CHAPTER 15

AMINOPEPTIDASES

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1. INTRODUCTION

Aminopeptidases hydrolyse peptide bonds at the N-terminus of proteins and polypeptides whereas carboxypeptidases hydrolyse peptide bonds at the C-terminus. Omega peptidase is an additional term referring to special types of aminopeptidases and carboxypeptidases that are capable of removing terminal residues lacking a free α -amino or α -carboxyl group, or include linkages other than the α -peptide type (*e.g.* pyroglutamyl peptidases; McDonald and Barret, 1986). Aminopeptidases can be subdivided into three groups: aminopeptidases in the strict sense which hydrolyse the first peptide bond in a polypeptide chain with the release of a single amino acid residue (aminoacyl- and iminoacyl peptidases [EC 3.4.11]); those that remove dipeptides or tripeptides (dipeptidyl- and tripeptidyl peptidases [EC 3.4.14]) from polypeptide chains; and those which only hydrolyse di- or tripeptides (dipeptidases [EC 3.4.15] and tripeptidases [EC 3.4.14.4]) (Sanderink *et al.*, 1988). Aminopeptidases are widely distributed among bacteria, fungi, plants and mammals (Gonzales and Robert-Baudouy, 1996; Sanz *et al.*, 2002; Tu *et al.*, 2003; Barret *et al.*, 2004). These enzymes are located in different subcellular compartments including the cytoplasm, lysosomes and membranes, and can also be secreted into the extracellular medium. Based on catalytic mechanism, most of the aminopeptidases are metallo-enzymes but cysteine and serine peptidases are also included in this group. Though some aminopeptidases are monomeric, most show multimeric structures particularly those from eukaryotic organisms (McDonald and Barret, 1986; Jones, 1991; Lowther and Matthews, 2002). The three-dimensional structures of some aminopeptidases have been solved, contributing to the understanding of their catalytic mechanism and

functions (Kim *et al.*, 1993; Bazan *et al.*, 1994; Joshua-Tor *et al.*, 1995; Lowther and Matthews, 2002). Aminopeptidases appear to act in concert with other peptidases to complete diverse proteolytic pathways. Thus, these enzymes can efficiently retrieve amino acids from dietary proteins and endogenous proteins degraded during protein turnover, thereby covering nutritional as well as other biological roles including protein maturation, hormone level regulation and cell-cycle control (McDonald and Barret, 1986; Christensen *et al.*, 1999). Many of the mammalian enzymes play important functions in cellular processes involved in health and disease and, as a consequence, constitute targets for the pharmaceutical industry (Scornik and Botbol, 2001; Holz *et al.*, 2003; Rigolet *et al.*, 2005; Inguibert *et al.*, 2005). Some aminopeptidases are also of great interest for their biotechnological and agro-industrial applications (Seppo *et al.*, 2003; FitzGerald and O'Cuinn, 2006).

2. CLASSIFICATION AND NOMENCLATURE OF AMINOPEPTIDASES

Aminopeptidases have been classified on the basis of their substrate specificity (broad or narrow), catalytic mechanism (metallo-, cysteine, and serine peptidases) and molecular structure (Gonzales and Robert-Baudouy, 1996; Barret *et al.*, 2004; Rawling *et al.*, this volume). The nomenclature of many (aminoacyl or iminoacyl peptidases) has been determined by their preferences or requirements for a particular N-terminal amino acid. Thus, an enzyme that for instance showed its highest rate of hydrolysis on N-terminal methionyl bonds was named methionyl aminopeptidase or aminopeptidase M. In an attempt to avoid ambiguity, the subcellular location (membrane, microsomal or cytosolic) has also been used to name aminopeptidases having similar specificities. In the cases of di- and tripeptidases their names have been based on substrate size requirements. In addition, the names of dipeptidyl (DPP) and tripeptidyl peptidases (TPP) were followed by a Roman number to differentiate between the various types described and this numbering convention has been retained. In the nomenclature of peptidases identified in lactic acid bacteria the term 'Pep' is used when the corresponding peptidase gene sequence is known, followed by a capital letter indicating the specificity and homology to other known peptidases, *e.g.* PepN for the homologue of aminopeptidase N (Tan *et al.*, 1993). Nevertheless, alternative names and abbreviations often appear in the literature. Recently, the MEROPS peptidase information database (<http://www.merops.sanger.ac.uk>; see chapter by Rawlings *et al.* in this volume) created a hierarchical structure-based classification of peptidases into families and clans. Members of a family are homologues, and families that are thought to be homologous are grouped together into clans. Clans consist of families of peptidases that share a single evolutionary origin, evidenced by similarities in their tertiary structures and/or the order of catalytic-site residues and common sequence motifs around the catalytic residues.

