



Purification and structural characterisation of lipid transfer protein from red wine and grapes

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ABSTRACT

Lipid transfer proteins (LTP) play a major role in plant defence and are of particular interest due to their known ability to cause allergic reactions. These proteins are expressed in grapes and also remain detectable after vinification, especially in red wine. However, it remains unknown whether the protein undergoes any changes during the vinification process. Here, we present a purification method for LTPs from Dornfelder grapes and wine. By liquid-chromatography–mass spectrometry (LC–MS/MS) we identified LTPs from two different species (*Vitis vinifera* and *Vitis aestivalis*). Additionally, the purified LTPs were characterised using spectrometric methods, confirming their high purity and structural stability during vinification. We conclude that LTPs are resistant to the alcohol content (13.5 vol%), acidic milieu of wine and other ingredients present during the vinification process, indicating that the allergenic potential of grape LTP is not diminished by the vinification process.

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1. Introduction

Lipid transfer proteins (LTP) are widely expressed throughout the plant kingdom and are involved in biotic and abiotic stress reactions (Kader, 1996). LTPs are alkaline proteins of about 9–12 kDa which are characterised by a high theoretical isoelectric point (IEP) of about 9 (calculated for grape LTP by using ProtParam, Gasteiger et al., 2005). A similar IEP was also described for maize LTP by isoelectric focussing (Pastorello et al., 2000). The amino acid sequences of various plant LTPs display a very high degree of sequence identity, indicating a very similar structure (Kader, 1996). Crystal structures of different LTPs were resolved, showing that they are organised mainly as an α -helices bundle (Charvolin, Douliez, Marion, Cohen-Addad, & Pebay-Peyroula, 1999; Cheng, Cheng, Peng, Lyu, & Sun, 2004; Han et al., 2001; Heinemann, Andersen, Nielsen, Bech, & Poulsen, 1996; Pasquato et al., 2006). Eight conserved cysteines form four disulphide bridges, resulting in a very compact and stable structure (Guerbette, Grosbois, Jolliot-Croquin, Kader, & Zachowski, 1999; Salcedo, Sanchez-Monge,

Diaz-Perales, Garcia-Casado, & Barber, 2004). In general, LTPs show resistance to heat treatment, acidic conditions and proteolysis, enabling them to survive the acidic and proteolytic conditions in the gastrointestinal tract (Vassilopoulou et al., 2006). These are the best preconditions for acting as an allergen by oral sensitisation (Salcedo et al., 2004).

Several lipid transfer proteins have already been identified as plant allergens (Salcedo et al., 2004). Allergic reactions against LTP are shown for fruits of the *Rosaceae* family, e.g. peach, apricot and cherry, in the Mediterranean area (Breiteneder & Mills, 2005). Additionally, LTPs have been described as allergens in pollen and some other fruits, including grapes (Breiteneder & Mills, 2005; Pastorello et al., 2003; Vassilopoulou et al., 2007). For example, Schäd et al. (2005) showed that a LTP was responsible for severe allergic reactions after consumption of fresh grapes, raisins and wine in a German patient. Thus, allergic reactions to LTP from grape and wine seem to be of importance in regions other than the Mediterranean area (Sanchez-Monge, Lombardero, Garcia-Selles, Barber, & Salcedo, 1999). Up to now, the LTP vit v1 is the only protein of *Vitis vinifera* listed in the allergen nomenclature of the International Union of Immunological Societies (www.allergen.org).

In previous experiments, we showed that LTP is present in higher amounts in Dornfelder red than in white wines, rosé wines and other varieties of red wines (Wigand, Tenzer, Schild, & Decker, 2009). Additionally, LTPs, or hydrolysis products thereof, were detected in various white wines, such as Chardonnay (Marangon, Van Sluyter, Haynes, & Waters, 2009; Okuda, Fukui, Takayanagi, & Yokotsuka, 2006). However, it remains unclear, whether the

Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; ESI, electrospray ionisation; FPLC, fast protein liquid chromatography; IEP, isoelectric point; LC–MS/MS, liquid-chromatography–mass spectrometry; LTP, lipid transfer protein; NaCl, sodium chloride; NaN₃, sodium azide; PDB, protein data base; PVP, polyvinylpyrrolidone; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TOF, time-of-flight; UPLC, ultra performance liquid chromatography.

