J. Gen. Appl. Microbiol.
Vol. 8, No. 1, 1962

PURIFICATION AND PROPERTIES OF DPNH PEROXIDASE IN *LACTOBACILLUS CASEI*

BY SHÔJI MIZUSHIMA AND KAKUO KITAHARA

(From the Institute of Applied Microbiology, University of Tokyo)

Received for publication, Nov. 10, 1961

It has repeatedly been reported that lactic acid bacteria contain a considerable amount of flavoprotein, and this fact has led some workers to the idea that the bacteria, which had been thought to be essentially anaerobic organisms, might have a capacity of performing aerobic respiration by virtue of flavoprotein acting as the terminal "oxidase". If this be true, we have to assume the formation of hydrogen peroxide as a product of the oxidation of reduced flavoprotein. However, most of the lactic acid bacteria thus far investigated have been known to be devoid of catalase (as well as of other hemoproteins such as cytochromes).

Recently DOLIN (*1*, *2*) found in a strain of *Streptococcus* the presence of a flavin nucleotide-linked DPNH peroxidase, which seems to be of significance as a consumer of hydrogen peroxide if it is formed in the aerobic metabolims of the bacterium.

In the present study we found the existence of a similar or an identical enzyme in *Lactobacillus casei*, another representative of the lactic acid bacteria. By the procedure to be described in detail it was purified to a considerable degree, and as shown to possess, besides the strong peroxidatic action in question, certain activities of catalysing the aerobic oxidation as well as anaerobic dehydrogenation of DPNH. Some other properties of the purified enzyme will also be described in this paper.

METHODS AND MATERIALS

Materials: Reduced diphosphopyridine nucleotide (DPNH) was prepared by reducing DPN (a sample from Nutritional Biochemicals Corporation) with alcohol and alcohol and dehydrogenase (*3*). Alcohol dehydrogenase was prepared from backer's yeast by the method of RACKER (*4*). Purified D-amino acid oxidase was kindly supplied from Mr. OZAWA, Faculty of Medicine, University of Nagoya. Calcium phosphate gel-cellulose was prepared according to the method of MASSAY (*5*).

Cultivation of bacterium: The bacterium was grown at 45° in a broth (pH 6.5) containing 2 per cent glucose, 2 per cent sodium acetate (anhydride), 1 per cent polypeptone, 1 per cent meat extract, 0.5 per cent sodium chloride and 0.1 per cent yeast extract in tap water. Usually the cultivation was carried out without aeration. The growth rate of the bacterium was measured using a nepherometer. The bacterium was harvested at an early

stage of stationary phase, in which the population density was about 300–400 mg dried cells per liter.

Measurement of DPNH oxidase and DPNH peroxidase activities The activity of DPNH oxidase was usually estimated by measuring the change of optical density at 340 mμ in an aerobic state. However, since the enzyme preparation always contained a high DPNH peroxidase activity, the value obtained by this method was about twice as the real value.

DPNH peroxidase activity was estimated by measuring the change of optical density at 340 mμ in the presence of DPNH and hydrogen peroxide. The value thus obtained represents the sum of DPNH peroxidase and oxidase activities.

Estimation of flavin nucleotide The amount of flavin compound contained in the sonicate and the purified enzyme was spectrophotometrically estimated from the optical density at 450 mμ.

RESULTS

Purification of DPNH perioxidase Bacterial cells obtained from 20 liters of the culture were washed once with 10 volumes of 0.5 per cent saline solution, suspended in 100 ml of M/15-phosphate buffer (pH 7.4) and then exposed to sonic oscillation (10 KC) for 20 minutes. The sonicate was centrifuged at 100,000×g for 2 hours to remove the intact cells and the subcellular particulate fraction. To 100 ml of the clear supernatant solution thus obtained, was added 235 g of solid ammonium sulfate, and the solution was allowed to stand for one hour at 4°. The resultant precipitate was collected by centrifugation, dissolved in 10 ml of M/15-phosphate buffer (pH 7.4) and was dialyzed against 100 volumes of distilled water at 4°. The dialyzed preparation was applied to a column of calcium phsophate gel-cellulose (5.3 cm^2×15 cm) containing 3 g of calcium phsophate and 20 g of cellulose powder. After charging the column with the enzyme solution, 0.25 M-ammonium sulfate was passed through the column at a rate of one ml per minute. The yellowish colored eluate which was not absorbed on the column was collected. The enzyme in the fraction was precipitated by the addition of ammonium sulfate (40 g per 100 ml). The precipitate thus obtained was dissolved in 10 ml of M/15-phosphate buffer (pH 7.5), dialyzed over night against 100 volumes of distilled water at 4° and was applied to the second calcium phosphate gel-cellulose column (conditions were the same as those described above). The elution curve is shown in Fig. 1. The eluates in tubes No. 23–30 were again salted out with ammonium sulfate and the precipitate was dissolved in one ml of M/5-Tris buffer (pH 7.4) and dialyzed against the same buffer solution. The ultracentrifugal patterns of the purfied preparation are shown in Fig. 2. Both the elution curve and the ultracentrifugal patterns provide strong evidence that the enzyme preparation obtained had a high degree (as high as about 90 per cent) of homogeneity.

