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iTRAQ-based quantitative proteomic analysis of longissimus muscle from growing pigs with dietary supplementation of non-starch polysaccharide enzymes*#

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Abstract: Non-starch polysaccharide enzymes (NSPEs) have long been used in the feed production of monogastric animals to degrade non-starch polysaccharide to oligosaccharides and promote growth performance. However, few studies have been conducted on the effect of such enzymes on skeletal muscle in monogastric animals. To elucidate the mechanism of the effect of NSPEs on skeletal muscle, an isobaric tag for relative and absolute quantification (iTRAQ) for differential proteomic quantitation was applied to investigate alterations in the proteome in the longissimus muscle (LM) of growing pigs after a 50-d period of supplementation with 0.6% NSPEs in the diet. A total of 51 proteins were found to be differentially expressed in the LM between a control group and the NSPE group. Functional analysis of the differentially expressed protein species showed an increased abundance of proteins related to energy production, protein synthesis, muscular differentiation, immunity, oxidation resistance and detoxification, and a decreased abundance of proteins related to inflammation in the LM of the pigs fed NSPEs. These findings have important implications for understanding the mechanisms whereby dietary supplementation with NSPEs enzymes can promote growth performance and improve muscular metabolism in growing pigs.

Key words: Non-starch polysaccharide enzymes (NSPEs), Longissimus muscle, Proteomics, Growing pigs
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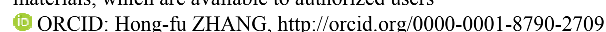
1 Introduction

Non-starch polysaccharide enzymes (NSPEs) are a group of exogenous enzyme mixtures, which have long been used in the feed production of monogastric animals to degrade non-starch polysaccharides (NSPs) to oligosaccharides (Silva and Smithard, 2002; Bindelle *et al.*, 2011; Walsh *et al.*, 2012; Willamil *et al.*, 2012; Kiarie *et al.*, 2013). Researchers have proved that the addition of exogenous enzymes has multiple benefits by reducing the impact of numerous antinutritional factors in corn and soybean meal based diets (Yang *et al.*, 2010; Zou *et al.*, 2013). Previous studies have demonstrated that NSPEs can enhance animal growth performance and improve nutrient absorption and immunity, indicating that NSPEs play a versatile role in regulating metabolic pathways (Ao *et al.*, 2010; Zduńczyk *et al.*, 2013). Most studies have shown multiple benefits in the small intestine of pigs from adding NSPEs to the diet (Gdala *et al.*, 1997; Yin *et al.*, 2001; O'Shea *et al.*, 2014). However, only a few studies have been conducted on the effects of NSPEs on skeletal muscle in



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monogastric animals (Wang *et al*., 2005; Buchanan *et al*., 2007; Hajati *et al*., 2009).

Skeletal muscle is an important organ in the body, representing 40%–50% of body mass in mammals, and is a critical regulator that integrates various biochemical pathways, such as heat homeostasis and carbohydrate metabolism (Abdul-Ghani and DeFronzo, 2010; Sandri, 2010). Other specialized functions have also been found among the complex cellular tasks of muscles because of the actions of a number of different proteins (Ohlendieck, 2011). Many of these are membrane-associated proteins including the nicotinic acetylcholine receptor (Fagerlund and Eriksson, 2009), acetylcholinesterase (Aldunate *et al*., 2004), the voltage-sensing dihydropyridine receptor (Arikkath and Campbell, 2003), the ryanodine receptor Ca^{2+}-release channel (Franzini-Armstrong, 2009), the dystrophin-glycoprotein complex (Michele and Campbell, 2003), and the respiratory chain (Hood, 2009). However, little is known about the exact molecular mechanisms of the action of NSPEs on skeletal muscle (Hajati *et al*., 2009).

Numerous studies have demonstrated a lack of correlation between mRNA and protein abundance because of RNA editing and post-translational modifications (Ohlendieck, 2011). Making the elucidation of protein expression more accurate is imperative (Wang *et al*., 2006). Recent research has applied advanced high throughput mass spectrometric proteomic technologies to the simultaneous measurement of the expression of hundreds of proteins (Kitteringham *et al*., 2010; Luo *et al*., 2013). A proteomic analysis of skeletal muscle in rainbow trout demonstrated that previously unrecognized proteins were involved in various functions, including energy production, protein biosynthesis and modification, inflammation, the immune response, and transcriptional regulation (Salem *et al*., 2010). There is great interest in using proteomic technology to elucidate the beneficial effects brought about by NSPEs in the skeletal muscle of growing pigs. Due to the large number of membrane-associated proteins and some highly-abundant proteins in the skeletal muscle, results from two-dimensional electrophoresis may be misleading. Therefore, in this study, we used a label-based isobaric tag for relative and absolute quantification (iTRAQ) procedure, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantitate the altered proteins that are induced differentially in the longissimus muscle (LM) of growing pigs fed NSPEs.

2 Materials and methods

2.1 Enzyme preparation

The NSP enzyme mixture used in the present study was supplied by the State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Beijing, China), and contains 7×10^5 U/g xylanase (EC 3.2.1.8), 1×10^5 U/g β-glucanase (EC 3.2.1.6) and 9000 U/g cellulase (EC 3.2.1.4). The activities of xylanase, β-glucanase, and cellulase were measured according the methods of Bailey and Poutanen (1989), Erfle *et al*. (1988), and Lowe *et al*. (1987), respectively.

