

**Studies on the effects of cholecystokinin on  
ghrelin, oxyntomodulin, growth hormone and  
metabolites in cattle**

**2012. 3**

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## ABSTRACT

We synthesized peptides of bovine CCK-8 (sulfated cholecystokinin-8 amide, non-sulfated CCK-8 amide, [Cys<sup>0</sup>]sulfated CCK-8 C-terminal free, [Tyr<sup>0</sup>]sulfated CCK-8 amide), bovine gastrin (sulfated gastrin-9 amide, non-sulfated gastrin-9 amide), bovine ghrelin [octanoyl ghrelin (O-ghrelin), decanoyl ghrelin (D-ghrelin) and decenoyl ghrelin, des-acyl ghrelin], growth hormone releasing hormone (GHRH), [Cys<sup>0</sup>] 1-29 human GHRH, [Cys<sup>0</sup>] 21-44 bovine GHRH, [Cys<sup>0</sup>] bovine amylin (7- 37) and [Tyr<sup>0</sup>]bovine amylin. These synthesized peptides were used for first antibody production, radioisotope labeling, cold standard preparation, first antibody specificity check and administration (Chapter-2). We got first antibodies of sulfated CCK-8, GHRH, amylin and decanoyl ghrelin (D-ghrelin) which generated in guinea pig and chicken. We got second antibody (goat anti-IgY serum which generated in goat). We validated bovine sulfated CCK RIA system which specifically recognized sulfated CCK-8 (CCK-8s). We studied the effect of ghrelin and oxyntomodulin (OXM) on plasma CCK release in cattle. We set up GHRH RIA and amylin RIA systems; however we cannot use these RIA systems for measuring of plasma GHRH and amylin levels due to low sensitivity of RIA (Chapter-2). First antibody of D-ghrelin recognized to D-ghrelin, O-ghrelin and decenoyl ghrelin. Therefore, this antibody cannot use for D-ghrelin RIA. For a better understanding of the effect of CCK on ghrelin, OXM, pancreatic polypeptide (PP), glucagon like peptide-1 (GLP-1), GH and metabolites (glucose and NEFA), we conducted experiment in 8 Holstein steers. Moreover, we studied the effects of acyl ghrelin and des-acyl ghrelin on plasma OXM, CCK, insulin, PP and GLP-1 secretions in steers. CCK-8s increased plasma acyl ghrelin and total ghrelin levels in steers. This result is first report for sulfated CCK stimulates ghrelin secretion in ruminant. CCK-8s stimulates PP release. CCK-8s also stimulates plasma PP, insulin, glucose and NEFA secretions. However, CCK-8s does not affect plasma OXM, GLP-1 and GH levels. In addition, ghrelin has no effect on plasma CCK, OXM, PP and GLP-1 secretions. OXM does not affect ghrelin and CCK secretions, and vice versa in ruminants (Chapter-3). We found that the effect of CCK-8s on plasma glucose and NEFA secretions in steer is different from sheep. In conclusion, sulfated CCK-8 has stimulatory effect on plasma ghrelin, insulin, PP, glucose and NEFA secretions in cattle.

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**Abbreviations****Full name**

AgRP	agouti-related peptide
ANOVA	analysis of variance
ARC	arcuate nucleus
BSA	bovine serum albumin
BW	body weight
CCK	cholecystokinin
CCK-8s	sulfated cholecystokinin octapeptide
CCK-33ns	non-sulfated cholecystokinin 33
CPM	count per minute
D-ghrelin	decanoyl ghrelin
EDT	ethanedithiol
EDTA	ethylenediaminetetraacetic acid
Fmoc	9-fluorenylmethoxycarbonyl
GH	growth hormone
GHRH	growth hormone releasing hormone
GHIH	growth hormone inhibiting hormone
GIT	gastrointestinal tract
GLP-1	glucagon like peptide -1
GLP-2	glucagon like peptide -2
HPLC	high performance liquid chromatography
IAPP	islet amyloid polypeptide
IgY	immunoglobulin Y
iv	intravenous
ip	intraperitoneal
icv	intracerebroventricular
LHN	lateral hypothalamic nucleus
mcKLH	mariculture keyhole limpet hemocyanin
MSH	melanocyte stimulating hormone
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
O-ghrelin	octanoyl ghrelin
OXM	oxyntomodulin

**Abbreviations**

pE

PEG

POMC

PEG

PP

PVN

PYY

RIA

rpm

SPPS

SPSS

TFA

TIS

**Full name**

pyroglutamic acid

polyethyleneglycol

pro-opiomelanocortin

polyethyleneglycol

pancreatic polypeptide

paraventricular nucleus

peptide tyrosine tyrosine

radioimmunoassay

rate per minute

solid phase peptide synthesis

statistical package for the social sciences

tri-fluoroacetic acid

Triisopropylsilane



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## **Chapter 1 (General introduction)**

### **1 Appetite regulatory hormones**

“Hormone” means “to excite” in Greek. After the discovery of hormones, scientists found the regulatory effect of hormones on appetite. A hormone is a chemical substance synthesized by particular endocrine glands and then enters the circulation to be carried to a target tissue, which has specific receptors that bind it (Squires 2003). Hormones are divided into different types according to the structure, steroid hormones, proteins, polypeptides and glycoprotein hormones, fatty acids and derivatives hormones (Squires 2003). Hormones are also classified into neuroendocrine hormones which synthesized by nervous tissue and carry in blood to the target tissue, neurocrine hormones which released into the synaptic cleft by neurons that are in contact with the target cells, paracrine hormones which diffuse to neighboring cells, and autocrine hormones which feedback on the cell of origin as self-regulation (Squires 2003). Hormones secreted from peripheral tissues bind to receptors in the hypothalamus and regulate appetite (Wynne et al. 2004).

Regulation of appetite is a complex mechanism in which many hormones secreted from brain and gastrointestinal tract are involved. In brain, the hypothalamus is mainly involved in the regulation of appetite (Vettor et al. 2002). There are many nuclei in the hypothalamus which regulate food intake including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic nucleus (LHN), the ventromedial nucleus and the dorsomedial nucleus (Heijboer et al. 2006). There are 2 main hypothalamic arcuate nucleus (ARC) neurons which involved in the regulation of food intake for energy homeostasis; appetite stimulating neuropeptide Y (NPY) and agouti-related peptide (AgRP) coexpressing neurons and appetite-inhibiting pro-opiomelanocortin (POMC) neurons (Williams et al. 2001).

Hormones released from the hypothalamus, gastrointestinal tract (GIT), pancreas

and adipose tissue are involved in the regulation of appetite. Neuropeptides Y (NPY), AgRP, POMC, orexin and galanin are released from hypothalamus and which regulates appetite. Appetite regulatory hormones mainly released from gastrointestinal tract (GIT) are secretin, gastrin, cholecystokinin (CCK), oxyntomodulin (OXM), peptide tyrosine tyrosine (PYY), glucagon like peptide-1, glucagon like peptide-2, ghrelin, somatostatin and obestatin. Other appetite regulatory hormones mainly released from pancreas are insulin, amylin, pancreatic polypeptide (PP) and glucagon. Moreover, leptin hormone is released from adipose tissue.

## **1.1 Hormones release from hypothalamus which regulate appetite**

### **1.1.1 Neuropeptide Y**

Neuropeptide Y (NPY) was isolated from bovine brain. It consists of 36 amino acids (Tatemoto 1982b). The amino acids sequence of neuropeptide Y is 70 % homology with peptide tyrosine tyrosine (PYY) and 50 % homology with pancreatic polypeptide (PP) (Tatemoto 1982a). It belongs to pancreatic polypeptide family (Tatemoto 1982a). Central administration of NPY increase food intake in rats (Clark et al. 1984, Stanley and Leibowitz 1985, Edwards et al. 1999). CCK decreases NPY mRNA concentration in rat (Bi and Moran 2002). However, intravenous injection of ghrelin stimulates NPY neurons and promotes secretion of NPY peptide (Kojima and Kangawa 2005).

### **1.1.2 Agouty related peptide (AgRP)**

Agouty relate peptide is 132 amino acids peptide. It is produced from hypothalamic arcuate nucleus neurons (Qian et al. 2002). Plasma AgRP is increased in fasted condition and decreased after eating in humans and rats (Shen et al. 2002). Administration of AgRP increases food intake in rats (Rossi et al. 1998) and mice

(Marsh et al. 1999, Qian et al. 2002). Central administration of ghrelin increases the AgRP mRNA level in the hypothalamus and intravenous injection of ghrelin also stimulates AgRP neurons and promotes secretion of AgRP peptide (Kojima and Kangawa 2005).

### **1.1.3 Pro-opiomelanocortin (POMC)**

Pro-opiomelanocortin (POMC) is a polypeptide. Alpha, beta and gamma-melanocyte-stimulating hormone (MSH) are post-translational products of POMC (Wilson and White 1998). Central administration of alpha-MSH decreased food intake in rats (Rossi et al. 1998). In addition, administration of alpha-MSH and gamma-2 MSH suppress feeding in fasted rats (Millington et al. 2001).

### **1.1.4 Orexin**

Orexin is an orexigenic hypothalamic neuropeptide. It is also called hypocretin (de Lecea et al. 1998). It has 2 subtype, orexin -A and orexin-B peptide. Orexin-A is 33 amino acids peptide and orexin- B is 28 amino acids peptide. Central administration of both orexin-A and orexin-B stimulate food intake in rodents (Sakurai et al. 1998, Edwards et al. 1999).

### **1.1.5 Galanin**

Galanin is 29 amino acids peptide (Tatemoto et al. 1983). It is widely distributed in brain and gut, with highest concentration in hypothalamus (Kyrkouli et al. 1986). Galanin has orexigenic effect and it last only 1 hour in rats (Kyrkouli et al. 1986). Administration of galanin stimulates food intake in rat (Edwards et al. 1999).

Galanin has relationship with some appetite regulatory hormones. Galanin inhibits glucose induced insulin in rodents (Ahrén et al. 2004). The effect of galanin on somatostatin is controversial. In vitro study of rainbow trout islets, galanin inhibits

somatostatin 25 release, however galanin stimulates somatostatin 14 at  $10^{-7}$  M in the present of 3 mM glucose (Eilertson et al. 1996). Some scientists reported that galanin did not affect somatostatin release from fragment of rat mediobasal hypothalamus (Hulting et al. 1991). Galanin suppresses somatostatin and insulin and stimulates pancreatic glucagon release in dog pancreas (Dunning et al. 1986), however does not influence on plasma glucagon levels in humans (Bauer et al. 1986). In vitro study, galanin does not affect glucagon secretion from the perfused rat pancreas (Silvestre et al. 1987).

Central and peripheral administration of galanin increases CCK in rats (Björkstrand et al. 1993). Administration of galanin decreases plasma PP secretion however does not influence on plasma glucagon levels in humans (Bauer et al. 1986). In vitro study, galanin inhibits GLP-1 secretion in rat ileal L-cells (Saïfia et al. 1998). There is few information about the effect of galanin on other appetite regulatory hormones in ruminant.

## **1.2 Hormones release from gastrointestinal tract (GIT) which regulate appetite**

### **1.2.1 Secretin**

Secretin was discovered in 1902 (Bayliss and Starling 1902). It is the first hormone discovered in human history. It is 27 amino acids peptide (Mutt et al. 1970) and mainly released from S-cells of duodenum (Polak et al. 1971). Secretin belongs to the vasoactive intestinal polypeptide/ Secretin/ glucagon/ growth hormone-releasing hormone peptide family (also called glucagon super family) (Vaudry et al. 2000). Secretin binds to secretin receptors which belong to secretin receptor family (Harmer 2001). Some scientists reported that secretin is related with feeding. Plasma concentration of secretin increased after eating in human (Chey et al. 1978), pre-ruminant and ruminant calves (Le Dréan et al. 1997) and sheep (Mineo et al. 1990).



There are controversial results for the effect of secretin on food intake. Some scientists reported that administration of secretin did not affect food intake in rat (Gibbs et al. 1973). However, some reported that intra-peritoneal infusion, and central injection of secretin decreases food intake in mice and the anorectic effect of secretin is mediated by melanocortin system (Cheng et al. 2011). Central administration of secretin has no effect on food intake in sheep (Della-Fera et al. 1980). However, Groum et al. (1981) reported that exogenous secretin decreases food intake in sheep.

Secretin has relationship with pancreatic polypeptide (PP), ghrelin and insulin in monogastric species. Because, administration of secretin increased plasma PP levels in human (Peracchi et al. 1999), and increased plasma insulin levels in humans (Chisholm et al. 1969) and dogs (Unger et al. 1969). In addition, secretin stimulates ghrelin release from rat stomach ghrelin cells (de la Cour et al. 2007). However, secretin has no relationship with ghrelin and GH release in steers because administration of secretin did not affect plasma ghrelin and GH levels in cattle (ThanThan et al. 2010). Moreover, secretin has no effect on somatostatin release in human (Holst et al. 1990). Secretin has no effect on fasting serum gastrin levels, however decreases in postprandial serum gastrin response in human (Thompson et al. 1972). In addition, secretin decreases fasting serum gastrin concentrations in dog (Thompson et al. 1972). Secretin produced a small release of PYY in rat colon (Plaisancié et al. 1995). However, administration of secretin does not affect glucagon like peptide-1 (GLP-1) release in rat colon (Plaisancie et al. 1994) and porcine ileum (Hansen and Holst 2002).

### **1.2.2 Gastrin**

Gastrin is mainly secreted from G-cells of the stomach (Buchan et al. 1979). There are various amino acid forms of gastrin, Gastrin-14, gastrin-17, gastrin-34 (Rehfeld et al. 1974) and gastrin-71 (Rehfeld 1994). It belongs to gastrin/ cholecystokinin family (Dockray et al. 1989). Gastrin binds to CCK-B/gastrin receptors (Rehfeld 1998, 2004).

Plasma concentration of gastrin increases after eating in rats (Lindén et al. 1989) and humans (Dockray and Taylor 1976). However, plasma gastrin did not increase in response to feeding in sheep and cows (Perry et al. 1988). Exogenous gastrin decreases food intake in rodent (Ibu et al. 1991) and sheep (Grosvum et al. 1981).

Gastrin has relationship with ghrelin, insulin and CCK. Gastrin stimulates plasma ghrelin release in rodents (Murakami et al. 2002, Fukumoto et al. 2008) and ruminants (Zhao et al. 2011). Lippl et al. (2004) reported that gastrin decreased ghrelin secretion in isolated rat stomach. Some scientist reported that intravenous administration of gastrin has no effect on plasma GH in rodents (Vijayan et al. 1978). However, administration of gastrin stimulates plasma concentration of GH in Holstein steers (Zhao et al. 2011). Although administration of gastrin decreased plasma CCK in humans (Rehfeld 1998), we did not observe changes of plasma CCK levels after administration of gastrin in Holstein steers (Yannaing et al. unpublished data). Gastrin stimulates plasma insulin levels in dogs (Kaneto et al. 1969) and humans (Kikachi et al. 1971, Rehfeld et al. 1978). However, injection of gastrin decreases insulin secretion in cattle (Zhao et al. 2011). Gastrin stimulates plasma glucagon secretion in humans (Rehfeld et al. 1978). However in our study, administration of gastrin has no effect on plasma glucagon levels in steers (unpublished data). Injeciton of gastrin stimulates plasma somatostatin secretion in sheep (Zavros and Shulkes 1997). Gastrin has stimulatory effect on pancreatic polypeptide (PP) release in dog (Guzman et al. 1987). However, Parks et al. (1979) reported that gastrin has no effect on PP secretion in dog. We did not observe changes of PP secretion after intravenous administration of bovine gastrin in steers (Yannaing et al. unpublished data).

### **1.2.3 Cholecystokinin (CCK)**

CCK is mainly secreted from intestinal I-cells (Buchan et al. 1978). It is also secreted from hypothalamus (Micevych et al. 1984). It belongs to Gastrin/

cholecystokinin family (Dockray et al. 1989). There are various amino acid forms of CCK, CCK-83, CCK-58, CCK-39, CCK-33, CCK-22, CCK-8, CCK-5 and CCK-4 (Rehfeld 1998, 2004). CCK binds to CCK receptors. There are two types of CCK receptors, CCK-A and CCK-B receptors (Rehfeld 2004). Sulfated CCK binds to CCK-A and CCK-B/gastrin receptors (Rehfeld 1998, 2004). However, non-sulfated CCK binds to CCK-B/gastrin receptors (Rehfeld 1998, 2004). Plasma concentration of CCK increased after eating in non-ruminants and humans (Lilja et al. 1984). CCK released from the small intestine during meal is a physiological control of satiation in both rat and human, but CCK's mode of action is paracrine in rat and endocrine in human (Geary et al. 2004). In fasted rat, intra-peritoneal administration of sulfated CCK-8 decreased food intake and subcutaneous injection did not affect food intake. The result showed that CCK mode of action to meal act in paracrine action to produce satiety (Ebenezer 1999). Plasma concentration of CCK did not change after feeding in lactating dairy cows (Furuse et al. 1991) and steers (Mir et al. 2000). In our experiment, we did not observe changes of plasma CCK after feeding in Holstein steers (Yannaing et al., In press). However, some scientists reported that plasma CCK increased after feeding in fatty acid supplement fed ruminants (Choi et al. 2000, Relling et al. 2007, 2010).

Exogenous CCK decreased food intake in rat (Gibb et al. 1973), dog (Inui et al. 1989) and pig (Parrott et al. 1991). CCK is firstly discovered hormone which related with appetite. The anorectic effect of CCK is mediated by the CCK-A receptor via the vagus nerve, and is abolished by vagotomy (Smith et al. 1981). Intracerebroventricular infusion of CCK decreases food intake in fasted sheep (Della-Fera et al. 1980). However, intravenous infusion of CCK did not affect food intake in sheep (Relling et al. 2011).

CCK has relationship with other appetite regulatory hormones. CCK stimulates plasma insulin release in non-ruminants (Ahrén et al. 1991) and ruminants (Baile et al. 1968, Mineo et al. 1997, Yannaing et al., In press). Administration of CCK decreased

plasma ghrelin in Humans (Brennan et al. 2007). However, CCK stimulates plasma ghrelin in rodents (Murakami et al. 2002, Friis-Hansen et al. 2005, Shrestha et al. 2009). Intravenous administration of sulfated CCK-8 increases ghrelin (acyl ghrelin and total ghrelin) secretions in cattle (Yannaing et al. 2012, In press). However, administrations of CCK-4 and non-sulfated CCK-33 have no effect on plasma ghrelin release in steers (Yannaing et al. unpublished data). Some scientists reported that administration of CCK has no effect on ghrelin release in human (Little et al. 2007).

Administration of CCK increased plasma somatostatin in non-ruminants (Ahrén et al. 1991) and ruminants (Zarvos and Shulkes 1997). In addition, CCK stimulates pancreatic polypeptide (PP) in mice (Ahrén et al. 1991, 1995), human (Schmid et al. 1989) and dog (Parks et al. 1979, Schusdziarra et al. 1986). Choi et al. (2000) reported that feeding induced endogenous increased CCK stimulates PP secretion in cattle. Moreover, intravenous injection of sulfated CCK-8 stimulates plasma PP secretion in Holstein steers, however non-sulfated CCK-33 and CCK fragment (CCK-4) have no effect on plasma PP release in steers (Yannaing et al. unpublished data).

Intravenous injection of CCK stimulates PYY release in human (Brennan et al. 2007, Little et al. 2007). Moreover, CCK produced a small release of PYY in rat colon (Plaisancié et al. 1995). However, Onaga et al. (2000) reported that exogenous CCK did not affect plasma PYY in sheep. The effect of CCK on plasma glucagon like peptide-1 (GLP-1) is controversial. Some scientists reported that administration of CCK did not affect plasma GLP-1 in rat colon (Plaisancie et al. 1994), human (Ahrén et al. 2000) and sheep (Relling et al. 2011). However, some reported that CCK stimulates GLP-1 secretion in human (Fieseler et al. 1995) and in porcine ileum (Hansen and Holst 2002). We do not observe changes of plasma GLP-1 levels after intravenous injection of sulfated CCK-8 and nonsulfated CCK-33 in cattle (Yannaing et al., unpublished data). Administration of CCK has no effect on plasma leptin in humans (MacIntosh et al. 2001). Exogenous CCK did not change plasma glucagon levels in human (Ahrén et al.

1991, 2000) and sheep (Mineo et al. 1997). However some scientists reported that CCK stimulates pancreatic glucagon release in vitro study of dog (Hermansen 1984). CCK has no relationship with OXM because CCK does not affect OXM secretion and OXM does not affect on CCK secretion in cattle (Yannaing et al., In press).

#### **1.2.4 Glucagon like peptide-1 (GLP-1)**

Glucagon like peptide-1 (GLP-1) is a proglucagon derived hormone. It is released from intestinal L-cells (Herrmann et al. 1995). It is 30 amino acids peptide (Ahrén 1998). It belongs to glucagon super family (Burrin et al. 2003). Postprandial concentrations of GLP-1 increased in human (Herrmann et al. 1995) and in fatty acid supplement diet fed dairy cow (Relling et al. 2010). However, plasma GLP-1 levels did not change after feeding in fatty acid fed sheep (Relling et al. 2011). Central or peripheral injection of GLP-1 decreases food intake in rats (Langhana et al. 1987). In addition, intravenous infusion of GLP-1 decreases food intake in wethers (Relling et al. 2011).

GLP-1 has relationship with other appetite regulatory hormones. Glucagon like peptide-1 decreased ghrelin secretion in isolated rat stomach (Lippel et al. 2004). In addition, GLP-1 suppress ghrelin secretion in human is via increased insulin secretion (Hagemann et al. 2007). However, some scientists reported that administration of GLP-1 has no effect on plasma ghrelin in humans (Brennan et al. 2007). GLP-1 stimulates insulin secretion in mice (Ahrén et al. 1995), rat pancreatic islets (Schmidt et al. 1985), human (Kreymann et al. 1987, Gutzwiller et al. 1999) and sheep (Martin and Faulkner 1993). GLP-1 stimulates insulin secretion in cattle (ThanThan et al. 2011). However, some scientists reported that GLP-1 inhibits glucagon secretion in human (Kreymann et al. 1987). Injection of GLP-1 decreased PYY release in human (Brennan et al. 2007). GLP-1 has no effect on CCK and leptin secretions in human (Gutzwiller et al. 1999). In addition, GLP-1 has no effect on PP secretion in mice (Ahrén et al. 1995).

GLP-1 stimulates somatostatin secretion in rat stomach (Eissele et al. 1990) and intestinal culture (Brubaker et al. 1997), and inhibits gastrin secretion in rat stomach (Eissele et al. 1990). In our study, intravenous administration of GLP-1 did not affect plasma CCK levels in Holstein steers (Yannaing et al. unpublished data). There is few information on the effect of GLP-1 on other appetite regulatory hormones in ruminant.

### **1.2.5 Glucagon like peptide-2 (GLP-2)**

Glucagon like peptide-2 (GLP-2) is another product from proglucagon. It is released from intestinal L-cells (Ørskov et al. 1987). It is 33 amino acids peptide hormone. It belongs to glucagon super family (Burrin et al. 2003). GLP-2 is released following ingestion of food in rat (Hartmann et al. 1995), pig (Nielsen et al. 2003) and human (Ørskov et al. 1987, Brubaker et al. 1997). Intracerebroventricular injection of GLP-2 decreases food intake in rats (Tang-Christensen et al. 2001). However, exogenous GLP-2 did not affect food intake in human (Schmidt et al. 2003, Sørensen et al. 2003). The effect of GLP-2 on feed intake is uncertain. Tsai et al. (1997) did not observe a decrease in feed intake in mice during a 9-d period of GLP-2 infusion; however, the route of infusion of the peptide on that study is not clear.

GLP-2 has relationship with some appetite regulatory hormones. Administration of GLP-2 increased plasma glucagon in humans (Sørensen et al. 2003, Meier et al. 2006). Banasch et al. (2006) reported that Glucagon-like peptide 2 inhibits ghrelin secretion in humans. GLP-2 may not be related with somatostatin and insulin secretions. Because, GLP-2 does not affect somatostatin secretion in rat intestinal culture (Brubaker et al. 1997). In addition, GLP-2 has no effect on insulin secretion from rat pancreatic islets (Schmidt et al. 1985). Moreover, intravenous infusion of GLP-2 has no effect on insulin and GLP-1 secretions in human (Sørensen et al. 2003). There is limited information about the relationship between GLP-2 and other appetite regulatory hormones in ruminant.

### **1.2.6 Glicentin**

Glicentin is a 69 amino acids peptide produced in the L-cells of the small intestine (Holst 1997). Glicentin contains the sequence of OXM and glucagon, which are the amino acids from 33 to 69, and 33 to 61, respectively. Dakin et al. (2001) reported that central administration of glicentin did not affect food intake in rats. The small intestine secretes glicentin. However, the mechanisms that regulate its secretion and the effect of glicentin on other appetite regulatory hormones are not known.

### **1.2.7 Oxyntomodulin (OXM)**

Oxyntomodulin (OXM) is like GLP-1 and GLP-2, a product of proglucagon. OXM is secreted from intestinal L-cells (Bataille et al. 1982). It is 37 amino acids peptide (Bataille et al. 1982). It has the glucagon sequence plus eight amino acids on the C-terminal end (Bataille et al. 1982, Holst 1997). Plasma concentration of OXM increased after eating in human (Hornnes et al. 1980). However, plasma OXM level does not change after feeding in sheep (Relling et al. 2010). Post-ruminal infusion of macronutrient also does not affect plasma OXM level in cattle (Relling et al. 2010). Exogenous OXM decreases food intake in rat and human (Cohen et al. 2003, Dakin et al. 2001, 2004). Central administration of OXM decrease food intake in fasted and non-fasted rat (Dakin et al. 2001). There is few information about exogenous OXM on food intake in ruminants. OXM level did not change after feeding in ruminant (Relling et al. 2010) In our experiment, we did not observe changes of plasma OXM level after feeding in steers (Yannaing et al., In press ).

OXM has relationship with ghrelin in non-ruminant. Administration of OXM decreases ghrelin release in non-ruminants (Cohen et al. 2003, Dakin et al. 2004, Patterson et al. 2009). However, OXM may not have relationship with ghrelin and CCK in ruminant because administration of OXM did not affect plasma ghrelin in cattle (ThanThan et al. 2010). Also, injection of ghrelin (acyl ghrelin and des-acyl ghrelin) did

not affect plasma OXM (Yannaing et al., In Press) in Holstein steers. In addition, OXM may not have relationship with CCK in ruminants. Administration of OXM did not change plasma CCK levels and, also CCK did not affect plasma OXM levels in steers (Yannaing et al., In press). Therefore, it is possible that OXM has no relationship with ghrelin and CCK in cattle. However, OXM has relationship with insulin in ruminant. Administration of OXM increased plasma insulin levels in Holstein cattle (ThanThan et al. 2010, 2011). In addition, injection of OXM stimulates insulin secretion in rat pancreas (Jarrousse et al. 1984). Injection of OXM does not affect glucagon release in cattle (ThanThan et al. 2010). Administration of OXM increases gastric somatostatin secretion in cats (Bado et al. 1993).

#### **1.2.8 Peptide tyrosine tyrosine (PYY)**

PYY is secreted from intestinal L-cells (Adrian et al. 1985). It is 36 amino acids peptide (Tatemoto 1982a). It is a member of the pancreatic polypeptide (PP) family (Tatemoto 1982a). There are two forms of PYY, PYY 1-36 and PYY 3-36 (Graint et al. 1994). PYY increases after feeding in human (Adrian et al. 1985). However, postprandial plasma PYY did not change in sheep (Onaga et al. 2000). Injection of PYY3-36 decreases food intake in rodent (Batterham et al. 2002), human (Challis et al. 2003) and pig (Ito et al. 2006). PYY inhibits feeding via the vagal afferent pathway. It is clarified that abdominal vagotomy abolished the anorectic effect of PYY3-36 in rats (Koda et al. 2005).

PYY has relationship with other appetite regulatory hormones. Peripheral administration of PYY reduces plasma ghrelin levels in humans (Batterham et al. 2003). PYY released by fat perfusion is CCK dependent in dog (Lin et al. 2000). In addition, injection of PYY increases gastric somatostatin secretion in cats (Bado et al. 1993). However, some scientists reported that administration of PYY did not affect plasma ghrelin in pigs (Ito et al. 2006) and mice (Adams et al. 2004). PYY did not change



either somatostatin or gastrin secretions from rat stomach (Eissele et al. 1990). PYY has inhibitory effects on glucose induced insulin secretion in rat pancreas (Bertrand et al. 1992). In addition, PYY inhibits stimulates insulin and glucagon secretion in mouse (Böttcher et al. 1989). There is limited information for the effect of PYY on other appetite regulatory hormones in ruminants.

### **1.2.9 Ghrelin**

Orexigenic hormone ghrelin is primarily released from X/A-like cells via stomach in monogastric animals (Kojima et al. 1999) and from abomasum in ruminants (Hayashida et al. 2001). It is a ligand for the growth hormone secretagogue receptor (GHS-R1a) (Kojima et al. 1999). There are two types of ghrelin cells in the gastrointestinal tract, opened-type cells which are in contact with the lumen and closed-type cells which do not have luminal connection (de la Cour et al. 2001, Sakata et al. 2002). Ghrelin also found in rumen of 12 weeks old corn-based calf starter feed ingesting calves (Gentry et al. 2003). It is 28 amino acids peptide (Kojima et al. 1999). There are two molecular types of ghrelin, ghrelin (28 amino acids peptide) and des-Gln 14-ghrelin (27 amino acids peptide) in rats (Hosoda et al. 2000a). Monogastric animals have both forms of peptide, however ruminants have only des-Gln 14-ghrelin (27 amino acids) (Dickin et al. 2004).

Ghrelin has two forms in the circulating, acyl ghrelin and des-acyl ghrelin (Hosoda et al. 2000b). Acyl ghrelin, its acylation to serine residue at position 3 is essential for binding to growth hormone secretagogue receptor-1a (GHS-R1a) and for stimulation of food intake and growth hormone releasing actions (Kojima and Kangawa 2005). Ghrelin stimulates on feeding is not depend on the growth hormone (GH) stimulatory pathway (Nakazato et al. 2001).

In addition, acyl ghrelin has 3 forms, octanoyl ghrelin, decanoyl ghrelin and decenoyl ghrelin. Octanoyl ghrelin (O-ghrelin) is acylated with octanoic fatty acids

(Kojima et al. 1999), decanoyl ghrelin (D-ghrelin) is acylated with decanoic fatty acids, and decenoyl ghrelin is acylated with decenoic fatty acids (Hiejima et al. 2009) at N-terminal serine 3 amino acid residue. Serine 3 residue of O-ghrelin is modified with fatty acids of (C8:0) an 8-carbon chain containing no double bond (Kojima and Kangawa 2005) and D-ghrelin is modified with fatty acids of (C10:0) a 10-carbon chain lacking double bonds (decanoyl ghrelin) and (C10:1) a 10-carbon chain containing with one double bond (decenoyl ghrelin) (Hosoda et al. 2004). D-ghrelin and O-ghrelin are co-localized in the same of stomach (Hiejima et al. 2009). In fasted condition, D-ghrelin increased in plasma and stomach. Plasma O-ghrelin is increased in fasted condition however, declined in stomach (Hiejima et al. 2009). There is some information on D-ghrelin in rats, human and cat (Hiejima et al. 2009). However, there is limited information about D-ghrelin in ruminants.

Plasma ghrelin levels increased before eating in non-ruminant (Kojima and Kangawa 2005) and restricted and scheduled meal fed ruminants (Sugino et al. 2002, Relling et al. 2010). In fasted condition, plasma ghrelin levels increased in rat (Murakami et al. 2002) and cattle (Wertz-Lutz et al. 2006) and decreased after eating in human (Kojima and Kangawa 2005) and ruminant (Wertz-Lutz et al. 2006). Some scientists reported that suppression of ghrelin release after eating is CCK dependent in human (Degen et al. 2007).

The non-acylated form of ghrelin is des-acyl ghrelin which exists at significant level in blood (Hosoda et al. 2000). It is produced not only in the gastric mucosa but also in plasma by de-acylation of the acyl ghrelin (Kojima et al. 2005). Des-acyl ghrelin does not bind to the ghrelin receptor GHS-R1a (Nakazato et al. 2001). Central or peripheral administration of des-acyl ghrelin has been shown to decrease food intake in rats (Inhoff et al. 2008). Intraperitoneally injected ghrelin induced food intake was blocked by peripherally injected des-acyl ghrelin in rats (Inhoff et al. 2008). Some scientists reported that central administration of des-acyl ghrelin stimulates feeding in

rodents; however, peripheral administration of des-acyl ghrelin has no effect on feeding (Toshinai et al. 2006).

Acyl ghrelin stimulates plasma GH secretion in non-ruminants (Kojima et al. 1999) and ruminants (Hashizume et al. 2005, ThidarMyint et al. 2006). However, des-acyl has no effect on plasma GH in non-ruminants (Toshinai et al. 2006) and ruminants (ThidarMyint et al. 2006, Yannaing et al, unpublished data). Bovine total ghrelin is the summation of acyl ghrelin, des-acyl ghrelin and all ghrelin fragments with an intact bovine C-terminal region (ThidarMyint et al. 2008). Des-acyl ghrelin has the inhibitory effect on acyl ghrelin induced food intake in rat (Inhoff et al. 2008). There is few information on the effect of des-acyl ghrelin on other appetite regulatory hormones. Administration of des-acyl ghrelin has no effect on plasma CCK and OXM secretions in ruminant (Yannaing et al., In press). In addition, des-acyl ghrelin does not affect plasma insulin, pancreatic polypeptide (PP) and GH release in steers (Yannaing et al, unpublished data).

Ghrelin has relationship with other appetite regulatory hormones. Intraduodenal injection of ghrelin increased plasma CCK in rats (Jawork 2006, Nawrot-Porabka et al. 2007). However, exogenous acyl ghrelin has no effect on plasma cholecystinin (CCK) and oxyntomodulin (OXM) in ruminants (Yannaing et al., In press). Administration of ghrelin increased plasma gastrin and insulin in rodents (Lee H-M et al. 2002). In addition, intravenous injection of acyl ghrelin increased plasma insulin in ruminants (ThidarMyint et al. 2006, Yannaing et al. unpublished data). Administration of ghrelin increased plasma PP in humans (Arosio et al. 2003, Tack et al. 2006) and rat (Sato et al. 2003). However, administrations of acyl ghrelin and des-acyl ghrelin have no effect on plasma PP secretion in Holstein steers (Yannaing et al. unpublished data). Ghrelin stimulates glucagon secretion transiently in lactating cow (Itoh et al. 2005). Adeghate and Parvez (2002) reported that ghrelin has no effect on glucagon secretion in rat pancreas. However, ghrelin stimulates glucagon release in

mouse islets (Chuang et al. 2011). The effect of ghrelin on somatostatin is controversial. Edigo et al. (2002) reported that ghrelin inhibits somatostatin release in rat pancreas. However, Arosio et al. (2003) reported that ghrelin stimulates somatostatin release in human. According to my knowledge, there is few information about the effect of ghrelin on GLP-1 in non-ruminant and ruminant. Our study in steers, administrations of acyl ghrelin and des-acyl ghrelin do not affect plasma GLP-1 release (Yannaing et al. unpublished data). The effect of ghrelin in the hypothalamus is opposite to the effect of leptin; ghrelin increases NPY and AgRP gene expression (Kojima and Kangawa, 2005).

