Progress in the field of aspartic proteinases in cheese manufacturing: structures, functions, catalytic mechanism, inhibition, and engineering

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REVIEW PAPER

Progress in the field of aspartic proteinases in cheese manufacturing: structures, functions, catalytic mechanism, inhibition, and engineering

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Abstract Aspartic proteinases are an important class of proteinases which are widely used as milk-coagulating agents in industrial cheese production. They are available from a wide range of sources including mammals, plants, and microorganisms. Various attempts have been made in order to get insights into enzyme structure/function relationships for designing improved biocatalysts. This review provides an overview of historical background and recent achievements on the classification and structural characteristics of such enzymes as related to their functional properties, mechanism of catalysis, pH, and temperature dependence, substrate specificities, mechanism of inhibition, enzyme engineering, and technological applications with the focus on cheese manufacturing.

Keywords Milk-clotting enzyme · Rennet · Coagulant · Aspartic proteinases · Cheese · Structure—function

1 Introduction

Enzymes are being demanded for an increasing number of applications in food, life-science, and chemical industries. Major sectors benefiting from enzyme utilization are the food, beverage, feed, detergent, pharmaceutical, chemical, leather, paper, pulp, and silk industries. It has been mentioned that in 2011, the global industrial enzyme market valued USD3.5 billion, with a year-on-year growth rate of 6.1% (Anonymous 2012). Proteinases are an important group of technical enzymes that constitutes a large product segment in the global industrial enzymes market.

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The term "peptidase" is recommended by Nomenclature Committee of International Union of Biochemistry and Molecular Biology to be used as synonymous with "peptide hydrolase" for any of the enzyme which hydrolyses peptide bonds. Peptidases are classified in group 3 (hydrolases) within the subgroup 4 (hydrolyses these are specific to peptide bonds). They are further divided into two major groups based on their cleavage pattern: (a) *exopeptidases* that cleave N- or C-terminal peptide bonds of a polypeptide chain, (b) *endopeptidases* that cleave internal peptide bonds. At present, the term "peptidase" is also used as synonymous with "protease" and "proteinase". It is noteworthy to indicate that previously in Enzyme Nomenclature (1984), "peptidase" was restricted to the enzymes included in sub-subclasses EC 3.4.11–19, the exopeptidases. The term "proteinases" is also used previously for the enzymes included in sub-subclasses EC 3.4.21–99 having the same meaning as "endopeptidase" (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/intro.html#EC34). However, the terms "protease" and "proteinase" are still preferred by many scientists. This review will use the term "proteinase" to describe the enzymes that hydrolyze the peptide bonds.

Proteinases have been divided into six subclasses on the basis of catalytic mechanism: aspartic proteinases, cysteine proteinases, serine proteinases, metalloproteinases, threonine proteinases, and unknown type proteinases (Yegin et al. 2011). Aspartic proteinases comprise a relatively small group within this classification. This has been linked to presence of aspartic acids in the active site of these proteinases since this feature limits their functions to an acidic environment which is a very rare case in living organisms (Tang 2010). Nevertheless, they are a unique class of enzymes because of their physiological and commercial importance. Aspartic proteinases are present in all living organisms. They have very important roles including protein processing, maturation, and degradation. For an overview of physiological functions of some of the aspartic proteinases, see Tang (2010).

Aspartic proteinases are the first enzymes discovered (Szecsi 1992) and industrial production of aspartic proteinases dates back to 1874, when Danish scientist Christian Hansen extracted chymosin from calves' stomachs for application in cheese manufacturing (Nielsen et al. 1994). Aspartic proteinases are the first crystalline protein ever analyzed by X-ray diffraction technique (Davies 1990). They are a very well-studied class of enzymes and current protein database include a large number of proteinase structures. Several reviews have already been published on aspartic proteinases with different focuses: the classification, structure, and function of aspartic proteinases (Davies 1990; Szecsi 1992; Chitpinityol and Crabbe 1998; Dunn 2002), plant aspartic proteinases (Mutlu and Gal 1999), properties of aspartic proteinases from different sources used in cheese making (Claverie-Martin and Vega-Hernandez 2007), different aspects of aspartic proteinases from *Mucor* sp. (Yegin et al. 2011), the advances in the application of coagulants from different sources with the focus on cheese chemistry and production technology (methods for analysis of coagulants, proteolysis, cheese yield, and quality) (Jacob et al. 2011). Based on the increased number of recent studies regarding the novel milk-clotting aspartic proteinases, we aimed to prepare an updated review on aspartic proteinases by combining different aspects of such enzymes at the same time. The objective of this review is to provide an overview of the historical background on aspartic proteinases and to summarize the recent achievements on the classification and structural characteristics of such enzymes as related to their functional properties, mechanism of catalysis, pH, and temperature dependence, substrate specificities, and mechanism of

inhibition. Engineering and technological applications of such enzymes from different sources with special focus on cheese manufacturing is also described.

2 Classification and structure of aspartic proteinases

Aspartic proteinases (EC 3.4.23), which are so called as acid proteinases or aspartyl proteinases include two aspartic acid residues within their active sites. These two aspartic acid residues play a critical role for their catalytic activity (Yegin et al. 2011). Aspartic proteinases have been grouped into three families, namely pepsin (A_1), retropepsin (A_2), and enzymes from pararetroviruses (A_3) (Rao et al. 1998). Most of the aspartic proteinases belong to the A1 pepsin family (Rawlings et al. 2004). A1 pepsin family is the most studied one and will be the focus of this review.

2.1 Primary structure

Most of the aspartic proteinases are single-chain enzymes with a molecular weight of approximately 35 kDa (Szecsi 1992). They consist of approximately 330 amino acids in length. Almost 5% sequence identity has been observed between all members of the pepsin family (Davies 1990). Porcine pepsin was the first aspartic proteinase sequenced. The pepsin sequence provides numbering of important residues for homologous alignment (Tang 2010). Both of the catalytic aspartic acid residues in pepsin sequence (Asp 32 and Asp 215) are found in the motif of Asp–Thr–Gly. This characteristic motif exists in almost all family members. However, there are some exceptional cases where one of the catalytic aspartic acid residues can be in the motif of Asp–Ser–Gly. Aspartic proteinases have characteristic sequences in the region of two catalytic aspartic acid residues: Asp–Thr–Gly–Ser in the N terminal domain and a corresponding Asp–Thr–Gly–Ser/Thr in the C terminal domain (Mutlu and Gal 1999). These aspartic acid residues are responsible for the activation of a water molecule which mediates the nucleophilic attack on the scissile peptide bond. Figure 1 shows the amino acid sequence alignment of several aspartic proteinases having milk-clotting activity from different sources. The sequences of *Mucor mucedo* (Yegin and Fernandez-Lahore 2013) and *Metschnikowia reukaufii* (Li et al. 2010) aspartic proteinases have very recently been deposited into the GenBank. As it can be seen from Fig. 1, the plant aspartic proteinase (Cardosin A) from *Cynara cardunculus* (Faro et al. 1999) contains an Asp–Thr–Ser motif in the C terminal domain which is a common observation within the sequence of plant aspartic proteinases. Similarly, the fungal aspartic proteinases from *M. reukaufii* (Li et al. 2010) and *Saccharomycopsis fibuligera* (Hirata et al. 1988) contain Asp–Thr–Ser motif in C terminal domain which is the exceptional case within the fungal aspartic proteinases as most of them contain Asp–Thr–Gly. The amino acid sequence of different commercial milk coagulants have been compared by Dekker (2007). It has been found that FDTGSSD/E motif specially recognizes the genes encoding the milk-clotting enzymes but is absent in other aspartic proteinases.

There are variable numbers of introns in the DNA sequence of aspartic proteinases. The lengths and positions of the introns are also different. For example, bovine chymosin includes nine exons separated by eight introns while the genes encoding the aspartic proteinases from *Rhizomucor miehei* and *Rhizomucor pusillus* do not contain any introns (Foltmann 1999). The aspartic proteinase gene from *Rhizopus niveus* (GenBank ID

X56964) comprises one intron, the one from *Penicillium janthinellum* (GenBank ID U81483) contains two introns, and both the genes of *Aspergillus saitoi* (GenBank ID D25318) and *Cryphonectria parasitica* (GenBank ID X53997) contain three introns.

Aspartic proteinases of the pepsin family are synthesized as preproenzymes (zymogen), inactive precursors of the active enzymes, which likely provide protection against proteolysis (Horimoto et al. 2009). The zymogen is autocatalytically converted to the active enzyme at acidic pH by removal of N-terminal pro-segment (Davies 1990). It has been mentioned that the pro-segment is responsible for stabilizing the inactive form of the enzyme (Horimoto et al. 2009) and it prevents the entry of the substrate into the active site. The pro-segment is thought to be important for correct folding, targeting, and control of the activation of zymogens (Koelsch et al. 1994). The zymogen forms usually have an N-terminal propeptide of up to 50 amino acids in length (Davies 1990) which is tend to be rich in basic amino acids (Inoue et al. 1996). A lysine residue is conserved in almost all aspartic proteinases with a few exceptions (e.g., lamb prochymosin) and this residue has been postulated to interact with catalytic aspartic acid residues in the zymogen molecule (Chitpinityol and Crabbe 1998). Inoue et al. (1996) studied the structure/function relationship of the prosegment of a putative proform of aspergillopepsin I (proteinase B) from *Aspergillus niger* var. *macrosporus* by site-directed mutagenesis studies in different expression systems. It has been suggested that only Lys56 is essential for the correct folding of proproteinase B among the eight basic residues in the sequence of prosegment. However, it has been mentioned that replacement of this residue with arginine and some of the other basic residues might also partly contribute to the folding. Similarly, Richter et al. (1999) generated three different mutant forms (Lys36Arg, Lys36Met, and Lys36Glu) of porcine pepsinogen A in order to elucidate the effect of Lys36 on activation of zymogen. It was shown that the mutants Lys36Met and Lys36Glu were extremely unstable and they were degraded very rapidly. It was concluded that Lys36 was important not only for stability of the pepsinogen but also for the correct alignment of the active-center residues.

