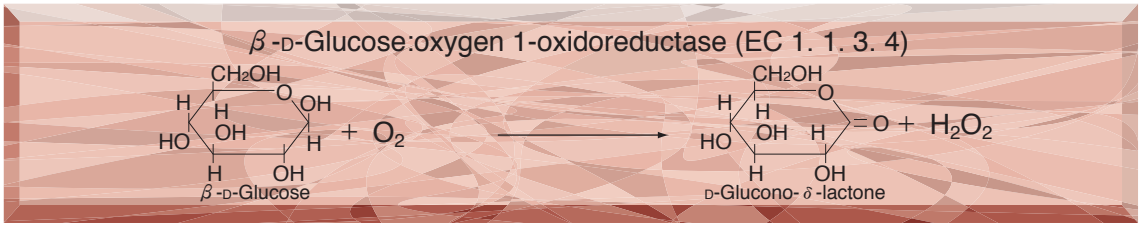
●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

GLUCOSE OXIDASE

from Aspergillus sp.



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized	
Activity	: Grade Ⅰ 180U/mg-solid or more	
	Grade Ⅱ 100U/mg-solid or more	
	(containing approx. 50% of stabilizers)	
Contaminant	: Catalase Grade Ⅰ $\leq 5.0\times10^{-3}$%	
	Grade Ⅱ ≤3.0%	
Stabilizers	: Potassium gluconate, sodium glutamate	

PROPERTIES [1,2]

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 153,000	
Michaelis constants	: 3.3×10^{-2}M (β-D-Glucose),[3]	
	6.1×10^{-2}M (2-Deoxyglucose)	
Structure	: Glycoprotein with 2 moles of FAD	
Inhibitors	: p-Chloromercuribenzoate,	
	heavy metal ions (Cu^{++}, Hg^{++}, Ag^{+})	
Optimum pH	: 4.5	(Fig.3)
Optimum temperature	: 40−50℃	(Fig.4)
pH Stability	: pH 4.5−6.0 (30℃, 20hr)	(Fig.5)
Thermal stability	: below 50℃ (pH 5.7, 1hr)	(Fig.6)
Substrate specificty	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS [4,5]

This enzyme is useful for enzymatic determination of glucose, and for amylase-activity assay when coupled with α-glucosidase (AGH-211, if maltooligosaccharide or modified starch is used as a substrate) in clinical analysis.

ASSAY

Principle:

β-D-Glucose + O_2 $\xrightarrow{\text{glucose oxidase}}$ D-Glucono-δ-lactone + H_2O_2

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

4-AA : 4-Aminoantipyrine

EHSPT : N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine

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. MES-Na buffer pH 5.7 : 0.1M [Dissolve 2.13g of 2-(N-morpholino) ethansulfonic acid (MW=213.25) in ca. 60ml of H_2O and, after adjusting the pH to 5.7 with 1N NaOH at 25℃, fill up to 100ml with H_2O] (Stable at 5℃ for one month)

B. Glucose solution : 15% [Dissolve 1.5g of β-D-glucose and fill up to 10ml with H_2O] for at least 2hrs before assay.) (Should prepare fresh)

C. 4-AA solution : 0.5% [50mg fo 4-aminoantipyrine (MW=203.25)/10ml of H_2O] (Stable at 5℃ in a brownish bottle for at least one week)

D. EHSPT solution : 40mM [118mg of N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (MW=295.3)/10ml of H_2O] (Stable at 5℃ in a brownish bottle for at least one week)

E. Peroxidase solution : 500U (purpurogalin unit)/ml of H_2O

F. Enzyme diluent : 10mM MES-Na buffer, pH 5.7, containing 0.1% Triton X-100

Procedure

1. Prepare the following working solution in a brownish bottle and store on ice.
   (Should be prepared fresh)

30 ml	Buffer solution	(A)
6 ml	Substrate solution	(B)
0.3ml	4-AA solution	(C)
0.3ml	EHSPT solution	(D)
0.3ml	POD solution	(E)

Concentration in assay mixture	
MES buffer	79 mM
D-Glucose	131 mM
4-AA	0.2mM
EHSPT	0.3mM
POD	ca.4 U/ml