#### **1.2.10 Obestatin**

Obestatin is 23 amino acids hormone (Zhang et al. 2005). Plasma level of obestatin does not change after feeding in rat. However, intra-peritoneal infusion of obestatin decreased food intake in mice (Zhang et al. 2005). Long term infusion of obestatin did not affect dry matter intake in bovine (Roche et al. 2008). It is possible that obestatin may not be a major functional appetite regulatory hormone in ruminants.

Qader et al. (2008) reported that, obestatin inhibited somatostatin and pancreatic polypeptide secretion and simulated glucagon release in isolated mouse and rat islets. The effect of obestatin on insulin secretion is controversial. Some scientists reported that obestatin inhibits insulin release in rat and mice islets (Qader et al. 2008). Some reported that obestatin does not affect insulin release in rat and rat pancreatic islets (Unniappan et al. 2008).

### **1.3 Hormones release from pancreas which regulate appetite**

#### **1.3.1 Glucagon**

Glucagon is 29 amino acids peptide (Bromer et al. 1956). It is discovered in 1923 (Kimball et al. 1923). It is secreted from A-cells of pancreas (Baum et al. 1962) and also

detected in the specific cells of stomach and intestine in some species (Baetens et al. 1976). It belongs to the vasoactive intestinal polypeptide/ secretin/ glucagon/ growth hormone-releasing hormone peptide family (also called glucagon super family) (Vaudry et al. 2000). There are controversial results on the effect of glucagon on food intake in rodents. Langhans et al. (1987) reported that injection of glucagon decreased food intake in rats. However, Dakin et al. (2001) reported that central administration of glucagon did not affect food intake in rats.

Glucagon has relationship with some appetite regulatory hormones. Administration of glucagon stimulates plasma insulin release in humans (Ahrén et al. 1987). In addition, intravenous injection of glucagon increases insulin secretion in cattle (ThanThan et al. 2011). Exogenous glucagon stimulates insulin and somatostatin secretions in canine pancreas (Wier et al. 1979). Moreover, central administration of glucagon increased hypophysial portal somatostatin secretions in rats (Abe et al. 1978). Injection of glucagon also decreased plasma ghrelin secretion in human (Hirsh et al. 2005).

### **1.3.2 Insulin**

Insulin is secreted from B-cells of pancreas (Coore et al. 1969). It composed of 51 amino acids (Sanger and Tuppy 1951). Central or peripheral injection of insulin decreases food intake in rats and baboons (Ikeda et al. 1986, Wood et al. 1979, 1984). Plasma insulin decreased after feeding in fatty acids supplement fed wethers (Relling et al. 2010).

Insulin has inhibitory effect on ghrelin. Administration of insulin decreases plasma ghrelin in humans (Saad et al. 2002) and rodents (McCowen et al. 2002.). In addition, insulin decreased ghrelin secretion in isolated rat stomach (Lippl et al. 2004, Shrestha et al. 2009). The effect of insulin on somatostatin and glucagon secretions is controversial. Insulin stimulates somatostatin release in chicken (Honey and Wier 1979). However, insulin has no effect on somatostatin secretion in dog pancreas (Wier et al. 1979). Some

scientists reported that administration of insulin increases glucagon secretion in mice (Kawamori et al. 2009). However, insulin has no effect on glucagon and pancreatic polypeptide (PP) release in dog pancreas (Wier et al. 1979). There is few information about the effect of insulin on plasma ghrelin in ruminants.

### **1.3.3 Somatostatin**

Somatostatin is also called somatotropin release inhibiting factor (SRIF) or growth hormone inhibiting hormone (GHIH). Somatostatin is released from pancereatic D-cells (Orci et al. 1975). It is also released from hypothalamus (Brazeau et al. 1973). It is also find in stomach, duodenum, jejunum and pancreas (Arimura et al. 1975). There are two forms of somatostain, SS-14 (Brazeau et al, 1973, Schally et al. 1976) and SS-28 (Polonsky et al. 1983). Somatostatin levels increased after eating in human (Polonsky et al. 1983). However, postprandial plasma somatostatin did not change in Holstein steers (Gaynor et al. 1995). Injection of somatostatin decreased food intake in rats and baboons (Lotter et al. 1981). But in fasted rats, injection of somatostatin increased food intake (Aponte et al.1984).

Somatostatin has relationship with other appetite regulatory hormones. Somatostatin decreased ghrelin secretion in rat stomach (Shimada et al. 2003, Lippl et al. 2004, Seoane et al. 2007). In addition, somatostatin inhibits CCK release in rats (Herzig et al. 1994) and dogs (Lloyd et al. 1994). Administration of somatostatin decreases plasma insulin and glucagon secretions in rats (Märki et al. 1982). In addition, intravenous administration of somatostatin inhibits PP, glucagon and insulin secretions in human (Macro et al. 1983). Somatostain restrains the secretion of GLP-1 and GLP-2 in porcine ileum (Hansen et al. 2000). In addition, infusion of somatostatin also decreases plasma GLP-1 concentration in sheep (Martin and Faulkner 1996).

#### **1.3.4 Pancreatic polypeptide (PP)**

Pancreatic polypeptide (PP) is primarily released by cells the periphery of the islets of Langerhans (Adrian et al. 1976). Later the cells were given the name as PP cells (Adrian et al. 1976) or F-cells (Ekblad and Sundler 2002). It is also secreted by the exocrine pancreas and distal gastrointestinal tract (Larsson et al. 1975). It is 36 amino acids polypeptide (Lin and Chance 1974). Plasma level of pancreatic polypeptide increases after eating in human (Adrian et al. 1976). It is a member of PP-fold family of peptides which also include PYY and NPY (Colon 2002). Administration of PP decrease food intake in rodents (Asakawa et al. 1999) and humans (Batterham et al. 2003a). Plasma PP transiently increased after feeding in sheep (Takahashi et al. 2010). It is possible that postprandial release of PP is CCK dependent in both non-ruminant and ruminant. Because, the study in human with CCK-A receptor antagonist (MK-329), CCK increases pancreatic polypeptide (PP) and finds that CCK receptor blockage selectively attenuates postprandial release in plasma PP concentration (Liddle et al. 1990). In addition, administration of CCK-A receptor antagonist (MK-329) decreases postprandial release of PP in dairy cows (Choi et al. 2000).

PP has relationship with other appetite regulatory hormones. Administration of PP decreased plasma ghrelin in 48 hr fasted sheep (Takahashi et al. 2010). In addition, administration of ghrelin increased plasma PP in humans (Arosio et al. 2003; Tack et al. 2006). PP and ghrelin do not affect each other on their secretions in cattle. We did not observe the effect of PP on ghrelin secretion and the effect of acyl ghrelin and des-acyl ghrelin on PP secretion in Holstein steers (Yannaing et al. unpublished data). Administration of PP has no effect on plasma insulin, ghrelin, PYY, GLP-1 and leptin in human (Batterham et al. 2003a). We do not find changes of plasma PP concentrations after injection of amylin in steers (Yannaing et al. unpublished data). In addition, we do not observe changes of plasma GLP-1 levels after administration of PP in cattle (Yannaing et al. unpublished data).

### **1.3.5 Amylin**

Amylin is secreted from beta-cells of pancreatic (Moore et al. 1991). It also called as islet amyloid polypeptide (IAPP) which is co-secreted with insulin (Moore et al. 1991, Bhavsar et al. 1998). It is 37 amino acids peptide (Cooper et al. 1987). Amylin may synergistically react with CCK for appetite control (Reidelberger et al. 2001). Amylin also found in gut endocrine cells (Mulder et al. 1994). Administration of amylin decreases food intake and body weight (Arnado et al. 1996). According to our knowledge, there is few information on the effect of amylin on other appetite regulatory hormones in ruminants.

Amylin has relationship with other appetite regulatory hormones. Administration of amylin decreased plasma glucagon secretion in cat (Furrer et al. 2010) and in mouse (Panagiotidis et al. 1992). In addition, injection of amylin increased plasma gastrin in rats (Funakoshi et al. 1992). Moreover, administration of amylin stimulates somatostatin in mice (Zaki et al. 2002). However, amylin inhibit ghrelin secretion in rats (Gedulin et al. 2004, Young et al. 2004). We did not observe the effect of amylin on plasma ghrelin, CCK, insulin, PP, GLP-1 and GH secretions in steers (Yannaing et al. unpublished data). Some scientists reported that amylin inhibits gastrin secretion in human, dog and rat stomach (Makhlouf et al. 1996). Amylin reduces insulin release but does not affect somatostatin release in rat (Peiró et al. 1991).

## **1.4 Hormone release from adipose tissue which regulate appetite**

### **1.4.1 Leptin**

Leptin is released from adipose tissue. It is 167 amino acids peptide (Zhang et al. 1994). Plasma leptin levels do not change before and after eating in human (Considine et al. 1996). Plasma levels of leptin do not change after feeding in hay fed sheep (Tokuda et al. 2000). However, some scientists reported that postprandial leptin



concentration was changed in hay and concentrated fed cows (Delavaud et al. 1999). Circulating leptin was affected by nutritional status of feed in ruminants. In cow and sheep, low energy diet feeding decrease postprandial leptin level and high energy diet feeding increase postprandial leptin level (Delavaud et al. 2002, Tokuda et al. 2002). Intracerebroventricular administration of leptin decreases food intake in obese rat, control rat (Niimi et al. 1999) and mice (Mistry et al. 2001). Intraperitoneal injection of leptin decreased food intake in rats and also showed peripheral leptin resistant in obese rat (Niimi et al. 1999).

Leptin has relationship with some appetite regulatory hormones. Leptin inhibits the secretion of ghrelin in rats (Kalra et al. 2005, Lippl et al. 2005). In addition, leptin inhibits glucose stimulated insulin secretion in rats (Muzumdar et al. 2003). However, some scientists reported that leptin has no effect on insulin and glucagon secretions in fed mice and leptin stimulates plasma insulin and glucagon in fasted mice (Ahren et al. 1999). Moreover, leptin does not influence glucagon or somatostatin release in rat pancreas (Silvestre et al. 2001). Leptin inhibits glucose stimulated amylin in mouse pancreas (Karlsson et al. 1998). Some scientists reported that leptin stimulates GLP-1 secretion from rodent and human intestinal L-cells (Anini et al. 2003). Leptin stimulates CCK release in rats (Guilmeau et al. 2003). There is limited information about the effect of leptin on other appetite regulatory hormones in ruminant.

### **1.5 Study on Cholecystokinin (CCK), ghrelin and Oxyntomodulin (OXM)**

There are many appetite regulatory hormones which release from brain, gastrointestinal tract and adipose tissue. In this thesis, I study only three major appetite regulatory hormones CCK, ghrelin and OXM in ruminants. Because, plasma levels of these 3 hormones are related with food intake (Cohen et al. 2003, Dakin et al. 2004, Gibbs et al. 1973), gastric acid secretion (Dubrasquet et al. 1982, Kojima and Kangawa 2005, Rehfeld 2004) and body weight (Dakin et al. 2004, Kojima and Kangawa 2005,

Rehfeld 2004) and pancreatic enzyme secretion (Ying et al. 2006, Nawrot-Porabka et al. 2007, Kerstens et al. 1985, Anini et al. 2000) in monogastric species. In addition, these hormones are related with energy status. Plasma ghrelin level is increased in fasted non-ruminant (Kojima and Kangawa 2005) and ruminants (Wertz-Lutz et al. 2006) and decreased after eating. Plasma CCK levels increased after feeding in non-ruminants (Lilja et al. 1984,) and fatty acids supplement fed ruminants (Relling et al. 2007, 2010). In addition, plasma OXM levels increased after feeding in non-ruminants (Hornnes et al. 1980).

Moreover cholecystokinin, ghrelin and OXM are related with energy homeostasis. In negative energy condition, plasma ghrelin is increased (Kojima and Kangawa 2005, Wertz-Lutz et al. 2006), and plasma CCK (Kanayama and Liddle 1991, Nowak et al. 1997, Ohgo et al. 1988) and OXM (Alain et al. 1998) is decreased, respectively.

In addition, these 3 hormones regulate pancreatic enzyme secretion. Ghrelin (Ying et al. 2006, Nawrot-Porabka et al. 2007) and CCK (Kerstens et al. 1985) stimulate pancreatic enzyme secretion in monogastric species. However, OXM inhibits pancreatic secretion in rats (Anini et al. 2000). Oxyntomodulin can suppress CCK induced pancreatic secretion (Anini et al. 2000). There is few information about the relationship among these hormones. Therefore, we study on CCK, ghrelin and OXM in bovine.

The information for the effect of appetite regulatory hormones on CCK secretion is limited because of difficult to set up bovine CCK radioimmunoassay system which only recognized CCK. The stimulatory effect of ghrelin on CCK secretion has been reported in rat (Jawork 2006, Nawrot-Porabka et al. 2007). However, there is no information for the effect of ghrelin on CCK secretion in ruminant. There are controversial results for the effect of CCK on ghrelin secretion in non-ruminant. CCK inhibits ghrelin release in human (Brennan et al. 2007), CCK has no effect on ghrelin release in human (Little et al. 2007) and CCK stimulates ghrelin secretion in rodents (Murakami et al. 2002). The inhibitory effect of OXM on ghrelin secretion has been reported in rat and human

(Cohen et al. 2003, Dakin et al. 2004, Patterson et al. 2009). However, there is no information for the effect of CCK on ghrelin and OXM secretions, and the effect of OXM on ghrelin and CCK secretions in ruminants.

### **1.6 Study on pancreatic polypeptide (PP)**

Pancreatic polypeptide (PP) is related with food intake in monogastric species (Asakawa et al. 1999). PP secretions increased after eating (Adrian et al. 1976). Scientists studied and reported the effect of CCK and ghrelin on PP secretions in non-ruminants. CCK stimulates pancreatic polypeptide (PP) in mice (Ahrén et al. 1991, 1995), human (Schmid et al. 1989) and dog (Parks et al. 1979, Schusdziarra et al. 1986). Choi et al. (2000) reported that feeding induced endogenous increased CCK stimulates PP secretion in cattle. In addition, administration of ghrelin increased plasma PP in humans (Arosio et al. 2003, Tack et al. 2006) and rat (Sato et al. 2003). However, there is limited information for the effect of CCK and ghrelin on PP secretions in ruminant.

### **1.7 Study on the effect of CCK on growth hormone (GH), glucose and NEFA**

The results of the effect of CCK on GH secretion are varied among human, non-ruminant and sheep. There are controversial results for the effect of CCK on plasma GH. Some scientists reported that CCK stimulates GH in humans (Calogero et al. 1993), pigs (Parrott et al. 1995) and rats (Matsumura et al. 1984). Karashima et al. (1984) reported that CCK decreased plasma GH in rats. Vijayan et al. (1979) reported that central administration of CCK increased plasma GH, however peripheral administration of CCK did not affect plasma GH in rats. Some scientists reported that administration of CCK did not affect plasma GH in sheep (Spencer et al. 1991, Della-fera and Baile 1985) and humans (Nair et al. 1984). There is no information for the effect of CCK on GH secretions in cattle.

In addition, the regulatory effects of CCK on plasma glucose and NEFA are varied

in human, rat and sheep. In rats, intra-duodenal administration of CCK decreases glucose production independent of changes in circulating insulin (Cheung et al. 2009). Central administration of CCK has no effect on plasma glucose in sheep (Della-Fera et al. 1985). In addition, intravenous infusion of CCK did not affect plasma glucose and NEFA in wethers (Relling et al. 2010, 2011). There is no information about the CCK on plasma glucose and NEFA in cattle.

The objectives of thesis are:

- to study the effect of cholecystokinin on plasma ghrelin (acyl ghrelin and total ghrelin), OXM, pancreatic polypeptide (PP), glucagon like peptide-1 (GLP-1), GH and metabolites (glucose and NEFA) secretions and
- to study the effects of acyl ghrelin and des-acyl ghrelin on plasma CCK, OXM, PP and GLP-1 secretions in cattle.

## **1.8 Discovery of Cholecystokinin**

In 1903, a year after the discovery of secretin hormone, French physiologists might have suggested the existence of bile-releasing hormone. Similarly, Okada almost predicted Cholecystokinin (CCK) when he noted that intestinal acidification stimulated the expulsion of bile into the intestine. (Jorpes and Mutt. 1966, Refeld 2004). CCK was discovered in 1928 which stimulated contraction of gallbladder in dog (Ivy et al. 1928). In 1943, Harper et al. reported a hormone which was stimulated pancreatic enzyme secretion in cat and called *pancreozymin*. (Harper et al. 1943). After 23 years later, Jorpes and Mutt proved that pancreozymin and cholecystokinin (CCK) are the same substance (Jorpes and Mutt 1966). In 1970, Grossman proposed to use the term cholecystokin instead of cholecystokinin-pancreozymin. CCK is released from intestinal I-cells (Buffa et al. 1976). In 1975, Vanderhaeghen et al. reported that CCK was present in the mammalian central nervous system as a gastrin-like immunoreactive material.

Scientists reported that CCK increased insulin secretion in monogastric animal and human (Ahrén et al. 1991). But for ruminants, Baile et al. (1968) has been reported that the intravenous injection of CCK increased the secretion of insulin. In sheep, intravenous injection of sulfated CCK-8 increased plasma insulin concentrations (Mineo et al. 1997) but did not affect plasma GH concentration (Spencer et al. 1991).

After finding the function of CCK on gallbladder contraction and pancreatic secretion and insulin secretion, Gibbs et al. (1973) demonstrated exogenously administered CCK decreases food intake in rats. The anorectic effects of CCK appear to be mediated by the CCKA receptor via the vagal nerve, and are abolished by vagotomy (Smith et al, 1981).

CCK binds to CCK receptors. Two types of cholecystokinin receptors have been identified (Boden et al. 1995). CCK-A receptors were mainly distributed in peripheral tissues, particularly in the gastrointestinal tract and CCK-B receptors were located mainly in the brain (Sartor et al. 2008). CCK receptors found in alimentary tract are CCK-receptors ('A' for alimentary) and CCK receptors found in brain are CCK-B receptors ('B' for brain ). CCK-A receptor is also called as CCK-1 receptor and CCK-B is called as CCK-2 receptor (Florence et al. 1999). Some scientists reported that CCK-B receptors and gastrin receptors are identical (Pisegna et al. 1992). Only tyrosyl-sulfated CCK-peptides are in physiological concentrations bound to the CCK-A receptor, and even though CCK peptides are also bound, with similar affinity, to the gastrin/CCK-B receptor, this binding has no impact in the periphery, because gastrins circulate in plasma in 10- to 20-fold higher concentrations than CCK peptides (Rehfeld 1998). CCK-B / gastrin receptor bind with non-sulfated CCK, gastrin and short C-terminal fragments with high affinity (Rehfeld 2004).

Biologically active forms (sulfated forms of CCK) CCK-83, CCK -58, CCK -33, CCK -22 and CCK -8 have been found in intestine, blood and brain of many animals (Paloheimo et al. 1994). Sulfated CCK-8 is the smallest biological form of CCK. The

most potent form in the brain of humans (Reeve et al. 1984), sheep (Dockray et al. 1978), pigs (Eng et al. 1982), rats and bovine (Barden et al. 1981). The half-life of exogenous CCK is about 2.5 in human (Thompson et al. 1975) and half-life of CCK-8 in dog is about 1.3 min (Hoffmann et al. 1993).

The primary biological actions of CCK are inhibition of gastric emptying, satiation, gallbladder contraction and pancreatic enzyme secretion (Sartor et al. 2008). The biological actions of CCK are mediated by cell-surface membrane receptors on its target cells (Liddle 1994, 1997). In ruminants, CCK-8 is found in brain and CCK-33 and CCK-39 are found in small intestine. However, there is no information about the main forms of CCK in blood. The anorectic effects of CCK appear to be mediated by the CCK-A receptor via the vagus nerve, and are abolished by vagotomy (Wank et al. 1994). Non-sulfated forms of CCK have been detected in brain or intestinal tract and cannot activate CCK-A receptor (Smith et al. 1981).

CCK-33 is degraded more slowly by the liver than CCK-8 (Doyle et al. 1984). The infusion of CCK antibody did not affect on food intake in rats (Reidelberger et al. 1994). In rats, intra-peritoneal (i.p) injection of CCK on food intake is more effective than intravenous injection of CCK (Strubbe et al. 1989). The i.p injected CCK arrived to the lymph and the lymphatic concentration of CCK is 30 times higher than plasma concentration, because the CCK in the lymph is protected from degradation of enzymes (Strubbe et al. 1989).

## **1.9 Preprocholecystokinin**

Amino acids sequence of preprocholecystokinin (preproCCK) is varied within species. Pre-proCCK is 115 amino acids in ruminants (Uniprot, Accession number P 41520), rats (Deschenes et al. 1984) and human (Takahashi et al. 1985), however, 114 amino acids in porcine (Gubler et al. 1984 ) and 130 amino acids in chicken (Jönson et

al. 2000). The bioactive fragments such as CCK-83, -58, -39,-33, -22, -8 and -5 (Rehfeld 2004) are derived from preproCCK. In the plasma of human, CCK-58, -33, -22 and -8 are predominant (Rehfeld 2004). CCK-58 is found in brain of bovine (Eng et al. 1990) and CCK-39 and -33 are also found in bovine intestine (Carlquist et al. 1985). However, the dominant form of plasma CCK in ruminants is still unknown. The processing of proCCK is cell-specific. Hence, endocrine cells contain a mixture of medium sized cholecystokinins, whereas neurons mainly release CCK-8 (Rehfeld 2004). Multiple forms of biologically active CCK are produced by the actions of endopeptidases. Until now, sulfation and amidation occur before or after endopeptidase events that from the amino terminus of CCK-58 or smaller forms of cholecystokinin is not known (Eberlein et al. 1991). After discovery of sulfated and amidated CCK-83, it shows that carboxyl terminal amidation to form biologically active peptides can occur after signal peptidases cleavage and before amino terminal processing of the prohormone (Eberlein et al. 1991). In human, proCCK is 95 amino acids residues (Rehfeld 1998). However, proCCK of ruminant is still unknown.

**Fig.1**

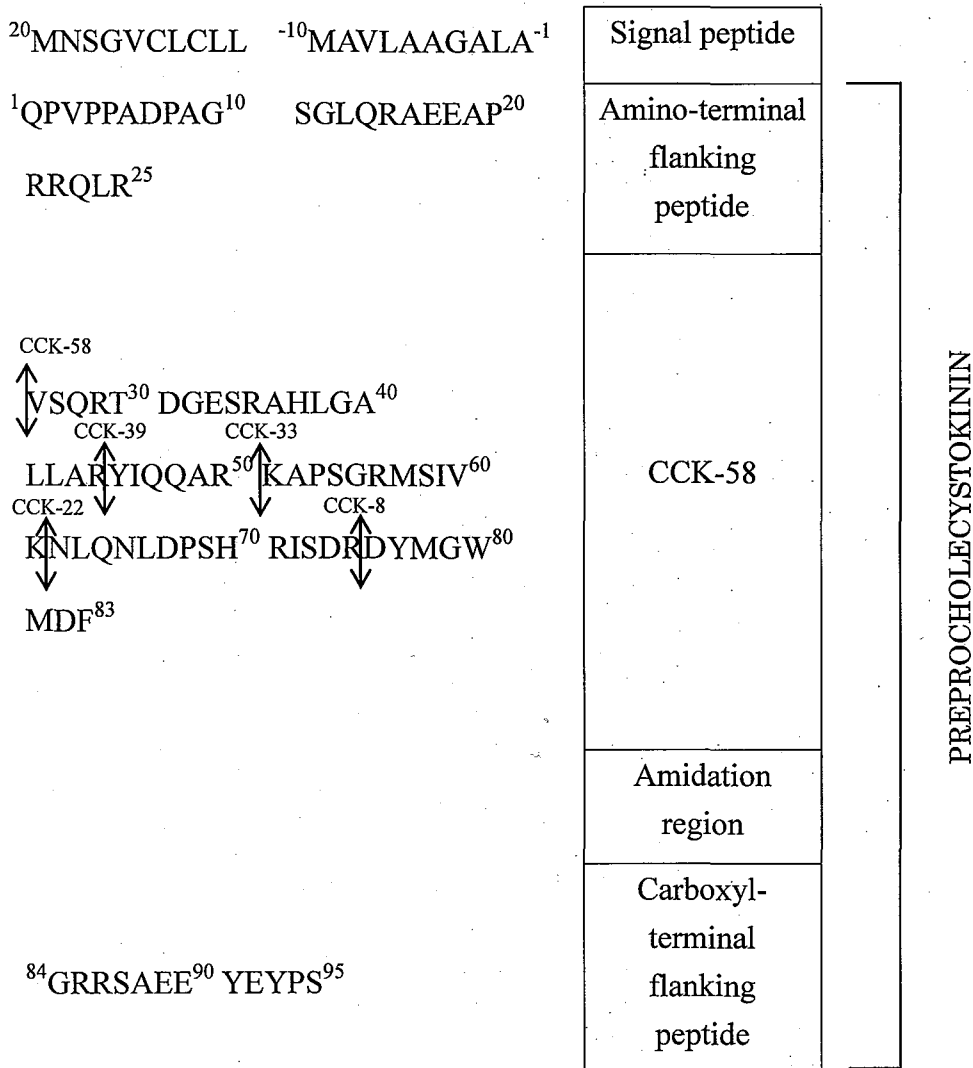


Figure 1. Predicted structure of preprocholecystokinin. (modified from Noble et al. 1990 and Eberlein et al. 1992). In human, the signal peptide includes residues -20 to -1. The amino terminal flanking peptide includes residues 1 to 25. The largest form from of CCK from brain and intestine is CCK-58 which consists of residues 26 to 83. Other active molecular forms are derived from this precursor, such as CCK-39, CCK-33, CCK-22, CCK-7 and CCK-5. Carboxyl terminal flanking peptide consists of residues 84 to 95. See table (1) for the translation of single-letter amino acid code.



**Table (1)** Translation of the single-letter amino acid code.

<b>Single-letter code</b>	<b>Three-letter code</b>	<b>Amino acid</b>
A	Ala	Alanine
C	Cys	Systeine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leusine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Threonine
Y	Tyr	Tyrosine

**Table (2)** The amino acid sequences of preprocholecystokinin. (Source- UniProt)

<b>Bovine</b>	<sup>1</sup> MNRGVCLCLL <sup>10</sup> MAVLAAGALA <sup>20</sup> QPMPHADPTG <sup>30</sup> PRAQQAEAP <sup>40</sup> RPQLRAVPRV <sup>50</sup> DDEPRAQLGA <sup>60</sup>
<b>Rat</b>	<sup>1</sup> <u>MKCGVCLCLV</u> <sup>10</sup> MAVLAAGALA <sup>20</sup> QP <u>VVPVEAVD</u> <sup>30</sup> <u>PMEERAEAP</u> <sup>40</sup> <u>RRQLRAVLRP</u> <sup>50</sup> <u>DSEPRARLGA</u> <sup>60</sup>
<b>Mouse</b>	<sup>1</sup> <u>MKSGVCLCVV</u> <sup>10</sup> MAVLAAGALA <sup>20</sup> QP <u>VVPAEATD</u> <sup>30</sup> <u>PVEQRAQEA</u> <sup>40</sup> <u>RRQLRAVLR</u> <sup>50</sup> <u>DGEPRARLGA</u> <sup>60</sup>
<b>Human</b>	<sup>1</sup> <u>MNSGVCLCLL</u> <sup>10</sup> MAVLAAGALT <sup>20</sup> QP <u>VPPADPAG</u> <sup>30</sup> <u>SGLQRAEAP</u> <sup>40</sup> <u>RRQLRVSQRT</u> <sup>50</sup> <u>DGESRAHLGA</u> <sup>60</sup>
<b>Pig</b>	<sup>1</sup> <u>MNGGLCLCLL</u> <sup>10</sup> MAVLAAGTLA <sup>20</sup> QP <u>VPPADPSG</u> <sup>30</sup> <u>VPGAQEEEAH</u> <sup>40</sup> <u>RRQLRAVQKV</u> <sup>50</sup> <u>DGESRAHLGA</u> <sup>60</sup>
<b>Chicken</b>	<sup>1</sup> <u>MYGGICICVL</u> <sup>10</sup> <u>LAALS</u> <u>VSSLG</u> <sup>20</sup> <u>QQPAGSHDGS</u> <sup>30</sup> <u>PVAELQQL</u> <sup>40</sup> <u>TEPHRHSRAP</u> <sup>50</sup> SSA GPLKPAP <sup>60</sup> <sup>61</sup> RLDGSEQRA <sup>70</sup> <u>TIGA</u> <sup>74</sup>

<b>Bovine</b>	<sup>61</sup> LLARYIQQAR <sup>70</sup> KAPSGRMSVI <sup>80</sup> KNLQSLDPSH <sup>90</sup> RISDRDYMGW <sup>100</sup> MDFGRRSAEE <sup>110</sup> FEYTS <sup>115</sup>
<b>Rat</b>	<sup>61</sup> LLARYIQQVR <sup>70</sup> KAPSGRMSVL <sup>80</sup> KNLQNLG <sup>90</sup> PSH <sup>90</sup> RISDRDYMGW <sup>100</sup> MDFGRRSAED <sup>110</sup> <u>YEYPS</u> <sup>115</sup>
<b>Mouse</b>	<sup>61</sup> LLARYIQQVR <sup>70</sup> KAPSGRMSVL <sup>80</sup> KNLQSLD <sup>90</sup> PSH <sup>90</sup> RISDRDYMGW <sup>100</sup> MDFGRRSAED <sup>110</sup> <u>YEYPS</u> <sup>115</sup>
<b>Human</b>	<sup>61</sup> LLARYIQQAR <sup>70</sup> KAPSGRMSIV <sup>80</sup> KNLQNLDP <sup>90</sup> SH <sup>90</sup> RISDRDYMGW <sup>100</sup> MDFGRRSAEE <sup>110</sup> <u>YEYPS</u> <sup>115</sup>
<b>Pig</b>	<sup>61</sup> LLARYIQQAR <sup>70</sup> KAPSGRVSMI <sup>80</sup> KNLQSLDPSH <sup>90</sup> RISDRDYMGW <sup>100</sup> MDFGRRSAED <sup>110</sup> <u>YEYPS</u> <sup>115</sup>
<b>Chicken</b>	<sup>75</sup> LLAKYLQ <sup>85</sup> QAR <sup>85</sup> <u>KGSTGRF</u> <u>SVLG</u> <sup>95</sup> <u>NRVQSIDPTH</u> <sup>105</sup> <u>RINDRDYMGW</u> <sup>115</sup> MDFGRRSAEE <sup>125</sup> <u>YEYSS</u> <sup>130</sup>

See (Table-1) for the translation of single-letter amino acid code.

Cholecystokinin 33 was firstly discovered in pig (Mutt and Jorpes 1968). The amino acid sequences of CCK-33 are varied within species.

**Fig.2**

	1			33
bovine	KAPSGRMSVI	KNLQSLDPSH	RISDRDYMGW	MDF
pig	-----V-M-	-----	-----	---
human	-----IV	---N---	-----	---
dog	-----	---N---	-----	---
rat	-----L	---G---	-----	---
mouse	-----L	-----	-----	---
monkey	-----I-	-----	-----	---

Figure 2. The alignment of the amino acid sequence of cholecystokinin-33. Amino acids sequence same with bovine are showed with symbol (—) and only showed different amino acids from bovine with single letter amino code. UniProt accession numbers for bovine is P41520; pig is P01356; human; P06307; rat, P01355; mouse, P09240; dog, Q9TS44; monkey, P23362. See (Table-1) for the translation of single-letter amino acid code.

Cholecystokinin is produced from a single gene however different molecular forms.

Fig.3

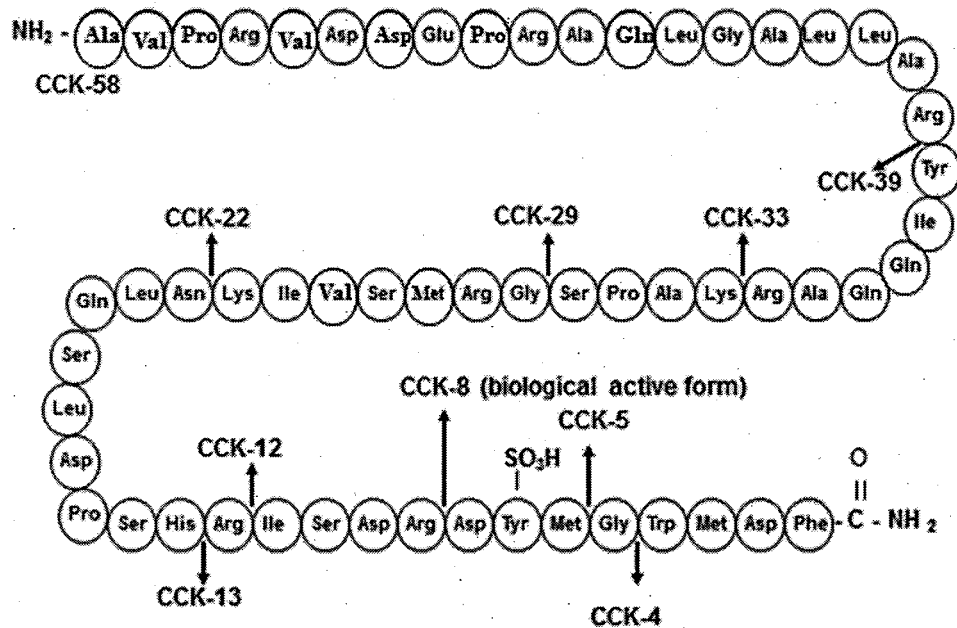


Figure 3. Amino acids sequence of bovine cholecystokinin (CCK)-4, -5, -8, -12, -13, -22, -29, -33, -39 and -58. UniProt accession number for bovine is P41520. See (Table-1) for the translation of three-letter amino acid code.

