CHAPTER V: DISCUSSION

Latex, generally a milky white exudate, is a typical characteristic of certain angiospermic plant families. Latex plays diverse roles in various physiological activities in vivo and is also known to have numerous biotechnological and pharmacological applications in vitro. Their in vivo activities involve regulation of water balance, storage of nutrients and providing defence against invading herbivorous insects and plant pathogens (Agrawal et al., 2009: 311-31). These varied roles have been attributed to the presence of heterogeneous classes of bioactive chemical components such as alkaloids, terpenes, tannins, sterols, glycosides, saponins, flavonoids as well as hydrolytic enzymes such as proteases, invertases, chitinases present in latexes (Konno 2011: 1510-30; Thakur et al., 2011: 17; Korpenwar 2012: 36-9). Large number of latex producing plants are traditionally used by tribals and folklores for healing of wounds and burns in addition to other ailments such as management of toothache/gum bleeding. They also find applications as antihelminthic, analgesic, antibiotic (an agent to clear skin infections) and as abortion inducer (Osoniyi et al., 2003: 101-5; Thankamma 2003: 971-2; Aderounmua et al., 2013: 574-9; Shivaprasad et al., 2016: 1-7).

Proteases, a ubiquitous class of hydrolytic enzymes, find specific applications in major domains such as wound management, cheese making, leather processing, stain removal, alcoholic beverage preparation, brewing, meat tenderization, pharmaceutical, and photographic industries etc (Neurath et al., 1976: 3825-32; Lopez-Otin et al., 2008: 30433-7; Li et al., 2013: 1155-63). Generally animals, plants and microbes serve as their sources with microbial proteases taking a lead on many of the industrial applications. Presently plant enzymes are attracting wide attention for diverse commercial applications (Turk 2006: 785-99; Sawant et al., 2014: 568-79).

Lattices from hundreds of plants belonging to various families consist of at least one proteolytic enzyme (Domsalla et al., 2008: 699-711; Gonzalez-Rabade et al., 2011: 983-96). Many such proteases from plant lattices have been isolated and characterized. Plant latex proteases are demonstrated to hydrolyze different protein-substrates, to name a few: casein, azocasein, fibrinogen, fibrin, gelatin, collagen, several synthetic substrates etc. (Kumar Dubey et al., 2003: 1057-71; Pande et al., 2006: 10141-50; Shivaprasad et al., 2016: 1-7). Proteolytic enzymes from plant latex are gaining attention for pharmaceutical and biotechnological applications. Purified latex proteases such as papain and ficin have been shown to have a broad range of applications (Li et al., 2010: e15168). More research is now focussed on identifying novel proteases with multiple application potentials.
In the present study, proteases from ten latex producing plants belonging to families Apocynaceae, Asclepiadaceae, Moraceae and Euphorbiaceae were screened for haemostatic, milk clotting and stain removal properties. Caseinolytic activity of latex enzymes were compared to that of standard proteases, papain and trypsin. It was evident from the results that caseinolytic activity progressively enhanced with increase in concentration of protein. Such an observation has been reported by many investigators (Rajesh et al., 2005: 84-92; Rajesh et al., 2006: 1313-22; Shivaprasad et al., 2009: 106-9; Chanda et al., 2011: 705-11; Pontual et al., 2012: 1848-54; Sequeiros et al., 2016: 332-46). Of the 10 plants studied, C. gigantea, T. divaricata and A. altilis exhibited significantly higher CA than both the positive controls.

