Assessment of the safety and efficacy of an additive of protease (subtilisin) produced by *Bacillus licheniformis* DSM 33099 (ProAct 360) as a feed additive for all growing poultry species

Reference number RP709

Regulated Products Risk Assessment Unit
Science, Evidence and Research Division, FSA

Risk Assessment Team
Science Division, FSS

Regulated Product Dossier Assessment
Assessment finalised: 15/03/2024

Contents

List of tables

Abbreviations

Acronym	Definition
ACAF	Advisory Committee on Animal Feedingstuffs
NFP	Quantification of the para-nitroaniline (pNA) released by the action of the protease on the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide substrate
EC	European Commission
TOS	Total organic solids
CFU	Colony forming units
NOAEL	No observed adverse effect level
pNA	Para-nitroaniline
RSDr	Relative standard deviations for repeatability
RSDip	Relative standard deviations for intermediate precision
EURL	European Reference Laboratory
EURL-FA	European Reference Laboratory for Feed Additives
Rrec	Recovery rates

Summary

An application was submitted to the Food Standards Agency in March 2021 from DSM Nutritional Products LTD (“the applicant”) for the new authorisation of an additive (ProAct 360) containing protease (subtilisin), under the category of “zootechnical additives” and functional group “digestibility enhancers”. The additive is proposed to be used in growing poultry, with a proposed inclusion rate of 30,000 NFP/kg of complete feed (12% moisture).

The Advisory Committee on Animal Feedingstuffs (ACAF) was asked to review the dossier and the supplementary information submitted by the Applicant, and to advise the Food Standards Agency and Food Standards Scotland (FSA/FSS) in evaluating the dossier.

The FSA/FSS concluded, based on the ACAF’s advice, that the additive was correctly identified and characterised. No causes for concern were raised in this section of the dossier.

The feed additive is safe for consumers, the target animal and the environment. The additive should be considered a potential respiratory sensitiser, dermal sensitiser and eye irritant. It is not a skin irritant.

Based on data from three efficacy studies in broilers, it was concluded that the additive ProAct 360 is efficacious in growing poultry when included in feed at a dose of 30,000 NFP/kg.

The views of ACAF have been taken into account in this safety assessment which represents the opinion of the FSA/FSS.

1. Introduction

The FSA/FSS have undertaken a risk assessment for a feed additive (ProAct 360, DSM Nutritional Products LTD, Wurmisweg 576, 4303 Kaiseraugst, Switzerland), consisting of protease (subtilisin) produced by *Bacillus licheniformis* DSM 33099, under Assimilated Regulation (EC) No 1831/2003[1] for a new authorisation under the category of "zootechnical additives" and functional group "digestibility enhancers" for its use in all growing poultry. To support the safety assessment, the ACAF provided advice to the FSA/FSS as outlined in this document.

In line with Article 8 of 1831/2003, the assessment has considered whether the feed additive complies with the conditions laid down in Article 5, including: safety considerations for human, animal and environmental health; efficacy of the additive for its intended effect; potential impairment of the distinctive features of animal products. This, and the guidance put in place by the European Food Safety Authority (EFSA) for the evaluation of feed additive applications, has formed the basis and structure for the assessment.

With thanks to the members of the ACAF during the course of the assessment, who were: Professor Nicholas Jonsson, Martin Briggs, Professor Emily Burton, Professor Katrina Campbell, Susan MacDonald, Professor Matthew Fisher, Hannah Kane, Christine McAlinden, Dr. Donald Morrison, Derek Renshaw, Dr. Oonagh Markey, Dr. Michael Salter, Dr. Helen Warren and Dr. Nick Wheelhouse. Dr. Adam Smith declared a direct conflict of interest and did not take part in the assessment.

The dossier was evaluated by the ACAF at their December 2022 meeting. Further information was provided by the applicant, after which a request for further information was communicated to the applicant. The applicant's response to this request and subsequent requests were evaluated at the ACAF February 2023 and September 2023 meetings.

This document sets out the findings of the Committee's assessment on the safety and efficacy of the feed additive, on which the FSA/FSS have made their opinion for the request of a new authorisation.