Temperature, pH, and salt concentration are important factors influencing the activation reactions of zymogens. For example, the activation of prochymosin is completed in 2 or 3 days at pH 5.0 and 25 °C, while the activation reaction takes only 5–10 min at pH 2.0 and 25 °C with the ionic strength of 0.1 $mg.L^{-1}$ (Chitpinityol and Crabbe 1998). Prochymosin exhibits a different activation pattern than the other aspartic proteinases. Activation of prochymosin at pH values below 2.5 results in formation of pseudochymosin by proteolytic cleavage of the bond 27–28. Pseudochymosin is 15 amino acid residues longer than the chymosin. Mature chymosin is formed after removal of the 15 amino acid peptide from pseudochymosin at pH values between 4 and 5 (Pedersen et al. 1979).

2.2 Three-dimensional structure

Aspartic proteinases are bilobal in structure with a large cleft separating these two domains. Each lobe contains one of the aspartic acid residues that are essential for catalytic activity in a psi-loop (Rawlings and Bateman 2009). The two lobes are structurally similar but there are considerable variations in the size of the loop regions (Nugent et al. 1996). The binding cleft is able to accommodate seven to eight amino

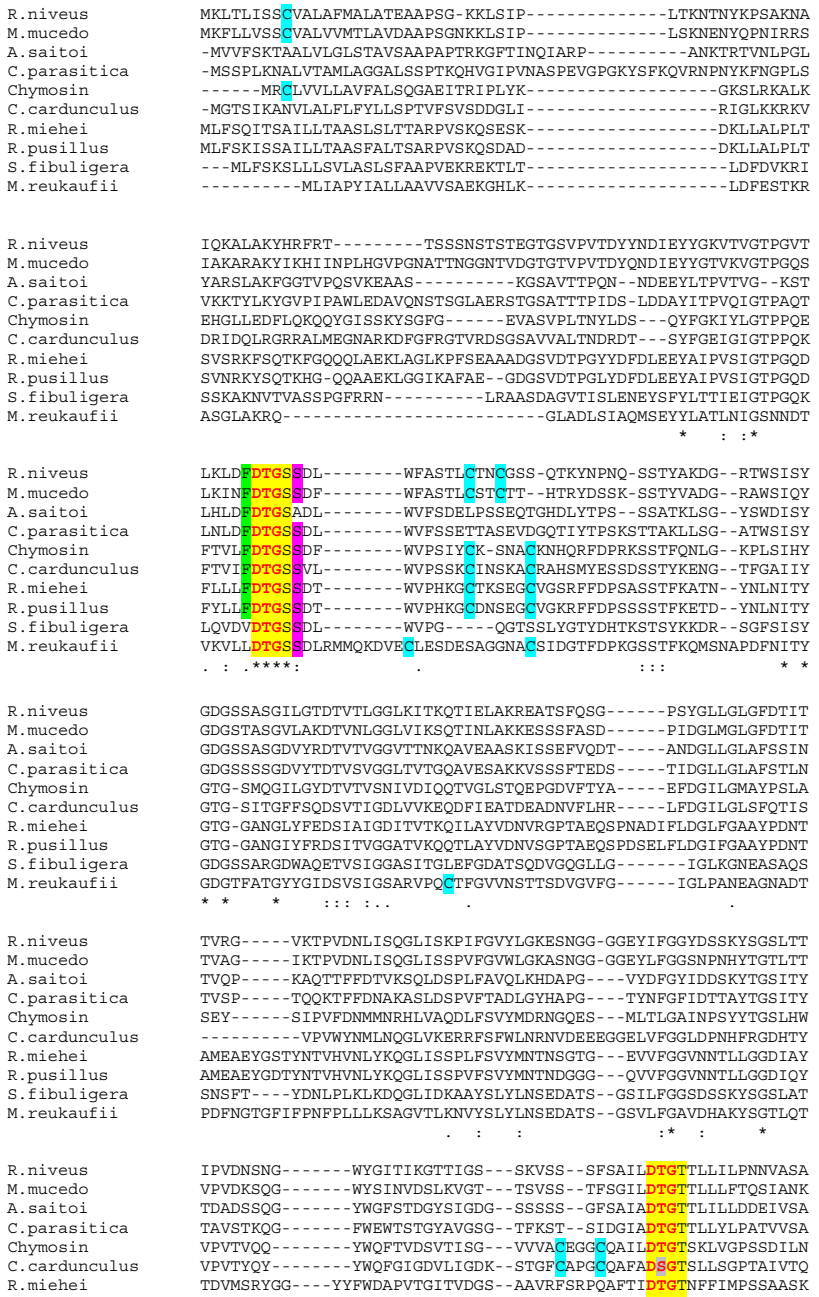

Fig. 1 Amino acid sequence alignment of aspartic proteinases having milk-clotting activity from different sources (*R. niveus*—GenBank ID: X56964.1, *M. mucedo*—GenBank ID: JN660818.1, *A. saitoi*—GenBank ID: D25318.1, *C. parasitica*—GenBank ID: X53997.1, chymosin—GenBank ID: NM_180994.1, *C. cardunculus*—GenBank ID: AJ132884.1, *R. miehei*—GenBank ID: M18411.1, *R. pusillus*—GenBank ID: AB018789.1, *S. fibuligera*—GenBank ID: D00313.1, *M. reukaufii*—GenBank ID: EU186020.1)

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R.niveus          VARSYG-ASDNG-------------------------------------------------
M.mucedo          VAAQYG-ATDNG-------------------------------------------------
A.saitoi          YYEQVSGAQESY-------------------------------------------------
C.parasitica      YWAQVSGAKSSS-------------------------------------------------
Chymosin          IQQAIGAT----------QNQYG-------------------------------------
C.cardunculus     INHAIGANGVMNQQCKTVVSRYGRDIIEMLRSKIQPDKICSHMKLCTFDGARDVSSIIES
R.miehei          IVKAALPDAT--------ETQQG-------------------------------------
R.pusillus        VVKAALPDAT--------ESQQG-------------------------------------
S.fibuligera      IGREYGTYSYSYG------------------------------------------------
M.reukaufii       IGAMMRGSYNETEG-----------------------------------------------


R.niveus          ------------------------------------------------------------
M.mucedo          ------------------------------------------------------------
A.saitoi          ------------------------------------------------------------
C.parasitica      ------------------------------------------------------------
Chymosin          ---------------------------------------------------------EFD
C.cardunculus     VVDKNNDKSSGGIHDEMCTFCEMAVVWMQNEIKQSETEDNIINYANELCEHLSTSSEELQ
R.miehei          ---------------------------------------------------------WV
R.pusillus        ---------------------------------------------------------YT
S.fibuligera      ------------------------------------------------------------
M.reukaufii       ------------------------------------------------------------


R.niveus          --DGTYTIDCDTSSFKPLVFSIGSSTFEVPADSLVFEQDGSTCY-AGFG----YGDYDFA
M.mucedo          --DGTYTISCNTANFKPLNFSINGAQFQVPVDSLIFEQSGSTCY-ASFG----YAGLDFA
A.saitoi          --EAGGYVFSCSTDLPDFTVVIGDYKAVVPGKYINYAPVSTGSS-TCYGGIQSNSGLGLS
C.parasitica      --SVGGYVFPCSATLPSFTFGVGSARIVIPGDYIDFGPISTGSS-SCFGGIQSSAGIGIN
Chymosin          IDCDNLSYMP---TVVFEINGKMYPLTPSAYTSQD----QGFCT-SGFQSENHSQK---W
C.cardunculus     VDCNTLSSMP---NVSFTIGGKKFGLTPEQYILKVGKGEATQCI-SGFTAMDATLLGPLW
R.miehei          VPCASYQNSKSTISIVMQKSGSSSDTIEISVPVSKMLLPVDQSN-ETCMFIILPDGGNQY
R.pusillus        VPCSKYQDSKTTFSLVLQKSGSSSDTIDVSVPISKMLLPVDKSG-ETCMFIVLPDGGNQF
S.fibuligera      -----GYVTSCDATGPDFKFSFNGKTITVPFSNLLFQNSEGDSE-CLVG--VLSSGSNYY
M.reukaufii       ---FIQVDCNYMTNTDSVIFDFSGAQISVPFSDLVFSDGSSCFLGLEPVDEDPVSDAPYA


R.niveus          IFGDVFLKNNYVVFN-QEVPEVQIAPIA---------------------------------
M.mucedo          ILGDVFLKNNYVVFN-QKVPQVQIAKSV--------------------------------
A.saitoi          ILGDVFLKSQYVVFN-SEGPKLGFAAQA--------------------------------
C.parasitica      IFGDVALKAAFVVFNGATTPTLGFASK---------------------------------
Chymosin          ILGDVFIREYYSVFD-RANNLVGLAKAI--------------------------------
C.cardunculus     ILGDVFMRPYHTVFD-YGNLLVGFAEAA--------------------------------
R.miehei          IVGNLFLRFFVNVYD-FGNNRIGFAPLASAYENE--------------------------
R.pusillus        IVGNLFLRFFVNVYD-FGKNRIGFAPLASGYENN--------------------------
S.fibuligera      ILGDAFLRSAYVYYD-IDNSQVGIAQAKY-------------------------------
M.reukaufii       ILGDNFLRHAYVVYD-LEDYEISLAQVKYNATENIEVVTSTIPLAVQASGYSSTFLAAEI
                  *.*:  ::     ::      : :*


