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Detection of cow milk adulteration in yak milk by ELISA

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ABSTRACT

In the current study, a simple, sensitive, and specific ELISA assay using a high-affinity anti-bovine β-casein monoclonal antibody was developed for the rapid detection of cow milk in adulterated yak milk. The developed ELISA was highly specific and could be applied to detect bovine β-casein (10–8,000 μg/mL) and cow milk (1:1,300 to 1:2 dilution) in yak milk. Cross-reactivity was <1% when tested against yak milk. The linear range of adulterant concentration was 1 to 80% (vol/vol) and the minimum detection limit was 1% (vol/vol) cow milk in yak milk. Different treatments, including heating, acidification, and rennet addition, did not interfere with the assay. Moreover, the results were highly reproducible (coefficient of variation <10%) and we detected no significant differences between known and estimated values. Therefore, this assay is appropriate for the routine analysis of yak milk adulterated with cow milk.

Key words: yak milk, adulteration, monoclonal antibody, indirect competitive ELISA

INTRODUCTION

Yak milk is an important income source for rural residents of Qinghai-Tibetan Plateau, China. As a result of its high levels of proteins, lactose, conjugated linoleic acids, and calcium, yak milk is considered to be a naturally concentrated milk (Sheng et al., 2008; Li et al., 2011; Livingstone et al., 2012). Yak milk and yak milk-derived products (e.g., yogurt and cheese) have become increasingly popular in recent years (Bai et al., 2011; Nikkhah, 2011). Yak is a species that lives in mountainous areas of Central Asia, at an altitude of 2,500 to 5,500 m (Zi et al., 2008). Yak lactation is seasonal, thus, yak milk production is quite limited (Wiener et al., 2003). Therefore, for legal, consumer protection, and consumer confidence reasons, it is important to be able to rapidly detect the presence of cow milk in yak milk to ensure yak milk quality.

To date, several analytical approaches have been developed to detect the adulteration of milk of different species in dairy products (Moatsou and Anifantakis, 2003; Hurley et al., 2004b). Infrared spectroscopy was applied to detect the adulteration of goat or sheep milk with cow milk (Rodriguez-Otero et al., 1997; Nicolaou et al., 2010). High-performance liquid chromatography coupled with electrospray ionization mass spectrometry was used to detect cow milk in goat milk (Chen et al., 2004). Brescia et al. (2004) used a nuclear magnetic resonance method to differentiate buffalo and cow milk samples according to species. Lee et al. (2004) detected the adulteration of cow milk in goat milk by PAGE. Two-dimensional electrophoresis was applied to detect bovine milk in buffalo Mozzarella cheese (Chianese et al., 1990). Enzyme-linked immunosorbent assay is a rapid, sensitive, and specific method that is widely used in the food industry (Giovannacci et al., 2004). Adulterated goat, sheep, or buffalo milk and milk products have been successfully detected by ELISA (García et al., 1990; Rodríguez et al., 1991; Haza et al., 1997, 1999; Hurley et al., 2004a, 2006; Song et al., 2011). However, no studies have applied ELISA to the detection of yak milk adulterated with cow milk.

To differentiate among different milk species, it is important to select species-specific antigens. Compared with whey protein, casein has higher heat stability, and β-CN has the highest specific antigenicity among caseins (Anguita et al., 1996). Therefore, we selected bovine β-CN as the target antigen in this study. The objectives of this study were to produce a high-affinity monoclonal antibody (**mAb 1–9B**), which is specific against bovine β-CN, and to develop a rapid and sensitive indirect competitive ELISA for the detection of cow milk in yak milk. Furthermore, we assessed the effect of different treatments (heat, acidification, and rennet addition) on the performance of the ELISA.

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MATERIALS AND METHODS

Reagents and Materials

Bovine β-CN (purity ≥98%), Freund's complete and incomplete adjuvant, hypoxanthine-thymidine, hypoxanthine-aminopterin-thymidine selective medium, 3,3′,5,5′-tetramethyl benzidine (**TMB**) liquid substrate, and Tween-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol (PEG) 4000 was obtained from Merck (Darmstadt, Germany), and Dulbecco's modified Eagle's medium (DMEM) culture medium was purchased from Invitrogen (Grand Island, NY). Peroxidase-conjugated Affinipure goat anti-mouse IgG (H^+L) was obtained from ZSGB-BIO (Beijing, China), and 96-well ELISA and cell culture plates were purchased from Costar (Cambridge, MA). The SP 2/0 myeloma cells were produced in our laboratory. Rennet (Stamix 1150, 1,070 international milk-clotting units/g) was purchased from Chr. Hansen (Hørsholm, Denmark). All other reagents were of analytical pure grade (Beijing Chemical Works, Beijing, China).

