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A kiwi juice aqueous solution as coagulant of bovine milk and its potential in Mozzarella cheese manufacture

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ABSTRACT

In this work a kiwi juice aqueous solution was prepared and used as coagulant enzyme in the manufacturing process of mozzarella cheese. The aqueous solution does exhibit high levels of milk-clotting activity probably due to the presence of the clotting enzyme actinidin in mixture with other kiwi fruit proteolityc enzymes. The mozzarella cheese was manufactured from bovine milk with a yield of 10.6% being in the range of that routinely obtained during cheese manufacture. A preliminary evaluation of the cheeses flavor showed positive results and lack of bitterness. Finally, in view of a future marketing of the kiwi juice aqueous solution as plant coagulant, it is shown that the aqueous solution stored for 20 days at −20 °C maintains both the clotting and proteolytic activities unaffected. Due to its properties as well as to the accessibility of the primary source for the preparation of the aqueous solution, it might represent a good candidate for calf rennet substitution, provided that further research on cheese properties will be carried out.

Keywords: Kiwi; Plant coagulant; Milk-clotting; SDS-PAGE; Cheese

1. Introduction

Vegetable extracts have been used as coagulants in cheese making since ancient times, although relatively few is known about their action. Cheeses made with plant coagulant can be found mainly in Mediterranean, west African and Southern European countries (Roseiro et al., 2003). Proteolytic enzymes extracted from plants, such as *Lactuca sativa* (Lo Piero and Petrone, 1999; Lo Piero et al., 2002), *Solanum elaeagnifolium* (Gutiérrez-Méndez et al., 2012), *Streblus asper* (Tripathi et al., 2011), among others, are a subject of growing interest in dairy technology. In fact, the use of traditional animal rennet is limited for religious (e.g. Judaism and Islam) or dietary (vegetarianism) reasons. Moreover, there are many steps involved in the extraction and subsequent purification of calf rennet from the animal stomachs, which make the enzyme supply very laborious and expensive. However, most of the above mentioned plant coagulants were found unsuitable for dairy industry applications because they produce cheeses characterized by extremely bitter tastes and poor textural characteristics. In fact, plant proteases exhibit high levels of proteolytic activity leading to the production of short peptides which affect both the flavor and the texture of the cheeses, resulting excessively acid and bitter (Oner and Akar, 1993). The aqueous extracts of *Cynara cardunculus* represent an exception to this general rule as the enzyme mixture extracted from cardoons is commonly utilized in Spain and Portugal for the manufacture of esteemed soft-bodied cheeses from ovine milk (Roseiro et al., 2003). As the quantity of added coagulant is usually empirical, excessive bitterness is a defect that can be avoided by better control of cheese making (Roseiro et al., 2003; Galán et al., 2012). Katsaros et al. (2010) reported the application of either kiwifruit powder rich in actinidin or kiwi juice in the production of dairy products as well as the use of high pressure as regulator of enzymatic activity. More recently, the cysteine protease actinidin purified from kiwifruit was characterized in view of its potential use as coagulant enzyme of bovine milk (Lo Piero et al., 2011). The data showed that actinidin exhibits the ability to form milk clots in which the casein coagulum is separated away from the whey proteins. Moreover, the enzyme dependence on pH and temperature and the stability profiles turn out to be fully suitable with the

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chemical–physical conditions adopted during the cheese making procedure (Lo Piero et al., 2011).

Mozzarella cheese is one of the most common types of pasta filata cheeses mainly used on pizza, characterized by its meltability and spreadability with a non-pronounced flavor. It is estimated that approximately 70% of mozzarella cheese is used in pizza making, so that the demand for mozzarella cheese is increasing in the global market (Kindstedt et al., 1995), as a result of a growing trend of fast foods. Pasta filata cheeses are prepared by an exceptional plasticizing and kneading treatment of the fresh curd in hot water which assigns to the finished product a characteristic fibrous structure as well as melting and stretching properties (Kindstedt, 1993). In cheese, casein molecules form a net that entraps fat and water, and proteolysis produces the softening of cheese body and changes its functional properties (Costabel et al., 2007). There is a very limited number of studies on manufacturing mozzarella cheeses using vegetable coagulant. In particular, the extract of dried berries of paneer booti (*Withania coagulans*) was successfully employed for the preparation of mozzarella cheese from buffalo milk characterized by acceptable taste and flavor (Nawaz et al., 2011). The authors showed that a better result in the curd formation is obtained using the proteolytic enzyme of *W. coagulans* extracted in 0.85% NaCl and at pH 4.25 (Nawaz et al., 2011).

