Asian-Aust. J. Anim. Sci.
Vol. 21, No. 9 : 1286 - 1295
September 2008

www.ajas.info

# Development of Bovine Specific Leptin Radioimmunoassay and Relationship of Plasma Leptin with Vitamin A and Age of Wagyu

**S. H. Yang[1,2,*], H. Kawachi[2], M. A. Khan[3], S. Y. Lee[3], H. S. Kim[1], Jong K. Ha[3], W. S. Lee[1], H. J. Lee[1], K. S. Ki[1], S. B. Kim[1], S. Sakaguchi[4], S. Maruyama[4] and H. Yano[2,5]**

[1] National Institute of Animal Science, R.D.A., Cheonan, 330-801, Korea

**ABSTRACT :** Leptin is produced by adipocytes and its role in the regulation of lipid metabolism, feed intake, productive and reproductive performance of domestic animal species has been greatly stressed and extensively investigated in recent years. This study was conducted to develop a radioimmunoassay (RIA) for the estimation of plasma bovine leptin and to determine plasma leptin concentration in fattening Japanese Black cattle (Wagyu) and its crossbreds at commercial farms. Relationships of plasma leptin with plasma vitamin A and age of crossbred cattle were also determined. Recombinant bovine leptin (rbleptin) was produced by the *E. coli* overexpressed leptin as a GST (glutathione S-transferase)-fusion protein. Then antiserum against bovine leptin was obtained by its immunization in rabbits. Using this antiserum, a bovine specific RIA was developed and plasma leptin level was determined in 120 crossbred fattening cattle (Wagyu×Holstein, 50:50) at commercial farms. The plasma leptin level increased with the age of cattle and its level was greater in the crossbred heifers than in the steers. Plasma vitamin A level was negatively correlated with plasma leptin level in crossbred heifers and steers. This relationship was stronger in heifers than in steers. Plasma leptin was gradually increased with advancing age in fattening Wagyu cattle. In conclusion, development of a bovine specific RIA to estimate plasma leptin will contribute to better understanding of the role of leptin in cattle. (**Key Words :** Bovine, Fattening, Leptin, RIA, Vitamin A, Wagyu)

## INTRODUCTION

Japanese black cattle (Wagyu) are well known for their potential to produce high marbled beef that fetch high price in Japanese market (Zenbayashi, 1994). Beef marbling that influences juiciness and flavor has been considered as one of the most important beef-quality traits in north-east Asia. Japanese and Korean used a special feeding system where the cattle are fed for longer fattening period to produce high marbled beef (Lee et al., 2007). However, higher accumulation of body fat in cattle during long fattening period usually depressed the appetite (Forbes, 1986). Reduced DM intake in fattening cattle negatively affects both body weight gain and marbling (Lee et al., 2007) thus increased the cost of beef production.

Leptin is synthesized and secreted from white adipose tissues in rodents, humans (Friedman and Halaas, 1998; Zhang et al., 1994) and bovine (Houseknecht et al., 2000; Yang et al., 2003). Intracerebroventricular (ICV) administration of leptin decreased voluntary DM intake in ruminants (Henry et al., 1999) through suppressing satiety center (Tokuda et al., 2000). Furthermore, plasma leptin down-regulated accretion of adipose tissue (Halaas et al., 1995; Pelleymounter et al., 1995; Levin et al., 1996) and its concentration was positively correlated with body fat in ruminants (Block et al., 2001). Concentration of plasma leptin was increased in cattle during fattening period (Kawakita et al., 2001; Tokuda and Yano, 2001; Vega et al., 2002). In recent past, many studies has been conducted to understand the role of leptin in growth biology, energy metabolism, milk synthesis, mammary system development, reproductive performance of dairy and beef cattle (Block et al., 2003; Kim and Baik, 2004; Vega et al., 2004; Thorn et al., 2007). Interest to further understand the role of leptin on

* Corresponding Author: Seung Hak Yang. Dairy Science Division, San-9, Eoyong-ri, Seonghwan-eup, Cheonan-si, Chungnam, 330-801, Korea. Tel: +82-41-580-3398, Fax: +82-41-580-3419, E-mail: y64h@rda.go.kr

[2] Graduate School of Agriculture, Kyoto University, Kyoto, 606-8502, Japan.

[3] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-741, Korea.

[4] GIFU Prefectural Livestock Research Institute, Gifu, 506-0051, Japan.

[5] National Livestock Breeding Center, Hukushima, 961-8511, Japan.

Received June 3, 2008, Accepted July 18, 2008

the biology of cattle is growing. Many efforts has also been made to develop enzyme linked ImmunoSorbent Assay (ELISA) or Radio Immuno Assay (RIA) to estimate plasma leptin in human and rodents (Ma et al., 1996; Considine et al., 1996; Imagawa et al., 1998). Concentrations of serum leptin in the bovine have been extensive studied using multi-species leptin RIA kit based on the protocol of Ma et al. (1996). However, Delavaud et al. (2000, 2002) demonstrated that multi-specie RIA method underestimate ovine and bovine leptin. Delavaud et al. (2000) developed an ovine specific leptin RIA and described it as an efficient method to estimate plasma leptin in ovine, bovine and caprine species. In later studies (Delavaud et al., 2002; Liefers et al., 2003; Becu-Villalobos et al., 2007) ovine specific RIA was used to estimate plasma leptin in cattle. Ehrhardt et al. (2000) developed a bovine specific RIA method to determine serum leptin in cattle. They used His-tag system to purify bovine specific leptin that need refolding procedure as previously demonstrated by Fawzi et al. (1996). However, protein refolding did not completely restore the denatured leptin to bioactive leptin (Varnerin et al., 1998) and thus they introduced an improved method of leptin refolding. However, recovery of bioactive leptin using refolding step remain at large. In addition, it is generally known that nothing is commercially available to determine bovine leptin level.

This study was conducted to develop bovine specific leptin RIA method using GST fusion system and to determine plasma leptin in the Wagyu cattle and its crossbreds. Furthermore, the relationships between plasma leptin, plasma vitamin A and age of Wagyu crossbred cattle reared on Japanese commercial farms were also estimated.

