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Dietary safety of a dual-enzyme preparation for animal feed: Acute and subchronic oral toxicity and genotoxicity studies

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ABSTRACT

Animal feed is routinely supplemented with exogenous enzymes to improve nutrient utilization, such as proteases to enhance protein hydrolysis *in vivo* and xylanases to alleviate feed related anti-nutritional factors. The present studies were conducted to evaluate the potential oral toxicity and genotoxicity of a dual-enzyme preparation, Vegpro® concentrate (VPr-C). Acute oral toxicity studies were conducted in adult male and female Sprague-Dawley Crl CD rats and CHS Swiss ICO:OFI (IOPS Caw) mice. Thirteen week preliminary and final subchronic oral toxicity studies were conducted in male and female rats. Genotoxicity was evaluated through a bacterial reverse mutation test (Ames test), an *in-vitro* mammalian chromosomal aberration test, and a mammalian micronucleus test. The LD_{50} was >2000 mg/kg of BW in mice and rats. In the 13-week oral toxicity study, the No Observed Adverse Effects Level (NOAEL) was 1000 mg/kg BW per day for females and 300 mg/kg BW per day for males. VPr-C showed no mutagenic activity in *Salmonella typhimurium*, did not induce significant chromosomal aberrations in cultured human lymphocytes, and did not increase the frequency or proportion of micronucleated immature erythrocytes in mice. There was no evidence of acute or subchronic toxicity or genotoxicity associated with the test article at these test dosages.

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1. Introduction

Efficient nutrient utilization is essential to the sustainability of food animal production. The routine practice of adding exogenous enzymes to animal feed to improve nutrient utilization has a relatively short history dating back less than 30 years (Choct, 2006). Enzymes are added to the vegetative feed of monogastric livestock to improve nutrient digestion, to enhance animal performance, and to reduce nutrient excretion (Adeola and Cowieson, 2011; Slominski, 2011). Phytases and carbohydrases, which comprise approximately 90% of the world feed enzyme market (Adeola and Cowieson, 2011), are added to feed to help unlock the nutrients in phytin and nonstarch polysaccharides. Because growth in poultry production has been constrained by the high cost of and limited access to traditional protein sources, interest has likewise increased in recent years concerning the broader use of exogenous proteases in feed (Chisoro, 2015). Vegetable protein is a major component of poultry feed, with approximately 70% of the world's soybeans being processed into soybean meal for animal feed (Potts et al., 2014). Proteases act as catalysts in the hydrolysis of protein, cleaving peptide bonds to generate smaller, more digestible peptides and amino acids (Isaksen et al., 2011). Various studies have shown that supplementing poultry feed with exogenous proteases can be beneficial in increasing protein hydrolysis and enabling more efficient nitrogen utilisation, while also reducing excretion of nitrogen to the environment (Doskovic et al., 2013; Oxenboll et al., 2011). In particular, performance benefits have been reported to be potentially significant in animals fed nutritionally marginal diets (Angel et al., 2011; Barekatain et al., 2013; Cowieson and Adeola, 2005; Cowieson et al., 2006; Ravindran, 2013; Ravindran and Son, 2011), and in young animals in which endogenous enzyme production is typically immature (Angel et al., 2011; Kiarie et al., 2013; Romero et al., 2013; Uni et al., 1999).

Although the potential agricultural benefits from exogenous proteases in feed are clear, the adoption of their use by the feed industry has lagged due, in part, to a history of mixed, unexplained physiologic responses when proteases are fed at effective concentrations (Liu et al., 2013; Bao et al., 2013; Zakaria et al., 2010). For example, one study conducted in broilers, a multi-enzyme with

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protease (10,000 U/g) as its primary activity resulted in significantly reduced growth rate and feed intake compared with the control (Zakaria et al., 2010). In another study conducted in pigs fed a basal diet containing rapeseed meal and wheat dried distillers grains with solubles, despite an improvement in apparent ileal digestibility, inclusion of protease (200 mg/kg of BW) in diet reduced average daily gain and feed consumption (O'shea et al., 2014). Exogenous feed enzyme development continues to present challenges due to incomplete understanding of the consequences of enzyme interactions during digestion and the variability of enzyme effectiveness in mixtures of feed ingredients (Bao et al., 2013).

A dual-enzyme preparation used in recent clinical studies (Kocher et al., 2015), Vegpro® concentrate (VPr-C), containing protease produced using solid-state fermentation along with xylanase activities, was shown to be well tolerated and effective in enhancing broiler performance (Kocher et al., 2015). Although numerous reviews have been published on the efficacy of exogenous proteases in animal feed (Adeola and Cowieson, 2011; Doskovic et al., 2013; Isaksen et al., 2011), the number of dose-response studies is limited (Kocher et al., 2015). Therefore, the present studies were designed to evaluate the safety profile of VPr-C as a feed additive. Oral toxicity studies (acute and 13-week subchronic) were conducted in mice and rats, and genotoxicity parameters were evaluated *in vitro* and *in vivo*. Studies were conducted in compliance with the Principles of Good Laboratory Practice regulations at CIT Safety and Health Research Laboratories, Evreux, France.

