PYRUVATE OXIDASE [POPG]

from *Aerococcus viridans*
(Pyruvate: oxygen oxidoreductase, phosphorylating, EC 1.2.3.3)

$$\text{Pyruvate} + \text{Pi} + O_2 \longrightarrow \text{Acetylphosphate} + CO_2 + H_2O_2$$

Preparation and Specification

Appearance	: Yellowish amorphous powder, lyophilized
Specific activity	: More than 25 U/mg solid
Contaminants	:
Lactate oxidase	Less than 0.002 % (U/U)
Total AST (GOT)	Less than 0.002 % (U/U)
Total ALT (GPT)	Less than 0.006 % (U/U)
Catalase	Less than 0.3 % (U/U)

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 155 kDa (gel filtration) 70 kDa (SDS-PAGE)	
Isoelectric point	: pH 4.0	
Michaelis constants	: Pyruvate 5.9×10^{-3}M (Mn^{2+}) Pyruvate 4.1×10^{-2}M (Mg^{2+}) Phosphate 2.0×10^{-3}M (Mn^{2+}) Phosphate 4.5×10^{-3}M (Mg^{2+})	
Optimum pH	: 6.5–7.0	Figure 1
pH stability	: 6.0–7.0 (37℃, 60 min, 10μM FAD)	Figure 2
Optimum temperature	: 40℃	Figure 3
Thermal stability	: Stable at 45℃ and below (phosphate buffer containing 10μM FAD, pH 6.5, 10 min)	Figure 4
Storage stability	: At least one year at −20℃	Figure 5
Stabilizer	: FAD	
Activators	: Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}	
Inhibitors	: EDTA	
Co-factors	: FAD, TPP	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **AST and ALT** when coupled with oxaloacetate decarboxylase (T-209) (in the case of AST) and POD.

$$\text{ALT: } \alpha\text{-KG} + \text{L-Alanine} \longrightarrow \text{L-Glutamate} + \text{Pyruvate}$$
$$\text{AST: } \alpha\text{-KG} + \text{L-Aspartate} \longrightarrow \text{L-Glutamate} + \text{Oxaloacetate}$$
$$\text{Oxaloacetate} \xrightarrow{\text{OACII}} \text{Pyruvate} + CO_2$$
$$\text{Pyruvate} + \text{Pi} + O_2 \xrightarrow{\textbf{POPG}} \text{Acetylphosphate} + CO_2 + H_2O_2$$
$$2\,H_2O_2 + \text{4-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

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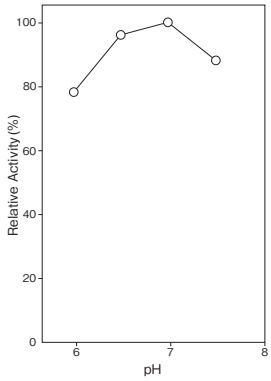
Table 1. Substrate specificity

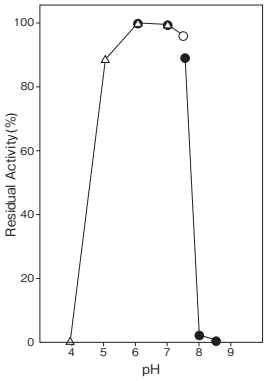
Substrate	Relative activity (%)
Pyruvate	100
2-Oxobutyrate	3
2-Oxoglutarate	0
Oxaloacetate	0
DL-Lactate	0
Acetate	0
L-Alanine	0
L-Aspartate	0

Fig.1 pH Optimum



Phosphate buffer

Fig.2 pH Stability



37℃, 60 min.
○ : Phosphate buffer
● : Tris-HCl buffer containing 10 μM FAD
△ : DMG-NaOH buffer

Fig.3 Optimum Temperature

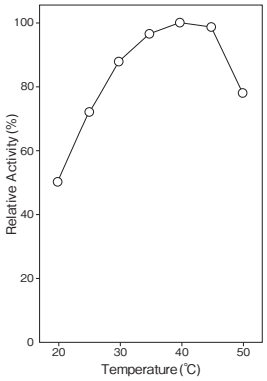
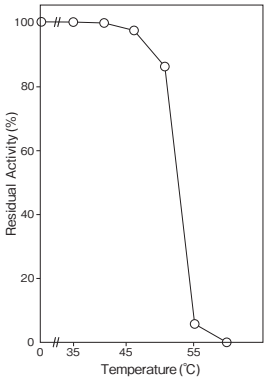
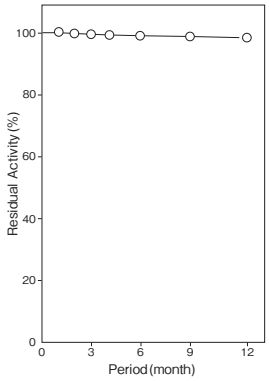


Fig.4 Thermal Stability



pH 6.5, 10 min.
10 mM phosphate buffer, containing 10 μM FAD.

Fig.5 Storage (lyophilized powder)



−20℃

Assay

■ Principle

The assay is based on the increase in absorbance at 565 nm as the formation of quinoneimine dye proceeds in the following reactions:

$$\text{Pyruvate} + O_2 + Pi \xrightarrow{\text{POPG}} \text{Acethylphosphate} + CO_2 + H_2O_2$$

$$2H_2O_2 + 4\text{-AA} + \text{DMA} + H^+ \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4H_2O$$

DMA: N, N-Dimethylaniline

■ Unit definition

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

■ Reagents

1. Reaction mixture Ⅰ

1 M KH_2PO_4-NaOH buffer pH 6.7	0.20 ml
10 mM Thiamine pyrophosphate	0.02 ml
1 mM FAD solution	0.01 ml
100 U/ml POD solution [1)]	0.05 ml
15 mM 4-AA solution	0.10 ml
Distilled water	0.22 ml

FAD: Flavine adenine dinucleotide
1): 100 U/ml POD solution
Dissolve 1,000 U (PPU) of POD with 10 ml of distilled water.