### 1.10 Homology with other hormones

The C-terminal 5 amino acids of CCK are identical with bovine sulfated gastrin, caerulein (isolated from the skin of the frog, *Hyla caerulea*), phyllocaerulein and cionin (extracted from *Ciona intestinalis*). The C-terminal 4 amino acids of gastrin/CCK like peptide (also called chicken gastrin) and the C-terminal 3 amino acids of leucosulphakinin (cockroach) and drosulphakinin (fruit fly) are homologous to C-terminal of CCK-8.

**Fig.4**

Sulfated cholecystokinin-8	DY(SO <sub>3</sub> H) <b><u>MGWMDF</u></b> -NH <sub>2</sub>
Bovine sulfated Gastrin-9	EAAY(SO <sub>3</sub> H) <b><u>GWMDF</u></b> -NH <sub>2</sub>
Caerulein	pEQDY(SO <sub>3</sub> H)Y <b><u>GWMDF</u></b> -NH <sub>2</sub>
Phyllocaerulein	pEEY(SO <sub>3</sub> H)Y <b><u>GWMDF</u></b> -NH <sub>2</sub>
Cionin	NY(SO <sub>3</sub> H)Y(SO <sub>3</sub> H) <b><u>MGWMDF</u></b> -NH <sub>2</sub>
Chicken gastrin	-QGNGAVEALHDFY <b><u>PDWMDF</u></b> -NH <sub>2</sub>
Leucosulphakinin	pEQFEDW <b><u>GHMDF</u></b> -NH <sub>2</sub>
Leucosulphakinin II	pESDDW <b><u>GHMDF</u></b> -NH <sub>2</sub>
Drosulphakinin I	FDDW <b><u>GHMDF</u></b> -G
Drosulphakinin II	GGDDQFDDW <b><u>GHMDF</u></b> -G

Figure 4. Homologous bioactive sequences of sulfated CCK-8, bovine gastrin-9, caerulein, cionin, chicken gastrin, leucosulphakinin and drosulphakinin. The identical C-terminal amino acids are bolded and underlined. (pE, pyroglutamic acid) (modified from Dockray 1989 and Rehfeld 2004). See (Table-1) for the translation of single-letter amino acid code.

### **1.11 Regulation of CCK secretion**

CCK is released from intestinal I- cells (Buchan et al. 1978). CCK secretion increased after eating in monogastric species (Lilja et al. 1984). The apical surfaces of I-cells are open type (Buchan et al. 1978) and these cells are likely to sense food in the intestinal lumen and release CCK into the circulation (Liddle 1997). It is possible that meal-induced secretion of CCK is modified by innervation of the I-cells (Rehfeld 2004). That regulation is difficult to examine *in vivo* because CCK may also be released into the plasma from nerves in the ileum and colon (Rehfeld 2004). The release of CCK from neurons has been examined in brain slices and synaptosomes. It is not known to what extent neuronal CCK over flows into the plasma (Rehfeld 2004). Scientists studied the regulation of CCK secretion with CCK releasing factor (trypsin sensitive releasing factor), monitor peptide, luminal releasing factor and cellular regulation of CCK secretion (Liddle 1997). The exact mechanisms which control CCK secretion have yet to be elucidated. It is possible that other appetite regulatory hormones may involve in the regulation of CCK secretion. Because, administration of ghrelin stimulates CCK secretion in rodents (Jawork 2006, Nawrot-Porabka et al. 2007). In addition, somatostatin inhibits CCK secretion in monogastric species (Herzig et al. 1994, Lloyd et al. 1994).

There is few information for the effect of ghrelin, oxyntomodulin and amylin on CCK secretion in ruminants. Therefore, we studied the effect of acyl ghrelin, des-acyl ghrelin, OXM and amylin on CCK secretion in Holstein steers.

### **1.12 Regulation of ghrelin secretion**

Ghrelin is mainly secreted from X/A like cells of stomach (de la Cour et al. 2001, Kojima and Kangawa 2005). In ruminants, ghrelin is mainly secreted from abomasum (Hayashida et al. 2001). Ghrelin secretion is increased in fasted condition and decreased after eating in ruminants (Wertz-Lutz et al. 2006) and non-ruminants (Kojima and Kangawa 2005).

Some scientists studied the secretion of ghrelin by nutrient infusion in rats. Infusion

of macronutrients into duodenum and jejunum also suppressed ghrelin levels (Overduin et al. 2005). There are 2 types of ghrelin cells, close type and open type. The number of open type cells increased in the direction from stomach to duodenum and jejunum (Sakata et al. 2002). Ghrelin cells are not able to sense food in the lumen of stomach because nutrients constrained within the stomach do not affect ghrelin levels (Williams et al. 2003). The mechanism which regulates ghrelin secretion is still need to clarify.

Some hormones regulate the secretion of ghrelin. CCK inhibits the secretion of ghrelin in humans (Brennan et al. 2007, 2008) however; CCK stimulates ghrelin secretion in rodents (Murakami et al. 2002, Friis-Hansen et al. 2005, Shrestha et al. 2009). In addition, secretin stimulates ghrelin release from rat stomach ghrelin cells (de la Cour et al. 2007).

In human, intraduodenal infusion of fat increased plasma CCK levels and observed CCK induced suppression of ghrelin (Degen et al. 2007). In addition, administration of insulin decrease plasma ghrelin in monogastric species (Saad et al. 2002, McCowen et al. 2002, Lippl et al. 2004). Amylin also inhibits ghrelin secretion in rats (Gedulin et al. 2004, Young et al. 2004). Moreover, administration of OXM decreased ghrelin secretion in fasted rodents (Dakin et al. 2004).

According to my knowledge, there is limited information the effect of appetite regulatory hormones on ghrelin secretion in ruminants. We recently reported that OXM has no effect on ghrelin secretion in steers (ThanThan et al. 2010). However, gastrin stimulates ghrelin secretion in rodents (Murakami et al. 2002) and ruminants (Zhao et al. 2011). We study the effects of CCK and amylin on ghrelin secretion in steers. We found that sulfated CCK-8 (CCK-8s) has stimulatory effect on ghrelin secretion. However, we do not observe the effect of amylin on ghrelin secretion in steers (Yannaing et al. unpublished data). In addition, administrations of non-sulfated CCK-33 and CCK-4 do not affect on ghrelin secretion in steers (Yannaing et al. unpublished data).

### **1.13 Regulation of oxyntomodulin secretion**

Oxyntomodulin is secreted from intestinal L-cells (Bataille et al. 1982). OXM

secretion increased after eating in monogastric species (Hornnes et al. 1980). However, plasma OXM did not change after feeding in ruminants (Relling et al. 2010). The apical surfaces of L-cells are exposed to the lumen of intestine (Deacon 2005). These cells are likely to sense food in the intestinal lumen (Anini et al. 1999). There is limited information about the effect of other appetite regulatory on OXM secretion in non-ruminant and ruminant. We find that administrations of ghrelin (acyl ghrelin and des-acyl ghrelin) and CCK did not affect OXM secretion in Holstein steers.

#### **1.14 Regulation of pancreatic polypeptide secretion**

Pancreatic polypeptide (PP) is primarily released by cells the periphery of the islets of Langerhans (Adrian et al. 1976). Plasma level of pancreatic polypeptide increases after eating in human (Adrian et al. 1976). Plasma PP levels increased after feeding in sheep (Takahashi et al. 2010) and in fatty acids supplement fed dairy cows (Choi et al. 2000). Scientists studied and reported the effects of other appetite regulatory hormones on PP secretion in non-ruminants. Administration of galanin decreases plasma PP secretion in humans (Bauer et al. 1986). Injection of secretin increased plasma PP levels in human (Peracchi et al. 1999). CCK stimulates pancreatic polypeptide (PP) in mice (Ahrén et al. 1991, 1995), human (Schmid et al. 1989) and dog (Parks et al. 1979, Schusdziarra et al. 1986). Choi et al. (2000) reported that feeding induced endogenous increased CCK stimulates PP secretion in cattle. In addition, GLP-1 has no effect on PP secretion in mice (Ahrén et al. 1995). Administration of ghrelin increased plasma PP in humans (Arosio et al. 2003, Tack et al. 2006) and rat (Sato et al. 2003). In addition, intravenous administration of somatostatin inhibits PP secretions in human (Macro et al. 1983). According to my knowledge, there is few information for the effect of CCK and ghrelin (acyl ghrelin and des-acyl ghrelin) on PP secretions in cattle.



## Chapter 2

### **Solid phase peptide synthesis (SPSS) and radioimmunoassay (RIA)**

The amino acid sequences of cholecystokinin (CCK) and gastrin among domestic animals, humans and ruminants are different. The CCK peptides of rat, mouse, pig and humans are commercially available. However, bovine sulfated gastrin-9 and bovine non-sulfated gastrin-9 are not commercially available and its amino acids sequences are different from other species (eg. pig, human, rat). Short amino acids peptide such as sulfated CCK-8 and non-sulfated CCK-8 are commercially available. Because, C-terminal amino acids sequence of these peptides are identical in human, ruminants and other animals (except guinea-pig and chinchilla). For administration study in bovine, mass volume of peptides are required. To study the effect of bovine CCK on other appetite regulatory hormones, we synthesized bovine sulfated CCK-8.

For measuring of plasma CCK levels, scientists used CCK-RIA kits, CCK-EIA kits and CCK-ELISA kits. These kits are specific for rat, mouse, pig and human, and commercially available. Only few samples can measure by using of these kits. In addition, these kits are expensive and are not specific to bovine. For radioimmunoassay (RIA), using of different amino acids sequence of peptides can decrease specificity of assay. Therefore we validated bovine CCK-RIA system to study the effects of other appetite regulatory hormones on CCK. For radioimmunoassay, we require first antibody of CCK (anti-CCK antiserum) and we need to label peptide with isotope ( $I^{125}$ ).

For first antibody production, scientist used pure peptides. Because, naturally prepared hormones are difficult to separate from other hormonally active or inert proteins and are not pure. To get high and specific titer of antibody, the purity of injected antigen (peptide) is important. Therefore, we synthesized peptide for first antibody production. Sulfated CCK-8 is small molecular weight peptide. Therefore, it needs to conjugate with carrier protein such as mKLLH. The amino acid cysteine in the sequence (added or part of the native sequence) of peptide is required for conjugation. Sulfated CCK-8s does not contain amino acid cysteine, therefore we synthesized

C-terminal cysteine attached, N-terminal free CCK-8s [CDY(SO<sub>3</sub>H)MGWMDF] for coupling with a carrier protein.

Radioisotope labeled CCK-8 is used for first antibody titer check, first antibody dilution check, standard check and specificity check. For labeling of peptide, amino acids tyrosine residue is needed in chloramine T-method. Sulfated CCK-8 has amino acid tyrosine, however it is already sulfated. We used sulfated CCK-8 for labeling. Therefore, we synthesized [Tyr<sup>0</sup>]CCK-8 amide for labeling of isotope. To check the specificity of first antibody against sulfated CCK-8, we synthesized bovine sulfated gastrin -9 and non-sulfated gastrin-9. In this experiment, we synthesized peptides with solid phase peptide synthesis system with manual base-labile Fmoc (9-fluorenylmethoxycarbonyl) strategy (Table-3).

**Table (3)** Synthesized peptides of CCK

No.	Synthesized Peptides	Amino acids sequence of peptides	Accession Number
1	Bovine sulfated CCK-8 amide	<sup>26</sup> DY(SO <sub>3</sub> H)MGWMDF <sup>33</sup> -NH <sub>2</sub>	P 41520
2	Bovine Cys <sup>0</sup> -sulfated CCK-8	C <sup>0</sup> - <sup>26</sup> DY(SO <sub>3</sub> H)MGWMDF <sup>33</sup>	P 41520
3	Bovine Tyr <sup>0</sup> -sulfated CCK-8 amide	Y <sup>0</sup> - <sup>26</sup> DY(SO <sub>3</sub> H)MGWMDF <sup>33</sup> -NH <sub>2</sub>	P 41520
4	Bovine sulfated gastrin-9 amide	<sup>84</sup> EAAY(SO <sub>3</sub> H)GWMD <sup>92</sup> -NH <sub>2</sub>	P 01352
5	Bovine non-sulfated gastrin-9 amide	<sup>84</sup> EAAYGWMD <sup>92</sup> -NH <sub>2</sub>	P 01352

### **2.1.1 SPSS for bovine sulfated CCK-8 amide, bovine sulfated gastrin-9 amide, bovine [Tyr<sup>0</sup>]sulfated CCK-8 amide and [Cys<sup>0</sup>]sulfated CCK-8**

To synthesized sulfated CCK-8, some scientists firstly synthesized non-sulfated CCK-8 and then sulfated at tyrosine amino acid residue with sulfuric acid (Nakahara et al. 1986). This method is only suitable for short form of sulfated peptides. The amino acids of long form of sulfated CCK can break by sulfuric acid and reduce the bioactivity of peptides. Therefore, we synthesized directly sulfated form of peptides. It does not require post- sulfation.

The peptides (sulfated CCK-8, sulfated bovine gastrin-9) were synthesized by Fmoc (9-fluorenyl-methoxycarbonyl) solid phase peptide synthesis procedures of Kitagawa et al. (2001). We synthesized peptide from C-terminal to N-terminal as routine method. To protect peptide aggregation, we used side-chain protected Fmoc-amino acids.

Two- chlorotriyl resin is highly acid-sensitive resin and which is weakly bind to synthesized peptide. Its N-terminal is free and ready for amino acid coupling. Therefore, it is no need to made deprotection for peptide coupling. The deprotection/coupling procedures were repeated until the desire peptide amino sequence was obtained.

We used Phe-pre-loaded 2-chlorotriyl resin (100- 200 mesh) for sulfated form of peptide synthesis. Therefore, we start synthesized sulfated form of peptide from C-terminal number 2 amino acids. A sequential protection and deprotection procedure was applied. In this experiment, the base-labile Fmoc group is used for N-terminal protection, and therefore repeated acid treatment incompatible with a Tyr(SO<sub>3</sub>H) residue is avoidable during the peptide synthesis (Kitagawa et al. 2001).

EDT (ethanedithiol) is significantly accelerated the de-sulfation of a Tyr(SO<sub>3</sub>H)-containing peptide (Yagami et al. 1993.). Therefore, we do not use EDT for peptide decomposition. TFA is a routinely used reagent for the final cleavage/ deprotection step in the Fmoc-SPPS, is not a destructive acid to a Tyr(SO<sub>3</sub>H) residue as long as the reaction was kept cold (Yagami et al. 1993). The presence of free amino

groups was tested by Kaiser method (Kaiser et al. 1970) with slight modification. A few resin beads was put into glass tube (size, 5 mL tube), then 33  $\mu$ L of phenol ethanol (4 g of phenol in ethanol 1 mL), 67  $\mu$ L of 98% pyridine (9.8 mL of pyridine in 0.2 mL of water) and 25 $\mu$ L of ninhydrin (0.5 g of ninhydrin in 10 mL of ethanol) were added to that glass tube. The mixture was heated by putting of glass tube into water bath for 5 min. After that check the color of resin beads, blue color resin beads is indicated as a positive test (de-protection). After coupling of amino acid, we check the attachment of amino acid by Kaiser test. Clear color resin beads indicate the attachment of amino acid. After synthesizing peptide, we keep the resin at 4° C without removing of Fmoc. For peptide purification, we firstly made decomposition of resin to separate resin and peptide. Then, the synthesized peptide was purified by HPLC.

To separate synthesized peptide and resin, we made decomposition of peptide. Resin plus synthesized peptide and stirring bar were put in the glass tube (with screw cap) and keep that glass tube in ice for 5 min. TFA, water and TIS were put into glass tube (with screw cap) and kept in ice. We prepared one beaker which containing ice and put on magnetic mixer. Glass tube which contain resin plus synthesized peptide was put into the ice-beaker and mix by stirring bar (which run by magnetic mixer) for 5 min. Then we add the mixture of cooled TFA, water and TIS into resin plus synthesized sulfated CCK-8 containing glass tube and put that glass tube in ice-beaker (which place on magnetic mixer) and mix (with stirring bar) for 8 hours.

After mixing, 5 ml of mixture (resin plus synthesized peptide, TFA, water and TIS) was put in the tube which including 45 ml of cooled methyl ether and mix by shaking. The tube was place in ice for 5 min. The color of the solution becomes cloudy and found precipitate within a few minutes. Then, these tubes are centrifuged 3000 rpm at 4° C for 10 min. The supernatant was removed and added 45 ml of ether and mix by shaking (by hand for a short time) and keep the tubes in ice. By adding of ether is to extract TFA from peptide. We do this procedure for 3 times.

Then, the sediment in plastic tube is added with 5 ml of ether and move to the glass tube. The total volume is about 10 ml in glass tube. Then, centrifuge with 3000 rpm at

4° C for 5 min. The supernatant is taken out and added 4 ml of 60% CH<sub>3</sub>CN plus 1% TFA, 2 ml of ether, after that mix by shaking. Then, centrifuge with 2000 rpm at 4°C for 5 min. After removing the supernatant, 3 ml of ether was added and centrifuge again (with 2000 rpm at 4°C) for 5 min. The supernatant is removed and added 2 ml of 0.1 % TFA, 4 ml of ether and 1ml of 100% CH<sub>3</sub>CN, and mix with vortex. After that, centrifuge with 2000 rpm at 4°C for 5 min and remove the supernatant. After that, 2 ml of ether is added and mixed by shaking, and centrifuge with 2000 rpm at 4°C for 5 min and supernatant was removed.

Then, 2 ml of ether was added to each glass tube, cover with paraffin and made one small hole on the middle part of paraffin to extract ether from this hole by high pressure vacuum pump. Put the glass tube which containing peptide, and glass tubes with water in high pressure vacuum pump. The pump runs until the ether was taken out. The glass tube from vacuum pump was taken out and added 4 ml of 100% CH<sub>3</sub> CN into the tube. After that, 1 ml of the mixture was separated from glass tube and kept in 1.5 ml polyethylene tubes. Then, polyethylene tubes which containing peptide are centrifuged at room temperature for 5 min. The supernatant fluid was collected in siliconized glass tube for HPLC purification. After decomposition of synthesized sulfated CCK-8, it is purified by HPLC. After purification, peptides were neutralized. To prevent oxidation of purified peptide's amino acids by water vapor, we put the purified peptide in glass tube which covered with paraffin and keep that glass tube in moisture free glass bottle and stored at -30°C.

### **2.1.2 SPSS for bovine non-sulfated gastrin-9 amide**

This peptide was synthesized by Fmoc (9-fluorenyl-methoxycarbonyl) solid phase peptide synthesis procedures. Rink amide MBHA resin (100-200 mesh) was used for peptide synthesis. We synthesized peptide as routine procedure from C-terminal to N-terminal. To protect peptide aggregation, we used side-chain protected Fmoc-amino

acids. The synthesized peptide is attached with resin.

To separate synthesized peptide and resin, we made decomposition of peptide. Resin plus synthesized peptide, 9.4 ml of 100% tri-fluoroacetic acid (TFA), 0.25 ml of distilled water, 0.25 ml of ethanedithiol (EDT) and 0.1 ml of triisopropylsilane (TIS) were put in a glass tube with cap (total 10 ml volume) and mix (by stirring bar which run by magnetic mixer) for 2.5 hours. The 45 ml of methyl ether was put in each plastic tube with cap (2 tubes) and these 2 tubes are kept in ice for cool down. After 2 hr and 30 min mixing, 5 ml of the solution (resin plus synthesized peptide, TFA, EDT and TIS) was put in the tube which including 45 ml of cooled methyl ether and mix by shaking. These 2 tubes were place in ice for 5 min. The color of the solution becomes cloudy and found precipitate within a few minutes. Then, these tubes are centrifuged with 3000 rpm at 4°C for 10 min. The supernatant was removed and added 45 ml of ether and mix by shaking and the tubes were kept in ice. By adding of ether is to extract TFA from peptide. After that the synthesized peptide plus resin was decomposed to separate peptide from resin. The procedure of decomposing of non-sulfated gastrin is same with sulfated CCK-8.

After decomposition of synthesized peptide, it is purified by HPLC. After purification, peptides were neutralized. To prevent oxidation of purified peptide's amino acids by water vapor, we put the purified peptide in glass tube which covered with paraffin and keep that glass tube in moisture free glass bottle and stored at -30°C.

### **2.1.3 Measuring of plasma concentration of CCK**

Cholecystokinin was found in 1928 (Ivy et al. 1928). However, plasma levels of CCK cannot measure until 1969. Plasma levels of CCK can measure after the development of the radioimmunoassay system (Young et al. 1969). Because of difficult to get the antibody that specifically recognize to CCK and difficulty of labeling of CCK. Carboxyl terminal (C-terminal) of 5 amino acid sequence (Gly-Trp-Met-Asp-Phe) of CCK and gastrin are identical. Therefore, it is difficult to get antibody that specifically

recognizes to CCK. Many scientists measure plasma CCK levels by using of RIA. In addition, plasma level of CCK is very low and plasma level of gastrin is 20 - 100 times higher than CCK (Liddle 1998). Therefore, using of CCK antibody which slightly reacts with gastrin cannot measure the plasma concentration of CCK accurately.

Most of their RIA systems can measure only in extracted plasma. Some of them used the first antibodies that cross react with gastrin. In blood, gastrin is 20-100 times higher than CCK (Liddle 1998). Therefore, it is difficult to measure exactly for plasma CCK levels. Mostly CCK used in RIA is CCK-8. Labeling also need special method because iodine I 125 attached at tyrosine. In sulfated CCK-8, tyrosine is sulfated. Only longer form CCK-39 has free tyrosine. Oxidative labeling destroys amino acid (methionine) and oxidation of CCK reduces its biological activity 100- to 1000-fold (Liddle 1998). Therefore, labeling of sulfated CCK-8 by oxidation procedures of chloramine T-method is difficult. We synthesized tyrosine attached sulfated CCK-8 amide [YDY(SO<sub>3</sub>H)MGWMDFNH<sub>2</sub>]. CCK-RIA kit, CCK-EIA kit and CK-ELISA kit are used for measuring of CCK. However, they are expensive and can measure few samples. Using of these kits also need to made extraction of plasma. Plasma extraction method is complicated and not practical for measuring of many samples. In addition, peptide can lose during extraction process. Therefore, we try to validate sulfated CCK RIA which can measure un-extracted plasma.

#### **2.1.4 First antibody production for sulfated CCK**

Naturally prepared hormones are difficult to separate from other hormonally active or inert proteins and are not pure. To get high and specific titer of antibody, the purity of injected antigen (peptide) is very important. Therefore, we synthesized peptide to get pure peptide. Most of the scientists used synthesized peptide for production of antibody. At present, synthesized sulfated CCK-8 amide [DY(SO<sub>3</sub>H)MGWMDF] can buy from company, but antibodies against it also reacted with gastrin. Therefore, it is difficult to get specific antibody against sulfated CCK-8 (CCK-8s). An immunogen requires both

an antigenic site and a T-cell (T-lymphocyte) receptor binding site, a minimum size is necessary (Germain 1986). Synthetic fragments of proteins may be able to bind to the surface of B-cells (B-lymphocytes), but do not stimulate an immune response (Adrian 2002). All small peptides/haptens must be conjugated to a carrier protein (mcKLH, Bovine Serum Albumin, Ovalbumin, thyroglobulin, mucopolysaccharides, poly-L-lysine, polyvinylpyrrolidone, etc) in order to get high titer antibody. If peptide does not conjugate with carrier protein, it does not become complete antigen. In the classical hapten carrier system T lymphocytes recognize processed carrier determinates and cooperate with B-cells which produce hapten-specific antibody response (Adrian 2002). The amino acid cysteine in the sequence (added or part of the native sequence) of peptide is required for a single point attachment of conjugation. Sulfated CCK-8 peptide does not contain amino acid cysteine, therefore we synthesized C-terminal cysteine attached, N-terminal free CCK-8s [CDY(SO<sub>3</sub>H)MGWMDF] for coupling with a carrier protein. We used mcKLH (Pierce # 77600) as a carrier protein for coupling of CCK-8s. The mcKLH was used as a potent immunogenic carrier protein stem from its high molecular weights, potent T-cell epitopes, and multiple sites for high density antigen conjugation.

Adjuvants are used for improving antibody response. It depots the injected antigen and controls slowly release of antigen, prevent dilution, degradation and elimination by host (Harold et al. 2005). There are over 100 preparations of adjuvant, Freund's adjuvant, TiterMax, Specol, RIVI Adjuvant System and Aluminum Salt adjuvant, etc (Harold et al. 2005). Most of the scientists used Freund's adjuvant for antibody production because of its high titer and specificity result. Therefore, we also used Freund's adjuvant for CCK-8s first antibody production.

Rabbits, guinea pigs, chicken and rat/mice are generally used for antibody production. To get higher specificity of antibody, the more remote the species used from that injecting the antigen. Most of mammals CCK-8s [DY(SO<sub>3</sub>H)MGWMDF-NH<sub>2</sub>] amino acid sequence is same except of guinea pig and chinchilla. The amino acid sequences of CCK-8 from guinea pig, chinchilla, gold fish and salmon are different



from other species (Fig. 5). In guinea pig and chincila, the amino acid at N-terminal no.3 Methionine is replaced with valine. Therefore, we used guinea pig for first antibody production. Some scientists also produced high titer and specificity CCK-8s antibody from rabbit, so we use 1 rabbit for CCK antibody production.

**Fig. 5**

1	8
DYMGWMDF	bovine
DYMGWMDF	chicken
DYMGWMDF	eastern gray kangaroo
DY <u>V</u> GWMD <b>F</b>	guinea pig
DY <u>V</u> GWMD <b>F</b>	chinchilla
DY <u>L</u> GWMD <b>F</b>	gold fish
DY <u>L</u> GWMD <b>F</b>	atlantic salmon
DY <u>N</u> GWMD <b>F</b>	atlantic salmon

Figure 5. The alignment of the amino acid sequence of cholecystokinin-8. Different amino acid is shown in bold and underline. UniProt accession numbers for references are; bovine, P41520; chicken, Q9PU41; guinea pig and chinchilla (Eng J. 1992), gold fish, O93464; atlantic salmon, B3IYK3; atlantic salmon, B3IYK4 and eastern gray kangaroo P0C229. See table (1) for the translation of single-letter amino acid code.

Hepaten are substances with low molecular weight peptides and small protein. Purified sulfated CCK-8 peptide [CDY(SO<sub>3</sub>H)MGWMDF] was coupled to maleimide-activated mariculture keyhole limpet hemocyanin according to the manufacturer's instruction (Piere # 77600). Hepaten-conjugated antigen was emulsified with same volume of Freund's complete adjuvant (FCA, Wako# 014-09541) for immunization. In guinea pigs, 0.25 mg of conjugated CCK-8s in 500  $\mu$ l of phosphate buffer was used for each injection and animals were subcutaneously injected 7 times with one week interval. The whole blood was collected at the following day of final (7 times) antigen injection. In rabbit, 1 mg of conjugated CCK-8s in 1 ml of phosphate buffer was used for injection. Rabbit was subcutaneously injected 6 times with 2 weeks interval. Blood (5-10 ml) was collected from the ear vein before antigen injection. The whole blood was collected the following day of final (6 times) antigen injection. The collected blood from guinea pigs and rabbit were kept at 4°C for 24 hours and then serum was separated after centrifugation with 3000 rpm at 4°C for 30 min. The titer of antibody was checked by RIA.

### 2.1.5 Labeling of sulfated CCK-8

[Tyr<sup>0</sup>] sulfated CCK-8 iodinated by chloramine-T method (McConahey et al. 1980). Two microliter of Na-I <sup>125</sup> (0.2 mCi), 1  $\mu$ l of Chloramine-T, 5mg/ml dissolved in Na-phosphate solution, pH 7.5 and 2  $\mu$ l of Tyr<sup>0</sup> sulfated CCK-8, 0.1 mg/ml in 50 % ethanol/ 1 mM HCl were put into silicon coated polystyrene tube, mix and wait for 30 seconds. Then, 12.5  $\mu$ l of Sodium metabisulfite, 5 mg/ml dissolved in Na-phosphate solution was added and wait for 90 seconds. After that, 2  $\mu$ l of tyrosine 0.1 mM dissolved in 1 mM of hydrochloric acid was added, mix and wait for 20 seconds. Then, 2 $\mu$ l of sodium iodide (1 mM in water) and 15.5  $\mu$ l of water were added to the tube and mix. After labeling, the I<sup>125</sup> labeled peptide was purified by HPLC with C-18 column. The tube which collect the fraction with peak concentration of CPM (400  $\mu$ l) was collected and neutralized by equal volume of Tri-base (400  $\mu$ l). Purified

radiolabeled CCK-8 was kept in - 30°C.

### **2.1.6 Sensitivity/titer check of sulfated CCK-8 antibody**

The assay was set up in room temperature. Two hundred milliliter of buffer (0.05 M NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, 0.9% NaCl, 0.025 M EDTA, 0.08% sodium azide, 1% BSA, pH 7.4), 100 uL of first antibody (1:2000) and 100 uL of I<sup>125</sup> labeled CCK-8s [10000 cpm/ 100 uL in assay buffer containing 1% carrier serum (normal guinea pig serum)] were put in each polystyrene tube and incubated at 4°C for 24 hr. After incubation, 1 ml of precipitation buffer which containing second antibody (goat anti-guinea pig serum) was added to each polystyrene tube and incubated again at 4°C for 30 min. Bound and free ligand were separated by centrifugation with 3000 rpm at 4°C for 30 min. Aspirate off all the supernatant (without touching the pellet) immediately after centrifugation. The radioactivity in the pellet was counted by gamma counter (ARC-1000, Aloka, Japan). All samples were assay in quintuplicate.

Sera obtained from guinea pig of batch 1 and rabbit do not show binding to I<sup>125</sup> CCK-8s, therefore antibody do not produce in these animals and the sera were discarded. The sera obtained from guinea pig of batch 2 (MASA), guinea pigs of batch 3 (TARO and YUTA) showed 30 - 50% binding to I<sup>125</sup> CCK-8s at the final dilution of 1:5000, 1:10000 and 1:2000, respectively (Fig. 6). We set RIA standard at room temperature. We set up cold standard of CCK-8s from 0.019 to 160 pg/ml. We used two day CCK RIA system. Standard/ plasma, first antibody (anti-CCK-8s serum) and tracer are put in polystyrene tubes and incubated for 24 hours at 4°C. After incubation, precipitation buffer which contains second antibody (goat anti-guinea pig serum) and incubated for 30 min at 4°C. Then, centrifuge with 3000 rpm at 4°C for 30 min, aspirate the supernatant and count the CPM of pellet. The antiserum of MASA highly recognized to non-sulfated CCK (Fig. 7) and the anti-serum of YUTA slightly recognized sulfated bovine gastrin and non-sulfated bovine gastrin (Fig. 8). Only the serum of TARO specifically recognizes sulfated CCK-8 (Fig. 9).

Fig.6

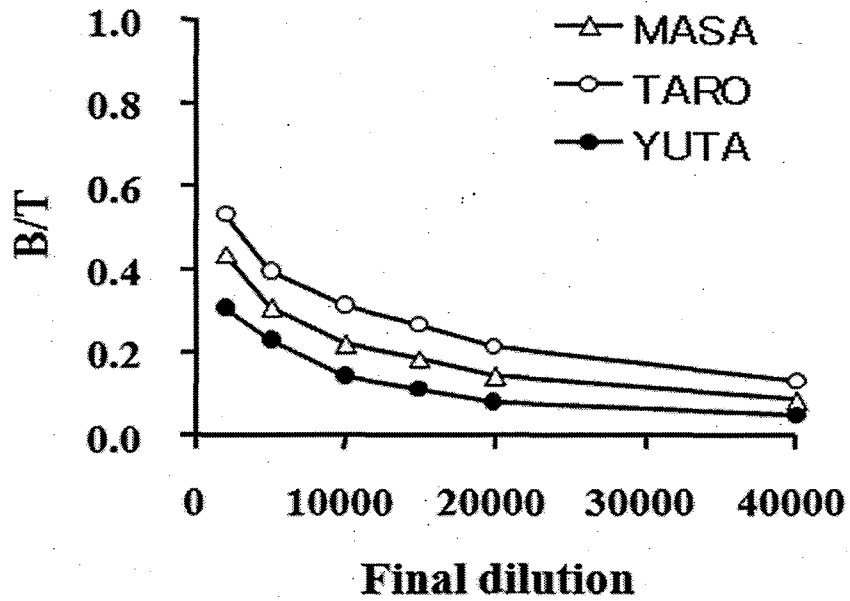


Figure 6. First antibody dilution curves of guinea pig anti-sulfated CCK-8 serum for guinea pig MASA ( $-\Delta-$ ), guinea pig TARO ( $-\circ-$ ) and guinea pig YUTA ( $-\bullet-$ ). The  $^{125}\text{I}$ -labeled ( $\text{Tyr}^0$ ) sulfated CCK-8 was binding to serial dilution of antiserum. Dilution of antiserum was prepared from 1: 2000 to 1: 40000 final dilutions. Each point is the mean of quintuplicate determinations.

### 2.1.7 Cholecystokinin Radioimmunoassay (CCK RIA)

[Tyr<sup>0</sup>]CCK-8 amide was iodinated by chloramine-T method (McConahey et al. 1980). To separate iodinated CCK and free iodide components, the <sup>125</sup>I-labeled [Tyr<sup>0</sup>]CCK-8 amide was immediately purified by reversed-phase HPLC with C-18 column and neutralized by Tris-base, and then stored at - 30°C. Standard concentrations of sulfated CCK-8 ranged from 0.019 to 160 ng/mL. Standards and plasma samples were incubated with antibodies diluted in assay buffer (0.05 M NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, 0.9% NaCl, 0.025 M EDTA, 0.08% sodium azide, 1% BSA, pH 7.4), and <sup>125</sup>I-labeled CCK-8 amide (8000 count per minute/100 µL assay buffer containing 1% carrier serum). The tubes were incubated for 24 h at 4°C. The next day, the second antibody was added to the tubes, and the tubes were incubated for 30 min at 4°C. After incubation, bound and free antigens were separated by centrifugation for 30 min at 1870 ×g and 4°C, aspiration of all the supernatant (without touching the pellet) immediately follow centrifugation.

The radioactivity in the pellet was measured by gamma counter (ARC-1000, Aloka, Japan). Triplicate standards and duplicate plasma samples were run within in a single assay. The average recovery rate of three known amounts of CCK-8 added to bovine pooled plasma was 92%. Sensitivity and the intra-assay coefficient of variation of this assay were 0.16 ng/mL and 6.6%, respectively.

The specific measurement of sulfated CCK in the plasma requires assays that recognize the C-terminal heptapeptide sequence [Y(SO<sub>3</sub>H)MGWMDF-NH<sub>2</sub>], irrespective of the N-terminal extension (Rehfeld JF 2004). The antibody should bind the active site of CCK without binding the homologous gastrin (Rehfeld JF 1998). The antibody used in our assays was specifically recognized sulfated CCK-8 amide and did not cross-react with non-sulfated CCK-8 amide, bovine gastrin-9 amide and bovine non-sulfated gastrin-9 amide (Fig.9). Therefore, the results from our RIA system represent the concentrations of sulfated CCK.

