VITAMIN B₂ ACTIVITY OF 7,8-DIMETHYL-10-(2,3,4-TRIHYDROXY-4-FORMYLIBUTYL)ISOALLOXAZINE IN LACTOBACILLUS CASEI¹

Sei Tachibana, Masanori Oka, Hitomi Tamura, Aisuko Kamei, Harumi Mukai, Chihoko Kanbayashi, and Izumi Shioiri²

Department of Chemistry, Faculty of Science and Engineering, Ritsumeikan University, Kyoto 603, Japan

(Received May 24, 1979)

Summary The microbial activities of vitamin B₂-aldehyde and vitamin B₂-acid, produced by Schizophyllum commune, a Basidiomycete, were studied. Lactobacillus casei ATCC No. 7469 was used as a test microorganism. B₂-aldehyde exhibited a good response curve in the growth of L. casei. B₂-acid had neither a stimulatory nor an inhibitory effect on the growth. When B₂-aldehyde was incubated with the homogenate of L. casei, it was converted to riboflavin. The flavin formed from B₂-aldehyde by the homogenate not only exhibited an equivalent response curve to authentic riboflavin in the growth of L. casei, but also showed the same Rf value as authentic riboflavin in any paper chromatogram, as far as tested. Hence, the microbial activity of B₂-aldehyde for L. casei seems to be ascribable to riboflavin which is a reduction product of B₂-aldehyde.

Keywords vitamin B₂-aldehyde, vitamin B₂-acid, Schizophyllum commune, microbial bioassay, Lactobacillus casei, reduction to riboflavin

The two flavin derivatives were found in a cultured broth of Schizophyllum commune, a Basidiomycete (1, 2). Each was identified as 7,8-dimethyl-10-(2,3,4-trihydroxy-4-formylbutyl)isoalloxazine, namely vitamin B₂-aldehyde, and 7,8-dimethyl-10-(2,3,4-trihydroxy-4-carboxybutyl)isoalloxazine, namely vitamin B₂-acid (3, 4). We have found that these flavins seemed to participate in L-malate accumulation with S. commune (1,5), and that they stimulated hydrocarbon fermentation by Candida lypolytica (6). Moreover, it was of interest to find these flavins widely distributed in edible Basidiomycetes: Agaricus bisporus, Flammulina

¹ A part of this paper was presented at the 30th Annual Meeting of the Vitamin Society of Japan in May, 1978, in Tokushima.

² 立花 晴, 岡 重則, 田村仁美, 亀井敦子, 向井治美, 神林千保子, 塩入いづみ

Abbreviations: B₂, riboflavin; PPC, paper partition chromatography.
velutipes and Pleurotus ostreatus and in some uncharacterized Basidiomycetes (7). This paper deals with their microbial activities, by using Lactobacillus casei ATCC NO. 7469.

MATERIALS AND METHODS

1. B2, B2-aldehyde and B2-acid. B2 was purchased from Merck and Company (New Jersey, United States).

B2-aldehyde and B2-acid were prepared from a cultured broth of S. commute according to a previous paper (3, 4) except that ethyl acetate–pyridine–water (10:3:3; upper layer) was used as a developer in PPC. This solvent system was the most suitable for the separation of B2-aldehyde and B2. B2-aldehyde is unstable at room temperature, so further purification of the B2-aldehyde preparation by PPC using ethyl acetate–pyridine–water (10:3:3; upper layer) was avoided. The purity of B2-aldehyde was greater than 80%. Most of the impurity was due to B2-acid. In the preparation of B2-aldehyde, B2 was not detected by paper chromatography. B2-acid was pure on paper chromatography. The concentration of each flavin was determined by using a Hitachi 124 spectrophotometer, postulating that the molecular extinction coefficients of B2-aldehyde and -acid at 448 nm are the same as the coefficient of B2.

