Short communication: Characterization of a monoclonal antibody for κ -casein B of cow's milk¹

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ABSTRACT

A monoclonal antibody (antik-B) against an oligopeptide of 23 AA corresponding to the region 131–153 of bovine κ -casein (κ -CN) B was generated using the Human Combinatorial Antibody Library (HuCAL) technology. Both AA substitutions distinguishing κ -CN A and B are located in that region (positions 136 and 148). In this study, the reactivity of antik-B to milk samples collected from cows previously genotyped as *CSN3*AA*, *CSN3*AB*, and *CSN3*BB* was tested. According to Western blot results, antik-B recognized κ -CN B and it showed no cross-reactivity toward κ -CN A and other milk proteins. Furthermore, a modified Western blot method, urea-PAGE Western blot, was set up to assess the reactivity of antik-B toward all isoforms of κ -CN B. In conclusion, antik-B was specific to κ -CN B in milk and it seemed to be reactive toward all its isoforms.

Key words: bovine milk, κ -casein B, monoclonal antibody

κ -Casein, which represents approximately 15% of total CN (Alais, 1984), is the most interesting and important CN component in that it is centrally involved in the formation and stabilization of CN micelles, the coagulation of milk by rennet, and many other technologically important functions of the milk protein system. The functional duality of κ -CN, which is to interact hydrophobically with other CN and at the

same time provide a hydrophilic and negatively charged surface on the micelle to stabilize the colloidal suspension, is strikingly reflected by its amphipathic primary structure (Swaisgood, 2003). Its hydrophilic and flexible C-terminal part (caseinomacropeptide, or **CMP**) is cleaved specifically by chymosin (between residues Phe105 and Met106), thus leading to the destabilization of the micelle [to which the highly hydrophobic and insoluble N-terminal part (para- κ -CN) remains anchored (De Kruif and Holt, 2003)] and the formation of the curd.

Thirteen genetic variants of κ -CN have been identified to date (for a review, see Formaggioni et al., 1999). The 2 widely diffused genetic variants are designated A and B (Baker and Manwell, 1980). κ -Casein B differs from the A variant by substitution of an Ile residue for Thr at position 136 and an Ala residue for Asp at position 148 (Grosclaude et al., 1972). Compared with κ -CN A, κ -CN B is associated with higher casein content (Ng-Kwai-Hang et al., 1986) with higher κ -CN proportion (Kroeker et al., 1985; Mariani et al., 1995), resulting in smaller micelles (Nielsen et al., 1982; Donnelly et al., 1984; Pearse et al., 1986), which in turn leads to improved curd properties (Niki and Arima, 1984; Pearse et al., 1986) and higher cheese yield (Mariani et al., 1976; Walsh et al., 1998a,b).

Because of the remarkable effects exerted by the B variant of κ -CN on the cheesemaking process, it appears useful to introduce κ -CN content B into milk quality payment systems. For this purpose, it is necessary to point out a practical, fast, and reliable system that allows the quantification of the presence of the B variant at the level of bulk milk samples. An indirect competitive ELISA rapid test was recently submitted for patenting by the Italian Brown Cattle Breeders Association and the Swiss Brown Cattle Breeders Federation. The key element of the test is a monoclonal antibody (**antik-B**) that is specific to the sequence 131–153 of the B variant of cow's milk κ -CN.

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This paper reports the characterization of the immunological behavior of antik-B in individual milk samples from cows previously genotyped as BB, AB, or AA at the κ -CN locus. Using the Human Combinatorial Antibody Library (HuCAL; www.morphosys.com/en/technologies/hucal-136.html) technology, antik-B against the sequence 131–153 of bovine κ -CN B (GenBank accession number AAQ87923.1) was generated by Morphosys (Martinsried, Germany). The antibody was tested against the milk of 9 Italian Brown cows that were previously genotyped at the κ -CN locus as BB (3 cows), AA (3 cows), and AB (3 cows) by means of urea-PAGE (Andrews, 1986). Individual milk samples from the 9 selected cows were collected during morning milking. Raw milk samples were transferred to the laboratory within 15 min and analyzed immediately. Milk pH and SCC were determined with a potentiometer (Crison Instruments, Barcelona, Spain) and Fossomatic (Foss, Hillerød, Denmark), respectively. Skim milk was obtained as follows: raw milk was warmed to 40°C and kept at this temperature for 10 min, and then centrifuged at $1,800 \times g$ for 15 min; the resulting fat layer was removed by gentle aspiration with a vacuum pump. Rennet-whey samples were obtained according to Verma and Sommer (1957). Briefly, 2 mL of a rennet solution (1:100,000, Chr. Hansen, Corsico MI, Italy) was added to 100 mL of a milk sample kept at 37°C. Ten minutes after the visible formation of the coagulum, rennet whey was separated by filtration of the soluble phase on filter papers (Whatman 1, Whatman International Ltd., Maidstone, UK). To prepare milk + rennet samples, rennet was added to raw milk as described previously. Four aliquots of 200 μ L (at 0 and 10, 20, and 30 min after rennet addition) were transferred to Eppendorf tubes containing 800 μ L of a 15% TCA solution. Western blot (WB) analysis was carried out as follows: skim milk, rennet whey, and milk + rennet samples were electrophoresed through 12% SDS-PAGE according to Laemmli (1970) and blotted onto a polyvinylidene difluoride membrane. After blotting, the polyvinylidene difluoride membrane was saturated for 1 h at 37°C with 5% BSA in Tris-buffered saline Tween [TBST; 50 mM Tris/HCl, 150 mM NaCl, 0.1% (vol/vol) Tween 20; pH 7.5] buffer. Each membrane was rinsed 3 times with 15 mL of TBST. Primary incubation was performed overnight at 4°C with antik-B in TBST containing 1% BSA. Membrane was then probed at 37°C for 1 h with horseradish peroxidase-labeled anti-human immunoglobulin G F(ab') antibody (AbD Serotec, Martinsried, Germany) in TBST containing 1% BSA and then visualized by enhanced chemiluminescence (ECL Kit, Pierce, Rockford, IL). Oligopeptides of 23 AA corresponding to the sequence 131–153 of both κ -CN