In our work, we have investigated the suitability of a kiwi juice aqueous solution as a prospective substitute for chymosin during the mozzarella cheese making procedure. We characterized the proteolytic activity of the kiwi solution with respect to the cheese manufacture technology by determination of milk-clotting activity of the enzyme preparation, and by electrophoretic analysis of the hydrolysis products after treatment of crude casein and milk with the kiwi aqueus solution. The stability of the potential plant coagulant was assessed by the estimation of the residual proteolytic activity as well as the clotting activity of the kiwi aqueus solution after storage at four different temperatures (25 °C, 4 °C, −20 °C and −80 °C) for 20 days. Finally mozzarella cheese manufactured using the kiwi extract as plant coagulant and a preliminary cheese tasting was also performed.

2. Materials and methods

2.1. Kiwi juice aqueous solution preparation

The kiwifruit (1 kg) (*Actinidia chinensis* Deliciosa), obtained from the University farm located in Catania, were peeled and chopped into small pieces. Aliquots (100 g) of kiwi flesh were immediately frozen with liquid nitrogen, powdered by mortar and pestle, and successively extracted with 150 mL of bidistilled water by vigorous shaking for 1 h at 4 °C in a 1:1.5 (w/v) ratio. The homogenate was filtered through three layers of cheesecloth and the resulting solution was used for further analysis and cheese manufacture.

2.2. Purified actinidin preparation

Actinidin was purified by the method described in Lo Piero et al. (2011). Briefly fruits (100 g) were homogenized and after filtration the homogenate was centrifuged at 24,400 $g \times 20$ min at 4 °C in a Beckman J2-HS centrifuge, rotor JA-20 (Beckman Instruments, Fullerton, CA, USA). The resulting supernatant was again centrifuged at 150,000 $g \times 60$ min at 4 °C in a Sorvall ULTRA PRO 80 ultracentrifuge (Sorvall, Ramsey, USA) and the supernatant (crude extract) was applied onto a DEAE-Sepharose CL 6B column (Pharmacia Uppsala, Sweden). Fractions of 2 mL were collected and assayed for proteolytic activity using as substrate a 2% solution of total casein from bovine milk, technical grade (Sigma, St. Louis, MO, USA), then the active proteolytic fractions were combined and precipitated with solid $(NH_4)_2SO_4$ at 70% of saturation. Finally the precipitate was redissolved and then dialysed two times for 4 h at 4 °C.

2.3. Milk-clotting activity

Milk-clotting activity was measured by the method described in Uchikoba and Kaneda (1996) based on the visual evaluation of the first clotting flakes appearance. Different amounts of the enzyme preparation (40, 100, 250 and 350 μg) were added to a 10% solution of skimmed milk powder in 67 mM NaH_2PO_4 pH 6.8 at 30 °C both in the presence and in the absence of $CaCl_2$ (5 mM) in a final volume of 3 mL. The time elapsing between the mixing of reagents and the first appearance of solid material against the background was measured (CT, clotting time). All the experiments were repeated four times on independent enzyme preparations.

2.4. Proteolytic activity assay

The proteolytic activity was assayed using total casein (Sigma, St. Louis, MO, USA), κ-casein (Sigma, St. Louis, MO, USA) and two types of milk (pasteurized whole milk and pasteurized semi-skimmed milk) as substrates as described in Lo Piero et al. (2002). The assay mixture (1 mL) contained 2% (w/v) of the substrates dissolved in 67 mM NaH_2PO_4 pH 7.2 and 2.5 mM DTT. The hydrolysis against different types of milk was measured by incubation of milk, in the amount corresponding to 20 mg of proteins as determined by the method described in Lowry et al. (1951). The milk types used in the experiments were pasteurized whole milk and pasteurized semi-skimmed milk. All the samples were incubated at 55 °C for 20 min, since the proteolytic activity of actinidin reaches a maximum at 55 °C (Lo Piero et al., 2011), then the reaction was stopped by adding 1.5 mL of TCA (trichloroacetic acid) 5% (w/v). After TCA precipitation, the supernatant was recovered by centrifugation at 9000 $g \times 10$ min in a benchtop centrifuge. The absorbance of supernatant was measured at 280 nm using a Shimadzu UV-VIS 1240 spectrophotometer (Shimadzu Corporation, USA). Blank samples were performed by adding the enzyme preparation at the end of the incubation time, just before TCA addition and precipitation. The enzyme preparation activity was expressed in unit defined as the amount of enzyme that yields a 0.001 absorbance change per min at 280 nm. All the experiments were repeated four times on independent enzyme preparations and the standard deviation (±SD) was calculated by the average of the four experiments.

2.5. Electrophoretic analysis

The hydrolysis of total casein (10 mg) and milk proteins (10 mg of proteins) was achieved by incubation of the substrates with the enzyme preparation (10 μg) for 20 min at 55 °C (final volume 0.3 mL) in 67 mM NaH_2PO_4 pH 7.2 (Lo Piero et al., 2011). The milk types used in the experiments were namely pasteurized whole milk and pasteurized semi-skimmed milk. After incubation, the samples (15 μg) were prepared for SDS-PAGE (15 cm × 20 cm) by adding an equal