Short Communication

The enzyme activity of a novel phytase from *Bacillus amyloliquefaciens* DS11 and its potential use as a feed pellet

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Monogastric animals such as poultry and pigs have virtually no phytase in their digestive tract, and thus cannot utilize phytate-phosphate in their feedstuff. In order to meet the phosphorus requirements in these animals, inorganic phosphorus has to be added to the animal feedstuff as an additional nutrient (Simons et al., 1990). As a result, undigested phytate and unabsorbed inorganic phosphate remain in manure, which can pose a serious phosphorus pollution problem, such as the eutrophication of surface water, in areas of intensive livestock production. Hence, adding microbial phytase into feedstuff can decrease the amount of supplemented inorganic phosphate by releasing phosphate from various phytate forms in feedstuff (Laboure et al., 1993; Reddy et al., 1982; Simons et al., 1990).

Many feedstuffs for poultry have been used as pellets produced by extruders at temperatures from 60 to 120°C in Korea. A number of marketed phytases have shown decreasing enzyme activity due to high temperature in the course of the pelleting process (Simons et al., 1990). Therefore, the enzymes have to be consid-

ered from the thermostability point of view. When animal feedstuff is pelleted, the temperature of the pellet machine is shown to range from 60 to 120°C. Wyss et al. (1998) reported the importance of thermostability and the stability of phytase in the pelleting process. They, however, have checked at two temperatures (75 and 85°C) without considering pelleting conditions, water content in pellets, or enzyme activity against calcium-phytate complex as substrate. It is difficult to determine thermostability of phytase in the pelleting process with the information obtained at those two temperatures because most pelleting for animal feed is conducted from 60 to 90°C for chickens and pigs and 120°C for fish.

We previously reported that a novel *Bacillus amyloliquefaciens* DS11 phytase (DS11 phytase) showed thermal stability in tubes at 90°C for 10 min and this enzyme exhibited optimal enzyme activity at 70°C (Kim et al., 1998a, b). This was higher than the optimum phytase activity at 50–60°C for *Enterobacter* sp., (Yoon et al., 1996) 60°C for *B. subtilis* (natto) N-77 (Shimizu, 1992), and 58°C for fungal phytase (Yamada et al., 1968).

DS11 phytase also showed a strong enzyme activity against insoluble Ca-phytate complex in the presence of 5 mM CaCl₂ at 90°C for 10 min retaining 50% of the

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initial activity in vitro, indicating that substantial increase in the thermal stability of DS11 phytase may be accompanied by conformational change of this protein (Oh et al., 2001). However, we were not certain whether or not DS11 phytase would be stable in the feed pelleting process. An important concern regarding the use of dietary enzymes is the thermal stability of DS11 phytase throughout the pelleting process because experiments performed in vitro may not properly reflect the conditions in the feed pelleting process. The purpose of the study, therefore, is to examine the thermostability of DS11 phytase and *Aspergillus ficcum* phytase (AF phytase) under temperatures prevalent during the pelleting process, and to investigate the post-pelleting enzymatic activity against insoluble Ca-phytate complex contained in normal chicken feedstuff.

AF phytase was purchased from Sigma Chemicals (St. Louis, MO) and adjusted to get the enzyme activity of 1,000 PU/ml in 0.1 M Tris-HCl buffer (pH 7.0). DS11 phytase was prepared from fermenting *B. amyloliquefaciens* DS11. To obtain DS11 phytase, the strain was cultured in a 5-L fermenter at 37°C and harvested 16 h later, when the enzyme activity reached its maximum (Fig. 1). During the culture of *B. amyloliquefaciens* DS11, dissolved oxygen concentration (DO) (%) was maintained above 20% in culture media for aerobic conditions.

DS11 phytase production medium consisted of 6% wheat bran, 0.04% NH_4NO_3 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1% casein hydrolysate, 0.05% KCl, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$. A basal diet for chickens consisting of corn (74%), soybean meal (20%), animal fat (1%), molasses (4%), salt (0.3%), L-lysine (0.3%) and vitamins (0.4%) was mixed with DS11 phytase or AF phytase by an automatic ribbon mixer (Jangwoo Machinery Co., Inchon, Korea). The mixed feedstuff was conveyed to an extruder to make pellets at temperatures from 60 to 120°C with 10°C intervals.

