



Therapeutic and biotechnological applications of substrate specific microbial aminopeptidases

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Abstract

Aminopeptidases (EC 3.4.11.) belongs to exoprotease family, which can catalyze the cleavage of peptide bond which connects the N-terminal amino acid to the penultimate residue in a protein. Aminopeptidases catalyze the process of removal of the N-terminal amino acids of target substrates by sequential cleavage of one amino acid residue at a time. Microbial aminopeptidase are of great acceptance as industrial enzymes with varying applications in food and pharma industry since these enzymes possess unique characteristics than aminopeptidases from other sources. This review describes the various applications of microbial aminopeptidases in different industrial sectors. These enzymes are widely used in food industry as a debittering agent as well as in the preparation of protein hydrolysates. In baking, brewing, and cheese making aminopeptidases are extensively used for removing the bitterness of peptides. The inhibitors of these enzymes are found great clinical applications against various diseases such as cancer, diabetes, and viral infections. Aminopeptidases are widely used for the synthesis of biopeptides and amino acids, and found to be efficient than chemical synthesis. These enzymes are capable of hydrolyzing organophosphate compounds, thus having biological as well as environmental significance.

Key Points

- Cleaves the amino-terminal amino acid residues from proteins and peptides.
- Microbial aminopeptidase are of great acceptance as both therapeutic and industrial enzyme.
- Review describes the potential applications of microbial aminopeptidases.

Keywords Debittering · Microbial aminopeptidases · Therapeutic enzyme · Peptide synthesis · Protein hydrolysate

Introduction

Microbial aminopeptidases—an overview

Enzymes that catalyze the hydrolysis of peptide bonds in protein are referred to as proteases or peptidases. The peptide bonds present in between amino acids of proteins are cleaved by specific proteases. Aminopeptidases (APs; EC 3.4.11) are a class of proteases that catalyze the cleavage of the amino-terminal amino acid residues from proteins and peptides.

These are exopeptidases and are widely distributed in prokaryotic and eukaryotic organisms, found in subcellular organelles, in the cytoplasm, and as membrane components (Jankiewicz and Bielawski 2003). Aminopeptidases form a large enzyme family in microbial world (Gonzales and Robert-Baudouy 1996) and are important in many biological functions such as post translational modification of proteins, its breakdown and maturation; thus, they perform both regulatory and housekeeping functions (Chandu and Nandi 2003; Taylor 1993a)). Aminopeptidases are predominantly present in cells where they have important roles in the processing of newly synthesized proteins, breakdown of peptide hormones, and the processing of various enzymes. Microbial aminopeptidases have major roles in the utilization of external protein substrates present in the medium as a source of essential amino acids (Gonzales and Robert-Baudouy 1996). Removal of the amino-terminal methionine by methionine aminopeptidase during protein translation is considered as a typical aminopeptidase activity occurring in the cells (Bradshaw et al. 1998; Li and Chang 1995).

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Aminopeptidases are classified into three major distinct classes based on the number of amino acids cleaved from polypeptide chains, substrate specificity, and sensitivity to various protease inhibitors (Fig. 1). Aminopeptidases which hydrolyze the first peptide bond in a polypeptide chain releasing a single amino acid residue are called aminoacylpeptidases (EC 3.4.11); some aminopeptidases remove dipeptides or tripeptides from polypeptide chains are named as dipeptidyl- and tripeptidyl peptidases (EC 3.4.14). Another class of enzymes which only act on di- or tripeptides are called dipeptidases (EC 3.4.15) and tripeptidases (EC 3.4.14.4). A novel prolyl tri/tetra-peptidyl aminopeptidase from *Streptomyces mobaraensis* that removes the tetra-peptide of pro-transglutaminase was reported (Umezawa et al. 2004). Aminopeptidases are further classified into two categories based on substrate specificity such as broad and narrow. Differences in the catalytic site of enzymes and enzyme binding pockets of substrates are majorly responsible for the substrate specificity of aminopeptidases, thus there are broad or narrow substrate specific aminopeptidases are present in microorganisms (Lowther and Matthews 2002; Holz et al. 2003). Based on the N-terminal amino acid specificities of aminopeptidases at the substrate site there are substrate specificities existing for prolyl aminopeptidase, X-prolyl dipeptidyl aminopeptidase, alanine aminopeptidase, methionyl aminopeptidase, arginine aminopeptidase, lysine aminopeptidase, leucine aminopeptidase, phenylalanine aminopeptidase, and so on. Based on catalytic mechanism and sensitivity to various protease inhibitors aminopeptidases are classified into metallo, cysteine, and serine peptidases. Metallo aminopeptidase are the largest group of aminopeptidases and are inhibited by metal-chelating agents such as EDTA, EGTA, and 1,10-phenanthroline. Cysteine aminopeptidase are inhibited by Hg^{2+} , iodoacetamide, N-ethylmaleimide, and *p*-

chloromercuribenzoate. Serine aminopeptidase are sensitive to phenyl methyl-sulfonyl fluoride and diisopropyl fluorophosphates. There are no ionic co-factors seen associated with cysteine and serine aminopeptidases, and its catalysis requires a highly reactive cysteine or serine residue. Among organisms, this class of aminopeptidases is found less diversified than metalloaminopeptidases. This includes cysteine peptidases of relatively broad specificity such as PepC and bleomycin hydrolase, and narrow specificity serine peptidases such as proline-specific peptidases (prolyl aminopeptidase or PepI, PepX, and dipeptidyl peptidase IV) (Gonzales and Robert-Baudouy 1996).