2.2 Reagents and chemicals

A Protein Assay Kit was purchased from Bio-Rad (Hercules, CA, USA). Reagent for total RNA isolation was obtained from Qiagen (Valencia, CA, USA). Reagents for quantitative polymerase chain reaction (qPCR) were obtained from TaKaRa Biotechnology (Dalian, China). All iTRAQ reagents and buffers were obtained from Applied Biosystems Inc. (ABI, Foster City, CA, USA). Other reagent grade chemicals used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA).

2.3 Animals and treatments

Forty-eight crossbred (Duroc×Landrace×Large White) growing pigs with similar initial body weights ((39.18±0.98) kg) were purchased from a local pig farm in Beijing. Pigs were randomly assigned to 2 groups according to their littermates and mean initial body weights, with 4 replicates in each group and 6 pigs in each replicate (3 males and 3 females). The 2 groups comprised a control group (CTRL) (fed basal diet) and a treatment group (NSPE) (fed basal diet supplemented with 0.6% NSPEs). Both diets were formulated to meet National Research Council (NRC, 2012) recommendations (Table 1). The animals were housed in a fermentation bed facility in 8 adjacent pens. Feed and water were provided *ad libitum* during the 50-d experimental period. The weight and feed intake of each pig were recorded at the beginning and the end of the experiment for the calculation of average daily gain

(ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). All procedures involving animals were evaluated and approved by the Animal Ethics Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

Table 1 Composition of the basal diet and calculated proximate composition of the diet

Composition	Proportion (%)*
Ingredients	
Corn	70.70
Soybean meal	19.82
Soybean oil	2.10
Wheat bran	5.00
Limestone	0.51
Calcium hydrophosphate	0.56
L-Lysine	0.01
Sodium chloride	0.30
Premix[a]	1.00
Total	100
Nutrient	
Digestible energy	14.22 (MJ/kg)
Ether extract	4.82
Crude protein	15.50
Calcium	0.50
Total phosphorus	0.45
Total lysine	0.75
Total methionine	0.25
Available phosphorus	0.24

* All data are expressed in g/kg dry weight except for digestible energy in MJ/kg. [a] Providing the following (g/kg fresh weight): vitamin A 8250 IU, vitamin D_3 825 IU, vitamin E 40 IU, vitamin K_3 4.0 mg, vitamin B_1 1.0 mg, vitamin B_2 5.0 mg, vitamin B_6 2.0 mg, vitamin B_{12} 25 μg, choline chloride 600 mg, nicotinic acid 35 mg, folic acid 2.0 mg, biotin 4.0 mg, Cu 50.0 mg, Fe 80.0 mg, Zn 100.0 mg, Mn 25.0 mg, Se 0.15 mg, iodine 0.5 mg

2.4 Sample collection and meat quality analysis

At Day 50 (a total 50 d of feeding treatment), all pigs were weighed after 12 h of fasting. One pig per replicate (n=8) was sacrificed by electrical stunning, and then exsanguinated. Blood samples were collected from the cervical vein before sacrifice. After collection, blood samples were centrifuged at 2000g for 30 min at 4 °C, then at 400g for 10 min at 4 °C. The sera obtained were stored at −20 °C for further analysis. The whole LM was obtained from the right side of the carcass, as previously described (Zhong *et al.*, 2011). LM samples collected for proteomic analysis were snap-frozen in liquid nitrogen and stored at −80 °C. The loin-eye area was measured by the method described by Turyk *et al.* (2014). The meat quality of the LM was analyzed as previously described (Liu *et al.*, 2014), including the pH values (after 45 min and 24 h), drip loss, and shear force. The chemical composition of the LM, including its intramuscular fat, moisture, protein, and ash content, was measured as described by Huang *et al.* (2014).

2.5 Serum biochemical analyses

For biochemical analysis, serum levels of total cholesterol, triglyceride, low density lipoprotein-cholesterol (LDL-C), and high density lipoprotein-cholesterol (HDL-C) were analyzed using a corresponding kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. The blood urea nitrogen (BUN) was measured by the Abbott Spectrum urea nitrogen test (Series II, Abbot Laboratories, Dallas, TX, USA).

2.6 Protein extraction

Protein extraction was performed as previously described with some modifications (Shao *et al.*, 2010; Ramadoss and Magness, 2012). LM samples (500 μg) were ground in a Dounce glass grinder using liquid nitrogen. Ground samples were precipitated with 10% (0.1 g/ml) trichloroacetic acid (TCA), in 90% ice-cold acetone at −20 °C for 2 h. The samples were then centrifuged at 20000g for 30 min at 4 °C. The supernatants were decanted and the pellets washed with ice-cold acetone. The pellets were lysed in lysis buffer consisting of 8 mol/L urea, 30 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 2 mmol/L ethylene diamine tetraacetic acid (EDTA), and 10 mmol/L dithiothreitol (DTT). The undissolved pellets in the crude tissue extracts were removed by centrifugation (20000g, 30 min, 4 °C). The tissue lysates were reduced with 10 mmol/L DTT for 1 h at 36 °C, and then alkylated with 55 mmol/L iodoacetamide (IAM) for 1 h at room temperature in the dark. After reduction and alkylation, 4 volumes of ice-cold acetone were added to the solution to precipitate proteins. The proteins were washed three times with ice-cold pure acetone and re-dissolved in buffer containing 50% tetraethyl ammonium bromide (TEAB) and 0.1% sodium dodecyl sulfonate (SDS).