R.niveus          ------------------------------------------------------------
M.mucedo          ------------------------------------------------------------
A.saitoi          ------------------------------------------------------------
C.parasitica      ------------------------------------------------------------
Chymosin          ------------------------------------------------------------
C.cardunculus     ------------------------------------------------------------
R.miehei          ------------------------------------------------------------
R.pusillus        ------------------------------------------------------------
S.fibuligera      ------------------------------------------------------------
M.reukaufii       LAIEQISTGIIQTGPAPSFMAHTSAVSGGLHTTAATTKASSSAASTSSASSTGTKLSSGI


R.niveus          ----------------------
M.mucedo          ----------------------
A.saitoi          ----------------------
C.parasitica      ----------------------
Chymosin          ----------------------
C.cardunculus     ----------------------
R.miehei          ----------------------
R.pusillus        ----------------------
S.fibuligera      ----------------------
M.reukaufii       KSTCVTKIVLYSVVCAAFFAYF
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Fig. 1 (continued)

acid residues of a substrate, equally divided on both sides of the catalytic aspartic acid residues (Szecsi 1992). The cleft is approximately 40 Å long (Szecsi 1992). The structures of aspartic proteinases mainly consist of β-sheet with very little α-helix (Nugent et al. 1996). This is one of the largest β-sheet structures observed within the structure of globular proteins (Szecsi 1992). Two antiparallel β-strands form a flexible loop (beta-hairpin loop) which is located at the entrance of the active site and is commonly known as the flap. The flap extends over the cleft and forms a channel where the substrate binds (Okoniewska et al. 1999). Another important feature of aspartic proteinases is a conserved network of hydrogen bonds stabilizing the active site which is called the "fireman's grip" that includes the hydroxyl groups of two threonine (serine) residues in the active site: Asp–Thr(Ser)–Gly (Ingr et al. 2003).

Loop regions joining the secondary structure of proteins play an important role in the structure, evolution, folding, and function of proteins (Nugent et al. 1996). The two types of loops (psi-loop and flap) are conserved in almost all sequences known or predicted to be active within the A1 pepsin family (Rawlings and Bateman 2009). Nugent et al. (1996) investigated the role of loop region in pepsin family by site-directed mutagenesis. The loop coding region of bovine chymosin B (155–164) was replaced by the loop coding region of *Rhizopus chinensis* pepsin and the mutant gene was actively expressed in *Trichoderma reesei*. Depending on the substrate employed, there were differences in the activity level of the mutant enzyme and the wild-type chymosin B. When a small synthetic peptide was used as a substrate, there was no effect on the specificity constant (k_{cat}/K_m) of the mutant and the wild-type enzyme. However, when a larger synthetic substrate was used, the mutation reduced the specificity constant 10-fold. It was concluded that the difference in activity level of the mutant and the wild-type enzyme might be a consequence of the different charge distribution of the mutated loop, its increased size and/or its different conformation.

The flap in aspartic proteinases has unique catalytic functions. It has been mentioned that the flap provides a more hydrophobic environment around the scissile bond (Tang 2010). Okoniewska et al. (1999) conducted a study to elucidate the role of the flap residue (Thr77) in the activation of pepsinogen and in the catalytic mechanism of active form. Three different mutants were constructed by changing the Thr77 residue to serine, valine, and glycine. Thr77Ser was activated at the same rate and had similar catalytic parameters as the wild-type protein. However, the activation rates of Thr77Val and Thr77Gly were slower and their catalytic efficiencies were lower than the wild-type protein. It was postulated that the hydroxyl group at position 77 provided an essential hydrogen bond that contributed to proper substrate alignment.

There are variable numbers of cysteine residues in the structures of aspartic proteinases. There is a potential for two disulphide bridges in the *Rhizomucor* and *Rhizopus* enzymes, a single disulfide bridge in *Endothia*, *Penicillium*, and *Aspergillus* enzymes, and no disulphide bridge in the *Irpex* aspartic proteinases (Chitpinityol and Crabbe 1998). It has been shown that the locations of the disulphide bridges are conserved when any disulfide bridge exists in the structure. For example, prochymosin and mature chymosin contain three disulfide bonds linking Cys45 to Cys50, Cys206 to Cys210, and Cys250 to Cys283 (pepsin numbering) while mucorpepsin from *R. miehei* has two disulfide bridges from Cys45 to Cys50 and Cys250 to Cys283, while endothiapepsin from *C. parasitica* has a single bridge between Cys250 and Cys283 (Chen et al. 2000). Table 1 depicts some of the aspartic proteinases

having milk-clotting activity available in protein database. Since a large amount of structural information on aspartic proteinases is avaliable in protein database, they provide excellent opportunity to investigate the structure–function relationship of proteins.

Members of the aspartic proteinases of the pepsin family may be glycosylated. Glycosylation of a secreted protein may increase the solubility of the protein and prevent aggregation of the protein in the secretory pathway. The effects of glycosylation on the secretion efficiency and activity are different for each protein and have to be determined by case-specific studies. Although the importance of glycosylation is not exactly known within aspartic proteinases, it has been suggested that it stabilizes the protein confirmation and therefore may result in higher thermostability (Machalinski et al. 2006). For example, a difference between calf and camel chymosin is that the latter is glycosylated and has a higher thermostability than the calf chymosin (Kappeler et al. 2006). This property of camel chymosin may be a disadvantage for industrial cheese production since it will be more difficult to inactivate the enzyme during whey pasteurization or during the cooking/stretching step in cheese making. The importance of thermostability of the milk-clotting aspartic proteinases in cheese manufacturing will be discussed in details in coming sections.

3 Mechanism of hydrolysis by aspartic proteinases

The mechanism of hydrolysis by aspartic proteinases is generally accepted to occur by nucleophilic attack on the peptide carbonyl carbon via a catalytic water molecule (Dunn 2002) but exact details of the mechanism is still controversy (Palmer et al. 2010).

Table 1 Some of the aspartic proteinases with milk-clotting activity available in Protein Data Bank (PDB)

Type	Name	Source	Resolution (Å)	PDB ID	Reference
Mammalian	Chymosin	Calf	2.2	4CMS	Newman et al. 1991
	Recombinant chymosin	Calf	2.3	1CMS	Gilliland et al. 1990
	Pepsin	Porcine	1.8	4PEP	Sielecki et al. 1990
Microbial	Aspergillopepsin	*Aspergillus oryzae*	1.9	1IZD	Kamitori et al. 2003
	Aspergillopepsin	*Aspergillus phoenicis*	2.18	1IBQ	Cho et al. 2001
	Endothiapepsin	*Cryphonectria parasitica*	2.1	1EPM	Blundell et al. 1990
	Penicillopepsin	*Penicillium janthinellum*	1.8	1APT	James et al. 1982
	Rhizopuspepsin	*Rhizopus chinensis*	1.8	3APR	Suguna et al. 1987
	Mucorpepsin	*Rhizomucor miehei*	2.15	2ASI	Yang et al. 1997
	Mucorpepsin	*Rhizomucor pusillus*	2.0	1MMP	Newman et al. 1993
	Trichodermapepsin	*Trichoderma reesei*	1.70	3C9X	Nascimento et al. 2008
Plant	Cardosin	*Cynara cardunculus*	1.72	1B5F	Frazao et al. 1999
Fungi (mushroom)	–	*Irpex lacteus*	1.3	1WKR	Fujimoto et al. 2004

According to the widely accepted mechanism for the hydrolysis of peptide bonds by aspartic proteinases, the two aspartic acid residues in the enzymes' active site are located on opposite sides of the scissile peptide bond. The first step involves a proton transfer from a water molecule to the aspartic dyad and concurrently another proton transfer from the dyad to the carbonyl oxygen of the scissile peptide bond. This is a general acid–base mechanism where the water molecule acts as a nucleophile that attacks the carbonyl of the scissile peptide bond. These protonation events result in formation of the tetrahedral intermediate. The breakdown of the intermediate to the products is generated by proton transfers to the dyad and from the dyad to the nitrogen atom of the substrate. The protonation of the substrate carbonyl and nucleophilic attack may occur simultaneously during the formation of the tetrahedral intermediate. Similarly, proton transfer from the intermediate to the dyad and the protonation of the nitrogen atom of the substrate during the cleavage of the resultant intermediate may occur at the same time (Davies 1990; Chitpinityol and Crabbe 1998).

Veerapandian et al. (1992) has proposed a catalytic mechanistic model as a result of X-ray structural studies of an endothiapepsin–difluorostatone inhibitor complex which was considered to be a transition state mimic. The model has been summarized in Fig. 2. According to this mechanism, the pro-R (statine-like) hydroxyl of the tetrahedral carbonyl hydrate is hydrogen bonded to the outer oxygen of Asp32 and Asp215 in the position occupied by a water molecule in the native enzyme. The second hydroxyl oxygen of the hydrate is hydrogen bonded only to the carboxyl oxygen of Asp32. The scissile bond carbonyl is protonated by Asp32 and concurrently attacked by a water molecule polarized into a nucleophilic state by Asp215. The rigid body movement in the enzyme–substrate complex may cause distortion of the amide function that facilitates the attack of the nucleophilic water on the polarized carbonyl. The resulting tetrahedral intermediate is stabilized by extensive hydrogen bonds to the negatively charged carboxyl of Asp32. Breakdown of the complex to form products is initiated by transfer of a proton to the leaving amino group either from Asp 215 or from bulk solvent (Chitpinityol and Crabbe 1998). A similar mechanism has been described by James et al. (1992) on the basis of X-ray crystallographic studies of complexes between penicillopepsin and difluorstatine or difluorostatone-containing peptides. These experimental data provide a basis for a model of the tetrahedral intermediate in aspartic proteinase-mediated cleavage of the amide bond. This indicates a mechanism in which Asp32 is the proton donor and Asp215 carboxylate polarizes a bound water molecule for nucleophilic attack. Crystallographic studies have provided important and valuable structural information to elucidate the catalytic mechanism of aspartic proteinases. Further studies by utilizing neutron diffraction studies provided more detailed information regarding the positions of protons on the catalytic aspartic residues. Coates et al. (2001) also showed that Asp32 is deprotonated and Asp215 is protonated on the outer oxygen atom in the transition state by the neutron diffraction studies utilizing the complex between endothiapepsin and hydroxyethylene-based inhibitor.

