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REVIEW

Chymosin and aspartic proteinases

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INTRODUCTION

Chymosin (rennin; EC 3.4.23.4) is a neonatal gastric aspartic proteinase and is of commercial importance in cheese-making. It belongs to the aspartic proteinase family which is widely distributed in many organisms and tissues with different physiological and functional properties. The nucleotide and amino acid sequences and three-dimensional structures of several aspartic proteinases are available and provide the information for protein engineering design of this family. Enzymes can now be produced recombinantly in various expression systems in sufficient amounts for structural and functional studies.

Proteolytic enzymes can be classified on the basis of their catalytic activities into one of four groups: the serine, cysteine, metallo- and aspartic proteinases (Kay, 1985). These enzymes have been isolated from virus, yeast, fungi, higher plants and mammals.

CHYMOSIN AND OTHER ASPARTIC PROTEINASES

Aspartic proteinases contain two aspartyl residues (Asp32 and Asp215, pepsin numbering) at the active site (Tang *et al.*, 1973). They are susceptible to inhibition by pepstatin, a pentapeptide naturally produced by *Streptomyces* strains (Umezawa *et al.*, 1970), and to affinity labeling at the catalytic aspartates either using diazoacetylnorleucinemethyl ester (DAN) in the presence of cupric ions (Rajagopalan *et al.*, 1966) or 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP).

Natural sources

Aspartic proteinases can be found throughout nature from viruses to higher plants and mammals. They are generally divided into two major groups; pepsin-like and retroviral enzymes. These enzymes have been isolated from five major sources:

(a) The stomach. Several types of gastric enzyme, i.e. pepsin A (EC 3.4.23.1), pepsin B (EC 3.4.23.2), gastricsin (EC 3.4.23.3) and chymosin (EC 3.4.23.4), are produced in the mucosa as inactive precursors, zymogens. Pepsin is the predominant proteinase in adult mammals (Tang *et al.*, 1973). Gastricsins are found in all parts of the mammal stomach, α-cells of pancreatic islets, prostate gland and seminal vesicles. Chymosin is produced early during gestation in the mucosa of new-born mammals including calf, piglet (Foltmann *et al.*, 1978), kitten (Jensen *et al.*, 1982), seal (Shamsuzzaman and Haard, 1984) and lamb (Baudys *et al.*, 1988*a*; Pungercar *et al.*, 1991). The production of these enzymes varies depending on age of animals and their feeding regimes.

(b) Lysosomes of many cell types contain cathepsin D and cathepsin E. Cathepsin E is found in gastric mucosa, thymus, spleen and blood cells (Kageyama, 1995). Human cathepsin D is possibly involved in the degradation of intracellular and endocytosed proteins, and is a prognostic indicator of breast tumour invasiveness.

(c) Tissues such as kidney and sub-maxillary gland produce renin (Kay, 1985).

(d) Plants, including squash, cucumber, tomato, barley, rice, wheat, sorghum, and lotus (Doi *et al.*, 1980; Morris *et al.*, 1985; Polanowski *et al.*, 1985; Belozersky *et al.*, 1989).

(e) Microorganisms. Several proteinases are secreted from fungi including the proteinases from *Endothia parasitica* (Sardinas, 1968), *Penicillium janthinellum* (Hofmann and Shaw, 1964), *Mucor pusillus* (Arima *et al.*, 1970), *Mucor miehei* (Sternberg, 1971), *Rhizopus chinensis* (Fumamoto *et al.*, 1967), *Aspergillus awamori* (Ostoslavskaya

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et al., 1986), *Aspergillus niger* (Koaze, *et al.*, 1964), and *Trichoderma reesei* (Pitts, 1992). Yeast proteinases have been found in *Saccharomyces cerevisiae* (MacKay *et al.*, 1988), *Candida tropicalis* (Tongi *et al.*, 1991) and *Yarrowia lipolytica* (Yamada and Ogrydziak, 1983). Thermopsin is secreted from *Sulfolobus acidocaldarius*, a thermophilic archaebacterium (Lin and Tang, 1990).

Retroviral proteinases are dimeric and each monomer is about half the size of a eukaryotic aspartic proteinase and carries only one catalytic aspartic residue. Retropepsins have been found in several viruses including human immunodeficiency virus (HIV), Rous sarcoma virus, avian myeloblastosis virus and simian immunodeficiency virus (SIV) (Toh *et al.*, 1985; Kotler *et al.*, 1989). These proteinases are required for processing for RNA dimerisation within the virion, and hence for infectivity.

PHYSICAL PROPERTIES AND STABILITY

Molecular weights and isoelectric points

Chymosin and aspartic proteinases have molecular weights between 32 and 39 kDa with a multiplicity of isoelectric points corresponding to a number of isozymes, auto-degradation and post-translationally modified products. N-linked glycosylation has been found in several proteinases such as cathepsin D (Asn67 and Asn183), *S. cerevisiae* proteinase A (Asn67 and Asn 263), mucorpepsin (Asn173) and human renin (Asn 67). Specific receptors for phosphorylation have been found in porcine pepsin at Ser68 (Tang *et al.*, 1973), bovine pepsin and human cathepsin D (Martin and Corre, 1984; Metcalf and Fusek, 1993).

Enzyme stability

Chymosin is most stable at pH values between 5.3 and 6.3. However, even at pH 2 chymosin is relatively stable (Foltmann, 1959*a*). Under acidic conditions (pH 3–4) the enzyme loses its activity rapidly, probably caused by auto-degradation, while at alkaline pH values (above 9.8), loss is due to an irreversible conformational change (Cheeseman, 1965). Loss of activity of chymosin A is higher than chymosin B (Foltmann, 1966). Chymosin is more stable at 2°C than at room temperature (Foltmann, 1959*b*). Kawaguchi *et al.* (1987) reported the rapid loss of the activity of chymosin when the temperature is increased from 45 to 55°C. Photo-oxidation of histidine as well as modification of ε-amino groups of lysine slightly affects the activity of chymosin (Hill and Laing, 1965; Smith *et al.*, 1991*b*,*c*). Chymosin loses approximately half of its activity after incubation with 4.6 M urea at 37°C for 30 min (Sugrue *et al.*, 1990). It has been shown that both the pro-part and cysteine residues are essential for refolding of chymosin after denaturation (Sugrue *et al.*, 1990; Huang *et al.*, 1992). Chymosin, in a crystalline form, appears to be very stable (Foltmann, 1992).

Prochymosin is more stable than chymosin at neutral pH (Foltmann, 1966). At pH values below 5.0 prochymosin is converted to chymosin whereas at pH values above 11.0 the stability of prochymosin is lost due to a conformational change. Pseudochymosin is stable at acidic pH for days but is quickly converted to chymosin if the pH is increased above 4.5 (Barkholt *et al.*, 1979).

Mucorpepsin, endothiapepsin and *S. cerevisiae* proteinase A are stable at pH 3.5–7.0 (Sardinas, 1968; Dreyer *et al.*, 1986; Bailey and Siika-aho, 1988). Pepsin shows greater general stability than chymosin; for example, after incubation with 6 M urea at 37°C for 30 min, only 10% of original activity is lost (Cheeseman, 1965). The thermostability of pepsin is decreased in solution at high pH, in the presence of urea or salt, but is increased in the presence of pepstatin (Privalov *et al.*, 1981). At pH 6.0, pepsin is more stable than pepsinogen. At pH values between 8.5 and 10.5, pepsinogen is less stable than prochymosin and cannot be converted to the active form in the acidic environment (McPhile, 1975).

Aspartic proteinases containing carbohydrate are more stable toward high temperature, denaturants and degradation (Aikawa *et al.*, 1990; Berka *et al.*, 1991; Brown and Yada, 1991). Glycosylation of mucorpepsin by both chemical and genetic modifications resulted in the loss of stability and the increase of C/P (milk clotting/proteolysis) ratio (Brown and Yada, 1991; Aikawa *et al.*, 1992). The stability of mucor was decreased by the pretreatment with acid, oxidation of methionine and modification of ε-amino groups of lysine (Hubble and Mann, 1984; Smith *et al.*, 1991*b*).

Enzyme solubility

The solubility of chymosin is affected by pH, temperature and ionic strength of the solution (Foltmann, 1959*b*). Non-crystallised chymosin is soluble in solution containing 1 M NaCl, and at pH 5.5. In a solution of > 2 M NaCl, chymosin appears insoluble. Crystallised chymosin shows higher solubility at 25°C than at 2°C; however, amorphous precipitates of chymosin are more stable at 2°C than at 25°C. At pH values close to the isoelectric point, chymosin is very insoluble at an ionic strength of 0.005; the solubility is increased by increasing ionic strength.

STRUCTURE OF CHYMOSIN AND OTHER ASPARTIC PROTEINASES

Gene sequence and primary structure

The genomic DNAs of avian and mammalian aspartic proteinases, chicken embryonic pepsinogen (Hayashi *et al.*, 1988), human renin (Miyasaki *et al.*, 1984),

bovine chymosin (Hidaka *et al.*, 1986) and human pepsinogen (Sogawa *et al.*, 1983), are comprised of nine exons separated by eight introns and all exon-intron junction points are highly conserved. These results support the belief that these enzyme genes have evolved from a common ancestral gene. Conversely, in several microbial aspartic proteinases including those of *S. cerevisiae*, *C. tropicalis*, *M. pusillus* (Tonouchi *et al.*, 1986) and *M. miehei* (Gray *et al.*, 1986), no intron was found in the primary structure of these enzymes. However, in the genes of *Rhizopus niveus* aspartic proteinase (Horiuchi *et al.*, 1988) and *A. awamori* aspartic proteinase (Berka *et al.*, 1990), one and three short introns were found, respectively, but their exon-intron junctions were at different positions from those in the genes from mammalian and avian aspartic proteinases.

Calf chymosin is found in three major forms: A, B and C, chymosin B being the most abundant. Chymosins A and B differ at only one amino acid position: chymosin A has an Asp residue at position 243 (pepsin numbering), whereas this is a Gly residue in B. Chymosin C appears to be a degradation product of chymosin A that lacks three residues, Asp244–Phe246 (Danley and Geoghegan, 1988). It is likely that chymosin A and chymosin B are synthesised from different alleles of the same polymorphic gene rather than a multiple gene family as only one locus of chymosin gene is found from the hybridisation of the calf genome with a chymosin gene (Donnelly *et al.*, 1986). Figure 1 illustrates the nucleotide and amino acid sequence of calf chymosin B cDNA. The secretion signals of aspartic proteinases are approximately 15–24 residues long with low sequence homology (Fig. 2). These secretion sequences tend to be rich in hydrophobic amino acids.

The known pro-regions of aspartic proteinases are shown in Fig. 3. The pro-peptides are 38–54 amino acid residues in length and rich in basic residues. Although sequence identity is high among the closely related enzymes, there are variables in the cleavage site between the pro-segment and mature enzymes. A lysine residue (Lys36P; pepsinogen numbering) is conserved in all proteinases, except lamb prochymosin and barley aspartic proteinase, and this residue has been postulated to interact with the catalyic aspartate residues in the zymogen molecule (James and Sielecki, 1986; Foltmann, 1988). The pro-segments are probably important for correct folding, targeting and control of the activation of enzyme zymogens (Koelsch *et al.*, 1994).

