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JB Review

New families of carboxyl peptidases: serine-carboxyl peptidases and glutamic peptidases

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Peptidases or proteinases are now classified into seven families based on the nature of the catalytic residues [MEROPS—the peptidase database (http://merops.sanger.ac.uk/)]. They are aspartic- (first described in 1993), cysteine- (1993), serine- (1993) metallo- (1993), threonine- (1997), glutamic- (2004) and asparagine-peptidase (2010). By using an S-PI (pepstatin Ac) as a probe, a new subfamily of serine peptidase, serine-carboxyl peptidase (sedolisin) was discovered in 2001. In addition, the sixth family of peptidase, glutamic peptidase (eqolisin) was also discovered in 2004. The former peptidase is widely distributed in nature from archea to mammals, including humans. One of these enzymes is related to a human fatal hereditable disease, Batten disease. In contrast, the distribution of the latter peptidases is limited, with most of them found in human or plant pathogenic fungi. One such enzyme was isolated from a fungal infection in an HIV-infected patient. In this review, the background of the findings, and crystal structures, catalytic mechanisms, substrates specificities and distribution of the new peptidase families are described.

Keywords: catalytic mechanism/glutamic peptidase/pepstatin/serine-carboxyl peptidase/structure/substrate specificity.

Abbreviations: Abz, *ortho*-aminobenzoic acid; AHMHA, 4-amino-3-hydroxy-6-methylheptanoic acid; DAN, diazoacetyl-DL-norleucine methylester; Dap, D-2,3-diamino propionic acid; Dnp, *N*-(2,4-dinitrophenyl)-ethylenediamine; DNP, 2,4-dinitrophenyl; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy) propane; FRET, fluorescence resonance energy transfer; iodoPhe, iodo-phenylalanine; MCA, peptidyl-4-methyl-coumaryl-7-amides; MeNHBz, 2-(*N*-methylamino)-benzoyl; Nph, *p*-nitro-L-phenylalanine; PDB, protein data bank; S-PI, acetyl pepstatin; Xaa, a mixture of 19 coded amino acids.

Carboxyl peptidases, formerly called acid proteinases, were classified into two groups on the basis of their sensitivity to inhibitors: pepstatin-sensitive and -insensitive carboxyl peptidases (*1*–*4*).

Pepstatin-sensitive carboxyl peptidases, represented by porcine pepsin, are blocked by inhibitors such as S-PI (pepstatin Ac found by Murao *et al*. (*5*), Ac-Val-Val-AHMHA-Ala-AHMHA, where AHMHA is 4-amino-3-hydroxy-6-methylheptanoic acid) (Fig. 1), pepstatin (isovaleryl-Val-Val-AHMHA-Ala-AHMHA, found by Umezawa *et al*. in 1970) (*6*), and the active site-directed affinity labelling reagents DAN (diazoacetyl-DL-norleucine methylester) (*7*) and EPNP [1,2-epoxy-3-(*p*-nitrophenoxy) propane] (*8*). In porcine pepsin, a pair of aspartic residues, Asp32 and Asp215, have been revealed to be essential for the catalytic function (*9*). Therefore, pepstatin-sensitive carboxyl peptidases are called aspartic peptidases (*9*). Extensive similarities are observed among the enzymes not only in their amino acid sequences, but also in their tertiary structures.

Murao and this author focused on the inhibitory spectrum of substrate-like inhibitor, S-PI that inhibited all carboxyl peptidases available at that time. An S-PI-insensitive carboxyl peptidase, if there was one, was thought by us to be distinct in substrate specificity and structure from any other known carboxyl peptidases. In 1972, we first demonstrated the presence of pepstatin-insensitive carboxyl peptidases from *Scytalidium lignicolum*, named scytalidopepsin A–C (*10*–*13*). None of them were inhibited by S-PI, pepstatin, DAN or EPNP, with the exception of scytalidopepsin B, which was inhibited by EPNP. These enzymes showed unique substrate specificities (*14*–*19*). The amino acid sequence of scytalidopepsin B is quite different from those of the aspartic peptidases (*20*). Similar carboxyl peptidases have been reported in fungi (*21*–*25*), bacteria (*26*, *27*), and even thermophilic bacteria (*28*–*31*).

Recently, the pepstatin-insensitive carboxyl peptidases were further classified into two groups: serine-carboxyl peptidase and glutamic peptidase. The former was identified as the S53 family of serine peptidase in 2001, and the latter was established to be the sixth family of peptidases, glutamic peptidase, in 2004. In terms of the optimum pH for the enzymatic action of peptidases, there are three major families of carboxyl peptidases, which are catalytically active in the acidic pH region. They are the aspartic peptidases, the serine-carboxyl peptidases and the glutamic peptidases.

Featured Article

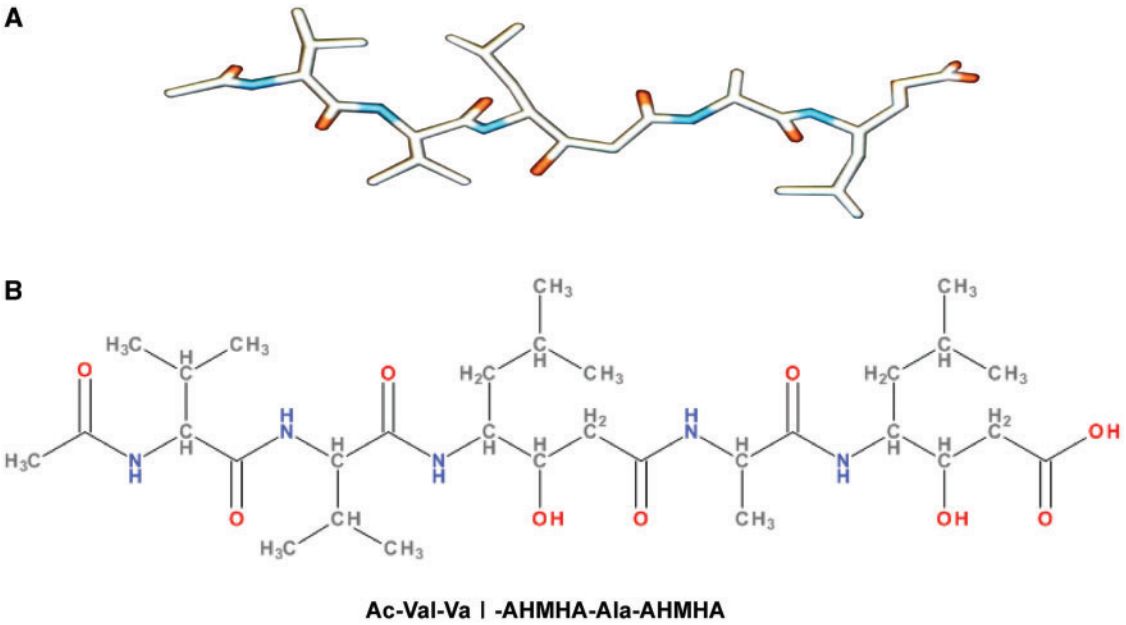

Fig. 1 Chemical structure of S-PI (pepstatin Ac, Ac-Val-Val-AHMHA-Ala-AHMHA, where AHMHA is 4-amino-3-hydroxy-6-methylheptanoic acid). (A) This structure was provided from the HIV-peptidase-S-PI complex. The chemical structure of pepstatin is isovaleryl-Val-Val-AHMHA-Ala-AHMHA.

