DOI:10.1021/jf903439q

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Application of Porcine Lipase Secreted by *Pichia pastoris* to Improve Fat Digestion and Growth Performance of Postweaning Piglets

Fang-Chueh Liu,[†,‡] Hsiao-Ling Chen,[§] Willie Lin,[∥] Yu-Tang Tung,[†] Cheng-Wei Lai,[†] A-Li Hsu,[‡] and Chuan-Mu Chen*[,†]

[†]Department of Life Sciences, National Chung Hsing University, Taichung 402, and School of Chinese Medicine, China Medical University, Taichung 404, Taiwan, [‡]Department of Animal Nutrition, Livestock Research Institute, Council of Agriculture, Tainan 712, Taiwan, [§]Department of Molecular Biotechnology, Da-Yeh University, Changhwa 515, Taiwan, and [∥]Program of Technology Management, Fu Jen Catholic University, Taipei County 242, Taiwan

The aim of the study was to use *Pichia pastoris* to express a recombinant porcine lipase gene (*pLip*). The expression−secretion cassette was constructed using the *P. pastoris GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) promoter and an 89-residue prepro-α-factor secretion signal fused to the *AOX1* terminator (the pGAPZαA vector). A total of 1,408 bp of pancreatic lipase cDNA was produced, which was located from the position of 4-nt upstream of ATG to 1408-nt inside the intact coding region of the *pLip* sequence. In an animal trial, three concentrations of recombinant lipase activity (0, 5,000 and 10,000 U/kg) were blended with the basal diet and fed to weaned piglets for six weeks. During the experimental period, the growth performance (bodyweight, feed intake, and feed efficiency) of the test groups was superior to that of the control group ($p < 0.05$). Furthermore, the group fed the diet blended with 10,000 U/kg of recombinant lipase showed significant ($p < 0.05$) increases in blood triglyceride (TG) concentration on the seventh day postweaning. These results suggested that the porcine lipase protein yielded by transformed yeast cells may improve fat digestibility and enhance the growth performance in postweaning piglets.

KEYWORDS: Porcine lipase; postweaning piglet; recombinant yeast; *Pichia pastoris*; animal feed; fat digestion

INTRODUCTION

The nutrients of sow's milk are suitable for efficient digestion in suckling pigs because the apparent digestibility of the milk fat can reach 96% (*1*). Although higher fat digestibility during the suckling period is seen, the most important lipolytic enzyme, pancreatic lipase, is still produced at low levels in suckling compared with fully developed pigs (*2*). Specifically, after weaning the milk fat digestibility will decline to 65 to 80%, leading to an energy deficiency phenomenon in postweaning piglets (*3*). Thus, we considered that the gastrointestinal system of postweaning piglets must adapt to the substantial changes in their feed to meet the digestion and absorption of the nutrients in the diet. In order to maintain caloric intake, several fat sources are often added to the diets of postweaning piglets, but these yield different results depending on the sources and amount of fat and feed consumption. Generally, fat sources derived from adipose are relatively inferior to those from plant fats, which include a higher level of middle-chain triglycerides (MCT) and unsaturated fatty acids, which are easier for postweaning piglets to digest and absorb (*3*,*4*).

The weaning process impacts the physiological response of pigs, including the gastrointestinal tract as it adapts to digest and absorb their feed and the considerable change of the dietary nutrients. An acceptable growth performance must be maintained, and during the postweaning period, the activation, secretion, and function of pancreatic digestive enzymes has not yet completely developed, in particular lipase and colipase. Cera et al. (*3*) pointed out that the weaning process affects the physiological responses of piglets because the height of intestinal villi is reduced and the depth of the crypts increased by the seventh day postweaning. The nutrient absorption of the piglet intestinal mucous membrane becomes weaker over the two weeks after weaning.

It has been demonstrated that the activation of pancreatic lipase in postweaning piglets is induced by an increase in the dietary fat content; commonly, increasing content 6-fold in the diet will increase lipase activity 1.8-fold (*5*). Most dietary formulas fed to postweaning piglets have fat contents from 1 to 6%, but this is not enough to satisfy their energy requirements and maintain a normal growth rate because of insufficient lipolytic enzyme (*6*). Robertson et al. (*7*) reported that postweaning piglets could not meet their energy requirement during the first two weeks, even though their energy uptake was sufficient in the suckling period. The critical cause was the lower lipolytic enzyme activity; thus, pancreatic lipase function was not fully developed for increased fat digestion (*2*). Jensen et al. (*8*) also pointed out

*To whom correspondence should be addressed. Tel: +886-4-2285-6309. Fax: +886-4-2287-4740. E-mail: chchen1@dragon.nchu.edu.tw.

 Published on Web 02/18/2010

that the main reason for lower energy uptake was derived from deficient pancreatic lipase activity. Therefore, the application of a recombinant lipase, secreted by *Pichia pastoris*, may be an alternative way to improve fat digestion and growth performance in postweaning piglets.

MATERIALS AND METHODS

Cloning and Construction of *pLip* cDNA in Yeast pGAPZαA Vector. Total RNAs were isolated from 1.0 g of the porcine pancreas of LYD three-breed conventional pig crosses with TRIzol reagent and using the protocol provided by the manufacturer (Invitrogen, San Diego, CA). Three hundred micrograms of total RNA was used to isolate the poly(A^+) mRNA using the FastTrack MAG mRNA isolation kit (Invitrogen). An aliquot of 5 μL of poly(A^+) mRNA was taken for first-strand cDNA synthesis by SuperScript III reverse transcriptase (Gibco/BRL, Burlington, ON). The recombinant 1.3-kb mature *pLipase* cDNA fragment, with a deleted 16-aa native signal peptide, was amplified by PCR with the following primers: pLip-U (forward), 5′-GAA TTC GGA AGC GAA GTC TGT TTC-3′; and pLip-D (reverse), 5′-TTC TAG ACA CGG TGT GAG GGT GA-3′ (underlined bases indicate the introduced restriction sites). The PCR product was cloned into the pGEM T-Easy Vector (Promega, Madison, WI), and the sequence was verified by nucleotide sequencing as described (*9*). The resulting pGEM-*pLip* was digested with *EcoR* I and *Xba* I (New England Biolabs, Beverly, MA) at the newly created sites, and then the *pLip* fragment was gel purified. The excised fragment was cloned as an *EcoR* I-Xba I fragment flanking the α-factor signal peptide into the pGAPZαA vector (Invitrogen). This construct plasmid was termed pGAPZαA-pLip.