Chymosin is a single polypeptide chain enzyme of 323 amino acid residues with a low content of basic residues, and rich in dicarboxylic and β-hydroxy amino acid residues (Foltmann *et al.*, 1978, 1979; Moir *et al.*, 1985; Hidaka *et al.*, 1986). The sequence alignment of chymosin with those of lamb chymosin, porcine pepsin, penicillopepsin, rhizopuspepsin and *S. cerevisiae* proteinase A is illustrated in Fig. 4.

There are variable numbers of cystein residues in their sequences but their positions, when present, are conserved. Therefore, there is the potential for two disulfide bridges in the *Rhizomucor* and *Rhizopus* enzymes, a single disulfide bridge in the *Endothia, Pennicillium* and *Aspergillus* enzymes, and no disulfide bridges in the *Irpex* aspartic proteinase.

Secondary structure

The secondary structure of chymosin consists mainly of β-sheet with a few small α-helical segments. The secondary structure of chymosin is illustrated in Fig. 5. The sheets and helices are named by analogy to the scheme adapted for endothiapepsin (Blundell *et al.*, 1985, 1990). The strands are named a_N, b_N, c_N, d_N, a'_N, b'_N, c'_N, d'_N, q_N and r_N in the N-terminal domain and a_C, b_C, c_C, d_C, a'_C, b'_C, c'_C, d'_C, q_C and r_C in the C-terminal domain. The helices are named h_N and h_C in the N- and C-terminal domain, respectively. The antiparallel β-strands form three well-defined sheets (Newman *et al.*, 1991). The sheets, 1_N and 1_C, have formed by 7 or 8 strands in a similar pattern in both lobes and are related by a topological two-fold axis. The b, c, b′, and c′ strands form sheets 2_N and 2_C which occur beneath 1_N and 1_C, respectively. The sheet 3 is formed by six of all antiparallel β-strands: a_N, r_N, q_N, q_C, r_C and a_C. This sheet resides beneath the strands forming the base of the active site cleft. In each lobe, strands labelled a, b, c, d are related to a′, b′, c′, d′ by the intra-lobe diad and these strands are related to their equivalents in the opposite lobe by the inter-lobe diad. The helices h_N, h'_N, h_C, and h'_C occur in topological intra- and inter-domain two-fold symmetry in that they all occur after the d strands. The fifth helix occurs between the c'_N and d'_N strands and the sixth in a large insertion in the C-terminal domain.

