Characteristics of Gene Structure of Bovine Ghrelin and Influence of Aging on Plasma Ghrelin

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ABSTRACT : Ghrelin is a novel growth-hormone-releasing acylated peptide, which has been purified and identified in rat stomach. In the present study, the full-length sequence of bovine ghrelin cDNA was cloned by RT-PCR. The bovine ghrelin cDNA sequence derived in the present study included a 348 bp open reading frame and a 137 bp 3'UTR. The putative amino acid sequence of bovine prepro-ghrelin consisted of 116 amino acids, which contained the 27-amino acid ghrelin. The sequence analysis of the bovine ghrelin gene revealed that an intron existed between Gln^{13} and Arg^{14} of ghrelin. This exon-intron boundary matched the GT-AG rule of the splicing mechanism. Compared with rats, which have two tandem CAG sequences in the 3'-end of intron, bovine ghrelin genome has only one CAG sequence. Therefore, although rats can produce 28 amino acid-ghrelin and 27 amino acid-des-Gln^{14}-ghrelin by alternative splicing, ruminant species, including bovines, might be able to produce only one type of ghrelin peptide, des-Gln^{14}-ghrelin. The influence of aging on plasma ghrelin concentration was also examined. Plasma ghrelin concentration increased after birth to approximately 600 days of age, and then remained constant. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 5 : 723-727)***

Key Words : Ghrelin, cDNA, Genomic DNA, Plasma Concentration, Cattle

INTRODUCTION

Growth hormone secretion by the somatotroph cells depends upon the interaction between hypothalamic regulatory peptides, e.g. growth hormone-releasing hormone and somatostatin. Recently a novel growth-hormone-releasing acylated peptide, ghrelin, has been purified and identified in rat stomach (Kojima et al., 1999). Since ghrelin was discovered, various physiological functions of ghrelin have been reported. The administration of synthetic ghrelin induced adiposity in mice (Tschop et al., 2000). Intracerebroventricular injections of ghrelin strongly stimulated feeding in rats and increased body weight gain (Nakazato et al., 2001). In ruminants, several reports have been published to investigate the physiological characteristics of ghrelin. The direct evidence of ghrelin to secrete growth hormone from anterior pituitary cells in cattle was offered in the *in vitro* study (Hashizume et al., 2003). Sugino et al. (2002a, b) showed that a transient surge in plasma ghrelin levels occurred just prior to a scheduled meal and pseudo-feeding in sheep, and that this transient surge was modified by the feeding regimen. Recently, they also reported that ghrelin secretion seems to be regulated by cholinergic neurons of the vagus and that cholinergic activity suppresses ghrelin secretion in sheep (Sugino et al., 2003). Although nutritional regulation like feeding regimens was investigated so far, the information associated with physiological factors like aging on plasma ghrelin levels has been limited.

It was revealed that the gene expression of stomach ghrelin was regulated by various factors, and the level of ghrelin mRNA was increased by fasting, insulin-induced hypoglycemia and leptin administration (Toshinai et al., 2001). However, compared to the study on rodents, there has been a little information about gene characteristics of ruminant ghrelin. Although Kojima et al. (2001) reported the partial sequence of bovine ghrelin cDNA, its full-length has not been cloned. In the present study, therefore, we cloned and characterized the full-length of bovine ghrelin cDNA and partial sequence of bovine ghrelin genome, and revealed the difference between ruminants and mono-gastric animals in the splicing mechanism for producing mature ghrelin peptide. Furthermore, in this study, influence of aging on plasma ghrelin concentration was also examined.

