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27669/27671 Collagenase Chromophore-Substrate Kit (for quantitative Collagenase-Determination)

Components:
Component A (Fluka 27667):
Collagenase Chromophore-Substrate: 4-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH dehydrate
Molecular Weight 812.91 g/mol
CAS Number 118081-33-7

Component B (Fluka 27668):
Standard: 4-Phenylazobenzyloxycarbonyl-Pro-Leu-OH
Molecular Weight 466.53 g/mol
CAS Number 98640-71-2

Enzymatic Assay of Collagenase

A specific and highly sensitive assay can readily be performed by means of the synthetic substrate.

Compound A is specifically cleaved by collagenase between the leucine and glycine residues. Digestian of the caloured hydrophylic substräte leads to the colaured fragment PAZ-Pro-Leu-OH (II) and the colourless tripeptide H-GJy-Pro-D-Arg-OH (III)[2]:

The orange-yellow acylpeptide PAZ-Pro-Leu-OH (II) 19 extremely soluble in organic solvents such as ethyl acetate or benzene and thus can be quantitatively extracted from the incubated mixture previously acidified with citric acid. The undigested substrate I remains in the acidic aqueous phase. The quantitative determination of the coloured fragment II is spectrophotometrically performed at 320 nm according to the Lambert-Beer law.

The C-terminal arginine residue increases the solubility of the substrate in acid and alkaline buffers and the Pro-D-Arg sequence protects the peptide against enzymatic degradations from the carboxylic end. The substrate has no toxic effects an cells in the concentrations used for the determination.

Principle:
Pz-Pro-Leu-Gly-Pro-D-Arg + H2O + Collagenase → Pz-Pro-Leu + Gly-Pro-D-Arg

Abbreviations used:
Pz = 4-Phenylazobenzyloxycarbonyl

Conditions: T = 25°C, pH = 7.1, A_{320nm}, Light path = 1 cm
Method: Spectrophotometric Stop Rate Determination

Reagents:

A. 1 M Tris HCl Buffer, pH 7.1 at 25°C
(Prepare 100 ml in deionized water using Trizma Base. Adjust to pH 7.1 at 25°C with 1 M HCl.)
B. Methanol
(Use Methanol, Absolute)
C. Pz-Pro-Leu-Gly-Pro-D-Arg Chromophore-Substrate (Component A)
D. Pz-Pro-Leu-OH Standard (Component B)
C. 1 M Calcium Chloride Solution ($CaCl_2$)
(Prepare 100 ml in deionized water using Calcium Chloride, Dihydrate)
E. 1 M Citric Acid Solution (Citric Acid)
(Prepare 100 ml in deionized water using Citric Acid, Free Acid, Monohydrate)
F. Ethyl Acetate (ETOAC)
G. Sodium Sulfate (Na_2SO_4)
H. Collagenase

Solutions:

1) Buffer
(80 ml 1 M Tris HCl Buffer, pH 7.1 at 25°C + 20 ml 1 M $CaCl_2$)

2) 1.3 mM Pz-Pro-Leu-Gly-Pro-D-Arg Substrate Solution (Component A)
(Prepare by dissolving 10 mg of Pz-Pro-Leu-Gly-Pro-D-Arg, Fluka 27667, in 0.2 ml of methanol. Bring to a total volume of 10 ml with Buffer)

3) Standard Solution (Component B)
(Prepare by dissolving 5.8 mg of Pz- Pro-Leu-OH Fluka 27668, in 0.2 ml of methanol. Bring to a total volume of 10 ml with Buffer)

4) 25mM Citric Acid
(Prepare by diluting 1 M Citric Acid Solution)

5) Collagenase Enzyme Solution
(Immediately before use, prepare a solution containing about 2-5 unit/ml of Collagenase in water)

Procedure:

Step 1:
Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank	Standard
Substrate Solution (Component A)	2.40	2.40	-
Standard Solution (Component B)	-	-	2.30
Buffer	-	0.10	0.20
Collagenase Solution	0.10	-	-

Immediately mix by swirling and incubate for exactly 15 minutes at 25°C.

Step 2:
Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank	Standard
Mixture from Step 1	0.50	0.50	0.50
25mM Citric Acid	1.00	1.00	1.00
Ethyl Acetate	5.00	5.00	5.00

Mix by shaking for 15 seconds. Remove the ethyl acetate phase (upper layer) and place into separate tubes, each containing 300 mg of Na_2SO_4. Mix by shaking and filter through Whatman #54 filter paper. Transfer the solutions to suitable cuvettes and record the A_{320nm} using a suitable spectrophotometer.

Calculations:

$$Units/ml = \frac{(A_{320nm}Test - A_{320nm}Blank)2.5 \cdot df}{15 \cdot 4.2 \cdot 0.5 \cdot 0.1}$$

2.5 [ml] = Total volume of assay in Step 1
df = Dilution factor
15 [min.] = Incubation time
4.2 [1/µmol] = extinction coefficient of 1 micromole of Pz-Pro-Leu in 5ml ethyl acetate and 1 cm light path (ε_{320nm} = 21.0 cm^2/µmol)
0.5 [ml] = Volume of reaction mixture in Step 1 used in Step 2
0.1 [ml] = Volume of enzyme used in Step 1 units/ml enzyme

$$\text{Units/mg solid} = \frac{\text{mg solid}}{\text{ml enzyme}}$$

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Units Definition:
One unit will liberate 1 µmol of Pz-Pro-Leu from Pz- Pro-Leu-Gly-Pro-D-Arg (Fluka 27667) in 1 minute at pH 7.1 at 25°C.

Note: The standard is to check the extinction coefficient (ε_{320nm} = 21.0 cm^2/µmol).

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