Tertiary structure

The three-dimensional structures of several aspartic proteinases have been solved by X-ray crystallography (Fig. 6). These include porcine pepsin (Andreeva *et al.*, 1984; Abad-Zapatero *et al.*, 1990; Cooper *et al.*, 1990; Sielecki *et al.*, 1990), pepsinogen (James and Sielecki, 1986; Hartsuck and Remington, 1988), human renin (Sielecki *et al.*, 1989), endothiapepsin (Blundell *et al.*, 1990), penicillopepsin (James and Sielecki, 1983), rhizopuspepsin and the retroviral proteinases (Lapatto *et al.*, 1989; Miller *et al.*, 1989; Wlodawer *et al.*, 1989). Crystals of chymosin were first obtained by Bunn *et al.* (1971). The space group was either $I222$ or $I2_12_12_1$, with one molecule in the asymmetric unit. The structure of recombinant bovine chymosin has been independently solved and refined at 2.3 Å resolution (Gilliland *et al.*, 1990) and at 2.2 Å resolution (Newman *et al.*, 1991). Crystallographic studies at 2.0 Å resolution have also been performed on a site-specific mutant of chymosin, where Val111 was replaced with Phe, and the structure has been refined to an *R*-factor of 19.5% (Strop *et al.*,

```
CCC AGA TCC AAG ATG AGG TGT CTC GTG GTG CTA CTT GCT GTC TTC GCT CTC TCC CAA GGC GCT
              M   R   C   L   V   V   L   L   A   V   F   A   L   S   Q   G   A
            PP1                                                               P1

GAG ATC ACC AGG ATC CCT CTG TAC AAA GGC AAG TCT CTG AGG AAG GCG CTG AAG GAG CAT GGG
 G   I   T   R   I   P   L   Y   K   G   K   S   L   R   K   A   L   K   E   H   G
                               P10                                   P20

CTT CTG GAG GAC TTC CTG CAG AAA CAG CAG TAT GGC ATC AGC AGC AAG TAC TCC GGC TTC * GGG
 L   L   E   N   F   L   E   K   E   E   Y   G   I   S   S   K   Y   S   G   F     G
                           P30                               P40               1

GAG GTG GCC AGC GTG CCC CTG ACC AAC TAC CTG GAT AGT CAG TAC TTT GGG AAG ATC TAC CTC
 E   V   A   S   V   P   L   T   N   Y   L   D   S   Q   Y   F   G   K   I   Y   L
                               10                                    20

GGG ACC CCG CCC CAG GAG TTC ACC GTG CTG TTT GAC ACT GGC TCC TCT GAC TTC TGG GTA CCC
 G   T   P   P   N   E   F   T   V   L   F   D   T   G   S   S   D   F   W   V   P
                           30                                    40

TCT ATC TAC TGC AAG AGC AAT GCC TGC AAA AAC CAC CAG CGC TTC GAC CCG AGA AAG TCG TCC
 S   I   Y   C   K   S   N   A   C   K   N   H   Q   R   F   D   P   R   K   S   S
                       50                                    60

ACC TTC CAG AAC CTG GGC AAG CCC CTG TCT ATC CAC TAC GGG ACA GGC AGC ATG CAG GGC ATC
 T   F   Q   N   L   G   K   P   L   S   I   H   Y   G   T   G   S   M   Q   G   I
                   70                                    80

CTA GGC TAT GAC ACC GTC ACT GTC TCC AAC ATT GTG GAC ATC CAG CAG ACA GTA GGC CTG AGC
 L   G   Y   D   T   V   T   V   S   N   I   V   D   I   Q   Q   T   V   G   L   S
                   90                                    100

ACC CAG GAG CCC GGG GCA GTC TTC ACC TAT GCC GAA TTC GAC GGG ATC CTG GGG ATG GCC TAC
 T   Q   E   P   G   D   V   F   T   Y   A   E   F   D   G   I   L   G   M   A   Y
               110                                   120

CCC TCG CTC GCC TCA GAG TAC TCG ATA CCC GTG TTT GAC AAC ATG ATG AAC AGG CAC CTG GTG
 P   S   L   A   S   E   Y   S   I   P   V   F   D   N   M   M   N   R   H   L   V
           130                                   140

GCC CAA GAC CTG TTC TCG GTT TAC ATG GAC AGG AAT GGC CAG GAG AGC ATG CTC ACG CTG GGG
 A   Q   D   L   F   S   V   Y   M   D   R   N   G   Q   E   S   M   L   T   L   G
     150                                   160

GCC ATC AAC CCG TCC TAC TAC ACA GGG TCC CTG CAC TGG GTG CCC GTG ACA GTG CAG CAG TAC
 A   I   N   P   S   Y   Y   T   G   S   L   H   W   V   P   V   T   V   Q   Q   Y
170                                   180                                   190

TGG CAG TTC ACT GTG GAC AGT GTC ACC ATC AGC GGT GTG GTT GTG GCC TGT GAG GGT GGC TGT
 W   Q   F   T   V   D   S   V   T   I   S   G   V   V   V   A   C   E   G   G   C
                               200                                   210

CAG GCC ATC TTG GAC ACG GGC ACC TCC AAG CTG GTC GGG CCC AGC AGC GAC ATC CTC AAC ATC
 Q   A   I   L   D   T   G   T   S   K   L   V   G   P   S   S   D   I   L   N   I
                               220                                   230

CAG CAG GCC ATT GGA GCC ACA CAG AAC CAG TAC GGT GAG TTT GAC ATC GAC TGC GAC AAC CTG
 Q   Q   A   I   G   A   T   Q   N   Q   Y   G   E   F   D   I   D   C   D   N   L
                           240                                   250

AGC TAC ATG CCC ACT GTG GTC TTT GAG ATC AAT GGC AAA ATG TAC CCA CTG ACC CCC TCC GCC
 S   Y   M   P   T   V   V   F   E   I   N   G   K   M   Y   P   L   T   P   S   A
                       260                                   270

TAT ACC AGC CAA GAC CAG GGC TTC TGT ACC AGT GGC TTC CAG AGT GAA AAT CAT TCC CAG AAA
 Y   T   S   Q   D   Q   G   F   C   T   S   G   F   Q   S   E   N   H   S   Q   K
                   280                                   290

TGG ATC CTG GGG GAT GTT TTC ATC CGA GAG TAT TAC AGC GTC TTT GAC AGG GCC AAC AAC CTC
 W   I   L   G   D   V   F   I   R   E   Y   Y   S   V   F   D   R   A   N   N   L
               300                                   310

GTG GGG CTG GCC AAA GCC ATC TGA TCACATCGCTGACCA.....
 V   G   L   A   K   A   I
           320         323
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Fig. 1. Nucleotide sequence of calf chymosin B cDNA (adapted from Moir *et al.*, 1982).

Secretion signal peptides		
Fungi:	*A. awamori*[1]	MVVFSKTAALVLGLSSAVSA*A
	A. orezae[2]	MVILSKVAAVAVGLSTVASA*L
	R. miehei[3]	MLFSQITSAILLTAASLSLTTA*R
	R. pusillus[4]	MLFSKISSAILLTAASFALTSA*R
	R. niveus[5]	MKFTLISSCVALAAMTLAVEAA*P
	R. chinensis[6]	MTFTLNSSCIAIAALAVAVNAA*P
Mammal:	Bovine chymosin[7]	MRCTVVLLAVFALSQG*A
	Lamb chymosin[8]	MRCLVVLLAVFALSQG*A
	Porcine pepsin[9]	MKWLLLLSLVVLSEC*L
	Human pepsin[10]	MKWLLLLGLVALSE*C
	Rat pepsin[11]	MKWMVVALLCLPLLEA*S
	Rat cathepsin D[12]	MQTPGVLLLILGLLDASS*S
Yeast:	*S. cerevisiae* (YPA)[13]	MFSLKALLPLALLLVSANQVAA*K
	S. cerevisiae (BAR1)[14]	MSAINHLCLYLILASFAIINTITA*L
	C. tropicalis[15]	MATIFLFTKNVFIA.LA.FA.L
Plant:	Barley APR[16]	MGTRGLALALLAAVLLQTVPAASEA*E

Fig. 2. Alignment of the secretion signal peptides of aspartic proteinases. The junctions between putative signal sequence and proenzyme are indicated by (*) and possible sites are indicated by (•). References: (1) Berka *et al.* (1990); (2) Ward and Kodama (1991); (3) Gray *et al.* (1986) and Boel *et al.* (1986); (4) Tonouchi *et al.* (1986); (5) Chen *et al.* (1991); (6) Horiuchi *et al.* (1988); (7) Harris *et al.* (1982) and Moir *et al.* (1985); (8) Pungercar *et al.* (1991); (9) Lin *et al.* (1989); (10) Hayano *et al.* (1988); (11) Ishihara *et al.* (1989); (12) Birch and Loh (1990); (13) Ammerer *et al.* (1986); (14) Mackay *et al.* (1988); (15) Togni *et al.* (1991); (16) Runeberg-Roos *et al.* (1991). (Adapted from Orprayoon, 1994).

1990). The molecules all display very similar secondary and tertiary structures.

The structure of authentic chymosin B described below was solved by Gilliland *et al.*, (1990) and by Newman *et al.* (1991). The crystals of of chymosin have the space group of 1222 with approximate overall dimensions of 40 × 60 × 65Å (Gilliland *et al.*, 1990). The protein has a bilobal folding pattern formed by the N-terminal and C-terminal domains divided by a deep active-site cleft. A 2.5 Å extended cleft contains the catalytic aspartates and the substrate binding pockets. These two lobes are related by an approximately 2-fold axis which passes between the two catalytic aspartates residue 32 and 215 and forms the approximate intramolecular symmetry (Fig. 7). High symmetry between the N- and the C-lobes is found inside the active site and core of the enzyme (Newman *et al.*, 1991). Intra-domain pseudo-diad axes in the N- and C-domains of chymosin have rotations of 180 and 177°, respectively, with negligible translations (Newman *et al.*, 1991).

There are three disulphide bridges at position 45...50, 206...210 and 249...282. In addition, several ion-pairs are found between Arg59...Asp57, Arg157...Glu308, Arg157...Ile326(COO^-), Arg307...Asp11 and Arg315...Asp138 (Gilliland *et al.*, 1990; Newman *et al.*, 1991). Chymosin also contains a single *cis*-proline, Pro23, on the π-turn connecting strands b_N to c_N (Gilliland *et al.*, 1990; Newman *et al.*, 1991). In mucorpepsin, endothiapepsin and porcine pepsin, a *cis*-proline is found at the identical position to that of chymosin (Blundell *et al.*, 1990; Cooper *et al.*, 1990; Newman *et al.*, 1993) while two residues are found at positions 23 and 324 in rhizopuspepsin and three residues are found at positions 111, 194 and 297 in human renin (Dhanaraj *et al.*, 1992).

Propeptide

Fungi :	
A. awamori APR[1]	APAPRTRKGFTINQIARPANKTRTINLPGMYARS-------LA-KFGGTVPQSVKEA-A*SK
A. oryzae APR[2]	LPTGPSHSPHARRGFTINQITRQTARVGPKTASFPAIYSRALA-KYGGTVPAKLKSAVA*GH
A. miehei APR[3]	RPVSKQSESKDKLLALPLTSVSRKFSQTKFGQQQ-------LAEKLAG-----LKPFSE*AA
M. pusillus APR[4]	RPVSKQSDADDKLLALPLTSVNRKYSQTKHGQQ--------AAEKLGG-----IK-A-F*AE
R. niveus APR[5]	PNGKKINIPLAKNN----SY-KPSA--KNALNKA------LA-KYNRRKVGSGGITTE*AS
R. chinensis APR[6]	PGEKKISIPLAKNP----NY-KPSA--KNAIQKA------IA-KYNKHKINTSTGGIV*AG
Mammals:	
Bovine prochymosin[7]	AEITRIPLYKGKSLRKAL-KEHGLLE-DFLQKQQYG-ISSKYS-------GF*GE
Lamb prochymosin[8]	AEITRIPLYKGKPLRKAL-KERGLLE-DFLQKQQYG-ISSEYS-------GF*GE
Chicken pepsinogen[9]	SIHRVPLKKGKSLRKQL-KDHGLLE-DFLKKHPYN-PASKYHPV------L*TA
