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Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine κ-casein

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Abstract

Milk-clotting activity found in ammonium sulfate-precipitated protein extracts from *Albizia lebbeck* and *Helianthus annuus* seeds was studied. Specific clotting activity of albizia seed extract was 15 times higher than that of sunflower seed extract. Zymogram analysis revealed several proteolytic bands in albizia seed extract and one diffuse proteolytic band for sunflower seed extract. Whole bovine casein was incubated with the plant seed extracts or chymosin and some breakdown products were characterized by reversed-phase high-performance liquid chromatography and electrophoresis. Similar to chymosin, the two seed extracts exhibited proteolytic activity toward κ-casein, α_s-casein and β-casein, with the highest activity observed for the albizia seed extract. Mass spectrometry analysis showed that the sunflower extract hydrolyzed κ-casein at the Phe_{105}–Met_{106} bond, as does chymosin. The albizia extract also displayed activity on κ-casein, but the Lys_{116}–Thr_{117} bond was its preferred target.

Keywords: Plant rennet; Milk-clotting activity; Bovine casein; κ-casein; Albizia; Sunflower

1. Introduction

Among the vast number of proteases with applications in the food industry, aspartic proteases such as chymosin (EC 3.4.23.4) are used for milk clotting in cheese-making. The primary cleavage occurs at Phe_{105}–Met_{106} bond of bovine κ-casein (κ-CN; Jollès, Alais, & Jollès, 1963) and causes destabilization of the casein micelles, resulting in milk coagulation to form the cheese curd.

Milk clotting can be achieved by a number of proteolytic enzymes from various sources, such as different animal (pig, cow, and chicken pepsins) and microbial species (*Rhizomucor miehei, R. pusillus* and *Cryphonectria parasitica*). Plant coagulants are of growing interest, as the use of animal rennet may be limited for religious reasons (e.g., Judaism and Islam), diet (vegetarianism), or consumer concern regarding genetically engineered foods (e.g., Germany, Netherlands and France forbid the use of recombinant calf rennet). More recently, the incidence of bovine spongiform encephalopathy has reduced both supply and demand for bovine rennet (Roseiro, Barbosa, Ames, & Wilbey, 2003).

Plant sources for milk-clotting enzymes have been identified from *Ananas comosus* (Cattaneo, Nigro, Messina, & Giangiacomo, 1994), *Calotropis procera* (Sanni, Onilude, & Momoh, 1999), *Opuntia phylloclades*, *Cereus triangularis, Euphorbia caducifolia, Ficus bengalensis*, *F. elastica*, *E. hista* (Umar Dahot, Yakoub Khan, & Memon, 1990), *Lactuca sativa* (Lo Piero, Puglisi, & Petrone, 2002), seven papilionoideae species (*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*,

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E. psoraleoides, *Adenolichos anchietae* and *Droogmansia megalantha*; Lopes, Teixeira, Liberato, Pais, & Clemente, 1998), the cardoons *Cynara scolymus* (Sidrach, Garcia-Canovas, Tudela, & Rodriguez-Lopez, 2005) and *C. cardunculus* (Sousa & Malcata, 2002), and *Helianthus annuus* (Park, Yamanaka, Mikkonen, Kusakabe, & Kobayashi, 2000). Unfortunately, most of these plant rennets have been found to be unsuitable because they produce extremely bitter cheeses. An exception to this general rule is the aqueous extracts of cardoons. Extracts of *Cynara* are used chiefly in the making of various Spanish cheeses, e.g., Torta del Casar, La Serena, Los Pedroches, Los Ibores, Flor de Guía, and Portuguese cheeses from sheep's milk, e.g., Serra da Estrela, Serpa, Azeitão, Nisa, Castelo Branco, Évora (Roseiro et al., 2003).

The flower of *C. cardunculus* contains aspartic proteases, cardosin A (GenBank accession No. CAB40134; Faro et al., 1999), the most abundant; and cardosin B (No. CAB40349; Vieira et al., 2001). Cardosin A has been studied in detail (Verissimo, Esteves, Faro, & Pires, 1995) and was shown to cleave bovine κ-CN at the same peptide bond, Phe_{105}–Met_{106}, as chymosin. Cardosin B, in comparison, is similar to pepsin, in terms of specificity and activity. Aspartic proteases have been found in the flower cells of *C. cardunculus* and of *C. scolymus* by other authors and named cyprosins A and B and cynarases A, B, and C, respectively (Cordeiro, Xue, Pietrzak, Pais, & Brodelius, 1994; Sidrach et al., 2005).