The undissolved pellets were removed from protein samples by centrifugation (20000*g*, 30 min, 4 °C), and protein quantitation was carried out using a Bio-Rad Bradford Protein Assay Kit (Hercules, CA, USA).

2.7 Trypsin digestion

Each protein sample was digested overnight at 37 °C by adding sequencing grade trypsin (Promega Co., Madison, WI, USA) at a 1:30 ratio (3.3 μg trypsin: 100 μg target) (Ramadoss and Magness, 2012).

2.8 iTRAQ labeling

The iTRAQ labeling procedure was described by Ramadoss and Magness (2012). All protein samples were labeled with iTRAQ tags (solubilized in 70 μl isopropanol). Control samples received tags 113, 114, 115, and 116, while treatment samples received tags 117, 118, 119, and 121. All labeled samples (organic composition >60% by adding isopropanol) were incubated at room temperature for 2 h.

2.9 Strong cation exchange chromatography

The strong cation exchange fractionation protocol followed previous reports (Olsen *et al*., 2006; Shao *et al*., 2010; Su *et al*., 2013) with slight modifications. Briefly, a total amount of 800 μg sample was loaded onto a strong cation exchange column (Phenomenex Luna SCX 100A) equilibrated with buffer A (10 mmol/L KH_2PO_4 in 25% acetonitrile, pH 3.0) using an Agilent 1100 system (Santa Clara, CA, USA). The peptides were separated using a linear gradient of buffer B (10 mmol/L KH_2PO_4 and 2 mol/L KCl in 25% acetonitrile, pH 3.0) increasing to 5% after 41 min, 50% after 66 min, and 100% after 71 min, at a flow rate of 1 ml/min. A total of 10 fractions were collected from the eluted peptides, and each fraction was desalted with a Strata X C18 column (Phenomenex, Torrance, CA, USA) and vacuum-dried using a Speedvac (Thermo Fisher Scientific, Bremen, Germany).

2.10 Mass spectrometry

The protocol for MS analysis was as described previously (Su *et al*., 2013) with slight modifications. Each SCX fraction was re-dissolved in buffer A (2% acetonitrile, 0.1% formic acid) and centrifuged at 20000*g* for 10 min. The final concentration of peptides in each fraction was about 0.25 μg/μl on average. Twenty microliters of supernatants were loaded by an auto sampler onto a C18 trap column (length 2 cm, inner diameter 200 μm) of an UltiMate® 3000 Nano LC system (Bannockburn, IL, USA). Peptides were eluted onto a resolving analytical C18 column (length 10 cm, inner diameter 75 μm, 5 μm particles, 30 nm) packed in-house. Samples were loaded at 15 μl/min for 4 min and eluted with a 45 min gradient at 400 nl/min from 5% to 60% buffer B (98% acetonitrile, 0.1% formic acid), separated with a 3-min linear gradient to 80% B, maintained at 80% B for 7 min, and finally returned to 5% B over 3 min. The peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (Q-Exactive, Thermo) coupled online to the nanoLC. Intact peptides were detected in the Orbitrap at a resolution of 70000 full width at half maximum (FWHM). Peptides were selected for MS/MS using the high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 28%; ion fragments were detected in the Orbitrap at 17500 FWHM resolution. A data-dependent acquisition mode that alternated between an MS scan and MS/MS scans was applied for the 10 most abundant precursor ions (2^+ to 4^+) above a threshold ion count of 20000 in the MS survey scan, with a following dynamic exclusion duration of 15 s (isolation window of *m/z* 2.0 and a maximum ion injection time of 100 ms). The electrospray voltage applied was 1.8 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the Orbitrap. The AGC target for full MS was 3e6 and 1e5 for MS2. For MS scans, the *m/z* scan range was 350–2000. For MS2 scans, the *m/z* scan range was 100–1800.

2.11 Data processing and analyses

MS/MS data for iTRAQ protein identification and quantitation were analyzed using Proteome Discover 1.3 (Thermo Fisher Scientific, Bremen, Germany) and searched using in-house MASCOT software (Matrix Science, London, UK; Version 2.3.0) against the database Uniprot_pig (Apr. 11th, 2014) with the following parameters: enzyme: trypsin; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M), gln-pyro-glu (N-term Q), iTRAQ 8-plex (N-term, K, Y); peptide mass tolerance:15 ppm; MS/MS tolerance: 20 mmu; maximum

missed cleavages: 1. Identified peptides had an ion score above the threshold of peptide identity established by Mascot, and protein identifications were accepted within the false discovery rate (FDR) of 1% in which at least one such unique peptide match was specific for the protein. Median ratio normalization was performed in intra-sample channels to normalize each channel across all proteins. Protein quantitative ratios for each iTRAQ labeled sample were obtained, using a sample in the control group (sample tagged with 113) as the denominator. Quantitative ratios were then log transformed to base 2 and presented as fold change relative to the denominator in the control group for final quantitative testing. Differentially expressed proteins were identified using Student's *t*-test corrected for multiple testing using the Benjamini and Hochberg correction (Hakimov *et al*., 2009). Proteins with a 1.2-fold change or greater were considered to be differentially expressed.

2.12 Bioinformatics analysis of protein differential abundance

Gene Ontology (GO) distributions for all of the proteins that were significantly altered in the LM in growing pigs fed NSPEs were classified using Blast2GO (http://www.blast2go.com) and WEGO (http://wego.genomics.org.cn) software provided by the Institute for Genomic Research (Ye *et al*., 2006; Zi *et al*., 2013).

