NIPRO ENZYMES

BILIRUBIN OXIDASE (BOD3)

[EC 1.3.3.5]

from *Trachyderma tsunodae*

$$2\ \text{Bilirubin} + O_2 \rightarrow 2\ \text{Biliverdin} + 2\ H_2O$$

SPECIFICATION

State	: Lyophilized	
Specific activity	: more than 100 U/mg protein	

PROPERTIES

Molecular weight	: ca. 60,000	(SDS-electrophoresis)	
	: ca. 80,000	(Gel filtration)	
Optimum pH	: 5.0		(Fig. 1)
pH stability	: 4.0 – 11.0	(4 °C, 24 hr)	(Fig. 2)
Isoelectric point (calculation)	: 3.8		
Optimum temperature	: 65 – 80 °C		(Fig. 3)
Thermal stability	: No detectable decrease in activity up to 50 °C.	(pH 7.0)	(Fig. 4, 5)
Michaelis constants	: See table 1		
Substrate specificity	: See table 1		

STORAGE

Stable at -20 °C for one year

APPLICATION

The enzyme is useful for enzymatic determination of bilirubin.
It could be used as a cathode catalyst in biofuel cells.

ASSAY

Principle

The change in absorbance is measured at 500 nm according to the following reaction.

$$\text{Phenol} + \frac{1}{2}O_2 + H_2O \xrightarrow{\text{BOD3}} \text{Quinone and/or Phenoxy radical} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\ H_2O$$

Unit Definition

One unit of activity is defined according to the calculation formula below.

Solutions

Ⅰ Buffer solution ; 300 mM Potassium phosphate buffer, pH7.0
Ⅱ 4-Aminoantipyrine (4-AA) solution ; 24.6 mM (0.25 g 4-AA / 50 mL distilled water)
Ⅲ Phenol solution ; 420 mM (1.98 g phenol/50mL distilled water)
Ⅳ Peroxidase*1 (POD) solution ; 240 U/mL (2,400 U/10mL distilled water)
*1POD: TOYOBO Co., LTD. Grade Ⅲ #PEO-302

Preparation of Enzyme Solution

Dissolve the lyophilized enzyme with distilled water and dilute to 15 to 60 U/mL with 10 mM potassium phosphate buffer, pH 7.0 containing 0.1 % BSA.

Procedure

1. Prepare the following reaction mixture and pipette 0.90 mL of reaction mixture into a cuvette.

Solution Ⅰ	4.00 mL
Solution Ⅱ	0.40 mL
Solution Ⅲ	0.40 mL
Solution Ⅳ	0.40 mL
H_2O	6.40 mL

2. Incubate at 37 °C for about 3 minutes.
3. Add 0.005 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 500 nm per minute (ΔAbs (test)) in linear portion of curve. Repeat the procedure 3 using distilled water in place of enzyme solution, and ΔAbs (blank) is obtained.

Calculation

$$\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs (test)} - \Delta\text{Abs (blank)}) \times (0.90 + 0.005)}{11.11 \times 0.005 \times 1/20} \times \text{d.f.}$$

$$\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)}^{*2}}$$

d.f. ; dilution factor
11.11 ; millimolar extinction coefficient of quinoneimine dye at 500 nm ($cm^2/\mu mol$)
1/20 ; coefficient of transformation for internal unit definition
*2Protein concentration ; determined by Bradford's method

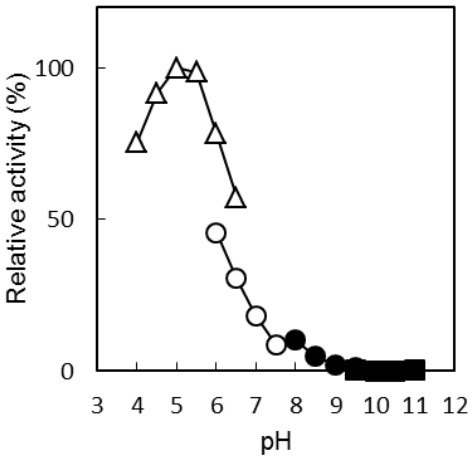

Fig. 1 pH profile

△ acetate, ○ phosphate
● Tris-HCl, ■ Glycine-KOH

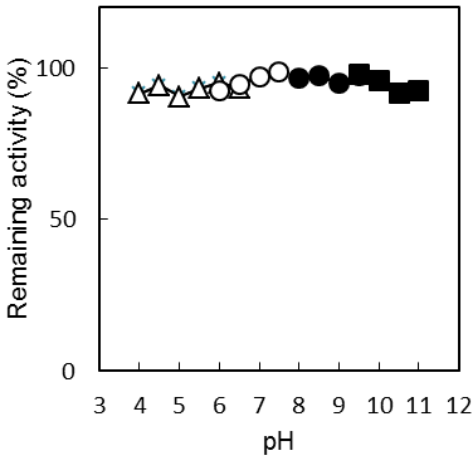

Fig. 2 pH stability

treated for 24 hr at 4 °C in the following buffer solution (50 mM);
△ acetate, ○ phosphate
● Tris-HCl, ■ Glycine-KOH

