Haze Formation and Application of Insect-derived Peptidases in Wine Fining

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Giessen zur Sicherung gutter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation.

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Abstract

Wine haze is a challenge for the wine industry as it causes global losses of more than 1 billion US dollars per year. During transportation and storage, non-fined bottled wines undergo temperature variations, which can result in the aggregation of thermolabile proteins such as thaumatin-like protein (TLP) and chitinase (CHI). Protein flocculation in wines generates particles large enough to disperse light and produce haze. To prevent this, bentonite clay is still widely employed as a fining agent, even though its detrimental effects on wine sensory. Since peptidases (or proteolytic enzymes) are able to degrade haze proteins, they could be alternatively applied as fining agents, although their activity is strictly dependent on the wine pH and temperature. Therefore, the present thesis is focused on understanding the chemistry behind the haze formation and the degradation of thermolabile proteins by peptidases to propose analytical and technological solutions for that challenge. By using advanced methods of mass spectrometry (MS), such as top-down proteomics (peptidomics), distinct cleavage spots along the structure of TLP or CHI were identified, and this technique was proposed as an analytical tool for the screening of peptidases. In addition, the cleavage profile performed by peptidases could be evaluated directly on purified recombinant TLP and CHI by heterologously expressing them in the yeast *Komagataella phaffii*. Thus, pure recombinant rTLP and rCHI were used as haze protein models and applied in combination with MS-based peptidomics for the screening of suitable peptidases for applications in wine fining. Insect-derived peptidases were considered as promising enzymes able to successfully cleave haze proteins under acidic conditions. Insects are organisms well adapted to diverse ecological niches and dietary habits and, consequently, they produce a broad variety of digestive enzymes along their gastrointestinal system. The spotted wing fly *Drosophila suzukii* is a natural pest of vine grapes and was considered as a potential source of proteolytic enzymes able to cleave wine haze proteins. The larval *D. suzukii* evolves and feeds on nutrients inside mature berries and, therefore, produces peptidases with activity at the acidic pH of grapes. Acid peptidases from *D. suzukii*, candidates to cleave wine haze proteins, could be here confirmed by identifying digested peptides from recombinant rTLP and rCHI via MS-based peptidomics. The combination of methods described in the present thesis can be applied for the identification and application of suitable peptidases in winemaking.

Zusammenfassung

Weintrübungen gelten als eine besondere Herausforderung für die Weinindustrie, da sie weltweit Verluste von mehr als einer Milliarde US-Dollar pro Jahr verursacht. Während des Transports und der Lagerung unterliegen nicht geschönte, in Flaschen abgefüllte Weine Temperaturschwankungen, die zur Aggregation von thermolabilen Proteinen wie Thaumatin-ähnlichem Proteinen (TLP) und Chitinasen (CHI) führen. Durch die Ausflockung von Proteinen in Weinen entstehen Partikel, die groß genug sind, um das Licht zu streuen und Trübungen zu verursachen. Um dies zu verhindern, wird nach wie vor Bentonit als Schönungsmittel eingesetzt, auch wenn er sich nachteilig auf die Sensorik des Weins auswirkt. Peptidasen könnten als wirksame Schönungsmittel zum Abbau von Trübungsproteinen genutzt werden, obwohl ihre Aktivität stark vom pH-Wert und der Temperatur des Weins und der Zugänglichkeit der Proteine abhängig ist. Die vorliegende Arbeit konzentrierte sich auf das Verständnis der Chemie hinter der Trübungsbildung und dem Abbau von thermisch instabilen Proteinen durch Peptidasen, um analytische und technologische Lösungen für diese Herausforderung vorzuschlagen. Durch den Einsatz von modernen Methoden der Massenspektrometrie (MS), wie der *Top Down Peptidomik* wurden Schnittstellen entlang der Struktur von TLP oder CHI identifiziert und ihre Verwendung als analytisches Werkzeug für das Screening von Peptidasen vorgeschlagen. Gleichzeitig konnte das von den Peptidasen durchgeführte Hydrolyseprofil direkt an gereinigten TLP- und CHI-Proteinmischungen in Weinen bewertet werden, indem diese rekombinant durch die Hefe *Komagataella phaffii* exprimiert wurden. So wurden reine rekombinante rTLP und rCHI als Trübungsproteinmodelle verwendet und in Kombination mit *MS-Peptidomik* für das Screening von proteolytischen Enzymen eingesetzt. Insekten-Peptidasen werden als vielversprechende Kandidaten für die erfolgreiche Hydrolyse von Weintrubproteinen unter sauren Bedingungen angesehen, weil Insekten in der Lage sind sich an verschiedene ökologische Nischen und Ernährungsgewohnheiten anzupassen, indem sie eine Vielzahl von Verdauungsenzymen in ihrem Verdauungssystem absondern. Die Kirschessigfliege *Drosophila suzukii* ist ein natürlicher Schädling von Weintrauben und wurde als potenzielle Quelle für proteolytische Enzyme, die Weintrübungsproteine spalten können, in Betracht gezogen. Die Larven von *D. suzukii* entwickeln sich im Inneren reifer Beeren und ernähren sich dort von Nährstoffen, weshalb sie Peptidasen exprimieren, die bei dem sauren pH-Wert der Trauben aktiv sind. Die Hydrolyse von Trübungsproteinen durch Peptidasen aus *D. suzukii* (Larve) unter sauren Bedingungen wurde durch den Nachweis von Peptiden aus rekombinantem rTLP und rCHI mittels *MS-Peptidomik* untersucht. Die in dieser Arbeit beschriebene Methodenkombination kann zur

Zusammenfassung

Identifizierung von Peptidasen eingesetzt werden, die für die Weinherstellung geeignet sind.

Peer-reviewed original publications

1. Albuquerque W.; Seidel L.; Zorn, H.; Will, F.; Gand, M. (2021) Haze formation and the challenges for peptidases in wine protein fining. *Journal of Agricultural and Food Chemistry*, 69 (48), 14402-14414.
2. Albuquerque, W.; Ghezellou, P.; Li, B.; Spengler, B.; Will, F.; Zorn, H.; Gand, M. (2021) Identification of intact peptides by top-down peptidomics reveals cleavage spots in thermolabile wine proteins. *Food Chemistry*, 363, 130437.
3. Albuquerque, W.; Sturm, P.; Schneider, Q; Ghezellou, P.; Seidel, L.; Bakonyi, D.; Will, F.; Spengler, B.; Zorn, H.; Gand, M. (2022) Recombinant thaumatin-like protein (rTLP) and chitinase (rCHI) from *Vitis vinifera* as models for wine haze formation. *Molecules*, 27 (19), 6409.
4. Albuquerque, W.; Ghezellou, P.; Lee, K.Z.; Schneider, Q.; Gross, P.; Kessel, T.; Omokungbe, B.; Spengler, B.; Vilcinskas, A.; Zorn, H.; Gand, M. (2023) Peptidomics as a tool to assess the cleavage of wine haze proteins by peptidases from *Drosophila suzukii* larvae. *Biomolecules*, 13(3), 451.
5. Albuquerque, W.; Ghezellou, P.; Seidel, L.; Burkert, J.; Will, F.; Schweiggert, R.; Spengler, B.; Zorn, H.; Gand, M. (2023) Mass spectrometry-based proteomic profiling of a Silvaner white wine. *Biomolecules*, 13(4), 650.
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Allasia, V.; Coustau, C.; Keller, H.; Spengler, B.; Thines, E.; Kogel, K.H. (2022) A key regulator with a MIF-like domain orchestrates cellular differentiation and virulence in the fungal pathogen *Magnaporthe oryzae*. bioRxiv.

10. Seidel, L.; Albuquerque, W.; Happel, K.; Ghezellou, P.; Gand, M.; Spengler, B.; Zorn, H.; Will, F.; Schweiggert R. (2023) Composition, ζ potential, and molar mass distribution of 20 must and wine colloids from five different cultivars obtained during four consecutive vintages. *Journal of Agricultural and Food Chemistry*.

11. Babkina, V.V.; Albuquerque, W.; Haiduk, Y.M.; Michalak, W.; Ghezellou, P.; Zorn, H.; Zhuk, T. (2023) Fungal lyophilisates as catalysts for organic synthesis: Preparative oxidations with the white-rot fungus *Bjerkandera adusta*. SSRN.

Conference contributions

Presentations

1. Albuquerque, W.; Ruhl, M.; Gand, M.; Zorn, H. A Peptidase from the bug *Spilostethus pandurus* for food processing. In: 4th International INSECTA 2018, 2018, Giessen.
2. Albuquerque, W.; Rühl, M.; Gand, M.; Zorn, H. Peptidases from insects for biotechnological applications. In: 11th Annual GGL Conference 2018, Giessen.
3. Albuquerque, W.; Zorn, H.; Gand, M. Cleavage of wine haze proteins by peptidases from *Drosophila suzukii*. In: 14th Annual GGL Conference 2021, Giessen.

Posters

1. Albuquerque, W.; Rühl, M.; Gand, M.; Zorn, H. Peptidasen aus der Fruchtfliege *Drosophila suzukii* beugen Weintrübungen vor?
   In: LOEWE Zentrum für Insektenbiotechnologie and Bioressourcen, Jahresbericht 2020, Giessen.

List of abbreviations

AGP - Aspergillopepsin

CHI - Chitinase

MS - Mass Spectrometry

NMR - Nuclear Magnetic Resonance

OIV - International Organisation of Vine and Wine

PDB - Protein Data Bank

PR – Pathogenic-related

PTM - Post Translational Modification

PVPP - Polyvinylpolypyrrolidone

TLP - Thaumatin-like protein

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1. Synopsis

Haze formation in white wines and attempts to prevent its economic impact have been discussed by wine researchers and winemakers for decades. Although, little progress has been achieved in terms of large-scale applications. New technological approaches based on the combination of the flash pasteurization and peptidases have been recently approved by the International Organisation of Vine and Wine (OIV), as an example that efficient methods to remove haze proteins during winemaking are still to be achieved. Meanwhile, advanced methods in protein analysis based on high-resolution mass spectrometry and molecular biology have become more easily accessible. These technologies have enabled more precise analyses of intramolecular mechanisms that can be used as tools to unravel the mechanisms that prevent protein aggregation and precipitation in white wines.

Insects are organisms adapted to diverse habitats and life conditions and they are considered promising sources of peptidases with activity at acidic pH conditions and low temperatures. Many proteolytic enzymes are secreted along their digestive tract and play a key role in their nutrition. In particular, the spotted wing fly *Drosophila suzukii* insert eggs in different fruits such as grapes and its larvae develop inside the berries in an acidic environment. Therefore, they are considered as potential producers of acid peptidases for applications in winemaking.

The present doctoral thesis combines theoretical concepts and perspectives with the use of analytical and technological tools to propose new strategies to cleave wine haze proteins by peptidases at acidic conditions. Therefore, the application of techniques in biochemistry, molecular biology and mass spectrometry associated with an extensive literature review has provided a better understanding of how to find methods of wine fining using peptidases (particularly insect-derived) for the purposes of the wine industry.

1.1. Wine haze and thermolabile wine proteins

Wine is a worldwide consumed beverage with an economic, social, and cultural significance (Legras et al., 2007). Its transparency is an important sensory quality parameter and a demand from consumers (Ferreira et al., 2001). However, protein aggregation and the consequent haze formation (Fig. 1) affects the wine clarity and leads to the rejection of the product due to alleged poor-quality (Batista et al., 2009). To avoid protein flocculation, wines have to undergo clarification processes, which are normally performed by the addition of large amounts of bentonite clay (Benucci et al., 2016).

Due to the low wine pH (around 3-4), most wine proteins become positively charged and adsorb to the negatively charged surface of bentonite clay (Sauvage et al., 2010). However, bentonite fining generates detrimental effects on the wine organoleptic properties by removing polyphenols and aroma compounds at the same time (Sauvage et al., 2010; Benucci et al., 2016). Therefore, a high need for alternative fining agents exists, which should be safe, cost-effective and do not affect the wine sensory. Proteolytic enzymes or peptidases have been studied as fining agents aiming to degrade proteins during the winemaking process (Marangon et al., 2012), but only in combination with heating procedures sufficient haze reduction could be achieved. In general, the usage of fining agents is regulated by international reglementary committees or laws like the OIV, European Community (EC) Regulation (European Community, Commission Regulation-EC, 2005), Australian Wine Research Institute – AWRI and others. Until now, each clarification method presents positive and negative aspects and many of them are only partially implemented at industrial scale (Waters et al., 2005).

Figure 1. Differences between a hazy (a) and a clear (b) white wine caused by protein aggregation. [Picture made in laboratory conditions with a heated (a) and non-heated (b) unfined wine]

Wine haze formation is a consequence of protein-protein interactions of thermolabile wine proteins, which can aggregate at temperatures above 40 °C, typically during transport or storage. As soon as these aggregates have reached a diameter of about 1 μm, turbidity can be optically perceived (Marangon et al., 2011). The protein concentrations in white wines are diverse and depend on factors such as grape cultivar, weather, year of production and others. The concentrations can reach values between 10 and 500 mg/L (Marangon et al.,

2011). However, the formed haze does not only depend on the total protein content, but mainly on specific proteins which contribute to haze formation (van Sluyter et al., 2015). The two main haze-forming proteins groups reported in the literature are thaumatin-like proteins (TLPs) and chitinases (CHIs) (Robinson and Davies, 2000). These proteins are also known as pathogenic-related (PR) proteins, for being involved in the plant defense against infections caused by viruses, bacteria or fungi. PR proteins are reported in the literature as having a compact structure, which provides protection against proteolysis under acidic conditions (Waters et al., 1996). Consequently, these proteins survive the fermentation process of wine, and if not fined, remain present in the bottled product (Marangon et al., 2011). CHIs are enzymes able to hydrolyze chitin, which is an essential cell wall component of fungi (Hamid et al., 2013) and so they protect grapevines from fungal infections. For TLPs, the exact antifungal mechanism is unknown (Wang et al., 2010).

1.1.1. Thaumatin-like proteins (TLPs) and chitinases (CHIs): Thermolabile wine proteins

TLPs and CHIs have distinct structures, which influences their aggregation behavior. CHIs denature irreversibly at lower temperatures (55 °C) than TLPs (Falconer et al., 2010), which are denatured at about 62 °C (Falconer et al., 2010). For TLPs, different isoforms have been reported in the literature, like the 4JRU, 4L5H and 4MBT (Protein Data Bank – pdb) with the isoform 4JRU being the most thermolabile (Marangon et al., 2014). Marangon et al., (2014) associated the unstable character of the isoform 4JRU to its high hydrophobicity and the presence of a free loop, which can trigger protein-protein aggregations. CHIs of the class IV are considered as very unstable, because their structures consist mainly of α-helices and irregular structures, which can be irreversibly denatured (Chaudet et al., 2014). Many isoforms of CHIs are also reported in the literature with different levels of hydrophobicity, which would facilitate hydrophobic interactions (Derckel et al., 1996).

1.1.2 Wine haze formation

In general, protein aggregation is generated when non-fined wines are exposed to temperatures above 40 °C. The increased temperatures cause conformational changes of TLPs and CHIs, which aggregate in the presence of other wine matrix components such as polyphenols and sulfites (van Sluyter et al., 2015). Under denaturation, haze proteins gain different degrees of freedom, and their secondary and tertiary structures unfold. This allows the exposure of hydrophobic cores (which were previously buried in the protein structure)

to the protein surface and subsequently (hydrophobic) amino acids are able to interact with phenolic compounds or rearrange their disulfide bonds.

pH conditions also are essential for protein aggregation, since they can affect the net charge of proteins and facilitate electrostatic interactions. Wine pH values range typically between 3.5 and 4, conditions at which most proteins are positively charged (Dufrechou et al., 2013). These positive charges can induce changes in protein conformations and promote denaturation (Chi et al., 2003).

Wine matrix components play essential roles in the haze formation. Polyphenolic compounds, polysaccharides and sulfite are the main cofactors related to processes of protein aggregation in wines. Sulfite is a common additive in wines (Pocock et al., 2007), because of its antimicrobial and antioxidant properties (Chagas et al., 2018), and it is responsible for reordering of disulfide bonds through S-sulfonation (Chan 1968). The reorganization of disulfide bridges among polypeptide chains can permanently denature proteins or trigger cross-linking reactions among proteins (Marangon et al., 2011). After consecutive protein denaturation-renaturation processes, covalent bonds (disulfide bridges) are differently reordered, resulting in irreversible conformational changes. As a consequence, haze proteins can no longer renature. Polyphenolic compounds are reported as the main contributor to the haze formation in wines (Marangon et al., 2010). After denaturation, proteins expose binding sites or specific amino acids that interact with polyphenols, which can act as a bridge between two proteins or intra-polypeptide chains (Albuquerque et al., 2021a). Polyphenols are also reported to interact through π-stacking of phenolic rings, which may connect different proteins.

1.1.3. Fining agents in winemaking

Bentonite fining is still the most commonly used method of wine fining (Benucci et al., 2016). The negatively charged surface of bentonite (Fig. 2) works as a cationic agent that adsorbs the positively charged wine proteins at low pH (Dawes et al., 1994). However, bentonite fining also removes other compounds, such as biogenic amines, amino acids, polyphenols, and aroma compounds (Dawes et al., 1994), affecting the organoleptic properties of wines (Benucci et al., 2016). Apart from that, the use of bentonite can reduce the original wine volume by approximately 10%, which has an additional economic impact (Waters et al., 1992).

Figure 2. Bentonite clay powder (made with microscope Leica S9i, Leica, Wetzlar, Germany).

Alternative fining agents should be safe, cost-effective and must comply with international regulatory laws. Casein, gelatin, egg albumin and polyvinylpolypyrrolidone (PVPP) are fining agents used to remove phenolic compounds from wines, although they are often associated with allergenicity and impact on the wine sensory (Weber et al., 2007).

Peptidases have been studied for their application in winemaking processes to prevent haze protein in wines. Recently, the OIV approved the use of proteolytic enzymes in combination with a heating process or flash pasteurization. A mixture of aspergillopepsin I and II from *Aspergillus niger* (called Proctase) in combination with a flash pasteurization approach (short-time at high-temperature up to 90 °C followed by a cooling process) was successful applied in the prevention of wine haze in white wines (Marangon et al., 2012), although winemakers are still skeptical about its effects on the wine organoleptic properties (Bartowsky, 2009). Effective proteolytic enzymes, which do not need a pre-heating step are in demand for application in wine fining.

1.2. Peptidases

The hydrolysis of peptides bonds in biological systems is catalyzed by peptidases or proteolytic enzymes. These hydrolytic enzymes represent approximately 2% of the total number of proteins present in all types of organisms (Polgár, 2005). In all organisms, they perform the same basic function: the cleavage of a carbon-nitrogen bond between two amino acids in a peptide or protein (Rawling and Bateman, 2019). These enzymes hydrolyze peptide bonds in different ways and their functions depend on many physicochemical and protein structural factors. Peptidases are commonly referred as proteases or proteinases and they are responsible for cellular functions in almost all living organisms, such as nutrition, cell growth, cell differentiation and cell death (apoptosis),

intracellular and extracellular protein synthesis and degradation, as well as protein activation (Sotiropoulou et al., 2009). Protein hydrolysis in cellular reactions is regulated by restricting the activity of peptidases by inhibitors. Such peptidase inhibitors can be peptides, proteins, small molecules, or, as in the case of metalloproteases, chelating agents (Abbenante and Fairlie 2005).

1.2.1. Classification

Peptidases belong to the class of hydrolases (EC 3) and are classified based on three aspects (Rawlings and Barrett, 1993): a) chemical mechanism of catalysis; b) details of the catalyzed reaction, and c) molecular structure and homology. Recently, peptidases have been classified based on the nature of their active site and considering structural aspects and evolutionary relationships (Rawlings and Bateman, 2019). Thus, they were classified by the nucleophile or chemical species, which donate electron pairs, represented by a particular amino acid residue. Six different amino acid residues are typically reported to assist the hydrolysis of peptide bonds: serine (serine peptidases); threonine (threonine peptidases); aspartic acid (aspartic peptidases); glutamic acid (glutamic peptidases); cysteine (cysteine peptidases); and metal ions (metallopeptidases). Mostly, the complete hydrolysis reaction is assisted by a triad of amino acid residues (Dodson, 1998).

With over 50 families per subclass, the serine and cysteine peptidases represent two of the largest subclasses of peptidases (MEROPS database, Rawlings et al. 2017). There is an identifier for each peptidase based on its catalyst type: A (aspartic), C (cysteine), M (metallo), S (serine), P (mixed catalytic type) and U (unknown type). For example, trypsin, chymotrypsin and elastase belong to family S1 (serine) of clan PA (proteases of mixed nucleophile, superfamily A), while papain and its relatives are listed in family C1 (cysteine) of clan CA (papain family and others generally containing Cys/His catalytic dyad) (Polgár 2005). The MEROPS database (Rawlings et al. 2017) contains known information about peptidases, such as the name, organism source and hydrolysis reaction.

Peptidases can also be classified according to cleavage sites along the polypeptide chain of the protein substrate as: endopeptidases, exopeptidases, aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, tripeptidyl-peptidases, peptidyl-dipeptidases and dipeptidases (Rawlings and Bateman, 2019). A third method of classification of peptidases is based on the similarity of their three-dimensional structure and their evolutionary correlations are separated in "clans" (Rawlings and Barrett, 1993).

1.2.2. Chemical mechanisms of catalysis

Hydrolysis of peptide bonds by peptidases proceeds similarly for different enzymes from the basic steps: 1) activation of a water molecule; 2) activation of the carbonyl group of the substrate peptide; 3) formation of tetrahedral intermediate; 4) protonation of the leaving group (Hedstrom, 2002). The catalytic mechanisms of the triads supporting these 3 steps differ for different enzymes and they are better explained in the next sections for serine, cysteine and metallopeptidases.

1.2.2.1. Serine peptidases

The catalytic triad of serine peptidases is formed by a *Ser*, a *His* and an *Asp* residue (*Ser-His-Asp*) to support the hydrolysis of peptide bonds (Polgár, 2005). The triad is located in the cleft of the enzyme's active site and proceeds in its catalytic mechanisms as described by Hedstrom (2002) (Fig. 3).

A *Ser* residue attacks the carbonyl carbon of a peptide substrate (Fig. 3a), which is assisted by a *His* residue by yielding a tetrahedral intermediate (Fig. 3b). At the same time, a residue *Asp* stabilizes the His through hydrogen bonding. Then the next step includes the amine residue released from the peptide bond (NHR') and the formation of an acyl enzyme complex (Fig. 3c). The imidazole ring in the *His* residue is used for proton shuttle. During the catalytic reaction, serine peptidases activate a molecule of water as a weak nucleophile. Afterwards, the water molecule attacks the acyl enzyme complex, returning to a tetrahedral intermediate with oxyanion (Fig. 3d). Since this intermediate is very unstable, the hydroxyl group of the *Ser* finally detaches from the peptide substrate and a carboxylic acid is formed with release of the peptide substrate (Fig. 3e).

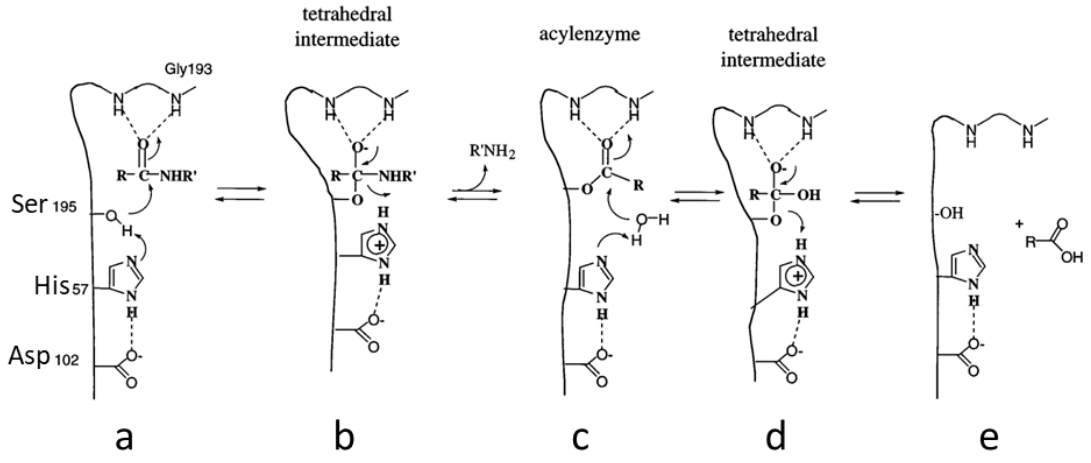

Figure 3. General mechanism of catalysis of a serine peptidase *Ser*: serine; *His*: histidine; *Asp*: aspartic acid [Reprinted with permission from Hedstrom (2002) Copyright 2023 American Chemical Society].

1.2.2.2. Cysteine peptidases

Cysteine peptidases catalyze the cleavage of peptide bonds (with high specificity to *Asp* residues) using the high nucleophilicity of a thiol group of a *Cys* residue (Stennicke and Salvesen, 1999) in its active site (Cstorer and Ménard, 1994). In cysteine endopeptidases, dyads (*Cys-His* pair) and not triads of amino acids assist the hydrolysis of peptide bonds (Stennicke and Salvesen, 1999).

Clark (2016) described the mechanism of reaction of cysteine endopeptidases as demonstrated in figure 4. The catalytic *Cys* residue donates a proton to a *His* residue (Fig. 4a). Subsequently, the sulfur atom of *Cys* attacks the carbonyl group of the peptide substrate, and a tetrahedral intermediate is formed (Fig. 4b). The amine residue of the peptide substrate is released after being protonated (Fig. 4c). When the molecule of water is added, a proton binds to the amine group of the *His* and the hydroxyl group attacks the carbonyl of the peptide substrate (Fig. 4c). This again creates a tetrahedral intermediate stage (Fig. 4d). Finally, the sulfur atom of *Cys* is protonated, a carboxylic acid is formed (with release of the peptide substrate), while *His* is reconstituted (Fig. 4e).

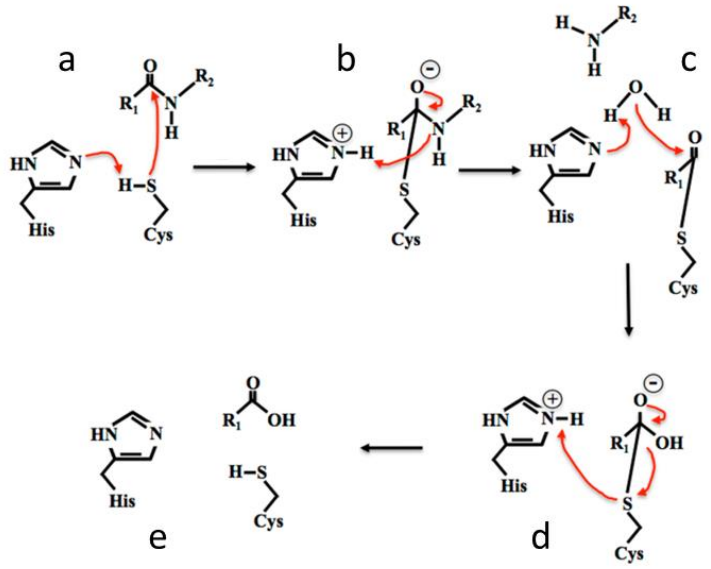

Figure 4. General mechanism of catalysis of a cysteine endopeptidase. *Cys*: cysteine, *His*: histidine [Reprinted (adapted) with permission from Clark (2016) Copyright 2023 American Chemical Society].

1.2.2.3. Metallopeptidases (MPs)

Metallopeptidases (MPs) cleave peptide bonds in a single-step reaction involving a solvent molecule, a general base and acid, and a catalytic metal site (mono or dinuclear) (Cerdà-Costa and Xavier Gomis-Rüth, 2014). Their active site contains a catalytic bivalent metal ion, which is mostly zinc, cobalt, manganese or nickel (Hooper, 1994). These metals are

anchored or attached in the active-site cleft of the enzyme by protein residue side chains (Cerdà-Costa and Xavier Gomis-Rüth, 2014). Since many of the catalytic zinc sites in metalloproteases have either two *His* and a *Glu* (or an *Asp*) or three *His* ligands, it is assumed that these residues play essential roles for the stability and catalytic function of the metal.

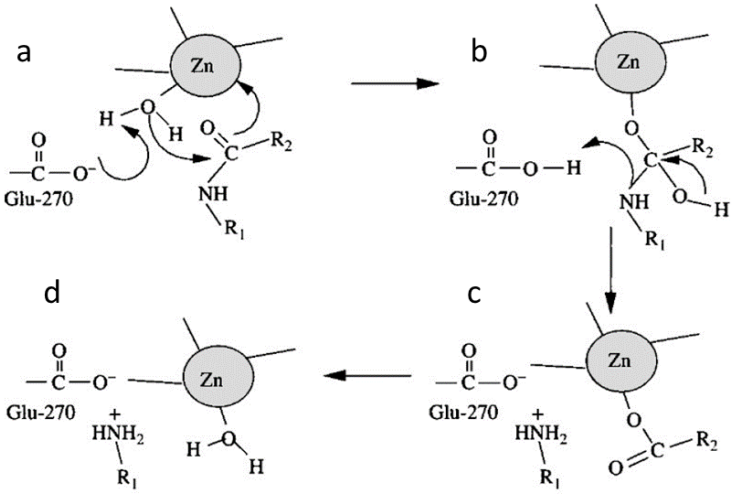

Figure 5. General mechanism of catalysis of a metallopeptidase. Zn: zinc; *Glu*: glutamate [Reprinted (adapted) with permission from Auld (2004) Copyright 2023 Elsevier].

The example described in figure 5 was proposed by Auld (2004) and uses a *Glu* residue in the catalytic site as the main residue responsible for the catalysis. In step *a* (Fig. 5a) the *Glu* residue acts by removing a proton from the metal-bound water, what allows the metal-bound hydroxide to attack the carbonyl group of the peptide substrate, forming a tetrahedral intermediate (Fig. 5b). Subsequently, *Glu* acts as an acid catalyst by donating a proton to the leaving amine (Fig. 5c). In the step *d* (Fig. 5d) the *N*-terminal product leaves, a molecule of water rebinds to the metal, and the formed carboxylic acid is released as part of the cleaved peptide substrate (Fig. 5d).

1.2.3. Peptidases in winemaking

Peptidases from fungal, bacterial, plant and animal origins have been tested as fining agents under wine physicochemical conditions (Albuquerque et al. 2021a). One important requirement is that such peptidases need to be functionally active at low pH and temperatures. Mostly, peptidases active under these conditions are aspartic, cysteine and glutamic peptidases. A mixture of glutamic peptidases from *Aspergillus* spp. (aspergillopepsin I and II), also commercially available as Proctase, was successful in reducing the amount of wine proteins by 20% (reduction of 20% of the total protein

content) (Marangon et al. 2012), but only when preceded by a flash pasteurization process.

Peptidases from microorganisms have also been considered as candidates to cleave wine haze proteins. A recombinant MpAPr1 aspartic peptidase from the yeast *Metschnikowia pulcherrima* could degrade wine proteins at moderate temperatures (25 °C) (Theron et al., 2017) and a peptidase produced by the yeast *Saccharomyces cerevisiae* could degrade wine proteins at 38 °C and pH 3.5 (Younes et al., 2011). Organisms, which are natural grape pathogens have been investigated as source of peptidases for application in wine clarification. BcAp8 peptidase from *Botrytis cinerea*, for example, could degrade CHIs, but only at 55 °C. In addition, plant peptidases like bromelain from *Ananus bracteratus* was able to reduce wine haze by 70% (reduction of 70% of the total protein content) (Benucci et al., 2014) and a porcine pepsin could cleave wine proteins at acidic pH (Pocock et al., 2003), but only after denaturation of the proteins. A list of peptidases previously investigated in winemaking can be found in Table 1.

1.3. Insects as sources of biomolecules

Insects are present in the most diverse habitats and ecological niches of the world. They adapt to extreme life conditions by producing enzymes to support their metabolism and nutrition from various sources. Insect-derived enzymes include peptidases, amylases, lipases, and β-D-glucosidases and their application in biotechnology has gained recently more importance (Mika et al., 2013). Many of those are digestive enzymes produced along the gut of insects and, for example, insect-derived peptidases are produced to convert proteins into absorbable peptides essential for insect nutrition.

1.3.1. Insect-derived peptidases

Insect peptidases are mostly represented by digestive enzymes or cellular enzymes participating in essential functions like embryogenesis, reproduction, and metamorphosis (Terra and Ferreira, 1994). Depending on the insect and its developmental stage, different pH values prevail in the various regions of the digestive tract, which can range from pH 3.1 to 10.4 (Terra and Ferreira, 1994). Most peptidases found in insects are serine peptidases, which include trypsin-like peptidases (Jagdale et al., 2017). These peptidases are used to digest protein nutrients and, depending on the insect diet, they are assisted by other proteolytic enzymes like cysteine peptidases (Ferrara et al., 2015). Such co-expression of peptidases was reported by Terra and Cristofoletti (1996) as a consequence of an evolutionary adaptation to the high expression of serine peptidase inhibitors in plants. Because of their potential application in agriculture, medicine and biotechnology,

peptidases from Orthoptera, Hemiptera, Dictyoptera, Coleoptera, Lepidoptera, Diptera and Hymenoptera have been well studied. Lazarević and Janković-Tomanić (2015) proposed that the profile of expressed peptidases is highly dependent on the insect taxon and feeding habits. Insect-derived peptidases can vary in different organisms, however they are predominantly reported as serine, cysteine and metallopeptidases (Shaw and Christeller, 2009, Terra and Ferreira, 1994), while aspartic peptidases are less often reported.

Table 1. Peptidases in winemaking, including their advantages and disadvantages in the winemaking process.

kingdom	peptidase	origin	results	advantage	disadvantage	reference
fungal	BcAp8	*Botrytis cinerea*	degrades CHIs at 22 or 40 °C within 21 days or 18 h of incubation time, respectively	effectively eliminated CHIs	did not statistically reduce the levels of TLP	van Sluyter et al., 2013
	glutamic peptidase AGP	*Aspergillus* sp.	reduces haze levels, after a heat step of 75 °C for approximately 1 min	reduction of 90% of haze for heated wines treated with AGP	use of pasteurization methods, preheating steps are necessary	Marangon et al., 2012
	supernatant	*Saccharomyces cerevisiae*	cleaves non-denatured HUPs at 38 °C and pH value of 3.5	active at low pH value	relative long incubation time (48 h)	Younes et al., 2011
	MpAPr1	*Metschnikowia pulcherrima*	cleaves non-denatured HUPs at 25 °C and long incubation time of at least 48 h	reduces about 50% of CHIs and TLPs	fermentation at 25 °C is too high for normal vinification processes	Theron et al., 2018
	proline-specific endopeptidases (Brewers Clarex)	*Aspergillus niger*	reduces the haze at different conditions	not specially stated for wine	tested in combination with a laccase and originally invented for beer	Mutsaers and Edens, 2014
plant	bromelain	pineapplestem (*Ananas comosus*)	reduces at least 70% of the wine haze with 24 h incubation time at 20 °C	immobilized peptidase	long reaction time (1 day), high enzyme load (1%)	Benucci et al., 2014
	papain	papaya latex (*Carica papaya)*	no activity shown against wine protein	activity in average wine pH value (3.2) and despite ethanol presence	not tested for real wines only synthetic peptide substrates	Esti et al., 2013
animal	trypsin and pepsin	bovine and porcine	degradation of wine protein in neutral pH values	up to 22% of cleavage (45 °C)	large scale treatments were not effective	Pocock et al., 2003

1.3.2. Peptidases from fruit flies and the grape pest Drosophila suzukii

Adult and larval fruit flies secrete peptidases in distinct parts of their gut to assist their digestion (Borges-Veloso et al., 2012). Serine and cysteine peptidases have been reported from the Mediterranean fly *Ceratitis capitata*, from the fruit fly *Drosophila melanogaster*, from the spotted wing drosophila *Drosophila suzukii* and others.

The fly *D. suzukii* (Fig. 6b) is a grape pest and it was first described in Japan in 1916 (Hauser, 2011). It can cause significant crop losses in fruit crops as the females lay their eggs in different fruits, in particular ripening berries. Even though *D. suzukii* can lay eggs in ripe fruits, studies have found that the fly prefers damaged fruits for oviposition and that depends on the ambient and fruit temperature (Zerulla et al., 2017). For that, they make use of an evolutionary advantage, a serrated ovipositor (Fig. 6c), to penetrate hard fruit skins (Rota-Stabelli et al., 2013). During the ripening process of fruits, there is an increase in the sugar content and in the pH and skin penetration resistance, what facilitates the oviposition (Ioriatti et al., 2015).

Figure 6. a) Microscopic picture (microscope Leica S9i, Leica, Wetzlar, Germany) of the larval *Drosophila suzukii*. b) Microscopic picture (Leica S9i, Leica) of the adult *Drosophila suzukii*. c) Ovipositor of *Drosophila suzukii* [figure c was reprinted (adapted) with permission from Asplen et al. (2015) Copyright 2023 Springer Nature].

During their lifetime, flies can lay hundreds of eggs into ripe fruits. In the case of the spotted wing fly *Drosophila suzukii*, a female can lay about 380 eggs in cherries, grapes, plums, blueberries and other fruits (Akutsu and Matsuo, 2022; Allocca et al., 2018). The eggs hatch inside of the fruits usually after one day, and the embryos develop into larvae (Fig. 7a and 7b) in three different stages (Allocca et al., 2018). The larval *D. suzukii* (Fig. 6a and 7a), and not the adult fly, feeds on grapes. Therefore, these organisms are supposed to produce acid peptidase with activity at the grape pH (between 3.5 and 4).

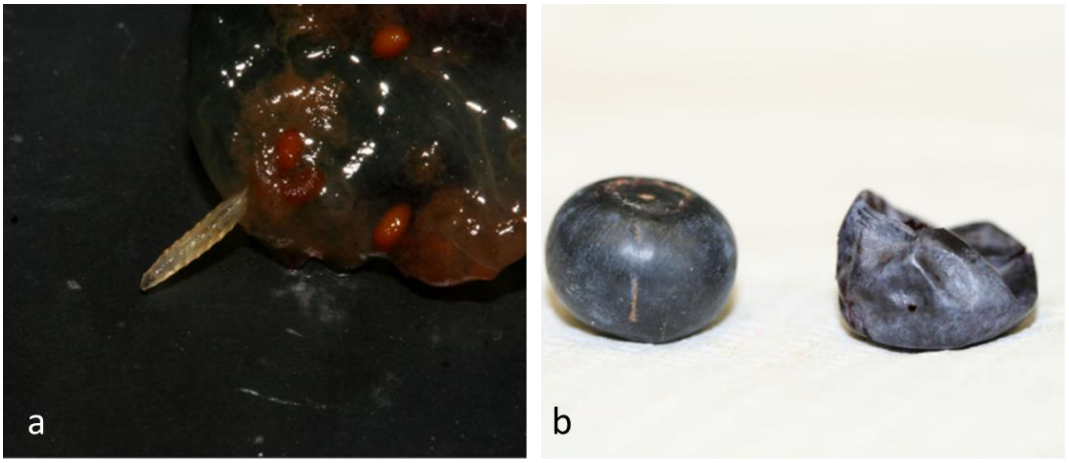

Figure 7. a) Larval *Drosophila suzukii* infesting blueberry tissues b) Comparison of blueberry, when undamaged (left) and damaged by *D. suzukii* (right). [reprinted (adapted) with permission from Asplen et al. (2015) Copyright 2023 Springer Nature].

Peptidases produced during developmental stages of *Drosophilae* species have already been reported (Hale, 1988). They are involved in many metabolic functions, signal peptide processing (Casso et al., 2005) and in cell growth (Patterson et al., 1949). These peptidases are reported, for example, as metallopeptidases (Vierstraete et al., 2003) and aspartyl peptidases (Casso et al., 2005) produced in the larval salivary gland (Patterson et al., 1949) or in the gut of larval *Drosophila* (Hale, 1988).

1.4. Molecular cloning and protein analytical tools

1.4.1. Molecular cloning and recombinant expression

The application of recombinant DNA has provided many advances in life sciences, including easy and replicable protein synthesis on a large scale. Recombinant protein expression has been applied in molecular research and in industrial settings for structural protein analysis, enzymatic assays, and in the pharma and biotech industries (Sørensen, 2010). The application of techniques such as ligation and restriction enzyme independent and recombinase-based cloning methods enabled the optimization and high-throughput protein expression (Celie et al., 2016). At the same time, it is possible to include the use of

affinity tags, which can facilitate protein purification (Arnau et al., 2006).

