การปรากฏของเซลล์หลั่งเลปทินไลค์เพปไทด์ในทางเดินอาหารของหอยเชอร์รี่

นางสาวปิยฉัตร ทรงวิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE PRESENCE OF LEPTIN-LIKE PEPTIDE-SECRETING CELLS IN THE GASTROINTESTINAL TRACT OF *Pomacea canaliculata*

Piyachat Songvijit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Sciences

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THE PRESENCE OF LEPTIN-LIKE PEPTIDE-SECRETING CELLS IN THE GASTROINTESTINAL TRACT OF *Pomacea canaliculata*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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วัตถุประสงค์หลักของการศึกษาในครั้งนี้ เพื่อศึกษากายวิภาคศาสตร์ของทางเดินอาหารของหอยเชอรี่ การปรากฏของเซลล์หลั่งเลปทินไลค์เพปไทด์ในทางเดินอาหารของหอย และการเปลี่ยนแปลงของระดับของเลปทินไลค์เพปไทด์ เปรียบเทียบระหว่างหอยที่ได้รับอาหารปกติและหอยที่อดอาหาร การศึกษามหกายวิภาคศาสตร์ของระบบทางเดินอาหารของหอยเชอรี่ พบว่าทางเดินอาหารประกอบไปด้วยหลอดอาหาร กระเพาะอาหาร ลำไส้ และไส้ตรง การศึกษาจุลกายวิภาคศาสตร์ของระบบทางเดินอาหารของหอยพบว่า ชั้นเยื่อเมือกของทางเดินอาหารถูกบุด้วยเซลล์บุผิวชนิดซิมเปิลคอลัมนาร์ และพบไมไครวิลไลที่บริเวณด้านบนของเซลล์บุผิวนี้ นอกจากนี้ยังพบเซลล์หลั่งมิวซิน ทั้งชนิดนิวทรัลมิวซิน เซียโลมิวซิน และซัลโฟมิวซิน ซึ่งเซลล์หลั่งมิวซินนี้พบแทรกอยู่ระหว่างเซลล์บุผิวตลอดทั้งทางเดินอาหาร ยกเว้นที่กระเพาะอาหาร การศึกษาด้วยวิธีอิมมูโนฮีสโตเคมมิสตรีโดยใช้แอนติบอดีต่อต้านต่อเลปทินของมนุษย์ พบการปรากฏของเซลล์หลั่งเลปทินไลค์เพปไทด์ ในหลอดอาหาร กระเพาะอาหาร ลำไส้ และไส้ตรง และพบมากที่สุดที่หลอดอาหาร โดยเลปทินไลค์เพปไทด์ที่พบมีมวลโมเลกุลเท่ากับ 16 กิโลดัลตัน ซึ่งเท่ากับมวลโมเลกุลของเลปทินของมนุษย์ที่ใช้เป็นพอสสิทีฟคอนโทล การศึกษาการเปลี่ยนแปลงของระดับของเลปทินไลค์เพปไทด์ในหลอดอาหาร เปรียบเทียบระหว่างหอยที่ได้รับอาหารปกติและหอยที่อดอาหาร พบว่าระดับของเลปทินไลค์เพปไทด์ในกลุ่มที่อดอาหารลดลงในวันที่ 5 และวันที่ 10 เมื่อเทียบกับกลุ่มที่กินอาหารปกติ อย่างไรก็ตาม ในวันที่ 15 ระดับของเลปทินไลค์เพปไทด์มีระดับใกล้เคียงกันในกลุ่มอดอาหารและกลุ่มที่กินอาหารปกติ การศึกษาครั้งนี้สามารถนำไปใช้เป็นความรู้พื้นฐานเกี่ยวกับระบบต่อมไร้ท่อของหอยซึ่งเกี่ยวข้องกับการควบคุมการกินอาหารของสัตว์ชนิดนี้

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PIYACHAT SONGVIJIT : THE PRESENCE OF LEPTIN-LIKE PEPTIDE SECRETING-CELLS IN THE GASTROINTESTINAL TRACT OF *Pomacea canaliculata*. THESIS ADVISOR : ASST. PROF. PIYADA NGERNGSOUNGNERN, Ph.D. 119 PP.

EPITHELIUM/ GASTROINTESTINAL TRACT/ IMMUNOHISTOCHEMISTRY/ LEPTIN/ MUCIN

The main objectives of the present study were to study anatomy of the gastrointestinal (GI) tract of the golden apple snail, the presence of leptin-like peptide-secreting cells in the GI tract, and changing of the leptin-like peptide levels compared between fed and fasted snails. Gross anatomy study showed that the snail GI tract consisted of esophagus, stomach, intestine, and rectum. Microanatomy study revealed that mucosa of the GI tract was covered with simple columnar epithelium with microvilli at the apical surface of the epithelial cells. Moreover, mucin-secreting cells including neutral mucin-, sialomucin-, and sulfomucin-secreting cells were observed to be scattered with the epithelial cells. The mucin-secreting cells were found in most parts of the GI tract, except in the stomach. Immunohistochemistry study using antibody that raised against human leptin showed the presence of leptin-like peptide-secreting cells in the esophagus, stomach, intestine, and rectum. The highest presence of leptin-like peptide secreting cells was observed in the esophagus. The leptin-like peptide had the molecular weight of 16 kDa, which is the same as that of human leptin (positive control). Study of changing of leptin-like peptide levels compared between fed and fasted snails revealed that the levels of the leptin-like

peptide decreased in the fasted snail at day 5 and 10. However, at day 15, levels of the leptin-like peptide were similar in both fed and fasted groups. Results of the present could provide basic knowledge on the endocrinology of the snail that related to its feeding control.

School of Preclinic Student's Signature Piyachat Songvijit

Academic Year 2015 Advisor's Signature P. Ngm

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Piyachat Songvijit

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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µg	=	Microgram
µm	=	Micrometer
BSA	=	Bovine serum albumin
^{o}C	=	Degree Celsius
CO_2	=	Carbon dioxide
cm	=	Centimeter
DAB	=	Diaminobenzidine
DI	=	Distilled water
ELISA	=	Enzyme-linked immunosorbent assay
hr	=	Hour
HRP	=	Horseradish peroxidase
H_2SO_4	=	Sulfuric acid
IgG	=	IgG immunoglobulin
KCl	=	Potassium chloride
KH_2PO_4	=	Potassium Dihydrogen Phosphate
M	=	Molar
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter

LIST OF ABBREVIATIONS (Continued)

ng/ml	=	Nanogram per milliliter
min	=	Minute
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate
$NaHCO_3$	=	Sodium bicarbonate
Na_2KPO_4	=	Disodium hydrogen phosphate
nm	=	Nanometer
PBS	=	Phosphate buffered saline
pH	=	Potential of hydrogen ion
PMSF	=	Phenylmethylsulfonyl fluoride
SD	=	Standard Definition
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	=	Scanning Electron Microscope
TEM	=	Transmission Electron Microscopy
TMB	=	3, 3', 5, 5'-Tetramethylbenzidine

CHAPTER I

INTRODUCTION

1.1 Introduction

The golden apple snail, *Pomacea canaliculata*, is organized in the subclass prosobranchia of the gastropoda. *P. canaliculata* is in order Mesogastropoda and family Ampullariidae (Bronson, 2002). A general characteristic of the golden apple snail is its shape which is round. Smooth rotation of the spiral shell is right and the spiral shells are separated clearly straight seam with a deep notch. The smallest at the top of the shell is called the apex. The spiral at the bottom to the largest of the body is where the internal organs locate. A large opening with a lid is called the operculum. Old juveniles can mate at any time for 2-3 months. After the juveniles mate for 5-7 days, female snails will spawn their pink eggs on land. The eggs are accumulated and arranged orderly. The length of the egg cluster is about 2-3 inches and each cluster consists of 200-3000 eggs. Hatching of the eggs can occur within 1 or 2 weeks after spawning (Bronson, 2002). For breeding, the golden apple snail competes with native species for limited resources, because it is able to eat all types of aquatic plants. A large consumption of vegetation modifies the natural balance of the ecology system. In the United States of America, It was reported that the golden apple snails reproduce rapidly because an absence of their hunters. The expulsion of their populations causes various problems. The elimination method of the snail has yet been discovered. There are still

no chemicals that are chose for direct elimination of the snail invasion. Famers usually use ducks to control the snail population (Bronson, 2002; Howells, 2003).

The digestive system of gastropod, like in most animals, is an esophagus leading from the mouth to the stomach, followed by a long intestine which ends at the anus. The stomach is embedded within a digestive gland which connects to the stomach by a series of ducts (Fretter and Graham, 1962; Voltzow, 1994).

The first objective of the present study was focus on anatomy of the gastrointestinal (GI) tract of the snail. Mucin-secreting cells in the GI tract were also identified. Mucin-secreting cell (also called goblet cell) is a glandular, modified simple columnar epithelial cell. The main function of this cell is to secrete gel-forming mucins, the major components of mucus. Mucin is viscoelastic gel that lubricates the intestinal mucosa and protects the epithelial layer from mechanical damage and pathogen invasion (Forstner and Forstner, 1994; Montagne et al., 2004; Pavelka and Roth, 2010). Mucin also provides a microenvironment for indigenous microflora resulting in the formation of a biofilm on the mucosal surface, which plays an essential role in the GI tract health of individuals (Probert and Gibson, 2002; Corfield and Shukla, 2003).

The presence of leptin-like peptide secreting cells in the GI tract and feeding control of the snail by the leptin-like peptide were also concerned in the present study. Feeding of snail is controlled by many hormones including leptin. Leptin is a signal that regulates food intake and body weight. The systems that control feeding behavior and energy balance appear to be comprised of short-term and long-term systems. The principal function of leptin is involved in the regulation of the digestive system. Leptin is an adiposity signal that acts directly on the central nervous system (CNS) via its receptor which is expressed at arcuate nucleus (ARC) of the hypothalamus. The ARC

contains neuropeptide Y (NPY) and agouti-related peptide (AGRP) neurons that stimulate food intake and these neurons are inhibited by leptin. In contrast, pro-opiomelanocortin (POMC) neurons that reduce food intake are stimulated by leptin (Elena et al., 2008).

1.2 Research objectives

1.2.1 To study the microstructure and ultrastructure of the GI tract of *P. canaliculata*.

1.2.1.1 To study histology of all parts of the GI tract (esophagus, stomach, intestine, and rectum) using hematoxylin and eosin staining.

1.2.1.2 To study microstructure and ultrastructure of the GI tract using scanning and transmission electron microscopies, respectively.

1.2.1.3 To identify mucin-secreteing cells in all parts of the GI tract.

1.2.2 To investigate the presence of leptin-like peptide in the GI tract of *P. canaliculata*.

1.2.2.1 To localize leptin-like peptide-secreting cells in the GI tract using immunoperoxidase technique.

1.2.2.2 To detect the expression of leptin-like peptide in the GI tract using Western immunoblotting technique.

1.2.2.3 To evaluate changing of the leptin-like peptide levels compared between fed and fasted snails using enzyme-linked immunosorbent assay (ELISA) technique.

1.3 Research hypotheses

1.3.1 The structure of the GI tract of *P. canaliculata* is similar to those of other closely gastropod species.

1.3.2 Mucin-secreting cells are observed along the GI tract of *P. canaliculata*.

1.3.3 Leptin-like peptide-secreting cells are localized in the GI tract of *P. canaliculata.*

1.3.4 Western immunoblotting shows an expression of leptin-like peptide in parts of the GI tract. Molecular weight of the leptin-like peptide is related to those of other species.

1.3.5 Level of leptin-like peptide is changed in the fasted animal.

1.4 Scope and limitation of study

1.4.1 Study the microstructure and ultrastructure of the cells throughout the GI tract using light and electron microscopies.

1.4.2 Classification of the mucin-secreting cells by histochemistry methods.

1.4.3 Examination of the appearance of leptin-like peptide-secreting cells in various parts of the GI tract, to confirm that leptin peptide is also found in invertebrates.

1.4.4 By mean of immunoblotting, the molecular weight of the leptin-like peptide could be related to that of the positive control.

1.4.5 Comparing the levels of the leptin-like peptide in the GI tract using ELISA technique. The study compared between the snails that were allowed to eat regular meals and snails that were fasts. This study could confirm that leptin-like peptide functions in the snail.

CHAPTER II

LITERATURE REVIEW

2.1 Golden apple-snail (*Pomacea canaliculata*)

Pomacea canaliculata (Lamarck, 1819), common name "golden apple snail", is organized in the subclass Prosobranchia of the Gastropoda which is dominant from the other subclass. *P. canaliculata* is in order Mesogastropoda and family Ampullariidae (Bronson, 2002). The snail has a distinct flat lid called an operculum which is used to close the mouth of the shell. The operculum is carried on the back of the snail body behind the shell and is pulled inwards to seal the shell when the snail withdraws inside (Robert, 2002). The golden apple snail is larger than other types of freshwater snail. It can be separated from other species by an observation of its egg shell which is porous or open umbilicus (Robert, 2003).

The shell of the golden apple snails is spherical and is separated by a deep, indented suture (the spiral seam marking). The shell aperture is large and is oval to round shape. Male snails are known to have a rounder aperture than those of females. The snails have yellow to brownish black body. Diameters of mature golden apple snails are ranged more than 3 cm (Robert and Thiengo, 2003) as shown in Figure 2.1.

Figure 2.1 Morphology of *Pomacea canaliculata*. Scale bar = 1 cm.

The golden apple snail can propagate and grow rapidly. Its diets are all types of plants. This species spends its days submerged and hidden under vegetation and is more active at night. The activity rate of this snail varies with the water temperature. Old juveniles can mate at any time for 2-3 months. After the juveniles mate for 5-7 days, female snails spawn their pink eggs on land. The eggs are accumulated and arranged orderly (Figure 2.2). The length of the egg cluster is about 2-3 inches and each cluster consists of 200-3000 eggs. Hatching can occur within 1 or 2 weeks after spawning. The eggs in a cluster are loosely bound to each other and are found on an object above the waterline for preventing of their predators. A previous study reported that the eggs of *P. canaliculata* contain proteins that are resistant to digestion and are a neurotoxic substance to mice. This could explain the lack of comparison of predators and bright colors warn (Dreon et al., 2010). Respiratory organs of the snail includes both gills and breathing air organs. The ability to breath in the air helps the snail to survive during the

irrigation. Snails usually dig deep in the soil in dry season and come out in the rice fields when flooded. Adult snails eat the above ground parts of rice seedlings (Bronson, 2002; Howells, 2003).

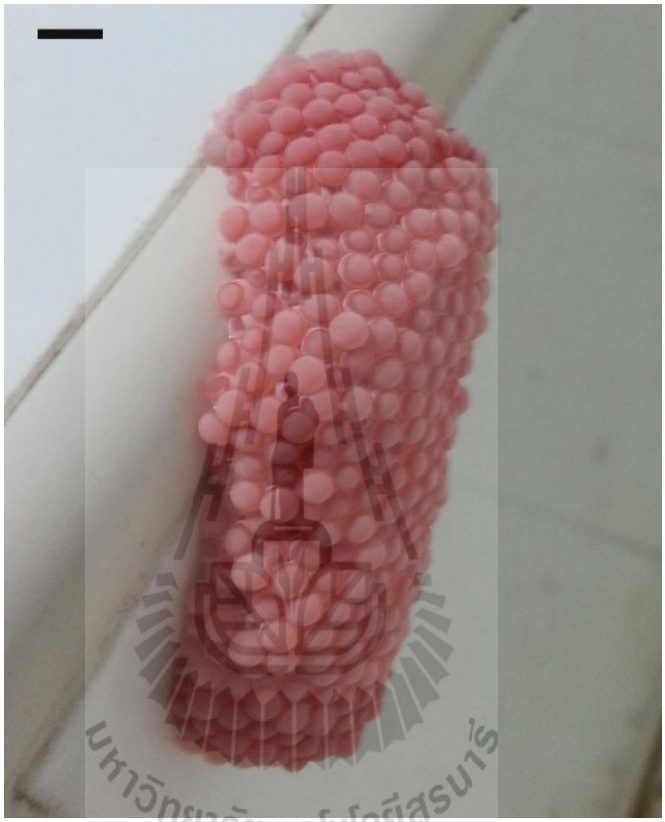

Figure 2.2 An egg cluster of *P. canaliculata*. Scale bar = 1 cm.

In 1980, the golden apple snail was brought to Taiwan for human food industry. After that, it became a serious pest by feeding on rice and corn. Subsequently, it quickly spread to Indonesia, Thailand, Cambodia, Hong Kong, South China, Japan, Philippines, and Hawaii (Bronson, 2002; Howells, 2003).

For breeding, the golden apple snail competes with native species for limited resources, because it is able to eat all types of aquatic plants. A large consumption of

vegetation modifies the natural balance of the ecology system. The rapid reproduction with the absence of its hunter in the United States made its populations exploded and caused further problems. The elimination method of snail has yet been discovered. There are no chemicals that are chosen for elimination of the snail invasion. In areas where snails are found and crops are found to be damaged. Famers usually use ducks to control the snail population (Bronson, 2002; Howells, 2003).

The temperature has been found to be a major factor affecting the biology of *P. canaliculata*. Its growth rate is highly dependent on temperature. The rest period is in winter which it mostly shows complete stop of growth. Females can store sperm for 140 days and lay up to 3,000 viable eggs along this period (Estebenet and Cazzaniga, 1998).

2.2 The snail gastrointestinal tract

The digestive system of gastropod, like in most animals, is an esophagus leading from the mouth to the stomach followed by a long intestine which ends at the anus. The stomach is embedded within a digestive gland which connects to the stomach by a series of ducts (Fretter and Graham, 1962; Voltzow, 1994). The esophagus is divided into an anterior, middle, and posterior regions. The stomach may or may not contain a crystalline style, a cellular gelatinous rod containing adsorbed digestive enzymes. When the crystalline style is presented, it may lie within the stomach or in a separate chamber (Graham, 1949; Owens, 1958). The rectum is the most variable part. It is a simple tube containing *Nucella lapillus* which is one of symbiotic bacteria that is used for the balance of microorganisms in the digestive tract (Brough and White, 1990; Andrews, 1991).

The gastrointestinal tract of *Megathura crenulata* subclass prosobranchia is composed of the lip, anterior esophagus, middle esophagus (called pharynx by Illingsworth, 1902; Figure 2.3), posterior esophagus, stomach, digestive gland, style sac, three regions of intestine, and rectum. The esophagus lies ventral to the visceral mass and enters the stomach at the posterior end to the right of the midline. The digestive gland, stomach, and intestine lie in a large ovoid visceral mass. The stomach is an ovoid sac diagonally orientated which opens to the intestine at the anterolateral left position. Posterior to the style sac is the intestine, the longest region of the gut. Three regions are labeled which are intestinal loop, middle, and posterior intestine (Martin et al., 2010). Anatomy of the gastrointestinal tract of *M. crenulata* is shown in Figure 2.4 (Martin et al., 2011).

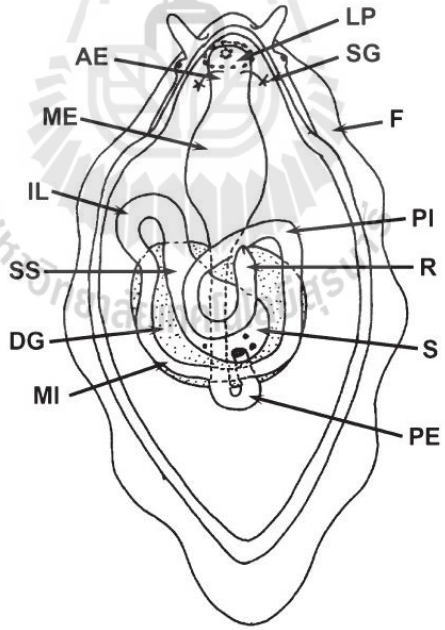

Figure 2.3 Schematic drawing of the snail gastrointestinal tract. AE: anterior esophagus; DG: digestive gland; F: foot; IL: intestine loop; LP: lips on ventral surface; ME: middle esophagus; MI: middle intestine; PE: posterior esophagus; PI: posterior intestine; R: rectum; S: stomach; SG: salivary gland; SS: style sac (Martin et al., 2010).

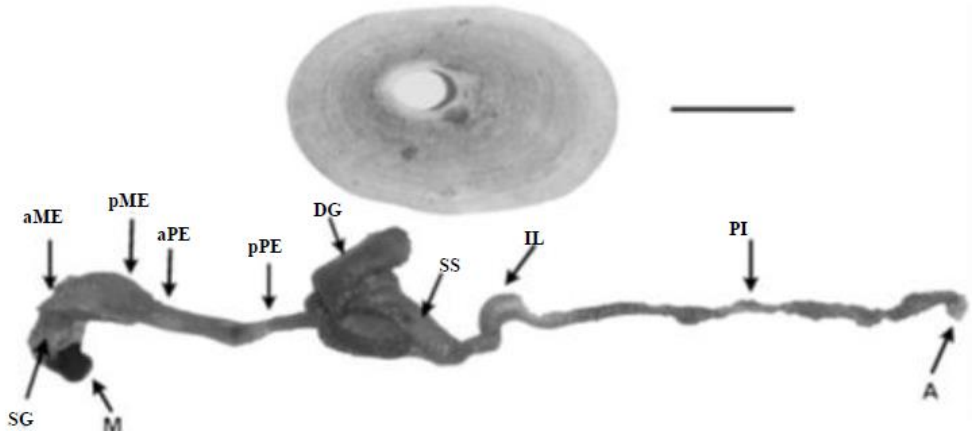

Figure 2.4 The gastrointestinal tract of *M. crenulata*. SG: salivary gland; aME: anterior region of middle esophagus; pME: posterior region of middle esophagus; aPE: anterior region of posterior esophagus; pPE: posterior region of posterior esophagus; DG: digestive gland; SS: style sac; IL: intestinal loop; and PI: posterior intestine. A: anus; M: mouth. Scale bar = 5 cm. Modified from Martin et al. (2011).

The esophagus is divided into 4 parts: anterior region of middle esophagus, posterior region of middle esophagus, anterior region of posterior esophagus, and posterior region of posterior esophagus. The tube from the anterior to the posterior end (Figure 2.4) shows the middle esophagus which opens along the mid-ventral axis. Three pairs of folds hang from the dorsolateral wall into the lumen. Using scanning electron micrograph (SEM), the surface of each fold appears quilted with cells organized into ovoid areas (Martin et al., 2010).

Epithelium of the esophagus contains two types of cells, mucus-secreting cells (located in posterior esophagus) which are inserted with the epithelial cells and are scattered between the more abundant apocrine secretory cells (ASC; Figure 2.5B and 2.5C; Martin et al., 2010). Each ASC is columnar in shape and contains a centrally

located nucleus with a prominent nucleolus. The cytoplasm around the nucleus and in the basal part of the cell contain rough endoplasmic reticulum (RER), Golgi bodies, mitochondria, ovoid electron lucent vesicles, and pigment granules. The apical cytoplasm (Figure 2.5C) contains numerous mitochondria, an occasional lipid droplet, and a variety of vesicles. They contain vesicles, cytoplasm, and granules. When the membrane of each bleb eventually lyses, the content is released into the gut lumen by an apocrine process (Figure 2.5B and 2.5C; Martin et al., 2010).

Mucus-secreting cells (Figure 2.5E and 2.5F) are tall cells and are the most abundant on the larger ridges. The nucleus is located in the basal third and the apical cytoplasm is filled with secretory granules that appear empty when examined under a light microscope (LM). The granules are stained with periodic acid-Schiff (PAS) and faintly with alcian blue. Using transmission electron micrograph (TEM), developing vesicles are electron-lucent with a punctate pattern of granules connected by fine filaments. Each vesicle also contains an eccentrically placed circular region of moderate electron density. The cytoplasm is not filled by mucus granules, but it contains numerous vesicles, RER, Golgi bodies, mitochondria, and secondary vacuoles (Martin et al., 2010).

