JOURNAL OF BACTERIOLOGY, Aug., 1965
Vol. 90, No. 2

Printed in U.S.A.

Isolation and Characterization of the Cyanide-Resistant and Azide-Resistant Catalase of *Lactobacillus plantarum*

M. A. JOHNSTON[1] AND E. A. DELWICHE

Division of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York

Received for publication 15 March 1965

ABSTRACT

JOHNSTON, M. A. (Cornell University, Ithaca, N.Y.), AND E. A. DELWICHE. Isolation and characterization of the cyanide-resistant and azide-resistant catalase of *Lactobacillus plantarum*. J. Bacteriol. **90**:352-356. 1965.—*Lactobacillus plantarum* T-1403-5 has been shown to possess a very active cyanide- and azide-resistant catalase. By means of fractional ammonium sulfate precipitation, removal of nucleic acids with protamine sulfate, adsorption on calcium phosphate gel, and *p*H gradient chromatography on diethylaminoethyl cellulose, the catalase "activity" was purified approximately 14-fold. The purified enzyme preparation was insensitive to the heme poisons cyanide and azide, the metal chelating agents ethylenediaminetetraacetate and *o*-phenanthroline, and the sulfhydryl binding agent *p*-chloromercuribenzoate. The purified enzyme moved at a uniform rate in the electrophoretic field (isoelectric point, *p*H 4.7). The ultraviolet-light absorption spectrum was negative for heme-iron components, and fluorescence measurements yielded negative results with regard to flavin components. Acriflavin and Atabrine had no effect on enzyme activity. The nonheme catalase displayed a much broader *p*H range of activity than the heme-iron catalase of a control culture of *Escherichia coli* and the azide-sensitive catalase developed by *L. plantarum* NZ48 when grown in the presence of preformed hematin. The nonheme catalase was more resistant to heat inactivation. No retention of the enzyme on a chromatographic column could be obtained with Sephadex 200, nor could the enzyme be separated from crystalline beef-liver catalase by the gel filtration technique. Sedimentation was obtained in a centrifugal field of 144,000 × *g* for 12 hr.

Nonheme catalase (Johnston and Delwiche, 1965; Jones, Deibel, and Niven, 1964; Whittenbury, 1964) is widely distributed among the members of Lactobacillaceae. In some strains, when they are grown in the presence of the heme component, this enzyme may coexist with a typical heme-iron catalase, and, by appropriate fractionation procedures, the two enzymes can be separated (Johnston and Delwiche, 1965). We describe herein some of the properties of a nonheme catalase obtained from *Lactobacillus plantarum* T-1403-5, with occasional reference to ancillary observations on a heme-type catalase obtained from *L. plantarum* NZ48.

MATERIALS AND METHODS

Bacterial strain and growth conditions. L. plantarum T-1403-5, a subject of previous investigations (Johnston and Delwiche, 1965), was found to be the most active producer of nonheme catalases yet described, and hence was selected as the organism of choice for purification and characterization of nonheme catalase.

The organism was cultured in a medium composed of 0.5% sodium citrate, 0.75% yeast extract (Difco), 1.25% Tryptone (Difco), 0.02% glucose, 0.5% NaCl, 0.014% $MnCl_2 \cdot 4H_2O$, 0.25% KH_2PO_4, 0.004% $FeSO_4 \cdot 7H_2O$, and 0.02% Tween 80 (*p*H 6.8).

Cell-free extracts. Aerobic shake cultures (30 C for 24 to 30 hr) were harvested by centrifugation, washed once with distilled water, suspended in 0.01 M potassium phosphate buffer (*p*H 6.9), and then disrupted by sonic oscillation in the 10-kc Raytheon apparatus (250 w for 30 min). Cell debris was removed by centrifugation (12,000 × *g* for 30 min), and the supernatant fraction was subjected to enzyme purification procedures.

Enzyme assay. Enzyme activity was determined by the direct titrimetric assay of residual peroxide in the test system (Johnston and Delwiche, 1965).

[1] Present address: Microbiology Section, Food and Drug Directorate, Department of National Health and Walfare, Ottawa, Ontario, Canada.