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structure of LTP is affected during and after the vinification process by the acidic, proteolytic and alcoholic environment of the wine, affecting the allergenic potential.

In the present study we characterised purified LTPs from Dornfelder red wine and grapes. LTP purification, close to homogeneity, is vitally important as a basic requirement for further characterisation concerning LTP stability during the wine making process. Due to their high IEP and intrinsic stability, LTP significantly differ from other wine proteins. Grape LTP (vit v1) is listed in the allergen database, underlining its medical importance.

Here we investigate the influence of vinification on structural changes which potentially affect the allergenic potential of grape LTPs.

2. Materials and methods

2.1. Materials

All chemicals were of high purity and purchased from commercial companies. Acrylamide, ammonium persulfate, 2-mercaptoethanol, SDS, Tris, glycine, di-sodium-hydrogen-phosphate, Coomassie brilliant blue G-250, sodium chloride, methanol, formic acid in acetonitrile (LC-MS grade), glacial acetic acid, citric acid and sodium citrate were from Roth (Karlsruhe, Germany), protein molecular weight standard was from Bio-Rad (Munich, Germany), sodium-di-hydrogen-phosphate from Merck (Darmstadt, Germany) and bromophenol blue and polyvinylpyrrolidone Polyclar^{AT} from Serva (Heidelberg, Germany).

2.2. Wine and concentration of proteins

The Dornfelder red wine 2009 (13.5 vol% alcohol, total acid content: 4.8 g/l, sugar content: 0.1 g/l, total sulphurous acid: 74 mg/l, pH value of 3.8) was obtained from a local winemaker in Mainz (Rhinehessen, Germany).

To remove low-molecular weight components, the wine was initially dialysed for at least 5 days against water at 4 °C with a volume/volume ratio of 1:20 in each step, using regenerated cellulose dialysis membranes of 3.5 kDa molecular mass cut off (Spectra/Por[®] from Roth, Karlsruhe, Germany). During dialysis, water was exchanged twice a day. The samples were spin-frozen at –30 °C to fully lyophilise to dryness for at least 24 h (Alpha 1-4 LSC from Christ, Osterode, Germany). About 300 mg of lyophilised wine powder were obtained from 250 ml of wine.

2.3. PVP-precipitation

To remove protein-interacting substances such as polyphenols, which may interfere with subsequent purification steps, the red wine concentrate was treated with polyvinylpyrrolidone (PVP). Three hundred milligrams of lyophilised wine powder were dissolved in 30 ml of 0.01 M phosphate buffer pH 7.0. 1.5 g (5%) PVP was added, stirred overnight and removed by centrifugation for 10 min at 5.000 rpm at 4 °C. The procedure was repeated, once, with the supernatant, using 10% PVP. Remaining PVP was removed by filtration through a 0.22 µm polyethersulphone-filter (Rotilabo sterile filter, Roth, Karlsruhe, Germany) before applying the sample to chromatography.

2.4. Grapes and concentration of proteins

The Dornfelder grapes were harvested, in 2010, in Harxheim (Rhinehessen, Germany). Grapes from this vineyard were used for Dornfelder winemaking. Immediately after vintage, grapes were frozen at –20 °C. Before use, 725 g of grapes were crushed

in a mixer, adding 362.5 ml of 0.1 M phosphate buffer pH 7.0 containing 20 mM EDTA, 3 mM NaN₃ and 0.5% Tween-20 and protease inhibitor cocktail for plant cell (Sigma Aldrich, Steinheim, Germany). Afterwards, the grape homogenate was filtered through a clean cloth strainer (washed before with distilled water, obtained by a local drapery shop) and centrifuged for 10 min at 5.000 rpm at 4 °C to obtain the supernatant. PVP-treatment of grape juice was conducted as described for wine.