Organisms such as bacteria, yeasts, mammalian cells or transgenic plant cells are normally used to produce recombinant proteins (Ma et al., 2003). Although methods of protein expression by procaryotic systems, such as *Escherichia coli* or *Bacillus subtilis*, are considered rapid and replicable, proteins derived from bacteria are prone to form inclusion bodies and low yields (Sørensen, 2010). Another disadvantage of bacterial expression systems is the limitation of providing post translational modifications (PTM) (Juturu and Wu, 2018). In contrast to bacteria, yeasts, as eukaryotic organisms, are able to form complex PTMs such as disulfide bridge formation, lipid additions, or glycosylation and such PTMs are important for the functionality of recombinant proteins (Cereghino and Cregg, 2000). The yeast *Komagataella phaffii*, formerly known as *Pichia pastoris*, for example, has advantages over other expression systems like rapid growth rate, ease of genetic manipulation and many post-translational modifications, including polypeptide folding, glycosylation, methylation and acylation (Li et al., 2007).

Schmidt (2004) proposed a few strategies to improve the expression rate and the secretion of proteins by recombinant organisms, based on: (1) enhancement of gene expression rates; (2) secretion signal sequences should be optimized; (3) co-expression of chaperones and foldases to improve the protein secretion, (4) prevention of protein degradation by the creation of protease deficient mutants (5) subsequent breeding and mutagenesis. Additional techniques have emerged providing ways to alter and analyze large genes for novel genetic properties and highly superior cloning techniques such Gateway cloning, plasmid fusion system, golden gate cloning and sequence and ligation independent cloning (Ashwini et al., 2016).

1.4.2. Mass spectrometry (MS)-based proteomics

Proteomics is the study of proteins and their interactions inside and outside of cells (Cho, 2007). Advances in methods and technologies have aided biological studies to evolve from simple biochemical analysis of single proteins to proteome-wide measurements (Patterson and Aebersold, 2003). Mass spectrometry (MS) has become essential for molecular and cellular biology and due to advances in MS in recent years, a larger fraction of proteins could be detected in complex matrices. In the last decades, advances in the resolution, mass accuracy, sensitivity, and scan rate of mass spectrometers used to analyze proteins were achieved (Zhang et al., 2013). That also allowed the studies of the native state of proteins without the need of previous chemical preparation and protein digestion for the MS

analysis. Therefore, MS-based protein analysis was divided into two proteomics approaches: bottom-up (with previous protein digestion and chemical modifications) and top-down (native protein) states.

In bottom-up, proteins are previously cleaved, and the peptides are analyzed by mass spectrometry, allowing the identification of the proteins (Bogdanov and Smith, 2005). Peptidases like trypsin and proteinase K are typically used for protein digestion (Zhang et al., 2013). For example, trypsin specifically cleaves proteins at the *C*-termini of the basic amino acids lysine and arginine if they do not have a subsequent proline residue (Olsen et al., 2004). In order to improve the digestion of proteins, reagents like detergents are used, which denature the proteins and facilitate the cleavage by peptidases (Zhang et al., 2013). A major disadvantage of bottom-up proteomics is the loss of information regarding PTMs due to previous chemical treatments and that not all peptides can be fully detected (Cui et al., 2011).

In top-down proteomics, large fragments of proteins are directly subjected to gas-phase fragmentation in MS instruments (Wehr, 2006). Thus, no information about PTMs is lost (Cui et al., 2011). For protein identification, *de novo* sequencing can be used to identify peptides and proteins from the peptide fragmentation patterns in a database-independent manner (Canas, 2006).

1.4.3. Homology based protein structure modeling

Homology modeling has become an essential tool in structural biology, linking the knowledge related to protein sequences and experimentally obtained structures (Waterhouse et al., 2018). It is a computationally developed technique to study the structural and sequence similarities of proteins or to predict the conformation of a newly characterized one. The fundamental principle to build a protein model is the use of amino acid sequences of a protein and the atomic coordinates of at least one other protein (Lee, 1992). Other softwares for protein prediction, such as AlphaFold (Kiersten and Rohit, 2021) have recently developed algorithms which predict protein structures from amino acid sequences without any reference protein.

According to Lee (1992), seven steps are necessary to build a protein model using homology techniques: (1) to search sequence databases and find similar proteins to the protein model to be built. (2) to select structurally conserved regions in the protein models. (3) to find similarities between the amino sequence of the model protein and the sequences

of the protein references by sequence alignment. (4) to align structural features of the model protein to be the same as to the reference proteins in each region. (5) to perform structural database searching to determine the conformations of the variable protein regions (6) The model should be completed by the coordinates for the *N*-and *C*-terminal residues. (7) Energy minimization and molecular dynamics needs to be performed to reduce tensions caused by misplaced side chains and inappropriate peptide chains.

Computational structural modeling methods complement experimental structural biology data and can provide additional understanding of protein functions. It provides the building of reliable three-dimensional *in silico* models of a protein and can be widely used in several applications, such as: virtual screening, designing site-directed mutagenesis experiments or rationalizing the effects of sequence variations (Bordoli et al., 2009).

2. Perspectives

The application of peptidases in winemaking could prevent haze and help to reduce the negative economic impact generated by the use of bentonite fining. Here, the use of peptidases from *Drosophila suzukii* (larvae) was proposed based on the following hypothesis: since its larvae grow in the acidic environment of grapes, it should be able to produce peptidases active at acid pH. In addition, analytical and biotechnological methods have been developed, resulting in three main alternative strategies to be applied as for the prevention of wine haze:

1. The use of MS-based peptidomics as an analytical tool to identify the degradation level in the structure of haze proteins. As proposed in the article: Albuquerque, W.; Ghezellou, P.; Li, B.; Spengler, B.; Will, F.; Zorn, H.; Gand, M. (2021). Identification of intact peptides by top-down peptidomics reveals cleavage spots in thermolabile wine proteins. *Food Chemistry*, 363, 130437.

2. The production and purification of recombinant haze proteins (rTLP and rCHI) to screen suitable acid peptidases. As described in the manuscript: Albuquerque, W.; Sturm, P.; Schneider, Q.; Ghezellou, P.; Seidel, L.; Bakonyi, D.; Will, F.; Spengler, B.; Zorn, H.; Gand, M. (2021) Recombinant thaumatin-like protein (rTLP) and chitinase (rCHI) from *Vitis vinifera* as models for wine haze formation. *Molecules*, 27 (19), 6409.

3. The heterologous expression of an identified acid peptidase Cathepsin L1 from the larval *D. suzukii*, which was described in the research article:
   Albuquerque, W.; Ghezellou, P.; Lee, K.Z.; Schneider, Q.; Gross, P.; Kessel, T.; Omokungbe, B.; Spengler, B.; Vilcinskas, A.; Zorn, H.; Gand, M. (2023) Peptidomics as a Tool to Assess the Cleavage of Wine Haze Proteins by Peptidases from *Drosophila suzukii* Larvae. Biomolecules, 13(3), 451.
   The referred peptidase is a promising candidate to cleave haze proteins, generating peptide products that can be detected by MS-based peptidomics. Evaluation of its potential as a fining agent and reducing haze in real wine samples remains to be studied. The heterologous expression of cathepsin L1 could also provide a large amount of the purified enzyme to be tested in wine samples as a fining agent.

The results obtained in this thesis provide alternative tools to be applied aiming at the successful leap of peptidases into winemaking processes. the evaluation of other potential peptidases by applying the proposed methods described here might be helpful to find ideal fining agents for the wine industry.

3. Research objectives

In the last two decades, wine researchers have made various efforts to replace the use of bentonite clay in wine clarification. Many of the proposed technological procedures applied temperature modulation or heating (Marangon et al., 2012), protein filtration (Mierczynska-Vasilev and Smith, 2015) and others. An alluring solution would be the use of peptidases to remove wine haze proteins without the need of fining agents, which negatively affect the wine sensory. Therefore, the first aim of this thesis was to review the scientific literature to understand the main challenges regarding wine haze formation. The results of this analysis were published in the following literature review:

Albuquerque, W.; Seidel, L.; Zorn, H.; Will, F.; Gand, M. (2021) Haze formation and the challenges for peptidases in wine protein fining, *Journal of Agricultural and Food Chemistry, 69*(48), 14402-14414. https://pubs.acs.org/doi/10.1021/acs.jafc.1c05427

To propose analytical methods and practical alternatives to solve the challenges created by the haze formation in white wines, the present thesis addressed: 1) The development of novel methods for detection of cleavage products of thermolabile wine proteins, based on the analysis of tryptic digested peptides by high resolution (HR) mass spectrometry (MS)-top-down peptidomics; 2) The production of the recombinant haze proteins thaumatin-like protein (TLP) and chitinase (CHI) to be used as model proteins; 3) The analysis of the proteome of a Silvaner Franken wine used as control for the application of peptidases for the cleavage of proteins from a real wine sample; 4) The screening of insect-derived peptidases with potential to cleave wine proteins at acidic pH. Finally, 5) The degradation of heterologously expressed TLP and CHI by promising insect-derived peptidases was evaluated by MS-based peptidomics. The combination of all proposed methods allowed the evaluation of insect peptidases with potential to successfully degrade thermolabile wine proteins at winemaking conditions and with potential to be applied in the wine industry. A summary of the planning and design of this thesis is presented in figure 8. Regarding the first objective, "top-down" peptidomics was applied to identify cleavage spots in TLPs and CHIs. The successful approach provided the comparison of tryptic cleavage of wine proteins at different pH and temperature conditions and allowed for the visualization in 3D protein models. Regarding the structural damage caused by tryptic digestion, α-helices and β-strands from CHIs and TLPs were cleaved after protein denaturation.

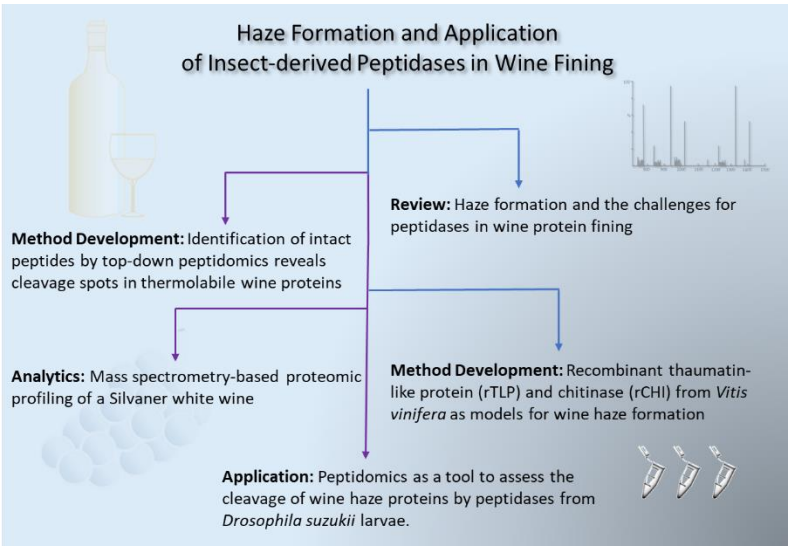

Figure 8. Graphic plan of the research objectives and design of the present thesis.

The degradation of haze proteins was semi-qualitatively confirmed by label-free peptide quantitation, showing higher cleavage rates of TLP and CHI under denaturing conditions. The proposed method could be used to identify suitable peptidases for wine clarification in screening processes for suitable proteolytic enzymes. The following conclusions were drawn: the cleavage of TLPs and CHIs is hindered mainly by acidic winemaking conditions rather than by their molecular rigidity and it was possible to modulate the cleavage of wine haze proteins by changes in the wine pH and temperature. The complete description of the methods and the main findings are publicly available as the research article:

Albuquerque, W.; Ghezellou, P.; Li, B.; Spengler, B.; Will, F.; Zorn, H.; Gand, M. (2021) Identification of intact peptides by top-down peptidomics reveals cleavage spots in thermolabile wine proteins. *Food Chemistry*, 363, 130437. https://doi.org/10.1016/j.foodchem.2021.130437

Regarding the second research objective, the recombinant expression of TLP and CHI as model haze proteins was proposed. By purifying them, the study of their cleavage by peptidases was possible, avoiding protein mixtures. For that, recombinant rTLP and rCHI were expressed by the yeast *Komagataella phaffii*. The recombinant proteins were able to form protein haze similar to native proteins from *Vitis vinifera*. Both recombinant proteins exhibited similar characteristics, such as melting points and aggregation potential when compared to their native analogues. These studies could also evidence that polyphenols and sulfite ions act as haze inducers, confirming their contribution to protein cross-linking reactions. This fact corroborates the relevance of wine matrix components for the haze formation. rTLP and rCHI are suggested to be applied as model haze proteins to help in the screening of peptidases as fining agents. The procedures of molecular cloning, haze

tests and the characterization of the recombinant proteins are available as the research article:

Albuquerque, W.; Sturm, P.; Schneider, Q.; Ghezellou, P.; Seidel, L.; Bakonyi, D.; Will, F.; Spengler, B.; Zorn, H.; Gand, M. (2021) Recombinant thaumatin-like protein (rTLP) and chitinase (rCHI) from *Vitis vinifera* as models for wine haze formation. *Molecules*, 27 (19), 6409. https://doi.org/10.3390/molecules27196409

The analysis of the proteome of a Silvaner Franken wine was proposed as the third research objective. For that, the combination of *in solution* and *in gel* protein digestion techniques allowed the identification of 154 proteins including high and low abundance proteins. In this thesis, the Silvaner wine was exemplarily used as wine sample and used for evaluating the degradation potential of the studied peptidases. The wine proteomics analysis is described in the manuscript:

Albuquerque, W.; Ghezellou, P.; Seidel, L.; Burkert, J.; Will, F.; Schweiggert, R.; Spengler, B.; Zorn, H.; Gand, M. (2023) Mass spectrometry-based proteomic profiling of a Silvaner white wine. Biomolecules, 13.

Insect-derived peptidases were studied as potential candidates for successful degradation because: 1) Insects are organisms adapted to harsh and diverse environments and able to produce varied enzymes; 2) Insects are fruit and cereal pests and express digestive enzymes able to degrade proteins with compact structure at acidic pH. In this thesis, peptidases from the larval *Drosophila suzukii* were investigated.

The fourth research objective proposed the combination of the techniques developed in the first (peptidomics analysis) and second (use of rTLP and rCHI) objectives to evaluate the degradation potential of peptidases from *Drosophila suzukii* (larvae). Peptidases purified from the larval *D. suzukii* were identified (MS-based proteomics) and were evaluated for their potential to cleave recombinant rTLP and rCHI at pH 3.5. The peptide products of the digestion of recombinant grape protein were detected by top-down peptidomics. An acidic peptidase, a cathepsin L1, as a digestive enzyme of *D. suzukii*, was suggested to be responsible for the cleavage for being active at low pH and for providing a broad specificity of cleavage. These results are presented in the following manuscript:

Albuquerque, W.; Ghezellou, P.; Lee, K.Z.; Schneider, Q.; Gross, P.; Kessel, T.; Omokungbe, B.; Spengler, B.; Vilcinskas, A.; Zorn, H.; Gand, M. (2023) Peptidomics as a tool to assess the cleavage of wine haze proteins by peptidases from *Drosophila suzukii* larvae. Biomolecules, 13(3), 451. https://doi.org/10.3390/biom13030451

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Review articles

Review article 1 – Haze formation and the challenges for peptidases in wine protein fining

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Review

Haze Formation and the Challenges for Peptidases in Wine Protein Fining

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ABSTRACT: To meet consumer expectations, white wines must be clear and stable against haze formation. Temperature variations during transport and storage may induce protein aggregation, mainly caused by thaumatin like-proteins (TLPs) and chitinases (CHIs), which thus need to be fined before bottling of the wine. Currently, bentonite clay is employed to inhibit or minimize haze formation in wines. Alternatively, peptidases have emerged as an option for the removal of these thermolabile proteins, although their efficacy under winemaking conditions has not yet been fully demonstrated. The simultaneous understanding of the chemistry behind the cleavage of haze proteins and the haze formation may orchestrate alternative methods of technological and economic importance in winemaking. Therefore, we provide an overview of wine fining by peptidases, and new perspectives are developed to reopen discussions on the aforementioned challenges.

KEYWORDS: *wine haze, peptidases, thaumatin-like protein, chitinase, protein aggregation*

1. INTRODUCTION

In the wine industry, fining is necessary to prevent turbidity and loss of product value in order to meet consumer expectations and quality standards. Bentonite fining is still the routinely used process to stabilize wines and prevent haze, although its negative effects on organoleptic properties and production costs are undesirable for winemakers. Because of its negative electrostatic character, bentonite clay is broadly adsorbing positively charged compounds, which impacts wine aroma and flavor profiles. Apart from that, its swelling and poor settling properties also lead to a loss of wine volume and generate wastes.[1] Additionally, there is still a risk of protein instability after bentonite fining, since proteins with an isoelectric point similar to the pH value of wine can hardly be fined. Since haze is typically originating from wine proteins, the use of peptidases to prevent haze formation seems to be plausible. The advantage of using peptidases as a fining agent would be higher product yields and no negative effects on organoleptic properties.[2] However, their use remains a challenge due to the adverse conditions of winemaking.

Diverse wine matrix elements and physicochemical conditions act together to trigger protein cross-linking and hinder the action of peptidases. The interaction between heat unstable proteins (HUPs), especially thaumatin-like proteins (TLPs), chitinases (CHIs), and sometimes β-(1,3)-glucanases,[3] and low molecular mass chemical compounds, such as (poly)-phenols, sulfites, metal ions, ethanol, and organic acids,[4] produces aggregates large enough to scatter light and thus causing a hazy appearance. Typically, winemaking conditions include a strongly acidic pH environment (around 3.5), low temperatures (10−20 °C),[5] and the presence of enzyme-inhibiting compounds such as high ethanol concentrations of up to 15%,[6] resulting from the fermentation process. Moreover, phenolic compounds,[7] preservatives, and antioxidants such as sulfur dioxide (SO_2) are present in the final product. Furthermore, peptidases generally hydrolyze linearized polypeptide chains, irregular structures, and exposed loops,[8] whereas TLPs and CHIs are widely known to be compact and lack structural flexibility in the physicochemical conditions that prevail in wine.[9]

Recent advances in understanding wine haze require evidence-based data to elucidate scientific concepts and facilitate the search for suitable peptidases for fining applications. A concise interpretation of the origin of the haze phenomenon and an overview of efficient enzymes and technological methods are provided here to motivate further studies and encourage winemakers to apply alternative clarification methods.

2. ORIGIN OF WINE HAZE

Wine haze is not promoted by any particular singular factor but rather by a combination of haze inducing elements and physicochemical conditions, which result in protein aggregation.[2,4,10] These factors include the (in)stability of different HUP isoforms, pH, ionic strength, temperature and concentration of phenolic compounds, sulfite ions, and polysaccharides.[11,12]

Although some controversies exist, there is a consensus in the literature that wine haze results from the subsequent steps

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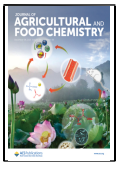

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of: (1) slow denaturation of HUPs;[13] (2) exposure of hidden hydrophobic domains and binding sites; (3) binding of chemical compounds (nonproteinaceous factors known as "factor x"); (4) improper refolding; and (5) finally protein cross-linking to form particulate aggregates. Each of these events is further discussed in this present review.

Some amino acids in HUPs that act as anchors for "factor x" are exposed to the protein surface under denaturing conditions. Moreover, they may trigger protein aggregation in cases where they are permanently exposed after an improper refolding process, where the proteins do not recover their native conformations. As a result, unfined commercial wines containing HUPs become turbid when exposed to defined moderate temperatures such as >40 °C or temperature changes during storage or transport.[14] This process can be experimentally accelerated by a so-called "heat test", which consists of heating wine samples up to 80 °C with subsequent cooling to different temperatures from 4 to 25 °C for different time intervals (from 0.5 to 18 h).[15,16]

Hydrophobic interactions are considered essential for protein−protein interactions. This was shown by Dufrechou et al.[17] when wine samples containing TLP isoforms with different levels of hydrophobicity also promoted different degrees of turbidity. Nonpolar residues assemble when hydrophobic cores are exposed to solvents,[18] induced by heating.[19] Corroborating this theory, Marangon et al.[20] found a proportional correlation between haze formation and protein hydrophobicity in the presence of tannins, and the authors discussed that haze originates from the exposure of tannin-binding sites after heating. With regard to protein composition and structure, some specific amino acids and protein features are reported to participate in haze formation mechanisms. Proline residues were considered sites for protein−phenolic interactions[21] as their content in all wine proteins has been proportionally related to haze levels, although neither TLPs nor CHIs are proline rich proteins. In the presence of polyphenolic compounds, HUPs with lower proline content showed a lower or complete absence of turbidity in model systems, which might result from less proline−phenolic-binding sites.[22] Such phenolic-binding sites have already been reported in a cleft located between the domains I and II of certain TLPs.[12,23] Other protein secondary structure elements such as loops in the TLPs can unfold upon heating and become prominent on the protein surface, exposing binding sites for phenolic compounds.[3,24]

The physicochemical wine environment plays an important role for the induction of protein aggregation.[11] The ethanol content is considered as a haze suppressive agent,[25] while ions in the wine matrix reduce the electrostatic repulsion between the proteins, facilitating their interaction.[3,26] As confirmed by Dufrechou et al.[17] the pH is considered as an important factor to induce protein aggregation. They observed that pH variation led to the exposure of buried protein residues, resulting in protein aggregation. Moreover, the pH has an influence on the proteins' net charge, which may affect their molecular conformation. Since protein−polyphenol interactions are proportional to protein hydrophobicity, increased net charges induced by a low pH, therefore, could reduce the haze formation.[12] In addition, pH may also affect the solubility of proteins with different isoelectric points.

3. PROTEINACEOUS FACTOR

Several factors such as grape cultivar,[27] climate,[10,27] soil conditions,[28] pathogen attack,[28] winemaking[3,28,29] and clarification processes[3,28,29] can affect the wine protein composition. The pathogen-related (PR) proteins TLPs and CHIs are expressed in response to pathogenic attacks[30] during all stages of grape development.[31] These proteins constitute the main protein fraction[32] of most wines,[33] and their thermolabile property makes them susceptible to denaturation and renaturation processes, which is fundamental to promote molecular aggregation.

TLPs have molecular masses (MM) between 21 and 24 kDa[31,33] and an amino acid sequence similar to that of the sweet-tasting protein thaumatin. Peng et al.[32] reported three different TLP fractions from a Sauvignon blanc wine: one major, VVTL1, and two minors, VVTL2 and VVTL3, with possible different roles in haze formation. Different TLP isoforms have been reported to have structural features that affect the protein folding/refolding process.[34]

CHIs are chitinolytic enzymes with MM between 27[33] and 34 kDa.[30,31] Different grape CHI isoforms have been identified such as a class IV endochitinase from *Vitis vinifera*,[35] the isoforms Fa and Ia[36] and up to 13 other isoforms (4 alkaline and 9 acidic) from vine plant tissues[37] all with similar haze potentials. Besides these two major players of haze formation, β-(1,3)-glucanases, the ripening-related protein Grip22, and the lipid transfer protein (LTP) have also been cited as relevant with respect to haze formation.[29,33,38]

TLPs and CHIs are considered to be the main haze promoters, but their contributions to protein aggregation are vastly different from each other. On one hand, some TLP isoforms are thermally unstable and reversibly denatured and interact preferentially with polysaccharides, phenolic compounds,[19] and sulfite ions;[39] on the other hand, CHIs are known for their irreversible unfolding[21] as they are more susceptible to the wine ionic strength.[40] Furthermore, particles formed by TLPs and CHIs have different sizes.[19]

Waters et al.[41] compared purified TLP and CHI fractions and their haze potential and observed that, when at the same concentration, TLP induced approximately 50% more haze than CHI. Vincenzi et al.[42] also reported a TLP (VVTL1) isoform as the main contributor to haze formation. In contrast, CHIs produce higher levels of aggregation[19] and are considered to be the most unstable wine proteins as they require lower temperatures (about 55 °C, in contrast to TLP, which unfolds at around 62 °C) to irreversibly unfold.[43] Since the amino acid composition can cause resistance to peptidases (see Section 5.1), TLPs and CHIs were examined in more detail. CHIs have a higher serine content, while TLPs contain a higher proportion of threonine, lysine, and arginine residues.[41] Neither of them is considered to be proline-rich and therefore they are not hydrolysis-protected by the particular structural properties of proline.[44]

4. FACTOR X: DO NONPROTEINACEOUS ELEMENTS CAUSE PROTEIN AGGREGATION?

Polyphenolic compounds, sulfite ions, organic acids, polysaccharides, and other matrix components can affect the wine stability.[45] Pocock et al.[46] discussed that HUPs do not form haze in the absence of low molecular mass components in the wine matrix, after observing that model solutions composed solely of proteins did not become turbid during heat tests.

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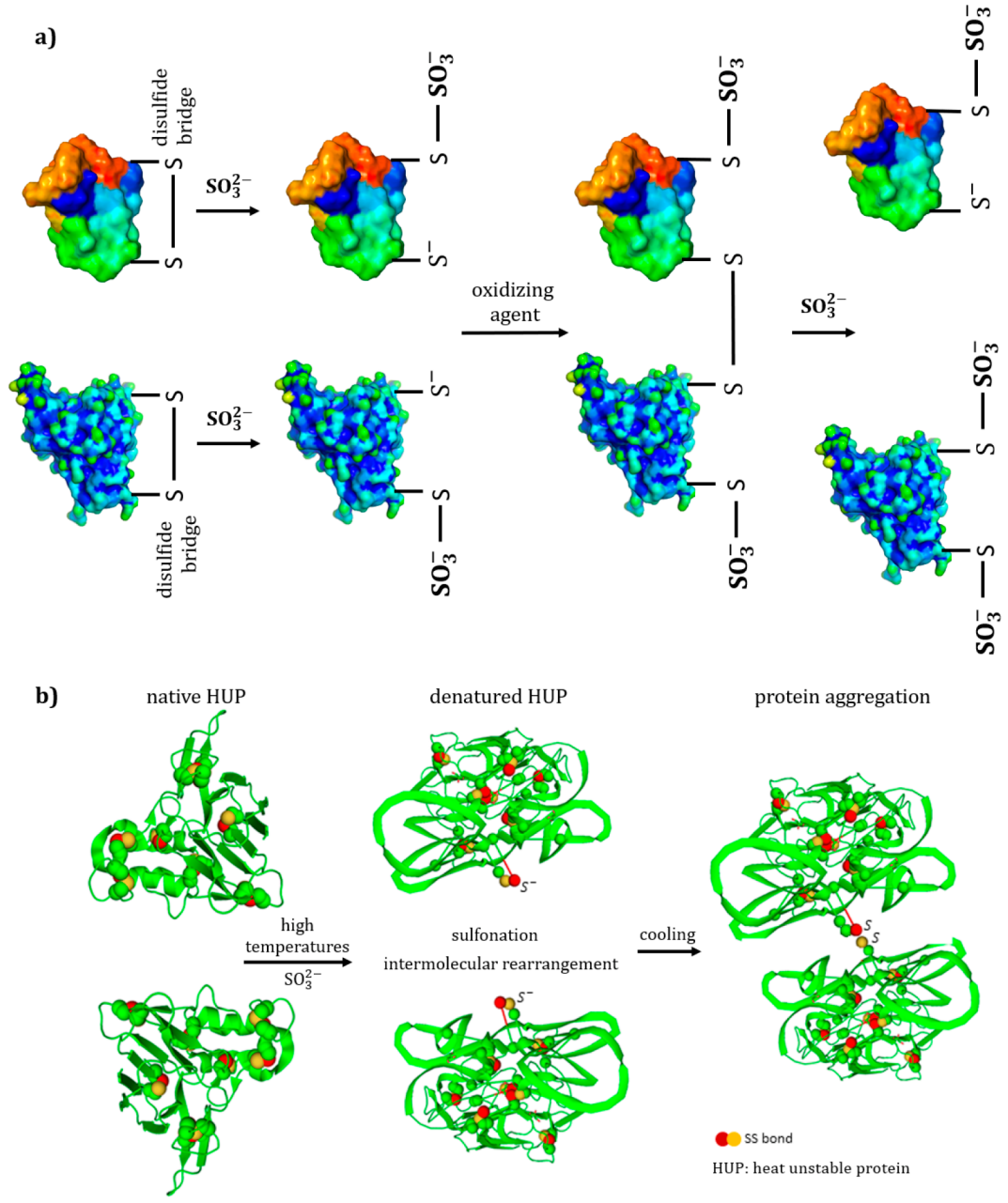

Figure 1. (a) Mechanism of sulfonation of disulfide bridges through SO_3^{2-}, resulting in free thiolates, which can then form new intermolecular S−S bonds (scheme based on Sugiyama et al.[52]). (b) Denaturation processes allow HUPs to expose their cysteine residues and undergo sulfonation reactions in the presence of SO_3^{2-} with the consequent formation of new disulfide bridges. A subsequent cooling step leads haze proteins in a non-native state with new intra- or intermolecular bonds.

Wine researchers have been hunting for a unique nonproteinaceous element responsible for protein aggregation, although it is likely that the so-called "factor x" is actually the interaction of factors that induce molecular cross-linking between hydrophobic residues, cysteine residues, and specific amino acids.

4.1. Sulfite Ions. Sulfur dioxide (SO_2) is a common additive used as an antioxidant and antimicrobial agent in winemaking,[47] and it is usually found as hydrogen sulfite (HSO_3^-) or in its oxidized form sulfate anion.[48] As these ions are commonly present in commercial wines, they were hypothesized to be a haze promoter. This has been demonstrated by Pocock et al.[46] when they showed that purified fractions of TLP and CHI (150 mg/mL) required about 150 and 15 mg/L sulfate, respectively, to become turbid.

Chagas et al.[39] described sulfur dioxide as an essential element for haze formation and proposed that, during the unfolding−folding dynamics of HUPs, they could act to alter the protein renaturation process. The authors suggested that sulfite ions trigger a cascade of events, which finally results in the formation of protein dimers, trimers, tetramers, etc. These events include the disruption of disulfide bridges, so-called sulfitolysis,[49] in HUPs by the formation of one S-sulfonated cysteine residue and one reactive sulfide group (eq 1). Van der Plank et al.[50] studied the role of SH−SS exchanges and hydrophobic interactions in the aggregation of egg white proteins and observed the exposure of thiol groups under

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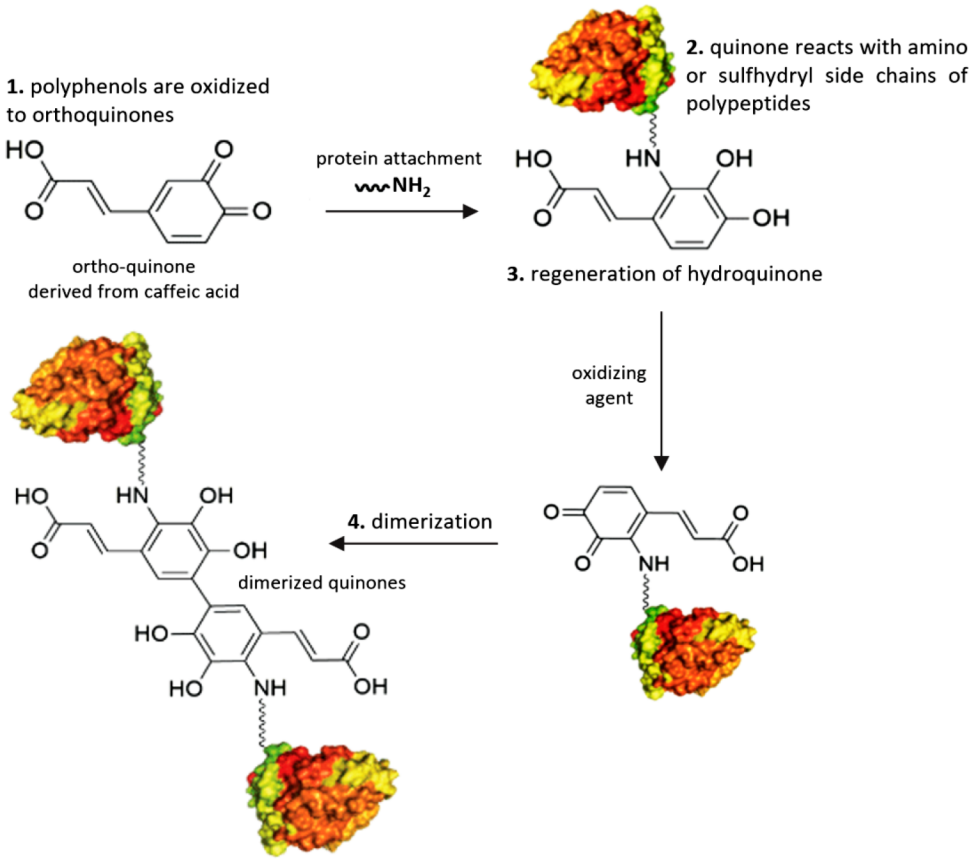

Figure 2. Model for the dimerization of phenolic compounds in solution, after their prior binding to wine haze proteins to form a crossing-linking chain. Scheme based on Strauss and Gibson.[61]

denaturing conditions and the formation of intra- and intermolecular covalent bonds via thiol−disulfide exchanges.

$$RS - SR \xrightarrow{SO_3^{2-}} RS - SO_3^- + RS^- \quad (1)$$

The disruption of disulfide bridges in proteins can be induced experimentally by exposing them to sodium sulfite in the presence of an oxidizing agent such as *o*-iodosobenzoate or tetrathionate.[51] In this process, sulfite ions would interact with the polypeptide chain promoting the reduction of S−S bonds, resulting in the formation of a sulfonated cysteine residue and a reactive thiolate, which can form new disulfide bridges. In summary, each disulfide bridge rupture by S-sulfonation generates at least one free thiolate "anchor" capable of binding to intra- or interprotein cysteine residues (Figure 1a), forming different aggregated forms[52] and causing a change in the pattern of disulfide bridges (angular torsions).[39] Such cascade events are logically increased if the entire protein is denatured allowing the possibility of various molecular conformations. To form a new disulfide bond via SH/SS exchanges, a protein needs to expose two cysteine residues in a favorable position for the reaction to take place, no matter if in its native or denatured state[53] (Figure 1b). Both CHIs and TLPs are reported to form aggregates that result from SH−SS exchanges. Van Sluyter et al.[3] discussed the existence of exposed loops in TLPs stabilized by a disulfide bridge, which can become exposed under heating and more susceptible to S-sulfonation. Gazzola et al.[19] reported that the presence of sulfate strongly affected CHI aggregation and the size of the formed particles, when model wines were heated.

4.2. Phenolic Compounds. Wine phenolic compounds correspond to a fraction of grape secondary metabolites, which contribute to essential sensory properties such as flavor, appearance, and aging capacity.[7] Simple phenolic acids to large polymeric flavonoids are present in wines,[54] and since they originate mostly from grape skins, their concentration depends on the grape variety, maceration process, vinification technique, and fermentation conditions.[55]

Haze and the size of aggregated particles[46] are proportional to the concentration of polyphenols in wines, with a certain threshold concentration leading to insolubility of the proteins.[25,56] Spanos and Wrolstad[57] proposed that the oxidation of phenolic compounds can form intermediate reactive compounds that are capable of binding to proteins and forming haze. Among the white wine phenolic compounds, proanthocyanidins/procyanidins (polymers of catechin and epicatechin monomers) are particularly important in the process of haze formation.[57] Many other phenolic compounds, such as (*E*)-*p*-coumaric acid, (*E*)-caffeic acid, vanillic acid, protocatechuic acid, syringic acid, gallic acid, ferulic acid, shikimic acid, *p*-coumaric acid ethyl ester, tyrosol, and quercetin, have been also found in wine protein precipitates.[58] Contrarily, Pocock et al.[46] reported that monomeric phenolic compounds and trimeric procyanidins are not essential for the haze formation and phenolic compounds such as caffeic acid, caftaric acid, epicatechin, epigallocatechin-*O*-galatate, gallic acid, and dp3 grape seed tannin[46] failed to promote protein haze.