It has been observed that various cellular compartments of microbial cells possess aminopeptidase activity. A large fraction of these enzymes are found in soluble forms in the cytoplasm or seen associated with in the cell wall or secreted into the exterior environment (Goldberg et al. 1997). A signal sequence which is a characteristic of exported proteins is present at the N-terminal end of an extracellular leucine aminopeptidase of *A. proteolytica* has been identified (Guenet et al. 1992). The microorganisms which are reported to be producing substrate specific aminopeptidase are mainly belong to genus *Aspergillus* (Matsushita-Morita et al. 2010), *Streptomyces* (Rahulan et al. 2009; Nandan et al. 2011; Wu et al. 2010), *Pseudomonas* (Wu et al. 2014), lactic acid bacteria (Tchorbanov et al. 2011), and *Bacillus* (Shen et al. 2011). Different classes of substrate specific extracellular aminopeptidases are produced from *Streptomyces griseus* (Hershcovitz et al. 2004), *Streptomyces gedenensis* (Rahulan et al. 2009), *Streptomyces lavendulae* (Nandan et al. 2011), *Aeromonas proteolytica* (Holz 2002), the filamentous fungi *Aspergillus oryzae* (Huang et al. 2015), and *Aspergillus sojae* (Chien et al. 2002). Cytoplasmic and soluble aminopeptidases are

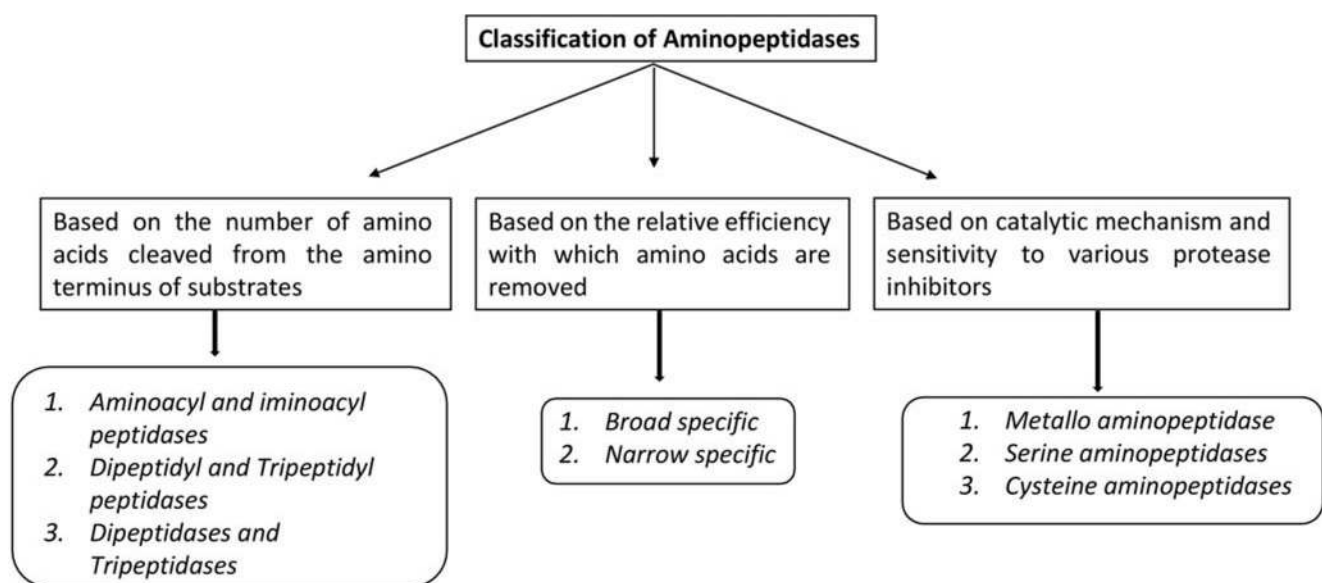


Fig. 1 Classification of aminopeptidases

reported from various *Lactobacillus* strains (Nandan et al. 2010; Vesanto et al. 1994; Klein et al. 1994). Aminopeptidases from lactic acid bacteria are of industrially important since they are widely used in food industry (Pan and Tanokura 2004; Choi et al. 1996).

Current research on aminopeptidases mainly focused on gene cloning and expression, protein purification and characterization, catalytic mechanisms, and in silico evaluations (Arima et al. 2004; Arima et al. 2006a; Sonoda et al. 2009; Nandan and Nampoothiri 2014; Arif et al. 2018; Labrie et al. 2019). The gene sequences of aminopeptidases and its biochemical functions are two determining factors of the substrate specificity of aminopeptidases (Rawlings et al. 2004). Numerous substrate-based library screening methods have been developed for the fast and unflinching determination of specificity of enzymes (Backes et al. 2000; Harris et al. 2000; Choe et al. 2006). A fast and reliable evaluation of the substrate specificity of individual aminopeptidase was developed by Drag et al. (2008) using solid phase chemistry with the substrate 7-amino 4-carbamoylmethylcoumarin fluorophore. Aminopeptidases are still an ongoing topic of research with its role already connected in explaining various vital processes such as protein processing and turnover, tissue invasion, regulation of peptide hormone synthesis, viral infections, and plant defense responses with reasonable confidence. Various attempts have been undertaken to study and determine the substrate specificity of aminopeptidases. One such attempt uses sequence similarity, which has its primary deficiency due to the lack of availability of sequence signatures (Petrovic et al. 2007). The mechanism of catalysis in aminopeptidase needs to be carefully studied for the successful utilization of the enzymes in the industry. Innovative technologies using recombinant DNA and site-directed mutagenesis have been raised for the development of stable aminopeptidases with improved substrate specificity (Nandan and Nampoothiri 2017a).

Applications of microbial aminopeptidases

Aminopeptidases have considerable applications in various fields because of their wide range substrate specificity, inflexible enantioselectivity, and high thermal stability (Arima et al. 2006b). As illustrated in Fig. 2, food industry is the primary sector which recognized and utilized the substrate-specific microbial aminopeptidases.

Therapeutic application

Aminopeptidases play important roles in diverse cellular processes such as protein modification, protein degradation, cell-cycle control, and hormone level regulation. Therefore, these enzymes play a significant role in many pathophysiological conditions from infections to cancer (Taylor 1993a; Taylor

1993b). Microorganisms are the major sources of high yield production of medically important aminopeptidases with economic feasibility. Pharmaceutical applications of aminopeptidases are directed to control the pathophysiological effects, in a way helps in the development of diagnostic tools such as biomarkers of these physiological pathways. Microbial aminopeptidases are potential targets for structure based drug design since these enzymes have high economic feasibility, high yields, regular availability, ease of modification, and high catalytic efficiency. Microbial aminopeptidases and their human counterparts share structural and functional similarity. The studies on the mechanism of co-catalytic active site of aminopeptidases are helpful in designing the new inhibitors of aminopeptidases acting as pharmaceuticals (Table 1) (Holz 2002; Lowther and Matthews 2002).

