Determination of Proteolytic Activity of CIBENZA® DP100 in Premixtures

Table of Contents

1 Foreword

N/A

2 Introduction

This method describes the procedure for measuring the proteolytic activity of the feed additive CIBENZA® DP100 in premixtures. 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® DP100.

3 Title

Proteolytic Activity in Premixtures 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 premixtures containing CIBENZA® DP100. Always wear gloves and a laboratory coat when working with premixtures containing CIBENZA® DP100 or solutions thereof. Avoid breathing CIBENZA® DP100 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 premixtures containing CIBENZA® DP100. The method should not be used to measure proteolytic activity of CIBENZA® feed additive or complete animal feeding stuffs containing CIBENZA® DP100.

Table 1 – Scope of Analytical Method

Method of chemical analysis	Active substances
Product to which it applies	CIBENZA® DP100 feed additive in premixtures
Limit of detection	15342 U/g
Limit of quantification	21699 U/g
Limitations	None
Interferences	None

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® DP100 contains a protease. Activity of the protease in CIBENZA® DP100 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.

One pNA unit is defined as the release of 1 micromole (μmole) of pNA per minute under the conditions specified in this procedure. Protease activity of CIBENZA® DP100 is reported as azocasein units (historical assay method) where pNA units are converted to azocasein units using a scaling factor.

9 Materials

9.1 Tris base; CAS # 77-86-1

9.2 Calcium Chloride, anhydrous; CAS # 10043-52-4

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 4-nitroaniline; CAS # 100-01-6

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

9.8 Hydrochloric Acid; CAS # 7647-01-0

9.9 Disposable serological pipettes

9.10 Microcentrifuge tubes – 2.0 ml capacity

9.11 Micropipette tips

9.12 Screw-cap centrifuge tubes – 50 ml capacity

9.13 Glass test tubes – 16 x 125 mm

9.14 Disposable cuvettes

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

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, weight 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 solublized (usually 2-4 min).
- Briefly (5 seconds) centrifuge the tube to collect the solution at the bottom of the tube.

10.2 Reaction Buffer

10.2.1 Composition

Tris base	12.114 g
Calcium chloride – anhydrous	0.222 g
Tween 20	5 g
Deionized water	1000 ml

10.2.2 Preparation

The following procedure yields a solution that contains 100 mM Tris, 2 mM CaCl, and 5 g/L Polysorbate 20 at a pH of 8.0

- Add approximately 900 ml deionized water to a beaker
- Add the Tris base and calcium chloride
- Adjust the pH to 8.0 ± 0.1 with hydrochloric acid
- Add the Tween 20
- Adjust the volume to 1000 ml with deionized water using a graduated cylinder
- Store at room temperature and check the pH before each use

10.3 Stop Solution

10.3.1 Composition

Glacial acetic acid	10 ml
Deionozed water	90 ml

10.3.2 Preparation

The following procedure yields a 100 ml/L solution of acetic acid:

- Add approximately 80 ml of deionized water to a beaker
- Carefully add 10 ml of glacial acetic acid
- Adjust volume to 100 ml with deionized water in a graduated cylinder

10.4 Standard pNA Solution

10.4.1 Composition

para-nitroanaline (pNA)	0.028 g
DMSO	10 ml

10.4.2 Preparation

The following procedure yields a 20.0 mM solution of pNA:

- Add approximately 8 ml of DMSO to a 10 ml volumetric flask.
- Weigh indicated amount of para-nitroanaline and quantitatively transfer to the volumetric flask.
- Mix the solution by swirling the flask until all the pNA is dissolved.
- Adjust volume to 10 ml with DMSO and mix thoroughly.
- Prepare the solution fresh each time a standard curve is generated.