3. ANIMOPEPTIDASE TYPES AND FUNCTIONS

3.1. Mammalian Aminopeptidases

Aminopeptidases were among the first proteases to be discovered in mammalian tissues and a large number have already been characterized (Barret *et al.*, 2004). The best characterized mammalian aminopeptidases and their biochemical properties are shown in Table 1. On the basis of specificity they can be divided into different groups: (i) aminopeptidases of broad specificity (*e.g.* leucyl aminopeptidase, and membrane and cytosol alanyl aminopeptidases); (ii) aminopeptidases of narrow specificity with preference for basic amino acid residues (aminopeptidase B), acid amino acid residues (glutamyl and aspartyl aminopeptidases), cysteine (cystinyl aminopeptidase), methionine (methionyl aminopeptidase), or bonds containing proline (prolyl aminopeptidase and aminopeptidase P); and (iii) dipeptidyl peptidases (DPP I, II, III, and IV) and tripeptidyl peptidases (TPP I and II) that release di- and tripeptides; respectively (Cunningham *et al.*, 1997; Sanz *et al.*, 2002; Albiston

Table 1. Main types and properties of mammalian aminopeptidases

Enzyme	EC number	Catalytic type	Specificity	Family
Aminopeptidase (AP)			$X-\downarrow-Y-(Z)_n$	
Leucyl AP	3.4.11.1	Metallo	X = Leu	M17
Membrane alanyl AP	3.4.11.2	Metallo	X = Ala, Phe, Tyr, Leu	M1
Cytosol alanyl AP	3.4.11.14	Metallo	X = Ala	M1
Aminopeptidase B	3.4.11.6	Metallo	X = Arg, Lys	M1
Glutamyl AP	3.4.11.7	Metallo	X = Glu, Asp	M1
Cystinyl AP	3.4.11.3	Metallo	X = Cys	M1
Methionyl AP	3.4.11.18	Metallo	X = Met	M24A
Aminopeptidase P	3.4.11.9	Metallo	X and Y = Pro	M24B
Prolyl AP (PIP)	3.4.11.5	Serine	X = Pro	S33
Bleomycin hydrolase	3.4.22.40	Cysteine	X = Met, Leu, Ala Bleomycin peptide	C1B
Dipeptidyl peptidases (DPP)			$X-Y-\downarrow-(Z)_n$	
DPP I	3.4.14.1	Cysteine	X $\neq$ Arg or Lys, Y or Z $\neq$ Pro	C1
DPP II	3.4.14.2	Serine	Y = Ala or Pro	S28
DPP III	3.4.14.4	Metallo	X-Y = Arg-Arg	M49
DPP IV	3.4.14.5	Serine	Y = Pro	S9B
Tripeptidyl peptidases (TPP)			$X-Y-T-\downarrow-(Z)_n$	
TPP I	3.4.14.9	Serine	Gly-Pro-T = hydrophobic	S53
TPP II	3.4.14.10	Serine	Ala-Ala-Phe T or Z $\neq$ Pro	S8

et al., 2004). The characteristics of the main aminopeptidases of broad and narrow specificities and proline-specific peptidases are briefly reviewed.

3.1.1. *Mammalian aminopeptidases of broad specificity*

Leucyl aminopeptidase (LAP) is a ubiquitous enzyme that has also been referred to as cytosol aminopeptidase and leucine aminopeptidase. It was the first cytosolic aminopeptidase to be identified (Linderstrom-Lang, 1929). LAP preferentially releases Leu located as the N-terminal residue of peptides, and can also release other amino acids including Pro but not Arg or Lys. This enzyme is a hexamer of identical chains and has a molecular mass of 324-360 (Kohno *et al.*, 1986). Human LAP is involved in the breakdown of the peptide products of intracellular proteinases and is one of the enzymes that trims proteasome-produced peptides for presentation by the major histocompatibility complex class I molecules. Expression of the encoding gene is promoted by interferon gamma (Beniga *et al.*, 1998).

