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Influence of dietary inclusion of xylanase from different sources on LingNan Chinese color-feathered chickens

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ABSTRACT

This study was conducted to research 2 single xylanase (XylnA, XylnD) (EC 3.2.1.8) cocktails. XylnA was from *Aspergillus* sp. and XylnD from *Trichoderma reesei*. Optimum temperature and pH of XylnA and XylnD was 45 °C and 4.5, respectively was 55 °C and 5.5 XylnA was inhibited by pepsin and trypsin. However XylnD was activated by pepsin and trypsin. XylnA was activated by Ca^{2+}, Cu^{2+} and Zn^{2+}. However XylnD was inhibited by Ca^{2+}, Zn^{2+} and Fe^{2+}. *In vitro* incubation of XylnA with XylnD at the ratio of 1:1 showed the highest synergistic effect of 1.84 on wheat cell wall degradation. The amounts of both reducing sugars and xyloses liberated from wheat cell walls were increased. 1800 one-day-old male LingNan Chinese color-feathered chickens were allotted to 5 treatments (a positive control diet (PC), a negative control diet (NC) with a lower energy density (approximately 0.66 MJ/kg feed) and 3 test diets where XylnA, XylnD and combination of XylnA and XylnD (1:1) were added to the negative control individually with the same enzyme activity (4000U/kg feed). Feed intake and body weight gain were significantly increased with XylnAD supplementation in comparison with NC and there was no difference in comparison with PC. However, growth performance was not significantly increased with single xylanase addition. It can be concluded that the combination of XylnA and XylnD supplementation to low-energy diet could improve growth performance over singe xylanase.

Key words: Enzyme, Feeding trial, *In vitro*, Xylanase

The main mechanism of the anti-nutritive activities of non-starch polysaccharides (NSP) was to increase gut viscosity and microbe quantities, depress feed nutrient value and apparent metabolic energy, integrate physiological activator and inhibit intrinsic enzymes. Preparation enzymes reversed the adverse effects and improved growth performance (Omogbenigun *et al*. 2004).

The effect of the mixture of two or more individual hydrolytic components is greater than the sum of the action of the individual components (Klyosov 1990), which was demonstrated by several studies (Wood *et al*. 1989, Vries *et al*. 2000, Sørensen *et al*. 2003). In addition, enzymes isolated from various sources differ in their molecular characteristics (Khucharoenphaisan *et al*. 2008, Pollet *et al*. 2009), produce different end products and have different number of binding sites (Meagher *et al*. 1988). Therefore, we presume that synergism exists among xylanase from different sources. However, purified enzymes are generally not available in feeding trials because of high production costs and low yield. Moreover, enzymes will show different activity depending upon the type of protein. Therefore, the available method to combine single enzymes was on the basis of enzyme activity.

In vitro method had a good estimate of *in vivo* digestibility (Zyla *et al*. 1995). Therefore, the combination of single xylanases from different sources was indentified through *in vitro* incubation. The screened enzymes were blended according to the optimum ratio and supplemented in feeding trial, which was conducted to determine if the administration of combination of xylanase to a low-energy wheat based diet could improve growth performance, similar to that of a high energy diet, over a single xylanase.

MATERIALS AND METHODS

Substrate, xylanases and animals: Birchwood xylan, trypsin, amylase, lipase, chymotrypsin and pepsin were purchased commerically. XylnA from *Aspergillus* sp. and XylnD from *Trichoderma reesei* were provided by Chinese firms. Wheat was in mashed form and passed through a 40 mesh screen. In feeding trial 1800 male LingNan Chinese Color-feathered chickens (BW 37.0±0.1) were used.

Enzymes extraction and enzyme assays: The proteins of

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enzymes were extracted with 100 ml 50 mM citrate-phosphate buffer (pH5.0). Xylanase activity was determined based on the amount of reducing sugars released from 1% birchwood xylan according to Miller (1959). One unit (U) of the enzyme liberates such an amount of the reducing sugars product from 1% birchwood xylan as to give 3,5 dinitrosalicylic acid (DNS) color equivalent to 1 µg xylose per min at 41°C and pH 5.0. The method of measuring the production of the sum of single xyloses and xyloses from oligosaccharides was according to the method of orcinol-pentose reaction (Albaum and Umbreit 1947).

Measure of optimal pH and temperature: The optimal pH was determined by monitoring xylanase (EC 3.2.1.8) activity at pHs ranging from 2.0–9.0 (50 mmol citrate-phosphate buffer). Enzymatic activity was measured by 30 min incubation at 41°C. The optimum temperature for two xylanases was determined in 50 mmol citrate-phosphate buffer at the optimal pH at temperatures between 30 and 80°C with the birchwood xylan.

