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Enzymatic milk clotting activity in artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus*) flowers. Immobilization of alpine thistle aspartic protease

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ABSTRACT

Two different milk clotting enzymes, belonging to the aspartic protease family, were extracted from both artichoke leaves and alpine thistle flowers, and the latter was covalently immobilized by using a polyacrylic support containing polar epoxy groups. Our findings showed that the alpine thistle aspartic protease was successfully immobilized at pH 7.0 on Immobeads IB-150P beads and that, under these experimental conditions, an immobilization yield of about 68% and a recovery of about 54% were obtained. Since the enzyme showed an optimal pH of 5.0, a value very similar to the one generally used for milk clotting during cheese making, and exhibited a satisfactory stability over time, the use of such immobilized vegetable rennet for the production of novel dairy products is suggested.

1. Introduction

Calf rennet is the most ancient milk clotting enzyme and still the most widely used biocatalyst in cheese making procedures. The coagulating properties of calf rennet are due to chymosin, an aspartic protease considered to be the best coagulating agent because of its high specificity for cleaving k-casein Phe105-Met106 bond (Ahmed, Morishima, Babiker, & Mori, 2009).

The worldwide increase in cheese production and consumption, together with the increase of calf rennet price, led to investigate new milk coagulating enzymes able to satisfactorily replace calf rennet in the manufacture of dairy products (Guiama, Libouga, Ngah, & Mbofung, 2010). Microbial rennet produced by genetically engineered bacteria has proven to be one suitable chymosin substitute, but the consumer constraints regarding genetically engineered foods, as well as religious or diet persuasion, have led to a growing interest toward vegetable coagulants (Ahmed et al., 2009; Egito et al., 2007). Therefore, even though plant extracts have been used in the manufacture of dairy products since ancient times, several new vegetable sources of milk clotting enzymes have been investigated in the last twenty years. Nowadays, the search for new potential coagulating enzymes from plants is in continuous progress to make them industrially useful following the increasing global demand for diversified and high quality cheese production (Hashim, Mingsheng, Iqbal, & Xiaohong, 2011).

The majority of these vegetable milk clotting enzymes belongs to the A1 aspartic protease family, being active at acidic pH, inhibited by pepstatin A and endowed with two aspartic acid residues responsible for their catalytic activity (Simoes & Faro, 2004). Plant sources for enzyme clotting (EC) activity have been identified in *Ficus carica* (El-Shibiny et al., 1973), *Calm viscera* (Gupta & Eskin, 1977), *Carica papaya* (Cabezas, Esteban, & Marcos, 1981), *Ananas comosus* (Cattaneo, Nigro, Messina, & Giangiacomo, 1994). Unfortunately, all of these enzymes were found unsuitable since they produce extremely bitter cheese as a consequence of their strong proteolytic activity (Ahmed et al., 2009; Lo Piero, Puglisi, & Petrone, 2002). An exception is represented by flower extracts of *Cynara* genus and, in particular, of *Cynara scolymus* and *Cynara cardunculus*, containing a multiplicity of aspartic protease molecular forms (Llorente, Obregón, Avilés, Caffini, & Vairo-Cavalli, 2014; Sarmento et al., 2009). However, the use of *C. scolymus* enzymes is extremely limited (Llorente et al., 2014), because globe artichoke is extensively consumed as food before blooming. Conversely, the most characterized plant aspartic proteases are the enzymes extracted from *C. cardunculus* flowers. In fact, they are successfully used in the manufacture of some traditional Portuguese (Serra and Serpa) and Spanish (Los Pedroches and Serena) dairy products (Roseiro, Barbosa, Ames, & Wilbey, 2003). These enzymes, cardosin A and cardosin B, are characterized by high clotting and strong

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proteolytic activity (Verissimo, Esteves, Faro, & Pires, 1995) producing cheeses with a creamy soft texture and genuine and slightly piquant aroma (Fernandez-Salguero & Sanjuan, 1999). Like chymosin, cardosin A cleaves bovine k-casein between Phe105 and Met106, while cardosin B is similar to pepsin in terms of specificity and activity (Prados, Pino, & Fernandez-Salguero, 2007).

Since artichoke processing industry generates large amounts of agricultural solid wastes, ~60% of which is represented by their leaves (FAO Statistical Database, 2011), we found of interest to test artichoke leaf as a possible source of milk EC activity, in the attempt to assign an added value to such waste, by improving the previously reported extraction procedure (Llorente, Brutti, & Caffini, 2004). Moreover, an additional potential source of vegetable rennet, obtainable at low cost, was identified in the flowers of a member of the Asteraceae family, *Carduus defloratus* subsp. *carlinifolius* Lam., commonly known as alpine thistle. The alpine thistle is a perennial widely distributed herbaceous plant belonging to the "wild flora". Its typical habitat is the meadow, but also the cloudy forest, walls, rocks and screes are possible environments for alpine thistle growth, being its preferred substrate both siliceous or calcareous with alkaline pH and basic nutritional value. In addition, the ability of growing on dry soil makes the alpine thistle suitable to be used as wildflower in recovery of marginal soils such as unproductive and/or intensely exploited agricultural lands. Namely, as wildflower, *C. defloratus* is easy to find and is very close in terms of systematic classification to *C. cardunculus* and *C. scolymus*.

Therefore, we found of great interest to investigate aspartic proteases possessing milk clotting properties both in *C. scolymus* leaves as well as in the flowers of *C. defloratus*, with the aim to proceed to the immobilization for the first time of a "plant rennet" possibly extracted from a low cost renewable source. In fact, to our knowledge, only immobilization procedures of rennets from *Mucor miehei* (Pessela et al., 2004), via covalent binding of their sugar chains on aminated supports, and from *Bacillus sphaericus* (El-Bendary, Moharam, & Ali, 2009), by adsorption on silica gel, have been thus far reported.

