Determination of Proteolytic Activity of CIBENZA® EP150 in Feed

Proteolytic Activity of CIBENZA® EP150 in Feed

Table of Contents

Table of Contents 2
1 Foreword 5
2 Introduction 5
3 Title 5
4 Warnings 5
5 Scope 5
Table 1 – Scope of Analytical Method 5
6 Normative References 6
7 Terms and definitions 6
7.1 Laboratory sample 6
7.2 Test sample 6
7.3 Test portion 6
7.4 Standard solution 6
8 Principle 6
9 Materials 6
10 Reagents 7
10.1 Substrate Solution 7
10.1.1 Composition 7
10.1.2 Preparation 7
10.2 Concentrated Sodium Hydroxide Solution 8
10.2.1 Composition 8
10.2.2 Preparation 8
10.3 Dilute Sodium Hydroxide Solution 8
10.3.1 Composition 8
10.3.2 Preparation 8
10.4 Stock Phosphate Buffer 9
10.4.1 Composition 9
10.4.2 Preparation 9
10.5 Tween® Solution 9
10.5.1 Composition 9
10.5.2 Preparation 9
10.6 SDS Solution 10
10.6.1 Composition 10
10.6.2 Preparation 10
10.7 Feed Extraction Buffer 10
10.7.1 Composition 10
10.7.2 Preparation 10
10.8 Enzyme Extraction Buffer 11
10.8.1 Composition 11
10.8.2 Preparation 11
10.9 Reaction Buffer 11
10.9.1 Composition 11
10.9.2 Preparation 11
10.10 Stop Solution 12
10.10.1 Composition 12
10.10.2 Preparation 12

Proteolytic Activity of CIBENZA® EP150 in Feed

11 Apparatus and glassware 12

12 Procedure 13
12.1 Standard curve 13
12.1.1 Preparation of working solution for standard curve 13
12.1.2 Generation of standard curve 14
12.2 Test portion preparation 15
12.3 Assay procedure 15

13 Calculations 16

14 Test report 17

Proteolytic Activity of CIBENZA® EP150 in Feed

1 Foreword

This is the second version of this analytical method.

2 Introduction

This method describes the procedure for measuring the proteolytic activity of the feed additive CIBENZA® EP150 in feed. The method is based on spectrophotometric measurement of a chromophore released from a synthetic peptide upon cleavage of an amide bond by the protease activity of CIBENZA® EP150.

3 Title

Proteolytic Activity in Feed by Hydrolysis of Succinyl-Ala-Ala-Pro-Phe-para-Nitroanilide

4 Warnings

WARNING — Persons using this method should be familiar with normal laboratory practice. Caution must be used when handling feed containing CIBENZA® EP150. Always wear gloves and a laboratory coat when working with feed containing CIBENZA® EP150 or solutions thereof. Avoid breathing CIBENZA® EP150 when weighing or transferring. Consult the MSDS for each substance used in this method for full safety information. This protocol does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national registry conditions.

5 Scope

This method is suitable only for analyzing proteolytic activity of complete animal feeds containing CIBENZA® EP150. The method should not be used to measure proteolytic activity of CIBENZA® EP150 feed additive or premixtures containing CIBENZA® EP150.

Table 1 – Scope of Analytical Method

Method of chemical analysis	Active substances
Product to which it applies	CIBENZA® EP150 feed additive in feed
Limit of detection (mash feed)	30 U/g
Limit of quantification (mash feed)	61 U/g
Limit of detection (pelleted feed)	41 U/g
Limit of quantification (pelleted feed)	128 U/g
Limitations	None
Interferences	None

Proteolytic Activity of CIBENZA® EP150 in Feed

6 Normative References

ISO 31 (all parts), Quantities and units

ISO 1000, SI units and recommendations for the use of their multiples and of certain other units

7 Terms and definitions

7.1 Laboratory sample

A sample as prepared for sending to the laboratory and intended for testing.