## MATERIALS AND METHODS

### Overexpression and purification of recombinant bovine leptin (rbleptin)

The constructed plasmid pGEX-4T-leptin, expressing GST-leptin fusion protein, was provided the Laboratory of Animal Breeding and Genetics, Kyoto University, Kyoto, Japan. This plasmid, pGEX-4T-leptin, was transformed into DH5alpha competent cells and selected by growth on Luria-Bertani (LB)/ampicillin plates. Overnight cultures grown at 37°C in LB medium with ampicillin (50 mg/ml) were used to inoculate 1 L of supplemented LB medium at a 1:100 dilution. The cells were incubated at 37°C to achieve OD600 nm = 0.5-0.6. The protein expression was induced with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and cells were harvested after overnight incubation at 22°C. All cells were harvested by centrifugation at 5,000×g for 10 min at 4°C and re-suspended in sonication solution i.e. phosphate buffered saline (PBS), pH 7.3, 1% Triton X-100, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), approximately 5 ml buffer/1 g wet cells. The cells were disrupted by sonication in the ice water for 6 min with 30 seconds (s) intervals (30 s on and 30 s off per cycle). Undisrupted cells and debris were removed by centrifugation at 10,000 g for 20 min at 4°C. The PBS containing 1% Triton X-100 pre-equilibrated glutathione-Sepharose 4B beads ((Amersham Pharmacia Biotech, UK) was added to the supernatants and then incubated overnight at 4°C on an orbital rotator. The beads were collected by centrifugation 3,000×g for 5 min at 4°C and were washed three times with PBS by repeated centrifugation. The beads were re-suspended in elution buffer i.e. 50 mM Tris-HCl, pH 7.5, 30 mM reduced glutathione (GSH), 1 mM PMSF. Subsequently beads mixture was incubated for 30 min at 4°C on an orbital rotator. After rotating, the beads mixture was centrifuged at 1,000×g for 5 min at 4°C and the supernatant was obtained. In order to remove GSH, the GST-fusion protein solution was dialyzed against the 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM PMSF for overnight. This solution was treated with thrombin protease (Amersham Pharmacia Biotech, UK) for 16 h at 22°C. To remove both GST tag and thrombin from the thrombin-treated contents, pre-equilibrated glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, UK) and Benzamidine Sepharose (Amersham Pharmacia Biotech, UK) were added to the thrombin-treated solution. After incubation for 30 min at 4°C, the mixture was loaded through Poly-Prep column (Bio-Rad Lab. Hercules, USA). The flow-through pure leptin was concentrated using Centriprep YM3 (Millipore, Bedford, MA, USA) by centrifugation at 3,000×g for 5 h at 4°C.

### Production of bovine leptin specific antiserum

Three male rabbits were immunized against rbleptin. Purified rbleptin of 200 μg in 0.5 ml 50 mM Tris-HCl pH 7.5 buffer was emulsified with 0.5 ml complete Freund's adjuvant (DIFCO, Detroit, MI) and this was then injected subcutaneously to rabbits at 2-week intervals for 5 times. Seven days after the last booster, blood was collected from the carotid artery. After centrifugation, rabbit sera were collected and stored at -80°C.

### SDS-PAGE and western blotting

Protein concentration was determined using Micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Cell extract from E. coli, the leptin-enriched fractions prepared from cell extract and rbleptin protein were subjected to SDS-PAGE using 12% or 13.5% polyacrylamide separating gel, respectively under reducing conditions according to method of Laemmli (1970). Separated proteins were visualized by Coomassie Blue

staining. Otherwise separated proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) for Western blotting. After blocking with 3% skim milk in PBS, the membrane was incubated with diluted bovine leptin antiserum (1:200) for 1 h, followed by incubation with biotinylated secondary antibody, i.e., anti-rabbit IgG (Amersham Pharmacia Biotech, UK; 1:200) for 30 min and then with alkaline phosphatase-conjugated third antibody (Amersham Pharmacia Biotech UK; 1:1,000) for 30 min. Visualization of the third antibody was performed by a color development reaction using alkaline phosphatase (Bestagno et al., 1987).

**Radioiodination of rbleptin**

Five μg rbleptin {Diagnostic Systems Laboratories (DSL), Inc., Webster, Tex., USA} was dissolved in 10 μl distilled water and labeled by 1 mCi Na125I (PerkinElmer Life and Analytical Sciences, Cambridge, UK) using the chloramine-T method (Hunter and Greenwood, 1962) as modified by Delavaud et al. (2000). The reaction mixture was applied to a gel filtration column (0.8×35 cm, AcA 54 Ultrogel (Sigma Inc., St. Louis, USA) for the separation of free 125I and 125I-labeled leptin. Radioactivity was measured by Cobra II gamma counter (Packard Instrument Co. Inc. Meriden, CT, USA). Leptin fractions were immediately diluted (V/V) with pure glycerol (ICN Biomedicals Ltd., Oxford-. shire, UK) and stored at -20°C.

**Determination of appropriate dilution ratio of the antiserum**

Leptin antisera were diluted with incubation buffer (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2 containing 0.1% gelatin, 0.02% sodium azide and 0.01% Tween 20) at a ratio of 1:100, 500, 1,000, 2,000, 5,000, 10,000 and 20,000. Four hundred μl incubation buffer were added to a tubes for non specific binding (NSB), and 300 μl were added to the other tubes with 100 μl diluted antiserum. Then, ~20,000 cpm of tracer (125I-leptin) diluted by incubation buffer was added to each tube, and the mixture was incubated for 44 h at 4°C. Bound and free tracer was separated by adding 500 μl of an anti-rabbit sheep Immunoglobulin G solution (Biogenesis Inc., Poole, UK). The mixtures were allowed to proceed at room temperature for 30 min. Precipitation of the antibody-antigen complexes was then accomplished by the addition of 2 ml of phosphate buffer (20 mM, pH 7.4), immediate centrifuged (3,000×g, 30 min, 4°C), and the unbound 125I-bovine leptin was removed by aspiration of the supernatant. The remaining radioactivity in the precipitate was counted with a Cobra II gamma counter (Packard Instrument Co. Inc. Meriden, CT, USA). Binding capacity was calculated with bound radioactivity (B)/total activity (T) as percentage.