Condensed tannins (oligomers and polymers of flavan-3-ol) classified as proanthocyanidins/procyanidins are reported as

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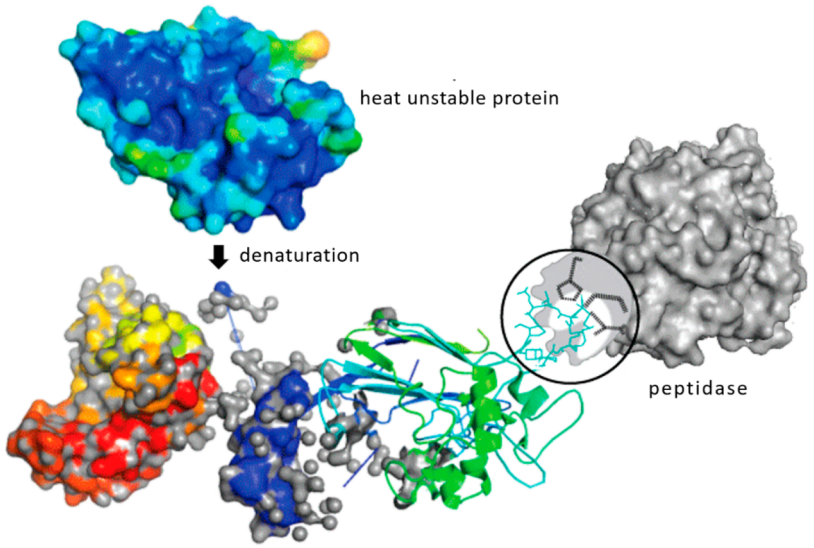

Figure 3. Demonstration of how the unfolded structure of a wine haze protein would make the peptide backbone more accessible for proteolysis. Loops and termini are then more accessible to the catalytic site of peptidases.

the main phenolic haze inducers. Their long polymeric chains, containing several phenol and hydroxyl groups, have been frequently related to protein binding and haze formation.[9,59,60] The amount of tannin-induced haze is proportional to the extent of hydrophobic surface exposed in TLPs,[34] but the protein hydrophobicity itself is insufficient to promote haze formation when tannins are removed from wines by ultrafiltration.[20] Hydrophobic interactions have long been reported to favor tannin−protein interactions,[46] and TLPs may have specific phenolic-binding sites that favor phenol−protein interactions by the stacking of polyphenol rings.[22]

Strauss and Gibson[61] discussed a mechanism for phenol−protein interactions in which the susceptibility of tannins to cross-link was mainly due to the high amounts of available hydroxyl groups, which can be oxidized to ortho-quinones forming reactive species capable of dimerization (Figure 2). The binding of phenolic compounds to nucleophilic groups in HUPs with phenolic compounds and their subsequent oxidation-induced dimerization is the reason for the formation of aggregates.[62] This hypothesis can be supported by findings that report the induction of cross-linkings in myofibrillar proteins by tannic acids and other phenolic compounds. However, in wines, this linking process is only triggered after heating HUPs in the presence of tannins.[20]

4.3. Ionic Strength and Other Wine Matrix Compounds Such As Organic Acids and Polysaccharides As Effectors of the Level of Haze Formation. Several other components of the wine matrix play a supplementary role in wine instability. Regarding the influence of ions, Pocock et al.[46] showed that the presence of anions (such Cl^- and PO_4^{3-}) and cations (such as Cu^{2+}) was not essential to promote haze formation but that haze could be induced by Fe^{2+} and Fe^{3+}. Metal ions may be introduced from natural (e.g., soil) and anthropogenic sources such as machinery used in winemaking including harvest machines, presses, pumps, and storage tanks. However, over the last three decades, the release of metal ions has been continuously reduced by the use of more inert materials. In general, ionic strength is considered to be a haze promoter, since it affects electrostatic interactions and consequently the interaction between charged residues in proteins. Haze formation by CHI was discussed to be strongly affected by increasing the ionic strength of wines.[40]

The major organic acids L-(+)-tartaric acid and L-(−)-malic acid are highly concentrated in wines and might act as wine stabilizers. Minor organic acids are citric acid, succinic acid, lactic acid, and gluconic acid as normal metabolism and fermentation products. Gluconic acid is only present in higher concentrations after *Botrytis cinerea* infection of the grapes, and malolactic fermentation induced by various lactic bacteria increases lactic acid concentrations. Tricarboxylic, dicarboxylic and monocarboxylic acids were shown to have protective effects against haze formation by attaching to free phenolic compounds and preventing their interactions with proteins in wines.[63] Polysaccharides are also considered as stabilizing agents and are supposed to prevent haze formation by directly binding to polyphenolic compounds to form protein−polyphenol−polysaccharide complexes and to reduce protein−phenolic interactions.[19] Sommer et al. reported that pectin, glucomannan, mannoprotein (proteoglycan), alginate, and carboxymethyl cellulose (CMC) have different potentials to inhibit haze formation, delaying polyphenol−protein interactions.[64] Among them, CMC had the highest capacity to compete for binding sites, while mannoproteins could reduce the size of particle aggregates.[65]

5. PEPTIDASES FOR WINE CLARIFICATION: A CONTINUING CHALLENGE

5.1. Proteolysis Efficiency under Winemaking Conditions. Peptidases have been proposed as an alternative to bentonite clay,[66] although many studies have discussed the impracticality of proteolysis for the prevention of protein aggregation, keeping in mind the conditions that exist during winemaking.[41] The acidity of wine is at least one relevant factor that hinders the degradation of HUPs.[36] In wines, this resistance is increased by the temperature conditions.[28] Moreover, since TLPs and CHIs are expressed in response to pathogenic attacks, it is postulated that they have a resistant structure against proteolysis.[9,13] Marangon et al.[34] described TLPs to have a compact structure tightly connected with eight disulfide bridges and with a lack of irregular structures or loops,

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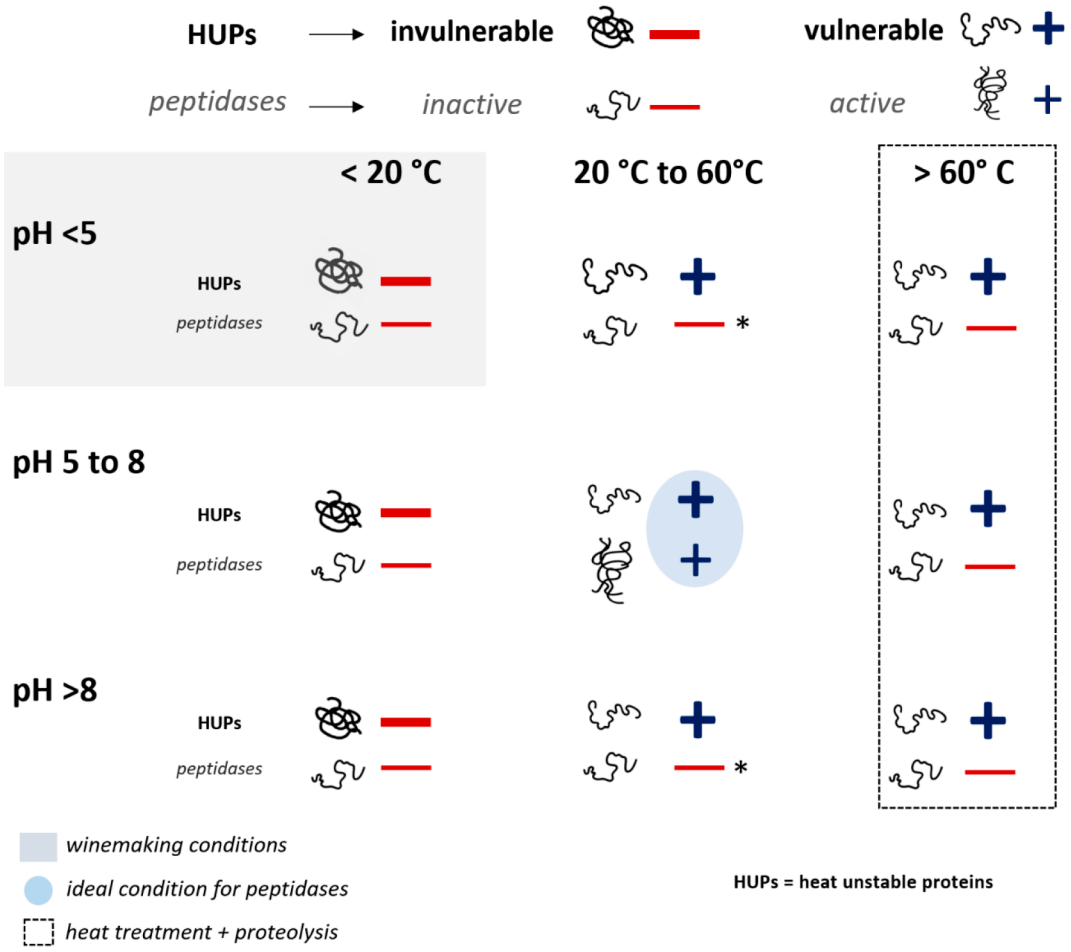

Figure 4. Combination of four factors (protein vulnerability, peptidase activity, temperature, and pH) interfering on the HUPs propensity to cleavage. The conformation of HUPs depends on the temperature and pH values and were classified as invulnerable and vulnerable (to the proteolytic action), while peptidases are classified as inactive and active. The gray rectangle highlights the pH and temperature at winemaking conditions. The blue ellipse highlights the conditions in which both substrates and enzymes are suitable to allow for protein hydrolysis. The dashed rectangle shows the temperatures conditions at which most proteins are denatured (flash pasteurization conditions) and only particular peptidases would be active. Asterisks show that acidic or alkaline peptidases can still be active at the respective pH ranges.

which prevents peptidases from accessing cleavage spots. It is discussed that the high amounts of asparagine, aspartic acid, glycine, serine, and threonine in TLPs and CHIs contribute to their resistance.[41] In addition, HUPs are highly glycosylated,[29] which may be an additional protective factor.[50] Proanthocyanidin oligomers have also been reported to increase HUP's resistance[41] and to inhibit peptidases such as trypsin and chymotrypsin.[67]

This in turn has discouraged winemakers to consider peptidases as a viable option. However, a few peptidases have already shown initial efficacy to cleave HUPs at acidic conditions.[68] But, to act as fining agents, they should be functional at low temperatures (around 10−20 °C) and in an alcoholic environment.[69] As an effective proteolysis depends on several factors such as the protein−substrate interaction, the exposure of secondary structures to facilitate access to peptidases[70] and the flexibility of these domains[71] to present certain peptide bonds to the peptidase. Loops and unstructured elements at the N- and C-termini of the substrate proteins are exposed to the protein surface and therefore are most susceptible to hydrolysis (Figure 3) due to their limited noncovalent interactions and propensity to unfold.[70] As temperature and pH are factors that can only be changed to a limited extent in the vinification process, which concomitantly affect the structure of substrates (HUPs) and catalysts (peptidases), the conditions for an effective proteolysis are limited (Figure 4). Figure 4 suggests hypothetical combinations of temperature and pH values to modulate an efficient proteolysis. While the low pH is inhibiting most peptidases, it has no significant effect on substrate denaturation.[17] Temperature, in contrast, may have denaturing effects on both HUPs and peptidases. In an ideal scenario, peptidases should be active at low temperatures and acidic pH, while HUPs need to be unfolded under these physicochemical conditions.

5.2. Peptidases as Fining Agents, What Has Been Achieved? Peptidases from different organisms applied under varied physicochemical conditions have been tested for wine clarification. We made a correlation regarding their characterization and source and shined a light into recent breakthroughs and drawbacks for the application of peptidases in wine fining. A brief summary is given in Table 1.

5.2.1. Peptidases from Yeasts and Fungal Grape Pathogens as an Alternative. Peptidases from organisms involved in fermentation processes or from grape pathogens have been explored as potential fining agents.[79] These microbial enzymes are commonly present in wine fermenta-

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Table 1. Tested Peptidases Including Their Advantages and Disadvantages in the Winemaking Process[a]

kingdom	peptidase	origin	results	advantage	disadvantage	reference
fungal	BcAp8	*Botrytis cinerea*	degrades CHIs at 22 or 40 °C within 21 days or 18 h of incubation time, respectively	effectively eliminated CHIs	did not statistically reduce the levels of TLP	68
	glutamic peptidase AGP	*Aspergillus* sp.	reduces haze levels, after a heat step of 75 °C for approximately 1 min	reduction of 90% of haze for heated wines treated with AGP	use of pasteurization methods, preheating steps are necessary	72
	supernatant	*Saccharomyces cerevisiae*	cleaves nondenatured HUPs at 38 °C and pH value of 3.5	active at low pH value	relative long incubation time (48 h)	33
	MpAPr1	*Metschnikowia pulcherrima*	cleaves nondenatured HUPs at 25 °C and long incubation time of at least 48 h	reduces about 50% of CHIs and TLPs	fermentation at 25 °C is still high for normal vinification processes	73
	proline-specific endopeptidases (Brewers Clarex)	*Aspergillus niger*	reduces the haze at different conditions	not specially stated for wine	tested in combination with a laccase and originally invented for beer	74
plant	bromelain	pineapplestem (*Ananas comosus*)	reduces at least 70% of the wine haze with 24 h incubation time at 20 °C	immobilized peptidase	long reaction time (1 day), high enzyme load (1%)	74,75
	papain	papaya latex (*Carica papaya*)	no activity shown against wine protein	activity in average wine pH value (3.2) and in spite of ethanol presence	not tested for real wines only synthetic peptide substrates	77
animal	trypsin and pepsin	bovine and porcine	degradation of wine protein in neutral pH values	up to 22% of cleavage (45 °C)	large scale treatments were not effective	78

[a]Abbreviations: AGP = aspergillopepsins I and II; CHI = Chitinase; HUP = heat unstable protein; TLP = thaumatin-like protein.

tions or during the grape infection and have an advantage to be adapted to grape-derived physicochemical conditions, because they are either active during the cultivation or the wine processing.[24] An extracellular peptidase from the *Saccharomyces cerevisiae* PIR1 strain was able to cleave native HUPs at moderate temperatures (38 °C) and acidic pH (3.5) when applied during different stages of wine processing[33] but only when incubated for 48 h. Theron et al.[80] reported a recombinant MpAPr1 aspartic peptidase secreted by the wine yeast *Metschnikowia pulcherrima* capable of degrading native CHIs, although still at moderate temperatures (25 °C), but they stil needed a minimum of 48 h of incubation. Because the peptidase was active at a low pH and moderate temperatures, the authors suggested their industrial application, but the protease provided a full degradation of CHIs and only 25% depletion of TLPs at pH 4.5 and 40 °C. From grape pathogens, a BcAp8 peptidase from *Botrytis cinerea* has been reported for its potential to degrade CHIs at moderate temperatures (at 55 °C and 18 h of incubation).[68] Peptidases from *Aspergillus niger* showed controversial results in fining applications.[66,79] But, glutamic peptidases from different *Aspergillus* spp. (aspergillopepsin I and II) could reduce the amount of wine proteins by 20% at wine pH and low temperatures.[72]

Some patents have claimed peptidases or methods for a successful wine clarification as an alternative to bentonite clay. Mutsaers and Edens[74] suggested that proline-specific endopeptidases from *Penicillium* and *Aspergillus* species were able to reduce wine haze levels. Sun and Harris[81] applied peptidases from the organisms *Aspergillus niger*, *Aspergillus oryzae*, *Rhizomucor meihei*, *Neosartorya fischeri*, *Candida olea*, and *Saccharomyces cerevisiae* in different stages of fermentation of musts of white grapes such as Chardonnay and Sauvignon blanc. They found a peptidase from *A. niger* active at pH 2.5 to 4 and low temperatures that could efficiently degrade wine proteins at concentrations between 30 and 899 mg/L. However, to the best of our knowledge, none of these peptidases have been used for fining wines to date.

5.2.2. Are Plant or Animal Peptidases Applicable in Wine Clarification? Plant peptidases have also shown efficacy in reducing turbidity in wines, e.g., an immobilized pineapple (*Ananus bracteratus*) stem enzyme, namely, bromelain, was able to reduce at least 70% of the wine haze formation in a stirred reactor at 20 °C for 24 h.[75] Papain from *Carica papaya* latex was also suggested for application in wine fining for its ability to hydrolyze synthetic substrates at pH 3.2, in the presence of ethanol.[77] However, in contrast, researchers have also discussed the inefficiency of plant peptidases under acidic conditions, such as ficin from *Ficus* sp. or the already mentioned papain and bromelain.[81] From animal sources, a porcine pepsin (from the stomach mucosa of pigs) showed potential applications as it was active at wine pH,[78] but only when wines were heated at 90 °C for 1 min (short-term heating) or at 45 °C for 1 day. Although plant-derived peptidases such as papain and bromelain can be active at pH values that exist in fruits (from 2.5 to 4),[81] they have not been recommended for wine fining applications so far,[82] contrary to enzymes from fungal origin.[78]

5.3. Peptidases as Fining Agents and Current Limitations. Wine quality is usually associated with oenological traditional methods, and the implementation of new fining agents, heating, and pH manipulations is not always well seen by winemakers. Although several aspartic and

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Table 2. Methods for Identification of Cleavage Products of Heat-Unstable Proteins (HUPs) and the Related Reference Ordered by the Year of Publication

method	description	reference
SDS-PAGE	comparison of protein bands in electrophoresis gels (molecular weight, band location and density)	Comuzzo et al.[89] (2020)
		Theron et al.[73] (2018)
		Younes et al.[83] (2013)
		Gazzola et al.[19] (2012)
		Marangon et al.[72] (2012)
		Ngaba-Mbiakop[90] (1981)
2-D electrophoresis	two-dimensional PAGE analysis of the products of cleavage	Marangon et al.[34] (2014)
HPLC	comparison of peak profiles and intensities in HPLC chromatograms	Gazzola et al.[19] (2012)
		Marangon et al.[72] (2012)
		Pocock et al.[78] (2003)
haze measurement	haze measurement by absorbance (at 540 nm/520 nm) or nephelometry	Sui et al.[91] (2021)
		Comuzzo et al.[89] (2020)
		Benucci et al.[76] (2014)
		Mutsaers and Edens[74] (2014)
		Gazzola et al.[19] (2012)
		Marangon et al.[66] (2012)
		Ngaba-Mbiakop[90] (1981)
heat test and protein quantification	Quantification of TLP/CHI or total protein (Bradford, HPLC, etc.) after proteolytic treatment or heat test	Sui et al.[91] (2021)
		Comuzzo et al.[89] (2020)
		van Sluyter et al.[68] (2013)
		Marangon et al.[72] (2012)
		Pocock et al.[78] (2003)
bentonite requirement	quantification of the amount of bentonite clay needed after fining by peptidases	Mutsaers and Edens[74] (2014)
		Pocock et al.[78] (2003)
LC−MS (bottom-up)	LC−MS identification of the haze proteins bands extracted from electrophoresis gel	Mutsaers and Edens[74] (2014)
		Gazzola et al.[19] (2012)
		Marangon et al.[72] (2012)
		Ngaba-Mbiakop[90] (1981)
top-down MS (peptidomics)	identification of intact peptides from haze proteins after proteolysis by using top-down MS peptidomics	Albuquerque et al.[85] (2021)

glutamic peptidases were found to be active in acidic conditions, they were only able to cleave wine proteins at moderate temperatures around 45[83] or 55 °C in significant amounts.[68] In addition, bentonite is also necessary for a complete wine clarification even after proteolytic treatments, which still implies a combination of techniques and the deleterious effects of bentonite fining. Finally, although some advances have been achieved, the OIV (International Organization of Vine and Wine) has not yet approved industrial applications of proteolysis for all kind of peptidases in winemaking, which makes its adoption difficult.

6. WHICH METHODS ARE AVAILABLE TO DETECT THE DEGRADATION OF WINE PROTEINS BY PEPTIDASES?

There is still a lack of ultrafast methods to identify degradation products of real wine proteins, which would facilitate the screening of peptidases from different organisms against wine proteins at various pH values and temperatures. Most of the analytical methods are based on measurements of turbidity levels before and after a heat test by absorbance or nephelometry,[72] comparison of the intensity of protein bands in electrophoresis gels (SDS-PAGE),[73,83] and chromatographic peaks of peptide cleavage products.[78] However, recent improvements in mass spectrometry (MS) instrumentation have led to the acquisition of analytical techniques that help in predicting the native state of the protein (top-down MS).[84] These new techniques can be used to understand cleavage sites in HUPs[85] and provide a tool for finding enzymes suitable for preventing wine haze. Another new method for wine protein analytics could be the fluorescence correlation spectroscopy (FCS).[86] Since it is applied for analyzing the conformation, aggregation, and concentration of fluorescent molecules, FCS might be an interesting tool for measuring the molecular and macromolecular aggregation without disturbing the wine system. The only drawback is that only fluorescently labeled molecules can be measured. For the best of our knowledge, the use of fluorescently labeled wine protein has not been reported until now. Another not well-explored tool for studying wine haze would be Fourier-transform infrared (FTIR) spectroscopy, which is nowadays a very powerful nondestructive technique that provides structural information on the molecular features of a large range of compounds. Its use in wine analytics provides the opportunity for the quantification of several matrix components such as organic acids, ethanol, or sugars[87] and might help with the characterization of protein structures in aqueous solutions.[88] Table 2 lists methods reported to detect cleavage products from HUPs.

7. NEW STRATEGIES FOR PROTEOLYTIC DEGRADATION IN WINES

7.1. Proteolysis of Heated Wine Proteins. Denaturation facilitates the degradation of wine proteins, and therefore, the pasteurization of wine or musts in combination with the

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Table 3. Literature Comparison of Successful Proteolytic Treatments Associated with Heating Procedures for the Reduction of Wine Haze

peptidase	concentration	temperature	duration	cooling to	results	reference
proctase, sumizyme or natuzyme	15 or 30 mg/L	60, 65, or 70 °C	1, 2, or 10 min	4 °C	protein removal of 30−96%	91
liquid preparations of aspergillopepsins	1 and 2 mL/L	75 ± 2 °C	2 min	25 °C	Δturbidity similar to a bentonite (200 mg/L) fined wine	89
proctase (aspergillopepsin 1 and 2)	15 mg/L	75 °C	1 min	3 °C	90% total protein reduction	72
trenolin blank or porcine pepsin	trenolin (10 mL/L) pepsin (100 mg/L)	90 °C	1 min	19−16 °C	40−80% of reduction of wine proteins	78

addition of peptidases have been proposed,[20] so HUPs could be successfully degraded.[72] Francis et al.[92] reported temperatures around 62 and 55 °C that were necessary to completely unfold most kinds of TLPs and CHIs, respectively. It was observed that only high temperatures can denature HUPs during industrial processes. Additionally, the heating duration is important to not affecting wine organoleptic characters, which was demonstrated by Ferenczy already in 1966.[93] Therefore, only a very short time frame for the heating is used in a flash pasteurization mode (from 1 to 10 min). An aspergillopepsin commercially named "Proctase", active at acidic pHs (2−4) and at high temperatures (50−70 °C), was able to reduce the concentration of TLPs and CHIs by about 90% when added to grape juices prior flash pasteurization steps.[72] Moreover, this heating process did not affect the wine sensory properties.[72] A more recent study with two different "Proctase" preparations could demonstrate a reduction of haze levels after flash pasteurization but no complete removal of HUPs.[89] A similar approach was the usage of peptidases in combination with preheating and ultrafiltration, where only the retentate fractions of wine were heated to 62 °C for 10 min.[91] Although the authors did not investigate the impact of the heating on the sensory profiles of the wine, they referred to another study, which stated that heating to 61 °C for up to 51 min did not result in perceivable changes in the wine aroma profile.[94] Similar results could be demonstrated by Pocock et al.[78] who found no adverse effects of heating (90 °C) on wine organoleptic properties. Nevertheless, although it has been partially proven that heating treatment does not severely impact wine sensory properties, it has not yet been widely adopted by oenologists.[34,91] Moreover, such methods are still criticized because of the preference to work at low temperatures to preserve wine quality.[78] A concise comparison of heating conditions and enzymes applied in combination with pasteurization processes is shown in Table 3.

7.2. Immobilization of Peptidases. Immobilization methods have been proposed to stabilize enzymes against harsh alcoholic and acidic environments that prevail in wine, and immobilized peptidases have already been shown to be active at winemaking conditions by the degradation of synthetic substrates.[95] Benucci et al.[96] demonstrated that the immobilization of bromelain could improve the enzyme's resistance against the wine environment including the acidic pH, phenolic compounds, and sulfur dioxide without affecting the sensory quality. The authors incubated seven unfined white wines for 24 h with 10 g/L of the chitosan-immobilized enzyme in a stirred reactor and observed a percentage of turbidity reduction from 59 to 96% and a protein removal from 14 to 68%. The same authors reported that chitosan-immobilized papain reduced the haze potential of white wines from 31 to 83%.[97] Agarose-immobilized acidic peptidases were also found to be stable under winemaking conditions when applied in stirred bioreactors.[66]

7.3. Other Organisms to Overcome the Limitations of Traditional Peptidase Sources. Since there is no peptidase reported to be active in alcoholic medium, at low temperatures, at acidic pH, and resistant to inhibitory elements of the wine matrix, there is a strong need to think out of the box of the current overview. This may include the following: (I) search for enzymes derived from organisms that survive the winemaking process such as the lactic acid bacterium *Oenococcus oeni*,[98] since their enzymes are adapted to conditions under which winemaking is carried out; (II) enzymes derived from organisms that survive in harsh conditions (extremophiles) and could be expressed and are active in a broad range of temperatures. The enzymatic toolbox of psychrophiles, for example, is adapted to low temperatures, similar to that present in wine[99] (III) Insects or insect-associated enzymes are organisms that have the ability to utilize grapes as a source of nutrition, such as the grape berry moth *Paralobesia viteana*[100] or the spotted wing *Drosophila suzukii*.[101]

7.4. Protein Engineering As a Method to Tailor Peptidases to the Needs of Winemakers. The use of protein engineering (PE) might be promising in generating catalysts for clarification purposes or by improving a particular enzyme property such as the stability of an active acidic peptidase. It has already been shown that the thermostability or the selectivity of peptidases could be improved by PE, but the challenges here are that (I) an initial activity would be necessary[102] and (II) depending on the PE technique used, there is a need for great screening efforts to successfully tailor the peptidase to the specific requirements of winemaking.[103]

8. DISCUSSION

Effective proteolysis under winemaking conditions is still challenging, and a better understanding of cleavage mechanisms of TLPs and CHIs could help reduce their resistance to peptidases. Over the past decades, various interpretations of the origin of wine haze have evolved. Initially considered as a result of electrical interactions of charges and bonds between phenolic compounds and basic amino acids,[90] the haze is now discussed as a consequence of cross-linking reactions between particular structural features in HUPs.[24,34] Recent advances in protein analytics, such as FTIR spectroscopy and high-resolution mass spectrometry, are still in an early phase of application in studying wine HUPs and have not been widely used.[85] Their application will probably improve the identi-

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fication of peptidases active at acidic pH and low temperatures. On one hand, they could shed light on how loops or exposed irregular structures contribute to the haze formation and how their removal would hinder cross-linking chains; on the other hand, they could help to reveal which spots of HUPs are more susceptible to proteolysis under winemaking conditions on the basis of the analysis of protein interaction networks and dynamics.[104]

Among the thermolabile wine proteins, studies in the literature mostly focus on TLPs, including their interaction with polyphenolic compounds, sulfite ions, and polysaccharides, and describing their full structural characteristics.[34] Furthermore, it is known that CHIs unfold irreversibly and generate higher turbidity levels and larger aggregate particles; however, not much is known about their structural properties and their structural contribution to haze formation mechanisms. One reason for the limited structural information might be the fact that CHIs are less stable, which makes it difficult to study their structure.

Since TLP and CHI unfold between 55 and 62 °C, respectively, the simultaneous use of moderate temperatures (instead of high temperatures) and acidic peptidases has been until now the most successful proteolysis strategy to reduce the amount of required bentonite in fining processes.

Using functional peptidases as a protein fining agent would minimize losses of wine volume and quality. According to an estimation by the Australian Wine Institute AWRI, the global loss of wine due to bentonite fining is more than 1 billion US dollars per year.[105] To overcome the problem of low activity of the known peptidases, new acidic peptidases from organisms living in harsh environments, from grape parasites or insects that feed on grapes, should be tested as potential fining agents to expand the portfolio of possible enzyme candidates, such as those suggested by Strauss et al.[106] A precise analysis of the different haze forming factors and relationship toward protease efficiency reveals that the reduction of wine protein levels is clearly the biggest issue, since higher amounts of HUPs cause higher haze levels. Albuquerque et al.[85] showed that peptidases can directly act on the main aggregation factor and prevent haze in wine protein solutions. The authors also proved that their haze potential was reduced proportional to the level of the degradation of HUPs. As maybe not one single peptidase can cleave all HUPs, an alternative would be the application of enzyme mixtures, such as different peptidases or a combination of proline-specific endopeptidases with polyphenol oxidases, as suggested by Mutsaers and Edens,[74] which could prevent protein−phenol interactions in wines. The reduction of protein−phenol interactions and S-sulfonationed peptides, probably the second important haze forming factors, would be facilitated by peptidases, since they can reduce the number of available binding sites in the proteins. Since the haze formation in wines is not triggered by an isolated factor, the key to the successful usage of peptidases could lie in a combination of different strategies mentioned above. A possible combination to overcome the limitations of the currently known peptidases could lie in immobilized, PE-optimized peptidase mixtures from a novel organism. A step in the right direction is the recent approval by the OIV of the use of "Proctase" combined with flash pasteurization, which is already one advance in the acceptance of peptidases as fining agent. We hope that the topics covered in the present review can provide a bridge to the successful leap of peptidases into industrial applications in winemaking.

9. CONCLUSION

This review mainly aimed to describe the advances of theories of wine haze formation and on the application of peptidases as an alternative to bentonite fining. We discussed both haze and proteolysis mechanisms to propose applications of peptidases at low pH values and temperatures. The search for ideal sources for enzymes, rapid screening methods, and the use of recent advances in protein analytics and engineering are suggested to improve the application of proteolysis for wine clarification.

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Notes

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Research articles

Research article 1 – Identification of intact peptides by top-down peptidomics reveals cleavage spots in thermolabile wine proteins

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Identification of intact peptides by top-down peptidomics reveals cleavage spots in thermolabile wine proteins

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ABSTRACT

Prevention of haze formation in wines is challenging for winemakers. Thermolabile proteins in wines, notably thaumatin-like proteins (TLPs) and chitinases (CHIs), undergo structural changes under varying physicochemical conditions, resulting in protein aggregation and visible haze in bottled products. Peptidases are an alternative fining method, although an effective proteolysis under typical winemaking conditions (acidic pH and low temperature) is difficult to achieve. In this study, tryptic peptides from TLPs and CHIs were identified by MS-based peptidomics (top-down proteomics) after exposure of scissile bonds on the protein surface. As proposed by the theory of limited proteolysis, protein conformational changes following temperature and pH variations allowed the detection of enzyme-accessible regions. Protein structure visualization and molecular dynamics simulations were used to highlight cleavage spots and provide the scientific basis for haze formation mechanisms. The described method offers a tool to the search for ideal enzymes to prevent wine haze.

1. Introduction

Protein haze in bottled wines is caused mainly by improper temperature conditions during transportation and storage, leading to rejection of the product by consumers. Insoluble haze particles are formed by cross-linking reactions between heat unstable proteins (HUPs), namely thaumatin-like proteins (TLPs) and chitinases (CHIs), through molecular bridges formed via phenol–protein interactions (Gazzola et al., 2012; van Sluyter et al., 2015) or disulfide bonds (Chagas et al., 2018; Pocock et al., 2007). Polysaccharides are also reported to influence the wine haze potential by interacting with wine proteins and forming stable protein/polysaccharide complexes, which reduce the aggregation rate of HUPs (Dufrechou et al., 2015). The structural conformations of these HUPs are directly affected by temperature and pH (Dufrechou et al., 2013), which can influence the exposure of hydrophobic cores. This leads to the induction of protein–protein interactions that are governed by hydrophobic attractive forces (Marangon et al., 2010), π-π stacking (di Gaspero et al., 2020; Mcrae & Kennedy, 2011) and occasionally, even the formation of covalent bonds through S-sulfonation of thiol residues (by SH-SS exchanges) (Chagas et al., 2018). The state-of-the-art technology for preventing haze is the usage of bentonite clay, which is reported to have deleterious effects on the sensory properties of wine and reduces the final product volume (Salazar et al., 2017). Since haze formation is induced by aggregation and precipitation of proteins, proteolytic enzymes (peptidases) may serve as an alternative to the traditional fining agents (van Sluyter et al., 2013), although typical winemaking conditions including strongly acidic pH, low temperatures and the presence of enzyme inhibiting compounds such as ethanol, sulfur dioxide (SO_2) and polyphenols are still challenging to overcome (Claus & Mojsov, 2018).

The theory of "limited proteolysis" (Hubbard, 1998; Novotný & Bruccoleri, 1987) proposes that only some prominent protein regions, such as loops and irregular structures, are accessible to proteolytic enzymes. Scissile bonds on protein surfaces are gradually exposed under slight temperature and pH changes, producing distinct detectable cleavage products. Therefore, physicochemical modifications promote the disruption of hydrogen bond networks and destabilization of protein

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tertiary conformations (Pace et al., 1996). As a consequence of the conformational changes, "sticky" hydrophobic amino acids are exposed on the protein surface and trigger different patterns of aggregates (Philo & Arakawa, 2009). Other effects of these modulations are intramolecular protein fluctuations and flexibility (Kamerzell & Middaugh, 2008), which may influence the proteins' susceptibility to enzymatic hydrolysis.

Cleavage spots inside the wine TLPs and CHIs have not yet been reported, and the analysis of degradation products has so far been restricted to chromatographic or electrophoretic methods (Marangon et al., 2012). Recent improvements in mass spectrometry (MS) instrumentation resulted in the acquisition of analytical techniques that help to predict the protein's native state. Top-down MS strategy allows the direct analysis of intact proteins and peptides without any preliminary chemical treatments, providing comprehensive data regarding the in-depth structural characterization of the proteins (Catherman et al., 2014; Ghezellou et al., 2019). In this study, we propose a method to combine top-down peptidomics with the theory of "limited proteolysis" under native and denaturing conditions. The goal is to identify and localize fragments from proteolytic digestion and linking them to molecular mechanisms of haze formation. With the identification of intact peptides from tryptic digested wine proteins and subsequent molecular dynamic simulations, further discussions about the formation of haze protein–protein interactions are fueled. The presented method is a leap forward in the fields of wine haze analytics and will be a key technology for the search of suitable proteolytic enzymes for wine clarification.

2. Materials and methods

2.1. Technical scale isolation of high protein-containing colloids from wine

Protein rich Silvaner Franken (250 L) wine was obtained from the Bavarian State Office for Wine and Horticulture (LWG) in Veitshoechheim, Germany. For heat test, which is usually carried out during wine production, the turbidity difference before/after the test should not exceed 2–3 NTU (nephelometric turbidity units), otherwise the sample is deemed to be unstable. The investigated Silvaner Franken turned out to be extremely unstable in the heat test (turbidity difference $>$ 50 NTU). For the heat test we measured the turbidity of the clear wine sample nephelometrically (Nephla LPG, Hach-Lange, Düsseldorf, Germany) before heating and after the procedure of heating and recooling. The heating was performed in a water bath to 80 °C for 3 h, subsequently the cool down to 5 °C in a refrigerator for at least 16 h, and a warming up to ambient temperature was done. The wine was sheet-filtered to remove coarse particles to prevent their co-isolation in the following ultrafiltration steps. A 40 × 40 cm stainless steel sheet filter (Pall-Seitz-Schenk, Bad Kreuznach, Germany) packed with 5 filter sheets (K 250, Pall-Seitz-Schenk) was used. After filtration, the clear products were subjected to ultrafiltration with a Sartocon beta system (Sartorius, Goettingen, Germany) equipped with a rotary pump and two 0.6 m^2 Sartocon Hydrosart cassettes [molecular weight cut-off (MWCO) of 10 kDa]. After reducing to approximately 5 L retentate, concentration factors of about 40–50 were achieved. The retentate was further diafiltered with 100 L of citrate buffer (5 g/L, pH 4.0) to remove phenolic substances. Afterwards, the buffer was diafiltered against 100 L of distilled water. The whole procedure was done under low pressure conditions (max. transmembrane pressure 0.1–0.15 MPa) to ensure a gentle process and to avoid thermal stress generated by friction heat. The final retentate was quantitatively removed from the system and lyophilized. The resulting hygroscopic colloid material was stored in airtight plastic containers.

2.2. Analysis of the colloids and the wine protein solutions by sugar determination and protein and amino acid quantification

The protein content of the lyophilized colloid was determined spectrophotometrically according to (Bradford, 1976) by the Roti-Nanoquant assay (Carl Roth, Karlsruhe, Germany) using bovine albumin fraction V (Carl Roth) as reference protein. Absorbance was measured at 595 nm on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT, USA) and the Gen5 software (v. 2.01, BioTek).

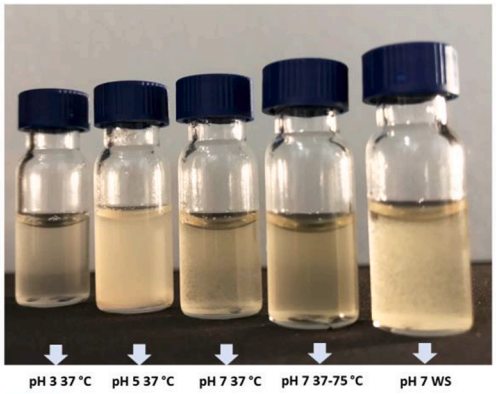
For the determination of neutral and acidic sugars, the freeze-dried colloids (10–15 mg) were treated by 125 µL 72% sulfuric acid (Bernd Kraft, Duisburg, Germany) for 45 min. After dilution with 1.35 mL water to 1 M sulfuric acid, the samples were heated to 120 °C in pressure-resistant reaction tubes with screw caps (16 × 100 mm Duran, Mainz, Germany) in a thermo block (TempContoller TR-L 288 Liebisch, Bielefeld, Germany) for 60 min. After hydrolysis, the samples were filled up to a volume of 50 mL with water and membrane filtered (0.2 µm PES filter, VWR, Darmstadt, Germany). Neutral and acidic sugars in the hydrolysates were analyzed by HPAEC-PAD on a Dionex/ThermoFisher BIOLC system (ICS 5000+, ICS 3000 SP, Thermo Fisher Scientific, Bremen, Germany) coupled with pulsed amperometric detection. The system was controlled by Chromeleon software (v. 7.2, Thermo Fisher Scientific). A 10 µL filtrated sample was injected into an anion-exchange Carbopac PA 100 column (250 × 4 mm; 8.5 µm, Thermo Fisher Scientific) with a corresponding guard column (4 × 50 mm). For neutral sugars, an isocratic elution with 0.012 mol/L NaOH (Bernd Kraft) at a flowrate of 0.6 mL/min was used. After separation, the column was conditioned with 0.5 mol/L NaOH (36.5–55 min) and re-equilibrated to 0.012 mol/L NaOH. Acidic sugars were separated under isocratic conditions in a second run with a mobile phase consisting of 0.5 mol/L NaOH and a flow rate of 0.5 mL/min. Quantification was carried out with external standard calibrations. D-rhamnose, L-arabinose and D-glucuronic acid, D-galactose, D-mannose and D-galacturonic acid were obtained from Fluka (Taufkirchen, Germany), and D-glucose from Carl Roth. Results are given in Supplementary Table 1 as the means of duplicates.

Isolated and freeze-dried wine colloids (40–50 mg) were hydrolyzed for 24 h with 2 mL 6 mol/L HCl (J. T. Baker, Deventer, Netherlands) in nitrogen-rinsed tubes with screw caps (16 × 100 mm, Duran) at 110 °C in a drying cabinet. After cooling to room temperature, samples were filtered, and aliquots of 200 µL were dried in a thermo block (1 h, 135 °C; TempController TR-L 288 Liebisch, Bielefeld, Germany), dissolved in 1 mL buffer (LiOH solution, pH 2.2, with norleucine as an internal standard). The membrane-filtered (0.45 µm) samples were used for amino acid analysis, which was performed with an amino acid analyzer (S433, Sykam GmbH, Eresing, Germany), using a Harzbett column (150 mm × 4.6) and postcolumn ninhydrin detection (Supplementary Fig. 3). Quantitation was performed based on the internal standard. Results are given in Supplementary Table 2 as the means of duplicates.

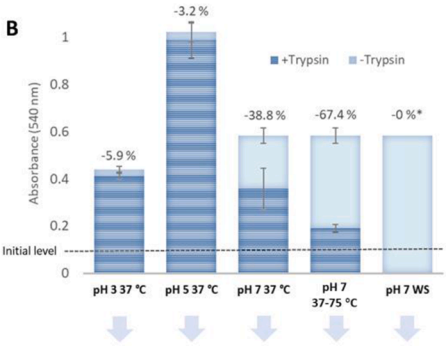
2.3. Heat test and haze measurement by spectrophotometry

Haze formation was evaluated by a heat test according to Pocock and Waters (2006), with modifications. Briefly, 1 mL of the resuspended wine colloid [with a protein concentration of approximately 1 mg/mL in buffers at pH values of 3, 5 [both in 0.1 M citrate buffer (Carl Roth, Karlsruhe, Germany) and 7 [in 0.1 M Tris-HCl buffer (Carl Roth)] was incubated with 50 µL of a trypsin solution (Trypsin gold #V5072, Promega, Germany, at 0.1 mg/mL in buffers at different pH values) for 24 h at 37 °C. The samples were then heated to 75° C (cooling-thermomixer HLC Biotech, Bovenden, Germany) for 20 min, followed by cooling down to room temperature and subsequent absorbance measurement at 540 nm (Marangon et al., 2014) (Spectrophotometer Biotek). Each experiment was performed both, in the presence and absence of trypsin, to estimate the effects of the proteolysis on haze formation. All analyses were performed in triplicates.

A



B



C

	Accession Number	Description	pH 3 37 °C	pH 5 37 °C	pH 7 37 °C	pH 7 75 °C-37 °C	pH 7 WS
1	**Q7XAU6**	**Class IV chitinase**	**X**	**X**	**Found**	**Found**	**X**
2	**B0FZ27**	**Class IV chitinase**	**X**	**X**	**Found**	**Found**	**X**
3	**A9CSP0**	**Putative thaumatin-like protein**	**X**	**X**	**Found**	**Found**	**X**
4	**A0A438I656**	**Glucan endo-1,3-β-glucosidase**	**X**	**X**	**Found**	**Found**	**X**
5	**A0A438DX78**	**β-fructofuranosidase, soluble isoenzyme I**	**X**	**X**	**Found**	**Found**	**X**
6	**A0A438K622**	**Aspartyl protease family protein 1**	**X**	**X**	**Found**	**Found**	**X**
7	**Q850K5**	**Non-specific lipid-transfer protein**	**X**	**X**	**Found**	**Found**	**X**
8	**A5BUW9**	**Uncharacterized protein**	**X**	**X**	**Found**	**Found**	**X**
9	**F6HZQ9**	**Expansin-like EG45 domain-containing protein**	**X**	**X**	**Found**	**Found**	**X**
10	**F6HAU0**	**Uncharacterized protein**	**X**	**X**	**Found**	**Found**	**X**
11	A0A438JJ58	Thaumatin-like protein	X	X	X	Found	X
12	A0A438HVS3	Endochitinase EP3	X	X	X	Found	X
13	A0A438BZL8	Thaumatin-like protein	X	X	X	Found	X
14	Q9SBY5	1,3 β-glucanase (Fragment)	X	X	X	Found	X
15	D7T227	Uncharacterized protein	X	X	X	Found	X
16	F6HXK4	Plasma membrane ATPase	X	X	X	Found	X
17	A0A438KGA8	Glucan endo-1,3-β-glucosidase	X	X	X	Found	X
18	A0A438HL46	Fasciclin-like arabinogalactan protein 10	X	X	X	Found	X
19	Q9M3U4	β 1-3 glucanase	X	X	X	Found	X
20	A0A438JKZ8	Glucan endo-1,3-β-glucosidase 8	X	X	X	Found	X
21	A0A438KIN6	PI-PLC X domain-containing protein	X	X	X	Found	X
22	A0A438DZR8	Non-specific lipid-transfer protein	X	X	X	Found	X
23	A0A438CS31	Glyceraldehyde-3-phosphate dehydrogenase	X	X	X	Found	X
24	F6HIB3	FAS1 domain-containing protein	X	X	X	Found	X
25	F6HTC9	PMEI domain-containing protein	X	X	X	Found	X
26	A0A438DEA6	Heat shock cognate 70 kDa protein	X	X	X	Found	X
27	F6HMA2	Uncharacterized protein	X	X	X	Found	X
28	A0A438JVD2	Peroxidase	X	X	X	Found	X
29	A5C0I8	Uncharacterized protein	X	X	X	Found	X
30	Q2QCI7	Non-specific lipid-transfer protein	X	X	X	Found	X
31	A0A438JSE9	Ubiquitin-60S ribosomal protein L40	X	X	X	Found	X
32	A0A438C3D6	LysM domain-containing GPI-anchored protein 1	X	X	X	Found	X
33	A0A438H4P5	Early nodulin-like protein 2	X	X	X	Found	X
34	A5B878	Nucleoside diphosphate kinase	X	X	X	Found	X
35	A0A438J6L0	11S globulin seed storage protein 2	X	X	X	Found	X
36	A0A438KNZ5	Putative inactive receptor kinase	X	X	X	Found	X
37	A0A438H2B0	Putative glutathione S-transferase	X	X	X	Found	X
38	A0A096ZEC7	Tonoplastic transporter 1	X	X	X	Found	X
39	A0A438F2P7	Glucan endo-1,3-β-glucosidase	X	X	X	Found	X
40	A0A438CZN1	Pectinesterase/pectinesterase inhibitor 3	X	X	X	Found	X
41	A0A438HIQ1	Aquaporin PIP2-2	X	X	X	Found	X
42	A0A438FNA6	Cysteine proteinase RD21A	X	X	X	Found	X
43	F6HVL7	Barwin domain-containing protein	X	X	X	Found	X
44	A5B118	Fructose-bisphosphate aldolase	X	X	X	Found	X
45	A0A438JSX9	LysM domain-containing GPI-anchored protein 2	X	X	X	Found	X
46	A0A438HJR5	Tetraspanin-8	X	X	X	Found	X
47	F6GU73	Protein kinase domain-containing protein	X	X	X	Found	X
48	A0A438I0P8	Uncharacterized protein	X	X	X	Found	X
49	A5B3K2	Uncharacterized protein	X	X	X	Found	X
50	A0A438GDG5	Glycerophosphodiester phosphodiesterase	X	X	X	Found	X
51	A5BQX9	Integrase catalytic domain-containing protein	X	X	X	Found	X
52	F6H7K8	X8 domain-containing protein	X	X	X	Found	X
53	F6I686	PINc domain-containing protein	X	X	X	Found	X
54	A0A438DS38	Profilin-1	X	X	X	Found	X
55	A5C048	Uncharacterized protein	Found	X	X	X	X
56	A0A438DP97	G-type lectin S-receptor-like serine/threonine-protein kinase	Found	X	X	X	X

Fig. 1. A comparison between the number of cleaved proteins and the haze formation levels at different physicochemical conditions. (A) Picture of the wine protein suspensions (after heat test) at different pH values along with the addition of trypsin (except pH 7 WS, which represents the wine protein solution without trypsin supplementation). The sample "pH 7 75–37 °C" was pre-denatured at 75 °C and afterwards incubated with trypsin at 37 °C. (B) Haze formation level at different temperatures and pH values. The dark blue bars represent the absorbance post tryptic digestion, the light blue bars show the absorbance levels of the same samples incubated without trypsin. The sample WS (without trypsin solution) is only represented by a light blue bar (*). The dashed line (about 0.1 abs) represents the initial levels of absorbance before the heat test. (C) List of 56 proteins with peptide fragments detected at different experimental conditions after the peptidomics analysis (see the complete list in the Supplementary Table 3). The reduction of the absorbance in at each experimental condition (B) is correlated with the number of peptides detected in the peptidomics analysis (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Top-down peptidomics

2.4.1. Sample preparation

As a positive control, a wine protein solution (pH 7) that was previously denatured at 75 °C was also incubated with trypsin. As a negative control, the wine protein solution without tryptic treatment was incubated under the same conditions. The reaction mixtures were subsequently filtered through 0.5 mL Amicon® ultra centrifugal filters (Merck Millipore, Darmstadt, Germany), with a MWCO of 10 kDa and collected. These were finally vacuum dried and subjected to mass spectrometric analysis (Supplementary Fig. 1).