Porcine pepsinogen[10]	LVKVPLVRKKSLRQNLIKD-GKLK-DFLKTHKHN-PASKYFPE---AAAL*IG
Human pepsinogen[11]	IMYKVPLIRKKSLRRTL-SERGLLK-DFLKKHNLN-PARKYFPQWE-APTL*VD
Human progastricsin[12]	AVVKVPLKKFKSIRETM-KEKGLLG-EFLRTHKYD-PASKYRFGD-----L*SV
Mouse prorenin[13]	TFSLPTRTATFERIPLKKMPSVREIL-EERG--V-DMIRLSAEWGVFTK----------R*PS
Human prorenin[14]	TFGLPTDTTTFKRIFLKRMPSIRESL-KERG--V-DMARLGPEWSQPMK----------R*LT
Human procathepsin D[15]	SALVRIPLHKFTSIRRTM-SEVGGSVEDLIAK----GPVSKYSQAV-PAVTE*GP
Rat procathepsin D[16]	SALIRIPLRKFTSIRRTM-TEVGGSVGDLI----LKGPITKYSMQSSPRTKE*PV
Yeast:	
S. cerevisiae APR[17]	KVHKAKIYKHELSDEMKEVTFEQHLAHLGQKYLTQFEKANPEVVFSREHPFFTE*GG
C. tropicalis APR[18]	LAFALFAQGLTIPD-----GIEKRTDKVVSLDFTVIRKPFNATAHR---LIQKR*SD
Plant:	
Barley APR[19]	EGLVRIALKKRP-IDRNSRVATGLSGGEEQP---LLSG------AN---PLR*SE

Fig. 3. Alignment of the propetides of aspartic proteinases. The junctions between proenzyme and mature enzyme are indicated by (*). References: (1) Berka *et al.* (1990); (2) Ward and Kodama (1991); (3) Gray *et al.* (1986) and Boel *et al.* (1986); (4) Tonouchi *et al.* (1986); (5) Horiuchi *et al.* (1988); (6) Chen *et al.* (1991); (7) Harris *et al.* (1982) and Moir *et al.* (1985); (8) Pungercar *et al.* (1991); (9) Baudys and Kostka (1983); (10) Lin *et al.* (1989); (11) Sogawa *et al.* (1983); (12) Wong and Tang (1986); (13) Holm *et al.* (1984); (14) Imai *et al.* (1983); (15) Faust *et al.* (1985); (16) Birch and Loh (1990); (17) Ammerer *et al.* (1986); (18) Togni *et al.* (1991); (19) Runeberg-Roos *et al.* (1991). (Adapted from Orprayoon, 1994).

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                    10         20         30         40         50
4CMS    GEVASVPLTNY-LDSQYFGKIYLGTPPNEFTVLFDTGSSDFWVPSIYCKSNAC-KNHQR
4PEP      IGDEPLENY-LDTEYFGTIGIGTPAQDFTVIFDTGSSNLWVPSVYCSSLAC-TNHNL
2APP    AASGVATNTPTANDIEEYIPVTIG--GTTLNLNFDTGSSDLWVFSTELP-ASQQSGHSV
2APR    AGVGTVPMTDYGNDIEYYGQVTIGTPGKKFNLDFDTGSSDLWIASTLCT--NCGSGQTK
4APE    STYSATTTPIDSLDDAYITPVQIGTPAQTLNLDFDTGSSDLWVFSSETTASE-VDGQTI
YPE     GGH-DVPLTNYLNA-QYYTDITLGTPPQNFKVILDTGSSNLWVPSNECGSLAC-FLHSK

              Strand a'          Strand b'           Strand c'
            60         70         80         90         100        110
4CMS    FDPRKSSTFQNL-GKPLSIHYGT-GSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTY
4PEP    FNPQDSSTYQST-SGELSITYGT-GSMTGILGYDTVQVGGISDTNQIFGLSETEPGSFLYY
2APP    YNPSA--TGKELSGYTWSISYGDGSSASGNVFTDSVTVGGVTAHGQAVQAAQQISAQFQQD
2APR    YDPNQSSTYQAD-GRTWSISYGDGSSASGILAKDNVNLGGLLIKGQTIELAKREAASFASG
4APE    YTPSKSTSTKLLSGATWSISYGDGSSSSSDVYTDTVSVGGLTVTGQAVESAKKVSSSFTED
YPE     YDHEASSSYKAN-GTEFAIQYGTG-SLEGYISQDTLSIGDLTIPKQDFAEATSEPGLTFAF

          Strand d'                          Strand q              Strand r
            120        130          140        150          160
4CMS    AEFDGILGMAYPSLASE---YSIPVFDNMMNRHLVAQDLFSVYMDRNG----QESMLTLG
4PEP    APFDGILGLAYPSISAS---GATPVFDNLWDQGLVSQDLFSVYLSSNG---DSGSVVLLG
2APP    TNNDGLLGLAFSSINTVQPQSQTTFFDTVKSS-L-AQPLFAVALKHQ-----QPGVYDFG
2APR    -PNDGLLGLGFDTITTVR--GVKTPMDNLISQGLISRPIFGVYLGKAKN--GGGGEYIFG
4APE    STIDGLLGLAFSTLNTVSPTSQQTFFDNAKA-S-LDSPVFTADLGY-----HAPGTYNFG
YPA     GKFDGILGLGYDTISVD---KVVPPFYNAIQQDLLDEKRFAFYLGDTSKDTENGGEATFG

              Strand a           Strand b      Strand c      Strand d
          170        180        190        200        210        220
4CMS    AIDPSYYTGSLHWVPVTV-QQYWQFTVDSVTISGVVVACEGGCQAILDTGTSKLVGPSSD
4PEP    GIDSSYYTGSLNWVPVSV-EGYWQITLDSITMDGETIACSGGCQAIVDTGTSLLTGPTSA
2APP    FIDSSKYTGSLTYTGVDNSQGFWSFNVDSYTAGSQ-SG-DG-FSGIADTGTTLLLDDSVV
2APR    GYDSTKFKGSLTTVPIDNSRGWWGITVDRATVGTSTVA-SS-FDGILDTGTTLLILPNNI
4APE    FIDTTAYTGGITYTAVSTLQHFWEWTSTGYAVGSGTFKSTS-IDGIADTGTTLLYLPATV
YPA     GIDESKFKGDITWLPVRRK-AYWEVKFEGIGLGDEYAELES-HGAAIDTGTSLITLPSGL

        Strand a'          Strand b'               Strand c'
          230        240        250        260        270          280
4CMS    ILNIQQAI-GATQNQ-YGEFDIDCDNLSYMPTVVFEINGKMYPLTPSAYTSQD---QGFC
4PEP    IANIQADI-GASENS-DGEMVISCSSIDSLPDIVFTIDGVQYPLSPSAYILQD---DDSC
2APP    VSQYYSQVSGAQQDSNAGGYVFDCST--NLPDFSVSISGYTATVPGSLINYGPSGDGSTC
2APR    AASVARAY-GASDNS-DGTYTISCDT-SAFKPLVFSINGASFQVSPDSLVFEEF--QGQC
4APE    VSAYWAQVSGAKSSSS-VGYVFPCSAT--LPSFTFGVGSARIVIPGDYIDFGPISTGSSC
YPA     AEMINAEI-GAKKGW-TGQYTLDCNTRDNLPDLIFNFNGYNFTIGPYDYTLEV---SGSC

        Strand d'          Strand q       Strand r
              290        300        310        320
4CMS    TSGFQSENHS----QKWILGDVFIREYYSVFDRANNLVGLAKAI
4PEP    TSGFEGMDVPTSSGELWILGDVFIRQYYTVFDRANNKVGLAPVA
2APP    LGGIQSNSGI----GFSIFGDIFLKSQYVVFDSDGPQLGFAPQA
2APR    IAGFGYG-NW----GFAIIGDTFLKNNYVVFNQGVPEVQIAPVAE
4APE    FGGIQSSAGIG----INIFGDVALKAAFVVFNGATTPTLGFASK
YPA     ISAITPMDFPEPVGPLAIVGAFLRKYYSIYDLGNNAVGLAKAI
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Fig. 4. The sequence of alignment of calf chymosin (4VMS, Newman *et al.*, 1991) with other aspartic proteinases based on three-dimensional structures. References: 4PEP: porcine pepsin (Sielecki *et al.*, 1990); 2APP: penicillopepsin (James and Sielecki, 1983); 2 APR: rhizopuspepsin (Suguna *et al.*, 1987*a*); 4APE: endothispepsin (Pearl and Blundell, 1984); YPA: *S. cerevisiae* proteinase A (Dreyer *et al.*, 1986). (Adopted from Orprayoon, 1994).

The active-site of aspartic proteinases is highly conserved and consists of residues Asp-Thr-Gly from each domain. Nine percent sequence identity is observed among the N- and C-terminal lobes of chymosin (Newman *et al.*, 1991).

A comparison of chymosin structure with other aspartic proteinases reveals a high degree of structural homology (Gilliand *et al.*, 1990). Chymosin has the closest structure to porcine pepsin. Of the fungal proteinase structures, compared to chymosin, the rhizopuspepsin molecule has higher structural homology than penicillopepsin and endothiapepsin.

Structural superposition of aspartic proteinases reveals that the N-terminal domain has greater structural similarity than the C-terminal domain (Gilliland *et al.*, 1990). The C-terminal domain is more separated from the rest of the molecule than the N-terminal domain and the rigid body movement appears in the C-terminal domain (residues 190–302) (Sali *et al.*, 1992). The greatest differences among these proteinases are in the surface loop regions. One remarkable difference is the position of the flap (residues 73–85 in chymosin). This region participates in the substrate binding specificity. In chymosin, the position of Tyr77 is stabilised by

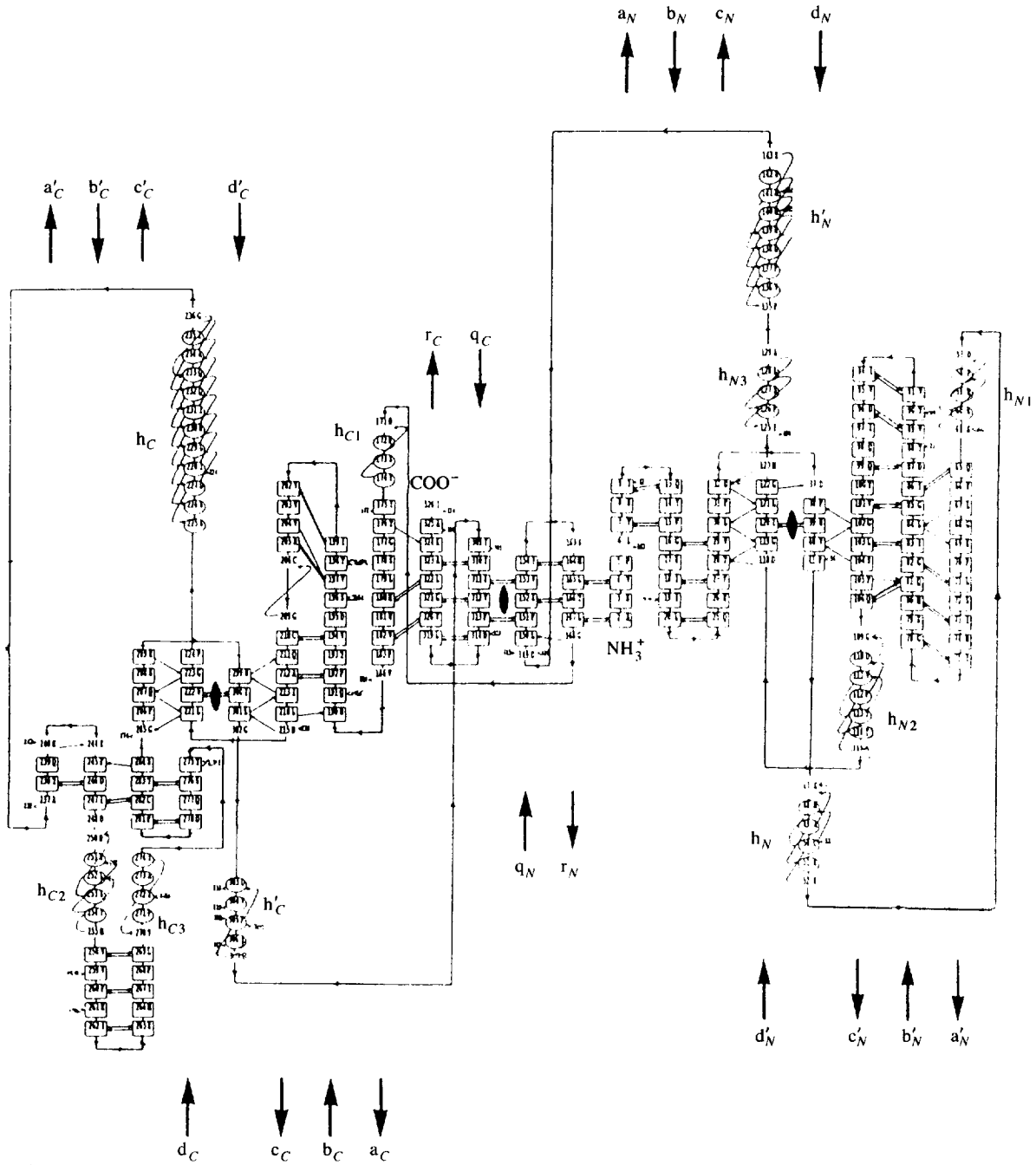


Fig. 5. A schematic diagram of secondary structure of chymosin. The directions of the strands are indicated by the large arrows. The inter- and intra-lobe 2-fold axes are shown as large and small diad markers. The main hydrogen bonds are indicated by arrows in the direction of donor to acceptor. (From Newman *et al.*, 1991)

the interaction with hydrophobic residues Phe119 and Leu32 (Gilliland *et al.*, 1990). In other aspartic proteinases, Tyr77 hydrogen bonds to NE1 of Trp39. In pepsin, the location of the hydroxyl group of Tyr77 is occupied by water molecule w424 in the chymosin crystal. This water molecule forms two hydrogen bonds with the hydroxyl group of Tyr75 and with the conserved water molecule, W403. In the Val111Phe mutant chymosin, the flap appears to occupy two different conformations corresponding to those found in native chymosin and pepsin (Strop *et al.*, 1990). This suggests that chymosin can exist in two alternative structural forms: the active form in which S1 and S3 binding pockets are free for a substrate binding and the self-inhibited form in which these pockets are occluded by its own Tyr77 residue (Andreeva *et al.*, 1992; Gustchina *et al.*, 1996). The conversion of chymosin from the self-inhibited to the active form can be promoted by an allosteric activator, the histidine-proline cluster (-His-Pro-His-Pro-His-) of κ-casein, thereby explaining the catalytic specificity of chymosin toward κ-casein.

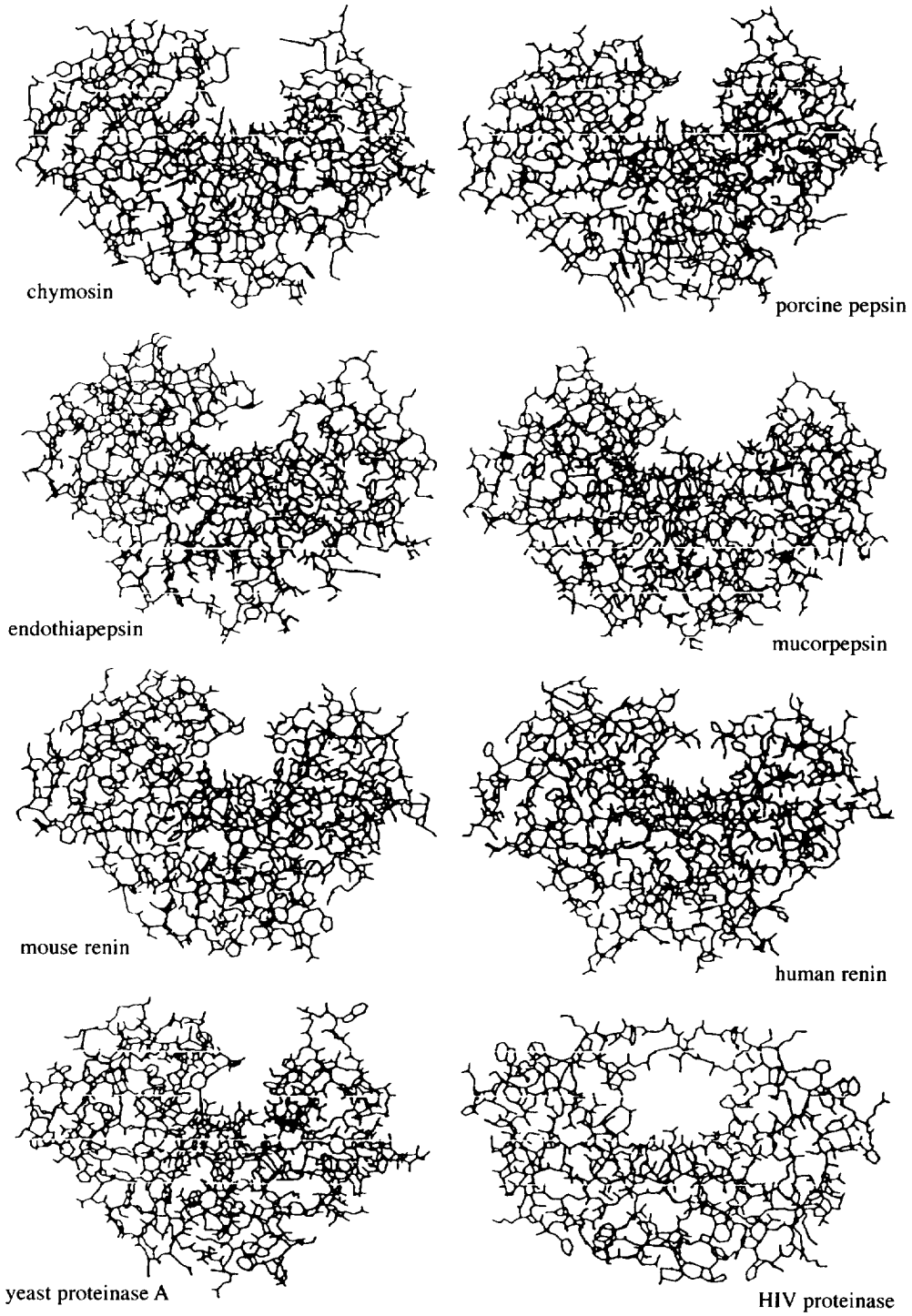


Fig. 6. Three-dimension structures of aspartic proteinases showing the high degree of structure homology among these proteinases. (From Pitts *et al.*, 1992).

Three-dimensional structures of homodimer retroviral proteinases are to a large extent similar and bear close resemblance to the structure of bilobal fungal and mammalian aspartic proteinases (Lapatto *et al.*, 1989; Miller *et al.*, 1989; Navia *et al.*, 1989; Wlodawer *et al.*, 1989). The structural superpositions between the retroviral enzymes and eukaryotic aspartic proteinases appear to be similar. It is not clear whether the eukaryotic proteinases are derived from a homodimer enzyme by gene duplication and fusion (Tang *et al.*, 1978) or evolved from a cellular gene by one or more deletion events (Rao *et al.*, 1991). Nervertheless, an engineered homodimer of the pepsin N-terminal lobe, which exhibits a general proteolytic activity, reveals the close relationship between these two aspartic proteinase families (Lin *et al.*, 1992).

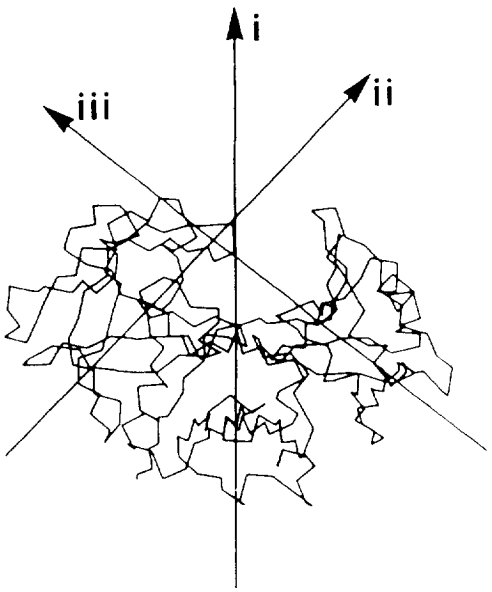


Fig. 7. A plot of the C^{α} position of chymosin. The approximate molecular symmetry axes are shown as following (i) the inter-lobe non-crystallographic 2-fold screw axis relating the N- and C-terminal lobes; (ii) the intra-domain axis for the N-terminal domain; (iii) the intra-domain axis for the C-terminal domain. (From Newman *et al.*, 1991).

THE ACTIVE SITE

