Optimization of Non-Starch Polysaccharide Enzyme Profile in Growing Pig Diets Using In Vitro Simulated Digestion Method Postprint

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Abstract

To address issues such as the massive screening workload for dietary non-starch polysaccharide (NSP) enzymes and poor comparability between animal trials, this study investigated the use of in vitro simulation methods to optimize NSP enzyme profiles for corn-soybean meal type diets and corn-miscellaneous meal type diets for growing pigs. Firstly, a single-factor randomized experimental design was employed to examine the relationship between NSP enzyme supplementation levels and in vitro dry matter digestibility (IVDMD) of diets. Six NSP enzymes—cellulase, xylanase, β-glucanase, β-mannanase, α-galactosidase, and pectinase—were added at different levels to corn-soybean meal type diets and corn-miscellaneous meal type diets to analyze the efficacy of each NSP enzyme on dietary IVDMD. Subsequently, a quadratic regression rotating orthogonal combination experimental design was adopted to screen for optimal enzyme profiles of the six NSP enzymes in the two diet types. The results indicated that: 1) A quadratic relationship existed between the supplementation levels of the six NSP enzymes and IVDMD of the two types of pig diets. 2) α-galactosidase yielded the greatest improvement in IVDMD for corn-soybean meal type diets, achieving an increase of 1.28%, while xylanase yielded the greatest improvement in IVDMD for corn-miscellaneous meal type diets, achieving an increase of 1.95%. 3) The optimal enzyme profile for corn-soybean meal type diets was: cellulase 533.6 U/kg, xylanase 9,983.7 U/kg, β-glucanase 1,014.4 U/kg, β-mannanase 4,080.6 U/kg, α-galactosidase 251.6 U/kg, and pectinase 107.3 U/kg. The optimal enzyme profile for corn-miscellaneous meal type diets was: cellulase 960.0 U/kg, xylanase 17,177.6 U/kg, β-glucanase 405.8 U/kg, β-mannanase 19,023.2 U/kg, α-galactosidase 307.2 U/kg, and pectinase 96.9 U/kg. 4) The optimized enzyme profiles increased IVDMD by 3.26% for corn-soybean meal type diets and by 3.75% for corn-miscellaneous meal type diets. These results demonstrate that the combined use of the six NSP enzymes can

more substantially improve the IVDMD of corn-soybean meal type diets and corn-miscellaneous meal type diets for growing pigs.

Full Text

Optimization of Non-Starch Polysaccharide Enzyme Complex for Growing Pig Diets Using an In Vitro Simulation Method

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Abstract: To address the challenges of massive screening workloads and poor comparability between animal trials for non-starch polysaccharide (NSP) enzymes, this study investigated the use of an in vitro simulation method to optimize NSP enzyme complexes for corn-soybean meal and corn-miscellaneous meal diets for growing pigs. First, a single-factor randomized experimental design was employed to examine the relationship between NSP enzyme supplementation levels and in vitro dry matter digestibility (IVDMD). Six NSP enzymes—cellulase, xylanase, β-glucanase, β-mannanase, α-galactosidase, and pectinase—were added at different levels to both diet types to analyze their effects on IVDMD. Subsequently, a quadratic regression rotation orthogonal combination design was used to identify optimal enzyme complexes for the two diets. The results demonstrated: (1) Quadratic relationships existed between the addition levels of all six NSP enzymes and IVDMD for both diet types. (2) α-Galactosidase produced the greatest improvement in IVDMD for the corn-soybean meal diet (1.28%), while xylanase showed the highest enhancement for the corn-miscellaneous meal diet (1.95%). (3) The optimal enzyme complex for the corn-soybean meal diet was: cellulase 533.6 U/kg, xylanase 9,983.7 U/kg, β-glucanase 1,014.4 U/kg, β-mannanase 4,080.6 U/kg, α-galactosidase 251.6 U/kg, and pectinase 107.3 U/kg. For the corn-miscellaneous meal diet, the optimal complex was: cellulase 960.0 U/kg, xylanase 17,177.6 U/kg, β-glucanase 405.8 U/kg, β-mannanase 19,023.2 U/kg, α-galactosidase 307.2 U/kg, and pectinase 96.9 U/kg. (4) The optimized enzyme complexes improved IVDMD by 3.26% for the corn-soybean meal diet and 3.75% for the corn-miscellaneous meal diet. These findings indicate that combined use of the six NSP enzymes can more effectively enhance the IVDMD of both corn-soybean meal and corn-miscellaneous meal diets for growing pigs.

