●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

ASCORBATE OXIDASE

from Cucumis sp.

L-Ascorbate:oxygen oxidoreductase (EC 1.10.3.3)

L-Ascorbic acid + $^1/_2O_2$ ⟶ Dehydroascorbic acid + H_2O

PREPARATION and SPECIFICATION

Appearance	: Light blue amorphous powder, lyophilized
Activity	: Grade Ⅲ 200U/mg-solid or more (containing approx. 70% of stabilizers)
Contaminants	: Catalase ≤1.0×10^{-1}%
	Phosphatase ≤2.0×10^{-2}%
Stabilizers	: BSA, borax, basic amino acids.

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: 132,000 [1], 140,000 [2]	
Isoelectric point	: between 6.0 and 7.8 [1], 8.2 [2]	
Michaelis constant	: 2.5×10^{-4}M (Ascorbate)	
Structure	: 8 copper atoms per enzyme molecule [1,2]	
Inhibitors	: cyanide, Na_2S, diethyldithiocarbamate (Na)	
Optimum pH	: 5.6	(Fig.4)
Optimum temperature	: approx. 30℃	(Fig.5)
pH Stability	: pH 7.0−10.0 (25℃, 17hr)	(Fig.6)
Thermal stability	: below 40℃ (pH 8.0, 30min)	(Fig.7)
Substrate specificity	: This enzyme oxidizes ascorbic acid and several ascorbic derivatives.[3]	
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of ascorbic acid and for eliminating the interference of ascorbic acid in clinical analysis.

ASSAY

Principle:

Ascorbic acid + $^1/_2O_2$ $\xrightarrow{\text{ascorbate oxidase}}$ Dehydroascorbic acid + H_2O

The disappearance of ascorbic acid is measured at 245nm by spectrophotometry.

Unit definition:

One unit causes the decrease of one micromole of ascorbic acid per minute under the conditions described below.

Method:

Reagents

A. Ascorbic acid solution : 1.0mM [Dilute the stock solution (10mM) to 10-fold volume with 0.2M KH_2PO_4 solution containing 1.0mM EDTA.] (Prepare freshly) Stock solution : 176mg L-ascorbic acid (MW=176.13)/100ml of 1.0mM HCl solution containing 1.0mM EDTA (Stable for one month if stored at 0−5℃)

B. Na_2HPO_4 solution : 10mM

C. HCl solution : 0.2N

D. Enzyme diluent : 10mM Na_2HPO_4 solution containing 0.05% BSA (Prepare freshly)

Procedure

1. Prepare the following reaction mixture in a test tube and equilibrate at 30℃ for about 5 minutes.

   0.5ml Substrate solution (A)

   0.5ml Na_2HPO_4 solution (B)

   (pH of the reaction mixture should be 5.6.)

Concentration in assay mixture	
KH_2PO_4	82 mM
Na_2HPO_4	5.5 mM
Ascorbic acid	0.45 mM
EDTA	0.45 mM
BSA	45.4 μg/ml

2. Add 0.1ml of the enzyme solution* and mix.
3. After exactly 5 minutes at 30℃, add 3.0ml of HCl solution (C) to stop the reaction and measure the optical density at 245nm against water (OD test).

   At the same time, prepare the blank by first mixing the reaction mixture with 3.0ml of HCl solution (C) after 5min-incubation at 30℃, followed by addition of the enzyme solution (OD blank).

* Dissolve the enzyme preparation in ice-cold distilled water (more than 60U/ml) and dilute to 0.15−0.25U/ml with ice-cold enzyme diluent (D), immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD\ (OD\ blank - OD\ test) \times Vt \times df}{10.0 \times 1.0 \times t \times Vs} = \Delta OD \times 0.820 \times df$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (4.1ml)

Vs : Sample volume (0.1ml)

10.0 : Millimolar extinction coefficient of ascorbic acid under the assay condition at pH 1.0 (㎖/micromole)

1.0 : Light path length (cm)

t : Reaction time (5 minutes)

df : Dilution factor

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

REFERENCES

1) T.Nakamura, N.Makino and Y.Ogura; ***J.Biochem., 64***, 189 (1968).

2) V.Ts.Aikazyan and R.M.Nalbandyan; ***FEBS LETTERS, 104***, 127 (1979).

3) G.A.White and F.G.Smith; ***Nature, 190***, 187 (1961).

Table 1. Effect of Various Chemicals on Ascorbate oxidase

[The enzyme dissolved in distilled water (60U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		88
$CaCl_2$		86
$Ba(OAc)_2$		86
$FeCl_3$		34
$CoCl_2$		83
$MnCl_2$		88
$ZnCl_2$		90
$CdCl_2$		87
$NiCl_2$		79
$CuSO_4$		90
$Pb(OAc)_2$		91
$AgNO_3$		3.7
$HgCl_2$		42
2-Mercaptoethanol	2.0	75
PCMB	1.0	26

Chemical	Concn.(mM)	Residual activity(%)
MIA	2.0	3.5
NEM	2.0	75
IAA	2.0	21
Hydroxylamine	2.0	81
EDTA	5.0	80
o-Phenanthroline	2.0	50
α,α'-Dipyridyl	1.0	78
Borate	50	75
NaF	2.0	84
NaN_3	2.0	85
Triton X-100	0.10%	84
Brij 35	0.10%	24
Tween 20	0.10%	19
Span 20	0.10%	97
Na-cholate	0.10%	80
SDS	0.05%	83
DAC	0.05%	88

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; NEM, N-Ethylmaleimide; IAA, Iodoacetamide; EDTA, Ethylenediaminetetraacetate; SDS, Sodium dodecyl sulfate; DAC, Dimethylbenzylalkylammonium chloride.

