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Protease activity of enzyme extracts from tamarillo fruit and their specific hydrolysis of bovine caseins

Zhao Li[a], Ken Scott[b], Yacine Hemar[a,c], Don Otter[d,*]

[a] School of Chemical Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[b] School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland 1143, New Zealand
[c] The Riddet Institute, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand
[d] Center for Dairy Research, College of Agriculture and Life Sciences, University of Wisconsin-Madison, 1605 Linden Drive, Madison 53706, USA

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ABSTRACT

The characterisation of a serine protease isolated from tamarillo (*Solanum betaceum*) fruit and its milk casein hydrolysis activity were investigated. Compared with calf rennet, a crude extract from tamarillo exhibited wider caseinolytic activity on sodium caseinate. The purified protease was named "tamarillin" and revealed proteolytic activity toward purified α-, β- and κ-casein. Similar to calf rennet, tamarillin preferably hydrolysed κ-casein, but, unlike calf rennet, it also displayed high proteolytic activity toward both α- and β-casein. The major peptide generated from κ-casein by tamarillin was analysed by gel electrophoresis and liquid chromatography mass spectrometry to confirm its molecular mass as 14,290 Da. The cleavage site was confirmed by in-gel tryptic digestion and time-of-flight mass spectrometry analysis to be at Asn_{123}-Thr_{124}. This was in contrast to the Phe_{105}-Met_{106} cleavage site of rennet hydrolysis.

1. Introduction

Milk coagulation is one of the basic steps in cheese making (Guinee & Wilkinson, 1992; Macedo, Faro, & Pires, 1993; Shieh, Thi, & Shih, 2009), and is usually achieved using chymosin (EC 3.4.23.4), the main protease component in calf rennet. Chymosin hydrolyses the Phe_{105}-Met_{106} bond of κ-casein with high specificity, resulting in instability of the casein micelles and subsequent milk coagulation (Egito et al., 2007; Jollès, Alais, & Jollès, 1963). Most commercial milk-clotting enzymes are aspartic proteases that display chymosin-like cleavage specificity. However, two proteases, from *Cryphonectria parasitica* and the microbial *Endothia parasitica* have been reported to have good milk-clotting activity with the hydrolysis site on κ-casein at Ser_{104}-Phe_{105} (Drohse & Foltmann, 1989; Jacob, Jaros, & Rohm, 2011).

Milk curd can also be produced using proteases from other animal, microbial, plant sources, and by using the chymosin genes from calf stomachs that have been genetically engineered into microbial hosts (Jacob et al., 2011). A world-wide shortage of calf rennet has been predicted due to the increased production of cheese and a simultaneous decrease in the general availability of suckling calves' stomachs (Jacob et al., 2011). The usage of animal rennet has been also restricted by religion in countries such as India and Israel (Gordin & Rosenthal, 1978; Whitaker, 1959), and because of vegetarianism and opposition against genetically engineered foods (e.g. Germany and Netherlands forbid the application of recombinant calf rennet) (Roseiro, Barbosa, Ames, & Wilbey, 2003). This has led to the search for alternative milk-clotting sources (Roseiro et al., 2003), particularly for proteases which are natural and cost effective, such as proteases isolated from plants (Nasr, Ahmed, & Hamid, 2016; Shah, Mir, & Paray, 2014).

The milk-clotting activities of a number of plant coagulants have been recently investigated. These include extracts from *Callotropis procera* (sodom apple) (Aworth & Muller, 1987), from artichoke (*Cynara scolymus*) flowers (Chazarra, Sidrah, López-Molina, & Rodríguez-López, 2007), from twigs of *Streblus asper* (Senthilkumar, Ramasamy, & Subramanian, 2006), from latex of *Euphorbia microsciadia* (Rezanejad, Karbalaei-Heidari, Rezaei, & Yousefi, 2015), actinidin from kiwifruit (Lo Piero, Puglisi, & Petrone, 2011), from bentong ginger (*Zingiber officinale* var. Bentong) (Nafi, Ling, Bakar, & Ghazali, 2014), extract from the root of *Jacaratia corumbensis O.* Kuntze (Duarte et al., 2009), a protease from the seed of *Solanum dubium* (Ahmed, Morishima, Babiker, & Mori, 2009), religiosin B from the latex of *Ficus religiosa* (Kumari, Sharma, & Jagannadham, 2012) and lettucine from the leaves of *Lactuca sativa* (Lo Piero, Puglisi, & Petrone, 2002). However, most of these plant coagulants have been found to be unsuitable for cheese making due to their broad, non-specific hydrolysis activity on caseins, resulting in low yields and a bitter taste in the final cheese product. The aspartic

* Corresponding author.
E-mail address: donotter1@gmail.com (D. Otter).