2. Detection of microbial activities. The microbial activities of flavins were estimated in terms of their effects on the growth of Lactobacillus casei ATCC No. 7469, an assay organism of B2. The procedure for microbial bioassay was based on the method of Robert and Snell (8) with some modifications. L. casei was precultivated in a medium consisting of glucose (0.5 g), CH3COONa (0.5 g), dried yeast extract (2.5 g) and tap water (100 ml) for 24 hr at 37°C. The cells were harvested by centrifugation, washed by 0.85% NaCl five times, and suspended in 0.85% NaCl so that the absorbance at 630 nm of the suspension might be 0.12 to 0.13. In order to avoid turbidity due to the precipitation of the hydroxide or phosphate of a metal ion in the experimental medium, the concentration of MnSO4 was decreased to one-fifth and the concentrations of other inorganic salts were decreased to a half of those in the basal medium of Robert and Snell (8). MgSO4 was added after adjusting the medium to pH 6. The medium containing flavin was sterilized in an autoclave at 1 atm for 20 min. NaCl, KH2PO4 and K2HPO4 were sterilized separately from other components and mixed just before use. The sterilized complete medium was inoculated with one drop of the cell suspension by an injector. L. casei was incubated at 37°C for 18 hr. The growth of the microorganism was estimated in terms of the turbidimetry at 630 nm using a Hitachi 124 spectrophotometer.

3. Reaction with the homogenate of L. casei. L. casei was cultured in medium containing glucose (0.5 g), CH3COONa (0.5 g), dried yeast extract (2.5 g) and tap water (100 ml) for 18 hr at 37°C. The cells were harvested by centrifugation and washed by 0.85% NaCl five times. The homogenate was prepared by grinding
the wet cells with a three-fold volume of 0.05 M phosphate buffer (pH 6.5) in a mortar. The reaction mixture contained the homogenate (1 ml), 0.05 M phosphate buffer (pH 6.5) and 37.5 µM B2-aldehyde preparation in a total volume of 2 ml. The incubation temperature was 37°C. Every 0.02 ml of the supernatant from the reaction mixture was applied to Toyo Roshi No. 2 after a certain incubation time. 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 spot on the paper was measured with a Shimadzu-Kotaki micro-reflecting fluorometer and the amount of flavin was determined from a calibration curve using B2 as a standard.

RESULTS AND DISCUSSION

1. Microbial activity of B2-aldehyde preparation

Curve 1 of Fig. 1 shows a response curve for B2. When the B2-aldehyde preparation was used instead of B2, significant growth of L. casei was observed as shown in curve 2 of Fig. 1. The apparent activity of B2-aldehyde was about 35% of B2. The activity of the B2-aldehyde preparation would not be due to B2 as an impurity, because no spot of B2 was detected on the paper chromatogram of the preparation.

2. Microbial activity of B2-acid

The B2-aldehyde preparation contained B2-acid as an impurity. The microbial activity of B2-acid for L. casei was studied and B2-acid was isolated and crystallized according to a previous paper (3,4). The result is shown in curve 3 of Fig. 1. Although the concentration of B2-acid was increased to 53.5 ng/ml, growth was not
observed. The effect of adding B2-acid together with B2 was also tested. As shown in curve 4 of Fig. 1, the response of L. casei was dependent on the concentration only of B2; therefore, B2-acid did not seem to have any effect on the growth of L. casei. This result suggested that the observed activity of B2-aldehyde was not influenced by any contaminating B2-acid.

3. Reduction of B2-aldehyde to B2 using a homogenate of L. casei

It is of interest to know why B2-aldehyde exhibited the microbial activity. We investigated the conversion of B2-aldehyde by using a homogenate of L. casei, relating to the microbial activity of B2-aldehyde. The preparation of homogenate and reaction conditions were as described in “MATERIALS AND METHODS.” The reaction products were identified by PPC using several developers such as ethyl acetate–pyridine–water (10:3:3; upper layer), n-butanol–pyridine–water (6:4:3), n-butanol–acetic acid–water (4:1:5; upper layer), water saturated with isoamyl alcohol, and 5% Na2HPO4. The B2 produced by the homogenate behaved identically to authentic B2 on PPC using any one of these developers. Figure 2 shows a paper chromatogram using ethyl acetate–pyridine–water as a developer. Figure 3 shows time-courses of B2 and B2-acid formation. The larger part of B2-aldehyde was converted to B2 as the B2-aldehyde concentration decreased, while a very little of the B2-acid was formed. The homogenate, which was boiled at 100°C for 10 min, was used as a control. The boiled homogenate did not reduce B2-aldehyde to B2. This result suggests that the activity of B2-aldehyde was attributable to B2 of a reduction product from B2-aldehyde.