Transformation of *pLip* Plasmid into *P. pastoris* GS115 Host Cells. The pGAPZαA-pLip expression cassette was digested with *Bgl*II prior to transformation into *P. pastoris*. For electroporation, a 50-mL culture of *P. pastoris* GS115 (*his4*$^-$) in YPD (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L dextrose) was grown at 30 °C to OD_{600} = 1.3 to 1.5 (~5 × 10^7 cells/mL). Cells were washed twice and resuspended in 1.5 mL of ice-cold 1 M sorbitol. An aliquot of competent GS115 cells (100 μL) was mixed with 10 μg of linearized transforming plasmid DNA and transferred to an ice-cold 2-mm electroporation cuvette. The cells were pulsed according to the parameters for yeast (1500 V, 25 μF, and 200 Ω; GenePulser, Bio-Rad, Hercules, CA) as previously described (*10*, *11*). The cells were incubated for 1 h at 30 °C and then spread on YPDS (YPD + 1 M sorbitol) containing 100 μg/mL Zeocin (Invitrogen). Transformants were visible after 2−3 days at 30 °C. Several hundred Zeocin-resistant transformants were identified during the first round of selection. In the second round, we selected for multicopy transformants by plating 200 Mut^s transformants on media with 2 mg of Zeocin per mL. Ten transformants resistant to high levels of Zeocin were arbitrarily selected for further analyses. A single colony of pGAPαA-pLip transformed GS115 cells was inoculated into 25 mL of YPD broth and grown at 29 °C in a shaker incubator until the culture reached an OD_{600} of 2−3 (approximately 36−48 h). The supernatant was decanted into a new tube and cell pellets separated as previously described (*12*). The extracellular secretions of the supernatant were then lyophilized and stored at −80 °C until assay.

Total RNA Extraction and RT-PCR Analysis. Total RNA from *Pichia* yeast transformants was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA). Briefly, yeast transformants were grown in 50 mL of YPD broth and incubated at 30 °C in a shaking incubator (250 rpm) for 72 h. Samples up to 5 × 10^7 yeast cells were harvested and centrifuged at 3,000*g* for 10 min at 4 °C. The cell walls were digested with 25 U of lyticase enzyme (Sigma, St. Louis, MO) and incubated at 30 °C with gentle shaking for 30 min. The cell lysates were applied for total RNA extraction according to the manufacturer's instructions (Qiagen). One microgram of total RNA was resuspended in 15 μL of diethylpyrocarbonate (DEPC)-treated water and used for synthesizing the first-strand cDNA with random primers and SuperScript III reverse transcriptase in a total volume of 25 μL (*13*, *14*). The reaction was carried out at 42 °C for 1 h. For further PCR amplification, an aliquot (1/10) of the RT product was adjusted to contain 0.1 μg of each primer, and additional buffer was added to a total volume of 50 μL. PCR was performed for 25 cycles (94 °C, 1 min; 55 °C, 2 min; 72 °C, 2 min). The pLip, pLip-R(+), 5′-TTA TTC GCA GCA TCC TCC GC-3′, and pLip-R(−), 5′-GCA AAT GGA ATT CTG ACA TCC-3′, are specific primers. A pair of α-tubulin-specific primers, α-tubulin-R(+), 5′-AGA TGG CCG ACC AAT GTG ACG-3′, and α-tubulin-R(−), 5′- TGT TCC AGG GTG GTA TGC GTG-3′, was used as an internal control. The transcription level of recombinant pLip mRNA was normalized to the α-tubulin mRNA by a semiquantitative method (*15*, *16*).

Immunoblot Analysis of pLipase Protein in the Cell Pellet and Supernatant. Cell pellets were lysed with a lysis buffer (pH 7.4, 50 mM sodium phosphate; 1 mM PMSF; 1 mM EDTA; 50 mL/L glycerol) and acid-washed glass beads (size 0.5 mm; Sigma). For assay of the extracellularly secreted recombinant porcine lipase, the culture medium was collected, filtered (Centriprep-10, Amicon, Danvers, MA), and fractionated by 4−12% NuPAGE Novex Bis-Tris gels electrophoresis under reducing conditions (Invitrogen). Proteins were electro-transferred from the gel to an immobile-P^{SQ} Transfer PVDF membrane (Millipore, Bedford, MA). To reduce nonspecific binding, the PVDF membrane was blocked with 5% nonfat skim milk in 1× PBS-Tween 20 buffer (*17*). A mouse anti-*myc* monoclonal antibody (Invitrogen) served as a primary antibody against the peptide sequence NH_3-Glu-Gln-Lys- Ile-Ser-Glu-Glu-Asp-Leu-COOH. Recombinant lipase was detected by this primary antibody (at a 1:3,000 dilution), and a second antimouse IgG antibody conjugated to horseradish peroxidase (1:3,000 dilution; Invitrogen) was added. The blot was developed with a chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech., Arlington Heights, IL) and a reaction time of 1 min to expose the membrane with Hyperfilm (*18*).

Quantitative pLipase Activity Assay with the pH Titration Method. The lipase activity was measured titrimetrically at a pH between 6.5 and 8.0, allowing 5 min at 25 °C with a pH-stat for equilibration. The standard assay procedure was determined according to the method reported by Gaskin et al. (*19*), using 0.5 mL of tributyrin (Sigma) as a substrate in a total of 30 mL of assay solution containing 2.5 mM Tris−HCl, 1 mM $CaCl_2$ (pH 8.5), emulsion with 4 mM sodium taurodeoxycholate (NaTDC), 50 pmol of colipase (Sigma), and 0.3 mL of the recombinant lipase supernatant. One unit of lipase activity was defined as 1 μmol free butyric acid released from tributyrin in 1 min at 25 °C.

Animals and Diets. A total of 48 hybrid LYD piglets at the age of 28 days, with an average body weight of 7.80 kg from 7 littermates were selected for animal trials. These piglets were obtained from the Animal Industry Division, Livestock Research Institute, Council of Agriculture. The animals were weaned at the 28 days of age and were randomly divided into 12 pens. The experimental piglets were raised in isolated pens of the nursery house, with each pen a standard size at 4 m^2 under the guidelines of the Animal Care Committee of the Council of Agriculture, Taiwan. The nutritional requirement for piglets was met according to the feeding standard of pigs. The secreted lipase used in the mixture of basal diet was respectively isolated with a 0.2 μm Milligard Cartridge Filter (Millipore Corporation, Billerica, MA) and condensed with a 10 K Pellicon 2 Fliter (Millipore). Prior to drying, we added the supernatant containing lipase by freeze drying to the glycerol and carrier to protect lipase bioactivity. Later, quantitative recombinant lipase activity was determined from the dried supernatant with the pH titration method, and the designed concentration was blended into the diet. The experimental diets for the test groups included 5,000 and 10,000 U/kg of recombinant lipase, while the same volume of yeast supernatant without lipase was added to the basal diet for the control group, as shown in **Table 1**. Furthermore, the yeast supernatants need the carriers to improve the homogeneity of the mixture during mixing. During the six weeks of the experimental period, animals were allowed access to food and water ad libitum. Food intake, blood samples, and body weight gains in each individual were recorded at one-week intervals.

