Status: Currently Official on 12-Feb-2025
Official Date: Official as of 01-Dec-2021
Document Type: General Chapter
DocId: GUID-2814E59A-96F2-4322-A7D6-2FDCA90B9AA2_2_en-US
DOI: https://doi.org/10.31003/USPNF_M7728_02_01
DOI Ref: k4d1v

⟨89⟩ ENZYMES USED AS ANCILLARY MATERIALS IN PHARMACEUTICAL MANUFACTURING

INTRODUCTION

The purpose of this chapter is to describe the quality attributes and associated tests with acceptance criteria for enzymatic preparations used in biopharmaceutical manufacturing. The quality of ancillary materials, including enzymes, used in biopharmaceutical manufacturing can have an impact on the therapeutic products. Several enzymes are used in this type of cell processing. Examples include trypsin, collagenase, pepsin, and papain. This chapter does not discuss applications of these enzymes but rather focuses on tests to assess their quality as process materials.

Recombinant Trypsin

IVGGYTCAAN SIPYQVSLNS GSHFCGGSLI NSQWVVSAAH CYKSRIQVRL
GEHNIDVLEG NEQFINAAKI ITHPNFNGNT LDNDIMLIKL SSPATLNSRV
ATVSLPRSCA AAGTECLISG WGNTKSSGSS YPSLLQCLKA PVLSDSSCKS
SYPGQITGNM ICVGFLEGGK DSCQGDSGGP VVCNGQLQGI VSWGYGCAQK
NKPGVYTKVC NYVNWIQQTI AAN

$C_{1020}H_{1597}N_{287}O_{321}S_{14}$ 23,463 (for β-Trypsin) CAS RN®: 9002-07-7.

DEFINITION

Recombinant trypsin, a key raw material in biopharmaceutical manufacturing, is a serine protease that cleaves peptide chains mainly at the carboxyl end of the amino acids arginine and lysine. The amino acid sequence of recombinant trypsin is identical to that of trypsin from porcine pancreas, and the recombinant trypsin is produced by methods based on recombinant DNA technology in the yeast *Pichia pastoris*. Therefore, the specifications described in this chapter apply only to recombinant porcine trypsin produced in yeast. Because of the recombinant production process, recombinant trypsin is free of chymotrypsin. Two active forms of trypsin are known: β-trypsin (23,463 daltons) and α-trypsin (23,481 daltons). Autolysis of β-trypsin at the peptide bond between Arg^{99} and Val^{100}, Lys^{125} and Ser^{126}, or Lys^{139} and Ala^{140} results in three possible isoforms of α-trypsin. All isoforms are held together by disulfide bridges and remain correctly folded. As a consequence of hydrolysis of a peptide bond, the molecular weight of α-trypsin is more than that of β-trypsin by 18 daltons. The peak area for β-trypsin is NLT 70%, and the peak area for α-trypsin is NMT 20% as determined by the HPLC procedure described in the test for *Purity*. The specific activity is NLT 180 Units/mg of protein using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate described in the *Assay*.

[Note—One Unit of trypsin activity using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate corresponds to 21 USP Trypsin Units. One USP Trypsin Unit is the activity causing a change in absorbance of 0.003/min under the conditions specified in the *Assay* in the *Trypsin* monograph using *N*-benzoyl-L-arginine ethyl ester hydrochloride as the substrate. Therefore, the specific activity of 180 Units/mg of protein using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate for recombinant trypsin corresponds to 3800 USP Trypsin Units/mg of protein.]

IDENTIFICATION

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

ASSAY

Change to read:

- **Procedure**

Buffer: Dissolve 1.21 g of tris(hydroxymethyl)aminomethane and 0.29 g of calcium chloride dihydrate in 100 mL of water, and adjust with 2 N hydrochloric acid to a pH of 8.0 (at 25 ± 1°).

Substrate stock solution: Dissolve 20 mg of carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate,[1] accurately weighed, in 3.0 mL of water. [Note—Use a freshly prepared solution only.]

Substrate solution: Prepare a solution by mixing 28 mL of *Buffer* and 2.8 mL of *Substrate stock solution*. [NOTE—Use a freshly prepared solution only.]

