

# Plant proteases as milk-clotting enzymes in cheesemaking: a review

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**Abstract** Plant proteases used as milk coagulants in cheesemaking are reviewed in this paper. Plant proteases have been used as milk coagulants in cheesemaking for centuries either as crude extracts or in purified form. These coagulants are an alternative to the calf rennet due to the limited availability and high price of rennet, religious factors, diet or ban on recombinant calf rennet in some countries. These enzymes are found in almost all kinds of plant tissues and can be obtained from their natural source or through in vitro culture to ensure a continuous supply of plant proteases. Almost all the enzymes used as milk coagulants belong to aspartic proteases, but enzymes from other groups such as cysteine and serine proteases have also been reported and possess the ability to clot milk under proper conditions. The excessive proteolytic nature of most plant coagulants has limited their use in cheese manufacturing due to lower yields of cheese, bitter flavors and texture defects. The search for new potential milk-clotting enzymes from plants still continues in order to meet the increasing global demand for diversified and good quality cheese production.

**Keywords** Plant protease · Milk-clotting activity · Cheese · Casein · Vegetable coagulant

## 1 Introduction

Milk coagulation is the main step for producing cheese, and coagulating enzymes, which are preparations of proteolytic enzymes, have been used in cheesemaking for thousands of years, and they seem to be the oldest known application of enzymes. The earliest indication of cheesemaking descends from cave paintings around 5000 BC (Harboe et al. 2010). Historically, most enzyme preparations used for cheese have been

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extracts from the stomachs of ruminants, but coagulants from microbes and plants were also used at very early dates (Harboe et al. 2010; Jacob et al. 2011). Ruminant stomach, especially that of the calf, is the source of rennet. It contains chymosin (EC 3.4.23.4) as the main enzyme component and has been the most widely used in cheesemaking. The cheese production increased by a factor of approximately 3.5 since 1961 but the rennet supply decreased due to the limited availability of ruminant stomachs (Jacob et al. 2011). Various factors such as high price of rennet, religious concerns (e.g., Islam and Judaism), diet (vegetarianism) or ban on recombinant calf rennet (in France, Germany and The Netherlands) have encouraged the search for alternative milk-clotting sources (Roseiro et al. 2003). The research has been directed towards discovering milk-clotting enzymes which would satisfactorily replace calf rennet in cheesemaking, including microbial, recombinant, and plant-based enzymes (Jacob et al. 2011). The most important substitutes which fulfill the requirements of cheese manufacture include microbial, recombinant, and plant-based enzymes which have been isolated and studied. Rennet substitutes produced by microorganisms and genetically engineered microorganisms have proven to be suitable substitutes for animal rennet, but increasing interest has been directed toward vegetable coagulants i.e., the milk-clotting enzymes extracted from plants. According to Tamer and Mavituna (1997), these enzymes are present in almost all kinds of plant tissues and it appears to be a general rule that all proteolytic enzymes have the ability to clot milk under appropriate conditions. Almost all the enzymes used as milk coagulants belong to aspartic proteases, but enzymes from other groups such as cysteine and serine proteases have also been used.

Plant extracts have been used as milk coagulants in cheesemaking since ancient times. Cheeses made with vegetable coagulant can be found mainly in Mediterranean, West African, and southern European countries. Spain and Portugal have the largest variety and production of cheeses using *Cynara* sp. as the vegetable coagulant (Roseiro et al. 2003). The extracts of *Cynara* spp. have been used in the making of Portuguese Serra and Serpa cheeses (Macedo et al. 1993) and Spanish Los Pedroches, La Serena (Roa et al. 1999) and Torta del Casar cheeses (from ewes' milk) as well as Los Iborens cheese (from goats' milk) and Flor de Guía cheese (from a mixture of ewes' and cows' milk) (Fernández-Salguero et al. 1991; Fernández-Salguero 1999; Sanjuán et al. 2002). In West African countries like Nigeria and the republic of Benin, extracts from *Calotropis procera* (Sodom apple) have been used in traditional cheesemaking (Roseiro et al. 2003). However, the excessive proteolytic nature of most vegetable coagulants has limited their use in cheese manufacturing due to lower cheese yield and defects in flavor and texture (Lo Piero et al. 2002). Therefore, the search for new potential milk-clotting enzymes from plants is in continuous process, so as to make them industrially useful and go with the increasing global demand for diversified and high quality cheese production (Hashim et al. 2011).