Effects of various salts: The effects of various inorganic salts ($CaCl_2$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$) at 1.0 mmol final concentration on the xylanase activities were determined by performing the assay under the conditions described at optimum pH and temperature.

Effects of pepsin and trypsin: XylnA and XylnD were treated with pepsin (EC 3.4.4.1) (200U/ml) for 90 min incubation at 41°C and pH 2.0 or trypsin (3.4.21.4) (200U/ml) for 120 min incubation at 41°C and pH 6.0 or both citrate-phosphate buffers. The control group was treated at pH 2.0 or treated at pH 6.0 or both citrate-phosphate buffers.

Measure of Michaelis constant (Km) value and maximum velocity: XylnA and XylnD Km values were measured at pH 5.0 by 15 min incubation at 41°C. Birchwood xylan was diluted. Substrate concentration was: 10.0 mg/ml, 5.0 mg/ml, 3.33 mg/ml, 2.5 mg/ml, 2.0 mg/ml, 1.66 mg/ml, and 1.42 mg/ml respectively. Maximum velocity (Vmax) and Michaelis constant (Km) were obtained by Lineweaver-Burk method.

Degradation of wheat with combination of enzymes: The wheat powder was incubated in triplicate, with the *in vitro* 3-stage (pH 4.36, 6 min; pH 2.66, 77 min; pH 6.0, 128 min; pepsin 0.51U/g; trypsin 1.18U/g; chymotrypsin 0.05U/g; amylase 338U/g; lipase 240U/g) enzyme incubation method, which was modified according to the method of Bedford *et al.* (1993), to measure the conversion of wheat xylan to xylanase by the production of two products: reducing sugars and total xyloses (the sum of single xyloses and xyloses from oligosaccharides). XylnA and XylnD were combined in different ratios (1:4, 2:3, 1:1, 3:2, 7:3, 4:1, 6:1, 9:1 and 19:1) with control groups of XylnA and XylnD at the same enzyme activity (4.17U/ml). A semi-permeable membrane was applied to prevent pentosan in this experiment. The molecular weight cut-off of semi-permeable membrane was 8000 to 12000.

Feeding trial: A feeding trial was conducted to investigate the effects of single xylanases and combination of xylanases on the growth of chicken fed diets containing high proportions of wheat. Basal diets for LingNan Chinese color-feathered chickens (the growth phase, 1–21 days of age) were formulated with wheat, maize and soybean meal. Basal diet 1 was formulated to meet Nutrient Requirements of Chinese color-feathered Chicken recommended by Ministry of Agriculture of the People's Republic of China (2005). To maintain energy and nitrogen ratio, metabolic energy and

Table 1. Ingredients of basal diet 1 and basal diet 2

Components (g/kg mixture)	Basal diet 1	Base diet 2
Corn[3]	645.90	407.0
Soybean meal[3]	216.00	178.5
Wheat[3]		223.4
Wheat bran[3]		75.2
Corn gluten meal(60CP)[3]	82.60	59.7
Dicalcium phosphate	20.10	18.3
Limestone	11.80	12.0
Premix(1%)[1]	10.00	10.0
Salt	4.80	4.40
Lysine (65%)	4.80	6.30
DL-Methionine (98%)	1.0	1.30
Threonine (98%)		0.90
Fungicides	2.00	2.00
Choline chloride	1.00	1.00
Nutrient by calculation[2]	12.41	11.75
Metabolisable energy (MJ/kg)	211.00	201.0
Crude protein (g/kg)	5.00	5.00
Salt	9.20	9.20
Calcium	6.60	7.00
Total phosphorus	4.50	4.50
Available phosphorus	12.70	12.60
Lysine	9.30	9.10
Met+Cys	2.20	2.20
Threonine	7.70	7.70
Tryptophan	12.20	11.30
Arginine		
Nutrients as analysis	873.10	876.4
Dry matter (g/kg)	209.00	202.0
Crude protein	22.80	22.5
Crude fat	43.40	59.8
NSP[4]		

