A METHOD FOR QUANTITATION OF VITAMIN B₂ BY USING AN ENZYME (PRODUCING VITAMIN B₂-ALDEHYDE) FROM *SCHIZOPHYLLUM COMMUNE*

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(Received April 14, 1980)

Summary A method for enzymatic determination of riboflavin was developed by enzymatic reduction of 2,6-dichlorophenolindophenol using a vitamin B₂-aldehyde-forming enzyme prepared from *Schizophyllum commune*. The enzymatic method enabled us to determine riboflavin in a mixture containing riboflavin, FMN and FAD without separation of riboflavin. Moreover, the total amount of flavins in the mixture was shown to be determined by the enzyme in the presence of phosphatase.

Keywords riboflavin, vitamin B₂-aldehyde, *Schizophyllum commune*, 2,6-dichlorophenolindophenol, determination, vitamin B₂-aldehyde-forming enzyme

The assay method of FR mixed with other flavin compounds has not been performed without the separation process (1-4) of FR from its derivatives.

During the course of study of flavin metabolism in *Schizophyllum commune* (5), we found an enzyme that oxidizes FR to B₂-aldehyde (6-8) in the same organism (9). DCIP serves as a hydrogen acceptor in the B₂-aldehyde-forming enzyme (10). The substrate specificity of the enzyme is high for FR, as far as tested (11). We tried to ascertain the possibility of enzymatic determination of FR by using the B₂-aldehyde-forming enzyme in a mixture containing FR, FMN and FAD without isolation of FR from other flavins. The result showed that the enzymatic determination of FR was possible. This paper deals with the establishment of direct determination of FR in a mixture containing FR, FMN and FAD by B₂-aldehyde-forming enzyme. In addition, the determination of total flavin by using the crude enzyme preparation from *S. commune* is reported.

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Abbreviations: FR, riboflavin; B₂-aldehyde, vitamin B₂-aldehyde, 7,8-dimethyl-10-(2,3,4-trihydroxy-4-formylbutyl)isoalloxazine; B₂-acid, vitamin B₂-acid, 7,8-dimethyl-10-(2,3,4-trihydroxy-4-carboxybutyl)isoalloxazine; DCIP, 2,6-dichlorophenolindophenol; A_{600} , absorbance at 600 nm; ϵ_{600} , molar extinction coefficient at 600 nm.

MATERIALS AND METHODS

1) *Chemicals*. FR was purchased from Merck and Company (New Jersey, United States). FAD was a gift from Wakamoto Pharmaceutical Co., Ltd. FMN, DCIP and other reagents were purchased from Nakarai Kagaku Co., or Wako Junyaku Co.

2) *Microorganism and cultivation*. *Schizophyllum commune* IFO No. 4928 was used. The culture medium was composed of 5% glucose, 0.05% peptone, 0.05% NH_4NO_3 , 0.1% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.13 mg of MnCl_2 , 0.75 μg of thiamine, 1% CaCO_3 and 100 ml of tap water. CaCO_3 was sterilized separately and added to the sterilized solution containing other components just before inoculation. Culture medium (60 ml) in a 500 ml-shaking flask was sterilized in an autoclave at 1 atm for 20 min and was inoculated with a few loopfuls of the stock culture. The shaking cultivation was carried out on a reciprocal shaker for 5 days at 30°C. The same medium (5 liter) in a 10 liter-jar-fermentor was sterilized at 1 atm for 20 min. The sterilized medium was inoculated with 300 ml of the pre-culture. Cultivation was carried out for 5 days at 30°C.

After cultivation, the mycelia were harvested by filtering the broth, washed by deionized water, and dried by washing with cold acetone. The acetone-dried mycelia were stored in an evacuated desiccator containing CaCl_2 at 5°C for at least 14 days and then used as the enzyme source.

3) *Preparation of enzyme*. The B_2 -aldehyde-forming enzyme was prepared from the acetone-dried mycelia of *S. commune*. The details of the procedure are reported elsewhere (11). The outline was as follows. One hundred grams of acetone-dried mycelia were suspended with 1,500 ml of 50 mM acetate buffer (pH 5.5) and homogenized in a glass/glass homogenizer. The homogenate was centrifuged at 12,000 g , for 30 min at 5°C. The supernatant was consecutively treated by $(\text{NH}_4)_2\text{SO}_4$ saturation fractionation, ethanol fractionation, and chromatographies on columns of Sephadex G-25, DEAE-Sephacel, and Sephadex G-100. The enzyme was purified about 1,400-fold over the crude cell-free extract. Unless otherwise mentioned, this purified enzyme was used in this study.

