# PRODUCTION OF RECOMBINANT PEPSIN, PANCREATIC LIPASE AND COLIPASE FROM *PICHIA PASTORIS* AS FEED ADDITIVE

Fang-Chueh Liu
Livestock Research Institute of Council of Agriculture,
Executive Yuan, Taiwan
e-mail: fcliu@mail.tlri.gov.tw

## ABSTRACT

*Enzyme feed additives are always added in animal nutrition to improve the quality of feeds, or to improve the animals' growth performance and health through intensifying digestibility of the feed ingredients. This study investigated the effect of adding the recombinant porcine pepsin, colipase and lipase, which were released by* Pichia pastoris *expression system, in diets on the digestion of high-protein or high-fat ingredients and growth performance of weanling piglets. A total of 978-bp, 300-bp and 1347-bp cDNA fragments encoding matured pepsin, pancreatic colipase and lipase were cloned from porcine stomach lining and pancreas, ligated into* Pichia pastoris *expression vector and transformed into yeast host cell. For the effect of adding recombinant pepsin in feeding experiment; when piglets were fed with diet supplemented with 5% fish meal (protein content of fish meal is equal to 9% of yellow corn and 9% of soybean meal) they served as positive control group, those fed without containing fish meal (dietary protein content is only out of soybean meal and yellow corn) as negative control group, and those fed with the negative control diet after addition of 1,000 U/kg recombinant pepsin activity as experimental group. Experimental results on growth performance of weaning piglets showed that there was no significant difference among groups, but the experimental group had a better trend in feed efficiency compared to the negative group. The concentrations of immunoglobulin showed that the amount of IgM in negative and experimental groups were lower than those in positive group, but concentrations of IgA and IgG were not remarkably different among groups. Thus, in accordance with animal feeding results it was demonstrated that 5% fish meal in formula could be replaced by 9% soybean meal and 9% yellow corn with complementing 1,000 U/kg recombinant pepsin. For the effect of adding recombinant colipase in feeding experiment, 32 weaning piglets designed to treat with 0 or 5000 U/kg recombinant colipase activity for 4 weeks. The results demonstrated that piglets fed with diet adding 5000 U/kg recombinant colipase had a significantly higher average daily gain (ADG) than those fed without adding recombinant colipase during the interval from 8 to 14 d after weaning, but other intervals didn't have a remarkable difference between without and with adding the recombinant colipase groups. Blood triglyceride (TG) concentration was not significantly different between both groups, but it could maintain at 11.98 mg/dL in group with adding recombinant colipase (at weaning level of TG at 12.82 ± 4.94 mg/dL) on the 7-day postweaning, but it was remarkably different compared to the group without adding recombinant colipase on the 28-day postweaning. Therefore, the outcomes suggested that adding recombinant colipase may have a function to improve fat digestibility and enhance the growth performance of weaning piglets. For the effect of adding recombinant lipase in feeding experiment, three amounts of recombinant lipase activity (0, 5,000 and 10,000 U/kg, respectively) were blended with the basal diet to raise weaning piglets for six weeks. The results showed that both levels of recombinant lipase in diets enhanced piglets' growth performance compared to the control group fed without adding recombinant lipase. Moreover, postweaning piglets fed with complementing 10,000 U/kg recombinant lipase had a significant boost in TG concentration on the seventh day after weaning. However, the results deemed that adding recombinant lipase activity above 5,000 U/kg in diet can improve fat digestion and enhance the growth performance of weaning piglets.*

Keywords**:** Recombinant Pepsin, Pancreatic Colipase, Pancreatic Lipase, Weaning Piglets

# INTRODUCTION

The global climate changes vigorously. In addition, the crude oil price gradually grows to even greater heights, while global population growth and demand of the energy resources increase continuously. Given this situation, the time to find effective ways to solve this problem has become an important and popular issue worldwide. Owing to grain crops such as corn, the impetus to develop biofuels moved quickly over the past 10 years. This has led to a successive increase in the price of corn. The phenomenon has seriously threatened the domestic animal industry due to the fast increasing cost of feeding diets. To date the price of corn in Taiwan has increased more than two timesover the past 10 years. In recent years, depending on several research topics and scientific technologies, there has been a new trend showing the use of the fermentation process to improve digestion of low-nutrition by-product or recombinant enzymes as ingredient to reduce the cost of animal feeds, inclusive of corn, soybean meal and fish meal. For that reason, we predict that addition of some recombinant pepsin in diets may be an effective way to cope with feeding diet without containing fish meal for postweaning piglets.

