

Microbial aspartic proteases: current and potential applications in industry

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Abstract Aspartic proteases are a relatively small group of proteolytic enzymes that are active in acidic environments and are found across all forms of life. Certain microorganisms secrete such proteases as virulence agents and/or in order to break down proteins thereby liberating assimilable sources of nitrogen. Some of the earlier applications of these proteolytic enzymes are found in the manufacturing of cheese where they are used as milk-clotting agents. Over the last decade, they have received tremendous research interest because of their involvement in human diseases. Furthermore, there has also been a growing interest on these enzymes for their applications in several other industries. Recent research suggests in particular that they could be used in the wine industry to prevent the formation of protein haze while preserving the wines' organoleptic properties. In this mini-review, the properties and mechanisms of action of aspartic proteases are summarized. Thereafter, a brief overview of the industrial applications of this specific class of proteases is provided. The use of aspartic proteases as alternatives to clarifying agents in various beverage industries is mentioned, and the potential applications in the wine industry are thoroughly discussed.

Keywords Microbes · Aspartic protease · Industrial applications · Beverage · Wine · Protein haze

Introduction

Proteases can be defined as enzymes which catalyze the cleavage of hydrolytic bonds within proteins, thereby

releasing peptides and/or amino acids. They make up the largest single family of enzymes and are mainly classified into six groups based on the mechanistic features consistent within each group. These proteolytic enzymes are of great biological importance and also find their use in several industrial applications which include the food, beverage, leather, pharmaceutical, medical and detergent industries (Gupta et al. 2002; Sumantha et al. 2006; Ward et al. 2009).

Generally, the term proteases can be used interchangeably with the terms proteinases and/or proteolytic enzymes, but the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) and the Enzyme Commission (EC) recommend that the term peptidases be used for all enzymes that hydrolyze peptide bonds (subclass E.C.3.4). Proteases have a major function in the global recycling of carbon and nitrogen from proteins. Proteins from dead organisms are indeed eventually hydrolyzed by microorganisms (in the process of decomposition) into peptides and amino acids. These products can be assimilated by the microorganisms that produced the proteases or by other organisms in the vicinity. For example, protease-producing microorganisms present in soil have been shown to regulate protease expression in response to carbon and nitrogen limitation (Sims and Wander 2002). In this context, proteases can be helpful in nitrogen-limited environments.

In organisms, proteases are known to carry out a vast array of physiological functions including cell division, signal transduction, sporulation, digestion of food proteins, blood pressure regulation, viral protein synthesis, apoptosis, processing of polypeptide hormones, degradation of incorrectly folded proteins, apoptosis, autolysis, protection against harmful peptides and enzymes amongst others (Barrett et al. 2004; Sandhya et al. 2005; Tyndall et al. 2005). Extracellular proteases play a critical role in the hydrolysis and the adsorption of proteinaceous nutrients (Kalisz 1988). As the latter can function in a variety of environments, not limited to the inner cell,

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they are of great commercial importance, and protein extracts prepared from the growth cultures of protease-producing microorganisms are commonly used as protein-degrading tools during various industrial processes (Kumar and Takagi 1999).

From a functional perspective, proteases can be subdivided into exopeptidases cleaving one or a few amino acids from the N- or C-terminus and endopeptidases which act on the internal polypeptide chain. Exopeptidases that act on the free C-terminus liberate single amino acid residues (carboxypeptidases) or dipeptides (peptidyl-dipeptidases). Those acting on the N-terminus liberate single amino acid residues, dipeptides or tripeptides and are commonly known as aminopeptidases, dipeptidyl-peptidases and tripeptidyl-peptidases, respectively. Another group, known as omega peptidases, also acts close to one or the other terminus but has no requirement for a charged terminal group. Instead, they are specific in removing terminal residues that are cyclized or linked by isopeptide bonds.

Endopeptidases are industrially more important than exopeptidases and are classified according to their molecular size, charge, substrate specificity, catalytic mechanism, three-dimensional structures and the amino acid residues present in their catalytic site (Beynon and Bond 1990; Sumantha et al. 2006). Each type of protease indeed exhibits a set of amino acid residues arranged in a specific configuration to produce its catalytic site. This gives them the characteristic ability to break certain peptide bonds (Barrett et al. 2004; Tyndall et al. 2005). Finally, a specific group of endoproteases, termed oligopeptidases, acts only on substrates smaller than proteins. Table 1 summarizes the classification of proteases and their modes of action.

The MEROPS database (<http://merops.sanger.ac.uk/>), a manually curated database dedicated to peptidases, divides peptidases into protein species, based on the main amino acid present at the catalytic domain. These species are then further subdivided into families according to the statistically significant similarities in their amino acid sequences. Protein species include aspartic/glutamate, cysteine, metallo, serine and the less characterized threonine peptidases (Madala et al. 2010). In the nomenclature of the NC-IUBMB (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>), endopeptidases which include serinepeptidase, cysteinepeptidase, asparticpeptidase, metallopeptidase and threonine endopeptidase are given the subclasses EC 3.4.21, EC 3.4.22, EC 3.4.23, EC 3.4.24 and EC 3.4.25, respectively. Figure 1 summarizes the abundance of these proteases found in nature.

All of these endopeptidases differ in their properties and response to environmental conditions. Table 2 shows the different species of endoproteases together with some additional information on their characteristics, sources and the industry they are used in. Briefly, serine proteases, which play an important role in digestion, possess a catalytic triad in their