Several studies have been performed using plant-derived enzymes for cheesemaking. Sousa and Malcata (2002) reviewed the role of plant coagulant (*Cynara cardunculus*) in vitro and during ripening of cheeses from several milk species, while as Roseiro et al. (2003) reviewed the use of plant extracts with special reference to *Cynara* species. Jacob et al. (2011) reviewed the important types of milk-clotting enzymes including animal rennet, microbial coagulants, recombinant coagulants, and plant-derived clotting enzymes. Yegin and Dekker (2013) have recently reviewed the progress in the field of aspartic proteinases from animal, plant and microbial origin with a special emphasis on

structures, functions, catalytic mechanism, inhibition and engineering. The objective of this review is to summarize the latest research findings on plant-derived clotting enzymes with special emphasis on enzyme chemistry, production and techno-functional properties.

## 2 Types and sources of plant proteases

Proteases are required by plants in all aspects of their life cycle. They are involved from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller 2004). Proteases have been divided into groups based on the catalytic mechanism used during the hydrolytic process. The main catalytic types are aspartate, serine, cysteine, and metalloproteases (Bah et al. 2006), but the plant proteases used as milk coagulants have been reported only from first three types (Table 1) and none from metalloproteases. Serine and cysteine proteases are catalytically very different from aspartic and metalloproteases in that the nucleophile of the catalytic site is part of an amino acid, whereas it is an activated water molecule in the other two groups (Bruno et al. 2006).

### 2.1 Aspartic proteases

Aspartic proteases have two aspartic residues at their catalytic site. They are most active at acidic pH and show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues responsible for the catalytic activity (Domingos et al. 2000).

Aspartic proteases with milk-clotting activity have been reported in artichoke (*Cynara scolymus* L.) (Llorente et al. 1997); milk thistle (*Silybum marianum* L. Gaertn.) (Vairo-Cavalli et al. 2005); *Onopordum turcicum* (Tamer 1993); rice kernels (Asakura et al. 1997); *Centaurea calcitrapa* (Domingos et al. 2000). Cardoon (*Cynara cardunculus*) flowers are traditionally used in the Mediterranean region for cheesemaking (Barros et al. 2003). It produces cardosins and cyprosin, aspartic proteases that have been found to accumulate in mature flowers (petals and pistils) but not in leaves or seeds (Cordeiro et al. 1998). Cardosin A is an abundant aspartic protease from pistils of *C. cardunculus*. Also, three cyprosin with milk-clotting activity, from dried flowers of *C. cardunculus*, were isolated, purified, and characterized by Heimgartner et al. (1990). These flowers contain aspartic proteases, previously described, which have been shown to share specificity and kinetic parameters with chymosin and pepsin (Verissimo et al. 1995, 1996).

### 2.2 Cysteine proteases

Cysteine proteases, also known as thiol proteases, and the catalytic mechanism of these enzymes involve a cysteine group in the active site. Cysteine proteases have great potential in the food, biotechnology, and pharmaceutical industries owing to their property of being active over a wide range of temperature and pH. Plants offer an attractive alternative for the production of CPs as they occur naturally in different tissues, in some cases in excessive amount (Gonzalez-Rabade et al. 2011).