The nutrients of sow's milk are suitable for a high efficient digestion of suckling pigs such as the apparent digestibility of the milk fat which can reach 96% (Cranwell *et al*., 1989). After weaning, the meal fat digestibility will decline to 65 to 80%, leading to an energy deficiency phenomenon in postweaning piglets (Cera *et al*., 1988). In addition, the weaning process can impact the physiological response of piglets, including the development of gastrointestinal tract which is not yet integrated. This can lead to the piglets' malnutrition owing to their intestines which can't digest and absorb feeds. The first point that needs to be noticed is the secretion and function of pancreatic digestive enzymes, which have not yet completely developed. Particularly, this refers to lipase and colipase (Jensen *et al*., 1997; Liu *et al*., 2008; Liu *et al*., 2010). Therefore, an addition of the recombinant lipase in diet may be an alternative way to improve fat digestion and growth performance of postweaning piglets.

## Production of recombinant pepsin, lipase and colipase by the yeast *Pichia pastoris*

The mature of 978-bp pepsin, 1347-bp lipase, and 285-bp cDNA fragments, respectively, was ligated into the *pichia pastoris* expression vector (Fig. 1). The yeast expression of recombinant pepsin, lipase and colipase, adopted yeast GS115 as host cells and transform those of secretion expressed cassettes by electroporation and get high copy number of pepsin, lipase and colipase of encoding region by adding zeocin$^{TM}$ antibiotic. Those integrated multiple copy of pepsin, lipase and colipase cDNA of yeast transformants were cultured in 50 ml YPD medium for more than 72 h, harvested their supernatants from culture medium by centrifuge, and surveyed their bioactivities. Extracellular secreted recombinant porcine pepsin, lipase and coliapse were fractionated by 4-12% Tris-Glycine gels with electrophoresis process. And then, recombinant proteins were transferred to PVDF membrane. A mouse Anti-His (C-term) monoclonal antibody served as primary antibody. Recombinant pepsin, lipase and colipase were detected by this primary antibody (1:5,000 dilution) and Anti-His (C-term)-HRP second antibody conjugated with horseradish peroxidase (1:10,000 dilution; invitrogen). The blot was developed with a chemiluminescence (ECL) detection system.

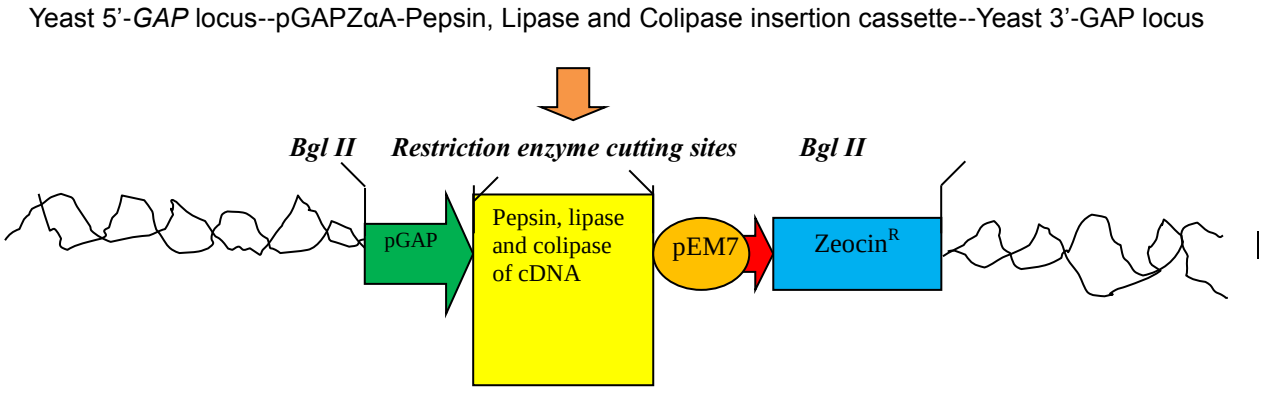


Fig 1. The secretion expressed cassette of recombinant pepsin, lipase and colipase with pGAP Forward and 3'-AOX primer set by *Pichia pastoris*

### Analysis of bioactivities of recombinant pepsin, lipase and colipase

The analytical method of the pepsin specific activity is as follows: using 0.1 N HCl to adjust pH value down to 2 in 100ul supernatant that contained recombinant pepsin, 0.5% fetal calf serum as substrate, reaction temperature at 37℃ incubator for 2 h, and addition of 0.5M TCA solution to stop reaction. The assay samples were collected by centrifuge at 12,092 × g for 20 min, and a supernatant was taken to survey the absorption value by Spectrophotometer at the wave length 280 nm (Qiao *et al.,* 2004). One unit of pepsin activity is defined as the absorption value equals 1.

Both of recombinant lipase and colipase activities were measured with titration at a pH from 6.5 to 8.0, permission 5 min at 25℃ with a pH-stat for equilibration. The standard assay procedure was determined according to the methods, which have been reported by Gaskin *et al*. (1982). One unit of lipase or colipase activities is defined as l μmol free butyric acid released from tributyrin in 1 min at 25°C.

