●TOYOBO ENZYMES●

(Biochemical Reagent Grade)

PROLINE SPECIFIC ENDOPEPTIDASE

from Flavobacterium sp.

Prolyl endopeptidase (EC 3.4.21.26)

X-Prolyl-peptide + H_2O ⟶ X-Proline + Peptide

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅰ 5.0U/mg-solid or more
Contaminants	: Leucine aminopeptidase ≤1.0×10^{-1}%
	Trypsin-like activity ≤1.0×10^{-1}%

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 78,000	
Isoelectric point	: 9.1	
Michaelis constants	: 2.5×10^{-5}M (Z-Gly-Pro-MCA)	
	1.4×10^{-4}M (Z-Gly-Pro-2NNap)	
Structure	: Monomer	
Inhibitors	: DFP, 3, 4-dichloroisocoumarin, Z-Gly-Pro-CH_2Cl	
Optimum pH	: 6.5	(Fig.2)
Optimum temperature	: 37℃ (40℃)[2]	(Fig.3)
pH Stability	: 5.5−8.5 (30℃,15hr)	(Fig.4)
Thermal stability	: below 40℃ (pH7.0,10min)	(Fig.5)
Substrate specificity	: Y-Pro(Ala)-X (Y, peptide or N-protected amino acid; X, amino acid, peptide, amide, or ester)(Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

This enzyme is useful for the determination of amino acid sequences of peptides and proteins containing proline residues.

ASSAY

Principle:

Carbobenzoxy-Gly-Pro-p-nitroanilide(Z-Gly-Pro-pNA)＋H_2O $\xrightarrow{\text{proline specific endopeptidase}}$ Z-Gly-Pro＋p-Nitroaniline

The appearance of p-nitroaniline is measured at 410nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of p-nitroaniline per minute under the conditions described below.

Method:

Reagents

A. K-Phosphate buffer, pH 7.0 : 0.1M

B. Z-Gly-Pro-pNA solution : 5mM [Dissolve 21.3mg of Z-Gly-Pro-pNA(MW＝426.43) in ca.8ml of 40% dioxane in a hot bath at 60℃, then cool down to 25℃, fill up to 10ml with 40% dioxane] (Should be prepared fresh)

C. Acetate buffer, pH 4.0 : 1M solution containing 10% of TritonX-100 (Store at 5℃)

D. Enzyme diluent : 50mM K-phosphate buffer, pH 7.0

Procedure

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

1.0 ml	K-Phosphate buffer	(A)
0.25ml	Substrate solution	(B)

Concentration in assay mixture	
Phosphate buffer	77.8 mM
Z-Gly-Pro-pNA	0.926mM

2. Add 0.1ml of enzyme solution* and mix by gentle inversion.
3. After exactly 5 minutes at 30℃, add 2.0ml of acetate buffer (C) to stop the reaction and measure the optical density at 410nm against water (OD test).

   At the same time, prepare the blank by first mixing the reaction mixture with 2.0ml of acetate buffer (C) after 5min-incubation at 30℃, followed by the addition of the enzyme solution (OD blank).

* Immediately before assay,dissolve the enzyme preparation in ice-cold enzyme diluent (D) and dilute to 0.05－0.2U/ml with the same buffer.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD (OD test}-\text{OD blank)}\times Vt\times df}{5.57\times 1.0\times t\times Vs} = \Delta\text{OD}\times 1.20\times df$$

Weight activity (U/mg) ＝ (U/ml)×1/C

Vt : Total volume (3.35ml)

Vs : Sample volume (0.1ml)

5.57 : Millimolar extinction coefficient of p-nitroaniline under the assay condition (㎠/micromole)

1.0 : Light path length (cm)

t : Reaction time (5 minutes)

df : Dilution factor

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

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Table 1. Substrate Specificity of Proline specific endopeptidase

Substrate ↓	Km (mM)	kcat (s^{-1})	kcat/Km (mM^{-1}・S^{-1})
Pro-2NNap		not hydrolyzed	
Z-Pro-2NNap		not hydrolyzed	
Gly-Pro-2NNap		not hydrolyzed	
Z-Gly-Pro-MCA	0.025	115	4600
Z-Gly-Pro-2NNap	0.14	169	1212
Z-Gly-Pro-pNP	0.125	102	816
Z-Ala-Pro-2NNap	0.08	642	834
Z-D-Pro-2NNap	0.20	0.142	0.73
Ala-			
Z-Ala-Gly-Pro-2NNap	0.29	192	664
Z-D-Ala-Gly-Pro-2NNap	0.14	38	271
Z-Gly-Pro-Leu	0.22	23	104
Z-Gly-Pro-Phe	0.74	180	250
Z-Gly-Pro-ALa	0.39	240	620
Z-Gly-Pro-D-Ala		not hydrolyzed	
Z-Gly-Pro-Leu-Gly	0.32	520	1600
Z-Gly-Pro-Leu-Ala	0.42	520	1100
Z-Gly-Pro-Leu-D-Ala	1.5	1600	1070
Z-Gly-Pro-Leu-Gly-Gly	1.4	700	500
Z-Gly-Pro-Leu-Gly-Ala	1.82	1000	550

Z, Carbobenzoxy; MCA, 4-Methyl-coumaryl-7-amide; 2NNap, β-Naphthylamide; pNP, p-Nitrophenyl ester.

