ALCOHOL OXIDASE [ALOD]

from *Candida* sp.
(Alcohol: oxygen oxidoreductase, EC 1.1.3.13)

$$R\text{-}CH_2OH + O_2 \longrightarrow R\text{-}CHO + H_2O_2$$

Preparation and Specification

Appearance : Light yellowish amorphous powder, lyophilized
Specific activity : More than 7 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 520 kDa (gel filtration) 75 kDa (SDS-PAGE)	
Isoelectric point	: pH 4.1	
Michaelis constants	: Methanol 2.9×10^{-3}M Ethanol 8.2×10^{-3}M	
Optimum pH	: 7.5–9.0	Figure 1
pH stability	: 6.0–9.5 (37℃, 60 min)	Figure 2
Thermal stability	: Stable at 40℃ and below (pH 7.5, 10 min)	Figure 3
Effect of chemicals	: See Table 2 and Table 3	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **blood alcohol.**

$$R\text{-}CH_2OH + O_2 \xrightarrow{\text{ALOD}} R\text{-}CHO + H_2O_2$$

$$2\,H_2O_2 + 4\text{-}AA + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

Table 1. Substrate specificity

Substrate	Relative activity (%)
Methanol	100
Ethanol	79.3
n-Propanol	46.5
iso-Propanol	25.8
n-Butanol	39.6
Form aldehyde	48.2
Acetoaldehyde	0

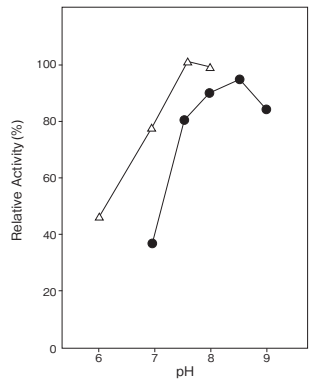
Table 2. Effect of metal ions on ALOD activity

Metal ion	Concentration (mM)	Relative activity (%)
None	–	100.0
KCl	10	103.4
NaCl	10	99.3
LiCl	10	99.3
NH_4Cl	10	95.2
$MgCl_2$	1	100.0
$CaCl_2$	1	93.1
$CoCl_2$	1	68.4
$BaCl_2$	1	69.8
$NiCl_2$	1	70.5
$MnCl_2$	1	100.6
EDTA	1	108.2

Table 3. Effect of detergents on ALOD activity

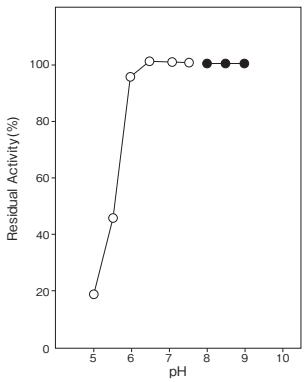
Detergent	Concentration (%)	Relative activity (%)
Nonidet P-40	0.1	96.3
Triton X-100	0.1	98.7
Adekatol PC-8	0.1	91.5
Adekatol SO-120	0.1	96.3
Tween 80	0.1	98.7
Briji 35	0.1	95.1
Deoxycholate	0.1	69.8

Fig.1 pH Optimum



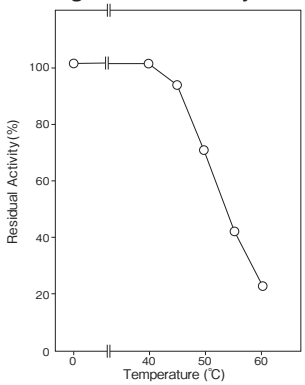
△ : Phosphate buffer
● : Tris-HCl buffer

Fig.2 pH Stability



(+0.2M NaCl) 37℃, 60 min.
○ : 3,3-Dimethylglutarate-NaOH buffer
● : Tris-HCl buffer

Fig.3 Thermal Stability



pH 7.5, 10 min.
10 mM Tris-HCl buffer

Assay

■ Principle

The assay is based on the increase in absorbance at 480 nm as the formation of quinoneimine dye proceeds in the following reactions:

$$CH_3OH + O_2 \xrightarrow{\text{ALOD}} HCHO + H_2O_2$$

$$2\,H_2O_2 + 4\text{-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

■ Unit definition

One unit is defined as the amount of enzyme which generates 1 μmole of H_2O_2 and formaldehyde from methanol per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture

0.2 M Tris-HCl buffer pH8.0	0.20 ml
15 mM 4-AA solution	0.10 ml
0.2% (W/V) Phenol solution	0.10 ml
2.0 M Methanol solution	0.25 ml

A

50 U/ml POD solution [1)] 0.10 ml
Distilled water 0.25 ml
1): 50 U/ml POD solution
Dissolve 500 U (PPU) of POD with 10 ml of distilled water.