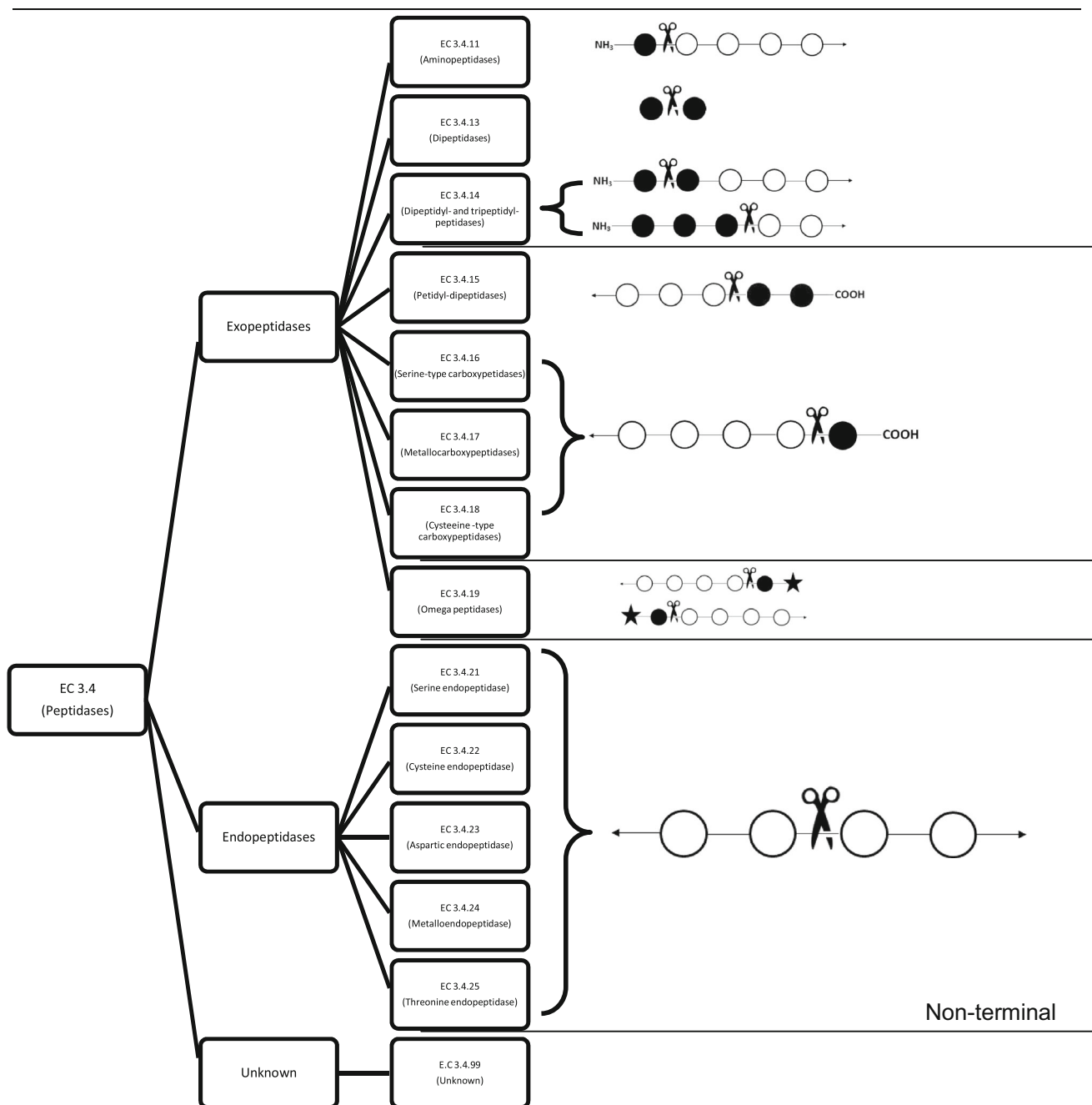
active site consisting of a serine, histidine and aspartic acid residues. They fall into two categories based on their structure: the chymotrypsin-like (serine protease I) and the subtilisin-like (serine protease II) proteases. Cysteine proteases, commonly used in meat tenderizers, have similar folds as the serine proteases but the catalytic dyad in their active site consists of cysteine and histidine residues. The metalloproteases, as the name suggests, are classified as any proteases whose catalytic mechanism involves a metal (usually divalent zinc ions). Threonine proteases are one of the newer classes of proteases described and harbour a threonine residue in their catalytic domain (Rao et al. 1998; Madala et al. 2010). The aspartic proteases, which will be discussed in more detail in the following paragraphs, have a tertiary structure consisting of two symmetrical lobes to form the catalytic site, each lobe harbouring an aspartic acid residue. With cysteine proteases, they are the only endoproteases active at acidic pH (Table 2). It is however worth mentioning that in 1990, Fusek et al. purified and cloned a thermophilic acid protease from *Sulfolobus acidocaldarius* (an archaeobacteria) which does not have an aspartyl residue in its active site nor does it show any apparent sequence homology to other acid proteases and therefore represents a new class (Fusek et al. 1990).

Aspartic proteases

Distribution

Aspartic proteases, commonly known as acid proteases, are distributed across all forms of life including vertebrates, plants, fungi, bacteria and also viruses (Fairlie et al. 2000; Cooper 2002). This relatively small group of enzymes has received much attention from the scientific community because of their involvement in human diseases. Some of these proteases indeed include the plasmepsins in malaria, HIV-1 peptidase in acquired immune deficiency syndrome (AIDS) and the secreted aspartic peptidases in *Candida* infections (Madala et al. 2010). From as early as 1989, crystal structures of aspartic proteases from retroviruses such as HIV and *Rous sarcoma* have been extensively studied and determined (Navia et al. 1989). The secreted aspartic proteases from *Candida albicans* have been intensively investigated due to their role in various forms of candidiasis. Since its discovery, the secreted proteolytic activity of *C. albicans* was discussed as a putative virulence factor. The major proteases secreted in vitro by *Candida* species have been termed Sap2, Sapp1 and Sapt1 from *C. albicans*, *Candida parapsilosis* and *Candida tropicalis*, respectively (Ruchel 1986; de Viragh et al. 1993; Monod et al. 1994). Their proposed functions during infection include the degradation of the host tissue barriers

Table 1 Classification of proteases



Scissors indicate the site where the enzyme will perform its action. Solid circles represent the terminal amino acids. Open circles signify amino acid residues in the polypeptide chain and stars represent blocked termini. Adapted from Ward et al. (2009)

during invasion and the destruction of the host defence molecules. Furthermore, they also have a role in nutrient supply by degrading proteins and releasing assimilable nitrogen sources (Naglik et al. 2003).

Aspartic proteases from other yeasts and fungi have also been studied extensively, and several have been purified and cloned for research and industrial purposes (Tonouchi et al.

1986; Horiuchi et al. 1988; Togni et al. 1991; De Viragh et al. 1993; Gomi et al. 1993; Jarai et al. 1994; Kakimori et al. 1996; Young et al. 1996; van Kuyk et al. 2000; Li et al. 2009, 2010; Radha et al. 2011; Shivakumar 2012). Several of these extra-cellular aspartic proteases from fungal species originate from *Aspergillus* species. Some of these species include: *Aspergillus oryzae* (Vishwanatha et al. 2009), *Aspergillus*

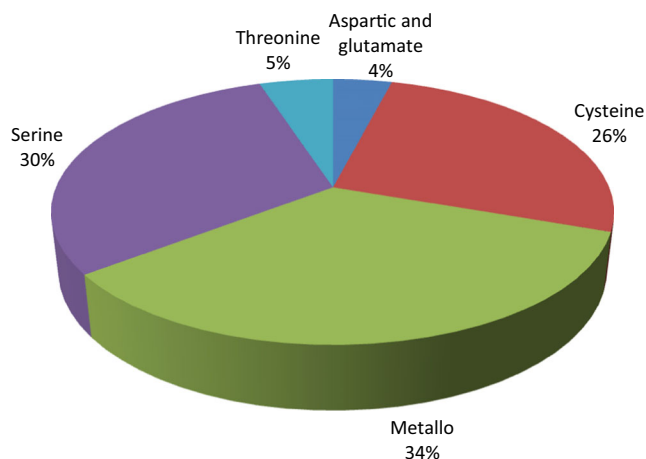


Fig. 1 Relative abundance of endoproteases in living organisms

fumigatus (Reichard et al. 1994), *Aspergillus saitoi* (Tello-Solis and Hernandez-Arana 1995), *Aspergillus awamori* (Moralejo et al. 2002) and *Aspergillus niger* (O'Donnel et al. 2001; Siala et al. 2009; Radha et al. 2011). Studies have also revealed that the aspergillopepsin I (PepI) and rhizopuspepsin of *A. fumigatus* and *Rhizopus* microspores are present in lung infections (Schoen et al. 2002).