Though latex from several plants in crude form have been studied earlier for their interference in hemostasis/wound healing/milk clotting/stain removal, only few of them have been purified. In the present study, the CEs of C. gigantea, T. divaricata and A. altilis, which showed relatively higher procoagulant, thrombolytic, milk clotting and blood destaining activity compared to other plants, were chosen for further purification through different purification steps such as ion exchange and gel exclusion chromatography. The observed protease activity recovery was 9.4%, 7.6% and 7.14% for T. divaricata, C. gigantea, and A. altilis respectively. The availability of multiple proteases in crude extract along with loss of activity during purification steps might be the reason for low observed activity recovery. Such a low recovery of proteases has also reported by other investigators (Singh et al., 2010: 399-406; Yadav et al., 2012: 1296-304). A single protein band in tricine SDS PAGE indicated homogeneity of these proteases after purification. Molecular weights of C. gigantea, T. divaricata and A. altilis proteases were 23.5, 24.11 and 81 kDa respectively. Activity band observed in zymogram corresponded to their respective molecular weights observed in non-denaturing tricine SDS–PAGE indicating the monomeric nature of these proteases. Inhibition studies indicated that C. gigantea and T. divaricata to be cysteine proteases and A. altilis as serine protease. The molecular weights of purified proteases from C. gigantea T. divaricata and A. altilis is comparable to many cysteine and serine proteases isolated from various plant lattices (Domsalla et al., 2008: 699-711). Many latex proteases reported so far belong to cysteine and serine family. Earlier studies have shown the presence of cysteine proteases in lattices of Asclepiadaceae and Apocyannaceae family and cysteine and serine protease in Moraceae families. Observations from the present study are in accordance with these reports (Domsalla et al., 2008: 699-711; Shivaprasad et al., 2010: 591-603; Siritapetawee et al., 2012: 907-12). A mention about occurrence of other types of proteases such as aspartic and
metalloprotease family in few plant latex can also be found in earlier reports (Domsalla et al., 2008: 699-711; Costa Jde et al., 2010: 1760-5; Shivaprasad et al., 2016: 1-7).

Main limitation of using soluble enzymes, in industrial and environmental applications, are their high cost and non-reusability. Enzyme immobilization is proposed to be one of the potential options for avoiding such drawbacks. Various technologies studied so far for immobilization of enzymes include ionic interaction, entrapment, adsorption and cross linking by nanomaterial/ polymers immobilization, etc. (Gürdağ et al., 2012: 904-11). PE was immobilized with 2% (w/v) sodium alginate beads. An approximate 80-85% loss in enzyme activity was observed with all PEs on immobilization, when assayed using casein as the substrate. Araujiain, a cysteine phytoprotease was immobilized within calcium alginate beads with 91% yield. However, the assay was performed with low molecular weight substrate BAPNA (Quiroga et al., 2011: 1029-34). The low yield reported in the study may be ascribed to poor substrate (casein) diffusion or denaturation of enzyme during immobilization. Similar low activity recovery have been reported for microbial and fish visceral proteases with casein as substrate (Adinarayana et al., 2005: E391-E7; Anwar et al., 2009: 1281-6; Geethanjali et al., 2013: 7; Paliwal et al., 2016: 18915-26).

5.1 Wound Healing and Latex Proteases

Modern medical system relies on utilization of indigenous substances used in traditional medicine for various healthcare services. The better understanding of scientific basis and associated molecular mechanisms has made traditional/folk medicine popular worldwide. Many of the present days drugs which are used on conventional basis have been derived from traditional medicine (Benzie et al., 2011). While molecular mechanisms of few traditional medicines are currently under study yet many are yet to be explored for their therapeutic potentials. The natural process of wound healing is known to be assisted by diverse external agents of plant origin. Several proteases from different sources are reported to be responsible for the pharmacological effect of inhibition/stoppage of bleeding on fresh cuts by application of plants which is practised by tribal and rural people of India (Richter et al., 2002: 1042-51; Thankamma 2003: 971-2).

Wound healing in human body is a natural process that involves precisely and highly regulated phases (Yariswamy 2013). Hemostasis and thrombolysis, the initial events in wound healing process, are brought about by the integrated action of several clotting factors and proteases such as thrombin and plasmin (Shivaprasad et al., 2009: 106-9). Blood coagulation cascade is a highly regulated pathway that includes many coagulation factors and platelet activation. Procoagulant activity is the first event of hemostasis that begins immediately after
an injury. It involves the creation of thrombogenic environment due to activation of platelets which further activates series of serine proteases that results in thrombin activation which then activates soluble plasma fibrinogen into an insoluble fibrin network (Davie et al., 1979: 277-318; Davie et al., 1991: 10363-70; Arnout et al., 2006: 1-41). The platelet and circulating cells get entrapped and enmeshed in fibrin network to forms a hemostatic plug that intensifies the process of coagulation (Hawiger 1987: 111-22; Mackman et al., 2007: 1687-93). Fibrinolysis or thrombolysis is a process in which the formed platelet plug is hydrolysed (Lijnen 2001: 226-36).