2.13 Validation of proteins of differential abundance

Real-time qPCR was used to verify six LM proteins of differential abundance at the mRNA level by the method of Huang *et al.* (2013) with slight modifications.

Total RNA from the LM was isolated using a Qiagen RNeasy Plus Mini Kit (Valencia, CA, USA). Agarose gel electrophoresis was used to test the RNA quality. The RNA quantity was verified with a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA). Then total RNA was reverse transcribed using the PrimeScrip™ Reverse Transcriptase, D2680A (TaKaRa Biotechnology, Dalian, China). All mRNA expression levels were analyzed by RT-qPCR using the PrimeScript™ RT reagent Kit, RR037A (SYBR Green) (TaKaRa Biotechnology, Dalian, China) and an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). RT-qPCR was performed under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Fluorescence data were detected at the last step of each cycle to monitor the amount of PCR product. The primer sequences are shown in Table S1. The relative fold-change was calculated by the $2^{-\Delta\Delta C_T}$ method (Huang *et al*., 2013).

2.14 Statistical analysis

Data on growth performance, serum parameters, and gene expression were analyzed by one-way analysis of variance (ANOVA; SAS Version 9.2, SAS institute Inc., Cary, NC, USA). A group difference was assumed to be statistically significant when $P<0.05$. All results are expressed as means±standard deviation (SD).

3 Results

3.1 Growth performance of growing pigs

In this study, all growing pigs had similar body weights ((39.18±0.98) kg) at the initiation of the experiment. During the entire experimental period (total 50 d), NSPE pigs increased their body weight by 5.9% and their ADG by 15.5% ($P<0.05$). However, the ADFI between the two groups was similar ($P>0.05$). Supplementation with NSPEs significantly improved FCR by 8.7% compared with the control group ($P<0.05$; Table 2).

Table 2 Effects of NSPEs on the growth performance of growing pigs

Group	Initial weight (kg)	Final weight (kg)	ADG (kg/d)	ADFI (kg/d)	FCR (kg feed/ kg weight gain)
CTRL	38.80±0.99	74.04±1.77^b	0.71±0.05^b	1.97±0.09	2.77±0.02^a
NSPE	39.55±0.63	78.42±1.06^a	0.82±0.05^a	2.07±0.06	2.53±0.03^b

CTRL: control; NSPE: non-starch polysaccharide enzyme; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio. Values are presented as mean±SD (n=24 for ADG; n=4 for ADFI and FCR). Values within a column having different superscript letters indicate a significant difference at $P<0.05$

3.2 Meat quality and composition of growing pigs

Most indexes of carcass characteristics, meat quality and meat chemical composition of pigs fed NSPEs were not different (P>0.05) from those of pigs fed the CTRL diet, except for loin-eye area, which was significantly greater in the NSPE group (P<0.05) (Tables 3 and 4).

3.3 Serum parameters of growing pigs

Among the serum parameters, only the level of LDL-cholesterol was significantly lower in the NSPE group than in the CTRL group (Table 5; P<0.05).

3.4 Identification and comparison of proteins of differential abundance

Using iTRAQ analysis, a total of 1167 proteins were identified within the FDR of 1% (supporting information, Table S2). Following statistical analysis, 51 proteins were found to be differentially expressed in the LM between CTRL and NSPE pigs, with 21 being up-regulated and 30 down-regulated (supporting information, Table S3).

A total of 38 proteins of differential abundance were grouped into seven classes based on putative functions: protein degradation, biosynthesis and modification (26.3%), transcriptional and translational regulation (18.4%), inflammatory and immune response (15.8%), miscellaneous (13.2%), lipid metabolism (10.5%), adenosine triphosphate (ATP) production and substrate metabolism (7.9%), and oxidative stress and detoxification (7.9%) (Fig. 1). Those related to protein degradation, biosynthesis and modification, transcriptional and translational regulation, and inflammatory and immune response were predominant, accounting for about 60% of the differentially-expressed proteins. A comparison of proteins of differential abundance with functional groupings between the two groups indicated that a smaller number of protein species were up-regulated in NSPE pigs (15 versus 23) (Table 6).

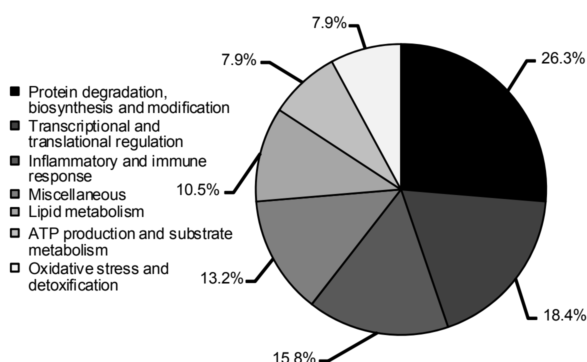

Fig. 1 Functional classification of proteins of differential abundance identified from the longissimus muscle of growing pigs

Table 3 Effects of NSPEs on the carcass characteristics of growing pigs

Group	Carcass length (cm)	Skin thickness (cm)	Average backfat (cm)	Loin-eye area (cm^2)	LM length (cm)
CTRL	101.7±5.0	0.92±0.16	2.46±0.57	41.85±3.14^b	82.00±1.87
NSPE	102.3±5.7	0.93±0.16	2.56±0.47	45.97±4.29^a	81.78±1.20

CTRL: control; NSPE: non-starch polysaccharide enzyme. Values within a column having different superscript letters indicate a significant difference at P<0.05. Values are presented as mean±SD (n=4)

