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# Pepsin

CAS RN<sup>®</sup>: 9001-75-6.—E.C. 3.4.23.1

## • ACTIVITY DETERMINATION

The activity determination of pepsin can be done by using a *Standard curve* or a *Single standard solution*. When the activity of pepsin is not known or is not expressed in USP Units/mg of weight, the activity is first determined by a *Standard curve*. If the correlation coefficient ( $r^2$ ) of the standard curve is NLT 0.99, the next activity determinations can be done by using the *Single standard solution* procedure.

### Standard curve

**Diluted hydrochloric acid solution:** Dilute 30 mL of [1 N hydrochloric acid](#) with [water](#) to 1000 mL. Adjust with [1 N hydrochloric acid](#) to a pH of  $1.6 \pm 0.1$ , if necessary.

**Trichloroacetic acid (TCA) solution:** 4.0% (w/v) [trichloroacetic acid](#) in [water](#)

**Substrate solution:** Transfer 4.0 g of [USP Hemoglobin Protease Substrate RS](#) to a 200-mL volumetric flask, and dissolve in 75 mL of *Diluted hydrochloric acid solution*. Once the hemoglobin is fully dissolved, adjust with [1 N hydrochloric acid](#) to a pH of  $1.6 \pm 0.1$ , if necessary. Dilute with *Diluted hydrochloric acid solution* to volume.

**Standard stock solution:** Quantitatively prepare a solution of [USP Pepsin for Assay RS](#) with about 1.25 USP Pepsin Units/mL in *Diluted hydrochloric acid solution*.

**Standard solutions:** Prepare four test tubes capable of holding a minimum of 6 mL, and label the tubes S1, S2, S3, and S4. Add 5 mL of the *Standard stock solution* to tube S4. Add 4 mL of the *Standard stock solution* and then 1 mL of *Diluted hydrochloric acid solution* to tube S3. Add 3 mL of the *Standard stock solution*, then 2 mL of *Diluted hydrochloric acid solution* to tube S2. Add 2 mL of the *Standard stock solution*, then 3 mL of *Diluted hydrochloric acid solution* to tube S1.

[NOTE—Prepare these solutions immediately before use.]

**Sample solution:** Quantitatively prepare a solution of pepsin in *Diluted hydrochloric acid solution* containing approximately 0.90–1.00 USP Pepsin Units/mL.

[NOTE—Prepare this solution immediately before use.]

**Procedure:** Prepare test tubes as follows (use tubes that can accommodate and allow mixing on a vortex mixer of a volume of NLT 16 mL). Separately transfer 1.0-mL aliquots of each of the *Standard solutions* into four test tubes (two tubes for the analysis of each *Standard solution* and two blanks for each *Standard solution*).

Prepare a substrate blank by transferring 1.0 mL of *Diluted hydrochloric acid solution* into a single test tube.

Separately transfer 1.0-mL aliquots of the *Sample solution* into four test tubes (two tubes for the analysis of the *Sample solution* and two blanks for each *Sample solution*).

To each of the blank tubes add 10.0 mL of *TCA solution*, and mix on a vortex mixer.

Place all test tubes in a water bath maintained at  $25 \pm 1^\circ$ , and allow equilibration for 5 min. Add 5.0 mL of the *Substrate solution* (previously equilibrated at  $25 \pm 1^\circ$ ) to each of the blank tubes, mix on a vortex mixer, and return the tubes to the water bath to incubate for 10 min.

At time equals zero, rapidly pipet 5.0 mL of the *Substrate solution* (previously equilibrated at  $25 \pm 1^\circ$ ) successively and at accurately timed intervals of at least 30 s into each of the tubes containing the *Sample solution* and *Standard solutions*. Mix each tube immediately after adding the *Substrate solution* by brief vortex mixing, and return the tube to the water bath. After 10.0 min, at the corresponding time intervals, stop the reaction by rapidly pipetting 10.0 mL of *TCA solution* into each tube. Mix each tube on a vortex mixer. Do not return the tubes to the water bath.

Allow all tubes, including blanks, to sit at room temperature for 25 min. After 25 min, mix the contents of each tube on a vortex mixer, and pass the contents through a fluted paper filter into clean tubes, discarding the first 5 mL of the filtrate. Refilter each of the filtrates through the same paper.

[NOTE—A suitable filter is the Whatman No. 41 ashless filter.]

Determine the absorbance of each filtrate using the following conditions.

**Wavelength:** 280 nm

**Cell:** 1 cm

**Blank:** Substrate blank

Calculate the average absorbance for each filtrate. Correct the absorbance of each *Standard solution*:

$$A_{SC} = A_S - A_{SB}$$

$A_S$  = average absorbance of the *Standard solution*

$A_{SB}$  = average absorbance of the *Standard solution* blank

Correct the absorbance of each *Sample solution*:

$$A_{UC} = A_U - A_{UB}$$

$A_U$  = average absorbance of the *Sample solution*

$A_{UB}$  = average absorbance of the *Sample solution* blank

Using linear regression, determine the correlation coefficient ( $r^2$ ), slope ( $m$ ) and  $y$ -intercept of the curve-corrected absorbance of the *Standard solutions* versus concentration (mg/mL).