**Table 1** Types and sources of milk-clotting plant proteases

Type of protease	Amino acid at catalytic site	Protease name	Source	References		
Aspartic	2 Aspartic acid residues	Cardosins and cyprosins	<i>Cynara cardunculus</i>	Silva et al. 2003; Silva and Malcata, 2005; Roa et al. 1999; Agboola et al. 2009; Barros et al. 2001; Esteves et al. 2002; 2003; Sanjuán et al. 2002; Low et al. 2006; Ordiales et al. 2012; Pino et al. 2009		
		Cynarase	<i>Cynara scolymus</i>	Llorente et al. 1997; 2004; Sidrach et al. 2005; Chazarra et al. 2007		
		Cardosin like Protein extract	<i>Cynara humilis</i> <i>Silybum marianum</i>	Esteves et al. 2003 Vairo-Cavalli et al. 2005; 2008		
		Onopordosin Protein extract	<i>Onopordum acanthium</i> <i>Onopordum turcicum</i>	Brutti et al. 2012 Tamer, 1993		
		Oryzasin Protein extract	<i>Oryza sativa</i> <i>Centaurea calcitrapa</i>	Asakura et al. 1997 Domingos et al. 2000; Salvador et al. 2006; Reis et al. 2000		
		Procirsin Protein extract	<i>Cirsium vulgare</i> <i>Solanum elaeagnifolium</i>	Lufrano et al. 2012 Nestor et al. 2012		
		Cysteine	Cysteine residue	Ficin	<i>Ficus racemosa</i>	Devaraj et al. 2008
				Caprifig Coagulant Protein extract	<i>Ficus carica sylvestris</i> <i>Albizia lebbek</i>	Faccia et al. 2012 Egito et al. 2007
				Protein extract	<i>Helianthus annuus</i>	Egito et al. 2007
				Actinidin	<i>Actinidia chinensis</i>	Katsaros et al. 2010
Serine	Serine residue			Cucumisin	<i>Cucumis melo</i>	Uchikoba and Kaneda 1996
		Neriifolin	<i>Euphorbia neriifolia</i>	Yadav et al. 2011		
		Neriifolin S	<i>Euphorbia neriifolia</i>	Yadav et al. 2012		
		Dubiumin	<i>Solanum dubium</i> Fresen	Ahmed et al. 2009a, b; 2010		
		Religiosin	<i>Ficus religiosa</i>	Kumari et al. 2010		
		Religiosin B	<i>Ficus religiosa</i>	Kumari et al. 2012		
		Religiosin C	<i>Ficus religiosa</i>	Sharma et al. 2012		
		Streblin	<i>Streblus asper</i>	Tripathi et al. 2011		
		Lettucine	<i>Lactuca sativa</i>	Lo Piero et al. 2002		
		Not specified	–	Hieronymain Protein extract	<i>Bromelia hieronymi</i> Mez <i>Moringa oleifera</i>	Bruno et al. 2010 Pontual et al. 2012

Ficin isolated from the latex of different *Ficus* species possess certain characteristic properties. A ficin isolated from the latex of *Ficus racemosa*, showed an ability to digest casein, suggestive of a milk-clotting property (Devaraj et al. 2008). Protein extracts from sunflower and albizia seeds were prepared to determine the milk-clotting activity and the

action of these milk-clotting plant extracts on bovine whole casein (Egito et al. 2007). Actinidin was isolated from ripe fresh kiwifruits (*Actinidia chinensis*) (Katsaros et al. 2010). Ginger protease was isolated from ginger rhizomes (*Zingiber officinale*) having a molecular mass of 36 kDa (Hashim et al. 2011).

### 2.3 Serine proteases

Serine proteases possess a serine residue in their active site and share a number of biochemical and physiological features. In plants, they are widespread among taxonomic groups, from trees and crops to legumes and herbs and present in almost all plant parts, but most abundant in fruits. Serine proteases from cucurbits, cereals, and trees are usually classified together (Rawlings and Barrett 2004).

Plant serine proteases have been found and extracted from latex, seeds, flowers, stems, leaves and roots. Neriifolin, a chymotrypsin-like serine protease, has been purified from the latex of *Euphorbia neriifolia* (Yadav et al. 2011). Another enzyme Neriifolin S, a dimeric serine protease of molecular mass 94 kDa with milk-clotting activity has been purified from the latex of *E. neriifolia* (Yadav et al. 2012). Religiosin (43.3 kDa), Religiosin B (63 kDa) and Religiosin C (80 kDa) were isolated from *Ficus religiosa* latex by Kumari et al. (2010), Kumari et al. (2012) and Sharma et al. (2012) respectively. Streblin, a thermostable enzyme having a molecular mass of 63 kDa was purified from *Streblus asper* (Tripathi et al. 2011). Dubiumin was purified from the seeds of *Solanum dubium*, having a molecular mass of 66 kDa (Ahmed et al. 2009b). Cucumisin from *Cucumis melo* (Uchikoba and Kaneda 1996) and lettuceine from *Lactuca sativa* (Lo Piero et al. 2002) were isolated and used as milk coagulants.

## 3 Production of plant proteases

Proteases used as milk coagulants have been identified and studied from almost every plant part whether it may be seed, flower, or latex. These enzymes can be obtained from their natural source or through in vitro culture to ensure a continuous supply of plant proteases (Gonzalez-Rabade et al. 2011).