In general, various constituents of plasma nonspecifically inhibit radioimmunoassay of small peptides. To solve this problem, some scientists have been

made extraction or acidification of plasma. Most of the previous studies (Furuse et al. 1991; Mir et al. 2000; Relling and Reynolds 2007) used ethanol-extracted plasma samples for measuring of CCK in bovine. Plasma (500 uL) was extracted with 96% ethanol (1 mL) and then centrifuge at  $1700 \times g$  for 15 min at 4°C. The supernatant was decanted to another tube, evaporated in a freeze-dryer overnight, and then reconstituted in assay buffer before analysis (Relling and Reynolds 2007). Extraction of plasma is complicated and time consuming. It is not convenient for measuring of many samples. In addition, the peptide in the plasma can lose during extraction procedures. Therefore, we validate CCK RIA system which can measure un-extracted plasma.

#### **2.1.8 Standard check and specificity check of CCK first antibody**

We made standard check and specificity check of CCK first antibody (anti-sulfated CCK serum) from 3 guinea pigs (MASA, YUTA, TARO) (Fig. 7, 8, 9). The procedure of standard check is described on section 2.1.7. In first antibody specificity check, we prepared standard of sulfated CCK-8, non-sulfated CCK-8, sulfated bovine gastrin-9 and non-sulfated bovine gastrin-9. In these standards, we used first antibody of CCK. The procedure is same as standard check preparation.

Fig.7

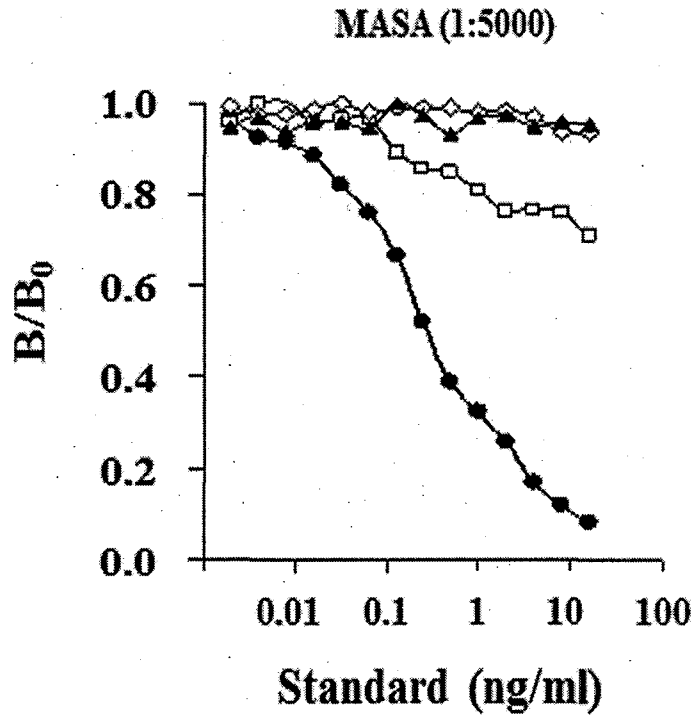


Figure 7. Standard check and specificity check (MASA). Standard RIA curves for sulfated CCK. Inhibition of  $^{125}\text{I}$ -labeled CCK-8 amide binding to guinea pig anti-CCK-8 antiserum by serial dilution of CCK-8 amide (—●—), non-sulfated CCK-8 amide (—□—), bovine gastrin-9 amide (—▲—) and bovine non-sulfated gastrin-9 amide (—◇—). Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

Fig. 8

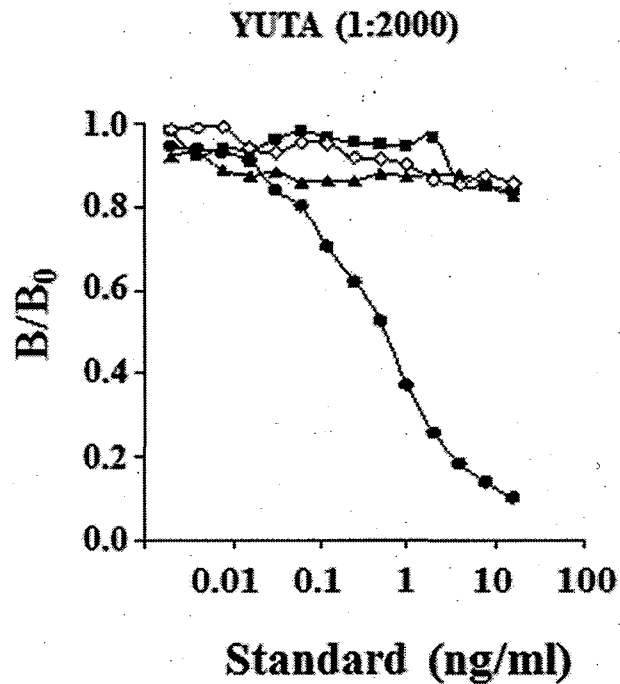


Figure 8. Standard check and specificity check (YUTA). Standard RIA curves for sulfated CCK. Inhibition of  $^{125}\text{I}$ -labeled CCK-8 amide binding to guinea pig anti-CCK-8 antiserum by serial dilution of CCK-8 amide (—●—), non-sulfated CCK-8 amide (—□—), bovine gastrin-9 amide (—▲—) and bovine non-sulfated gastrin-9 amide (—◇—). Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.



Fig.9

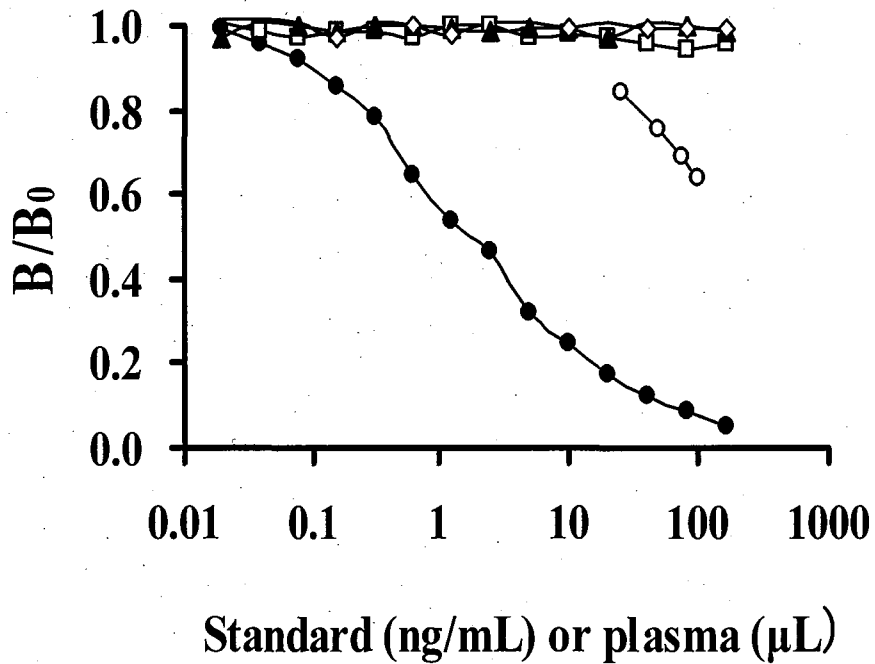


Figure 9. Standard check and specificity check (TARO). Standard RIA curves for sulfated CCK. Inhibition of <sup>125</sup>I-labeled CCK-8 amide binding to guinea pig anti-CCK-8 antiserum by serial dilution of CCK-8 amide (—●—), non-sulfated CCK-8 amide (—□—), bovine gastrin-9 amide (—▲—) and bovine non-sulfated gastrin-9 amide (—◇—). Inhibition of <sup>125</sup>I-labeled CCK-8 amide binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control CCK-8 amide as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

## **2.2 Growth Hormone Releasing Hormone (GHRH) Radioimmunoassay**

In my experiment, sulfated CCK-8 increased ghrelin secretion, however, that endogenous increased ghrelin did not affect plasma GH in steers. It is well known that GHRH and ghrelin stimulate GH secretion in non-ruminants (Evans et al. 1985, Kojima et al. 1999) and ruminants (Hashizume et al. 2005, Itoh et al. 2005, ThidarMyint et al. 2006). GHRH binds to GHRH receptor and stimulates GH secretion (Lin-Su and Wajnrajch 2002). Ghrelin binds to growth hormone secretagogue receptor-1s (GHSR-1a) and stimulates GH secretion (Kojima et al. 1999). Therefore, the mechanism of GHRH and ghrelin which stimulate GH secretion is different. It is possible that CCK may inhibit GHRH and GHRH may not stimulate GH secretion in cattle.

### **2.2.1 Solid phase peptide synthesis of GHRH**

We synthesized human GHRH (1-29), Cys<sup>0</sup>-human GHRH (1-29) and Tyr<sup>0</sup>-bovine GHRH by solid phase peptide synthesis (Table- 4) (ThidarMyint et al. 2008). For peptide synthesis, rink amide MBHA resin 4-(2,4-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin, 100- 200 mesh, 1.37 mmoles per gram for loading) was used. A major problem in SPSS was peptide chain aggregation due to either hydrophobic interactions or interchain hydrogen bonding. This typically occurs between 5- 15 residues from the C-terminus and can lead to incomplete coupling and deprotection. Therefore, side-chain protected Fmoc-amino acids were used. Resin 1 mmoles (0.7138 mmoles/g) was primarily used and side-chain protected Fmoc-amino acids were Fmoc-Ther(tBU)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBU)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(otBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc- His(Trt)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Ser(tBu)-OH. Synthesized peptides were purified by RP-HPLC and the peptide containing elution were lyophilized and stored at - 30°C.

**Table (4)** Synthesized peptides of GHRH

No.	Synthesized Peptides	Amino acids sequence of peptides	Accession Number
1	Human GHRH (1-44)	<sup>31</sup> YADAIFTNSY      RKVLGQLSAR KLLQDIMNRQ      QGERNQEQGA KVRL <sup>74</sup>	P 01286
2	Cys <sup>0</sup> -human GHRH (1-29)	<sup>30</sup> CYADAIFTNSY      RKVLGQLSAR KLLQDIMSR <sup>59</sup>	P 01286
3	Cys <sup>20</sup> -bovine GHRH (21-44)	<sup>50</sup> CKLLQDIMNRQ      QGERNQEQGA <sup>70</sup> KVRL <sup>74</sup>	P 63292

### 2.2.2 First antibody production of GHRH

GHRH is 44 amino acids peptide (Grossman et al. 1986). The amino acid sequence from number 1 to 27 are identical in human and bovine (Table-5) (Uniprot accession no. P 01286, P 63292). We used synthesized (Cys<sup>0</sup>) 1-29 human GHRH peptide and (Cys<sup>0</sup>) 21-44 bovine GHRH for first antibody production. We generated GHRH first antibody with Cys<sup>0</sup>-bovine GHRH (1-29) in 2 laying hens (batch 1, chicken no.7 and no.8) and Cys<sup>20</sup>-bovine GHRH (21-44) in 1 laying hen (batch 2, chicken no. 23). We selected chicken for immunization because amino acid sequence of chicken is different from human and bovine (Table-5). For bovine GHRH, we used from Phoenix Pharmaceuticals Inc. Burlingame, USA.

**Table (5)** Amino acids sequence of GHRH

Human GHRH	<sup>31</sup> YADAIFTNSY      RKVLGQLSAR      KLLQDIM <u>S</u> RQ QGES <u>N</u> QEQGA KVRL <sup>74</sup>	Accession No. P 01286
Bovine GHRH	<sup>31</sup> YADAIFTNSY      RKVLGQLSAR      KLLQDIM <u>N</u> RQ QGER <u>N</u> QEQGA KVRL <sup>74</sup>	Accession No. P 63292
Chicken GHRH	<sup>83</sup> HADGIFSK      AYRLLGQLS      ARNYLHSLMA KRVGGASSGL GDEAEPLS <sup>128</sup>	Accession No. P 41534

GHRH peptide (hepaten) was conjugated with carrier protein, mariculture keyhole limpet hemocyanin (mcKLH) (PIERCE Prod # 77600, Lot # HJ 104537).

Generally, reacting equal mass amounts of hepaten and carrier protein. For conjugation of peptide/hepaten with carrier protein, it needs to activate carrier protein. To activate carrier protein, we put mcKLH 20 mg dissolved with 1 ml of deionized water was added with 5 mg of sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) (Thermo scientific) dissolved with 1 ml of deionized water were put into the glass bottle. Sulfo-SMCC is sulfhydryl crosslinker which used to create sulfhydryl-reactive, maleimide-activated carrier proteins for coupling of sulfhydryl containing haptens. The glass bottle was put on rotator for 60 min at room temperature (for regular gentle mixing). The suspension cannot make vortex and heating because which can cause precipitation of mcKLH. To avoid precipitation of mcKLH, we made gentle mixing with rotator. After 60 min rotating, the color of liquid changes to blue color. The mixture of mcKLH and SMCC was gel filtrated by PD-10 column to remove excess SMCC.

We used inject purification buffer salt (Thermo scientific # 77159, Lot # JG125242) which contains 0.083 M sodium phosphate, 0.9 M NaCl, with a proprietary stabilizer in gel filtration. Cysteine attached peptide is sulfhydryl containing hepaten. We prepared inject purification buffer with 60 ml of deionized water. We put 1 ml of (Cys<sup>0</sup>) 1-29 human GHRH (4.1 mg) and 0.3 ml of dimethyl sulfoxide (DMSO) into the tube (1.5 mL) and mix. After that, the mixture (GHRH and DMSO), 0.9 ml of mcKLH (3.9 mg), 0.133 mL of DMSO and 0.01 ml of EDTA (0.1M, pH 8.5) were put quickly into the glass bottle with cap and mix gently by hand. Adding of EDTA is to prevent oxidation with air. The glass bottle was place on rotator for 120 min at room temperature for regular gentle mixing. The suspension cannot make vortex and heating because which can cause precipitation of mcKLH. After 120 min rotating, the color of liquid changes to blue color and found some precipitate.

We centrifuged the mixture for 10 min, and collect the supernatant fluid and save the precipitate. The volume of supernatant is 2.1 ml. We make purification of

supernatant fluid by PD - 10 column to remove DMSO and EDTA. Before purification, PD-10 column (Sephadex G-25) was washed 2 times with 5 ml of distilled water. After that the column was washed with inject purification buffer for 2 times. We used inject purification buffer salt (Thermo scientific #77159) for purification because which will preserve the conjugated GHRH during freeze-thaw cycles.

We prepared 10 glass-tubes for collection of filtrate. We put 500  $\mu$ l of the supernatant of conjugated GHRH into PD-10 column and collect the filtrate with glass tube. The total volume of supernatant is 2100  $\mu$ l. Therefore, after putting of 500  $\mu$ l of the supernatant into PD-10 column for 2 times, the volume 100  $\mu$ l of supernatant was left for next purification. Therefore, we add 400 $\mu$ l of inject purification buffer to 100  $\mu$ l of supernatant and the final volume becomes 500  $\mu$ l. Then, we put 500  $\mu$ l of this mixture into the column and collect the filtrate. After that, PD-10 column was washed with 500  $\mu$ l of inject purification buffer and collect filtrate. We washed 8 times to PD-10 column with 500  $\mu$ l of inject purification buffer after sample loading and collect filtrate. We used 10 glass tubes for collecting of filtrate. We collect the filtrate from tube no. 6 to no. 10. Each tube contains 500 $\mu$ l of filtrate. Therefore, total 2.5 ml of filtrate were collected.

The collected filtrate (2.5 mL) was mixed with precipitate which saved from GHRH conjugate after centrifugation. The mixture includes 4.1 mg of conjugated GHRH. We divided 2.5 ml of this conjugated GHRH into 1.25 ml and keep in polyethylene tubes (size 1.5 ml) and freezed by liquid nitrogen. After freezing, conjugated GHRH were stored at - 30°C. The 1.25 ml of conjugated GHRH which keeps in polyethylene tube contains 2.05 mg of peptide. We used 1.025 mg of conjugated peptide for immunization of one chicken. We prepared antigen for second time antibody production in chicken No. 23. We used Cys<sup>20</sup>-bovine GHRH (21 - 44) which has disulfide bond at number 31 amino acid and at number 36 amino acid.

Conjugated GHRH was emulsified with same volume of Freund's complete adjuvant for initial immunization. In chicken, 2.05 mg of conjugated GHRH in 1.25 ml of purification buffer was emulsified with 1.25 ml of Freund's adjuvant by sonifier. The

immunogens (GHRH + mcKLH + Adjuvant) were used for 2 chickens. The immunogen was injected subcutaneously at the back of each chickens' neck. We injected 6 times of immunogen to chicken with two weeks interval. Two to five milliliter of blood from each chicken (wing vein) was collected before injection of immunogen and after injection of immunogen for antibody titer check. The collected blood samples were store in 4°C for 24 hours. Then, the samples were centrifuged and decant serum. The collected sera were stored in -30°C.

In second time of bovine GHRH first antibody generation, we used 0.35 ml of conjugated bovine GHRH emulsified with 0.35 ml of Freund's adjuvant (immunogen) for immunization. We injected immunogen to chicken no. 23 for 7 times with one week interval.

We checked the titer of GHRH antibody by RIA. We sampled all blood from hens one day after final injection of antigen. The collected blood was stored at 4°C for 24 hours and then centrifuged. The serum was separated and stored at - 30°C. For GHRH first antibody titer check, we set up the assay at room temperature. We prepared T-tubes (only put radioisotope labeled GHRH). Two hundred milliliter of buffer (glucagon assay buffer, 0.05 M glycine, 0.03M EDTA, 0.08% sodium azide and 1% BSA; pH 8.8), 100 µL of GHRH first antibody (final dilution of 1: 5000) and 100µL of I125 labeled GHRH (8000 cpm/ 100µL in assay buffer containing 1% normal chicken serum), were put in polystyrene tubes and incubate for 24 hours at 4°C. After incubation, we add 1 ml of precipitation buffer containing 4% second antibody (goat anti-IgY serum) and incubate for 30 min at 4°C. Then, we made centrifugation 3000 rpm, at 4°C for 30 min to separate bound and free ligand. Remove all of the supernatant (without touching pellet) immediately after centrifugation. The radioactivity of pellet was counted by gamma counter (ARC-1000, Aloka, Japan). All samples were assay in quintuplicate. All hens produced antibody (Fig.18,19) (Table- 14, 15), however, the titer of hen no. 8 (batch 1) is very low. Therefore, we selected anti-GHRH serum of hen no. 7 and no.23 for radioimmunoassay.

### **2.2.3 Labeling of GHRH**

We used synthesized human GHRH (1-29) peptide for labeling. Number one N-terminal amino acid sequence of human GHRH is tyrosine. Therefore, this peptide is no need to made N-terminal tyrosine attachment for labeling. GHRH was iodinated by chloramine-T method (McConahey et al. 1980). We labeled human GHRH (1-29) for first batch. We used chloramine T-method for labeling of bovine GHRH (1-44) for second batch. I<sup>125</sup> labeled peptide was purified by HPLC. The peak fraction tube (400  $\mu$ L) was collected and mixed with 400  $\mu$ L of 60% CH<sub>3</sub>CH/ 0.1% TFA for neutralization. Purified I<sup>125</sup> labeled peptide was used as tracer in first antibody titer check, first antibody dilution check, standard check and specificity check.

### **2.2.4 Extraction of chicken egg IgY**

We made extraction of IgY for immunization and first antibody titer check. We use the method of Akita and Nakai (1993) with slight modification. We crack the egg shell, remove and put egg-yolk with albumin into the beaker. We remove the albumin beside the egg yolk by sucking with pipette. After that, we washed the egg-yolk with distilled water by 3 times. Then, we moved the egg-yolk from the beaker into the measuring cylinder and removed the outer cover of egg-yolk by tweezers. The volume of egg-yolk is about 20 mL. We put 180 mL of distilled water and stir-bar into cylinder which contain 20 mL of egg-yolk and mix by stirring (stirring bar was run by magnetic mixer) for 24 hours at 4°C. After that, the mixture is moved into plastic bottle and centrifugation with 10000 rpm for 25 min at 4°C. We only collect the supernatant, that supernatant was filtered with cotton filter and the filtrate was put into the beaker. Then, we put the beaker on hot water at 40°C and mix by glass-rod. When the temperature inside the beaker arrives to 30°C, we put 19 % of sodium phosphate into the beaker and mix by glass rod for 5 min. After that, put on room temperature for 2 hours. Then, the mixture was put into plastic bottle and centrifuge with 10000 rpm at 30°C for 25 min.

After centrifugation, we pour out the supernatant and put 20 mL of distilled water with 14% of sodium phosphate into the plastic bottle and mix by shaking. After that, centrifuge again and remove the supernatant. The precipitate was mixed with 2 mL of distilled water and put into the dialysis-tube. The dialysis-tube and stirring-bar were put into the beaker which contain 200 mL of distilled water and stir for 30 min. After that, we changed the water inside the beaker with new 200 mL of distilled water and put dialysis-tube and stirring-bar and stir for 30 min again. We changed the water inside the beaker with 200 mL of distilled water with 1.8 g of sodium chlorite. Then, we put dialysis-tube and stirring-bar into the beaker and stir for 24 hours at 4°C. After stirring, we collect the liquid which is IgY into the polystyrene tubes and keep at - 30°C.

### **2.2.5 Second antibody (goat anti-IgY) production**

We generated second antibody in goat. We used extracted egg IgY for immunization. We prepared immunogen for immunization. Firstly, we covered the tip of disposable syringe (size 5 mL) with parafilm. After that, we put 1.5 mL of Freund's adjuvant, 1 mL of extracted IgY and 0.5 mL of purification buffer were put into the syringe. The syringe which contains IgY, adjuvant and purification buffer is placed on ice and conjugated with sonifier machine (we set sonifier of Out put control at 3 and Duty cycle % at 30) until the mixture in the syringe becomes hard. This adjuvant conjugated IgY was injected subcutaneously to goat with 1 week interval. Blood samples were collected and keep in 4°C for 24 hours and separate serum by centrifugation with 8000 rpm for 30 min at 4°C. The collected serum was kept in - 30°C. The antibody (second antibody) titer was checked by titration method. The percentage of second antibody used in radioimmunoassay was chose and adjusted according to the condition of pellet hardness. We used 4% of second antibody in radioimmunoassay.

### **2.2.6 Second Antibody (goat anti- IgY) titer check**

Goat anti-IgY (2<sup>nd</sup> antibody) titer was checked by titration method. Second



antibody titer check was prepared in room temperature. We put 300 $\mu$ l of assay buffer (0.05 M phosphate, 0.01 M EDTA, 0.08 % sodium azide, 0.1% gelatin, 0.25 % BSA; pH 6.85) into polystyrene tubes. Then, we add 100 $\mu$ l of assay buffer which include normal chicken serum, 4  $\mu$ l to 16  $\mu$ l of goat anti-IgY (second antibody) and precipitation buffer (0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4). After that, the tubes which contain mixture are incubated at room temperature for 30 min and check the turbidity of the mixture.

### **2.2.7 Sensitivity/titer check of GHRH first antibody**

We made titer check for anti-human GHRH serum of chicken from first batch and for anti-bovine GHRH serum of second batch by RIA (Fig. 10, Fig. 11). The assay was prepared at room temperature. Firstly we put 200  $\mu$ l of glucagon assay buffer (0.05 M glycine, 0.03M EDTA, 0.08% sodium azide and 1% BSA; pH 8.8) into the polystyrene tube. After that, we prepared first antibody at 1:5000 final dilution in assay buffer with 1.5 % carrier serum and added to the tubes which contain buffer. Then, we add the tracer (about 6000 CPM) which was prepared in assay buffer contained 1% normal chicken serum. Vortex mix and incubate for 24 hours. After incubation, we add 1 ml of precipitation buffer (0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4 ) with 4 % second antibody (goat anti-IgY serum) and incubate again for 30 min. Then, centrifuge with 3000 rpm at 4°C for 30 min and count CPM of the pellet. In first batch, chicken no. 7 was produced antibody (Table-6). In second batch, chicken no. 23 produced antibody (Table-15).

**Table (6)** Sensitivity/titer check for chicken No. 7 and No. 8 anti-GHRH serum

<b>Chicken no.</b>	<b>Days after initial immunization</b>	<b>Bound/Total (B/T)</b>
7	0	0
7	21	0
7	35	0.12
7	49	0.57
7	56	0.63
7	70	0.5
7	80	0.44
8	0	0
8	21	0
8	35	0
8	49	0.07
8	56	0.02
8	70	0.12
8	80	0.07

**Table (7)** Sensitivity/titer check for chicken No. 23 anti-GHRH serum

<b>Chicken no.</b>	<b>Days after initial immunization</b>	<b>Bound/Total (B/T)</b>
23	0	0
23	14	0
23	22	0.08
23	31	0.2
23	38	0.51
23	42	0.6
23	48	0.51

Fig. 10

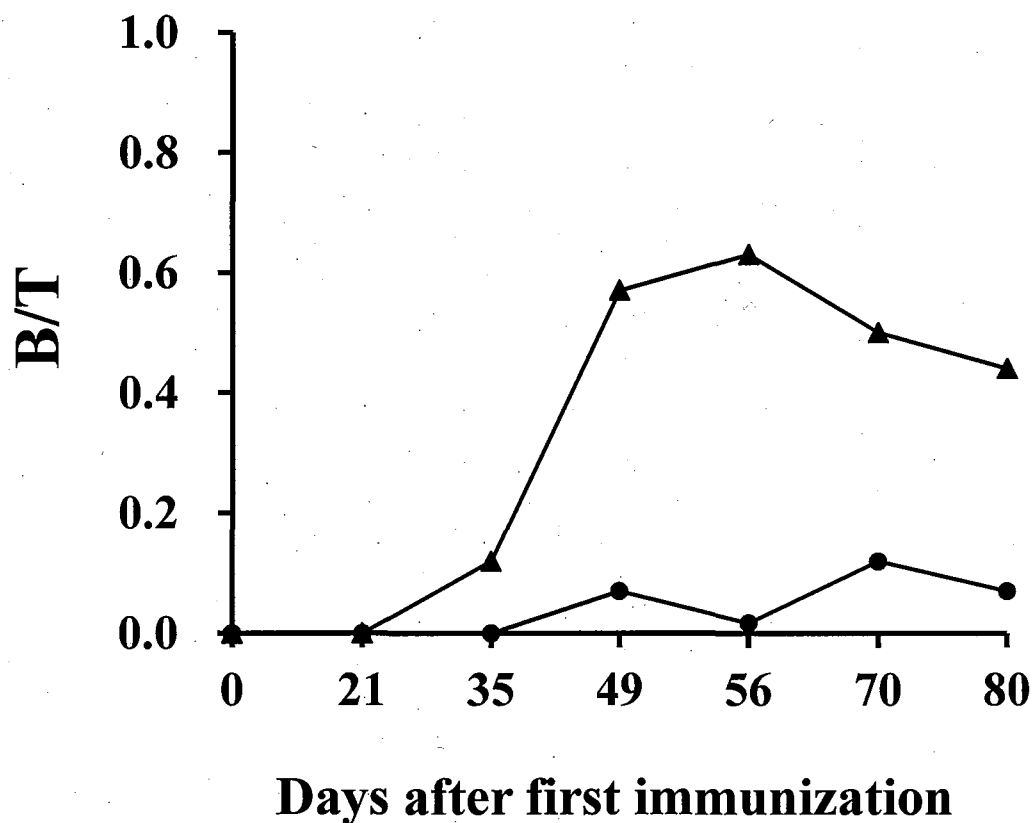


Figure 10. Sensitivity/titer check for in chicken no. 7 (-▲-) and chicken no. 8 (-●-) anti-human GHRH serum. Serum samples were collected from before antigen injection to until whole blood sampling. First antibodies were used 1: 5000 final dilution and each point is the mean of quintuplicate serum samples in this assay. B/T = Bound/ Total.

Fig. 11

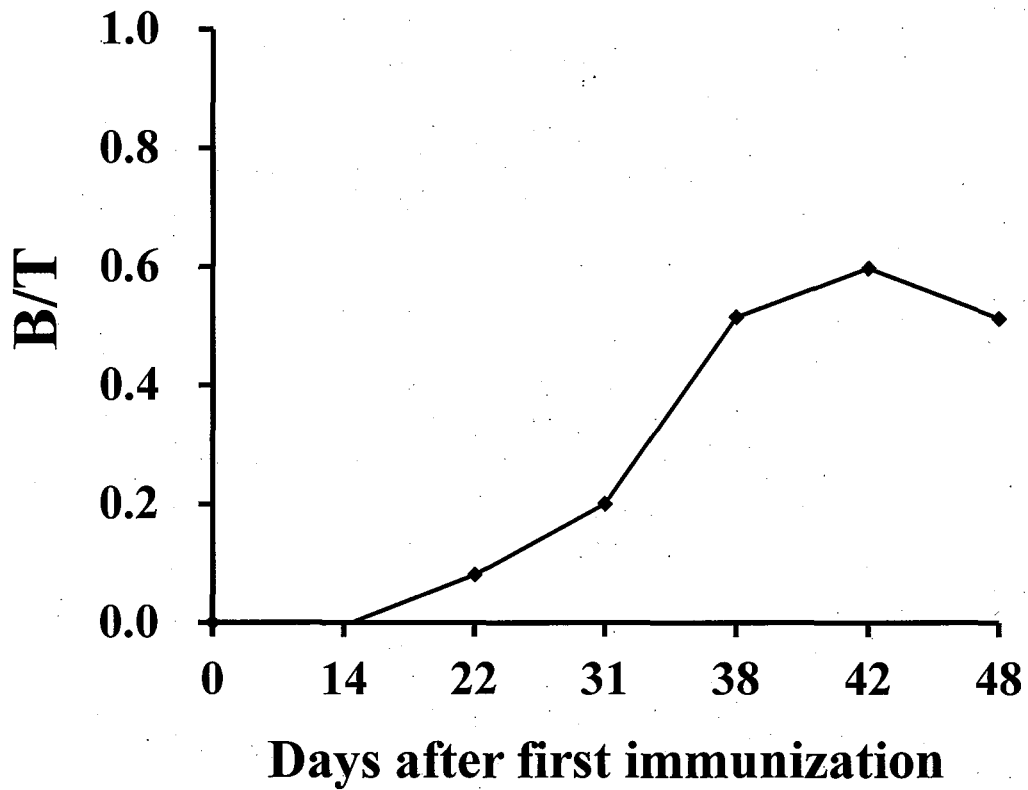


Figure 11. Sensitivity/titer check for chicken no. 23 (—◆—) anti-bovine GHRH serum. Serum samples were collected from before antigen injection to until whole blood sampling. First antibodies were used 1: 5000 final dilution and each point is the mean of quintuplicate serum samples in this assay. B/T = Bound/ Total

### 2.2.8 Sensitivity/titer dilution check of GHRH first antibody

We set the assay at room temperature. In first antibody (chicken anti-bovine GHRH serum) dilution check, we used the tracer 6000 CPM (with 1 % normal chicken serum in assay buffer) and final dilution of first antibody from 1: 5000 to 1: 20000 (Table-8, 9, Fig. 12, 13).

**Table (8)** Sensitivity/titer dilution check for chicken No. 7 anti-GHRH serum

<b>First antibody final dilution</b>	<b>Chicken no. 7 (B/T)</b>
5000	0.35
10000	0.25
15000	0.17
20000	0.11

**Table (9)** Sensitivity/titer dilution check for chicken No. 23 anti-GHRH serum

<b>First antibody final dilution</b>	<b>Chicken no. 23 (B/T)</b>
5000	0.51
10000	0.37
15000	0.28
20000	0.15

Fig. 12

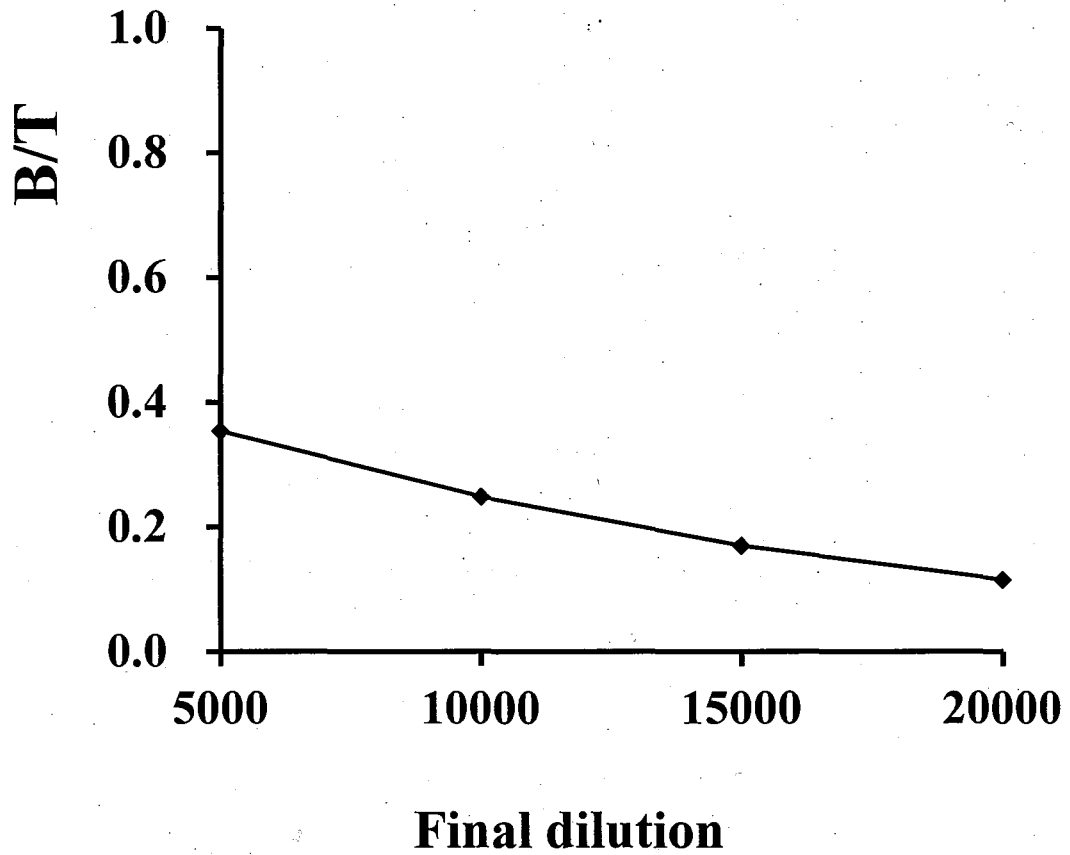


Figure 12. Specificity/titer dilution check for chicken no. 7 (—▲—) anti-human GHRH serum. First antibodies were used from 1: 5000 to 1: 20000 final dilution and each point is the mean of quintuplicate serum samples in this assay. B/T = Bound/ Total.