2.4.2. Liquid chromatography high-resolution tandem mass spectrometry (LC-HR-MS/MS)

Peptide mixtures were separated using an UltiMate 3000 RSLC UHPLC system (Ultrahigh-Performance Liquid Chromatography, Thermo Fisher Scientific) on a bioZen™ LC Column (3.6 μm, 50 × 2.1 mm, pore size of 200 Å; Intact XB-C8, Phenomenex, CA, USA) coupled to a Q Exactive HF-X (Thermo Fisher Scientific). Chromatographic analysis was performed at a flow rate of 250 μL/min using water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) as eluents. The following gradient elution profile was applied as follows: isocratic (2% B) for 3 min, followed by 2–40% B over 77 min, 40–70% B over 10 min, and re-equilibration (2% B). The mass spectrometer was operated in data-dependent acquisition mode (top-10 DDA) using the following parameters in full MS scans: mass range of m/z 350 to 1800, resolution of 120,000, AGC target of 3×10^6, IT of 50 ms, and MS/MS scans: mass range of m/z 200 to 2000, resolution of 30,000, AGC target of 1×10^5, IT of 120 ms, isolation window m/z 1.3 and dynamic exclusion of 60 s.

2.4.3. Data analysis

MS raw files were processed by the software Proteome Discoverer (PD v. 2.2, Thermo Fisher Scientific) (Dorfer et al., 2014). The obtained sequences were searched against the UniProtKB databases, which were taxonomically set to *Vitis vinifera* (organism from which haze forming proteins are derived). The following parameters were further set: two missed cleavage sites of trypsin digestion; minimum peptide length of six amino acids; MS^1 and MS^2 tolerances of 10 ppm and 0.5 Da, respectively. The percolator node was used to validate identified peptide-spectrum matches (PSMs) and to filter the data with parameters of a strict target FDR (false discovery rate) of 0.01 and a relaxed target FDR of 0.05. The MaxQuant contaminant database was used to mark contaminants in the results file. Protein identification was considered valid when the MS spectra matched to one or more unique peptides and only the highest scored peptides (with high statistical confidence) ranked by the software PD were considered for the analysis. The semi-quantitative values of the targeted peptides and proteins were achieved based on relative MS signal intensities and peak areas using the MZmine2 software (Pluskal et al., 2010). For this purpose, the subsequent steps of peak detection (mass detection, chromatogram building and peak deconvolution), isotope grouping, peak alignment and filtering were applied to process the raw MS spectra, and then the MS ion intensities corresponding to the analyzed peptides were compared. The software PatternLab for Proteomics 4.0 and Byonic, Protein Metrics were used to generate the peptide lists available as supplementary information and the data for the deposition to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD024723 and https://doi.org//10.6019/PXD024723.

2.4.4. Label-free peptide quantification

For the label-free peptide quantification, extracted ion chromatograms (EIC) from the full scan mass spectrometry data (MS^1) that were obtained from the peptidomics analysis were processed. A reduction of background noise was carried out by peak filtration. For that, ion peaks were filtered (mass tolerance of 0.1 and retention time tolerance of 0.5) at the m/z range of 1087.5 (for the peptide LDSGQSWTITVNPGTTNAR, from a putative TLP, ID: Q9M4G6) and 1153.1 (for the peptide AAFLSALNSYSGFGNDGSTDANKR, from a class IV CHI, ID: Q7XAU6). For better visualization, each peak was represented in a three-dimensional Cartesian coordinate system, with the m/z ratio represented on the X-axis, the retention time on the Y-axis and the intensity (peak height) on the Z-axis.

2.5. Preparation of structure models of TLP and CHI

The molecular structure of the TLP from *Vitis vinifera,* obtained from Protein Data Bank (PDB) [ID: 4JRU, (Marangon et al., 2014)], was used for the visualization. For comparison of the different TLP isoforms identified by the peptidomics analysis a structural alignment was performed and provided in Supplementary Fig. 2. Due to the non-availability of a crystallographic structure of a wine class IV CHI, a three-dimensional model was constructed by Modeller (v. 9.23) (Šali & Blundell, 1993), which makes use of the homology modeling technology. The template was a class IV CHI from *Zea mays* (PDB ID: 4MCK) with 68.34% identity compared to a class IV CHI from *Vitis vinifera* by BLAST (Basic Local Alignment Search Tool) (Chaudet et al., 2014). The DOPE (Discrete Optimized Protein Energy) method was used to assess the obtained model (Shen & Sali, 2006). All structures were visualized with PyMol v.2.3.2.

2.6. Molecular dynamics simulation

The three-dimensional model of CHI was further optimized by molecular dynamics (MD) simulations. More importantly, MD was employed to study the structural change of wine haze proteins under denaturation and renaturation conditions systemically. MD simulations were performed using NAMD (v. 2.14b) (Vanommeslaeghe et al., 2009). For all MD simulations, the proteins (TLP or CHI) were incubated in a cubic TIP3P water box of $94 \times 94 \times 94$ $Å^3$ under NPT (normal pressure and temperature) system. The particle-mesh Ewald (PME) method, as an efficient full electrostatics method, was employed for use with periodic boundary conditions (Ewald, 1921). The non-bonded cut-off for the van der Waals interaction was set to 11 Å and other parameters were set as default values. The minimization consisted of 5000 steps conjugate gradient energy minimization to relax all atoms.

To evaluate the model at the reaction temperature of 37 °C, the temperature of the system was gradually raised to 310 K (36.85 °C) in a 200 ps relaxation. Then, the MD simulation was carried out within a time scale of 60 ns. The last frame (under the equilibrium state) was taken for the further analysis. To study the structural change under the denaturation and renaturation conditions (75 °C to 37 °C), the optimized structures of TLP and CHI were used. The whole MD simulation involved two parts. First, the temperature of the system was raised to 348 K (74.85 °C) after which MD was carried out with the time scale of 200 ns. The last frame from the first simulation was picked and the temperature was set to 310 K (36.85 °C). MD was carried out again within a time scale of 200 ns. Eventually, the last frame (under the equilibrium state) was taken for further analysis.

3. Results

3.1. Effects of molecular alteration on haze formation

To investigate the haze formation and peptidase-mediated cleavage spots in wine proteins, the protein-containing colloids from a white wine were isolated and further processed by heat testing, tryptic digestion and subsequent mass spectrometric analysis (Supplementary Fig. 1). Four different conditions were investigated for analyzing the effect of proteolysis on haze formation, which were generated by applying three different pH values and one heat pretreatment. Fig. 1A shows the visual aspect of each sample after a heat test. Absorbance levels were reduced by 38.8% for the native samples at pH 7 and they were almost

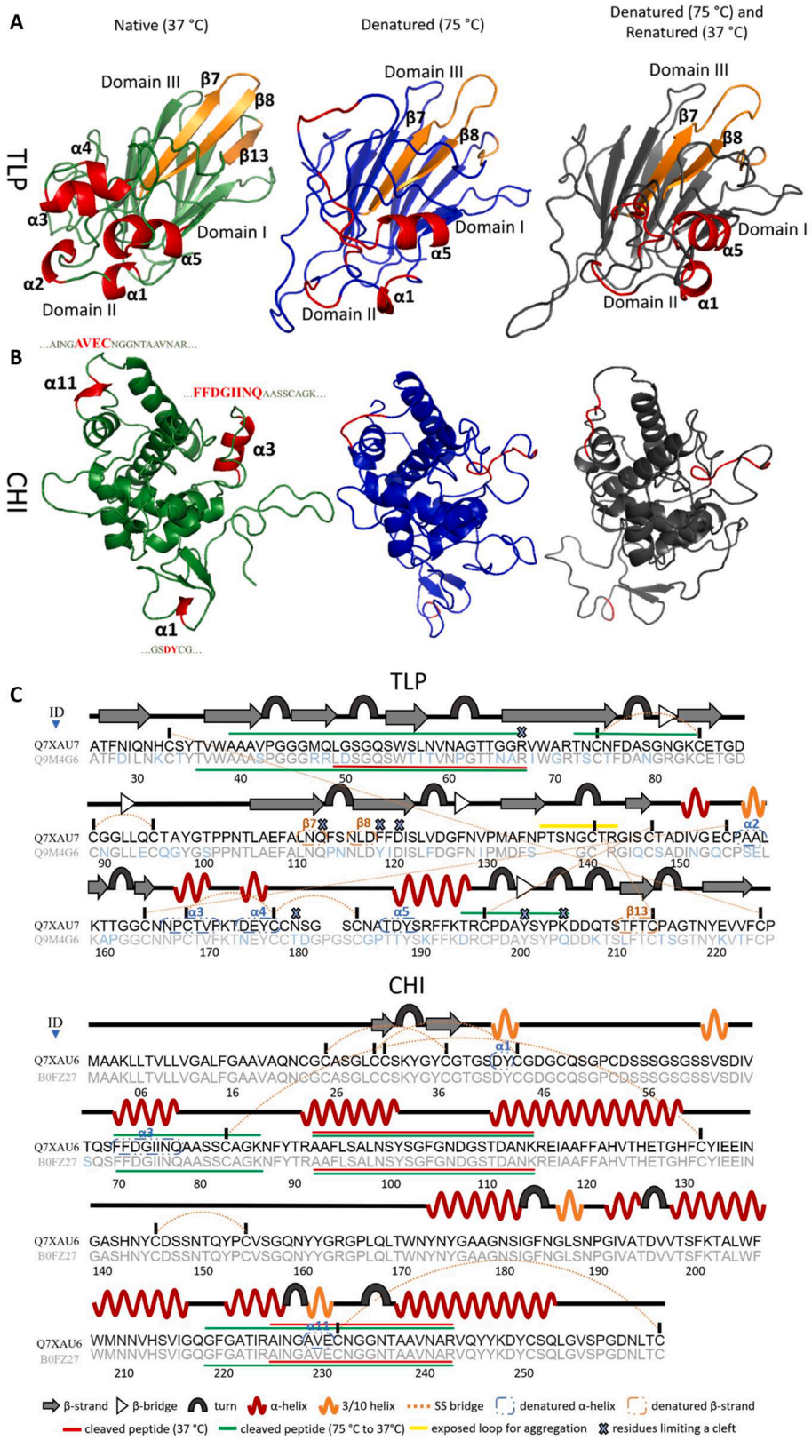


Fig. 2. Simulated molecular conformations of TLP and CHI models at pH 7 and at different temperatures, at 37 °C (native state, in green), at 75 °C (denatured state, in blue), from 75 °C to 37 °C (denatured-renatured, in gray), the latter model shows structural changes which are reversible/irreversible. (A) The TLP structure was divided in 3 domains, from which domain II was the most affected by heating. (B) For CHI, α-helices were the most affected secondary structures. In both (A) and (B), the structures highlighted in red show specific motifs, which undergo modifications under denaturing conditions. (C) Amino acid sequences of TLPs (Q7XAU7 in bold and Q9M4G6 in gray from Uniprot database) and CHIs (Q7XAU6 in bold and B0FZ27 in gray) and their secondary structure elements based for TLP on the Q9M4G6 (PDB ID: 4JRU) or for the CHI from the modelled structure are displayed. Cleaved fragments are underlined in red (when cleaved at native conditions) and in green (when cleaved after a denaturing/refolding process). Some semi-tryptic peptides (not cleaved at their *N*-terminal after K or R) were also found under denaturing conditions. For TLP, the amino acids limiting a hydrophobic cavity are marked with an "X" and the loop previously reported for promoting aggregation is underlined in yellow. For CHI, the α-helices which were permanently unfolded and posteriorly detected in the cleaved fragments are circled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

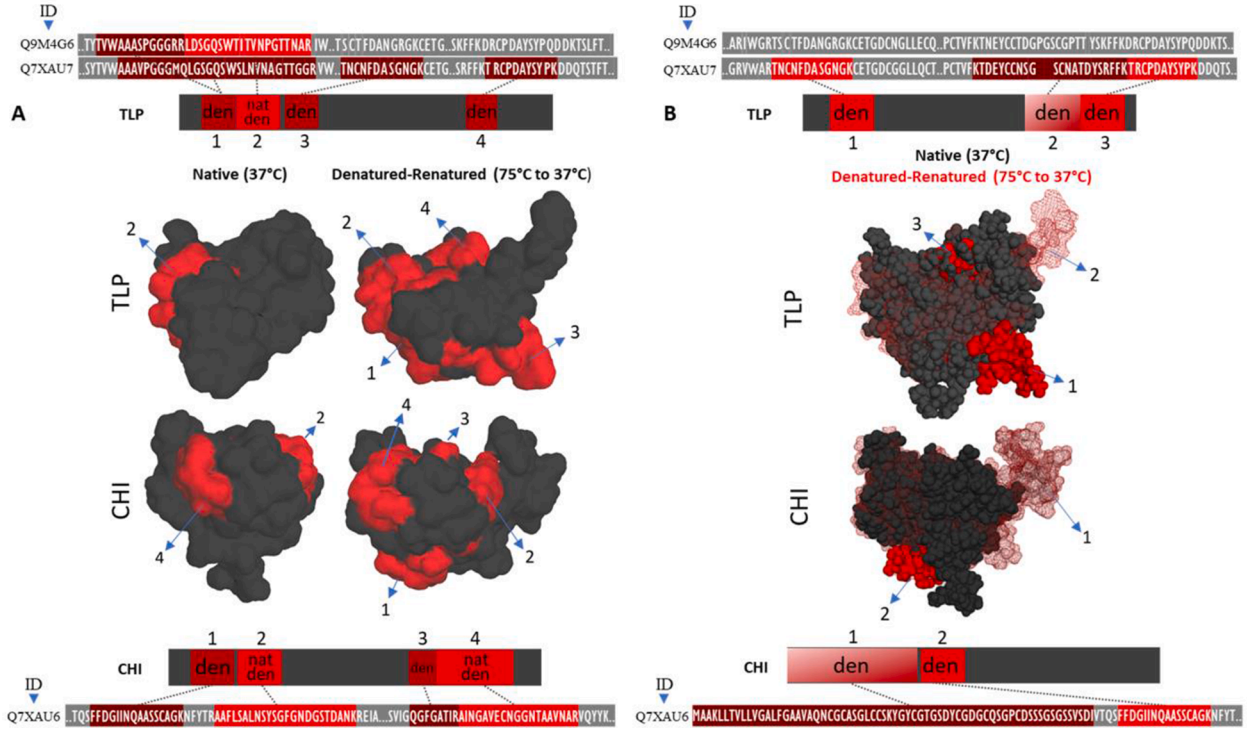


Fig. 3. Surface analysis of TLP and CHI, along with the marked cleaved regions. (A) Comparison of the cleaved regions of the surface of TLP (upper part) and CHI (lower part) pre (37 °C) and post denaturation (75 °C to 37 °C) b) Overlapping of the two protein molecular conformations of TLP (upper part) and CHI (lower part) under denatured (red) and native (gray) states. The scheme illustrates how the protein structures were affected by the heating and how the denaturation resulted in cleavage. The dashed red regions in (B) show the differences between the proteins' conformations at the two experimental conditions, the highlighted dark red spheres demonstrate how these differences after denaturation resulted in cleaved protein fragments. The respective TLP (ID: Q9M4G6 and Q7XAU7) and CHI (ID: Q7XAU6) amino acid sequences are given, and the cleaved fragments obtained at native and denaturing-renaturing conditions are highlighted either in dark red if they were found only under the denatured condition or light red if they were found in denatured and the native conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diminished (-67.4%) when the proteins were pre-denatured (Fig. 1B). At pH 3 and pH 5 (when trypsin is inactive) and in the negative control, the wine protein solutions (pre- or post-addition of trypsin) presented similar haze levels (Fig. 1B).

3.2. Identification of intact peptides (LC-MS top-down proteomics)

The highest scored tryptic protein fragments (see Supplementary Table 3) were identified exclusively in experiments performed at pH 7 (optimal pH), while the number of cleavage products varied between the native and heat-denatured samples. The wine protein solution in the absence of trypsin was used as negative control for the assays and showed, as expected, no detectable peptides. TLP and CHI were enzymatically hydrolyzed only at pH 7, and therefore further comparative analyses were performed only for these proteins (ID: Q9M4G6 for TLP and ID: Q7AUX6 for CHI). Fig. 1C displays the protein matches to intact peptides identified at native (37 °C) and denatured-renatured (75 °C – 37 °C) conditions, both at pH 7. At pH 3, pH 5 and in the negative control, no high scored peptides were detected. The results of proteolysis at different physicochemical conditions from the peptidomics analysis were compared to the absorbance levels after a heat test. Reductions of haze formation in the wine protein solutions were found to be directly proportional to the number of the detected peptides (blue dashes in Fig. 1B). The complete list of identified peptides (including small peptides with less than six amino acids and low scored peptides) is available in the supplementary information (Supplementary Table 4–8).

3.3. Molecular dynamics (MD) of TLP and CHI

The structure models of native TLP and CHI (37 °C, at pH 7) were compared to their denatured (75 °C) and denatured-renatured models (75 °C to 37 °C, at pH 7) to identify exposed regions of each protein chain which are susceptible to hydrolysis. Fig. 2A displays the TLP structure divided in three domains, as reported by Marangon et al. (2014), and simulated at three different conditions. Directly after denaturation, domain II presented a loss of α-helical motifs (α-helices 2, 3, 4 and 5 partially), with consequent exposure of unfolded regions on the protein surface. The denaturation of the β-strand 13, as well as a shortening of the β-strands 7 and 8 (Fig. 2A), were observed in domain I. The β-strand 5 was also shorted in domain III (Fig. 2A).

Models of CHI at three conditions are presented in Fig. 2B. Since no crystallographic structure is available, the secondary structures are labelled following the CHI's amino acid sequence (Fig. 2C). After heating at 75 °C, the α-helical motifs α1 and α3 were unfolded and loops were exposed to the protein exterior. A complete rearrangement of the CHI's *N*-terminal region was also observed as an irreversible conformational change.

Irreversible denaturation was also noticed on the protein surface as shown in Fig. 3 and Supplementary Movies 1 and 2. While the TLP model was not severely affected during the denaturation and renaturation processes (reversible folding, Supplementary Movie 1), the CHI model increased its surface area (Fig. 3 and Supplementary Movie 2), leading to the exposure of protein domains, which included scissile regions in the α3 helix. These cleaved regions could be confirmed via peptidomics analysis (Fig. 2C).

3.4. Identification of intact peptides and cleavage spot analysis

Peptides from 56 different protein species were identified under denaturing conditions, while only ten of them were found under native

Table 1
Peptides identified by MS-based peptidomics after tryptic hydrolysis of wine proteins under native and denatured conditions. The peptides in bold are those found under both experimental conditions (native and denatured); these peptides were further used for the semi-quantitative analysis and are marked in red for TLP and in blue for CHI.

	Protein	Peptide Sequence	Accession number
Native wine protein solution (pH 7)			
TLP			
1	Putative thaumatin-like protein	**LDSGQSWTITVNPGTTNAR**	Q9M4G6
CHI			
1	Class IV chitinase	**AAFLSALNSYSGFGNDGSTDANK**	Q7XAU6
2	Class IV chitinase	**AINGAVECNGGNTAAVNAR**	Q7XAU6 & B0FZ27
Denatured wine protein solution (pH 7)			
TLP			
1	Putative thaumatin-like protein	**LDSGQSWTITVNPGTTNAR**	Q9M4G6
2	Putative thaumatin-like protein	TVWAAASPGGGR	Q9M4G6
3	Thaumatin-like protein	LGSGQSWSLNVNAGTTGGR	Q7XAU7
4	Thaumatin-like protein	AAAVPGGGMQLGSGQSWSLNVNAGTTGGR	Q7XAU7
5	Thaumatin-like protein	TNCNFDASGNGK	Q7XAU7
6	Thaumatin-like protein	TRCPDAYSYPK	Q7XAU7
CHI			
1	Class IV chitinase	**AAFLSALNSYSGFGNDGSTDANK**	Q7XAU6 & B0FZ27
2	Class IV chitinase	**AINGAVECNGGNTAAVNAR**	Q7XAU6 & B0FZ27
3	Class IV chitinase	FFDGIINQAASSCAGK	Q7XAU6 & B0FZ27
4	Class IV chitinase	WNYNYGAAGNSIGFNGLSNPGIVATDVVTSFK	Q7XAU6 & B0FZ27
5	Class IV chitinase	NNVHSVIGQGFGATIR	Q7XAU6 & B0FZ27

conditions (Fig. 1C). Two fragments were also identified at pH 3. A putative TLP (ID: Q9M4G6) and class IV CHIs (ID: Q7XAU6; B0FZ27) were found simultaneously at denaturing and native conditions, while a thaumatin like protein (ID: Q7XAU7) was found only after preheating of the samples. Peptides originating from different TLPs and CHIs that were identified by top-down peptidomics are listed in Table 1, while all identified peptides are available in Supplementary Table 3–8.

With respect to TLPs, the peptide LDSGQSWTITVNPGTTNAR from a putative thaumatin-like protein (ID: Q9M4G6), was observed at native conditions (37 °C), while a denaturation step prior to the hydrolysis enabled the release of two peptides (LDSGQSWTITVNPGTTNAR and TVWAAASPGGGR) from the same putative TLP (ID: Q9M4G6) and four peptides (LGSGQSWSLNVNAGTTGGR, AAAVPGGGMQLGSGQSWSLNVNAGTTGGR, TNCNFDASGNGK, TRCPDAYSYPK) from another TLP (ID: Q7XAU7).

For the CHIs, two peptides (AAFLSALNSYSGFGNDGSTDANK and AINGAVECNGGNTAAVNAR) were detected after proteolysis under native conditions (ID: Q7XAU6 and B0FZ27), while the pre-denaturation step promoted the release of five different peptides (AAFLSALNSYSGFGNDGSTDANK, AINGAVECNGGNTAAVNAR, FFDGIINQAASSCAGK, WNYNYGAAGNSIGFNGLSNPGIVATDVVTSFK and NNVHSVIGQGFGATIR), all originating from a class IV CHI (ID: Q7XAU6 and B0FZ27) (Table 1).

All structural elements of TLPs (based on the map of the isoform 4JRU) and CHIs (based on our modelled structure), reported in the literature for generating haze, are graphically mapped in Fig. 2C. The amino acid sequences are displayed following the distribution of secondary structural elements, and a correlation between the cleavage spots identified in the peptidomics analysis is presented. The peptide sequences of the main identified protein species are aligned to illustrate the origin of peptides from different protein isoforms.

3.5. Label-free peptide quantitation

For quantification, only peptides found under both native (37 °C, pH 7) and denatured-renatured (75–37 °C, pH 7) conditions were used. Moreover, only one peptide per HUP was considered. Their ion intensities were comparatively measured between these two experimental conditions. The estimation of the relative peptide abundance was based on both, the signal intensity and the integrated peak area (Fig. 4A). In all cases, the calculated peak areas and peak heights of peptides from TLP (Fig. 4B upper part and Fig. 4B) and from CHI (Fig. 4B lower part and Fig. 4C) were higher when the samples were previously denatured.

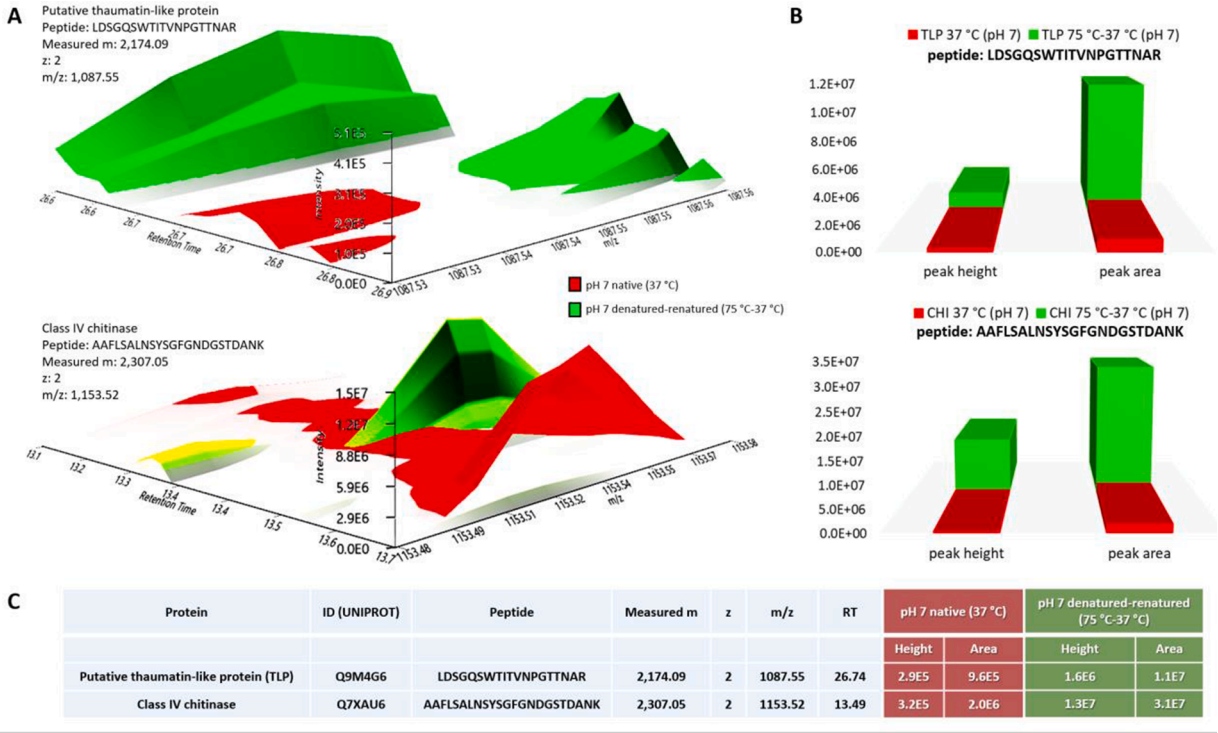
4. Discussion

4.1. Haze levels and proteolysis at different pH values

Our results (Fig. 1A and B) demonstrate that different pH values affect the intensity of haze in wine protein solutions, with higher haze being observed at pH 5. (Dufrechou et al., 2013) reported similar observations indicating that even small variations in pH value could affect the structure and stability of wine proteins by exposing their hydrophobic residues. This phenomenon has already been discussed by (Dufrechou et al. 2012), who hypotheses that the protein aggregation for pH $\leq$3.5 led to different final hazes due to electrostatic repulsions between aggregates, and lower haze is formed at pH 3 compared to pH 5. Moreover, Batista et al. (2009) demonstrated that the haze levels at pH 7 are lower than those at pH 5. We could further establish that wine proteins were cleaved at pH 7 (37 °C) and haze formation was inhibited, while the reduction of the haze at pH 3 and 5 was negligible. Since TLPs and CHIs are the main proteins responsible for wine haze and are widely known to be resistant, compact, and lacking structural flexibility (Waters et al., 1992, 1996), peptidases must be hindered to interact with the polypeptide chain of these substrate proteins. However, a significant reduction of haze formation at pH 7 (37 °C) was found, suggesting that the rigidity of the haze proteins (van Sluyter et al., 2015; Waters et al., 1992) does not prevent their proteolysis, but the acidic conditions during the vinification process do. The reduction of the turbidity levels under native conditions may be attributed to the reduction of cross-linking spots from haze proteins or a decrease in the total amount of proteins. Moreover, since pre-denaturing of wine proteins at 75 °C leads to greater reduction of the haze levels, it may be possible that at least some structural parts of the wine proteins were inaccessible under native conditions. Further, the haze reduction was directly proportional to the number of released peptides, as shown in Fig. 1B and C, indicating that all wine proteins can contribute to the formation of aggregates and that TLPs and CHIs create an interconnected network *via* molecular "bridges".

4.2. Identification of intact peptides confirms limited proteolysis

For both proteins - TLP and CHI, the intact peptides found in the peptidomics analysis originated from the protein surface (Fig. 3), and the results confirm that the denaturing conditions made it possible to extend the length of the cleaved peptide sequence (in comparison with native conditions) (Fig. 5). This phenomenon may be explained by an improved enzymatic accessibility to other nearby cleavage sites. In TLP, the peptide identified (TVWAAASPGGGR) was directly adjacent to the peptide LDSGQSWTITVNPGTTNAR, while in CHI the peptide



C

Protein	ID (UNIPROT)	Peptide	Measured m	z	m/z	RT	pH 7 native (37 °C)		pH 7 denatured-renatured (75 °C-37 °C)	
							Height	Area	Height	Area
Putative thaumatin-like protein (TLP)	Q9M4G6	LDSGQSWTITVNPGTTNAR	2,174.09	2	1087.55	26.74	2.9E5	9.6E5	1.6E6	1.1E7
Class IV chitinase	Q7XAU6	AAFLSALNSYSGFGNDGSTDANK	2,307.05	2	1153.52	13.49	3.2E5	2.0E6	1.3E7	3.1E7

Fig. 4. Semi-quantitative analysis based on MS peak signals and comparison of the relative peptide abundances from a putative TLP (peptide LDSGQSWTITVNPGTTNAR) and a class IV CHI (peptide AAFLSALNSYSGFGNDGSTDANKR) at pH 7, under native (37 °C) and denaturing conditions (75–37 °C). (A) The peptides peaks are represented in a Cartesian system where the coordinates x, y and z refer to mass-to-charge-number ratio (*m/z*), retention time and intensity, respectively. (B) The peak intensities are represented in terms of peak height and area for the peptides from TLP (upper part) and CHI (lower part). (C) The table shows the peak identification and the values of peak height and area for each peptide under the two different experimental conditions.

FFDGIINQAASSCAGK was almost adjacent to AAFLSALNSYSGFGNDGSTDANK, although a short peptide NFYT (showed in the Supplementary Table 7) is missing in between (Fig. 2C). The denaturation steps enhanced the number of exposed regions of haze proteins that were susceptible to cleavage (Fig. 4). As protein flexibility is greatly impacted by physicochemical conditions, proteolysis can be markedly influenced by pH and temperature. Moreover, our findings are in line with the theory of limited proteolysis (Ahmad et al., 2012; Fontana et al., 1997), which proposes that peptide bonds are more prone to hydrolysis when they are superficially exposed in form of loops, termini and irregular structures, in contrast to the native protein folding, in which steric constraints of the tertiary structure impede the access of peptidases to the internal scissile bonds.

4.3. Molecular dynamics simulation and analysis of cleaved fragments

4.3.1. TLP

The degradation of HUPs by proteolysis has been reported (Dizy & Bisson, 1999), and their susceptibility to enzymatic hydrolysis is intrinsically dependent on the amino acid sequence and structural features (Waters et al., 1998). Some TLP structures were described by Marangon et al. (2014) as consisting of three domains along with a cleft located between domains I and II. These two domains and the protein core are mainly formed by α-helices and β-strands, while domain III consists mainly of small loops and β-strands. The entire TLP structure is held together tightly by eight disulfide bridges, amongst which only one loop is said to be exposed. More precisely, a hydrophilic loop [formed by the sequence PTSNGCTR (from Pro135 to Arg142)] located between the β-strands 9 and 10 (Marangon et al., 2014) is more exposed in a particular TLP isoform (F2/4JRU) and may act as a destabilizing element for the entire structure, resulting in protein–protein aggregation. Its hydrophilic character propels it to the protein surface, exposing neighboring hydrophobic regions that are able to form weak interactions with other proteins (Marangon et al., 2014). A disulfide bridge located in this loop (at Cys140) is also considered to be a possible trigger point for cross-linking. Despite its exposed location and the presence of the Arg142 residue, no peptide was identified as originating from that loop (Fig. 5A and 2C). The molecular dynamic simulations showed that domains I and II were denatured which led to the unmasking of irregular structures (Fig. 5). Furthermore, the denaturation of the β-strand 3 (β3) probably facilitated the access of trypsin to the cleavage spots, resulting in the extension of the length of the cleaved peptides (Fig. 5C and 2C). Additionally, the denaturation of the β-strands β7, β8 and β13 did not appear to facilitate the cleavage of TLP, while the release of the peptide TRCPDAYSYPK may be promoted by the partial melting of the α-helix α5. No peptide products were generated from the denaturation of the α-helices α2, α3 and α4.

The cleft between domains I and II of the TLP appears to possess an acidic character due to the presence of the amino acids Glu107, Asp120, Asp125 and Asp206 and may favor the binding of small molecules, such as phenolic compounds (di Gaspero et al., 2020). After denaturation, this cleft is presumed to be exposed and vulnerable to cleavage. In fact, the peptide TRCPDAYSYPK found in the peptidomics analysis is partially located in that region and its cleavage may affect protein-phenol–protein interactions and reduce aggregation. The mentioned cavity specifically contains the amino acids Arg67, Gln112, Phe118, Asp120, Asn179, Tyr200 and Lys204 (Toledo et al., 2017) and from these amino acids, the cleaved peptides LDSGQSWTITVNPGTNAR and TRCPDAYSYPK detected in our peptidomics analysis were found to contain the residues Arg67, Tyr200 and Lys204. On the other hand, under native conditions (pH 7, 37 °C), tryptic digestion led to the exclusive generation of the free peptide LDSGQSWTITVNPGTTNAR, which includes only the Arg67 residue. Fig. 5A-C illustrate the comparative analysis of how specific amino acids or irregular structures in TLP (anchorage points for protein–protein interactions) would be affected by denaturation and become prone to cleavage. In Fig. 2C, the residues limiting a buried cleft

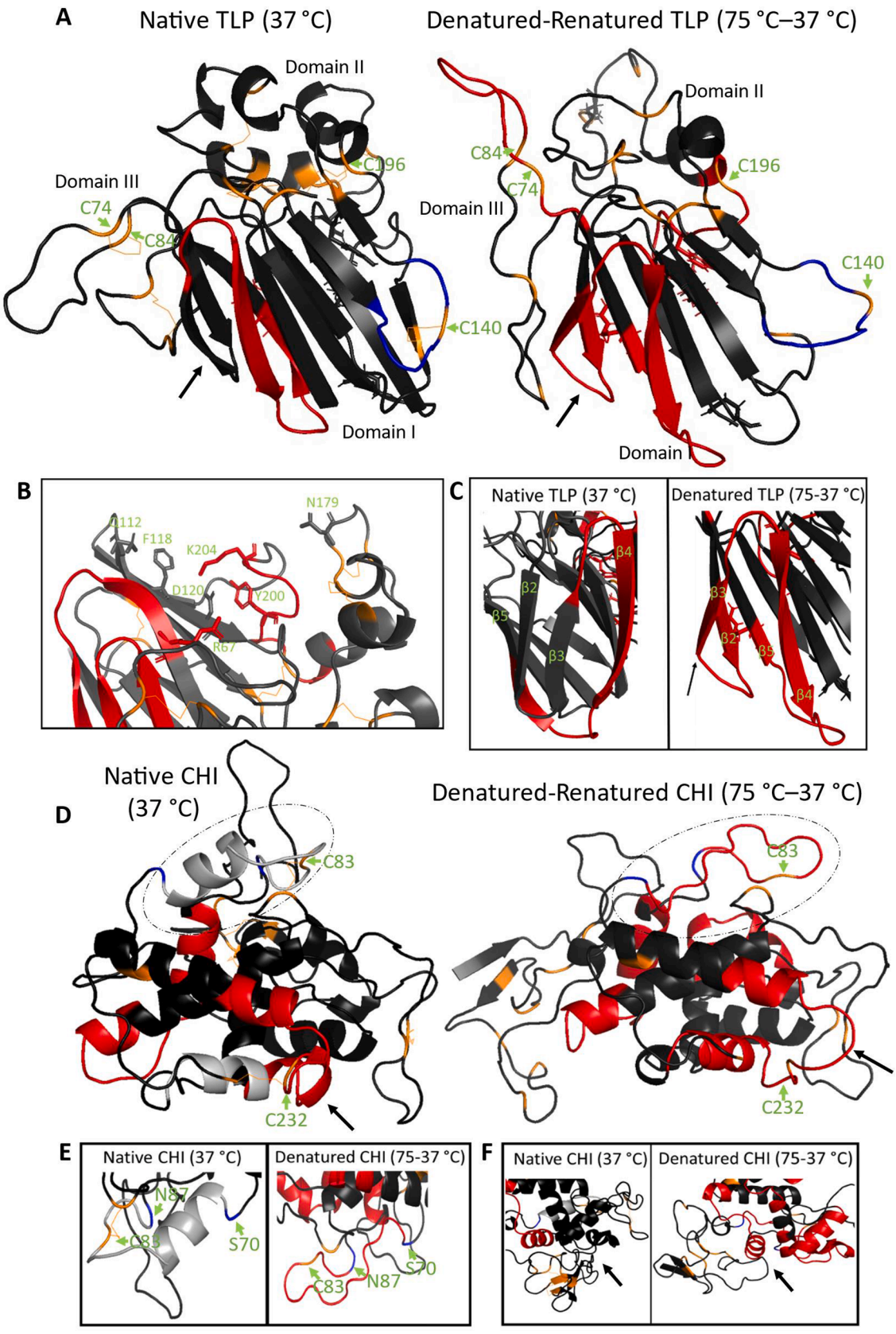


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Fig. 5. Molecular analysis of cleavage spots along the TLP (4JRU) and the modelled CHI structures and the comparison between native (37 °C, on the left) and denatured/refolded (75–37 °C, on the right) conditions. (A) Cleavage spots (in red), cysteine residues (in yellow) and an exposed loop (in blue) are highlighted. Disulfide bridges are only displayed in the native models. (B) Specific amino acid residues which limit a cleft that is favorable to binding small molecules have their organic structure represented. From these amino acids, the ones found in the cleaved peptides (Arg67, Tyr200 and Lys204) are highlighted in red. (C) The denaturation of the β-sheet 3 (β3) would make the local structure unstable and would extend the cleaved peptide chain. (D) Cleavage spots (in red) and cysteine residues (in yellow) are highlighted in the CHI structure. The CHI molecule is divided in regions I, II and III, the dashed circles highlights the region, which was completely denatured. (E) The blue segments (Ser70 and Asn87) mark the beginning and end of a cleaved segment which include an α-helix (native CHI, 37 °C) melted into a disordered structure (denatured-renatured CHI, 75 °C to 37 °C) containing a Cys83 residue which forms at native conditions a disulfide bridge to Cys140. (F) A displacement of a complete domain at the *N*-terminal of CHI allowed the prolongation of the protein cleavage, possibly due to a better access of the enzyme to the cleavage spots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the TLP structure, the exposed loop between the β-strands 9 and 10, and the cysteine residues that form disulfide bridges are highlighted and can be comparatively related to the cleaved fragments. Since loops are considered accessible regions for peptidases, we analyzed six most vulnerable fragments (located between the residues Thr72-Lys83, Glu85-Thr104, Pro135-Arg142, Lys159-Asn166, Cys178-Thr186 and Cys196-Thr210) where trypsin might have easier access to the native and denatured-renatured TLP structures. From all of them, only the loop containing the fragment TNCNFDASGNGK (Thr72-Lys83) was cleaved, particularly after denaturation. This implies that the possibility of cleavage is not limited to direct accessibility; possibly other structural elements adjacent to the loops also influence proteolysis. It is also known that removal of peptides containing disulfide bridges can reduce the number of protein cross-linking points by preventing the formation of new covalent bonds between cysteine residues (Chagas et al., 2018). From the total eight disulfide bridges in the TLP structure, two were located within the detected cleavage fragments of the peptidomics analysis under denaturing conditions: the bridge between the residues Cys74 and Cys84 in an exposed loop in the domain III and the bridge between the residues Cys146 and Cys196, located in the domain II (Figs. 5A and 2C).