Extrusion was performed by a corotating and intermeshing type twin-extruder (Bhuler, Biex-DNDL44, Uzwil, Switzerland) with barrel diameter=44 mm and barrel length/diameter=12. The barrel was sectioned in 176 mm lengths and equipped with a jacket for cooling. Feed was metered with a K-tron twin-screw volumetric feeder (K-tron Corp., Pitman, NJ). The screw configuration was 3×150 mm forward+1×100 mm kneading disk+3×100 mm forward+33 mm reverse+5×75 mm forward element from the feed to the die.

The die was a single nozzle 8 mm in diameter. The barrel temperature was maintained between 12°C and 17°C throughout the experiment by the circulation of cooling water through the jacket. Stimulus-response technique was used to determine the residence time distribution in the extruder. A feed material was fed to the extruder by a feeder, and dye tracer particles were added at steady state as a pulse through a side opening to the extruder feed hopper. Extrudate samples were collected at 1.5-s intervals after the tracer injection, and the color intensity of the dye (Cr_2O_3) was determined with a Hunter calorimeter.

The feed pellets were ground with a mortar and pestle, aliquots of 10 g were suspended in 100 ml of 200 mM Tris-HCl buffer (pH 7.0), and each suspension was stirred for 30 min at 4°C. After centrifugation for 10 min at 3,000×g and extensive dialysis of the supernatant against 200 mM Tris-HCl buffer (pH 7.0) at 4°C, phytase activity was calculated by measuring the rate of increase of inorganic orthophosphate (Pi) according to the method of Fiske and Subbarow (1925). In short, a reaction mixture containing 100 µl enzyme preparation, 400 µl of 2 mM sodium phytate in 0.1 M Tris-HCl buffer (pH 7.0) with 2 mM CaCl_2 for DS11 phytase and 0.1 M Tris-HCl buffer (pH 5.0) for AF phytase, was incubated at 37°C for 30 min. The reaction was stopped by adding 500 µl of 15% trichloroacetic acid. The released inorganic phosphate was measured at A_{820} nm by incubation with 4 ml of reagent A (1 : 1 : 1 : 2 ratio of 6 N H_2SO_4 /2.5% ammonium molybdate/10% ascorbic acid/ H_2O) at 37°C for 30 min. One unit (PU) of phytase activity was defined as the amount of enzyme required to liberate one micromole of phosphate per min under the assay conditions.

After feed pelleting, the remaining enzyme activities

Fig. 1. Phytase activity (PU, ▲), dissolved oxygen concentration (DO×10, ●) and growth (CFU/ml, ■) of *Bacillus amyloliquefaciens* DS11 during fermentation in synthetic medium at 37°C.

of DS11 and AF phytase were compared and expressed as mean $\pm$ SD. Student's *t* test was applied in the statistical analysis, with $p < 0.05$ as the significance level.

Our previous study has demonstrated that the half-life of DS11 and AF phytases are 42 min and 0.2 min at 80°C in tube, respectively (Kim et al., 1998a, 1999). With this result, it is difficult to determine whether DS11 phytase can be available to the pelleting process. To learn the possibility of pelletization for chickens and pigs, the remaining enzyme activity of DS11 and AF phytase after feed pelleting at various temperatures (from 60 to 120°C) was checked (Fig. 2). The results demonstrated that DS11 phytase retained >85% of its enzyme activity at pelleting temperatures from 60 to 90°C, as compared with that before pelleting. In contrast to DS11 phytase, AF phytase showed rapid decreasing enzyme activity at more than 70°C. This higher thermal stability of DS11 phytase may be attributable to high calcium content in feedstuff, because calcium plays a critical role in the thermal stability of DS11 phytase (Ha et al., 1999, 2000), but not of AF phytase (Wyss et al., 1998). These results suggested that DS11 phytase is quite stable and suitable for the pelleting process in the feed industry.

