

Biochemical, molecular and physiological characterization of a new β -casein variant detected in Korean Cattle

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Summary

There are seven known genetic variants of bovine β -casein (β -CN) – A1, A2, A3, B, C, D and E. In this study, we identified a new genetic variant (named β -CN H) which migrates slower than the other variants in acidic starch gel electrophoresis. We confirmed through protein and DNA sequence analyses that the H variant differs at five residues from the A2 sequence: Arg25/Cys, Leu88/Ile, Gln117/Glu, Glu175/Gln and Gln195/Glu. Of these substitutions the 25th residue was contained in the casein phosphopeptide (CPP) region. In rats, calcium solubilizing effect of the CPP of bovine variant H was increased by $\approx$ 23% compared with that of the CPP of non-H. Using extensive Korean *Bos taurus* pedigrees, we confirmed that β -CN H was controlled by a codominant allele.

Keywords: β -casein, variant, casein phosphopeptide region, Korean cattle

The primary structure of β -casein (β -CN) has been elucidated by Ribadeau-Dumas *et al.* (1972). Bonsing *et al.* (1988) reported the characterization and complete DNA sequence of the bovine β -CN. The β -CN A2 variant consists of a single polypeptide chain containing 209 residues, with a calculated molecular weight of 23 983. Shown in Table 1, the other genetic variants (A1, A3, B, C, D and E) differ from A2 by one to three residue substitutions (Grosclaude *et al.* 1972, 1974a,b; Egle *et al.* 1984). The casein phosphopeptide (CPP) is a tryptic hydrolysate of bovine casein including β -casein, and consists of a polypeptide chain containing 25 residues (Li *et al.* 1989). The CPP prevents precipitation of calcium phosphate and increases the concentration of soluble calcium *in vitro* and in the lumen of the small intestine (Meisel & Frister 1989; Sato *et al.* 1986).

In this paper, we report the protein and DNA sequence, genetic inheritance and biological function of the β -CN H variant. This new β -CN variant had the slowest migration in acidic starch gel electrophoresis when compared to other β -CN variants (A1, A2, A3 & B). In Korean cattle (*Bos taurus*) ten β -casein (*CSN2*) genotypes, A1A2, A1A3, A1B, A1H, A2A3, A2B, A2H, A3B, A3H and BH, were identified and the allele frequencies of A1, A2, A3, B and H were 0.21, 0.66, 0.05, 0.07 and 0.01, respectively. C, D and E alleles were not identified in the studied population.

In order to obtain protein sequence for the β -CN H variant, β -CN A2H and BH fractions were purified by FPLC using an anion exchange column (Mono Q, Pharmacia, Uppsala, Sweden) with imidazol-HCl buffer containing 3.3 M urea and 10 mM 2-mercaptoethanol. The β -CN A2, B and H fractions were divided using a cation exchange column (Mono S, Pharmacia, Uppsala, Sweden) with Na acetate buffer of pH 5.0 (Barrefors *et al.* 1985; Hollar *et al.* 1991). Purified β -CN H protein was digested by N-Tosyl-L-phenylalanine Chloromethyl Ketone (TPCK)-treated trypsin at 38 °C for 12 h in 0.1 M Na phosphate buffer (pH 7.5), and the reaction was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF). Enzymatic digests were analyzed by HPLC on a C18 column using a linear gradient from 100% solvent A (0.01% TFA) to 60% solvent B (0.01% TFA and 90% CH₃CN), and sequenced using an automatic protein sequencer (Applied Biosystems). Residues 31–209 from the N-terminal were characterized (PIR accession nos. A59068, B59068; see also Swiss-Prot: P02666). We found four substitutions of Leu88/Ile, Gln117/Glu, Glu175/Gln and Gln195/Glu compared to the A2 variant. However, protein sequence was not obtained for the N-terminal peptides (1–30 residues). Therefore, the sequence of this region was deduced from DNA sequence data.

DNA was prepared from whole blood according to Sambrook *et al.* (1989) from individuals carrying *CSN2* A2H and BH genotypes. We amplified 443 and 640 bp fragments of the β -

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Table 1. Location of amino acid substitution in β -casein variants

	Position of sequence											
Variants*	18	25	35	36	37	67	88	106	117	122	175	195
A1						His						
A2	Ser-P	Arg	Ser-P	Glu	Glu	Pro	Leu	His	Gln	Ser	Glu	Gln
A3								Gln				
B						His				Arg		
C			Ser		Lys	His						
D	Lys											
E				Lys								
H†		Cys					Ile		Glu		Gln	Glu

*Grosclaude *et al.* (1972 1974a,b); Egle *et al.* (1984).