4.3.2. CHI

The molecular dynamic simulations for CHI under denaturation (75 °C) and refolding (37 °C) exposed different regions of permanent denaturation that might be considered as irreversible modifications (Fig. 2B-C and 5D-F). The polypeptide sequence and the three-dimensional model of a CHI (Fig. 2B) clearly showed a structure dominated by α-helices and tied by five disulfide bridges. These results corroborate experiments performed by circular dichroism (CD) spectroscopy (Falconer et al., 2010), which claimed that the CHI structure was irreversibly denatured after heating. The irreversible denaturation of the α-helix α3, formed from Phe71 until Gln78 (Fig. 5E) allowed the cleavage of the peptide exposed on the loop fragment, which remains in accordance with the concepts of limited proteolysis. In addition, after the melting of the α-helix α11, a prolonged peptide was observed under denaturing conditions (Fig. 2C). As discussed for TLP, cysteine residues are considered cross-linking points for aggregation, and their removal would affect the protein aggregation potential. Disulfide bridges related to the residues Cys83 and Cys232 would be affected by an eventual cleavage of those protein segments (Fig. 2C and 5D-F), both under native and denaturing conditions.

A long peptide segment of CHI was cleaved after denaturation including the peptides WNYNYGAAGNSIGFNGLSNPGIVATDVVTSFK and NNVHSVIGQGFGATIR, with the short peptide TALWF missing in between (found in a further analysis and showed in the Supplementary Table 7) and that was attributed to discrepancies of the MS settings (minimum peptide length of six amino acids) (Fig. 2C). The above-mentioned peptide has already been reported by Vincenzi et al., (2014), when the authors found the peptide TALWFWMNNVHSVIGQGFGATIR by performing a LC-MS bottom-up analysis of a CHI class IV protein. Moreover, the cleavage of a protein segment located between residues Phe71 and Lys114 of CHI was probably facilitated by the denaturation step, which may have promoted a rearrangement of the proteins' *N*-terminus, allowing for a better accessibility of the peptidase to previously buried cleavage spots, as observed in Fig. 5F.

4.4. Peptide quantitation

The identified peptide ion peaks showed higher intensities for the denatured TLP and CHI, confirming that wine proteins are more susceptible to hydrolysis once they are unfolded, in accordance with the theoretical prediction (Hubbard, 1998; Novotný & Bruccoleri, 1987). The relatively high quantities that were obtained under denaturing conditions are also in line with the successful clarification of Chardonnay and Sauvignon Blanc musts carried out by Marangon et al. (2012), wherein they applied a mixture of Aspergillopepsins I and II, active at acidic pH and high temperature, to prevent protein aggregation after a flash pasteurization procedure. In our study, peptides from native wine protein at pH 7 were also detected in considerable amounts, indicating that their cleavage is plausible without an additional preheating step and demystifying the protective character caused by structural rigidity (van Sluyter et al., 2015).

Structural rigidity seems not to be the main reason why unstable wine proteins are not cleaved by peptidases under native conditions, since peptides from TLP and CHI were found at pH 7 without previous heating steps. This assumption implies that the challenge that needs to be overcome is the finding of suitable (acidic) peptidases that are functional under the winemaking conditions (acid pH and low temperatures). With this work, we confirmed the presence of cleavage spots in the TLP and CHI proteins and found degraded regions on protein surfaces, whereas even longer fragments were obtained by simple temperature modulations. Protein motifs organized in secondary structures were cleaved after becoming exposed as irregular structures, validating the principle of limited proteolysis. Haze might be a phenomenon involving all the proteins present in wine, but mostly triggered by HUPs. The methods described in this study can further be applied for the search of ideal enzyme candidates to fine beverages or for other proteolysis applications.

5. Conclusions

A combination of mass spectrometry and molecular dynamics revealed cleavage spots in unstable wine proteins and leads to the demystification that structural rigidity is the main reason, why these proteins are not cleaved by peptidases under winemaking conditions. The method here described proved that modulations on pH and temperature can affect the way that TLPs and CHIs are cleaved and open perspectives to find an ideal peptidase for clarification.

CRediT authorship contribution statement

Wendell Albuquerque: Methodology, Validation, Investigation, Visualization, Formal analysis, Writing - original draft. **Parviz Ghezellou:** Investigation, Data curation. **Binglin Li:** Visualization, Software. **Bernhard Spengler:** Funding acquisition, Resources. **Frank Will:** Resources, Formal analysis. **Holger Zorn:** Funding acquisition, Supervision. **Martin Gand:** Conceptualization, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130437.

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Research article 2 – Recombinant thaumatin-like protein (rTLP) and chitinase (rCHI) from *Vitis vinifera* as models for wine haze formation

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Recombinant Thaumatin-Like Protein (rTLP) and Chitinase (rCHI) from *Vitis vinifera* as Models for Wine Haze Formation

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Abstract: Cross-linking net aggregates of thermolabile thaumatin-like proteins (TLPs) and chitinases (CHIs) are the primary source of haze in white wines. Although bentonite fining is still routinely used in winemaking, alternative methods to selectively remove haze proteins without affecting wine organoleptic properties are needed. The availability of pure TLPs and CHIs would facilitate the research for the identification of such technological advances. Therefore, we proposed the usage of recombinant TLP (rTLP) and CHI (rCHI), expressed by *Komagataella phaffii*, as haze-protein models, since they showed similar characteristics (aggregation potential, melting point, functionality, glycosylation levels and bentonite adsorption) to the native-haze proteins from *Vitis vinifera*. Hence, rTLP and rCHI can be applied to study haze formation mechanisms on a molecular level and to explore alternative fining methods by screening proteolytic enzymes and ideal adsorptive resins.

Keywords: thaumatin-like protein; chitinase; haze; sulfite; polyphenols; wine; protein

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1. Introduction

Wine haze is generated by insoluble protein aggregates large enough to scatter light and lead to a loss of transparency. Such protein flocculation result from the thermolabile grape pathogenesis-related (PR) proteins or heat-unstable proteins (HUPs), predominantly thaumatin-like proteins (TLPs) and chitinases (CHIs). Moreover, these protein-protein interactions are influenced by numerous wine matrix components such as polyphenols [1], metal and sulfite ions [2], organic acids [3] as well as specific physicochemical conditions such as moderate temperature [4,5], high ionic strength [6] and acidic pH [7]. Although TLPs are reported to have hydrophobic spots that bind polyphenolic compounds [8] and irregular structures that interact via intermolecular disulfide bridges [9,10], CHIs are reported to be less stable and to denature irreversibly [11]. In addition, minor variations in pH conditions and ionic strength can affect the interaction of polyphenols with hydrophobic residues of HUPs, since their conformational states depend on their isoelectric points (pI) and the net charges that are present on the protein surfaces [12]. Therefore, the understanding of the interaction between thermolabile wine proteins and other wine matrix components is a fundamental step in the search for novel clarification methods [9].

Since haze in non-fined wines is mainly induced by temperature variations during transportation and storage, it eventually causes consumer aversion due to an unpleasant appearance [5,13]. To avoid this, high amounts of bentonite clay are still applied in clarification processes, although its adsorptive cationic character has deleterious effects on wine aroma, taste, and volume [14,15]. Due to its economic impact, winemakers have considered replacing bentonite with alternative cost-effective fining methods, demanding

research and technological advances. However, isolation of purified wine haze protein fractions on a technical scale is too cumbersome to become a commonly applied method. The "large-scale" availability of pure TLP and CHI would facilitate the screening of novel fining agents for the selective removal of HUPs and prevent detrimental effects on other wine matrix components.

We proposed the comparison of recombinant TLPs and CHIs (rTLP and rCHI) to serve as alternative models of wine haze proteins. Therefore, those proteins were heterologously expressed by *Komagataella phaffii* and compared with their corresponding native-host proteins in terms of glycosylation, melting point, aggregation potential, adsorption by bentonite and functionality (CHI activity). Moreover, we studied the recombinant proteins as models for their haze potential influenced by haze inducers such as polyphenols and sulfite ions employing heat tests [16–18]. Hitherto, such tests have been applied in experiments that studied haze formation using HUPs directly isolated from *V. vinifera* under the influence of other wine matrix components [1,19]. The use of rCHI and rTLP may be crucial for the search of proteolytic enzymes and adsorptive agents as alternative fining agents, providing the opportunity to find suitable and profitable alternatives for winemakers. With such recombinant haze models, a better understanding of haze mechanisms and future alternative strategies to bentonite fining, such as proteolytic treatments, can be conveniently achieved and aid in resolving the "haze challenge" in the wine industry.

2. Results

2.1. Validation of Transformants

After plasmid isolation from Escherichia coli cells, the correctness of the plasmids was confirmed by DNA sequencing (Supplementary Materials: Tables S1–S4) and by agarose gel electrophoresis. The DNA excised from gel bands at the molecular size of approximately 8000 bp and 3000 bp (BglII digestion) and 10,000 bp (SacI digestion) was integrated into the *K. phaffii* genome (Figure S4). Afterward, the transformed *K. phaffii* cells were grown on a dextrose medium deficient in histidine (MD-His) and geneticin agar plates (Figures S5 and S6, respectively). All *K. phaffii* transformants were considered to have a Mut^+ phenotype as they grew in both MD and MM media (Figure S7) and harbor an intact AOX1 gene (Section 2.2.2 of the Supplementary Materials, Figure S8).

2.2. Heterologous Expression of rTLP and rCHI

In total, 80 clones (exemplified in Figure S9), 20 for each of the four electroporation batches (rTLP plasmid digested by BglII (1), and by SacI (2), rCHI plasmid digested by BglII (3), and by *Sac*I (4) (see Table S7) from the transformations (Table S5), were screened. The clones with the highest protein expression levels (based on the protein band intensities on WB membranes) were selected for further analysis (9 clones for rTLP and 6 for rCHI). SDS-PAGE analysis of the selected clones showed dense bands of expressed protein with approximately 23 kDa (Figure 1a) for rTLP, and a double protein band at 27–32 kDa representing rCHI (Figure 1a,b). The correlation between the visualized bands, the clones, and the respective electroporation batches is shown in Tables S6 and S7. Protein bands representing rTLP and rCHI were further identified by WB (Figure 1b).

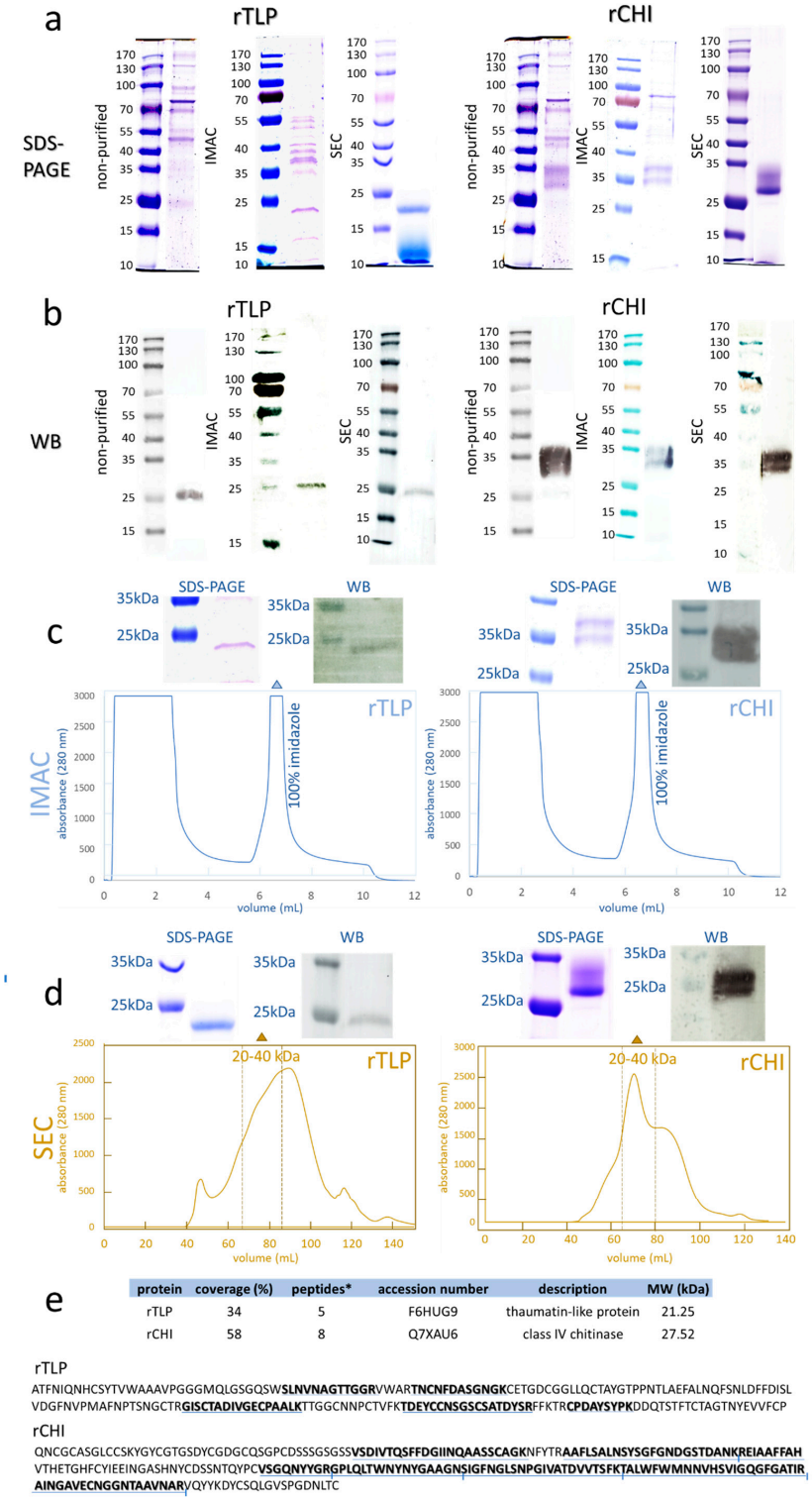

Figure 1. Analysis of recombinant TLP (rTLP) and CHI (rCHI). (**a**) Electrophoresis gels (SDS-PAGE) show the separation of the non-purified protein fractions and the protein fractions purified by IMAC and SEC. (**b**) WB membranes showing the detected his-tagged rTLP and rCHI in the non-purified fractions and the protein fractions purified by IMAC and SEC. (**c**) IMAC chromatograms of the fermentation extracts (selected clones) of rTLP and rCHI. (**d**) SEC chromatogram of the eluted peak (100% imidazole) of rTLP and rCHI (in Figure 1c). (**e**) Protein identification based on MS analysis of tryptic peptides and their identification (underlined in blue) in the amino acid sequences of rTLP and rCHI. * Means unique peptides.

2.3. Purification of rTLP and rCHI and Characterization by MS-Based Bottom-Up Proteomics

IMAC chromatograms exhibited a major peak of proteins eluted by an imidazole-containing buffer (Figure 1c) for both rTLP (Figure S10) and rCHI (Figure S11). Their purification was confirmed by SDS-PAGE and WB, as shown in Figure 1c. A further SEC [calibrated with protein standards (Figure S12)] purification provided a peak with a retention time corresponding to molecular masses of 20 to 40 kDa (Figures S13 and S14, Table S8) for both recombinant proteins, which was also confirmed by SDS-PAGE and WB (Figure 1d).

The LC-MS/MS analysis of the digested protein bands confirmed the expression of rTLP (5 unique peptides) and rCHI (8 unique peptides) confidently. The identified peptides and their locations of conformity in the corresponding sequences are presented in Figures 1e, S15 and S16, and Tables S9 and S10.

2.4. TLP and CHI: Recombinant Versus Native Proteins

2.4.1. Glycosylation Analysis

Figure 2a,b show the comparison of the recombinant proteins (rTLP and rCHI), both treated (for cleavage of attached *N*-glycans) and not treated with PNGase Endo H. The bands observed corresponded to rTLP (24 kDa), rCHI (27 kDa) and PNGase (34 kDa). No significant MW shifts were observed for rTLP. However, for rCHI, other protein bands were observed between 20 and 27 kDa after treatment by PNGase Endo H (Figure 2b). Figure 2c shows the separation of rTLP and rCHI in an electrophoresis gel after Schiff staining. A dense band corresponding to the highly glycosylated mucin protein was observed at 200 kDa, and a faint protein band compared to rCHI was detected at about 27–32 kDa. The computational analysis (performed by NetNGlyc v.1 [20] and NetOGlyc v.4.0 [21] of potential glycosylation sites is shown in Figure 3d,e. Moreover, two glycans [$(Xyl)_1$-$(GlcNAc)_3$-$(Man)_4$ and $(Man)_3$] attached to a tryptic peptide (KDYCSQLGVSPGDNLTC) of rCHI were detected by LC-MS/MS analysis (see Figure 3f and Section 2.5 of Supplementary Materials and Table S11). No glycans attached to peptides from rTLP were detected in the MS-based analysis.

2.4.2. Chitinolytic Activity

The chitinolytic activity of rCHI was evidenced by visualization of a degradation halo of chitin (Section 2.6 of Supplementary Materials) embedded in an agarose gel, as shown in Figure 2d. The radii of the halos (proportional to the enzyme activity) were 1.5 cm for rCHI, 1 cm for cCHI and 0.9 cm for the SF wine protein samples (Figures 2g and S17). The denatured cCHI (marked as a control in Figure 2d) did not show any degradation zone. The quantitative analysis of the chitinolytic activities by DNS assays is shown in Figure 2h. For both chitosan and chitin substrates, high levels of rCHI activity against the substrates were observed, and the activities were similar to those of CHI from *S. griseus* and of CHIs present in the SF wine. When cCHI was heat-denatured, its activity was strongly reduced or almost completely abolished (Figure 2h).

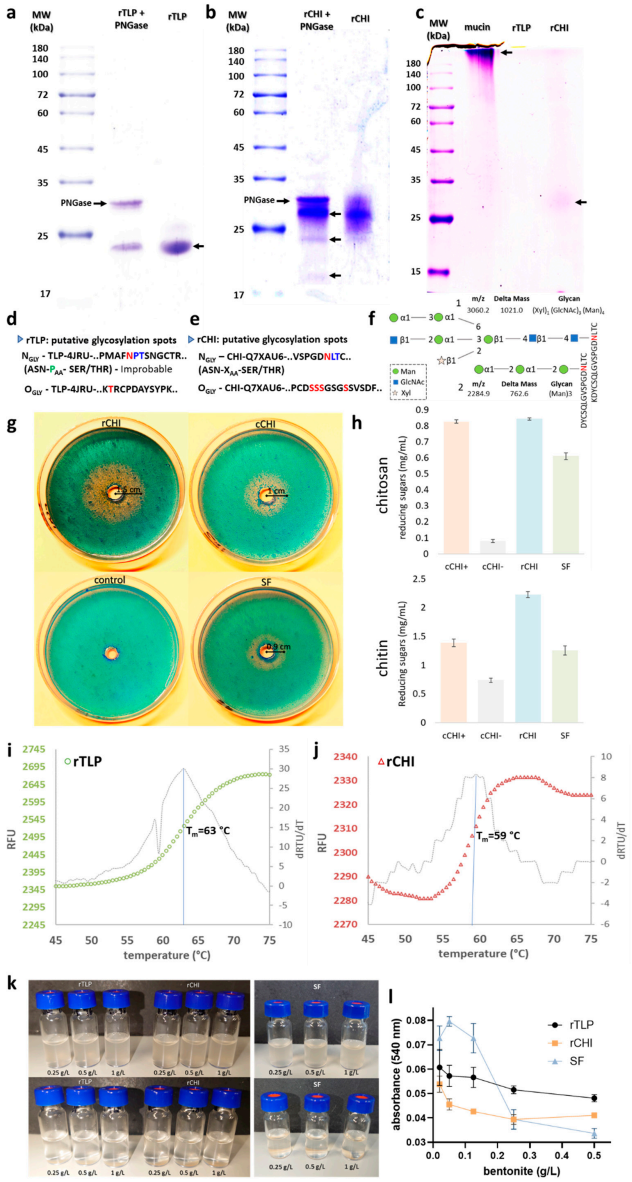

Figure 2. Characterization of rTLP and rCHI in terms of glycosylation, activity (CHI), melting point and adsorption by bentonite. The glycosylation analysis of rTLP and rCHI by (**a**) comparison of PNGase treated and non-treated rTLP. (**b**) Comparison of PNGase treated and non-treated rCHI (protein bands are indicated with arrows). (**c**) Identification of glycoproteins by the Schiff-reagent method after SDS-PAGE (the highly glycosylated mucin protein was used as control and protein bands are indicated with arrows). The predicted glycosylations are presented in (**d**,**e**) by showing the putative glycosylation sites for rTLP and rCHI, respectively. (**f**) two glycans (numbered 1 and 2) identified by MS-based analysis were found attached to the Asn (N) residue (highlighted in red) of rCHI. The mass-to-charge ratio (m/z) and the differences in the peptide masses (due the glycan attachment) are also shown (**g**) Chitinolytic activity of rCHI assessed by the agar diffusion method and stained by the calcofluor white stain reagent. A commercial chitinase from *Streptomyces griseus* (cCHI) was used as positive control. A pre-heated chitinase (denatured cCHI, used as negative control) and chitinases present in proteins from the Silvaner Franken wine (SF). (**h**) DNS assays with rCHI, cCHI, cCHI-(denatured) and SF using two chitinous substrates (chitin and chitosan). CPM fluorescence signal of rTLP and of rCHI are shown in (**i**,**j**) with their respective first derivatives. (**k**) Buffered solutions (pH 4) of rTLP, rCHI and SF (on the right) with addition of bentonite pre (upper part) and post (lower part) a heat test. (**l**) Quantitative analysis of the adsorption of the protein (rTLP, rCHI and SF) to bentonite with haze threshold concentrations (0.02 to 0.5 g/L).

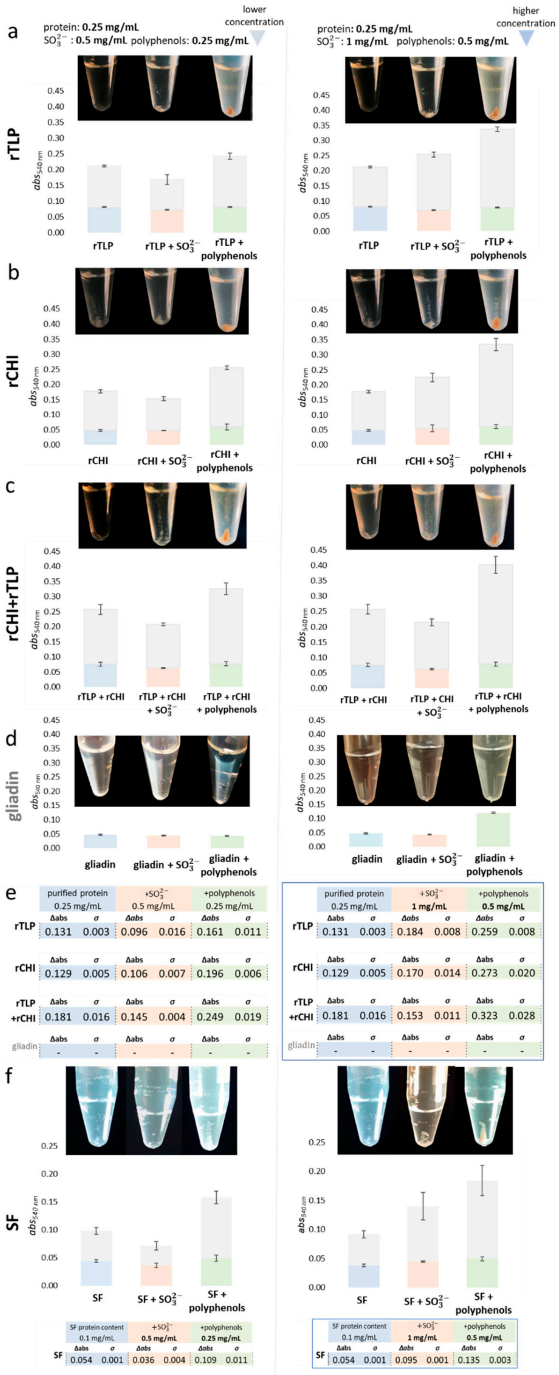

Figure 3. Influence of SO_3^{2-} and polyphenols on the protein aggregation and haze levels of rCHI, rTLP and controls. Aggregation levels are presented in terms of absorbance (in bars) and the visible residual pellet formed (at the bottom of micro tubes); the experimental variants are divided into purified protein, supplied with sulfite (+ SO_3^{2-}), and supplied with polyphenols. The gray segment of each bar represents the absorbance after the heat test (haze formation) and the colored part represents the final absorbance after centrifugation. The columns on the left and right display experiments with lower and higher concentrations of matrix compounds or additive (sulfite and polyphenols), respectively. The letters on the horizontal axis show the results for different proteins: (**a**) TLP, (**b**) CHI, (**c**) CHI+TLP and (**d**) gliadin (used as control). (**e**) The tables show the absorbance values of formed haze (at 540 nm) under the different experimental conditions and the highlighted table (outlined by blue square) shows the absorbance values (540 nm) at higher concentrations of sulfite and polyphenols. Haze levels of protein solutions from the SF wine under the same experimental conditions, are shown in (**f**).

2.4.3. Thermostability of rTLP and rCHI

The melting curves of rTLP and rCHI based on the CPM fluorescence signal are shown in Figure 2i,j, respectively. For both proteins, the fluorescence started to increase (protein melting) at approximately 55 °C. The curve for rTLP (Figure 2i) presented a sigmoidal form with an exponential phase from 58 to 68 °C, reaching a plateau afterwards. The sigmoidal-shaped curve of rCHI (Figure 2j) started its exponential phase at about 54 °C and reached its steady state at 63 °C. The first derivative of each melting curve (Figure 2i,j) defined melting temperatures of 63 °C and 59 °C for rTLP and rCHI, respectively.

2.4.4. Adsorption of the Proteins to Bentonite (Bentonite Fining)

Both rTLP and rCHI did not form haze under a heat test after treatment with bentonite at final concentrations of 0.25, 0.5 and 1 g/L (Figure 2k). Likewise, proteins from the SF wine could also be fined by the three different bentonite concentrations tested (Figure 2k). The correlation between different concentrations of the applied bentonite (0.5, 0.25, 0.125, 0.05, 0.02 g/L) and the residual haze formed is shown in the Figures 2l and S22. Haze levels increased following a decrease in the bentonite concentration, more distinctly from 0.25 g/L to 0.02 g/L.

2.4.5. Influence of Polyphenols and Sulfite Ions on the Haze Potential of rTLP and rCHI

Figure 3 correlates the aggregation potential of rTLP and rCHI to the haze levels (at 540 nm) of the respective solutions (and the residual pellet formed) after a heat test. Both rTLP and rCHI (at 0.25 mg/mL) formed hazy solutions and residual protein pellets after a heat test (Figure 3a,b), even in the absence of haze inducers. This effect was enhanced if both proteins were used (Figure 3c), although the same solutions containing pure gliadins (negative control) did not form haze (Figure 3d). The SF colloids (Figure 3e) formed haze similarly to rTLP and rCHI, with protein aggregation highly induced by sulfite ions (at 1 mg/mL) and polyphenol extracts (at concentrations of 0.25 mg/mL and 0.5 mg/mL).

The identified polyphenolic compounds (Figure S18 and Table S12) in the extracts were: caffeic acid [2.9 mg/g (milligram per gram of the grape juice dry extract)], caftaric acid (3.9 mg/g), catechin (6.1 mg/g), coutaric acid (4.9 mg/g), epicatechin (10.7 mg/g), fertaric acid (1.1 mg/g), grape reaction product (GRP, 2-*S*-glutathionyl caftaric acid) (1.6 mg/g), *p*-coumaroyl–glucosyl–tartrate (*p*-CGT) (0.5 mg/g), procyanidin B1 (1.5 mg/g), procyanidin B2 (6.3 mg/g), procyanidin C1 (6.7 mg/g), protocatechuic acid (1.6 mg/g) and quercetin-3-*O*-glucoside (Que-3-glc) (0.7 mg/g). Moreover, the monosaccharide content was 17.97% (Figures S19 and S20, Table S13). In particular, adding the polyphenol extract to rTLP and rCHI model solutions promoted a two-fold increase in haze formation (compared to the samples in the absence of polyphenols). Under such conditions, the total haze formed was slightly higher for rCHI than for rTLP, reaching differences between them of about 21.7% and 5.4%, at polyphenol concentrations of 0.25 and 0.5 mg/mL, respectively (Figure 3e). When both proteins were combined (rTLP + rCHI) haze levels were strongly enhanced, up to 37.5% (at a polyphenol concentration of 0.25 mg/mL) and 78.4% (at 0.5 mg/mL), as shown in Figure 3c,f. The protein aggregates were visualized as a pellet after centrifugation (Figures 3 and S21).

The haze levels of model solutions of rTLP and rCHI increased at higher concentrations of sulfite ion (1 mg/mL). At the same time, the absorbances were comparable to or slightly lower than those of the pure proteins without additives at 0.5 mg/mL (Figure 3f). At high sulfite concentrations, samples containing rTLP were 8.2% more turbid than those containing rCHI. The combination of two HUP species (rTLP + rCHI) was not crucial to induce higher haze levels under the influence of sulfite ions.

3. Discussion

3.1. Molecular Characterization and Comparison with Native Proteins

Transformants of *K. phaffii* (GS115) successfully expressed high levels of thermolabile rTLP and rCHI. Using a eukaryotic host organism provided the cell machinery to obtain

recombinant HUPs similar to the native ones from *V. vinifera* in terms of glycosylation, melting point, and functionality (enzymatic activity). Furthermore, their ability to aggregate in acidic solutions (pH 4) demonstrated their potential to be regarded as haze-forming model proteins. Such histidine-tagged haze proteins have the advantage that they can be produced on a large scale and may be easily purified.

Grape HUPs play an essential role in fungal defense processes, which are regulated via post-translational modifications [22,23] including potential glycosylation reactions, which was reported by Palmisano et al. [24]. Using tandem MS analysis, the authors identified glycopeptides belonging to a putative thaumatin-like protein (accession: gi|7406714, from *V. vinifera*), a class IV chitinase (accession: gi|164699029, *Vitis pseudoreticulata*) and a class IV endochitinase [accession: gi|2306813, *V. vinifera*) from a Chardonnay white wine. In our experiments, the identified double "his-tagged" protein bands in the purified rCHI (SDS-PAGE) indicated a partial glycosylation (also detected by the Schiff method). In addition, the presence of other protein bands with a lower molecular mass after incubation with the enzyme PNGase suggests the presence of glycosylated, non-glycosylated and partially deglycosylated forms. LC-MS/MS analysis of the "his-tagged" double bands of rCHI (about 27–30 kDa) also confirmed that both bands are related to class IV chitinases. Furthermore, the peptide with amino acid sequence of KDYCSQLGVSPGDNLTC from rCHI (the one with a potential *N*-glycosylation site) was also experimentally found to be glycosylated, which was confirmed by the presence of different glycans attached to the Asn (N) residue. What proves the capability of *K. phaffii* to provide similar glycosylation levels of rCHI as *V. vinifera*. This is crucial as differences in the glycosylation level between native and heterologous HUPs would influence their functionality, since glycans (part of *N*-glycosylated residues) can interfere with protein folding [25] and inhibit protein aggregation, thermolysis and proteolysis [24]. The glycosylation analysis using NetNGlyc and NetOGlyc showed that both proteins are putatively glycosylated, and up to five potential glycosylation sites in TLP and CHI were observed. For a putative TLP from a Chardonnay white wine, Palmisano et al., [24] identified the Asn134 residue as being glycosylated. Asn134 of rTLP is followed by Pro135 and Thr135, and *N*-glycosylation is probably hindered by the presence of the close proline residue of the recombinant protein. Additionally, three putative *O*-glycosylation sites are located at the residues Thr170, Thr171 and Thr193 (Figure 2d).

For rCHI, an *N*-glycosylation site was identified at Arg261 (experimentally confirmed in the rCHI and also mass spectrometrically identified by Palmisano et al. [24]) and four *O*-glycosylation sites at the residues Ser55, Ser56, Ser57 and Ser62 (Figure 2e). Landim et al. [26] reported *O*-glycosylation in a class I chitinase belonging to the glycoside hydrolase family 19 with one *N*-terminal carbohydrate-binding module (CBM) of the family 18 from the plant *Vigna unguiculata* expressed in *K. phaffii*. However, plant class IV CHIs, to which the rCHI reported in this study also belongs to, are classified as proteins of the glycoside hydrolase family 19 with one *N*-terminal CBM of the family 18. They are also reported to have shorter sequences (also fewer subsides at the catalytic cleft) than other CHI classes [27]. Similar to the class I CHI of *V. unguiculata*, the functionality of the rCHI was confirmed by its potential to degrade chitinous substrates. In our case, the acid-hydrolyzed chitin was embedded in agar, comparable with the chitinolytic activity of chitinases present in an SF colloid (Figure 2g). Moreover, rCHI exhibited high chitinolytic activity, similar to that of a commercial CHI in the DNS assays, confirming a proper folding, which is necessary for the enzymatic activity.

The CPM fluorogenic dye binds to free and exposed sulfhydryl groups, revealed under protein denaturation and, therefore, can be applied in thermofluor assays [28]. According to Eilers et al. [29], Cys residues mediate helix interactions (they are often located at helix-helix interaction sites) and they can work as sensors for protein denaturation [30]. Cys residues of wine haze proteins are exposed during protein denaturation and can cross-link proteins under *S*-sulfonation [9,31]. The increase in the CPM fluorescence signal under gradual denaturation of rTLP and rCHI evidences the exposure of Cys residues, which can

participate in protein S-S exchanges from temperatures above 55 °C, rearranging disulfide bridges along the polypeptide chains and consequently promoting aggregation [9]. In the thermal shift assays, rCHI showed a lower T_m (59 °C) than rTLP (63 °C). This has already been reported by Falconer et al. [11], who found a T_m of approximately 55 °C for CHI and a T_m of approximately 62 °C for TLP isoforms from a Sauvignon blanc wine, by performing differential scanning calorimetry (DSC). These findings underline the active folding of both recombinantly produced proteins. The CHI structure is reported to have distinctly higher amounts of helices than TLP. About 65% of CHI's structure is composed of α-helices, whereas TLP has 31% of helical secondary structures [7]. CHI and TLP contain 15 and 16 Cys residues in their polypeptide chains, respectively, which are mostly located outside of helices [32], and could participate in helix-helix interactions [30]. The reduction of the fluorescence signal (from about 65 to 70 °C) for rTLP and rCHI illustrates the quenching of the CPM dye fluorescence caused by protein aggregation and supports the fact that removal of HUPs by proteolytic treatments requires temperatures above 70 °C, as in the case of heat tests [17] or flash pasteurization [33].

rTLP, rCHI and SF colloids could be adsorbed by bentonite clay in concentrations routinely used in winemaking [34]. A bentonite concentration of 0.25 g/L was detected as a threshold value for the loss of the capability to prevent haze formation for the SF wine colloids (probably caused by the presence of other wine matrix components). Different concentrations of alternative adsorptive compounds or resins could be evaluated to bind selectively to rTLP and rCHI in the future. Fining agents such as casein, egg albumin, chitosan and polyvinylpolypyrrolidone (PVPP) were already tested as alternatives to bentonite clay [35], but other agents such as synthetic polymers have still not been well studied [36]. Recently, Sommer and Tondini [37] applied different potential fining agents such as saccharomyces paradoxus (with high concentrations of chitin in its cell wall), polystyrene, chitosan and carboxymethyl cellulose (CMC) to remove wine proteins with low levels of instability.

3.2. Influence of Polyphenols and Sulfite Ion on the Haze Potential of TLP and CHI

Both class IV CHI and the TLP isoform 4JRU from *V. vinifera* undergo irreversible denaturation processes, leading to conformational changes, which expose specific amino acids that bind to other polypeptide chains and form aggregates [38]. None of the rTLP or rCHI individually induced more haze than their combination in the presence of polyphenols. The assumption that wine haze is triggered by different classes of proteins was also discussed by Esteruelas et al. [39], when they found a protein mixture in the precipitates formed after heating Sauvignon Blanc wines.

As previously reported in the literature [10], polyphenols induced aggregation of rTLP and rCHI under heating (Figure 4), confirming their role as main haze factors. The concentrations of polyphenols differ in white wines depending on the grape variety and vintage [40] in a range between 220 and 500 mg/L [41]. The cross-linking net formed as a result of the interaction between the polyphenols and the proteins could be visually confirmed by the color of the protein aggregates (Figure 4). In theory, hydrophobic residues in the protein backbone, which are exposed after a denaturation process, can interact with polyphenols through various reactions [9,41]. According to Pocock et al. [19], gallic acid and caffeic acid are haze inducers, although caftaric acid, epicatechin and ferulic acid did not affect haze levels in model solutions. In addition, putative binding sites in TLP have already been identified for quercetin and caffeic acid [42]. Marangon et al. [8] showed the potential of tannins from a Pinot Grigio wine to promote protein aggregation. The authors discussed that the conformational mobility of phenolic molecules seems to be essential for the polyphenol-protein binding associated with stacking-stacking interaction between the planar proline residues in proteins and the phenolic rings [14,43].

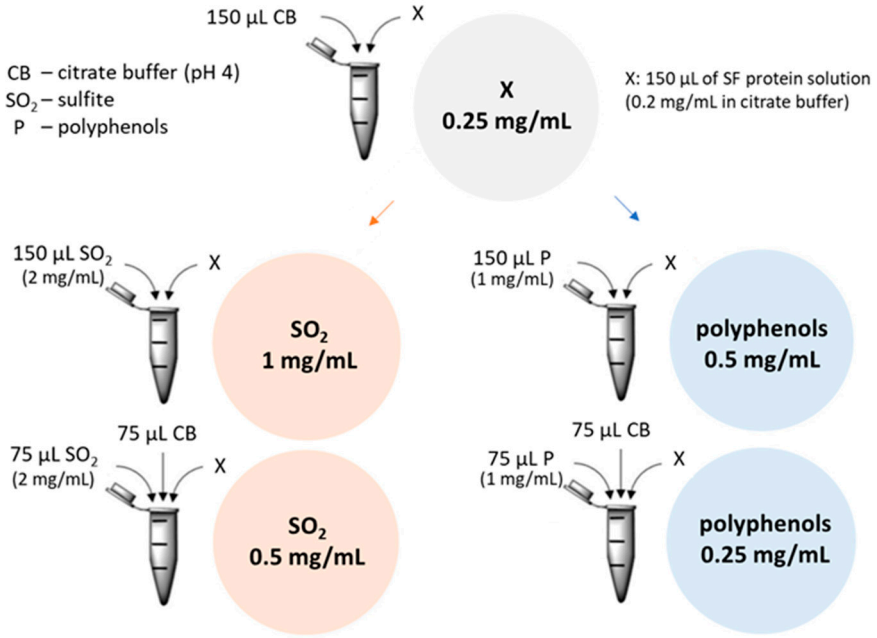

Figure 4. Recombinant TLP and CHI at different concentrations with sulfite ions and polyphenols at different concentrations were used to measure their influence on the haze potential. CB: 0.1 M citrate buffer; SO_3^{2-}: Sulfite ion (from Na_2SO_3 solution) (2 mg/mL); P: Polyphenol extract solution (2 mg/mL); x: 150 µL of protein solution (rTLP, rCHI or gliadin at 0.5 mg/mL) or 75 µL of TLP and 75 µL of CHI solutions in case of combination.

Sulfur dioxide is a common additive in winemaking, and it is normally concentrated between 0.05 and 1.8 mg/mL [19]. These authors added different concentrations (0, 0.5, 1, 1.5 and 2 mg/mL) of potassium hydrogen sulfate to model wines containing purified HUPs and observed a concentration of 1 mg/mL as crucial to developing haze. In our experiments, sulfite ions showed a secondary role in haze formation. For the induction of protein aggregation, high concentrations of sulfite ions were required. Moreover, haze levels also did not increase in the presence of sulfite ions when the recombinant proteins were combined (rTLP + rCHI).