Fig. 13

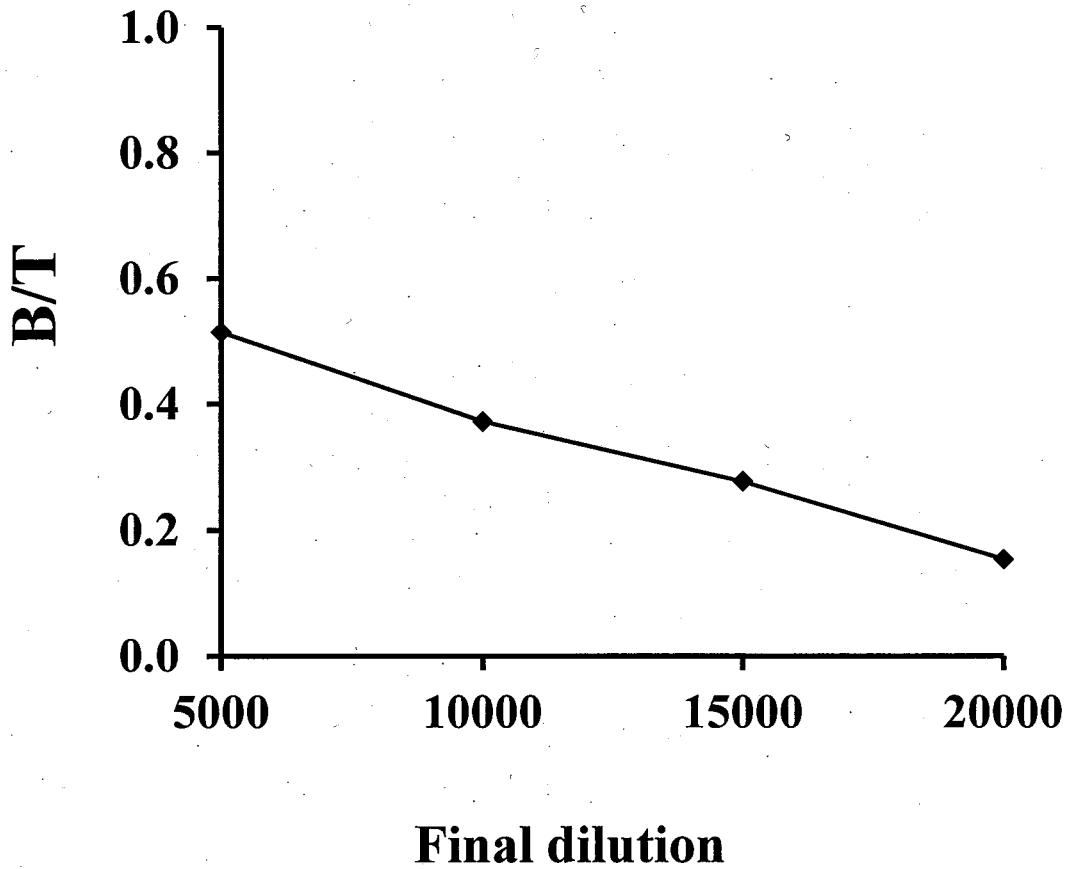


Figure 13. Sensitivity/titer dilution check for chicken no. 23 (—◆—) anti-GHRH serum. Serum samples were collected from before antigen injection to until whole blood sampling. First antibodies were used from 1: 5000 to 1: 20000 final dilutions and each point is the mean of quintuplicate serum samples in this assay. B/T = Bound/ Total

### **2.2.9 GHRH radioimmunoassay**

Standard concentrations of GHRH ranged from 0.19 to 100 ng/mL was set up at room temperature. Standards and plasma samples were incubated with antibodies diluted in glucagon assay buffer (0.05 M glycine, 0.03M EDTA, 0.08% sodium azide and 1% BSA; pH 8.8) for 24 hours. After 24 hours incubation, <sup>125</sup>I-labeled GHRH amide (8000 count per minute/100 µL assay buffer containing 1% normal chicken serum) was added to the tubes. The tubes were incubated again for 24 h at 4°C. The next day, the second antibody (4% goat anti-IgY prepared in precipitation buffer, 0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4) was added to the tubes, and the tubes were incubated for 30 min at 4°C. After incubation, bound and free antigens were separated by centrifugation for 30 min at 1870 ×g and 4°C, aspiration of all the supernatant (without touching the pellet) immediately follow centrifugation.

The radioactivity in the pellet was measured by gamma counter (ARC-1000, Aloka, Japan). Triplicate standards and duplicate plasma samples were run within in a single assay.

### **2.2.10 Standard check of GHRH first antibody**

We made standard check and specificity check of anti-bovine GHRH serum of first batch of chicken no. 7 and second batch of chicken no. 23. After that, we set up cold standard from 0.19 ng/ml to 100 ng/ml. The sensitivity of GHRH RIA (used human GHRH first antibody, chicken No. 7) is 0.47 pg/ml and intra-assay coefficient of variation is 11.6 %. Plasma level of GHRH in this standard is 77 - 85 pg/ml (Fig. 14). The sensitivity of GHRH RIA (used bovine GHRH first antibody, chicken No. 23) is 51.25 pg/ml and intra-assay coefficient of variation is 25.6 %. Plasma level of GHRH in this standard is 49 - 83 pg/ml (Fig. 15). Our GHRH RIA systems can measure basal plasma level in cattle.



Fig. 14

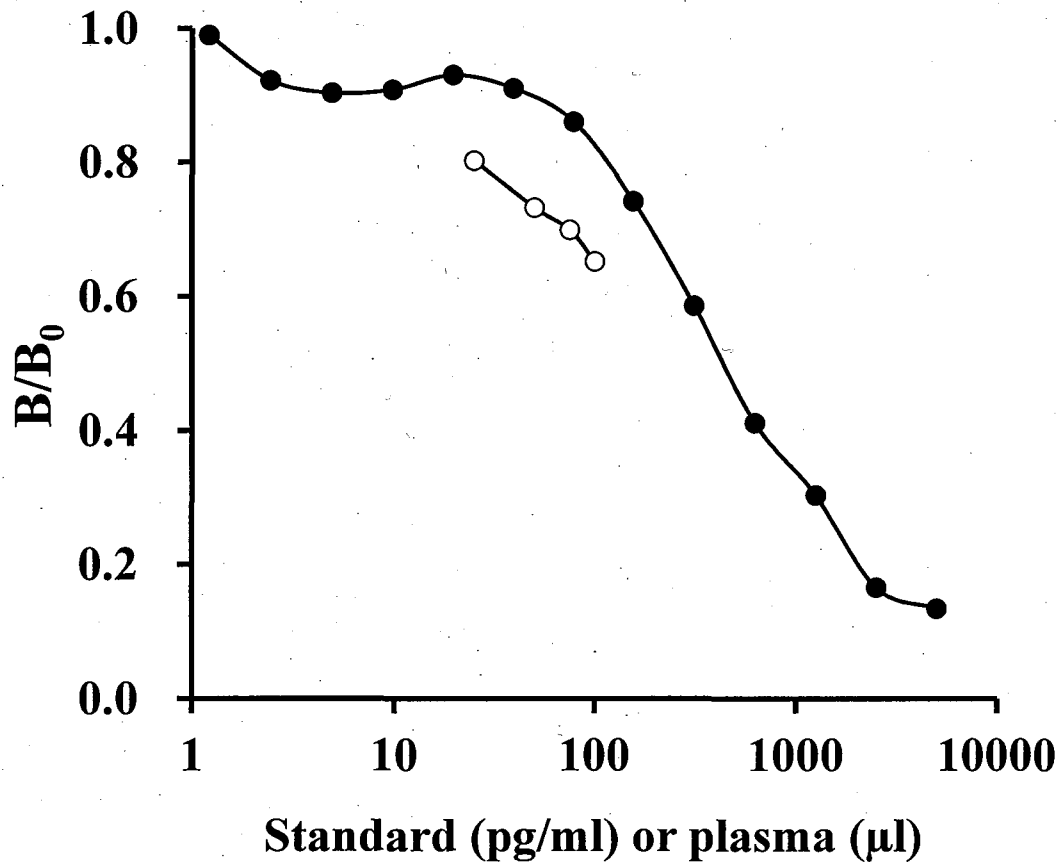


Figure 14. Standard RIA curves for human GHRH. Inhibition of  $^{125}\text{I}$ -labeled GHRH binding to chicken anti-GHRH antiserum by serial dilution of GHRH (—●—). Inhibition of  $^{125}\text{I}$ -labeled GHRH binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine GHRH as standard. Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

Fig. 15

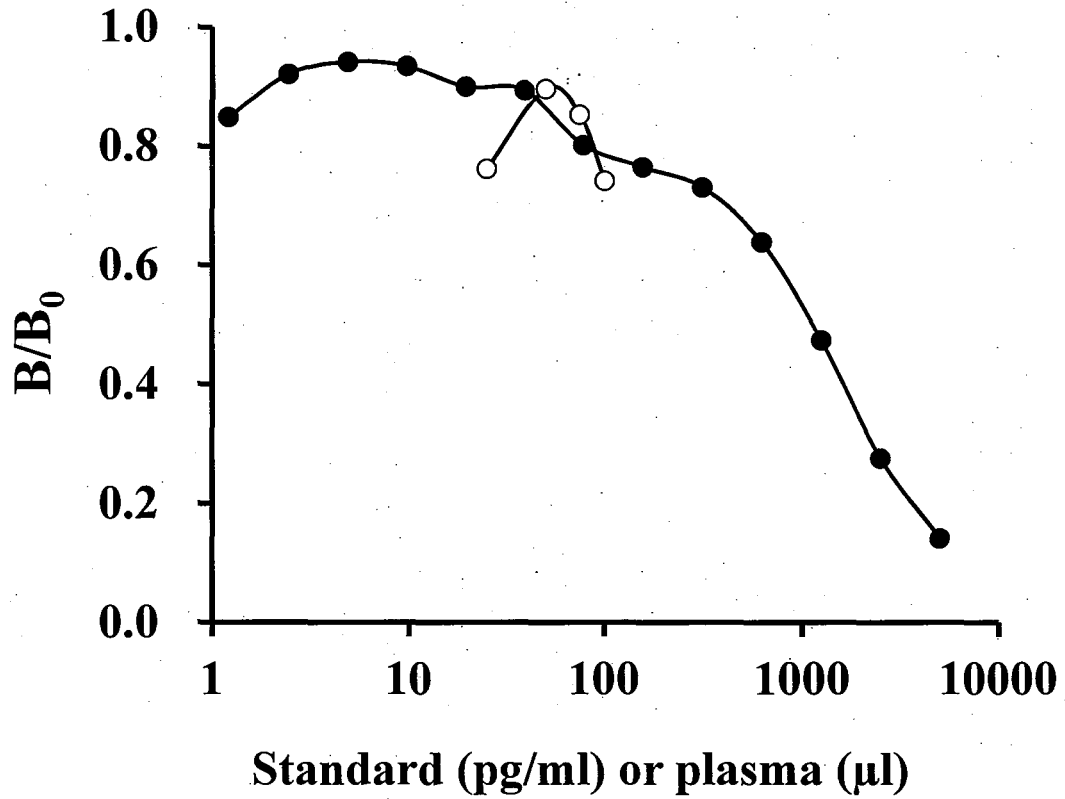


Figure 15. Standard RIA curves for bovine GHRH. Inhibition of <sup>125</sup>I-labeled GHRH binding to chicken anti-GHRH antiserum by serial dilution of GHRH (—●—). Inhibition of <sup>125</sup>I-labeled GHRH binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine GHRH as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

### 2.3 Amylin radioimmunoassay

Amylin is 37 amino acids peptide (Cooper et al. 1987). The amino acid sequence of amylin between bovine and guinea pig are different (Table-10). Therefore, we generated first antibody production in guinea pig.

**Table (10)** Amino acids sequence of amylin

Peptide	Amino acids sequence of peptides	Accession Number
Bovine Amylin	<sup>37</sup> KCGTA TCETQRLANF LAPSSNKLGA IFSP TKMGSN TY <sup>72</sup>	Q 28207
Guinea pig Amylin	<sup>37</sup> <b>K</b> <u>C</u> <b>N</b> <u>T</u> A <b>T</b> <u>C</u> <b>A</b> <u>T</u> QRL <b>T</b> <u>N</u> F <b>L</b> <u>V</u> <b>R</b> <u>S</u> <b>S</b> <u>H</u> <b>N</b> <u>L</u> G <b>A</b> <b>A</b> <u>L</u> <b>L</b> <u>P</u> <b>T</b> <u>D</u> <b>V</b> <u>G</u> <b>S</b> <u>N</u> TY <sup>73</sup>	P 12966

Amino acids different from bovine amylin show with bold and underline. See (Table-1) for single letter code translation.

#### 2.3.1 Solid phase peptide synthesis of amylin

In our study, CCK-induced endogenous ghrelin does not stimulate GH in steers. We assume that CCK may stimulate amylin and that amylin may inhibit GH release in cattle. Because, amylin inhibits GHRH stimulated GH release in rat (Netti et al. 1995). We synthesized amylin peptides by solid phase peptide synthesis. We synthesized bovine amylin (1-37), Cys<sup>0</sup>-bovine amylin (7-37) and Tyr<sup>0</sup>-bovine amylin (Table-11).

**Table (11)** Synthesized peptides of amylin

No.	Synthesized Peptides	Amino acids sequence of peptides	Accession Number
1	Bovine Amylin (1-37) amide	<sup>37</sup> KCGTA TCETQRLANF LAPSSNKLGA IFSPTKMGSN TY <sup>72</sup>	Q 28207
2	Cys <sup>0</sup> -bovine amylin (7-37)	<sup>42</sup> CETQRLANF LAPSSNKLGA IFSPTKMGSN TY <sup>72</sup>	Q 28207
3	Tyr <sup>0</sup> -bovine amylin	<sup>36</sup> YKCGTA TCETQRLANF LAPSSNKLGA IFSPTKMGSN TY <sup>72</sup>	Q 28207

### 2.3.2 First antibody production of amylin

We generated amylin first antibody in guinea pigs. We used synthesized (Cys<sup>0</sup>)7-37 amylin for conjugation. In guinea pigs, 0.5 mg of conjugated amylin (mcKLH (Cys<sup>0</sup>)7-37 amylin) in 0.375 ml of phosphate buffer was emulsified with equal volume of Freund's adjuvant buffer. We raised amylin first antibody in first batch of 2 guinea pigs and in second batch of 2 guinea pigs. Immunogen (mcKLH (Cys<sup>0</sup>)7-37 amylin+ Freund's adjuvant) was injected subcutaneously for 7 times with one week interval. In first batch, we did not collect blood samples for titer check. We collected the whole blood after the final (7<sup>th</sup> time) administration of antigen. In second batch of guinea pigs, we collected blood samples (0.5 mL) at 24 days, 31 days and 38 days after first immunization for titer check. We collected the whole blood at 38 days after first immunization.

### 2.3.3 Labeling of amylin

We labeled synthesized (Tyr<sup>0</sup>)-bovine amylin with chloramine T-method (McConahey et al. 1980). Labeled peptide was purified with HPLC. The peak fraction

tube (400  $\mu$ L) was collected and mixed with 400  $\mu$ L of 60%  $\text{CH}_3\text{CH}/$  0.1% TFA for neutralization. Purified  $\text{I}^{125}$  labeled peptide was used as tracer in first antibody titer check, first antibody dilution check, standard check and specificity check.

#### **2.3.4 Sensitivity/titer check of amylin first antibody**

We made titer check for anti-bovine amylin serum of guinea pigs from first batch and second batch. We checked the titer of bovine amylin antibody by RIA. The assay was prepared at room temperature. Firstly we put 200  $\mu$ l of assay buffer (0.05 M sodium dihydrogen phosphate dihydrate, 0.9% saline, 0.025M EDTA, 0.08% sodium azide and 1% BSA; pH 7.4) into the polystyrene tube. After that, we prepared first antibody at 1:5000 final dilution in assay buffer with 1.5 % carrier serum and added to the tubes which contain buffer. Then, we add the tracer (about 8000 CPM) which was prepared in assay buffer contained 1% normal guinea pig serum. The tubes were vortex mixed and incubated for 24 hours. After incubation, we add 1 ml of precipitation buffer (0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4 ) with 3.5 % second antibody (goat anti-guinea pig serum) and incubate again for 30 min. Then, the tubes were centrifuged with 3000 rpm at 4°C for 30 min, remove the supernatant, and count CPM of the pellet in each tube.

First batch of 2 guinea pigs was produced antibody and their antibody titer is high. It can use in RIA standard. In second batch of 2 guinea pigs, guinea pig no. 2 only produced antibody however its titer is low (Table-12, Fig. 16). We do not made dilution check of 1<sup>st</sup> antibody from 2<sup>nd</sup> batch guinea pigs, because of very low binding with tracer.

**Table (12)** Sensitivity/titer for guinea pig No. 1 and No.2 (second batch)  
anti-amylin serum

<b>Guinea Pig no.</b>	<b>Days after initial immunization</b>	<b>Bound/Total (B/T)</b>
1	0	0
1	24	0
1	31	0.01
1	38	0.01
2	0	0
2	24	0
2	31	0.14
2	38	0.22

Fig. 16

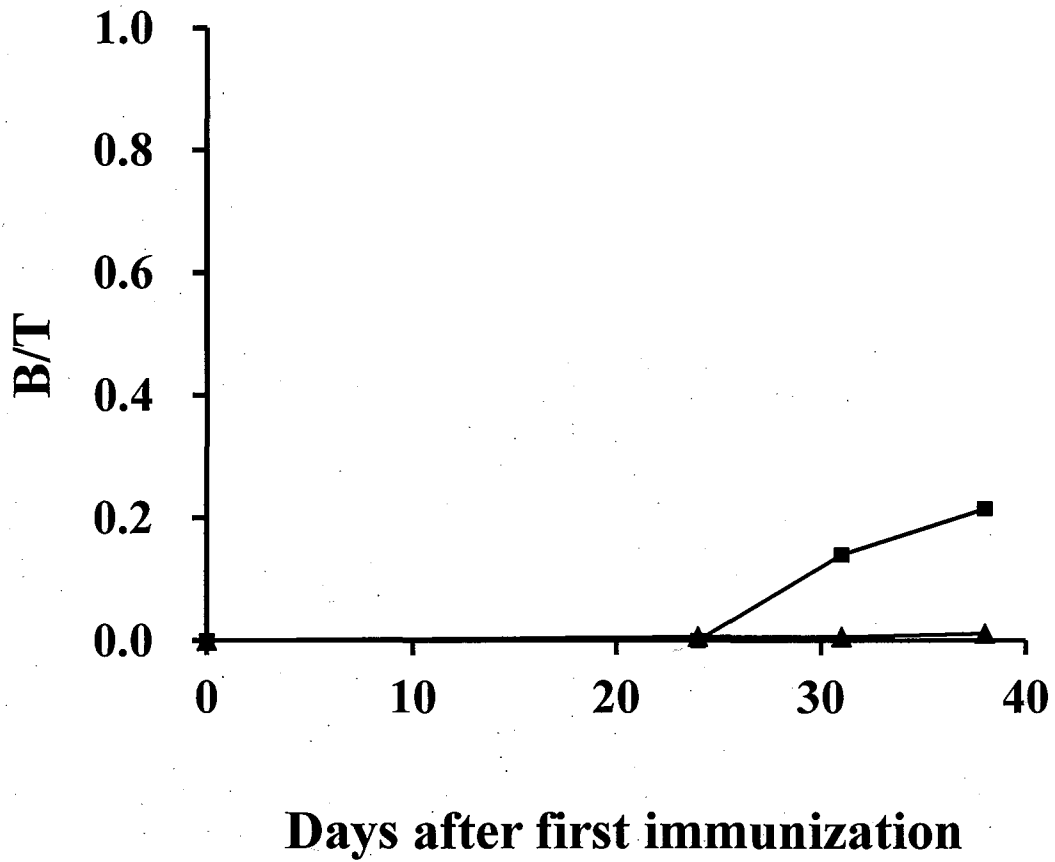


Figure 16. Specificity/titer check for guinea pig no. 1 ( $\blacktriangle$ ) and guinea pig no. 2 ( $\blacksquare$ ) (second batch) anti- bovine amylin serum. Serum samples were collected from before antigen injection to until whole blood sampling. First antibodies were used 1: 5000 final dilution and each point is the mean of quintuplicate serum samples in this assay.

### 2.3.5 Sensitivity/titer dilution check of bovine amylin antibody

In first antibody (guinea pig anti-bovine amylin serum) dilution check, we used the tracer 9000 CPM (with 1.5 % normal guinea pig serum in assay buffer) and final dilution of first antibody from (first batch) 1: 20000 to 1: 40000 (Table-13, Fig. 17), and final dilution of first antibody from (second batch) is only 1:5000 (Table-14) . The binding of first antibody of guinea pig 1 (second batch) serum with tracer is very low and its binding is only 0.1 % at 1:5000 final dilution. Therefore, we select the first antibody of guinea pig 2 for radioimmunoassay.

**Table (13)** Dilution check of guinea pig No.1 and No.2 (first batch) anti-amylin serum

<b>First antibody final dilution</b>	<b>Guinea Pig no. 1 (B/T)</b>	<b>Guinea Pig no. 2 (B/T)</b>
0	0	0
20000	0.64	0.64
30000	0.45	0.42
40000	0.36	0.35

**Table (14)** Dilution check of guinea pig No.1 and No.2 (second batch) anti-amylin serum

<b>First antibody final dilution</b>	<b>Guinea Pig no. 1 (B/T)</b>	<b>Guinea Pig no. 2 (B/T)</b>
0	0	0
5000	0.01	0.22



Fig. 17

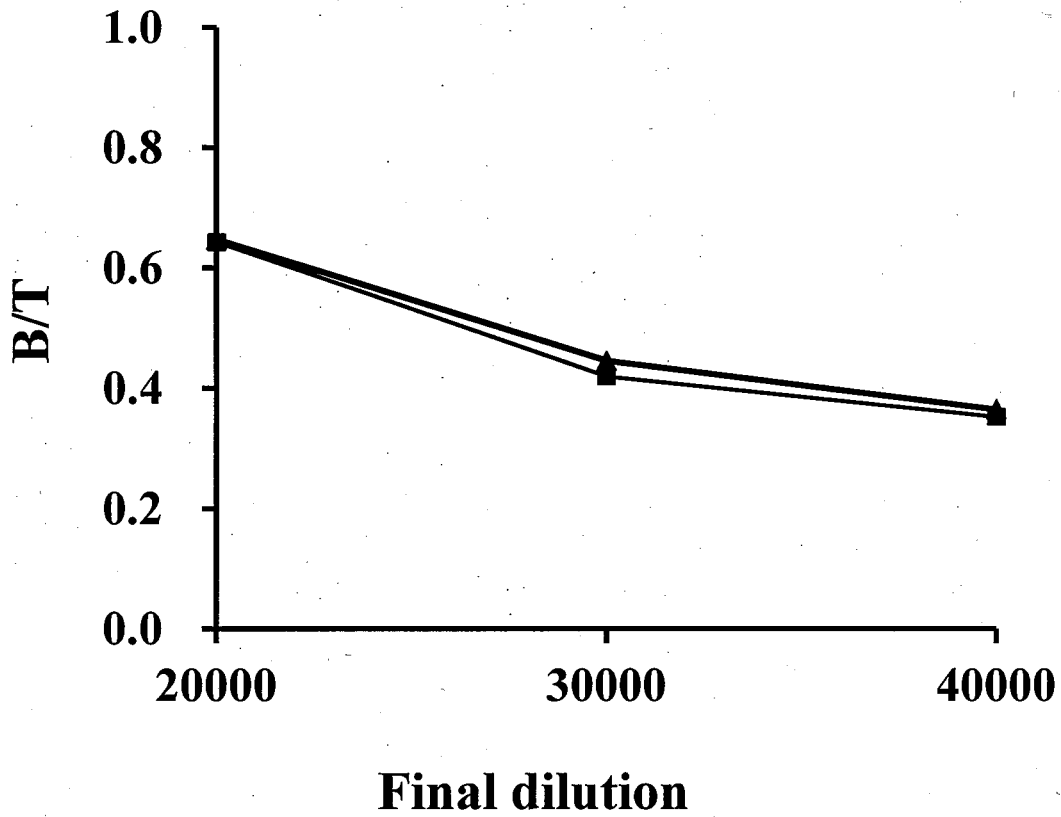


Figure 17. Sensitivity/titer dilution check for guinea pig no. 1 (—▲—) and guinea pig no. 2 (—●—) (first batch) anti-bovine amylin serum. First antibodies were used from 1: 20000 to 1: 40000 final dilutions and each point is the mean of quintuplicate serum samples in this assay.

### **2.3.6 Amylin radioimmunoassay**

Standard concentrations of amylin ranged from 0.19 to 100 ng/mL was set up at room temperature. Standards and plasma samples were incubated with antibodies diluted in insulin assay buffer (0.05 M glycine, 0.03M EDTA, 0.08% sodium azide and 1% BSA; pH 8.8) and <sup>125</sup>I-labeled amylin (10000 counts per minute/100 µL assay buffer containing 1.5 % normal guinea pig serum) was added to the tubes. The tubes were incubated again for 24 h at 4°C. The next day, the second antibody (3.5 % goat anti-IgY prepared in precipitation buffer, 0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4) was added to the tubes, and the tubes were incubated for 30 min at 4°C. After incubation, bound and free antigens were separated by centrifugation for 30 min at 1870 ×g and 4°C, aspiration of all the supernatant (without touching the pellet) immediately follow centrifugation.

The radioactivity in the pellet was measured by gamma counter (ARC-1000, Aloka, Japan). Triplicate standards and duplicate plasma samples were run within in a single assay.

### **2.3.7 Standard check and specificity check of amylin first antibody**

We made standard check and specificity check of anti-bovine amylin serum of first batch of 2 guinea pigs and guinea pig no.2 from second batch. In standard check and titer check of first antibody from second batch, we used first antibody of 1: 4000 final dilution. After we set up standard of amylin we made titer check. The procedure of specificity check is same as standard check. In specificity check we used human amylin amide (Phoenix Pharmaceuticals, Inc.) as standard. The sensitivity of bovine amylin RIA (used first antibody of guinea pig -1, batch 1 ) is 0.4 ng/ml. Plasma level of amylin in this standard is 0.6 - 0.7 ng/ml and intra assay coefficient of variation is 10.2 % (Fig. 18). The sensitivity of bovine amylin RIA (used first antibody of guinea pig -2, batch

1 ) is 0.1 ng/ml. Plasma level of amylin in this standard is 0.88 – 1.1 ng/ml. Intra assay coefficient of variation is 12.8 % (Fig. 19). The sensitivity of bovine amylin RIA (used first antibody of guinea pig -2, batch 2 ) is 0.027 ng/ml. Plasma level of amylin in this standard is 2.21- 2.99 ng/ml and Intra assay coefficient of variation is 16.2 % (Fig. 20). We made specificity check for first antibody of amylin of guinea pig no.1 and no.2 from first batch and antibody of guinea pig no. 2 from batch 2. All first antibody of amylin from 3 guinea pigs specifically recognized to bovine amylin and no cross reaction to human amylin (Fig. 21, 22, 23). Our amylin RIA systems cannot measure basal plasma level of amylin in steers because of low sensitivity.

Fig. 18

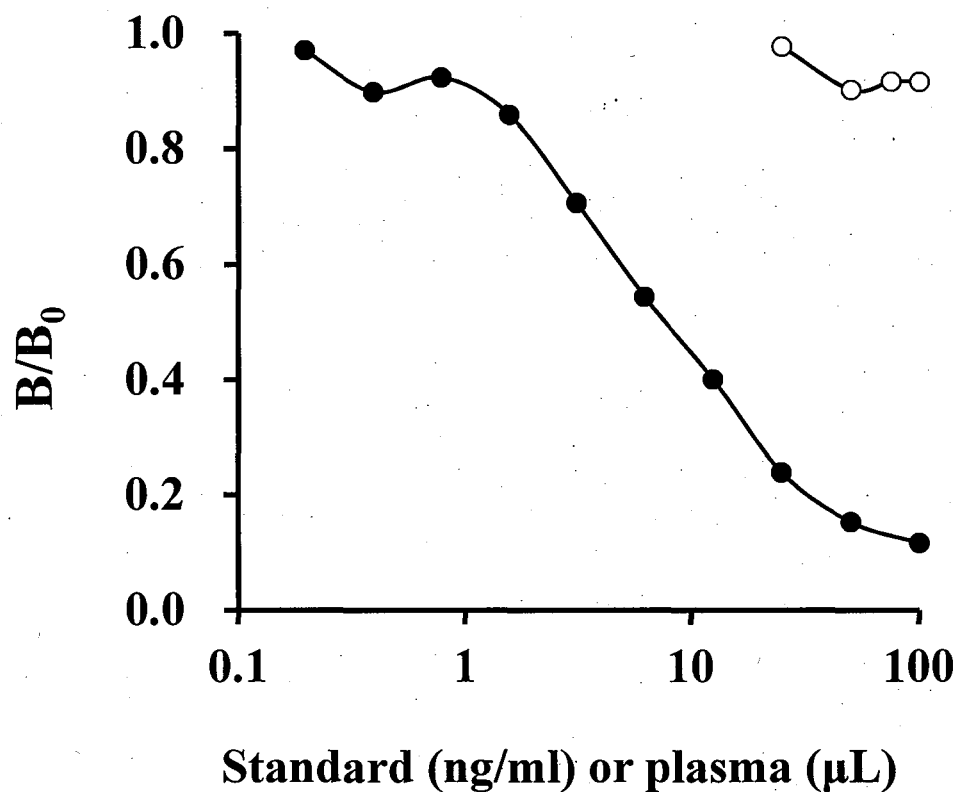


Figure 18. Standard RIA curves for bovine amylin. Inhibition of <sup>125</sup>I-labeled amylin binding to guinea pig anti-bovine amylin antiserum (first batch, guinea pig No.1) by serial dilution of bovine amylin (—●—). Inhibition of <sup>125</sup>I-labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine amylin as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

Fig. 19

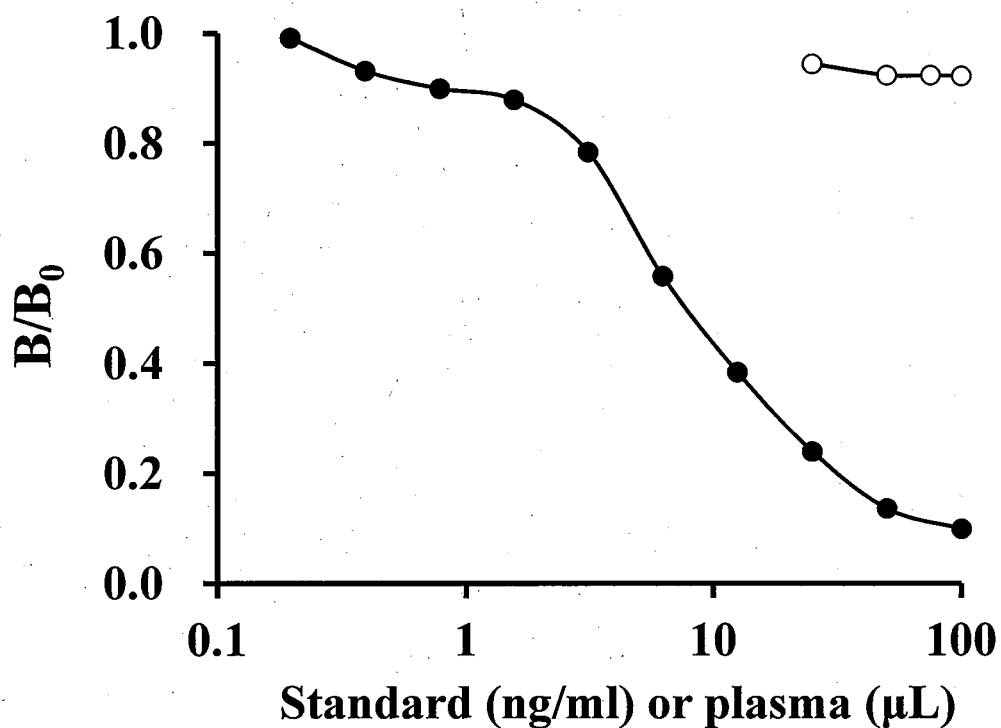


Figure 19. Standard RIA curves for amylin. Inhibition of <sup>125</sup>I-labeled amylin binding to guinea pig anti-amylin antiserum (first batch, guinea pig No. 2) by serial dilution of amylin (—●—). Inhibition of <sup>125</sup>I-labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control amylin as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

Fig. 20

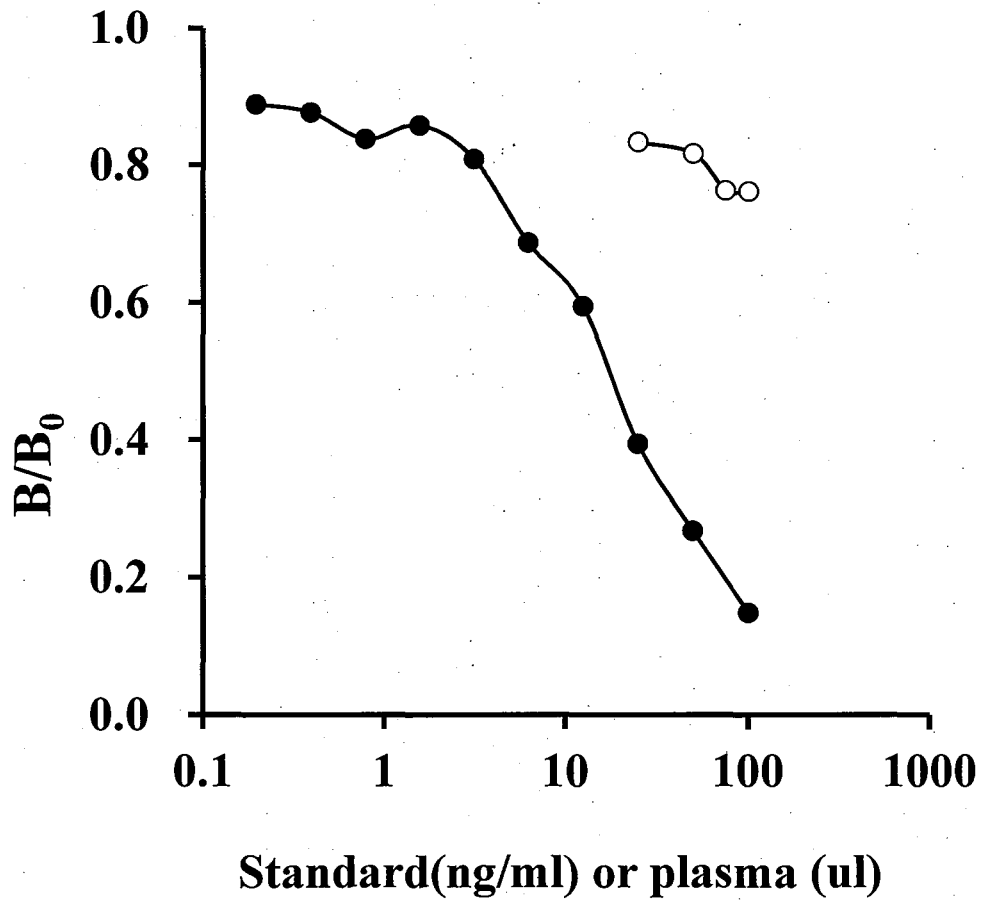


Figure 20. Standard RIA curves for bovine amylin. Inhibition of  $^{125}\text{I}$ -labeled amylin binding to guinea pig anti-bovine amylin antiserum (second batch, guinea pig No. 2) by serial dilution of bovine amylin (—●—). Inhibition of  $^{125}\text{I}$ -labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine amylin as standard. Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

