Facoltà di Agraria, Dipartimento di Scienze e Tecnologie Agroalimentari
(AGR/15)

CORSO DI DOTTORATO DI RICERCA
Biotecnologia Degli Alimenti - XXIII ciclo

Removal of unstable proteins from white wine by immobilized acid protease

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ABSTRACT

This PhD thesis research project was aimed at assessing and optimizing different immobilization procedures of pineapple stem bromelain, in order to develop an innovative biotechnological technique, alternative to bentonite fining, useful to removal selectively unstable proteins from white wines.

Stem bromelain activity was assessed on a suitable synthetic substrate at a reference pH value (3.2), this being the average minimum pH value of wine. Protease was covalently immobilized on different supports by various procedures and the best biocatalyst was chosen measuring immobilization percentage, kinetic parameters and half-life (in model wine buffer). Moreover, the influence on free and immobilized protease activity of potential inhibitors naturally present in wine, such as ethanol, tannins and sulphur dioxide (SO_2) over the average range concentration of wine, was investigated. Finally a kinetic study was carried out using 6 artisan and unrefined white wines, spiced with the synthetic substrate, in order to compare catalytic properties of free and immobilized bromelain. Immobilized protease activity, then, was tested in these wines both on total proteins and on unstable ones.

Covalent immobilization reduced bromelain catalytic properties. All kinds of procedures applied at pH 7 allowed the highest immobilization yield. Nevertheless, biocatalysts immobilized at pH 3.2 showed the best catalytic performance. Stem bromelain was successfully immobilized on chitosan beads without glutaraldehyde at pH 3.2, obtaining the most interesting and food-safe biocatalyst, which was used for all other experiments.

Inhibition study proved that all the inhibitors tested resulted to be reversible for stem bromelain activity. Nevertheless, the immobilized enzyme was lesser affect respect to the free one. Free sulphur dioxide was the strongest inhibitor, being a mixed-type for free stem bromelain and an uncompetitive inhibitor for the immobilized one.

Kinetic study of free and immobilized protease in different artisan and unrefined white wines, showed that the catalytic efficiency of immobilized one, as measured with

respect to the synthetic substrate, paralleled that on wine proteins, varied depending on the different inhibitors content in the tested wines.

In terms of turbidity haze, bromelain immobilized on chitosan beads exerted its useful proteolytic activity on unstable white wine proteins in almost the same way whatever their nature and content, in a 24-h treatment.

RIASSUNTO

Questa tesi di dottorato ha avuto per oggetto l'applicazione e l'ottimizzazione di differenti procedure per l'immobilizzazione di bromelina estratta da gambo d'ananas, al fine di sviluppare una biotecnologia innovativa, alternativa al trattamento con bentonite, in grado di rimuovere selettivamente le proteine instabili presenti nei vini bianchi.

L'attività della bromelina è stata valutata impiegando un substrato sintetico ad un pH di riferimento (3.2), considerato il valore minimo medio di pH del vino. La proteasi è stata immobilizzata covalentemente su differenti supporti applicando varie procedure e, fra i biocatalizzatori ottenuti, è stato individuato quello migliore valutando la resa di immobilizzazione, i parametri cinetici e l'emivita in vino modello. E' stata inoltre studiata l'influenza sull'attività della proteasi libera ed immobilizzata di potenziali inibitori naturalmente presenti nel vino, quali etanolo, tannini e anidride solforosa (SO_2), testandone l'effetto nel range di concentrazione tipica del vino. Le proprietà catalitiche della bromelina libera ed immobilizzata sono state, infine, confrontate mediante uno studio cinetico, condotto in 6 differenti vini bianchi artigianali, arricchiti con il substrato sintetico. In tali vini è stata poi valutata l'attività della proteasi immobilizzata, sia sulle proteine totali che su quelle instabili.

Dai risultati ottenuti è emerso che l'immobilizzazione covalente ha inciso negativamente sulle proprietà catalitiche della bromelina, indipendentemente dal pH di immobilizzazione (3.2 o 7). Fra le procedure impiegate, quelle applicate a pH 7 hanno permesso di ottenere le più alte rese d'immobilizzazione, anche se le migliori performance catalitiche sono state rilevate per tutti i biocatalizzatori immobilizzati a pH 3.2. La bromelina da gambo d'ananas è stata immobilizzata con successo su sfere di chitosano a pH 3.2 senza l'impiego di glutaraldeide, ottenendo così un biocatalizzatore *food-safe* che, risultando il più interessante dal punto di vista catalitico, è stato impiegato per i successivi esperimenti.

Lo studio di inibizione ha dimostrato che l'attività della proteasi è inibita in modo reversibile da tutti i composti testati e che l'immobilizzazione ha reso l'enzima più resistente alla loro azione. L'anidride solforosa ha ridotto notevolmente l'attività

catalitica della bromelina risultando, fra i composti testati, il più forte degli inibitori. La sua azione inibente è risultata di tipo misto nei confronti della bromelina libera e acompetitiva nei confronti di quella immobilizzata.

Dallo studio cinetico condotto nei vini bianchi artigianali, è emerso che la proteasi immobilizzata esercita un'efficace azione catalitica sul substrato sintetico, così come nei confronti delle proteine presenti nei vini testati, in modo diverso a seconda del loro differente contenuto in inibitori.

In seguito ad un trattamento di 24-h, la bromelina immobilizzata su sfere di chitosano è risultata inoltre utile nel rimuovere le proteine instabili presenti nei vini bianchi in esame, indipendentemente dalla loro natura e dal loro contenuto.

Nomenclature

ε	molar absorptivity (mM^{-1} cm^{-1})
A.S.	specific activity (I.U. mg^{-1} of protein)
IY	immobilization yield (%)
V_{max}	maximum velocity at which enzyme catalyze reaction (I.U. mg^{-1})
K_M	Michaelis-Menten constant (μM)
k_{cat}	($V_{max}/[E]_{tot}$), turnover number (min^{-1})
K_a	(k_{cat}/K_M), affinity constant ($min^{-1}\mu M^{-1}$)
K_i	inhibition constant
K_i'	inhibition constant
E_t	amount of enzyme added to the assay (μl)
BSA	Bovine Serum Albumin
E	free enzyme
ES	enzyme-substrate complex
I	inhibitor
EI	enzyme- inhibitor complex
ESI	enzyme-substrate- inhibitor complex
$V_{max(app)}$	apparent maximum velocity at which enzyme catalyze reaction in presence of an inhibitor (I.U. mg^{-1})
$K_{M(app)}$	apparent Michaelis-Menten constant in presence of an inhibitor (μM)
$k_{cat(app)}$	($V_{max(app)}/[E]_{tot}$), apparent turnover number in presence of an inhibitor (min^{-1})

Contens

INTRODUCTION

Among the different nitrogenous substances present in white wines, proteins, in spite of their small concentration, varying from about 10 to 500 mg l^{-1} (Sauvage *et al*., 2010; Batista *et al*., 2009; Ferreira *et al*., 2002), are of primary importance for the colloidal stability and clarity of white wine (Batista *et al*., 2009; Waters *et al*., 1991). Haze or deposit formation in bottled wines is due to protein aggregation during storage and is a common defect of commercial wines, this make them unacceptable to consumers.

The traditional method applied to stabilize white wine against haze formation is based on bentonite fining. The bentonite particles are negatively charge and interact electrostatically with the wine proteins, allowing their removal (Ferreira *et al*., 2002). Though effective, this non-specific treatment generates several problems because of the non-specific adsorption properties of bentonite.

To the best of our knowledge, several studies have been carried out to remove unstable wine proteins, using different procedures, such as ultrafiltration (Hsu *et al*., 1987; Peri *et al*., 1988; Flores *et al*., 1990) and flash pasteurization (Hsu & Heatherbell, 1987b). No other techniques have been so far developed, since their application under standard winemaking conditions is neither viable economically nor qualitatively.

For these reasons, increasing attention is given today to the development of alternative practices for white wine protein stabilization, which would maintain wine quality, reducing costs and being more sustainable (Waters *et al*., 2005).

The main aims of this PhD thesis were the assessment and the optimization of different immobilization procedures of pineapple stem bromelain, in order to develop an innovative biotechnological technique, alternative to bentonite fining, useful to removal selectively unstable proteins from white wines.

In this PhD thesis a six-step experimental procedure was set up by performing in sequence the following activities:

i) assessment of stem bromelain activity on a suitable synthetic substrate at a reference pH value, this being the average minimum pH value of wine (3.2);

ii) determination of free protease kinetic, both in reference buffer (McIlvaine) and in model wine (tartaric buffer);

iii) different immobilization procedures were applied and the best biocatalyst was chosen measuring immobilization percentage, kinetic parameters and half-life (in model wine buffer);

iv) the influence on free and immobilized protease activity of potential inhibitors naturally present in wine, such as ethanol, tannins and sulphur dioxide (SO_2) over the average range concentration of wine, was investigated;

v) a kinetic study was carried out in different wines, using synthetic substrate, in order to compare catalytic properties of free and immobilized bromelain;

vi) immobilized bromelain activity was studied in unstable white wines to test proteolytic action both on total proteins and on unstable ones.

CHAPTER 1: White wine proteins

1.1 Proteins: function and structure

Proteins are macromolecules, defined as linear polymers of amino acids linked by peptide bonds. The main subunit of a protein is an amino acid, which consists of a central carbon atom (C_α), an amino group (NH_2), a hydrogen atom (H), a carboxyl group (COOH), and a side chain (R), which are bound to the α-Carbon atom, as shown in Figure 1.

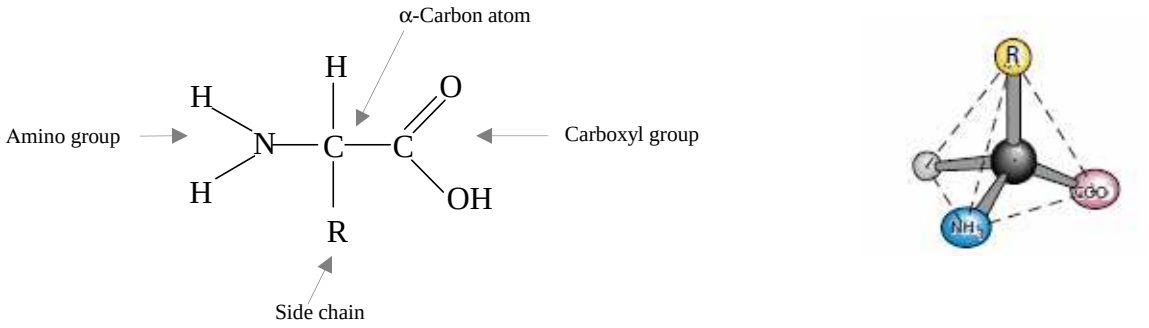


Figure 1: Amino acid structure, consisting of amino group (NH_2), carboxyl group (COOH), hydrogen atom (H) and side chain (R).

In nature, there are twenty different types of amino acids, which can be group, according to the structure of their side chain, into different classes: apolar, polar, uncharged and charged.

Four levels of structure are usually distinguished: primary, secondary, tertiary and for some proteins also quaternary structure. The primary structure is the sequence of amino acids in the polypeptide chain. The secondary structure is the bending and hydrogen bonding of a protein backbone to form repeating patterns. The elements of the secondary structure are subdivided into α-helices and β-sheets (Figure 2). The way in which twist or bends of whole polypeptide are folded together is called tertiary structure (Figure 2). The association of proteins by non-covalent bonds in order to form larger units is termed quaternary structure. Many globular proteins (monomers) form dimmers,

trimers or larger aggregates. Secondary, tertiary and quaternary structures together determine the conformation of the protein.

The proteins in general are amphoteric molecules, since they contain both acidic and basic moieties, and amphiphilic molecules, since they contain also both hydrophilic and hydrophobic moieties.

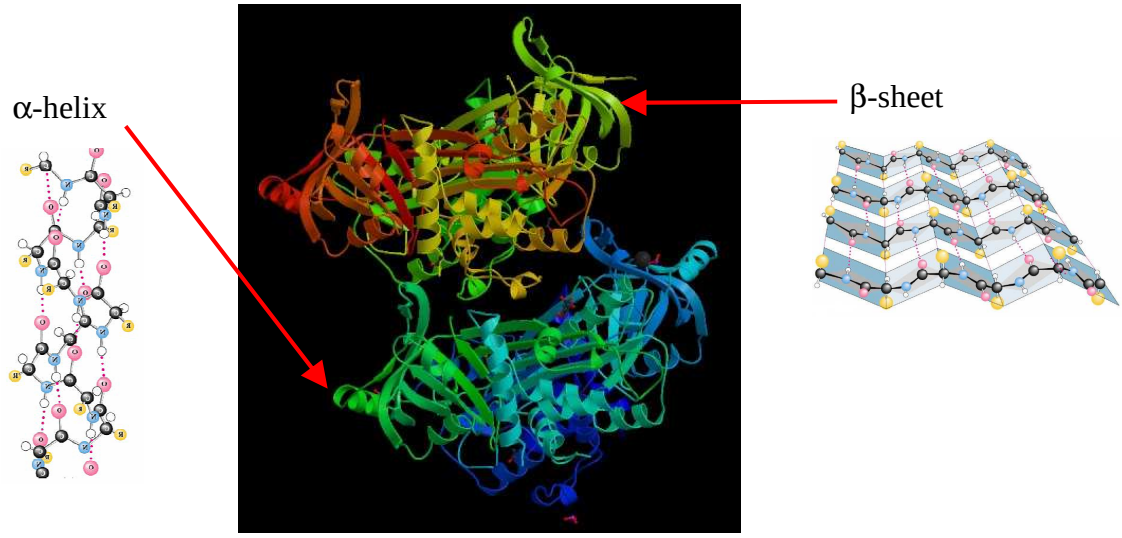


Figure 2: Crystal structure of uncleaved chicken egg albumin at 1.95 Å resolution (Stein *et al*., 1991).

Hydrophilic amino acid side groups reside predominantly at the surface of protein, while hydrophobic ones in the core of the structure. This chemical particularity allows them to be absorbed on a wide range of different surfaces (Martin, 2003; Engel, 2004; Rezwan, 2005).

1.2 Origin of wine proteins

Wine proteins have long been considered as a mixture of grape proteins and proteins from autolyzed yeast.

Yeasts may affect wine protein composition in two ways: by transferring proteins to wine during their autolysis and/or by the action of their exocellular protease, that hydrolyze must proteins (Feuillat *et al*., 1980). More recently, Ferreira *et al*. (2000)

employed immunological methods to show that at least the vast majority of polypeptides present in wines derive entirely from the grape pulp.

Wines contain polypeptides ranging in molecular mass from 9 to 62 kDa and isoelectric points from 3 to 9 (Brissonet & Maujean, 1993; Lamikanra & Inyang, 1988). However, the vast majority of wine proteins exhibit low molecular masses (20-30 kDa) and low isoelectric points ($4.1<pI<5.8$), possessing a net positive charge at wine pH values (Brissonet & Maujean, 1993; Ferreira *et al*., 2000). A simple denaturing electrophoretic analysis suggests that wine total protein fraction is mainly composed of only a few polypeptides. However, a more detailed examination reveals the presence of many tens of polypeptides with similar molecular masses but subtle differences in electric charge (Monteiro *et al*., 2001). Immunological and N-terminal sequencing experiments revealed that these polypeptides are structurally related, possibly deriving from one or a few common precursors synthesized during grape formation, which may have undergone limited proteolysis in the later stages of grape maturation or during vinification, to generate the variety of distinct polypeptides present in wines (Monteiro *et al*., 2001).

Colby was the first to point out, in 1896, that the grape variety, the environment where the grapes are grown, *terroir*, and the fermentation process are the three main factors affecting wines stability. The variations in protein content detected among wines are primarily caused by factors that influence the total nitrogen in grapes, such as climate, soil and the variety itself (Bayly & Berg, 1967). *Terroir* and biotic stresses influence not only the quantity but also the quality of proteins synthesized during grape development and maturation. Dorrestein et al., (1995) obtained variety specific chromatograms when the protein profiles of four different varietal wines were analysed by FPLC ion exchange chromatography. Other studies showed that fruit maturity also relates to the irregular occurrence of protein clouding in wines (Murphey *et al*., 1989). Indeed, the soluble proteins in juice and wine increase with increasing grape maturity (Murphey *et al*., 1989). Protein synthesis proceeds rapidly after veraison and parallels the rapid accumulation of sugars (Luis, 1983). Nevertheless, the proteins present in wine do not correspond to a representative fraction of the pulp proteins, since most of these are lost during winemaking (Ferreira *et al.*, 2000). It is well known that protein configuration is subjected to several modifications during winemaking, as suggested by wine proteins

electrophoretic bands. In fact, while their molecular masses are the same of those found in the juices, their isoelectric points differ (Lamikanra & Inyang, 1988; Murphey *et al*., 1989; Pueyo *et al*., 1993). Fermentation is primarily responsible for the difference between grape juice and wine protein content (Murphey *et al*., 1989). The lower protein levels typically found in wine are mainly due to proteolysis and denaturation of the grape proteins during fermentation, caused by proteases and changes in the pH, respectively (Bayly & Berg, 1967; Feuillat *et al*., 1980; Murphey *et al*., 1989). In fact, the proteins that end up in wine are those that are highly resistant to proteolysis and to the low pH values characteristic of this beverage (Waters *et al*., 1992). Moreover, during vinification, part of the soluble grape proteins precipitate via interaction with tannins (Figure 3), (Somers & Ziemelis, 1973).

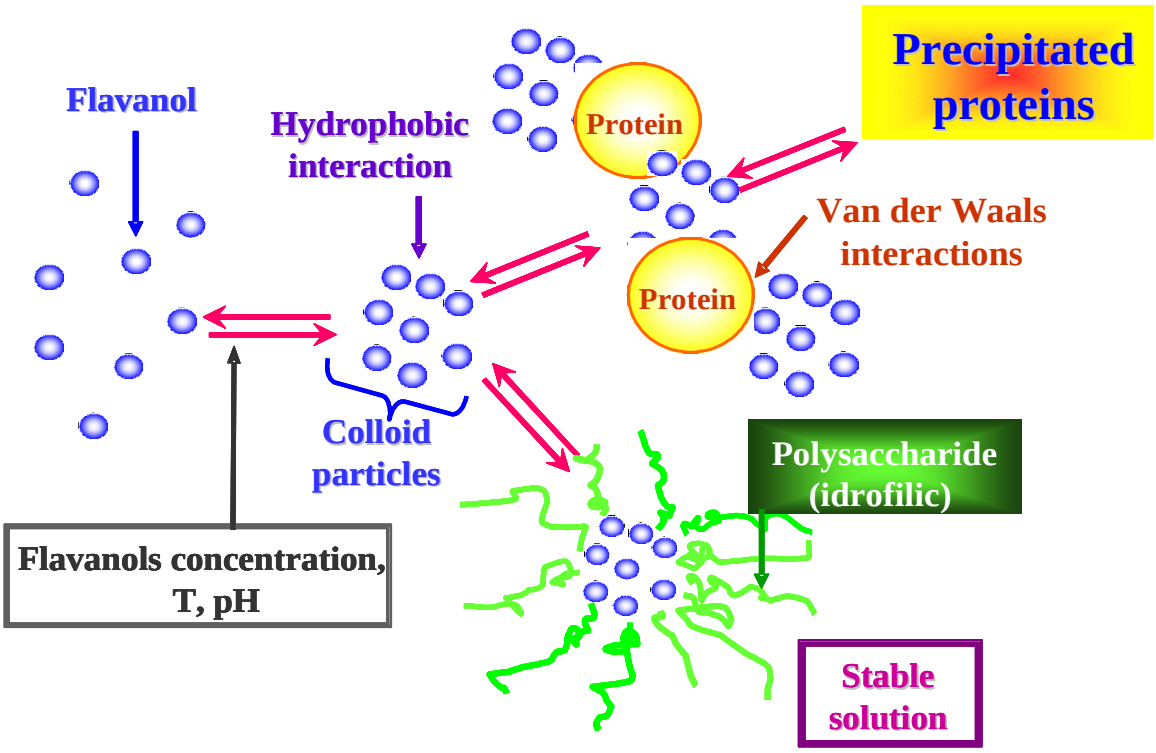


Figure 3: Proteins precipitation via interaction with tannins.

In addition, it has been estimated that approximately one-half of total wine protein is bound to grape phenols via different interaction, as described in Figure 4.

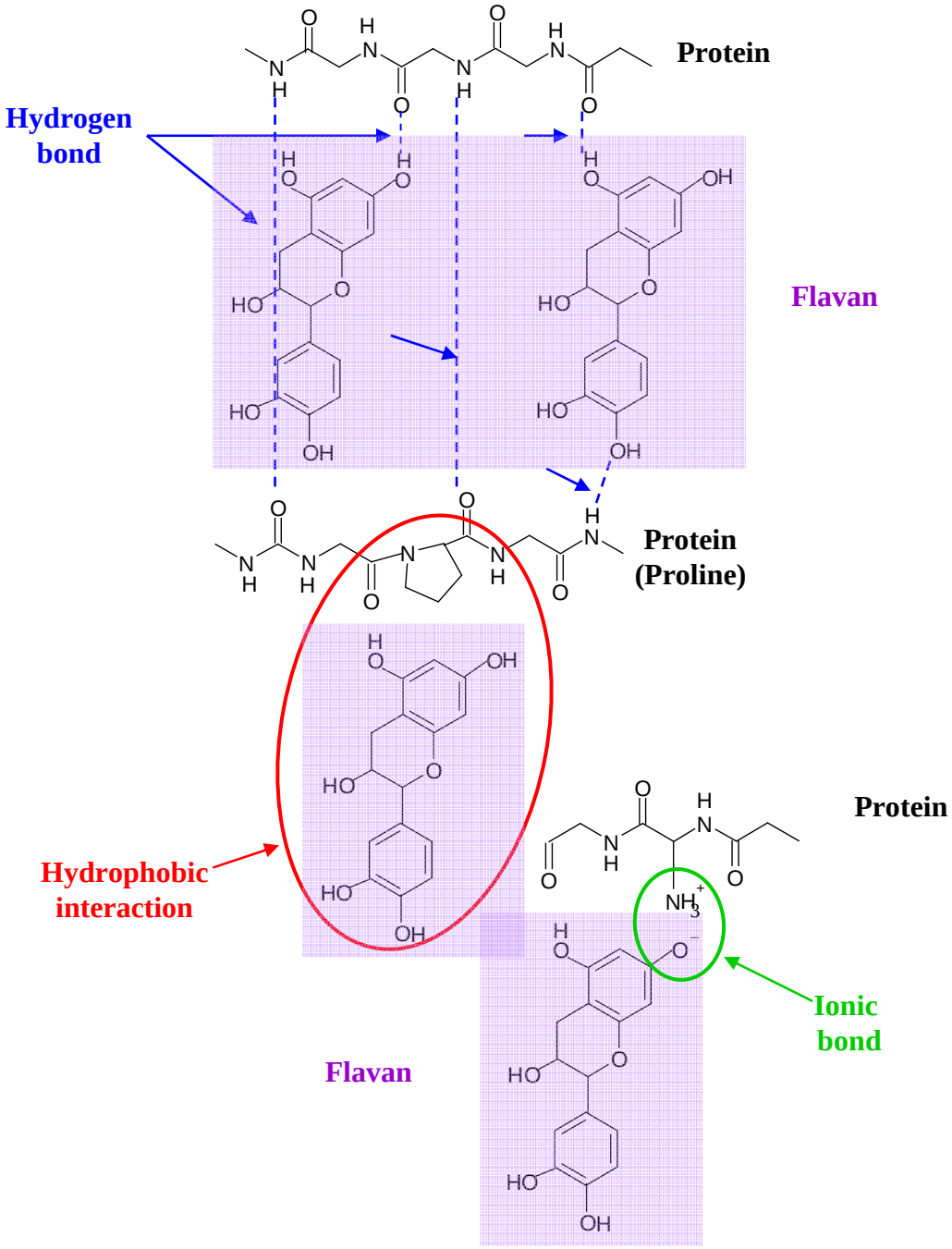


Figure 4: Interaction between wine proteins and polyphenols.

1.3 Haze in bottled white wine

Limpidity (translucency) is of vital importance to wine quality because this visual attribute make the first impression on the consumer (Ferreira *et al.*, 2004). For these

reason, protein precipitation in bottled white wines reduces their commercial value, indicating that they are unstable and therefore unacceptable for sale (Bayly & Berg, 1967; Hsu & Heatherbell, 1987a; Waters *et al*., 1991, 1992).

The measurement of limpidity is related with the turbidity, which corresponding to the optical phenomenon known as the Tyndall effect, caused by the presence of particles in suspension that deflect light from its normal path (Ribéreau-Gayon *et al*., 2000), (Figure 5).

Figure 5: Example of protein haze formation in white wine. On the left side a white wine protein unstable and on the right side a white wine clarified and stabilized.

It is possible to find different types of hazes in bottled wine, which can have microbiological or chemical origin. The haze induced by microorganism can occur due to the action of spoilage yeast (*Zygosaccharomyces* and *Brettanomyces*) and bacteria (lactic acid bacteria, acetic acid bacteria and *Bacillus* species), causing off-flavours and changes in wine quality (Rankine & Pilone, 1973; Van de Waters, 1985). The haze induced by chemical factors can occur by crystallization of metal salts (iron and copper), including potassium bitartrate (Dunsford & Boulton, 1981; Rodriguez-Clemente *et al*., 1990; Lubbers *et al*., 1993) and calcium tartrate (Clark *et al*., 1988). Polysaccharides and polyphenols are also involved in the induced-haziness, producing color changes and precipitates (Somers & Ziemelis, 1985; Siebert *et al*., 1996; Waters *et al*., 1996; Pellerin *et al*., 1994).

