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Safety evaluation of a novel muramidase for feed application

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ABSTRACT

Safety evaluation of a muramidase produced by a *Trichoderma reesei* strain (safe lineage), expressing a muramidase gene isolated from *Acremonium alcalophilum* is presented. Intended use in feed of this enzyme is as digestive aid in broiler chickens.

Muramidase 007, was non-mutagenic and non-clastogenic *in vitro*, and no adverse effects were observed in 90-day subchronic toxicity studies in rats at doses up to 1132 mg TOS/kg body weight/day. The enzyme did not exhibit, *in vitro*, skin, nor eye irritation potential. Acute aquatic toxicity evaluated on daphnia and algae showed absence of effect of the enzyme at the standard doses tested.

Muramidase 007 was fully tolerated by broiler chickens in a 6-weeks tolerance study showing no adverse effects in any of the dietary treatments (0, 1×, 5× and 10× maximum recommended dose).

In conclusion, Muramidase 007 is found to be toxicologically inert, and there are no worker's safety concerns if standard precautions are instituted and a non-dusty formulation is employed. Muramidase 007 is well tolerated by the target species (broiler chickens) and cause no harm to the environment. The beneficial safety evaluation of Muramidase 007 is in line with this type of enzyme that is found ubiquitously in nature.

1. Introduction

Adding enzymes to animal feed is a common practice in modern feed formulation. Enzymes help to increase nutrient digestibility and optimize nutritional value of the diet. An extensive range of enzymes is available to nutritionists to optimize nutrient utilization from feed ingredients. The use of phytases, glucanases, xylanases, hemi-cellulases (Adeola and Cowieson, 2011; Dersjant-Li et al., 2015) or proteases (Cowieson and Roos, 2016), for example, allows enhancing the availability of key nutrients for the animal and/or for its intestinal microbiota (Józefiak et al., 2010; Bedford and Cowieson, 2012; Kiarie et al., 2013). Beyond the direct nutritional interest, employing enzymes allows optimizing the use of feed for efficient production of animal protein. Therefore, enzymes are essential contributors to the sustainability rating of animal production for meat production purposes (Bundgaard et al., 2014; Leinonen and Kyriazakis, 2016).

Muramidases (EC 3.2.1.17), also known as lysozymes or N-acetylmuramidases, are enzymes that recently gained interest as potential feed additive (Nyachoti et al., 2012; May et al., 2012, Oliver and Wells, 2015; Maga et al., 2012; Cooper et al., 2014; Liu et al., 2010; Long et al., 2016). Muramidases belong to the family of glycosyl hydrolytic enzymes, as do glucanases or hemi-cellulases (Strynadka and James, 1996; Korczynska et al., 2010). These enzymes cleave the β-1, 4 glycosidic linkages between N-acetylmuramic acid and N-acetyl glucosamine in the carbohydrate backbone of peptidoglycan, which is a major component of microbial cell debris present in the digestive tract of animals. The catalysis of the depolymerisation of peptidoglycans obtained with dietary muramidase supplementation seems to be beneficial to the animal in consideration of the positive effect recorded for feed efficiency (May et al., 2012; Oliver and Wells, 2013; Oliver et al., 2014).

We have studied the potential dietary application of a muramidase as a digestive aid for broiler chicken. While in literature,

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the effects of the inclusion, in feed, of hen egg white lysozyme (Nyachoti et al., 2012; Oliver and Wells, 2015; Long et al., 2016) or human lysozyme (Maga et al., 2012; Cooper et al., 2014) were evaluated, we have considered a new microbial muramidase from *Acremonium alcalophilum* expressed in a production organism (*Trichoderma reesei*).

Fleming first described muramidase in 1922 (Fleming, 1922) to be present in human nasal secretions. Muramidases are ubiquitous enzymes mostly found in animal secretions like saliva, tiers, milk (Jollès and Jollès, 1967), in microorganisms (Morgavi et al., 1994; Martinez-Fleites et al., 2009; Ai-Riyami et al., 2016) and in plants (Sytwala et al., 2015; Audy et al., 1988; Manikandan et al., 2015). One hen egg contains around 100 mg of muramidase as natural constituent in the albumen (Vidal et al., 2005; Stevens, 1991). The ubiquity of muramidases means that feeding them to animals does not constitute a novel enzymatic exposure.

Here we report a series of toxicological studies that were undertaken to document the safety profile of the selected muramidase, designated as Muramidase 007, as suggested by Pariza (Pariza and Johnson, 2001; Pariza and Cook, 2010) for feed enzymes, and required by the European Regulation (EC) No 1831/2003 (EC, 2003) and its corresponding guidelines.

The objective of these studies was to demonstrate the safety of Muramidase 007 as a feed additive compiling the safety information on the production strain, and the results of a toxicological study in rats, relevant *in vitro* tests, and a target animal tolerance study in broiler chickens, as prescribed by the European Food Safety Authority (EFSA, 2008).