Triglyceride (TG) and Blood Urea Nitrogen (BUN) Concentration Assay from Piglet Blood Samples. Blood samples were collected from the venous sinus on days 1, 7, and 28 after the start of the trial. EDTA was used as an anticoagulant as the plasma specimens were used for TG and BUN measurements (*20*). Blood TG and BUN concentration was analyzed by using a TG and BUN diagnostic kit (Pointe Scientific Inc., Michigan, IA) according to the manufacturer's protocol.

Statistical Analysis. All data were analyzed in the randomized complete block design using the General Linear Model procedures of SAS (SAS Institute Inc., Cary, NC). Statistical significance was indicated by $p < 0.05$ (*) and was assessed using least-squares means (LS-Means).

Table 1. Composition of the Basal Diet for Experimental Postweaning Piglets

items	basal diet
ingredients (%)	
corn, yellow	67.75
soybean meal, 44%	19.0
dried skim milk, 35%	2.0
whey, dried	2.0
fish meal, 61.2%	5.0
dicalcium phosphate	1.6
limestone, pulverized	0.8
salt, iodized	0.5
vitamin premix[a]	0.1
mineral premix[b]	0.15
soybean oil	1.0
chloride-choline, 50%	0.1
total	100
calculated values	
crude protein (%)	18.49
digestible energy (kcal/kg)	3550
lysine (%)	1.19
calcium (%)	0.88
phosphorus (%)	0.69
analyzed value	
dry matter (%)	87.77
crude protein (%)	18.52
crude fat (%)	3.58

[a] Vitamin premix provided the following vitamins per kg of diet: vitamin A, 8000 IU; vitamin D_3, 800 IU; vitamin E, 30 IU; vitamin K_3, 1.0 mg; thiamin, 2.0 mg; riboflavin, 5.0 mg; vitamin B_{12}, 25 μg; Ca-pantothenate, 12 mg; niacin, 18 mg; folic acid, 0.4 mg; biotin, 0.06 mg; and choline, 120 mg. [b] Mineral premix provided the following minerals per kg of diet: Cu, 10 mg; Fe, 100 mg; Zn, 100 mg; Mn, 10 mg; and Se, 0.1 mg.

RESULTS

Molecular Cloning and Transformation of Recombinant Porcine Lipase into *P. pastoris*. The full-length, 1,395-bp porcine pancreatic lipase (*pLip*) mRNA encodes a 16-aa signal propeptide followed by a 449-aa mature polypeptide (**Figure 1**). In this study, RT-PCR was applied to identify and clone a pancreatic lipase cDNA fragment from porcine pancreas tissue. A total of 1,408-bp of pancreatic lipase cDNA was produced, which was located from the position at 4-nt upstream of ATG to 1,408-nt into the *pLip* cDNA sequences with an intact coding region (Supporting Information Figure 1). We applied Blastx (Basic Local Alignment Search Tool) at the NCBI Web site (National Center for Biotechnology Information) to search their protein database. We found amino acid identity of up to 98% of the cloned pancreatic cDNA sequence with *Sus scrofa* pancreatic triacylglycerol lipase (Genbank accession no. gi:6686288). The alignments of encoded amino acids from the cloned porcine pancreatic lipase cDNA with *Homo sapiens* and *Mus musculus* lipase showed 87% and 78% identities, respectively (**Figure 1**).

On the basis of this intact coding region of *pLip* cDNA clone, a pair of degenerate primers was designed containing *EcoR* I and *Xba* I enzyme-recognition sites in the forward and reverse primers, respectively (**Figure 2A**). The sites allowed us to further reconstruct a mature form of *pLip* cDNA by deleting the 16-aa signal peptide from its N-terminus and then cloning it as a fragment flanking the α-factor signal peptide and the 3′-*AOX1* terminator sequence (**Figure 2B**). After introducing the *pLip* gene into *P. pastoris*, a Zeocin-resistant gene was used as the selectable marker in the screening processes. Several hundreds of transformed yeast clones were obtained with a Zeocin-resistant phenotype.

Identification of Integrated pGAPZαA-pLip Expression Cassettes in the *P. pastoris* Genome. The secreted expression cassette was constructed using the *P. pastoris GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) promoter, and an 89-residue pre-pro-α-factor secretion signal fused to the *AOX1* terminator (pGAPZαA vector). The 1,347-bp *pLip* cDNA was inserted as an *Eco*RI−*XbaI* fragment flanking the α-factor signal peptide and the 3′-*AOX1* terminator sequence (**Figure 2**). After introducing the *pLip* gene into *P. pastoris*, the resulting transformants carried the pGAPZαA-pLip expression cassette inserted into the innate yeast *GAPDH* gene locus via homologous recombination. In order to confirm the recombination of the *pLip* gene target into the yeast *GAPDH* locus, a PCR amplification of the insertion flanking sequences with upstream pGAPDH forward and *pLip* 5′-CDS reverse primers was performed to screen hundreds of *pLip*-transformed Zeocin-resistant yeast colonies. As a representative example (Supporting Information Figure 2), all of the examined yeast transformants exhibited a predominant band of 1,935-bp, indicating that the homologous recombination of the *pLip* foreign gene insertion was successfully targeted into the *GAPDH* gene locus.

One hundred transformants bearing the integrated pGAPαB-pLip cassette were selected and further spread on selective plates containing a 20-fold range of Zeocin concentrations for identification of those clones containing multicopy recombinants in the *P. pastoris* yeast genomes. Eleven transformants (clone nos. 2, 8, 9, 22, 24, 26, 31, 38, 53, 60, and 61) exhibiting high-tolerance in the 20-fold Zeocin antibiotic selection were chosen for further analyses.

pLip mRNA Transcriptional Efficiency in Different *P. pastoris* Transformants. To characterize the mRNA expression level of recombinant porcine lipase transcripts, the isolated pLip-transformants were cultured in 50 mL of YPD media, and the total yeast RNA was extracted from cell pellets at 72 h of culture. RT-PCR analysis was performed using two pairs of primer sets including the pLip-R(+) and pLip-R(−), specific to the porcine lipase cDNA, and the α-*tubulin* primer set as an internal control. In **Figure 3A**, the 1,347-bp *pLip* transcription product was found in the 11 transformed *P. pastoris* clones. No homologous transcript was detected in the *P. pastoris* clones transformed with the empty vector (**Figure 3A**, lane NC).