MATERIALS AND METHODS

cDNA cloning

Total RNA was purified from bovine abomasum and ghrelin cDNA was amplified by reverse transcriptase polymerase chain reaction (RT-PCR). Firstly, a 229-bp fragment which codes a part of prepro-ghrelin peptide was obtained using the following primers; forward 5'- GCC ATGGCAGGCTCCAGCTT -3', reverse 5'- TTGGCCTCT TCCCAGAGGAT -3'. The forward and reverse primers were the partial sequence of human ghrelin cDNA (GenBank accession no. AB029434). Secondly, a 385-bp fragment including the stop codon was derived by rapid

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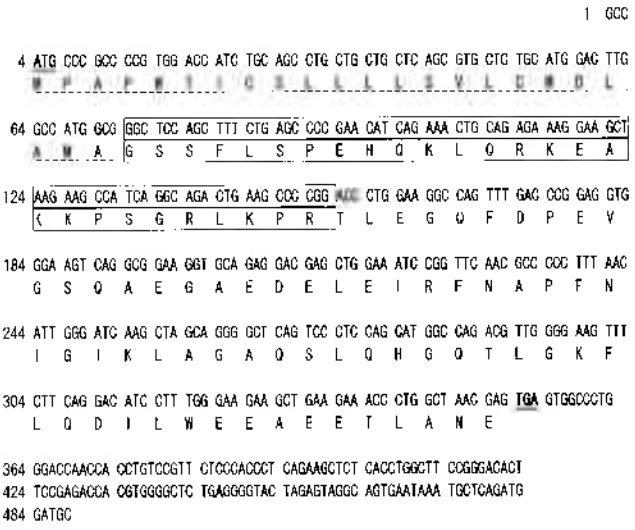

Figure 1. Nucleotide sequence of bovine ghrelin cDNA. The inferred amino acids are shown below the sequence. The translation initiation (ATG) and stop (TGA) codons are shown in bold and underlined. The dotted line indicates a signal peptide. Ghrelin sequences are boxed.

amplification of cDNA end (3'RACE). The forward primer was the same to that used in the first RT-PCR for the 229 bp fragment. The reverse primer was M13 Primer M4 in a commercial RT-PCR kit (RNA PCR kit (AMV) ver 2.1, Takara Shuzo Co. Ltd., Shiga, Japan). The 3'RACE products were purified and applied for nested PCR using the following primers: forward 5'- TCTGAGCCCCGAA CATCAGA -3', reverse M13 Primer M4. The forward primer was the partial sequence of bovine ghrelin cDNA fragment of the above 229 bp length. Finally, a 207 bp fragment which including start codon was obtained by RT-PCR using the following primers; forward 5'- CTGTCTG CAACCCAGCTGAG -3', reverse 5'- CAATGTTAAAGG GGGCGTTG -3'. The forward primer was the partial sequence of human ghrelin cDNA (GenBank accession no. AB029434) and the reverse primer was the partial sequence of bovine ghrelin cDNA derived from 3'RACE in the present study. The PCR products were purified and applied for nested PCR using the same forward primer and the reverse primer as follows; 5'- AACCGGATTTCCAGCT CGTC -3'. The reverse primer was the partial sequence of bovine ghrelin cDNA confirmed in the present study. To confirm the full sequence of complete bovine ghrelin cDNA, RT-PCR was conducted using sense and antisense primers based on the sequence derived in the present study as follows: forward 5'- ATGCCCGCCCCGTGGACCAT -3', reverse 5'- GCATCCATCTGAGCATTTAT -3'. Sequencing reactions were carried out on the above primers, using an ABI 310 PRISM automated DNA sequencer and the accompanying software (Perkin Elmer Japan, Yokohama, Japan).

Genomic DNA cloning

The partial fragment of ghrelin genomic DNA including intron 1 was purified from bovine abomasum. The partial ghrelin gene was amplified by PCR with the following primers; forward 5'- ATGCCCGCCCCGTGGACCAT - 3', reverse 5'- AACCGGATTTCCAGCTCGTC -3'. These primers were based on the sequence of full-length ghrelin cDNA derived in the present study. Other procedures were the same to those for cDNA cloning.

