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Short communication

An immunoassay to assess lamb and kid rennets authenticity

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ABSTRACT

The potential adulteration of kid or lamb rennet with calf rennet is of interest for some Protected Designation of Origin cheeses producers and those looking for a specific cheese typicality. The approach proposed here for the authentication of kid or lamb rennet is based on the immuno-detection of bovine pepsin possibly present in calf rennet in varying quantities. The developed immunoassay (indirect ELISA) used a monoclonal antibody (mAb) raised against bovine pepsin. This mAb was found to be specific as it didn't cross-react with the pepsin of animal species other than bovine (kid, lamb, pig) and with other milk-clotting enzymes (chymosin and microbial enzymes). Adulteration tests were conducted with kid and lamb rennets spiked with a wide range of calf rennet (from 0 to 100% v/v). The presence of bovine pepsin was detected at low levels down to 6 mg/L in kid and lamb rennets. Good linear relationships were obtained between added bovine pepsin concentration and the absorbance values over the range 1.25–120 mg/L. Results showed that indirect ELISA proved to be an interesting tool for testing rennets authenticity targeting bovine pepsin as an indicator of the bovine adulteration of kid and lamb rennets.
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1. Introduction

In cheese production, milk is coagulated by addition of an enzyme preparation containing at least one aspartic proteinase inducing destabilization and aggregation of milk casein micelles. In traditional and Protected Designation of Origin (PDO) cheese making, the most widely used coagulant enzyme preparations are rennets extracted from the abomasa of suckling ruminants, calf, kid, and lamb, with calf rennet being the most widely used. Kid and lamb rennets are very similar to calf rennet. They are principally used in Southern Europe where goats and ewes are the main dairy animals. They are best suited for clotting milk of their own species (Chitpinityol & Crabbe, 1998; Foltmann, 1993).

Rennets are commercially available from a number of rennet companies in a variety of liquid, powder or pastes formulations, or they are still prepared on a small scale by cheese makers using traditional local procedures and for their own use (Jacob, Jaros, & Rohm, 2011). Their price depends on the supply which in turn has to do with events on the global market for cow, goat, and lamb meat products. In France, according to current regulation (decree n°69-475 of May 14th, 1969, Journal Officiel de la République Française), a commercialized rennet can be produced from one animal species (calf, kid, or lamb) or from a mixture of animal species. Technological process, together with the species origin of milk or the preferred cheese typicality can affect the cheese maker's choice on rennet depending on its composition. Moreover, specifications of some PDO cheeses can stipulate the animal origin of rennets to be used for manufacturing. However, rennet producers have no legal obligation to specify if the rennet results from abomasa of the same animal species or from different animal species abomasa in mixture or from different animal species rennets in mixture (Granday, 2015). Today, the authenticity of the animal source of rennet is not guaranteed by reliable analytical methods.

A variety of established and specialized analytical methods have been used for testing food authenticity. These methods have been described in several reviews dealing with different food (Aparicio, Morales, Aparicio-Ruiz, Tena, & García-González, 2013; De la Fuente & Juarez, 2005; Primrose, Woolfe, & Rollinson, 2010; Reid, O'Donnell, & Downey, 2006; Sentandreu & Sentandreu, 2014). Among these methods, immunoassays have proved to be useful in detecting different types of molecules recognized as suitable

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indicators of food authenticity. Immunoassays, in particular Enzyme-linked immunosorbent assays (ELISA), are sensitive tests that use the ability of antibodies to bind to an extremely wide range of biomolecules, low- and high-molecular-weight, with a remarkable specificity which enables low concentration of analyte to be assayed in the presence of many closely related substances (Asensio, González, García, & Martín, 2008). It has successfully been applied to milk adulteration for the identification of milk species in milk mixtures and cheese (Pizzano, Nicolai, Manzo, & Addeo, 2011). Adulterated buffalo, donkey, ewe, goat, or yak milk has been detected using ELISA (Hurley, Coleman, Ireland, & Williams, 2006; López-Calleja et al., 2007; Pizzano & Salimei, 2014; Ren et al., 2014; Song, Xue, & Han, 2011). The performance of such assay relies on the ability of the raised antibodies to specifically detect the target molecule characteristic of a particular animal species. As gastric proteinases are highly homologous proteins (Chitpinityol & Crabbe, 1998), one proteinase from different animal species may show pronounced immunochemical cross-reactions due to common antigenic determinants, giving rise to false positive cases. This limitation should be overcome to develop reliable immunoassays.

Rennets mainly contain two proteinases, chymosin and pepsin, in varying amounts depending on the age and the previous diet of the animals from which the extract is obtained (Robinson & Wilbey, 1998, pp. 146–164). Commercialized calf rennets contain from 50 mg to more than 800 mg of active chymosin/L of solution with a mass of active chymosin/mass of active bovine pepsin ratio higher than or equal to 1.38 according to French current regulation (Ministerial decree DQ/SRFCQ/C.81 of January 20th, 1981). When calf rennet is added to other animal species rennet, both bovine chymosin and pepsin can be found in the mixture of rennets. Because pepsin shows greater general stability than chymosin (Chitpinityol & Crabbe, 1998), it was chosen as the target protein to indicate the presence of added calf rennet in other animal species rennet. In the present study, monoclonal antibodies raised against bovine pepsin were characterized and the most specific one was used in an indirect ELISA for the qualitative detection of added bovine pepsin in kid and lamb rennets.