7.2 Test sample

A sample prepared from the laboratory sample and from which the test portions will be taken

7.3 Test portion

The quantity of material drawn from the test sample (or from the laboratory sample if both are the same) and on which the test or observation is actually carried out.

7.4 Standard solution

Solution of accurately known concentration of an element, an ion, a compound or a group derived from the substance used for its preparation.

8 Principle

CIBENZA® EP150 contains a protease. Activity of the protease in CIBENZA® EP150 is measured by spectrophotometric detection of the chromophore para-nitroanaline (pNA), which is released from the synthetic peptide substrate (Succinyl-Ala-Ala-Pro-Phe-pNA) upon cleavage of the peptide bond that links the chromophore to the peptide. The free chromophore is detected by measuring the optical density at 410 nm.

Protease activity of CIBENZA® EP150 is reported as azocasein units (historical assay method).

9 Materials

9.1 Sodium Tetraborate, decahydrate; CAS # 1303-96-4

9.2 Sodium Phosphate, dibasic anhydrous; CAS # 7558-79-4

9.3 N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (S-AAPF-pNA peptide); CAS # 70967-97-4

9.4 Polysorbate 20 (Tween® 20); CAS # 9005-64-5

9.5 Dimethyl Sulfoxide; CAS # 67-68-5

9.6 Sodium Dodecyl Sulfate; CAS # 151-21-3

9.7 Glacial acetic acid; CAS # 64-19-7

9.8 Sodium Hydroxide; CAS # 1310-73-2

9.9 CIBENZA® EP150

9.10 Disposable serological pipettes

9.11 Disposable standard 7.5 ml transfer pipette (VWR #414004-004 or equivalent)

9.12 Microcentrifuge tubes – 1.5 ml capacity

9.13 Micropipette tips

9.14 Disposable cuvettes

9.15 Calibration standard solutions for pH meter – minimum of pH 7.0 and 10.0 solutions

9.16 Magnetic stir bars

10 Reagents

10.1 Substrate Solution

10.1.1 Composition

S-AAPF-pNA peptide	0.0313 g
DMSO	1 ml

10.1.2 Preparation

Prepare only enough substrate solution to be used in one day. Prepare the 50 mM S-AAFP-pNA solution as follows:

- For each ml of Substrate Solution required, weigh out 0.0313g of S-AAPF-pNA peptide directly into the appropriate size tube.
- Add the appropriate volume of DMSO – 1 ml for each 0.0313 g of substrate weighed. A fraction of 1 ml should be added if a fraction of 0.0313 g is weighed.
- Intermittently vortex the tube vigorously until the peptide has been completely solubilized (usually 2-4 min).
- Briefly (5 seconds) centrifuge the tube to collect the solution at the bottom of the tube.

Proteolytic Activity of CIBENZA® EP150 in Feed

10.2 Concentrated Sodium Hydroxide Solution

10.2.1 Composition

Sodium hydroxide	50 g
Deionized water	to volume

10.2.2 Preparation

Prepare a 500 g/L solution of sodium hydroxide as follows:

- Add the sodium hydroxide to a beaker
- Add approximately 60 ml of water
- Stir at room temperature until dissolved
- Adjust volume to 100 ml with water using a graduated cylinder
- Transfer to a storage container and store at room temperature

10.3 Dilute Sodium Hydroxide Solution

10.3.1 Composition

Sodium hydroxide	4 g
Deionized water	100 ml

10.3.2 Preparation

Prepare a 1 N sodium hydroxide solution as follows:

- Add approximately 80 ml of water to a beaker
- Add the sodium hydroxide and stir at room temperature until to dissolve
- Adjust the volume to 100 ml with water using a graduated cylinder
- Transfer to a container and store at room temperature

Proteolytic Activity of CIBENZA® EP150 in Feed

10.4 Stock Phosphate Buffer

10.4.1 Composition

Sodium phosphate, anhydrous	28.4 g
Deionized water	to 1000 ml

10.4.2 Preparation

Prepare a stock of Sodium Phosphate buffer at pH 10.0 as follows:

- Add approximately 900 ml of water to a beaker
- Add the anhydrous sodium phosphate and stir at room temperature to dissolve
- Adjust the pH to 10.0 ± 0.05 with 1 N sodium hydroxide solution (10.3)
- Adjust the volume to 1000 ml with deionized water using a graduated cylinder
- Transfer to a bottle and store at room temperature
- Check pH before each use and adjust to 10.0 ± 0.05 if necessary

10.5 Tween® Solution

10.5.1 Composition

Tween® 20	10 g
Deionized water	to 100 ml

10.5.2 Preparation

Prepare a 100 g/L solution of Tween® 20 as follows:

- Weigh 10 g of Tween® 20 directly into a 100 ml graduated cylinder
- Adjust the volume to 100 ml with deionized water
- Cover the top of the cylinder securely with Parafilm® and invert the cylinder until the solution is homogeneous
- Transfer the solution to an amber bottle and store in the dark at room temperature
- The solution can be used for up to one week

Proteolytic Activity of CIBENZA® EP150 in Feed

10.6 SDS Solution

10.6.1 Composition

Sodium dodecyl sulfate	10 g
Deionized water	to 100 ml

10.6.2 Preparation

Prepare a 100 g/L solution of sodium dodecyl sulfate (SDS) as follows:

- Add approximately 80 ml of deionized water to a beaker
- Add the SDS and stir at room temperature until the SDS is completely dissolved
- Adjust the volume to 100 ml with deionized water using a graduated cylinder
- Transfer to a bottle and store at room temperature

10.7 Feed Extraction Buffer

10.7.1 Composition

Sodium tetraborate, decahydrate	38.14
Concentrated sodium hydroxide solution (10.2)	as needed
Tween® 20 solution (10.5)	50 ml
Deionized water	to volume

10.7.2 Preparation

The following procedure yields a solution that contains 100 mM sodium tetraborate and 5 g/L Tween® 20 at a pH of 10.0

- Add approximately 800 ml water to a beaker
- Add the sodium tetraborate and stir until dissolved
- Adjust pH to 10.0 ± 0.05 with concentrated sodium hydroxide solution (10.2)
- Add Tween® 20 solution (10.5)
- Adjust to 1000 ml with water using a graduated cylinder

- Transfer to a bottle and store at room temperature – check pH before use each day and adjust to 10.0 ± 0.05 if necessary
- The solution can be used for up to one week

10.8 Enzyme Extraction Buffer

10.8.1 Composition

Stock sodium phosphate solution (10.4)	50 ml
Tween® 20 solution	5 ml
Deionized water	45 ml

10.8.2 Preparation

The following procedure yields a solution that contains 100 mM sodium phosphate and 5 g/L Tween® 20 at a pH of 10.0

- Add 50 ml of Stock sodium phosphate solution (10.4) to a graduated cylinder
- Add 5 ml of Tween® 20 (10.5) solution to the graduated cylinder
- Adjust the volume to 100 ml with water
- Transfer the contents of the graduated cylinder to a beaker and mix thoroughly
- Check pH and adjust to 10.0 ± 0.05 if necessary
- Store at room temperature
- The solution can be used for up to one week

10.9 Reaction Buffer

10.9.1 Composition

Stock sodium phosphate buffer (10.4)	50 ml
Deionized water	50 ml

10.9.2 Preparation

The following procedure yields a solution that contains 100 mM sodium phosphate at a pH of 10.0

- Add the stock sodium phosphate buffer (10.4) to a graduated cylinder
- Adjust the volume to 100 ml with deionized water
- Transfer contents of the graduated cylinder to a beaker and mix thoroughly
- Transfer to a bottle
- Check the pH before each use and adjust to pH 10.0 ± 0.05 if necessary
- Store at room temperature

10.10 Stop Solution

10.10.1 Composition

Glacial acetic acid	10 ml
Sodium dodecyl sulfate	10 ml
Deionized water	80ml

10.10.2 Preparation

The following procedure yields a solution that contains 100 ml/L of glacial acetic acid and 10 g/L sodium dodecyl sulfate:

- Add approximately 70 ml of deionized water to a beaker
- Carefully add 10 ml of glacial acetic acid and 10 ml Sodium dodecyl sulfate solution (10.6)
- Adjust volume to 100 ml with deionized water using a graduated cylinder
- Transfer the contents of the graduated cylinder to a beaker and mix thoroughly
- Transfer to a bottle and store at room temperature

11 Apparatus and glassware

11.1 Water bath or dry bath set at 37°C ± 1°C

11.2 Multi-position stir plate or at least 3-4 individual stir plates

11.3 Analytical balance – qualification and calibration must be complete and on file, capable of measuring to 0.001 g

11.4 Visible range spectrophotometer set at 410 nm

11.5 Fritsch rotor speed mill – model Pulverisette 14 (or equivalent) with 1 mm screen

11.6 Timer capable of counting up

11.7 Microcentrifuge capable of generating a force of 14000 x g

11.8 Vortex mixer

11.9 pH meter accurate to 0.01 pH unit

11.10 Micropipettes capable of delivering 50 µl, 400µl, and 500 µl

11.11 Grade A glassware – 250 or 400 ml beakers

12 Procedure

12.1 Standard curve

A standard curve of CIBENZA® EP150 in feed must be prepared each day the analysis is performed and for every type of diet to be analyzed. The standard curve MUST be prepared using a control diet that is identical to the sample diet except that it lacks CIBENZA® EP150. The standard curve for a mash diet must be prepared using the corresponding mash control diet. Likewise, the standard curve for a pelleted diet must be prepared using the corresponding pelleted control diet.

12.1.1 Preparation of working solution for standard curve

Prepare a CIBENZA® EP150 working solution as detailed below:

— If not already known, determine the protease activity of one lot of CIBENZA® EP150 using Novus International Method 260140-3 ("Proteolytic Activity by Hydrolysis of Succinyl-Ala-Ala-Pro-Phe-para-Nitroanilide").

— Calculate the weight of CIBENZA® EP150 needed to prepare 50 ml of a 1500 U/ml solution (need a total of 75,000 Units)

— Weigh the amount of CIBENZA® EP150 calculated above to the nearest mg and quantitatively transfer to a 50 ml volumetric flask

— Adjust the volume to 50 ml with Enzyme Extraction Buffer (10.8)

— Cap tightly and shake the flask to dissolve – shake for 10-15 seconds every couple of minutes for a total of 10-15 minutes

— Use this CIBENZA® EP150 working solution to prepare the in-feed standards as described in Step 12.1.2

Table 2 — Preparation of CIBENZA® EP150 In-feed Standards

Standard Number	Feed (g)	CIBENZA® EP150 Working Solution (ml)	Feed Extraction Buffer (10.7) (ml)	CIBENZA® EP150 Concentration (U/ g feed)
1	5	0.0	200.0	0
2	5	0.5	199.5	150

3	5	1.0	199.0	300
4	5	1.5	198.5	450
5	5	2.0	198.0	600

12.1.2 Generation of standard curve

NOTE 1 The maximum number of test portions that can be analyzed at one time depends on the number of spaces present in the microcentrifuge. All reaction tubes from a single test portion must fit in the microcentrifuge at one time.

EXAMPLE If the microcentrifuge has 20 positions, then a maximum of four test portions can be analyzed at one time (5 reaction tubes per portion x 4 portions = 20 reaction tubes.

NOTE 2 To perform analysis of a large number of test portions, preparation of a new set of 3 - 4 feed extracts can be initiated every 5 minutes.