To quantify the amount of *pLip* mRNA, a densitometric analysis was performed to compare the intensity of the 1,347-bp band (*pLip* mRNA) in each lane, which was then normalized to the product of α-*tubulin* in the same reaction. The highest amount of the *pLip* transcripts was found in transformed clone no. 26 (**Figure 3B**). This clone was then selected for further protein expression, activity analysis, animal trials, and functional assays.

Expression and Secretion of Recombinant Porcine Lipase in *P. pastoris*. To assay and purify the expression of recombinant porcine lipase protein in *P. pastoris*, transformed clones no. 26 and no. 38 were cultivated in a shaken flask with 50 mL of YPD culture medium. Aliquots of culture broth were removed at intervals of 24 h over a period of five days of continuous culture. The supernatants and cell pellets in the culture media were collected by centrifugation and then subjected to protein purification and characterization. The expression of recombinant lipase protein in *P. pastoris* transformants was analyzed with 4−12% NuPAGE and by Western blot, as shown in **Figure 4A** and **B**, respectively. A higher density of the 49.6-kDa recombinant pLip protein was observed in clone no. 26 compared to that of other transformants using an immunoblot assay against a mouse anti-*myc* antibody. The time course of pLip protein expression in transformed clone no. 26 was monitored over four days of yeast culture. Data showed that the expression level and accumulation

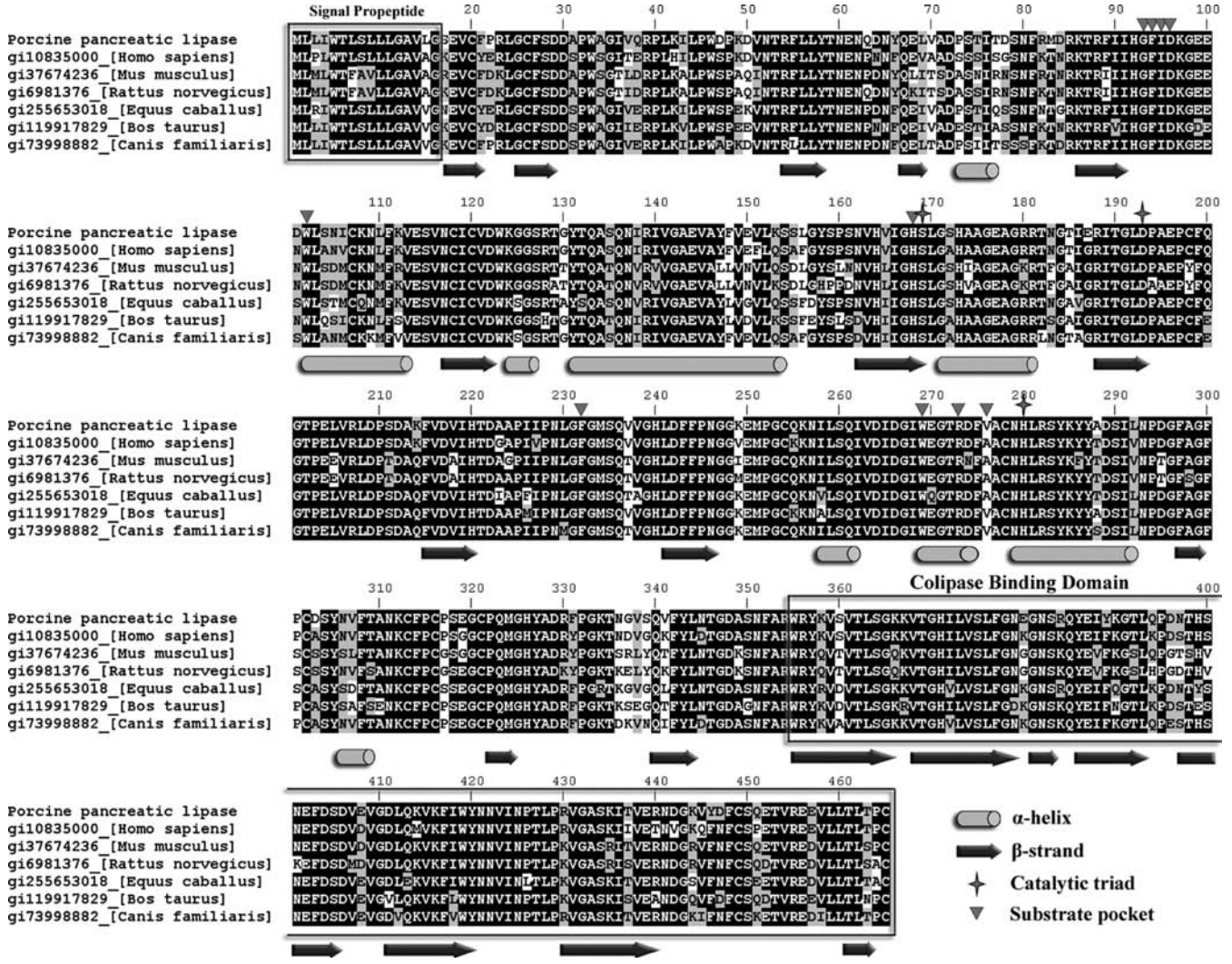

Figure 1. Porcine pancreatic lipase amino acids predicted by cloned pancreatic lipase cDNA were aligned with six different species. The N-terminus of 16 amino acids was identified as a signal peptide, and the C-terminus of 111 amino acids comprised the colipase binding domain. This newly cloned sequence of porcine pancreatic lipase cDNA has been deposited in GenBank (accession no.: 1308322). The α-helix and β-strand were assigned according to the *Sus scrofa* pancreatic lipase protein structure 1ETH. The catalytic triad, Ser^{169}, Asp^{193}, and His^{280}, formed the substrate binding pocket with Gly^{93}, Phe^{94}, Ile^{95}, Asp^{96}, Trp^{102}, His^{168}, Phe^{232}, Trp^{269}, Arg^{273}, Val^{276}.

of pLip protein in the transformed yeast culture medium was stably maintained after 96 h of culture (**Figure 4C**). A total of 288 μg/mL of recombinant lipase was secreted and detected by Western blot analysis in the culture medium of clone no. 26 transformed yeast after 72 h culture (**Figure 4D**).

The activities of recombinant porcine lipase in the supernatant were 102.2, 180.3, 300.4, and 305.8 U/300 μL in clone no. 26 yeast cultures at 24, 48, 72, and 96 h of culture, respectively. However, there was no lipase activity observed in the supernatant of native GS115 yeast cells. Accordingly, Western blot and titration assays also demonstrated that the recombinant lipase was secreted successfully into the yeast culture medium and remained their throughout the three-day culture period. Therefore, the time-course assay indicated that the recombinant lipase protein exhibited relative stability using these culture conditions. Following an animal trial, the yeast culture medium from recombinant clone no. 26 was harvested at 72 h for the maximum pLip protein yields as the source of supplementary porcine lipase.

