A study on plant-derived small proteins and peptides with antiyeast activity and preliminary investigations in food materials

Giulia Mignone
Department of Biological Sciences, Munster Technological University, Cork, Ireland,
giulia.mignone@mycit.ie

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A study on plant-derived small proteins and peptides with antiyeast activity and preliminary investigations in food materials

Giulia Mignone
Al mio Papà, la mia ispirazione e il mio scienziato preferito

To my Dad, my inspiration and my favourite scientist
A study on plant-derived small proteins and peptides with antiyeast activity and preliminary investigations in food materials

A dissertation presented to Munster Technological University
for the degree of
Doctor of Philosophy

by

Giulia Mignone, BSc, MSc

Department of Biological Sciences,
Munster Technological University,
Rossa Ave, Bishopstown,
Cork, Ireland.

Research supervisors:
Prof. Aidan Coffey

Submitted to Munster Technological University,
31st May 2022
Declaration

I declare that this thesis, which I submit to Munster Technological University, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged, such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in Munster Technological University or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

[Signature]

Student Number: R00158428

Date: 01/06/2022

Supervisor Signature

[Signature]

Date: 01/06/2022
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Authorship statement

The conceptualisation and the drafting of this thesis were supervised by the project Principal supervisor Prof. Aidan Coffey (F.I.R.M., Project Reference 15/F/731); the author contributions for each Chapter are listed below:

**General Introduction, Chapter 1 – Literature Review, Chapter 3, Chapter 6, Chapter 7 and General conclusion**

These Chapters were exclusively the candidate work

**Chapter 2 and 5**

These Chapters are exclusively the candidate work; although some experiments were carried out by the candidate in University College of Cork in the laboratory of Elke Ardent with the guidance of Laila Shwaiki, who were collaborator partners of the project.

**Chapter 4**

This Chapter was exclusively the candidate work, apart from the fact that some of the bioinformatics data presented were performed by one undergrad student (Dean Grimes) under the direct supervision of the candidate. Nevertheless, all the analysis were repeated by the candidate.
Abbreviations

2S       Seed Storage
3D       Three Dimensional
aa       Amino acid
AMPs     Antimicrobial Peptides
Arg      Arginine
BAMPs    Botanic Antimicrobial Peptides
BCA      Bicinchoninic Acid
CaM      Calmodulin
CB       Chitin-binding
CCK      Cys-knot
CFU      Colony forming unit
CRKs     Cysteine-rich Receptor-like Kinases
CRPs     Cysteine Rich Peptides
CRRSPs   Cysteine-rich Receptor-like Secreted Proteins
CTPP     C terminal pro-peptide
CWI      Cell Wall Integrity
Cys      Cysteine
DAFM     Department of Agriculture, Food and the Marine
DNA      Deoxyribonucleic acid
DSMZ     Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT      Dithiothreitol
EDTA     Ethylenediaminetetraacetic Acid
EFSA     European Food Safety Authority
ER       Endoplasmic Reticulum
EUCAST   European Committee on Antimicrobial Susceptibility Testing
GASA     Gibberellic Acid Stimulated in Arabidopsis
GlcCer   Glycosphingolipid glucosylceramide
Gly      Glycine
GRAS     Generally Recognised As Safe
GRPs     Glycine-rich peptides
<table>
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<td>h</td>
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<tr>
<td>HDPs</td>
<td>Host Defense Peptides</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HLPs</td>
<td>Hevein-type antimicrobial peptides</td>
</tr>
<tr>
<td>HOG</td>
<td>High Osmolarity Glycerol</td>
</tr>
<tr>
<td>KTIs</td>
<td>Kunitz-type inhibitor</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LTPs</td>
<td>Lipid transfer proteins</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>M(IP)2C</td>
<td>Mannosyldiinositolphosphorylceramide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein Kinase</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
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<tr>
<td>PDB</td>
<td>Potato Dextrose Broth</td>
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<tr>
<td>PDLPs</td>
<td>Plasmodesmata-localised Proteins</td>
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<tr>
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<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PIs</td>
<td>Protease Inhibitors</td>
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<td>RLKs</td>
<td>Receptor-like Kinases</td>
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<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>s</td>
<td>seconds</td>
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<tr>
<td>SAMPs</td>
<td>Synthetic Antimicrobial Peptides</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SDA</td>
<td>Sabouraud-Dextrose Agar</td>
</tr>
<tr>
<td>SDB</td>
<td>Sabouraud-Dextrose Broth</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>SPIs</td>
<td>Serine Protease Inhibitors</td>
</tr>
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<td>Seed Storage Proteins</td>
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<td>TLPS</td>
<td>Thaumatin-like proteins</td>
</tr>
<tr>
<td>Tris–HCl</td>
<td>Tris Hydrochloride</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-high-temperature processing</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Extract Peptone Dextrose</td>
</tr>
</tbody>
</table>
Thesis abstract

The discovery of novel antimicrobial molecules would benefit considerably different biotechnological fields, including medicine, agriculture and the food industry, on which this thesis work focuses. Specifically, here are presented fundamental studies on plant-derived antimicrobial proteins for the reduction of food spoilage caused by yeast microorganisms. Small proteins and peptides from the plant innate immune system possess several characteristics which could make them ideal candidates for a novel and natural antispoilage agent. The first Chapter reviews proteins families associated with the plant innate immune system that exhibit antiyeast activity and low molecular weight (<30 kDa), focusing on their structure and antiyeast mode of action.

Chapter 2 investigates two peptides, Pn-AMP1 and Pn-AMP2 belonging to the hevein-type peptides family; peptides were synthetically generated using their native amino acid sequence. Synthetic Pn-AMP1 showed fast action activity against common food spoilage yeasts, it resulted potentially safe for consumption, and it was successfully incorporated in UHT milk and Fanta Orange, where it contained the growth of Kluyveromyces lactis and Zygosaccharomyces bailii, respectively.

A different family of antimicrobial plant proteins (2S albumins) was studied in Chapters 3, 4, 5 and 6. Chapter 3 describes the selective extraction of an antiyeast protein (labelled WMS1 in this thesis work) of ~14 kDa from white mustard (Brassica hirta) seeds. The protein was purified in a relatively high yield, using a simple protocol that included only one chromatographic method step. In Chapter 4, bioinformatics tools and wet-lab techniques are used to study the structure and classify the antiyeast protein. The WMS1 antiyeast protein was identified as an isoform of the Napin protein Allergen Sin a 1 belonging to the 2S albumins family, characterised by high structural stability which donates to the protein resistance heat treatment and proteolytic degradation. Chapter 5 describes the antimicrobial spectrum of WMS1, its antiyeast mechanism of action and its validity as preservative agent. Although WMS1 resulted potentially safe for consumption and inhibited the growth of Z. bailii in several beverage systems, its biotechnological potential is limited by its allergic properties. In Chapter 6, the protein WMS1 was cloned in a pET28a-MBP vector and expressed in an E. coli BL21 system. Interestingly, recombinant WMS1 and native WMS1 showed similar activity against the yeast Z. bailii.
Chapter 7 includes a protocol for the rational design of ultra-short antimicrobial peptides. Since ultra-short antimicrobial sequences can be found within the sequence of any larger protein, the protocol was applied using WMS1 primary sequence, and seven ultra-short SinA-AMPs were chemically generated. Among the various SinA-AMPs, the peptide labelled SinA-pepIII had in vitro activity against several common yeast spoilage strains via membrane permeabilisation and it could be employed as a food preservative as it was pH- and heat- stable, potentially safe in terms of human consumption and it prevented the growth of *Z. bailii* in cranberry juice.

The results obtained in this work offer new perspectives in the research for novel and clean-label food preservatives to fight the food waste phenomenon.
General Introduction

Yeast

Yeasts are fungi that predominantly exist as unicellular organisms (Stöckheim, 1972); they have evolved from multicellular ancestors (Kurtzman and Piškur, 2006), and they can reproduce both sexually and asexually. During sexual reproduction, a single cell undergoes meiosis and produces haploid spores; however, most yeasts reproduce asexually by mitosis. Many do so by the asymmetric division process known as "budding" (Dujon, 2010). Moreover, several yeast species can develop multicellular characteristics by forming strings of connected budding cells known as "pseudohyphae" or false hyphae (O’Leary, 2001). Yeast morphologic and physiologic characteristics can vary greatly depending on species and environment; also, some fungi (dimorphic) can alternate between a yeast phase and a hyphal phase, depending on environmental conditions. It is clear that the term yeast is not easily defined taxonomically; Lodder, in 1971, described yeasts as "microorganisms in which the unicellular form is conspicuous, and which belong to the kingdom Fungi" and this definition is still valid nowadays and represent a cornerstone for the studies of this heterogeneous assembly of often unrelated fungi.

Yeasts are classified as eukaryotes, and indeed they possess all the major classical traits of other eukaryotic cells, including the presence of the cytoskeleton, a nucleus and various membrane-bound subcellular organelles (e.g., endoplasmic reticulum, the Golgi apparatus, mitochondria and microbodies) (Walker and Dundee, 2009). The internal environment of yeasts is protected by a layered thick envelope consisting of a distinct wall (outer layer) and an internal plasma membrane with a periplasm space in between the two (Figure 1). The cell wall is the primary contact site between the yeast cell and the outside world; in Saccharomyces cerevisiae, it occupies about 15% of the total cell volume (Stewart and Stewart, 2017). It determines the cell shape and integrity and provides the cell with mechanical strength to resist changes in the environmental osmotic pressure and other stresses. The major components are β-glucans (50%–60% of the cell wall mass) and mannoproteins (40–50%), and chitin (1–3%) (Lipke and Ovalle, 1998). The periplasm layer is a non-continuous and irregular space with interrupting invaginations in the plasma membrane and the inner surface of the cell wall; it is characterised by the presence of essential enzymes (e.g. invertase and acid phosphatase) (Harsay and Bretscher, 1995). The inner layer of the yeast envelope is the plasma membrane.
which contains a high proportion of negatively charged phospholipids (unlike mammalian cells) and membrane proteins. It possesses numerous functions: it is a barrier to the free diffusion of solutes, it catalyses specific change reactions, stores energy as transmembrane ions and solute gradients, regulates the rate of energy dissipation, provides sites to bind specific molecules for catabolic signalling pathways and sites of enzyme pathways involved in the biosynthesis of cell components (Anwar et al., 2017; Kalebina and Rekstina, 2019).

Figure 1. Schematic representation of the yeast cell with a focus on the envelope composition. Reproduced from Coradello and Tirelli (2021).

**Yeast spoilage of foods and beverages**

Although bacteria and moulds (strictly filamentous fungi) are recognised as the most prevalent cause of spoilage in foods, yeasts contribute significantly. Preservation techniques as low-temperature storage and the use of preservatives or chemical additives can prevent bacterial and fungal growth; however, certain yeast species can overcome such measures and cause spoilage outbreaks (Table 2). Products rich in hexose sugars, vitamins, minerals and salt, with high acid content (pH 5 or lower) or/and preserved with weak acids and long-term frozen products can constitute an ideal substrate for yeast growth. Usually, noticeable spoilage signs appear when yeast growth reaches $10^4/10^5$ cells or CFU per gramme or millilitre. These include
carbon dioxide gas production (causes the containers of packaged products to swell and sometimes explode), the appearance of colonies/film/slimy biomass on the exterior of solid products or floating on the surface of liquid products, development of yeasty, alcoholic, and other off-odours and off-flavours, decolouration of the products, presence of cell sediments and turbidity (Booth and Stratford, 2003; Fleet, 2011; Leyva Salas et al., 2017; Davies et al., 2021)

Table 1. Yeast species and symptoms of spoilage in various food. Table reproduced from Hernández et al. (2018).

<table>
<thead>
<tr>
<th>Yeast species</th>
<th>Foodstuff</th>
<th>Symptoms of spoilage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brettanomyces bruxellensis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pichia guilliermondii</td>
<td>Wine</td>
<td>Production of 4-ethylphenol, 4-ethylguaiaol, and 4-ethylcatechol: off-flavours described as animal odours, farm, horse sweat, medicine and animal leather</td>
</tr>
<tr>
<td>Pichia manshurica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida ishiwadai</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trigonopsis cantarellii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida wickerhamii</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pichia membranifaciens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pichia anomala</td>
<td>Cider, beer, soft drinks</td>
<td></td>
</tr>
<tr>
<td>Candida spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dekkera anomala</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Saccharomyces cerevisiae</strong></td>
<td>Green table olives</td>
<td>Deep softening (pectinolytic activity)</td>
</tr>
<tr>
<td>Pichia manshurica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida boidinii</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pichia manshurica</strong></td>
<td>Fermented cucumber</td>
<td>Consumption of organic acids and subsequent increase of pH</td>
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<tr>
<td><strong>Issatchenckia occidentalis</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Zygosaccharomyces bailii,</strong></td>
<td>Fruit juices</td>
<td>Film formation, softening and generation of off-odours</td>
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<td><strong>Zygosaccharomyces rouxii</strong></td>
<td></td>
<td>Visible growth on the surface and fermentations</td>
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<td><strong>Saccharomyces rouxii</strong></td>
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<td>Producing ethanol or film formation</td>
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<td><strong>Saccharomyces spp.</strong></td>
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<td></td>
</tr>
<tr>
<td>Pichia spp.</td>
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</tr>
<tr>
<td><strong>Meyerozyma guilliermondii</strong></td>
<td>Yoghurt</td>
<td>Bubble formation and swelling of the packages</td>
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<td>Yarrowia lipolytica</td>
<td>Cheese</td>
<td>Undesirable pigment production</td>
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<tr>
<td>Debaryomyces hansenii</td>
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<td>Strong gas producers in close texture cheeses</td>
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<td>Candida catenulata</td>
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<td>Kluyveromyces lactis</td>
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<td>Pichia guilliermondii</td>
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<tr>
<td><strong>Kazachstania psychrophila</strong></td>
<td>Vacuum-packed beef</td>
<td>Discolouration, off-odours and gas swelling</td>
</tr>
</tbody>
</table>

5
**Plant-derived peptides (and small proteins) for the reduction of yeast spoilage**

Almost all living organisms can produce small proteins and peptides with antimicrobial properties to respond to pathogen attacks. These types of molecules are typically labelled Antimicrobial Peptides (AMPs) or Host Defense Peptides (HDPs) (Anunziato and Costantino, 2020); and they are known for displaying a broad spectrum of biological activities (both *in vivo* and *in vitro*), including antiviral, antifungal, anti-mitogenic, anticancer, and anti-inflammatory. Unlike traditional antimicrobial drugs or preservatives, AMPs exert their activity by interacting with microbial cell membranes causing disruption in the phospholipid bilayer and leading to cell death. This membrane lysis type of attack is often irreversible for bacteria or fungi, consequently reducing the possibility of developing drug resistance (Nawrot *et al.*, 2014). Moreover, this type of proteins are generally characterised by low toxicity, good thermal stability and water solubility (Divyashree *et al.*, 2020). Thus, it is no surprise that the scientific community’s interest in these types of molecules is increasing.

This thesis focuses on host defense peptides (and small proteins) from plant origin with antiyeast properties and their possible exploitation as novel and natural food preservatives against yeast spoilage. Botanic AMPs (BAMPs) application in food can be advantageous as their natural origin may be seen as a more conservational and healthier solution to current preservatives.

Antimicrobial small proteins (or peptides) are traditionally extracted from organic materials and subsequently characterised. Databases containing known plant antimicrobial sequences can be exploited; BAMPs can be chemically synthesised based on the sequence of the wild-type peptide or expressed and produced in a heterologous system. Moreover, *in silico* tools can predict ultra-short peptides with antimicrobial activity within known sequences (Figure 2).

Producing BAMPs, whether natural, synthetic or *heterologous* generated, in quantities where they can be beneficial in the food industry can be costly, laborious and time-consuming. As the technologies improve and develop, BAMPs could be exploited to a fuller extent and the research being conducted in this thesis work could represent foundation for future development.
Extraction from plant materials

Plant native small protein/peptide with antimicrobial activity

Identification of the primary sequence

Synthetic production

Synthetic plant-derived Small protein/peptide

Heterologous production

Plant-derived Small protein/peptide

Identification antimicrobial ultra-short peptides within the sequence

Figure 2. Workflow for the production of plant-derived antimicrobial small proteins and peptides.

Bibliography


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Chapter 1

Review on plant defense-related small proteins and peptides with antiyeast activity
Abstract

Plants are a rich source of defense-related small weight proteins that can display *in vivo* and *in vitro* activity against a broad spectrum of microorganisms, pests and cancerous cells. The scientific community interest in these evolutionarily conserved proteins has notably increased in recent years. Focusing on land plant sources, this review aims to summarise and discuss various families of small proteins and peptides with proven ability to inhibit the growth of yeast strains. Most of the protein and peptide families here described present an overall positively charged structure that includes hydrophobic and hydrophilic features enabling the molecule to be soluble in aqueous environments and express activity by binding and interacting with the negatively charged yeast cell membranes. However, this antiyeast activity is modulated by proteinic structural diversity among the various families (*e.g.*, presence of binding sites for specific components on the fungal cell membrane or the number of stabilizing disulphide bridges), which inevitably results in a different spectrum of activity as well as different sensitivity to external perturbances such as pH variation, salt concentrations and protease action. Small proteins and peptides from plant defense systems are here grouped based on their cysteine (Cys) content; Cys-rich families are classified in: defensins, thionins, lipid transfer, hevein-type, thaumatin-like, 2S albumin, snakins, knottins, cyclotides, DUF26 proteins, vicilin-like and protease inhibitors; while proteins that are relatively poor in Cys include glycine (Gly)-rich peptides and lectins.

Natural antiyeast compounds from the plant kingdom could represent a viable alternative to traditional antifungal drugs or chemical preservatives; advantages derived by their application in biotechnological sectors include the reduced possibility of microbial resistance occurrence and increased appeal in a green consumerism perspective.
1. Introduction

Plants as sessile organisms are constantly exposed to a wide array of pathogenic fungi, viruses, bacteria and parasitic nematodes. Plants use preformed (structural and biochemical) and inducible defense responses to fight biotic stresses (Selitrennikoff, 2001). Preformed or constitutive defenses include cutin, waxes, rigid cell walls and the production of antimicrobial molecules like phytoanticipins. In the event of a pathogen crossing this first defense barrier, the plant inducible defense response is activated (Verma et al., 2016). The inducible plant defense response includes hypersensitive response followed by generation of reactive oxygen species (ROS), cell wall cross-linking, synthesis of antimicrobial molecules like phytoalexins, and eventually the production of pathogenesis-related (PR) proteins (Sels et al., 2008). Typically, the interaction between secondary messengers and the genome of an infected cell leads to changes in the up and down-regulation, often resulting in an accumulation of products toxic for the pathogen (Dzhavakhiya et al., 2007).

Is it clear that plants can produce a wide range of genetically divergent molecules active against phytopathogens; whatever the origin or the structure, these molecules will be generally labelled as defense-related proteins and peptides throughout this review work.

The classification of plant defense-related proteins and peptides can be complex. Typically, the term "peptides" is used to indicate proteins of less than 100 amino acid residues and with a molecular weight not greater than 10 kDa. Plant defense-related peptides with antimicrobial activity can be categorised as antimicrobial peptides (AMPs); AMPs exist in almost all lifeforms and AMPs from plant origin can be also called Botanic AMPs (BAMPs). Most BAMPs are positively charged at physiological pH with molecular weights ranging from 2–10 kDa; they have diverse functions, structures, and expression patterns, as well as specific targets (Ali et al., 2018). They typically contain 4–12 Cys residues forming disulfide bonds, which can make them exceptionally stable to chemical, thermal and enzymatic degradation by stabilizing their tertiary and quaternary structures (Annunziato and Costantino, 2020). BAMPs classification, although problematic, is usually based on their sequence similarity, presence of Cys motifs, and tertiary structures. Several families of BAMPs can be classified as PR proteins families. In the past, all kinds of stress-infection induced proteins were collectively designated as PR proteins. A more precise definition was introduced by van Loon (van Loon et al., 2006) to identify originally intended PR proteins as “inducible defense related proteins”. PR proteins
are widely distributed in healthy plants in trace amounts, but their production increase significantly following infection (De Lucca et al., 2005). They were first discovered in the leaves of the virus-infected tobacco plant and classified into five families, PR-1 to PR-5; different proteins designated with the same number are serologically related, have a close molecular weight, and possess partial similarity in amino acid sequence (Stintzi et al., 1993). Since they were discovered in the early 1970s, the classification of PR-protein was updated from time to time; various PR-proteins were found in many other plant species, and novel groups of proteins were identified as PR-proteins. Currently, it comprises 17 families of PR-proteins, PR-1–PR-17 (Moosa et al., 2018). For the purpose of clarity, this review will discuss families of plant defense-related peptides (BAMPs) with known antiyeast properties and the discussion is extended to several families of defense-related antiyeast small proteins (<30 kDa) which generally share BAMPs features e.g., Cys-rich structure and cationic net charge. However, the discussion will include also defense-related antiyeast small proteins with more divergent characteristics.

The majority of the proteins discussed possess amphiphilic properties and provoke lysis of yeast cells as a consequence of protein-yeast membrane interaction. In general, there are three methods or models used to describe the interaction mechanism between the antimicrobial peptide and the fungal (or bacterial) membrane: carpet model, barrel stave model, and toroidal pore model (Figure 1). Briefly, “Carpet model” peptides are electrostatically attracted to the negatively charged phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. Their accumulation on the membrane surface generates tension between the two leaflets of the bilayer, which eventually leads to disintegration/rupture of the membrane. In contrast, “Barrel-stave model” and “Toroidal-pore model” peptides are characterised by pore forming activity. In the first case, helices orient themselves in the membrane in such a manner that the hydrophobic portion of the peptide aligns with the hydrophobic lipid chains in the core of the bilayer, whereas the hydrophilic portion forms the interior region of the pore. This topology can be compared with a barrel composed of helical peptides as the staves. In the “Toroidal-pore model”, antimicrobial peptide helices insert themselves perpendicularly into the membrane to relieve the curvature strain caused by peptide binding. In this process, they induce the monolayers to continuously bend causing the water core to be lined by both the inserted peptides and lipid head groups (Oliveira et al., 2013; Matsuzaki, 2019).
Figure 1. Three models used to describe the interaction mechanism between the antimicrobial peptide and the fungal (or bacterial) membrane. Reproduced from Shwaiki et al., (2021).

To summarise, plant defense-related peptides and small proteins are here presented on a family basis exploring their diversity in structure, antiyeast mechanism of action, cytotoxic potential and sensitivity to salt concentrations, pH variation and heat treatment. Proteins and peptides of interest are listed in Table 1 and their mechanism of action is summarised in Figure 7 (at the end of the discussion).
Chapter 1

2. Defensins / (PR-12) / BAMPs

Plant defensins are ubiquitous throughout the plant kingdom, and they constitute the PR-12 family. Most plant defensins isolated to date are seed-derived; although, they have also been identified in other tissues, including leaves, pods, tubers, fruit, roots, bark and floral organs (Sher Khan et al., 2019). They are either constitutively expressed in storage and reproductive organs or produced upon pathogenic attack or injury as part of a systemic defense response (Biotica et al., 2012).

Their activity is primarily directed against fungi; in some cases, they also possess antibacterial, proteinase and insect amylase inhibitor activity. Defensins have been reported to be active against phytopathogenic fungi (e.g. Fusarium culmorum and Botrytis cinerea), human pathogenic fungi (e.g. Candida albicans) and S. cerevisiae (Thomma et al., 2002). However, they are generally non-toxic to human and plant cells (Pinheiro Da Silva and MacHado, 2012). In the early 1990s, the first family members of plant defensins were isolated from wheat and barley grains and called γ-thionins because their size and Cys content were found to be similar to the Thionins. Terras (1992) and his colleagues isolated two new antifungal peptides, Rs-AFP1 and Rs-AFP2 and noticed that the plant peptides’ structural and functional properties were very similar to insect and mammalian defensins, and therefore termed the family of peptides “plant defensins” (Stotz et al., 2009).

Interestingly, most of the reported defensins do not exhibit a broad range of anti-yeast activity, limited, in most cases, to inhibition of S. cerevisiae or C. albicans or both. Still, it must be noted that antimicrobial assays were only conducted in these yeasts, so it has not been verified that the anti-yeast activity is limited to these species. Thus, complete screening and characterisation of plant defensins with anti-yeast activity are still non-existent.

2.1. Structure of Defensins

Plant defensins are small (~5 kDa, 45 to 54 amino acids), highly basic and Cys-rich peptides. The structures of these peptides vary greatly but are all built around a conserved and Cys-stabilised core scaffold, which comprises a triple-stranded β-sheet with an α-helix in parallel (CSαβ) in which two Cys residues separated by one turn of the α-helix are connected to two Cys, which are located a single amino acid apart in the third β-strand (Figure 2). In general, the structure has eight Cys that form four structure-stabilising disulfide bridges. These
disulfide bonds render the molecule stable to proteases and extreme pH and temperature, and the spacing and connectivity of the eight highly conserved Cys define the plant defensin family (Mohd and Gupta, 2011). Plant defensins can further be classified into two distinct classes based on their precursor structures. In the first and largest group (Class I), defensins are synthesised with a signal sequence and a mature defensin domain. The signal sequence targets the protein to the endoplasmic reticulum, where it is folded and subsequently directed to the secretory pathway; their destiny is to accumulate in the cell walls or the extracellular space (Ganz, 2003).

In contrast, in the less common second class (Class II), defensins are produced from larger precursors with an endoplasmic reticulum (ER) signal sequence, together with a mature defensin domain and a C terminal pro-peptide (CTPP) of 27–33 amino acids which carries vacuolar targeting information. The CTPP also protects plant cells from the cytotoxic activity of this class of defensins during transport to the vacuole. In the vacuole, they are proteolytically processed to release the mature defensin and are stored (Vriens et al., 2014). Mature defensins of both classes share a highly similar fold centred on the family defining Cys-rich core.

Figure 2. Alignment of defensins’ amino acid sequences and schematic representation of the conserved defensins’ secondary structure. Reproduced from Vriens, Cammue and Thevissen (2014).

2.2. Defensins antiyeast mode of action

Most plant defensins have a powerful fungicide action; it has been demonstrated that defensins can specifically interact with the fungal host membrane compounds such as sphingolipids and
phospholipids. For example, the radish antifungal defensin RsAFP2 interacts directly with fungal glycosphingolipid glucosylceramide (GlcCer) inducing membrane permeabilisation in *C. albicans* and *P. pastoris* (Thevissen et al., 2004). While the defensin DmAMP1 from *Dahlia merckii* interacts with *S. cerevisiae* sphingolipids of the mannosyldiinositolphosphorylceramide (M(IP)2C) class (Aerts et al., 2006). Following interaction with their target, plant defensins are either internalised by the fungal cell and interact with intracellular targets or remain at the cell surface and induce cell death through induction of a signalling cascade (Thevissen et al., 1996). Studies on the mechanism of antiyeast action of Rs-AFP2 show that after the binding to the GlcCer in the cell wall, the peptide does not enter the cell and induce cell wall stress activating the Cell Wall Integrity (CWI) - MAP (mitogen-activated protein) kinase cascade (Figure 3). Significant symptoms of the CWI pathway activation are ROS production and hence oxidative stress, induction of ion fluxes, accumulation of ceramides (Phyto-C24) and activation of caspases, all of which contribute to fungal cell death (Parisi et al., 2019a). Similar to Rs-AFP2, DmAMP1 activate the CWI pathway after binding to sphingolipids in the yeast cell wall, insertion into the membrane results in potassium efflux and calcium uptake, and fungal growth arrest occurs (Vriens et al., 2014). Not all the defensins induce the activation of the CWI pathway. Studies on the filamentous fungi *Fusarium oxysporum* and the yeasts *S. cerevisiae* and *C. albicans* suggest a different mechanism of action for the Class II solanaceous defensin NaD1 (Van Der Weerden et al., 2008). Unlike RsAFP2 and DmAMP1, after the binding to the cell wall, NaD1 moves to the cytoplasm by endocytosis (Hayes et al., 2018), where it interacts with the phospholipid PI(4,5)P2 of the inner leaflet of the plasma membrane and triggers the production of ROS and nitric oxide (NO) (Figure 3). The high osmolarity glycerol (HOG) pathway is also activated in response to sub-lethal concentrations of NaD1. HOG is a MAPK pathway that regulates stress response in yeast, leading to permeabilisation of the plasma membrane, granulation of the cytoplasm and cell death (Hayes et al., 2014; Parisi et al., 2019b).
Defensins represent one of the largest families of defense-related plant proteins; in the completely sequenced plant genomes, dozens of novel defensin-like sequences were discovered (Odintsova et al., 2020). However, the functions of the vast majority of them are still poorly understood; and only a few defensins have been tested for antiyeast activity. The literature available on antiyeast activity of defensins shows that their toxic effects are often achieved by two main mechanisms of action: one where the peptide passes through the cell wall and the plasma membrane and is internalised in the cytoplasm, and one where the peptide
mediates the toxic effects without entering the cell. All ultimately lead to disruption of the plasma membrane, production of ROS and induction of programmed cell death pathways. Due to multiple mechanisms and interactions with essential fungal membrane structures, the frequency of resistance occurrence is low. However, mutants of *S. cerevisiae* show resistance towards the defensin HsAFP1 (from *Heuchera sanguinea*). The HsAFP1 mechanism of action requires a properly working yeast respiratory chain (Thevissen *et al.*, 2007; Aerts *et al.*, 2011), and *S. cerevisiae* can generate respiration deficient mutants. Overall, the plant defensins family possess potent antimicrobial activity and a non-toxic nature towards animal cells; moreover, they have been reported to be stable in extreme conditions, including high temperature, low pH, oxidative and proteolytic environments. However, some defensins are sensitive to the effect of cations, limiting their potential for biotechnological applications. For example, the inhibitory effect of the alfalfa defensin Ms-Def1 is reduced in the presence of calcium ions (Kim *et al.*, 2009).

### 3. Thionins / PR-13 / BAMPs

The thionin proteins family is also classified as PR-13. Thionins are found in a wide range of plants species; all thionins are present in almost every crucial plant tissue from endosperm to leaves (Stec, 2006). The prototypic thionin with antimicrobial activity, α-purothionin, was isolated in the endosperm of wheat. Following the discovery of α-purothionin, subsequent thionins isolated from other plants are labelled with descending letters of the Greek alphabet in the order of their discover (Zhao, 2011). The biological activities recognised for thionins are plant protection, seed maturation, dormancy, germination, packaging of storage proteins, and acting as secondary messengers during signal transduction (Tam *et al.*, 2015). Thionins are expressed as precursor proteins processed to produce the final peptide. The precursor peptide consists of an N-terminal signal sequence, the mature peptide sequence and an acidic C-terminal coiled region; the expression of thionins is inducible by external stimuli (Bohlmann, 1994). They have been active against bacteria and many pathogenic fungi, including yeast such as several *Candida* species and *S. cerevisiae* (Taveira *et al.*, 2014, 2016). Moreover, some thionins display toxic activity against mammalian cells, *e.g.*, the pyrularia thionin from mistletoe (*Pyrularia pubera*) showed anticancer activity against cervical cancer cells (HeLa) and mouse melanoma cells (B16) (Hayes and Bleakley, 2018).
3.1. Structure of Thionins

Thionins are small proteins (~5 kDa) containing 45–47 amino acids rich in arginine (Arg), Lys and Cys residues, mainly positively charged at neutral pH and distinguished into two distinct groups: α-/β-thionins and γ-thionins. In this review, γ-thionins are referred to as plant defensins. There are five classes of α/β-thionins (I–V) classified depending on their different properties like length, overall net charge and number of disulfide bonds. Type I α/β-thionins have 45 amino acids, 8 Cys forming four disulfide bonds plus ten basic residues. Type II thionins are 46–47 amino acids in length; they form four disulfide bonds and are slightly less basic than type I with seven basic residues. Type III (viscotoxins and phoratoxins) thionins feature three disulfide bonds and are 45–46 amino acids long. They are as basic as type II thionins. Thionins of type IV (crambins) are typically 46 amino acids long, and they have no charge at neutral pH and three disulfide bonds. Type V thionins are truncated forms of type I thionins. However, all types appear to have similar three-dimensional structures and be highly homologues at the aminoacidic level (Figure 4). The mature peptide conserves a structural architecture consisting of a gamma (Г) fold of two antiparallel α-helices, an antiparallel double-stranded β-sheet and six or eight Cys forming three or four disulphide bridges. The presence of disulphide bridges and the residue Arg10 ensure the overall stability of the protein’s structure; Arg10 is an abundant source of hydrogen bonds between the secondary structures (Rao, Hassan and Hemple, 1994; De Lucca et al., 2005; Sels et al., 2008; Zhao, 2011; Nawrot et al., 2014; Yan et al., 2015)
3.2. Thionins antiyeast mode of action

It is generally accepted that thionins, unlike defensins, inhibit fungal growth due to direct protein-membrane interactions. It is thought that the positively charged thionin interacts with the negatively charged head groups of phospholipids in the fungal membrane. This first electrostatic binding event is followed by either pore formation or a specific interaction with a particular domain in the membrane and culminates in increased permeability, imbalance of ion homeostasis and final cell death (Nawrot et al., 2014).

One of the most studied thionins for their toxic activity against yeasts is CaThi, a plant-derived peptide isolated from Capsicum annuum fruits. It has been proposed (Taveira et al., 2018) that CaThi may trigger apoptosis in yeast cells, involving a pH signalling mechanism. In S. cerevisiae CaThi interact with the plasma membrane and causes membrane permeabilisation, changes in hydrogen ion gradient and increased ROS production. Morphological alterations and structural membrane damage are evident also on K. marxiannus and C. albicans treated with this peptide (Ribeiro et al., 2012). The cell surface pH increases significantly when the Candida tropicalis cells are exposed to CaThi caused by inhibition of extracellular hydrogen ion efflux. Moreover, CaThi induces other typical apoptotic markers in C. tropicalis cells, such
as phosphatidylserine externalisation in the outer leaflet of the cell membrane and activation of caspases (Taveira et al., 2018). More studies are needed to understand if other thionins can promote an imbalance in pH homeostasis during yeast cell death playing a modulatory role in the hydrogen ion transport systems.

Thionins’ biotechnological potential is limited as the first purely electrostatic interaction of these peptides with fungal membranes can be inhibited by divalent cations such as calcium and phosphates (Stec, 2006).

