●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

D-FRUCTOSE DEHYDROGENASE

from Gluconobacter sp.

D-Fructose:(acceptor) 5-oxidoreductase (EC 1.1.99.11)[1~2)]

D-Fructose + Acceptor ⟶ 5-Keto-D-fructose + Acceptor(red)

PREPARATION and SPECIFICATION

Appearance	: Red-yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 20U/mg-solid or more (containing approx. 80% of stabilizers)
Stabilizers	: Sugars, amino acids, BSA

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 140,000 (by gel filtration)	
Isoelectric point	: 5.0±0.1	
Michaelis constant	: 5×10^{-3}M (D-Fructose)	
Inhibitors	: Ag^{+}, Hg^{++}, SDS	
Optimum pH	: 4.0	(Fig.2)
Optimum temperature	: 37℃	(Fig.3)
pH Stability	: pH 4.0−6.0 (25℃, 16hr)	(Fig.4)
Thermal stability	: below 40℃ (pH 4.5, 15min)	(Fig.5)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS [3)]

This enzyme is useful for enzymatic determination of D-fructose in clinical analysis.

ASSAY

Principle:

D-Fructose + 2Potassium ferricyanide $\xrightarrow{\text{D-fructose dehydrogenase}}$ 5-Keto-D-fructose + 2Potassium ferrocyanide

2Potassium ferrocyanide + Ferric sulfate ⟶ 2Prussian blue + $3K_2SO_4$

Prussian blue : $KFe^{III}[Fe^{II}(CN)_6]$

The appearance of prussian blue formed by chelate reaction is measured at 660nm by spectrophotometry.

Unit definition:

One unit causes the oxidation of one micromole of D-fructose (the formation of two micromoles of prussian blue) per minute under the conditions described below.

Method:

Reagents

A. McIlvaine buffer, pH 4.5 : Prepare by mixing of 0.1M citric acid and 0.2M disodium phosphate, at 25°C

B. D-Fructose solution : 1.0M (1.80g D-fructose (MW=180.16)/10ml McIlvaine buffer (A) contg. 0.1% Triton X-100)

C. Potassium ferricyanide solution : 0.1M (0.33g potassium ferricyanide (MW=329.25)/10ml McIlvaine buffer (A) contg. 0.1% Triton X-100)

D. Ferric sulfate-SDS solution : 5.0g $Fe_2(SO_4)_3 \cdot H_2O$, 3.0g SDS (sodium dodecyl sulfate), 95ml 85% phosphoric acid/1,000ml of H_2O

E. Enzyme diluent : McIlvaine buffer (A) contg. 0.1% Triton X-100 and 0.05% BSA

Procedure

Concentration in assay mixture	
McIlvaine buffer	×1
Triton X-100	0.1%
D-Fructose	0.1M
Potassium ferricyanide	10mM

1. Pipette 0.7ml of Reagent E, 0.1ml of Reagent B and 0.1ml of the enzyme solution* into a test tube and equilibrate at 37℃ for about 5 minutes.
2. Add 0.1ml of Raegent C and mix.
3. After exactly 5 minutes at 37℃, add 0.5ml of Reagent D to stop the reaction, and then incubate at 37℃ for further 20 minutes.
4. Add 3.5ml of distilled water and measure the optical density at 660nm against water (OD test).
   At the same time, prepare the blank by using the same method as the test except that Reagent E (0.1ml) is used instead of the Reagent B (OD blank).

* Dissolve the enzyme preparation in ice-cold enzyme diluent and dilute to 1.0−3.0U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD\ (OD\ test - OD\ blank) \times Vt \times df}{2.0 \times 2 \times t \times 1.0 \times Vs} = \Delta OD \times 2.5 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (5.0ml)

Vs : Sample volume (0.1ml)

2.0 : Millimolar extinction coefficient of prussian blue under the assay conditions (㎠/micromole)

2 : Factor based on the fact that oxidation of one mole of D-fructose produces two moles of prussian blue

t : Reaction time (5 minutes)

1.0 : Light path length (cm)

df : Dilution factor

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

REFERENCES

1) M.Ameyama, E.Shinagawa, K.Matsushita and O.Adachi; *J.Bacteriol., 145,* 814 (1981).

2) M.Ameyama; *Methods in Enzymology,* vol.89, p.20 (1982).

3) K.Nakashima, H.Takei, O.Adachi, E.Shinagawa and M.Ameyama; *Clinica Chimica Acta, 151,* 307 (1985).

Table 1. Substrate Specificity of D-Fructose dehydrogenase

Substrate	Concn.(mM)	Relative activity(%)
D-Fructose	100	100
D-Galactose	100	0.1
D-Glucose	100	0.1
D-Mannose	100	0.4
L-Sorbose	100	0
D-Arabinose	100	0.3
D-Xylose	100	0.2
D-Ribose	100	0.2
D-Rhamnose	100	0
Sucrose	100	0
Lactose	20	0
Maltose	100	0
Raffinose	10	0
D-Sorbitol	100	0

Substrate	Concn.(mM)	Relative activity(%)
D-Mannitol	100	0
D-Xylitol	100	0
Glucose-1-phosphate	20	0
Fructose-6-phosphate	12.5	0
Fructose-1.6-diphosphate	12.5	0
Glycerol	100	0
D-Glyceraldehyde	20	0
D-Dihydroxyacetone	100	0.1
Ethanol	100	0
Malic acid	100	0
3α -Hydroxy-n-butyric acid	100	0
Choline chloride	100	0.1
Potassium gluconate	100	19

