CHAPTER III

MATERIALS AND METHODS

This present study had 2 trials. The first trial was to determine mRNA expressions of amino acid transport systems; L and heterodimer (LAT2 and 4F2hc), system A (ATA2), system $B^{0,+}$ ($ATB^{0,+}$) and system y^+ (CAT2B) in lactating mammary tissues. The second trial was to compare the quantity of mRNA expressions of amino acid transporters in sows fed with deficient protein diet (8.2% CP) and normal protein diet (18.2% CP) in parallel with growth performance, plasma amino acid concentrations and physical and histological changes in sow's mammary gland at lactating period.

Experimental design and animals

Animal procedures used in this study were approved by the Animal Care and Use committee, Faculty of Veterinary Science, Chulalongkorn University (No. 27/2549).

Eleven multiparous crossbred lactating sows were randomly selected from the herd at the veterinary student training center, unit of animal husbandry department for the study based on their genetic backgrounds, mammary gland conformation and farrowing day. Sows were moved to farrowing crates 2 weeks prior to farrow and kept individually in farrowing crates. After farrowing (farrowing day = d 0), piglets were cross-fostered as soon as possible after birth to achieve equal number of piglet per sow. Dead piglets showing evidence of delayed growth would be assumed to have died from starvation and consequently would not replaced, because this indicated a nonfunctional gland.

Sows were randomly assigned to one of two dietary treatments groups, either a deficiency (8.2 %CP) protein or normal (18.2 %CP) protein diet (Table 3.1). Feeding of the experimental diets was started after farrowing. They were fed four times per day at 4 kg per sow. Throughout lactation, sows and piglets had free access drinking water at all

time from nipple drinkers. Their piglets were received sow's milk throughout lactation and given a starter diet (creep feed) ad libitum from d 18 of lactation until weaned day on d 28 of lactation.

Sows were weighed and recorded on d 1 postpartum, d 14 of lactation, and d 28 of lactation. In addition, all piglets were individually weighed and recorded on d 1 postpartum and every three days until they were weaned. Feed intake of sows and piglets was recorded daily during d 0 until d 28 and during d 18 until d 28 of lactation, respectively.

The description of experimental management was shown in Figure 3.1.

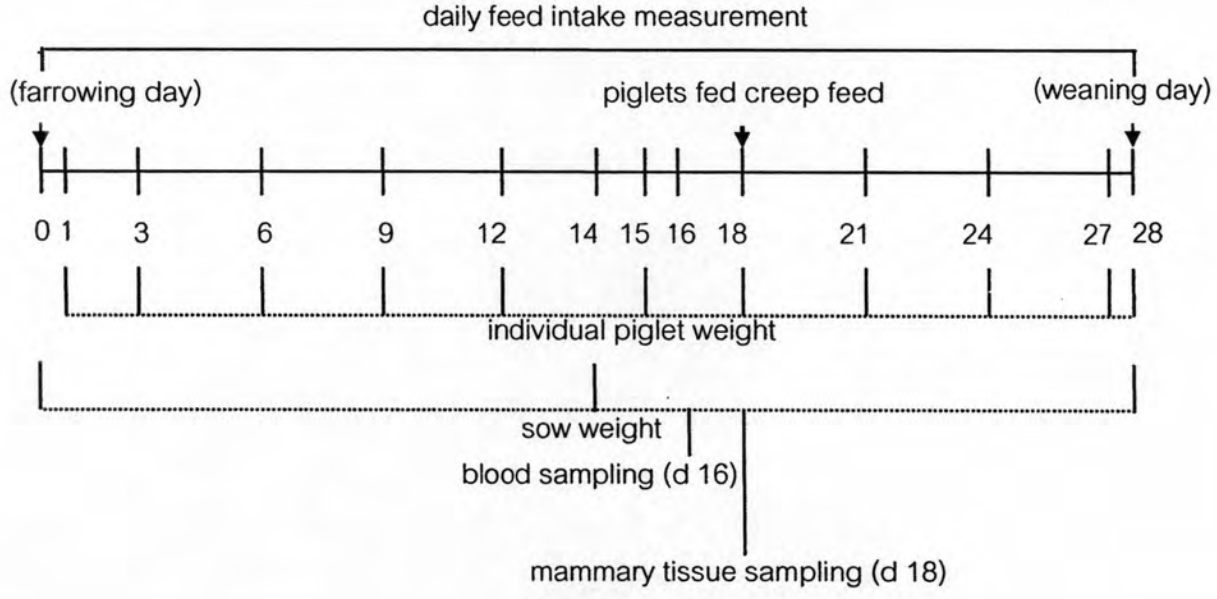


Figure 3.1 Experimental management chart

Diets

Throughout lactation, the two experimental diet groups were differed in the crude protein (CP); normal protein diet 18.2% CP (NRC, 1998) and deficient protein diet 8.2% CP (Guan et al., 2004a). However, both diets contained similar energy, mineral, vitamin and other nutrient compositions which met recommendations of NRC (1998).