4. Lipid transfer proteins (LTPs or ns-LTPs) / PR-14 / BAMPs

Lipid transfer proteins (LTPs) are a group of cationic small plant proteins belonging to the class PR-14 (Moosa et al., 2018). As their name suggests, they have been firstly discovered for their ability to transfer non-specifically phospholipids between membranes of cytoplasmic organelles; for this reason, LPTs are also known as ns-LPTs (Kader, 1996). The biological role of these proteins in plants is still a matter of discussion; excluding plant protection against biotic and abiotic stresses, they are believed to be involved in angiosperms fertilisation, adhesion of pollen, somatic embryogenesis, lipid metabolism, formation of the cuticle and cell death (Maximiano and Franco, 2021). LTPs occur in all structures of plants, but their most common localisation is in the cuticle-coated epidermal cells. These molecules are commonly localised in the extracellular cell wall but are also found in the vacuole and vesicles. Large gene families encode for LPT proteins, and it has been proposed that the genes of multiple LTP isoforms performing different functions have been generated by mutations occurring during the duplications of the ancestral gene. Diversification of isoform functions is a powerful tool of the plant defense system (Amador et al., 2021). LTP genes occur in all land plants; they have been isolated from various species such as seeds and leaves of ginseng (Cai et al., 2016), pea (Bogdanov et al., 2016), mung beans (Liu and Lin, 2003) and barley (Lindorff-Larsen et al., 2001). LPTs have been reported to have in vitro activity against a large spectrum of microorganisms (including fungi and bacteria) as well as antiproliferative activity against tumour cells in vitro (Amador et al., 2021). Their antimicrobial activity is described to be more potent against fungi; however, only a few have been tested for antiyeast activity. They are also important allergens in fruits or nuts (Egger et al., 2010).
4.1. Structure of LTPs

LTPs are traditionally subdivided into two families according to their observed molecular masses: LTP1 (~9–10 kDa) and LTP2 (~7 kDa). They conserve several residues such as valine (Val), Gly and serine (Ser), but almost lack tryptophan (Trp) residues (Yeats and Rose, 2008). Basic LTP peptides contain 90 to 94 amino acids organised in a three-dimensional fold that includes four to five α-helixes and a central internal cavity for loading of hydrophobic molecules or lipids. The eight conserved Cys residues linked by four disulfide bonds makes the overall peptide structure stable and resistant to high temperature. The disulfide pairing patterns among LTP1 and LTP2 are different. LTP1 presents 4 helices and a tunnel-like shaped hydrophobic cavity, while LTP2 exhibits 3 parallel α- helices which form a triangular hollow box topology cavity (Carvalho and Gomes, 2007) (Figure 5).

Figure 5. Spatial structure and hydrophobic cavity of LTPs. (A) LTP1 (PDB ID: 3GSH) presents 4 parallel α- helices (H1–H4). (B) LTP1 structures form a hydrophobic cavity with large and small entrances. (C) LTP2 (PDB ID: 1L6H) presents 3 parallel α- helices (H1–H3). (D) LTP2 forms a hydrophobic cavity with a triangular structure. Reproduced from Maximiano and Franco (2021).
4.2. LTPs antiyeast mode of action

To date, the toxic mechanism of LTP has not been elucidated yet; however, the capacity of LTPs to interact with several lipids seems to be involved in activity against phytopathogens. LTPs are also capable of interacting with fungal cell membrane proteins, and studies have suggested that these molecules can modulate the influx of ions across the fungal membrane, causing permeabilisation. It has been recently proposed that some LTP proteins could insert themselves into the fungal cell membrane, and the central hydrophobic cavity forms a pore, allowing a process of bleaching out the lipids from the membrane (Madni et al., 2019).

Studies on antiyeast activity of LTPs have been done on the peptide Cc-LTP1 (Carvalho and Gomes, 2007) from the coffee plant (Coffea canephora) and Ca-LTP1 (Diz et al., 2006) isolated from chilli pepper (Capsicum annuum); they promoted membrane permeabilisation, induced the production of ROS and caused several morphological changes (e.g., formation of pseudohyphae) in yeasts such as S. cerevisiae, C. albicans and C. tropicalis. In particular, Cc-LTP1 can interfere in a dose-dependent manner with glucose-stimulated, H^+/ATPase pumps dependent acidification of medium and permeabilises yeast plasma membranes (Zottich et al., 2011). The antifungal activity of LTPs from Brassicaceae species and mung beans has been reported to be thermally, pH-, and proteolytic treatment-stable (Lin et al., 2007).

5. Hevein-type AMPs (HLPs or CB-HLPs) / PR4 / BAMPs

Hevein-type antimicrobial peptides (HLPs) are a small group of plant defense-related peptides classified as PR-4 (Van Damme et al., 1999). Hevein, the prototypic member of this group, is a 43 amino acid antimicrobial Cys-rich peptide isolated from the latex of the rubber tree (Hevea brasiliensis) (Archer, 1960; Van Parijs et al., 1991). The hevein protein sequence contains a chitin-binding (CB) domain. Heveinlike AMPs have been found in mono and dicotyledonous plants from different families, and they all possess the ability to bind chitin (Wong et al., 2016). Chitin is a major cell wall component of pathogens (fungi, insects, and nematodes), absent in plants. HLPs are ribosomally synthesised and post-translationally modified; their precursors are known to undergo an extensive post-translational process to release the mature peptides (Loo et al., 2021). Almost all HLPs are reported to be toxic, at their micromolar concentrations, against a wide range of phytopathogenic fungi, few of them are active against
bacteria, and so far, a limited number of HLPs has been identified with antiyeast activity (Koo et al., 2004).

5.1. Structure of HLPs

HLPs are usually short basic peptides (29–45 amino acids), and they share several conserved Cys, Gly, and aromatic amino acid residues. Typically they have 8 Cys residues; however, there are forms with 6 or 10 Cys (Tam et al., 2015). Hevein contains a characteristic structural motif including an α helix–β1–β2–α helix–β3, a central β sheet with antiparallel β chains being surrounded by two small helices stabilised by disulfide bridges (Wong et al., 2016). Six of these residues are generally founded in a conservative position (CXnCXnCCXnCXnC), and the disulfide bond formation occurs between the following Cys residues: C1–C4, C2–C5, and C3–C6. Their main distinctive feature is the conserved CB domain (UniProt database accession no. PS00026) characterised by an SXϕGϕ sequence in intercysteine loop 3, followed by GXXXXΦ in loop 4, where X represents any amino acid, and Φ represents aromatic acid residues (Slavokhotova et al., 2017).

5.2. HLPs antiyeast mode of action

HLPs mode of action is still partially unclear. Traditionally, it is considered that the primary mechanism of toxicity is correlated with the peptide ability to interact with the chitin in the growing hyphae of fungi, thus disrupting the normal cell wall structure (Slavokhotova et al., 2017). The presence of aromatic residues plays a major role in the interaction between the hydrophobic groups of chitin and the π-electron systems (Beintema, 1994). It has been suggested that HLPs and chitinase proteins could work together since they are structurally similar and have a common target of action. In response to the fungal infection of Fusarium verticilloide, the wheat plant expresses a chitinase that binds and degrades hyphal chitin. In response, the fungus produces an enzyme called fungalysin that cleaves the chitinase, suppressing its function. Then the plant begins to produce hevein-like peptides (WAMP) that bind to fungalysin and inhibit its activity (Slavokhotova et al., 2014). It is unclear whether all the hevein-like peptides share this synergetic mechanism, or it occurs only for some of them. Moreover, more studies are needed to prove if yeasts are subjected to a similar toxic process as filamentous fungi.
The short peptide (40 acidic amino residues) Pn-AMP1 represents an exception since its mechanism of action against yeasts *S. cerevisiae* and *C. albicans* has been observed and partially understood. Pn-AMP1 contains 8 Cys residues; it was isolated from the seeds of morning glory (*Pharbitis nil*) and possessed a remarkable antifungal activity against fungi either containing or not containing chitin in their cell walls (Koo *et al.*, 1998). It was shown that this peptide penetrates very rapidly into yeasts false-hyphae, causing actin depolarisation and the burst of hyphal tips, followed by membrane disruption and the damaging of cytoplasm integrity. Interestingly, although Pn-AMP1 possesses CB activity, yeast growth arrests is mediated through cell wall mannoproteins rather than chitin (Koo *et al.*, 2004). It can be assumed that hevein-like peptides might be active against more than one host target.

Moreover, peptides known as HLPs vaccatides, from *Vaccaria hispanica*, maintained their antifungal activity after heat-treatment and in acidic (pH 2.0) conditions (Wong *et al.*, 2017). Nevertheless, the antifungal activity of EAFP1 and EAFP2, from the bark of *Eucommia ulmoides*, is antagonised by calcium ions (Huang *et al.*, 2002).

### 6. Thaumatin-like Proteins (TLPs) / PR-5

Thaumatin-like (TL) proteins are classified in the PR-5 family; they are named after an intensely sweet peptide called Thaumatin isolated from the West African bush *Thaumatococcus daniellii* (Robertson, 1986). Thaumatin-like proteins (TLPs) are a highly complex protein family associated with host defense and developmental processes. TLPs are widely distributed in plants, including green algae, bryophytes, gymnosperms, and angiosperms, and homologous of these plant proteins have been observed in major kingdoms of living organisms such as animals, plants and fungi (Shatters *et al.*, 2006). In plants, TLPs have a variety of properties associated with their structural diversity. They are primarily associated with responses to biotic and abiotic stresses (de Jesús-Pires *et al.*, 2019); an increase in their expression was observed when the plant suffers from various stresses such as pathogen attack, wounding, salinity, drought, and freezing. In addition, TLPs seems to play a role in plant physiological processes, including floral formation, fruit ripening, seed germination, and senescence (Sakamoto *et al.*, 2006).
Several TLPs isolated from various plant species, including tobacco, maize, barley, chestnut and pumpkin, have shown \textit{in vitro} activity against phytopathogenic fungi and yeasts (Cheong \textit{et al.}, 1997; Yun \textit{et al.}, 1998; Higuchi \textit{et al.}, 2016).

6.1. Structure of TLPs

Generally, TLPs have molecular masses ranging from 21 to 26 kD and possess 16 Cys conserved residues that form eight S-S bonds. The majority of TLPs share a similar amino acid sequence (De Lucca \textit{et al.}, 2005), and they can be categorised into three subclasses according to their isoelectric point (Koiwa \textit{et al.}, 1994). Their 3D model consists of three characteristic domains held in a compact antiparallel β-sheet fold by disulfide-bridges donating structural stability to different pH environments, proteases, and heat denaturation (Faillace \textit{et al.}, 2019). Another characteristic of TLPs structure is a cleft between domains I and II; this gap space can be acidic, neutral, or basic, depending on the nature of the ligands. The acidic cleft is known to confer antifungal activity due to the conserved signature motif composed of Arg, Glu (glutamic acid), and three aspartic acid residues (Tachi \textit{et al.}, 2009). Due to the high similarity among an amino acid sequence of known TLPs, it can be assumed that all the proteins belonging to this family would share common structural features and differences in 3D structures might lead to diverse individual functions (de Jesús-Pires \textit{et al.}, 2019).

6.2. TLPs antiveast mode of action

Many PR-5 thaumatin-like proteins have been evaluated for their antifungal activity, considering their proposed primary biological function is protection against phytopathogens. However, only a few have been recorded for antiveast activity. The main TLPs toxicity mechanism is fungal membrane permeabilisation followed by intracellular contents leakage and cell apoptosis. Fungal susceptibility to TLPs is regulated by protein composition on the cell wall; thus, these proteins display varying activity depending on the differences in host wall composition. Osmotin is one of the most studied TLPs for its toxicity mechanism against yeasts. Osmotin is a basic protein of 24 kDa expressed in the tobacco plant (\textit{Nicotiana tabacum} L.) and inhibits the growth of \textit{S. cerevisiae} (Yun \textit{et al.}, 1998). The phosphate group in cell-wall phosphomannoproteins was described as the initial target of osmotin’s binding to yeast (Ibeas \textit{et al.}, 2001). The osmotin-phosphomannans binding is pH-dependent, possibly
determined by the acidic cleft region on TLP and the calcium ions seems to play a role in mediating the toxin's concentration on the yeast cell (Salzman et al., 2004). Subsequently, this linkage eases the osmotin diffusion in the plasma membrane and potentially in the cytoplasm. Osmotin can activate the MAPK pathway for signal transduction (Yun et al., 1997) and initiates proapoptotic signalling via suppression of the RAS2/cAMP stress response pathway, promoting the intracellular accumulation of ROS and eventually leading to death by apoptosis (Narasimhan et al., 2001).

7. Seed storage albumin proteins (2S albumins)

2S albumins proteins are a group of essential seed storage proteins (SSPs); SSPs are essential to seeds at early and late developmental stages by providing amino acids and other nutrients during germination and seed defense (Shewry et al., 1995). SSPs were first classified based on their solubility, and albumin was merely the name given to SSPs soluble in pure water (Osborne, 1924). Nowadays, 2S albumins represent a multifunctional class of seeds storage proteins classified as part of the Prolamin superfamily, which includes: ns-LTPs, bifunctional α-amylase, inhibitors/serine proteases and cereal prolamines (Byczyńska and Barciszewski, 1999). 2S albumins are widely present in seeds of mono- and dicotyledons. Generally, the 2S albumin protein’s group is encoded by a multigenic family, resulting in high polymorphism. During biosynthesis, they are first synthesised as a polypeptide of 18–21 kDa, and they undergo post-translational modifications, with proteolysis being the most common (Souza, 2020). In ungerminated seeds, the location of 2S albumins has been reported in protein bodies, composing 40% of total seed protein. 2S albumins start to be degraded upon seed germination, providing amino acids for seed development. Several 2S albumins also displayed in vitro antifungal properties (Agizzio et al., 2003; Agizzio et al., 2006), and the 2S albumin from pumpkin seeds also had anti-cancer properties (Tomar et al., 2014). They are also important food allergens in Brassicaceae plants (Moreno and Clemente, 2008).

7.1. Structure of 2S albumins

Mature 2S albumins are positively charged proteins with low molecular mass; typically, they are composed of 2 polypeptide subunits: a small chain of 3/4 kDa and a large chain of 9/10
kDa (Barciszewski et al., 2000). The amino acid composition of 2S albumins provides evidence of their importance in seedlings germination. Because they are rich in Arg and glutamine, 2S albumins are an excellent nitrogen source. Also, the high content of Cys and methionine (Met) provides suitable levels of sulfur (Terras et al., 1993). The structure of 2S albumin is rich in α-helices and characterised by a well-conserved skeleton of 8 Cys residues forming intrachain and interchain SS bridges (Ullah et al., 2015). This type of fold confers stability towards high temperature, pH variations and protease action (Shewry and Pandya, 1999).

7.2. 2S albumins antiyeast mode of action

Several studies reported the activity of 2S albumins against filamentous fungi; however, only a few members have been tested for their ability to impact yeast growth. The α-helical structure and the overall positive net charge of 2S albumins are thought to be the determinant for permeabilizing the fungal membrane (Terras et al., 1992); specifically, the electrostatic interaction between proteins and anionic phospholipids of the microbial membrane mediate the direct membrane disruption, leading to cytoplasmic leakage and cell death (Neumann et al., 1996).

Ca-Alb isolated from chilli pepper (Capsicum annuum) and Pf-Alb from passion fruit (Passiflora edulis) showed activity against several yeasts, this 2S albumin permeabilises the fungal membrane, leading to an impairment of the H+/ATPase pump and the inhibition of the glucose-stimulated acidification of the media. In addition, the growth of yeasts Kluyveromyces marxianus and C. parapsilosis is inhibited via NO induction in the presence of Ca-Alb. In general, yeasts cells treated with this type of protein tend to show apoptotic-like behaviour, including shrinking cytosol and cell wall deformation (Agizzio et al., 2006; Pelegrini et al., 2006; Ribeiro et al., 2012; Meneguetti et al., 2017)

The 2S albumins showed sensitivity to high salt concentrations, and generally, a large concentration is necessary to induce a significant microbial inhibition (Thery et al., 2020).

8. Snakins / BAMPs

Snakins are Cysteine Rich Peptides (CRPs); the first defined Snakin peptide, Snakin-1 (StSN1), was purified in potato (Solanum tuberosum) by Segura et al. (1999), who reported that it had
some sequence motifs in common with snake venoms and named it Snakin. Due to their sequence similarity to GASA (Gibberellic Acid Stimulated in Arabidopsis) proteins, the snakins were subsequently classified as members of the snakin/GASA family. The genes encode these peptides with a signal sequence of approximately 28 amino acid residues, a variable region, and a mature peptide sequence of approximately 60 residues. Snakin proteins have been reported to be involved in diverse biological processes, including cell division, cell elongation, cell growth, transition to flowering, signalling pathways and defense. Snakins may be expressed in different plant parts, like stems, leaves, flowers, seeds, and roots, both constitutive or induced by biotic or abiotic stresses. In vitro activity was observed against various fungi, bacteria, and nematodes, acting as a destabiliser of the plasma membrane (Daneshmand et al., 2013; Su et al., 2020; Li et al., 2021).

8.1. Structure of Snakins

Snakin family peptides have 12 Cys residues at constant positions in the GASA conserved domain at the C-terminal region. They also have a putative signal peptide at the N-terminus and a variable region in the middle of their sequences. The amino acid sequence of the GASA domain in Snakins consists of a peculiar Cys-motif “XnCX3CX2RCX8(9)CX3CX2CCX2CXCVPGX2GNX3CPCYX10(14)KCP” (where X is any residue except for Cys, R is Arg, V is Val, P is Pro (proline), G is Gly, Y is Try (tyrosine) and K is Lys (lysine)), in which the number and arrangement of Cys residues is highly conserved. The 12 conserved Cys residues maintain the 3D structure of the peptide through disulfide bonds, besides providing stability to the molecule when the plant is under stress (Su et al., 2020).

8.2. Snakins antiyeast mode of action

There is a lack of studies on the antiyeast activity of native Snakins, and in general, the antifungal mechanism of action has yet to be elucidated (Wink and Herbel, 2016). The recombinant form of Snakin-2 (Sn2 or StSN2) from tomato (Solanum lycopersicum) was able to agglomerate single S. cerevisiae cells prior to killing and form pores in the membrane of target cells (Herbel et al., 2015). The synthetic form of Snakin-1 (Sn1 or StSN1) from potato (Solanum tuberosum L.) showed activity against spoilage yeasts, and mechanisms of
membrane permeabilisation and leakage of cytoplasmatic components were noticed (Shwaiki, Arendt and Lynch, 2020). The Snakin-Z peptide derived from *Zizyphus jujuba* fruits showed anticandidal activity and no toxic effect towards human erythrocytes (Daneshmand *et al.*, 2013).

**9. Knottins – Cyclotides / BAMPs**

Knottins are a family of peptides featuring various biological actions such as toxic, inhibitory, antimicrobial, insecticidal, cytotoxic, anti-HIV, or hormone-like activities. As the name suggested, knottins proteins are characterised by a unique knotted topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and interconnecting peptide backbones (Heitz *et al.*, 2008). This Cys-knot (CCK) donates remarkable stability to the proteins, making the overall structure amphipathic (Grage *et al.*, 2017). The knottin scaffold is found in almost 30 different protein families, among which are the plant cyclotides. Cyclotide proteins exist in *Cucurbitaceae*, *Fabaceae*, *Rubiaceae*, *Solanaceae*, and *Violaceae* families, and they display activity against multiple groups of pathogens, including the yeast *C. albicans* (Strömstedt *et al.*, 2017; Grover *et al.*, 2021). In plants, the natural role of cyclotides is to provide a defense mechanism against pests, including insects and nematodes.

**9.1. Structure of cyclotides**

Cyclotides usually range in size from 25 to 40 amino acids and feature a unique head-to-tail macrocyclic structure containing the CCK motif. The six highly conserved Cys residues in the CCK (C1-C4, C2-C5, C3-C6) plus the amino acid sequence between two successive Cys residues form the six loops of the cyclic backbone (Figure 6). Based on structural similarities, cyclotides are divided into several subfamilies, *e.g.*, Mobius, the bracelets and a hybrid Mobius named kalata isolated from *Oldenlandia affinis* (Pelegrini, Quirino and Franco, 2007). Cyclotides can tolerate high sequence variation in the non-conserved Cys residues, and cyclotides of the Möbius and bracelet types contain well-defined hydrophilic and hydrophobic patches, leading to an amphiphilic property similar to that of classical antimicrobial peptides (Grover *et al.*, 2021). However, the variation of these hydrophobic patches differs among
individual cyclotides, resulting in different membrane-binding properties (Greenwood et al., 2007).

![Schematic representation of kalata B1, a prototypic cyclotide. Reproduced from Srivastava et al. (2021).](image)

9.2. Cyclotides antifungal mode of action

The cyclotides’ antifungal mechanism of action has not been elucidated yet; however, their activity seems to be connected to membrane disruption. Cyclotides are known to target and disrupt membranes containing phosphatidylethanolamine (PE). Nevertheless, it has been suggested that general electrostatic and hydrophobic parameters are more critical for broad-spectrum cyclotides; while, a phospholipid-specific mechanism of membrane permeabilisation, through interaction with PE lipids, is essential for cyclotides active primarily on Gram-negative bacteria (Slazak et al., 2018). More studies are necessary to understand the antifungal potential of this exciting family of plant peptides.

10. Other Cysteine-rich protein families

10.1. Domain of unknown function 26 [DUF26] proteins

The domain of unknown function 26 [DUF26] makes up a large gene family encoding for signalling proteins characterised by the plant-specific Cys-rich motif. The motif was initially found in the extracellular region of Cys-rich receptor-like kinases (CRKs) and Cys-rich receptor-like secreted proteins (CRRSPs) from Arabidopsis; subsequently, it has been found in receptor-like kinases (RLKs) plasmodesmata-localised proteins (PDLPs) (Vaattovaara et al., 2019).

Ginkobilobin2 (Gnk2) is a seed storage protein in gymnosperm that possesses antifungal activity and a plant-specific Cys-rich motif [DUF26] (Sawano et al., 2007). The Gnk2-homologous
sequence is also observed in an extracellular region of CRKs that function in response to biotic and abiotic stresses. The Gnk-2 structure comprises 108 amino acid residues, three disulfide bridges, two $\alpha$-helices and a five-stranded antiparallel $\beta$-sheet; in addition, in Gnk-2, the DUF26 region is found at the C-terminal side. Gnk-2 exhibits antiyeast activity by interacting exclusively with mannan polysaccharides in \textit{S. cerevisiae} cell wall; three residues (Asn-11, Arg-93, and Glu-104) on Gnk-2 bind the hydroxy group on mannose (a building block of mannan) by hydrogen bonds (Miyakawa \textit{et al.}, 2014). This mannose-binding interaction is directly related to the ability of Gnk2 to inhibit fungal growth.

10.2. Vicilin-like antimicrobial peptides / BAMPs

Vicilins are seed storage proteins of the 7S globulin class, present in seeds of legumes and other plants. These proteins are characterised as oligomers of 150–170 kDa formed by three similar subunits (Tai \textit{et al.}, 2001). It is generally accepted that vicilins serve as amino acid storage for seedling development; however, they can show antimicrobial properties, \textit{e.g.} a vicilin protein from cowpea seeds inhibited glucose-stimulated acidification of the medium by \textit{S. cerevisiae} by up to 60\% (Gomes \textit{et al.}, 1998). Vicilins bind to chitin-containing structures of yeast cells, and such interaction could inhibit hydrogen ion pumping, cell growth and spore formation. Marcus \textit{et al.} (1999) reported the discovery of a low molecular weight (5 kDa) peptide from nut kernels produced from a large precursor protein related to 7S globulins in other plants. The peptide was labelled MiAMP2c, and it inhibited various plant pathogenic fungi \textit{in vitro}. Wang \textit{et al.} (2001) described two small antifungal peptides (CW-2 and CW-3) isolated from cheeseweed seeds with high homology to vicilins from cotton seeds.

Plant peptides that share substantial sequence homology with vicilin proteins can be labelled as Vicilin-like AMPs. An 8 kDa vicilin-like AMP from melon seeds (Ribeiro \textit{et al.}, 2007) and two low-molecular mass proteins from \textit{Capsicum baccatum} seeds (Bard \textit{et al.}, 2014) showed activity against yeasts, including \textit{S. cerevisiae}, \textit{C. albicans}, \textit{Candida tropicalis} and \textit{K. marxianus}. At present, information on the antiyeast mechanism of action of vicilin-like peptides is minimal. However, it has been suggested that the fungal growth inhibition caused by this type of AMPs might be mediated by interaction with components of the cell surface (possibly chitin) followed by either inhibition of fungal plasma membrane H$^+$/ATPase pumps
or an increase in the hydrogen ion membrane permeability (Ribeiro et al., 2007; Bard et al., 2014).

10.3. Plant Serine Protease Inhibitors (SPIs)

The protease inhibitors (PIs) are widely distributed in living organisms such as bacteria, fungi, plants, and humans; they are essential for components for cellular homeostasis and survival. In plants, PIs participate in many biological processes related to metabolism and cell physiology; they have been related to the mobilisation of storage proteins, regulation of endogenous enzymatic activities, modulation of apoptosis and programmed cell death and stabilisation of defense proteins or compounds against animals, insects and microorganisms (Clemente et al., 2019). Generally, PIs can be classified into four main families based on the specific reactive site present in the sequences: Cys protease inhibitors, metalloid protease inhibitors, aspartic protease inhibitors, and serine protease inhibitors (SPIs). Plant SPIs exist in many members of the plant kingdom; however, most of the studies regard SPIs from Fabaceae, Solanaceae and Gramineae families (Kim et al., 2009). SPIs represent between 1% and 10% of the total proteins in storage organs (e.g., seeds and tubers). They are categorised into different groups according to their function of structural and biochemical properties, including Bowman-Birk serine protease inhibitors, cereal trypsin/α-amylase inhibitors, mustard trypsin inhibitors, potato type I protease inhibitors, potato type II protease inhibitors, serpins, Kunitz-type inhibitors, and squash serine inhibitors (Pesoti et al., 2015).

Among these SPIs, the Kunitz-type inhibitor (KTIs, also known as soybean trypsin inhibitors) are the most described group regarding antiyeast properties. The typical KTIs structure features a single peptide folded in a β-trefoil manner containing four Cys residues forming two disulfide bridges and a molecular mass of about 10–25 kDa (Bendre et al., 2018). However, a growing number of Kunitz inhibitors that have been characterised do not present a unique pattern of disulfide bridge numbers and polypeptide chains. They have plasticity in their structure, which allows them to interact with peptidases with different specificities simultaneously; nevertheless, KTIs usually show the highest affinity to trypsin. KTIs are commonly believed to be stable in a broad range of pH and at temperatures of 100°C (Losso, 2008). EtTI (de Oliveira et al., 2018) and IETI (Dib et al., 2019) are 20 kDa KTIs isolated from the seeds of Enterolobium timbouva and Inga edulis (plant trees belonging to the Fabaceae family). They
showed in vitro activity against Candida spp.; their mechanism of action triggered disturbances on the integrity of yeast plasma membrane and morphological alterations, presumably mediated via apoptosis.

11. Other antifeast plant-defense related protein families

11.1. Glycine-rich peptides (GRPs) / BAMPS

Glycine-rich peptides (GRPs) are plant storage proteins characterised by a high content of repetitive sequences containing Gly residues. The expression of these proteins is modulated by both abiotic and biotic factors. GRPs display a different pattern of expression and different subcellular localisation, suggesting involvement in diverse physiological processes (Mousavi and Hotta, 2005). Defense against pathogens has been reported among the various biological functions attributed to GRPs, specifically towards filamentous fungi, gram-negative bacteria and viruses (Mangeon et al., 2010). GRPs comprise an abundant content of Gly (more than 60%) and a specific hydrophobic character. The structure of this peptide consists of α-helices at C and N-termini, with the presence of Arg residues at their extremity. Typically, GRPs contains no Cys residues, and thus the peptide is unable to form disulphide bonds (Sachetto-Martins et al., 2000). In most GRPs, a signal peptide has been identified and other specific structures, such as cold shock response domains or RNA-binding regions (Zottich et al., 2013).

There is a lack of knowledge on GRPs antifungal and antiyeast mechanisms of action. The peptide Cc-GRP isolated from Coffea canephora seed prevented colony formation by C. albicans and C. tropicalis. It was suggested that CC-GRP might interact with the receptor protein kinase WAK1; in yeast, such receptors are directly involved in the regulation of cell proliferation (Zottich et al., 2013).

11.2. Lectins

Lectins are a class of proteins characterised by at least one non-catalytic domain which binds specifically and reversibly to mono- or oligosaccharides. This feature is associated with their endogenous biological function as well as with multiple applications, including remarkable antimicrobial effects, notably against bacteria, fungi and protozoa, in addition to modulating
host immunity (Han et al., 2018). Plant lectins are classified into 12 subfamilies; plants are undeniably an important natural source of these proteins. They have demonstrated activity against various microbial strains; in vivo, they modulated host immunity, signalling and activating defense cells, while in vitro, plant lectins can inhibit microbial development and morphology. Although several lectins displayed antiyeast activity, the discussion will exclude those with a molecular weight > 30 kDa.

The antifungal lectin known as C-25 from chickpea had activity against several Candida spp. strains resistant to the antifungal agent fluconazole; C-25 induced bleb-like surface changes (protrusions), and yeast cell wall disruption. Moreover, no toxic effect was found on normal human peripheral blood mononuclear cells (Kumar et al., 2014). The Helianthus annuus jacalin (Helja) protein, belonging to the jacalin-related lectins (JRLs) subfamily, showed activity against Candida spp (Pinedo et al., 2015). Jacalin proteins were first isolated from jack fruit seeds, and they are widely distributed among the plant kingdom. Cells of C. albicans treated with 0.1 µg/µl of Helja showed a drastic decrease in yeast survival (Del Rio et al., 2019); the lectin interacted with the mannoproteins on the yeast cell wall affecting cell integrity. It induced the production of hydrogen peroxide and inhibited the morphological transition from yeast to filamentous forms. Helja caused a significant reduction of surface hydrophobicity in the fungal cells.

12. Unclassified antiyeast proteins

A 14.3 kDa protein isolated from the seeds of butterfly pea (Clitoria ternatea) and labelled as Ct protein exhibited activity against several clinically relevant yeast such as C. neoformans, C. albicans and C. parapsilosis. Ct proteins also displayed an inhibitory activity on mycelial growth in several moulds and lytic activity against the bacteria Micrococcus luteus. Moreover, Ct protein did not possess haemolytic properties (Ajesh and Sreejith, 2014).

Mo-CBP2 is 13.3 kDa CB protein isolated from Moringa oleifera seeds. Mo-CBP2 is a basic glycoprotein (isoelectric point (pI): 10.9) and possesses in vitro antifungal activity against C. albicans, C. parapsilosis, C. krusei, and C. tropicalis. Mo-CBP2 increased the cell membrane permeabilisation and ROS production in C. albicans and promoted degradation of circular plasmid DNA (pUC18) from Escherichia coli. Mo-CBP2 presents sequence similarities to Mo-
CBP3 (a 2S albumin) and flocculating proteins. Moreover, Mo-CBP2 did not display hemagglutinating and haemolytic activities upon rabbit and human erythrocytes (Oliveira et al., 2017).

Potide-J is a peptide derived from the proteolytic digestion of a 15 kDa protein named AFP-J isolated from the potato tuber (*Solanum tuberosum* L. cv. Jopung). AFP-J had 10 Cys residues and Potide-J amino acid sequence was determined to be Ala-Val-Cys-Glu-Asn-Asp-Leu-Asn-Cys-Cys. It displayed an Minimum Inhibitory Concentration (MIC) of 6.25 μg/ml against *Candida albicans*; however, no haemolytic activity was detected (Lee et al., 2012).

The rose *Impatiens balsamina* produces for small Cys-rich peptides made of 20 residues, named Ib-AMP1, Ib-AMP2, Ib-AMP3 and Ib-AMP4 (Tailor et al., 1997). Although Ib-AMPs have CB activity, they don’t fit in any of the protein families above described. They are highly basic and present a loop structure stabilised by two disulphide bonds plus a distinctive Glu residue at the N terminus. Studies of Ib-AMP1 analogs without disulfide bonds have shown that these bonds are not essential for their antimicrobial activity. Ib-AMPs are active against fungal phytopathogens, including yeasts *P. pastoris* and *S. cerevisiae*. Filamentous fungi are more sensitive than yeasts, probably due to a higher content of chitin. The peptides bind to the hyphal walls and are not thought to act via pore formation because they do not significantly disrupt artificial liposomes (Thevissen et al., 2005). Native Ib-AMPs show no haemolytic nor toxic activity.

