PAPER

CHARACTERIZATION OF CHITINASE ISOFORMS FROM GRAPE JUICE

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ABSTRACT

Grape chitinases are recognized as being mainly responsible for protein haze formation in white wines. *Vitis vinifera* L. cv. Manzoni Bianco grape juice proteins were fractionated using anion exchange and hydrophobic interaction chromatographies. According to SDS-PAGE and zymography, six protein bands with chitinolytic activity were subjected to mass spectrometry (MALDI-TOF/TOF MS), which assigned all the bands to *Vitis vinifera* class IV chitinases. These grape chitinase isoforms showing different electrophoretic and chromatographic behaviours are likely to be also distinct in their functionality in wine. This could be relevant to understand the involvement of single chitinase components in wine hazing and to develop specific winemaking techniques for their removal from wine.

Keywords: chitinase, electrophoresis, glycol chitin, grape juice, isoform, mass spectrometry

1. INTRODUCTION

The problem of protein haze formation in white wines is still unresolved, despite wine hazing being a serious quality defect because consumers perceive hazy wines as faulty products. Protein haze is caused by the presence of relatively low concentrations (from 15 to 700 mg/L) of Pathogenesis-Related (PR) proteins, namely Thaumatin-Like Proteins (TLPs) and chitinases (FERREIRA *et al.*, 2001; WATERS *et al.*, 2005; VINCENZI *et al.*, 2005; VAN SLUYTER *et al.*, 2015).
Chitinases are the most active protein components in causing wine turbidity (FALCONER *et al.*, 2010; MARANGON *et al.*, 2011). These proteins derive from grapes, are present in different isoforms (MARANGON *et al.*, 2011; GAZZOLA *et al.*, 2012), are tolerant to low pH in juice and wine and are resistant to proteolytic enzymes, as most of the PR proteins (FERREIRA *et al.*, 2001; WATERS *et al.*, 2005; VAN SLUYTER *et al.*, 2015).
The first step of the mechanism leading to haze formation in wines should involve protein denaturation (VAN SLUYTER *et al.*, 2015). Grape chitinases can denature within minutes at temperatures >40°C, compared to weeks for TLPs under the same conditions, with a predicted half-lives of only 14 hours at a realistic temperature of 35°C (FALCONER *et al.*, 2010). Moreover, these grape enzymes seem to maintain their activity in wine at least for some months after alcoholic fermentation (MANTEAU *et al.*, 2003) and the consequences of this activity on wine quality are unknown. Chitinases have antifungal properties resulting from their activity toward chitin, a major structural component of many fungal cell walls (GRAHAM and STICKLEN, 1994). However, a chitinase purified from *Vitis vinifera* L. cv. Manzoni Bianco grape juice, although showing both endo- and exo-chitinase activities, was not able to inhibit wine yeast growth (VINCENZI *et al.*, 2014).
Chitinases have been successfully purified by others (MARANGON *et al.*, 2009; VAN SLUYTER *et al.*, 2009; DUFRECHOU *et al.*, 2013), despite their low concentration and strong interaction with endogenous polyphenols and other non-protein compounds (FERREIRA *et al.*, 2001; GAZZOLA *et al.*, 2012). In spite the interest of these type of wine components, in-depth knowledge surrounding them is still incomplete. Therefore it is important to develop robust systems for a better characterisation of these components and in particular to establish the role of each chitinase isoform found in grape in wine haze formation and development. In this paper, the purification, the electrophoretic characterisation and the Mass Spectrometry identification of some chitinase isoforms from Manzoni Bianco grape juice is described.

2. MATERIALS AND METHODS

2.1. Protein extraction from grape juice

According to VINCENZI *et al.* (2014) with minor modifications, fifteen kg of *Vitis vinifera* L. cv. Manzoni Bianco berries were manually crushed and treated with 7.5 g/kg polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO), 0.15 g/kg ascorbic acid (Baker, Deventer, Holland) and 0.375 g/kg potassium metabisulfite (Carlo Erba, Milano, Italy). The grape juice (10 L) was treated overnight at 4°C with 3 g/L of pectolytic enzymes (Pectazina DC, Dal Cin SpA, Milano, Italy), and centrifuged (5000 g, 20 min, 4°C). The free run juice was dialysed (3500 Da cut-off) against distilled water, concentrated by ultrafiltration (3000 Da cut-off) and freeze-dried.