Table 2. Effect of Various Chemicals on D-Fructose dehydrogenase

[The enzyme dissolved in McIlvaine buffer, pH 4.5(3U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		96
$CaCl_2$		98
$Ba(OAc)_2$		98
$FeCl_3$		88
$CoCl_2$		95
$MnCl_2$		80
$ZnSO_4$		91
$Cb(OAc)_2$		82
$NiCl_2$		93
$CuSO_4$		92
$Pb(OAc)_2$		82
$AgNO_3$		0.20
$HgCl_2$		0.07

Chemical	Concn.(mM)	Residual activity(%)
NaF	2.0	96
NaN_3	2.0	88
EDTA	4.0	81
o-Phenanthroline	2.0	88
α,α'-Dipyridyl	1.5	83
Borate	40	89
IAA	2.0	95
NEM	2.0	92
Hydroxylamine	2.0	88
PCMB	1.5	87
MIA	2.0	91
Triton X-100	0.10%	89
Brij 35	0.10%	98
Na-cholate	0.10%	101
SDS	0.05%	6.5
DAC	0.05%	69

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

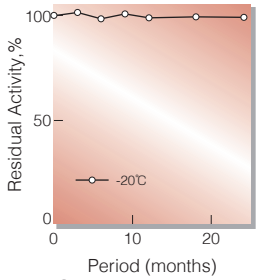

Fig.1. Stability (Powder form)
〔kept under dry conditions〕

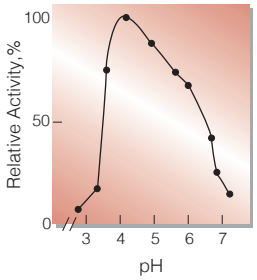

Fig.2. pH-Activity
〔37℃,5min-reaction in McIlvaine buffer solution〕

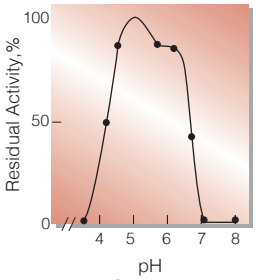

Fig.4. pH-Stability
〔25℃,16hr-treatment with McIlvaine buffer solution〕

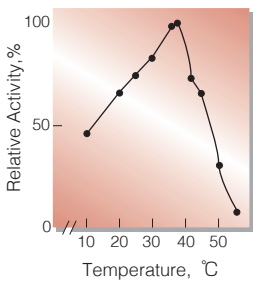

Fig.3. Temperature activity
〔in McIlvaine buffer,pH4.5〕

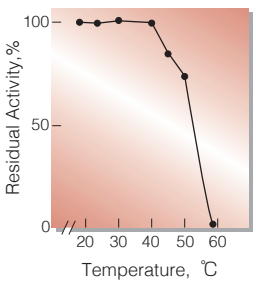

Fig.5. Thermal stability
〔15min-treatment with McIlvaine buffer pH4.5. enzyme concn. :3U/ml〕

活性測定法（Japanese）

1.原理

D-Fructose＋2Potassium ferricyanide
$\xrightarrow{\text{D-fructose dehydrogenase}}$ 5-Keto-D-fructose＋2Potassium ferrocyanidee
2 Potassium ferrocyanide＋Ferric sulfate ⟶
2 Prussian blue＋$3K_2SO_4$
Prussian blue : $KFe^{III}[Fe^{II}(CN)_6]$
プルシャンブルーの生成量を660nmの吸光度で測定する。

2.定義

下記条件下で1分間に1マイクロモルのD-フラクトースが酸化される(2マイクロモルのプルシャンブルーが生成される)酵素量を1単位 (U)とする。

3.試薬

A. McⅠlvaine緩衝液,pH4.5:0.1Mのクエン酸と0.2Mリン酸ナトリウムを混合して,pHを4.5に調整する。
B. 1.0M D-フラクトース溶液:1.80gのD-フラクトース(MW＝180.16)を0.1%トリトンX-100を含むMcⅠlvaine緩衝液 (A)に溶解し,10mℓとする。
C. 0.1Mフェリシアン化カリ溶液:0.33gのフェリシアン化カリ(MW＝329.25)を0.1%トリトンX-100を含むMcⅠlvaine緩衝液 (A)に溶解し,10mℓとする。
D. 硫酸第二鉄-SDS溶液:5.0gの硫酸第二鉄・xH_2O,3.0gのSDS(sodium dodecyl sulfate)及び,95mℓの85%リン酸を蒸留水に溶解し1,000mℓとする。
E. 酵素希釈液:0.1%トリトンX-100と0.05%のBSAを含むMcⅠlvaine緩衝液 (A)。

酵素溶液：酵素標品を予め氷冷した酵素希釈液 (E)で溶解し,分析直前に同希釈液で1.0～3.0 U/mℓに希釈する。

4.手順

①試験管に試薬E 0.7mℓ,試薬B 0.1mℓ,酵素溶液0.1mℓを採り,37℃で約５分間予備加温する。
②試薬Cを0.1mℓを加えて，反応を開始する。
③37℃で正確に5分間反応させた後,試薬Dを0.5mℓ加えて反応を停止させ,37℃で更に20分間静置する。
④蒸留水3.5mℓを加え,水と対照にして660nmの吸光度を測定する(ODtest)。
⑤盲検は試薬Bの代わりに試薬E (0.1mℓ)を加え,上記同様に操作を行って吸光度を測定する(ODblank)。

5.計算式

$$U/m\ell = \frac{\Delta OD\ (OD\ test - OD\ blank) \times 5.0(m\ell) \times 希釈倍率}{2.0 \times 2 \times 5(分) \times 1.0 \times 0.1(m\ell)}$$
$$= \Delta OD \times 2.5 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C
2.0 ： プルシャンブルーの上記測定条件でのミリモル分子吸光係数(cm²/micromole)
2 ： 1モルのD-フラクトースの酸化から生成するプルシャンブルーは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)