**Animal, management, sampling and analyses**

Jugular blood samples of crossbred (Wagyu×Holstein, 50:50) cattle (82 steers and 38 heifers) raised on the 10 commercial farms (Aichi, Japan) were collected between 10 to 30 month of age using vein puncture to determine plasma leptin, cholesterol and vitamin A concentrations.

Ten pure Wagyu steers aged 12 months were used in fattening trial for 12 months and were fed a diet consisting of concentrate mixture and rice straw. The diet contained 76% TDN and 10% DCP based on the Japanese feeding standard by the Japan Livestock Industry Association (2000). The DM intake in Wagyu was ranged from 6.6 kg to 8.9 kg/d during the experimental period. Blood samples were collected from jugular vein at 12, 19 and 24 months of age from each cattle to determine plasma leptin.

Wagyu steers (n = 10) were slaughtered at 26 months of age. The crossbred steers (n = 27) and heifers (n = 18) were slaughtered at 21 months of age. The carcasses evaluation of animals was done in accordance with Japan Meat Grading Association (1988). Beef marbling standard (BMS; 1-12, No.1 is the lowest grade) was applied to determine the meat quality. High performance liquid chromatography (HPLC) was used to determine plasma vitamin A (Makimura et al., 1991). Total cholesterol was determined using Cholesterol *E*-test (Wako chemicals, Kanagawa, Japan). Care of animals was in accordance with the Guide for the Care and Use of Experimental Animals (Animal Care Committee, National Institute of Livestock and Grassland Science, Tsukuba, Japan).

**Validation of RIA to determine plasma bovine leptin**

The assay was performed using a double-antibody method under disequilibrium conditions as previously described by Delavaud et al. (2000). Rbleptin (DSL, Inc., Webster, Tex., USA) was used for preparation of standards (0.5, 1, 2, 4, 8, 16, 32, 64 ng/ml diluted in incubation buffer). The tubes for total count, non specific binding (NSB), B0, standards and samples were set. Standard solutions and samples were added to each tube in volume of 100 μl, respectively. Two hundred μl incubation buffer was added to standard and sample tubes, and 300 μl to NSB and B0 tubes. Finally, 100 μl of a 1:2,000 working dilution of leptin antiserum (diluted in incubation buffer containing 1:100 normal rabbit serum) were added to all tubes except for total and NSB tubes to achieve a total volume of 400 μl in incubation buffer. After incubation overnight at 4°C, ~20,000 cpm of tracer (125I-leptin) in incubation buffer were added to each tube, and the mixture was incubated for 44 h at 4°C. Bound and free 125I were separated and bound 125I was determined by the same method as described above for antiserum.

The binding of 125I-bovine leptin to antiserum was expressed as logit, i.e.,

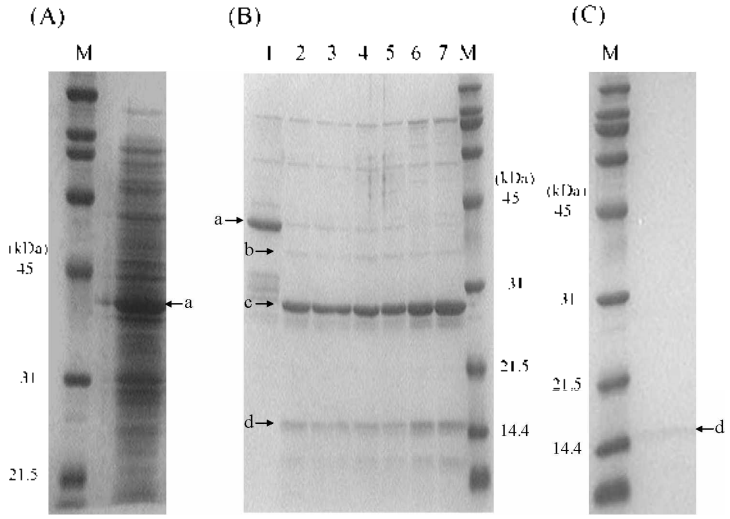


**Figure 1**. (A) SDS-PAGE on 12% gel separation of proteins of induced *E. coli* cells carrying plasmid pGEX-4T-leptin. This figure shows the resolution of whole-cell proteins from *E. coli* strains DH5alpha containing plasmid pGEX-4T-leptin. The position of migration of fusion protein (a) is indicated by arrow. Lane M, molecular weight markers (21.5, 31 and 45 kDa). (B) SDS-PAGE separation of GST-leptin fusion protein and GST, leptin and thrombin protease mixture on 13.5% gel. After GST-leptin fusion protein (a) was purified from *E. coli* using glutathione-Sepharose 4B beads (Lane 1), GST-leptin fusion protein was digested with thrombin protease. GST (c), leptin (d) and thrombin (b) (Lane 2-7) were indicated by arrows. (C) SDS-PAGE analysis of recombinant bovine leptin (rbleptin) purified. This recombinant leptin (d) at the final step of the purification was indicated by arrow. The rbleptin was concentrated in order to use the immunization into rabbit. All samples were stained with Coomassie Brilliant Blue. The lane marked M contains molecular weight markers for size indicated.

$$\text{Logit } (B/B_0) = \log\left[\frac{(B\text{-NSB})/(B_0\text{-NSB})}{\left[1-((B\text{-NSB})/(B_0\text{-NSB})\right]}\right]$$

**Statistical analysis**

The regression between plasma leptin levels and age of cattle was analyzed by GLM procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC). As a preliminary analysis, a covariance model was used to access the effect of sex and the interaction of sex and animal age on the regression. Because the effect of sex was significant, the regression analysis was individually performed in each sex. Carcass characteristics were also analysed by the ANOVA procedure of SAS in the finishing animal studies. The changes in plasma leptin concentration over fattening period were analysed by the MIXED procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC) in the fattening trial. The Tukey's multiple range test was used to identify the difference among the means. The differences were considered significant at $p<0.05$.