The majority of cells lining the posterior esophagus are ASC. These cells are as tall as the mucus secreting cells, but are narrower. They are connected by intermediate junctions. Their apices bear microvilli and cilia (Figure 2.5F). From the base of each cilium is a long striated rootlet that extends into an accumulation of electron dense stained mitochondria when observed by TEM (Martin et al., 2010).

Cells in all regions of the esophagus produce blebs which are expanded from their apices. They release cytoplasm containing vesicles into the gut lumen by an

apocrine secretion (Figure 2.6A). As the apex of each cell bulges into the lumen, the microvilli shorten and may disappear. The cytoplasmic bulges pinch off to form spherical blebs viewing by SEM, which can be seen to cover the gut surface (Figure 2.6B; Martin et al., 2010).

The stomach consists of four major regions; the sorting area, gastric shield, roof, and style sac that extends into the intestine (Figure 2.7A; Martin et al., 2010). Near the esophageal opening are three ducts connecting to the digestive gland. The opening of the esophagus into the stomach contains a circular muscular area (Martin et al., 2010).

Ventral surface of the stomach contains large ridges that run parallel to the esophageal opening and form the sorting area (Figure 2.7B; Martin et al., 2010). The epithelium is composed of a single type of ciliated columnar cell (Figure 2.7C; Martin et al., 2010), with centrally located elongate nuclei. These cells bear very long and densely packed cilia on their apices (Figure 2.7D; Martin et al., 2010). The cytoplasm beneath the nuclei lacks distinctive vesicles, and RER. Mitochondria are especially abundant along the base of the cell. To the right of the esophageal valve along the posterior wall of the stomach is the gastric shield (Figure 2.7A; Martin et al., 2010). It composes of an epithelium covered by a chitinous layer that stains with PAS and dissolves in chitinase (Martin et al., 2010).

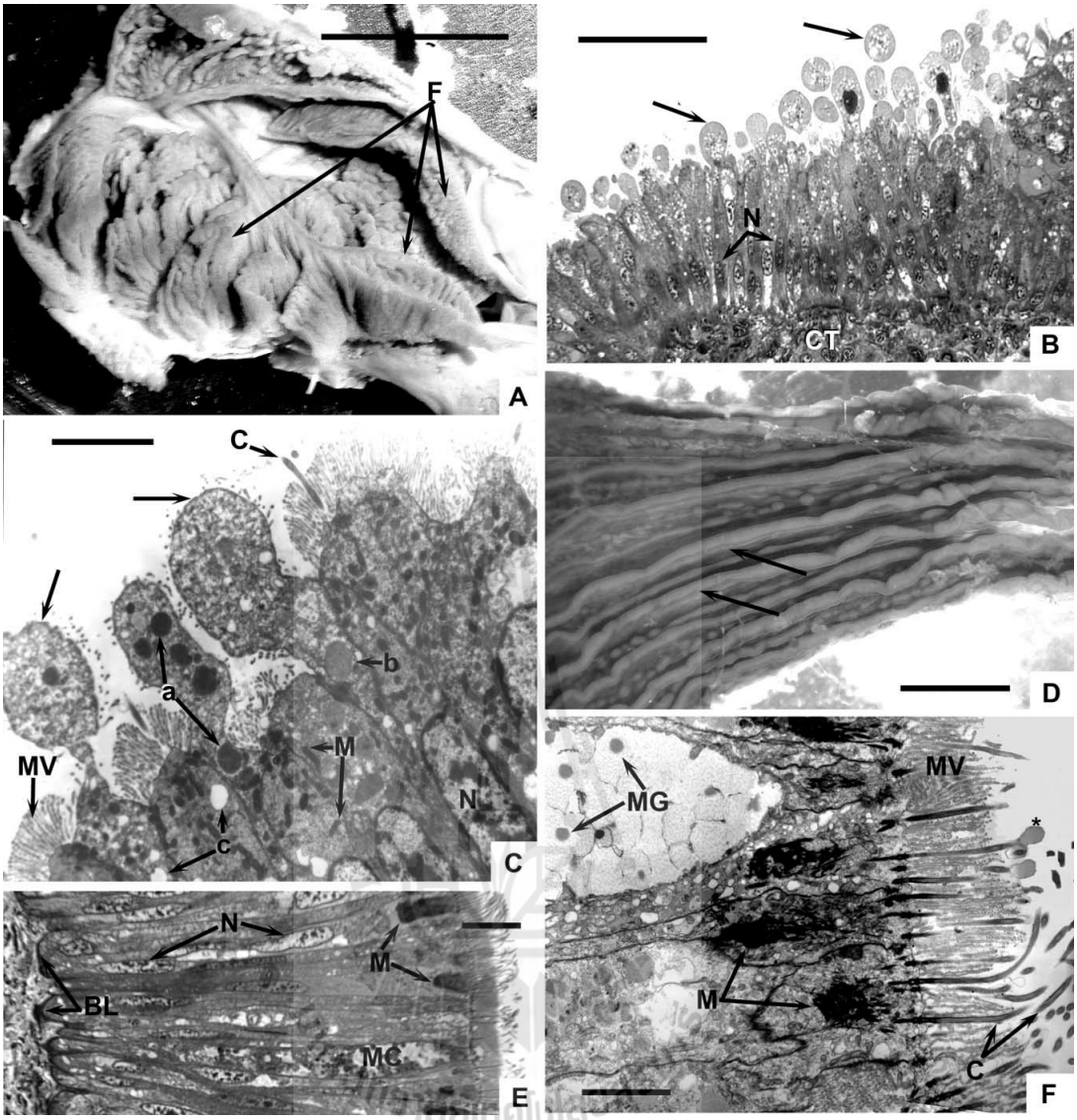

Figure 2.5 The middle and posterior parts of the esophagus. A: The middle esophagus showing folds (F) which project into the lumen. The anterior (left) and the posterior (right). Scale bar = 10 mm. B: Apocrine (arrows); secretory cells of middle esophagus; connective tissue (CT); nuclei (N). Scale bar = 20 µm. C: TEM micrograph showing cover (arrows) extending from secretory cells; electron-dense (a), intermediate (b) and electron-lucent (c) granules; cilia (C); mitochondrion (M); microvilli (MV); nucleus (N). Scale bar = 5 µm. D: The posterior esophagus showing longitudinal ridges (arrows), the anterior (left). Scale bar = 10 mm. E: Light microscopy (LM) picture

showing the epithelium of posterior esophagus; mucus-secreting cell (MC); mitochondria (M); basal lamina (BL); nuclei (N). Scale bar = 10 µm. F: TEM micrograph showing apex of posterior esophagus; mucus granules (MG); electron-dense mitochondria (M). The cilium with a dilated tip (*), cilia (C); microvilli (MV). Scale bar = 5 µm. (Martin et al., 2010).

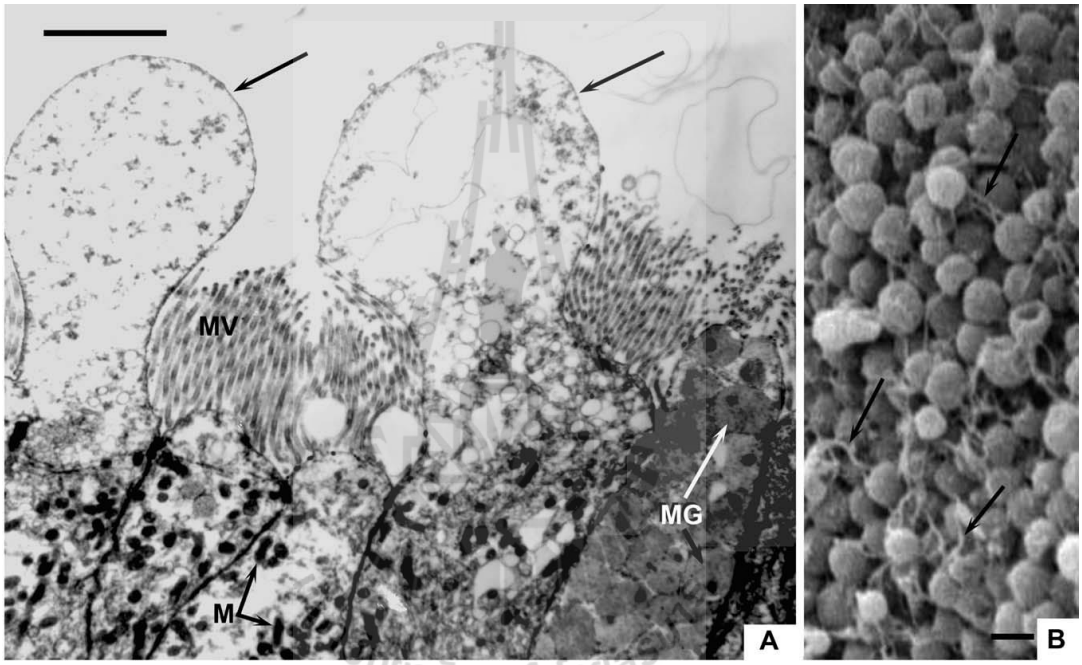

Figure 2.6 TEM and SEM micrographs of the anterior esophagus A: TEM picture showing apocrine secretions (arrows) from posterior esophagus; mitochondria (M); mucus granules (MG); microvilli (MV). Scale bar = 5 µm. B: SEM of the posterior esophagus covered with spherical blebs released by apocrine secretion; cilia (arrows). Scale bar = 10 µm. (Martin et al., 2010).

Adjacent cells of the stomach are connected by intermediate junctions and possess extremely long and wavy microvilli that span nearly the entire thickness of the gastric shield (Figure 2.7F; Martin et al., 2010). The matrix between the microvilli contains filaments which are located in the bottom half of each cell (Martin et al., 2010).

The posterior end of the stomach tapers to the anterior and left side of the animal and forms the style sac. This region is typically filled by a crystalline style that extends into the stomach. Such that the anterior end of the style is directed towards the posterior of the animal and presses against the gastric shield (Figure 2.8A; Martin et al., 2010). The style is cream in color, shiny, cylindrical, and stains with eosin and PAS. The sides of the cylinder are smooth and the anterior end has a rough surface and fits into the ducts leading into the digestive gland. The style is composed of lamellae and debris and often embedded in the layers (Figure 2.8B; Martin et al., 2010).

The style sac has a large C-shaped typhosole which wraps around the crystalline style and a smaller typhosole which runs along the ventral surface of the style sac. There are two types of epithelial cells on the wall of the style sac including both the concave and convex surfaces of the typhosole; typical mucus secreting cells and ASC. The ASC are similar to those seen in the sorting area and the esophagus. They are columnar cells which contain punctate heterochromatin and a prominent nucleolus, elongate mitochondria, and RER (Figure 2.9A; Martin et al., 2010). Their apices are covered with a dense brush border. The apical cytoplasm of these cells contains pigment granules which to the narrowness of these cells often which appear to be lined up in rows. The ASCs protrude their microvilli and cilia into the lumen (Figure 2.9B; Martin et al., 2010). At the base of the epithelium along the basal lamina and between adjacent

cells are irregularly shaped spaces containing flocculent material as same as that observed in other gastropods (Graham, 1949).

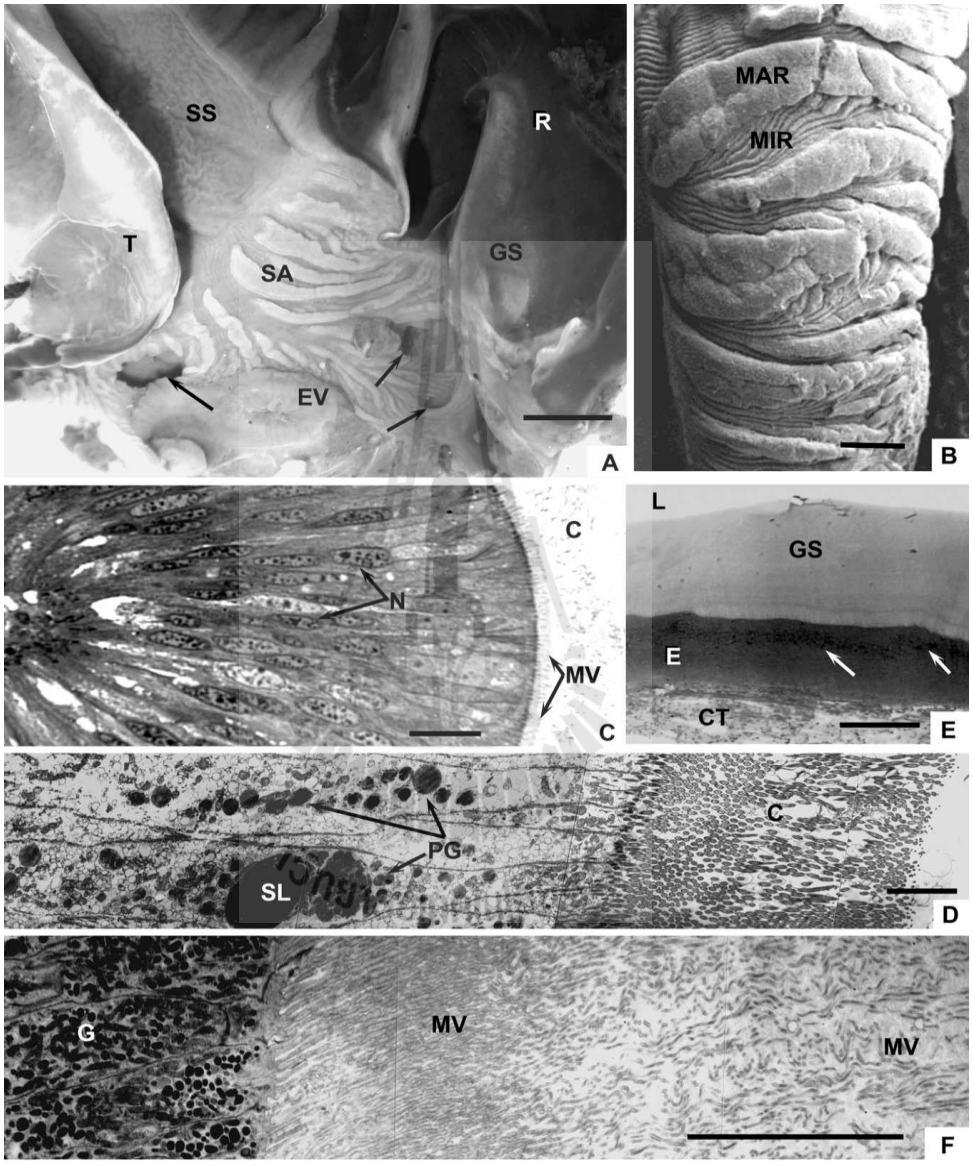

Figure 2.7 LM, SEM and TEM micrographs of the stomach. A: Morphology of the stomach; esophageal valve (EV); sorting area (SA). The left of the sorting area is the major typhosole (T) bounding the style sac (SS), and to the right is the gastric shield (GS) merging into the chitin-lined roof (R), and openings of stomach into the digestive

gland (arrows). Scale bar = 5 µm. B: SEM image of sorting area showing major (MAR) and minor (MIR) ridges. Scale bar = 2 mm. C: LM image showing elongate cells; cilia (C), microvilli (MV), and elongate nuclei (N). Scale bar = 15 µm. D: TEM image showing apex of ciliated cell from sorting area; long cilia (C); pigment-granules (PG), and secondary lysosomes (SL). Scale bar = 5 µm. E: LM image showing gastric shield (GS); epithelial cells (E). The granules in the apical half of the cells (arrows); connective tissue (CT); lumen (L). Scale bar = 300 µm. F: TEM image showing microvilli (MV) embedded in chitinous matrix; granules (G). Scale bar = 10 µm. (Martin et al., 2010).

Intestine is the longest part of the gut. In the loop, the major typhosole from the style sac is still presented. The minor typhosole is also reduced and looks like one of the many ridges that run parallel to the long axis of the gut and continue into the rectum. A pair of larger ridges forms the food groove (Figure 2.9C; Illingsworth, 1902). The middle intestine is at least partially embedded in the digestive gland. The posterior intestine separates from the digestive gland, and then extends dorsally into the mantle cavity where it forms a short tube, the rectum. The simple columnar epithelium of the intestine (Figure 2.9D; Martin et al., 2010) is similar throughout the length of the intestine with only minor variations in the height of the cells and is composed of two types of cells; mucus-secreting cells and ASCs (Martin et al., 2010).

The rectum is a short tube. Its lumen is reduced and its wall has an irregular shape to several major ridges that project into the lumen (Figure 2.10A; Martin et al., 2010). The ridges are continuations of the folds seen in the intestine. The epithelial cells are shorter than in the intestine and have a similar width. There are two types of

cells found in the rectum, is mucus-secreting and ciliated cells (Figure 2.10B; Martin et al., 2010). They have the typical apical region of microvilli (Martin et al., 2010).

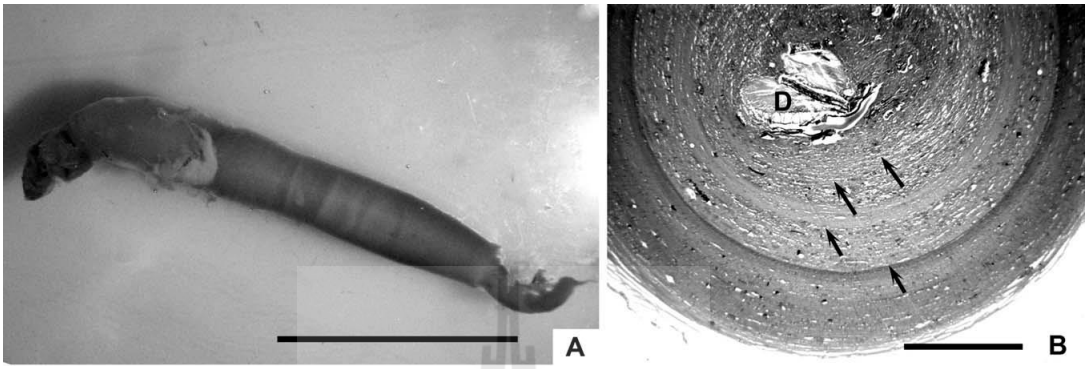

Figure 2.8 The crystalline style A: The crystalline style tapered at the posterior end (to the right). Scale bar = 15 mm. B: Cross section of crystalline style showing lamellae (arrows) and embedded debris (D). Stained with PAS. Scale bar = 0.5 mm. (Martin et al., 2010).

Although ASCs in the rectum look very similar to those seen in other regions of the gut, apocrine secretion was not observed in these cells. Clusters of microfilaments are seen in the cytoplasm extending from the base of the cilia to the basal plasma membrane. The mucus-secreting cells of the epithelium are similar to those seen in other regions of the gut. The epithelium rests on a typical basal lamina (Martin et al., 2010).

In the stomach, on the right side of the esophageal valve are the openings of two ducts carrying materials into the digestive gland, and on the right side is a single opening and duct. All three ducts branch repeatedly and end in acini. Ducts have the simple columnar epithelium which is composed of a single type of cell (Figure 2.10C;

Martin et al., 2010). The apex of the cell has a brush border and adjacent cells are connected by intermediate junctions (Martin et al., 2010).

The lumen of the rectum may be nearly eliminated by folds of the epithelium. The epithelium is composed of two types of cells. The major type of cells, digestive cells, have a basal nucleus and eosinophilic cytoplasm. The basal half of each cell is filled with spherical vacuoles (Figure 2.10E). These are presumably phagocytic vacuoles and contain material of various densities. The apex of the cells may lack microvilli or if microvilli are presented, they are spared. The second type of cell in the acini is called the secretory cell. Its cytoplasm appears denser and stained darker with H&E than the digestive cell (Figure 2.10F; Martin et al., 2010).

2.3 Mucin

Mucin is secreted from goblet cell. It is likely a proteoglycans consisting of a polysaccharide chains linked protein core (Gendler and Spicer, 1995). The carbohydrate content of a mucin may probably be up 90% of its molecular weight. Mucins are classified into neutral and acid mucins, according to the net charge of the molecule (Deplancke and Gaskins, 2001). The polysaccharide chains of the mucins vary from neutral mucin or weakly acidic mucin (sialomucin) to strongly acidic (sulfomucin). The neutral mucin composes of monosaccharides, for example mannose, galactose, and galactosamine. The sialic acid is a group of nine-carbon monosaccharide that contents a carboxylate group at the carbon molecule (Schauer, 1982; Varki et al., 1999). Carboxyl group is ionized at a physiological pH and forms an overall negative charge on the molecule (Varki et al., 1999). In additional sialomucin composes of N- or O-acetylated group and sulfomucin composes of a group of N-acetylated type (Filipe, 1979).

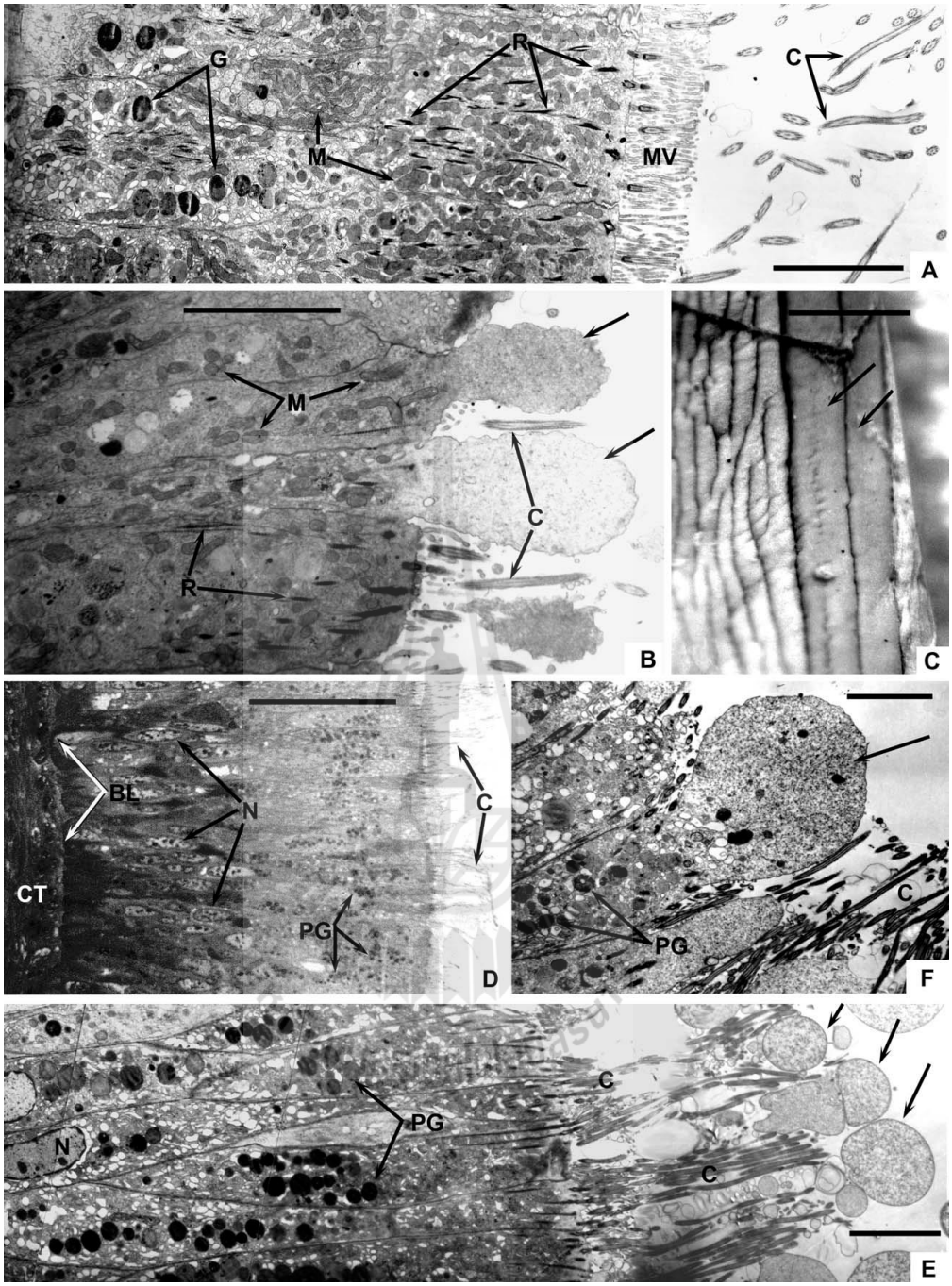

Figure 2.9 LM and TEM images of the intestine. A: TEM picture of style sac; microvilli (MV); cilia (C); mitochondria (M); granules (G); and striated rootlets (R). Scale bar = 3 µm. B: TEM showing apocrine (arrows) in the style sac; cilia (C); mitochondria (M); striated rootlets (R). Scale bar = 5 µm. C: Intestinal loop showing longitudinal ridges and two larger ones (arrows) which are continuations of the style

sac. Scale bar = 300 µm. D: LM image showing the intestine; basal lamina (BL); Cilia (C); connective tissue (CT); nuclei (N); pigment granules (PG). Scale bar = 50 µm. E: TEM image of apocrine secretory cells from intestine showing blebs detached from the cells (arrows), cilia (C), and pigment granules (PG); nucleus (N). Scale bar = 8 µm. F: TEM image showing apocrine secretion (arrow) of the intestine; cilia (C); pigment granules (PG). Scale bar = 5 µm. (Martin et al., 2010).

