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A New Enzymic Method for the Determination of FAD[1)]

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A new enzymic method has been developed for the determination of FAD by using deflave xanthine oxidase. It was possible to measure FAD concentration in the pico mole range by this method. This method is accurate, simple and more sensitive than existing enzymic methods.

Keywords——flavin adenine dinucleotide; deflavo xanthine oxidase; xanthine oxidase; hydrogen peroxide; enzymic determination

Several methods for the determination of flavin adenine dinucleotide (FAD) concentration in biological materials have been reported, and they fall into two major categories: (1) chemical methods and (2) enzymic methods.

Most of the chemical methods require a preliminary separation of FAD from other flavins and then the FAD concentration is determined by spectrophotometric methods or fluorescence methods.[2)] They are therefore time-consuming and require large amounts of samples.

A rapid fluorescence method without preliminary separation, based on the different behavior of FAD and riboflavin-5′-phosphate (FMN) fluorescence at two pH values, has been described.[3)] However, this method cannot be used when riboflavin and other fluorescent compounds are present.

On the other hand, methods using D-amino acid oxidase apoenzyme,[4)] glucose oxidase apoenzyme[5)] and apoflavodoxin[6)] have been reported, but these methods are not satisfactory as regards sensitivity.

We developed a new method for the measurement of xanthine oxidase activity based on the formation of hydrogen peroxide.[7)] Hydrogen peroxide is measured by oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone hydrochloride and N,N-dimethylaniline in the presence of peroxidase.

Deflavo xanthine oxidase does not produce hydrogen peroxide, unlike xanthine oxidase, and this enzyme activity is recovered by the incubation of deflavo xanthine oxidase with FAD. This apoenzyme binds specifically with FAD, and has no detectable reaction with FMN or riboflavin.[8)]

This paper describes a sensitive enzymic method for FAD determination based on the properties of deflavo xanthine oxidase.

Materials and Methods

Materials——Xanthine and peroxidase (POD) were purchased from Sigma Chemical Co., Ltd. and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), 4-aminoantipyrine (4-AA), N,N-dimethylaniline (DMA) and FAD from Wako Pure Chemical Industries, Ltd. (Japan). D-Amino acid oxidase (D-AOD) was obtained from Boehringer Mannheim Co., Ltd. All reagents were of the highest grade of purity commercially available. Xanthine oxidase (XO) was purified from bovine milk by the method of Nathans and Kirby-Hade,[9)] and its homogeneity was confirmed by disc electrophoresis.

Preparation of Deflavo Xanthine Oxidase——The method of Komai *et al.*[8)] was used with a minor modification. One ml of XO solution (670 μg/ml) and 4.0 ml of 2.55 M $CaCl_2$ solution ($CaCl_2$ was dissolved in 0.1 M Tris solution and adjusted to pH 8.0 with 1 N HCl) were mixed and incubated at 20° for 2 hr and then dialyzed against frequent changes of 0.1 M Tris-HCl buffer (pH 8.5) at 4°.

The Proposed Method for the Determination of FAD——One hundred μl of sample solution, 1.0 ml of deflavo XO solution (250 μg/ml) and 1.0 ml of 0.1 M phosphate buffer (pH 8.5) were incubated at 37° for 60 min. Then, 0.1 ml of xanthine solution (2 mg/ml) was added to the above incubation mixture and the whole was incubated at 37° for 5 min. The enzyme reaction was stopped by the addition of 0.5 ml of 0.4 M citric acid solution, and after the addition of 2.0 ml of the color reagent solution (9.6 mg of MBTH, 0.3 ml of DMA and 400 units of POD dissolved in 100 ml of 0.2 M McIlvaine buffer (pH 3.5)) the mixture was allowed to stand at 37° for 10 min. The absorbance of the resulting solution was measured at 600 nm.