Table I. Serine-carboxyl peptidases studied by the author's group.

	Sedolisin (PSCP)	Sedolisin-B (XSCP)	Kumamolisin (kumamolysin)	Kumamolisin-As	Kumamolisin-B (J-4)	TPP-1 (CLN2)
Origin	*Pseudomonas*	*Xanthomonas*	*Bacillus* novosp.	*Alicyclobacillus sendaiensis*	*Bacillus coagulans*	Homo sapiens
Opt. pH	3.0	2.7	3.0	3.9	3.0	3.5
MW	38,431	40,222	36,966	36,702	37,168	39,790
Structure (amino acid)	N-prepro: 215 Mature: 372	N-prepro: 237 Mature: 398 C-pro: 192	N-prepro: 188 Mature: 364	N-prepro: 189 Mature: 364	N-prepro: 191 Mature: 368	N-prepro: 195 Mature: 368
Identity (%)	100	53	37	31	36	31
Catalytic residues	Ser287 Glu80 Asp84	Ser307 Glu75 Asp79	Ser278 Glu78 Asp82	Ser278 Glu78 Asp82	Ser279 (Glu79)[a] (Asp83)[a]	Ser280 Glu77 Asp81
References	(*26*, *36*, *37*)	(*27*)	(*28*, *29*, *41*)	(*33*, *42*)	(*34*)	(*48*, *50*, *51*)

[a]Not determined yet.

In this review, the background of the findings of the latter two peptidases, as well as their crystal structures, catalytic mechanisms, substrates specificities and distribution are described.

Serine-carboxyl peptidases

In 1997, Lobel and colleagues (*32*) reported the existence of a pepstatin-insensitive lysosomal carboxyl peptidase in human brain, relating to the fatal neurodegenerative disease, Batten disease, which they named CLN2 (current name TPP-1: tripeptidyl peptidase I). That report motivated this author and his colleagues to study this mammalian enzyme, as well as its bacterial homologues such as sedolisin (an earlier name: pseudomonapepsin) from *Pseudomonas* sp. (*26*), sedolisin-B from *Xanthomonas* (*27*), kumamolisin from a thermophilic bacterium, *Bacillus* novosp. (*28*, *29*), kumamolisin-As with collagenase activity from *Alicyclobacillus sendaiensis* (*33*) and kumamolisin B from *Bacillus coagulans* (*34*) (Table I).

The pH optima of all these peptidases are around pH 3 and the molecular masses are ~40 kDa. These enzymes have a large amino-terminal pro-region composed of about 200 amino acid residues. Although primary structures of serine-carboxyl peptidases are highly similar to one another, they have no sequence similarity to any aspartic peptidases. In order to identify the catalytic residues, screening for inhibitors was carried out by using microorganisms as targets. In 1989, a new inhibitor of sedolisin, tyrostatin, has been isolated from microorganisms (*35*). The chemical structure of tyrostatin is isovaleryl-tyrosyl-leucyl-tyrosinal and its inhibition constant against sedolisin is in the nano molar range. In addition, a pseudo-tyrostatin with no leucine residue was synthesized

and shown to also have inhibitory activity. Such activity requires the presence of the carboxyl-terminal aldehyde.

Structures and catalytic mechanisms

Sedolisin. Three-dimensional structures of sedolisin were solved at 1.0 Å resolution in 2001 (*36*, *37*) (Figs 2A and 3A). The fold of the protein was unexpected, since sedolisin is a single domain protein resembling the serine peptidase subtilisin, not a two-domain structure as observed for the aspartic peptidases. In addition, the catalytic residues of sedolisin have been shown to form a new type of a catalytic triad composed of the side chains of serine, glutamic acid and aspartic acid (Ser287, Glu80 and Asp84), not a pair of aspartic acid residues as present in the aspartic peptidases. There have been no previous reports of a glutamic acid residue in the catalytic triad of a peptidase. Therefore, we named this enzyme a serine-carboxyl peptidase and the *Pseudomonas* enzyme was named as PSCP. In 2003, PSCP was renamed as sedolisin, based on the single-letter code for the catalytic residues, Ser–Glu–Asp (SED in single-letter notation) and its family as the sedolisin family (sedolisins) (*38*).

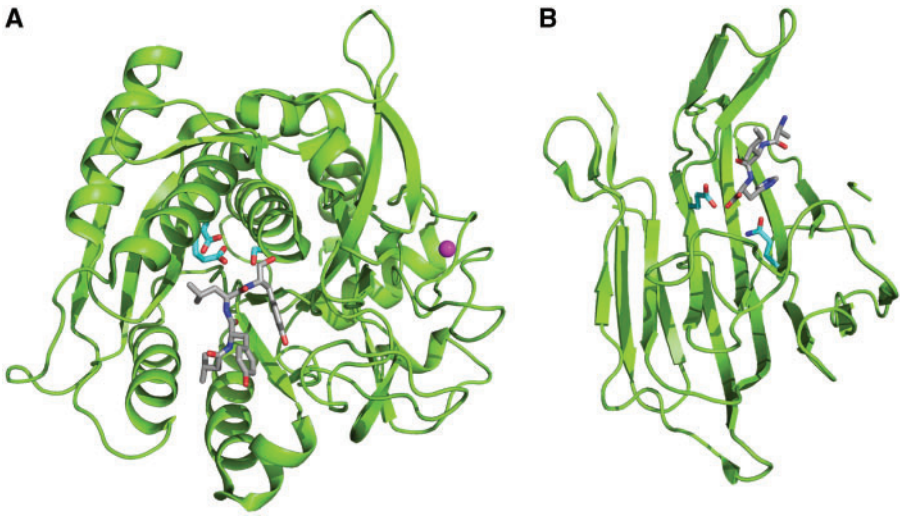

Fig. 2 Crystal structures of sedolisin and eqolisin. (A) Sedolisin [Wlodawer, A., *et al*., 2001, *Nat. Struct. Biol.* PDB (protein data bank) accession code 1GA1, IGA4, 1GA6]. The carbon atoms of the active-site residues Ser287, Glu80 and Asp84 of sedolisin are shown in blue colour, the Ca^{2+} is reddish purple and the carbon atoms of the inhibitor found in the crystals are grey, respectively. (B) Eqolisin (Fujinaga, M., *et al*., 2004, *Proc. Natl Acad. Sci. USA* PDB accession code 1S2B, 1S2K). The active-site residues, Gln53 and Glu136, are shown in stick representation and the carbon atoms are coloured blue. The carbon atoms of the product tripeptide (Ala–Ile–His) bound in the active site are coloured grey.

