A Feed Serine Protease Improves Broiler Performance and Increases Protein and Energy Digestibility

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Effects of a novel serine protease, RONOZYME® ProAct (RPA) on protein solubilisation, hydrolysis and digestibility were tested in a gut simulation model. Furthermore, the effects of RPA (supplemented at 15,000 PROT/kg feed) on growth performance and nutrient digestibility were tested in two in vivo broiler experiments each arranged in a 2 x 2 factorial design (enzyme x sex in experiment 1 and enzyme x protein level in experiment 2). Each dietary treatment had 12 replicates. In experiment 1, birds were fed 12.7 MJ ME per kg iso-energetic diets in 2 phases. Each diet was fed without or with RPA (C or C + RPA, respectively) to either males or females. In experiment 2, two diets were fed in four treatments. Diet 1 (211 and 200 g CP per kg feed in the starter and grower phases respectively) was fed without or with RPA (NP or NP + RPA, respectively). Diet 2 (200 and 190 g CP per kg feed in the starter and grower phases respectively), was fed without or with RPA (LP or LP + RPA, respectively). At the end of each experiment, eight male birds per treatment were randomly selected and used for ileal digestibility measurements. In vitro, RPA significantly increased the degree of protein hydrolysis, solubilisation and digestibility. In males, C + RPA was significantly better than C in WG (82.8% vs. 76.9%) and FCR (1.60 vs. 1.65). In females, C + RPA was better than C in FCR (1.37 vs. 1.39) in the starter phase. The FCR of LP + RPA was significantly better than LP. In experiment 1, RPA significantly increased ileal protein (76.6% vs. 75.5%) digestibilities. In LP, RPA significantly improved energy digestibility (77.8% vs. 70.6%). These results suggest that RPA can improve broiler performance by enhancing protein and energy digestibility.

Key words: broiler, growth performance, nutrient digestibility, protein hydrolysis, serine protease

Introduction

Nitrogen emission from farm animals is part of humanity’s unfriendly environmental footprint. The EU, through the European Directive 2001/81/EG (National Emission Ceilings) has set targets to reduce the total nitrogen excretion throughout its member states. Using dietary supplements to influence the performance of farm animals is an old art in animal nutrition. The use of exogenous enzymes in poultry diets, though not as old as the use of vitamins and minerals, is also not a new concept and has been extensively studied and reported (Campbell and Bedford, 1992; Leeson et al., 1996; Leeson and Summers 1997; Seskevicience et al., 1999; Smits and Annison, 1996). An effective protease may not only reduce production costs for the farmer, by increasing the efficiency of feed utilization by the animals, but will also reduce the total content of nitrogen in manure and thereby meet the societal demand for a decreased nitrogen excretion.

Exogenous enzymes such as phytases and carbohydrases, especially of microbial origin, have been shown to be effective and are widely used in animal production. The positive effects of using enzyme cocktails of carbohydrases, phytases and proteases in broiler diets have been reported (Kocher et al., 2002; Cowieson and Adeola, 2005), and pretreatment of feed with protease has also been demonstrated to be beneficial for broilers (Ghazi et al., 2002). Positive effects on growth performance of broilers has been reported upon inclusion of keratinase PWD-1 (Odetallah et al., 2003; Wang et al., 2008) or the keratinase-based feed additive Versazyme (Odetallah et al., 2005; Wang et al., 2006).

In vitro digestion models are essential tools in targeting development of effective feed enzymes. Digestion models allow comparison of a high number of feed enzyme candidates prior to selecting only the most promising candidates for in vivo performance studies. In the recent decade, development of well established digestion models have provided more stable and higher performing enzymes to the feed market, as well as a better understanding of their
mode of action.

Recently a novel serine protease expressed in Bacillus licheniformis was introduced into the market for broilers. This feed protease is claimed to act through solubilisation and hydrolysis of dietary proteins, and to have an unspecific mode of action on a broad range of dietary proteins.

The objective of the present work was to study the effects of this protease on the digestibility of protein, fat, and energy, as well as on the growth performance of broiler chickens. The effects were studied in both in vitro and in vivo experiments.