Description and mechanism of action

The molecular weight of aspartic acid proteases typically ranges between 35 and 50 kDa usually consisting of 320 to 340 amino acid residues. These enzymes have isoelectric points in the range of 3 to 4.5. Analysis of various aspartic proteases by X-ray crystallography shows that they are mostly composed of β -strand secondary structures. β -Strands are

found at the base of the active-site cleft and contain the catalytic aspartic residues. In porcine pepsin and endothiapepsin, these aspartic residues have been identified at Asp³² and Asp²¹⁵ (Coates et al. 2001; Veerapandian et al. 1992). A water molecule is found hydrogen bonded between both aspartate carboxyls and is thought to take part in the catalytic mechanism (Pearl and Blundell 1984). Interestingly, these structures represent some of the largest β -strand structures observed in globular proteins (Claverie-Martin and Vega-Hernandez 2007). The majority of aspartic proteases are also known to have at least one flap made up of a β -hairpin that completes their active site (Madala et al. 2010). The flap region can be visualized in Fig. 2a highlighted in green. The flaps serve as a mechanism that upon closing, squeezes all the components into the correct geometry and holds the substrate in place enabling the catalytic process to begin. Well-known examples of aspartic proteases include rennet, cathepsin D, cathepsin E and pepsin. The Protein Data Bank (PDB) and MEROPS database classify eight subfamilies within the aspartic proteases with the sequence Asp-Thr(Ser)-Gly in their active site. Subfamilies differ according to the position of their catalytic site, the specific residues in their active site, the number of disulphide bridges present within the structure and optimal pH at which the enzyme functions (Cascella et al. 2005; Rawlings et al. 2009; Rawlings and Bateman 2009).

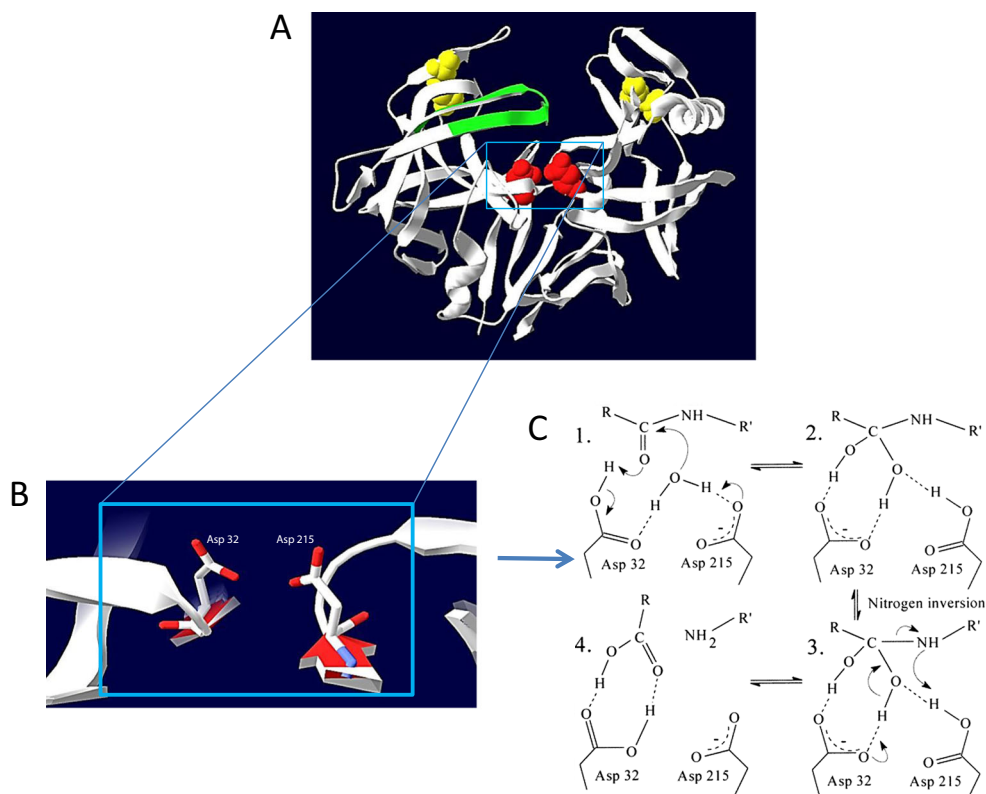
In a catalytic mechanism proposed by Veerapandian et al. (1992) which was based on the X-ray structure of a difluoroketone inhibitor bound to endothiapepsin, these enzymes perform their action through general acid-base catalysis where the one aspartic residue (Asp³²) acts as a base, accepting a proton, while the other (Asp²¹⁵) acts as an acid,

Table 2 The different species of endoproteases, their principal properties, main sources or isolation and industries in which these proteases have found applications

Family	Cofactors	Characteristic active site	Optimal pH range	Inhibitors	Source	Industrial applications
Serine proteases	Ca ²⁺	Asp, Ser, His	7–11	PMSF, EDTA, phenol, triamino acetic acid	<i>Bacillus</i> , <i>Aspergillus</i> , animal tissue (gut)	Detergent, medical and pharmaceutical
Metallo proteases	Zn ²⁺ , Ca ²⁺	Glu, Try	7–9	Chelating agents such as EDTA, EGTA	<i>Bacillus</i> , <i>Aspergillus</i> , <i>Penicillium</i> , <i>Pseudomonas</i> , <i>Streptomyces</i>	Food, medical and pharmaceutical
Cysteine proteases	N.d.	Cys, His, Asp	2–3	Indoacetamide, p-CMB	<i>Aspergillus</i> , <i>Streptomyces</i> , <i>Clostridium</i>	Food, medical and pharmaceutical
Aspartic proteases	Ca ²⁺	Asp, Asp	2.5–7	Pepstatin, EPNP, DAN	<i>Aspergillus</i> , <i>Mucor</i> , <i>Rhizopus</i> , <i>Penicillium</i> , animal tissue (stomach)	Food and beverage
Threonine proteases	N.d.	Thr	Neutral	DON	<i>Thermoplasma</i> , <i>Escherichia</i> , <i>Saccharomyces</i>	Food

DAN diazoacetyl-DL-norleucine methyl ester, *DON* 5-diazo-4-oxonorvaline, *PMSF* phenylmethylsulfonyl fluoride, *PCMB* pchloromercuribenzoic acid, *EDTA* ethylenediaminetetraacetic acid, *EGTA* ethylene glycol tetraacetic acid, *EPNP* 1,2-epoxy-3-(*p*-nitrophenoxy)propane, *N.d.* not determined (Rao et al. 1998; Sumantha et al. 2006)