Plasma ghrelin

Japanese Black cattle (21 female and 4 male) and F1 back-cross cattle (F1 (Holstein×Japanese Black)×Japanese Black) (10 female and 10 male), bred in University Farm of Nagoya University, Japan, were used. Female cattle were 192 to 4,093 days old and male cattle were 176 to 842 days old. All cattle were fed on Italian ryegrass silage prepared in the farm, and diets were given at 9:00 and 16:00. Cattle were allowed free access to drinking water and trace-mineralized salt blocks (Cow candy, Mercian Co. Ltd., Tokyo, Japan). At 14:00, blood samples were taken by jugular venipuncture and added aprotinin with the final concentration of 500 KIU/ml. Plasma was separated and stored at -80°C until analyzed. The 1 ml of plasma sample was mixed with 1 ml of 1% (w/w) trifluoroacetic acid and then centrifuged at 13,000×g for 20 min at 4°C. The supernatant was loaded C_{18} Sep-Pak column (Waters, Milford, MA, USA) and washed with 3 ml of 1% (w/w) trifluoroacetic acid twice. The fraction caontaining ghrelin was eluted with 3 ml of 60% (w/w) of acetonitrile containing 1% (w/w) trifluoroacetic acid, and the collected eluent was dried by a centrifugal evaporator. The concentration of ghrelin was determined by radioimmunoassay (Peninsula Laboratories, Inc., Belmont, CA, USA).

Statistical analysis

Data was analyzed by NLIN procedure in a commercial statistical package SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

The full-length sequence of cDNA and putative amino acid of bovine ghrelin are shown in Figure 1. The full-length bovine ghrelin cDNA sequence derived in the present study showed 488 bp long with 3 bp 5'UTR, followed by a 348 bp open reading frame and a 137 bp 3'UTR. The putative amino acid sequence of bovine prepro-ghrelin consisted of 116 amino acids, which contained the 27-amino acid ghrelin (Figure 2). The comparison of amino acid sequence of matured bovine ghrelin with that of swine showed 81.5% identity. Although the peptide sequence of

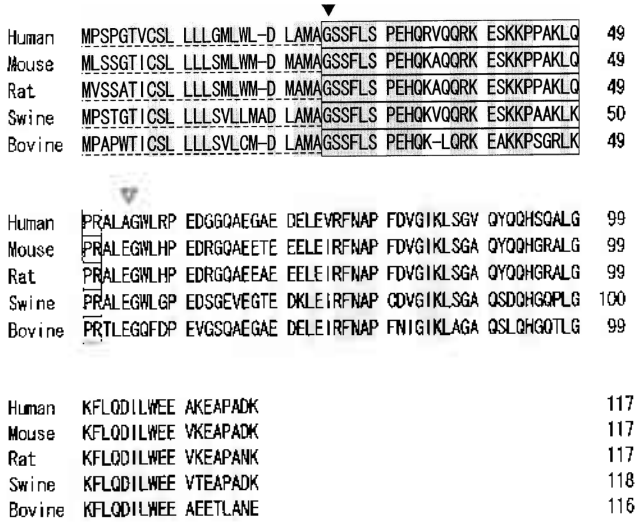

Figure 2. The comparison of the inferred amino acid sequences of ghrelin. The hatching parts are common amino acids conserved. The dotted line indicates a signal peptide. The filled arrowhead indicates a cleavage site of a signal peptide. The open arrowhead indicates a C-terminal-processing site. Ghrelin sequences are boxed.