2.5. Cation-exchange chromatography on FPLC

To purify lipid transfer protein, fast protein liquid chromatography (FPLC) was performed on a Bio-Rad HR-apparatus, using cation-exchange chromatography (UNO S-6, Bio-Rad, Munich). The S-6 column was equilibrated in buffer A (0.01 M sodium phosphate buffer, pH 7.0) for 6 min, followed by buffer B (0.01 M sodium phosphate buffer, pH 7.0, containing 1 M NaCl) for 6 min, completing by buffer A for 6 min at 2 ml/min. Previously, both buffers were filtered through a 0.22 µm polyethersulphone-filter and degassed. The wine and grape samples were used directly after PVP-treatment. Per run, 4 ml of sterile filtered sample were injected into the S-6 column after 6 min of initial equilibration at a flow rate of 2 ml/min. Proteins were eluted by a linear gradient of buffer B, from 0% to 50% (corresponding to 0.5 M NaCl) for 15 min, with a flow rate of 2 ml/min, followed by a gradient step to 100% (corresponding to 1 M NaCl). Elution was monitored by UV-absorbance at 214 nm at 20 °C.

2.6. SDS–PAGE

SDS–PAGE of fractions obtained by cation-exchange chromatography was performed, using the method of Laemmli (1970). For the SDS–Page, homemade 15% polyacrylamide gels (pH 8.8) with 3% stacking gels (pH 6.8) were used.

Due to the low protein content, the samples were concentrated tenfold, using regenerated cellulose-centrifugal filters (Amicon[®] from Millipore, Eschborn, Germany), with a 3 kDa molecular weight cut-off. Before SDS–PAGE, samples were mixed, in equal amounts, with SDS sample buffer (25% (v/v) 0.5 M Tris (pH 6.8)/20% (v/v) glycerol/10% (v/v) 2-mercaptoethanol/4% (w/v) SDS with a small amount of bromophenol blue) and denaturated for 10 min at 95 °C. As protein standard, the Precision Plus Protein[™] Standard from Bio-Rad was used. A voltage of 100 V was applied to the gels for 90 min at room temperature and the proteins were visualised by staining with Coomassie brilliant blue R-250 (Sigma Aldrich, Steinheim, Germany; staining solution: 0.2% Coomassie brilliant blue R-250, 50% methanol, 40% water, 10% glacial acetic acid) for at least 2 h and destained (40% methanol, 50% water, 10% glacial acetic acid) for 30 min.

2.7. Fluorescence spectrometry

Emission spectra were monitored in 0.01 M sodium phosphate buffer, pH 7.0, with a Hitachi F-4500 fluorescence spectrophotometer (Uwe Binniger Analytik, Schwäbisch-Gemünd, Germany) between 290 and 410 nm. For this, excitations of 280 and 295 nm were used. Excitation spectra between 250 and 300 nm were monitored for the following emission wavelength of 310, 320 and 350 nm at 20 °C.

2.8. Circular dichroism spectrometry

For secondary structure analysis of the purified LTP, spectra were monitored in 0.01 M sodium phosphate buffer, pH 7.0, with a J-810 spectropolarimeter spectrometer (Jasco GmbH Germany,

Paris, France). The spectra were monitored at 22 °C from 195 to 260 nm as an average of five scans after baseline-subtraction.

2.9. In-gel digest and mass spectrometry

SDS–PAGE gels were stained with Coomassie blue G-250, destained for 30 min and the relevant protein bands excised and sliced into small pieces. After destaining, gel slices were reduced with 2 mM DTT at 55 °C and alkylated with 15 mM iodoacetamide at room temperature in the dark for 1 h each. After washing and drying with acetonitrile, trypsin digests were done at 37 °C overnight (1 µg of trypsin per gel slice). The resulting peptides were transferred into an autosampler vial for peptide analysis via LCMS.