2. Materials and methods

2.1. Materials

Rennet from *Mucor pusillus*, var. Lindt (28 U/μl) was purchased from Ditta Rappelli (Tecnolatte, Lodi, Italy). *C. scolymus*, L. subsp. *scolymus* leaves were obtained from plants grown in Salerno province (Campania, Italy). Flowers of *C. defloratus* L. subsp. *carlinifolius*, Lam. were harvested in Gallicchio (PZ), a municipality of National Park "Appennino Lucano Val d'Agri Lagonegrese" (Basilicata, Italy). Viscozyme, a multi-enzyme mixture containing a wide range of carbohydrases, including arabinase, cellulase, β-glucanase, hemicellulase, and xylanase, was purchased from Novozyme (Bagsvaerd, Denmark). The Immobead IB-150P beads (particle size 150–300 μm) were a kind gift from ChiralVision (The Netherlands). Electrophoresis reagents and nonfat dry milk were purchased from BIO-RAD (Milano, Italy). All other chemicals and reagents of analytic grade were purchased from Sigma–Aldrich (Milano, Italy).

2.2. Protein extraction

Crude plant protein extracts were obtained by using Ultra-Turrax (IKA-WERKE, Germany) homogenizer under different conditions. Fresh artichoke leaves were homogenized (1:3, w/v) with one of the following three buffers: 0.1 M sodium citrate/pH 3.0, 0.1 M sodium acetate/pH 5.0, 0.1 M Tris–HCl/pH 7.0. Where indicated, artichoke leaves (1:5, w/v) were incubated in 0.1 M sodium acetate buffer/pH 5, containing 13% (w/v) sorbitol and different amounts of Viscozyme (3%, 6%, 12% or 24%, v/w of leaves) for different times (5, 10, 20 and 40 min). The enzymatic hydrolysis was carried out in an orbital incubator (K*i*nher, Switzerland) at 200 rpm and 37 °C and then homogenized. Treatment with 6% Viscozyme for 20 min was found sufficient to extract all soluble proteins.

Fresh thistle flowers were homogenized in 0.1 M sodium acetate/pH 5.0 (1:3, w/v) according to Chen, Zhao, and Agboola (2003). All homogenates were centrifuged at 30,000×*g* for 30 min at 4 °C and the collected supernatants were filtered through Whatman n° 1 filter papers and then brought to different ammonium sulfate saturations (0–20%, 20–50% and 50–80%) by slowly adding the appropriate salt amount during continuous stirring at 4 °C. The precipitates were collected by centrifugation at 30,000×*g* for 10 min at 4 °C and redissolved in the minimum volume of the homogenization buffer. Finally, all the fractions were dialyzed 3 times for 6 h against 100 volumes of the same buffer.

2.3. Immobilization procedure

Either 1.12 mg of *M. pusillus* enzyme (11.2 U) or 2.08 mg of thistle flower protein (20–50% ammonium sulfate fraction) (16 U) were added to 100 mg of dry Immobead IB-150P beads suspended in 0.1 M phosphate buffer at pH 5.0, 6.0 or 7.0 (0.4 mL final volume) according to Mateo et al. (2003). The mixtures were shaken for 3 h at 4 °C on the rotary mixer and then the vials were let stand for 13 h at 4 °C in order to complete the immobilization reaction and to allow the decantation of the beads. The supernatants containing the free (unbound) enzyme molecules were then removed and used to determine both protein content and EC activity. The sedimented beads were washed four times for 5 min with 1.5 mL of buffer with the pH appropriate for the free enzyme and each washing solution was analyzed for protein content and EC activity. Finally, the EC activity of the washed beads was also determined as described below.

2.4. Milk clotting activity

EC activity of the crude enzyme extracts isolated from *C. scolymus* or *C. defloratus* was qualitatively evaluated according to the method called "caseogram prints", a kind of zymogram described by Foltman, Szecsi, and Tarasova (1985). The sample was thus allowed to electrophoretically migrate in a polyacrylamide gel in non-denaturing conditions at 20 mA for 50 min in Tris–HCl buffer, pH 8.8. After this step, the gel was equilibrated with 0.15 M sodium acetate buffer, pH 5.0, for 30 min and then placed on the top of a previously prepared 1% (w/v) agarose gel, containing 1% (w/v) skim-milk powder in 0.1 M sodium acetate buffer, pH 5.3. The assembled system was finally incubated at 37 °C for 20 min to allow the appearance of the clotting signals in correspondence of the electrophoretic bands.

For quantitative measurements of EC activity a spectrophotometric assay was performed as previously described (Douillard & Ribadeau-Dumas, 1970; Penzol, Armisen, Fernandez-Lafuente, Rodes, & Guisan, 1998). The enzyme source was incubated at 25 °C in a disposable spectrophotometer cuvette (pathlength 1 cm) containing 1.0 mL of different buffers (0.1 M sodium acetate, pH 5.0; 0.1 M sodium phosphate, pH 7.0) and 0.1% k-casein as substrate, in the absence or presence of 1 μM pepstatin A. When immobilized enzyme was used, 180 mg of enzyme containing wet beads (corresponding to 100 mg of starting dry beads) were incubated at 25 °C in 1.0 mL of either 0.1 M sodium phosphate buffer, pH 7.0 (*M. pusillus* enzyme) or 0.1 M sodium acetate buffer, pH 5.0 (*C. defloratus* enzyme) in the presence of 0.1% k-casein. Both for