11 Apparatus and glassware

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

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

11.3 Visible range spectrophotometer set at 410 nm

11.4 Timer capable of counting up

11.5 Microcentrifuge capable of generating a force of 14000 x g

11.6 Vortex mixer

11.7 pH meter accurate to 0.01 pH unit

11.8 Tris-compatible pH electrode

11.9 Micropipettes capable of delivering 50 µl, 500 µl, and 800 µl

11.10 Grade A glassware

12 Procedure

12.1 Standard curve

A standard curve must be prepared if any of the following conditions apply:

- Each analyst must generate his/her own standard curve
- Absorbance of samples will be measured using a spectrophotometer other than the one used to prepare the previous standard curve
- When the standard curve is > 3 months old

12.1.1 Preparation of working stocks for standard curve

Prepare fresh Standard pNA Solution (10.4). Prepare working stocks of pNA by making eight consecutive serial 2-fold dilutions as shown in Table 2:

Table 2 — Preparation of pNA Working Stocks for Making a Standard Curve

Starting pNA Concentration (mM)	Volume of pNA Solution (ml)	Volume of DMSO (ml)	Ending Concentration of pNA (mM)
20.0	2.5	2.5	10.0
10.0	2.5	2.5	5.0
5.0	2.5	2.5	2.5
2.5	2.5	2.5	1.25
1.25	2.5	2.5	0.625
0.625	2.5	2.5	0.131
0.313	2.5	2.5	0.157
0.157	2.5	2.5	0.078

12.1.2 Generation of standard curve

12.1.2.1 For each pNA solution shown in Table 3, prepare duplicate "mock reaction" tubes by adding the following to glass test tubes:

- 450 µl of Reaction Buffer (10.2)
- 50 µl of pNA working stock solution (Table 3)
- 500 µl of Stop Solution (10.3)

12.1.2.2 Vortex the tubes to ensure the contents are completely mixed

12.1.2.3 Transfer the contents of each tube to a cuvette

12.1.2.4 Measure the absorbance at 410 nm

12.1.2.5 Calculate the mean absorbance for each concentration of pNA standard

12.1.2.6 Plot the mean absorbance on the y-axis *vs.* the concentration of pNA in the "mock reaction" (µmole/ml) (Table 3) on the x-axis

- Derive an equation using linear regression analysis
- Do not force the line through zero
- If the correlation coefficient is < 0.98, then prepare a new standard curve

Table 3 — Final pNA Concentrations for Standard Curve

Working Stock Concentration (mM)	pNA Concentration in "Mock Reaction" (µmoles/ml)
5.0	0.25
2.5	0.125
1.25	0.0625
0.625	0.0313
0.313	0.0156
0.157	0.0078
0.078	0.0039

12.2 Test sample preparation

12.2.1 Weigh a 1.0 ± 0.01 g portion of the laboratory sample and transfer into a 50 ml screw-cap tube

12.2.2 Add 40 ml of Reaction Buffer (10.2) and tightly close the cap

12.2.3 Shake the tube vigorously for 20 to 30 seconds

NOTE If clumps of dry test sample are still present in the tube, turn the tube upside down and forcibly tap the tube on the bench top until the entire sample is wetted

12.2.4 Let the test sample stand for 10 to 15 minutes, shaking occasionally (4-5 times)

12.2.5 Dilute the test sample to 6.25 µg/ml by serially diluting 320 fold (see Table 4) with Reaction Buffer (10.2). Be sure to shake the test sample immediately prior to removing a portion for dilution.

Table 4 — Dilution Scheme for Sample Preparation

Step Number	Step Dilution Factor	Volume of Test Sample (ml)	Volume of Diluent (ml)	Total Dilution Factor
1	10	0.5	4.5	10
2	8	0.5	3.5	80
3	4	1.0	3.0	320

NOTE Make sure the entire amount of test sample is delivered to the diluent, especially for step number 1.

12.3 Assay procedure

12.3.1 Prepare a set of four microcentrifuge tubes for each test sample to be analyzed by adding 400 µl of Reaction Buffer (10.2). One tube serves as an enzyme blank and the remaining three tubes are for triplicate analysis of a test portion.