The active-site aspartates, Asp32 and Asp215, are situated on the corners of the two extended loops (ψ-structures within sheets $c_N d_N$ and $c_C d_C$) in the N- and C-terminal domains. The side chain of these two aspartates are oriented toward each other around the pseudo-interlobe diad axis in a complicated hydrogen-bonding network known as the 'fireman's grip' (Pearl and Blundell, 1984) shown in Fig. 8. This network is formed by the interaction of two loops (residues 31–35 and residues 214–218) and a central water molecule. The side chain of Thr33 and its symmetry-related Thr126 form hydrogen bonds across the dyad axis; to the carbonyl oxygens of Leu214 and Phe31, respectively, and to the peptide N atoms of Thr216 and Thr33, respectively. The carboxyl oxygens of Asp32 and Asp215 are hydrogen-bonded with nitrogen atoms of the conserved Gly34 and Gly217, respectively. In addition, the side chains of Ser35 and Thr218 also form hydrogen-bonds with the outer oxygen atoms of Asp32 and Asp215, respectively. There are some conserved Gly residues in eukaryotic aspartic proteinases which are believed to be important; among them, Gly34 and Gly217 are conserved in all aspartic proteinases. Side-chains in these positions would interfere sterically with the catalytic aspartates. Residue Asp303 is conserved among all proteinases with an acidic pH optimum. However, in renins which have more neutral pH optima, this residue is replaced by an alanine. The effect of the side-chain at this position on the pK_a has been revealed by site-directed mutagenesis in renin (Yamauchi *et al.*, 1988) and in chymosin (Mantafounis and Pitts, 1990). The hydrogen bond between Asp303 and Thr216 may affect the pK_a of Asp215 via the peptide dipole of Thr216-Gly217 (Pearl and Blundell, 1984).

CATALYTIC MECHANISMS

The catalytic mechanism of aspartic proteinases has been modelled on the structural analysis of several aspartic proteinase-inhibitor complexes. Early mechanisms (James *et al.*, 1977, 1982; James and Sielecki, 1985) proposed that the catalysis was initiated by protonation of the carbonyl oxygen of the substrate by a proton from Asp215, followed by the nucleophilic attack on the carbonyl carbon of substrate of Asp by hydroxide ion generated from water after donation of its proton to Asp32. These protonation events led to the formation of the tetrahedral intermediate. The breakdown of the intermediate was generated by protonation of the nitrogen atom, either from bulk solvent or from the

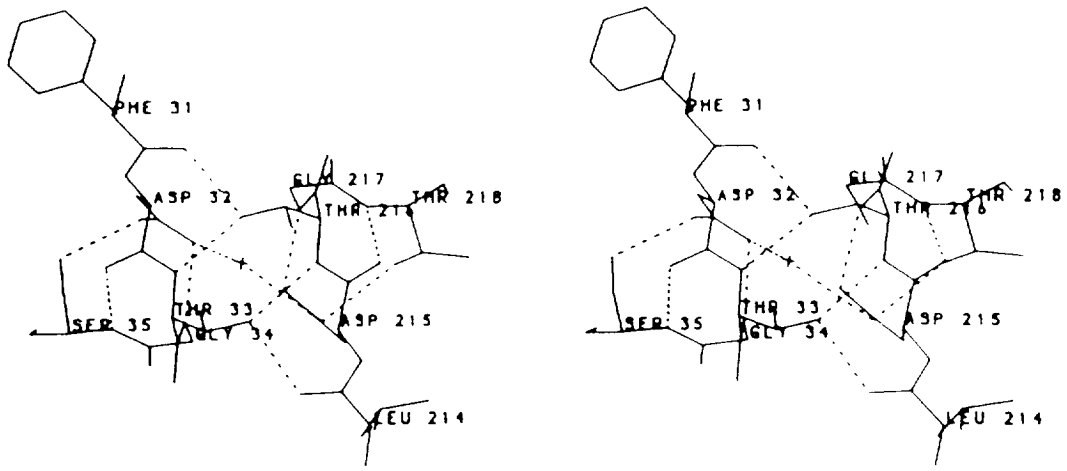


Fig. 8. The 'fireman's grip' at the active-side of chymosin. Hydrogen bonds (broken lines) involved are Thr216 N...Thr33 $O^{\gamma 1}$(2.8 Å), Thr33 $O^{\gamma 1}$...Lys214 O (2.7 Å), Thr33 N...Thr216 $O^{\gamma 1}$ (2.9 Å) and Thr216 $O^{\gamma 1}$...Phe31 O (2.8 Å). Other hydrogen bonds contributing to the stability of Asp32 and Asp215 are also shown. (From Newman *et al.*, 1991).

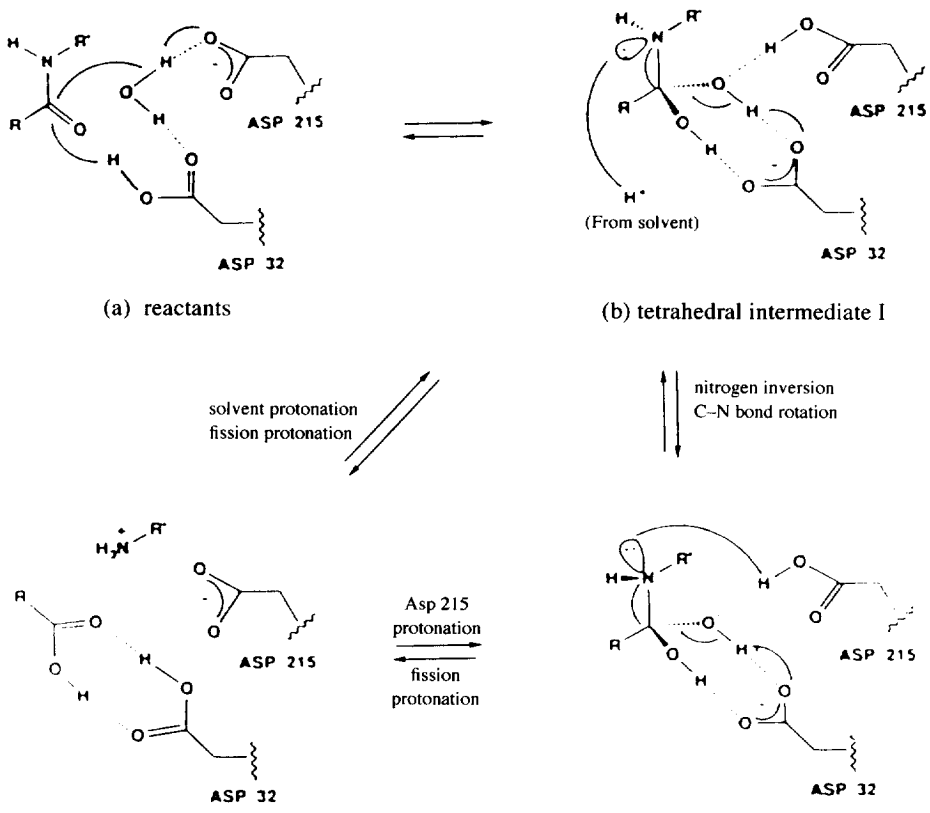
catalytic carboxyl group of Asp215. The protonation of substrate carbonyl and nucleophilic attack may appear simultaneously during the formation of the tetrahedral intermediate. Similarly, proton transfer from the intermediate to the diad may occur at the same time as the protonation of the nitrogen atom of substrate during the cleavage of the resultant intermediate (Polgár, 1987). Pearl (1987) suggested that the distortion of the scissile bond toward the enzyme–substrate binding may facilitate the collapse of the intermediate by generating lone pair orbitals antiperiplanar to the C–N bond but not to the hydroxyl C–O bond. Therefore, the leaving product is free amine rather than the original nucleophile. In addition, the charged oxygen of a solvent molecule forms hydrogen bonds with residues Asp32 and Ser35 or residues Gly 76, Asp77 or Tyr75 on the flap (Blundell *et al.*, 1987; Pearl, 1987).

Veerapandian *et al.* (1990) has proposed a catalytic mechanistic model as outlined in Fig. 9. The pro-R(statine-like) hydroxyl of the tetrahedral carbonyl hydrate is hydrogen-bonded to the outer oxygen of Asp32 and Asp215. The second hydroxyl oxygen of the hydrate is hydrogen-bonded only to the carboxyl oxygen of Asp32. The scissile bond carbonyl is protoned by Asp32 and concurrently attacked by a water molecule polarised into a nucleophilic state by Asp215. The rigid movement in the enzyme–substrate complex may impel distortion of the amide bond and facilitate the attack of nucleophilic water on the polarised carbonyl. Thus, in the tetrahedral intermediate I, the negatively charged Asp31 is stabilised by extensive hydrogen bonding. The amide nitrogen will have been pyramidalised with the new arrangement favouring protonation. A proton can be transferred from bulk solvent or from Asp215. A similar mechanistic proposal have been described by James *et al.* (1992).

Pepsin and chymosin have been shown to catalyse peptide synthesis (Fruton, 1982 Abdel Malak, 1992). Formation of peptide is catalysed by chymosin optimally at pH 4-5 which is similar for peptide hydrolysis (Abdel Malak, 1992). The pH optimum for the peptide synthesis catalysed by pepsin is different from those for peptide hydrolysis. The activity of the enzyme is sensitive to the amino acid residues flanking the bond to be formed or hydrolysed as well as to the nature of adjacent amino acid residues.

ZYMOGEN ACTIVATION

The structure of porcine pepsinogen has been refined at high-resolution (James and Sielecki, 1986; Sielecki *et al.*, 1991; Hartsuck *et al.*, 1992). Structural comparisons between pepsin and pepsinogen suggest that the enzyme and proenzyme structures are very similar. Most of the differences occur in the proximity of the cleft which, in



(a) reactants

(b) tetrahedral intermediate I

(d) products

(c) tetrahedral intermediate II

Fig. 9. A proposed catalytic mechanism for aspartic proteinase by Veerapandian *et al.* (1990).

pepsinogen, is covered and filled by the propart (1P-44P) and the first 13 residues of pepsin. The extension of 13 residues adopts completely different conformations in the active and zymogen forms (James and Sielecki, 1986).

The secondary structure of the zymogen consists mainly of β-sheet, with an approximate 2-fold axis of symmetry (James and Sielecki, 1986). The activation peptide packs into the active site cleft, and the N-terminus (2P-9P) occupies the position of the mature N-terminus (2–9) since the first 10 amino acids of the pro-part form β-strand a_N of pepsinogen. Therefore, changes upon activation include excision of the activation peptide and proper relocation of the mature N-terminus. At neutral or alkaline pH, the pro-segment of pepsin binds and is stabilised across the active site between the two lobes by electrostatic, hydrogen-bonding and hydrophobic interactions which contribute to the binding between the pro-segment and the rest of the protein (Sielecki *et al.*, 1991). Lowering of pH protonates acidic residues on the mature enzyme portion of the molecule, thereby disrupting favourable electrostatic interactions with positively charged amino acid residues on the pro-segment. Subsequent conformational changes of the zymogen lead to intramolecular proteolytic cleavages that liberate the pro-segment from the zymogen (McPhile, 1972).

The mechanisms of activation of aspartic proteinase zymogens are different and depend on the pH. At pH below 2.5, conversion of pepsinogen is primarily by an intramolecular mechanism. The propeptide is cleaved monomolecularly at positions Met16P-Glu17P, giving active pseudo-enzyme. The pseudo-enzyme is enzymatically active and may form a complex with the released pro-segment. At pH values below 4.0, the leu 44P-Ile1 bond is unsusceptible to proteolytic cleavage but becomes susceptible at higher pH. At low pH, the cleavage sites differ among the aspartic proteinases; Phe27P-Leu28P for calf prochymosin, human progastricsin and chicken pepsinogen, Met16P-Glu17P for porcine pepsinogen B and Leu26P-Ile27P for procathepsin D (Barkholt and Foltmann, 1975; Barkholt *et al.*, 1979; Turk *et al.*, 1985; Foltmann, 1993; Larsen *et al.*, 1993). The removal of entire pro-peptide predominantly occurs at pH 3–4 through an intermolecular mechanism. It has been suggested that the cleavage of the Phe42P-Gly1 bond of prochymosin is more active at pH 2 than at pH 4.5 (Barkholt *et al.*, 1979). The recombinant rhizopuspepsinogen produced in *E. coli* can convert to the active rhizopuspepsin in the acidic medium by a mechanism similar to pepsinogen (Chen *et al.*, 1991). The pseudo-rhizopuspepsin and rhizopuspepsin were generated by the cleavage at Asn38P-Thr39P and Val45P-Ala1, respectively. Moore *et al.* (1995) have studied the crystal and molecular-structures of human progastricsin at 1.62 Å resolution and suggest that human progastricsin has a conformational structure and mechanism of activation analogous to those of pepsinogen.

Site-directed mutagenesis at the two sites for autoproteolysis of prochymosin suggests that these processing sites can function independently of one another (McCaman and Cummings, 1988). The changing of prochymosin sequence from Phe27P-Leu28P-Gln29P-Lys30P-Gln31P to Phe27P-Pro28P-Arg29P-Gln30P resulted in the partially activated form of zymogen at pH 2 but lack of proteolysis was found while, at pH 4.5, normal activation processing and proteolytic processing occurred. Conversely, when the seven residues including the processing site at pH 4.5 were removed, a new cleavage site (Ser37P-Val38P) was generated at pH 4.5 while the processing site at pH 2 was not affected (McCaman and Cummings, 1988).

