⟨89.1⟩ COLLAGENASE I

IANTNSEKYD FEYLNGLSYT ELTNLIKNIK WNQINGLFNY STGSQKFFGD
KNRVQAIINA LQESGRTYTA NDMKGIETFT EVLRAGFYLG YYNDGLSYLN
DRNFQDKCIP AMIAIQKNPN FKLGTAVQDE VITSLGKLIG NASANAEVVN
NCVPVLKQFR ENLNQYAPDY VKGTAVNELI KGIEFDFSGA AYEKDVKTMP
WYGKIDPFIN ELKALGLYGN ITSATEWASD VGIYYLSKFG LYSTNRNDIV
QSLEKAVDMY KYGKIAFVAM ERITWDYDGI GSNGKKVDHD KFLDDAEKHY
LPKTYTFDNG TFIIRAGDKV SEEKIKRLYW ASREVKSQFH RVVGNDKALE
VGNADDVLTM KIFNSPEEYK FNTNINGVST DNGGLYIEPR GTFYTYERTP
QQSIFSLEEL FRHEYTHYLQ ARYLVDGLWG QGPFYEKNRL TWFDEGTAEF
FAGSTRTSGV LPRKSILGYL AKDKVDHRYS LKKTLNSGYD DSDWMFYNYG
FAVAHYLYEK DMPTFIKMNK AILNTDVKSY DEIIKKLSDD ANKNTEYQNH
IQELADKYQG AGIPLVSDDY LKDHGYKKAS EVYSEISKAA SLTNTSVTAE
KSQYFNTFTL RGTYTGETSK GEFKDWDEMS KKLDGTLESL AKNSWSGYKT
LTAYFTNYRV TSDNKVQYDV VFHGVLTDNA DISNNKAPIA KVTGPSTGAV
GRNIEFSGKD SKDEDGKIVS YDWDFGDGAT SRGKNSVHAY KKAGTYNVTL
KVTDDKGATA TESFTIEIKN EDTTTPITKE MEPNDDIKEA NGPIVEGVTV
KGDLNGSDDA DTFYFDVKED GDVTIELPYS GSSNFTWLVY KEGDDQNHIA
SGIDKNNSKV GTFKSTKGRH YVFIYKHDSA SNISYSLNIK GLGNEKLKEK
ENNDSSDKAT VIPNFNTTMQ GSLLGDDSRD YYSFEVKEEG EVNIELDKKD
EFGVTWTLHP ESNINDRITY GQVDGNKVSN KVKLRPGKYY LLVYKYSGSG
NYELRVNK

$C_{5099}H_{7771}N_{1329}O_{1610}S_{14}$
113,897 daltons (for β subtype)
[9001-12-1]

DEFINITION

Collagenase I (EC 3.4.24.3), isolated from *Clostridium histolyticum* and encoded by *colG* gene (GenBank accession number BAA77453.1), is a key raw material used in the dissociation or destruction of a broad range of tissue types. Collagenase I is a metalloprotease that acts as an endoprotease and also exhibits a tripeptidylcarboxypeptidase activity. It shows endopeptidic activity with the main cleavage site found in front of the human collagen duplex amino acids glycine–proline. Hydrolysis takes place near the ends of the triple helical domains of collagen.

Collagenase I is also known as class I collagenase and consists of three subtypes: α, β, and γ. Collagenase I β is the full-length enzyme while collagenase I α (68,000 Da) and collagenase I γ (79,000 Da) are thought to be proteolytic degradation products of collagenase I β caused by other proteases present in *C. histolyticum* (mainly a trypsin-like enzyme and clostripain/endoproteinase Arg C).

Collagenase I can be provided in a liquid formulation consisting of 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 1 mM calcium chloride pH 7.5, and stored as a frozen liquid. The specific activity of collagenase I is 0.10–0.60 units/mg of protein using 4-phenylazobenzyloxycarbonyl (PZ)-Pro-Leu-Gly-Pro-D-Arg as the substrate described in the *Assay*. The peak area for collagenase I is NLT 90% as determined by HPLC described in the test for *Purity*. The test for *Clostripain Activity* is used to assess the activity of the clostripain impurity and the acceptance criterion is NMT 0.5 units/mg of protein. The test for *Trypsin Activity* is used to assess the activity of the trypsin-like enzyme impurity and the acceptance criterion is NMT 0.5 units/mg of protein.

