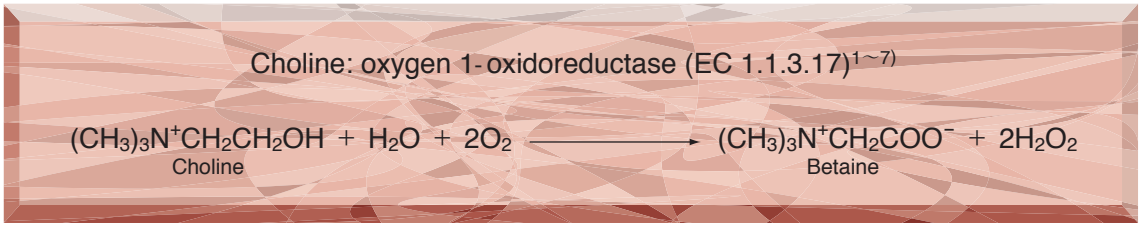
●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

CHOLINE OXIDASE

from Alcaligenes sp.

Choline: oxygen 1-oxidoreductase (EC 1.1.3.17)[1~7)]



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 10U/mg-solid or more (containing approx. 20% of stabilizers)
Contaminant	: Catalase ≤1.0×10^{2}%
Stabilizers	: EDTA, BSA, amino acids (glycine, sodium glutamate, etc.)

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 95,000	
Isoelectric point	: 4.1±0.1	
Michaelis constants	: 2.84×10^{-3}M (Choline), 5.33×10^{-3}M(Betaine aldehyde)	
Structure	: One mol of FAD is covalently bound to mol of the enzyme [8)]	
Inhibitors	: p-Chloromercuribenzoate, Cu^{++}, Co^{++}, Hg^{++}, Ag^{+}	
Optimum pH	: 8.0−8.5	(Fig.4)
Optimum temperature	: 40−45℃	(Fig.5)
pH Stability	: pH 7.0−9.0 (30℃, 2 hr)	(Fig.6)
Thermal stability	: below 37℃ (pH 7.5, 10min)	(Fig.7)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of phospholipids when coupled with phospholipase D and for choline esterase-activity in clinical analysis.[9~11)]

ASSAY

Principle:

$$\text{Choline} + H_2O + 2O_2 \xrightarrow{\text{choline oxidase}} \text{Betaine} + 2H_2O_2$$

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

The appearance of quinoneimine dye is measured at 500nm 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. Choline chloride solution : 2.1% [2.1g choline chloride/100ml of Tris-HCl buffer (D)] (Should be prepared fresh)
B. 4-AA solution : 1.0% (1.0g 4-aminoantipyrine/100ml of H_2O)(Store at 4℃ in a brownish bottle)
C. Phenol solution : 1.0% (1.0g phenol/100ml of H_2O)(Store at 4℃ in a brownish bottle)
D. Tris-HCl buffer : 0.1M Tris-HCl buffer, pH 8.0 [Dissolve 12.1g of Tris (MW=121.14) in ca.800ml of H_2O and, after adjusting the pH to 8.0 at 25℃ with 2.0 N HCl, fill up to 1,000ml with H_2O.]
E. Enzyme diluent : 10mM Tris-HCl buffer, pH 8.0 contg. 2mM EDTA and 1.0% KCl.

Procedure

1. Prepare the following working solution (100ml) in a brownish before use and store on ice in a brownish bottle.

97 ml	Substrate solution	(A)
1.0ml	4-AA solution	(B)
2.0ml	Phenol solution	(C)
5.0mg	Peroxidase from horseradish (110 purpurogallin units/mg)(Toyobo GradeⅢ)	

Concentration in assay mixture	
Tris buffer	97 mM
Choline chloride	0.14 M
EDTA	33 μM
KCl	2.2 mM
4-Aminoantipyrine	0.48 mM
Phenol	2.1 mM
POD	ca.4.92U/ml

2. Pipette 3.0ml of working solution into a cuvette (d=1.0cm) and equilibrate at 37°C for about 5 minutes.
3. Add 0.05ml of the enzyme solution* and mix by gentle inversion.
4. Record the increase in optical density at 500nm against the working solution for 3 to 4 minutes in a spectrophotometer thermostated at 37℃, and calculate the ΔOD per minute from the initial linear portion of the curve.

* Dissolve the enzyme preparation in ice-cold Tris-HCl buffer (D) and dilute to 0.1−0.5U/ml with enzyme diluent (E).

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min \times Vt \times df}{12.0 \times 1/2 \times 1.0 \times Vs} = \Delta OD/min \times 10.17 \times df$$

$$\text{Weight activity (U/mg)} = (U/ml) \times 1/C$$

Vt : Total volume (3.05ml)
Vs : Sample volume (0.05ml)
12.0 : Millimolar extinction coefficient of quinoneimine dye under the assay conditions (㎠/micromole)
1/2 : Factor based on the fact that one mole of H2O2 produces half a mole of quinoneimine dye.
1.0 : Light path length (cm)
df : Dilution factor
C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

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Table 1. Effect of Various Chemicals on Choline oxidase

[The enzyme dissolved in 10mM Tris-HCl buffer, pH 8.0 contg. 2mM EDTA and 1.0% KCl (5U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		87
$CaCl_2$		92
$Ba(OAc)_2$		89
$FeCl_3$		87
$CoCl_2$		89
$MnCl_2$		91
$ZnCl_2$		88
$CdCl_2$		92
$NiCl_2$		91
$CuSO_4$		92
$Pb(OAc)_2$		87
$AgNO_3$		80
$HgCl_2$		48
2-Mercaptoethanol	2.0	90
PCMB	1.0	13
MIA	2.0	87
NEM	2.0	100
IAA	2.0	95
Hydroxylamine	2.0	77
EDTA	5.0	92
o-Phenanthroline	2.0	90
α,α'-Dipyridyl	1.0	91
Borate	50	94
NaF	2.0	92
NaN_3	2.0	92
Triton X-100	0.10%	96
Brij 35	0.10%	92
Tween 20	0.10%	95
Span 20	0.10%	94
Na-cholate	0.10%	96
SDS	0.05%	95
DAC	0.05%	91