### 13. Recombinant plant defense-related proteins and peptides

Traditionally, novel antifungal compounds are isolated from plant materials; however, an alternative method is to isolate the gene encoding the novel antifungal compound and express the relevant protein in a heterologous system. It is worth mentioning that several plant defense-related proteins and peptides with antiyeast activity have been identified using cloning methodologies. For instance, the recombinant form of the 5 kDa defensin named OsAFP1 from rice (*Oryza sativa*) displayed anticandidal activity through interaction with PI(3)P phospholipids followed by membrane permeabilisation (Ochiai et al., 2020). However, cloned proteins will not be discussed further since the scope of this work is exploring the diversity of plant native proteins directly isolated from organic materials.
Table 1. Plant native defense-related small proteins and peptides with antiyeast activity. N.d. stands for Not Determined.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type member</th>
<th>Plant species</th>
<th>Tissue</th>
<th>Size (kDa)</th>
<th>Cys</th>
<th>Yeast target</th>
<th>Described mode of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RsAFP2</td>
<td>Defensins</td>
<td>Radish (Raphanus sativus)</td>
<td>Seeds</td>
<td>5</td>
<td>8</td>
<td>Candida albicans, Candida krusei, Candida glabrata, Pichia pastoris</td>
<td>Interaction with GlcCer followed by membrane permeabilisation and ROS production</td>
<td>(Terras et al., 1992; Thevissen et al., 2004; Aerts et al., 2007)</td>
</tr>
<tr>
<td>DmA MP1</td>
<td>Defensins</td>
<td>Dahlia (Dahlia merckii)</td>
<td>Seeds</td>
<td>5.5</td>
<td>8</td>
<td>Saccharomyces cerevisiae</td>
<td>Interaction with (M(IP)_2C) followed by membrane permeabilisation</td>
<td>(Osborn et al., 1995; Thevissen et al., 2000; Fujimura et al., 2004)</td>
</tr>
<tr>
<td>NaD1</td>
<td>Defensins</td>
<td>Tobacco (Nicotiana alata)</td>
<td>Flower</td>
<td>5</td>
<td>8</td>
<td>Candida albicans, Saccharomyces cerevisiae, Cryptococcus neoformans, Cryptococcus gatti</td>
<td>Interaction with PA and (PI(4,5)P_2) followed by membrane permeabilisation</td>
<td>(Hayes et al., 2013)</td>
</tr>
<tr>
<td>HsAFP1</td>
<td>Defensins</td>
<td>Coral bell (Heuchera sanguinea)</td>
<td>Seeds</td>
<td>5</td>
<td>8</td>
<td>Candida albicans, Candida krusei, Saccharomyces cerevisiae</td>
<td>Interaction with PA followed by membrane permeabilisation and ROS production</td>
<td>(Osborn et al., 1995; Aerts et al., 2011; Vriens et al., 2015)</td>
</tr>
<tr>
<td>Psd1</td>
<td>Defensins</td>
<td>Pea (Pisum sativum)</td>
<td>Seeds</td>
<td>5</td>
<td>8</td>
<td>Candida albicans</td>
<td>Interaction with GlcCer followed by membrane permeabilisation</td>
<td>(Gonçalves et al., 2017)</td>
</tr>
<tr>
<td>Mj-AMP</td>
<td>Defensins</td>
<td>Four o'clock (Mirabilis jalapa)</td>
<td>Seeds</td>
<td>4</td>
<td>6</td>
<td>Saccharomyces cerevisiae</td>
<td>Not described</td>
<td>(Cammue et al., 1992; Terras et al., 1992)</td>
</tr>
<tr>
<td>Ha-DEF1</td>
<td>Defensins</td>
<td>Sunflower (Helianthus annuus)</td>
<td>Seeds</td>
<td>5.8</td>
<td>8</td>
<td>Saccharomyces cerevisiae</td>
<td>Membrane permeabilisation</td>
<td>(De Zélicourt et al., 2007; Carvalho and Gomes, 2009)</td>
</tr>
<tr>
<td>Name</td>
<td>Type member</td>
<td>Plant species</td>
<td>Tissue</td>
<td>Size (kDa)</td>
<td>Cys</td>
<td>Yeast target</td>
<td>Described mode of action</td>
<td>Reference</td>
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<tr>
<td><strong>PvD1</strong></td>
<td>Defensins</td>
<td>Common bean <em>(Phaseolus vulgaris L.)</em></td>
<td>Seeds</td>
<td>6</td>
<td>8</td>
<td><em>Candida albicans</em> <em>Candida parapsilosis</em> <em>Candida tropicalis</em> <em>Candida guilliermondii</em> <em>Kluyveromyces marxianus</em> <em>Saccharomyces cerevisiae</em></td>
<td>Interaction with GlcCer followed by membrane permeabilisation</td>
<td>(Games <em>et al.</em>, 2008; Salas <em>et al.</em>, 2015)</td>
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<td><strong>PsDef1</strong></td>
<td>Defensins</td>
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<td>Seedling</td>
<td>5.6</td>
<td>8</td>
<td><em>Candida albicans</em></td>
<td>Not described</td>
<td>(Kovaleva <em>et al.</em>, 2009)</td>
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<tr>
<td><strong>CcDef3</strong></td>
<td>Defensins</td>
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<td>Fruit</td>
<td>Nd</td>
<td>Nd</td>
<td><em>Candida buinensis</em> <em>Candida albicans</em> <em>Candida tropicalis</em></td>
<td>Membrane permeabilisation</td>
<td>(Aguieiras <em>et al.</em>, 2021)</td>
</tr>
<tr>
<td><strong>CaDef2.1</strong></td>
<td>Defensins</td>
<td>Chili Pepper <em>(Capsicum annuum)</em></td>
<td>Fruit</td>
<td>Nd</td>
<td>Nd</td>
<td><em>Candida buinensis</em> <em>Candida tropicalis</em> <em>Candida albicans</em> <em>Candida parapsilosis</em></td>
<td>Membrane permeabilisation</td>
<td>(da Silva Gebara <em>et al.</em>, 2020)</td>
</tr>
<tr>
<td><strong>CaDef2.2</strong></td>
<td>Defensins</td>
<td>Chili Pepper <em>(Capsicum annuum)</em></td>
<td>Fruit</td>
<td>Nd</td>
<td>Nd</td>
<td><em>Candida buinensis</em> <em>Candida tropicalis</em> <em>Candida albicans</em> <em>Candida parapsilosis</em></td>
<td>Membrane permeabilisation</td>
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<td>Not described</td>
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| **Hevein** | Hevein | Rubber tree *(Hevea brasiliensis)* | Latex | 4.7 | 8 | *Candida albicans*  
*Candida tropicalis*  
*Candida krusei* | Not described | *(Kanokwiroon et al., 2008)* |
| **Pn-AMP1** | Hevein-like | Morning glory *(Pharbitis nil)* | Seeds | 4.3 | 6 | *Saccharomyces cerevisiae*  
*Candida albicans* | Actin depolarisation followed by membrane permeabilisation | *(Koo et al., 1998; Fujimura et al., 2004; Koo et al., 2004)* |
| **Fa-AMP1** | Hevein-like | Buckwheat *(Fagopyrum esculentum)* | Seeds | 3.9 | 8 | *Geotrichum candidum* | Not described | *(Fujimura et al., 2003)* |
| **Fa-AMP2** | Hevein-like | Buckwheat *(Fagopyrum esculentum)* | Seeds | 3.9 | 8 | *Geotrichum candidum* | Not described | *(Fujimura et al., 2003)* |
| **Ay-AMP** | Hevein-like | Prince feather *(Amaranthus hypochondriacus)* | Seeds | 3.1 | 6 | *Geotrichum candidum*  
*Candida albicans* | Not described | *(Rivillas-Acevedo and Soriano-García, 2007)* |
<p>| <strong>Jackin</strong> | Hevein/Lectins | <em>Jackfruit</em> <em>(Artocarpus integrifolia)</em> | Seeds | 14 | 6 | <em>Saccharomyces cerevisiae</em> | Interaction with chitin followed by membrane permeabilisation | <em>(Trindade et al., 2006)</em> |
| <strong>Frutacki n</strong> | Hevein/Lectins | <em>Breadfruit</em> <em>(Artocarpus incisa)</em> | Seeds | 14 | 6 | <em>Saccharomyces cerevisiae</em> | Interaction with chitin followed by membrane permeabilisation | <em>(Trindade et al., 2006)</em> |</p>
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Figure 7. Diversity of mechanisms used by natural antiyeast peptides isolated from plants. This representation is a modification of the figure (and described mechanisms) presented by Van Der Weerden et al. (2013). Yeast cell wall is marked by the colour pink while the yeast cell membrane is in light blue.
13. Concluding remarks

Plants can produce many antimicrobial proteins and peptides, which are integral components of a universally conserved defense mechanism found in all higher organisms. These molecules’ genomic and proteinic structure can be variable and characterised by isoform diversity that translates into homologous proteins showing a broad spectrum of activity. In plants, they are generated with greater diversity and abundance when compared to other kingdoms. Classification and comparison of plant defense-related proteins and peptides can be challenging even in the “Omics age”; moreover, it can be speculated that plants might shelter yet undescribed families. This review described families of peptides and small proteins, members of which have displayed *in vitro* activity against yeast strains; nevertheless, it must be noticed the existence of further antifungal families that have been excluded from the discussion due to a lack of data on their activity against yeasts (*e.g.*, α-hairpinin and puroindolines).

The advances and improvement of existing bioinformatics tools have led to more efficient approaches concerning the molecular modelling and identification of antiyeast proteins; however, most novel plant antifungal compounds and their antimicrobial spectrum are typically investigated by employing wet-lab techniques regarding single candidates. High throughput *in silico* methods has the potential to transform this scenario, revealing many new antimicrobial candidates with desirable features for a biotechnological application, such as structure stability, low cytotoxicity and reduced sensitivity to salts and pH variation.

This review and numerous reports highlight the potential benefits of employing these molecules as alternatives to antimicrobial agents in therapeutic, agricultural and industrial sectors. In this regard, this thesis represents a preliminary investigation work on the validity of plant defense-related small proteins/peptides as potential antiyeast food preservative agents.

Like other naturally produced antimicrobial agents (*e.g.*, essential oils), plant defense-related proteins/peptides have gained significance as a possible novel generation of bio-preservatives. Their incorporation in food and beverages could help in the fight against food waste and loss provoked by spoilage microbial strains since these proteins are usually thermostable with good water solubility, broad-spectrum activity and a mechanism of action which would limit the occurrence of microbial resistance. However, their application in food preservation is still under review and continues in the process of overcoming technical limitations, such as large-scale production. To our knowledge, thus far, no plant AMPs have been approved for use in the food industry, although AMP-producing lactic acid bacteria, such as *Lactobacillus*...
fermentum, can be applied as food preservatives since they are commonly used as starter cultures in fermented products. According to the FDA “Guidance for Industry: Microbiological Considerations for Antimicrobial Food Additive Submissions”, several studies as needed for an antimicrobial food additive to be classified as Generally Recognised As Safe (GRAS). A detailed description of the antimicrobial effect and clear identification of targeted microbes (including complete reports of the efficacy studies) and a detailed description of the conditions of use (e.g., type of foods, proposed use level, temperature range of use and method of application) are necessary and required for the approval process to witch AMPs must be subjected by regulatory agencies (Keymanesh et al., 2009; Rai et al., 2016; León Madrazo and Segura Campos, 2020; Food and Drug Administration, 2021; Sultana et al., 2021).

As highlighted by the literature review, the current knowledge regarding the applicability of plant defense-related proteins/peptides is deficient, with the majority of the studies focusing on increasing cytotoxic effects and proteolytic resistance for application in therapeutics and drug development. Concerning their potential application in food preservation, more research needs to be conducted on antimicrobial potential and overall safety. In addition, a better understanding of how these molecules interact with the food and beverages matrices is necessary to regulate their use in industrial settings as preservative agents.

The work described in this thesis mainly aims to set a foundation for possible future exploitation of plant defense-related antimicrobial proteins and peptides in food preservation. The main objectives of the work further discussed in this thesis were to investigate the activity of these molecules against spoilage yeast strains and if this activity is retained in food and beverages samples and under conditions typical of food manufacturing settings (such as pH variation, salts presence and high-temperature treatments). Moreover, this thesis describes and explores different sources for obtaining large quantities of pure protein products, including natural extraction, chemical synthesis and heterologous production, comparing the advantages and disadvantages of each technique. In addition, preliminary tests were conducted to establish if these molecules would be safe for human ingestion.
14. Bibliography


HAN, Y. juan et al. (2018) ‘Evolutionary analysis of plant jacalin-related lectins (JRLs) family and expression of rice JRLs in response to Magnaporthe oryzae’, *Journal of Integrative
Chapter 1


Ibeas, J. I. et al. (2001) ‘Resistance to the plant PR-5 protein osmotin in the model fungus Saccharomyces cerevisiae is mediated by the regulatory effects of SSD1 on cell wall


Chapter 2

Inhibitory activity of two synthetic *Pharabitis nil* L. antimicrobial peptides against common spoilage yeasts

A manuscript based on this chapter has been submitted to the journal “Applied food Research” ISSN: 2772-5022
Abstract

Fighting food waste caused by spoilage yeasts and developing more natural forms of preservatives are critical points for the food industry. Plant antimicrobial peptides have biological activity against a broad spectrum of microorganisms, which could represent a valid alternative to the current preservative agents. In this work, the *Pharabitis nil* antimicrobial peptides Pn-AMP1 and Pn-AMP2 were chemically synthesised, and the potency of their antiyeast activity against common spoilage yeasts was measured. Pn-AMP1 showed a better inhibitory effect than Pn-AMP2; therefore, it was further characterised. Pn-AMP1 displayed fast inhibitory activity against yeast strains *Kluyveromyces lactis* and *Zygosaccharomyces bailii*, and the antiyeast mechanism of membrane permeabilisation was detected. Its safety in terms of human consumption was studied and no adverse effects were found. Lastly, the stability of the peptide in different environments and conditions, such as heat, high salt, and a range of pH was studied, in addition to antiyeast activity in different food matrices such as a soft drink and dairy product, further supporting Pn-AMP1’s potential for use in food preservation.
1. Introduction

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics; in industrialised countries, microbial spoilage can cause the loss of up to 30% of stored foods (FAO, 2011). Although bacteria are described as the most common spoilage microorganisms, yeasts and filamentous fungi growth can be favoured by low pH or high osmolarity environments typical of preserved foods (Booth and Stratford, 2003). Low-temperature storage and the use of preservatives such as weak organic acids can be sufficient to prevent fungal spoilage. Nevertheless, certain yeast species can overcome these measures and grow to cause spoilage outbreaks (Davies et al., 2021). Thus, there is a need to discover more effective antiyeast agents to be applied in the food industry. Moreover, consumer perception of the current forms of preservatives has also become a leading factor in developing more natural forms of preservatives.

Plants are a rich source of small proteins and peptides with natural activity against yeasts. Usually these proteins are part of the plant immune system and can be labelled as plant antimicrobial peptides (AMPs) or plant pathogenesis-related (PR) proteins (Ali et al., 2018). Plant AMPs can be isolated from organic tissues through purification and extraction methods, and pure peptides derived from known amino acid sequences can be produced via chemical synthesis (Rizza et al., 2008). Typically, AMPs mainly target the cytoplasmic membrane of microorganisms, disrupting its structural integrity and eventually leading to cell death. This antimicrobial mode of action reduces the incidence of antimicrobial resistance; therefore, several classes of AMPs are being considered as alternatives to commonly used antimicrobial drugs or preservatives.

Hevein-type antimicrobial peptides or PR-4 are short basic peptides (29-45 amino acid residues) found in mono and dicotyledonous plants from different families (Van Damme et al., 1999). Normally, they are characterised by 8 Cys residues, six of which are normally found in conservative position with disulfide bond formation (from three to five) occurring between the following residues: C1–C4, C2–C5, and C3–C6 (Tam et al., 2015). The main distinctive feature of all hevein-like AMPs is a conservative chitin binding site containing the amino acid sequence SXFGY/SXYGY, where X is any amino acid residue; overall, the typical structure include an α helix–β1–β2–α helix–β3 motif and a central β sheet with antiparallel β chains surrounded by two small helices stabilised by disulfide bridges (Slavokhotova et al., 2017). Almost all hevein-like peptides are reported to be toxic at their micromolar concentrations.
against a wide range of phytopathogenic fungi. In particular, two peptides isolated from Japanese morning glory (*Pharbitis nil*) seeds named Pn-AMP1 and Pn-AMP2 showed antiyeast activity. Pn-AMP1 and Pn-AMP2 are small peptides of 41 and 40 amino acid residues, respectively. The amino acid sequences of both peptides are identical with the exception that Pn-AMP1 carries one additional serine residue at the C-terminus; moreover, their chitin binding domains display high homology with other hevein proteins (Koo *et al.*, 1998).

Plant AMPs have been examined for their application in the fight against microbial spoilage, and few studies considered the potential use of these proteins as food preservative agents both in native (Thery *et al.*, 2020) and synthetic (Shwaiki *et al.*, 2020a) forms. Theoretically, native Pn-AMP1 and Pn-AMP2 possess several desirable characteristics for a bio-preservative application (e.g. they are found in edible seeds, they have potent antifungal activity and a stable Cys-rich structure); however, native peptides are extracted in low amounts using a laborious protocol. According to Koo *et al.*, (1998) the yields of Pn-AMP1 and Pn-AMP2 from 50 g of *P. nil* are reported to be 1.43 and 2.06 mg, respectively. Thus, it was chosen to test the two peptides in their synthetic form since the synthetic production enables for analysis higher quantity of pure product.

For the purpose of this study, the chemical synthesis of Pn-AMP1 and Pn-AMP2 was carried out. Main objectives of this work are to evaluate the peptides’ activity against five common food spoilage yeast strains and to investigate their potential as possible preservative agents. In this prospective, the peptides’ mechanism of antiyeast action was studied alongside their stability under different conditions. The safety and incorporation of the peptides into different food matrices was also explored.
2. Material and Methods

2.1. Generation of synthetic peptides

Two synthetic peptides were created based on the sequence (Table 1) of the antifungal peptides isolated from seeds of *Pharbitis nil* (Koo et al., 1998) and labelled Pn-AMP1 and Pn-AMP2. GLBiochem (Shanghai) Ltd. synthesised the two peptides to a purity of >90%, as indicated by the supplier. Each peptide was resuspended in sterile distilled water at 4 mg/ml concentration and stock that -20°C.

<table>
<thead>
<tr>
<th>Table 1. Amino acids sequences of the “Pharbitis nil L. antimicrobial peptides”.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pn-AMP1</strong></td>
</tr>
<tr>
<td><strong>Pn-AMP2</strong></td>
</tr>
</tbody>
</table>

2.2. Yeast strains

Four strains representative of common food/feed spoilage yeasts were used, namely *Kluyveromyces lactis* ATCC 56498, *Zygosaccharomyces bailii* Sa 1403, *Zygosaccharomyces rouxii* ATCC 14679 and *Debaryomyces hansenii* CBS 2334. In addition, the type strain *Saccharomyces cerevisiae* DMS 70449 was used to further characterise the antiyeast activity of the peptides. All the strains were obtained either from the Leibniz Institute DSMZ collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) or the MTU strains collection. Stock cultures were maintained at -80 °C, and strains were grown aerobically in malt extract agar (MEA) at 30 °C. Overnight cultures were obtained from inoculating a single yeast colony in 5 ml of malt extract broth and incubating the culture at 30°C under gentle agitation. All media and chemicals used in this work were purchased from Sigma Aldrich (MO, USA) unless otherwise stated.

2.3. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of synthetic Pn-AMP1 and Pn-AMP2 was determined using a micro broth dilution method and following the guidelines of the "EUCAST
Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts" (Rodriguez-Tudela, 2008). Test were performed in a flat-bottom 96-well microtiter plate (Sarsdedt, Nümbrecht, Germany). The method is based on the preparation of serially diluted peptide solutions in 100 µl volumes/well with the addition of a yeast inoculum (5x10⁵ cells/ml) in a volume of 100 µl. The concentration of yeast cells was calculated using a haemocytometer (Improved Neubauer Counting Chamber, Sigma Aldrich). Pn-AMP1 and Pn-AMP2 solutions were tested at two serially diluted series concentrations: from 1000 to 62.5 µg/ml and from 400 to 25 µg/ml. Sabouraud-Dextrose Broth (SDB) was employed as the growth media, and controls contained distilled water (dH₂O) instead of peptide solution. The prepared plates were incubated for 48 h at 28 °C in a microtiter plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) with gentle shakes between readings. The optical density (600 nm) was measured at 2 h intervals. The MIC was determined as the lowest concentration of peptide required to inhibit yeast growth.

2.4. Spoilage yeasts susceptibility to synthetic Pn-AMP1

To evaluate susceptibility of the four spoilage yeast strains toward peptide Pn-AMP1, a 10⁵ cells/ml yeast suspension was prepared in SD Broth for each strain and incubated at 30 °C under gentle shaking with different concentrations of synthetic peptide (25, 50, 100, 200 and 400 µg/ml). The 10⁵ cells/ml yeast concentration was used to represent the number of cells commonly found to spoil food and beverage products (Fleet, 2011). Ten microlitres of each suspension was spotted on SD agar after 1, 2 and 3 h of incubation. Positive controls contained dH₂O instead of peptide solution. Inhibition of the yeast growth was assessed visually.

2.5. Colony count assay

The time necessary for Pn-AMP1 to impact the growth of 10⁴ CFU/ml of the yeast Z. bailii was evaluated with a colony count assay as described by Shwaiki et al. (2020a). Briefly, a yeast suspension was inoculated at 30 °C with 125, 250 and 500 µg/ml concentrations of the peptide and incubated for 6 h. Each hour, 100 µl of the suspensions were spread onto SD agar plates and subsequently incubated for 48 h at 30 °C. The control consisted of a yeast suspension with dH₂O without peptide.
2.6. Membrane permeabilisation

The ability of synthetic Pn-AMP1 to permeabilise the cell membrane of the yeast *S. cerevisiae* was examined as a possible antiyeast mechanism of action following the protocol by Shwaiki *et al.*, 2019. A yeast suspension (10^5 cells/ml) was prepared in 1X Phosphate Buffered Saline (PBS) from an overnight culture. Ninety microlitres of this inoculum was seeded in a flat-bottom 96-well microtiter plate in conjunction with 10 µl of the peptide at three different concentrations (125, 250 and 500 µg/ml). Simultaneously, 6 µM of propidium iodide (PI) were added to each well. PI is a dye that can produce fluorescence upon binding with nucleic acids of cells; since it is excluded from viable cells, the bond occurs only when the cell membrane has been damaged. Triton X-100 (0.1%) and water were used instead of PN-AMP1 as the positive and negative control, respectively. The extent of membrane permeabilisation was measured using a VarioscanLUX plate reader at the maximal excitation (λEx) and maximum emission (λEm) wavelengths of 535 nm and 617 nm, with readings every 10 min up to 5 h.

2.7. Synthetic Pn-AMP1 stability

Pn-AMP1’s antiyeast potency against *K. lactis* was tested in different environmental conditions which may be encountered in food and beverage products. A microtiter growth curve assay was used to test the antiyeast activity of Pn-AMP1 at 500 and 1000 µg/ml under varying salts conditions, in a range of pHs, and after heat treatment. Salt solutions of 1 and 5 mM magnesium chloride (MgCl2) and 50 and 150 mM potassium chloride (KCl) were added to the growth medium (SD broth), and the antiyeast assay was carried out, including controls of modified broth, yeast and no peptide. A peptide sample was heated for 15 min at 100 °C and left to cool for 30 min before carrying out the antiyeast test; this was done to determine if Pn-AMP1 is resistant to thermal degradation. The effect of different pH on the peptide was conducted by changing the pH of the SD broth used to perform the antiyeast assay. pH 3, 5, 7, 9 and 11 were tested by adjusting the media with 0.1 M hydrochloric acid and 1 M sodium hydroxide. Controls of media modified to the different pH containing the yeast and no peptide were used.

2.8. Haemolytic assay

A haemolytic assay was carried out to evaluate Pn-AMP1’s safety for human consumption. The release of haemoglobin from mammalian red blood cells due to the presence of the peptide
was calculated following the protocol outlined by Shwaiki et al. 2020b. Briefly, a 4% solution of defibrinated sheep erythrocytes (Oxoid™) was incubated for 1 h at 37 °C with 125, 250, 500 and 1000 µg/ml peptide concentrations. Samples incubated with 0.1% Triton X-100 and PBS were used as the positive and negative controls, respectively. Afterwards, samples were centrifuged at 1000 g for 10 min, and the OD at 405 nm of the supernatant was measured. Results are expressed as a percentage of haemolysis, with 10% being the threshold in the data interpretation; if >10%, the protein was considered haemolytic, and if <10%, it was not haemolytic. The calculations were made using the measured absorbance values and the formula below:

\[
\% \text{ Haemolysis} = \frac{(A_{405 \text{ protein treatment}}) - (A_{405 \text{ PBS}})}{(A_{405 \text{ 0.1% Triton X-100}}) - (A_{405 \text{ PBS}})}
\]

2.9. Proteolytic assay

Pn-AMP1’s resistance to proteolytic digestion was tested with α-chymotrypsin, a digestive enzyme of the human gut. The experimental protocol (Shwaiki et al., 2020) was intended to simulate the gastrointestinal environment that the peptide may encounter after ingestion, thus evaluating the peptide's safety. Briefly, peptide samples were incubated for 4 h at 37 °C in conjunction with α-chymotrypsin at different peptide:enzyme molar ratios (60:1, 250:1 and 2500:1). Afterwards, the enzyme was heat-inactivated at 80 °C for 10 min. An antiyeast growth curve assay against K. lactis was carried out with the digested peptide samples at 125, 250, 500 and 1000 µg/ml. The enzyme was stored in solution in a digestion buffer consisting of 50 mM tris hydrochloride (Tris–HCl) (pH 7.4) and 5 mM calcium chloride (CaCl₂).

2.10. Synthetic Pn-AMP1 application in different food matrices

The peptide’s inhibitory activity was tested in milk UHT, pH 7 (Łaciate Milk 3.2%, fat) against the yeast K. lactis and in Fanta Orange, pH 3.1 (Coca-Cola, Ireland) against the yeast Z. bailii. Usually, yeast populations less than 10³ CFU/ml are reported in dairy products; however, some yeast species, such as Kluyveromyces spp, could grow to 10⁶-10⁹ CFU/ml due to their ability to utilise milk lactose (Fleet, 2011). Pn-AMP1 at concentrations of 62.5, 125, 250 and 500 µg/ml were incubated at 30 °C in conjunction with 1 ml of milk spiked with 10² CFU/ml of K. lactis. After 4 h, 100 µl of each solution was plated on Yeast Extract Peptone Dextrose (YPD) agar and colonies were counted after 2 days of incubation at 30 °C. Samples of spiked milk
without peptide and just milk were used as the positive and negative control, respectively. Due to their salt and sugar content, soft drinks represent a favourable environment for the growth of the spoilage yeast Z. bailii (Davies et al., 2021). Pn-AMP1 was tested via the growth curve method using a $10^3$ cells/ml suspension of yeast made up in filter sterilised solutions of the Fanta. The peptide was tested at concentrations ranging from 62.5 to 500 µg/ml, and its anteyes activity was observed over 48 h at 30 °C by measuring the optical density at 620 nm every 2 h. Afterwards, 100 µl of solutions in each well was seeded in YPD agar plates to verify yeast death. Controls consisting of the beverages inoculated with the same concentration of yeast without the peptide were also included.

2.11. Data presentation

All the tests were performed in duplicate or triplicate, and results in the tables and graphs are presented as means ± standard deviation.

3. Results

3.1. Determination of Minimum Inhibitory Concentration (MIC)

The MIC for Pn-AMP1 and Pn-AMP2 were measured with a growth curve assay, and results are reported in Table 2. The yeast K. lactis was inhibited by 400-500 µg/ml concentration of Pn-AMP1 and 500-1000 µg/ml of Pn-AMP2. Similar results were obtained for Z. bailii. The yeast Z. rouxii was not sensitive to any of the tested concentrations for the two peptides, while D. hansenii was killed only at 1000 µg/ml of Pn-AMP1. The growth of type strain S. cerevisiae DMS 70449 was impacted by 500 µg/ml of Pn-AMP1 and 1000 µg/ml of Pn-AMP2. In light of these results, only Pn-AMP1 was characterised for its potential application as a food preservative.

Table 2. Range of MIC measured for Pn-AMP1 and Pn-AMP2 against five yeast strains

<table>
<thead>
<tr>
<th></th>
<th>K. lactis</th>
<th>D. hansenii</th>
<th>Z. bailii</th>
<th>Z. rouxii</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pn-AMP1</td>
<td>400-500 µg/ml</td>
<td>1000 µg/ml</td>
<td>400-500 µg/ml</td>
<td>No inhibition</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>Pn-AMP2</td>
<td>500-1000 µg/ml</td>
<td>No inhibition</td>
<td>1000 µg/ml</td>
<td>No inhibition</td>
<td>1000 µg/ml</td>
</tr>
</tbody>
</table>
3.2. Spoilage yeasts susceptibility to synthetic Pn-AMP1

For each of the four spoilage yeasts, a $10^3$ cells/ml solution was prepared and exposed to 25, 50, 100, 200 and 400 µg/ml concentrations of Pn-AMP1. The yeasts’ susceptibility to the peptide were checked every hour for 3 h by spotting 10 µl of each sample on SD agar. Yeasts *K. lactis* and *Z. bailii* were the most impacted by Pn-AMP1, coherently with data obtained for the MIC. After 3 h, 400 and 200 µg/ml of the peptide completely prevented the yeast growth (Figure 1.A and 1.C), and only a few colonies of *K. lactis* were seen at 100 µg/ml. Yeasts *D. hansenii* and *Z. rouxii* were inhibited by 400 µg/ml of Pn-AMP1 (Figure 1.B and 1.D). Since *K. lactis* and *Z. bailii* were the most sensitive strains to Pn-AMP1, they were chosen as reference yeast strains in the following experiments.

![Figure 1. The sensitivity of various yeast spoilage strains (A) *K. lactis* (B) *D. hansenii* (C) *Z. bailii* (D) *Z. rouxii* exposed to Pn-AMP1 (25-400 µg/ml). Yeasts were tested at $10^3$ cells/ml concentrations, and all the strains showed a growth decrease over only 3 h of exposure to the peptide. Tests were done in triplicates.](image)

### Pn-AMP1 concentrations (µg/ml)

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>200</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>1H</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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</tr>
<tr>
<td>2H</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>3H</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>

3.3. Colony count assay

The colony count assay evaluated the impact of synthetic Pn-AMP1 on *Z. bailii* cells ($10^4$ CFU/ml starting concentration) over time. High concentrations (250 and 500 µg/ml) of Pn-AMP1 induced an immediate decline in the yeast growth (Figure 2). After only 1 h incubation, less than $5\times10^2$ CFU/ml were counted in the 500 µg/ml sample. After 6 h of incubation, no
colonies were present in the 250 and 500 µg/ml samples; a steady growth was observed for the 125 µg/ml and the control samples that reached 2.3x10^4 and 3.2x10^4 CFU/ml, respectively.

![Graph showing yeast colony count assay demonstrating the rate of Z. bailii inhibition caused by 125, 250 and 500 µg/ml concentrations of Pn-AMP1. After only 1 h, a decrease in the yeast growth is evident at 250 and 500 µg/ml levels. Tests were done in triplicate (bars represent standard deviation).](image)

3.4. Membrane permeabilisation

Membrane permeabilisation is a common mechanism by which AMPs target microbial cells (Lei et al., 2019). *S. cerevisiae* cells were incubated with different concentrations of Pn-AMP1 (125, 250 and 500 µg/ml) and PI in a microtiter plate to follow the degree of membrane permeabilisation. As predicted, the highest fluorescence intensity was read for the control Triton-X 0.1% and the lowest intensity was recorded for the control with water; 500 and 250 µg/ml of Pn-AMP1 resulted in a steady increase of the measured fluorescence over 6 h, indicating the occurrence of membrane permeabilisation (Figure 3).
Figure 3. Kinetics indicating the rate of membrane permeabilisation of *S. cerevisiae* yeast in the presence of synthetic Pn-AMP1. All the peptide concentrations tested produced a detectable fluorescence: Pn-AMP1 causes membrane permeabilisation in yeasts. Tests were done in triplicate (bars represent standard deviation).

3.5. Synthetic Pn-AMP1 stability

Pn-AMP1’s stability was examined to evaluate if its antiyeast activity is maintained under conditions that may be encountered if the peptide was to be applied as a food preservative. Results are summarised in Table 3; Pn-AMP1’s antiyeast potential was retained at pH 5, 7 and 9 with 500 and 1000 µg/ml causing complete yeast inhibition. At pH 3 and 11, no yeast growth was observed in the controls. After being heated at 100 °C for 15 min, the peptide showed a decrease in their antiyeast activity: only 1000 µg/ml was lethal for *K. lactis*. The Pn-AMP1 inhibitory activity was partially affected by the salts; in fact, at 500 µg/ml yeast growth was observed in all the conditions tested, and at 1000 µg/ml for 150 mM KCl. However, at 1000 µg/ml, Pn-AMP1 caused yeast cell death when tested with 1 and 5 mM of MgCl₂ and 50 KCl.
Table 3. Pn-AMP1 inhibitory activity against *K. lactis* in varying environmental conditions. The symbol (-) means \( \text{OD}_{600\text{nm}} < 0.2 \); (+) means \( \text{OD}_{600\text{nm}} \) between 0.2 and 0.8; (++) means \( \text{OD}_{600\text{nm}} > 0.8 \)

<table>
<thead>
<tr>
<th>Environmental conditions</th>
<th>( K. lactis ) growth</th>
<th>Control with no Pn-AMP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000 µg/ml Pn-AMP1</td>
<td>500 µg/ml Pn-AMP1</td>
</tr>
<tr>
<td>pH 3</td>
<td>Growth inhibited (-)</td>
<td>Growth inhibited (-)</td>
</tr>
<tr>
<td>pH 5</td>
<td>Growth inhibited (-)</td>
<td>Growth inhibited (-)</td>
</tr>
<tr>
<td>pH 7</td>
<td>Growth inhibited (-)</td>
<td>Growth inhibited (-)</td>
</tr>
<tr>
<td>pH 9</td>
<td>Growth inhibited (-)</td>
<td>Growth inhibited (-)</td>
</tr>
<tr>
<td>pH 11</td>
<td>Growth inhibited (-)</td>
<td>Growth inhibited (-)</td>
</tr>
<tr>
<td>Heat (15 min at 100°C)</td>
<td>Growth inhibited (-)</td>
<td>No inhibition (+)</td>
</tr>
<tr>
<td>MgCl(_2) 1mM</td>
<td>Growth inhibited (-)</td>
<td>No inhibition (+)</td>
</tr>
<tr>
<td>MgCl(_2) 5mM</td>
<td>Growth inhibited (-)</td>
<td>No inhibition (++)</td>
</tr>
<tr>
<td>KCl 50 mM</td>
<td>Growth inhibited (-)</td>
<td>No inhibition (++)</td>
</tr>
<tr>
<td>KCl 150 mM</td>
<td>No inhibition (+)</td>
<td>No inhibition (++)</td>
</tr>
</tbody>
</table>

3.6. Haemolytic assay

This assay was carried out to observe the potential of 125, 250, 500 and 1000 µg/ml of synthetic Pn-AMP1 to rupture mammalian red blood cells. Results are reported as the percentage of haemolysis (Figure 4). Only at the highest concentration tested (1000 µg/ml), Pn-AMP1 resulted haemolytic with 18% haemolysis measured. For 125, 250 and 500 µg/ml, the percentage of haemolysis calculated was 2.3%, 2.7% and 9.3%, respectively.
3.7. Proteolytic assay

Pn-AMP1’s ability to be digested by the gut proteolytic enzyme α-chymotrypsin was evaluated at three different molar ratios of peptide:enzyme (60:1, 250:1 and 2500:1). Pn-AMP1 resulted in being digested by all the three concentrations of α-chymotrypsin used as K. lactis growth was always recorded. The protease fully degraded Pn-AMP1 and its antiyeast activity was eliminated even at the MIC and double the MIC levels (500 and 1000 µg/ml) (data not shown).

3.8. Synthetic Pn-AMP1 application in different food matrices

Pn-AMP1 was applied in two different food matrices at concentrations ranging from 62.5 to 500 µg/ml. The peptide’s antiyeast activity against K. lactis (10^2 CFU/ml) was tested in UHT milk for 4 h at 30 °C. An observable inhibition in yeast growth was recorded in all Pn-AMP1 concentrations tested compared to yeast in the control sample that grew to reach 3.3 x 10^3 CFU/ml (Figure 5.A). No differences were noticed amongst the different peptide concentrations, and no growth was observed in the control consisting of milk alone. Pn-AMP1 was tested in a microtiter plate against the Z. bailii using Fanta Orange as a growth medium, and, as was observed in the milk samples, every concentration examined caused yeast inhibition compared to the control (Figure 5.B). Spreading the content of each well on agar plates confirmed that yeast cells were still present in the 62.5 µg/ml sample; however, complete
inhibition was recorded in the 125, 250 and 500 µg/ml samples. The reduction of different yeast strains in the different food/beverage matrices can be considered a proof of concept for applying synthetic Pn-AMP1 as a potential food preservative.