Standard solutions: Precool USP Trypsin Recombinant Porcine RS and water to approximately 4°. Start preparing *Standard solutions* immediately when the temperature has reached 4°. Prepare each *Standard solution* by diluting USP Trypsin Recombinant Porcine RS with water, at 1:68,921 dilution. Prepare at least five *Standard solutions* in parallel. Assay each *Standard solution* in duplicate. [NOTE—Use an adjustable pipettor for each measurement and dilution operation. Use ▲low protein binding▲ (USP 1-Dec-2021) test tubes for preparing *Standard solutions* and *Sample solutions*, and use ▲low protein binding▲ (USP 1-Dec-2021) pipet tips containing ▲low protein binding▲ (USP 1-Dec-2021) filters to transfer samples. The pipet tip should not be wet before transfer, and each pipet tip should be used only for transferring one sample.][NOTE—A dilution of 1:68,921 can be achieved by three dilution steps in which each step has a 1:41 dilution (1:41/1:41/1:41). For example, for the initial dilution withdraw 0.1 mL of USP Trypsin Recombinant Porcine RS using a pipettor with a ▲low protein binding▲ (USP 1-Dec-2021) tip containing ▲low protein binding▲ (USP 1-Dec-2021) filters, wipe off the outside of the tip to remove any residual solution, add the Reference Standard to a ▲low protein binding▲ (USP 1-Dec-2021) test tube containing 4.0 mL of precooled water, rinse the tip by pipetting the solution up and down for 2–3 times, discard the tip, and mix the solution on a vortex mixer for approximately 2 s with maximum speed. For the second and third dilution steps, proceed as described for the initial dilution except for transferring 0.1 mL of solution from the previous dilution step.]

Sample solutions: Prepare at least five *Sample solutions* of recombinant trypsin in parallel as directed for the *Standard solutions* to obtain a final concentration of at least 0.16 Units/mL using precooled water, as the diluent. Each *Sample solution* is assayed in duplicate.

Instrumental conditions

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

Mode: UV

Analytical wavelength: 405 nm

Path length: 1 cm

Temperature: 25°

System suitability

Samples: *Standard solutions* and *Sample solutions*

Suitability requirement: Δ*A*/min (calculated below) should be 0.03–0.07 for *Standard solutions* and *Sample solutions*. The average calculated activity for the *Standard solutions* is 90%–110% of the value on the label.

Analysis

Samples: *Standard solutions* and *Sample solutions*

Transfer 1.10 mL of *Substrate solution* into a ▲low protein binding▲ (USP 1-Dec-2021) semimicro cuvette, allow the temperature to stabilize, check the cuvette for the specified temperature, and wait for 10 min. Start the reaction by adding 0.020 mL of *Standard solution* or *Sample solution*. Record the absorbance for at least 5 min, and determine the change in absorbance per minute, Δ*A*/min, from the linear range of the reaction.

Calculate the activity of recombinant trypsin in Units/mL:

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

V_T = volume of the reaction mixture, 1.12 mL

ε = extinction coefficient for 405 nm, 10.4 ($\text{mmol}^{-1} \cdot 1\ \text{cm}^{-1}$)

V = volume of *Standard solution* or *Sample solution*, 0.020 mL

B = absorption path length, 1 cm

$\Delta A/\text{min}$ = change in absorbance per minute

D = dilution factor

[NOTE—One Unit will release the equivalent of 1 mmol of 4-nitril aniline from carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate per min under the conditions of the *Assay*.]

Calculate the specific activity of recombinant trypsin in Units/mg of protein:

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

Activity = activity of recombinant trypsin as previously calculated (Units/mL)

C = protein concentration of recombinant trypsin (mg/mL)

Acceptance criteria

Specific activity: NLT 180 Units/mg of protein

Relative standard deviation: NMT 5% for the activities determined from 5 replicates

PURITY

• **Procedure**

Solution A: Dilute 1 mL of phosphoric acid (85%) with water, to 1000 mL.

Solution B: Dilute 1 mL of phosphoric acid (85%) with acetonitrile to 1000 mL.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
25	55	45
30	10	90
34	10	90
35	75	25
45	75	25

Standard solution: Thaw 100 µL of USP Trypsin Recombinant Porcine RS at room temperature for about 1 h, mix, and transfer to an HPLC vial. The target protein concentration should be 70 ± 10 mg/mL.