2.2. Protein separation by chromatography

A two-step chromatographic separation was performed using an ÄKTA purifier FPLC (GE-Healthcare, Uppsala, Sweden) equipped with an UV detector. Data were processed by the Unicorn 5.11 software (GE-Healthcare). Each solution used and samples to load were previously filtered with 0.20 μm cellulose acetate filters (Millipore, Vimodrone, Italy).
The first chromatographic step was Anion Exchange Chromatography (AEC). ≈ 50 mg of freeze-dried extract were dissolved in 20 mM Tris-HCl pH 9.0 (buffer A) and loaded onto a Tricorn MonoQ 5/50 column (GE-Healthcare) equilibrated with buffer A at a flow rate of 1 mL/min. Bound proteins were eluted at 1 mL/min with a gradient of buffer B (20 mM Tris-HCl, 1 M NaCl, pH 9.0) as follows: 0 to 14% B in 70 min and 14 to 100% B in 3 min (VINCENZI *et al.*, 2011 with minor modifications). AEC fractions were pooled on the basis of 280 nm elution profiles and analysed by SDS-PAGE after being concentrated and dialysed against water (Vivaspin 50, 3000 Da cutoff, Sartorius, Göttingen, Germany).
The second purification step was performed by Hydrophobic Interaction Chromatography (HIC) according to VINCENZI *et al.* (2014). The pooled and selected AEC fractions were fractionated at 0.5 mL/min on a HIC BioSuite Phenyl 10 μm HIC 7.5 x 7.5 mm column (Waters, Milford, MA) with a 60 min linear gradient to 100% buffer B (20 mM tartaric acid pH 3.5) in buffer A (20 mM tartaric acid pH 3.5 containig 1.25 M ammonium sulfate).

2.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analyses were performed according to LAEMMLI (1970) in a Mini-Protean III apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Samples were prepared by precipitating proteins from 5 to 50 μL (depending on protein concentration on samples) of pooled chromatographic fractions by the KDS method (VINCENZI *et al.*, 2005; GAZZOLA *et al.*, 2015). Precipitated proteins were solubilized in 20 μL of 0.5 M Tris-HCl buffer, pH 6.8, containing 15% (w/v) glycerol and 1.5% (w/v) SDS (Bio-Rad Laboratories) and heated at 100°C for 5 minutes before loading. In order to detect the presence of disulphide linked protein aggregates, SDS-PAGE was performed also in reducing conditions. This was done by adding 4% (v/v) 2-mercaptoethanol to the loading buffer.
Electrophoresis was carried out at 25 mA constant current until the tracking dye Bromophenol Blue ran off the gel. The molecular weight standard proteins were the Broad Range Molecular Weight Markers (Bio-Rad Laboratories). 1.5 mm thick gels were prepared with T = 12% (SDS-PAGE in Fig. 5) or 14% (acrylamide-N, N' metilenbisacrylamide 29:1; Sigma-Aldrich) and stained with Colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich) or with the PAS (Periodic Acid-Schiff) method for glycocompounds detection (SEGREST and JACKSON, 1972).