Bacterial methionine aminopeptidases have been implicated as potential targets for developing broad spectrum antibacterial drugs (Helgren et al. 2016; Schiffmann et al. 2006). Inhibitors of methionine aminopeptidase are having therapeutic function since they are reported to have regulatory roles in angiogenesis and tumor progression (Selvakumar et al. 2005; Zhong and Bowen 2006). The inhibitors of glutamyl aminopeptidase (aminopeptidase A) act as potential antihypertensive compounds, thus mediating the conversion of angiotensin II into angiotensin III, which ultimately regulating the arterial blood pressure (Cogolludo et al. 2005; Inguibert et al. 2005). *Lactobacillus delbrueckii* is an efficient producer of aspartyl/glutamyl aminopeptidase (Stressler et al. 2016). Inhibitors of alanyl aminopeptidase (aminopeptidase N) are being used in the treatment and regulation of inflammatory diseases, cancer, leukemia, diabetic nephropathy, and rheumatoid arthritis (Bauvois and Dauzonne 2006; Ansorge et al. 2006). Aminopeptidase N acts as a membrane receptor for many types of corona viruses in human host (Fehr and Perlman 2015). The inhibitors of these membrane-bound aminopeptidases can function as antiviral agents, and thus can be considered as a treatment strategy for the viral diseases. It has been reported that *E. coli* is a potent microbial producer of aminopeptidase N, and its crystal structure revealed that methionine 260 serves as a substrate recognition residue (Ito et al. 2006). The design of inhibitors of dipeptidyl peptidase IV (DPP IV) and related proline-specific aminopeptidases are used in the treatment of type 2 diabetes and immunological disorders (Augustyns et al. 2005; Mest 2006). The inhibition of X-prolyl dipeptidyl aminopeptidase (PepX) is helping in treating against infection by *Streptococcus gordonii* (Goldstein et al. 2001). Rigolet et al. (2005) conducted a comparative study between the structures of *Lactococcus lactis* PepX and its human counterpart. They have identified the key residues and projected them as drug targets since PepX is involved in many bacterial infections. Dipeptidyl peptidase and aminopeptidases from periodontopathic rods inhabiting human oral microflora are useful diagnostic tool by acting as markers of

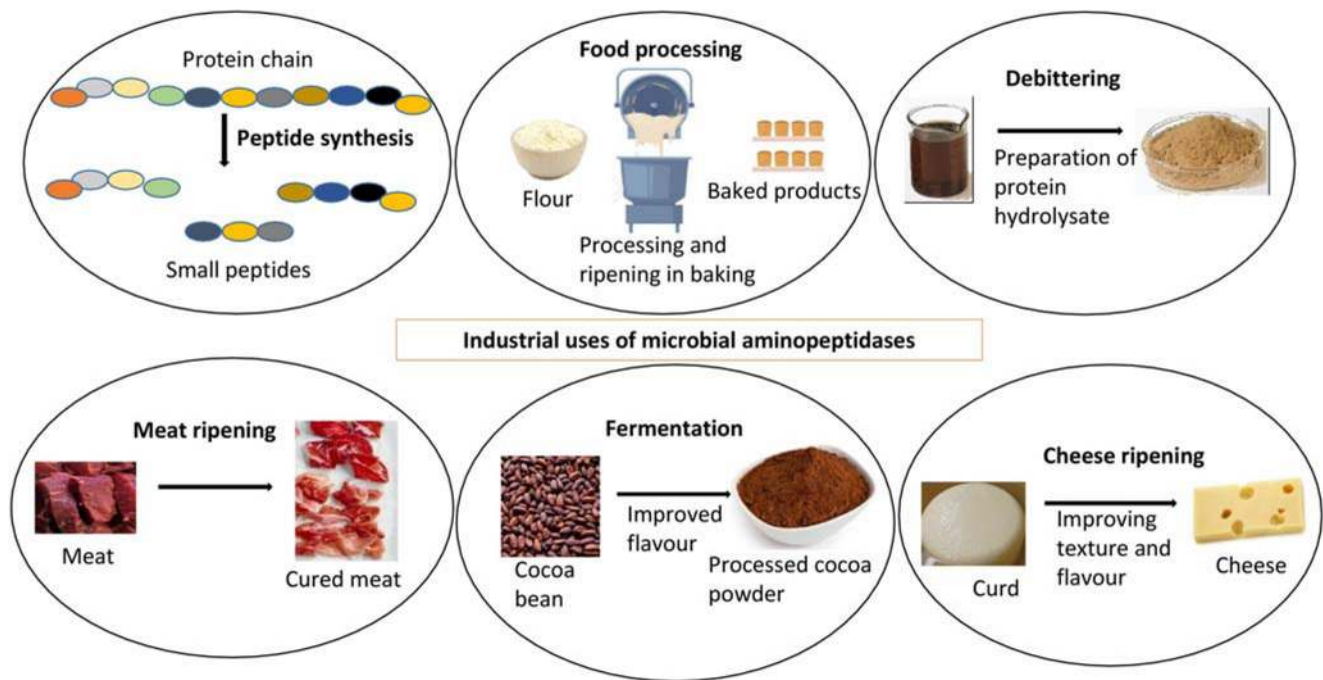


Fig. 2 Illustration of various industrial applications of microbial aminopeptidases

periodontopathic bacteria (Nemoto et al. 2018). An antibiotic of microbial origin called bestatin act a potent inhibitor of most of the aminopeptidases including alanine aminopeptidase, cystinyl aminopeptidase, aminopeptidase B, and aminopeptidase N (Scornik and Botbol 2001). Apstatin, an inhibitor of a membrane-bound aminopeptidase P function as a vasodilator by potentiating the effect of bradykinin. Bradykinin is a neuropeptide having role in the blood vessel dilation and is cleaved by aminopeptidase P (Maggiore et al. 1999). Bacterial aminopeptidases are also perfect enzymes for structure–function analysis, since they can efficiently produce and purified as recombinant proteins. A novel immune regulatory function for *Streptococcus pneumoniae* aminopeptidase in generating CD8+T cell population with reduced effector function gave rise to the possibility of direct regulation by pneumococcal components. This study elucidates a novel mechanism by which these enzymes can directly modulate host T cell effector function and may play a vital role in pneumococcal disease (Blevins et al. 2017).