The calculated metabolizable energy of both experimental diets was 3.2 ME, Mcal/kg. Nutrient and amino acid compositions of the diets are shown in Table 3.1, 3.2, and 3.3.

Table 3.1 Ingredient composition of the experimental diets, as fed basis

Ingredients	Dietary CP, %	
	8.2 (deficiency)	18.2 (control)
Broken rice	20.00	18.00
Corn	15.00	17.00
Cassava	25.00	10.00
Palm oil	3.35	3.00
Fatpack	1.60	2.00
Raw rice bran	18.50	7.00
Rice solvent bran	10.00	10.00
Soybean 48% CP	0.70	21.00
Full fat soybean meal	1.00	7.00
L-lysine	0.35	0.30
DL-methionine	0.10	0.30
Calcium carbonate	1.50	1.50
Monocalcium phosphate (P_{21})	2.30	2.30
Salt	0.35	0.35
Trace mineral and vitamin premix [1]	0.25	0.25
Total	100.00	100.00

[1] *Each kilogram of the trace minerals and vitamins contained the following: vitamin A, 6.0 IU; vitamin D_3, 0.8 IU; vitamin E, 12,000 IU; vitamin K_3, 0.8 g; vitamin B_1, 0.6 g; vitamin B_2, 1.4 g; vitamin B_6, 0.8 g; vitamin B_{12}, 0.008 g; pantothenic acid, 6.0 g; niacin, 8.0 g; folic acid, 0.12 g; biotin, 0.12 g; choline, 120.0 g; selenium, 0.12 g; iron, 40.0 g; manganese, 12.0 g; zinc, 24.0 g; copper, 8.0 g; cobalt, 0.08 g; iodine, 0.20 g.*

Table 3.2 Calculated values of nutrient composition in the experimental diets, as dry matter basis

Nutrient composition	Dietary CP, %	
	8.2	18.2
ME, kcal/kg	3.25	3.22
Crude protein, %	8.15	18.24
Fat, %	8.98	8.47
Fiber, %	4.59	4.47
Calcium, %	1.05	1.08
Total phosphorous, %	0.47	0.48
Lysine, %	0.58	1.18
Methionine + cysteine, %	0.35	0.85
Methionine, %	0.25	0.58
Threonine, %	0.25	0.66
Tryptophan, %	0.70	0.22

Sample sampling

Blood sampling

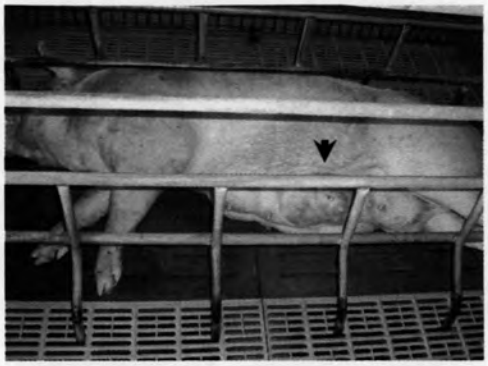
Blood samples obtained from each sow and her randomly selected 4 piglets per litter were collected on d 16 of lactation. Sows were fed 4 h before blood sampling. Blood samples of sows and their piglets were collected via anterior vena cava puncture, and put into heparinized tubes. Approximately 4 ml and 1 ml of blood were collected per sow and piglet, respectively; blood samples of piglets were pooled together. All blood samples were kept on ice until centrifugation. Plasma was separated by centrifugation at 3,000xg for 15 min at 4°C, then transferred to new tube and stored at -20°C until analysis of amino acid concentration according to method documented by Reverter et al. (1997).

Moreover, on d 16 of lactation, two sow's blood samples from each experimental group were randomly selected for analysis of hematological profile; Red blood cells (R.B.C.), hemoglobin, hematocrit, White blood cells (W.B.C.) (differential W.B.C.: neutrophils, eosinophils, lymphocytes, monocytes), and blood parasite. Then hematological values were used as an indicator of sow's health.