Mucin is generally associated with the epithelial surface of the respiratory, digestive, and reproductive systems. The difference of carbohydrate structures may affect the presence of mucin (Hattrup and Gendler, 2008; Thornton et al., 2008). The function of mucin in mucous layer is to lubricate the intestinal mucosa and protects the epithelial layer from mechanical damage and pathogen infection (Forstner and Forstner 1994; Montagne et al., 2004). These mucins may be participate in the control of cellular functions, cell proliferation, and cell adhesion (Wesseling et al., 1995; Moniaux et al., 1999; Schroeder et al., 2001). The neutral mucin occurs in greater quantities in the gastric mucosa, whereas the acidic mucin dominants in the intestinal epithelium. Acidic mucins are more tolerant to degradation by bacterial glycosidases and host proteases and they show higher viscosities and acid compared to neutral mucins (Deplancke and Gaskins, 2001). The distribution of mucin can differ according the number of cells secretion, cell type, region, anatomical, pathology, conditions, and species (Scillitani et al., 2007).

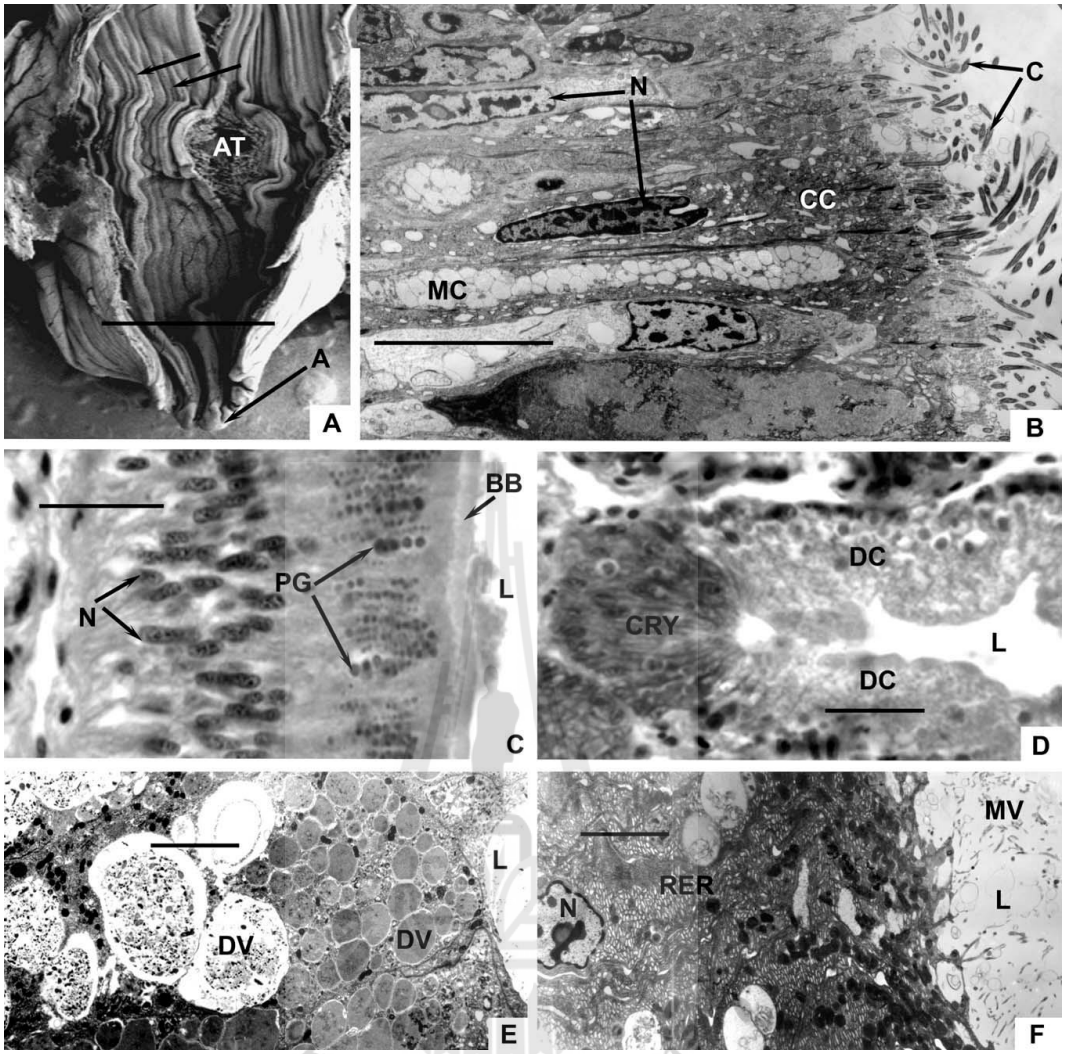

Figure 2.10 SEM and TEM micrographs of the rectum A: SEM picture of rectum showing ridges (arrows) and artifact tear (AT); anus (A). Scale bar = 2 mm. B: TEM picture of rectal epithelium showing two cell types; mucus-secreting (MC) and ciliated cells (CC); cilia (C); nuclei (N). Scale bar = 10 µm. C: The ducts in the digestive gland; pigment granules (PG); brush border (BB); lumen (L); nuclei (N). Scale bar = 25 µm. D: LM picture of digestive gland showing crypt (CRY) and digestive cells (DC); lumen (L). Scale bar = 50 µm. E: TEM picture of digestive cell; digestive vacuoles (DV); lumen (L). Scale bar = 5 µm. F: TEM picture of secretory or crypt cells; lumen (L); microvilli (MV); nucleus (N). Scale bar = 5 µm. (Martin et al., 2010).

Goblet cell or mucin-secreting cell were identified in the GI tract of various species. In the Southern African spiny mouse (*Acomys spinosissimus*), the body (corpus) of the stomach contained predominantly neutral mucins. In Hottentot golden mole (*Amblysomus hottentotus*), the surface mucous cells and the proximal mucous neck cells of the gastric glands consisted of mixed mucins (neutral and acid), whereas the distal mucous neck cells were stained intensely for neutral mucins. Sialomucins were presented in surface and neck mucous cells of *A. hottentotus*, while few mixed sialomucins and sulfomucins were presented in the corpus of the stomach. Neutral mucin-secreting goblet cells in the small intestines of *A. spinosissimus* and Reddish gray musk shrew (*Crocidura cyanea*) appeared to dominate the acid mucin. Moreover, in *A. hottentotus*, the acid mucin secreting goblet cells were predominantly located in the small intestine. The small intestine of various animals (mouse, rat, hamster, guinea pig, rabbit and cat) also contained mucin-secreting cells (Boonzaier et al., 2013; Sheahan and Jervis, 1976).

2.4 Feeding hormone

There are many hormones that are released during food ingestion. Some of these hormones are important in regulating of an appetite. The appetite is found to be regulated by a complex system of central and peripheral signals which interact in order to modulate the individual response to nutrient ingestion. Peripheral regulation includes satiety and adiposity signals, whereas central control is accomplished by several effectors, including the neuropeptidergic, monoaminergic and endocannabinoid systems. Satiety signals, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), originated from the gastrointestinal tract during a meal and via the

vagus nerve reaching the nucleus of tractus solitarius (NTS) in the caudal brainstem. From NTS, afferent fibers project to the arcuate nucleus (ARC), where satiety signals are integrated with adiposity signals, namely leptin and insulin, and also with several hypothalamic systems (Elena et al., 2008).

Concerning the neuropeptidergic system, neuropeptide Y (NPY) is the most powerful central enhancer of appetite. Its expression is predominant in ARC, from which NPY neurons project to second-order neurons located in paraventicular nucleus (PVN), lateral hypothalamic area (LHA), perifornical area (PFA), ventromedial (VMN), dorsomedial (DMN) nuclei, and also to other brain regions, setting in motion of the anabolic pathway (Ramos et al., 2005). Furthermore, 90% of NPY co-expresses with agouti-related peptide (AGRP; Schwartz et al., 2007). Low leptin levels are found in hypoglycemia, hypoinsulinemia, and conditions of negative energy balance can enhance NPY mRNA expression in ARC. Central administration of NPY inhibits thermogenesis, enhances food intake and promotes adipogenesis in rats (Williams et al., 2004). To date, six NPY receptors have been isolated, two of which (Y1 and Y5) seem to mediate the NPY anabolic effects. Y1 and Y5 antagonists are under investigated as antiobesity agents, although blockade of NPY activity might be associated with side effects such as arterial hypertension, analgesia, impairment of pituitary hormone secretion and hypoglycemia (Parker et al., 2002).

AGRP is another potent orexigenic peptide. Releasing of AGRP by ARC is inhibited by leptin infusion, while its expression is upregulated in ob/ob leptin-deficient mice. AGRP influences food intake mainly through the competitive antagonism of central melanocortin receptors (Ollmann et al., 1997). Alternative mechanisms of action might be mediated by orexin or opioid receptors (Schwartz et al., 2000). AGRP

secretion appears to be triggered chiefly by any impairment of energy balance (Harrold et al., 1999). High circulating levels of AGRP have been documented in human obesity (Katsuki et al., 2001), A polymorphism in the human AGRP gene, which seemed to be correlated with late-onset obesity, has recently been described (Argyropoulos et al., 2002).

Pro-opiomelanocortin (POMC) is the precursor of several molecules including α-melanocyte-stimulating hormones, which represents the main regulator of energy balance in this family. A study in rats showed that involuntary overfeeding followed by a significant increasing in POMC expression and by anorexia increased higher than 5% of body weight. This pattern was reversed by intracerebroventricular administration of a melanocortin-receptor antagonist (Schwartz et al., 2000).

The anorexigenic effect of melanocortin is mediated by two receptors, MC3R and MC4R, which are highly expressed in the brain and particularly in ARC. In humans, more than 5% of the cases of morbid nonsyndromic obesity are associated with mutations of the MC4R gene. Heterozygous mutations are characterized by severe obesity, hyperphagia, increased fat free mass, hyperinsulinemia and acceleration of linear growth, while homozygous mutations exhibit an even more severe phenotype (Farooqi et al., 2003). A mutation of the MC3R gene, responsible for obesity and insulin resistance, had recently been found in a child and his father (Lee et al., 2002). The key role of the melanocortinergic system as a mediator of anorexigenic signaling is encouraging the experimental use of agonist or antagonist molecules for the treatment of eating disorders. Due to their long-lasting anorexigenic effect, some MC4R analogs, such as melanotan II (Hansen et al., 2005), are good candidates as antiobesity drugs. Syndecans are a family of four transmembrane heparan sulfate proteoglycans that act

as coreceptors for a variety of cell-surface ligands and receptors. Interestingly, syndecan-1 and syndecan-3 enhance the activity of AGRP (Reizes et al., 2001). Thus, inhibitors of syndecan-3 have been proposed as a possible treatment of obesity and are now under investigations (Hansen et al., 2005).

Ninety percent of cocaine- and amphetamine-regulated transcript (CART) neurons are co-localized with POMC neurons in ARC and project their fibers to second-order neurons which are likely mediating the anorexigenic effect of leptin. The anorexigenic action of CART seems to be mediated by central release of glucose-like peptide 1 (GLP-1), since blockade of GLP-1 receptors inhibits CART induced hypophagia (Aja et al., 2001). Both ob/ob and fa/fa mice show reduced CART expression, while CART central administration reduces food intake in rats dose-dependently (Vettor et al., 2002). A missense mutation in the CART gene has recently been described, which causes severe obesity and reduction of resting energy expenditure in humans (Yanik et al., 2006). CART null mice developed an increase in food intake and obesity while on a high fat diet, whereas, unexpectedly, food intake was reduced in the heterozygous model (Kokkotou et al., 2005). This suggests a dual effect of CART on eating behavior, is likely dependent on different sites of action at the hypothalamus. Indeed, 95% of CART neurons located in the lateral hypothalamic area (LHA) co-expresses the orexigenic peptide melanin-concentrating hormone (MCH; Kong et al., 2003).

Melanin-concentrating hormone (MCH) is expressed in a discrete sub-population of neurons situated in the zona incerta and LHA. In ob/ob leptin-deficient mice, MCH mRNA levels increased and the administration of leptin reduces MCH expression. Infusion of MCH in rats induced a significant hyperphagia with an

increasing in body weight (Williams et al., 2004). MCH knock-out mice were resistant to diet-induced obesity, increasing of energy expenditure, and locomotor activity (Kokkotou et al., 2005). Several MCHR1 antagonists are under investigation as anti-obesity drugs (Kokkotou et al., 2005). Among these compounds, SNAP-7941 has proved that it is able to reduce food intake in rat, counteracting the effects of central MCH administration. Given chronically, it decreased palatable food consumption and body weight (Elena et al., 2008).

Orexin A (OXA) and orexin B (OXB) are two peptides derived from the common precursor prepro-orexin, identified in 1998 (Sakurai et al., 1998). They activate two closely related G protein-coupled receptors known as OX1 and OX2. OXA, which appears to exert a more prominent orexigenic effect compared with orexin B, is expressed in neurons of the PFA, LHA and dorsomedial nucleus, with projections to neighboring hypothalamic nuclei and extra-hypothalamic areas including NTS. A single intracerebroventricular injection of OXA increased feeding when administered during the light phase, but not at the beginning of the dark phase, indicating that sensitivity of OXA to orexins might be subjected to circadian variations. Similarly, chronic administration of OXA over 8 days in rats increased food intake during daytime, but caused a compensatory reduction of nighttime feeding, leaving daily food intake and body weight unchanged (Rodgers et al., 2001). Studies on the potential use of OXA antagonists to reduce appetite in obese patients are in progress (Ehrstrom et al., 2005). Central administration of OXA antagonist to rats decreased feeding and accelerated the attainment of satiety and resting (Rodgers et al., 2001), while peripheral injection of OXA in humans did not affect eating behavior, though it reduced leptin levels and slowed gastric emptying (Ehrstrom et al., 2005).

Monoaminergic neurotransmitters interact with neuropeptides to control satiety mechanisms and eating behaviors. Serotonin, produced in the dorsal raphe nucleus, reduces food intake and body weight by diminishing appetite and increasing energy expenditure (Schwartz et al., 2000). Based on these properties, several antiobesity agents have been developed, for example, the serotonin agonists; fenfluramine and dexfenfluramine, and the inhibitors of serotonin reuptake, fluoxetine and sertraline. However, fenfluramine and dexfenfluramine have been withdrawn from the market for serious adverse cardiovascular effects. Sibutramine, which is a combined serotonin and norepinephrine (NE) reuptake inhibitor, has proved efficacious in human obesity, causing a weight loss of 15% after 1 year of treatment. The drug is contraindicated in non-adequately controlled arterial hypertension and in psychiatric disorders (Proietto et al., 2000). NE, synthesized in the dorsal vagal complex and the locus coeruleus, stimulates food intake through the activation of the α_2-receptor, whereas stimulation of α_1-, β_2- and β_3-receptors shows the opposite effect (Ramos et al., 2005). In ob/ob leptin-deficient mice, circulating levels of NE are elevated, suggesting a modulation of NE release by leptin as one of the mechanisms of action of the adiposity signal (Schwartz et al., 2000). The effects of dopamine (DA) on food intake depend on the numerous receptor subtypes and brain sites of action. Indeed, DA signaling appears to suppress food intake in ARC and LHA, and to stimulate it in VMH (Ramos et al., 2005). Furthermore, mesolimbic pathways seem to be associated with the "rewarding" effects of palatable food through the activation of the D5 subtype receptor (Pothos et al., 1995). Repeated systemic treatment with D1, D2 or D1/D2 receptor agonists reduce food intake. In ob/ob mice, leptin deficiency is accompanied by reduced DA levels. However, the observation that incubation of rat hypothalamic extracts with leptin leads

to the inhibition of DA release contrasts with the hypothesis of a direct correlation between leptin and the DA system (Brunetti et al., 1999).

In the regulation of eating behavior, endogenous cannabinoids are emerging as important "carriers" of metabolic information from the central nervous system to the periphery and vice versa. The orexigenic effect of exogenous cannabinoids, exerted through G-protein-coupled cannabinoid (CB) type 1 and type 2 receptors, is well known. The two principal endocannabinoids in the brain are anandamide (AEA), derived from membrane phospholipids and 2-arachidonoylglycerol (2-AG), and from triglycerides. They act as retrograde messengers, being secreted by postsynaptic cells. They bind their receptors on nerve terminals to inhibit synaptic transmission in either excitatory glutamatergic or inhibitory GABAergic axons (Alger, 2004). Cannabinoids are also important players in the reward circuitries, since they interact with opioid and dopaminergic systems to enhance satisfaction coming from the ingestion of palatable food (Pagotto et al., 2006). Ob/ob leptin-deficient mice display elevated levels of hypothalamic endocannabinoids, which are reduced by leptin administration. An effect also documented in normal mice (Van et al., 2005). Circulating levels of endogenous cannabinoids are also increased in obese women compared with lean controls (Engeli et al., 2005). Confirming previous reports showed that weight loss and improvement of metabolic features were found in obese animals treated with endocannabinoid antagonists (Tonstad et al., 2006). The recently published RIO (Rimonabant in Obesity) which studied in the treatment of human obesity demonstrated the efficacy of the CB1 blocker SR141716 (rimonabant) given at the dose of 20 mg daily in addition to hypocaloric diet. This antagonist was able to induce a decrease in body weight by 5% in 67% of the patients and by 10% or more in 39% of the cases, together with a

significant reduction of waist circumference. Additionally, the drug significantly decreased the prevalence of dyslipidemia and metabolic syndrome. Owing to the interactions of endogenous cannabinoids with a number of suprahypothalamic, hypothalamic and peripheral signals, the use of rimonabant, at the moment the most promising anti-obesity agent, may be accompanied by mood disturbances, especially depression, and gastrointestinal dysfunctions (Van et al., 2005).

Cholecystokinin (CCK) is secreted by duodenal and ileal cells when nutrients enter the lumen. It binds to specific receptors (CCK-1R) located on vagal sensory terminals delivering to NTS a sense of fullness. Under some experimental conditions, exogenous CCK elicited satiety and reduced meal size in different species (Gutzwiller et al., 2001; Geary, 2004). Indeed, intravenous infusion of physiological doses of CCK-33 significantly reduced the size of a single-food test meal as well as the degree of post-prandial hunger in humans (Lieverse et al., 1995). However, the CCK agonist, cerulean, failed to modify food intake both in lean and obese Zucker rats (Schwanke et al., 1998) and in obese women (Cavagnini et al., 1987). In contrast, intravenous infusion of loxiglumide, a CCK-1R antagonist, inhibited the satiating effect of intraduodenal administration of fat emulsions in healthy men (Matzinger et al., 1999). The same drug was able to reverse the premeal reduction of hunger induced by CCK-8 to human (Elena et al., 2008).

Glucose-like peptide 1 (GLP-1) is another gut hormone released in response to food intake. It enhances glucose-induced stimulation of insulin synthesis and secretion. In contrast, it suppresses glucagon secretion and delaying gastric emptying. Moreover, its infusion to rats decreases food intake and body weight (Meeran et al., 1999). In a recent meta-analysis of seven studies, a significant dose-dependent decrease in ad

libitum caloric intake was shown both in lean and obese subjects (Verdich et al., 2001). In this latter study, a diminished postprandial GLP-1 release has also been demonstrated. These notions represent a rationale for the use of GLP-1 in the treatment of obesity and diabetes mellitus. Unfortunately, the clinical employment of this molecule is limited by its short half-life (1-3 min), since it is rapidly inactivated by the enzyme dipeptidyl peptidase IV (DPPIV). Long-acting GLP-1 agonists, as well as inhibitors of DPPIV, are under investigation to circumvent this limitation. In healthy volunteers, intravenous infusion of exendin-4 (exenatide), a potent long-acting agonist of GLP-1, decreased 21% in total daily food intake, as well as a reduction in fasting and post- prandial glucose level. Exenatide is now available on the market as an adjunctive therapy to improve glycemic control in patients with type 2 diabetes mellitus treated with conventional oral antidiabetic drugs (Bray, 2006). While some studies have shown a beneficial effect of dipeptidyl peptidase IV (DPPIV), inhibitors on glucose metabolism in patients with type 2 diabetes mellitus, there is no evidence as yet on the efficacy of such drugs on weight loss in human (Ahren et al., 2002; Bays, 2004).

Peptide YY (PYY_{3-36}) is mostly released by L-cells of the distal segments of the gut in amounts correlated with the ingested calories (Batterham et al., 2003). Most, but not all, published data indicate that peripheral infusion of PYY_{3-36} reduced food intake and prolonged inter- meal intervals in several animal models. Recently, a PYY_{3-36} deficiency has been demonstrated in obesity (Le et al., 2005; Koda et al., 2005). Peripheral infusion of doses of the peptide reproducing postprandial concentrations was able to significantly reduce caloric intake in obese as well as in lean subjects (Batterham et al., 2003). PYY_{3-36} is hypothesized to act at the hypothalamus, at least in part via vagal pathways afferent to NTS (Koda et al., 2005). Its effect might

be mediated by excitement of POMC neurons and activation of anorexigenic circuits (Woods, 2004). Although PYY agonists are under active investigation, further information is still needed about the relative contribution of PYY to satiety and its potential use for weight reduction (Elena et al., 2008).

Ghrelin is the only orexigenic GI peptide isolated so far. This 28-amino-acid acylated peptide is mainly secreted by the "A-X like" cells of the oxyntic glands of the stomach and represents the chief endogenous ligand for growth hormone-secretagogue receptors (GHS-Rs). It is also synthesized by the placenta, kidney, heart, thyroid and Leydig cells (Van et al., 2004). A mounting body of evidence has demonstrated that ghrelin, in addition to its powerful GH-releasing and orexant effects, plays a remarkable role in the control of ACTH and prolactin secretion, glucose and lipid metabolism, gastric motility and acid secretion, heart function, sleep, and reproduction. In addition, ghrelin exerts antiproliferative effects both *in vivo* and *in vitro* (Van et al., 2004). Type 1a ghrelin receptor (GHS-R1a) is chiefly located in the hypothalamus-pituitary unit, especially on the NPY and GHRH neurons (Lucidi et al., 2005). In rats, ghrelin enhanced food intake in a dose dependent manner. In human, intravenous infusion of ghrelin at physiological doses induced hunger and caused short-term enhancement of food intake (Wren et al., 2001). Circulating ghrelin increases almost two-fold just before a meal and rapidly falls down postprandially (Tschop et al., 2001). Ghrelin levels elevated in anorectic patients (Tolle et al., 2003) and the levels were low in obese subjects (English et al., 2002). However, the negative feedback physiologically exerted by food on ghrelin release is lacking (Vettor et al., 2002). Notably, the reduction of caloric intake observed in obese patients following infusion of PYY_{3-36} was accompanied by a decrease in circulating ghrelin (Batterham et al., 2003). Interestingly,

the only form of human obesity characterized by elevated circulating ghrelin described so far is the one associated with Pradere-Willi syndrome (Delparigi et al., 2002) and a contribution of hyperghrelinemia to the hyperphagia of these patients has been suggested. Ghrelin action is mediated by the enhancement of NPY/AGRP pathways and the inhibition of POMC neurons (Lucidi et al., 2005) in a way opposite to that of leptin. Vagus nerve is likely to be an important mediator of ghrelin action (Van et al., 2004). Prolonged ghrelin administration to rodents was followed by an increase in fat mass and body weight, likely a result of the decreasing of lipid oxidation (Tschop et al., 2001). Antagonists of ghrelin may be a potential approach to limit food intake and reduce fat mass in obesity. However, the administration of the novel GHS-R1a antagonist BIM-28163 to rodents led to a paradoxical weight gain accompanied to the expected inhibition of GH release (Halem et al., 2005). Another peptide also derived from proghrelin and named obestatin has recently been isolated from rat stomach. Administered to rats, this peptide has been found to bind the orphan receptor known as GPR39, to suppress food intake and decrease body weight (Zhang et al., 2005).

