

## Determination of Xanthine Oxidase

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### Introduction

Xanthine oxidoreductase is widely distributed among mammalian species and is expressed mainly in vascular endothelial cells, hepatocytes, epithelial cells of the intestine, and the secretory cells of the mammary gland. Expression of the enzymatic activity of XO/XD depends on the animal species. XO/XD activity is, for example, high in the blood of mice and rats, whereas very low levels of XO/XD activity are detected in human blood. This enzyme, however, is also distributed in human tissues, particularly in endothelial cells. We must, therefore, employ a highly sensitive and selective method for detection of the activity of this enzyme in human specimens.

It is well known that this enzyme produces the active oxygen species (superoxide anion radical and hydrogen peroxide,  $O_2^-$ ,  $H_2O_2$ ) which play important roles in the pathogenesis of ischaemia-reperfusion injury, and inflammatory and virus-induced tissue injury. It has, moreover, been suggested that these active oxygen species interact with nitric oxide produced by the endothelial type of nitric oxide synthase. One of the most intriguing features of the enzyme xanthine oxidoreductase is that under physiological conditions it exists in the form xanthine dehydrogenase (XD), which utilizes  $NAD^+$  as a coenzyme, and is converted to xanthine oxidase (XO) which utilizes  $O_2$  as an electron acceptor. Xanthine oxidoreductase also catalyses the metabolism of hypoxanthine and xanthine to uric acid and metabolizes some other naturally occurring heterocyclic compounds.

Widely employed assays for xanthine oxidoreductase activity are spectrophotometric quantitation of uric acid, the final product of the reaction [1], quantitation of hypoxanthine, xanthine or uric acid by high performance liquid chromatography (HPLC) [2], fluorimetric quantitation of isoxanthopterin, the metabolite of pterin by XO/XD [2,3], and quantitation of radiolabelled uric acid after thin-layer chromatography (TLC) of the reaction mixture of radioactive hypoxanthine/xanthine and the enzyme [2,4]. These methods are introduced briefly.

XO/XD catalyses two steps of the reactions as shown in Figure 1.

Xanthine is not only the reaction product derived from hypoxanthine but also the substrate for uric acid formation. The XO/XD activity can, therefore, be determined by measuring the amount of final product-uric acid or both xanthine and uric acid from hypoxanthine. XD is converted to XO by limited proteolysis. Oxidation of the thiol moieties in the XD molecule also causes conversion to XO. Conversion of XD to XO induces a dramatic change in the dependence of redox coenzymes for its enzymatic reaction.

### Spectrophotometric Quantification of Uric Acid Formation

#### Protocol

XO/XD activity is easily evaluated by quantifying the 'formation of uric acid by measuring the absorbance at 290 nm ( $A_{290}$ ), an absorption

maximum of uric acid. This method is, however, not sufficiently sensitive to be applicable to the measurement of biological samples which contain only low levels of XO/XD activity. Because XO/XD occurs in the cytosol fractions of cells, we usually quantify the enzymatic activity of some tissues by using the cytosol fractions obtained by ultracentrifugation (100,000 g) of the tissue (organ) homogenates.

1. Mix sample or XO/XD enzyme solution, the substrate (hypoxanthine, 50  $\mu$ M final concentration), and sodium phosphate buffer (pH 7.8, 50 mM), total volume 1.0-3.0 mL, in a cuvette.
2. Record the time-dependent increase in the absorbance at 290 nm during incubation of the reaction mixture at 25 or 37°C.
3. Determine XD activity in a sample by adding  $NAD^+$  to the reaction mixture.

### Calculation

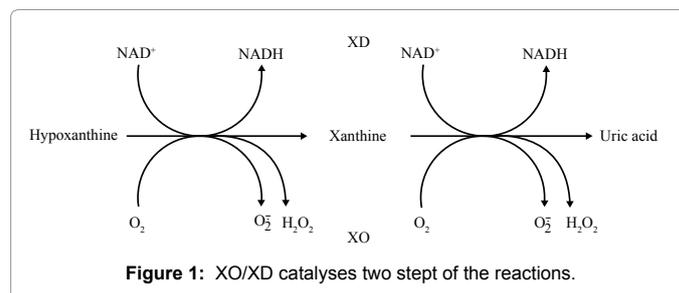
Because 1 unit of XO/XD activity is defined as the velocity of the formation of 1  $\mu$ mol uric acid  $min^{-1}$ , the enzymatic activity in each sample can be calculated by use of the equation:

$$\text{units/mg protein} = (\Delta A / \text{min} \times 1000) / (1.22 \times 10^4 \times \text{mg mL}^{-1} \text{ reaction mixture})$$

In which the molar absorption coefficient of uric acid is  $1.22 \times 10^4 M^{-1}cm^{-1}$ .

### Measurement of XO/XD activity by HPLC

The sensitivity and specificity of spectrophotometric detection of uric acid formation for XO/XD assay can both improved by use of HPLC (column, e.g., Asahipak GS-320; mobile phase, sodium phosphate buffer (pH 7.4, 0.01 M) containing NaCl (0.15 M) (PBS); the elution



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time of uric acid can be adjusted by modifying the concentration of sodium chloride in the mobile phase). The detection limit of uric acid in this method is 1  $\mu\text{M}$  or more.