Enzyme purification. Table 1 outlines the principal procedure involved in the purification of the enzyme. Solid ammonium sulfate was added to the buffered crude cell-free preparation to 80% saturation. This preliminary step effected complete precipitation of the enzyme, which was then separated by centrifugation and dissolved in 0.05 M potassium phosphate buffer. Dialysis against 50 volumes of cold buffer (*p*H 6.8) removed residual ammonium sulfate and facilitated the subsequent removal of nucleic acids, which was accomplished by the addition of protamine sulfate to a concentration of 10 mg/ml. The protamine sulfate treatment was repeated if more than 10% of the original nucleic acid content remained, as determined by ultraviolet-light absorption. Further purification was accomplished by adsorption on calcium phosphate gel at *p*H 5.0 followed by elution at *p*H 8.0. It was usual to repeat the gel procedure once. The final step in the purification involved a gradient *p*H elution system on diethylaminoethyl (DEAE) cellulose with potassium phosphate buffer as the mobile phase. DEAE cellulose (Brown Co., New York, N.Y.) was washed thoroughly, adjusted to *p*H 7.5, and packed in a column (1.5 by 13 cm). The partially purified enzyme preparation obtained after the calcium phosphate gel treatment was dialyzed against 0.01 M phosphate buffer (*p*H 7.5), concentrated with Carbowax 6000 (Union Carbide Corp., Chemicals Division, New York, N.Y.), dialyzed again, and then placed on the DEAE cellulose column. Gradient elution was accomplished by the controlled admixture of 0.1 M KH_2PO_4 to an initial reservoir of approximately 9.5 ml of 0.01 M potassium phosphate buffer (*p*H 7.5). Elution (10 to 20 ml/hr) of a volume of approximately 100 ml caused a *p*H shift from *p*H 7.5 to 5.6, resulting in the complete removal of protein from the column, and yielded a purified enzyme fraction in the range of *p*H 6.8 to 6.3.

Absorption spectrum. The absorption spectrum of the most highly purified preparation was determined in the Beckman DU spectrophotometer and compared with that displayed by a sample of crystalline beef-liver catalase of comparable activity.

Flavin analyses. Flavin determinations by means of fluorescence analyses were made on purified enzyme fractions. Concentrated and dialyzed preparations were deproteinized with trichloroacetic acid and adjusted to *p*H 7.0 after the removal of the precipitated protein by centrifugation. Fluorescence was measured in the Aminco-Bowman Spectro-photofluorometer, with an excitation maximum of 450 mμ and a fluorescence maximum of 535 mμ. After this measurement of total fluorescence, nonflavin fluorescence was determined on an identical sample which had been reduced with sodium hydrosulfite, this latter value being used to correct the value obtained with the first sample. The method was made quantitative by the use of a riboflavine standard.

Electrophoresis. Electrochromatography was performed on purified and concentrated enzyme fractions. For these procedures, the Precision Ionograph power supply was employed with the Gelman chamber, and cellulose polyacetate strips were used for the supporting material. One of two strips for each sample was stained with acidic Ponceau (200 mg of dye in 100 ml of 5% trichloroacetic acid) to locate direction and extent of mobility. The other strip was retained for sectioning and individual section elution with 0.05 M potassium phosphate buffer (*p*H 7.0).

Molecular size. Sephadex 200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was suspended in 0.01 M potassium phosphate buffer (*p*H 7.0), aged for 24 hr, and packed uniformly in a column (1.0 by 35 cm). Purified and concentrated enzyme preparations were placed on the top of the column and eluted slowly (5 to 10 ml/hr) with 0.01 M potassium phosphate buffer (*p*H 7.0).

RESULTS

Purification. Table 1 summarizes the purification procedure. In the final step, which involved the use of a DEAE cellulose column (1.5 by 13 cm) and a gradient elution system (Materials and Methods), 96 mg of protein contained in a concentrated partially purified preparation were eluted in the column, with the first protein ap-

TABLE 1. *Purification of catalase from Lactobacillus plantarum T-1403-5*

Fraction	Protein	Enzyme*	Specific activity†	Yield	Purification (fold)
	mg	*units*		%	
Sonic extract	1,774	157,884	89	100	—
$(NH_4)_2SO_4$, 80%	1,207	148,461	123	93	1.4
After protamine sulfate	558	141,732	254	90	2.8
$Ca_3(PO_4)_2$ gel	286	136,136	476	86	5.3
$Ca_3(PO_4)_2$ gel	96	88,320	920	55	10
DEAE cellulose	15	18,930	1262	12	14

* One unit of enzyme is defined as that amount of enzyme which will decompose 1 μmole of H_2O_2 in 1 min.