Figure 5. Application of Pn-AMP1 in different food matrices. (A) Antiyeast activity is recorded for all the peptide concentrations in this growth curve assay in Fanta orange against Z. bailii and (B) K. lactis inhibition is also observed after 4 h exposure to the peptide in UHT milk. Tests were done in triplicate (bars represent standard deviation).
4. Discussion

The chemical synthesis of plant AMPs is a methodology that can be utilised to fast produce pure compounds as an alternative to the isolation of the analogous native compounds from plant tissues. In this work, the *Pharbitis nil* antimicrobial peptides Pn-AMP1 and Pn-AMP2 were chemically synthesised using the peptides' native amino acid sequences and tested for their antiyeast activity against common spoilage yeast strains. As the first step, the MIC values were determined; *K. lactis* was found to be susceptible to 400-500 µg/ml of Pn-AMP1 and 500-1000 µg/ml of Pn-AMP2. Similar results were obtained for *Z. bailii* and *S. cerevisiae* where complete inhibition was observed at 400-500 µg/ml of Pn-AMP1 and 1000 µg/ml of Pn-AMP2. Pn-AMP1 was only lethal for *D. hansenii* at 1 mg/ml concentrations, and it did not show any inhibitory activity towards *Z. rouxii*. Pn-AMP2 was not active against these two yeast strains. Considering the difference in the antiyeast activity between the two peptides, further characterisation was only conducted on Pn-AMP1. Interestingly, according to Koo *et al.* (1998), in the native form, Pn-AMP2 resulted more effective towards the yeast *S. cerevisiae* EGY48 (IC50: 8 µg/ml) compared to Pn-AMP1 (IC50: 14 µg/ml).

All the hevein-type peptides carry the potential of binding to the fungal chitin and consequently disrupting the cell wall synthesis. It has been shown that endogenous Pn-AMPs strongly bind chitin, however they exhibit considerable antifungal activity against a broad spectrum of fungi, including those that do not contain chitin in their cell walls (Sun Lee *et al.*, 2003). Specifically, Pn-AMP1 possesses the ability to penetrate into *S. cerevisiae* and *Candida albicans* pseudo-hyphae inducing actin depolarisation in the cytoskeleton, membrane disruption and leakage of cytoplasmic materials. In this case, mannans, rather than chitin, are responsible for this antiyeast mechanism of action (Koo *et al.*, 1998). Moreover, it has been revealed that the conserved disulfide linkage between Cys residues is necessary for Pn-AMP1 to display antifungal and chitin-binding activity (Sun Lee *et al.*, 2003). It can be speculated that the higher dosage required for synthetic Pn-AMPs to induce antiyeast activity compare to the native ones lies in a lack of corrected SS bonds formation or the presence of mismatched ones.

To better understand synthetic Pn-AMP1 mechanism of action a PI intake assay was performed, and the rate of membrane disruption induced by the peptide on *S. cerevisiae* cells was measured. As expected, the mechanism of yeast membrane permeabilisation was detected. Synthetic Pn-AMP1 and native Pn-AMP1 share the cationic nature of their overall net charge (+2.2) due to the presence of four positively charged Arg residues. In general, cationic AMPs
can bind and interact with the negatively charged microbial cell membrane, leading to changes in the cell electrochemical potential and thus inducing damage and the consequent permeation of larger molecules, destroying cell morphology and eventually resulting in cell death (Lei et al., 2019). Both endogenous and synthetic Pn-AMP1 can cause membrane disruption in yeast; however, the chemical synthesis might have interfered with the peptide’s natural ability to bind with specific components of the fungal cell walls.

In food products, when yeast are present at 10⁴/10⁵ cells per gram or millilitre, signs of spoilage such as carbon dioxide gas production, the appearance of slimy biomass and off-putting odours and flavours are usually noticeable (Leyva Salas et al., 2017). Since, the MIC measured on 10⁵ cells/ml yeast concentrations (following EUCAST guideline), to evaluate whether Pn-AMP1 can prevent the growth of yeast in stored food products, a second antiyeast assay was carried out using lower yeast concentrations (10³ cells/ml). The growth of all the spoilage yeasts under investigation was impacted by the presence of varying concentrations of Pn-AMP1 (25, 50, 100, 200 and 400 µg/ml). After only 3 h of exposure, 100 µg/ml of peptide was sufficient for inducing an almost complete inhibition of the yeast K. lactis, and 200 µg/ml was lethal for Z. bailii, while D. hansenii and Z. rouxii were dead at 400 µg/ml. The colony count assay confirmed the fast action of the peptide where 250 and 500 µg/ml induced a clear decrease in Z. bailii viability (10⁴ cells/ml) after only 1 hour of exposure.

Due to the proposed use of Pn-AMP1 in food preservation, it is key to study if its antiyeast activity can withstand different treatments and environments to which the peptide might be exposed. Following treatment at high temperatures, a standard process in the food industry, synthetic Pn-AMP1 activity was partially affected, causing yeast inhibition only at the highest concentration tested (1 mg/ml). Changing the pH of the growth medium did not affect the peptide; indeed, complete inhibition of K. lactis growth was always observed. Pn-AMP1 showed salt sensitivity; a loss in the antiyeast activity was recorded in the presence of salts MgCl₂ and KCl. An explanation could lie in the possible interaction between the cations present in the solution and the yeast cell membrane that could potentially shift Pn-AMP1’s overall charge and thus modify its structure (Baldauf et al., 2013), leading to the peptide’s reduced antiyeast activity.

The peptide safety in terms of human consumption was also studied; Pn-AMP1 resulted haemolytic only at the highest concentration tested (1000 µg/ml), while at the MIC level (500 µg/ml) and lower concentrations, less than 10% haemolysis was measured. This is a positive
attribute if the peptides were to be applied in food. Moreover, Pn-AMP1 was sensitive to proteolytic action even at double the MIC levels (1000 µg/ml); this is an essential feature for a preservative agent. The proteolysis of the peptide predicted its degradation after ingestion, safeguarding that the Pn-AMP1 will not be active after the gut digestion process.

Finally, applying Pn-AMP1 in different matrices showed that UHT milk and Fanta Orange were suitable food media for the peptide’s application as an antiyeast agent. In the soft drink Fanta Orange, up to 62.5 µg/ml concentration of the peptide halved the growth of the yeast Z. bailii. In UHT milk, 62.5, 125, 250, and 500 µg/ml concentrations of synthetic Pn-AMP1 reduced the growth of the yeast K. lactis. Thus, showing that Pn-AMP1’s antiyeast activity can be maintained in a complex matrix such as milk that favours the growth of lactose-fermenting species as K. lactis.

In recent years, the scientific interest around plant AMPs is increasing; these peptides have been studied and tested for their ability to inhibit the growth of different microorganisms, including food spoilage pathogens, and thus hold great promise as possible bio-preservative agents. However, the potential of these small peptides is yet to be fully understood and exploited; in general, plant AMPs have not been widely researched for their application in food preservation (Thery et al., 2019). The synthetic plant AMP called Pn-AMP1 and generated from the natural sequence of its native form represents a potential novel food preservative. This study describes a possible approach for developing new, plant-based and consumer-friendly preservatives to fight the food loss phenomenon caused by spoilage yeasts. Although the costs of chemically synthesised peptides are currently prohibitive for an industry-scale application (e.g., according to Shwaiki et al., 2021) a 40 amino acid peptide can cost as high as $1600 per gram), the synthetic production of these molecules is faster and less laborious compared to the purification of the corresponding natural form from organic sources. Additionally, high purity can be achieved, and peptides can be produced free of any contaminants. In conclusion, the approach illustrated in this work provides a proof of concept for preliminary investigations regarding the applicability of synthetic plant AMPs as novel food preservative agent in industrial settings, which may become more practicable in the future as the cost of chemical synthesis reduces.
Bibliography


Chapter 3

Extraction, purification, and aminoacidic sequence identification of a white mustard (Brassica hirta) protein (named WMS1 in this study) with antiyeast activity

The work described in this chapter has been published as part of the article “Isolation of the mustard Napin protein Allergen Sin a 1 and characterisation of its antifungal activity” in the journal “BB Reports” ISSN: 2405-5808
Abstract

Fungi and yeast strains resistant to the commercially available antifungal molecules represent a concern to human health, agricultural production and food industries. The protein-based plant immune system can be an excellent source of novel antifungal molecules. Plant antimicrobial proteins are easy to target since they are often found accumulated in tissues, and they are usually characterised by low molecular weight and amphiphilic and cationic properties. However, protein isolation from plant materials can be time-consuming and complex due to the degradation activity of secondary plant products. In this study, a simple protocol designed and optimised for the isolation of small and cationic proteins was successfully carried out on white mustard (Brassica hirta) seeds. As a result, a 14 kDa protein with activity against Saccharomyces cerevisiae was selectively extracted with ammonium sulphate precipitation and purified with only one step of chromatographic separation (cation-exchange) on an FPLC system (AKTA start). Five extraction trials were conducted, and between 0.9 and 1.5 mg of purified protein were isolated from 1 g of seeds in each trial. The pure protein product was labeled WMS1; MS and nano LC-MS-MS methods were used to identify WMS1 primary structure. In the end, a high sequence homology with the Napin mustard protein Allergen Sin a 1 was suggested.
1. Introduction

In recent years, a rapid rise of fungal (and yeast) strains resistant to the commonly used antifungal agents has been recorded; these microbes can present a worldwide threat to human health, agricultural production and food industries (Fisher et al., 2018). Alternatives to the commercially available antifungal molecules are scarce; thus, it is crucial to discover and characterise new products to fight the emergence of these troublesome microorganisms. Different approaches exist; organic materials are the traditional target for the isolation and identification of new antimicrobial molecules, however in silico tools can be used to predict and design new potential antimicrobial sequences. Lastly, characterisation studies can be done on native products purified from natural sources, on chemically synthetised molecules or on recombinant proteins expressed in a host system.

Natural antifungal compounds are present in many animal, plant and bacterial organisms that have evolved to produce biologically active substances to respond to pathogenic environmental fungi. Kingdom Plantae is an excellent source of biologically active antimicrobial proteins and peptides showing divergent genetic origins and structures; however, they are generally characterised by low molecular weight and amphiphilic and cationic properties (Lei et al., 2019). These molecules mainly belong to the plant innate immunity system, and they are often found accumulated in different plant tissues; therefore, they represent an ideal target for the investigation of new antifungal and antiyeast compounds (Barashkova and Rogozhin, 2020). However, the isolation of these molecules from vegetable materials can be complex. Protocols for plant protein isolation must be developed considering the presence of rigid cellulose cell walls and vacuoles containing secondary plant products, organic acids and proteinases; specific extraction methods are essential to protect proteins from the degradation activity of components leaked after the cell wall disruption and the consequent vacuole breakage (Laing and Christeller, 2004).

The plant family Brassicaceae (or Cruciferae) includes valuable crops such as cabbage, broccoli, cauliflower, kale, Brussels sprouts, turnip, rocket salad, watercress, radish, horseradish, wasabi, rapeseed and white, Indian and black mustard. Several of these plants have been investigated for their antimicrobial, antioxidant and anticancer properties (Favela-González et al., 2020). In particular, antifungal proteins and peptides have been discovered in the seeds of radish (Terras et al., 1992), broccoli (Thery et al., 2020) and rapeseed (Terras et al., 1993).
The main objective of this study is to isolate pure novel antiyeast protein product form edible white mustard (Brassica hirta or Sinapis alba) seeds, since this type of molecules could have great biotechnological potential. In this chapter, a protocol specifically designed to extract small, amphiphilic and cationic proteins with potential antimicrobial activity was successfully applied to white seeds. In addition, the antifungal activity of the purified protein against the yeast Saccharomyces cerevisiae and its primary structure were investigated.

2. Material and Methods

2.1. Homogenisation, extraction and purification of the WMS1 protein

The WMS1 isolation protocol, described in Figure 1, was designed following the indications by (Koo et al., 1998) with few significant modifications. Major modifications in the protocol concern centrifugations (force and time), amount of water used in the heat denaturation step and number and type of chromatographic separation protocol used. Following the protocol optimisation, a total of five WMS1 protein isolation trials were carried out.

![Figure 1. Flow chart of the main steps in WMS1 isolation protocol: from the plant seeds to the purified protein.](image)
2.1.1. Homogenisation and extraction

Dry white mustard seeds (Fruit Hill Farm/Veyranno Ltd T/A, Bantry, Co. Cork, Ireland) were homogenated with the help of a coffee grinder (Blade KG49, DeLonghi, Italy), seeds were milled for circa 30 seconds. The resulting flour was resuspended with a 1:10 ratio in cold buffer composed of 15 mM Na₂HPO₄, 10 mM NaH₂PO₄, 100 mM KCl, 1 mM EDTA and 1 mM thiourea dissolved in dH₂O. All chemicals in this chapter were purchased from Sigma Aldrich (MO, USA) unless stated otherwise. The sample was stirred for 3 h at 4°C; subsequently, solid materials were removed by centrifugation for 45 min at 7000 x g (CL30R, Thermo Scientific, MA, USA). The resulting supernatant was passed through sterile Whatman filter paper Grade 595 (GE Healthcare, Uppsala, Sweden). Solid ammonium sulphate (NH₄)₂SO₄ was next added to the solution until 30% of relative saturation, and the sample was stirred at 4°C. After 24 h, the resulting precipitate was removed by centrifugation for 45 min at 7000 x g. The supernatant was adjusted with solid ammonium sulphate to reach 70% of relative saturation, and the final precipitate formed overnight at 4°C under gentle stirring was collected by centrifugation (45 min at 7000 x g) and re-dissolved in dH₂O. The quantity of water to be used would depend on the initial quantity of seed flour; for example, if the trial started with 50 g of seeds flour, at this step, 75 ml of water will be used. The solution was heated in a water bath (Clifton range, Nickel-Electro Ltd., North Somerset, UK) at 80°C for 15 min to denature proteins. Heat-denatured protein precipitates were removed by centrifugation for 45 min at 7000 x g. The supernatant was extensively dialysed against distilled water in dialysis tubing with a molecular mass cut-off of 2,000 Da (Sigma-Aldrich, MO, USA). After three days, the proteins and peptides solution obtained was clarified once again through a sterile Whatman filter paper Grade 595 and collected. The extraction steps are summarised in Table 1.
2.1.2. Purification

The WMS1 purification was achieved after one cycle of cation-exchange chromatography in an ÄKTA-start system (GE Healthcare, Uppsala, Sweden). The description of the basics of the methods is illustrated in Figure 2. The peptides and proteins solution was adjusted to 10 mM phosphate buffer (pH 6.0) and 25 mM NaCl and loaded on a HiPrep SP HP cation-exchange column (GE Healthcare, Uppsala, Sweden). Fractions were eluted with a linear gradient of 0–100% buffer B (10 mM PBS and 520 mM NaCl, pH 6) in buffer A (10 mM PBS and 25 mM NaCl, pH 6) at a flow rate of 5 ml/min within 60 min. Salt was removed from the collected fraction by dialysis on Slide-A-Lyzer MINI Devices with MWCO of 3.5 kDa (Thermo Scientific, MA, USA).
Figure 2. Description of the method ion exchange chromatography on the ÄKTA-start system. The first step of an ion exchange process regards loading the proteins-peptide extract sample onto the column. The sample is sucked into the system through a sample wire (1) and, with the help of a pump (2), is applied to the column (3). The column features an ion charged resin that attracts only proteins with positive or negative net surface charge (in the case of cation exchange, the resin is negatively charged, and the attracted proteins are positive charged), unbounded proteins are directed to the waste bottle (5). A UV monitor (4) constantly measures the absorbance of the liquid exiting the column at a set wavelength of 280 nm. The run is monitored with the aid of the software UNICORN start that generates a real-time graph (UV absorbance vs ml of liquid entering the system). Once the whole sample has been applied to the column, the second step (elution phase) begins, buffer A (6) starts being applied to the column along with a gradually increase quantity of buffer B (6). Proteins lose their affinity with the column resin depending on the quantity of NaCl salt passing through the column, and after the absorbance is read, they are eluted in the fraction collector (7).

2.2. Antiyeast test

Desalted fractions eluted from the cation exchange were tested for antiyeast activity with a growth curve assay following the "EUCAST Definitive Document EDef 7.1" guidelines. This method was performed in a flat-bottom 96-well microtiter plate (Sarsdett, Nümbrecht, Germany), mixing 100 µl of the solution under investigation with 100 µl of a 5x10^4 cells/ml yeast inoculum, prepared from an overnight culture of Saccharomyces cerevisiae DSM-70449 (DSMZ, Germany). The concentration of yeast cells was calculated using a haemocytometer (Improved Neubauer Counting Chamber, Sigma Aldrich). Positive controls contained 100 µl
of dH₂O instead of the fraction solution. The plates were incubated for 48 h at 30°C in a microtiter plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) with gentle shakes between readings. The yeast growth was monitored, measuring the optical density (600 nm) at 2 h intervals.

2.3. Quantification

Active antiyeast fractions were combined and dried in a freeze dryer (benchtop K VirTis, SP Industries, MO, USA). The resulting powder was dissolved in distilled water to reach 2 mg/ml concentration, filter-sterilised (pore size: 45 µm) and stored at -20°C for further analysis. The protein concentration was determined by a BCA (Bicinchoninic Acid) assay for total protein quantification (QuantiPro BCA Assay Kit, Sigma Aldrich, MO, USA). The specific of the method are described in Figure 3.

Figure 3. Description of the method BCA (Bicinchoninic Acid) assay for total protein quantification. In the figure, ten Eppendorf are displayed; seven standards are composed by increasing known albumin protein concentration (from 0 to 30 µg/ml), and the three samples are composed with unknown and serial diluted protein concentrations prepared from antiyeast protein powder collected after the freeze-dryer step. The principle of this colourimetric method is that proteins can reduce copper salt from Cu²⁺ to Cu¹⁺ in an alkaline solution; the amount of Cu²⁺ that is reduced is proportional to the amount of protein present in the solution and result in a purple colour (with strong absorbance at 562 nm) formation by bicinchoninic acid.

2.4. Gel electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) assay was performed according to Laemmli to verify the purity of the isolated protein sample. This method allows protein separation by mass. Protein samples were prepared in a dilution 1:1 with
2x SDS gel loading buffer (in absence of a reducing agent) and boiled for 10 min at 95°C (Applied Biosystems Thermal Cycler, Thermo Scientific, MA, USA). Proteins samples so prepared were loaded at the top of a polyacrylamide gel matrix sandwiched between two glasses. The SDS role is to unfold proteins and eliminate the influence of structure and charge, so when a constant electric field is applied to the gel, the proteins migrate towards the anode at different speeds, solely depending on their molecular weight. E.g. smaller proteins migrate faster due to less resistance from the gel matrix. The gel was prepared using TGX FastCast Acrylamide Starter Kit, 12% (Bio-Rad, Hercules, CA, USA) and following manufacturer's instructions. After the run, protein bands were stained with EZBlue Gel Staining Reagent. One gel was prepared loading six samples collected in different isolation-process steps: after cold buffer lysis, after salting-in with 30% (NH₄)₂SO₄, after salting-out with (NH₄)₂SO₄, after heat denaturation, after dialysis and after the final cation-exchange. The second gel was prepared loading purified WMS1 protein samples isolated from the five different isolation trials.

2.5. Aminoacidic sequence identification

The aminoacidic chain sequencing of the WMS1 protein was carried out commercially using two different strategies. Specifically, SDS-gel bands were submitted to Proteome Factory AG (Berlin, Germany) for a “protein identification by Mass Spectronomy” and to Creative Proteomics (New York, USA) for a “de novo protein sequencing analysis”. In the first approach, gel bands were digested by Trypsin proteolytic enzyme. Trypsin cleaves at C-term of arginine and lysine residues and the peptides generated were identified by Mass Spectrometry. The list of the experimental peptide mass data obtained was compared to the theoretical peptide mass list for each protein in the Viridiplantae (Green Plants) NCBInr database using MASCOT software. In the second approach, gel bands were digested by six different enzymes: Trypsin, Chymotrypsin, Glu-C, Arg-C, Lys-N, and Pepsin. Each protease has different specific cleavage sites. The aminoacidic sequence of each peptide fragment is determinate by nanoscale Liquid Chromatography coupled to tandem Mass Spectrometry (nano LC-MS/MS); this technique combines the separating power of LC with the highly sensitive and selective mass analysis capability of MS/MS. Data from peptide mapping analysis are explored with the software PEAKS STUDIO Desktop Version 8.5. to identify the whole protein sequence. Conclusively, the WMS1 primary structure was subjected to further BLASTp search on the NCBI database to determine the percentage of identity with existing sequences.
3. Results

3.1. Homogenisation, extraction and purification of the WMS1 protein

The whole isolation protocol was performed five times; trials 1, 2, 3 and 4 were carried out on 50 g of starting amount of seed flour, while Trial 5 was done with 100 g. After the cold buffer extraction of mustard seeds, the proteins and peptides solution obtained was purified on an FCLP system (ÄKTA-start) using one cycle of cation-exchange chromatography. Chromatograms for the five trials can be found in Figures 4 and 5. The five chromatograms produced share many similarities, especially regarding eluted peaks, meaning that the proteins and peptides solution loaded into the column had a similar composition throughout the five different trials.

![Chromatogram of one cycle of cation exchange on an ÄKTA-start system (Trial 1).](image)

Figure 4. Chromatogram of one cycle of cation exchange on an ÄKTA-start system (Trial 1). The chromatogram was generated by the program UNICORN start; in the ordinate axis is the absorbance unit for the liquid exiting the column and on the abscissa axis is the quantity (ml) of liquid exiting column (system flow). 70 ml of the clarified dialyzed sample were loaded on the HiPrep SP HP column. The first peak (1) represents the negative charged
unbounded proteins. When the totality of the sample was loaded onto the column, the elution step started with buffer B (pink line) being gradually applied to the column. Consequently, proteins with different net surface charges are released at different times (peak 2, 3 and 4) and collected as eluted fractions (in red).

**Figure 5.** Chromatograms of Trials 2, 3, 4 and 5. The chromatograms in this figure are produced uniformly with the chromatogram of Trial 1 in Figure 4. In black circles are highlighted the fractions that showed antiyeast activity. The similarity proves the constancy of the purification among all the chromatograms; the antiyeast fractions are always detected in the last peak, and their elution starting and ending point occurs between 45% and 99% gradient concentration of buffer B. In Trial 5, 150 ml of peptides and proteins solution was loaded into the column compared to 75 ml of Trials 1, 2, 3 and 4.
3.2. Antiyeast test

Following the elution, fractions and unbounded protein solution were desalted and tested for antiyeast activity with a growth curve assay against the yeast *S. cerevisiae*. Relevant results for the products obtained from Trial 1 are shown in Figure 6. Yeast cells died when exposed to samples collected from fractions T16 to T23, while yeast growth can be observed when the cells are exposed to other cation exchange products. Antiyeast fractions with comparable characteristics were detected as well in Trials 2, 3, 4 and 5.

![S. cerevisiae growth graph](image)

Figure 6. Antiyeast test on cation exchange products of Trial 1 (Figure 4). The graph is expressed as Absorbance 600 nm vs time (h). Lines represent the growth of yeast cells inoculated in microtiter plate wells with products of the cation exchange or dH2O for control (Yeast). The unbounded proteins sample was taken from peak 1; peak 2 sample was taken from fraction T3, peak 3 from fraction T7, peak 4 from fraction T18. After 48 h, the yeast cells were dead only in well with peak 4 sample. All the fractions were tested (data not shown), and fraction between T16 and T23 showed antiyeast activity. Each fraction was tested in duplicate (bars represent standard deviation).

3.3. Quantification

Active fractions were polled together, freeze-dried and resuspended in dH2O. The total amount of WMS1 protein isolated was quantified with the coulometric BCA assay. As a result, 75 mg of protein were recovered from Trial 1, 60 mg for Trial 2 and 3, 55 mg for Trial 4 and 90 mg for Trial 5. Results are summarised in Table 2.
Table 2. Summary of the results regarding the extraction yield for the WMS1 protein obtained in the five trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Quantity of seed flour at start point</th>
<th>Total of antiyeast protein extracted</th>
<th>mg of protein / g of seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 g</td>
<td>~75 mg</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>50 g</td>
<td>~60 mg</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>50 g</td>
<td>~60 mg</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>50 g</td>
<td>~55 mg</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>100 g</td>
<td>~90 mg</td>
<td>0.9</td>
</tr>
</tbody>
</table>

3.4. Gel electrophoresis

Two SDS-PAGE gels were prepared to confirm that the isolation process has been carried out correctly. One gel (Figure 7) was prepared with six samples taken from each of the six different extraction and purification steps. During the run, samples migrate on their respective lanes and proteins with different MW separate making it possible to estimate if one sample contains one or more types of protein. As expected, only the sample collected after the chromatographic purification presented one protein band, meaning that the protein sample was pure. A second gel (Figure 8) was prepared with five samples containing the final purified protein isolated from the five different trials. All the gel bands were formed in the same position (around 14 kDa); thus, it can be assumed that the protein isolated was always the same throughout the five trials.
Figure 7. SDS-PAGE gel with samples collected from the six steps of the isolation process. Lanes from 1 to 6 correspond to the six progressive steps of the protein isolation procedure; on lane 7 a protein ladder was loaded to improve the visual comparison. 6: sample collected after cold buffer lysis, 5: after salting-in with 30% (NH₄)₂SO₄, 4: after salting-out with (NH₄)₂SO₄, 3: after heat denaturation, 2: after dialysis, 1: after cation-exchange. Sample in lane 1 produced only one visible band and it contains only one type of protein (15–14 kDa), while sample 2 and 3 produced faded bands (highlighted in the circles).

Figure 8. SDS-PAGE gel with purified samples from the five trials. The five samples loaded are from the five different isolation trials (lanes 2, 3, 4, 5 and 6); on both sides (lanes 1 and 7), a protein ladder was loaded to improve the visual comparison. Protein bands are visible in the same position; thus, the constancy of the isolation process is confirmed.
3.5. Amino acid sequence identification

The protein identification by Mass Spectrometry performed by the Berliner company consists of three main steps: in gel Trypsin digestion of the WMS1 protein samples, acquisition of the mass spectrum of the peptide mixture by Mass Spectrometry, search against the green plant subset of NCBIInr with the Mascot database. The WMS1 experimental peptide mass list matched 64% of the aminoacidic sequence of the protein “Allergen Sin a 1 1.0108” (NCBI accession number: CAA62908.1) (Figure 9). The USA company's de novo protein sequencing analysis successfully identified the whole primary structure sequence of the WMS1 protein (Figure 10). The gel sample bands were first hydrolysed to small peptides using six different proteases, and LC-MS/MS mapped the peptides. In this case, the protein identification did not rely on the available databases to match experimental Mass Spectrometry data; instead, the peaks in the MS/MS spectrum were analysed with a software package called PEAKS that extracted information on the best amino acid sequence for each peptide with confidence scores for the entire protein sequence. The sequence obtained was submitted to a BLASTp analysis (data not shown), and it resulted in having 96% sequence identity with the protein Allergen Sin a 1 1.0106 (NCBI accession number: CAA62911.1). In the light of these results, it can be concluded that the protein WMS1 isolated from white mustard seeds shares a high similarity in the primary structure with the protein Allergen Sin a 1.

**Figure 9.** Protein identification by Mass Spectrometry results. 64% of the aminoacidic sequence of the protein Allergen Sin a 1 matched the peptides obtained from the WMS1 protein sample. Matched peptides are shown in red.

**Figure 10.** WMS1 de novo sequencing results. The amino acid sequence of the protein WMS1 proposed after the de novo sequence analysis. The sequence possesses a 96% identity with the protein Allergen Sin a 1 sequence.

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PAGPFGIPKC RKEFQQAAQLH RACQQWHLKQ AMQSGSGPSW TLDDEFDFED
DMENPQQGQQK PPLLQQCCCN ELHQEEPLCV CPTLGASKA VKQQVRQQLG
QQGQQGPQQVQ HVISRIYQTA THLPKVCNIPQ VSVCPFKKT MPGPS
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PAGPFGIPKC RKEFQQAAQLH RACQQWHLKQ AMQSGSGPSL ALYGEFEDDM
ENPQQGQQRP PLLQQCCNEL HQEELCVCP TLKGASKAVK QQVRQQLGQQ
GQQGPQVQHV ISRIYQTA THEPKVCNQPV SVCPFKKTMP GPS
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4. Discussion

Resistant fungal strains represent a rising concern in human health, food industries and agriculture. Therefore, there is a need to discover new antifungal molecules to be used as alternatives to the current commercially available antifungal drugs, preservatives and fungicides. This work focused on investigating the antimicrobial activity of mustard (*B. hirta*) seeds. Mustard seeds are known for their antimicrobial properties; however, there is a lack of studies on purified mustard protein's antimicrobial activity (Rahman *et al.*, 2020).

A protocol designed to target small, cationic and amphiphilic proteins with potential antimicrobial activity was applied on white mustard seeds; a ~14 KDa protein with activity against *S. cerevisiae* was isolated and called WMS1. Afterwards, WMS1 primary sequence was identified (MS and nano LC-MS-MS), and high sequence homology with the mustard protein *Allergen Sin a 1* was suggested. After all the protein isolation protocol steps, an average amount of 60 mg of antifungal protein was obtained from 50 g of dry seeds, specifically between 0.9–1.5 mg of purified WMS1 were isolated from 1 g of seeds in each trial. This data is in line with the amount of *Sin a 1* contained in white mustard seeds, where the protein/seed ratio was measured to be 0.82–2.94 mg/g depending on the type of mustard line (Shim and Wanasundara, 2008). The total protein quantity present in the whole *B. hirta* seed is around 30% (Wanasundara *et al.*, 2012); consequently, the yield of extraction for WMS1 was between 0.3–0.5%, while for *Sin a 1* is 0.4–0.7%. The protein *Sin a 1* is Storage Seeds Protein (SSPs) belonging to the 2S albumin family; it is mainly studied for its allergenic properties (Menéndez-Arias *et al.*, 1988), and it has never been reported for antimicrobial activity.

Although the allergenicity of *Sin a 1* could negatively impact the biotechnological potential of the antiyeast protein WMS1, these types of small antimicrobial proteins normally hold great promise as possible novel antifungal agents. For centuries, mustard seeds have been incorporated in foods as additive for expanding products’ shelf-life (Rahman *et al.*, 2020); thus, WMS1 could be employed as novel preservatives. WMS1 could also be applied to industrial cleaning supplies or paints to prevent fungal growth on a manufacturing floor or to prevent any mould growth on manufacturing building's walls. Restricting it to industrial use will limit its concern as an allergen as only select people will be in contact with the protein. Moreover, it could be employed in the agricultural sector as antifungal protection agent for growing crops.
In general, more studies are necessary to 1) investigate the relationship between Allergen Sin a 1 and WMS1 and 2) characterise the antimicrobial potential of WMS1.

Isolating antimicrobial peptides or small proteins from plant material generally require efficient and sophisticated techniques; well established methodologies currently employed for the isolation of plant antimicrobial peptides normally include: extraction in a buffer solution at low temperatures, salt precipitation methods and a combination of ion-exchange and Reverse-phase high-performance liquid chromatography (RP-HPLC) (Cilia et al., 2009). The RP-HPLC technique is a commonly used option for the purification of these type of molecules since they are often resistant to the various organic solvents usually used as mobile phases (Daneshmand et al., 2013). However, achieving cost- and time-effective procedures for the isolation of these type of molecules remain a major challenge. Remarkably, the protocol presented in this chapter included only one step of chromatographic separation (ion-exchange) which reduced the time, cost and machinery normally utilised in these isolation practices; thus, it could be used for the isolation of further novel antimicrobial molecules from different Brassicaceae seeds.
Bibliography


Chapter 4

Characterisation of WMS1 structure and its relationship with the protein *Allergen Sin a 1: in silico and in vitro tests*

The work described in this chapter has been published as part of the article “Isolation of the mustard Napin protein *Allergen Sin a 1* and characterisation of its antifungal activity” in the journal “BB Reports” ISSN: 2405-5808
**Abstract**

WMS1 is a protein previously isolated from white mustard seeds, it displayed antiyeast activity, and its primary structure was identified in Chapter 3. This work investigates the relationship between the purified protein product labelled WMS1 and the mustard Napin protein *Allergen Sin a 1*. The study has been conducted firstly analyzing WMS1 amino acid sequence with bioinformatic *de novo* tools, this was followed by establishing homology relationships among WMS1 and other protein sequences present in online databases, and finally, experimental data on WMS1 structure and stability were collected to corroborate the information gathered by bioinformatics analysis. Models computed with *de novo* and homology tools predicted a polypeptide structure organized in two chains, rich in α-helices structures and characterised by eight cysteine residues forming four S-S bonds in positions C1-C5, C2-C3, C4-C7 and C6-C8. Laboratory data confirmed the presence of two chains and a stable structure resistant to proteolytic digestion and thermal degradation. All the data collected on WMS1 matched literature data on *Allergen Sin a 1*; specifically, the isoform *Allergen Sin a 1.0106* resulted in the closest homologous relative of WMS1. No differences were detected in the amino acid sequence of two bio-functional chains between WMS1 and *Allergen Sin a 1.0106*. In light of these results, it has been concluded that WMS1 is almost certainly an isoform of the protein *Allergen Sin a 1*. 
1. Introduction

Proteins are the most versatile macromolecules in living systems; they not only compose structural and motor elements in the cells, but they also serve as the catalysts for virtually every biochemical reaction that occurs. This astoundingly diverse range of biological functions derives from each protein’s unique linear amino acid sequence that determines the final folded shape and the conformation of each protein. The investigation of a protein’s three-dimensional structure is fundamental in the understanding of a protein’s natural functions (Grunert and Labudde, 2015).

A variety of methodologies are available to characterise a protein structure from its amino acid sequence; in this work, bioinformatics techniques (in silico tests) and laboratory experimental data (in vitro tests) were employed to study the structure of the antiyeast protein WMS1. The description of WMS1 isolation from white mustard (Brassica hirta) and its amino acid sequence identification is illustrated in Chapter 3. Moreover, protein identification reports have suggested a high sequence similarity between the protein WMS1 and the protein Allergen Sin a 1. Allergen Sin a 1 is a well-characterised Seed Storage Albumin. It belongs to the Napin/Bra allergen family (Shewry et al., 1995), and although several Napin proteins have shown antibacterial and antifungal activity (Nioi et al., 2012), Allergen Sin a 1 antimicrobial potential has never been reported.

Aims of this work are to characterise the secondary and tertiary structure of the antiyeast protein WMS1 and to investigate the degree of homology with the Allergen Sin a 1 protein and with other antimicrobial Napin proteins. The investigation has been conducted firstly analyzing WMS1 amino acid sequence with bioinformatic de novo tools, this was followed by establishing homology relationships among WMS1 and other protein sequences present in online databases and finally experimental data on WMS1 structure and stability were collected to corroborate the information gathered by bioinformatics analysis.
2. Material and Methods

2.1. Bioinformatic (in silico) tests

Bioinformatics (or in silico) tools were used to investigate the WMS1 structure from its amino acid sequence. Depending on whether similar proteins have been experimentally solved, protein structure prediction methods can be grouped into two categories. If proteins with similar structures are identified from the Protein Data Bank (PDB) library (https://www.rcsb.org/pages/about-us/index), the target model can be constructed by copying and refining the framework of the solved proteins (templates). To avoid confusion, in this work, this procedure is called "homology modelling". If protein templates are not available, the protein 3D models must be built by de novo (or ab initio) protein modelling.