Sample solution: Thaw 100 µL of recombinant trypsin at room temperature for about 1 h, mix, and transfer to an HPLC vial. The target protein concentration should be 70 ± 10 mg/mL.

[Note—Keep *Standard solution* and *Sample solution* at 2°–8° if they are not ready to be injected immediately after preparation.]

Chromatographic system

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

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 3-µm packing L1 with a 200 Å pore size

Column temperature: 40°

Flow rate: 1.0 mL/min

Injection volume: 1 µL

System suitability

Sample: *Standard solution*

[Note—The retention time for the main peak of recombinant trypsin is 12–17 min.]

Suitability requirements

Resolution: NLT 1 between the peaks of α-trypsin and β-trypsin

Analysis

Sample: *Sample solution*

Record the chromatograms, and measure the peak areas. Evaluate the purity of trypsin using the area percentage method. Time of integration is 25 min. The blank should be considered for integration. Peaks that elute as a fronting shoulder of α-trypsin are integrated by perpendicular dropping if and only if a minimum is formed. Peaks that elute as a tailing shoulder of β-trypsin are evaluated by tangential integration if and only if a minimum is formed.

Acceptance criteria: NLT 70% for the peak area of β-trypsin and NMT 20% for the peak area of α-trypsin

SPECIFIC TESTS

Change to read:

• **Protein Content**

4 N hydrochloric acid solution: Mix 10.4 mL of 25% hydrochloric acid with 9.6 mL of water.

Storage buffer: Dissolve 2.9 g of calcium chloride dihydrate in water, add 2.5 mL of *4 N hydrochloric acid solution*, and dilute with water, to a final volume of 1000 mL. Adjust with *4 N hydrochloric acid solution* to a pH of 2.0 ± 0.2, if necessary.

Sample solutions: Add 0.025 mL of recombinant trypsin to 3 mL of *Storage buffer*. Prepare at least in triplicate.

Blank solution: *Storage buffer*, 3 mL

Instrumental conditions

▲(See *Ultraviolet-Visible Spectroscopy ⟨857⟩*.)▲ (USP 1-Dec-2021)

Mode: UV

Analytical wavelength: 280 nm

Path length: 1 cm

System suitability

Sample: *Sample solutions*

Suitability requirement: ΔA (as calculated below) is in the range of 0.13–1.8.

Analysis

Samples: *Sample solutions* and *Blank solution*

▲Calculate the change in absorbance (ΔA):▲ (USP 1-Dec-2021)

$$\Delta A = A_U - A_B$$

A_U = absorbance of the *Sample solution*

A_B = absorbance of the *Blank solution*

Calculate the protein concentration in mg/mL:

$$\text{Result} = \left[\frac{(\Delta A \times F)}{A_{280\,\frac{1\%}{1\text{cm}}}}\right] \times D$$

▲ΔA = change in absorbance, as calculated previously▲ (USP 1-Dec-2021)

F = conversion factor from % to mg/mL, 10

$A_{280\,\frac{1\%}{1\text{cm}}}$ = extinction coefficient for trypsin, 13.6

D = dilution factor

Change to read:

• **Microbial Enumeration Tests ⟨61⟩:** The total ▲aerobic microbial count is NMT 10^2 cfu/mL,▲ (USP 1-Dec-2021) the test being performed on 1 mL of recombinant trypsin in duplicate.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Store in closed containers at −15° to −25°.

• **Labeling:** The labeling states that the material is of recombinant DNA origin, along with the product number and lot number, storage conditions, and expiration date.

• **USP Reference Standards ⟨11⟩**

USP Trypsin Recombinant Porcine RS

[1] A suitable carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate is Chromozym TRY from Roche ▲CustomBiotech▲ (USP 1-Dec-2021) (catalog number 10378496103) or equivalent.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
<89> ENZYMES USED AS ANCILLARY MATERIALS IN PHARMACEUTICAL MANUFACTURING	Jennifer Tong Sun Senior Scientist II	BIO2 Biologics Monographs 2 - Proteins

Most Recently Appeared In:
Pharmacopeial Forum: Volume No. 46(3)

Current DocID: GUID-2814E59A-96F2-4322-A7D6-2FDCA90B9AA2_2_en-US

DOI: https://doi.org/10.31003/USPNF_M7728_02_01

DOI ref: k4d1v