The Conventional Method for the Determination of FAD——The method of Huennekens *et al.*[4a] was used with a minor modification. One hundred μl of sample solution and 50 μl of D-AOD apoenzyme solution (500 μg/ml) were added to 1.0 ml of 0.1 M phosphate buffer (pH 8.5) and the whole was incubated at 37° for 60 min. Then, 0.1 ml of DL-α-alanine solution (100 mg/ml) and 2.0 ml of the color reagent solution (8 mg of 4-AA, 20 μl of DMA and 400 units of POD dissolved in 100 ml of 0.1 M phosphate buffer (pH 8.5)) were added to the incubation mixture and the whole was incubated at 37° for 5 min. The enzyme reaction was stopped by the addition of 0.5 ml of 0.3 M citric acid solution and the absorbance of the resulting solution was then measured at 550 nm.

Results

Effect of Enzyme and Reagent Concentrations on the Determination of FAD

It was found that the concentrations of xanthine and deflavo XO required to obtain a maximum value were 100 μg and 150 μg in the reaction mixture, respectively, as shown in Fig. 1A.

As shown in Fig. 1B, we found that the absorbance at 600 nm was maximum with above 125 μg of MBTH, 2 μl of DMA and 2 units of POD (one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 and at 20°) per tube.

Calibration Curve

The calibration plots for FAD obtained by the proposed method were curved and the absorbance at 600 nm was about 0.5 with 30 pmol of FAD as shown in Fig. 2.

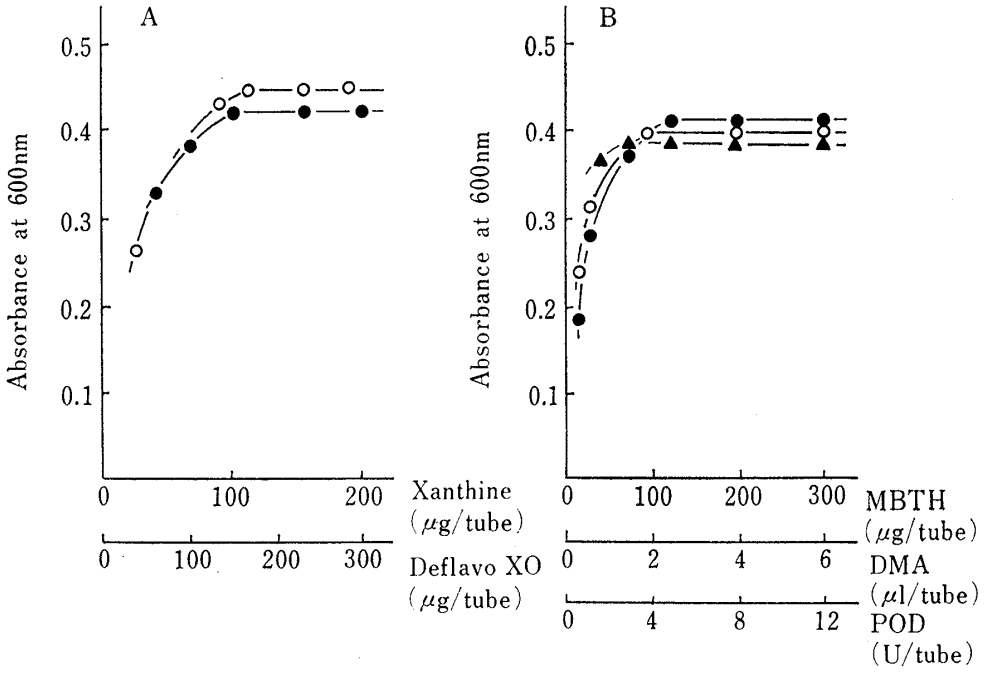

Fig. 1. A. Effect of Reagent Concentration on Final Absorbance
B. Effect of Color Reagent Concentration on Final Absorbance

Xanthine (—●—); deflavo XO=deflavo xanthine oxidase (—○—).
MBTH=3-methyl-2-benzothiazolinone hydrazone hydrochloride (—●—); DMA=N,N-dimethylaniline (—○—); POD=peroxidase (—▲—).