2.4. Chitinolytic activity detection on SDS-PAGE gels

Chitinolytic activity detection was assayed according to VINCENZI and CURIONI (2005). Samples were prepared with the same reagents used for SDS-PAGE and loaded into a gel (T = 14%) containing glycol-chitin (0.01% or 0.05% w/v). After protein separation, the gels were incubated overnight at room temperature in a 50 mM sodium acetate buffer pH 5.5 containing 1% (w/v) Triton X-100 (Sigma-Aldrich). Afterwards, gels were incubated for 20 minutes in 0.5 M Tris-HCl buffer pH 8.9, containing 0.01 % (w/v) Calcofluor White MR2 (Sigma-Aldrich), followed by a wash in distilled water (for at least 1 h). Protein bands with chitinolytic activity were digitized with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.5. Protein identification by MALDI-TOF/TOF MS

The selected bands were excised from SDS-PAGE gels, dehydrated with acetonitrile for 10 min and dried in Speed Vac concentrator. Disulphide bridges were reduced with 10 mM dithiotreitol (1 h, 56°C, in the dark) and cysteines were alkylated with 55 mM iodoacetamide (1 h, room temperature, in the dark). Gel bands were repeatedly washed with 50 mM NH_4HCO_3 and acetonitrile, and dried under vacuum. In gel protein digestion was performed using sequencing grade modified trypsin (Promega, Madison, WI). 10 μL of trypsin (12.5 ng/μL in 50 mM NH_4HCO_3) were added to each band, and digestion was carried out at 37°C overnight. Peptides were extracted with 50 μL of 50% acetonitrile and 1% formic acid (3 times), dried under vacuum and dissolved in 10 μL of 0.1% formic acid. The digested sample was mixed with an equal volume of matrix solution (a-cyano-4-hydroxycinnamic acid, 5 mg/mL in 70% acetonitrile, 0.1% trifluoroacetic acid) and 1 μL was spotted on a 384-well AB OptiTOF MALDI stainless steel target plate (SHEVCHENKO *et al.*, 2006). Samples were analysed using a MALDI-TOF/TOF 4800 Analyzer (Applied Biosystems, Toronto, Canada) with 4000 Series Explorer v3.5.3 software. MS data were acquired automatically over a mass range of 900–3500 Da in the positive-ion reflector mode. In the MS spectrum, the 10 most abundant MS peaks were selected for MS/MS.
MS/MS data were searched using the Mascot search engine (Matrix Science, London, UK) against the MSDB database (3239079 sequences; 1079594700 residues; Taxonomy: Viridiplantae, 247880 sequences). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm for the precursor ion and 0.3 Da for the fragment ions and carbamidomethylcysteine as fixed modification.

3. RESULTS AND DISCUSSIONS

3.1. SDS-PAGE analysis of the grape juice proteins

Vitis vinifera L. cv. Manzoni Bianco (Riesling Renano x Pinot Bianco) was used for the grape protein extraction, as this variety shows a high protein content and gives wines generally requiring fining treatments with significant amounts of bentonite for protein stabilization (VINCENZI *et al.*, 2011).
From the free run juice, 2.6 g of grape juice macromolecular powder (crude extract, CE) was obtained. An aliquot of CE was analyzed by SDS-PAGE, showing main protein bands in the region between 20 and 30 kDa (Fig. 1a).
These bands have been previously identified as grape Pathogenesis-Related (PR) proteins including Thaumatin-Like Proteins (TLPs) and chitinases (WATERS *et al.*, 1996; MONTEIRO *et al.*, 2007; VAN SLUYTER *et al.*, 2015). High molecular weight protein bands were also evident in the 45-80 kDa range. The protein with relative molecular mass (*Mr*) of 65 kDa is likely to be the grape vacuolar invertase which is known to be one of the most abundant proteins in grape juice and wine, reaching 14% of Chardonnay wine proteins (DAMBROUCK *et al.*, 2005). The minor bands with *Mr* ranging from 45 to 60 kDa have also been identified by proteomic analysis in a Semillon grape juice as (*Vitis vinifera*) "unnamed protein product" and class IV chitinase (MARANGON *et al.*, 2009). Finally, the band of 12 kDa is likely to correspond to the Lipid Transfer Protein (LTP), whose presence has already been reported in grapes where was indicated as one of the major allergens (PASTORELLO *et al.*, 2003). Staining the gel for sugar residues confirmed the presence of glycocompounds, probably polysaccharides, which barely entered the gel (Fig. 1b) (VINCENZI *et al.*, 2012).