A number of proteases that interfere in haemostasis have been separated and well characterized from different plants and animals. Scientific study validating the applications of latex proteases in the process of wound healing commenced only after 1930s with reporting of a cysteine protease from *C. papaya* having procoagulant activity through fibrinogen clotting effect (Eagle et al., 1937: 543). After a gap of six decades, subsequent studies on different plant lattices (several at the crude level and over 10 purified proteases-including the cysteine- and serine-proteases) have been reported for their involvement in blood coagulation/fibrinolysis (Rajesh et al., 2005: 84-92; Rajesh et al., 2006: 1313-22; Rajesh et al., 2007: 1061-7; Shivaprasad et al., 2009: 106-9; Shivaprasad et al., 2010: 591-603; Ramos et al., 2012: 455-63; Siritapetawee et al., 2012: 907-12; Shivaprasad et al., 2016: 1-7).

In the present study, ten latex producing plants were screened for their procoagulant and thrombolytic potential. CEs of 9 plant latex exhibited pronounced and progressive increase in procoagulant activity of platelet poor plasma and induced fibrin clot formation. CEs of 6 plants exhibited clot formation in less than 100 seconds. Calcium ideally acts as a co-factor in both intrinsic and extrinsic pathways for the conversion of prothrombin to thrombin (Davie et al., 1975: 799-829; Spronk et al., 2003: 1220-8). Similar to Shivaprasad et al., (2009) observation on selected Asclepiadaceae members, calcium independent fibrin clot induction was observed with CEs of *C. gigantea* and *T. divaricata* in the present study. These observation points towards the possible bypassing of calcium requiring steps and direct action of these enzymes on the terminal step of blood coagulation cascade (Rajesh et al., 2005: 84-92). However the CEs of remaining plants did not appear to possess this property and may be operating via the calcium dependent coagulation cascade. The procoagulant effect of CEs was further assessed by fibrinogen polymerization and fibrinogen agarose plate assays. There was an increase in the absorbance (at 540nm) values of fibrinogen solution with the increase in protein concentration of CE, suggesting the ability of the enzyme/enzymes in hydrolyzing fibrinogen and its subsequent polymerization to form fibrin threads. Fibrinogen agarose plate assays also supported the spectrophotometric results. Direct incubation of crude
latex enzyme with human fibrinogen induced zone of precipitation which was comparable to the one given by the positive control (human thrombin). These results of the present study support the earlier reports on thrombin like activity which is also reported for other latex proteases (Badgujar 2014: 733-9; Shivaprasad et.al., 2016: 1-7). The proteases involved in induction of fibrin clot resembling at least partially to thrombin mediated fibrinogenolysis are termed as thrombin like enzymes.

Apart from its usage to stop bleeding, plant latex has been used since time immemorial in folk medicine as remedy for wound healing (Rajesh et.al., 2005: 84-92). Although fibrin clot supports initial events of wound healing, subsequent breakdown leading to removal of clot is a pre-requisite for the events of repair phase that involve tissue repair and its remodelling (Clark 1996: 3-50; Green et.al., 2008: 2092-101). Clot dissolution process in vivo is mediated by the serine protease, plasmin. Proteases from plant lattices have been shown to exhibit blood clot dissolving properties indicating their plasmin like behaviour. The dual activities by some latex proteases have projected them as good alternatives in the management of fresh cuts/wounds (Siriritapetawee et.al., 2012: 907-12). In the present study CEs of 6 plants exhibited efficient hydrolysis of fibrin leading to its dissolution. Comparison of mean % clot lysis revealed C. gigantea to be having the highest thrombolytic effect followed by T. divaricata and then A. altilis whereas others showed negligible reduction in blood clot weight. 30,000 I.U of streptokinase treatment under similar conditions used in this study could bring about 70.80 % of clot lysis whereas in the present study 100 µg of CEs from C. gigantea, T. divaricata and A. altilis could induce clot lysis of 91.5%, 86.35% and 78.60% respectively indicating their potency towards thrombolysis.

