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Characterization of chitinase isoforms from grape juice

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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 (WATERS *et al.*, 2005).
Chitinases are the most active protein components in causing wine turbidity (MARANGON *et al.*, 2011). These proteins derive from grapes and are present in different isoforms (MARANGON *et al.*, 2011).
Chitinases have been successfully purified by others, despite their low concentration and strong interaction with endogenous polyphenols and other non-protein compounds. In spite of the interest of these type of wine components, in-depth knowledge surrounding them is still incomplete.

Experimental

Protein extraction. Fifteen kg of *Vitis vinifera L.* cv. Manzoni Bianco berries were manually crushed and treated with 7.5 g/kg PVPP, 0.15 g/kg ascorbic acid and 0.375 g/kg potassium metabisulfite. The grape juice was centrifuged, dialysed (3500 Da cut-off), concentrated (3000 Da cut-off) and freeze-dried. **Protein separation by chromatography**. A two-step chromatographic separation was performed using an ÄKTA purifier FPLC equipped with an UV detector. The first chromatographic step was Anion Exchange Chromatography (AEC) onto a Tricorn MonoQ 5/50 colum (loading in 20 mM Tris-HCl pH 9.0 and elution with 20 mM Tris-HCl, 1 M NaCl, pH 9.0) (VINCENZI et al., 2011) followed by Hydrophobic Interaction Chromatography (HIC) on a HIC BioSuite Phenyl column (loading in 20 mM tartaric acid pH 3.5 containing 1.25 M ammonium sulfate, elution with same buffer without ammonium sulphate). **SDS-PAGE.** SDS-PAGE analyses were performed according to Laemmli (1970) and stained with Colloidal Coomassie Brilliant Blue G-250 or with the PAS (Periodic Acid-Schiff) method for glycocompounds detection. **Chitinolytic activity detection.** The procedure was performed as reported in VINCENZI and CURIONI, (2005), incorporating in the separation gel 0.01% or 0.05% w/v of glycol chitin. **Protein identification by MALDI-TOF/TOF MS.** The selected bands were excised, reduced and alkylated, then digested with trypsin. Peptides were extracted, 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. Samples were analysed using a MALDI-TOF/TOF 4800 Analyzer. 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 against the MSDB database (3239079 sequences; 1079594700 residues; Taxonomy: Viridiplantae, 247880 sequences).

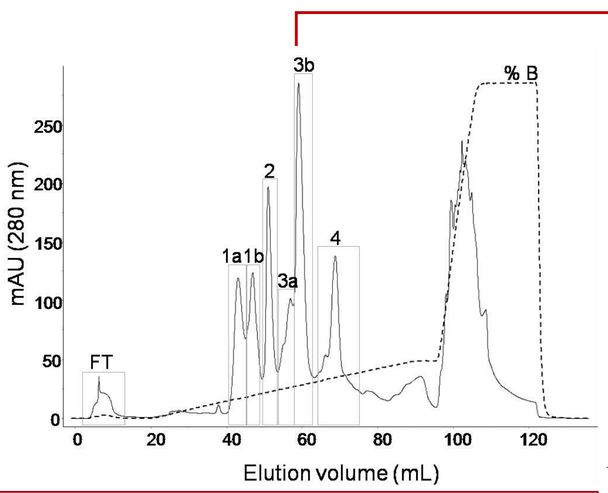


Figure 1 - Anion exchange chromatogram for Manzoni Bianco crude extract (50 mg). Collected fractions are indicated by numbered boxes. The dotted line indicates the salt gradient.

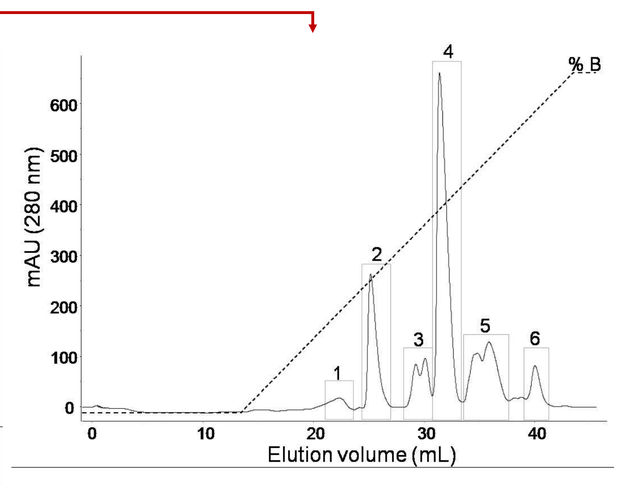


Figure 2 - Hydrophobic interaction chromatogram of AEC fraction 3 (pooled fractions 3a and 3b). Collected fractions are indicated by numbered boxes. The dotted line indicates the linear gradient.