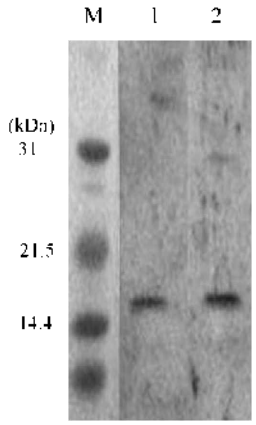


**Figure 2.** Western blot analysis of recombinant bovine leptin with anti-bovine leptin rabbit antiserum. Purification of recombinant bovine leptin (lane 2) was confirmed by comparison with commercial bovine recombinant leptin (Diagnostic Systems Laboratories, Webster, Tex., USA, lane 1) Proteins were visualized by Western blot analysis using anti bovine leptin rabbit antiserum generated. The lane marked M contains molecular weight markers for size indicated.

## RESULTS AND DISCUSSION

The SDS-PAGE showed approximately 42 kDa protein in the extract of *E. coli* overexpressed GST-leptin fusion protein (Figure 1A). Fractionation using GSH-affinity beads separated the protein having 42 kDa molecular weight (Figure 1B). Proteins with 16 kDa and 26 kDa were produced using thrombin treatments which were corresponding to the molecular weights of leptin and GST, respectively (Figure 1B). Purified 16 kDa rbleptin fraction was obtained using glutathione-affinity beads and presented in Figure 1C. The purified rbleptin and commercially available bovine leptin showed the same single band on the

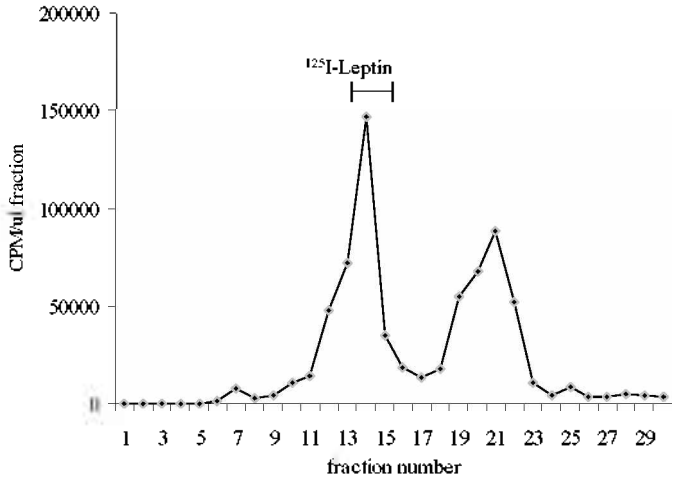


**Figure 3.** Fractionation of $^{125}$I-labeled leptin and free $^{125}$I by gel filtration. Radioactivity of each fraction was measured at a dilution of 1:100 with incubation buffer. Fraction number 14 and 15 were adopted for RIA, fraction number 21 and 22 proved to be unusable for RIA.

gel (Figure 2). Generally, when many GST-fusion proteins are overexpressed in *E. coli*, these proteins are partially or completely insoluble and make inclusion bodies because of protein aggregation. Once these aggregates are formed, it is very difficult to solubilize them. Therefore, *E. coli* overexpressed GST-leptin fusion protein was cultured under the mild induction conditions, such as low temperature (22°C) and for longer time (overnight) or semi-mild condition (at 30°C for 8 h), for suppressing the production of inclusion body. We decided the optimal temperature for producing rbleptin efficiently. Consequently, we could acquire the soluble GST-leptin fusion protein to our expectation as depicted in Figure 1A).

For validation of RIA, commercial rbleptin was labeled by 125I. Free and protein bound 125I were separated by a gel filtration chromatography (Figure 3). The specific radioactivity was 40-60 μCi/μg in the 125I-labeled leptin. The binding of 125I-labeled leptin and the diluted antiserum was determined for deciding appropriate dilution ratio of antiserum using RIA (Figure 4A). The binding of labeled leptin was decreased with the dilution (from 1:100 to 1:5,000) of antiserum. The radioactivity of bound/total labeled rbleptin was 33% at the dilution of 1:2,000. Therefore, the dilution ratio at 1:2,000 was adopted for RIA. The immunoreactivity of obtained antisera had shown

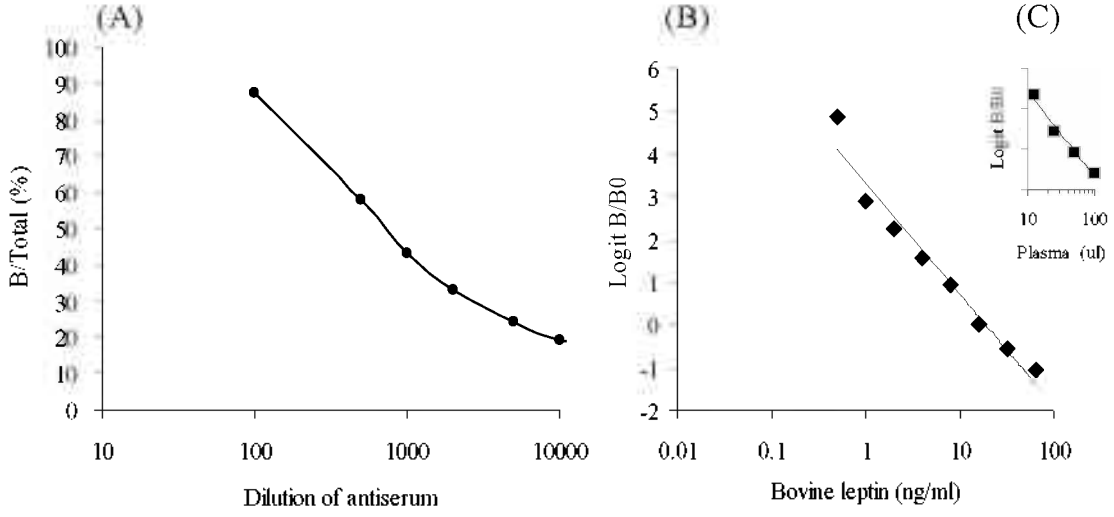


**Figure 4.** Binding capacity of labeled rbleptin against diluted anti-bovine rabbit antiserum (A), standard curve of rbleptin (B) and dilution curve of bovine plasma samples (C). Antiserum was diluted by incubation buffer at 1:100, 500, 1,000, 2,000, 5,000, 10,000 and 20,000. The ratio of bound to total labeled rbleptin (B/T) was shown on the vertical axis in A. Standard curve of rbleptin (♦) was derived using 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng leptin/tube). Dilution curve of bovine plasma (■) was derived using 12.5, 25, 50 and 100 μl plasma samples/tube. Data were expressed as log-logit method (Rodbard et al., 1969).

enough binding activity to radio-labeled rbleptin in the antiserum-dilution test. The rbleptin antisera from other purification method using chicken didn't respond to standard curve test in the final RIA validation step. Additionally, commercial rbleptin not purified rbleptin was used only for the standard indicator because of high price and objectivity.