Fig. 2 Three-dimensional structure and mechanism of action of a typical aspartic protease. Secreted aspartic proteinase (SAPT; Accession number: 1j71) from *Candida tropicalis* (Symersky et al. 1997) was used to construct these pictures as visualized through Swiss-PDbViewer (v4.0.4). **a** Representation of the structural elements: active site (in red), disulphide bounds (in yellow) and flap region (in green). **b** Close-up of the active-site cleft. **c** Catalytic mechanism as represented by Coates et al. (2001) according to a model proposed by Veerapandian et al. (1992)



donating a proton. In other terms, the former residue has a relatively low pKa value and the latter a relatively high pKa value. Figure 2a, b illustrates the three-dimensional structure of a typical aspartic protease and details the molecular mechanism of action (C) as proposed by Veerapandian et al. (1992). Following exposure to low pH, cleavage events lead to conformational rearrangement. Firstly, a water molecule is bound to the two aspartic residues through hydrogen bonds and acts as a nucleophile that attacks the carbonyl carbon of the peptide scissile bond. The aspartic residue that acts as a general base removes one proton from the water molecule which is followed by a nucleophilic attack of the water molecule to the carbonyl carbon of the substrate scissile bond. At the same time, the other aspartic acid residue, acting as a general acid, donates a proton to the carbonyl oxygen atom of the peptide scissile bond. This leads to the formation of a tetrahedral intermediate. Thus, the aspartic residue acting as a base is hydrogen bonded to the attacking oxygen atom, while the hydrogen remaining on that oxygen is hydrogen bonded to the oxygen of the aspartic residue acting as an acid. During the final stages, a reversal of the configuration occurs around the nitrogen atom of the scissile bond of the substrate with the transfer of a hydrogen atom from the aspartic acid residue acting as a base to the nitrogen atom. In parallel, a proton is transferred from the oxygen atom of aspartic acid acting as an acid to the carbonyl oxygen on the peptide bond being cleaved. This leads to the C-N bond breaking and releasing

the two peptide products. Consequently, the aspartic acid that acted as a base is negatively charged at this stage and is therefore ready for the next round of catalysis (Coates et al. 2001; Dunn 2002).

In 2001, Northrop proposed an alternative mechanism based on the same principle as described above but in which a low-barrier hydrogen bond (not present in the former proposed mechanism) is formed between the two aspartic residues present in the catalytic site (Northrop 2001). Another major difference is that the final step involves the binding of a water molecule and the reformation of the low-barrier hydrogen bond. However, there have been disagreements with this proposal based on the angle between the two inner oxygen of the aspartic residues being too wide for hydrogen-bond formation (Andreeva and Rumsh 2001; Dunn 2002). Nevertheless, all authors agree on the occurrence of a covalent intermediate.

The aspartic proteases are typically inhibited by pepstatin, a hexapeptide containing the rare amino acid statine. This molecule, which was originally isolated from various species of *Actinomyces*, has the remarkable ability to inhibit pepsin at picomolar concentrations (Umezawa et al. 1970; Marciniuszyn et al. 1976). There have however been reports of pepstatin-insensitive acid proteases isolated from bacteria including *Xanthomonas* sp., *Pseudomonas* sp., *Bacillus* sp. (Oda et al. 1987; Prescott et al. 1995) and more recently from *Thermoplasma volcanium* (Kocabiyik and Ozel 2007). Rao

et al. (1998) reported that aspartic proteases are also sensitive to diazoketone compounds such as 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN) in the presence of copper.

The pepstatin-sensitive aspartic proteases are divided into two families: the retroviral and eukaryotic pepsin-like-type proteases. The retroviral types consist out of β -homodimers possessing aspartic residues located within the two loops at the monomer interface with two β -hairpins covering the active site (Sielecki et al. 1991). The eukaryotic pepsin-like protease has a tertiary structure consisting of two approximately symmetrical lobes (α/β monomers) with each lobe carrying an aspartic acid residue in order to form the catalytic site. In the N-terminal domain, the characteristic sequence Asp³²-Thr-Gly-Ser can be found with a corresponding Asp²¹⁵-Thr-Gly-Ser/Thr in the C-terminal domain (De Viragh et al. 1993). Because of their twofold symmetry, it is the general consensus that these domains possibly arose through ancestral gene duplication. A flap made of a β -hairpin covers the catalytic site constituting the active-site cleft. This cleft is located perpendicular to the largest diameter of the molecule and can accommodate seven to eight amino acid residues, equally divided on both sides of the catalytic aspartic residues (Szecsi 1992; Dunn 2002). The number and position of disulphide bonds throughout the protein have been suggested to have a strong impact on the native state stability of the enzyme (Cascella et al. 2005; Friedman and Caflich 2010). Members of the aspartic proteases family generally have one to three disulphide bridges that are located at the position between amino acids 251 and 286. This position is conserved across all members of the family (Machalinski et al. 2006). The disulphide bonds play an important role in the folding and stability of the protein and can be visualized in Fig. 2a, highlighted in yellow. In general, most aspartic proteases from microbial origin exhibit a broad-based specificity towards regions in the peptides that contain six hydrophobic residues at specific substrate positions (Dash et al. 2003).

Proteases in industry

Some of the earlier applications of proteolytic enzymes found their use as milk-clotting agents for the manufacturing of cheese. These were probably first indirectly discovered when animal skins and inflated organs were used as storage containers for a range of foodstuffs. For instance, when milk is stored in the stomach of calves, it results in the formation of curd and whey because of the rennet present in the stomach (which contains several enzymes including chymosin). In Asian countries, proteases were used in the early production of natto, which is produced through the fermentation of soy beans with *Bacillus* species. Proteases involved in this process

are important for the development of the main flavours associated with natto through the hydrolysis of the soy bean proteins (Ward et al. 2009; Borah et al. 2012).

The involvement of proteases in the life cycle of many pathogens makes them important to the pharmaceutical and medical industry. Inhibition of various proteases has also become a valuable approach for studying neurodegenerative diseases, infections and various parasitic diseases (Rao et al. 1998). The most essential property of protease action resides in their ability to control and limit cleavage to intended substrates without degradation of functional proteins. Moreover, 2 % of functional genes found in the human genome encode proteolytic enzymes, thus they have become important therapeutic targets and are also used in diagnostics (Craik et al. 2011).

Proteases of all categories are also extensively applied in several research applications, some which include peptide synthesis and sequencing, digestion of unwanted proteins in purified samples (for example in nucleic acid purification), preparation of antibodies, production of Klenow fragments and removal of affinity tags from proteins in recombinant techniques (Mótyán et al. 2013). The study and production of proteases are also motivated by their use in several fields of industry. In 2010, it was estimated that the world market for industrial enzymes reached 3.3 billion dollars and that proteases form the largest segment of this market.

