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Comparison of the milk-clotting properties of three plant extracts

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ABSTRACT

Several proteases from plant sources have been proposed as milk coagulants, however, limited research has been done on their milk-clotting properties. The effect of temperature on the milk-clotting activity of kiwi fruit, melon and ginger extracts was evaluated, as well as the effects of the different extracts on curd properties. Melon extracts showed high milk-clotting activity over a broad temperature range (45–75 °C) while kiwi fruit and ginger extracts showed high activity over a narrower temperature range, with a maximum at 40 and 63 °C, respectively. Curds produced using kiwi extracts had textural properties comparable with those obtained using commercial rennet, while melon extracts produced a fragile gel and low curd yield. The milk-clotting behavior of the three plant extracts was related to the protease specificity present in these extracts. The kiwi proteases displayed chymosin-like properties and thus hold the best potential for use as a milk coagulant in cheese production.

1. Introduction

The coagulation of milk by enzymatic methods is a basic step in the manufacture of most cheeses. Chymosin is the principal milk-clotting protease present in the natural calf rennet and has been used for centuries as an aid in the cheese making process. Nowadays, most commercial rennet used in the cheese industry comes from recombinant sources or from microbial origin, and only 20–30% comes from its natural source (Jacob, Jaros, & Rohm, 2011). The decay in the natural rennet supply (stomach of calves) and the steady increase in world cheese production in recent years have led to an increased demand for new rennet substitutes, motivating a search for new sources of proteases with rennet-like properties.

Proteases from plant sources offer a high potential as processing aids in production of cheese, food (e.g., production of novel dairy products, meat tenderizers and protein hydrolyzates production) and medicine (e.g., digestive and anti-inflammatory agents) (Huang, Chen, Luo, Guo, & Ren, 2011; Katsaros, Tavantzis, & Taoukis, 2010). The common plant proteases papain, ficin and bromelain (from papaya, fig and pineapple, respectively) have a low milk clotting activity/proteolytic activity (MCA/PA) ratio and have often been mentioned as the principal obstacle to their utilization in cheese making (Feijoo-Siota & Villa, 2011). A broad proteolytic specificity and the nonspecific hydrolysis of caseins affect texture, flavor and yield of cheese (Garg, Johri, & Pages., 1994; Jacob et al., 2011). However, some proteases that are highly specific for milk proteins are attractive as milk-clotting and/or ripening agents in cheese production. Dried cardoon flowers of *Cynara cardunculus* L. and *Cynara humillis* L. have been used for centuries in the Iberian Peninsula to prepare certain varieties of cheese with a creamy soft-texture and exquisite flavor. The Spanish and Portuguese ewe's milk cheeses, such as Serra da Estrella, Serpa and Azeitao, are highly appreciated and protected by a denomination of origin (POD) (Roseiro, Barbosa, Ames, & Wilbey, 2003). The aspartic proteases cardosins and cyprosins (also called cynarases) are the main milk-clotting proteases present in the cardoon extracts (Verissimo, Esteves, Faro, & Pires, 1995). Other sources of proteases include the berries of the plant *Solanum dobium*, several papilionoideae species used to produce the traditional white-soft cheese "Jibna beida" in Sudan, and soft dairy products in Angola (Lopes, Teixeira, Liberato, Pais, & Clemente, 1998; Mohamed-Ahmed, Morishima, Babiker, & Mori, 2009; Yousif, McMahon, & Shammet, 1996). In addition, the juice from the leaves of sodom apple (*Calotropis procera*) has been used in the African regions of Nigeria and Benin for traditional cheese-making (Aworh & Muller, 1987) and, in the south of China, the rhizome ginger juice is used to produce a ginger milk curd which is highly appreciated due to its tofu pudding-like, sweet-snack characteristics (Su, Huang, & Wang, 2009).

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The potential for using proteases from other plant sources as milk coagulants has been reported, including those obtained from the seeds of *Balanites* (Beka et al., 2011), *Solanum* (Guiama et al., 2010) and *albizia* (Egito et al., 2007), from asparagus stem (Yonezawa, Kaneda, & Uchikoba, 1998) and from fruits such as kiwi and melon (Sugiyama, Ohtsuki, Sato, & Kawabata, 1997; Uchikoba & Kaneda, 1996).

The sarcocarp of melon fruit (*Cucumis melo* L.) contains high concentrations of serine proteases, known as cucumisins. These proteases possess high proteolytic activity and milk-clotting activity, which are comparable with papain, a cysteine protease from papaya (Uchikoba & Kaneda, 1996). Kiwi fruit (*Actinidian* L) and ginger rhizomes (*Zingiber officinale*) also contain high amounts of the cysteine proteases actinidin (or kiwellin) and zingibaine (ginger protease, or GP), respectively. These could be useful as a meat tenderizing agent and rennet substitute due to their activity against collagen and caseins (Huang et al., 2011; Su et al., 2009; Uchikoba & Kaneda, 1996). While the potential exists for using plant proteases more extensively in food processing and other biotechnological processes, information regarding their development as processing aids, particularly their utility in cheese-making, is scarce. Hence, the objective of the present study was to evaluate the milk-clotting activities of ginger, melon and kiwi extracts in terms of temperature dependence and their performance on yield and textural properties of curds during cheese-making, compared to commercial chymosin.