Effect of Growth Performance for the Dietary Administration of pLip-Enriched Yeast Culture in Postweaning Piglets. The growth rate of postweaning piglets fed with a diet supplemented with 5,000 and 10,000 U/kg of recombinant pLip derived from yeast culture was monitored and compared with that of an age-matched control group fed nonrecombinant yeast culture. The pLip-added test group ($n = 16$) had significantly higher bodyweight than piglets in the control group when measured at days 7, 14, 21, 28, 35, and 42 of postweaning, but there were no significant differences between added 5,000 and 10,000 U/kg of added recombinant lipase in the diets, as shown in **Table 2**. The average daily gain (ADG) of piglets in the test groups both showed a remarkable difference from the control group when measured at 1, 2, 3, 4, 5, and 6 weeks of postweaning, but there was no significant difference between the test groups. The average daily feed intake (ADFI) of piglets in the test group had a significantly higher amount than the control group when measured at 1 and 2 weeks of postweaning. The feed efficiency (feed intake/body-weight gain) of piglets in the test group showed no significant difference between test groups and the control group in each week. During the overall period, the growth performance (bodyweight, feed intake, and feed efficiency) of piglets in the test group was superior to that of the control group.

Effect of Oral Administration of pLip-Enriched Yeast Culture on Dietary Fat Digestion in Postweaning Piglets. To assay the biological activity of recombinant lipase fed to postweaning

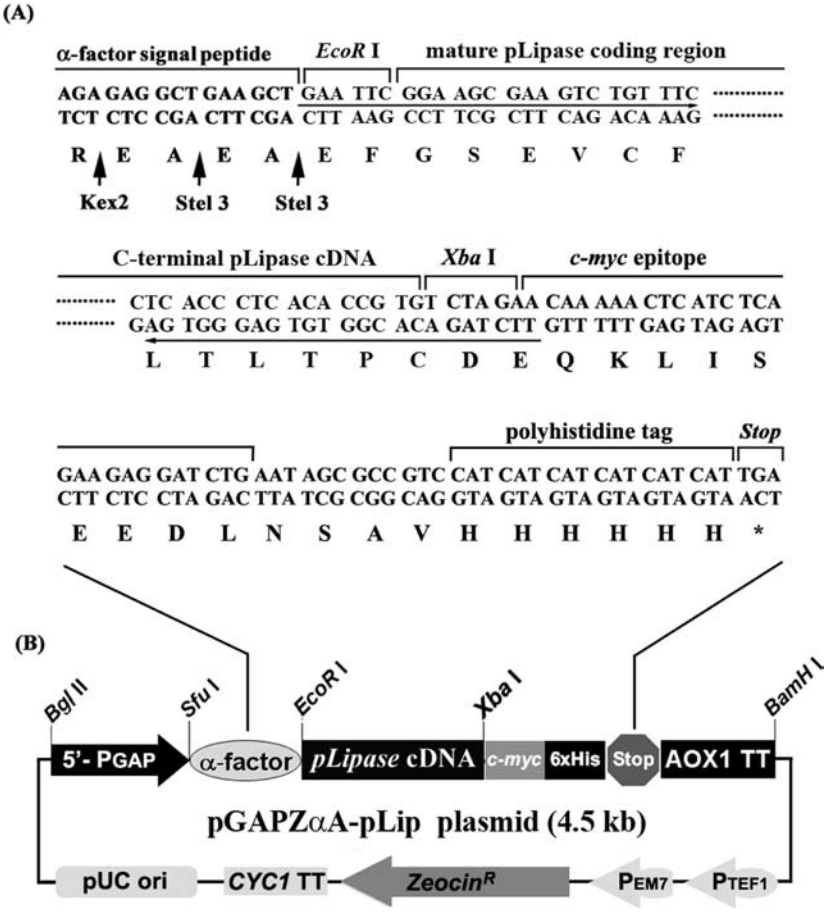

Figure 2. Structure map and sequence of the *P. pastoris* pGAPZαA-pLip expression vector. (**A**) Generation of the recombinant pLip fragment. The sequence of the 5′ and 3′ degenerate PCR primer set is underlined. The rLipase encoding region is flanked by the *EcoR* I and *Xba* I restriction sites. The lower line shows the predicted amino acid sequence with the α-factor leader sequence, the KEX2 protease cleavage site fused to the N-terminus, and the stop codon after the C-terminal *myc* epitope-6 X His tag. (**B**) Construction of the recombinant porcine lipase expression plasmid pGAPZαA-pLip. The pLip *EcoR* I-*Xba* I fragment was amplified using degenerate primer PCR and cloned between the *EcoR* I and *Xba* I sites of the pGAPZ vector flanking the α-factor leader sequence. The pGAPZ vector is an integrating vector utilizing the GAPDH promoter and Zeocin selectable marker.

piglets, blood triglyceride (TG) and blood urea nitrogen (BUN) concentrations were measured to monitor the efficiency of dietary fat digestion, the amount of nitrogen in the blood in the form of urea, and renal function. Blood samples were collected from all test and control piglets on days 1, 7, and 42 during the animal trial period. The levels of blood TG concentration were significantly increased in the experimental test group supplemented with 10,000 U/kg of recombinant lipase on the seventh day postweaning compared with that of the control group, as shown in **Table 3**, but the blood TG concentration levels of the group with 5,000 U/kg of recombinant lipase added were not significantly different compared with that of either the control group or the 10,000 U/kg group in the experimental period. This result is in accordance with the previous results suggesting that supplementation of 10,000 U/kg of recombinant lipase resulted in better fat digestibility in postweaning piglets. The BUN concentration levels of the control group, 5,000 U/kg, and 10,000 U/kg group are shown in **Table 2**. The blood BUN concentration levels of the groups were not significantly different in the experimental period. This result revealed that the renal function upon supplementation of recombinant lipase remains normal.

DISCUSSION

In a normal physiological situation, dietary fat could be absorbed by the intestinal mucous membrane prior to being hydrolyzed by lipase into glycerol, diacylglyceride, and monoacylglyceride. Thus, postweaning piglets would preferably ingest the energy equivalent of suckling sow's milk on the previous day at weaning, but they must wait for two weeks to adapt to their feed (*7*). The related factors (feed intake reduction, weaning stress, and feed composition) in declined pancreatic lipase activity were sought by Lindemann et al. (*2*). In a conventional diet, newly weaned piglets are usually fed fat sources such as rendered fish oil, coconut oil, or olive oil to supplement their energy intake, but during this time, their lipase activity is still insufficient during the first two-week period after weaning (*21*). Therefore, the addition of exogenous lipase in the diet may be one feasible way to alleviate energy ingestion problems for postweaning piglets.