Bioactive peptide synthesis

Enzymatic peptide synthesis presents a useful and helpful strategy because it can perform specified reactions under milder conditions than those used in chemical synthesis. The peptide synthesis applications of microbial aminopeptidases are already been reported in literature (Table 2). From the view point of biotechnology, proline aminopeptidases might be a perfect tool for synthesizing peptides containing proline by catalyzing aminolysis reaction. The application of proline aminopeptidase

(PAP) from *Streptomyces thermoluteus* in synthesizing proline containing peptides was demonstrated using variants of PAP (Yamamoto et al. 2010). Proline containing peptides are reported to be having nutraceutical properties. Studies on prolyl hydroxyproline (Pro-Hyp) proved that they can stimulate the growth of fibroblasts from mouse skin (Shigemura et al. 2009). The peptide Pro-Arg is well-known for its defensive activity against hydrogen peroxide induced cell death and other oxidative stresses. In another study, the milk peptides Val-Pro-Pro and Ile-Pro-Pro showed activity against the transmembrane aminopeptidase angiotensin-converting enzyme (ACE) (Meyer et al. 2009). Proline rich diketopiperazine such as cyclo (Pro-Pro) showed antibacterial activity against *Micrococcus luteus* and *Pseudomonas aeruginosa* (Huberman et al. 2007). An executioner caspase-3 was activated by cyclo (Pro-Phe) in HT-29 cells was studied (Brauns et al. 2005). Whey protein hydrolysate treated with exopeptidases showed ACE inhibitory activity, thus reducing systolic pressure in spontaneously hypertensive rats (Cheung et al. 2015). ACE inhibitory peptide generation from skimmed milk hydrolysate using aminopeptidase was explained by Pan et al. (2005). Since majority of bioactive peptides are produced by exopeptidases such as aminopeptidases, the impact of these enzymes in determining the properties of these bioactives are of much concern. The bioactive peptide profiles of three European dry fermented sausages were analyzed and explained the role of microbial peptidases in producing bioactive peptides (Gallego et al. 2018). Many intracellular peptidases have been reported from the starter cultures such as *L. sakei*, *L. helveticus*, *L. delbrueckii*, *L. curvatus*, *L. plantarum*, *L. brevis*, *L. casei*, and *L. paracasei*. These cultures have been

Table 1 List of microbial aminopeptidases reported as potential targets of enzyme inhibitors

Targeted aminopeptidase	Functions	Reference
Glutamyl aminopeptidase (aminopeptidase A)	Act as potential antihypertensive compounds thus mediating the conversion of angiotensin II into angiotensin III	Cogolludo et al. 2005; Inguibert et al. 2005; Stressler et al. 2016
Methionine aminopeptidase	Regulatory roles in angiogenesis and tumor progression, antibacterial drug development	Helgren et al. 2016; Selvakumar et al. 2005; Zhong and Bowen 2006
Alanyl aminopeptidase (aminopeptidase N)	Treatment and regulation of inflammatory diseases, cancer, leukemia, diabetic nephropathy, rheumatoid arthritis, and antiviral agents	Bauvois and Dauzonne 2006; Ansorge et al. 2006; Fehr and Perlman 2015; Ito et al. 2006
Proline-specific aminopeptidase	Treatment of type 2 diabetes and immunological disorders	Augustyns et al. 2005; Mest 2006
X-prolyldipeptidyl aminopeptidase (PepX)	Treatment against infection by <i>Streptococcus gordonii</i>	Goldstein et al. 2001; Rigolet et al. 2005
Aminopeptidase P	Reducing hypertension	Maggiora et al. 1999
Aminopeptidase from <i>Streptococcus pneumonia</i>	Treatment against pneumococcal disease	Blevins et al. 2017

reported to exert high exopeptidase activity by producing enzymes such as dipeptidase, tripeptidase, aminopeptidase, arginine aminopeptidase, PepA, PepX, PepP, and X-prolyl dipeptidyl peptidase etc, thus showed an increased amount of bioactive peptides in the medium (Toldra et al. 2018).

Degradation of organophosphorus compounds

Organophosphorus poisoning is considered as a major health problem across the world. These compounds are known for their toxicological effects such as the inactivation of the enzyme acetylcholinesterase and the subsequent loss of nerve function. Thus, the safety and protection from the detoxification process of these compounds is of much concern to the general public. Scientists are looking for an efficient ecofriendly biodegradation method for the efficient removal of these compounds from the environment. Various microbial sources are explored for the biological degradation of organophosphate pesticides (Sidhu et al. 2019). Organophosphorous acid anhydrolase isolated from *Alteromonas undina* was purified which can detoxify organophosphorous compounds has been reported (Cheng et al. 1993). Aminopeptidase P was found to catalyze the hydrolysis of a wide range of organophosphate triesters. The activity of aminopeptidase P was promoted in the presence of Mn^{2+} . Mutant proteins of aminopeptidase P was made and tested for the hydrolytic activity against organophosphorous compounds. The study concluded the structural details of aminopeptidase P to facilitate the hydrolysis of organophosphate tri esters (Jao et al. 2004). A proline-specific aminopeptidase P was found to be sharing 31% sequence similarity with the acid anhydrolase capable of degrading organophosphorus compounds was identified from *E. coli* (Singh and Walker 2006). The catalytic activities of *E. coli* aminopeptidase P showed

Table 2 Functional roles of bioactive peptides synthesized by aminopeptidases

Bioactive peptides	Functional applications	Reference
Pro-containing peptides	Nutraceutical properties	Yamamoto et al. 2010
Pro-Hyp	Stimulates growth of fibroblasts from mouse skin	Shigemura et al. 2009
Pro-Arg	Defensive activity against oxidative stress and cell damage	Meyer et al. 2009
Val-Pro-Pro	Angiotensin I-converting enzyme inhibition	Meyer et al. 2009
Ile-Pro-Pro		
Cyclo (Pro-Pro)	Antibacterial activity against <i>Micrococcus luteus</i> and <i>Pseudomonas aeruginosa</i>	Huberman et al. 2007
Cyclo (Pro-Phe)	Caspase 3 activation	Brauns et al. 2005
Bioactive peptides from skimmed milk hydrolysate	Angiotensin-converting enzyme inhibition	Pan et al. 2005
Bioactive peptides from whey protein hydrolysates	Angiotensin-converting enzyme inhibition	Cheung et al. 2015

substrate preference towards organophosphorus compounds and methylphosphonate derivatives (Fig. 3) (Hsu et al. 2008).