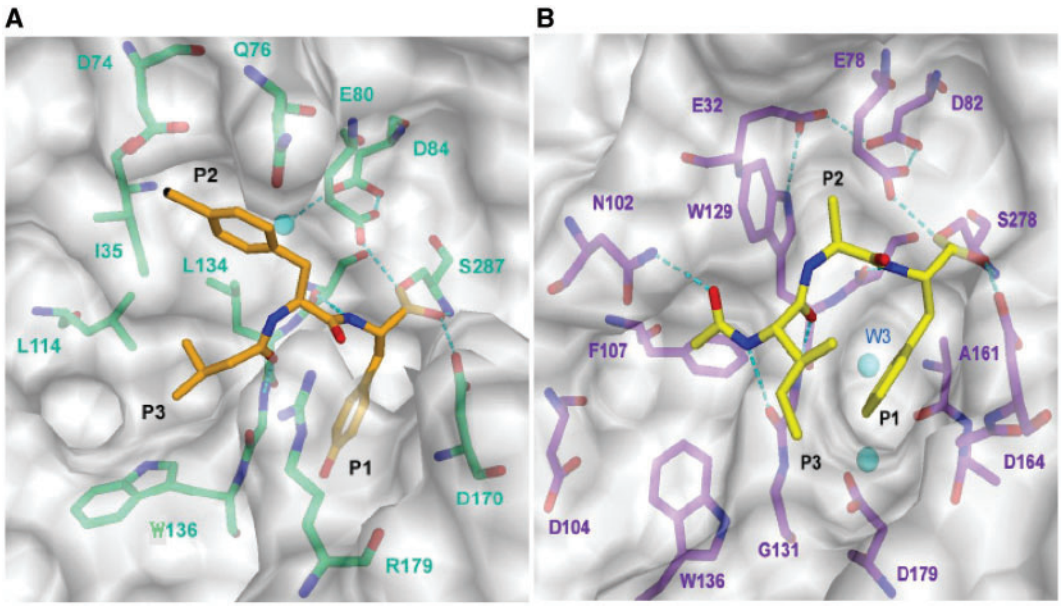

Fig. 3 Active-site structures of sedolisin and kumamolisin. (A) Sedolisin (PDB accession code 1KE1). The covalently bonded inhibitor, pseudo-tyrostatin is shown in gold. (B) Kumamolisin (PDB accession code 1GTJ). The covalently bonded inhibitor, Actyl-Ile-Ala-Phenylalaninal is shown in yellow. (Wlodawer, A., *et al*., 2003, *Acta Biochimica Polonica*).

There is some evidence indicating that these three residues are involved in the catalytic function (Fig. 3A). Firstly, the carboxyl-terminal aldehyde of pseudo-tyrostatin made a hemi-acetal linkage with the hydroxyl group of Ser287. Second, Ser287 is very close to two residues, Glu80 and Asp84. Each interatomic distance among these amino acid residues is ~2.6 Å, which indicates the presence of hydrogen bonds. Third, Asp170 forms part of the oxyanion hole, in a manner similar to that of Asn155 in subtlisin. The fourth line of evidence is a result of site-directed mutagenesis (*38*, *39*). By using an *Escherichia coli* expression system, the wild-type enzyme was expressed as a 62-kDa precursor protein and was processed to an inactive 43-kDa protein by *E. coli* peptidases. After acid treatment, the inactive protein was processed to the mature form having peptidase activity. In contrast, mutants in which Ser287 or Glu80 is converted to Ala (S287A or E80A) did not show any autocatalytic or peptidase activity. The D84A mutant showed very weak auto-processing activity and the peptidase activity was only 0.04% of that of wild-type enzyme. D170A and D170N mutants did not show any processing activities or any peptidase activities. The D328A mutant also did not show either activity since Asp328 is involved in the binding of a Ca^{++}, which is essential to maintain the structure (*36*).

Taken together, it was concluded that sedolisin has a new type of catalytic triad composed of Ser287, Glu80 and Asp84. This can be compared to the catalytic triad of subtilisin composed of Ser221, His64 and Asp32 with Asn155 forming an oxyanion hole. Asp170 in sedolisin is involved in the formation of an oxyanion hole in a manner similar to that of subtilisin Asn155. Thus significant differences between sedolisin and subtilisin are the general base and the amino acid residue involved in the formation of the oxyanion hole. These two structural differences are likely the cause of the differences in optimal pH for their catalytic reactions: the optimal pH of subtilisin and sedolisin are at alkaline pH and at acidic pH, respectively.

The catalytic mechanism for sedolisin was proposed by Wlodawer *et al*. (*36*, *37*) and more detailed studies of the mechanism of sedolisins were subsequently presented by Guo *et al*. (*40*) (Fig. 4A). Quantum mechanical/molecular mechanical (QM/MM) free energy simulations were applied in order to understand the mechanism of the acylation reaction, leading to the acyl-enzyme and first product from the enzyme-catalysed reaction. The proposed roles of some key residues in the catalysis were confirmed through free energy simulations. Glu80 was found to act as a general base to accept a proton from Ser287 during the nucleophilic attack and then as a general acid to protonate the leaving group (N-H of P1′-Phe) during the cleavage of the scissile peptide bond. Another acidic residue forming the oxyanion hole, Asp170, acts as a general acid catalyst to protonate the carbonyl oxygen of P1-Glu during the formation of the tetrahedral intermediate and as a general base for the formation of the acyl-enzyme. The free energy simulations supported the importance of proton transfer from Asp170 to the carbonyl of P1-Glu in the stabilization of the tetrahedral intermediate and the formation of a low-barrier hydrogen bond between the carboxyl group of P1-Glu and Asp170 in the lowering of the free-energy barrier for the cleavage of the peptide bond (*40*).

Kumamolisin and kumamolisin-As. Three-dimensional structures of thermostable enzymes, kumamolisin and kumamolisin-As, were determined in 2002 (*41*) and in 2004 (*42*), respectively. The overall structure of kumamolisin is very close to that of sedolisin. The catalytic triad is also composed of Ser278, Glu78 and Asp82 residues, as was observed in sedolisin (*43*). One of the unique points associated with this enzyme is that there are extensive hydrogen-bond networks among the members of the catalytic triad that extend through Glu32 to Trp129 (Fig. 3B). In order to clarify the role of these residues, which are not present in the sedolisin molecule, a mutational analysis was carried out. The E32A and W129A mutants showed weak autocatalytic activity, respectively. In addition, they showed 4–6% of peptidase activity compared with that of the wild-type enzyme. Based on these data, the additional two residues, Glu32 and Trp129 are speculated to be involved in order to enhance the catalytic function at high temperature. Asp164 residue is involved in the formation of oxyanion hole and Asp316 is involved in the binding of a Ca^{++}.

