

# Investigating the impact of MpAPr1, an aspartic protease from the yeast *Metschnikowia pulcherrima*, on wine properties

by

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## Declaration

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## Summary

Protein removal is a key step during the production of white wine in order to avoid the possible appearance of a harmless but unsightly haze. Alternatives to the use of bentonite are actively sought because of technological, organoleptic and sustainable issues associated with its use. Acid proteases that are able to break down proteins under winemaking conditions could be one such alternative. Recent literature reports the successful outcome of the addition of fungal aspartic proteases from *Aspergillus* and *Botrytis*. In this study, MpAPr1, an extracellular aspartic protease previously isolated and partially characterised from the yeast *Metschnikowia pulcherrima*, was cloned and expressed heterologously in *Komagataella pastoris*. Enzymatic properties of MpAPr1 were initially ( $K_m$ ,  $V_{max}$ ,  $K'_i$ , optimal pH and temperature for protease activity, impact of minerals, sugars and ethanol on protease activity) characterised in a crude extract. After several attempts using different techniques, MpAPr1 was successfully purified via cation exchange chromatography. Its activity against haze-forming grape proteins was initially tested in a model solution under optimal environmental conditions (for MpAPr1 activity) and under those occurring during winemaking (pH 3.5 and 25°C). Thereafter, MpAPr1 activity was evaluated in grape must and throughout alcoholic fermentation. These experiments showed that MpAPr1 was able to degrade certain haze-forming proteins, especially chitinases, under optimal conditions and to a lesser extent under winemaking conditions. Prior denaturation of the target proteins by heat treatment was also not required. Moreover, MpAPr1 was able to degrade yeast proteins in a model solution under both conditions. Finally, the presence of MpAPr1, supplemented to grape must, resulted in the partial degradation of grape proteins throughout fermentation and ultimately in a slight difference in the wine's volatile compound composition. Winemaking conditions limited its impact and it is thus proposed that future work focus on enhancing MpAPr1 activity to make it a viable alternative to bentonite. The study nevertheless provides further evidence that aspartic proteases could represent a potential alternative to bentonite for the wine industry and that non-*Saccharomyces* yeasts such as *M. pulcherrima* could have a beneficial impact on wine properties.

## Opsomming

Proteïen verwydering is 'n belangrike stap tydens die vervaardiging van witwyn en vermy die voorkoms van 'n onooglike maar skadelose wasigheid. Alternatiewe vir die gebruik van bentoniet is aktief begeerd as gevolg van tegnologiese, organoleptiese en volhoubare kwessies wat verband hou met die gebruik daarvan. Suur proteases wat die afbraak van proteïene kan fasiliteer onder wynmaak toestande kan dus as sulke alternatiewe dien. Onlangse literatuur beskryf die suksesvolle gebruik van swam-afkomstige aspartiensuur proteases vanaf *Aspergillus* en *Botrytis*. In hierdie studie was MpAPr1, 'n ekstrasellulêre aspartiensuur protease voorheen geïsoleer en gedeeltelik gekenmerk vanaf die gis *Metschnikowia pulcherrima*, gekloneer en uitgedruk in *Komagataella pastoris*. Ensiem kenmerke ( $K_m$ ,  $V_{max}$ ,  $K_i$ , optimale pH and temperature vir protease aktiwiteit, impak van minerale, suiker en etanol) was aanvanklik bepaal vanaf 'n ru-ekstrak. Na verskeie pogings deur gebruik te maak van verskeie tegnieke, is MpAPr1 suksesvol gesuiwer via kationuitruilings chromatografie. Ensiem aktiwiteit teen waas-vormende druif proteïene was aanvanklik getoets in 'n model oplossing onder optimale omgewings toestande (vir MpAPr1 aktiwiteit) en dié wat gedurende wynmaak voorkom (pH 3.5 en 25 °C). Daarna was MpAPr1 aktiwiteit geëvalueer in druiwemos tydens alkoholiese fermentasie. Eksperimente het getoon dat MpAPr1 verskeie waas-vormende proteïene, veral chitinases, afbreek onder optimale omstandighede en in 'n mindere mate onder wynmaak toestande. Voorafgaande denaturasie van die teiken proteïene deur hitte behandeling was nie benodig nie. Verdermeer was MpAPr1 in staat om gis proteïene af te breek in 'n model oplossing onder beide toestande. Ten slotte, die teenwoordigheid van MpAPr1 in druiwesap het gelei tot die gedeeltelike afbraak van proteïene tydens fermentasie asook tot 'n effense verskil in die uiteindelijke vlugtige verbinding samestelling. Wynmaak toestande het gelei to beperkte ensiem impak en dus word dit voorgestel dat toekomstige werk fokus op die verbetering van MpAPr1 aktiwiteit sodat dit as 'n lewensvatbare alternatief vir bentoniet behandeling kan dien. Hierdie studie stel nogtans bewyse voor dat aspartiensuur proteases 'n moontlike alternatief vir bentoniet kan wees in die wynbedryf en dat nie-*Saccharomyces* giste soos *M. pulcherrima* voordelige impakte op wyn einskappe kan hê.

## Résumé

L'élimination des protéines est une étape-clef durant la production du vin blanc afin d'éviter l'apparition d'un voile inoffensif mais disgracieux, causé par la dénaturation de ces protéines (un phénomène connu sous le nom de "casse protéique") au cours du vieillissement ou du stockage de ces vins en conditions sous-optimales. Des alternatives à l'utilisation de la bentonite, une argile couramment employée pour ses propriétés adsorbantes, sont activement recherchées pour des raisons technologiques, organoleptiques et durables associées à son utilisation. Les protéases acides capables de dégrader les protéines en conditions de vinification pourraient constituer une telle alternative. La littérature récente rapporte les succès obtenus lors de l'addition de protéases aspartiques d'origine fongique isolées d'*Aspergillus* et de *Botrytis*. Dans cette étude, MpAPr1, une protéase aspartique extracellulaire de la levure *Metschnikowia pulcherrima*, précédemment isolée et partiellement caractérisée, a été clonée et exprimée de manière hétérologue chez la levure *Komagataella pastoris*. Les propriétés enzymatiques de MpAPr1 ( $K_m$ ,  $V_{max}$ ,  $K'_i$ , pH et température optimaux d'activité, impact de minéraux, sucres et éthanol sur l'activité enzymatique) ont été initialement caractérisées sur un extrait brut. Après de nombreux essais utilisant diverses techniques, elle a été purifiée avec succès par chromatographie échangeuse de cations. Son activité contre les protéines de raisin responsables de casse protéique a été tout d'abord évaluée en solution modèle en conditions environnementales optimales pour son activité et en conditions telles que celles trouvées lors de la vinification (pH 3.5 et 25°C). Par la suite, l'activité de MpAPr1 a été évaluée dans du moût de raisin et au cours de la fermentation alcoolique. Ces expérimentations ont montré que MpAPr1 est capable de dégrader certaines protéines responsables de casse, particulièrement les chitinases, en conditions optimales, mais aussi, bien que de manière moindre, en conditions de vinification. La dénaturation préalable des protéines-cibles par traitement à la chaleur n'a pas été pas requis. De plus, MpAPr1 est capable de dégrader les protéines de levure en solution modèle dans les deux conditions. Enfin, la présence de MpAPr1, supplémentée dans du moût de raisin, a résulté en la dégradation partielle des protéines du raisin au cours de la fermentation et à la fin, en une légère différence dans la composition en composés volatils du vin. Les conditions œnologiques ont limité son impact et il est donc proposé que de futurs travaux se concentrent sur l'amélioration de l'activité de MpAPr1 afin d'en faire une alternative viable à la bentonite. L'étude a toutefois renforcé l'idée que les protéases aspartiques pourraient représenter une alternative potentielle à la bentonite pour l'industrie viti-vinicole et que les levures non-*Saccharomyces* telles que *M. pulcherrima* pourraient impacter positivement sur les propriétés technologiques du vin.

*“Do the difficult things while they are easy and do the great things while they are small. A journey of a thousand miles must begin with a single step”*

-Lao Tzu

## **Biographical sketch**

Louwrens Wiid Theron was born in the Western Cape, South Africa on the 17th of November 1988 and was raised in the town of Paarl. He matriculated at Paarl Boys' High School in 2006 and commenced his undergraduate studies at the University of Stellenbosch in 2008 where he enrolled for a BSc degree in Molecular Biology and Biotechnology. After graduating in 2010 he pursued post-graduate studies at the Institute for Wine Biotechnology. Obtaining a BScHons degree in Wine Biotechnology in 2011, he started his MSc degree in Wine Biotechnology in 2012. He obtained his MSc degree in 2013 and the following year enrolled for a PhD degree. The specific PhD program he enrolled for was part of a cotutelle international PhD program between the University of Stellenbosch (South Africa) and the University of Bordeaux (France).

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## Preface

This dissertation is presented as a compilation of seven chapters.

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## List of Abbreviations

2D PAGE	Two dimensional polyacrylamide gel electrophoresis
AGP	Aspergillopepsins
AIDS	Autoimmune deficiency syndrome
APSm1	Aspartic protease from <i>Stenocarpella maydis</i>
Eap1	Aspartic protease from
BCA	Bichonic acid
BcAP8	<i>Botrytis cinerea</i> aspartic protease
BSA	Bovine serum albumin
CaAPr1	<i>Candida apicola</i> aspartic protease
Cap1	<i>Cryptococcus</i> spp. S-2 aspartic protease
CPGR	Centre for Proteomic and Genomic Research
DAN	Diazoacetylnorleucinemethyl
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DON	5-diazo-4-oxonorvaline
DTT	1,4-Dithiothreitol
EC	European council
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EPNP	1,2-epoxy-3-(p-nitrophenoxy)propane
FOSS	Fourier-transform mid-infrared spectroscopy
FP	Flash pasteurised
GAP	Glyceraldehydes-3-phosphate dehydrogenase
GC-FID	Gas chromatography - Flame ionization detection
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric focusing
IMAC	Immobilized Metal Affinity Chromatography
IPG	Immobilized pH gradient
IWBT	Institute for Wine Biotechnology
$K_i$	Inhibitor constant
$K_m$	Michaelis constant
LB	Luria Bertani
LC-MS/MS	Liquid chromatography – mass spectrometry

MCAP	Extracellular aspartic protease from <i>Mucor circinelloides</i>
MEGA	Molecular evolutionary genetic analysis
MpAPr1	<i>Metschnikowia pulcherrima</i> aspartic protease
MWCO	Molecular weight cut off
NCBI	National Centre for Biotechnology Information
IUBMB	International Union of Biochemistry and Molecular Biology
p-CNB	p-chloromercuribenzoic acid;
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis related
rSAP6	Aspartic protease from <i>Metschnikowia reukauffii</i>
SAP	Secreted aspartic protease
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SIOS	Scanning Ion Occlusion Sensing
TCA	Trichloroacetic acid
TLP	Thaumatococcus-like protein
$V_{max}$	Maximum velocity
VvTl	<i>Vitis vinifera</i> thaumatococcus-like protein
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose
YPDS	Yeast Peptone Dextrose Sorbitol

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# Chapter 1

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## Introduction and project aims

## Chapter 1 - General introduction and project aims

### 1.1 Introduction

Several non-*Saccharomyces* species naturally occurring within the wine making environment have been shown to secrete extracellular enzymes of oenological interest. One such genus, namely *Metschnikowia*, does not display strong fermentation capacity but is believed to possess enzymes of oenological relevance (e.g. esterases, glucosidases, proteases). Indeed, various authors have either noted an increase in certain esters in wine fermented by a combination of *Metschnikowia* and *Saccharomyces* (Parapouli et al. 2010, Varela et al. 2016) or performed plate assays revealing active extracellular enzyme activity of oenological interest (Strauss et al. 2001, Reid et al 2012). Nevertheless, these enzyme activities have never been thoroughly investigated. In particular, acid proteases are of interest to the wine industry, because of their activity at low pH values present during grape juice fermentation.

Acid proteases find their application in various industries including the medicine, pharmaceutical, leather, food and beverage industries (Rao et al. 1998, Theron and Divol 2014). Within the beverage industry, these enzymes have especially gained attention because of their potential to degrade haze-forming proteins. They are also widely used as meat tenderizers (Bekhit et al. 2014), in flour for baking and cheese manufacturing (Rao et al. 1998). In the brewing industry, acid proteases are used to extract peptides and amino acids from malts and barley (Lei et al. 2013) and are also utilised as tools to degrade proteins that can form an unsightly haze. Similarly, the broader beverage industry makes use of acid proteases to degrade proteins responsible for turbidity in fruit juices and alcoholic beverages.

In the wine industry, proteases are being investigated for their ability to degrade haze-forming proteins in order to eliminate or at least reduce the need for fining agents such as bentonite (van Sluyter et al. 2015). The latter is a type of clay that possesses the property to adsorb high amounts of proteins. Despite the successful outcome in terms of protein elimination, the use of bentonite comes with a number of disadvantages: it is expensive, it reduces yield because of the large layer of precipitated lees obtained and consequently increases the amount of mechanical treatments of the wine as the lees must be removed (Waters et al. 2005, Pocock et al 2011). Furthermore, nutrients and aroma compounds might be carried along in the precipitates, thereby decreasing the flavour complexity of wine (Sanborn et al 2010, Moio et al. 2004). Finally, bentonite is not recyclable and this therefore creates environmental and sustainable issues. Consequently, there is a growing demand in the wine industry for the release of alternative means to eliminate haze-forming proteins that would not damage wine quality and reduce volumes.

The total concentration of proteins in wines varies generally from 15 to 230 mg/L (Monteiro et al. 2001, Ferreira et al. 2002, Waters et al. 2005) and mainly consists of grape proteins but may also originate from yeast and bacterial autolysis. Modern proteomic techniques have allowed enumerating and identifying more than 100 proteins in wine (D'Amato et al. 2011). The vast majority of these proteins are pathogenic-related proteins (viz  $\beta$ -1,3-glucanases, thaumatin-like proteins and chitinases), but those of yeast origin (mostly cell wall components as well as lipid transfer proteins) are also present (Esteruelas et al. 2009, Giribaldi 2013, Sauvage et al. 2010). The most abundant class of haze-forming proteins are chitinases and thaumatin-like proteins (TLPs). These specific proteins are generally small, possessing globular structures and being positively charged at low pH such as that occurring in grape juice/wine (Marangon et al. 2014). Because of their abundance and their damaging capacity, they have been extensively studied over the past decade, but other proteins (although less abundant) such as  $\beta$ -glucanases has also been shown to contribute to haze formation (Esteruelas et al. 2009, Sauvage et al. 2010).

In the past, the isolation and characterisation of proteins from grape juice and wine have proved problematic mainly due to the complex mixture of proteins and their degradation products present during or after fermentation (Vincenzi et al. 2011). However, advances in research, such as the release of the *Vitis vinifera* genome (Velasco et al. 2007) and improved protein purification strategies, have significantly improved and made possible the isolation and characterisation of some proteins associated with winemaking (Marangon et al. 2009, van Sluyter et al. 2009, Giribaldi et al. 2010, Palmisano et al. 2010, Le Bourse et al. 2011, Cilindre et al. 2014). Proteins responsible for haze formation are identified and classified as pathogenesis-related proteins although they have been found to be expressed constitutively throughout berry development (Pocock et al. 2000). Furthermore, it has been reported that their concentrations vary significantly between cultivars, vintage, disease pressure and even harvest conditions (Hayasaka et al. 2001, Monteiro et al. 2003, Girbau et al. 2004) and can reach high concentrations regardless of pathogen exposure. Because of their physical structure and properties, grape proteins (especially TLP's) are very resilient and are not or poorly degraded during the course of fermentation. Following an increase in temperature during ageing (in the cellar or in the bottle), they may denature and cause a visible haze, wrongly interpreted as spoilage by the consumer. Their removal from grape juice or wine before bottling has therefore become a standard step of the winemaking process.

Theoretically, proteases would constitute an ideal alternative to bentonite. They would indeed break down the haze-forming proteins (as well as other proteins) into peptides that could no longer denature and form haze while being able to be assimilated by the yeasts during fermentation as yeast assimilable nitrogen. Due to the harsh physico-chemical conditions occurring in wine (low pH, low temperature, presence of inhibitors such as ethanol

and phenolic compounds), most of the microbial enzymes are however not suitable. Proteases from *Aspergillus niger*, widely used in the beer and pharmaceutical industries, are for instance not active in wine (Bakalinsky and Boulton, 1985). However, the proteolytic activity of *Botrytis cinerea* has been shown to be effective in efficiently degrading PR proteins (Girbau et al 2004, van Sluyter et al. 2013). Preliminary reports have shown that the use of proteases in wine is an efficient way of reducing protein haze formation without being detrimental to wine quality (Lagace and Bisson 1990, Pocock et al. 2003), but heating needs to be coupled with the treatment in order to denature the heat-unstable proteins prior to degrade them. Pocock et al. (2003) indeed demonstrated that the combined treatment of heat (90°C for 1 min or 45°C for 24 h) and proteolysis (using Trenolin blank) reduced bentonite requirements. The use of AGP, a mixture of Aspergillopepsins I and II isolated from *A. niger* var. *macrosporus*, was tested by Marangon et al (2012) together with flash pasteurisation at 72°C for 1 min. The results showed that the treatment was very effective: all heat unstable proteins (i.e. accounting for 90% of wine proteins and including those responsible for haze formation) were degraded and the main physicochemical parameters and sensorial characteristics remained unchanged. The use of AGP coupled with flash pasteurisation eliminated the need for bentonite treatment, therefore preserving wine organoleptic quality. This enzyme is now commercialised under the commercial name Proctase and has been approved for use in oenology in Australia and New Zealand.

In addition to protein degradation, protease activity can lead to the release of assimilable nitrogen sources and is therefore hypothesised to have a direct impact on the production of yeast secondary metabolites such as higher alcohols and esters and also indirectly by influencing population dynamics. Indeed, this has been noticed in jujube wines (fermented with *S. cerevisiae*) treated with proteases before yeast inoculation (Zhang et al. 2016). Furthermore, some grape varietal aroma precursors such as thiols are present as cysteine- or glutathione-conjugates (Roland et al 2010) and these could potentially be cleaved off by the action of proteases. It has also been recently noticed that yeast peptides could be responsible for the perception of sweetness in dry wines (Marchal et al 2011). Finally, through the elimination or reduction of bentonite, the use of proteases could indirectly increase yields.

At the Institute of Wine Biotechnology, the exploration of the oenological properties of non-*Saccharomyces* yeasts isolated from grape juice/wine led to the identification of yeasts displaying extracellular acid protease activity. One such yeast species, *Metschnikowia pulcherrima* IWBT Y1123, was found to display strong activity against BSA, casein and grape proteins at low pH conditions (Reid et al. 2012). The protease-encoding gene, named *MpAPr1*, was isolated by the latter authors and tentatively identified as an aspartic protease, based on sequence similarities with other known aspartic proteases. In follow-up study (Theron 2013), expression of *MpAPr1* in *Escherichia coli* and subsequent purification were attempted, but

recovery of an active enzyme remained unsuccessful due to the formation of inclusion bodies and refolding issues after protein extraction. Thus the characterisation and assessment of MpAPr1 remains to be elucidated as the use of aspartic proteases in oenology and new enzymes is becoming more sought after.

## 1.2 Scope and aims of study

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The aim of this study was to characterise and investigate the oenological potential of the aspartic protease MpAPr1 through the expression of the *MpAPr1* gene in a eukaryotic host and the characterisation of its enzymatic properties. A further objective was to purify MpAPr1 by means of chromatography and assess its impact on grape proteins and oenological parameters.

**The specific objectives of this study were the following:**

1. Clone *MpAPr1* into a suitable eukaryotic host for heterologous expression
2. Optimise expression and purify the recombinant enzyme
3. Characterise MpAPr1's enzymatic properties
4. Assess the ability of MpAPr1 to degrade grape proteins, including pathogenesis-related proteins
5. Investigate the impact of MpAPr1 activity on the fermentation proceedings and the broader wine properties

# Chapter 2

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## Literature review

### Microbial aspartic proteases: current and potential applications in industry

Paragraphs 2.1 to 2.5 of this chapter were published as a review article  
in

#### **Applied Microbiology and Biotechnology**

Theron, L.W. & Divol, B. (2014) *Appl Microbiol Biotechnol* 98: 8853-68  
doi:10.1007/s00253-014-6035-6

Paragraph 2.6 provides a brief update of the literature published  
between 2014 and 2016.

## Chapter 2 - Microbial aspartic proteases: current and potential applications in industry

### 2. Abstract

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

Keywords Microbes. Aspartic protease, Industrial applications, Beverage, Wine, Protein haze

### 2.1 Introduction

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

Generally, the term proteases can be used interchangeably with the terms proteinases and/or proteolytic enzymes, but the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) and the Enzyme Commission (EC) recommend that the term peptidases be used for all enzymes that hydrolyse peptide bonds (subclass E.C.3.4). Proteases have a major function in the global recycling of carbon and nitrogen from proteins. Proteins from dead organisms are indeed eventually hydrolysed by

microorganisms (in the process of decomposition) into peptides and amino acids. These products can be assimilated by the microorganisms that produced the proteases or by other organisms in the vicinity. For example, protease-producing microorganisms present in soil have been shown to regulate protease expression in response to carbon and nitrogen limitation (Sims and Wander 2002). In this context, proteases can be helpful in nitrogen-limited environments.

In organisms, proteases are known to carry out a vast array of physiological functions including cell division, signal transduction, sporulation, digestion of food proteins, blood pressure regulation, viral protein synthesis, apoptosis, processing of polypeptide hormones, degradation of incorrectly folded proteins, autolysis, protection against harmful peptides and enzymes amongst others (Barrett et al. 2004, Sandhya et al. 2005, Tyndall et al. 2005). Extracellular proteases play a critical role in the hydrolysis and the adsorption of proteinaceous nutrients (Kalisz 1988). As the latter can function in a variety of environments, not limited to the inner cell, they are of great commercial importance, and protein extracts prepared from the growth cultures of protease-producing microorganisms are commonly used as protein-degrading tools during various industrial processes (Kumar and Takagi 1999).

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

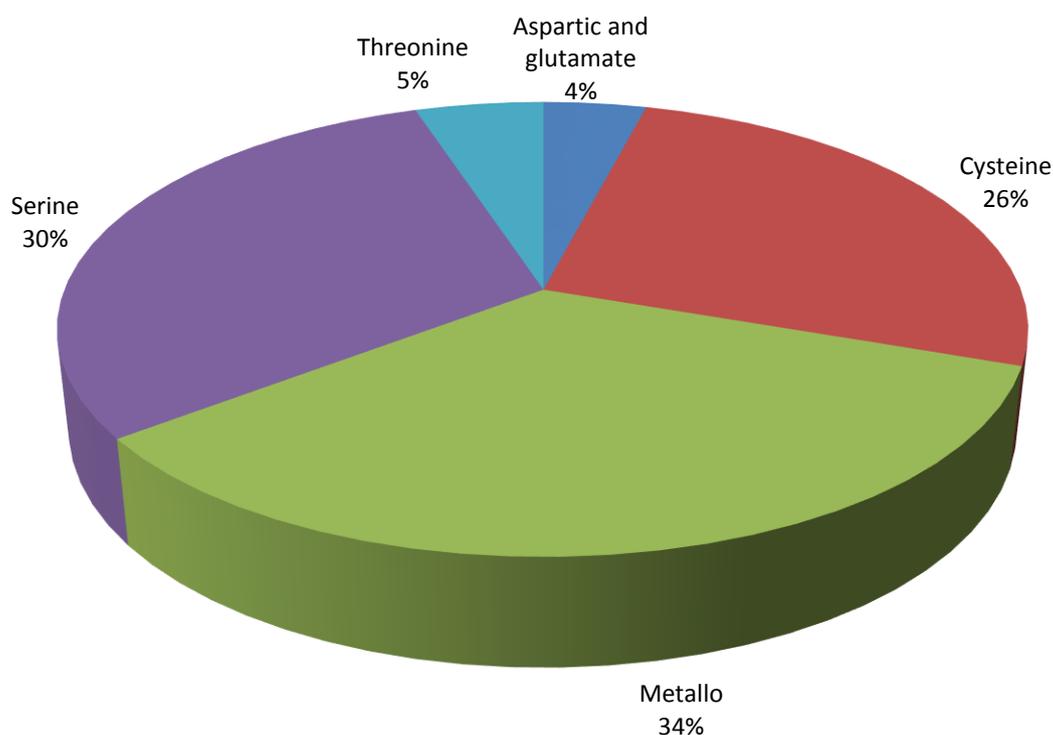
Endopeptidases are industrially more important than exopeptidases and are classified according to their molecular size, charge, substrate specificity, catalytic mechanism, three dimensional structures and the amino acid residues present in their catalytic site (Beynon and Bond 1990, Sumantha et al. 2006). Each type of protease indeed exhibits a set of amino acid residues arranged in a specific configuration to produce its catalytic site. This gives them the characteristic ability to break certain peptide bonds (Barrett et al. 2004, Tyndall et al. 2005). Finally, a specific group of endoproteases, termed oligopeptidases, acts only on substrates smaller than proteins. Table 2.1 summarizes the classification of proteases and their modes of action.

**Table 2.1:** Classification of proteases. Solid circles represent the terminal amino acids. Open circles signify amino acid residues in the polypeptide chain.

Proteases	Mode of action	Cleavage site	
<b>EC 3.4 (Peptidases)</b> Exopeptidases	EC 3.4.11 (Aminopeptidases) 	Free N-terminus	
	EC 3.4.13 (Dipeptidases) 		
	EC 3.4.14 (Dipeptidyl- and tripeptidyl-peptidases) 		
	EC 3.4.15 (Peptidyl-dipeptidases) EC 3.4.16 (Serine-type carboxypeptidases) EC 3.4.17 (Metallocoarboxypeptidases) EC 3.4.18 (Cysteine-type carboxypeptidases)	Free C-terminus	
	EC 3.4.19 (Omega peptidases)		Blocked N- or C-terminus
	EC 3.4.21 (Serine endopeptidase) EC 3.4.22 (Cysteine endopeptidase) EC 3.4.23 (Aspartic endopeptidase) EC 3.4.24 (Metalloendopeptidase) EC 3.4.25 (Threonine endopeptidase)		
	EC 3.4.99 (Unknown)		Non-terminal

The MEROPS database (<http://merops.sanger.ac.uk/>), a manually curated database dedicated to peptidases, divides peptidases into protein species, based on the main amino acid present at the catalytic domain. These species are then further subdivided into families according to the statistically significant similarities in their amino acid sequences. Protein

species include aspartic/glutamate, cysteine, metallo, serine and the less characterized threonine peptidases (Madala et al. 2010). In the nomenclature of the NC-IUBMB (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>), endopeptidases which include serinepeptidase, cysteinepeptidase, asparticpeptidase, metallopeptidase and threonine endopeptidase are given the subclasses EC 3.4.21, EC 3.4.22, EC 3.4.23, EC 3.4.24 and EC 3.4.25, respectively. Figure 1 summarizes the abundance of these proteases found in nature.



**Figure 2.1:** Relative abundance of endoproteases in living organisms.

All of these endopeptidases differ in their properties and response to environmental conditions. Table 2.2 shows the different species of endoproteases together with some additional information on their characteristics, sources and the industry they are used in. Briefly, serine proteases, which play an important role in digestion, possess a catalytic triad in their active site consisting of a serine, histidine and aspartic acid residues. They fall into two categories based on their structure: the chymotrypsin-like (serine protease I) and the subtilisinlike (serine protease II) proteases. Cysteine proteases, commonly used in meat tenderizers, have similar folds as the serine proteases but the catalytic dyad in their active site consists of cysteine and histidine residues. The metalloproteases, as the name suggests, are classified as any proteases whose catalytic mechanism involves a metal (usually divalent zinc ions). Threonine proteases are one of the newer classes of proteases described and harbour a threonine residue in their catalytic domain (Rao et al. 1998, Madala et al. 2010). The aspartic

proteases, which will be discussed in more detail in the following paragraphs, have a tertiary structure consisting of two symmetrical lobes to form the catalytic site, each lobe harbouring an aspartic acid residue. With cysteine proteases, they are the only endoproteases active at acidic pH (Table 2.2). It is however worth mentioning that in 1990, Fusek et al. purified and cloned a thermophilic acid protease from *Sulfolobus acidocaldarius* (an archaeobacteria) which does not have an aspartyl residue in its active site nor does it show any apparent sequence homology to other acid proteases and therefore represents a new class (Fusek et al. 1990).

**Table 2.2:** The different species of endoproteases, their principal properties, main sources or isolation and industries in which these proteases have found applications.

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

DAN, diazoacetyl norleucinemethyl; DON, 5-diazo-4-oxonorvaline; PMSF, phenylmethylsulfonyl fluoride; PCMB, (pchloromercuribenzoic acid; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane), Nd., Not determined. (Rao et al. 1998, Sumantha et al. 2006)

## 2.2 Aspartic proteases

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### 2.2.1 Distribution

Aspartic proteases, commonly known as acid proteases, are distributed across all forms of life including vertebrates, plants, fungi, bacteria and also viruses (Fairlie et al. 2000, Cooper 2002). This relatively small group of enzymes has received much attention from the scientific community because of their involvement in human diseases. Some of these proteases indeed include the plasmepsins in malaria, HIV-1 peptidase in acquired immune deficiency syndrome (AIDS) and the secreted aspartic peptidases in *Candida* infections (Madala et al. 2010). From as early as 1989, crystal structures of aspartic proteases from retroviruses such as HIV and Rous sarcoma have been extensively studied and determined (Navia et al. 1989). The secreted aspartic proteases from *Candida albicans* have been intensively investigated due to their role in various forms of candidiasis. Since its discovery, the secreted proteolytic activity of *C. albicans* was discussed as a putative virulence factor. The major proteases secreted in vitro by *Candida* species have been termed Sap2, Sapp1 and Sapt1 from *C. albicans*, *Candida parapsilosis* and *Candida tropicalis*, respectively (Ruchel 1986, de Viragh et al. 1993, Monod et al. 1994). Their proposed functions during infection include the degradation of the host tissue barriers during invasion and the destruction of the host defence molecules. Furthermore, they also have a role in nutrient supply by degrading proteins and releasing assimilable nitrogen sources (Naglik et al. 2003). Aspartic proteases from other yeasts and fungi have also been studied extensively, and several have been purified and cloned for research and industrial purposes (Tonouchi et al. 1986, Horiuchi et al. 1988, Togni et al. 1991, De Viragh et al. 1993, Gomi et al. 1993, Jarai et al. 1994, Kakimori et al. 1996, Young et al. 1996, van Kuyk et al. 2000, Li et al. 2009, 2010, Radha et al. 2011, Shivakumar 2012). Several of these extracellular aspartic proteases from fungal species originate from *Aspergillus* species. Some of these species include: *Aspergillus oryzae* (Vishwanatha et al. 2009), *Aspergillus fumigatus* (Reichard et al. 1994), *Aspergillus saitoi* (Tello-Solis and Hernandez-Arana 1995), *Aspergillus awamori* (Moralejo et al. 2002) and *Aspergillus niger* (O'Donnel et al. 2001, Siala et al. 2009, Radha et al. 2011). Studies have also revealed that the aspergillopepsin I (Pep1) and rhizopuspepsin of *A. fumigatus* and *Rhizopus* microspores are present in lung infections (Schoen et al. 2002).

### 2.2.2 Description and mechanism of action

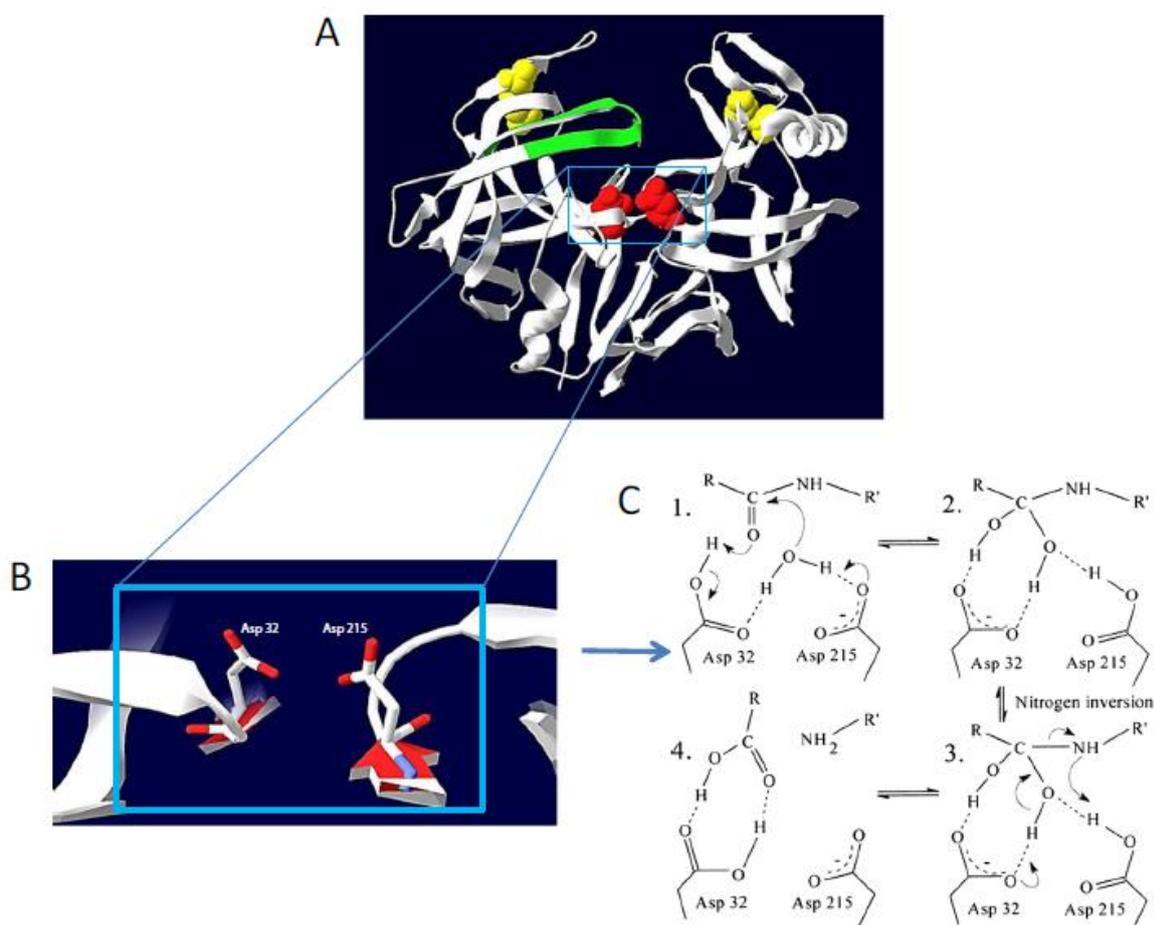
The molecular weight of aspartic acid proteases typically ranges between 35 and 50 kDa usually consisting of 320 to 340 amino acid residues. These enzymes have isoelectric points in the range of 3 to 4.5. Analysis of various aspartic proteases by X-ray crystallography shows that they are mostly composed of  $\beta$ -strand secondary structures.  $\beta$ -Strands are found at the base of the active-site cleft and contain the catalytic aspartic residues. In porcine pepsin and

endothiapepsin, these aspartic residues have been identified at Asp32 and Asp215 (Coates et al. 2001, Veerapandian et al. 1992). A water molecule is found hydrogen bonded between both aspartate carboxyls and is thought to take part in the catalytic mechanism (Pearl and Blundell 1984). Interestingly, these structures represent some of the largest  $\beta$ -strand structures observed in globular proteins (Claverie-Martin and Vega-Hernandez 2007). The majority of aspartic proteases are also known to have at least one flap made up of a  $\beta$ -hairpin that completes their active site (Madala et al. 2010). The flap region can be visualized in Figure 2.2 is highlighted in green. The flaps serve as a mechanism that upon closing, squeezes all the components into the correct geometry and holds the substrate in place enabling the catalytic process to begin. Well-known examples of aspartic proteases include rennet, cathepsin D, cathepsin E and pepsin. The Protein Data Bank (PDB) and MEROPS database classify eight subfamilies within the aspartic proteases with the sequence Asp- Thr(Ser)-Gly in their active site. Subfamilies differ according to the position of their catalytic site, the specific residues in their active site, the number of disulphide bridges present within the structure and optimal pH at which the enzyme functions (Cascella et al. 2005, Rawlings et al. 2009, Rawlings and Bateman 2009).

In a catalytic mechanism proposed by Veerapandian et al. (1992) which was based on the X-ray structure of a difluoro ketone inhibitor bound to endothiapepsin, these enzymes perform their action through general acid-base catalysis where the one aspartic residue (Asp32) acts as a base, accepting a proton, while the other (Asp215) acts as an acid, donating a proton. In other terms, the former residue has a relatively low pKa value and the latter a relatively high pKa value. Figure 2a, b illustrates the three-dimensional structure of a typical aspartic protease and details the molecular mechanism of action (C) as proposed by Veerapandian et al. (1992). Following exposure to low pH, cleavage events lead to conformational rearrangement. Firstly, a water molecule is bound to the two aspartic residues through hydrogen bonds and acts as a nucleophile that attacks the carbonyl carbon of the peptide scissile bond. The aspartic residue that acts as a general base removes one proton from the water molecule which is followed by a nucleophilic attack of the water molecule to the carbonyl carbon of the substrate scissile bond. At the same time, the other aspartic acid residue, acting as a general acid, donates a proton to the carbonyl oxygen atom of the peptide scissile bond. This leads to the formation of a tetrahedral intermediate. Thus, the aspartic residue acting as a base is hydrogen bonded to the attacking oxygen atom, while the hydrogen remaining on that oxygen is hydrogen bonded to the oxygen of the aspartic residue acting as an acid. During the final stages, a reversal of the configuration occurs around the nitrogen atom of the scissile bond of the substrate with the transfer of a hydrogen atom from the aspartic acid residue acting as a base to the nitrogen atom. In parallel, a proton is transferred from the oxygen atom of aspartic acid acting as an acid to the carbonyl oxygen on the peptide bond

being cleaved. This leads to the C-N bond breaking and releasing the two peptide products. Consequently, the aspartic acid that acted as a base is negatively charged at this stage and is therefore ready for the next round of catalysis (Coates et al. 2001, Dunn 2002).

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



**Figure 2.2:** Three-dimensional structure and mechanism of action of a typical aspartic protease. Secreted aspartic proteinase (SAPT, Accession number: 1j71) from *Candida tropicalis* (Symersky et al. 1997) was used to construct these pictures as visualized through Swiss-PDBViewer (v4.0.4). A: Representation of the structural elements: active site (in red), disulphide bounds (in yellow) and flap

region (in green). B: Close-up of active-site cleft. C: Catalytic mechanism as represented by Coates et al. (2001) according to a model proposed by Veerapandian et al. (1992).

The aspartic proteases are typically inhibited by pepstatin, a hexapeptide containing the rare amino acid statine. This molecule, which was originally isolated from various species of *Actinomyces*, has the remarkable ability to inhibit pepsin at picomolar concentrations (Umezawa et al. 1970, Marcinišzyn et al. 1976). There have however been reports of pepstatin sensitive acid proteases isolated from bacteria including *Xanthomonas* sp., *Pseudomonas* sp., *Bacillus* sp. (Oda et al. 1987, Prescott et al. 1995) and more recently from *Thermoplasma volcanium* (Kocabiyik and Ozel 2007). Rao et al. (1998) reported that aspartic proteases are also sensitive to diazoketone compounds such as 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN) in the presence of copper. The pepstatin-sensitive aspartic proteases are divided into two families: the retroviral and eukaryotic pepsin-like-type proteases. The retroviral types consist out of  $\beta$ -homodimers possessing aspartic residues located within the two loops at the monomer interface with two  $\beta$ -hairpins covering the active site (Sielecki et al. 1991). The eukaryotic pepsin-like protease has a tertiary structure consisting of two approximately symmetrical lobes ( $\alpha/\beta$  monomers) with each lobe carrying an aspartic acid residue in order to form the catalytic site. In the N-terminal domain, the characteristic sequence Asp32-Thr-Gly-Ser can be found with a corresponding Asp215-Thr-Gly-Ser/Thr in the C-terminal domain (De Viragh et al. 1993). Because of their twofold symmetry, it is the general consensus that these domains possibly arose through ancestral gene duplication. A flap made of a  $\beta$ -hairpin covers the catalytic site constituting the active-site cleft. This cleft is located perpendicular to the largest diameter of the molecule and can accommodate seven to eight amino acid residues, equally divided on both sides of the catalytic aspartic residues (Szecsi 1992, Dunn 2002). The number and position of disulphide bonds throughout the protein have been suggested to have a strong impact on the native state stability of the enzyme (Casella et al. 2005, Friedman and Caflich 2010). Members of the aspartic proteases family generally have one to three disulphide bridges that are located at the position between amino acids 251 and 286. This position is conserved across all members of the family (Machalinski et al. 2006). The disulphide bonds play an important role in the folding and stability of the protein and can be visualized in Figure 2.2, highlighted in yellow. In general, most aspartic proteases from microbial origin exhibit a broad-based specificity towards regions in the peptides that contain six hydrophobic residues at specific substrate positions (Dash et al. 2003).

## 2.3 Proteases in industry

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Some of the earlier applications of proteolytic enzymes found their use as milk-clotting agents for the manufacturing of cheese. These were probably first indirectly discovered when animal skins and inflated organs were used as storage containers for a range of foodstuffs. For instance, when milk is stored in the stomach of calves, it results in the formation of curd and whey because of the rennet present in the stomach (which contains several enzymes including chymosin). In Asian countries, proteases were used in the early production of natto, which is produced through the fermentation of soy beans with *Bacillus* species. Proteases involved in this process are important for the development of the main flavours associated with natto through the hydrolysis of the soy bean proteins (Ward et al. 2009, Borah et al. 2012). The involvement of proteases in the life cycle of many pathogens makes them important to the pharmaceutical and medical industry. Inhibition of various proteases has also become a valuable approach for studying neurodegenerative diseases, infections and various parasitic diseases (Rao et al. 1998). The most essential property of protease action resides in their ability to control and limit cleavage to intended substrates without degradation of functional proteins. Moreover, 2 % of functional genes found in the human genome encode proteolytic enzymes, thus they have become important therapeutic targets and are also used in diagnostics (Craik et al. 2011).