Table 4 Effects of NSPEs on meat quality traits and chemical composition of the longissimus muscle of growing pigs

Group	Meat quality traits				Chemical composition			
	$pH_{45\ min}$	$pH_{24\ h}$	Drip loss (%)	Shear force (kg)	Moisture (%)	Intramuscular fat (%)	Crude protein (%)	Ash (%)
CTRL	5.66±0.21	5.45±0.08	9.31±2.30	3.57±0.17	75.50±1.71	1.36±0.09	24.80±1.00	1.11±0.12
NSPE	5.66±0.20	5.54±0.06	9.12±2.01	3.12±0.22	75.70±2.10	1.42±0.11	24.40±1.30	1.12±0.13

CTRL: control; NSPE: non-starch polysaccharide enzyme. Values are presented as mean±SD (n=4)

Table 5 Effects of NSPEs on serum parameters of the longissimus muscle of growing pigs

Group	BUN (mmol/L)	Triglyceride (mmol/L)	Cholesterol (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
CTRL	7.09±1.69	0.62±0.23	2.66±0.42	1.60±0.25^a	1.09±0.12
NSPE	7.07±1.23	0.52±0.08	2.31±0.41	1.22±0.19^b	1.00±0.23

CTRL: control; NSPE: non-starch polysaccharide enzymes; BUN: blood urea nitrogen; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol. Values within a column having different superscript letters indicate a significant difference at P<0.05. Values are presented as mean±SD (n=4)

Table 6 Differentially expressed proteins with GO identifiers in the longissimus muscle from the NSPE group and the CTRL group identified by iTRAQ labeling-based proteomics

Accession	Description	Gene symbol	$\log_2$ fold change	P-value	Biological process GO term
ATP production and substrate metabolism					
O19069	Succinyl-CoA ligase [ADP/GDP-forming] subunit α	SUCLG1	−0.14	0.0194	GTP binding
F1SDJ8	Uncharacterized protein	GLRX3	0.08	0.0382	Electron carrier activity
P11708	Malate dehydrogenase, cytoplasmic	MDH1	0.12	0.0132	Tricarboxylic acid cycle
Lipid metabolism					
F1SJT7	Apolipoprotein A-IV	APOA4	−0.07	0.0307	Cholesterol transporter activity
M9TGS8	Mitochondrial δ3,δ2-dienoyl-CoA isomerase	ECI1	0.17	0.0282	Fatty acid β-oxidation
P49924	Fatty acid-binding protein	FABP1	1.03	0.0045	Lipid binding
F1STB2	Uncharacterized protein	HINT2	0.19	0.0407	Mitochondrial lipid metabolism and respiration
Transcriptional and translational regulation					
Q53DY6	Histone H1.3-like protein		−1.05	0.0051	DNA binding
B6V8C8	40S ribosomal protein S3a	RPS3A	−0.30	0.0070	Structural constituent of ribosome
A1XQU3	60S ribosomal protein L14	RPL14	−0.26	0.0341	Structural constituent of ribosome
I3LVI8	60S ribosomal protein L7a	RPL7A	−0.24	0.0446	Ribosome biogenesis
Q29293	60S ribosomal protein L3	RPL3	0.36	0.0253	Structural constituent of ribosome
I3LKZ4	Uncharacterized protein	HNRNPR	−0.26	0.0308	Nucleotide binding
I3LIL4	Uncharacterized protein	MYO1C	−0.44	0.0345	Motor activity
Protein degradation, biosynthesis and modification					
K7GKE7	Haptoglobin		−1.03	0.0184	Proteolysis
I6LNT8	Glycogen synthase kinase 3 α	GSK3A	−0.31	0.0158	Protein serine/threonine kinase activity
G9F6X9	Dihydropyrimidinase-like 2		−0.29	0.0177	Hydrolase activity
I3LD20	Dystroglycan	DAG1	−0.38	0.0055	Structural constituent of muscle
P37111	Aminoacylase-1	ACY1	0.15	0.0007	Cellular amino acid metabolic process
Q29549	Clusterin	CLU	−0.26	0.0128	Ubiquitin protein ligase binding
D0G0C8	Chaperonin containing TCP1, subunit 2 (β)	CCT2	−0.19	0.0390	Actin-binding
I3LUK9	Ubiquitin-conjugating enzyme E2 D2	UBE2D2	−0.15	0.0334	Ubiquitin-protein ligase activity
F1SID4	Uncharacterized protein	PSMC3	−0.15	0.0325	Ubiquitin-dependent process
D0G0C9	Chaperonin containing TCP1, subunit 7	CCT7	−0.14	0.0303	Protein folding
Inflammatory and immune response					
K9J6K1	Tapasin	TAPBP	−0.59	0.0228	Antigen processing
F1SH92	Inter-α-trypsin inhibitor heavy chain H4	ITIH4	−0.47	0.0018	Acute-phase response
F1SHR2	Uncharacterized protein	BCL2L13	−0.26	0.0002	Regulation of apoptotic process
Q29014	α-1 acid glycoprotein	AGP	−0.19	0.0454	Acute-phase response
L8B180	IgG heavy chain	IGHG	0.51	0.0469	Immune response
K7GQ48	Uncharacterized protein	A2M	0.88	0.0393	Complement and coagulation cascades
Oxidative stress and detoxification					
I3LEF2	Uncharacterized protein	NXN	−0.46	0.0275	Thioredoxin-disulfide reductase activity
Q9N1F5	Glutathione *S*-transferase ω-1	GSTO1	0.09	0.0066	Metabolism of xenobiotics by cytochrome P450
F1SR94	Uncharacterized protein		0.40	0.0319	Metabolism of xenobiotics by cytochrome P450