Keywords: non-starch polysaccharide enzyme; growing pig; diet; in vitro dry

matter digestibility; optimization

Introduction

Non-starch polysaccharides (NSP) function as anti-nutritional factors that frequently impair nutrient digestion in livestock feed. NSP enzymes have attracted increasing attention for their ability to mitigate these anti-nutritional effects. Numerous studies have demonstrated that NSP enzymes can reduce the anti-nutritional activity of NSP, improve nutrient utilization, and enhance intestinal health. However, other research has found no effects of NSP enzymes on pig performance or nutrient utilization. Because NSP content and composition vary substantially among different diets, and NSP enzymes are biological catalysts with substrate specificity, rational enzyme combination is critical for maximizing NSP degradation. Nevertheless, screening appropriate NSP enzyme combinations for different diets through animal trials is not only labor-intensive but also yields highly variable results, failing to meet practical needs.

In recent years, researchers have employed in vitro simulation methods to evaluate NSP enzyme efficacy, providing an efficient and rapid approach for optimizing enzyme combinations. Wang et al. and He et al. used a two-step pepsin-pancreatin in vitro digestion method to assess NSP enzymes in poultry diets, achieving favorable production outcomes. Narasimha et al. successfully identified optimal combinations of cellulase, xylanase, and β-glucanase for several feed ingredients using in vitro digestion. However, no studies have reported the use of in vitro methods to optimize NSP enzyme complexes for different growing pig diets. Furthermore, pancreatin is a multi-enzyme complex extracted from porcine pancreas with variable composition between batches, which prevents reproducible simulation of intestinal digestion. Our laboratory previously developed a simulated porcine small intestinal fluid based on the composition of pig intestinal secretions, which effectively simulates the digestive process with in vivo-in vitro correlation coefficients exceeding 0.95. Therefore, this study utilized a two-step in vitro digestion method in Erlenmeyer flasks based on physiological porcine digestive secretions to investigate the effects of six NSP enzymes on two types of growing pig diets, explore methods for optimizing enzyme combinations, and provide a basis for efficient NSP enzyme application in pig diets.

1.1 NSP Enzymes and Activity Assays

Six NSP enzymes were selected: cellulase, xylanase, β-glucanase, β-mannanase, α-galactosidase, and pectinase. Cellulase activity was determined according to NY/T 912-2004, xylanase activity according to GB/T 23874-2009, and β-glucanase activity according to NY/T 911-2004. β-Mannanase activity was measured using the reducing sugar colorimetric method, defined as the amount

of enzyme required to release 1 mol of reducing sugar per minute from a 3 mg/mL mannan solution (Sigma G0753) at 37°C and pH 5.5, measured at 540 nm. α-Galactosidase activity was determined using a colorimetric method, defined as the enzyme quantity releasing 1 mol of p-nitrophenol per minute from a 10 mmol/L p-nitrophenyl-α-D-galactopyranoside solution (Sigma N0877) at 37°C and pH 5.5, measured at 400 nm. Pectinase activity was measured using the reducing sugar colorimetric method, defined as the enzyme amount releasing 1 mol of galacturonic acid per minute from a 4 mg/mL polygalacturonic acid solution (Sigma P9135) at 37°C and pH 5.5, measured at 540 nm. The measured activities were: cellulase 6,867 U/g, xylanase 33,290 U/g, β-glucanase 12,076 U/g, β-mannanase 49,283 U/g, α-galactosidase 2,753 U/g, and pectinase 1,129 U/g.

1.2 Experimental Diets

The experiment was conducted at the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, from 2014-2015. Two diets—corn-soybean meal and corn-miscellaneous meal—were formulated according to NRC (2012) nutrient requirements for growing pigs. Diet composition and nutrient levels are shown in Table 1 . Dietary NSP content was determined using the method of Huang et al., with results presented in Table 2 .

1.3.1 Reagent Preparation

Gastric buffer and small intestinal buffer were prepared according to Zhao et al., with the small intestinal buffer pH adjusted to 7.0 using KOH. Simulated digestive fluids were prepared as follows:

Simulated porcine gastric fluid: 184.38 kU pepsin (Sigma, P7000) was dissolved in 250 mL gastric buffer solution (pH 2.0, calibrated at 39°C) with gentle stirring until dissolved (prepared immediately before use).