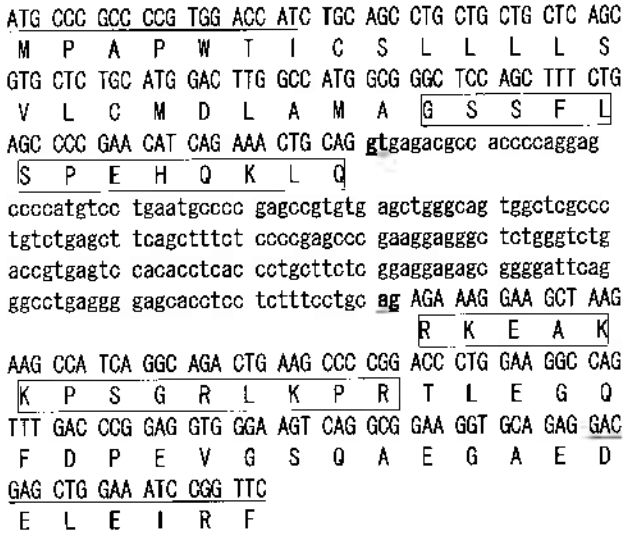

Figure 3. Partial gene sequence (exon 1-exon 2) of bovine ghrelin. Intron, untranslated regions, sequences are in lower case. Upper cases show exon 1 and exon 2. One letter below the sequence shows the inferred amino acids. Ghrelin sequences are boxed. Intron/exon junctions are shown in bold and underlined. The underlines indicate primer sequences.

matured ghrelin consisted of 28 amino acids in rat, mouse, swine and human, bovine ghrelin contained 27 amino acids in this study.

The partial sequence (exon 1-exon 2) of genomic DNA of bovine ghrelin ia represented in Figure 3. The length of intron 1 was 203 bp. Figure 4 shows the comparison of partial genomic structure between bovine and rat ghrelin. Sequence analysis of the bovine ghrelin gene revealed that

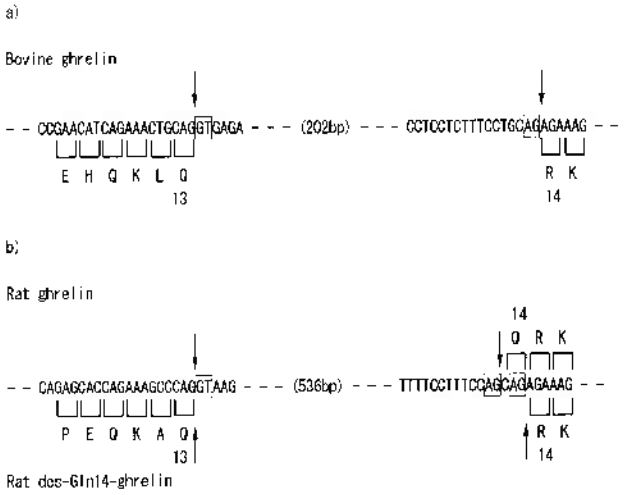

Figure 4. The comparison between the splice junction of bovine ghrelin gene (a) and that of rat (b).The arrows indicate the splicing signals, GT for the 5'-side and AGs for the 3'-side of the intron, are boxed.

an intron existed between Gln^{13} and Arg^{14} of ghrelin and that matured peptide of bovine ghrelin consisted of only 27 amino acids excluding Gln^{14}.

Figure 5 represented the influence of aging on plasma ghrelin concentration. Plasma ghrelin concentration increased after birth to approximate 600 days of age, and then reached the constant level.

DISCUSSION

We report here the characterization of the full-length of bovine ghrelin cDNA (Figure 1). The complete sequence of cDNA of the human (Kojima et al., 1999; GenBank accession no. AB029434), rat (Kojima et al., 1999; GenBank accession no. AB029433), mouse (GenBank accession no. AB035701) and swine (GenBank accession no. AB035703) have been identified so far. Although the partial sequence of bovine ghrelin cDNA has been cloned (GenBank accession no. AB035702), the complete sequence has not been clarified. The full-length bovine ghrelin cDNA sequence derived in the present study showed 488 bp long with 3 bp 5'UTR, followed by a 348 bp open reading frame and a 137 bp 3'UTR. Putative bovine prepro-ghrelin was consisted of 116 amino acids, which contained the 27-amino acid ghrelin.