The most abundant haze problem in bottled white wine is still due to precipitation of unstable proteins (Waters *et al*., 2005), that is considered one of the most common non-microbial defects of commercial wines (Bayly & Berg, 1967; Hsu & Heatherbell, 1987a; Waters *et al*., 1992). Slow denaturation of wine proteins, resulting from unfavourable storage conditions, probably originate protein aggregation and flocculation into a hazy suspension, leading to the appearance of a haze or deposit in bottled wine.

1.4 Proteins responsible for wine turbidity

Despite significant advances in wine proteins research, the involved factors and the precise molecular mechanism of protein haze formation in bottled white wines, remain largely to be elucidated.

For a number of years, the study of protein haze formation in bottled white wines was essentially focused on the proteins themselves. It was initially proposed that wine instability is solely related to its protein content as reported by Somers & Ziemelis (1973) and Anelli (1977). Supporting this hypothesis is the study of Koch and Sajak (1959), who used paper electrophoresis to show that wine contains two major protein fractions, both of which decrease upon heat treatment.

However, other studies have shown that protein instability does not correlate well with the wine total protein content, and, therefore, the potential of wine to form haze is not predictable from its protein concentration (Berg & Akioshi, 1961; Moretti & Berg, 1965). Afterwards, Bayly & Berg (1967) supported this assertion demonstrating the different heat sensitivity of proteins in must. Therefore, two alternative hypotheses have been advanced to explain the insolubility of proteins in wine:

a) individual proteins behave differently in their sensitivity to heat denaturation, contributing differentially to haze formation; only part of the protein mixture is responsible for instability rather than the entire protein content;
b) although protein-dependent, the development of turbidity in wines is controlled by one or more factors of non-protein origin (Ferreira *et al*., 2002).

There is also conflicting evidence in the literature as to which proteins are responsible for haze and deposit formation. Thus, some reports suggest that the lower molecular

mass, lower pI proteins are the major and most important fractions contributing to protein instability in wines (Hsu & Heatherbell, 1987a, 1987b; Hsu *et al*., 1987; Mesrob *et al*., 1983). Other studies indicate that the lower molecular mass and higher pI (Heatherbell *et al*., 1984; Lee, 1986; Ngaba & Heatherbell, 1981) or the higher molecular mass proteins contribute mostly to heat instability. Other investigations revealed that all the major wine protein fractions are present in wine hazes and all have been shown to be heat unstable (Waters, 1991; Waters & Høj, 1999; Waters *et al*., 1990, 1991, 1992).

Recently, the attention of wine researchers moved towards compounds of non-protein nature. Indeed, the observations that wines are essentially composed of identical sets of polypeptides, identified as PR proteins, and that the haze forming wine proteins are PR proteins apparently similar in wines vinified from different grape varieties (Dawes *et al*., 1994; Ferreira *et al*., 2000; Monteiro *et al*., 2001; Pueyo *et al*., 1993; Waters *et al*., 1992, 1996), support the view that protein insolubility is not determined by the protein molecules themselves. It, probably, depends on some other non-protein factor, such as polyphenols, wine pH and the presence of polysaccharides. In other words, the presence of PR proteins in wine is certainly a pre-requisite for haze formation, even if it results affected by factors of non-protein origin (Ferreira *et al*., 2002).

1.5 Pathogenesis-related proteins

Pathogenesis-related proteins (PRs) are now widely considered as a rich source of allergens. The PRs are encoded by the plant genome and induced specifically in response to infections by pathogens such as fungi, bacteria, viruses, or by adverse environmental factors (Stintzi *et al*., 1993; Edreva, 2005; Kiba *et al*., 2007). These proteins include chitinases, thaumatinlike proteins and osmotins (Monteiro *et al*., 2001; Waters *et al*., 1998), which are particularly stable under winemaking conditions (low pH, proteolysis), passing selectively into the wine. The actual pattern of polypeptides that accumulate in mature grapes and wines is determined by the environmental and pathological conditions that prevail during vegetative growth (Ferreira *et al*., 2004). Infection with common grapevine pathogens or skin contact, such as occurs during

transport of mechanically harvested fruit, results in enhanced concentrations of PR proteins in wine (Cilindre *et al*., 2007; Waters *et al*., 2005).

Pathogenesis-related proteins do not constitute a super-family of proteins but represent a collection of unrelated protein families, which contribute to the plant defence system. Today, PRs are classified into 14 families. Many important plant food allergens are homologues to proteins that are members of PR families (Van Loon & Van Strien, 1999; Hoffmann-Sommergruber, 2002). The family 5 of PRs comprises unique proteins with diverse functions. Because of the sequence homologies between PR-5 proteins and thaumatin, an intensely sweet tasting protein isolated from the fruits of the West African rain forest shrub *Thaumatococcus daniellii*, members of this family of proteins are referred to as thaumatin-like proteins (TLPs). TLPs can be classified into three groups, (i) those produced in response to pathogen infection, (ii) those produced in response to osmotic stress, also called osmotins, and (iii) antifungal proteins present in cereal seeds (Breiteneder, 2004).

1.6 Protein fining treatments

The term ‘fining’ is used in winemaking to describe the deliberate addition of an adsorptive compound that is followed by the settling or precipitation of partially soluble components from wine.

The mechanisms of fining wine are based on the charge cancellation between the suspended particles and the fining agent ones, allowing the colloid suspension to agglomerate and flocculate by gravity. Other mechanism can be the absorption of the suspended particles on the surface of the fining agent.

Commonly, fining agents used in winemaking are proteins of animal origin, such as egg albumin, blood albumin, casein, isinglass, gelatins or plant proteins sourced from cereals and legumes.

Nevertheless, the traditional method of stabilizing white wine against haze formation is based on bentonite fining. This material is a montmorillonite clay, which contains exchangeable cations. The adsorption of wine proteins onto bentonite is due to the cationic exchange capacity of this clay, depending on the amount of displacement of aluminium ions by sodium, calcium, or magnesium ions (Figure 6).

Bentonite, carrying a net negative charge at the wine pH, interacts electrostatically with the positively charged wine proteins, producing their flocculation (Hsu & Heatherbell, 1987a; Lamikanra & Inyang, 1988).

The relationship between wine pH and protein isoelectric point governs the cationic nature of the wine proteins and thus their tendency to be adsorbed electrostatically by the negatively charged plate surfaces of bentonite (Bayly & Berg, 1967; Hsu & Heatherbell, 1987a; Moretti & Berg, 1965; Somers & Ziemelis, 1973).

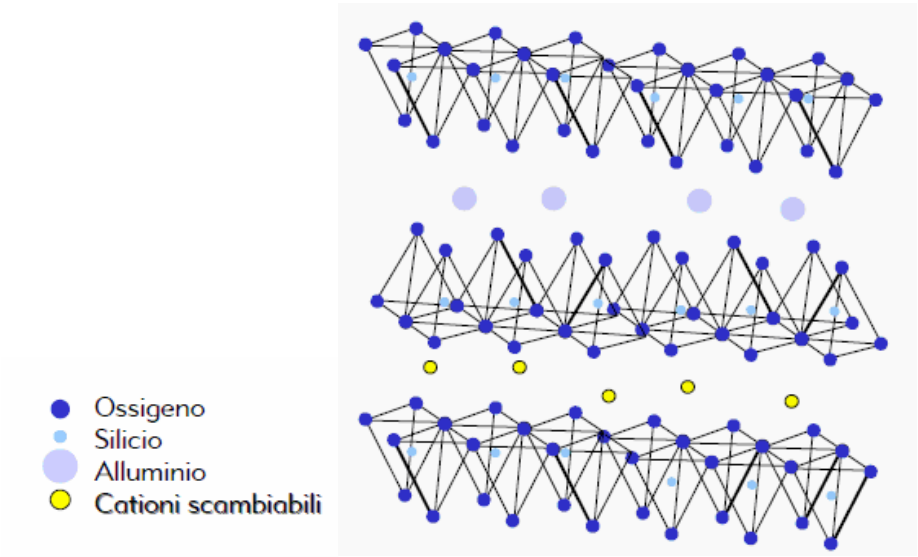


Figure 6: Bentonite structure.

However, bentonite is not equally effective in removing all proteins from wine. Various studies showed that different protein fractions may require distinct bentonite concentrations for removal (Hsu & Heatherbell, 1987a; Murphey *et al*., 1989; Paetzold *et al*., 1990). Dawes et al. (1994) observed that the amount of protein depletion correlates approximately linearly with the level of bentonite added. The amount of bentonite required to stabilize wines has increased over the last 10-20 years. Doses of 0.2–0.4 g l^{-1} bentonite were generally enough to stabilize wines. However, nowadays, to ensure a secure stabilization, doses of 0.8–1 g l^{-1} bentonite are often employed (Hsu & Heatherbell, 1987a; Paetzold *et al*., 1990).

As a cation exchanger, bentonite adsorption is not specific for proteins, removing other charged species or aggregates involved as aroma and *flavour* compounds, inducing

significant aroma loss and, occasionally, colour alteration (Cabaroglu *et al*., 2002). Bentonite fining also causes substantial volume losses (between 3% and 10%) (Hoj *et al*., 2001) and the disposal of spent bentonites constitute a non-negligible source of waste. Finally, bentonite handling is also of concern on account of occupational health and safety issues (Sauvage *et al*., 2010).

Several alternative techniques to bentonite fining, as well as the use of others fining agents, to removal haze-forming wine proteins have been studied. Some alternative techniques studied have been ultrafiltration (Hsu *et al*., 1987; Flores, *et al*., 1990), flash pasteurization (Pocock *et al*., 2003) and use of others adsorbents materials such as resin (Sarmento *et al*., 2000) and metal oxides (Pashova *et al*., 2004a; Pashova *et al*., 2004b; Salazar *et al*., 2006; Salazar *et al*., 2007).

The addition of glycoproteins, such as mannoproteins (Waters *et al*., 1991; Waters *et al*., 1994a; Waters *et al*., 1994b), of polysaccharides extracted from seaweeds (Cabello-Pasini *et al*., 2005), as well the use of immobilized phenolic compound, such as proanthocyanidins (Weetall *et al*., 1984; Powers *et al*., 1988), has been tested as alternative to reduce the protein haze in white wine. The results obtained to date have not been successful on industrial scale and, thus, bentonite is still the only commercially acceptable practical solution to avoid protein haze in bottled white wines (Waters *et al*., 2005).

CHAPTER 2: Enzyme immobilization

2.1 Enzymes in food industry

Enzymes exhibit a number of features that make their use advantageous as compared to conventional chemical catalysts (Krajewska, 2004).

Enzymes have a high level of catalytic efficiency, often far superior to chemical catalysts, and a high degree of specificity that allows them to discriminate not only between reactions but also between substrates (substrate specificity), similar parts of molecules (regio-specificity) and between optical isomers (stereo-specificity). These specificities warrant that the catalyzed reaction is not perturbed by side-reactions, resulting in the production of one wanted end-product, whereas production of undesirable by-products is eliminated. This provides substantially higher reaction yields reducing material costs (Bullock, 1995; Woodley, 1992; Van de Velde *et al*., 2002). In addition, enzymes generally operate at mild conditions of temperature, pressure and pH with reaction rates of the order of those achieved by chemical catalysts at more extreme conditions, resulting in substantial process energy savings and reducing manufacturing costs (Krajewska, 2004).

For many thousands of years, man has used naturally occurring microorganisms, bacteria, yeasts and their enzymes to make foods such as bread, cheese, beer and wine. In bread-making, for example, amylase is used to break down flour into soluble sugars, which are transformed by yeast into alcohol and carbon dioxide, making the bread rise. Today, enzymes are used for an increasing range of applications in food industries, such as bakery, cheese making, starch processing, production of fruit juices and other drinks (Table 1), in order to improve food texture, appearance and nutritional value, generating desirable *flavours* and aromas.

Like all proteins, enzymes can cause allergic reactions when people have been sensitized through exposure to large quantities. For this reason, enzyme immobilization results useful in order to allow the prevention of protein contamination in food products (Krajewska, 2004).

Table 1: Enzyme applications in food industry.

Market	Enzyme	Purpose / function
Dairy	Rennet (protease)	Coagulation in cheese production
	Lactase	Hydrolysis of lactose to give lactose-free milk products
	Protease	Hydrolysis of whey proteins
	Catalase	Removal of hydrogen peroxide
Brewing	Cellulase, beta-glucanase, alpha-amylase, protease, maltogenic amylase	For liquefaction, clarification and to supplement malt enzymes
Alcool production	Amyloglucosidase	Conversion of starch to sugar
	Alpha-amylase	Breakdown of starch, maltose production
Baking	Amyloglycosidase	Saccharification
	Maltogen amylase	Delays process by which bread becomes stale
	Protease	Breakdown of proteins
	Pentosanase	Breakdown of pentosan, leading to reduced gluten production
	Glucose oxidase	Stability of dough
Wine and fruit juice	Pectinase	Increase of yield and juice clarification
	Glucose oxidase	Oxygen removal
	Beta-glucanases	
Meat	Protease	Meat tenderising
Starch	Alpha amylase, glucoamylase, hemicellulase, maltogenic amylase, glucose isomerase	Modification and conversion (to dextrose or high fructose syrups)
Inulin	Inulinase	Production of fructose syrups

2.2 Enzyme immobilization

In addition to the unquestionable advantages, there exist a number of practical problems in the use of enzymes. To these belong: the high cost of their isolation and purification, the instability of their structures, once they are isolated from their natural environments, and enzymes sensitivity both to process conditions other than to trace levels of substances that can act as inhibitors, resulting in enzymes short operational lifetimes. Moreover, unlike conventional heterogeneous chemical catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems, contaminating the product. Consequently, they cannot be recovered in the active form from reaction mixtures for further uses.

Several methods have been proposed to overcome these limitations, one of the most successful is enzyme immobilization (Krajewska, 2004, Tischer & Wedekind, 1999; Scouten *et al*., 1995). Immobilization is achieved by fixing enzyme to or within solid supports. By mimicking the natural mode of occurrence in living cells, where enzymes for the most cases are attached to cellular membranes, the systems stabilize the structure of enzymes, hence their activities (Figure 7).

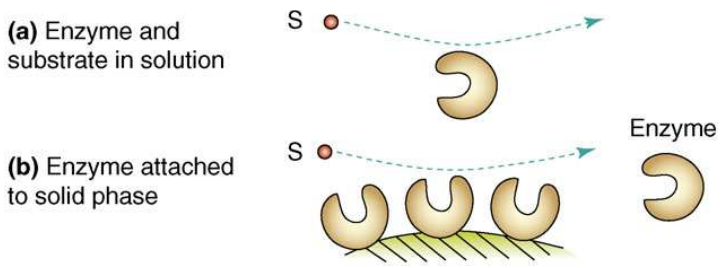


Figure 7: Different types of enzymatic reactions. Enzyme catalysis has commonly been studied in solution phase **(a)**. For practical reasons, enzymes can be immobilized on a polymeric support, which is also well documented **(b)**, (Laurent *et al*., 2008).

Thus, as compared to free enzymes in solution, immobilized ones are more robust and resistant to environmental changes (Krajewska, 2004).

In details, enzyme immobilization provides the following advantages:

- enhanced stability;
- repeated or continuous use;

- easy separation from the reaction mixture;
- possible modulation of the catalytic properties;
- prevention of protein contamination in the product;
- prevention of microbial contaminations.

However, regardless of its nature or preparation, an immobilized enzyme, by definition, has to perform two essential functions, namely:

i. the non-catalytic functions (NCFs), that are designed to aid separation (e.g. isolation of catalysts from the application environment, reuse of the catalysts and particularly control of the process);
ii. the catalytic functions (CFs), that are designed to convert the targeting compounds (or substrates) within a desired time and space (Cao, 2005).

Enzymes can be immobilized by a variety of methods, which may be broadly classified as physical, where weak interactions between support and enzyme exist, and chemical, where covalent bonds are formed with the enzyme (Krajewska, 2004; Tischer & Wedekind, 1999; Scouten *et al*., 1995; Bullock, 1995; Woodley, 1992), as described in Figure 8.

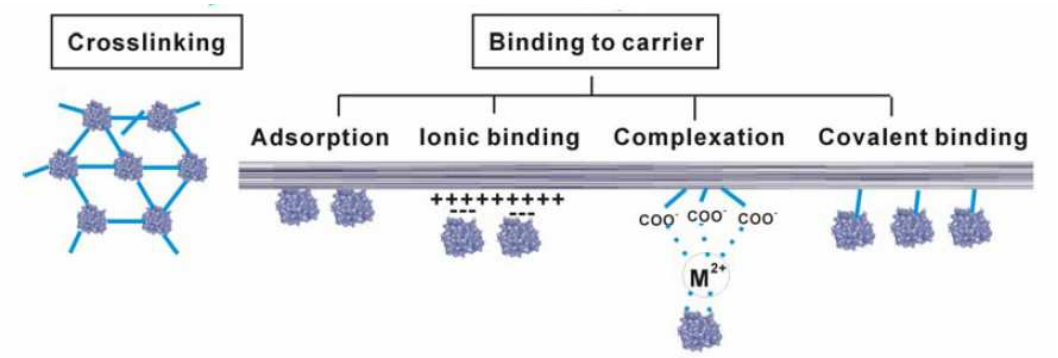


Figure 8: Schematic classification of immobilization methods.

To the physical methods belong: (i) containment of an enzyme within a membrane reactor, (ii) adsorption (physical, ionic) on a water-insoluble matrix, (iii) inclusion (or gel entrapment), (iv) microencapsulation with a solid membrane, (v) microencapsulation with a liquid membrane, and (vi) formation of enzymatic Langmuir-Blodgett films. The chemical immobilization methods include: (i) covalent attachment to a water-insoluble matrix, (ii) crosslinking with use of a multifunctional, low

molecular weight reagent, and (iii) co-crosslinking with other neutral substances, e.g. proteins. All these approaches are a compromise between maintaining high catalytic activity while achieving the advantages of immobilization. Numerous other methods, which are combinations of the ones listed or original and specific of a given support or enzyme, have been devised. However, no single method and support is best for all enzymes and their applications. Besides, all of the methods present advantages and drawbacks. Adsorption is simple, cheap and effective but frequently reversible; covalent attachment and cross-linking are effective and durable, but expensive and easily worsening the enzyme performance; in membrane reactor-confinment, entrapment and microencapsulations generate diffusional problems (Krajewska, 2004). Consequently, as a rule the optimal immobilization conditions for a chosen enzyme are found empirically by a process of trials, in order to ensure the highest activity retention, operational stability and durability. Nevertheless, immobilization affects enzyme performance (Krajewska, 2004; Tischer & Wedekind, 1999; Scouten *et al*., 1995; Bullock, 1995; Woodley, 1992). Immobilized enzyme, usually, presents both lower activity and higher Michaelis constant respect to the free one. These alterations result from enzyme structural changes, due to the immobilization procedure application and from the creation of a microenvironment, different from the bulk solution, in which enzyme works. The latter is strongly dependent on the reaction taking place, the nature of the support and on the design of the reactor. Furthermore, being two phase systems, the immobilized enzyme systems suffer from inevitable mass transfer limitations, producing unfavorable effects on their overall catalytic performances. These, however, may be reduced by applying appropriate reactor designs (Krajewska, 2004).

2.3. Supports for enzyme immobilization

The properties of immobilized enzymes depend both on enzyme and support material properties (Krajewska, 2004; Tischer & Wedekind, 1999). In Figure 9, the most used immobilization supports are schematized.

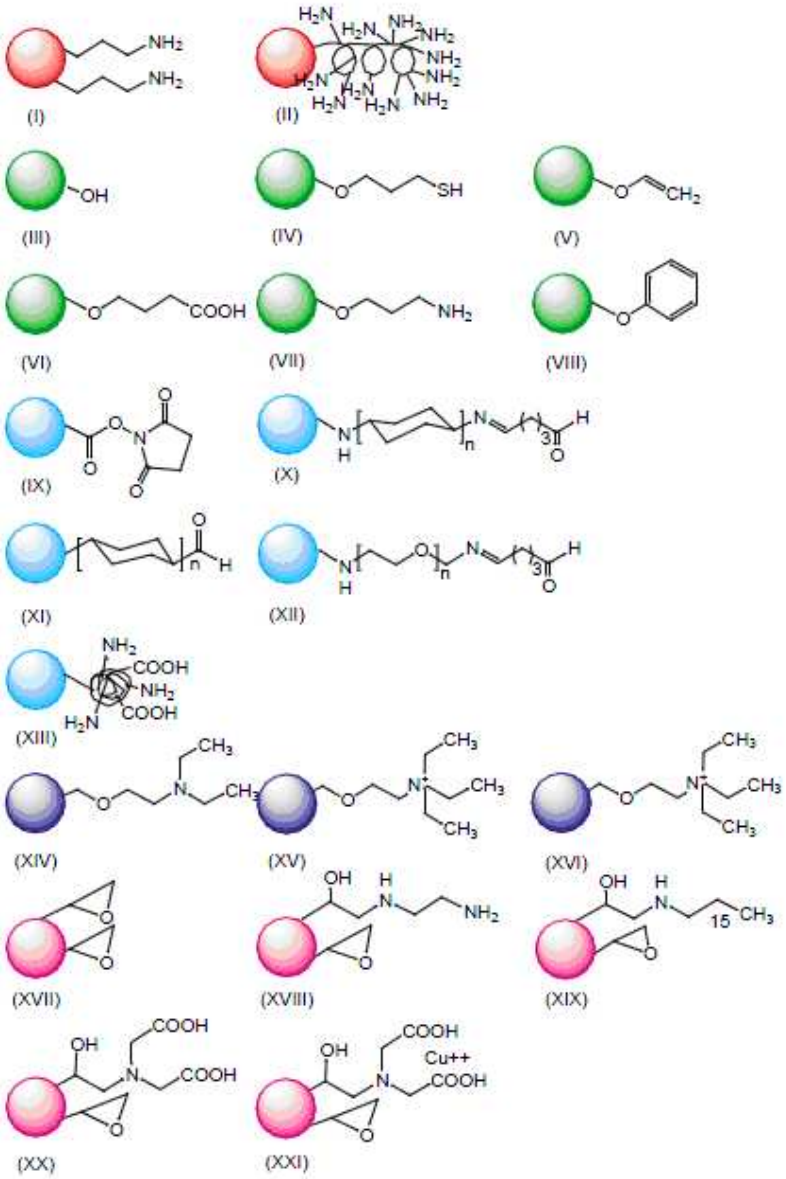


Figure 9: The most used immobilization supports (Cao, 2005).

Silica and derivatives. (I) Silanized silica and (II) PEI-coated silica; Mesoporous zeolite: (III) SAB-15, (IV) MPTMS, (V) VTES, (VI) TSBN-COOH, (VII) APTES, (VIII) PTMS; hydrophobic carriers: (IX) no spacer, (X) amino dextran as spacer, (XI) aldehyde dextran as spacer, (XII) amino PEG as spacer, (XIII) BSA as spacer; polysaccharide-based carriers: (XIV) DEAE-sephadex, (XV) TEAE-Cellulose, (XVI) Ecetola-cellulose; functionalized Eupergit C: (XVII) Eupergit C; (XVIII) DAE-Eupergit C, (XIX) dodecyl-Eupergit C, (XX) IDA-Eupergit C, (XXI) Cu++-IDA-Eupergit C.

Although it is recognized that there is no universal support for all enzymes and their applications, a number of desirable characteristics should be common to any material considered for immobilizing enzymes. These include: high affinity to proteins, availability of reactive functional groups for direct reactions with enzymes and, for chemical modifications, hydrophilicity, mechanical stability, rigidity and regenerability. Understandably, for food, pharmaceutical, medical and agricultural applications, non-toxicity and biocompatibility of the materials are also required. Furthermore, to respond to the growing public health and environmental awareness, the materials should be biodegradable, economical and inexpensive (Krajewska, 2004). In the last decades, numerous organic and inorganic materials have been proposed as carriers for enzyme immobilization, among these, silica materials, epoxy-activated acrylic resins and chitosan have been largely studied for a wide range of industrial applications.

2.3.1. Silica materials

Among various inorganic carriers, silica materials, have been widely used as the stationary phases for HPLC. Recently, mesoporous silica materials have gathered a significant attention in both academic and industrial areas because of its well-ordered and adjustable pore structures as well as abundant surface silanol groups. Their large surface area and their high pore volume allow the reusability of immobilized enzymes, enhancing their activity, selectivity as well as operational stability. For these reasons silica materials have been largely applied for enzyme immobilization.

Recently, a wide range of enzyme has been immobilized on silica materials. For the first time, Wainer and co-workers immobilized trypsin on silica particles modified by hydrophilic polymers and glutaraldehyde in sequence (Ma *et al*., 2009). Bonneil et al. proposed an immobilized trypsin reactor with porous glass beads (80–120 mesh; 700Å average pore size) as carrier, bonding trypsin via diisothiocyanate (Bonneil *et al*., 2000; Bonneil & Waldron, 2000). Later, the immobilization of lipase on several kinds of mesoporous silica has been well investigated (Gao *et al*., 2010; Lee *et al*., 2005; Hartmann, 2005; Kim *et al*., 2007; Li *et al*., 2009a). Moreover, it has been reported that the pore-size of support materials, especially the ratio pore-size *vs* enzyme molecule size, may greatly influence the enzyme adsorption (Lu *et al*., 2008; Kang *et al*., 2007)

and the ideal size of pores was found to be 3–5 times of the protein size (Serra *et al*., 2008; Gritti and Guiochon, 2007).