2.1.1. De novo (or ab initio) protein modelling

De novo approaches were used to calculate physiochemical properties of WMS1, to predict WMS1 secondary structures and to build de novo WMS1 three-dimensional models.

Approaches used to calculate physiochemical properties of WMS1

The amino acid sequence of WMS1 was used to calculate the theoretical physicochemical properties of the protein. Each amino acid possesses well-known quantitative physicochemical properties such as size, charge and hydropathy, and therefore, similarities and differences in these properties can be measured and indexed. The WMS1 amino acid composition, theoretical hydropathy index, aliphatic index, protein charge, molecular weight and isoelectric point were investigated with the tool R (package "peptide"). R is a free software environment for statistical computing and graphics (RStudio Team, 2020). WMS1 hydrophobicity was further investigated on the free online tool Expasy's ProtScale (https://web.expasy.org/protscale/), it allowed to compute and represent (in the form of a two-dimensional plot) the profile produced by any amino acid scale on the selected protein (Khodadad et al., 2020). An amino acid scale is defined by a numerical value assigned to each type of amino acid. WMS1 hydrophobicity was calculated using hydrophobicity scales KytoDoolittle and HoppWoods.
Approaches used to predict WMS1 secondary structure

The secondary structure of a protein refers to a folded structure resulting from polypeptide interactions between atoms on the backbone of a polypeptide chain, most common secondary structures are α-helices and β-strands. The WMS1 secondary structure prediction was carried out with the help of three different programs: Predict Protein, PSIPRED and PHD. These tools work on the same principle: examine a protein's sequence and align, compare, and predict a protein's structure based on current protein databases (Kashani-Amin et al., 2019). Predict Protein (https://predictprotein.org/) is a server that creates alignments with a query protein and predicts segments of a protein's function and structure by searching through various current databases. PSIPRED (PSI-blast based secondary structure prediction) is a protein secondary structure prediction server created using machine learning algorithms. Its algorithm is divided into three stages, generating a sequence profile, initial secondary structure prediction and prediction structure filtering. PHD servers (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_phd.html) predicted WMS1 secondary structure, residue solvent accessibility and helical transmembrane regions. The results are presented as percentages rather than amino acid ranges like the PSIPRED and Predict Protein.

Approach used to build de novo 3D model for WMS1

The overall three-dimensional arrangement of a protein's polypeptide chain in space is referred to as the tertiary structure. The overall WMS1 three-dimensional structure (tertiary structure) was predicted with Robetta (https://robetta.bakerlab.org/submit.php). Robetta is an automated structure prediction and analysis tool that has been created and maintained by Baker Lab (Park et al., 2018). It can predict a protein's tertiary structure using de novo approaches. The predicted 3D models were downloaded as a Protein Data Bank (pdb) file format; this type of file provides for description and annotation of protein and nucleic acid structures including atomic coordinates, secondary structure assignments, as well as atomic connectivity. The open-source Pymol (https://pymol.org/2/) was used to visualise and align the predicted 3D structures of WMS1 protein.

2.1.2 Homology protein modelling of WMS1

Homology modelling, also known as comparative modelling, refers to the set of bioinformatic tests aimed at constructing the three-dimensional structure of a target protein from the
experimental three-dimensional structure of related homologous proteins. Two proteins are homologous if they share a common ancestor, and they may share structures, functions or sequences.

**Approaches used for determining WMS1 homologues**

Since evolutionarily related proteins have similar sequences and naturally occurring homologous proteins have similar protein structure, the first step is to identify WMS1 family and homologous proteins. Firstly, the WMS1 amino acid sequence was submitted into NCBI Conserved Domains IT program (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to locate conserved domains and therefore identify the family to which WMS1 belongs (Blum et al., 2021). A conserved domain is a recurrence of components within protein sequences as sustained blocks of amino acid residues with specific functions; the search was done on conserved domain databases in Interpro (https://www.ebi.ac.uk/interpro/) and NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Afterwards, the WMS1 amino acid sequence was investigated with homology identification tools. In this type of analysis, the protein sequence is compared to other known sequences searching for similarities. Homology occurs when two sequences or structures share more similarity than would be expected by chance; when excess similarity is observed, the simplest explanation for that excess is that the two sequences did not arise independently; they arose from a common ancestor. Determining homologues of a protein involves employing the database NCBI BLASTp. BLASTp (Basic Local Alignment Search Tool for proteins) is a tool that compares a protein's primary sequence to a database of amino acid sequences and identifies sequences that resemble the query sequence submitted. BLASTp displays the percentage similarity between the sequences and aligns the protein's respective amino acid sequences belongs (Blum et al., 2021). Data received from BLASTp were conveyed on R, and R was used to create dot-plots and a phylogenetic tree. Dot-plots visualise similarities or differences between two protein or nucleic acid sequences. Ten dot-plots were created to illustrate the similarity between WMS1 and some of the hits received from BLASTp. Phylogenetic trees contain information regarding the inferred evolutionary relationships between different sets of organisms; a distance-based phylogenetic tree was constructed using R (packages required MSA, seqinr and ape). The amino acid sequences of the closest related protein to WMS1 were downloaded in FASTA format from the BLASTp hits received and aligned with the help of the online tool Clustal Omega – Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/). This software builds a
multiple sequence alignment from a series of pairwise alignments (Khodadad et al., 2020); the protein sequences are analysed as a whole, a distance matrix is generated, and sequences are aligned by similarity. These steps are carried out automatically by the software, and the results are expressed with output symbols. In particular, the asterisk (*) means that the amino acid position has a single and fully conserved residue, the colon (:) means that the position presents conservation between groups of strongly similar properties, and the period (.) means that the position has conservation between groups of weakly similar properties. Moreover, this tool was used to visualise the position of Cys residues in WMS1 and its homologous.

**Approach used to build homology 3D model for WMS1**

Once the closest relative to WMS1 had been identified, the homology modelling software SWISS-MODEL was used to create a possible WMS1 3D model and evaluate its quality. The final structure was visualised with the open-source PyMol.

### 2.2. In vitro tests

Experimental data on WMS1 structure were produced after reducing the protein with dithiothreitol (DTT) and testing the protein for resistance to proteolytic digestion and heat denaturation.

#### 2.2.1. WMS1 stability to heat and proteolysis

Purified protein samples were subjected to heat treatment or to digestion with α-chymotrypsin to assess if WMS1 possesses resistance to proteolysis activity and heat denaturation. All the chemicals used in this work were purchased from Sigma (St Louis, MO, USA) unless stated otherwise. The WMS1 sample subjected to heat treatment was heated for 15 min at 100°C and left to cool for 30 min. WMS1 samples were digested following the protocol of Shwaiki et al. (2020); briefly, samples were incubated at 37°C in conjunction with α-chymotrypsin at different WMS1:enzyme molar ratios of 60:1, 250:1, 2500:1. After 4 h, the α-chymotrypsin was inactivated by heat at 80°C for 10 min. The α-chymotrypsin was stored in solution in a digestion buffer consisting of 50 mM Tris–HCl (pH 7.4) and 5 mM CaCl2. Afterwards, native and treated samples were used for two different tests: SDS-PAGE (as described in Chapter 3 section 2.4) and an antiyeast growth curve test against the strain *Zygosaccharomyces bailii* (as
described in Chapter 3 section 2.2). These tests aim to evaluate if WMS1 structure and properties are affected by heat and proteolytic digestion.

2.2.2. Dithiothreitol (DTT) reduction assays

Dithiothreitol (DTT) is a potent redox agent also known as Cleland's reagent, and it was used to break down WMS1 intra- and inter-molecular disulfide bonds between Cys residues. Briefly, reduced WMS1 samples were produced by adding 100 mM DTT to the 2x SDS buffer and a SDS gel run was set up with reduced and nonreduced WMS1 samples as described in Chapter 3. This technique is used to verify if a protein is composed of one or more chains; whenever a protein with multiple chains held together by disulfide bridges is reduced, it will appear on the SDS gel as multiple bands since polypeptide subunits will be separated according to their molecular weight.

2.3. Literature search

A literature search on known and described characteristics and properties regarding the protein Allergen Sin a 1 was conducted using PubMed (https://pubmed.ncbi.nlm.nih.gov/).
3. Results

3.1. Bioinformatics \textit{(in silico)} tests

3.1.1. \textit{De novo} (or \textit{ab initio}) protein modelling of WMS1

\textit{Physiochemical properties of WMS1}

In total, WMS1 is composed of 143 amino acid residues and it contains all the twenty natural amino acids at varying amounts (Table 1). Glu was the most prevalent (25 times or 17.5%), this is an essential amino acid; it can produce a site for attachment for glutamine binding sites and stabilise a protein's structure through the formation of hydrogen bonds. Over 50% of WMS1 was composed of only six different amino acids, and these were glutamine, proline, glycine, leucine, lysine and alanine. Besides structure, glutamine and proline in high concentrations have been reported to be responsible for a protein to develop allergenic properties. Another amino acid that is present in relative amounts (8 times or 5.5%) in WMS1 is Cys. Cys is a sulfur-containing amino acid; it is necessary to form a disulphide (S-S) bond or bridge, a stable and covalent interaction between two Cys residues. S-S bonds are essential building blocks in proteins' secondary and tertiary structures, acting as inter-and intra-subunit cross-links.

Each amino acid contains a unique side chain (R group) which determine each amino acid's physical and chemical characteristics. WSM1 physical and chemical parameters such as aliphatic index, protein charge, molecular weight, isoelectric point and polarity were calculated based on amino acid composition (Table 2). The isoelectric point of a protein is defined as the pH at which no net migration occurs in an electric field and an isoelectric point of approximately 8.50 was measured for WMS1. Hence, WMS1 carries a positive charge below this pH, and above this pH, WMS1 possesses a negative charge. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains. WMS1 achieved an aliphatic index of 62.72, which indicates that the protein is thermally stable and contains a significant amount of hydrophobic amino acids. The molecular weight of WSM1 was calculated to be 15.89 kDa. Regarding polarity, the chemical type of a protein or a particular region of a protein can be determined by knowing if the amino acid side chains possess hydrophobic or hydrophilic properties. The hydrophobic or hydrophilic character of a protein is referred to as hydropathy. WSM1 is composed of almost 50% non-polar or hydrophobic amino acids and 50% polar or hydrophilic amino acids. This result seems to indicate that...
WSM1 is possibly amphipathic, with alternating hydrophilic and hydrophobic amino acid residues. For more accurate results, WMS1 hydropathy index of WSM1 was calculated using two different methods: Kyte & Doolittle and Hopp-Woods. The Kyte & Doolittle scale measured hydrophobic amino acids and the plot showed approximately eight amino acid groups recorded a score above 0 (Figure 1. a); however, the majority of the amino acids were below the origin, indicating WMS1 overall protein sequence to be hydrophilic. The hydropathy index value obtained was 0.701. Similar results were obtained with the Hopp-Wood scale which measured hydrophilic amino acids (Figure 1. b), seven hydrophobic groups/segments of amino acid residues were recorded, but most of the plot remained in the hydrophilic region and a hydropathy value of -0.002 was calculated. Additionally, some segments or groups of hydrophobic amino acids nearly stretched to 20 amino acids in length (Figure 1. b), providing some insight into the possible secondary structure of the protein. If a stretch of about 20 amino acids shows positive for hydrophobicity, these amino acids may be part of an α-helix spanning across a lipid bilayer composed of hydrophobic fatty acids. On the other hand, for stretches of amino acids that fall within the hydrophilic portion of the graph, these segments are often in contact with water molecules and, therefore, possibly reside on the outer surface of the protein. To conclude, WMS1 has an overall amphipathic structure, and with hydrophilic residues (possibly on the outer surface of the protein) and with 7 or 8 areas recorded as hydrophobic.
Table 1. Amino acid count and percentage of WMS1. Both high Glutamine and Proline concentrations are often associated with allergenic proteins.

<table>
<thead>
<tr>
<th>Amino Acid Letter</th>
<th>Amino Acid Name</th>
<th>Count</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>Glutamine</td>
<td>25</td>
<td>17.48</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
<td>16</td>
<td>11.19</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
<td>11</td>
<td>7.69</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
<td>11</td>
<td>7.69</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
<td>9</td>
<td>6.29</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
<td>8</td>
<td>5.59</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
<td>8</td>
<td>5.59</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
<td>8</td>
<td>5.59</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic Acid</td>
<td>7</td>
<td>4.90</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
<td>7</td>
<td>4.90</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
<td>5</td>
<td>3.50</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
<td>5</td>
<td>3.50</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
<td>4</td>
<td>2.80</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
<td>4</td>
<td>2.80</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
<td>4</td>
<td>2.80</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
<td>3</td>
<td>2.10</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
<td>3</td>
<td>2.10</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
<td>2</td>
<td>1.40</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic Acid</td>
<td>2</td>
<td>1.40</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
<td>1</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Table 2. Physiochemical properties of WMS1’s amino acid sequence.

<table>
<thead>
<tr>
<th>Properties</th>
<th>No of AA residues</th>
<th>Molecular %</th>
<th>Additional Physiochemical results of WMS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiny</td>
<td>38</td>
<td>26.57</td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>67</td>
<td>46.85</td>
<td>Theoretical Net charge = 4.90</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>31</td>
<td>21.67</td>
<td>Aliphatic Index = 62.72</td>
</tr>
<tr>
<td>Aromatic</td>
<td>12</td>
<td>8.39</td>
<td>Isoelectric point (PI) = ~8.50</td>
</tr>
<tr>
<td>Non-polar</td>
<td>76</td>
<td>53.14</td>
<td>Molecular Weight = 15.89 kDa</td>
</tr>
<tr>
<td>Polar</td>
<td>67</td>
<td>46.85</td>
<td>Hydropathy Index Results</td>
</tr>
<tr>
<td>Charged</td>
<td>28</td>
<td>19.58</td>
<td>KytoDoolittle = -0.7013986</td>
</tr>
<tr>
<td>Basic</td>
<td>19</td>
<td>13.28</td>
<td>HoppWoods = -0.002097902</td>
</tr>
<tr>
<td>Acidic</td>
<td>9</td>
<td>6.29</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Hydrophobicity plots created using ProtScale. The plots have WMS1 amino acid sequence on x-axis and a) degree of hydrophobicity (Kyte & Doolittle) or b) degree of hydrophilicity (Hopp & Woods) on its y-axis. The majority of WMS1 sequence is plotted in the hydrophilic area.
Secondary structure of WMS1

The bioinformatics tools Predict Protein, PSIPRED and PHD were used to investigate WMS1 secondary structure and it was determined to be solely composed of α-helices and coils. The α-helices are a spiral conformation in which every backbone carbonyl oxygen (C=O) group forms a hydrogen bond with the backbone amide (N-H) group of the amino acid four residues ahead of it in the helix; pulling the backbone of the peptide chain into a helical or ribbon-like structure. PSIPRED predicted 55% of WMS1 to be composed of α-helices and the remainder of the structure was determined to be composed of coils (Figure 2). PSIPRED predicted a total of eight different α-helical structures between amino acids: 8-16, 21-31, 40-41, 47-50, 60-72, 75-78, 70-97 and 107-124 with varying degrees of confidence, in particular, the three smallest helices (40-41, 47-50 and 75-77) were predicted with the lowest confidence level meaning that they may have been misplaced. After removing α-helices with a low confidence level, 48% of the WMS1 sequence was predicted to be composed by α-helices. Prediction Protein tool prediction showed five different α-helices between amino acid residues; 8-31, 61-71, 78-78, 80-93, 106-120 (data not shown). It calculated the remainder of the sequence to be composed of coils and determined that approximately 42% of WMS1 is composed of α-helices. Predict protein provided additional information (data not shown), such as the presence of 4 disulphide bonds and the occurrence of over 60 conserved regions indicating that a substantial portion of the protein has remained unchanged over time. Finally, PHD was used to consolidate the previous findings regarding WMS1’s secondary structure. PHD determined that 45% or 65 amino acid residues formed α-helices while the rest (78 amino acids) formed coils (data not shown). Overall, the results show that over 40% of WMS1’s sequence is composed of α-helices, with the remainder being random coils.
Figure 2. WMS1 secondary structure prediction using PSIPRED. The predicted secondary structures are displayed in conjunction with WMS1’s corresponding protein sequence. Each prediction’s confidence level is shown above the structure with a dark blue colour illustrating a high confidence level that the prediction is correct, whereas grey indicates a poor confidence level. Additionally, any α-helical structures present are highlighted in pink, and any random coils are coloured grey.

De novo 3D model of WMS1

The protein structure prediction service Robetta, was used to define a de novo tertiary structure model of WMS1. Five models of WMS1 tertiary structure were created with a corresponding graph illustrating the prediction error rate at different amino acid positions. All the models produced only contained α-helices and coils as expected. Model one and four contained seven α-helices, while models two, three and five had six α-helices. All five models were calculated with an overall confidence level of 74%. Model one displayed the least error (eight angstroms), while model five showed the highest. However, all models have high error rates at the start and end of WMS1’s sequence and the level of error for each prediction spiked around amino acid positions fifty and one hundred. To illustrate differences in error between the predictions, all five models were aligned using Pymol (Figure 3), and then the best three representations of
WMS1 were aligned (Figure 4). When examining all five models of WMS1, misalignment can be immediately seen with models four and five; a considerable amount of both the coils and α-helices do not align together. In comparison, all the α-helices for models one, two and three align with the variation shown in some coil segments. Additionally, WMS1’s disulphide bonds were predicted to be four (Figure 5) and cross-linking amino acids in positions C1-C5 (10-77), C2-C3 (23-66), C4-C7 (67-125) and C6-C8 (79-133).

Figure 3. Alignment of the five WMS1 de novo models predicted by Robetta. Models #4 and #5 (yellow and red) have the highest error rates.
Figure 4. Alignment of the best three WMS1 de novo models predicted by Robetta.

Figure 5. Model #1 of WMS1 tertiary structure with its disulphide bridges highlighted in red.
3.1.2 Homology protein modelling

WMS1 homologues

Homology protein modelling is a branch of bioinformatics that focuses on the search of similarities among a protein "target" (in this case, WMS1) and other related protein "templates" available in a protein database. Before finding the correct template, it is crucial to identify WMS1 conserved domains, families and homologous. A conserved domain was identified between amino acids 10 and 135 on the WMS1 sequence. The domain identified is AAI_LTSS: Alpha-Amylase Inhibitors (AAI), Lipid Transfer (LT) and Seed Storage (2S), meaning that WMS1 belongs to this superfamily. This superfamily is unique to higher plants and represents a large collection of diverse but related proteins characterised by the following properties: nutrient storage, lipid transportation and defense against pathogens. WMS1 was recorded to be a member of the family Napin/Bra, which includes major allergens, trypsin inhibitors, and natural anti-fungal proteins. WMS1's homologous were searched using NCBI's BLASTp analysis tool. Proteins are considered highly homologous if they present an E value less than 1E-50 to a query sequence. In Table 3 are recorded all WMS1 highly homologous proteins; hypothetical, unnamed and predicted proteins were excluded from the list. All homologues of WMS1 were members of the Brassicaceae family and belonged to Sinapis, Brassica or Raphanus genera. The highest identity match was an Allergen Sin a 1 protein with a percentage identity of 95.86% and an e-value of 3e-92. Overall, WMS1 is homologous to Sin a 1, Napin, Napin-like and 2S albumin storage proteins. In addition, all these proteins are members of the Napin/Bra allergen family.

Table 3. BLASTp hits received that had an e-value less than 1e-50. All WMS1 homologs determined by BLASTp are members of the Brassicaceae family.

<table>
<thead>
<tr>
<th>Description</th>
<th>Scientific Name</th>
<th>Accession number</th>
<th>E value</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergen Sin a 1.0106</td>
<td>Sinapis alba</td>
<td>CAA62911.1</td>
<td>3E-92</td>
<td>95.86</td>
</tr>
<tr>
<td>Allergen Sin a 1.0105</td>
<td>Sinapis alba</td>
<td>CAA62910.1</td>
<td>1E-79</td>
<td>93.79</td>
</tr>
<tr>
<td>Napin-like</td>
<td>Brassica rapa</td>
<td>XP_009143149.2</td>
<td>3E-76</td>
<td>91.03</td>
</tr>
<tr>
<td>Napin-like</td>
<td>Brassica rapa</td>
<td>XP_009143171.2</td>
<td>6E-76</td>
<td>89.66</td>
</tr>
<tr>
<td>Protein Type</td>
<td>Species</td>
<td>Accession Number</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------------</td>
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<td>XP_009143128.1</td>
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Further bioinformatic analyses were carried out to quantify the similarity among WMS1 and its homologous. Dot plots were then used to illustrate the sequence similarity between WMS1 and a select amount of the received homologs from BLASTp. The sequences of eight napin/napin-like and two Sin a 1 proteins were aligned against WMS1 using dot-plots. WMS1 was plotted on the x-axis with the recorded BLASTp results on the y-axis. Only three out of ten chosen proteins will be discussed; these are Allergen Sin a 1.0106 (AC: CAA62911.1), Napin-like (AC: XP_009143149.2) and Napin A1-like (AC: XP_013738180.1) proteins. The protein Allergen Sin a 1.0106 showed the highest similarity to WMS1’s sequence, which is heavily illustrated in its dot-plot (Figure 6). After reducing the plot’s noise, only one mutation region and one inverted region can be seen. The mutation is nine amino acid residues in length and is situated between amino acids 40 and 48. A small, inverted repeat of two amino acids in length can be seen between amino acids 99 and 101. In comparison, a large number of mutations, insertions and deletions are spotted between the Napin-like protein isolated from Brassica rapa and WMS1 (Figure 7). These mutations are illustrated at approximately amino acid positions 6, 38-43, 46-47, 97-101, 104-110 and 133-135. Additionally, a shift was present in axis ranging from amino acid position 38-47 and 97-110; the first shift indicates that there has been an insertion of nucleotides within that range, as the alignment line moves away from the x-axis, whereas the second shift suggests there has been a deletion of nucleotides. Between amino acid positions 102-104, there is more than one diagonal line in that same region of the sequence, indicating repeats. The Napin-like protein is less similar to WMS1 than the Allergen Sin a 1.0106 protein. In contrast to both the Sin a 1 and Napin-like protein, the dot-plot highlighted the poor sequence similarity of the Napin A1-like protein isolated from Brassica napus to WMS1 (Figure 8). Ten short sequences appear on the diagonal line, with some of the segments only being three amino acids in length. The longest sequence matched is only approximately five amino acids in size compared to the Sin a 1 protein, which matched approximately 95 amino acids residues. Many deletions, insertions, and mutations are present in the Napin A1-like protein, illustrating that it has evolutionarily changed compared to WMS1 and other members of its family.
Figure 6. Dot plot comparing the sequence similarity between WMS1 and the protein Allergen Sin a 1.0106. Only one mutation can be seen between WMS1’s and Allergen Sin a 1.0106's sequence.

Figure 7. Dot plot comparing the sequence similarity between WMS1 and the Napin-like protein isolated from the organism Brassica rapa. Notable differences can be seen between their sequences from mutations and insertion.
Figure 8. Dot plot comparing the sequence similarity between WMS1 and the Napin 1A-like protein isolated from Brassica napus. Even though these proteins are homologs, their sequences vary considerably with numerous insertions, deletions and mutations.

Understanding historical relationships between genes, proteins, and organisms is central to evolutionary biology, with the phylogenetic tree serving as a key tool for analysis and visualisation. A distance-based phylogenetic tree was created to illustrate further similarities and differences between WMS1 and its homologs (Figure 9). A distance-based approach was chosen due to its generality and speed when reconstructing evolutionary trees of proteins. The phylogenetic tree presented a total of 19 and 23 internal and external nodes, with all sampled proteins rooted to a common ancestor. Three distinct branches extended from the root, which separated the proteins into three groups; with the first, Group 1, mainly composed of 2S storage, napin embryonic and napin-B proteins, the second one by a prepropeptide 2S storage and napin proteins, while the last group is composed of isoforms of Sin a 1 proteins and WMS1. WMS1 resulted to have a close common ancestor to both Sin a 1.0108 and Sin a 1.0106, with its sequence only deviating by approximately 5.68%. Additionally, these three proteins (on average) only differ from the other Sin a 1 proteins by approximately 1.43%, illustrating their high sequence similarity to each other. It is essential to note that no napin proteins are located
in the same group as WMS1, yet some share high sequence similarities to WMS1. These results suggested a highly probability that WMS1 is an isoform of the protein *Allergen Sin a 1*. An alignment was created on Clustal Omega server among WMS1 and all the other five natural isoforms of *Allergen Sin a 1* (Figure 10). This alignment was done to show the natural polymorphism of the protein *Sin a 1* and to evaluate if WMS1 could be an isoform. The six sequences alignment showed a total of 90% of fully conserved amino acid positions, highlighted in Figure 10 by the asterisk (*), while only 5% of non-conserved positions, represented by a blank space. Particular attention was paid to the Cys residues present in entirely conserved positions among WMS1 and the *Sin a 1* proteins. As already outlined, Cys residues play a fundamental role in the final folded protein stability.

![Diagram of phylogenetic tree](image)

**Figure 9.** Distance-based phylogenetic tree of WMS1 and its homologs received from BLASTp. A distance of 0.05 is shown on the right to allow for distances between proteins to be approximated. WMS1 (reported in red) shares close common ancestors with isoforms of the protein *Allergen Sin a 1* (reported in orange).
Figure 10. Cluster Omega alignment of the sequences of WMS1 and the natural isoforms of Allergen Sin a 1. CAA62911.1 represents the accession number for Allergen Sin a 1.0106, CAA62910.1 is Allergen Sin a 1.0105, CAA62912.1 is Allergen Sin a 1.0107, CAA62909.1 is Allergen Sin a 1.0104 and CAA62908.1 is Allergen Sin a 1.0108. 90% of the alignment results to be conserved amino acid positions and Cys residues are in an identical conserved position.

To summarise, WMS1 shares 96% identity and a common close ancestor with Allergen Sin a 1.0106, moreover the dot plot in Figure 6 highlights the presence of only one mutation, of nine amino acid residues, situated between amino acids 40 and 48. To examine the impact of this mutation in the sequence of WMS1, a further alignment was created between the sequences of Allergen Sin a 1.0106 and WMS1 (Figure 11), using as reference the paper of Hummel et al. (2015). The alignment highlights that Sin a 1 is composed of two different chains and a linker peptide that is excised during the maturation process of the protein. WMS1 sequence was arranged according, and the mutation seen in Figure 6 is reported to be in the linker peptide. The two functional chains results are composed of identical amino acids residues. The linker peptide is not part of the biologically active structure of the mature protein, so it can be suggested that WMS1 has an identical structure to Allergen Sin a 1.0106.
Figure 11. Alignment between Allergen Sin a 1.0106 (A) and WMS1 (B). In green is highlighted the small chain, in light blue is the large chain, and grey is the linker peptide where all the differences between the two proteins are reported (red).

Homology 3D model of WMS1

Unfortunately, none of the isoforms of Allergen Sin a 1 possess a validated structure template added to the Protein Data Bank; therefore, a homology 3D model for WMS1 was built with the help of the tool SWISS-MODEL, an automated homology modelling server developed within the Swiss Institute of Bioinformatics (https://swissmodel.expasy.org/). Firstly, possible target models for WMS1 were computed by the SWISS-MODEL server homology modelling pipeline, which relies on an in-house comparative modelling engine that extracts initial structural information from the template structure database. Final candidates are then identified using statistical potentials of mean force scoring methods. Nine potential target templates were proposed; they matched the WMS1 sequence at varying degrees. WMS1 3D structure was built on the best template available: 1pnb.1 “STRUCTURE OF NAPIN BNIB, NMR, 10 STRUCTURES”. The Napin template was validated (Rico et al., 1996) with NMR, and the PDB file was downloaded and visualised with the software RasMol (Figure 13). The built WMS1 3D homology template presented an overall 57.28% sequence identity between the target (Napin) and WMS1; it covered 74% of the Small chain (A) and 81% of the Large chain (B). In the absence of an experimental reference structure, it is crucial to estimate the quality of a 3D protein structure model prediction. Several 3D model evaluation parameters are available on the SWISS-MODEL server, and relevant results obtained from the evaluation of the WMS1 model are reported in the following list:

- GMQE (Global Model Quality Estimation): 0.55. This is a coverage-depdant measurement with higher numbers indicating higher expected quality.
-QSQE (Quaternary Structure Quality Estimation): 0.81. This score reflects the expected accuracy of the interchain contacts for a model built based on a given alignment and template; it is expressed as a number between 0 and 1, and a value above 0.7 can be considered reliable to follow the predicted quaternary structure in the modelling process.

-QMEANDisCo global score: 0.5 ± 0.8. This is the average per-residue score and the provided error estimate; a score threshold of 0.5 significantly reduces the likelihood of importing low-quality models. The overall results are reported in Figure 12.

Figure 12. Global local quality estimation for the WMS1 model built using as target the structure of Napin. WMS1 model is constituted of two polypeptide chains; the small chain (A) of the model covers WMS1 amino acid residues from 7-35 (74%), while the large chain (B) covers the range 2-86 (81%). In the graph, the yellow line represents the long chain; the grey is the small one. According to the model estimated error score, a colour code is assigned to the amino acid sequence and the 3D figure of the WMS1 model. Scores below 0.5 (orange) are considered low quality. The area presenting significant errors located in the “hypervariable region” in the long chain.

To summarise, it can be assumed that the model built is overall reliable; nevertheless, local areas with high errors are present, especially between residues 40 and 65 in the large chain. This area showed a high degree of error in the 3D model produced with the de novo approach. According to literature, this area is known as the “hypervariable region” which has been described to be the most important antigenic region of the protein family 2S albumins; it is an exposed segment linking α-helices III and IV and it does not play any role in determining the
folded structure (Moreno and Clemente, 2008). WMS1 3D model built with SWISS-MODEL was downloaded as a PBD file and visualised with the software RasMol (Figure 13) and PyMol (Figure 14). It was possible to identify secondary structure features such as four S-S bonds and five helices; helices are marked according to the indication collected from Moreno and Clemente (2008). Since the 3D model presented errors, experimental data needed to be collected to improve conclusions on the WMS1 structure.

Figure 13. RasMol ribbon visual of the protein Napin (PDB file ID: 1pnb.1). The closest WMS1 homologous with a solved validated template.
Figure 14. PyMol ribbon visualisation of WMS1 homology 3D model. A) Polypeptide chains and helices are highlighted B) In red are highlighted the four S-S bonds.
3.2. In vitro analysis of WMS1

3.2.1. WMS1 stability to heat and proteolysis

Experimental data were collected to assess if WMS1 possessed resistance to proteolysis and heat denaturation. Purified samples of WMS1 were subjected to heat treatment for 15 min at 100°C or proteolytic digestion for 4 h at 37°C with α-chymotrypsin at different protein:enzyme molar ratios of 60:1, 250:1, 2500:1. If WMS1 were to exhibit resistance to heat and proteolytic digestion, it would retain its folding and antisyent activity after treatment. An SDS-PAGE gel was loaded with native and treated WMS1 samples (Figure 15). All the samples showed only one band on the gel, all the bands appeared around 14 kDa, and no visible difference was detectable between the WMS1 native sample and the treated ones. This outcome suggested that WMS1 retained its native fold after heat treatment or digestion with α-chymotrypsin. The antisyent activity of native and treated WMS1 samples was tested with a microdilution growth curve assay against the yeast strain Z. bailii; WMS1 concentrations tested were 150, 75 and 37.5 µl/ml (Figure 16). Native WMS1 showed antisyent activity at 150 µl/ml and 75 µl/ml concentrations, while yeast cells grew in the wells with 37.5 µl/ml of WMS1 and in the control wells with water. Identical results were recorded for WMS1 samples subjected to heat treatment, while a slight loss in antisyent potency was recorded for WMS1 samples digested with α-chymotrypsin. Yeast growth was observed for protein concentration of 75 µl/ml for all three different WMS1:enzyme molar ratios (2500:1, 250:1 and 60:1) and antisyent activity was always retained for concentrations of 150 µl/ml. These results suggested that WMS1 is resistant to proteolysis; however, the digestion affected the protein antisyent potential, with a one-fold reduction in the antisyent activity against Z. bailii. Overall, the experimental data collected suggested a high WMS1 tolerance for heat denaturation since, after being treated at 100°C for 15 min, the protein showed no changes in its "behaviour" (appearance on SDS gel and antisyent potential) compared to the native WMS1. WMS1 was shown to possess resistance to proteolytic digestion as well, it maintained its structural integrity after being digested, and the antisyent activity was only reduced of one-fold.
Figure 15. SDS-PAGE gel with native (lane 2) and treated (lanes: 3, 4, 5, 6) WMS1 samples, lanes 1 and 7 are the MW ladder. No differences were observed among the bands; WMS1 protein retains its native structure with the 4 SS bonds after heat treatment and proteolytic digestion.
Figure 16. Growth curves of *Zygosaccharomyces bailii* exposed to different concentrations of native and treated WMS1. (1) The native WMS1 possessed antiyeast activity at 150 µg/ml (blue line) and 75 µg/ml (orange line) concentrations, while at 37.5 µg/ml (grey line), the yeast cells have a growth comparable to the control with water (yellow line). (2) After being heated at 100°C for 15 min, WMS1 retains its antiyeast activity unchanged. (3) WMS1 was digested with three different WMS1:enzyme molar ratios of 2500:1 (3A), 250:1 (3B) and 60:1 (3C). In this case, WMS1 antiyeast activity is slightly affected, yeast growth was recorded at 75 µg/ml, while no growth was recorded at 150 µg/ml for all the WMS1:α-chymotrypsin molar ratio tested, meaning that 1 fold-loss in the antiyeast potency was documented. WMS1 possess resistance against heat treatment and proteolysis. Test were conducted in triplicate (bars represent standard deviation).
3.2.2. Dithiothreitol (DTT) reduction assays

As already described in this work, disulphide bridges play a significant role in the final fold of a protein, and the presence of four S-S bonds in WMS1 have been predicted by bioinformatics analysis. Nonreduced and reduced WMS1 samples were prepared and loaded onto an SDS gel. Treatment with DTT was used to prepare reduced samples; it is necessary to break down disulphide bonds that participate in the protein tertiary structure. After the run, the nonreduced WMS1 sample appeared on the gel as a single band of 14 kDa (Figure 17), while the WMS1 sample reduced with DTT appeared as two bands, one band as 9/10 kDa and the second one at 5/4 kDa. It can be concluded that WMS1 is composed of two polypeptide chains held together by S-S bonds.

![Figure 17. SDS gel with reduced and non-reduced WMS1 samples. In lanes 1 and 4, a molecular weight ladder was loaded; in lane 2, WMS1 was not reduced and in lane 3, the reduced sample. The non-reduced sample appears as a single band at 14 kDa, while the sample in lane 3 appears as two bands, one at 9/10 kDa and one at 4/5 kDa, which means that the DTT broke the S-S bonds.](image)
3.3. Literature search

Data collected from the literature search on the mustard napin protein *Allergen Sin a 1* vastly matched data gathered in this chapter for WMS1. Results are summarised in Table 4.