Collection of mammary tissues

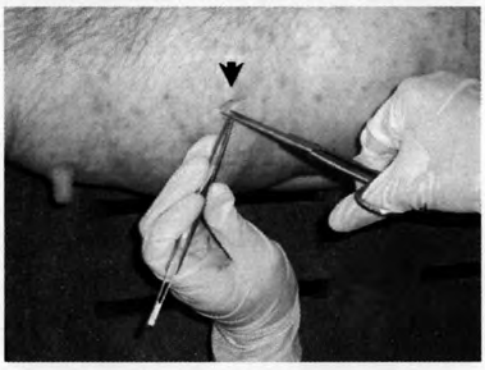
Mammary tissue was obtained by incisional biopsy in the five anterior glands of the udder that were usually suckled throughout lactation. The lactating sows on d 18 of lactation were anesthetized by intramuscular injection of Azaperone (4 mg/kg body weight; Stresnil®; JANSSEN Pharmaceutical N.V., Belgium). The selected functional glands were sampled by surgical technique. Before the incision biopsy, the udders were washed, disinfected with ethanol, iodine, and locally infused by 2% xylocaine (OLIC, Thailand) at the incision site. The incision site was made approximately midway (4 to 5 cm) between the teat and the upper line of the udder. Care was taken not to perforate blood vessels visible on the gland. The incision was continued through the subcutaneous tissues and fascia layers to expose the underlying mammary tissues. Approximately 15-mm elliptical incision at a depth of 5-10 mm, mammary tissues (1-5 g) were collected and immediately placed in RNAlater tissue protection kit® (Qiagen, Hilden, Germany) to prevent RNA degradation. In addition, approximately 0.5-1 g of the mammary tissues collected were stored in 10% buffer formalin for later histological evolution. The subcutaneous tissue and skin were sutured with coated Vicryl®; Ethicon Division of Johnson & Johnson, NSW, Australia). An antibiotic was administered immediately after surgery and during post-care. The surgical wounds were cleaned and disinfected with alcohol and iodine throughout post-care period.

Finally, the samples of mammary tissues kept in RNAlater tissue protection were stored at -20°C for later determination of gene expression and samples kept in 10% buffer formalin were stored at room temperature for later determination of histological changes. The picture of mammary biopsy is provided in Figure 3.2.



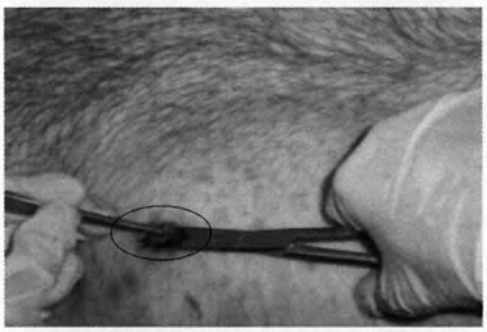
1. The lactating sow was anesthetized by intramuscular injection of Stresnil® and the mammary glands were cleaned up for surgical preparation.

Arrow indicates site of incision.



2. The 15-mm incision was made approximately 4 to 5 cm above the plica lateralis in the arterior part of mammary glands.

Arrow indicates that the fat and fascia layers were dissected.



3. Mammary tissues were collected (1-5 g) at a depth of 5-10 mm.

4. Mammary tissue sample was immediately placed in RNAlater tissue protection kit®.

5. Samples were immediately placed on ice and were then stored at -20°C.

6. The subcutaneous and skin layers were sutured with standard surgical procedure.

Figure 3.2 Procedure of the porcine mammary tissue sampling

Chemical analysis and composition

Diets

Feed samples of two experimental groups were randomly collected and used as a representative for each group. Nutrient composition of both experimental diets was analyzed by proximate analysis method (AOAC, 1990). Amino acid concentrations in feed samples were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) method as described by Reverter et al. (1997). Chemical analysis was conducted in duplicate.

Plasma

Plasma samples obtained from sows and piglets were diluted with HCl (0.1 *M*) and deproteinized by addition of 30% cold sulphosalicilic acid. The treated plasma samples were centrifuged at 2,500xg at 4°C for 30 min. Plasma amino acid

concentrations were determined on each sample in duplicate by RP-HPLC at the Central Instrument Facility (Mahidol University, Thailand) as described by Reverter et al (1997).