In this study, we cloned the porcine lipase cDNA, having a 1,395-bp open reading frame preceded by 4 bp of 5′-untranslated region and followed by 9 bp of 3′-untranslated region. The open reading frame encodes a 465-amino acid protein at a calculated molecular mass of 51,150 Da. The first 16 amino acids at the N-terminus encode signal pro-peptide and the following 449 amino acid residues the mature porcine pancreatic lipase, which was previously purified by De Caro et al. (*22*). The alignments of encoded amino acids from the cloned porcine pancreatic lipase cDNA with *Homo sapiens* and *Mus musculus* lipase showed 87% and 78% identities, respectively. The amino acids which composed the pancreatic lipase catalytic triad and substrate binding pocket were also highly conserved between cloned pancreatic

lipase in this study and the other five mammalian lipases (**Figure 1**). A signal peptide of 16 amino acids is predicted at the cloned cDNA encoded amino acid's N-terminus, and the mature protein has 449 amino acids, giving a calculated molecular mass of ~49,558 Da, which is the same as the molecular weight of the recombinant lipase (**Figure 4A**). Although this sequence is shorter than that of most signal peptides (*23*), the current initiation consensus sequence, CCACGATGC compared to CCACCATGG (*24*), suggests that this is an acceptable translation initiation site.

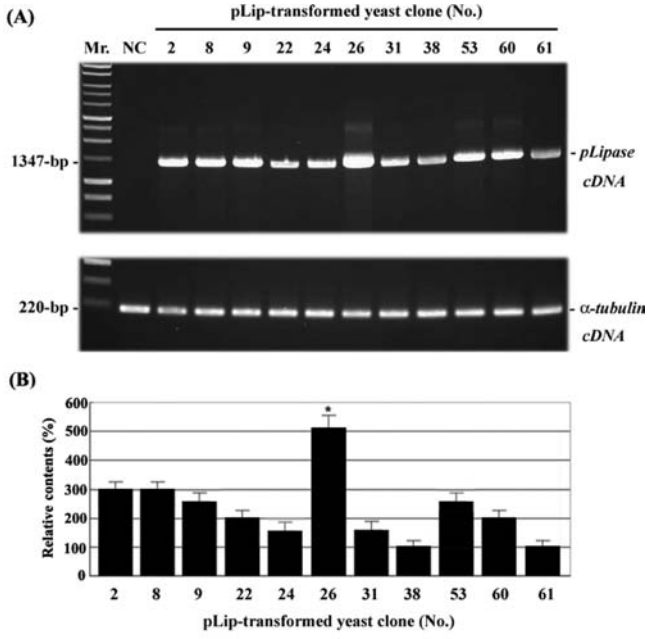

Figure 3. Transcription levels of pLipase mRNA in different yeast transformants. (**A**) Reverse transcription−polymerase chain reaction (RT-PCR) analysis of the *GAPDH* promoter-driven pLip transcripts cultured for 72 h of *P. pastoris* transformants using a pair of pLip-specific primers. A yeast α-tubulin cDNA primer set was used as an internal control. NC: a native GS115 host cell transformed with an empty pGAPZαA plasmid was used as a negative control. (**B**) The relative pLip transcript contents were plotted by densitometric computer analysis (Bio-Rad densitometer). The pLip/α-tubulin ratio was adjusted to 100% in the lowest expression transformed clone (no. 61). All results were expressed as mean ± SD ($n = 3$). The start symbol (*) stands for the significant difference ($p < 0.05$) of the mRNA expression level.

Huguet et al. (*25*) found the pancreatic lipase activity of a fifth-day postweaning piglets is about 80% lower than that on the first weaning day. Therefore, we added 5,000 and 10,000 U/kg of recombinant lipase protein to the diets of postweaning piglet. Animals fed with mixed recombinant lipase protein showed significantly higher bodyweight and average daily gain than the control group during the experimental period, but no remarkable difference in feed efficiency was seen among the test groups (**Table 2**). We found that the feed intake of piglets was also higher in those fed with pLip protein than the control group from 1 to 14 days after weaning.

The blood TG concentrations were not significantly different on the weaning day, but increased by the seventh day postweaning in the experimental test group fed 10,000 U/kg of recombinant lipase (**Table 3**). Therefore, this suggested that the recombinant lipase protein in the diet possessed a specific functionality to improve fat absorption in postweaning piglets. The concentration of BUN showed no significant difference between supplemented and control groups. The primary results were consistent with those of Gaskin et al. (*19*).

Dietary fat hydrolyzed in the intestinal lumen using the addition of recombinant lipase to the diet to raise postweaning piglets would be a better way than injection or implantation, both for animal welfare and fodder digestion. These experimental data showed that the administration of recombinant porcine lipase as a feed additive in the diet provided an alternative approach of enhancing growth performance (**Table 2**) and elevating fat digestibility (**Table 3**) of postweaning piglets. Lipase production and purification from animals or cultured cells are time-consuming and laborious processes (*26*); expression and secretion of

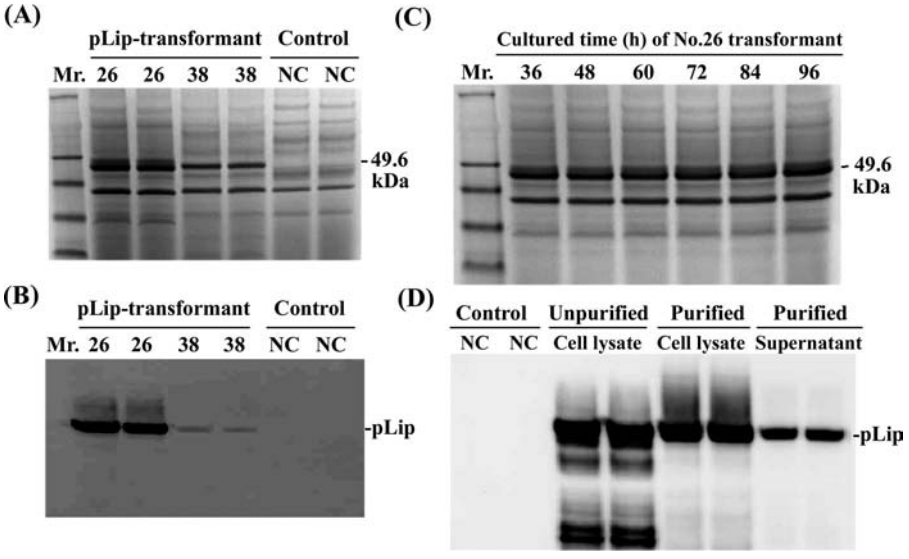

Figure 4. Recombinant pLipase protein expression and purification from the cell lysate and supernatant of yeast transformants. (**A**) The expression of pLip protein in *P. pastoris* cells cultured for 72 h was analyzed with 12% NuPAGE. (**B**) A myc tag-specific antibody was used to detect a 49.6-kDa recombinant pLip protein by Western blot. (**C**) Time-course expression assay of pLip protein production in *P. pastoris* transformant no. 26 during four days of culture. (**D**) Recombinant pLip protein was purified from the cell pellets and supernatant of *P. pastoris* transformant no. 26 cultured for 72 h by a Hitrap polyhistidine-affinity column (Clontech, CA) and applied to Western blot analysis. The samples of negative control (NC) in panels **A**, **B**, and **D** were proteins collected from GS115 yeast supernatants as the negative controls.