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

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

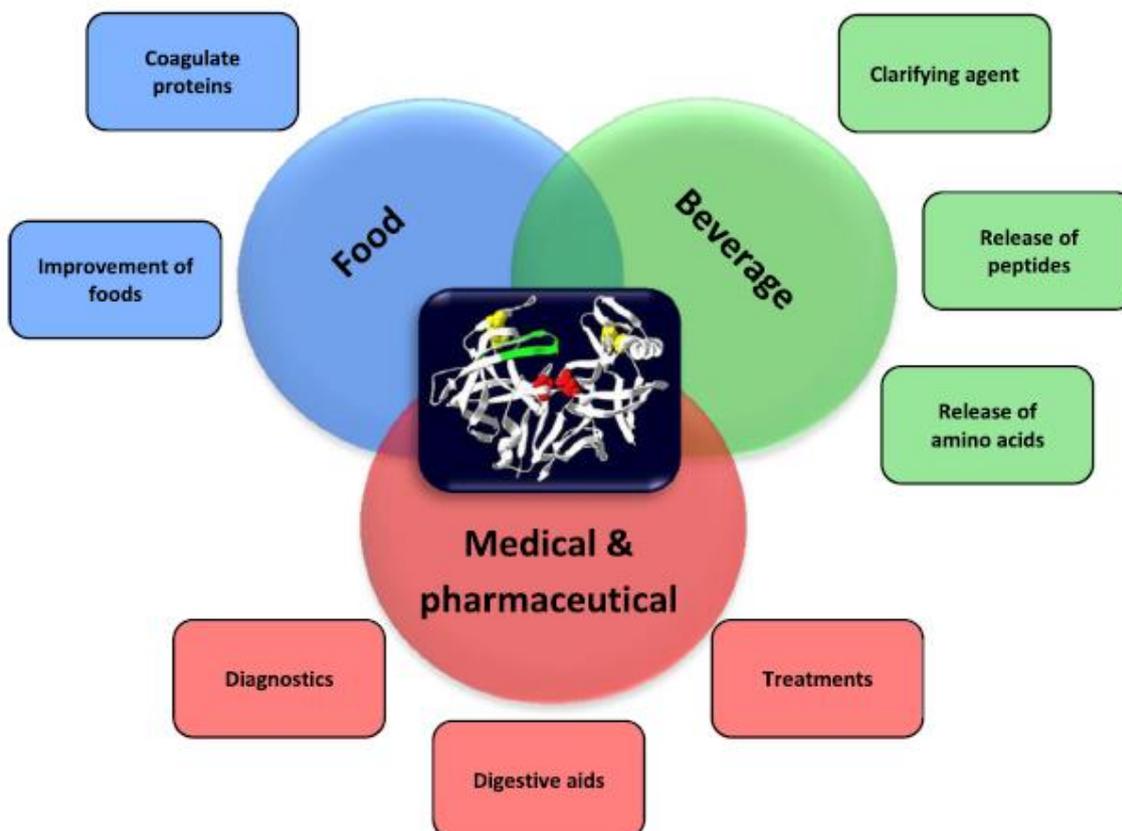
Alkaline proteases are active at basic pH range and make up the largest share of the enzyme market because of their use in household detergents. Most of the proteases used in this industry are alkaline or neutral proteases from *Bacillus* species. Some of the most important to the detergent industry are the serine alkaline proteases. Highly alkaline detergents use proteases from alkalophilic species such as *Bacillus halodurans* and *Bacillus clausii*, whereas proteases from *Bacillus licheniformis* are used in low-pH detergents. Three main product categories exist: (1) the low pH (7.5–9.0), low ionic strength liquid detergents containing no bleach, (2) the high pH (9.5–10.5), high ionic strength powders which contains bleach, and finally (3) the high pH (9.5–10.5) compact powders that contain sodium sulphate (Ward et al. 2009). The use of alkaline/neutral proteases has also received much attention in terms of replacing of harsh or harmful chemicals.

Alkaline proteases are also used in the leather industry. The major components of leather are proteins, including elastin, keratin and collagen. The principal steps in the processing of leather include soaking, dehairing, bating and tanning. The purpose of the soaking step is to swell the hide, and this is usually achieved by use of an alkaline reagent. Conventional methods for dehairing include treatment with extremely alkaline chemicals followed by treatment with hydrogen sulphate. This solubilizes and removes the proteins from the hair root. These conventional methods used in the leather industry thus involve the use of harsh chemicals which creates safety risks, disposal problems and chemical pollution (Khan 2013). Collagen exists in hides and skin in association with various globular proteins such as albumin, globulin, mucoids and fibrous proteins such as elastin, keratin and reticulin. The extent to which the non-collagenous constituents are removed determines the characteristics of the final leather such as durability and softness. The success of detergent enzymes has led to their being used in a number of other applications including pest control (Kim et al. 1999), degumming of silk (Kanehisa 2000, Puri 2001), isolation of nucleic acid (Kwon et al. 1994), lens cleaning (Nakagawa 1994), delignification of hemp (Dorado et al. 2001), cleaning of surgical instruments (Gupta et al. 2002), production of peptides (Cheng et al. 1995) and silver recovery from X-ray films (Fujiwara et al. 1991). The different industrial applications of alkaline proteases have been recently reviewed (Anwar and Saleemuddin 1998, Horikoshi 1999, Gupta et al. 2002, Saeki et al. 2007, Fujinami and Fujisawa 2010, Li et al. 2013, Mienda et al. 2014) and will therefore not be detailed further in this review.

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

## 2.4 Applications of microbial acid proteases

Acid proteases of microbial origin are mostly found in three industries: food, beverage and pharmaceutical. In each of these industries, they are used for a variety of purposes (Figure 2.3) that will be discussed further in the following paragraphs.



**Figure 2.3:** Summary of the current and potential uses of aspartic proteases in industry. The picture in the middle represents a typical aspartic protease (SAPT from *Candida tropicalis*) as visualized through SwissPdbViewer (v 4.0.4). The structural elements are represented as indicated in Figure 2.2.

### 2.4.1 Food industry

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

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

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

#### **2.4.2 Medical and pharmaceutical industry**

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

#### **2.4.3 Beverage industry**

Most industrially processed fruit-based beverages are clarified in order to prevent haze and turbidity. In the making of fruit juices and certain alcoholic beverages, acid proteases from *A. saitoi* (aspergillopepsin I) are used to degrade the proteins that cause turbidity (Sumantha et al. 2006). In the fermentation of sake, an alcoholic beverage of Japanese origin, acid proteases from *A. oryzae* determine the taste of the final product because of the manner in which they hydrolyse the proteins from the steamed rice in order to liberate peptides and amino acids (Shindo et al. 1998). Addition of fungal proteolytic enzymes from *A. niger* to kiwi

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

In the brewing industry, acid proteases have also been investigated as tools to degrade proteins that can form haze during storage. Haze formation can be due to glucan from modified malt, dead bacteria from malt, oxalate from calcium deficient worts, residual starch, carbohydrates and proteins from autolyzed yeasts (Steiner et al. 2010). Two forms of haze occur: chill haze and age-related haze (sometimes referred to as permanent haze). Chill haze, also known as cold break haze, forms at 0°C when polypeptides and polyphenols are non-covalently bound. Chill haze, also known as cold break haze, forms at 0°C when polypeptides and polyphenols are non-covalently bound. Age-related haze is initially formed in the same manner, but strong covalent bonds are formed during storage leading to insoluble complexes. Unlike in chill haze, the complexes formed over time cannot dissolve upon heating (Siebert et al. 1996, Steiner et al. 2011). In a study by Lopez and Edens (2005), it was found that addition of proline-specific proteases from *A. niger* effectively prevented chill-haze formation in beer, suggesting that the hydrolysis of proline-rich proteins resulted in a peptide fraction that is unable to interact with the polyphenols. Haze particles can show different appearances and have been classified into three main categories according to Glenister (1975). The first encompasses native particles which originate from beer by coagulation and/or precipitation. The second includes process particles originating from materials added during the brewing process. The last category comprises foreign particles which can enter into contact with beer as accidental contaminants (Glenister 1975, Bamforth 1999, Steiner et al. 2010).

Similarly to the brewing industry, protein haze is also a very challenging problem during the production of white wine. Like in beer, the presence of haze is usually perceived as

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

Slow denaturation of these proteins is thought to cause protein aggregation and flocculation eventually resulting in the appearance of haze. This is possibly due to unsuitable transport and storage conditions (Batista et al. 2009). It is generally accepted that the higher the total protein content of a wine, the higher tendency it has to become hazy (Mesquita et al. 2001). Thus for several years, studies on haze formation have focused on the proteins themselves. However, despite significant advances, the molecular mechanism of protein haze formation is still not fully understood. Initially, it was thought that instability solely related to protein content (Anelli 1977, Somers and Ziemelis 1973), but studies have shown that the potential of a wine to form haze is not predictable from its protein concentration alone (Bayly and Berg 1967, Moretti and Berg 1965). Wine composition (pH, ethanol content, ionic strength, sulphate ions polyphenols and polysaccharides) and temperature have all been shown to play a role (Waters et al. 1995, Dupin et al. 2000, Mesquita et al. 2001, Carvalho et al. 2006, Pocock et al. 2007, Dufrechou et al. 2010, Marangon et al. 2011b, c), but their actual involvement, possibly in combination with each other, in protein aggregation and flocculation remains unclear. Indeed, there are only a few studies investigating the impact that pH and ionic strength (salts) have on protein stability relating to haze formation, despite their strong influence (Sarmiento et al. 2000a, Dufrechou et al. 2010). The overall effect that the ionic strength of a solution can have on proteins can be both stabilizing and destabilizing, depending on the charge distribution within the protein (Von Hippel and Wong 1964, Kohn et al. 1997,

Record et al. 1998). Protein-protein interactions are favoured when the net charge of the molecule is reduced. Thus, at conditions with high ionic strengths or at pH values close to the isoelectric point of the protein, interaction is favoured (Boye et al. 1995, Chi et al. 2003). Normal wine ionic strength ranges between 10 and 100 mM (Cabanis et al. 1998). At these values, it is expected to strongly influence electrostatic interactions (Israelachvili 1991, van Oss 1994). Furthermore, the specific ion type that is present may also influence interaction following a different mechanism. Pocock et al. (2007) indicated that a sulphate anion is an essential factor required for haze formation.

Moreover, in a recent study performed by Gazzola et al. (2012), the authors examined the aggregation behaviour of five purified wine proteins and measured the size and concentration of individual particles formed in the presence and absence of phenolics and/or polysaccharides using scanning ion occlusion sensing (SIOS). The study revealed that chitinases are indeed the proteins most prone to cause haze formation and that polysaccharides and phenolics present in wine do not have a significant effect on their aggregation behaviour. Furthermore, it was observed that the TLP isoforms tested varied in their interaction with the polysaccharides and phenolics present and thus also their susceptibility to cause haze formation. Phenolic compounds present in wine have been associated with haze formation as they interact with haze-forming proteins (Somers and Ziemelis 1973, Yokotsuka et al. 1983) and have been found to be present in haze (natural and heat induced) studied in several white wines (Waters et al. 1995, Esteruelas et al. 2011). Some studies suggest that the major mode of interaction between the phenolic compounds and the proteins present is hydrophobic, particularly when the proteins are in their unfolded state (Oh et al. 1980, Siebert et al. 1996, Marangon et al. 2010). In literature, some contradicting results have been reported about the stabilizing effect of polysaccharides. Mesquita et al. (2001) found that polysaccharides could negatively affect wine stability, whereas other authors (Waters et al. 1994b, Brown et al. 2007, Pellerin et al. 1994) found that some polysaccharides can have a stabilizing effect towards heat-induced protein haze. However, the polysaccharide concentration used during these studies was much greater than that found in wine (Doco et al. 2003). Studies involving interactions between wine molecules and proteins responsible for haze formation require the characterization of the size and concentration of the protein aggregates responsible for haze formation (Gazzola et al. 2012). Dynamic light scattering (DLS) techniques have mainly been used to study protein aggregation (Dufrechou et al. 2010, 2012, Marangon et al. 2011b), whereas studies on the particle sizes use methods involving gel electrophoresis (Alberts et al. 1994), electron microscopy (Ito et al. 2004) and disc centrifugation (Bondoc and Fitzpatrick 1998).

Currently, the most effective tool that winemakers have to eliminate haze is treatment with bentonite. This montmorillonite clay has a net negative charge and serves as a cation

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

Preliminary reports have shown that the use of proteases in wine is an efficient way of reducing protein haze formation without being detrimental to wine quality (Lagace and Bisson 1990, Pocock et al. 2003), but heating needs to be coupled with the treatment in order to denature the heat-unstable proteins prior to their degradation that degrade them. This would be in agreement with the study claiming that, without prior denaturation, haze-forming proteins are resistant to proteolysis (Conterno and Delfini 1994). In 2003, Pocock et al. indeed demonstrated that combining heat treatment and proteolytic enzymes reduced the requirement for bentonite by 50–70 % without affecting the sensory profile of final wine. Treatment consisted of exposing the wine for 1 min at 90 °C and adding Trenolin® blank (Erbslöh, Geisenheim, Germany) which is a commercially available aspergillopepsin. The idea behind this dual treatment is that exposure to heat denatures the haze-forming proteins allowing access for proteolytic enzymes to hydrolyse the proteins into smaller peptides. Nevertheless, despite these encouraging results, it was concluded that a more efficient protease was needed. Recently in 2012, Marangon et al. investigated the use of an acid protease isolated from *A. niger var. macrosporus* (Koaze et al. 1964), namely aspergillopepsin I and II (AGP), together with flash pasteurization to degrade haze proteins in white wine. The

sole addition of AGP directly to the fermentation resulted in a 20 % reduction in proteins. However, maximum effects were obtained when the juice was treated by combining AGP addition with flash pasteurization (75 °C for 1 min). It was found that, under the conditions tested, the chitinases and TLPs were almost completely degraded in Chardonnay and Sauvignon blanc wines thereby eliminating the need for bentonite. In a study performed by Reid et al. (2012), two extracellular aspartic protease-encoding genes were retrieved and sequenced. The two genes, MpAPr1 and CaAPr1, were isolated from two separate yeast species of oenological origin, *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. Furthermore, MpAPr1 production was shown to be constitutive in the native host, and secretion of this enzyme was confirmed in the presence of bovine serum albumin (BSA), casein and grape juice proteins. Very recently, van Sluyter et al. (2013) also demonstrated that an acid protease from *Botrytis cinerea*, named BcAP8, was able to effectively reduce haze at winemaking temperatures and to remain active after fermentation was completed. Although it was found that the enzyme was not able to remove all the PR proteins, showing more activity against chitinases than the TLPs, it was shown that it could still benefit winemakers by reducing bentonite requirements. The success of previously mentioned reports encourages further investigations into proteases of wine-related non-*Saccharomyces* yeasts followed by the assessment of their potential use in wine.

Proteins present in wine have been found to account for up to 2% of the total nitrogen content (Feuillat 2005). Apart from preventing protein haze, proteases present and active during the winemaking process may potentially increase the assimilable nitrogen necessary for microbial growth as well as amino acids, purine and pyrimidine syntheses (Bell and Henschke 2005) during fermentations by breaking down these proteins. However, the main wine yeast *Saccharomyces cerevisiae* is unable to utilize proteins as a nitrogen source as it is not known to actively secrete aspartic proteases. Only one study reported recently on the occurrence of an actively secreted acid protease in *S. cerevisiae* (Younes et al. 2011), but this phenomenon has only been noted in one strain. However, some of *S. cerevisiae*'s intracellular aspartic proteases (e.g. Pep4) have been reported to occur in the extracellular matrix (i.e. the wine) upon autolysis, and the consequent release of amino acids and peptides via the activity of the liberated Pep4 was hypothesized to impact on malolactic fermentation carried out by lactic acid bacteria (Guilloux-Benatier et al. 2006). The main lactic acid bacterium responsible for malolactic fermentation, *Oenococcus oeni*, has also been shown to secrete an aspartic protease (Farías and Manca de Nadra 2000) and an uncharacterized acid protease (Folio et al. 2008) which could potentially play a role in the bacterial growth and release of flavour compounds. Furthermore, insufficient nitrogen sources may lead to fermentations that become slow or stop, which are referred to as sluggish and stuck, respectively. A shortage in sulphur-containing amino acids also leads to the production of hydrogen sulphide which is

known to have a negative effect on sensory attributes. Proteases, either naturally secreted by indigenous microorganisms or added from external sources, could prevent the production of this off flavour compound.

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

## 2.5 Conclusion and future outlooks

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

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

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

proteases and their use in industrial applications is an exciting field of research, and various new findings are to be expected, especially in the beverage industries.

## **2.6 A brief review of the literature published since 2014 on haze forming proteins in wine and the application of aspartic proteases in the wine industry**

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Since the publication of the previous paragraphs as a review article in 2014, some, albeit limited, developments and advances have been made in the pursuit of understanding protein haze formation in wine and the role that aspartic proteases might play in circumventing this issue. Research in this area has mainly focused on developing methods for grape protein purification, quantification and identification in an effort to better understand the mechanisms of haze formation. Significant attention has also been paid to improving bentonite efficiency and finding alternative stabilisation strategies as well as predicting haze potential. Furthermore, several studies have also directed their attention to evaluating novel aspartic proteases from various organisms including non-*Saccharomyces* yeast species for winemaking purposes. This paragraph will summarise these recent developments and findings.

In 2014, Marangon et al. performed an in-depth investigation into the molecular structure of three TLPs (termed I/4L5H, H2/4MBT and F2/4JRU) isolated from Sauvignon Blanc juice. The crystal structure along with some physico-chemical parameters were determined and interestingly, it was found that the different isoforms display distinct aggregation behaviours and therefore have different hazing potentials. Furthermore, despite belonging to different classes, the proteins had very similar structures and while protein I/4LH5 was heat stable and did not form haze, protein F2/4JRU did. The differences in properties were attributable to the composition of the flanking regions and the difference in conformation of a single loop (located between  $\beta$ -stands 9 and 10). Information gathered about the structure of these proteins could be helpful in making the search for proteases more targeted.

Following the discoveries made over the past decade, van Sluyter et al. compiled in 2015 all the experimental data in an extensive review on the mechanisms of protein haze formation and prevention thereof in wine. The review suggests and describes a new three-stage mechanism model as opposed to the previously accepted two-stage model. The new model includes a protein unfolding step, followed by protein aggregation and finally cross-linking of aggregates that form what is known as haze. Emphasis is also placed on other wine factors and components such as pH, presence of sulphates and polyphenols that can affect protein aggregation in the second and third stage. This is a significant step towards the understanding and prediction of haze formation in wine that should allow for the development of more targeted techniques in the quest to prevent haze formation in wine. The investigation

into and discovery of novel fining agents is also reviewed. These include seaweed polysaccharides (Cabello-Pasini et al. 2005, Marangon et al. 2013, Marangon et al. 2012), chitin (Vincenzi et al. 2005, Chagas et al. 2012), zirconium dioxide (Marangon et al. 2011, Lucchetta et al. 2013). However, all of these fining agents either remove favourable compounds or require high dosages thereby limiting their commercial viability and making proteases still one of the most viable options for haze removal and alternatives to bentonite fining. The combination treatment developed by Marangon et al. (2014) involving heat treatment in the presence of a heat tolerant protease prior to fermentation has proved to be effective on an industrial scale (Robinson et al. 2012). Furthermore, the use of Aspergilloglutamic peptidase (AGP, formerly known as Aspergillopepsin II), also commercially known as Proctase, has recently been approved for use in Australia and New-Zealand for winemaking.

Based on the promising success of proteases to eliminate haze, the search for novel aspartic proteases for applications in winemaking has intensified and expanded from mainly fungal sources to include plant and yeast sources. Benucci et al. (2014) investigated the activity of free and immobilised stem bromelain (from pineapples) in unfinned white wines. In order to evaluate activity, a synthetic tripeptide chromogenic substrate was added to the wine after which samples were incubated at 20°C for 24 h and the hazing potential determined via the heat test. Overall it was found that an amount of 10 g l<sup>-1</sup> enzyme (stem bromelian immobilised on chitosan beads) was able to reduce wine hazing potential by 70%. The same research group studied the effect of potential inhibitors in a follow-up paper (Esti et al. 2015). The activity of the enzyme against a synthetic tripeptide was evaluated in a wine-like acidic medium (pH 3.2) in presence of 0, 12 and 18% (w/v) ethanol. Ethanol only had a significant inhibitory effect on enzyme activity at a concentration of 18% (w/v). Furthermore, activity was also evaluated in the presence of a wine like acidic medium (pH 3.2) with ethanol constant at 12% (v/v) with the addition of free SO<sub>2</sub>, skin tannins and seed tannins, separately. Briefly, free SO<sub>2</sub> had a significant inhibitory effect on enzyme activity at the lowest concentration tested (10 mg l<sup>-1</sup> free SO<sub>2</sub>) and both sources of tannins exerted similar negative effects on enzyme activity. Using similar techniques, the same research group (Benucci et al. 2015) also further investigated the effect of potential inhibitors found in wine on papain from papaya latex. Similar trends could be identified and the authors concluded that strongest inhibition was exerted by free SO<sub>2</sub> which acted as a mixed type inhibitor, similar to the grape skin and seed tannins used. Although interesting results have been generated, further testing in real winemaking scenarios are required to further elucidate the actual benefit and activity of these enzymes during or after winemaking. Indeed, because of the complexity of protein haze formation in wine, the actual impact on haze forming proteins remains to be investigated.

Several yeast species naturally occurring in the vineyard or in grape juice such as non-*Saccharomyces* species have also been screened for acid protease production. Lopez et al. (2015) screened twenty-six *Hanseniaspora* spp. strains, yeasts commonly occurring in grape juice, for enzymes with potential applications in winemaking. The authors screened for protease activity by means of plate assays using casein as a substrate. From the collection of strains tested, only one (*H. guilliermondii* HG2) displayed moderate activity and fifteen displayed weak activity against casein after 7 days at 28°C. In 2016, Albertin et al. investigated thirty strains of *H. uvarum* and ten strains of *Hanseniaspora* spp. for extracellular enzyme activity that could be of oenological interest. Protease activity was also screened using plate assay and a similar substrate for 72 h at 30°C. Of all the strains tested, only three *H. uvarum* strains showed weak protease activity with strain YB 783 displaying the largest halo of 4 mm. These studies reveal that extracellular acid protease activity is strain-specific and usually weak in this yeast genus. Furthermore, screening for protease activity via plate assays has some drawbacks in that it is tedious to prepare media and it sometimes takes several days before activity can be visualized and measured. A new method was proposed for measuring protease activity during fermentation by which azocasein is added directly to the grape juice and degradation thereof measured using spectroscopy (Chasseriaud et al. 2015). Such methods could potentially be used to measure extracellular protease activity more quantitatively and directly in the medium of choice for screening purposes.

A large number of authors have actually screened a variety of non-*Saccharomyces* yeasts for protease activity. Macias and Mateo (2015) summarised the potential enzymatic contribution of non-*Saccharomyces* yeasts in wine production. The authors briefly reviewed the use of aspartic proteases as an alternative to bentonite fining. Furthermore, the potential of aspartic protease to increase the assimilable nitrogen content of the surrounding media through their activity is briefly mentioned and it is proposed that this action might be beneficial in preventing sluggish fermentations and lead to an increase production of esters higher alcohols and volatile fatty acids

The latter hypothesis was recently investigated in jujube wine. Indeed, Zhang et al. (2016) performed a study in which the chemical composition of jujube wines treated with protease (acid protease from Imperial Jade Biotechnology Co., Ltd, China - acid proteases derived from fermentation and refinement of *Aspergillus niger*) was investigated. Free amino acid content, in particular alanine, threonine, valine, proline, phenylalanine and aspartate, was significantly enriched in wines that were protease-treated. A significant increase in the production of fusel alcohols, namely n-propanol, isobutanol, isoamyl alcohol, phenylethyl alcohol and 3-methoxythiopropanol resulting from the degradation of aspartate/threonine, valine, leucine, phenylalanine and methionine through the Ehrlich pathway, respectively, could also be observed. A further significant increase in 3-ethoxy-1-propanol could also be

observed. Sensory evaluation performed in this study showed that protease treatment could increase/improve the intensity and complexity of wine aroma which the authors ascribed to the higher levels of ethyl esters and fusel alcohols observed. The authors further hypothesised that this outcome was due to the increase in assimilable nitrogen following the break-down of proteins by the supplemented protease which could consequently lead to the increased production of volatile compounds.

In a recent review, Mandujano-González et al. (2016) summarised extracellular fungal aspartic proteases (SAP) with a specific focus on their secretion pathways, inhibitors and regulation of the genes encoding these enzymes. Chemical and kinetic mechanisms of catalysis are also described. Application of these proteases is briefly discussed with a specific focus on their application in the dairy industry, especially for cheese making. Although mentioned, very little is discussed on the use of aspartic proteases to remove haze formation and reduce proteins that cause turbidity in juices and wine

In conclusion, much has been discovered about the factors present and mechanisms involved in protein haze formation in white wine. A new model for haze formation has been proposed providing a better understanding of this phenomenon, however the exact conditions and mechanisms whereby proteins unfold and form cross-linking is yet to be elucidated. The use of protease for the degradation of haze-forming proteins as an alternative to bentonite fining has also become more sought after. The search has extended to various organisms including non-*Saccharomyces* species from wine. However, few authors studied these enzymes in-depth and information on their action against grape proteins remains scarce. Focus is also shifting from the use of these enzymes with the sole purpose to prevent haze (in an effort to reduce or replace bentonite treatment) toward additional benefits including the release of yeast assimilable nitrogen and its positive consequences such as the release/production of aroma compounds, prevention of sluggish and/or stuck fermentations.

# Chapter 3

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## Materials and Methods

## Chapter 3 - Materials and Methods

### 3.1 Microbial strains, plasmids and culture conditions

Forty-three strains of *Metschnikowia* spp. were used in this study and are listed in Table 3.1. The main strain studied was the grape derived yeast *Metschnikowia pulcherrima* IWBT Y1123. It forms part of the Institute for Wine Biotechnology (IWBT) culture collection (Stellenbosch University, South Africa) and was deposited in the Centre de Ressources Biologiques Œnologiques, Villenave d'Ornon, France under the reference CRBO L1601. It was maintained at -80°C in 30% glycerol prior to experimental use.

*Escherichia coli* DH5 $\alpha$  [F- $\phi$ 80/*lacZ* $\Delta$ M15  $\Delta$  (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK-, mK+) *phoA supE44*  $\lambda$ - *thi-1 gyrA96 relA1*] (GIBCO-Invitrogen Life Technologies, Mowbray, South Africa) was employed to propagate the plasmids carrying the cloned genes and *Komagataella pastoris* X33 [wild type, selection for zeocin resistant vectors] (Invitrogen, Carlsbad, CA) as the host strain for gene expression. Plasmids pGEM $\text{\textcircled{R}}$ -T Easy (Promega, Whitehead Scientific, Cape Town, South Africa) and pGAPZ $\alpha$ A (Invitrogen) were used as cloning and expression vectors, respectively. *Saccharomyces cerevisiae* VIN13 [commercial wine yeast] (Anchor Yeast, Cape Town, South Africa) was used throughout the fermentation trials. Yeast strains were grown at 30°C on yeast extract peptone dextrose (YPD) agar (Biolab diagnostics, Wadenville, South Africa). Prior to experimental use, yeast strains were freshly cultured in YPD broth (Biolab diagnostics) at 30°C. *E. coli* was grown at 37°C on Luria Bertani (LB) agar (Biolab diagnostics) and freshly cultured before its use in downstream experiments. Chemicals and antibiotics were supplemented at the following concentrations where appropriate: 100  $\mu$ g/ml ampicillin (Sigma-Aldrich, Aston Manor, South Africa), 80  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal; Sigma-Aldrich), 25 – 1000  $\mu$ g/ml zeocin (Invitrogen).

**Table 3.1:** Strains of *Metschnikowia* spp. used in this study and their protease activity. All strains were from the microbial culture collection of the Institute for Wine Biotechnology, Stellenbosch University, South Africa with the exception of the CBS 5833 type strain that is deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. SA: South Africa. CA: California.

Species	Strain	Source, origin and year of isolation	Protease activity*
<i>M. chrysoperlae</i>	†Y955	Cabernet sauvignon grape juice, Stellenbosch, SA (2014)	+
<i>M. fructicola</i>	Y1005	Chardonnay grape juice, Paarl, SA (2009)	+
	†Y1423	Chardonnay grape juice, Paarl, SA (2009)	+
<i>M. pulcherrima</i>	Y932	Sauvignon blanc grapes, Elgin, SA (2012)	+
	Y956	Cabernet sauvignon grape juice, Stellenbosch, SA (2014)	+
	Y1063	Cabernet Sauvignon grape juice, Paarl, SA (2009)	+
	†Y1065	Cabernet Sauvignon grape juice, Paarl, SA (2009)	+
	Y1067	Shiraz grape juice, Paarl, SA (2009)	+
	Y1072	Chardonnay grape juice, Paarl, SA (2009)	+
	Y1075	Chardonnay grape juice, Sir Lowry's Pass, SA (2009)	+
	Y1093	Tinta barroca grape juice, SA (2009)	+
	Y1094	Tinta barroca grape juice, SA (2009)	+
	Y1102	Shiraz grape juice, Robertson, SA (2009)	+
	Y1103	Shiraz grape juice, Robertson, SA (2009)	++
	†Y1108	Shiraz grape juice, Robertson, SA (2009)	+
	Y1112	Irsai olivér grape juice, SA (2009)	+
	†Y1113	Irsai olivér grape juice, SA (2009)	++
	†Y1114	Sauvignon blanc and Chardonnay grape juice blend, Somerset West, SA (2009)	++
	Y1115	Sauvignon blanc and Chardonnay grape juice blend, Somerset West, SA (2009)	+
	Y1118	Sauvignon blanc and Chardonnay grape juice blend, Somerset West, SA (2009)	++
	Y1120	Sauvignon blanc and Chardonnay grape juice blend, Somerset West, SA (2009)	+
	†Y1123**	Sauvignon blanc grape juice, Somerset West, SA (2009)	+++
	†Y1124	Sauvignon blanc grape juice, Somerset West, SA (2009)	+
	Y1125	Sauvignon blanc grape juice, Somerset West, SA (2009)	+
	†Y1174	Shiraz grape juice, Paarl, SA (2009)	+
†Y1176	Shiraz grape juice, Paarl, SA (2009)	+	
Y1183	Shiraz grape juice, Robertson, SA (2009)	+	
Y1188	Shiraz grape juice, Robertson, SA (2009)	+	
Y1191	Shiraz grape juice, Robertson, SA (2009)	+	
†Y1195	Sauvignon blanc grape juice, Somerset West, SA (2009)	+	
†Y1208	Chardonnay grape juice, Sir Lowry's pass, SA (2009)	+	
†Y1213	Chardonnay grape juice, Sir Lowry's pass, SA (2009)	+	
†Y1217	Chardonnay grape juice, Sir Lowry's pass, SA (2009)	+	

Y1270	Chardonnay grape juice, Somerset West, SA (2009)	+
Y1271	Chardonnay grape juice, Somerset West, SA (2009)	+
Y1325	Chardonnay grape juice, Somerset West, SA (2009)	+
Y1337	Grape juice, SA (2009)	+
Y1413	Unknown	+
Y1424	Sauvignon blanc grape juice, Somerset West, SA (2012)	+
†Y1425	Shiraz grape juice, Robertson, SA (2009)	+
†Y1430	Shiraz grape juice, Robertson, SA (2009)	+
†CBS 5833 <sup>T</sup>	Berries of <i>Vitis labrusca</i> (Concord grape), CA (1939)	+

\* As determined from skim plate assay through measurement of the clear zone (i.e. halo) around the colony:  
+++ : 7 mm (very strong activity), ++ : 2.5-5 mm (strong activity), + : 1 mm (detectable activity).

\*\* Also deposited in the Centre de Ressources Biologiques Œnologiques (Villenave d'Ornon, France) under the reference CRBO L1601

† Strains used to generate phylogenetic tree

## 3.2 DNA techniques

### 3.2.1 Genomic DNA extraction

Genomic DNA from yeast strains was isolated from overnight cultures grown at 30°C in 10 ml YPD broth (Biolab Diagnostics), using mechanical cell disruption and phenol-chloroform extraction as described in Current Protocols in Molecular Biology (2008) according to Hoffman and Winston (1987). Plasmid DNA was extracted from bacterial cultures using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA).

### 3.2.2 PCR amplification of *MpAPr1*

All primers were synthesised by Integrated DNA Technologies (IDT, Whitehead Scientific) and are listed in Table 3.2. All PCRs were performed using TaKaRa Ex Taq™ (TaKaRa, Shiga, Japan). PCR programmes were performed in an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA). Primers were designed according to the sequence of the *MpAPr1* gene (NCBI accession number: JQ677912).

For genetic screening purposes, primers MpAPr1-F and MpAPr1-R (Reid et al. 2012) were used to amplify the *MpAPr1* gene with the following PCR programme: 94°C for 2 min, 35 cycles of the denaturation at 94°C for 20s, annealing at 58°C for 20 s, elongation at 72°C for 30 s, followed by a final elongation step at 72°C for 7 min.

To facilitate cloning and production purposes, a new forward primer was designed on the *MpAPr1* sequence (MpAPr1Fwd) and the reverse primer was designed with an additional thrombin site (MpAPr1Rev) as shown in table 3.2. The PCR programme used was as follows: 94°C for 2 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 45 s, elongation at 72°C for 1 min and 30s, followed by a final elongation step at 72°C for 5 min.

All PCR products were resolved in 0.8% agarose gel using TAE buffer. Gels were stained with 0.2 µg/ml ethidium bromide (Sigma-Aldrich) and visualised through UV transillumination. The gels were photographed using a G: Box (Syngene, Cambridge, United Kingdom) with the software provided by the manufacturer (Genesnap Syngene, version 7.09).

**Table 3.2:** Primers used in this study

Primer	Sequence 5' - 3'*	Restriction site	Reference
MpAPr1 - F	GGATCCATGCAATTCCTCACTCTCTTTC	<i>Bam</i> HI	Reid et al. 2012
MpAPr1 - R	CTCGAGTTAAGCACTTATGATGTTTGACGA	<i>Xho</i> I	Reid et al. 2012
MpAPr1Fwd	GAATTCATGGCCATCCCTGGGC	<i>Eco</i> RI	This study
MpAPr1Rev	GCGGCCGCGCTGCGCGCGGCACCAGAGCACTTATGATGTTTGA CGA	<i>Not</i> I	This study

\*Underlined sequence indicates thrombin site and italicised sequences the restriction sites

### 3.2.3 Sequencing and sequence analysis

PCR amplicons were cloned with the pGEM®-T Easy vector system (promega) according to the manufacturer's specifications. After plasmid extraction, insertion was verified through restriction digest experiments and visualised on an agarose gel as described above. DNA strands (i.e. plasmids) were sequenced in an ABI 3130XL Genetic Analyzer at the Central Analytical Facility (Stellenbosch University) using the SP6 and T7 primers (Promega).

*In silico* transformation of nucleotide sequences as well as the determination of theoretical molecular weights and isoelectric points were performed through various online software of the ExPASy bioinformatics resource portal (<http://web.expasy.org/>). Amino acid sequences were analysed and aligned using the online software Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) after which a phylogenetic tree was generated on selected strains according to the Maximum Likelihood method using MEGA 7.0.18 (<http://www.megasoftware.net/>).

### 3.2.4 *MpApr1* cloning and heterologous expression in *Komagataella pastoris*

After amplification of *MpAPr1*, the gene was excised from an agarose gel and cloned into pGEM®-T Easy, followed by transformation into *E. coli* DH5α using the heat shock method. Positive transformants were selected on LB agar containing 100 µg/ml ampicillin and 80 µg/ml X-gal. After plasmid extraction, *MpAPr1* was excised by restriction digest with *Eco*RI and *Not*I

(Roche Diagnostics, Randburg, South Africa). Thereafter, the gene was again excised and isolated from an agarose gel and ligated into corresponding sites within the multiple cloning site of the pGAPZ $\alpha$ A vector using T4 DNA ligase (Promega). The resulting plasmid, hereinafter referred to as pGAPZ $\alpha$ A-MpAPr1, was transformed into *E. coli* DH5 $\alpha$  using the heat shock method. All DNA fragments were extracted from agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, Whitehead Scientific) according to the manufacturer's protocol. Positive transformants were randomly selected on low-salt LB agar containing 25  $\mu$ g/ml zeocin as specified by the pGAPZ $\alpha$ A vector manufacturer's protocol.

Single colonies were selected and grown in 5 ml low-salt LB broth containing 25  $\mu$ g/ml zeocin. After plasmid extraction and validation of the sequence by sequencing (Central Analytical Facility, Stellenbosch University), pGAPZ $\alpha$ A-MpAPr1 was transformed into *K. pastoris* X33 using electroporation according to the provider's protocol (Invitrogen). Positive transformants were randomly selected on YPD agar containing 1 M sorbitol (YPDS) supplemented with 100  $\mu$ g/ml zeocin.

Furthermore, positive transformants were serially cultivated on YPDS agar containing increasing amounts of zeocin until a final concentration of 1000  $\mu$ g/ml to select for multi-copy recombinants, as described in the manufacturer's manual. One positive transformant was randomly selected and cultured in YPD for 24 h. The culture was collected and stored in 15% glycerol at stored at -80°C prior to experimental use.

### **3.3 Protein expression and analysis**

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#### **3.3.1 Production of protein crude extract**

Cultures were plated on YPD agar and a single colony randomly selected and grown in 10 ml YPD broth for 24 h at 30°C and inoculated into 200 ml growth medium (1% yeast extract, 2% peptone, 4% glucose) at an optical density of 0.1 at 600 nm ( $OD_{600nm} = 0.1$ ) in a 500-ml baffled Erlenmeyer flask. Cultures were incubated at 20°C with vigorous shaking and after 72 h (Yegin and Fernandez-Lahore 2013) centrifuged at 2370 *g* for 5 min. The supernatant was collected and concentrated 10 times using an Amicon stirred cell Model 8200 (Millipore, Randburg, South Africa) fitted with a 30 kDa molecular weight cut off membrane filter (Millipore). The concentrated supernatant will be referred to as the crude extract hereinafter.

#### **3.3.2 Optimization of MpAPr1 expression in *Komagataella pastoris***

The same yeast culture as selected for above was inoculated into 10 ml YPD test tube(s) and incubated for 24 h at 30°C. Minimal expression medium (per litre: 5 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g CaCl<sub>2</sub>, 0.3 g MgCl<sub>2</sub>, 10 g peptone, 40 g glucose and 2 ml PTM trace salts as described in the Pichia Fermentation Process Guidelines, Invitrogen) was inoculated at an  $OD_{600nm} = 0.1$  in a 500-ml baffled Erlenmeyer flask and incubated with shaking at 145 rpm. In order to

determine the adequate environmental parameters for optimal expression, cultures were incubated at various temperature (20°C, 25°C and 30°C) and pH (4 and 6) values. Samples were taken at 24 h, 48 h and 72 h and the total protein concentration was determined as described below.

Activity assays were also performed using azocasein (Megazyme, Bray, Ireland) as a substrate (as described below) in order to calculate specific activity which was used to compare expression. Specific activity was expressed in units per milligramme of total protein. The supernatant was harvested by centrifugation at 2370 *g* and concentrated 10 times using a Amicon stirred cell Model 8200 (Millipore, Randburg, South Africa) fitted with a 10 kDa molecular weight cut off membrane filter (Millipore). The concentrated supernatant was stored at 4°C until further downstream purification experiments.

### **3.3.3 Purification of MpAPr1**

The MpAPr1 enzyme was purified from a concentrated crude extract harvested from cultures grown in minimal medium (as described above). Chromatographic experiments were performed using different equipment throughout. Immobilised metal affinity chromatography (IMAC) was performed using a BioLogic Duoflow™ Chromatographic System (Bio-Rad) connected to a Model 2110 fraction collector (Bio-Rad) and monitored using the software provided (BioLogic Duoflow).

Initial cation exchange chromatography was also performed on this system. Upon returning to South Africa the BioLogic LP™ Low-Pressure Chromatography System connected to a Model 2128 fraction collector (Bio-Rad) was used and purification monitored using LP Data View software. Purification was further optimised using the NGC™ Chromatography System (Bio-Rad) connected to a fraction collector (Bio-Rad) and monitored via the software provided. Final purification was performed on the ÄKTA Pure Chromatography System (GE Healthcare) connected to a Model 2128 fraction collector (Bio-Rad) and monitored using the software provided (Unicorn™ V 7.0.1).

#### **3.3.3.1 Immobilised metal affinity chromatography (IMAC)**

Purification was first attempted by means of immobilised metal affinity chromatography (IMAC) using a 1-ml HiTrap™ IMAC HP column (GE Healthcare Bio-sciences, Uppsala, Sweden) according to the manufacturer's specifications. During IMAC experiments, the system was set up in such a way that the sample was loaded using the buffer B line (represented by the black line in the figures below). Moreover, all samples and buffers were filtered through a 0.45-µM syringe filter prior to their use in downstream experiments. Initially, the supernatant from rich medium (SRM) was harvested through centrifugation and, after filtration through a 0.2-µm membrane, dialysed (three times) using Buffer 2 (20 mM sodium phosphate, 0.5 M NaCl, 20

mM imidazole, pH 7.5). This buffer was also used to wash the column after sample injection and before elution using Buffer 1 (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.5). Furthermore, were appropriate the column stripped and recharged with  $\text{CoCl}_2$  and  $\text{ZnCl}_2$ , separately. The flow rate throughout the experiment was 0.5 ml/min and all fractions were collected above  $\text{OD}_{280\text{nm}}$  of 0.1 in 1 ml aliquots.

### 3.3.3.2 Cation exchange chromatography

Following the repeated lack of success of IMAC, cation-exchange chromatography was used to purify MpAPr1 using the Bio-Rad DuoFlow System. Note that for cation exchange experiments, the system was setup in such a way that the sample (and the wash buffer) was applied using the buffer A line and elution was performed using the buffer B line (represented by a black line in the figure below). Moreover, all samples and buffers were filtered through a 0.45- $\mu\text{m}$  syringe filter prior to their use in purification. Initially the SRM was concentrated via ultrafiltration (using a 30-kDa cut-off membrane) while simultaneously buffer-exchanging with Buffer A (20 mM McIlvaine's buffer at pH 3.0). 20 ml sample was loaded onto a 1-ml HiTrap SP HP column after which elution was performed over a 30-ml gradient using Buffer B (20 mM McIlvaine's buffer at pH 3.0, 1 M NaCl). MpAPr1 was firstly purified from the supernatant obtained from rich medium (SRM) using five 1-ml HiTrap™ HP SP (GE Healthcare Biosciences) columns connected in series. The concentrated protein extract was adjusted to pH 3.0 after which 5 ml was injected into a previously equilibrated column. Columns were equilibrated with 25 ml Buffer A (20 mM McIlvaine's buffer at pH 3.0) prior to injection. After the sample was loaded, the column was washed with 30 ml Buffer A. Elution of proteins was performed over a linear gradient of 50 ml with Buffer B (20 mM McIlvaine's buffer at pH 3.0, 1 M NaCl). Fractions were collected above an  $\text{OD}_{280\text{nm}}$  of 0.1 in 1 ml aliquots. The flow rate throughout the purification process was 0.5 ml/min and all collected fractions were stored at 4°C.

