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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

Muramidase produced by Trichoderma reesei DSM 32338

(FAD-2017-0046; CRL/170035)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2017-0046 - CRL/170035**
Name of Product / Feed Additive:	***Muramidase produced by Trichoderma reesei DSM 32338***
Active Agent (s):	**Muramidase**
Rapporteur Laboratory:	**Austrian Agency for Health and Food Safety (AGES), Austria**
Report prepared by:	**Irmengard Strnad, Elisabeth Reiter, Magdalena Wagner**
Report checked by: Date:	**Stefano Bellorini (EURL-FA)** **09/02/2018**
Report approved by: Date:	**Christoph von Holst** **12/02/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) of Regulation (EC) No 1831/2003 for a preparation of *muramidase* under the category/functional group 4(d) 'zootechnical additives'/'other zootechnical additives'. Specifically, authorisation is sought for chickens for fattening and minor poultry species for fattening.

According to the Applicant the *active substance* in the preparation is *muramidase* (lysozyme) *produced by Trichoderma reesei DSM 32338.* The activity of *muramidase* is expressed in LSU(F) units. One LSU(F) unit is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labelled peptidoglycan per minute at pH 6.0 and 30 °C by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer I.

The product is intended to be marketed as solid and liquid formulation having a guaranteed minimum *muramidase* activity of 60000 LSU(F)/g or ml. *Muramidase* is intended to be used directly in *feedingstuffs* or through *premixtures* to obtain a minimum activity of 25000 LSU(F)/kg *feedingstuffs*.

For the quantification of *muramidase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified fluorometric enzyme assay.
Based on the performance characteristics available, the EURL recommends for official control this method.

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

Muramidase, *lysozyme*, zootechnical additives, other zootechnical additives, chickens and minor poultry species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new use) of the Regulation (EC) No 1831/2003 for a preparation of *muramidase* under the category/functional group 4(d) 'zootechnical additives'/'other zootechnical additives' [1,2]. Specifically, authorisation is sought for the use of *feed additive* for chickens for fattening and minor poultry species for fattening [2,3].

Muramidase is produced by fermentation with a genetically modified strain of *Trichoderma reesei* (DSM 32338) [4]. According to the Applicant the product is intended to be marketed as solid (granulated powder, GT) and liquid (L) formulation [3].

The *muramidase* activity is expressed in LSU(F) units, where one LSU(F) unit is defined as "the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labelled peptidoglycan per minute at pH 6.0 and 30 °C by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer I" [5].

According to the Applicant the active substance in the preparation is *muramidase* (lysozyme) with a minimum guaranteed enzyme activity of 60000 LSU(F)/g [3]. The product is intended to be used directly in *feedingstuffs* (GT and L) or through *premixtures* (GT) to obtain a minimum activity of 25000 LSU(F)/kg *feedingstuffs* [3]. The recommended dose range is between 25000 and 45000 LSU(F)/kg *feedingstuffs* [2,3].

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 *muramidase produced by Trichoderma reesei DSM 32338* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [6].

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

For the quantification of the activity of *muramidase* in the *feed additive*, the Applicant proposed a single-laboratory validated and further verified fluorometric enzyme assay method [7-10]. The *feed additive* is weighed, extracted and further diluted with citrate-phosphate buffer. The sample extract and the substrate (peptidoglycan), which is available from the Applicant upon request, are pipetted into microtiter plate wells and fluorescence is measured from 0-30 min for each minute at 30 °C. In this assay, the *muramidase* hydrolyses ß-1,4 bonds between N-acetylmuramic acid and N-acetylglucosamine in fluorescein-conjugated peptidoglycan macromolecules from *Micrococcus lysodeiktus*. The fluorescence intensity of the bound fluorescein is reduced by quenching. Depolymerization of the fluorescein-conjugated peptidoglycan by *muramidase* releases high fluorescent labelled fragments which can be measured with a microplate fluorescence reader. The substrate is excited at 484 nm and the emission is recorded at 528 nm. The increase in fluorescence over time (slope) is linear proportional to the *muramidase* activity which is quantified against a calibration curve prepared from a reference *muramidase* standard (available from the Applicant upon request) with known activity [8].