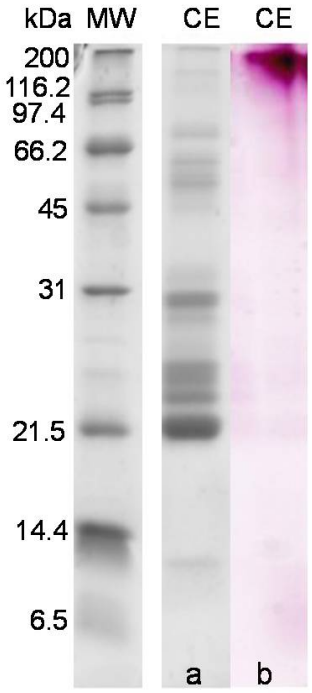

Figure 1 Non-reducing SDS-PAGE of the grape berries crude extract (CE), stained for proteins (a) and glycocompounds (b). Molecular weight standard proteins are on the left.

3.2. Protein fractionation and characterization

Starting from the CE, the grape juice macromolecules (>3.5 kDa) were initially fractionated by Anion Exchange Chromatography (AEC). Since the grape proteins have very similar MWs and different pI (MONTEIRO *et al.*, 2001), this chromatographic technique proved to be very effective at this stage. AEC has already been applied previously to those compounds (WATERS *et al.*, 1992; DORRESTEIN *et al.*, 1995; PASTORELLO *et al.*, 2003), allowing to obtain a good resolution of protein peaks.
A representative AEC chromatogram for grape juice macromolecules (≈ 50 mg) is shown in Fig. 2.
The material not retained by the column (Flow through) was very little or at least had a low UV absorption. Almost all peaks were eluted at relatively low NaCl concentrations (0.08-0.12 M), while a last peak was obtained with a high NaCl concentration (1 M), indicating that at pH 9 the eluting fractions have different charge properties.
Six separated fractions were collected (Fig. 2) and analysed by SDS-PAGE in non-reducing conditions (Fig. 3).
As expected, the Flow through did not contain any proteins. The first two peaks (1a and 1b) both displayed a band at ≈ 20 kDa. Fraction 1b contained also some minor bands around 25 kDa. All these bands probably correspond to TLPs according to literature data (WATERS *et al.*, 1996; MARANGON *et al.*, 2011; MARANGON *et al.*, 2014). Moreover, peak 1b showed a 40 kDa band which could correspond to a β-glucanase (ESTERUELAS *et al.*, 2009; SAUVAGE *et al.*, 2010). Peak 2 contained only one band that showed a *Mr* similar to that of TLPs (WATERS *et al.*, 1996; MARANGON *et al.*, 2011; MARANGON *et al.*, 2014). Peaks 3a and 3b showed several bands with *Mr*s similar to that of TLPs and a protein of ≈ 66 kDa, probably corresponding to invertase (MARCHAL *et al.*, 1996).

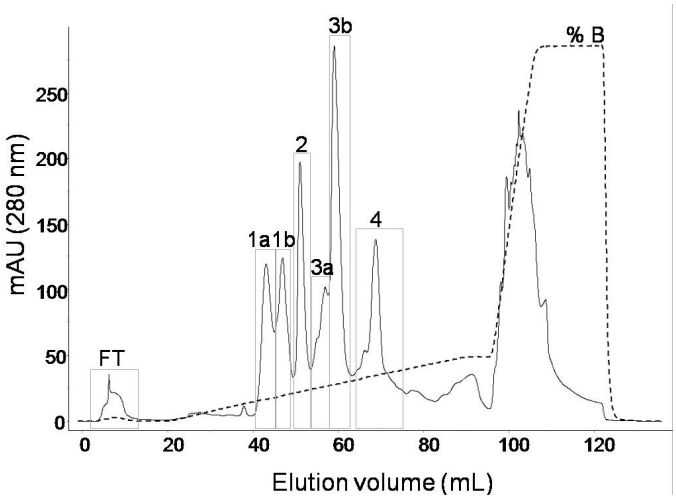

Figure 2. Anion exchange chromatogram for Manzoni Bianco crude extract (50 mg). Collected fractions are indicated by numbered boxes. The dotted line indicates the salt gradient.