When body weight augments, insulin resistance occurs with attendant increase in insulin secretion. Insulin enters the brain in proportion to its circulating levels, contributing to reduce energy intake through the activation of catabolic pathways (Schwartz et al., 2000). Central administration of insulin significantly reduced feeding and body weight in animal models (Vettor et al., 2002). Insulin and leptin both activate POMC neurons, but they seem to differentially regulate AGRP, with leptin inhibiting and insulin stimulating its synthesis (Wanting et al., 2005). In any case, insulin deficiency is associated with increased NPY, while insulin administration inhibits hypothalamic NPY expression (Vettor et al., 2002). Indirect and direct evidence

suggests that the two adiposity signals, leptin and insulin, not always act in concert in regulating food intake (Sindelar et al., 1999).

Glucagon is a twenty-nine amino acid peptide that reduces food intake after peripheral administration (Penick and Hinkle, 1961; Geary and Smith, 1983; Geary, 1990). Following portal vein administration in experimental animals, glucagon produces a dose-dependent inhibition of food intake. Antibodies that bind to glucagon increase food intake, suggesting that the signals generated by pancreatic glucagon act in the liver and may be physiologically relevant in modulating feeding. Glucagon decreased food intake in human subjects when given alone, but not when given simultaneously with CCK (Geary et al., 1992). Glucagon-like peptide-1 is produced by the post-translational processing of pro-glucagon, and is thought to be one signal that enhances insulin release in response to glucose incretin (gastric inhibitory peptide and/or glucagon-like peptide; Nauck et al., 1993). Infusion of glucagon-like peptide-1 peripherally in human subjects significantly reduced food intake (Flint et al., 1998).

Somatostatin is a fourteen amino acid peptide that is present in the pancreas, gastrointestinal tract and brain. Somatostatin serves to inhibit gastrointestinal motility as well as exocrine and endocrine secretions. In experimental animals somatostatin decreased food intake (Lotter et al., 1981). Somatostatin also decreased food intake in healthy human subjects (Lieverse et al., 1995). During the first 1 h of somatostatin infusion, there was a significant decrease in feelings of hunger. When an intraduodenal fat load was given at this time, it tended to reverse the feelings of satiety. The intake of sandwiches at 90 min after the fat load tended to be higher during the somatostatin infusion than during the saline infusion. Feelings of hunger were less in the 5 h after terminating the somatostatin infusion than with the control infusion (George, 2002).

Peripheral injection of serotonin reduced food intake and specifically decreased fat intake (Bray and York, 1972; Gambil and Kanarek, 1982). Since the majority of serotonin is located in the gastrointestinal tract, it seems that serotonin receptors in this tissue play an important role in the modulation of food intake, in response to enteral signals, or to the rate of gastric emptying. Tryptophan, the precursor of serotonin, also reduced food intake in human subjects (Cangiano et al., 1992).

At least four pituitary peptides have been shown to modulate food intake. The first of these is vasopressin, the anti-diuretic hormone that enhances water re-absorption from the renal tubal. Vasopressin significantly reduced food intake over a 4 h period in experimental animals. The reduction in food intake, particularly in the first 30 min of feeding, was not significantly impaired by vagotomy, suggesting that its peripheral mechanism of action is different from that of CCK or enterostatin (Langhans et al., 1991).

Following treatment with growth hormone, hypophysectomized animals increased their food intake and grow. Whether this finding is a direct effect of growth hormone on feeding centers or a consequence of the enhanced flux of amino acids into new protein and a second stimulation of feeding is unclear, but the latter appears to be a more reasonable hypothesis. Lactation increases food intake, suggesting that prolactin may increase feeding. Gettens et al. (1989) found a dose-dependent increase in food intake in response to treatment with prolactin. Injection of prolactin into the cerebroventricular system of pigs also increased food intake. Bromocriptine, a dopamine (3, 4-dihydroxyphenylethylamine)-agonist that reduces prolactin secretion, has been reported to modulate the seasonal fattening of hibernating and migratory animals. The clinical relevance of prolactin to human obesity has not been established.

The fact that prolactinomas do not produce obesity would argue against an important role (George, 2000).

Calcitonin decreased food intake in genetically-obese (db/db) and in non-obese animals (Morley et al., 1982). A strong dose-dependent suppressive effect of calcitonin on food intake can be demonstrated in animals whose feeding has been stimulated by tail pinching, a technique that is used for increasing food intake (Levine and Morley, 1981). As with β-casomorphin, the effect of calcitonin is blocked by vagotomy, suggesting vagally-transmitted afferent messages to the central nervous system (George, 2000).

2.5 Leptin

Leptin is a polypeptide produced mainly by adipocytes and liver cells. Leptin circulates as a peptide of relative molecular weight of 16 kDa. The previous study reported that the plasma levels of leptin were highly correlated with adipose tissue mass and fall in both humans and mice after weight loss. The levels of leptin increased in obese humans and induced forms of rodent obesity. Leptin acts via its receptor in the brain to regulate energy balance. Leptin concentrations in the blood are correlated with body weight and body mass index (BMI). Circulating leptin serves to communicate the state of body energy repletion to the central nervous system (CNS) in order to suppress food intake. Many effects of leptin were reported, particularly in the hypothalamus, a site of high leptin receptor. In the hypothalamus, leptin acts on neurons that regulate levels of circulating hormones, such as thyroid hormone, sex steroids, and growth hormone. Leptin does not increase significantly after a meal and does not, by itself, lead to the termination of a meal. Leptin appears to function largely within the long-

term system and influences the quantity of food consumed relative to the amount of energy expended. However, leptin was depleted from the stomach of rats after a meal or administration of CCK, indicating that it could also function in the short-term system or locally in the gastrointestinal tract (Stunkard et al., 1990; Maffei et al., 1995; Bado et al., 1998).

Leptin, the *ob gene* product, is produced mainly in the adipose tissue and enters the brain in proportion to its plasma levels. Leptin maintains long-term control on adiposity and regulates adaptive metabolic changes in response to modifications of nutritional (Ahima and Osei, 2004). Leptin is also able to regulate short-term energy intake, modulating meal size according to changes in energy balance: with negative energy balance. Low leptin signaling activates anabolic and inhibits catabolic circuits, enhances NPY/AGRP release, and blocks the activity of POMC/CART neurons which increase in meal size and decrease in energy expenditure (Schwartz et al., 2000). The opposite occurs with positive energy balance. Genetic absence of either leptin or its receptor is associated with severe obesity and hyperphagia. These features improve both in animal and human models after restoration of physiological plasma leptin levels. An 18-month treatment of recombinant human leptin to adult human leptin-deficient patients caused a weight loss greater than 40% and an initial 49% reduction of food intake (Licinio et al., 2004). Congenital leptin or leptin receptor deficiencies, however, are extremely rare in human, and the beneficial effect of leptin in essential obesity appears to be only transient (Geary, 2004; Ahima and Osei, 2004; Heymsfield et al., 1999; Rosenbaum et al., 2002). Most obese subjects display increased levels of circulating leptin, indicating obesity as a state of leptin resistance (Considine et al.,

1996). Elucidation of the molecular mechanisms underlying this alteration could provide clues for successful treatment of obesity (Elena et al., 2008).

Leptin replacement corrected abnormalities, implying that *ob* mice exist in a state of "perceived starvation" and that the resulting biological response in the presence of food leads to obesity. The idea that decreased plasma leptin levels signal nutrient deprivation was supported by the observation that exogenous leptin attenuated the neuroendocrine response to food restriction (Ahima et al., 1996). These observations led to a speculation that main physiological role of leptin is to signal nutritional status during periods of food deprivation. However, leptin role in preventing excess weight gain has been shown to be physiologically significant (Friedman and Halaas, 1998).

The effect of leptin on adipocyte metabolism has been published related to an investigation of the effects of leptin on adipocyte metabolism compared with the large number of studies that have examined the effects of central or peripheral leptin administration on whole animal energy balance. Adipose tissue expresses both long- and short-form receptors which exert direct metabolic effects of leptin on adipocytes. The change in metabolism may also be achieved indirectly through modification of release of metabolically active hormones, changes in response to these hormones or by increasing the activity of sympathetic afferents to the fat cell. Administration of leptin to leptin-responsive animals reduced body fat mass (Kielar et al., 1998; Harris et al., 2001).

Leptin receptors have been found in several hypothalamic nuclei, including the ARC, VMH, LH, DMH and PVN. NPY is the most potent orexigenic agent known when administered intrathecally. The LH and VMH project both within and outside the hypothalamus and modulate activity of the parasympathetic and sympathetic nervous

systems, respectively. The DMH also sends inputs to the parasympathetic nervous system and may be involved in integrating information among the VMH, LH, and PVN. The PVN controls secretion of peptides from both the posterior and anterior pituitary and projects to nuclei with sympathetic or parasympathetic efferents. Leptin concentrations are sensed by groups of neurons in the hypothalamus. During starvation, leptin levels fall, thus activating behavioral, hormonal and metabolic responses that are adaptive when food is unavailable. Weight gain increases plasma leptin concentration and elicits a different response, leading to a state of negative energy balance. It is not yet known whether the same (or different) neurons respond to increasing and decreasing leptin levels. The range of leptin effects is likely to be complex, as different thresholds exist for several of leptin actions (Ioffe et al., 1998).

Also, the mechanism by which centrally administered leptin leads to lipolysis and the loss of adipose tissue mass is unknown. The metabolic response to leptin is markedly different from the response to reduced food intake. Whereas food restriction (dieting) leads to the loss of both lean body mass and adipose tissue mass. Leptin induced weight loss is specific for the adipose tissue mass. Leptin also prevents the reduced energy expenditure normally associated with decreased food intake. Moreover, in contrast to food-restricted (pair-fed), a marked increase in serum-free fatty acids was shown. Hyperleptinemia animals underwent a rapid period of weight loss fail to show a rise in serum-free fatty acids or ketones (Halaas et al., 1995; Pelleymounter et al., 1995).

However, recent evidence suggests that leptin was also expressed in lower vertebrates including fish. Leptin-like immunoreactive material was detected in blood and tissues of amphibians (Muruzabal et al., 2002) and reptiles (Muruzabal et al., 2002;

Paolucci et al., 2001). Peripheral injections of murine leptin decreased food intake and increase metabolic rates in lizards (Niewiarowski et al., 2000).

Leptin-like immunoreactive material was detected in several species of fish (Johnson et al., 2000; Mustonen et al., 2002; Nieminen et al., 2003; Yaghoubian et al., 2001). With western blot analysis using anti-human leptin antibodies, a leptin-like peptide with the molecular weight of 15 kDa was identified in salmon adipocytes (Vegusdal et al., 2003). Leptin-treated green sunfish, *Lepomis cyanellus*, displayed an increase in the enzymatic activity of several indicators of intracellular fat metabolism, suggesting that leptin induces an overall increase in fat metabolism (Londraville and Duvall, 2002). In teleosts, leptin was first identified in pufferfish (*Takifugu rubripes*), using genomic synteny around the human leptin gene. Subsequent identification of leptin in other teleosts included carp, goldfish (*Carassius auratus*), Atlantic salmon (*Salmo salar*), and zebrafish (*Danio rerio*) (Kurokawan et al., 2005; Huising et al., 2006; De Pedro et al., 2006; Murashita et al., 2008; Runnestad et al., 2010; Gorissen et al., 2009).

In goldfish, higher peripheral doses of leptin were required to cause a decrease in food intake than when administered centrally, suggesting that in fish, as in mammals, leptin acts primarily in the brain to control energy homeostasis (Volkoff et al., 2003). Also in goldfish, central injections of leptin accentuated the anorexigenic effects of CART and CCK and inhibited both NPY and orexin induced food consumption, and these actions were associated with an increase in both CCK and CART mRNA expression and a decrease in NPY mRNA expression in brain (Volkoff and Peter, 2001a; Volkoff et al., 2003). These data suggest that in fish, as in mammals, leptin interacts with hypothalamic pathways to inhibit food intake. As mentioned previously,

data suggest that the actions of leptin in fish are mediated in part by CCK (Volkoff et al., 2003).

In yellow catfish (*Pelteobagrus fulvidraco*), the distributions and expression levels of leptin and leptin receptor were examined in pituitary, heart, liver, spleen, intestine, white muscle, mesenteric fat, ovary, and testis. The leptin gene was expressed in a wide range of tissues, but their expression levels varied. The leptin mRNA expression levels were highest in liver, followed by ovary, mesenteric fat, and spleen, and lowest in intestine, heart, muscle, pituitary, and testis. The leptin receptor mRNA levels were highest in pituitary, intermediary in mesenteric fat, liver, ovary, muscle and spleen, and were lowest in heart, intestine, and testis, respectively (Yuan et al., 2013).

Leptin immunoreactivity was found to be scattered in stomach cells of non-mammalian vertebrate species including trout, frog, lizard, and snake. Leptin immunolabeling was identified in oxyntic glands of frog and reptilian species, but not in trout. In trout and lizard, leptin immunoreactivity was found scattered in cells located at typical of endocrine cells. Leptin immunoreactivity was found in epithelium of the enteric nervous system. Leptin immunoreactivity was also observed in myenteric plexus of trout, as well as those of frogs and snakes (Muruzabal et al., 2002).

Immunohistochemical analysis revealed the expression of leptin and leptin receptors in large and roundish cells organized in clusters of human and rats. Immunostaining of these cells was observed. In rat carotid bodies, cytoplasmic staining for leptin and leptin receptors were diffusely positive. The immunoreactivity of both leptin and leptin receptors was intense and widely distributed in the cytoplasm. Immunostained cells were found in both the center and periphery of lobules in carotid body (Porzionato et al., 2011).

In Chinese mitten crab, *Eriocheir sinensis*, the leptin receptor is a single transmembrane glycoprotein with homology to interleukin 6 that belongs to the class I cytokine receptor superfamily (Morash et al., 2003). Isoforms of leptin receptors with cytoplasmic regions of varying lengths is due to alternative splicing. The previous study identified a partial EST clone from the hepatopancreas library in *E. sinensis* that shares high sequence identity to *lepr* gene from other species (Jiang et al., 2009). This was the first report of a crustacean *lepr* gene, which may regulate male reproduction in this species, similar to its role in other animals. The *lepr* cDNA contains a 402 nucleotide open reading frame (ORF) encoding a putative polypeptide of 133 amino acids with a predicted molecular weight of 14.42 kDa. Alignment of *E. sinensis lepr* gene with other *lepr* amino acid sequences from vertebrates and invertebrates showed that they shared similarities (Hui et al., 2010).

Quantitative real-time RT-PCR was employed to investigate the distribution of *lepr* expression in different tissues from healthy immature *E. sinensis*. The results of these experiments showed that *lepr* was expressed in all of the tissues examined, but the expression levels varied between tissues. *Lepr* expression levels were highest in intestine, thoracic ganglia, gonad and accessory gonad, moderate in hepatopancreas and cranial ganglia, and only trace levels of expression were detected in muscle, gill, heart, haemocytes and stomach (Hui et al., 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Animal preparation

Male and female snails were collected from rice fields in Chum Phuang sub-district, Nakhon Ratchasima province (15°20’6” N 102°44’31” E) in April 2014. They were housed in soil-made containers filled with de-chlorinating water in the ratio of 8:1 (female:male), and fed daily with morning glories. They were acclimatized for at least 2 weeks prior to experiments. The snails were kept under a normal day/light cycle (12/12 h) and the temperature was ranged between 25-28ºC. Adult snails were used in the experiments. For the classification of the snail ages, sizes of operculum were measured. The adult sizes are usually more than 3 cm (Pain, 1960). Female snails were used in the experiments, because they consumed more food.

3.2 Methods

3.2.1 Tissue preparations for light and electron microscopies

3.2.1.1 Light microscopy

After euthanization by placing the snails at -20ºC for 10 min, the gastrointestinal (GI) tract was removed and separated into parts; esophagus, stomach, intestine, and rectum. The tissues were then fixed in Bouin’s solution (Bio-optica, Milano, Italy) for 24 hr. dehydrated with graded series of ethanol (70%-100%), and

finally embedded in paraffin. The tissue blocks were cut serially with a rotary microtome at the thickness of 5 µm. The sections were stained with hematoxylin and eosin (Bio-optica) using the conventional method. Briefly, the sections were immerged in xylene (Fisher Chemical, New Jersey, NY) for an elimination of paraffin. Subsequently, the sections were rehydrated by immerging in graded series of ethanol (100-70%) and then distilled water, followed by staining with hematoxylin and eosin (Bio-optica). Finally, the samples were dehydrated with graded series of ethanol (70% -100%), cleared with xylene, and mounted with permount (Fisher Chemical). The experiments were repeated using tissues from five different individuals.

Heights of the epithelial cells in parts of the GI tract were measured according to the method modified from Mustafa and Qasem (2016). Five hematoxylin and eosin stained sections were selected from each part of the GI tract. From each section, 10 microscopic fields were photographed (a microscopic field area equaled 0.12 nm^2). The measurements were conducted from the basement membrane to the apical surface of the epithelial cells using a computerized image analysis program (Cell^D software; licensed to Suranaree University of Technology).

3.2.1.2 Scanning electron microscopy

Conventional preparation for tissue viewing under a scanning electron microscopy was performed according to the method modified from Martin et al. (2010). After euthanization of the snails, parts of the GI tract (as described above) were removed and fixed with 4% paraformaldehyde (Acros Organics, New Jersey, NY) overnight at 4°C. The tissues were washed with 0.1 M phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and post-fixed with 1% osmiumtetroxide (Electron Microscopy Sciences, Hatfield, PA) in PBS at 4°C for

4 hr. After washing with PBS, the tissues were dehydrated through a graded ethanol series. Critical point drying (Samdri PVT-3B, Tousimis Research Corporation, Rockville, MA) with liquid CO_2 was then performed, followed by coating the tissues with gold by ion sputtering JFC-1100E. Finally, the coated tissues were viewed under a JEOL-JSM-6010LV scanning electron microscope.

3.2.1.3 Transmission electron microscopy

Conventional preparation for tissue viewing under a transmission electron microscopy was performed according to the method modified from Lobo-da-Cunha (2001). Parts of the GI tract (as described above) were fixed with 4% paraformaldehyde (Acros Organics), overnight at 4^{o}C. The tissues were washed with PBS and then post-fixed with 1% osmiumtetroxide (Electron Microscopy Sciences) in PBS at 4^{o}C for 4 hr. The tissues were subsequently dehydrated through a graded ethanol series and embedded in Araldite 502 resin (Electron Microscopy Sciences). Semi-thin and ultrathin sections were cut. The semi-thin sections were stained with methylene blue and the ultra-thin sections were stained with lead citrate and uranyl acetate. The ultra-thin sections were examined and photographed using a JEOL-JEM-2010 transmission electron microscope.

3.2.2 Study of the presence of mucin-secreting cells in the GI tract

Paraffin sections from each part of the GI tract were used to study the presence of neutral and acidic mucins. Staining methods were performed according to the procedures modified from Mcmanus (1946) and Mowry (1956). The sections were deparaffinized with xylene, rehydrated with graded series of ethanol, and then stained with Periodic acid-Schiff (PAS) or alcian blues, pH 1.0 and 2.5 (Sigma Aldrich, St. Louis, MO). Subsequently, nuclei of the cells were stained with hematoxylin (Bio-

optica) or nucleus fast red (Sigma Aldrich). Alcian blue, pH 1.0 is able to detect sulfomucins, while alcian blue at pH 2.5 stains sialomucins. In addition, the sections were also counter-stained with alcian blue, pH 2.5 and PAS. Finally, the stained sections were then dehydrated, cleared, mounted, and observed under a light microscope.

Percentages of mucin-secreting cells from parts of the GI tract were calculated. Five sections were selected from each part of the GI tract (esophagus, intestine, and rectum). From each section, 10 microscopic fields (each field equaled 0.12 mm^2) were photographed. Percentages of the mucin-secreting cells were then calculated as described below.

$$\text{Percentage of mucin-secreting cells} = \frac{\text{Number of mucin-secreting cells}}{\text{Total number of epithelial cells}} \times 100$$

Percentages of the mucin-secreting cells were compared between parts of the GI tract by one-way analysis of variance (ANOVA), followed by a Tukey Post Hoc multiple comparison. The probability value less than 0.05 ($p<0.05$) was used to indicate a significant difference.

3.2.3 Study of the localization of leptin-like peptide-secreting cells in the GI tract using immunoperoxidase method

Immunoperoxidase technique is a method that uses labeled antibodies to localize antigens within a tissue. The antibody is conjugated to an enzyme which will catalyze a color-producing reaction. In order to determine the localization of leptin-like peptide in the GI of the snails, the method was performed according to a previous study (Gambadella et al., 2010). The paraffin sections were deparaffinized, rehydrated,

followed by an elimination of endogenous peroxidase and picric acid using 1% hydrogen peroxide and 1% lithium carbonate (Sigma Aldrich), respectively. Free aldehyde was eliminated by 0.1 M glycine (Acros Organics), and cell membrane was broken by 1% Triton X-100 (Fibertech Co., Ltd, Seoul, Korea). Subsequently, the sections were placed in citrate buffer (pH 6, Acros Organics) and heated in a microwave oven for 10 min, 3 times, for an increasing of the binding between the antigen and the antibody. The non-specificity was blocked by emerging the sections in 0.1 M PBS containing 4% bovine serum albumin (BSA, Acros Organics) for 1 hr at room temperature. The sections were then incubated with rabbit anti-human leptin polyclonal antibody (Abcam, Cambridge, MA), at the dilution of 1:100, and left at 4^{o}C, overnight. The sections were extensively washed with PBS containing 0.1% Tween-20 (Amresco, Solon, OH), twice (10 min, each), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) (1:500) for 1 hr at room temperature. After extensively washing, the antigen-antibody complex was visualized by adding the substrate, 3, 3'-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA). After stopping of the enzyme-substrate reaction, the sections were dehydrated, cleared with xylene, mounted using permount, and observed under a light microscope. Negative control was performed by omitting the primary antibody.