2.10. UPLC configuration

Capillary liquid chromatography of tryptic peptides was performed with a Waters NanoAcquity UPLC system equipped with a 75 µm × 150 mm BEH C18 reversed phase column and a 2.6 µl PEEKIL-sample loop (SGE, Darmstadt, Germany). The aqueous mobile phase (mobile phase A) was H₂O (LC–MS Grade, Roth, Freiburg, Germany) with 0.1% formic acid. The organic mobile phase (mobile phase B) was 0.1% formic acid in acetonitrile (LC–MS grade, Roth). Samples (2.6 µl injection) were loaded onto the column in direct injection mode with 3% mobile phase B for 15 min at 400 nl/min, followed by an additional 10 min wash (3% B) for 10 min at 300 nl/min. Peptides were eluted from the column with a gradient from 3% to 35% mobile phase B over 90 min at 300 nl/min, followed by a 20 min rinse of 80% mobile phase B. The column was immediately re-equilibrated, under initial conditions (3% mobile phase B), for 20 min. [Glu¹]fibrinopeptide was used as lockmass at 300 fmol/µl. Lockmass solution was delivered from the auxiliary pump of the NanoAcquity system, at 400 nl/min, to the reference sprayer of the NanoLockSpray™ source. Samples were analysed in triplicate.

2.11. Mass spectrometer configuration

Mass spectrometry analysis of tryptic peptides was performed using a Waters Q-TOF Premier API system, operated in V-mode with typical resolving power of $R = 10,000$. All analyses were performed with positive mode ESI, using a NanoLockSpray source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a [Glu¹]fibrinopeptide solution (300 fmol/µl), delivered through the reference sprayer of the NanoLockSpray source. For fragment identification, the instrument was run in data-directed acquisition mode, selecting the three most intense peaks for MS–MS fragmentation analysis. Fragmentation of the parent ion was achieved by collision with argon atoms. Collision energy was varied from 15 to 35 eV, dependent on precursor ion mass and charge. The integration time for the TOF analyzer was 1 s, with an interscan delay of 0.1 s.

2.12. Data processing and protein identification

The liquid chromatography tandem MS (LCMSMS) data were processed and searched by using PROTEINLYNX GLOBAL SERVER, Ver. 2.3. (Waters). Protein identifications were assigned by searching a database containing all known protein sequences from *V. vinifera*, *Saccharomyces cerevisiae* and known possible contaminants (trypsin, human keratins) with the precursor and fragmentation data afforded by the LCMSMS acquisition method. The mass error tolerance values were typically <5 ppm. Peptide identifications were restricted to tryptic peptides with no more than one missed cleavage and cysteine carbamidomethylation. For identification of homologous peptides, an Automod-Query was used, allowing up to two amino acid exchanges per peptide. All identi-

fied peptide sequences were verified by manual interpretation of the fragment spectra.

3. Results and discussion

3.1. LTP purification and identification

Since LTPs are characterised by their high IEP (calculated by using ProtParam, Gasteiger et al., 2005) and therefore carry a positive charge at neutral pH, we selected cation-exchange chromatography for purifying lipid transfer proteins from Dornfelder red wine and grapes. Prior to cation-exchange chromatography Dornfelder red wine and grape proteins were isolated and concentrated, as described in the Materials and methods section. After PVP-precipitation, for removing the main part of interacting compounds such as polyphenols, the protein solution was directly applied to chromatographic separation.

The resulting elution profiles of protein mixtures from wine and grapes are shown in Fig. 1A. The absorbance at 214 nm was recorded to facilitate the detection of small protein concentrations. As most proteins have a IEP < 7, they carry a negative charge at neutral pH. Therefore, they do not interact with the cation-exchange chromatography column at pH 7.0 and are found in the flow-through fractions (W1, G1, and G2 in Fig. 1A). For the elution of wine proteins, we applied a salt gradient of 0–25% (corresponding to 0.25 M NaCl) and collected six peak fractions (W2–W7) in the elution range of 19–28 min (Fig. 1A). Most peak profiles contain shoulders, indicating that co-elution of other proteins is occurring. In order to analyse separation efficiency, several elution fractions were analysed by the SDS–PAGE. One strong band (about 11 kDa) was detectable in all fractions and an additional weak band with a lower apparent molecular weight (Fig. 1B). Elution profiles of grape proteins were similar, except that only three peaks (G3–G5) were observed in a similar elution range (Fig. 1A).