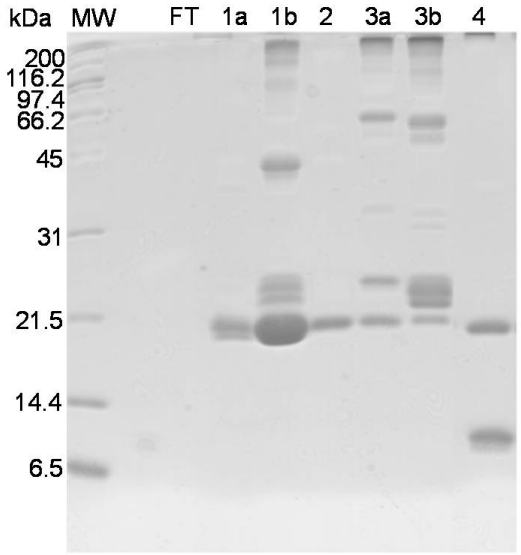

Figure 3. Non-reducing SDS-PAGE of the fractions collected from Anion Exchange Chromatography. Molecular weight standard proteins are on the left.

The presence of faint bands with MWs of ≈ 31 and ≈ 32 kDa is noteworthy, which could correspond to grape chitinases (WATERS *et al.*, 1996; MARANGON *et al.*, 2009). Peak 3b also contained a ≈ 52 kDa band, whose identity was investigated in this work. Finally, peak 4 showed another band with the same mobility of TLPs and a low MW band that could correspond to the grape LTP (PASTORELLO *et al.*, 2003) (Fig. 3). According to this chromatographic behaviour it is clear that different protein isoforms assigned to both TLPs and chitinases on the basis of their SDS-PAGE mobility show different charge properties at pH 9 being eluted from the AEC column at different NaCl concentrations (from ≈ 0.08 to ≈ 0.12 M). This is obviously related to a heterogeneity of the grape juice proteins at the amino acid level (MONTEIRO *et al.*, 2001) which is likely to affect their net

charge also at the low pH of the wine. Since charge is one of the main factors involved in protein functionality in terms of colloidal behavior (VINCENZI *et al.*, 2011), it is likely that different forms of the same protein detected by SDS-PAGE in the different AEC fractions play specific roles in the phenomena leading to haze formation in wine. Indeed the pH-dependent variation of protein charges has been indicated as one of the factors that strongly affect protein aggregation in wine (DUFRECHOU *et al.*, 2012). The chitinase-containing peaks (3a and 3b in figures 2 and 3), from 15 chromatographic separations of 50 mg of protein each, all giving the same results (not shown), were combined and freeze dried. Since this sample (from now on named "peak 3") was contaminated by other proteins (Fig. 3, lanes 3a and 3b), a further purification step involving Hydrophobic Interaction Chromatography (HIC) was used, resulting in the separation of protein fractions differing in surface hydrophobicity (VAN SLUYTER *et al.*, 2009). The protein peak 3 from AEC gave six peaks after HIC (Fig. 4).
SDS-PAGE analysis of the proteins of HIC peaks under reducing and non-reducing conditions, showed several protein bands, differing in both *Mr* and staining intensity (Fig. 5).
Also in this case, proteins with the same SDS-PAGE mobility were detected in more than one peak, indicating differences in surface hydrophobicity of components showing very similar charges (those of fractions 3a and 3b of the AEC) and apparent molecular weight (by SDS-PAGE). Hydrophobicity is also an important property affecting the interaction of a protein with other components (SIEBERT *et al.*, 1996). Therefore the propensity to form haze in wine can be different for wine protein isoforms with different hydrophobicity, as demonstrated, for example, by studying the reactivity of wine protein fractions differing for this parameter with tannins (MARANGON *et al.*, 2010).