Food processing applications

Protein hydrolysate and debittering

Food protein hydrolysate preparation is considered as one of the leading industrial applications of aminopeptidases. Protein hydrolysates derived from milk, soy, meat, and cereals are essentially prepared by the combined action of endo and exopeptidases (Fig. 4) (Meyer-Barton et al. 1994; Chevalet et al. 2001; Scharf et al. 2006). These food protein hydrolysates are widely used for the generation of bioactive peptides with nutritional and pharmacological importance. These hydrolysates are the rich source of pre-digested ingredients which can be easily utilized during intestinal absorption (FitzGerald and O’Cuinn 2006). As previously described, the substrate specificity of aminopeptidases contributes to the hydrolysis efficiency which determines the properties of the hydrolysates which is being prepared. The substrate specificity of an aminopeptidase from *Bacillus licheniformis* was tested by checking its efficiency in hydrolyzing proteins such as peanut protein isolate and zein both with different percentages of leucine content. Peptide profiling results suggested that the enzyme is a leucine specific aminopeptidase (Lei et al. 2018). A comparative study was conducted to estimate the hydrolysis efficiency of leucine aminopeptidase from *Streptomyces gedenensis* and other two commercially available enzymes such as pepsin and trypsin. This result reflects the substrate specificity of *S. gedenensis* aminopeptidase to hydrolyse peptides with amino-terminal hydrophobic and aromatic residues is more when compared with commercial enzymes (Rahulan et al. 2012). Moreover, the studies confirmed that the bioactivity of peptide hydrolysates is determined by the amount of free fatty acids (FAAs) present (Rahulan et al. 2012). Researchers emphasized that bitterness is a major limitation in food industry which limits the consumption and utilization of food protein hydrolysates.

Aminopeptidases are capable of reducing the bitterness by increasing the degree of hydrolysis thus releasing free amino acids (Giesler et al. 2013). The debittering property of aminopeptidases was demonstrated in various studies. The degree of hydrolysis and free fatty acid contents were increased when treated with recombinant aminopeptidase (alcalase) from *A. sojae* GIM3.30. Thus, it reduced the bitterness of casein and soy protein hydrolysates (Huang et al. 2015). An aminopeptidase from *B. licheniformis* improves the hydrolysis and debittering efficiency of soy protein isolate (Lei et al. 2017). Bacterial strains such as *Bacillus* spp., *Streptomyces* spp., *Aeromonas* spp., and various fungal strains are producers of leucine aminopeptidases whose debittering activity was extensively studied (Stressler et al. 2013; Nampoothiri et al. 2005; Lin et al. 2004). Furthermore, aminopeptidases from *A. oryzae* and *A. sojae* are regarded as food safe enzymes and have a long history of use as debittering enzymes. Hydrolysis with *A. sojae* recombinant leucine aminopeptidase increased the degree of hydrolysis thereby decreased the bitterness of casein and soy protein hydrolysates (Huang et al. 2015). Extracellular proteome analysis of *A. oryzae* during soy sauce fermentation using iTRAQ method resulted in the identification of dipeptidase, dipeptidyl aminopeptidase, and leucine aminopeptidase. These enzymes have important implications for soy sauce fermentation (Zhao et al. 2018). The debittering effect of *Aeromonas cavieT-64* is reflected in the hydrolysis of milk casein and soy protein. The bitterness of the solutions were remarkably reduced by the release of free amino acids (Izawa et al. 1997). The release of free amino acids such as tyrosine, phenyl alanine, leucine, isoleucine, and valine had reduced the bitterness of milk protein and soy protein hydrolysate when treated with aminopeptidases from the fungal strain *Grifola frondosa* (Nishiwaki et al. 2002). Aspartyl aminopeptidases have specificity towards N-terminal aspartic and glutamic acids had been isolated from *A. oryzae*, yeast, *E.coli*, and *L. delbrueckii*. Food proteins rich with the glutamyl or aspartyl group when treated with aspartyl aminopeptidases from *A. oryzae* leads to the production of flavored hydrolysates by the release of free aromatic amino acids

Fig. 3 *E. coli* Aminopeptidase P catalyzed hydrolysis of methylphosphonate derivatives

