Note

Enzymatic Assay for Biotin Using Biotinyl-CoA Synthetase

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Biotin has recently been attracting increasing interest not only as a medicine¹⁾ and a biochemical reagent²⁾ but also as a food and feedstuff supplement.³⁾ The vitamin is known to occur in various natural sources and to be synthesized by microorganisms⁴⁾ and plant cells.⁵⁾ Therefore, a biotin assay is of great importance in these research fields. There are some methods for the determination of biotin and its vitamers: microbiological, chemical and radioactive assay methods.⁶⁾ We previously developed microbiological assay methods for biotin using lyophilized cells of *Lactobacillus plantarum* and glycerol-suspended cells of *Saccharomyces cerevisiae*, and also established an assay method using *Escherichia coli* C 162.⁷⁾

However, an unavoidable problem is that, since a bioassay is based upon the growth of microorganisms, it takes much time (about 10~30hr) to obtain results. Recently, extensive studies concerning the enzymatic determination of various biological compounds have been reported.⁸⁾ Enzymatic determinations are rapid and have high specificity and sensitivity.

On the other hand, we first purified biotinyl-CoA synthetase (BCS) from a soil bacterium, *Mycoplana* sp.,^{9,10)} and applied the enzyme to the production of α -dehydrobiotin in combination with acyl-CoA oxidase (ACO).¹¹⁾ In this reaction system, another product is

hydrogen peroxide. Hydrogen peroxide is well known to be a good probe for the enzymatic determination of various biological compounds when oxidases are used. So, we tried to apply the reactions of BCS and ACO to the enzymatic determination of biotin.

On the basis of the above consideration, we proposed the following assay procedure for biotin using three enzymes: BCS, ACO and peroxidase (POD).

The final product (II) was a highly fluorescent compound, which has a λ_{ex} of 315 nm and a λ_{em} at 425 nm.¹²⁾ In this paper, we describe the fluorometric determination for biotin using BCS, ACO and POD.

Biotinyl-CoA was synthesized as described previously.¹¹⁾ BCS was purified from *Mycoplana* sp. No. 166 as described in a previous paper,⁹⁾ and the enzyme obtained at the first FPLC step (6.6 U/mg) was used throughout this work. ACO (8.76 U/mg for palmitoyl-CoA) was a kind gift from Toyobo Co., Japan. POD (250 U/mg) from horseradish (EC 1.11.1.7) was purchased from Boehringer Mannheim.

Other chemicals were usual commercial preparations and used without further purification.

Fluorescence measurements were made at a λ_{ex} of 315 nm and a λ_{em} of 425 nm with a Hitachi 204R fluorescence spectrophotometer. Fluorescence intensity was daily standardized with a 0.1 $\mu\text{g}/\text{ml}$ solution of quinine sulfate (λ_{ex} = 350 nm, λ_{em} = 450 nm). The bioassay was performed with the biotin assay organism, *L. plantarum*, and the detailed procedure was described previously.⁷⁾

After determining the optimum assay conditions, the

FIG. 1. Standard Assay Procedure for Biotin.

FIG. 2. Standard Curve for Biotin with the Enzymatic Method.

The reaction was carried out under the conditions given in Fig. 1.

FIG. 3. Correlation between the Results for Biotin Levels Determined by the Enzymatic and Bioassay Methods.

Various amounts of biotin were dissolved in a 0.1% yeast extract solution. The enzymatic method was performed as described in Fig. 1.

standard procedure with BCS, ACO and POD were finally established (Fig. 1). For the reaction procedure, the concentration of homovanillic acid was critical: above the optimal concentration, the fluorescence intensity became unstable and below it became low. Within the time used here the reaction reached the end point and the intensity of

fluorescence was found to be stable.

This method requires only about 2 hr for the determination of biotin. This suggests that we can determine amounts of biotin much more rapidly using the enzymatic method than the bioassay method.

The sensitivity of the enzymatic method and the linearity of standard curve for it are shown in Fig. 2. Strict proportionality was observed between the concentration of biotin and the fluorescence intensity over the range of 5 nmol/ml to 200 nmol/ml. The sensitivity of this enzymatic assay method was slightly lower than that of the paper disk plate bioassay method using *L. plantarum* (about 1 nmol/ml to 50 nmol/ml). However, if a micro-cuvette is used instead of the 3-ml cuvette usually used for the measurement of fluorescence intensity, the sensitivity could become several times higher.

A comparison of the bioassay method with the present enzymatic method is shown in Fig. 3. The results when various concentrations of biotin were dissolved in a yeast extract solution followed by determination by the end point enzymatic assay method were almost the same as those with the bioassay method. The calculated linear regression and correlation coefficient were: $y = 0.981x + 4.525$ and $r = 0.997$, respectively.

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