12.1.2.1 Grind an approximately 100 g test sample of control diet in the Fritsch rotor speed mill (11.4) using a 1 mm screen

12.1.2.2 Weigh five 5 g test portions of ground control diet.

12.1.2.3 Place five beakers on a multi-position stir plate or on five individual stir plates

12.1.2.4 Add 200 ml of Feed Extraction Buffer (10.7) to each beaker.

12.1.2.5 Prepare feed extracts for a set of 3 - 4 standards as follows (depending on the number of spaces present in the microcentrifuge; see NOTE 1 above):

12.1.2.5.1 While the buffer is stirring, quickly add one pre-weighed ground feed portion followed rapidly by the appropriate volume of CIBENZA® EP150 working solution (Table 2) to each of 3 - 4 beakers.

12.1.2.5.2 Allow the samples to mix for exactly 10 min.

12.1.2.5.3 While stirring, remove 1-2 ml of extract using a transfer pipet and transfer to a microcentrifuge tube.

12.1.2.5.4 Centrifuge each sample for 1 min at 2400 x g at room temperature.

12.1.2.5.5 Immediately begin the Assay Procedure (12.3) before preparing another set of feed extracts.

12.1.2.6 If necessary, continue repeating Steps 12.1.2.5.1 through 12.1.2.5.5 for the remaining standards.

NOTE 1 Only begin preparing additional feed extracts after Step 12.3.7 is completed for the current set of feed extracts.

NOTE 2 Extracts of test portions can be prepared at the same time as the standards.

12.1.2.7 Calculate the corrected mean optical density for each standard according to Equation 1.

12.1.2.8 Plot the corrected optical density for each standard on the Y-axis and the CIBENZA® EP150 concentration (azocasein units per gram of feed) from Table 2 on the X-axis.

12.1.2.9 Use linear regression to derive the equation of the curve; do not force the intercept through zero.

12.1.2.10 The correlation coefficient (R^2) must be ≥0.95 for the standard curve to be valid. If the R^2 value is less than 0.95, then standard curve must be repeated.

Proteolytic Activity of CIBENZA® EP150 in Feed

12.2 Test portion preparation

NOTE 1 The maximum number of test portions that can be analyzed at one time depends on the number of spaces present in the microcentrifuge. All reaction tubes from a single test portion must fit in the microcentrifuge at one time.

EXAMPLE If the microcentrifuge has 20 positions, then a maximum of four test portions can be analyzed at one time (5 reaction tubes per portion x 4 portions = 20 reaction tubes.

NOTE 2 To perform analysis of a large number of feed test portions, preparation of a new set of 3 - 4 feed extracts can be initiated every 5 minutes.

12.2.2.1 Grind an approximately 100 g test sample of test diet in the Fritsch rotor speed mill (11.4) using a 1 mm screen

12.2.2.2 Weigh three 5 g test portions of each ground test sample to be analyzed.

12.2.2.3 Place one beaker for each test portion on a multi-position stir plate or on individual stir plates.

12.2.2.4 Add 200 ml of Feed Extraction Buffer (10.7) to each beaker.

12.2.2.5 Prepare feed extracts for a set of 3 - 4 test portions as follows (depending on the number of spaces present in the microcentrifuge; see NOTE 1 above):

12.2.2.5.1 While the buffer is stirring, quickly add one pre-weighed ground test portion to each of 3 - 4 beakers.

12.2.2.5.2 Allow the samples to mix for exactly 10 min.

12.2.2.5.3 While stirring, remove 1-2 ml of extract using a transfer pipet and transfer to a microcentrifuge tube.

12.2.2.5.4 Centrifuge each sample for 1 min at 2400 x g at room temperature.

12.2.2.5.5 Immediately begin the Assay Procedure (12.3) before preparing another set of feed extracts.

12.2.2.6 Continue repeating Steps 12.2.2.5.1 through 12.2.2.5.5 for the remaining test portions to be analyzed.

NOTE Only begin preparing additional feed extracts after Step 12.3.7 is completed for the current set of feed extracts.

12.3 Assay procedure

12.3.1 Prepare a set of five microcentrifuge tubes for each feed extract to be analyzed by adding 400 µl of Reaction Buffer (10.9). Two tubes serve as enzyme blanks and the remaining three tubes are for triplicate analysis of an extract.