2. Materials and methods

2.1. Samples

Fresh kiwifruit (*Actinidia deliciosa*), melon (*Cucumis melo*) and ginger rhizomes (*Zingiber officinale*) were obtained from a local market (Hermosillo, Sonora, Mexico). Commercial pasteurized low-fat (1%) bovine milk was purchased in a local market in four different occasions. Milk samples presented a standard composition typical of cow's milk, but reduced in fat content. All treatments were tested in duplicate by using the same milk batch. Rennet was obtained from Cuamex (Chr. Hansen de México S.A de C.V. Mexico D.F, Mexico). All chemical reagents were from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. Preparation of extracts

Water extracts of kiwi and ginger were prepared from slices of peeled kiwi or ginger rhizomes by adding one equal part (w/v) of 20 mM sodium phosphate buffer (pH 7.2), followed by homogenization in a model 450-10 Osterizer blender (Sunbeam Mexicana, S.A. de CV Acuña Coahuila, Mexico). Melon juice was obtained by homogenizing slices of melon mesocarp directly without adding buffer. The homogenized samples were then centrifuged at 5000*g* for 30 min at 4 °C using a model J2-21 Beckman centrifuge (Beckman Instruments NC. Palo Alto, CA, USA) and filtered through double cheesecloth (Guevara, Daleo, & Oliva, 2001; Mutlu, Pfeil, & Gal, 1998) to remove suspended particles. Fresh extracts were stored at 4 °C and were either used the same day, for protein and milk-clotting activity determination, or were frozen at −40 °C and lyophilized using a Labconco freeze drier (Labconco Corporation. Kansas City, MO, USA). Powder samples were kept at −15 °C until further use.

2.3. Effect of temperature on milk-clotting activity (MCA)

MCA was determined using the method described by Arima, Yu, and Iwasaki (1970), with slight modifications. Briefly, one ml of fresh plant extract (at 25 °C) was added to 10 ml of low fat (1%) pasteurized milk (containing 0.02% $CaCl_2$) that was pre-equilibrated at the appropriated temperature depending on the activity of each extract (35–50 °C for kiwi, 35–80 °C for melon and 40–70 °C for ginger). The mixture was incubated at the desired temperature until coagulation was observed. The assay was performed at least in triplicate. Coagulation times lower than 60 min were considered positive and were included in the data set of the temperature effect on MCA. The MCA was defined in terms of Soxhlet units (SU) as the quantity of protein needed to coagulate 1 ml of low-fat milk in 40 min (2400 s) at the temperature evaluated:MCA (SU) = $2400/t \times S/E$ where t = clotting time (sec); S = volume of milk (ml); E = volume of extract (ml)

2.4. Proteolytic activity

Proteolytic activity was determined by the method of Kunitz (1947) using bovine serum albumin (BSA) or casein as the substrate. Briefly, 450 μl of 1% protein substrate solution (0.1 M Phosphate buffer, pH 7.0) was mixed with 50 μl of coagulant and incubated for 60 min. Incubation temperatures were 40, 50 and 60 °C for kiwi, melon and ginger extracts, respectively. Chymosin (control) was incubated at 34 °C. After incubation, the reaction was stopped by the addition of 500 μl of 5% (w/v) trichloroacetic acid. The mixture was vortexed using a Mini Vortex MV-1 (VWR Scientific Products, Radnor PA, USA), left to stand on ice for 30 min and then centrifuged at 20,800*g* for 20 min using a model 5417R Eppendorf centrifuge. To measure soluble nitrogen, 100 μl of TCA extract were mixed with 200 μl of 0.2 N NaOH followed by 100 μl of phenol reagent (Folin–Ciocalteu phenol solution/water 1:2). Color was developed at 35 °C for 15 min and the optical density (OD) was measured at 660 nm using a Cary 50Bio spectrophotometer (Varian, Palo Alto, CA, USA). One unit of enzyme activity (U) was defined as the amount of protein that gave rise to an increase in the absorbance by one unit at 660 nm under the described conditions.

2.5. Protein determination

The protein content of plant extracts was determined using the DC Protein Assay kit from BioRad following the microplate protocol (Biorad Laboratories, Hercules, CA, USA). The absorbance at 590 nm was measured using an OPSYS MR microplate reader (DYNEX Technologies, Chantilly, VA, USA) and using BSA as standard at concentrations of 0.25, 0.50, 0.75, 1.00, 1.25 and 1.47 mg ml^{-1}.

2.6. Manufacture of miniature cheese

Cheese curds were produced following the protocol for the manufacture of miniature cheeses (Hynes, Ogier, & Delacroix-Buchet, 2000; Shakeel-Ur-Rehman, McSweeney, & Fox, 1998) adapted for fresh cheese to obtain a moisture level of 65–70% in the curd (Torres-Llanez, Vallejo-Cordoba, Díaz-Cinco, Mazorra-Manzano, & González-Córdova, 2006). Four batches of miniature cheese were prepared with two independent replications for all treatments. Briefly, 300 ml of milk was placed in a wide-mouth centrifuge bottle and mixed with the amount of coagulant necessary to clot the milk within 40–60 min.

In order to reduce the natural variability on coagulant strength between different batches of fresh plant extracts, the extracts were lyophilized and then the required amount of coagulant needed to coagulate the milk (300 ml), were re-dissolved in a minimum volume of water (3–4 ml), at 25 °C. Milk coagulation was performed at 63 °C for ginger extract, 40 °C for kiwi and melon extracts, and 34 °C for commercial rennet (control).