Andreeva and Rumsh (2001) compared the crystal structures of pepsin-like enzymes, their complexes with inhibitors and, their zymogen forms in order to elucidate the role of amino acid residues adjacent to the catalytic site of such enzymes. They found another water molecule around the active groups other than the water molecule located between the active carboxyls acting as a nucleophile during catalytic reaction. It has been mentioned that this water molecule plays an essential role in the formation

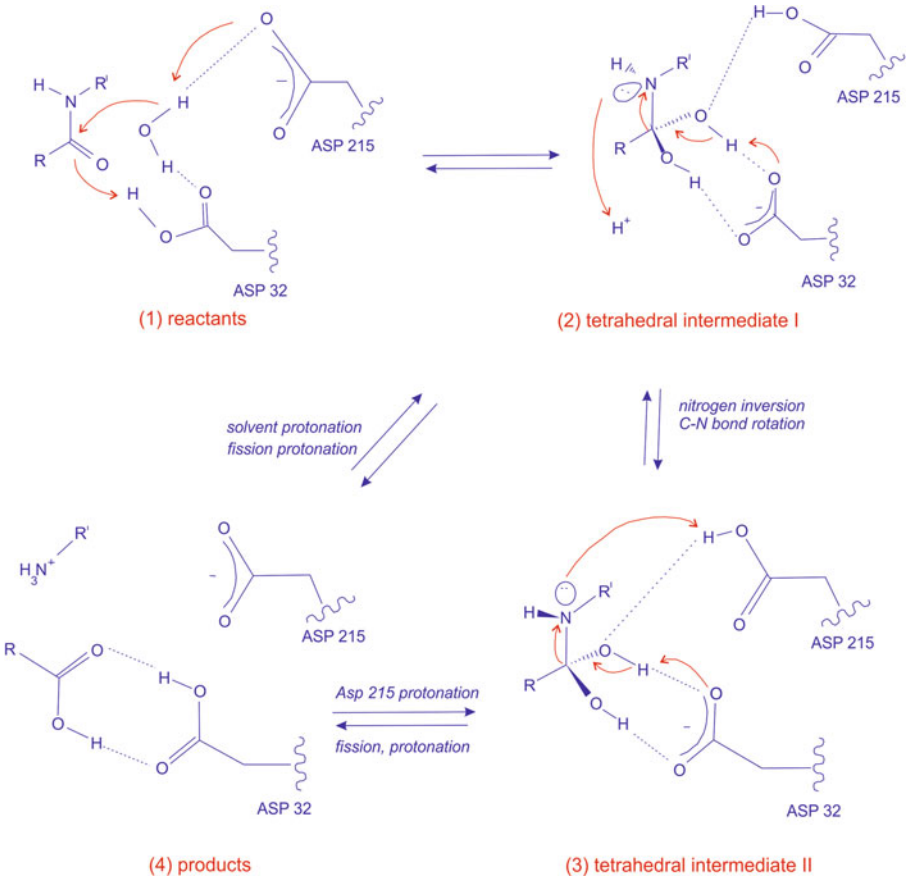

Fig. 2 A proposed catalytic mechanism for aspartic proteinase by Veerapandian et al. (1992)

of a chain of hydrogen-bonded residues between the active site flap and the active carboxyls on ligand binding.

Another reaction mechanism that involves the formation of a low-barrier hydrogen bond between Asp32 and Asp215 has been suggested by Northrop (2001). The mechanism includes a rearrangement of protons around a 10-membered cyclic intermediate that is proposed to occur by quantum tunneling. Although different mechanistic models have been proposed, it can be concluded that all agreed on the occurrence of a covalent intermediate.

4 pH and temperature dependence of the aspartic proteinases

pH and temperature dependence of aspartic proteinases are very important factors for the selection of milk-clotting enzymes since milk coagulation strongly depends on the pH of the milk and the temperature. A decrease in the pH of milk from 7.0 to 5.2 causes a significant decrease in the clotting time. The pH optimum for the hydrolysis of κ-casein has been reported as 5.1–5.3 (Nájera et al. 2003).

Most aspartic proteinases exhibit maximal activity at low pH values generally between 3 and 5. They have isoelectric points in the range of 3 to 4.5 (Yegin et al. 2011). The neutron diffraction studies utilizing the complex between endothiapepsin and hydroxyethylene-based inhibitor confirm that the proteinase has a number of buried ionized carboxylate groups which are likely to give the molecule a net negative charge even at very low pH values, thereby accounting for its low pI (Coates et al. 2001). It has been proposed that the optimum pH of each aspartic proteinase is determined by the electrostatic potential of the active site which in turn is determined by the position and orientation of all residues near the active site (Tanaka and Yada 2001). Modifications in overall surface charge of proteins can alter the optimal pH of the enzymes (Rao et al. 1998). Protein engineering may provide tools to tailor the pH dependence of the enzymes adjusted to the requirements of the industrial processes, e.g., by introducing point or systematic multiple mutations on the surface of the proteins.

Inside the cell, all proteins are synthesized at an environment with neutral pH, therefore their natural conformational state and functionality exists in this environment (Tanaka and Yada 2001). However, most aspartic proteinases are stable under acidic conditions and become inversely denaturated at neutral pH values. Tanaka and Yada (2001) studied the possible factors responsible for the different pH stability profile of porcine pepsin and its zymogen since the active enzyme was unstable at neutral pH values while the zymogen form was stable. According to the findings of this study, mutations to change the number and the distribution of positive charges on the surface had a minor effect on the conformational stability. However, replacement of five amino acid residues in the first 13 residues in the N-terminal fragment had a major impact on stability. At pH 7.0, this mutant was inactivated 5.8 times slower than the wild-type protein. The introduction of a disulfide bond between the N-terminal fragment and the enzyme body prevented the enzyme from denaturation. It was concluded that limiting the N-terminal mobility resulted in limited denaturation of pepsin at neutral pH.

From another perspective, it is noteworthy to mention that the optimum pH for aspartic proteinases is not an absolute value. The optimum pH values depend on the experimental conditions such as denaturation of the substrates, ionic strength of the solutions, duration, temperature of the experiments, and the method used to follow the progress of the proteolysis. The pH optima of pepsins have been stated as 2 while the pH optima of chymosin and fungal aspartic proteinases have been determined as 3–4 under common conditions (Foltmann 1999). Similarly, the observed values for pH stability may also depend on the experimental conditions. Chymosin is stable at pH values between 5.3 and 6.3. However, even at pH 2, chymosin is relatively stable. Mucorpepsin, endothiapepsin, and *Saccharomyces cerevisiae* proteinase A are stable at pH 3.5–7.0 (Foltmann 1999).

The velocity of coagulum formation increases gradually from 20 to 40–42 °C; however, at higher temperature values, the velocity of coagulum formation slows down. It has been observed that the temperature of the milk remarkably affects the rate of gel firming (Nájera et al. 2003) indicating the importance of the temperature optima of the aspartic proteinases employed in cheese manufacturing. The optimum temperature for milk-clotting activity of the aspartic proteinases varies to a large extent. Optimum temperature for purified bovine chymosin has been reported to be between 30 and 40 °C (Kumar et al. 2010). Table 2 summarizes the pH and temperature dependence of the recently discovered aspartic proteinases. Thermostability is an important criterion for the selection of the coagulant in cheese manufacturing. High thermal stability results in

Table 2 pH and temperature dependence of novel milk-clotting enzymes from recent studies

Enzyme source	Substrate	Optimum temperature (°C)	Optimum pH	Thermostability	pH stability	Reference
Mucor mucedo	Milk	35–40	5.0–5.6	Complete activity loss after incubation at 55 °C for 10 min	Stable at pH 5.0–5.5 The pH values beyond 6.0 resulted in a marked decrease in the activity, even after short exposure times (10 min incubation at respective pH at 25 °C)	Yegin et al. 2012
Piptoporus soloniensis	Milk	35–40	5.5	Complete activity loss after incubation at 60 °C for 30 min	Stable at pH 3.0–5.0 The enzyme retained about 70% of its activity with a maximum stability at pH 6. At pH values beyond 6.0 the enzyme lost its activity (22 h incubation at respective pH at 25 °C)	El-Baky et al. 2011
Cirsium vulgare—expressed in *E. coli*	–(MCA)Lys–Lys–Pro–Ala–Glu–Phe–Phe–Ala–Leu–Lys(DNP)–	–	4.0	Stabile at 30–37 °C 90% of activity loss at 65 °C (10 min incubation at respective temperature)	–	Lufrano et al. 2012
Bacillus licheniformis 5A5	Milk	75	–	Complete activity loss after incubation at 80 °C for 75 min	–	Ahmed and Helmy 2012
Aloe variegata	Milk	85	–	63.8% of activity loss after incubation at 80 °C for 75 min	–	Ahmed and Helmy 2012
Amylomyces rouxii	Hemoglobin	50	3.5	70% of activity loss after incubation at 50 °C for 60 min	Active at pH 2.0–4.5	Marcial et al. 2011
Metschnikowia reukaufii—expressed in *E. coli*	Hemoglobin	40	3.4	The enzyme was stable up to 40 °C, but inactivated rapidly at temperatures above this. 92% of activity loss after incubation at 70 °C for 30 min.	The enzyme activity was decreased significantly at pH values higher than 5.0 or lower than 2.6 (1 h incubation at respective pH at 4 °C)	Li et al. 2010
Thermomucor indicae-seudaticae N31	Milk	70	5.7	Stabile at 40–45 °C No activity has been detected after 60 °C (1 h incubation at respective temperature)	Stabile at pH 3.5–4.5 Almost no activity has been detected beyond pH 7.0 (24 h incubation at respective pH at 25 °C)	Merheb-Dini et al. 2010