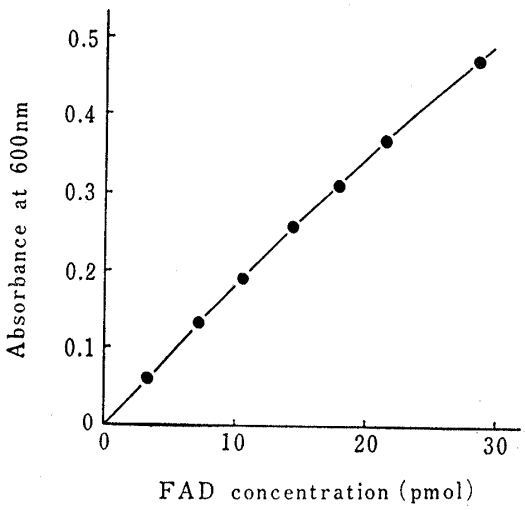

Fig. 2. Calibration Curve for FAD Concentration

Table I. Comparison of FAD Concentration Determined by the Proposed Method and the Conventional Method

Sample	FAD concentration (nmol/ml)	
	Proposed method[a]	Conventional method
FAD solution	0.74	0.69
	1.06	1.06
	1.46	1.35
	1.79	1.79
	2.51	2.58
D-Amino acid oxidase (10 μg)	1.82	1.79
Glucose oxidase (10 μg)	0.97	0.96

a) In the proposed method, the sample solutions for the conventional method were diluted 10-fold, and then the FAD concentration was determined.

Reproducibility and Day-to-Day Precision

We studied the within-day precision of the proposed method with 11.0 pmol of FAD, and the coefficient of variation was 1.27%. Further daily studies of precision were carried out with 11.2 pmol of FAD and the coefficient of variation was 2.65%.

Effects of FMN and Riboflavin on the Proposed Method

We observed that FMN (below 10 μg per tube) and riboflavin (below 10 μg per tube) had no effect on the proposed method. Thus, it was suggested that FMN and riboflavin did not inhibit the reconstitution process even when present in 1000-fold excess over FAD.

Comparison of FAD Concentrations Determined by the Proposed Method and by the Conventional Method

We compared the FAD concentrations determined by the proposed method and the conventional method (Table I). The conventional method required ten times as much sample solution for the determination. The calculated correlation coefficient between these two methods was 0.998. We measured the FAD concentration in D-AOD and glucose oxidase by both methods. The values estimated by the proposed method were indentical with the values obtained by the conventional method.

Comparison of *K*d value for FAD

The dissociation constant (*K*d) of deflavo XO for FAD was determined to be 7.7×10^{-8} M, and this value was significantly lower than the value of 3.0×10^{-7} M of D-AOD apoenzyme. From these results, it appeared that deflavo XO has a higher affinity for FAD than D-AOD apoenzyme.

Discussion

In this paper, we describe a new enzymic method for the determination of FAD based on the reactivation of deflavo XO by incubation with FAD. The procedure is simple and the results are reproducible; furthermore, no interference of FMN and riboflavin is observed.

The XO reaction is a mixed type of one-electron transfer and two-electron transfer,[10] and the superoxide anion radical is produced to some extent in addition to hydrogen peroxide when the oxygen molecule serves as the electron acceptor.[11] In the previous paper,[7] we presented a new method for the assay of XO activity. In this method, we stopped the enzyme reaction in the acidic region by the addition of citric acid solution, and then developed the

color. In this case, the superoxide anion radical formed by XO (which is unstable in the acidic region) was converted to hydrogen peroxide.[12)]

Deflavo XO has been shown to be devoid of xanthine oxygen reductase activity, and can be reconstituted by a short incubation with FAD. Reconstitution of activity is also obtained with FMN, but the binding of FMN to the deflavo XO is several orders of magnitude weaker.[8)]

This method does not require preliminary separation of FAD from other flavins even when they are present in 1000-fold excess over FAD. In addition, deflavo XO has a higher affinity for FAD than D-AOD apoenzyme, since the *K*d value of deflavo XO for FAD is lower than that of D-AOD apoenzyme, and so this method is more sensitive than the conventional enzymic method.

References and Notes

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