Histological procedure and determination

Sow mammary tissues designated for histological examination were fixed in buffered neutral formalin for 48 hours. Subsequently, samples were dehydrated and embedded in paraffin. After sections of tissue samples (4-6 µm) were made, thick sections cut on a microtome were stained with Harris' Alum Hematoxylin and counter-stained with eosin to confirm the presence of mammary epithelial tissues. Histological photomicrographs were taken with microscope at X 40, X 100, and X 200 magnification (Kensinger et al., 1982).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis for determination of amino acid transporter gene expressions

Isolations of total RNA

Isolations of total RNA were extracted from mammary tissue with Aurum™ Total RNA Fatty and Fibrous Tissue Kit (BioRad, Hercules, USA) in accordance with the manufacturer's instruction. The small chunk of the frozen mammary tissues (up to 100 mg) was transferred into 1 ml of PureZOL reagent and immediately disrupted and homogenized by passing the tissue samples through an 18-gauge needle and syringe until homogeneous. Once the samples were completely disrupted in PureZOL, then lysate were incubated at room temperature for 5 min to allow the complete dissociation of protein complexes. Chloroform (2.0 ml) was added to the lysate, mixed for 15 sec, incubated for 5 min at room temperature, and centrifuged at 12,000 g for 15 min at 4°C to separate the organic and the aqueous phases. Then the aqueous phase, contained the RNAs, was immediately transferred to 2.0 ml microcentrifuge tube and equal amount of 70% ethanol was added to this tube. The sample was then passed through a silica membrane packed in the Aurum RNA binding mini column, where nucleic acids were bound. The membrane in Aurum RNA binding mini column selectively bound to mRNA

and larger rRNAs, while small RNA molecule (less than 200 nucleotides) such as tRNA, was removed. Wash steps were performed to removed proteins and other cellular debris. The RNA was eluted with elution solution.

Reverse transcription and the first strand cDNA synthesis

Total RNA samples were reverse transcribed into cDNA (complementary DNA) using random-primer. This reverse transcription step was performed by using iScript™ Select cDNA Synthesis kit (BioRad, Hercules, USA). A 20-μl of reverse transcription reaction mixture, containing 10 mM random-primer, 1 pg to 1μg total RNA sample and 11 μl nuclease-free water, was sequentially incubated at 65°C for 5 min, at 25°C for 5 min. 5x iScript selected reaction mix (containing dNTPs, magnesium chloride, and stabilizers) and iScript reverse transcriptase were added for 4 μl and 1 μl in each reaction and incubated sequentially at 25°C for 5 min, at 42°C for 30 min, at 85°C for 5 min, and then cooled on ice. Finally, the synthesized cDNA products were used for RT–PCR.

Polymerase chain reaction (PCR)

The synthesized cDNA products were used for RT–PCR, using IQ™ Supermix kit (BioRad, Hercules, USA). The PCR amplification in a 50-μl reaction mixture composed of 25 μl IQ™ Supermix (containing 100 mM KCl, 40 mM Tris-HCl, 1.6 mM dNTPs, iTaq DNA polymerase, 50 units/ml, 6 mM $MgCl_2$, and stabilizers), 0.5 μl forward and backward primers, autoclaved ultra pure water 22 μl, and 2 μl cDNA template. The PCR primers were designed from the published human 4F2hc, human LAT2, rat ATA2, human CAT2B, human $ATB^{0,+}$, pig 18S rRNA, and human CAT2A. The primer pairs used in the subsequent PCR and the expected size of the PCR products were summarized in Table 3.3. The PCR conditions were: initial step at 95°C for 3 min, amplification step at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min (40 cycles for amino acid transporters and 30 cycles for 18S rRNA), and then followed by stop reaction at 72°C for 10 min. The PCR products were subjected to electrophoreses to size-fractionate on a

1.8% agarose gels in Tris Acetic acid EDTA (TAE) buffer for 67 min at 70 voltage. The PCR products were loaded for 18 μl / well. DNA bands were visualized with ethidium bromide staining. A single band of each target gene was observed at expected size. The nucleotide sequences of the PCR fragments were identified and sequenced with an automated ABI Prism 377 DNA sequencer (Perkin-Elmer Corp). The CAT2A and 18S rRNA were used as negative control and positive control, respectively.

Table 3.3 Primer sequence used in region PCR amplification for detecting transcripts of porcine 4F2hc, LAT2, ATA2, CAT2B, $ATB^{0,+}$, 18S rRNA, and CAT2A mRNA expression in lactating mammary tissues.

