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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2008-431 FAD-2008-0026 CRL/080009
Product name:	Ronozyme ProAct CT and L
Active Substance(s):	Serine Protease (EC 3.4.21.-)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
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Date:	05/01/2009
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Date:	06/01/2009

EXECUTIVE SUMMARY

The current application authorisation is sought for *Ronozyme ProAct CT and L,* in accordance with article 4(1) of Regulation (EC) No 1831/2003. Authorisation is sought to use *Ronozyme ProAct CT and L* as a digestibility enhancer for chicken for fattening under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. The product is intended to be marketed as solid (*Ronozyme ProAct CT*) and as liquid (*Ronozyme ProAct L*) formulations.

The active agent of *Ronozyme ProAct CT and L* is *serine protease* produced by submerged batch-fed pure culture fermentation of a genetically modified strain of a *Bacillus licheniformis* denoted "Rh-3". The enzymatic activity is expressed in protease unit (PROT) where 1 PROT is the amount of *serine protease* that liberates 1 µmol para-nitroaniline (pNA) from 1mM Suc-Ala-Ala-Pro-Phe-pNA ($C_{30}H_{36}N_6O_9$) substrate per minute at pH = 9.0 and at 37 ^oC. The solid and liquid formulations have a target activity of 75 000 PROT/g. The solid formulation (*Ronozyme ProAct CT*) is intended to be mixed into *premixtures* and/or *feedingstuffs* to provide an enzyme activity of 15 000 PROT per kg of *feedingstuffs*, whereas the liquid formulation (Ronozyme ProAct *L*) is directly sprayed onto the compound feed to obtain an enzyme activity of 15 000 PROT per kg of *feedingstuffs*.

For the determinations of *serine protease* activity in the *feed additive*, in the *premixtures* and in the *feedingstuffs*, the applicant proposes two in-house validated colorimetric methods based on the same principle, where the amount of yellow complex (para-nitroaniline, pNA) released by *serine protease* enzyme from the substrate "Suc-Ala-Ala-Pro-Phe-pNA" at pH = 9.0 and at 37 ^oC. The enzyme activity of the unknown sample is quantified against certified Ronozyme ProAct ™ *serine protease* standard with known enzyme activity.

For determination of the *serine protease* activity in the *feed additives* a relative standard deviation for repeatability (RSD_r) of 1.1% and a relative standard deviation for within-laboratory reproducibility (RSD_R) of 3.8% were reported. Based on the obtained method performance characteristics the method is considered suitable for the intended purpose.

For determination of the *serine protease* in the *premixture*s, the following performance characteristics were reported: a percentage recovery rate of 102%, a RSD_r of 6.8 % and a RSD_R of 6.4%. The validation experiments were conducted on *premixture* samples covering an activity range of 400 to about 36.000 PROT/g. The method is considered suitable for the intended purpose within the activity range covered by the validation study.

For determination of the *serine protease* activity in the *feedingstuffs* the following performance characteristics were reported: a limit of quantification (LOQ) of 1000 Prot/kg, a percentage recovery rate of 101%, a RSD_r of 8.9 % and a RSD_R of 11.7%. The performance characteristics were determined in feedingstuffs samples containing enzyme activity levels close to the target levels of this application. Based on acceptable performance characteristics, the proposed method is considered suitable for official control purposes for determination of *serine protease* activity in *feedingstuffs* for chicken for fattening within the frame of authorisation.

No further testing or method validation is considered necessary.

KEYWORDS

Ronozyme ProAct CT&L, *serine protease*, *Bacillus licheniformis,* digestibility enhancer, chickens for fattening.

1. BACKGROUND

Ronozyme ProAct CT and L is a product for which authorisation as feed additive is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains *serine protease* (EC 3.4.21.- not yet fully classified) as the active substance [2], produced by a strain *Bacillus licheniformis* denoted "Rh-3" (synonyms NNO49921, DSM 19670), which has been deposited at DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany [3]. The activity of *serine protease* is expressed as protease unit (PROT). According to the applicant, one PROT is the amount of *serine protease* that liberates 1 µmol para-nitroaniline (pNA) from 1mM Suc-Ala-Ala-Pro-Phe-pNA substrate per minute at pH = 9.0 and 37 ^{o}C [2]. The product is intended to be marketed in two forms [4]:

- A solid formulation, *Ronozyme ProAct CT,* with a target *serine protease* activity of 75 000 PROT/g, to be mixed first into premixture and then into compound feed. Alternatively, it can be mixed together with the other ingredients directly into compound feed;
- A liquid formulation, *Ronozyme ProAct L,* with a target *serine protease* activity of 75 000 PROT/g, to be sprayed onto the feed.

Both formulations are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a minimum enzyme activity levels of 15 000 PROT/kg in *feedingstuffs* for chickens for fattening [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis, submitted in connection with *Ronozyme ProAct CT and L*, (EFSA-Q-2008-431), and their suitability to be used for official controls in the frame of authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of impurities in the *additive* (e.g. arsenic, cadmium, mercury and lead) are available from the respective Community Reference Laboratories [6].

Description of the analytical methods for the determination of the active agent in the feed additive, premixtures and feedingstuffs

For the determinations of active agent(s) in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes two colorimetric methods based on the same principle,

where the amount of yellow complex (para-nitroaniline, pNA) released by *serine protease* enzyme from the substrate "Suc-Ala-Ala-Pro-Phe-pNA ($C_{30}H_{36}N_6O_9$)" at pH = 9.0 and at 37 ^oC is related to the enzymatic activity measured at 405 nm. The *serine protease* activity is determined by a standard curve of a certified Ronozyme ProAct ™ serine protease standard [7, 8].