3.2.4 Detection of expression of leptin-like peptide in the gastrointestinal tract using Western immunoblotting technique

Western immunoblotting method was performed according to a previous study (Gambadella et al., 2010). Parts of the GI tract (esophagus, stomach, intestine, and rectum) were separated, immediately immerged in liquid nitrogen, and then kept at -80^{o}C until used. After thawing, cells in each tissue were lysed in lysis buffer (10 mM

Tris-HCl, 150 mM NaCl, 0.5% Triton X-100) containing protease inhibitors; 1 mM methylenediaminetetraacetic acid (EDTA) and 100 mM phenymethylsulfonyl fluoride (PMSF, Sigma Aldrich) using a hand homogenizer. After the homogenization, the extracts were centrifuged at 15,000 rpm for 20 min, at 4^{o}C. The supernatants containing crude protein were collected, and the protein concentrations were measured using Bradford kit (Thermo Scientific). The crude proteins (20 μg, each) and leptin standard (positive control, 2 μg, Biovision, Mountain View, CA) were mixed with loading dye, boiled at 95^{o}C for 5 min, and then loaded in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 20 milliamps, constantly). The proteins were subsequently transferred to a nitrocellulose membrane (constant at 100 volts for 1 hr). The membrane was then emerged in 0.1 M PBS containing 5% skim-milk (Fluka Analytical, St. Louis, MO), for an elimination of non-specific binding (1 hr, at room temperature). Rabbit anti-human leptin (1:1,000, Abcam) was then applied to the membrane and incubated overnight at 4^{o}C. After extensively washing twice with PBS containing 0.1% Tween-20, HRP-conjugated goat anti-rabbit IgG (1:5,000, Invitrogen) was added to the membrane, followed by extensively washing. The presence of antigen-antibody complex was visualized by DAB enhanced liquid substrate system (Vector Laboratories). Negative control was performed by using pre-adsorbed primary antibody (50 μg leptin peptide/0.1 ml primary antibody at working dilution).

Analysis of intensities of leptin-like peptide immunoreactive bands in the parts of the GI tract (esophagus, stomach, intestine, and rectum) was conducted using Image J software. A box of 50 × 50 pixels was generated and placed over the immunoreactive bands. In each immunoreactive band, 5 areas were randomly selected and the intensities

were then analyzed. The experiments were repeated using tissues from 5 different individuals. The intensities were shown as mean ± S.D. The means among groups were compared using one-way analysis of variance (ANOVA), followed by a Tukey Post Hoc multiple comparison. The probability value less than 0.05 ($p<0.05$) was used to indicate a significant difference.

3.2.5 Measurement of levels of leptin-like peptide compared between fed and fasted snails using enzyme-linked immunosorbent assay (ELISA)

3.2.5.1 Animal grouping

The snails were divided into 2 groups, fed and fasted groups. In both groups, the snails were euthanized at days 0, 5, 10, and 15 (n = 6, each). Result of preliminary study (Western immunoblotting) indicated that the highest level of leptin-like peptide was observed in the esophagus. The whole length of the esophagus was then used in ELISA assay.

3.2.5.2 ELISA assay

The objective of an ELISA is to determine levels of particular proteins presented in samples. Esophagus of fasted and fed animals was collected at days 0, 5, 10, and 15. After dissection, the tissues were immediately immerged in liquid nitrogen, and kept at -80^{o}C until used. Subsequently, crude proteins from the esophagus were extracted in lysis buffer containing protease inhibitors; 1 mM EDTA and 100 mM PMSF using a hand homogenizer. After the homogenization, the samples were centrifuged at 15,000 g for 20 min, at 4^{o}C. The supernatants (crude proteins) were collected, and the protein concentrations were measured using Bradford kit (Thermo Scientific). ELISA assays were performed according to the method described by

Ngernsoungnern et al. (2012). Human leptin (Biovision) was used as the protein standard. The standard was serially diluted with ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) to the concentrations of 12.5, 6.25, 3.125, and 1.5625 ng/ml. The crude proteins were also diluted with ELISA coating buffer to the amount of 20 μg. The diluted standard and crude proteins were then coated onto an ELISA plate in triplicates and left at 4ºC, overnight. After that, the plate was washed twice with 0.01 M PBS containing 0.05% Tween-20 (Amresco), followed by incubating with 0.25% BSA (Acros Organics) in PBS. The primary antibody, rabbit anti-human leptin (1:5,000, Abcam) was applied to the plate and incubated at 37ºC for 2 hr. After washing, HRP-conjugated goat anti-rabbit IgG (Invitrogen) was added and the plate was left at 37ºC for 1 hr, followed by washing. Subsequently, 3, 3', 5, 5'-tetramethylbenzidine (TMB, Thermo Scientific) was added for developing of color. The reaction was stopped with 2 M sulfuric acid (H_2SO_4) and to color was read spectrophotometrically at 450 nm. The sensitivity of the assay was 0.125 ng/ml.

3.2.5.3 Statistical analysis

Levels of leptin-like peptide were shown as mean ± S.D. from triplicates. The means were compared between fasted and fed groups collected at the same day by one-way analysis of variance (ANOVA), followed by a Tukey Post Hoc multiple comparison. The probability value less than 0.05 ($p<0.05$) was used to indicate a significant difference.

CHAPTER IV

RESULTS

4.1 Light microscopic study of the gastrointestinal tract

The gastrointestinal (GI) tract of the snail composed of the esophagus which connected the mouth to the stomach (Figure 4.1). The esophagus situated ventrally to the buccal mass and entered the rostral part of the stomach. Salivary glands were situated along the sides of the esophagus. The stomach is embedded within a digestive gland. Follow the length of the stomach was the intestine. The distal part of the intestine connected to the rectum, and the ended part of the GI tract was the anus.

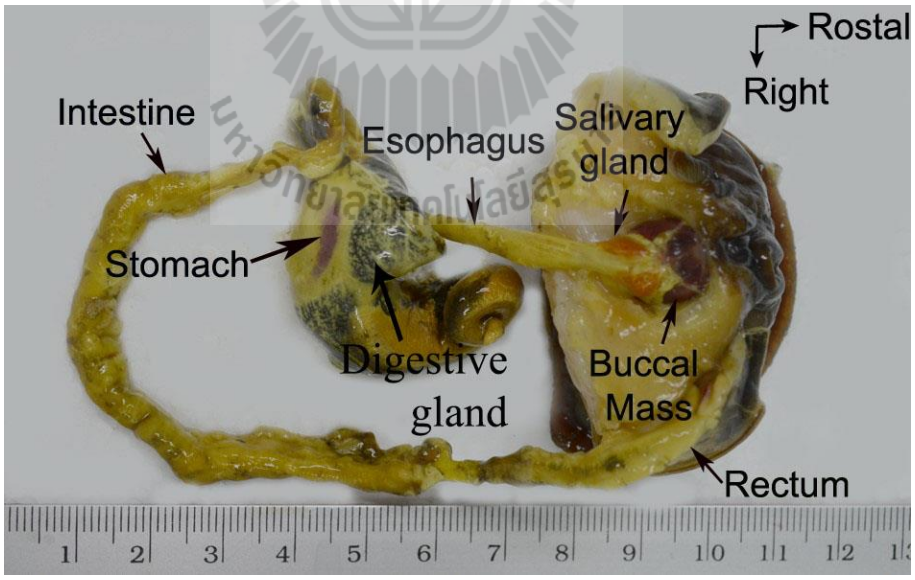

Figure 4.1 Gross structure of the GI tract. The GI tract composed of the esophagus, stomach, intestine, and rectum.

All parts of the GI tract were lined with simple columnar epithelium with microvilli presents on the apical region of the cells (Figure 4.2). All cells were rested on the same basement membrane and their nuclei were located at the basal region. In the intestine, nuclei of the cells appeared to locate in different levels giving an appearance of pseudostratified columnar epithelium. However, all cells were in contact with the basement membrane.

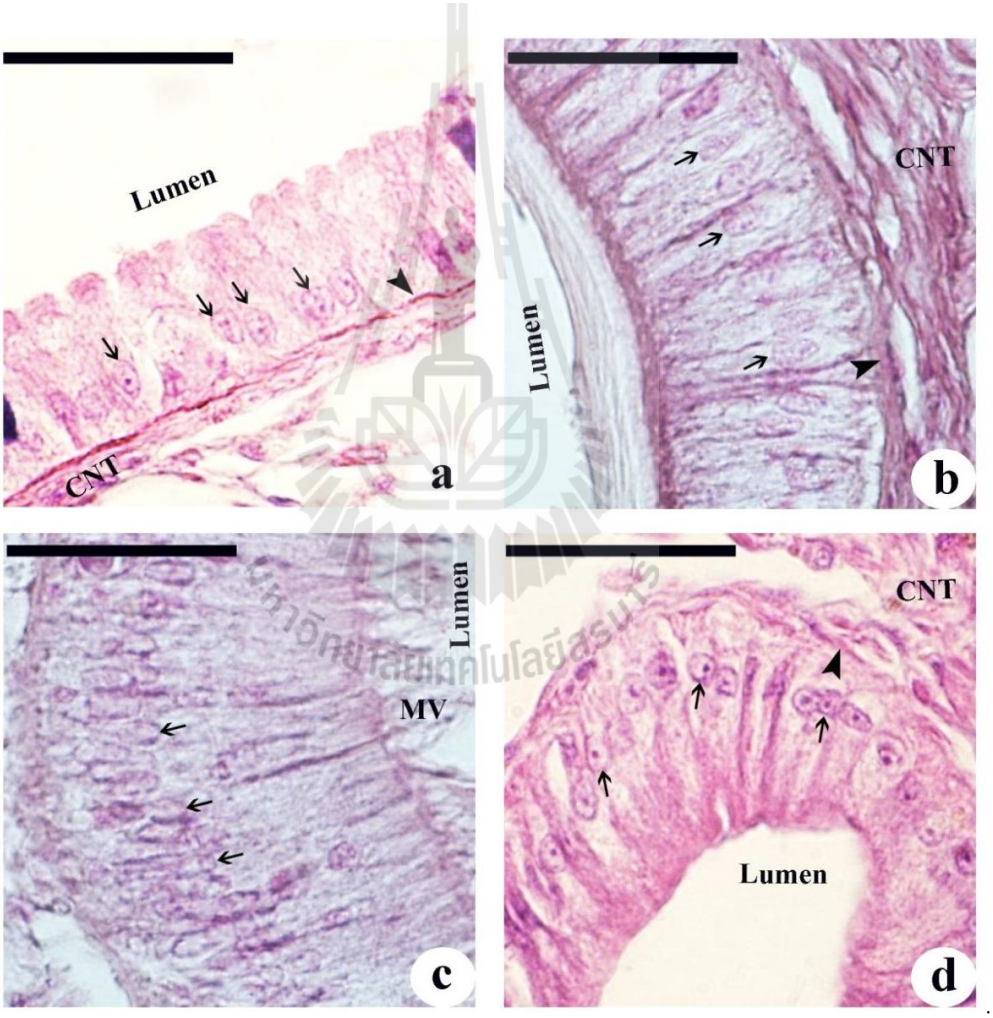

Figure 4.2 Photographs showing histology of parts of the GI tract. a = esophagus; b = stomach; c = intestine; d = rectum; arrows = nuclei; arrowheads = basement membrane; CNT = connective tissue; MV = microvilli. Scale bars = 100 µm.

Cell^D software was used as the tool for measuring heights of the epithelial cells lining parts of the GI tract. It was shown that the epithelial cells of the intestine had the height of 2.00 ± 0.19 µm which was significantly greater ($p<0.05$) than those of the esophagus (0.08 ± 0.05 µm), stomach (1.10 ± 0.06 µm), and rectum (1.00 ± 0.07) (Figure 4.3).

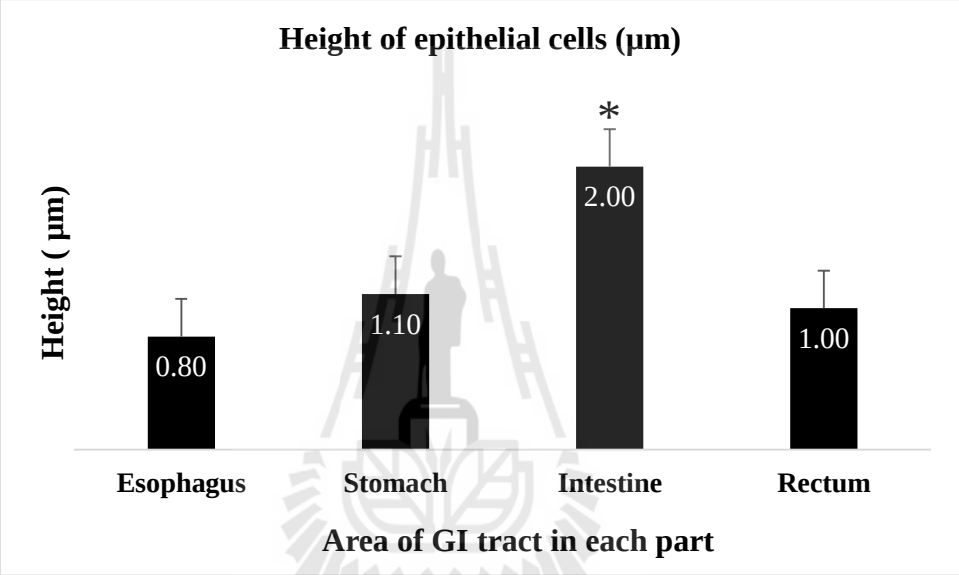

Figure 4.3 Heights of epithelial cells in parts of the GI tract.

4.2 Scanning electron microscopic study of the gastrointestinal tract

Scanning electron micrographs of the GI tract showed similarities in the structures of all parts of the GI tract. In the esophagus, the mucosa consisted of numerous mucosal folds (Figure 4.4a). The mucosal surface of the esophagus was lined by simple columnar epithelium which inserted with mucous-secreting cells (Figure 4.4b). The stomach showed similarity in the structure compared to that of the esophagus. Large numbers of the mucosal folds were observed in the stomach. However, the height of the mucosal folds of the stomach was greater than those of other

regions (Figures 4.4c and d). The mucosa of the intestine also covered with simple columnar epithelial cells (Figure 4.5a). In the rectum, numerous primary and secondary mucosal folds and crypts were observed (Figure 4.5c). The intestine and rectum showed the microvilli which covered the apical surface of epithelial layer (Figures 4.5b and d). The length of the microvilli was markedly higher in the intestine than those of other regions (Figures 4.5b and d).

4.3 Transmission electron microscopic study of the gastrointestinal tract

Transmission electron micrographs revealed that mucosa of the esophagus was lined with simple columnar epithelium (Figure 4.6a). Numerous microvilli were covered the apical surface of the epithelial cells (Figure 4.6a and b). Nuclei of the epithelial cells contained euchromatins. Mucous-secreting cells were found inserted within the epithelial cells (Figure 4.6c). Moreover, collagen fiber and fibrocytes were identified in the laminar propia (Figures 4.6a, c and d).

Mucosa of the intestine also lined with simple columnar epithelium with microvilli covered at the apical surface of the cells (Figure 4.7a). Cell nuclei were in different levels (Figure 4.7b). Euchromatins were observed within the epithelial cells nuclei. Golgi apparatus and rough endoplasmic reticulum were prominence in the cytoplasm. Other cell organelles, for example, mitochondria were also identified (Figure 4.7c)

Epithelial cells of the rectum were also simple columnar with microvilli. Mucous-secreting cells were inserted with the epithelial cells (Figure 4.8a). Tight junctions and desmosomes were found connected the adjacent epithelial cells, and

adherens junctions were observed between the epithelial cells and the mucin-secreting cell. The epithelial cells nuclei also contained euchromatins. Numerous lysosomes were found in the cytoplasm (Figure 4.8c).

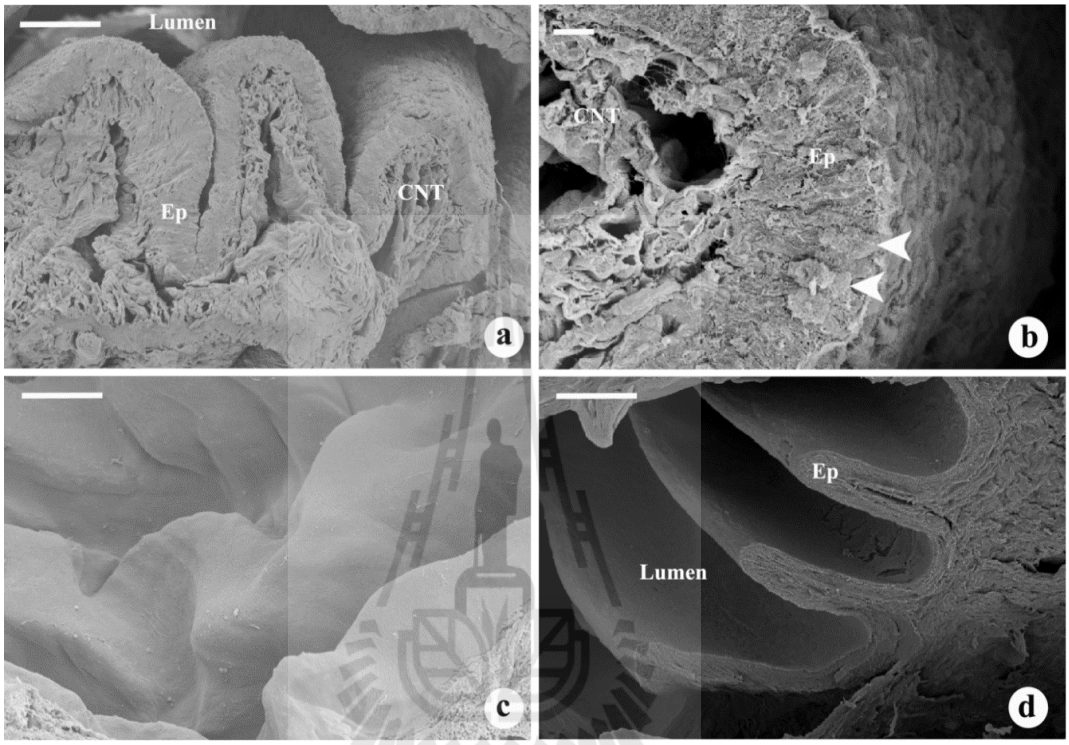

Figure 4.4 Scanning electron micrographs of the mucosa of the esophagus (a and b) and stomach (c and d). **a)** Mucosal folds were observed in the esophagus. **b)** Simple columnar epithelium of the esophagus was inserted with mucin-secreting cells (arrowheads). **c)** Mucosal folds of the stomach. **d)** Mucosal folds of the stomach. Ep = epithelium; CNT = connective tissue. Scale bars: a and c = 100 µm, b = 10 µm, d = 50 µm.

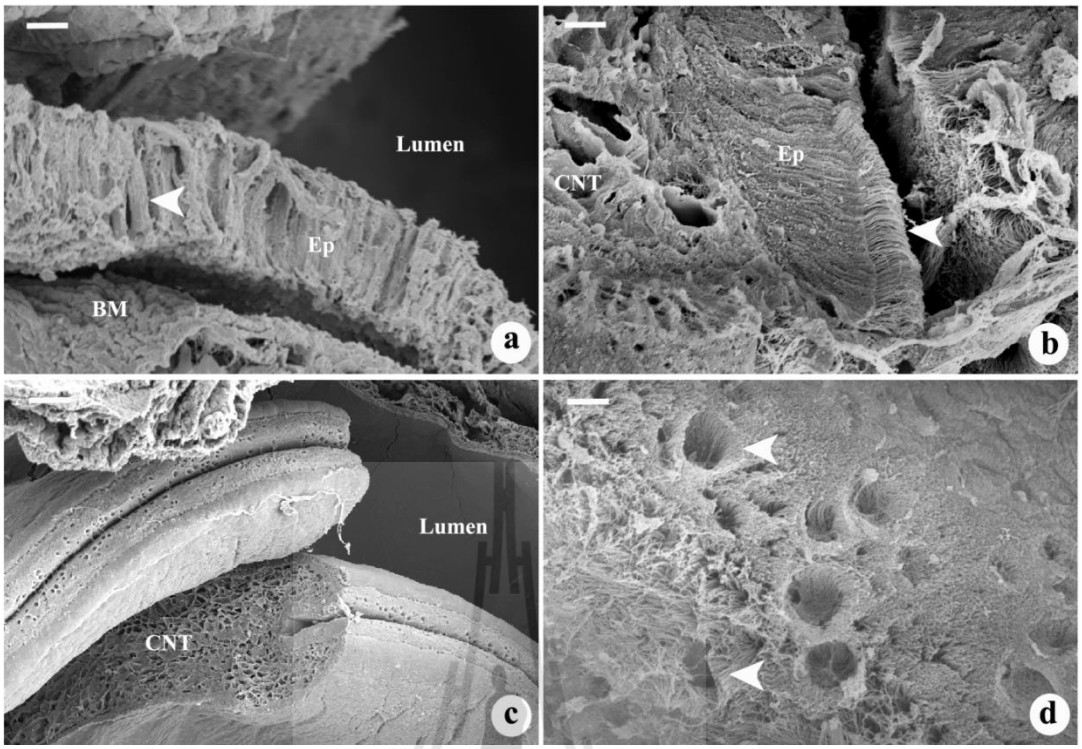

Figure 4.5 Scanning electron micrographs of the mucosa of the intestine (a and b) and rectum (c and d). **a**) Epithelial cells lined of the intestine were simple columnar (arrowhead). **b**) Apical surface of the intestine epithelial cells was covered with microvilli (arrowhead). **c**) Mucosal folds of the rectum and the center of the folds showed crypts. **d**) Apical surface of the rectum epithelial cells contained microvilli (arrowheads). BM = basement membrane; CNT = connective tissue. Scale bars: a, b, and d = 10 µm, c = 100 µm.

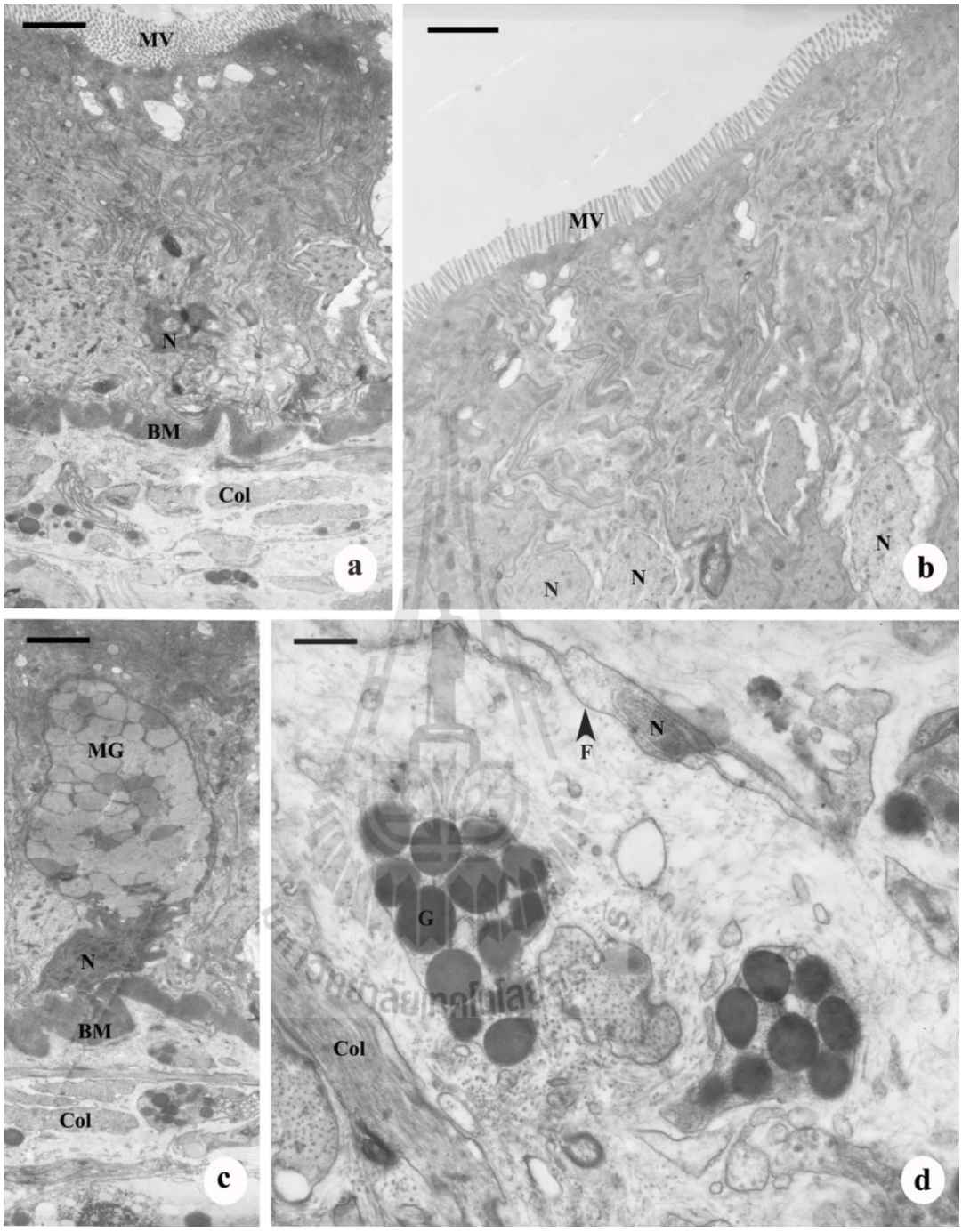

Figure 4.6 Transmission electron micrographs of the mucosa of the esophagus. BM, basement membrane; Col = collagen fiber; F = fibrocyte; G = granule; MG = mucous granule; MV = microvilli; N = nucleus. Scale bar: a-c = 2 μm; d = 500 nm.