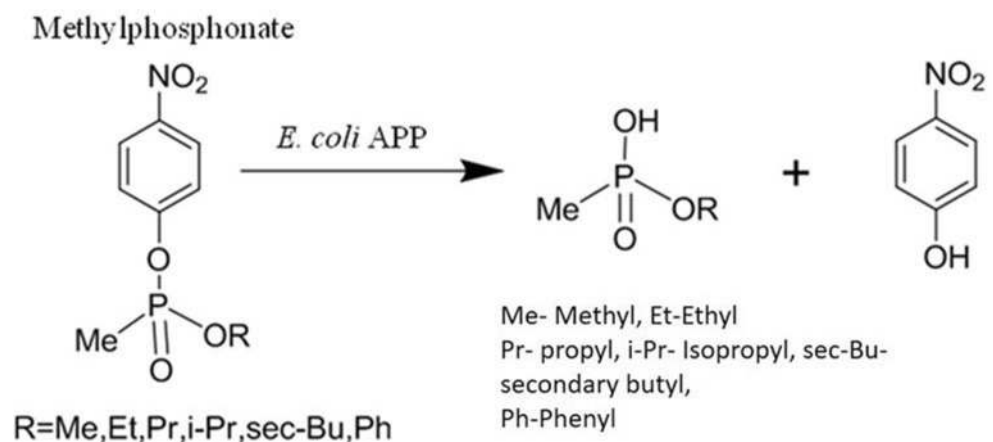
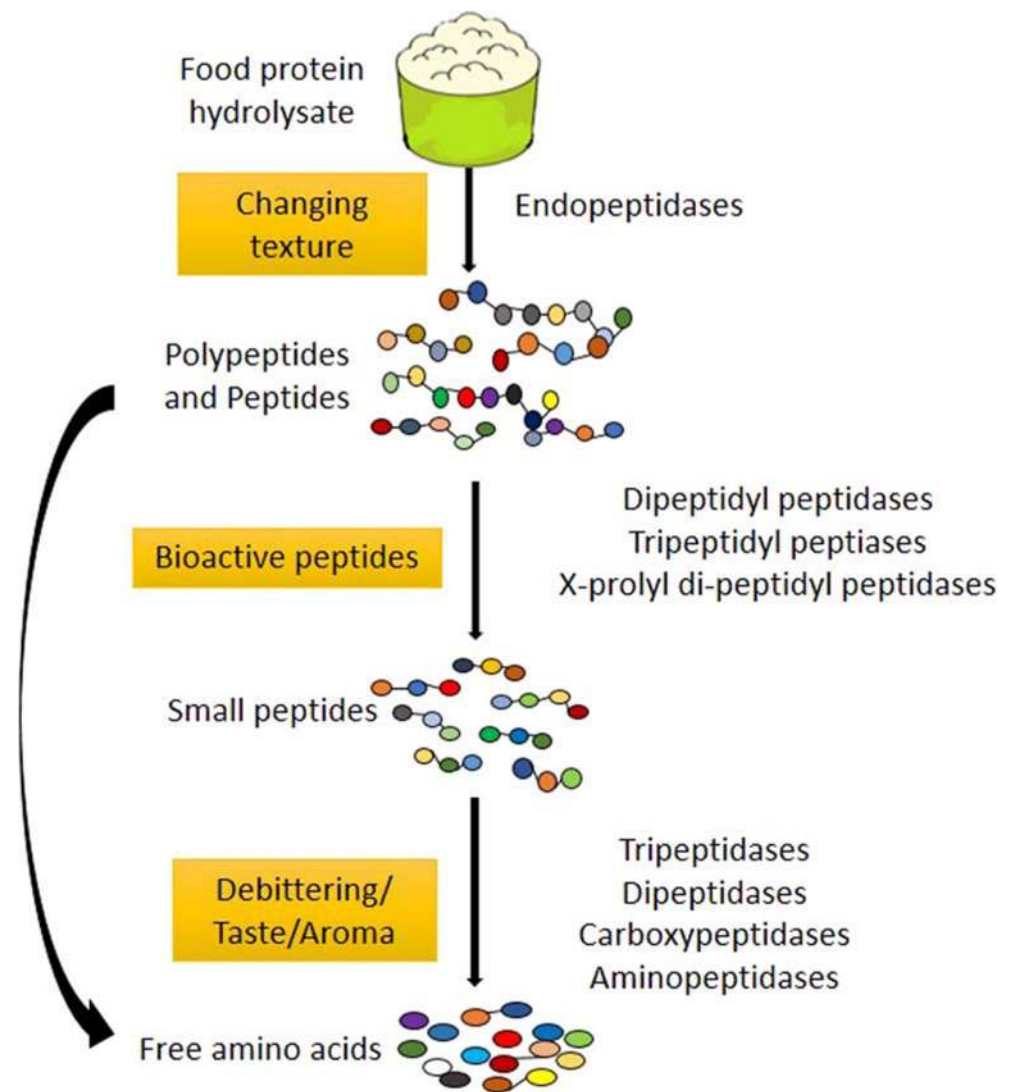


Fig. 4 Schematic representation of the enzymes involved in the process of food protein hydrolysis



(Kusumoto et al. 2008; Yokoyama et al. 2006; Watanabe et al. 2007). Salt tolerant aspartyl aminopeptidase was isolated from *A. oryzae* (Gao et al. 2018). Aspartyl aminopeptidase can also contribute to the umami taste production of hydrolysate from wheat gluten, casein, and fermented soy products (Stressler et al. 2016).

Cured meat products

Dry-cured meat products are prepared by various processes involving drying and ripening of meat. Aminopeptidase appears to be responsible for the increase in free amino acids during dry-curing. In an analysis of proteases in the fresh pork muscle during ripening, the release of free amino acids from bitter peptides were found to be greater in number (Virgili et al. 1998). The overall process of meat protein processing involved the use of both endopeptidases and exopeptidases. During processing, meat proteins are extensively hydrolyzed

by muscle endopeptidases followed by exopeptidases. Endopeptidases such as calpains and cathepsins hydrolyze the proteins into smaller peptides and oligopeptides. Exopeptidases such as aminopeptidases and carboxypeptidases are responsible for the release of free amino acids capable of contributing flavor in dry-cured products (Mora et al. 2018). Microbial starter cultures such as lactic acid bacteria, *Staphylococci*, yeasts are rich source of several peptidases like aminopeptidases, dipeptidases, and tripeptidases (Bintsis et al. 2003; Macedo et al. 2003; Bolumar et al. 2003). The prolyl aminopeptidase, arginyl aminopeptidase, and aminopeptidase I, II, and IV of cell-free extracts from *L. sakei* were proved to enhance the sensory qualities of dry-fermented sausages (Bolumar et al. 2006). *Leuconostoc mesenteroides* and *L. curvatus* strains have been reported to show elevated activity of X-prolyl dipeptidyl aminopeptidase (Zotta et al. 2007). Most of the exopeptidases are reported to be involved in the release of small peptides, free amino acids, and some bioactive