Microbes are the most abundant source of enzymes and extensively studied for their application in industry. One of the first reports on this dates back to 1894, by Jhokichi Takamine who pioneered the industrial production of digestive enzymes prepared from *A. oryzae* for the treatment of digestive disorders. As reported by Ward et al. (2009) and Khan (2013), proteases were for the first time used in 1914 as additives to detergents, and since then, this industry has seen tremendous growth and development. Furthermore, proteases derived from plant and animal species are unable to meet the current world demand and are not diverse enough to meet industrial requirements thus creating a consistently growing interest in microbial proteases. Microbes can also easily be manipulated into producing enzymes at high amounts. Because of the large biodiversity amongst microbes, they represent an unparalleled source of enzymes with a wide spectrum of characteristics.

Alkaline proteases are active at basic pH range and make up the largest share of the enzyme market because of their use in household detergents. Most of the proteases used in this industry are alkaline or neutral proteases from *Bacillus* species. Some of the most important to the detergent industry are the serine alkaline proteases. Highly alkaline detergents use proteases from alkalophilic species such as *Bacillus halodurans* and *Bacillus clausii*, whereas proteases from *Bacillus licheniformis* are used in low-pH detergents. Three main product categories exist: (1) the low pH (7.5–9.0), low ionic strength liquid detergents containing no bleach, (2) the high

pH (9.5–10.5), high ionic strength powders which contains bleach, and finally (3) the high pH (9.5–10.5) compact powders that contain sodium sulphate (Ward et al. 2009). The use of alkaline/neutral proteases has also received much attention in terms of replacing of harsh or harmful chemicals.

Alkaline proteases are also used in the leather industry. The major components of leather are proteins, including elastin, keratin and collagen. The principal steps in the processing of leather include soaking, dehairing, bating and tanning. The purpose of the soaking step is to swell the hide, and this is usually achieved by use of an alkaline reagent. Conventional methods for dehairing include treatment with extremely alkaline chemicals followed by treatment with hydrogen sulphate. This solubilizes and removes the proteins from the hair root. These conventional methods used in the leather industry thus involve the use of harsh chemicals which creates safety risks, disposal problems and chemical pollution (Khan 2013). Collagen exists in hides and skin in association with various globular proteins such as albumin, globulin, mucoids and fibrous proteins such as elastin, keratin and reticulin. The extent to which the non-collagenous constituents are removed determines the characteristics of the final leather such as durability and softness. The success of detergent enzymes has led to their being used in a number of other applications including pest control (Kim et al. 1999), degumming of silk (Kanehisa 2000; Puri 2001), isolation of nucleic acid (Kwon et al. 1994), lens cleaning (Nakagawa 1994), delignification of hemp (Dorado et al. 2001), cleaning of surgical instruments (Gupta et al. 2002), production of peptides (Cheng et al. 1995) and silver recovery from X-ray films (Fujiwara et al. 1991). The different industrial applications of alkaline proteases have been recently reviewed (Anwar and Saleemuddin 1998;

Horikoshi 1999; Gupta et al. 2002; Saeki et al. 2007; Fujinami and Fujisawa 2010; Li et al. 2013; Mienda et al. 2014) and will therefore not be detailed further in this review.

As reported above, acid proteases are active at acidic pH range and although they are not as popular as the alkaline/neutral proteases, they are used in a number of industrial applications.

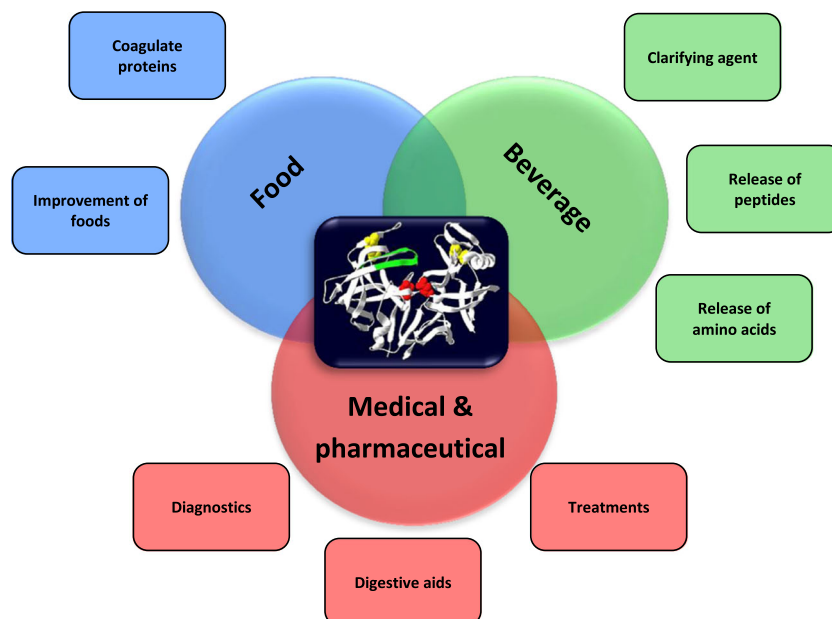
Applications of microbial acid proteases

Acid proteases of microbial origin are mostly found in three industries: food, beverage and pharmaceutical. In each of these industries, they are used for a variety of purposes (Fig. 3) that will be discussed further in the following paragraphs.

Food industry

The significant ability of acid proteases to coagulate proteins, especially milk proteins, is the main reason for their high demand in the food industry. Indeed, the major application of acid protease in this industry is the manufacturing of cheese where milk proteins are coagulated thereby forming solid masses, or curds, from which cheese is prepared after the removal of whey (Neelakantan and Mohanty 1999). Basically, four categories of milk-coagulating enzymes exist. They include animal rennets, microbial milk coagulate, genetically engineered chymosin and vegetable rennet (Ward et al. 2009). As the human population and the demand for cheese increased, the cheese-making industry was hindered by a worldwide shortage of calf rennet which became even scarcer

Fig. 3 Summary of the current and potential uses of aspartic proteases in industry. The picture in the middle represents a typical aspartic acid protease (SAPT from *Candida tropicalis*) as visualized through Swiss-PdbViewer (v 4.0.4). The structural elements are represented as indicated in Fig. 2



because of resistance from animal rights lobbies (Furia 1980). This triggered a search for alternative milk coagulation proteins, and proteins of microbial origin started to receive more attention. A primary characteristic of enzymes involved in cheese production is the ability to hydrolyze the specific peptide bond (Phe¹⁰⁵-Met¹⁰⁶ in bovine casein) to generate para-casein and macromolecules (Rani et al. 2012). In the 1980s, Genecor International expressed recombinant calf chymosin (rennin) on a large scale using *A. niger* var. *awamori* as host. Commercially, the most important native enzyme for cheese making is isolated from the mold *Rhizomucor miehei* (Ward et al. 2009).