2.3.2. Epoxy-activated acrylic resins

The commercial products EUPERGIT C and EUPERGIT C 250 L are amongst the most extensively studied matrixes for enzyme immobilization (Boller *et al*., 2002). Both resins are microporous, epoxy-activated acrylic beads with a diameter of 100-250 μm. They differ in the content of oxirane groups and in their porosity. While EUPERGIT C has an average pore size of r = 10 nm and an oxirane density of 600 μmol/g dry beads, EUPERGIT C 250 L has larger pores (r = 100 nm) and a lower oxirane density (300 μmol/g dry beads).

Immobilization of enzymes on these resins is rapid and easy both at laboratory scale and at industrial scale. There is no need for additional reagents or special equipment. As a standard procedure, the enzyme is simply dissolved in aqueous buffer and, after addition of the EUPERGIT beads, left to stand at room temperature or at 4 °C for 12-72 h.

Guisan et al. have proposed a two-step binding mechanism for this process (Mateo *et al*., 2000). It is assumed that, in the first step, the enzyme is physically adsorbed on the carrier by hydrophobic interactions. This brings amino and thiol groups on the surface of the enzyme in close proximity to the oxirane groups of the carrier. In the second step they react with the oxirane groups by nucleophilic attack. In this way, very stable C-N and C-S bonds are formed. Lasch and Janowski showed that there was no detectable protein leakage from Azocasein-EUPERGIT C conjugates once residual, non-covalently bound protein had been removed (Lasch & Janowski, 1988).

The high density of oxirane groups on the surface of the support promotes “multipoint attachment”. It is assumed that such multipoint attachment leads to an increase in conformational stability of the enzyme and hence improves longterm operational stability of the immobilized biocatalyst. Other advantages of covalent immobilization onto EUPERGIT are increased thermostability, immunisation against aldehydes, and increased stability against denaturing polar organic solvents. Due to its compatibility

with a wide range of different enzymes, it is also frequently used for the immobilization of enzymes in academic and industrial research. Different classes of enzymes, such as oxidoreductases (E.C.1), transferases (E.C. 2), hydrolases (E.C. 3), and lyases (E.C. 4), have been successfully immobilized on these supports (Boller *et al*., 2002).

2.3.3. Chitin- and chitosan-based materials

Of the many carriers that have been considered and studied for immobilizing enzymes, organic or inorganic, natural or synthetic, chitin and chitosan are of interest since their characteristics. Chitin and chitosan are natural polyaminosaccharides (Krajewska, 2004; Peter, 1995; Krajewska, 1991). Chitin is one of the world's most plentiful, renewable organic resources; it is the major constituent of crustaceans shell, insects exoskeleton and fungi cell walls, providing strength and stability. Chitin is estimated to be synthesized and degraded in the biosphere in the vast amount of at least 10 Gt each year. Chemically, chitin is composed of β (1 → 4) linked 2-acetamido-2-deoxy-β-D-glucose units (or N-acetyl-d-glucosamine) (Peter, 1995), forming a long chain linear polymer (Figure 10).

Chitosan, the principal derivative of chitin, is obtained by N-deacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetyl-D-glucosamine and D-glucosamine. Chitin and chitosan can be chemically considered as analogues of cellulose, in which the hydroxyl at carbon-2 has been replaced by acetamido and amino groups, respectively. Chitosan is insoluble in water, but the presence of amino groups renders it soluble in acidic solutions below pH about 6.5. Chitin and chitosan are not single chemical entities, but vary in composition depending on the origin and manufacture process. Chitosan can be defined as chitin sufficiently deacetylated to form soluble amine salts, the degree of deacetylation necessary to obtain a soluble product being 80–85% or higher (Krajewska, 2004). Commercially, chitin and chitosan are obtained at a relatively low cost from shells of shellfish (mainly crabs, shrimps and lobsters), wastes of the seafood processing industry (Hudson & Smith, 1998; Tharanathan & Kittur, 2003). Basically, the process consists of deproteinization of the raw shell material with a dilute NaOH solution and decalcification with a dilute HCl solution. To result in chitosan, the obtained chitin is

subjected to N-deacetylation by treatment with a 40–45% NaOH solution, followed by purification procedures. Thus, production and utilization of chitosan constitutes an economically attractive means of crustacean shell wastes disposal sought worldwide.

Chitosan possesses distinct chemical and biological properties (Peter, 1995; Krajewska, 1991). In its linear polyglucosamine chains of high molecular weight, chitosan has reactive amino and hydroxyl groups, amenable to chemical modifications (Peter, 1995; Tharanathan & Kittur, 2003; Dutta *et al*., 2002).

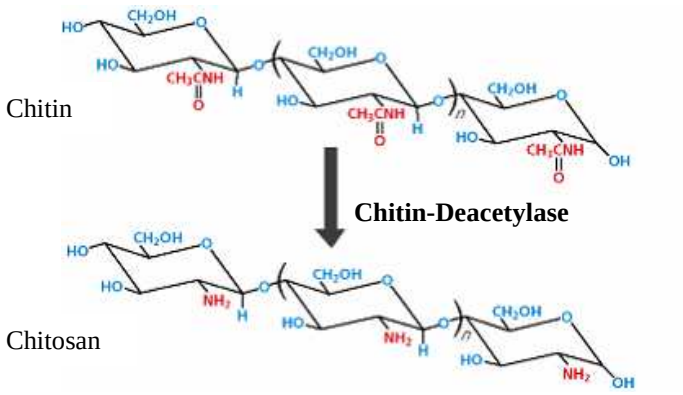


Figure 10: Chitin and chitosan structures.

Additionally, amino groups make chitosan a cationic polyelectrolyte (pKa ≈ 6.5), one of the few found in nature. This basicity gives chitosan singular properties: chitosan is soluble in aqueous acidic media at pH < 6.5 and when dissolved it possesses high positive charge on $-NH_3^+$ groups, it adheres to negatively charged surfaces, it aggregates with polyanionic compounds, and it chelates heavy metal ions. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties. Along with unique biological properties that include biocompatibility, biodegradability to harmless products, non-toxicity, physiological inertness, remarkable affinity to proteins, hemostatic, fungistatic, antitumoral and anticholesteremic properties. Chitin and chitosan, as yet underutilized, offer an extraordinary potential in a broad spectrum of applications which are predicted to grow rapidly once the standardized chitinous materials become available. Crucially, as biodegradable polymers chitin/chitosan materials are eco-friendly, safe for humans and

the natural environment. Increasingly over the last decade chitin- and chitosan based materials have been examined and a number of potential products have been developed for areas such as (Peter, 1995; Dutta *et al*., 2002; No & Meyers, 2000) wastewater treatment (removal of heavy metal ions, flocculation/coagulation of dyes and proteins, membrane purification processes), the food industry (anticholesterol and fat binding, preservative, packaging material, animal feed additive), agriculture (seed and fertilizer coating, controlled agrochemical release), pulp and paper industry (surface treatment, photographic paper), cosmetics and toiletries (moisturizer, body creams, bath lotion).

Moreover, owing to the unparalleled biological properties, the most exciting uses of chitin/chitosan-based materials are those in the area of medicine and biotechnology (Khor, 2002; Paul & Sharma, 2000; Singla & Chawla, 2001). In medicine they may be employed as bacteriostatic and fungistatic agents, drug delivery vehicles, drug controlled release systems, artificial cells, wound healing ointments/dressings, haemodialysis membranes, contact lenses, artificial skin, surgical sutures and for tissue engineering. In biotechnology, on the other hand, they may find application as chromatographic matrices, membranes for membrane separations, and notably as enzyme/cell immobilization supports.

As enzyme immobilization supports chitin- and chitosan based materials are used in the form of powders, flakes and gels of different geometrical configurations. Chitin/chitosan powders and flakes are available as commercial products among others from Sigma-Aldrich and chitosan gel beads (Chitopearl) from Fuji Spinning Co. Ltd. (Tokyo, Japan). Otherwise the chitinous supports are laboratory manufactured. Preparation of chitosan gels is promoted by the fact that chitosan dissolves readily in dilute solutions of most organic acids, including formic, acetic, tartaric and citric acids, to form viscous solutions that precipitate upon an increase in pH and by formation of water-insoluble ionotropic complexes with anionic polyelectrolytes. In this way, chitosan gels in the form of beads, membranes, coatings, capsules, fibres, hollow fibres and sponges can be manufactured (Krajewska, 2004).

2.4. Immobilized enzymes applications

To date, several immobilized enzyme-based processes have been implemented on a larger scale, mainly in food industry, where they replace free enzyme-catalyzed processes, in chemical and pharmaceutical manufactures, where asymmetric synthesis or resolution of enantiomers to produce optically pure products are involved (Bullock, 1995; van de Velde *et al*., 2002; Wiseman, 1993). A selection of currently used immobilized-enzyme processes, in the approximate order of the decreasing scale of manufacture, is given in Table 2.

Areas of present and potential future applications of immobilized enzyme systems other than industrial include: laboratory scale organic synthesis, analytical and medical applications (Bullock, 1995; van de Velde *et al*., 2002; Scouten *et al*., 1995). In analytical applications immobilized enzymes are used chiefly in biosensors (Wilson & Hu, 2000; Krajewska, 2004) and to a lesser extent, in diagnostic test strips. Biosensors are constructed by integrating biological sensing systems, e.g. enzyme(s), with transducers. These obtain a chemical signal produced by the interaction of the biological system with an analyte and transduce it into a measurable response. Different kinds of transducers have been employed in biosensors, viz-potentiometric, amperometric, conductometric, thermometric, optical and piezo-electric, most of the current research being placed on the first two. Enzymes for the most cases are immobilized either directly on a transducer's working tip or in/on a polymer membrane tightly wrapping it up. In principle, due to enzyme specificity and sensitivity biosensors can be tailored for nearly any target analyte, and these can be both enzyme substrates and enzyme inhibitors. Advantageously, their determination is performed without special preparation of the sample. Meeting the demand for practical, cost-effective and portable analytical devices, enzyme-based biosensors have enormous potential as useful tools in medicine, environmental in situ and real time monitoring, bioprocess and food control, and in biomedical and pharmaceutical analysis. Their use, impaired as yet by not quite satisfactory reliability, is predicted to become widely accepted once their storage and operational stabilities have been improved. The most extensively studied enzymes for the application in enzyme-based biosensors are presented in Table 3.

An overview of enzymes immobilized on chitin- and chitosan-based materials for applcation in food industry, is presented in Table 4.

Table 2: Some of the most important industrial applications of immobilized enzyme systems (Krajewska, 2004).

Enzyme (EC number)	**Substrate**	**Product**
Glucose isomerase (5.3.1.5)	Glucose	Fructose (high-fructose corn syrup)
β-Galactosidase (3.2.1.23)	Lactose	Glucose and galactose (lactose-free milk and whey)
Lipase (3.1.1.3)	Triglycerides	Cocoa butter substitutes
Nitrile hydratase (4.2.1.84)	Acrylonitrile	Acrylamide
	3-Cyanopyridine	Nicotinamide
	Adiponitrile	5-Cyanovaleramide
Aminoacylase (3.5.1.14)	D, L-Aminoacids	L-Amino acids (methionine, alanine, phenylalanine, tryptophan, valine)
Raffinase (3.2.1.22)	Raffinose	Galactose and sucrose (raffinose-free solutions)
Invertase (3.2.1.26)	Sucrose	Glucose/fructose mixture (invert sugar)
Aspartate ammonia-lyase (4.3.1.1)	Ammonia + fumaric acid	L-Aspartic acid (used for production of synthetic sweetener aspartame)
Thermolysin (3.4.24.27)	Peptides	Aspartame
Glucoamylase (3.2.1.3)	Starch	D-Glucose
Papain (3.4.22.2)	Proteins	Removal of “chill haze” in beers
Hydantoinase (3.5.2.2)	D,L-Amino acid hydantoins	D,L-Amino acids
Penicillin amidase (3.5.1.11)	Penicillins G and V	6-Aminopenicillanic acid (precursor of semi-synthetic penicillins, e.g. ampicillin)
β-Tyrosinase (4.1.99.2)	Pyrocatechol	L-DOPA

Table 3: Some of the most frequently studied enzymes for enzyme-based biosensors (Krajewska, 2004).

Enzyme (EC number)	**Substrate**	**Application**
Glucose oxidase (1.1.3.4)	Glucose	Diagnosis and treatment of diabetes, food science, biotechnology
Horseradish peroxidase (1.11.1.7)	H_2O_2	Biological and industrial applications, inhibition-based determination of heavy metal ions and pesticides
Lactate oxidase (1.13.12.4)	Lactate	Sports medicine, critical care, food science, biotechnology
Tyrosinase (1.14.18.1)	Phenols, polyphenols	Determination of phenolic compounds in foods, inhibition-based determination of carbamate pesticides
Glutamate oxidase (1.4.3.11)	Glutamate	Food science, biotechnology
Urease (3.5.1.5)	Urea	Medical diagnosis, artificial kidney, environmental monitoring
Alcohol dehydrogenase (1.1.1.1)	Ethanol	Food science, biotechnology
Acetylcholinesterase (3.1.1.7)	Acetylcholine, acetylthiocholine	Inhibition-based determination of organophosphorus and carbamate pesticides
Choline oxidase (1.1.3.17)	Choline	Enzyme used in conjunction with acetycholinesterase
Lactate dehydrogenase (1.1.1.27)	Lactate	Sports medicine, critical care, food science, biotechnology
Cholesterol oxidase (1.1.3.6)	Cholesterol	Medical applications
Penicillinase (3.5.2.6)	Penicillins	Pharmaceutical applications
Alliinase (4.4.1.4)	Cysteine sulfoxides	Food industry (garlic-, onions- and leek-derived products)

Table 4: Applications of enzymes immobilized on chitin- and chitosan-based materials in food industry (Krajewska, 2004).

Enzyme (EC number)	**Application**	**Support**	**Immobilization**
α-Amylase (3.2.1.1)	Hydrolysis of starch for glucose syrup	Chitin powder	III
β-Amylase (3.2.1.2)	Production of high maltose syrup from starch	Chitosan beads	I
α-L-Arabinofuranosidase (3.2.1.55)	Aromatization of musts, alcoholic beverages and fruit juices	Chitosan powder	I, II , III
Catalase (1.11.1.6)	Removal of H_2O_2 from food	Chitosan powder	I, IV, II
Cellulase (3.2.1.4)	Decrease in viscosity of fruit/vegetable juices	Chitin powder	IV
α-Galactosidase (3.2.1.22)	Raffinose hydrolysis in beet molasses	Chitin powder	IV
β-Galactosidase (3.2.1.23)	Hydrolysis of lactose (lactose-free dairy products)	Chitin powder	III
		Chitosan powder	III
		Chitosan beads	I, III
		Chitosan precipitate	II
Glucoamylase (3.2.1.3)	Hydrolysis of starch (ethanol production)	Chitin powder	III
		Chitosan magnetite beads	I
		Chitosan powder	I
		Chitosan beads	I, III
α-Glucosidase (3.2.1.20)	Hydrolysis of maltose (food/feed additives)	Chitosan beads	III

β-Glucosidase (3.2.1.21)	Wine making and juice processing	Chitosan powder	III, II
β-Glycosidase (3.2.1.group)	Cellobiose hydrolysis for glucose production	Chitosan powder	II
		Chitosan powder	I, III, IV
		Chitosan microbeads	V
Invertase (3.2.1.26)	Hydrolysis of sucrose (production of invert sugar)	Chitosan-organosilane particles	I
		Chitosan-magnetite beads	I
Isoamylase (3.2.1.68)	Hydrolysis of starch (glucose and maltose)	Chitin powder	III
Lysozyme (3.2.1.17)	Cheesemaking	Chitosan powder	I
Neutral proteinase (3.4.24.28)	Hydrolysis of soybean protein	Chitosan precipitate	II
Papain (3.4.22.2)	Removal of “chill haze” in beers	Chitin powder	II
Pectin lyase (4.2.2.10)	Reduction of fruit/vegetable juices’ viscosity	Chitin powder	III
Proteases (3.4.groups)	Casein hydrolysate debittering	Chitin powder	III
		Chitin film	II
		Chitin powder	III
		Chitosan-magnetite particles	IV
Pullulanase (3.2.1.41)	Hydrolysis of starch (glucose/maltose syrup)	Chitosan powder	I, III
		Chitosan beads	I

		Chitin powder	II
α-L-Rhamnopyranosidase (3.2.1.40)	Aromatization of musts, alcoholic beverages and fruit juices	Chitosan powder	III, II
		Chitosan particles	V
		Chitin powder and colloidal chitin	III, I
Tannase (3.1.1.20)	Hydrolysis of tea tannins	Chitosan precipitate	III
		Chitosan-triphosphate beads	V
Transglutaminase (2.3.2.13)	Deamidation of food proteins	Chitosan beads	III
Trypsin (3.4.21.4)	Hydrolysis of proteins	Chitosan-magnetite particles	I
		Chitosan-PVA capsules	V
		Chitosan-PGMA precipitate	I
Urease (3.5.1.5)	Removal of urea from beverages and food	Chitosan-coated alginate beads	V
		Chitosan-organosilane particles	I

Immobilizations are presented as five techniques: (I) adsorption of enzyme on support; (II) adsorption of enzyme on support followed by cross-linking with glutaraldehyde (reticulation); (III) covalent binding of enzyme to glutaraldehyde-activated support; (IV) covalent binding of enzyme to support activated with agents other than glutaraldehyde; (V) gel inclusion.

CHAPTER 3: Bromelain from pineapple stem

3.1 Proteases

The large family of peptide-bond-cleaving hydrolases, the peptidases (= proteases, EC 3.4), can be classified as endopeptidases (= proteinases, EC 3.4.21-99) and exopeptidases (EC 3.4.11-19), according to the point at which they break the peptide chain (Otto & Schirmeister, 1997). These enzymes can be ordered further, according to the reactive groups at the active site involved in catalysis, into serine (EC 3.4.21), aspartic (EC 3.4.23), metallo (EC 3.4.24) and cysteine-endopeptidases (EC 3.4.22), as shown in Figure 11.

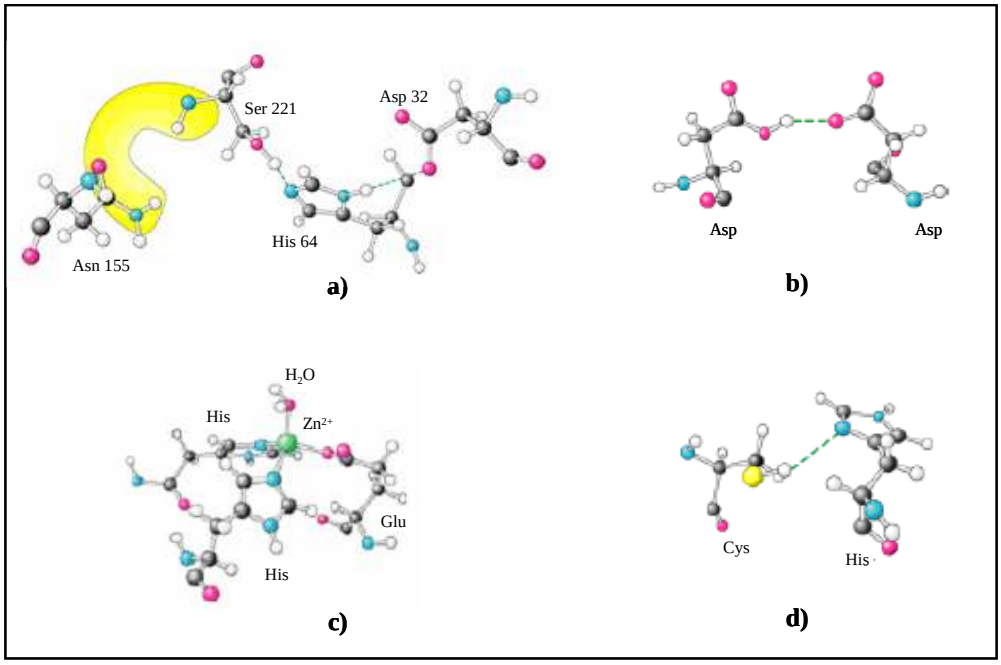


Figure 11: Reactive groups at the active site of serine **a)**, aspartic **b)**, metallo **c)** and cysteine peptidases **d)**.

Cysteine proteases have been found in viruses and prokaryotes as well as in higher organisms such as plants and mammals, including humans. Mammalian cysteine

proteinases fall into two classes: caspases (Rzychon *et al*., 2004; Barrett *et al*., 1998) and the papain superfamily comprising the papain family, calpains and bleomycin hydrolases (Otto & Schirmeister, 1997; Barrett *et al*., 1998; McGrath *et al*., 1999).
Cysteine proteinases participate in varied biological processes. The cathepsins alone are involved in protein breakdown in lysosomes, antigen presentation, proteolytic processing of proenzymes and prohormones, fertilization, cell proliferation, differentiation and apoptosis (Chapman *et al*., 1997; Grzelakowska-Sztabert, 1998; Berdowska & Siewiński, 2000).

3.2 Catalytic mechanism of cysteine proteases

The proteases of this group are most commonly exemplified by papain, being a well described plant enzyme isolated from the latex of *Carica papaya* fruit (Rzychon *et al*., 2004).
The proteolytic activity of all cysteine proteases arises from the presence of the catalytic Cys and His residues in the enzyme active centre. In the case of papain-like cysteine proteinases, the catalytic centre is complemented with Asn that ensures an orientation of the His imidazole ring optimal for successive stages of hydrolysis. The crucial step of the catalytic process involves formation of a reactive thiolate/imidazolium ion pair (Cys-S–/His-Im^+), which results from proton transfer between Cys-25 and His-159 (papain numbering). In principle, the thiolate anion attacks the carbonyl carbon of the scissile peptide bond, and the double bond between the carbon and the oxygen converts into a single one (Figure 12A). The oxygen assumes a negative net charge allowing formation of the first tetrahedral transition state. The oxyanion is stabilized by hydrogen bonding to the NH groups of Gln-19 side chain and Cys-25 backbone, which is likely to result in the formation of an oxyanion hole (Figure 12B) (Menard *et al*., 1991; Menard *et al*., 1995; Harrison *et al*., 1997; Otto & Schirmeister, 1997). Subsequent rotation of the His residue enables proton transfer from the imidazolium cation to the nitrogen of the peptide bond being hydrolyzed, and cleavage occurs. The newly formed substrate amine is hydrogen bonded to His-159, whereas the substrate carboxylic part is linked to Cys-25 via a thioester bond, forming acyl enzyme (Figure 12C). The next reaction step involves dissociation of the aminic part of the substrate and its replacement with a water

molecule. The imidazole nitrogen contributes to polarization of the water molecule that in turn attacks the carbonyl carbon of acyl enzyme (Figure 12D). This is followed by formation of the second tetrahedral intermediate (Figure 12E). In the final step, thioester deacylation leads to reconstruction of the carboxyl group in the hydrolyzed substrate, which is concerted with the release of an active enzyme (Figure 12F) (Menard *et al*., 1991; Otto & Schirmeister, 1997; Rzychon *et al*., 2004).

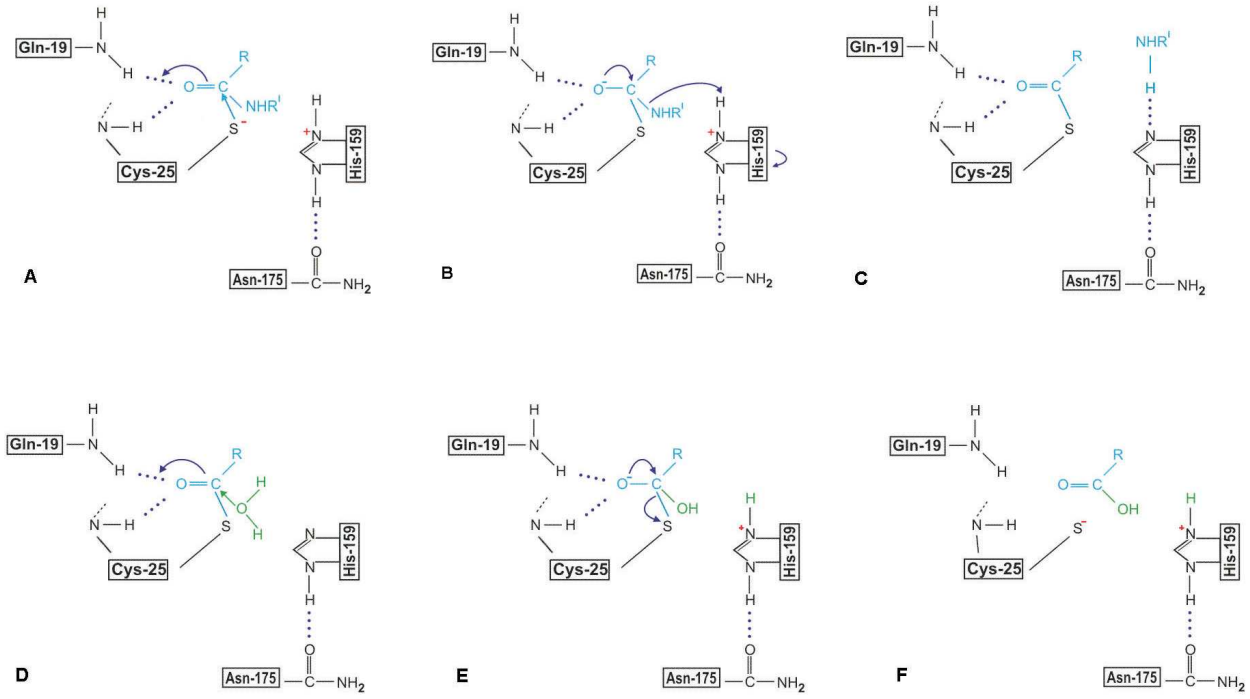


Figure 12: Catalytic mechanism of cysteine proteinases as exemplified by papain (Rzychon *et al*., 2004).