Chagas et al. [2], Marangon et al. [6] and Pocock et al. [19] observed that the turbidity of wine model solutions increased proportionally to the protein and sulfite concentrations. That was explained by a rearrangement of disulfide bonds between proteins caused by sulfonation reactions that eventually resulted in aggregation. The authors comparatively verified that the TLP isoform 4JRU from *V. vinifera* aggregates more in the presence of sulfite than the class IV CHI.

3.3. Heterologous rTLP and rCHI as Haze-Forming Protein Models for Research and Applications

Recently, many technological advances for the analysis of wine haze proteins have been made [44] and they are still in demand to facilitate the research of adapted clarification processes. Since pure recombinant haze proteins can be produced large scale, their use has a high potential to evaluate various fining agents such as adsorptive compounds [37], resins [45,46], and peptidases [32]. Nano (magnetic) particles with functionalized surfaces have been recently developed for clarification purposes [47]. Acrylic-acid plasma-polymer-coated magnetic nanoparticles (AcrAppMNP) have been applied for the removal of TLPs and CHIs from wines in a fast method and without affecting organoleptic properties [44]. Yang et al. [48] confirmed the haze potential of recombinant GRIP32 as a novel haze protein in wines and the influence of polyphenols as haze inducers. The authors demonstrated that procyanidins (PCs) or epigallocatechin gallate (EGCG) interacted with the GRIP32 proteins to form aggregates and studied the roles of polysaccharides in hindering protein-protein interactions.

The recent application of top-down proteomics as an advanced MS technique showed a promising strategy for characterizing intact wine proteins [32], which can be applied to rTLP and rCHI to locate cleavage spots by various peptidases comprehensively. The use of recombinant proteins in the study of haze formation offers enormous possibilities to study novel clarification methods, especially by applying agents that offer high affinities to bind both TLP and CHI, preventing the concomitant removal of polyphenolic compounds or organic acids that are deemed essential for the taste of wine. The screening for peptidases, which possess the ability to cleave TLP and CHI in acidic pH and low temperatures specifically, would be facilitated by the improved availability of pure haze proteins. Alternatively, rTLP and rCHI could be used as experimental models to test different variations of pH, temperature, ionic strength, and different concentrations of wine matrix components (polysaccharides, sulfite, and polyphenols) aiming to find conditions of high protein aggregation, which should be avoided in real winemaking processes.

4. Materials and Methods

4.1. Plasmid Amplification and Isolation

The codon-optimized genes for a TLP (UniProt ID: F6HUG9) and a CHI (UniProt ID: Q7XAU6) with nucleotides coding for a hexahistidin-tagged at the 5′-end, encoding a thermo-labile thaumatin-like protein isoform (PDB code 4JRU) and a class IV chitinase from *V. vinifera*, respectively, were synthesized by BioCat GmbH (Heidelberg, Germany) and cloned into a pPIC9K vector [with *Eco*RI/*Not*I restriction sites (plasmid vectors are shown in Figures S1 and S2)]. The plasmids were transformed into *Escherichia coli* competent cells (NEB® 10-beta cells, New England Biolabs, Ipswich, MA, USA), cultured in 5 mL of Lysogeny Broth (LB)-ampicillin medium (Section 1.1.2 of Supplementary Materials) with a working concentration of 100 µg/mL (overnight at 37 °C and 180 rpm), followed by the main culture in 100 mL of LB-Amp (ampicillin) medium (for 6 h at 180 rpm and 37 °C). After harvesting cells by centrifugation (4000× *g*, 20 min, 4 °C), the plasmids were subsequently: (1) isolated with a PureLink™ HiPure Midiprep kit (Thermo Fisher Scientific, Waltham, MA, USA); (2) sequenced by Sanger sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany); (3) linearized by restriction enzymes (*Bgl*II and *Sac*I both from Thermo Fisher Scientific); and (4) separated by 1% agarose gel electrophoresis. Plasmid fragments (excised from gels) were dissolved in a binding buffer (NTI buffer) and the DNA was extracted with a NucleoSpin® Gel (PCR Clean-up) Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and quantified by nanophotometry at 260/280 nm (Pearl spectrophotometer, Implen GmbH, Munich, Germany).

4.2. Transformation into K. phaffii, Selection of Transformed Cells and Phenotype Determination

Linearized DNA plasmids were transformed into the *K. phaffii* strain GS115 (Invitrogen AG, Carlsbad, CA, USA) by electroporation. For that, yeast cells were: (1) cultivated in 100 mL of yeast-peptone-dextrose (YPD) liquid medium (at 30 °C and 200 rpm, until the OD_{600} reached 1.2); (2) harvested by centrifugation (4000× *g*, 20 min, 4 °C); (3) resuspended in 250 mL ice-cold water; (4) centrifuged again (4000× *g*, 20 min, 4 °C); and (5) finally resuspended in 1 mL of ice-cold 1 M sorbitol. Electroporation was performed by adding 10 µL (1 µg/µL) of the linearized DNA plasmid solution to 80 µL of the cell suspensions in chilled sterile cuvettes in which an electroporator (Eporator®, Eppendorf SE, Hamburg, Germany) was inserted following standard protocols [49].

Transformants were screened by cultivation on his-deficient selective agar plates (his-selective medium, at 30 °C for 72 h) and by their antibiotic resistance, culturing them on geneticin agar plates (at 30 °C for 96 h). For stable storage, the clones were grown on YPD agar (see Section 1.1.4 of Supplementary Materials) plates and stored at 4 °C.

For phenotype determination, transformants were cultured (at 30 °C for 48 h on methanol minimal medium (MM) and dextrose minimal medium (MD) agar plates (Section 1.1.6 of Supplementary Materials) for selecting phenotypes Mut^+ (active *AOX1* and *AOX2* genes) or Mut^S (p*AOX1* gene knocked out). The insertion of the gene p*AOX*1 was further

evaluated by PCR amplification by lysing the transformants and following standard PCR procedures, using the primers Pichia_*AOX1*_fw and Pichia_*AOX1*_rv.

4.3. Recombinant Expression, Purification and Identification of rTLP and rCHI

4.3.1. Recombinant Expression

Transformants were picked from stocks and inoculated in 50 mL (in 250 mL baffled Erlenmeyer flasks) buffered glycerol-complex medium (BMGY) (at 30 °C and 200 rpm, for 24 h). Subsequently, 1 mL of the BMGY cultures was used to inoculate 100 mL (in 500 mL baffled flasks) of buffered methanol-complex medium (BMMY) (at 30 °C and 200 rpm, for 3 days), with a daily feeding of 1% methanol. Cultures were further scaled up to 600 mL (in 2 L-Erlenmeyer flasks) with an initial inoculum volume of 4 mL (from BMGY cultures). Cells were separated by centrifugation (4000× *g*, 20 min, 4 °C) and the culture supernatant was concentrated by pressure-operated dialysis (Vivaflow ultrafiltration, Sartorius AG, Göttingen, Germany) with molecular mass cut-off (MWCO) of 10 kDa.

4.3.2. Protein Purification

His-tagged proteins were purified through immobilized metal affinity chromatography (IMAC) by using a HiTrap™ IMAC FF (5 mL, Cytiva Europe GmbH, Freiburg, Germany) column coupled to an FPLC system (NGCTM chromatography system, Bio-Rad Laboratories, Munich, Germany). Proteins were eluted at a flow rate of 1 mL/min using a his-elution buffer containing 250 mM imidazole (Carl Roth KG, Karlsruhe, Germany) at pH 7 (Section 1.3.1 of Supplementary Materials). The eluted proteins were concentrated and desalted by centrifugal filters with a 10 kDa MWCO (Merck KGaA, Darmstadt, Germany). A second purification step was performed by size exclusion chromatography (SEC) with a HiLoad 16/600 Superdex 75 column (Cytiva Europe GmbH, Freiburg, Germany) with a flow rate of 1 mL/min using 0.1 M TRIS-HCl buffer (pH 7) as mobile phase. Proteins were quantified according to Bradford [50], separated by 12% SDS-PAGE [51] under denaturing conditions, and visualized by Coomassie blue staining. His-tagged proteins were further identified by electroblotting onto a polyvinylidene fluoride (PVDF) membrane through western blot (WB) (Bio-Rad Laboratories). Protein detection was performed by incubation with a primary 6×-his tag monoclonal antibody (HIS.H8) (Thermo Fisher Scientific, Bremen, Germany) and a secondary anti-mouse IgG antibody horseradish-peroxidase (HRP) conjugate (Thermo Fisher Scientific, Bremen, Germany). The bands of the his-tagged proteins were revealed by using an Opti-Dilut 4CNTM substrate kit (Bio-Rad Laboratories).

4.3.3. MS-Based Proteomics Analysis

The recombinant proteins were verified by MS-based proteomics following sample preparation described in Section 1.4 of the Supplementary Materials. The peptides were separated using a Kinetex C18 (2.1 × 100 mm, 2.6 µm, 100 Å, Phenomenex, Torrance, CA, USA) column through an ultra-high-pressure liquid chromatography (UHPLC) system (Dionex UltiMate 3000 RSL, Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with instrumental parameters as described by Ghezellou et al. [52]. The recorded raw files were searched against the UniProt database, taxonomically set to *Vitis vinifera*, using Proteome Discoverer software version 2.5 (Thermo Fisher Scientific, Bremen, Germany). The parameters were set to two maximum missed cleavage sites of trypsin digestion, minimum peptide length of 6, MS1 and MS2 tolerances of 10 ppm and 0.5 Da, respectively. The dynamic modification was set to oxidation (+15.995 Da [M]) and static modification to carbamidomethyl (+57.021 Da [C]). Percolator node was used to validate identified peptide-spectrum matches (PSMs) and filter the data with parameters of a strict target FDR (false discovery rate) of 0.01 and a relaxed target FDR of 0.05. The MaxQuant contaminant database was used to mark contaminants in the results file. The MS data are deposited to the ProteomeXchange Consortium via the PRIDE partner repository [53] with the dataset

identifier PXD035796 and https://doi.org//10.6019/PXD035796 (accessed on 4 August 2022).

4.4. Protein Glycosylation

For removal of possibly-attached glycans, rTLP and rCHI were incubated overnight at 37 °C with PNGase endo H (New England Biolabs), and the molecular masses were compared with those of the non-treated proteins by SDS-PAGE under denaturing conditions. Additionally, the periodic acid-Schiff staining [54] method was performed to detect glycoproteins. The highly glycosylated porcine mucin protein (MW about 200 kDa, Carl Roth KG) was used as a control. In addition, LC-MS/MS data recorded from in-gel digestion of the purified rTLP and rCHI (gel proteins bands) samples were analyzed by the software SimGlycan (Premier Biosoft, Palo Alto, CA, USA) [55] for glycan modifications based on an internal database of carbohydrates.

4.5. Chitinolytic Activity

The chitinolytic activity of rCHI was evaluated by degrading chitin from crab shells (Carl Roth KG), which were acid-hydrolyzed as described before [56], and then embedded in agarose gels (agar diffusion method) as described by Zou et al. [57] with minor modifications (Section 2.6 of Supplementary Materials). Circular holes (of approximately 1 cm radius) were made in the gel and filled with 20 µL of the following protein (0.5 mg/mL) samples: (a) purified rCHI; (b) native (non-heated) CHI from *Streptomyces griseus* (cCHI, a commercial chitinase, which was used as positive control, Merck KGaA); (c) denatured (heated at 80 °C) CHI from *S. griseus* (0.5 mg/mL) (used as negative control); and (d) lyophilized protein fractions of a Silvaner Franken (SF) wine (in 0.1 M Tris-HCl buffer pH 7) (used as a control). The samples were incubated overnight at 37 °C. Afterward, chitin was stained by incubation with calcofluor white stain (0.1 g/mL, Merck KGaA) for 10 min, washed with distilled water and left to rest for 1 h at room temperature. In addition, a quantitative estimation of the chitinolytic activity was performed as described by Breuil & Saddler [58] and Brandt et al. [59] by DNS (3,5-dinitrosalicylic acid) assays (Section 1.5 of Supplementary Materials). All assays were performed in triplicate and a standard curve was established by using different concentrations of *N*-acetylglucosamine (Merck KGaA).

4.6. Analysis of Protein Thermostability by Differential Scanning Fluorimetry (DSF)

Protein thermostability was evaluated by differential scanning fluorimetry (DSF) performed according to Alexandrov et al. [30] and Wang et al. [60], in a real-time PCR device (CFX96 system, Bio-Rad Laboratories). For this purpose, 1 µL of a 50 mM solution of the fluorogenic dye CPM [7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin] (Merck KGaA) was mixed with 30 µL of the purified protein solutions (about 50 µg/mL of rTLP or rCHI) in a sealed 96-well PCR plate (Bio-Rad Laboratories). Both, the dye and proteins, were solved in dimethyl sulfoxide (DMSO, Carl Roth) concentrated at 10% [61]. A gradient of temperature from 45 °C to 75 °C was set, and measurements were performed at each 0.5 °C with fluorescence excitation and emission wavelengths set at 387 nm and 463 nm, respectively.

4.7. Bentonite Fining

rTLP, rCHI and proteins from a SF wine were bentonite-fined according to Pocock et al. [34] and Pocock and Waters [17]. For that, bentonite powder (Merck KGaA) was dissolved (5%, *w*/*v*) in pre-heated distilled water. The stock solution was further diluted to three different concentrations (0.25, 0.5 and 1 g/L) and volumes of 0.8 mL were aliquoted and mixed with 0.2 mL of the rTLP or rCHI solutions (at 0.5 mg/mL). The samples were subsequently left to rest at room temperature for 2 h, centrifuged (at 1500× *g* for 20 min) and the supernatants were submitted to a heat test, as described in Section 4.8.1. To measure the adsorption of the samples to the bentonite quantitatively, a correlation between different final bentonite concentrations (0.5, 0.25, 0.125, 0.05 and 0.02 g/L) and the residual haze

(after a heat test) was made by performing heat tests with buffered solutions (0.1 M citrate buffer, pH 4) of the rTLP, rCHI and the SF colloids.

4.8. Influence of Haze-Inducing Agents on Protein Aggregation

4.8.1. Haze Test

Heat tests were performed according to Pocock and Waters [17]. rTLP, rCHI, gliadin and proteins from a SF wine in buffered solutions (Section 4.8.3, were heated to 75 °C in a heater device HLC (DITABIS AG, Pforzheim, Germany) for 20 min, followed by cooling down to 25 °C. The samples were pipetted into 96 well plates (150 µL per well) and the absorbance values were measured at 540 nm in a microplate reader (Agilent Biotek, Winooski, VT, USA).

4.8.2. Extraction and Analysis of Polyphenols and Monosaccharides from Grape (*V. vinifera*) Juices

Polyphenols from a white grape juice (Niehoffs-Vaihinger Fruchtsaft GmbH, Lauterecken, Germany, 2.5 L) were eluted from a chromatography column [250 mL, Amberlite XAD16 adsorber resin (Merck KGaA) pre-washed with water (5 L)] by adding 250 mL methanol [subsequently removed by evaporation (rotary evaporator, Heidolph Instruments, Schwabach, Germany]. The polyphenol extract was dried by lyophilization for further utilization. The dry extract was dissolved (2 mg/mL) in water (with 1% methanol) and analyzed using a Luna 3u C18(2) column (Phenomenex) through a UHPLC system (Thermo Scientific Ultimate 3000) with and a low-pressure gradient of water with acetic acid and acetonitrile with water and acetic acid (Section 1.6 of the Supplementary Materials) at a flow rate of 0.25 mL/min at 40 °C. Chromatograms were recorded at 280, 320 and 360 nm and visualized by the software ChromeleonTM (Thermo Scientific). Monosaccharides were determined after hydrolysis of 10 mg of the extract by 125 µL of sulfuric acid (72%, Carl Roth) and 1.35 mL water at 120 °C for 1 h. The samples were filled up to 50 mL water and subsequently filtered (0.2 µm pore size, PSE, Avantor Inc., Darmstadt, Germany). Monosaccharides were analyzed via HPAEC-PAD with a Carbo Pac PA-100 column (250 mm × 4 mm, Thermo Fisher) coupled to a Dionex Bio-LC system. The separation of neutral and acid sugars followed different protocols, as described in Section 1.6 of the Supplementary Materials. Calibrations were performed by using polyphenol and monosaccharide standards.

4.8.3. Aggregation Assays

rTLP and rCHI were incubated at 25 °C for 1 h with different concentrations (Figures 4 and S3) of sodium sulfite (Na_2SO_3, Carl Roth KG), to obtain its dissociated form SO_3^{2-}, and polyphenol extracts (both dissolved in 0.1 M citrate buffer pH 4). Aggregation experiments combined 0.25 mg/mL of protein (rTLP, rCHI and gliadin as control) solutions at two different concentrations (1 mg/mL and 0.5 mg/mL, based on Pocock et al. [19]) of sodium sulfite and two concentrations (0.5 and 0.25 mg/mL, based on Gazzola et al. [1]) of polyphenol extracts (Figure 4). Subsequently, haze tests were performed as described in Section 4.8.1 and the absorbance was measured pre (haze) and post (supernatant) centrifugation (12,000× *g* for 10 min) and compared with the visual residual pellets formed. Controls were performed with gliadin (a protein with less haze potential) and solutions without proteins (only polyphenols or sulfite) were used as blanks. The same experiments were performed with lyophilized samples of a SF wine (see Section 2.8 of Supplementary Materials) to compare the haze potential of recombinant proteins with that of native wine proteins (from *V. vinifera*) under the same experimental conditions. All experiments were performed in triplicate.

5. Conclusions

Recombinant proteins (rTLP and rCHI) were able to form haze and can be applied as haze-forming protein models for future research. rTLP and rCHI can be produced and purified by affinity tag chromatography, which is much faster than the tedious purification

steps of HUPs from *V. vinifera*. The heterologously expressed proteins presented similar characteristics in terms of glycosylation, melting point, adsorption by bentonite and activity (CHI) in comparison to the native proteins. The exposure of thiol groups was evidenced under denaturation of rTLP and rCHI and might be associated with *S*-sulfonation reactions and rearrangement of disulfide bridges. Sulfite ions and polyphenols were confirmed as haze inducers, and polyphenols clearly participate in protein cross-linking reactions. The combination of the two different haze proteins increased the haze levels, ensuring that haze in wines is caused by the aggregation of several proteins from the grape plant and also possibly from yeast.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27196409/s1, Figures S1–S22 and Tables S1–S13. (Citation [17,34,50,51,56–59]).

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Research article 3 – Mass Spectrometry-Based Proteomic Profiling of a Silvaner White Wine.

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Mass Spectrometry-Based Proteomic Profiling of a Silvaner White Wine

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Abstract: The comprehensive identification of the proteome content from a white wine (cv. Silvaner) is described here for the first time. The wine protein composition isolated from a representative wine sample (250 L) was identified via mass spectrometry (MS)-based proteomics following *in-solution* and *in-gel* digestion methods after being submitted to size exclusion chromatographic (SEC) fractionation to gain a comprehensive insight into proteins that survive the vinification processes. In total, we identified 154 characterized (with described functional information) or so far uncharacterized proteins, mainly from *Vitis vinifera* L. and *Saccharomyces cerevisiae*. With the complementarity of the two-step purification, the digestion techniques and the high-resolution (HR)-MS analyses provided a high-score identification of proteins from low to high abundance. These proteins can be valuable for future authentication of wines by tracing proteins derived from a specific cultivar or winemaking process. The proteomics approach presented herein may also be generally helpful to understand which proteins are important for the organoleptic properties and stability of wines.

Keywords: Silvaner; proteomics; wine; proteins; mass spectrometry; *Vitis vinifera*

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1. Introduction

The white grape Silvaner (synonym Grüner Silvaner) is an autochthonous cultivar from Austria, originating from a genetic crossing of the cultivars Traminer and Österreichisch-Weiß [1]. Being only marginally important in today's Austria, the grape variety is of highest importance in the region of Franconia (Franken, in German), where it was introduced at the end of the seventeenth century. Thus, Silvaner can be considered as a very old grape variety [2]. In 2021, 4535 ha of vineyards in Germany are planted with Silvaner, which corresponds to 6.5% and 4.4% of the white (70,138 ha) and total wine growing area (103,421 ha) in Germany, respectively [3]. Moreover, Silvaner is cultivated in various countries, including France (Alsace), Romania, Slovakia, Croatia, Italy (Trentino-Alto Adige), Austria, the United States and Australia. Wines of the cultivar are generally characterized to have mild acidity and subtle aromas. The on-going climate change has also been shown to affect the quality of Franconian Silvaner wines, particularly increasing sugar levels and decreasing acidity, thereby altering the wines' sensory characteristics [4]. Furthermore, increased temperatures and decreased precipitation amounts, a frequent consequence of climate change in many wine growing regions, increased the risk for protein haze formation in the wine [5]. Proteins that survive the vinification process can interact with other wine components (e.g., ethanol) to influence the wine aroma, flavor, texture astringency, and color [6]. Additionally, wine proteins and

their interactions with other wine components affect the product stability [7,8] and foaming properties [9]. Although wine proteins represent only minor components of wines, they can act as antioxidants by interacting with polyphenols [10] and some of them are likely to be allergens [11]. In addition, the remaining proteins may contribute to wine authentication by providing information about the winemaking process [12] and grape cultivation [13].

Most wine proteins originate from the plant *Vitis* spp. (less abundant fractions are derived from fermentative organisms or parasites), and therefore, factors such as soil conditions, weather and plant stress can influence the wine proteome [14,15]. Moreover, it has been discussed that the state of maturity of the grape berries highly influences the efficiency of the protein expression [16,17]. The total wine protein content also depends on a plethora of different and variable processing unit operations during harvest and in the winery [18,19]. For example, the protein concentrations of Silvaner wines from a single winery varied over four consecutive years from rather low to high levels (0.10–0.22 mg/L) compared to other wines (0.03–0.26 mg/L) [20]. In addition, proteins from microorganisms, typically from the yeast *Saccharomyces cerevisiae* [21] or grape pathogens, such as *Botrytis cinerea* [9,22], have been reported to survive the vinification process. Further proteins, such as casein, lysozyme, gelatin, and isinglass may be applied as clarification or preservation agents and may partly be transferred into the bottled wines [23]. In brief, the wine proteome is expected to be highly diverse. Among all grape proteins, a major research focus is on thermolabile proteins, such as thaumatin-like proteins (TLPs) and chitinases (CHIs), which are assumed to be responsible for major economic losses through their key role as wine haze promoters [24,25].

In the last decades, mass spectrometry-(MS)-based proteomics has evolved as a powerful research technology that has also been exploited in oenology [12]. MS techniques based on liquid chromatography coupled to electrospray ionization (LC-ESI-MS) and matrix-assisted laser desorption ionization (MALDI)/-time of flight (TOF) have been successfully applied for the characterization of proteins of different wine varieties such as Chardonnay, Semillon, Sauvignon blanc, Pinot noir and others [13]. For example, Flamini and de Rosso [26] applied MALDI-TOF for the identification of *V. vinifera* grape varieties and tissue extracts. High resolution (HR)-MS-based proteomics analysis has provided advances in terms of accurate protein identification and enough sensitivity to study even low abundance species [27]. However, this potential has not yet been fully exploited in studies on wine proteomes and applications of recent advances in MS on wine research are still emerging [28].

Proteomics commonly refers to the mass spectrometric identification and sometimes quantification of the comprehensive set of proteins present in a system [29]. Complementary sample preparation steps, such as chromatography, one dimensional (1D) or two dimensional (2D) electrophoresis, dialysis, ultrafiltration, isoelectric focusing and immunodetection are usually applied prior to mass spectrometric analysis [30].

In addition, protein digestion techniques, either *in-gel* or *in-solution*, are routinely applied in bottom-up MS analyses before sample analyses by LC-MS [31], supporting the identification of proteins. *In-solution* digestion is a gel-free and less demanding method in terms of sample preparation, whereas the *in-gel* digestion is reported to be robust, reproducible and effective, however, being known to cause protein losses due to the fractionation of the protein mixture by gels [31]. Protein separation by LC and gel electrophoresis has often been employed in MS-based proteome analyses of wines [12,22], increasing the sensitivity (by reducing protein mixtures) and thus the number of identified proteins [26].

To date, the proteomic profile of Silvaner wine has not been reported in the literature. Here, we describe for the first time the comprehensive protein identification of a Silvaner wine using the combination of two MS-based bottom-up approaches based on *in-gel* and *in-solution* digestion. The analytical approach here described might be applied to determine protein "fingerprints" for wine authentication.

2. Materials and Methods

2.1. Chemicals

High-performance liquid chromatography (HPLC)-grade water was purchased from Thermo Fisher Scientific (Bremen, Germany). Rapigest SF surfactant was obtained from Waters (Milford, MA, USA). TRIS and TRIS-hydrochloride were obtained from Carl Roth (Karlsruhe, Germany). Ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), trifluoroacetic acid (TFA) and acetonitrile (ACN, gradient grade) were obtained from Merck (Darmstadt, Germany), while MS-grade trypsin was purchased from Promega (Madison, WA, USA).

2.2. Silvaner Wine

Silvaner grapes were harvested from the "Würzburger Pfaffenberg" vineyard (Würzburg, Germany) on 19 September 2018 and processed to must and wine by the Bavarian State Institute for Viticulture and Horticulture (LWG, Veitshöchheim, Germany). The pH of the must and wine sample was measured using a titrator (TitroLine alpha plus with TA20 plus, TM 125 and Titrisoft 3.1 SI Analytics, Mainz, Germany). The must had a measured weight of 99°Oe (DMATM 35, Anton Paar, Graz, Austria), a total acidity of 5.0 g/L (as tartaric acid) and a pH value of 3.5 (after adding 1.5 g/L tartaric acid to lower the pH). The grapes were not destemmed and only lightly crushed (crush roller, Scharfenberger Maschinenbau, Bad Dürkheim, Germany). The maceration time was 4 h at 16 °C. The solid-liquid separation was performed using a pneumatic, partially slotted tank press with a volume of 900 L (Europress P9, Scharfenberger Maschinenbau). Pectinase treatment was carried out at the must stage with 2 mL/hL (Trenolin Rapid, Erbslöh, Geisenheim, Germany). After enzymation, the must sedimented for 12 h at 16 °C and then the clear supernatant was drawn off and used for fermentation. For better nutrition of the yeast, 200 mL/hL of Vitamon Liquid (Erbslöh) was added as a yeast nutrient (combination nutrient of vitamin B_1 and diammonium phosphate). The commercial yeast strain "Oenoferm Terra" (Erbslöh) was used at 20 g/hL to ferment the must for 21 days at 17 °C, while in the last third of fermentation the temperature was increased to 18 °C to obtain a safe final fermentation. The obtained wine had an alcohol content of 11.31%, fermentable sugars of 3.4 g/L, total acidity of 5.1 g/L (calculated as tartaric acid), a pH of 3.35, volatile acid content of 0.24 g/L, free SO_2 (incling reductones) content of 102 mg/L, reductone levels of 66 mg/L, and an effective content of free SO_2 at 36 mg/L. The bentonite (NaCalit PORE-TEC, Erbslöh) requirement, determined by a heat test (4 h at 80 °C in a drying oven (UNB 200, Memmert, Büchenbach, Germany), subsequent cooling and then evaluation with turbidity meter (Turb 430 IR, WTW, Weilheim, Germany)) was extremely high (450 g/hL), which indicated a high content in proteins and proteinaceous colloids.

2.3. Technical Scale Isolation and Analysis of Silvaner Wine Colloids

The ultrafiltration of the protein-rich colloid of the Silvaner wine (250 L) was performed as described by Albuquerque et al. [32]. Briefly, the wine was firstly sheet-filtered by using a stainless steel sheet filter (40 cm × 40 cm, Pall-Seitz-Schenk, Bad Kreuznach, Germany) packed with 5 filter sheets (K 250, Pall-Seitz-Schenk). Ultrafiltration was subsequently performed with a Sartocon beta system (Sartorius, Göttingen, Germany) equipped with two 0.6 m^2 Sartocon Hydrosart cassettes with a molecular mass cut-off (MWCO) of 10 kDa. A subsequent diafiltration step, performed with citrate buffer (5 g citric acid per L, pH 4) and water, aimed to remove low molecular weight substances. However, still low molecular weight wine components bound to the colloids may remain in the isolated colloids. After the lyophilization of the retentate, the resulting powder was hygroscopic and, thus, stored in airtight containers at room temperature.

The carbohydrate content of the isolated colloids was determined by quantitation of neutral sugars and uronic acids released after hydrolysis with sulphuric acid by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described beforehand [32]. Additionally, the total protein content of the isolated colloids was determined after colloid hydrolysis by measuring the released amino acids by anion

exchange chromatography according to Ahlborn et al. [33]. The wine colloids contained 47.1% of carbohydrates and 34.7% of protein in the dry matter. Residual moisture, determined by a moisture analyzer (ML-50, AND, Tokyo, Japan) at 120 °C with 0.5 g sample, was 8.9%. Based on the yield of the ultrafiltration and the residual moisture, the studied Silvaner wine contained 0.63 g colloid per L wine [20].

2.4. Protein Content and Visualization

Protein in the isolated colloid and from chromatographic runs (see Section 2.5.1) were quantified according to Bradford [34], with bovine serum albumin (Carl Roth) as standard. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (12% polyacrylamide gel) according to Laemmli [35] under denaturing conditions. After separation, protein spots were visualized by Coomassie blue staining (Thermo Fisher Scientific).

2.5. MS-Based Proteomics Analysis of Proteins from a Silvaner Wine

The aforementioned isolated colloid was submitted to size exclusion chromatography (SEC) and subsequent *in-solution* and *in-gel* digestion, as described in Figure 1.

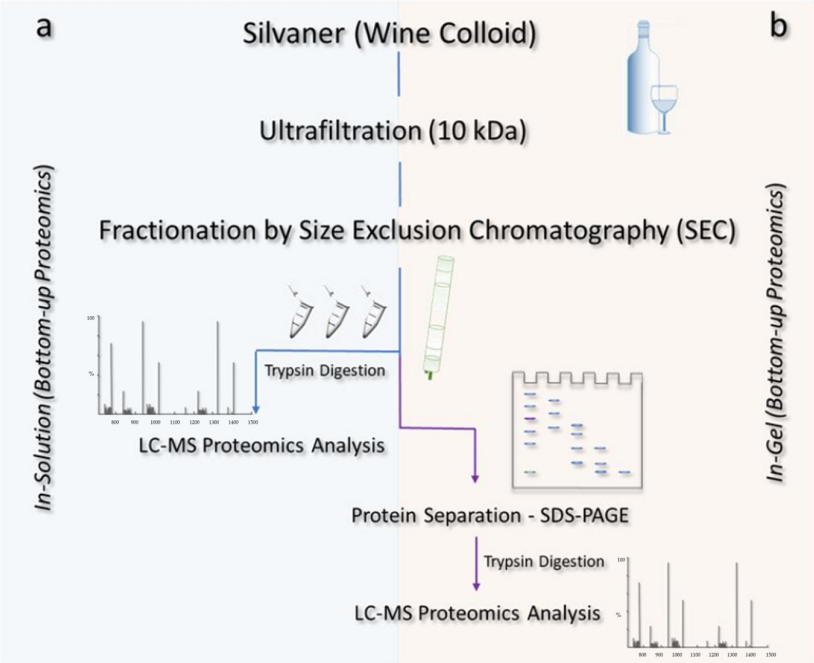

Figure 1. Illustrative scheme of the methods applied for the isolation and identification of proteins from a Silvaner wine. After fractionation via size exclusion chromatography (SEC), the wine proteins were subjected to distinct methods of digestion: (**a**) *in-solution*, in which the samples were directly tryptically digested and submitted to LC-MS analyses after the SEC fractionation step; and (**b**) *in-gel*, whereby the proteins were further fractionated by SDS-PAGE and then tryptically digested prior to the LC-MS analysis.

2.5.1. *In-solution* Digestion: Protein Fractionation by SEC Chromatography

The proteins present in the isolated wine colloid with 0.5 ± 0.1 mg/mL were fractionated using a HiLoad 16/60 Superdex 200 prep grade size-exclusion chromatography column (GE Healthcare Biosciences, Uppsala, Sweden) on a fast protein liquid chromatography (FPLC) system (Bio-rad NGC™ Quest Plus, Feldkirchen, Germany), using 50 mM Tris-HCl (pH 7, containing 150 mM NaCl) as eluent at 1 mL/min. Proteins were detected at 280 nm and automatically collected by a fraction collector (BioFrac™, Bio-Rad). The % of the yield from the protein fractions after FPLC fractionation is shown in Figure S1. The retention time was correlated to the molecular mass based on gel filtration protein

standards (from 1350 kDa to 670,000 kDa, Bio-Rad) using the software ChromLab version 6.1.29 (Bio-Rad).

To perform the *in-solution* digestion, aliquots of 25 µL of wine proteins collected from the SEC (standardized at 1 µg/µL by vacuum concentration or dilution) were mixed with 5 µL of a 50 mM ammonium bicarbonate solution and 20 µL of a RapiGest solution (0.1% dissolved in ABC) and vortexed. Subsequently, the mixture was incubated with 5 µL of 5 mM dithiothreitol dissolved in ABC at 60 °C for 15 min. Protein alkylation was performed by incubation with 5 µL of 200 mM iodoacetamide dissolved in ABC for 30 min at 25 °C. Trypsin digestion was performed by the addition of 1.25 µL trypsin/Lys-C mix (0.5 µg/µL in ABC buffer), further incubation at 37 °C for 16 h, and then stopped by the addition of 2 µL of 100% formic acid. The samples were centrifuged (15 min at 4 °C and about 13,000× *g*) and concentrated using a vacuum concentrator (Eppendorf, Hamburg, Germany). The obtained digestates were resuspended in 100 µL of ultrapure water, desalted by ZipTip C18 pipette tips (Merck), vacuum concentrated and stored for further analysis.

2.5.2. *In-gel* Digestion: Proteins Fractionated by Gel Electrophoresis

Proteins were further separated by SDS-PAGE based on their molecular mass, as described in Section 2.4. After protein separation, the bands were excised from the gels with a scalpel and the gel pieces were subsequently supplemented with 30 µL of 50% ACN for 15 min, 20 µL of 0.1 M ABC solution for 5 min and 30 µL of a 100% ACN solution for 15 min. After vacuum concentration, the gel pieces were incubated in 50 µL of a 10 mM DTT solution (dissolved in 0.1 M ABC solution) for 45 min at 56 °C, 30 µL of a solution of 55 mM iodoacetamide (in 0.1 M ABC) for 30 min at 25 °C and 20 µL of a 0.1% RapiGest solution (dissolved in 50 mM ABC solution) for 30 min at 37 °C. The gel pieces were dried again and a trypsin solution (0.5 µg/µL solved in 50 mM ABC) was added for protein digestion for 16 h at 37 °C. Afterwards, the samples were centrifuged (13,000× *g*, 10 min, 4 °C) and the supernatants were used for further analysis.

2.5.3. Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

The digested peptides were separated using a UHPLC system (UltiMate 3000 RSLC HPLC system, Ultra-High-Performance Liquid Chromatography, Thermo Fisher Scientific). A Kinetex C18 (2.1 mm × 100 mm, 2.6 µm 100 Å particle size) column (Phenomenex, CA, Torrance, USA) was used to separate the digests at a flow rate of 250 µL/min following an optimized gradient of the solvents A (aqueous 0.1% (*v*/*v*) water) and B (ACN/0.1% formic acid): isocratic flow (2% B) for 5 min, followed by a gradient of 2–40% (B) for 70 min, 40–50% (B) over 5 min and 50–98% (B) for 2 min. Re-equilibration was obtained by an isocratic flow at 2% of B for 10 min. The HPLC system was coupled to a Q Exactive HF-X (Thermo Fisher Scientific) mass spectrometer. The MS device was operated in data-dependent acquisition (top-10 DDA) mode with the following parameters for full MS scans: mass range of *m/z* 350 to 1800, resolution of 120,000 (at *m/z* 200), automatic gain control (AGC) target of 3×10^6, injection time (IT) of 50 ms; and MS/MS scans: mass range of *m/z* 200 to 2000, mass resolution of 30,000 (at *m/z* 200), AGC target of 1×10^5, IT of 120 ms, isolation window *m/z* 1.3 and dynamic exclusion duration set to 60 s.

2.5.4. MS Data Analysis

Protein sequences were obtained through shotgun searching performed by the software Proteome Discoverer (PD) version 2.4 (Thermo Fisher Scientific). The organisms *Vitis vinifera* and *Saccharomyces cerevisiae* were taxonomically set for the search. Protein sequences from both organisms were downloaded from the UniProt protein database [36] and used as a personal database. Other organisms, which are pathogens or participate in the fermentative process, were included in the database search (see Section 3.2., i.e., the methylotrophic bacterium *Methylobacterium* sp., which has epiphytic interactions with grapes and can survive during the wine production [37]). The peptide precursor and fragment ion mass tolerance in PD were set to 10 and 0.5 ppm, respectively. The pa-

rameters were assigned to a maximum of two missed cleavage sites of trypsin digestion and a minimum peptide length of 6. The dynamic modification was set to an oxidation (+15.995 Da (M)) and static modification to carbamidomethyl (+57.021 Da (C)). Percolator node was used to validate the identified peptide-spectrum matches (PSMs) and filter the data with parameters of a strict target FDR (false discovery rate) of 0.01 and a relaxed target FDR of 0.05. The MaxQuant contaminant database was used to mark the contaminants in the results file and proteins with at least one identified unique peptide were considered in the survey. "Characterized" proteins were considered those with annotated functional information in the database.

3. Results

3.1. Protein Fractionation and Visualization

Proteins (Figure 2a) separated by size exclusion chromatography (SEC) were collected in four main fractions (A, B, C and D), with the proteins represented by the largest peak in the range of 20–70 kDa and collected in fraction C. The collected proteins from each chromatographic peak were subjected to *in-solution* digestion bottom-up MS-based proteomics and were further separated according to their molecular mass (also described as molecular weight (MW)) by SDS-PAGE, resulting in a total of 16 protein bands (Figure 2b). Fraction A from SEC showed a single protein band greater than 170 kDa, fraction B showed two bands between 130 and 55 kDa, fraction C showed the densest protein bands, with a total of 12 spots from 72 to 20 kDa and finally fraction D revealed two bands from 17 to 10 kDa.

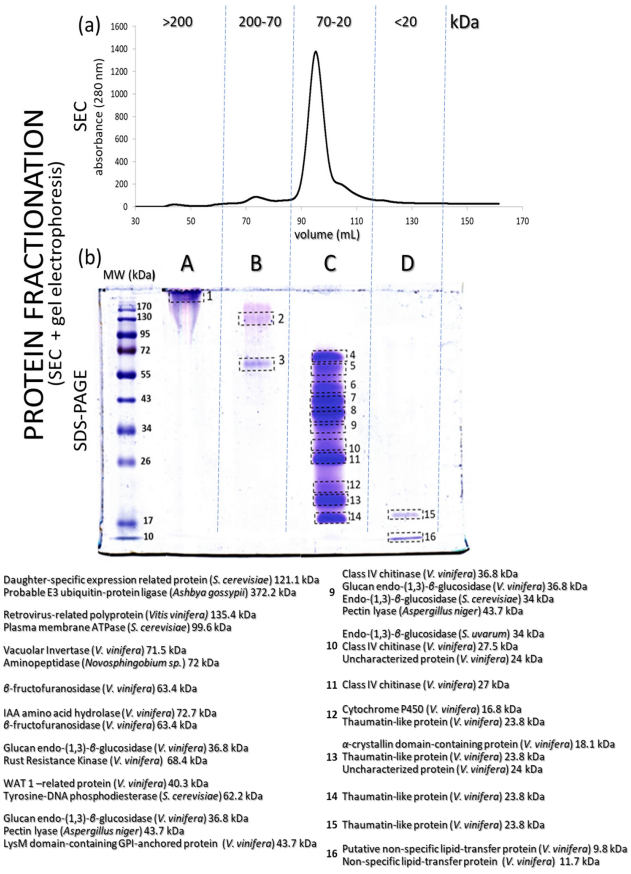

Figure 2. (**a**) SEC chromatogram of proteins from a Silvaner wine (separated according to molecular mass). (**b**) SDS-PAGE profile of the four main protein fractions obtained from the SEC chromatographic step shown in (**a**). Some of the identified proteins (sorted by molecular mass) are described in (**b**).