The activation reactions are dependent on pH, salt concentration, and temperature. At pH 5 and room temperature the activation is completed in 2 or 3 days (Rand and Ernstrom, 1964), while at pH 2, room temperature, and ionic strength of 0.1, the activation is completed in 5–10 min (Foltmann, 1962). However, autoproteolysis alone may not be able to generate the mature form of the enzymes as shown in procathepsin D, which cannot autoactivate to the mature enzyme at acid pH (Larsen *et al.*, 1993). Prochymosin is also activated by proteolytic enzymes including plasmin, *Legionella pneumophila* metalloproteinase and *Aspergillus oryzae* thermolysin (Stepanov *et al.*, 1990).

Alpha B crystallin can form a complex with prochymosin; after activation, once chymosin is recovered without bound alpha crystallin, which itself acts as a chaperone-like protein (Plater *et al.*, 1996; Chitpinityol, 1996).

SUBSTRATE-BINDING POCKETS AND SPECIFICITY

High concentrations of NaCl or $(NH_4)_2SO_4$ raised the hydrolytic activity of pepsin and retroviral proteinases in addition to broadening their specificities (Kotler *et al.*, 1989; Tropea *et al.*, 1992). Aspartic proteinases have extended sites of substrate-binding pockets that can accommodate at least seven amino acid residues. Detailed structural studies of aspartic proteinase-inhibitor complexes have been used to identify the amino acid residues in each subsite (Bott *et al.*, 1982; Andreeva *et al.*, 1984; James *et al.*, 1985; Blundell *et al.*, 1987; Cooper *et al.*, 1987; Foundling *et al.*, 1987; James and Sielecki, 1987; Suguna *et al.*, 1987*a*). In chymosin, the subsites S_1 and S_1' are shallow pockets within the active site cleft. The S_1 subsite (for Phe105 binding) is greater specificity than S_1' and is blocked by Tyr75 (Gililland *et al.*, 1990). Therefore, a significant movement of the flap is essential to allow the binding of substate. The S_1 is quite hydrophobic compared to S_1' in which an additional charged residue Glu290 is near to the κ-casein Met106 site chain. The S2 pocket is of low specificity and allows the peptide side chains to adopt a range of

Table 1. The substrate-binding pockets of chymosin. Chymosin residues involved in the interactions with the corresponding residues of the substrate are shown together with a sequence identical to that of the κ-casein cleavage site. (From Gilliland *et al.*, 1990; Newman *et al.*, 1991)

Subsite	κ-Casein residues	Chymosin residues
S_4	His102	Ser219, Lys220, Gln288
S_3	Leu103	Ser12, Gln13, Tyr75, Phe117, Gly217, Thr218, Ser219
S_2	Ser104	Gly76, Thr77, Gly217, Thr218, Lys220
S_1	Phe105	Leu30, Asp32, Gly34, Tyr75, Gly76, Phe117, Ile120, Asp215, Gly217, Thr219
S_1'	Met106	Gly34, Tyr189, Asp215, Thr218, Glu289, Ile301
S_2'	Ala107	Gly34, Ser35, Tyr189
S_3'	Ile108	Tyr189

conformations whereas at subsites S_1 and S_3, the conformations of the side chain are strongly restricted (Dhanaraj *et al.*, 1992) The specificity pockets for chymosin to the κ-casein cleavage site is shown in Table 1. There are two differences in the S_1 subsites of chymosin and the fungal proteinases that promote more hydrophobic S1 subsites (Gilliland *et al.*, 1990). The first one is the position of the flap region which is due to the reorientation of Tyr75 and a deletion of one amino acid residue in this loop. Another difference is the substitution of Leu30 in chymosin for the Asp30 and Asn30 of the rhizopuspepsin and penicillopepsin, respectively. In human cathepsin E, the important specificity-determining interactions are found in the S_3 (Glu13) and S_2 (Thr222, Glu287, Leu289, Ile300) subsites (Raonaik *et al.*, 1995). Figure 10 summarises the results of cleavage of the B chain of oxidised insulin by chymosin and some related acid proteinases. In chymosin, the S_1 subsite has favourable interactions with aromatic amino acids at P_1 whereas the S_1' is less specific (Bang-Jensen *et al.*, 1964; Foltmann, 1964; Guillou *et al.*, 1991; Nedjar *et al.*, 1991).

The fungal and yeast proteinases have an S_1 subsite with a deeper pocket and broader specificity. Therefore, the S_1 pocket can accomodate lysine as well as hydrophobic residues at P_1 (Oka *et al.*, 1973; Hofmann *et al.*, 1984; Newman *et al.*, 1993). However, in mucorpepsin, specificity for Lysine at P_1 was not observed due to the absence of polar residues at positions 30 and 111. In retroviral aspartic proteinases, the primary specificity for HIV-1 and HIV-2 aspartic proteinases at P1 are Leu, Met, Tyr and Phe and at P_1' are Pro, Met, Phe and Ala (Poorman *et al.*, 1991).

Among the isozymes of chymosin, Chymosin A has a significantly higher specific activity which may be the result of the enhanced binding affinity of κ-casein through, possibly, the stronger electrostatic interactions between the substrate and chymosin A. In addition, these two isozymes have different pH optima, 4.2 and 3.7 for chymosin A and chymosin B, respectively. These different values may be the result of an extensive hydrogen-bonding network nearby the two catalytic aspartates. The optimum pH for proteolysis of aspartic proteinases is dependent on the species of enzymes and the substrate used (Table 2). HIV-1 proteinase and renin have high pH optima among aspartic proteinases. The residues Ser35, Thr218 and Asp303 have been postulated to play a role in the pH profile of aspartic proteinases. *In vitro* mutagenesis of Ala35Ser of HIV-1 proteinase (Ala28Ser in HIV-1 numbering) showed a lowering of pK_{a2} by 1.2 units but no effect was found in the pK_{a1} value (Ido *et al.*, 1991). In contrast, mutations of Ser35Ala in porcine pepsin resulted in the lowering of pK_{a1} and pK_{a2} but the raising of both values in rhizopuspepsin. Site-directed mutagenesis of Thr218 to Ala in porcine pepsin, chymosin and rhizopuspepsin resulted in a shift of pH optimum by 0.2–0.5 units (Mantafounis and Pitts, 1990; Tang *et al.*, 1992). Mutation of Ala303 to Asp in renin lowered the pH optimum by 0.5 units (Yamauchi *et al.*, 1988). Similarly, the replacing of Asp303 to Ala in chymosin resulted in raising of the optimum pH by 0.6 units (Mantafounis and Pitts, 1990). The double mutations Thr218ala/Asp303Ala in chymosin effected the pH optimum similarly to that of Asp303Ala mutagenesis (Pitts *et al.*, 1993).

The substrate specificities of aspartic proteinases are affected by the operating pH and the presence of salts (Kotler *et al.*, 1989; Athauda *et al.*, 1991; Tropea *et al.*,

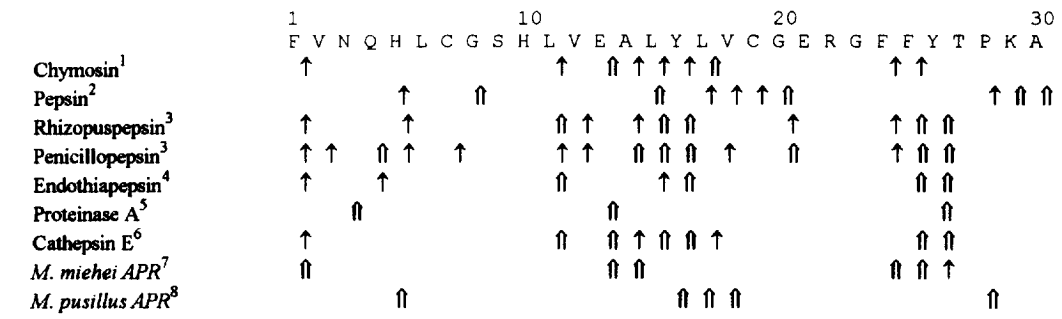


Fig. 10. Comparison of the cleveage specificity of chymosin toward the B-chain of oxidised insulin with those of some aspartic proteinases. References : (1) Foltman, 1964; (2) Sanger and Tuppy, 1981; (3) Oka *et al.*, 1973; (4) Williams *et al.*, 1972; (5) Takahashi, 1995; (6) Athauda *et al.*, 1991 (pH 3.0); (7) Rickert, 1971; (8) McCullough and Whitaker, 1971. Legends: (⇑) main cleavage site and (↑) other sites of actions.

Table 2. pH optima for general proteolysis by chymosin and other aspartic proteinases

Enzymes	Substrates	Optimum pH	References
Chymosin	Acid-denatured haemoglobin	3.7	Berridge (1945); Fish (1957)
	Bovine serum albumin	3.4	Foltmann (1959*a*)
	Oxidised B-chain of insulin	3.5	Fish (1957)
	α-,β-caseins	4.5	Lindqvist and Storgard (1960)
	Milk clotting activity	6–6.3	Okigbo *et al.* (1985*a*)
	Synthetic peptides	4.7	Raymond *et al.* (1972)
	κ-casein	5.5	van Hooydonk *et al.* (1984)
	H-P-H-P-H-L-S-F-M-A-I-P-P-K-K	5.4	Visser *et al.* (1976, 1987)
Pepsin	Oxidised B-chain of insulin	2	Fish (1957)
Penicillopepsin	Trypsinogen	3–4	Hofmann and Shaw (1964)
Endothiapepsin	Haemoglobin	2–2.5	Williams *et al.* (1972)
Mucorpepsin	Haemoglobin	4.0	Arima *et al.* (1970)
	κ-casein	4.5	Arima *et al.* (1970)
	Milk clotting	5.5	Arima *et al.* (1970)
	Hammerten casein	3.5	Arima *et al.* (1970)
	z-Phe-Leu-Ala-Ala	3-4	Oka *et al.* (1973)
S. cerevisiae proteinase A	Acid-denatured haemoglobin	3.2	Dreyer *et al.* (1986)
A. niger proteinase A	Haemoglobin	1.1	Takahashi (1995)

1992). The pH dependence of hydrolysis using synthetic substrates demonstrates that secondary specificities occur in subsite S3 of mammalian aspartic proteinase whereas less specificity is found in microbial proteinases (Dunn *et al.*, 1986). In chymosin, Ile or Val are favoured at P3 and Tyr, Val or Ser at P2 (Guillou *et al.*, 1991). The favourable interaction between Lys220 (NH_3^+) of chymosin and Glu (COO^-) in P2 of the substrate is suggested to cause the pH effects on the hydrolysis (Dunn *et al.*, 1987). The specificity at P2 toward both Lys220 and Gln 288 has been determined by *in vitro* mutagenesis (Suzuki *et al.*, 1990; Quinn *et al.*, 1991).

We have studied the effect of replacing Thr77 of chymosin to Asp (mutant T77D), as well as the addition of two residues (-His-Gly) (mutant PC+2) to the C-terminus of the protein, on the activity of the enzyme using a synthetic hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe as substrate (Chitpinityol, 1996; Chitpinityol *et al.*, 1996). For the recombinant wild type, the pH optimum was pH 3.7, similar to that reported for the authentic chymosin B using the same substrate (Martin *et al.*, 1980). The PC+2 mutant exhibited a similar optimum pH to the native enzyme although the k_{cat}/Km was lowered from 49.7 m^{-1} s^{-1} in the native enzyme to 3.8 m^{-1} s^{-1}. The optimum pH of T77D mutant chymosin was shifted toward neutrality by 1 pH unit to pH 4.7 from 3.7. The optimum temperature of activity of the T77D mutant was increased relative to the wild type enzyme, from approximately 45°C for the wild type and PC+2 mutant, to 55°C for the T77D mutant; milk clotting activity was reduced although there was little change in k_{cat} or k_{cat}/Km for the synthetic substrate. These changes may be due to the increased negative charge at the 'flap' region which may have altered the network of hydrogen bonding and influenced the substrate recognition of the enzyme.

INHIBITORS