After a minimal media was optimised (as described below) and the supernatant obtained (SMM-Op-30C) purification was attempted on the Bio-Rad DuoFlow System as described above. The apparatus was set up in such a way that the sample was loaded using the buffer A line and elution was performed using the buffer B line (represented by a black line in the chromatograms).

For the purification of MpAPr1 using the BioLogic LP Low Pressure Chromatography System the enzyme was produced in optimised conditions as determined above. Furthermore, prior to purification, it was decided to concentrate 10x using a 10-kDa cut-off filter (hereinafter referred as SMM-Op-10C) instead of a 30-kDa cut-off filter. Note that this system (BioLogic LP™ Low-Pressure Chromatography System) was different from that used in paragraph above in that it uses peristaltic pumps and a different software to analyse the data.

Furthermore, the sample could be directly injected into the system instead of loading it in through the use of the buffer A line. Using this system 20 ml SMM-Op-10C was injected onto five 1-ml HiTrap SP HP columns connected in series and eluted over a 50-ml gradient. The flow rate throughout the purification process using this system was 0.5 ml/min and all collected fractions were stored at 4°C.

In purification using the NGC™ Chromatographic System 20-ml sample (SMM-Op-10C) was injected onto five 1-ml HiTrap columns connected in series and eluted over a linear gradient of 50 ml. The flow rate throughout the purification process using this system was 1 ml/min and all collected fractions were stored at 4°C.

Final purification experiments were carried out (performed on the ÄKTA Pure Chromatography System) using two 5-ml HiTrap HP SP (GE Healthcare Bio-sciences) columns connected in series. This state-of-the-art system utilises two high precision piston pumps able to maintain 25 ml/min and includes real time monitoring of pump status, flow rate and pressure readings. The system utilises a sample loop through which the sample is injected directly onto the column, but sample volume is limited to 10-ml. Finally, the Unicorn software was used to generate and analyse chromatograms. Columns were equilibrated with 50 ml Buffer A prior to injection after which 10 ml sample was loaded. Elution of proteins was carried out as follows: linear gradient 20% Buffer B using 20 ml, hold step for 20 ml, from 20% to 100% Buffer B using 80 ml linear gradient. The flow rate throughout the purification process was 5 ml/min.

### **3.3.4 Protein visualisation and identification**

#### **3.3.4.1 Protein quantification**

Protein concentrations were determined by means of the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, South Africa). Bovine serum albumin (BSA) standards were prepared by the standard microplate procedure as described in the manufacturer's instructional document. Of all the standards and samples 25 µl were pipetted in triplicate into the wells of a 96-well microtitre plate to which 200 µl BCA working reagent was added. Plates were incubated at 37°C for 30 min and allowed to cool down to room temperature after which absorbance was measured at 540 nm through use of a PowerWave™ Microplate Scanning Spectrophotometer (BioTek Instruments Inc, Vermon, USA). In some cases, in order to obtain a more accurate reading, both BSA and the protease from *Aspergillus saitoi* (Sigma-Aldrich) were used as standards to quantify the concentration of proteins.

#### **3.3.4.2 SDS-PAGE and protein identification**

Proteins were visualised through the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Laemmli 1970). Prior to gel loading

samples were added to SDS-PAGE loading sample buffer (0.25 M Tris-HCl pH 6.8, 15% SDS 50% glycerol, 25%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue) in a 4:1 ratio and mixed by pipetting. From the fractions collected during purification experiments gels were loaded on according to a volume basis, thus 40  $\mu$ l total volume was loaded into each well. Gels containing 12% bis-acrylamide were run on a Bio-Rad Mini-Protean® Tetra Cell System (Bio-Rad Laboratories, Hercules, CA) or a Bio-Rad PROTEAN II XL cell (Bio-Rad Laboratories) (for enhanced resolution). Electrode chambers were filled with Tris-Glycine buffer (50 mM Tris, 200 mM glycine, 0.2% SDS). Gels were stained overnight in staining solution (1 g Coomassie blue R250 (Merck, Darmstadt, Germany) in 50% (v/v) ethanol, 10% acetic acid (v/v)). Gels were destained until all background was removed using 12.5% isopropanol and 10% (v/v) acetic acid.

SDS-PAGE gels were documented using the Gel Doc™ XR+ System (Bio-Rad Laboratories) and densitometric analysis of SDS-PAGE gels were carried out using the software package Image Lab™ version 5.1.

Selected protein bands were excised from the bis-acrylamide gels and were sequenced by LC-MS/MS after trypsin in-gel digestion at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa). Identification was derived from finding the best match to a large protein database. Database interrogation was performed with the MASCOT algorithm (Matrix Science, London, U.K., version 2.3) using Saccharomyces UniProt KB sourced both reviewed and unreviewed sequences. All identified peptides had an ion score of 99% and a low false discovery rate of 1%.

#### **3.3.4.3 2D-PAGE protein visualisation**

In order to prepare proteins for 2D-PAGE samples were concentrated 10 times using 2-ml Amicon centrifugal devices with a 10 kDa MWCO filter (Sigma-Aldrich). Briefly, 2 ml samples were centrifuged at 7000 *g* for 10 min after which the filtrate was removed and 2 ml McIlvaine's buffer (pH 3.5) added followed by another centrifugation step. The buffer exchange process was repeated three times after which the retentate was removed and stored at 4°C.

Prior to isoelectric focusing (IEF), samples were precipitated using the ReadyPrep™ 2-D Cleanup Kit (Bio Rad) according to the manufacturers protocols and resuspended in 300  $\mu$ l rehydration buffer (Bio-Rad Laboratories). Subsequently, the sample(s) was loaded onto a IEF focusing tray and overlaid with a 17-cm ReadyStrip IPG strip pH 3 – 10 (Bio-Rad Laboratories) according to the manufacturer's specifications and allowed to passively rehydrate at room temperature overnight. IEF was performed using the PROTEAN IEF Cell (Bio-Rad Laboratories) and was carried out according to the following programme: 250 V for 15 min, linear increase of the voltage from 250 to 10000 V for 3 h, and a final focusing step at 10 000 V for 60 000 Vh. Immobilised focused IPG strips were washed for 10 min in 6 ml

equilibration buffer I (6 M urea, 50 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2% DTT) and subsequently for 10 min in 6 ml equilibration buffer II (6 M urea, 50 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2.5% iodoacetamide).

IPG strips were loaded onto a 12 % bis-acrylamide gel as previously described using the PROTEAN II XL cell (Bio-Rad Laboratories). Gels were documented using a Molecular Imager® Gel Doc™ System (Bio-Rad Laboratories) using the software provided. Densitometric analysis of PAGE gels were carried out using the software package Image Lab™ version 5.1.

#### **3.3.4.4 Grape protein identification and quantification using RP-HPLC**

Grape protein identification and quantification was performed by Sarco (Floirac, France) according to the reversed phase – high performance liquid chromatography method described by Marangon et al. (2009).

### **3.4 MpAPr1 characterisation**

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#### **3.4.1 Milk clotting assay**

In order to obtain evidence that the protease retained activity in the crude extract, milk clotting reactions were carried out in test tubes using 1 ml crude extract added to 4 ml fresh milk. Reactions were incubated at 30°C without agitation for 3 days. Milk clotting activity was visualised after centrifugation of the reaction mixtures at 230 g for 10 s. Negative controls consisted of crude extract prepared from untransformed *K. pastoris* X33 cells and McIlvaine's buffer (pH 4.5). Reactions containing 0.1 mg/ml of protease from *Aspergillus saitoi* were included as positive controls.

#### **3.4.2 Protease activity assay**

##### **3.4.2.1 Visualisation and semi-quantification (for screening purposes) of MpAPr1 activity**

Yeast cells were cultivated in 5 ml YPD until an OD<sub>600nm</sub> of 1 was reached and 5 µl were spotted on skim milk plates at pH 3.5 as described by Charoenchai et al. (1997) and incubated for 4-7 days at 30°C. Protease activity was visualised through a zone of clearance around the colony. Intensity of activity was estimated by measuring the diameter of the zone of clearance. Negative and positive controls were included as untransformed *K. pastoris* X33 and *M. pulcherrima* IWBT Y1123, respectively.

### **3.4.2.2 Determination of MpAPr1 properties**

#### **3.4.2.2.1 Liquid assay**

Protease activity was estimated by measuring the release of acid-soluble material from azocasein. Briefly, 5 mg/ml substrate (azocasein dissolved in McIlvaine's buffer pH 4.5) was incubated with 0.1 mg/ml crude extract. In order to stop the reaction, 150  $\mu$ l was removed at specific time points and added to 150  $\mu$ l 20% TCA solution. The mixture was briefly vortexed and allowed to stand at room temperature for 10 min and centrifuged for 10 min at 9300 *g*. The supernatant was collected in 100  $\mu$ l aliquots in a 96-well plate before absorbance was measured at an optical density of 440 nm according to the manufacturer's specifications using a PowerWave™ Microplate Scanning Spectrophotometer (BioTek). All experiments were performed in triplicate. One acid protease unit was defined as the amount of protease causing an increase in absorbance at 440 nm of 0.001 under the experimental conditions used.

#### **3.4.2.2.2 Effect of pH and temperature**

The optimal pH for the crude extract was determined by measuring activity at 40°C for 12 h over the pH range 3 – 8 using McIlvaine's buffer as assay buffer. Optimal temperature for protease activity was determined through assays performed at 10°C - 70°C for 12 h in McIlvaine's buffer at pH 4.5. The maximum observed activity under any of the conditions tested was defined as 100% and the relative activities were calculated as a fraction of this value.

#### **3.4.2.2.3 Effect of metal ions, pepstatin A and EDTA**

To determine whether the enzyme activity is metal dependent, an assay was performed with ethylene diamine tetra acetic acid (EDTA) at concentrations of 5, 10 and 50 mM. Enzyme activity was monitored in the presence CaCl<sub>2</sub>, CuCl<sub>2</sub>, FeCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub> and ZnCl<sub>2</sub> added to a final concentration of 1 mM. Additionally, activity was also measured in the presence of 2 mM CaCl<sub>2</sub> and 5 mM MgCl<sub>2</sub>. Pepstatin A (an aspartic protease inhibitor) was included (at a final concentration of 100 nM) in the experiment to serve as a control. Relative activity is expressed as a percentage of enzyme activity with no added compound.

#### **3.4.2.2.4 Effect of sugars and ethanol**

The effect of sugar was assessed at the concentrations of 300, 200, 100 and 2 g/l. In order to determine the effect of ethanol on enzyme activity, different amounts were added to the substrate at a final concentration of 6% (v/v), 12% (v/v) and 15% (v/v) prior to enzyme addition. Relative activity was expressed as a percentage of enzyme activity with no added compound. As a control, pepstatin A was also included (at a final concentration of 100 nM) in the

experiment as a known aspartic protease inhibitor. All experiments described above were conducted in McIlvaine's buffer (pH 4.5) at 40°C.

#### 3.4.2.2.5 Determination of kinetic constants

MpAPr1's kinetic constants were determined on the protein crude extract as well as on the purified enzyme. The crude extract was assayed against increasing substrate concentration of 2.5 and 20 mg/ml in McIlvaine's buffer at pH 4.5. The reaction was as follows: 100 µl of crude extract was added to 900 µl substrate and incubated for 12 h at 40°C. This experiment was repeated using pepsin (Sigma-Aldrich) and the protease from *Aspergillus saitoi* (Sigma-Aldrich) at a concentration of 0.1 mg/ml to serve as a comparison.

The hydrolytic activity of the crude extract was also measured in the presence of 2.5, 5, 10 and 20 mg/ml azocasein in McIlvaine's buffer (pH 4.5) in the presence of pepstatin A (Sigma-Aldrich) at concentrations of 2.5 - 25 nM at 40°C for 12 h. The inhibition constant was calculated graphically according to Cornish Bowden (1974).

Purified MpAPr1 at a final concentration of 0.1 mg/ml was assayed against increasing amounts of substrate (azocasein) concentrations (0, 1, 2, 3, 5, 7.5, 10, 12.5, 15 and 20 mg/ml) in McIlvaine's buffer (pH 4.5). Reactions were incubated at 40°C during which samples were taken at 0, 2, 4, 7, 12 and 24 h. Protease activity was calculated as described above. Controls included a protease from *Aspergillus saitoi* (Sigma-Aldrich) at a final concentration of 0.1 mg/ml in order to serve as a comparison. Data was analysed and the  $K_m$  and  $V_{max}$  calculated using the Prism software (GraphPad, La Jolla, CA).

### 3.5 Impact of MpAPr1 on wine properties

#### 3.5.1 Impact of MpAPr1 on grape proteins

Purified MpAPr1 was assayed against grape proteins. Pure grape proteins isolated from Pinot Grigio grape juice were generously donated by Dr Simone Vincenzi (University of Padua, Italy), specifically chitinases and thaumatin-like proteins (TLP). In order to more closely resemble the grape protein profile found in grape juice, the proteins were mixed to a 1/3 chitinase to 2/3 TLP ratio prior to experimental use. In a first experiment, 0.1 mg/ml MpAPr1 was assayed against 0.1 mg/ml grape proteins in McIlvaine's buffer (pH 4.5) and incubated at 40°C for 48 h. Thereafter, the experiment was repeated in McIlvaine's buffer pH 3.5 and incubation at 20°C in order to simulate wine making conditions.

#### 3.5.2 Fermentation trial layout

Sauvignon Blanc grapes were harvested at the Welgevallen experimental vineyard (Stellenbosch University), crushed, destemmed and pressed at the experimental cellar (Stellenbosch University) in 2016. No enzyme addition was made during or after pressing was

stored at  $-20^{\circ}\text{C}$ . After thawing and prior to experimental use, the grape juice was first centrifuged at  $5000\text{ g}$  for  $10\text{ min}$ . The juice was further filtered through a  $1.2\text{-}\mu\text{M}$  membrane filter (Sartorius, Göttingen, Germany) and subsequently through a  $0.45\text{-}\mu\text{M}$  membrane filter (Sartorius). Sugar concentration ( $215.7\text{ g/l} \pm 0.197$ ), pH ( $3.5$ ) and total acidity ( $3.67\text{ g/l} \pm 0.045$ ) were analysed using Fourier-transform mid-infrared spectroscopy (FOSS WineScan, Hillerød, Denmark) as described by Nieuwoudt et al. (2006).

$0.1\text{ mg/ml}$  MpAPr1 was added to grape juice and incubated at  $25^{\circ}\text{C}$  for  $48\text{ h}$  after which the juice was inoculated with the yeast *Saccharomyces cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) and allowed to ferment to dryness. Grape juice with no addition of MpAPr1 was also inoculated separately as a control.

Fermentations were carried out in triplicate at  $25^{\circ}\text{C}$  with  $80\text{ rpm}$  agitation in  $100\text{-ml}$  cylindrical bottles fitted with airlocks that were filled with  $75\text{ ml}$  grape juice with or without prior treatment with MpAPr1. Yeast cell cultures were pre-cultured in YPD broth at  $30^{\circ}\text{C}$  overnight before inoculation at  $10^6\text{ cells/ml}$ .

Fermentation kinetics were monitored by measuring weight loss three times per day (as a estimation of  $\text{CO}_2$  released) and yeast population dynamics by plating on YPD agar once a day throughout the course of fermentation. Ten millilitre samples were taken at time  $0\text{ h}$  and  $48\text{ h}$  for activity assays, SDS-PAGE, 2D-PAGE and HPLC analyses (as described above and below). Twenty millilitre samples were also taken at the end of fermentation for protease activity assays, heat stability assays, SDS-PAGE, HPLC, major volatile compounds, sugars and nitrogen compounds analyses (as described above and below). After activity assays were performed in liquid assays (as described above), all samples were stored at  $-20^{\circ}\text{C}$  prior to experimental use.

### **3.5.3 Analytical techniques**

#### **3.5.3.1 Major volatile compounds**

Samples were prepared and major volatile compounds were determined by gas chromatography - flame ionisation detection (GC-FID) as described by Rossouw et al. (2008). Analyses were carried out on a Hewlett Packard 5890 Series II GC coupled to an HP 3396A auto-sampler and injector. A Lab Alliance organic coated column with fused silica capillary ( $60\text{ m} \times 0.32\text{ mm}$  internal diameter with a  $0.5\text{ }\mu\text{m}$  thick coating). Hydrogen was used as the carrier gas for flame ionisation detection (held at  $250^{\circ}\text{C}$ ). The following conditions were used: Injection temperature at  $200^{\circ}\text{C}$ , split ratio  $20:1$ , flow rate  $15\text{ ml/min}$ . Oven temperature was increased from  $35^{\circ}\text{C}$  to  $230^{\circ}\text{C}$  at  $3^{\circ}\text{C/min}$ .

### 3.5.3.2 Sugars and nitrogen compounds

Before and after fermentation, the concentrations of various compounds were measured as follows: residual glucose and fructose concentrations were measured using the D-Glucose/D-Fructose Kit (R-Biopharm, Roche). Primary amino nitrogen and free ammonium concentrations were determined using the appropriate enzyme kits from Megazyme (Bray, Ireland) and Enzytec (r-Biopharm), respectively, in an Arena 20XT Photometric Analyzer (Thermo Scientific).

### 3.5.4 Protein haze assay

Heat stability or protein haze potential of grape juice and wine samples were determined as described by Pocock and Waters (2006). All measurements were performed in triplicate with the appropriate controls. Briefly, samples were filtered through a 0.45- $\mu\text{m}$  syringe filter (GVS Filter Technology, Bologna, Italy) prior to remove cells. Absorbance was read at 520 nm using a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA). Samples were then incubated at 80°C for 2 h and subsequently cooled at 4°C overnight. Absorbance at 520 nm was measured after acclimatisation at room temperature for 30 min. Haze formation was determined by calculating the difference in absorbance before and after heating of the sample. Samples were considered unstable (i.e. prone to form haze) when the difference in absorbance was greater than 0.02 absorbance unit (Pocock et al. 2007).

### 3.5.5 Amino acid analyses

Amino acids were quantified via high performance liquid chromatography (HPLC) as previously described by Henderson and Brooks (2010). HPLC was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with a Poroshell HPH-C18, 4.6 x 150 mm, 2.7  $\mu\text{m}$  column (Agilent Technologies).

## 3.6 Statistical analysis

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When appropriate, statistical analyses were performed using computer software StatSoft STATISTICA. Data sets were compared using the Student's t-test for independent samples. Graphical analyses were performed on Excel (Microsoft, Redmond, USA). All experiments were performed in triplicate, with three independent measurements.

# Chapter 4

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## Results and Discussion

## Chapter 4 – Results and Discussion

### 4.1 Introduction

Although all part of the same study, different sections compose this chapter. The first part of this study focuses on screening for and sequencing the *MpAPr1* gene in several strains of *Metschnikowia* spp. (section 4.2) in order to evaluate the correlation between intensity of enzyme activity and gene sequence. The second part (section 4.3) relates the cloning of the *MpAPr1* gene into a eukaryotic host for overexpression in a rich medium. A concentrated supernatant (referred to as “the crude extract” in this part) was obtained and used to characterise the enzymatic properties of MpAPr1 because initial challenges faced during purification trials delayed its purification (section 4.4). Section 4.5 focuses on optimising the expression of the recombinant *MpAPr1* in a minimal medium and details the challenges faced to obtain a pure enzyme. Once the pure MpAPr1 enzyme was obtained, its activity was first evaluated against pure grape proteins in a buffered medium under optimal and sub-optimal (i.e. resembling those of wine fermentation) pH and temperature conditions. Finally, the holistic impact of the pure MpAPr1 enzyme added to grape juice was evaluated in fermentation trials (section 4.6).

Each section is subdivided into smaller units where the results of experiments are highlighted. A discussion follows at the end of each section.

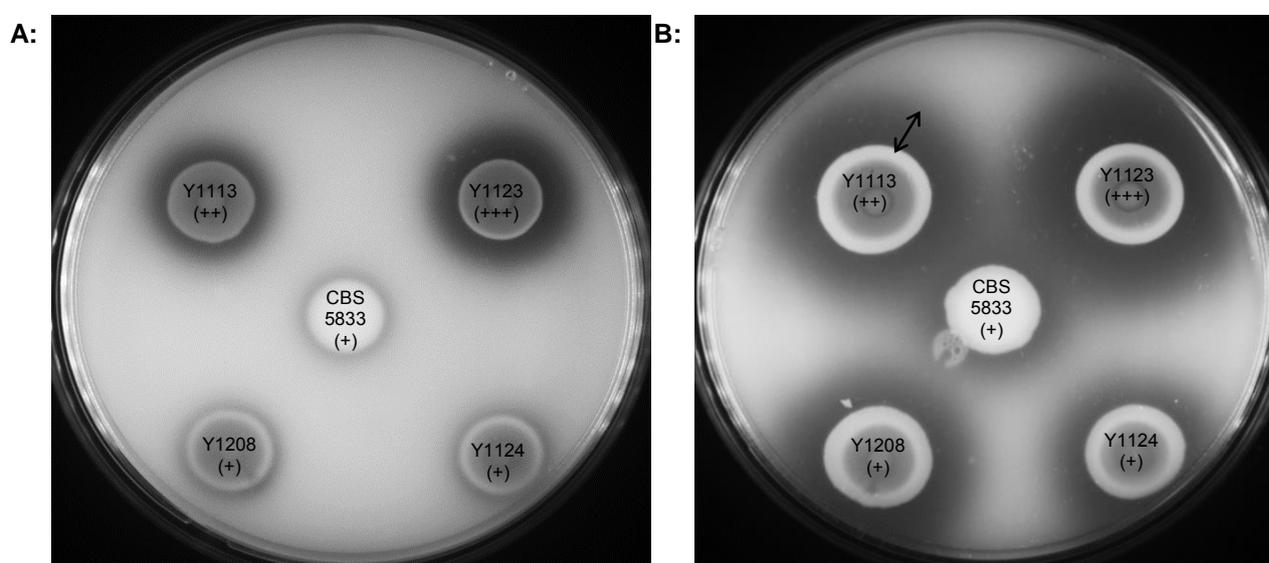
### 4.2 Genetic and phenotypic screening of *Metschnikowia* spp. for acid protease activity and strain selection

#### 4.2.1 Extracellular protease activity screening and cloning of *MpAPr1* genes

A collection of forty-three strains of *Metschnikowia* spp. (forty from *M. pulcherrima*, two from *M. fructicola* and one from *M. chrysperlae*) was screened for extracellular protease activity by performing plate assays at pH 3.5 using casein, a commonly used substrate for acid protease activity screening. *Saccharomyces cerevisiae* VIN 13 and *K. pastoris* X33 were included as negative controls and as no detectable protease activity against casein could be detected. Activity was revealed through a clear zone (i.e. halo) around the spot. The level of activity of the different strains were determined semi-quantitatively by measuring the size of the distance from the spot to the edge of the halo (indicated by a double arrow in Figure 4.1). The strains were grouped with respect to their protease activity as follows: +++ 7 mm (very strong activity), ++ 2.5-5 mm (strong activity), + ≤1 mm (detectable activity). The results are captured in Table 3.1 (in Chapter 3). Briefly, only one strain displayed very strong protease activity, namely *Metschnikowia pulcherrima* IWBT Y1123, while four displayed strong activity and thirty eight detectable activity. Furthermore, all strains exhibiting visible protease activity belonged to the

species *pulcherrima* and two of the strains displaying weak activity belonged to the species *fructicola*.

As a visual example, Figure 4.1 illustrates the protease activity, of one strain from each group of activity, on skim milk plates. After 4 days of incubation (Figure 4.1, A) only two strains display observable activity *M. pulcherrima* IWBT Y1123 (+++) very strong and *M. pulcherrima* IWBT Y1113 (++) strong, and only weak activity can be observed for the other strains. After 7 days of incubation (Figure 4.1, B) *M. pulcherrima* IWBT Y1123 and *M. pulcherrima* IWBT Y1113 still show the strongest activity (respectively) but the other strains tested also started to show slightly stronger activity. A few strains from each of the four activity groups were selected in order to investigate whether intensity of activity was connected to a specific genetic feature(s).



**Figure 4.1:** Skim milk plate(s) used in screening for extracellular protease activity. Note that photos were taken in black and white and activity is visualised as a dark halo (shaded area) around the spot (white area) in the photo (note that contrast was enhanced to emphasize halo). Panel A and B show spots after 4 and 7 days of incubation at 30°C, respectively. Spot selected out of each activity group are labelled as follows: (Y1123 +++) *M. pulcherrima* IWBT Y1123, (Y1113 ++) *M. pulcherrima* IWBT Y1113, (Y1208 +) *M. pulcherrima* IWBT Y1208, (Y1124 +) *M. pulcherrima* IWBT Y1124, *M. pulcherrima* CBS 5833 (CBS 5833 +). The mathematical symbols indicate the intensity of activity as populated in Table 3.1. The double black arrow indicates the distance measured to evaluate activity.

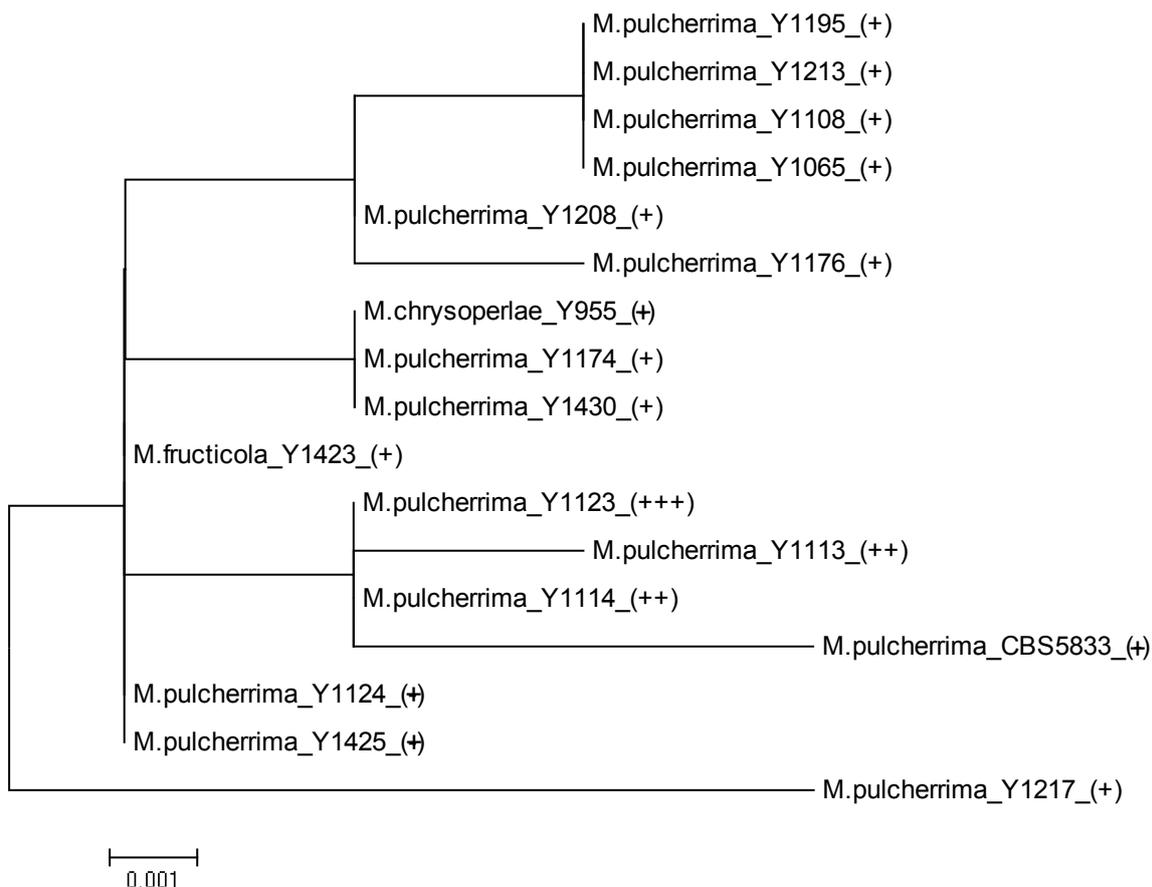
#### 4.2.2 Sequence alignment and phylogenetic tree

In order to investigate the presence of the *MpAPr1* gene, genomic DNA was extracted from the selected strains and PCR was performed as previously described by Reid et al. (2012). Strains were selected from the different activity groups as follows: one from the very strong

(+++), two from the strong (++) , fourteen from the detectable (+). The PCR amplification was successful in all the strains (data not shown) showing the presence of the *MpAPr1* gene. The amplicons were cloned into the pGEM-T easy and transformed in to *E. coli*. After cultivation plasmids were extracted and sequenced. The respective nucleotide sequences were obtained, *in silico* translation was performed and the amino acid sequences obtained were aligned and compared. Differences between strains are summarised in Table 4.1. A few single mutations were observed between the different MpAPr1 amino acid sequences but they seemed to be of minor importance and differences observed in activity appeared unrelated to these mutations. Indeed, most of the mutations are located in non-essential regions of the protein (i.e. no mutation in the secretion signal, active sites, glycosylation sites or flap region), with the exception of site 11 which is located within the secretion signal. Furthermore, substitution mostly occurred between closely related amino acids (including that occurring within the secretion signal, i.e. Val<sup>11</sup> to Ile in certain strains), thereby minimising the likelihood of structural changes that would in turn impact protein sorting, secretion or enzyme activity. Only one substitution, namely Lys<sup>47</sup> to Met in strain IWBT Y1217, resulted in the substitution with a sulphur-containing amino acid. A dendrogram was drawn to further illustrate the relationship between the amino acid sequences (Figure 4.2). Although the strains displaying the strongest activity grouped together, a strain with poor activity clustered in the same group. This particular grouping seems to be driven by the presence of a valine instead of an alanine in position 18, a minor mutation considering that valine and alanine belong to the same family of amino acids and that this mutation is not located within a specific feature of the protein/enzyme.

**Table 4.1:** Summary of the single mutations found in the MpAPr1 amino acid sequences of several strains of *Metschnikowia* spp. after sequence alignment. (-: no change from the MpAPr1 amino acid sequence of strain IWBT Y1123 used as reference). Note that Y955 is a *M. fructicola* strain while the rest are *M. pulcherrima* strains.

Strains <i>Metschnikowia</i> spp	Position and identity of single amino acid mutations										
	11	18	26	41	47	108	136	147	178	191	235
Y1123 (+++)	Val	Ala	Asp	Val	Lys	Pro	Tyr	Pro	Ser	Gly	Gly
Y1113 (++)	-	-	-	-	-	-	-	-	Leu	-	-
Y1114 (++)	-	-	-	-	-	-	-	-	-	-	-
Y1065 (+)	-	Val	-	-	-	Ser	-	-	-	-	Val
Y1108 (+)	-	Val	-	-	-	Ser	-	-	-	-	Val
Y1124 (+)	-	Val	-	-	-	-	-	-	-	-	-
Y1174 (+)	Ile	Val	-	-	-	-	-	-	-	-	-
Y1176 (+)	Ile	Val	-	-	-	-	-	-	-	-	Val
Y1195 (+)	-	Val	-	-	-	Ser	-	-	-	-	Val
Y1208 (+)	-	Val	-	-	-	-	-	-	-	-	Val
Y1213 (+)	-	Val	-	-	-	Ser	-	-	-	-	Val
Y1217 (+)	-	Val	-	Ile	Met	-	His	-	-	Val	-
Y1423 (+)	-	Val	-	-	-	-	-	-	-	-	-
Y1430 (+)	Ile	Val	-	-	-	-	-	-	-	-	-
Y1425 (+)	-	Val	-	-	-	-	-	-	-	-	-
Y955 (+)	Ile	Val	-	-	-	-	-	-	-	-	-
CBS 5833 (+)	-	-	Val	-	-	-	-	Ser	-	-	-
<b>Comment:</b>	-	-	-	-	Aliphatic to sulphur containing	Aliphatic to hydrophilic	Aromatic to basic	Aliphatic to hydrophilic	Hydrophilic to aliphatic	-	-



**Figure 4.2:** Dendrogram obtained using the Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (-1181.3443) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 378 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

### 4.2.3 Discussion and partial conclusion

The screening of 43 *Metschnikowia* spp. for extracellular acid protease activity confirmed that a majority of strains display activity against casein on solid medium, but strain-specific differences were observed in the intensity of this enzymatic activity as well as in the respective gene sequences of *MpAPr1*, the gene encoding this activity in *M. pulcherrima* (Reid et al. 2012). This result was not surprising since a similar observation was made by previous authors in other yeast genera such as *Hanseniaspora* (Lopez et al. 2015, Albertin et al. 2016). Furthermore, the results confirmed that all strains investigated possess the *MpAPr1* gene, as

previously noted on fewer strains by Reid et al. (2012), but this study revealed that the presence of *MpAPr1* is unrelated to the level of secretion and activity.

Mutations were observed between strains but likewise, no clear connection between level of activity and presence of specific mutations could be established. Although the strains displaying the strongest activity grouped together, a strain with poor protease activity was present in the same group. Furthermore, the fact that these strains possess the same mutation definitely invalidate the hasty hypothesis that this minor mutation could induce stronger activity. The groupings are therefore likely to be linked to the general evolution of the strains' genomes and their relatedness to each other rather than protease activity specifically. Further investigation comparing the promoter regions and transcription factors would be required to further unravel the differences observed. Finally, the plate screening assay used to determine extracellular activity could be potentially replaced with a liquid assay in which the substrate is added to the medium and protein degradation is measured in real time. Such methods are already proposed for measuring protease activity during grape juice fermentation (Chasseriaud et al. 2015) and could be used to measure protease activity more quantitatively during screening experiments.

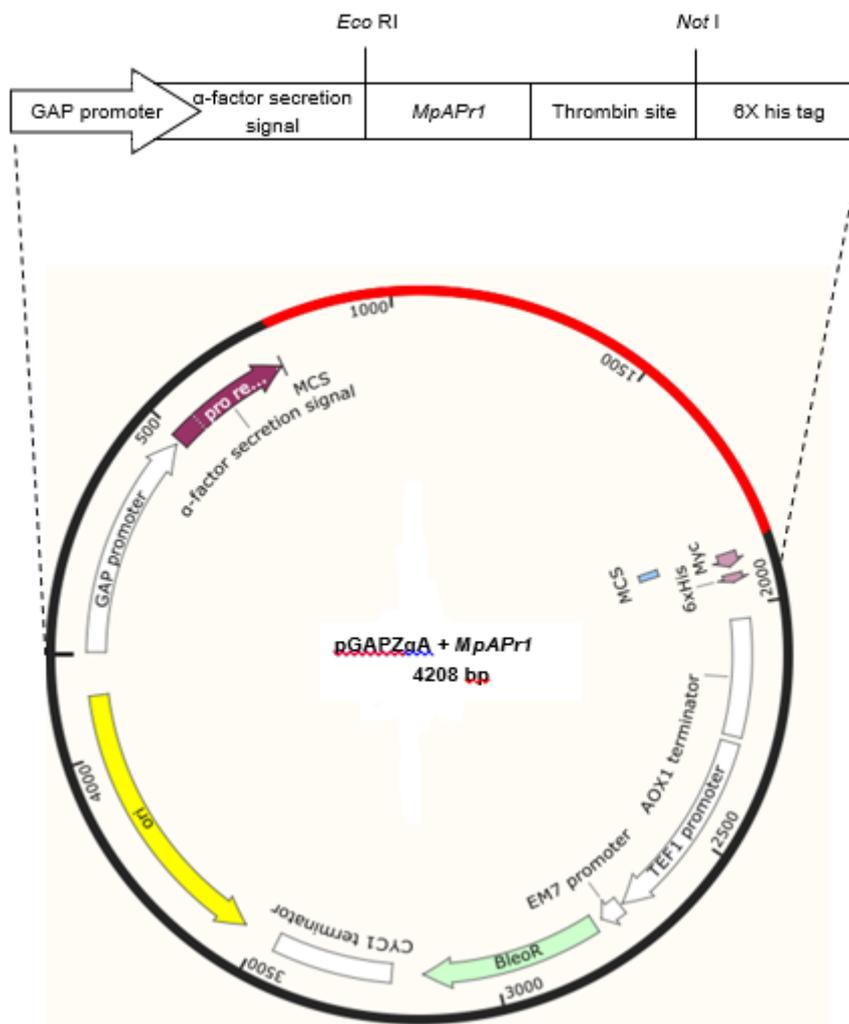
The strain displaying the strongest activity (i.e. *M. pulcherrima* IWBT Y1123) was nevertheless selected to determine the properties of *MpAPr1* and to assess its potential application in winemaking.

### **4.3 Heterologous expression of *MpAPr1* in *Komagataella pastoris***

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#### **4.3.1 Construction of *MpAPr1* expression cassette**

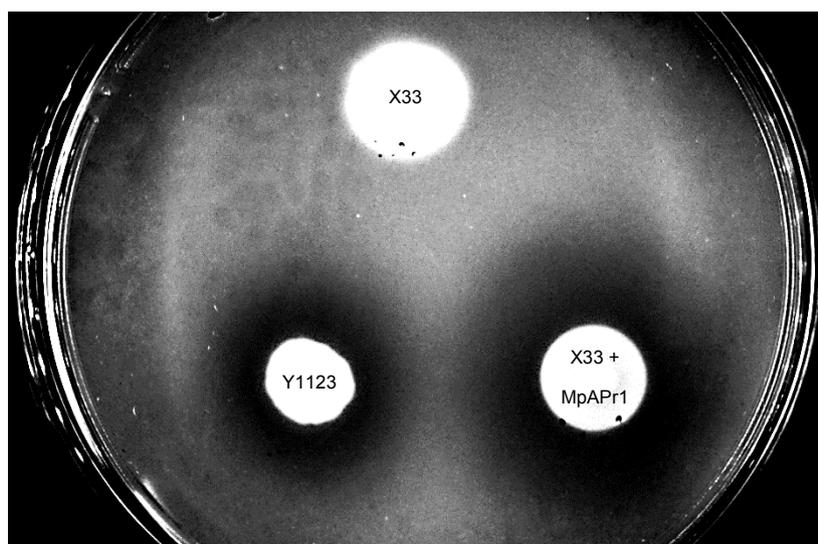
The *MpAPr1* gene was amplified without its native secretion signal and with an additional thrombin site fused to the C-terminal region, for downstream expression purposes. The cassette was cloned into pGAPZ $\alpha$ A to be placed under the control of the *GAP* promoter. Furthermore, an  $\alpha$ -factor secretion signal (that encodes native *Saccharomyces cerevisiae*'s  $\alpha$ -factor secretion signal and that allows for secretion of most proteins in *Komagataella*) was fused to the N-terminal region of the enzyme together with an additional 6x histidine tag that was fused to the C-terminal region (following the thrombin site). The resulting plasmid was named pGAPZ $\alpha$ A + *MpAPr1*. An illustration of the plasmid with a magnified view of the expression cassette can be viewed in Figure 4.2. This specific plasmid was further chosen for its compatibility with both bacteria and yeast expression systems using zeocin resistance (resulting from the presence of the *Sh ble* gene on the plasmid) as a selectable marker.



**Figure 4.3:** Map of the expression vector pGAPZαA + *MpAPr1*. An out-of-scale expression cassette (displaying added features) with the cloned *MpAPr1* gene (shown in red on the map) is presented above the plasmid map.

After propagation in *E. coli* DH5α, the plasmid was extracted. Thereafter, the expression cassette was amplified by PCR and sequenced to verify the construct. The size and sequence of the cloned gene (without the added features; α-factor secretion signal, thrombin site and 6x histidine tag) were confirmed to be identical to those of the *MpAPr1* gene (accession number: JQ677912) (data not shown). Thereafter, the plasmid was transformed into *K. pastoris* X33 for heterologous overexpression in a eukaryotic host and a positive transformant growing on YPD supplemented with zeocin was randomly selected. After confirmation of extracellular expression and activity, positive transformants were serially plated on YPD containing increasing amounts of zeocin until a final concentration of 1 mg/ml was reached. Transformants exhibiting tolerance to high amounts of zeocin were selected as it was speculated that they contained multiple copies the *MpAPr1* gene. However, number of copies,

gene expression and protein production were not compared or evaluated. Expression of *MpAPr1* along with secretion and activity of the corresponding enzyme were further confirmed through spotting of the cells on skim milk plates (Figure 4.3). Evidently, the assay revealed that the transformed strain showed stronger activity than the natural host (*M. pulcherrima* IWBT Y1123) and confirmed that the untransformed *K. pastoris* X33 cells (negative control) displayed no activity.

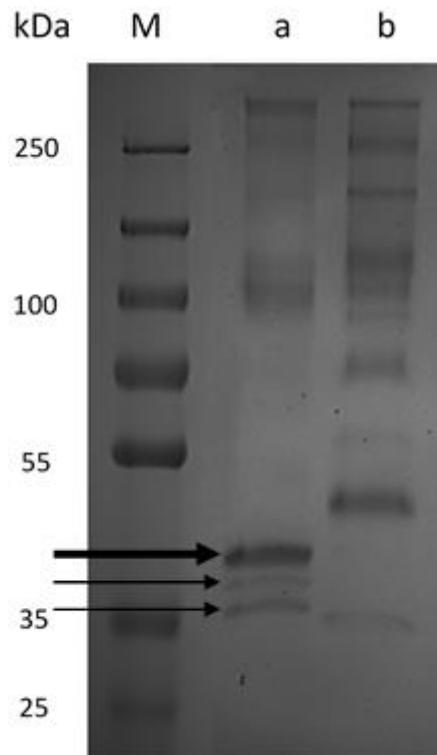


**Figure 4.4:** Skim milk plate assay for extracellular protease activity. Note that photos were taken in black and white and activity is visualised as a dark halo (shaded area) around the spot (white area) in the photo (note that strong contrast was applied to accentuate the halos). X33: *K. pastoris* X33, X33 + MpAPr1: *K. pastoris* X33 + *MpAPr1*, Y1123: *M. pulcherrima* IWBT Y1123.

