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A preliminary study of continuous milk coagulation using *Cynara cardunculus* flower extract and calf rennet immobilized on magnetic particles

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ABSTRACT

The aim of this study was to develop a bioreactor design for continuous milk coagulation using a biocatalyst composed of immobilized animal and vegetable rennet on aminated magnetic particles, which has been proven to be an appropriate carrier for enzyme immobilization. Calf and vegetable (*Cynara cardunculus*) rennets were covalently immobilized on CLEA® magnetic supports and the immobilization procedure was optimized in batch mode, by evaluating protein loading, caseinolytic activity and the coagulation properties of skim milk powder and cow's milk. Subsequently the optimal temperature of immobilized coagulant was defined and a technically-friendly enzyme bioreactor was developed in order to carry out a continuous milk coagulation process with the aim of producing soft cheese.

1. Introduction

Milk coagulation is one of the most important steps in cheese manufacturing. For centuries calf rennet (CR), the conventional milk clotting enzyme obtained from the fourth stomach of suckling calves, has been the most widely used coagulant for manufacturing most types of cheeses worldwide (Shieh, Thi, & Shih, 2009). Chymosin (E.C. 3.4.23.4) is an enzyme found in calf rennet whose primary function is to coagulate milk. It is an aspartic protease which is considered to be the best coagulating agent due to its high specificity for cleaving k-casein Phe105-Met106 bond (Ahmed, Wehaidy, Ibrahim, El Ghani, & El-Hofi, 2016; Vasbinder, Rollema, Bot, & De Kruif, 2003). The worldwide increase in cheese production and consumption, as well as the increase in the price of calf rennet, have emphasized the need to find new milk coagulating enzymes that can satisfactorily replace calf rennet dairy product production (Guiama et al., 2010). Plant extracts have been used for manufacturing dairy products ever since ancient times. The best-known vegetable coagulant is thistle (*Cynara cardunculus* L.) extract (CC), which has been successfully used for manufacturing some traditional Portuguese (Serra and Serpa) and Spanish (Los Pedroches and Serena) ewe milk cheeses (Roseiro, Barbosa, Ames, & Wilbey, 2003). The thistle contains cardosin A and cardosin B enzymes, which have high milk clotting and proteolytic activity (Veríssimo et al., 1996) and produces cheeses with a creamy soft texture and genuine and slightly piquant aroma (Fernández-Salguero & Sanjuán, 1999). Similarly to chymosin, cardosin A cleaves bovine k-casein between Phe105 and Met106, while cardosin B is comparable to pepsin in terms of specificity and activity (Prados, Pino, & Fernández-Salguero, 2007).

Production costs can be reduced by implementing new technologies e.g. by using immobilized enzymes, which is currently arousing considerable interest (Liese & Hilterhaus, 2013). Immobilized enzyme systems generally improve the control and flexibility of processes, more specifically, it is easier to separate the enzyme from the product than with soluble enzyme systems, biocatalysts can be used repeatedly (Kim, Grate, & Wang, 2008) and in a continuous process (Benucci, Esti, Liburdi, & Garzillo, 2012). There has recently been renewed interest in using immobilized enzymes for milk clotting (Barouni et al., 2016; El-Bendary, Moharam, & Ali, 2009; Esawy & Combet-Blanc, 2006; Esposito, Di Pierro, Dejonghe, Mariniello, & Porta, 2016). Using immobilized chymosin in cheese production is deemed to be a promising approach due to the shortage of calf rennet that covers only 20–30% of the world demand for milk coagulant (Jacob, Jaros, & Rohm, 2011). Moreover, immobilization of vegetable coagulant may enable us to overcome the disadvantages of vegetable rennets, i.e. bitter cheese production, either by keeping the enzyme separate from the reaction products or by removing the immobilized enzyme to avoid its

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incorporation prevent it from being incorporated into the cheese curd matrix and consequently to avoid undesirable excess proteolysis before or during cheese ripening (Esposito et al., 2016).

In this study, we focused on the immobilization of calf and vegetable rennets on magnetic supports by means of covalent binding. Moreover, the aim of the study was to analyze and optimize the performance of a stable and reusable technically-friendly enzyme bioreactor at both laboratory and industrial scale, in order to develop a continuous milk coagulation process for cheese production.

2. Materials and methods

2.1. Materials

2.1.1. Milk-clotting enzymes

Calf rennet was purchased from Caglificio clerici spa (130 I.M.C. U. g^{-1}) (Como, ITALY). Cardoon extract was prepared from *Cynara cardunculus* dried flowers supplied by Agricoltura Nuova Coop (Rome, Italy).

2.1.2. Chemicals

Casein from bovine milk, sodium phosphate monobasic (99–102%), sodium phosphate dibasic (≥99.0%), trichloroacetic acid (>99.0%), acetic acid (99.7%) and glutaraldehyde (25%) were purchased from Sigma-Aldrich (Milano, Italy). Sodium hydroxide and sodium acetate were supplied by CARLO ERBA Reagents (Milano, Italy). Skimmed milk powder was purchased from Graziano s.a.s (Cosenza, Italy). Pasteurized whole cow's milk was supplied by Centrale del latte di Roma (Rome, Italy). The magnetic carrier was kindly donated by CLEA Technologies (Delft, Netherlands).

2.2. Experimental procedures

2.2.1. Extraction of Cynara cardunculus

The crude enzyme extract was prepared by macerating 6 g of stylets and stigmae of dried *C. cardunculus* flowers in 80 ml of distilled water for 20 min. Following vacuum centrifugation at 4 °C for 10 min at 19,000*g* in a Beckman J2-21 centrifuge, the supernatant was collected and frozen at −20 °C. It was then lyophilized in a VirTis AdVantage freeze-dryer.