3.3 Selection of bromelain from pineapple stem

The most extensively investigated plant cysteine protease is papain (EC 3.4.22.2) from the latex of *Carica papaya* fruit. Papain is a monomeric polypeptide consisting of a chain of 212 amino acid residues with three disulfide bridges (Cys22-Cys63, Cys56-Cys95, Cys153-Cys200). Other plant cysteine proteases whose known fragment sequences indicate a relationship to papain are: chymopapains A and B from *Carica*

papaya, ficin (EC 3.4.22.3) from the latex of various *Ficus* species and bromelain (EC 3.4.22.4) from pineapple steam.

Stem bromelain (EC. 3.4.22.32) is distinguished from fruit bromelain (EC. 3.4.22.33), previously called bromelin (Rowan & Buttle, 1994; Maurer, 2001). Heinecke and Gortner (1957) demonstrated that bromelain concentration in pineapple stem is greater than that revealed in the fruit. Moreover, pineapple fruit is normally used as food, whereas stem, being a waste by-product results an inexpensive and suitable source for bromelain extraction (Tochi *et al*., 2008).

Stem bromelain has multiple uses in food processing, including meat tenderization and applications in baking industry (Lyons, 1982). Moreover, this protease, active at the normal beer pH (about 4.5), is widely used for haze prevention of this beverage (Ash & Ash, 2002). In light of all these considerations, among the plant cysteine proteases, bromelain from pineapple stem was chosen for the present work. In order to the develop an alternative practices for white wine protein stabilization that would maintain quality, reduce costs and be more sustainable, immobilization of this protease could ensure a repeated/continuous use as well as an easy separation from the reaction mixture so as to prevent protein contamination of treated wine.

3.4 Bromelain in pineapple plant

Bromelain is a crude, aqueous extract from stem and immature fruit of pineapple plant (*Ananas comosus*), constituting an unusually complex mixture of different thiol-endopeptidases and other not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others (Maurer, 2001).

The presence of bromelain proteinases in pineapple plants is a big mystery in plant science. The carnivorous plants get their supply of nitrogen and phosphorus by degradation of organic matter (insects, microbes) by using proteinases and other digestive enzymes. Pineapple plants, native to Central and South America, grow as epiphyte in forest of several tropical and subtropical countries including Hawaii, India, China, Kenya, South Africa, Malaysia, the Philippines and Thailand. Normally, they grow on other plants, which do not provide any nutritional support. The rosette like

arrangements of pineapple leaves develop funnel-type rain water reservoirs. This so-called phytotelmata are always filled with water, as well as with nitrogen and phosphorus suppliers. This hypothesis is supported by recent findings that leaves react to mechanical stimuli by producing protein-kinases (Bhattacharyya, 2008).

Pineapple has been largely used as a medicinal plant in several native cultures and bromelain has been chemically known since 1876 (Tochi *et al*., 2008). Bromelain obtained from stem of pineapple plants contains all the soluble components of the pineapple stem in their original properties, which may involve malignant cell growth, thrombus formation, inflammation, control of diarrhoea, dermatological and skin debridement (Kelly, 1996; Maurer, 2001; Tochi *et al*., 2008).

3.5 Bromelain extraction from pineapple plant

Today bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration and lyophilization. The process yields an enzimatic preparation that appears as a yellowish powder; its enzymatic activity is usually determined using different substrates, such as casein, gelatine or chromogenic tripeptides (Filipova *et al*., 1984; Harrach *et al*., 1995; Maurer, 2001). In aqueous solution, bromelain rapidly deteriorates through self-digestion, which can be prevented by the addition of serum containing α_2-macroglobulin. By high-resolution fast protein liquid chromatography (FPLC) and other biochemical methods, basic (stem bromelain, ananain, comosain) and acidic thiol-proteinases have been isolated from crude bromelain, partially or fully sequenced and characterized in more detail (Harrach *et al*., 1995; Maurer, 2001). They mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kDa, and isoelectric points between > 10 and 4.8. Two major basic proteinases, F4 and F5, were further characterized, showing molecular masses of 24397 and 24472 Da, respectively (Harrach *et al*., 1995; Maurer, 2001) (Table 5).

Stem bromelain has broad substrate specificity and hydrolyses a great variety of natural and synthetic substrates. Casein and haemoglobin are the most widely used natural substrates for this protease (Vanhoof & Cooreman, 1997). Stem bromelain rapidly digests casein over the pH range 7.0–8.5 (Minami, *et al*., 1971), while the optimum for

hydrolysing haemoglobin is around pH 5.0 (Murachi, 1976). Synthetic substrates are also very useful for assaying bromelain, as reported by Vanhoof and Cooreman (1997). The most commonly used are N-benzoyl-l-Arg-ethyl ester (BAEE) and N-benzoyl-l-Arg-amide (BAA), for which the pH optimum falls in the pH range 5.0–6.0 and 5.0–8.0, respectively (Yamada, *et al*., 1975).

Table 5: Cysteine proteinases (bromelains) from pineapples (*Ananas comosus*), (Maurer, 2001).

Name (EC Number)	**Abbreviation**	**Molecular mass (Dalton)**	**Isoelectric point**	**Sequences**	**Glycosylation**
		From pineapple stems:			
Stem bromelain (EC 3.4.22.32)	F4 and F5	23800 (sequence + sugar)	> 10	Completely sequenced (212 amino acids)	glycosylated
Ananain (EC 3.4.22.31)	F9	23464 (sequence)	> 10	Completely sequenced (216 amino acids)	not glycosylated
Comosain	F9/b	24509 and 23569	> 10	N-term. sequence	glycosylated
	SBA/a and SBA/b	23550 and 23560	4.8 and 4.9	N-term. sequence highly	glycosylated
		From pineapple fruits:			
Fruit bromelain (EC 3.4.22.33)		23000	4.6	N-term. sequence	not glycosylated

3.6 Stem bromelain structure

Ritonja et al. (1989) revealed the complete amino acid sequence of stem bromelain. Their results showed that bromelain exists as a single polypeptide chain with 211 or 212 residues, depending on the presence of the N-terminal Ala. Based on the amino acid composition, the molecolar weight of the longer form of bromelain was calculated to be 22828 Daltons (Figure 13). Stem bromelain is a glycoprotein with one oligosaccharide moiety per molecule, which is covalently attached to the peptide chain by the residues Asn-Asn[117] -Glu-Ser. The total molecolar weight of the glycosylated form of bromelain, with an oligosaccharide chain of 1000 Dastons, containing fucose, mannose, xylose and N-acetylglucosamines, is about 23800 Daltons.

The alignment of the amino acid sequence of bromelain with those of other cysteine proteinases (Figure 14) shows unequivocally that the enzyme is a member of the papain superfamily, although it is not very closely related to any other known member. Percentages (in parentheses) of identical residues when the sequences are aligned as in Figure 14, decrease in the order: papaya proteinase III (43), actinidin (42), human cathepsin L (38), papain (38), human cathepsin H (33) and human cathepsin B (22) (Ritonja *et al*., 1989).

When the amino acid sequence of bromelain is compared with those of papain, human cathepsin H and human cathepsin L all tightly bound by cystatin (Barrett *et al*., 1986) as in Figure 14, a number of differences that must affect the geometry of the active-site cleft are apparent (Figure 15). Perhaps the most striking is the deletion of Asn-175 and two adjacent residues, and the mutation of the highly conserved Ser-176 to Lys. Asn-175 has been found in all other papain homologues sequenced previously, with the exception of the calpains, which incidentally are unaffected by most cystatins (Barrett *et al*., 1986). In papain, Asn-175 is hydrogen-bonded to His-159, and orients it for its catalytic function (Ritonja *et al*., 1989). The substitution of Ser-21 by Pro, and of the highly conserved Ser-24 by Ala may well also affect the orientation of the catalytic Cys-25. In view of these factors, the putative catalytic thiolate-imidazolium pair at the active site of bromelain is likely to have a different conformation from that in the cysteine proteinases that are tightly inhibited by cystatin.

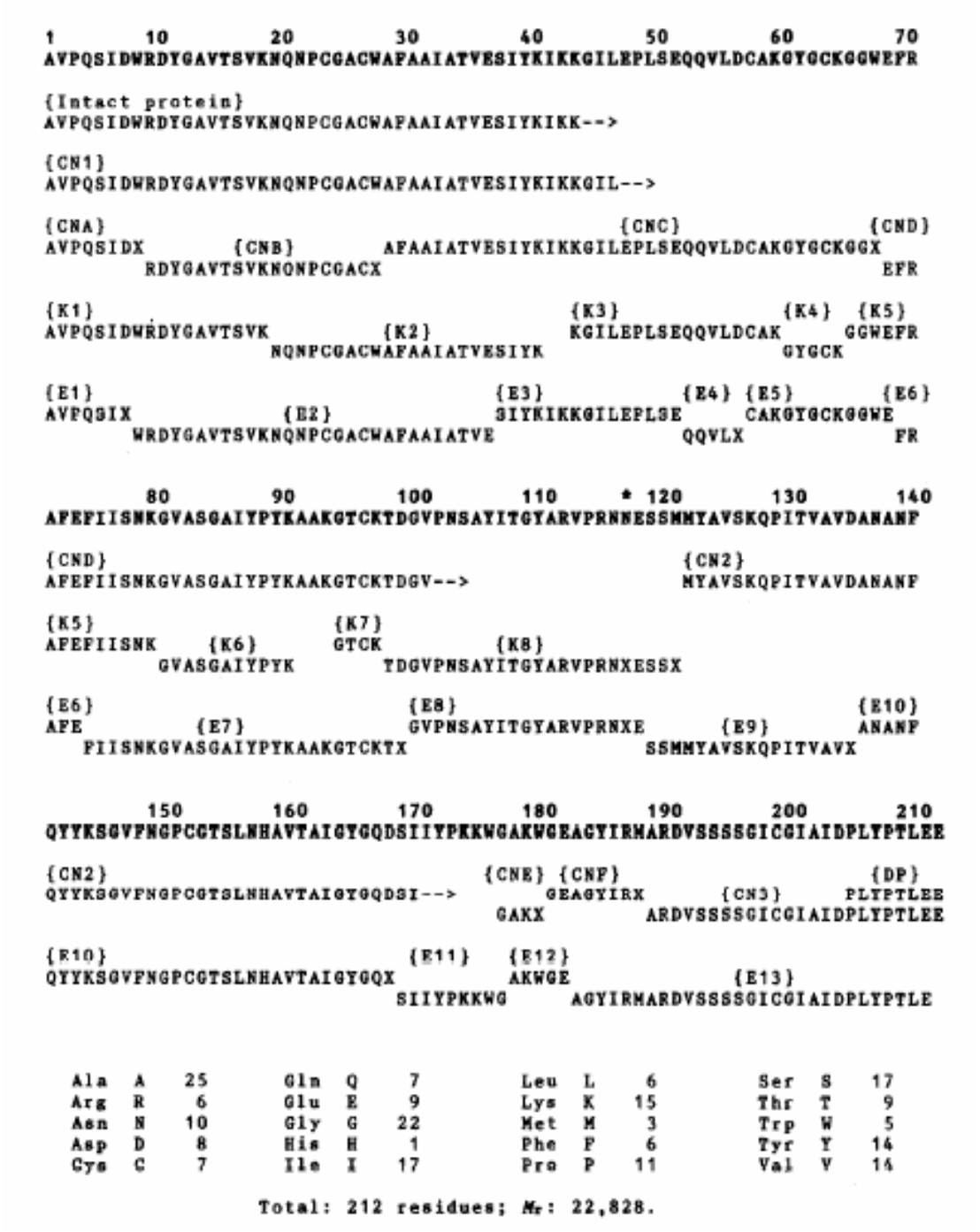


Figure 13: Amino acid sequence and composition of bromelain, and strategy of the sequence determination, (Ritonja *et al*., 1989).

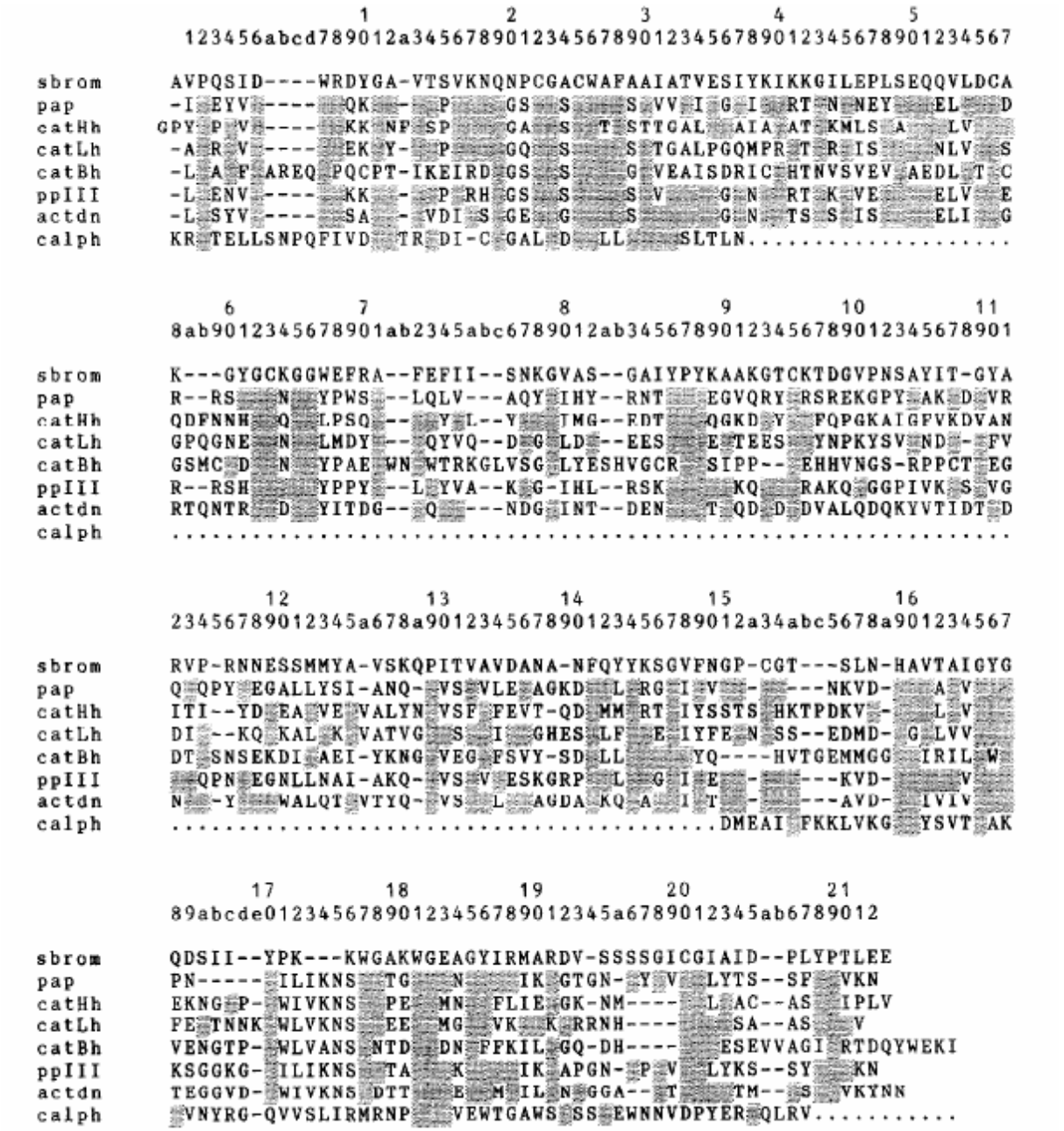


Figure 14: Alignment of the bromelain (sbrom) sequence with those of papain (pap), human cathepsin H (catHh), human cathepsin L (catLh), human cathepsin B (catBh), papaya proteinase III (ppIII), actinidin (actdn) and human calpain II (calph), (Ritonja *et al.*, 1989).

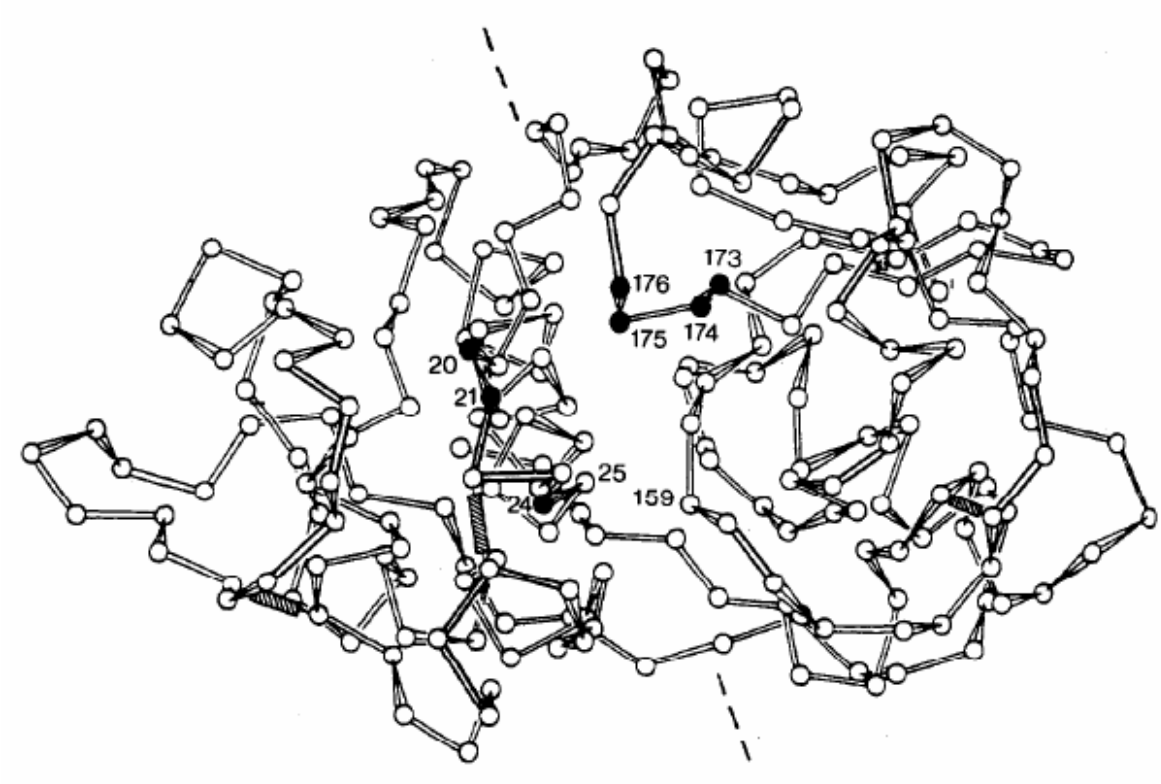


Figure 15: Perspective drawing of the structure of papain emphasising residues that contribute to the conformation of the catalytic site, and are mutated or deleted in bromelain (closed circles). The broken lines mark the approximate line of the active-site cleft. Residues 25 and 159 are the cysteine and histidine residues that form the catalytic thiolateimidazolium pair (Ritonja *et al*., 1989).

CHAPTER 4: Materials and methods

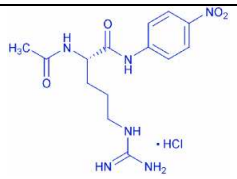
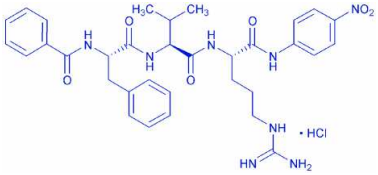
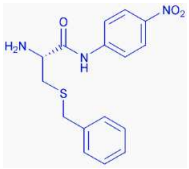
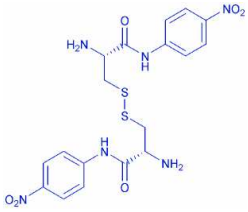
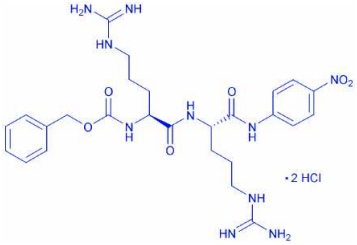
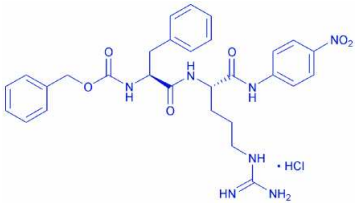
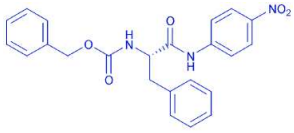
4.1 Enzyme and chemicals

A single lot (ref No. 068K0692) of stem bromelain (EC 3.4.22.32), obtained from Sigma-Aldrich (Milan, Italy) and containing 45% of protein w/w (Bradford), was used.
Synthetic peptide substrates (Table 6), Ac-Arg-p-nitroaniline (pNA), Bz-Phe-Val-Arg-pNA, H-Cys(Bzl)-pNA, $(H\text{-}Cys\text{-}pNA)_2$, H-Val-Ala-pNA, Suc-Ala-Ala-Val-pNA, Suc-Ala-Pro-Leu-Phe-pNA, Suc-Phe-Leu-Phe-pNA, Z-Arg-Arg-pNA, Z-Phe-Arg-pNA and Z-Phe-pNA were purchased from Bachem, Germany.
Skin, seed, gallic and ellagic tannins, as preparations intended for enological use, were kindly supplied by EVERINTEC (Venice, Italy). All other reagents were obtained from Sigma-Aldrich (Milan, Italy).

4.2 Buffer preparation

Bromelain immobilization and proteolytic activity study were carried out both in Mc Ilvaine and tartaric buffer (model wine).
The McIlvaine buffer consists of 0.1 M monohydrated citric acid and 0.2 M disodium hydrogenphosphate; these two solutions were mixed in different volume ratios to obtain the final pH, from 2.6 to 12.0, as reported by Schmidt et al. (2006). This was used as reference buffer, in presence or not of ethanol.
In order to simulate wine conditions a model wine buffer, containing ethanol 12% v/v at the average minimum pH value in wine (3.2), was used. The model wine consisted of tartaric acid/Na tartrate 0.03 M and ethanol 12% v/v with pH adjusted to 3.2, using 1 N NaOH (Nikolantonaki *et al*., 2010).

Table 6: Synthetic peptide substrates purchased from Bachem, Germany.

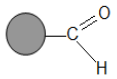
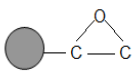
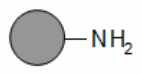
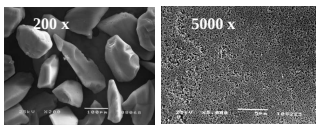
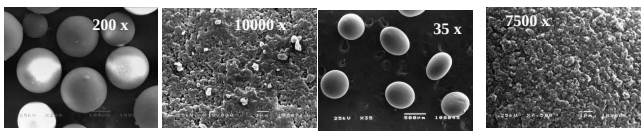
Substrate	**Structural formula**	**Structure**
Ac-Arg-pNA	$C_{14}H_{20}N_6O_4 \cdot HCl$	
Bz-Phe-Val-Arg-pNA	$C_{33}H_{40}N_8O_6 \cdot HCl$	
H-Cys(Bzl)-pNA	$C_{16}H_{17}N_3O_3S$	
$(H\text{-}Cys\text{-}pNA)_2$	$C_{18}H_{20}N_6O_6S_2$	
H-Val-Ala-pNA	$C_{14}H_{20}N_4O_4$	-
Suc-Ala-Ala-Val-pNA	$C_{21}H_{29}N_5O_8$	-
Suc-Ala-Pro-Leu-Phe-pNA	$C_{33}H_{42}N_6O_9$	-
Suc-Phe-Leu-Phe-pNA	$C_{34}H_{39}N_5O_8$	-
Z-Arg-Arg-pNA	$C_{26}H_{36}N_{10}O_6 \cdot 2\ HCl$	
Z-Phe-Arg-pNA	$C_{29}H_{33}N_7O_6 \cdot HCl$	
Z-Phe-pNA	$C_{23}H_{21}N_3O_5$	

4.3 Stem bromelain immobilization

4.3.1 Immobilization supports

The supports employed for protease immobilization were: aldehydic controlled pore glass beads (Prosep-9CHO from Millipore, Milan, Italy), epoxy-activated acrylic beads (Eupergit®-250L from Degussa Rohm GmbH&Co., Darmstadt, Germany) and chitosan beads (Chitopearl BCW-3010 from Wako Chemicals GmbH, Neuss, Germany). A Scanning Electron Microscope (SEM, mod. Jeol-JSM-5200) was employed to observe the supports surfaces. Physico-chemical characteristics of these supports and SEM images are reported in Table 7.