B and A were used as positive and negative controls, respectively, in WB analysis. Both oligopeptides were conjugated to transferrin (TRF) and were produced by Morphosys. A modified WB procedure (urea-PAGE WB) was performed as well. In this method, skim milk samples were electrophoresed through urea-PAGE according to Andrews (1986). Prior to blotting, the gel was equilibrated as follows: 15 min in 50 mL of 1M Tris (pH 8), 10 min in 50 mL of 10 $\times$ transfer buffer (25 mM Tris, 196 mM glycine, 10% methanol), and 10 min in 50 mL of transfer buffer containing 20% methanol. After this step, the gel underwent standard WB procedure.

Milk pH values (mean = 6.71, minimum = 6.62, maximum = 6.76) were in the normal range for cow's milk. Eight out of 9 milk samples showed an SCC below 400,000 cells/mL, which is the European threshold limit for milk destined for cheesemaking. One milk sample was characterized by a somatic cell value above this limit (552,000 cells/mL). However, because both the milk sample and cow's udder did not show clinical signs of mastitis and because at this somatic cell level κ -CN should not be affected by proteolysis, this sample was also analyzed. The WB analysis of negative and positive control, skim milk, and rennet whey from *CSN3*BB*, *CSN3*AB*, and *CSN3*AA* cows is reported in Figure 1. A specific peptide (**pep1**) was detected by antik-B in skim milk samples from *CSN3*BB* and *CSN3*AB* cows. According to SDS-PAGE migration profile, pep1 was positioned in the zone of κ -CN and it was detectable only in milk samples from cows carrying the B allele at the *CSN3* locus. Furthermore, the band corresponding to pep1 was more clearly marked in the milk sample from the *CSN3*BB* cows than in that from the *CSN3*AB* cows. On the basis of these observations, pep1 could be hypothetically assigned to κ -CN B. A second peptide (**pep2**) was recognized by antik-B in rennet whey from *CSN3*BB* and *CSN3*AB* cows. According to migration pattern, pep2 should be smaller than pep1. Because pep2 was detected only in rennet whey of *CSN3*BB* and *CSN3*AB* cows, it could be hypothetically assigned to CMP arising from the cleavage of κ -CN B by chymosin. In fact, the antibody recognizes a sequence (131–153) that is included in CMP fragment. No peptides were recognized by antik-B in skim milk and rennet whey samples from the *CSN3*AA* cows. To verify the hypothetical assignation of pep1 and pep2 to κ -CN B and the corresponding CMP, respectively, the milk sample from the *CSN3*BB* cows was treated with rennet (Figure 2). In normal milk, the formation of curd takes place when >90% of the κ -CN is specifically hydrolyzed by the chymosin (the clotting enzyme present in rennet; Dalgleish, 1979); in the conditions adopted here, it takes about 20 min.

Figure 1. Characterization of a monoclonal antibody specific to bovine κ -CN B: Western blot of individual milk samples and rennet whey. Lane 1: oligopeptide of 23 AA, corresponding to the region 131–153 of κ -CN A, conjugated to transferrin. Lane 2: oligopeptide of 23 AA, corresponding to the region 131–153 of κ -CN B, conjugated to transferrin. Lanes 3 and 4: rennet whey and skim milk, respectively, from a *CSN3*AB* cow. Lanes 5 and 6: rennet whey and skim milk, respectively, from a *CSN3*BB* cow. Lanes 7 and 8: rennet whey and skim milk, respectively, from a *CSN3*AA* cow. pep = peptide.

In this experiment, rennet was added to milk and 4 aliquots were collected at 0 and 10, 20, and 30 min after rennet addition. Each aliquot was immediately treated with TCA to denature and precipitate proteins and, thus, stop reaction between κ -CN and chymosin.