2. Reaction mixture Ⅱ

0.2 % (V/V) DMA soultion	0.20 ml
0.1 M $MgCl_2$ solution	0.10 ml

3. Substrate solution
   1 M Potassium pyruvate solution
4. Reaction stopper
   McIlvaine buffer pH 5.50-5.55 containing 0.1 M EDTA
   EDTA: Ethylenediamine tetraacetic acid
5. Enzyme dilution buffer
   10 mM KH_2PO_4-NaOH buffer pH 7.0 containing 10 μM FAD
6. Reagents
   Thiamine pyrophosphate (Cocarboxylase): FUJIFILM Wako Pure Chemical Corporation #031-03833
   4-AA: NACALAI TESQUE, INC. Special grade #01907-52
   POD: Sigma Chemical Co. Type Ⅱ #P-8250
   DMA: FUJIFILM Wako Pure Chemical Corporation Special grade #044-02763
   FAD (2Na): Kyowa Hakko Co., Ltd.
   Potassium pyruvate: FUJIFILM Wako Pure Chemical Corporation for biochemistry #166-08351
   EDTA ($2Na \cdot 2H_2O$): KISHIDA CHEMICAL Co., Ltd. #060-29133

■ 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 solution buffer to adjust the concentration as required.

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■ Procedure

1. Mix reaction mixture Ⅰ, substrate solution and reaction mixture Ⅱ in a ratio of 6 : 1 : 3. Pipette accurately 1.0 ml of the mixture into a small test tube and preincubate at 37℃.
2. After 5 min, add exactly 20 μl of enzyme solution and mix to start the reaction at 37℃.
   ※ In the case of a test blank, add 20 μl of enzyme dilution buffer in place of enzyme solution.
3. At 10 min after starting the reaction, add 2.0 ml of the reaction stopper to stop the reaction.
4. After 5 min, measure the absorbance at 565 nm.

Absorbance sample : As
blank : Ab
△A = (As − Ab) ≦ 0.400 Abs

■ Calculation

$$\text{Activity (U/mg of powder)} = \frac{\triangle A/10}{23.56 \times 1/2} \times \frac{3.02}{0.02} \times \frac{1}{X}$$

23.56 : millimolar extinction coefficient of quinoneimine dye at 565 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
10 : reaction time (min)
3.02 : final volume (ml)
0.02 : 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. Enzyme activity will be retained for at least one year under this condition (Figure 5).

References

1. Harger, L. P., Geller, D. M. and Lipmann, F. (1954) Fed. Proc., **13**, 734–738.
2. Lipmann, F. (1940) J. Biol. Chem., **134**, 463–464.
3. Harger, L. P. and Lipmann, F. (1955) Methods Enzymol., **Vol. 1**, 482.
4. Sedewitz, B., Schleifer, K. H. and Gotz, F. (1984) J. Bacteriol., **160**, 273–278.
5. Sedewitz, B., Schleifer, K. H. and Gotz, F. (1984) J. Bacteriol., **160**, 462–465.

POPG 活性測定法 (Japanese)

Ⅰ. 試薬液

1. 反応試薬混合液 I

1M KH_2PO_4 – NaOH 緩衝液 pH6.7	0.20 ml
精製水	0.22 ml
15mM 4–AA 溶液	0.10 ml
100U/ml POD 溶液 [1)]	0.05 ml
10mM チアミンピロリン酸溶液	0.02 ml
1mM FAD 溶液	0.01 ml

1): 100U/ml POD 溶液
POD 1,000 単位 (PPU) を精製水 10ml で溶解する。

2. 反応試薬混合液Ⅱ

0.2% (V/V) DMA 溶液	0.20 ml
0.1M 塩化マグネシウム溶液	0.10 ml

3. 基質溶液
1M ピルビン酸カリウム溶液
4. 反応停止液
0.1M EDTA を含むマックイルバイン緩衝液 pH5.50〜5.55
5. 酵素溶解希釈用液
10 μ M FAD を含む 10mM KH_2PO_4 – NaOH 緩衝液 pH7.0
6. 試薬
チアミンピロリン酸 (コカルボキシラーゼ):
富士フイルム和光純薬製 #031–03833
POD: シグマ製 Type Ⅱ #P–8250
4–AA: ナカライテスク製 特級 #01907–52
DMA (N,N'– ジメチルアニリン):
富士フイルム和光純薬製 特級 #044–02763
FAD (フラビンアデニンジヌクレオチド·2Na):
協和発酵製
ピルビン酸カリウム:
富士フイルム和光純薬製 生化学用 #166–08351
EDTA (エチレンジアミン四酢酸·2Na·$2H_2O$):
キシダ化学製 #060–29133

Ⅱ. 酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液で溶解して全容 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. 反応試薬混合液 I と基質溶液及び反応試薬混合液Ⅱを 6:1:3 に混合し、その混合液 1.0ml ずつを正確に小試験管へ分注して 37℃で予備加温する。
2. 5 分経過後、酵素試料液 20 μl を加えて混和し、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 20 μl を加える。
3. 10 分経過後、反応停止液 2.0ml を加えて混和し、反応を停止する。
4. 5 分後、565nm 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。

$$\Delta A = (As - Ab) \leqq 0.400 \text{ Abs}$$

Ⅳ. 計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{23.56 \times 1/2} \times \frac{3.02}{0.02} \times \frac{1}{X}$$

23.56: キノンイミン色素の 565nm 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
10 : 反応時間 (min)
3.02: 反応総液量 (ml)
0.02: 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)

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