2. Reaction stopper
Ethanol

3. Enzyme dilution buffer
10 mM Tris-HCl buffer pH 8.0

4. Reagents
4-AA: NACALAI TESQUE, INC. Special grade #01907-52
POD: Sigma Chemical Co. Type Ⅱ #P-8250
Ethanol: FUJIFILM Wako Pure Chemical Corporation
Japanese Pharmacopoeia Grade #324-00015

■ **Enzyme solution**

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration as required.

■ **Procedure**

1. Pipette accurately 1.0 ml of reaction mixture into a small test tube and preincubate at 37℃.
2. After 5 min, add exactly 50 μl of enzyme solution and mix to start the reaction at 37℃.
※ In the case of a test blank, add 50 μl of enzyme dilution buffer in place of enzyme solution.
3. At 5 min after starting the reaction, add 2.0 ml of the reaction stopper to stop the reaction.
4. Measure the absorbance at 480 nm.
Absorbance sample : As
blank : Ab
△A = (As − Ab) ≦ 0.20 Abs

■ **Calculation**

$$\text{Activity (U/mg of powder)} = \frac{\triangle A/5}{17.17 \times 1/2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

17.17 : millimolar extinction coefficient of quinoneimine dye at 480 nm (cm^2 / μmole)
1/2 : a multiplier derived from the fact that 2 mole of H_2O_2 produces 1 mole of quinoneimine dye
5 : reaction time (min)
3.05 : final volume (ml)
0.05 : volume of enzyme solution (ml)
X : concentration of the sample in enzyme solution (mg/ml)

Storage

Storage at −20℃ in the presence of a desiccant is recommended.

References

1. Fujii, T. and Tonomura. K. (1972) Agric. Biol. Chem., **36**, 2297-2306.
2. Sahm, H. and Wagner, F. (1973) Eur. J. Biochem., **36**, 250-256.
3. Kato, N., Omori, Y., Tani. Y. and Ogata. K. (1976) Eur. J. Biochem., **64**, 341-350.
4. Tani, Y., Miya, T., Nishikawa. H. and Ogata. K. (1972) Agric. Biol. Chem., **36**, 68-75.

ALOD 活性測定法 (Japanese)

Ⅰ. 試薬液

1. 反応試薬混合液
0.2Mトリス -HCl 緩衝液 pH8.0 0.20 ml
15mM 4-AA 溶液 0.10 ml
0.2% (W/V) フェノール溶液 0.10 ml
2M メタノール液 0.25 ml
50U/ml POD 溶液 [1)] 0.10 ml
精製水 0.25 ml
1): 50U/ml POD 溶液
POD 500 単位 (PPU) を精製水 10ml で溶解する。

2. 反応停止液
エタノール原液をそのまま使用する。

3. 酵素溶解希釈用液
10mM トリス-HCl 緩衝液 pH8.0

4. 試薬
4-AA:ナカライテスク製 特級 #01907-52
POD:シグマ製 Type Ⅱ #P-8250
エタノール:富士フイルム和光純薬製
日本薬局方 #324-00015

Ⅱ. 酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液に溶解して全容 20ml とする。
その液を酵素溶解希釈用液で適宜希釈する。

Ⅲ. 測定操作法

1. 小試験管に反応試薬混合液 1.0ml を正確に分注して 37℃で予備加温する。
2. 5 分経過後、酵素試料液 50 μl を正確に加えて混和後、37℃で反応を開始する。
※盲検は酵素試料液の代わりに酵素溶解希釈用液 50 μl を加える。
3. 5 分経過後、反応停止液 2.0ml を正確に加え反応を停止する。
4. 480nm における吸光度を測定する。
求められた吸光度を試料液は As、盲検液は Ab とする。
ΔA = (As − Ab) ≦ 0.20 Abs

Ⅳ. 計算

$$\text{活性 (U/mg)} = \frac{\Delta A/5}{17.17 \times 1/2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

17.17 : キノンイミン色素の 480nm におけるミリモル分子吸光係数 (cm^2/ μmole)
1/2 : H_2O_2 2 モルからキノンイミン色素 1 モルが生成することによる係数
5 : 反応時間 (min)
3.05 : 反応総液量 (ml)
0.05 : 反応に供した酵素試料液量 (ml)
X : 酵素試料液中の検品濃度 (mg/ml)