The method proposed by the Applicant for the quantification of the *muramidase* activity in *premixtures* and in *feedingstuffs* is based on the same principle as the method described for the *feed additive* with the introduction of slight modifications in the experimental procedure [7,11]. The method was validated and further verified by an external laboratory [12,13].

The sample is extracted at room temperature and afterwards diluted with citrate-phosphate buffer. The extract is pipetted into microtiter plate wells and is pre-incubated at 30 °C before the peptidoglycan (25 µg/ml; 30 °C) is added. Fluorescence is measured from 0-20 minute at each minute at 30 °C. In a microplate fluorescence reader the substrate is excited at 484 nm and the emission is recorded at 528 nm. The *muramidase* activity is calculated using a calibration curve prepared from a reference *muramidase* standard (available from the Applicant upon request) with known activity [11].

Table 1 presents the performance characteristics calculated by the Applicant based on experimental data obtained in the frame of the validation and verification studies [9-10,12-13]. Additionally, the Applicant reported a limit of quantification (LOQ) in *feedingstuffs* of around 2000 LSU(F)/kg [14].

The EURL recalculated the performance characteristics for one *feedingstuff* data set applying ANOVA. The results obtained are in the range of those reported by the Applicant [12,15].

Table 1. Performance characteristics presented by the Applicant in the frame of validation and verification studies for the quantification of *muramidase* activity in the liquid and granulated *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

	R_{Rec} (%)		RSD_r (%) (*)		RSD_{ip} (%) (*)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA [9-10]	96.7-100	97.1	4.4	1.9-4.4	5.8	4.6-6
PM [12-13]	96.5-100.7	99	3-7.3	4.4-8.7	4.5-9.1	5-7.3
FS [12-13]	96.4-100.7	99-107	4-11.8	5.4-12.9	5.6-9.1	7.7-11.5

R_{Rec}: *recovery* rate (%); RSD_r:and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*;

(*) The applicant assessed the precision under repeatability and intermediate conditions on data obtained from two separate sets of experiments and not according to a design of ANOVA. While the RSDr is expected to be below or equal to the RSDip, the specific experimental design selected by the Applicant may lead to the situation that the RSDr is slightly above RSDip. This is observed for FS, but the difference is minor and therefore considered acceptable.

Based on the performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified method based on a fluorometric enzyme assay for the quantification of *muramidase* 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 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 fluorescence-based enzyme assay method for the quantification of *muramidase* in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *muramidase* activity in the *feed additive*, *premixtures* and *feedingstuffs*:

- Fluorescence-based enzyme assay method that determines the enzyme-catalyzed depolymerisation of a fluorescein-labelled peptidoglycan preparation at pH 6.0 and 30 °C

One LSU(F) unit is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labelled peptidoglycan per minute at pH 6.0 and 30 °C by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer I.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *muramidase produced by Trichoderma reesei DSM 32338* 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_FWD. APPL. 1831-0035-2017
[2] *Application, Proposal for Register Entry - Annex A
[3] *Technical dossier, Section II: 2.5. Condition of use of the additive
[4] *Technical dossier, Section II: 2.2. Characterisation of the enzyme
[5] *Technical dossier, Section II: 2.6. Methods of analysis and reference samples
[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 to Community Reference Laboratories
[7] *Technical dossier, Section II: 2.6. Methods of analysis and reference samples
[8] *Technical dossier, Annex II-36
[9] *Technical dossier, Annex II-37
[10] *Technical dossier, Annex II-38
[11] *Technical dossier, Annex II-40
[12] *Technical dossier, Annex II-41
[13] *Technical dossier, Annex II-42
[14] *Supplementary information, validation of method LYS-10/02E
[15] Results ANOVA Annex 2-41 table 9.xlsx

*Refers to Dossier no: FAD-2017-0046

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the Österreichische Agentur für Gesundheit und Ernährungssicherheit - Austrian Agency for Health and Food Safety (AGES), Wien Austria. 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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- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)