Figure 2. Characterization of a monoclonal antibody specific to bovine κ -CN B: Western blot of an individual milk sample from a *CSN3*BB* cow analyzed at 10-min intervals from rennet addition. Lane 1: milk added with rennet (milk + rennet) at time 0. Lane 2: milk + rennet after 10 min. Lane 3: milk + rennet after 20 min. Lane 4: milk + rennet after 30 min. Lane 5: rennet whey from a *CSN3*BB* cow. Lane 6: oligopeptide of 23 AA, corresponding to the region 131–153 of κ -CN B, conjugated to transferrin. Lane 7: oligopeptide of 23 AA, corresponding to the region 131–153 of κ -CN A, conjugated to transferrin. pep = peptide.

Figure 3. Characterization of a monoclonal antibody specific to bovine κ -CN B: urea-PAGE Western blot of individual milk samples. Lane 1: skim milk from a *CSN3*AA* cow. Lane 2: skim milk from a *CSN3*BB* cow. Lane 3: skim milk from a *CSN3*AB* cow.

The aliquots were then analyzed in SDS-PAGE WB with antik-B. The result of this experiment is reported in Figure 2. As time from rennet addition increased, pep1 decreased in intensity, and it disappeared after 30 min. On the other hand, pep2, which was not detectable at time 0, increased in intensity from 10 to 30 min after rennet addition. According to these results, pep1 and pep2 corresponded to native κ -CN B and the correspondent CMP, respectively. To complete the characterization of the monoclonal antibody, its reactivity toward all the isoforms of κ -CN present in milk was checked as well. It is well known that the major fraction of κ -CN is present in a nonglycosylated form (Mariani et al., 1995). However, even if in a lesser amount, there are minor fractions of κ -CN with a different degree of glycosylation (isoforms). To employ the monoclonal antibody in a quantitative method, it was necessary to test the affinity of the antibody toward all the isoforms of κ -CN B. To this end, milk samples from *CSN3*BB* and *CSN3*AA* cows were analyzed in urea-PAGE WB (Figure 3). The migration patterns of κ -CN and its glycosylated isoforms in urea-PAGE electrophoresis were reported by Egito et al. (2001). These authors proved that in urea-PAGE κ -CN isoforms give rise to several fast-migrating bands compared with the nonglycosylated form. The presence of several bands was caused by the different degree of glycosylation of κ -CN. According to these electrophoretic profiles, if the antibody was specific against only 1 isoform of κ -CN, a discrete band (as the one observed in SDS-PAGE WB) was expected to appear. Otherwise, if the antibody recognized all the glycosylated forms of κ -CN, a smear band was expected. A smear band was observed in milk

samples with the B variant of κ -CN (lane 2, Figure 3). According to its intensity, the reaction of the monoclonal antibody was clearly higher in κ -CN BB milk (lane 2, Figure 3) than in κ -CN AB milk (lane 3, Figure 3). The differences of intensity were probably related to the higher content of κ -CN B in milk from *CSN3*BB* cows than in milk from *CSN3*AB* cows. The shape of the band (a smear) is likely a result of the reaction of the monoclonal antibody with the different isoforms of κ -CN B. The AA sequence 131–153 of κ -CN B used to select the monoclonal antibody is shared by the variants C (Miranda et al., 1993) and J (Mahé et al., 1999) of κ -CN. Furthermore, the variant B² (characterized only at cDNA level) differs in that region for a single AA substitution: Thr in 153 (Gorodetskij and Kaledin, 1987). These variants are potentially recognizable by antik-B, particularly C and J. The presence of κ -CN C was reported with very low frequencies in milk of Grey Alpine (Di Stasio and Merlin, 1979) and Italian Brown (Caroli et al., 2004) cows (0.007 and 0.003, respectively), and it was not detected at all in milk of Italian Friesian cows (Caroli et al., 2004). Other potentially cross-reactive variants—B² and, in particular, J—are rare variants in dairy cattle, detected in populations with very limited diffusion and with low allele frequencies. For these reasons, the possibility of overestimating the content of κ -CN B in bulk milk samples owing to the presence of potentially recognizable genetic variants should be considered very low. Based on the results, the monoclonal antibody, employed in the ELISA test, is specific toward κ -CN B and its isoforms, and it shows no cross-reactivity toward κ -CN A and other milk proteins. This would permit the introduction of κ -CN B content into quality payment systems for cheese milk. For example, in Parmigiano-Reggiano cheese production, the current pricing of milk is based on quantity of milk delivered to the cheese farm and on a milk quality payment system at herd milk level. The quality score is assessed on the basis of the values of some herd milk characteristics (mainly fat and casein content, titratable acidity, total bacterial count, and somatic cell content) recorded each 15 d. Because of the positive effects on milk processability, cheese yield, and quality, κ -CN B content would be a promising parameter to introduce into milk quality payment system scoring.

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