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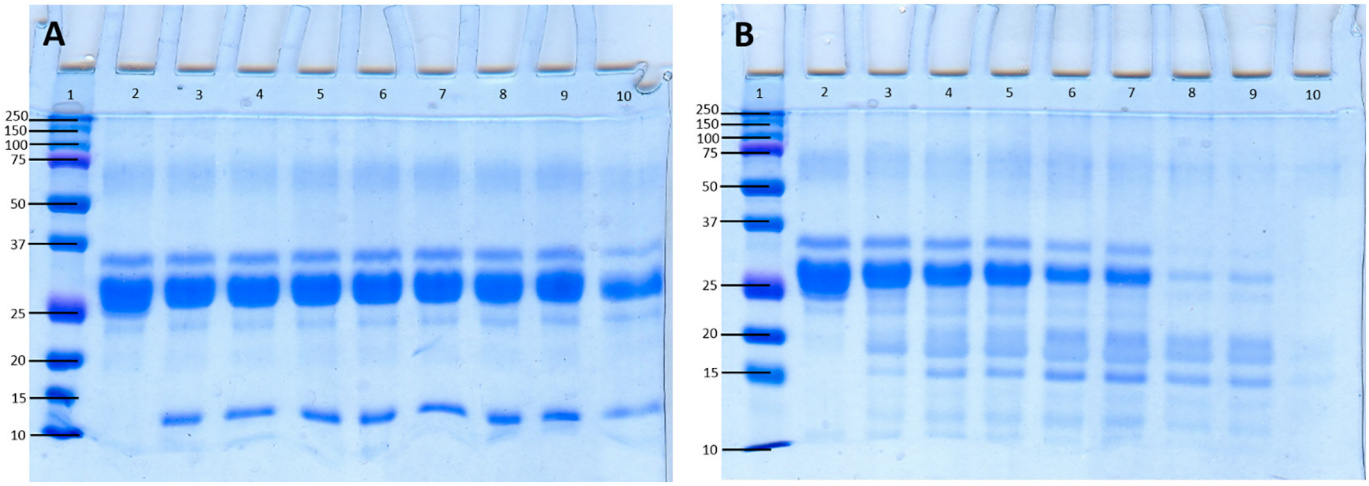

Fig. 1. SDS-PAGE (5% stacking gel; 12% resolving gel) of bovine sodium caseinate (10 μL of 2.5 mg/mL) hydrolysed at different times by rennet (A) and tamarillo crude extract (B). Lane 1: protein marker; Lane 2: sodium caseinate; Lane 3: 15 min hydrolysis; lane 4 and 5: 30 min hydrolysis; lane 6 and 7: 1 h hydrolysis; lane 8 and 9: 4 h hydrolysis and lane 10: 24 h hydrolysis.

protease cardosin A, extracted from the flowers of *Cynara carduculus,* has been used as a coagulant in ovine cheese making in Portugal and Spain (Carmona, Sanjuán, Gómez, & Fernández-Salguero, 1999; Fernández-Salguero & Sanjuán, 1999; Macedo et al., 1993). Cardosin A has similar characteristics to rennet with a highly specific cleavage on the Phe_{105}-Met_{106} site of κ-casein (Macedo et al., 1993). Unlike rennet however, cardosin A has also been found to hydrolyse α- and β-casein (Silva & Malcata, 1998; Silva & Malcata, 1999). Therefore, cardosin A has not been used for the production of bovine milk cheese due to its broad hydrolytic activity, resulting in a bitter taste of the cheese made using bovine milk (Macedo, Faro, & Pires, 1996).

In this paper we report for the first time the effect of a serine protease extracted from tamarillo (*Solanum betaceum*) fruit (tamarillin) on bovine caseins. The effect of crude tamarillo extract on caseinates and that of the purified tamarillo extract on individual caseins are investigated by SDS-PAGE to determine the extent of hydrolysis in comparison with rennet. The molecular masses of the peptides resulting from the hydrolysis of κ-casein by the tamarillin were characterised using mass spectrometry, and its specific cleavage site on κ-casein was elucidated using time-of-flight mass spectrometry (TOF-MS).

