BILIRUBIN OXIDASE (BOD3)

[EC 1.3.3.5]

from Trachyderma tsunodae

2 Bilirubin + O₂ → 2 Biliverdin + 2 H₂O

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 (Fig. 2)
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.

\[
\text{Phenol} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{BOD3}} \text{Quinone and/or Phenoxyl radical} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4-\text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4 \text{H}_2\text{O}
\]

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

Solutions
I Buff solution : 300 mM Potassium phosphate buffer, pH 7.0
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/50 mL distilled water)
IV Peroxidase*1 (POD) solution : 240 U/mL (2,400 U/10 mL distilled water)
*1 POD: 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
   H₂O  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
Volume activity (U/mL) = \( \frac{(\Delta \text{Abs (test)} - \Delta \text{Abs (blank)}) \times (0.90 + 0.005)}{11.11 \times 0.005 \times 1/20} \times \text{d.f.} \)

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

\text{d.f.} \quad \text{dilution factor}
11.11 \quad \text{millimolar extinction coefficient of quinoneimine dye at 500 nm (cm}^2/\mu\text{mol)}
1/20 \quad \text{coefficient of transformation for internal unit definition}
*2 \quad \text{Protein concentration} \quad \text{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 °C, ● 55 °C, □ 60 °C, ■ 65 °C

Fig. 6 pH profile (ABTS)*
Fig. 7 pH profile (Bilirubin C)*
Fig. 8 pH profile (Bilirubin F)*

** 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

<table>
<thead>
<tr>
<th></th>
<th>Phenol</th>
<th>ABTS</th>
<th>Bilirubin C</th>
<th>Bilirubin F</th>
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<tbody>
<tr>
<td>Optimum pH</td>
<td>5.0</td>
<td>4.0</td>
<td>6.0</td>
<td>6.0</td>
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<tr>
<td>Michaelis constants (μM)</td>
<td>41</td>
<td>39</td>
<td>26</td>
<td>26</td>
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<tr>
<td>Relative activity (%)</td>
<td>100</td>
<td>427</td>
<td>36</td>
<td>8</td>
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<tr>
<td>Wavelength for Measurement (nm)</td>
<td>500</td>
<td>405</td>
<td>450</td>
<td>450</td>
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<tr>
<td>Extinction Coefficient (cm²/μmol)</td>
<td>11.11</td>
<td>29</td>
<td>74</td>
<td>32</td>
</tr>
</tbody>
</table>

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.