Subsequent purification of CEs of C. gigantea, T. divaricata and A. altilis could considerably enhance the observed procoagulant and thrombolytic performance. Thrombin-like enzymes represents group of proteases that have similar fibrinogen hydrolytic pattern as mediated by thrombin and involve in formation of fibrin clots. Thrombin, a specific fibrinogenolytic enzyme breaks down Aα and Bβ chains and forms fibrin clot by releasing fibrinopeptide A and B with partially maintaining the γ band intact (Rajesh et.al., 2006: 1313-22; Magalhães et.al., 2007: 565-75; D Raghavendra Gowda et.al., 2011: 2578-88). CEs mimicked hydrolytic pattern of thrombin. Similar behaviour was observed by earlier works on Synandenium grantii and Wrightia tinctoria crude enzyme (Rajesh et.al., 2007: 1061-7). Even with most of the reported fibrinogenolytic proteases from plant latex, snake venom, insects, algae and caterpillar γ subunit appear to be resistant to hydrolysis (Swenson et.al., 2005: 1021-39; Kim et.al., 2006: 622). Purification in current study appears to enhance the proteolytic activity leading to hydrolysis of all the three subunits (initially targeting Aα and Bβ chains). In the
present work, 10µg of *C. gigantea*, *T. divaricata* and *A. altilis* latex PE preferentially hydrolyzed Aα within 5 min and Bβ within 15-20 min. Extended incubation for a period of 60 minutes resulted hydrolysis of even γ subunit. Time of incubation and concentration dependency of many latex enzyme during fibrinogen hydrolysis have been studied extensively to distinguish whether these enzymes share a common molecular mechanism with thrombin or not. A few such studies have shown a positive correlation between higher doses and extended incubation time with γ chain hydrolysis (Shivaprasad *et al.*, 2010: 591-603; Shivaprasad *et al.*, 2016: 1-7). Earlier reports with purified plant latex proteases such as AMP48, *Synadenium grantii* latex protein the susceptibility of fibrinogen subunits seem to follow Aα > Bβ >> γ chains. The pattern of fibrinogenolysis by yet another purified latex protease, hirtin, demonstrated its preferential specificity to Aα and Bβ subunits’ hydrolysis and releasing fibrinopeptides to favour fibrin clot formation even with its mild γ subunit hydrolysis (Komori *et al.*, 1985: 127-30; Rajesh *et al.*, 2006: 1313-22; Patel *et al.*, 2012: 104-11; Siritapetawee *et al.*, 2012: 907-12). However, activity of cysteine protease ‘Pergularain e I’ and serine protease ‘Eumilin’ was similar to thrombin as hydrolysis of γ was not observed even after prolonged incubation (Fonseca *et al.*, 2010: 708-15; Shivaprasad *et al.*, 2010: e100-e5). Ficin, a cysteine protease obtained from the plant latex of *F. carica*, was reported to activate human factor X and induce blood clotting (Bolay 1979: 97-112). Russel Doolittle deciphered detailed mechanism of thrombin like activity of papain from *C. papaya*. Papain is known to act in similar fashion as that of thrombin and also additionally possesses factor XVIIIa like activity that stabilizes fibrin monomers and assist in coagulation process (Doolittle 2014: 6687-94). Thrombin-like enzymes have been isolated and characterized from other sources like snake venoms. However they do lack coagulation factor XIIIa-like activity as observed in papain (Vu *et al.*, 2013: 16862-71).