†Present study.

casein gene (*CSN2*) which included exons 3, 4, 5 and 6 (Bonsing *et al.* 1988) using two primer sets: β -CN 1 sense strand, 5'-TACCCA-TAAAAACTCTTCATT-3'; antisense strand, 3'-CGACATAATAGAAAAACACAA-5'; β -CN 2 sense strand, 5'-CAACAGCCTTATTCAGAA-GAGTGG-3'; antisense strand 3'-GTCACCC-TACTGTCTTTCATCAGCATATCC-5'. Amplifications were performed with the Gene E thermal cycler (Techne LTD, U.K.). The PCR was performed using 5 μ l 10X PCR reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.8), 15 mM MgCl₂, and 1% Triton X-100], 200 μ M of each dNTP, 0.1 μ M of each primer, 1 unit of *Taq* DNA polymerase, and 20 ng genomic DNA. The volume was adjusted to 50 μ l with double-distilled, filter-sterilized water. Thirty cycles of 40 s denaturation at 94 °C, 40 s annealing at 57 °C and 40 s elongation at 72 °C were followed by an additional 5 min at 72 °C. The PCR products were directly sequenced using an automatic DNA sequencer (Applied Biosystems). In the results of *CSN2* HDNA sequencing (GenBank accession nos. AF104928 &

AF104929), the 13th base in the exon 5 was substituted from cytosine to thymine. This difference would result in residue Arg 25 (variant A2) being replaced with Cys. Because the Cys contains a sulfhydryl group, the first 30 residues could not be analyzed by protein sequencing. The β -CN H variant has a negative charge due to substitutions of five residues.

The region from the N-terminal to Arg 25 of β -CN contains three phosphoserines and two glutamines, which produce a significant negative charge. Thus this region has calcium solubilizing effects and is referred to as the casein phosphopeptide (CPP) region (Meisel & Frister 1989; Sato *et al.* 1986). The calcium solubilizing effect *in vivo* of the CPP H was measured as in Hirayama *et al.* (1992). Male Wistar rats (Charles River, Japan) weighing 120 g were offered a diet containing 44% corn starch, 20% potato starch, 5.0% cellulose powder, 5.0% plant oil, 20% protein, 5.0% mineral mixture, and 1.0% vitamins. The diet contained 0.56% calcium and 0.53% phosphorus. After 12 days on this diet, two groups

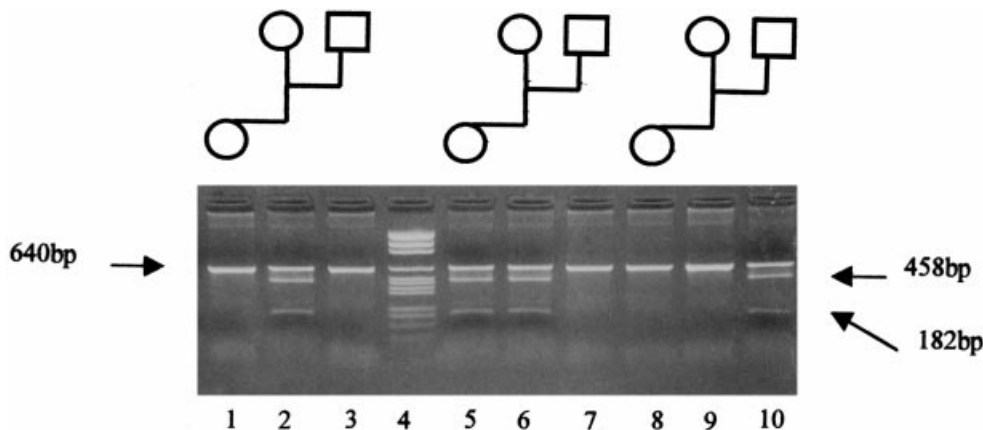


Fig. 1. Agarose gel electrophoresis of amplified DNA following digestion with *NisI*. Animals in lanes 1–3 and 5–10 are *CSN2* A2B, BH, A2A2, A2H, A2H, A2B, A2B, A2A2 and BH, respectively. Lane 4 is a molecular weight marker (pGEM DNA digested with *HinfI*, *RsaI* and *SinI*).

Table 2. Segregation ratio of the *CSN2* *H* allele in five matings of Korean cattle

