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Quantification of pepsin in rennet using a monoclonal antibody-based inhibition ELISA

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ABSTRACT

Pepsin and chymosin are milk-clotting enzymes found in rennets. They differ in their pH and temperature sensitivities and their milk-clotting activity (MCA)/proteolytic activity ratio which impact cheese technology. Therefore, characterization of rennet should not be limited to its total MCA, but also its enzymatic composition. Monoclonal antibodies against pepsin, obtained from mice immunized with purified pepsin, were characterized. A specific inhibition enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of pepsin in rennets. The limit of quantification was 143 ng pepsin/mL. The precision within runs ranged from 7.0 to 9.4%, for rennets with low and high pepsin concentrations, respectively. The precision among runs also ranged from 8.8 to 11.4%. Satisfying recovery, from 84.7 to 100.3%, was found with spiked samples. The applicability of the developed inhibition ELISA for pepsin quantification was assessed by analyzing commercial rennets, and compared to the standard chromatographic method 110A of International Dairy Federation. The relation and agreement between methods were evaluated using Deming regression analysis and Bland-Altman plot. A good agreement was found with a fixed bias. By means of bias subtraction, inhibition ELISA could be a reliable alternative for rapid and sensitive determination of pepsin in rennet.

1. Introduction

Pepsin (EC 3.4.23.1) is a natural aspartic proteinase secreted in the stomach of mammals and also found in various birds, reptiles, amphibians and fishes. For the cheese manufacturing process, the abomasum of ruminants serves as a source of enzymes. Once extracted from gastric tissues, these enzymes, known as rennet, are traditionally used for milk clotting. Pepsin is closely related to chymosin (EC 3.4.23.4), which secretion predominates in the abomasa of young unweaned animals but dramatically falls down after weaning. Therefore, pepsin becomes the major enzyme in gastric juices of adult ruminants. Most commercial calf rennets do in fact contain mixtures of chymosin and bovine pepsin in varying amount. Chymosin/pepsin ratio may differ by the source (cow, lamb, goat), the age, and the feeding of the animal from which the rennet extract is obtained (Rossano et al., 2003; Scott, 1986; Zhang, Chen, Yang, & Li, 2005). Rennet extracts from young milk-fed calves contain 88–94% chymosin and about 6–12% pepsin, while extracts from adult bovines contain 90–94% pepsin and 6–10% chymosin. In France, according to current regulation, rennets must have a mass of active chymosin/mass of active bovine pepsin ratio higher than or equal to 1.38 (decree of March 25th, 1924, section 24, supplemented by the decree n°69–475 of May 14th, 1969, Journal Officiel de la République Française).

Pepsin is similar to chymosin on certain points. Both enzymes i) are gastric acid proteinases, ii) derive from zymogens, pepsinogen and prochymosin, respectively, iii) have about the same molecular weights, 35.0 and 35.6 kDa, respectively, iv) act similarly on the

Abbreviations: CI, confidence interval; IDF, International Dairy Federation; IMCU, international milk-clotting unit; LoA, limit of agreement; MCA, milk-clotting activity; RAM, rabbit antimouse; R_{max}, theoretical maximum binding capacity of the surface; RU, response unit; SPR, surface plasmon resonance.

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peptide bond of κ-casein. However, they differ in their pH and temperature sensitivities, and their milk-clotting activity (MCA)/proteolytic activity ratio (Fox, Guinee, Cogan, & Mc Sweeney, 2000). Pepsin is less specific and hydrolyses any peptide bond with phenylalanine, tyrosine, leucine, or valine residues. These properties are of great importance for the cheese making industry due to their effect on cheese yield and quality (Guilloteau, Corring, Toullec, & Robelin, 1984). So, rennets with the same global activity but a different enzymatic composition may have a different behaviour in vat. The ratio of chymosin to pepsin in a rennet is of major importance since it affects its technological properties and proteolytic activity. Therefore, for economic and technological reasons, cheesemakers need to know not only the MCA, which is a measure of a global activity, but also the relative enzymatic composition of the rennet. The enzymatic composition is usually determined by an International Dairy Federation (IDF) standard method based on the separation of chymosin and pepsin from rennet by ion exchange chromatography (Standard IDF 110B/ISO 15163, 2012; Standard IDF 110A, 1987) followed by the measurement of the MCA of each enzyme fraction (Standard IDF 157/ISO 11815, 2007). Results are expressed in percentage of activity (Standard IDF 110B/ISO 15163, 2012; Standard IDF 110A, 1987) or in mg of active enzyme/L (Journal Officiel de la République Française, 1981). This 2-steps method is time-consuming with pretreatment procedures and based on visual assessment of the flocculation of a standard milk substrate. Moreover, it provides relative proportions of pepsin and chymosin which values are affected by the pH of the standard milk used for MCA measurement (Guinee & Wilkinson, 1992) (pH 6.5 (Standard IDF 110B/ISO 15163, 2012; Standard IDF 110A, 1987) or pH 6.3 (Journal Officiel de la République Française, 1981)). Immunoassay may provide an alternative method, as it is a highly sensitive, specific, and versatile tool, able to detect protein/enzymes in complex mixtures. Moreover, it has the advantage of a high sample throughput and a minimum sample treatment. In a previous study, polyclonal antibodies against bovine pepsin and chymosin were generated and used for specific inhibition of the enzyme in the rennet sample (Berankova, Rauch, & Kas, 1989). The authors measured bovine pepsin percentage of activity after the immunochemical inhibition of chymosin. Because bovine pepsin and chymosin represent 97–100% of the total MCA of bovine rennets, the authors considered that the residual activity of the rennet sample was a measure of the activity of pepsin. On the contrary, they measured chymosin percentage of activity after the immunochemical inhibition of pepsin. Enzyme-linked immunosorbent assays (ELISA) have been successfully applied to detect porcine pepsin in fresh soft cheeses (Boudjellab, Grosclaude, Zhao, & Collin, 1998) and in coagulants (Zahran et al., 1994). Moreover, ELISA based on monoclonal antibodies (mAbs) has been demonstrated to be suitable for the quantification of chymosin in rennet samples (Rolet-Répécaud et al., 2015).