Membrane alanyl aminopeptidase has also been referred to as aminopeptidase M due to its membrane localization since there is a cytosolic counterpart, and also as aminopeptidase N due to its preference for neutral amino acids. The amino acid residue preferentially released is Ala, but most amino acids including Pro may be hydrolysed by this enzyme. When a terminal hydrophobic residue is followed by Pro, the two may be released as an intact X-Pro dipeptide (McDonald and Barret, 1986). In most species the native enzyme is a homodimer with a molecular mass of 280-300 and is glycosylated. The mammalian enzyme plays a role in the final digestion of peptides generated from proteins by gastric and pancreatic protease hydrolysis. It is also important for the inactivation in the kidney of blood-borne peptides such as enkephalins and the neuropeptide 'substance P'. Furthermore, it is a regulator of IL-8 bioavailability in the endometrium and therefore may contribute to the regulation of angiogenesis. This aminopeptidase is also the myeloid leukaemia marker CD13 and serves as a receptor for human coronavirus (Shimizu *et al.*, 2002; Albiston *et al.*, 2004).

3.1.2. *Mammalian aminopeptidases of narrow specificity*

Glutamyl aminopeptidase is also referred to as aminopeptidase A, angiotensinase A and aspartate aminopeptidase. It releases N-terminal Glu (and to a lesser extent Asp) from a peptide. It is generally a membrane-bound enzyme involved in the formation of the brain heptapeptide angiotensin III which exerts a tonic stimulatory effect on the central control of blood pressure and is a regulator of blood vessel formation (Fournie-Zaluski *et al.*, 2004.).

Methionyl aminopeptidase has also been named methionine aminopeptidase and peptidase M. It releases N-terminal amino acids, preferentially methionine, from peptides but only when the second residue is small and uncharged. In eukaryotes, two types of methionyl aminopeptidases exist due to protein synthesis occurring in the mitochondria (type I) and in the cytoplasm (type II). The type I peptidase is similar to the bacterial methionyl aminopeptidase whereas type II resembles the enzyme from archaea (Arfin *et al.*, 1995). The mammalian enzymes are involved in

the regulation of protein synthesis and in the processing of those proteins required for the formation of new blood vessels in normal development, tumour growth and metastasis (Yeh *et al.*, 2000; Selvakumar *et al.*, 2005; Zhong *et al.*, 2006).

3.1.3. Proline-specific peptidases

This group includes two aminopeptidases: prolyl aminopeptidase and X-Pro aminopeptidase. The prolyl aminopeptidase has variously been named Pro-X aminopeptidase, proline aminopeptidase and proline iminopeptidase. It releases N-terminal proline from a peptide. This enzyme was first detected in *E. coli* but is widely distributed in nature and also present in the cytosol of mammalian cells (Matsushima *et al.*, 1991). In contrast to the bacterial form, the mammalian enzyme is not specific for prolyl bonds (Cunningham *et al.*, 1997). X-Pro aminopeptidase has also been termed aminopeptidase P and proline aminopeptidase. It releases any N-terminal amino acid residue, including proline, from oligopeptides and even dipeptide and tripeptides in which the penultimate N-terminal residue is proline. The preferred substrates have a hydrophobic or basic residue at the N-terminus. The mammalian enzyme exists in membrane-bound and cytosolic forms (Cottrell *et al.*, 2000). It appears to contribute to the processing of bioactive peptides involved in the cardiovascular and pulmonary systems, and the degradation of collagen products (Yaron and Naider, 1993; Yoshimoto *et al.*, 1994).

Of the mammalian peptidyl peptidases, dipeptidyl peptidase IV (DPP IV) is the best known. It releases an N-terminal dipeptide from polypeptides in which, the penultimate residue is Pro (preferentially but not exclusively) and provided that the antepenultimate residue is neither Pro nor hydroxyproline (Leiting *et al.*, 2003). This enzyme is anchored to the cell membrane and expressed in various cell types. It has a calculated molecular mass of 88 kDa and the native enzyme is a homodimer. DPP IV plays a key role in various regulatory processes, acting on a number of bioactive oligopeptides including neuropeptides, endomorphins, circulating peptide hormones, glucagon-like peptides (GLP-1 and GLP-2), gastric inhibitory peptide (GIP) and paracrine chemokines, leading to modification of their biological activities or even their inactivation (Augustyns *et al.*, 2005).