### 3.1 Production from natural sources

Generally, these enzymes have been extracted from their natural source by aqueous maceration of various plant organs such as flowers, seeds, roots and leaves. There are several different ways of preparing the aqueous extract of the plant material. The dried whole or crushed cardoon flowers are soaked in water at room temperature for a variable time period. Then, the filtrate is collected and this crude extract is used as coagulant (Roseiro et al. 2003). An alternative method of extraction is grinding the dried flowers with crude kitchen salt, laying the paste on a cotton cloth (which acts as a strainer) and solubilizing the enzymes by percolation with warm milk (Sousa and Malcata 2002). The crude extract can also be further purified to obtain partially purified enzyme or pure enzyme depending upon the degree of purification. Precipitation with ammonium sulfate is an effective way to produce substantial amounts of active proteases from the flowers of *C. cardunculus* (Barros et al. 2001).

Cardosin A and B were extracted from the stigmata and stylets of dried flowers of *C. cardunculus* (Silva et al., 2003). Proteases were extracted from stigmas of *C. scolymus* (Sidrach et al. 2005), dried flowers of *Moringa oleifera* (Pontual et al. 2012), fresh flowers *Silybum marianum* (L.) Gaertn. (Vairo-Cavalli et al. 2005, 2008). A partially purified enzyme extract, named onopordosin was obtained from the upper portions (stigmas and styles) of fresh flowers from *Onopordum acanthium* (Brutti et al. 2012).

Protein extracts were obtained from *Bromelia hieronymi* fruits (Bruno et al. 2002) and the preparation was named hieronymain (Bruno et al. 2010). Peeled ginger rhizomes were used to obtain the enzyme extract (Hashim et al. 2011). Seeds of different plants have also been used to prepare plant extracts for cheesemaking. Protease extracts were obtained from the seeds of *Solanum dubium* (Ahmed et al. 2009a, 2010), and peeled seeds of sunflower (*Helianthus annuus*) and whole albizia (*Albizia lebbek*) seeds (Egito et al. 2007). Nestor et al. (2012) obtained the enzyme extract from the berries of *Solanum elaeagnifolium*. Also, proteases were obtained from the latex of fig tree and evaluated for the milk-clotting properties (Kumari et al. 2012; Sharma et al. 2012)

### 3.2 In vitro production

Milk-clotting proteases have also been produced by in vitro techniques. Callus and cell suspension cultures have been studied by several authors (Table 2). Tamer and Mavituna (1997) used the culture of *Mirabilis jalapa* to produce proteases and found that the proteolytic yield was higher with proteases produced in vitro as compared to the proteases from the intact plant. Cells from the cell suspension culture of *Centaurea calcitrapa* were homogenized with a buffer at pH 8.1. The homogenate was centrifuged and the supernatant was then lyophilized to obtain the protein extract (Reis et al. 2000). Cimino et al. (2006) have carried out research to optimize the conditions for the production of protease from the callus culture of *Silybum marianum*. Oliveira et al. (2010) established *C. cardunculus* callus culture to produce and characterize aspartic proteases.

**Table 2** Milk-clotting plant proteases produced in vitro

Type of culture	Source	Type of protease	Reference
Cell suspension	<i>Cynara cardunculus</i>	Aspartic	Lima-Costa et al. 1996; Cordeiro et al. 1998
	<i>Centaurea calcitrapa</i>	Aspartic	Domingos et al. 1992; Reis et al. 2000; Raposo and Lima-Costa, 2006; Raposo and Domingos 2008; Lourenco et al. 2002
	<i>Mirabilis jalapa</i>	–	Tamer and Mavituna, 1997
	<i>Silybum marianum</i>	Aspartic	Cimino et al. 2006
Callus	<i>Cynara cardunculus</i>	Aspartic	Lima-Costa et al. 1996; Cordeiro et al. 1998; Oliveira et al. 2010
	<i>Mirabilis jalapa</i>	Aspartic	Tamer and Mavituna 1997
	<i>Silybum marianum</i>	Aspartic	Cimino et al. 2006

In vitro techniques have several advantages. In vitro production of enzymes has the potential of overcoming the low enzyme yield and difficulties in extraction from the natural sources. The problems due to climate and season conditions, and heterogeneity of the product obtained from plant parts can also be solved by in vitro techniques (Gonzalez-Rabade et al. 2011). Plant cell cultures have shown to be a feasible alternative to obtain milk-clotting aspartic proteases from *Centaurea calcitrapa* (Raposo and Domingos 2008; Raposo and Lima-Costa 2006).