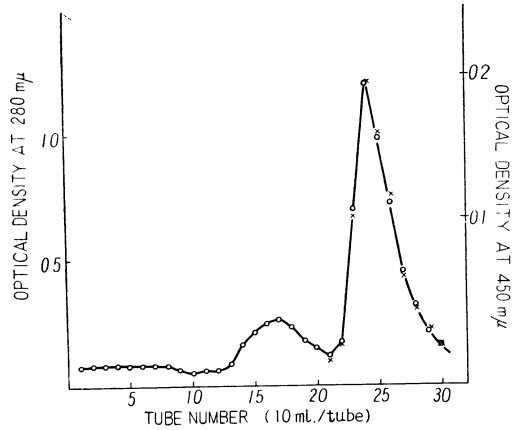

Fig. 1. Chromatography of the enzyme on a column of calcium phosphate gel-cellulose.

Amount of total protein and flavoprotein were estimated from optical densities at 280 mμ and 450 mμ, respectively. Flow rate; 1 ml. per minute. Temperature; 20°C.
○——○ Total protein ×——× Flavoprotein.

Specificity of enzyme: The enzyme preparation, whose high degree of homogeneity had been confirmed by the data given in Figs. 1 and 2, was found to have a strong activity of oxidizing DPNH in the presence of hydrogen peroxide. It should be remarked that the preparation was also found to be capable of catalysing the dehydrogenation of DPNH under anaerobic (in the presence of 2, 6-dichlorophenolindophenol as hydrogen

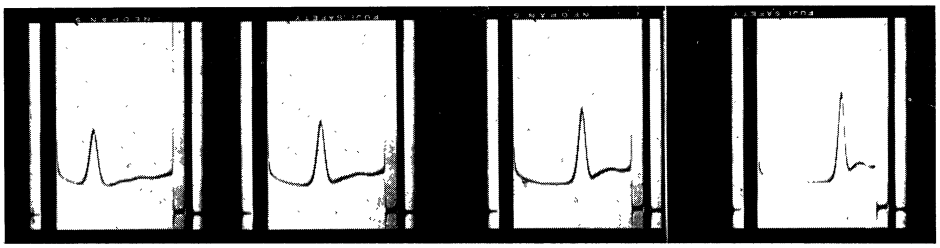

Fig. 2. Sedimentation patterns of purified enzyme in a ultracentrifuge. Buffer 0.2 M-Tris buffer, pH 8.4. Temp., 15°C, speed 59,780 rpm, bar angle 65°. Each of the four exposures was spaced 32 minutes apart. Yellowish portion was always found on left hand side of the main boundary.

acceptor) as well as aerobic conditions. These latter activities were, however, considerably low compared with the peroxidatic activity mentioned above. In Table 1 are compared these three activities of the purified enzyme preparation in terms of the turnover number of flavin which was assumed to be the common redox agent operating in those reaction. Con-

Table 1. Turnover Numbers of Purified Enzyme

Emzyme action	pH	Buffer	Turnover number per mole of FAD per min. at 20C
DPNH oxidation	6.6-8.3	M/15-phosphate	40
DPNH dehydrogenation	6.5-7.0	〃	30
	8.3	〃	93
DPNH peroxidation	6.6	M/5-phosphate	400
	4.6	M/5-acetate	1500-2000

Reaction conditions: DPNH oxidation; each photocell contained 0.2 ml of diluted enzyme and 2.6 ml. of buffer. DPNH dehydrogenation; each photocell contained 0.2 ml. of diluted enzyme, 0.2 ml. of 10^{-3} M-2, 6-dichlorophenol-indophenol and 2.4 ml. of buffer (anaerobic). DPNH peroxidation; each photocell contained 0.2 ml. of diluted enzyme, 0.2 ml. of M/5-H_2O_2 and 2.4 ml. of buffer. Reactions were started by addition of 0.2 ml. of 2×10^{-3} M-DPNH.