3.2. Microbial Aminopeptidases

The first studies on microbial aminopeptidases were carried out over 40 years ago, and since then a large number of aminopeptidases of microbial origin have been characterized (Gonzales and Robert-Baudouy, 1996; Jones, 1991; Kunji *et al.*, 1996; Christensen *et al.*, 1999; Sanz and Toldrá, 2002; Sanz *et al.*, 2002; Barret *et al.*, 2004; Nampoothiri *et al.*, 2005; Savijoki *et al.*, 2006). The main types of microbial activity characterized to date as well as their properties are summarized in Table 2. These enzymes can be divided according to their specificities into groups similar to those described for the mammalian enzymes: (i) general aminopeptidases showing broad specificity (PepN, PepC, LAP and PepS); (ii) aminopeptidases of narrow specificity that selectively hydrolyse certain amino acid residues such

Table 2. Main types and properties of microbial aminopeptidases

Enzyme	EC number/ homologous	Catalytic type	Specificity	Family
Aminopeptidases (AP)			X- ↓-(Y)-(Z) _n	
PepN/ Lysyl aminopeptidase	Mammalian AP N	Metallo	X = Lys, Leu,	-
Bleomycin hydrolase GAL6/BLH1/YCP1	3.4.22.40	Cysteine	X = Arg, Tyr Bleomycin	C1B
PepC/aminopeptidase C	Bleomycin hydrolase	Cysteine	X = Lys, Glu, Ala, Met, Leu X or Y ≠ Pro	C1B
PepA/aminopeptidase A	-	Metallo	X = Glu, Asp, Ser	M42
PepS	-	Metallo	X = Arg, Trp	M29
CAP/PepA	3.4.11.10	Metallo	X = Leu, Met, Phe, Arg	M17
Leucine aminopeptidase				
Aminopeptidase Y/yscI	3.4.11.15	Metallo	X = Arg, Lys, Ala	M28
Aminopeptidase M/MAP	3.4.11.18	Metallo	X = Met	M24A
PepP/AminopeptidaseP	3.4.11.9	Metallo	Y = Pro	M24B
PepI/Proline iminopeptidase	3.4.11.5	Serine	X = Pro	S33
D-stereospecific aminopeptidase/DppA	-	Serine	X = D-Ala, D-Ser or D-Thr	S12
Dipeptidases			X-↓-Y	
PepV/peptidase V	-	Metallo	X = Lys, Leu, Met βAla-dipeptides	M20A
PepD/PepDA	-	Cystein	X = Lys, Met, Leu	C69
PepQ/prolidase	3.4.13.19	Metallo	Y = Pro	M24B
PepR/prolinase	-	Serine	X = Pro	-
Tripeptidases			X-↓-Y-Z	
PepT/Peptidase T	-	Metallo	Leu-Gly-Gly	-
Dipeptidyl-peptidases			X-Y-↓-(Z) _n	
PepX/X-Pro-dipeptidyl peptidase	3.4.14.11	Serine	Y = Pro	S15

as acidic residues (PepA) and methionine (MAP), D-amino acid residues (DppA) or peptide bounds containing proline (PepI and PepP); (iii) dipeptidases hydrolysing peptide bounds containing proline (PepQ and PepR); (iv) dipeptidases (PepV and PepDA) and tripeptidases (PepT) of broad specificity that only hydrolyse dipeptides or tripeptides, respectively; and (v) dipeptidyl peptidases showing specificity for N-terminal X-Pro.

Microbial aminopeptidases play important roles in the utilization of exogenous proteins as a source of essential amino acids that can be utilized for protein synthesis, the generation of metabolic energy and the recycling of reduced cofactors (Christensen *et al.*, 1999). They are also implicated in the final steps of protein turnover and in more specific cellular functions such as the processing of newly synthesized proteins and high copy number plasmid stabilization (Gonzales and Robert-Baudouy, 1996).

3.2.1. Microbial aminopeptidases of broad specificity

PepN aminopeptidases, also known as lysyl aminopeptidases, have been identified in numerous bacterial species (e.g. *E. coli*, *Pseudomonas*, and lactic acid bacteria. Gonzales and Robert-Baudouy, 1996; Christensen *et al.*, 1999). In most micro-organisms these are monomeric enzymes of about 95 kDa. Their primary sequences are homologous to mammalian aminopeptidase N and conserve the signature sequence of zinc-dependent metallo-peptidases (Gonzales and Robert-Baudouy, 1996; Kunji *et al.*, 1996). In lactic acid bacteria this enzyme is involved in the utilization of caseins as an exogenous source of amino acids (Kunji *et al.*, 1996).

LAPs (PepA in *E. coli*) are also zinc-metallo aminopeptidases of broad specificity identified in Gram-negative bacteria and fungi (Gonzales and Robert-Baudouy, 1996; Nampoothiri *et al.*, 2005). These enzymes show sequence homology with bovine lens leucine aminopeptidase and similar specificity (Gonzales and Robert-Baudouy, 1996). Pep L aminopeptidases identified and partially characterized in different species of *Lactobacillus* seem, however, to be serine peptidases (Sanz *et al.*, 1997; Christensen *et al.*, 1999). PepC and bleomycin hydrolases are cysteine aminopeptidases of relatively broad specificity identified in lactic acid bacteria and yeast, respectively. Both exhibit similarity to mammalian bleomycin hydrolases (Kunji *et al.*, 1996).