[1]Added per kg of diet: DL-methionine, 1200 mg; retinylpalmitate 5.5 mg; cholecalciferol, 0.05 mg; DL-α-tocopheryl acetate, 40 mg; menadione, 3 mg; thiamin, 3 mg; riboflavin, 4.5 mg; pyridoxine, 7 mg; cyanocobalamin, 0.03 mg; nicotinic acid, 50 mg; Ca-pantothenate, 8 mg; folic acid, 1.5 mg; choline chloride, 600 mg; Mn 80 mg as $MnSO_4.H_2O$ Fe, 80 mg as $FeSO_4.H_2O$ Zn, 50 mg as $ZnSO_4.H_2O$; Cu, 10 mg as $CuSO_4.5H_2O$; Co, 0.4 mg as $CoSO_4$; iodine, 0.35 mg as KI; Se, 0.25 mg as $NaSeO_3$. [2]Calculated according to *Feed Composition and Nutritive Values in China*. [3]The crude protein content of corn, soybean meal, wheat, wheat bran and corn gluten meal was 85 g/kg; 479 g/kg; 139 g/kg; 157 g/kg; 635 g/kg respectively. [4]NSP non-starch polysaccharides.

crude protein were lowered in basal diet 2. The dietary crude protein content amounted to 211 in basal diet 1 and 201 g/kg in basal diet 2. Energy densities were calculated as 12.41 and 11.75 MJ/kg, respectively. Basal diet 1 did not contain wheat and basal diet 2 contained 223.4 g/kg wheat and 75.2 g/kg wheat bran. All diets were in mashed form and diet formulations were presented in Table 1. A total of 5 experimental diets were created, viz. 2 controls (Basal diets 1 and 2 with no enzyme supplementation) and 3 enzyme-supplemented diets (basal diet 2 with XylnA, XylnD and XylnAD (XylnA: XylnD=1: 1) respectively) with the same enzyme activity (4000U/kg feed).

Day-old single sex (male) LingNan Chinese color-feathered chickens (1800), average BW 37.0±0.1 g, were used in the performance trial and chickens were randomized among 30 floor pens each with 8 m^2 pen containing 60 chickens, i.e. 6 replicates (pens) per dietary treatment. Clean wood shavings were used as litter. The chickens had free access to feed and water. Lighting was given for 24 h. The temperature inside the room was gradually reduced from 30 to 21°C.

Experimental measurements: Feed intake (FI) per pen was recorded daily throughout the trial and body weight data was recorded at day 21. Feed conversion ratio (FCR) were calculated from this data. Mortality and its cause were registered and analyzed.

Chemical analytical methods: The chemical composition of the diets was determined according to AOAC (2000) methods and NSP by orcinol-pentose reaction (Albaum and Umbreit 1947).

Statistical analysis: The study was conducted in a randomized complete block design. All obtained data were evaluated statistically by one-factorial ANOVA using SAS. The differences between treatments for all parameters were tested according to the following statistical model:

$$y_{ij}=\mu + a_i + e_{ij}$$

Where y_{ij} means the variance associated with a parameter; μ is the overall mean; a_i is the treatment effect; e_{ij} is the error term. For basal performance (body weight gain, BWG; feed intake, FI; feed conversion ratio, FCR and mortality), the means for pens were treated as experimental units for statistical evaluation and differences between treatment means were analyzed for significance ($P<0.05$). The study was approved by the College of Animal Science and Technology Animal Protocol Review Committee. Housing and care of the animals conformed to the guidelines of Chinese Department of Agriculture.

RESULTS AND DISCUSSION

Properties of XylnA and XylnD: The optimal pH for XylnA and XylnD was 4.5, 5.5 respectively. The optimal temperature for XylnA and XylnB was 45°C, 55°C respectively (Fig.1). XylnA was more acid stable and still active (19.4% relative activity) at *p*H 2.0 and lost activity at *p*H 8.5, while XylnD was active at *p*H 8.5 (16.19% relative activity) and lost activity at *p*H 2.0.