### Scale up and preparation of recombinant pepsin, lipase and colipase to do feeding tests

The yeast transromants for pepsin, lipase and colipase were inoculated, respectively, in 10 liters of fermenter for more than 72 h, and the supernatants were harvested and condensed by 0.22 um filter and 3 kDa molecular weight of condenser, and then blended with 3% cornstarch and dried by dryer, frozen and stored at -70$^{o}$ freezer. After running activity analysis of pepsin, lipase and colipase, they were adopted to become feed additives and to examine their potential functions in the following experiments.

### Running animal feeding experiments

#### Application of recombinant pepsin to improve the digestion of dietary protein out of soybean meal and corn for postweanig piglets

In feeding experiment of weaning piglets with ages of 26 - 28 days and, a total of 24 heads, consisting of half males and half females which were adopted and assigned to 12 nursing pens. In each pen 1 male and 1 female piglets were raised depending on their bodyweight and gender. From the traditional creep-feed, there is always a fish meal to improve piglets' growth performance and it was to be the positive control group in this experiment. On the other hand, in the negative control group without fish meal the protein source consists of soybean meal and yellow corn. The group without fish meal but supplemented with 1,000 U/kg of pepsin activity in the diet was to be the experimental group (dietary formula shown in Table 1), amounting to 4 weeks during the trial period. In every animal body weight was weighed and feed intake of each animal raised in isolated pen was recorded weekly. Meanwhile, animal blood samples were drawn on the initial day and the last day of raising when immunoglobulin concentration was analyzed. After that we would apply those of datum served as monitoring indicator to examine the effects on the growth performance and the digestion of dietary protein out of soybean meal and corn (the whole plant protein) for postweaning piglets.

#### Application of recombinant lipase to improve the digestion of dietary fat for postweaning piglets

A total of 48 hybrid LYD 28-day piglets with the average body weight of about 7.80 kg from 7 littermates were selected for animal trials. The animals were weaned at 28 days of age and were randomly divided into 12 pens. The piglets were raised in isolated pens and each pen with a standard size at 4 $m^2$. The nutritional requirement for piglets in diets was according to the feeding standard of pigs in Taiwan (1990). The experimental diets were supplemented with 5,000 and 10,000 U/kg of recombinant lipase in the basal diet as the test groups and adding same volume of yeast supernatant without lipase in the basal diet for the control groups as shown in Table 2. During 6 weeks of the experimental period, animals were allowed *ad libitum* access to water and diets. Feed intakes, blood samples and body weight gains in each individual were recorded weekly. Blood samples were collected from venous sinus on days 1, 7, and 42 after trial started. As the plasma specimens were taken for measurement of TG concentration. Blood TG concentration was analyzed by using a TG diagnostic kit (Pointe Scientific Inc., Michigan, IA), and measurement procedure was according to the manufacturer's instruction for surveying the concentration of TG in blood plasma.

Table 1. Composition of diets for experimental postweaning pigletswith and without fish meal supplement and addition of recombinant pepsin

| Trial group<br>Items | With 5% fish meal<br>(positive control group) | Without fish meal<br>(negative control group) | Adding 1,000U/kg pepsin in without fish meal<br>(experimental group) |
|---|---|---|---|
| Ingredients, % | | | |
| Corn, yellow, kg (CP 7.5%) | 597.3 | 507.3 | 507.3 |
| Soybean meal, kg (CP 44%) | 200 | 290 | 290 |
| Dried skim milk, kg(CP35%) | 50 | 50 | 50 |
| Whey, dried, kg | 50 | 50 | 50 |
| Fish meal, kg (CP 61.2%) | 50 | - | - |
| Dicalcium phosphate, kg | 15 | 15 | 15 |
| Limestone , pulverized, kg | 10 | 10 | 10 |
| Salt, iodized, kg | 4 | 4 | 4 |
| Vitamin premix[a], kg | 1 | 1 | 1 |
| Mineral premix[b],kg | 1.5 | 1.5 | 1.5 |
| Soybean oil, kg | 20 | 20 | 20 |
| Corn starch, kg | - | 50 | 50 |
| Chloride-choline, kg (50%) | 1.2 | 1.2 | 1.2 |
| Total | 1,000 | 1,000 | 1,000 |
| Calculated values | | | |
| Crude protein , % | 18.89 | 18.69 | 18.69 |
| Digestible energy , kcal/kg | 3550 | 3450 | 3450 |
| Lysine , % | 1.19 | 1.16 | 1.16 |
| Calcium , % | 0.88 | 0.88 | 0.88 |
| Phosphorus , % | 0.69 | 0.69 | 0.69 |
| Analyzed value | | | |
| Dry matter ,% | 87.97 | 87.87 | 87.87 |
| Crude protein , % | 18.62 | 18.42 | 18.42 |
| Crude fat , % | 3.68 | 3.46 | 3.46 |

[a]Vitamin premix provided the following vitamins per kg of diet: vitamin A, 8000 IU; vitamin D3, 800 IU; vitamin E, 30 IU; vitamin K3, 1.0 mg; thiamin, 2.0 mg; riboflavin, 5.0 mg; vitamin B12, 25 μg; Capantothenate, 12 mg; niacin , 18 mg; folic acid, 0.4 mg; biotin , 0.06 mg and choline, 120 mg.
[b] Mineral permix 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.