Apart from their extensive use in the dairy industry, fungal-derived acid proteases have also been extensively applied in the production of food seasonings and the improvement of protein-rich foods such as bread and related foodstuffs. Gluten found in wheat flour is an insoluble protein that determines the properties of the dough. Enzymatic treatment of dough facilitates its handling and also reduces the mixing time. Furthermore, proteases from *A. oryzae* are used to modify wheat gluten resulting in an increased loaf volume and the production of a wider range of products (Rao et al. 1998).

Medical and pharmaceutical industry

As recently reviewed by Chanalia et al. (2011), aspartic proteases are utilized as digestive aids, commercially available as Nortase and Luizym, for the treatment of certain lytic enzyme deficiency syndromes (Rao et al. 1998). Several aspartic proteases from *Candida* species have also been extensively studied because of their involvement in infections (Tsushima et al. 1994; Cutfield et al. 1995; Fallon et al. 1997; Pichova et al. 2001; Aoki et al. 2012). This has led to the development of aspartic protease inhibitors of interest for the treatment of infections caused by these yeast species, as thoroughly reviewed by Ghosh (2010). Considering that the application of aspartic proteases in the medical and pharmaceutical industries has been recently described extensively, this topic will not be discussed further in this review. Readers are nevertheless invited to consult the reviews cited above for further information.

Beverage industry

Most industrially processed fruit-based beverages are clarified in order to prevent haze and turbidity. In the making of fruit juices and certain alcoholic beverages, acid proteases from *A. saitoi* (aspergillopepsin I) are used to degrade the proteins that cause turbidity (Sumantha et al. 2006). In the fermentation of sake, an alcoholic beverage of Japanese origin, acid proteases from *A. oryzae* determine the taste of the final product because of the manner in which they hydrolyze the proteins from the steamed rice in order to liberate peptides and amino

acids (Shindo et al. 1998). Addition of fungal proteolytic enzymes from *A. niger* to kiwi fruit juice decreases the immediate turbidity and retard haze formation during cold storage (Dawes et al. 1994). Haziness is due to the aggregation and precipitation of proteins leading to light dispersing particles that can be perceived by the naked eye and is usually interpreted as microbial spoilage by consumers (Bayly and Berg 1967; Falconer et al. 2010). In cherry juice, Pinelo et al. (2010) found that addition of a commercial protease (ENZECO fungal acid protease) from *A. niger* resulted in a significant reduction in the immediate turbidity but also noted that it had a low impact on clarification during cold storage. These observations were also made in the production of black currant juice where commercially available acid proteases from *A. niger* (amino acid protease A, Deapsin 2P, ENZECO fungal acid protease) and *Mucor miehei* (Novozyme 89L) were used (Landbo et al. 2006). More recently, similar observations were also made in the production of banana wine in which commercially available proteases (Zumizyme) were added (Byarugaba-Bazirake et al. 2013). In the latter study, it was found that when compared to the controls, the wines prepared from juices that underwent protease treatment displayed a significantly lower turbidity. It was observed that a longer period of incubation led to greater reduction in turbidity. Furthermore, the addition of proteases was shown to have a significant reductive effect on protein haze.

In the brewing industry, acid proteases have also been investigated as tools to degrade proteins that can form haze during storage. Haze formation can be due to glucan from modified malt, dead bacteria from malt, oxalate from calcium-deficient worts, residual starch, carbohydrates and proteins from autolyzed yeasts (Steiner et al. 2010). Two forms of haze occur: chill haze and age-related haze (sometimes referred to as permanent haze). Chill haze, also known as cold break haze, forms at 0 °C when polypeptides and polyphenols are non-covalently bound. Chill haze, also known as cold break haze, forms at 0 °C when polypeptides and polyphenols are non-covalently bound. Age-related haze is initially formed in the same manner, but strong covalent bonds are formed during storage leading to insoluble complexes. Unlike in chill haze, the complexes formed over time cannot dissolve upon heating (Siebert et al. 1996, 2011). In a study by Lopez and Edens (2005), it was found that addition of proline-specific proteases from *A. niger* effectively prevented chill-haze formation in beer, suggesting that the hydrolysis of proline-rich proteins resulted in a peptide fraction that is unable to interact with the polyphenols. Haze particles can show different appearances and have been classified into three main categories according to Glenister (1975). The first encompasses native particles which originate from beer by coagulation and/or precipitation. The second includes process particles originating from materials added during the brewing process. The last category comprises foreign particles which can enter into contact with

beer as accidental contaminants (Glenister 1975; Bamforth 1999; Steiner et al. 2010).

Similarly to the brewing industry, protein haze is also a very challenging problem during the production of white wine. Like in beer, the presence of haze is usually perceived as microbial spoilage by consumers and results in a reduction of the commercial value of the wine (Waters et al. 2005). In white wine, this phenomenon occurs when proteins of grape origin become unstable under certain conditions and aggregate thereby rendering the wine hazy (Hsu et al. 1987; Waters et al. 1992; Marangon et al. 2012). The proteins involved have been identified as pathogenesis-related (PR) proteins, more specifically β -glucanases, chitinases and thaumatin-like proteins (TLPs) which exhibit molecular weights ranging from 15 to 30 kDa (Waters et al. 1996, 1998; Van Sluyter et al. 2009; Le Bourse et al. 2011; Marangon et al. 2011c). They have been shown to be stable at acidic pH and resistant to proteolytic hydrolysis because of their compact globular structure preventing access to the protease enzymes (Conterno and Delfini 1994). In literature, some studies indicated that TLPs are the major wine haze proteins (Esteruelas et al. 2009; Vincenzi et al. 2010), whereas other authors indicated that chitinases are the major proteins responsible for haze formation (Vincenzi et al. 2005; Sauvage et al. 2010). Recently, the two classes of proteins have been demonstrated to have separate unfolding temperatures, 55 and 62 °C for chitinases and TLPs, respectively. The unfolding behaviour of the proteins was also found to differ in that once heated, TLPs refold upon cooling while chitinases remain unfolded (irreversible refolding) (Falconer et al. 2010). This finding revealed that chitinases are thus more prone to cause haze in wine.