Fig.21

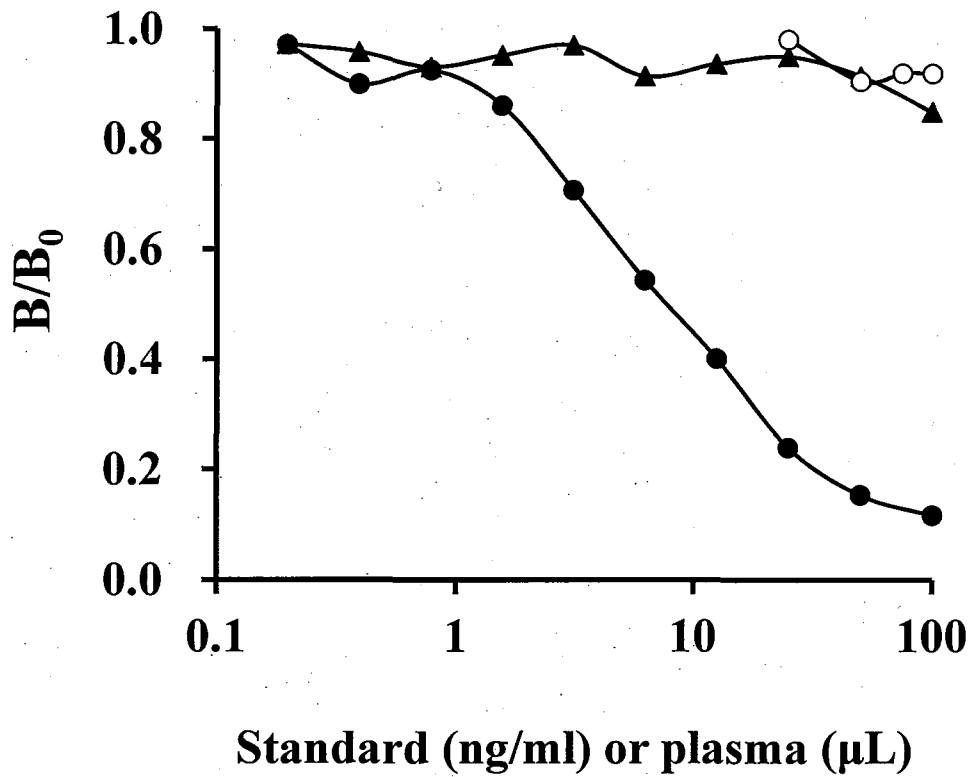


Figure 21. Specificity check of amylin antibody (first batch, guinea pig No. 1). Standard RIA curves for bovine amylin. Standard RIA curves for bovine amylin of <sup>125</sup>I-labeled amylin binding to guinea pig anti-amylin antiserum by serial dilution of bovine amylin (—●—) and human amylin (—▲—). Inhibition of <sup>125</sup>I-labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine amylin as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

Fig. 22

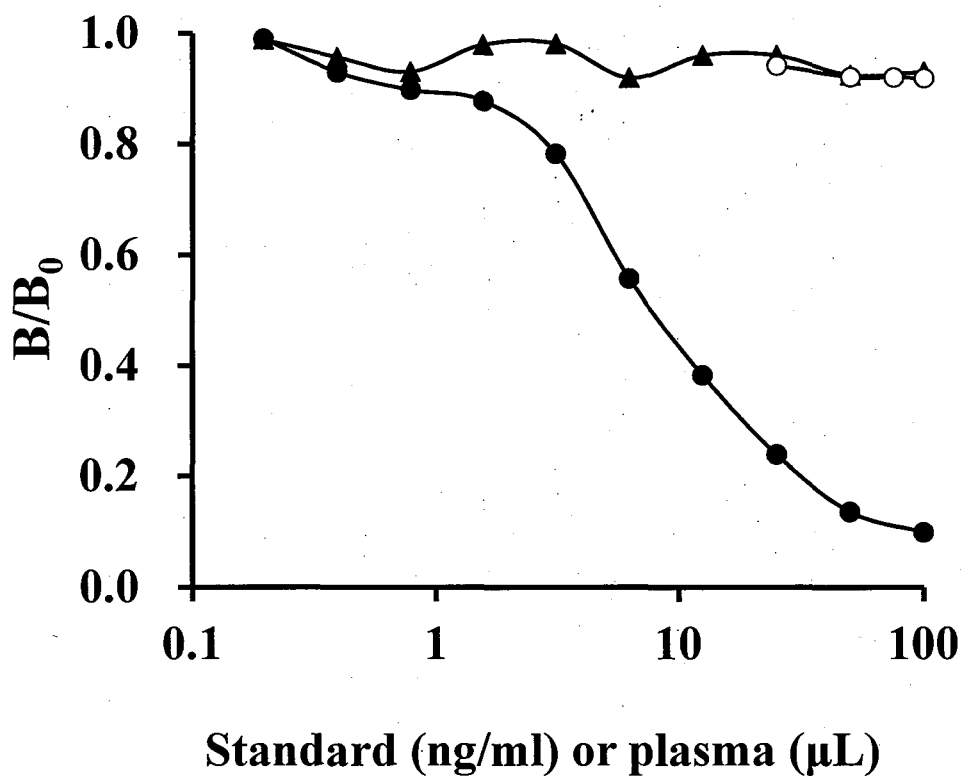


Figure 22. Specificity check of amylin antibody (first batch, guinea pig No. 2). Standard RIA curves for bovine amylin of <sup>125</sup>I-labeled amylin binding to guinea pig anti-amylin antiserum by serial dilution of bovine amylin (—●—) and human amylin (—▲—). Inhibition of <sup>125</sup>I-labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine amylin as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.



Fig. 23

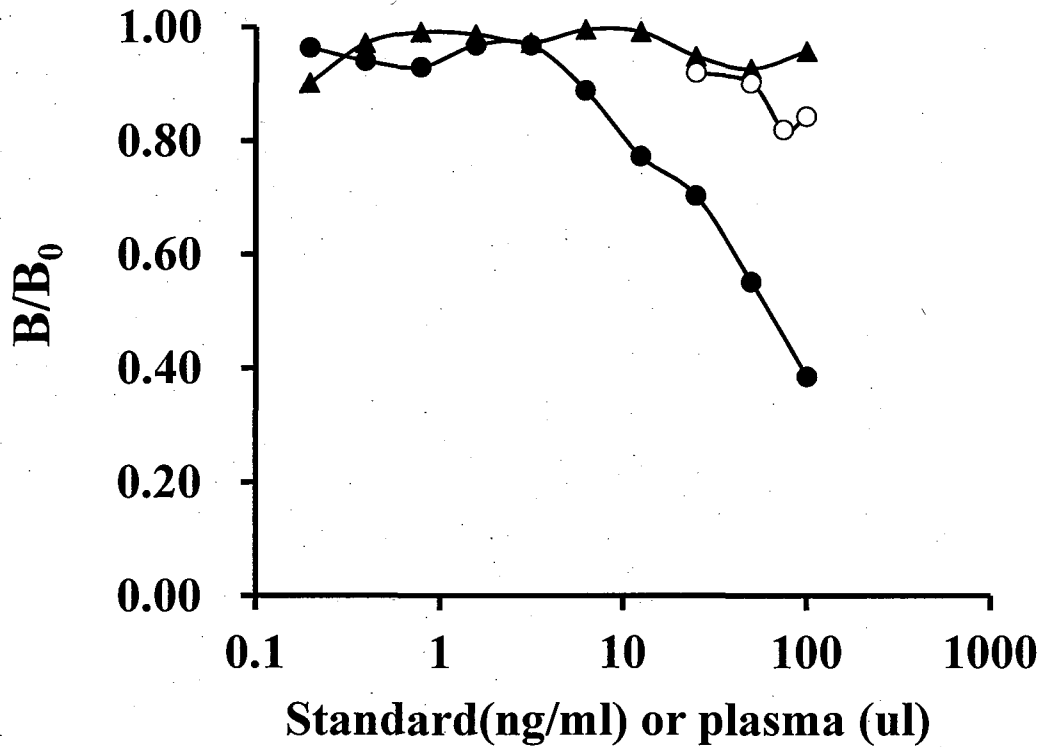


Figure 23. Specificity check of amylin antibody (second batch, guinea pig No. 2). Standard RIA curves for bovine amylin. Inhibition of <sup>125</sup>I-labeled amylin binding to guinea pig anti-amylin antiserum by serial dilution of bovine amylin (—●—) and human amylin (—▲—). Inhibition of <sup>125</sup>I-labeled amylin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine amylin as standard. Each point is the mean of triplicate determinations. B/B<sub>0</sub>, bound/bound in zero standard.

## 2.4 Decanoyl ghrelin (D-ghrelin) radioimmunoassay

Decanoyl ghrelin (D-ghrelin) is acylated with decanoic acids at N-terminal serine 3 amino acid residue (Hiejima et al. 2009). The amino acid sequence of octanoyl ghrelin, decanoyl ghrelin and des-acyl ghrelin are similar only different of acylation at serine 3 residue. Serine 3 residue of O-ghrelin is modified with fatty acids of (C8:0) an 8-carbon chain containing no double bond (Kojima and Kangawa 2005) and D-ghrelin is modified with fatty acids of (C10:0) a 10-carbon chain lacking double bonds and decenoyl ghrelin (C10:1) a 10-carbon chain containing with one double bond (Hosoda et al. 2004). There is no information about D-ghrelin in ruminant. We suggest that D-ghrelin may present in cattle.

### 2.4.1 Solid phase peptide synthesis of D-ghrelin

We synthesized O-ghrelin, des-acyl ghrelin and D-ghrelin. The procedures of peptide synthesis for O-ghrelin and des-acyl ghrelin are same with ThidarMyint et al. (2006) (Table-15). Peptide synthesis of D-ghrelin is similar to O-ghrelin, only different in acylation of serine 3 residue with octanoic fatty acid and decanoic fatty acid.

Decanoyl ghrelin was synthesized using Wang resin (p-Benzyloxybenzyl alcohol resin, 200- 400 mesh eq. to 75- 38  $\mu\text{m}$  in diameter). Side chain protected Fmoc-amino acids; Fmoc-Arg(Pbf)-OH, Fmoc-Glu(otBu), Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH and Boc-Gly were used. Fmoc-Ser-OH was chosed for Ser<sup>3</sup> because it needed acylation with n-decanoic acid. Acylation procedure was done repeatedly 3 times. The synthesized peptide was purified by RP-HPLC, semi-preparative column: TSK-GEL ODS-120A, pore size 10 $\mu\text{m}$ , 7.8 mm I.D  $\times$  30 cm, TOSOH). After purification, peptides were lyophilized and stored at -30°C.

**Table (15) Synthesized peptides of ghrelin**

No.	Name of Synthesized Peptides	Amino acids sequence of peptides	Accession Number
1	Bovine des-acyl ghrelin	<sup>1</sup> GSSFLSPEHQ <sup>10</sup> KLQRKEAKKP <sup>20</sup> SGRLKPR <sup>27</sup>	Q6SLG7
2	Bovine (O-ghrelin)	◆ <sup>1</sup> GSSFLSPEHQ <sup>10</sup> KLQRKEAKKP <sup>20</sup> SGRLKPR <sup>27</sup>	Q6SLG7
3	Bovine (D-ghrelin) Decanoyl	• <sup>1</sup> GSSFLSPEHQ <sup>10</sup> KLQRKEAKKP <sup>20</sup> SGRLKPR <sup>27</sup>	
4	Bovine (D-ghrelin) Decenoyl	• <sup>1</sup> GSSFLSPEHQ <sup>10</sup> KLQRKEAKKP <sup>20</sup> SGRLKPR <sup>27</sup>	

- ◆ Acylation with octanoic acid,
- Acylation with decanoic acid

#### 2.4.2 First antibody production of D-ghrelin

We generated first antibody production in 2 guinea pigs and 2 rabbits. Synthesized bovine (Cys<sup>0</sup>)-D-ghrelin was conjugated with mCKLH. The hepten-conjugated antigen was emulsified with same volume of Freund's adjuvant (FCA, Wako # 014- 09541 ) for immunization. In rabbit, 1 mg of conjugated D-ghrelin was used for each injection and animals were subcutaneously injected 7 times with two weeks interval. In guinea pig, 0.28 mg of conjugated D-ghrelin in 250 µL of phosphate buffer was used for each injection and animals were subcutaneously injected 7 times with one week interval. The whole blood was collected at the following day of final (7<sup>th</sup> time) antigen injection. During immunization, one guinea was died at 25 days after

initial antigen injection. The collected blood is stored at 4°C for 24 hours and then centrifuges and collects serum. The collected serum was kept in -30°C. We do not get antibody of D-ghrelin generated in 2 rabbits.

#### **2.4.3 Labeling of D-ghrelin**

We labeled synthesized (Tyr<sup>0</sup>)-bovine D-ghrelin with chloramine T-method. Labeled peptide was purified with HPLC. The peak fraction tube (400 µL) was collected and mixed with 400 µL of 60% CH<sub>3</sub>CH/ 0.1% TFA for neutralization. Purified I<sup>125</sup> labeled peptide was used as tracer in first antibody titer check, first antibody dilution check, standard check and specificity check.

#### **2.4.4 Sensitivity/titer check of D-ghrelin first antibody**

We checked the titer of D-ghrelin antibody by RIA. The assay was prepared at room temperature. Firstly, we put 200 µl of assay buffer into the polystyrene tube. After that, we prepared first antibody contained serum at 1:500 final dilution in ghrelin assay buffer (0.05 M phosphate, 0.01 M EDTA, 0.08 % sodium azide, 0.1% gelatin, 0.25 % BSA: pH 6.85) and added to the tubes which contain buffer. Then, we add the tracer (about 8000 CPM) which was prepared in assay buffer contained 1% normal guinea pig serum. Vortex mix and incubate for 24 hours. After incubation, we add 1 ml of precipitation buffer (0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4 ) and incubate again for 30 min. Then, centrifuge and count CPM of pellet. We checked serum samples (from before immunization to 46 days after immunization) of 2 guinea pigs for titer/sensitivity. The highest binding of D-ghrelin first antibody with tracer is 26 % for guinea pig 1 and 51 % in guinea pig-2 (Table-16, Fig. 24).

**Table (16)** Titer/sensitivity check for guinea pig No.1 and No.2 anti-D-ghrelin serum

<b>Guinea Pig Number</b>	<b>Days after initial immunization</b>	<b>Bound/Total (B/T)</b>
1	0	0
1	22	0.25
1	25	0.26
2	0	0
2	22	0.43
2	25	0.46
2	28	0.51
2	36	0.45
2	39	0.44
2	42	0.47
2	46	0.42

Fig. 24

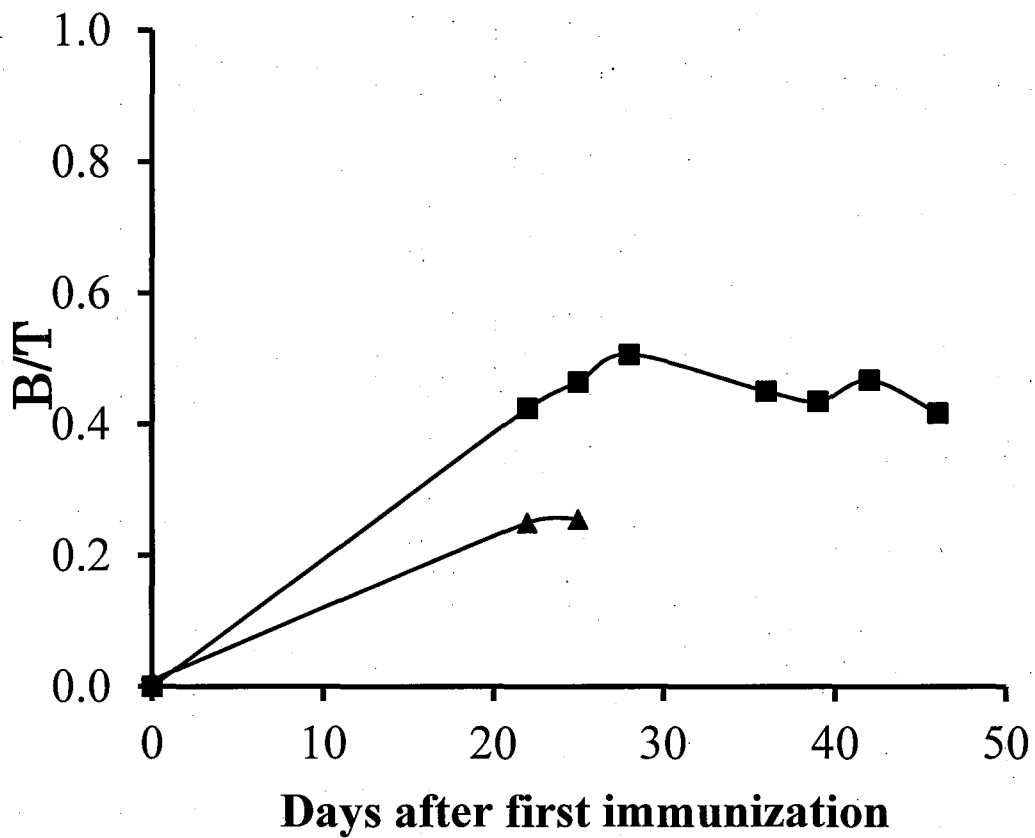


Figure 24. Sensitivity/titer check for guinea pig 1 (-▲-) and guinea pig 2 (-■-) anti-bovine D-ghrelin serum. The  $^{125}\text{I}$ -labeled D-ghrelin binding to guinea pig anti-D-ghrelin antiserum, by serial dilution of D-ghrelin (-●-). Inhibition of  $^{125}\text{I}$ -labeled GHRH binding to antiserum by serial dilution of pooled bovine plasma (-○-) was obtained using control bovine D-ghrelin as standard. Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

#### 2.4.5 Sensitivity/titer dilution check of bovine D-ghrelin antibody

In first antibody (guinea pig anti-bovine D-ghrelin serum) dilution check, we used the tracer with 10000 CPM and final dilution of first antibody from 1: 5000 to 1: 120000 (Table-17). The binding of first antibody of guinea pig 1 serum with tracer is very low, it's binding is only 4 % at 1:5000 final dilution (Table-17, Fig. 25). Therefore, we selected the first antibody of guinea pig 2 for radioimmunoassay.

**Table (17)** Dilution check of guinea pig No.1 and No.2 anti-D-ghrelin serum

<b>First antibody final dilution</b>	<b>Guinea Pig no. 1 (B/T)</b>	<b>Guinea Pig no. 2 (B/T)</b>
0	0	0
5000	0.04	0.35
10000	0.03	0.3
15000	0.02	0.24
20000	0.02	0.20
30000	0.01	0.16
40000	0.01	0.13
60000	0.01	0.09
80000	0.01	0.08
120000	0.01	0.06

Fig.25

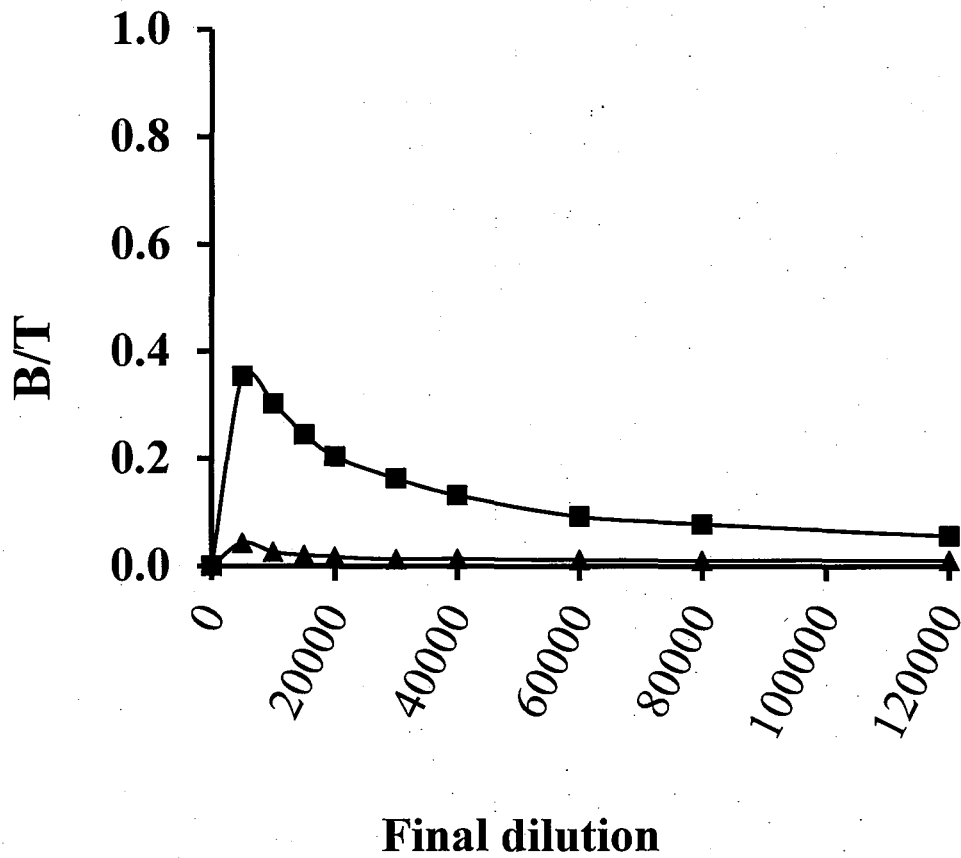


Figure 25. Sensitivity/titer dilution check for guinea pig 1 (-▲-) and guinea pig 2 (-■-) anti-bovine D-ghrelin serum. The  $^{125}\text{I}$ -labeled bovine D-ghrelin was binding to serial dilution of antiserum. Each point is the mean of quintuplicate determinations.



#### **2.4.6 D-ghrelin radioimmunoassay**

Standard concentrations of D-ghrelin ranged from 0.15 to 160 ng/mL was set up at room temperature. Standards and plasma samples were incubated with antibodies diluted in ghrelin assay buffer (0.05 M phosphate, 0.01 M EDTA, 0.08 % sodium azide, 0.1% gelatin, 0.25 % BSA: pH 6.85) for 24 hours at 4°C. After 24 hours incubation, <sup>125</sup>I-labeled D-ghrelin (8000 count per minute/100 µL assay buffer containing 1% normal guinea pig serum) was added to the tubes. The tubes were incubated again for 24 hours at 4°C. The next day, the second antibody (4% goat anti-guinea pig serum prepared in precipitation buffer, 0.05 M phosphate, 0.9 % saline, 0.025 M EDTA, 0.08 % sodium azide, 0.05% Triton X-100, 3 % PEG; pH 7.4) was added to the tubes, and the tubes were incubated for 30 min at 4°C. After incubation, bound and free antigens were separated by centrifugation for 30 min at 1870 ×g and 4°C, aspiration of all the supernatant (without touching the pellet) immediately follow centrifugation.

The radioactivity in the pellet was measured by gamma counter (ARC-1000, Aloka, Japan). Triplicate standards and duplicate plasma samples were run within in a single assay.

#### **2.4.7 Standard check and specificity check for first antibody of D-ghrelin**

We set up the standard of bovine D-ghrelin after dilution check of first antibody (anti-bovine D-ghrelin). We set up cold standard of D-ghrelin at room temperature. We used 1: 10000 final dilution of first antibody of guinea pig 1 and the tracer of 10000 CPM in the assay. We can set up D-ghrelin standard (Fig. 26). After that, we made specificity check of D-ghrelin first antibody. We prepared the dilution of standards (D-ghrelin, O-ghrelin, des-acyl ghrelin) from 0.15 ng/ mL to 160 ng/ mL with triplicate samples in this assay. In specificity check, we set up D-ghrelin standard, O-ghrelin (N-octanoyl ghrelin) standard and des-acyl ghrelin standard. We found that the antibody of D-ghrelin recognized to D-ghrelin, O-ghrelin and decenoyl ghrelin (Fig. 27). This antibody cannot use for measuring of plasma D-ghrelin with RIA in cattle.

Fig. 26

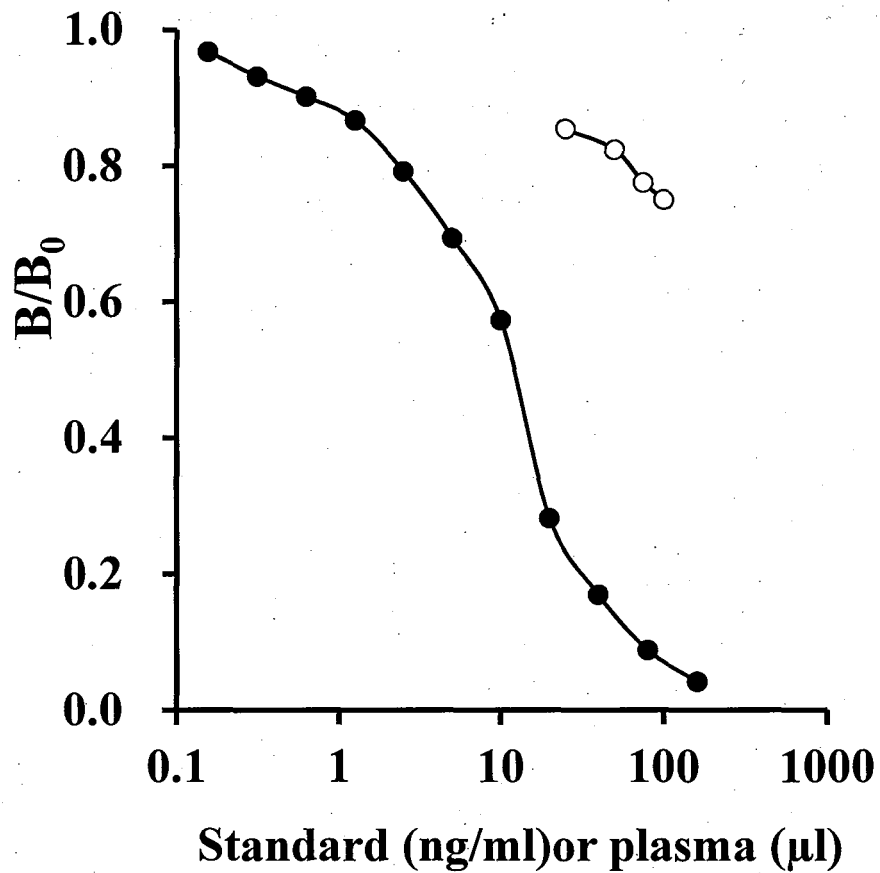


Figure 26. Standard RIA curve for D-ghrelin. Inhibition of  $^{125}\text{I}$ -labeled D-ghrelin binding to guinea pig anti-D-ghrelin antiserum by serial dilution of D-ghrelin (—●—). Inhibition of  $^{125}\text{I}$ -labeled D-ghrelin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control D-ghrelin as standard. Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

Fig. 27

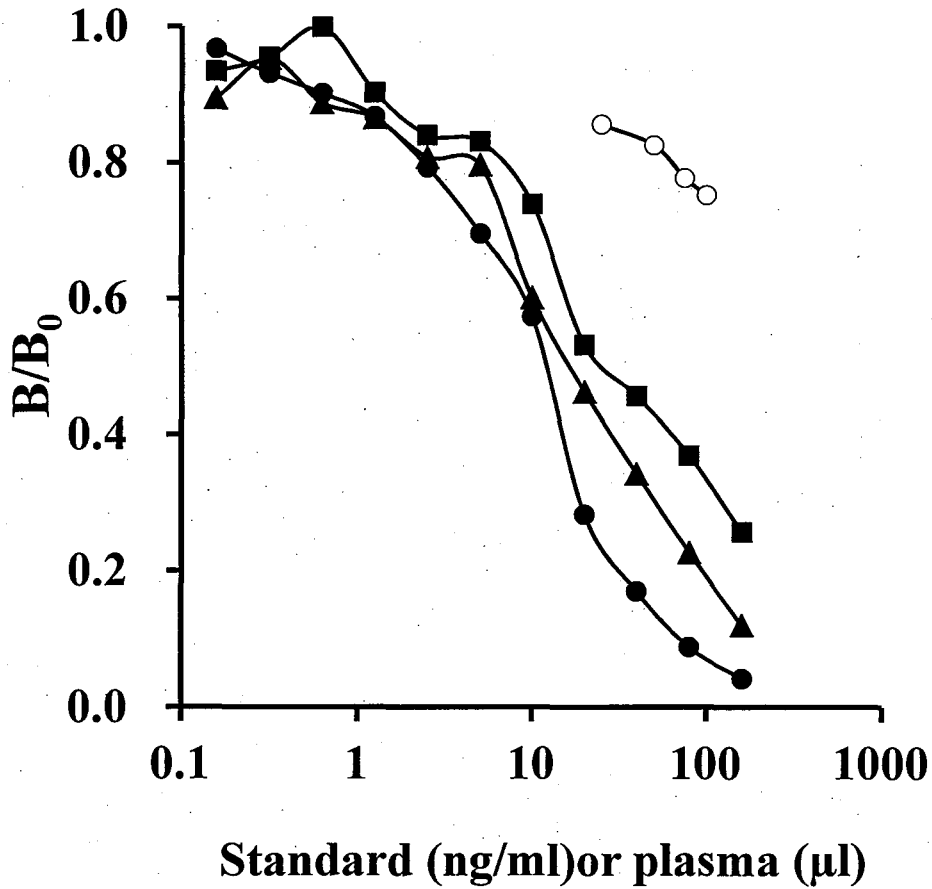


Figure 27. Specificity check of D-ghrelin antibody. Standard RIA curves for D-ghrelin (—●—), O-ghrelin (—▲—) and des-acyl ghrelin (—◻—). Inhibition of  $^{125}\text{I}$ -labeled D-ghrelin binding to guinea pig anti-bovine D-ghrelin by serial dilution of bovine D-ghrelin, bovine O-ghrelin and decenoyl ghrelin.  $^{125}\text{I}$ -labeled D-ghrelin binding to antiserum by serial dilution of pooled bovine plasma (—○—) was obtained using control bovine D-ghrelin as standards. Each point is the mean of triplicate determinations.  $B/B_0$ , bound/bound in zero standard.

## 2.5 Discussion

We produced first antibody of CCK which specifically recognized only sulfated CCK-8 and did not recognize to non-sulfated CCK-8, bovine sulfated gastrin-9 and bovine non-sulfated gastrin-9. We validated sulfated CCK radioimmunoassay which can measure sulfated CCK in un-extracted plasma.

It is well known that GHRH and ghrelin stimulate GH secretion in non-ruminant (Evans et al. 1985, Kojima et al. 1999) and ruminants (Hashizume et al. 2005, Itoh et al. 2005, ThidarMyint et al. 2006). GHRH binds to GHRH receptor and stimulates GH secretion (Lin-Su K and Wajnrajch MP 2002) and ghrelin binds to growth hormone secretagogue receptor -1a (GHSR-1a) and stimulates GH secretion (Kojima et al. 1999). In our experiment CCK stimulates ghrelin secretion in steers. However, CCK induced endogenous increased ghrelin does not stimulate GH secretion in steers. We recently reported that GHSR-1a receptor may not be involved in secretion of GH by gastrin, because administration of D-Lys<sup>3</sup>GHRP-6 (GHSR-1a receptor antagonist) did not affect GH secretion which stimulated by gastrin induced endogenous ghrelin in steers (Zhao et al. 2011). Therefore, CCK may not regulate GH secretion GHSR-1a receptor pathway and may regulate GHRH receptor pathway or other. It is possible that CCK may affect GHRH which stimulates GH secretion in ruminant. To study the effect of CCK on GHRH, we try to validate GHRH RIA system. There are 2 reports for plasma GHRH level in human. Basal level of plasma GHRH in human is  $11.8 \pm 1.1$  pg/ml (Foot et al. 1990) and  $20.5 \pm 6.5$  pg/ml (Zhang et al. 1991). Hypophysial portal concentration of GHRH in sheep is  $6.6 \pm 1.4$  pg/ml (Thomas et al. 1991) and in rat is approximately 200 to 800 pg/ml (Plotsky and Vale 1985). Basal plasma level of GHRH in human, sheep and rat are very low, only picogram level. We can set up GHRH RIA system, however we cannot measure plasma GHRH in cattle due to low sensitivity of our GHRH RIA system.

Amylin has inhibitory effect on GHRH-induced GH secretion in monogastric species (Netti et al. 1995). It is possible that CCK may stimulate plasma amylin

secretion and amylin may inhibit GH secretion in steers. To study the effect of CCK on plasma amylin secretions in bovine, we try to validate amylin RIA system. Basal plasma level of amylin in cat is 70 - 133 pmol/L (about 273- 519 pg/ml) (Lutz and Rand 1996). Basal plasma level of amylin in human is 11.9 +/- 3.5 ng/l (11.9 +/- 3.5 pg/ml) (Hartter et al. 1991). Although we can set up amylin RIA, we cannot measure plasma amylin in bovine. Because of low sensitivity of our amylin RIA system,

In our experiment (Chapter 3), CCK-8s stimulates acyl ghrelin and total ghrelin secretions in steers. It is possible that CCK-8s may stimulate plasma D-ghrelin in ruminant. To study the effect of CCK on D-ghrelin we try to validate D-ghrelin RIA system. There is some information of D-ghrelin in rats (Hiejima et al. 2009). However, there is no information about D-ghrelin in ruminants. We can produce D-ghrelin first antibody and can set up standard. The first antibody of D-ghrelin recognize to D-ghrelin, O-ghrelin and decenoyl ghrelin. Therefore, this first antibody cannot use for measuring of bovine D-ghrelin, because it cannot differentiate D-ghrelin from other acyl forms.