**Materials and Methods**

**Enzyme Characteristics**

The tested enzyme (RONOZYME® ProAct (RPA), DSM Nutritional Products) claimed to be a purified monocomponent serine protease is expressed in Bacillus licheniformis. For the in vitro studies, a purified non-formulated protease was used while for the in vivo studies, a heat stable formulated product containing 75,000 PROT/g was used. One PROT is one protease unit, and is defined as the amount of enzyme that releases 1 mmol of p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pNA) per minute at pH 9.0 and 37°C.

The enzyme was selected as feed enzyme candidate because of its encouraging intrinsic characteristics. At peptic and acidic conditions (pH 2), the enzyme retained more than 90% residual activity after 2 hrs at 40°C.

**Experimental Design**

**In vitro Studies**

The performance of the protease was studied in an in vitro digestion model simulating digestion in monogastric animals. The protease was tested for its ability to improve solubilisation and digestion of a mixture of maize and soybean meal (SBM) proteins. The in vitro system consisted of bottles placed on a magnetic stirrer (500 rpm) in a water bath at 40°C. Initially 10 g maize/SBM (in a 60/40 ratio) were pre-incubated in 41 mL of 0.105 M HCl (pH 3 for 30 min). Subsequently 5 mL of 0.105 M HCl containing 6,000 U/mL porcine pepsin (SP7000, Sigma-Aldrich) was added to simulate gastric digestion (pH 3 for 60 min). Small intestinal digestion was then simulated by further addition of 23 mL of 0.39 M NaOH and 5 mL of 1 M NaHCO3 containing 16 mg/mL porcine pancreatin (8 xUSB, P-7545, Sigma-Aldrich) at pH 6.8 for 240 min. Five bottles were incubated with the feed protease dosed at 100 mg enzyme protein (EP) per kg feed (maize/SBM mixture) equal to 150,000 PROT/kg, which was added at the start of the gastric digestion phase, another five bottles were likewise incubated with the feed protease at 50 mg EP/kg, and finally five bottles served as blanks (control). During incubation, pH was monitored at 60, 150 and 330 min. After complete in vitro digestion, the incubations were terminated and the samples were placed on ice before centrifugation (10,000 x g for 10 min at 4°C). Supernatants were stored at 20°C before analysis. All samples were analysed to determine the degree of protein hydrolysis as well as the content of solubilised and digested protein.

**Degree of Hydrolysis (DH):**

The DH of protein in different samples was determined using a colorimetric assay based on the o-phthaldialde-hyde (OPA) method according to Nielsen et al. (2001). All in vitro supernatants were diluted (1:100, v/v) in de-ionized water using an automated Tecan dilution station (Männedorf, Switzerland). Microtiter plates containing eight replicates each of 25 µL of blank or sample were loaded onto an iEMS MF reader (Labsystems, Finland), and 200 µL of OPA reagent was automatically dispensed. Plates were shaken (for 2 min at 700 rpm), absorbance was measured at 340 nm, and DH was calculated according to Nielsen et al. (2001). DH is defined as the percentage of cleaved peptide bonds:

$$DH(\%) = \frac{100 \times h}{h_{tot}},$$

where $h_{tot}$ is the total number of peptide bonds per protein equivalent, and $h$ is the number of hydrolyzed bonds. Calculation of $h_{tot}$ is based on the amino acid sequence of the raw material. In this study the value for soy was used (7.8 g equivalents per kg protein) according to Adler-Nissen (1986). The expression for $h$ in the OPA method is:

$$h = \frac{(\text{serine} - \text{NH}_2 - \beta)}{\alpha \text{meqv/g protein}},$$

where $\alpha = 0.970$ and $\beta = 0.342$ according to Adler-Nissen (1979). Serine-NH2 is calculated as:

$$\text{Serine-NH}_2 = \frac{(\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}})}{(\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}) \times 0.9516 \text{meqv/L}},$$

$$\times 10^{-1}/X \times P,$$

where serine-NH2 = meqv serine-NH2/g protein; X = g sample; P = protein% in sample and 0.1 is the sample volume in litres (L).

