●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

LACTATE OXIDASE

from Microorganism

L-Lactate:oxygen oxidoreductase (EC 1.13.12.4)

L-Lactate + O_2 ⟶ Pyruvate + H_2O_2

PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized	
Activity	: Grade Ⅲ 80U/mg-solid or more	
Contaminants	: Pyruvate oxidase	≤1.0×10^{-3}%
	Cholesterol oxidase	≤1.0×10^{-3}%
	Uricase	≤1.0×10^{-3}%
	Glucose oxidase	≤1.0×10^{-3}%

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 160,000 (by gel filtration)	
Isoelectric point	: 4.3±0.2	
Michaelis constant	: 1.0×10^{-3}M (L-Lactate)	
Inhibitors	: Fe^{+++}, SDS	
Optimum pH	: 7.5	(Fig.2)
Optimum temperature	: 35−40℃	(Fig.3)
pH Stability	: 4.0−9.8 (25℃, 16hr)	(Fig.4)
Thermal stability	: below 50℃ (pH 7.0, 10min)	(Fig.5)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of L-lactate.

ASSAY

Principle:

L-Lactate + O_2 —(lactate oxidase)→ Pyruvate + H_2O_2

$2H_2O_2$ + 4-Aminoantipyrine + EHSPT —(peroxidase)→ Quinoneimine dye + $4H_2O$

The appearance of quinoneimine dye is measured at 555nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute under the conditions described below.

Method:

Reagents

A. DL-Lactate solution : 0.125M [240mg of DL-lithium lactate (MW=96.01)/20ml of 50mM K-PB pH7.5] (Should be prepared fresh)

B. 4-AA solution : 0.5% (500mg of 4-aminoantipyrine/100ml of H_2O) (Store at 4℃ in a brownish bottle)

C. EHSPT(TOOS) solution : 20mM [591mg N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (MW=295.3)/100ml of H_2O] (Store at 4℃ in a brownish bottle)

D. Peroxidase solution : 25U/ml [ca. 23mg of horseradish peroxidase (Toyobo GradeⅢ, 110 purpurogallin units/mg)/100 ml of H_2O]

E. SDS solution : 0.25% (500mg sodium dodecyl sulfate/200ml of H_2O)

F. Enzyme diluent : 20mM K-PB, pH7.0 containing 0.1%(w/v) sodium cholate

Procedure

1. Prepare the following working solution (20 tests) in a brownish bottle, and store on ice.

8.0ml	DL-Lactate solution	(A)
1.2ml	4-AA solution	(B)
0.8ml	EHSPT solution	(C)
2.0ml	Peroxidase solution	(D)
8.0ml	distilled water	

Concentration in assay mixture	
K-phosphate buffer	20 mM
DL-Lactate	48 mM
4-Aminoantipyrine	1.2 mM
EHSPT	0.76 mM
Peroxidase	2.4 U/ml

2. Pipette 1.0ml of working solution into a test tube and equilibrate at 37℃ for about 5 minutes.
3. Add 0.05ml of the enzyme solution* and mix.
4. After exactly 15minutes at 37℃, add 2.0ml of SDS solution (E) to stop the reaction and measure the optical density at 555nm against water (ODtest).
   At the same time, prepare the blank by using the same method as the test except that the enzyme diluent (F) is used instead of the enzyme solution (ODblank).

* Dissolve the enzyme preparation in ice-cold 20mM ACES-NaOH pH7.0 containing 1mM EDTA and 0.5%(w/v) sodium cholate, and dilute to 0.04−0.1 U/ml with the enzyme diluent (F) immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD\ (OD\ test - OD\ blank) \times Vt \times df}{34.3 \times 1/2 \times t \times 1.0 \times Vs} = \Delta OD \times 0.237 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.05ml)

Vs : Sample volume (0.05ml)

34.3 : Millimolar extinction coefficient of quinoneimine dye under the assay condition (cm²/micromole)

1/2 : Factor based on the fact that one mole of H_2O_2 produced half a mole of quinoneimine dye

t : Reaction time (15minutes)

1.0 : Light path length (cm)

C : Enzyme concentration in dissolution (C mg/ml)