#### 4.3.2 Expression of recombinant MpAPr1

Positive transformant (*K. pastoris* X33 + *MpAPr1*) was selected for on YPD agar containing 1 mg/ml zeocin pre-cultured in YPD and grown for 24 h at 30°C. Cultures were then transferred into growth medium, incubated for 72 h at 20°C, the supernatant collected and concentrated via ultrafiltration. SDS-PAGE analysis showed the presence of a band corresponding to the recombinant MpAPr1 (the expected molecular mass with added features was 43.3 kDa) in the concentrated protein crude extract from transformed cells (Figure 4.5, lane a indicated by thick black arrow). These bands were absent from the concentrated supernatant of untransformed *K. pastoris* X33 cultures (Figure 4.5, lane b). Furthermore, this band was manually excised from the bis-acrylamide gel, trypsin digested and analysed through LC-MS/MS, in order to confirm identification by mass fingerprinting. Two additional bands, just below that described above ranging between 35 kDa and 55 kDa (Figure 4.5, lane a indicated by thin black arrows)

were also visible on the SDS-PAGE gels. Since these bands did not appear in the supernatant of untransformed X33, they were also excised and mass fingerprinted. Identity was confirmed as MpAPr1 (accession number: I3QI66) for the three bands respectively. Mascot scores and the number of significant peptides identified were as follows, respectively: top band 4347 and 12; middle band 1962 and 13; bottom band 974 and 13.



**Figure 4.5:** Image of SDS-PAGE indicating extracellular protein profile of (a) *K. pastoris* X33 + MpAPr1 and (b) *K. pastoris* X33. (M) Molecular weight marker (molecular masses are indicated in kDa on the left). Black arrows indicate bands that were excised for mass fingerprinting analyses.

#### 4.3.3 Discussion and partial conclusion

In a previous study (Theron, 2013), a prokaryotic host (*E. coli* Rosetta-gami) was used to express the *MpAPr1* gene, but expression led to the formation of inclusion bodies and the enzyme could not be retrieved in an active state. However, purification was possible and pure enzyme (although inactive and denatured) could be obtained using the Ni-NTA spin columns based on IMAC. In this study, it was thus decided to express *MpAPr1* in a eukaryotic host in order to obtain a properly folded and active enzyme for characterisation experiments. Successful cloning of the *MpAPr1* gene from *M. pulcherrima* IWBT Y1123 into the pGAPZa A vector was followed by the transformation and subsequently integration into *K. pastoris* X33's genome. Expression, secretion and activity were confirmed via plate assay and SDS-PAGE.

Analysis confirmed that the untransformed strain had no detectable extracellular acid protease activity and that the activity observed in the supernatant of the recombinant strain was due to successful expression and secretion of MpAPr1. Furthermore, SDS-PAGE analysis combined with mass fingerprinting revealed the presence of three bands between the regions of 35 to 50 kDa (not present in the untransformed culture supernatant), the top band being thicker than the smaller two bands. All 3 bands positively identified as MpAPr1. The expected molecular mass of the native mature MpAPr1 protease (without its native secretion signal) is 39.2 kDa, thus the recombinant MpAPr1 is predicted to have an increased molecular weight of 43.3 kDa because of the presence of the added thrombin and hexa-histidine tag. Consequently, the band corresponding to the expected molecular mass of 43.3 kDa was determined to be the correct version of MpAPr1 with its additional features (indicated by the thick black band in Figure 4.4). The two additional bands (indicated by thin black arrows in Figure 4.4) are suspected to be different isoforms of MpAPr1 being expressed (e.g. glycosylated and non-glycosylated) or perhaps truncated versions. Similar results have been reported in literature when an extracellular aspartic protease from *Mucor circinelloides* (MCAP) was expressed in *K. pastoris* X33 (Salgado et al. 2013). Indeed, the latter authors describe two bands around the expected size of the recombinant protease (MCAP) following SDS-PAGE analysis. It was confirmed that a non-glycosylated and glycosylated form of MCAP was expressed with molecular weights of 33 and 37 kDa, respectively. This hypothesis is investigated in section 4.5 (paragraph 4.5.2.2.2). Finally, it can be observed that some protein bands disappeared within the culture supernatant when comparing the extracellular protein profiles. This is speculated to be due to the activity of the recombinant protease resulting in the breakdown of proteins from *K. pastoris* and/or proteins from the medium used within the culture supernatant (e.g. other extracellular proteins, intracellular proteins originating from autolysis and proteins present in YPD).

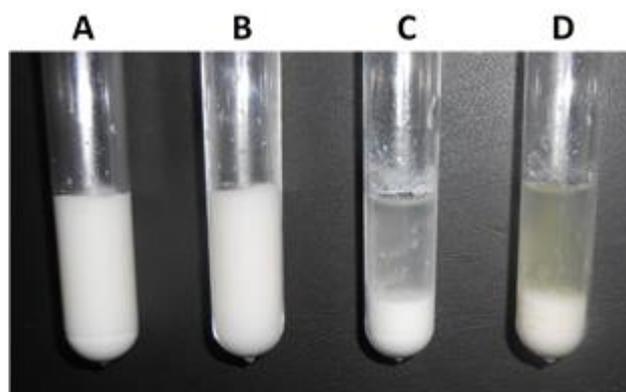
Since the culture supernatant of the untransformed strain showed no detectable activity and could serve as a control it was decided to characterise some of the enzyme properties using the concentrated supernatant which will be referred to as the crude extract hereinafter.

#### **4.4 Determination of MpAPr1 properties within crude extract**

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##### **4.4.1 Confirmation of protease activity**

In order to verify proteolytic activity within the crude extract after ultrafiltration, its ability to clot milk was investigated (Figure 4.6). After visual inspection, it was found that the crude extract could clot milk to the same observable degree as the positive control (commercial acid protease from *Aspergillus saitoi*) (Figure 4.6, tubes D and C respectively). Furthermore, the concentrated supernatant from *K. pastoris* X33 had no detectable activity (Figure 4.7, tube B).



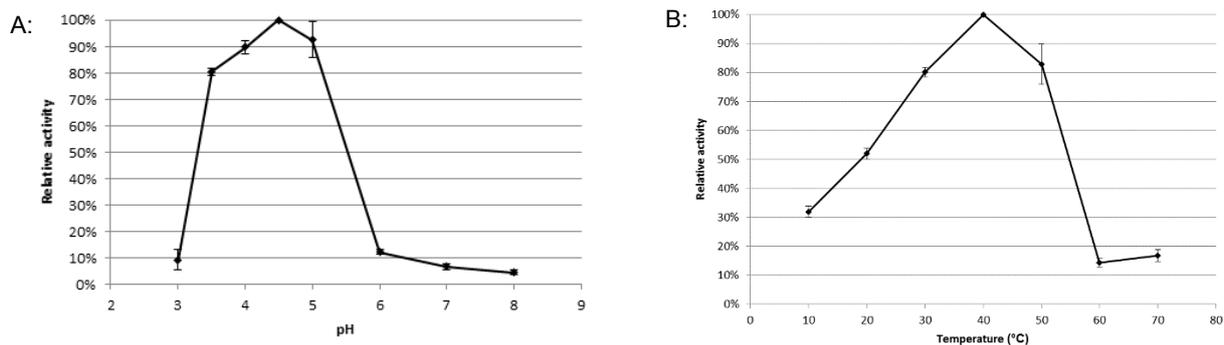
**Figure 4.6:** Milk clotting test of crude extracts obtained from untransformed and transformed *K. pastoris* X33 (+ *MpAPr1*) transformants. (A) Milk and water, (B) Milk and crude extract from untransformed strain, (C) Milk and commercial protease from *Aspergillus saitoi*, (D) Milk and crude extract from positive transformants of *K. pastoris* X33 + *MpAPr1*.

#### 4.4.2 Determination of optimal pH and temperature

Considering the confirmed activity of MpAPr1 against casein, the properties of MpAPr1 were determined using azocasein as a convenient substrate. Indeed, azocasein (i.e. casein conjugated to an azo-dye) is a chromogenic substrate that serves as a general substrate for endopeptidases (Biver et al 2013, Chasseriaud et al. 2015).

Proteolytic activity of the crude extract was measured against azocasein in various pH and temperature conditions in order to determine maximal activity (Figure 4.7). In all the conditions tested, the crude extract from untransformed *K. pastoris* X33 was included and displayed no detectable activity. Maximum activity was detected at pH 4.5 and favourable pH conditions were in the range of 3.5 to 5 between which relative activity remained above 80%. Below pH 3.5, activity rapidly decreased to barely detectable levels. Similarly, at pH 6, enzyme activity decreased by 88% and steadily decreased further with increasing pH values (Figure 4.7, A).

Favourable temperature conditions were identified in the range of 30°C to 50°C where above 80% relative activity was retained with maximum activity observed at 40°C (Figure 4.8, B). Below 30°C, relative activity decreased steadily but still retained above 30% activity at the lowest temperature tested (10°C). At and above 60°C, activity was severely affected with a decrease of 87%.



**Figure 4.7:** Graphical illustration of the proteolytic activity determined for the crude extract against azocasein at different pH and temperature (°C) conditions. (A) Effects of pH were determined in Mcllvaine's buffer after 12 h at 40°C (B) Effects of temperature was determined at various temperatures in Mcllvaine's buffer pH 4.5 after 12 h. The data points shown are means of three independent experiments and the highest observed activity was defined as 100%. Error bars indicate standard deviation between triplicates.

#### 4.4.3 Effect of metal ions, pepstatin A and EDTA

In order to investigate the effect that metal ions, pepstatin A and EDTA have on the proteolytic activity of MpAPr1 within the crude extract, optimal pH and temperature conditions (pH 4.5 and 40°C) were used during the assays.

All metal ions used were salts conjugated to  $\text{Cl}_2$  and were tested at a final concentration of 1 mM. Furthermore,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are commonly found in grape juice at concentrations of 2 mM and 5 mM, respectively, and were thus included in the test at these higher concentrations. The results are shown in Table 4.2. The presence of  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  had no significant effect on enzyme activity. In contrast, the presence of  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  had a significant inhibitory impact on activity. Indeed,  $\text{Mn}^{2+}$  slightly inhibited activity whereas  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  inhibited activity by 47% and 25%, respectively.

Pepstatin A, a known inhibitor of aspartic proteases, was tested at several concentrations and was found to completely inhibit enzyme activity at the lowest final concentration of 0.1 mM

The chelating agent ethylenediaminetetraacetic acid (EDTA) had a weak inhibitory effect on proteolytic activity at lower concentrations of 5 mM and 10 mM, leading to a decrease in activity of 8.14% and 17.06% respectively. This effect was much more severe at 50 mM leading to an 82.27% decrease in activity.

**Table 4.2:** Summary of the effects of metal ions, pepstain A and EDTA on proteolytic activity of the crude extract following optimal assay conditions (pH 4,5 and 40°C). Data shown are the means of three independent experiments with standard deviations shown after the activity value. The control (no added compounds) was defined as 100% activity.

Compound	Concentration (mM)	Activity (AU)*	Relative activity (%)
None		0.913 ± 0.020 a	100
CaCl <sub>2</sub>	1	0.853 ± 0.044 a	93.49 ± 2.27
	2	0.946 ± 0.031 a	103.64 ± 1.35
CuCl <sub>2</sub>	1	0.479 ± 0.024 b	52.49 ± 2.07
FeCl <sub>2</sub>	1	0.896 ± 0.053 a	98.16 ± 1.51
MgCl <sub>2</sub>	1	0.846 ± 0.035 a	92.57 ± 1.91
	5	0.925 ± 0.049 a	101.39 ± 2.89
MnCl <sub>2</sub>	1	0.869 ± 0.013 b	96.43 ± 0.71
NiCl <sub>2</sub>	1	0.689 ± 0.015 b	75.54 ± 3.05
ZnCl <sub>2</sub>	1	0.848 ± 0.035 a	92.88 ± 1.97
Pepstain A	0.1	0.0 b	0
EDTA	5	0.839 ± 0.01	91.86 ± 0.98
	10	0.757 ± 0.028	82.94 ± 3.09
	50	0.098 ± 0.004	10.73 ± 0.48

\*Letters indicate significant differences between samples as determined by t-test (independent samples,  $p \leq 0.05$ )

#### 4.4.4 Effect of ethanol and sugar

The effect of ethanol and sugar (glucose and fructose to a 1:1 ratio) was investigated on the proteolytic activity of the crude extract. Experiments were conducted at concentrations for ethanol and sugar resembling those of grape juice fermentation. The results are summarised in Table 4.3. Both ethanol and sugar exhibited an inhibitory effect on MpAPr1 activity. For both compounds, inhibition was more severe with increasing concentrations. Indeed, at 6% (v/v) ethanol, more than 50% of activity was lost and at higher concentrations of 12% (v/v) and 15% (v/v), up to 83% and 92% of activity was lost, respectively. Low sugar concentrations of 2 g/l had no significant effect on enzyme activity. However, at higher concentrations of 100 g/l and 200 g/l, activity decreased by 11% and 26%, respectively. A concentration of 300 g/l sugar inhibited activity by up to 40%.

**Table 4.3:** Summary of the effects of ethanol and sugar concentration (resembling those found during grape juice fermentation) on the proteolytic activity of the crude extract. Data shown are the means of three independent experiments with standard deviations shown after the activity value. The control (no added compounds) was defined as 100% activity.

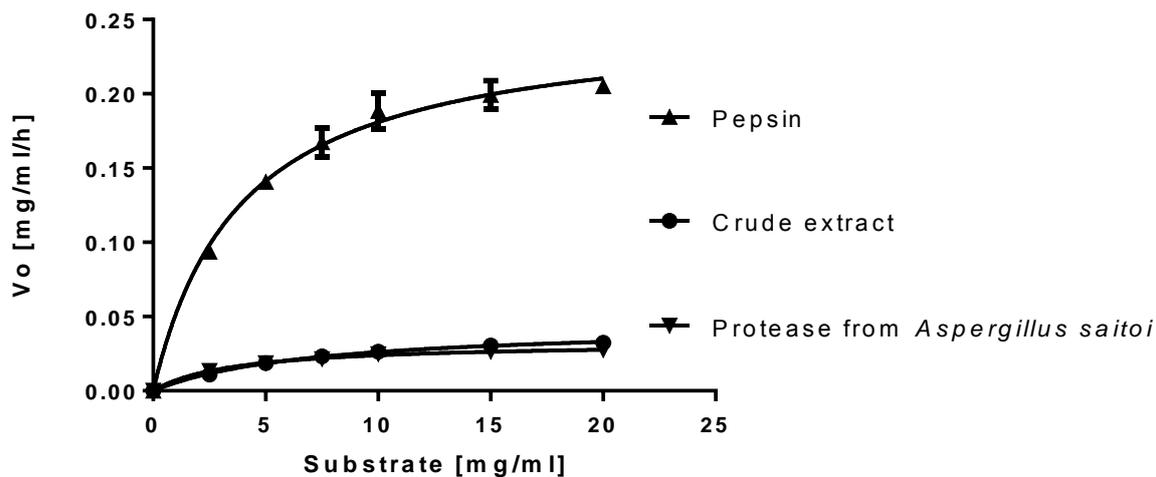
Compound	Concentration	Activity (AU)	Relative activity (%)
None		0.894 ± 0.043 a	100
Ethanol	6% (v/v)	0.426 ± 0.009 b	47.72 ± 2.22
	12% (v/v)	0.153 ± 0.003 c	17.11 ± 0.82
	15% (v/v)	0.070 ± 0.019 d	7.85 ± 2.05
None		0.842 ± 0.033 a	100
Sugar	2 g l <sup>-1</sup>	0.870 ± 0.045 a	103.31 ± 2.86
	100 g l <sup>-1</sup>	0.746 ± 0.042 b	88.49 ± 2.21
	200 g l <sup>-1</sup>	0.620 ± 0.049 c	73.57 ± 3.44
	300 g l <sup>-1</sup>	0.498 ± 0.038 d	59.09 ± 2.38

Letters indicate significant differences between samples as determined by t-test (independent samples,  $p \leq 0.05$ )

#### 4.4.5 Determination of kinetic constants on crude extract

Initial kinetic assessment of the proteolytic activity within the crude extract was performed using azocasein as a substrate under optimal pH (4.5) and temperature (40°C) conditions as determined above. Two commercial acid proteases, namely pepsin and the protease from *Aspergillus saitoi* were included in the experiment to serve as a basis for comparison of kinetic parameters  $K_m$  and  $V_{max}$ .

The parameters  $K_m$  and  $V_{max}$  were graphically calculated by plotting the velocity “v” of the reaction against the substrate concentration “s” (Figure 4.8) using GraphPad Prism computer software. Kinetic parameters obtained are summarised in Table 4.4. The Michaelis constant of MpAPr1 was 1.81 times and 1.93 times higher than that of pepsin and the protease from *Aspergillus saitoi*, respectively. Moreover, the  $V_{max}$  value of MpAPr1 was 5.64 times less than that of pepsin but 1.36 times more than that of the protease from *Aspergillus saitoi*.

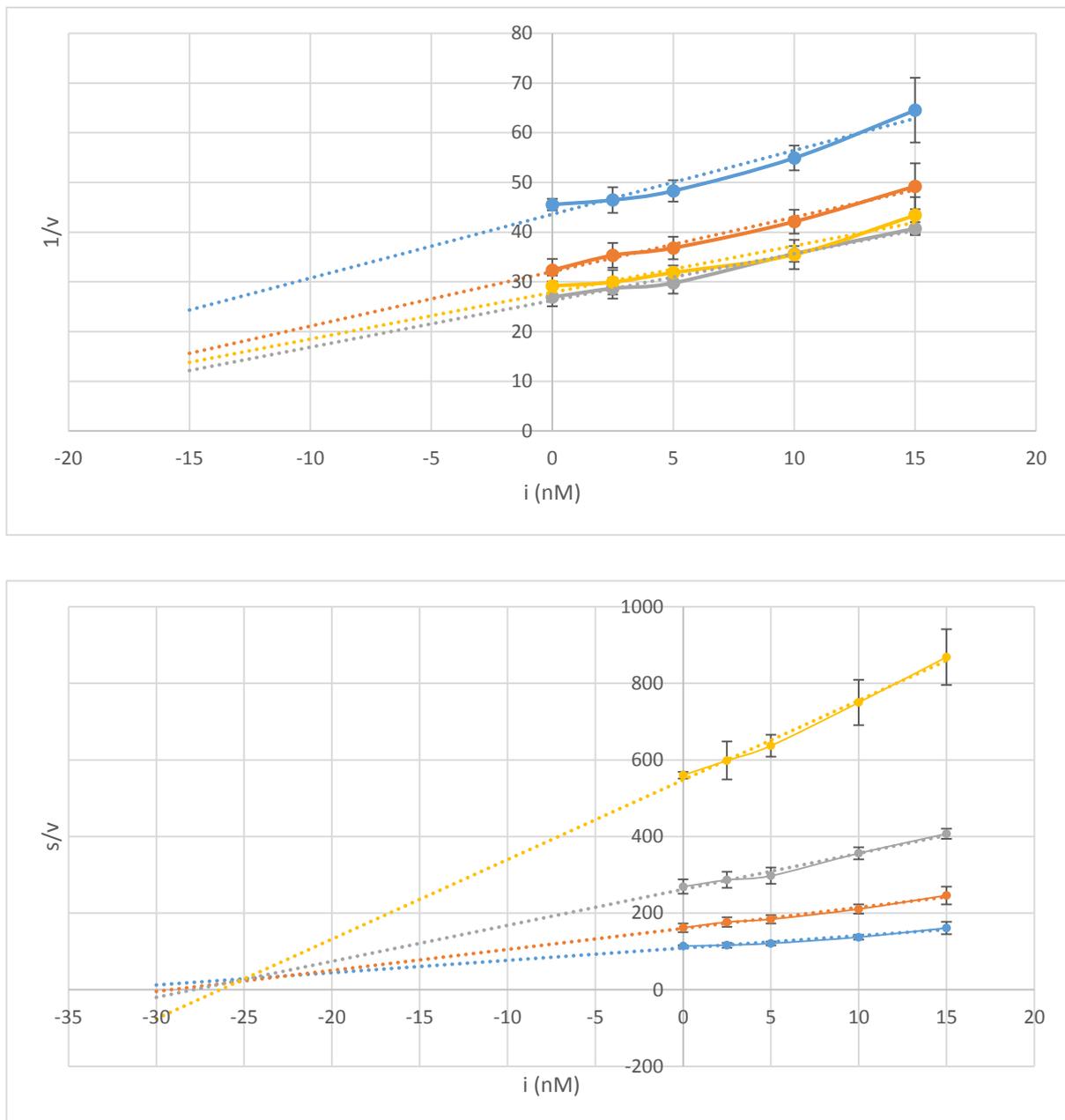


**Figure 4.8:** Plots of  $v$  against  $s$ . The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates.

**Table 4.4:** Summary of  $K_m$  and  $V_{max}$  values as calculated through GraphPad Prism computer software by plotting  $v$  against  $s$  (Figure 4.8).

Source	$K_m$ (mg/ml)	$V_{max}$ (mg/ml/h)
MpAPr1 within concentrated supernatant	$7.1 \pm 0.6026$	$0.045 \pm 0.0015$
Pepsin	$3.9 \pm 0.34$	$0.25 \pm 0.0067$
Protease from <i>Aspergillus saitoi</i>	$3.7 \pm 0.2281$	$0.033 \pm 0.0006$

Inhibition kinetics of the crude extract by pepstatin A was determined under the same conditions as above (i.e. optimal pH and temperature conditions using azocasein as a proteolytic substrate). Through varying substrate and inhibitor ( $i$ ) concentrations and keeping enzyme concentration constant the  $K_i$  could be calculated graphically by plotting  $1/v$  against  $i$  (Dixon plot) and plotting  $s/v$  against  $i$  (Figure 4.9).  $K_i$  was calculated to be  $-25.4$  (nmol/l/h).



**Figure 4.9:** Plots of  $1/v$  against  $i$  (Dixon plots) and  $s/v$  against  $i$ . The intersection point in the plot  $s/v$  against  $i$  provides a measure of  $K'_i$ . The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates.

#### 4.4.6 Discussion and partial conclusion

MpAPr1 protease activity within the crude extract concentrated by ultrafiltration was first assessed via its ability to clot milk at 30°C. Indeed, the ability to clot milk has been identified as a common characteristic of all aspartic proteases (Kumar et al. 2005). As visualised in Figure 4.6, the crude extract containing MpAPr1 clotted the milk while no observable clotting could be observed in samples treated with the concentrated supernatant from untransformed *K. pastoris* X33. This result confirmed the presence and activity of MpAPr1 within the crude

extract and was therefore used in downstream experiments to characterise its enzymatic properties. Furthermore, it was decided not to remove the hexa-histidine tag (using thrombinase) as the activity was considered high enough when compared to that of the natural host (Figure 4.4) and to that of a commercially available enzyme (Figure 4.6).

In order to perform further characterisation experiments, a chromogenic substrate was used, azocasein. The degradation of this substrate leads to the release of free dye which was quantified by colorimetry. Proteolytic activity of the crude extract was found to be favoured at values between pH 3.5 and 5 with maximum activity displayed at pH 4.5, above which activity decreased drastically. At pH 3, relative activity decreased to as low as 10%, but this is a questionable result as most acid proteases display activity at this pH and even lower pH values (Siala et al. 2009, Li et al. 2010, Hsiao et al. 2014). The decrease in activity may rather be ascribed to substrate limitation as the solubility of casein decreases with lower pH. Indeed, it was found that azocasein did not dissolve at pH 3.0 during the assay explaining the result obtained. In order to overcome this substrate limitation and test activity at low pH values haemoglobin can be used as a substrate. However in this study only azocasein was used throughout in order to compare with downstream experiments (haemoglobin is also not available in South Africa at this time). In general, the pH optima for aspartic proteases from fungal species is in the pH range 4 to 4.5 (Rao et al. 1998, Theron and Divol 2014) but some have been reported to exhibit optimum pH values in a broader range (between 3 and 5.5). Examples include acid proteases from fungi such as *Penicillium camembertii* (pH 3.5) (Chrzanowska et al. 1995) and *Rhizopus oryzae* (pH 5.5) (Kumar et al. 2005) and *Aspergillus niger* NRRL 1785 (pH 4) (Olajuyigbe et al. 2003). An acid protease from *Aspergillus niger* I1 had also been isolated and shown to exhibit an optimal pH of 3 but its activity significantly decreases below and above this pH (Siala et al. 2009). It was observed that similar pH optima have also been found for other non-*Saccharomyces* yeast species. The rSAP6 aspartic protease from *Metschnikowia reukauffii* has a pH optimum at 3.4 (Li et al. 2010). Furthermore, the pH optimum of five acid proteases (Sap 1, Sap1, Sap4, Sap5 and Sap6) from *Candida albicans* (Aoki et al. 2011) and Cap1 from *Cryptococcus* spp. S-2 (Rao et al. 2011) is 5. An acid protease produced by *Saccharomyces lipolytica* CX161-1B had been found to exhibit an optimal pH of 4.2 (Yamada and Ogrzydziak 1983). Overall, the acceptable and optimum pH range where MpAPr1 displayed activity is similar to that of other yeast and fungal aspartic proteases. Activity at lower pH values is a sought-after property especially for application in the wine, brewing and cheese making industries.

Elevated temperatures of 60°C and above had a severe effect on enzyme activity. Maximum activity was observed at 40°C while lower temperatures affected activity negatively. Identical temperature optimum has been found for the rSAP6 protease from *M. reukauffii* (Li et al. 2010) and similar results have also been found for aspartic proteases from other yeast

species. Indeed, the Cap1 from *Cryptococcus* sp. S-2 showed maximal activity between 30°C to 40°C (Rao et al. 2011). Acid proteases from fungi *Amylomyces rouxii* (Marcial et al. 2011) *Trichoderma harzianum* (Liu and Yang 2007), *Neosatorya fischeri* var. *spinosa* IBT 4872 (Wu and Hang 1998) and *Aspergillus niger* NRRL 1785 (Olajuyibe et al. 2003) also showed optimal activity between 40°C and 50°C. In contrast to MpAPr1, optimum temperature from other fungi such as *R. oryzae*, *P. duponti* K1014 (Hashimoto et al. 1973), *P. oxalicum* (Hashem 1999) and *A. niger* I1 (Siala et al. 2009) display maximum activity between 50°C and 60°C. Generally, in the wine making and brewing processes, lower temperatures are used during the course of fermentation while cheese making involves temperatures closer to that of MpAPr1 optimum temperature. Once optimal pH and temperature were established, further characterisation experiments were conducted at these set parameters.

Metal ions, chosen for their high frequency in several beverages such as grape juice, wort etc., were tested at a final concentration of 1 mM as a standard basis for comparison with literature. The presence of FeCl<sub>2</sub> CaCl<sub>2</sub>, MgCl<sub>2</sub> and ZnCl<sub>2</sub> had no effect, while MnCl<sub>2</sub> had a slight inhibitory effect on proteolytic activity. These findings are similar to those found for other aspartic proteases (Sharma et al. 2005, Siala et al. 2009, Hsaio et al. 2014) with the exception of Fe<sup>2+</sup> that had a strong activating effect on an acid protease purified from *A. niger* BCRC 32720 (Alessandro and Federico 1980). Furthermore, both NiCl<sub>2</sub> and CuCl<sub>2</sub> had a strong inhibitory effect on proteolytic activity. Several other acid proteases from species such as: *P. vivax*, *C. albicans*, *M. reukauffi* and *Aspergillus niger* are also inhibited by Cu<sup>2+</sup> (Alessandro and Federico 1980, Sharma et al. 2005, Siala et al. 2009, Li et al. 2010). Generally, the effects that metal ions have on the activity of MpAPr1 are similar when compared to other sources. Moreover, Cu<sup>2+</sup> and Ni<sup>2+</sup> levels found in winemaking are generally low and therefore should not have an impact on MpAPr1 activity. Considering the mean concentrations of the metal ions tested in grape juice, milk and wort the results suggest that MpAPr1 would be suitable for application in these matrices.

Proteolytic activity of the crude extract was strongly inhibited by pepstatin A, a hexapeptide inhibitor that specifically and irreversibly binds in the active site within the aspartic protease, at a concentration of 0.1 µM. Two aspartic proteases isolated from fungal sources, *Aspergillus niger* I1 and *Rhizopus oryzae* was completely inhibited at low concentration of 1.5 µM (Siala et al. 2005) and 1 µM (Hsiao et al. 2014), respectively. Furthermore, aspartic proteases isolated from bio-trophic fungi involved with plant infections, APSm1 and Eap1 from *Stenocarpella maydis* (Mandujano-González et al. 2015) and *Sporisorium reilianum* (Mandujano-González et al. 2013), respectively, was completely inhibited at a final concentration of 5 µM. Conversely, a more resistant aspartic protease from the malarial parasite *Plasmodium vivax* could still display 15% activity at concentrations of 10 µM (Hsiao et al. 2014). Thus the concentration at which inhibition occurs seems to vary between aspartic

proteases from different sources. Because of its sensitivity to pepstatin, ability to clot milk along and with activity against azocasein, it is proposed that MpAPr1 be classified as part of the pepstatin-sensitive aspartic proteases. Moreover, other protease inhibitors, such as PMSF (serine protease), iodoacetic acid (cysteine protease) and DON (threonine protease), could be included in a follow-up experiment to ensure its specific inhibition by pepstatin A.

In an attempt to investigate if the enzyme was metal-dependent, the chelating agent EDTA was added to the substrate prior to protease treatment. The presence of EDTA had an inhibitory effect on activity which became more evident with increasing concentrations. Similar result to that obtained for treatment with lower concentrations of EDTA (5 mM and 10 mM) has been found for other aspartic proteases such as those of *Plasmodium vivax* (Sharma et al. 2005), *Rhizopus oryzae* (Hsaio et al. 2014), *Aspergillus niger* I1 (Siala et al. 2005), *Stenocarpella maydis* (Mandujano-González et al. 2015) and *Sporisorium reilianum* (Mandujano-González et al. 2013). At 50 mM, EDTA had a very strong inhibitory effect on MpAPr1 activity. Although very few studies tested EDTA at such high concentrations, it is in contrast with an aspartic protease obtained from *Plasmodium vivax* where the treatment with 50 mM EDTA had only slight inhibitory effects (Sharma et al. 2005). In our study, the potential inhibition of MpAPr1 through the presence of EDTA was assessed through the addition of the compound to the reaction. Thus inhibition was most likely due the increase in ionic strength interfering with ionic bonds causing denaturation or inhibition of the active site. Indeed, most enzymes are not tolerant to high salt concentrations above 500 mM. Indeed low salt concentration are generally found in wine, beer and milk and activity should thus not be affected by the ionic strength found in these matrices.

With the focus on possible applications in the wine making environment, the impact of sugar and ethanol concentrations resembling those occurring during grape juice fermentation was tested. Indeed, both high levels of sugar and ethanol had an inhibitory effect on the proteolytic activity of MpAPr1 but at moderate to low concentrations, activity was retained to an acceptable degree. Furthermore, the effect of ethanol was more severe (leading to a more than 50% decrease at only 6% (v/v)) than when compared to sugar which was more subtle leading to a decrease in activity of only 26.43% at 200 g/l (>50%, total sugar). Overall, the results suggest that the enzyme treatment be applied at the beginning of fermentation in order to give the enzyme the best advantage to degrade proteins.

Kinetic constants  $K_m$  and  $V_{max}$  were calculated for the crude extract using azocasein as a substrate to complete MpAPr1 properties. In order to interpret these properties and compare them in a more sensible way, two commercial proteases, pepsin and the protease from *Aspergillus saitoi*, were also included. Not much literature has been published on these parameters and comparison with MpAPr1 is made even more difficult because of inconsistencies in the use of various substrates. Nevertheless, the results indicate that when

compared to the other 2 aspartic proteases tested, MpAPr1 has the lowest affinity for the substrate and catalyses the reaction at a similar rate as the protease from *Aspergillus saitoi*.

The  $K_i$  was determined graphically by Dixon plots and methods proposed by Cornish Bowden (Cornish Bowden 1973). Both methods were used because the Dixon plot does not always distinguish competitive from mixed inhibition whereas the plot  $s/v$  against  $i$  does not always distinguish uncompetitive from mixed inhibition. Thus, if both plots are used the pattern of results provides an indication of the type of inhibition. In this case, the results indicate that pepstatin is an uncompetitive inhibitor to MpAPr1. In contrast, it has been found that pepstatin is a competitive inhibitor of pepsin (Marciniszyn et al. 1976).

It should be considered that in all of the above mentioned characterisation experiments, although proper controls were used throughout, both the substrate and protease may be affected by environmental factors. For instance, the presence of other proteins within the crude extract might influence the substrate or even with MpAPr1 activity (lowering activity against azocasein). Sugar and ethanol might also make the substrate inaccessible for protease degradation therefore resulting in false negatives.

As a side note, commercial rennet preparations used in the making of cheese usually contain unwanted tertiary proteolytic activity arising from aspartic proteases, usually chymosin (leading to too much activity). This leads to the degradation of curd proteins which can in turn result in the formation of bitter peptides and dissolution of the curd. Another unwanted feature somewhat linked to the former is the high thermal stability of aspartic proteases used which allows for extended action on milk proteins after coagulation has occurred resulting in low cheese yields and poor quality (Sousa et al. 2001, Yegin et al. 2010). This is particularly true in the making of soft and semi-hard cheeses (Hynes et al. 2001). An alternative to the technical challenges described above is the identification of milk clotting enzymes with higher specificity and reduced tolerance to high temperatures. The ideal situation would result from a protease enzyme with limited proteolytic activity when compared to that of chymosin (either with a slower reaction rate and/or more specific not degrading random proteins and peptide, and subsequent rapid destruction of the enzyme in downstream operation (such as heating) (Fernandez-Lahore et al. 1999). Thus although not its initial intended use, MpAPr1 might perform well within the dairy (cheese) industry however this hypothesis remains to be further explored.

Considering the composition of different potential matrices (i.e. grape juice, wort, milk etc.), MpAPr1 should display moderate activity considering no other parameters than pH, temperature, ions, sugar and ethanol are taken into account. However, the targets to be degraded in these matrices are not azocasein and therefore activity of MpAPr1 need to be tested against these specific targets. Nevertheless, the properties found for MpAPr1 suggest that it be a suitable candidate for further experimentation in order to elucidate its potential use

in industrial applications and in particular wine making. The remainder of this thesis will focus on grape proteins and the potential application of MpAPr1 in grape juice/fermenting grape juice. To achieve this, MpAPr1 needed to be purified first.

#### **4.5 Purification and analysis performed using MpAPr1**

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This paragraph relates the various purification attempts that were required to obtain a pure recombinant MpAPr1. The different trials are summarised in Table 4.5 and detailed below. Briefly, purification was attempted using both IMAC and cation exchange chromatography. Purification was performed on supernatant obtained from cultures grown either in rich medium (1% yeast extract, 2% peptone and 4% glucose) hereinafter referred to as SRM, or in minimal medium as described in paragraph 4.5.2.1, hereinafter referred to as the SMM. Different columns and chromatography systems were used until purification was achieved. After a pure enzyme was obtained, the kinetic parameters ( $K_m$  and  $V_{max}$ ) were determined and compared to those determined in the crude extract (paragraph 4.4.5).

**Table 4.5:** Summary of the different trails performed to obtain pure MpAPr1.

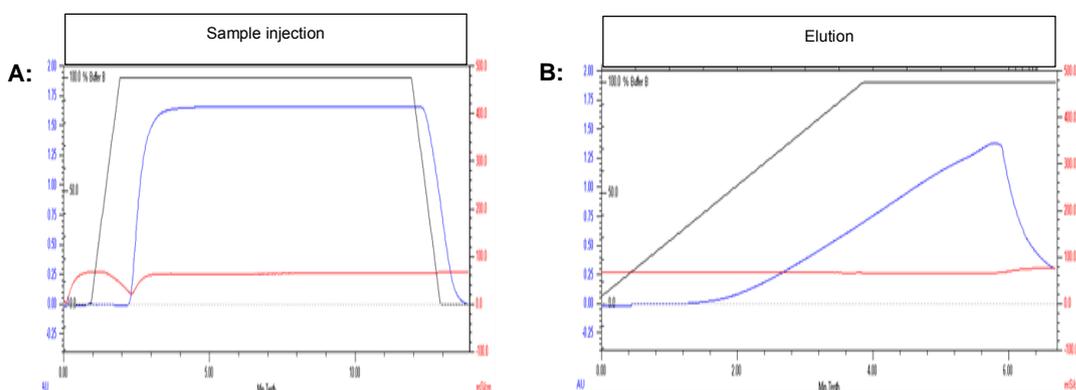
Technique	Apparatus	Column	Method	Medium	Buffers	Conclusion	
IMAC	BioLogic DuoFlow™ Chromatographic System	1-ml HiTrap IMAC HP (NiCl <sub>2</sub> )	10 ml sample injection with a 30 ml elution gradient	SRM	Buffer 1 (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.5) Buffer 2 (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.5)	After elution no proteins could be detected via SDS-PAGE analysis. Furthermore, the presence of substances interfering with optical density readings was noted. No binding to the columns occurred and the method was deemed unsuccessful.	
		5-ml HiTrap IMAC HP (NiCl <sub>2</sub> )	20 ml sample injection with a 50 ml elution gradient	SRM concentrated 10x (30 kDa*)			
		1-ml HiTrap IMAC HP (NiCl <sub>2</sub> )	10 ml sample injection with a 30 ml elution gradient				
		1-ml HiTrap IMAC HP (CoCl <sub>2</sub> )					
		1-ml HiTrap IMAC HP (ZnCl <sub>2</sub> )					
Cation exchange chromatography	BioLogic DuoFlow™ Chromatographic System	1-ml HiTrap SP HP	20 ml sample injection with a 30 ml elution gradient	SRM Concentrated 10x (30 kDa*)	Buffer A (20 mM Mcllvaine's buffer at pH 3.0) Buffer B (20 mM Mcllvaine's buffer at pH 3.0, 1 M NaCl <sub>2</sub> )	Purification of MpAPr1 from SMM-Op-30C was successful, but the presence of two faint bands corresponding to MpAPr1 was observed.	
		Five 1-ml HiTrap SP HP connected in series	20 ml sample injection with a 50 ml elution gradient	SMM Concentrated 10x (30 kDa*)		SMM Concentrated 10x (10 kDa*)	Purification was successful and single band could be observed, but the apparatus used was too slow for purification of high amounts of enzyme
	BioLogic LP™ Low-Pressure Chromatography System						NGC™ Chromatography System
				ÅKTA Pure Chromatography System			
	5-ml HiTrap SP HP		10 ml sample injection with 100 ml elution gradient				Larger column volume resulted in satisfactory yields of ± 1 mg/ml per pure fraction obtained
	Two 5-ml HiTrap SP HP connected in series						

\* pore size used for ultrafiltration.

#### 4.5.1 Purification from rich medium

##### 4.5.1.1 Purification using IMAC

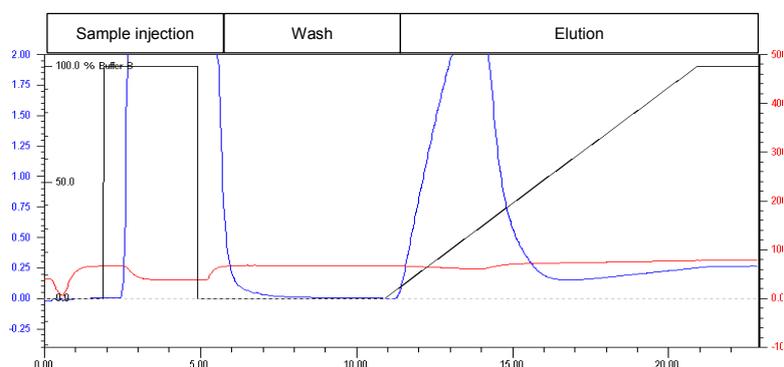
Purification was initially attempted using immobilised metal affinity chromatography (IMAC) using the BioLogic DuoFlow™ Chromatography System (Bio-Rad Laboratories). Initially, a 10 ml sample was loaded onto a 1-ml HiTrap IMAC HP column charged with NiCl<sub>2</sub>. The complete run from sample loading to elution (30-ml gradient) is illustrated in Figure 4.10. An elongated peak starting from 2 min into elution could be observed. The fractions corresponding to this peak were collected and analysed through BCA protein assay and SDS-PAGE (data not shown). Although high protein concentrations (>1 mg/ml) were quantified with the assay, no bands could be observed following SDS-PAGE analysis. This suggested that certain substances present in the medium interfered with the BCA protein determination results, either absorbing at 562 nm and/or reacting with the working reagent of the kit. Purification was unsuccessful and further optimisation thus required.



**Figure 4.10:** IMAC chromatogram obtained during initial purification conditions (10 ml SRM loaded onto a 1-ml HiTrap IMAC HP column). Panel A indicates sample application and panel B sample elution. Note that the buffer B line (represented by the black line) was used for sample loading shown in panel A. Furthermore, absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and the buffer B line (%) in black.

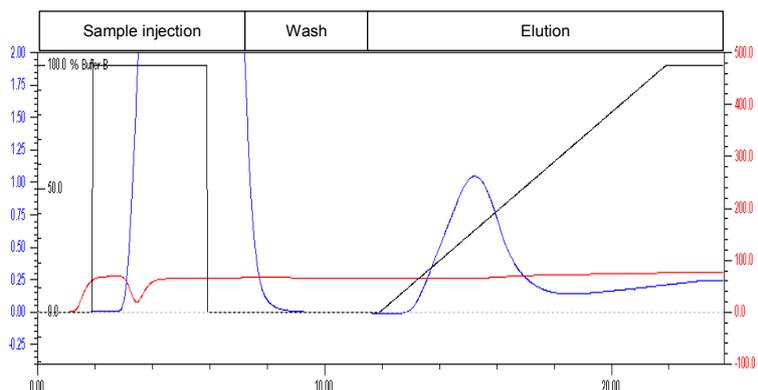
Since purification was unsuccessful, it was decided to concentrate the SRM via ultrafiltration using a 30 kDa filter while simultaneously buffer-exchanging with Buffer 1. The same procedure for purification described above was followed and the complete run from sample loading to completion is illustrated in Figure 4.11. At sample loading (from 2 min to 5 min) the detection limit for optical density (at 280 nm) was quickly reached. A somewhat sharper and large peak also reaching detection limit could be observed at elution between 11.5 min and 15 min. Fractions were collected under the elution peak area and analysed via BCA protein determination and SDS-PAGE. Once again, protein quantification revealed high

concentrations (> 1 mg/ml) in all fractions, but when visualised on SDS-PAGE, no bands could be observed. At this point, it was suspected that the column might be faulty.