IDENTIFICATION

- **A.** It meets the requirements in the *Assay*.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Purity*.

ASSAY

- **PROCEDURE**

Tris buffer: 0.1 M Tris pH 7.1, prepared as follows. Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (Tris) in 400 mL of water, and adjust with 2 N hydrochloric acid to a pH of 7.1 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

Substrate solution: Dissolve 10 mg of PZ-Pro-Leu-Gly-Pro-D-Arg in 0.2 mL of methanol, and dilute this solution with *Tris buffer* to a final volume of 10 mL. [NOTE—Use a freshly prepared solution only.]

Calcium chloride solution: 0.1 M, prepared as follows. Weigh 1.47 g of calcium chloride dihydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

Citric acid solution: 0.025 M, prepared as follows. Weigh 525 mg of citric acid monohydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

Extraction mixture: To one test tube per sample to be assayed, pipette 5.0 mL of ethyl acetate and 1.0 mL of *Citric acid solution*. [NOTE—Use a freshly prepared mixture only.]

Drying tube: Into one test tube per sample to be assayed, add 0.35–0.40 g of sodium sulfate anhydrous. Seal the test tube with parafilm.

Standard solution: Dilute USP Collagenase I RS with *Tris buffer* in the range of 1:50 to 1:100 (v/v). [NOTE—Avoid freezing and thawing USP Collagenase I RS. After withdrawing USP Collagenase I RS, wipe off the outside of the plastic pipette tips to remove any residual solution.]

Sample solutions: Dilute collagenase I with *Tris buffer* to an appropriate dilution to achieve the absorbance range of 0.3–0.9 from the *Analysis*. Prepare in triplicate. [NOTE—Avoid freezing and thawing the collagenase I sample. After withdrawing the collagenase I sample, wipe off the outside of the tip to remove any residual solution.]

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 320 nm

Path length: 1 cm

Temperature: 25°

Analysis

Samples: *Standard solution* and *Sample solutions*

Transfer 1.0 mL of *Substrate solution* and 0.2 mL of *Calcium chloride solution* into a test tube, and equilibrate the test tube to 25°. Start the reaction by adding 0.05 mL of *Standard solution* or each *Sample solution*. Prepare a blank by replacing the *Standard solution* or *Sample solution* with 0.05 mL of *Tris buffer*. Mix and incubate for exactly 15 min at 25°. Transfer 0.5 mL of the reaction to the test tube containing 6.0 mL of *Extraction mixture*. Vortex immediately for 20 s. Transfer 3 mL of the ethyl acetate phase (upper layer) into a *Drying tube* using a glass pipette, and vortex thoroughly. Transfer the supernatant to a disposable, semi-micro cuvette suitable for UV absorbance with a Pasteur pipette. Record the absorbance.

Calculate the activity of collagenase I in units/mL:

$$\text{Result} = (A - A_B) \times [V_T \times V_E/(\varepsilon \times V \times V_R \times B \times T)] \times D$$

A = absorbance of the *Standard solution* or *Sample solution*
A_B = absorbance of the blank
V_T = volume of the reaction mixture, 1.25 mL
V_E = volume of ethyl acetate in the *Extraction mixture*, 5.0 mL
ε = extinction coefficient for 320 nm, 21 ($1\ cm^2 \cdot \mu mol^{-1}$)
V = volume of the *Standard solution* or *Sample solution*, 0.05 mL
V_R = volume of the reaction transferred to *Extraction mixture*, 0.5 mL
B = absorption path length, 1 cm
T = incubation time, 15 min
D = dilution factor

[NOTE—One unit will release the equivalent of 1 µmol of PZ-Pro-Leu from PZ-Pro-Leu-Gly-Pro-D-Arg per minute under the conditions of the *Assay*.]