A summary of putative amino acid sequence comparisons between several animal species is represented in Figure 2. The comparison of amino acid sequence of matured bovine ghrelin with that of swine showed 81.5% identity. Although the peptide sequence of matured ghrelin consisted of 28 amino acids in rat, mouse, swine and human, bovine ghrelin contained 27 amino acids in this study. This was agreed with previous report by Kojima et al. (2001).

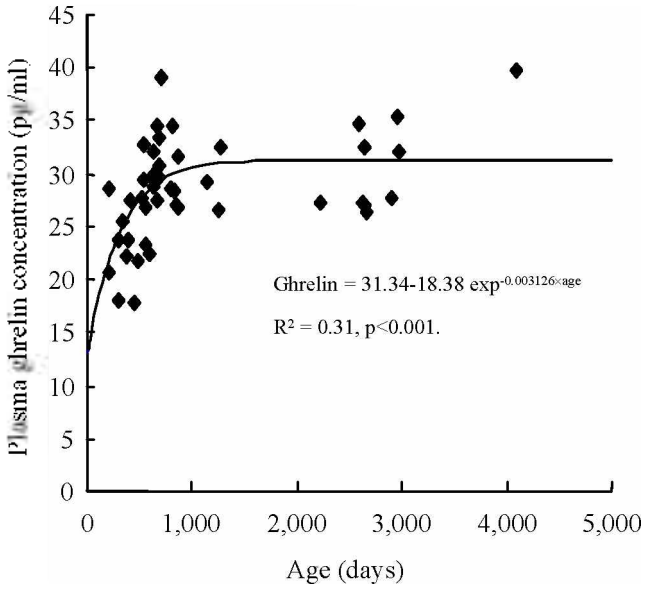

Figure 5. Influence of aging on plasma ghrelin concentration in cattle.

Recently, it was reported that a 27-amino acid peptide, whose sequence was identical to ghrelin except for one amino acid Gln^{14}, was purified and characterized as the second endogenous ligand of growth hormone secretagogue recepter in rats (Hosoda et al., 2000). In this report, genome sequence of rat ghrelin was also analyzed, and it was revealed that an intron existed between Gln^{13} and Gln^{14} and the 3'-end of the intron had two tandem CAG sequences. Two tandem CAG sequences of the exon-intron boundary matched the GT-AG rule of splicing mechanism (McKeown 1992), resulting in two types of ghrelin peptides coding 27 and 28 amino acids produced by alternative splicing. In the present study, we also analyzed the partial sequence of bovine ghrelin genome and revealed that the 3'-end of intron including only one CAG sequence existed between Gln^{13} and Arg^{14} (Figure 3). This finding indicated that matured peptide of bovine ghrelin consisted of only 27 amino acids excluding Gln^{14} without alternative splicing (Figure 4). As Kojima et al. (2000) reported that matured peptide of ovine ghrelin also had 27 amino acids like bovine, ruminants might have only one type of matured ghrelin peptide, des-Gln^{14}-ghrelin, differently from monogastric animals.

As shown in Figure 5, plasma ghrelin concentration increased after birth to approximate 600 days of age, and then reached the constant level. This is the first report to indicate the influence of ageing on the alteration of plasma ghrelin concentration in ruminants. It was reported that gene expression of rat ghrelin in stomach, which was thought to be the main site of ghrelin production, was very low in the postnatal period but then increased until 56 days of age (Sakata et al., 2002). Moreover, Rigamonti et al. (2002) reported that plasma concentration of ghrelin in young woman was significantly higher than that of old woman. From these results, it can be suggested that plasma ghrelin concentration increases rapidly after birth to sexual maturity, then reaches plateau and finally may decline gradually toward the late stage of life.

In the present study, it was revealed that an intron of bovine ghrelin gene existing between Gln^{13} and Arg^{14} suggested to be the existence of only one type of ghrelin peptide, des-Gln^{14}-ghrelin in ruminant species and that plasma ghrelin concentration increased after birth to approximate 600 days of age, and then reached the constant level.

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