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

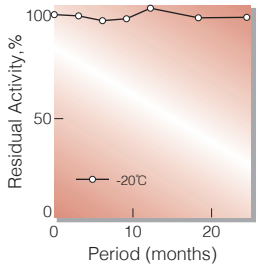

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

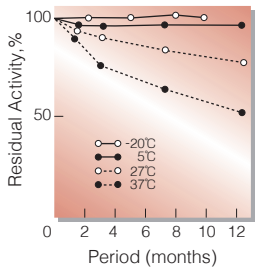

Fig.2. Stability (Powder form)

〔kept under dry conditions〕

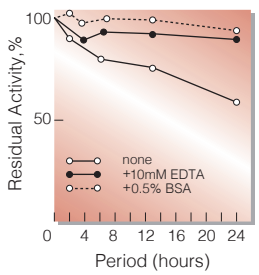

Fig.3. Stability (Liquid form at 37℃)

〔enzyme concentration: 1.0mg/ml
buffer composition: 0.1M K-phosphate buffer, pH7.5〕

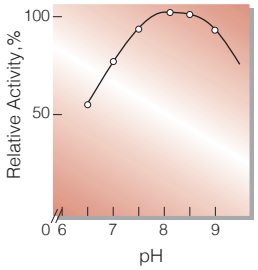

Fig.4. pH-Activity

〔37℃, in 50mM K-phosphate buffer〕

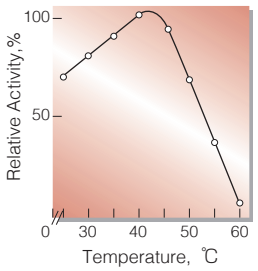

Fig.5. Temperature activity

〔in 50mM K-phosphate buffer, pH7.5〕

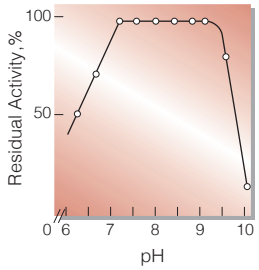

Fig.6. pH-Stability

〔30℃,2hr-treatment with 50mM buffer solution:pH6.0-9.0, K-phosphate; pH9.0-10.0, glycine-NaCl-NaOH.〕

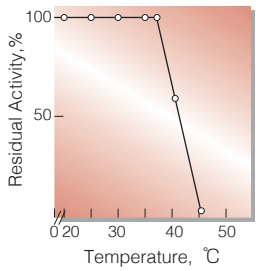

Fig.7. Thermal stability

〔15min-treatment with 50mM K-phosphate buffer, pH7.5〕

活性測定法（Japanese）

1.原理

Choline＋H_2O＋$2O_2$ $\xrightarrow{\text{choline oxidase}}$ Bataine＋$2H_2O_2$
$2H_2O_2$＋4-Aminoantipyrine＋phenol $\xrightarrow{\text{peroxidase}}$
Quinoneimine dye＋$4H_2O$
4-AminoantipyrineとPhenolの酸化縮合生成物であるQuinoneimine色素を500nm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. 2.1%塩化コリン溶液(2.1gの塩化コリンを0.1M Tris-HCl緩衝液,pH8.0で溶解し100mℓとする)
B. 1.0% 4-AA水溶液(1.0gの4-アミノアンチピリンを蒸留水に溶解して100mℓとする)(褐色瓶中で4℃保存)
C. 1.0%フェノール水溶液(1.0gのフェノールを蒸留水に溶解して100mℓとする)(褐色瓶中で4℃保存)
D. 0.1M Tris-HCl緩衝液, pH8.0〔12.1gのトリス(MW＝121.14)を約800mℓの蒸留水で溶解し,2.0N HClでpH8.0(25℃)に調製した後1000mℓにする〕

酵素溶液：酵素標品を予め氷冷した0.1M Tris-HCl緩衝液,pH8.0で溶解し,2.0mM EDTAと1.0%のKClを含む10mM Tris-HCl緩衝液,pH8.0で0.1～0.5 U/mℓに希釈する。

4.手順

①下記反応混液を調製する(褐色瓶にて氷冷保存)。

97.0mℓ	基質溶液	(A)
1.0mℓ	4-AA水溶液液	(B)
2.0mℓ	フェノール水溶液	(C)
5.0mℓ	peroxidase(110プルプロガリン単位/mg)	

②反応混液3.0mℓをキュベット(d＝1.0cm)にとり,37℃で約5分間予備加温する。
③酵素溶液0.05mℓを添加し, ゆるやかに混和し,反応混液を対照に37℃に制御された分光光度計で500nmの吸光度変化を3～4分間記録し,その初期直線部分から1分間あたりの吸光度変化を求める(ΔOD/min)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min \times 3.05(mℓ)}{12.0\times1/2\times1.0\times0.05(mℓ)} \times 希釈倍率$$
$$= \Delta OD/min \times 10.17 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

12.0 ： 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 ： 酸素反応で生成したH_2O_2の1分子のから形成するQuinoneimine色素は$^1/_2$分子である事による係数。
1.0 ： 光路長(cm)
C ： 溶解時の酵素濃度(c mg/mℓ)