† Specific activity is defined as micromoles of H_2O_2 decomposed per minute per milligram of protein.

pearing in the effluent immediately after clearance of the void volume. Approximately 15 mg of protein were recovered with the active enzyme fraction which appeared in the 25- to 50-ml fraction over the gradient range of *p*H 6.8 to 6.3. An approximate 14-fold purification was achieved, with repeated chromatography on DEAE cellulose not yielding further enzyme purification. The application of the electrophoretic technique to the purest fraction likewise was ineffective in accomplishing further purification, but the technique did prove useful in determining the isoelectric point of the purified enzyme. Figure 1 represents the electrophoretic mobility of the enzyme as a function of *p*H. From the curve, it can be determined that the isoelectric point is in the vicinity of *p*H 4.7. When tested under a variety of different conditions of hydrogen ion concentration, the protein of crude preparations did not move uniformly over the strip, but with the purified fractions virtually all of the protein moved as a uniform band. Sectioning of the protein band resulted in substrips carrying varying degrees of enzyme activity, but in no case did any of the subfractions display a specific enzyme activity greater than that of the starting material. In spite of these latter observations, it should be emphasized that the results of the electrophoretic technique as applied to the purified fractions suggested a considerable degree of homogeneity in the preparation.

Absorption spectrum. The absorption spectrum of the purified *Lactobacillus* catalase was examined over the range of 220 to 470 mμ and was compared with that of a sample of crystalline beef-liver catalase of approximately the same activity (Fig. 2). Typical of the heme-iron prosthetic group, the beef-liver catalase displayed an absorption peak in the 390- to 410-mμ region (Soret band), whereas this absorption peak was absent in the case of the catalase obtained from *L. plantarum*. In other experiments, we have been unable to demonstrate a Soret band in catalase-positive preparations obtained from pediococci and streptococci. When *L. plantarum* NZ48 was grown on a medium containing hematin (Johnston and Delwiche, 1965), however, the typical heme-iron absorption spectrum could be observed.

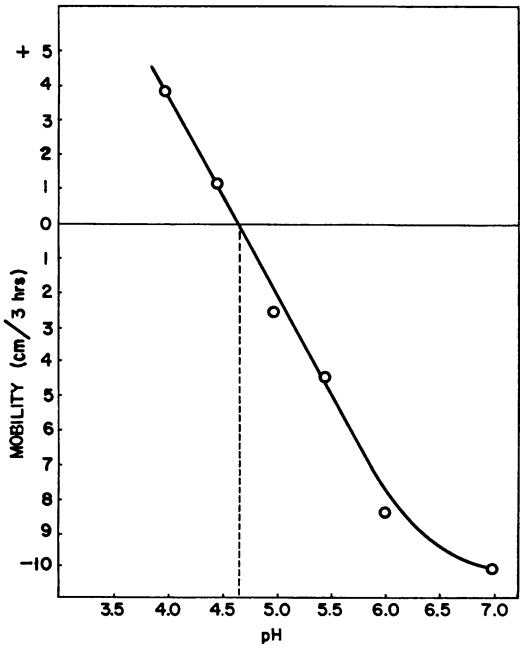

FIG. 1. *Electrophoretic mobility of purified azide-insensitive catalase (Lactobacillus plantarum T-1403-5). Each mobility measurement was made in a 150-v field on approximately 0.27 mg of protein; 0.01 M acetate buffer was used in all cases except for the mobility determination at pH 6.0 and 7.0, in which 0.01 M phosphate buffer was employed. Polyacetate strips (2.4 by 17 cm) served as the support media.*

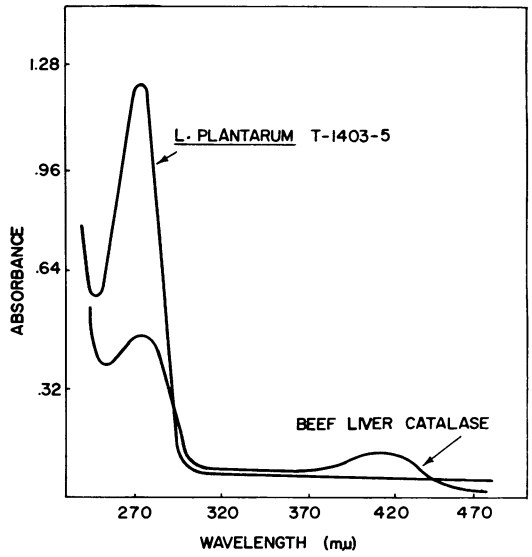

FIG. 2. *Absorption spectrum of a purified preparation of azide-insensitive catalase (Lactobacillus plantarum T-1403-5) compared with the spectrum of crystalline beef-liver catalase. Absorption was measured with a 1-cm light path in a Beckman model DU spectrophotometer. The two preparations displayed approximately the same specific catalase activity (950 μmoles of H_2O_2 decomposed per mg of protein per min). The bacterial preparation contained approximately 0.28 mg of protein per ml and the beef-liver catalase contained approximately 0.05 mg/ml.*

TABLE 2. *Flavin content and enzyme activity of purification fractions*

Fraction	Specific activity*	Flavin (μg/mg of protein)
After treatment with		
Protamine sulfate	223	0.025
Calcium phosphate gel	893	0.011
DEAE cellulose	1,530	0.006

* Specific activity is defined as micromoles of H_2O_2 decomposed per minute per milligram of protein.