Table 2. 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 | 0.80 |
| Salt, iodized | 0.5 |
| Vitamin premixa | 0.10 |
| Mineral premixb | 0.15 |
| Soybean oil | 1.00 |
| 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 D3, 800 IU; vitamin E, 30 IU; vitamin K3, 1.0 mg; thiamin, 2.0 mg; riboflavin, 5.0 mg; vitamin B12, 25 µg; Capantothenate, 12 mg; niacin , 18 mg; folic acid, 0.4 mg; biotin , 0.06 mg and choline, 120 mg.
[b] Mineral permix 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.

Application of recombinant colipase to improve the digestion of dietary fat for postweaning piglets

A total of 32 hybrid 28-days-old LYD piglets with an average body weight of 7.32 kg from 6 littermates were selected for the animal trials. The animals were weaned at 28 days of age and were randomly divided into eight pens. The experimental piglets were raised in isolated pens of nursery house and each unit reared four piglets (two males and two females). Each pen was a standard size of 4 $m^2$. The nutritional requirements for piglets were met according to the feeding standard for pigs in Taiwan (1990). The experimental diet for the test group included 5,000 U/kg of recombinant colipase while the same volume of yeast supernatant without colipase was added to the basal diet for the control group, dietary formula was the same as Table 2. During the 4 weeks of the experimental period, animals were allowed to access to food and water *ad libitum*. Blood samples were collected from venous sinus after a fasting period of 12 h in all experimental and control groups. Body weights were measured individually and food intakes in each isolated pen were also recorded weekly. Blood samples were collected from the venous sinus on Days 1, 7, and 28 after the start of the trial. Blood TG concentration was analyzed by using a TG diagnostic kit (Pointe Scientific, Canton, MI) according to the manufacturer's protocol.

**Growth performance, protein digestion, immunoglobulin concentration and blood traits after dietary administration of recombinant pepsin in postweaning piglets**

Yeasts transformant-integrated secretion expressed cassettes of recombinant pepsin were cultured into YPD culture medium for more than 72 h. The supernatant were harvested through centrifuge from culture medium, and ran analysis of pepsin activity. Among 9 yeast transformants could find their possession of capacity on expressing pepsin protein and their bioactivities range from 18 to 46 U/mg, as showed in Table 3. The yeast transromant No. 4-3 was selected and inoculated in 10 liters of fermenter for secreting recombinant pepsin in this study to examine effect on improving the whole plant protein of digestion for postweaning piglets.

In animal experiment, there were no significant differences on growth performance of experimental animals among groups (in Table 4), but body weight trended to be higher in piglets fed with diet supplemented with5% fish meal than those fed with diet without fish meal or without fish meal but supplemented with 1,000 U/kg pepsin during the initial 3 weeks. In the fourth week, piglets fed with diet without fish meal but with 1,000U/kg pepsin activity showed a higher body weight trend than those fed without fish meal, the level up to 0.7 kg/d. ADG of piglets tended to be higher when they were fed in the diet without fish meal but with pepsin than when fed without fish meal and pepsin. Piglets' feed intake in with or without fish meal diets tended to be higher than that in without fish meal but with pepsin diet during the whole experimental period. Piglets' feed efficiency in without fish meal but with pepsin diet also tended to be higher than that in with or without fish meal and pepsin diets. About piglets' immunoglobulin (Ig) content in blood samples, IgM concentration in piglets fed with fish meal had a higher level than those fed with both without fish meal and without fish meal but with pepsin **(**Table 5), however, concentrations of IgA and IgG were very close among piglets. The piglets' plasma nitrogen urea content in blood samples was significantly lower when they were fed in without fish meal and with pepsin diet than when they were fed in without fish meal and pepsin diet. In accordance with results from piglets' growth performance and immuneglobulin contents, it was revealed that supplementation of 1,000U/kg pepsin in diet without fish meal could improve postweaning piglets' growth performance and digestion of the whole plant dietary protein out of soybean and corn. Therefore, we may deem that when the diet without fish meal is served as creep-feed for postweaning piglets, it is recommend to add 1,000U/kg of recombinant pepsin activity in the diet with soybean and corn to maintain their growth performance; this creep-feed can reduce feed cost due to without containing the high price fish meal (Neil and Alan 1993; Pearson *et al*., 1986).