2. Materials and methods

2.1. Characterisation of the muramidase

2.1.1. Construction of the production strain

The wild type muramidase gene from the fungus *Acremonium alcalophilum* (strain CBS 114.92) was PCR amplified using genomic DNA from the donor organism as template. The construct was cloned into *E. coli* using standard vectors with strictly defined and well-characterised DNA sequences that are known not to encode or express any harmful or toxic substances, to create a plasmid containing the muramidase expression cassette. The muramidase expression cassette was introduced into the *Trichoderma reesei* recipient strain that derives from RUT-C30 (ATCC 56765) by incubating protoplasts with plasmid DNA, using a standard transformation procedure.

The *amdS* selection marker from *Aspergillus nidulans* strain biA1 Glasgow present in the muramidase expression cassette was used for selection, allowing the transformants to grow on medium containing acetamide as sole nitrogen source (Kelly and Hynes, 1985). Transformants were subsequently evaluated by gene sequencing to assess incorporation of the muramidase expression cassette and to ensure that no unintended sequences were incorporated into the genome of the selected production strain. The muramidase protein expressed from the introduced gene in the final production strain was verified by mass spectroscopy to be 100% identical to the protein sequence encoded by the donor gene.

The safety of *Trichoderma reesei* has been discussed in several review papers and described not to produce any mycotoxins or antibiotics of concern under conditions used for enzyme production (Nevalainen et al., 1994; Blumenthal, 2004; Kubicek et al., 2007; Peterson and Nevalainen, 2012). The Environmental Protection Agency (EPA, 2012) acknowledged in this assessment that under normal submerged fermentation conditions, peptaibols (e.g. paracelsin) are not produced. Absence of paracelsin was confirmed for the muramidase product by the respective assay (data not shown).

2.1.2. Preparation of the muramidase test substance

The Muramidase preparation evaluated in the present study was carried out in an industrial pilot biotechnological set-up certified according to ISO 9001 and in accordance with the procedures used for the manufacturing of commercial enzyme products. In brief, the genetically modified *Trichoderma reesei* production strain described in Section 2.1.1, was cultivated in a bioreactor using a medium made of sterilised food-grade ingredients with pH adjustment. At the end of the process, the product was separated from the production organism using a series of filtration an evaporation steps. Finally, a number of chemical and microbial analysis were carried out to characterise the muramidase preparation.

A dried solid form of this product was produced by granulation following well-established industrial production practices for use in a tolerance study in chickens.

2.1.3. Characterisation of the muramidase activity

Muramidase 007 activity is expressed in muramidase units (LSU(F). One LSU(F) unit is defined as the amount of enzyme that increases the fluorescence of a 12.5 μg/mL fluorescein-labelled peptidoglycan suspension by a value that corresponds to the fluorescence of 0.077 mM fluorescein isothiocyanate (FITC), per minute at pH 7.5 and 30 °C. In brief, Muramidase 007 was also analysed for chemical and microbial status (Table 1) using standard methods. Total organic solids (TOS) from the fermentation consist mainly of protein and carbohydrate components and was calculated as follows: TOS (%) = 100 - water (%) - ash (%). The TOS content of Muramidase was 10.8% (w/w). The main activity was 36 700 LSU(F)/g for the toxicological studies. For the tolerance study with chickens, the typical commercial granulated form used for the 1× dose had 65 555 LSU(F)/g and the liquid preparation used for providing the 5× and 10× Muramidase 007 doses had 75 000 LSU(F)/g as described in 2.3.

2.2. Toxicological studies

All toxicological studies were carried out in accordance to the current OECD guidelines and with Good Laboratory Practice (OECD, 1998a). The *in vivo* studies were also conducted in agreement to the regulations and ethical guidelines on use of experimental animals

Table 1
Composition analyses of Muramidase 007 for toxicological studies.

Composition analyses	
Enzyme activity (LSU(F)/g)	36 700
Carbohydrate (anthron) (g/kg)	40.3
Carbohydrate (tryptophan) (g/kg)	42.5
Water (Karl Fisher) (% w/w)	87.9
Total Organic Solids (% w/w)	10.8
Dry Matter (% w/w)	12.1
Ash (% w/w)	1.3
N_{tot} (Keldahl, mg/L)	10 200 mg/L
Pb (ppm)	<0.5
As (ppm)	<0.3
Cd (ppm)	<0.05
Hg (ppm)	<0.05
Cu (ppm)	2.72
Total viable count CFU/g	200
6.1.1.1.1 *Salmonella* CFU/25 g	ND
Coliform bacteria CFU/g	<4
Enteropathogenic *E. coli* CFU/25 g	ND
Sulphur-reducing clostridia CFU//g	<10
Staphylococcus aureus CFU/g	ND
6.1.1.1.2 *Trichoderma reesei* (Production strain detection)	ND

ND: Not detectable.