Based on the structural analysis, the following acylation scenario was suggested (*41*) (Fig. 3B): upon the approach of a polypeptide substrate, the scissile peptide bond will be presented towards the Ser278Oγ with its carbonyl oxygen inserted into the oxyanion hole. This would allow the nucleophilic Ser278Oγ to attack the polarized carbonyl carbon atom of the scissile peptide bond to form a tetrahedral intermediate and to pass into the transition state, under simultaneous proton transfers from the acidic Ser278 oxonium cation to the Glu78 carboxylic group, and from the latter to Asp82, and from Asp82 to Glu32. In the second acylation step, this proton would be transferred back to the leaving group nitrogen of the substrate, allowing cleavage of the P1–P1′ scissile bond, formation of the acyl-ester intermediate and the dissociation of the C-terminal substrate fragment. Thus, Glu78 and Asp82 of kumamolisin might serve as pure proton shuttles, while the Glu32 carboxylate group might represent the general base/acid proton: accepting the proton from Ser278Oγ and shuttling the proton back to the leaving group, respectively.

Recently, the reaction mechanism of kumamolisin-As, which has almost the same structure as kumamolisin (*42*), has been studied by the group of Guo by using the QM/MM molecular dynamics and each step of a tetrahedral complex formation, the acylation- and the deacylation reactions have been clarified (*44–46*) (Fig. 4B). The importance of the general acid/base role of Asp164 of kumamolisin-As (corresponding to Asp170 in sedolisin) was pointed out.

In addition, the 1.2 Å crystal structure of the pro-kumamolisin was solved in 2004 (*47*). The structure exhibited a half-β sandwich core docking to the catalytic domain similarly as the equivalent subtilisin

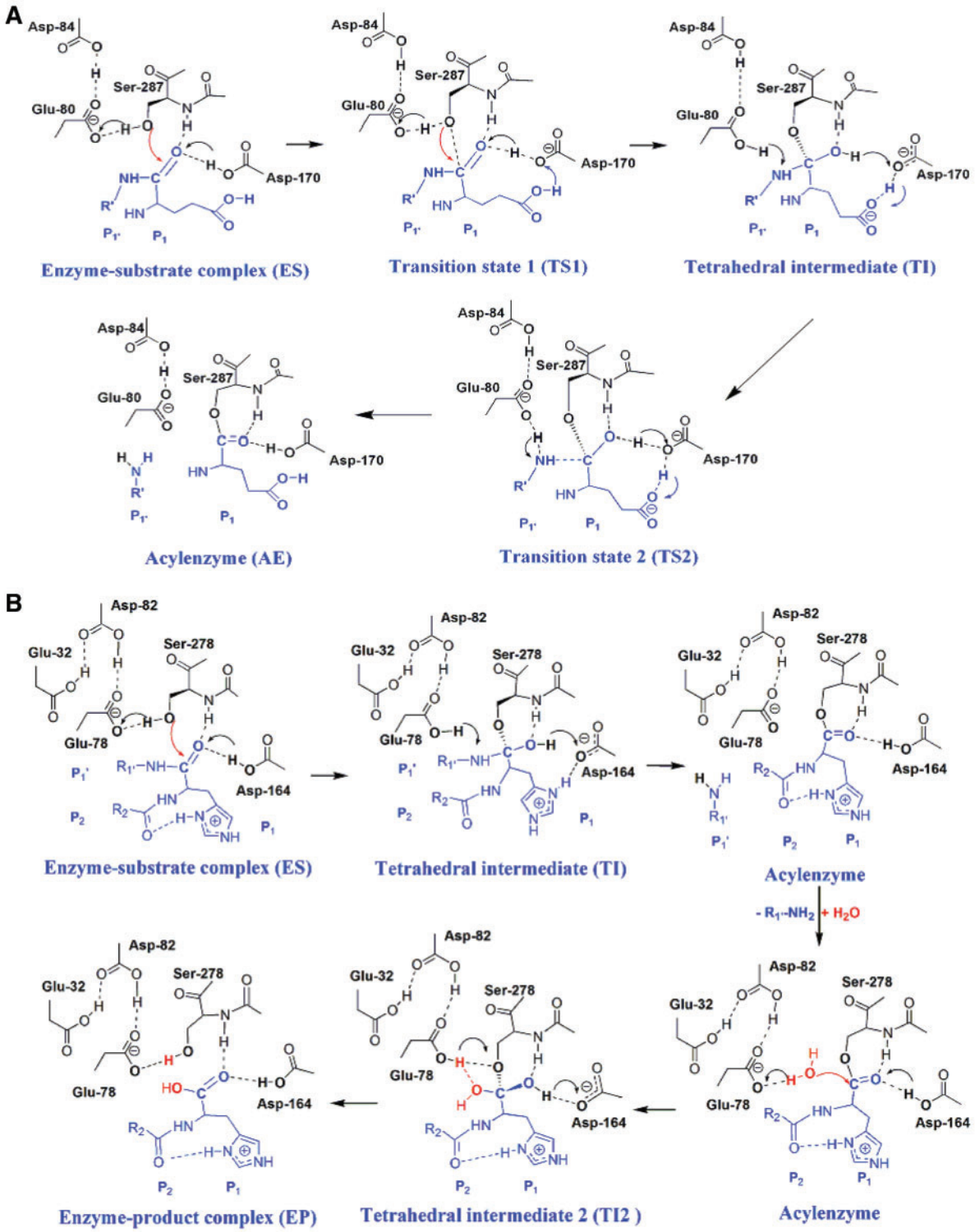

Fig. 4 Catalytic mechanism of sedolisin and kumamolisin-As through QM/MM free energy simulations. (A) Sedolisin (Xu, Q., *et al*., 2011, *J. Phys. Chem. B*). A catalytic mechanism of the acylation reaction of a peptide substrate (Leu-Leu-Glu#Phe-Leu) catalysed by sedolisin that is consistent with QM/MM free energy simulations. The black bend arrows indicate the directions of the proton transfers and red bend arrows the nucleophilic attacks. (B) Kumamolisin-As (Xu, Q., *et al*. 2010, *J. Phys. Chem.B*). Catalytic mechanism and the role of the key active-site residues for both acylation and deacylation reactions of kumamolisin-As based on computer simulations. The black arrows indicate the directions of the proton transfers and red arrows the nucleophilic attacks. The atoms from the enzyme, substrate and water are coloured in black, blue and red, respectively.

pro-domains in their catalytic domain complexes. The pro-kumamolisin structure displayed, for the first time, the uncleaved linker segment running across the active site and connecting the pro-domain with the properly folded catalytic domain. The structure strongly indicated an initial intramolecular activation cleavage in subtilases, as presumed for pro-subtilisin and -furin.

TPP-I. TPP-I plays a crucial role in lysosomal protein degradation and a deficiency in this enzyme leads to a fatal neurodegenerative disease (Batten's disease) (*32*). Recombinant human TPP-I and its mutants were analysed in order to clarify the biochemical role of TPP-I and its mechanism of activity. Ser280, Glu77 and Asp81 were identified as the catalytic residues based on substitutional analyses, inhibition studies and sequence similarities with other family members (*48*) (Table I). In the homology model of TPP-I, it was shown that the carboxyl group of Asp132 extended out into the active-site cleft and could act as an anchor for the N-terminus of the substrate (*49*). In 2009, two independently determined crystal structures of TPP-I precursor have been reported (*50*, *51*).