12.3.2 For every test sample to be analyzed, add a 50 µl test portion to the three tubes for triplicate analysis.

NOTE DO NOT ADD A TEST PORTION TO THE ENZYME BLANK TUBES AT THIS TIME

12.3.3 Pre-incubate the 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 (10.3) is added.

12.3.4 Carefully add 50 µl of Substrate Solution (10.1) to the first microcentrifuge tube containing the pre-warmed test portion and mix by pipetting up and down for at least 4 seconds.

NOTE 1 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 2 DO NOT VORTEX TUBES: mix by pipetting up and down until the substrate is homogeneously distributed – at least 4 seconds.

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.

NOTE 1 DO NOT VORTEX TUBES: mix by pipetting up and down until the substrate is homogeneously distributed – at least 4 seconds.

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 Allow the reactions to proceed for **exactly** 10 min at 37°C.

12.3.8 Using the same pre-defined time interval as for step 12.3.6, add 500 µl of Stop Solution (10.3) to each microcentrifuge tube.

12.3.9 Vortex briefly and place tubes at room temperature.

12.3.10 Add a 50 µl test portion of the appropriate test sample to the corresponding enzyme blank tube and vortex to mix.

NOTE If a precipitate is present, centrifuge the tubes in a microcentrifuge for 5 min at a minimum of 14000 x g.

12.3.11 Carefully decant the reaction mixture into a cuvette. If the tubes were centrifuged, be careful to not disturb any pellet that may be present.

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

12.3.13 Measure the optical density at 410 nm. Absorbance values must be between 0.1 and 2.6 after subtraction of the optical density obtained from the corresponding enzyme blank tube. If the absorbance value for any tube is outside this range, then the assay for that particular test sample must be repeated.

NOTE Steps 12.3.8 through 12.3.13 should be completed within a 20 min window.

12.3.14 Calculate the enzyme activity as described in Section 13.

13 Calculations

Calculate the number of pNA units and then convert to the number of azocasein units (historical assay units). One pNA unit of proteolytic activity is defined as the release of 1 µmole of pNA per minute under the conditions specified in this method.

8.1 Calculate the mean optical density (OD_s) for each sample using Equation 1:

Equation 1 Mean optical density

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

8.2 Convert the sample OD to µmoles/ml of pNA released according to Equation 2 using the equation derived from the standard curve:

Equation 2 pNA released

$$\mu\text{moles/ml of pNA released} = \frac{\text{OD}_S - Y_0}{\text{m}}$$

where

OD_S is calculated in Equation 1

Y_0 is the Y-intercept of standard curve

m is the slope of standard curve

8.3 Calculate the number of pNA units/g according to Equation 3:

Equation 3 pNA units

$$pNA\,units/g = \frac{(\mu moles/ml)\,x\,(DF)\,x\,(V_R/V_{TP})}{(\min)\,x\,(W/V_{TS})}$$

where

µmole/ml is calculated in Equation 2

DF is the dilution factor (DF = 320)

V_R is the total reaction volume in ml (V_r = 1.0)

V_{TP} is the volume of test portion in the reaction in ml (V_R = 0.05)

min is the time of reaction in miutes (min = 10)

W is the weight of the test sample in grams

V_{TS} is the volume of test sample in ml before dilution (V_{TS} = 40)

8.4 Covert pNA protease units to azocasein protease units (historical assay method) by applying the scaling factor shown in Equation 4 and then round off to the nearest whole number:

Equation 4 Azocasein units

$$Azocasein\,units\,/\,g = pNA\,units\,/\,g\;x\;SF$$

where

pNA units/g is calulated in Equation 3

SF is the scaling factor (61.8 - determined emperically)

14 Quality assurance control

Each time the method is performed, a single sample of known proteolytic activity (check standard) must be analyzed to assure that the method is working properly. The activity value of the current check standard must be within ± 10% of the running mean of the check standard. If the check standard is not within ± 10% of the running mean, then the assay is not valid and all of the samples must be assayed again.

The running mean for the check standard is calculated by summing all activity values obtained with a particular lot of protease and then dividing by the number of assays performed.

If the check standard values do not yet exist, analyze a protease sample 3 independent times over the course of at least three days. Calculate the mean activity value.

15 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