2. Materials and methods

2.1. Test article

The test enzyme concentrate that forms Vegpro®, VPr-C (Alltech Inc., Nicholasville, KY, USA) contains the protease aspergillopepsin I (EC 3.4.23.18), as its main activity produced by solid state fermentation with *Aspergillus oryzae* (SD154) and xylanase (EC 3.2.1.32) produced by *Trichoderma longibrachiatum* rifar (IMI 387064). The VPr-C batches used for the studies herein each comprised a mixture of samples from 3 separate solid-state fermentations. The production process (including fermentation, downstream processing, and product formulation) meets the requirements for ISO 9001:2008 certification. The preparation complies with the specifications for food-grade enzymes recommended by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) and Food Chemical Codex (FCC). The first batch, which was used in the acute oral toxicity studies in rats and mice, the 13-week preliminary subchronic oral toxicity study in rats and in all genotoxicity testing, had a protease activity of 43,870 HUT/ml and a xylanase activity of 1338 XU/ml. The batch had a density of 1.74 g/ml giving an activity of 25.2 HUT/mg and 0.77 XU/mg for protease and xylanase respectively. The second batch, which was used in a 13-week subchronic oral toxicity study in rats, had a protease activity of 48,540 HUT/ml and a xylanase activity of 880 XU/ml. The batch had a density of 1.74 g/ml giving an activity of 27.9 HUT/mg and 0.51 XU/mg for protease and xylanase respectively. One HUT (hemoglobin unit on a tyrosine basis) is the amount of enzyme that liberates in 1 min under specific conditions an hydrolysate with an absorbance at 275 nm equal to that of a solution containing 1.10 μg per ml of tyrosine in 0.006 N hydrochloric acid (Dillon et al., 2015a). One XU is the amount of enzyme that liberates 1 μmole of xylose per minute at pH 5.3 and 50 °C (Dillon et al., 2015b). The test item was free was *Salmonella* and *Enterobacteriaceae* and was free from mycotoxins (Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, Zearalenone, Ochratoxin, Fumonisin B1, Deoxynivalenol and T-2 Toxin; LODs: 0.5 μg/kg, 0.5 μg/kg, 0.5 μg/kg, 0.5 μg/kg, 5 μg/kg, 1 μg/kg, 50 μg/kg and 0.08 μg/kg, respectively) and heavy metals (Arsenic, Lead, Cadmium and Mercury; LODs: 0.05 mg/kg, 0.05 mg/kg, 0.01 mg/kg and 0.005 mg/kg, respectively).

2.2. Animals

Animal care complied with the relevant animal welfare regulations in place at the time of the study (Department of Agriculture, 1989; EEC Council, 1986). CIT LAB has an internal ethics committee and is AAALAC accredited. This review has been documented in the Ethical Committee Database under the reference number 00030.

Healthy CHS Swiss ICO:OFI (IOPS Caw) mice and specific-pathogen-free Sprague-Dawley Crl CD rats (Charles River Laboratories, L'Arbresle, France) of both sexes were used in the studies herein. Female animals were nulliparous and not pregnant. Mice were used in acute oral toxicity and micronucleus genotoxicity testing; rats were used in acute and subchronic oral toxicity testing. Animals were acclimated at least 5 days before the initiation of testing. At (initial) dosing animals were approximately 6 weeks of age. Rats or mice used in acute oral toxicity and genotoxicity studies were housed in polycarbonate cages (5 animals of the same sex and treatment group per cage), whereas rats used in the 13-week subchronic oral toxicity study were housed in suspended wire-mesh cages (2 animals of the same sex and treatment group per cage). All cages contained autoclaved sawdust bedding (SICSA, Alfortville, France). The animal room temperature was maintained at 22 ± 2 °C at a relative humidity of 30–70% with a 12-hour light-dark cycle. All animals had free access to pelleted maintenance diets (SSNIFF Spezialdiäten GmbH, Soest, Germany) distributed weekly and to bottled, filtered (0.22 μm) tap water.

2.3. Acute oral toxicity

Toxicity of the test article was assessed after a single oral administration in mice or rats. Animals were fasted (mice 3 h; rats 18 h) before treatment. Access to food was restored 4 h post-treatment. Preliminary tests in mice (1 male, 1 female) and rats (1 male, 1 female) were conducted to define the treatment concentrations. In the main experiments, mice (2 groups of 5 males and 5 females each) and rats (2 groups of 5 males and 5 females each) were administered the test article at 2000 mg/kg body weight (BW) (equivalent to 50,400 HUT/kg BW and 1540 XU/kg BW) or the vehicle control (reverse osmosis (RO) water). Treatments (10 ml/kg BW) were administered a single dose by oral gavage. The single-dose administration on the morning of day 1 was followed by a 4-day (preliminary test) or 14-day (main test) observation period in which mortality or signs of morbidity were recorded for each animal. Animals were weighed immediately before administration of the test article on day 1 and again on days 8 and 15. No statistical analysis was performed in the event of an absence of mortality, clinical signs of morbidity, or macroscopic abnormalities in the main organs of the control and high-dose groups.

2.4. Preliminary subchronic oral toxicity

One group of 10 male and 10 female Sprague-Dawley rats received the test item at 1000 mg/kg per day (equivalent to 25,200 HUT/kg BW and 770 XU/kg BW) by oral gavage for 90 days. Treatments were administered via oral gavage at a volume of 5 ml/kg BW per day for at least 13 weeks. The test item was administered as a solution in the vehicle (purified water). The test item was mixed with the required quantity of vehicle in order to achieve the concentration of 200 mg/mL and then homogenized using a magnetic stirrer. The test item dosage forms were prepared weekly, stored at +4 °C and then delivered at room temperature. Each animal was

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