Substrate specificities

Sedolisin. Based on the published results (*52–54*), the substrate specificity of sedolisin was studied in detail by using the following two combinatorial libraries: the P1 library; Lys–Pro–(Xaa)-Glu-P1#Nph–(Xaa)–Leu and the P1′ library; Lys–Pro–Ile–(Xaa)–Nph#P1′–Gln–(Xaa), where Xaa, '#' and Nph and indicate a mixture of 19 coded amino acids, the point of cleavage and *p*-nitro-L-phenylalanine, respectively (*55*). Each pool contained 361 peptides and the 19 pools of the P1 or the P1′ library totaled 6,859 peptides each. The preference of sedolisin cleavage at each position can be summarized as follows: sedolisin preferred Pro (100%), Leu (80%) and Val (72%) at the P3 position (number in parenthesis is the ratio to a maximum of 100%); Leu (100%), Glu (30%) and Tyr (20%) at the P2 position; Glu (100%), Gly (95%) and Asp (80%) at the P1 position; Tyr (100%), Phe (90%) and Ile (80%) at the P1′ position; and Arg (100%), Leu (95%) and Nle (95%) at the P2′ position. The numbering of amino acid residues is obeyed by the system of Schechter and Berger (1970).

Taken together, these data and the binding mode of isovaleryl-iodoPhe (iodo-phenylalanine)-tyrosinal to sedolisin, the following nature of each subsite was elucidated (Fig. 3A): (1) the S3 and S2 subsites are large enough to accommodate bulky, hydrophobic residues, (2) the S1 subsite has an Arg179 residue able to make an ion pair with the carboxylate of the P1-Glu substrate, (3) the S1′ subsite has a hydrogen bond between the P1′-Tyr hydroxyl and side-chain amide of Gln 76, (4) at the S2′ subsite, the hydrophobic part of the side chain of the P2′-Arg could interact with the indole of Trp231, while the guanidinium group might make a salt bridge to the carboxylate of Glu222, (5) at the S3′ subsite, a Leu side chain of the substrate could easily interact with Ile283 (*55*).

A new fluorescence peptide substrate for sedolisin, MCA-Lys–Pro–Pro–Leu–Glu#Tyr–Arg–Leu–Gly–Lys(DNP)–Gly, where MCA and DNP are peptidyl-4-methyl-coumaryl-7-amides and 2,4-dinitrophenyl, was synthesized based on the result described above. The kinetic parameters of sedolisin for the substrate were: $K_m = 0.12 \pm 0.011\,\mu M$, $k_{cat} = 73 \pm 5\,s^{-1}$ and $k_{cat}/K_m = 608 \pm 85\,\mu M^{-1}\,s^{-1}$ (pH 3.5, 37°C) (*55*). Thus, a highly sensitive and specific substrate for sedolisin was developed.

Kumamolisin. Substrate specificity of kumamolisin was studied using a series of 74 oligopeptide substrates having the general structure P5-P4-P3-P2-Phe#Nph-P2′-P3′ (*56*). Kumamolisin hydrolysed Lys–Pro–Ile–Pro–Phe#Nph–Arg–Leu most effectively among them. The kinetic parameters for this peptide were $K_m = 41 \pm 5\,\mu M$, $k_{cat} = 176 \pm 10\,s^{-1}$ and $k_{cat}/K_m = 4.3 \pm 0.6\,\mu M^{-1}\,s^{-1}$ (pH 3.5, 60°C). These systematic analyses revealed the following features (Fig. 3B): (1) kumamolisin has a unique preference for the P2 position. It preferentially cleaves peptides with an Ala or Pro residue at the P2 position; this was also observed for the putative sedolisin from *B. coagulans* J-4 (kumamolisin-B) (*34*). Other serine-carboxyl peptidases, including sedolisin and sedolisin-B, prefer peptides having a hydrophobic and bulky amino acid residue such as Leu at the P2 position. (2) Kumamolisin prefers charged amino acid residues such as Glu or Arg at the P2′ position, suggesting that the S2′ subsite of kumamolisin has hydrophilic residues, similar to that of sedolisin, sedolisin-B and kumamolisin-B. The hydrophilic nature of the S2′ subsite was confirmed to be a distinguishing feature of sedolisins from prokaryotes. Thus, the substrate specificity of kumamolisin is notably different from those of sedolisin and sedolisin-B.

TPP-I. TPP-I hydrolysed Ala–Arg–Phe#Nph–Arg–Leu most effectively among all substrates tested (*48*). Kinetic parameters of TPP-I for this peptide were $K_m = 4.02\,\mu M$, $k_{cat} = 11.8\,s^{-1}$, and $k_{cat}/K_m = 2.94\,\mu M^{-1}\,s^{-1}$ (pH 4.0, 37°C). The k_{cat}/K_m value was 40 times higher than that for Ala–Ala–Phe–MCA, which had previously been used as a general substrate for TPP-I (*57*). The substrate-binding cleft of TPP-I appeared to be composed of a total of six subsites, S3–S3′. The S1 subsite of TPP-I is large enough to accommodate bulky amino acid residues. TPP-I preferentially cleaved peptides having Arg, Ala or Asp residue at the P2 position, whereas sedolisin and kumamolisin did not exhibit any preference for peptides having charged amino acid residues at the P2 position (*52–54*). The electrostatic nature of the S2 pocket in TPP-I might be different from those of sedolisin and kumamolisin. The human lysosomal aspartic peptidase, cathepsin D, is involved in the normal degradation of intracellular and endocytosed proteins. It accommodates large hydrophobic residues in the P2 position of the substrate, but does not prefer such positively charged residues as Lys or Arg in this position (*52*). TPP-I preferentially hydrolysed a peptide with a P3-Ala substitution, suggesting that TPP-I prefers small amino acid residues at the P3

Table II. List of the most suitable substrate for each peptidase.

Enzyme	Substrates	k_{cat}/K_m(μM^{-1} s^{-1})	Remarks	References
Sedolisin	MCA–Lys–Pro–Pro–Leu–Glu#Tyr–Arg–Leu–Gly–Lys(DNP)-Gly	608 ± 85	pH 3.5, 37°C	(*55*)
Kumamolisin	Lys–Pro–Ile–Pro–Phe#Nph–Arg–Leu	4.3 ± 0.6	pH 3.5, 60°C	(*56*)
Kumamolisin-As	Met–Gly–Pro–Arg#Gly–Phe–Pro–Gly–Ser	1.64	pH 4.0, 60°C	(*33*)
TPP-1	Ala–Arg–Phe#Nph–Arg–Leu	2.94	pH 4.0, 37°C	(*48*)
Eqolisin	D-Dap(MeNHBz)– Gly–Phe–Lys–Phe–Phe# Ala–Leu–Arg–Lys(Dnp)–D-Arg–D–Arg	535	pH 4.0, 37°C	(*61*)

position, whereas lysosomal cathepsin D prefers large, hydrophobic residues at this position (*52*, *53*).