REFERENCES

1) A. Toda, and Y. Nishiya; *J. Ferment. Technol., 85,* 507 (1998)

Table 1. Effect of Various Chemicals on Lactate oxidase

[The enzyme solution dissolved in 20mM ACES-NaOH, pH7.0 (50U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		100
$CaCl_2$		101
$Ba(OAc)_2$		101
$FeCl_3$		5
$CoCl_2$		100
$MnCl_2$		100
$ZnCl_2$		94
$Cd(OAc)_2$		91
$NiCl_2$		99
$CuSO_4$		94
$AgNO_3$		54
MIA	2.0	94
NEM	2.0	99

Chemical	Concn.(mM)	Residual activity(%)
IAA	2.0	90
Hydroxylamine	2.0	99
EDTA	5.0	94
o-Phenanthroline	2.0	100
α,α'-Dipyridyl	2.0	94
Borate	50.0	97
NaF	2.0	99
NaN_3	2.0	100
Triton X-100	0.10%	98
Brij 35	0.10%	86
Tween 20	0.10%	81
Span 20	0.10%	96
Na-cholate	0.10%	101
DAC	0.05%	76
SDS	0.05%	0

Ac, CH_3CO; MIA, Monoiodoacetate; NEM, N-ethylmaleimide; IAA, Iodoacetate; EDTA, Ethylenediaminetetraacetate; SDS, Sodium dodecyl sulfate; DAC, Dimethylbenzylalkylammonium chloride.

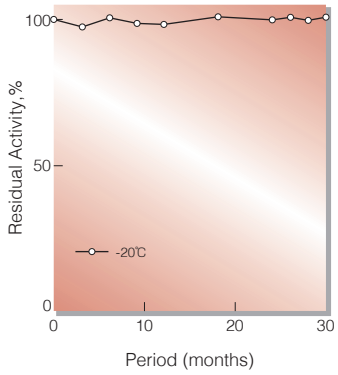

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

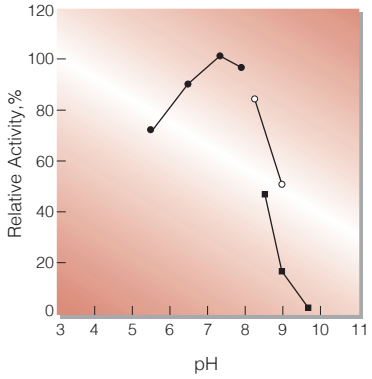

Fig.2. pH-Activity

〔37℃, in 20mM buffer solution
●, pH5.7-7.9 K-phosphate;
○, pH8.2-9.0 borate;
■, pH8.6-9.6 glycine-NaOH〕

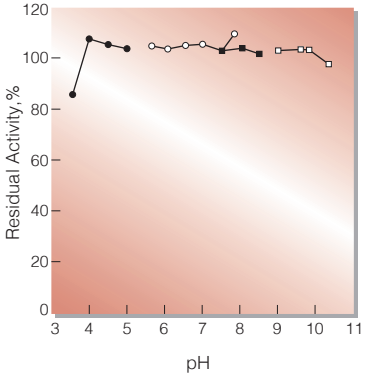

Fig.4. pH-Stability

〔25℃, 16hr-treatment with 50mM buffer solution:
●, pH3.6-5.0 acetate;
○, pH5.6-7.8 K-phosphate;
■, pH7.5-8.5 Tris-HCl;
□, pH8.9-10.2 glycine-NaOH〕