**Estimation of Solubilised and Digested protein:**

The content of solubilised protein in supernatants from digested samples was estimated by quantifying crude protein (CP) using High Performance Liquid Chromatography (HPLC). In vitro supernatants were thawed, filtered through 0.45 µm polycarbonate filters (Sartorius Biotech land), and of OPA reagent was automatically dispensed. Plates were shaken (for 2 min at 700 rpm), absorbance was measured at 340 nm, and DH was calculated according to Nielsen et al. (2001). DH is defined as the percentage of cleaved peptide bonds:

$$DH(\%) = \frac{100 \times h}{h_{tot}},$$

where $h_{tot}$ is the total number of peptide bonds per protein equivalent, and $h$ is the number of hydrolyzed bonds. Calculation of $h_{tot}$ is based on the amino acid sequence of the raw material. In this study the value for soy was used (7.8 g equivalents per kg protein) according to Adler-Nissen (1986). The expression for $h$ in the OPA method is:

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$$\times 10^{-1}/X \times P,$$

where serine-NH2 = meqv serine-NH2/g protein; X = g sample; P = protein% in sample and 0.1 is the sample volume in litres (L).
peak area corresponding to peptides and amino acids having a molecular mass ≤1500 Da. To determine the 1500 Da dividing line, the gel filtration column was calibrated using Cytochrome C (Boehringer, Germany), aprotinin, gastrin I, and substance P (Sigma Aldrich, USA) as molecular mass standards.

In vivo Studies

In an attempt to confirm the protein hydrolysing effects of RPA that were recorded in the in vitro study, two in vivo feeding experiments were carried out at the Research Centre for Animal Nutrition and Health (DSM Nutritional Products, France) in accordance with the official French norms for experiments with live animals. Day-old broiler chickens (Ross PM3) were procured from a commercial hatchery. Birds were housed in deep littered (wood shavings) floor pens in an environmentally controlled room. The room temperature was adapted according to the age specific requirements of the birds. Birds were given ad libitum access to feed and tap water. From day old, they were divided by weight into groups of 20 birds, which were kept in a pen that served as an experimental unit and as a replicate of their respective treatment. The group weights and feed intake were recorded on days 1, 22 and 35. In both trials, chickens were fed diets based on maize and SBM (48% crude protein, SBM 48). Titanium dioxide (TiO₂) was added to the grower feed as an indigestible marker at a concentration of 1 g per kg feed (1,000 ppm). The experimental diets were pelleted (3×25 mm) with a conditioning temperature of about 70°C. Before pelleting, mash feed samples were collected, and together with samples of pellets were analysed for proximate composition and added enzyme to estimate recovery. At the end of the feeding trial, birds were randomly selected from each group and sacrificed by cerebral dislocation. The birds were dissected, and the content of the terminal ileum, defined as the region between 17 cm and 2 cm proximal to the ileo-caecal junction, was collected. The digesta from chickens in the same pen was pooled. The digesta was immediately frozen (−18°C) and subsequently freeze dried. The contents of CP, fat and energy as well as the concentration of TiO₂ in the digesta samples were determined.

The analyses of the nutrients in the feed and representative ileal samples were performed according to standard methods (VDLUFA, 1976). Crude protein was determined as N × 6.25 by a LECO apparatus according to the Dumas method. Fat was analysed using the Caviezel® method for total fat determination (Pendl et al., 1998). The energy determinations were performed using an IKA® Werke Calorimeter (C 2000 basic). Crude protein, total fat and gross energy in the rest of the ileal samples were determined by NIRS predictions using NIRS calibrations developed by Philippes et al. (2004). The results were confirmed by results from representative ileal samples that were analysed by standard methods for proximate analyses (VDLUFA, 1976). TiO₂ was determined by ICP according to DIN EN ISO 11885: 1997 (DIN EN ISO 1998) after H₂SO₄/Na₂SO₄ solubilisation.