Simulated porcine small intestinal fluid: Amylase (Sigma, A3306) 60.89 kU, trypsin (Amresco, 0785) 19.00 kU, chymotrypsin (Amresco, 0164) 2.39 kU, and lipase (Sigma, L3126) 919.2 U were dissolved in 25 mL deionized water with gentle stirring until dissolved (prepared immediately before use).

1.3.2 In Vitro Digestion Procedure

Approximately 1 g of feed sample (passed through 60-mesh sieve, weighed to 0.0001 g precision) was placed in a 50 mL Erlenmeyer flask. Sixteen mL of simulated porcine gastric fluid was added and mixed carefully. The flask was sealed with parafilm and incubated in a 39°C water bath shaker (180 r/min) for exactly 4 hours to simulate gastric digestion.

After gastric digestion, the parafilm was removed and 4 mL of small intestinal buffer was added. After 5 minutes of shaking, 2 mL of pre-prepared simulated

porcine small intestinal fluid was added. The flask was resealed and incubated at 39°C (180 r/min) for 22 hours to simulate small intestinal digestion. Following incubation, flasks were left at room temperature for 30 minutes, then the digesta residue was vacuum-filtered through pre-dried and weighed filter paper (Whatman 1541, pore size 22-25 m). The residue and filter paper were dried at 105°C to constant weight.

1.4 Experimental Design

A single-factor design was used to evaluate different levels of six NSP enzymes in both diets. A two-step in vitro flask method based on porcine physiological digestive secretions (simulated gastric fluid + simulated small intestinal fluid) was employed to determine IVDMD and analyze enzyme effects on different diet types. Subsequently, a six-factor quadratic regression rotation orthogonal combination design was used to screen optimal enzyme combinations. The six NSP enzymes served as factors, each with five coded levels (-2.384, -1, 0, 1, 2.384) based on enzyme supplementation levels, with IVDMD as the response variable for optimization.

1.5 Data Processing

SAS 9.2 GLM procedure was used to analyze the regression relationship between single NSP enzyme supplementation levels and dietary IVDMD. The RSREG procedure was used for the six-factor quadratic orthogonal rotation combination design data.

Results

2.1 Nutrient Content and NSP Composition of Experimental Diets

As shown in Table 1, the corn-soybean meal diet contained 17.2% crude protein and 16.46 MJ/kg gross energy, similar to the corn-miscellaneous meal diet (17.7% and 16.42 MJ/kg). However, due to higher corn content, the corn-soybean meal diet had greater starch content (44.2%) but lower neutral detergent fiber (10.5%) and acid detergent fiber (2.8%) compared to the corn-miscellaneous meal diet (36.8% starch, 13.1% NDF, and 6.1% ADF).

Table 2 reveals that the corn-miscellaneous meal diet had higher total, insoluble, and soluble NSP contents than the corn-soybean meal diet. Most NSP components were also higher in the corn-miscellaneous meal diet, with arabinoxylan being the major component in both diets, followed by glucose.

2.2 Effects of Single NSP Enzymes on IVDMD of Corn-Soybean Meal Diet

Table 3 shows that at low supplementation levels, IVDMD of the corn-soybean meal diet increased with enzyme addition, reaching maximum values at cellulase 60 g/g, xylanase 200 g/g, β-glucanase 80 g/g, β-mannanase 60 g/g, α-galactosidase 80 g/g, and pectinase 100 g/g, beyond which no further improvements occurred.

Table 4 demonstrates quadratic relationships ($P<0.01$) between all six NSP enzyme supplementation levels and IVDMD, indicating that IVDMD does not continuously increase with enzyme addition. The calculated maximum IVDMD values from models closely matched measured values. Among individual enzymes, α-galactosidase produced the greatest IVDMD improvement (1.28%), followed by β-mannanase (1.05%), while cellulase showed the smallest effect (0.62%).

2.3 Effects of Single NSP Enzymes on IVDMD of Corn-Miscellaneous Meal Diet

Similar to the corn-soybean meal diet, IVDMD of the corn-miscellaneous meal diet increased with enzyme supplementation at low levels, reaching maxima at cellulase 100 g/g, xylanase 400 g/g, β-glucanase 80 g/g, β-mannanase 300 g/g, α-galactosidase 100 g/g, and pectinase 100 g/g (Table 5).