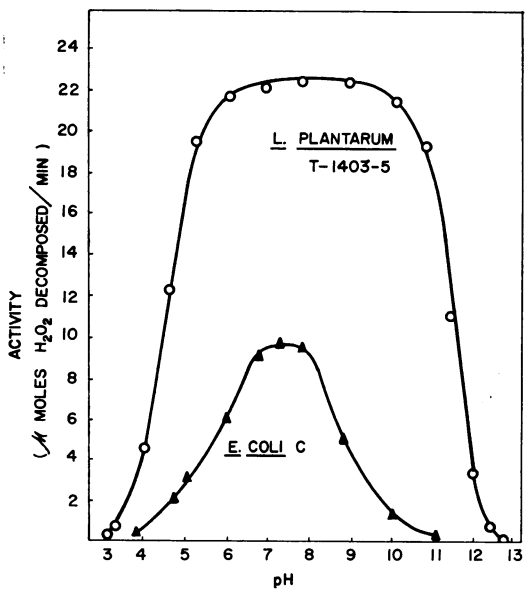

FIG. 3. *Catalase activity as a function of hydrogen ion activity. The test system was composed of 150 μmoles of H_2O_2 contained in 3.0 ml of 0.1 M buffer (pH 3.0 to 5.5 acetate buffer; pH 5.6 to 7.8 potassium phosphate buffer; pH 8.0 to 13.0 sodium borate-HCl and sodium borate-NaOH buffer), 0.3 mg of protein in the case of the Lactobacillus plantarum preparation, and 1.4 mg of protein in the Escherichia coli preparation. Incubation was for 10 min at 25 C.*

Absence of flavin. The flavin content of partially purified preparations, as measured by the fluorescence technique, bears an inverse relationship to their specific enzyme activity (Table 2). The most active fractions when examined spectrophotometrically for the presence of a specific flavin-absorption spectrum yielded completely negative results. Flavin adenine dinucleotide and flavin mononucleotide or riboflavine neither stimulated nor inhibited the enzyme activity of crude

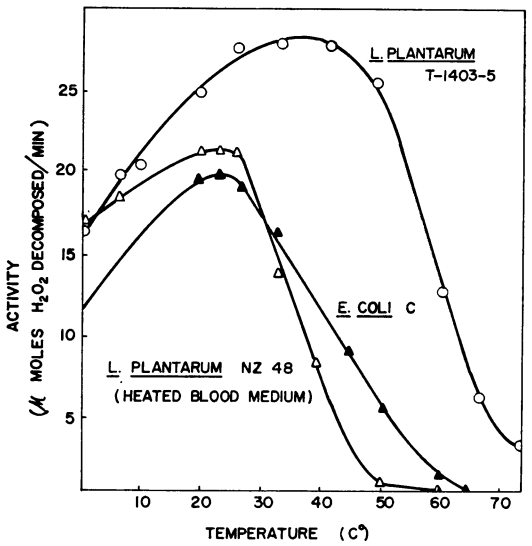

FIG. 4. *Effect of temperature on the activity of cell-free preparations of azide-sensitive and azide-insensitive catalases. The experimental system was composed of 150 μmoles of H_2O_2 contained in 3.0 ml of 0.01 M potassium phosphate buffer (pH 7.0). For the cell-free preparation of Lactobacillus plantarum T-1403-5, 0.3 mg of protein was present in each test; 3.0 mg of protein were present in each case in the tests with L. plantarum NZ48 and Escherichia coli C. Incubation was for 10 min at each temperature. Activity was calculated from the straight line portion of the activity curve with the exception of the two high extremes where average figures were used.*

or highly purified preparations. Acriflavine and Atabrine likewise had no effect.

Effect of metabolic inhibitors. Purified preparations of the nonheme catalase were found to be insensitive to the metal-chelating agents, ethylenediaminetetraacetate and *o*-phenanthroline, insensitive to the sulfhydryl inhibitor *p*-chloromercuribenzoate, and, as repeatedly stated, completely insensitive to the heme poisons, cyanide and azide. It was even possible to grow the organism in the presence of 0.001 M azide and to obtain cells with normal catalase activity.

