Distribution and Characteristics of the Catalases of Lactobacillaceae

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ABSTRACT

JOHNSTON, M. A. (Cornell University, Ithaca, N.Y.), and E. A. DELWICHE. Distribution and characteristics of the catalases of Lactobacillaceae. J. Bacteriol. 90:347-351. 1965.—Certain strains of lactobacilli and pediococci incorporated hematin during growth, with the concomitant formation of cyanide- and azide-sensitive catalase. Three of five strains of lactobacilli and five of 25 strains of pediococci were capable of this biosynthesis. The pediococci required the heme component of blood, whereas the lactobacilli could incorporate the heme component in the form of purified and solubilized hemin or from blood. In all cases where inhibitor-sensitive enzyme was produced, it was accompanied by the production of inhibitor-insensitive enzyme. In the absence of hematin, only insensitive enzyme was obtained. Two catalase-positive strains of Streptococcus faecalis were found incapable of the synthesis of a heme-type enzyme, as was one member of the genus Leuconostoc. Iron and manganese in the growth medium stimulated the production of the insensitive catalase, but significant quantities of these metals could not be found in a purified enzyme preparation obtained from Lactobacillus plantarum. Aeration had little or no effect on growth, but it consistently doubled the amount of cyanide- and azide-resistant catalase. By means of conventional enzyme fractionation techniques, it was possible to separate the two different enzymes present in the cell-free extract of a strain of Pediococcus homari which had been grown in the presence of blood.

The previously unsuspected and undetected catalases of Lactobacillaceae have been extensively described within the past 5 years and have been adequately documented (for recent literature reviews, see Johnston and Delwiche, 1962; Jones, Deibel, and Niven, 1964; Whittenbury, 1964).

Whittenbury (1964), in a broad survey of lactobacilli, leuconostocs, streptococci, pediococci, and aerococci, reported no correlation between peroxide formation and a preference for aerobic or anaerobic conditions. As reported by Whittenbury (1960), two distinctive catalase "activities" existed. One activity was insensitive to cyanide and azide, as reported earlier (Delwiche, Bacteriol. Proc., p. 117, 1959; p. 168, 1960), and appeared to be characteristic of those catalase-positive species which also displayed a negative benzidine test (Deibel and Evans, 1960) when cultured in the absence of preformed iron-porphyrin compounds. The other activity was described as sensitive to the heme poisons, and it was characteristic of some of the strains when they were cultured or preincubated prior to test in the presence of hematin or heated blood. Whittenbury's study offers convincing evidence that stains possessing this type of activity can synthesize an apocatalase capable of combining with the preformed heme prosthetic group to produce an active catalase. In all important characteristics, it appears that this enzyme may be regarded as identical with the ubiquitous "classical" catalase.

Jones et al. (1964) made an intensive study of the cyanide- and azide-resistant catalase formed in two strains of Streptococcus faecalis. They recorded a loss of activity by culture in stationary broth tubes, but maintenance of activity under aerobic culture. Supplementation of the growth medium with iron, manganese, and zinc ions enhanced activity, but the addition of these metallic ions to the cell suspension or cell-free extracts had no effect. Purified preparations (185-fold) were not inhibited by cyanide or azide, and in no case by independent test could the iron-porphyrin coenzyme of classical catalase be detected.

In a previous note (Johnston and Delwiche,
1962) we described catalase activity in Lactobacillaceae with primary attention being given to the distribution of the enzymes among the various species and the sensitivity of the enzymes to sodium azide. These studies suggested the application of the Whittenbury (1960, 1964) technique to our strains, i.e., the incorporation of the preformed iron-porphyrin group to produce inhibitor-sensitive enzyme. We report herein our investigations in this area, including observations on the simultaneous occurrence of the two types of enzyme in the same culture. The role of metals in the development of azide-insensitive catalase (Jones et al., 1964) has also been made a subject of investigation with one very active catalase producer. Evidence is presented which seems to preclude iron and manganese as structural components of the enzyme.