The standard bovine curve exhibited almost linear relationship (Figure 4B), which allowed the determination of plasma leptin concentration between 1 ng/ml to 64 ng/ml. About 100 μl bovine plasma sample was used in this RIA which has a sensitivity to detect 1 ng leptin/ml of plasma.

The measured B/B0 in diluted bovine plasma sample showed the parallelism with the standard curve (Figure 4C), although any linear trend in the dilution test of this RIA with chicken, goat, horse and rabbit serum was not detected (data not shown). Other studies using ruminant-specific leptin RIA showed that plasma leptin concentration was 4-7

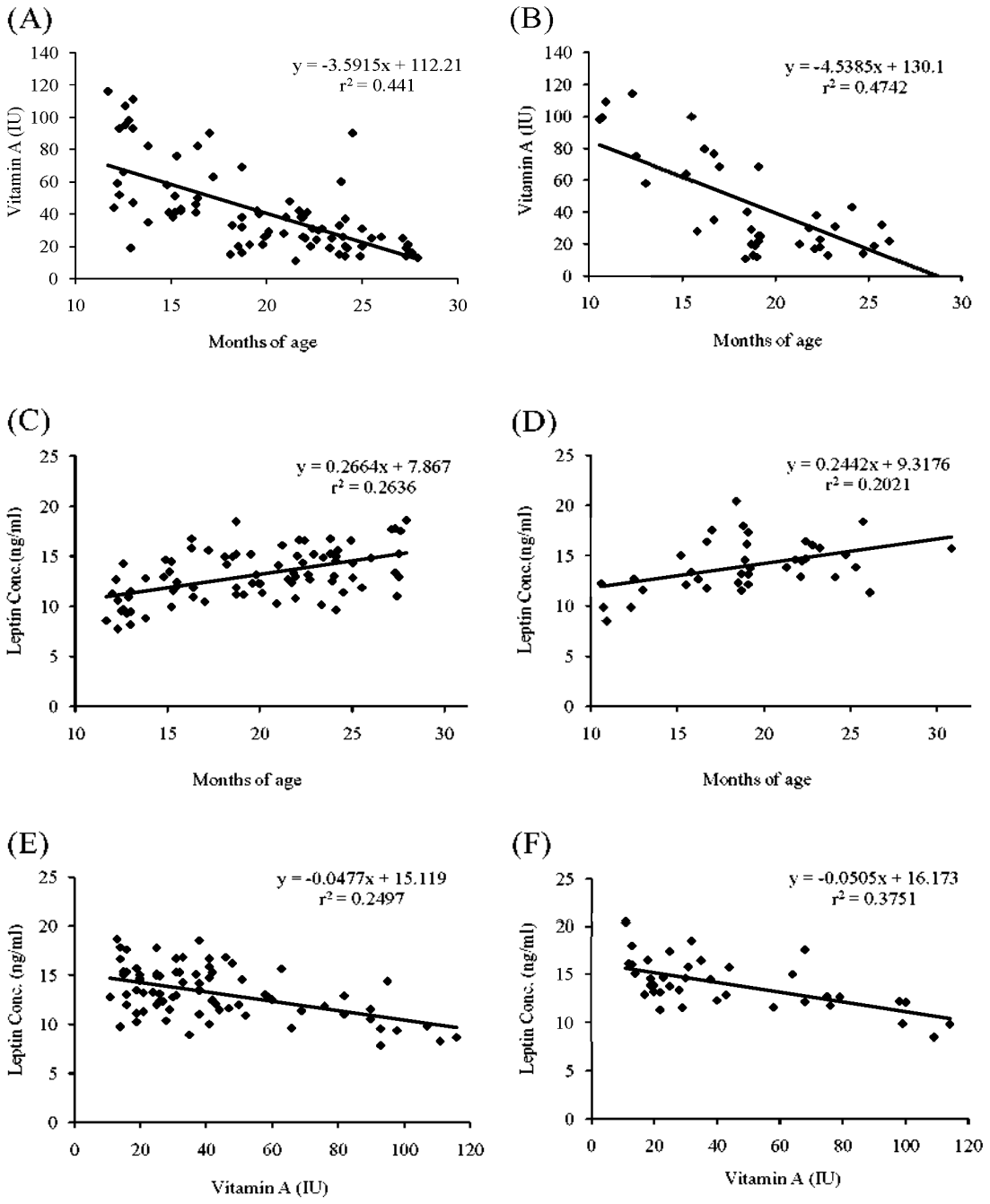


**Figure 5.** Regression within plasma leptin, vitamin A and age in the crossbred Wagyu steers (A, C, E; n = 82) and heifers (B, D, F; n = 38). The intercept for all parameters were significantly different ($p<0.05$) between steers and heifers.

ng/ml in calves (Ehrhardt et al., 2000) or ranged between 3.4 and 13.7 ng/ml in adult cattle (Delavaud et al., 2002). Additionally, it was proposed that the limited range might cause the difference of the effect significance in the results that the detectable range with multi-species RIA was practically below 5 ng/ml (Ehrhardt et al., 2000; Delavaud et al., 2002). Therefore, the analytical range of the present RIA is likely to satisfy the determination of plasma leptin concentration in cattle.

The recovery of added rbleptin into bovine plasma was 95.4±6.5 percent. The intra-assay coefficient of variation (CV) was 4.12% and the inter-assay CV was 7.37%. The results of recovery test and plasma dilution test verified that plasma constituents did not affect the determination of leptin.

