Communication

Inhibitory Effect of Vitamin E on Vitamin B₂-Aldehyde-Forming Enzyme in *Schizophyllum commune*

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(Received September 29, 1980)

Key Words vitamin E, DL- α -tocopheryl acetate, vitamin B₂-aldehyde-forming enzyme, *Schizophyllum commune*, inhibition

The novel enzyme of *Schizophyllum commune* which converts the 5' hydroxymethyl group of riboflavin to the aldehyde group has been found to be unable to use NAD(P)⁺, FMN, FAD and Cyt *c* as electron acceptor for the conversion, as reported previously (1, 2). During further investigation on the reaction mechanism of the enzyme, we have found a significant inhibition of the enzyme by α -tocopherol, as detailed in this paper.

The enzyme was purified about 100-fold from a cell-free extract of *S. commune* IFO No. 4928 by means of ammonium sulfate fractionation, ethanol fractionation, Sephadex G-25 column chromatography, and DEAE-Sephacel column chromatography and the activity of the enzyme was assayed in terms of the fluorescence intensity of B₂-aldehyde produced by the method described in the previous papers (3, 4).

By utilizing the capability of β -cyclodextrin of forming an inclusion complex with fat-soluble compound as guest (5), the inclusion complex of α -tocopheryl acetate with β -cyclodextrin was prepared as follows. Seven hundred and forty milligrams of DL- α -tocopheryl acetate was suspended in 200 ml of 1% β -cyclodextrin containing a few drops of toluene, and shaken in a flask at room temperature for about 20 hr, until it became turbid. The resulting precipitate was collected by centrifugation at $12,000 \times g$ for 20 min at 5°C, and successively washed with deionized water, acetone, and ether at 0°C, and then dried under reduced pressure. The white powder obtained, the inclusion complex, was stored in a desiccator at 5°C.

The reaction mixture contained 50 mM sodium acetate buffer, pH 5.5, 53.4 μ M

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Abbreviations: FR, riboflavin; B₂-aldehyde, 7,8-dimethyl-10-(2,3,4-trihydroxy-4-formylbutyl)isoalloxazine; B₂-acid, 7,8-dimethyl-10-(2,3,4-trihydroxy-4-carboxybutyl)isoalloxazine; DCIP, 2,6-dichlorophenolindophenol.

Table 1. The effect of α -tocopherol on B₂-aldehyde-forming enzyme. Experimental details have been described in the text.

The specific activity of the preparation of B₂-aldehyde-forming enzyme was 0.18 unit/mg of protein. One unit of the enzyme activity was defined as the amount of enzyme which formed 1 μ mole of B₂-aldehyde per minute.

The inclusion complex of DL- α -tocopheryl acetate (mM)	B ₂ -aldehyde formed (μ M/hr)	Inhibition (%)
None	32.6	—
0.1	26.4	19
0.5	19.2	42
1.0	8.8	73
5.0	4.8	85
10.0	1.6	95

FR, 100 μ M DCIP, 0.1 ml of the B₂-aldehyde-forming enzyme preparation (5.4×10^{-4} units), and the inclusion complex in a total volume of 1 ml. After the mixture was continuously shaken during the incubation for 1 hr at 37°C, 0.02 ml of the reaction mixture was applied to Toyo Filter Paper No. 2. The paper was developed with a solvent system of ethyl acetate–pyridine–water (10:3:3; upper layer) by the ascending method. The fluorescence intensity of each flavin spot on the paper was measured by a Shimadzu-Kotaki micro-reflecting fluorometer.

The results in Table 1 show that the inhibitory effect of the inclusion complex of α -tocopherol on the B₂-aldehyde-forming enzyme was dependent on the concentration of inclusion complex in the range from 0.1 to 10 mM. Furthermore, the significant inhibition by the inclusion complex was also observed when an unidentified cofactor from the mold was used in place of DCIP.

In order to investigate the effect of α -tocopherol on B₂-aldehyde formation *in vivo*, the experiment under culture conditions was carried out. *S. commune* IFO No. 4928 or IAM No. 9006 which is a most preferable strain for ethanol(6) was cultivated on a reciprocal shaker at 30°C in a medium containing 2 g of glucose or 2 ml of ethanol, 0.1 g of NH₄NO₃, 0.1 g of KH₂PO₄, 0.05 g of MgSO₄·7H₂O, 0.05 g of KCl, 0.13 mg of MnCl₂, 0.5 μ g of thiamine, 2 or 1.5 g of CaCO₃, 5 mg of FR, 0.6 ml of DL- α -tocopheryl acetate solution in ethanol (finally 0.1 mM) and 100 ml of tap water. After cultivation, 0.02 ml of the culture broth was applied to Toyo Filter Paper No. 2. The flavin compounds were determined as described above.

The data presented in Table 2 demonstrate that in the case of the culture medium containing α -tocopherol the conversion of riboflavin to B₂-aldehyde by the mold was markedly inhibited.

Accordingly, it was concluded that α -tocopherol inhibits the B₂-aldehyde formation by *S. commune*. The inhibitory effect of α -tocopherol on the enzyme might suggest a participation of a radical mechanism in the enzyme reaction,

Table 2. The effect of α -tocopheryl acetate on B₂-aldehyde formation by *S. commune* under culture conditions.

Experimental details have been described in the text. In the case of the culture of *S. commune* IAM No. 9006, 2 g of glucose and 2 g of CaCO₃ in the medium for *S. commune* IFO No. 4928 were replaced by 2 ml of ethanol and 1.5 g of CaCO₃, respectively. The culture time was 5 days. B₂-aldehyde-forming activity was expressed as the coefficient of conversion ratio of riboflavin to B₂-aldehyde (%) per mycelial weight (mg).

Strain of <i>S. commune</i>	DL- α - Tocopheryl acetate	Mycelial wt.	Conversion	B ₂ -aldehyde- forming activity	Inhibi- tion
			ratio: B ₂ -aldehyde formed/B ₂ added		
	(mm)	(mg/100 ml)	(%)	(%/mg)	(%)
IFO No. 4928	None ^a	83	33.7	0.41	—
	0.1	64	15.8	0.25	39
IAM No. 9006	None	122	11.1	0.09	—
	0.1	225	6.2	0.03	67

^a Ethanol (0.6 ml) added.

because α -tocopherol reacts with organic free radicals (7).

In summary, vitamin E (α -tocopherol) was found to protect to some extent vitamin B₂ (riboflavin) from the enzymatic oxidation of the 5'-site which is indispensable for the formation of FMN or FAD in *Schizophyllum commune*.

We appreciate the generous gift of DL- α -tocopheryl acetate from Dr. G. Katsui, Eisai Co., Ltd., Tokyo. We also wish to thank Mr. T. Imai, H. Uehashi, I. Takeuchi and S. Nakagawa for their technical assistance.

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