LWT - Food Science and Technology 50 (2013) 459–468

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Characterization of the non-coagulating enzyme fraction of different milk-clotting preparations

Odile Rolet-Répécaud [a], Françoise Berthier [a], Eric Beuvier [a], Stéphane Gavoye [b], Eric Notz [b], Sébastien Roustel [c], Valérie Gagnaire [d], Christine Achilleos [a,*]

[a] INRA, UR342 Technologie et Analyses Laitières, F-39800 Poligny, France
[b] Actilait, F-25620 Mamirolle, France
[c] ISBA Franche-Comté, ENILBio, F-39800 Poligny, France
[d] INRA-Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France

ARTICLE INFO

Article history:
Received 10 February 2012
Received in revised form
6 August 2012
Accepted 24 August 2012

Keywords:
Cheese
Coagulation
Milk-clotting preparation
Rennet
Chymosin

ABSTRACT

Proteolytic milk-clotting enzymes are extracted from various sources (animals, plants, fungi) and processed according to various methods that are specific to each manufacturer or cheese-maker. Chemical composition and polypeptide patterns of 24 milk-clotting preparations from animal and fungal sources: 10 commercial rennets, 9 artisanal calf rennets, 2 recombinant chymosin preparations and 3 microbial preparations, were compared in order to identify differences according to both their manufacturing process and their source. The preparation from *Cryphonectria parasitica* had the highest ammonia and small peptide content. Commercial rennets and preparations from *Rhizomucor miehei* had the highest NaCl and pH values while artisanal rennets had the lowest and recombinant chymosins were intermediate. In comparison with the other commercial preparations, commercial rennets had the highest variability in chemical composition and their polypeptide profiles showed numerous protein bands ranging from 15 kDa to 197 kDa, like artisanal rennets. Protein composition of commercial rennets revealed the presence of bovine serum albumin, either native or degraded, and degraded chymosin. The results indicated that the source of coagulating enzymes and the conditions applied for enzyme extraction led to specific chemical compositions, polypeptide patterns and protein composition which are described in this article.

1. Introduction

The cheese making process involves many different steps including coagulation, acidification, curd cutting, salting, moulding and ripening (Bennett & Johnston, 2004). The earliest and most crucial step is the conversion of liquid milk to a semi-solid gel by aggregation of the casein, the main milk protein, which is also called milk clotting. Milk clotting can be achieved using proteolytic enzymes from various sources such as animals (calf, lamb, kid, pig, chicken), plants (*Cynara cardunculus*, *Ficus carica*, *Arctium minus* and *Solanum dobium*) and fungi (*Rhizomucor miehei, Rhizomucor pusillus* and *Cryphonectria parasitica*). Milk-clotting enzymes are aspartic proteases (EC 3.4.23.) selected for their high milk-clotting activity and their low general proteolytic activity. Jacob, Jaros, and Rohm (2011) and Kumar, Grover, Sharma, and Batish (2010) recently reviewed the properties of the different milk-clotting enzymes and also compared the results with regard to cheese yield and cheese quality, such as texture, aroma and flavour. Sousa, Ardö, and McSweeney (2001) reviewed the role of milk-clotting enzymes, according to their source, on the initial hydrolysis of caseins in cheese – Cheddar, blue cheese, Parmigiano Reggiano, Serra da Estrela and Feta.

Traditionally, cheese makers extensively use calf rennet, a clotting preparation extracted from calf abomasum that contains two enzymes: chymosin and pepsin. Today, only 20–30% of the world demand for milk-clotting preparations can be covered by calf rennet (Jacob et al., 2011), leading to the wide use of other kinds of milk-clotting preparations. Among the numerous milk-clotting preparations available for cheese making, a preparation is selected according to many criteria. On top of being practical, it has to comply with rules and regulations in force in the country, technological and economic constraints as well as the target market (kosher certification, organic or vegetarian approval). In France, preparations from animal and fungal sources are used most. In

* Corresponding author. INRA, UR342 Technologie et Analyses Laitières, BP 20089, F-39800 Poligny, France. Tel.: +33 3 84 73 63 00; fax: +33 3 84 37 37 81.
E-mail address: Christine.Achilleos@poligny.inra.fr (C. Achilleos).

0023-6438/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2012.08.021

2010, rennet represented 33% of demand for milk-clotting preparations, recombinant chymosin 14% and microbial coagulants 53% with 35% for *R. miehei* and 18% for *C. parasitica* (SPPAIL, 2010). Meanwhile, 80–90% of cheeses in the USA and Great Britain are manufactured using recombinant chymosin (GMO Compass, 2010).

Various methods exist to extract and process milk-clotting enzymes as recently reviewed by Jacob et al. (2011), Kumar et al. (2010) and Mistry (2006), from small-scale production to large-scale standardized commercial production. For traditional cheese making, especially in southern Europe, rennet and plant-derived coagulants are still produced on a small scale directly at the dairy according to a process that is specific to the cheese maker using traditional local procedures and is for his own use (Jacob et al., 2011). Valles and Mocquot (1972) reported the influence of the quality and the quantity of abomasa as well as the extraction conditions, pH and temperature, on the properties of artisanal calf rennets. They demonstrated that the extraction of the enzymes and the activation of chymosin were higher with a higher acidification rate. Small quantities of abomasum, from 5 to 10 g/L, used for chymosin extraction had no effect on the extraction yield in contrast with higher quantities, 100 g/L, which led to a decrease in the extraction yield. The influence of the manufacturing process was particularly investigated for lamb rennet, either in the form of commercial liquid preparations or artisanal pastes. Addis, Piredda, and Pirisi (2008) reviewed how the physical quality of the raw materials and the production conditions largely determine the milk-clotting, proteolytic and lipolytic activities of lamb rennet paste. The age and diet of the lambs at slaughter directly affect both the quantity and the quality of rennet paste enzymes. Chymosin and lipase activity are favoured when rennet is prepared with the abomasum of young suckling ruminants, the animal being slaughtered rapidly after suckling.