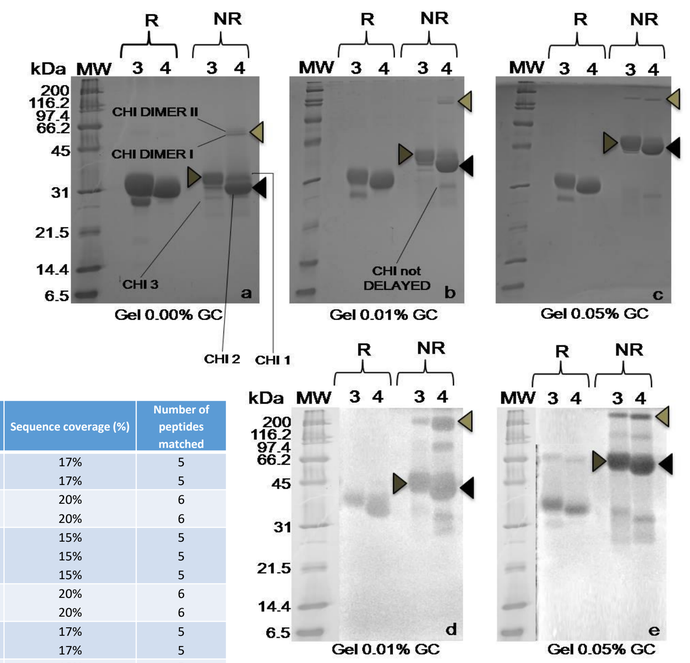
Protein purification

From 15 kg of grapes, 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 (not showed), previously identified as Thaumatin-Like Proteins (TLPs) and chitinases. These macromolecules were initially fractionated by AEC, allowing to obtain six separated fractions (Fig. 1). In non-reducing conditions, peaks 3a and 3b showed several bands, including MWs of ≈ 31 and ≈ 32 kDa, which could correspond to grape chitinases (MARANGON *et al.*, 2009). The chitinase-containing peaks (3a and 3b), were further purified by Hydrophobic Interaction Chromatography (HIC), obtaining six peaks (Fig. 2).

Chitinase identification

Peaks 3 and 4 after HIC were those containing chitinases and were further characterized by SDS-PAGE and for chitinolytic activity.

Figure 3 - 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.



Sample	Protein identification name	NCBI accession number	Sequence coverage (%)	Number of peptides matched
CHI 1	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	17%	5
	class IV chitinase [Vitis vinifera]	>gi|33329392|gb|AAQ10093.1|	17%	5
CHI 2	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	20%	6
	class IV chitinase [Vitis vinifera]	>gi|33329392|gb|AAQ10093.1|	20%	6
CHI 3	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	15%	5
	class IV endochitinase [Vitis vinifera]	>gi|2306813|gb|AAB65777|	15%	5
	class IV chitinase [Vitis vinifera]	>gi|33329392|gb|AAQ10093.1|	15%	5
CHI not DELAYED	class IV chitinase [Vitis vinifera]	>gi|33329392|gb|AAQ10093.1|	20%	6
	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	20%	6
CHI dimer I	class IV chitinase [Vitis vinifera]	>gi|33329392|gb|AAQ10093.1|	17%	5
	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	17%	5
CHI dimer II	class IV endochitinase [Vitis vinifera]	>gi|2306811|gb|AAB65776.1|	18%	5

When grape chitinases were analysed in non-reducing conditions a minor band of ≈ 52 kDa was detected, suggesting the presence of a disulphide-linked protein (Fig. 3). When tested for chitinolytic activity on gel (VINCENZI and CURIONI, 2005), peaks 3 and 4 confirmed to contain active chitinases at the MW expected for grape chitinases (CHI 1, CHI 2, CHI 3 and CHI not delayed) (30-32 kDa) but also at ≈ 52 kDa (CHI dimer I and II) (Fig. 3). The presence of glycol chitin caused a mobility shift of the chitinase bands CHI 1, CHI 2, CHI 3 (Fig. 3), indicating an interaction with the substrate during the electrophoretic migration, likely due to the presence of the chitin-binding domain typical of the type IV chitinases (VINCENZI and CURIONI, 2005). Only one minor chitinase isoform (CHI not delayed) did not show the same behaviour. Also the ≈ 52 kDa band was retarded, and this result suggest that this protein could be a dimer of chitinases linked by S-S bonds. The six bands showing chitinolytic activity (CHI 1, 2 and 3, CHI dimers I and II, and CHI not delayed, Fig. 3) 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. In most of the cases the analysed proteins were assigned to two isoforms of class IV chitinases. Two hypotheses can explain why bands with different behaviour are recognised as chitinases corresponding to the same isoforms: i) the MS data do not provide complete coverage; ii) the proteins could be modified forms of the same original chitinase isoforms.

Conclusions

Grape chitinases are considered as one of the main protein components involved in protein haze formation in wines. Here we have confirmed that these proteins are present in the grape juice as different isoforms, including a S-S-linked dimer. Since all these characteristics can be related to different functional properties, it is likely that the different isoforms have different hazing potential in wines. This can be useful also for practical purposes. For example, the identification of the most unstable protein components will help to design stabilisation treatments tailored to specifically remove the desired proteins. 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.

REFERENCES
Waters E.J., Alexander G., Muhlack R., Pocock K.F., Colby C., O'Neill B.K., Hoj P.B. and Jones P. 2005. Aust. J. Grape Wine R. 11 (2): 215.
Marangon M., Van Sluyter S.C., Neilson K.A., Chan C., Haynes P.A., Waters E.J. and Falconer R.J. 2011. J. Agric. Food Chem. 59 (2): 733.
Vincenzi S. and Curioni, A. 2005. Electrophoresis. 26 (1): 60.
Marangon M., Van Sluyter S.C., Haynes P.A. and Waters E.J. 2009. J. Agric. Food Chem. 57 (10): 4415.