Fresh yak milk was obtained from Wushaoling town of the Tianzhu grassland, on the Qinghai-Tibetan Plateau. Fresh cow milk was obtained from Beijing San Yuan Foods Co. Ltd. (Beijing, China). Before analyses, all raw milk samples were first stored at 4°C and then frozen at −20°C within 1 h. Before the experiments, milk samples were skimmed by centrifugation at 3,000 × *g* for 20 min at 4°C and filtered through glass wool to remove lipids.

Immunization

The antigen solution was prepared by resuspending bovine β-CN in PBS (0.5 mg/mL) and stored at −20°C. The antigen solution was emulsified with an equal volume of Freund's complete adjuvant and injected (50 μL) both subcutaneously and intraperitoneally into female BALB/c mice (6–8 wk old). Booster injections with Freund's incomplete adjuvant were performed 3 times with the same dosage at 2-wk intervals. Blood samples were obtained from the tail 7 to 10 d after each booster injection. Antiserum was obtained by centrifugation at 10,000 × *g* for 3 min at 4°C.

Monitoring Antibody Titers by Indirect ELISA

The collected antiserum was assayed by indirect ELISA for antibody titer: each ELISA well was coated with 100 μL of bovine β-CN diluted in coating buffer (15 m*M* Na_2CO_3 and 35 m*M* $NaHCO_3$, pH 9.6) at 37°C for 2 h. Following the 2-h incubation, the wells were washed 3 times with PBS plus 0.05% Tween 20 (150 μL/well; **PBST**) for 2 min on a plate shaker and coated with 100 μL of 5% defatted milk for 1 h at 37°C. Following 3 successive washes with PBST, 100 μL/well of antiserum diluted in antibody diluents (PBST with 0.1% gelatin) was added to the wells and the plates incubated for 1 h at 37°C. The plates were washed as described above. Following the addition of 100 μL of peroxidase-conjugated Affinipure goat anti-mouse IgG diluted 1:5,000 in antibody diluent, the plates were incubated for 30 min at 37°C and washed with PBST. Then, TMB was added to each well (100 μL/well) and the plates were incubated for 15 min at 37°C. The reaction was stopped with 2 *M* H_2SO_4 (50 μL/well). Absorbance was measured by using a microplate reader (model 680, Bio-Rad, Hercules, CA) at 450 nm.

Production of the Monoclonal Antibody

The mouse with the highest antibody immunization titer was injected intraperitoneally with 100 μL of antigen solution without adjuvant 3 d before fusion. The spleen was removed for hybridoma production (Devi et al., 1999). After fusion, hybridoma cells were screened by indirect ELISA for the presence of antibodies against bovine β-CN. Positive hybridoma cells were also tested against casein extracted from yak milk. Then, hybridomas selected to produce specific antibody were subcloned by the limiting dilution method (Köhler and Milstein, 1975). Cells with the highest antibody titer and specificity were selected during each subcloning until a single positive hybridoma was obtained. Subcloning was repeated 5 times to obtain positive monoclonal antibody (**mAb**)-producing cells. Monoclonal antibody 1–9B was produced using the mouse ascites method (Cho et al., 2005). Animals were killed by cervical dislocation under the anesthesia. All animal maintenance and experimental procedures were conducted in accordance with the principles and specific guidelines presented in *Guidelines for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010) and approved by the Animal Ethics Committee of China Agricultural University. The resulting antibodies were purified by ammonium sulfate precipitation (Walker, 1996).

Detection of β-CN and Cow Milk and Assessment of Cross-Reactivity

Following optimization of the assay by checkerboard titration, mAb 1–9B was used to detect β-CN and cow milk by indirect competitive ELISA.

The inhibitory concentration was calculated as follows: % inhibition = $\%B/B_0$, where B and B_0 are the absorbance in the presence and absence of the com-