peptides. These free amino acids are contributing to the development of aroma. The proteomic profile of small peptides in dry-cured meat resulted in the identification of amino acids such as alanine, lysine, serine, tyrosine, arginine, and valine released as a result of combined action of microbial and muscle aminopeptidases (Mora et al. 2015). Free amino acids are non-volatile compounds that contribute to the improvement of both meat taste and flavor. *Micrococcus roseus* produced two intracellular aminopeptidases with affinity for nonpolar amino acids, L-Pro, and L-Arg. *M. roseus* aminopeptidase appears to release amino acids and thereby contribute to flavor development in cured bacon (Hinrichsen et al. 1994). Lactic acid bacteria proteases have also been studied in sausage products. Three *L. plantarum* strains were screened for endopeptidase and aminopeptidase activities to evaluate them as starters in sausage. Whole cells of *L. plantarum* CRL 681 generated hydrophilic peptides while whole cells when used with cell-free extract produced hydrophobic peptides from both sarcoplasmic and myofibrillar proteins. *L. curvatus*, *L. sake*, and *L. casei* are the most common microorganisms in dry-fermented sausages, and their use as starter cultures is also widespread (Sanz et al. 1999). Fadda et al. (1999) had studied the aminopeptidase activities of *L. curvatus* and *L. sake* in dry-fermented sausages. The activity of *L. curvatus* resulted mainly in the generation of large amounts of glutamic acid, b-alanine, arginine, histidine, and lysine while the activity of *L. sake* increased the levels of glutamic acid, b-alanine, g-aminobutyric acid, alanine, threonine, phenylalanine, leucine, and ornithine. In the preparation of sausages, microbial aminopeptidases such as aminopeptidase 1 and 2 from *L. sake* can contribute to the release of free amino acids. Sodium chloride was used as a curing agent which could activate arginine aminopeptidase and aminopeptidase 2 from *L. sake* (Flores et al. 1998).

Ripened cheeses

The conversion of fresh cheese curd into mature cheese is largely determined by the progress of proteolysis. For the development of an admissible cheese flavor, a well-balanced breakdown of milk protein casein into small peptides and amino acids is necessary. Casein is rich with proline residues, the peptide bond-containing proline are resistant towards most of the proteases; thus, the peptidases specific for proline has a crucial role in the degradation of casein proteins. A great variety of proline-specific aminopeptidases such as proline iminopeptidase, aminopeptidase P, and prolyl dipeptidase have been found in starter organisms commonly used in cheese manufacture. Caseins are also rich in glutamine residues which are hydrolyzed by microbial aminopeptidase A (Visser 1993). The bitterness in cheese is contributed by the medium-sized peptides resulting from the enzymatic digestion of casein. Various studies observed that the most important

role in bitterness is played by the starter cultures (Lemieux and Simard 1991). During cheese ripening aminopeptidases from the thermophilic lactic acid bacteria remains to be active throughout the process (Gatti et al. 1999). Prolyl aminopeptidases from *Penicillium camemberti* plays an important role in the ripening of Camembert-type cheese (Fuke and Matsuoka 1993). The activities of PepX and lysine aminopeptidase of *Lactococcus lactis* were found to be increased during the ripening of Saint Paulin cheese (Chapot-Chartier et al. 1994). El-Kholy et al. (1998) studied the strong aminopeptidase and dipeptidyl aminopeptidase activities of thermophilic *lactobacilli* in Ras-type cheese. Lactic acid bacterial proteolytic enzymes are particularly important in the formation of flavors during cheese ripening (Visser 1993). The meta-omics analysis data of surface ripened cheese community revealed the differential expression of proline-specific aminopeptidase gene from *Hafnia alvei* and cysteine aminopeptidase gene from *Geotrichum candidum* (Dugat-Bony et al. 2015). The optimum time for ripening of a traditional Brazilian cheese named as artisanal Minas cheese was studied and the data indicated that higher temperatures accelerate the process of cheese ripening due to the presence of high amount of aminopeptidases (Martins et al. 2015).

Fermented fish products

Fish fermentation is the conversion of organic substances into simple compounds such as peptides, amino acids, and other nitrogenous compounds either by the action of microorganisms or their endogenously producing enzymes. **Fermented fish** products are very popular particularly in Southeast Asian countries contributing significantly to the protein intake of population. These products have distinctive characteristics, mostly in terms of aroma, and texture during fermentation process. A bacterial dynamic study had isolated a total of 210 bacterial species from a dry fermented fish product Ngari. The dominant bacteria inhabited were *Staphylococcus carnosus*, *L. pobuzihii*, *Bacillus indicus*, *Enterococcus faecium*, and *Tetragenococcus halophilus* (Devi et al. 2015). Fish fermentation is a natural process mainly depends both on naturally occurring bacteria (in the muscle or the intestinal tract) as well as its enzymes. The activities of three endogenous aminopeptidases such as arginine aminopeptidase, alanyl aminopeptidase, and leucyl aminopeptidases were reported in the processing of dry-salted fish. The levels were significantly increased during the final stage of fish processing (Wu and Cao 2018). There are many studies on the quality improvement of fish sauce by optimizing the starter inocula strains. Zheng et al. (2017) have found that inoculation of *Psychrobacter* sp. SP-1 during meat processing significantly increased the protease activity and also promoted the umami taste and meaty aroma. Fish sauce is a prime seasoning for Asian cuisines and contains salt concentrations around

25% to 30%. Therefore, studies are focusing on designing of halophilic starter cultures for preparing quality fish sauce. A novel *Staphylococcus* sp. has been isolated from fermented fish sauce and suggested to be applied as a starter culture to increase the free amino acid content, and thus improves the umami and aroma of the fish sauce (Udomsil et al. 2015). The recovery of flavor molecules from fermented fish products mostly relies on the use of commercial protease preparations such as flavourzyme which is a leucyl aminopeptidase and protamex (Suresh and Prabhu 2013). These commercial enzymes are widely used during the processing of various fermented fish products which helps in quality improvement and process acceleration (Giyatni and Irianto 2017). The endogenous aminopeptidase activity in fish sauce was studied by Vo-Van et al. (1984). These studies described the identification and purification of the aminopeptidase appearing during sardine fish sauce fermentation and changes of this enzyme activity during the entire process. Thus, aminopeptidases plays an important role in contributing to free amino acids in fish sauce.