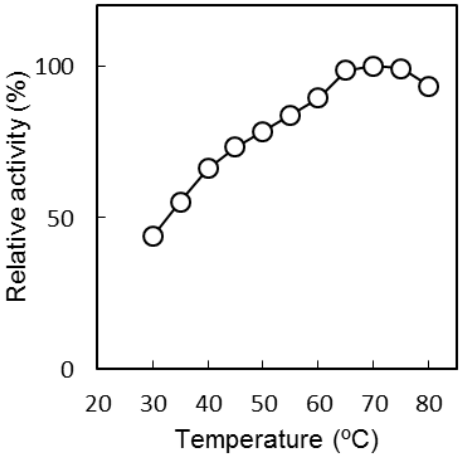

Fig. 3 Thermal activity

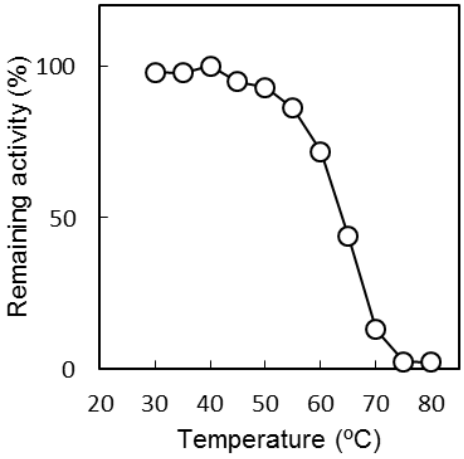

Fig. 4 Thermal stability

treated for 15 min in 20 mM potassium phosphate buffer, pH 7.0

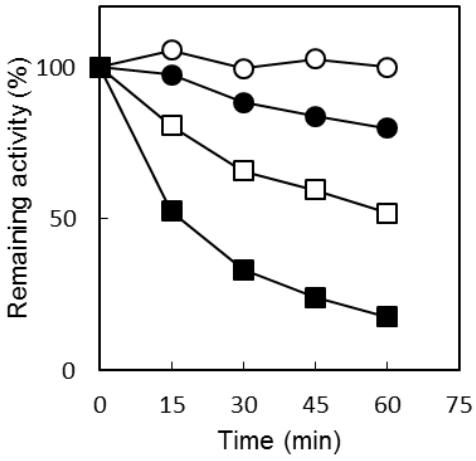

Fig. 5 Thermal stability

(treated in 20 mM potassium phosphate buffer, pH 7.0
○ 50 ºC, ● 55 ºC, □ 60 ºC, ■ 65 ºC)

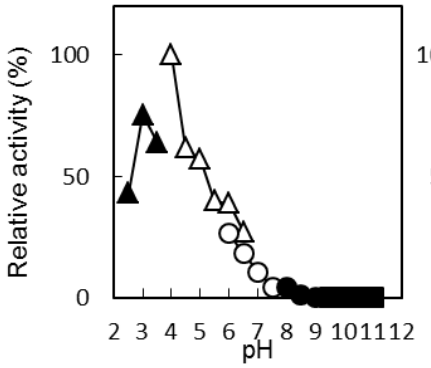

Fig. 6 pH profile (ABTS*3)

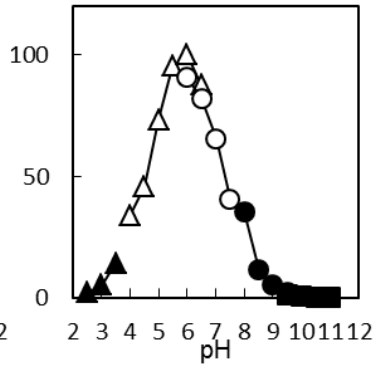

Fig. 7 pH profile (Bilirubin C*4)

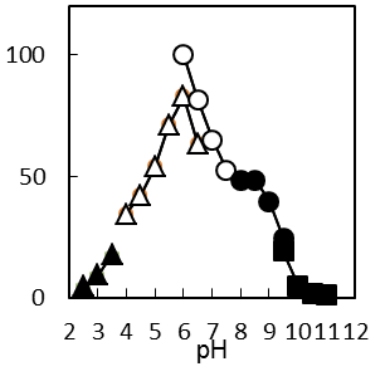

Fig. 8 pH profile (Bilirubin F*4)

(Measured in 20 mM buffer.
▲ Glycine-HCl, △ acetate, ○ phosphate, ● Tris-HCl, ■ Glycine-KOH)

*3 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic Acid) Diammonium Salt
*4 Bilirubin C (conjugated type) and Bilirubin F (free type) are from [Interference Check. A Plus] (Sysmex, Kobe, Japan).

Table 1. Substrate specificity of BOD3

	Phenol	ABTS	Bilirubin C	Bilirubin F
Optimum pH	5.0	4.0	6.0	6.0
Michaelis constants (μM)	41	39	26	26
Relative activity (%)	100	427	36	8
Wavelength for Measurement (nm)	500	405	450	450
Extinction Coefficient (cm^2/μmol)	11.11	29	74	32

Michaelis constants and activity of phenol were defined at pH 7.0. They were defined at each optimum pH when the substrate was ABTS, Bilirubin C, or Bilirubin F,.