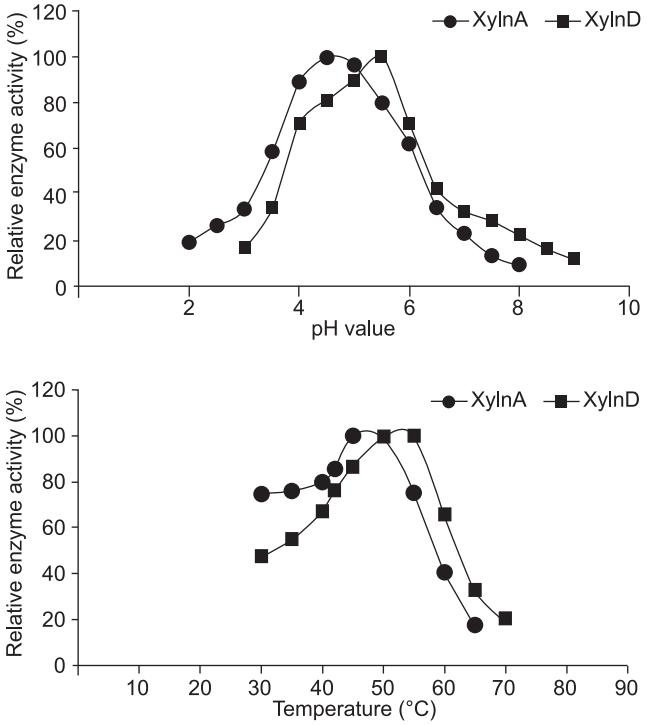

Fig. 1. Effects of pH (A) and temperature (B) on the activities of XylnA and XylnD. The maximal activities of XylnA and XylnD as depicted by each curve were defined as 100% relative activity.

XylnA was activated by Cu^{2+}, Ca^{2+}, Zn^{2+}; and there was no inhibition with Fe^{2+}. XylnD was inhibited by Ca^{2+}, Zn^{2+}, Fe^{2+}; while Cu^{2+} showed no effect. After treated by pepsin or trypsin or both, the activity of XylnA was lowered; the relative enzyme activity was 92.8, 79.2 and 62.8% respectively. However, the activity of XylnD was improved when treated by pepsin, trypsin, or both; the relative enzyme activity was 159.0, 124.0 and 162.6% respectively (Fig. 2).

Km value of XylnA and XylnD was 16.25 mg/ml and 5.27 mg/ml, respectively. Maximum velocity was 16.65 and 6.05μmol/min.ml, respectively. XylnA and XylnD showed different affinities for birchwood by measure of Km value. Different xylanases showed different optimum pH values and temperatures (Wood and McCrae 1986), inhibition or activation by metal ions (Frederick *et al*. 1985), different spatial structures (Wood *et al*. 1989), active sites and affinities for substrate (Khucharoenphaisan *et al*. 2008, Pollet *et al*. 2009), and produce different end products (Meagher 1988). In this study, XylnA and XylnD showed different properties. Therefore, it can be presumed that there was affinity difference for the same substrate between XylnA and XylnD.

Degradation of wheat with combinatiion of enzymes: The mixture of XylnA and XylnD at an enzyme activity ratio of 1:1 showed the highest synergistic effect of 1.84 on wheat xylan degradation. The amounts of both reducing sugars and

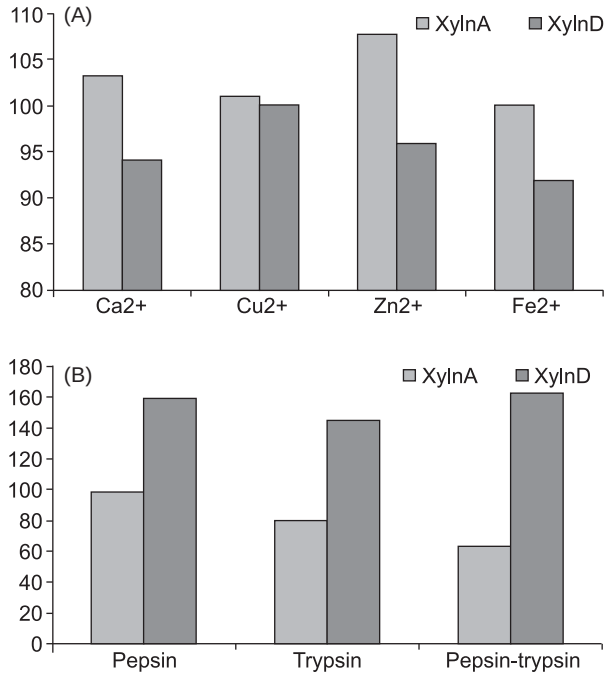

Fig. 2. Effects of 4 inorganic salts (A) and digestive enzymes (B) on the activities of XylnA and XylnD. The enzyme activity in the absence of additives was defined as 100% relative activity.

total xyloses liberated from wheat xylan were increased by the synergistic action of XylnA and XylnD (Fig. 3).