Slow denaturation of these proteins is thought to cause protein aggregation and flocculation eventually resulting in the appearance of haze. This is possibly due to unsuitable transport and storage conditions (Batista et al. 2009). It is generally accepted that the higher the total protein content of a wine, the higher tendency it has to become hazy (Mesquita et al. 2001). Thus for several years, studies on haze formation have focused on the proteins themselves. However, despite significant advances, the molecular mechanism of protein haze formation is still not fully understood. Initially, it was thought that instability solely related to protein content (Anelli 1977; Somers and Ziemelis 1973), but studies have shown that the potential of a wine to form haze is not predictable from its protein concentration alone (Bayly and Berg 1967; Moretti and Berg 1965). Wine composition (pH, ethanol content, ionic strength, sulphate ions polyphenols and polysaccharides) and temperature have all been shown to play a role (Waters et al. 1995; Dupin et al. 2000; Mesquita et al. 2001; Carvalho et al. 2006; Pocock et al. 2007; Duffrechou et al. 2010; Marangon et al. 2011c, b), but their actual involvement, possibly in combination with each other, in protein aggregation and flocculation remains unclear. Indeed, there are only a few studies

investigating the impact that pH and ionic strength (salts) have on protein stability relating to haze formation, despite their strong influence (Sarmiento et al. 2000a; Duffrechou et al. 2010). The overall effect that the ionic strength of a solution can have on proteins can be both stabilizing and destabilizing, depending on the charge distribution within the protein (Von Hippel and Wong 1964; Kohn et al. 1997; Record et al. 1998). Protein-protein interactions are favoured when the net charge of the molecule is reduced. Thus, at conditions with high ionic strengths or at pH values close to the isoelectric point of the protein, interaction is favoured (Boye et al. 1995; Chi et al. 2003). Normal wine ionic strength ranges between 10 and 100 mM (Cabanis et al. 1998). At these values, it is expected to strongly influence electrostatic interactions (Israelachvili 1991; van Oss 1994). Furthermore, the specific ion type that is present may also influence interaction following a different mechanism. Pocock et al. (2007) indicated that a sulphate anion is an essential factor required for haze formation.

Moreover, in a recent study performed by Gazzola et al. (2012), the authors examined the aggregation behaviour of five purified wine proteins and measured the size and concentration of individual particles formed in the presence and absence of phenolics and/or polysaccharides using scanning ion occlusion sensing (SIOS). The study revealed that chitinases are indeed the proteins most prone to cause haze formation and that polysaccharides and phenolics present in wine do not have a significant effect on their aggregation behaviour. Furthermore, it was observed that the TLP isoforms tested varied in their interaction with the polysaccharides and phenolics present and thus also their susceptibility to cause haze formation. Phenolic compounds present in wine have been associated with haze formation as they interact with haze-forming proteins (Somers and Ziemelis 1973; Yokotsuka et al. 1983) and have been found to be present in haze (natural and heat induced) studied in several white wines (Waters et al. 1995; Esteruelas et al. 2011). Some studies suggest that the major mode of interaction between the phenolic compounds and the proteins present is hydrophobic, particularly when the proteins are in their unfolded state (Oh et al. 1980; Siebert et al. 1996; Marangon et al. 2010). In literature, some contradicting results have been reported about the stabilizing effect of polysaccharides. Mesquita et al. (2001) found that polysaccharides could negatively affect wine stability, whereas other authors (Waters et al. 1994b; Brown et al. 2007; Pellerin et al. 1994) found that some polysaccharides can have a stabilizing effect towards heat-induced protein haze. However, the polysaccharide concentration used during these studies was much greater than that found in wine (Doco et al. 2003). Studies involving interactions between wine molecules and proteins responsible for haze formation require the characterization of the size and concentration of the protein aggregates responsible for haze formation (Gazzola et al. 2012). Dynamic light scattering (DLS) techniques have mainly been used to study

protein aggregation (Dufrechou et al. 2010, 2012; Marangon et al. 2011b), whereas studies on the particle sizes use methods involving gel electrophoresis (Alberts et al. 1994), electron microscopy (Ito et al. 2004) and disc centrifugation (Bondoc and Fitzpatrick 1998).

Currently, the most effective tool that winemakers have to eliminate haze is treatment with bentonite. This montmorillonite clay has a net negative charge and serves as a cation exchanger adsorbing proteins (Ferreira et al. 2002). Bentonite has been widely used in oenology as a fining agent since as early as the 1930s (Saywell 1934). Despite its widespread use, the application of bentonite has several negative attributions; some of which include the removal of positive flavour compounds, high handling costs, loss of colour and disposal issues leading to environmental concerns associated with sustainability (Lagace and Bisson 1990; Waters et al. 2005). Because of these negative impacts, several alternatives to bentonite treatment have been investigated, including the use of pasteurization (Ferenczy 1966), flash pasteurization (Francis et al. 1994; Pocock et al. 2003) and ultrafiltration (Hsu et al. 1987). The use of other adsorbents has also been investigated, some of which include low swelling adsorbing clays, ion exchange resins, silica gels, alumina, hydroxyapatite, chitin and trisacryl (Sarmiento et al. 2000b; Vincenzi et al. 2005; de Bruijn et al. 2009). Furthermore, the use of immobilized phenolic compounds, such as proanthocyanidins (Weetall et al. 1984; Powers et al. 1988) and the addition of polysaccharides (mannoproteins and seaweed extracts), has also been proposed as an alternative to bentonite fining (Waters et al. 1991, 1994a, b; Cabello-Pasini et al. 2005). The use of metal oxide materials (such as zirconium oxide) has also proved to be a promising alternative (Waters et al. 2005; Pashova et al. 2004a, b; Salazar et al. 2006; Marangon et al. 2011a; Lucchetta et al. 2013). However, none of these alternative treatments are fully optimal either with regard to their efficiency to eliminate haze or to their absence of negative impact on the organoleptic properties of wine. In this context, an ideal solution to address the issue of haze formation would be to use enzymes able to degrade haze-forming proteins at winemaking temperatures.

Preliminary reports have shown that the use of proteases in wine is an efficient way of reducing protein haze formation without being detrimental to wine quality (Lagace and Bisson 1990; Pocock et al. 2003), but heating needs to be coupled with the treatment in order to denature the heat-unstable proteins prior to their degradation that degrade them. This would be in agreement with the study claiming that, without prior denaturation, haze-forming proteins are resistant to proteolysis (Conterno and Delfini 1994). In 2003, Pocock et al. indeed demonstrated that combining heat treatment and proteolytic enzymes reduced the requirement for bentonite by 50–70 % without affecting the sensory profile of final wine. Treatment consisted of exposing the wine for 1 min at 90 °C

and adding Trenolin® blank (Erbslöh, Geisenheim, Germany) which is a commercially available aspergillopepsin. The idea behind this dual treatment is that exposure to heat denatures the haze-forming proteins allowing access for proteolytic enzymes to hydrolyze the proteins into smaller peptides. Nevertheless, despite these encouraging results, it was concluded that a more efficient protease was needed. Recently in 2012, Marangon et al. investigated the use of an acid protease isolated from *A. niger* var. *macrosporus* (Koaze et al. 1964), namely aspergillopepsin I and II (AGP), together with flash pasteurization to degrade haze proteins in white wine. The sole addition of AGP directly to the fermentation resulted in a 20 % reduction in proteins. However, maximum effects were obtained when the juice was treated by combining AGP addition with flash pasteurization (75 °C for 1 min). It was found that, under the conditions tested, the chitinases and TLPs were almost completely degraded in Chardonnay and Sauvignon blanc wines thereby eliminating the need for bentonite. In a study performed by Reid et al. (2012), two extracellular aspartic protease-encoding genes were retrieved and sequenced. The two genes, *MpAPr1* and *CaAPr1*, were isolated from two separate yeast species of enological origin, *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. Furthermore, *MpAPr1* production was shown to be constitutive in the native host, and secretion of this enzyme was confirmed in the presence of bovine serum albumin (BSA), casein and grape juice proteins. Very recently, Van Sluyter et al. (2013) also demonstrated that an acid protease from *Botrytis cinerea*, named BcAP8, was able to effectively reduce haze at winemaking temperatures and to remain active after fermentation was completed. Although it was found that the enzyme was not able to remove all the PR proteins, showing more activity against chitinases than the TLPs, it was shown that it could still benefit winemakers by reducing bentonite requirements. The success of previously mentioned reports encourages further investigations into proteases of wine-related non-*Saccharomyces* yeasts followed by the assessment of their potential use in wine.