Table 3. The recombinant pepsin of activity analysis from yeast transformants

| Yeast Transformant No. | Specific activity (U*/100μL) | Yeast Transformant No. | Specific activity (U/100μL) |
|---|---|---|---|
| 1-1 | 4.6 | 3-1 | 4.8 |
| 1-2 | 5.8 | 3-2 | 5.8 |
| 1-3 | 5.4 | 3-3 | 4.6 |
| 2-1 | 5.1 | 4-1 | 4.5 |
| 2-2 | 4.9 | 4-2 | 5.6 |
| 2-3 | 5.4 | 4-3 | 11.3 |

*One unit of pepsin activity is defined as when the absorption value equals 1.

Table 4. Growth performance of postweaning piglets after administration of recombinant pepsin in the diet

| Trial group<br>Items | With 5% fish meal (positive control group) | Without fish meal (negative control group) | Adding 1,000U/kg pepsin in without fish meal (experimental group) |
|---|---|---|---|
| Body weight, kg | | | |
| Day 1 postweaning | 7.93 ± 0.89 | 7.86 ± 1.05 | 7.89 ± 0.96 |
| Day 7postweaning | 9.13 ± 0.94 | 8.84 ± 1.09 | 9.0 ± 1.10 |
| Day 14postweaning | 11.74 ± 0.83 | 11.36 ± 1.21 | 11.56 ±1.12 |
| Day 21 postweaning | 15.16 ± 0.86 | 14.61 ± 1.62 | 14.98 ± 1.17 |
| Fay 28 postweaning | 19.58 ± 0.78 | 18.95 ± 1.78 | 19.65 ± 1.32 |
| **Growth performance from 1 to 28 postweaning day** | | | |
| Average daily gain, kg/d | | | |
| Day 1-7 postweaning | 0.17 ± 0.05 | 0.14 ± 0.04 | 0.16 ± 0.07 |
| Day 8-14 postweaning | 0.37 ± 0.08 | 0.36 ± 0.04 | 0.37 ± 0.07 |
| Day15-21postweaning | 0.49 ± 0.11 | 0.46 ± 0.09 | 0.49 ± 0.08 |
| Day 22-28 postweaning | 0.63 ± 0.09 | 0.62 ± 0.10 | 0.67 ± 0.10 |
| Day 1-28 postweaning | 0.42 ± 0.04 | 0.40 ± 0.03 | 0.42 ± 0.04 |
| Average daily feed intake kg/d | | | |
| Day 1-7 postweaning | 0.18 ± 0.07 | 0.18 ± 0.07 | 0.15 ± 0.03 |
| Day 8-14 postweaning | 0.54 ± 0.04 | 0.56 ± 0.11 | 0.51 ± 0.06 |
| Day15-21postweaning | 0.77 ± 0.12 | 0.71 ± 0.07 | 0.68 ± 0.05 |
| Day 22-28 postweaning | 1.34 ± 0.15 | 1.37 ± 0.18 | 1.36 ± 0.10 |
| Day 1-28 postweaning | 0.71 ± 0.07 | 0.71 ± 0.09 | 0.67 ± 0.05 |
| Feed efficiency (Feed/gain) | | | |
| Day 1-7 postweaning | 1.17± 0.50 | 1.31 ± 0.32 | 1.03 ± 0.49 |
| Day 8-14 postweaning | 1.50 ± 0.28 | 1.56 ± 0.37 | 1.40 ± 0.21 |
| Day15-21postweaning | 1.62 ± 0.35 | 1.56 ± 0.25 | 1.41 ± 0.25 |
| Day 22-28 postweaning | 2.13 ± 0.12 | 2.23 ± 0.24 | 2.05 ± 0.10 |
| Day 1-28 postweaning | 1.60 ± 0.16 | 1.67 ± 0.14 | 1.47 ± 0.10 |

Table 5. Immunoglobulin concentration and blood traits of postweaning piglets after dietary administration of recombinant pepsin in the diet

| Trial group<br>Items | With 5% fish meal (positive control group) | Without fish meal (negative control group) | Adding 1,000U/kg pepsin in without fish meal (experimental group) |
|---|---|---|---|
| IgA $\times 10^6$, ng/mL | 0.43 ± 0.20* | 0.49 ± 0.15 | 0.51 ± 0.13 |
| IgM $\times 10^6$, ng/mL | 2.14 ± 0.24[a] | 1.74 ± 0.16[b] | 1.76 ± 0.23[b] |
| IgG $\times 10^6$, ng/mL | 4.66 ± 0.51 | 4.55 ± 0.88 | 4.68 ± 0.72 |
| Total potein, g/dL | 5.47 ± 1.90 | 5.39 ± 0.14 | 5.19 ± 0.14 |
| Creatinine, g/dL | 1.54 ± 0.15 | 1.38 ± 0.06 | 1.48 ± 0.06 |
| Plasma nitrogen urea, g/dL | 12.53 ± 3.51[ab] | 14.88 ± 1.51[a] | ± 1.51[b] |

* Mean ± SD.

[a,b] Within the same rows with the different superscripts represent significantly ($P<0.05$).