Calculate the activity of pepsin in the sample:

$$\text{Activity (Units/mg)} = [(A_{UC} - b) \times P] / (m \times C_U)$$

$A_{UC}$  = absorbance of each *Sample solution*

$b$  =  $y$ -intercept of the standard curve

$P$  = activity of the [USP Pepsin for Assay RS](#) (Units/mg)

$m$  = slope of the standard curve

$C_U$  = concentration of the *Sample solution* (mg/mL)

### Single standard solution

**Diluted hydrochloric acid solution:** Dilute 30 mL of [1 N hydrochloric acid](#) with [water](#) to 1000 mL. Adjust with [1 N hydrochloric acid](#) to a pH of  $1.6 \pm 0.1$ , if necessary.

**TCA solution:** 4.0% (w/v) [trichloroacetic acid](#) in [water](#)

**Substrate solution:** Transfer 4.0 g of [USP Hemoglobin Protease Substrate RS](#) to a 200-mL volumetric flask, and dissolve in 75 mL of *Diluted hydrochloric acid solution*. Once the hemoglobin is fully dissolved, adjust with [1 N hydrochloric acid](#) to a pH of  $1.6 \pm 0.1$ , if necessary. Dilute with *Diluted hydrochloric acid solution* to volume.

**Standard solution:** Using the [USP Pepsin for Assay RS](#), prepare a solution containing 1.0 USP Pepsin Unit/mL in *Diluted hydrochloric acid solution*.

[NOTE—Prepare this solution immediately before use.]

**Sample solution:** Quantitatively prepare a solution of pepsin in *Diluted hydrochloric acid solution* containing approximately 0.90–1.00 USP Pepsin Units/mL.

[NOTE—Prepare this solution immediately before use.]

**Procedure:** Prepare test tubes as follows (use tubes that can accommodate and allow mixing on a vortex mixer of a volume of NLT 16 mL). Separately transfer 1.0-mL aliquots of the *Standard solution* into four test tubes (two tubes for the analysis of the *Standard solution* and two blanks for the *Standard solution*).

Prepare a substrate blank by transferring 1.0 mL of *Diluted hydrochloric acid solution* into a single test tube.

Separately transfer 1.0-mL aliquots of the *Sample solution* into four test tubes (two tubes for the analysis of the *Sample solution* and two blanks for each *Sample solution*).

To each of the blank tubes add 10.0 mL of *TCA solution*, and mix on a vortex mixer.

Place all test tubes in a water bath maintained at  $25 \pm 1^\circ$ , and allow equilibration for 5 min. Add 5.0 mL of the *Substrate solution* (previously equilibrated to  $25 \pm 1^\circ$ ) to each of the blank tubes, mix on a vortex mixer, and return the tubes to the water bath to incubate for 10 min.

At time equals zero, rapidly pipet 5.0 mL of the *Substrate solution* (previously equilibrated to  $25 \pm 1^\circ$ ) successively and at accurately timed intervals of at least 30 s into each of the tubes containing the *Sample solution* and the *Standard solution*. Mix each tube immediately

after adding the *Substrate solution* by brief vortex mixing, and return the tube to the water bath. After 10.0 min, at the corresponding time intervals, stop the reaction by rapidly pipetting 10.0 mL of *TCA solution* into each tube. Do not return the tubes to the water bath. Allow all tubes, including blanks, to sit at room temperature for 25 min. After 25 min, mix the contents of each tube on a vortex mixer, and pass the contents through a fluted paper filter into clean tubes, discarding the first 5 mL of the filtrate.

[NOTE—A suitable filter is the Whatman No. 41 ashless filter.]

Determine the absorbance of each filtrate using the following conditions.

**Wavelength:** 280 nm

**Cell:** 1 cm

**Blank:** Substrate blank

Calculate the average absorbance for each filtrate. Correct the absorbance of each *Standard solution*:

$$A_{SC} = A_S - A_{SB}$$

$A_S$  = average absorbance of the *Standard solution*

$A_{SB}$  = average absorbance of the *Standard solution* blank

Correct the absorbance of each *Sample solution*:

$$A_{UC} = A_U - A_{UB}$$

$A_U$  = average absorbance of the *Sample solution*

$A_{UB}$  = average absorbance of the *Sample solution* blank

Calculate the activity of pepsin in the sample:

$$\text{Activity (Units/mg)} = (A_{UC}/A_{SC}) \times (C_S/C_U) \times P$$

$A_{UC}$  = absorbance of each *Sample solution*

$A_{SC}$  = absorbance of each *Standard solution*

$C_S$  = concentration of the *Standard solution* (mg/mL)

$C_U$  = concentration of the *Sample solution* (mg/mL)

$P$  = activity of the [USP Pepsin for Assay RS](#) (Units/mg)

One Pepsin Unit is defined as the quantity of enzyme that produces the equivalent of 1  $\mu\text{mol}$  of tyrosine per min under the conditions of the activity determination procedure.

**Auxiliary Information** - Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
PEPSIN	<a href="#">Margareth R.C. Marques</a> Principal Scientific Liaison	HDQ Headquarters

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