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A New Method for the Assay of Xanthine Oxidase Activity[1)]

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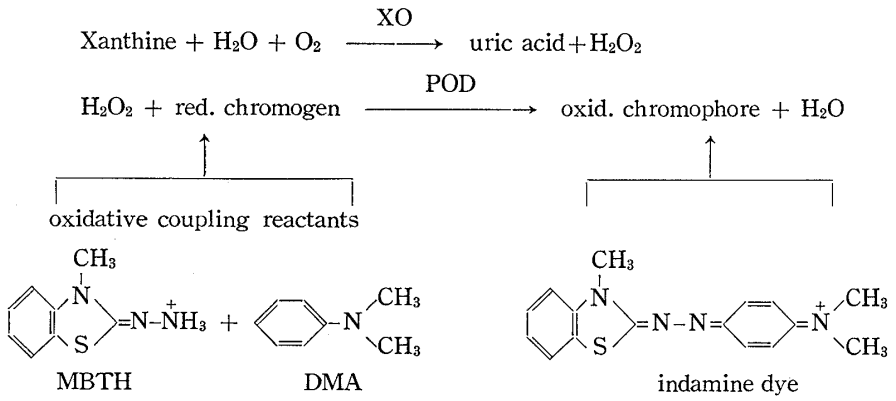
A colorimetric method for the assay of xanthine oxidase activity, based on the production of hydrogen peroxide, is described.



The precision, accuracy, sensitivity and specificity of the method were found to be satisfactory for the rapid and reliable determination of xanthine oxidase activity.

Keywords——xanthine oxidase; hydrogen peroxide; colorimetric method; superoxide anion radical; peroxidase

Xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) is one of the most complex of the flavoproteins, and contains 2 g-atoms of molybdenum, 2 molecules of FAD and 8 g-atoms of iron per molecule of the enzyme.[2)]

In addition, its substrate specificity is very low: xanthine, purine, aldehyde and NADH serve as electron donors,[3)] and the oxygen molecule, ferricyanide, cytochrome *c* and some artificial dyes serve as electron acceptors.[4)]

The methods currently employed for the assay of xanthine oxidase activity can classified into spectrophotometric assays based on the production of uric acid, and methods measuring the reduced electron acceptor.[4b-d)]

When the oxygen molecule serves as the electron acceptor, the superoxide anion radical is partially produced in addition to hydrogen peroxide,[5)] and this superoxide anion radical is a strong inhibitor of peroxidase. Yamazaki and Piette proposed that peroxidase reacts with superoxide anion radical to yield compound III.[6)] In 1970, this proposal was repeated and in 1972, it was definitely established.[7)] It is known that compound III is less reactive than the other forms of peroxidase.[8)] However, the superoxide anion radical is unstable under acidic conditions, being converted to hydrogen peroxide.[9)]

We have developed a new method for determining xanthine oxidase activity, and the details are presented in this paper.

Materials and Methods

Materials——Xanthine and peroxidase (POD) were purchased from Sigma Chemical Co. Ltd., and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and N,N-dimethylaniline (DMA) were

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purchased from Wako Pure Chemical Industries, Ltd. (Japan). Xanthine oxidase (XO) was purified from bovine milk by the method of Nathans *et al.*[10]

Conventional Method for Assay of Xanthine Oxidase Activity——XO activity was measured as follows: the uric acid production was measured by following the increase in absorbance at 293 nm;[11] and 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) reduction was measured in terms of the increase in absorbance at 500 nm.[4d] The substrate concentration, pH of the buffer and temperature were identical to those used in the standard method.

Standard Assay of Xanthine Oxidase Activity——A color reagent solution was prepared as follows before use; 9.6 mg of MBTH, 0.3 ml of DMA and 400 units (one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 and at 20°) of POD were dissolved in 100 ml of 0.2 M McIlvaine buffer (pH 3.5).

One ml of 0.1 M phosphate buffer (pH 8.5) containing 1 mM sodium azide and 0.2 ml of 6 mM xanthine solution were mixed and preincubated at 37° for 5 min. Fifty μl of the enzyme solution was added to the substrate mixture and the whole was incubated at 37° for 10 min. The enzyme reaction was stopped by the addition of 0.5 ml of 0.2 M citric acid solution, then 2 ml of the color reagent solution was added, and the reaction mixture was incubated at 37° for 10 min. The absorbance was measured at 600 nm.

One unit of XO activity was defined as the amount which produces 1 μmol of hydrogen peroxide per min in the standard assay system.