## Growth performance and blood traits after dietary administration of recombinant lipase in postweaning piglets

The activities of recombinant porcine colipase were 102.2, 180.3, 300.4, 305.8 U/300µl of clone No. 26 yeast cultures at the time-course of 24, 48, 72, 96 h post-culture, respectively. However, there is no lipase activity observed in the supernatant of native GS115 yeast cells. Western blot and titration assay demonstrated that the recombinant lipase was secreted successfully into yeast culture medium and it remained a constant activity for the three-day's culture period in the examined samples. Therefore, the time-course assay indicated that the recombinant protein of lipase exhibited a relative stability using this culture condition. Following an animal trial, the yeast culture medium of recombinant clone No. 26 was harvested at 72 h to get the maximum recombinant lipase protein yields to be used as the source of supplementary porcine lipase. The growth rate of postweaning piglets fed with a diet administrated with 5,000 and 10,000 U/kg of recombinant lipase derived from yeast culture (the lipase administrated test groups) was monitored and compared with that of age-matched non-recombinant yeast culture fed control group. The lipase administrated test groups (n=16) had significantly heavier bodyweight than those of the control group when measured at Day 7, 14, 21, 28, 35and 42 of postweaning, but had no difference between blended 5000 and 10000 U/kg of recombinant lipase groups as shown in Table 6. ADG of piglets in the lipase administrated test group showed a significant difference from control group when measured at 1, 2, 3, 4, 5 and 6 wk of after weaning; however, both lipase administrated test groups had no significant difference in ADG. The feed intake of piglets in test groups was significantly higher than that in the control group when measured at 1 and 2 wk of postweaning. The feed efficiency (feed intake/body weight gain) of piglets in the test and control groups had no significant difference. During the overall period, the growth performance (bodyweight, feed intake and feed efficiency) of piglets in the test groups exhibited superior to that of control group. To assay the biological activity of recombinant lipase fed with postweaning piglets, blood TG concentration was measured for monitoring the efficiency of dietary fat digestion. Blood samples were collected from all test and control piglets on days 1, 7, and 42 during the period of animal trial. The level of blood TG concentration had significantly increased in experimental test group fed with additional 10,000 U/kg body weight of recombinant lipase on the 7 day of postweaning when comparing with that of the control group as shown in Table 7. But the addition of 5000 U/kg of recombinant lipase and 10,000 U/kg of recombinant lipase had no effects on TG concentration in the full period. Therefore, in accordance to the previous results it was suggested that addition of 10,000 U/kg of recombinant lipase in the diet will have a better fat digestibility of postweaning piglets.

## Growth performance and blood traits after dietary administration of recombinant colipase in postweaning piglets

Recombinant porcine colipase activity in supernatant from clone No. 2-5 was 126, 130, 190, and 198 U/300 lL at 24, 48, 72, and 96 h of culture, respectively. However, there was no colipase activity in the supernatant of native GS115 yeast cells. Western blot and titration assays demonstrated that the recombinant colipase was secreted successfully into yeast culture medium and retained a constant level of activity throughout the 4-day culture period. Therefore, the time course assay indicated that the recombinant colipase protein exhibited relative stability in this culture condition. For the animal trials, the yeast culture medium of recombinant clone No. 2-5 was harvested at 72 h for the maximum recombinant colipase protein yields to be used as the source of supplementary porcine colipase.

Table 6. The effect of adding recombinant lipase in the diet on the growth performance of postweaning Piglets

