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TECHNICAL INFORMATION

Catalog Number: 190000
Bilirubin Oxidase

CAS # 80619-01-8

Source: *Myrothecium* species

Bilirubin oxidase is a purified bilirubin oxidase preparation using a strain belonging to *Myrothecium* species.

Bilirubin oxidase oxidizes bilirubin to biliverdin and decreases the specific color at 440 nm of bilirubin as follows:

Bilirubin + 1/2 O_2 --------> Biliverdin + H_2O

Furthermore, bilirubin oxidase can oxidize biliverdin to an unidentified material at the rate of 2% in comparison with that of bilirubin.

Bilirubin oxidase is very useful for laboratory diagnostic determination as mentioned below.

It is possible to determine two types of bilirubin, glucuronide-conjugated and unconjugated bilirubin separately. Also in various enzymatic assay using oxidase-peroxidase such as "Glucose oxidase system", "Cholesterol oxidase system", "Acyl coenzyme A oxidase system", and "Glycerol-3-phosphate oxidase system", the accuracy of the assay system can be increased as the interference of bilirubin is removed by oxidation of bilirubin in body fluid with bilirubin oxidase.

Principle

The activity of bilirubin oxidase can be detected by measuring the decrease in the optical density at 440 nm as bilirubin has maximal absorption spectrum at 440 nm.

Procedure:

A. Substrate Solution

5 mg of bilirubin is suspended in 1 ml of deionized water and dissolved by adding a drop of 2 N NaOH, then filled up to 50 ml with 0.2 M Tris-HCl buffer (pH 8.4).

The above solution should be diluted 5-fold with 0.2 M Tris-HCl buffer as substrate solution.

– The substrate solution before 5-fold dilution has 1.7 - 1.8 of optical density at 440 nm.

– The substrate solution is stable one hour at 25ºC and/or 15 minutes at 37ºC.

B. Enzyme Solution

Bilirubin oxidase is dissolved and diluted with deionized water so that the resulting decrease in optical density at 440 nm becomes 0.100 - 0.050 per minute. For example, bilirubin oxidase having activity of 7 units per milligram is usually diluted

400,000 - 700,000 fold.

– The enzyme solution is stable for 2 hours at 25ºC.

– Powdered bilirubin oxidase perparation should be stored below freezing.

– The aqueous solution of bilirubin oxidase is stable under repeated freezing and thawing.

3 ml of substrate solution (A) preincubated at 37ºC and 0.3 ml of enzyme solution (B) are mixed will in a cuvette (f 10 nm) and incubated at 37ºC. The decrease in the optical density at 440 nm is recorded for 1 - 2 minutes. Then, the decrease in the optical density per minute is calculated by reading the recorded chart.

Calculation of units

$$\text{Units} = \frac{\text{O.D.} \times 3.3}{56.3 \times 0.3} \times n$$

O.D. : The decrease in optical density at 440 nm per one minute.
56.3 : Molecular extinction coefficient of bilirubin in the above condition.
3.3 : Volume of the reaction mixture.
0.3 : Volume of the enzyme solution.
n : Dilution multiple of the enzyme.

Definition of Unit: One unit of bilirubin oxidase is defined as the enzyme quantity which oxidizes one micromole of bilirubin per one minute under the above condition.

1. Substrate Specificity

It is known that two types of bilirubin exist in human body fluid. One is "Glucuronide-conjugated bilirubin", the other is "Unconjugated bilirubin". Bilirubin oxidase by itself is able to oxidize rapidly the "Glucuronide-conjugated bilirubin", but has no activity on "Unconjugated bilirubin."

Nevertheless, by the addition of various detergents such as sodium cholate, sodium dodecylsulfate, salicylic acid, sulfosalicylic acid, and p-toluenesulfonate, bilirubin oxidase is able to oxidize "Unconjugated bilirubin."

As a result of the above, it is possible to determine two types of bilirubin, respectively, with and without detergent.

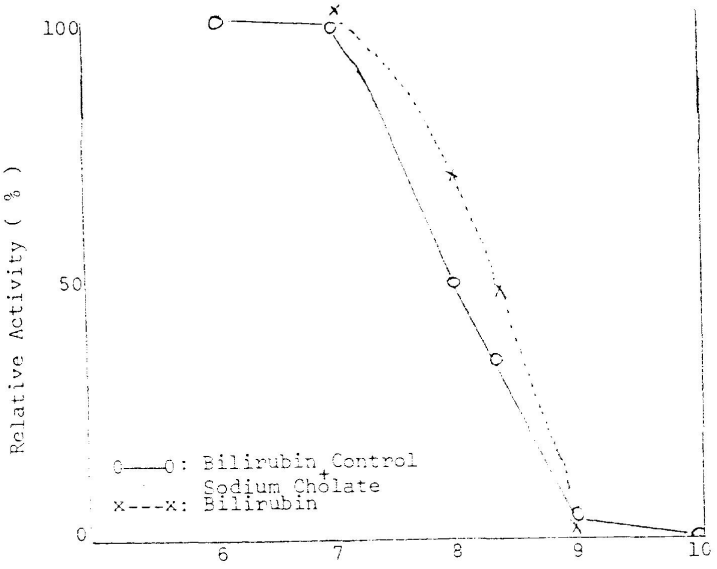
Relative reaction rate of bilirubin oxidase against various material is summarized below.

