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Physico-chemical characterization of dairy gel obtained by a proteolytic extract from *Calotropis procera* – A comparison with chymosin

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ABSTRACT

Chymosin is the major enzyme used in cheesemaking but latex enzymes are also used. The aim of this work was to characterize the composition and the structure of dairy gel obtained by an extract of *Calotropis procera* leaves in comparison with those obtained by chymosin. The biochemical and mineral compositions of the curds and the cheese yields obtained by using *Calotropis procera* extract or chymosin were relatively similar. Quantitative and qualitative evaluations of proteolysis after milk coagulation, determined by the non-protein nitrogen content and chromatography coupled to mass spectrometry, indicated that *Calotropis procera* extract was more proteolytic than chymosin and that κ-casein was proteolyzed. The main consequence of proteolysis by *Calotropis procera* extract or chymosin was the formation of a similar and regular network with the presence of aggregates of casein micelles. These results support that *Calotropis procera* extract can be used as effective coagulant in cheesemaking.

1. Introduction

Coagulation of milk is an important step in cheesemaking and its success is an important parameter to have a cheese of high quality (Fox, McSweeney, Cogan, & Guinee, 2004). Different coagulant agents are used in cheesemaking but the most commonly used in the world is the chymosin. Today, the action of chymosin is relatively well described and understood. It corresponds to the hydrolysis of κ-casein with a release of caseinomacropeptide, followed by a destabilization of the paracasein micelles leading to the milk coagulation.

Other enzymes from vegetable origin also possess the property to clot milk and different results focusing on their potentiality to be used in cheese technology are reported (Mahajan & Badgujar, 2010; Mahajan & Chaudhari, 2014; Shah, Mir, & Paray, 2014). Among these enzymes, those present in the extract of *Calotropis procera* are especially used in West Africa to make a traditional soft cheese. The traditional name of this cheese depends on the country of the production: «Warankasi» or «Wara» in Nigeria, «Wagassirou», «Waragachi» or «Waoagachi» in the Republic of Benin, or « Wagachi» in Ghana. This West African soft cheese is usually manufactured at artisanal scale by women belonging to Fulani or Yoruba communities (Akinyosoye, 2006). From practical point of view, *Calotropis procera* extract is incorporated into a heated milk until formation of the curd. Then, curd is drained and molded (O'Connor, 1993). For traditional processing, fresh whole cow milk is directly heated on wood fire after milking without any standardization. For other groups, the fresh leaves of *Calotropis procera* are crushed, mixed with a small quantity of water or fresh milk and the mixture is used as coagulant. Sometimes, a whole branch including leaves, flowers and stems of the plant is directly immersed in the heated milk. Then the mixture is heated until a visible milk coagulation. The whey is clear, yellowish or greenish and the curd crumbly with a slight vegetal taste. The curd is not pressed but rather left to drip during many hours in traditional molds made with raffia. The operation is ended when the manufacturer judges that the consistence and moisture content of the cheese are correct. Sometimes, the fresh cheese is boiled and colored with red sorghum (*Sorghum vulgaris*) extract. In general way, the manufacture of this type of cheese is empirical and not

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well standardized (Adetunji & Babalobi, 2011; Olapido & Jadesimi, 2013; O'Connor, 1993). This cheese is locally appreciated and directly consumed or incorporated into traditional food recipes in substitution to meat or fish especially in rural areas. It is consumed fresh, without ripening, as a sandwich filling or fried cake (Omotosho, Oboh, & Iweala, 2011). The application of leaves extract in excess lead to a bitter taste making the cheese inappropriate for consumption. The presence of several proteases with proteolytic activities and release of peptides are responsible for this bitter taste.

Some researches focused on the proteolytic activity in *Calotropis procera* and revealed the presence of several proteases. Thus, Freitas et al. (2007) characterized the laticifer proteins of *Calotropis procera* by mass spectrometry and reported the presence of cysteine proteases, aspartic proteinases and chitinase. Among these enzymes, procerain is the most described and exists in two different forms. Both of these forms were extracted and characterized from the latex of *Calotropis procera*. The first, named procerain, is a cysteine protease of 28.8 KDa with optimal pH of 8 and temperature of 60 °C. The second, a cysteine protease, was also purified and named procerain B (Singh, Shukla, Jagannadham, & Dubey, 2010). The molecular weight, isoelectric point and cleavage recognition site, are different from those of the first procerain (Aworh, Kasche, & Apampa, 1994; Dubey & Jagannadham, 2003; Singh & Dubey, 2011). Singh, Yadav, and Dubey (2013) have cloned, characterized this protein from a structural point of view and determined a high sequence homology with other papain's like plant cysteine proteases of peptidase C1A superfamily. In 2013, Ramos et al. reported new insights into the complex mixture of latex cysteine peptidases in *Calotropis procera*. These authors indicated the presence, in the latex of *Calotropis procera,* of three distinct cysteine proteases from procerain and procerainB. These new described enzymes have minor differences in their molecular weights, ordered structures and activities. Authors suggested that they came from the same initial protein which is differently modified during post-translational processes. The activity of procerain was relatively stable over a wide range of pH and temperature higher than 65 °C (Baba-Moussa et al., 2007; Ogundiwin & Oke, 1983; Oseni & Ekperigin, 2013a, 2013b; Singh et al., 2010). Oseni and Ekperigin (2013a) demonstrated that addition of cysteine and calcium enhanced the proteolytic activity of the enzymes. Procerain B was also stable in presence of 35% acetonitrile, 30% methanol, 8 M urea and different detergents (Singh et al., 2010). It was reported that the proteolytic activity was effective on a variety of denatured proteins such as azoalbumine and azocasein but also on native intact caseins (Singh et al., 2010). However, the peptide bonds hydrolyzed by procerain are not precisely known. Recently, Freitas et al. (2016) reported that fractions of *Calotropis procera* proteolyzed κ-casein with the production of peptides of 16 KDa.