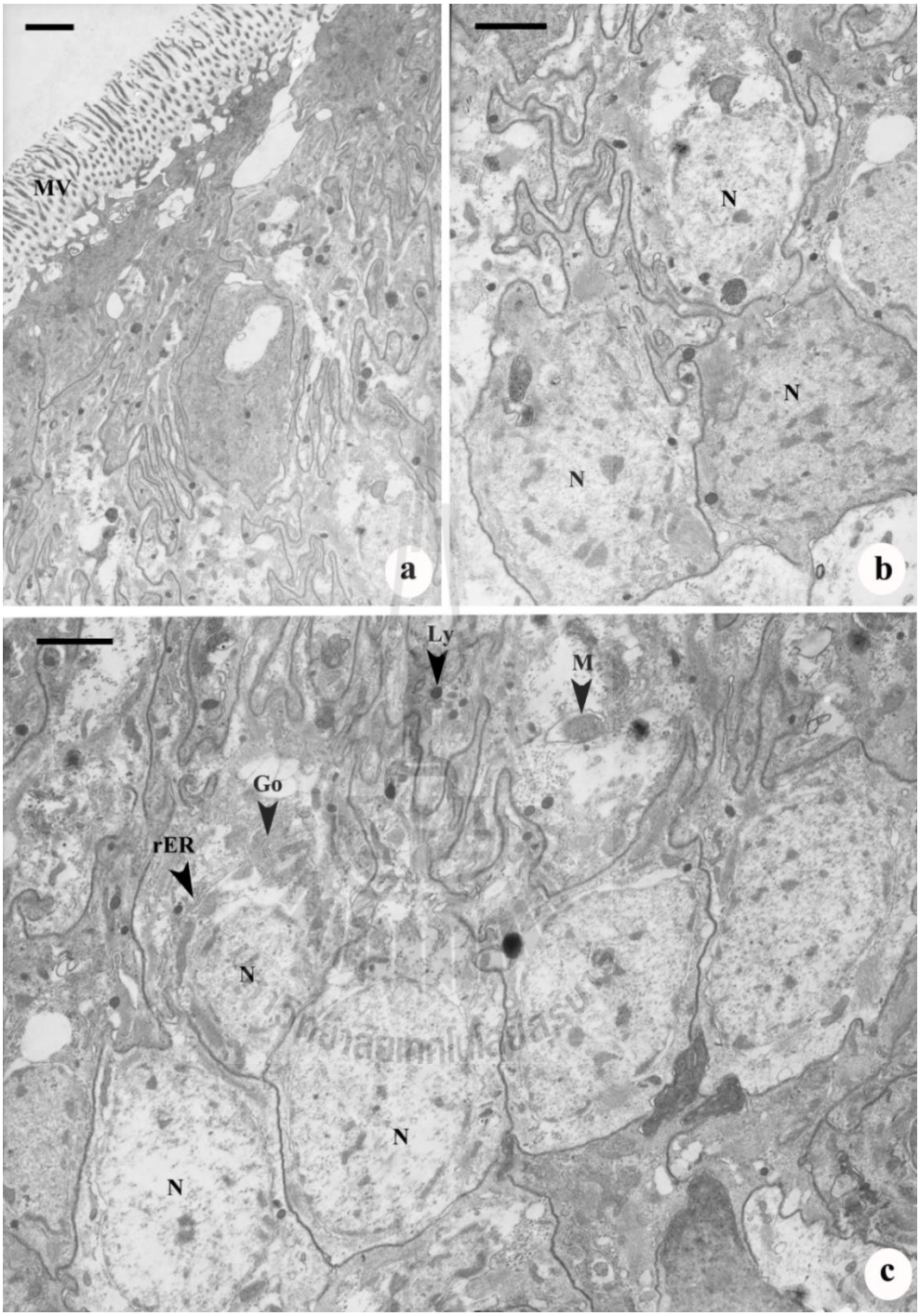

Figure 4.7 Transmission electron micrographs of the mucosa of the intestine. Go = Golgi apparatus; Ly = lysosome; M = mitochondria; MV = microvilli; N = nucleus; rER = rough endoplasmic reticulum. Scale bar = 1 μm.

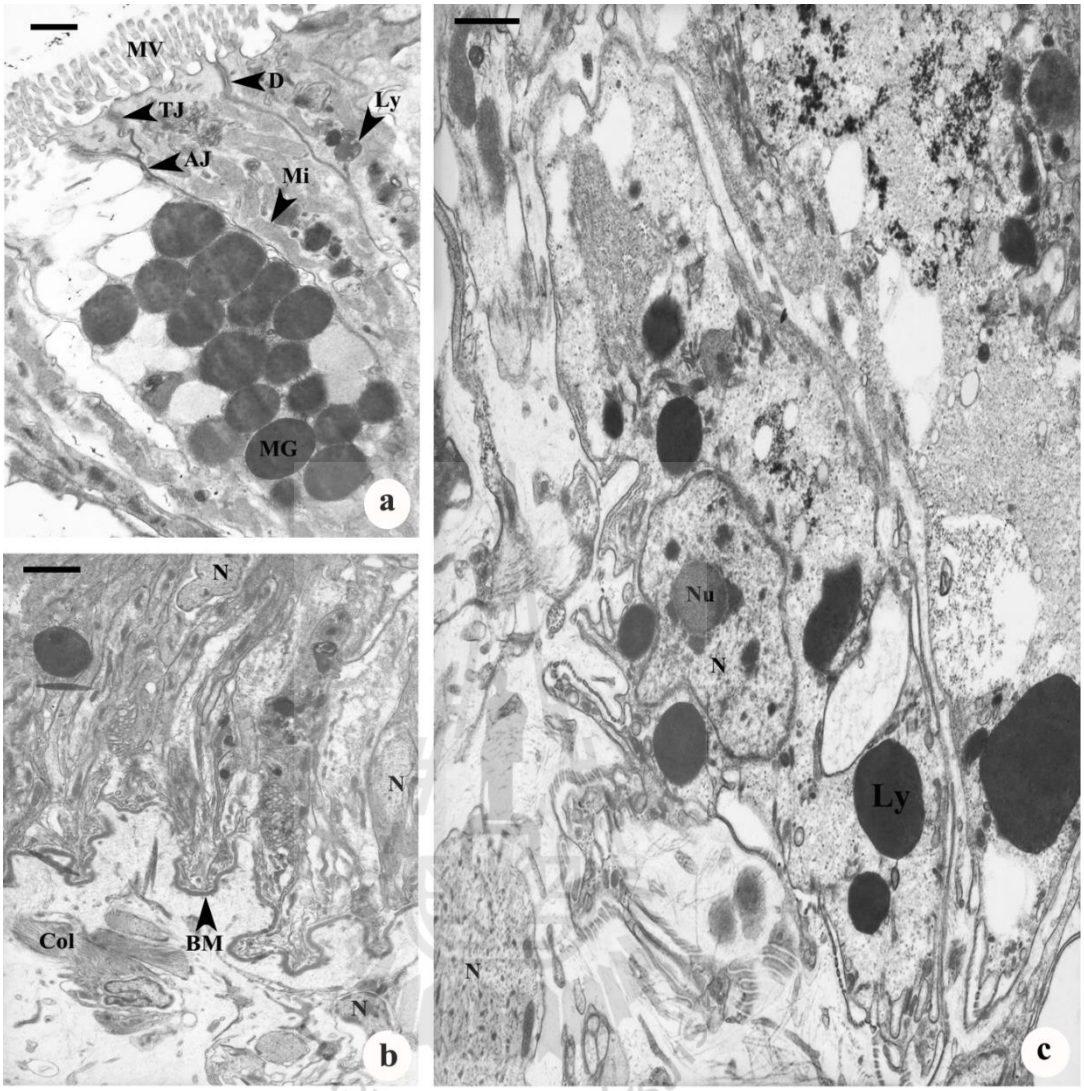

Figure 4.8 Transmission electron micrographs of the mucosa of the rectum. AJ = adherens junction; BM = basement membrane; Col = collagen fiber; D = desmosomes; Ly = lysosome; Mi = mitochondria; MG = mucous granule; MV = microvilli; TJ = tight junction; N = nucleus. Scale bars: a = 500 nm; a = 2 µm; a = 1 µm.

4.4 Presence of mucin-secreting cells in the gastrointestinal tract

Using periodic acid-Schiff (PAS) staining technique, the result showed that neutral mucin-secreting cells were observed throughout the GI tract, except in the stomach (Figure 4.9). The neutral mucin-secreting cells were in pear-shaped. The apical part of the cells was in contact with the lumen of the GI tract indicating the release of mucin substances into the GI tract lumen.

Alcian blue pH 2.5 staining showed numerous sialomucin-secreting cells in the esophagus, intestine, and rectum, but not in the stomach (Figure 4.10). The sialomucin-secreting cells were also in pear-shaped and their apical regions were in contact with the GI tract lumen.

Alcian blue pH 1.0 was able to stain numerous sulfomucin-secreting cells in the esophagus, intestine, and rectum (Figure 4.11). However, no sulfomucin-secreting cell was observed in the stomach.

Counter-staining of PAS and alcian blue, pH 2.5 detected the presence of both neutral and acidic mucin-secreting cells (Figure 4.12). The cells were found in all parts of the GI tract, except in the stomach. In addition, the neutral and acidic mucins were localized in different cells.

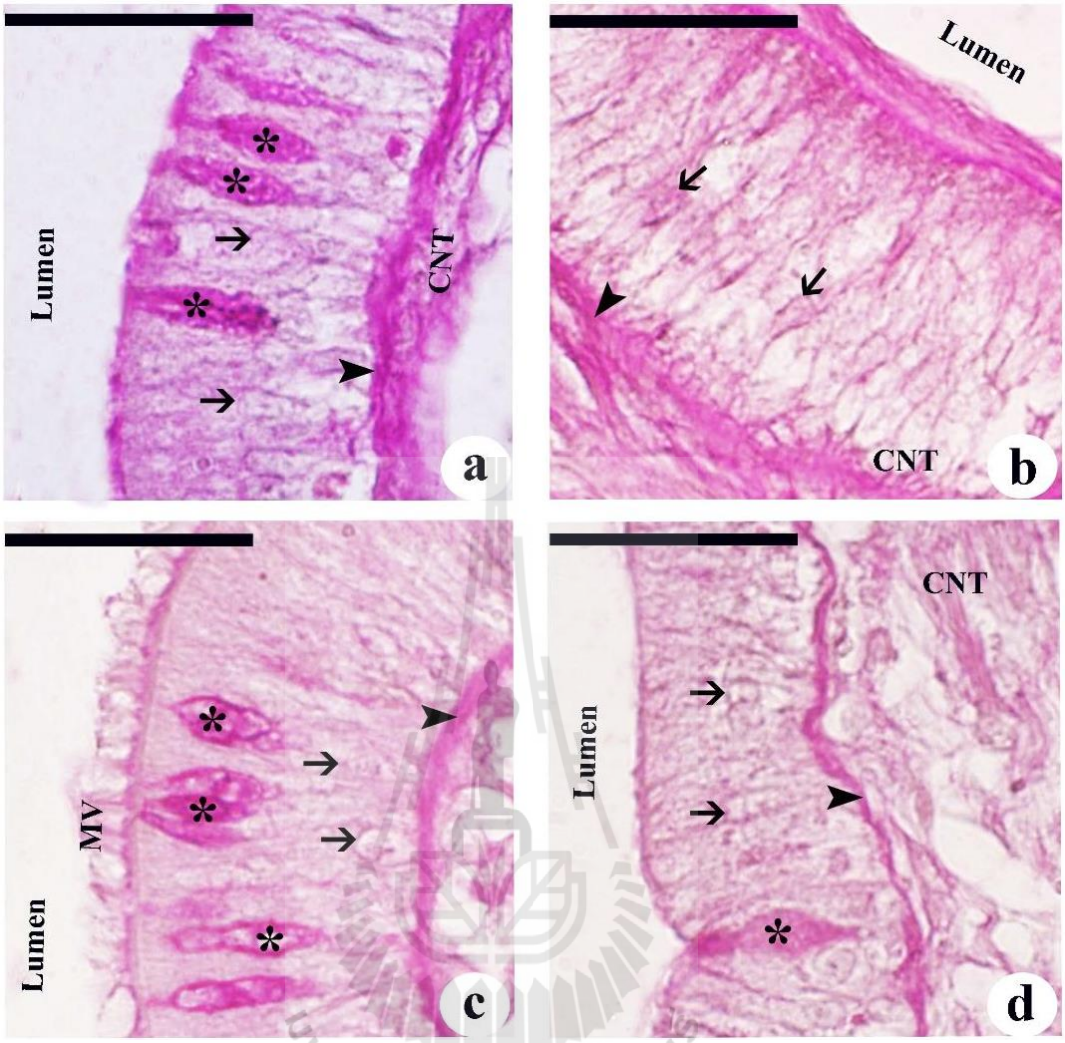

Figure 4.9 Photographs showing neutral mucin-secreting cells stained with PAS. Asterisks indicate the mucin-secreting cells. a = esophagus; b = stomach; c = intestine; d = rectum; arrows = nuclei; arrowheads = basement membrane; CNT = connective tissue; MV = microvilli. Scale bars = 100 μm.

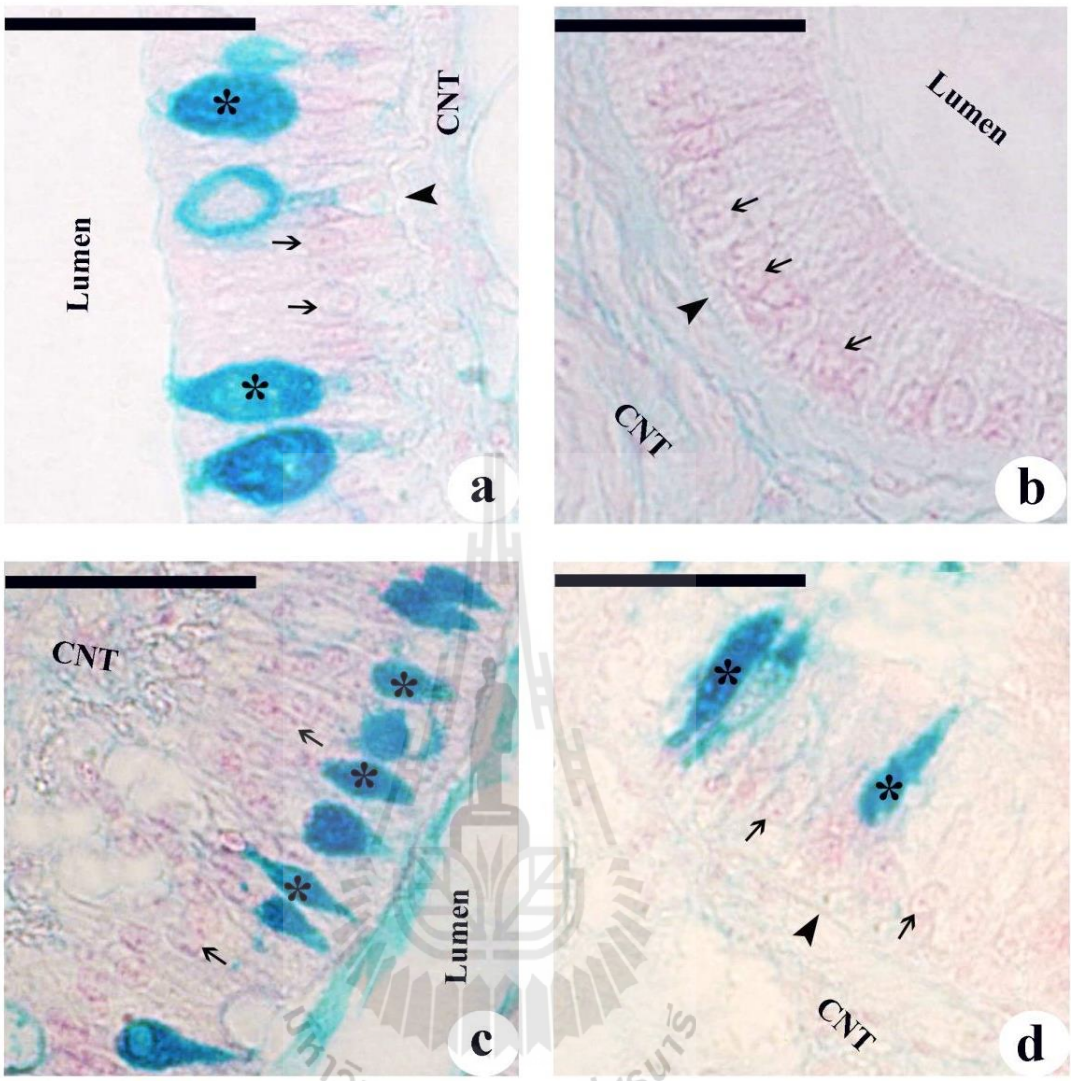

Figure 4.10 Photographs showing sialomucin-secreting cells stained with alcian blue pH 2.5. Asterisks indicate the mucin-secreting cells. a = esophagus; b = stomach; c = intestine; d = rectum; arrows = nuclei; arrowheads = basement membrane; CNT = connective tissue; MV = microvilli. Scale bars = 100 µm.

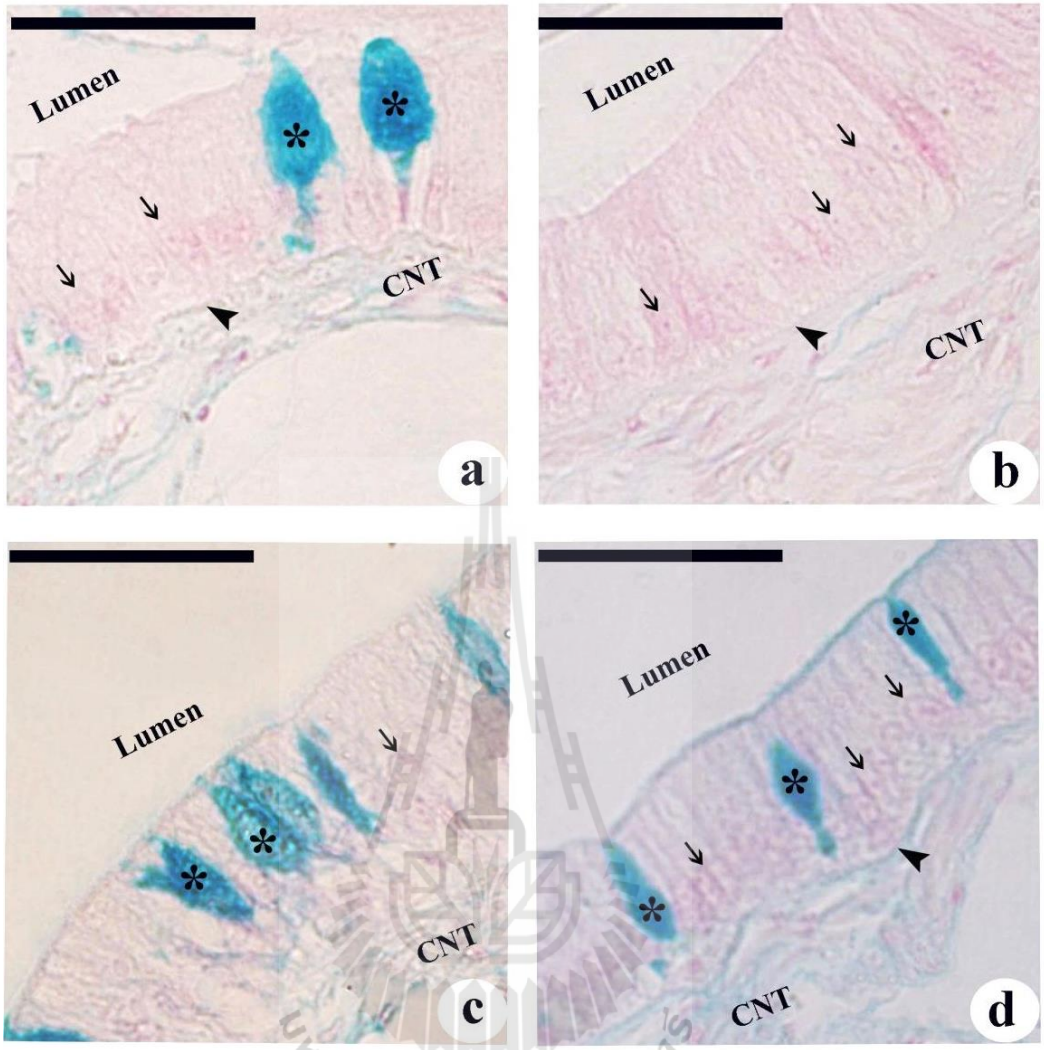

Figure 4.11 Photographs showing sulfomucin-secreting cells stained with alcian blue pH 1.0. Asterisks indicate the mucin-secreting cells. a = esophagus; b = stomach; c = intestine; d = rectum; arrows = nuclei; arrowheads = basement membrane; CNT = connective tissue; MV = microvilli. Scale bars = 100 µm.

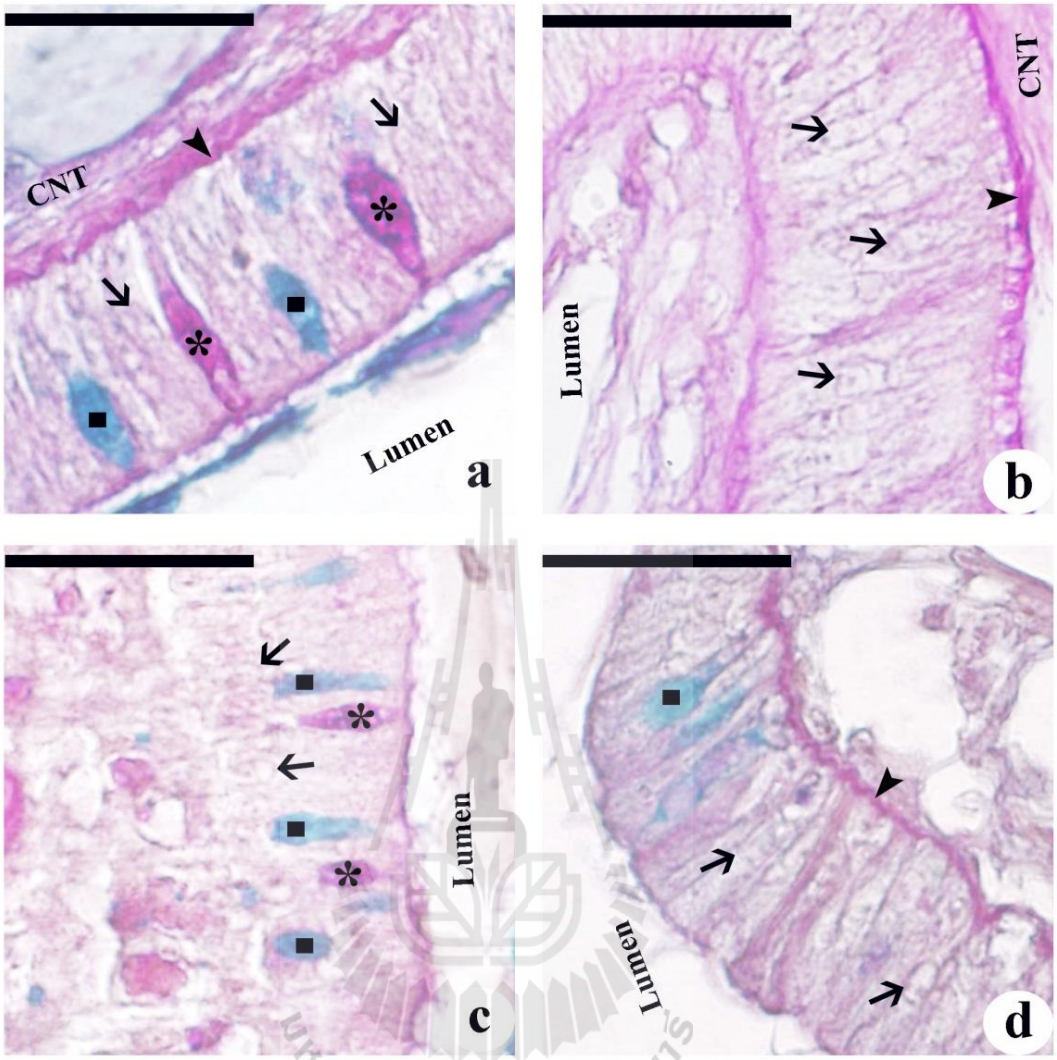

Figure 4.12 Photographs showing mucin-secreting cells in the GI tract. a = esophagus; b = stomach; c = intestine; d = rectum. Asterisks indicate cells stained with PAS and rectangles indicate cells stained with alcian blue, pH 2.5. Arrows = nuclei; arrowheads = basement membrane; CNT = connective tissue; MV = microvilli. Scale bars = 100 µm.