## Chapter 3

### **Sulfated cholecystokinin-8 increases ghrelin and pancreatic polypeptide secretions but does not affect oxyntomodulin and glucagon like peptide-1 in Holstein steers.**

#### **3.1 ABSTRACT**

The effect of appetite regulatory hormone cholecystokinin (CCK) on the secretions of oxyntomodulin (OXM), ghrelin, pancreatic polypeptide (PP) and glucagon like peptide-1 (GLP-1), and the effect of ghrelin on the secretions of CCK, OXM, PP and GLP-1 were studied in ruminants. Eight Holstein steers, 7 months old, 243±7 kg body weight (BW), were arranged to an incomplete Latin square design (8 animals × 4 treatments × 4 days of sampling). Steers were intravenously injected 10 µg of sulfated CCK-8/kg BW, 20 µg of acyl ghrelin/kg BW, 100 µg of des-acyl ghrelin/kg BW or vehicle. Blood samples were collected from - 60 min to 120 min relative to time of injection. Plasma concentrations of ghrelin, sulfated CCK and OXM were measured by double-antibody radioimmunoassay. Plasma acyl ghrelin was increased to peak level (428.3± 6 pg/mL) at 60 min after injection of sulfated CCK-8 compared with pre-injected levels (203.3±1 pg/mL). These results showed for the first time, that intravenous bolus injection of CCK increased ghrelin secretion in ruminants. In contrast, injections of acyl ghrelin and des-acyl ghrelin did not change CCK, OXM, PP and GLP-1 secretions. Intravenous injection of sulfated CCK-8 increases plasma PP, glucose and NEFA levels, however does not affect plasma GLP-1 and GH levels. In conclusion, our results show that administration of CCK increases ghrelin and PP secretions but does not affect OXM and GLP-1 release in cattle. Ghrelin did not affect on the secretions of CCK, OXM, PP and GLP-1 in steers.

Key words: cholecystokinin, ghrelin, oxyntomodulin, pancreatic polypeptide, glucagon like peptide-1, ruminant.

### 3.2 INTRODUCTION

Anorexigenic hormone cholecystokinin (CCK) is mainly secreted from intestinal I-cells (Buchan et al. 1978). It is also secreted from the hypothalamus (Micevych et al. 1984). Another anorexigenic hormone oxyntomodulin (OXM) is secreted from intestinal L-cells (Bataille et al. 1982). Orexigenic hormone ghrelin is mainly secreted from X/A like cells of stomach (de la Cour et al. 2001, Kojima and Kangawa 2005). In ruminants, ghrelin is mainly secreted from abomasum (Hayashida et al. 2001). The apical surfaces of intestinal I-cells (Buchan et al. 1978) and L-cells (Deacon 2005) are exposed to the lumen of the intestine. These cells are likely to sense food in the intestinal lumen, and release CCK and OXM into the circulation after ingestion (Anini et al. 1999, Liddle 1994). However, ghrelin cells are not able to sense food in the lumen of the stomach because nutrients constrained within the stomach do not affect ghrelin levels (Williams et al. 2003). Therefore, postprandial change of ghrelin is not a direct stimulatory effect of ingested food. Plasma concentrations of CCK and OXM increased after eating in monogastric animals and humans (Hornnes et al. 1980, Lilja et al. 1984). In human, postprandial ghrelin concentration decreased after eating (Kojima and Kangawa 2005). The regulatory mechanisms for secretions of CCK, OXM and ghrelin are still need to clarify.

After clarifying the regulatory effects of CCK, OXM and ghrelin on food intake, gastric acid secretion and body weight, the interest of research is shifted to investigate the counter regulatory effects among anorexigenic and orexigenic hormones. CCK and ghrelin exert opposite effect on intestinal motility, gastric acid secretion, body weight and food intake (Rehfeld 2004, Kojima and Kangawa 2005). In rats, pre-administration of CCK blocked orexigenic effect of ghrelin (Date et al. 2005, Kobelt et al. 2005) and pre-administration of ghrelin blocked anorexigenic effect of CCK (Date et al. 2005). In humans, intra-duodenal infusion of fat increased plasma concentration of CCK and observed CCK induced suppression of ghrelin (Degen et al. 2007). Moreover, in fasted human, intravenous infusion of CCK decreased plasma ghrelin (Brennan et al. 2007, Brennan et al. 2008). However, some studies reported that exogenous CCK increased



ghrelin in fasted rodents (Murakami et al. 2002, Friis-Hansen et al. 2005, Shrestha et al. 2009). Brennan et al. (2007) and Shrestha et al. (2009) did not describe the form (sulfated CCK-8 or non-sulfated CCK-8) of injected CCK in their studies. Their experiments were done in fasted humans and rodents. Plasma concentrations of ghrelin in fasted humans, rats and ruminants are higher than non-fasted humans and animals (Kojima and Kangawa 2005, Wertz-Lutz et al. 2006). In addition, plasma CCK levels of fasted rats (Kanayama and Liddle 1991), 3-day-old lambs (Nowak et al. 1997), humans (Ohgo et al. 1988) and cows (Samuelsson et al. 1996) were low. Therefore, basal plasma ghrelin and CCK levels between fasted condition and non-fasted condition are different. There is few report on the regulatory effect of CCK on ghrelin secretion in non-fasted (normal physiological condition) monogastric animals and ruminants. Some scientists reported that administration of ghrelin increased CCK release in rats (Jawork 2006, Nawrot-Porabka et al. 2007). According to our knowledge, however, there is no report on the effect of ghrelin on the secretion of CCK in non-fasted ruminants. We injected sulfated CCK-8 into steers to clarify the effect of CCK on ghrelin secretion.

In rats and humans, it is well established that OXM and ghrelin exert opposite effects on food intake (Cohen et al. 2003, Dakin et al. 2004, Kojima and Kangawa 2005), gastric acid secretion (Dubrasquet et al. 1982, Kojima and Kangawa 2005) and body weight (Dakin et al. 2004, Kojima and Kangawa 2005). On the other hand, the inhibitory effect of OXM on ghrelin release in humans (Cohen et al. 2003) and rats (Dakin et al. 2004, Patterson et al. 2009) has been reported. We have recently reported that administration of OXM did not change plasma concentration of ghrelin in non-fasted (normal physiological condition) pre-ruminant and ruminant calves (ThanThan et al. 2010). However, there is still limited information on the effect of ghrelin on the secretions of CCK and OXM, and the effect of CCK on the secretion of OXM in ruminants.

Another anorexigenic hormone pancreatic polypeptide (PP) is primarily released by cells of the islets of Langerhans (Adrian et al. 1976). It is also secreted by the

exocrine pancreas and distal gastrointestinal tract (Larsson et al. 1975). Plasma level of pancreatic polypeptide increases after eating in human (Adrian et al. 1976). Scientists reported stimulatory effect of CCK on PP secretion. Administration of CCK stimulates PP in mice (Ahrén et al. 1991, 1995), human (Schmid et al. 1989) and dog (Parks et al. 1979, Schusdziarra et al. 1986). Stimulated CCK cause a substantial rise in PP in peripheral blood of dog, and the mechanism of PP release may involve CCK (Lonovics et al. 1981). In addition, Choi et al. (2000) reported that supplement fatty acids feeding induced endogenous increased CCK stimulates PP secretion in dairy cow. Moreover, scientists reported that administration of ghrelin increased plasma PP release in humans (Arosio et al. 2003, Tack et al. 2006) and rat (Sato et al. 2003). However, there is limited information for the effects of CCK and ghrelin on PP secretions in cattle.

Moreover, another anorexigenic hormone glucagon like peptide-1 (GLP-1) is released from intestinal L-cells (Herrmann et al. 1995). Postprandial concentrations of GLP-1 increased in human (Herrmann et al. 1995) and after fatty acid supplement diet fed dairy cow (Relling et al. 2010). Scientists reported the controversial results for the effect of CCK on plasma GLP-1. Some scientists reported that CCK stimulates GLP-1 secretion in non-ruminant (Fieseler et al. 1995, Hansen and Holst 2002). However, some scientists reported that administration of CCK did not affect plasma GLP-1 in rat colon (Plaisancie et al. 1994), human (Ahrén et al. 2000) and sheep (Relling et al. 2011). According to my knowledge, there is few information about the effect of CCK on GLP-1 in cattle. GLP-1 has no effect on CCK in human (Gutzwiller et al. 1999). However, the effect of GLP-1 on CCK secretion in ruminant is still need to clarify. In addition, there is limited information for the effect of ghrelin and CCK on GLP-1 secretions in ruminant.

Most of the previous studies (Furuse et al. 1991; Mir et al. 2000; Relling and Raynolds 2007) used ethanol-extracted plasma samples for measuring of CCK in bovine but that method is not practical to measure over thousand numbers of serial samples. In this regard, we validated the in-house radioimmunoassay (RIA) system for the measurement of plasma CCK concentrations in un-extracted bovine plasma.

There are controversial results for the effect of CCK on plasma GH. Some scientists reported that CCK stimulates GH in humans (Calogero et al. 1993), pigs (Parrott et al. 1995) and rats (Matsumura et al. 1984). Karashima et al. (1984) reported that CCK decreased plasma GH in rats. Vijayan et al. (1979) reported that central administration of CCK increased plasma GH, however peripheral administration of CCK did not affect plasma GH in rats. Some scientists reported that administration of CCK did not affect plasma GH in sheep (Spencer et al. 1991, Della-fera and Baile 1985) and humans (Nair et al. 1984). According to my knowledge, there is no information about the effect CCK on plasma GH in cattle.

Ghrelin has direct effects on glucose metabolism, such as increasing hepatic glucose production and decreasing the glucose disposal rate (Sun et al. 2006, Vestergaard et al. 2008). In human, administration of ghrelin increased plasma glucose and NEFA (Vestergaard et al. 2008). However, the regulatory effect of ghrelin on plasma glucose is varied in cattle (Itoh et al. 2006). Ghrelin stimulates plasma NEFA levels in human (Vestergaard et al. 2008) and cattle (ThidarMyint et al. 2006). Another appetite regulatory hormone CCK has regulatory effect on plasma glucose and NEFA levels. In rats, intra-duodenal administration of CCK decreases glucose production is independent of changes in circulating insulin (Cheung et al. 2009). Central administration of CCK has no effect on plasma glucose in sheep (Della-Fera et al. 1985). In addition, intravenous infusion of CCK did not affect plasma glucose and NEFA in wethers (Relling et al. 2010, 2011). There is few information about the effect of CCK on plasma glucose and NEFA in cattle.

The objective of this experiment is to study the effect of CCK on the secretions of ghrelin, OXM and PP, and the effect of ghrelin on the secretions of CCK, OXM and PP in ruminants. In addition, we study the effect of CCK on plasma GH, glucose and NEFA levels in steers.

### **3.3 MATERIALS AND METHODS**

All animal procedures were approved by the Institutional Animal Experimentation

Ethics Committees of Obihiro University of Agriculture and Veterinary Medicine, Japan.

### **3.3.1 Animals**

Eight Holstein steers, 7 months old (castrated at 3 months old),  $243 \pm 7$  kg BW, were housed under a natural light-darkness cycle and fed 3 kg of concentrate (crude protein 14.5%, crude fat 2%, crude fiber 10% and crude ash 9%, Nisshin Marubeni, Japan) mixed with 90 g of calcium salts of fatty acids (Nichiyu Solution Inc, Japan) per head per day, twice daily (at 09.00 and 16.00 hours). The amount of feed (concentrate) is calculated by the nutrient requirements of dairy cattle (NRC, seventh revised edition, 2001) and it is enough for energy requirement of steers. Timothy hay and water were supplied *ad libitum* during experiment. One day before experiment, body weight was measured and a catheter was inserted into the external jugular vein of each steer. The patency of catheter was maintained with heparinized saline (10 IU heparin/mL saline). To get the minimum residual effect of hormone injection, a recovery period of 24 h was allowed. The steers were loosely chained to stanchion during hormone injection and blood sampling. Each steer received all treatments. At the end of experiment, steers were  $249 \pm 7$  kg BW.

### **3.3.2 Peptides**

Sulfated CCK-8 amide (chapter 2), bovine acyl ghrelin and des-acyl ghrelin (ThidarMyint et al. 2006) are synthesized by solid phase peptide synthesis. In this study, injection of acyl ghrelin increased plasma concentration of GH (Fig. 31) and injection of CCK increased plasma concentration of insulin (Fig.30). These results show that the synthesized peptides are biologically active.

### **3.3.3 Peptide injection and sample collection procedures**

Animals were arranged to an incomplete Latin square design (8 animals  $\times$  4 treatments  $\times$  4 days of sampling). Blood sampling and peptide injection were done from

the jugular catheter. Peptides for injection were freshly dissolved on the day of experiment. Acyl ghrelin and des-acyl ghrelin were dissolved in de-ionized water at a concentration of 10 mg/mL and 50 mg/mL, respectively. Lyophilized CCK-8 was dissolved in 1 mM NaHCO<sub>3</sub> at a concentration of 5 mg/mL. These dissolved peptides were diluted in 0.1% BSA saline to get a 5 mL of total injection volume. Steers were intravenously injected with 20 µg/kg of acyl ghrelin, 100 µg/kg of des-acyl ghrelin, 10 µg/kg of sulfated CCK-8 or vehicle (0.1% BSA saline as control). After hormone or vehicle injection, the catheter was immediately flushed with 5 mL of heparinized saline.

Blood samples were collected at - 60 (feeding), - 45, - 30, - 15, 0 (injection), 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min relative to time of injection. Plasma ghrelin levels increase before eating and then decrease to stable level after eating (Sugino et al. 2002; Kojima & Kangawa 2005). We want to study the effect of CCK on plasma ghrelin when ghrelin level is stable. Therefore, we injected peptides to steers 1 h after feeding of concentrate diet. Blood sampling for - 60 min was done at 1 min before feeding (feeding time is 09.00 hour). Steers consumed concentrate within 15 min. Blood samples were put into pre-ice-chilled heparinized tubes. All tubes were put on ice after sample collection until they were centrifuged for plasma collection. Plasma was isolated from blood by centrifugation (at 4 °C for 30 min at 1870 ×g) and stored at - 30 °C. For ghrelin assays, 600 µL of plasma was acidified with 30 µL of 1 M HCl and stored at - 30°C.

## **Measurement of plasma hormones**

### **3.3.4 RIA for ghrelin, OXM and GH**

Plasma concentrations of acyl ghrelin, total ghrelin, OXM and GH were measured by in-house double antibody RIA, as previously described (ThidarMyint et al. 2006; ThanThan et al. 2010, Lee et al. 2000). The average recovery rates of three known amounts of hormones added to bovine pooled plasma were 107%, 116%, 106% and 000% for acyl ghrelin, total ghrelin, OXM and GH respectively. Sensitivity of acyl

ghrelin, total ghrelin, OXM and GH assays were 17.11 pg/mL, 0.27 ng/mL, 1.10 ng/mL and 0.13 ng/mL respectively. The intra-assay coefficients of variation for acyl ghrelin, total ghrelin, OXM and GH were 8.6%, 11%, 9.1% and 9.3% respectively. The inter-assay coefficients of variation for acyl ghrelin, total ghrelin and OXM were 10%, 12.2% and 12.7%, respectively.

### **3.3.5 RIA for CCK**

Plasma level of sulfated CCK was measured by RIA (chapter 2). We prepared standard dilution from 0.019 ng/mL to 160 ng/mL. Triplicate standards and duplicate plasma samples were run within in a single assay. The average recovery rate of three known amounts of CCK-8 added to bovine pooled plasma was 92%. Sensitivity and the intra-assay coefficient of variation of this assay were 0.16 ng/mL and 6.6%, respectively.

The specific measurement of sulfated CCK in the plasma requires assays that recognize the C-terminal heptapeptide sequence [Y(SO<sub>3</sub>H)MGWMDF-NH<sub>2</sub>], irrespective of the N-terminal extension (Rehfeld 2004). The antibody should bind the active site of CCK without binding the homologous gastrin (Rehfeld 1998). The antibody used in our assays was specifically recognized sulfated CCK-8 amide and did not cross-react with non-sulfated CCK-8 amide, bovine gastrin-9 amide and bovine non-sulfated gastrin-9 amide (Fig. 9). Therefore, the results from our RIA system represent the concentrations of sulfated CCK.

### **3.3.6 RIA for pancreatic polypeptide (PP) and glucagon like peptide-1 (GLP-1)**

Plasma level of PP and GLP-1 were measured by RIA. Plasma GLP-1 was measured by inhouse double antibody RIA as previously described (ThanThan et al. 2011). We prepared standard dilution of PP from 0.039 ng/mL to 20 ng/mL, and standard dilution of GLP-1 from 0.19 ng/mL to 100 ng/mL. Triplicate standards and duplicate plasma samples were run within in a single assay. Sensitivity of PP and GLP-1 assays was 0.095 ng/mL and 0.6 ng/mL, and the intra-assay coefficient of variation of

assays were 12.6 % and 11.9 %, respectively.

### **3.3.7 Measurement of plasma metabolites**

Plasma levels of glucose and non-esterified fatty acids (NEFA) were measured by commercially available kits (Code nos 439-90901 and 276-75411 respectively).

### **3.3.8 Statistical analysis**

The results were expressed as means  $\pm$  SEM. Statistical differences between averaged concentrations of hormone before (at - 60, - 45, - 30, -15, and 0 min) and after injection at each time point were analyzed by repeated measure one way ANOVA. All data were analyzed by using of SPSS for windows, version 16.0, and  $P < 0.05$  was considered statistically significant.

## **3.4 RESULTS**

### **3.4.1 Effect of CCK-8s on ghrelin and insulin**

Plasma concentration of CCK was increased to peak level ( $26.2 \pm 4$  ng/mL,  $P < 0.05$ ) at 5 min following its administration and decreased to pre-injected level ( $0.3 \pm 0.1$  ng/mL,  $P < 0.05$ ) within 15 min (Fig. 28a). We did not find changes of CCK secretion after feeding (Fig. 28b).

Fig. 28 a

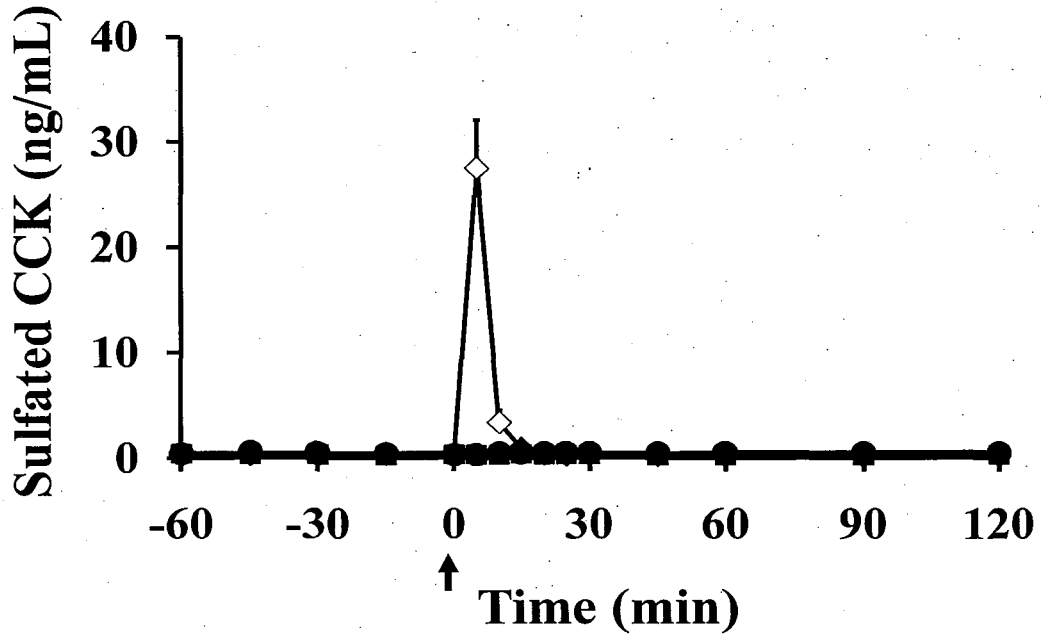


Figure 28 (a). Changes in plasma sulfated CCK concentration in response to sulfated CCK-8 and vehicle in Holstein steers. Animals were intravenously injected sulfated CCK-8 (10  $\mu\text{g}/\text{kg}$  BW,  $-\diamond-$ ), acyl ghrelin (20  $\mu\text{g}/\text{kg}$  BW,  $-\triangle-$ ), des-acyl ghrelin (100  $\mu\text{g}/\text{kg}$  BW,  $-\square-$ ) or vehicle (0.1% BSA saline,  $-\bullet-$ ). The arrow indicates the time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).



Fig. 28b

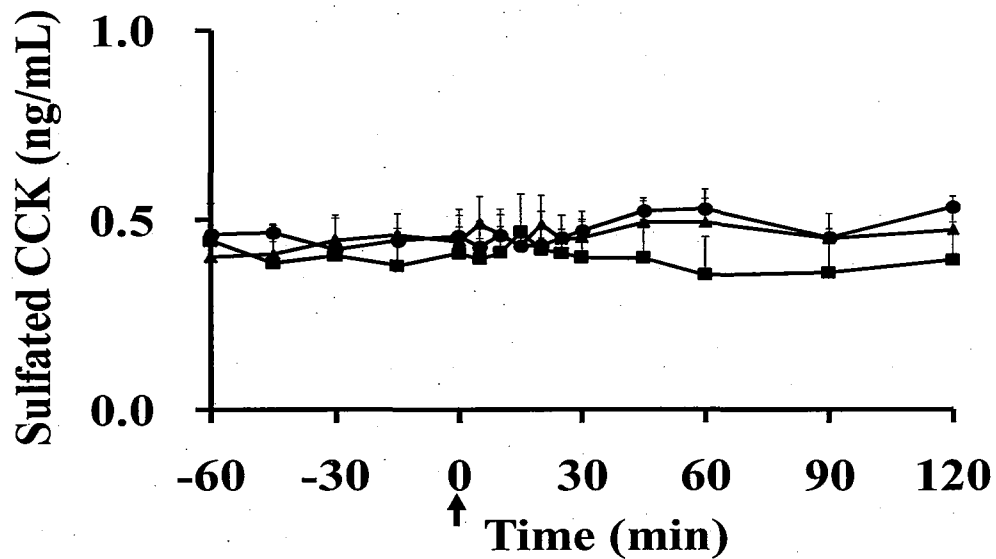


Figure 28 (b). Changes in plasma sulfated CCK concentration in response to acyl ghrelin, des-acyl ghrelin and vehicle in Holstein steers. Animals were intravenously injected sulfated CCK-8 (10 µg/kg BW, -◆-), acyl ghrelin (20 µg/kg BW, -▲-), des-acyl ghrelin (100 µg/kg BW, -■-) or vehicle (0.1% BSA saline, -●-). The arrow indicates the time of injection. Values are the means ± S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference (P<0.05) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.2 Effect of CCK-8s on acyl ghrelin

The response of acyl ghrelin concentrations to exogenous CCK-8 is shown in Fig. 29a. Significantly higher concentration of acyl ghrelin ( $295.9 \pm 32$  pg/mL,  $P < 0.05$ ) was seen at 20 min after injection of 10  $\mu$ g/kg of CCK compared with pre-injected levels ( $203.3 \pm 11$  pg/mL,  $P < 0.05$ ). Elevated plasma acyl ghrelin concentration reached to peak level ( $428.3 \pm 60$  pg/mL,  $P < 0.05$ ) at 60 min after injection of CCK and decreased to pre-injected level within 90 min post-injection. This peak value was approximately 2 times higher than pre-injected level. Vehicle injection did not change the concentration of acyl ghrelin.

Fig. 29 a

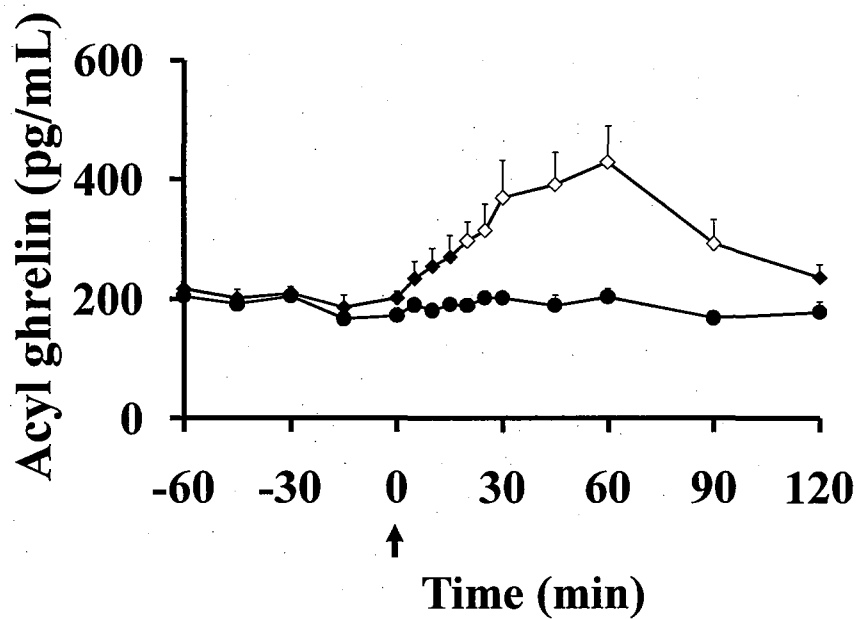


Figure 29 (a). Changes in plasma concentrations of acyl ghrelin in response to sulfated CCK-8. Animals were intravenously injected sulfated CCK-8 (10  $\mu$ g/kg BW,  $\blacklozenge$ ) or vehicle (0.1% BSA saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.3 Effect of CCK-8s on total ghrelin

Similarly, administration of CCK also increased concentration of total ghrelin in plasma (Fig. 29b). Significantly higher concentration of total ghrelin ( $1.4 \pm 0.3$  ng/mL,  $P < 0.05$ ) was seen at 20 min after injection of CCK compared with pre-injected level ( $0.7 \pm 0.1$  ng/mL,  $P < 0.05$ ). The peak concentration of total ghrelin ( $1.5 \pm 0.3$  ng/mL,  $P < 0.05$ ) was seen at 30 min after injection of CCK. Vehicle injection did not change the concentration of total ghrelin.

Fig. 29 b

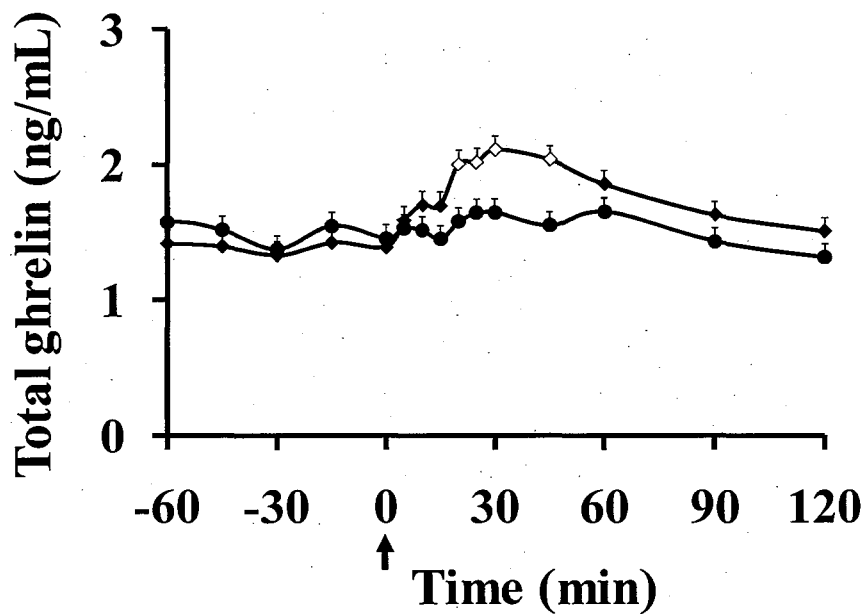


Figure 29 (b). Changes in plasma concentrations of total ghrelin in response to sulfated CCK-8. Animals were intravenously injected sulfated CCK-8 ( $10 \mu\text{g}/\text{kg}$  BW,  $\blacklozenge$ ) or vehicle ( $0.1\%$  BSA saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.4 Effect of CCK-8s on insulin

Injection of CCK increased plasma concentration of insulin in steers. The peak concentration of insulin ( $0.66 \pm 0.05$  ng/mL,  $P < 0.05$ ) was seen at 5 min after injection of CCK. Changes of plasma insulin feeding are not observed in steers (Fig. 30).

Fig. 30

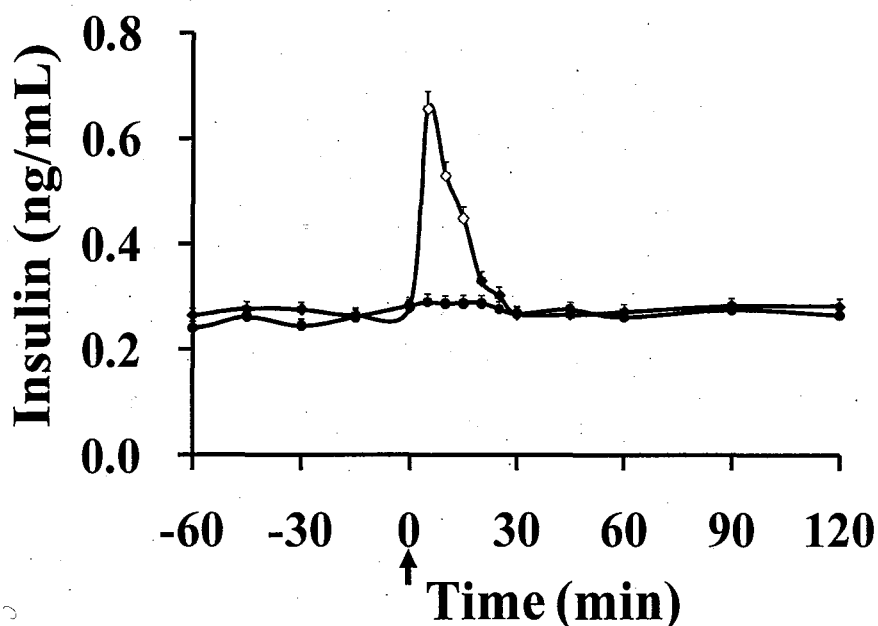


Figure 30. Changes in plasma insulin concentration in response to sulfated CCK-8 and vehicle in Holstein steers. Animals were intravenously injected sulfated CCK-8 ( $10 \mu\text{g/kg BW}$ ,  $-\diamond-$ ) or vehicle ( $0.1\%$  BSA saline,  $-\bullet-$ ). The arrow indicates the time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for  $-60$  min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations ( $-60$  to  $0$  min).

### 3.4.5 Effect of CCK-8s and ghrelin on GH

The response of plasma GH to exogenous ghrelin and CCK is shown in Fig. 31. Intravenous bolus injection of 10  $\mu\text{g}/\text{kg}$  of sulfated CCK-8, 100  $\mu\text{g}/\text{kg}$  of des-acyl ghrelin or vehicle injection did not change plasma concentration of GH. Administration of acyl ghrelin only increased plasma concentration of GH in steers (Fig. 31).

Fig. 31

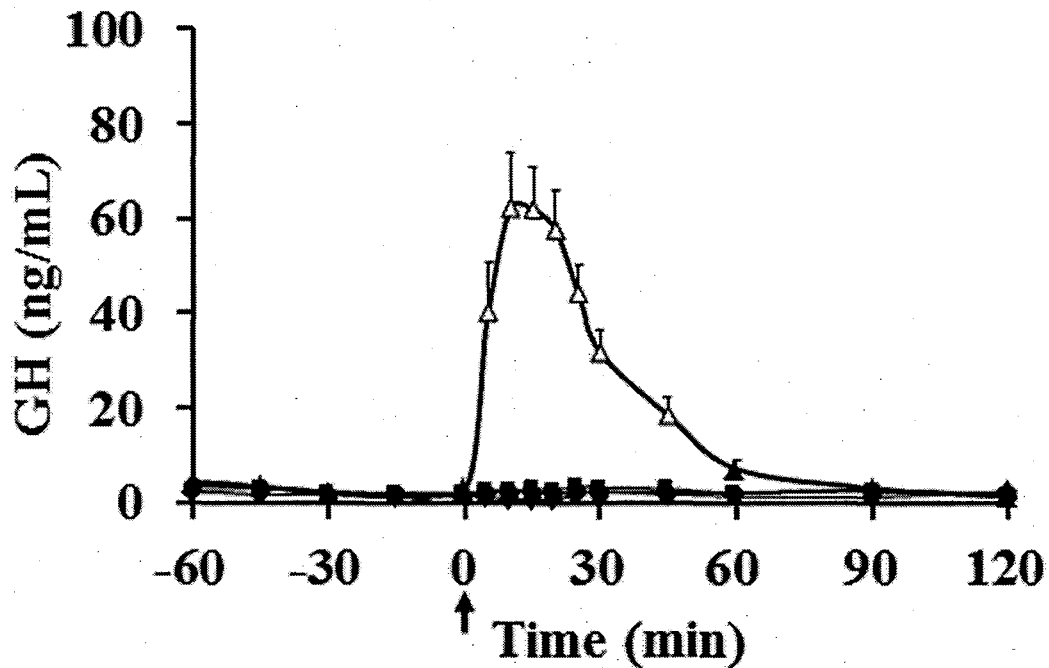


Figure 31. Changes in plasma concentration of growth hormone (GH) in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin (20  $\mu\text{g}/\text{kg}$  BW,  $\blacktriangle$ ), des-acyl ghrelin (100  $\mu\text{g}/\text{kg}$  BW,  $\blacksquare$ ), sulfated CCK-8 (10  $\mu\text{g}/\text{kg}$  BW,  $\blacklozenge$ ) or vehicle (0.1% BSA-saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.6 Effects of CCK-8s and ghrelin on OXM

Figure 32 shows the effects of exogenous CCK and ghrelin on OXM secretion in steers. Injection of 10  $\mu\text{g}/\text{kg}$  of CCK, 20  $\mu\text{g}/\text{kg}$  of acyl ghrelin, 100  $\mu\text{g}/\text{kg}$  of des-acyl ghrelin or vehicle injection did not change concentration of OXM in plasma. An average basal concentration of OXM in plasma was  $2.1 \pm 0.4$  ng/mL in the vehicle injected group. We did not find changes of OXM secretion after feeding (Fig. 32).

