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Determination of bovine lactoferrin concentrations in cheese with specific monoclonal antibodies

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Abstract

In order to determine bovine lactoferrin concentration in cheese, bovine lactoferrin-specific monoclonal antibodies have been raised and an ELISA has been developed to determine lactoferrin concentrations in milk, whey and experimental soft, semi-hard and Swiss-type cheeses made with raw or pasteurised milk. The lactoferrin concentration in cheese was shown to depend on the cheese-making process, with higher values in Swiss-type and semi-hard cheeses than in soft cheeses. Furthermore, Western-blotting analysis of lactoferrin in cheese showed that this protein stayed intact throughout ripening in raw milk cheese, whereas it was partially hydrolysed in cheeses made with pasteurised milk. Based on these observations, we propose that cheese may constitute a natural dairy source of lactoferrin beneficial to health.

Keywords: Lactoferrin; Pasteurisation; Cheese; Ripening; Bovine lactoferrin-specific monoclonal antibody

1. Introduction

Lactoferrin is an approximately 80 kDa glycoprotein found in milk of various mammals and other secretions including tears, saliva, seminal fluids, mucins and the secondary granules of neutrophils (Farnaud & Evans, 2003). Bovine milk contains between 20 and 200 $\mu g\,mL^{-1}$ of lactoferrin, depending on the lactation period (Law & Reiter, 1977).

Lactoferrin possesses two metal-binding sites, each of which is able to bind a ferric ion (Fe^{3+}) together with a bicarbonate ion (HCO^{3-}) (Baker, Baker, & Kidd, 2002). In the native state, lactoferrin is only partly saturated with iron (15–20%), which is physiologically important because iron can be chelated and thus inhibit bacterial growth by an iron chelating effect (Vorland, 1999; Steijns & van Hooijdonk, 2000). Apo-lactoferrin (iron free) has a bactericidal effect against a wide range of microorganisms: Gram-positive, Gram-negative as well as yeasts. Some peptides generated from lactoferrin hydrolysis, such as lactoferricin (Bellamy, Takase, Wakabayashi, Kawase, & Tomita, 1992; Wakabayashi, Takase, & Tomita, 2003) and lactoferrampin (Van der Kraan et al., 2004), have been shown to exhibit microbicidal activity.

Besides its effect on microbial growth, lactoferrin is known to possess a diverse range of biological properties, including immuno-modulating and anti-inflammatory properties (Farnaud & Evans, 2003; Legrand, Elass, Pierce, & Mazurier, 2004). Furthermore, Van Belzen (2002) pointed out that lactoferrin probably lowered the risk of cancer.

For these reasons, lactoferrin, purified from bovine milk or cheese whey, is nowadays commercially available for human nutrition throughout the world. Dairy products, such as cows' milk, are also natural sources of lactoferrin. However, in many dairy products, lactoferrin is likely to be extensively denatured by the industrial production process. For instance, lactoferrin is totally heat-denatured by UHT treatment (Paulsson, Svensson, Kishore, & Naidu, 1993).

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It is also quite probable that heating of milk will denature lactoferrin during yoghurt manufacture. Whilst lactoferrin has been quantified in cheese whey, in cheese itself, to our knowledge, lactoferrin has never been quantified.

Lactoferrin has been quantified in milk using different techniques such as RP-HPLC (Palmano & Elgar, 2002) and capillary electrophoresis (Riechel et al., 1998). Several immunoassays have also been developed for this purpose. A competitive ELISA for lactoferrin quantification in biological fluid was reported to be more sensitive than chromatographic or electrophoretic techniques (Shinmoto, Kobori, Tsushida, & Shinokara, 1997). This immunoassay showed a detection limit of 10 ng mL^{-1}. Recently, Indyk and Filonzi (2005) developed a quantitative immunoassay on an optical biosensor and applied it to lactoferrin determination in milk, colostrums and infant formulas. Since lactoferrin is probably present at very low concentrations in cheese, immunoassays seem to be the most appropriate technique for lactoferrin determination in cheese.

