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Feed Additives

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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Protease (Subtilisin) I.U.B 3.4.21.62

(FAD-2021-0025; CRL/200088)

European Union Reference Laboratory
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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2021-0025 - CRL/200088**
Name of Product:	***Protease (Subtilisin)***
Active Agent (s):	**Protease**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **19/08/2022**
Report approved by: Date:	**Christoph von Holst** **19/08/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *protease (subtilisin*) under the category / functional group 4(a) "zootechnical additives / digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for all growing poultry species.

The *feed additive* is intended to be marketed under the trade name *PRoAct 360* as a solid formulation with a minimum *protease* activity of 600000 NFP / g *feed additive*.

According to the Applicant, one protease unit (NFP) is defined as the amount of enzyme that releases 1 μmol of p-nitroaniline from 1 mM substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) per minute at pH 9.0 and 37°C.

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a recommended minimum *protease* activity of 30000 NFP / kg complete *feedingstuffs*.

For the quantification of the *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted methods based on the quantification of the para-nitroaniline (pNA) released by the action of the *protease* on the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide substrate. These methods have been single-laboratory validated and further verified and the Applicant reported relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_{ip}) ranging from 1.9 to 14.9 %, and recovery rates (R_{rec}) ranging from 74 to 113 %.

Based on the overall available performance data, the EURL recommends for official control the above mentioned single-laboratory validated and further verified colorimetric methods for the quantification of the *protease (subtilisin)* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Protease (subtilisin), PRoAct 360, zootechnical additives, digestibility enhancers, all growing poultry species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *protease (subtilisin*) under the category / functional group 4(a) "zootechnical additives / digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the authorisation is sought for all growing poultry species [2].

According to the Applicant, the *feed additive* contains as an active substance *subtilisin protease (protease)* produced by a genetically modified strain of a *Bacillus Licheniformis* production strain (DSM 33099) [3].

The *feed additive* is intended to be marketed under the trade name *PRoAct 360* as a solid formulation with a minimum *protease* activity of 600000 NFP / g *feed additive* [3].

According to the Applicant, one protease unit (NFP) is defined as the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 mM substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) per minute at pH 9.0 and 37°C [4].

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a recommended minimum *protease* activity of 30000 NFP / kg complete *feedingstuffs* [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *protease (subtilisin)* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of the *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted single-laboratory validated [6-8] and further verified [9-10] colorimetric methods based on the quantification of the para-nitroaniline (pNA)

released by the action of the *protease* on the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide substrate [11-13].

For the quantification of the *protease* activity in the *feed additive*, the sample (between 0.28 and 1.0 g) is dissolved in Tris-buffer (pH 9) by stirring the mixture between 30 to 90 min and further diluted with the Tris-buffer. Then, the enzymatic analysis is carried out using the following microtiter method. The 96 well plate and the substrate are preheated at 37 ^{o}C for 20 to 30 min. Then, the standard and the samples are diluted in a deep well plate, covered and shaken for 4 min. Aliquots (50 µl) of the samples and the standards, are placed in the microtiter plate and mixed in the wells with the substrate (150 µl), the plate is then placed on the plate carrier and the reading is started. The absorbance of the samples and the standards are measured at 405 nm [11] and used for the determination of the concentration of pNA.

For the quantification of the *protease* activity in *premixtures* the samples (around 5 g), are treated with the extraction buffer (100 mM glycine-sodium hydroxide buffer, pH 9, containing 1.5 % EDTA with 0.01 % Tween 20) for 1 h. An aliquot of the extract is centrifuged for 3 min and the supernatant is properly diluted. Aliquots (0.1 ml) of the diluted extracts, are mixed with a preheated substrate solution and incubated at 65 °C for 60 min. After the incubation, 0.2 ml of ethanol are added. The tubes are then mixed and centrifuged for 3 min. The clean supernatant is then measured at 405 nm and the *protease* activity is determined using a standard calibration curve prepared with a reference *protease* enzyme of known activity, available from the Applicant upon request [12].