Results

Effect of Reagent Concentration on Color Development

The effects of various concentrations of reagents on the final absorbance were investigated by the standard method. As shown in Fig. 1, the absorbance was maximum at concentrations of above 125 μg of MBTH, 2 μl of DMA and 2 units of POD per tube.

Calibration Curve

The calibration curve for the XO activity was linear, passing through the zero point, and the absorbance at 600 nm was about 1.0 with 13 μg of XO. There was a linear relationship between the absorbance and reaction time up to 30 min. This relationship between the absorbance and XO activity was maintained up to an absorbance of 1.0.

Reproducibility and Day-to-Day Precision

The within-batch precision for the determination of XO activity by the standard assay system was studied, and the coefficient of variation was found to be 1.4%. Daily study of precision was also carried out, and the coefficient of variation was 1.5%.

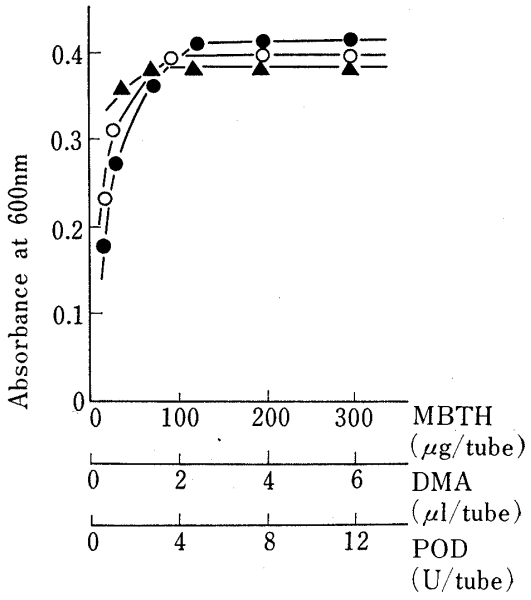

Fig. 1. Effects of the Concentrations of Reagents on the Final Absorbance

MBTH: 3-methyl-2-benzothiazolinone hydrazone hydrochloride (—●—); DMA: N,N-dimethylaniline (—○—); POD: peroxidase (—▲—).

Comparison of the New and Conventional Assays for Xanthine Oxidase Activity

XO activity was determined by the standard method and by conventional methods (measuring uric acid production in terms of the increase in the absorbance at 293 nm or INT reduction in terms of the increase in the absorbance at 500 nm). The calculated correlation coefficients were 0.996 and 0.988, and the linear regression equations were $Y=0.960X+0.001$ and $Y=0.936X+0.002$, respectively, indicating good agreement between the proposed method and the conventional methods.

The molar extinction coefficient (ε) for the oxidized chromophore in the proposed method was calculated to be 3.5×10^4 and this value was significantly higher than the value of 1.5×10^4

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for INT formazan, 9.0×10^3 for uric acid. From these results, it appears that the proposed method is more sensitive than the conventional methods.

Discussion

The methods currently used for the assay of XO activity are spectrophotometric methods based on the production of uric acid or colorimetric methods measuring the reduced electron acceptors. However, the former method cannot be applied to the assay of enzyme activity in serum, because biological materials, for example uric acid or albumin, in serum result in a high blank value. The latter methods are not satisfactory for clinical tests as regards sensitivity.

It has been reported that the production of hydrogen peroxide by XO is not stoichiometric, and so there has been no report of a method for XO activity assay based on the production of hydrogen peroxide.

Since XO activity was reduced in the presence of color reagent, it was suggested that POD in the color reagent solution formed compound III with the superoxide anion radical produced by XO under these conditions. We thus stopped the enzyme reaction in the acidic region (approx. pH 3.0) by the addition of citric acid solution, and then developed the color. In this case, the superoxide anion radical formed by XO was converted to hydrogen peroxide through the dismutation reaction. Moreover, the dye system used in this method was most stable under weakly acid conditions.

We have examined the new method for the assay of XO activity in this study, and the results mentioned above indicate that the proposed method is accurate and more sensitive than the conventional methods. In the proposed method, we added sodium azide to the substrate mixture to inhibit catalase, which is present in biological materials, and the average recovery of hydrogen peroxide in the serum in this assay system was $97.1 \pm 2.58\%$. Thus, it appears that hydrogen peroxide was stable in this assay system.

Using the proposed method, we are now investigating the concentrations of purine metabolites and associated enzyme activities in the sera of patients with various diseases.

References and Notes

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