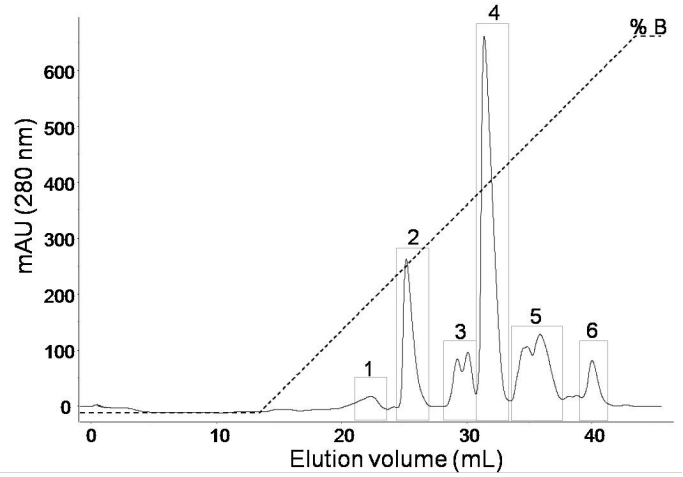

Figure 4. Hydrophobic interaction chromatogram of AEC fraction 3 (pooled fractions 3a and 3b). Collected fractions are indicated by numbered boxes. The dotted line indicates the linear gradient.

The bands of HIC fractions 3 and 4 were found to be almost pure when analysed by SDS-PAGE in reducing conditions (Fig. 5a). However, when the same samples were analysed in non-reducing conditions a variation of the electrophoretic patterns was noted.
In addition to a shift of the main bands to a slightly higher apparent molecular weight, a minor low mobility band of ≈ 52 kDa was detected when the gel was run under non-reducing conditions (fig. 5b). Similar bands of 50-52 kDa found by analysing the proteins present in the natural wine haze, and not directly in the grape juice as done here, where

identified by NanoLC-MS/MS as *Vitis vinifera* class IV chitinase (MARANGON *et al.*, 2011). However, in that study it was not clear the origin of these high MW chitinase bands, which could be artefacts produced during the extraction procedure (MARANGON *et al.*, 2011), but also the result of the protein aggregation leading to haze. In contrast, the 52 kDa bands here detected, which were obtained directly from the grape juice, are clearly due to the presence of disulphide-linked proteins, being present only when the fractions were not treated with a reducing agent (fig. 5b).

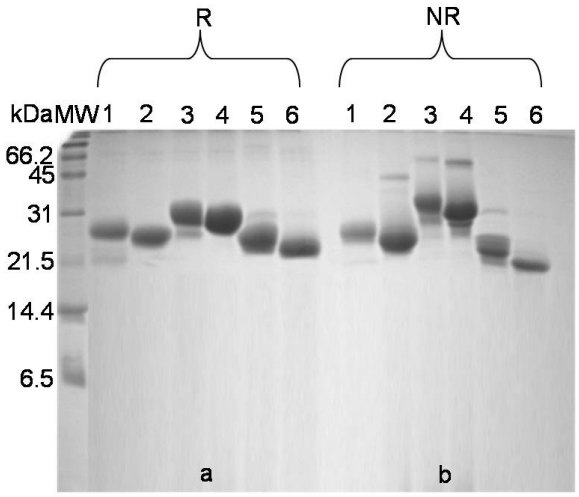

Figure 5. Reducing (a) and non-reducing (b) SDS-PAGE of the fractions (1 to 6) from hydrophobic interaction chromatography. Molecular weight standard proteins are on the left.

When tested for chitinolytic activity on gel (VINCENZI and CURIONI, 2005), peaks 3 and 4 confirmed to contain active chitinases corresponding to the bands with the MW expected for grape chitinases (CHI 1, CHI 2, CHI 3 and CHI not delayed) (in the range 30-32 kDa) but also to that of the disulphide linked components of ≈ 52 kDa (CHI dimer I and II) (Fig. 6).

In contrast the other chromatographic fraction did not show bands with well marked chitinolytic activity (not shown).