Mating type			Genotypes of offspring							
Sire	Dam	<i>n</i>	<i>A2A2</i>	<i>A2B</i>	<i>BB</i>	<i>A2H</i>	<i>BH</i>	<i>HH</i>	χ^2	<i>P</i>
<i>A2A2</i> × <i>A2H</i>		15	8	0	0	7	0	0	0.0666	0.90 > <i>P</i> > 0.75
<i>A2H</i> × <i>A2A2</i>		10	4	0	0	6	0	0	0.4000	0.75 > <i>P</i> > 0.50
<i>A2A2</i> × <i>BH</i>		6	0	4	0	2	0	0	0.6666	0.50 > <i>P</i> > 0.25
<i>A2H</i> × <i>A2B</i>		7	2	2	0	2	1	0	0.4285	0.95 > <i>P</i> > 0.90
<i>A2H</i> × <i>A2H</i>		6	2	0	0	3	0	1	0.3333	0.75 > <i>P</i> > 0.50

of ten rats each were fed with one of two experimental diets, containing the bovine CPP H or CPP non-H fractions. Protein in the basal diet was substituted with 10% whey protein isolate and 10% bovine CPP H or CPP non-H. After 1 h from the end of feeding, the rats were anesthetized with ether and the entire small intestine was divided into two equal lengths containing the upper and lower intestine, respectively. The contents were centrifuged and the supernatants were analyzed for the amount of soluble calcium using an atomic absorption spectrometer (Perkin Elmer, USA). The mean concentration of soluble calcium in the CPP H group was 68.20 ± 2.2 p.p.m., which was 23.1% higher ($P > 0.05$) than that of the CPP non-H group (55.38 ± 2.1). In the lower intestine where 80% of the intestinal solubilized calcium is absorbed, the average concentration of soluble calcium in the CPP H group was 51.02 ± 3.1 p.p.m., which was 30% higher ($P > 0.05$) than that of the CPP non-H group (39.33 ± 2.8). The Arg 25/Cys substitution resulting in a change in charge and the addition of a sulfhydryl group could be involved in these differences. These results are interesting and require further study.

For the analysis of inheritance of the *CSN2* *H* allele, a mating experiment was performed in our experimental farm using 200 Korean cattle and the PCR-RFLP method was used to determine *CSN2* genotypes. The PCR products using the β -CN 2 primer pair were digested with 5 units of *NisI* enzyme as described by the manufacturer (Promega, USA). Digested fragments were separated by electrophoresis (80 V, 45 min) in 2% agarose gels and stained by EtBr (Fig. 1). DNA fragments containing the *CSN2* *H* allele were cleaved into fragments of 182 bp and 458 bp while genotypes including the alleles *A1*, *A2*, *A3* and *B* were not cut (Fig. 1). Family data for *CSN2* *H* are presented in Table 2. In the 44 offspring from five matings, 22 offspring were identified containing *CSN2* *H* in heterozygous and homozygous forms, and the segregation ratio of this allele in the offspring was in good agreement with the expected values, thereby confirming the codominant inheritance of the *CSN2* *H* allele.

References

- Barrefors P., Ekstrand B., Fagerstem L., Larsson-Raznikiewicz M., Schaar J. & Steffner P. (1985) Fast protein liquid chromatography (FPLC) of bovine caseins. *Milchwissenschaft* **40**, 257.
- Bonsing J., Ring J.M., Stewart A.F. & Mackinlay A.G. (1988) Complete nucleotide sequence of the bovine β -casein gene. *Australian Journal of Biological Science* **41**, 527.
- Eagle W.N., Butler J.E., Ernstrom C.A. *et al.* (1984) Nomenclature of proteins of cow's milk: Fifth revision. *Journal of Dairy Science* **67**, 1599–31.
- Grosclaude F., Mahe M.-F. & Mercier J.-C. (1974a) Comparaison du polymorphisme genetique des lactoproteines du zebu et des bovines. *Annales de Genetique et de Selection Animale* **6**, 305–29.
- Grosclaude F., Mahe M.-F., Mercier J.-C. & Ribadeau Dumas B. (1972) Caracterisation des variants genetiques des caseine α - et β bovines. *European Journal of Biochemistry* **26**, 328–37.
- Grosclaude F., Mahe M.-F. & Voglino G.-F. (1974b) Le variant β E et le code de phosphorylation des caseines bovines. *FEBS Letters* **45**, 3–5.
- Hirayama M., Toyota K., Hidaka H. & Naito H. (1992) Phosphopeptide in rat intestinal digests after ingesting casein phosphopeptides. *Bioscience, Biotechnology and Biochemistry* **56**, 1128–9.
- Hollar C.M., Law A.J.R., Dalgleish D.G. & Brown R.J. (1991) Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *Journal of Dairy Science* **74**, 2403.
- Li Y., Tom D. & Desjeux I.F. (1989) Indirect effect of casein phosphopeptides on calcium absorption in rat ileum *in vitro*. *Reproduction, Nutrition, Development* **29**, 227–33.
- Meisel H. & Frister H. (1989) Chemical characterization of bioactive peptides from *in vivo* digests of casein. *Journal of Dairy Research* **56**, 343–9.
- Ribadeau-Dumas B., Brignon G., Grosclaude F. & Mercier J.-C. (1972) Structure primaire de la casein β bovine. *European Journal of Biochemistry* **25**, 505–14.
- Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
- Sato R., Noguchi T. & Naito H. (1986) Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *Journal of Nutritional Science and Vitaminology* **32**, 67–76.