Table 7: Physico-chemical characteristics of employed supports and SEM images.

Support	Prosep-9CHO	Eupergit®-250L	Chitopearl BCW-3010
Material	aldehydic glass	acrylic copolymer	Chitosan
Functional group			
Diameter (μm)	200	100-200	840-1190
SEM images			

4.3.2 Immobilization procedures

The immobilization procedures described in Table 8, were performed with 100 mg of supports and 1 ml of enzyme preparation (5 mg ml^{-1}) in two different buffer solutions, incubating with slow tilt rotation, at different time and temperature according with cited

procedures. At the end, supports were washed with 2 M NaCl solution in order to remove all non-covalently bound proteins.

Table 8: Description of applied immobilization procedures.

Support	Cross-linker	Cysteine addition	Oriented Immobilization	pH	References	Label
Prosep-9CHO	-	-	-	3.2	Millipore corporation	PRO 3.2
	-	-	-	7		PRO 7
Eupergit®-250L	-	-	-	3.2	Boller *et al.*, 2002	EU 3.2
	-	-	-	7		EU 7
Chitopearl BCW-3010	X	-	-	3.2	Oliveira *et al.*, 2001	BCW 3.2-CL
	X	-	-	7		BCW 7-CL
	-	-	-	3.2		BCW 3.2
	-	-	-	7		BCW 7
	-	X	-	3.2	Hale *et al.*, 2005	BCW 3.2-cys
	-	X	-	7		BCW 7- cys
	-	-	X	3.2	Mahmood *et al.*, 2007	BCW 3.2-OI
	-	-	X	7		BCW 7- OI

4.3.3 Determination of immobilization yield

Immobilization yield (IY, %) was determined according to Bradford's method (Bradford, 1976), using Coomasie brilliant blue reagent and reading absorbance at 595 nm on a spectrophotometer Perkin-Elmer Lambda 25 UV/VIS (Beaconsfield Buks), (Figure 16). BSA was used as standard.

The amount of bound proteins was determined indirectly, from the difference between the amount of proteins in solution before and after immobilization. All measurements were made in triplicate and the standard deviations were reported.

Figure 16: Spectrophotometer Perkin-Elmer Lambda 25 UV/VIS.

4.4 Bromelain activity assay

Free and immobilized bromelain activity was determined measuring the change in absorbance *vs* time for 3 and 30 min, respectively, using a Perkin-Elmer Lambda 25 UV/VIS (Beaconsfield Buks). Cleavage of the substrate results in release of free pNA that was detected colorimetrically at 410 nm. Bromelain activity was calculated in I.U. of pNA produced, using a molar absorptivity of 8.480 mM^{-1} cm^{-1} at 410 nm for pNA (Hale *et al*., 2005). A blank correction was used, by a sample not containing enzyme. Specific activity (A.S.) was calculated as I.U. mg^{-1} of protein. All measurements were made in triplicate and the standard deviations are reported.

4.4.1 Selection of synthetic substrate to test bromelain activity at wine pH

In order to select the synthetic substrate suitable to study the proteolitic activity at the average minimum pH value of wine (3.2), different model peptide substrates for free bromelain were tested in McIlvaine buffer at various pH. The proteolytic activity was assessed against all the synthetic peptide substrates (370 μM final concentration) listed in the previous paragraph. Assays were performed in McIlvaine buffer, containing 5 mM cysteine and adjusted to various pH values (2.6, 3.2, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0 and 12.0) at 25 °C. In all experiments the final bromelain concentration was 0.02 mg ml^{-1} (0.84 μM).

4.4.2 Bromelain assay optimisation

Assay optimisation of stem bromelain was performed by using Bz-Phe-Val-Arg-pNA as substrate (370 μM). The buffer used was McIlvaine at pH 3.2 and 8.0, in presence or not of cysteine (5 mM) or EDTA (5 mM).

4.5 Half-life determination

Half-life ($t_{1/2}$), defined as time (h) in which enzyme looses half of its original activity, has been calculated by extrapolation of the fitted curves. Free and immobilized bromelain were maintained in model wine at room temperature.

4.6 Kinetic studies

Kinetic studies were carried out using Bz-Phe-Val-Arg-pNA substrate, varying its concentration (0-510 μM) at 25°C in presence of free or immobilized bromelain. All measurements were made in triplicate and the standard deviations were reported.

4.6.1 Kinetic study of free bromelain in different buffer solution

The kinetic study of free bromelain was initially carried out in McIlvaine buffer, containing ethanol at the average concentration of wine (12% v/v), at different pH values: 3.2 (average minimum value in wines), 4.0, 5.0 and 7.0 (optimum pH), using Bz-Phe-Val-Arg-pNA substrate (0-510 μM). In order to evaluate the buffer composition effect on protease activity, the kinetic study was also performed comparing bromelain activity in McIlvaine (pH 3.2, ethanol 12% v/v) and in model wine buffer (pH 3.2, ethanol 12% v/v).

4.6.2 Kinetic study of free and immobilized bromelain in model and real wines

Kinetic study of free or immobilized bromelain was also carried out using the same substrate (Bz-Phe-Val-Arg-pNA) at different concentrations (0-510 μM), both in model wine and in 6 different artisan and unrefined white real wines, previously filtered through a PES membrane filter with a pore size of 0.45 μm.

4.6.3 Determination of kinetic parameters

Kinetic parameters (k_{cat}, K_M, K_a) of free and immobilized stem bromelain were determined according to Michaelis–Menten equation (Nelson & Cox, 2008), using a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.).

4.7 Inhibition study of free and immobilized bromelain

The influence on protease activity of potential inhibitors naturally present in wine, such as ethanol, sulphur dioxide (SO_2), skin, seed, gallic and ellagic tannins over the average range concentration of wine, was investigated.

Kinetic study was carried out as previously described in model wine buffer, containing one of the following potential inhibitors at different concentrations: ethanol (0, 12, 18% v/v), free SO_2 (0, 10, 25 mg l^{-1}), skin tannin preparation (0, 0.5, 2 g l^{-1}), seed tannin preparation (0, 0.5, 2 g l^{-1}), gallic tannin preparation (0, 3, 5 g hl^{-1}) and ellagic tannin preparation (0, 5, 10 g hl^{-1}). An “inhibitor” can be defined as any substance that reduces the velocity of an enzyme-catalyzed reaction. Irreversible and reversible inhibition may be distinguished by plotting ΔA/min *vs* $[E_t]$, where $[E_t]$ represents the amount of enzyme added to the assay. For a reversible inhibitor, the “plus inhibitor” curve has a smaller slope than the control curve and goes through the origin. If an irreversible inhibitor is present, the “plus inhibitor” curve has the same slope as the control curve, but intersects the horizontal axis at a position equivalent to the amount of enzyme that is irreversibly inactivated (Segel, 1975), (Figure 17).

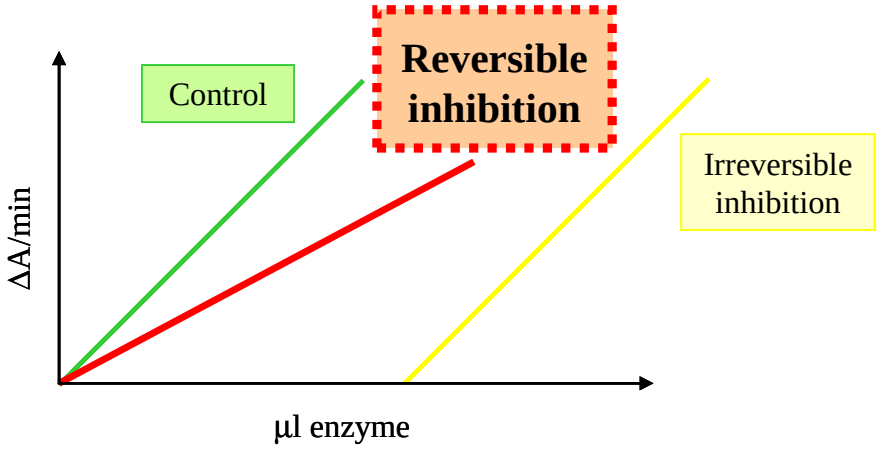


Figure 17: Different kinds of inhibition.

The interaction between a reversible inhibitor (I) and free enzyme (E), or enzyme-substrate complex (ES) can be described by different inhibition models (competitive, uncompetitive and mixed-type inhibition), assuming that only a single substrate is involving in the reaction and that only one type of inhibitor is present at any time. The equilibria:

$$E + I \underset{K_i}{\rightleftharpoons} EI \quad (1)$$

$$ES + I \underset{K_i'}{\rightleftharpoons} ESI \quad (2)$$

are defined by the thermodynamic constants, K_i or K_i', respectively:

$$K_i = \frac{[E]\,[I]}{[EI]} \quad (3)$$

$$K_i' = \frac{[ES]\,[I]}{[EI]} \quad (4)$$

These definitions and equilibria describe the different types of enzyme inhibition listed below. In each inhibition model, the kinetic equation used is a modification of Michaelis-Menten, in which the kinetic parameters K_M and V_{max} are replaced by the corresponding apparent kinetic parameters $K_{M(app)}$ and $V_{max(app)}$ (Segel, 1975).

4.7.1 Competitive inhibition

A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, often because of true competition for the same site (Figure 18).

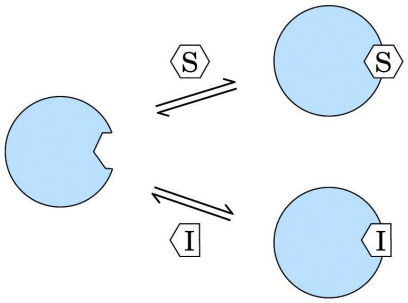


Figure 18: Schematic representation of competitive inhibition.

$$\begin{array}{l} E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_p} E + P \\ + \\ I \\ K_i \Big\| \\ EI \end{array} \qquad (5)$$

A competitive inhibitor acts only to increase the K_M for the substrate. The V_{max} remains unchanged, but in the presence of a competitive inhibitor a much greater substrate concentration is required to attain any given fraction of V_{max}.

The K_i value is determined by a replot of $K_{M(app)}$ *vs* [I], that has intercepts of K_M (on the $K_{M(app)}$ -axis) and - K_i (on the [I] -axis). The lower the value of K_i, the greater is the degree of inhibition at any given [S] and [I]. The $K_{M(app)}$ is a linear function of the inhibitor concentration, as shown below (Segel, 1975):

$$K_{M(app)} = \frac{K_M}{K_i}[I] + K_M \qquad (6)$$

4.7.2 Uncompetitive inhibition

An uncompetitive inhibitor binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex (Figure 19).

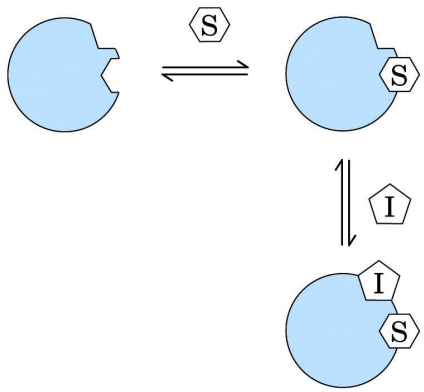


Figure 19: Schematic representation of uncompetitive inhibition.

$$\begin{array}{l} E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_p} E + P \\ \qquad\quad + \\ \qquad\quad I \\ \qquad K_i \Big\| \\ \qquad\quad ESI \end{array} \qquad (7)$$

An uncompetitive inhibitor decreases V_{max} and K_M to the same extent. A replot of $1/K_{M(app)}$ *vs* [I], being linear with intercepts of $1/K_M$ (on the $1/K_{M(app)}$ -axis) and - K_i (on the [I] -axis), is used for the determination of the K_i value determination (Segel, 1975):

$$\frac{1}{K_{M(app)}} = \frac{1}{K_i K_M}[I] + \frac{1}{K_M} \qquad (8)$$

4.7.3 Mixed-type inhibition

A mixed-type inhibitor binds reversibly both to free enzyme and to enzyme-substrate complex. EI has a lower affinity than E for S, and the ESI complex is non-productive (Figure 20).

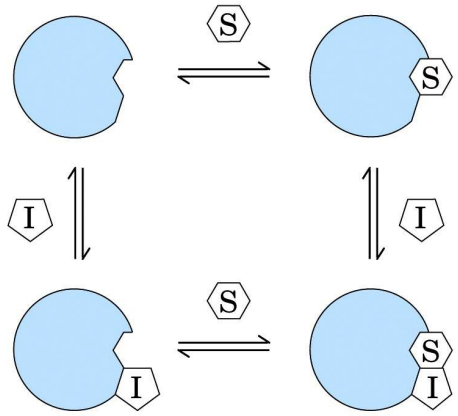


Figure 20: Schematic representation of mixed-type inhibition.

The system can be considered a mixture of partial competitive inhibition and pure non-competitive inhibition; the equilibria describing this system are shown below:

$$\begin{array}{ccccc} E + S & \underset{K_s}{\rightleftharpoons} & ES & \xrightarrow{k_p} & E + P \\ + & & + & & \\ I & & I & & \\ {\scriptstyle K_i}\uparrow\downarrow & & {\scriptstyle K_i'}\uparrow\downarrow & & \\ EI + S & \underset{K_s'}{\rightleftharpoons} & ESI & & \end{array} \qquad (9)$$

$$\alpha = 1 + \frac{[I]}{K_i} \qquad (10)$$

$$\alpha' = 1 + \frac{[I]}{K_i'} \qquad (11)$$

$$K_{M(app)} = K_M \frac{\alpha}{\alpha'} \qquad (12)$$

$$V_{max(app)} = \frac{V_{max}}{\alpha'} \qquad (13)$$

A replot of $1/V_{max(app)}$ *vs* [I], being linear with intercepts of $1/ V_{max}$ (on the $1/V_{max(app)}$ -axis) and - K_i' (on the [I] -axis), is used to determine the K_i' value determination. A second replot of $K_{M(app)}/V_{max(app)}$ *vs* [I], being linear with intercepts of K_M/V_{max} (on the $K_{M(app)}/V_{max(app)}$ -axis) and - K_i (on the [I] -axis), is used to determine the K_i value (Segel, 1975).

4.8 Total phenol content of tannin preparations

The total phenol content of the above mentioned tannin preparations was measured at 700 nm using an UV-visible spectrophotometer (Perkin-Elmer Lambda 25, Beaconsfield Buks) according to the Folin-Ciocalteu method (Di Stefano *et al.*, 1989). Results were expressed as gallic acid equivalents (g $l^{-1}_{gallic\ acid\ eq}$). A calibration curve was constructed on the basis of solutions at known and increasing concentrations of gallic acid.

4.9 Stabilization of wine proteins using immobilized bromelain

Six artisan and unrefined white wines, with a wide range of heat turbidity index (18-93 NTU), were used. Aliquots of these wines were filtered through PES membrane filter with a pore size of 0.45 μm. Stabilization treatment was carried out incubating 100 mg of BCW 3.2 in 10 ml of wine, at 20°C for 24 h, using an incubator shaker (Zhicheng ZHWY 200B), (Figure 21).

4.9.1 Total protein content

Total protein content in wines was detected by Bradford's method, using Coomasie brilliant blue reagent and reading absorbance at 595 nm after 45 minutes of incubation (Bradford, 1976). The protein content was expressed as mg l^{-1} of BSA.
Total protein removal yield (TPRY, %) by immobilized bromelain was calculated, after a 24 h treatment, as the percentage of proteins removed from treated white wines.

Figure 21: Incubator shaker, ZHWY 200B (Zhicheng).

4.9.2 Protein heat stability test

Unstable proteins in wine were determined according to heat test. Wine filtered samples were heated at 80 ºC for 30 min in a thermostat bath (Kottermann) and than cooled for 45 min (Figure 22).

Figure 22: Thermostat bath (Kottermann).

The turbidity, measured by nephelometry, was expressed in NTU. Nephelometric principle passage of a light ray through a turbid medium results in scattering and apparent energy loss in the incident beam. In fact, energy is not lost but undergoes directional changes as a result of scattering. This scattering effect may be measured at any angle relative to the plane of incident light. As seen in Figure 23, nephelometric measurements are made at 90° to the incident light beam:

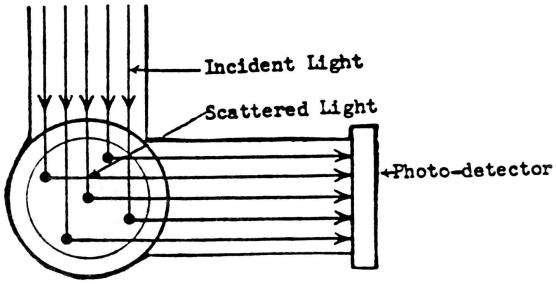


Figure 23: Schematic representation of a nephelometric measurement.

The degree of scattering depends primarily upon particle number, size, and shape. These parameters are, themselves, dependent upon several variables, including temperature, pH, concentration of reagents, and mixing procedures.

The difference in turbidity before and after the thermal test, measured using a turbidimeter HD 25.2 Delta Hom (Figure 24), was assumed to be proportional to protein instability. The wines were considered stable if this difference did not exceed 2 NTU (Vincenzi *et al.*, 2005). Each measurement was the average of three replicates. Turbidity removal yield (TRY, %) by immobilized bromelain was calculated, after a 24 h treatment, as the percentage of turbidity removed from treated white wines.

Figure 24: Turbidimeter HD 25.2 (Delta Hom).

4.9.3 Wine composition

The Official Methods of Analysis (EEC, 1990) were applied to evaluate the main chemical-physical characteristics of the 6 artisan and unrefined white wine tested.

CHAPTER 5: Results and discussion

5.1 Selection of synthetic substrate to test bromelain activity at wine pH

Stem bromelain has a broad substrate specificity and hydrolyses a great variety of natural and synthetic substrates. Casein and haemoglobin are the most widely used natural substrates for this protease (Vanhoof and Cooreman, 1997). Stem bromelain rapidly digests casein over the pH range 7.0–8.5 (Minami *et al*., 1971), while the optimum for hydrolysing haemoglobin is around pH 5.0 (Murachi, 1976). Synthetic substrates are also very useful for assaying bromelain, as reported by Vanhoof and Cooreman (1997). The most commonly used are N-benzoyl-l-Arg-ethyl ester (BAEE) and N-benzoyl-l-Arg-amide (BAA), for which the pH optimum falls in the pH range 5.0–6.0 and 5.0–8.0, respectively (Yamada *et al*., 1975).

In this study, bromelain activity *vs* pH has been determined using 11 synthetic peptide substrates, in order to select the most suitable one to then test protease activity at the average minimum pH value in wine (3.2). For each substrate the optimum pH and the specific activity, both at optimal pH and at pH 3.2, were determined (Table 9).

Table 9: Optimum pH and specific activity (A.S.) of stem bromelain (at optimal pH and at pH 3.2) tested toward synthetic substrates in McIlvaine buffer (25°C) at different pH values.

Substrate	**pH optimum**	**A.S. (I.U. mg^{-1}) at optimal pH**	**A.S. (I.U. mg^{-1}) at pH 3.2**
Suc-Phe-Leu-Phe-pNA	4.0	0.73 ± 0.02	0.37 ± 0.11
Suc-Ala-Pro-Leu-Phe-pNA	5.0	0.16 ± 0.04	*no activity*
$(H\text{-}Cys\text{-}pNA)_2$	4.0	0.010 ± 0.001	0.0048± 0.0006
Z-Arg-Arg-pNA	8.0	2.023 ± 0.008	0.011± 0.002
Bz-Phe-Val-Arg-pNA	8.0	1.02 ± 0.04	0.50 ± 0.04

Among the 11 substrates tested, stem bromelain cleaved five of them (Figure 25), showing maximal activity toward Z-Arg-Arg-pNA at the optimal pH of 8.0. No activity was shown towards the following substrates: Ac-Arg-pNA, H-Cys(Bzl)-pNA, H-Val-Ala-pNA, Suc-Ala-Ala-Val-pNA, Z-Phe-Arg-pNA and Z-Phe-pNA.

Nevertheless, the highest protease activity observed at the average minimum pH value of wine (3.2) was obtained with Bz-Phe-Val-Arg-pNA (Benucci *et al.*, 2011), and thus this substrate has been used in all other experiments.

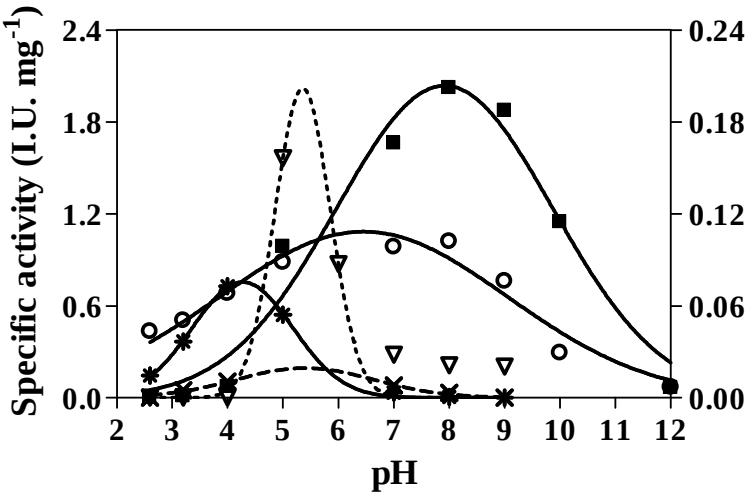


Figure 25: Specific activity of stem bromelain in McIlvaine buffer (25°C) *vs* pH towards 5 model substrates. On left Y axis: (○) Bz-Phe-Val-Arg-pNA, (✱) Suc-Phe-Leu-Phe-pNA, (■) Z-Arg-Arg-pNA; on right Y axis: (×) $(H\text{-}Cys\text{-}pNA)_2$, (▽) Suc-Ala-Pro-Leu-Phe-pNA.

5.2 Optimisation of assay conditions

Stem bromelain activity depends on the thiol group (SH) of a cysteine residue that is frequently activated by reducing agents (Minami *et al.*, 1971). For this reason, the effect on bromelain activity of either cysteine as a reducing agent or EDTA as a metal chelator was investigated. The influence of EDTA and cysteine was tested using Bz-Phe-Val-Arg-pNA as substrate in McIlvaine buffer at pH 3.2 and 8.0.

There was a negligible influence on protease activity by using EDTA, indicating that metals which influence bromelain activity, were not present in the buffer (Figure 26A).The specific activity of stem bromelain increased significantly in the presence of

cysteine at both pH values tested (37% and 53% at pH 3.2 and 8.0, respectively), as shown in Figure 26B (Benucci *et al*., 2011). The enzyme was activated by cysteine, because the Cys at the active site, essential for enzyme activity, is maintained in the reduced form by the presence of free Cys in the medium (Minami *et al*., 1971).

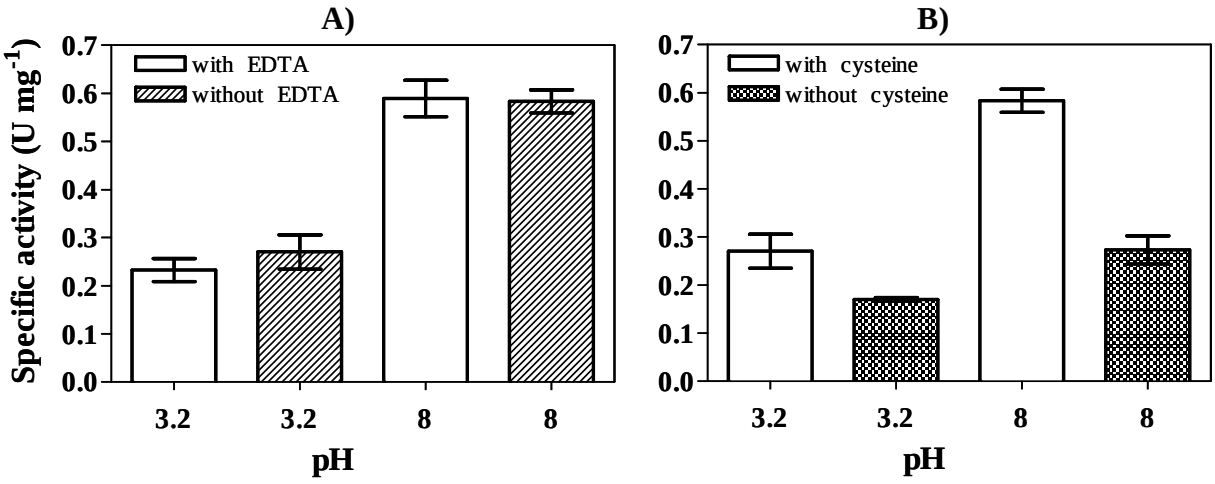


Figure 26: Specific activity of stem bromelain toward the Bz-Phe-Val-Arg-pNA substrate in McIlvaine buffer (25°C) at pH 3.2 and 8.0: (**A**) with 5 mM EDTA and without EDTA; (**B**) with 5 mM cysteine (cys) and without cysteine.

5.3 Kinetic study of free bromelain in different buffer solution

A kinetic study allowed us to obtain more significant and reliable results with respect to the activity levels observed at a fixed substrate concentration. In fact, a determination of kinetic parameters, although requiring a larger number of assays, more time and higher consumption of reagents, supplies useful information relative to the whole catalytic process.