Proteins present in wine have been found to account for up to 2 % of the total nitrogen content (Feuillat 2005). Apart from preventing protein haze, proteases present and active during the winemaking process may potentially increase the assimilable nitrogen necessary for microbial growth as well as amino acids, purine and pyrimidine syntheses (Bell and Henschke 2005) during fermentations by breaking down these proteins. However, the main wine yeast *Saccharomyces cerevisiae* is unable to utilize proteins as a nitrogen source as it is not known to actively secrete aspartic proteases. Only one study reported recently on the occurrence of an actively secreted acid protease in *S. cerevisiae* (Younes et al. 2011), but this phenomenon has only been noted in one strain. However, some of *S. cerevisiae*'s intracellular aspartic proteases (e.g.

Pep4) have been reported to occur in the extracellular matrix (i.e. the wine) upon autolysis, and the consequent release of amino acids and peptides via the activity of the liberated Pep4 was hypothesized to impact on malolactic fermentation carried out by lactic acid bacteria (Guilloux-Benatier et al. 2006). The main lactic acid bacterium responsible for malolactic fermentation, *Oenococcus oeni*, has also been shown to secrete an aspartic protease (Fariás and Manca de Nadra 2000) and an uncharacterized acid protease (Folio et al. 2008) which could potentially play a role in the bacterial growth and release of flavour compounds. Furthermore, insufficient nitrogen sources may lead to fermentations that become slow or stop, which are referred to as sluggish and stuck, respectively. A shortage in sulphur-containing amino acids also leads to the production of hydrogen sulphide which is known to have a negative effect on sensory attributes. Proteases, either naturally secreted by indigenous microorganisms or added from external sources, could prevent the production of this off-flavour compound.

Finally, the metabolism of nitrogen-containing compounds leads to the production of several important aroma compounds that contribute to the fermentation bouquet in wine (Fleet 2003). Such compounds include higher alcohols which are produced via the Ehrlich pathway (Bell and Henschke 2005) and their corresponding esters and fatty acids. Organic acids present together with these alcohols provide substrates for ester formation which are known to positively influence the wine quality (Lambrechts and Pretorius 2000). Protein and peptide utilization as sources of nitrogen has been reported in some non-*Saccharomyces* species (Milewski et al. 1988; Shallow et al. 1991). Proteases active under winemaking conditions, either naturally present or added as external enzymes, could liberate peptides and amino acids, thus contributing to the overall nitrogen content that is instrumental to the formation of flavour-active compounds. Furthermore, some grape varietal aroma precursors such as thiols are present as cysteine or glutathione conjugates (Roland et al. 2010), and these could potentially be cleaved off by the action of proteases. It has also been recently noticed that yeast peptides could be responsible for the perception of sweetness in dry wines (Marchal et al. 2011). The presence of proteases could therefore play a role in this release of peptides. Further research is however needed to ascertain these hypotheses.

Conclusion and future outlooks

Proteases represent a unique class of enzymes that are of significant commercial interest since they possess both synthetic and degrading properties. Proteases are produced by all living organisms, but microbes are the preferred source of enzymes for industrial applications. Microbes have received

much attention in this regard because of their rapid growth and ease of genetic manipulation. Furthermore, limited space is required for their cultivation. Microbial proteases are used in many industrial applications and have been extensively exploited in the food, dairy and detergent industries since ancient times. Moreover, they have also found their use in many research applications especially in the field of molecular biology. Although the alkaline and neutral proteases are the most widely used proteases in industry because of their addition to household detergents, acid proteases are also utilized in several industrial applications. Generally, these enzymes range between 35 and 50 kDa and are mostly composed of β -strand secondary structures. In eukaryotes, these enzymes usually exhibit a tertiary structure consisting of two symmetrical lobes, each carrying an aspartic acid residue in order to form the catalytic site. The proposed mechanism of action is based on a general acid-base catalysis where the one residue acts as an acid (donating a proton) and the other as a base (donating a proton).

The development of recombinant rennin and its commercialization constitutes an excellent example of one of the first successful applications of acid proteases in industry. Furthermore, since the turn of the twenty-first century, the food, dairy, medical and pharmaceutical industries seem to show a growing interest in these proteases. In the beverage industries especially, these proteases are receiving much attention because of their use as an alternative to currently employed clarifying agents. The wine industry is particularly interested in aspartic proteases because of their potential to remove haze-forming proteins. These proteins have been confirmed to be PR proteins, mainly chitinases and TLPs. Although much has been elucidated about the mechanism of haze formation in wine, and the molecules responsible for this phenomenon, there are still areas that are not fully understood. Further investigations into the mechanism of haze formation are essential as its understanding could lead to the development of improved predictive tools and more targeted approaches in the struggle to prevent haze formation. The most commonly used method for removing proteins susceptible to form haze currently is treatment of the wine with bentonite. This treatment has several negative effects on the wine quality, and alternatives are therefore sought but although several have been proposed, none has been efficient enough to replace bentonite. The removal of these proteins through the use of aspartic proteases would be an ideal alternative and has been shown not to influence the wine's organoleptic properties negatively. Furthermore, some studies suggest that treatment with aspartic protease has the added benefit of releasing beneficial secondary products. Through the metabolism of nitrogen-containing compounds, higher alcohols and esters can indeed be produced by the yeast during alcoholic fermentation and thereby potentially impact positively on the wine bouquet. However, most of the commercially available aspartic proteases of

fungal origin tested in various studies are unable to meet the current requirements, and for most of them, a combined heat treatment is required for the enzymatic treatment to be effective. Therefore, further investigations are needed in order to find other aspartic proteases that could alleviate the need for bentonite addition.

In this context, the search for novel aspartic proteases, such as those secreted by organism naturally present in the winemaking environment, has begun and shows great promises. Furthermore, the exploitation of biodiversity through the use of indigenous aspartic proteases-producing yeasts as starter cultures in inoculated fermentations is also gaining attention and might be a good alternative. Moreover, protein engineering strategies can also be employed in order to design enzymes that are more suitable, although this field has not received so much attention yet. However, with the continued advancement of microbiology and biotechnology, a favourable environment is created for the development of more efficient novel acid proteases and their application in several industries to improve the quality of life. Although there are several areas that lack complete understanding, the study of aspartic proteases and their use in industrial applications is an exciting field of research, and various new findings are to be expected, especially in the beverage industries.

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