Table 2 (continued)

Enzyme source	Substrate	Optimum temperature (°C)	Optimum pH	Thermostability	pH stability	Reference
Aspergillus niger I1	Casein	60	3.0	Highly stable at temperatures below 40 °C after incubation for 60 min No activity has been detected after incubation for 30 min at 50 °C	Stabile at pH 3.0–6.0 No activity has been detected at pH 2.0 and values beyond pH 7.0 (1 h incubation at respective pH at 4 °C)	Siala et al. 2009
Synergistes sp.	Casein	30–45	5.5–6.0	Stabile at 30–45 °C Inactive at temperatures above 50 °C		Kumar et al. 2008
Centaurea calcitrapa	FITC-casein	52	5.1	Fully active after 6 h of incubation at 4 and 25 °C Complete activity loss after 6 h of incubation at 70 °C	–	Raposo and Domingos 2008
Ficus racemosa (*L.*)	Azocasein	60	4.5–6.5	The enzyme was stable up to 60 °C after incubation for 15 min	Stabile at pH 4.0–7.0 Rapid decrease in activity at pH 8.0 (2 h incubation at respective pH at 25 °C)	Devaraj et al. 2008

FITC fluorescein isothiocyanate labeled

persistence of the enzyme after cooking of the curd and may generate off-flavors during the cheese ripening period due to the extensive breakdown of the caseins. Additionally, a high thermostability will prevent the inactivation of the enzyme by whey pasteurization. The thermal stability of the aspartic proteinases strongly depends on the pH. The presence of other proteins often also has a stabilizing effect. Thunell et al. (1979) tested the stability of six different milk-clotting aspartic proteinases over a pH range of 5.2 to 7.0 and heat application at 68.3 and 73.9 °C. The proteinase from *R. miehei* was the most heat stable followed in order by *R. pusillus* proteinase, calf rennet, bovine pepsin, *C. parasitica* proteinase, and porcine pepsin. By decreasing the pH, the heat stability of all proteinases increased with the exception of *C. parasitica* proteinase where pH had little effect.

5 Inhibition

All aspartic proteinases are inhibited by pepstatin, a peptide from species of *Streptomyces* with six amino acids (isovaleryl–Val–Val–Sta–Ala–Sta), two of which are the unusual amino acid statine. The positions of amino acid residues of pepstatin are defined as P_4, P_3, P_2, P_1, $P_{1'}$, and $P_{2'}$. Aspartic proteinases generally have a broad substrate specificity, favoring hydrophobic amino acid residues at positions P_1 and $P_{1'}$ (Kamitori et al. 2003) but pepstatin has an inhibitory group (–CH(OH)CH_2–) at this cleavage site. The inhibition by pepstatin occurs as a result of binding of the hydroxyl group of statine to the two catalytic aspartic acid residues. It has been reported that inhibition studies of rhizopuspepsin and penicillopepsin by pepstatin showed that the hydroxyl group of the first statine was bound between the carboxyl oxygen of both aspartic acid residues, effectively replacing the water molecule seen in the native enzyme (Dunn 2002). Besides, aspartic proteinases are also inhibited by diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2 epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions (Rao et al. 1998). Pepstatin has been proposed to be a transition state analog (Davies 1990), while these diazoketone compounds are not (Tang 2010). It has been proven that the inhibition by DAN is due to esterification of Asp-215 whereas the inhibition with EPNP preferentially occurs by esterification of Asp-32 (Foltmann 1999). All X-ray crystallographic studies indicate that different types of peptide inhibitors all bind to the cleft (Davies 1990).

Recently, novel aspartic proteinase inhibitors have been isolated from different microbial and plant sources. Table 3 depicts some of the properties of these novel inhibitors. More information is needed on these novel inhibitors in order to get the full advantage of this type of inhibitors in structural studies.

Table 3 Novel microbial aspartic proteinase inhibitors

Inhibitor producing source	Aspartic proteinase	IC_{50} (nM)	K_i (nM)	Reference
Penicillium sp. VM24	*Aspergillus saitoi*	1,800	850	Menon and Rao 2012
Vigna radiata	*Vigna radiata*	11	34	Kulkarni and Rao 2009
Bacillus licheniformis	Pepsin	4.0	3.83–5.31	Kumar and Rao 2006
Bacillus sp.	*Aspergillus saitoi*	–	3,250	Dash et al. 2001

6 Enzyme engineering and technological applications with the focus on cheese industry

Aspartic proteinases available from a wide diversity of sources have biotechnological potential in different industrial processes other than cheese manufacturing. There are several other application areas: production of seasonings products (Kanlayakrit and Maweang 2006), production of casein hydrolysates (Phelan et al. 2009), drug design (Eder et al. 2007), meat tenderization (Ashie et al. 2002), peptide synthesis (Kumar and Bhalla 2005; Filippova and Lysogorskaia 2003), leather processing (Thanikaivelan et al. 2004), and fiber hydrolysis from feed stocks (Abbas and Bao 2009). However, it is noteworthy to indicate that commercially available aspartic proteinase preparations are mainly formulated for cheese industry.

Milk coagulation by aspartic proteinases is one of the most critical steps in cheese manufacturing. Coagulation process consists of two phases. In the first phase, the enzyme specifically cleaves the Phe^{105}–Met^{106} bond of κ-casein and splits the protein in two fragments: the (hydrophobic) para-κ-casein and the (hydrophilic) casein glycomacropeptide. The glycomacropeptide diffuses into the milk serum and its stabilizing effect becomes lost. The second phase consists of Ca^{2+} induced aggregation of the casein micelles that have been destabilized by the proteolytic attack to form a gel. This phase is extremely sensitive to protein concentration, temperature, pH, and Ca^{2+} ion concentration. Any variation in the chemical environment can affect the coagulation process at a large extent (Yegin et al. 2011). For example, milk does not clot at temperatures below 15 °C. This is due to the loss of enzymatic activity and also the slow coagulation rate of renneted micelles at or below this temperature (Dalgleish 1999).

Several attempts have been made in order to elucidate the specificity of aspartic proteinases towards the Phe–Met bond. It has been indicated that di-, tri-, or tetrapeptides containing a Phe–Met bond are not hydrolyzed. However, in the form of pentapeptide (Ser–Leu–Phe–Met–Ala–OMe), the Phe–Met bond can be hydrolyzed. Therefore, the length of the peptide and the residues around Phe–Met bond are important for the enzyme–substrate interactions (Fox 2007). It has been mentioned that the residues around the cleavage site function to hold the substrate in its correct orientation in the active site of the enzyme (Dalgleish 1999). Replacement of Ser by Gly or Ala in the above pentapeptide made the Phe–Met bond very resistant to hydrolysis by chymosin but not by pepsins. Substituting D-Ser with L-Ser in this pentapeptide significantly reduced the sensitivity of the Phe–Met bond while extension of the above pentapeptide from the N- and/or C-terminal to reproduce the sequence of κ-casein, increased the efficiency of hydrolysis of the Phe–Met bond by chymosin (Fox 2007).

Apart from the main function of milk-clotting enzymes in digestion of κ-casein, they play a very important role in the initiation of cheese ripening (Upadhyay et al. 2004). Hydrolysis of the caseins in cheese by rennet provides substrates for flavor formation by starter bacteria during cheese ripening. This "primary proteolysis" plays an essential role in the maturation of many hard and semi-hard cheese varieties. Although proteolysis is essential for ripened cheese, the ideal milk-clotting enzyme for manufacturing of young cheeses should possess a high ratio of clotting activity (specificity to Phe–Met bond) to general proteolytic activity at pH and temperature values which the cheese is stored. Severe proteolytic action on milk protein fractions

other than the specific action on the κ-casein is considered undesirable especially for young cheese varieties since it may lead to texture changes and off-flavor generation during storage. However, the general proteolytic actions of thermolabile enzymes can be eliminated after milk coagulation during further processing steps (e.g., curd cooking, stretching/molding step of pasta filata cheeses). Thus, sufficient thermolability is important criterion especially for those aspartic proteinases having higher general proteolytic activity.

It may be possible to engineer aspartic proteinases to better meet the industrial requirements for activity, substrate specificity, and temperature stability. In the next sections several engineering possibilities for different types of aspartic proteinases used in the cheese industry for the purpose of milk clotting will be discussed.

6.1 Bovine chymosin and other chymosins

Chymosin (EC 3.4.23.4) is a gastric digestive aspartic peptidase that is responsible for the coagulation of milk in the abomasum of unweaned calves (Fox and McSweeney 1999). The proteinase extracted from the abomasum of calves is a combination of chymosin and pepsin. This crude extract is also called as calf rennet. The composition of calf rennet changes depending on the age of animals when slaughtered. It is often desired to have a high proportion of chymosin in the preparation for cheese making since pepsin has more unspecific proteolytic activities other than milk-clotting activity. The proportion of chymosin to pepsin is often 80:20 in regular calf rennets.