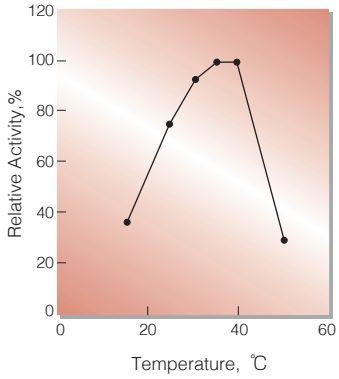

Fig.3. Temperature activity

〔in 20mM K-phosphate , pH7.5〕

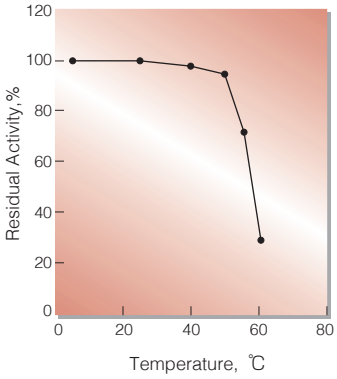

Fig.5. Thermal stability

〔10min-treatment with 20mM ACES-NaOH, pH7.0.
Enzyme concentration: 100U/ml〕

活性測定法（Japanese）

1.原理

L-Lactate＋O_2 $\xrightarrow{\text{lactate oxidase}}$ Pyruvate＋H_2O_2

$2H_2O_2$＋4-Aminoantipyrine＋EHSPT $\xrightarrow{\text{peroxidase}}$ Quinoneimine dye＋$4H_2O$

4-AminoantipyrineとEHSPTの酸化縮合物であるQuinoneimine色素を555nmで測定し，上記反応で生成したH_2O_2量を定量する。

2.定義

下記条件下で1分間に1マイクロモルのH_2O_2を生成する酵素量を1単位(U)とする。

3.試薬

A. 0.125M DL-乳酸溶液〔240mgのDL-乳酸リチウム(MW=96.01)を50mM K-PB pH7.5に溶解し，最終液量を20mℓとする。〕 (用時調製)

B. 0.5% 4-AA水溶液 (500mgの4-アミノアンチピリンを蒸留水に溶解し，最終液量を100mℓとする。) (褐色瓶中で4℃保存)

C. 20mM EHSPT(TOOS)水溶液〔591mgのEHSPT (MW＝295.3)を蒸留水に溶解し，最終液量を100mℓとする。〕 (褐色瓶中で4℃保存)

D. 25U/mℓ POD水溶液 〔約23mgの西洋ワサビ由来ペルオキシダーゼ(東洋紡製，GradeⅢ) (110プルプロガリン単位/mg)を冷蒸留水に溶解し，最終液量を100mlとする。〕

E. 0.25% SDS水溶液〔500mgのドデシル硫酸ナトリウム(SDS)を蒸留水に溶解し，最終液量を200mℓとする。〕

酵素溶液：酵素標品を予め氷冷した1.0mM EDTA，0.5%(w/v)コール酸ナトリウムを含む20mM ACES-NaOH，pH7.0で溶解し,分析直前に0.1%(w/v)コール酸ナトリウムを含む20mM K-リン酸緩衝液，pH7.0で希釈する。

4.手順

①下記反応混液(20テスト分)を調製する(褐色瓶にて氷冷保存)

8.0mℓ	DL-乳酸溶液	(A)
1.2mℓ	4-AA水溶液	(B)
0.8mℓ	EHSPT水溶液	(C)
2.0mℓ	POD水溶液	(D)
8.0mℓ	蒸留水	

②反応混液1.0mℓを試験管に採り，37℃で約5分間予備加温する。

③酵素溶液0.05mlを加え，反応を開始する。

④37℃で正確に15分反応させた後，SDS水溶液(E) 2.0mlを加えて反応を停止させる。この液につき水を対照に555nmにおける吸光度を測定する(ODtest)。

⑤盲検は酵素溶液の代わりに酵素希釈液〔0.1%(w/v)コール酸ナトリウムを含む20mM K-リン酸緩衝液，pH7.0〕を用い，上記同様に操作を行って吸光度を測定する(ODblank)。

5.計算式

$$U/mℓ = \frac{\Delta OD\ (OD\ test - OD\ blank) \times 3.05(mℓ) \times 希釈倍率}{34.3 \times 1/2 \times 15(分) \times 1.0 \times 0.05(mℓ)}$$

$$= \Delta OD \times 0.237 \times 希釈倍率$$

$U/mg = U/mℓ \times 1/C$

34.3 ：Quinoneimine色素の上記測定条件下でのミリモル分子吸光係数(㎠/micromole)

1/2 ：酵素反応で生成したH_2O_2の1分子から形成するQuinoneimine色素は1/2分子であることによる係数

1.0 ：光路長(cm)

C ：溶解時の酵素濃度(c mg/mℓ)