The aim of this study was to describe the physico-chemical compositions of curds obtained by *Calotropis procera* extract. Microstructures of curds were examined by scanning electron microscopy. In the same time, the released peptides from casein micelles especially those coming from κ-casein after milk coagulation were identified.

2. Materials and methods

2.1. Materials

2.1.1. Milk

Low heat skimmed milk powder (Lactalis, Retiers, France) was used as raw material. Milk was reconstituted at 10% (w/w) in distilled water. Reconstituted milk was stored at 4 °C overnight to have a good dissolution of the milk powder.

2.1.2. Calotropis procera extract and chymosin

Fresh *Calotropis procera* leaves were collected from Niger, washed with water and dried in the shade at ambient temperature (25–35 °C) for 7 days. The leaves were frequently turned to avoid mold development. Before use, the leaves were milled in an electric crusher to obtain a rough powder. Before milk coagulation, 50 g of powder of *Calotropis procera* were added to1 L of reconstituted milk and stored 1 h at 25 °C to facilitate the extraction of enzymes. Then the liter of milk was filtered and added to 9 kg of milk.

The chymosin used (CHY-MAX 200) was purchased from Chr. Hansen (Saint-Germain-lès-Arpajon, France). Just before addition to milk, the chymosin stock solution was diluted 10-fold in distilled water. The amount added to the reconstituted milk was 40 mL kg^{-1} of milk.

2.2. Manufacture of curds with Calotropis procera extract or chymosin

The flow charts with *Calotropis procera* extract and chymosin are presented in Fig. 1A and B, respectively. The cheesemaking was repeated 4 times independently. Milk coagulation by chymosin was conducted in similar conditions (pH 6.7 and without addition of calcium) to those used for *Calotropis procera* extract except for the temperature. It was not possible to coagulate milk with chymosin at 75 °C due to its loss of activity. So, a classical temperature of 30 °C was used for this enzyme. After coagulation, cutting, draining and pressing (Fig. 1), the curds were recovered for physico-chemical analyses and scanning electron microscopy observation. Whey liquids were collected for physico-chemical and reversed phase liquid chromatography coupled to mass spectrometry analyses.

2.3. Composition of milk, curds and wheys. Cheese yield and rates of recovery of constituents

Dry matter (DM) was determined according to IDF (1982,1987). Ash content was determined according to the protocols described by IDF (1964) and AFNOR (1989). Contents in calcium, magnesium, sodium and potassium were determined by atomic absorption spectrometry as described by Brulé, Maubois, and Fauquant (1974).

Total nitrogen content of milk, whey liquids and curds was determined after their mineralization (destruction of organic matter in nitrogen) by Kjeldahl method. A converting factor of 6.38 was used to convert nitrogen content into protein content. The content in Non Protein Nitrogen (NPN), corresponding to soluble nitrogen in 12% trichloroacetic solution, was determined according to IDF (1993). The NPN content was converted into equivalent protein contents (g kg^{-1}) using 6.19 as converting factor. The experimental error was ±0.1 g of nitrogen expressed in g of protein per kg (IDF, 1993).

Normal and corrected cheese yields and the coefficients of recovery for DM, ash and total nitrogen were calculated. The normal cheese yield was expressed as the percentage of the weight of the curd per liter of milk used for cheesemaking. The formula used was as follows: Yield = (mass of the curd (kg))/(mass of milk (kg)) × 100. The corrected cheese yield was calculated by taking into account DM as the following formula: (Milk DM (g kg^{-1}) – Whey liquid DM (g kg^{-1}))/(Curd DM (g kg^{-1}) – Whey liquid DM (g kg^{-1})) × 100. The rates of recovery of dairy components (DM, ash or protein) corresponded to the ratios of their amounts (in g) in curd and milk.

2.4. Reversed-phase high performance nano liquid chromatography (RP-nano HPLC) coupled to tandem mass spectrometry (MS/MS) analysis

Whey liquids were recovered to qualitatively analyze their compositions in peptides. These liquids were sampled 1 h after