3.2.2. Aminopeptidases of narrow specificity

MAPs of microbial origin show high levels of similarity with mammalian MAP, conserving all five metal-binding residues and also maintaining similar specificity. The enzymes from prokaryotes and yeasts seem to be monomers of 29 kDa and 44 kDa, respectively. They play critical biological roles since their inactivation in *E. coli*, *S. typhimurium* and *S. cerevisiae* result in lethal phenotypes (Gonzales and Robert-Baudouy, 1996). A homologue to mammalian MAP type 2 is also present in yeast and shows subtle differences in its peptide substrate specificity (Chen *et al.*, 2002).

Aminopeptidase A, also referred to as glutamyl aminopeptidase and PepA, was identified in *Lactococcus lactis*. The genetic and physicochemical properties of this enzyme are not related to other aminopeptidases in prokaryotes or eukaryotes of similar specificity except for the enzyme purified from *Streptococcus thermophilus*. It specifically hydrolyses Glu and Asp residues, and to a lesser extent Ser residues, from the N-terminus of oligopeptides. In most cases the native enzyme seems to be a hexamer with a molecular mass of 240 kDa although other values (440–520) have been reported. The lactococcal enzyme was not demonstrated to be essential for growing on milk caseins but is thought to be important for flavour generation in dairy products (I'Anson *et al.*, 1995).

3.2.3. Proline-specific aminopeptidases

A set of peptidases specialised in the hydrolysis of proline-containing peptides has been detected in lactic acid bacteria, and is thought to be necessary for the complete degradation of caseins since they have a high proline content (Kunji *et al.*, 1996).

This group includes: two aminopeptidases (PepP and PepI), two dipeptidases (PepQ and PepR) and a dipeptidyl-peptidase (PepX), all of which conserve the consensus signatures of their catalytic types (metallo or serine peptidases). PepX has also been detected in streptococci and is thought to play a role in the pathological processes caused by *Streptococcus gordonii* and *S. agalactiae*, such as endocarditis, neonatal sepsis and meningitis (Rigolet *et al.*, 2005).

3.3. Plant Aminopeptidases

Several aminopeptidases have also been identified in plants. These enzymes are believed to play biological roles in protein turnover, stress responses, protein mobilization from cotyledons after germination, protein maturation and meiosis. The aminopeptidases identified in plants also include enzymes of the broad and narrow specificities previously described. Among them, the LAP from tomato (*Lycopersicon esculentum*) is one of the best characterized aminopeptidases of broad specificity. At least two distinct LAPs have been identified which seem to have different expression patterns and roles. The best-known enzyme (LAPa-A) is a wound-induced metallo aminopeptidase which preferentially hydrolyses substrates having N-terminal Leu, Arg or Met residues and with a homo-hexamer structure (Tu *et al.*, 2003). Aminopeptidase N has also been identified in cucumber (*Cucumis sativus* L. *suyo*) and *Arabidopsis thaliana*. This is a metalloenzyme with similar specificity and sequence homology to that of aminopeptidases N which is classified into family M1 (Yamauchi *et al.*, 2001). Among the aminopeptidases of narrow specificity, methionine aminopeptidases have also been identified in diverse plant species such as *Arabidopsis thaliana*, and are required for normal plant development (Ross *et al.*, 2005). Aminopeptidase P has been identified in tomato (*Lycopersicon esculentum*) and is more than 40% identical to mammalian aminopeptidase P. It hydrolyses the amino terminal X-Pro bonds of bradykinin and also shows some endoproteolytic activity (Hauser *et al.*, 2001).