In the present work, we report the production and characterization of mAbs against bovine pepsin. One selected specific mAb was used to develop an inhibition ELISA for the quantification of pepsin in milk-clotting solutions. The described assay was applied to the analysis of rennet samples and compared to the IDF standard chromatographic method 110A (Standard IDF 110A, 1987).

2. Materials and methods

2.1. Chemicals and samples

Analytical-grade chemicals were purchased from VWR International (Fontenay-sous-Bois, France). Purified chymosin and porcine pepsin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Caprine pepsin was purified in our laboratory from kid rennet. Adult Bovine Rennet Reference, calf, kid and lamb rennets, recombinant chymosin and microbial coagulants, were obtained from different suppliers (Chr. Hansen, Denmark; Danisco, Denmark; Laboratoires ABIA, France).

2.2. Purification of bovine pepsin

Bovine pepsin used for the production of mAbs was purified from Adult Bovine Rennet Reference (Chr. Hansen, Denmark) by ion exchange chromatography. Adult Bovine Rennet Reference (5.6 g) was dissolved in 20 mL 25 mM piperazine-HCl buffer pH 5.3, and dialyzed overnight in a dialysis bag (molecular weight cut-off 6–8 kDa) against 25 mM piperazine-HCl buffer (3 L) at 4 °C. After dialysis, the solution was filtered through a 0.45 μm PVDF 3D syringe filter and loaded onto an ion exchange column HiPrep DEAE FF 16/10 (GE Healthcare, Uppsala, Sweden), which was equilibrated with 25 mM piperazine-HCl buffer (pH 5.3). The bound proteins were eluted with an increasing gradient of NaCl (0.2 M pH 5.3; 0.5 M pH 5.5; 2 M pH 5.7) in the same buffer, at a flow rate of 5 mL/min. The eluted fractions were collected and monitored at 280 nm (Pharmacia Biotech Uvicord SII). The total volume of isolated pepsin (140 mL) was dialyzed overnight in a dialysis bag (molecular weight cut-off 6–8 kDa) against 25 mM piperazine-HCl buffer (80 L) at 4 °C. The dialyzed pepsin was concentrated using Centrifugal Devices Macrosep (cut-off 10 kDa; PALL Life Sciences, Saint-Germain-en-Laye, France) centrifuged at 3000*g* for 1 h 30 at 5 °C. The concentrated fraction of pepsin (13.5 mL) was further purified using gel filtration chromatography (three times 4.5 mL). Concentrated pepsin (4.5 mL) was loaded onto a HiLoad 16/600 Superdex 75 pg column (GE Healthcare, Uppsala, Sweden), which was equilibrated with 25 mM piperazine-HCl buffer (pH 5.3) and eluted with the same buffer at a flow rate of 0.75 mL/min. The eluted fractions were monitored at 280 nm and characterized for their milk clotting activity (Journal Officiel de la République Française, 1981), their concentration (BC Assay Protein Quantitation Kit Uptima (Interchim, Montluçon, France)), and their purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (see below).

Once mixed with the sample buffer (2:1) (1.2 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 1% (v/v) DTT, 20% (v/v) glycerol and 1% (v/v) bromophenol blue) and boiled for 2 min at 97 °C, the eluted fractions were loaded onto 12% acrylamide Novex® Tris-Glycine gel (Life Technologies SAS, Saint-Aubin, France) in fixed volume (10 μL) in each lane. SDS-PAGE was performed at 125 V, variable intensity, for 2 h at 10 °C. After SDS-PAGE, the gel was stained overnight at 4 °C according to the procedure of Blakesley and Boezi (1977). Protein patterns were then visualized after destaining 6 h in distilled water. Immediately after separation, Western blotting was performed using a 0.2-μmpore-size nitrocellulose membrane (Protran BA83 NC, Whatman GE Healthcare Life Sciences – Europe GmbH, Velizy-Villacoublay, France) as described by Rolet-Répécaud et al. (2015). To check for the absence of chymosin in the eluted fraction of pepsin, anti-chymosin specific mAbs (Rolet-Répécaud et al., 2015) were used for incubation. Precision Plus Protein Kaleidoscope standards (Life Technologies SAS, Saint Aubin, France) were used as the molecular weight standard.

2.3. Production, purification and isotyping of mAbs

Antibody production was performed as described by Rolet-Répécaud et al. (2015). Two female BALB/c mice were immunized with 20 μg of purified pepsin (3 mg/mL diluted 3.75-fold in physiological water) emulsified in Freund's complete adjuvant (1:1 v/v) (Difco laboratories, Detroit, MI, USA). Culture supernatants obtained after cell fusion were screened by indirect ELISA for the