Materials and Methods

Cultural methods, cell preparations, and general procedures. For the production of cells containing the heme-type catalase, a basal medium supplemented with blood was used. It contained 0.5% peptone (Difco), 0.5% yeast extract (Difco), 1.0% yeast autolysate (Difco), 0.05% MnSO₄·4H₂O, 0.06% Tween 80, 0.5% sodium citrate, and 0.5% glucose (pH 6.8). Bovine blood was added to a final concentration of 5% after having first been lysed by the addition of an equal volume of sterile distilled water and boiled for 15 min to destroy blood catalase. In certain experiments, blood was replaced with hemin (solvulized in 0.067 M Na₂HPO₄) at a final concentration of 5 μg/ml. Cells used for the metal determinations were obtained from a medium composed of 0.75% yeast extract, 1.25% Tryptone, 0.02% glucose, 0.02% Tween 80, 0.5% sodium citrate, 0.25% KH₂PO₄, 0.5% NaCl, 0.014% MnCl₂·4H₂O, and 0.004% FeSO₄·7H₂O (pH 6.8). This medium, with and without the manganese or iron salts, was also employed in testing the effect of manganese and iron on growth and enzyme activity. Aerobic shake culture techniques were employed. After harvest, the cells were washed with distilled water and suspended in 0.1 M potassium phosphate buffer (pH 6.9). Cell-free extracts were prepared by disrupting the cells in a Raytheon sonic oscillator (10 kc) operated at 250 w for 30 min at 0 to 5 C. Cell debris was removed by centrifugation. Protein was determined turbidimetrically after combination with trichloroacetic acid, colorimetrically by a quantitative biuret procedure, or by ultraviolet-light absorption.

Catalase assay. In the enzyme assay procedure, residual peroxide was determined by the iodometric method of Herbert (1965). The assay system, in Wasserman tubes, consisted of 1.0 ml of 0.1 M potassium phosphate buffer (pH 6.5), 0.3 ml of 0.01 M sodium azide (when included), and 0.5 ml of the cell suspension or cell preparation, all contained in a total volume of 2.5 ml. The reaction was initiated by the addition of 0.5 ml of H₂O₂ solution of appropriate concentration and terminated by the addition of 2.0 ml of 1 N H₂SO₄. The acidified reaction mixture was then assayed directly for residual peroxide by the iodometric procedure.

Enzyme separation and purification. Cells were harvested from blood-agar plates (30 C for 36 to 48 hr), washed twice with 0.1 M phosphate buffer (pH 7.0), and disrupted by sonic oscillation. Supernatant protein was determined by the biuret method. The protein was totally precipitated with ammonium sulfate and dissolved in 0.1 M phosphate buffer. Nucleic acids were removed by the addition of protamine sulfate to a concentration of 10 mg/ml, with the treatment being repeated if more than 10% of the original nucleic acid content remained. Enzyme separation and purification were achieved by conventional ammonium sulfate fractionation procedures.

Metal determination. Iron determinations were made on purified preparations by the o-phenanthroline method described by Ballentine and Burford (1967). Crystalline beef-liver catalase served as a positive control. Similar preparations were assayed for manganese by the Willard-Burford (1957).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conc of NaN₃</th>
<th>Activity of cells after growth on Heated blood-agar</th>
<th>Basal agar medium with hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus plantarum</td>
<td>0.001</td>
<td>394</td>
<td>352</td>
</tr>
<tr>
<td>L. plantarum NZ 48</td>
<td>0</td>
<td>766</td>
<td>598</td>
</tr>
<tr>
<td>Pedicoccus homari 7764</td>
<td>0.001</td>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>Streptococcus faecalis T91</td>
<td>0.001</td>
<td>8.2</td>
<td>2.3</td>
</tr>
<tr>
<td>S. faecalis P318</td>
<td>0.001</td>
<td>7.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Leuconostoc sp. T19</td>
<td>0.001</td>
<td>4.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Lactobacillus casei C</td>
<td>0.001</td>
<td>4.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Escherichia coli C</td>
<td>0.001</td>
<td>20.9</td>
<td>22.4</td>
</tr>
</tbody>
</table>

* Activity expressed as micromoles of H₂O₂ decomposed per milligram of cells during the first 10 min of assay.

† Basal medium was supplemented with 5% bovine blood or hemin (6 μg/ml) and solidified with 1.5% agar. Cultures were incubated at 30 C for 30 hr.
Greathouse (1917) technique as modified by Bal-\text{'}lentine and Burford (1957).