The natural function of chymosin in the body is the hydrolysis of κ-casein once the milk is in the calf's stomach, leading to the formation of a coagulum that slows down gastric passage and thereby increases digestion. The first step in the biosynthesis of chymosin (323 amino acids, 35.6 kDa) by the cells of the gastric mucosa is the synthesis of preprochymosin, a polypeptide of 381 amino acids and 42.1 kDa. Preprochymosin is secreted as an inactive precursor, known as prochymosin, with 365 amino acids and 40.8 kDa, produced by cleavage of the N-terminal signal peptide (Foltmann 1999). Prochymosin is activated by autocatalytic removal of the 42-amino acid prosegment in the acidic environment of the gastric lumen. Calf chymosin has been found in three major forms: A, B, and C, chymosin B being the most abundant. Chymosin A and B differ from each other by a single amino acid substitution. Chymosin A has an Asp residue whereas chymosin B has a Gly residue at position 243. Chymosin C has been reported as to be a degradation product of chymosin A (Chitpinityol and Crabbe 1998) but recently it has been proven that this form is genetically distinct and a product of a different allele (Rampilli et al. 2005). It has been reported that chymosin A has 20% higher milk-clotting activity than chymosin B but chymosin B is preferred in the industry because of the longer shelf life (Palmer et al. 2010).

Chymosin is a globular protein. The secondary structure consists of 13% helical segments (9 helices and 44 residues) and 48% β-sheet (29 strands and 158 residues) (Newman et al. 1991). Chymosin is monomeric, with a 9% sequence identity between N- and C-terminal domains. The catalytic Asp34 and Asp216 residues occur in conserved Asp–Thr–Gly motifs. A water molecule is observed between the catalytic Asp residues in all crystal structures of pro-chymosin like many other pepsin family members. The protein contains three disulfide bridges (Cys45–Cys50, Cys206–

Cys210, and Cys250–Cys283). Chen et al. (2000) demonstrated that Cys250–Cys283 is indispensable for correct refolding of prochymosin, whereas Cys45–Cys50 is dispensable but has some contribution to the stability and substrate specificity of the enzyme. Figure 3 shows the three-dimensional structure of chymosin.

Traditionally, bovine chymosin is employed for cheese manufacturing. Bovine chymosin is the industrial gold standard for cheese manufacturing because it is highly specific for the Phe^{105}–Met^{106} bond of κ-casein. Recently, Palmer et al. (2010) performed a pioneering study to develop models of residues 97–112 of bovine κ-casein complexed with bovine chymosin, using ligand docking, conformational search algorithms, and molecular dynamics simulations. In agreement with experimental data obtained from neutron and X-ray crystallographic studies and site-directed mutagenesis studies, the proposed model has indicated that the substrate binds in an extended conformation with charged residues on either side of the scissile bond. It has been observed that Lys111 and Lys112 bind to the N-terminal domain of chymosin and takes the place of the conserved water molecule. The same researchers have pointed out that a cluster of histidine and proline residues (His98–Pro99–His100–Pro101–His102) in κ-casein binds to the C-terminal domain of the protein, where adjacent conserved arginine residue (Arg97) has been found to be important for stabilizing the binding pose. Further studies are required for different proteinases to take full advantage of such information.

Chymosins from other animal sources (e.g., lamb, goat, buffalo, and camel) have also been the focus of different studies as an alternative to bovine chymosin. The detailed structural studies on animal rennet extracts from other ruminants are scarce. These studies mainly focused on determination of the primary structure, purification, and biochemical characterization of the enzyme preparations. The primary structures of animal rennets from other ruminants are very similar to that of bovine chymosin. Sequencing of the cDNA fragments of lamb (Pungercar et al. 1990), buffalo (Vallejo et al. 2008) and camel (Kappeler et al. 2006) preprochymosins revealed similarities of

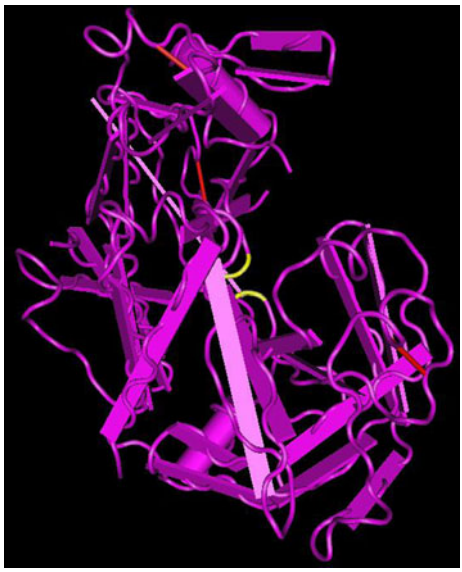

Fig. 3 Three-dimensional structure of chymosin (PDB ID: 4CMS; Newman et al. 1991). The DTG residues that are critical for catalytic activity are highlighted in yellow. The disulphide bridges are highlighted in red

95%, 97.9%, and 87%, respectively to that of bovine preprochymosin. Some of the biochemical properties of purified chymosins from other animal sources have also been reported. It has been shown that buffalo chymosin has a molecular weight of 35.6 kDa and its partial N-terminal amino acid sequence is identical to that of bovine chymosin. Thermostability studies revealed that the buffalo chymosin is more stable than the bovine chymosin (Mohanty et al. 2003). The pH dependence of buffalo and bovine chymosin has been compared by utilizing hemoglobin as a substrate. It has been found that buffalo chymosin (pH 4.0) has slightly higher pH optimum than bovine chymosin (pH 3.2) (Abdel Malak et al. 1996). Some of the characteristics of rennet extracted from camel stomach in comparison with buffalo rennet have also been reported. It has been shown that camel rennet is more thermostable than the buffalo rennet. The proteolytic activity of camel rennet has been found higher than buffalo rennet towards both camel and cows' milk at pH 6.0 (Elagamy 2000).

Lamb or kid rennet pastes are other alternatives to bovine chymosin. They are preferentially used in the manufacture of some ewe's or goat's milk cheeses in certain areas of the Mediterranean countries (Etayo et al. 2006). These rennet preparations are not highly commercialized and usually prepared at artisanal level. They contain variable amounts of lipolytic activities together with chymosin and pepsin. The use of lipase-containing lamb rennet pastes causes the accumulation of short chain free fatty acids during cheese ripening which impart a characteristic "pungent" flavor (Etayo et al. 2006). The cheese characteristic prepared with lamb rennet and bovine rennet pastes have been compared by different researchers (Irigoyen et al. 2002; Vicente et al. 2000; Calandrelli et al. 1997) and contradictory results have been demonstrated. The possible explanations for that could be the lack of standardization of enzymatic activities in the paste preparations and the difference in the hygienic conditions and microbial load of the paste preparations. In the light of these possible reasons, Bustamante et al. (2003) performed a study to compare the cheeses made with lamb rennet paste and bovine rennet paste after providing standardization of the enzyme activities of both rennet pastes. It was concluded that a higher level of activity resulted in higher levels of free amino nitrogen (higher level of proteolysis) for cheese made with both rennet pastes. The type of rennet significantly affected the percent of β-casein which was higher at all times in cheeses made with lamb rennet paste than in cheeses made with bovine rennet paste.

Although different types of animal rennets have been the focus of several studies to replace bovine chymosin, none of them are widely applied in industrial cheese production. Since cheese is a very popular dairy product all over the world, the production of different cheese varieties has been following an increased trend. Consequently, the availability of bovine chymosin has become limiting. Calf rennet is also relatively expensive and there are some consumer constraints against the enzymes of animal origin (e.g., fear for animal-borne diseases, vegetarian reasons). Therefore, the recombinant forms of animal rennets and alternatives from different microbial sources have since been developed by industry (Yegin and Fernandez-Lahore 2013).

6.2 Recombinant chymosin

The first enzyme for food processing produced with recombinant DNA technology which was registered by the U.S. Food and Drug Administration was recombinant

chymosin usually denoted as fermentation produced chymosin (FPC) (Flamm 1991; Jacob et al. 2011). It has been reported that currently FPC comprises 70–80% of the global rennet market (Johnson and Lucey 2006; Jacob et al. 2011).

Different microorganisms have been used as a host for expression of chymosin. Calf prochymosin has been expressed in *Escherichia coli* and the structure of the gene and properties of the recombinant enzyme was analyzed. The proenzyme was produced as insoluble inclusion bodies in *E. coli* resulting in aggregate formation and low activity. The enzyme obtained after denaturation and renaturation of the inclusion bodies was however indistinguishable from native calf chymosin (Foltmann 1999). In order to prevent these complicated and expensive procedures, the enzyme has been expressed in secreted form in yeast (e.g., *S. cerevisiae* and *Kluyveromyces lactis*). Mellor et al. (1983) cloned the chymosin without the pro-segment and observed no milk-clotting activity from the clones containing the chymosin gene. The results suggested that pro-segment was essential for correct folding of the protein. Similar observation was obtained by Vallejo et al. (2008) when buffalo chymosin was expressed in *Pichia pastoris*. Prochymosin has also been expressed in *Aspergillus nidulans* and *A. niger* as glucoamylase-prochymosin fusion protein and *Tricoderma reesei* as cellobiohydrolase I-prochymosin fusion protein (Foltmann 1999). In all cases, the primary translation product was processed to a polypeptide having a molecular weight similar to bovine chymosin and the secreted polypeptides were enzymatically indistinguishable from bovine chymosin. Currently, recombinant chymosin B is produced with the fungus *A. niger* (Chymax from Chr. Hansen) and the dairy yeast *K. lactis* (Maxiren from DSM Food Specialties) and both products are commercially available in different purity grades for cheese makers.

