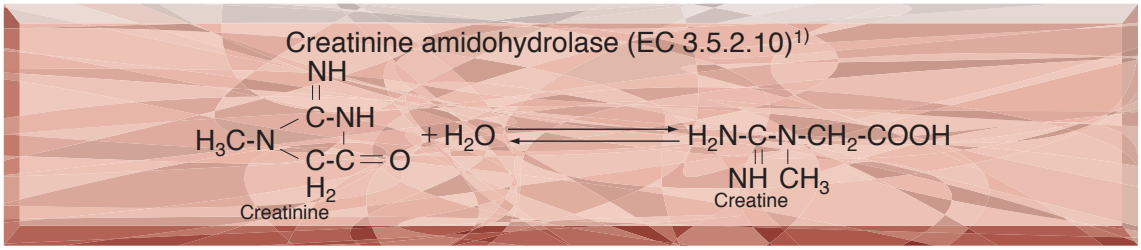
●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

CREATININE AMIDOHYDROLASE

from Microorganism



PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅱ(−211) 450U/mg-solid or more
	Grade Ⅲ(−311) 150U/mg-solid or more
Contaminants	: NADH oxidase ≤5.0×10^{-2}%
	Catalase ≤2.0%
Stabilizers	: Sucrose, BSA

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1,2)
Molecular weight	: approx. 175,000 [1)]	
Isoelectric point	: 4.7 [3)]	
Michaelis constants	: 3.2×10^{-2}M (Creatinine), 5.7×10^{-2}M (Creatine)	
Structure	: 6 subunits per enzyme molecule (One zinc is bound to each subunit) [3)]	
Inhibitors	: Ag^{+}, Hg^{++}, N-bromosuccinimide, EDTA	
Optimum pH	: 6.5−7.5	(Fig.5)
Optimum temperature	: 70℃	(Fig.6)
pH Stability	: pH 7.5−9.0 (5℃, 16hr)	(Fig.7)
Thermal stability	: below 70℃ (pH 7.5, 30min)	(Fig.8)
Effect of various metals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of creatinine when coupled with creatine amidinohydrolase (CRH-211, CRH-221, CRH-229) and sarcosine oxidase (SAO-351) in clinical analysis.

ASSAY

Principle:

Creatine $\xrightarrow{\text{creatinine amidohydrolase}}$ Creatinine + H_2O

Creatinine + Picric acid $\xrightarrow{OH^-}$ Orange dye

The appearance of creatinine-picrate (orange dye based on Jaffe's reaction) is measured at 520nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of orange dye per minute under the conditions described below.

Method:

Reagents

A. Creatine solution : 0.1M [1.49g creatine (Nacalai tesque)/100ml of 50mM phosphate buffer, pH 7.5] (Should be prepared fresh)

B. NaOH solution : 0.5N

C. Picric acid solution : 1.0% (W/V)

D. Enzyme diluent : 50mM phosphate buffer, pH 7.5

Procedure

Concentration in assay mixture	
Phosphate buffer	45mM
Creatine	90mM

1. Pipette 1.0ml of the substrate solution (A) into a test tube and equilibrate at 37℃ for about 5 minutes.
2. Add 0.1ml of the enzyme solution* and mix.
3. After exactly 10 minutes at 37℃, immediately transfer an aliquot (0.1ml)(1/11 volume) of the reaction solution to 2.0ml of NaOH solution (B).
4. Add 1.0ml of picric acid solution (C) and incubate at 25℃ for 20 minutes.
5. Measure the optical density at 520nm against water (OD test).

   At the same time, prepare the blank by transferring an aliquot (0.1ml) of the reaction solution into NaOH solution (2.0ml) just after the addition of the enzyme solution into ice-cold substrate solution, and carry out the same procedure as the test (procedure 4 and 5)(OD blank).

* Dissolve the enzyme preparation in ice-cold 50mM phosphate buffer, pH 7.5 and dilute to 1.8–2.4U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD\ (OD\ test - OD\ blank) \times Vt \times 11 \times df}{4.65 \times 1.0 \times t \times Vs} = \Delta OD \times 7.33 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.1ml)

Vs : Sample volume (0.1ml)

4.65 : Millimolar extinction coefficient of creatinine-picrate (orange dye)(㎤/micromole)

11 : Volume of reaction solution (1.1ml)/Sampling volume (0.1ml)

1.0 : Light path length (cm)

t : Reaction time (10 minutes)

df : Dilution factor of enzyme solution

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

REFERENCES

1) D.Tsuru; *Nucleic Acid and Amino Acids, 35,* 31 (1977).

2) D.Tsuru; *Rinsho Kensa, 22,* 1331 (1978).

3) K.Rikitake, I.Oka, M.Ando, T.Yoshimoto and D.Tsuru; *J.Biochem., 86,* 1109 (1979).

4) K.Yamamoto, M.Oka, T.Kikuchi and S.Emi; *Biosci. Biotech. Biochem., 59 (7),* 1331 (1995).

Table 1. Effect of Various Metals on Creatinine amidohydrolase
[The enzyme dissolved in 50mM K-phosphate buffer, pH 7.5 was incubated with each metal (0.2mM) for 30 minutes at 25℃.]

Metal	Residual activity(%)	Metal	Residual activity(%)
None	100	$MgCl_2$	82.8
$FeSO_4$	91.0	$NiCl_2$	82.8
$FeCl_3$	93.0	$CoSO_4$	78.5
$HgCl_2$	1.4	$BaCl_2$	71.2
$Zn(OAc)_2$	79.9	$Pb(OAc)_2$	88.0
$Cu(OAc)_2$	22.7	$AgNO_3$	0
$Ca(OAc)_2$	96.8	$CdCl_2$	80.0
$MnCl_2$	94.0		

Ac, CH_3CO.