TPP-I is the only member of the sedolisin family that has been shown to exhibit tripeptidyl peptidase activity. In addition, the P2 and P3 preferences are quite different from those of bacterial homologues and from cathepsin D. The substrate-binding cleft of TPP-I might have evolved in order to share a proteolytic function with cathepsin D in the lysosomal compartment. Table II shows the list of the most suitable substrate for each peptidase described here.

Biological functions and application prospects

As for the biological functions, physarolisin isolated from slime mold is reported to be involved in the morphological change of the bacteria (*58*). TPP-I is related to the human fatal hereditary disease, Batten disease. Defects of the expression of the *TPP1* gene in the lysosomes of human brain cause accumulation of mitochondrial ATP synthetase subunit C in the neurons, and this causes the fatal disease at a young age (*32*). Kumamolisin and kumamolisin-As exhibit collagen-hydrolytic activity (*33*). These enzymes are useful for digesting collagen at high temperature and acidic pH, which might be effective to protect the food processing from microbial contamination. In addition, some strains are also useful to make composts using food wastes, particularly, having a high content of carbohydrates, because these strains can grow in acidic pH conditions and at high temperature.

Distribution

It should be emphasized that the sedolisin family is widely distributed among archea, bacteria, fungi, slime mold, amoeba and animal kingdom including amphibians, fish and mammals (*38*, *59*) (also see the MEROPS—the peptidase database). In order to elucidate the biological functions of these enzymes as well as their evolutionary relationships, further studies are necessary.

Glutamic peptidases

Scytalidoglutamic peptidase (eqolisin, an earlier name: scytalidopepsin B) (*60–63*) is the first identified member of the newly discovered G1 family of glutamic peptidases (MEROPS) (Table III). Eqolisin is most active at pH 2.0 when casein is used as substrate (*12*).The activity of eqolisin is not inhibited by S-PI, pepstatin or DAN, but it is inhibited by EPNP. It is a single polypeptide composed of 204 amino acid residues, with a molecular weight of 21,969 and three intramolecular disulphide bridges (*20*). A chromosomal DNA fragment of *S. lignicolum* was cloned and its nucleotide sequenced determined (*64*, *65*). Eqolisin is synthesized as a precursor protein consisting of a prepro-region (54 amino acid residues) and the mature enzyme region (206 amino acid residues). An expression and secretion system for eqolisin was constructed in yeast (*66*). In 2004, the James group in Canada solved the crystal structures of eqolisin in the unbound form and in a form having a hydrolytic peptide product from angiotensin II bound in the active site. In addition, the crystal structures of eqolisin in complex with two transition state peptide analogues designed to mimic the tetrahedral intermediate of the peptidase reaction have been determined in 2007 (*62*).

Structures and catalytic mechanisms

The scytalidoglutamic peptidase molecule adopts a β-sandwich tertiary structure composed of two seven-stranded anti-parallel β-sheet (*60*) (Fig. 2B). The crystal structures revealed a new fold for peptidases and a unique catalytic dyad consisting of residues Gln53 and Glu136. Based on these unique features, this enzyme has been established in 2004 to be a founding member of the sixth family of peptidases, the glutamic peptidase (G1) family in the MEROPS. This family has been named the eqolisins in the same manner as sedolisins (*60*). The three-dimensional structure of aspergilloglutamic peptidase, which is very similar to that of eqolisin was reported at the end of 2004 (*67*).

As mentioned above, eqolisin was presumed to have a novel catalytic dyad composed of Glu136 and Gln53 (*60*). In order to confirm this presumption, site-directed mutagenesis has been carried out. The E136A, Q53A and Q53E mutants of eqolisin lost both the auto-processing and the enzymatic activities of the wild-type enzyme. Coupled with the results from the structural analysis of eqolisin, these biochemical and structural studies confirmed that Glu136 and Gln53 are the catalytic residues (*61*).

The most likely hydrolytic mechanism involves nucleophilic attack of a general base (Glu136) activated water (OH^-) on the *si*-face of the scissile peptide carbonyl-carbon atom to form a tetrahedral intermediate (*60*) (Fig. 5A). Electrophilic assistance and oxyanion stabilization is provided by the side-chain amide of Gln53. Protonation of the leaving-group nitrogen can be accomplished by the general acid function of the protonated carboxyl group of Glu136 (*60*). Consistent with our earlier proposal (*60*), the catalytic

Table III. Glutamic peptidase.

Origin	Scytalidoglutamic peptidase (eqolisin) *Scytalidium lignicolum*	Aspergilloglutamic peptidase *Aspergillus niger* var. *macrosporus*	PMAP-1 *Penicillium marneffei*	TGP1 *Talaromyces emersonii*	EapC *Cryphonectria parasitica*	Acp1 *Scerotinia sclerotiorum*	BcACP1 *Botrytis cinerea*	PepG1 *Alicyclobacillus sp. (Bacteria)*
Opt. pH	2.0	2.0	3.0	3.5	3.0	2.0		3-4
MW (kDa)	22	22.3	24	21.4	21.5	20.7	20.6	28
Structure (amino acid)	N-prepro: 54 Mature: 204	N-prepro: 59 Mature: 39 (L), 173 (H)		N-prepro: 56 Mature: 208	N-prepro: 63 Mature: 206	N-prepro: 52 Mature: 200	N-prepro: 39 Mature: 200	
Identiy (%)	100	~50		47	49	50	45	23.5
Catalytic Residues	Gln53 Glu136	GlnB24 GluB110	(Gln109, Glu196)[a]	(Gln116, Glu201)[a]	(Gln121, Glu206)[a]		(Gln108, Glu194)[a]	(Gln117, Glu199)[a]
Substrate	Glu13–Ala14	Asn3–Gln4	Glu13–Ala14	Glu13–Ala14				
Specificity	Phe24–Phe25	Glu13–Ala14	Phe24–Phe25					
(insulin B chain)	Tyr26–Thr27	Tyr26–Thr27	Phe25–Tyr26	Tyr26–Thr27				
References	(*60–63*)	(*74*)	(*71*)	(*72*)	(*75*)	(*76*)	(*77*)	(*78*)

[a]The data within parenthesis indicates number from the amino-terminal of the N-prepro part.

mechanism has been analysed and discussed in detail based on the structural analysis of the several inhibitor complexes (*62*). The non-hydrolysable scissile bond analogue of the inhibitor is located in the active site forming close contacts with Gln53 and Glu136. The nucleophilic water molecule is displaced and a unique mode of binding is observed with the *S*-OH of the inhibitor occupying the oxyanion binding site of the proposed tetrahedral intermediate (*62*). The catalytic mechanism was further demonstrated by the analysis of the solvent kinetic isotope effects and by the proton inventory experiments (*63*). Sasaki H *et al*., have reported that in aspergilloglutamic peptidase, the catalytic glutamic acid acts as a general acid in the first phase of catalysis (*68*).