3.2. MS-Based Proteomics Analysis

A total of 154 proteins (with different identification numbers, but not 154 proteins with different functions) were identified by combining the data obtained from the *in-solution* and *in-gel* protein digestion methods. The identified proteins were further classified as "characterized" (with characteristics or functions described in the database) and "uncharacterized" (when no properties or functions were found in the database). Among these proteins, 88 were only identified with the *in-gel* digestion method (48 characterized and 40 uncharacterized), while 45 other proteins were exclusively found with the *in-solution* digestion approach (38 characterized and seven uncharacterized). Moreover, 21 further proteins were commonly found after both digestion methods (16 characterized and five uncharacterized) (Figure 3). Table 1 (characterized) and Table 2 (uncharacterized) list all identified proteins, according to the respective digestion method applied. Some proteins were repeatedly found; therefore, only the those with the highest coverage and identified unique peptides are presented. The complete protein list is available as Supplementary Data S1. The proteins had molecular masses ranging from 6.4 to 372.2 kDa. Figure 2 shows the correlation of each spot in the gel (spots 1 to 16) with some of the identified proteins by MS proteomics analysis (*in-gel* analysis). The complete list of identified proteins for each gel spot (Figure 2) is available in the Supplementary Data S1. The organism source and MW for each protein are given and the characterized proteins have a description associated with their accession numbers. Proteins from 10 additional organisms were included in the database of *Saccharomyces cerevisiae*, because they are eventually found as grape pathogens or fermentative organisms. Among them, we identified proteins from *Ashbya gossypii* (n = 5), *Cyberlindnera fabianii* (n = 4), *Kazachstania saulgeensis* (n = 2), *Methylobacterium* sp. (n = 2), *Novosphingobium* sp. (n = 2), *Pichia kudriavzevii* (n = 2), *Geotrichum candidum* (n = 1), *Aspergillus niger* (n = 1) and *Penicillium citrinum* (n = 1).

Table 1. Characterized proteins identified by MS-based proteomics of a colloid isolated from a Silvaner wine.

	IN-GEL (Exclusively Identified by *in-gel* Digestion)					
	Accession	**Gel Band**	**Description**	**Organism**	**MW (kDa)**	**Reported by (Ref *)**
1	C8ZG69	1	Ygp1p	*Saccharomyces cerevisiae*	37.3	5
2	G2WD47	1	K7_Spt2p	*Saccharomyces cerevisiae*	38.5	-
3	H0GMG3	1	Ygp1p	*S. cerevisiae x S. kudriavzevii*	37.3	5
4	A0A438HVN1	1 and 12	Endochitinase EP3	*Vitis vinifera*	27.2	1,2,3,4,6
5	A0A438ENJ7	2 and 6	Retrovirus-related Pol polyprotein from transposon TNT 1-94	*Vitis vinifera*	33.7	-
6	C8Z7L9	3	EC1118_1F14_0100p	*Saccharomyces cerevisiae*	53.7	-
7	G2WEU0	3	K7_Zpr1p	*Saccharomyces cerevisiae*	55.1	-
8	A0A061ASV5	3	CYFA0S02e01574g1_1	*Cyberlindnera fabianii*	34.6	-
9	A0A1V2L9U0	3	Cytokinesis protein sepH	*Cyberlindnera fabianii*	116.3	-
10	I9C1P4	3	Aminopeptidase	*Novosphingobium* sp.	72	-
11	A0A1V2LS96	3	Putative lipase ATG15	*Pichia kudriavzevii*	56.8	-
12	A6ZPP5	5	Pathogen-related protein	*Saccharomyces cerevisiae*	30.6	-
13	C8ZFH3	5	EC1118_1M3_5204p	*Saccharomyces cerevisiae*	12.8	-
14	A0A438EI04	5 and 13	IAA-amino acid hydrolase ILR1-like 4	*Vitis vinifera*	72.7	-
15	A0A438F5Y0	5	Retrovirus-related Pol polyprotein from transposon TNT 1-94	*Vitis vinifera*	10.1	-
16	A0A438HFW8	5	UDP-glycosyltransferase 85A8	*Vitis vinifera*	20.5	-
17	A0A438HSQ5	6	Rust resistance kinase Lr10	*Vitis vinifera*	68.4	-
18	I9WYJ6	6	6-carboxy-5,6,7,8-tetrahydropterin synthase	*Methylobacterium* sp.	13.5	-
19	A0A438JNK9	7	WAT1-related protein	*Vitis vinifera*	40.3	-
20	A6ZLG3	7	Tyrosine-DNA phosphodiesterase	*Saccharomyces cerevisiae*	62.2	-
21	A6ZMC5	7	Conserved protein	*Saccharomyces cerevisiae*	104.7	-
22	A0A438C3D6	8	LysM domain-containing GPI-anchored protein 1	*Vitis vinifera*	43.7	-
23	A0A0M3M4Y7	8 and 9	Pectin lyase A	*Aspergillus niger*	39.7	5
24	O24531	8 and 11	Class IV endochitinase (fragment)	*Vitis vinifera*	27	1,2,3,4,6
25	A6ZQF9	9	Killer toxin resistant protein	*Saccharomyces cerevisiae*	30	-
26	A0A1X7QY33	9	Similar to *Saccharomyces cerevisiae* YHR098C SFB3 component of the Sec23p-Sfb3p heterodimer of the COPII vesicle coat	*Kazachstania saulgeensis*	106.6	-
27	A0A1X7R1P0	9	Similar to *Saccharomyces cerevisiae* YJL170C ASG7 protein that regulates signaling from a G protein β-subunit Ste4p	*Kazachstania saulgeensis*	25.7	-

Table 1. *Cont.*

28	A0A438F8T9	10	Ethylene-overproduction protein 1	*Vitis vinifera*	113.4	-
29	A0A1V2LQA7	10 and 11	Nuclear GTP-binding protein NUG1	*Pichia kudriavzevii*	58.7	-
30	A0A438F497	11	Protein HUA2-like 3	*Vitis vinifera*	187.4	-
31	H0GDF3	11	Non-specific serine/threonine protein kinase	*S. cerevisiae x S. kudriavzevii*	120	-
32	A6ZWD3	12	ATP-dependent RNA helicase DBP1	*Saccharomyces cerevisiae*	67.9	-
33	A0A438FBU5	12	Cytochrome P450 81E8	*Vitis vinifera*	16.9	-
34	A3QRB5	12, 13 and 14	Thaumatin-like protein	*Vitis vinifera*	23.9	1,2,3,4,5
35	Q75E94	13	AAR186Wp	*Ashbya gossypii*	25.8	-
36	H0GH06	13	Yor1p	*S. cerevisiae x S. kudriavzevii*	166.7	-
37	H0GRW5	13	Mak32p	*S. cerevisiae x S. kudriavzevii*	36.3	-
38	A0A438CAI5	13	Retrovirus-related Pol polyprotein from transposon RE1	*Vitis vinifera*	73.7	-
39	A0A438F753	13	5′-nucleotidase SurE	*Vitis vinifera*	39.7	-
40	A0A438KCF4	13	α-Crystallin domain-containing protein 22.3	*Vitis vinifera*	18.1	-
41	A0A438KHH5	13	RNA exonuclease 4	*Vitis vinifera*	44.2	-
42	A0A0J9X743	13	Similar to *Saccharomyces cerevisiae* YGL131C SNT2 DNA binding protein with similarity to the *S. pombe* Snt2 protein	*Geotrichum candidum*	153.2	-
43	I9C4L4	13	Protein ImuA	*Novosphingobium* sp.	29.1	-
44	A0A438FPT4	13	Retrovirus-related Pol polyprotein from transposon 17.6	*Vitis vinifera*	98.5	-
45	A0A1V2L627	15	Sensitive to high expression protein 9, mitochondrial	*Cyberlindnera fabianii*	42.6	-
46	H0GZX2	15	Prm1p	*S. cerevisiae x S. kudriavzevii*	73.2	-
47	A0A438IBY2	15	Retrovirus-related Pol polyprotein from transposon opus	*Vitis vinifera*	144.6	-
48	A0A438IP20	15	Putative ribonuclease H protein	*Vitis vinifera*	16.6	-
	IN-SOLUTION (exclusively identified by the *in-solution* digestion method)					
	Accession	**SEC Fraction**	**Description**	**Organism**	**MW (kDa)**	**Reported by Ref ***
49	A6ZL40	A	Acid phosphatase	*Saccharomyces cerevisiae*	52.9	1
50	B3LP15	A	Protein YGP1	*Saccharomyces cerevisiae*	37.3	5
51	A6ZM69	A	Lysophospholipase	*Saccharomyces cerevisiae*	71.6	-
52	F8KAD2	A	Exo-(1,3)-β-glucanase of the cell wall	*Saccharomyces uvarum*	51.2	1
53	A6ZQA6	A	Cell wall mannoprotein	*Saccharomyces cerevisiae*	29.6	-
54	A0A438EWP8	A	Plasma membrane ATPase	*Vitis vinifera*	105.8	-
55	H0GZ48	A	Lysophospholipase	*S. cerevisiae x S. kudriavzevii*	75.4	-
56	A0A438F6R5	A	Pentatricopeptide repeat-containing protein	*Vitis vinifera*	104.7	-
57	A0A438JSE9	A	Ubiquitin-60S ribosomal protein L40	*Vitis vinifera*	80.1	-
58	C7GRZ8	A	YJL171C-like protein	*Saccharomyces cerevisiae*	42.9	-
59	C8Z9T5	A	Sps100p	*Saccharomyces cerevisiae*	34.2	-
60	H0GRF2	A	Tos1p	*S. cerevisiae x S. kudriavzevii*	48.2	4
61	G2WLU7	A	K7_Ygp1p	*Saccharomyces cerevisiae*	37.3	5
62	H0GVA1	A	Glycosidase	*S. cerevisiae x S. kudriavzevii*	54.8	4,5
63	A0A438CXL6	A	Transposon Ty3-I Gag-Pol polyprotein	*Vitis vinifera*	59.1	-
64	Q753A2	A	AFR422Wp	*Ashbya gossypii*	39.2	-
65	Q758V6	A	AEL320Wp	*Ashbya gossypii*	112.9	-
66	A5ANX3	A and B	Cysteine proteinase inhibitor	*Vitis vinifera*	11.2	-
67	A0A438HVZ7	A and C	Endochitinase EP3	*Vitis vinifera*	28.6	1,2,3,4,6
68	A6ZVW2	A, C and D	Seripauperin	*Saccharomyces cerevisiae*	17.7	5
69	A0A438DZR8	A, C and D	Non-specific lipid-transfer protein	*Vitis vinifera*	11.7	3,4,6
70	A7A1R6	B	Cell wall mannoprotein	*Saccharomyces cerevisiae*	23.3	-
71	G2WE85	B	Plasma membrane ATPase	*Saccharomyces cerevisiae*	99.6	-
72	Q9P963	B	ACC synthase	*Penicillium citrinum*	48.2	-
73	A0A438J3Y1	B	Retrovirus-related Pol polyprotein from transposon TNT 1-94	*Vitis vinifera*	135.4	-
74	H0GGT5	B	Glycosidase	*S. cerevisiae x S. kudriavzevii*	53.7	1,4,5
75	C8ZED9	B	Sma2p	*Saccharomyces cerevisiae*	40.8	-
76	A6ZLA4	B and C	Target of Sbf	*Saccharomyces cerevisiae*	47.9	1
77	H0GYP4	C	Ccw14p	*S. cerevisiae x S. kudriavzevii*	25	-
78	A6ZPT3	C	GTPase-activating protein	*Saccharomyces cerevisiae*	53.9	-
79	A6ZVC9	C	Histidine kinase osmosensor that regulates an osmosensing MAP kinase cascade	*Saccharomyces cerevisiae*	134.5	-
80	H0GWM4	C	Cis3p	*S. cerevisiae x S. kudriavzevii*	23.3	-
81	H0GL37	C	Asi1p	*S. cerevisiae x S. kudriavzevii*	71.4	-
82	A0A438DEP9	C	Retrovirus-related Pol polyprotein from transposon TNT 1-94	*Vitis vinifera*	169.1	-
83	G2WJP1	C	K7_Sen1p	*Saccharomyces cerevisiae*	252.5	-

Table 1. *Cont.*

84	Q2QCI7	D	Non-specific lipid-transfer protein	*Vitis vinifera*	11.8	3,4,6
85	I9WWM7	D	PAS domain-containing protein	*Methylobacterium* sp.	21.3	-
86	Q752D0	D	AFR645Wp	*Ashbya gossypii*	44.7	-
	IN-GEL/IN-SOLUTION (identified by *in-gel* and *in-solution* digestion)					
	Accession	*Gel Band*/**SEC fraction**	**Description**	**Organism**	**MW (kDa)**	**Reported by Ref ***
87	A6ZSE1	*1*/**A**	Daughter-specific expression-related protein	*Saccharomyces cerevisiae*	121.1	1
88	C7GQJ1	*1* and *2*/**A, B**	Cell wall protein ECM33	*Saccharomyces cerevisiae*	43.8	1
89	A0A438I656	*1, 2, 4, 5, 6, 8, 9* and *10*/**A, B, C**	Glucan endo-(1,3)-*β*-glucosidase	*Vitis vinifera*	36.8	-
90	Q9S944	*1, 3* and *8*/**D**	Vacuolar invertase 1	*Vitis vinifera*	71.5	1,2,3,4,6
91	Q7XAU6	*1, 4, 5, 6, 8, 9, 10, 11, 12* and *13*/**A, B, C, D**	Class IV chitinase	*Vitis vinifera*	27.5	2,3,4,6
92	A6ZVQ6	*2*/**A, B**	Cell wall mannoprotein	*Saccharomyces cerevisiae*	26.6	-
93	A0A438I659	*1, 2, 4, 5, 6, 8, 9* and *10*/**A, B, C**	Glucan endo-(1,3)-*β*-glucosidase	*Vitis vinifera*	23.9	-
94	A0A438DX78	*4* and *5*/**A, B**	*β*-Fructofuranosidase, soluble isoenzyme I	*Vitis vinifera*	23.9	-
95	A0A438JJ75	*4, 5, 6, 8, 9, 10, 11, 12, 14* and *16*/**A, B, C, D**	Thaumatin-like protein	*Vitis vinifera*	23.9	1,2,3,4,5,6
96	A0A438BZP1	*6, 8, 9, 10, 11, 12, 13, 14* and *15*/**B, C, D**	Thaumatin-like protein	*Vitis vinifera*	36.8	1,2,3,4,5,6
97	Q756G2	*8, 9* and *14*/**C**	Probable E3 ubiquitin-protein ligase TOM1	*Ashbya gossypii*	372.2	-
98	A0A438JJ53	*8, 9, 12, 13* and *14*/**C, D**	Thaumatin-like protein	*Vitis vinifera*	23.9	1,2,3,4,5,6
99	F8KAD7	*9*/ **B**	Endo-(1,3)-*β*-glucanase	*Vitis vinifera*	34	1,2,6
100	F8KAD8	*10* and *11*/**C**	Endo-(1,3)-*β*-glucanase	*Vitis vinifera*	63.5	1,2,6
101	A0A438GZ57	*16*/ **D**	Putative non-specific lipid-transfer protein AKCS9	*Vitis vinifera*	9.8	3,4,6
102	Q850K5	*16*/**C, D**	Non-specific lipid-transfer protein	*Vitis vinifera*	11.7	3,4,6

* Ref. means References in which a protein or a similar one was identified. 1: Kwon [30]; 2: Cilindre et al. [22]; 3: Marangon et al. [38]; 4: Wigand et al. [15]; 5: D'Amato et al. [39]; 6: D'Amato et al. [12].

Table 2. Uncharacterized proteins identified by the MS-based proteomics of a Silvaner wine.

	IN-GEL (Exclusively Identified by *in-gel* Digestion)				
	Accession	**Gel Band**	**Description**	**Organism**	**MW (kDa)**
1	A0A438J4X9	1	Uncharacterized protein	*Vitis vinifera*	67.3
2	F6HUG6	1, 4 and 5	Uncharacterized protein	*Vitis vinifera*	25.3
3	A0A438HSP1	2 and 9	Uncharacterized protein	*Vitis vinifera*	32.6
4	A0A438J6G3	2	Uncharacterized protein	*Vitis vinifera*	77.5
5	A5AP16	2	Uncharacterized protein	*Vitis vinifera*	61.5
6	A0A438HTJ6	3	Uncharacterized protein	*Vitis vinifera*	26.6
7	A5B108	3	Uncharacterized protein	*Vitis vinifera*	101.2
8	A5BPD3	3	Uncharacterized protein	*Vitis vinifera*	93.1
9	A5BUH4	3 and 6	Uncharacterized protein	*Vitis vinifera*	73.7
10	D7SRI7	3	Uncharacterized protein	*Vitis vinifera*	44.4
11	A5BGP0	4	Uncharacterized protein	*Vitis vinifera*	42.1
12	A5BD73	4	Uncharacterized protein	*Vitis vinifera*	73.2
13	A5BWA5	4	Uncharacterized protein	*Vitis vinifera*	28.7
14	A5AD63	4, 9 and 13	Uncharacterized protein	*Vitis vinifera*	71.8
15	F6GZ16	5	Uncharacterized protein	*Vitis vinifera*	98.2
16	A0A438IVS9	7	Uncharacterized protein	*Vitis vinifera*	88.7
17	A5AYX1	7	Uncharacterized protein	*Vitis vinifera*	73.9
18	A5B6K0	9	Uncharacterized protein	*Vitis vinifera*	91.9
19	A5BKS0	9	Uncharacterized protein	*Vitis vinifera*	71.5
20	A5BW59	9	Uncharacterized protein	*Vitis vinifera*	91.8
21	A5BX40	9	Uncharacterized protein	*Vitis vinifera*	147.5
22	A0A1V2L6J1	9	Uncharacterized protein	*Cyberlindnera fabianii*	105.9
23	A0A438JPS2	9	Uncharacterized protein	*Vitis vinifera*	76.1
24	A5BRN8	9	Uncharacterized protein	*Vitis vinifera*	38.3
25	D7SL13	9	Uncharacterized protein	*Vitis vinifera*	6.4
26	A5AVZ0	9	Uncharacterized protein	*Vitis vinifera*	168.4
27	A5BVR4	10	Uncharacterized protein	*Vitis vinifera*	38.6
28	F6HAW3	11	Uncharacterized protein	*Vitis vinifera*	32
29	A5B6N1	11	Uncharacterized protein	*Vitis vinifera*	54.9
30	D7SVF8	12	Uncharacterized protein	*Vitis vinifera*	16.8
31	A0A438I1U6	13	Uncharacterized protein	*Vitis vinifera*	10.8
32	F6I094	13	Uncharacterized protein	*Vitis vinifera*	58.7
33	A5AK33	14	Uncharacterized protein	*Vitis vinifera*	36.1
34	A5B9R1	14	Uncharacterized protein	*Vitis vinifera*	248.6
35	A5B1A9	15	Uncharacterized protein	*Vitis vinifera*	69.3
36	A0A438JBK9	15	Uncharacterized protein	*Vitis vinifera*	24.9
37	A5BEX7	15	Uncharacterized protein	*Vitis vinifera*	118.5
38	A5BUI9	15	Uncharacterized protein	*Vitis vinifera*	40.2

Table 2. *Cont.*

39	A5CAU1	15	Uncharacterized protein	*Vitis vinifera*	84.7
40	A5AT89	16	Uncharacterized protein	*Vitis vinifera*	65.6
	IN-SOLUTION (exclusively identified by *in-solution* digestion)				
	Accession	**SEC Fraction**	**Description**	**Organism**	**MW (kDa)**
41	F6H9W6	A	Uncharacterized protein	*Vitis vinifera*	133.1
42	A5BP85	B	Uncharacterized protein	*Vitis vinifera*	113.1
43	A5BY31	C	Uncharacterized protein	*Vitis vinifera*	125.3
44	D7TT81	C	Uncharacterized protein	*Vitis vinifera*	47
45	F6H4B3	C	Uncharacterized protein	*Vitis vinifera*	58.1
46	A5BYL8	D	Uncharacterized protein	*Vitis vinifera*	103.5
47	A0A438FVB3	D	Uncharacterized protein	*Vitis vinifera*	22.2
	IN-GEL/IN-SOLUTION (identified by *in-gel* and *in-solution* digestion)				
	Accession	**Gel Band/SEC fraction**	**Description**	**Organism**	**MW (kDa)**
48	F6HMA2	*1*/ **A, B**	Uncharacterized protein	*Vitis vinifera*	60.7
49	F6HAU0	*4, 5, 6, 9, 10, 11* and *12*/**A, B, C**	Uncharacterized protein	*Vitis vinifera*	60
50	F6HUH1	*4, 5, 6, 8, 9, 10, 11, 12, 13* and *14* /**B, C, D**	Uncharacterized protein	*Vitis vinifera*	24
51	A5C9F1	*10, 11* and *16*/**A, B**	Uncharacterized protein	*Vitis vinifera*	23.8
52	D7TXF5	*10, 11* and *16*/**D**	Uncharacterized protein	*Vitis vinifera*	15.1

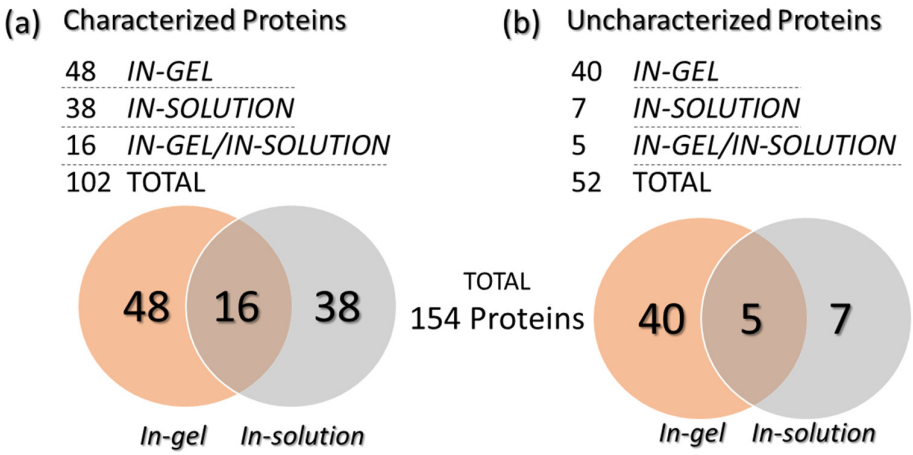

Figure 3. Venn diagrams presenting the number of characterized (**a**) and uncharacterized (**b**) proteins identified after *in-gel* or *in-solution* digestion.

4. Discussion

With the availability of high-throughput and rapid screening methods and HR-MS techniques, the evaluation of wine processing and an overview of the metabolism and defense mechanisms of grapes are feasible [26]. Therefore, MS-based proteomics may be applied to authenticate wines as a "proteome signature" to avoid fraudulent products in the wine market [12] in addition to other methods such as polyphenolic profiling (based on HPLC coupled with ultraviolet (UV) and MS analysis (HPLC-UV-MS/MS)) [40] and fluorescence spectroscopy [41]. The proteomics data reported here might serve in the future (after authenticity requirements) for a comparative authentication of Silvaner wine based on identifying particular proteins. A comparative analysis of wine proteomes showed that some proteins are commonly reported, and generally present across different cultivars. These include proteins from the vine plant *V. vinifera* (TLPs, CHIs, vacuolar invertase, (1,3)-β-glucanase, lipid transfer protein), from fermentative organisms, i.e., *S. cerevisiae* (acid phosphatase, seripauperin, protein YGP1, glycosidases, protein Tos1p, daughter-specific expression-related protein, and cell wall proteins) and from grape pathogens such as *A. niger* (pectin lyase).

Eventually, the reported proteins might be useful for a comparative analysis between cultivars (similarly to the analyses presented in the Tables 1 and 3) and therefore, protein matches with at least one unique peptide were considered in the present study. In this study, the combination of two different protein fractionation steps, the HR-MS analysis and the complementary *in-solution* and *in-gel* digestion techniques allowed for a high-score level of identified proteins. In total, from the 154 proteins identified from a Silvaner wine, 80% orig-

inated from *V. vinifera* and *S. cerevisiae*, and roughly 20% from other organisms, which are frequently found to be associated with wine and grapes (Figure 4a). Protein species, which can survive the vinification process may influence the wine organoleptic properties and haze formation in wines [17]. Similar compositions of proteins from different organisms have been reported in the literature. However, the methods and the HR-MS analysis in this study provided a higher number of identified proteins compared to other studies (Table 3). Marangon et al. [38] combined hydrophobic interaction chromatography with reversed-phase liquid chromatography using HPLC and nano-LC-MS/MS analyses to improve the protein purity and the quality of the proteomics analysis of Semillon grape juice and wine. The *in-gel* digestion allowed the identification of proteins after an additional step of separation (gel electrophoresis) and had the advantage of reducing the mixture of proteins that are digested by trypsin and further fragmented during the MS analysis. However, some proteins were still detected in unexpected molecular masses (Supplementary Data S1). The number of identified proteins after *in-gel* digestion was higher than that after the *in-solution* method, which was also observed by Choksawangkarn et al. [31]. In contrast, the *in-solution* approach allowed the direct LC-MS/MS analysis of the digested peptide mixtures, avoiding the risk of protein losses during further fractionation steps. Approximately one-third of the identified proteins in this present study were exclusively found using the *in-solution* digestion method. Additionally, methods of protein extraction are compared in Table 3. Sample isolation such as the MWCO of membranes, precipitation method and pellet resuspension can reduce the final protein content and influence the proteome analysis.

Table 3. Comparison of wine proteomics results in terms of wine type, methods of separation, MS analysis and protein digestion, and the number of identified proteins found in the literature.

Wine	Protein Extraction	Protein Separation	MS Analysis	Digestion Method	Identified Proteins (n)	% of Grape + Yeast Proteins	Reference
Sauvignon blanc	Cellulose acetate membrane (MWCO—5 kDa) Precipitation [$(NH_4)_2SO_4$]	SDS-PAGE	Nano-LC-MS Ion trap MS	*In-gel*	**Total: 20** 5 (grape) 12 (yeast) 1 (fungi) 2 (bacteria)	85%	Kwon [30]
Chardonnay	Polysulfone membrane (MWCO—10 kDa) Precipitation (85%—C_2H_6O + 15% $C_2HCl_3O_2$)	Isoelectric Focusing (IEF) SDS-PAGE	Nano-LC-MS/MS Ion trap MS	*In-gel*	**Total: 13** 10 (grape) 1 (yeast) 2 (fungi)	84.6%	Cilindre et al. [22]
Semillon	Precipitation [$(NH_4)_2SO_4$]	Hydrophobic interaction chromatography (HIC) Reversed phase HPLC SDS-PAGE	Nano-LC-MS/MS TOF-MS	*In-gel* *In-solution*	**Total: 10** 10 (grape)	100%	Marangon et al. [38]
German Portugieser	Cellulose membrane (MWCO—3.5 kDa)	SDS- PAGE	LC-MS TOF-MS	*In-gel*	**Total: 18** 12 (grape) 6 (yeast)	100%	Wigand et al. [15]
Valpolicella	Protein adsorption (ProteoMiner beads) Protein desorption (Laemmli buffer)	SDS-PAGE	LC-MS TOF-MS	*In-gel*	**Total: 23** 1 (grape) 4 (yeast) 13 (fungi) 2 (bacteria) 3 (bovine)	17.3%	D'Amato et al. [39]
Recioto	Protein adsorption (ProteoMiner beads)	SDS-PAGE	Nano-LC-MS/MS	*In-gel*	**Total: 106** 95 (grape) 11 (yeast)	100%	D'Amato et al. [12]
Silvaner	**Ultrafiltration Cellulose membrane (MWCO—10 kDa)**	**Size exclusion chromatography (SEC) SDS-PAGE**	**LC-MS Quadrupole Orbitrap**	*In-gel* *In-solution*	**Total: 154 91 (grape) 47 (yeast) 12 (fungi) 4 (bacteria)**	**89.6%**	**Present study**

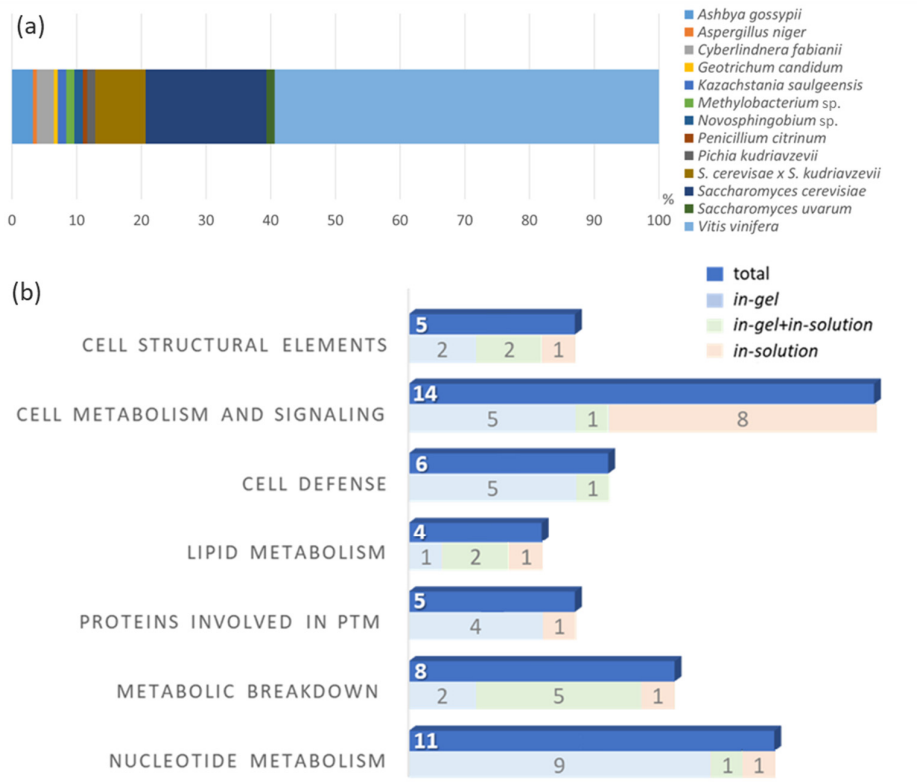

Figure 4. (**a**) Stack-bar blot of the percentage distribution of the found protein to the organisms (**b**) Quantitative comparison of the identified proteins from a Silvaner wine divided per cellular function. The number of proteins identified by each digestion technique is also presented. PTM means post-translational modifications.

The identification of low-abundance proteins originated from eventual grape infections, contaminations, distinct fermentative organisms and others are difficult to reproduced in different wine analyses, even if these grapes are from the same cultivar. The eventual presence of organisms such as pathogens [37,42], fermentative bacteria or yeasts [17,43] and factors such as differential gene expression induced by abiotic and biotic stress including climatic aspects [44,45] or protein contaminants [46,47] can greatly influence the variability of the proteomic analysis of wine. Righetti et al. [48] discussed that the wine composition and age might be affected by the presence of additives and, therefore, low-abundance proteins can evidence the vinification process. In addition, proteins from the fermentation process or added as fining agents such as egg white, as potential allergens, can influence the protein composition and may participate in the formation of haze particles [49]. The proteomics of wines has already been established as a tool for product authentication and avoiding food fraud. Ortea et al. [50] highlighted that not only vintages or cultivars, but also protein additives could be traced and characterized by proteomics analysis. Since such proteins were not identified, their absence in the clarification process of the analyzed Silvaner wine was confirmed.

Table 4 shows the classification of the characterized proteins based on their cellular functions. In total, eleven proteins were related to gene regulations and nucleotide metabolism: eight, five, and four proteins were described as participating in the metabolism of carbohydrates, proteins and lipids, respectively. Six proteins were identified as participating in the cell defense of *V. vinifera* and *S. cerevisiae*, including the pathogenesis-related TLPs and CHIs. Six proteins were related to cell structural functions, and 14 proteins (the most abundant group) are responsible for metabolic and cell signaling functions. Several proteomics studies have classified wine proteins in different classes, including the proteins involved in sugar metabolism (such as vacuolar invertases) and in stress response or plant

defense (such as the pathogenesis-related proteins TLPs, CHIs and osmotin-like proteins) as well as proteins from yeast and other fungal origins [22]. In general, the distribution of the proteins of berries is known to vary with the stages of their development. In late growth stages (i.e., at full maturity, during harvesting periods), an increase in the levels of proteins involved in stress response, metabolism, plant defense, and cytoskeleton formation is significant [51].

Table 4. Characterized proteins from a Silvaner wine identified by MS-based proteomics. The proteins are classified by cell function, organism source, and molecular mass (MW).

n°	Protein Description	Organism	MW (kDa)	Digestion Method
Gene expression and nucleotide metabolism				
1	DNA binding protein	*Geotrichum candidum*	153.2	*In-gel*
2	6-carboxy-5,6,7,8-tetrahydropterin synthase	*Methylobacterium* sp.	13.5	*In-gel*
3	Nuclear GTP-binding protein NUG1	*Pichia kudriavzevii*	58.7	*In-gel*
4	ATP-dependent RNA helicase DBP1	*Saccharomyces cerevisiae*	67.9	*In-gel*
5	Tyrosine-DNA phosphodiesterase	*Saccharomyces cerevisiae*	62.2	*In-gel*
6	Daughter-specific expression-related protein	*Saccharomyces cerevisiae*	121.1	*In-gel+In-solution*
7	Putative ribonuclease H protein	*Vitis vinifera*	16.6	*In-gel*
8	Retrovirus-related Pol polyprotein from transposon RE1	*Vitis vinifera*	73.7	*In-gel*
9	RNA exonuclease 4	*Vitis vinifera*	44.2	*In-gel*
10	Transposon Ty3-I Gag-Pol polyprotein	*Vitis vinifera*	59.1	*In-solution*
11	5′-nucleotidase SurE	*Vitis vinifera*	39.7	*In-gel*
Metabolic breakdown and formation of carbohydrates				
12	Pectin lyase A	*Aspergillus niger*	39.7	*In-gel*
13	Glycosidase	*S. cerevisiae x S. kudriavzevii*	53.7	*In-solution*
14	Endo-(1,3)-β-glucanase	*Saccharomyces uvarum*	34	*In-gel+In-solution*
15	Exo-(1,3)-β-glucanase of the cell wall	*Saccharomyces uvarum*	51.2	*In-gel+In-solution*
16	Glucan endo-(1,3)-β-glucosidase	*Vitis vinifera*	36.8	*In-gel+In-solution*
17	UDP-glycosyltransferase 85A8	*Vitis vinifera*	20.5	*In-gel*
18	Vacuolar invertase 1	*Vitis vinifera*	71.5	*In-gel+In-solution*
19	β-fructofuranosidase, soluble isoenzyme I	*Vitis vinifera*	63.5	*In-gel+In-solution*
Proteins involved in post-translational modifications				
20	Aminopeptidase	*Novosphingobium* sp.	72	*In-gel*
21	Non-specific serine/threonine protein kinase	*S. cerevisiae x S. kudriavzevii*	120	*In-gel*
22	Cysteine proteinase inhibitor	*Vitis vinifera*	11.2	*In-solution*
23	IAA-amino acid hydrolase ILR1-like 4	*Vitis vinifera*	72.7	*In-gel*
24	α-Crystallin domain-containing protein 22.3	*Vitis vinifera*	18.1	*In-gel*
Lipid metabolism				
25	Putative lipase ATG15	*Pichia kudriavzevii*	56.8	*In-gel*
26	Lysophospholipase	*Saccharomyces cerevisiae*	71.6	*In-solution*
27	Putative non-specific lipid-transfer protein AKCS9	*Vitis vinifera*	9.8	*In-gel+In-solution*
28	Non-specific lipid-transfer protein	*Vitis vinifera*	11.7	*In-gel+In-solution*
Cell defense				
29	Killer toxin resistant protein	*Saccharomyces cerevisiae*	30	*In-gel*
30	Pathogen-related protein	*Saccharomyces cerevisiae*	30.6	*In-gel*
31	Class IV endochitinase (Fragment)	*Vitis vinifera*	27	*In-gel*
32	Endochitinase EP3	*Vitis vinifera*	27.2	*In-gel*
33	LysM domain-containing GPI-anchored protein 1	*Vitis vinifera*	43.7	*In-gel*
34	Thaumatin-like protein	*Vitis vinifera*	23.9	*In-gel+In-solution*
Cell metabolism and signaling				
35	Probable E3 ubiquitin-protein ligase TOM1	*Ashbya gossypii*	372.2	*In-gel+In-solution*
36	Cytokinesis protein sepH	*Cyberlindnera fabianii*	116.3	*In-gel*
37	Protein that regulates signaling from a G protein β subunit Ste4p	*Kazachstania saulgeensis*	25.7	*In-gel*
38	ACC synthase	*Penicillium citrinum*	48.2	*In-solution*
39	PAS domain-containing protein	*Methylobacterium* sp.	21.3	*In-solution*
40	Acid phosphatase	*Saccharomyces cerevisiae*	52.9	*In-solution*
39	GTPase-activating protein	*Saccharomyces cerevisiae*	53.9	*In-solution*
40	Histidine kinase osmosensor that regulates an osmosensing MAP kinase cascade	*Saccharomyces cerevisiae*	134.5	*In-solution*
41	Cytochrome P450 81E8	*Vitis vinifera*	16.9	*In-gel*
42	Ethylene-overproduction protein 1	*Vitis vinifera*	113.4	*In-gel*
43	Pentatricopeptide repeat-containing protein	*Vitis vinifera*	104.7	*In-solution*
44	Plasma membrane ATPase	*Vitis vinifera*	105.8	*In-solution*
45	Rust resistance kinase Lr10	*Vitis vinifera*	68.4	*In-gel*
46	Ubiquitin-60S ribosomal protein L40	*Vitis vinifera*	80.1	*In-solution*
Cell structural elements				
47	Sensitive to high expression protein 9, mitochondrial	*Cyberlindnera fabianii*	42.6	*In-gel*
48	Component of the Sec23p-Sfb3p heterodimer of the COPII vesicle coat	*Kazachstania saulgeensis*	106.6	*In-gel*
49	Cell wall mannoprotein	*Saccharomyces cerevisiae*	29.6	*In-gel+In-solution*
50	Cell wall protein ECM33	*Saccharomyces cerevisiae*	43.8	*In-gel+In-solution*
51	Seripauperin	*Saccharomyces cerevisiae*	17.7	*In-solution*

A graphical comparison of the number of identified proteins (classified by their cellular functions) and the digestion method used (*in-gel*, *in-solution*, and *in-gel+in-solution*) is presented in Figure 4b. In our findings, the highest number of proteins was associated with basic cellular functions related to metabolism and cell signaling. According to Kuang et al. [51], such protein profiles are more related to late stages of berry development, which is in agreement with the fact that wines are produced from ripe fruit. Proteins related to basic cellular functions were also found by Marsoni et al. [52], when they isolated and identified 15 proteins from different grape tissues and verified that most of them were involved in the regulatory and secondary metabolism such as energy metabolism. The classes of proteins or enzymes participating in the metabolism of proteins, nucleotides and lipids were also well represented in our findings. Sarry et al. [53] identified 67 proteins from six *V. vinifera* grape varieties and classified the proteins by their cellular functions: 34% of them were involved in energy metabolism, 19% had functions in the cell defense and in the response to stress, while 13% participated in the primary metabolism.

Particularly important for the deleterious haze formation are the pathogenesis-related (PR) proteins, which exert defensive functions in diverse plant species [17]. In *V. vinifera*, they are commonly expressed on a basal level during ripening or mechanical stress, while their expression level is upregulated during plant infection [54]. The highest fraction of these PR proteins is represented by TLPs and CHIs [18,45]. These two protein species are often reported as the main contributors for haze formation and wine instability [8,55]. Many isoforms of heat unstable proteins (HUPs), such as TLPs and CHIs, as well as other proteins such as β-glucanases [56] are also involved in haze formation and they are often reported to have molecular masses in the range of 20–30 kDa [17].

We previously used top-down proteomics to detect peptides obtained by tryptic digestion of the same proteinaceous substance studied herein [32]. A total of nine proteins (including high and low-abundances) from our earlier study could be identified in the present study (Supplementary Data S2, Table S1). Kwon [30] found a total of 20 proteins from a Sauvignon blanc wine by nano-LC-MS analysis. From these, five proteins were from grape, twelve from yeast, two from bacteria and one of fungal origin. The author emphasized that the MS analysis provided a sensitive and selective analysis for the protein identification. Okuda et al. [57], for example, detected vacuolar invertases (with a MW of approximately 66 kDa) and a lipid transfer protein (LTP, with 13 kDa) in Chardonnay wines by sequencing the *N*-terminal amino acid sequences of protein spots from 2D electrophoresis gels (electroblotted onto a Polyvinylidene fluoride (PVDF) membrane). Although the authors found approximately 150 protein spots on a 2D electrophoresis gel, most of which were related to TLP, osmotin-like protein, invertase, LTP, and their hydrolysis products. As expected, yeast proteins were also often reported as part of the wine proteome. Cilindre et al. [22] reported ten different proteins in a wine from healthy grapes and eight different proteins in a wine from grapes infected with Botrytis sp. (two protein bands probably secreted by *B. cinerea*), including a cell-wall mannoprotein from *S. cerevisiae* and two pectinolytic enzymes from *Botryotinia fuckeliana* (teleomorph of *B. cinerea*).