Milk-clotting enzymes are used in the form of a liquid, powder or paste. However, only a few studies have examined the influence of the other components, or the non-clotting enzyme fraction, present in the milk clotting preparation on the final quality of the cheese. Besides milk clotting activity, lipolytic activity was measured in artisanal lamb rennet pastes (Bustamante et al., 2000; Pirisi et al., 2007), mesophilic microflora was found in artisanal lamb and kid rennets (Etayo et al., 2006; Flórez, Hernández-Barranco, Marcos, & Mayo, 2006; Gil et al., 2007; Moschopoulou, Kandarakis, & Anifantakis, 2007) and thermophilic microflora in artisanal calf rennets (Valles & Mocquot, 1972). Total nitrogen, salt, phosphate and ammonia have been quantified in some commercial calf rennets and fungal protease preparations (Granday, Mietton, Olsson, Perrod, & Quiblier, 1987). These authors suggested that salt and phosphate from the non-clotting enzyme fraction had an influence on the rheological behaviour of milk curd and on soft cheese yield. There was evidence for an increased cheese yield when using commercial calf rennets or fungal protease preparations with lower salt and higher phosphate; the milk-clotting enzymes being equal in the compared preparations.

The objectives of the current study were (i) to determine the chemical composition of 24 milk-clotting preparations representative of the diversity of liquid milk-clotting preparations from animal and fungal sources and (ii) to identify differences in chemical compositions according to both the manufacturing process and the sources of these preparations.

2. Materials and methods

2.1. Artisanal calf rennets

Nine different liquid artisanal calf rennets, AR1 to AR9, including the variability observed in French dairies, were prepared from a fresh whey obtained at a local dairy (Fig. 1). Identical volumes were immediately processed at the laboratory according to the nine procedures. Size of the added calf abomasa varied according to the age of the animals when slaughtered: small abomasa resulted from younger calves than large ones. Three replicates were obtained over a three-month period from three different samples of fresh whey. The 27 processed rennets were stored at −20 °C until analysis.

2.2. Commercial milk-clotting preparations

Fifteen commercial liquid preparations representative of the diversity available on the market were analysed, i.e. calf rennets CR1 to CR7, lamb rennet LR1, kid rennets KR1 and KR2, microbial coagulants M1 to M3 and recombinant chymosins R1 and R2 (Caglificio Clerici S.p.A, Italy; Chr. Hansen, Denmark; Danisco, Denmark; DSM Food Specialties, The Netherlands; Laboratoires ABIA, France; Laboratoire Central des Présures S.A.S., France; Österreichische Laberzeugung Hundsbichler GmbH, Austria). Within each type of milk-clotting preparation, numbers represented a different manufacturer. Moreover, calf rennet CR1, lamb rennet LR1 and kid rennet KR1 were from the same manufacturer. Similarly, CR6 and KR2 were from the same manufacturer. Commercial milk-clotting preparations were sampled directly from different dairies. Each preparation was sampled from non-open batches at three different times over a six-month period, each specimen being sampled from different batches.

2.3. Chemical composition

2.3.1. Minerals and ash content

Milk-clotting preparations were analyzed for sodium (Na), phosphorus (P) and chloride (Cl). Na and P content were determined using an inductively coupled plasma optical emission spectrophotometer (ICP-OES PerkinElmer 7300 DV) (PerkinElmer, Waltham, MA, USA). Cl content was measured using a Corning Chloride Analyzer 926 (Humeau, La Chapelle sur Erdre, France). Ash content was determined according to NF Standard V 04-404 (NF., 2001).

2.3.2. pH

pH was measured using a WTW 526 digital pH meter by direct reading using a glass insertion electrode (WTW, Weilheim, Germany).

2.3.3. Nitrogen fractions

The ammonia (NH_3) content was determined spectrophotometrically using a Boehringer Mannheim NH_3 kit (n°11112732). The phosphotungstic-acid-soluble nitrogen fraction (NPT) was determined according to IDF Standard 224 (IDF, 2008). Free amino groups (free NH_2) were measured using molecular absorption spectrometry according to the ability of trinitrobenzene sulfonic acid (TNBS) to react with primary amino groups. The resultant trinitrophenylated amine complex was detected at 420 nm. A standard absorbance curve versus α amine nitrogen was prepared using glycine as a standard.

2.3.4. Data analysis

Principal Component Analysis (PCA) was used to capture the diversity between the milk-clotting preparations and to obtain a graphical representation of similarities and differences in chemical data. Newman–Keuls multiple means comparison was used to assess statistically significant differences between preparations (95% confidence interval). Statistical analyses were performed using Xlstat software, version 2010.3.01 (Addinsoft, Paris, France).