Calculate the specific activity of collagenase I in units/mg of protein:

$$\text{Result} = Activity/C$$

$Activity$ = activity of collagenase I (units/mL)
C = protein concentration (mg/mL)

System suitability

Samples: *Standard solution* and *Sample solutions*

Suitability requirements

Average calculated activity: 85%–115% of the value on the label, *Standard solution*

Absorbance: 0.3–0.9, *Standard solution* and *Sample solutions*

Acceptance criteria: 0.10–0.60 units/mg of protein

PURITY

• **PROCEDURE**

Solution A: 20 mM Tris and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

Solution B: 20 mM Tris, 1 mM calcium chloride, and 1 M sodium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris, 147 mg of calcium chloride dihydrate, and 58.44 g of sodium chloride in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
22	85	15
32	0	100
34	100	0
40	100	0

Storage buffer: 5 mM HEPES and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 1.19 g of HEPES and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 4 N sodium hydroxide solution to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

Standard solution: Thaw USP Collagenase I RS at room temperature shortly before use and mix. Store on ice or at 5°. Dilute with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL. Transfer to an HPLC vial and keep at 5°. Prepare in duplicate, and inject each duplicate once.

Collagenase II solution: Thaw USP Collagenase II RS at room temperature shortly before use and mix. Store on ice or at 5°. Dilute with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL. Transfer to an HPLC vial and keep at 5°. Prepare in duplicate, and inject each duplicate once.

Sample solution: Dilute collagenase I with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL and keep at 5°.

Blank: *Storage buffer*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 5-mm × 5-cm; 10-µm packing L91

Temperatures

Column: 25°

Autosampler: 5°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirement: The chromatogram from the *Standard solution* corresponds to the typical chromatogram provided with USP Collagenase I RS.

Analysis

Sample: *Sample solution*

The *Blank* should be considered for integration. Identify the peak corresponding to collagenase II by comparing its retention time to the main peak from the *Collagenase II solution*. Evaluate the purity of collagenase I using the area–% method but excluding peaks associated with the *Blank*. All shoulders in the fronting and tailing of the main peak are integrated by dropping a perpendicular line at the inflection points and considered as separate impurities. Disregard any peaks having retention times greater than 25 min.

Acceptance criteria: NLT 90% for the main peak of collagenase I and NMT 3% of collagenase II

IMPURITIES

• **CLOSTRIPAIN ACTIVITY**

Potassium phosphate buffer: 0.1 M pH 7.6, prepared as follows. Dissolve 1.36 g of monobasic potassium phosphate in water, and dilute to 100 mL. Dissolve 2.28 g of dibasic potassium phosphate trihydrate in water, and dilute to 100 mL. Adjust the pH of the second solution to 7.6 with the first solution.

Dithiothreitol solution: 0.194 M, prepared as follows. Dissolve 60 mg of dithiothreitol (DTT) in 2 mL of *Potassium phosphate buffer*.

Calcium chloride solution: 0.01 M, prepared as follows. Dissolve 147 mg of calcium chloride dihydrate in 100 mL of water.

Substrate stock solution: 38 mM, prepared as follows. Dissolve 13 mg of *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl) in 1 mL of *Potassium phosphate buffer*.

Substrate solution: 0.73 mM BAEE · HCl, 7.8 mM DTT, and 0.4 mM calcium chloride, prepared as follows. Transfer 0.5 mL of *Substrate stock solution*, 1.0 mL of *Dithiothreitol solution*, and 1.0 mL of *Calcium chloride solution* to a 25-mL volumetric flask, and dilute with *Potassium phosphate buffer* to volume.

Sample solution: Prepare in such a way that ΔA/min lies in the 0.02–0.06 range. Dilute with ice-cold *Potassium phosphate buffer* if necessary.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 255 nm

Path length: 1 cm

Temperature: 25°

Analysis

Sample: *Sample solution*

Transfer 3.0 mL of *Substrate solution* into a cuvette, and equilibrate the cuvette to 25°. Start the reaction by adding 0.05 mL of *Sample solution*. Prepare a blank by replacing the *Sample solution* with 0.05 mL of *Potassium phosphate buffer*. Mix well. Determine the change in absorbance (ΔA/min) from the linear range of the reaction. Assay the *Sample solution* in triplicate.