The flow-through fraction contains two separate peaks (G1 and G2). Both peaks contain an intensive protein band with a molecular mass of 27 kDa, which was identified as class IV endochitinases from *V. vinifera*. Additionally, we identified thaumatin-like proteins (Fig. 1B; Suppl. Table 1) by mass spectrometry in fraction G1.

Four peaks were obvious and detected in the elution profile of grape proteins (G3–G6) at a wavelength of 214 nm.

Analysis by SDS–PAGE revealed a protein band in the smallest peak, G4, when stained by Coomassie brilliant blue. However, no protein bands were detectable using Coomassie staining in the other peak fractions, even though they displayed a higher absorbance at 214 nm. Therefore these peaks may result from eluting polyphenols or polysaccharides which also absorb at 214 nm. The same result was observed after silver staining, as a more sensitive method.

Analysis of wine sample eluates revealed several peaks as well. Most of them (W3–W6) contain a protein band with very similar molecular masses (11 kDa) but in increasing concentrations (Fig. 1B). As for grapes, these bands appear as double bands with one main band and a minor smeared one below it. The front peak, W1, contains different proteins with molecular masses ranging from 13 to 150 kDa. The most intense bands range from 22 to 30 kDa. These contain different thaumatin- and osmotin-like proteins and chitinases from *V. vinifera* (Suppl. Table 1). Peak W2 contains small amounts of proteins with molecular masses of about 37–45 kDa, which were both identified as polygalacturonase from *S. cerevisiae*. Peaks W7 and W8 do not contain any protein. As described for grape, they most likely contain polyphenols or polysaccharides.

For further analysis, we focussed on the elution peaks W3–W6 and G4, containing proteins with a molecular mass of 11 kDa. Using mass spectrometry, the protein sample W6 was identified