Table 2. Effect of Recombinant Porcine Lipase As a Feed Additive by Oral Administration in the Growth Performance of Postweaning Piglets[a]

	level of added recombinant lipase (units/kg)			
items	0	5000	10000	MSE
		body weight (kg)		
1 DPW	7.84[a]	7.84[a]	7.72[a]	0.061
7 DPW	8.67[a]	9.36[b]	9.36[b]	0.106
14 DPW	10.27[a]	11.80[b]	11.92[b]	0.085
21 DPW	12.53[a]	14.93[b]	15.21[b]	0.051
28 DPW	15.72[a]	19.04[b]	19.22[b]	0.102
35 DPW	19.39[a]	23.39[b]	23.79[b]	0.126
42 DPW	24.16[a]	28.82[b]	29.56[b]	0.137
		ADG (kg)		
1−7 DPW	0.12[a]	0.22[b]	0.23[b]	0.008
8−14 DPW	0.23[a]	0.35[b]	0.37[b]	0.010
15−21 DPW	0.32[a]	0.45[b]	0.47[b]	0.009
22−28 DPW	0.46[a]	0.59[b]	0.57[b]	0.013
29−35 DPW	0.52[a]	0.62[b]	0.65[b]	0.016
36−42 DPW	0.68[a]	0.78[ab]	0.82[b]	0.020
		ADFI (kg)		
1−7 DPW	0.20[a]	0.34[b]	0.32[b]	0.017
8−14 DPW	0.42[a]	0.55[b]	0.54[b]	0.016
15−21 DPW	0.62[a]	0.77[a]	0.75[a]	0.029
22−28 DPW	0.82[a]	0.95[a]	0.92[a]	0.025
29−35 DPW	1.03[a]	1.05[a]	1.11[a]	0.019
36−42 DPW	1.32[a]	1.34[ab]	1.43[b]	0.022
		FE (feed/gain)		
1−7 DPW	1.80[a]	1.58[a]	1.39[a]	0.107
8−14 DPW	1.87[a]	1.58[a]	1.48[a]	0.076
15−21 DPW	1.94[a]	1.73[a]	1.61[a]	0.091
22−28 DPW	1.82[a]	1.63[a]	1.61[a]	0.071
29−35 DPW	1.97[a]	1.69[a]	1.71[a]	0.052
36−42 DPW	1.93[a]	1.72[a]	1.75[a]	0.046
		overall period (1−42 d postweaning)		
ADG	0.39[a]	0.50[b]	0.52[b]	0.002
ADFI	0.73[a]	0.83[b]	0.85[b]	0.008
FE	1.89[b]	1.67[a]	1.63[a]	0.015

[a] Within the same rows, the different superscripts represent significantly different ($p < 0.05$) values. DPW, day postweaning; ADG, average daily gain; ADFI, average daily feed intake; FE, feed efficiency; MSE, mean standard error.

Table 3. Effect of Adding Recombinant Lipase on the Blood Traits of Postweaning Piglets[a]

	level of added recombinant lipase (units/kg)			
blood traits	0	5,000	10,000	MSE
		triglyceride (mg/dL)		
1 DPW	33.0[a]	25.5[a]	29.4[a]	2.1
7 DPW	19.8[a]	25.3[ab]	30.9[b]	2.0
42 DPW	54.3[a]	53.3[a]	55.5[a]	6.3
		BUN (mg/dL)		
1 DPW	11.4[a]	10.7[a]	11.4[a]	0.6
7 DPW	13.4[a]	13.0[a]	15.7[a]	0.8
42 DPW	11.2[a]	11.8[a]	12.5[a]	1.0

[a] Within the same rows, different superscripts represent significantly different ($p < 0.05$) values. DPW, day postweaning; BUN, blood urea nitrogen; MSE, mean standard error.

recombinant porcine lipase in *P. pastoris* cell culture are more efficient. Feeding postweaning piglets with pLip-enriched yeast culture is also a more convenient and safe way of adding lipase and is conformable to animal welfare and fodder digestion. This approach not only requires no complicated procedures for isolation of inclusion bodies, renaturation, or further processing of pLip but also causes no stress to piglets while being treated. Therefore, we suggest that feeding lipase-enriched yeast cultures is a more convenient method to elevate dietary fat digestibility and absorption, and to promote the growth performance of postweaning piglets. This allows the large-scale use of yeast culture containing enriched recombinant lipase in the pig-farming industry.

Supporting Information Available: Detailed information for the yield of porcine lipase cDNA products amplified reverse transcription (RT)-PCR (Figure 1) and the existence of pGAPZαB-pLip secretion−expression cassette in the *GAPDH* gene locus of yeast transformants detected by PCR amplification (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Cranwell, P. D.; Moughan, P. J. In *Biological Limitations Imposed by the Digestive System to the Growth Performance of Weaned Pigs*; Barnett, J. L., Hennessy, D. P., Eds.; Manipulating Pig Production II, Australiasian Pig Science Association: Werribee, Australia, 1989; pp 140−165.

(2) Lindemann, D.; Gornelius, S. G.; Kandellegy, S. M. Effect of age, weaning, and diet on digestive enzyme levels in the piglet. *J. Anim. Sci.* **1986**, *62*, 1298–1307.

(3) Cera, K. R.; Mahan, D. C.; Reinhart, G. A. Weekly digestibilities of diets supplemented with corn oil, lard or tallow by weanling swine. *J. Anim. Sci.* **1988**, *66*, 1430.

(4) Cera, K. R.; Mahan, D. C.; Reinhart, G. A. Evaluation of various extracted vegetable oils, roasted soybeans, medium-chain triglyceride and an animal-vegetable fat blend for postweaning swine. *J. Anim. Sci.* **1990**, *68*, 2756–2765.

(5) Malika, O.; Bertrand, S.; Anikn, G. G.; Genevieve, A. Differential regulation of lipase and colipase in the pancreas by dietary fat and protein. *J. Nutr.* **1980**, *110*, 2302–2309.