Other unique features revealed by the structural analysis are as follows: (i) Topological and three-dimensional structural comparisons reveal that the β-sandwich fold of eqolisin is similar to the members of the concanavalin A-like lectins/glucanases superfamily (*69*). (ii) Conformational rearrangements are seen in a disulphide bridged surface loop (Cys141–Cys148), which moves inwards upon inhibitor or substrate binding, thus partially closing the open substrate binding cleft of the native enzyme. As a result of this movement, the maximum displacement in the C^{α} position of Ser143 is 8 Å.

Substrate specificity and subsite structure

Eqolisin cleaves the B chain of oxidized insulin at the Tyr26–Thr27 bond as well as at other positions such as the Phe24–Phe25 bond (*18*) (Table III). No other known carboxyl peptidase cleaves the Tyr26–Thr27 bond. Eqolisin also cleaves the His6–Pro7 bond of angiotensin I (*19*), another unique characteristic of this enzyme.

The substrate specificity of eqolisin was further investigated by using a novel type of FRET (fluorescence resonance energy transfer) substrate (*61*). The substrate specificity of eqolisin is unique, particularly in the substrate preferences at the P3, P1′ and P3′ positions. Eqolisin prefers a positively charged amino acid (Arg or Lys) at the P3 and P3′ positions and a smaller amino acid (Ala, Gly or Thr) at the P1′ position. In contrast, porcine pepsin preferentially hydrolysed peptides containing Ile, Ala or Thr as the P3 residue, Leu or Ala as the P3′ residue and Tyr or Phe at the P1′ position. These results suggest that eqolisin may have one or more negatively charged residues in the S3 and S3′ subsites, and that it has a narrow space or limited access to the S1′ subsite (*61*).

Based on the substrate specificities described above, two substrates were synthesized (*61*). They were sub-1; D-Dap(MeNHBz)-)–Gly–Phe–Lys–Phe–Phe#Ala–Leu–Arg–Lys(Dnp)–D-Arg–D-Arg and sub-2; D-Dap(MeNHBz)–Gly–Phe–Lys–Phe–Phe#Ala–Phe–Pro-Lys(Dnp)–D-Arg–D-Arg), where Dap, MeNHBz and Dnp are D-2,3-diamino propionic acid, 2-(*N*-methylamino)-benzoyl and *N*-(2,4-dinitrophenyl)-ethylenediamine, respectively. Substrate sub-1 was designed to reflect the preferences at the P3–P3′ sites, while sub-2 was designed to reflect the preferences at the P3–P1 sites. The k_{cat}/K_m values of eqolisin

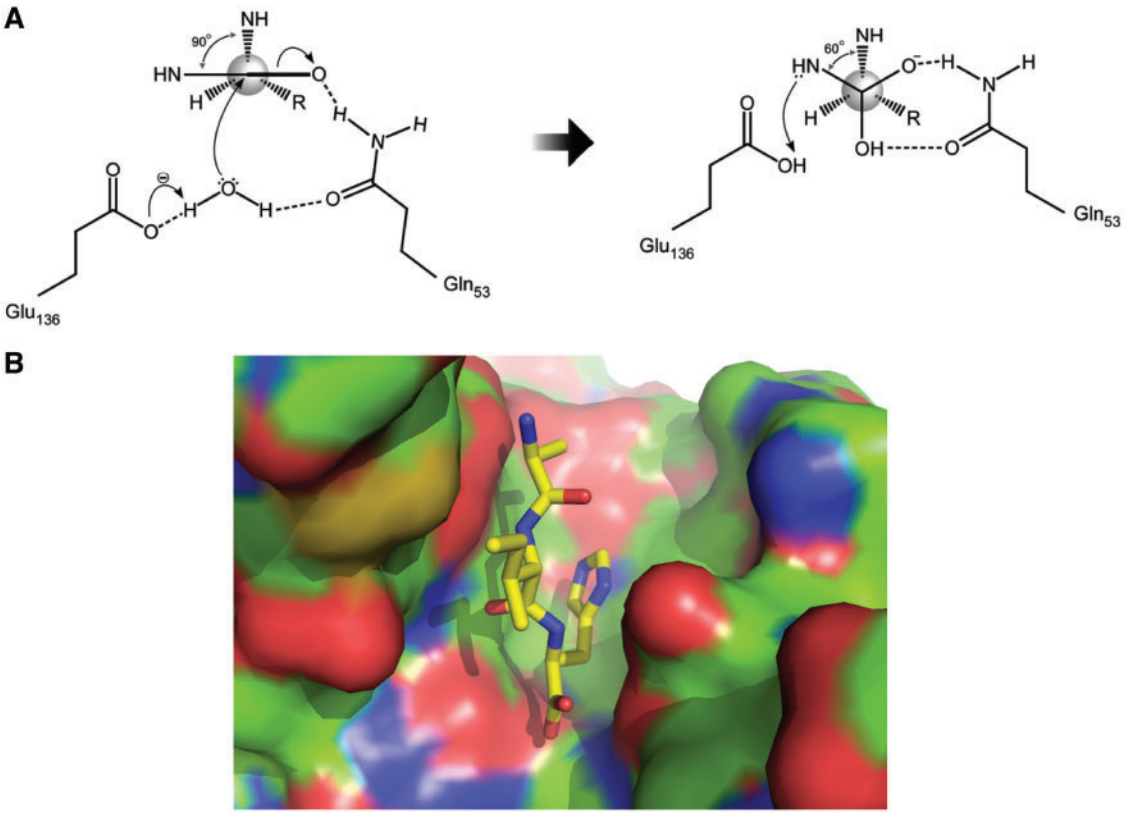

Fig. 5 Catalytic mechanism of eqolisin. (A) The proposed catalytic mechanism of eqolisin. The water molecule hydrogen bonded to both Glu136 and Gln53 is the nucleophile. The general base is the carboxylate of Glu136. The side-chain amide of Gln53 assists in the nucleophilic attack and stabilizes the tetrahedral intermediate by hydrogen bonding. (B) A model of the product Ala-Ile-His-COO^- bound in the active site of eqolisin. The nucleophilic attack by the activated OH^- ion would be on the *si*-face of the scissile peptide; it's position coincides with the oxygen atom of the carboxyl group closest to the enzyme surface in the above figure. The surface of eqolisin is represented and coloured according to the underlying atoms (slate, carbon; blue, nitrogen; red, oxygen). (Fujinaga, M., *et al*., 2004, *Proc. Natl Acad. Sci. USA*).

for sub-1 and sub-2 were $535\,\mu M^{-1}\,s^{-1}$ and $31.0\,\mu M^{-1}\,s^{-1}$, respectively (pH 4.0, 37°C). Not only the non-prime-side residues, but also the prime-side residues of substrates are important for substrate recognition and catalysis. In addition, two putative inhibitors were synthesized (*61*). Ac-Phe–Lys–Phe–(*3S*, *4S*)-phenylstatinyl–Leu–Arg–NH_2 showed potent inhibitory activity. The K_i value for eqolisin was 1.2×10^{-10} M. In contrast, Ac-Phe–Lys–Phe–(*2R*, *3S*)-phenylisoseryl-Ala–Leu–Arg–NH_2 did not show any inhibitory activity against eqolisin. These results suggest that the steric orientation of the scissile bond between P1 and P1′ is crucial for making the enzyme-inhibitor complex.