\textbf{Results}

\textit{Hematin incorporation.} Three catalase-positive strains of lactobacilli and 5 of 25 strains of catalase-positive pediococci were found to be capable of incorporating hematin during growth to produce what appears to be a typical heme-iron catalase (Whittenbury, 1964); but, when cultured in the absence of the preformed hematin component, they produced only the azide-insensitive enzyme. Unlike the observation of Whittenbury (1964), the incubation of resting cells or cell-free extracts with hematin did not result in the production of active heme enzyme. The catalase-positive lactobacilli were identified as strains of \textit{Lactobacillus plantarum}. The pediococci were identified by us as strains of \textit{Pediococcus homari} and \textit{P. cerevisiae}. Pertinent data are summarized in Table 1. When \textit{L. plantarum T-1403-5} is grown in a suitable medium containing either hematin or blood, active catalase can always be detected, with a substantial portion of the activity being demonstrable even in the presence of 0.001 M sodium azide. In the case of \textit{L. plantarum} NZ48, most of the activity appears as the azide-sensitive, heme-containing enzyme. \textit{P. homari} 7764 also produces catalase of both types, but does so only in the presence of blood. Included in the table are data obtained with two strains of \textit{S. faecalis}, one strain of the genus \textit{Leuconostoc} (unclassified with regard to species), one strain of \textit{Lactobacillus casei}, and one strain of \textit{Escherichia coli}. The \textit{Streptococcus} and \textit{Leuconostoc} species display a low level of azide-insensitive catalase activity and are incapable of the synthesis of a heme-iron enzyme. \textit{L. casei} C was included as a catalase-negative control. \textit{E. coli} C, the positive control culture, always produced active catalase, but of the azide-sensitive heme type only.

\textit{Separation of the two types of catalase.} Cell-free extracts from \textit{Pediococcus} cultures on heated blood-agar were fractionated, in an effort to detect and to separate the two catalases. The data obtained from the fractionation of an extract of \textit{P. homari} 7764 are presented in Table 2. All of the catalase could be precipitated by the addition of ammonium sulfate to 90\% saturation, and less than 5\% of the total activity in the extract was

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Fraction\text{*}} & \textbf{Protein (mg)} & \textbf{Total activity (units)\textdagger}} & \textbf{Specific activity \textsuperscript{\ddagger}} & \textbf{Recovery (\%)} & \textbf{Purification (fold)} \\
\hline
& & \textbf{Azide-insensitive enzyme} & \textbf{Azide-sensitive enzyme} & \textbf{Azide-insensitive enzyme} & \textbf{Azide-sensitive enzyme} & \textbf{Azide-insensitive enzyme} & \textbf{Azide-sensitive enzyme} \\
\hline
Cell-free extract & 189 & 37.8 & 246.0 & 0.2 & 1.3 & 100.0 & 100.0 & 0 & 0 \\
0-90 & 185 & 37.0 & 241.1 & 0.2 & 1.3 & 98.0 & 98.0 & 0 & 0 \\
Protamine-treated & 151 & 30.3 & 182.0 & 0.2 & 1.2 & 80.2 & 74.0 & 0 & 0.9 \\
extract & & & & & & & & & \\
0-80 & 61 & 7.0 & 28.0 & 0.1 & 0.5 & 18.5 & 11.4 & 0.5 & 0.4 \\
30-60 & 23 & 11.5 & 80.5 & 0.5 & 3.5 & 30.4 & 32.7 & 2.5 & 2.7 \\
60-80 & 9 & 18.2 & 31.8 & 2.0 & 3.5 & 48.2 & 12.9 & 10.0 & 2.7 \\
\hline
30-60 fraction refractionated & & & & & & & & & \\
0-50 & 6.3 & 0.6 & 10.5 & 0.1 & 1.6 & 1.6 & 4.2 & 0.5 & 1.2 \\
50-55 & 1.0 & 0.5 & 18.5 & 0.5 & 18.5 & 1.3 & 7.5 & 2.5 & 14.2 \\
55-60 & 1.6 & 1.0 & 47.0 & 0.6 & 29.4 & 2.6 & 19.1 & 3.0 & 22.6 \\
60-65 & 0.9 & 1.0 & 15.0 & 1.1 & 16.7 & 2.6 & 6.1 & 5.5 & 12.8 \\
\hline
60-80 fraction refractionated & & & & & & & & & \\
0-65 & 0.7 & 0.6 & 3.1 & 0.8 & 4.4 & 1.6 & 1.3 & 4.0 & 3.4 \\
65-70 & 2.2 & 10.8 & 0.2 & 4.9 & 0.1 & 28.6 & 0.1 & 24.5 & 0.1 \\
70-75 & 2.6 & 2.0 & 0 & 0.8 & 0 & 0.5 & 0 & 4.0 & 0 \\
75-80 & 0 & - & - & - & - & - & - & - & - \\
\hline
\end{tabular}
\caption{Separation and purification of the two catalases produced by \textit{Pediococcus homari} 7764}
\end{table}

\textsuperscript{*} Figures refer to range (per cent) of ammonium sulfate saturation.
\textsuperscript{\dagger} One unit of enzyme (U) is defined as that amount of enzyme which will decompose 1 \(\mu\) mole of \(H_2O_2\) in one minute.
\textsuperscript{\ddagger} Specific activity is defined as \(\mu\) moles of \(H_2O_2\) decomposed per minute per mg of protein.
destroyed by the procedure. The most active catalase fraction precipitated between 30 and 60% saturation and contained a larger amount of azide-sensitive enzyme than the subsequent fraction. More precise fractionation yielded fractions (50 to 55%, 55 to 60% saturation) which were azide-sensitive, and a fraction (65 to 70% saturation) which was insensitive to the inhibitor.