The objective of the present study was to quantify lactoferrin in milk and cheese. To reach this goal, we developed a sensitive sandwich ELISA using a monoclonal and a polyclonal antibody. Lactoferrin was found in all cheeses manufactured in our pilot plant. The lactoferrin concentration in cheese was shown to depend on the cheese-making process used, but not on the heat treatment applied to milk prior to manufacture.

2. Material and methods

2.1. Chemicals

Unless otherwise stated, analytical grade chemicals were purchased from Sigma (Sigma-Aldrich, Saint-Quentin Fallavier, France).

2.2. Cheese manufacture

Experimental soft, semi-hard and Swiss-type cheeses were manufactured in duplicate at pilot plant scale using either 11.65 L of raw milk (1-d-stored milk collected in a dairy plant located near INRA) or 11.65 L of the same raw milk pasteurised for 15 s at 72 °C, producing a total of 12 cheeses.

Soft cheeses were manufactured using MA 400 (blend of *Lactococcus lactis* and *Streptococcus thermophilus* strains) and MY 800 (blend of *S. thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and subsp. *lactis* strains) as starters. KL 71 (*Kluyveromyces lactis*) and Pen. Neige (*Penicillium candidum*) were used as secondary starters (Danisco France, Dangé Saint Romain, France). Before analysis, they were ripened for 10 d at 12.5 °C followed by 20 d at 7 °C. Semi-hard cheeses were manufactured using MA 400, MY 800 as starters and were ripened for 32 d at 12.5 °C. Finally, Swiss-type cheeses were manufactured using SRTA mesophilic starters at 0.1%, *S. thermophilus* (STC) at 0.2% and *L. helveticus* (LBA) at 0.1% as starters (INRA, Poligny, France). They were ripened for 32 d at 12.5 °C, followed by 42 d at 17 °C and 49 d at 7 °C. Milk clotting was achieved with 2.15, 3.5 and 3.5 mL calf rennet containing 700 mg L^{-1} of chymosin for soft, semi-hard and Swiss-type cheeses, respectively.

During cheese manufacture, whey was collected for lactoferrin determination and was weighed. Cheese weight was also determined before ripening.

2.3. Polyclonal antibodies

Polyclonal antibodies (Pabs) directed against bovine lactoferrin were produced according to the procedure previously described (Senocq, Dupont, Rolet-Repecaud, Faurie, & Levieux, 2001).

2.4. Production of monoclonal antibodies

Female BALB/c mice (IFFA-CREDO, St-Germain sur l'Arbresle, France) were immunised with 20 μg of bovine lactoferrin (approximately 90%) suspended in complete Freund adjuvant (Difco laboratories, Detroit, MI, USA), distributed equally between the rear foot pads. After 14 d, mice were immunised using the same procedure in incomplete Freund adjuvant (Difco laboratories). On day 17, draining lymph popliteal nodes were removed and pooled. The polyclonal sera of the mice were collected and tested. Fusion of lymphocytes and myeloma cells SP2-O-Ag14 (Shulman, Wilde, & Köhler, 1978) was carried out with 1 mL of 45% PEG 1000 following the procedure described by Köhler and Milstein (1975). Supernatants of hybrid clones were assayed by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) (see below).

Ascites from interesting monoclonal antibodies (Mabs), Lf-2 and Lf-10, were obtained according to the procedure of Jones, Cox, and Pearson (1990).

2.5. Characterisation of Mabs

2.5.1. By ACP-ELISA

This method was used to test the hybrid clones against bovine lactoferrin and to determine possible cross-reactions of the antibodies. Briefly, 100 μL lactoferrin or other milk proteins at 0.5 μg mL^{-1} in 0.1 M sodium bicarbonate buffer, pH 9.6, were coated onto a microtitre plate (NUNC F96 Maxisorp, Dominique Dutscher, Brumath, France) and incubated for 90 min at 37 °C. The remaining binding sites were blocked by incubating 250 μL bovine gelatin (VWR International, Fontenay-sous-bois, France) at 10 g L^{-1} in phosphate-buffered saline (PBS), 0.05% Tween 20 (PBS–T) for 1 h at 37 °C. Culture supernatants (50 μL) were diluted 1:2 in PBS–T and incubated for 1 h at 37 °C. Bound immunoglobulins were detected by incubating 100 μL of donkey anti-mouse immunoglobulin alkaline