All aspartic proteinases are inhibited by pepstatin, by the binding of the hydroxyl group of statine to the two catalytic aspartates (Marciniszyn *et al.*, 1976*a*,*b*). The inhibition constant (K_i) of pepstatin for chymosin determined at pH 6.0 and 3.2 is 2.2×10^{-7} M and 3.2×10^{-8} M, respectively (Powell *et al.*, 1985). Pepsin and cathepsin also show pH-dependency of the inhibitory effect (Knight and Barrett, 1976; Baxter *et al.*, 1990), and psuedochymosin is more sensitive to pepstatin than chymosin (McCaman *et al.*, 1985).

As pepstatin is relatively ineffective towards calf chymosin, analogue inhibitors have been developed. A series of inhibitors have been designed by Powell *et al.* (1985) including R(CO)NH-Leu-Ser-Sta-Ala-Ile-Pro-Pro-Lys-Lys (R = acyl group) which has a K_i value for chymosin almost 20-fold better than pepstatin at pH 6.0 and approximately 10-fold better at pH 3.1 than pepstatin. Chymosin is inhibited by the pro-part of chicken pepsinogen (K_i value of 8×10^{-8} M at pH 5.6) but not by its own pro-segment (Strop *et al.*, 1990).

MECHANISM OF MILK CLOTTING

In milk, primary soluble proteins are the whey proteins α-lactalbumin and β-lactoglobulin. The insoluble proteins found in the colloidal particles include casein micelles. κ-Casein is a calcium-insensitive protein which forms a protective layer around the calcium-sensitive caseins (α_S1-, α_S2-, β- and γ-), resulting in stable casein micelles. In the presence of chymosin, milk clotting occurs in two separate steps.

The first phase starts with the cleavage of κ-casein at the Phe105-Met106 bond which results in a release of a

hydrophilic glycopeptide (106–169 component) which passes into the whey, and para-κ-casein which remains in the micelles. Para-κ-casein becomes positively charged at neutral pH and causes the decreasing of electric repulsive forces between casein micelles (Green, 1973).

The hydrolysis of other proteins in milk including α_S1-casein, α_S2-casein, β-casein and α-lactalbumin monomer by chymosin have been reported with a much slower rate of proteolysis (Carles and Dumas, 1985; Miranda *et al.*, 1989). The proteolytic actions of microbial proteases on κ-casein have been reported (de koning, 1967; Yu *et al.*, 1968; Larson and Whitaker, 1970). Porcine pepsin A and C, and *R. miehei* proteinase cleave the same bond as chymosin, but *C. parasitica* proteinase cleaves the Ser104-Phe105 bond (Drønse and Foltmann, 1989). Chymosin has a limited hydrolysis on κ-casein, only with formation of κ-macropeptide and para-κ-casein, while in the case of fungal proteinases, an extensive nonspecific hydrolysis of both κ-casein and para-κ-casein occurs (Shammet *et al.*, 1992).

Visser *et al.* (1980) have suggested that other residues near the cleaved bond are also involved in the hydrolytic reaction. From studies with synthetic peptides, the two additional residues at both sides of the hydrolysable bond are required for appreciable reaction (Raymond *et al.*, 1972). The peptide corresponding to the residues 98-111 of κ-casein (His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys) was found to provide a complete requirement for hydrolysis (Visser *et al.*, 1987, 1988).

Initially, the stability of the micelle is destroyed by the addition of chymosin. This is followed by a nonenzymatic secondary phase in which the aggregation of para-κ-casein and other casein components occurs under the association of Ca^{2+} ions and eventually results in the gel formation (Bringe and Kinsella, 1986*a*; Merin *et al.*, 1989). The formation of a clot is Ca^{2+} dependent. The primary and secondary phases of milk-clotting overlap as the aggregation of micelles begins before the enzymatic process is complete (Brown and Collinge, 1986; Bringe and Kinsella, 1986*b*).

There are several factors that influence the milk-clotting process including pH, temperature, ionic strength, enzyme concentration and salts (Bringe and Kinsella, 1986*a,b*; Okigbo *et al.*, 1985*a,b*). The reaction is pH-dependent; at high pH (6.6–6.7) the clotting time and the curd firmness is reduced (Okigbo *et al.*, 1985*a*), while at low pH (3–4) the hydrolytic activity is high and a decrease of curd yield occurs. Generally, milk-clotting is performed at pH 5.5–6.3. The rate of milk-clotting increases with temperature as long as the enzyme and milk components are stable (Berridge, 1942). Increasing the temperature above 30–32°C or reducing the pH from 6.6 permits flocculation at a lower percentage of κ-casein hydrolysis (Dalgleish, 1982). However, the induction of gel formation at 35°C and 12°C requires approximately 65 and 95% of κ-casein hydrolysis, respectively (Carlson *et al.*, 1986). The differences in milk constituents (both proteins and other chemicals) as well as the pre-treatment process can affect the rate of the primary enzymatic stage. The time taken for clotting milk decreases with increasing enzyme concentration, but the formation and firmness of the gel does not alter (Bringe and Kinsella, 1986*a*). Milk-clotting activity is also dependent on the source of chymosin; for example, porcine chymosin was eight times more active with porcine milk than with bovine milk, calf chymosin had only half its activity against porcine milk than against bovine milk and the activity of lamb chymosin was about 20% higher against ovine milk than against bovine milk (Foltmann, 1992). Calcium ion concentration affected milk-clotting by forming chemical bridges between micelles to form the coagulum and stabilised activity caused by inconsistency of milk composition (Berridge, 1952; Bringe and Kinsella, 1986*b*). However, Pyne (1955) reported that other ions such as strontium, magnesium and barium could affect the Ca^{2+} requirement for coagulation. Milk-clotting was also inhibited by anions (Bringe and Kinsella, 1986*b*).

While synthetic substrates have been used to reveal the hydrolytic mechanism of chymosin and other aspartic proteinases (Raymond *et al.*, 1972; Martin *et al.*, 1980; Visser *et al.*, 1987, 1988), milk-clotting is much more complicated than the hydrolysis of a synthetic substrate. For cheese making, the appropriate enzyme should have a high ratio of milk-clotting activity to proteolytic activity (C/P) (Dalgleish, 1982). The C/P ratio of chymosin is higher than for other enzymes; over 2× higher than mucorpepsin, 4× higher than endothiapepsin and over 25× higher than pepsin, trypsin and papain (Martin *et al.*, 1980; Yada and Nakai, 1986).

RECOMBINANT CALF CHYMOSIN

Chymosin has been used as the milk-clotting enzyme for industrial production of cheese. Several rennet substitutes have been developed including bovine rennet from adult cows, fungus proteinases and other proteolytic enzymes. However, they have a much greater level of non-specific proteolytic activity, and higher thermostability which more completely degrade the milk proteins to peptides, leading to a reduction in yield and poor flavour development in some types of cheese. Consequently, there have been numerous attempts to produce chymosin in microorganisms.

Prokaryotic expression

The first report of an attempt at chymosin production in *E. coli* was carried out by Beppu and coworkers in 1980 (Uchiyama *et al.*, 1980). Efforts to express prochymosin cDNA in *E. coli* led to intracellular accumulation of inactive chymosin in the form of inclusion bodies (Emtage *et al.*, 1983; Nishimori *et al.*, 1984; McCaman *et al.*, 1985; Kawaguchi *et al.*, 1987; Chitpinityol *et al.*, 1996). Generally, chymosin was synthesised in the form

of Met-prochymosin or N-terminal fusion proteins under the control of *E. coli lac* promoter (Nishimori *et al.*, 1984; McCaman *et al.*, 1985), *trp* promoter (Beppu, 1983; Emtage, *et al.*, 1983; Marston *et al.*, 1984; Kawaguchi *et al.*, 1984; Nishimori *et al.*, 1984), *tac* promoter (McCaman *et al.*, 1985; Strop *et al.*, 1990), λP_R promoter, *pho* A promoter (Little *et al.*, 1989) or *T7* promoter (Chitpinityol, 1996).

In *E. coli* expression systems, the recombinant prochymosin was expressed at a high level which resulted in the accumulation of highly refractive inclusion bodies (Emtage *et al.*, 1983; Kawaguchi *et al.*, 1984; Shoemaker *et al.*, 1985). The inclusion bodies produced were up to 40% of the total cell mass and organised in an irregular mass without any obvious membrane-like boundary, with 0.5–1 μm average diameters (Marston *et al.*, 1984; McCaman *et al.*, 1985; Strop *et al.*, 1990; Kaprálek *et al.*, 1991). The synthesis of prochymosin as intracellular inclusion bodies causes a fragilility of cell membranes and the loss of cell respiratory activity and their ability to multiply (Marston *et al.*, 1985; Kaprálek *et al.*, 1991). The production of inclusion bodies can be improved by the plasmid construction, plasmid stability, host strain, the composition of cultivation medium and growth temperature (Kawaguchi *et al.*, 1986, 1987; Kaprálek *et al.*, 1991). The N-terminal methionine of Met-prochymosin can be removed together with the pro-part during acid activation.

The insoluble form of prochymosin requires denaturing condition (8 M urea or 6 M guanidine HCl) to solubilise prochymosin followed by renaturation to generate correctly folded protein that can be activated (Shoemaker *et al.*, 1985). The deletion of disulfide bonds from prochymosin showed that the presence of disulfide bonds was not reponsible for inclusion body formation (McCaman, 1989).