Bilirubin	100
Biliverdin	2
Pyrogallol	trace
Guaiacol	trace
Hemoglobin	0

2. Physico-chemical Properties

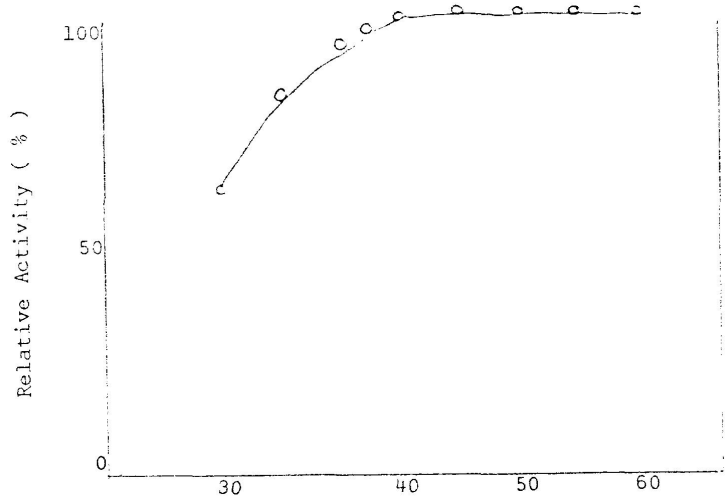
Molecular Weight	52,000
Isoelectric point	pH 4.1
Km	190 micro Mole
Copper Content	1 atom per molecule

3. pH and Activity



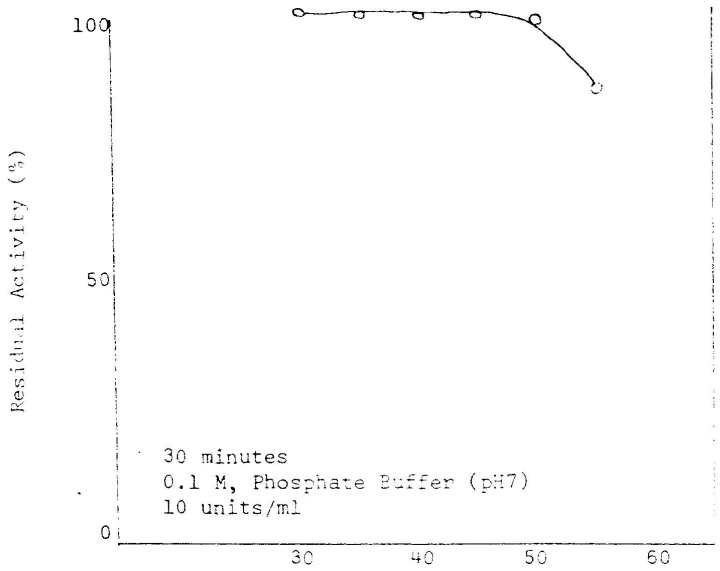
pH

4. Temperature and Activity



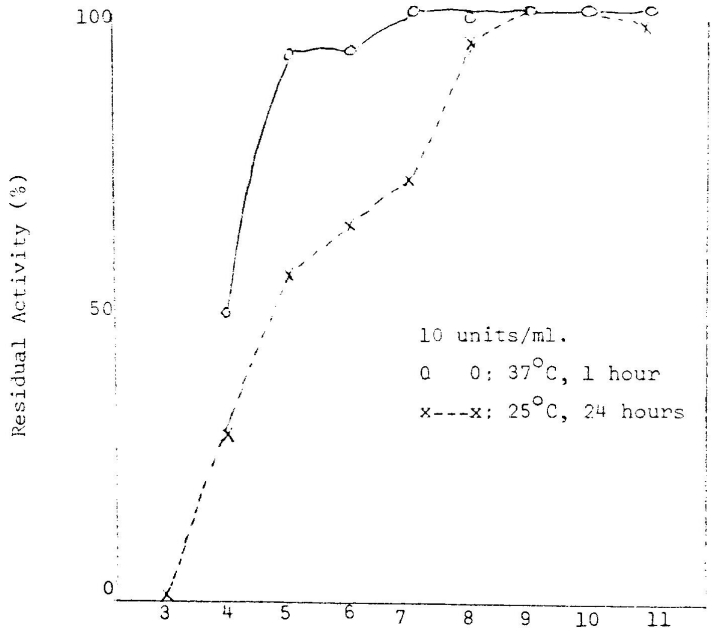
Temperature (°C)

5. Temperature and Stability



Temperature (°C)

6. pH and Stability



pH 3,4,5: Phosphate-Citrate Buffer
pH 6,7,8: Phosphate buffer
pH 9,10: Na_2CO_3-Na_2HCO_3 buffer
pH 11: Na_2HPO_4-NaOH buffer

Example 1: Method for the Quantitative Determination of Two Types of Bilirubin

Determination of conjugated bilirubin

To 0.1 ml each of aqueous solutions containing 0, 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 milligrams of bilirubin per deciliter were added 3 ml of 0.1 M phosphate buffer (pH 7.0) containing bilirubin oxidase (0.3 u/3 ml). The mixtures were incubated at 37ºC for 30 minutes and the decreases of absorbance at 440 nm was measured.