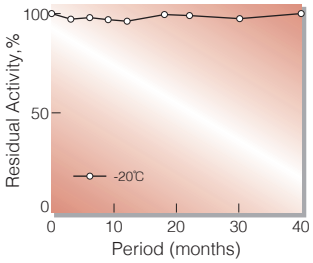

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

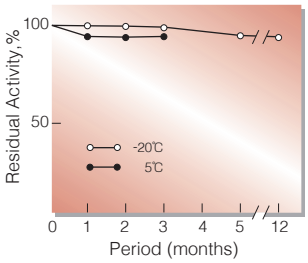

Fig.2. Stability (Powder form)

〔kept under dry conditions〕

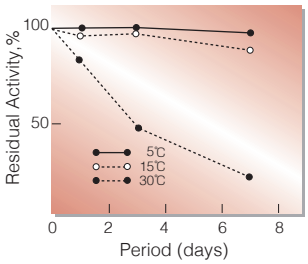

Fig.3. Stability (Liquid form)

〔enzyme concn. :8,000U/ml
buffer composition:45mM borate buffer,pH7.8〕

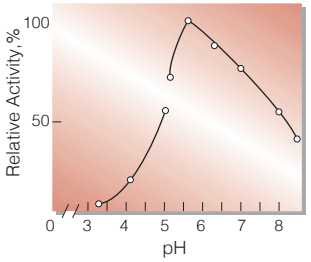

Fig.4. pH-Activity

〔30℃ in 0.33 M buffer solution: pH3.0-6.0, acetate; pH5.0-8.0, phosphate〕

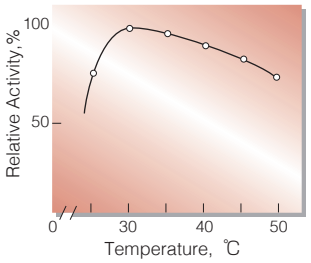

Fig.5. Temperature activity

〔in 0.33M phosphate buffer,pH5.6〕

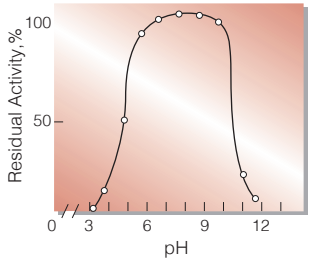

Fig.6. pH-Stability

〔25℃, 17hr-treatment with Britton-Robinson's buffer〕

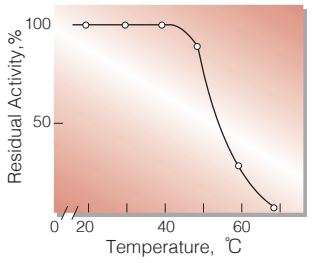

Fig.7. Thermal stability

〔30min-treatment with 50mM phosphate buffer,pH 8.0
enzyme concn.: 12U/ml〕

活性測定法（Japanese）

1.原理

Ascorbic acid+$^1/_2$ O_2 $\xrightarrow{\text{ascorbate oxidase}}$ Dehydroascorbic acid+H_2O

Ascorbic acidの消失量を245nmの吸光度の変化で測定する。

2.定義

下記条件下で1分間に1マイクロモルのアスコルビン酸を酸化する酵素量を1単位(U)とする。

3.試薬

A. 1.0mMアスコルビン酸溶液〔保存溶液(10mMのL-アスコルビン酸溶液)を1.0mM EDTAを含む0.2M KH_2PO_4溶液で10倍希釈する〕(用時調製)
保存溶液は176mgのL-アスコルビン酸 (試薬特級,MW=176.13)を精秤し1.0mM EDTAを含む1.0mM HCl溶液100mlに溶解して調製する(0～5℃保存で1ケ月は使用可能)。

B. 10mM Na_2HPO_4溶液

C. 0.2N HCl溶液

酵素溶液：酵素標品を予め氷冷した蒸留水で溶解(60U/ml以上)し, 分析直前に0.05% BSAを含む10mM Na_2HPO_4溶液(氷冷)で0.15～0.25U/mlに希釈する。

4.手順

①試験管に下記反応混液を調製し,30℃で約5分間予備加温する。

0.5ml	基質溶液	(A)
0.5ml	Na_2HPO_4溶液	(B)

(反応混液のpHは5.6)

②酵素溶液0.1mlを加え, 反応を開始する。

③30℃で正確に5分間反応させた後, HCl溶液(C)3.0mlを加えて反応を停止させる。この液につき245nmにおける吸光度を測定する(ODtest)。

④盲検は反応混液①を30℃で5分間放置後, HCl溶液(C)3.0mlを加えて混和し, 次いで酵素溶液0.1mlを加えて調製する。以下同様に吸光度を測定する(ODblank)。

5.計算式

$$U/ml = \frac{\Delta OD\ (OD\ blank - OD\ test) \times 4.1(ml) \times 希釈倍率}{10.0 \times 1.0 \times 5(分) \times 0.1(ml)}$$

$$= \Delta OD \times 0.82 \times 希釈倍率$$

$$U/mg = U/ml \times 1/C$$

10.0 : アスコルビン酸の上記測定条件下(pH1.0)でのミリモル分子吸光係数 (cm^2/micromole)

1.0 : 光路長(cm)

C : 溶解時の酵素濃度(c mg/ml)