Proteomic profile might be comparatively used to detect differences in products from different wineries and years and validate authentication marker proteins. Proteins such as TLP, CHI, vacuolar Invertase, and protein Ygp1, detected in the Silvaner wine, are regularly found in other wine samples. Other low-abundance proteins identified in this study could be characterized as protein markers from now on. Some examples could be a cysteine proteinase inhibitor (A5ANX3) and a plasma membrane ATPase (A0A438EWP8), which are originated from the plant *V. vinifera* (to evidence a protein from the cultivar Silvaner and not from fermentative organisms), they were found here with three and two unique peptides (respectively) and were not previously identified in literature-reported wine proteomics. However, to validate the hypothesis that these proteins may be used as qualitative markers, several wines from different cultivars and geographical regions and years have to be analyzed by the same method described herein. A comparison of proteins reported from different white wines, which were also identified in the present study, can be

found in the Supplementary Data S2 (Table S2). Rešetar et al. [58] emphasized the increase in fraud on the wine market in recent years and discussed the need for guidelines and laws to regulate standard production procedures and ensure quality parameters such as geographical origin. Chambery et al. [59] presented the concepts of food traceability based on the EU General Food Law Regulation as a form to guarantee food quality and safety. Recent advances and the availability of MS techniques could be applied in the proteomics analyses of different wines and become a powerful tool to provide information about food additives, allergenic proteins, fining agents, and haze potential to validate products and prevent commercial counterfeiting. Such methods are also recommended for the validation of suitable marker proteins based on the evaluation of many different vineyards, cultivars, years, drought, grape pathogens, and plant stress conditions.

5. Conclusions

The two-step protein fractionation and subsequent HR-MS techniques allowed the analysis of the comprehensive proteome profiling of a Silvaner wine for the first time. In addition, combining *in-solution* and *in-gel* protein digestion techniques enabled sufficient sensitivity to detect a high number (154 different accession numbers) of identifiable proteins. The functions of 50 proteins were described and classified according to their roles in cell metabolism, signaling, defense and structure. Such a combination of methods can improve the characterization of wine proteomes and be helpful to obtain traces of wine's origin and processing as an authentication method for future applications.

Supplementary Materials: All data needed to evaluate the conclusions are presented in the paper and in the Supplementary Materials and can be found in the online version, at https://www.mdpi.com/article/10.3390/biom13040650/s1. Supplementary data are available as Supplementary Data S1 (A list of all identified proteins from the Silvaner wine) and Supplementary Data S2 (Table S1. Comparison of proteins from the same Silvaner wine identified in the present study and proteins from a Silvaner wine identified by Albuquerque et al. (2021); Table S2. Comparison of proteins from different white wines reported in the literature and identified in the present study; and Figure S1. Quantification of the % of yield from the sample to protein fraction after FPLC fractionation based on the relative area calculated by the software ChromLab v6.1.29). The raw MS data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD040172.

Author Contributions: W.A. was responsible for the experimental design, conceptualization, investigation, data curation and writing. P.G. participated in the sample preparation, investigation and data curation. L.S. and J.B. participated in the sample preparation and data curation. B.S. was involved with resources and review. F.W., R.S. and H.Z. were involved with resources, funding acquisition, supervision, review, and editing of the final manuscript. M.G. was responsible for the project administration, conceptualization, supervision and writing. All authors have read and agreed to the published version of the manuscript.

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Research article 4 – Peptidomics as a Tool to Assess the Cleavage of Wine Haze Proteins by Peptidases from *Drosophila suzukii* Larvae.

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Peptidomics as a Tool to Assess the Cleavage of Wine Haze Proteins by Peptidases from *Drosophila suzukii* Larvae

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Abstract: Thermolabile grape berry proteins such as thaumatin-like proteins (TLPs) and chitinases (CHIs) promote haze formation in bottled wines if not properly fined. As a natural grapevine pest, the spotted-wing fly *Drosophila suzukii* is a promising source of peptidases that break down grape berry proteins because the larvae develop and feed inside mature berries. Therefore, we produced recombinant TLP and CHI as model thermolabile wine haze proteins and applied a peptidomics strategy to investigate whether *D. suzukii* larval peptidases were able to digest them under acidic conditions (pH 3.5), which are typically found in winemaking practices. The activity of the novel peptidases was confirmed by mass spectrometry, and cleavage sites within the wine haze proteins were visualized in 3D protein models. The combination of recombinant haze proteins and peptidomics provides a valuable screening tool to identify optimal peptidases suitable for clarification processes in the winemaking industry.

Keywords: peptidases; peptidomics; *Drosophila suzukii*; thaumatin-like proteins; chitinases; recombinant proteins; wine haze

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1. Introduction

The formation of turbidity in wines results from the denaturation of thermolabile proteins, which then interact and form cross-links, resulting in particle agglomeration and flocculation at moderate temperatures [1]. Protein aggregation in white wines is also promoted by ingredients such as polyphenols and sulfite ions [2], which link the hydrophobic protein cores and/or rearrange disulfide bridges. The most prominent thermolabile proteins in wine are thaumatin-like proteins (TLPs) and chitinases (CHIs), which are constitutively expressed at a basal level but are induced in response to fungal infections [3]. Accordingly, they are also described as pathogen-related (PR) proteins. Such proteins are stable under acidic conditions and have a compact structure that hinders degradation and facilitates their survival during vinification [4].

Haze formation in bottled wines is prevented by clarification or fining steps during winemaking [5]. Clarification typically involves the addition of bentonite clay, a cation exchanger that binds the wine haze proteins but also negatively affects the organoleptic properties [6,7]. Other fining methods such as the use of animal proteins (casein, gelatin and albumin) and ultrafiltration can be used to remove polyphenolic compounds, but they also reduce the levels of tannin [8].

A promising alternative is the in situ degradation of thermolabile proteins using peptidases. However, peptidase activity is highly dependent on the pH, temperature, and the exposure of buried protein structural features [2,9]. Recently, cleavage sites have been traced along the TLP and CHI polypeptide backbones by high-resolution LC-MS/MS, an approach known as top–down peptidomics [10]. This is based on the analysis of native proteins by high-resolution mass spectrometry (HR-MS) without previous chemical treatments [11], thus preserving their post-translational modifications [12]. Alternatively, when the cleavage profiles of peptidases are unknown, de novo sequencing algorithms [13] can be used to identify peptides and proteins based on peptide fragmentation patterns [14,15].

Although peptidases are ubiquitous and responsible for essential cellular functions in all living organisms, not all peptidases have been studied in detail, particularly those from insects [16,17]. Given their ecological diversity, insects provide an immense and mostly untapped source of novel and uncharacterized proteolytic enzymes [18]. Insects secrete digestive enzymes such as serine, cysteine, metallo-, and aspartate peptidases into their gut or saliva [19–21]. The expression of such enzymes in polyphagous insects is influenced by their diverse food sources [22,23]. For example, the spotted-wing fly *Drosophila suzukii* (Diptera: Drosophilidae) is an invasive pest of fruit crops that has adapted to thrive under many environmental conditions [24]. It causes significant crop losses by laying eggs in ripe or ripening fruits such as cherries, plums, and grape berries [25]. Indeed, *D. suzukii* infests ripe grape berries to exploit the increasing sugar content, lower pH, and lower skin penetration resistance [26]. Because *D. suzukii* larvae can survive in this acidic environment, they offer a promising source of acidic peptidases that remain active under typical winemaking pH conditions.

The application of peptidases with residual activity under acidic conditions could facilitate wine clarification. Therefore, we extracted peptidases from *D. suzukii* larvae and characterized the purified enzymes to evaluate their ability to cleave haze-forming proteins at pH 3.5. Recombinant TLP and CHI (rTLP and rCHI) were expressed, purified [27], and digested with the larval peptidases at pH 3.5. The cleaved peptides were detected by LC-MS/MS analysis, and the resulting peptidomics data were analyzed by de novo sequencing. The cleavage sites were visualized using 3D protein models. Finally, we tested the potential of the purified peptidases to degrade native wine proteins isolated from a Silvaner Franken wine in vitro. The combination of methods described herein would be suitable for screening enzyme candidates that can be used as haze preventers in the wine industry.

2. Materials and Methods

2.1. Wine Proteins

We expressed rTLP and rCHI, each bearing a His_6-tag, in the yeast *Komagataella phaffii* (formerly *Pichia pastoris*). Plasmid vectors (described in Albuquerque et al. [27]) containing the genes encoding a thermolabile TLP (4JRU isoform) and a class IV CHI were introduced into *K. phaffii* cells by electroporation. The yeast cells were cultured for 4 days in buffered methanol-complex medium (BMMY) with a daily feed of 1% methanol. The proteins were recovered from the culture medium and purified by chromatography, as previously described [27]. Briefly, the culture supernatant was loaded onto a 5-mL HiTrap IMAC FF column (Cytiva, Freiburg, Germany) and eluted with 0.2 M phosphate buffer (pH 6.0) containing 250 mM imidazole (Carl Roth, Karlsruhe, Germany) directly into a HiLoad 16/600 Superdex 75 column (Cytiva) mounted on an NGC fast protein liquid chromatography (FPLC) system (Bio-Rad Laboratories, Munich, Germany). Size exclusion chromatography (SEC) was carried out at a flow rate of 1 mL/min in 0.1 M Tris-HCl (pH 7.0). The eluted proteins were quantified as described by Bradford [28]. The proteins were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in a 12% polyacrylamide gel under denaturing conditions [29] and visualized by staining with Coomassie Brilliant Blue (AppliChem, Darmstadt, Germany) and by Western blot

(Bio-Rad Laboratories) using antibodies specific for His_6-tag (Thermo Fisher Scientific GmbH, Bremen, Germany). The tag was then removed by incubating the recombinant proteins with tobacco etch virus peptidase (Biozol Diagnostica, Eching, Germany) for 2 h at 37 °C. The proteins were stored at 4 °C. Colloids were obtained from a Silvaner Franken wine by ultrafiltration, as described by Albuquerque et al. [10].

2.2. Rearing of Drosophila suzukii

Adult *D. suzukii* flies were reared on a sterile soymeal and cornmeal medium comprising 9% (*w*/*v*) soymeal and cornmeal mix, 1.8% (*w*/*v*) brewer's yeast, 0.8% (*w*/*v*) agar, 8% (*w*/*v*) malt, 2.2% (*w*/*v*) molasses, 0.2% (*w*/*v*) nipagin in 70% ethanol. and 0.625% (*v*/*v*) propionic acid in distilled water and kept in a climate chamber (Regineering, Preith, Germany) at 26 °C, 60% relative humidity, with a 12-h photoperiod. *D. suzukii* eggs were kept in the growth medium before hatching, and the larvae (Figure 1) were harvested after 9–11 days by floating on a solution of 50 mM sucrose. Larval samples were rinsed in tap water on a 300 mic test sieve (Retsch, Haan, Germany) and stored at –80 °C in reaction tubes for further experiments.

Figure 1. Microscopic image of *Drosophila suzukii* (larval stage).

2.3. Extraction of Peptidases from D. suzukii Larvae

Frozen larval samples (estimated at 4000 larvae per extraction) were macerated under liquid nitrogen and the homogenate was incubated overnight at 4 °C in 20 mL of extraction buffer (0.1 M Tris-HCl pH 7.0, 0.15 M NaCl, and 1% Triton X-100) for cell lysis. The cell debris was removed by centrifugation at 12,000× *g* (for 10 min at 4 °C) using an Allegra X-15R device (Beckman Coulter, Brea, CA, USA) and the supernatants were concentrated in microcentrifuge tubes with a molecular weight cut-off (MWCO) of 10 kDa (Merck, Darmstadt, Germany).

2.4. Proteolytic Activity

The activity of the extracted peptidases was quantified by a rapid spectrophotometric method (spectrophotometer BioTek Synergy, Agilent Technologies, Santa Clara, CA, USA) using azocasein as a substrate and according to Leighton et al. [30]. The degradation of native wine proteins was analyzed by the agar diffusion assay [31]. Briefly, we prepared 1% (*w*/*v*) agarose (Biozym Scientific, Oldendorf, Germany) in 0.1 M citrate buffer (pH 3.5) by boiling and then cooling to 50 °C before mixing with the proteins (1 mg/mL in 0.1 M citrate buffer, pH 3.5) isolated from a Silvaner Franken wine. The solution was poured (20 mL) into Petri dishes and 1 mM ampicillin was added to avoid microbial contamination. Holes measuring 1 cm in diameter (made with a sterile scalpel) were punched into the polymerized agar gels before adding 20 µL of the cell lysate or purified peptidase. The plates were incubated overnight at 37 °C and stained with Coomassie Brilliant Blue to detect halos. Zymograms were prepared by embedding casein (Carl Roth) in SDS-PAGE gels under semi-native conditions (pH 8.8). Degradation bands were observed after staining the gels with Coomassie Brilliant Blue.

2.5. Purification and Characterization of Peptidases from D. suzukii Larvae

2.5.1. Chromatography

Peptidases in the cell lysates were purified by anion-exchange (AEX) chromatography on a HiPrep DEAE FF column 16/10 (GE Healthcare, Chicago, IL, USA) and SEC on a Superdex 75 column 10/300 GL (GE Healthcare) mounted on the NGC FPLC system. AEX chromatography was carried out at a flow rate of 1 mL/min and the proteins were eluted by isocratic flow in a mixture of buffer A (0.1 M Tris-HCl pH 7.0) and buffer B (0.1 M Tris-HCl pH 7.0 containing 0%, 20%, 50% or 100% 1 M NaCl). For SEC, the samples were eluted at 1 mL/min using an isocratic flow of 0.1 M Tris-HCl (pH 7.0) containing 0.15 M NaCl. The azocasein and agar diffusion assays described above were used to identify peaks containing peptidase activity. The protein samples were pooled, desalted by dialysis in float-A-Lyzer devices (in 0.1 M Tris-HCl, pH 7.0, under magnetic stirring and with a membrane with MWCO of 10 kDa, LubioScience, Zürich, Switzerland) and concentrated in microcentrifuge tubes with a MWCO of 10 kDa. Conductivity was monitored during each chromatographic step to ensure the complete desalting of the samples. Protein stability was preserved by keeping the samples at 4 °C during chromatography and on ice during sample handling.

2.5.2. LC-MS/MS Analysis

The protein fractions from each chromatography step were digested with trypsin (Promega, Madison, WI, USA) and the peptides were concentrated and desalted using C18 ZipTip pipette tips (Merck). The peptides were then separated on a Kinetex C18 column (2.1 mm × 100 mm, 2.6 µm, 100 Å; Phenomenex, Torrance, CA, USA) mounted on a Dionex UliMate 3000 RSL UHPLC system (Thermo Fisher Scientific GmbH). This was connected to a Q Exactive HF-X orbital trapping mass spectrometer (Thermo Fisher Scientific GmbH). LC-MS/MS was carried out using the parameters described by Albuquerque et al. [10]. The MS raw data were used to screen the UniProt database (taxonomically restricted to *Drosophila suzukii*) using Byonic v4.2 (Protein Dynamics, Cupertino, CA, USA) and Proteome Discoverer v2.2 (Thermo Fisher Scientific GmbH). The following parameters were applied: two missed cleavage sites; minimum peptide length six amino acids; MS1 and MS2 tolerances of 10 ppm and 0.5 Da, respectively. A strict target FDR (false discovery rate) of 0.01 and a relaxed target FDR of 0.05 were used to validate the identified peptide-spectrum matches and to filter the final output.

2.5.3. Gene Expression Analysis by Quantitative RT-PCR

The gene sequences matching the identified peptidases were obtained from a public transcriptome dataset of *D. suzukii* (NCBI database) and were amplified by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). *D. suzukii* total RNA was isolated using TRI Reagent (Zymo Research, Freiburg, Germany) and reverse transcribed

using the iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was amplified using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific GmbH) and the primers listed in Table 1. A gene encoding actin was used as the reference gene.

Table 1. Primers used to analyze the expression of genes encoding the *D. suzukii* larval peptidases.

Peptidase	ID (NCBI)	Forward Primer	Reverse Primer
Glutamyl aminopeptidase-like	XP_016943864.1	5′-TGTGCATCATTGTGTCCGAC-3′	5′-TCGATCTGATGGGAAGTGGC-3′
Caspase-3	XP_016923550.1	5′-GACTGCCAGGACGCCAAC-3′	5′-CGCTCGCAATTCTCGTATGT-3′
Serine protease 1/2-like	XP_016934104.1	5′-GCGACAACACTATCTGCACC-3′	5′-CTGACTCCCACCAGCTTGTT-3′
Dipeptidyl peptidase III	XP_016925042.1	5′-CGAGCACTACATCCGATCCT-3′	5′-TCCCTTGTCCTTGATCCACC-3′
Cathepsin L1	XP_016943011.1	5′-CAACTGCAATCGTTCCCCAA-3′	5′-TCGTCCGAGTATACCTTGCC-3′

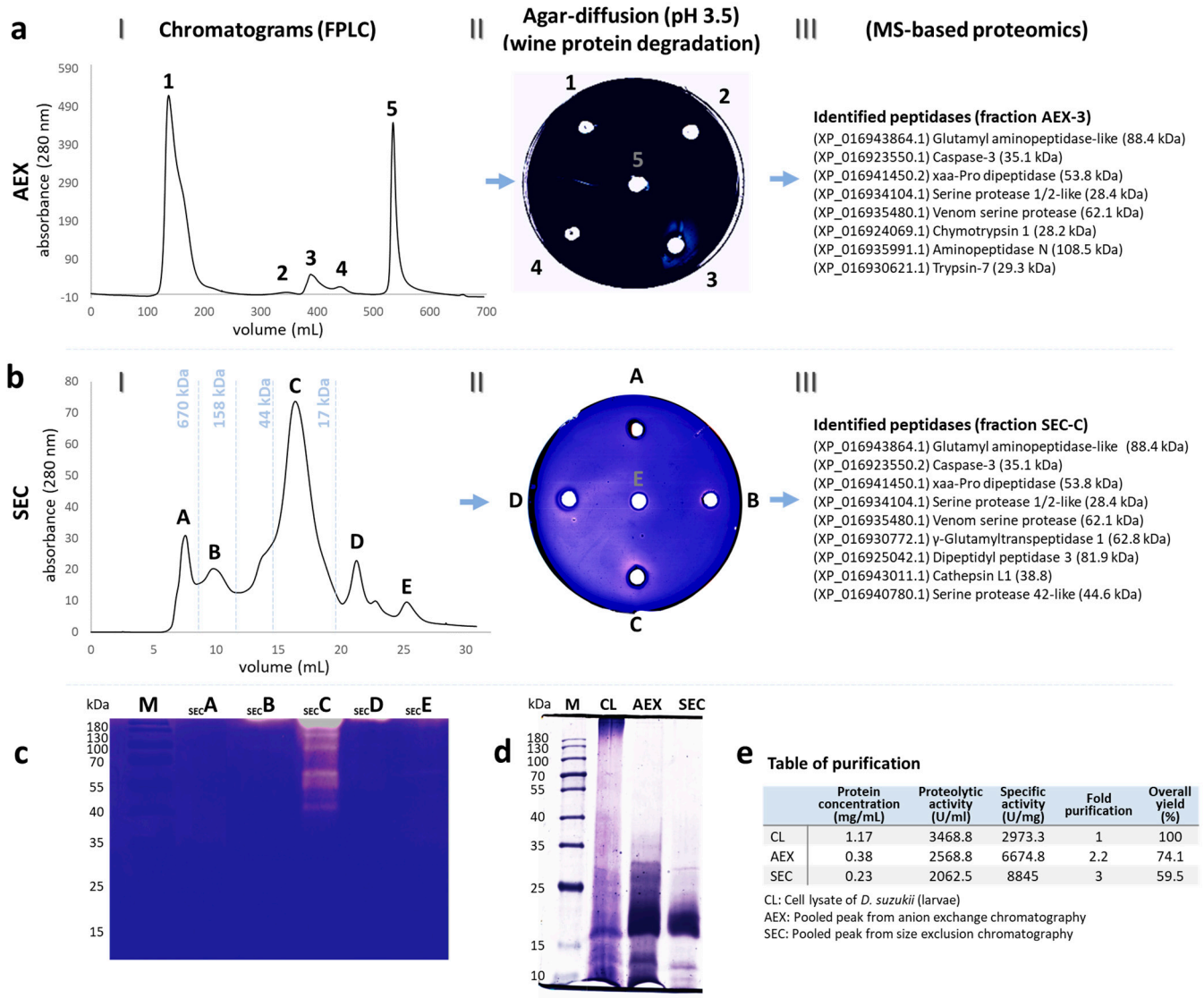
2.6. Analysis of Cleavage Sites in rTLP and rCHI

Purified and vacuum-dried rTLP and rCHI were dissolved in 0.1 M citrate buffer (pH 3.5) to a concentration of 0.25 mg/mL and incubated (in a heating-thermomixer HLC, DITABIS AG, Pforzheim, Germany) with the purified peptidases at 37 °C for 18 h. The cleaved peptides were collected by filtration by using Amicon filters with a MWCO of 10 kDa. Native peptides produced by the digestion of rTLP and rCHI with *D. suzukii* larval peptidases were identified by LC-MS/MS (peptidomics). Raw MS data were analyzed using Peaks Studio vX+ (Bioinformatics Solutions, Waterloo, ON, Canada) based on de novo sequencing [32]. The obtained sequences were searched against the UniProtKB databases (and further correlated to NCBI accession numbers) taxonomically restricted to *Vitis vinifera.* Protein visualization and cleavage site analysis were carried out using Pymol v2.0 (Schrödinger, New York, NY, USA) with 3D models of thermolabile TLP (PDB code 4JRU) and a class IV CHI homology model [10]. For control analysis, rTLP and rCHI were filtered without degradation (incubated at the same pH and temperature but without peptidases) to confirm that no digested peptides were produced without enzymatic reactions.

3. Results

3.1. Purified Peptidases

Five protein peaks were detected in the AEX chromatogram of the *D. suzukii* larval cell lysates (Figure 2(aI)). The proteolytic activity of peak 3 (eluted with an isocratic flow of 20% 1 M NaCl) showed a clear halo of degradation around the wine proteins embedded in agar at pH 3.5 (Figure 2(aII)) and the specific activity against azocasein was 6674.8 U/mg. After desalting, fraction 3 from the AEX step was pooled for SEC, resulting in six further peaks (Figure 2(bI)). Fraction C showed a visible halo of degradation around the wine proteins in the agar diffusion assay at pH 3.5 (Figure 2(bII)) and the specific activity toward azocasein was 8845 U/mg. Moreover, we observed distinct degradation bands in a casein zymogram (Figure 2c) from ~38 kDa to ~180 kDa. The protein composition after each chromatography step was compared by electrophoresis (Figure 2d), and the degree of purification was calculated in terms of the yield and purification factor (Figure 2e). The proteins identified by MS are shown in Figure 2(aIII,bIII).



	Protein concentration (mg/mL)	Proteolytic activity (U/ml)	Specific activity (U/mg)	Fold purification	Overall yield (%)
CL	1.17	3468.8	2973.3	1	100
AEX	0.38	2568.8	6674.8	2.2	74.1
SEC	0.23	2062.5	8845	3	59.5

Figure 2. Partial purification of peptidases from the *D. suzukii* larvae. (**aI**) Anion exchange chromatography (AEX) of the cell lysates (CL) of the *D. suzukii* larvae. (**aII**) The proteolytic activity of each peak was assessed by the degradation of proteins from a Silvaner Franken wine in an agar diffusion assay. (**aIII**) Peptidases in peak III identified by MS-based proteomics. (**bI**) Size exclusion chromatography (SEC) of peak 3 from the AEX step. (**bII**) Degradation of wine proteins assessed by the agar diffusion assay. (**bIII**) Peptidases in peak C identified by MS-based proteomics. (**c**) Protein peaks purified by SEC tested by casein zymography. (**d**) SDS-PAGE analysis to characterize protein separation during each purification step. (**e**) Table of purification efficiency and comparison of the activity and protein content during the purification process.

3.2. Characterization of Peptidases by MS-Based Proteomics

Twelve unique peptidases were identified in the purified protein fractions (in both AEX and SEC), each representing different levels of protein coverage (Table 2). We identified candidates representing the glutamyl aminopeptidase-like (NCBI: XP_016943864.1), caspase-3 (XP_016923550.1), Xaa-Pro dipeptidase (XP_016941450.2), serine protease 1/2-like (XP_016934104.1), and venom serine protease (XP_016935480.1) families after both chromatography steps, which provide additional confidence in the results. We identified aminopeptidase N (XP_016935991.1), chymotrypsin 1 (XP_016924069.1), and trypsin-7 (XP_016930621.1) exclusively after the AEX step, whereas γ-glutamyltranspeptidase 1 (XP_016930772.1), dipeptidyl peptidase 3 (XP_016925042.1), serine protease 42-like

(XP_016940780.1), and cathepsin L1 (XP_016943011.1) proteins were identified exclusively after the SEC step. The identification of three unique peptides representing glutamyl aminopeptidase-like (XP_016943864.1, coverage 4%) and serine protease 1/2-like proteins (XP_016934104.1, coverage 5%) as well as γ-glutamyltranspeptidase 1 (XP_016930772.1, coverage 4.1%) increased our confidence in their identification following the SEC step. A list of all identified peptidases and their closest relatives is provided in Supplementary Data File S1.

Table 2. Peptidases from the *D. suzukii* larvae identified following the AEX and SEC purification steps (entries in bold were identified after both steps). The identification score is represented in terms of the percent coverage and unique peptides. The identified enzymes and their peptides detected by LC-MS/MS are related to the NCBI accession number. The catalysis type and molecular masses are provided for each enzyme based on the NCBI data.

n	Accession No. (NCBI)	Description	Peptides	Type	Coverage [%]	Unique Peptides	MW (kDa)
			AEX step				
1	XP_016943864.1	**Glutamyl aminopeptidase-like**	R.QAFPCFDEPALK.A K.YNIEWLAR.N K.WWNDLWLNEGFAR.F	Metallo	4	3	88.4
2	XP_016923550.1	**Caspase-3**	R.TYDDLTFSDINDK.L	Cysteine	4	1	35.1
3	XP_016941450.2	**Xaa-Pro dipeptidase**	K.SLYNTDVDYVFR.Q	Metallo	2	1	53.8
4	XP_016934104.1	**Serine protease 1/2-like**	K.VELPSYNDR.Y	Serine	5	1	28.4
5	XP_016935480.1	**Venom serine protease**	K.FLQQDFVGMNPFVAGWGAVK.H	Serine	4.1	1	62.1
6	XP_016924069.1	Chymotrypsin 1	R.ILGGEDVEQGEYPWSASVR.Y	Serine	8.6	1	28.2
7	XP_016935991.1	Aminopeptidase N	K.QLIDPIFNK.I	Metallo	1	1	108.5
8	XP_016930621.1	Trypsin 7	R.EWLEETIEANK.D	Serine	4	1	29.3
			SEC step				
1	XP_016943864.1	**Glutamyl aminopeptidase-like**	R.QAFPCFDEPALK.A K.YNIEWLAR.N K.WWNDLWLNEGFAR.F	Metallo	4	3	88.4
2	XP_016923550.1	**Caspase-3**	R.TYDDLTFSDINDK.L	Cysteine	4	1	35.1
3	XP_016941450.2	**Xaa-Pro dipeptidase**	K.SLYNTDVDYVFR.Q	Metallo	2	1	53.8
4	XP_016934104.1	**Serine protease 1/2-like**	K.VELPSYNDR.Y	Serine	5	1	28.4
5	XP_016935480.1	**Venom serine protease**	K.FLQQDFVGMNPFVAGWGAVK.H	Serine	4.1	1	62.1
6	XP_016930772.1	γ-Glutamyltranspeptidase 1	R.YGILPWK.R R.LFEPSIK.L K.EIYDGGETGR.K	Cysteine	4.1	3	62.8
7	XP_016925042.1	Dipeptidyl peptidase 3	K.IFDK.V	Metallo	2	2	81.9
8	XP_016943011.1	Cathepsin L1	R.LGVNPLADMTR.K	Cysteine	3.1	1	38.8
9	XP_016940780.1	Serine protease 42-like	K.DGEYQVILK.K K.LWNIDPK.Y	Serine	3.9	2	44.6

3.3. Gene Expression Analysis

The expression profiles of genes encoding the identified glutamyl aminopeptidase-like (metallopeptidase), caspase-3 (cysteine peptidase), cathepsin L1, dipeptidyl peptidase III (metallopeptidase), and a serine protease 1-like (serine peptidase) proteins are summarized in Supplementary Data File S2.

3.4. Identification of Intact Peptides (LC-MS/MS Top–Down Proteomics)

The rTLP and rCHI proteins were enzymatically hydrolyzed at pH 3.5 under native conditions (without the use of denaturing agents) by peptidases from the *D. suzukii* larvae. The peptides detected by MS are highlighted in the 3D structures of rTLP and rCHI, which show the surface features and secondary structures alongside the anticipated trypsin cleavage pattern at pH 7.0 (Figure 3). Only one peptide cleavage product was found for rTLP, whereas the structure of rCHI was largely degraded. The secondary structures cleaved by the purified peptidases are shown in Figure 3(aI,bI) for rTLP and rCHI, respectively. The cleavage sites are also highlighted in the corresponding amino acid sequences.

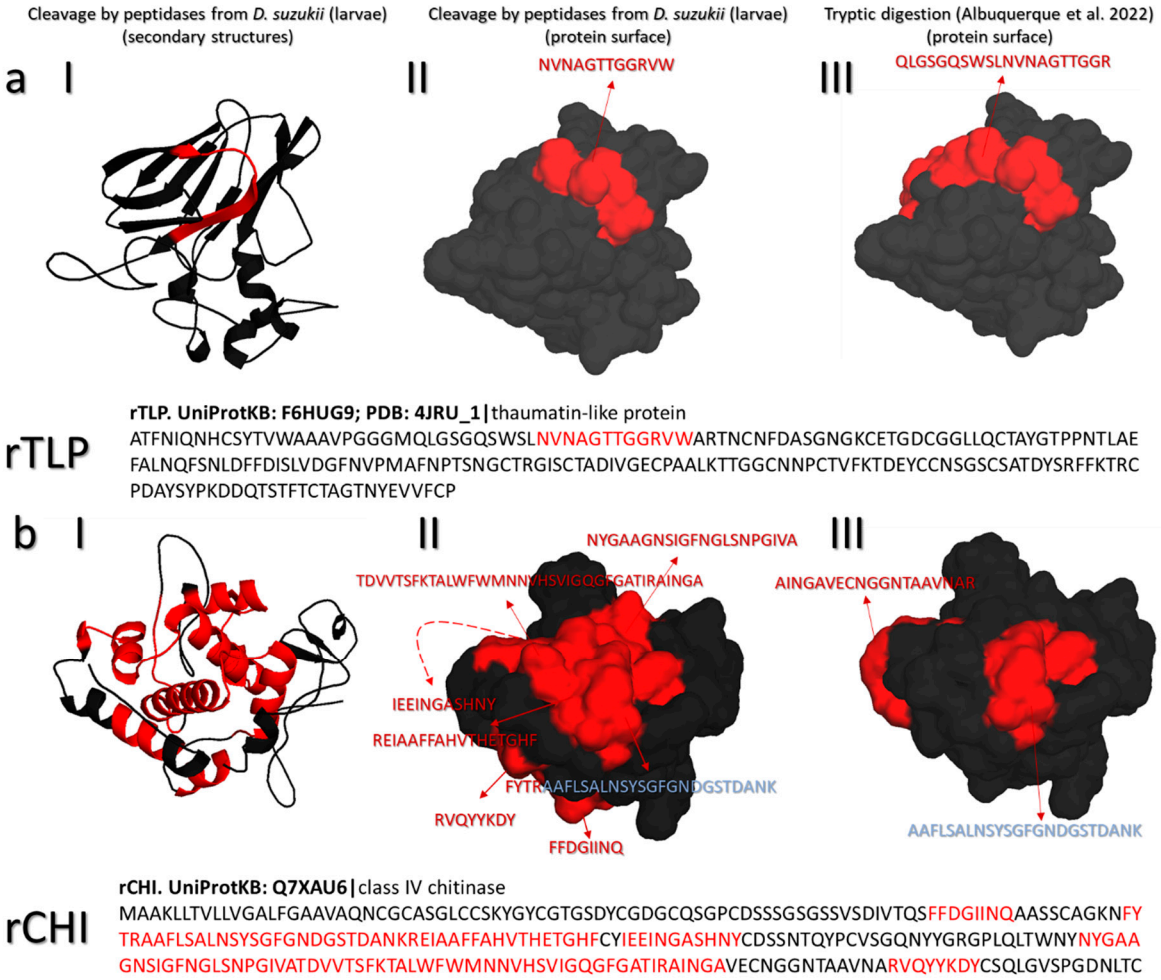


Figure 3. Cleavage sites in rTLP and rCHI detected after digestion with the purified peptidases from the *D. suzukii* larvae. The cleaved peptides from (**a**) rTLP and (**b**) rCHI are displayed separately as (**I**) cleaved peptides identified in secondary structures and (**II**) cleaved peptides identified on the protein surface. (**III**) The same protein structure is shown cleaved by a trypsin. For each recombinant protein, the identified peptides are also highlighted in red in the amino acid sequence.

4. Discussion

We identified at least 12 peptidases from the *D. suzukii* larvae with molecular masses ranging from 28.2 to 108.5 kDa. This number of peptidases may explain the complex pattern of degradation bands observed in the casein zymograms (Figure 2c). The purified peptidases were assigned to three different classes based on their active sites: five serine peptidases (trypsin-7, chymotrypsin 1, venom serine protease, serine protease 1/2-like, and serine protease 42-like), four metallopeptidases (glutamyl aminopeptidase-like, aminopeptidase N, Xaa-Pro dipeptidase, dipeptidyl peptidase 3), and three cysteine peptidases (caspase-3, γ-glutamyltranspeptidase 1, and cathepsin L1).

The *D. suzukii* peptidases were able to cleave rTLP and rCHI under acidic conditions (pH 3.5) at 37 °C, and the resulting peptides were detected by MS-based peptidomics. The use of purified recombinant proteins rather than a complex mixture of wine proteins made it possible to study the direct action of the peptidases on the structure of haze proteins. TLP is a compact protein because it features several disulfide bridges and β-sheet secondary structures [3]. In contrast, class IV CHI is mainly composed of α-helices and loops [33], which facilitates its irreversible denaturation at temperatures above 50 °C [34]. As a consequence, rCHI was completely degraded by the purified *D. suzukii* peptidases, producing ~45 distinct peptides (Figure 3b, Supplementary Table S1 and Supplementary

Figure S1), suggesting that the rCHI structure was more accessible to the peptidases under native conditions (37 °C). In contrast, the rTLP structure was only cleaved at one specific site, releasing the peptide NVNAGTTGGRVW (Figure 3a, Supplementary Table S1 and Supplementary Figure S2).

Not every *D. suzukii* larval peptidase is likely to be active at pH 3.5, and the cleavage of rTLP and rCHI therefore probably reflects the combined action of the acidic peptidases. Furthermore, the metabolic function of each peptidase must be taken into account when considering their potential to cleave wine haze proteins efficiently. Digestive peptidases, for example, are typically endopeptidases with broad cleavage specificity [35]. Some of the identified peptidases have a luminal digestive function including trypsin-7 [36], chymotrypsin 1 [37], Xaa-Pro dipeptidase or prolidase [38], and cathepsin L1 [39].

Serine peptidases are essential digestive enzymes in the insect gut [40,41] and are supported by cysteine peptidases and others as an evolutionary strategy to overcome the production of serine peptidase inhibitors by plants [42]. Serine proteases are typically endopeptidases with optimal activity under neutral to alkaline conditions (pH 7–10) at moderate temperatures (20–50 °C), and they have distinct functions in insect development, reproduction, and metabolism [43–45]. Many other insect metallopeptidases are classified as aminopeptidases, which act as *N*-terminal exopeptidases [46,47]. These enzymes favor alkaline pH conditions and temperatures of 30–60 °C [48–50]. In contrast, cysteine peptidases have an optimal pH range of 4–6 [51,52] and they complement serine peptidases in insect nutrition [42]. Among the *D. suzukii* larval peptidases we identified, γ-glutamyltranspeptidase 1 (XP_016943864.1), caspase-3 (XP_016923550.1), and cathepsin L1 (XP_016943011.1) are cysteine peptidases, which in *Drosophila* species have been associated with apoptosis and the digestion of cytoplasmic components. Caspase-3 is a cysteine aspartic peptidase (cleaving after aspartic acid residues) that participates in death signaling and apoptosis [53]. Caspases are most active at pH~4, implying that they are located in vacuoles rather than the cytosol [54]. In *Drosophila* cells, the degradation of filamentous actin, α-tubulin, α-spectrin, and nuclear lamins coincides with caspase-3 activity [55]. Furthermore, γ-glutamyltranspeptidase 1 is a transmembrane glycoprotein [56] catalyzing the transpeptidation and hydrolysis of the γ-glutamyl group of glutathione [57]. This is achieved by cleaving the γ-glutamyl bond (γ-Glu-Cys-Gly), releasing free glutamate and the dipeptide cysteinyl-glycine [58]. This enzyme also regulates apoptosis depending on the levels of intracellular glutathione [59]. The expression and activity of bacterial γ-glutamyltranspeptidase was found to be induced at pH ~4 by the addition of glutamine and salts [56].

Cathepsins belong to the papain family and feature a cysteine residue in their active site [60]. They are active at pH 3–4.5 [61] and have a broad specificity for protein cleavage sites according to the MEROPS database [62]. This broad activity was confirmed by using nano-LC-MS/MS to measure the frequency and distribution of cleavage sites when *Fasciola hepatica* cathepsin L1 was used for the complete degradation of hemoglobin [61]. Cathepsin L1 is a digestive peptidase in many organisms, transforming proteins into absorbable peptides [63]. Insects secrete cathepsins from epithelial cells into the gut [64], although they are only active in acidic regions [65]. For example, cathepsin L1 has been described as an acidic endopeptidase that is unstable at neutral pH [66]. The cathepsin L1 we identified in the *D. suzukii* larvae may be responsible for the observed cleavage profile of rTLP and rCHI (Figure 3), given its broad specificity as a typical digestive enzyme.

The cleavage of rTLP and rCHI should also be tested under winemaking conditions, specifically a low pH and temperature, high ionic strength, and adequate concentrations of ethanol, sulfite, and polyphenols. Therefore, the following workflow should be implemented: (1) expression of recombinant enzyme candidates and scaled-up production for the most promising peptidases such as cathepsin L1; (2) purification of the peptidases using appropriate tags; and (3) evaluation of their ability to reduce haze formation in real wine samples. A peptidase (or a mixture) that reduces wine haze under typical winemaking conditions would constitute a real breakthrough in wine research. Innovative methods for

haze prevention are still in demand. Recently, the use of an enzymatic mixture known as Proctase combined with flash pasteurization was approved by the International Organization of Vine and Wine (OIV) for market applications [67]. Furthermore, the combination of rTLP and rCHI and the identification of cleavage sites by MS-based peptidomics and de novo sequencing is an innovative tool to screen peptidase candidates for their ability to cleave thermolabile grape berry proteins at low pH and temperatures suitable for winemaking, providing more insights into the effects of peptidases in wine haze reduction. Our workflow could remove hurdles preventing the use of peptidases in industrial wine fining.

5. Conclusions

Peptidases purified from the *D. suzukii* larvae were identified by MS-based proteomics and evaluated regarding their potential to cleave wine proteins under acidic conditions. The identified peptidases cleaved recombinant rTLP and rCHI proteins at pH 3.5, and the digestion products were detected by top–down MS-based peptidomics. Acid peptidases such as cathepsin L1 are likely to be responsible for the observed cleavage profile given their activity at low pH, broad cleavage specificity, and natural function as digestive enzymes. The methods discussed herein can be used to screen for peptidases that are optimal for eventual winemaking applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom13030451/s1, Figure S1: Peptide products from the cleavage of rCHI (UniProt ID: QX7AU6) following incubation with peptidases from the *D. suzukii* larvae are available; Table S1: Peptide sequences for rTLP and rCHI identified by top-down peptidomics following incubation with peptidases from *Drosophila suzukii* larvae at pH 3.5. Figure S2: Peptide products from the cleavage of rTLP (PDB ID: 4JRU) following incubation with peptidases from *D. suzukii* larvae. Supplementary Data File S1: list of all identified peptides from the peptidases and their closest relatives, Supplementary Data File S2: primer and the expression profiles of the genes encoding peptides are presented. The raw MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD039025 and 10.6019/PXD039025. Additional data related to this paper may be requested from the authors.

Author Contributions: W.A.: Methodology, Validation, Investigation, Visualization, Formal analysis, Writing-Original draft preparation; P.G. (Parviz Ghezellou), K.-Z.L. and Q.S.: Investigation, Data Curation; P.G. (Phillip Gross), T.K. and B.O.: Investigation; B.S.: Funding acquisition, Resources; A.V.: Resources, Formal analysis; H.Z.: Funding acquisition, Supervision, Writing—Reviewing and Editing; M.G.: Conceptualization, Supervision, Project administration, Writing-Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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