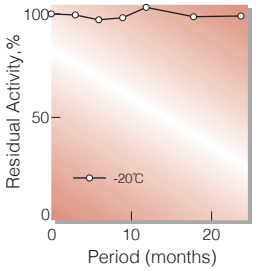


Fig.1. Stability (CNH-211) (Powder form)
〔kept under dry conditions〕

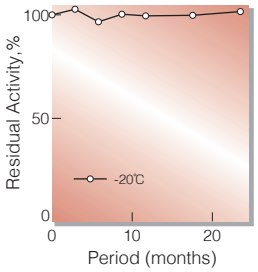


Fig.2. Stability (CNH-311) (Powder form)
〔kept under dry conditions〕

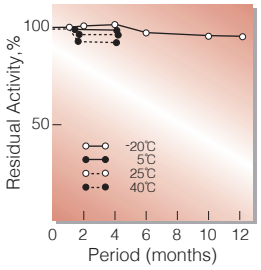


Fig.3. Stability (Powder form)
〔kept under dry conditions〕

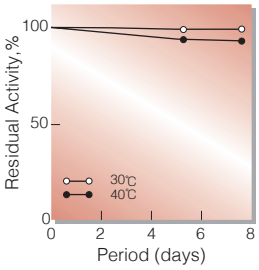


Fig.4. Stability (Liquid form)
〔in 50 mM Tris-HCl buffer solution pH7.5〕

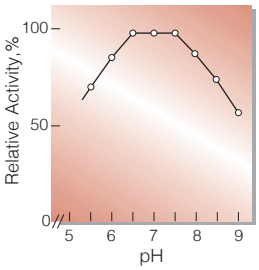


Fig.5. pH-Activity
〔37℃, 10min-reaction in 50mM buffer solution: pH5.5, acetate;pH6.0-8.0,phosphate; pH8.5-9.0,carbonate〕

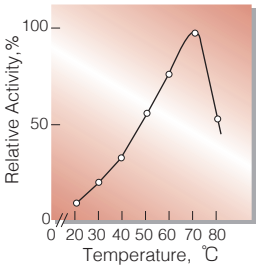


Fig.6. Temperature activity
〔10min-reaction in 50mM phosphate buffer, pH7.4〕

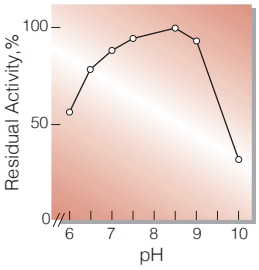


Fig.7. pH-Stability
〔25℃,16hr-treatment with 50mM buffer solution: pH6.0-8.0, phosphate; pH8.5-9.0, carbonate〕

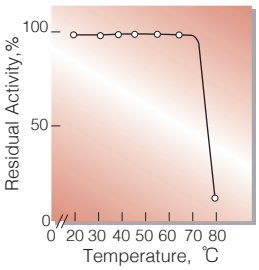


Fig.8. Thermal stability
〔30min-treatment with 50mM phosphate buffer, pH7.4〕

活性測定法（Japanese）

1.原理

Creatine $\xrightarrow{\text{creatinine amidohydrolase}}$ Creatinine＋H_2O

Creatinine＋Picric acid $\xrightarrow{OH^-}$ Orange dye

生成したクレアチニンをアルカリ性下ピクリン酸と反応させ生じた橙色色素を比色定量する。

2.定義

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

3.試薬

A. 0.1Mクレアチン溶液〔1.49gのクレアチン(ナカライテスク製)を50mMリン酸緩衝液 pH7.5に溶解し, 100mℓとする〕(用時調製)

B. 0.5N NaOH溶液(2.0gのNaOHを蒸留水に溶解し100mℓとする)

C. 1.0%ピクリン酸溶液(1.0gのピクリン酸を蒸留水に溶解し100mℓとする)

酵素溶液：酵素標品を予め氷冷し50mMリン酸緩衝液,pH7.5で溶解し,分析直前に同緩衝液で1.8～2.4U/mℓに希釈する。

4.手順

①試験管に基質溶液(A)1.0mℓを採り,37℃で約5分間予備加温する。

②酵素溶液0.1mℓを加え,反応を開始する。

③37℃で正確に10分間反応させた後,直ちに反応液0.1mℓ(1/11容量)を採り,予め準備したNaOH溶液(B)2.0mℓ中へ加える。

④ピクリン酸溶液(C)を1.0mℓ加え,25℃で20分間放置後,520nmにおける吸光度を測定する (ODtest)。

⑤盲検は氷冷基質溶液に酵素溶液を添加後直ちに反応液の0.1mℓを採り,NaOH溶液(2.0mℓ)の中へ加えて調製する。以下手順④を操作して吸光度を測定する(ODblank)。

5.計算式

$$U/m\ell = \frac{\Delta OD\ (OD\ test - OD\ blank) \times 3.1(m\ell) \times 11 \times 希釈倍率}{4.65 \times 1.0 \times 10(分) \times 0.1(m\ell)}$$

$$= \Delta OD \times 7.33 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

4.65 ： Creatinine-picrate(Orange dye)のミリモル分子吸光係数 (cm^2/micromole)

11 ： 反応液容量(1.1mℓ)/採取液量(0.1mℓ)

1.0 ： 光路長(cm)

C ： 溶解時の酵素濃度(c mg/mℓ)