DS11 and AF phytase extracted from the pellet, however may have different enzymatic activity due to inorganic salts, such as calcium and phosphate normally distributed in feedstuff used for chickens, unlike purified phytases. We further tested enzyme activities of both enzymes extracted from feedstuff pelleted at 60°C in the pH ranges of 5–8, and in the presence and absence of 5 mM phosphate. As shown in Fig. 3, A and B, neither pH nor phosphate changed the activity of DS11 phytase. In contrast, the activity of AF phytase significantly decreased below 120 PU (20%) at pH > 7 as compared with 1,000 PU at pH 5 without 5 mM phosphate (Fig. 3A). In the presence of 5 mM phosphate and pH of 5, the enzyme activity of AF phytase was remarkably diminished to 140 PU (14%) as compared with the enzyme activity shown at pH 5 without 5 mM phosphate (Fig. 3B). These results implied that AF phytase could be inhibited by high pH and phosphate. These characteristics of AF phytase may constitute disadvantages for an additive to animal feedstuff, whereas the activity of DS11 phytase was not affected by pH or 5 mM phosphate, indicating broad site of action in the digestive tract as compared with AF phytase (Park et al., 1999).

Fig. 2. Comparison of the enzymatic activity of DS11 phytase and AF phytase following pelleting at various temperatures.

■, DS11 phytase. □, AF phytase. These experiments were performed in triplicate. ^{a)} Paired *t*-test: $p < 0.05$.

Fig. 3. Comparative enzyme activity of DS11 phytase and AF phytase extracted from feedstuff pelleted at 60°C in the pH range of 5–8 in the absence (A) and presence (B) of 5 mM phosphate.

■, DS11 phytase. □, AF phytase. These experiments were performed in triplicate. ^{a)} Paired *t*-test: $p < 0.05$.

The enzyme activity of both phytases was also determined against various substrates, such as 1 mM magnesium phytate (Mg-phytate), 1 mM calcium phytate (Ca-phytate), 1 mM potassium phytate (K-phytate) and 1 mM sodium phytate (Na-phytate) in soluble state at pH 7 as well as further determined against insoluble state Ca-phytate complex by adding 5 mM calcium chloride. Phytate in nature binds minerals, such as calcium, zinc and iron, to form metal-phytate complexes,

especially insoluble Ca-phytate complex form (Ca-phytate) due to electrostatic force (Maenz et al., 1999). From a practical point of view, therefore, it may be important to check the enzyme activity against insoluble Ca-phytate complex. Phytase activity against the phytate forms may play a key role in the utilization of phosphate in the animal intestine. The enzyme activity of DS11 phytase at pH 7 was compared with that of AF phytase at pH 5, which showed the maximal enzyme activity for this enzyme (Kim et al., 1999). In the present study, the enzymatic activity of DS11 and AF phytase extracted from pellet showed the mean enzyme activity of 1,190, 890, 900 and 1,000 PU, and of 987, 870, 1,000, 890 PU against 1 mM Mg-, Ca-, K- and Na-phytate at pH 7, respectively (Fig. 4A). Adding 5 mM calcium chloride to all the above substrates makes them change to insoluble Ca-phytate complex by electrostatic force (Oh et al., 2001). The enzyme activity of AF phytase was inhibited against insoluble Ca-phytate complex, indicating that AF phytase could not hy-

drolyze against insoluble Ca-phytate complex (Fig. 4B). These findings suggest that DS11 phytase is a more suitable enzyme than AF phytase as a feed supplement, because most of the phytate is present as insoluble Ca-phytate complex due to electrostatic force (Maenz et al., 1999) in the intestine and in the feedstuff of animals (Oh et al., 2001). In the previous report, we also demonstrated that Ca-phytate, but not other phytate forms, was a true substrate for DS11 phytase (Oh et al., 2001).

Taking into consideration the results of present study, we strongly suggest that the novel thermostable DS11 phytase could be used in the feed pelleting process for animal feed as a potent industrial enzyme on the basis of its higher thermostability, absence of reduction of enzymatic activity at higher concentration of phosphate, and wide enzyme activities against various natural Ca-phytate complexes.

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Fig. 4. Comparative enzyme activity of DS11 phytase and AF phytase extracted from feedstuff pelleted at 60°C against magnesium phytate (Mg-phytate), calcium phytate (Ca-phytate), potassium phytate (K-phytate) and sodium phytate (Na-phytate) in soluble state (A) and insoluble state (B) at pH 7.

■, DS11 phytase. □, AF phytase. These experiments were performed in triplicate. ^{a)} Paired *t*-test: *p* < 0.05.

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