2.2.2. Immobilization procedure

Calf rennet proteins and cardoon coagulants were covalently immobilized onto aminated magnetic particles, activated with glutaraldehyde as reported by Benucci et al. (2016). In short, 1 g of the aminated magnetic particles (CLEA®), functionalized with 1 ml of glutaraldehyde solution (3% v/v), was incubated for 2 h (h) at room temperature with constant agitation and washed five times in 1 ml of the same buffer used for the enzyme immobilization procedure. The CC and CR coagulant solutions were dissolved in phosphate buffer (0.2 M pH 7) in order to obtain a concentration of 5 mg_{BSA}/ml of protein in CC and CR coagulants. Increasing CC and CR volumes (0.1, 0.2, 0.4, 0.8, 1.0 and 1.25 ml) were added to the CLEA® supports in order to examine the effect of protein concentration on the efficiency of the immobilized biocatalyst. More specifically, the immobilization procedure was optimized by adding the different volumes of the CC and CR solutions to 100 mg of CLEA®; the phosphate buffer solution (0.2 M pH 7) was used to reach the final volume of 1.5 ml. After shaking at 25 °C for 4 h, the unbound proteins were removed from the supports by washing thoroughly with ammonium sulphate (2 M) and distilled water. The amount of immobilized protein was indirectly determined as the difference between the concentration of protein in the coagulant solutions before and after immobilization. The protein concentration was determined using the Bradford Protein Assay (Bradford, 1976), with Coomassie brilliant blue reagent and measuring absorbance at 595 nm. BSA was used as standard protein.

2.2.3. Caseinolytic activity

In soluble and immobilized forms, the caseinolytic activity (CA) of the calf rennet and *C. cardunculus* extracts was determined as described by Anusha, Singh, and Bindhu (2014) with some modifications. For the soluble extracts (CC and CR), 0.25 ml of appropriately diluted coagulant solution was incubated with 0.25 ml of substrate (1 % w/v casein in 0.05 M NaOH) and 0.5 ml of 0.1 M phosphate buffer (pH 6.5) for 20 min at 40 °C. The reaction was stopped by adding 0.5 ml of 15 % (w/v) TCA and was then allowed to stand for 25 min at room temperature and centrifuged. For the immobilized forms, 500 mg of CC_{CLEA}® and CR_{CLEA}® were incubated with the same amount of substrate and buffer for 20 min at 40 °C. At the end of the incubation, the supernatant was removed from the immobilized coagulant and the reaction was then stopped by adding 0.5 ml of 15 % (w/v) TCA.

Casein hydrolysis was assayed according to the colorimetric method developed by Anson (1938) and Folin and Ciocalteu (1927). 7.5 ml of NaOH (0.5 M) and Folin Ciocalteau reagent (diluted 1:2 with distilled water) were added to 0.5 ml of supernatant and incubated for 20 min in the dark. Absorbance of supernatant was measured at 660 nm with a UV–Vis spectrophotometer; model UV–Vis 1240 from Shimadzu (Kyoto, Japan). One unit of the protease activity was defined as the mg of the enzyme that liberated 1 μg of tyrosine (Tyr) under standard assay conditions.

2.2.4. Milk clotting activity

The milk clotting activity (MCA) was evaluated for the CC, CR, CC_{CLEA}® and CR_{CLEA}® samples, as reported by Anusha et al. (2014), in reconstituted skim milk prepared by dissolving 0.25 g of skim milk powder in 0.75 ml of 0.05 M sodium acetate buffer (pH 5.5) and in pasteurized whole cow's milk. MCA was assayed by adding 0.5 ml of the coagulant solution and 500 mg of CC_{CLEA}® and CR_{CLEA}® to 1.5 ml of skim milk after heating it to the optimum temperature of the coagulant for 10 min in a water bath. Similarly, 0.25 ml of coagulant solution and 500 mg of CC_{CLEA}® and CR_{CLEA}® were added to 3 ml of pasteurized whole milk. The time required for the milk to clot was recorded. One unit of milk-clotting activity was defined as the volume of milk that can be clotted by one unit volume of enzyme within 40 min at 37 °C.

$$\mathrm{MCA, U/mg_{BSA}} = [(2,400/\mathrm{T}) \times (\mathrm{S/E})]/\mathrm{mg_{BSA}} \quad (1)$$

where *T* = time for the curd formation (seconds), *S* = Volume of the milk (ml) and *E* = Volume of the enzyme (ml).

The milk clotting index, that is the ratio of milk-clotting activity (MCA) to caseinolytic activity (CA), was calculated as given below:

$$\mathrm{MCI} = \mathrm{MCA/CA} \quad (2)$$

2.2.5. Optimum pH and temperature

The effects of temperature on the caseinolytic activities of calf rennet and *C. cardunculus* coagulants, in both free and immobilized forms, were investigated in 0.1 M phosphate buffer (pH 6.5) under the same assay conditions described in paragraph 2.2.4.

In order to determine the optimum pH level, the CA of the coagulants was measured in McIlvaine buffer at various pH values at substrate concentration (casein 1%) as described above (paragraph 2.2.4). The intervals chosen for pH (4.8–6.8) and temperature (15–65 °C) were restricted according to the real optimal operating conditions of cheesemaking, which depend on the properties of the milk and coagulant. Optimum pHs and temperatures were investigated in both the soluble and immobilized forms of the coagulants.