To be continued

Table 6

Accession	Description	Gene symbol	$\log_2$ fold change	*P* value	Biological process GO term
Miscellaneous					
I3LUN7	Uncharacterized protein	KCNS3	−3.50	0.0184	Voltage-gated potassium channel activity
I3LJ91	Uncharacterized protein	FHOD1	0.13	0.0251	Actin filament organization
F1SRI8	Uncharacterized protein	MYBPC1	0.13	0.0420	Muscle contraction
C1L369	Parvalbumin	PVALB1	0.51	0.0274	Calcium ion binding
B3STX9	Prothrombin		0.19	0.0127	Calcium ion binding

CTRL: control; NSPE: non-starch polysaccharide enzyme. NSPE supplementation significantly altered 51 proteins with 21 being up-regulated and 30 down-regulated. A total of 38 proteins of differential abundance were grouped into seven classes based on putative functions. Protein expression is represented as $\log_2$ fold relative to the control

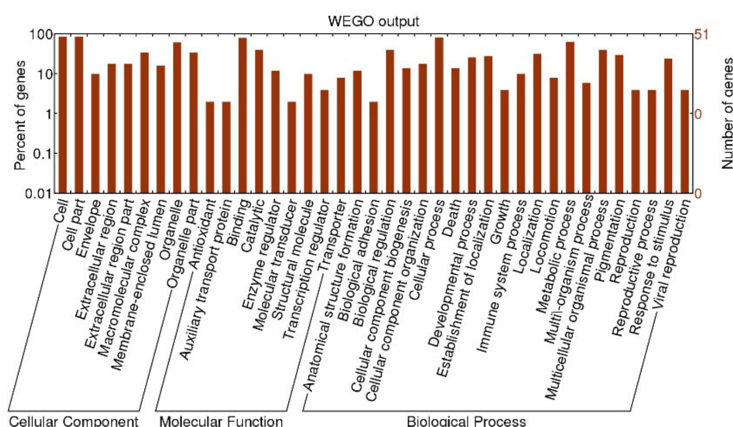

Fig. 2 GO distribution analysis of differentially expressed proteins in the longissimus muscle from the treatment group and the control group
The number of proteins for each GO annotation is shown on the right axis, and the proportion of proteins for each GO annotation is shown on the left axis

3.5 GO annotations and functional analysis

In the cellular component group, the differentially expressed proteins were concentrated in intracellular organelles and the cytoplasm (Fig. 2). In the molecular functional group, the differentially expressed proteins that are metabolic enzymes (hydrolase, oxidoreductase, or ligase activity) and binding proteins (protein, ion, or nucleoside binding) were ranked at the top of the category, suggesting that the relevant functions were important in the LM of pigs (Fig. 2). In the biological process category, the proteinsthat participate in cellular processes (protein metabolic process), metabolism (nitrogen compound metabolic process), and biological regulation (signal transduction), had the highest ratios among the differentially expressed proteins (Fig. 2), suggesting that supplementation with NSPEs results mainly in changes in the protein metabolism in the LM of pigs.

3.6 Validation of proteins of differential abundance

Six differentially expressed proteins (aminoacylase-1 (ACY1) involved in protein biosynthesis, prothrombin involved in the differentiation of skeletal

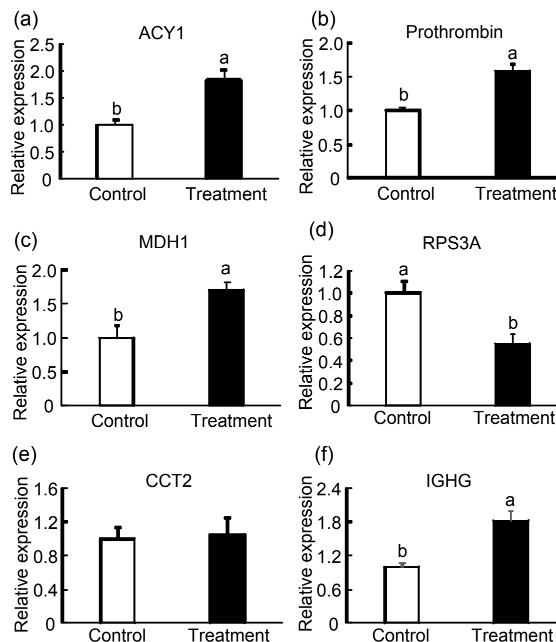

Fig. 3 qPCR validation of six proteins of differential abundance from the longissimus muscle of growing pigs at the mRNA level
Samples were normalized with the reference gene *β-actin*. Data are presented as mean±SD (n=4). Mean values with different letters are significantly different (P<0.05)

muscle, malate dehydrogenase (MDH1) involved in ATP production, 40S ribosomal protein S3a (RPS3A) involved in transcriptional and translational regulation, chaperonin containing TCP1, subunit 2 (β) (CCT2) involved in protein modification and immunoglobulin heavy chain γ polypeptide (IGHG) involved in immune response) were selected for validation of proteomic data at the mRNA level using qPCR (Fig. 3). Most protein levels were consistent with their mRNA expression levels, except for CCT2.