For the quantification of the *protease* activity in *feedingstuffs,* the samples (around 50 g), are treated with the extraction buffer (100 mM sodium chloride containing 0.01 % Tween 20) for 1 h. An aliquot of the extract is centrifuged for 3 min and the supernatant is diluted 50 times with the assay buffer (pH 9). Aliquots (0.1 ml) of the diluted extracts, are mixed with a preheated substrate solution and incubated at 65 °C for 60 min. After the incubation, 0.2 ml of ethanol is added. The tubes are then mixed and centrifuged for 3 min. The clean supernatant is then measured at 405 nm and the *protease* activity is determined using a calibration curve prepared with a blank feed sample supplemented with a reference *protease* enzyme of known activity, available from the Applicant upon request. The calibrant solutions undergo a similar procedure as the *feedingstuffs* samples [13].

This approach requires the use of blank feed sample for the quantification of the *protease* activity in *feedingstuffs*. Therefore, whenever a blank feed sample is not available, the Applicant upon request of the EURL, proposed to prepare the calibration curve replacing the blank feed by a *feedingstuffs* sample submitted beforehand to a heating treatment (at 130 ^{o}C for 17 h) to ensure that any enzyme present is deemed inactive [14].

Table 1. Performance characteristics of the methods for the quantification of *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs*

	Feed additive		***Premixtures***		***Feedingstuffs***	
	Validation	**Verification**	**Validation**	**Verification**	**Validation**	**Verification**
Activity, NFP/g	713639	879836	1000-15000	15000	5 - 150	10 - 150
RSD_r, %	3.7	1.9	4.9 - 14.9	3.6 - 6.7	3.1 - 9.3	1.5 - 14.3
RSD_{ip}, %	4.0	1.9	6.3 - 14.5	6.6	3.8 - 10.5	3.2 - 13.9
R_{Rec}, %	98-102	100	96 - 106	113	96 - 105	74 - 113
Reference	[6]	[9]	[7]	[10]	[8]	[10]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively;
R_{Rec}: a recovery rate

Furthermore, the Applicant also provided another test using a different feed blank i.e. without protease, which lead to protease activity values closer to the activities obtained when using the blank feed from the protease trial. The EURL considers both approaches as suitable alternatives to determine the *protease* enzyme activity in *feedingstuffs.*

The performance characteristics reported by the Applicant in the frame of the validation and verification studies [6-10] for the quantification of the *protease* activity in the *feed additive*, *premixtures* and *feedingstuffs* are presented in Table 1. In addition, the Applicant reported a limit of detection (LOD) and a limit of quantification (LOQ) of 321 and 2194 NFP / kg *feedingstuffs*, respectively [8], which are far below the minimum *protease* activity recommended by the Applicant [5].

Based on the overall available performance data, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods described above for the quantification of the *protease (subtilisin)* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not relevant for the present application.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods based on the quantification of the para-nitroaniline (pNA) released by the action of *protease* on the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide substrate for the determination of the *protease (subtilisin)* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of the *protease (subtilisin)* activity in the *feed additive*, *premixtures* and *feedingstuffs:*

- colorimetric methods based the enzymatic reaction of *protease* on the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide substrate

One protease unit (NFP) is defined as the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 mM substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) per minute at pH 9.0 and 37°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *protease (subtilisin) I.U.B 3.4.21.62* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE/E5: FORW. APPL. 1831-0019-2021
[2] *Application, Annex 1 – submission number 1614690324626-2848
[3] *Technical dossier, Section II: 2.1 Identity of the additive
[4] *Technical dossier, Section II: 2.5 Control methods
[5] *Technical dossier, Section II: 2.4 Conditions of use of the additive
[6] *Technical dossier, Section II – Annex_2-24
[7] *Technical dossier, Section II – Annex_2-33
[8] *Technical dossier, Section II – Annex_2-32
[9] *Technical dossier, Section II – Annex_2-25
[10] *Technical dossier, Section II – Annex_2-34
[11] *Technical dossier, Section II – Annex_2-1
[12] *Technical dossier, Section II – Annex_2-31
[13] *Technical dossier, Section II – Annex_2-30

[14] Supplementary information «DSM ProAct 360 SIn 2022 - Annex pdf» »

*Refers to Dossier no: FAD-2021-0025

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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