**Figure 4.11:** IMAC chromatogram showing purification profile of concentrated SRM (10 ml was injected onto a 1-ml HiTrap IMAC HP column). Absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and buffer B line (%) in black.

The experiment was repeated as described above, but using a 5-ml HiTrap IMAC HP column (charged with  $\text{NiCl}_2$ ) and injecting 20 ml concentrated (30 kDa) SRM. The complete run from sample application to completion is illustrated in Figure 4.12. Similarly, upon sample injection detection limit was quickly reached. However, a smaller hyperbolic peak could be observed at sample elution between 12.5 min and 14.5 min. Fractions collected under the peak area were investigated by means of BCA protein determination and SDS-PAGE analysis. However, as described in the above paragraph results were inconclusive and no bands could be observed. High protein estimations obtained from BCA protein determination method were ascribed to interfering compounds, derived from the yeast extract within the SRM, resulting in an overestimation of protein concentrations within samples. It was concluded that the 1-ml HiTrap IMAC HP column was indeed functional and that metal ions, other than  $\text{Ni}^{2+}$ , should be investigated to charge the column for protein binding.



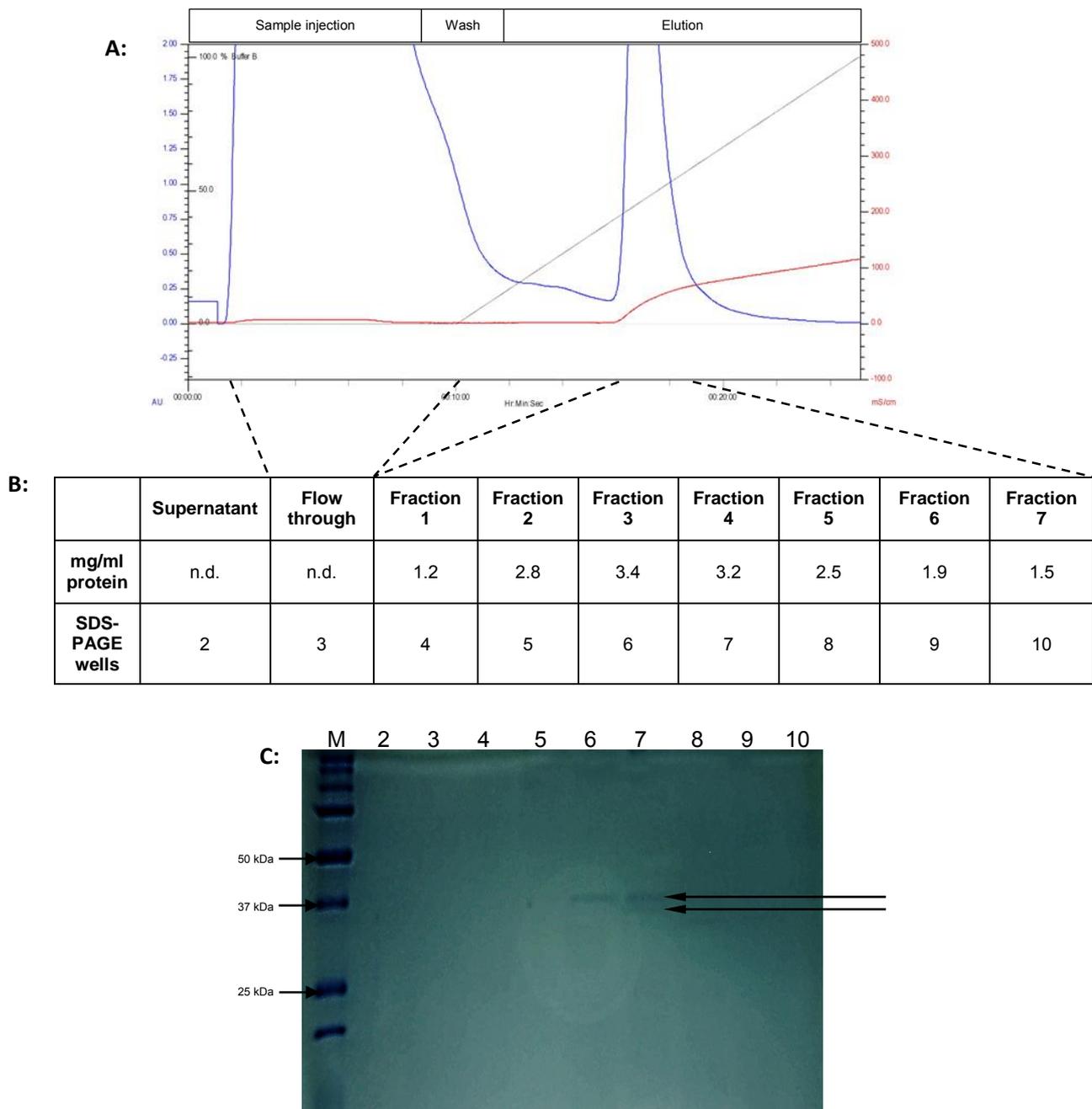
**Figure 4.12:** IMAC chromatogram showing purification profile of concentrated SRM (20 ml was injected onto a 5-ml HiTrap IMAC HP column). Absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and the buffer B line (%) in black.

As a final attempt with IMAC, it was decided to utilise two different metal ions (other than  $\text{Ni}^{2+}$ ) for charging the column prior to sample loading. Between purification attempts, the 1-ml HiTrap IMAC HP column was stripped and recharged with  $\text{CoCl}_2$  and  $\text{ZnCl}_2$ , separately. However, despite several attempts, purification remained unsuccessful and similar results to those described above were obtained. It was hypothesised that either the histidine tag was inaccessible or that binding was outcompeted by contaminating compounds. Therefore, it was decided to investigate a different technique such as ion exchange chromatography that relies on the charge of the protein rather than a fused amino acid tag.

#### 4.5.1.2 Preliminary attempts to purify MpAPr1 using ion exchange chromatography

Initially, in order to conduct purification using cation exchange chromatography, the BioLogic DuoFlow™ Chromatography System (Bio-Rad Laboratories) was used. Initially a 20 ml sample was loaded onto a 1-ml HiTrap SP HP column after which elution was performed over a 30-ml gradient using Buffer B (20 mM McIlvaines's buffer at pH 3.0, 1 M NaCl). The complete run from sample application to completion, BCA protein determination and SDS-PAGE analysis are illustrated in Figure 4.13. Upon sample application, detection limit was quickly reached and column washing was not complete when the elution gradient started. Between 16 min and 19 min, a fairly sharp peak can be observed that is above detection limit. Fractions corresponding to the elution peak were collected and protein concentration was determined (Figure 4.13, B). The highest concentrations (>3 mg/ml) were observed in fractions 3 and 4. Furthermore, fractions were loaded onto an SDS-PAGE gel according to the fraction number indicated in the table (Figure 4.13, C). Two faint bands could be observed in lanes 6 and 7 (i.e. fractions 3 and 4, respectively) that corresponded to the theoretical expected size of MpAPr1 (43.3 kDa). Indeed, the experiment displayed results indicating that protein binding

and elution were being achieved, albeit protein concentrations were very low as confirmed by SDS-PAGE analysis. High protein estimations observed when using the BCA protein determination kit were ascribed, once again, to contaminating compounds derived from yeast extract interfering with reagents used in this method. Furthermore, it was also determined that this/these compound(s) interfered with optical density readings at 280 nm and 562 nm and should thus be avoided in further purification experiments. Optimising expression of MpAPr1 in a minimal medium (medium containing as little as possible substances that might interfere with optical density readings and/or reagents used in protein estimation methods) was therefore essential in order to minimise interference before attempting purification anew.

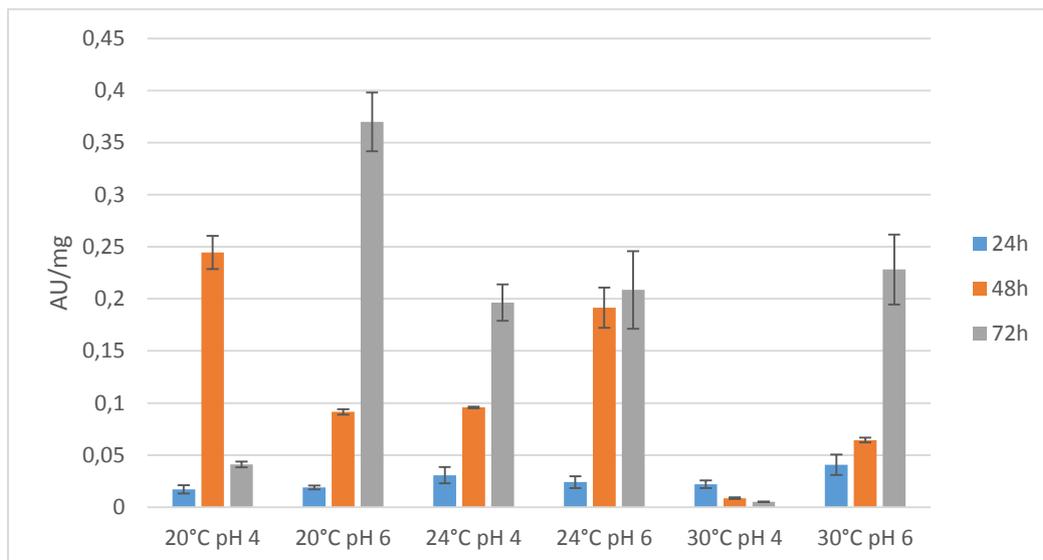


**Figure 4.13:** Summary of purification performed using cation exchange chromatography (20 ml concentrated SRM injected onto 1-ml HiTrap SP HP column). A: Chromatogram of run from sample application to completion: absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and the buffer B line (%) in black. B: Table summarising results obtained following BCA protein determination on fractions obtained and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing sample before application (lane 2), flow through (lane 3) and fractions obtained at elution (lanes 4-7). Two bands corresponding to MpAPr1 are indicated by the thin black arrows. Lane M: Molecular weight marker (Precision Plus Protein™ All Blue Prestained Protein Standard Bio-Rad).

## 4.5.2 Optimisation of expression media and cation exchange chromatography

### 4.5.2.1 Optimisation of MpAPr1 expression in minimal media

In an attempt to establish optimal expression conditions, recombinant *K. pastoris* X33 was grown in minimal expression medium at different pH (4 and 6) and temperature (20°C, 25°C and 30°C) conditions. The supernatant of all the samples was collected at different time points (24 h, 48 h and 72 h) and the total protein concentration along with the activity was determined as previously described. The specific protease activity in each sample was calculated and compared (Figure 4.14). The result shows that expression after 24 h was the highest at 30°C in pH 6, but was eventually considered low relative to activity displayed at longer incubation times of 48 h and 72 h. After 48 h of incubation under the various conditions, expression varied. At a pH of 4, the maximum specific activity was observed at 20°C and decreased significantly at increased incubation temperatures. At a pH of 6, the highest specific activity was observed at 25°C and lowest at 30°C. Expression after 72 h varied as well, but was generally higher than for the other incubation times (with the exception at a media pH of 4 and incubation temperatures of 20°C and 30°C). At a pH of 4 expression was favoured at 25°C. Maximum expression was found in a pH of 6 at 20°C while lower similar values could be observed at 25°C and 30°C. Consequently, the enzyme was produced under optimal expression conditions: 72 h incubation at 20°C in minimal medium adjusted to a pH of 6 prior to inoculation. The supernatant was collected and stored at 4°C until further purification experiments.



**Figure 4.14:** Specific activity (in AU/mg total proteins) calculated for supernatant samples taken at different time points from *K. pastoris* X33 cells (transformed with pGAPzαA-MpAPr1) incubated at different physicochemical conditions.

#### 4.5.2.2 Purification using cation exchange chromatography on different systems

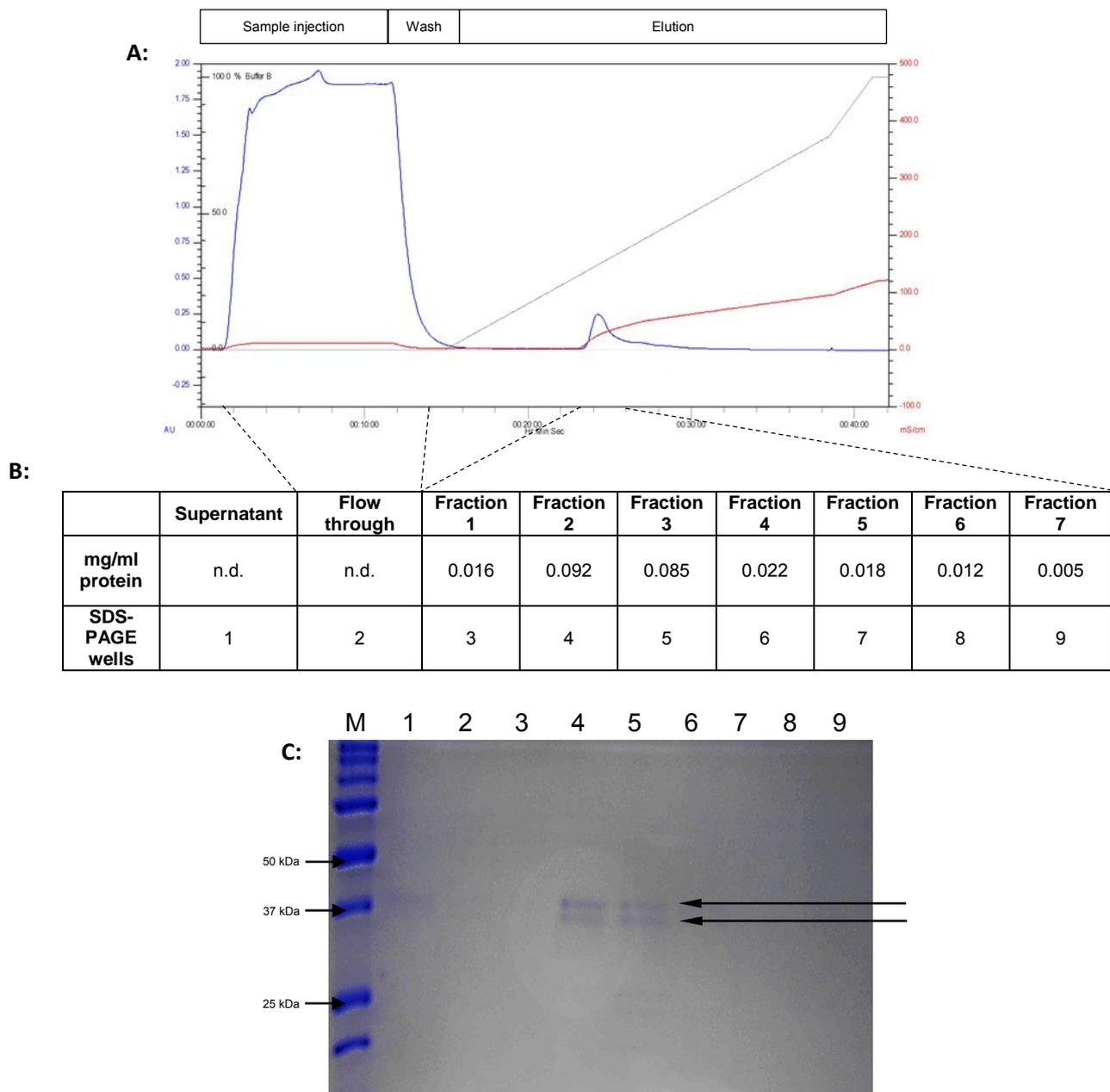
First attempts to purify MpAPr1 from SMM using cation exchange chromatography were performed using the BioLogic DuoFlow™ Chromatography System (Bio-Rad Laboratories). Although purification could be achieved on this system (using the optimised expression media), two bands were still present by the end of the trails. At this point in time, the author of this thesis had to return to South Africa. This meant that purification had to be tested and further optimised using a different system or machine to facilitate the process. Initially, at the Institute for Wine Biotechnology (IWBT), the BioLogic LP™ Low-Pressure Chromatography System was used to purify proteins. Purification could be achieved and resulted in a pure band corresponding to MpAPr1 (see below). However, this system was not optimal as purification runs took extended periods of time mainly due the peristaltic pumps used in this system to drive the purification process. It was decided to contact other departments within Stellenbosch University to find an alternative system as large amounts of MpAPr1 were required for downstream fermentation experiments. Indeed, the Biochemistry department had in their possession a Bio-Rad NGS Chromatographic system. The system uses pumps that can accommodate 10 ml/min and could thus easily achieve the necessary pressure and flow required. Indeed, the same results could be achieved and purification time was reduced, but because it was located in another department, logistics were sometimes troublesome. Fortunately, at this point in time, the IWBT received an ÄKTA Pure Chromatography System

for protein purification purposes. Final optimisation on purification of MpAPr1 from concentrated SMM was achieved using this system.

#### 4.5.2.2.1 Purification using Bio-Rad DuoFlow System

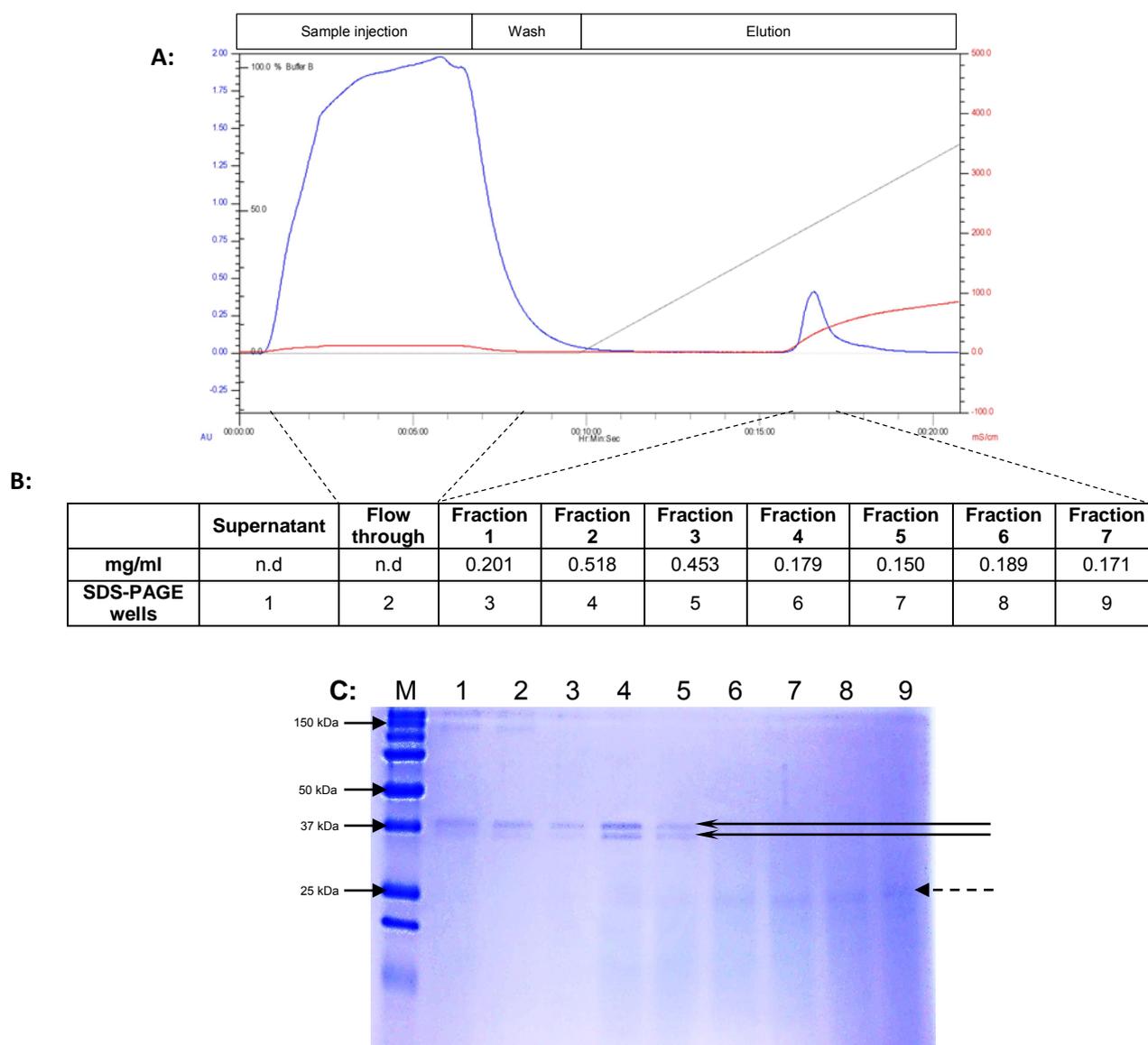
Prior to sample loading, in all experiments using the BioLogic DuoFlow™ Chromatography System (Bio-rad Laboratories), the supernatant obtained following optimal expression conditions (as described above in paragraph 4.5.2.1) was concentrated 10x via ultrafiltration using a 30 kDa cut-off filter. This concentrate will be referred as the SMM-Op-C30 hereinafter. Furthermore, samples were simultaneously buffer exchanged while concentrating with 20 mM Mcllvaine's buffer at pH 3. All samples and solutions were filtered through a 0.45- $\mu$ m membrane prior to their use in downstream purification experiments. The apparatus was set up in such a way that the sample was loaded using the buffer A line and elution was performed using the buffer B line (represented by a black line in the chromatograms).

Initially, 20 ml SMM-Op-C30 was loaded onto a 1-ml HiTrap SP HP column and eluted using a 30-ml gradient. A summary of the purification following BCA protein determination and SDS-PAGE analysis is illustrated in Figure 4.15. The chromatogram (Figure 4.15, A) shows a large elongated peak at sample loading (flow-through). A single peak at sample elution between 22 min and 26 min is observed reaching a maximum of 0.249 in  $A_{280nm}$ . Fractions were collected over the elution peak area and their respective concentrations determined (Figure 4.15, B). The highest concentrations were measured in fractions 2 and 3, respectively, with a maximum of 0.092 mg/ml in fraction 2. The supernatant, flow-through and fractions 1 to 7 were loaded onto a SDS-PAGE gel according to the table (Figure 4.14, C and B). Two distinct bands could be observed ca. 37 kDa corresponding to the expected size of MpAPr1 (43.3 kDa) in lanes 4 and 5 (fractions 2 and 3, respectively). Furthermore, when compared and evaluated, the results from BCA protein determination and SDS-PAGE analysis seem to match more closely. These results were regarded as encouraging and it was decided to increase column volume.



**Figure 4.15:** Summary of purification performed using cation exchange chromatography of 10x concentrated SMM-Op-30C (20 ml loaded onto a to 1-ml HiTrap SP HP column). A: Chromatogram of run from sample loading to completion: absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and Line B (%) in black. B: Table summarising results obtained following BCA protein determination on fractions obtained and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing sample before application (lane 1), flow through (lane 2) and fractions obtained at elution (lanes 3-9). Bands corresponding to MpAPr1 in lane 5 are indicated by the thin black arrows. Lane M: Molecular weight marker (Precision Plus Protein™ All Blue Prestained Protein Standard Bio-Rad).

In order to increase column volume five 1-ml HiTrap SP HP columns connected in series to which 20 ml sample was injected onto and eluted over a 50 ml gradient. The purification process of this concentrated sample (from sample application to completion) along with BCA protein determination and SDS-PAGE analysis are illustrated in Figure 4.16. The chromatogram (Figure 4.16, A) shows a single peak at elution between 16 min and 17 min reaching a maximum in  $A_{280}$  of 0.427. Fractions were collected over the elution peak area and the protein concentration was estimated using the BCA protein determination kit (Figure 4.16, B) and visualised via SDS-PAGE (Figure 4.16, C). Similarly as determined above, the highest concentrations were observed in fractions 2 and 3 displaying 0.518 mg/ml and 0.453 mg/ml, respectively. Samples were loaded onto an SDS-PAGE gel according to the indicated number in the table. The SMM-Op-30C (10X) showed the presence of four very faint bands, two at ca. 150 kDa and two corresponding to ca. 37 kDa. The supernatant and flow-through showed identical profiles in lanes 1 and 2, respectively. Fractions 1, 2 and 3 (in lanes 3, 4 and 5, respectively) show the presence of two distinct bands corresponding to MpAPr1 (indicated by the black arrows). Furthermore, fraction 2 displays the brightest bands indicating the highest concentration of proteins between fractions as corroborated by BCA protein determination results. In lanes 6, 7, 8 and 9 (representing fractions 4, 5, 6 and 7, respectively), a band at ca. 25 kDa can be observed (shown by a dashed arrow in Figure 4.16) that is not visible within the sample before sample application. Nevertheless, time constraints did not allow for further optimisation using this system (at the University of Bordeaux, France) and new systems had to be optimised (at Stellenbosch University, South Africa).

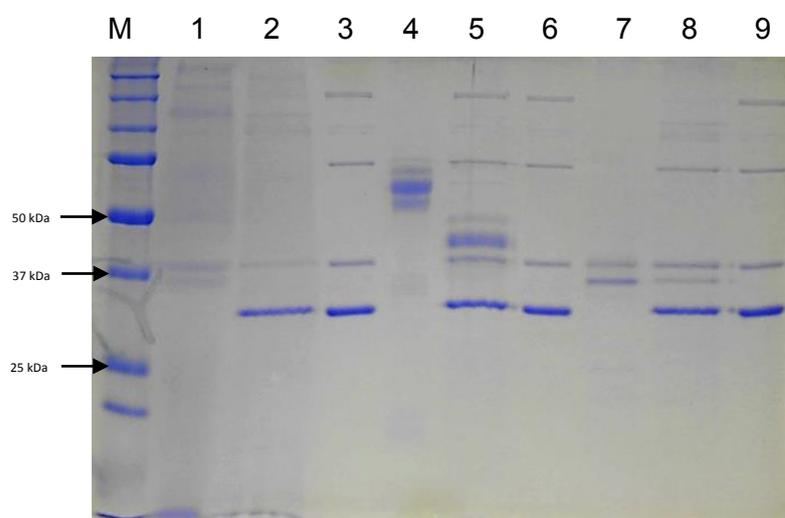


**Figure 4.16:** Summary of purification performed using cation exchange chromatography of 10x concentrated SMM-Op-30C (20 ml loaded onto five 1-ml HiTrap SP HP columns connected in series). A: Chromatogram of run from sample loading to completion: absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and Line B (%) in black. B: Table summarising results obtained following BCA protein determination on fractions obtained and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing sample before application (lane 1), flow through (lane 2) and fractions obtained at elution (lanes 3-9). Bands corresponding to MpAPr1 in lane 5 are indicated by the thin black arrows. Lane M: Molecular weight marker (PageRuler™ Prestained Protein Ladder).

#### 4.5.2.2.2 Deglycosilation

Prior to optimising a different system, it was decided to test the hypothesis discussed in paragraph 4.3.4 regarding the nature of the 2 bands as 2 differently glycosylated isoforms of MpAPr1. Indeed, one potential region for N- glycosylation has previously been tentatively

identified within MpAPr1 sequence (Reid et al. 2012). In order to investigate this hypothesis, the concentrated supernatant and fraction 2 obtained from cation exchange chromatography (from above mentioned purification trail) was de-glycosylated by enzymatic means and visualised via SDS-PAGE (Figure 4.17). The de-glycosylation enzymes (i.e. a mixture of N-deglycosylases and O-deglycosylases) were able to completely de-glycosylate Fetuin, a positive control included in the experiment (lane 4 and 5). Results obtained for the supernatant are unclear and bands are too faint to draw conclusions (lanes 1 and 2). It should be noted that one of the deglycosylation enzymes display the same molecular weight as the top band identified as MpAPr1, to be specific Neuraminidase (also known as Sialidase) since it has an approximate molecular weight of 43 kDa. Nevertheless, no difference in the bands in fraction 3 could be observed before (lane 7) and after (lane 8) treatment with deglycosylation enzymes.

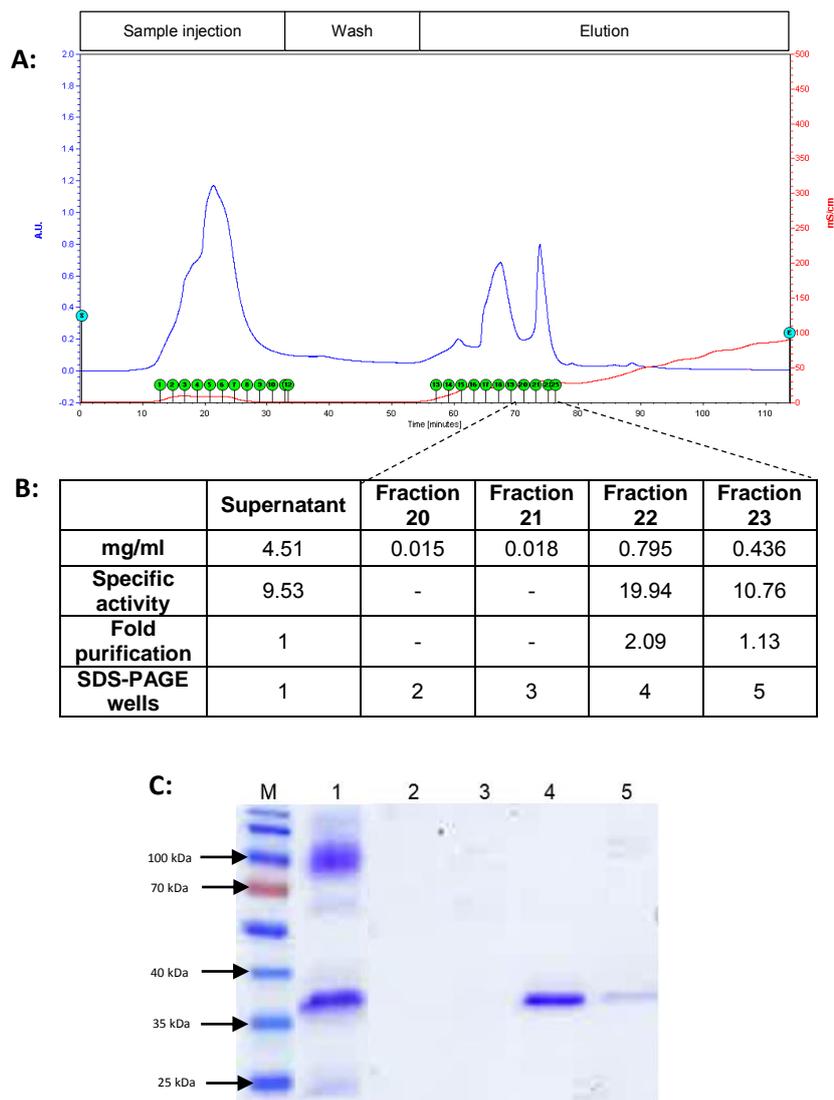


**Figure 4.17:** SDS-PAGE of de-glycosylation assay. Lane 1: Concentrated supernatant, lane 2: Concentrated supernatant treated with de-glycosylation enzymes, lane 4: Fetuin (control), lane 5: Fetuin treated with deglycosylation enzymes, lane 7: fraction 3, lane 8: fraction 3 treated with deglycosylation enzymes. Lanes 3, 6 and 9: deglycosylation enzymes. Lane M: molecular weight marker (Precision Plus Protein™ All Blue Prestained Protein Standard Bio-Rad).

#### 4.5.2.2.3 Purification on BioLogic LP™ Low-Pressure Chromatography System

Upon returning to the IWBT, MpAPr1 was produced in optimised conditions as determined above, but note that although the same chemicals and stocks were used some were from different batches and manufacturers. 20 ml SMM-Op-10C was injected onto five 1-ml HiTrap SP HP columns connected in series and eluted over a 50-ml gradient. The complete run from sample application along with analysis is summarised in Figure 4. 19. Over the course of elution (starting at 55 min), one small peak and two large peaks could be observed (Figure

4.18, A). Fraction 22 (0.795 mg/ml) had the highest protein concentration as estimated by BCA protein determination (Figure 4.18, B). Enzyme purity in this fraction was confirmed as visualised on SDS-PAGE gel. Unlike with previous attempts a pure enzyme could be obtained and a single band migrated as a single with an apparent molecular weight of ca. 40 kDa (Figure 4. 18, C). Furthermore, after purity was confirmed activity assays were performed using azocasein as substrate in order to calculate specific protease activity within the samples. It was found that when compared to the SMM-Op-10C, fraction 22 displays a 2.09 fold in purification. Although purification to the point where only one band corresponding to MpAPr1 could be visualised on SDS-PAGE gels, could be achieved using this system it was not efficient to yield high amounts of pure enzyme due to the time taken per purification run. Long purification runs were mainly due to the peristaltic pumps being unable to pump fast enough and maintain the appropriate pressure. It was thus decided to look for another more modern purification system in order to speed up production.

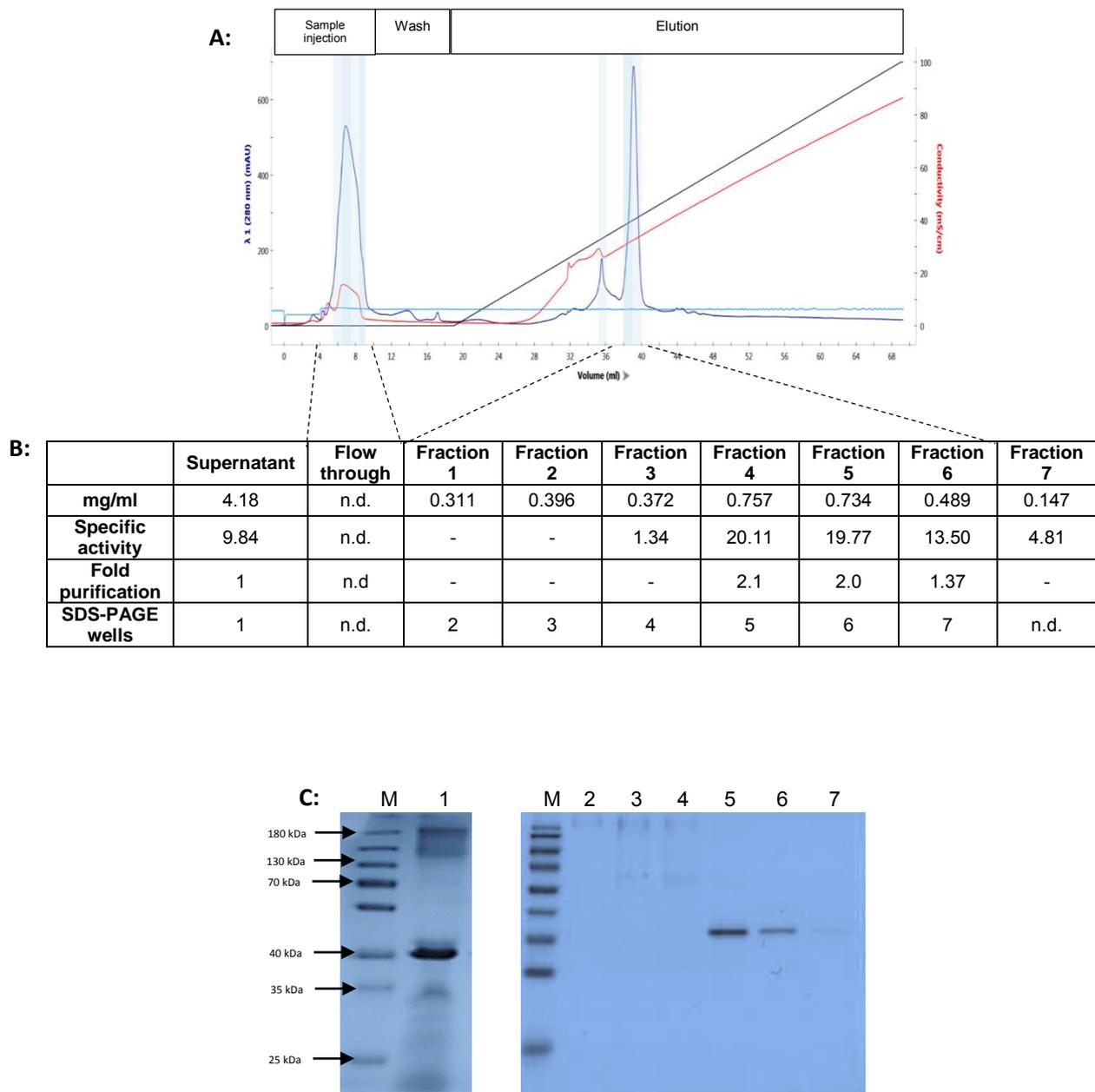


**Figure 4.18:** Summary of purification performed on the BioLogic LP™ Low-Pressure Chromatography System using cation exchange chromatography (20 ml SMM-Op-10C injected onto five 1-ml HiTrap SP HP columns connected in series). A: Chromatogram of run from sample injection to completion: absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and Line B (%) in black. B: Table summarising analysis and of samples obtained following BCA protein determination (mg/ml), specific activity (AU/mg) and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing sample before application (lane 1) and fractions obtained at elution (lanes 2-5). Lane M: Molecular weight marker (PageRuler™ Prestained Protein Ladder).

#### 4.5.2.2.4 Purification using the NGC™ Chromatographic System

At the time of this assay, the Biochemistry department acquired a NGC™ Chromatographic System for protein purification purposes. A 20-ml sample was injected onto five 1-ml HiTrap columns connected in series and eluted over a linear gradient of 50 ml. The complete run from sample application to completion along with protein estimation, specific activity calculation and

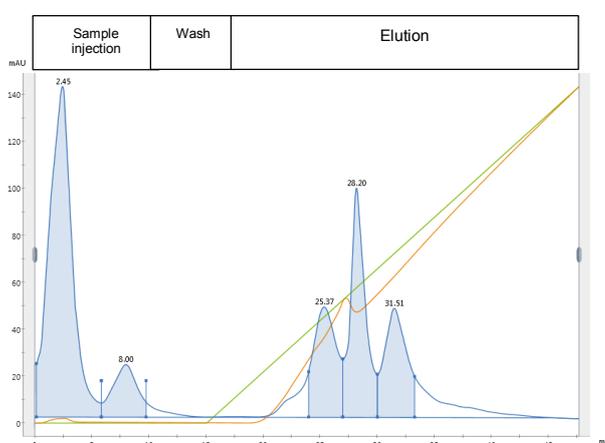
SDS-PAGE analysis are illustrated in Figure 4.19. Two peaks are observed at elution, the first being much smaller than the second sharp peak. The protein concentrations as determined by the BCA protein determination kit of the sample before injection and fractions obtained are shown in Figure 4.19, B. The highest concentrations were detected in fractions 4 and 5 displaying 0.757 mg/ml and 0.734 mg/ml, respectively. Activity was determined for all samples using azocasein as substrate after which specific activity was calculated and is also shown in the table (Figure 4.19, B). Maximum activity was observed in fraction 4 and was responsible for 2.1 fold increase in purification. Samples were loaded onto an SDS-PAGE gel and purity was visually confirmed (Figure 4.19, C). Pure bands could be observed in lanes 5, 6 and very faintly 7 corresponding to fractions 4, 5 and 6, respectively. Purification could easily be achieved with this system and similar fold in purification could be obtained. Furthermore run time was reduced from 120 min to 70 min, but above all system cleaning, column equilibration and system setup time were greatly reduced. However, due to logistical challenges faced, purification using this system was tedious.



**Figure 4.19:** Summary of purification performed on the NGC™ Chromatographic System using cation exchange chromatography (20 ml SMM-Op-10C injected onto five 1-ml HiTrap SP HP columns connected in series). A: Chromatogram of run from sample injection to completion: absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in red and Line B (%) in black. B: Table summarising analysis and of samples obtained following BCA protein determination (mg/ml), specific activity (AU/mg) and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing sample before application (lane 1) and fractions obtained at elution (lanes 2-7). Lane M: Molecular weight marker (PageRuler™ Prestained Protein Ladder).

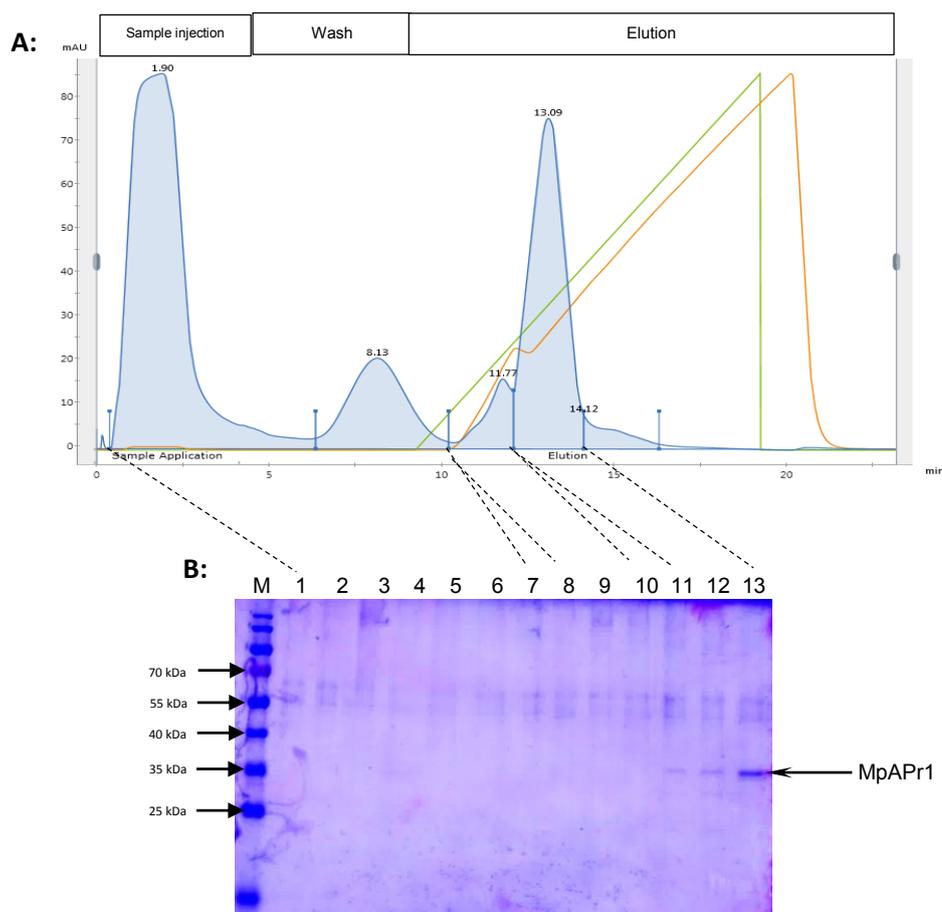
#### 4.5.2.2.5 Purification on the ÄKTA Pure Chromatography System

Fortunately, at this point in time, the IWBT received an ÄKTA Pure Chromatography System for protein purification purposes. Initially, 10 ml was injected onto five 1-ml columns connected in series and eluted over a 50 ml linear gradient. The chromatogram is shown in Figure 4.20. Similarly to results previously obtained using the BioLogic LP™ Low-Pressure Chromatography System, three peaks were identified at elution, from which the third peak eluting at 31.5 min (25.49 mS/cm) revealed a pure band corresponding to MpAPr1 (data not shown). At this time, 5-ml HiTrap SP HP columns were obtained and it was decided to optimise purification capacity.



