphyrin IX prepared from blood hemin. The two curves were identical.

RESULTS AND DISCUSSION

It proved impossible when working with 1-liter quantities of culture material to obtain by previous methods enough enzyme to use for isolation of the prosthetic group. Preparation of catalase by the above procedure produced a greater yield of enzyme than could be obtained with other methods (1, 4). The increased
yield resulted from the summation of two steps in these methods. The steps eliminated were the second partition in ethanol and (NH$_4$)$_2$SO$_4$ and the second (NH$_4$)$_2$SO$_4$ fractionation, and the introduction of repeated extractions with ethanol of the lower layer appearing after the first ethanol-(NH$_4$)$_2$SO$_4$ partition, as well as extensive dialysis of the (NH$_4$)$_2$SO$_4$ fraction. The data presented in Table I demonstrate that the enzyme prepared by our procedure has activity and purity similar to the preparations of Herbert and Pinsent (1).

Crystallization results in about a one-third increase in activity, but also considerably reduces the yield of enzyme (4). Catalase prepared by our procedure was not crystalline. However, evidence obtained from ultracentrifugal analyses demonstrated that the purity of our preparation was similar to the crystalline enzyme. Samples of our preparation showed a single homogeneous component with some heavier and lighter impurities. The heavier material moved away from the principal component as the length of centrifugation time increased. These results are similar to those reported by Cecil and Ogston (14) for the crystalline enzyme prepared from M. lysodeikticus (1).

Table II presents the absorption spectra values obtained for protoporphyrin and its derivatives prepared from M. lysodeikticus and human red blood cells. The spectra for hemin were determined in pyridine solution; those of protoporphyrin, in 5% HCl. Chu and Chu (7) reported maximal absorption values for blood hemin of 527 and 557 m$\mu$. Although these values are not in exact agreement with those of Table II, they are within the same range. Attempts to further characterize the hemin samples by the chromatographic procedures of Chu and Chu (7) were unsuccessful.

As can be seen from Table II the absorption spectrum of neither hemin nor protoporphyrin prepared from bacterial catalase was in exact accord with the same substances obtained from hemoglobin. Therefore to establish that the two substances were identical, and that the compound from bacteria was indeed protoporphyrin IX, the porphyrins were esterified. Comparisons between the dimethylesters are shown in Table II. The paper chromatographic $R_F$ values and melting points of the two esters are in agreement. The $R_F$ values also agree with those presented by Bogorad and Granick (12) for the dimethylester of protoporphyrin IX, and the melting point figures are in agreement with that reported for the compound (10, 15).

Since the absorption data indicated that the bacterial protoporphyrin might not be quite pure, the pure dimethylester of the compound was hydrolyzed with 0.5 m NaOH. The spectrum of the liberated protoporphyrin then was compared to that of protoporphyrin prepared by hydrolysis of esterified material obtained from blood. Table II demonstrates that the spectrum of the two substances was identical.

The data presented are evidence that the porphyrin isolated from the catalase of M. lysodeikticus is protoporphyrin IX. The procedure outlined provides a simple method for isolating 10 to 15 mg of protoporphyrin IX from the bacterial catalase obtained from 1 liter of culture medium. The use of a combination of both ethanol extraction and precipitation with (NH$_4$)$_2$SO$_4$ in the purification and isolation of catalase is essential when working with small quantities of the enzyme.

### Table II

<table>
<thead>
<tr>
<th>Source</th>
<th>Compound*</th>
<th>Absorption maxima $\mu$</th>
<th>$R_F$ values in solvent A</th>
<th>Melting point $^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial catalase</td>
<td>I</td>
<td>408, 555, 605</td>
<td>0.81</td>
<td>228.5*</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>409, 557, 600</td>
<td>0.81</td>
<td>228.5*</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>408, 555, 600</td>
<td>0.81</td>
<td>228.5*</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>530, 557</td>
<td>0.81</td>
<td>228.5*</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>I</td>
<td>408, 555, 602</td>
<td>0.82</td>
<td>229*</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>409, 555, 602</td>
<td>0.82</td>
<td>229*</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>408, 556, 600</td>
<td>0.82</td>
<td>229*</td>
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<tr>
<td></td>
<td>IV</td>
<td>528, 560</td>
<td>0.82</td>
<td>229*</td>
</tr>
</tbody>
</table>

* I = protoporphyrin; II = dimethyl ester of protoporphyrin; III = protoporphyrin obtained by hydrolysis of dimethyl ester; IV = hemin.

† Solvent A: Kerosene-chloroform (100:65); Solvent B: Kerosene-n-propyl alcohol (100:20) (11).

A procedure is reported whereby 10 to 15 mg of protoporphyrin can be isolated from the catalase obtained from 1 liter of culture medium of Micrococcus lysodeikticus. The method included enzymatic lysis of the bacterial cells, (NH$_4$)$_2$SO$_4$ and ethanol precipitation, ethanol extraction, and (NH$_4$)$_2$SO$_4$ fractionation of the enzyme. Chromatographic, melting point, and spectrophotometric analyses carried out on the isolated prophyrin and its derivatives demonstrated that the porphyrin prepared from the catalase was protoporphyrin IX.

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### REFERENCES


Isolation and Characterization of Protoporphyrin IX from Bacterial Catalase
Steve Miller, Davis Hawkins, Jr. and Robert P. Williams