An aspartic protease from sunflower seeds displaying a milk-clotting activity has been identified and its primary structure has been deduced from gene sequence as No. AB025359 (Park et al., 2000); a comparison with the sequence of a cynarase of *C. cardunculus* (No. X69193; Cordeiro et al., 1994) shows 78% identity with the sunflower aspartic protease (Park et al., 2000). However, the sunflower enzyme displays a negligible value of milk-clotting activity, whereas the cynarase has high milk-clotting activity (Park et al., 2000). To the best of our knowledge, the specific action of sunflower proteolytic enzymes toward caseins is not known.

Similarly, the seeds of the tree *Albizia julibrissin* have been shown to possess proteolytic enzymes which clotted milk readily, without developing any bitterness in cheese after 3 months of ripening (Otani, Matsumori, & Hosono, 1991). Surprisingly, no other work has been performed to study more extensively the clotting activity of any *Albizia* species.

The aim of the present work was to study the potential ability of protein extracts from *A. lebbeck* and *H. annuus* seed to coagulate milk and to determine the action of these milk-clotting plant extracts on bovine whole casein and, in particular, κ-CN.

2. Materials and methods

2.1. Preparation of crude and protein extracts

Dried seeds of *A. lebbeck* and *H. annuus* (variety EMBRAPA 122-V2000) were obtained in experimental farms of the Brazilian Agricultural Research Co. (EMBRAPA) located in the regions of Sobral and Londrina, respectively. Ten grams of peeled sunflower seeds and of whole albizia seeds were ground in a coffee grinder, and aqueous extracts were prepared by soaking the seed powders in 100 mL of distilled water containing 1% (w/v) NaCl and 0.02% (w/v) sodium azide. The aqueous mixtures were maintained for 24 h at 4 °C with agitation, and then the samples were filtered to give crude extracts.

Proteins were precipitated from the crude extracts by using ammonium sulfate at 40% saturation, and the mixture was kept at 4 °C for 45 min before centrifugation (15,000 × *g* at 4 °C for 10 min). The pellets were discarded, and ammonium sulfate was added to the supernatants to reach 60% saturation in the case of sunflower and 70% in the case of albizia. After 45 min of incubation at 4 °C, the mixtures were again centrifuged (15,000 × *g* at 4 °C for 10 min). The pellets were dissolved in 20 mL of pure water, dialyzed for 48 h at 4 °C to remove salts, and finally freeze-dried to give protein extracts of sunflower and albizia seeds, respectively.

2.2. Milk-clotting experiments

The clotting activities of plant extracts were determined according to the method of Berridge (1952). Crude and protein extracts were dissolved at 20 mg mL^{-1} in 10 mM $CaCl_2$, and the clotting time was measured using 100 μL of each solution mixed with 1 mL of reconstituted milk (12%, w/v, commercial low-heat skim milk powder at pH 6.5 dissolved in 10 mM $CaCl_2$; Régilait, Saint-Martin-Belle-Roche, France) and incubated at 37 °C until milk clotting occurred. One unit (1 U) was defined as being the quantity (mg) of crude or protein extract needed to coagulate 1 mL of reconstituted skim milk powder in 1 min at 37 °C.

2.3. Zymogram analysis

Enzyme activities of plant extracts were detected by zymography, adapted from the method of Dib, Chobert, Dalgalarrondo, Barbier, and Haertlé (1998). A quantity of 3 mg of each of plant crude extract or protein extract or chymosin was added to 1 mL of 0.125 M Tris-HCl buffer, pH 6.8, containing 5% (w/v) SDS, 1% (w/v) sucrose, and 0.05% (w/v) bromophenol blue. A volume of 10 μL of each solution was loaded onto SDS-PAGE gels containing 0.1% (w/v) gelatin. Electrophoresis (SDS-PAGE) was performed with a 4.9% (w/v) polyacrylamide stacking gel in 0.125 M Tris-HCl buffer, pH 6.8 and with a 15.4% (w/v) polyacrylamide resolving gel in 0.38 M Tris-HCl buffer, pH 8.8 containing 0.1% (w/v) SDS, at 4 °C for 150 min at 500 V, 60 mA, and 30 W (Laemmli & Favre, 1973). After electrophoretic migration, the gel was washed two times with 2% (v/v) Triton X-100 for 30 min. The hydrolysis reaction then proceeded inside the gel during incubation at 37 °C for 48 h in a bath of 0.05 M Tris-HCl buffer, pH 7.5, containing 15 mM $CaCl_2$. The active enzymes were revealed