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SSCP polymorphism within 5' region of bovine lactoglobulin (LGB) gene

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Abstract. In the paper the detection of the SSCP polymorphism within the 5' fragment of bovine beta-lactoglobulin (LGB) gene is described. The 5' fragment of LGB gene (209 bp) was PCR-amplified and then subjected to electrophoresis allowing the detection of SSCP polymorphism. Among 124 animals (50 cows and 74 bulls) six SSCP patterns were identified and named R1, R2, R3, R4, R5 and R6, which occured with the frequency of 0.32, 0.51, 0.09, 0.06, 0.01and 0.01, respectively. The PCR-SSCP method is simple, fast, and relatively inexpensive. The SSCP polymorphism reported in the paper may be useful in looking for the associations between different SSCP patterns and LGB gene expression and milk properties.

Key words: beta-lactoglobulin, cattle, polymorphism, SSCP.

Introduction

Beta-lactoglobulin (LGB) is the major whey protein secreted in the cow's milk (FOX 1992) and is considered a quantitative trait locus in dairy cattle breeding (GRAML et al. 1989, JAKOB, PUHAN 1992, MAO et al. 1992).

The gene encoding bovine LGB is located on chromosome 11 at q 28 (HAYES, PETIT 1993, EGGEN, FRIES 1995). The complete genome sequence of bovine LGB gene has been characterised by ALEXANDER et al. (1993).

So far, 8 protein variants of LGB are known (A, B, C, D, E, F, G, W) (BELL et al. 1970, BRAUNITZER et al. 1973, BRIGNON, RIBADEAU-DUMAS 1973, BELL et al. 1981, CONTI et al. 1988, GODOVAC-ZIMMERMANN et al. 1990). The A and B alleles are the most common in Holstein cattle.

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Lately, several DNA variants located within the LGB 5' flanking region have been identified (ALEXANDER et al. 1993, WAGNER et al. 1994).

In this study we tried to find the SSCP (Single Stranded Conformation Polymorphisms) in the 5' region of LGB gene. Mutations located in this region might potentially influence the yield of gene transcription and in consequence, some properties of milk.

Material and methods

Fifty unrelated cows and 74 bulls of Polish Black and White cattle were included into the analysis. From each cow, 25 mL of milk were taken to isolate somatic cells which naturally occur in milk. The cells were washed 3 times in PBS buffer to remove milk. The cell pellet was then subjected to DNA isolation with the use of Wizard Genomic Purification Kit (Promega).

One semen portion has been washed 3 times in 1 mL of PBS buffer for 10 min and then centrifuged in the microcentrifuge for 5 min to remove semen plasma. The third washing was done in the solution of 980 μL of PBS buffer and 20 μL of Tween 20 for 30 min in the rotating wheel. The samples were then subjected to DNA isolation with Wizard Genomic Purification Kit.

Genomic DNA was used to amplify the fragment of LGB gene by PCR. PCR reaction mix was as follows: 3.0 μL 10 × PCR buffer (15 mM $MgCl_2$), 2.0 μL dNTP-mix (2 Mm each), 0.7 μL of primer R-LGB 1 and 0.7 μL of primer R-LGB 2 (100 pmol/μL each), 1 U Taq polymerase (Promega), 400 ng of DNA template and H_2O to a volume of 30 μL. PCR program was as follows: initial denaturation – 3 min 94°C followed by 35 cycles of: 30 sec 94°C, 30 sec 60°C, 40 sec 72°C and finished by 5 min in 72°C. Based on the sequence published by ALEXANDER et al. (1993) and using the software Primer3 available in Internet (server www.genome.wi.mit.edu), we designed the following PCR primes: R- LGB 1: 5' GCAGAGCCCTCGATACTGAC 3',

R- LGB 2: 5' GACAAGGACAGGTCAGAGCC 3'.

The PCR products were electrophoresed in 1.5% agarose/ethidium bromide gel. Gels were documented with GDS 7500 system (UVP).