Experiment 1

The experiment consisted of a 2×2 factorial arrangement (2 levels of enzyme and sexes). Birds with an average initial body weight of 52±5g were fed a starter diet (day 1–22 of age) containing 210 g CP and 12.7 MJ ME per kg feed, and a grower diet (day 22–35 of age) containing 200 g CP and 12.7 MJ ME per kg feed. There was a control treatment (C) without enzyme supplementation and a test treatment (C+RPA) which was the C plus 15,000 PROT RPA/kg feed (200 ppm of formulated product) (Table 2). Each treatment was replicated by 12 groups of males and 12 groups of females. At the end of the experiment (day 36), eight male birds from each treatment were randomly selected and individually sampled to collect ileal digesta.

Experiment 2

The trial consisted of a 2×2 factorial arrangement (2 levels of protein with or without the addition of protease). Two diets were fed in four treatments. The first diet (NP) contained a normal protein level (211 and 200 g CP per kg feed in the starter and grower phases respectively). The diet was fed without protease supplementation to a treatment (NP) or supplemented with 15,000 PROT RPA per kg diet to another treatment (NP+RPA). The second diet (LP) contained lower protein level (200 and 190 g CP per kg feed in the starter and grower phases respectively) than the NP. This diet was fed without protease supplementation to a third treatment (LP) or with the supplementation of 15,000 PROT RPA/kg diet to a fourth treatment (LP+RPA). Each treatment was replicated by 12 pens of male broiler birds. The composition of the experimental diets is shown in Table 2. At the end of the trial, eight birds per replicate were randomly selected from the low protein treatments (LP and LP+RPA) for individual collection of ileal digesta.

Statistical Analyses

Data from the in vitro studies were subjected to one way ANOVA to evaluate the effect of the enzyme on protein digestion. Comparison of means were done using the Tukey test (α = 0.05) provided by the ANOVA procedure (SAS Institute, 2003). For the in vivo studies, a 2-way ANOVA (Experiment 1 - enzyme supplementation x sex; Experiment 2 - enzyme supplementation x protein level) was carried out, using the statistical analysis software ‘Stat Box Pro’, version 5.0 (Grimmersoft, 1995). Results were considered different if P<0.05 and Newman-Keuls post-hoc test was used to compare differences between treatment means.

Results and Discussion

In vitro Studies

The degree of hydrolysis, protein solubility as well as protein digestibility were significantly increased (P<0.05) in samples incubated with pepsin, pancreatic enzymes and RPA compared to samples incubated with only pepsin and pancreatic enzymes (Table 1). In control samples, 26.8%
of the peptide bonds were hydrolysed and this number was significantly increased by 5.1% upon addition of RPA at 100 mg EP/kg. The level of soluble protein in control samples was 90.1% of total protein, whereas only 54.1% of the total protein was found to be 'digestible', defined as peptides with molecular weights below 1,500 Da. Both the level of soluble and digestible protein was significantly increased by RPA in a dose dependent manner, reaching 5.2% and 8.9% respectively at 100 mg EP/kg feed. At 50 mg EP/kg feed, there was significant improvement in the soluble and digestible protein. The data showed that on top of the digestive proteases RPA improved DH as well as levels of both solubilised and digested crude protein. The increase in solubilisation and hydrolysis of protein in vitro indicate that RPA might act complementary to the endogenous proteases in the digestive tract and thereby enhance protein and amino acid digestibility in vivo.

**In vivo Studies**

The analysed chemical composition and the ME, calculated on the basis of analysed nutrients (EC-equation, EEC, 1986), of the experimental diets for both trials are shown in Table 2. In experiment 1, the targeted CP and ME levels in the feed were achieved. In experiment 2, the CP levels and ME levels targeted were achieved, except in the grower feed where the CP was targeted at 190 g/kg feed but 171 g/kg feed was realised. Despite this difference, the composition of the diets was within acceptable ranges. Since there was only one batch of basal diet compounded for each growth phase, which was subsequently divided into two lots, one serving as the control and the other supplemented with the enzyme to serve as the test diet, possible effects can be attributed to the enzyme. The protease activity recovery in the diets either as mash or pellet are also presented in Table 2. With a target of 15,000 PROT per kg feed, recovery in test diets ranged between 14,858 to 16,839 PROT/kg feed (102% to 112% recovery) and 14,354 to 16,300 PROT/kg feed (96% to 109% recovery) for the mash and pellet feeds respectively. The differences between the enzyme activities in the pellet feed compared to those in the mash feed demonstrate the high pelleting stability of the enzyme product as only about 4% of the enzyme activity was lost through pelleting.