#### 4 Techno-functional aspects

Plant extracts as a coagulant for cheesemaking has been largely used for the production of hard and semi-hard cheese products from ovine and caprine milk. Cheeses prepared with plants coagulants have a characteristic soft texture and a slightly bitter flavor (Roseiro et al. 2003). Their application to bovine milk has sometimes resulted in poor quality cheeses, especially in terms of texture and flavor. The plant coagulants such as cardoon extract can be used for production of bovine cheeses if the cow's milk could be modified to resemble to those of sheep milk (Agboola et al. 2009). The most significant difference between sheep and cow milk is protein content, an important component directly involved in coagulation. Ultrafiltration (UF) is an established process for manipulating the protein level in cheese milk. Cheese made using UF milk and calf rennet was found to be hard and crumbly, with less developed flavors (Renner and Abd El-Salam 1991). The effects of plant coagulants such as ficin, papain and cardoon extract on UF milk samples whose protein level had been increased up to four times that in the regular milk were studied, comparing their clotting properties and products of enzymatic proteolysis to those obtained using commercial calf rennet. It was shown that the undesirable proteolysis inherent in the application of plant coagulants to regular cow's milk can be significantly reduced when concentrated UF milk was employed as the milk source (Low et al. 2006).

Concentrated cow's milk, obtained by either limited ultrafiltration or by mixing ultrafiltered milk with regular milk was used to manufacture cheeses coagulated with calf rennet or aqueous extract from *C. cardunculus* L. (cardoon). Proteolytic, textural, and sensory studies showed that while UF may have reduced the extent of milk and cheese proteolysis in the presence of cardoon extract, it did not result in improved sensory qualities unless a significant amount of regular milk was present (Agboola et al. 2009).

Milk-clotting activity is the most important property of enzymes used in cheesemaking. It is the capability of the enzyme for specific  $\kappa$ -casein hydrolysis (Jacob et al. 2011). It can be measured by different methods such as Soxhlet, Berridge, and international standard method and the units used are Soxhlet units, Berridge, or Rennet units and International Milk Clotting Units, respectively (Harboe et al. 2010). The large number of methods, different units at different conditions used by different authors has made it difficult to compare the units for milk-clotting activity. However, some authors have compared the milk-clotting activity of plant proteases with calf rennet in the same conditions. Nestor et al. (2012) compared the milk-clotting activity of enzyme extract from *Solanum elaeagnifolium* berries with calf rennet and found that the milk-clotting activity was 39.4 and 2,474 milk-clotting unit (MCU) at 32 °C respectively. Ahmed et al. (2009a)

compared the milk-clotting activity of enzyme extract from *Solanum dubium* with calf rennet and found that the milk-clotting activity was 880 and 2,496 MCU at 37 °C, respectively. Kumari et al. (2012) compared the milk-clotting activity of enzymes Religiosin and Religiosin B from *Ficus religiosa* with rennet and found that the milk-clotting activity was 387, 803, and 4,989 MCU at 37 °C respectively.

A good milk-clotting enzyme is characterized by a high specific caseinolytic activity and a low general proteolytic activity, since the proteolysis strongly affects the sensory properties of cheese. When a potential rennet substitute is studied, it is particularly important to evaluate adequately the degradation patterns of the caseins because of their effects on yield, consistency and flavor of the final cheese (Fox 1989). One of the most adequate and expeditious methods to monitor proteolytic processes is polyacrylamide gel electrophoresis. The electrophoretic techniques currently used in the study of milk proteins (SDS-PAGE, alkaline urea PAGE, acid urea PAGE) usually do not allow a clear identification of all casein components and hence an improved electrophoretic technique (Tricine SDS-PAGE) is recommended (Bruno et al. 2010).

A serine protease named dubiumin, isolated from *Solanum dubium* seeds, having *pI* value of 9.3 and optimum pH 11.0 worked efficiently under different salts concentration and at different pH levels. It is also thermostable retaining complete activity at 60 °C after 1 h and acts optimally at 70 °C for 30 min. Furthermore, it is highly stable in the presence of various denaturants and organic solvents (Ahmed et al. 2009b). It hydrolyzes casein fractions in separate forms or in a whole casein form very efficiently at different conditions. This property could be useful in the dairy industry both for milk clotting, as an alternative or with calf rennet, and for the acceleration of cheese ripening to reduce the time and costs of storage and maturation (Ahmed et al. 2010).