The optimization of SSCP procedure was based on the rules described by ORITA et al. (1989) and TADASHI et al. (1993). The amount of 3.5 μL of PCR product was briefly mixed with 12.5 μL of the denaturation solution (50 mM NaOH, 1 mM EDTA) and 1 μL of loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol, denatured for 12 min at 85°C, rapidly chilled in ice block and then loaded onto precast polyacrylamide

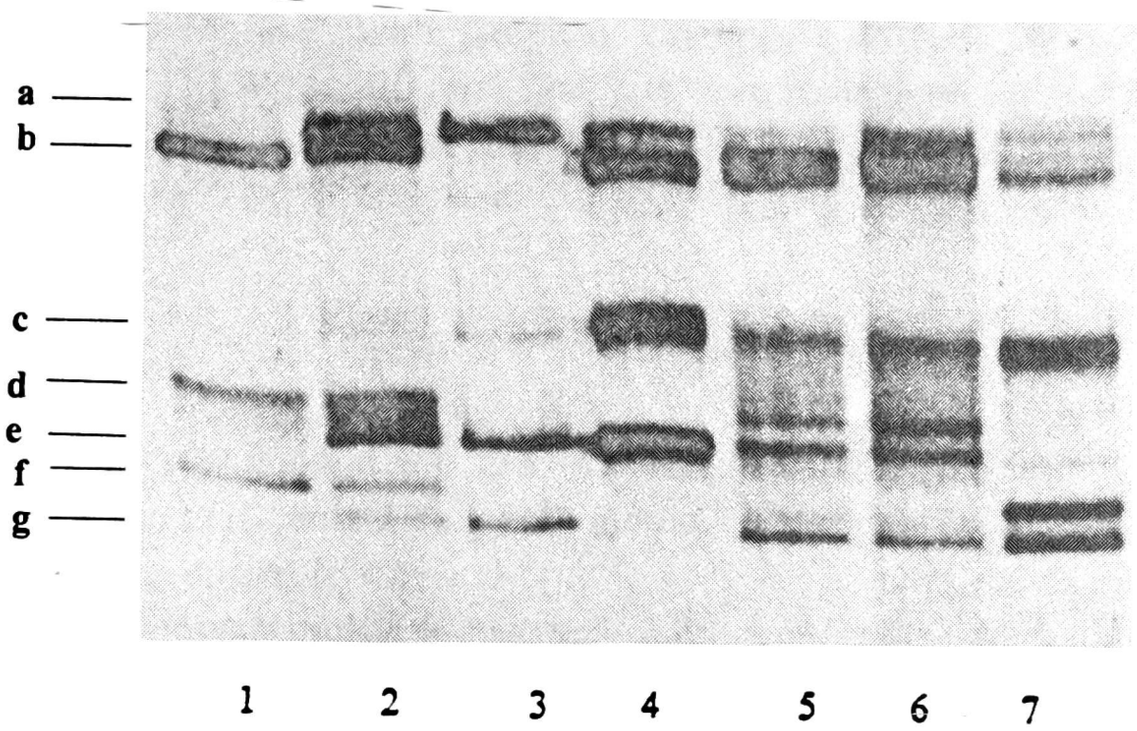

Figure 1. Detection of single stranded conformation polymorphism (SSCP) of 209 bp PCR products derived from 5' region of the bovine LGB gene. Six different SSCP patterns were detected, which are shown in the following lanes:

Lane 1 – R1 (bands: b, d, f), lane 2 – R2 (bands: a, b, d, e, f, g), lane 3 – R3 (bands: a, e, g), lane 4 – R4 (bands: a, b, c, e), lanes 5, 6 – R5 (bands: b, c, d, e, g), lane 7 – R6 (bands: b, c, f, g)

CleanGel 36S (ETC-Electrophorese Technik). The samples were electrophoresed in Multiphor II Electrophoresis System (Pharmacia Biotech). A termostatically controlled refrigerated circulator (MultiTemp III, Pharmacia Biotech) was used to maintain constant temperature of the gel (6°C). Electrophoresis was performed in Delect gel buffer (ETC). The gel was run in the following conditions: preelectrophoresis (without samples): 200 V for 10 min, electrophoresis (with samples): 200 V, 20 mA, 10 W for 20 min; 375 V, 30 mA, 20 W for 170 min. The gels were stained with Silver Staining Kit (Pharmacia Biotech). The patterns of SSCP bands were observed and photographed by GDS7500 System (UVP).