Plasma concentration of Vitamin A decreased with advancing age of Wagyu crossbred steers and heifers (Figures 5A, B). Plasma concentration of Vitamin A has shown strong inverse relation with the age of heifers ($r^2$ = 0.474) and steers ($r^2$ = 0.441). In agreement to present findings, several studies (Kumar and Scarpace, 1998; Kumar et al., 1999; Bonet et al., 2000) demonstrated that retinoic acid inhibited leptin secretion from adipose tissue of rodents, indicating that leptin expression was directly under negative control by retinoids. However, Tokuda et al. (2001) reported that plasma leptin was not related with vitamin A level in cattle. Our data suggested that the plasma leptin level is related with plasma vitamin A. This relationship have special meaning for Japanese commercial beef farms where dietary vitamin A concentration considered important to control marbling index in fattening cattle.

The plasma leptin concentrations in Wagyu crossbred steers and heifers are presented in Figures 5C and 5D. Plasma leptin level was directly related to the age of Wagyu crossbred steers ($r^2$ = 0.264) and heifers ($r^2$ = 0.202). A plasma leptin concentration was reported to be positively correlated with adipose tissue mass in sheep (Delavaud et al., 2000) and in cattle (Ehrhardt et al., 2000). The previous study reported that the expression of leptin mRNA was higher in larger adipocytes in beef cattle (Yang et al., 2003) and recent study indicated that bovine Ob-Ra, Ob-Rb and Ob-Rc mRNA were strongly expressed in the 3 adipose depots (intermuscular, perirenal and subcutaneous) of fattening cattle (Kawachi et al., 2007), suggesting that adipose tissue might be vigorously controlled by leptin signaling. Because the adipocyte size is increased during fattening and the accumulation of body fat was mainly result from enlargement of adipocytes (Cianzio et al., 1985), fattening probably increases plasma leptin concentration through increasing adipocyte size. In the present study, the plasma leptin concentration also increased gradually ($p<0.001$) with the age of fattening Wagyu steers (Figure 6).

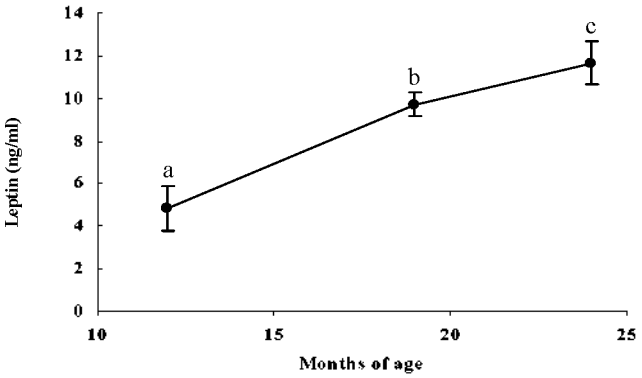


**Figure 6.** Plasma (mean±SE) leptin concentrations in Wagyu steers (n = 10) during fattening period. Means with the different letter (a, b, c) are significantly different ($p<0.001$).

The highest concentration of plasma leptin (11.6±1.03 ng/ml) in the Wagyu steers was noticed at 25 months of age. In contradiction to present finding, some studies using the multi-species RIA showed lower circulating plasma leptin concentrations (8 ng/ml) in 27 month old Wagyu steers (Kawakita et al., 2001; Tokuda and Yano, 2001). Although in the present study the plasma leptin was not determined using the multi-species RIA, it may be postulated that the multi-species RIA underestimated the bovine plasma leptin in previous studies (Delavaud et al., 2002). Ehrhardt et al. (2000) also demonstrated that plasma leptin concentrations measured by a bovine specific RIA were higher than those measured by the multi-species RIA in growing cattle.

During experimental period, mean concentration of plasma leptin was higher in heifers (14.0±0.4 ng/ml) than in steers (13.1±0.3 ng/ml). However, the results of covariate analysis indicated that the intercept for plasma leptin in heifers was significantly ($p<0.05$) higher than steer, i.e., the plasma leptin level was higher in the heifers than in the steers but the rate of increase did not differ between the heifers and the steers. An inverse relationship between plasma concentration of leptin and vitamin A was noticed in both steers and heifers (Figures 5E, 5F). This relationship was stronger in heifers ($r^2$ = 0.375) than in steers ($r^2$ = 0.249). Generally, the heifers accumulate more body fat than steers, which probably induced the high plasma leptin concentration in heifers (Murphey et al., 1985; Tokuda and Yano, 2001). Other studies (Havel et al., 1996; Saad et al., 1998) reported that plasma leptin levels were higher in women than in men, regardless of fat mass. Estrogen is known to stimulate leptin production and secretion (Tanaka et al., 2001). Testosterone was reported to inhibit leptin secretion (Wabitsch et al., 1997). There results suggested that different sex hormones may explain the high levels of leptin in heifers. Further studies are necessary for clarifying

**Table 1.** Mean (±SE) carcass characteristics, plasma vitamin A and total cholesterol in the Wagyu and its crossbreds

| | Crossbred[1] steers | Crossbred[1] heifers | Wagyu steers | p-value |
|---|---|---|---|---|
| n | 27 | 18 | 10 | |
| Age, mo | 21.4±0.6 | 20.8±0.8 | 26.6±0.5 | |
| Slaughter weight (kg) | 503.4±9.5[a] | 430.5±10.1[ab] | 418.8±11.1[b] | 0.0001 |
| Ribeye area ($cm^2$) | 49.2±0.8[a] | 48.0±1.5[a] | 51.7±1.2[a] | 0.1624 |
| Dressing yield (%) | 70.3±0.3[a] | 71.0±0.3[a] | 73.6±0.3[b] | 0.0001 |
| Subcutaneous fat (cm) | 2.4±0.3[a] | 2.6±0.2[a] | 2.7±0.2[a] | 0.4613 |
| BMS[2] | 3.4±0.3[a] | 4.2±0.4[a] | 5.3±0.4[b] | 0.0033 |
| Vitamin A (IU/dl) | 28.2±2.3[a] | 27.3±4.3[a] | 41.2±1.1[b] | 0.0241 |
| T-cholesterol (mg/dl) | 138.5±5.4[a] | 125.5±5.9[a] | 185.7±9.8[b] | 0.0001 |

[a,b] Mean within same row bearing different superscripts are different at the given p value.
[1] F1 of Wagyu×Holstein. [2] Beef marbling standard (BMS; 1-12, No.1 is the lowest grade).

why a plasma leptin level is higher in heifers than in steers.