Confirming what was previously reported (VINCENZI and CURIONI, 2005), the presence of glycol chitin in the non-reducing SDS-PAGE gel caused a *Mr* decrease of the chitinase bands CHI 1, CHI 2, CHI 3 and this shift was proportional to the quantity of glycol chitin incorporated (Fig. 6). This result indicates that grape chitinases interact with the substrate during the electrophoretic migration if not reduced, likely due to the presence of the chitin-binding domain typical of the type IV chitinases (COLLINGE *et al.*, 1993). However, one minor chitinase isoform (CHI not delayed) did not show the same behaviour (Fig. 6), suggesting that the chitin-binding domain involved in the interaction with chitin was lacking in this component.

It is also interesting to note that the ≈ 52 kDa band was retarded in these conditions, showing the same behaviour of the main bands with higher *Mr*. This result and the disappearance of the ≈ 52 kDa band in reducing conditions suggest that this protein could be a dimer of chitinases linked by S-S bonds. As a matter of fact, all these bands, including that at ≈ 52 kDa, showed chitinolytic activity after staining the gels for its detection (Fig. 6).

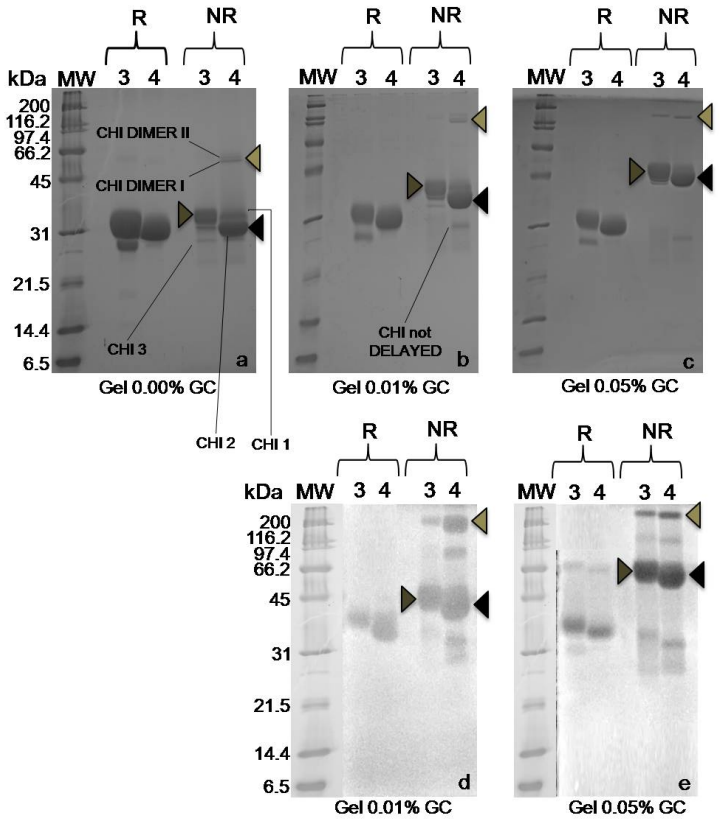

Figure 6. SDS-PAGE analysis of HIC fractions 3 and 4 under reducing (R) and non-reducing (NR) conditions. Gels contained 0 (a), 0.01 (b, d), and 0.05 (c, e) % glycol chitin (GC). Panels a-c: staining for proteins. Panel d-e: staining for chitinolytic activity. The arrowheads indicate bands retarded in the presence of glycol chitin. The bands selected for MALDI-TOF/TOF MS analysis are indicated in panels a and b. Molecular weight standard proteins are on the left.

3.3. MS protein identification

The six bands showing chitinolytic activity (CHI 1, 2 and 3, CHI dimers I and II, and CHI not delayed, Fig. 6) were excised from the SDS-PAGE gels and analysed by MALDI-TOF/TOF MS.