4. CATALYTIC MECHANISM AND STRUCTURE OF AMINOPEPTIDASES

4.1. Metalloaminopeptidases

Metalloaminopeptidases, which constitute the largest group of aminopeptidases, are hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule that is activated by a divalent metal cation (Barret *et al.*, 2004). Some aminopeptidases require a single metal ion for catalysis (*e.g.* MAP) while others require two metal ions (*e.g.* LAP from bovine lens; Lowther and Matthews, 2002). The known metal ligands of metallopeptidases are His, Glu, Asp or Lys residues. In addition to these metal ligands at least one additional residue, which can be Glu, Lys or Arg, is required for catalysis (Barret *et al.*, 2004). Despite the differences in structure and metal centres among the metalloaminopeptidases, overall they utilize a

similar reaction mechanism. The carbonyl group of the substrate binds to the active site interacting with metal site 1 and a conserved enzyme residue. The N-terminus of the substrate also interacts either with metal site 2 or with one or more acidic enzyme residues. The scissile peptide bond is attacked by a solvent molecule that has been activated by its interaction with the metal ion and an enzyme residue that functions as a general base. Breakdown of the intermediate is most likely promoted by the addition of a proton to the leaving amino group donated by the general base. Differences in the binding pockets are responsible for the differences in substrate specificity, being broad or restrictive. Conserved amino acid side chains and the backbone atoms that are adjacent to the metal centre also provide key interactions. The oligomeric nature of some of the active enzymes also appears to be important for substrate specificity (Lowther and Matthews, 2002; Holz *et al.*, 2003).

Metalloaminopeptidases have been subdivided into six clans (MA, MF, MG, MH, MN and MQ) on the basis of their folds, their active site architectures and the identities of active metal ions (see Rawling *et al.* in this volume). The best-known metalloaminopeptidases are found in clans MA (subclan MA(E)) – those enzymes which have only one catalytic metal ion -, and MF and MG, which have co-catalytic metal ions.

Amongst others, subclan MA(E) contains zinc-dependent peptidases which belong to peptidase family M1 and include bacterial lysyl aminopeptidase (PepN), and the mammalian enzymes membrane alanyl aminopeptidase (aminopeptidase N) and leukotriene A4 hydrolase, the latter possessing aminopeptidase and epoxyhydrolase activities. The peptidases of family M1 have a conserved His-Glu-X-X-His (HEXXH) motif involved in catalysis; they are also dependent on a single zinc ion for activity. The catalytic zinc ion is bound by the two histidines in the motif and the glutamate is a catalytic residue. The tertiary structures of members of this family show a two-domain structure with the active site in the cleft between them (Turner *et al.*, 2004). The structure of leukotriene A4 hydrolase has been solved revealing a three-domain protein in which the catalytic domain is the middle one. This domain contains an antiparallel β -sheet and α -helices, similar to that of thermolysin which is the type example of subclan MA(E) (Thunnissen *et al.*, 2001).

Clan MF is comprised of peptidase family M17 that includes LAPs from eukaryotes and bacteria (PepA). These enzymes require co-catalytic metal ions for activity (Barret *et al.*, 2004). The three-dimensional structure of bovine lens LAP has been solved (Burley *et al.*, 1990; Kim *et al.*, 1993; Cappiello *et al.*, 2006), revealing that the protein is a homohexamer and that each monomer contains two domains: the N-terminal and the catalytic C-terminal domain, the latter containing the metal centre. Both domains contain α and β structures, with β -sheets in an $\alpha/\beta/\alpha$ layering. The monomers within the hexamer are arranged as two layers of trimers. The two metal ions Zn1 and Zn2 are coordinated by the side chains of conserved amino acid residues of the enzyme (Lipscomb and Sträter, 1996). Zn2 binds the N-terminus of the substrate; Zn1 is also thought to provide critical binding and stabilizing interactions for the substrate and transition stages (Sträter and Lipscomb, 1995). The three-dimensional structure of the *E. coli* enzyme (Fig. 1)

has also been solved showing a hexameric quaternary structure similar to that of bovine lens LAP, but containing two manganese ions in the active site (Sträter *et al.*, 1999).

Clan MG includes peptidases of family M24 which is itself split into two subfamilies: M24A which includes the methionyl aminopeptidases and M24B which includes the aminopeptidase P and prolidase (X-Pro dipeptidase or PepQ) type peptidases. They have two cobalt or two manganese ions in their active centres. The narrow specificity of these enzymes is related with a common pitta-bread fold which contains a metal centre flanked by well-defined substrate binding pockets (Bazan *et al.*, 1994). The structure of the *E. coli* enzyme revealed the two metal ions to be sandwiched between two β -sheets surrounded by four α helices, yielding a structure with pseudo-2-fold symmetry (Roderick and Matthews, 1993). The restricted specificity suggests that these enzymes play roles in regulatory processes rather than in general protein degradation (Lowther and Matthews, 2002).