Table 4. Summary of comparative study between protein *Allergen Sin a 1* and WMS1

<table>
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<th>Parameters</th>
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<th>WMS1</th>
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<td></td>
<td><em>In silico</em></td>
<td><em>In vitro</em></td>
</tr>
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<td>S-S bonds</td>
<td>Present in C1-C5, C2-C3, C4-C7, C6-C8 (2)</td>
<td><em>De novo</em> and homology: present in C1-C5, C2-C3, C4-C7, C6-C8 (2)</td>
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<tr>
<td>Helices</td>
<td>5 (overall 40-50%) 2 Sc. + 3 Lc (2)</td>
<td><em>De novo</em>: 5/6 (40-50%) Homology: 2 Sc + 3 Lc</td>
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<td>Isoelectric Point</td>
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<td>Hydrophobic / Hydrophilic</td>
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<td>Resistance to heat</td>
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<tr>
<td>Resistance to proteolysis</td>
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4. Discussion

WMS1 is the name given to an antiyeast protein isolated from white mustard seeds in Chapter 3. The protein's primary structure was identified, and first reports showed a potentially high degree of identity between the protein WMS1 and the protein Allergen Sin a 1. In this chapter, the relationship between these two proteins has been investigated with bioinformatic tools and experimental data; it has been concluded that WMS1 is almost certainly an isoform of the protein Allergen Sin a 1.

WMS1 possess a Glu-rich primary structure, and it is overall composed of 143 amino acid residues. The calculated MW was 15.89 kDa, and it appeared on 12% SDS-PAGE as a 14 kDa single band in a non-reducing environment and as two bands of 9/10 and 4/5 kDa in reducing conditions. Eight Cys residues were found in WMS1 in conserved position compared to isoforms of Sin a 1, and four S-S bonds in positions C1-C5, C2-C3, C4-C7 and C6-C8 were predicted by both de novo and homology modelling. This is not surprising since, in the 2S albumin family, the pattern of eight Cys in a specific order (C1 and C2 located on the small chains while C3, C4, C5, C6, C7 and C8 on the large one) and the relative inter and intra-chain disulphide bridge links are highly conserved and represent the structural scaffold of the tertiary structure. In general, Sin a 1 proteins are characterised by a compact Glu-rich primary structure stabilised by four S-S bridges that link eight conserved Cys residues. They are originally synthesised as a single larger precursor polypeptide, and the correct fold is taken into the lumen of the endoplasmic reticulum. The folded protein is then transported into the vacuole, where it is subsequently processed to a single polypeptide of 145 amino acid residues and finally to the large and small subunits of 39 and 91 amino acid residues, respectively. Sin a 1 calculated MW is 16.49 kDa, while the appearance of the protein on SDS-PAGE is as a single band of circa 14 kDa in non-reducing conditions and as two bands of 9–10 kDa and 3–4 kDa in reducing conditions (Menéndez-arias et al., 1988; Moreno and Clemente, 2008; Marambe et al., 2015; L’Hocine et al., 2019).

Another secondary structure feature of the 2S albumin family is the presence of α-helices; de novo analysis predicted WMS1 to be composed by 40%–50% of α-helices structures, and the homology 3D model predicted two helices in the small chain and three on the large one. Models built with de novo methods predicted 6/7 helices; however, since the model did not predict the presence of the two separate subunits, it is not accurate. Moreover, de novo tools calculated WMS1 to possess a pI of 8.5 and to be amphipathic, these data matched values present in the
literature for Sin a 1. Significant is the pI data since different 2S albumins show a broad range of pI due to their variable amino acid composition (Bueno-Díaz et al., 2021). Both WMS1 and Sin a 1 present hydrophobic and hydrophilic portions, making their structure overall amphipathic (although hydrophilic areas are slightly more numerous). Hydrophobic helices are known for being fundamental for AMPs, including 2S albumins; indeed, Neumann et al., (1996) indicated a possible involvement of the α-helical structure of Brassicaceae Napins in CaM (calmodulin) antagonism. Moreover, the presence of hydrophilic portion in the structure of WMS1 and Sin a 1 confer to the proteins water solubility.

The search on conserved domain databases (Interpro and NCBI) recognised WMS1 as a member of the Napin/Bra allergen family; in detail, the BLASTp search and the phylogenetic tree clearly showed the isoform Allergen Sin a 1.0106 being the closest homologous relative of WMS1. Additionally, the alignment and the dot plot in reported no differences in the amino acid sequence of the small and large chains between WMS1 and Allergen Sin a 1.0106; variances were only recorded in the linker peptide. The protein Sin a 1 is a 2S albumin; specifically, it belongs to the Napin/Bra allergen family, and their main biological function is suggested to be nutrients storage. Several other properties have been recorded for proteins belonging to this family, including allergenicity for humans, trypsin inhibition, resistance to heat degradation and defense against pathogens (Shewry and Pandya, 1999). The protein Sin a 1 is the major allergen present in mustard; its compact structure donates to the protein resistance to heat degradation and trypsin proteolysis allowing the protein to pass the digestion process (Moreno and Clemente, 2008). While Napin from rapeseed (Brassica napus) has been studied from their antimicrobial activity (Nioi et al., 2012), no record exists about the antimicrobial activity of Sin a 1. Further in vitro experiments verified that WMS1 retains other characteristics typical of Sin a 1, such as resistance to proteolytic digestion and heat degradation. Finally, 2S Albumins are known for the presence of a "hypervariable region" located in an exposed loop between the IV and V helix in the large chain; this area represents the important antigenic region for these proteins, and epitope mapping studies have localised an epitope in the hypervariable region of Sin a 1 with a histidine residue (H-58) (Monsalve et al., 1993) and a tyrosine (Y-64) (Menéndez-Arias et al., 1990) playing a crucial role in the immune-dominant portion of the allergen. Regarding WMS1, the immune-dominant residues H-58 and Y-64 are present on its large chain since it shares the same amino acid sequence of Sin a 1. The presence of the hypervariable region in WMS1 was suggested by both de novo and homology 3D models when a high degree of error was predicted between residues 40 and 65 in the large chain.
To conclude, in this chapter it has been found that the antiyeast protein WMS1 isolated from mustard seeds in the previous chapter is a new isoform of the protein known as Allergen Sin a 1. Sin a 1 has never been characterised for antimicrobial potential and its biotechnological potential has never been exploited, although the allergenicity of Sin a 1 could represent a drawback for WMS1 potential application involving human ingestion (e.g., food preservatives or medical drug).
Bibliography


Marambe, H. K. et al. (2015) ‘Structural stability and Sin a 1 anti-epitope antibody binding ability of yellow mustard (Sinapis alba L.) napin during industrial-scale myrosinase


Chapter 5

The characterisation of WMS1 killing activity proves the antimicrobial potential of the protein *Allergen Sin a 1*

The work described in this chapter has been published as part of the article “Isolation of the mustard Napin protein *Allergen Sin a 1* and characterisation of its antifungal activity” in the journal “BB Reports” ISSN: 2405-5808
Abstract

Several Napins (name given to the 2S albumins in *Brassicaceae*) proteins have been described for their antimicrobial *in vitro* properties; however, the mustard Napin, better known as *Allergen Sin a 1*, has been studied mainly for its allergenic properties. The main aim of the work described in this Chapter is to investigate for the first time the antimicrobial potential of this protein. All data were collected using the purified protein product labelled WMS1 which is an isoform of *Sin a 1* (Chapter 3 and 4). The Minimum inhibitory concentration was established against several strains (two bacteria, two filamentous fungi, and nine yeasts); WMS1 possessed antifungal and antiyeast *in vitro* activity, but no antibacterial activity was recorded. The yeasts *Zygosaccharomyces bailii* Sa 1403 and *Saccharomyces cerevisiae* DSM 70449 and the filamentous fungi *Fusarium culmorum* FST 4.05 resulted in being among the most sensible strains to WMS1 (MICs range 50-100 µg/mg). Mechanism membrane permeabilisation was detected and suggested as a possible killing mode of action. In general, the antiyeast activity of WMS1 seemed to be expressed in a dose-dependent manner. Data collected confirmed WMS1 to be a stable and compact protein; it displayed insensitivity to pHs variation or salts presence. In addition, a cytotoxicity assay against human CaCo2 cells and a haemolytic assay against sheep's red blood cells showed that the protein is not harmful to mammalian cells.
1. Introduction

Seed storage proteins (SSPs) are proteins that accumulate considerably in the developing seed; they are stored in deposits called protein bodies, and during germination, they are mobilised and used in seedling growth, maintenance and defence (Shewry et al., 1995).

SSPs were first classified based on their solubility and albumin was merely the name given to SSPs soluble in pure water (Osborne, 1924). Nowadays, Seed Storage (2S) albumin proteins represent a multifunctional class of seed storage proteins classified as part of the Prolamin superfamily which includes: 2S albumins, non-specific lipid transport proteins (nsLTPs), bifunctional α-amylase, inhibitors/serine proteases and cereal prolamines (Byczyńska and Barciszewski, 1999). The 2S albumin are one of the most abundant type of SSPs found in plants of the Brassicaceae family and they are called Napin in oilseed rape (Brassica napus); the term Napin can be also found associated to 2S albumin proteins of mustard (B. juncea, B. nigra and B. hirta) (Wanasundara et al., 2012).

Several 2S albumins proteins have been described for diverse biological and biotechnologically exploitable functions, for example the Napin from rapeseed (B. napus) possesses antimicrobial activity (Nioi et al., 2012) and the 2S albumin from pumpkin seeds has anticancer properties (Tomar et al., 2014). However, the White Mustard Napin is officially known under the name of Allergen Sin a 1 and it has been studied mainly for its allergenic properties.

In the two previous chapters, the isolation and the identification of a small protein from the seeds of White Mustard (Brassica hirta) have been described; this protein was called WMS1, and it displayed killing activity against the yeast Saccharomyces cerevisiae DSM-70449 (Chapter 3) and was proven to be an isoform of the protein known as Allergen Sin a 1 (Chapter 4). This chapter work aims to investigate for the first time the antimicrobial potential of the protein Allergen Sin a 1 and this was achieved by characterizing the antiyeast, antifungal and antibacterial activity of the purified protein product WMS1. For continuity with previous chapters, the protein under investigation Allergen Sin a 1 will be called WMS1 in the rest of the work. Initially, the Minimum inhibitory concentration of WMS1 was established against several micoorganisms (two bacteria, two filamentous fungi, and nine yeasts); afterwards, WMS1 killing mechanism of action was analyzed and whatever its antimicrobial activity is challenged by different conditions or incorporation into different food matrices. Moreover, WMS1 toxicity towards mammalian cells was evaluated and finally, potential applications were discussed.
2. Material and Methods

2.1. Microbial strains

Several microbial strains were employed in this work, including two bacteria, two filamentous fungi, and nine yeasts. Specifically, the bacteria utilised were a Gram-negative (*Escherichia coli* ATCC 25922) and a Gram-positive (*Micrococcus luteus* CIT3). Among the fungal strains used, there were four yeast strains often associated with food/feed spoilage: *Kluyveromyces lactis* ATCC56498, *Zygosaccharomyces bailII* Sa1403, *Zygosaccharomyces rouxii* ATCC14679, *Debaryomyces hansenii* CBS2334, as well as three different strains of *Saccharomyces cerevisiae*: DSM 70449 (type strain, isolated from top-fermenting beer in 1990), MTU 01P (commercially available instant active dry Baker’s Yeast) and NNCYC 77 (or NCTC 815, Baker’s Yeast deposited in 1921), two human pathogenic strains *Candida albicans* CUH 001 and *Aspergillus fumigatus* DSM 15966 and a phytopathogen *Fusarium culmorum* FST 4.05. All stock cultures were maintained at -80°C and the microbial strains used were present in the MTU collection or purchased from DMSZ (Germany).

2.2. WMS1 protein

The WMS1 protein was purified from white mustard seeds as described in Chapter 3 sections 2.1. Protein’s samples at concentration of 2 mg/ml were stocked in distilled water at -20°C.

2.3. Minimum inhibitory concentration

The antimicrobial potency of WMS1 was investigated, calculating the minimum inhibitory concentration (MIC) for the bacterial and fungi strains listed above. The MIC for each strain was found using broth dilution methods where the growth of each strain, exposed to a serially diluted concentration of WMS1, was monitored by a microtiter plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) in a flat bottom 96-well microtiter plate. Tests were done in triplicate on two WMS1 concentration series ranging from 1000 µg/ml to 31.25 µg/ml and from 800 µg/ml to 25 µg/ml. Water without WMS1 was always used as a control. The MIC was determined as the lowest concentration of WMS1 that prevented visible growth of the microorganism after incubation.
Antibacterial assay

The antibacterial activity of WMS1 was assessed following the method of Thery et al. (2020). Bacteria were cultured on Mueller-Hinton agar for 24 h at 37°C. One colony was transferred to Tryptic Soy Broth, incubated 2 h and diluted with PBS until the OD of the media reached McFarland 1 (DEN-1 McFarland densitometer, Biosan Limited, UK). Mueller-Hinton broth (Sigma-Aldrich) was used as the growth medium, and 2.5 μl of the bacterial solution was added to each well. The OD 600 nm was monitored continuously for 24 h at 37°C.

Antifungal assay

The activity against filamentous fungi was assessed following Thery et al. (2020) with some modifications. Moulds were cultured on Potato Glucose Agar for 5/7 days at 30 °C; afterwards, fungal spores were collected using 10 ml of dH2O and a cell strainer. The final inoculum was prepared in half-strength Potato Dextrose Broth (½ PDB) at a 10^5 spores/ml concentration, and 100 μl of spore solution was added to each well; the spore concentration was calculated using a haemocytometer (Improved Neubauer Counting Chamber, Sigma Aldrich). Fungal growth was monitored at 28°C for 48 h, and absorbance was followed by spectrophotometry at 600 nm at 2 h intervals.

Antiyeast assay

Antiyeast assays were carried out following the guidelines of the "EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts" (Rodriguez-Tudela, 2008), as described in Chapter 2.

2.4. Colony count assay

A colony count assay was performed to determine the time necessary for WMS1 to exhibit its antiyeast activity against the type strain S. cerevisiae DSM 70449. A yeast suspension of 10^4 CFU/ml was prepared in conjunction with 400, 200, 100, 50 and 25 μg/ml of the WMS1 protein. 100 μl of each suspension was spread every hour onto SD agar plates; this was repeated every hour for a total period of 6 h. Colonies were counted after incubating plates for 48 h at 30 °C.
2.5. Total nucleotide leakage

The total nucleotide leakage of the type strain *S. cerevisiae* DSM 70449 resulting from the activity of different concentrations of WMS1 was calculated according to Shwaiki *et al.* (2020). Briefly, a $10^6$ cells/ml yeast suspension was prepared, washed twice in PBS and incubated at 30 °C for 5 h with WMS1 concentrations of 25, 50, 100, 200 and 400 μg/ml. Afterwards, yeast cells were removed via filtration through a 0.22 μm filter, and the OD260nm of the filtrate was recorded. 0.1% Triton X-100 and water were used as positive and negative controls, respectively.

2.6. Membrane permeabilisation assay

The ability of WMS1 to permeabilise the membrane of the yeast *S. cerevisiae* DSM 70449 was studied as a possible fungicidal mechanism of action. Propidium iodide (PI) is a fluorescent dye which possess the ability to bind nucleic acids of cells; since it is excluded from viable cells, the bond occurs only when the cell membrane has been permeabilised. The protocol of Canelli *et al.* (2020) was followed with some modifications; briefly, a $10^6$ cells/ml suspension was prepared in PBS from an overnight culture in SD broth and exposed to 50, 100, 200 and 400 μg/ml of WMS1. Yeast cells treated with 0.1% Triton X-100 and water were used as positive and negative controls, respectively. After 5 h incubation at 30 °C, the cells were washed in PBS twice. Subsequently, samples were incubated in dark condition for 5 min at room temperature in conjunction with 200 μl of PI (6 μM). Then, treated cells were washed twice again with PBS and the pellets resuspended in 250 μl of PBS and 4 μl of each sample was visualised with the microscope EVOS®FL Auto Imaging System (Life Technologies - Thermo Fisher Scientific, MA, USA). Images were captured at 40X magnification under the fluorescent channel RFP (531/40 nm excitation; 593/40 nm emission) edited with phase contrast. Moreover, the rate of yeast membrane permeabilisation was evaluated in a microtiter plate on 100 μl of samples prepared as described above with the difference that yeast cells were treated with the PI dye and WMS1 concentrations simultaneously. The fluorescence was measured for 5 h in a VarioscanLUX plate reader at the maximal excitation ($\lambda_{Ex}$) and maximum emission ($\lambda_{Em}$) wavelengths of 535 nm and 617 nm, respectively.
2.7. Haemolysis assay

The release of haemoglobin from defibrinated sheep erythrocytes due to the presence of WMS1 was calculated following the protocol outlined in Shwaiki et al. (2020). Briefly, a 4% red blood cell (Oxoid™) solution was incubated for 1 h at 37 °C with 200, 100 and 50 μg/ml concentrations of WMS1. Samples incubated with 0.1% Triton X-100 and PBS were used as positive and negative controls, respectively. Afterwards, samples were centrifuged at 1000 g for 10 min, and the OD at 405 nm of the supernatant was measured. Results are expressed as a percentage of haemolysis, with 10% being the threshold in the data interpretation, if >10% WMS1 was considered hemolytic and if <10% not haemolytic. The calculations were made using the measured absorbance and the below formula:

\[
\text{% Haemolysis} = \frac{(A_{405 \text{ WMS1 treatment}}) - (A_{405 \text{ PBS}})}{(A_{405 0.1\% \text{ Triton X-100}}) - (A_{405 \text{ PBS}})}
\]

2.8. Cytotoxicity assay

The cytotoxicity of WMS1 protein was tested by measuring cell viability using a MTT cell viability kit (Cell proliferation Kit I MTT, Sigma Aldrich, MO, USA). The protocol was performed as described by Shwaiki et al., (2020). Human colonic cells, Caco-2 cells (ECACC) were maintained and passaged in Dulbecco's Modified Eagle Media (DMEM) supplemented with 1% non-essential amino acids and 10% Fetal Bovine Serum (FBS). A 200 μl cells inoculum (1×10^5 cells/ml) was added into wells of a flat-bottom 96 well microtiter plate and incubated for 24 h at 37°C with 5% CO₂ allowing cells to reach confluence. After removing the media, the protein was added at concentrations of 100, 200, 300, 400, 500, 600 and 700 μg/ml in conjunction with DMEM and 2.5% FBS. Untreated cells served as positive control and wells without cells were used as negative control (0% viability), since the assay is based on the reduction of 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by living cells. After 24 h incubation in the same conditions, the media was removed, and 100 μl of DMEM plus 10 μl of MTT labelling reagent (Cell proliferation Kit I MTT, Sigma Aldrich, MO, USA) were added to each well. A further four h incubation followed this step before adding 100 μl of solubilisation buffer. Next, the plate was incubated overnight, and the viability of the cells was measured using a fluorometric spectrophotometer at 570 nm with a background reading of 690 nm.
2.9. Stability tests: pHs and salts

The stability of WMS1 under varying pH and salt conditions was determined by examining the effect on its inhibitory activity against the yeast *Z. bailii*. The experimental protocols were developed in accordance with the WMS1 thermal stability assay described in section 2.2.1. in Chapter 3. In this test, the broth microdilution assays were performed with modified SD broth. 1 M sodium hydroxide (NaOH) and 0.1 M hydrochloric acid (HCl) were used to adjust the medium to the following pHs: 3, 5, 7, 9 and 11; controls consisted of regular SD pH-adjusted broth without added WMS1. MgCl₂ and KCl at two different concentrations: 1mM and 5 mm, and 50mM and 150 mM, respectively, were added to the medium. SD broth containing the salts, but without WMS1 was used as control. WMS1 was tested at three concentrations: ½ MIC (37.5 µg/ml), MIC (75 µg/ml) and 2MIC (150 µg/ml).

2.10. WMS1 application in different food matrices

WMS1 was applied in several food matrices spiked with the yeast *Z. bailii*; this yeast is commonly known to spoil foodstuffs and drinks (Kuanyshev *et al.*, 2017), and among all the microbial strains tested, *Z. bailii* resulted in being the most susceptible to WMS1. The anteyeast WMS1 activity was tested in a soft drink Fanta Orange (Coca-Cola, Ireland) and two fruit juices cranberry juice (Kelkin, SuperValu, Ireland) and apple juice (Squeez, SuperValu, Ireland). The anteyeast methodology of choice was a microtiter plate assay using filter sterilised beverage inoculated with 10³ cells/ml yeast from an overnight culture of SD broth, and the yeast growth was monitored over 48 h at 30°C with OD readings of 600 nm every 2 h. WMS1 was tested at 200, 100, 50 and 25 µg/ml concentrations. WMS1 was applied in a complex beverage; the vegan white wine Mosel Riesling (Kendermanns, Germany). In this case, a 1 x 10⁵ cells/ml yeast suspension was resuspended in 1 ml of wine in conjunction with 2, 1, 0.5 and 0.250 mg/ml of WMS1 and incubated at 30 °C for 48 h; the growth was monitored in two complementary ways: firstly spotting 20 µl of the solution on SD agar after 1, 3, 5, and 24 h, secondly spreading 100 µl of the solution onto SD agar plates after 48 h incubation. Plates were checked after two days of incubation at 30°C. Controls of each beverage consisted of the corresponding beverage inoculated with yeast and no WMS1 protein. A similar assay as the one described for wine was used for more food viscous matrices: one savoury product *i.e.*, salad dressing (Heinz salad cream, UK) and one high in sugar product *i.e.*, maple syrup (Buckwud Organic Canadian Maple Syrup, Canada).
2.11. Statistical analysis

All the tests were run in triplicate or quadruplicate and results reported in tables and graphs are presented as means ± standard deviation. Statistical tests were performed on the program Analysis ToolPak (Microsoft Excel). In general, the statistical significance of the difference between behaviours observed in samples with protein and controls without protein was calculated with a student’s t-test. A probability of p<0.05 was considered statistically significant.

3. Results

3.1. Minimum inhibitory concentration

WMS1 possessed antifungal activity, but no antibacterial activity was recorded; results are summarised in Table 1. WMS1 was able to completely inhibit the growth of all the yeasts and filamentous fungi tested with variable MIC values depending on the fungal strain. Regarding yeasts, the highest WMS1 antifungal potency was recorded for Z. bailii with 50/100 µg/ml, while the lowest was for C. albicans when only the highest tested WMS1 concentration (1000 µg/ml) was effective. The variability in WMS1 antifungal potency is evident when examining results for the yeast S. cerevisiae where the three strains tested resulted in different MIC values; the industrial strain of Baker's yeast MTU 01P was susceptible only to 1000 µg/ml WMS1, while the strains DSM 70449 and NCYC 77 were susceptible to smaller quantities of WMS1 (100 or 200 µg/ml). The four spoilage yeast strains, K. lactis, D. hansenii, Z. bailii and Z. rouxii, were completely inhibited by 200–250 µg/ml, 150–200 µg/ml, 50–100 µg/ml and 400–500 µg/ml, respectively. Concerning the growth of filamentous fungi, F. culmorum and A. fumigatus were inhibited by WMS1 at 50–100 µg/ml and 1000 µg/ml, respectively.
Table 1. MICs range for yeasts, moulds and bacteria tested with WMS1 protein samples. Tests were conducted in triplicates.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Kingdom</th>
<th>Type</th>
<th>MICs range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Kluyveromyces lactis</em> ATCC 56498</td>
<td>Fungi</td>
<td>Spoilage Yeast</td>
<td>200–250</td>
</tr>
<tr>
<td><em>Debaryomyces hansenii</em> CBS 2334</td>
<td>Fungi</td>
<td>Spoilage Yeast</td>
<td>150–200</td>
</tr>
<tr>
<td><em>Zygosaccharomyces bailii</em> Sa 1403</td>
<td>Fungi</td>
<td>Spoilage Yeast</td>
<td>50–100</td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> ATCC 14679</td>
<td>Fungi</td>
<td>Spoilage Yeast</td>
<td>400–500</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> DSM 70449</td>
<td>Fungi</td>
<td>Yeast</td>
<td>100</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> NCYC 77</td>
<td>Fungi</td>
<td>Yeast</td>
<td>100–200</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> MTU 01P</td>
<td>Fungi</td>
<td>Yeast</td>
<td>1000</td>
</tr>
<tr>
<td><em>Candida albicans</em> CUH 001</td>
<td>Fungi</td>
<td>Pathogen Yeast</td>
<td>1000</td>
</tr>
<tr>
<td><em>Fusarium culmorum</em> FST 4.05</td>
<td>Fungi</td>
<td>Phytopathogen mould</td>
<td>50–100</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> DSM 15966</td>
<td>Fungi</td>
<td>Pathogen mould</td>
<td>1000</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 25922</td>
<td>Bacteria</td>
<td>Gram - bacterium</td>
<td>No inhibition</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> CIT3</td>
<td>Bacteria</td>
<td>Gram + bacterium</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

3.2. Colony count assay

In the presence of WMS1, *S. cerevisiae* growth was always affected compared to the control with water that reached $9.08 \times 10^4$ CFU/ml after 6 h (Figure 1). In the presence of the smallest WMS1 concentrations tested (25 and 50 µg/ml), *S. cerevisiae* growth was impacted; however, an increase in the growth was observed over time. A visible decrease in the growth was noted for WMS1 at the MIC and double MIC levels (100 and 200 µg/ml); however, the decrease was delayed compared to the highest concentration tested (400 µg/ml), where no yeast growth was recorded after 4 h. The antiyeast potential of WMS1 seemed to be expressed in a dose-
dependent manner, with a faster decrease in the yeast growth at higher concentrations of WMS1.

![Graph showing colony count assay for S. cerevisiae inhibition by WMS1.](image)

Figure 1. Colony count assay demonstrating the rate of \textit{S. cerevisiae} inhibition by WMS1. Test was done in triplicate (bars represent standard deviation).

3.3. Total nucleotide leakage

The amount of nucleotide leakage generated by cells of \textit{S. cerevisiae} after 5 h of exposure to WMS1 showed a positive correlation with protein concentrations (Figure 2). The highest OD at 260 nm recorded in this experiment was the Triton-X 0.1% control with 0.559, and the highest WMS1 concentration tested (400 μg/ml) produced a significant high OD (0.253) compared to the control with water (0.015). The OD measurement recorded for 200, 100, 50, and 25 μg/ml were 0.130, 0.059, 0.041 and 0.017, respectively, suggesting a dose-dependent correlation between the amount of antiyeast protein WMS1 and yeast cells lysis. Moreover, with the exclusion of the 25 μg/ml sample, all the protein samples produced an OD significantly higher than the control with water.
Figure 2. Degree of total nucleotide leakage caused by the presence of WMS1 at different concentrations; increasing amounts of protein translated to greater nucleotide leakage from *S. cerevisiae* cells. Test was conducted in triplicate and the significance of the difference between protein samples and the negative control without protein was calculated with student’s t-test (mean ± SD; n = 3; * p < 0.05, ** p < 0.01, *** p < 0.001 and ns: no significant difference).

3.4. Membrane permeabilisation

In this assay, WMS1 ability of inducing membrane permeabilisation in *S. cerevisiae* was evaluated. In general, WMS1 was found to cause damage in the yeast membrane at all the concentration tested with the level of permeabilisation decreasing as the concentration of the protein was lowered. After 5 h exposure to 50, 100, 200 and 400 µg/ml of WMS1, yeast cells were treated with the fluorescent dye PI and the fluorescence emitted by cells subjected to membrane permeabilisation was visually observed with fluorescence microscopy (Figure 3) and quantified by fluorescence spectroscopy (Figure 4). As expected, *S. cerevisiae* showed the highest fluorescence when treated with Triton-X 0.1% and the lowest in the control with water. Yeast cells with damage in their membrane where visibly present in all the protein samples tested and they produced a fluorescence intensity significantly higher compared to the sample control with dH₂O, meaning that membrane permeabilisation was detected as WMS1 antiyeast mode of action.
Figure 3. Permeabilisation effects of WMS1 on *S. cerevisiae* cells observed with fluorescence microscopy. A, B, C and D display WMS1 samples of 400 μg/ml, 200 μg/ml, 100 μg/ml and 50 μg/ml, respectively. E shows the positive control (Triton-X 0.1%) and F the negative control (water).

Figure 4. Permeabilisation effects of WMS1 on *S. cerevisiae* cells calculated by fluorescence spectroscopy. Test were conducted in triplicates, the significant difference between protein samples and the control with water was established with student’s t-test (mean ± SD; n = 3; * p < 0.05, ** p < 0.01, *** p < 0.001 and ns: no significant difference).
3.5. Haemolysis assay

This assay was carried out to observe the potential of 200, 100 and 50 μg/ml of WMS1 to rupture mammalian red blood cells. According to the MIC results, these WMS1 concentrations were lethal for several fungi and yeast strains and this test was performed to characterise WMS1 safety for mammalian cells. All the WMS1 concentrations tested resulted in no haemolytic activity with all the percentages of haemolysis recorded to be less than 10%, and no differences were noticed among different WMS1 concentrations (Figure 5).

![Haemolysis assay graph](image)

**Figure 5.** Percentage of haemolysis of 200, 100 and 50 μg/ml of WMS1 on sheep erythrocytes. Test was conducted in triplicates (bars represent standard deviation).

3.6. Cytotoxicity assay

The cytotoxicity assay was carried out to characterise WMS1 safety for human cells. The results indicated that WMS1 was not cytotoxic for Caco-2 cells at any of the concentrations tested. This is because no major variance in the cell viability was noticed between WMS1 treated cells and the control with water (Figure 6).
Figure 6. Percentage viability of Caco-2 cells in the presence of increasing concentrations of WMS1. Since the assay is based on the reduction of MTT by living cells, the viability percentage was calculated using wells without cells as negative control (0% viability) and untreated cells as positive control. Tests were conducted in quadruplicate (bars represent standard deviation).

3.7. Stability tests: pHs and salts

The antiyeast activity of WMS1 was not affected by any of the pHs tested and only slightly altered by salts; 150 μg/ml of WMS1 was able to inhibit completely the growth of Z. bailii in every condition tested (Figure 7). In particular, the pH changes of the media caused no reduction in WMS1 antiyeast potency since no yeast growth was recorded at the MIC level, while Z. bailii grew at the MIC level in the presence of both MgCl2 (5 mM) and KCl (50 and 150 mM). To summarise, WMS1 antiyeast activity against the yeast Z. bailii is impacted by pH change and is decreased by the presence of salts.
3.8. WMS1 application in different food matrices

WMS1 was tested for its ability to inhibit the growth of the spoilage yeast *Z. bailii* in different food and beverage matrices, and the results are summarised in Table 2. Regarding Fanta Orange and the fruit juices, WMS1 was effective in causing complete inhibition of yeast growth up to 100 μg/ml for apple juice and Fanta Orange and up to 50 μg/ml for the cranberry juice (Figure 9). In white wine, over 24 h, WMS1 caused a visible reduction in the yeast growth for all of the concentrations tested (Figure 8); however, after 48 h, *Z. bailii* was fully inhibited only at 2 and 1 mg/ml. In salad dressing, the antiyeast effect of WMS1 was visible only after 48 h of exposure and only for the highest concentration tested (2 mg/ml). In maple syrup, WMS1 was incapable of preventing the growth of *Z. bailii* at any of the concentrations tested.
Figure 9. Growth of *Z. bailii* in different beverage matrices exposed to 200, 100, 50, 25 and 0 µg/ml of WMS1. Tests were conducted in triplicate (bars represent standard deviation).

Table 2. Summary of the food trials with WMS1 incorporated in several foodstuffs along with different concentrations of WMS1; pHs and yeast *Z. bailii* inoculum concentrations are reported. The N/A the symbol means non applicable (not tested). All tests were conducted in triplicate.
4. Discussion

The protein known as Allergen Sin a 1 has been mainly studied for its allergenic potential, and it is indeed considered a major allergen (Sharma et al., 2019). The primary biological function described for Sin a 1 is as a nutrient reservoir; however, several proteins belonging to the same family (2S albumins, called Napins in Brassicaceae plants) have been studied for their in vitro antimicrobial properties, and defense against phytopathogens has been suggested as secondary biological function. This work reports the first description and characterisation of Sin a 1 antimicrobial potential. The protein was purified and labelled WMS1 in Chapter 3, WMS1 was identified as an isoform of Sin a 1 in Chapter 4, and for simplicity and continuity with the rest of the work, Allergen Sin a 1 is here called WMS1.

WMS1 antimicrobial potential was evaluated against 13 microorganisms, including two bacteria, two filamentous fungi, and nine yeasts. WMS1 showed antifungal and antiveast activity but no antibacterial activity was detected. Among the most sensitive strains, the type strain of the yeast S. cerevisiae (DSM 70449), the spoilage yeast Z. bailii Sa 1403 and the phytopathogenic fungus F. culmorum FST 4.05 had the lowest MIC ranges reported (50–100 µg/ml). Results obtained are in agreement with data on the antifungal activity of other Napin proteins, e.g. in the work by Thery et al. (2020), a Napin protein from broccoli seeds (Brassica oleracea var. italica) showed a MIC of 37 µg/ml against F. culmorum, while the Napin protein from rapeseeds meal (Brassica napus) showed an IC50 value of 70 µM against the fungi Fusarium langsethiae (Nioi et al., 2012).

The two most sensitive yeasts strains (S. cerevisiae DSM 70449 and Z. bailii Sa 1403) were used to characterise the antiveast activity of WMS1 further. Experimental findings suggested that the WMS1 mode of action involved yeast membrane permeabilisation as it caused leakage of cytoplasmatic components. Additionally, the membrane permeabilisation and cytoplasm displacement rate were positively correlated to the quantity of WMS1 present. A colony count assay confirmed that the protein’s antiveast potency over time depended on the amount of protein, suggesting a dose-dependent relationship. In general, a faster yeast inhibition was observed in samples exposed to high doses of WMS1; 400 µg/ml was lethal after only 4 h, while in the 100 µg/ml (MIC) sample, yeast cells were still present after 6 h. Similar results were obtained with a total nucleotide leakage assay where it was determined that a high dose of WMS1 induced greater leakage of intracellular nuclear acids. Yeast cells exposed to high concentrations of WMS1 for 5 h showed membrane damage, suggesting that membrane
permeabilisation was a killing mode of action for the protein. The antifungal mechanism of other Napin proteins has been associated with membrane damage (Terras et al., 1993); moreover, the report of Neumann et al. (1996) indicated a possible involvement of the amphipathic α-helical structure of Brassicaceae Napins in CaM (calmodulin) antagonism; thus these proteins possess the potential for forming pores on the fungal membrane.