4) *Enzyme assay*. The B_2 -aldehyde-forming enzyme oxidizes FR to B_2 -aldehyde using DCIP as a hydrogen acceptor. The absorption maximum of DCIP is at 600 nm, where FR, FMN and FAD do not show absorbance. Hence, the decrease of absorbance at 600 nm could be employed as an indication of the enzyme activity. Then the activity was assayed in terms of the decrease in absorbance at 600 nm with reduction of DCIP. One unit was defined as the amount of enzyme which reduces 1 nmole of DCIP per min.

The determination of FR was carried out by the end-point method. The reaction mixture contained 100 mM phosphate buffer (pH 6.0), 75 μM DCIP, enzyme and flavin in a total volume of 2 ml. The reaction was initiated by the addition of the enzyme. The reduction of DCIP was recorded with a Hitachi 124 spectrophotometer equipped with a Hitachi 056 recorder. The reaction was carried

out at room temperature (approximately 25°C). The end point was defined as the point where the decrease of absorbance at 600 nm was not longer observed. The difference between the absorbance at the initiation point of the reaction and at the end point; ΔA_{600} , was measured (Fig. 1). FR concentration was calculated from ΔA_{600} based on the molar extinction coefficient of DCIP.

RESULTS AND DISCUSSIONS

1) Determination of FR in FR solution

Figure 1 shows the time course of the DCIP reduction carried out by using 8.3 units of enzyme and 40.4 μM of FR. When A_{600} reached a minimum after 20 min incubation, the concentration of reduced DCIP was estimated to be 40.5 μM based on 12.8×10^3 of molar extinction coefficient of DCIP at pH 6.0 in Fig. 2, which shows a significant pH-dependency of molar extinction coefficient of DCIP in the range from pH 4 to 7, suggesting that pH should be more carefully controlled. The amount of reduced DCIP was almost equal to that of FR added, showing that an end-point method is useful for the enzymatic determination of FR using DCIP as hydrogen acceptor, within 20 min of the start of the reaction. Furthermore, the reduced DCIP was shown to be stable for about 40 min.

Next, an experiment to investigate the relation between FR concentration and ΔA_{600} was carried out. Figure 3 shows that the value of ΔA_{600} stoichiometrically corresponded to the amount of FR up to 75 μM .

From the results described above, we concluded that FR could be determined enzymatically by using a B₂-aldehyde-forming enzyme from *S. commune*.

2) FR determination in the mixture of FR, FMN and FAD

B₂-Aldehyde-forming enzyme is specific for FR (11). The direct determination

Fig. 1. Time-course of 75 μM DCIP reduction at 600 nm with B₂-aldehyde-forming enzyme. Experimental details were described in "MATERIALS AND METHODS."

Fig. 2. The pH dependency of molar extinction coefficient of DCIP at 600 nm. Absorbance at 600 nm of 40.9 μM DCIP was measured in a buffer solution at respective pHs. Molar extinction coefficient at each pH was calculated from the absorbance, based on the value of 19.1×10^3 at pH 7 (12). $\square$, 100 mM acetate buffer; $\bullet$, 100 mM phosphate buffer; $\circ$, 100 mM Tris-HCl buffer.

Fig. 3. Proportionality of ΔA_{600} of DCIP to FR concentration. Experimental details were described in "MATERIALS AND METHODS." Enzyme activity used was 5.6 units and the reaction time was 30 min.

of FR was expected in the mixture of FR, FMN and FAD without separating them. We applied this enzymatic assay to samples containing much more FMN and FAD than FR. Three samples were selected for the experiment. They contained FR, FMN and FAD in the following μM concentrations, respectively: (1) 13.5, 109.5, 27.3, (2) 13.5, 69.5, 63.7, (3) 13.5, 29.9, 100.1. The enzyme preparation used was paper-chromatographically analysed to exhibit no FR-liberating activity

from FMN or FAD in the reaction mixture, as described in "MATERIALS AND METHODS." Table 1 shows the results of determination of 13.5 μ M FR in these samples. Although FMN and FAD were contained in the sample at concentrations ten-fold that of FR, the competitive inhibition by FMN and FAD was not detected and FR could be determined with error of only $\pm 6\%$. Consequently, it was substantiated that FR could be determined without the separation procedure of FR in the mixture containing FMN and FAD.