Different comparative studies have been performed to figure out the differences between the recombinant chymosin and standard calf chymosin. O'Sullivan and Fox (1991) compared the cheeses produced by calf chymosin and its recombinant form expressed in *K. lactis*. It has been proven that both enzymes behaved similarly under all environmental conditions studied and there was no difference between the cheeses obtained with respect to sensory and chemical criteria. Similarly, Brome and Hickey (1990) observed no differences between the Cheddar cheeses produced by using FPC and standard calf chymosin after comparing the results of compositional, microbiological, and proteolytic analyses.

Chymosins originating from other mammalian species such as lamb (Rogeli et al. 2001), goat (Vallejo et al. 2012), buffalo (Vallejo et al. 2008), and camel (Kappeler et al. 2006) have also been produced in recombinant forms. Recently, Vallejo et al. (2012) performed a direct comparative study of 4 different recombinant chymosins (goat and buffalo chymosins expressed in *P. pastoris*, and bovine and camel chymosin expressed in *A. niger*). They have pointed out that recombinant goat chymosin exhibited the best catalytic efficiency compared with the buffalo, bovine, or camel recombinant enzymes. Moreover, recombinant goat chymosin exhibited the best specific proteolytic activity, and a wider pH range of action, than the other 3 enzymes. It was proposed that recombinant goat chymosin represents a very good alternative to recombinant bovine chymosin for use in the cheese making. Although these chymosins have a high sequence identity, the difference in catalytic efficacy is not well understood on molecular level. Elucidation of the basis for the biochemical differences seen between the chymosins from different mammals awaits the elucidation of the crystal structure of chymosin–κ-

casein complexes. In this sense, a very detailed study on relationship between the structure and cheese making properties of recombinant camel and bovine chymosins expressed in *A. niger* have very recently been conducted by Jensen et al. (2013). Thermal differential scanning calorimetry studies showed a slightly higher thermal stability of camel chymosin compared with bovine chymosin. Comparison of the crystal structure of a doubly glycosylated variant of camel chymosin (1.6 Å resolution) and the crystal structure of unglycosylated bovine chymosin (1.8 Å resolution) indicated that camel and bovine chymosin share the same overall fold, except for the antiparallel central β-sheet connecting the N-terminal and C-terminal domains. It has been observed that in bovine chymosin the N-terminus forms one of the strands which is lacking in camel chymosin. This difference has been linked to an increase in the flexibility of the relative orientation of the two domains in the camel chymosin. It has been concluded that the improved electrostatic interactions arising from variation in the surface charges and the greater malleability both in domain movements and substrate binding contribute to the good milk-clotting activity of camel chymosin towards bovine milk.

Bansal et al. (2009) compared the properties of Cheddar-type cheeses produced with recombinant camel chymosin and recombinant bovine chymosin. No significant differences were observed in the composition and pH values between the cheeses made with either coagulant. The extent of primary proteolysis was significantly lower in cheeses made with camel chymosin than in cheeses made with bovine chymosin. The cheeses produced with recombinant camel chymosin were characterized by lower flavor intensities, lower smoothness and mouthcoating, and less cohesiveness and adhesiveness than bovine chymosin. It has been concluded that camel chymosin may be suitable for making Cheddar cheese with lower levels of proteolysis but with acceptable flavor.

6.3 Microbial aspartic proteinases

Apart from the production of FPC, different microbial strains have also been efficiently utilized to produce milk-clotting enzymes. Rennets from fungal origin have found wide acceptance in the dairy industry as an alternative to chymosin and FPC. *R. pusillus*, *R. miehei*, and *C. parasitica* are common industrial sources of microbial rennets. However, the first generation fungal rennet formulations were partially appropriate for cheese making due to their excessive proteolytic activity and higher thermostability. The main limitation for the use of first generation rennet formulations from *Rhizomucor* sp. was their high thermal stability which resulted in the inability to inactivate the residual enzyme activity by pasteurization. These enzymes exhibit the highest levels of thermal stability among the aspartic proteinases. Residual proteolytic activity in the pasteurized cheese whey causes undesired proteolysis resulting in loss of protein, lower yield, and off-flavor generation and has thereby hampered the application of unmodified microbial rennet preparations. Therefore, the main application of the first generation rennet formulations from *Rhizomucor* sp. was production of young cheeses. Because of the mentioned limitation, attempts have been made to reduce the thermostability of rennet preparations from *Rhizomucor* sp. by different approaches, e.g., chemical modification(s) and genetic engineering tools. As a first approach, some chemical procedures have been employed to reduce the thermal stability of these enzymes. Havera and Humphreys (1988) reported that a methionine-oxidizing treatment with H_2O_2 in combination with maleic anhydride addition resulted in increased milk-

clotting activity and decreased thermostability for *R. pusillus* rennet. It has also been mentioned that modification of ε-amino groups by treatment with an ethylene/maleic anhydride co-polymer provided a decreased ratio of milk clotting to proteolytic activity and lower thermostability for *R. miehei* rennet (Smith et al. 1991). As a result of these studies, it became also possible to produce excellent ripened cheeses with the modified fungal rennet preparations. At present, almost all commercially available microbial rennets are destabilized using chemical modifications.

As a second approach, genetic engineering tools have been utilized. Yamashita et al. (1994) have generated different mutant forms of *R. pusillus* aspartic proteinase having decreased thermal stability. Mutant genes (Gly186Asp and/or Ala101Thr) have been expressed in *S. cerevisiae*. All the mutations caused a significant decrease in thermal stability of the enzyme. The double mutant showed the lowest thermal stability without any change in the enzymatic activity. In another study, Park et al. (1996) showed that replacement of Tyr75 in the flap by Asn reduced the unspecific proteolytic activity of the same enzyme, leading to a considerable enhancement of the specificity towards Phe–Met bond. In addition, mutation of Glu13Ala of the same gene resulted in a 5-fold increase in the ratio of clotting versus proteolytic activity without any significant loss in milk-clotting activity (Aikawa et al. 1990). Residue Glu13 seemed to play a critical role in forming the correct hydrogen bond network around the active center. From another perspective, the thermal stability of rennets from *Rhizomucor* sp. has been linked to the degree of protein glycosylation. The carbohydrate moieties in glycoproteins protect them from proteolytic attack and may stabilize the protein conformation resulting in high level of thermal stability (Yang et al. 1997). Deglycosylation studies on rennet preparations from *Rhizomucor* sp. by endo-β-*N*-acetylglucosaminidase H and by mutation of asparagine residues within the glycosylation signal sequence revealed that removal of N-linked carbohydrate groups decreased the thermal stability of the enzyme. A significant increase in milk-clotting activity and decrease in general proteolytic activity have also been observed after the removal of carbohydrate moieties (Aikawa et al. 1990).

C. parasitica proteinase has greater proteolytic activity than the rennet preparations from *Rhizomucor* sp. Specificity studies of *C. parasitica* proteinase towards bovine κ-casein showed that the proteinase cleaves the Ser^{104}–Phe^{105} bond, in contrast to the other microbial rennets and chymosin where the Phe^{105}–Met^{106} bond is split. This difference in cleavage site in comparison with other aspartic proteinases does not seem to affect clotting (Egito et al. 2007). *C. parasitica* proteinase is a very thermolabile enzyme therefore generally used in the manufacture of Emmental and Italian style cheeses varieties (Claverie-Martin and Vega-Hernandez 2007).

Apart from the above-mentioned commercially available fungal rennet preparations that have been improved by different approaches, there have been many studies to discover new microbial strains having a higher ratio of milk clotting to proteolytic activity and a lower thermostability. Fraile et al. (1981) have proven that the aspartic proteinases from mesophilic *Mucor* sp. are more thermolabile than those obtained from thermophilic *Rhizomucor* sp. After incubation at 55 °C for 10 min, the aspartic proteinase from *Mucor bacilliformis* exhibited 31% of the initial milk-clotting activity while *R. pusillus* proteinase exhibited 71% of the initial activity. Further studies performed by Yegin et al. (2012) indicated that the enzyme from *M. mucedo* was more sensitive to thermal treatment and the enzyme activity was completely lost after

application of heat at 55 °C for 10 min. Several other reports described the efficient biosynthesis of aspartic proteinases by *Mucor* sp. strains (Fraile et al. 1981; Fernandez-Lahore et al. 1999; Andrade et al. 2002; Machalinski et al. 2006; Yegin et al. 2011). It is still a challenge to discover microbial milk-clotting enzymes that are more thermolabile and having higher milk clotting to proteolysis ratio in order to compete with chymosin. Recently, Yegin et al. (2012) has shown that *M. mucedo* DSM 809 milk-clotting enzyme exhibits interesting technological properties, including a remarkable sensitivity to thermal treatment and a higher milk clotting to total proteolysis ratio which are very important characteristics for cheese industry. Further studies by the same group (Yegin and Fernandez-Lahore 2013) focused on identification of the aspartic proteinase gene of *M. mucedo* DSM 809 and functional expression of the gene in *P. pastoris* X-33 to solve the problems faced due to the fungal morphology during scale up of the fermentation process. This expression system also provided an increase in milk-clotting activity without leading to a change in the degree of thermolability.