Improvements in the production of recombinant chymosin in *E. coli* have been successively developed by selection of host strain, the modification of plasmids, and the optimization of cultivation conditions (Kawaguchi *et al.*, 1986; Kaprálek *et al.*, 1991). It also has been found that the high yield recovery of active recombinant calf chymosin can be achieved by optimisation of solubilisation and renaturation conditions (Tichy *et al.*, 1993; Yonezawa *et al.*, 1993; Chitpinityol, 1996; Chitpinityol *et al.*, 1996). In our experiments, recombinant enzyme can be refolded by a modified procedure based on that of Marston *et al.* (1984). Table 3 shows that the yield of chymosin was maximal when the urea mixture was diluted 25-fold (0.32 M final urea concentration). If the solubilisation mixture was diluted over 25-fold, the yield of chymosin was dependent on the protein concentration in the alkaline buffer. Table 4 shows that 0.25 mg ml^{-1} protein was optimal under the refolding conditions used. This optimised procedure improves yield of recombinant enzyme nearly 3-fold.

Since insoluble proteins require a further refolding process prior to regaining enzymatic activity, there have been attempts to produce prochymosin extracellularly. The N-terminal sequence of prochymosin was fused to a signal peptide of the outer membrane protein A signal; this resulted in cell lysis after induction. Holland *et al.*, (1990) reported that the fusion of hemolysin *Hly* A^S signal sequence to the C-terminal of prochymosin resulted in the production of hybrid protein up to 25% of the total cell proteins, of which 0.8% was a soluble hybrid product. Recently an expression system for the production of soluble porcine pepsinogen A was developed by fusing the pepsinogen and thioredoxin gene and then expressing the fused product in *E. coli* (Tanaka and Yada, 1996).

Other bacterial expression systems used to produce prochymosin include *Lacto bacillus lactis*, *Bacillus subtilis* and L forms of *Proteus mirabilis* (Parente *et al.*, 1991; Simons *et al.*, 1991; Kaprálek *et al.*, 1991). In *Lacto bacillus lactis*, cDNA of prochymosin was expressed under the control of proteinase *prtP* promoter by fusion with various lengths of *L. lactis* cell envelope-located protease (Simons *et al.*, 1991). Under the control of the T5 phage promoter and the induction of

Table 3. Effect of dilution ratios on the yield of chymosin recovered by refolding

Dilution ratio of urea mixture in buffer pH 10.7	Final urea concentration (M)	Initial concentration of proteins in buffer (mg ml^{-1})	Final amount of activated chymosin (mg)
1:10	0.80	0.79	0.46
1:20	0.40	0.39	0.49
1:25	0.32	0.32	0.50
1:30	0.27	0.26	0.40
1:40	0.20	0.20	0.26

Washed inclusion pellets (protein concentration 7.9 mg ml^{-1}) were solubilised in 8 M urea buffer, pH 8. The urea mixture was incubated at 25°C for 1 h before the insoluble molecules were removed by centrifugation. The urea solution was then diluted in a high pH buffer (pH 10.7) for renaturation of prochymosin. The protein concentrations were determined by using a BCA Protein Assay Reagent.

Table 4. Effect of protein concentration on the refolding of recombinant prochymosin

Initial protein concentration (mg ml^{-1})	Initial amount of protein (mg)	Final amount of activated chymosin (mg)	% of refolding
0.32	1.58	0.33	20.9
0.28	1.42	0.31	21.9
0.25	1.26	0.37	29.0
0.22	1.11	0.28	25.5
0.19	0.95	0.18	18.7

Inclusion bodies solubilised in 8 M urea were diluted at various protein concentrations in phosphate buffer pH 10.7. The urea final concentrations were kept at 0.32 M. The protein concentrations were determined by using a BCA Protein Assay Reagent.

a two-cistron sequence at the 5′ region of the gene, prochymosin was synthesised as an insoluble form aggregated in *B. subtilis* cells but the yield was still low (Parente *et al.*, 1991). The extracellular production of prochymosin in *B. subtilis* can be achieved by fusing prochymosin gene to the *B. subtilis* subtilisin signal sequence and production reached up to 100 μg l^{-1} (Parente *et al.*, 1991). Using L forms of a *P. mirabilis* expression system, the fusion of prochymosin cDNA minus codon 1 to 4 to streptococcal pyrogenic exotoxin type A gene (*speA′*) sequences resulted in the secretion of fusion prochymosin up to 40 μg ml^{-1} of cell-free culture fluid (Kaprálek *et al.*, 1991).

Eukaryotic expression

Several eukaryotes including yeast, fungi, insect and mammalian cells have been employed for the production of prochymosin and chymosin. In *Saccharomyces cerevisiae*, the gene either coding for preprochymosin, prochymosin or chymosin has been expressed under control of phosphoglycerate kinase (*pgk*), galactosidase (*gal 1* and *gal 10*) and triosephosphate isomerase (*tpi*). The proteins, synthesised mainly as insoluble forms, accumulated in the cells and were difficult to activate (Mellor *et al.*, 1983; Goff *et al.*, 1984; Moir and Davidow, 1991). Expression of preprochymosin cDNA did not allow the secretion of chymosin, while substituting yeast invertase signal peptide for the chymosin secretion signal led to the secretion of approximately 10% of the total prochymosin made (Moir *et al.*, 1985). The secretion of prochymosin is critical for obtaining soluble activatable proteins. The failure or the incorrect formation of disulfide bonds is characterised by insoluble prochymosin produced in the cytoplasm of both yeast (Smith *et al.*, 1985) and *E. coli* (Shoemaker *et al.*, 1985). Using yeast secretion signals, integration of the transcriptal units into yeast genome and mutations of the host genome, the secretion of prochymosin increased at least 80-fold, which allowed the production of activatable prochymosin to a level of 20 mg l^{-1} of culture medium (Smith *et al.*, 1985; Moir and Davidow, 1991). *Kluyveromyces lactis* has been developed as an alternative host to *S. cerevisiae* in the expression of recombinant proteins. It has been successfully used to secrete prochymosin under various signal sequences. Efficient synthesis and secretion of prochymosin to more than 95% was achieved by using the *K. lactis* lactase gene (*Lac4*) (van den Berg *et al.*, 1990). The commercially viable yields have been obtained from this species by Gist-Brocades nv. The yeast *Yerrowia lipolytica* has also expressed prochymosin using either the *Leu2* or the alkaline protease XPR2 promoters (Franke *et al.*, 1988). All prochymosin produced by these systems was readily activated to mature chymosin.

Filamentous fungi have also been used as hosts for the production of chymosin. In *Aspergillus nidulans*, chymosin was synthesised as an active extracellular enzyme using the glucoamylase (*glaA*) promoter from *A. niger* (Cullen *et al.*, 1987). However, the yield was low with 90% of the total enzyme activity produced being secreted in this host. The commercial strain of *A. niger* var. *awamori* has been used to expressed prochymosin cDNA under different expression cassettes (Ward, 1989; Ward *et al.*, 1990). The level of active extracellular chymosin was 250 mg l^{-1} when prochymosin cDNA was fused with the entire coding sequence for glucoamylase and expressed in the host which has been deleted for aspergillopepsin A gene (*pepA*) (Ward *et al.*, 1990). The introduction of an N-linked glycosylation site on the flap region resulted in a 10× the production of secreted glycosylated chymosin, more than that of the wild type chymosin, possibly as a result of improved secretion efficiency. Milk clotting activity of glycosylated chymosin was reduced to about 20% of the native enzyme. However, almost all of the activity was recovered after endoglycosidase H treatment (Berka *et al.*, 1990). The production of chymosin in *A. niger* var. *awamori* has been obtained up to 1.3 g l^{-1} by combining a mutagenesis and an efficient screening program (Dunn-Coleman *et al.*, 1991).

Chymosin has been produced up to about 10 mg l^{-1} in *A. oryzae* using a host α-amylase promoter (Boel *et al.*, 1987) whereas in the same expression system, greater than 3 g l^{-1} of mucorpepsin was obtained (Christensen *et al.*, 1988).

The production of chymosin has also been reported from *Trichoderma reesei*, using chymosin signal peptide, cellobiohydrolase I (*cbh I*) sequence or the fusion of *cbh I*-chymosin signal sequence (Harkki *et al.*, 1989). Chymosin A was produced at levels of 40 mg l^{-1} (Harkki *et al.*, 1989). A number of chymosin mutants cloned in *T. reesei* have been reported to exhibit novel properties, including mutants with pH optima shift, substrate-specificity pocket and altering surface loop (Pitts *et al.*, 1991, 1993). The prochymosin secreted by *T. reesei* was readily activated to chymosin.

In HeLa cells, calf preprochymosin cDNA has been expressed under the *CMV-SV* promoter (Kolmer *et al.*, 1991). The product was processed into prochymosin prior to secretion into the cultivation medium at levels of 10–20 mg l^{-1} and readily activated to active chymosin by acid treatment.

Recombinant chymosin is now produced in large scale commercial operations using *E. coli* (California Biotechnology and Pfizer, USA), yeast *Kluyveromyces lactis* (Gist-Brocades, The Netherlands) and mammalian cells (Upjohn, USA) as the hosts (Hodgson, 1993). Many firms, including Genencor/Genentech, Celltech, Hansen and Novo produce commercial recombinant enzymes. Varieties of cheese have been made from recombinant chymosin and evaluated in comparison to cheese produced using the natural enzyme. No significant differences could be detected among them, regarding recovery of milk solids, the rate of proteolysis during ripening, or in the characteristics of the final

cheese products (Green *et al.*, 1985; Kawaguchi *et al.*, 1987; Hicks *et al.*, 1988; Bines *et al.*, 1989; Flamm, 1991; Ward *et al.*, 1991).

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