Gene	Primer pair 5' ⟶ 3'		GenBank accession no.	Expected size (bp)
4F2hc	CACAAGAACCAGAAGGATGA	(F)	J02939	183
	AGTGTCAACCTGAGTGGAGA	(R)		
LAT2	TTTCCAGGAACCTGACATCG	(F)	AF171669	200
	ACATTGCAGTGACATAAGCG	(R)		
ATA2	AATCTGACCAATGCGATTGTG	(F)	BC040342	164
	AATAAAGACCCTCCTTCGTTG	(R)		
CAT2B	CCCAATGCCTCGTGTAATCT	(F)	U76369	121
	TGCCACTGCACCCGATGATAAAGT	(R)		
$ATB^{0,+}$	GGTGGTCCATTTTGGTCCATAT	(F)	AF151978	86
	GTGATCGTTTCAATCGAAGCAA	(R)		
18S rRNA	CCGCGGTTCTATTTTGTTGGTTTT	(F)	AF102857	399
	CGGGCCGGGTGAGGTTTC	(R)		
CAT2A	CAAGACGGGGTCTGCATATT	(F)	U76368	368
	TGCCACATTTCCTTTCACAA	(R)		

Semiquantitative reverse transcription-polymerase chain reaction for quantitative determination of amino acid transporter genes

The relative abundance of amino acid transporter mRNA was determined by semiquantitative RT-PCR using IQ™ Supermix kit. In multiplexed PCR, 18S rRNA was used as an internal control that was amplified with target gene in multiplexed PCR reaction. Total RNA samples were reverse transcribed into cDNA using random-primer. The cDNA product concentration of each sample, which were diluted to 140 time (consisted of 5 µl of concentrated cDNA and 695 µl of autoclaved ultra pure water), was then measured the concentration with SPECTRONIC® GENESYS™ spectrophotometer (Spectronic Instruments, INC,. N.Y.) at a wavelength of 260 nm (in the UV spectrum). DNA concentration was calculated by using the equation (Birren et al., 1997) as shown below.

$$\text{DNA concentration } (\mu g/\mu l) = \left[\text{measured } OD_{260} \times \frac{50\ \mu g/ml \times \text{dilution factor}}{1\ OD_{260}}\right] / 1000$$

OD_{260} : UV absorbance of DNA was measured at a wavelength of 260 nm.
Dilution factor : time number of diluted DNA solution

The final DNA concentration of each sample was adjusted and equalized by diluted DNA sample to 1 µg/µl with autoclaved ultra pure water. Multiplexed PCR reactions were then set up with a gene-specific primer pair (Table 3.4) and 18S rRNA primer to allow simultaneous amplification of the gene of interest and the internal control in a single tube. Preliminary experiments were conducted to determine both the optimal amount of internal control primers and interested gene primers ratio and the number of PCR cycles that would enable detected PCR amplification within the linear range of the PCR reaction for both groups of sows fed with deficient and normal protein diet.

The data of preliminary were used to set the protocol of multiplexed PCR reaction. The PCR protocol consisted of 3 minutes at 95°C, followed by 33 cycles of denaturing at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at

72°C for 60 seconds, and finally, stop reaction at 72°C for 10 minutes. The resultant PCR products were size-fractionated in 1.8% agarose gels, stained with ethidium bromide. The PCR products were loaded for 18 μl/well. The voltage of electrophoresis was set up at 70 voltage for 67 min. The intensities of both the amino acid transporter and 18S rRNA band for each sample were measured by densitometry using Scion Image software program (www.scioncorp.com). The expression levels of each transporter gene which were normalized to the18S were calculated by the equation below. The relative quantitative RT-PCR of each transporter gene was compared between control and deficient group. All assays were conducted in duplicate or triplicate.

$$\text{Intensity of amino acid transporter gene expression} = \frac{\text{Expression level of transporter gene}}{\text{Expression level of 18S rRNA}}$$

Calculation of the growth performance

The data of feed intake and individual body weight were collected and used to calculate the average daily gain (ADG) of piglets, average daily feed intake (ADFI) of sows and their piglets, and body weight change of sows. These formulas are shown below.

$$\text{Average daily gain (ADG, g/day)} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Days}}$$

$$\text{Average daily feed intake (ADFI)} = \frac{\text{Feed intake}}{\text{Days}}$$

$$\text{Body weight change (kg)} = \text{Final body weight} - \text{Initial body weight}$$

Statistical analysis

Data of growth performance were presented as means ± SD. The relative gene expressions obtained from semiquantitative RT-PCR determination were presented as

means ± SE which were shown as bar chart. Differences between groups were analyzed by unpair t-test using the commercially computer program GraphPad Prism (Prism3) and results were considered statistically significant at $P \leq 0.05$.