| Trial group | The level of adding recombinant lipase, units/kg | | | |
|---|---|---|---|---|
| Items | 0 | 5000 | 10000 | MSE |
| Body weight, kg | | | | |
| 1 d postweaning | 7.84 | 7.84 | 7.72 | 0.061 |
| 7 d postweaning | 8.67[b] | 9.36[a] | 9.36[a] | 0.106 |
| 14 d postweaning | 10.27[b] | 11.80[a] | 11.92[a] | 0.085 |
| 21 d postweaning | 12.53[b] | 14.93[a] | 15.21[a] | 0.051 |
| 28 d postweaning | 15.72[b] | 19.04[a] | 19.22[a] | 0.102 |
| Average daily gain, kg/d (ADG) | | | | |
| 1-7 d postweaning | 0.12[b] | 0.22[a] | 0.23[a] | 0.008 |
| 8-14 d postweaning | 0.23[b] | 0.35[a] | 0.37[a] | 0.010 |
| 15-21 d postweaning | 0.32[b] | 0.45[a] | 0.47[a] | 0.009 |
| 22-28 d postweaning | 0.46[b] | 0.59[a] | 0.57[a] | 0.013 |
| 29-35 d postweaning | 0.52[b] | 0.62[a] | 0.65[a] | 0.016 |
| 36-42 d postweaning | 0.68[b] | 0.78[ab] | 0.82[a] | 0.020 |
| Average daily feed intake, kg/d (ADFI) | | | | |
| 1-7 d postweaning | 0.20[b] | 0.34[a] | 0.32[a] | 0.017 |
| 8-14 d postweaning | 0.42[b] | 0.55[a] | 0.54[a] | 0.016 |
| 15-21 dpostweaning | 0.62 | 0.77 | 0.75 | 0.029 |
| 22-28 d postweaning | 0.82 | 0.95 | 0.92 | 0.025 |
| 29-35 d postweaning | 1.03 | 1.05 | 1.11 | 0.019 |
| 36-42 d postweaning | 1.32[b] | 1.34[ab] | 1.43[a] | 0.022 |
| Feed efficiency (Feed/gain) (FE) | | | | |
| 1-7 d postweaning | 1.80 | 1.58 | 1.39 | 0.107 |
| 8-14 d postweaning | 1.87 | 1.58 | 1.48 | 0.076 |
| 15-21 d postweaning | 1.94 | 1.73 | 1.61 | 0.091 |
| 22-28 d postweaning | 1.82 | 1.63 | 1.61 | 0.071 |
| 29-35 d postweaning | 1.97 | 1.69 | 1.71 | 0.052 |
| 36-42 d postweaning | 1.93 | 1.72 | 1.75 | 0.046 |
| Overall period (1-42 d postweaning) | | | | |
| ADG, kg/d | 0.39[b] | 0.50[a] | 0.52[a] | 0.002 |
| ADFI, kg/d | 0.73[b] | 0.83[a] | 0.85[a] | 0.008 |
| FE (Feed /gain) | 1.89[a] | 1.67[b] | 1.63[b] | 0.01 |

[a,b] Within the same rows the different superscripts represent significant difference ($P<0.05$).

Table 7. The effect of adding recombinant lipase in the diet on the blood traits of postweaning piglets

| Trial group | The level of adding recombinant lipase, units/kg | | | |
|---|---|---|---|---|
| Items | 0 | 5000 | 10000 | MSE |
| Triglyceride, mg/Dl | | | | |
| 1 d postweaning | 33.0 | 25.5 | 29.4 | 2.1 |
| 7 d postweaning | 19.8[b] | 25.3[ab] | 30.9[a] | 2.0 |
| 42 d postweaning | 54.3 | 53.3 | 55.5 | 6.3 |
| BUN, mg/dL | | | | |
| 1 d postweaning | 11.4 | 10.7 | 11.4 | 0.6 |
| 7 d postweaning | 13.4 | 13.0 | 15.7 | 0.8 |
| 42 d postweaning | 11.2 | 11.8 | 12.5 | 1.0 |

[a,b] Within the same rows the different superscripts represent significant difference (P<0.05).
BUN is the abbreviation of plasma urinary nitrogen.
MSE is the abbreviation of mean standard error.

The growth rate of postweaning piglets receiving a diet with 5,000 U/kg body weight of recombinant colipase derived from yeast culture was monitored and compared with that of age-matched nonrecombinant yeast culture fed controls. The colipase administered test group (n= 8) gained significantly more weight than piglets in the control group when measured at Day 15 (2nd week of postweaning; 11.84 ± 0.70 *vs*. 10.59 ± 0.39 kg, P<0.05), Day 22 (3rd week of postweaning; 15.84 ± 0.95 *vs*. 14.32 ± 0.59 kg, P<0.01), and Day 28 (4th week of postweaning; 20.19 ± 1.47 *vs*. 18.54± 0.92 kg, P<0.01) as shown in Table 8**.** Moreover, there were no significant differences in the ADG, average daily feed intake (ADFI), and feed efficiency between the control group (no addition of recombinant colipase) and test group (added 5,000 U/kg of recombinant colipase). To assay the biological activity of recombinant colipase fed to postweaning piglets, TG concentration was used to monitor the efficiency of dietary fat digestion. Blood samples were collected from all test and control piglets on Days 1, 7, and 28 during the period under investigation. The blood TG levels significantly increased in the experimental test group on the 28th day of postweaning compared with that of the control group (32.50 *vs*. 16.37 mg/dL; P<0.0001) as shown in Table 9.

Table 8. The Effect of recombinant porcine colipase as feed additive by oral administration in growth performance of postweaning piglets (n=8)

| Trial group | Added Recombinant Colipase Level | | |
|---|---|---|---|
| Items | 0 | 5,000(U/kg) | P- Value |
| Body weight (kg) | | | |
| Day 1 postweaning | 7.37 ± 0.16 | 7.28 ± 0.09 | 0.86 |
| Day 8 postweaning | 8.06 ± 0.23 | 8.49 ± 0.38 | 0.50 |
| Day 15 postweaning | 10.59 ± 0.39 | 11.84 ± 0.70 | 0.02* |
| Day 22 postweaning | 14.32 ± 0.59 | 15.84 ± 0.95 | 0.005** |
| Day 28 postweaning | 18.54 ± 0.92 | 20.19 ± 1.47 | 0.003 |
| Growth performance from 1 to 28 postweaning day | | | |
| ADG, kg[a] | 0.40 ± 0.04 | 0.46 ± 0.08 | 0.174 |
| ADFI, kg[b] | 0.77 ± 0.08 | 0.86 ± 0.13 | 0.386 |
| Feed/gain weight | 1.93 ± 0.17 | 1.87 ± 0.34 | 0.570 |

[a]ADG is abbreviated from average daily gain. [b]ADFI is abbreviated from average daily feed intake. * P<0.05; ** P<0.01.