Fibrinolysis is the immediate second significant proteolytic event after fibrin clot formation which favours the process of wound healing. This process involves plasmin as main enzyme that breaks both fibrin clot and haemostatic plug to facilitate wound healing (Lijnen 2002: 92-8). Interestingly, most of the plant latex proteases that have procoagulant activity also possess fibrinolytic/ plasmin-like activity. PEs and CEs under study hydrolyzed crude fibrin of plasma clot resulting in the production of small molecular weight protein bands as seen in tricine PAGE. Percentage blood clot lysis along with plasma clot hydrolytic PAGE pattern in the current study revealed probable thrombolytic (plasmin like) nature associated with *C. gigantea*, *T. divaricata* and *A. altilis*. These results are in accordance with earlier reports on plasmin like activity by latex proteases from selected plant species (Rajesh *et al.*, 2006: 1313-22; Dayanand *et al.*, 2010: 645-55; Uday *et al.*, 2017: 316-22).
Coexistence of the dual characteristics of fibrinogenolysis and fibrinolysis favouring wound healing has been reported earlier in the latex of several plants. Present study could also observe fibrinogenolytic and fibrinolytic activity with CEs of 6 plants. The dual activities by some latex proteases have projected them as good alternatives in the management of not only fresh cuts but also for wounds. Figure 5.1 depicts the possible involvement by latex proteases in the coagulation and fibrinolysis cascade. A similar study on *Asclepias curassavica* L., *Pergularia extensa* R.Br., and *Cynanchum puciflorum* R.Br., belonging to the family Asclepiadaceae, explained the involvement of cysteine proteases in both blood coagulation and fibrinolysis. (Rajesh *et al.*, 2006: 1313-22; Shivaprasad *et al.*, 2009: 106-9; Singh *et al.*, 2015: 43-9).

![Figure 5.1: Latex Proteases and their involvement in coagulation and fibrinolysis cascade](image)

Cysteine proteases in the latex of *C. procera* exhibited thrombin and plasmin-like activities *in vivo* suggesting its healing potential in diverse coagulation abnormalities (Ramos *et al.*, 2012: 455-63). 34 kDa serine protease (hirtin) from *Euphorbia hirta*, was studied for its fibrinolytic activity and its potential industrial and therapeutic applications (Patel *et al.*, 2012: 104-11).
Purified latex proteases, AMP48 (serine) and Pergularine el (cysteine) exhibited thrombolytic activities in addition to their procoagulant effect. Majority of the proteases isolated till date from mammalian source and snake venoms which interfere in blood coagulation are either serine or metalloproteases (Iwasaki et al., 1990: 822-8). The dual action mechanism by latex proteases needs further exploration to understand the exact order of coagulation factor hydrolysis/involvement.

This dual thrombin like and plasmin like activity is unique only to plant latex proteases and is not observed in proteolytic systems of mammals and snake venom (Rajesh et al., 2007: 1061-7; Costa Jde et al., 2010: 1760-5; Shivaprasad et al., 2016: 1-7; Uday et al., 2017: 316-22). This unique property of plant latex proteases might play a role therapeutically in enhancing the overall process of wound healing by preventing blood loss, stoppage of bleeding and dissolving the deposition of fibrin around the wound.

5.2 Latex Proteases as Vegetable Milk Coagulant

Proteases are the key mediators of milk coagulation in cheese making. Cheese is traditionally prepared using rennin, an aspartic protease from 4th stomach of unweaned calf which specifically cleaves the bovine κ casein Phe105–Met106 bond (Egito et al., 2007: 816-25). Growing demand for cheese, inadequate ruminant source availability and its escalating cost, concerns pertaining to religious aspects, localized ban on genetically engineered rennin, ethical issues etc. directed research focus on alternate sources of rennin (Shah et al., 2013: 5-16). Initially the focus was on microbial rennet. Plant sources are being explored as source of proteases for milk coagulation because of their relative safety, high abundance, inexpensiveness, simple isolation and purification processes, general acceptance by various consumer populations. In addition, their use could be beneficial for vegetarians in improving their nutritional intake (Tavaria et al., 1997: 3760-5; Sousa et al., 2001: 327-45; Sousa et al., 2002: 151-70; Roseiro et al., 2003: 76-85). Proteolytic enzymes are generally found in majority of plant species. They have been characterized and purified from a variety of tissues such as seeds, flowers, leaves, roots and stems. Under optimum conditions, these enzymes are known to clot milk (Tamer 1993: 427-32; Domsalla et al., 2008: 699-711). Latex is important source of milk clotting protease compared to other plant parts.