A protease named hieronymain, obtained from unripe fruits of *Bromelia hieronymi*, was capable of clotting milk and hydrolyzing bovine casein and milk whey proteins. The  $\kappa$ -casein fraction, directly involved in clotting formation, began to be degraded after 10 min of reaction, while the degradation of the other casein fractions proceeds slowly enough as to guarantee the production of a firm curd, with no evidence of extensive hydrolysis. In the case of whey proteins, bovine serum albumin and  $\alpha$ -lactalbumin were quickly degraded after 30 min, while  $\beta$ -lactoglobulin was considerably degraded only after 60 min at 50 °C. Miniature cheeses were prepared both with chymosin and hieronymain and analyzed by a taste panel, who found acceptable both cheeses. It was concluded that hieronymain might be appropriate for cheesemaking, as well as for the production of milk protein hydrolysates (Bruno et al. 2010).

A partially purified enzyme preparation (onopordosin) obtained from *Onopordum acanthium* L. flowers was used as coagulant agent for cheesemaking. It is an aspartic protease of optimum pH 2.5 and *pI* 4.4. The electrophoretic profiles of onopordosin in early stages of bovine milk coagulation showed a similar degradation behavior of  $\alpha$ s<sub>1</sub>-casein and  $\beta$ -casein compared with that of chymosin. The sensory quality of the cheeses made with onopordosin compared with other two commercial cheeses was similar to that of the latter, but with some differential characteristics. It was concluded that onopordosin was a suitable plant coagulant which can be used alone or in combination with a commercial starter to assure a limited proteolysis (Brutti et al. 2012).

Extract of artichoke (*Cynara scolymus* L.) flowers have been exploited as a source of enzymes to be used in cheesemaking and it was found that the coagulation activity



was highly dependent upon milk pH and temperature. The milk-clotting activity of this extract increased hyperbolically with increasing concentrations of calcium, and the concentration was saturated at 50 mM, while sodium chloride had no significant effect (Chazarra et al. 2007).

The extract of *C. scolyms* was purified and yielded three proteases (cynarases A, B, and C). All three cynarases are glycoproteins with milk-clotting activity (Sidrach et al. 2005). Purification led to a decrease in the specific coagulant activity relative to that of the crude extract in the case of cynarases A and C, whereas cynarase B increased its specific clotting activity. Moreover, whereas the cynarases A and C showed a slight increase in specific peptidase activity relative to the initial extract, the specific peptidase activity of cynarase B was much higher. The results indicate the possibility of the use of this enzyme in cheesemaking (Chazarra et al. 2007).

The seeds of the tree *Albizia julibrissin* have been shown to possess milk-clotting activity without developing any bitterness in cheese after 3 months of ripening (Otani et al. 1991). Milk-clotting activity found in ammonium sulfate-precipitated protein extracts from *Albizia lebbbeck* and *Helianthus annuus* seeds were studied by Egito et al. (2007) and found that specific clotting activity of albizia seed extract was 15 times higher than that of sunflower seed extract. Similar to chymosin, the two seed extracts exhibited proteolytic activity toward  $\kappa$ -casein,  $\alpha_s$ -casein and  $\beta$ -casein, with the highest activity observed for the albizia seed extract. Mass spectrometry analysis showed that the sunflower extract hydrolyzed  $\kappa$ -casein at the Phe105–Met106 bond, as does chymosin. The albizia extract also displayed activity on  $\kappa$ -casein, but the Lys116–Thr117 bond was its preferred target (Egito et al. 2007).

## 5 Conclusion

Milk coagulants are essential for the production of cheese and plant proteases have been isolated from several plant sources and studied for milk-clotting ability. These enzymes can be obtained from their natural sources or through in vitro culture. Extraction of milk-clotting proteases from intact plants parts is labor intensive and so, plant cell cultures (in vitro) is a viable alternative to obtain clotting enzymes. The crude extracts can be further purified to obtain partially purified or pure enzyme depending upon the degree of purification.

Coagulation studies are important in the manufacture and maturation of cheeses and a good milk-clotting enzyme is characterized by a high specific caseinolytic activity and a low general proteolytic activity, since the proteolysis strongly affects the textural and sensory properties of cheese. When a potential rennet substitute is studied, it is particularly important to evaluate its milk-clotting activity. Different authors have used different methods and different units which makes it difficult to compare the milk-clotting activity of different plant coagulants. These enzymes do, however, differ in molecular structure and in proteolytic activity, and there are still reports that cheese yield and cheese quality is negatively affected. The understanding of the action of the enzymes during  $\kappa$ -casein cleavage and subsequent milk coagulation has increased substantially, and is still going on.

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