2. Material and methods

2.1. Chemicals and milk-clotting enzymes

Bovine pepsin used for the production of mAbs, goat pepsin and microbial enzymes were purified in our laboratory from Adult Bovine Rennet Reference (Chr. Hansen, Denmark), kid rennet and microbial coagulants, respectively. Purified bovine chymosin and pig pepsin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Analytical-grade chemicals were purchased from VWR International (Fontenay-sous-Bois, France).

2.2. Rennet samples

Calf, lamb, and kid rennets were obtained from different suppliers (Laboratoires ABIA, France; Laboratoire Central des Présures S.A.S., France). Calf rennet was added to lamb and kid rennets with varying amounts of bovine pepsin ranging from 1.25 to 260 mg/L. Samples were prepared in 2 independent batches in which calf rennet and lamb or kid rennet were mixed at varying percentages: 0, 1, 2, 5, 10, 20, 50, 100% (v/v). Samples were analyzed in duplicate and detected by indirect ELISA as described in 2.4.

2.3. Monoclonal antibody

Hybridomas to bovine pepsin were obtained from mice immunized by injection of purified bovine pepsin; antibody-producing cells being fused in a 5:1 ratio with Sp2/O-Ag14 myeloma cells as described by Rolet-Répécaud et al. (2017). All animals were maintained in accordance with the French Ministry of Agriculture ethical guidelines for care and use of laboratory animals (B 21 231 010 EA). Hybridomas were subcloned by the limiting dilution method and then cryopreserved in liquid nitrogen. The isotype of the mAb was determined by a mouse monoclonal antibody isotyping kit (Rd-Biotech, Besançon, France) according to the manufacturer's instructions. The specificity for bovine pepsin and the possible cross-reactivity against pepsin from other animal species and other milk-clotting enzymes, bovine chymosin and microbial enzymes, were checked by indirect ELISA and Western blotting.

2.4. Indirect ELISA

Indirect ELISA was performed as previously described (Rolet-Répécaud et al., 2015). Briefly, microtiter plates (NUNC F96 Maxisorp, 4000 Roskilde, Denmark) were coated with 100 μL/well of purified milk-clotting enzymes (1 μg/mL): pepsin from different animal species, bovine chymosin, and microbial enzymes, and of rennet samples (diluted 1:2) in 100 mM sodium bicarbonate buffer, pH 9.6. The plates were incubated for 90 min at 37 °C. After washing, wells were blocked (10 g/L gelatin in PBS 0.05% Tween 20 (PBS-T)). Then, 100 μL of anti-bovine pepsin antibody, supernatant diluted 1:150 in PBS-T, and 100 μL of donkey anti-mouse immunoglobulin alkaline phosphatase conjugate, 1:3000 diluted in PBS-T, were successively added to each well. After each step, the plates were incubated 1 h at 37 °C and washed four times 15 s (250 μL/well PBS–T). The colorimetric reaction was then developed by incubating the plates with 100 μL/well of chromogenic substrate for 30 min. The absorbance at 405 nm (A_{405nm}) was read against a blank (substrate only) using a NanoQuant Infinite F200 Pro microplate spectrophotometer (TECAN, Männedorf, Switzerland).

The limit of detection (LOD), defined as the smallest amount of bovine pepsin that could be differentiated from unadulterated rennet (blank sample), was estimated as the A_{405nm} corresponding to the average measured absorbance of 10 blank samples plus 3 times the standard deviation of the average value. A value of A_{405nm} higher than this limit clearly indicates the presence of bovine pepsin.

2.5. SDS-PAGE and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of pepsin and rennets from different animal sources and Western blotting were performed as described by Rolet-Répécaud et al. (2015). In short, samples were separated in a 12% acrylamide Novex Tris glycine gel. Following the electrophoresis, the proteins were transferred onto a 0.2 μm pore size nitrocellulose membrane. The membrane was incubated 1 h at room temperature in buffer (10 mM Tris-HCl, 500 mM NaCl, 0.5 mM DTT, 0.275 g/L Tween 20, pH 8.0) with successively heat-inactivated horse serum (1:20 v/v), bovine pepsin-specific mAb (1:2 v/v), and donkey anti-mouse immunoglobulin-alkaline phosphatase conjugate (1:2500 v/v). Between each step, the membrane was washed in the same buffer (four times 5 min soaking). The immunoreactive bands were visualized with a mixture of Fast-Red TR salt and Naphthol AS-MX phosphate disodium salt.

3. Results and discussion

3.1. MAb specificity

The specificity describes the ability of the mAb to produce unequivocally a measurable response for the analyte of interest,

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