**Experiment 1**

Results of the growth performance of the birds are presented in Table 3. The total mortality registered in the trial was within acceptable range (<5%), although the male animals seemed to have had a higher mortality (4.6%) than the females (1.3%). Enzyme supplementation did not affect mortality. In the first 21 days, the addition of the enzyme significantly ($P=0.002$) improved the WG of the birds especially in the males (>5% improvement). Feed conversion ratio was also significantly ($P=0.001$) improved by the addition of the enzyme, especially in the male birds (about 4% improvement). On feeding a broad spectrum protease (keratinase) at 1,000 ppm to broiler chickens for 26 days, Odetallah et al. (2003) reported significant improvement in FCR (from 2.14 with the control treatment to 2.04 with enzyme supplemented treatment). Comparative results were also reported by Wang et al. (2006). In the grower period (day 22 - day 35), the effects of the addition of the enzyme on WG and FCR were not statistically significant, although numerical improvements were observed in the male group. Similar results were recorded by Odetallah et al. (2005) when they fed 1,000 ppm of Versazyme (a protease enzyme) and reported significant effects of the enzyme in the first 21 days, which became non significant at 35 days.

From 1 to 35 days, RPA significantly improved WG and FCR with the males responding more than females. In terms of growth performance, male broilers are significantly ($P<0.001$) better than females confirming previous reports by Howlider and Rose (1992). As female...

**Table 1. Degree of hydrolysis (DH), soluble and digestible crude protein of samples incubated with pepsin, pancreatic enzymes and RPA in vitro**

<table>
<thead>
<tr>
<th>RPA (mg EP/kg feed)</th>
<th>Percentage of total</th>
<th>Relative to blank (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of Hydrolysis (DH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26.84±0.69</td>
<td>100.0*</td>
</tr>
<tr>
<td>100</td>
<td>28.21±0.35</td>
<td>105.1*</td>
</tr>
<tr>
<td>Soluble crude protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>90.1±1.1</td>
<td>100.0*</td>
</tr>
<tr>
<td>50</td>
<td>93.2±1.4</td>
<td>103.4*</td>
</tr>
<tr>
<td>100</td>
<td>94.8±0.9</td>
<td>105.2*</td>
</tr>
<tr>
<td>Digestible crude protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>54.1±1.1</td>
<td>100.0*</td>
</tr>
<tr>
<td>50</td>
<td>57.7±1.1</td>
<td>106.7*</td>
</tr>
<tr>
<td>100</td>
<td>58.9±0.8</td>
<td>108.9*</td>
</tr>
</tbody>
</table>

Data in the percentage of total represent the Mean±SD (Standard Deviation) of five replicates.
Different superscript letters within the same analytical parameter indicate significant differences (1-way ANOVA, Tukey-Kramer test, $P<0.05$).

*In vitro*
Table 2. Composition of experimental diets and enzyme recovery in test diets

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet C</td>
<td>Diet NP</td>
<td>Diet LP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Starter</td>
<td>Grower</td>
<td>Starter</td>
<td>Grower</td>
</tr>
<tr>
<td>Maize</td>
<td>58.42</td>
<td>61.40</td>
<td>58.02</td>
<td>61.28</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>35.20</td>
<td>32.30</td>
<td>35.10</td>
<td>32.30</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2.50</td>
<td>2.20</td>
<td>3.30</td>
<td>2.80</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.08</td>
<td>0.15</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>DCP</td>
<td>2.15</td>
<td>2.16</td>
<td>1.95</td>
<td>1.95</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.45</td>
<td>0.54</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.20</td>
<td>0.15</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>TiO₂</td>
<td>—</td>
<td>0.10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Premix²</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Enzyme: 15,000 PROT/kg</td>
<td>—/+</td>
<td>—/+</td>
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</table>