Diet is a very complex substrate, which is composed of different feed materials. Moreover, different enzymes generally show different catalysis with the same substrate for their different design patterns. Therefore, the cocktails of single enzymes can show higher degradation on substrate than single enzyme. In addition, many enzymes are likely to be strongly inhibited by the products of the reaction by means of Michaelis-Menten kinetic retro-action (Schmidt *et al.* 1983). Therefore, combination of enzymes showed higher activity and decomposition efficiency for the lower end product concentration than single enzyme. For the mixture of different kinds of enzymes showing the highest synergistic effect on corn cell wall degradation (Murashima

Table 2. Feed intake (FI), mortality, body weight gain (BWG) and feed conversion ratio (FCR) of LingNan color-feathered chickens (one-way ANOVA) for the whole 21 days period

Meal	Group	FI (g/head)	BWG (g/head)	FCR	Mortality
Diet 1	PC	471±4^{a}	236±3^{a}	2.00±0.02^{b}	1.53±0.79^{a}
Diet 2	NC	456±8^{b}	215±5^{c}	2.12±0.02^{a}	2.30±1.04^{a}
Diet 2	XylnA	462±3^{ab}	219±3^{bc}	2.11±0.02^{a}	2.82±1.10^{a}
Diet 2	XylnD	465±3^{ab}	223±2^{bc}	2.09±0.01^{a}	2.80±0.62^{a}
Diet 2	XylnAD	477±5^{a}	227±4^{ab}	2.08±0.02^{a}	2.46±0.79^{a}

^{abc} Values within a column with no common superscripts differ significantly (P < 0.05). Values are means±SE (n = 6).

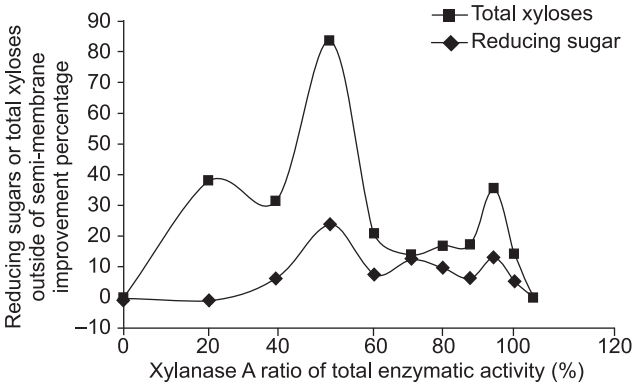

Fig. 3. Curve of released xyloses and reducing sugars from wheat xylan in the presence of XylnA and XylnD mixture with different proportions.

et al. 2003), we presume that optimum ratio exists between enzymes. In this study, combination of xylanases produced maximum reducing sugars and xyloses in the ratio of 1:1 in comparison with single xylanases at the same enzyme activity under the same conditions.

Feeding trial: Growth performance of chickens (1–21 days) is shown in Table 2. Feed intake (FI), feed conversion ratio (FCR) and body weight gain (BWG) were significantly affected (P<0.05) by meal types. Birds of positive control showed better performance than negative control. FI and BWG were significantly (P<0.05) increased with XylnAD supplementation in comparison with NC and there was no difference (P>0.05) in comparison with PC. However, growth performance was not significantly enhanced with single xylanase addition and there was difference (*P*<0.05) between PC and single enzyme groups. FCR was not significantly increased with xylanase addition and there was differcence (*P*<0.05) between birds fed diet 1 and birds fed diet 2. The birds fed diet 2 had higher mortality than birds fed diet 2, which was caused by weak chicken, the sudden death of heavy birds because of accrued stress during and after weighing and wheat addition which often caused wet litter and enteritis. However, no significant difference (*P*>0.05) was observed.

NSP enzyme addition in feed could improve nutrient absorption and NSP digestibility (Owusu-Asiedu *et al.* 2010) especially in young animals for their under developed digestive systems (Fang *et al.* 2008) and sensitivity to the addition of enzymes compared with more mature animals. Therefore, the chickens were adopted in the feeding trial. In this study, FI and BWG of chickens were significantly (*P*<0.05) improved with combinative xylanase addition. In contrast, the study of Owusu-Asiedu *et al.* (2010) showed that carbohydrase supplementation significantly (P<0.05) improved FCR and there was no difference (P>0.05) in weight gain and feed intake among treatments. Therefore, adding a combination of xylanase cocktails and other NSP enzymes should disrupt the physical barrier improving NSP

hydrolysis and subsequently improve animal performance. In addition, the results confirmed combination of xylanase was more effective than single xylanase. The positive effects on laying hens were observed in the study of Zhang *et al.* (2010). In addition, for the difference of substrate specificity and decomposition capacity, different xylanase showed different efficiency in the same enzyme activity (Choct *et al.* 2004) and the same results were observed in this study. Therefore more effective methods which could evaluate enzymatic degradation capacity should be established.

In conclusion, combination of xylanase from different sources (XylnA from *Aspergillus* sp.; XylnD from *Trichoderma reesei*), had higher decomposition efficiency, produced more end products and enhanced animal growth performance than single enzymes.

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