A cytotoxicity assay against human CaCo2 cells and a haemolytic assay against sheep's red blood cells showed that WMS1 is not harmful to mammalian cells at concentrations toxic for several fungal strains. WMS1 was proven to be a stable protein as it retained its antiyeast activity when exposed to various pHs. In a previous report, Napin from Brassica napus showed minimal structural changes at different pHs (Krzyzaniak et al., 1998). Conversely, WMS1 showed salt sensitivity; however, only a one-fold loss in the MIC levels was recorded in the presence of 5 µM of MgCl₂, 50 and 150 µM of KCl.

In this chapter, the antiyeast activity of the Napin protein WMS1 (isoform of Allergen Sin a 1) from white mustard seed has been explored, and data collected confirmed the antifungal potential of Napins from Brassicaceae. The antifungal potency, the non-toxic nature against mammalian cells and the structure stability are desirable features for possible biotechnological applications. In this prospect, WMS1 was applied in a series of beverages and foodstuff to evaluate its potential as a natural preservative against spoilage yeast. Most interesting, protein concentrations of 50-100 µg/ml were able to prevent the growth of the spoilage yeast Z. bailii in several acidic beverage matrices such as soft drinks (Fanta Orange) and fruit juices (apple and cranberry); these types of sweet products represent ideal substrates for yeast growth (Deak, 2007). In contrast, WMS1 was applied in more complex matrices as white wine, salad dressing and maple syrup; however, higher concentrations of WMS1 (1 and 2 mg/ml) were necessary to induce inhibition of Z. bailii in white wine and salad dressing, while no yeast inhibition was in maple syrup samples. It can be assumed that viscous matrices (e.g., maple syrup) are less favourable compared to liquid systems for WMS1 to exhibit its antiyeast activity or they represent better substrates for Z. bailii growth.

Even if food trials data suggest a potential use of WMS1 in beverage systems, the industry application of this protein would be limited by its allergenic nature. In general, other potential applications involving humans must be excluded; however possible use in agriculture can be explored. Additionally, the WMS1 sequence can be investigated within in silico tools to determine novel antifungal AMPs.
Bibliography


Chapter 6

Design and production of a recombinant WMS1-derived protein in *Escherichia coli* and evaluation of its antiyeast activity
Abstract

Napin and Napin-like proteins are an interesting group of seed storage proteins; endogenous Napins can display desirable features for potential biotechnological applications such as a remarkably stable structure, solubility in water, low molecular weight, antifungal and antimicrobial activity, protease inhibitor properties and little cytotoxicity. Thus far, several studies have focused on the recombinant production of this type of protein, and often the cloned protein has shown properties comparable to the native one. In this work, the white mustard Napin Sin a 1 was successfully cloned in a pET28a-MBP vector and expressed in an E. coli BL21 expression system. The recombinant form of Sin a 1 is labelled R-WMS1 since it was obtained using the primary sequence of WMS1 which has been proved to be an isoform of Sin a 1 in Chapter 4. The recombinant Napin protein R-WMS1 showed activity against the yeast Zygosaccharomyces bailii Sa 1403, similar to the native form of WMS1 which has previously isolated from white mustard seeds (Chapter 3).
1. Introduction

Seed storage proteins (SSPs) play a crucial role in the seedlings development by providing amino acids and other essential nutrients and participating in the plant defense against pathogens. The SSPs Napin and napin-like belong to the 2S albumin family, and they have been associated with several defensive mechanisms, including antifungal, antimicrobial, trypsin inhibitor, and calmodulin antagonist activity (Nioi et al., 2012 and Neumann et al., 1996). The endogenous white mustard (Brassica hirta) Napin protein WMS1 (which is an isoform of Allergen Sin a 1) showed in vitro antifungal, antiyeast and trypsin-inhibition properties; additionally, it was non-toxic against mammalian cells, and its antiyeast activity was retained in different environmental conditions (Chapter 5).

Recombinant protein production can represent a safer and cost-effective alternative to extracting proteins from natural sources; indeed, heterologous gene expression systems can be exploited to produce large quantities of pure proteins. Several studies are available on the recombinant production of Sin a 1; its cloning has been carried out using both Escherichia coli (González De La Peña et al., 1996) or Pichia pastoris (Palomares et al., 2005) as expression host, and generally, the recombinant protein presented comparable structural, immunological and protease-inhibitor properties of the native one. Moreover, the Napin-like protein of Momordica charantia showed similar antifungal activity both in the native and recombinant form (Vashishta et al., 2006). To our knowledge, no recombinant form of mustard Napin protein has ever been tested for antimicrobial activity.

The amino acid sequence of antifungal mustard protein WMS1 (isolated from seeds materials in Chapter 3 and already identified as isoform of Sin a 1 in Chapter 4) was used to develop a cloning strategy carried out by GenScript (Leiden, The Netherlands). For the sake of continuity, in this chapter, the protein Sin a one will be labelled as WMS1. The main scope of this work is to clone and express WMS1 in an E. coli host system and to assess if the recombinant form of WMS1 (R-WMS1) retained similar antiyeast properties recognised for the native protein.
2. Material and Methods

2.1. Sequence optimisation

The amino acid sequence of WMS1 (Chapter 3) was translated to a nucleotide sequence and analysed with various bioinformatics programs to optimise expression levels. The sequence was checked for the presence of common restriction enzyme recognition sites using "REDseq", and functional sequences such as cis-regulatory elements, Rnase splice sites or nucleotide repeats were checked using the DSOpt sequence analysis tool (GenScript, Leiden, The Netherlands). Additionally, the GC content was measured using the R studio programs, "seqinr" and "ape". Calculations were made using the following AT/GC ratio equation:

\[ GC(\%) = \frac{G+C}{A+T+G+C} \times 100 \]

2.2. Gene design, synthesis and cloning

The synthetic gene was designed as a fusion construct to maximise protein recovery. The designed gene included enzyme restriction sites at the construct’s ends (HindIII site at 5’ gene end and XhoI site at 3’ end of the gene), His tag composed of six histidine residues, the sequence of *E. coli* maltose-binding protein (MBP) to function as a tag and as a solubilising agent, a TEV cleavage site composed of the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly (ENLYFQG) and the optimised WMS1 nucleotide sequence. The construct’s DNA was chemically synthesised (GenScript Handbook, 2019) and then inserted into the HindIII (AAGCTT) and XhoI (CTCCAG) cloning sites of the pET28a plasmid vector (Figure 1). Recombinant plasmids containing the synthesised gene were transformed in *E.coli* BL21 Star™ (DE3) competent cells, and transformants were cultured onto Luria-Bertani (LB) agar plates (with Kanamycin antibiotic) at 30°C until colonies appeared.
Figure 1. Plasmid vector pET28a. The positions of its promoters, open reading frames, primers, terminators and restriction enzyme sites are highlighted in cyan, green, red and blue. Black arrows highlight HindIII and XhoI sites.

2.3. Expression and purification of the fusion protein

A single transformant colony was inoculated into LB broth medium (with 50 μg/ml Kanamycin), and cultures were incubated at 37°C with shaking at 200 rpm. Once the cell density had reached OD=0.6-0.8 at 600 nm, 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was introduced to promote the induction. Two IPTG induction conditions were tested: IPTG for 16h at 15°C (slow induction) and 4 h at 37°C (fast induction), and both were compared with a control without IPTG. Following expression, cells were harvested by centrifugation and lysed by sonication. After cell disruption, the cell lysate was centrifuged and filtered to achieve a clear supernatant. The presence of the fusion protein was
confirmed by SDS PAGE (12%) and Western blot analysis. Upon confirmation of its presence, the fusion protein was isolated through affinity chromatography (amylose column).

2.4. R-WMS1 Recovery

The fusion protein sample was digested with a His-tagged TEV (Tobacco Etch Virus) protease, which recognises the TEV site (ENLYFQG) present on the fusion protein and cleaves between the Gln and Gly residues. The sample was then run through a Nickel column to trap the MBP and TEV protease, which both have a His-tag. The WMS1 was collected in the flow through. Purity and the concentration of the final product were assessed by SDS PAGE and Bradford method, respectively.

2.5. Antiyeast assay

The antiyeast activity was measured with a colony count assay against the yeast Zygosaccharomyces bailii Sa 1403. R-WMS1 was stocked in a storage buffer composed of 50 mM Tris-HCl, 150 mM NaCl and 10% glycerol (pH 8.0), and this buffer was used as the designated medium for this test. Samples of 2 ml were prepared in the buffer mentioned above; a sample containing 100 µg/ml of R-WMS1, a sample with 100 µg/ml of control protein (native WMS1 isolated protein product from Chapter 3) and a negative control sample with water instead of protein products. Yeast cells were cultivated in Malt Extract (ME) Broth overnight at 30°C with gentle shaking, washed twice in PBS and inoculated in each sample at the concentration of 1x10^4 CFU/ml. Samples were incubated at 30°C for 6 h; each hour, 100 µl of each suspension was spread onto Yeast Extract-Peptone-Dextrose (YPD) Agar plates, in duplicate. After 48 h of incubation (30°C), the colonies formed on each plate were counted.

3. Results and discussion

3.1. Sequence optimisation

The nucleotide sequence for R-WMS1 was obtained from the native amino acid sequence (Fig 2) as determined in Chapter 3 of this thesis. The amino acid sequence comprises 143 residues divided into two subunits (small chain of 39 residues and large chain of 91 residues) and a thirteen amino acid linker peptide. Like other Napin proteins, mature endogenous WMS1
possesses a heterodimeric structure due to a complex posttranslational process in which several sequences are proteolytically cleaved from a larger single precursor. The final cleavage would be at the linker peptide region. Artificial generation of this heterodimeric structure from the \textit{E. coli}-generated recombinant peptide was not possible. Also, a report from Palomares \textit{et al.} (2005) suggested that the presence of the linker peptide is crucial during the recombinant production of mustard Napin to shape the protein into the correct folding, and its presence afterwards had little effect on the physiological features of the cloned protein. Thus, it was decided to express R-WMS1 as a single chain with large and small chains joined together by the linker peptide as shown.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sequence.png}
\caption{Nucleotide and amino acid sequence of the natural form of the WMS1 polypeptide. It is divided into three regions namely the 39-amino acid small peptide (blue), the thirteen amino acid linker peptide (black) and the 91-amino acid large peptide region (red).}
\end{figure}

The WSM1 GC content was determined to be 62.47\%. This result was close to being out of the recommended range for PCR amplification (40 to 60\% GC content); therefore, a more precise
representation of the distribution of guanine and cytosine residues throughout the sequence was necessary. The sequence was divided into 42 "strings" of 10 nucleotides each, the GC content was calculated for each string (percentage values were converted in a 0 to 1 scale), and each GC value obtained was plotted against its respective nucleotide position. Results are shown in Figure 3; the first ten nucleotides have a GC content of 1 with ten other frames with a GC content above 0.8, and the lowest score recorded was 0.2 between residues 401 and 410. It was necessary to note these regions of high GC content, as they could cause complications during the amplification of the WSM1’s nucleotide sequence, which was avoided by increasing the annealing temperature to 95°C during the gene synthesis step.

**Figure 3.** Distribution of guanine or cytosine residues within WMS1’s nucleotide sequence. Each point on the graph represents ten nucleotides of WMS1’s sequence. Eleven out of 42 dots were recorded with high GC content (over 0.8).

It was observed that WMS1’s cis-regulatory elements had already been removed from its sequence. Also, the WMS1 nucleotide sequence was noticed to contain three total repeats which had the potential to introduce secondary structure complexes, using the GenScript proprietary DSOpt sequence analysis tool, such complications were ruled out. The sequence
was also checked for restriction sites that might compromise cloning (Figure 4), and it was confirmed that neither XhoI nor HindIII was present. Besides noting the GC content, no other sequence optimisation was required for WMS1.

Figure 4. Common restriction enzymes sites detected on WMS1’s nucleotide sequence to be avoided during cloning.

3.2. Gene design, synthesis and cloning

The synthetic gene designed for R-WMS1 cloning (Figure 5) was planned to encode a fusion protein consisting of R-WMS1 and MBP-His tagged peptides linked by the cut site sequence for TEV protease. The fusion protein gene was generated synthetically from stepwise addition of multiple oligonucleotide fragments, which were then enzymatically assembled via polymerase chain assembly to form the complete gene (Genscript). The correct linear DNA was cloned into the pET28a vector using HindIII and XhoI restriction and ligation enzymes, which were built into the termini of the synthetic gene. In pET28a, the enzyme cut sites (or restriction sites) for HindIII and XhoI are adjacent to the Lac operon site and downstream of the T7 promoter. E.coli BL21 cells were transformed with the recombinant vector and cultivated on LB agar plates.
3.3. Expression and purification of the fusion protein

After IPTG induction of cells, samples of cell lysate, supernatant of cell lysate and pellet of cell lysate, collected from cultures exposed to fast and slow inducing conditions, were tested for the fusion protein presence with SDS-PAGE Western blot assays (Figure 6).

The calculated molecular weight for the fusion protein was 60.04 kDa, and, as expected, a prominent protein band of ~60 kDa was evident on the gels (Figure 6), indicating that the fusion protein was stable and soluble outside the cytoplasmic environment. Indeed, quality control tests (GenScript) indicated a good level of solubility (>50%). The protein presence was detected in all the samples induced with IPTG but not in the non-induced controls. The best-expressed condition was when bacterial cells were induced with IPTG for 16 h at 15°C (slow induction). SDS-PAGE showed a protein of the predicted size of 60 kDa (Figure 6). The expression level was 20 mg/L.

The fusion protein was then purified via affinity chromatography exploiting the attraction between the MBP protein and the amylose resin. The immobilised MBP tag-fused recombinant proteins were then eluted by the addition of maltose to the column. The ammino acid sequence of the fusion protein is reported in Figure 7.
Figure 6. SDS-PAGE (left) and Western blot (right, using anti-His antibody (GenScript, Cat. No. A00186)) analysis of the cloned and expressed fusion protein (highlighted by an arrow). Lane M1: Protein marker, Lane M2: Western blot marker, Lane PC1: BSA (1μg), Lane PC2: BSA (2μg), Lane NC: Cell lysate without induction, Lane 1: cell lysate with induction for 16 h at 15 °C (best condition), Lane 2: cell lysate with induction for 4 h at 37 °C, Lane NC1: Supernatant of cell lysate without induction, Lane 3: Supernatant of cell lysate with induction for 16 h at 15 °C, Lane NC2: Pellet of cell lysate without induction, Lane 5: Pellet of cell lysate with induction for 16 h at 15 °C, Lane 6: Pellet of cell lysate with induction for 4 h at 37 °C.
3.4. R-WMS1 Recovery

The eluted protein was cleaved with TEV protease and subjected to another round of affinity chromatography resulting in the retention of the MPB region (and also the TEV protease) on the Nickel column. The R-WMS1 component was eluted and analysed by SDS-PAGE under reducing conditions (Figure 8). It was observed that the protein was not isolated to homogeneity, having a final purity value of ≥ 35%. Nevertheless, there was a prominent band at ~16 kDa corresponding with the R-WMS1 protein predicted size of 15946.4 Da. The single band appearance of R-WMS1 confirmed its production as a single polypeptide. The R-WMS1 sequence was composed of 144 amino acid residues and resulted identical to endogenous WMS1 plus an initial Gly residue derived from the TEV proteolytic cut (Figure 9). The isoelectric point (pI) of R-WMS1 was calculated as 8.60, similarly to the native WMS1 pI of 8.50. In the end, the final amount of recovered R-WMS1 was calculated with the Bradford method, and, in total, 500 µg of protein was recovered (from 1 L of bacterial culture).
3.5. Antiyeast assay

After exposure to R-WMS1 and native WMS1 as a control (100 µg/ml) for 6 h, a yeast (Z. bailii) colony count assay was performed to evaluate the antiyeast potential using $10^4$ cells/ml. Results are displayed in Figure 10. At the beginning (time 0 h), a similar number of colonies was counted in all the samples (between 750 and 830 CFU/ml). Throughout the 6 h, steady but slow growth was observed in the control sample (water instead of protein), and it reached $1.8 \times 10^4$ cells/ml. Samples with recombinant and native WMS1 followed a very similar trend to each other; after 6 h of incubation, the number of yeasts cells present were halved compared to time zero ($4.0 \times 10^3$ CFU/ml for WMS1 sample and $3.9 \times 10^3$ CFU/ml for the R-WMS1 sample). The activity results for WMS1 are in line with the findings reported in Chapter 5 for *Saccharomyces cerevisiae* DSM 70449. It must be noted that the medium utilised in this assay...
differs from those typically used in susceptibility tests (reason mentioned earlier). Nevertheless, Tris-HCl has previously been used as an alternative buffer for antifungal sensibility tests (de Sousa et al., 2021). To conclude, under the conditions of the experiment, the R-WMS1 exhibited similar anti-yeast potency against Z. bailii as WMS1 despite the differences in their primary structures, with the former composed of a single chain and an extra initial Gly residue compared to the heterodimeric native WMS1.

Figure 10. Yeast colonies count assay demonstrating the rate of Z. bailii inhibition caused by 100 µg/ml concentration of native and R-WMS1 compared to the control with water. The test was carried out in triplicate (bars represent standard deviation)
4. Conclusion

Mustard seeds are known for possessing antimicrobial properties; they have been used as natural food preservatives for decades. Recently, interest has gathered around purified mustard protein products (Rahman et al., 2020). Our group isolated the endogenous protein Sin a 1 from white mustard seeds, specifically a new isoform of the protein which was labelled WMS1. WMS1’s potent antifungal activity has been characterised (Chapter 3, 4 and 5) and in this chapter, it was successfully produced in a recombinant form (R-WMS1) as a single polypeptide from an E. coli B12 expression system using the plasmid pET28a as the cloning vector. E. coli expression systems are one of the earliest and most commonly used hosts for heterologous protein production in both laboratory and industrial settings; advantages include rapid growth, fast expression, ease of cultivation, and high product yields (Ma et al., 2020). Expressing antimicrobial plant proteins in an E. coli system could be used as a fast, secure and low-cost alternative for obtaining higher quantity of product compared to purifying endogenous products from plant sources. The recombinant form of WMS1 was obtained in soluble form with an extraction concentration of 0.5 mg/L. Significantly, the recombinant form of Sin a 1 (R-WMS1) showed comparable antiyeast properties to endogenous Sin a 1. This study confirms the antiyeast potential of the recombinant white mustard Napin proteins. The strategy used in this study may thus be employed for recombinant expression of other Napin and Napin-like proteins.
Bibliography


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Chapter 7

Antiyeast activity by ultra-short synthetic peptides bioinspired to the mustard Napin protein *Sin a 1*

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Food loss caused by the growth of spoilage yeast strains represents a challenge for the food industry; in this respect, synthetic AMPs have been suggested to overcome this problem. Novel ultra-short AMPs can be found within the sequence of larger antimicrobial proteins. This chapter provides a straightforward workflow for the prediction and characterisation of this type of molecule using online free servers. The protocol was applied, and seven ultra-short peptides were designed from the white mustard antifungal protein WMS1 (an isoform of the protein known as Sin a 1). SinA-AMPs were chemically synthesised and tested for their activity against several spoilage strains. Among the various synthetic SinA-AMPs, the peptide labelled SinA-pepIII (FGIPKCRKEF) had the best in vitro antiyeast activity and it was further characterised. SinA-pepIII caused yeast inhibition via membrane permeabilisation and cytoplasm displacement mechanisms; it was found to be pH- and heat-stable and generally safe in terms of human consumption. In addition, up to 3.2 µg/ml of the peptide completely prevented the growth of the spoilage yeast Zygosaccharomyces bailii in a fruit juice matrix. In conclusion, SinA-pepIII could be employed to prevent yeast spoilage outbreaks. The work proposed in this chapter can be used as proof of principle and lay the foundation for future research and development of novel food preservatives against yeast spoilage microorganisms.
1. Introduction

Food loss due to fungal spoilage is a global problem; using preservatives and a clean manufacturing environment mitigates food spoilage caused by many fungal species. However, certain spoilage yeasts are characterised by extreme resistance to preservatives and can persist and grow in storage environments (e.g., products frozen or preserved with weak acids) hostile to unspecialised microorganisms (Wareing and Davenport, 2007). Moreover, several chemical preservatives that are extensively used in the food industry to improve the shelf life of food products have been associated with possible side effects (Devi et al., 2008). It is thus imperative that we move toward natural and safer alternatives to address the area's specific needs.

Antimicrobial peptides (AMPs) are important constituents of the innate immune system of all living organisms. Endogenous AMPs from plants, also called Botanic Antimicrobial peptides (BAMPs), are described as low molecular weight proteins with cationic properties; most commonly, they act by interfering and disrupting the microbial membrane structure, leading to cell lysis (Lei et al., 2019). Nevertheless, a remarkable quality of many BAMPs is that they show antimicrobial activity against different microorganisms via multiple mechanisms and can have numerous intracellular biological targets that are distinct from targets of the traditional antimicrobial molecules. Thus, BAMPs could represent novel and natural alternatives to common preservatives; however, in many cases, poor solubility, low stability, time-consuming extraction methods, and cost of production are limiting the use of native BAMPs in food applications (Ahmed and Hammami, 2019).

In this prospective, synthetic AMPs (SAMPs) chemically designed based on naturally occurring antimicrobial proteins are increasingly attracting more attention; SAMPs have emerged due to various advantages, such as potent activity, low production cost and no or very low toxicity (Shwaiki et al., 2021). SAMPs can be engineered for better efficacy, or de novo designed to include functionally interesting features in their structure (Vishweshwaraiah et al., 2021). In particular, ultra-short SAMPs have been considered for possible biotechnological applications due to their enhanced biocompatibility, biodegradability and ease of synthesis and modification (Eckhard et al., 2014; Carratalá et al., 2020). Most of the efforts, thus far, have focused on specific applications in therapeutics and have largely overlooked the potential of these molecules in food preservation.
In this study, seven ultra-short synthetic AMPs have been designed based on the amino acid sequence of the mustard Napin protein WMS1 (isoform of Allergen Sin a 1), a naturally occurring antifungal protein. SinA-AMPs were designed to meet the most desirable prerequisites of an effective, low-cost antiyeast preservative agent (e.g., low molecular mass, positive charge and high hydrophobicity, high stability and low toxic potential against mammalian cells). Subsequently, SinA peptides were chemically synthesised, and their activity against five food spoilage yeasts, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* was investigated. In addition, the most promising SinA peptide was further characterised, and its mechanism of antiyeast action was studied alongside its stability under different conditions. The safety and incorporation of the peptide into a food matrix was also explored.

2. Material and Methods

2.1. Rational design of Sin a 1 ultra-short AMPs

Synthetic peptides used in this work were designed using as a template the antimicrobial Napin protein from mustard WMS1 (isoform of Allergen Sin a 1); the whole amino acid sequence (Figure 1) was used excluding residues between H-109 and Y-115 where the epitope of allergenicity lies (Shim and Wanasundara, 2008). Guidelines of Souza et al. (2020) were followed to develop and design SinA-AMPs and all the software and servers employed are freely available online. At the start, WMS1 sequence was loaded into the database CAMP\textsubscript{R3} (http://www.camp.bicnirrh.res.in/predict_c/), this tool used four antimicrobial predictor algorithms to perform *in silico* cleavage on WMS1 sequence, and potential AMPs of 10, 11 and 12 residues were obtained. At this stage, potential AMP sequences were processed for their overall quality with the Antimicrobial Peptide Database (APD3) tool (https://wangapd3.com/design/design_improve.php) and their antifungal potential with the iAMPpred tool (http://cabgrid.res.in:8080/amppred/); the iAMPpred tool computes a score ranging between 0 and 1 and values near to 1 indicate a peptide with high probability to present activity. In general, screening analysis led to the selection of potential AMPs with the following features: positive net charge, Boman index \( \leq 2.5 \), total hydrophobic ratio \( \geq 40\% \) and \( > 0.7 \) scores for the antifungal potential. An additional screening was carried out to identify Sin a 1 AMPs with the best chemical and physical-biological properties. Desirable characteristics
include an alkaline Isoelectric Point, a total mass <1300 Da, no allergenic potential, no haemolytic potential, no potential to penetrate human cells, only 1 or 2 cysteines residues (to avoid unnecessary disulphide bonds formation), high stability, a half-life <2 s in the intestine environment and predicted cleavage points for trypsin and pepsin. The in silico tools used are listed here: CellPPD (http://crdd.osdd.net/raghava/cellppd/multi_pep.php), Predicted Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl), HemoPI (http://crdd.osdd.net/raghava/hemopi/design.php) and HLP (http://crdd.osdd.net/raghava/hlp/interactive.htm). The most promising peptides (7) were chemically synthesised (GLBiochem Ltd., Shanghai) with >90% purity, as indicated by the supplier. The workflow of this protocol is presented in a schematic way in Figure 2.

**Small chain (1-39):**
PAGPGIPKRCKEFQQAQLRCQQLHLKQAMQSGGPS

**Linker peptide:**
LALYGEFEDD--MEN

**Large chain (55-145):**
POGPQRPLQQCLCNHELHDEELVCPTKGAASKVQQVQVLQQGQQGPQQHVISRYQVTATHLPKVCNPQVSVPFKKTMPGPS

*Figure 1.* The amino acid sequence the protein *Allergen Sin a 1*. Composed of two bio-functional chains. The sequence highlighted shows the allergenic epitope and it was excluded from the AMPs rational design.

### 2.2. Antiveast potency of synthetic AMPs

The minimum inhibitory concentration (MIC) of peptides was determined using a micro broth dilution method following the guidelines of the "EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts" (Rodriguez-Tudela, 2008). Tests were performed in a flat-bottom 96-well microtiter plate (Sarsdedt, Nümbrecht, Germany). The method is based on the preparation of serially diluted solutions of the peptide under investigation in 100 µl volumes / well with a yeast inoculum also in a volume of 100 µl. The concentration of yeast cells was calculated using a haemocytometer (Improved Neubauer Counting Chamber, Sigma Aldrich). The synthetic peptides solutions were serially diluted in Sabouraud-Dextrose (SD) broth to produce six different concentrations of each peptide (800 µg/ml to 25 µg/ml). The inoculum was prepared in SD broth from an overnight culture adjusted to 5x10^5 cells/ml. Controls contained dH₂O
instead of peptide solution. The plates were incubated for 48 h at 28 °C in a microtiter plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) with gentle shakes between readings. The optical density (600 nm) was measured at 2 h intervals. The MIC was determined as the lowest concentration of peptide required to inhibit yeast growth and confirmed by subsequently spotting 100 μl of yeast/peptide suspension from the microtiter plate onto SD agar and incubated at 30 °C for 48–72 h, depending on the optimal incubation time of the yeast. *Saccharomyces cerevisiae* DMS 70449, *Zygosaccharomyces bailii* Sa 1403 and *Debaryomyces hansenii* CBS 2334 yeast colonies were counted after 48 h while *Kluyveromyces lactis* ATCC 56498 and *Zygosaccharomyces rouxii* ATCC 14679 were incubated for 72h. If the agar plate showed either no growth or less than three colonies, the peptide activity is fungicidal; more colonies categorise the peptide as fungistatic. Unless stated otherwise, all chemicals and kits used in this work were purchased from Sigma Aldrich (MO, USA).

### 2.3. Characterisation of SinA-pepIII

SinA-pepIII was selected as the best peptide; it was subjected to further characterisation of its antiyeast activity, and its potential as a preservative agent was also evaluated.

#### 2.3.1. Colony count assay

The time required for SinA-pepIII to impact *Z. bailii* growth was assessed with a colony count assay as described by Shwaiki *et al.* (2020) Briefly, a yeast suspension of $10^4$ CFU/ml was inoculated with 50, 100 and 200 μg/ml concentrations of the peptide and incubated for 6 h. At 1 h intervals, 100 μl of the suspensions were spread onto SD agar plates and subsequently incubated for 48 h at 30 °C. The control consisted of a yeast suspension with dH₂O without peptide.

#### 2.3.2. Total nucleotide leakage

The total nucleotide leaked from *Z. bailii* cells after exposure to SinA-pepIII was measured following the protocol by Shwaiki *et al.* (2020). Yeast cells from an overnight culture were washed twice in phosphate buffered saline (PBS) before being resuspended at $10^4$ cells/ml
concentration. 50, 100 and 200 µg/ml of the peptides were added to the yeast solution and incubated at 30 °C. After 4 h, yeast cells were removed from samples through filtration with a 0.22 µm filter, and the OD at 260 nm was measured for each concentration. Positive and negative controls of 0.1% Triton X-100 and dH2O were used, respectively.

2.3.3. Membrane permeabilisation assay

The ability of SinA-pepIII to permeabilise the membrane of the yeast Z. bailii was studied as a possible antiyeast mechanism of action. Propidium iodide (PI) is a fluorescent dye that can bind nucleic acids; since it is excluded from viable cells, the bond occurs only when the yeast cell membrane is damaged. The protocol by Canelli et al. (2020) was followed with some modifications. Briefly, 10^6 cells/ml suspensions (500 µl) were prepared in PBS from an overnight culture and exposed to 50, 100 and 200 µg/ml of the peptide. Yeast cells treated with 0.1% Triton-X and water were used as positive and negative controls, respectively. After 5 h incubation at 30 °C, the cells were washed in PBS twice. Subsequently, samples were incubated in dark conditions for 5 min at room temperature in conjunction with 200 µl of PI (6 µM). Then, treated cells were washed twice again with PBS, and the pellets were resuspended in 250 µl of PBS, and 4 µl of each sample was visualised with the microscope EVOS®FL Auto Imaging System (Life Technologies - Thermo Fisher Scientific, MA, USA). Images were captured at 40X magnification under the fluorescent channel RFP edited with phase contrast (531/40 nm excitation; 593/40 nm emission).

2.3.4. Resistance to proteolytic digestion

In order to evaluate SinA-pepIII safety for consumption, its resistance to proteolytic digestion was tested with α-chymotrypsin, a common digestive enzyme found in the human gut. The experimental protocol (Shwaiki et al., 2019) was designed to mimic conditions that the peptide may face after ingestion. Briefly, peptide samples were incubated with α-chymotrypsin at different peptide:enzyme molar ratios of 60:1, 250:1, 2500:1, for 4 h at 37 °C. Afterwards, α-chymotrypsin was inactivated by heat (80 °C for 10 min), and an antiyeast growth curve assay against Z. bailii was carried out with digested protein at concentrations of 50, 100 and 200
µg/ml. The enzyme was stored in solution in a digestion buffer consisting of 50 mM Tris–HCl (pH 7.4) and 5 mM calcium chloride (CaCl$_2$).

2.3.5. Haemolytic assay

SinA-pepIII ability to cause rupture of red blood cells was measured with relevance to its potential application as a food preservative. Following the indication of (Shwaiki et al., 2019), a 4% solution of defibrinated horse erythrocytes (Analab, Lisburn, Northern Ireland) was incubated for 1 h at 37 °C in conjunction with 50, 100 and 200 µg/ml concentrations of SinA-pepIII. Samples incubated with 0.1% Triton X-100 and PBS were used as positive and negative control. Following a centrifugation step (1000 g x 10 min), the supernatant of the samples was transferred into a 96-well plate, and OD at 405 nm was measured. The percentage of haemolysis was calculated using the formula below:

$$\% \text{ Haemolysis} = \frac{(A_{405 \text{ protein treatment}}) - (A_{405 \text{ PBS}})}{(A_{405 \text{ 0.1% Triton X-100}}) - (A_{405 \text{ PBS}})}$$

2.3.6. Peptide stability

SinA-pepIII inhibitory activity against *Z. bailii* was tested in different conditions simulating environments that may be encountered in food and beverage products; specifically, 50, 100 and 200 µg/ml peptide concentrations were tested for salt sensitivity, resistance to heat degradation and stability in a range of pHs. Salt solutions of 1 and 5 mM MgCl$_2$ and 50 and 150 mM KCl were added to the growth medium (SD broth), and a micro broth dilution antiyeast assay (as described in section 2.2.) was carried out, including controls of modified broth, yeast and no peptide. To determine if SinA-pepIII is resistant to thermal degradation, a peptide sample was heated for 15 min at 100 °C and left to cool for 30 min before performing the antiyeast assay. The effect of different pH on the peptide was conducted by changing the pH of the SD broth used to perform the antiyeast assay. pH 3, 5, 7, 9 and 11 were tested by adjusting the media with 0.1 M hydrochloric acid and 1 M sodium hydroxide. Controls of media modified to the different pH containing the yeast and no peptide were used.
2.3.7. Application in a beverage matrix

The ability of SinA-pepIII to inhibit yeast growth was evaluated in cranberry juice, pH 3.05 (Kelkin, SuperValu, Ireland) spiked with Z. bailii. Due to their high sugar and salt content, fruit juices and soft drinks represent an ideal substrate for the growth of this spoilage strain (Leyva Salas et al., 2017). The methodology of choice was a microtiter plate assay using filter sterilised beverage inoculated with $10^3$ cells/ml yeast, and the yeast growth was monitored over 48 h with OD readings of 600 nm every 2 h. SinA-pepIII was tested at 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml concentrations; controls consisted of the beverage inoculated with yeast but without peptide.

2.4. Data presentation

All the tests were performed in triplicate, and results in the tables and graphs are presented as means ± standard deviation.
3. Results

3.1. Rational design of *Sin a 1* ultra-short AMPs

Using the CAMP$_{R3}$ tool, 154 sequences classified as AMPs were predicted within the amino acid sequence of the antifungal protein WMS1; 21 sequences presented low Boman index, positive charge, and high hydrophobicity and high antifungal potential. Additional *in silico* tools were used to screen potential AMPs, and 7 sequences were selected for further *in vitro* tests (Table 1). The chemical and physical-biological properties of the 7 selected peptides include alkaline PI, small mass, no allergenic or toxic potential and high stability to proteolysis in the intestinal environment, with a half-life smaller than 2 s in the intestine environment and presence of pepsin and trypsin cleavage sites. Peptides under investigation were chemically synthesised and stored (-20°C) in dH$_2$O at a 2 mg/ml concentration.