Calculated content

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<tr>
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</thead>
<tbody>
<tr>
<td>ME₅ (MJ/kg)¹</td>
<td>12.7</td>
<td>12.7</td>
<td>12.7</td>
</tr>
<tr>
<td>Crude protein (g/kg)</td>
<td>210</td>
<td>200</td>
<td>211</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>1.18</td>
<td>1.09</td>
<td>1.17</td>
</tr>
<tr>
<td>Methionine+Cystein (%)</td>
<td>0.77</td>
<td>0.81</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Analysed content

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Crude protein (g/kg)</td>
<td>211</td>
<td>203</td>
<td>200</td>
</tr>
<tr>
<td>Total Fat (g/kg)</td>
<td>56</td>
<td>56</td>
<td>63</td>
</tr>
<tr>
<td>Starch (g/kg)</td>
<td>405</td>
<td>418</td>
<td>409</td>
</tr>
<tr>
<td>Sugar (g/kg)</td>
<td>43</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td>ME₅ (MJ/kg)²</td>
<td>12.5</td>
<td>12.6</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Enzyme Recovery³ in test diets (PROT/kg)

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<thead>
<tr>
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<tbody>
<tr>
<td>Targeted amount</td>
<td>15,000</td>
<td>15,000</td>
<td>15,000</td>
</tr>
<tr>
<td>Mash feed + enzyme</td>
<td>16,839</td>
<td>15,227</td>
<td>15,182</td>
</tr>
<tr>
<td>Pellet feed + enzyme</td>
<td>16,300</td>
<td>14,765</td>
<td>14,354</td>
</tr>
</tbody>
</table>

¹TiO₂ as indigestible marker was included in the feed mixture (grower).
²Including Avatec (vitamin and mineral premix supplied in % (Ca = 17.3, Mg = 0.8, Na = 11.7), in UI/kg (Vit. A = 11000000, Vit. D₃ = 3000000, Vit. E = 4000), in mg/kg (Vit B₁ = 250, Vit B₂ = 800, Cal panth. = 1200, Vit. B₆ = 500, Vit B₁₂ = 2.5, Vit. PP = 5000, Vit. C = 100000, Vit. K₃ = 300, Biotin = 15, Folic acid = 150, Choline = 50004, Fe = 6000, Ag = 3000, Zn = 5400, Mn = 8000, I = 124, Co = 60, Se = 29.7, Las. = 9000).
³Calculated with EC-equation.
⁴Calculated with EC-equation based on analysed crude nutrients.
⁵Recovery in the treatments without enzyme were all below detection limit.

Birds grow older, they start depositing fat as a sign of maturity, due to oestrogen effects on fat metabolism (Yannakopoulos et al., 1995), and therefore it is hypothesized that fat absorption and accretion becomes more relevant than protein in this phase. This may have affected protein digestibility and therefore account for the interaction of enzyme and the animal sex as the birds got older (grower phase). Sex x diet interactions were also reported by Reece et al. (1985).

Digestibility of nutrients is presented in Table 5. In experiment 1, the RPA significantly improved protein digestibility by about 8%. Total fat digestibility was also significantly improved by 3%. As a result, ileal digestible energy was also significantly increased. The increase in protein digestibility confirms the results that were obtained in the in vitro studies. Because RPA is a pure protease and does not have any lipase activity, the improvement of fat digestibility recorded is most probably a secondary effect of protein degradation. By degrading large protein molecules in a chyme complex, there might be better access to the total surface area of the lipid molecules for micelle formation.

**Experiment 2**

Performance parameters for animals in experiment 2 are presented in Table 4. Mortality was within the acceptable range and had no trends that could be linked to dietary protein level. However, birds that were fed the RPA supplemented diets had lower mortality compared to the birds without the enzyme supplementation. Whether the enzyme really plays a role in the livability of the birds is subject to further investigation, as this observation is not corroborated by experiment 1.