Vishwanatha et al. (2010) also reported the production of a milk-clotting enzyme with a high degree of milk clotting to proteolytic activity and low thermal stability on solid state fermentation by *Aspergillus oryzae* MTCC 5341. Biosynthesis of milk-clotting enzymes from bacterial strains has also been reported, mainly from *Bacillus* sp. and others like *Myxococcus* sp. (Poza et al. 2003) and *Nocardiopsis* sp. (Cavalcanti et al. 2005). For example, *Bacillus licheniformis* USC13 has been utilized for the production of milk-clotting enzyme but further inhibition studies revealed that the enzyme was a serine type proteinase (Ageitos et al. 2007). Recently, Ding et al. (2012) also reported production of a milk-clotting enzyme by *Bacillus amyloliquefaciens* JNU002 but it has also been mentioned that the enzyme was not an aspartic type of proteinase. Cheese making industry seeks for novel microbial enzyme sources; however, more studies are needed for elucidation of structural characteristic of these enzymes and their proteolytic patterns during cheese ripening.

6.4 Plant aspartic proteinases

Most of the plant aspartic proteinases belong to the A1 family. They have an extra insertion between the N-terminal and C-terminal domains consisting approximately 100 amino acids which is called as plant-specific insert (PSI). This structural feature is only specific to plant aspartic proteinases within the A1 family (Simões and Faro 2004) and is highly similar to the structures of saposins and saposin-like proteins (Chen et al. 2002). Figure 4 depicts the difference in the primary structure of plant

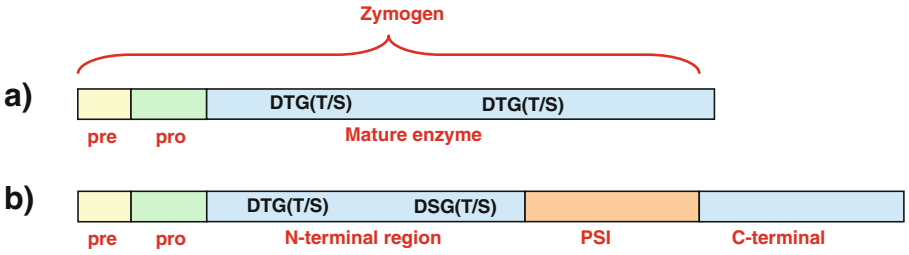

Fig. 4 **a** Schematic representation of the primary structure of mammalian and microbial aspartic proteinase. **b** Schematic representation of the primary structure of plant aspartic proteinase (PSI: plant specific insert)

aspartic proteinases and other aspartic proteinases. The biological function of PSI has not been completely established. The PSI contains six conserved cysteines, several hydrophobic residues, and a consensus glycosylation site. Almost all plant aspartic proteinases identified so far are synthesized as preproenzymes like other aspartic proteinases but the plant aspartic proteinases are subsequently converted to mature two-chain enzymes while animal aspartic proteinases are converted to single-chain enzymes (Costa et al. 2010). In most aspartic proteinases, the catalytic Asp residues are contained in a common Asp–Thr–Gly motif in both lobes of the enzyme, but plant aspartic proteinases contain Asp–Ser–Gly at one of the sites (Mutlu and Gal 1999). At present, the evolutionary and biological importance of this variation in plant enzymes is also not known. Several plant aspartic proteinases have been localized to the vacuoles and there is biochemical evidence that some of them are secreted (Faro et al. 1999).

Plant extracts represent potential as coagulants in the cheese industry. Cheeses made with plant coagulants are mainly found in Portugal, border regions of Spain, and West African countries. In these regions, rennets from plant origin have been used for centuries in the manufacture of raw ovine and/or caprine milk cheeses (Raposo and Domingos 2008). However, their application in cheese making from bovine milk has been mostly unsuccessful due to their excessive proteolytic cleavage patterns (Walstra et al. 1999). The major limitations for the widespread use of plant aspartic proteinases in cheese making are the low yield of the final product, the heterogeneity in proteinase profile of the flowers and the seasonal flowering. Besides, extraction of milk-clotting proteinases from intact plants is labor intensive and therefore expensive. An alternative for the intensive production of these proteinases could be the use of in vitro plant cell/tissue culture for biomass production (Raposo and Domingos 2008). The plant aspartic proteinase cyprosin B has also been successfully expressed in *S. cerevisiae* (Sampaio et al. 2008) in an attempt to overcome the limitations described above.

Milk-clotting enzyme from different plant sources have been reported as indicated in Table 4. Unfortunately, most of these plant rennets have been found to be unsuitable since the cheeses obtained by the use of these coagulants were found to be extremely bitter. On the other hand, several plant aspartic proteinases are used for

Table 4 Examples of milk-clotting aspartic proteinases from plants

Species	Organ source	Reference
Cynara cardunculus	Cell suspension, callus	Cordeiro et al. 1998
Cynara scolymus L.	Flower	Sidrach et al. 2005
Moringa oleifera	Flower	Pontual et al. 2012
Onopordum acanthium L.	Flower	Brutti et al. 2012
Cirsium vulgare	Flower	Lufrano et al. 2012
Bromelia hieronymi	Fruits	Bruno et al. 2010
Centaurea calcitrapa	Cell suspension	Raposo and Domingos 2008
Albizia lebbeck	Seed	Egito et al. 2007
Helianthus annuus	Seed	Egito et al. 2007
Silybum marianum	Flower	Vairo-Cavalli et al. 2005
Oryza sativa	Seed	Asakura et al. 1997

specialized local cheese varieties since broader proteolytic activity is desirable in order to obtain the typical organoleptic characteristics accepted by the consumers of such type of cheeses. For example, the aqueous extracts of *Cynara* sp. 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, and Éora (Roseiro et al. 2003; Egito et al. 2007). It is also important to mention that cheeses made with plant coagulants are produced on an artisanal scale, in a farmhouse or small dairy. Some of them (e.g., Serra cheeses from Portugal) have the Protected Denomination of Origin (PDO) designation (Roseiro et al. 2003).

Since *Cynara* sp. extract has been widely used for centuries for making traditional Portuguese and Spanish ewe's milk cheeses, further investigations have been carried out to elucidate the structural characteristics of aspartic proteinases from *Cynara* sp. Verissimo et al. (1996) isolated two proteinases cardosin A and cardosin B from stigma of the cardoon *Cynara cardunculus* L. Both cardosins were found to be active at low pH utilizing a synthetic peptide. They were inhibited by pepstatin, with inhibition constant values of 3 nM for cardosin A and 1 nM for B, indicating that they are aspartic type proteinases. From analysis of the structural and kinetic properties of these cardosins, it became clear that they are the products of distinct genes which have probably arisen by gene duplication. Cardosin A consists of two subunits with apparent molecular weight of 31 and 15 kDa, whereas cardosin B consists of two subunits with apparent molecular weights of 34 and 14 kDa (Silva and Malcata 1999). Cardosin A has been studied in detail in terms of specificity towards κ-casein and it has been shown that it cleaves the same peptide bond, Phe^{105}–Met^{106}, like chymosin. Cardosin B, in comparison, has been mention to be similar to pepsin, in terms of specificity and activity (Egito et al. 2007). A unique feature of cardosin A, among the other plant aspartic proteinases, is the presence of a functional Arg–Gly–Asp (RGD) sequence which is well known as an integrin-binding sequence. Faro et al. (1999) reported the first evidence for the involvement of a proteinase in RGD-dependent recognition in plants. It has been shown that the RGD motif functions as cell-surface binding receptor. Cardosin B lacks the RGD motif and includes an additional putative N-glycosylation site which is generated by the replacement of an Asp by Asn within this motif. The cDNA-derived amino acid sequence of Cardosin B has 73% similarity with that of cardosin A (Vieira et al. 2001). Other aspartic proteinases have also been found in the flower cells of *C. cardunculus* and *Cynara scolymus* by other authors and named as cyprosins A and B and cynarases A, B, and C (Egito et al. 2007).

Recently, Lufrano et al. (2012) performed a study on cloning, expression, and characterization of a novel aspartic proteinase precursor from the flowers of *Cirsium vulgare*. The isolated cDNA encoded a protein with 509 amino acids which was termed cirsin. The pro-form of cirsin was expressed in *E. coli*. It has been shown that in contrast to several plant aspartic proteinases, the novel protein was active without removal of its pro-segment. This may indicate the existence of structural differences between the plant aspartic proteinases. The recombinant procirsin exhibited typical proteolytic features of aspartic proteinases such as optimum acidic pH, inhibition by pepstatin, and strict dependence on two catalytic Asp residues for activity. Procirsin was also exhibiting milk-clotting activity, suggesting that it might be effective vegetable rennet.

7 Conclusions and future remarks

The knowledge on aspartic proteinases has expanded greatly as a result of recent comparative investigations conducted by utilizing classical mutation, screening technologies, genome shuffling procedures, gene identification, and cloning procedures and other protein engineering tools. However, there are still questions which remain unclear regarding the biosynthesis, correct folding, secretion, mechanism of activation of zymogens. Currently, research on microbial rennets is still being directed towards the discovery of enzymes that are more thermolabile and having higher milk-clotting to proteolysis ratio in order to compete with bovine chymosin: the industrial gold standard for cheese making. It is especially important for cheese industry to study the ways to modulate the specificity towards κ-casein for cheese industry as milk-clotting is a very specific proteolytic activity towards Phe^{105}–Met^{106} bond of κ-casein. By using the knowledge on aspartic proteinase structure and the details on the interaction with different substrates, κ-casein, and other caseins, coagulants may also be fine-tuned for the specific requirements in the production of different cheese varieties. Although the cheese production industry is a conservative market, with the current state of knowledge and technology available on protein modification and application, it should be possible to see the first examples of engineered rennet substitutes other than recombinant chymosin in the market in the near future.

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