Carcass evaluation, plasma vitamin A and cholesterol in Wagyu steers and crossbred cattle are presented in Table 1. Wagyu steers has lower slaughter weight, plasma vitamin A and cholesterol concentrations than crossbred steers and heifers. Higher dressing yield and BMS score were noticed in Wagyu cattle than in crossbred steers and heifers. Ribeye area and subcutaneous fat were similar in Wagyu steers and crossbred cattle. Similar to present result Lee et al. (2007) reported lower slaughtering weight and carcass yield in Jeju native cattle, a close relative of Wagyu, than its crossbreds.

## CONCLUSIONS

In conclusion, a bovine specific RIA method was successfully developed to determine the plasma circulating leptin in cattle. This RIA method would offer an opportunity to estimate plasma leptin more precisely and will probably replace the multi-species RIA method. Plasma leptin in Wagyu cattle and its crossbreds increased with advancing age. Plasma vitamin A has shown an inverse relationship with plasma leptin concentration in Wagyu crossbred steers and heifers which may have special meanings for Japanese commercial beef farms where dietary vitamin A concentration is important to control marbling index in cattle.

## ACKNOWLEDGMENT

We gratefully appreciate Dr. Mitsugu Ito (the AKABANE Animal Clinic) for giving me the bovine plasma samples and the technical information. We are thankful to Assistant Professor Yukio Taniguchi (Laboratory of Animal Breeding and Genetics of Kyoto University) for providing the constructed plasmid expressing GST-leptin fusion protein. This work was partly supported by a Grant-in Aid from the ministry of Education, Science, Sports and Culture of Japan and by research project (No.1673) for utilizing advanced technologies in agriculture, forestry and fisheries.

## REFERENCES

Becu-Villalobos, D., I. Garcia-Tornadu, G. Shroeder, E. E. Salado, G. Gagliostro, C. Delavaud, Y. Chilliard and I. M. Lacau-Mengido. 2007. Effect of fat supplementation on leptin, insulin-like growth factor I, growth hormone, and insulin in cattle. Can. J. Vet. Res. 71:218-225.

Bestagno, M., A. Cerino, S. Riva and G. C. Astaldi Ricotti. 1987. Improvements of western blotting to detect monoclonal antibodies. Biochem. Biophys. Res. Commun. 146:1509-1514.

Block, S. S., J. M. Smith, R. A. Ehrhardt, M. C. Diaz, R. P. Rhoads, M. E. Van Amburgh and Y. R. Boisclair. 2003. Nutritional and developmental regulation of plasma leptin in dairy cattle. J. Dairy Sci. 86:3206-3214.

Block, S. S., W. R. Butler, R. A. Ehrhardt, A. W. Bell, M. E. Van Amburgh and Y. R. Boisclair. 2001. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. J. Endocrinol. 171:339-348.

Bonet, M. L., J. Oliver, C. Pico, F. Felipe, J. Ribot, S. Cinti and A. Palou. 2000. Opposite effects of feeding a vitamin A-deficient diet and retinoic acid treatment on brown adipose tissue uncoupling protein 1 (UCP1), UCP2 and leptin expression. J. Endocrinol. 166:511-517.

Cianzio, D. S., D. G. Topel, G. B. Whitehurst, D. C. Beitz and H. L. Self. 1985. Adipose tissue growth and cellularity: changes in bovine adipocyte size and number. J. Anim. Sci. 60:970-976.

Considine, R. V., M. K. Sinha, M. L. Heiman, A. Kriauciunas, T. W. Stephens, M. R. Nyce, J. P. Ohannesian, C. C. Marco, L. J. McKee and T. L. Bauer. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N. Engl. J. Med. 334:292-295.

Delavaud, C., A. Ferlay, Y. Faulconnier, F. Bocquier, G. Kann and Y. Chilliard. 2002. Plasma leptin concentration in adult cattle: effects of breed, adiposity, feeding level, and meal intake. J .Anim. Sci. 80:1317-1328.

Delavaud, C., F. Bocquier, Y. Chilliard, D. H. Keisler, A. Gertler and G. Kann. 2000. Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. J. Endocrinol. 165: 519-526.

Ehrhardt, R. A., R. M. Slepetis, J. Siegal-Willott, M. E. Van Amburgh, A. W. Bell and Y. R. Boisclair. 2000. Development of a specific radioimmunoassay to measure physiological

changes of circulating leptin in cattle and sheep. J. Endocrinol. 166:519-528.

Fawzi, A. B., H. Zhang, M. van Heek and M. P. Graziano. 1996. Purification of milligram quantities of human leptin from recombinant *E. coli*. Horm. Metab. Res. 28:694-697.

Forbes, J. M. 1986. Dietary factors affecting intake. In: The voluntary feed Intake of farm animals (Ed. J. M. Forbes). Butterworths, London, pp. 85-113.

Friedman, J. M. and J. L. Halaas. 1998. Leptin and the regulation of body weight in mammals. Nature 395:763-770.

Halaas, J. L., K. S. Gajiwala, M. Maffei, S. L. Cohen, B. T. Chait, D. Rabinowitz, R. L. Lallone, S. K. Burley and J. M. Friedman. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Sci. 269:543-546.

Havel, P. J., S. Kasim-Karakas, G. R. Dubuc, W. Mueller and S. D. Phinney. 1996. Gender differences in plasma leptin concentrations. Nat. Med. 2:949-950.

Henry, B. A., J. W. Goding, W. S. Alexander, A. J. Tilbrook, B. J. Canny, F. Dunshea, A. Rao, A. Mansell and I. J. Clarke. 1999. Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: evidence for a dissociation of effects on appetite and neuroendocrine function. Endocrinol. 140:1175-1182.

Houseknecht, K. L., C. P. Portocarrero, S. Ji, R. Lemenager and M. E. Spurlock. 2000. Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression. J. Endocrinol. 164:51-57.

Hunter, W. M. and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495-496.

Imagawa, K., Y. Matsumoto, Y. Numata, A. Morita, S. Kikuoka, M. Tamaki, C. Higashikubo, T. Tsuji, K. Sasakura, H. Teraoka, H. Masuzaki, K. Hosoda, Y. Ogawa and K. Nakao. 1998. Development of a sensitive ELISA for human leptin, using monoclonal antibodies. Clin. Chem. 44:2165-2171.