**Figure 4.20:** Chromatogram obtained from of cation exchange chromatography (10 ml sample injected onto five 1-ml HiTrap SP HP columns connected in series). Peak areas are highlighted in blue and their retention time (min) in shown on the top of the peak. Absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in orange and Line B (%) in green.

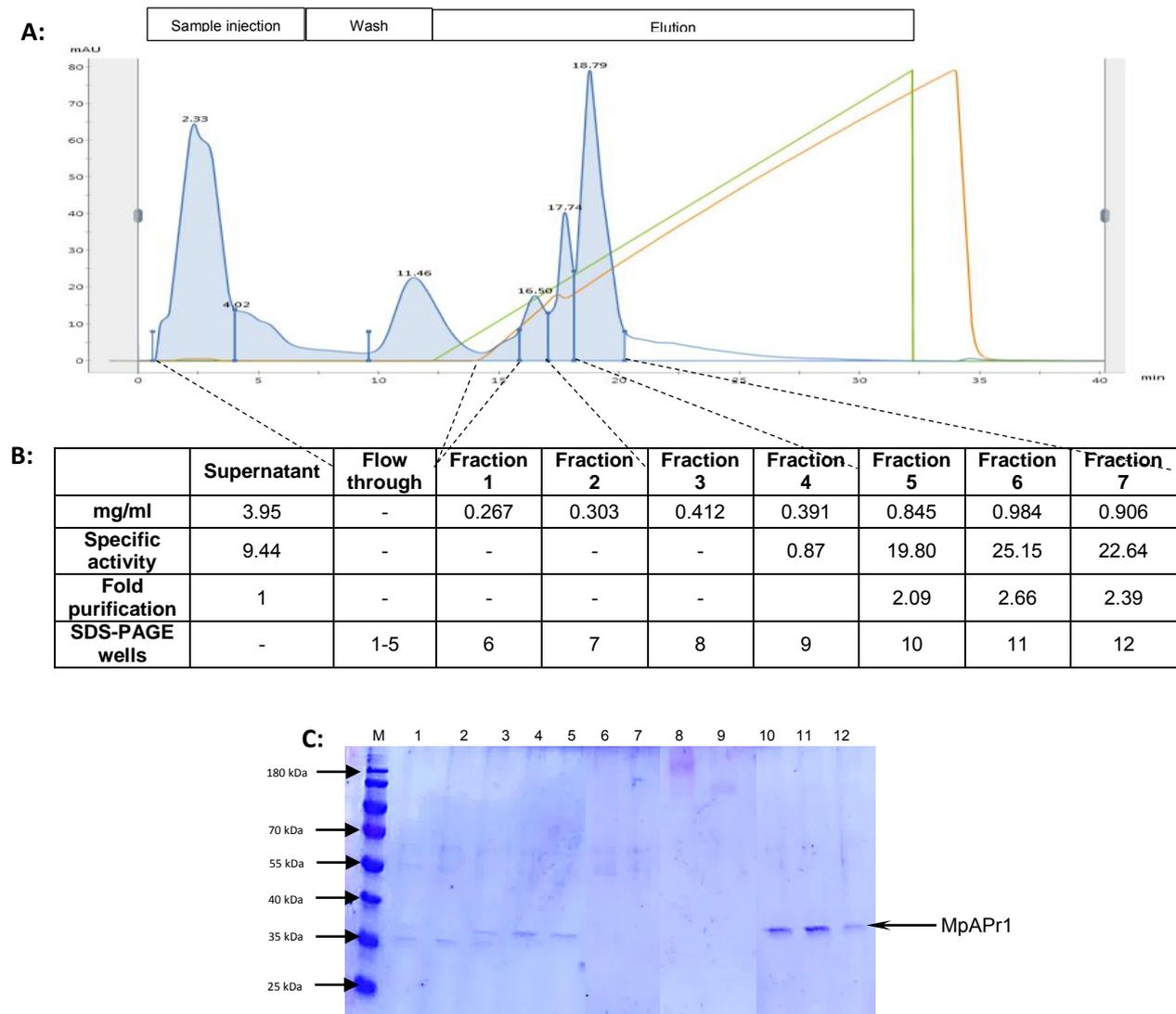
At first, 10 ml SMM-Op-10C was injected onto a 5-ml HiTrap SP HP column and eluted using a 50-ml linear gradient. The chromatogram is shown below (Figure 4.21). At elution, only two peaks could be observed, the second larger peak eluting at 13.1 min (24.58 mS/cm) corresponded to MpAPr1. The flow through along with the fractions obtained over the peak area was analysed via SDS-PAGE. Although MpAPr1 could be obtained (as indicated in Figure 4.21), other proteins were also present in these fractions.



**Figure 4.21:** Summary of purification on the ÄKTA Pure Chromatography System using cation exchange chromatography (10 ml sample injected onto a 5-ml HiTrap SP HP column). A: Chromatogram in which peak areas are highlighted in blue and their retention time (min) is shown on the top of the peak. Absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in orange and Line B (%) in green. B: SDS-PAGE analyses: Lane(s) 1-7: Flow through, Lane(s) 8-13: fractions collected over elution area, Lane M; Molecular weight marker (PageRuler™ Prestained Protein Ladder).

Finally, purification was optimised using two 5-ml HiTrap SP HP columns connected in series. 10 ml sample was injected and eluted over a 100 ml gradient. The complete run from sample injection to completion along with BCA protein determination, specific activity calculation and SDS-PAGE analyses is shown in Figure 4.22. Three peaks could be observed at elution and fractions 5, 6 and 7 collected over the third peak (eluting at 18.79 min and 24.57 mS/cm) displayed the presence of MpAPr1 (Figure 4.22, A). These fractions also had the highest protein concentration from all the fractions collected over the elution area with maximum protein concentration found in fraction 6 (0.984 mg/ml). Activity against azocasein was tested and specific activity was calculated and estimated a 2.66 fold in purification (Figure 4.22, B). SDS-PAGE analyses revealed a pure band corresponding to MpAPr1 in fractions 5, 6 and 7 (lanes 10, 11 and 12, respectively). Furthermore, fractions 1 and 2 showed the

presence of two faint bands at ca. 55 kDa, and fractions 3 and 4 the presence of bands at ca. 180 kDa. Purification of higher amounts of MpAPr1 from SMM-Op-10C could be achieved using the ÄKTA Pure Chromatography System and the two 5-ml HiTrap SP HP columns connected in series. These results were encouraging and suggested it would be possible to purify enough MpAPr1 for calculating  $K_m$  and  $V_{max}$  parameters and for evaluating activity against pure grape proteins and fermentation trails with *Saccharomyces cerevisiae*.



**Figure 4.22:** Summary of purification on the ÄKTA Pure Chromatography System using cation exchange chromatography (10 ml SMM-Op-10C onto two 5-ml HiTrap SP HP columns connected in series). A: Chromatogram of run from sample injection to completion. Peak areas are highlighted in blue and their retention time (min) in shown on the top of the peak. Absorbance (at 280 nm) is shown in blue, conductivity (mS/cm) in orange and Line B (%) in green. B: Table summarising analysis and of samples obtained following BCA protein determination (mg/ml), specific activity (AU/mg) and indicating the well that it is loaded on the SDS-PAGE gel. C: SDS-PAGE gel showing flow through (lane 1-5) and fractions obtained at elution (lanes 6-12). Lane M: Molecular weight marker (PageRuler™ Prestained Protein Ladder).

### 4.5.3 Determination of $K_m$ and $V_{max}$ of pure MpAPr1 and a commercial protease

Assessment of the kinetic properties of the purified recombinant MpAPr1 was performed under optimal pH and temperature conditions using azocasein as a substrate (similar to experiments performed on the crude extract paragraph 4.4.5). A calculated  $K_m$  and  $V_{max}$  of 5.9 mg/ml and 0.025 mg/ml/h was obtained for purified MpAPr1, respectively (Table 4.6). For comparison purposes, a commercially available protease (protease from *Aspergillus saitoi*) was included in the experiment. The  $K_m$  value of MpAPr1 was 2.50 times higher than that of the protease of *Aspergillus saitoi*. No statistical difference could be observed for calculated  $V_{max}$  values.

**Table 4.6:** Summary of  $K_m$  and  $V_{max}$  values as calculated through GraphPad Prism computer software by plotting v against s.

Kinetic rate constant	MpAPr1	Protease from <i>Aspergillus saitoi</i>
$K_m$	5.88 mg/mL $\pm$ 0.62 <sup>a*</sup>	2.35 mg/mL $\pm$ 0.39 <sup>b</sup>
$V_{max}$	0.025 mg/mL $\pm$ 0.00097 <sup>a</sup>	0.022 mg/mL $\pm$ 0.00098 <sup>a</sup>

\*Letters a and b indicate significant differences ( $K_m$  values were compared and  $V_{max}$  values were compared)

### 4.5.4 Discussion and partial conclusion

First introduced in the 1970's, metal affinity chromatography has a broad application within the field of protein biochemistry. Furthermore, the incorporation of histidine tags (either to the C or N terminus of the protein) has become increasingly popular for purification of the recombinant proteins via the covalent bonds which form between metal chelating amino acid residues and divalent metal ions, such as those of cobalt, copper, iron, nickel and zinc. For this reason, in this study, MpAPr1 was expressed in *K. pastoris* X33 with a fused hexa-histidine tag to the C-terminal of the protein. In order to exploit this hexa-histidine tag, purification was initially attempted via immobilised metal affinity chromatography (IMAC).

Initially, prior to purification using IMAC, MpAPr1 was heterologously expressed in a rich medium (2% yeast extract, 1% peptone and 4% glucose) referred to as SRM. However, even after multiple attempts purification was unsuccessful via this method. Furthermore, BCA protein determination also estimated high amounts of protein in the fractions obtained. Yet, following SDS-PAGE analysis, no bands could be visualised. High protein estimations obtained using the BCA protein determination method was ascribed to contaminating

compounds derived from yeast extract with the SRM resulting in an overestimation of protein concentrations within samples.

It was thus decided to first concentrate the medium via ultrafiltration using a 30-kDa cut-off filter in an attempt to eliminate at least some contaminating substance and concentrate MpAPr1. Furthermore, purification was attempted using both a 1-ml and a 5-ml HiTrap IMAC HP column (respectively). However, similar results were obtained and after several attempts also purification remained unsuccessful. As a final attempt, it was decided to incorporate different metal ions to charge the column ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$ ) prior to sample loading in an attempt to increase protein binding. Once again, similar results were obtained and purification remained unsuccessful. Throughout these experimental trails the presence of a yellow/orange compound present within the samples could be noted. This compound is derived from the yeast extract that forms part of the rich culture. It was suspected that this compound was interfering with the results obtained from optical density readings and reagents used in the chromatography apparatus (280 nm) and the protein determination assays (562 nm).

These results were somewhat contradictory to those obtained in a previous study where MpAPr1 was expressed in *E. coli*. Indeed, the enzyme could be purified by means of IMAC using the Ni-NTA spin columns (Theron, 2013). However, purification was only possible under denaturing conditions and activity could not be retained after several refolding attempts. It must be noted that in the latter study, the hexa-histidine tag was located on the N-terminal of the protein and in this study, it was fused to the C-terminal. The histidine tag might thus be in such a conformation that it is hidden or folded within the protein making it unavailable to bind to the charged resin. Indeed, protein modelling performed using online software revealed that both the C and N termini are distant from the active site and should thus not result in complications. Nevertheless, protein modelling was performed based on the protein sequence from an extracellular aspartic protease from *Candida tropicalis* (accession number: 1J71) to which MpAPr1 shared 41.1 sequence identity. Thus, the model might not be a good representation of MpAPr1 leading to the hasty conclusion about the conformation of the fused hexa-histidine tag. A few other possible explanations may thus be ventured. (1) The high pH of the buffers used during purification might have induced protein conformation change to such a degree that the histidine tag became inaccessible. (2) Contaminating proteins from the rich medium used for MpAPr1 expression might have outcompeted for binding to the charged resin and/or might have bound to the hexa-histidine. Indeed, similar findings have been observed by other authors (Siala et al. 2009). In the latter study, a hexa-histidine tag was added to the recombinant protein, but the protein was eventually purified by means of anion exchange chromatography; possible reasons or explanations are however not discussed by the authors. Nevertheless, it was decided to utilise cation exchange chromatography to purify the recombinant MpAPr1. This technique was chosen as it relies on the charge of the protein

rather than a fused amino acid tag and because of the low theoretical pI calculated for MpAPr1 (pI of 4.2).

Initially, purification of MpAPr1 from SRM was attempted using cation exchange chromatography. Similarly to the observations made using IMAC, high protein concentrations were determined by BCA protein assay, but SDS-PAGE analysis revealed low concentrations. Conversely, SDS-PAGE analysis revealed the presence of two faint bands corresponding to ca. 37 kDa in fractions 3 and 4. This result was promising as it showed that purification or at least protein binding was possible using cation ion exchange chromatography. However, it became abundantly clear that an expression medium for purification purposes would have to be optimised as the presence of contaminating substances within the SRM was interfering with both optical density readings and reagents used.

Minimal medium expression was derived from literature (*Pichia* Fermentation Process Guidelines, Invitrogen) and comparison to other minimal media generally used in for yeast cultivation (i.e. YNB). Before inoculation, this medium is almost colourless with a very slight yellow tinge derived from the added peptone. Initial expression in minimal medium using the above-mentioned conditions resulted in unsatisfactory quantities of protein. Expression was thus optimised in terms of pH, incubation temperature and time. Optimal expression of MpAPr1 in *K. pastoris* X33 was found at a culture medium of pH 6 incubated for 72 h at 20°C with shaking (120 rpm). Similar results in terms of incubation time and temperature have been found for expression of aspartic proteases in *K. pastoris* (Salgado et al. 2013, Yegin et al. 2013). The concentrated supernatant obtained from optimised conditions (SMM-Op) was used in downstream experiments.

Once a minimal medium and expression conditions could be optimised, the SMM-Op was initially concentrated and buffer exchanged via ultrafiltration using a 30-kDa cut off filter (SMM-Op-30C). Initially, a 1-ml HiTrap SP HP column was used for purification purposes. Two bands could be observed in the fractions collected over the elution peak area, demonstrating that partial purification was achieved. Furthermore, the yields obtained were very low and consequently five 1-ml HiTrap SP HP columns were connected in series to increase resin volume and potentially increase yields. Indeed, protein concentration were increased, but the presence of two bands was once again observed following SDS-PAGE analyses.

It was suspected that one or potentially both of the two bands obtained following cation exchange chromatography (from SMM-Op-30C) might be glycosylated to different extents (as previously hypothesised). Subsequently, the concentrated supernatant and the semi-pure fraction obtained via cation ion exchange chromatography were treated with de-glycosylation enzymes. Unlike the MCAP protease studied by Salgado et al. (2013), de-glycosylation of bands corresponding to MpAPr1 did not result in a further migration on SDS-PAGE gel when compared to non-treated samples suggesting that the proteins corresponding to the 2 bands

are not (or weakly) glycosylated upon expression in *K. pastoris*. Note that one of the de-glycosylation enzymes (Neuramidase) has a similar MW as MpAPr1 i.e. 43 kDa resulting in difficulty to distinguish bands on SDS-PAGE gels and should thus be avoided in future similar experiments. Nevertheless, it could be concluded that the recombinant MpAPr1 is not (or rather weakly) glycosylated. The presence of the additional band was thus rather thought to be a truncated version or an isoform of MpAPr1 being expressed. Nevertheless, time was restricted on this instrument (BioLogic DuoFlow™ Chromatography System located at the University of Bordeaux) and purification had to be further optimised in South Africa.

The cultures were grown under optimised conditions as determined above and concentrated via ultrafiltration but using a 10-kDa cut-off filter (SMM-Op-10C). Initially, the BioLogic LP™ Low-Pressure Chromatography System was made use of to facilitate purification. Initially, the same procedures were followed as described in the paragraphs above for cation exchange chromatography (five 1-ml HiTrap SP HP columns connected in series). In contrast, three peaks could be observed after elution. Proteins could be purified satisfactorily and a single band corresponding to MpAPr1 could be observed following SDS-PAGE analysis estimated to have 0.795 mg/ml BSA equivalents as estimated by BCA protein determination. Furthermore, activity assays performed using azocasein as substrate confirmed protease activity the fractions obtained. Fraction 4 displaying the highest activity resulted in a 2.09 fold in purification as determined through comparing the calculated specific activity. The disappearance of the “second” band corresponding to MpAPr1 (as seen in previous attempts) is noted not only before sample injection, but also after elution. The disappearance of this band is not clear at this stage and is discussed later. Although purification could be achieved using this system, the efficiency was not optimal mainly due to the peristaltic pumps within the system used to drive purification.

At the Biochemistry department the NGC system was used satisfactorily to achieve higher purification yields and faster run times. Indeed, yields of 0.757 mg/ml could be obtained that had a higher specific activity than the previous attempt resulting in a fold purification of 2.11. Furthermore, run time could be reduced by 60 min.

At the Institute for Wine Biotechnology, an ÄKTA Pure Chromatography System for protein purification purposes was received in 2016. Similar results could initially be obtained for purification of MpAPr1 from SMM-Op-10C using five 1-ml HiTrap SP HP columns connected in series. In order to increase purification yield 5-ml HiTrap SP HP columns were utilised. Finally, two 5-ml HiTrap columns were connected in series and MpAPr1 was purified from 10 ml SMM-Op-10C. Yields of  $\pm 1$  mg/ml pure fractions could be obtained in which calculated specific activity showed a fold purification of 2.66. Furthermore, run time was reduced to 30 min per run. Note that SDS-PAGE analyses also revealed the presence of other bands within the fractions obtained over the course of elution. These proteins are most

probably other extracellular proteins secreted by *K. pastoris*, as identified by Mattanovich et al. (2009), and also because of protease activity within the sample resulted in the degradation of such proteins. When inspecting the flow-through the presence of the “second” band corresponding to MpAPr1, that seemed to disappear in earlier experiments, was indeed noticed but in low amounts. Possible reasons include structural instability and possible proteolytic activity, but this remains to be investigated. Nevertheless, MpAPr1 could be purified to satisfaction and further characterisation and application experiments could be envisaged.

Kinetic constants  $K_m$  and  $V_{max}$  were determined for both MpAPr1 and a commercially available acid protease from *Aspergillus saitoi* for comparison. For MpAPr1, a  $K_m$  of 5.88 mg/ml was 2.50 times lower demonstrating a lower affinity toward the substrate (azocasein). Moreover,  $V_{max}$  values obtained for both proteases were similar suggesting that both enzymes catalyse the reaction at a similar rate. These results were similar to those previously obtained for the crude extract (paragraph 4.4.5) and this suggests that results obtained from the characterisation of MpAPr1 in the crude extract would have been similar as well, should these experiments have been carried out using the purified enzyme. However, it is indeed noted that exact values were not obtained and this is ascribed to experimental error and assay limitations.

In conclusion, pure active MpAPr1 could be obtained from SMM-Op-10C via cation exchange chromatography. Furthermore, purification methods and equipment could be optimised to achieve satisfactory yields (and run times). This laid the foundation for obtaining sufficient MpAPr1 in order to evaluate and assess the effect of its enzymatic activity on haze-forming proteins (from grape berries) and also its holistic impact during grape juice fermentation.

## **4.6 Investigating the holistic impact of MpAPr1 activity during alcoholic fermentation on wine properties**

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After a pure enzyme was obtained, the ability of MpAPr1 to degrade grape pathogenesis-related proteins could be investigated. Moreover, the impact of its global activity in grape juice could also be assessed more holistically.

### **4.6.1 Estimation of pure MpAPr1 concentration for further analyses**

In order to test specific substrate:enzyme ratios, the concentration of the purified MpAPr1 needed to be determined. The Pierce BCA protein assay kit was used to determine concentration of pure MpAPr1 and grape proteins in buffered media and in grape juice. At first, BSA was used to establish a standard curve for protein determination (as described in the manufacturer’s protocol), but the data revealed a large discrepancy between the amount of proteins determined through this assay and that estimated on SDS-PAGE gels (as reported

in section 4.5). In an attempt to obtain a more accurate quantification, it was decided to establish a standard curve using the commercial protease from *Aspergillus saitoi* instead of BSA. Although widely used, BSA has several drawbacks when used as a standard to calculate pure proteins. If the protein to be measured does not interact with the dye in a similar way as the standard, false results can be obtained. The protease from *Aspergillus saitoi* resembles MpAPr1 more closely and was therefore tentatively used as an alternative standard. Although the quantification appeared more accurate (i.e. it was somewhat closer to the visual estimation on the SDS-PAGE gel) than when using BSA as a standard, a fairly large discrepancy was still evident. It was therefore decided to use SDS-PAGE analysis to estimate protein concentrations using the protease from *Aspergillus saitoi* as a reference rather than the BCA assay. Using this method, it was estimated that the working stock solution of pure MpAPr1 was ca. 0.5 mg/ml before its use in downstream experiments. Similar estimation of protein concentration was utilised in downstream experiments in order to evaluate protein degradation as it proved difficult to establish a standardised assay for grape protein degradation as none exists to date.

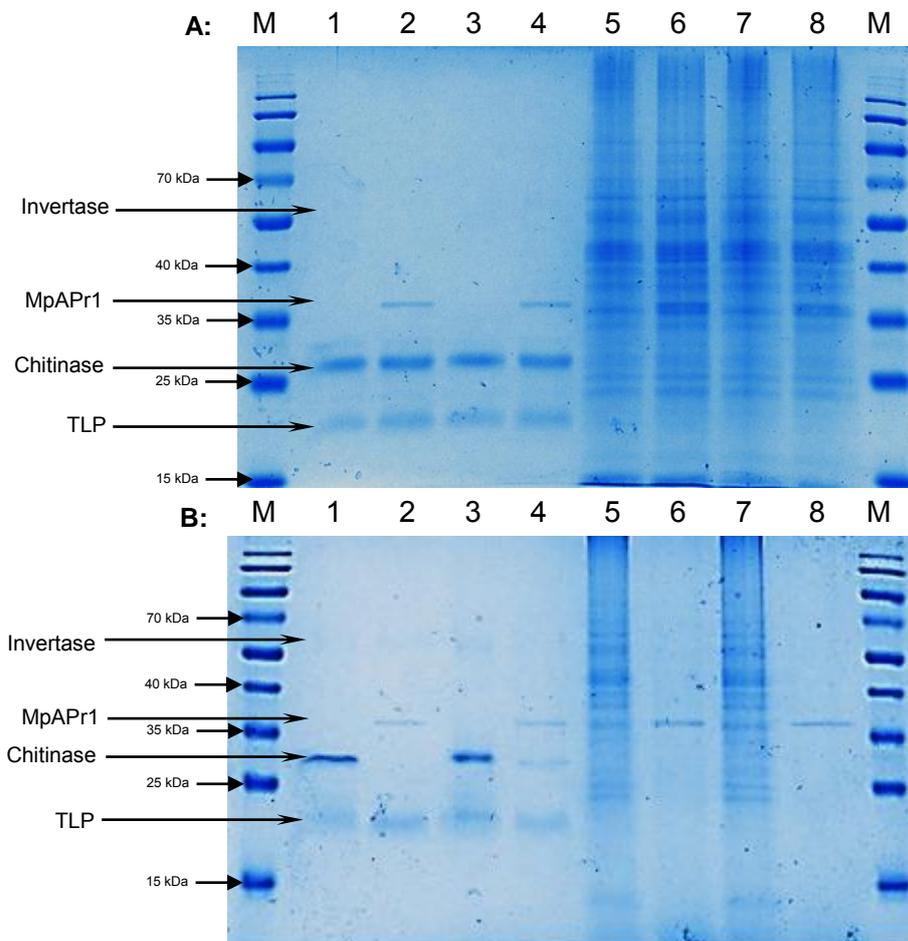
#### **4.6.2 Impact of MpAPr1 on pure grape proteins in a buffered medium**

Partially purified grape proteins from Pinot grigio were generously donated by Prof Andrea Curioni and Dr Simone Vincenzi (University of Padua, Italy). They were used as substrates to test MpAPr1 activity before commencing with fermentation trials. Furthermore, experiments using pure grape proteins in a buffered medium made it possible to establish assay pH and temperature with which fermentations trials were then carried out (paragraph 4.6.3).

Grape proteins were received in a freeze-dried form in two separate aliquots: the first contained chitinases and the second a pool of proteins (mainly consisting out of TLP and invertases). Stock solutions of the first and second aliquots were dissolved in McIlvaine's buffer at the desired pH (either 4.5 or 3.5 for optimal and sub-optimal conditions, respectively) to a final concentration of 2 mg/ml. A final mixture of total grape proteins was prepared and consisted of a mixture of 1/3 chitinase to 2/3 pool of proteins. This ratio was chosen in order to obtain similar amounts of TLP and chitinases. This was confirmed via SDS-PAGE (as seen in paragraphs 4.6.1.1 and 4.6.1.2 below). Furthermore, where appropriate, SDS-PAGE gels were evaluated using densitometry in order to calculate the percentage of relative degradation of the different bands tentatively identified based on their apparent molecular weight as shown previously (van Sluyter et al. 2015, Le bourse et al. 2011). In other words, protein profiles were compared after 48 h with or without the addition of MpAPr1, single bands were identified and compared. In each case, the band in the lane containing proteins without the addition of MpAPr1 was regarded as 0% (i.e. no degradation).

#### **4.6.2.1 MpAPr1 activity against grape proteins under optimal pH and temperature conditions of activity**

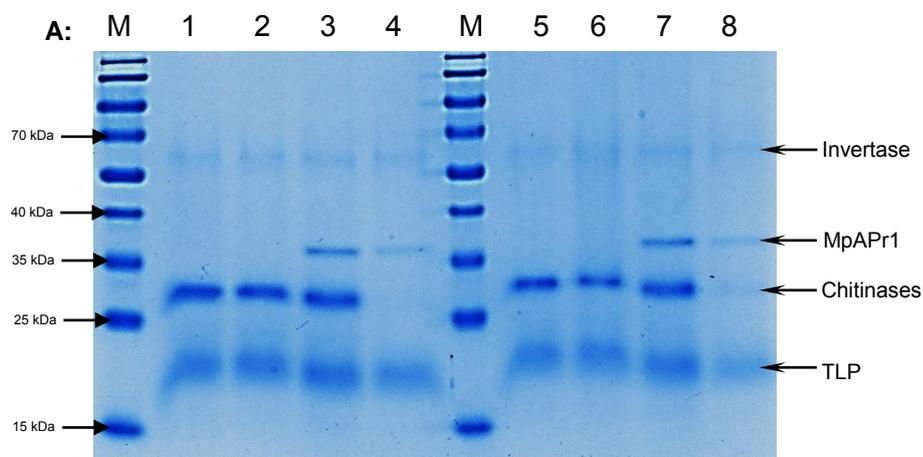
As determined above in paragraph 4.4.2, MpAPr1 activity is optimal at a pH of 4.5 and 40°C. These conditions were therefore initially chosen to evaluate protease activity against grape proteins. Flash pasteurisation (FP) of proteins prior to enzymatic treatment was also investigated and achieved by rapidly heating proteins to 72°C for 1 min and cooling to 4°C for 5 min prior to addition of MpAPr1. A mixture of chitinase and a pool of proteins (TLP and invertase) was prepared as described above to a final concentration of 0.9 mg/ml (0.3 mg/ml chitinase and 0.6 mg/ml pool of proteins) and MpAPr1 (in McIlvaine's buffer at pH 4.5) was added to a final concentration of 0.15 mg/ml. The mixture was incubated for 48 h at 40°C. Opti-white, a commercial product from Lallemand (Blagnac, France), is a blend of inactive dried whole yeast cells containing several proteins and peptides of yeast origin sometimes used in the production of white wine. It was also included in the experiment at a final concentration of 6.25 mg/ml. Controls included samples without the addition of MpAPr1 prior to incubation in order to evaluate degradation. Samples were taken at time 0 h and after 48 h for SDS-PAGE analysis in order to evaluate protein degradation (Figure 4.23). Slight degradation of grape proteins (lanes 1 and 3) and Opti-white (lanes 5 and 7) could be observed after 48 h. Furthermore, no differences could be observed between protein samples that were flash-pasteurised (FP) before enzyme addition (lanes 3 and 4: grape proteins, lanes 7 and 8: Opti-white). In samples containing grape proteins, three bands were identified by means of molecular weight comparison with literature, as indicated by the thin black bands in Figure 4.23 (Invertase, chitinase and TLP). In all samples containing grape proteins, bands corresponding to invertases, although very faint, were unaffected. Bands corresponding to chitinases disappeared completely in samples treated with MpAPr1 whereas TLP's were unaffected. Unidentified protein bands present in Opti-white were completely degraded following incubation of MpAPr1.



**Figure 4.23:** SDS-PAGE gel showing the outcome of the incubation of grape proteins and Opti white with and without MpAPr1 (0.15 mg/ml) after 48 h at optimal conditions. A: Samples at 0 h, B: Samples at 48 h. Note that lanes 1-2 and 5-6, proteins untreated prior to enzyme addition, and lanes 3-4 and to 7-8 show proteins that were flash-pasteurised prior to MpAPr1 addition. Lanes 1 and 3: grape proteins, lanes 2 and 4: grape proteins + MpAPr1, lanes 5 and 7: Opti White, lanes 6 and 8: Opti White + MpAPr1. Lane(s) M: molecular weight marker (PageRuler™ Prestained Protein Ladder). Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

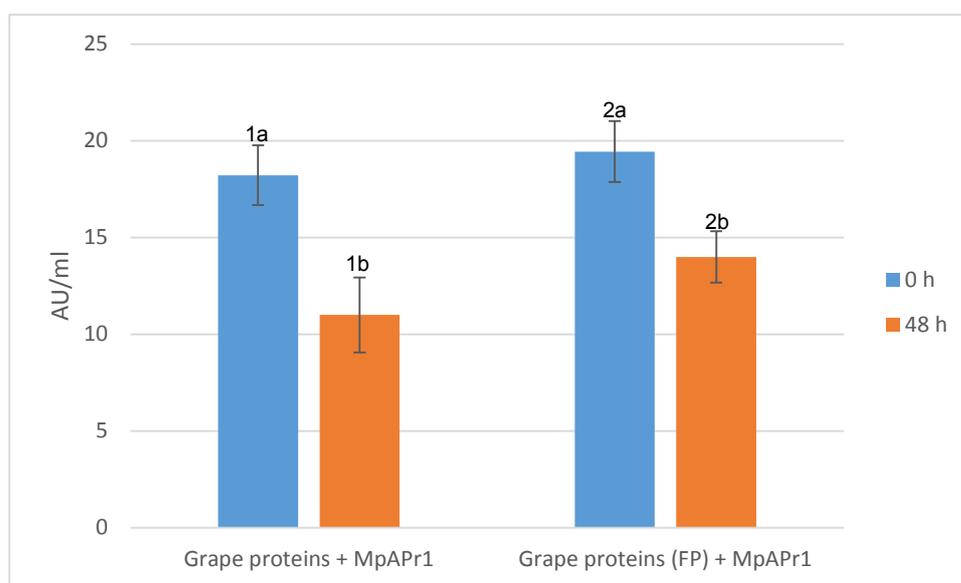
It was decided to increase the ratio of MpAPr1 to grape proteins as the enzyme was difficult to visualise on the gel. This also aimed to evaluate whether enzyme to substrate ratio has an impact on grape protein degradation. The ratio was adjusted to add double the amount of MpAPr1. The final concentration was thus 0.3 mg/ml MpAPr1 to 0.9 mg/ml grape proteins (0.3 mg/ml chitinase and 0.6 mg/ml TLP's). The experiment was repeated as described above using grape proteins (with or without FP prior to MpAPr1 treatment). SDS-PAGE analyses are illustrated in Figure 4.24 along with the percentage of degradation of protein bands identified

following densitometry analyses. Three bands were identified by means of molecular weight comparison with literature, as indicated by the thin black bands (invertase, chitinases and TLP). As observed above, the results obtained for both unheated and flash-pasteurised samples were similar. Following densitometry analyses (Figure 4.24), it was determined that some natural degradation of grape proteins occurred. According to densitometry analysis, approximately 20% natural degradation of all proteins could be observed after 48 h. Furthermore, in all samples treated with MpAPr1, chitinase was fully degraded. The band corresponding to invertase were degraded ca. 40% in samples without FP, but was unaffected in samples with FP). In contrast, TLP was slightly degraded in untreated samples and degraded ca. 55% in FP samples. Furthermore, degradation of MpAPr1 could also be observed in both treatments after incubation leading to ca. 59.5% degradation in untreated samples and ca. 68.2% degradation in FP samples. These results were similar to those shown in the previous paragraph and would suggest that the increase in MpAPr1 concentration did not have an effect on degradation. Nevertheless, they confirmed that MpAPr1 was able to degrade grape proteins, especially chitinase at optimal conditions. Furthermore, it was able to do so without the FP of grape proteins prior to enzyme treatment.



**Figure 4.24:** SDS-PAGE gel showing the outcome of the incubation of grape proteins with and without MpAPr1 (0.3 mg/ml) after 48 h at optimal conditions. Lanes 1 to 4 indicate grape proteins that were unheated and lanes 5 to 8 indicate grape proteins that were flash-pasteurised (FP) prior to (or without) addition of MpAPr1. Lanes 1 and 5: Grape proteins at 0 h, Lanes 2 and 6: Grape proteins at time 48 h, Lanes 3 and 7: Grape proteins with MpAPr1 at 0 h, Lanes 4 and 8: Grape proteins with MpAPr1 at 48 h, Lane(s) M: molecular weight marker (PageRuler™ Prestained Protein Ladder). Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

In order to determine if MpAPr1 was still active after 48 h of incubation, the residual protease activity was evaluated using azocasein as a substrate (Figure 4.25). Upon addition, the protease activity was the same for grape juice samples untreated and FP. Protease activity could still be detected after 48 h, but decreased significantly when compared to activity immediately after addition (0 h). Specifically a decrease 40% and 28% could be observed in samples untreated and FP, respectively.



**Figure 4.25:** Residual protease activity of MpAPr1 (against azocasein) at 0 h and after 48 h of incubation (at optimal conditions). (FP): Proteins flash pasteurised prior to addition of MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

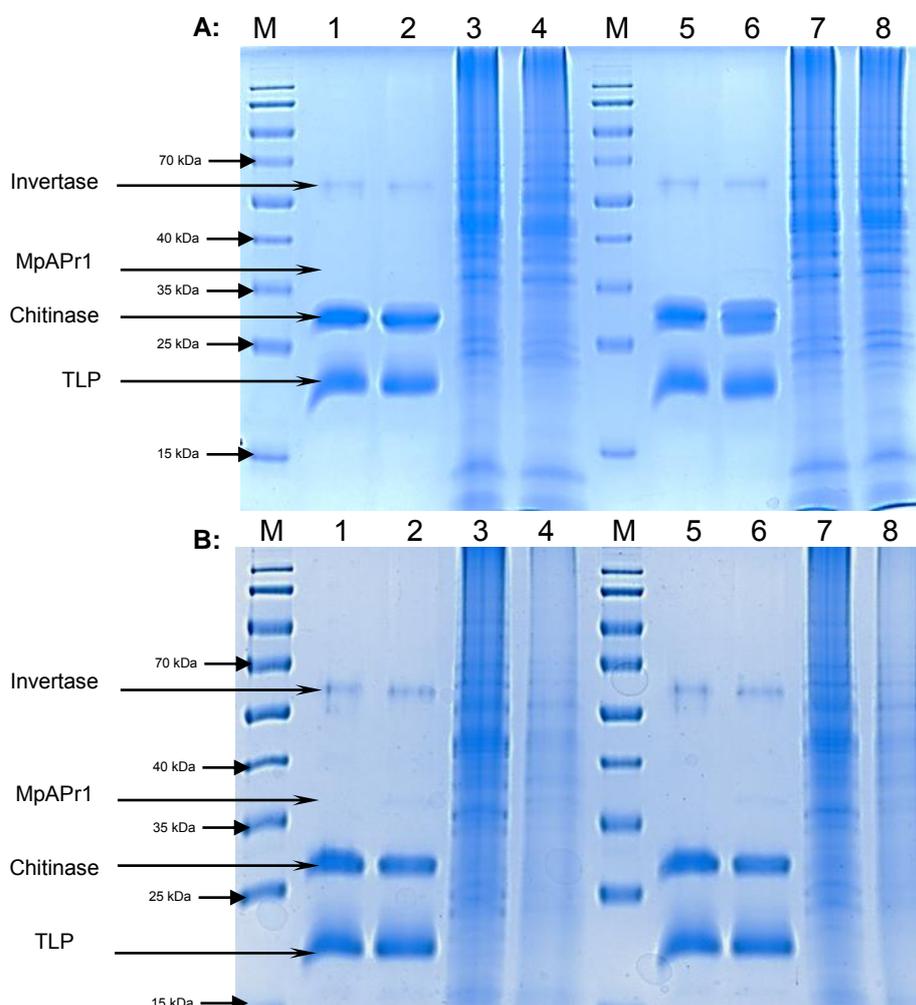
The pH and temperature conditions of these experiments (pH 4.5 and 40°C) are not those occurring during grape juice fermentation and it was therefore decided to test the impact of MpAPr1 under more industry-relevant conditions.

#### 4.6.2.2 MpAPr1 activity against grape proteins under oenological pH and temperature conditions

The conditions chosen to simulate grape juice fermentation were 20°C for 48h in buffer with a pH adjusted to 3.5. According to the results obtained in paragraph 4.4.2, MpAPr1 should exhibit moderate activity under these conditions.

Grape protein to enzyme ratios were the same as described above. MpAPr1 (0.15 mg/ml) was initially added to the grape protein mixture (0.9 mg/ml) and incubated for 48 h at 20°C. Opti-white was also included as described above. Samples were taken at 0 h and 48 h

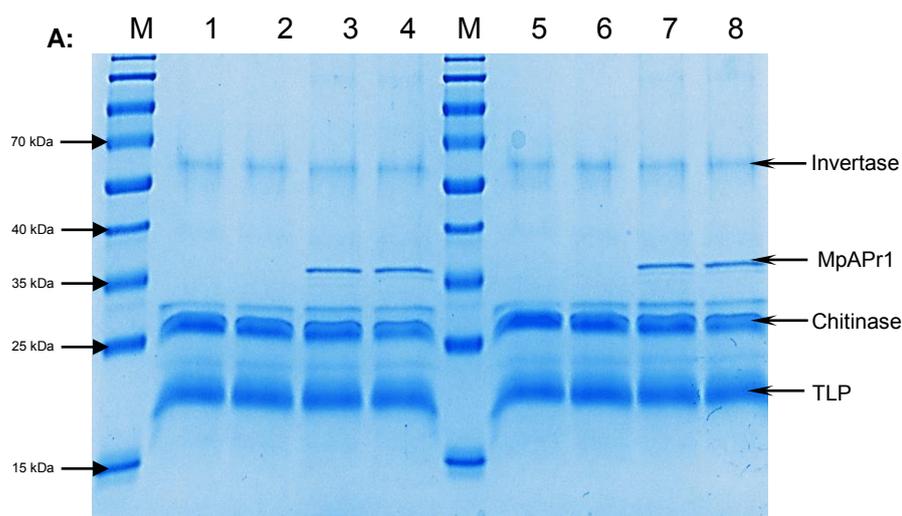
for SDS-PAGE analysis and are shown in Figure 4.26. Three bands were identified, by means of molecular weight comparison with literature, as indicated by the thin black arrows (invertase, chitinases and TLP). Results obtained for both unheated and flash-pasteurised samples were similar. No natural degradation of proteins could be observed over the course of 48 h at 20°C in samples without addition of MpAPr1 (lanes 1 and 3, lanes 5 and 7). Slight degradation could be observed in samples with the addition MpAPr1. Chitinase degraded slightly, but TLP and invertase were unaffected (lanes 2 and 6). Proteins from Opti white were degraded in the presence of MpAPr1, but degradation was not complete. Visualisation of MpAPr1 was difficult and it was suspected that the enzyme might have degraded during storage. Nevertheless, it was decided to purify more enzyme and repeat the experiment with a higher concentration of MpAPr1.



**Figure 4.26:** SDS-PAGE gel showing the outcome of the incubation of grape proteins and Opti white with and without MpAPr1 (0.15 mg/ml) after 48 h at sub-optimal conditions. A: Samples at 0 h, B: Samples at 48 h. Lanes 1 to 4 show proteins untreated prior to enzyme addition and lane 5 to 8 show proteins that were flash-pasteurised prior to MpAPr1 addition. Lanes 1 and 5: grape proteins, lanes 2 and 6: grape proteins + MpAPr1, lanes 3 and 7: Opti White, lanes 4 and 8: Opti white + MpAPr1. Lane(s) M: molecular weight marker (PageRuler™ Prestained Protein Ladder). Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

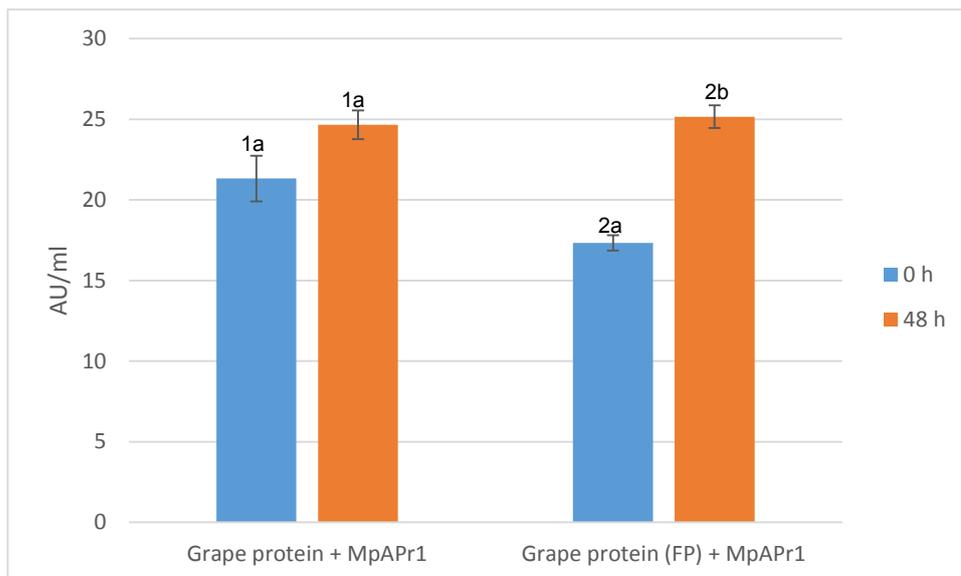
The experiment was repeated (as described above) with a higher ratio of MpAPr1 to grape proteins. Final concentrations were 0.3 mg/ml MpAPr1 to 0.9 mg/ml grape proteins. Samples were taken at 0 h and 48 h for protease activity and SDS-PAGE analysis (Figure 4.27). Note that wells were loaded exactly as in Figure 4.26. Natural degradation of proteins occurred in both samples unheated and FP samples. Invertase was degraded by ca. 13.7% in untreated samples but was unaffected in FP samples along with TLP. Chitinase naturally

degraded in both samples at ca. 17%. Invertase and chitinase were degraded by ca. 19.6% and ca. 31.2%, respectively in samples of grape proteins (untreated) with MpAPr1 while TLP was unaffected. In samples of grape proteins (FP) with MpAPr1, invertase and chitinase were degraded by ca. 30% and ca. 26%, respectively, while TLP also remained unaffected. Interestingly, in samples of grape proteins (untreated) MpAPr1 degraded by ca. 5.3% while in grape protein samples (FP) MpAPr1 was unaffected.