### Fluorimetric Assay

The sensitivity of the XO/XD assay can be improved further without the use of radiolabelled compounds (substrates). As mentioned above, XO/XD efficiently metabolizes pterin (2-amino 4-hydroxypteridine) into a fluorescent product, isoxanthopterin (2-amino-4,7-pterinediol), which enables the highly sensitive quantitation of the activity of the enzyme.

It is necessary to remove endogenous substrates from biological samples, because these often interfere with the oxidation of pterin by XO/XD, by dialysis or molecular sieve column chromatography on, e.g., Sephadex G25.

### Protocol

1. Mix sample (without endogenous substrates) or enzyme solution, the substrate (pterin; Sigma; 10 $\mu\text{M}$  final concentration), and sodium phosphate buffer (pH 7.8, 50 mM), total volume 1.0 mL.
2. Incubate the reaction mixture for 10-60 min at 37°C.
3. Quantify the amount of isoxanthopterin produced by spectrofluorimetry.

The fluorescence intensity of isoxanthopterin is measured with excitation at 345 nm and emission at 390 nm and quantitation achieved by use of a calibration curve constructed by use of authentic isoxanthopterin (Aldrich, Milwaukee, WI, USA).

Because XD cannot use oxygen in the solution efficiently as a coenzyme, little pterin is oxidized by XD. XD activity is, therefore, evaluated by measuring the increase in the total activity after addition of methylene blue (final concentration 9 $\mu\text{M}$ ) as a coenzyme (an electron acceptor) of XD [2]. It is also recommended that inhibition by allopurinol, a specific inhibitor of XO/XD, is examined to confirm the specificity of the reaction.

### Radiochemical Method

Although the procedure is somewhat complicated, the method using radiolabelled hypoxanthine ( $^{14}\text{C}$ hypoxanthine) is the most

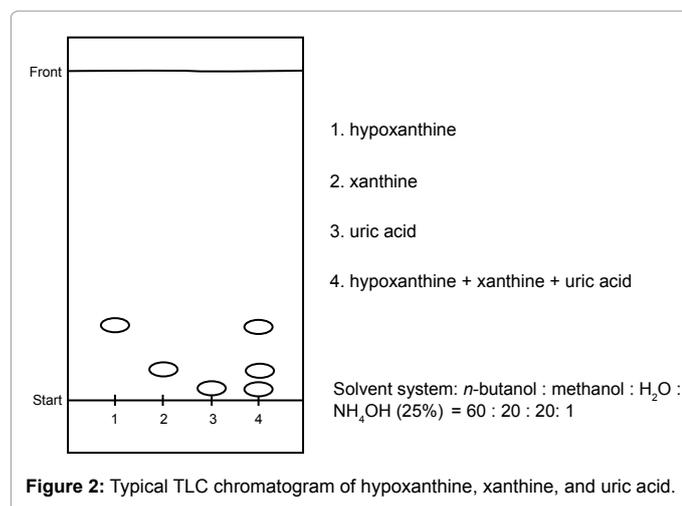


Figure 2: Typical TLC chromatogram of hypoxanthine, xanthine, and uric acid.

sensitive way of determining XO/XD. The principle of this method is essentially the same as those of the spectrophotometric quantitation of uric acid formation and the measurement of XO/XD activity by HPLC. In this method the reaction products [ $^{14}\text{C}$ ]hypoxanthine and [ $^{14}\text{C}$ ]uric acid are separated from the substrate by TLC (Cellulose F; E. Merck, Darmstadt, Germany). Figure 2 shows the chromatogram obtained from these compounds. To enable identification of the spot of each compound on the plate under UV illumination, non-radiolabelled hypoxanthine, xanthine and uric acid should also be applied to the plate.

### Protocol

1. Mix [ $^{14}\text{C}$ ]hypoxanthine (57 mCi mmol<sup>-1</sup>, 200  $\mu\text{M}$  final concentration), sample, and Tris-HCl buffer (pH 7.8, 50 mM), total volume 100  $\mu\text{L}$ .
2. Stop the reaction by adding perchloric acid (0.15 M).
3. Centrifuge to remove proteins.
4. Apply a mixture of non-radiolabelled hypoxanthine, xanthine and uric acid at each sample application site of the TLC plate (at levels sufficient to be detected under UV illumination). After drying the plate, apply the reaction mixture to each spot.
5. Develop the plate with 60:20:20:1 (v/v) *n*-butanol-methanol-water. 25% (v/v) aqueous ammonia.
6. Determine the positions of hypoxanthine, xanthine and uric acid separated on the TLC plate under UV illumination then measure the radioactivity of [ $^{14}\text{C}$ ]xanthine and [ $^{14}\text{C}$ ]uric acid on the plate by use of a scintillation counter.

### Other Methods

Expression of XO/XD in the organs and tissues can be detected by Western blotting or by immunohistochemical techniques-by use of polyclonal and/or monoclonal antibodies against XO/XD. Because the cDNAs for XO/XD have been cloned by Amaya et al. [5] and Terao et al. [6], expression of mRNA of this enzyme can be detected by RT-PCR or Northern blotting.

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