The K_M (Michaelis–Menten constant) value reflects the enzyme-substrate complex formation, whereas k_{cat} (turnover number) measures the number of substrate molecules turned over per enzyme per minute. Moreover, k_{cat} is indicative of the product release velocity, representing the maximum number of moles of substrate converted to the product per number of moles of catalyst per unit time. This parameter can be obtained from the equation $k_{cat} = V_{max}/[E]_{tot}$, where $[E]_{tot}$ is the enzyme molar concentration. In

addition, the K_a (affinity constant), being the ratio k_{cat}/K_M, indicates the affinity of the enzyme toward the substrate. It is indicative of both reaction steps and expresses the overall catalytic efficiency.

Bromelain activity at different pH values (3.2, 4.0, 5.0 and 7.0) was evaluated in McIlvaine buffer in the presence of 12% v/v ethanol by using Bz-Phe-Val-Arg-pNA as substrate. Experimental data extracted using the Michaelis–Menten equation (Figure 27) and the curves, obtained at four different pH values, are grouped in two sets: pH 3.2–4.0 and 5.0–7.0, respectively (Benucci *et al*., 2011).

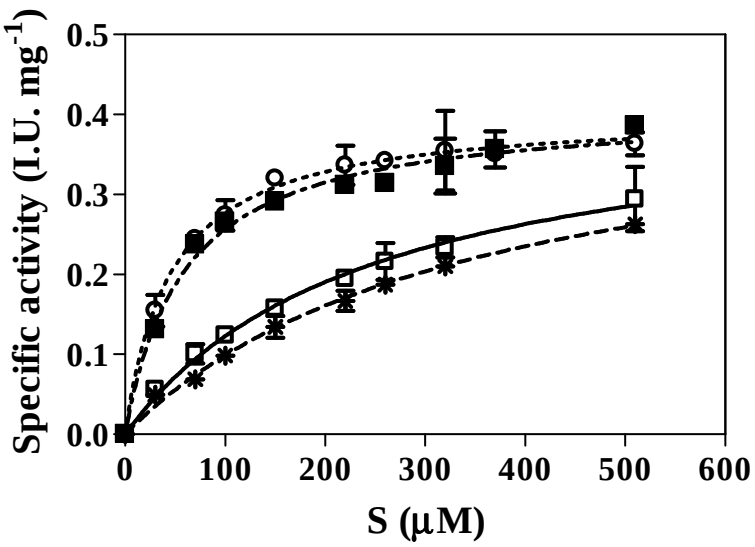


Figure 27: Specific activity of stem bromelain toward Bz-Phe-Val-Arg-pNA substrate in McIlvaine buffer (ethanol 12% v/v), at pH 3.2 (✱), 4 (□), 5 (○) and 7 (■).

Kinetic parameters (Table 10), obtained by means of non-linear regression, showed similar values of k_{cat}, indicating that the product release is not greatly pH-dependent, being only slightly higher at acidic pH. However, an interesting differential behaviour was observed relative to the K_M, that reflects the enzyme-substrate complex formation; these values were about 6-fold lower at higher pH, indicating a higher affinity for the substrate at pH 5.0–7.0. Obviously, the K_a reflects the K_M differences, indicating an affinity for the substrate about 5-fold higher at pH 5.0–7.0. This may be due to the imidazole group of the His in the active site that polarises the SH-group of Cys and enables the deprotonation at neutral and weakly acidic pH (Otto & Schirmeister, 1997).

Our kinetic measurements lead to the conclusion that for bromelain, as well as some other cysteine proteinases such as papain, actinidin and cathepsin, a further group with a pKa of 4.0–5.5 influences the reactivity. This conclusion comes from the log [S] *vs* log K_M and log [S] *vs* log k_{cat} plots, that indicate a deprotonation occurring at pH 4.5 that influences both enzyme-substrate complex formation and product release. In terms of the possibility to utilise bromelain for removing proteins in wine applications, these results indicate that bromelain can be very effective at the average minimum pH values of wine, precisely due to its higher value of k_{cat}. All these experiments were performed in McIlvaine buffer, which has a different composition relative to that of wine.

Table 10: Kinetic parameters of stem bromelain toward the Bz-Phe-Val-Arg-pNA substrate in McIlvaine buffer (ethanol 12% v/v), at different pH: 3.2, 4.0, 5.0 and 7.0.

pH	**V_{max} (I.U. mg^{-1})**	**K_M (μM)**	**k_{cat} (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
3.2	0.44 ± 0.02	342.6 ± 31.3	1154.03 ± 0.02	3.37 +0.34/-0.28
4.0	0.42 ± 0.03	245.5 ± 31.0	1121.51 ± 0.03	4.57 +0.66/-0.51
5.0	0.403 ± 0.009	45.6 ± 4.8	1065.18± 0.01	23.35 +2.78/-2.24
7.0	0.41 ± 0.01	59.2 ± 6.0	1078.67 ± 0.01	18.23 +2.07/-1.68

In order to further evaluate the proteolytic activity of bromelain relative to its potential application for winemaking, a comparison between the activity in McIlvaine buffer, *vs* that in a model wine buffer was carried out. It is known that, among the organic acids naturally present in wine, tartaric acid is the main component, contributing strongly for the overall acidity of this beverage; its concentration can vary within the average range of 1.5-5.0 g l^{-1} (Fernandes *et al*., 2006). Thus, in order to simulate a wine medium, the tartaric acid content in the model wine buffer used in our experiments was similar to the highest average concentration found in wine. The kinetic curves obtained with two different buffers are reported in Figure 28.

A significantly higher affinity of bromelain towards synthetic substrates was revealed in the model wine buffer, as can be seen from the K_M and K_a values reported in Table 11 (Benucci *et al*., 2011).

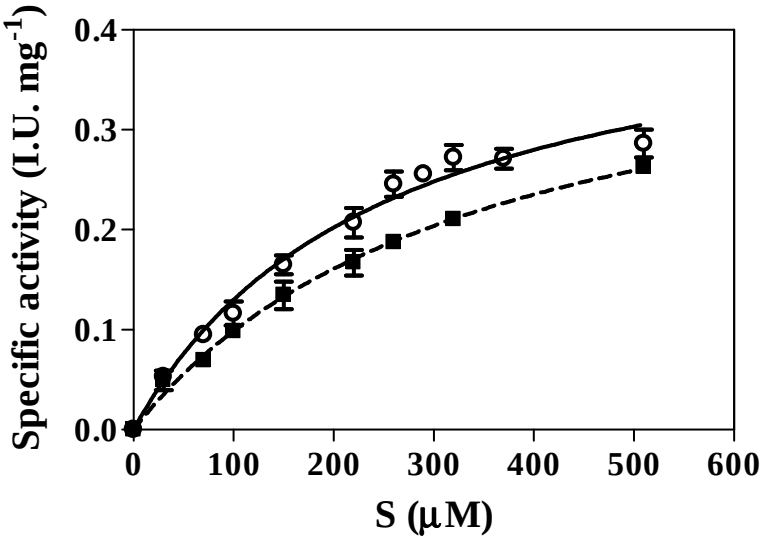


Figure 28: Specific activity of stem bromelain toward Bz-Phe-Val-Arg-pNA substrate in two different buffer solution at pH 3.2, containing ethanol 12% v/v: McIlvaine (■) and tartaric buffer (○).

Table 11: Kinetic parameters of stem bromelain toward the Bz-Phe-Val-Arg-pNA substrate in two different buffer solutions at pH 3.2, each containing ethanol 12% v/v: McIlvaine and tartaric buffer.

Buffer, pH 3.2	**V_{max} (I.U. mg^{-1})**	**K_M (μM)**	**k_{cat} (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
McIlvaine	0.44 ± 0.02	342.6 ± 31.3	1154.04 ± 0.02	3.37 +0.34/-0.28
Tartaric	0.46 ± 0.02	249.7 ± 27.1	1201.90 ± 0.02	4.81 +0.58/-0.47

5.4 Kinetic study of immobilized bromelain in model wine

As shown in Table 12, immobilized bromelain, with the exception of a few procedures, showed higher half-life respect to the free one. Nevertheless, all immobilization procedures applied affected catalytic properties, since V_{max} and K_a became smaller than the free enzyme.

All kinds of immobilization procedures applied at pH 7 allowed to obtain the highest immobilization yield (IY,%), observing the maximum value for Eupergit®-250L (81%). Nevertheless, all biocatalysts immobilized at pH 3.2 showed the highest V_{max} and k_{cat} value, indicating a good product release velocity.

Immobilization on chitosan beads in presence of cysteine (BCW-cys) improved both bromelain half-life and kinetic parameters. These evidences could be due to the presence of free cysteine in immobilization medium that maintains the Cys at the active site in the reduced form. Oriented immobilization allowed to obtain a significantly higher half-life, but no positive effect was revealed in terms of kinetic properties. This results could be explained considering that the number of covalent/non-covalent linkages between bromelain oligosaccharide chain and support influences the stability of enzymes against to various forms of inactivation with stability increasing with the number of linkages. Stem bromelain was successfully immobilized on chitosan beads without glutaraldehyde, obtaining the most interesting and food-safe biocatalyst. This procedure applied at pH 3.2 (BCW 3.2) allowed to obtain the best catalytic properties respect to all other ones.

Bromelain immobilized on chitosan (BCW 3.2) showed a lower K_M value, indicating a higher affinity for substrate than that of free bromelain. This may be due to the mild immobilization process, which slightly affected the structural or conformational integrity of the enzyme (Tan *et al.*, 2008). For that reason, this biocatalyst (BCW 3.2) was used for all other experiments.

Table 12: Immobilization yield (IY), kinetic parameters (mean ± SD) and half-life of free or immobilized bromelain toward Bz-Phe-Val-Arg-pNA substrate in model wine buffer.

	IY (%)	**V_{max} (I.U. mg^{-1})**	**K_M (μM)**	**k_{cat} (min^{-1})**	**K_a ($min^{-1}μM^{-1}$)**	**Half-life (h)**
Free bromelain	-	0.46 ± 0.02	249.7 ± 27.1	1201.90 ± 0.02	4.81 +0.58/-0.47	40
PRO 3.2	19 ± 1	0.118 ± 0.006	103.5 ± 10.3	10.184 ± 0.006	0.098 +0.009/-0.008	25
PRO 7	57 ± 2	0.0073 ± 0.0004	74.4 ± 9.1	0.0359 ± 0.0004	0.00048 +0.00007/-0.00006	24
EU 3.2	36 ± 4	0.149 ± 0.008	153.5 ± 15.2	8.249 ± 0.008	0.054 +0.005/-0.005	37
EU 7	81 ± 2	0.072 ± 0.004	111 ± 14	1.624 ± 0.004	0.015 +0.002/-0.002	64
BCW 3.2-CL	41 ± 2	0.059 ± 0.009	304.9 ± 66.1	2.132 ± 0.009	0.0070 +0.0004/-0.0003	50
BCW 7-CL	68 ± 2	0.018 ± 0.001	77.15 ± 16.28	0.364 ± 0.001	0.0047 +0.0013/-0.0008	39
BCW 3.2	26 ± 2	0.243 ± 0.009	49.49 ± 5.45	17.359 ± 0.009	0.35 +0.04/-0.03	50
BCW 7	70 ± 1	0.066 ± 0.004	48.45 ± 9.11	1.664 ± 0.004	0.034 +0.005/-0.004	66
BCW 3.2 - cys	41 ± 3	0.30 ± 0.02	136.2 ± 18.1	12.76 ± 0.02	0.09 +0.01/-0.01	74
BCW 7- cys	66 ± 5	0.109 ± 0.008	91.91 ± 15.06	3.314 ± 0.008	0.036 +0.009/-0.006	176
BCW 3.2-OI	46 ± 3	0.0210 ± 0.0009	108.5 ± 19.4	2.9205 ± 0.0009	0.027 +0.035/-0.021	137
BCW 7- OI	57 ± 2	0.0026 ± 0.0007	907.7 ± 469.6	0.0127 ± 0.0007	0.000014 +0.000016/-0.000005	664

5.5 Inhibition study of free stem bromelain

Wine contains various compounds that could have an inhibitory effect on enzymatic activity. In light of these considerations and in order to propose a future biotechnological application of stem bromelain in winemaking, the influence on protease activity of the potential inhibitors naturally present in wine was investigated. For each compound the first step was to identify the inhibition type: reversible or irreversible (Figure 29 A and B) by analysis of the kinetic data as described in the experimental section.

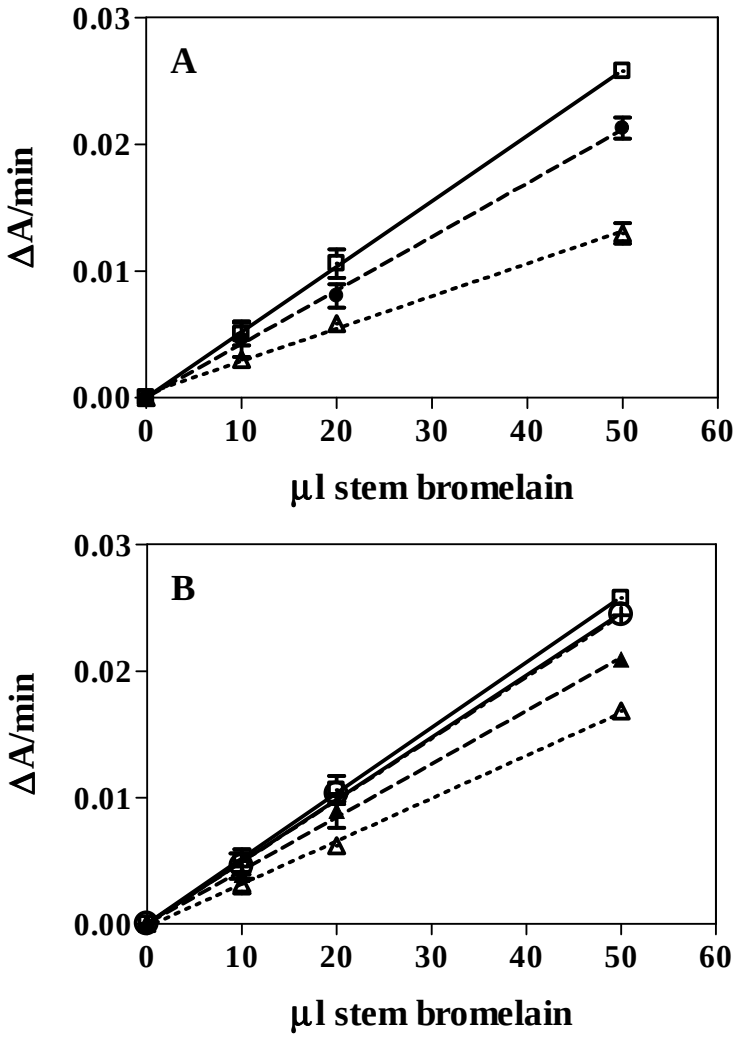


Figure 29: ΔA/min *vs* free stem bromelain assay volume (µl) against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in absence (control, □) and in presence of different inhibitors: (**A**) ethanol (●) and SO_2 (Δ); (**B**) skin (▲), seed (Δ), gallic (○) and ellagic tannins (+).

The “inhibitor added” curves have a smaller slope than the control curve and they pass through the origin (Segel, 1975). The ethanol inhibitory effect was rather limited, while sulphur dioxide strongly affected protease activity. Gallic and ellagic tannins showed no significant inhibitory effect, while skin and seed tannins proved to be reversible inhibitors. In summary all inhibitors tested turned out to be reversible for stem bromelain activity.

5.5.1 Inhibitory effect of ethanol on free stem bromelain activity

Ethanol in wine derives from the alcoholic fermentation of grape sugars and its concentration can range from 8% to 18% v/v in dry white and red wines (Moreno-Arribas *et al.*, 2009). The kinetic curves obtained in the presence of different ethanol concentrations (0, 12, 18% v/v) are reported in Figure 30.

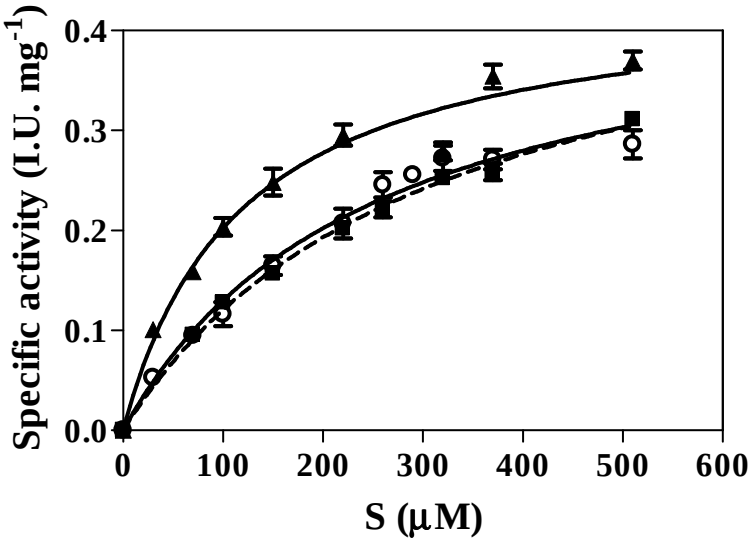


Figure 30: Specific activity (I.U. mg^{-1}) of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in absence (control, ▲) and in presence of different ethanol concentration: 12% v/v (○) and 18% v/v(■).

Kinetic parameters (Table 13) show that V_{max} does not change in the presence of ethanol, while $K_{M(app)}$ increases significantly and K_a decreases. In light of these data, ethanol is shown to be a competitive inhibitor of stem bromelain activity.

Table 13: Kinetic parameters of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) containing different concentration of ethanol: 0% v/v, 12% v/v and 18% v/v.

Ethanol (% v/v)	**$V_{max(app)}$ (I.U. mg^{-1})**	**$K_{M(app)}$ (μM)**	**$k_{cat(app)}$ (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
0	0.44 ± 0.02	118 ± 10	1166.20 ± 0.02	9.89 +1.20 / -0.97
12	0.46 ± 0.02	250 ± 27	1201.90± 0.02	4.81 + 0.59/-0.47
18	0.48 ± 0.02	305 ± 20	1287.05± 0.02	4.22 +0.30 / -0.26

The K_i value was determined by a replot of $K_{M(app)}$ *vs* ethanol concentration (%, v/v), as previously described. As shown in Figure 31, the K_i value was 11.4% v/v, indicating that the ethanol inhibition effect was rather limited. In fact, at a concentration closer to the average ethanol content in wine, the ratio K_i/[I] corresponding to the ratio [E]/[EI] (eq. 3), indicates that about a 50% of stem bromelain remains in free active form [E]. In light of these considerations, the protease activity remained sufficient to support its use in winemaking.

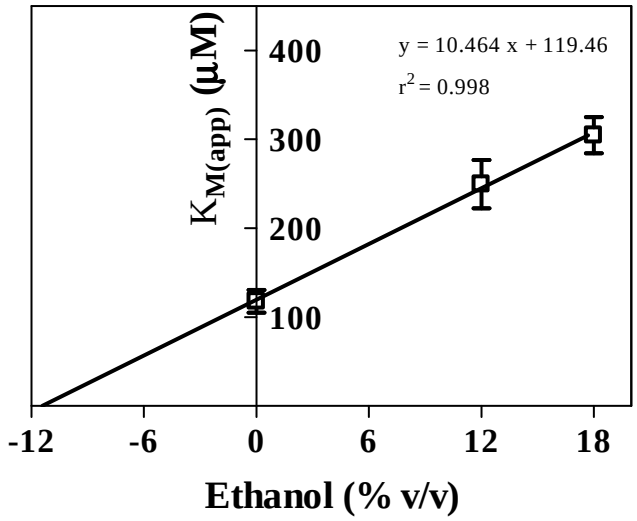


Figure 31: Secondary plot of $K_{M(app)}$ *vs* ethanol concentration.

5.5.2 Inhibitory effect of tannins on free stem bromelain activity

In order to study inhibitory effect of tannins on stem bromelain activity, different enological preparations, at various concentrations (g l^{-1}), were used. Even though dependent on the content of proanthocyanidic tannins in the grapes, the phenolic composition of a wine is strongly affected by winemaking techniques and oenological practices (Baiano *et al*., 2009).

5.5.2.1 Total phenolic content of tannins preparations

Total phenolic content of the different tannin preparations, expressed as grams of gallic acid equivalents per liter of model wine solution is shown in Table 14. Among the enological preparations tested, gallic and seed tannins presented the highest total phenolic content (67% and 60%, respectively), while skin and ellagic tannins showed the lowest values (50% and 40%, respectively).

Table 14: Total phenolic content of enological tannin preparations used, at different concentration (g l^{-1}), expressed as gram of gallic acid equivalents of model wine solution (g $l^{-1}_{\text{gallic acid eq}}$).

Tannins	**Experimental concentration used (g l^{-1})**	**Total phenolic content (g $l^{-1}_{\text{gallic acid eq}}$)**
Skin	0.5	0.25
	2	0.99
Seed	0.5	0.30
	2	1.20
Gallic	0.03	0.02
	0.05	0.03
Ellagic	0.05	0.02
	0.1	0.04

5.5.2.2 Skin and seed grape tannins

Most of grape seed and skin proanthocyanidic tannins are oligomeric and polymeric forms of (+)-catechin and (-)-epicatechin. Moreover, some epigallocatechin is found in skin, whereas epicatechin gallate is a small but significant proportion of seed tannins (Waterhouse, 2002). As shown in Figure 32 A and B, distinct kinetic hyperbolic curves were obtained in the presence of different amount of skin and seed tannins in the model wine buffer.

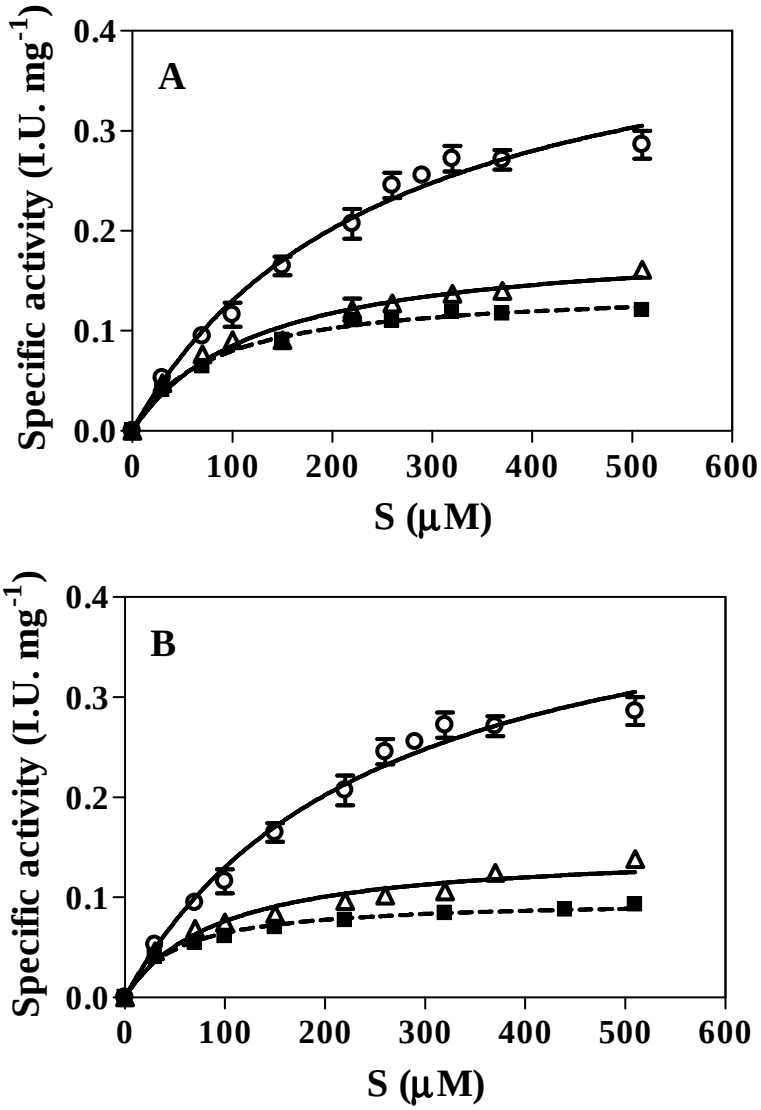


Figure 32: Specific activity (I.U. mg^{-1}) of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in absence (control, ○) and in presence of different concentration of: (**A**) skin tannins (0.25 g $l^{-1}_{\text{gallic acid eq}}$ (Δ); 0.99 g $l^{-1}_{\text{gallic acid eq}}$ (■)) and (**B**) seed tannins (0.30 g $l^{-1}_{\text{gallic acid eq}}$ (Δ) and 1.20 g $l^{-1}_{\text{gallic acid eq}}$ (■)).

The estimated kinetic parameters $V_{max(app)}$ and $K_{M(app)}$ decrease to the same extent; however K_a does not change, indicating that both proanthocyanidic tannins are uncompetitive inhibitors (Table 15).

Table 15: Kinetic parameters of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of skin and seed tannins.