Table 6 shows quadratic relationships ($P<0.01$) between supplementation levels and IVDMD for all enzymes except xylanase and β-glucanase, though IVDMD did not continuously increase for these two enzymes either. Calculated maximum IVDMD values closely matched measured values. Xylanase and β-mannanase produced the greatest improvements (1.95% and 1.90%, respectively), while β-glucanase had the smallest effect (0.63%).

2.4 Establishment of IVDMD-Enzyme Complex Regression Models and Screening of Optimal Combinations

The supplementation level at which IVDMD reached maximum (no further increase with additional enzyme) was designated as the 0 coded level. For the corn-soybean meal diet, these levels were: cellulase 60 g/g, xylanase 200 g/g, β-mannanase 60 g/g, α-galactosidase 80 g/g, β-glucanase 80 g/g, and pectinase 100 g/g. For the corn-miscellaneous meal diet: cellulase 100 g/g, xylanase 400 g/g, β-glucanase 80 g/g, β-mannanase 300 g/g, α-galactosidase 100 g/g, and pectinase 100 g/g. According to the six-factor quadratic orthogonal rotation combination design requirements, coded values and corresponding levels were determined (Table 7).

Fifty-three experimental runs were conducted for each diet. Based on results, six-factor quadratic regression equations were established between IVDMD (Y,

%) and enzyme supplementation levels (X, g/g), with equations and parameters shown in Table 8 .

Analysis of pairwise enzyme interactions (data not shown) revealed positive synergistic effects ($0.05<P<0.10$) in the corn-soybean meal diet between cellulase and xylanase (P=0.0133), cellulase and β-mannanase (P=0.0503), xylanase and β-glucanase (P=0.0540), and β-mannanase and α-galactosidase (P=0.0540). Significant antagonistic effects were observed between cellulase and β-glucanase (P=0.0146). In the corn-miscellaneous meal diet, no significant interactions were detected ($P>0.10$). Both regression equations were significant ($P<0.05$), validating the models for optimization analysis to obtain optimal enzyme combinations (Table 9).

Validation of the optimized enzyme complexes (Table 10) showed that for the corn-soybean meal diet, the predicted IVDMD was 83.17% (theoretical improvement 3.82%) while the measured value was 82.61% (actual improvement 3.26%). For the corn-miscellaneous meal diet, predicted IVDMD was 74.37% (theoretical improvement 3.51%) and measured value was 74.61% (actual improvement 3.75%).

Discussion

3.1 Importance of Screening Pig Diet NSP Enzyme Complexes Based on Dietary NSP Composition

Building on our laboratory' s established methods for determining dietary NSP content and composition, this study measured NSP profiles of both diets. The corn-miscellaneous meal diet containing rapeseed meal, cottonseed meal, and sugar beet pulp had higher NSP content than the corn-soybean meal diet. Both diets contained NSP components including arabinoxylan, glucose, uronic acid, mannose, galactose, and rhamnose, with low fucose content. Most NSP degradation in pig diets occurs through microbial fermentation in the large intestine, so anti-nutritional effects impact nutrient digestion and absorption in the stomach and small intestine. The extent of NSP degradation depends on solubility, polymer type, and structural associations with other cell wall components. While some studies show NSP enzymes improve dry matter, nitrogen, and energy digestibility in pig diets, others report no effects. These inconsistent results largely stem from mismatches between diet composition and enzyme selection. Therefore, adding appropriate NSP enzymes based on dietary NSP composition and content can fully exploit feed nutritional potential.

In vitro simulation methods offer rapidity, good repeatability, and high correlation with biological methods. Bedford et al. successfully predicted xylanase effects in broiler wheat-based diets using pepsin-pancreatin two-step digestion based on digesta viscosity. Malathi et al. found different NSP enzyme sources varied in their effects on feed viscosity and total sugar release. Saleh et al. re-

ported pectinase produced the greatest IVDMD improvement in soybean meal. Narasimha et al. screened optimal three-enzyme combinations for different ingredients using in vitro digestion. These methods were based on Boisen' s "Erlenmeyer flask + shaker" system, which shows high correlation with in vivo digestibility (r=0.94 for organic matter digestibility, r=0.81 for barley energy digestibility). Enzymatic determination of digestible energy has been adopted in feeding standards of Denmark, Netherlands, and France.