According to database searching using Mascot, all bands were found to belong to *Vitis vinifera* class IV chitinases, including that named "CHI not delayed". As mentioned above, these chitinases are characterised by the presence of a chitin binding domain of the hevein type in the N-terminal region (COLLINGE *et al.*, 1993), and this would be the reason for being retarded in glycol chitin containing gel (VINCENZI and CURIONI, 2005). Therefore, since the electrophoretic migration of the minor "CHI not delayed" band was unaffected by the presence of glycol chitin in the gel, it is likely that this grape chitinase has in common with the typical class IV chitinases only one part of its structure, but not the chitin binding domain.

In most of the cases the analysed proteins were assigned to two isoforms of class IV chitinases (accessions>gi|2306811|gb|AAB65776.1| and >gi|33329392|gb|AAQ10093.1|). Only for the band named 'CHI DIMER II' there was only one sequence matched (>gi|2306811|gb|AAB65776.1|), and three in the case of 'CHI 3' (Table 1).

Table 1. Selected proteins identified by MALDI-TOF/TOF MS.

Sample	Protein identification name	NCBI accession number	Sequence coverage (%)	Number of peptides matched
CHI 1	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	17%	5
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	17%	5
CHI 2	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	20%	6
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	20%	6
CHI 3	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	15%	5
	class IV endochitinase [*Vitis vinifera*]	>gil2306813lgblAAB65777l	15%	5
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	15%	5
CHI not DELAYED	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	20%	6
	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	20%	6
CHI dimer I	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	17%	5
	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	17%	5
CHI dimer II	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	18%	5

Two reasonable hypotheses can be given to explain why bands with different electrophoretic and chromatographic behaviour are recognised as chitinases corresponding to the same isoforms: i) the MS data do not provide complete coverage of any sequence and therefore a precise identification of the proteins is not possible. In addition, because of the partial lack of available grape protein sequences, there is a chance that the selected peptides do not exactly match corresponding database entries; ii) the proteins could be modified forms of the same original chitinase isoforms, affecting only the chromatographic and electrophoretic behaviour but not the catalytic activity or the capacity to bind chitin. Indeed, it has been shown that some partial modification of the chitinases could occur during juice preparation (WATERS *et al.*, 1998; MANTEAU *et al.*, 2003; DAHIYA *et al.*, 2006). Overall, the results of the MS analysis are the same of those reported for the proteins found in natural wine haze (MARANGON *et al.* 2011), confirming that grape protein components related to type IV chitinases are actually those mainly involved in haze formation in wines.

4. CONCLUSIONS

Grape chitinases are considered as one of the main protein components involved in protein haze formation in wines (FALCONER *et al.*, 2010; MARANGON *et al.*, 2011). Here we have confirmed that these proteins are present in the grape juice as different isoforms, which, although sharing common amino acid sequences related to type IV chitinases, can be distinguished on the basis of their electrophoretic and chromatographic behaviours. Moreover, chitinases are present in the grape juice also in the form of S-S-linked dimers, and also in a form apparently lacking the chitin-binding domain. Since all these characteristics can be related to differences in the functional properties of the single components, it is likely that the different chitinase isoforms found in grape juice have different impacts on their hazing potential in wines, as it has been demonstrated for TLPs (GAZZOLA *et al.*, 2012; MARANGON *et al.*, 2014). In particular, differences in charge, hydrophobicity and also molecular weight can affect the interactions of the single chitinase components when they are present in a complex colloidal system as wine, leading to different tendencies to form haze. This can be an important point to be clarified, not only to better understand the mechanisms of wine hazing, but also for practical pourposes. For example, the identification of the most unstable protein components will help to develop protein instability tests much more specific than those currently in use, thus allowing the winemaker to be more precise in applying the wine stabilisation treatments. Moreover, also these treatments can be improved by a deep knowledge of the molecular characteristics and functionality of the single wine protein components, which will allow to design stabilisation treatments tailored to specifically remove the desired proteins and not the others. For example, the discovery that wine chitinases are able to bind chitin was the rational basis for the application of chitin as a specific adsorbent to remove these unstable proteins from wine (VINCENZI *et al.*, 2005). In conclusion, the biochemical and molecular characterisation of the different protein components of grape, as done here, can be of great help to develop "precision" winemaking techniques aimed to improve wine quality.

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