**Figure 4.27:** SDS-PAGE gel showing the outcome of the incubation of grape proteins and Opti white with and without MpAPr1 (0.3 mg/ml) after 48 h under sub-optimal conditions. Lanes 1 to 4 indicate grape proteins that was unheated prior to incubation and lanes 5 to 8 indicate grape proteins that were flash-pasteurised prior to incubation. Lanes 1 and 5: Grape proteins at 0 h, Lanes 2 and 6: Grape proteins at time 48 h, Lanes 3 and 7: Grape proteins with MpAPr1 at 0 h, Lanes 4 and 8: Grape proteins with MpAPr1 at 48 h, Lane(s) M: molecular weight marker (PageRuler™ Prestained Protein Ladder). Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

Finally, in order to investigate if MpAPr1 was still active after 48 h under sub-optimal conditions of activity, protease activity was measured using azocasein as substrate (Figure 4.28). Immediately upon addition, protease activity was similar for grape juice samples untreated and FP. Protease activity did not decrease after 48 h of incubation but significantly increased only in grape juice samples that were FP before enzyme addition. These results were encouraging and suggested that MpAPr1 is able to degrade haze forming proteins, at least to certain extent, under sub-optimal conditions simulating those occurring during fermentation of grape must. Consequently, experimentation in grape juice including fermentation with *Saccharomyces cerevisiae* could now be envisioned in order to evaluate the overall impact of MpAPr1 on wine properties.



**Figure 4.28:** Residual protease activity of MpAPr1 (against azocasein) at 0 h (immediately after addition) and after 48 h of incubation (under sub-optimal conditions). (FP): Proteins flash pasteurised prior to addition of MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

#### 4.6.3 Impact of MpAPr1 on grape proteins and wine properties of Sauvignon Blanc

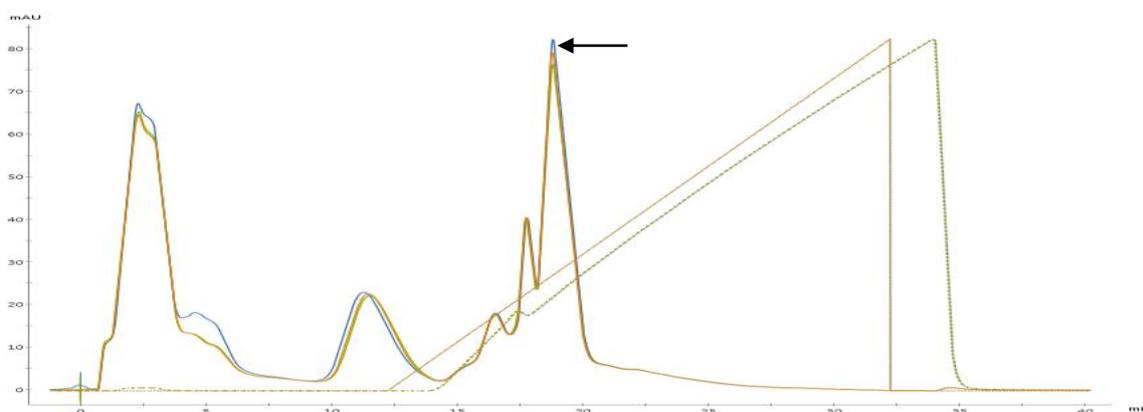
Grape juice (Sauvignon Blanc) was obtained from pressing Sauvignon Blanc grapes berries with the addition of  $\text{SO}_2$ , but without the addition of external enzymes (i.e. pectinases). After the juice was collected,  $\text{SO}_2$  was added and stored at  $-20^\circ\text{C}$  before use in downstream experiments. All fermentation trials were performed using the yeast *Saccharomyces cerevisiae* VIN 13. This commercial strain was chosen for its common use in wine fermentation and the fact that it displays no extracellular protease activity (as determined in section 4.1).

Once additional amounts of MpAPr1 could be purified by means of cation exchange chromatography, the fermentation trials could commence. These were set up in such a way that it would grant MpAPr1 an advantage thereby allowing it time to perform protein degradation before inoculation with *S. cerevisiae* VIN 13. Thus, after addition of MpAPr1, the samples were incubated for 48 h after which inoculation occurred. After inoculation, the samples were allowed to complete fermentation. Fermentation kinetics and yeast population dynamics were monitored until the end of fermentation via calculating  $\text{CO}_2$  production from weight loss and plating, respectively. Completion was verified via enzymatic analysis of the residual sugars. Controls included grape juice samples that were untreated with MpAPr1 in addition to un-inoculated samples. The impact that MpAPr1 has on grape proteins was investigated via HPLC, PAGE (1D and 2D) and heat stability assays. Furthermore, the impact

of MpAPr1 activity on the chemical composition of wine (with a specific emphasis on amino acids and fermentative aroma compounds) was assessed via GC-FID, HPLC and enzymatic analyses.

#### 4.6.3.1 Mass purification of MpAPr1 on ÄKTA system

Purification of a high amount of MpAPr1 from SMM-Op-10C was achieved as optimised in paragraph 4.5 via cation exchange chromatography using the ÄKTA Pure Chromatography System with two 5-ml HiTrap SP HP columns connected in series (Figure 4.29). In each run, MpAPr1 (indicated by the black arrow) was eluted over the third peak (as demonstrated in paragraph 4.5.2.2.4). These fractions were pooled and a total of 45 mg MpAPr1 (corresponding to 15 runs) was obtained for the fermentation trial that was to follow.



**Figure 4.29:** Overlay of several chromatograms obtained following cation exchange purification of MpAPr1 from SMM-Op-10C using the ÄKTA Pure Chromatography System. The black arrow indicates the peak containing MpAPr1.

#### 4.6.3.2 Fermentation kinetics

Alcoholic fermentation conditions (25°C) were chosen to resemble those occurring during white wine fermentation. It should be noted that 25°C is somewhat high for the fermentation of white wine yet still within the acceptable limit. Nevertheless, these conditions were chosen in order to keep MpAPr1 partially active; lower temperatures would indeed compromise its activity severely. Fermentations, after inoculation with *S. cerevisiae* VIN 13, were monitored by calculating CO<sub>2</sub> production from weight loss measurements taken over the course of fermentation three times per day. The alcoholic fermentation kinetics for *S. cerevisiae* VIN 13 in grape juice was similar to the fermentation in grape juice treated with MpAPr1 (for 48 h prior to inoculation) (data not shown). Fermentations reached dryness 144 h after yeast inoculation.

Yeast population dynamics of fermentations were monitored through plating on YPD agar medium once a day and counting colonies after 3 days of incubation. *S. cerevisiae* VIN 13 was inoculated from a wet pre-culture to an initial concentration of  $2.0 \times 10^6$  cfu/ml and after 24 h of incubation reached a maximum population of  $2-3 \times 10^7$  cfu/ml. Population numbers were similar in both fermentations (data not shown). The population increased exponentially in the first 24 h after which it became stable.

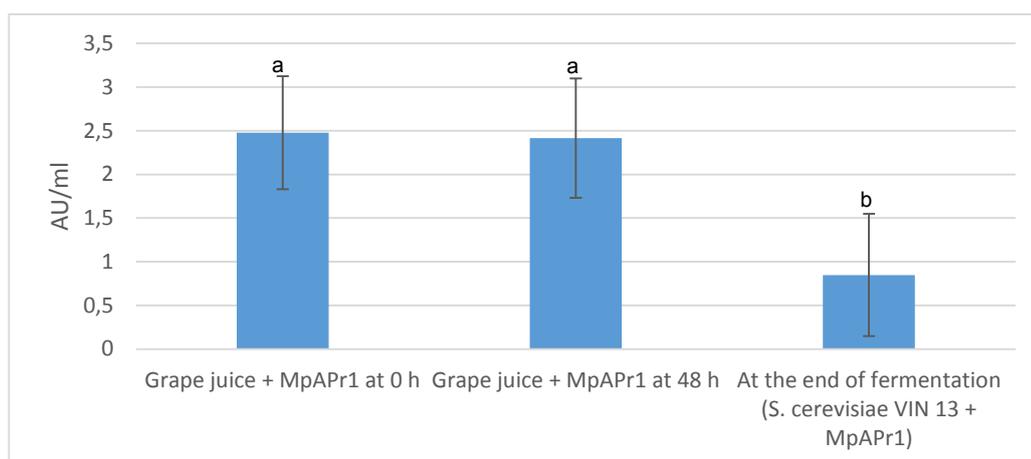
Glucose and fructose concentrations were determined enzymatically in order to ensure that fermentations were complete.

#### **4.6.3.3 Impact of MpAPr1 on grape and wine proteins**

After the addition of MpAPr1, the grape juice was allowed to stand at 25°C for 48 h prior to alcoholic fermentation. Samples were taken at 0 h and 48 h (before inoculation) for HPLC, residual protease activity, SDS-PAGE and 2D PAGE analyses in order to investigate the impact on extracellular proteins present within the samples. After the 48 h incubation period, specific samples were inoculated with *S. cerevisiae* VIN 13 and allowed to complete fermentation at 25°C (as described above). Controls included un-inoculated samples and therefore MpAPr1 activity was also assessed over the time course of fermentation (without yeast inoculation). After the end of fermentation the samples were left for an additional 120 h at 25°C (thus all analyses were performed 264 h in total after addition of MpAPr1). At the end of fermentation samples were taken for HPLC, SDS-PAGE, 2D-PAGE, protease activity, free ammonium, primary amino nitrogen, glucose and fructose concentrations, heat stability and GC-FID analyses in order to investigate impact on proteins and chemical composition of wine produced.

##### **4.6.3.3.1 Residual protease activity**

Residual protease activity of MpAPr1 in grape juice and wine was evaluated using azocasein as a substrate. Activity in grape juice was evaluated at 0 h and 48h after the addition of MpAPr1 and after fermentation with *S. cerevisiae* VIN 13 (Figure 4.30). Proteolytic activity was not significantly lost after 48 h, but after fermentation, very slight to no detectable activity could be observed. In order to further investigate the impact of MpAPr1 on proteins present in grape juice and wine, PAGE techniques were used.

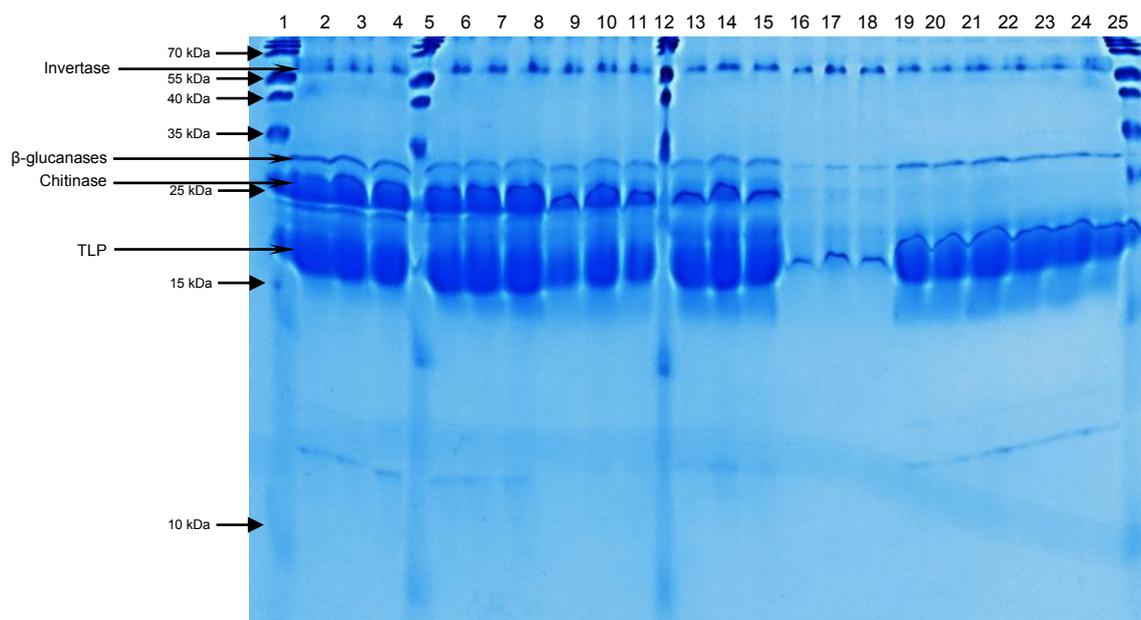


**Figure 4.30:** Residual activity of MpAPr1 against azocasein (AU/ml) in grape juice and after fermentation. Note that 0 h and 48 h are from grape juice samples and after fermentation with *S. cerevisiae* VIN 13. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

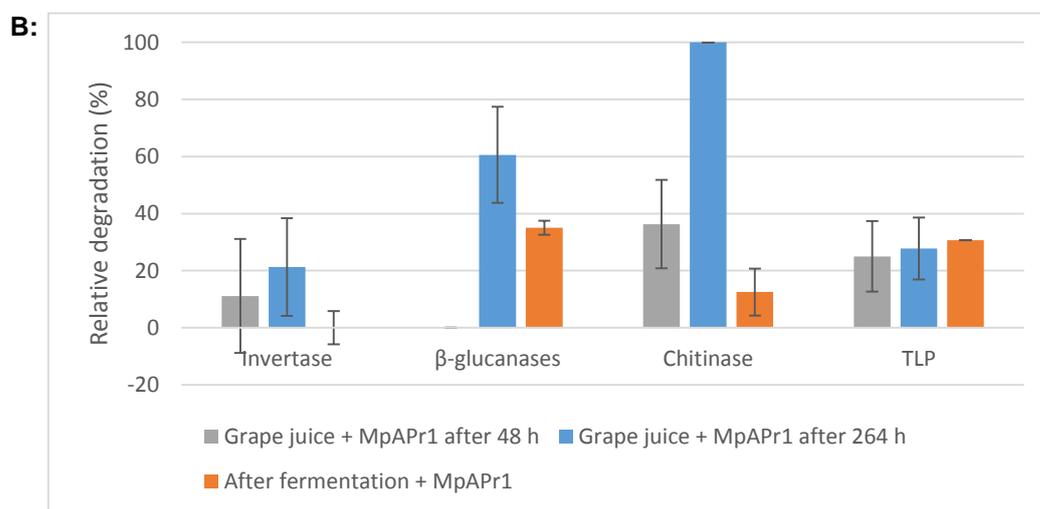
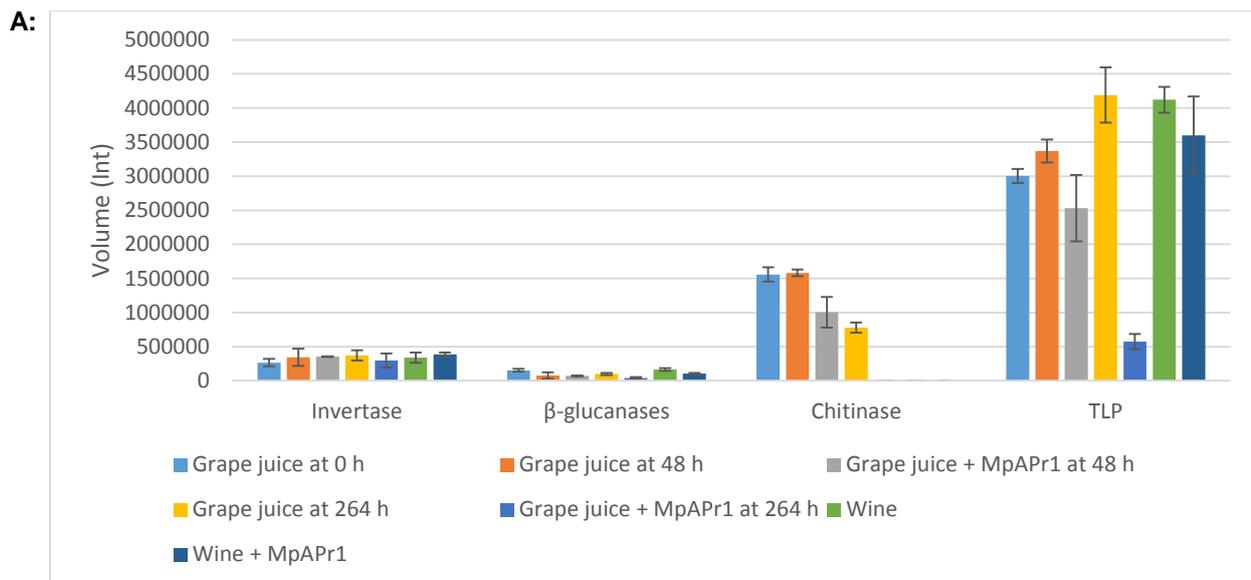
#### 4.6.3.3.2 SDS-PAGE

Prior to SDS-PAGE analysis, samples were concentrated ten times and simultaneously buffered exchanged via ultrafiltration using a centrifugal filter with a 10-kDa cut-off filter. A large SDS-PAGE gel (18.5 x 20 cm) was utilised in order to load all relevant samples on a single gel (Figure 4.31). Relative abundance of grape proteins was determined via densitometry (Figure 4.32, A) and the percent relative degradation of identified proteins was calculated by comparing untreated samples and samples treated with MpAPr1 (Figure 4.32, B). Lanes 2 – 4 displaying grape juice at time 0 h before the addition of MpAPr1 was mainly included as a control to ensure that no natural degradation of proteins were taken place over the duration of the experiment. After 48 h of incubation (lanes 6 – 8), no natural degradation of grape proteins could be observed. However, after incubation of grape juice at 25°C for 264 h (lanes 13 -15) slight degradation of the band corresponding to chitinase could be observed. In order to compare samples containing MpAPr1 the relative degradation was calculated (e.g. grape juice vs. grape proteins + MpAPr1, wine vs. wine + MpAPr1). Firstly, samples after 48 h showed no degradation of the bands corresponding to invertase and  $\beta$ -glucanases, but a degradation of 36% and 25% could be observed for bands corresponding to chitinases and TLP, respectively. Grape juice samples at 264 h showed degradation of all the bands identified: invertase 21%,  $\beta$ -glucanases 61%, chitinase 100% and TLP 27.8%. Interestingly, when comparing with the wine samples (i.e. at the end of fermentation), those treated with MpAPr1 prior to inoculation showed no degradation of invertase, but a degradation of 35%,

12.5% and 30.6% could be observed for bands corresponding to  $\beta$ -glucanases, chitinases and TLP. However, the same degree of protein degradation took place over the course of fermentation and natural degradation of  $\beta$ -glucanase, chitinase and TLP, but the band corresponding to invertase was unaffected. It should be noted that MpAPr1 could be visualised in these gels upon destaining, but when destaining was satisfactory to perform densitometry analysis, the band could no longer be observed. Smaller gels able to accommodate larger volumes per well were used in order to further investigate the degradation of grape proteins.



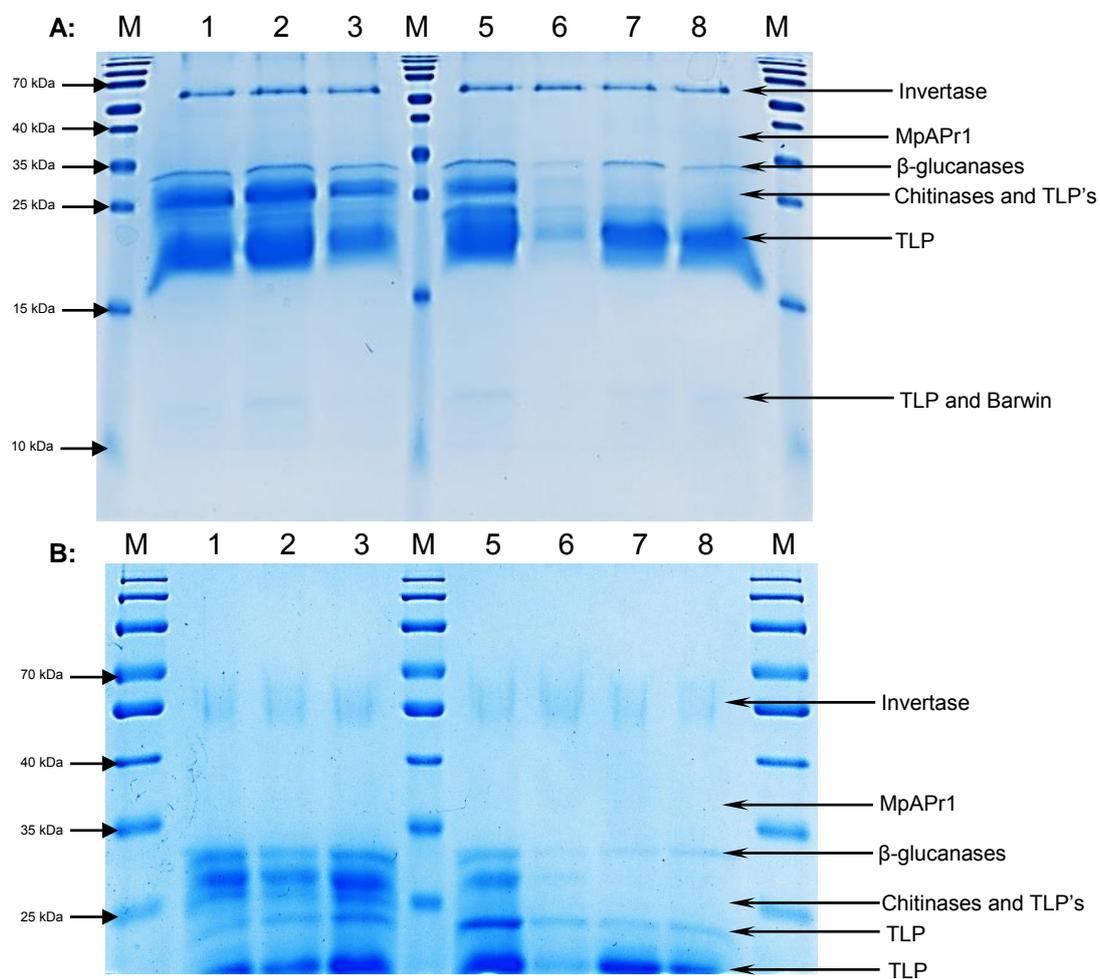
**Figure 4.31:** SDS-PAGE analysis of grape juice and wine samples treated with MpAPr1. A: 15% gel large gel. Lanes 2 - 4: grape juice at 0 h, lanes 6 - 8: grape juice at time 48 h, lanes 9 - 11: grape juice + MpAPr1 at time 48 h, lanes 13 - 15: grape juice at 264 h, lanes 16 - 18: grape juice + MpAPr1 at 264 h, lanes 19 - 21: samples after fermentation with *S. cerevisiae* VIN 13, lanes 22 - 24: samples after fermentation with *S. cerevisiae* VIN 13 + MpAPr1. Lanes 1, 5, 12 and 25: molecular weight marker (PageRuler™ Prestained Protein Ladder). Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).



**Figure 4.32:** Densitometry analysis of SDS-PAGE gel (Figure 4.31). A: Abundance of identified bands. B: Relative degradation of identified bands calculated by comparing untreated samples and samples treated with MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates.

A 15%- gel and a 12% bisacrylamide gel were loaded and are shown in Figure 4.33 panel A and B, respectively. Note that in panel B, lane 2 and lane 3 should be swapped around. After 48 h of incubation with MpAPr1, a slight degradation of all bands except for invertase can be viewed. After 264 h, similar impact was observed but more severe leading to degradation of bands between ca. 35 kDa – 10 kDa. When comparing the samples after alcoholic fermentation, the protein profiles are similar and degradation of the band corresponding to chitinase is observed, regardless of MpAPr1 addition prior to fermentation. This suggests that natural protein degradation (i.e. not facilitated by protease activity) took

place over the course of fermentation. Degradation of proteins could be visualised using SDS-PAGE and it was decided to further investigate grape juice samples treated with MpAPr1 via 2D-PAGE.



**Figure 4.33:** SDS-PAGE image of grape proteins. A: 15% gel B: 12% gel. Lane 1: grape juice at 0 h, lane 2: grape juice at 48 h, lane 3: grape juice + MpAPr1 at 48 h, lane 5: grape juice at 264 h, lane 6: grape juice + MpAPr1 at 264 h, lane 7: sample after fermentation with *S. cerevisiae* VIN 13, lane 8: sample after fermentation with *S. cerevisiae* VIN 13 + MpAPr1. Lane(s) M: molecular weight marker (PageRuler™ Prestained Protein Ladder). Note that in B (12% gel) lanes 2 and 3 should be swapped around. Thin black arrows indicate protein bands identified as grape proteins through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

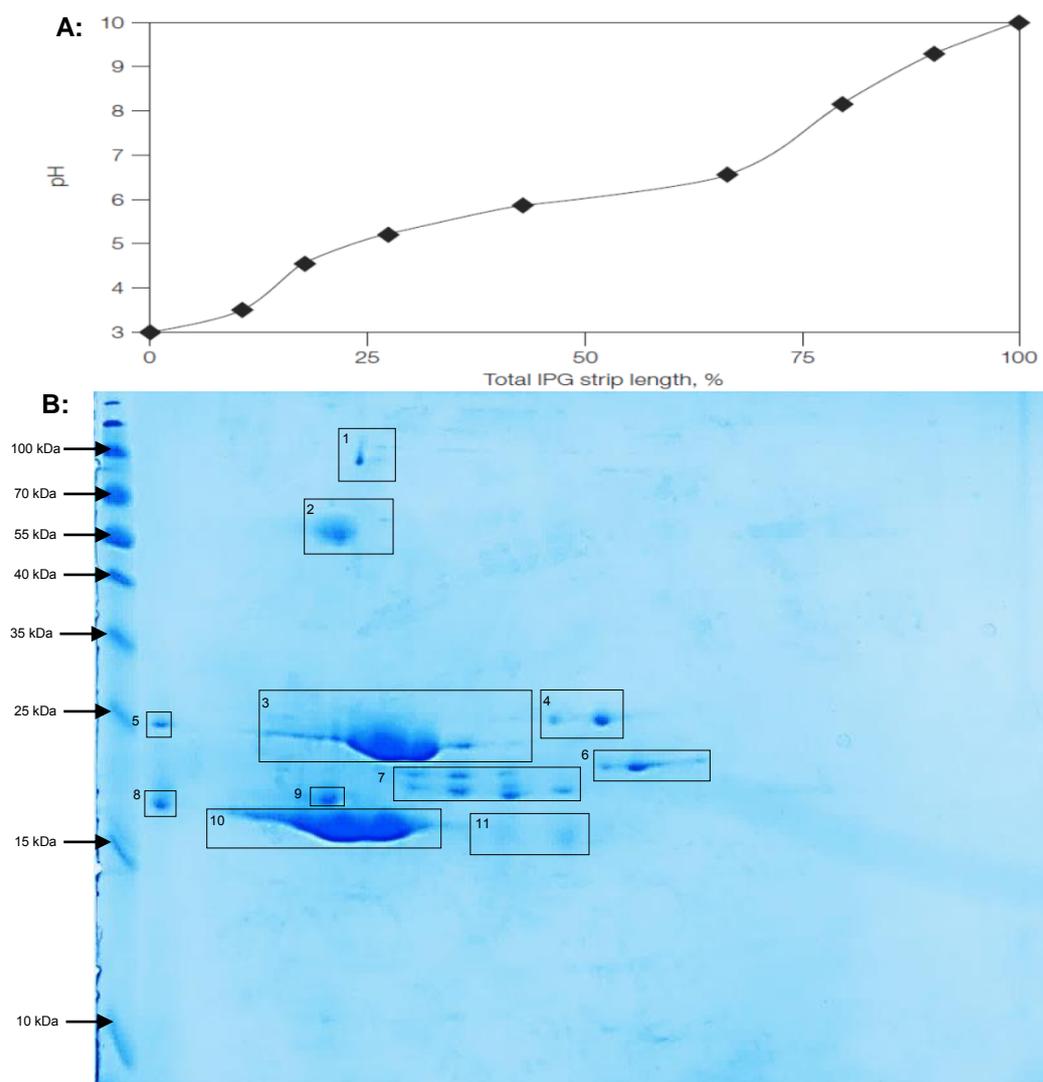
#### 4.6.3.3.3 2D-PAGE

In an attempt to get better resolution and separation of proteins, the 2D-PAGE technique was utilised. In all cases, samples were precipitated and cleaned up prior to sample application. Samples were first separated by their isoelectric points and then through their molecular

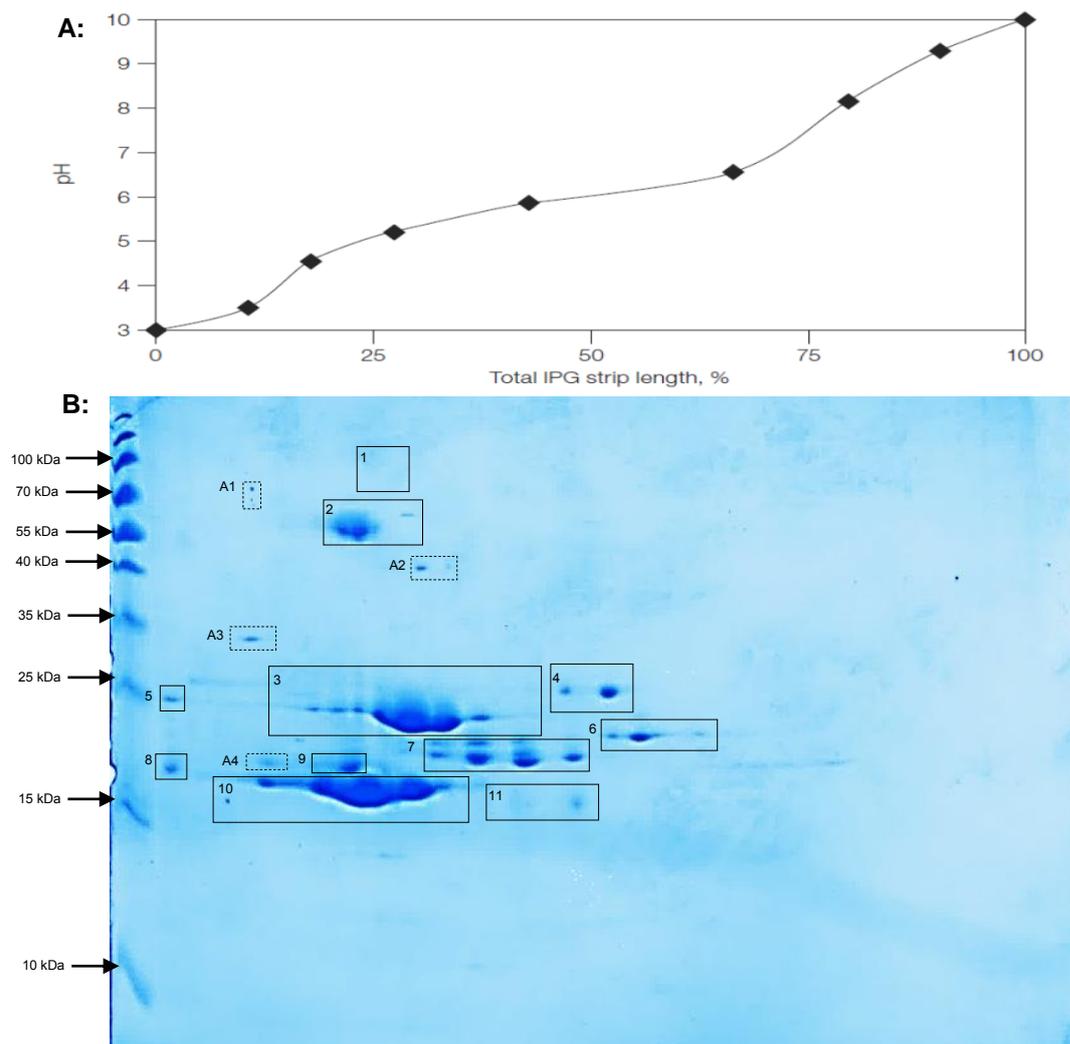
weight. Three samples, namely 1) grape juice at 0h, 2) grape juice + MpAPr1 at 48h and 3) after fermentation (*S.cerevisiae* VIN13 + MpAPr1) were analysed via 2D-PAGE and the images along with protein identification (through comparison with literature data on pI and molecular weight) are shown in Figures 4.34, 4.35 and 4.36, respectively. Note that in order to simplify identification of proteins and comparison of gels, spots are indicated by a black boxes on the image of the gel. The boxes were numbered and the same number designates the same protein or the same group of proteins. Furthermore, description and possible protein identity within the boxes are summarised in Table 4.7. In grape juice samples at 0 h, a total of 11 boxes are identified. When these 11 boxes are compared to grape juice + MpAPr1 at 48 h, the following is observed: in box 1, the spot disappeared, only a slight degradation of the spots in boxes 3, 7 and 10 (tentatively identified as chitinases and TLPs) was observed and no degradation could be visualised in any of the other boxes. Furthermore, boxes marked A1 – A4 designate protein spots that appeared after 48 h and MpAPr1 can be seen as a single spot in box A3. These concluded experiments and analyses performed on grape proteins and further experimental analyses aimed to assess the effect of MpAPr1 on wine chemical composition with an emphasis on wine aroma compounds.

**Table 4.7:** Identification of boxes on as illustrated on 2D-PAGE gels below. Spots in boxes were tentatively identified through comparison of molecular weight (van Sluyter et al. 2015, Le bourse et al. 2011).

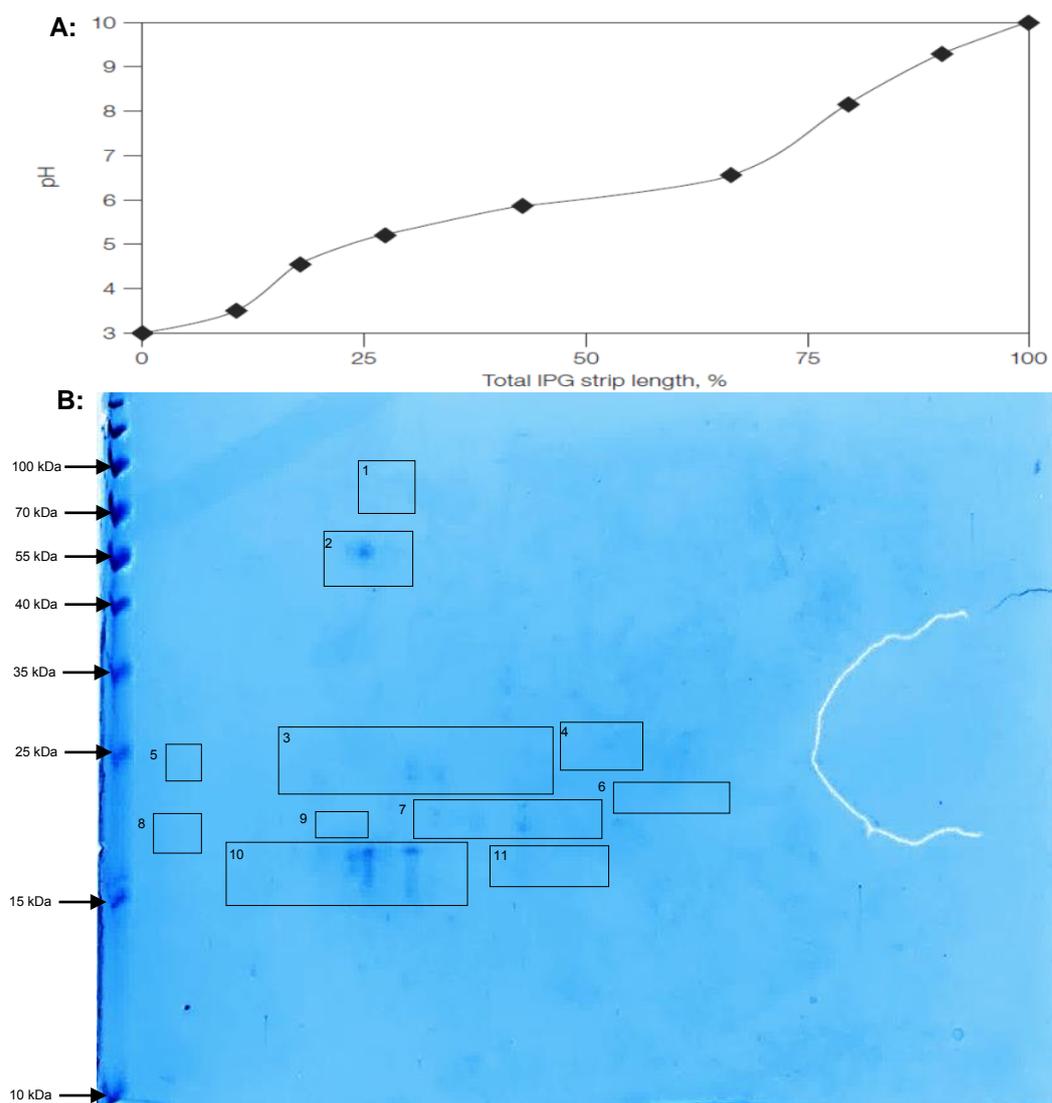
<b>Box</b>	<b>ca. MW(kDa)/pI</b>	<b>Description</b>	<b>Possible protein identities</b>
1	100/ 4.5 - 5.5	One small spot	?
2	55/ 4.5 – 5.5	One big spot and one small spot	Invertase
3	25/ 3.5 - 6	One big blob spot with three small spots to the right and one small spot the left	Chitinase and TLP
4	25/ 6 – 6.5	Two small spots	
5	25/ 3	One small spot	
6	25/ 6 – 6.5	Three small spots	
7	15 - 25/ 5.5 - 6	Seven small spots	
8	15/ 3	One small spot	
9	15/ 4.5 -5.5	One small spot	TLP
10	15/ 3.5 – 5.5	One big blob	
11	15/ 5.5 – 6	Two small spots	
A1	70/ 3.5	Two small spots	?
A2	40/ 5 – 5.5	Two small spots	?
A3	35/ 3.5	One small spot	MpAPr1
A4	15/ 3.5	One small spot	TLP



**Figure 4.34:** 2D PAGE analysis of proteins extracted from grape juice at time 0 h (without addition of MpAPr1) A: Graph showing pH vs. length relationship (Bio-Rad Laboratories). B: Image of gel after second dimension. Lane M: molecular weight marker (PageRuler™ Prestained Protein Ladder).



**Figure 4.35:** 2D PAGE analysis of proteins extracted from grape juice with the addition of MpAPr1 after 48 h of incubation at 25°C. A: Graph showing pH vs. length relationship (Bio-Rad Laboratories). B: Image of gel after second dimension. Lane M: molecular weight marker (PageRuler™ Prestained Protein Ladder).

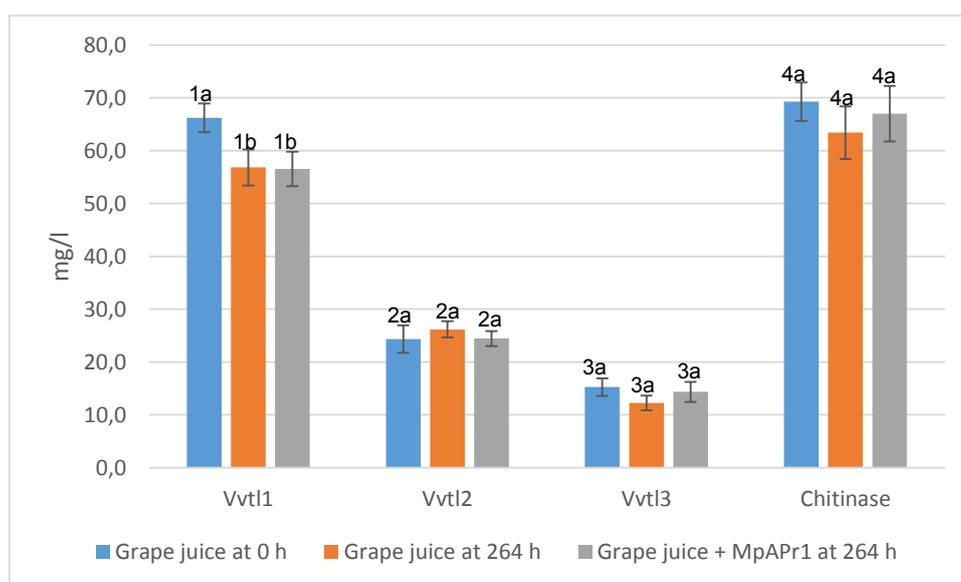


**Figure 4.36:** 2D PAGE analysis of proteins extracted from grape juice with the addition of MpAPr1 after 264 h of incubation at 25°C. A: Graph showing pH vs. length relationship (Bio-Rad Laboratories). B: Image of gel after second dimension. Lane M: molecular weight marker (PageRuler™ Prestained Protein Ladder).