Table 2. Comparison of DPNH Oxidation and Peroxidation Activities in Crude Sonicate and in Purified DPNH Peroxidase

	Relative activity	
	DPNH peroxidase	Crude sonicate
DPNH oxidation	0.3	7.5
DPNH peroxidation	100	100

Reaction conditions were similar to those given in Table 1. M/5-phosphate buffer (pH 5.9) was used in this case.

sidering the high degree of chemical homogeneity shown by the enzyme preparation, it may be reasonable to assume that the oxidation and dehydrogenation of DPNH were due to the secondary activities of the DPNH peroxidase itself, and not due to the contamination of other enzymes in the preparation used in the experiment. As may be seen from the data given in Table 2, crude sonicates of original bacterial cells showed a fairly strong activity of oxidizing DPNH under aerobic conditions (without added hydrogen peroxide), which can hardly be accounted for by the oxidatic action of the purified preparation of DPNH peroxidase. It is therefore, assumed that the bacterial cells contain a certain DPNH oxidizing enzyme which is different from the DPNH peroxidase purified in the present study.

Fig. 3 shows the effect of pH on the different enzyme activities shown by the *purified* preparation of DPNH peroxidase. Whereas the optimum pH for the peroxidatic action was at around 5, that for anaerobic DPNH dehydrogenation in Tris buffen was at 8-8.5, and the rate of aerobic DPNH oxidation remained the same in the pH range from 6.6 to 8.3.

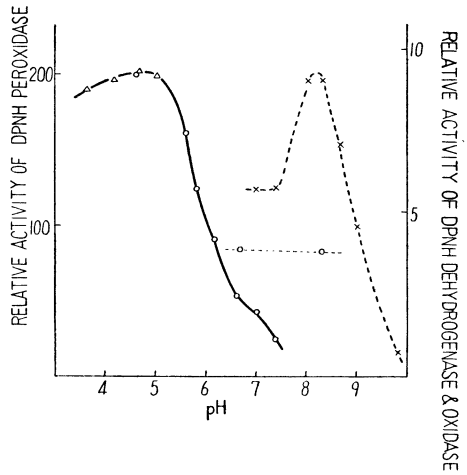

Fig. 3. Effect of pH on enzyme activity —DPNHperoxidase.....DPNH oxidase,- - - DPNH dehydrogenase. △M/5-acetate buffer, ○M/5-Phosphate buffer. ×M/S Tris buffer.
Conditions of measurement of activities were the same as given in Table 1.

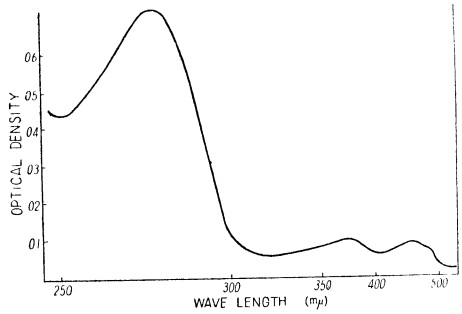

Fig. 4. Absorption spectrum of DPNH peroxidase in M/5 phosphate buffer (PH 7.4)

Prosthetic group: In Fig. 4 is presented the absorption spectrum of the purified enzyme which is typical of flavoprotein showing characteristic maxima at 270, 370 and 446 mμ. The prosthetic group of the enzyme was thought to be FAD, since the heated preparation reactivated the apo-D-amino acid oxidase to the same degree as authentic FAD when compared in terms of their absorption at 450 mμ.

When DPNH was added to the enzyme solution under anaerobic conditions, a broad absorption peak around 550 mμ appeared concomitantly with a partial disappearance of the absorption at around 450 mμ. The results are shown in Fig. 5. By the addition of hydrogen peroxide to the mixture

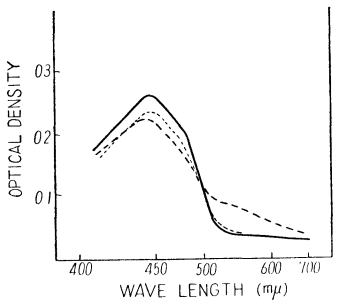

Fig. 5. Effect of DPNH and hydrogen peroxide on absorption spectrum of enzyme.