Table 9. Triglyceride Concentrations in Blood from Postweaning Piglets Fed a Basal Diet with and without Added Recombinant Colipase (n=32)

| Trial Group Items | Added Recombinant Colipase Level | | | |
|---|---|---|---|---|
| | w/o Colipase P-value[a] (0 U/kg) | w/Colipase[b] (5,000 U/kg) | MSE[c] | P- Value |
| 1st day of postweaning | 15.71 | 12.82 | 1.72 | 0.49 |
| 7th day of postweaning | 7.59 | 11.98 | 0.91 | 0.21 |
| 28th day of postweaning | 16.37 | 32.50 | 2.95 | <0.0001** |

[a] Native yeast culture without (w/o) recombinant colipase protein was added to the diet of the control group.
[b] Transformaned yeast culture with (w/colipase) recombinant colipase protein was added to the diet of the test group (5,000 U/kg of feed).
[c] MSE: mean of standard error.
** P<0.01.

## CONCLUSION

When postweaning piglets are raised with the whole plant protein (soybean and corn) diet, it has detrimental effect on their growth performances. The addition of appropriate activity of recombinant pepsin in the diet may be an alternate approach to maintaining normal growth situation of postweaning piglets.

These experimental data showed that the use of recombinant porcine lipase or colipase as a dietary supplement provided an alternative approach for improving fat digestion and enhancing growth performance in postweaning piglets. Therefore, we suggest that feeding with colipase-enriched yeast cultures is a convenient method to elevate dietary fat digestion and absorption and to promote the growth performance of postweaning piglets. It allows the large-scale use of yeast culture containing enriched recombinant lipase or colipase in the pig farming industry.

## REFERENCES

Council of Agriculture. 1990. The feeding standard of pigs (Taiwan). Pig Research Institute, Taiwan.

Cera, K. R., D. C. Mahan, and G. A Reinhart. 1988. Weekly digestibilities of diets supplemented with corn oil, lard or tallow by weanling swine. *J. Anim. Sci*., 66: 1430 - 1437

Cranwell, P. D., P. J. Moughan. 1989. 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, pp 140 - 165.

Gaskin, K. J., P. R. Durie, R. E. Hill, L. M. Lee, and G. G. Forstner. 1982. Colipase and maximally activated pancreatic lipase in normal subjects and patients with steatorrhea. *J. Clin. Invest*. 69(2): 427 - 434.

Hedemann, M. S., A. R. Pedersen, and R. M. Engberg. 2001. Exocrine pancreatic secretion is stimulated in piglets fed fish oil compared with those fed coconut oil or lard. J. *Nutri*. 131(12) : 3222 - 3226

Huguet, A., G. Savary, E. Bobillier, Y. Lebreton, and I. Le Huerou-Luron. 2006. Effects of level of feed intake on pancreatic expcrine secretions during the early postweaning period in piglet. *J. Anim. Sci.* 84: 2965 - 2972.

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

Liu, F. C., H. L. Chen, K. Y. Chong, A. L. Hsu, and C. M. Chen. 2008. Production of
recombinant porcine colipase secreted by *pichia pastoris* and its application to improve dietary fat digestion and growth of postweaning piglets. Biotech. Prog. 24: 1333 - 1341.

Liu, F. C., H. L. Chen, W. Lin, Y. T. Tung, C. W. Lai, A. L. Hsu, and C. M. Chen. 2010. Application of porcine lipase secreted by pichia pastoris to improve fat digestion and growth performance of postweaning piglets. *J. Agric. Food Chem.* 58(6): 3322 - 3329.

Neil, D. R. and J. B. Alan. 1993. Evolutionary families of peptidases. *Biochem. J.* 290: 205 - 218.

Pearson, J. P., R. Ward, A. Allen, N. B. Roberts and W. H. Taylor. 1986. Mucus degradation by pepsin: comparison of mucolytic activity of human pepsin 1 and pepsin 3: implications in peptic ulceration. Gut 27(3): 243 - 248.

Qiao, Y., X. Lin, J. Odle , A. Whittaker and T. A. van Kempen. 2004. Refining in vitro digestibility assays: fractionation of digestible and indigestible peptides. *J Anim. Sci.* 82(6): 1669 - 1677.

SAS Institute. 2003. Statistics User Guide. Version 9.1. SAS Inst., Inc., Cary, NC.