![Fig. 2. Paper chromatogram of the homogenate reaction mixture. The reaction conditions were as described in “MATERIALS AND METHODS.” The sample which was incubated for 6 hr at 37°C was applied to Toyo Roshi No. 2. The developer was ethyl acetate–pyridine–water (10:3:3; upper layer).]
Fig. 3. Time-course of B₂ formation by the homogenate of L. casei. Experimental details were as described in "MATERIALS AND METHODS." ○—○, B₂-aldehyde; •—•, B₂; ▲—▲, B₂-acid.

4. Microbial activity of the B₂ produced from B₂-aldehyde with the homogenate of L. casei

Some isomers of B₂ have been studied with respect to their biological activities. For example, it was reported that araboflavin had some activity for L. casei (9, 10). We studied the microbial activity of B₂ produced by the homogenate (the sample B₂), comparing it with authentic B₂ in order to obtain information as to whether the sample B₂ was a true D-riboflavin. If the product from B₂-aldehyde with the homogenate is D-riboflavin, its activity should be equivalent to that of B₂.

The sample B₂ in the homogenate reaction mixture was separated by PPC using ethyl acetate–pyridine–water as a developer and then was eluted from the

Fig. 4. Microbial activities of the B₂ produced by the homogenate of L. casei and authentic B₂. Experimental details were as described in "MATERIALS AND METHODS." ○—○, authentic B₂ treated by PPC; •—•, the B₂ produced by the homogenate of L. casei.
paper with deionized water. The eluate was used as the sample B2 without further purification. Authentic B2 was charged on the same filter paper, and treated by PPC in the same way as the sample B2 as described above, so that the influence of impurities during the preparation of the sample B2 might be compensated.

As shown in Fig. 4, the sample B2 showed the same response curve in the growth of L. casei as the authentic B2, D-riboflavin. Therefore, it is almost conclusive that the product from B2-aldehyde is D-riboflavin.

From the results of these studies it could be determined that B2-aldehyde possessed the stimulatory activity for growth of B2-requiring L. casei, and that the microbial activity of B2-aldehyde seems attributable to the normal metabolism of B2 which was produced by an enzymatic reduction of B2-aldehyde in L. casei.

REFERENCES

1) TACHIBANA, S. (1972): Metabolism of riboflavin in Schizophyllum commune. J. Vitaminol., 18, 210–212.
2) TACHIBANA, S., and MURAKAMI, T. (1975): The isolation and some properties of new flavins (“Schizoflavin”) formed by Schizophyllum commune. J. Nutr. Sci. Vitaminol., 21, 61–63.
3) TACHIBANA, S., MURAKAMI, T., and NINOMIYA, T. (1975): Identification of the chemical structures of Schizoflavins as 7,8-dimethyl-10-(2,3,4-trihydroxy-4-formylbutyl)isoalloxazine and 7,8-dimethyl-10-(2,3,4-trihydroxy-4-carboxybutyl)isoalloxazine. J. Nutr. Sci. Vitaminol., 21, 347–353.
4) TACHIBANA, S., and MURAKAMI, T.: Isolation and identification of Schizoflavins, in Methods in Enzymology, ed. by Colowick, S. P., and Kaplan, N. O., Academic Press, New York and London, Vol. 66, Part E, in press.
5) TACHIBANA, S., and MURAKAMI, T. (1974): Effects of flavins on L-malate fermentation utilizing ethanol by Schizophyllum commune. J. Ferment. Technol., 52, 511–516.
6) TACHIBANA, S. (1973): On the physiological activities of noncoenzyme-type, phosphate of flavin. Vitamins (in Japanese), 47, 328.
7) TACHIBANA, S., MURAKAMI, T., and OKA, M. (1974): Schizoflavin and L-malate accumulation of Schizophyllum commune. Proceedings of the Ninth International Scientific Congress on Cultivation of Edible Fungi, Mushroom Science (Part 1), pp. 761–769.
8) ROBERT, E. C., and SNELL, E. E. (1946): An improved medium for microbiological assay with Lactobacillus casei. J. Biol. Chem., 163, 499–509.
9) SNELL, E. E., and STRONG, F. M. (1939): The effect of riboflavin and certain synthetic flavins on the growth of lactic acid bacteria. Enzymologia, 6, 186–193.
10) LAMBOOY, J. P. (1975): Biological activities of analogs of riboflavin, in Riboflavin, ed. by Rivlin, R. S., Plenum Press, New York and London, pp. 313–318.