4 Discussion

The value of providing NSPEs to promote growth performance has been reported in many studies (Bindelle *et al.*, 2011; Willamil *et al.*, 2012; Kiarie *et al.*, 2013). However, the effects of NSPEs on meat quality and chemical composition were not obvious in the present study, which is consistent with previous studies (Kim *et al.*, 2011; Świątkiewicz *et al.*, 2013). As an important organ, skeletal muscle plays vital roles in multiple metabolic pathways, such as energy metabolism, which are highly relevant to growth (Suryawan and Davis, 2014). Hence, it is possible that the alteration of metabolism in the skeletal muscle is related to supplementation with NSPEs in the diet. However, the underlying mechanisms are largely unknown. In the current study, we observed that several specific pathways were altered in the LM of growing pigs via supplementation with NSPEs, including energy metabolism, protein degradation, biosynthesis and modification, inflammation and immune response, oxidative stress and detoxification, and some other functions. The proteomic data obtained from the present study were also verified by mRNA expression levels. Most of the protein level were consistent with their corresponding mRNA expression levels, except for CCT2. Possible reasons for the inconsistent result may be the existence of RNA editing and post-translational modifications (Ohlendieck, 2011; Luo *et al.*, 2013).

Energy is a major resource needed for maintaining the normal physiological function of skeletal muscle, and is critical for muscle growth, development, and contraction (Gordon *et al.*, 2000; Ohlendieck, 2011). In some pathological or stress conditions, for instance diabetes and fasting, the expression of proteins related to glucose use and the production of ATP is reduced, which triggers atrophy of skeletal muscle (Lecker *et al.*, 2004). In the present study performed *in vivo* with pigs fed NSPEs, we found a greater abundance of proteins, such as glutaredoxin 3 (GLRX3) and MDH1, that are involved in ATP production. GLRX3 may serve as an electron carrier in the respiratory chain for ATP synthesis (Cha and Kim, 2009). MDH1 is an important enzyme in the tricarboxylic acid cycle that may provide H^+ for producing ATP in the next step (Minárik *et al.*, 2002). Increased abundance of GLRX3 and MDH1 in NSPE supplemented pigs could contribute to enhanced production of ATP in the LM. In contrast, succinyl-CoA ligase [ADP/GDP-forming] subunit α (SUCLG1), which serves as a GTP-binding protein in the formation of metabolic intermediates, was down-regulated in the treatment group. This requires additional research (Ottaway *et al.*, 1981). Protein species related to lipid metabolism were also up-regulated in pigs fed NSPEs. Of these proteins, mitochondrial δ3,δ2-dienoyl-CoA isomerase (ECI1) acts as an auxiliary enzyme in the β-oxidation of polyunsaturated fatty acids (Gurvitz *et al.*, 1998). Histidine triad nucleotide binding protein 2 (HINT2) is positively responsible for regulating mitochondrial lipid metabolism and respiration in the livers of mice (Martin *et al.*, 2013). Fatty acid-binding protein 1 (FABP1) facilitates the transportation of lipids in the body (Furuhashi and Hotamisligil, 2008). Increased expression of these proteins may enhance the catabolism of lipids in the LM to stimulate the production of energy for growth. Apolipoprotein A-IV is associated with the secretion of triglyceride in the liver (VerHague *et al.*, 2013). Down-regulation of this protein was observed in the LM of NSPE-supplemented pigs, whereas the serum level of triglyceride was not different between the two groups. This behavior is consistent with previous research which showed that supplementation with NSPEs had no effect on serum level of total triglyceride (Kim *et al.*, 2013).

Interestingly, down-regulated expression of proteins related to transcriptional and translational regulation, such as histone (histone H1.3-like protein) and ribosomal proteins (RPS3A, 60S ribosomal protein L14 and 60S ribosomal protein L7a), was observed in the LM of pigs fed NSPEs (Ramadoss and Magness, 2012). Salem *et al.* (2010) demonstrated

that protein synthesis is reduced during stress or pathological conditions, while expression of proteins related to transcriptional and translational regulation is elevated. Based on the results of the current study, we speculate that supplementation with NSPEs in the diet of growing pigs may reduce the possibility of muscle atrophy.

Furthermore, the lower abundance of haptoglobin, glycogen synthase kinase 3 α (GSK3A), and dihydropyrimidinase-like 2 observed in the LM of pigs fed NSPEs may further protect proteins from proteolysis under stress (Wassler and Fries, 1993; Woodgett, 1994; Wang and Strittmatter, 1997). Protein species that participate in the modification of protein, including clusterin (CLU), CCT2, ubiquitin-conjugating enzyme E2 D2 (UBE2D2), PSMC3 (proteasome (Prosome, Macropain) 26S subunit, ATPase, 3), and CCT7 (chaperonin containing TCP1, subunit 7), were down-regulated in the treatment group (Lecker *et al*., 2004). This pattern is in concert with expression of proteins related to transcriptional and translational regulation, and indicates that the two clusters of protein in the treatment group may have a similar function in preventing muscle atrophy.

The growth of the LM depends mainly on protein synthesis. ACY1 plays an important role in protein synthesis by increasing the availability of amino acids in the serum (Welberry Smith *et al*., 2013). Up-regulated expression of this protein was observed in the LM of growing pigs supplemented with NSPEs, which may explain why the loin-eye area was greater in pigs fed NSPEs compared with those in the control group. This outcome is consistent with previous research which showed that supplementation with NSPEs in the diet improved the loin-eye area in growing pigs (Uthai *et al*., 2004).