Figure 1. Overall structure of hexameric *E. coli* leucyl aminopeptidase (Sträter *et al.*, 1999)

4.2. Cysteine and Serine Aminopeptidases

Cysteine and serine aminopeptidases have no ionic co-factors associated with their structures. Catalysis requires a highly reactive cysteine or serine residue. In both cases the reaction begins with a nucleophilic attack on the carbon of the carbonyl group involved in the peptide bond of the substrate. In the case of cysteine aminopeptidases the attack is made by the sulphur of the sulphhydryl group whereas in the serine aminopeptidases the attack is made by the oxygen of the hydroxyl group (Gonzales and Robert-Baudouy, 1996). These types of enzymes are less abundant than the metalloaminopeptidases and include cysteine peptidases of relatively broad specificity such as bleomycin hydrolase and PepC, and serine peptidases of narrow specificity such as proline-specific peptidases (PepI or prolyl aminopeptidase, PepX and DPP IV).

Cysteine aminopeptidases are included in clan CA and family C1B. They show the signature sequences of the catalytic site of the papain superfamily, and the amino acid residues important for catalysis (Gln, Cys, His, and Asn/Asp). The crystal structures of yeast bleomycin hydrolase (GAL6) and the human enzyme have been solved and show overall similarity (Zheng *et al.*, 1998; Joshua-Tor *et al.*, 1995; O'Farrell *et al.*, 1999). The proteins are hexameric, the six identical subunits forming barrel structures with the active sites embedded in a prominent central channel (Zheng *et al.*, 1998). The monomers have a papain-like polypeptide fold as the core, with additional structural and functional modules inserted into loop regions. The crystallographic model of *Lactococcus lactis* PepC reveals that it is a homohexamer the subunits of which leave a narrow channel restricting the access to peptides. The projection of the C-terminal arm into the active site is a major difference relative to papain which, together with the overall architecture of the hexamer, limits the access to the active site cleft and may explain why peptidase activity observed *in vitro* has been restricted to small peptides (Joshua-Tor *et al.*, 1995). This carboxyl-terminal arm, also conserved in bleomycin hydrolases, is critical for oligomerization and aminopeptidase activity but not for endopeptidase activity (Mistou *et al.*, 1994; Joshua-Tor *et al.*, 1995; Mata *et al.*, 1999).

Serine aminopeptidases do not belong to the main group of serine proteolytic enzyme families represented by trypsin and subtilisin. Peptide sequence analysis revealed that both prolyl aminopeptidase (PIP), PepI, DPP IV and PepX contain a catalytic triad which consists of Ser, His and Asp, and are related to prolyl oligopeptidases (Engel *et al.*, 2005). The three-dimensional structures of the PIPs of several bacteria have been solved (Yoshimoto *et al.*, 1999; Engel *et al.*, 2005). The PIP protein is folded into two contiguous domains. The larger domain shows the general topology of the α/β hydrolase fold, with a central eight-stranded β -sheet flanked by two helices and the 11 N-terminal residues on one side, and by four helices on the other. The smaller domain is located above the larger and consists of six helices (Fig. 2). The catalytic triad (Ser 113, His 296, and Asp 268) is located near the large cavity at the interface between the two domains. The residues which make up the hydrophobic pocket line the smaller domain, and the specificity of the exo-type enzyme originates from this smaller domain (Yoshimoto *et al.*, 1999).

Figure 2. Monomer of prolyl aminopeptidase from *Serratia marcescens* showing its two distinct domains: the larger α/β domain in the bottom part of the figure and the smaller one, composed of six helices, on top (Yoshimoto *et al.*, 1999)

The crystal structures of lactococcal PepX and a number of mammalian DPP IV enzymes have also been solved as a result of the interest generated by their key roles in diverse regulatory processes and the therapeutic potential of DPP IV inhibitors (Engel *et al.*, 2003). The mammalian enzyme is an α/β -hydrolase that is secreted as a mature monomer but requires oligomerization to display normal proteolytic activity. Each monomer (Fig. 3) consists of an N-terminal β -propeller domain and an α/β -hydrolase domain enclosing an internal cavity that harbours the active site. The cavity is connected with the external environment through two different openings, the “propeller opening” and a “side opening” (Engel *et al.*, 2005). The lactococcal enzyme is a homodimer with 2-fold symmetry. It folds into four distinct contiguous domains. The α/β -hydrolase fold is the largest domain and contains the catalytic site. The shortest domain is involved in oligomerization and binding specificity (Chich *et al.*, 1995).