#### 4.6.3.3.4 HPLC

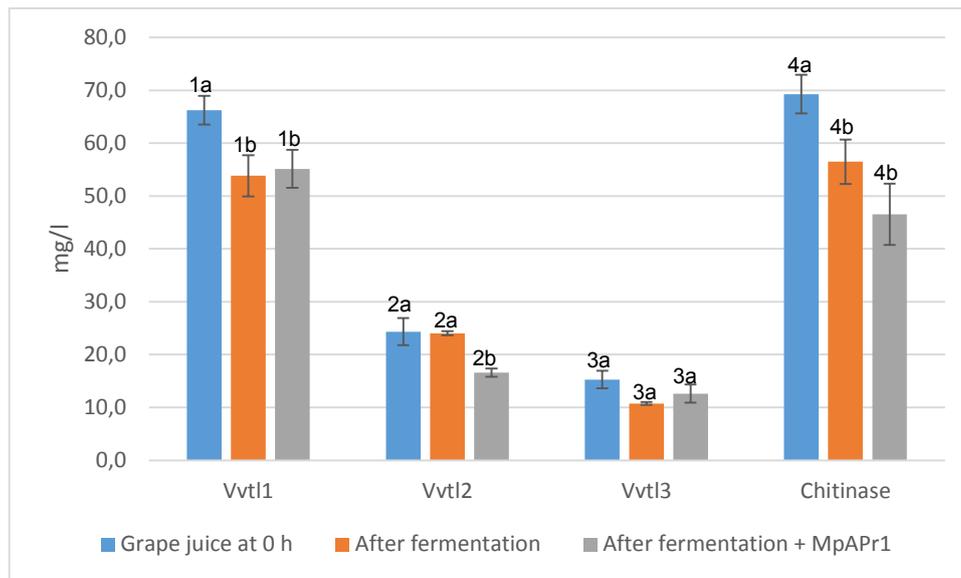
In order to investigate specific degradation of haze-causing proteins an HPLC method was used. This method specifically measures the concentrations of Vvt1, Vvt2, Vvt3 (thaumatin-like proteins) and chitinase. Samples were taken at 0 h before addition of MpAPr1 and at 48 h after addition of MpAPr1, but before inoculation with *S. cerevisiae* VIN 13. Controls included grape juice without the addition of MpAPr1. After 48 h protein profiles were similar and no significant natural degradation or because of MpAPr1 activity could be observed in these proteins (data not shown).

After the 48 h incubation period some grape juice samples (with or without MpAPr1) were inoculated and allowed to ferment to dryness. HPLC analyses were performed on samples after fermentation (Figure 4.37) and/or un-inoculated controls incubated for the same amount time (Figure 4.38). Although Vvt1 was significantly degraded in un-inoculated controls, natural degradation occurred and the addition of MpAPr1 did not result in additional degradation. No significant degradation, natural or because of MpAPr1 activity could be observed in any of the other proteins analysed.



**Figure 4.37:** Protein concentration (mg/l) determined by HPLC of specific haze-causing grape proteins after 264 h of incubation at 25°C with or without MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

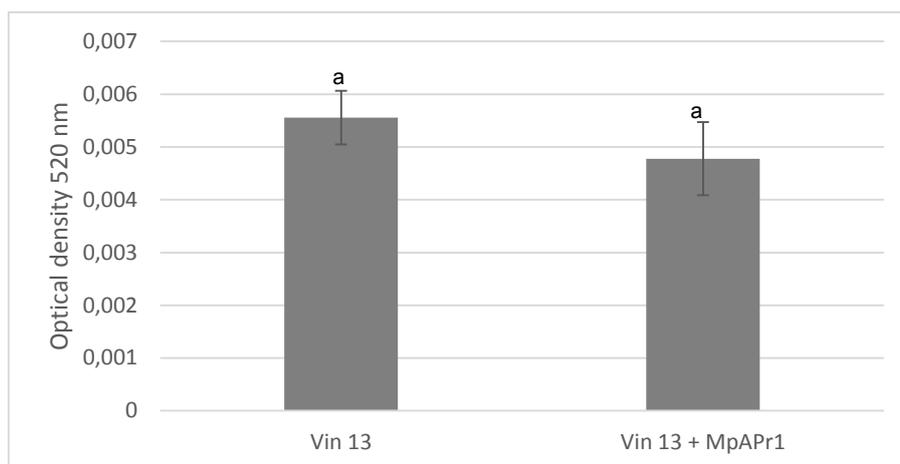
At the end of fermentation with *S. cerevisiae* VIN 13, specific grape proteins namely Vvt1 and chitinase were significantly degraded because of naturally means. The addition of MpAPr1 resulted in a significant degradation of Vvt2, but in no measurable degradation of the other grape proteins (Figure 4.38).



**Figure 4.38:** Protein concentration (mg/l) determined by HPLC of specific haze-causing grape proteins after fermentation with *S. cerevisiae* VIN 13 of incubation at 25°C with or without MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

#### 4.6.3.3.5 Protein haze assay

In order to estimate the protein stability or ability to form haze, the heat test was used. After 48 h and 264 h of incubation with MpAPr1 no differences could be observed when compared to untreated grape juice samples (data not shown). Although the results was not statistically significant the addition of MpAPr1 did result in a lower measurement in optical density (Figure 4.39).



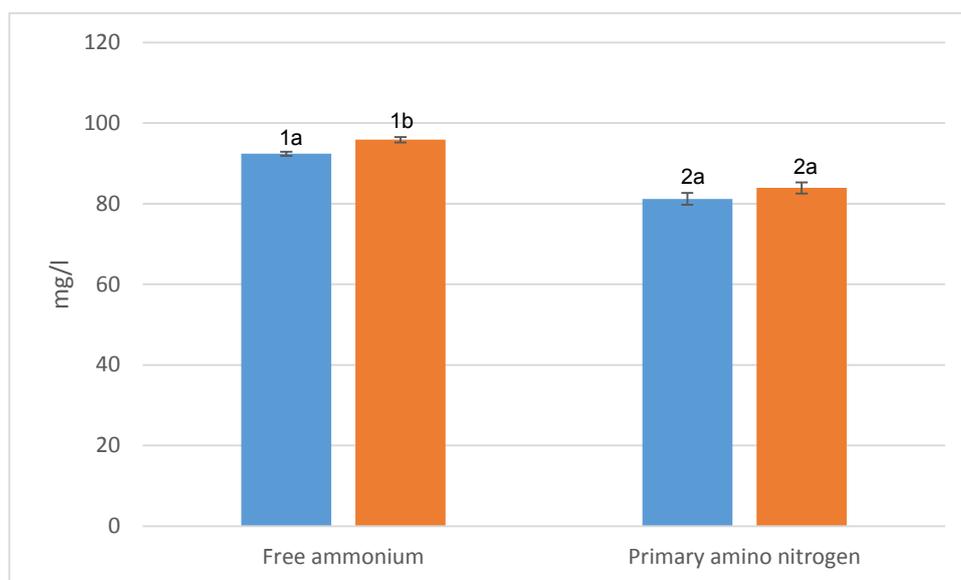
**Figure 4.39:** Heat stability of grape juice fermented with *S. cerevisiae* VIN 13 with or without MpAPr1 treatment prior to fermentation. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

#### 4.6.3.4 Impact of MpAPr1 on wine chemical properties

Enzymatic analysis was performed to evaluate whether a release of yeast assimilable nitrogen compounds within the samples after treatment with MpAPr1 occurred. The release of nitrogen containing compounds in the surrounding matrix can potentially be catabolised by the yeasts to produce sought-after aroma compounds. Furthermore, major volatile compounds were also analysed through the use of Gas chromatography – Flame Ionisation Detection (GC-FID) in order to investigate if such aroma compounds (originating from nitrogen and sometimes carbon metabolism) were produced in samples treated with MpAPr1.

##### 4.6.3.4.1 Analysis of nitrogen containing compounds

Free ammonium and primary amino nitrogen were measured via enzymatic assay kits. Measurements were performed at 48 h after incubation with MpAPr1 but before inoculation with *S. cerevisiae* VIN 13 in order to determine if MpAPr1 treatment could release sufficient nitrogen containing compounds prior to fermentation (Figure 4.40). Grape juice samples treated with MpAPr1 had a significant but low increase in ammonia concentration, but no significant increase in primary amino nitrogen concentration could be detected.

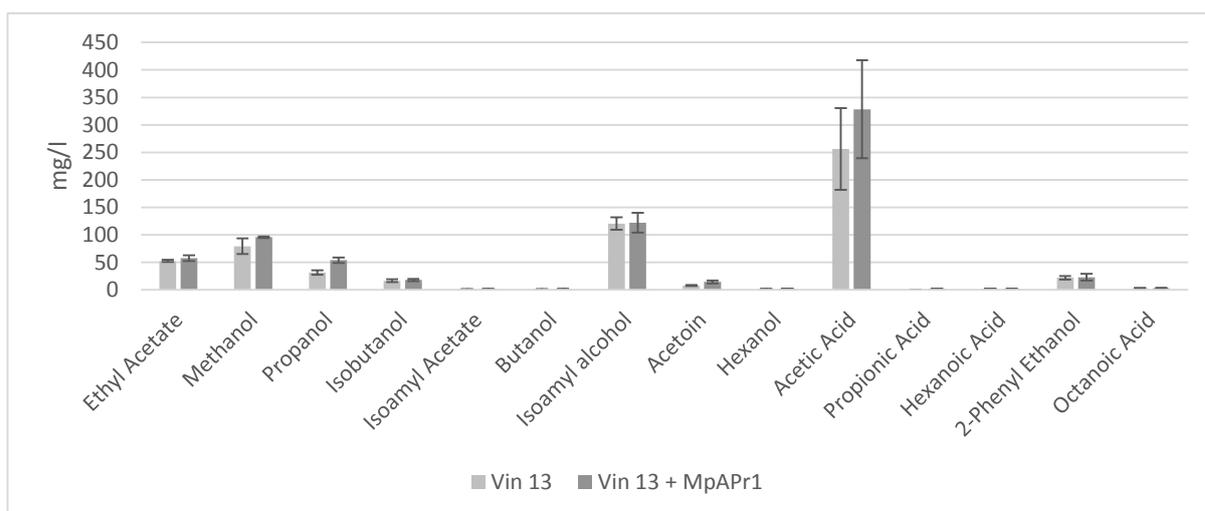


**Figure 4.40:** Free ammonium and primary amino nitrogen measurements (mg/l) of grape juice with or without the treatment of MpAPr1 after 48 h at 25°C. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

This was further confirmed by measuring the concentration of individual amino acids through HPLC (data not shown). Grape juice treated with or without MpAPr1 was inoculated and after fermentation the major volatile compounds inspected.

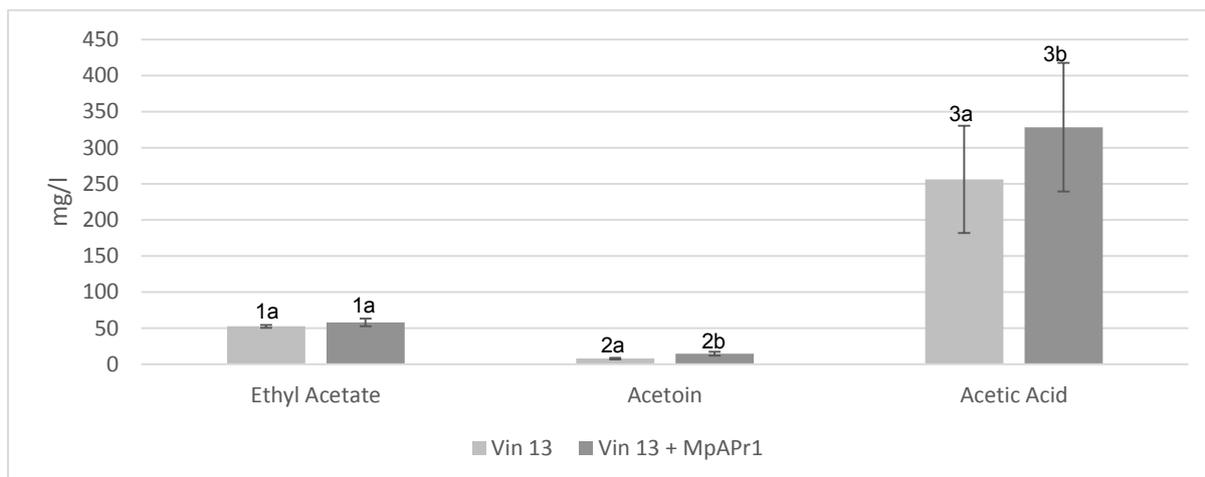
#### 4.6.3.4.2 Major volatile compounds

A total of 14 compounds were measured after fermentation via GC-FID (Figure 4.41). The results revealed that in the presence of MpAPr1, concentrations of certain volatile compounds typically associated with yeast alcoholic fermentation were affected.



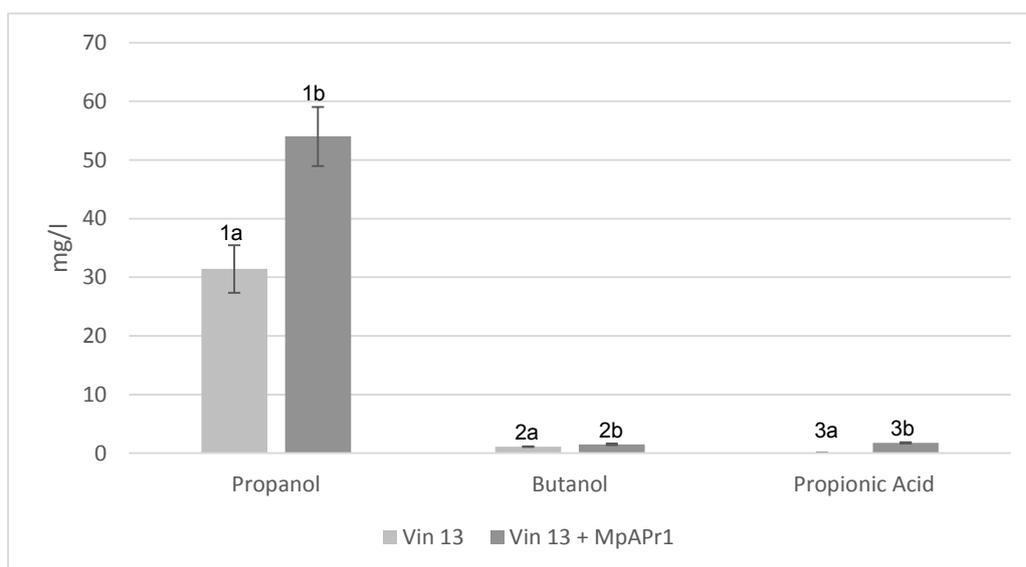
**Figure 4.41:** Graph showing measurement of major volatile compounds (determined by GC-FID) in wine samples fermented with *S. cerevisiae* VIN 13 with and without the addition of MpAPr1. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates.

Two groups of compounds can be identified. The first group, directly linked to carbon metabolism, comprises ethyl acetate, acetoin and acetic acid (Figure 4.42). No significant difference in ethyl acetate concentration could be observed. However, acetoin and acetic acid concentrations were significantly increased after fermentation in samples treated with MpAPr1.



**Figure 4.42:** Graph showing compounds linked to carbon metabolism in yeast. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

The second group comprises compounds that can be linked to amino acid catabolism for the production of higher alcohols and fusel acids via the Ehrlich pathway. Amongst the compounds belonging to this group propanol, butanol and propionic acid showed a significant increase fermentation in samples treated with MpAPr1 (Figure 4.43). No difference in concentrations could be observed in the other compounds tested.



**Figure 4.43:** Graph showing compounds linked to amino acid metabolism. The data points shown are means for three independent experiments and error bars indicate standard deviation between triplicates. Letters indicate significant differences between samples as determined by t-test ( $p \leq 0.05$ ).

#### 4.6.4 Discussion and partial conclusion

Initially, MpAPr1 concentration was estimated via the BCA assay using BSA as standard. The BCA assay is a colorimetric assay that makes use of the biuret reaction in which the protein backbone chelates  $\text{Cu}^{2+}$  ions and reduces them to  $\text{Cu}^+$  ions. The  $\text{Cu}^+$  ions then react with bicinchoninic acid (BCA) to form a purple-coloured product that absorbs at 562 nm. The assay procedure is similar to the well-known Bradford assay in which a standard curve is created based on a series of known concentrations of protein standards, in this case BSA. The colour formation using the BCA assay is thus dependent on the macromolecular structure of the protein, the number of peptide bonds and the presence of three amino acids (cysteine, tryptophan and tyrosine). Thus, false reading can be obtained if these parameters do not match well, and hence statistically this problem gets smaller the larger the protein or if mixtures of proteins are being measured. This means that for a purified protein the best standard to use for accurate quantification is the protein itself or a very similar one. For this reason, the protease from *Aspergillus saitoi* was used as alternative standard in an attempt to determine purified concentrations of MpAPr1 more accurately. Although accuracy could be increased, discrepancies were still evident and it was thus decided to estimate protein concentrations via visualisation on SDS-PAGE gels. Although MpAPr1 could be observed on SDS-PAGE gels and its concentration estimated, this estimation was at best semi-quantitative as some aspartic proteases (e.g. pepsin) bind an unusually low amount of Coomassie stain per molecule (Tal et al. 1985), leading to the under-estimation of the actual concentration. Moreover, results obtained via densitometry should be taken with caution as they are directly subjected to the limitation of the imaging software. Certain discrepancies may therefore occur between visual inspection of protein bands and their intensity as detected by the software. Therefore, protein concentrations could be estimated semi-quantitatively, but a quantitative method could be optimised for future investigation.

Grape proteins have been shown to be resistant to hydrolysis by fungal enzymes and Pocock et al. (2003) demonstrated that fungal protease (and pepsin) are able to remove PR proteins at 90°C. As further demonstrated by Marangon et al. (2012), heat treatment at 75°C for 1 min can make grape proteins more susceptible to protease activity. In order to test a possible application of MpAPr1, the ability of the pure enzyme was assessed against grape proteins under optimal conditions for the enzyme activity (as determined previously) as well as under average conditions occurring during winemaking in a buffered medium. MpAPr1 was found to be more active under optimal conditions where it fully degraded chitinases, one of the main proteins responsible for haze in white wine. Under conditions occurring during winemaking, MpAPr1 degraded proteins, but only partially (although again chitinases were more affected than the other proteins), suggesting that a longer incubation or higher concentration is necessary to achieve a somewhat more complete degradation. Previous

studies have indicated that chitinases are indeed the main proteins responsible for haze formation (Marangon et al. 2011) considering that they are more prone to form aggregates (Marangon et al. 2011b). It is interesting to note that MpAPr1 activity dropped over the 48-h period under optimal conditions for the enzyme. This would suggest that, although the enzyme activity is optimal, the enzyme itself is not stable under these pH and temperature conditions. Under sub-optimal conditions (i.e. winemaking conditions), the enzyme appeared to be more stable. Interestingly, protease activity increased over time under these conditions this was an unexpected result observed repeatedly. It is speculated that this could be due to certain peptides and proteins acting as substrate competitor for azocasein and/or reversible inhibitors of MpAPr. Furthermore, increasing the amount of MpAPr1 did not seem to result in any further degradation of the grape proteins. It should also be noted that further degradation of grape proteins (specifically TLP's) could be observed upon heat treatment, but to a lower degree than seen in studies performed by Marangon et al. (2012), probably due to difference in scale and equipment used for heat treatment.

Unlike the aspergillopepsins previously tested for application in the wine industry (Marangon et al. 2012), MpAPr1 was active against grape chitinases without the need of prior denaturation of these proteins via flash-pasteurisation. Similar results have been obtained by van Sluyter et al. (2013) where under typical winemaking conditions BcAP8 was able to degrade chitinase without the need for prior heat treatment. In this study, incubation with MpAPr1 was set at 48 h in order to mimic a possible maceration time prior to yeast inoculation, but since the enzyme was shown to remain active after 48 h, it is likely that it is able to continue degrading proteins during the course of fermentation, before natural degradation (i.e. not mediated by protease activity) occurs and/or the rising ethanol concentration can cause inhibition or denaturation.

Degradation of grape proteins without the need for prior heat treatment could be a great advantage for the wine industry. Furthermore, it should be noted that Marangon et al. (2012) applied heat treatment in the presence of the protease while in this study, heat treatment was applied without the presence of the enzyme. Flash pasteurisation was performed in this way as it was found that heat treatments above 50°C (paragraph 4.4.2) neutralise MpAPr1's protease activity possibly due to denaturation. Furthermore, aspergillopepsins were tested in grape juice and not in a buffered medium and thus the presence of other compounds might still have had an influence on MpAPr1 activity. Nevertheless, results demonstrated that MpAPr1 was able to partially degrade grape proteins under fermentation conditions.

Sufficient amounts of MpAPr1 could be purified using cation exchange chromatography as previously optimised in paragraph 4.5.2.2.4. In order to evaluate the impact of MpAPr1 during winemaking, Sauvignon Blanc juice was used for inoculation with *S. cerevisiae*. Similarly to the previous experiments, MpAPr1 was incubated with grape juice for 48 h prior

to inoculation in order to give the enzyme time to degrade proteins and possibly lead to an increase in yeast assimilable nitrogen that could be further utilised by the yeast and impact fermentation kinetics, population dynamics and/or aroma production.

No differences could be observed in fermentation kinetics or population dynamics between grape juices treated with or without MpAPr1 prior to yeast inoculation. Furthermore, the yeast population dynamics were also similar.

Protease activity of MpAPr1 was not impacted after the 48-h incubation period suggesting that the enzyme is stable at these conditions in grape juice, as already noticed in the model solution. However, after fermentation, protease activity was completely lost. This result combined with the inhibitory impact of ethanol reported in paragraph 4.4 suggest that although active at the beginning of fermentation MpAPr1 loses its activity some time during fermentation because of rising ethanol levels and/or natural degradation.

PAGE techniques were used to visualise degradation of proteins that were tentatively identified by comparison of molecular weights using available literature (van Sluyter et al. 2015, Marangon et al. 2012, Le Bourse et al. 2009). MpAPr1 was able to slightly degrade chitinase and TLP after 48 h and degradation was even more severe at the end of fermentation (thereby confirming that MpAPr1 remained active at least during part of the fermentation). After fermentation, with or without prior addition of MpAPr1, chitinases were fully degraded and slight degradation of  $\beta$ -glucanases and TLP could be observed while invertases remained unaffected. This indicated that protein degradation took place naturally over the course of alcoholic fermentation. Slight differences could nevertheless be observed when comparing the samples treated with MpAPr1 and those untreated thereby suggesting that MpAPr1 contributed to protein degradation, but only to a limited extent. Similar natural degradation of a band corresponding to ca. 25 kDa as well as the resilience of invertases to protease activity have both been previously observed in fermentations performed with Sauvignon Blanc and Chardonnay juice (Marangon et al. 2012).

2D-PAGE analyses of grape samples treated with MpAPr1 confirmed the results obtained from SDS-PAGE gels. However, the appearance of some bands could be visualised (irrespective of the presence of MpAPr1) after 48 h of incubation. These bands could potentially be degradation products as a consequence of protease activity. At the end of fermentation, all bands experienced some degree of degradation, but degradation of chitinases was complete and only spots corresponding to TLP and invertases could be observed. It should be noted that in both the SDS-PAGE gels and 2D-PAGE gels, the presence of MpAPr1 is difficult to observe, suggesting that the intended addition of ca. 0.15 mg/ml had been underestimated (highlighting the protein determination issues once again) and could indicate that the enzyme might have degraded upon storage before use in

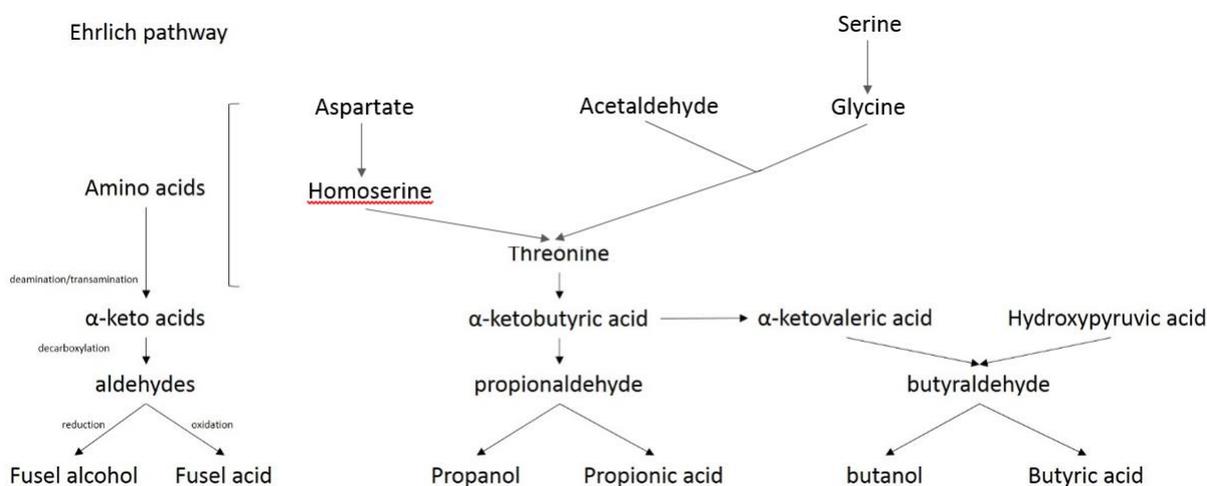
fermentation trials. Nevertheless, concentrations were high enough to observe a certain impact.

HPLC analysis was used for determination of specific grape proteins, however the specific method used is limited to measuring only four proteins, namely Vvlt1, Vvlt2, Vvlt3 and chitinase. No degradation of specific haze-causing grape proteins (Vvlt1 - 3 and chitinase) could be observed, in grape juice after 48 h and at the end of fermentation only Vvlt1 was slightly degraded following incubation with MpAPr1. Although degradation of certain proteins could be visualised on the SDS-PAGE gels, not all protein bands appeared fully degraded at the end of fermentation and the latter likely correspond to the specific grape proteins (Vvlt1, Vvlt2, Vvlt3 and chitinase) determined by HPLC methods. This could explain the discrepancy between the results from the SDS-PAGE visual inspection and those from the HPLC analysis. For instance, the band tentatively identified as a 35-kDa chitinase on the gels is not the chitinase detected by the HPLC, which is a 24-kDa class IV chitinase. After fermentation, natural degradation of Vvlt1 and chitinase could be observed in all samples regardless of MpAPr1 addition. However, in samples with MpAPr1 addition, Vvlt2 was significantly degraded. This indicated that MpAPr1 targeted only specific TLP proteins. Somewhat similar results have been obtained for BcAP8 (van Sluyter et al. 2013) that caused only a slight reduction in the TLP levels under winemaking conditions.

Despite degradation of some grape proteins, MpAPr1 was not able to significantly reduce haze formation according to the results of the heat test performed. After 48 h, no differences could be observed in the grape juice samples treated with or without MpAPr1. Moreover, similar observations were found in samples after fermentation and no significant differences could be obtained. Similar results have been previously obtained for Aspergillopepsins I and II (Marangon et al. 2012). However, because of the conditions under which the heat test is conducted (80°C for 2 h), all wine proteins are precipitated (even those that are considered heat stable) and this can lead to false interpretation (Esteruelas et al. 2009, Falconer et al. 2010). In contrast, van Sluyter et al. (2013) found that after treatment by BcAP8, protein haze could be significantly reduced. However the heat test was performed under gentler conditions (i.e. 55°C for 18 h) and this could explain apparent differences between the two studies.

Degradation of grape proteins can potentially lead to the release of nitrogen-containing compounds that can in turn be used by the yeasts during fermentation and result in the production of sought-after aroma compounds. Enzymatic analysis of grape juice treated with MpAPr1 revealed a significant (but slight) increase in the concentration of nitrogenous compounds in grape juice after 48 h. When fermentation was performed using MpAPr1-treated grape juice, increased levels of acetoin, acetic acid, propanol, propionic acid and butanol could be observed at the end of fermentation. The first two compounds are directly connected to

central carbon metabolism and their increase might possibly indicate that the protease exhibits some kind of stress on the yeast during fermentation. The second group of compounds (propanol, propionic acid and butanol) arise from the Ehrlich pathway and originate specifically from the metabolism of aspartate, threonine and serine as illustrated in Figure 4.44. Note that both aspartate and threonine can lead to the production of propanol, propionic acid, butanol and butyric acid while the metabolism of serine can lead to the production of glycine which can be converted to threonine before yielding the compounds described above. Thus, the increased levels observed for these compounds in the presence of MpAPr1 could be linked to a greater availability of aspartate and/or threonine and possibly serine. It should be noted that all volatile compounds tested were under sensory detection threshold, according to Guth (1997), with the exception of acetic acid and ethyl acetate (200 mg/l and 7.5 mg/l, respectively).



**Figure 4.44:** Production of higher alcohols and fusel acids from amino acids aspartate, threonine and serine via the Ehrlich pathway.

In a recent study, similar findings have been observed (Zhang et al. 2016). In the latter authors' study, protease treatment led to an increase of specific free amino acids (namely alanine, threonine, valine, phenylalanine and aspartate) and a significant increase in the production of propanol, isobutanol and isoamyl alcohol. The authors did however not provide a specific explanation.

Upon aspartic protease activity, grape proteins might be cleaved in such a way that it facilitates liberation of these specific amino acids. Indeed, the structure of class IV chitinase isolated from grape vine (*Vitis vinifera*) is rich in serine (10.6%) and TLP in threonine (10.7%) (Figure 4.45) and their degradation could account for a significant increase in these amino acids. Furthermore, *in silico* analysis using the online software ExPASy PeptideCutter revealed

that the break-down of the class IV chitinase by an endo-aspartic protease such as MpAPr1 is likely to result in aspartate residues being exposed (Figure 4.46). This could have resulted in an increase in the concentrations of aspartate that ultimately led to the observed increase in propanol, butanol and propionic acid upon assimilation by the yeasts. Although no increase in individual amino acid levels was observed after 48 h of incubation, it is possible that these amino acids were released later during fermentation and immediately assimilated by the yeasts. The increase in volatile compounds reported is unlikely to have an impact on sensory properties of the finished wines because all compounds detected, with the exception of acetic acid and ethyl acetate were below individual sensory detection threshold limits (Guth 1997), but even then, the concentrations remain low and acceptable according to oenological standards. However, a production of these compounds may be viewed as an indirect proof of protease activity. Furthermore, sensory evaluation performed in studies using AGP (Marangon et al. 2012) found no differences in aroma but this observation could have originated from a similar scenario where protein degradation indirectly resulted in the production of some volatile compounds whose concentration remained under sensory detection thresholds.

**A:** >gi|33329392|gb|AAQ10093.1| class IV chitinase [*Vitis vinifera*]  
 MAAKLLIIVLLVGFALFGAAVAQNCGCASGLCCSKYGYCGIGSDYCGDGCQSGPCDSSSGSGSSVSDIVIQSFFDGI  
 INQAASSCAGKNFYIRAAFLSALNSYSGFGNDGSTDANKREIAAFFAHVTHEIGHFCYIEEINGASHNYCDSSNI  
 QYPCVSGQNYGRGPLQLIWNYNAGAAGNSIGFNGLSNPGIVAIDVVTISFKIALWFWMMNVHSSVIGQGFGAIRAI  
 INGAVECNGGNIAAVNARVQYYKYCSQLGVSFGDNLIC

**B:** >gi|33329390|gb|AAQ10092.1| thaumatin-like protein [*Vitis vinifera*]  
 MGLCKILSISSFLLIIFALFFTPSYAATFNIQNHCSYIVWAAAVPGGGMQLGSGQSWSLNVNAGTGGRVWARTNCN  
 FDASGNGKCEIIGDCGGLLQCIAYGIPPNILAEFALNQFSNLDFFDISLVDGFNVPMFNPISNGCIRGISCIADI  
 VGECPAALKITGGCANNPCIVFKIDEYCCNSGSCNADYSRFFKTRCPDAYSYPKDDQTSIFTCPAGINYEYVFC

**Figure 4.45:** Protein sequence of (A) chitinase class IV and (B) thaumatin-like protein isolated from *V. vinifera*. Aspartate is highlighted in yellow, serine in green and threonine in red.



# Chapter 5

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## General conclusion and future prospects

## Chapter 5 – General conclusion and future prospects

### 5.1 Summary of the main results

Wine haziness, especially in white and rosé wines, is a fault affected by poor storage conditions (especially too high temperatures) during ageing. The removal of the proteins responsible for haze prior to bottling is thus an essential step of the winemaking process, but it can be challenging for winemakers. Indeed, this is usually achieved via the addition of bentonite. Several issues have been identified with the use of this clay and consequently, alternatives are sought. Considering the mechanisms of wine protein haze formation, several strategies are being investigated. One of the most promising is to degrade grape proteins with enzymes. This is particularly appealing since enzymatic treatment would minimise wine volume loss and stripping of aroma compounds. Thus, the search for enzymes able to serve as alternative has become more popular and has extended to include non-*Saccharomyces* yeasts as potential producers. Those occurring naturally in grape juice/wine could be ideal candidates in the current context of trying to minimise the addition of chemicals or biological agents of external origin throughout the winemaking process.

One such organism naturally present in grape juice has been isolated at the Institute for Wine Biotechnology. Indeed, *Metschnikowia pulcherrima* IWBT Y1123 was found to display strong extracellular acid protease activity against BSA, casein and grape proteins at pH 3.5. The protease-encoding gene, named *MpAPr1*, was isolated and tentatively identified as an aspartic protease (Reid et al. 2012). Further studies involved cloning the gene into a bacterial host for over-expression and eventual purification (Theron 2013). However, extraction was only possible under denaturing conditions and following purification activity could not be regained.

The aim of this study was to investigate MpAPr1's oenological potential with a specific focus on its ability to degrade the grape proteins responsible for haze. In order to study the enzyme, heterologous expression was realised in a eukaryotic host (*Komagataella pastoris*) commonly used for expression of recombinant enzymes. Indeed, expression was successful but the presence of three bands could be observed corresponding to MpAPr1 following SDS-PAGE analysis. Initial purification attempts were unsuccessful and characterisation of MpAPr1 properties were performed within a protein crude.

Optimal conditions for protease activity were identified at pH 4.5 and 40°C. Of all metal ions tested, only Ni<sup>2+</sup> and Cu<sup>2+</sup> had a significant effect on enzyme activity. Pepstatin completely inhibited activity at nM concentrations similarly to other aspartic proteases and followed an uncompetitive inhibition strategy. Ethanol and sugar had an inhibitory effect on protease activity at high levels such as those occurring in wine and grape juice, respectively. When kinetic parameters are compared to those of commercially available aspartic proteases,

MpAPr1 had the lowest affinity amongst the aspartic proteases tested towards casein, but could catalyse its hydrolysis at a similar rate as the protease from *Aspergillus saitoi*. Characterisation experiments demonstrated that, although not optimal, MpAPr1 should be moderately active under winemaking conditions and was proposed as a suitable candidate for potential application in oenology.

In order to elucidate MpAPr1's potential application in wine, purification on a larger scale had to be achieved. However, expression had to be optimised first in a minimal medium for purification purposes. Optimal expression of MpAPr1 in *K. pastoris* was found at 20°C and pH 6. Initial attempts using IMAC proved unsuccessful and exploitation of the hexa-histidine tag fused to the enzyme could not be achieved. Eventually, purification was achieved via cation exchange chromatography. MpAPr1 could be purified and the presence of a single band could be observed following SDS-PAGE analysis.

Pure grape proteins could be obtained and their degradation via MpAPr1 investigated in a buffered model solution. Under optimal conditions for enzyme activity MpAPr1 could completely degrade chitinases, but under sub-optimal conditions only partial degradation of proteins could be achieved. Furthermore, protease activity decreased following the incubation period at optimal conditions while no activity was lost at wine making conditions. Finally, MpAPr1 was able to degrade grape chitinases without the need for prior denaturation via flash pasteurisation.

Sufficient amounts of MpAPr1 could be purified for use in grape juice/fermentation experiments. Sauvignon Blanc juice was treated with MpAPr1 prior to inoculation and its impact evaluated after 48 h after which inoculation occurred using *S. cerevisiae* VIN13. Partial degradation of proteins (especially chitinases) could be achieved and at the end of fermentation an increase in specific volatile compounds could be detected, confirming that MpAPr1 degraded certain proteins at least partially. Nevertheless, protease activity was limited under grape juice fermentation conditions. Further optimisation would be required to attain a satisfactory level of activity.

## 5.2 Concluding remarks and future prospects

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Future work should focus on establishing an appropriate ratio of MpAPr1 to grape proteins to investigate if optimised degradation of grape proteins can be achieved. Although an increase in stability was observed under fermentation conditions, further studies need to be performed in order to determine MpAPr1's stability at pH and temperature conditions (and a combination thereof). Protease activity could also be monitored throughout fermentation in order to determine exactly at which point activity is lost and if a slow decline or a sudden drop in activity occurs.

Future prospects should include purification of MpAPr1 on a larger scale in order to evaluate the enzyme's performance on pilot scale fermentations. Furthermore, economic analysis of MpAPr1's ability to reduce bentonite requirements needs to be calculated and compared in order to determine if the can enzyme provide a significant financial savings in operation costs. Prior to performing large scale purifications, further studies could focus on optimising expression and enhancing MpAPr1 activity against grape proteins. The latter should include optimisation possibly by enhanced expression cassettes using inducible promoters that can perhaps enhance expression and/or optimising growth an expression conditions via bioreactor in order facilitate large amounts of MpAPr1 production. Although purification could be achieved using cation exchange chromatography, other strategies could also be employed such as the use of different affinity tags. Furthermore, purification on a larger scale should be envisaged by employing larger columns able to house larger volumes of binding resins.

Assessment of protease activity using azocasein as substrate was sufficient, but it suffers from solubility issues at low pH values. Furthermore, characterisation of MpAPr1's enzymatic properties should be performed at low pH values using a different substrate i.e. haemoglobin. Although both substrates are efficient at determining protease activity their use as substrates does not provide information about protease activity against grape proteins. The production or synthesis of suitable substrates for use in oenological analysis should therefore be investigated (i.e. the use of grape proteins with a conjugated dye such as azo). Such substrates could also be used in fermentation in order to evaluate activity and progression throughout.

PAGE techniques should be further exploited and coupled with HPLC and LC-MS/MS techniques in order to elucidate which specific proteins are degraded, either by protease activity or by other means. The exact mechanism of MpAPr1 activity (i.e. cleavage sites) on target proteins also needs to be unravelled and the mechanism by which free amino acid residues are potentially released elucidated. The production of volatile compounds in the presence of MpAPr1 needs be further investigated through sensory evaluation of the finished wine.

The efficiency and degradation of grape proteins should be evaluated and compared to other existing or commercial aspartic proteases (e.g. Proctase) in order to evaluate if MpAPr1 is a viable candidate for commercialisation for use in the wine industry. Furthermore, it would seem that protease from different sources envisaged for oenological purposes (e.g. BcAP8) are effective at different parameters and are able to degrade different grape proteins. Thus, the use of a cocktail of aspartic proteases from non-*Saccharomyces* species and/or fungal species should be investigated for optimal efficiency.

Investigations into the use of non-*Saccharomyces* yeasts combined with *S. cerevisiae* starter cultures are already available commercially and has shown promising results. The use of non-*Saccharomyces* yeasts displaying strong protease activity (e.g. *M. pulcherrima* IWBT Y1123) can thus also be envisaged and further investigations should be launched in order to evaluate their commercial application. Furthermore, the search for and isolation of proteases for oenological purposes should be extended to other non-*Saccharomyces* species as some (other than *Metschnikowia pulcherrima*) have been shown to also display strong activity at oenological pH and temperatures. Furthermore, novel proteases might be able to degrade additional proteins (other than or the same as MpAPr1) and result in the release of different peptides and or amino acids resulting in the production of different aroma compounds following fermentation.

Finally, evaluation of MpAPr1 for applications in other industries (other than the wine industry), such as the brewing industry where similar parameters are dealt with or the cheese making industry in which parameters used are more suited for MpAPr1 activity, should also be investigated in order to broaden the enzyme's applications and commercial value.

# Chapter 6

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## Chapter 6 – Bibliography

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Zhang W, Zhang L, Xu C (2016) Chemical and volatile composition of jujube wine fermented with *Saccharomyces cerevisiae* with and without pulp contact and protease treatment. *Food Sci Technol* 36:204-9

# Chapter 7

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## Scientific communications

## Chapter 7 – Scientific communications

### 7.1 Peer-reviewed publications

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Theron LW, Bely M, Divol B (2017) Characterisation of the enzymatic properties of MpAPr1, an aspartic protease secreted by the wine yeast *Metschnikowia pulcherrima*. *J Sci Food Agric* In press doi: 10.1002/jsfa.8217

Theron LW, Divol B (2014) Microbial aspartic proteases: current and potential applications in industry. *Appl Microbiol Biotechnol* 98:8853-68

Reid VJ, Theron LW, Du Toit M, Divol B (2012) Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. *Appl Environ Microbiol* 19:6838-49

### 7.2 Oral communications

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Theron LW, Bely M, Divol B (2016) Investigating the potential application of MpAPr1, an aspartic protease isolated from *Metschnikowia pulcherrima* IWBT Y1123, for wine making purposes. 38<sup>th</sup> Conference of the South African Society for Enology and Viticulture, Somerset west, South Africa

Theron LW, Bely M, Divol B (2015) Enzymatic characterisation of an oenological relevant protease isolated from *Metschnikowia pulcherrima*. Oeno 2015, 10e Symposium International d'Œnologie de Bordeaux, Bordeaux, France

Theron LW, Bely M, Divol B (2014) Characterization of aspartic proteases from non-*Saccharomyces* yeasts to assess their oenological potential. 36<sup>th</sup> Conference of the South African Society for Enology and Viticulture (SASEV), Somerset west, South Africa

Theron LW, Zietsman JJ, Divol B (2013) Optimizing recombinant expression of an aspartic protease from *Metschnikowia pulcherrima*. 18<sup>th</sup> biennial Conference of the South African Society of Microbiology (SASM), Bela-Bela, South Africa

Theron LW, Zietsman JJ, Divol B (2013) Utilisation of acid protease produced by non-Saccharomyces yeast to prevent haze and release assimilable nitrogen. 35<sup>th</sup> Conference of the South African Society for Enology and Viticulture (SASEV), Somerset west, South Africa

### **7.3 Poster communications**

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Theron LW, Bely M, Divol B (2016) Investigating an aspartic protease isolated from *Metschnikowia pulcherrima* and its potential application in wine. 25<sup>th</sup> International ICFHM Conference, Dublin, Ireland

Theron LW, Bely M, Divol B (2015) Enzymatic characterisation of an oenological relevant protease isolated from *Metschnikowia pulcherrima*. Oeno 2015, 10e Symposium International d'Œnologie de Bordeaux, Bordeaux, France