2. Assessment

2.1. Section II: Identity, characterisation and conditions of use

The additive is a preparation containing protease (subtilisin) produced by a genetically modified strain of *Bacillus licheniformis* DSM 33099. The applicant provided data from five batches supporting the composition values outlined below (Table 1):

Table 1: Identity table protease (subtilisin)

Composition		**Approx. Composition (w/w)**
Granulation	TOS	12 %
	Enzymatic activity	600000 NFP/g
	Cellulose	6 %
	Dextrin	7 %
	Sodium sulphate	45 %
Coating	Calcium carbonate	17 %
	Palm oil, hydrogenated	8 %
	Magnesium sulphate	4 %
	Water	≤ 1 %
Physico-chemical		
Dusting potential	21 - 30 mg/m^3	
Particle size	0.15 - 0.85 mm (88 %) > 0.85 mm (12 %) No particles of diameter < 150 µm	
Purity		
Coliform bacteria	<30 CFU/g	
Salmonella spp.	Negative in 25 g	
E.coli	Negative in 25 g	
Total yeast	Not tested	
Filamentous fungi	Not tested	
Bacillus cereus	Not tested	
Mycotoxins	Not tested	
Lead	≤ 5 mg/kg	
Mercury	≤ 0.5 mg/kg	
Cadmium	≤ 0.5 mg/kg	
Arsenic	≤ 3 mg/kg	

During the first evaluation, the Committee noted that a comprehensive description of the genetic modification had been provided, however, antimicrobial susceptibility had not been sufficiently demonstrated. Furthermore, the pelleting stability trials did not contain adequate detail for assessment. These queries were raised to the applicant and an appropriate response was received and further evaluated by the Committee.

The Committee concluded that the applicant's response adequately addressed the queries raised and the information provided was suitable for assessment.

The additive aims to supply a minimum of 30,000 NFP/kg feed for growing (fattening) poultry. The proposed conditions of use of the additive are described in Table 2:

Table 2: Proposed conditions of use of protease (subtilisin)

Proposed mode of use in animal nutrition				
Additive	Protease (subtilisin)			
Registration number/ EC No/No	Not available			
Category(-ies) of additive	4. Zootechnical			
Functional group(s) of additive	a. Digestibility enhancers			
Description				
Composition description	**Purity criteria**	**Method of analysis**		
Preparation of protease (subtilisin)	600,000 NFP/g	Single laboratory validated method		
Trade name (if appropriate)		ProAct 360		
Name of holder authorisation (if appropriate)		DSM Nutritional products LTD		
Conditions of use				
Species or category of animal	**Min-max Age**	**Min. content**	**Max. content**	**Withdrawal period**
		Protease (subtilisin) in complete feed at 12% moisture		
All growing poultry	--	30,000 NFP/kg	--	--

2.1.1. Conclusions on Section II

The ACAF concluded that the additive was correctly identified and characterised.

No further concerns were raised for Section II of the dossier.

2.2. Section III: Safety

2.2.1. Safety for the target species

The Committee evaluated a subchronic (90-day) oral toxicity study performed on rats, noting that the full dataset had not been provided, therefore, claims that the formulations used were stable could not be confirmed. The applicant responded to the Committee's queries and provided the relevant data to allow a comprehensive assessment of the study. Following receipt of these data, the Committee agreed with the established NOAEL of 438.6 mg Total Organic Solids (TOS)/kg BW/day. When

applying an uncertainty factor of 100 to the NOAEL to account for species differences, this would result in a maximum safe dose of 4.4 mg TOS/kg BW/day. The Committee concluded that further tolerance studies were not required for assessment.

2.2.2. Safety for the consumer

The applicant provided three studies to demonstrate safety for the consumer:

- Bacterial reverse mutation assay
- *In vitro* micronucleus test
- Vero cell assay (cytotoxicity)

The Committee discussed the use of a 'less purified' batch in each of the studies rather than the additive in its final formulation. This was deemed acceptable in this case owing to the demonstration of equivalence between the batch used and the final product. Following assessment of these studies, the Committee concluded that the additive is non-genotoxic and non-cytotoxic.