Kawachi, H., S. H. Yang, A. Hamano, T. Matsui, S. B. Smith and H. Yano. 2007. Molecular cloning and expression of bovine (*Bos taurus*) leptin receptor isoform mRNAs. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 148:167-173.

Kawakita, Y., H. Abe, K. Hodate, A. Iguchi, M. Kobayashi, T. Mori, K. Kasai, Y. Tanai, Y. Kanbe, H. Mashiyama, T. Asada and T. Miyashige. 2001. The relation between plasma leptin concentrations and carcass lipid contents in Japanese Black steers. Lives. Prod. Sci. 73:25-34.

Kim, K. S. and M. G. Baik. 2004. Production of leptin in *E. coli* and its effect on glucose and acetate transport and expression of uncoupling protein-2 gene in adipose tissues of Korean cattle (Hanwoo). Asian-Aust. J. Anim. Sci. 17:1062-1068.

Kumar, M. V. and P. J. Scarpace. 1998. Differential effects of retinoic acid on uncoupling protein-1 and leptin gene expression. J. Endocrinol. 157:237-243.

Kumar, M. V., G. D. Sunvold and P. J. Scarpace. 1999. Dietary vitamin A supplementation in rats: suppression of leptin and induction of UCP1 mRNA. J. Lipid Res. 40:824-829.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee, W. S., W. Y. Oh, S. S. Lee, M. A. Khan, M. S. Ko, H. S. Kim and Jong K. Ha. 2007. Growth performance and carcass evaluation of Jeju native cattle and its crossbreds fed for long fattening period. Asian-Aust. J. Anim. Sci. 20:1909-1916.

Levin, N., C. Nelson, A. Gurney, R. Vandlen and F. de Sauvage. 1996. Decreased food intake does not completely account for adiposity reduction after ob protein infusion. Proc. Natl. Acad. Sci. USA. 93:1726-1730.

Liefers, S. C., R. F. Veerkamp, M. F. te Pas, C. Delavaud, Y. Chilliard and T. van der Lende. 2003. Leptin concentrations in relation to energy balance, milk yield, intake, live weight, and estrus in dairy cows. J. Dairy Sci. 86:799-807.

Ma, Z., R. L. Gingerich, J. V. Santiago, S. Klein, C. H. Smith and M. Landt. 1996. Radioimmunoassay of leptin in human plasma. Clin. Chem. 42(Pt 1):942-946.

Makimura, S., S. Matsuo, M. Usui and H. Katayama. 1991. Simultaneous determination of retinol, β-carotene and α-tocopherol in serum and liver of Japanese Black beef cattle by high performance liquid chromatography. J. Japanese Vet. Med. Asso. 44:328-332.

Murphey, C. E., D. D. Johnson, G. C. Smith, H. C. Abraham and H. R. Cross. 1985. Effects of sex-related differences in external fat deposition on subjective carcass fatness evaluations - Steer versus Heifer. J. Anim. Sci. 60:666-674.

Pelleymounter, M. A., M. J. Cullen, M. B. Baker, R. Hecht, D. Winters, T. Boone and F. Collins. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Sci. 269:540-543.

Saad, M. F., M. G. Riad-Gabriel, A. Khan, A. Sharma, R. Michael, S. D. Jinagouda, R. Boyadjian and G. M. Steil. 1998. Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. J. Clin. Endocrinol. Metab. 83:453-459.

Tanaka, M., S. Nakaya, T. Kumai, M. Watanabe, T. Tateishi, H. Shimizu and S. Kobayashi. 2001. Effects of estrogen on serum leptin levels and leptin mRNA expression in adipose tissue in rats. Horm. Res. 56:98-104.

Thorn, S. R., M. J. Meyer, M. E. Van Amburgh and Y. R. Boisclair. 2007. Effect of estrogen on leptin and expression of leptin receptor transcripts in prepubertal dairy heifers. J. Dairy Sci. 90:3742-3750.

Tokuda, T. and H. Yano. 2001. Blood leptin concentrations in Japanese black cattle. Anim. Sci. 72:309-313.

Tokuda, T., S. Kono and T. Fujihara. 2001. The relationships between leptin and vitamin A levels in plasma of fattening cattle supplemented with vitamin A. Anim. Sci. 73:433-441.

Tokuda, T., T. Matsui and H. Yano. 2000. Effects of light and food on plasma leptin concentrations in ewes. Anim. Sci. 71:235-242.

Varnerin, J. P., T. Smith, C. I. Rosenblum, A. Vongs, B. A. Murphy, C. Nunes, T. N. Mellin, J. J. King, B. W. Burgess, B. Junker, M. Chou, P. Hey, E. Frazier, D. E. MacIntyre, L. H. Van der Ploeg and M. R. Tota. 1998. Production of leptin in Escherichia coli: a comparison of methods. Protein Expr. Purif. 14:335-342.

Vega, R. A., H. G. Lee, H. Kuwayama, N. Matsunaga and H. Hidari. 2002. Age-related changes in plasma Leptin from early growing to late finishing stages of castrated Holstein steers: Utilizing multi-species Leptin RIA. Asian-Aust. J. Anim. Sci. 15:725-731

Vega, R. A., H. Hidari, H. Kuwayama, M. Suzuki and D. D.

Manalo. 2004. The relationships of plasma leptin, backfat thickness and TDN intake across finishing stage of Holstein steers. Asian-Aust. J. Anim. Sci. 17:330-336.

Wabitsch, M., W. F. Blum, R. Muche, M. Braun, F. Hube, W. Rascher, E. Heinze, W. Teller and H. Hauner. 1997. Contribution of androgens to the gender difference in leptin production in obese children and adolescents. J. Clin. Invest. 100:808-813.

Yang, S. H., T. Matsui, H. Kawachi, T. Yamada, N. Nakanishi and H. Yano. 2003. Fat depot-specific differences in leptin mRNA expression and its relation to adipocyte size in steers. Anim. Sci. J. 74:17-21.

Zenbayashi, M. 1994. Effects of nutritional planes and breeds on intramuscular-lipid deposition in M. longissimus dorsi of steers. Meat Sci. 38:367-374.

Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold and J. M. Friedman. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-432.