12.3.2 For each feed extract prepared, add 50 µl of feed extract to the three tubes for triplicate analysis.

NOTE DO NOT ADD A FEED EXTRACT TO THE ENZYME BLANK TUBES AT THIS TIME

12.3.3 Pre-incubate all tubes at 37°C for 5 min

NOTE Once the test portion is added to the tubes, do not remove the tubes from the water (or dry) bath until the Stop Solution (10.10) is added.

12.3.4 Carefully add 50 µl of Substrate Solution (10.1) to the <u>first</u> microcentrifuge tube containing the pre-warmed feed extract and mix by pipetting up and down 3-4 times being very careful to avoid touching the side of the tube with the pipette tip.

NOTE 1 DO NOT VORTEX TUBES: mix by pipetting up and down 3-4 times.

NOTE 2 Be aware that excess substrate may cling to the outside of the pipette tip. If this occurs, carefully wipe off the excess from the tip before adding the substrate to the reaction tube.

NOTE 3 Do not allow the tip to touch the side of the tube when withdrawing the tip from the tube.

12.3.5 Immediately start a timer counting up.

12.3.6 At a pre-defined interval, start carefully adding 50 µl of Substrate Solution (10.1) to the remaining microcentrifuge tubes containing feed extract.

NOTE 1 DO NOT VORTEX TUBES: mix by pipetting up and down 3-4 times.

NOTE 2 Analysts with little or no experience performing this method should use an interval of 20-30 seconds. Analysts with more experience with this method may use a 10-20 seconds interval.

12.3.7 After adding substrate to all tubes containing feed extract, add 50 µl of substrate to all of the enzyme blank tubes.

NOTE DO NOT VORTEX TUBES: mix by pipetting up and down 3-4 times.

12.3.8 Allow the reactions to proceed for **exactly** 60 min at 37°C.

12.3.9 Using the same pre-defined time interval as for step 12.3.6, add 500 µl of Stop Solution (10.10) to every microcentrifuge tube containing feed extract.

12.3.10 Vortex briefly and place tubes at room temperature.

12.3.11 Add 500 µl of Stop Solution (10.10) to every enzyme blank tube.

12.3.12 Add a 50 µl feed extract of the appropriate test portion or standard to both corresponding enzyme blank tubes and vortex to mix. Place at room temperature.

12.3.13 Centrifuge each tube for 5 minutes at 14000xg.

12.3.14 Carefully decant the supernatant into a cuvette. Be careful to not disturb the pellet that at the bottom of the tube.

12.3.15 Set the spectrophotometer to read at 410 nm and then zero it against water.

12.3.16 Measure the optical density at 410 nm.

NOTE Steps 12.3.9 through 12.3.16 should be completed within a 20 minute window.

12.3.17 Calculate the protease activity as described in Clause 13.

13 Calculations

Calculate the number of azocasein units per gram of feed by comparing the optical density of the test sample to the standard curve.

13.1 Calculate the mean optical density (OD_s) for each sample extract (test or standard) using Equation 1:

Proteolytic Activity of CIBENZA® EP150 in Feed

Equation 1 Mean optical density

$$OD_S = \left(\frac{OD_{tube\#1} + OD_{tube\#2} + OD_{tube\#3}}{3} \right) - \left(\frac{OD_{enzyme\,blank\#1} + OD_{enzyme\,blank\#2}}{2} \right)$$

13.2 Convert the sample OD to units of protease activity per gram of feed (U/g feed) according to Equation 2 using the equation derived from the standard curve:

Equation 2 Protease Activity

$$Units/g\ of\ feed = \frac{OD_S - Y_0}{m}$$

where

OD_S is calculated in Equation 1

Y_0 is the Y-intercept of standard curve

m is the slope of standard curve

14 Test report

The test report shall include the following information:

a) all information necessary for identification of the sample tested

b) a reference to this method

c) the results of the test as calculated in Clause 13

d) any deviations from the procedure

e) any unusual features observed during the procedure

f) the date of the test