In the starter period (day 1–22), enzyme supplementation had no significant effects on WG of the birds irrespec-
Table 3. **Performance of male and female broiler chicks in experiment 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ANOVA-2 (P-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>day 1 – 22</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>942b</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>1296</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.38b</td>
</tr>
<tr>
<td>day 22 – 35</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>1321</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>2433b</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.85</td>
</tr>
<tr>
<td>overall, day 1 – 35</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>2262b</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>3723b</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.65b</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Data represents Mean of 12 replicates per treatment and SEM = pooled standard error mean across treatments. Newman-Keuls test: Means within a row of a same sex, not sharing a common superscript, are significantly different (P<0.05).

Table 4. **Performance of male broiler chicks in experiment 2**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>ANOVA-2 (P-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
</tr>
<tr>
<td>day 1 – 22</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>986</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>1443</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.47</td>
</tr>
<tr>
<td>day 22 – 35</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>1368</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>2434</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.78</td>
</tr>
<tr>
<td>Overall, day 1 – 35</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/bird)</td>
<td>2353</td>
</tr>
<tr>
<td>Feed intake (g/bird)</td>
<td>3873</td>
</tr>
<tr>
<td>Feed conversion ratio (g feed/g gain)</td>
<td>1.65</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Data represents Mean of 12 replicates per treatment and SEM = pooled standard error mean across treatments. Newman-Keuls test: Means within a row of a same diet (diet vs. diet+enzyme), not sharing a common superscript, are significantly different (P<0.05).

Table 5. **Apparent ileal digestibility (%) of protein, fat and energy of male broiler chickens at day 36 in Experiment 1 and 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>C + RPA</td>
</tr>
<tr>
<td>Protein</td>
<td>76.9b</td>
<td>82.8b</td>
</tr>
<tr>
<td>Fat</td>
<td>88.6b</td>
<td>91.1b</td>
</tr>
<tr>
<td>Energy</td>
<td>70.6b</td>
<td>77.8b</td>
</tr>
</tbody>
</table>

Data represents Mean of 8 replicates per treatment and SEM = pooled standard error mean across treatments. Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different (P<0.05).
tive of the dietary protein level, but contrary to the first trial, the birds fed enzyme supplemented diets tended ($P=0.055$) to consume less feed than those without enzyme. As expected, there was a significant effect of protein level on WG, feed consumption and FCR. In the grower phase (day 22–35), though the effects of the enzyme were not significant, there were improvements of WG with up to about 3% (1,269 g vs. 1,309 g) in the low protein diets. These cumulative improvements in WG and reduced feed consumption translated to a significant ($P=0.001$) improvement in FCR (1.98 vs 1.88). In the grower phase, the dietary protein level significantly ($P<0.001$) influenced WG, feed consumption and FCR. Birds on the low protein diet consumed more feed, gained less weight and had a poorer FCR. Significant reductions of feed consumption and significant improvements in FCR were observed as a result of protease supplementation. These effects were stronger in the low protein diet than in the normal protein diet. Overall, the normal protein diet demonstrated a significant advantage over the low protein diet.

As a consequence of the performance observed, ileal digestibility was carried out only in the low protein diet. The protease significantly ($P<0.05$) improved energy digestibility as a cumulative effect of increased protein and fat digestibility.

In both experiments, the addition of the enzyme had significant benefits on the performance of the animals, mainly by increasing the digestibility of CP and consequently digestible energy. The activity of the exogenous protease can complement that of the endogenous protease. Therefore, in feeds with lower nutrient digestibility, the effect of the exogenous enzyme seems to be more pronounced, such as was the case in experiment 1.

In conclusion, the protease demonstrated an ability to act on top of pepsin and pancreatic enzymes by hydrolyzing and solubilising protein in vitro. This was confirmed by in vivo studies, where it improved protein and energy digestibility which lead to significant improvements in broiler performance. Proteases could help address some of the current challenges faced by the livestock industry as feed cost and demands for sustainable farming.

References


Wang H, Yuming G and Shih JCH. Effects of dietary supplementation of keratinase on growth performance, nitrogen