3) *Conversion to B₂-aldehyde from FMN or FAD by using crude enzyme preparation*

FR, FMN or FAD was incubated with the crude preparation of B₂-aldehyde-forming enzyme. The crude preparation used was an active pool of DEAE-Sephacel column chromatography step, before Sephadex G-100 step. The reaction mixture was composed of 50 mM acetate buffer (pH 5.5), 7.5 μ M DCIP, 18.5 units of enzyme of the crude preparation, and substrate in a total volume of 1 ml. Flavin as a substrate was 53.8 μ M FR, 59.7 μ M FMN, or 54.6 μ M FAD. The reaction was allowed to proceed at room temperature (approximately 25°C). After incubation for 10 min, 0.02 ml of the reaction mixture was applied to Toyo Filter Paper No. 2 and was developed with a solvent system of ethyl acetate-pyridine-water (10:3:3; upper layer) by an ascending method. As shown in Fig. 4, it was found that FMN or FAD, as well as FR was converted to B₂-aldehyde by the crude preparation of B₂-aldehyde-forming enzyme which might contain phosphatase, the activity of which should be sufficiently high to decompose FMN or FAD within 10 min under the conditions employed above.

Some B₂-acid formation from FR or FMN was shown as in Fig. 4, but it was elucidated that no DCIP reduction coupled with enzymatic or nonenzymatic oxidization of B₂-aldehyde was observed when B₂-aldehyde was used instead of FR.

Table 1. Effect of FMN and FAD on FR determination. The determination of FR was carried out according to "MATERIALS AND METHODS."

Enzyme activity used was 8.3 units and the reaction time was 20 min.

Sample			ΔA_{600} at pH 6.0	Determined FR (μ M)	Recovery (%)
FAD (μ M)	FMN (μ M)	FR (μ M)			
27.3	109.5	13.5	0.183	14.3	106
63.7	69.7	13.5	0.177	13.8	102
100.1	29.9	13.5	0.168	13.1	97

Fig. 4. Paper chromatogram of the products from FR, FMN and FAD by the crude preparation of B₂-aldehyde-forming enzyme of *S. commune*. Experimental details were described in the text. Substrate used: A, FR; B, FMN; C, FAD.

Table 2. Determination of total flavin using crude enzyme preparation. Enzyme used was DEAE-Sephacel pool which contained B₂-aldehyde forming activity of 54 units/ml. The reaction mixture contained 50 mM acetate buffer (pH 5.5), 75 μ M DCIP, 5.4 units of the enzyme and flavin in a total volume of 2 ml. Flavin concentration was determined from ΔA_{600} based on 7.60×10^3 of a molar extinction coefficient of DCIP at pH 5.5. The reaction time was 30 min.

Sample			Sample total flavin (μ M)	ΔA_{600} at pH 5.5	Determined total flavin (μ M)	Recovery (%)
FAD (μ M)	FMN (μ M)	FR (μ M)				
37.3			37.3	0.266	35.0	94
	51.8		51.8	0.397	52.2	101
		53.8	53.8	0.402	52.9	98
6.2	31.9	3.9	42.0	0.314	41.3	98
14.5	20.3	3.9	38.7	0.288	37.9	98
22.8	8.7	3.9	35.4	0.273	35.9	101

4) Determination of total flavin using crude enzyme preparation

Figure 4 suggests that total flavin in the mixture of FR, FMN and FAD may be determined and that the sum of FMN and FAD could then be calculated. This possibility was examined quantitatively. The crude preparation used was the DEAE-Sephacel pool described above. The reaction was allowed to proceed according to "MATERIALS AND METHODS," except that 50 mM acetate buffer (pH

5.5) was employed instead of 100 mM phosphate buffer (pH 6.0), because phosphate inhibited phosphatase. The molar extinction coefficient of DCIP was 7.60×10^3 at pH 5.5 (Fig. 2). As shown in Table 2, total flavin was recovered at a rate of nearly 100%. Then it was shown that total flavin could be determined by using the enzyme preparation containing phosphatase from *S. commune*.

From the results of these studies, it was demonstrated that FR could be determined specifically in the mixture of FR, FMN and FAD by using Sephadex G-100 pool which was the purest B₂-aldehyde-forming enzyme obtained by us, and that total flavin could be assayed by using the preparation containing phosphatase from *S. commune*. Moreover, the sum of FMN and FAD could be calculated by subtracting the FR from the total flavin.

This new enzymatic determination of FR is rapid, simple, accurate and does not require apparatus, reagents and efforts for separating FR from other flavins. This method might become more available for determination of FR in medicine, food or feed, while it is obvious from Fig. 3 that a fluorometric determination is useful at a concentration of flavin lower than $1 \mu\text{M}$. A study on the improved method of this determination by immobilization of the enzyme is in progress, and it is hoped to shift the optimum pH 5.5 of the enzyme to pH 8, exhibiting maximum A_{600} of DCIP as shown in Fig. 2.

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