Cholesterol Oxidase (CHO-PEWL)

from recombinant *E. coli*

Cholesterol : oxygen oxidoreductase, EC 1.1.3

\[
\text{Cholesterol} + 2 \text{O}_2 \rightarrow 6\beta\text{-hydroperoxycholest-4-en-3-on} + \text{H}_2\text{O}_2
\]

**SPECIFICATION**

<table>
<thead>
<tr>
<th>Appearance</th>
<th>liquid form (30% sucrose solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>( \geq 200 \text{ U/ml} )</td>
</tr>
<tr>
<td>Storage</td>
<td>at (-20^\circ\text{C})</td>
</tr>
</tbody>
</table>

**PROPERTIES**

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>ca. 59 kDa (gel filtration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>monomer of 60 kDa (SDS-PAGE)</td>
</tr>
<tr>
<td>Michaelis constants</td>
<td>(1.9 \times 10^{-5} \text{ M} ) (cholesterol)</td>
</tr>
<tr>
<td>pH Optimum</td>
<td>6.5–8.0 ( \text{ (Fig. 1) } )</td>
</tr>
<tr>
<td>pH Stability</td>
<td>3.5–8.5 ( \text{ (Fig. 2) } )</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>55(^\circ\text{C}–65(^\circ\text{C}) ( \text{ (Fig. 3) } )</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>below 70(^\circ\text{C} ) ( \text{ (Fig. 4) } )</td>
</tr>
<tr>
<td>Stability (liquid form)</td>
<td>stable at 25(^\circ\text{C} ) for at least one month (Fig. 5)</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>(\text{Ag}^+, \text{Hg}^{2+})</td>
</tr>
</tbody>
</table>
ASSAY PROCEDURE

Principle

\[
\text{Cholesterol oxidase} \quad \text{cholesterol} + 2 \text{O}_2 \rightarrow 6\beta\text{-hydroperoxycholest-4-en-3-one} + \text{H}_2\text{O}_2
\]

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

The appearance of quinoneimine dye is measured spectrophotometrically at 500 nm.

Definition of unit

One unit (U) is defined as the amount of enzyme which produces 1 μmol of hydrogen peroxide per min at 37°C and pH 7.0 under the conditions described below.

Reagents

A. Potassium phosphate buffer, 0.1 M; pH 7.0: mix 0.1 M KH₂PO₄ solution and 0.1 M K₂HPO₄ solution to make a pH 7.0 solution.

B. Cholesterol solution, 0.5%: dissolve 500 mg of cholesterol in 5.0 ml of Triton X-100 with heating. Add 90 ml of distilled water to the hot cholesterol-Triton X-100 solution by slowly pouring. Stir and boil for 30–60s. Cool under running water with gentle agitation. Dissolve 4.0 g of sodium cholate in the cholesterol-Triton X-100 solution and dilute with distilled water to 100 ml. This solution is stable for about one week at room temperature. If it becomes cloudy, warm slightly with stirring until becomes clear.

C. 4-Aminoantipyrine (4-AA) solution, 1.76%: 1.76 g of 4-AA/100 ml of distilled water.

D. Phenol solution, 6.0%: 6.0 g of phenol/100 ml of distilled water.

E. Peroxidase (POD) solution, 1150 U/ml: 75 mg of POD (200 guaiacol U/mg)/100 ml of potassium phosphate buffer (Reagent A).

F. Enzyme dilution buffer: 20 mM potassium phosphate buffer, pH 7.0, containing 0.2% bovine serum albumin (BSA).

Sample: dissolve the enzyme preparation to a volume activity of 0.1–0.5 U/ml with ice-cold enzyme dilution buffer (Reagent F).

Procedure

1. Pipette the following reagents into a cuvette (light path: 1 cm)

\[
\begin{align*}
2.55 \text{ ml} & \quad \text{Potassium phosphate buffer} \quad \text{(Reagent A)} \\
0.20 \text{ ml} & \quad \text{Cholesterol solution} \quad \text{(Reagent B)} \\
0.05 \text{ ml} & \quad \text{4-AA solution} \quad \text{(Reagent C)} \\
0.10 \text{ ml} & \quad \text{Phenol solution} \quad \text{(Reagent D)} \\
0.10 \text{ ml} & \quad \text{POD solution} \quad \text{(Reagent E)}
\end{align*}
\]

2. Equilibrate at 37°C for about 5 min.

3. Add 0.05 ml of sample and mix.

4. Record the increase of absorbance at 500 nm in a spectrophotometer thermostated at 37°C, and calculate the ΔA per min using the linear portion of the curve (ΔAₜ). The blank solution is prepared by adding enzyme dilution buffer (Reagent F) instead of sample (ΔAₒ).
Calculation

Activity can be calculated by using the following formula:

\[
\text{Volume activity (U/ml)} = \frac{(\Delta A_t - \Delta A_i) \times 3.05 (\text{ml}) \times df}{13.8 \times 1/2 \times 0.05 (\text{ml})} = \Delta A \times 8.84 \times df
\]

13.8 : Millimolar extinction coefficient of quinoneimine dye under the assay conditions (cm²/μmol)
1/2 : Factor based on the fact that 1 mol of hydrogen peroxide produces 1/2 mol of quinoneimine dye
\(df\) : Dilution factor

APPLICATIONS

The enzyme is useful for the determination of cholesterol in clinical analysis.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}
\]

\[
\text{Cholesterol} + 2 \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} 6\beta\text{-hydroperoxy} + \text{H}_2\text{O}_2
\]

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

REFERENCES

EXPERIMENTAL DATA

Fig. 1  pH Optimum

![Figure 1: pH Optimum](image1)

Relative activity (%) vs. pH

- ▲ 0.1 M acetate buffer
- ● 0.1 M phosphate buffer
- ○ 0.1 M Tris-HCl buffer
- □ 0.1 M NaHCO₃-NaOH buffer

Treatment: 25°C, 20 h

Fig. 2  pH Stability

![Figure 2: pH Stability](image2)

Residual activity (%) vs. pH

- ▲ 0.1 M acetate buffer
- ● 0.1 M phosphate buffer
- ○ 0.1 M Tris-HCl buffer
- □ 0.1 M NaHCO₃-NaOH buffer

Treatment: 25°C, 20 h

Fig. 3  Optimum temperature

![Figure 3: Optimum temperature](image3)

Relative activity (%) vs. °C

Buffer: 0.1 M phosphate buffer, pH 7.0

Fig. 4  Thermal stability

![Figure 4: Thermal stability](image4)

Residual activity (%) vs. °C

Treatment: 0.1 M phosphate buffer, pH 7.0, 15 min

Fig. 5  Stability (liquid form)

![Figure 5: Stability (liquid form)](image5)

Residual activity (%) vs. Day

Buffer: 0.1 M phosphate buffer, pH 7.0

- ▲ 25°C
- ● 37°C