Effect of hydrogen ions. The nonheme catalase has a much broader optimal *p*H range than has the heme catalase of *Escherichia coli* (Fig. 3). In other experiments, the heme-type catalase which can be developed in *L. plantarum* NZ48, when cultured in the presence of hematin, displays a *p*H activity curve similar to that displayed by the catalase of *E. coli*. Irreversible denaturation of the nonheme enzyme from *L. plantarum* T-1403-5 was obtained by exposure to *p*H 2.5. Precipitation of the enzyme occurred at *p*H 4.0, but brief exposure to this condition did not de-

stroy activity, since most of the original activity was regained after readjustment to *p*H 7.0.

Heat inactivation. Cell-free preparations of heme catalases are much more susceptible to heat inactivation than are their nonheme counterparts. In the series of experiments designed to test the effect of temperature (Fig. 4), the test systems were maintained at various temperatures over the range of 0 to 80 C. The heme-type catalase of *E. coli* and *L. plantarum* NZ48 displays optimal activity at 25 C and virtually complete inactivity at 60 to 65 C. The nonheme catalase of *L. plantarum* T-1403-5 displays a broad optimal-temperature range of 30 to 45 C, and displays considerable activity at temperatures as high as 70 C. In other experiments, it was observed that heating of the enzyme before test required a treatment of 80 C for approximately 10 min to bring about heat inactivation of the nonheme preparation of *L. plantarum*, whereas the catalase of *E. coli* and the heme-type enzyme obtained from *L. plantarum* NZ48 were destroyed by a treatment of 50 to 55 C for 10 min.

Molecular size. An approximation of the molecular size of the nonheme catalase of *L. plantarum* was made possible through the use of Sephadex. No retention of the enzyme in a chromatographic column could be observed with Sephadex 200. A mixture of the purified bacterial enzyme and solubilized crystalline beef-liver catalase (molecular weight, 248,000) moved with the void volume of the column, and enzyme separation could not be obtained. These results suggest a molecule at least as large as that of beef-liver catalase. The active enzyme of crude preparations was poorly sedimented by high-speed centrifugation, but, when present in purified fractions, it was sedimented in a centrifugal field of 144,000 $\times$ *g* for 12 hr.

DISCUSSION

In this study, it has been our aim to bring about such concentration and purification of an azide-insensitive catalase that meaningful observations could be made regarding its composition, structure, and properties.

The inhibitor data, in conjunction with previously reported analytical data (Johnston and Delwiche, 1965) regarding the absence of metals in the *Lactobacillus* enzyme, offer convincing evidence for the existence of a nonheme, nonmetallo-catalase. It is also only remotely possible that a flavin prosthetic group could be present. Flavin adenine dinucleotide has been described by Dolin (1957) as the coenzyme of a reduced nicotinamide adenine dinucleotide peroxidase in *Streptococcus faecalis*, and in fluorometric analyses of highly purified preparations he was able to detect significant amounts (0.5 to 1.0 μg/mg of protein) of the cofactor, but these amounts were only sufficient to account for one molecule per molecule of enzyme. Partially purified catalase from *L. plantarum* yielded only traces of flavin (0.006 μg/mg of protein), and successive fractions in the purification process showed increasing activity with decreasing flavin content.

Purification of active *Lactobacillus* catalase beyond 15- to 20-fold has not been achieved. There is evidence, however, that specific protein isolation is occurring in our procedure but that concomitant loss of catalytic activity occurs in the final steps. Attention is directed to the apparent electrophoretic homogeneity of the enzyme fraction and also to the behavior of the partially purified preparation when subjected to the DEAE cellulose gradient elution system. In the elution process, it was noted that a very marked protein "peak" coincided with the activity peak, thus suggesting that the unexpectedly low purification value was a reflection of enzyme inactivation and not the result of failure to isolate a specific (albeit inactive) enzyme protein. As a working hypothesis for future investigations, we have entertained the concept of a dissociable cofactor which has undergone partial separation in passage through the chromatographic column.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-0-3198-04 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

DOLIN, M. I. 1957. The *Streptococcus faecalis* oxidases for reduced diphosphopyridine nucleotide. III. Isolation and properties of a flavin peroxidase for reduced diphosphopyridine nucleotide. J. Biol. Chem. **225**:557–572.

JOHNSTON, M. A., AND E. A. DELWICHE. 1965. Distribution and characteristics of the catalases of Lactobacillaceae. J. Bacteriol. **90**:347–351.

JONES, D., R. H. DEIBEL, AND C. F. NIVEN, JR. 1964. Catalase activity of two *Streptococcus faecalis* strains and its enhancement by aerobiosis and added cations. J. Bacteriol. **88**:602–610.

WHITTENBURY, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. **35**:13–26.