Table 2. Effect of Various Chemicals on Proline specific endopeptidase
[The enzyme dissolved in 50mM K-phosphate buffer, pH 7.0 (2.5U/ml) was incubated with each chemical at 25 ℃ for 30min.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		72.1
$CaCl_2$		74.6
$BaCl_2$		74.3
$FeCl_3$		52.6
$CoCl_2$		62.7
$MnCl_2$		68.9
$ZnSO_4$		56.8
$Cd(OAc)_2$		31.9
$NiCl_2$		66.8
$CuSO_4$		45.2
$Pb(OAc)_2$		59.8
$AgNO_3$		55.7
$HgCl_2$		0
PCMB	2.0	71.9
MIA	2.0	78.1
NaF	2.0	76.9
NaN_3	20.0	77.3
DFP	1.0	2.7
o-Phenanthroline	2.0	84.9
α,α′-Dipyridyl	1.0	84.9
Borate	50	73.4
IAA	2.0	62.8
NEM	2.0	74.4
Hydroxylamine	2.0	77.4
3,4-Dichloroiso-		
coumarin	2.0	9.0
Triton X-100	0.10%	86.4
Brij 35	0.10%	84.8
Tween 20	0.10%	84.1
Span 20	0.10%	81.9
Na-cholate	0.10%	84.1
SDS	0.05%	73.0

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; DFP, Diisopropylphosphorofluoridate; IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate.

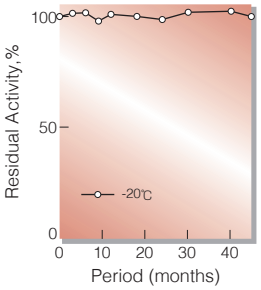

Fig.1. Stability (Powder form)
〔kept under dry conditions〕

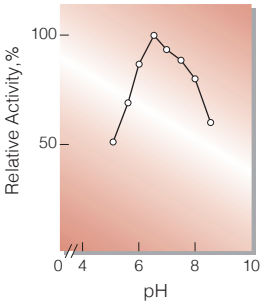

Fig.2. pH-Activity
〔30℃ in 50mM phosphate buffer〕

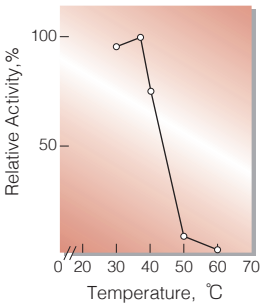

Fig.3. Temperature activity
〔in 50mM phosphate buffer, pH 7.0〕

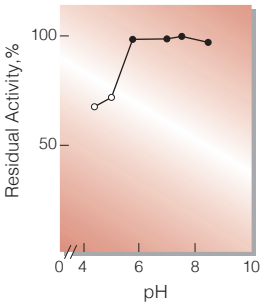

Fig.4. pH-Stability
〔30℃, 15hr-treatment with buffer solution: pH4.5-7.0, 50mM acetate;pH6.0-8.5, 0.1M phosphate〕

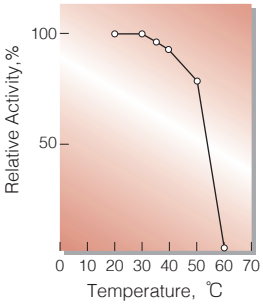

Fig.5. Thermal stability
〔10min-treatment with 0.1M phosphate buffer, pH7.0 enzyme concn.:5U/ml〕

活性測定法（Japanese）

1.原理

Carbobenzoxy-Gly-Pro-p-nitroanilide(Z-Gly-Pro-pNA)＋H_2O $\xrightarrow{\text{proline specific endopeptidase}}$ Z-Gly-Pro＋p-Nitroaniline

p-Nitroanilineの生成量を410nmにおける吸光度の変化で測定する。

2.定義

下記条件で1分間に1マイクロモルのp-Nitroanilineを生成する酵素量を1単位(U)とする。

3.試薬

A. 0.1M K-Phosphate緩衝液，pH7.0
B. 5mM Z-Gly-Pro-pNA溶液〔21.3mgのZ-Gly-Pro-pNA(MW＝426.43)を60℃の湯浴中で約8mlの40%ジオキサン蒸留水に溶解する。本溶解液を25℃まで冷却した後,40%ジオキサンで10mlとする〕(用時調製)
C. 10% トリトンX-100を含む1M酢酸緩衝液，pH4.0
D. 酵素希釈液〔50mM K-Phosphate緩衝液，pH7.0〕

酵素溶液：酵素標品を予め氷冷した酵素希釈液(D)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。

1.0 ml	K-Phosphate緩衝液	(A)
0.25ml	基質溶液	(B)

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，酢酸緩衝液(C)2.0mlを加えて反応を停止させる。この液につき410nm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分間加温後,酢酸緩衝液(C)2.0mlを加えて混和し,次いで酵素溶液0.1mlを加えて調製する。この液につき以下上記同様に吸光度を測定する(ODblank)。

5.計算式

$$U/ml = \frac{\Delta OD\ (OD\ test - OD\ blank) \times 3.35(ml) \times 希釈倍率}{5.57 \times 1.0 \times 5(分) \times 0.1}$$

$$= \Delta OD \times 1.20 \times 希釈倍率$$

U/mg ＝U/ml×1/C

5.57 ：p-Nitroanilineのミリモル分子吸光係数(㎠/micromole)
1.0 ：光路長(cm)
C ：溶解時の酵素濃度(c mg/ml)