Stability and activity of plant proteases to act in a broad range of pH and temperature is an added advantage of using plant proteases over rennin. However, research focussing on plant latex as a rennet source is sparse with most of such studies limiting their work only till the crude level without dwelling much on purification and molecular mechanism in detail. Latex of several plants from Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae
and Euphorbiaceae families possess milk clotting proteases (Domsalla et al., 2008: 699-711; Jacob et al., 2011: 14-33; Kumari et al., 2012: 1295-303; Cleverson D.T. Freitas et al., 2016: 50–9). However, most of these studies are with crude enzymes without dwelling much on purification and molecular mechanism.

Studies with the crude and purified latex proteases of selected plants in the current study indicated that CEs of C. gigantea, T. divaricata and A. altilis possessed relatively high milk clotting activity and significantly high milk clotting index. CEs of P. alba, P. rubra, E. antiquorum showed relatively low caseinolytic activity resulting in enhanced MCI values even with low MCA compared to C. gigantea, T. divaricata and A. altilis. Purification of CEs of C. gigantea, T. divaricata and A. altilis resulted in 81.19%, 63.73% and 61.19% enhancement of milk clotting activity and index. MCI values of C. gigantea, T. divaricata and A. altilis are similar to that reported for Religiosin B and Religiosin, purified proteases from Ficus religiosa (Kumari et al., 2010: 8027-34; Kumari et al., 2012: 1295-303). Ficin isolated from the latex of various Ficus spp. possesses characteristic properties. Casein hydrolysis by ficin from the latex of Ficus racemosa suggested its potential in clotting milk (Devaraj et al., 2008: 647-55). Torres et al., reported cysteine protease from Vasconcellea quercifolia latex with milk clotting potential higher than that of papain (M.Jose Torres et al., 2010: 11027–35; Torres et al., 2012: 1471-84). Neriifolin, a chymotrypsin-like serine protease and Neriifolin S, a 94 kDa dimeric serine protease purified from the latex of Euphorbia neriifolia possessed comparable milk clotting ability as observed in the present study (Yadav et al., 2011: 1654-62; Yadav et al., 2012: 1296-304). Streblin (63 kDa), a milk coagulating thermostable enzyme was purified from Streblus asper (Tripathi et al., 2011: 1005-12). Efficiency of milk coagulant enzymes from Calotropis procera have been evaluated by many investigators (Kumari et al., 2012: 1295-303; Oseni et al., 2013: 24-7; Shah et al., 2013: 5-16). Latex of C. procera was found to possess better ratio of MCA to proteolytic activity and are used in commercial production of local cheese "Warankasi/Wara" in West Africa (Belewu 2001: 93-5; Vairo-Cavalli et al., 2005: 271-5). Wara is also produced using leaf extracts of Carica papaya and Calotropis procera assessment of their nutritive values have been carried out. However, cheese from C. procera extracts are preferred to C. papaya for its sweet flavour (Adetunji et al., 2008: 3360-2; Adetunji et al., 2011: 5573-85). A comparative assessment of nutritional values of purified latex proteases from C. procera, procerain and procerain B are extensively explored for their milk clotting potential. cDNA profiling and immobilization of these proteases have shown their potentiality to be used in dairy industry (Kumar Dubey et al., 2003: 1057-71; Singh et al., 2011: 6256-62). Jacaratia corumbensis O. kuntze was identified as another vegetable source of milk clotting enzyme by Duarte et al., Study suggested that the
proteases obtained from the latex of *J. corumbensis* root might be a potential source for milk clotting and were useful in dairy applications (Dawson 1954: 39-63; Duarte *et al.*, 2009: 1-9; Meghwal *et al.*, 2017). Studies on a cysteine protease from *Euphorbia nivulia* latex indicated it to be a promising candidate as vegetable coagulant (Badgujar *et al.*, 2014: 391-8).