Tannins	**Concentration ($g\ l^{-1}_{gallic\ acid\ eq}$)**	**$V_{max(app)}$ ($I.U.\ mg^{-1}$)**	**$K_{M(app)}$ (μM)**	**$k_{cat(app)}$ (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
	0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 + 0.59/-0.47
Skin	0.25	0.191 ± 0.008	125.2 ± 15.8	506.147 ± 0.008	4.04 + 0.58/-0.45
	0.99	0.143 ± 0.003	79.14 ± 6.52	378.42 ± 0.003	4.78 + 0.43/-0.36
Seed	0.30	0.149 ± 0.006	95.28 ± 13.02	393.229± 0.006	4.13 + 0.60/-0.49
	1.20	0.098 ± 0.002	52.64 ± 3.92	259.235 ± 0.002	4.92 +0.40/-0.34

K_i value, determined by a replot of $1/K_{M(app)}$ *vs* [I] was 0.59 $g\ l^{-1}_{gallic\ acid\ eq}$ and 0.46 $g\ l^{-1}_{gallic\ acid\ eq}$ for skin and seed tannins, respectively (Figure 33 A and B).

These results indicate that the inhibitor effect of seed tannins was higher than that for those from skin. In any case the inhibitory effect of both tannins is not limiting for bromelain application in winemaking, since at the highest tannin concentration tested (1.200 $g\ l^{-1}_{gallic\ acid\ eq}$) about 50% of stem bromelain remains in the free active form [E].

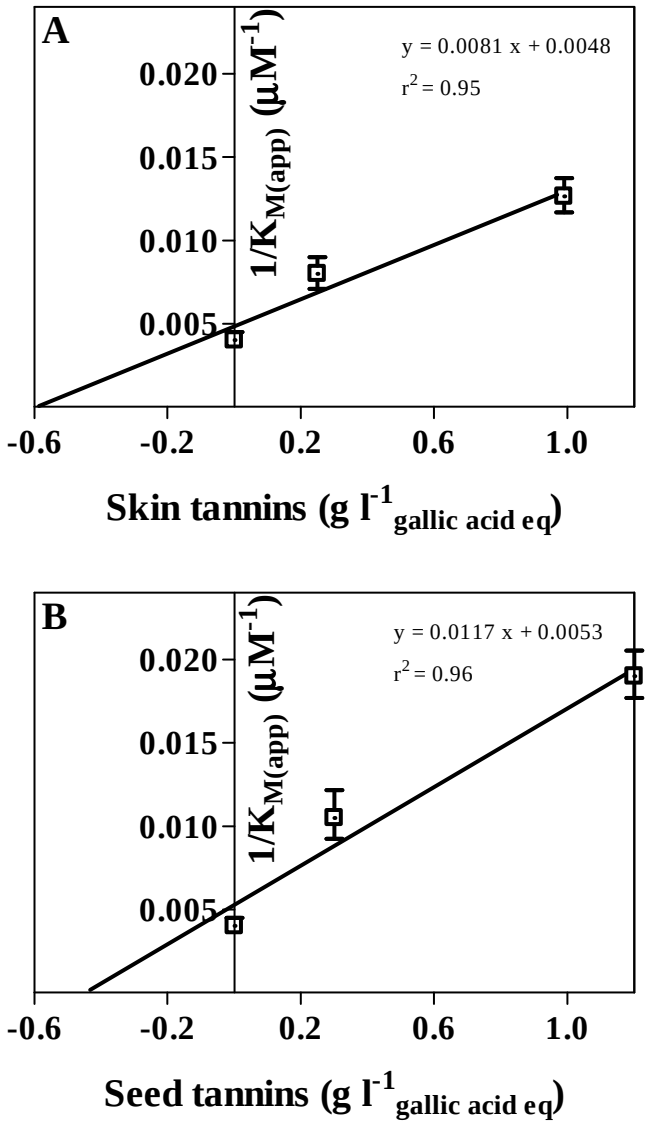


Figure 33: Secondary plot of $1/K_{M(app)}$ *vs*: (**A**) skin tannins concentration and (**B**) seed tannins concentration.

5.5.2.3 Gallic and ellagic tannins

Gallotannins and ellagitannins are, respectively, gallic and ellagic acid esters with glucose or other sugars (Waterhouse, 2002). The kinetic curves (Figure 34 A and B) and the unchanged kinetic parameters $V_{max(app)}$, $K_{M(app)}$ and K_a obtained in presence of different gallic and ellagic tannin concentrations (Table 16), indicate that these phenolic compounds have no inhibitory effect on stem bromelain activity.

This conclusion is reached in accordance with the discussion of results represented in Figure 29, given that both the “plus gallic” and the “plus ellagic” tannin curve were about superimposable with the control one, indicating that these tannins didn’t have any significant inhibitory effect at the levels tested.

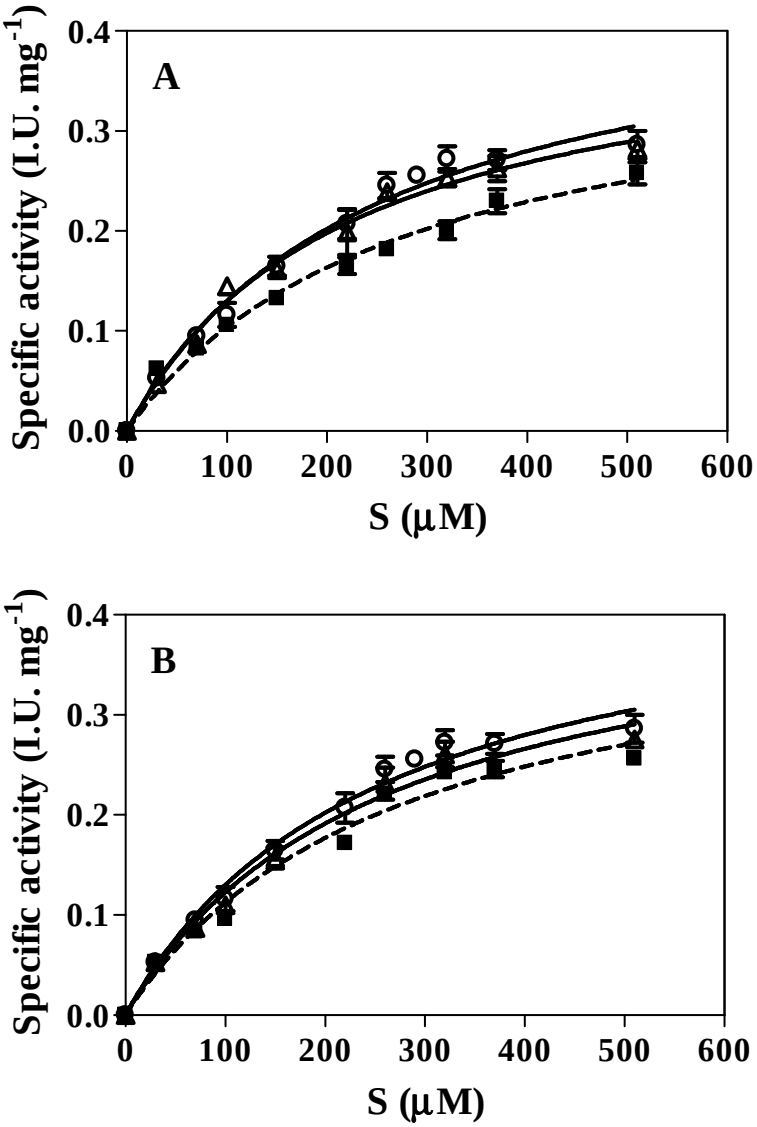


Figure 34: Specific activity (I.U. mg^{-1}) of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in absence (control, ○) and in presence of different concentration of: (**A**) gallic tannins (0.02 g $l^{-1}_{\text{gallic acid eq}}$ (Δ); 0.03 g $l^{-1}_{\text{gallic acid eq}}$ (■) and (**B**) ellagic tannins (0.02 g $l^{-1}_{\text{gallic acid eq}}$ (Δ); 0.04 g $l^{-1}_{\text{gallic acid eq}}$ (■)).

Table 16: Kinetic parameters of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of gallic and ellagic tannins.

Tannins	Concentration ($mg\ l^{-1}_{gallic\ acid\ eq}$)	$V_{max(app)}$ ($I.U.\ mg^{-1}$)	$K_{M(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)
	0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 + 0.59/-0.47
Gallic	0.02	0.42 ± 0.02	220.7 ± 22.5	1101.15± 0.02	4.99 +0.56/-0.46
	0.03	0.3844 ± 0.02	270.5 ± 29.45	1016.52± 0.02	3.76 +0.46/-0.37
Ellagic	0.02	0.44 ± 0.02	255.8 ± 29.6	1153.51 ± 0.02	4.51 + 0.59/-0.47
	0.04	0.42 ± 0.03	270.2 ± 36.5	1100.09 ± 0.03	4.07 +0.63/-0.48

5.5.3 Inhibitory effect of free SO_2 on free stem bromelain activity

Sulphur dioxide is added to wine, especially white wine, during winemaking process to prevent undesirable microbial growth and oxidation processes (Segundo *et al*., 2001; Toniolo *et al*., 2010). To the best of our knowledge, sulphur dioxide inhibition of bromelain in wine-like conditions is still unknown, although its inhibitory effect on the activity of various enzymes, such as trypsin and plant metabolic ones, has been studied (Wedzicha *et al*., 1987; Malhotra *et al*., 1976). It was demonstrated that sulphur dioxide is able to inactivate many enzymes by splitting their disulphide linkages; in particular, both sulphur dioxide and H_2SO_3 are able to convert disulphide bonds of enzymes or proteins to thiosulphonates and thiols (Malhotra *et al*., 1976).

Hyperbolic kinetic curves for bromelain were obtained in presence of different free sulphur dioxide concentration in model wine buffer (Figure 35).

The estimated kinetic parameters ($V_{max(app)}$, $K_{M(app)}$ and K_a) decrease, indicating that sulphur dioxide is a mixed-type inhibitor (Table 17).

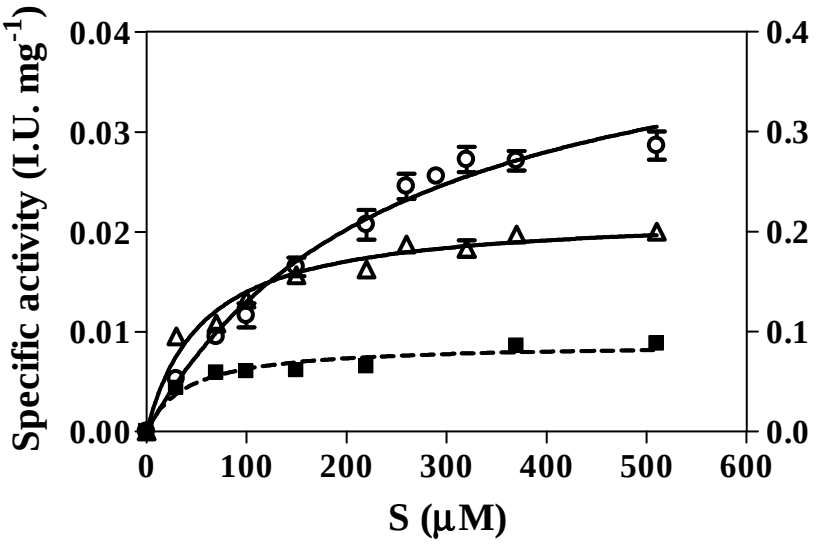


Figure 35: Specific activity (I.U. mg^{-1}) of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in absence (control, on right Y axis, O) and in presence of different concentration of free SO_2: 10 mg l^{-1} (Δ) and 25 mg l^{-1} (■) on left Y axis.

Table 17: Kinetic parameters of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of free SO_2:0 mg l^{-1}, 10 mg l^{-1} and 25 mg l^{-1}.

SO_2 (mg l^{-1})	**$V_{max(app)}$ (I.U. mg^{-1})**	**$K_{M(app)}$ (µM)**	**$k_{cat(app)}$ (min^{-1})**	**K_a ($min^{-1}µM^{-1}$)**
0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 + 0.59/-0.47
10	0.0218± 0.0006	56.3± 6.3	57.7282 ± 0.0006	1.03 +0.13/-0.10
25	0.0088 ± 0.0003	40.0 ± 6.5	23.2420± 0.0003	0.58 +0.11/-0.08

The K_i value, determined by a replot of $K_{M(app)}/V_{max(app)}$ *vs* [I], was 4.55 mg l^{-1} (Figure 36A). A second replot of $1/V_{max(app)}$ *vs* [I], was used to determine the K_i' value, which was 0.40 mg l^{-1}(Figure 36B). These K_i and K_i' values show that free sulphur dioxide strongly inhibits stem bromelain activity.

In the biological phases of winemaking (alcoholic and malolactic fermentation), before the aging and fining phases, the free SO_2 level is kept lesser then 25 mg l^{-1} in order to allow yeast and lactic-acid bacteria metabolism. Our data indicate that stem bromelain can be suitably applied in all the early winemaking phases. In fact, the K_i value (4.55 mg l^{-1}, corresponding to 68 μM) indicates that just the 30% of enzyme is in the free form (eq. 3); a stronger inhibitory effect was indicated by the K_i' value (0.4 mg l^{-1} corresponding to 0.4 μM) that implies that the ratio E/EI is strongly forced toward the EI complex (eq. 4).

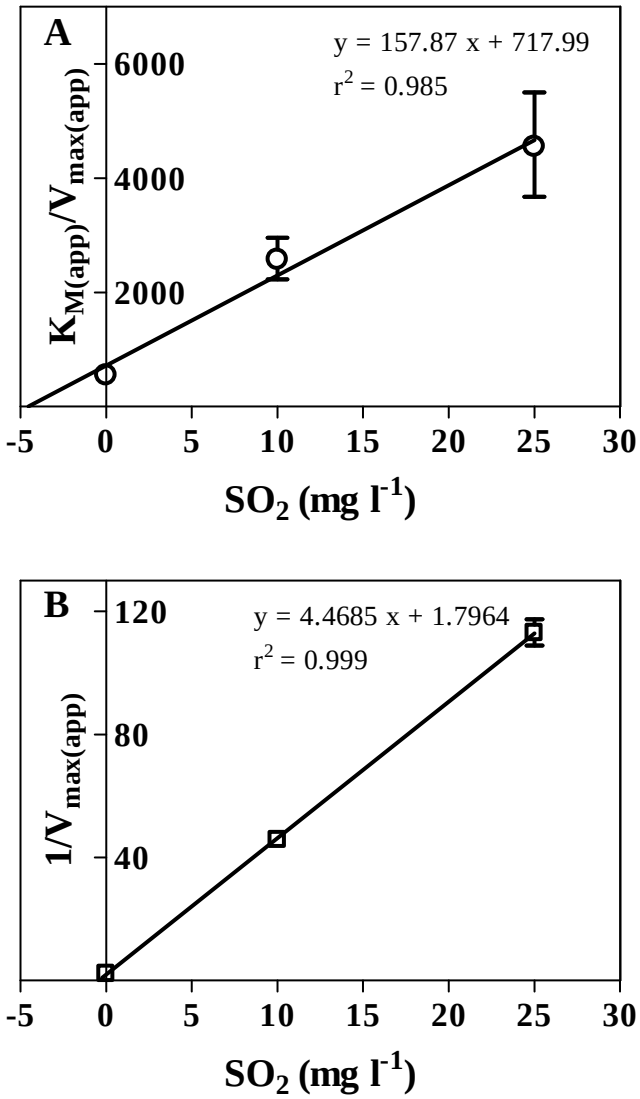


Figure 36: Secondary plot of: $K_{M(app)}/V_{max(app)}$ *vs* SO_2 concentration (**A**) and of $1/V_{max(app)}$ *vs* SO_2 concentration (**B**).

5.6 Inhibition study of immobilized stem bromelain

5.6.1 Inhibitory effect of ethanol on immobilized stem bromelain activity

The kinetic curves obtained in the presence of different ethanol concentrations (0, 12, 18% v/v) are reported in Figure 37.

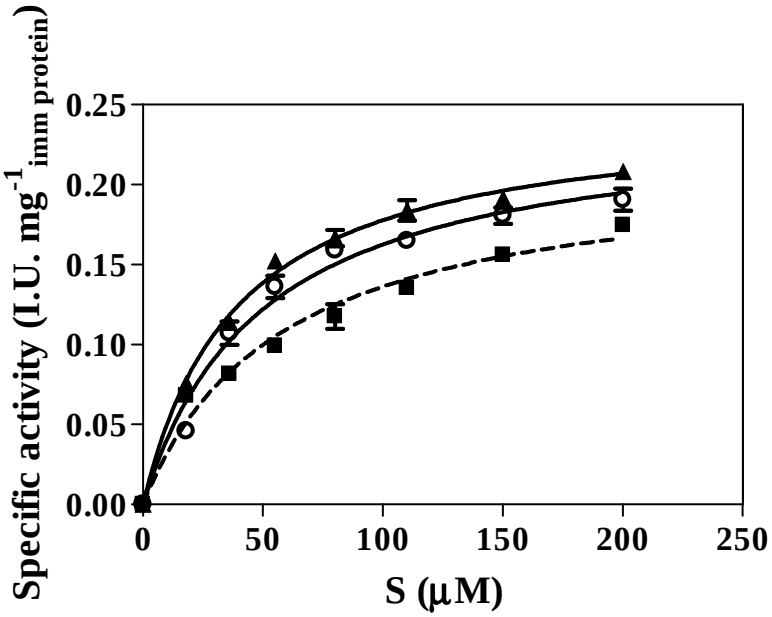


Figure 37: Specific activity (I.U. $mg^{-1}_{immobilized\ protein}$) of immobilized protein against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in absence (control, ▲) and in presence of different ethanol concentration: 12% v/v (○) and 18% v/v(■).

Kinetic parameters (Table 18) showed that $V_{max(app)}$ does not change in the presence of ethanol, while $K_{M(app)}$ increases significantly and K_a decreases. In light of these data, ethanol is shown to be a competitive inhibitor for immobilized stem bromelain such as for free protease.

Table 18: Kinetic parameters of immobilized stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) containing different concentration of ethanol: 0% v/v, 12% v/v and 18% v/v.

Ethanol (% v/v)	**$V_{max(app)}$ (I.U. mg^{-1})**	**$K_{M(app)}$ (μM)**	**$k_{cat(app)}$ (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
0	0.247 ± 0.004	39.19 ± 2.09	27.644 ± 0.004	0.71 +0.14/-0.10
12	0.243 ± 0.009	49.5 ± 5.5	17.359 ± 0.009	0.35 +0.04/-0.04
18	0.214± 0.009	57.31 ± 6.48	23.945 ± 0.009	0.42 +0.05/-0.04

The K_i value was determined by a replot of $K_{M(app)}$ *vs* ethanol concentration (%, v/v), as previously described. As shown in Figure 38, the K_i value obtained for immobilized bromelain (39.4% v/v) was higher than that obtained for free enzyme (11.4% v/v, see paragraph 5.5.1), indicating that immobilized protease resulted more resistant to the ethanol inhibition effect respect to the free one.

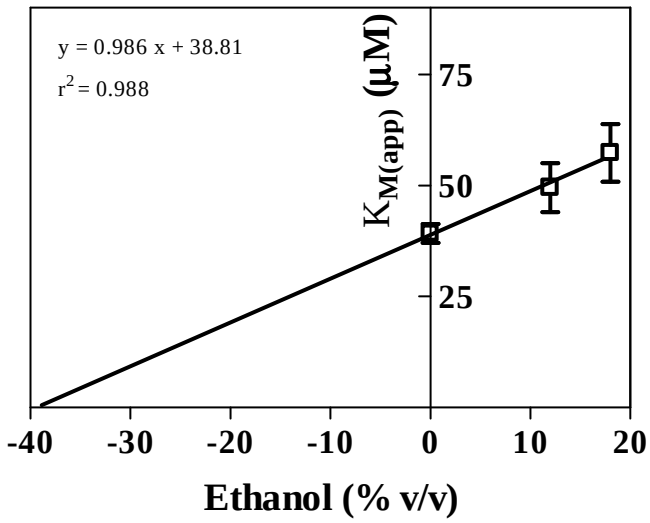


Figure 38: Secondary plot of $K_{M(app)}$ *vs* ethanol concentration.

5.6.2 Inhibitory effect of tannins on immobilized stem bromelain activity

5.6.2.1 Skin and seed grape tannins

Distinct kinetic hyperbolic curves were obtained in the presence of different amount of skin and seed tannins in the model wine buffer (Figure 39A and 39B).

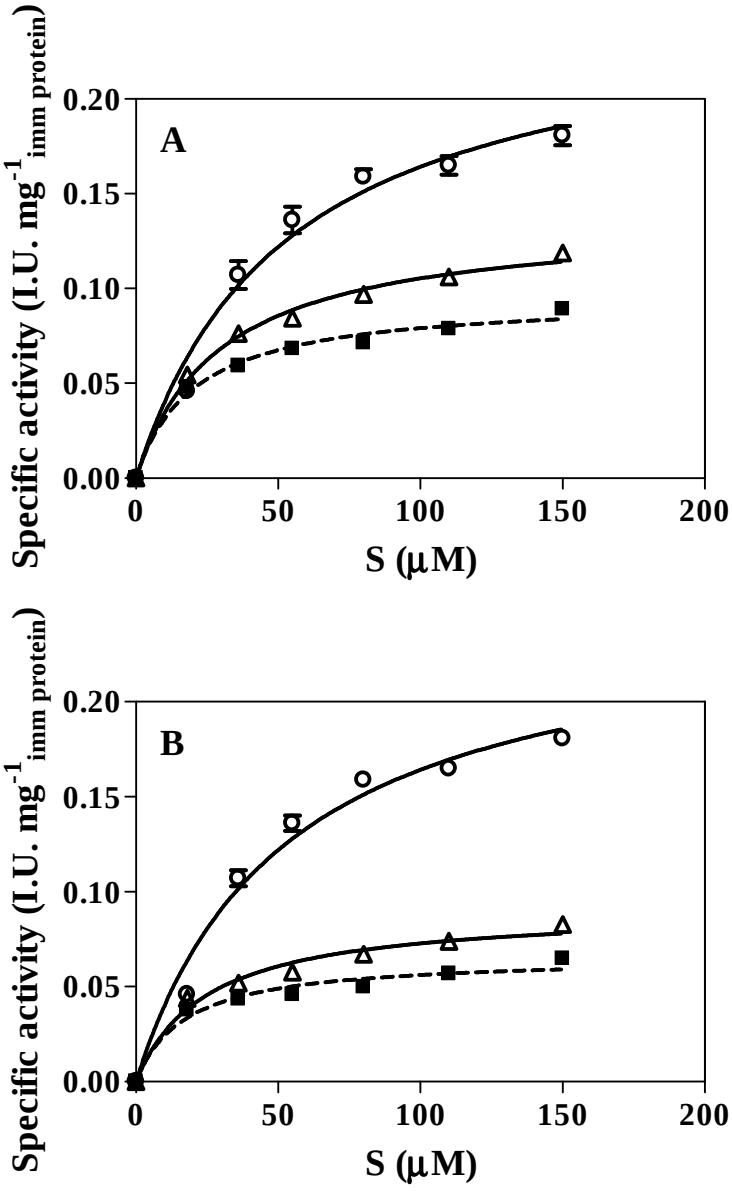


Figure 39: Specific activity (I.U. $mg^{-1}_{\text{immobilized protein}}$) of immobilized stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in absence (control, O) and in presence of different concentration of: (**A**) skin tannins (0.25 g $l^{-1}_{\text{gallic acid eq}}$ (Δ); 0.99 g $l^{-1}_{\text{gallic acid eq}}$ (■)) and (**B**) seed tannins (0.30 g $l^{-1}_{\text{gallic acid eq}}$ (Δ) and 1.20 g $l^{-1}_{\text{gallic acid eq}}$ (■)).

The estimated kinetic parameters $V_{max(app)}$ and $K_{M(app)}$ decrease to the same extent; consequently, K_a does not change, indicating that both proanthocyanidic tannins result uncompetitive inhibitors (Table 19) such as for free protease.

Table 19: Kinetic parameters of free stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of skin and seed tannins.

Tannins	**Concentration** ($g\ l^{-1}_{gallic\ acid\ eq}$)	$V_{max(app)}$ ($I.U.\ mg^{-1}$)	$K_{M(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)
	0	0.243 ± 0.009	49.5 ± 5.5	17.359 ± 0.009	0.35 +0.04/-0.04
Skin	0.25	0.137 ± 0.003	30 ± 2	9.187 ± 0.003	0.30 +0.02/-0.02
	0.99	0.095 ± 0.002	20.7 ± 2.1	6.403 ± 0.002	0.31 +0.03/-0.03
Seed	0.30	0.091± 0.003	25 ± 3	6.099 ± 0.003	0.25 +0.03/-0.03
	1.20	0.065± 0.003	17.4 ± 2.8	4.428 ± 0.003	0.25 +0.05/-0.04

K_i, determined by replot of $1/K_{M(app)}$ *vs* [I] was 0.87 $g\ l^{-1}_{gallic\ acid\ eq}$ and 0.90 $g\ l^{-1}_{gallic\ acid\ eq}$ for skin and seed tannins, respectively (Figure 40 A and B).

Both values were higher than those obtained for the free protease (0.59 $g\ l^{-1}_{gallic\ acid\ eq}$ and 0.46 $g\ l^{-1}_{gallic\ acid\ eq}$ for skin and seed tannins, respectively see paragraph 5.5.2.2), indicating that immobilized bromelain was more resistant both to seed and skin tannins inhibitor effect respect to the free one.