For the determination of *serine protease* activity in the *feed additive* and *feedingstuffs* the applicant proposed the same protocol [7]. 0.2 g of liquid samples (Ronozyme ProAct ™ L) and 0.2-1.0 g of solid samples (Ronozyme ProAct ™ CT) are added to a 100 ml of glycine buffer (100 mmol/L, pH = 10.0) and stirred for 30-60 minutes. The samples are further diluted with the buffer to a final concentration of 0.0005-0.0015 PRO/mL. 50.0 g of mash and pellet samples of the feedingstuffs are added to 500 ml glycine buffer (100 mmol, pH = 1.6), and stirred at 40 ^oC for 60 min. The extracted samples are measured in triplicates together with two blank samples. The substrate Suc-Ala-Ala-Pro-Phe-pNA is incubated at 37 ^oC for 60 min. Finally the extract is spectrophotometrically measured at 405 nm. The *serine protease* activity in the samples is calibrated against a standard curve prepared with a certified protease standard with known activity. The following performance characteristics for the determination of the enzyme activity in the feed additive were reported: a relative standard deviation for repeatability (RSD_r) of 1.1% and a relative standard deviation for within-laboratory reproducibility (RSD_R) of 3.8% [9]. The method is considered suitable for the intended purpose.

A similar method is proposed for the *serine protease* activity in the *premixtures* [8]. 5 g of premix sample containing Ronozyme ProAct ™ CT are added to 100 ml of glycine buffer with ETDA (100 mmol/L, pH = 10.0) and stirred for 60 min at room temperature. 0.2 ml of 2mM substrate solution containing the Suc-Ala-Ala-Pro-Phe-pNA is added and incubated at 37 ^0C for 60 min. The extracted samples are measured in triplicates together with two blank samples. The substrate Suc-Ala-Ala-Pro-Phe-pNA is incubated at 37 ^oC for 60 min. Finally the extract is spectrophotometrically measured at 405 nm. The *serine protease* activity in the samples is calibrated against a standard curve prepared with a certified protease standard with known activity. The following performance characteristics were reported: a percentage recovery rate of 102%, a RSD_r of 6.8 % and a RSD_R of 6.4%. The validation experiments were conducted on *premixture* samples covering an activity range of 400 to

about 36.000 PROT/g [10]. The method is considered suitable for the intended purpose within the activity range covered by the validation study.

For the determination of *serine protease* activity in the *feedingstuffs* the following performance characteristics were reported: a limit of quantification (LOQ) of 1000 PROT/kg [7], a percentage recovery rate of 101%, a RSD_r of 8.9 % and RSD_R of 11.7%. The method performance characteristics were performed on feedingstuffs samples containing enzyme activity levels close to the target levels of this application [9]. Based on the above mentioned performance characteristics, the proposed method [7] is considered suitable for official control purposes for determination of *serine protease* activity in *feedingstuffs* for chicken for fattening within the frame of the authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

For official control of the *serine protease* activity in feedingstuffs at or around the proposed minimum and maximum content (15000 PROT/ kg complete feedingstuffs) the CRL recommends the method proposed by the applicant.

No further testing or method validation is considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Characterisation of the active agent in the *feedingstuffs*:

Colorimetric method measuring yellow complex paranitroalanine (pNA) released by the enzyme from "Suc-Ala-Ala-Pro-Phe-pNA" at pH = 9.0 and at 37 ^{o}C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme ProAct CT&L* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Reference SANCO/D/2 Forw. Appl. 1831/023-2008.

[2] *Section II, 2.1.2. Qualitative and quantitative composition.

[3] *Appendix 2–26. DSMZ: Safe Deposit Certificate for *Bacillus licheniformis* strain NN049921– DSM 19670. September 13, 2007

[4] *Annex III, Proposal of Register entry.

[5] *Section II, 2.4.2. Zootechnical Additives

[6] COMMISSION REGULATION (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards Community reference laboratories, Official Journal of the European Union L 136

[7] *Appendix 2-36 Report No. 2000659: Method SOY-101/01E: "Determination of RONOZYME® ProAct protease Activity in Tel Quel (per se) and Feed Samples". M.Schritz, K.Vogel. November 27, 2007.

[8] *Appendix 2-37 Report No. 2000660: Method SOY-102/01E: "Determination of RONOZYME® ProAct protease Activity in Premix Samples". M. Schritz, N. Venker, K.Vogel. November 27, 2007.

[9] *Appendix 2-38 Report No. 2000661: Validation of Method SOY-101/01E: "Determination of RONOZYME® ProAct protease Activity in Tel Quel (per se) and Feed Samples". M. Chan, N. Venker, K.Vogel. November 27, 2007.

[10] *Appendix 2-39 Report No. 2000662: Validation of Method SOY-102/01E: "Determination of RONOZYME® ProAct protease Activity in Premix Samples". M. Chan, N. Venker, K.Vogel. November 27, 2007.

***Refers to Dossier number: FAD-2008-0026**

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

8. ACKNOWLEDGEMENTS

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- Plantedirektoratets Laboratorium, Lyngby, DK.
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- Państwowy Instytut Weterynaryjny, Puławy, Poland.
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Austria.