5. INDUSTRIAL APPLICATIONS OF AMINOPEPTIDASES

5.1. The Pharmaceutical Industry

Aminopeptidases play important roles in diverse cellular processes. As a consequence, pharmaceutical applications are being directed to control their activity in pathophysiological processes as well as the development of diagnosis tools and markers of physiological pathways (Brown, 2005). Most of the applications

Figure 3. Mammalian (pig kidney) dipeptidyl peptidase (Engel *et al.*, 2003). Panel **A** shows a view of the protein facing the N-terminal β -propeller domain. Panel **B** represents the protein from a perpendicular orientation showing the β -propeller domain in the upper part and α/β domain harbouring the catalytic site at the bottom

are oriented to the design of inhibitors for specific aminopeptidases. Selective inhibitors of glutamyl aminopeptidase (aminopeptidase A) constitute potential anti-hypertensive agents due to the role of this enzyme in the conversion of angiotensin II into angiotensin III, which plays an essential role in control of arterial blood

pressure (Cogolludo *et al.*, 2005; Inguibert *et al.*, 2005). The design of inhibitors of methionine aminopeptidases is also considered to be of therapeutic potential due to the role of these enzymes in angiogenesis and tumour growth (Selvakumar *et al.*, 2005; Zhong and Bowen, 2006). Inhibitors of the expression of alanyl aminopeptidase (aminopeptidase N), which is deregulated in inflammatory diseases, cancer, leukaemia, diabetic nephropathy and rheumatoid arthritis, are also being developed to try to control these disorders (Bauvois and Dauzonne, 2006; Ansorge *et al.*, 2006). The design of inhibitors of DPP IV and related proline-specific peptidases is currently under investigation since these enzymes are involved in peptide metabolism of members of the PACAP/glucagon peptide family, neuropeptides and chemokines. The most promising applications of these agents are in the treatment of type 2 diabetes and immunological disorders (Augustyns *et al.*, 2005; Mest, 2006). The inhibition of other aminopeptidases such as PepX (involved in infections by *Streptococcus gordonii*), the stereospecific DppA aminopeptidase (involved in peptidoglycan synthesis) and methionyl aminopeptidase, also constitute potential pharmaceutical targets to control microbial infections (Holz *et al.*, 2003; Rigolet *et al.*, 2005; Schiffmann *et al.*, 2006).

5.2. Biotechnological and Food Industrial Applications

One of the main industrial applications of aminopeptidases and their microbial producer strains is the manufacture of protein hydrolysates and protein-rich fermented products derived from soy, meat, milk, cereals, *etc.* (Meyer-Barton *et al.*, 1994; Suchiibun *et al.*, 1993; Chevalet *et al.*, 2001; Scharf *et al.*, 2006). Food protein hydrolysates are manufactured for diverse purposes such as the fortification of foods and beverages, the elaboration of pre-digested ingredients for enteral/parenteral nutrition, and the generation of bioactive peptides and healthcare products (FitzGerald and O'Cuinn, 2006). The use of aminopeptidases in these industrial processes not only contributes to the improvement of nutritional value but also the flavour of the final product by promoting the degradation of hydrophobic peptides which have undesirable tastes and the release of other peptides of agreeable taste characteristics and free amino acids. The application of these strategies to cheese ripening has been thoroughly investigated due to the high content of hydrophobic amino acid residues (*e.g.* proline) present in milk caseins (Meyer-Barton *et al.*, 1994; Savijoki *et al.*, 2006). The use of proline-specific peptidases together with aminopeptidases of broad specificity (*e.g.* LAP) has been especially successful in the food industry (Raksakulthai and Haard, 2003). Some of the commercial aminopeptidases that are used to reduce bitterness in food are LAPs from lactic acid bacteria, *Rhizopus oryzae*, *Aspergillus oryzae* and *Aspergillus sojae* (Nampoothiri *et al.*, 2005). The use of lactic acid bacteria expressing specific peptidase activities during food protein processing is also being explored for reducing the levels of toxic and allergenic epitopes present in milk and cereal proteins (Di Cagno *et al.*, 2004). A similar approach has also been used for the generation of bioactive peptides with antihypertensive, immunomodulatory and

antimicrobial properties (Meisel, 2004). Recently, the peptidases of *Lactobacillus helveticus* R211 and R389 have been found to generate casein-derived peptides that inhibit the angiotensin converting enzyme and are active *in vivo* (Leclerc *et al.*, 2001; Seppo *et al.*, 2003).

The application of combinations of peptidases to hydrolyse collagen for cosmetic uses has also been developed (Shigeri *et al.*, 2005). In addition, thermostable high-activity aminopeptidases constitute alternatives for biotechnological applications such as the processing of recombinant proteins (Gilboa *et al.*, 2001).

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