(6) Flores, C. A.; Brannon, P. M.; Bustamante, S. A.; Bezerra, J.; Butler, K. T.; Goda, T.; Koldovsky, O. Effect of diet on intestinal and pancreatic enzyme activities in the pig. *J. Pediatr. Gastroenterol. Nutr.* **1988**, *7*, 914–921.

(7) Robertson, A. M.; Clark, J. J.; Bruce, J. M. Observed energy intake of weaned piglets and its effect on temperature requirement. *Anim. Prod.* **1985**, *40*, 475–479.

(8) Jensen, M. S.; Jensen, S. K.; Jakobsen, K. Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. *J. Anim. Sci.* **1997**, *75*, 437–445.

(9) Chen, H. L.; Lai, Y. W.; Yen, C. C.; Lin, Y. Y.; Lu, C. Y.; Yang, S. H.; Tsai, T. C.; Lin, Y. J.; Lin, C. W.; Chen, C. M. Production of recombinant porcine lactoferrin exhibiting antibacterial activity in methylotrophic yeast, *Pichia pastoris*. *J. Mol. Microbiol. Biotechnol.* **2004**, *8*, 141–149.

(10) Chen, H. L.; Yen, C. C.; Tsai, T. C.; Yu, C. H.; Liou, Y. J.; Lai, Y. W.; Wang, M. L.; Chen, C. M. Production and characterization of human extracellular superoxide dismutase (ECSOD) in the methylotrophic yeast *Pichia pastoris*. *J. Agric. Food Chem.* **2006**, *54*, 8041–8047.

(11) Chen, H. L.; Yen, C. C.; Lu, C. Y.; Yu, C. H.; Chen, C. M. Synthetic porcine lactoferricin with 20-residue peptide exhibits antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. *J. Agric. Food Chem.* **2006**, *54*, 3277–3282.

(12) Liu, F. C.; Chen, H. L.; Chong, K. Y.; Hsu, A. L.; Chen, C. M. Production of recombinant porcine colipase secreted by *Pichia pastoris* and its application to improve dietary fat digestion and growth of postweaning piglets. *Biotechnol. Prog.* **2008**, *24*, 1333–1341.

(13) Chen, H. L.; Huang, J. Y.; Chu, T. W.; Tsai, T. C.; Hung, C. M.; Lin, C. C.; Liu, F. C.; Wang, L. C.; Chen, Y. J.; Lin, M. F.; Chen, C. M.

Expression of VP1 protein in the milk of transgenic mice: As a potential oral vaccine against for enterovirus 71 strain infection. *Vaccine* **2008**, *26*, 2882–2889.
(14) Cheng, W. T. K.; Lee, C. H.; Hung, C. M.; Chang, T. J.; Chen, C. M. Growth hormone gene polymorphisms and growth performance traits in Duroc, Landrace and Tao-Yuan pigs. *Theriogenology* **2000**, *54*, 1225–1237.
(15) Cheng, W. T. K.; Chen, B. C.; Chiou, S. T.; Chen, C. M. Use of nonautologous microencapulated fibroblasts in growth hormone gene therapy to improve growth of midget swine. *Hum. Gene Ther.* **1998**, *9*, 1995–2003.
(16) Wu, S. C.; Chen, H. L.; Yen, C. C.; Kuo, M. F.; Yang, T. S.; Wang, S. R.; Weng, C. N.; Chen, C. M.; Cheng, W. T. K. Recombinant porcine lactoferrin expressed in the milk of transgenic mice enhances offspring growth performance. *J. Agric. Food Chem.* **2007**, *55*, 4670–4677.
(17) Yen, C. C.; Lin, C. Y.; Chong, K. Y.; Tsai, T. C.; Shen, C. J.; Lin, M. F.; Su, C. Y.; Chen, H. L.; Chen, C. M. Lactoferrin as a natural regimen of selective decontamination of the digestive tract: Recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonates from pathogen challenges in the gastrointestinal tract. *J. Infect. Dis.* **2009**, *199*, 590–598.
(18) Chen, C. M.; Chen, H. L.; Hsiau, T. H.; Hsiau, A. H. A.; Shi, H.; Brock, G. J. R.; Wei, S. H.; Caldwell, C. W.; Yan, P. S.; Huang, T. H. M. Methylation target array for rapid analysis of CpG island hypermethylation in multiple tissue genome. *Am. J. Pathol.* **2003**, *163*, 37–45.
(19) Gaskin, K. J.; Durie, P. R.; Hill, R. E.; Lee, L. M.; Forstner, G. G. Colipase and maximally activated pancreatic lipase in normal subjects and patients with steatorrhea. *J. Clin. Invest.* **1982**, *69*, 427–434.
(20) Hung, C. M.; Yeh, C. C.; Chong, K. Y.; Chen, H. L.; Kao, S. T.; Yen, C. C.; Yeh, M. H.; Lin, M. S.; Chen, C. M. Gingyo-San enhances peripheral lymphocyte proliferation and assists infectious Bursal disease vaccination in native chicken. *Evid. Complement. Alter. Med.* [Onlines early access]. DOI: 10.1093/ecam/nep021. Published Online: Mar 22, **2009**.
(21) Hedemann, M. S.; Pedersen, A. R.; Engberg, R. M. Exocrine pancreatic secretion is stimulated in piglets fed fish oil compared with those fed coconut oil or lard. *J. Nutr.* **2001**, *131*, 3222–3226.
(22) De Caro, J.; Boudouard, M.; Bonicel, J.; Guidoni, A.; Desnuelle, P.; Rovery, M. Porcine pancreatic lipase. Completion of primary structure. *Biochim. Biophys. Acta* **1981**, *671*, 129–138.
(23) von Heijne, G. Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **1983**, *133*, 17–21.
(24) Kozak, M. Evaluation of the "scanning model" for initiation of protein synthesis in eukaryotes. *Cell* **1980**, *22*, 7–8.
(25) Huguet, A.; Savary, G.; Bobillier, E.; Lebreton, Y.; Le Huerou-Luron, I. Effects of level of feed intake on pancreatic expcrine secretions during the early postweaning period in piglet. *J. Anim. Sci.* **2006**, *84*, 2965–2972.
(26) Thirstrup, K.; Carriere, F.; Hjorth, S.; Rasmussen, P. B.; Woldike, H.; Nielsen, P. F.; Thim, L. One-step purification and characterization of human pancreatic lipase expressed in insect cells. *FEBS Lett.* **1993**, *327*, 79–84.

Received for review September 30, 2009. Revised manuscript received January 12, 2010. Accepted February 2, 2010. This research was supported in part by grant NSC-97-2313-B-005-012 from the National Science Council, grant COA-96-6.2.1-U1(9) from the Council of Agriculture, and the Ministry of Education, Taiwan, Republic of China, under the ATU plan.