Percentages of mucin-secreting cells in parts of the GI tract are shown in Figure 4.13. Neutral mucin-secreting cells (stained with PAS) were found mostly in the intestine (31.00 ± 0.08%), and the percentage was decreased in the esophagus (24.86 ± 0.08%). In the rectum, the percentage of neutral mucin-secreting cells was significant lower (22.80 ± 0.08%) compared to those of the esophagus and intestine. Sialomucin mucin-secreting cells (stained with alcian blue, pH 2.5), were found mostly in the esophagus (26.32 ± 0.10%), medium in the intestine (18.42 ± 0.10%), and significant least in the rectum (13.45 ± 0.10%). Moreover, the highest percentage of sulfomucin-secreting cells (stained with alcian blue, pH 1.0) was observed in the intestine (24.49 ± 0.07%). Percentages of sulfomucin-secreting cells in the esophagus and rectum were 17.96 ± 0.07% and 16.71 ± 0.07%, respectively, which were not significantly different from that of the intestine.

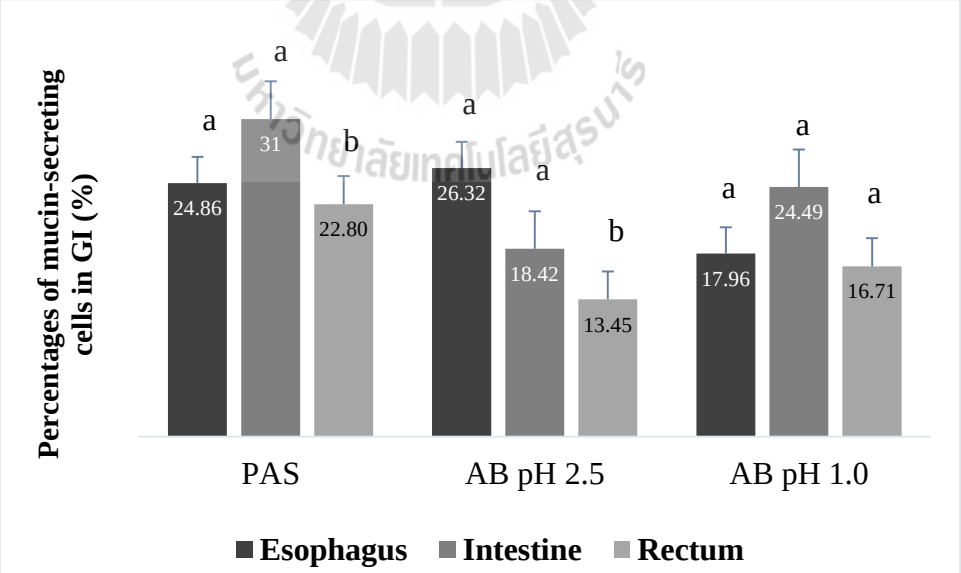

Figure 4.13 Percentages of mucin-secreting cells in parts of the GI tract. Different letter indicates a significant difference between groups.

4.5 Localization of leptin-like peptide immunoreactive cells in the gastrointestinal tract

Immunohistochemical study revealed leptin-like peptide immunoreactive cells in all parts of the snail GI tract (Figure 4.14). The immunoreactive cells were most numerous in the esophagus (Figure 4.14a), but were least in the stomach (Figure 4.14b). Medium immunoreactive cells were detected in the intestine (Figure 4.14c) and rectum (Figure 4.14d). The immunoreaction was observed elongate in some epithelial cells of the esophagus, intestine, and rectum. The apical surfaces of these immunoreactive cells were in contact with the lumen of the GI tract. In contrast, the immunoreaction was found only at the basal region of the some epithelial cells of the stomach and rectum. In negative control, there was no immunoreactivity was observed when the primary antibody was omitted from the staining procedure (Figure 4.14e).

4.6 Expression of leptin-like peptide in parts of the gastrointestinal tract

Expression of leptin-like peptide in parts of the GI tract was identified using Western immunoblotting. Leptin-like peptide was expressed in all parts of the GI tract (Figure 4.15). Molecular weights of the leptin-like peptides were at ~16 kDa which were the same as that of the molecular weight of leptin peptide which used as the positive control. No immunoreactive peptide band was detected in the negative control (data not shown).

Analysis of immunoreactive band intensities revealed that the highest intensity was observed in the esophagus (132.37 ± 3.44 arbitrary units (AU)) which was

significantly higher ($p<0.05$) than those of the intestine (107.47 ± 4.5 AU), stomach (89.10 ± 2.98 AU), and rectum (86.44 ± 2.99 AU) (Figure 4.16).

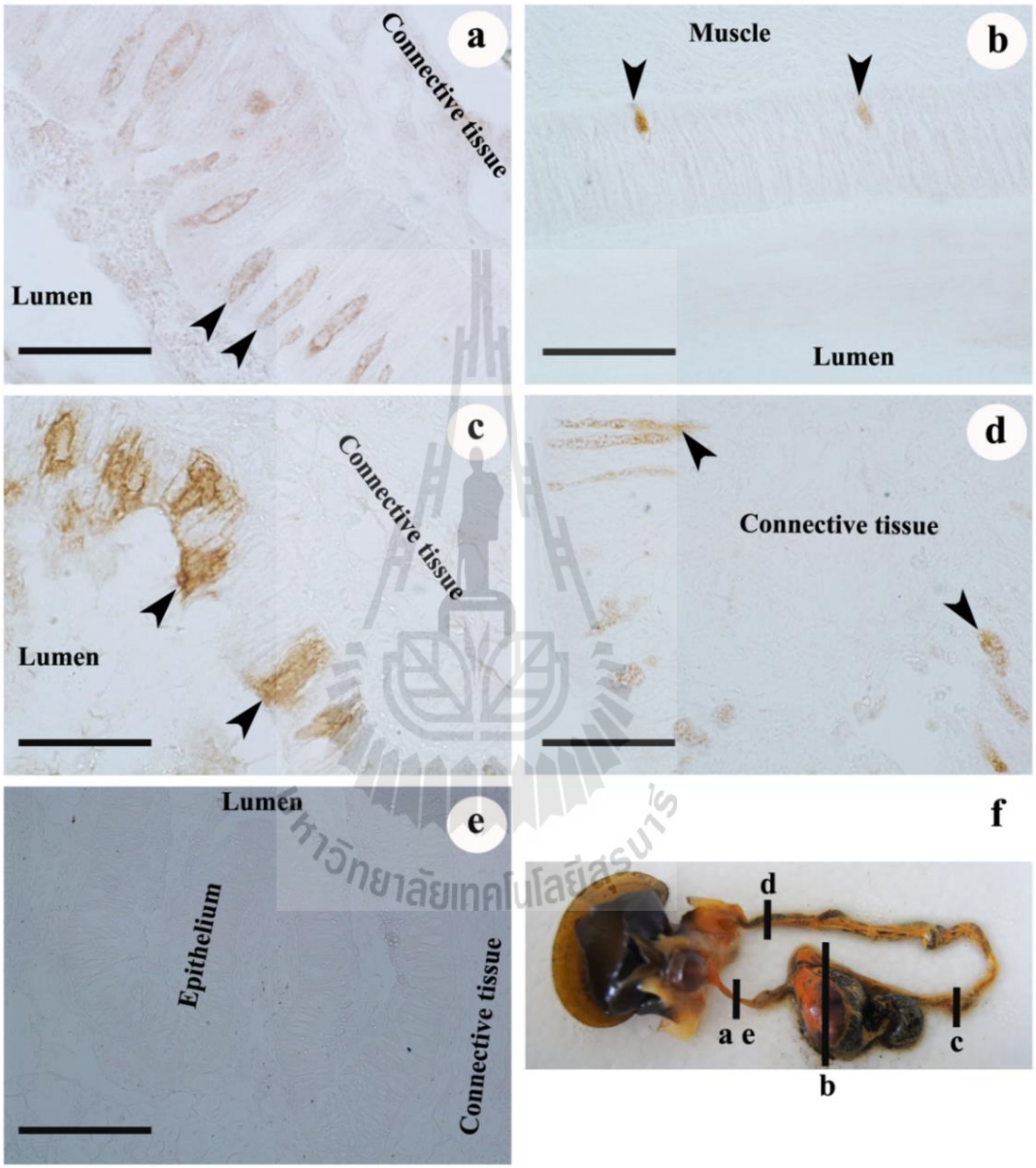

Figure 4.14 Leptin-like peptide immunoreactive cells in parts of the GI tract; esophagus (a), stomach (b), intestine (c) and rectum (d). Negative control showed no immunoreactivity (e). Scale Bars = 100 µm.

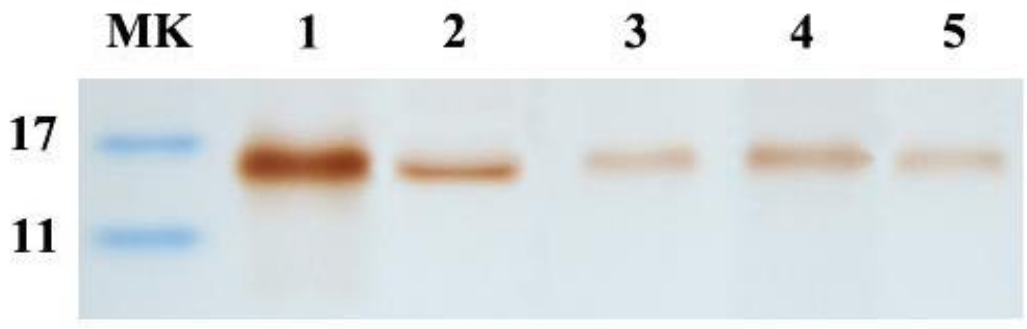

Figure 4.15 Expression of leptin-like peptide in parts of the GI tract. MW = Molecular weight marker; Lane 1 = positive control; Lane 2 = esophagus; Lane 3 = stomach; Lane 4 = intestine; Lane 5 = rectum.

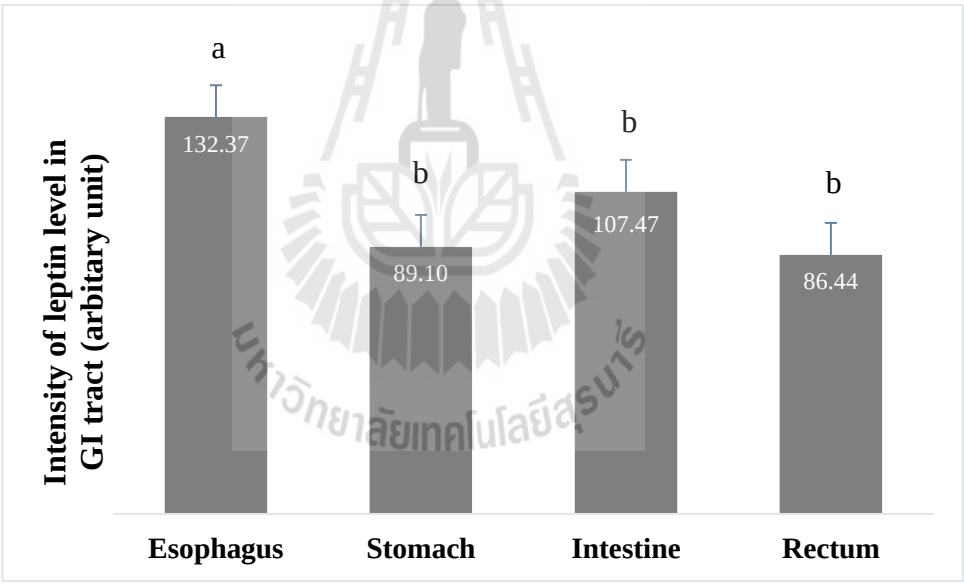

Figure 4.16 Intensities of leptin-like peptide immunoreactive bands in parts of the GI tract. Different letter indicates a significant difference between groups.

4.7 Levels of leptin-like peptide compared between fed and fasted groups

To determine whether levels of leptin-like peptide are related to feeding conditions of the snails, ELISA assay was conducted. At day 0, levels of leptin-like peptide were similar in fed and fasted groups (2.26 ± 0.52 and 2.26 ± 0.52 ng/0.1 ml, respectively) (Figure 4.17). In contrast, at day 5, level of leptin-like peptide of the fasted group was significantly decreased (0.28 ± 0.19 ng/0.1 ml) compared to the level of the fed group (2.17 ± 0.53 ng/0.1 ml). Similarly, at day 10, level of leptin-like peptide of the fasted group was significantly reduced when compared to that of the fed group (0.3 ± 0.13 and 2.12 ± 0.49 ng/0.1 ml, respectively). However, levels of leptin-like peptide in fed and fasted groups were come back to be similar at day 15 (2.05 ± 0.68 and 2.06 ± 0.79 ng/0.1 ml, respectively).

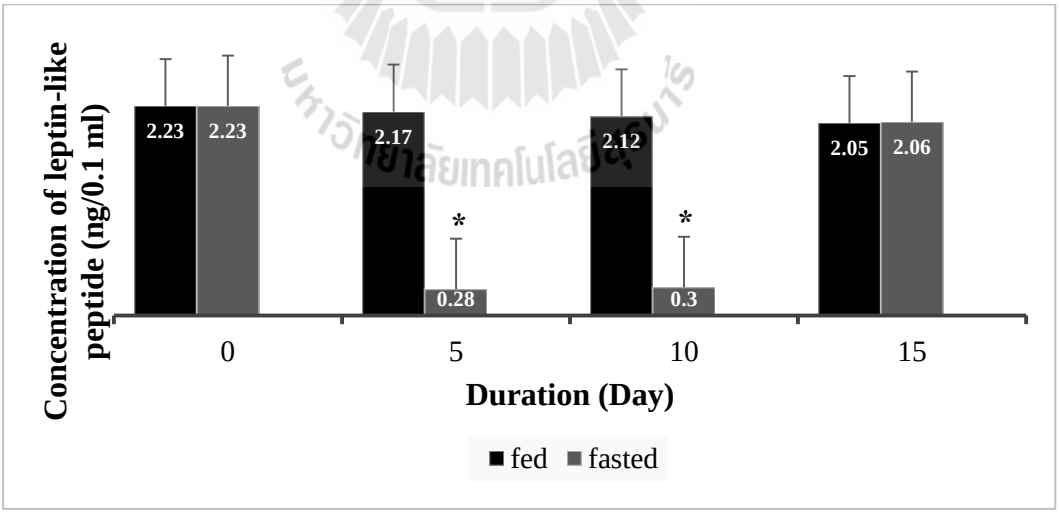

Figure 4.17 Levels of leptin-like peptide of fed and fasted groups. Data is presented as mean ± S.D. Asterisk indicates a significant difference compared to the control.

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Gross structure of the gastrointestinal tract

In the present study, gross structure of the GI tract of *P*. *canaliculata* was examined. The snail GI tract composed of esophagus, stomach, intestine and rectum. This is similar to the structure of the GI tract of other gastropods. A study in a limpet, *Megathura crenulata*, indicated that its GI tract composed of the esophagus leading from the mouth to the stomach followed by a long intestine which connected to the rectum. The rectum ended at the anus (Martin et al., 2010). It was found that the stomach of *P*. *canaliculata* was embedded in the digestive gland. Similarly, the stomach of *Bulla striata* was also embedded in the digestive gland, but its esophagus was located between the buccal mass and gizzard. However, the gizzard was not found in *P*. *canaliculata* (Lobo-da-Cunha et al., 2010; Lobo-da-Cunha et al., 2011).

In *P*. *canaliculata*, no apparent crop. However, the crop was found in some gastropods, such as, a garden snail (*Helix aspersa*) (Charrier and Rouland, 2001), a sea hare (*Aplysia depilans*) (Lobo-da-Cunha and Batista-Pinto, 2005), and a land snail (*Achatina fulica*) (Cardoso et al., 2012). This indicates some differences among the structure of the GI tract of gastropods. It was reported in *A*. *depilans* that the crop consisted of a wide lumen which was used to support the food ingestion.

5.2 Microstructure and ultrastructure of the gastrointestinal tract

In *P. canaliculata*, all parts of the GI tract were lined with simple columnar epithelium with microvilli presented on the apical region of the cells. All cells were rested on the same basement membrane and their nuclei were located at the basal region. Similarly, the stomach, intestine, and rectum of *M. crenulata* were also lined with simple columnar epithelium with microvilli (Martin et al., 2010). The epithelium of the esophagus, crop, and intestine of *A. depilans* consisted of a single layer of columnar cells with apical microvilli, and some of them also possessed cilia (Lobo-da-Cunha and Batista-Pinto, 2005; Martin et al., 2010). In addition, in *B. striata*, epithelium covered the ridges of the esophagus and those of the stomach and intestine were also ciliated columnar epithelium. The apical surface of the columnar epithelial cells was covered with microvilli which embedded in a thick cuticle made of finely granular material (Lobo-da-Cunha et al., 2010; Lobo-da-Cunha et al., 2011).

Scanning electron micrographs showed a large number of mucosal folds of the stomach and the height of the folds was greatest than those of other regions. In the rectum, there were primary and secondary folds. This might relate to the increasing in surface area of the stomach and rectum. A study by scanning electron microscopy in *M. crenulata* reported that the stomach also showed major and minor ridges, and numerous ridges were found in the rectum (Martin et al., 2010).

Transmission electron micrographs showed numbers of Golgi apparatus and rough endoplasmic reticulum in the cytoplasm of the intestinal epithelial cells. This indicates the high level of protein synthesis in the epithelial cells of the intestine. Tight junctions were found in the rectum. This could be related to the protection property of the rectal epithelial cells. Moreover, desmosomes and adherens junctions were also

found in the rectum indicating the communications between adjacent cells. A study in *B. striata* reported that mitochondria, several vesicles, and multivesicular bodies were presented in the apical region of the epithelial cells. In addition, the flask-shaped subepithelial secretory cells which were inserted in the connective tissue consisted of the cytoplasm filled with electron-lucent secretory vesicles and Golgi stacks. Some of these cells contained many rough endoplasmic reticulum cisternae (Lobo-da-Cunha et al., 2010). Golgi stacks were formed by a large number of flat cisternae, mainly located around the nuclei (Lobo-da-Cunha et al., 2011). Large numbers of spherical secretory vesicles containing electron-dense material were also found in the intestine. These vesicles were very abundant in the cytoplasm above the nuclei (Lobo-da-Cunha et al., 2011). The presence of the organelles mentioned above indicates the secretory property of the epithelial cells.

5.3 Mucin-secreting cells in the gastrointestinal tract

Mucins are classified according to their ability to form a gel, namely gel forming (secreted) or non-gel-forming (membrane-bound) mucins (Devine and McKenzie, 1992). Mucins are also classified into neutral or acid mucins, according to the net charge of the molecule. Acid mucins are further differentiated based on their histochemical properties into sulfate-containing mucins (sulfomucins) and sialicacid-containing mucins (sialomucins) (Filipe, 1979). In the present study, mucin-secreting cells were found throughout the GI tract of *P. canaliculata*, but not in the stomach. Neutral mucin and sulfomucin-secreting cells were found mostly in the intestine, whereas sialomucin-secreting cells were mostly identified in the esophagus. Cells that secrete mucins were found in the GI tract of various gastropods, for example, *Marisa*

cornuarietis (Demian and Michelson, 1971), *Bulinus africanus* (Brackenbury, 1999), *A. depilans* (Lobo-da-Cunha and Batista-Pinto, 2005), and *M. crenulata* (Martin et al., 2010). This suggests that the secretory processes concerning with digestion occurs in most regions of the GI tract of gastropods.

Both neutral and acidic mucins were observed in parts of the GI tract of *P. canaliculata.* In a common limpet (*Patella vulgate*), nine types of mucin-secreting cells were found by a characterization by PAS, alcian blue pH 2.5, and alcian blue, pH 1.0. Four cell types were found to secrete neutral and acidic, sulfated mucins (Grenon and Walker, 1978). It was reported that neutral mucins were found in the stomach and duodenum, whereas acid mucins were distributed throughout the GI tract (Awad and Abdul Raheem, 2014). Neutral mucins are known to help for reducing the pH and toxicity of substances that pass through the GI tract, whereas acidic mucins contain chelating agents and have antibacterial and antiviral properties (Nikumbh et al., 2012). Moreover, it is known that mucous cells are important in the physical and immunological protections against invasive microorganisms in mostly carnivorous freshwater species. Other functions of mucins in the GI tract are known, such as lubrication, absorption, digestion, controlling of infection and colonization of the dangerous or opportunistic microorganisms (Leknes, 2011; Zaccone et al., 1989; Loretz, 1995; Domeneghini et al., 2005). The mucin oligosaccharides in the mucous gel represent a direct source of peptides, carbohydrates, and nutrients that allow the colonization of bacteria in the mucous layer of the GI tracts (Deplancke and Gaskins, 2001). The distribution of the GI mucins can differ according to the number of secreting cells, cell type, anatomical region, pathological condition and species (Scillitani et al., 2007).

Mucins have become an important element in the study of the GI tract physiology, pathology and even taxonomy (Scillitani et al., 2007; Cao and Wang, 2009). The different types of mucins (neutral, sulfomucins, and sialomucins) have also been implicated in the colonization of the GI tract. The mucin oligosaccharides provide a source of carbohydrates and peptides which can supply bacteria with the necessary nutrients to improve colonization. In addition, mucins may function as epitopes with which bacteria can interact to colonize the mucosal layer (Deplancke and Gaskins, 2001).

Result of the present study showed that no mucin-secreting cell was identified in the stomach. However, the microvilli of the epithelial cells of the stomach were stained with PAS. This indicates the presence of neutral mucin that covered the inner wall of the stomach. Because there was no mucin-secreting cell found in the stomach, the mucin covered that inner wall of the stomach could be transferred from the esophagus to the stomach during the passage of food.