Results and discussion

The PCR-SSCP method was used to find polymorphism within 5' region flanking bovine LGB gene. Using PCR method, we were able to amplify DNA sequence of the desirable size 209 bp.

We found polymorphisms observed as the appearance of six distinctly different SSCP patterns, which we named R1, R2, R3, R4, R5 and R6 (Figure 1). The number of bands and their position in the gel show very clearly the occurrence of LGB gene polymorphism. Sometimes the gels showed additional weak bands comprised of reannealed double-stranded DNA or metastable "ghost" bands. These bands are the effect of incompletely denatured single strands of DNA. But this did not affect the distinguishing of six distinct SSCP patterns. In the preliminary studies, different polyacrylamide precast and manually prepared gels and buffers were tested. The best results were achieved using CleanGel 36S and Delect buffer (ETC).

Milk as a source of genomic DNA turned out to be relatively effective and convenient. DNA samples were suitable enough for effective PCR amplification. Sometimes unsuccessful DNA isolations occurred, most probably due to considerable differences in the number of somatic cells and other unidentified factors (LIPKIN et al. 1993).

We identified 40, 63, 11, 7, 2 and 1 animals with R1, R2, R3, R4, R5 and R6 SSCP pattern, respectively. The overall frequency of identified SSCP pattern was 0.32, 0.51, 0.09, 0.06, 0.01 and 0.01 for R1, R2, R3, R4 and R5 and R6, respectively. It was shown that R2 and R1 SSCP patterns are the most frequent ones.

So far, the results showing the polymorphisms within 5' fragment of bovine LGB gene have only been reported by WAGNER et al. (1994). They found

16 point mutations within entire 5' fragment of LGB gene and exon 1. In our experiment we decided to narrow the region to be analysed. We chose the part of the gene promoter (from –501 to –293, according to the sequence published by ALEXANDER et al. 1993). This region is the most conservative fragment in the entire 5' part of LGB gene (95% homology with ovine beta-lactoglobulin) (WAGNER et al. 1994). Additionally, it contains nearly all of the potential binding sites for hormone receptors and therefore may have particular influence on hormonal stimulation on LGB synthesis in mammary glands of ruminants. Five point mutations identified by WAGNER et al. (1994) are located within our PCR product. Four of these mutations occur within consensus sequences for hormonal receptors (TRE-thyroid receptor element, PRE-RC – progesterone receptor element reverse complement, AP-2 – activator protein 2, MAF – mammary cell activator factor, AP3 – activator protein 3). Our studies may help in explaining the phenomenon of allele-dependent synthesis of LGB heterozygous animals which was observed on protein level (MCLEAN et al. 1984, GRAML et al. 1989, KIM et al. 1996, NG-KWAI-HANG, KIM 1996) and in mRNA level (FORD et al. 1993).

In the screening studies, the method used to identify polymorphism is very important. In such studies, DNA sequencing, which was applied by WAGNER et al. (1994) is rather laborious and expensive. The PCR-SSCP method presented in this report is simple, fast, relatively inexpensive and available in most molecular biology laboratories. A disadvantage of the PCR-SSCP method is the requirement of a rather short PCR product (150-300 bp) and rigorous conditions of electrophoresis (SHEFIELD et al. 1993).

Further studies should show whether the identified SSCP polymorphisms are associated with different yield of LGB gene expression and with different milk properties.

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