2. Materials and methods

2.1. Materials and sodium caseinate preparation

Casein standards, α-, β- and κ-casein, were purchased from Sigma-Aldrich Ltd., (Auckland, New Zealand). Calf rennet (280 International Milk-clotting Units (IMCU) mL^{-1}) was supplied by RENCO New Zealand Laboratory (RENCO New Zealand, Eltham, New Zealand). Low heat skim milk powder (SMP) was obtained from Synlait Milk Ltd., Rakaia, New Zealand. All chemicals were of analytical grade.

Sodium caseinate was prepared by the method of Lucey, Srinivasan, Singh, and Munro (2000) with some modifications. Skim milk powder (10% w/w) was mixed with ultrapure water for 2 h using a magnetic stirrer, then the reconstituted milk was kept in the fridge one day before use to ensure full hydration. The reconstituted skim milk was acidified to pH 4.6 by adding 2 M HCl drop-wise at room temperature. The resulting curd was separated from the whey, then washed five times with ultrapure water and dewatered using two layers of cheesecloth. The washed curd was re-dissolved with ultrapure water in a 1:1 ratio, and the pH of the mixture was adjusted to 6.8 using 2 M NaOH under slow stirring. The sodium caseinate solution was freeze-dried for future use.

2.2. Tamarillo crude and purified protease extracts

The method for preparing a crude tamarillo protease extract was based on McDowall (1970) with some modifications (Li, Scott, Hemar, Zhang, & Otter, 2018). Laird's Large (*Cyphomandra betacea*) tamarillo fruit (~300 g) were homogenized for 20 min using a Polytron PCU2 laboratory homogeniser (Brinkmann Instruments, Luzern, Switzerland), followed by filtration through two layers of cheesecloth to remove insoluble material. Tamarillo filtrate (~85 mL) was mixed with 80 mL 0.05 M pH 5.5 sodium citrate buffer by gentle stirring, then centrifuged at 15,000 × *g* for 20 min at 4 °C. Protein from the supernatant (155 mL) was precipitated overnight at 4 °C using a 65% saturated ammonium sulphate solution (61.69 g). The precipitated protein was centrifuged at 15,000 × *g* for 20 min at 4 °C, and then dissolved in 30 mL 0.05 M sodium citrate buffer (pH 5.5). One volume of the resulting solution was dialyzed 3 times against five volumes of the same buffer for 24 h at 4 °C. The dialysed solution was freeze-dried and called the crude extract. The protein concentration of the crude extract solution was measured based on Bradford (1976), which was 1.17 ± 0.03 mg/mL.

Tamarillin was purified and prepared as described in Li et al., 2018. Crude tamarillo extract solution (40 mL) was loaded onto a DEAE-Sepharose Fast Flow ion exchange column (22 × 5 cm) pre-equilibrated with 0.05 M sodium citrate buffer (pH 5.5). The individual proteins were eluted using a 200 mL linear gradient of 0–1.0 M NaCl at a 1.0 mL/min flow rate. Fractions (10 mL) were collected and their protein content was measured by absorbance at 280 nm using a spectrophotometer (UVmini-1240, Shimadzu Corporation, Australia). Fractions corresponding to peaks of high caseinolytic activity (method based on Li et al., 2018) were pooled and dialysed three times against ultrapure water for 24 h at 4 °C to remove salt. The principal pooled protease fraction was then freeze-dried and stored at −80 °C. The purified tamarillo protease was given the name “tamarillin”.

2.3. Hydrolysis of caseins by rennet and tamarillo extracts

Casein hydrolysates were prepared based on Egito et al. (2007). For sodium caseinate hydrolysed by crude tamarillo extract or rennet, the sodium caseinate was dissolved in 100 mM sodium phosphate buffer, pH 6.5 using a magnetic stirring for 2 h at room temperature to obtain a concentration of 10 mg/mL. Tamarillo crude extract (350 μL) or rennet (10 μL, diluted 10-fold with ultrapure water) were added to 3 mL sodium caseinate solution to make a final sodium caseinate concentration of 10 mg/mL and then 200 μL aliquots were transferred into individual HPLC sample vials (Interlab, Wellington, New Zealand). The hydrolysis