Plant parts of the latex producing plants have also been explored for their milk clotting potential. Crude enzyme from the plant parts of *C. procera* studied for their MCA showed similarity to rennin in casein hydrolytic pattern, opening a new source of vegetable coagulant. All parts of this plant except root were found to possess milk clotting potential (Oseni *et al.*, 2013: 24-7). Leaves of *C. procera* are used till date in manufacture of wara cheese in western African regions. Many attempts to study proteolytic activity associated with this plant and manufacture of laboratory cheese produced satisfactory quality/yield and it was suggested suitable for small scale cheese production. However, the cheese produced was slightly different in its flavour to that of cheese produced from calf rennet (Aworh *et al.*, 1987: 71-9; Raheem 2006: 32). Dubiumin (66 kDa) a protease with high milk clotting potential was purified from the seeds of *Solanum dubium* (Ahmed *et al.*, 2009: 395-400). Cucumisin from *Cucumis melo* and lettuceine from *Lactuca sativa* were isolated and utilized as potential milk clotting agents (Uchikoba *et al.*, 1996: 325-30; Lo Piero *et al.*, 2002: 2439-43). Fruits and latex from leaf petiole of *A. fruticosa* were studied for their casein hydrolytic pattern and concluded to be alternative milk coagulant source (Pardo *et al.*, 2010: 211-21). Fruits of Kiwi, melon and flowers of *Moringa* spp. have been ascertained by studies to be potential milk coagulants (Lo Piero *et al.*, 2011: 517-24; Pontual *et al.*, 2012: 1848-54; Da Silva 2017: 1-19).

In order to evaluate the enzyme’s behaviour during the cheese making process, casein hydrolysates with each enzyme preparations are analysed. Different casein fractions were reported to be responsible for distinct cheese properties. The α-caseins, β-casein and κ-casein were found to be associated with cheese texture, bitterness and milk-clotting respectively. Tricine SDS PAGE was conducted to evaluate casein hydrolysis pattern of PEs along with that of rennin and Enzeco in the present study. Tricine-SDS PAGE pattern could visualize α1, α2, β and κ-caseins subunits of casein. The degradation pattern of whole casein hydrolysate after one hour incubation showed that all crude enzymes invariably hydrolysed all the casein subunits suggesting significantly high proteolytic activity, whereas PEs hydrolysed casein in systematic manner. The hydrolytic pattern given by PEs mimicked that exhibited by enzeco to a greater extent on visual analysis. Specificity of protease to κ-casein is a significant factor for ideal milk coagulant. All the PE hydrolyzed κ-casein significantly. To limit the hydrolysis and for efficient milk coagulation, time and dose dependent studies are needed. Similar preference
for κ-casein was exhibited by proteases from *Silybum marianum* flowers, *Bromelia hieronymi*, *Moringa oleifera* and *Albizia* seeds (Bruno *et al.*, 2003: 127-34; Vairo-Cavalli *et al.*, 2005: 271-5; Egito *et al.*, 2007: 816-25; Pardo *et al.*, 2010: 211-21; Pontual *et al.*, 2012: 1848-54). In addition, the study on *Silybum marianum* flowers could even observe rennin like casein subunit hydrolytic pattern with reduction in incubation time to 30 min (Vairo Cavalli *et al.*, 2008: 997-1003). The latex peptidases from *C. procera*, *C. grandiflora*, and *C. papaya* exhibited non-specific hydrolysis on all caseins after five min of incubation (Freitas *et al.*, 2016: 50-9). Enzyme mediated hydrolysis of casein subunits brought about by all CEs and PEs in comparison with rennin and enzeco presented κ-casein as preferred substrate in the present study. Casein bands of both crude and purified enzymes tended to disappear as incubation progressed to 24 hours for an earlier study on *Helianthus annus* (Egito *et al.*, 2007: 816-25). Considerable amount of work has been gone in to understand the extent of specificity of peptide bonds acted upon by the milk coagulants, the event that governs the initial phase of hydrolysis. Casein micellar destabilization is accelerated by rapid hydrolysis of the peptide bond (Phe105–Met106 bond) in κ-casein by proteases eventually leading to speedy coagulation of milk. Complete breakdown of casein fractions was also reported on prolonged proteolytic action or during substrate limitation. (Sienkiewicz *et al.*, 1994; Krause *et al.*, 2001: 146-9).

