Crystalline DPNH Oxidase from *Lactobacillus plantarum* No. 11¹⁾

Sir:

In a previous paper²⁾ it was presented that the yellow color in the cells of *Lactobacillus plantarum* No. 11 was remarkably enhanced when the organism was grown in a medium containing D-gluconate or D-ribose as a carbon source in place of glucose. During growth in such a medium, the ability of oxidative fermentation on D-ribose was also induced. From the studies on the absorption spectrum of the cells, the yellow pigment was identified as a flavin compound which could be converted into reduced form by the addition of D-ribose as a hydrogen donor²⁾. The reduced flavin compound was readily reoxidized by aeration. Flavin in these cells was found to be present mostly as FAD in a bound form and its concentration was estimated to be 10^{-4} M or higher¹⁾.

The present paper deals with a DPNH oxidase which constitutes the main part of yellow protein in the gluconate grown cells of *L. plantarum* No. 11. The crystalline preparation of DPNH oxidase could be isolated from the soluble fraction of sonicate of this bacterium by means of DEAE cellulose column chromatography and salting-out method.

As the characteristic properties of the purified enzyme, the followings were found:

1) Molecular weight of the enzyme was calculated to be approximately 170,000 from the values of sedimentation constant ($S_{20,w} = 7.02 \times 10^{-13}$ cm sec⁻¹) and diffusion constant ($D_{20,w} = 4.12 \times 10^{-7}$ cm²sec⁻¹).

2) By employing paper chromatography, spectrophotometric method and D-amino acid

oxidase assay, one mole of this enzyme was proved to contain two moles of FAD as the prosthetic group (Fig. 1).

FIG. 1. Absorption Spectra of Crystalline DPNH Oxidase from *Lactobacillus plantarum* No. 11 (Beckman DK II).

— Oxidized Enzyme
 - - - Reduced Enzyme
 2 mg enzyme/ml in M/100 KH₂PO₄ solution;
 Thunberg-type anaerobic cells were used;
 Enzyme in reduced form was prepared by addition of DPNH.

Plate 1. Crystalline DPNH Oxidase $\times 2,600$

3) At 20°C, optimum pH is 4.4~4.8, Michaelis constant of the enzyme for DPNH 1.2×10^{-4} M, and turnover number of DPNH oxidation 6,600 moles/minute/mole enzyme.

4) Reduced form of this enzyme is readily auto-oxidized by atmospheric oxygen producing water but no hydrogen peroxide. Manometric experiments (Fig. 2) support the

1) S. Fukui, Presented at 13th annual meeting of Symposium on Enzyme Chemistry, July, 1961 (Tokyo).

2) S. Fukui, and A. Oi, *J. Gen. Appl. Microbiol.*, **4**, 120 (1958).

following equation for the enzymatic oxidation of DPNH.

FIG. 2. Oxidation of DPNH by the Crystalline DPNH Oxidase from *L. plantarum* No. 11.

Reaction mixture:

	Curve 1 (solid line)	Curve 2 (dotted line)
DPNH	1.0 ml (3.6 μmoles)	1.0 ml (3.6 μmoles)
H ₂ O ₂	0	0.1 (2.6 μmoles)
M/10 KH ₂ PO ₄	0.2	0.2
DPNH oxidase	0.2 (400 units)	0.2 (400 units)
Catalase	0.1	0.1
Dist. water	0.5	0.4

Reaction temp. 30°C.

TABLE I. FORMATION OF HYDROGEN PEROXIDE DURING THE OXIDATION OF DPNH BY MODIFIED DPNH OXIDASE.

	Original Preparation	Modified Preparation*
H ₂ O ₂ formed (μmoles)	Trace	1.2
DPNH consumed (μmoles)	1.5	1.5

Reaction mixture: M/10 KH₂PO₄ 0.075 ml
 DPNH 0.4 (1.5 μmoles)
 DPNH oxidase 0.25 ml (250 units)
 Dist. water 0.075 ml
 Total volume 0.8 ml

After 30 minutes incubation at 30°C, hydrogen peroxide formed was estimated by manometric method.

* Modified DPNH oxidase was prepared by the treatment with 2 M urea solution under the following conditions: pH 5.0, 30°C, 30 minutes. The enzyme preparation thus treated showed approximately one half activity of the original one.

5) The oxidase is not inhibited by M/100 CN⁻ or hydrogen peroxide, but is strongly inhibited by low concentrations of *p*-chloromercuribenzoate and Cu²⁺.

6) Treatment with 2 M urea solution converts the enzyme into a modified form which produces hydrogen peroxide on auto-oxida-

tion (Table I).

It is a well known fact^{3,4} that true lactic acid bacteria belong to anaerobes or facultative aerobes which do not contain any cytochrome-dependent terminal oxidases but show oxidative metabolism on glucose. In these bacteria, therefore, flavin enzymes have been considered to function as terminal oxidases. On the other hand, in his investigation on the cytochrome-independent electron transport in bacteria, Dolin^{5,6} has demonstrated the presence of a specific DPNH oxidase in the cells of *Clostridium perfringens*. Partially purified oxidase of Dolin is stated to contain also FAD as prosthetic group and not to form free hydrogen peroxide as an intermediate during the oxidation of DPNH. In his case, however, hydrogen peroxide and *p*-chloromercuribenzoate were found to be strong inhibitors. For the mechanism of water formation with the enzymatic oxidation of DPNH, two schemes were proposed by Dolin⁶. There appears to be, however, no experimental evidence for the schemes, and his enzyme preparation is not yet purified enough to permit the discussion on the mechanism of water formation.

Now, the present author would like to

(I)+(II) is $2 \text{ DPNH} + 2 \text{ H}^+ + \text{O}_2 \longrightarrow 2 \text{ DPN}^+ + 2 \text{ H}_2\text{O}$.

* $\begin{array}{c} \text{F} \\ \vdots \\ \text{F} \end{array}$: Oxidized form of DPNH oxidase; $\begin{array}{c} \text{FH}_2 \\ \vdots \\ \text{FH}_2 \end{array}$: Reduced form

3) M.A. Farrel, *J. Bact.*, **29**, 47 (1935).

4) C.F. Strittmatter, *J. Biol. Chem.*, **234**, 2789, 2794 (1959).

5) M.I. Dolin, *Bacteriol. Proc.* (Sec. Am. Bacteriologists) p. 105 (1958).

6) M.I. Dolin, *J. Bact.*, **77**, 383, 393 (1959).

show the following formulas in order to explain the water formation:

Two molecules of FAD in one molecule of the enzyme are assumed to be situated so close to each other that they are able to combine simultaneously with one molecule of oxygen as shown in formula (II). As a result, one mole of the reduced enzyme is converted non-enzymatically into the oxidized form by one mole of oxygen with the liberation of 2 moles

of water. Since the enzyme could be converted into the hydrogen peroxide forming form by treatment with urea, the molecular structure of the enzyme proposed above is believed to be held by S-S bridges and/or tertiary structure.

Sakuzo FUKUI

*The Institute of Applied Microbiology,
University of Tokyo, Tokyo.*

Received October 14, 1961