2.2.3. Safety for the user

The applicant presented the following tests to evaluate the safety of the additive for the user/worker:

- *In vitro* skin irritation potential (EPISKIN model)
- *In vitro* acute eye irritation potential (isolated chicken eye (ICE) test)

Based on the data presented, the ACAF concluded that the additive is not an irritant to the skin and has the potential to be an irritant to the eyes. In the absence of data, the additive should be considered a dermal sensitiser and, as the product is an enzyme, the Committee concluded that the additive should be considered a potential respiratory sensitiser.

The dusting potential and particle size distribution data indicated that the additive was unlikely to form a respirable dust when handled by operators.

2.2.4. Safety for the environment

For this enzyme preparation, environmental safety studies are not required for assessment.

2.2.5. Conclusions on safety

The ACAF concluded that the additive can be considered safe for the target animal with an established NOAEL of 438.6 mg TOS/kg BW/day. The additive is not mutagenic and can be considered safe for consumers and the environment.

The additive should be considered a potential respiratory sensitiser, skin sensitiser and eye irritant. The additive is not a skin irritant.

2.3. Section IV: Efficacy

The Committee Section IV of the dossier, containing three efficacy trials in broiler chickens.

2.3.1. Efficacy studies in broiler chickens

- Study 1 was carried out on 870 broiler chickens for 35 days, with a targeted dosage of 30,000 NFP/kg.
- Study 2 was carried out on 1056 broiler chickens for 35 days, with a targeted dosage of 30,000 NFP/kg.
- Study 3 was carried out on 576 broiler chickens for 35 days, with a targeted dosage of 30,000 NFP/kg.

The Committee evaluated the reports presented and noted that the studies were performed to an acceptable standard. The results of the studies are summarised in Table 3. The ACAF concluded that the additive can be considered efficacious in growing poultry when included in feed at a dose of 30,000 NFP/kg.

Table 3: Effects of protease (subtilisin) on growing poultry

Study	Number of animals	Concentration in feed (NFP/kg)	Daily feed intake (g)	Final live weight (g)	Daily weight gain (g)	Feed/gain ratio
1	870	0 30,000	90.0 89.0	2,180 2,190	61.1 61.3	1.48 1.45**
2	1056	0 30,000	104.0 103.0	2,110 2,200**	59.0 61.5***	1.79 1.70*
3	576	0 30,000	92.0 95.0*	2,260 2,530***	63.0 67.0**	1.48 1.40**

2.3.2. Conclusions on efficacy

Based on the information presented in three efficacy trials, the ACAF concluded that the additive ProAct 360 can be considered efficacious in growing poultry.

3. Analytical methods evaluation

Conclusions on the analytical methods are presented here as an extract from the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for ProAct 360[2]:

> "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 (RSDr) and intermediate precision (RSDip) ranging from 1.9 to 14.9%, and recovery rates (Rrec) 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."

FSA/FSS accepts the EURL analytical method evaluation reports. FSA/FSS determined the analytical method as appropriate for official controls for this feed additive.

4. Conclusions

The FSA/FSS have considered and agree with the conclusions reached by the ACAF on the safety and efficacy of the feed additive.

The ACAF concluded that the additive was correctly characterised and no major causes for concern were identified.

The additive can be considered safe to the consumer, the target animal and the environment. The additive is not an irritant to the skin and has the potential to be an irritant to the eyes. The additive should be considered a potential respiratory sensitiser and dermal sensitiser.

The ACAF concluded that ProAct 360 can be considered efficacious in growing poultry when included in feed at a dose of 30,000 NFP/kg.

The FSA/FSS agree with the conclusions reached by the ACAF. FSA/FSS accepts the EURL analytical method evaluation reports. FSA/FSS determined the analytical method as appropriate for official controls for this feed additive.

5. References

1. EC (European Commission), 2003. Regulation No 1831/2993 of the European Parliament and of the Council on additives for use in animal nutrition. Available at Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance) (legislation.gov.uk)

2. EURL-FA (European Reference Laboratory for Feed Additives), 2022. 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). Available at: finrep_fad-2021-0025_proact360-protease.pdf (europa.eu)

For enquiries about this publication, contact the Food Standards Agency.

Follow us on Twitter: @foodgov

Find us on Facebook: facebook.com/FoodStandardsAgency