Fig. 32

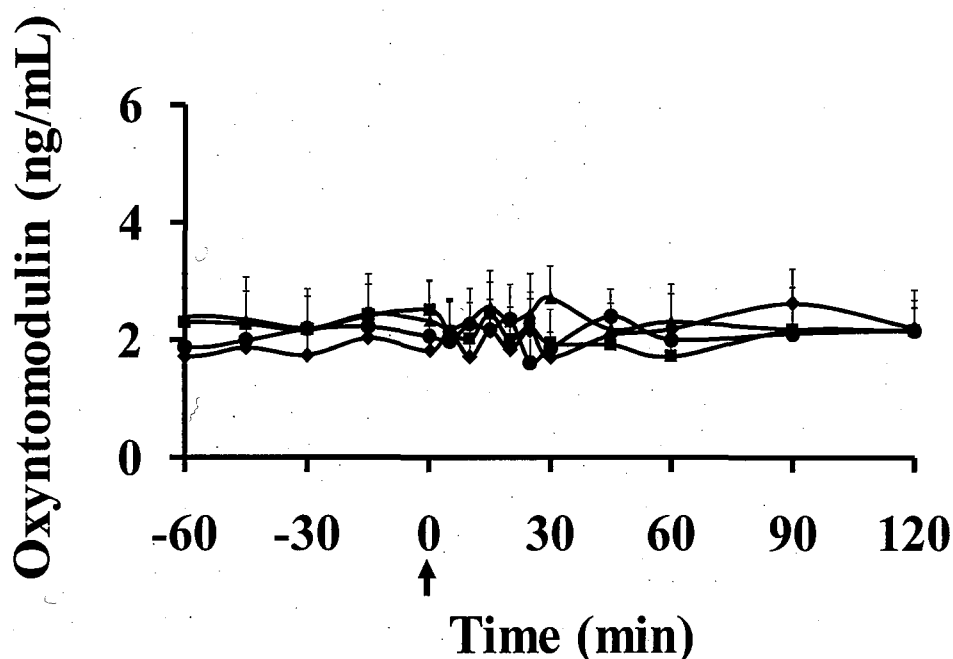


Figure 32. Changes in plasma concentration of oxyntomodulin in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin (20  $\mu\text{g}/\text{kg}$  BW,  $\blacktriangle$ ), des-acyl ghrelin (100  $\mu\text{g}/\text{kg}$  BW,  $\blacksquare$ ), sulfated CCK-8 (10  $\mu\text{g}/\text{kg}$  BW,  $\blacklozenge$ ) or vehicle (0.1% BSA-saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.7 The effect of CCK-8s and ghrelin on PP

Administration of sulfated CCK-8 increased plasma PP levels. The peak concentration of PP ( $0.5 \pm 0.08$  ng/mL,  $P < 0.05$ ) was seen at 5 min after injection of CCK compared the preinjected level ( $0.25 \pm 0.04$  ng/mL). We did not find changes of plasma PP levels by administration of acyl ghrelin and des-acyl ghrelin in steers (Fig. 33).

Fig.33

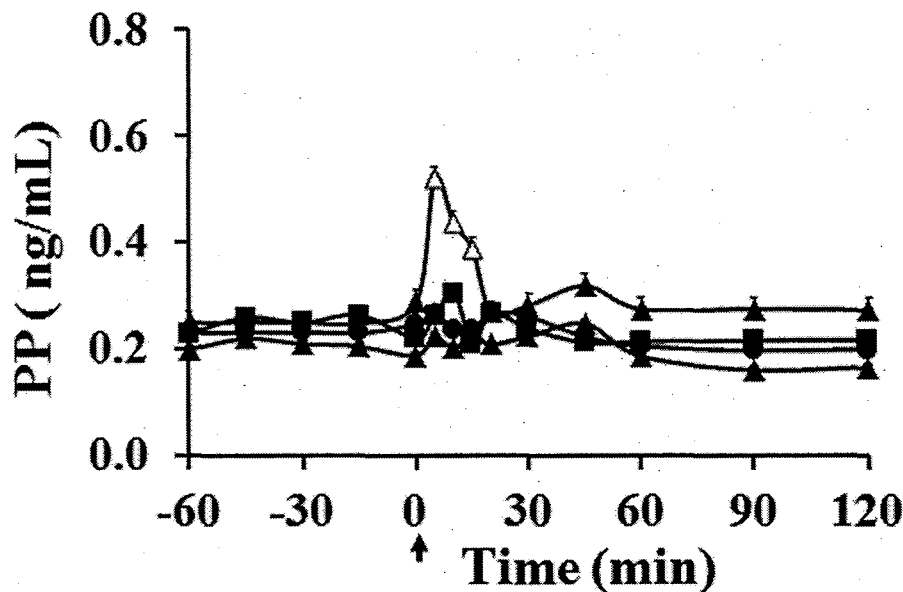


Figure 33. Changes in plasma concentration of PP in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin (20 µg/kg BW, -▲-), des-acyl ghrelin (100 µg/kg BW, -■-), sulfated CCK-8 (10 µg/kg BW, -◆-) or vehicle (0.1% BSA-saline, -●-). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).



### 3.4.8 The effect of CCK-8s and ghrelin on GLP-1

Administrations of sulfated CCK-8, acyl ghrelin and des-acyl ghrelin did not affect plasma GLP-1 levels in steers (Fig. 34).

Fig.34

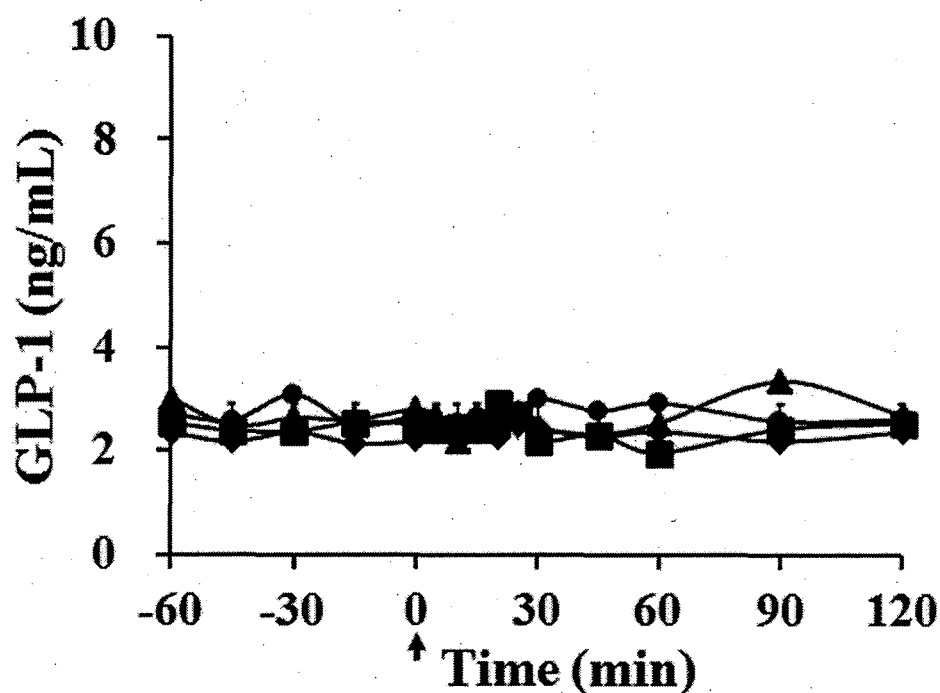


Figure 34. Changes in plasma concentration of GLP-1 in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin (20  $\mu\text{g}/\text{kg}$  BW,  $\blacktriangle$ ), des-acyl ghrelin (100  $\mu\text{g}/\text{kg}$  BW,  $\blacksquare$ ), sulfated CCK-8 (10  $\mu\text{g}/\text{kg}$  BW,  $\blacklozenge$ ) or vehicle (0.1% BSA-saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.9 The effect of CCK-8s on glucose

Administration of sulfated CCK-8 increased plasma glucose levels. The peak concentration of glucose ( $136 \pm 6$  mg/dL,  $P < 0.05$ ) was seen at 5 min after injection of CCK. We did not find changes of plasma glucose levels by administration of acyl ghrelin and des-acyl ghrelin in steers (Fig. 35).

Fig.35

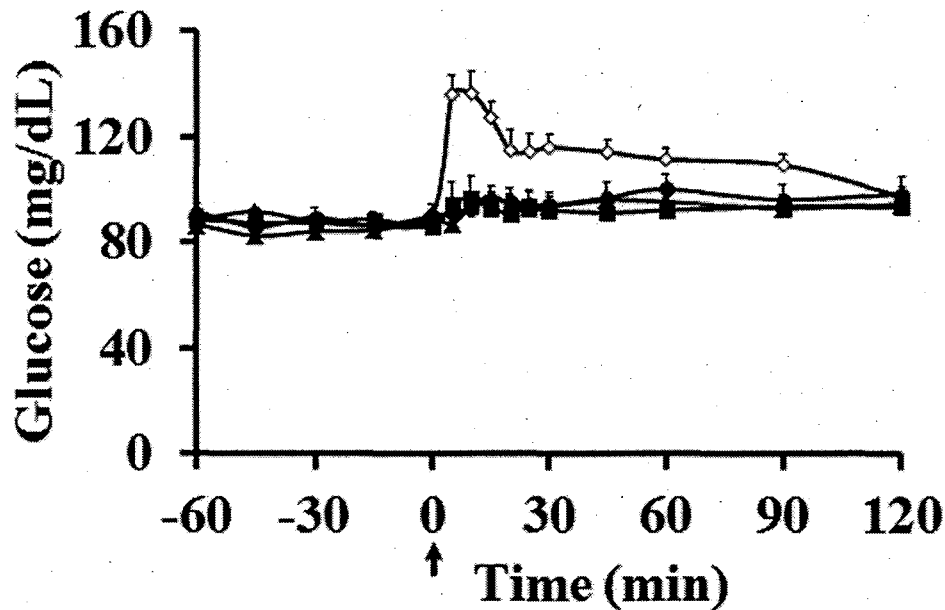


Figure 35. Changes in plasma concentration of glucose in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin (20 µg/kg BW, -▲-), des-acyl ghrelin (100 µg/kg BW, -■-), sulfated CCK-8 (10 µg/kg BW, -◆-) or vehicle (0.1% BSA-saline, -●-). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.4.10 The effect of CCK-8s on NEFA

Administration of sulfated CCK-8 and acyl ghrelin increased plasma NEFA levels in steers. The peak concentration of NEFA ( $256 \pm 31 \mu\text{Eq/L}$ ,  $P < 0.05$ ) was seen at 5 min after injection of CCK. Acyl ghrelin stimulates plasma NEFA levels however des-acyl has no effect on plasma NEFA levels (Fig. 36).

Fig. 36

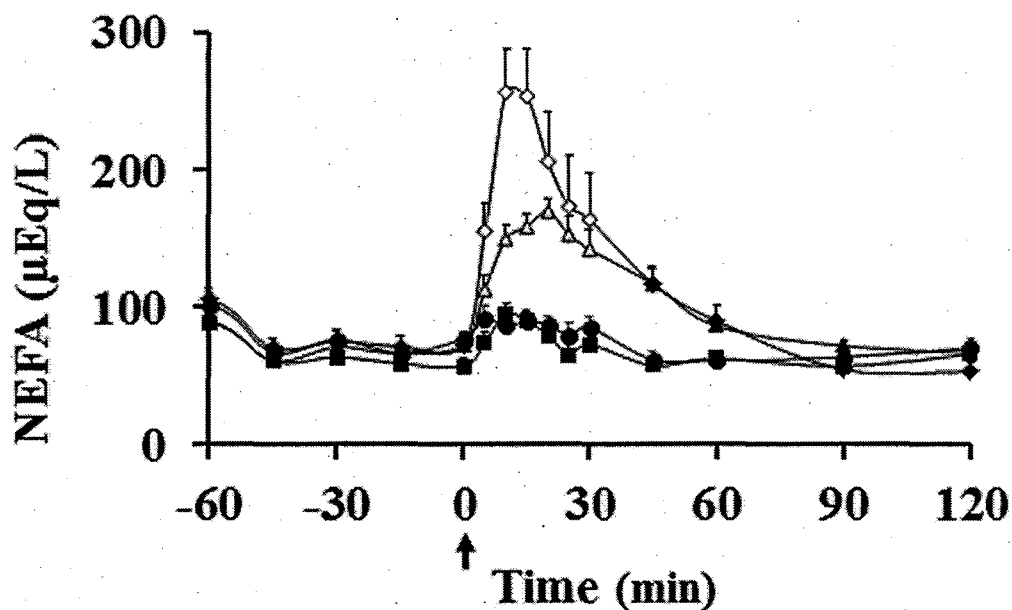


Figure 36. Changes in plasma concentration of NEFA in response to bovine acyl ghrelin, des-acyl ghrelin and sulfated CCK-8 injection. Animals were intravenously injected acyl ghrelin ( $20 \mu\text{g/kg BW}$ ,  $\blacktriangle$ ), des-acyl ghrelin ( $100 \mu\text{g/kg BW}$ ,  $\blacksquare$ ), sulfated CCK-8 ( $10 \mu\text{g/kg BW}$ ,  $\blacklozenge$ ) or vehicle (0.1% BSA-saline,  $\bullet$ ). The arrow indicates time of injection. Values are the means  $\pm$  S.E.M of 8 animals. Animals were fed at 1 min after sampling for - 60 min. Open symbols indicate a significant difference ( $P < 0.05$ ) compared with mean pre-injected concentrations (- 60 to 0 min).

### 3.5 DISCUSSION

Administration of CCK increased ghrelin release in rodents (Murakami et al. 2002; Friis-Hansen et al. 2005; Shrestha et al. 2009). To our knowledge, the effect of CCK on ghrelin secretion has not been reported previously for ruminants. Our present result showed for the first time, that intravenous bolus injection of CCK increased ghrelin secretion in ruminants (Fig. 29a,b). Murakami et al. (2002) reported that intraperitoneal injection of CCK increased ghrelin secretion in 12 h fasted rats. Friis-Hansen et al. (2005) also reported that 2 or 6 days subcutaneous infusion of CCK increased ghrelin release in anaesthetized mice. In addition, vascular perfusion of CCK increased ghrelin secretion in isolated stomach of 36 h food deprived rats (Shrestha et al. 2009). In rodents, administration of CCK increased ghrelin secretion in fasted condition. Our study showed that intravenous injection of CCK increased ghrelin secretion in steers under normal physiological condition (non-fasted).

Ghrelin is mainly released from the stomach (Kojima et al. 1999; Kojima and Kangawa 2005). In the stomach of mice, CCK-A receptors are expressed in fundic ghrelin cells (Friis-Hansen et al. 2005). CCK presumably stimulates fundic ghrelin secretion directly via the CCK-A receptors on the ghrelin cells (Friis-Hansen et al. 2005). On the other hand, expression of CCK-A receptors is different in adult and pre-weaning calves. In adult Holstein males, expression of CCK-A and of CCK-B receptors was slight in the rumen and fundus of abomasum, respectively (Yonekura et al. 2002). CCK-A receptor was expressed in the rumen of 3 week old calves (Yonekura et al. 2002). Therefore, we hypothesize that the mechanism of stimulatory effect of sulfated CCK-8 on ghrelin release in ruminants may be different from rodents. In the present study, we demonstrated the stimulatory effect of exogenous CCK on plasma ghrelin levels but the mechanism in which sulfated CCK-8 induced ghrelin release is difficult to explain because sulfated CCK-8 can bind to both CCK-A and CCK-B receptors (Rehfeld 1998, 2004). It is possible that sulfated CCK-8 binds to CCK-A receptors and increased the secretion of ghrelin in steers.

Injection of non-sulfated CCK-8 increased ghrelin secretion in rats (Murakami N,

personal communication). Moreover, administration of gastrin increased plasma ghrelin levels in rodents (Fukumoto et al. 2008; Murakami et al. 2002). In rodents, administration of gastrin may stimulate ghrelin secretion by acting directly on ghrelin cells because oxyntic ghrelin cells express CCK-B receptors on their plasma membrane (Fukumoto et al. 2008). Sulfated CCK, non-sulfated CCK and gastrin can bind to CCK-B receptors (Rehfeld 1998, 2004). Therefore, it is possible that sulfated CCK-8 binds to CCK-B receptors and increased ghrelin release in steers.

Brennan et al. (2007) reported that intravenous infusion of CCK (1.8 pmol/kg/min for 2 h) decreased ghrelin release in humans. However, Little et al. (2007) reported that intravenous infusion of CCK (2 ng/kg/min, approximately 1.7 pmol/kg/min for 45 min) did not change plasma ghrelin level in lean subjects. In a separate study by Friis-Hansen et al. (2005), subcutaneous infusion of CCK (15 nmol/kg/min for 2 or 6 days) increased ghrelin secretion in rats. The dose of CCK used in the study of Friis-Hansen et al. (2005) is higher than Brennan et al. (2007). In our study, intravenous bolus injection of CCK (10 µg/kg BW) increased ghrelin release in steers (Fig. 29 a). Therefore, it can be assumed that the dose is responsible for the controversial effect of CCK on plasma ghrelin concentrations in humans, rats and ruminants.

In steers, plasma ghrelin levels increased after injection of CCK may not be feedback regulation of ghrelin to suppress CCK, because plasma CCK is not affected by administration of ghrelin. The interpretation of our result is limited, because we administered only one dose of sulfated CCK-8. In rodents, ghrelin secretion is increased in both gastric emptying and filling conditions (Murakami et al. 2002). However in ruminants, high plasma ghrelin level is observed in fasted condition (Wertz-Lutz et al. 2006) and before feeding time of restricted and scheduled meal fed ruminants (Sugino et al. 2002; Relling et al. 2010). Therefore, CCK-induced ghrelin release in ruminant is probably related with initiation of feeding.

Ghrelin and OXM are oppositely regulated on food intake (Cohen et al. 2003; Dakin et al. 2004; Kojima and Kangawa 2005), gastric acid secretion (Dubrasquet et al. 1982; Kojima and Kangawa 2005) and body weight (Dakin et al. 2004; Kojima and

Kangawa 2005). However, our result (Fig.32) showed that ghrelin had no effect on plasma concentration of OXM in steers. We have recently reported that OXM did not affect plasma ghrelin level in bovine (ThanThan et al. 2010). We found that ghrelin and OXM did not affect each other's secretion in steers. Intravenous injection of sulfated CCK-8 also did not affect plasma concentration of OXM in steers (Fig. 32). Injection of OXM did not change plasma concentration of CCK in steers (unpublished data, data not shown). Therefore, we hypothesize that CCK and OXM have no effect on their secretions.

CCK (Lilja et al. 1984) and OXM (Hornnes et al. 1980) are released after eating in pig, dog and human. In contrary, plasma concentrations of CCK (Furuse et al. 1991) and OXM (Relling et al. 2010) did not change after feeding in ruminants. Our present study in steers also showed that plasma concentrations of CCK (Fig. 28b) and OXM (Fig. 32) are not changed after feeding. According to the digestive system of ruminants, the food cannot arrive quickly to the abomasum and small intestine. Also, ruminants have a steady outflow of digesta to the abomasum and small intestine. Therefore, in ruminants postprandial concentrations of CCK and OXM cannot increase as rapidly as in monogastric animals. In the study of direct infusion of nutrients into abomasum, postprandial changes of CCK were observed (Relling & Reynolds 2008). Therefore, CCK release after feeding may depend on food which arrived to the abomasum.

CCK stimulates pancreatic polypeptide (PP) in mice (Ahrén et al. 1991, 1995), human (Schmid et al. 1989) and dog (Parks et al. 1979, Schusdziarra et al. 1986). In addition, Choi et al. (2000) reported that fatty acids supplement feeding induced endogenous increased CCK stimulates PP secretion in dairy cow, and CCK induced PP release was inhibited by CCK-A receptor antagonist (MK-329). In our study, intravenous injection of sulfated CCK-8 increases plasma PP secretion in Holstein steers (Fig. 33). Our result shows exogenous sulfated CCK-8 stimulates PP in cattle and it is in agreement with previous reports in mice, human and dog. Non-sulfated CCK and CCK C-terminal fragment do not have relationship with PP, because administration of

non-sulfated CCK (CCK-33ns) and CCK fragment (CCK-4) did not affect plasma PP release in steers (data not shown). It is possible that sulfated CCK-8 binds to CCK-A receptors and stimulates PP secretion, and CCK-B receptors may not involve. Because, administration of CCK-A receptor antagonist inhibits endogenous CCK induced PP release in cattle (Choi et al. 2000).

Administration of ghrelin increased plasma PP release in humans (Arosio et al. 2003, Tack et al. 2006) and rat (Sato et al. 2003) were reported. However, in our study, administrations of acyl ghrelin and des-acyl ghrelin have no effect to PP release in steers (Fig. 33). In addition, administration of PP also does not affect ghrelin release in steers (unpublished data). Therefore, ghrelin and PP do not affect each other on their secretions in cattle.

The effect of CCK on plasma glucagon like peptide-1 (GLP-1) is controversial. Some scientists reported that CCK stimulates GLP-1 secretion in human (Fieseler et al. 1995) and in porcine ileum (Hansen and Holst 2002). However, some scientists reported that administration of CCK did not affect plasma GLP-1 in rat colon (Plaisancie et al. 1994), human (Ahrén et al. 2000) and sheep (Relling et al. 2011). In our study, administration of sulfated CCK-8 does not affect plasma GLP-1 release in steers (Fig. 34). Injection of nonsulfated CCK-33 has no effect on GLP-1 secretions in steers (data not shown). Our result is in agreement with the results in rat, human and sheep. Some scientists reported that GLP-1 does not affect CCK secretion in human (Gutzwiller et al. 1999). In our study, administration of GLP-1 has no effect on plasma sulfated CCK in steers (unpublished data). Our result is in agreement with the result in human. CCK and GLP-1 does not affect each other on their secretions in cattle.

According to my knowledge, there is few information about the effect of ghrelin on GLP-1 in non-ruminant and ruminant. Our study in steers, administrations of acyl ghrelin and des-acyl ghrelin do not affect plasma GLP-1 release (Fig. 34). The effect of GLP-1 on ghrelin secretion in cattle is still need to clarify.

Some scientists reported that CCK stimulates GH in monogastric species (Calogero et al. 1993, Parrott et al. 1995, Matsumura et al. 1984). However, some

scientists reported that CCK decreased plasma GH in rats (Karashima et al. 1984) and some reported that peripheral administration of CCK did not affect plasma GH in rats (Vijayan et al. 1979), humans (Nair et al. 1984) and sheep (Spencer et al. 1991, Della-fera and Baile 1985). The results of the effect of CCK on plasma GH are controversial. In our study, intravenous administration of sulfated CCK-8 has an effect on plasma GH levels in steers (Fig. 31). We found that CCK stimulates ghrelin release however, that endogenous increased ghrelin did not stimulate plasma GH.

Acyl ghrelin stimulates NEFA secretion however does not affect plasma glucose. This result is in agreement with previous report (ThidarMyint et al. 2006). In addition des-acyl ghrelin has no effect on plasma glucose and NEFA secretion in steers (Yannaing et al. unpublished data). It is possible that des-acyl ghrelin may not have function in cattle. Another appetite hormone CCK has regulatory effect on plasma glucose and NEFA levels. In rats, intra-duodenal administration of CCK decreases glucose production independent of changes in circulating insulin levels (Cheung et al. 2009). In addition, CCK reduces the increase in circulating glucose after meal ingestion in humans (Ahrén et al. 2000). However, some scientists reported that CCK has no effect on plasma glucose (Della-Fera et al. 1985, Relling et al. 2010, 2011) and NEFA (Relling et al. 2010, 2011) in sheep. In our study, administration of CCK increases plasma glucose levels in steers (Fig. 35). In cattle, plasma glucose and NEFA can be influenced by stress. Plasma glucose and NEFA significantly increased by stress of handling (Leroy et al. 2011). In sheep, modification of feeding regimen (feeding once per day, twice per day and ad libitum feeding) did not significantly differ on plasma glucose (Udum et al. 2008). In our study, we do not observe changes of glucose levels after feeding in steers (Fig. 35). Plasma NEFA levels increased in fasted condition of before feeding and decreased after eating (Udum et al. 2008). In fasted ruminant, plasma glucose level decreased and plasma NEFA level increased, respectively (Bowden 1971). In fasting ewe, blood glucose levels decline more slowly than NEFA increased, and that increased in NEFA was not directly due to hypoglycemia (Annison 1960). In addition, administration of glucose decreased plasma NEFA levels in fasted



ewes (Annison 1960). It is possible that the increment of plasma NEFA may not be related with glucose in cattle.

Some scientists reported that administration of CCK did not affect plasma NEFA levels (Relling et al. 2010, 2011) in sheep. In our study, administration of CCK stimulates plasma NEFA levels in steers (Fig. 36). Increased plasma level of NEFA found in negative energy condition (Wertz-Lutz et al. 2006) and after eating of fatty acids supplement diet (Choi et al. 2000, Relling et al. 2010) in cattle. The interpretation of CCK increased glucose and NEFA release in steers (non-fasted) is difficult. Because, increased plasma level of glucose and NEFA in cattle can see at stress condition.

In conclusion, administration of CCK increased plasma concentrations of ghrelin and PP but did not affect OXM and GLP-1 in cattle. On the other hand, exogenous ghrelin has no effect on plasma CCK, OXM, PP and GLP-1 secretions. OXM does not affect ghrelin and CCK secretions, and vice versa in cattle. CCK stimulates plasma glucose and NEFA levels but does not affect plasma GLP-1 and GH levels.

## Chapter 4

### General summary

Measuring of hormones in bovine is limited, because of not commercially available and measuring kits are not specific for bovine. In addition, it is costly to measure many samples. These facts inhibit to study the effect of one appetite hormone to other appetite regulatory hormone in bovine. Most of the kits can measure hormone in extracted plasma. The procedure of plasma extraction is complicated, time consuming and hormone can loss during process. Therefore, RIA system which can measure un-extracted plasma is practical for measuring of over thousands of samples.

To study the effect of CCK on plasma ghrelin, oxyntomodulin (OXM), pancreatic polypeptide (PP), glucagon like peptide-1 (GLP-1), GH, glucose and NEFA secretion, we synthesized peptide (sulfated CCK-8). Because of mass amount of peptide is required for administration to cattle. It requires various forms of peptide for each hormone to validate radioimmunoassay (RIA) system. For CCK RIA, various forms of CCK peptide are needed to generate first antibody body production (anti-sulfated CCK-8 serum), for cold standard, for labeling and for specificity check. We synthesized sulfated CCK-8 amide, (Cys<sup>0</sup>)sulfated CCK-8 C-terminal free, (Tyr<sup>0</sup>)sulfated CCK-8 amide, bovine sulfated gastrin-9 amide, non-sulfated bovine gastrin-9 amide, (Cys<sup>0</sup>) 1-29 human GHRH peptide, (Cys<sup>0</sup>) 21-44 bovine GHRH, bovine amylin, Cys<sup>0</sup>-bovine amylin (7- 37), Tyr<sup>0</sup>-bovine amylin (1-37), bovine octanoyl ghrelin (O-ghrelin), bovine des-acyl ghrelin and bovine decanoyl ghrelin (D-ghrelin). We try to validate sulfated CCK-8, GHRH, amylin and decanoyl ghrelin (D-ghrelin) RIA systems (chapter 2). We can produce first antibody of sulfated CCK-8 which specifically recognized to sulfated CCK and did not recognize to non-sulfated CCK-8, bovine sulfated gastrin-9 amide and bovine non-sulfated gastrin-9 amide. We validated sulfated CCK RIA system which can measure un-extracted plasma. This RIA system provides us new information on the effect of other appetite regulatory hormone on CCK secretion. We can set up GHRH, amylin and D-ghrelin RIA systems. However, these RIA systems cannot use for

measuring of plasma GHRH, amylin and D-ghrelin levels (chapter 2).

For a better understanding of the effect of appetite regulatory hormone CCK on ghrelin, OXM, PP, GLP-1, GH and metabolites (glucose and NEFA), we conducted experiment in 8 Holstein steers. Moreover, we study the effect of ghrelin on CCK, OXM, PP and GLP-1 secretions.

In chapter 3, administration of sulfated CCK-8 increased plasma acyl ghrelin, total ghrelin and PP secretions. CCK also stimulates plasma glucose and NEFA in cattle. This result is different from sheep, rats and humans. However, CCK injection has no effect on plasma OXM, GLP-1 and GH levels. Intravenously injected acyl ghrelin and des-acyl ghrelin have no effect on plasma CCK, OXM, PP and GLP-1 secretions. OXM does not affect ghrelin and CCK secretions, and vice versa in steers. This is the first report on the effect of sulfated CCK-8 on plasma ghrelin secretion in ruminant (Animal Science Journal, in press). In conclusion, CCK has stimulatory effect on plasma ghrelin, insulin, PP, glucose and NEFA secretions in cattle.

## Japanese General Summary

ウシ・ペプチドホルモンで測定できるものは、限られた状況にある。それは、利用可能な市販の測定キットがウシ・ペプチドホルモンに対して特異的に反応しないためである。このような状況は、ひとつの採食制御ホルモンが他の採食制御ホルモンに及ぼす効果を明らかにする本研究において、大きな障害である。そこで、本論文の第二章では、ウシ・硫酸化CCK、成長ホルモン放出ホルモン (GHRH)、ウシ・アミリン、ウシ・デカノイルグレリン (D-グレリン) についてのラジオイムノアッセイによる特異的測定法を検討し、その精度と特異性を明らかにした。それらの測定系は、本研究を進めるための重要な手がかりとなった。

第二章では、血漿におけるグレリン、オキシントモジュリン (OXM)、腓ポリペプチド (PP)、成長ホルモン (GH)、グルコース、およびNEFAの濃度に及ぼすCCKの効果を調べるため、活性ペプチド (硫酸化CCK-8) を化学合成した。これは、ウシで投与実験するために不可欠な大量のペプチドを用意するためである。また他方では、CCK測定系を確立して反芻動物におけるグレリンやOXMによるCCK分泌変動を調べるためである。我々は、測定系を構築するために一次抗体 (抗硫酸化CCK-8) を産生するモルモットに免疫する抗原ペプチド、測定での希釈用標準ペプチド、さらに、反応特異性を調べるための標識ペプチドを合成した。それらは、硫酸化CCK-8アミド、(Cys<sup>0</sup>)硫酸化CCK-8、(Tyr<sup>0</sup>)硫酸化CCK-8アミド、硫酸化ウシ・ガストリン-9アミド、非硫酸化ウシ・ガストリン-9アミドなどである。その結果、硫酸化CCK-8に対するモルモット一次抗体として、硫酸化CCKを特異的に識別可能な血清が得られた。CCKの特異抗体によるRIAの構築に成功することにより、CCK分泌に及ぼす採食量制御ホルモンの効果を研究可能となった。さらには、採食制御ホルモン分泌や血中代謝産物濃度に及ぼすCCKの効果を調べるのが可能になった。

論文では、ホルスタイン去勢牛をもちいる動物実験を実施し、採食量制御ホルモンCCKが、グレリン、OXM、腓ポリペプチド (PP)、GH、および、血中代謝産物 (グルコースおよびNEFA) に及ぼす効果を調べ、さらに、グレリンおよびOXMが、血漿CCK濃度に及ぼす効果を研究した。

第三章では8頭のホルスタイン去勢牛に静脈内投与して、血漿グレリンとOXMの分泌に対する硫酸化CCK-8の効果、および、血漿CCKとOXM分泌に対するグレリンの効果調べた。同時に、血漿GHの分泌や代謝産物濃度 (グルコースおよびNEFA) におよぼすCCKの効果も検討した。硫酸化CCK-8の投与は血漿アシルグレリンおよび全グレリンの分泌を増加させた。また、CCKはウシにおいて血漿グルコースと血漿NEFAの濃度を増加させた。この結果は、ヒツジ、ラット、およびヒトでの報告結果と異なる。一方、CCK投与は血漿のOXMやグルカゴン様ペプチド-1 (GLP-1) GHの濃度には効果がなかった。また、ウシ静脈内に投与したアシルグレリンまたはデスアシルグレリンは、血漿のCCKやOXMの分泌に何ら影響を及ぼさなかった。反芻家畜ではOXMは

グレリンやCCKに影響しないし、また、逆にグレリンやCCKはOXMに影響しなかった。グレリンは膵ポリペプチド (PP)、グルカゴン様ペプチド-1 (GLP-1) に対して影響を与えなかった。硫酸化CCK-8が血漿グレリン分泌を増加させるという我々の発見は、反芻動物では最初である (Animal Science Journal、印刷中)。反芻動物におけるCCKはグレリン、インスリン、膵ポリペプチド (PP)、グルコース、およびNEFAに影響を及ぼすことが示された。

## **Acknowledgement**

I would like to acknowledge many people who helped me for my further study and to finish my PhD study. However, it is impossible to express all of them.

First of all, I would like to express my heartfelt to my supervisor, Professor Dr. Hideto Kuwayama, Department of Life Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine, Japan for his kind advice, giving ideas and discussion throughout my study and various supports. In addition, I wish to extend my deep thanks to my advisors Dr. Tsutomu Hashizume (Professor) Animal Reproduction Department, Faculty of Agriculture, Iwate University, Morioka, Iwate, Japan and Dr. Mutsuyo Kadohira (Professor) Animal Health Department, Obihiro University of Agriculture and Veterinary Medicine, Japan for their guidance and advices.

Dr. Hnin Thidar Myint, Researcher from Livestock Breeding and Veterinary Department, Ministry of Livestock and Fishery, Myanmar and ex-Director General U Maung Maung Nyunt from Livestock Breeding and Veterinary Department, Ministry of Livestock and Fishery, Myanmar who help me for my further study in Japan.

I would like to express my sincere expression to all students from our laboratory for their help. I would like to extend my thanks to the staff of the United Graduate school of Agricultural Sciences, Iwate University and Obihiro University of Agriculture and Veterinary Medicine for their assistance and kind help. I am thankful to examiners, for their comments and suggestions.

I also wish acknowledge to Obihiro University of Agriculture and Veterinary Medicine (Kouenkai shihi gaikokujin ryugakuesi ikuei shougakuhi) for granting me one year scholarship, and Japan Student Services Organization (JASSO) for granting me one year scholarship. Moreover, I am thankful to United Graduate school of Agricultural Sciences, Iwate University (UGAS) for granting me scholarship of 50 % free for entrance fees and tuition fees. I would like to thank Japanese Ministry of Education, Science, Sports, Culture and Technology, Global Centers of Excellence (GCOE) program giving me the chance to work as research associate for 3 years and

partly financial support for my research.

Special thanks to my uncle U Min Lwin and his wife Daw Tin Win Mar for their kindly support and help. Finally, my greatest gratitude goes to my father U Khin Maung Swe, my mother Daw Khin Win May and my two younger sisters Swe Hlaing Win and Swe Zin Win for trusting my choice and supporting me.





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