2. Pipette 3.0ml of working solution into a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.
3. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
4. Record the increase in optical density at 555nm against water for 2 to 3 minutes in a spectrophotometer thermostated at 37℃, and calculate the ΔOD per minute from the initial linear portion of the curve (ΔOD test).
   At the same time, measure the blank rate (ΔOD blank) by using the same method as the test except that enzyme diluent (F) is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice cold enzyme diluent (F) and dilute to 0.05−0.2U/ml with the same buffer, immediately before the assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min}\ (\Delta\text{OD test} - \Delta\text{OD blank}) \times \text{Vt} \times \text{df}}{32.8 \times 1/2 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 1.89 \times \text{df}$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.1ml)

Vs : Sample volume (0.1ml)

32.8 : Millimolar extinction coefficient of quinoneimine dye under the assay conditions (㎤/micromole)

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

1.0 : Light path length (cm)

df : Dilution factor

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

REFERENCES

1) *The Enzymes, Vol.XIIB,* P.421 (P.D.Boyer, ed.), Academic Press (1975).

2) *Method in Enzymology, Vol.IX,* p.82 (S.P.Colowick and N.O.Kaplan, ed.),Academic Press (1966).

3) B.E.P.Swoboda and V.Massay; *J.Biol.Chem., 240,* 2209 (1965).

4) P.J.Auses, S.L.Cook and J.T.Maloy; *Anal.Chem., 47,* 244 (1975).

5) D.C.Williams, G.F.Huff and W.R.Gaitz; *Clin.Chem., 22,* 372 (1976).

Table 1. Substrate Specificity of Glucose oxidase
[0.1M of Substrate, 79mM MES buffer, pH 5.7, at 30℃]

Substrate (0.1M)	Relative activity(%)	Substrate (0.1M)	Relative activity(%)
D-Glucose	100	Fructose	0.24
2-Dexy-D-glucose	16.2	Xylose	0.93
Glucono-1,5-lactone	0.06	Ribose	0.00
L-Glucose	0.00	Maltose	0.69
Galactose	3.10	Lactose	0.00
Mannose	2.10		

Table 2. Effect of Various Chemicals on Glucose oxidase
[The enzyme dissolved in 0.1M MES buffer, pH 5.7 (10U/ml) was incubated with each chemical for 2hr at 25℃.]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	NaN_3	20	96.3
Metal salt	2.0		EDTA	5.0	97.3
$MgCl_2$		92.6	o-Phenanthroline	2.0	95.3
$CaCl_2$		93.6	α,α'-Dipyridyl	2.0	99.5
$BaCl_2$		94.4	Borate	50.0	96.1
$CoCl_2$		98.1	IAA	2.0	96.1
$MnCl_2$		95.1	MIA	2.0	101.1
$ZnSO_2$		94.3	Hydroxylamine	10.0	98.3
$FeCl_2$		96.8	Sodium bisulfite	10.0	100.0
$NiCl_2$		91.7	hydrazine	10.0	103.1
$CuSO_4$		71.6	Triton X-100	0.1%	111.2
$AgNO_3$		58.6	Brij 35	0.1%	108.0
$HgCl_2$		0.7	Tween 20	0.1%	110.7
PCMB	2.0	31.6	Span 20	0.1%	106.7
MIA	2.0	96.8	Na-Cholate	0.1%	106.1
NaF	2.0	97.1	SDS	0.1%	113.1

PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediamimetetraacetate;
IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate.

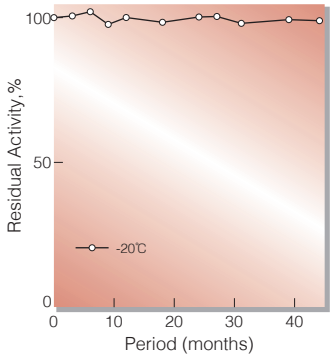


Fig.1. Stability (Powder form)
〔 kept under dry conditions 〕

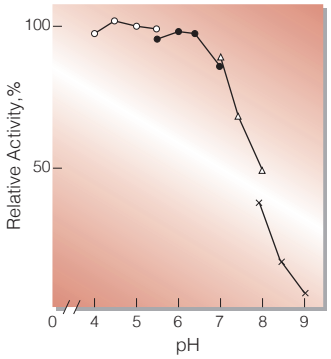


Fig.3. pH-Activity
〔37℃,5min-reaction in 79mM buffer solution : ○—○ . acetate: ●—● MES;△—△, BES;×—×, BICINE〕