The measured values were plotted against the corresponding concentrations of the above bilirubin solutions to obtain a calibration curve. Then, 0.1 ml of a serum sample was treated in the same manner as described above, and the decrease of absorbance at 440 nm was measured.

The "Glucuronide-conjugated bilirubin" concentration in the sample was calculated by comparing the control value of the calibration curve.

Determination of total bilirubin

To 0.1 ml of a serum sample was added 3 ml of 0.1 M phosphate buffer (pH 7.0) containing bilirubin oxidase (0.3 u/3 ml) and sodium cholate (14 mM). The mixture was incubated at 37ºC for 30 minutes and the decrease in absorbance at 440 nm was measured.

The total bilirubin ("Glucuronide-conjugated bilirubin" + "Unconjugated bilirubin") was calculated by comparing the control value of the above calibration curve.

The "Unconjugated bilirubin" concentration in the serum sample was calculated by subtracting the value of "Glucuronide-conjugated bilirubin" from the value of total bilirubin.

Example 2: Method of Quantitative Determination of Cholesterol Using Cholesterol Oxidase together with Bilirubin Oxidase.

To 0.2 ml of control serum and 0 - 0.7 ml of bilirubin control (20 mg/deciliter), were added 3 ml of 0.1 M phosphate buffer (pH 7.5) containing the following reagents:

4-aminoantipyrine	0.4 mM
phenol	15 mM
Cholesterol Esterase	1 u/ml
Cholesterol Oxidase	2 u/ml
Peroxidase	6 u/ml
Triton X-100	0.1%
Sodium Cholate	14 mM
Sodium Fluoride	2.5 mM
Bilirubin Oxidase	0.1 u/ml

The reaction mixtures were incubated at 37ºC for 20 minutes and their absorbance at 505 nm were measured.

In the same manner, the absorbance of the reaction mixtures without Bilirubin Oxidase were measured.

As shown in the following figure, the absorbances at 505 nm was almost constant when Bilirubin oxidase was added in the assay system using cholesterol oxidase.

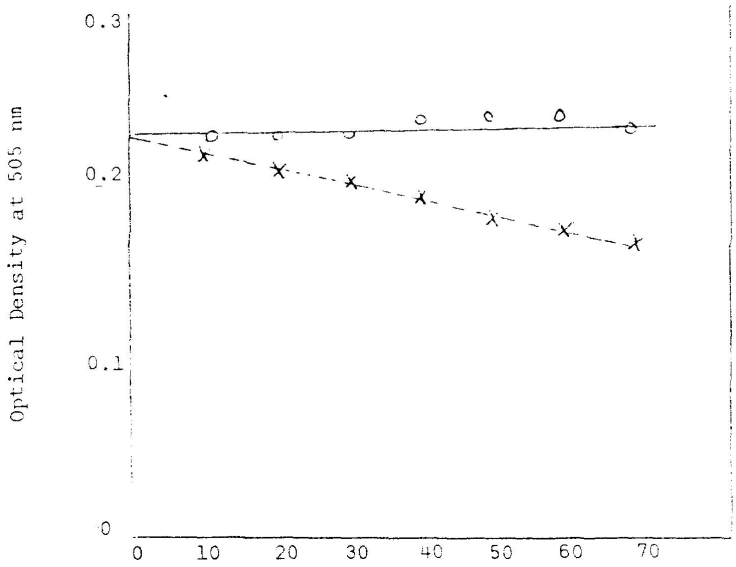
Note:

Bilirubin oxidase was found to have an activity to make chromogens more color active. 0.3 unit of bilirubin oxidase was added to several chromogens and incubated at 37oC for 40 minutes, respectively. As shown below, each absorbance of chromogen color was increased though each reaction was somewhat different among the chromogens.

Bilirubin Oxidase		
	0 unit	0.3 unit
4-Aminoantipyrine + Phenol	0.011	0.244
4-Aminoantipyrine + p-Chlorophenol	0.006	0.108
4-Aminoantipyrine + 4,6-dichloro-o-cresol	0.021	0.083
4-Aminoantipyrine + Diethylaniline	0.013	< 2

Many substances were studies to try and eliminate this problem, and it was found out sodium fluoride and phenanthroline were able to inhibit the coloration when more than 2 mM was added.

Effect of Bilirubin Oxidase to assay System Using Cholesterol Oxidase



Microliter of "Bilirubin Control" (20 mg/dl)