Cocoa processing

Cocoa is cultivated in tropical regions around the world, and its fruit is the main component in chocolate production. Cocoa has astringent bitterness by the content of tannin and polyphenols. An important phase of cocoa processing is fermentation that reduces bitterness and fermentation. During the fermentation process, the pulp of fruit is degraded by the action of various microbes such as lactic acid bacteria, acetic-acid bacteria, and yeasts naturally occurring in the environment. Enzymatic reactions play important roles in protein hydrolysis in cocoa almonds to produce flavoring precursor compounds. Proteolysis is very important for cocoa flavor development (Voigt et al. 1994a, b). Therefore, in the production of a standardized chocolate mass, the addition of enzyme can be used in the treatment of cocoa (Gray 2011). The study by Oliveira et al. (2011) showed that the better quality of the chocolate were produced by the action of protease and carboxypeptidase (flavor protease) used in the processing of cocoa almonds. Merz et al. (2015) identified the eight enzymes in the commercial preparation of Flavourzyme from *Aspergillus oryzae*. Flavourzymes is a widely used enzyme cocktail in cocoa fermentation. Among the eight enzymes, two were reported as leucine aminopeptidase A and leucine aminopeptidase 2. The protease activity in the pulp and seed of cocoa during the fermentation of two cocoa cultivars were compared by Sousa et al. 2016. The result showed same isoenzyme behavior for both the cultivars with regard to the aminopeptidase and carboxypeptidase. The use of a proteolytic enzyme with an activity of 1000 leucine aminopeptidase

units (LAPU) g^{-1} provided by Novo Nordisk A/S (Bagsvaerd, Denmark) was useful in improving cocoa flavor precursors and affected the flavor perception in their products (De Brito et al. 2004). Among cocoa proteins, proline content (0.72–1.97 g/100 g of cocoa) showed maximum, and because of its specific structure, it possesses many limitations on the conformational aspects of peptides and proteins, and thus proline-specific peptidases takes the role for hydrolyzing such proteins (Kratzer et al. 2009). It is stated that both endogenous enzymes present in cocoa seeds as well as exogenous enzyme derived from microorganisms have importance in the processing of cocoa and thus developing cocoa flavor precursors.

Commercial aminopeptidases and market

The global enzymes market value was \$7082 million in 2017, and is predicted to reach \$10,519 million in 2024, registering a compound annual growth rate (CAGR) of 5.7% from 2018 to 2024. The crucial factors driving the enzyme market are the growing array in enzyme applications, its products and strict environmental norms suppressing the use of chemicals (Research and Markets 2019). Proteases are the most valuable commercial enzyme covering 60% of total enzyme market. The flavourzyme (Novo Nordisk), debitraser (Imperial Biotechnology Ltd), corolase (Rohm GmbH), and pronase (Calbiochem) are some of the various trade names of industrially available aminopeptidases (Nandan and Nampoothiri 2017b). Microorganisms are preferred sources of industrial enzymes as they are economic, effective, and have a controllable enzyme reaction mechanism. Table 3 shows some of the patents claims on aminopeptidases either to enhance the enzymatic activity or to improve various industrial applications. Most of the accepted patents on aminopeptidases are from *Aspergillus* stain as shown in table. Patented enzymes are widely used for various industrial applications. The global market size of industrial enzymes has been segmented on the basis of type, source, application of enzymes and its sources. According to the report published by Markets and Markets (2019), the top level manufacturers in the food enzymes market include Associated British Foods (UK), DowDuPont (US), Novozymes (Denmark), DSM (Netherlands), and Chr. Hansen (Denmark). These companies have a wide range of product portfolios and advanced technologies for food enzymes at major strategic locations. The increasing interest in using microbial enzymes especially proteases for the production of various industrially important products in the market is of huge importance. In order to find a cost-effective way, scientists are in search of suitable source for enzyme production. Microbial proteases are and will very likely remain the most utilized enzymes at both academic and applied levels.

Table 3 List of patents developed on microbial aminopeptidases

Patent no.	Organism	Aminopeptidase	Application	Reference
WO 96/285-42	<i>Aspergillus oryzae</i>	Leucine AP	Reduction of bitter taste in protein hydrolysate	Kauppinen 1996
US 6,271,013 B1	<i>Aspergillus niger</i>	Phenylalanine AP Leucine AP valine AP	Food industry in the preparation of bread dough or cheese	Chevalet et al. 2001
EP 2206 777B1	Aminopeptidase gene in <i>Vibrio proteilytica</i> cloned in <i>E.coli</i>	Aminopeptidase P	Selectively remove N-terminal amino acids when the penultimate residue is proline. Used in the production of medicines and food stuff, in particular proteins such as human growth hormone, G-CSF, and IL-2	Sonoda et al. 2014
US 7,098,018 B2	Recombinant <i>Bacillus licheniformis</i> aminopeptidase	Methionine AP	Enzymatic preparation of industrially important protein such as human growth hormones	Lee et al. 2006
US 3,405,034	<i>Aspergillus</i>	Leucine AP	Protein hydrolysis	Wenzel et al. 1968
US 5,013,662	<i>Escherichia coli</i>	Methionine AP	N-terminal methionine processing	Ben-Bassat et al. 1991
WO 97/041-08	<i>Aspergillus sojae</i>	Leucine AP	Preparation of protein hydrolysate	Schuster et al. 1997
US 6,127,161	<i>Aspergillus sojae</i>	Leucine AP	Food products such as seasoning	Umitsuki and Abe 2000
EP 1,629,719 A1	<i>Aspergillus nidulans</i>	Glutamyl/aspartyl AP	Producing foods and/or beverages having improved taste and/or flavor	Tomohiro et al. 2006

Conclusion

This review focuses on the application of microbial aminopeptidases in different sectors. Microbial aminopeptidases provide a greater amount of catalysis with a wide range of applications across many industries such as food and pharma. Biocatalysis and structural studies of these enzymes will provide a greater platform for better screening of novel inhibitors. An aminopeptidase-mediated bioactive peptide synthesis is more preferred because the process is ecofriendly and economic. The substrate specific aminopeptidases from various strains of microorganisms and their applications are yet to be studied. Various molecular and biochemical approaches are being carried out to improve the characteristics of the substrate specific aminopeptidases. Aminopeptidase profiles of several bacterial and fungal strains that are widely used as starter cultures are yet to elaborate and find application in novel bioprocesses in food processing. The substrate specificity of microbial aminopeptidase has to be exploited further for their commercial interest and industrial applications.

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Author's contributions AN, the first author collected all the data and wrote the article. KMN, the corresponding author conceived the topic, drafted it, and did critical reading to prepare the manuscript. Both authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest regarding this manuscript.

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