The yield, consistency and flavour of cheese are generally determined by the rate of this hydrolysis. Slow degradation of α and β-caseins in presence of chymosin is preferred for the formation of firm curd (Fox 1989: 1-53; Fox *et al.*, 1996: 163-328; Pardo *et al.*, 2010: 211-21). Plant rennet brings about extensive caseinolysis, conferring bitter taste to the cheese produced. Therefore they are generally less preferred with an exception for cheese obtained from Cyanara spp. (Sousa *et al.*, 2002: 151-70; Lo Piero *et al.*, 2011: 517-24; Shah *et al.*, 2013: 5-16). These reports point towards the notion that time of incubation is a critical factor for casein hydrolysis by latex enzyme. The present study could evaluate the PEs performance on casein subunits after one hour incubation. Further analysis of casein hydrolysis with different incubation time interval may reveal more details on the optimum time required for the controlled proteolysis by these proteases.

An ideal vegetable coagulant should have minimum proteolytic activity and maximum milk clotting capacity to substitute rennin. The enhanced substrate specificity towards κ-casein is considered as a suitable quality for a vegetable coagulant to function as an effective rennin substitute. (Jacob *et al.*, 2011: 14-33). The replacement of rennet with such an alternative coagulant could help in bringing down its production cost. Cloning, expression and characterization of a novel proteases from different plant source that display high specificity...
towards κ-casein and milk-clotting activity is a recent research advancement (Lufrano et.al., 2012: 7-18). Recent rapid progress in some of the above mentioned areas may lead to application of plant proteases within the foreseeable future.

**5.3 Detergent additive prospect of Latex Proteases**

One of the well-known areas of enzyme applications in biotechnology is their use in laundry and dishwashing detergents. Although amylases and lipases are increasingly used for removal of carbohydrate and lipid based stains, proteases have dominated the detergent market. Proteases being the first enzymes to be used in detergents, do find huge application in laundry industries, where they help remove protein based stains from clothing (Saperas et.al., 2011: 1702-6). Significant research on microbes as source of detergent enzymes has led to the commercial application, whereas plant proteases especially latex is just emerging (Sherry et.al., 1959: 343-82; Kumar et.al., 1998: 1312-8; Banerjee et.al., 1999: 213-9; Banik et.al., 2004: 135-40; Maurer 2004: 330-4; Kumar et.al., 2008: 661-72; Hasan et.al., 2010: 4836-44; Kanmani et.al., 2011: 157-63). Current study gives evidence for the presence of such proteases in the CEs and PEs of selected latex producing plants. The PEs were highly stable with detergents and enhanced blood stain removal efficiency of commercially available detergents supporting their suitability as alternative detergent enzymes. Procerain B, a purified cysteine protease, from *C. procera* efficiently hydrolyzed blood stain and was found to have potential application in detergent industries (Singh et.al., 2010: 399-406). Studies by Badguzar et. al., suggested utility of glycosylated cysteine proteases from *Euphorbia nivulia* Buch.-Ham for blood stain removal and detergent compatibility along with dehairing properties supporting its eco-friendly applications (Badgujar et.al., 2013: 716545). Protease extract from *Araujia hortorum* latex fulfilled all the requirements for its application as additive for laundry detergents (Barberis et.al., 2013: 3). Studies by Mahajan et.al., on lattices of *P. tithymaloides*, *E. tirucalli*, *E. nivulia*, and *E. nerifolia* of Euphorbiaceae family could reveal remarkable destaining property associated with them (Mahajan et.al., 2016: 333-7). Accumulated data suggests the possible role of plant latex as a source of cost-effective biocompatible eco-friendly detergent enzyme/s.