However, the commonly used pepsin-pancreatin system has limitations. Pancreatin' s variable composition between batches compromises reproducibility, and its use across different species lacks physiological justification. Our laboratory developed a simulated porcine small intestinal fluid based on pig digestive physiology, achieving in vivo-in vitro correlations above 0.95. This study employed the "flask + physiologically-based simulated digestive fluid" approach to evaluate six NSP enzymes in two diet types. Results showed IVDMD did not continuously increase with enzyme levels, indicating optimal rather than maximal enzyme addition. The critical supplementation levels differed between diets due to varying NSP contents. For instance, the optimal individual level for xylanase was 400 g/g in the corn-miscellaneous meal diet versus 200 g/g in the corn-soybean meal diet, and for β-mannanase, 300 g/g versus 60 g/g. These differences underscore the importance of screening enzyme combinations specific to diet type.

3.2 Effects of Single NSP Enzyme Supplementation on Dietary IVDMD

Different diet compositions result in varying NSP contents and profiles. In this study, optimal supplementation levels for all six enzymes were generally higher in the corn-miscellaneous meal diet, particularly for xylanase and β-mannanase. Xylanase improved IVDMD of the corn-miscellaneous meal diet by 1.95% compared to 0.68% for the corn-soybean meal diet, while β-mannanase improved it by 1.90% versus 1.05%. This is because corn contains only about 8% NSP, whereas the miscellaneous meal diet includes rapeseed meal (~24% NSP), cottonseed meal (~30% NSP), and sugar beet pulp (~65% NSP). Total NSP content was 18.43% in the corn-miscellaneous meal diet versus 13.29% in the corn-soybean meal diet, with xylan (6.79%) and mannan being major components. Degradation of these high levels resulted in greater IVDMD improvements. Interestingly, α-galactosidase and β-mannanase were more effective than xylanase and cellulase in the corn-soybean meal diet despite its higher xylan and glucose content, likely due to enzyme substrate specificity and differences in NSP backbone structures and cross-linking groups among ingredients. This further demonstrates that enzyme supplementation cannot be based solely on dietary NSP content.

3.3 Enhancement of Dietary IVDMD by NSP Enzyme Complexes

Dietary NSP forms complex structures through various chemical bonds, and only by breaking these connections can anti-nutritional effects be eliminated and nutritional potential realized. The complexity of NSP components and structures means single enzymes are rarely effective, while multiple enzymes can synergistically degrade chain structures, release nutrients, and improve feed value. The optimized enzyme complexes in this study produced greater IVDMD improvements than any single enzyme, indicating synergistic degradation of NSP. Malathi et al. found that a xylanase+cellulase+pectinase+β-glucanase combination significantly increased total sugar release from soybean meal compared to other combinations. Saleh et al. reported that a multi-enzyme complex produced higher sugar release from corn-soybean meal diets than individual enzymes. The greater improvement in the high-NSP corn-miscellaneous meal diet (3.75%) versus the corn-soybean meal diet (3.26%) aligns with Zhao' s findings in laying hens and He et al.' s results in broilers, confirming that NSP enzymes are more effective in high-NSP diets.

Conclusion

This study demonstrates that a two-step in vitro digestion method based on porcine physiological digestive secretions provides a feasible approach for rapidly optimizing NSP enzyme complexes for growing pig diets and can help preliminarily predict enzyme efficacy in practice. Under the experimental conditions, the optimal NSP enzyme complex for corn-soybean meal diets was: cellulase 533.6 U/kg, xylanase 9,983.7 U/kg, β-mannanase 4,080.6 U/kg, α-galactosidase 251.6 U/kg, β-glucanase 1,014.4 U/kg, and pectinase 107.3 U/kg. For corn-miscellaneous meal diets, the optimal complex was: cellulase 960.0 U/kg, xylanase 17,177.6 U/kg, β-mannanase 19,023.2 U/kg, α-galactosidase 307.2 U/kg, β-glucanase 405.8 U/kg, and pectinase 96.9 U/kg. These optimized complexes improved IVDMD by 3.26% in corn-soybean meal diets and 3.75% in corn-miscellaneous meal diets.

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Note: Figure translations are in progress. See original paper for figures.