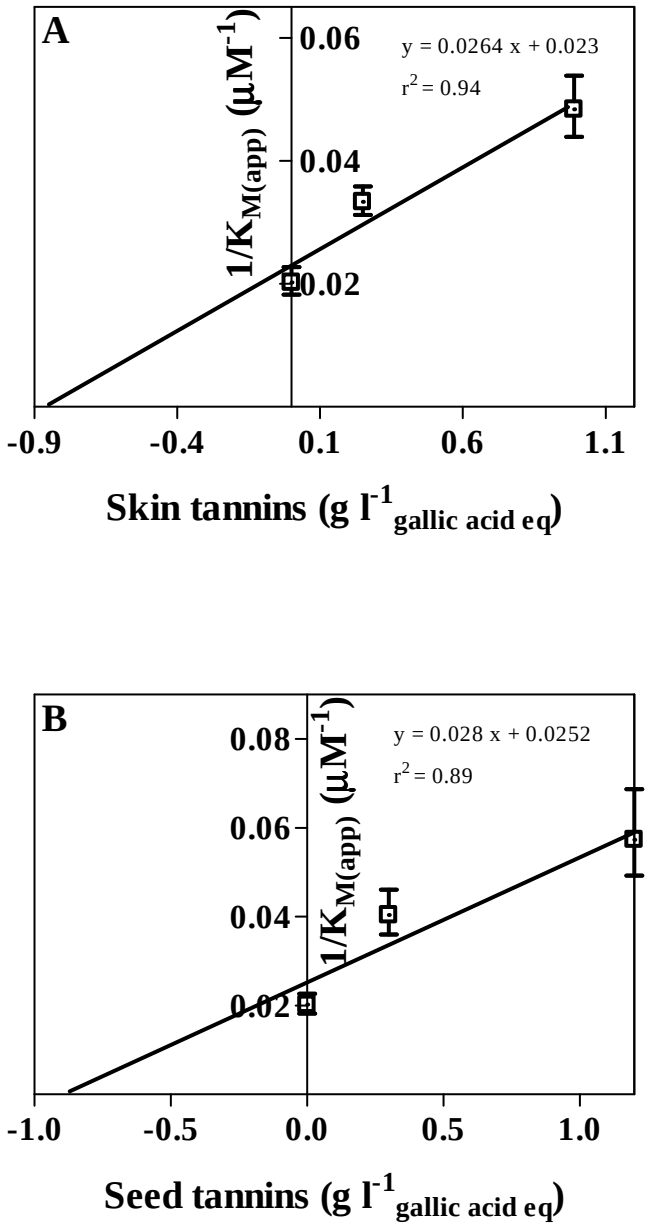


Figure 40: Secondary plot of $1/K_{M(app)}$ *vs*: (**A**) skin tannins concentration and (**B**) seed tannins concentration.

5.6.2.2 Gallic and ellagic tannins

Gallic and ellagic tannins didn't affect free bromelain activity, for this reason their effect was not tested on immobilized one.

5.6.3 Inhibitory effect of free SO_2 on immobilized stem bromelain activity

Hyperbolic kinetic curves for bromelain were obtained in presence of different free sulphur dioxide concentration in model wine buffer (Figure 41).

The estimated kinetic parameters $V_{max(app)}$ and $K_{M(app)}$ decrease to the same extent; consequently, K_a does not change (Table 20), indicating that SO_2 behaved as a uncompetitive inhibitor for immobilized bromelain.

The different inhibition effect of sulphur dioxide on free and immobilized protease is probably due to the different interaction mechanisms between inhibitor and free or immobilized enzyme. That occurs in consequence of enzyme conformational changes, resulting from the bromelain-support covalent bound.

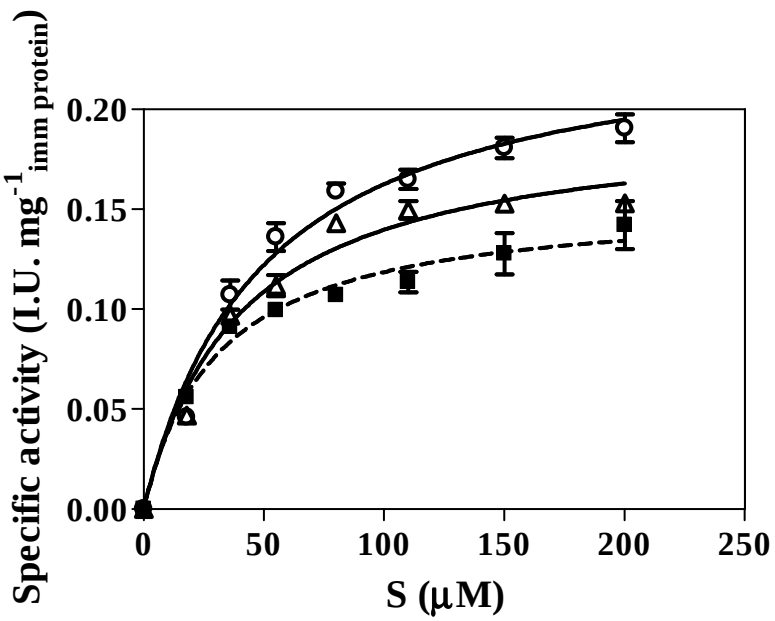


Figure 41: Specific activity (I.U. mg^{-1} $_{immobilized\ protein}$) of immobilized stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in absence (control, O) and in presence of different concentration of free SO_2: 10 mg l^{-1} (Δ) and 25 mg l^{-1} (■).

Table 20: Kinetic parameters of immobilized stem bromelain against Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of free SO_2: 0 mg l^{-1}, 10 mg l^{-1} and 25 mg l^{-1}.

SO_2 (mg l^{-1})	**$V_{max(app)}$ (I.U. mg^{-1})**	**$K_{M(app)}$ (μM)**	**$k_{cat(app)}$ (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
0	0.243 ± 0.009	49.5 ± 5.5	17.359 ± 0.009	0.35 +0.04/-0.04
10	0.195 ± 0.007	39.9 ± 4.6	15.70 ± 0.007	0.39 +0.05/-0.04
25	0.155± 0.005	30.63± 3.64	12.44 ± 0.005	0.41 +0.07/-0.05

K_i, determined by a replot of $1/K_{M(app)}$ *vs* [I], was 40.40 mg l^{-1} (Figure 42). This value was greater then K_i and K_i' (4.55 mg l^{-1} and 0.40 mg l^{-1}, respectively) obtained for free bromelain (see paragraph 5.5.3), indicating that immobilized enzyme was less inhibit by sulphur dioxide respect to the free one.

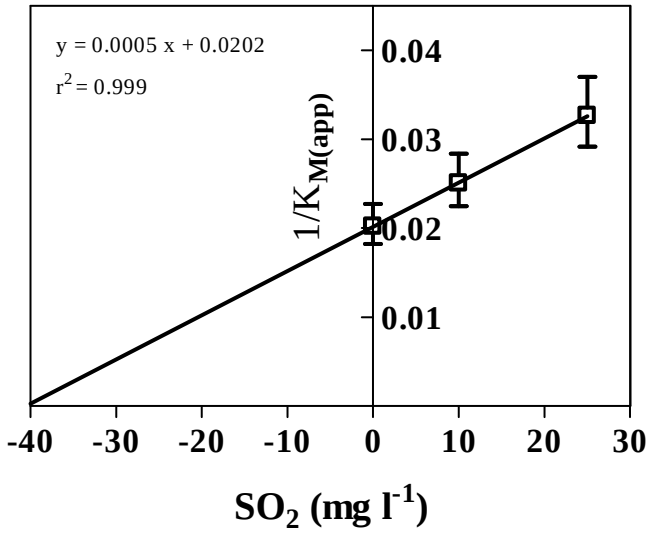


Figure 42: Secondary plot of: $1/K_{M(app)}$ *vs* SO_2 concentration.

5.7 Kinetic study of free and immobilized bromelain in real wines

Kinetic study of free and immobilized bromelain was carried out in 6 different artisan and unrefined white wines spiced with the synthetic substrate. In all wines tested, immobilized bromelain (BCW 3.2) exhibited V_{max} values greater than the free one (Figure 43 and Table 21), with the only exception of wine 6.

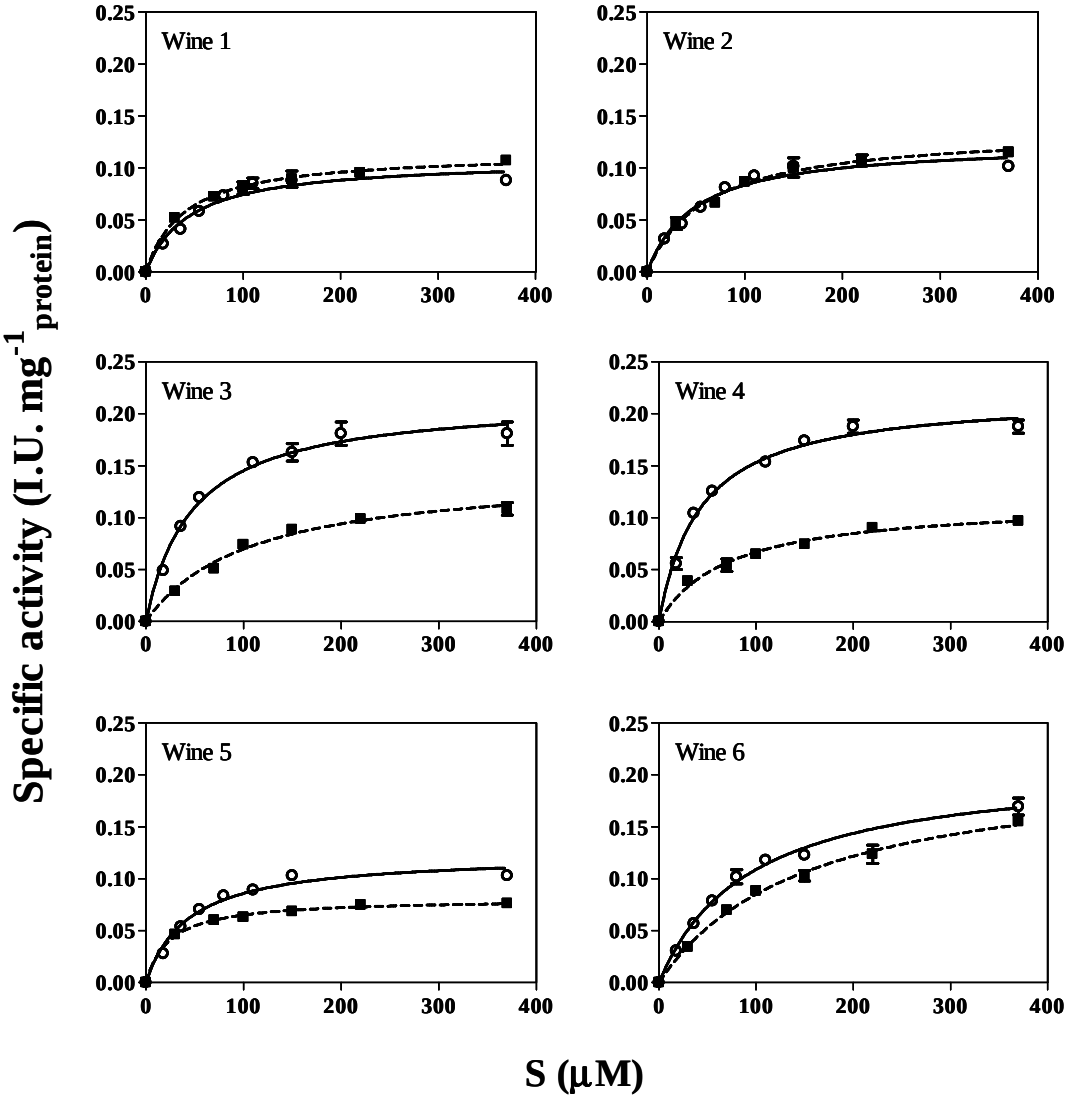


Figure 43: Specific activity (I.U. mg^{-1} $_{protein}$) of free (■) and immobilized stem bromelain (○) in 6 different wines spiced with the synthetic substrate (Bz-Phe-Val-Arg-pNA).

Moreover, immobilized bromelain exhibited both lower k_{cat} and K_a values than the free one, indicating a worse product release velocity and a lower specificity toward the substrate. The catalytic efficiency of immobilized bromelain with respect to the

synthetic substrate varied depending on the wines tested, probably due to the presence of inhibitors, such as sulphur dioxide and phenols (Table 22). In particular, immobilized bromelain showed the best catalitic properties in wine 3 and 4, which presented the lowest content in total sulphur dioxide (15 ppm) and phenols (825 ppm), respectively.

Table 21: Kinetic parameters (mean ± SD) of free or immobilized bromelain in unrefined white wines toward Bz-Phe-Val-Arg-pNA substrate.

Wine	**Stem bromelain**	**V_{max} (I.U. mg^{-1})**	**K_M (μM)**	**k_{cat} (min^{-1})**	**K_a ($min^{-1}\mu M^{-1}$)**
1	Free	0.115 ± 0.003	39.8 ± 3.7	304.111 ± 0.003	7.65 +0.78/-0.65
	Imm	0.137.± 0.006	74.1 ± 7.4	6.803 ± 0.006	0.092 +0.005/-0.004
2	Free	0.136 ± 0.004	61.4 ± 6.5	360.967 ± 0.004	5.88 +0.69/-0.56
	Imm	0.157 ± 0.006	79.7 ± 5.9	7.497 ± 0.006	0.094+0.007/-0.007
3	Free	0.145 ± 0.006	107.0 ± 11.2	382.122 ± 0.006	3.57 +0.42/-0.34
	Imm	0.230± 0.006	56 ± 4	17.298 ± 0.006	0.31 +0.07/-0.05
4	Free	0.116 ± 0.004	73.1 ± 7.1	306.227 ± 0.004	4.19 +0.45/-0.37
	Imm	0.230 ± 0.005	48.49 ± 3.35	18.750 ± 0.005	0.39 +0.06/-0.05
5	Free	0.081 ± 0.001	24.24 ± 2.03	214.015 ± 0.001	8.83 +0.81/-0.69
	Imm	0.146 ± 0.004	63.44 ± 4.34	6.705 ± 0.004	0.106 +0.008/-0.007
6	Free	0.214 ± 0.009	151.9 ± 13.2	566.440 ± 0.009	3.73 +0.36/-0.30
	Imm	0.20 ± 0.01	87.5 ± 9.6	9.51 ± 0.01	0.11 +0.01/-0.01

Table 22: Chemical-physical characteristics of 6 artisan and unrefined white wines.

		Wine samples					
		1	**2**	**3**	**4**	**5**	**6**
pH		3.54	4.05	3.88	3.39	3.46	3.67
Alcohol level	**% v/v**	11.43	11.35	10.82	12.65	10.24	12.50
Reducing sugars	**g l^{-1}**	< 2	< 2	< 2	< 2	< 2	< 2
Extract	**g l^{-1}**	22	27	20	18	17	19
Total acidity	**g $l^{-1}_{\text{tartaric acid}}$**	4.32	5.80	5.47	5.51	4.21	4.28
Volatile acidity	**g $l^{-1}_{\text{acetic acid}}$**	0.52	0.72	0.70	0.38	0.44	0.76
Tartaric acid	**g l^{-1}**	1.53	1.38	1.63	2.21	2.43	1.76
L(-)-Malic acid	**g l^{-1}**	0.82	0.21	0.35	1.64	1.53	0.32
L(+)-Lactic acid	**g l^{-1}**	1.03	2.49	1.79	0.28	0.30	1.37
Citric acid	**g l^{-1}**	0.11	0.16	0.11	0.44	0.55	0.14
Glycerol	**g l^{-1}**	6.5	7.8	7.7	5.6	5.9	7.2
K	**g l^{-1}**	1.3	1.6	1.0	0.8	0.7	1.1
Ash	**g l^{-1}**	2.46	3.15	1.64	2.04	2.13	2.25
Total SO_2	**mg l^{-1}**	135	76	15	62	81	119
Total phenols	**mg l^{-1}_{catechin}**	1174	1351	1285	825	914	1081

5.8 Immobilized bromelain activity toward total and unstable wine proteins

Finally, immobilized bromelain activity was tested toward total and unstable wine proteins. As shown in Figure 44, after 24-h treatment, the total protein removal yield (TPRY) varied from 59 to 93% depending on the initial protein content.

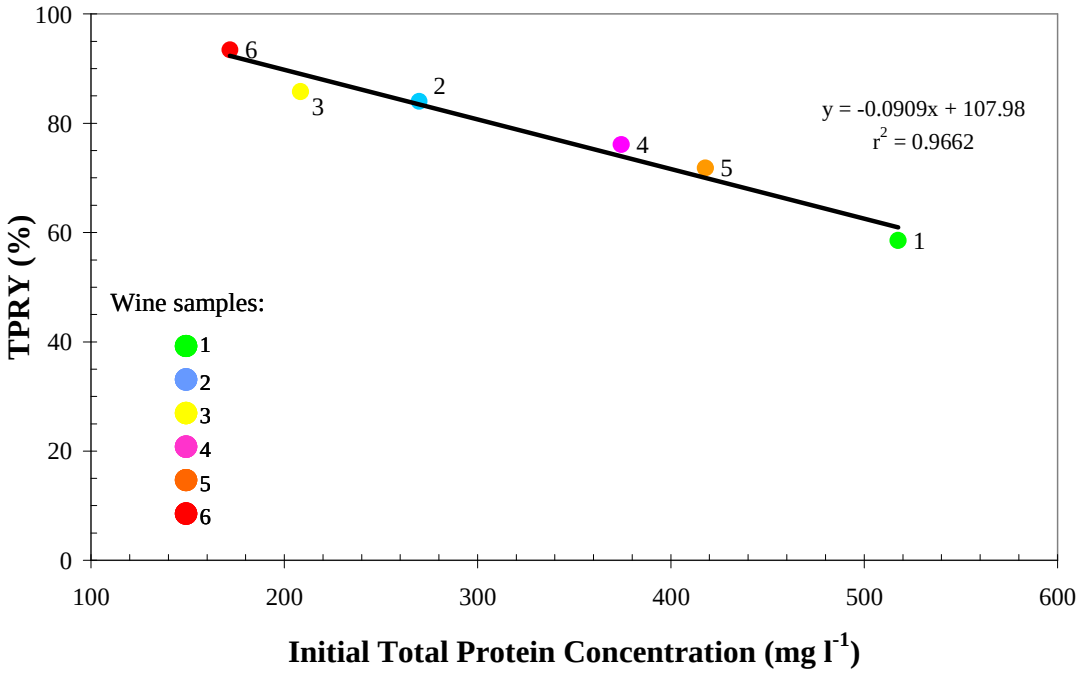


Figure 44: Total protein removal yield (TPRY, %) in 24 h by immobilized bromelain in white wines with different initial protein content, expressed as mg l^{-1} BSA equivalent.

On the contrary, the turbidity removal yield (TRY) was practically constant (66 ± 7 %) for all the 6 wines tested independently of the initial NTU index (Figure 45). This confirmed that immobilized bromelain exerted its useful proteolytic activity on unstable wine proteins in almost the same way whatever their nature and content.

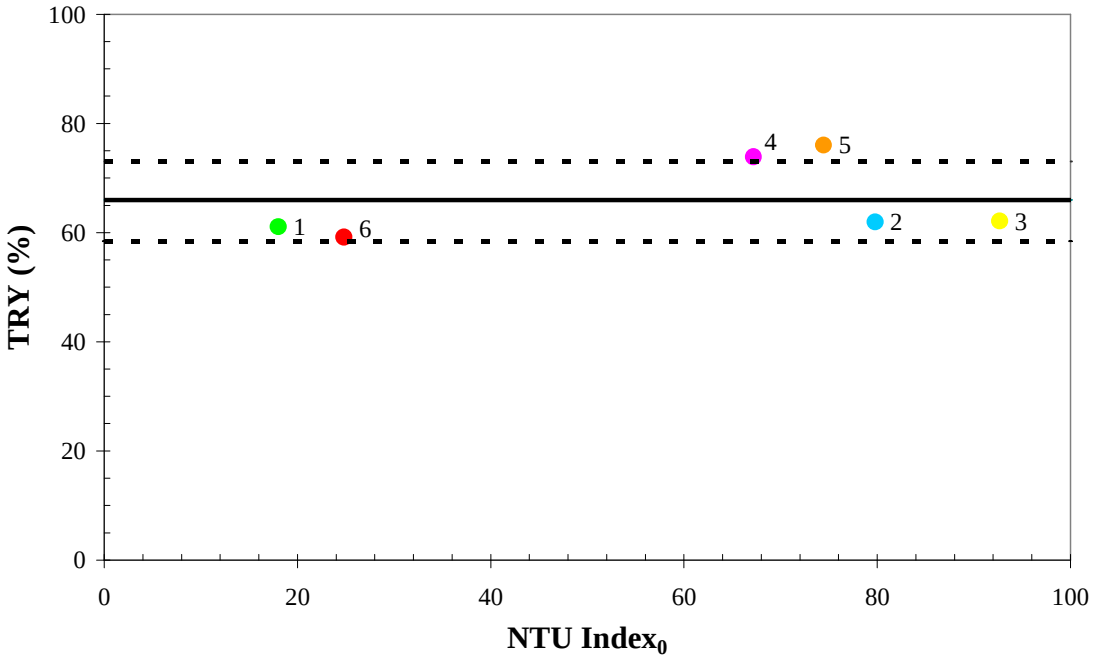


Figure 45: Turbidity removal yield (TRY, %) in 24 h by immobilized bromelain, in white wines with different initial turbidity index, expressed as NTU.

Conclusions

Free pineapple stem bromelain activity was assessed on different synthetic substrates. Among these, Bz-Phe-Val-Arg-pNA turned out to be the most suitable one for evaluating protease activity at a reference pH value (3.2), this being the average minimum pH value of wine. Free enzyme showed high activity both in McIlvaine buffer and in model wine, at the same pH 3.2.

The enzyme was covalently immobilized on different supports (Prosep-9CHO, Eupergit®-250L, Chitopearl BCW-3010), even if all procedures applied affected its catalytic properties. Immobilization at pH 7 allowed the highest immobilization yield. Nevertheless, biocatalysts immobilized at pH 3.2 showed the best catalitic performance. Stem bromelain was successfully immobilized on chitosan beads without glutaraldehyde cross-linking (BCW 3.2), thus improving the food safety of the biocatalyst of concern. By referring to the selected synthetic substrate, the catalytic parameters (V_{max}, k_{cat}, K_a) of immobilized enzyme, in model wine solution, resulted to be lower than those of free one, even if the lower K_M value indicates a higher affinity for substrate than that of free bromelain. This may be due to the mild immobilization process, which slightly affected the structural or conformational integrity of the enzyme (Tan *et al.*, 2008). For that reason, this biocatalyst (BCW 3.2) was used for all other experiments.

Inhibition study proved that all inhibitors tested resulted to be reversible for stem bromelain activity. In detail, even if ethanol was a competitive inhibitor both for free and for immobilized bromelain, its effect was rather limited, thus allowing a satisfactory application of bromelain in winemaking.

Among the enological preparations tested and independently of their phenolic content, gallic and ellagic tannins exhibited no inhibitory effect on stem bromelain activity, while seed and skin tannins resulted to be uncompetitive inhibitors for free and immobilized bromelain. Moreover, the inhibitory effect of seed tannins was higher than that of skin ones, even if this not affected its application in winemaking. The strongest inhibition effect was due to sulphur dioxide, that behaved as a mixed-type inhibitor for free enzyme and a uncompetitive inhibitor for immobilized one. Nevertheless, whatever the inhibitors tested, immobilized bromelain was less affected than the free one.

Kinetic study of free and immobilized bromelain was carried out in using 6 artisan and unrefined white wines spiced with the synthetic substrate. The catalytic efficiency of immobilized protease varied depending on the different inhibitors content in the tested wines. Immobilized bromelain (BCW 3.2) exhibited V_{max} values greater than the free form for all wines examined, except one. Moreover, immobilized bromelain exhibited lower k_{cat} and K_a values than the free one, indicating a worse product release velocity and a lower specificity toward the substrate.

In terms of turbidity haze, bromelain immobilized on chitosan beads exerted its activity even on unstable white wine proteins whatever their nature and content. An average reduction of about 66% of the initial NTU index, this ranging from 20 to 90 NTU, was generally obtained after a 24-h treatment.

Further work will be directed to maximise the rate of turbidity haze removal by varying the biocatalyst loading per unit volume of wine to be treated, as well as to optimise the architecture of the bioreactor system to be industrially applied.

Acknowledgements

This PhD thesis research was supported by financial backing of the Italian Ministry of Agriculture, Food and Forestry.

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Ringraziamenti

Questa tesi di Dottorato è il frutto di un lavoro durato 3 anni che ho cercato di svolgere con impegno e passione anche nei momenti difficili, sostenuta sempre dall'affetto dei miei genitori, di mia sorella Simona e di Pierluigi.

Grazie alle mie colleghe, le Dottoresse Katia Liburdi e Sara Torresi che sono le migliori amiche che potessi trovare, pronte in ogni momento ad affrontare e risolvere i problemi con il sorriso e l'ironia.

Desidero ringraziare la Professoressa Anna Maria Vittora Garzillo, che oltre ad essere un'ottima insegnante, è stata per me un punto di riferimento, una guida materna e un esempio da seguire.

Un ringraziamento al Professor Mauro Moresi per l'aiuto con l'inglese e per i suoi preziosi consigli.

Infine grazie al Professor Marco Esti che in questi anni ha creato un gruppo di lavoro unito e affiatato, trasmettendo a me e Katia il suo modo di fare e facendoci crescere sia professionalmente che umanamente.