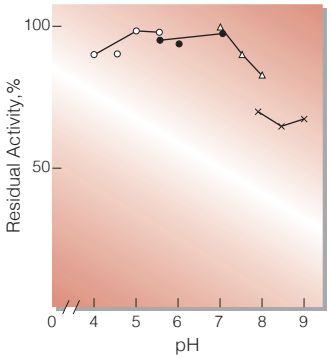


Fig.5. pH-Stability
〔30℃,20hr-treatment with 0.1M buffer solution : ○—○ . acetate : ●—● MES : △—△, BES : ×—×, BICINE〕

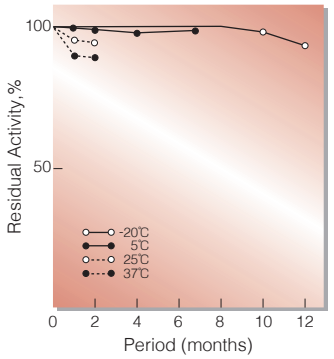


Fig.2. Stability (Powder form)
〔 kept under dry conditions 〕

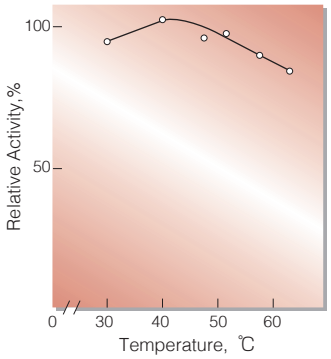


Fig.4. Temperature activity
〔5min-reaction in 79mM MES buffer, pH5.7〕

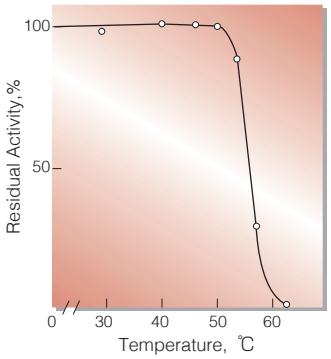


Fig.6. Thermal stability
〔1hr-treatment in 79mM MES buffer , pH5.7 〕

活性測定法（Japanese）

1.原理

β-D-Glucose＋O_2 $\xrightarrow{\text{glucose oxidase}}$ D-Glucono-δ lactone＋H_2O_2

$2H_2O_2$＋4-AA＋EHSPT(TOOS) $\xrightarrow{\text{POD}}$ Quinoneimine dye＋$4H_2O$ 4-AminoantipyrineとEHSPTの酸化縮合生成物である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，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. 0.1M MES-Na緩衝液，pH5.7 (5℃保存で1カ月間使用可能)
B. 15%グルコース溶液 (使用前に2時間以上放置する)(用時調製)
C. 0.5% 4-AA溶液 (褐色瓶中5℃保存で1週間安定使用可能)
D. 40mM EHSPT (TOOS)溶液 (褐色瓶中5℃保存で1週間安定使用可能)
E. 500PU/mℓ POD溶液

酵素溶液：酵素標品を予め氷冷した0.1% Triton X-100を含む10mM MES-Na緩衝液 (A)で溶解し,分析直前に同緩衝液で0.05～0.2U/mℓに希釈する。

4.手順

①褐色瓶中に下記反応混液を調製し氷冷保存する。
(用時調製)

30 mℓ	MES-Na緩衝液	(A)
6 mℓ	基質溶液	(B)
0.3mℓ	4-AA水溶液	(C)
0.3mℓ	EHSPT(TOOS)水溶液	(D)
0.3mℓ	POD水溶液	(E)

(褐色瓶にて氷冷保存)

②反応混液3.0mℓをキュベット(d＝1.0cm)に分注し37℃で約5分間予備加温する。
③酵素溶液0.1mℓを添加しゆるやかに混和後,水を対照に37℃に制御された分光光度計で555nmの吸光度変化を2～3分間記録し，その直線部分から1分間あたりの吸光度変化を求める(ΔOD test)。
④盲検は反応混液①に酵素液の代りに酵素希釈液〔MES-Na緩衝液(A)〕0.1mℓを加え，上記同様に操作を行い，1分間当りの吸光度変化を求める(ΔOD blank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.1 \times df}{32.8 \times 1/2 \times 1.0 \times 0.1}$$
$$= \Delta OD/min \times 1.89 \times df$$

U/mg ＝U/mℓ×1/C

32.8 ：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)