System suitability

Sample: *Sample solution*

Suitability requirement: 0.02–0.06 for ΔA/min

Calculate the activity of clostripain in units/mL in the portion of collagenase I taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{min} \times D$$

V_T = volume of the reaction mixture, 3.05 mL
ε = extinction coefficient for 255 nm, 0.81 (1 $cm^2 \cdot mmol^{-1}$)
V_U = volume of the *Sample solution*, 0.05 mL
B = absorption path length, 1 cm
ΔA/min = change in absorbance from the linear range of the reaction
D = dilution factor

Calculate the specific activity in units/mg of protein:

$$\text{Result} = \textit{Activity}/C$$

Activity = activity of clostripain (units/mL)
C = protein concentration (mg/mL)

Acceptance criteria: NMT 0.5 units of clostripain activity per mg of protein

• **TRYPSIN ACTIVITY**

Buffer: 0.1 M Tris and 0.02 M calcium chloride pH 8.0, prepared as follows. Dissolve 6.05 g of Tris and 1.45 g of calcium chloride dihydrate in 400 mL of water. Adjust with 2 N hydrochloric acid to a pH of 8.0 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

Substrate stock solution: Dissolve 10 mg of carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate,[1] accurately weighed, in 1.5 mL of water. Store on ice. [NOTE—Use freshly prepared solution only.]

Substrate solution: Prepare a solution by mixing 9.2 mL of *Buffer* and 1.0 mL of *Substrate stock solution*. Store on ice. [NOTE—Use freshly prepared solution only.]

Sample solution: Undiluted collagenase I solution

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 405 nm

Path length: 1 cm

Temperature: 25°

Analysis

Sample: *Sample solution*

Transfer 1.02 mL of *Substrate solution* into a polystyrene, semi-micro cuvette, allow the temperature to stabilize, equilibrate the cuvette to 25°, and wait for 10 min. Start the reaction by adding 0.10 mL of *Sample solution*. Start recording the absorbance and continue for at least 5 min after the addition of the *Sample solution*. Determine the change in absorbance (ΔA/min) from the linear range of the reaction. Assay the *Sample solution* in triplicate. [NOTE—Use polyethylene pipette tips to transfer the *Sample solution*. The pipette tip should not be wet before transfer, and each pipette tip should be used only for transferring one sample. After withdrawing the *Sample solution*, wipe off the outside of the tip to remove any residual solution. After adding the *Sample solution* to the *Substrate solution*, rinse the tip by pipetting the solution up and down 2–3 times, discard the tip, and mix.]

System suitability

Sample: *Sample solution*

Suitability requirement: >0.01 for ΔA/min

Calculate the activity of trypsin in units/mL in the portion of collagenase I taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{min} \times D$$

V_T = volume of the reaction mixture, 1.12 mL
ε = extinction coefficient for 405 nm, 10.4 (1 $cm^2 \cdot mmol^{-1}$)
V_U = volume of the *Sample solution*, 0.10 mL
B = absorption path length, 1 cm
ΔA/min = change in absorbance from the linear range of the reaction
D = dilution factor

[1] A suitable carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate is Chromozym TRY from Roche Applied Science (catalog number 10378496103) or equivalent.

Calculate the specific activity in units/mg of protein:

$$\text{Result} = Activity/C$$

Activity = activity of trypsin (units/mL)
C = protein concentration (mg/mL)

Acceptance criteria: NMT 0.5 units of trypsin activity per mg of protein

SPECIFIC TESTS

- **PROTEIN CONTENT**

Sample solutions: Dilute collagenase I in water. Prepare at least in triplicate. [NOTE—Prepare the dilution using plastic pipette tips and not glass pipettes. Carefully wipe off the outside of the tip to remove any residual solution.]
Blank solution: Water
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Analytical wavelength: 280 nm
Path length: 1 cm
System suitability
Samples: *Sample solutions*
Suitability requirement: Absorbance is in the range of 0.10–1.00.
Analysis
Samples: *Sample solutions* and *Blank solution*
Determine the net absorbance of *Sample solutions* by subtracting the absorbance of the *Blank solution* from the absorbance of each *Sample solution*. Determine the average net absorbance of *Sample solutions*.
Calculate the protein concentration in mg/mL:

$$\text{Result} = A_U \times D/\varepsilon$$

A_U = average net absorbance of the *Sample solutions*
D = dilution factor
ε = extinction coefficient (A_{280}0.1%/cm) for collagenase, 1.4

- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** NMT 50 USP Endotoxin Units/mg of protein
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩**:** The total bacterial count is NMT 100 cfu/mL.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in closed containers at –15° to –25°.
- **LABELING:** The labeling states that the material is derived from *Clostridium histolyticum* along with the lot number, product or catalog number, and storage conditions.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Collagenase I RS
  USP Collagenase II RS
  USP Endotoxin RS