IGHG, an important antibody subunit, is composed of an antibody with an immunoglobulin light chain, and can be produced with the initiation of an immune response (Janeway *et al*., 2001). α-2-Macroglobulin (A2M) may be involved in the pathway of complementation and coagulation cascades to eliminate pathogens (Turnberg and Botto, 2003). The abundance of these two proteins was elevated in the LM of pigs fed NSPEs. This indicates that supplementation with NSPEs could enhance immunity in animals. In contrast, as a transporter associated with antigen processing, the abundance of tapasin was lower in the LM of pigs supplemented with NSPEs. This behavior is consistent with previous research showing the overexpression of tapasin in muscle atrophy (Salem *et al*., 2010). Moreover, inter-α-trypsin inhibitor heavy chain H4 (ITIH4) and α-1 acid glycoprotein (AGP) are acute-phase proteins (APP), which are induced by inflammation-related cytokines (Fournier *et al*., 2000; Piñeiro *et al*., 2004). Also, BCL2-like 13 (apoptosis facilitator) (BCL2L13) is a member of the Bcl-2 family of proteins, and its overexpression can induce apoptosis (Kataoka *et al*., 2001). Reduced abundance of these proteins could attenuate inflammation in the LM of the NSPE group. Along with the impact of immunity and inflammation, we also noticed that supplementation with NSPE had effects on the expression of proteins related to the stress response and detoxification. Nucleoredoxin (NXN) can regulate the formation of reactive oxygen species (ROS) (Hirota *et al*., 2000; Funato and Miki, 2007). Reduced expression of NXN in NSPE pigs attenuated oxidative stress in the LM. Glutathione *S*-transferase ω 1 (GSTO1) plays an important role in the detoxification of different drugs, carcinogens, and endogenous compounds by catalyzing the conjugation of glutathione with electrophilic substrates (Mukherjee *et al*., 2006). Thus, a higher abundance of GSTO1 in NSPE pigs may facilitate the elimination of toxins in the LM.

Notably, increased abundance of two calcium binding proteins, parvalbumin and prothrombin, was observed in the treatment group in our study. Parvalbumin is found in fast-twitch fibers and binds cytosolic Ca^{2+}, enabling rapid relaxation after contraction (Ottaway *et al*., 1981). Prothrombin is highly expressed by skeletal and vascular smooth muscle (McBane *et al*., 1997), and its expression is up-regulated in murine skeletal muscle cells in response to differentiation (Kim and Nelson, 1998). Combined with the results of the current study, we assume that elevated expression of parvalbumin and prothrombin in NSPE-supplemented pigs may help the skeletal muscle maintain normal physiological function and stimulate differentiation.

5 Conclusions

In summary, the results of this study provide the first evidence for an altered abundance of proteome in the LM of growing pigs supplemented with NSPEs.

Though the meat quality and chemical composition of the LM were not affected by NSPEs, protein species related to protein metabolism were significantly changed, which may be associated with the growth of skeletal muscle. Furthermore, supplementation with NSPEs resulted in additional benefits by facilitating an enhanced immune response, oxidation resistance and detoxification in the LM of pigs. In contrast, protein species relevant to inflammation were down-regulated in pigs fed NSPEs. These novel findings elucidate the mechanisms whereby dietary supplementation with NSPEs promotes growth performance and improves muscular metabolism in growing pigs.

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Compliance with ethics guidelines

Ji-ze ZHANG, Yang GAO, Qing-ping LU, Ren-na SA, and Hong-fu ZHANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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List of electronic supplementary materials

Table S1 qPCR primers used for verification of the differentially expressed genes of longissimus muscle in growing pigs

Table S2 List of all proteins (n=1167) identified in the study

Table S3 List of all differentially expressed proteins (n=51) identified in the study

中文概要

题 目：基于 iTRAQ 定量蛋白质组学技术分析日粮中添加非淀粉多糖酶对生长猪背最长肌中蛋白表达影响

目 的：通过日粮中添加非淀粉多糖酶，运用同位素标记相对和绝对定量技术（iTRAQ 技术）分析非淀粉多糖酶对生长猪背最长肌蛋白质表达有何影响，为饲料中添加非淀粉多糖酶提供理论基础。

创新点：采用 iTRAQ 定量蛋白质组学技术，通过对生长猪背最长肌蛋白质表达进行高通量分析，发现日粮中添加非淀粉多糖酶可影响许多功能蛋白表达，从分子水平阐述了其发挥作用的机理。

方 法：将体重约 39 kg 生长猪（48 头）随机分为两个处理，每个处理 4 个重复，每个重复 6 头猪。对照组饲喂基础日粮，试验组在基础日粮中添加 0.6% 非淀粉多糖酶。50 天试验期后，每个重复屠宰 1 头猪（n=8），取背最长肌，通过 iTRAQ 定量蛋白质组学技术分析肌肉组织中差异蛋白表达。

结 论：iTRAQ 定量蛋白质组学分析结果显示，试验组与对照组相比，共发现 51 个差异蛋白，其中 38 个可进行生物学功能定位（图 1，表 6）。差异表达蛋白中与能量生成、蛋白质合成、肌肉分化、免疫、抗氧化和解毒相关蛋白表达量上调，而与炎症反应相关蛋白表达量下调。综上所述，生长猪日粮中添加非淀粉多糖酶不仅可改善生产性能，同时还可调节肌肉中诸多代谢功能。

关键词：非淀粉多糖酶；背最长肌；蛋白质组学；生长猪