The inhibitors described above have been used in order to gain insight into the unique substrate specificity and catalytic action of eqolisin (*62*). In both structures of the transition state analogues inhibitor-bound eqolisins, the phenyl ring at the P1 position is sandwiched between residues Phe138 and Trp67. In the S1 subsite, Asp57 can provide hydrogen-bonding interactions with the side-chain hydroxyl of the P1-Tyr. The S1′ subsite is sterically restrained by close packing of residues Trp6, Ile51, Gln53 and Glu136, accounting for the preference of a smaller amino acids like Ala, Gly or Thr at the P1′ position. Eqolisin prefers a positively charged residue, Lys or Arg, at the P3 position. Three acidic residues, Asp57, Asp65 and Asp77, form a negatively charged pocket (S3) for binding the positively charged side-chains of Lys or Arg in the peptide substrate (*62*).

Hydrolysis of peptide bonds with Pro in the P1′ position is a rare event among peptidases, including eqolisin. It was investigated by using a series of FRET peptides, Abz–Lys–Leu-X#Pro–Ser–Lys–Gln–Glu–Asp–Dnp and Abz–Lys–Leu–X#Ser–Ser–Lys–Gln–Glu–Asp–Dnp, where Abz is *ortho*-aminobenzoic acid (*63*). The preference observed in these two series for X = Phe and His over X = Leu, Ile, Val, Arg and Lys, seems to be related to the structure of the S1 subsite of eqolisin. These results and the pH profiles of eqolisin activity showed that its S1 subsite can accommodate the benzyl group of Phe at pH 4 as well as the positively charged imidazolium group of His. In the pH range 2–7, eqolisin maintains its structure and activity, but at pH 8 or higher it is irreversibly denatured. The intrinsic fluorescence of the Trp residues of eqolisin is sensitive to the titration of carboxyl groups having low pK values; this can be attributed to the buried Asp57 and/or Asp43 (*63*) as described in the articles on the eqolisin three-dimensional structure (*62*).

Substrate specificities of other G1 family enzymes for insulin B chain are summarized in Table III.

Aspergilloglutamic peptidase cleaves at Asn3–Gln4, Glu13–Ala14 and Tyr26–Thr27 (*70*). PMAP-1 from *Penicillium marneffei* cleaves at Glu13–Ala14, Phe24–Phe25 and Phe25–Tyr26 (*71*). TGP1 from *Talaromyces emersonii* cleaves at Glu13–Ala14 and Tyr26–Thr27 (*72*). Thus, the common cleavage sites of these enzymes against the oxidized insulin B chain are Glu13–Ala14, and Tyr26–Thr27 except for PMAP1. In addition, it was reported that TGP1 preferred large residues with the exception of Trp or Tyr at the P1 site, and smaller amino acids such as Gly, Ser and Ala at the P1′ site (*72*). These data are comparable with the subsite specificity profiling of eqolisin, which indicates that cleavage is favoured between bulky P1 residues (Phe > Tyr > His) and small P1′ residues (Ala > Gly/Thr > Ser). Accordingly, it can be said that the glutamic peptidases prefer bulky amino acid residues at the P1 site and small amino acid residues at the P1′ site.

Establishment of the sixth family of peptidase, glutamic peptidase

Several reasons exist to place the eqolisins into a new family of peptidases (*60*). (1) They have a unique fold not previously observed for peptidases. No other known peptidases have a β-sandwich as the tertiary fold. (2) They have a unique catalytic dyad of a glutamate and a glutamine to activate the nucleophilic water and to stabilize the tetrahedral intermediate on the hydrolytic pathway, respectively. (3) The nucleophilic attack on the carbonyl-carbon atom is from the *si*-face of the scissile peptide. This feature is in common with the papain-like peptidases but distinct from the serine-, aspartic- and metallo-peptidases that attack the scissile peptide from the *re*-face. (4) The conformational angle ψ of the P1 residue of eqolisin substrates is unique among peptidase families. Thus, eqolisin (scytalidoglutamic peptidase) was established to be the first known member of the sixth family of peptidases, the glutamic peptidase family (Family G1 in MEROPS).

Distribution and their biological aspects

Most known glutamic peptidases are from the filamentous fungal species of the *Ascomycota* phylum (*73*) (Table III). Their sequence identities compared to eqolisin are shown in Table III: aspergilloglutamic peptidase; ∼50% (*74*), EapC from *Cryphonectria parisitica*; 49% (*75*), Acp1 from *Sclerotina sclerotiorum*; 50% (*76*), BcACP1 from *Botrytis cinerea*; 45% (*77*) and TGP1 from *Talaromyces emersonii*; 47% (*72*). Quite recently, Jensen *et al*. found that thermoacidophilic bacteria, *Alicyclobacillus* sp. produced a G1 family peptidase having an amino acid sequence identity of 23.5% to that of eqolisin (*78*). Among these peptidases, the catalytic residues, Glu136 and Gln53, are well conserved. As for the putative glutamic peptidase, please see MEROPS.

Scytalidium lignicolum is a wood-destroying fungus, and the biological function of these enzymes is assumed to be the degradation of protein in the wood of dead trees. Most of the peptidases described above except TGP1 from *Talaromyces emersonii,* and the bacterial peptidase, PepG1, from *Alicyclobacillus* sp., are pathogens for human or plants. In 2001, it was shown that the glutamic peptidase is produced during plant infection by *Sclerotina sclerotiorum*, a white mold fungus that infects a wide range of cultivated plants (*76*).

Conclusions

In 1972, Murao and the author first demonstrated the presence of pepstatin Ac-insensitive carboxyl peptidases in the culture filtrate of *S. lignicolum* ATCC 24568 (*11–14*). Such pepstatin-insensitive peptidases have been found not only from *S. lignicolum*, but also in fungi (*21–25*), bacteria (*26*, *27*) and even in thermophilic bacteria (*28–31*, *33*). Accordingly, we proposed that carboxyl peptidases could be divided into two groups: pepstatin-sensitive (aspartic peptidase) and pepstatin-insensitive carboxyl peptidases (*1–4*). The author would like to emphasize that the new families of peptidases described here, the sedolisins and the eqolisins, were found from the pepstatin-insensitive carboxyl peptidases.

Further Reading

For further reading see Murao and Oda (*1*), Oda and Murao (*2*), Oda *et al*. (*3*), Oda *et al*. (*4*), Wlodawer *et al*. (*38*) and James (*79*).

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Conflict of Interest

None declared.

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