5.4 Leptin-like peptide immunoreactive cells in the gastrointestinal tract

The present study is the first report for the presence of leptin-like peptide in the GI tract of gastropod species. Leptin is a peptide which was currently reported to be synthesized mostly by adipocytes. Leptin can also be found in other organs, such as the brain and placenta. In the present study, leptin-like peptide immunoreactive cells were identified in the esophagus, stomach, intestine, and rectum. Similarly, leptin immunoreactivity was found to be scattered in stomach cell of non-mammalian vertebrate species, for example trout, frog, lizard, and snake. Leptin immunolabeling

was also identified in oxyntic glands of frog and reptilian species. Immunoreactivity of leptin was found to be scattered in endocrine cells of a lizard, and in the myenteric plexus of trout, as well as frogs and snakes (Muruzabal et al., 2002). Previous studies found leptin immunoreactive cells in the pepsinogen-secreting chief cells of humans' stomach (Bado et al., 1998; Sobhani et al., 2000) and gastric fundic mucosa of rats (Buyse et al., 2004; Cammisotto et al., 2005). These studies suggested that leptin was secreted by chief cell after the onset of food intake in according to the short-term controlling of food intake (Cammisotto et al., 2005). It also be hypothesized for the presence of leptin immunoreactive cells in oxintopeptic cells that could be related to the stimulation of mucus secretion for anti-acid gastrointestinal protection (Plaisancie et al., 2006). A previous study in the Chinese mitten crab (*Eriocheir sinensis*) reported the presence of leptin receptor. Expression of the leptin receptor was highest in the intestine of mature crabs, indicating that a role of leptin in nutrient absorption (Jiang et al., 2010). The function of leptin is related to the GI tract function. It is suggested that direct leptin signaling in the intestine may be involved in the regulation of nutrient absorption. Leptin is also able to decrease feeding (Buyse et al., 2001).

5.5 Expression of leptin-like peptide in the gastrointestinal tract

The molecular weight of leptin-like peptide in all parts of the GI tract was at 16 kDa which was corresponded to that of the synthetic human leptin peptide. This suggests that the leptin-like peptide in *P. canaliculata* could be structurally similar to that of the human leptin peptide. The molecular weight of the leptin-like peptide was also corresponded to those reported in the previous studies. In *Scyliorhinus canicula*, a cartilaginous fish, leptin immuno-reactive band observed in the gastric pit was also at

16 kDa (Gambardella et al., 2010). Similarly, leptin detected in the GI tract of a chicken had the molecular weight of 16 kDa (Neglia et al., 2007). In the juvenile sea bass (*Dicentrarchus labrax*), Western blot analysis showed a leptin-like molecule with the molecular weight of 16 kDa (Gambardella et al., 2010). Moreover, in the rainbow trout (*Oncorhynchus mykiss*), leptin determined by western blot analysis shows a single band at 16 kDa (Kling et al., 2009).

In *P. canaliculata*, high expressions of the leptin-like peptide were detected in the esophagus and intestine. In contrast, the expressions of the leptin-like peptide were lower in the rectum and were least in the stomach. This result was related to the result of the immunoperoxidase which showed the highest number of leptin-like peptide immunoreactive cells in the esophagus, and the least number of the immunoreactive cells was detected in the stomach.

5.6 Levels of leptin-like peptide compared between fasted and fed snails

Levels of leptin-like peptide of the fasted groups were significantly lower compared to those of the fed group at days 5 and 10. In green sunfish, plasma leptin was also reduced by 2 weeks fasting (Johnson et al., 2000). It is now known that leptin can decrease feeding (Buyse et al., 2001). In the fed group, the high level of leptin could be related to the adiposity signal and the suppression of ghrelin level which increased food intake (Gil-Campos et al., 2010). After food intake, increasing level of lipids in adipocytes and therefore increased the size of these cells leads to the secretion of the leptin which subsequently binds to the receptors in the hypothalamic arcuate nucleus (ARC). The association with the receptors leads to inhibition of both

neuropeptide Y (NPY) and agouti-related protein (AGRP) which are appetite stimulating factors. This may trigger the stopping of eating and the feeling of satiety (Linjawi and Hussain, 2012). It was reported that leptin treatment might inhibit melanocyte-stimulating hormone that decreased the release of AGRP (Breen et al., 2005). Peripheral injections of murine leptin decreased food intake and also increased metabolic rates in lizards (Niewiarowski et al., 2000). It is also known that leptin mainly involves in the GI function, for example, GI tract motility and nutrient absorption. It is assumed that direct leptin signaling in the intestine may be involved in the regulation of nutrient absorption and intestine motility (Buyse et al., 2001).

The result of the present study revealed that level of leptin-like peptide in the fasted group at day 15 increased to a similar level compared to that of the control group. Similarly, the plasma leptin level of the rainbow trout was higher than those of the fed group in week 3 (Kling et al., 2009). Leptin level increase and falls in direct proportion to energy stores at all levels of adiposity. It is well known that the main site of leptin synthesis is adipocytes (Zhang et al., 1994). The re-increasing of the leptin-like peptide during day 15 could come from adipolysis from the snail body or visceral organs. This could be related to the controlling of the body into a state of hibernation (Myers et al., 2008).

In conclusion, we demonstrate that the microstructure and ultrastructure of the mucosa of the snail GI tract was similar to those of other gastropod species. Three types of mucin-secreting cells were found scattered within the epithelial cells lining the GI tract. Leptin-like peptide was identified in parts the GI tract of *P. canaliculata*. Moreover, level of the leptin-like peptide was decreased in fasted snails. This finding could provide a basic knowledge on the structure of the snail GI tract and its endocrinology related to the feeding control.

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APPENDICES

APPENDIX A

STATISTICAL ANALYSIS

Table 1A Statistic comparison of heights of the epithelia cells in GI tract.

(I) organ	(J) organ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Esophagus	Stomach	-.23100	.09911	.132	-.5146	.0526
	Intestine	-1.16820*	.09911	.000	-1.4518	-.8846
	Rectum	-.09930	.09911	.751	-.3829	.1843
Stomach	Esophagus	.23100	.09911	.132	-.0526	.5146
	Intestine	-.93720*	.09911	.000	-1.2208	-.6536
	Rectum	.13170	.09911	.559	-.1519	.4153
Intestine	Esophagus	1.16820*	.09911	.000	.8846	1.4518
	Stomach	.93720*	.09911	.000	.6536	1.2208
	Rectum	1.06890*	.09911	.000	.7853	1.3525
Rectum	Esophagus	.09930	.09911	.751	-.1843	.3829
	Stomach	-.13170	.09911	.559	-.4153	.1519
	Intestine	-1.06890*	.09911	.000	-1.3525	-.7853

*The mean difference is significant at the 0.05 level.

Table 2A Statistic comparison of percentages of the mucin-secreting cells stained with Periodic acid-Schiff (PAS).

(I) organ	(J) organ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
esophagus	Intestine	-1.00000	1.51438	.788	-4.7548	2.7548
	rectum	3.80000*	1.51438	.047	.0452	7.5548
Intestine	esophagus	1.00000	1.51438	.788	-2.7548	4.7548
	rectum	4.80000*	1.51438	.010	1.0452	8.5548
rectum	esophagus	-3.80000*	1.51438	.047	-7.5548	-.0452
	Intestine	-4.80000*	1.51438	.010	-8.5548	-1.0452

* The mean difference is significant at the 0.05 level.

Table 3A Statistic comparison of percentages of the mucin-secreting cells stained with alcian blue pH 2.5.

(I) organ	(J) organ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
esophagus	Intestine	-2.30000	1.24186	.172	-5.3791	.7791
	rectum	3.30000*	1.24186	.034	.2209	6.3791
Intestine	esophagus	2.30000	1.24186	.172	-.7791	5.3791
	rectum	5.60000*	1.24186	.000	2.5209	8.6791
rectum	esophagus	-3.30000*	1.24186	.034	-6.3791	-.2209
	Intestine	-5.60000*	1.24186	.000	-8.6791	-2.5209

* The mean difference is significant at the 0.05 level.

Table 4A Statistic comparison of percentages of the mucin- secreting cells stained with alcian blue pH 1.0.

(I) organ	(J) organ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
esophagus	Intestine	-1.30000	1.22957	.548	-4.3486	1.7486
	rectum	.00000	1.22957	1.000	-3.0486	3.0486
Intestine	esophagus	1.30000	1.22957	.548	-1.7486	4.3486
	rectum	1.30000	1.22957	.548	-1.7486	4.3486
rectum	esophagus	.00000	1.22957	1.000	-3.0486	3.0486
	Intestine	-1.30000	1.22957	.548	-4.3486	1.7486

Table 5A Statistic comparison of intensities of leptin-like peptide immunoreactive bands in parts of the GI tract.

(I) Organ	(J) Organ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Esophagus	Stomach	43.27120*	2.23737	.000	36.8700	49.6724
	Intestine	24.90320*	2.23737	.000	18.5020	31.3044
	Rectum	45.93220*	2.23737	.000	39.5310	52.3334
Stomach	Esophagus	-43.27120*	2.23737	.000	-49.6724	-36.8700
	Intestine	-18.36800*	2.23737	.000	-24.7692	-11.9668
	Rectum	2.66100	2.23737	.642	-3.7402	9.0622
Intestine	Esophagus	-24.90320*	2.23737	.000	-31.3044	-18.5020
	Stomach	18.36800*	2.23737	.000	11.9668	24.7692
	Rectum	21.02900*	2.23737	.000	14.6278	27.4302
Rectum	Esophagus	-45.93220*	2.23737	.000	-52.3334	-39.5310
	Stomach	-2.66100	2.23737	.642	-9.0622	3.7402
	Intestine	-21.02900*	2.23737	.000	-27.4302	-14.6278

* The mean difference is significant at the 0.05 level.

Table 6A Statistic comparison of leptin-like peptide levels in fed snails.

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
D0	D5	.08584	.32295	.993	-.8181	.9897
	10	.13233	.32295	.976	-.7716	1.0362
	15	.21102	.32295	.913	-.6929	1.1149
D5	D0	-.08584	.32295	.993	-.9897	.8181
	10	.04650	.32295	.999	-.8574	.9504
	15	.12518	.32295	.980	-.7787	1.0291
10	D0	-.13233	.32295	.976	-1.0362	.7716
	D5	-.04650	.32295	.999	-.9504	.8574
	15	.07868	.32295	.995	-.8252	.9826
15	D0	-.21102	.32295	.913	-1.1149	.6929
	D5	-.12518	.32295	.980	-1.0291	.7787
	10	-.07868	.32295	.995	-.9826	.8252

Table 7A Statistic comparison of levels of the leptin-like peptide in fasted snails.

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
D0	D5	1.97783*	.31021	.000	1.1096	2.8461
	10	1.74106*	.31021	.000	.8728	2.6093
	15	.20029	.31021	.916	-.6680	1.0686
D5	D0	-1.97783*	.31021	.000	-2.8461	-1.1096
	10	-.23677	.31021	.870	-1.1050	.6315
	15	-1.77754*	.31021	.000	-2.6458	-.9093
10	D0	-1.74106*	.31021	.000	-2.6093	-.8728
	D5	.23677	.31021	.870	-.6315	1.1050
	15	-1.54077*	.31021	.000	-2.4090	-.6725
15	D0	-.20029	.31021	.916	-1.0686	.6680
	D5	1.77754*	.31021	.000	.9093	2.6458
	10	1.54077*	.31021	.000	.6725	2.4090

*. The mean difference is significant at the 0.05 level.

APPENDIX B

INFORMATION FOR ANALYSIS

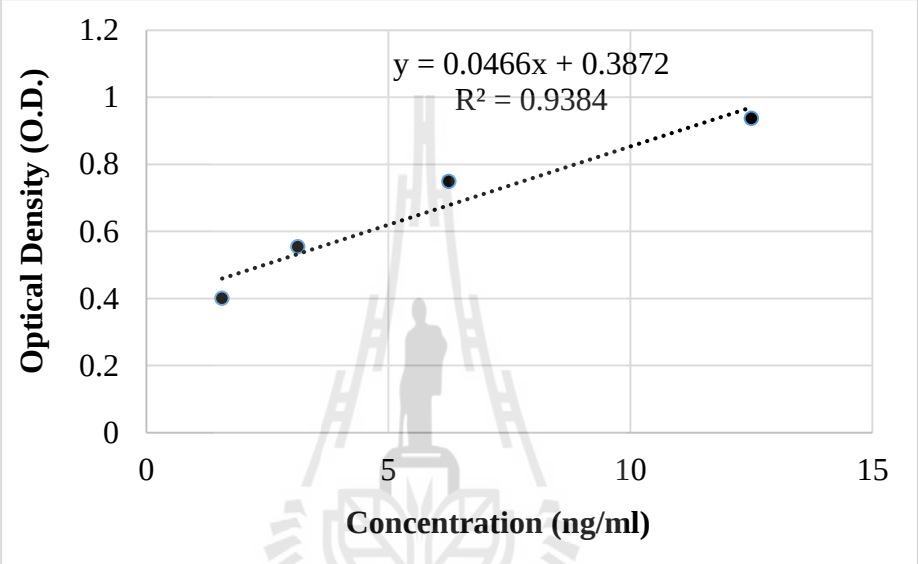

Figure 1B Standard graph for human leptin.

APPENDIX C

THE PREPARATIONS OF REAGENTS

Phosphate buffer saline pH 7.4

Chemicals

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	0.2 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.44 g
Potassium dihydrogen Phosphate (KH_2PO_4)	0.24 g
Distilled water	1 L

Preparation

Add chemical compounds one by one into 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 liter. Sterilization by autoclaving (20 min, 121^{o}C, liquid cycle). Store at room temperature.

Coating buffer (carbonate buffer), 0.05 M pH 9.6

Chemicals

15 mM Sodium carbonate (Na_2CO_3)	1.59 g
35 mM Sodium bicarbonate ($NaHCO_{3)}$	2.93 g
Distilled water	1 L

Preparation

The total chemical compounds were mixed in distilled water. pH was adjusted to 9.6. Sterilization by autoclaving (20 min, 121^{o}C, liquid cycle).

Lysis buffer

Chemicals

10 mM TRIS hydrochloride (Tris-HCl) pH 7.2	0.16 g
150 mM Sodium chloride (NaCl)	0.87 g
1 mM Ethylenediaminetetraacetic acid (EDTA)	0.04 g
0.5% Triton X-100	25 µl
1 mM Phenylmethylsulfonyl fluoride (PMSF)	50 µl
Distilled water	100 ml

Preparation

Prepare stock solution by adding Tris-HCl, NaCl, and EDTA into 80 ml distilled water. Adjust pH to 7.2 and then add distilled water to a total volume of 100 ml. Sterilization by autoclaving (20 min, 121^{o}C, liquid cycle program). Prepare working solution by adding PMSF and Triton X-100 into 4.945 ml stock solution.

Electrophoresis buffer pH 8.3

Chemicals

Tris (hydroxymethyl) aminomethane (Tris base)	3.02 g
Glycine	14.4 g
Sodium dodecyl sulfate (SDS)	1 g
Distilled water	1 L

Preparation

The total chemical compounds were mixed in 1 L distilled water.

Transfer buffer

Chemicals

Tris (hydroxymethyl) aminomethane (Tris base)	2.93 g
Glycine	5.81 g
Sodium dodecyl sulfate (SDS)	0.375 g
Methanol	200 ml
Distilled water	1 L

Preparation

The total chemical compound was mixed in distilled water. Ready for use.

Separating gel

Chemicals

Autoclaved H_2O	5.68 ml
30% acrylamide	12 ml
Separating gel buffer	6 ml
10% Sodium dodecyl sulfate (SDS)	240 µl
10% Ammonium persulfate (APS)	120 µl
(N, N, N, N, -tetramethyl ethylenediamine) TEMED	8 µl

Preparation

Mix all chemical compounds together, except TEMED which was added at the final process.

Stacking gel

Chemicals

Autoclaved H_2O	6 ml.
30% acrylamide	1.34 ml.
Stacking gel buffer	2.5 ml.
10% Sodium dodecyl sulfate (SDS)	100 µl.
10% Ammonium persulfate (APS)	50 µl.
(N, N, N, N, -tetramethyl ethylenediamine) TEMED	5 µl.

Preparation

Mix all chemical compounds together, except TEMED which was added at the final process.

Gel Setting

Reagents	**12.5% separating gel**		**15% separating gel**		**4% stacking gel**	
	2 sides	**4 sides**	**2 sides**	**4 sides**	**2 sides**	**4 sides**
30% acrylamide	5 ml	10 ml	6 ml	12 ml	0.67 ml	1.34 ml
Separating gel buffer	3 ml	6 ml	3 ml	6 ml	-	-
Stacking gel buffer	-	-	-	-	1.25 ml	2.5 ml
10% SDS	120 µl	240 µl	120 µl	240 µl	50 µl	100 µl
Autoclaved H_2O	3.84 ml	7.68 ml	2.84 ml	5.68 ml	3 ml	6 ml
10% APS	60 µl	120 µl	60 µl	120 µl	25 µl	50 µl
TEMED	4 µl	8 µl	4 µl	8 µl	2.5 µl	5 µl

APPENDIX D

PROCEEDING PRESENTATION

Proceedings of the APMC11 / MST33 / AAT39 Conference

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PA22

Histological Study of the Gastrointestinal Tract of *Pomacea Canaliculata*

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Abstract

In the present study, histology of epithelial cells lining the gastrointestinal tract of the *Pomacea canaliculata* was studied. It was observed that the snail's gastrointestinal tract consisted of the esophagus, stomach, intestine, and rectum. The esophageal crop was not apparently seen. Using conventional hematoxylin and eosin staining, the result showed that morphology of the epithelial cells was similar in all parts of the gastrointestinal tract. Each part of the gastrointestinal tract was covered by simple columnar epithelium with microvilli presented at the apical region of the cells. Moreover, both neutral and acidic mucin-secreting cells were observed in parts of the snail's gastrointestinal tract.

Keywords Gastrointestinal tract, golden apple snail, *Pomacea canaliculata*

Background

The golden apple snail, *Pomacea canaliculata*, is a freshwater snail that is commonly found in South America. Since 1980, the snail has been brought to many countries in Asia for human food consumption instead of abalone which is more costly. However, this species escapsed or was released into natural environment. The snail has then become a cause of major problems in agriculture as a wetland pest (Hayes et al., 2008).

Morphology of the gastrointestinal (GI) tract compared between gastropods was similar. The GI tract consists of the esophagus leading from the mouth to the stomach followed by the long intestine. The stomach is embedded within the digestive gland. The caudal part of the GI tract is the rectum which ended at the anus. However, some gastropods have the crop located between the esophagus and stomach (Voltzow, 1994).

P. canaliculata has been brought to Thailand for over many decades. The snail can propagate and grow quickly because its diets are all types of plants. Moreover, the snail consumes more food than other snail species. The reason why the snail is a good survivor is still undefined. Moreover, knowledge on the snail's biology is still limited. The main objective of the present study was thus to investigate the snail's biology emphasizing on the histology of its GI tract.

Materials and Methods

After euthanization of the snails, the GI tracts were removed and parts of the GI tract were classified. Each part of the GI tract was then processed conventionally for embedding in paraffin wax. Subsequently, the tissue blocks were cut and the sections were stained with hematoxylin and eosin. The sections were then dehydrated, cleared, mounted, and finally observed under a light microscope.

Mucus-secreting cells in parts of the GI tract were also identified. According to the methods suggested by the manufacturer, the paraffin sections of the GI tract were counterstained with Periodic acid-Schiff (PAS) and alcian blue, pH 2.5 for neutral and acidic mucins, respectively. The sections were then dehydrated, cleared, mounted, and finally visualized under a light microscope.

Results, Discussion and Conclusion

The GI tract of *P. canaliculata* composed of the esophagus which connected the mouth to the stomach (Fig.1). The esophagus situated ventrally to the buccal mass and entered the rostral part of the stomach. Follow the length of the esophagus was the intestine. The distal part of the intestine connected to the rectum, and the ended part of the GI tract was the anus. The stomach is embedded within a digestive gland. This is similar to the structure of the GI tract of other gastropods. A study in *Megathura crenulata* indicated that its GI tract was divided into the esophagus, stomach and intestinal loop which terminated at the anus (Martin et al., 2010). In *P. canaliculata*, no apparent crop was seen. However, the crop was found in some gastropods, such as, *Helix aspersa* (Charrier and Rouland, 2001), *Aplysia depilans* (Lobo-da-Cunha and Batista-Pinto, 2005), and *Achatina fulica* (Cardoso et al., 2012). This indicates some differences among the structure of the GI tract of gastropods.

In *P. canaliculata*, all parts of the GI tract were lined with simple columnar epithelium with microvilli presents on the apical region of the cells (Fig.2). All cells were rested on the same basement membrane and their nuclei were located at the basal region. Similarly, the stomach, intestine, and rectum of *M. crenulata* were also lined with simple columnar epithelium with microvilli (Martin et al., 2010). Moreover, the epithelium of the esophagus and crop of *A. depilans* consisted of a single layer of columnar cells with apical microvilli, and some of them also possessed cilia (Lobo-da-Cunha and Batista-Pinto, 2005).

Mucus-secreting cells were observed in parts of the GI tract of *P. canaliculata*, but not in the stomach (Fig.3). Cells that secrete mucins were found in the GI tract of various gastropods, for example, *Marisa cornuarietis* (Demian and Michelson, 1971), *Bulinus africanus* (Brackenbury, 1999), *A. depilans* (Lobo-da-Cunha and Batista-Pinto, 2005), and *M. crenulata* (Martin et al., 2010). This suggests that the secretory processes concerning with digestion occurs in most regions of the GI tract of gastropods.

Both neutral and acidic mucins were observed in parts of the GI tract of *P. canaliculata*. It was reported that neutral mucins were found in the stomach and duodenum, whereas acid mucins were distributed throughout the GI tract (Awad and Abdul Raheem, 2014). Neutral mucins are known to help for reducing the pH and toxicity of substances that pass through the GI tract, whereas acidic mucins contain chelating agents and have antibacterial and antiviral properties (Nikumbh et al., 2012).

In summary, the structure of the GI tract of *P. canaliculata* is similar to those of the other gastropods with some minor differences. The mucosa of the GI tract was lined by simple columnar epithelium with apical microvilli. In addition, mucus-secreting cells were scattered within the epithelial cells. Results of the present study could be useful for further studies conducting to understand how this snail species is a good survivor.

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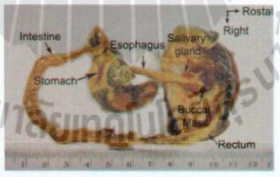

Fig.1 Gross structure of the GI tract of *P. canaliculata*. The GI tract composed of the esophagus, stomach, intestine, and rectum.

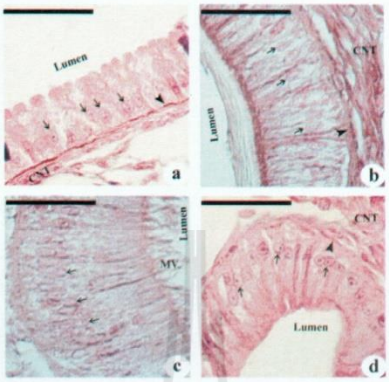

Fig.2 Histology of parts the GI tract of *P. canaliculata*; a, esophagus; b, stomach; c, intestine; d, rectum. Arrows, cell nuclei; arrow heads, basement membranes; CNT, connective tissue; MV, microvilli. Scale bars = 100 µm.

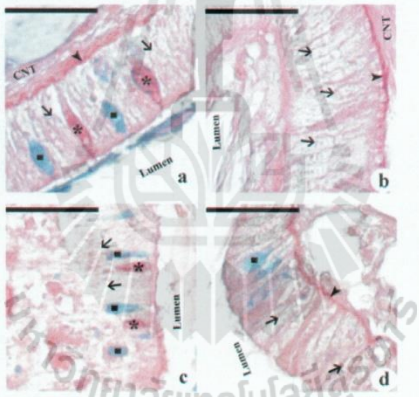

Fig.3 Mucus-secreting cells in the GI tract of *P. canaliculata*: a, esophagus; b, stomach; c, intestine; d, rectum. Asterisks indicate cells stained with PAS and rectangles indicate cells stained with alcian blue, pH 2.5. Arrows, cell nuclei; arrow heads, basement membrane; CNT, connective tissue; MV, microvilli. Scale bars = 100 µm.

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