![Figure 2. Workflow for the design of the 7 SinA-AMPs from the primary sequence of the protein WMS1.](image-url)
Table 1. The seven *Sin a 1* ultra-short peptides and their chemical and physical-biological features.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence a</td>
<td>VCPTLKGA</td>
<td>VCPTLKGA</td>
<td>FGIPKCRKEF</td>
<td>LCVCPTLKGA</td>
<td>AGPFGIPKCR</td>
<td>VCPTLKGA</td>
<td>VCFFKKTMPG</td>
</tr>
<tr>
<td>Position in <em>Sin a 1</em></td>
<td>78-87</td>
<td>78-88</td>
<td>5-14</td>
<td>76-85</td>
<td>2-11</td>
<td>78-89</td>
<td>132-141</td>
</tr>
<tr>
<td>Antifungal potential b</td>
<td>0.94</td>
<td>0.94</td>
<td>0.93</td>
<td>0.93</td>
<td>0.92</td>
<td>0.86</td>
<td>0.82</td>
</tr>
<tr>
<td>Antibacterial potential b</td>
<td>0.97</td>
<td>0.96</td>
<td>0.94</td>
<td>0.81</td>
<td>0.92</td>
<td>0.94</td>
<td>0.86</td>
</tr>
<tr>
<td>Net charge c</td>
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<td>+2</td>
<td>+2</td>
<td>+3</td>
<td>+2</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>Total hydrophobic ratio c</td>
<td>40%</td>
<td>45%</td>
<td>40%</td>
<td>40%</td>
<td>40%</td>
<td>50%</td>
<td>40%</td>
</tr>
<tr>
<td>Boman index c</td>
<td>0.4</td>
<td>0.2</td>
<td>1.97</td>
<td>1.2</td>
<td>0.76</td>
<td>-0.14</td>
<td>0.2</td>
</tr>
<tr>
<td>Cell penetrating potential d</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
<td>Non-CPP</td>
</tr>
<tr>
<td>Allergenic potential e</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>MW (Da) f</td>
<td>1003.4</td>
<td>1074.4</td>
<td>1224.6</td>
<td>1004.4</td>
<td>1045.4</td>
<td>1173.6</td>
<td>1107.5</td>
</tr>
<tr>
<td>Haemolytic potential f</td>
<td>no (0.5)</td>
<td>no (0.45)</td>
<td>no (0.49)</td>
<td>no (0.49)</td>
<td>no (0.48)</td>
<td>no (0.47)</td>
<td>no (0.49)</td>
</tr>
<tr>
<td>Half-life intestine (s) g</td>
<td>1.178</td>
<td>1.178</td>
<td>1.856</td>
<td>1.091</td>
<td>1.764</td>
<td>1.178</td>
<td>1.976</td>
</tr>
<tr>
<td>Stability g</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
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<tr>
<td>Cleavage points for trypsin g</td>
<td>2</td>
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<td>2</td>
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</tr>
<tr>
<td>Cleavage points for pepsin g</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Data obtained from the following free online tools: a CAMP3; b iAMPpred; c APD3; d CellPPD; e Predicted Antigenic Peptides; f HemoPI; g HLP
3.2. Antiyeast potency of synthetic AMPs

Two out of seven peptides tested showed *in vitro* antiyeast activity (Table 2). SinA-pepI was able to inhibit the growth of the yeast *Z. bailii* and *D. hansenii* only at the high concentration of 800 µg/ml, while SinA-pepIII showed MIC level of 50-100 µg/ml against *Z. bailii*, 100 µg/ml against *D. hansenii* and 400 µg/ml against *S. cerevisiae*. At the MIC, SinA-pepIII was found to have fungicidal action against *Z. bailii* and *S. cerevisiae*, while it was fungistatic against *D. hansenii*.

Table 2. MICs for the seven synthetic peptides against selected yeasts. Tests were conducted in triplicate.

<table>
<thead>
<tr>
<th></th>
<th><em>K. lactis</em></th>
<th><em>D. hansenii</em></th>
<th><em>Z. bailii</em></th>
<th><em>Z. rouxii</em></th>
<th><em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>SinA-pepI</td>
<td>No inhibition</td>
<td>800 µg/ml Fungistatic</td>
<td>800 µg/ml Fungistatic</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>SinA-pepII</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>SinA-pepIII</td>
<td>No inhibition</td>
<td>100 µg/ml Fungistatic</td>
<td>50-100 µg/ml Fungicidal</td>
<td>No inhibition</td>
<td>400 µg/ml Fungicidal</td>
</tr>
<tr>
<td>SinA-pepIV</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>SinA-pepV</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>SinA-pepVI</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>SinA-pepVII</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

3.3. Characterisation of SinA-pepIII

SinA-pepIII showed the highest antiyeast potency among the seven synthetic peptides under investigation. It was subjected to a further characterisation of its antiyeast activity and its mode of action was examined. Moreover, SinA-pepIII was evaluated as potential food preservative; characterisation studies included: evaluation of peptide stability in different environmental conditions, ability to cause direct harm toward mammalian cells, sensibility to proteolytic digestion and application in food. Investigations were carried out on the spoilage yeast *Z. bailii* which was the most sensitive to the peptide.
3.4. Colony count assay

The colony count assay evaluated the impact of SinA-pepIII on *Z. bailii* cells over time. After 6 h incubation, yeast cells were still present at all the peptide concentrations tested (50, 100 and 200 µg/ml); however, yeast growth was heavily decreased in all peptide samples compared to the control with water that reached $4.2 \times 10^4$ CFU/ml (Figure 3). The 200 µg/ml sample showed a slow and steady decline in yeast cell number; in the 100 µg/ml sample, the decline was visible after 4 h and after 5 in the 50 µg/ml sample.

![Figure 3](image)

*Figure 3.* Yeast colony count assay demonstrating the rate of *Z. bailii* inhibition caused by 50, 100 and 200 µg/ml concentrations of SinA-pepIII. In the presence of the peptide, yeast growth was always inhibited compared to the control, which showed a steady growth increase over 6 h. Test was conducted in triplicate (bars represent standard deviation).

3.5. Total nucleotide leakage

Total nucleotide leakage assay was performed to study the consequences that SinA-pepIII has on the integrity of yeast cells; this was done by measuring the total amount of nucleotide released from *Z. bailii* solutions exposed to 50, 100 and 200 µg/ml of the peptide. The highest concentration (200 µg/ml) tested gave a substantially higher OD (0.147) compared to the
negative control (0.02); while the OD measured for the 50 and 100 µg/ml samples was 0.097 and 0.110, respectively (Figure 4).

Figure 4. Total nucleotide leakage from yeast cells caused by the presence of SinA-pepIII at different concentrations as determined by spectrophotometric analysis of filtered Z. bailii cultures at 260nm. Test was conducted in triplicate (bars represent standard deviation).

3.6. Membrane permeabilisation assay

SinA-pepIII possible antiyeast mechanism of action was studied by observing, via fluorescent microscopy, its ability to cause damage to the yeast membrane. The peptide's potential to cause permeabilisation can be detected by the capability of the PI dye to enter permeabilised cells. The peptide displayed permeabilisation activity against the membrane of Z. bailii at all the concentrations tested compared to the control with water (Figure 5). Similar emitted florescence was visually observed for the 100 and 200 µg/ml samples, both concentrations of the peptide are fungicidal for Z. bailii. The level of permeabilisation was lower at reduced (50 µg/ml) concentration of peptide.
Figure 5. Fluorescent microscopic analysis of *Z. bailii* cells after treatment with SinA-pepIII in the presence of Propidium Iodide dye. Yeast membrane disruption was observed at all peptide concentrations tested (50, 100 and 200 µg/ml). The RFP channel indicates the location of fluorescent (and damaged) cells. Red fluorescence in the Overlay option (right) indicates damaged cells compared to the sample without peptide (water control). Tests were conducted in triplicate.

3.7. Resistance to proteolytic digestion

SinA-pepIII safety for human consumption was studied by looking at the peptide’s ability to be digested by α-chymotrypsin, a proteolytic gut enzyme. Results are shown in Figure 6. The peptide was found to resist proteolysis at the enzyme’s lower concentrations (peptide:enzyme molar ratio of 2500:1 and 250:1). At these concentrations of α-chymotrypsin, SinA-pepIII was able to cause yeast inhibition at 200 µg/ml (double the MIC) but not at its MIC level (100 µg/ml). At the higher concentrations of the enzyme (60:1), the peptide was fully degraded as,
even at the highest SinA-pepIII concentration tested of 200 μg/ml, no inhibition of *Z. bailii* was observed.

Figure 6. The antiyeast activity of SinA-pepIII after digestion with α-chymotrypsin is reduced compared to the MIC levels (50, 100 and 200 μg/ml of non-digested SinA-pepIII are lethal for *Z. bailii*). Tests were conducted in triplicate (bars represent standard deviation).
3.8. Haemolytic assay

This assay was carried out in order to observe the peptide's potential to rupture mammalian red blood cells and therefore characterise its safety in terms of human consumption. The haemolytic activity of SinA-pepIII against erythrocytes was determined at different concentrations of the peptide (Fig. 7). All the peptide’s concentrations tested showed less than 10% haemolysis; at the highest concentration of 200 μg/ml, the percentage of haemolysis was observed to be approximately 8%, while at 100 and 50 μg/ml it was 1.9% and 0.9%, respectively.

![Graph showing haemolysis percentage](image)

**Figure 7.** Percentage of haemolysis showed by 50, 100 and 200 μg/ml of SinA-pepIII. Test was conducted in triplicate (bars represent standard deviation).

3.9. Peptide stability

SinA-pepIII stability in salt concentrations, different pHs and high heat was tested to evaluate if its antiyeast activity is retained under conditions that may be encountered if the peptide was applied as a food preservative. SinA-pepIII antiyeast activity was affected by the presence of salts; among all the conditions examined, Z. bailii growth was inhibited only at 200 μg/ml peptide in 1 mM of MgCl$_2$ (Figure 8.A). Subjecting SinA-pepIII to heat treatment did not influence its activity (Figure 8.B), as no yeast growth was recorded at any of the peptide concentrations under investigation (50, 100 and 200 μg/ml). SinA-pepIII inhibitory activity was maintained at pH 3, 5 and 7, with 50, 100 and 200 μg/ml concentrations of peptide fully...
inhibiting yeast growth. At pH 9 and 11, no yeast growth was observed in the controls (Figure 8.C).

![Figure 8](image)

**Figure 8.** Spectrophotometric analysis of cultures of *Z. bailii* after 48 h in the presence of 50, 100 and 200 µg/ml of SinA-pepIII at different concentrations of MgCl₂ and KCl (A). Heat treatment of the SinA-pepIII prior to addition to the yeast cultivated over the 48 h did not inactivate the peptide (B). SinA-pepIII retained its activity at different pHs (C). Tests were conducted in triplicate (bars represent standard deviation).

### 3.10. Application in a beverage matrix

To evaluate the quality of SinA-pepIII as a potential food preservative, an antiyeast assay was carried out in filter sterilised cranberry juice inoculated with *Z. bailii* (10³ cells/ml) and serially
diluted concentrations of the peptide (1.562, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml). The yeast growth was monitored in a microtiter plate; in Figure 9, results are reported as the OD (600 nm) measurements, after 48 h incubation, minus the blank (only cranberry juice). Up to 3.125 µg/ml concentrations of peptide prevented yeast growth compared to the control without peptide.

![Figure 9](image.png)

**Figure 9.** Up to 3.125 µg/ml of SinA-pepIII inhibited the growth of 10^3 cells/ml of Z. bailii in cranberry juice. Test was conducted in triplicate (bars represent standard deviation).

### 3. Discussion

Throughout documented history, man has always profited from biochemical products of yeast metabolism. Yeasts provide us with the biotechnological conversion processes needed to make bread, wine and other alcoholic beverages. Nevertheless, under certain circumstances, yeast growth in food and beverages can result in spoilage outbreaks compromising the products’ visual appearance, texture, taste, and aroma (Fleet, 2011). It has been suggested that the integration of AMPs derived from plants (BAMPs) in food and beverage goods could limit the occurrence of yeast spoilage outbreaks (Thery *et al.*, 2019). In addition, BAMPs application in foods can be advantageous in terms of consumer perception as their natural origin might be seen as a more environmentally friendly and healthier option when compared to current preservatives (Ahmed and Hammami, 2019).
Members of typical BAM families are characterised by having less than 100 amino acid residues and a molecular weight not greater than 10 kDa (e.g., defensins and thionins); however, slightly larger protein families (e.g., thaumatin-like and 2S albumins) can show BAMP properties such as a cysteine-rich structure and antimicrobial activity through membrane permeabilisation. The Napin protein *Sin a 1* (WMS1) from white mustard (*B. hirta*) seeds is a 2S albumin that possesses *in vitro* antiyeast activity, low cytotoxicity towards mammalian cells and a highly stable structure (Chapter 3, 4 and 5); unfortunately, the biotechnological potential of WMS1 is limited by its allergenic properties. The application in industrial sectors of native BAMPs can suffer from other drawbacks such as laborious extraction protocols requiring expensive purification equipment or difficulties in obtaining a sufficient amount of purified product. In this regard, chemically synthesising BAMPs can be advantageous over natural extractions, as large quantities of pure protein can be obtained rapidly (Shwaiki *et al.*, 2021). Most interestingly, recent advances in the bioinformatics field have facilitated for synthetic BAMPs to be rationally designed and thus generated with predicted more desirable features than native ones (e.g., shorter sequence, enhanced antimicrobial activity or reduced allergenic potential). These *de novo* rationally designed BAMPs can be derived from amino acid sequence modification of the original peptide or formed by sequences found within the primary structure of larger antimicrobial proteins (Vishweshwaraiah *et al.*, 2021).

In this work, seven novel BAMBs were designed bioinspired on the 2S albumin *Sin a 1*; moreover, SinA-AMPs here described were ultra-short (less than 12 amino acid residues), a beneficial feature that would contain the cost in case of large-scale production. In general, all the generated SinA-AMPs were predicted to be antimicrobial, not toxic and safe for consumption. Among the various SinA-AMPs, SinA-pepI and SinA-pepIII were the only ones to show *in vitro* antiyeast activity, and SinA-pepIII was the most promising, as it presented the best features to behave as an antiyeast preservative agent.

SinA-pepIII displayed activity against the spoilage yeast strains *Z. bailii*, *D. hansenii* and *S. cerevisiae* with MICs of 50-100, 100 and 400 µg/ml, respectively. A colony count assay demonstrated the peptide killing efficacy against *Z. bailii*; after only 2 h of exposure, 50, 100, and 200 µg/ml of SinA-pepIII severely impacted the yeast growth compared to the control without the peptide. Moreover, *Z. bailii* cells treated for 5 h with the same amounts of SinA-pepIII showed permeabilisation in their plasma membrane, and the leakage of nucleotide material was detected. The peptide's antiyeast mode of action was verified using propidium
iodide as the indicator for detecting compromised plasma membranes. This assay confirmed that the SinA-pepIII cationic nature enabled interaction with the negatively charged yeast membrane lipids causing its disruption. SinA-pepIII application for use in human consumption can be assumed to be safe since the neutral nature of mammalian membranes renders them unaffected by this type of cationic peptide.

SinA-pepIII safety for human consumption was also evaluated in a simulated gastrointestinal environment. The peptide antiyeast potency was considerably reduced after being digested with the digestive gut enzyme α-chymotrypsin; this is an essential feature for a preservative agent (Shwaiki et al., 2020). It can be assumed that the peptide will be degraded after ingestion and thus not active after the human digestion process (Shwaiki et al., 2020). In this regard, the peptide was specifically designed to be generally stable in the intestinal environment but with a half-life smaller than 2 s and its primary structure contained two predicted cleavage sites for trypsin and one for pepsin. Another aspect of safety is the ability of the peptide to rupture red blood cells, representative of the peptide effect on human cells. The haemolytic assay determined less than 10% haemolysis at SinA-pepIII concentrations of 200 µg/ml and below, suggesting that the peptide would be safe if consumed.

Due to the proposed use of SinA-pepIII in food preservation, it is fundamental to evaluate if its antiyeast potency is maintained in different environmental conditions to which the peptide might be exposed. Notably, SinA-pepIII antiyeast activity withstood treatment at high temperature (15 min at 100°C), a standard process in the food industry; changing the pH of the growth medium did not influence the peptide ability to prevent yeast growth. Overall, SinA-pepIII concentrations of 50, 100 and 200 µg/ml were demonstrated to possess heat- and pH-stability. Unfortunately, the peptide showed salt sensitivity in the presence of salts as KCl and MgCl₂, a common drawback for many antifungal BAMPs (Chu et al., 2013) possibly due to structure modification, hydrophobicity reduction, or decreased electrostatic interaction induced by mono and divalent cations (Baldauf et al., 2013). A general loss in SinA-pepIII antiyeast potential was recorded; 200 µg/ml of the peptide were not sufficient to inhibit Z. bailii growth in the presence of salts such as MgCl₂ (5 mM) and KCl (50 and 150 mM).

Finally, SinA-pepIII potential as a food preservative agent was also evaluated in a beverage matrix as cranberry juice. Sugary fruit juices represent a favourable environment for the growth of Z. bailii (Davies et al., 2021), visible signs of spoilage (e.g., the appearance of slimy biomasses and off-putting odours) are usually noticeable when yeast growth reaches
concentrations of $10^3/10^5$ cells/ml (Leyva Salas et al., 2017). Up to 3.2 µg/ml of SinA-pepIII completely inhibited the growth of Z. bailii in cranberry juice samples spiked with $10^3$ cells/ml of yeast, suggesting that the peptide could be employed to prevent yeast spoilage outbreaks in this type of beverage products.

In conclusion, the synthetic peptide SinA-pepIII, a short fragment of the antifungal protein Sin a 1, possesses the potential to be used as a novel preservative to fight food loss caused by yeast. The peptide demonstrated potent activity against several yeast spoilage strains; it was pH- and heat- stable and possibly safe in case of ingestion by humans. Incorporating synthetic AMPs as food preservatives in an industrial-scale setting is not currently feasible due to the high costs associated with the chemical synthesis of proteins. However, the application of ultra-short antimicrobial AMPs combined with advances in technology could rapidly change this scenario, since the cost of synthesis mainly depends on the peptide length (Shwaiki et al., 2021). In this respect, the peptide SinA-pepIII could represent a low-cost and effective antiyeast preservative agent, thus this work demonstrates proof of principle for applying synthetic ultra-short BAMPs in food preservation.

Besides food preservation, ultra-short antimicrobial synthetic peptides such as SinA-pepIII can in principle be employed for a variety of applications, for instance, topical medical treatments or surface sterilisation. Moreover, this work describes a straightforward protocol that could be used by other research groups seeking new ultra-short AMPs from entire existing antimicrobial proteins and provides a deeper knowledge of the key determinants of the antiyeast activity of the protein Sin a 1.
Bibliography


General Conclusions

Spoilage caused by yeasts is often overlooked since there are limited foods and beverages where yeasts outcompete food-spoilage moulds or bacteria. However, yeast microorganisms can cause spoilage outbreaks in products with low pH, low water activity, and/or high carbohydrate content, for instance, acidic beverages such as soft drinks and fruit juices (Deak, 2007). Yeasts are ubiquitous microorganisms that form part of the microbiota of most if not all natural ecosystems, thus they are normally present in numerous types of foods and beverages; however, the selective pressure exercised by the clean environment in manufacturing facilities reduces the natural yeast diversity to a limited number of well-adapted species. The results of yeast spoilage of foods can be diverse (pectinolytic activity on fresh fruit and vegetables, appearance of a powdery or slimy coat on solid products, formation of films or unnatural turbidity in liquid goods), but all are effects of the yeast growth in the food and the consequent metabolic activity (Food and Beverage Spoilage Yeast, 1998; Fleet, 2011; Hernández et al., 2018). Spoilage of foods and beverages by yeast causes undesirable changes in food products, these changes are mostly of a sensory nature, and they are likely to be grossly underreported precluding a true estimation of the levels of yeast spoilage; however, the costs must run into millions, possibly billions of euros per year (Stratford, 2006; Krisch et al., 2016). All types of spoilage yeasts cause economic loss, their occurrence contributes significantly to the food loss phenomenon, making the prevention of undesirable growth of yeasts a crucial problem for the food and beverage industry (Fleet, 2011).

The plant innate immune system is one of the most significant natural sources of antimicrobial low-weight proteins presenting a broad spectrum of activities and multiple mechanisms of action; a large body of literature focuses on the discovery and characterisation of new antimicrobial agents from plants and online databases are constantly updated with novel sequences. The biotechnological potential of these types of molecules is virtually untapped; in this regard, the work discussed in this thesis supports the integration in foods of small proteins and peptides from plant origin to reduce the occurrence of yeast spoilage outbreaks.

Several plant defense-related small proteins/peptides families have been proven to be antifungal; however, specific data on their antiyeast activity are scarce, limited for the most part to the inhibition of *S. cerevisiae* and *C. albicans*. Therefore, Chapter 1 present a literature
General Conclusions

review with an exclusive focus on families with recognised antiyeast properties and their antiyeast mechanism of action. Many of the antiyeast proteins described had potent in vitro activity via membrane permeabilisation, low cytotoxic potential and high stability; thus, they are promising candidates for future industrial applications. Possible disadvantages include reduced antiyeast activity in salt-rich environments, difficulties in obtaining large amounts of pure protein from plant tissues and possible allergenic activity. However, the knowledge gained from the characterisation of these compounds in their native form and the development of bioinformatics tools can enable the generation and prediction of novel active antiyeast compounds that could be integrated into food and beverages.

The use of natural antimicrobials for food preservation, either as an ingredient or combined with traditional techniques, is not a new concept; spices and herbs have been used since ancient times to improve foods sensory characteristics and shelf life (Gottardi et al., 2016). In more recent times, few reports have discussed the potential of antimicrobial plant proteins/peptides as conservative agents, whereas natural (Thery, Lynch and Arendt, 2019) or synthetic (Shwaiki, Arendt and Lynch, 2021). In addition, antimicrobial plant proteins/peptides can be produced in recombinant form using host systems (e.g., E. coli). However, there is a lack of knowledge on the large-scale application of these types of compounds for food preservation.

Research involving BAMBs mainly had focused on the inhibition of food-borne pathogens and only a tiny number of bacteriocins (AMPs from bacteria) have been regulated for modern bio-preservation techniques (e.g., the nisin peptide isolated from the bacterium Lactococcus lactis) (Rai et al., 2016). Although data on the applicability of BAMPs in the food industry are scarce to non-existent, the work discussed in this thesis sustains the potential of BAMPs as potential novel preservative agents; nevertheless, the work is here presented with a future prospective. More research is needed before BAMPs can be awarded the GRAS (generally recognised as safe) status and be considered as antimicrobial additives by the food industry.

Since the development of solid-phase technology in the 1960s (Takashima, Vigneaud and Merrifield, 1968), the synthetic production of proteins has been continually improved. The first objective of this research was to test the activity of synthetic BAMPs against yeast spoilage strains. The chemical synthesis was carried out on Pharabitis nil antimicrobial peptides (Pn-AMP1 and Pn-AMP2) belonging to the hevein-type class discussed in the literature review, and the characterisation of the activity of the two peptides is presented in Chapter 2. Synthetic Pn-AMP1 possessed promising properties for a potential application as a preservative agent. It displayed fast inhibitory activity against the spoilage yeast strains K. lactis and Z. bailii,
General Conclusions

reducing their growth in UHT milk and Fanta Orange beverage. In addition, Pn-AMP1 resulted safe for consumption as concentrations found inhibitory for yeasts were neither haemolytic nor resistant to digestion by α-chymotrypsin. Natural and synthetic Pn-AMP1 mechanism of action involved the permeabilisation of the yeast plasma membrane. However, the antiyeast activity of synthetic Pn-AMP1 was weaker than its native form; this might be explained by the absence of native disulfide bonds, which are described as fundamental for hevein-type chitin-binding activity.

One of the major advantages of chemical synthesis over extraction from organic sources is the possibility of producing large amounts of compound with high purity; nevertheless, the current cost of synthesis (€100-600/gram, depending on the length and the chemistry used for synthesis) are prohibitive for industrial-scale applications. Over time, as the technology advances and the cost of synthesis begins to drop, the chemical synthesis of peptides could represent part of the solution for controlling food waste. Since the lack of familiarity in the general population toward food additives affects their acceptance, the use of synthetic BAMPs as bio-preservatives could be challenged by consumers’ perception of their chemical origin. Natural extracts are more likely to be approved, and the use of proteins directly isolated from plant tissues with limited use of chemical processes appears as the most consumer-friendly approach.

Mustard has been known as one of the oldest condiments and one of the most widely grown and multifunctional plants in the world for thousands of years; currently, the use of the seeds of the mustard species in the food and beverage industry is further growing due to their nutritional and functional properties. Mustards are commercially available as whole seeds, ground/cracked seeds, meals or flour forms and are widely used in condiments, salad dressings, pickles, sauces, processed meats, and plant-based substitutes for egg ingredients. Moreover, mustard seeds are known for their antimicrobial properties, and they have been used to provide protection of raw and processed foods against pathogenic and spoilage microorganisms (Rahman et al., 2020; Lietzow, 2021). It is known that mustard seed glucosinolates (a class of secondary metabolites) and their derived product isothiocyanates display antimicrobial and anticancer properties (Aarabi et al., 2021). However, it has been suggested that other seeds defense-related proteins/peptides contribute to mustard antimicrobial properties (Rahman et al., 2020); these proteins could represent an ideal novel and natural preservative agent.
In order to characterise antiyeast compounds involved in the activity of mustard seeds, a simple isolation protocol was applied to white mustard seeds (B. hirta), and a ~14 kDa protein with inhibitory activity against S. cerevisiae was selectively extracted. The protein product was labelled WMS1 and with the help of bioinformatics tools coupled with wet-lab techniques, WMS1 was identified as an isoform of the protein known as Allergen Sin a 1 belonging to the 2S albumins family (described in the literature review). Finally, WMS1 antimicrobial spectrum, antiyeast mode of action and potency was characterised for the first time. Chapters 3, 4, 5 and 6 respectively describe isolation, identification, characterisation and recombinant production (in an E. coli system) of WMS1. The protein WMS1 possessed several suitable properties for a green food preservative agent. It is present in a relatively high abundance in seeds (0.82-2.94 mg/g). Its purification required only one step of chromatographic separation; it displayed potent in vitro activity against spoilage yeast strains, and it successfully inhibited Z. bailii growth in several beverage matrices including fruit juices (cranberry and apple), soft drinks (Fanta Orange) and wine. Like most plant defense-related proteins, WMS1 mode of action involved yeast membrane permeabilisation as it caused leakage of cytoplasmatic components. Moreover, the protein did not show cytotoxicity towards mammalian cells and displayed resistance to heat denaturation and insensitivity to pH variations and the presence of salts. In addition, the recombinant form of WMS1 displayed similar antiyeast properties compared to the native one. Unfortunately, the high structural stability of Sin a 1 renders the protein unaffected by protease action in the digestive system, where it can provoke IgE-mediated allergic reactions in sensitive individuals. Thus, there are limited prospects for the biotechnological use of WMS1 in its native form.

Despite being an allergenic protein, WMS1 can promote the development of novel and beneficial antiyeast molecules since the amino acidic sequences of antimicrobial proteins can be scanned for the rational design of smaller antimicrobial peptides. Therefore, the objective of the work presented in Chapter 7 was the design and characterisation of antiyeast peptides bioinspired to WMS1. Among the various SinA-AMPs, a peptide called SinA-pepIII had desirable prerequisites of an effective and low-cost antiyeast preservative agent. SinA-pepIII demonstrated potent activity against yeast spoilage strains such as Z. bailii, D. hansenii and S. cerevisiae via membrane permeabilisation; in addition, it was pH- and heat- stable and potentially safe in terms of human consumption. Most notably, even small amounts of SinA-pepIII (3.2 µg/ml) prevented the growth of the spoilage yeast Z. bailii in a beverage matrix (cranberry juice). SinA-pepIII was composed of only 10 amino acid residues (FGIPKCRKEF);
ultra-short synthetic peptides could represent a solution that would contain the expenses of chemical synthesis. The design of these types of peptides is enabled by the availability of free and accessible databases and predictor servers; the potential of modern bioinformatics tools in peptides design is not limited to the discovery of ultra-short AMP sequences within larger antimicrobial proteins/peptides; indeed, they can be employed for the design of optimised peptides with improved activity and stability.

In conclusion, scientific interest in the possible biotechnological applications of natural AMPs and their synthetic and/or cloned derivates has dramatically increased during the last two decades. This thesis work explores the possible use of these types of molecules as antiyeast agents to be incorporated in food and beverages to overcome the phenomenon of food loss and waste. Preservatives have been incorporated into foods for centuries; however, modern preservation techniques mainly consist of temperature control, vacuum packaging, water content reduction, acidification and chemical preservation. Some yeast species are known to survive the acidification methods, and the use of chemicals has become unappealing for many consumers, creating a demand for more naturally occurring forms of food preservatives that are generally considered healthier and safer. Throughout the following decades, food preservation will change and evolve, antimicrobial agents will continue to be needed, and defense-related proteins/peptides (either in their native form or chemically synthesised) from plants could make their way into the market as plant-based alternatives. Both natural and chemical low molecular weight proteins discussed in this thesis' work are characterised by antimicrobial potential combined with low cytotoxic activity and, most outstandingly, a mode of action that would prevent the occurrence of resistant strains since it involves a lethal attack to the microorganism’s membrane integrity. In general, naturally extracted compounds are more likely to be accepted; however, they can present downsides such as laborious extraction methods and difficulties in obtaining sufficient amounts of pure product. Synthetic peptides bioinspired to naturally occurring proteins could represent a more feasible solution for large-scale applications; however, current high costs of synthesis and consumers' view on chemical protein production could limit their application as food preservative agents. The work carried out in this thesis lays the foundation for the future use of plant-derived antiyeast peptides and small proteins in the food industry; Table 1 provides a list of all the antiyeast molecules described in this thesis, Table 2 summarises the properties of the three potential preservative agents discussed (synthetic Pn-AMP1, naturally extracted Sin a 1, and ultra-short chemically synthetised SinA-pepIII), while and Table 3 concerns all the preliminary food trials conducted.
Future objectives

The research conducted during this project represents a foundation for the future development of plant-derived small proteins and peptides as novel classes of bio-preservatives. Protocols described in various Chapters could be employed to discover and characterise other candidates with biotechnological potential. Moreover, the preliminary analysis carried out here highlights how the food industry could benefit from the applications of these types of molecules as alternatives to the current methods of preservation; however, careful studies on natural, chemical or recombinant additives must be conducted to evaluate their overall safety since unknown problems may result from increased ingestion of such compounds. The approval of plant-derived antimicrobial peptides/proteins as bio-preservative by regulatory agencies such as the FDA (U.S. Food and Drug Administration) and EFSA (European Food Safety Authority) will rely on sufficient data on their cytotoxicity and behaviour in the gastrointestinal environment; thus, future objectives mainly regard the detailed exploration of the safety and toxicity of these plant-derived compounds. In general, the evaluation of a food additive involves the examination of the chemical structure and characteristics of the additive, including its specifications, impurities and the potential breakdown products in its intended use. Toxicological data from in vivo analysis and animal testing must be examined to identify and characterise possible health hazards; in addition, tests must be carried out using higher doses of the additive than would be used in food for humans and the data are then used to determine safe levels of intake for consumers (FSAI, 2015). Moreover, regulatory agencies normally require data on the potential exposure of consumers from the intended uses in foods in order to determine whether consumer intake could potentially be above the safe limit (Ahmed and Hammami, 2019; León Madrazo and Segura Campos, 2020; Shwaiki, et al., 2021; Soltani et al., 2021).
Table 1. The antyeast compounds presented in the thesis.

<table>
<thead>
<tr>
<th>Antiyeast protein</th>
<th>Origin</th>
<th>Yeast species inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pn-AMP1</td>
<td>Synthetic; sequence of native Pn-AMP1</td>
<td><em>Kluyveromyces lactis</em>, <em>Debaryomyces hansenii</em>, <em>Zygosaccharomyces bailii</em>, <em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>Pn-AMP2</td>
<td>Synthetic; sequence of native Pn-AMP2</td>
<td><em>Kluyveromyces lactis</em>, <em>Zygosaccharomyces bailii</em>, <em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td><em>Sin a 1</em></td>
<td>Naturally extracted from white mustard (<em>B. hirta</em>) seeds</td>
<td><em>Kluyveromyces lactis</em>, <em>Debaryomyces hansenii</em>, <em>Zygosaccharomyces bailii</em>, <em>Zygosaccharomyces rouxii</em>, <em>Saccharomyces cerevisiae</em>, <em>Candida albicans</em></td>
</tr>
<tr>
<td>R-WMS1</td>
<td>Recombinant form of <em>Sin a 1</em></td>
<td><em>Zygosaccharomyces bailii</em></td>
</tr>
<tr>
<td>SinA-pepI</td>
<td>Synthetic; ultra-short sequence present on native <em>Sin a 1</em></td>
<td><em>Debaryomyces hansenii</em>, <em>Zygosaccharomyces bailii</em></td>
</tr>
<tr>
<td>SinA-pepIII</td>
<td>Synthetic; ultra-short sequence present on native <em>Sin a 1</em></td>
<td><em>Debaryomyces hansenii</em>, <em>Zygosaccharomyces bailii</em>, <em>Saccharomyces cerevisiae</em></td>
</tr>
</tbody>
</table>
Table 2. Properties of the 3 antiyeast potential preservatives presented in the thesis.

<table>
<thead>
<tr>
<th></th>
<th>Pn-AMP1</th>
<th>WMS1</th>
<th>SinA-pepIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa residues</td>
<td>41</td>
<td>143</td>
<td>10</td>
</tr>
<tr>
<td>MW (kDa)</td>
<td>4.2</td>
<td>14</td>
<td>1.2</td>
</tr>
<tr>
<td>Antiyeast mode of action</td>
<td>Membrane permeabilisation</td>
<td>Membrane permeabilisation and cytoplasm displacement</td>
<td>Membrane permeabilisation and cytoplasm displacement</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>pH stability</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Salt sensitivity</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Haemolytic potential</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>α-chymotrypsin action</td>
<td>Completely digested</td>
<td>Resistant to digestion</td>
<td>Digested</td>
</tr>
</tbody>
</table>

Table 3. Summary of the most relevant preliminary test in foods. The ability of Pn-AMP1, WMS1 and SinA-pepIII of reduce (yeast are alive after 48 h but in reduced concentrations compared to the control) or prevent (yeast are dead after 48 h) spoilage yeast growth in different beverages systems. N. t. means “not tested”.

<table>
<thead>
<tr>
<th>Spillage yeast growth</th>
<th>K. lactis</th>
<th>Z. bailii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pn-AMP1</td>
<td>WMS1</td>
</tr>
<tr>
<td>UHT milk</td>
<td>Up to 62.5 µg/ml Reduced</td>
<td>N. t.</td>
</tr>
<tr>
<td>Fanta Orange</td>
<td>N. t.</td>
<td>Up to 62.5 µg/ml Reduced</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>N. t.</td>
<td>N. t.</td>
</tr>
<tr>
<td>Apple juice</td>
<td>N. t.</td>
<td>N. t.</td>
</tr>
<tr>
<td>White wine</td>
<td>N. t.</td>
<td>N. t.</td>
</tr>
</tbody>
</table>
Bibliography


Appendix i

Figure 1. Protein sequence coverage for WMS1, from the *de novo* sequencing report produced by the company Creative Proteomics (New York, USA).
Appendix iii – Dissemination

Journal Papers Accepted


Journal Papers in Review

Mignone, G. Shwaiki, L.N. and Coffey, A. “Inhibitory activity of two synthetic Pharabitis nil L. antimicrobial peptides against common spoilage yeasts”. Submitted to the journal “Applied food Research” ISSN: 2772-5022 (Currently under review)

Manuscripts submitted

Mignone, G. and Coffey, A. “Antiyeast activity and potential use as preservative agent by a short synthetic peptide bioinspired to the mustard Napin protein Sin a 1” submitted to the journal “BBA – General Subjects” ISSN: 0304-4165

Conference Abstracts


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