These results demonstrate that the two different types of catalase exist in P. homari 7764 and that they can be separated. Additional studies demonstrated that when the organism is grown in the absence of blood there is but one active fraction, an azide-insensitive enzyme activity, precipitating at 65 to 70% ammonium sulfate saturation.

Conditions for optimal growth and production of azide-insensitive enzyme by L. plantarum T-1403-5. Ferrous and manganous ions enhanced growth and the production of azide-insensitive catalase, as was observed with S. faecalis by Jones et al. (1964). No attempt was made to establish the quantitative aspects of these metal ion effects; but, when the culture medium was prepared without manganese, the cell crop of L. plantarum T-1403-5 (36 hr at 30 C) was one-third less than that observed when the manganese salt was present. Specific enzyme activity of the crude cell-free preparation was reduced by one-half. The effect of ferrous ion on growth was not so dramatic. Both growth and specific enzyme activity were reduced by approximately one-quarter when the ferrous salt was omitted from the medium. In both cases, appreciable quantities of the metallic ions were present in the absence of added salts because of the undefined constituents of the medium. Aeration had little or no effect on total growth, but it consistently doubled the specific activity.

Iron and manganese determinations on partially purified azide-insensitive catalase. A partially purified enzyme preparation of azide-insensitive catalase obtained from L. plantarum T-1403-5 was subjected to iron and manganese analyses, with crystalline beef-liver catalase as a positive analytical control. In accordance with published data on the iron content of beef-liver catalase (Sumner and Somers, 1947), 50 mg of beef-liver catalase protein yielded approximately 45 µg of iron. A 50-mg amount of the L. plantarum preparation gave a value of 0.55 µg. The very sensitive manganese analysis yielded consistently negative data.

DISCUSSION

None of our data is in serious conflict with the currently published work regarding catalases in Lactobacillaceae (Jones et al., 1964; Whittenbury, 1964), although there are three variances immediately apparent when our data are compared with the observations of Whittenbury (1964). In no case have we been able to obtain evidence for the existence of a preformed enzyme component capable of hematin activation, even after having examined over 30 different cultures isolated or obtained from widely different sources. Subtle differences in our culture techniques may be responsible for this observation, but it seems more reasonable to conclude that this biosynthetic ability is not of common occurrence, and that our cultures are lower in the scale of "physiological evolution" (Dolin, 1961) or, expressed in terms of Whittenbury's concept, they have moved farther in the direction of "physiological retrogression." Both of these concepts imply the characteristic of more limited biosynthetic ability. The inability of any of our pediococci to utilize hematin instead of blood is the second variance from the data presented by Whittenbury (1964), and it lends some support to our interpretation of limited biosynthetic ability. Perhaps the most interesting difference in the observations of the two groups pertains to the finding in our laboratory that the two different catalases can exist in the same culture and that they are separable. Any speculation on the evolutionary significance of this observation would seem to be too ephemeral to yield any useful hypotheses.

The data regarding the absence of manganese in the active enzyme are convincing, since the tests were consistently negative and the analytical method was of adequate sensitivity. Since a positive test was obtained with the iron determination on a purified preparation, some interpretation is necessary in the attempt to rule iron as a structural component or as merely extraneous. The partially purified Lactobacillus catalase had a specific enzyme activity which was approximately 10% of that displayed by the beef-liver catalase, yet it contained less than 1% of the iron contained in the pure beef-liver catalase. The partially purified preparation may have contained very active enzyme with structural iron, but this condition would assume a catalase several times more active than beef-liver catalase. In view of the very high turnover number of classical catalase, and in consideration of the inhibitor data, it could be cautiously concluded that the enzyme molecule does not contain iron. It is germane to the argument to point out that independent criteria (Johnston and Delwiche, 1965) suggest that the enzyme molecule is at least as large as the bovine catalase, and it could be larger. These results are convincing but not unequivocal.
We direct attention to the very high catalase activity of L. plantarum T-1403-5, with particular reference to the activity of the azide-insensitive enzyme (i.e., the activity in the presence of 0.001 M sodium azide). In comparison with the control culture of E. coli, it is more than 10-fold greater. We take issue in this context to the usage of the term "pseudocatalase" (Whittenbury, 1964). There is nothing "false" about this catalytic activity. The term "pseudo" is misused in the differentiation of the two catalase types, even though they may differ widely in composition and structure.

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LITERATURE CITED