All reactions were carried out under anaerobic conditions. Thunberg type photocell, provided with two side arms, contained 0.2ml. of enzyme solution, 2.5 ml. of M/5-phosphate buffer (PH 6.6) in the main compartment, 0.2 ml. of DPNH (0.6 μ mole) in the first side arm and 0.2 ml. of M/5-H_2O_2 in the second side arm. Total volume 3.1 ml.

To the enzyme solution (solid line) was added first DPNH (broken line) then H_2O_2 (dotted line).

the spectrum was again reversed to that of the typical flavoprotein. In the absence of DPNH, no shift of absorption spectra was observed on the addition of hydrogen peroxide, a fact indicating that no complex was formed between the enzyme and hydrogen peroxide.

DISCUSSION

A flavin nucleotide-linked DPNH peroxidase has already been obtained by DOLIN in a partially purified state from a *Streptococcus* (1). The results obtained above have shown that the peroxidase isolated in almost homogeneous state from *Lactobacillus casei* was similar to, or presumably identical with, that of *Streptococcus*. Throughout the course of the purification, large parts of yellowish colored fractions were always accompanied by the DPNH peroxidase activity, indicating that most part of flavoprotein in the bacterial cells was that of DPNH peroxidase. The purified preparation of the peroxidase was found to possess a weak activity of catalyzing dehydrogenation of DPNH both under anaerobic (in the presence of 2,6-dichlorophenolindophenol as hydrogen acceptor) and aerobic conditions. It was found that the crude sonicate preporation obtained from the bacterial cells showed a strong DPNH oxidase activity, which was hardly explicable by the secondary oxidase activity shown by the DPNH peroxidase itself. No doubt, the bacterial cells contain a DPNH oxidase which is different from the DPNH peroxidase dealt with in the present study. It should be remarked that the presence of a flavin-linked DPNH oxidase in *Streptococcus* has already been reported by Dolin who separated the enzyme also in a partially purified state. By applying the method used by DOLIN, however, we could not obtain the same enzyme from *Lactobacillus casei*.

As shown in Fig. 5, the peroxidase shows a broad absorption at around 550 mμ in the presence of reduced DPN. A similar spectrum was observed with *Streptococcal* DPNH peroxidase by DOLIN (*2*) who thought to be that of enzyme-DPNH complex. A strikingly similar spectrum has been observed with xanthine oxidase (*7*) which has, however, been attributed to the FAD of semi-quinone form. Further studies on the spectrophotometric properties of the enzyme as they change under various experimental conditions seem to be highly relevant.

SUMMARY

1. From the cells of a strain of *Lactobacillus casei* a flavin nucleotide-linked DPNH peroxidase was isolated in an almost homogeneous state, and it was revealed to have flavin adenine dinucleotide as prosthetic group. The preparation possessed weak activities of DPNH oxidase and dehydrogenase.

2. The enzyme itself showed absorption maxima at 370 and 446 mμ, and in the presence of DPNH there appeared a broad absorption at around 550 mμ conconitantly with partial disappearance of the absorption at around 450 mμ. The absorption at around 450 mμ. The absorption at around 550 mμ disappeared on addition of hydrogen peroxide together with DPNH to the enzyme.

REFERENCES

(*1*) M. I. DOLIN: *J. Biol. Chem.* **225,** 557 (1957)
(*2*) M. I. DOLIN: *J. Biol. Chem.* **235,** 544 (1960)
(*3*) S. P. COLOWICK and N. O. KAPLAN: Method in Enzymology, Academic Press, Inc. New York. Vol. III. P. 887 (1957)
(*4*) E. RACKER: *J. Biol. Chem.* **184,** 313 (1950)
(*5*) V. MASSAY: *Biochem. et Biophys. Acta* **37,** 310 (1960)
(*6*) M. I. DOLIN: *Arch. Biochem. Biophys.* **55**, 415 (1959)
(*7*) H. KUBO, H. WATARI, K. SHIGA, A. ISOMOTO, M. UOZUMI, *Symp. Enzyme Chem.* (Japan) **15,** in press (1961)