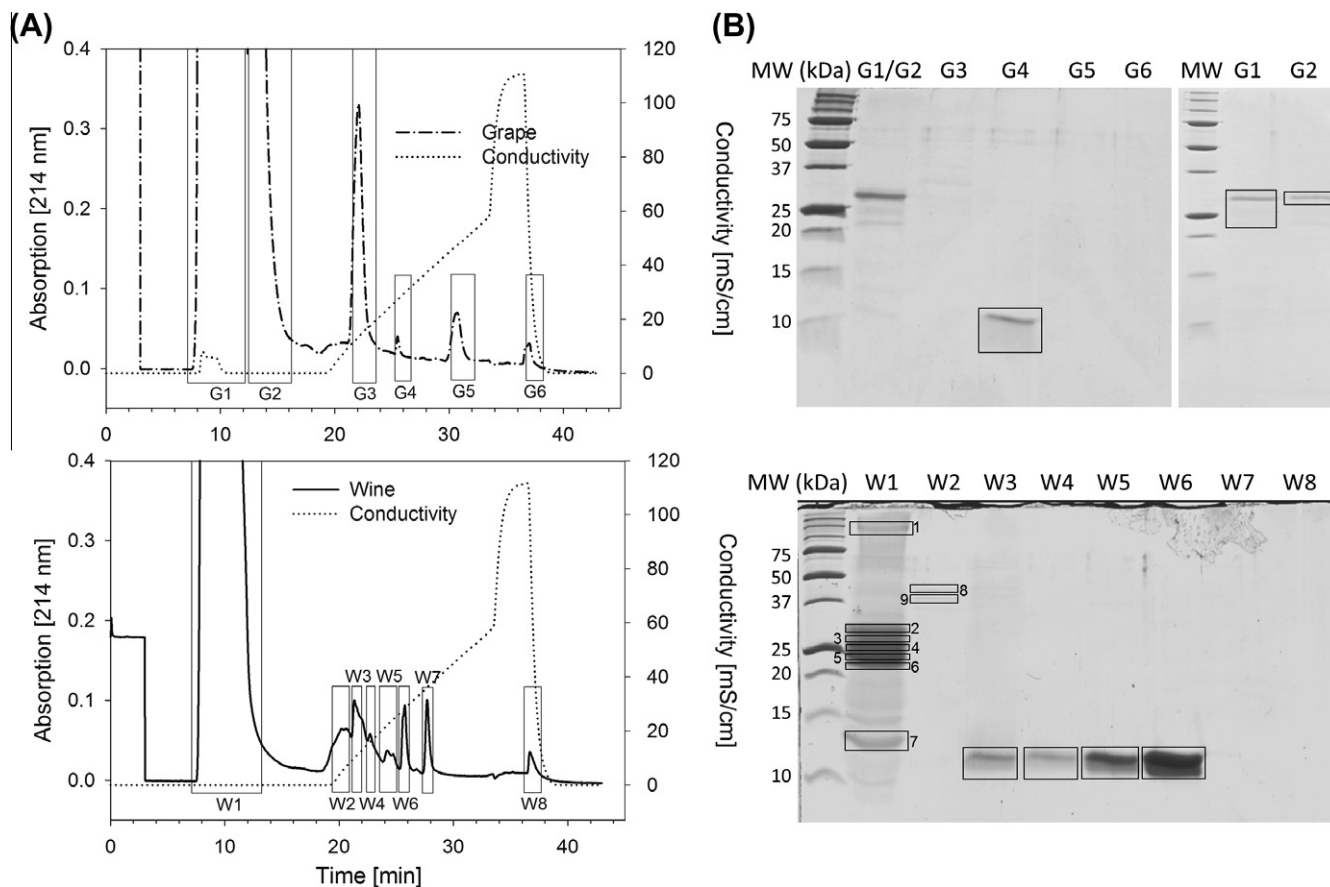


Fig. 1. Purification and detection of LTP. (A) Cation-exchange chromatogram of Dornfelder red wine and grapes. 4 ml of sample were applied in phosphate buffer; a linear 1 M NaCl gradient, up to 50%, followed by a step to 100% NaCl. The squares indicate the collected and subsequently concentrated fractions; (B) Coomassie-stained SDS-PAGE (15%) of the purified and concentrated protein fractions from wine and grape samples. The eluted samples were concentrated by a factor of 10 before applying to SDS PAGE. The squares around the bands indicate the excised sample for the LC-MS/MS analysis. Due to the low protein concentration and the absence of tryptophan in LTP, the absorption was monitored at 214 nm, observing peptide bonds.

with a coverage of 77.2% to the LTP isoform 4 from *V. vinifera* (GenBank ID: AAO33394) and with a coverage of 34.8% to the LTP from *Vitis aestivalis* (GenBank ID: AAQ96338). These LTPs were also identified in the other elution peaks, W3–W5 and G4 (Fig. 2).

The analysed peptides differ in one position (S116T) at the C-terminal (Fig. 2). These data reveal that the detected peptides from wine (W3–W6) and grapes (G4) are derived from LTPs from two different species, *V. vinifera* and *V. aestivalis* (Suppl. Table 2). The origin of the LTP from *V. aestivalis* is not known, but could also be explained as a so-far-undescribed isoform of *V. vinifera* LTP with a single amino acid exchange at position 116 (S116T).

The high purity of the two LTPs can be confirmed by fluorescence spectroscopy. In contrast to most proteins, e.g. thaumatin-like-proteins, the LTP amino acid sequences contain no tryptophan. This is also supported by Fig. 3, displaying the excitation and emission spectra of protein fraction W6. The maximum wavelength of the excitation spectra is recorded at about 278 nm and the maximum intensity of the emission spectrum is found at a wavelength of 306 nm, while almost no fluorescence emission is detectable around 350 nm, which is specific for tryptophan. Taken together, the fluorescence spectra indicate presence of tyrosine residues and complete absence of tryptophan as expected for highly purified LTPs.

3.2. Structural characterisation and stability

Based on the sequence of the wine LTP isoform 4, a homology model was reconstructed, using Swiss Model (Arnold, Bordoli,

Kopp, & Schwede, 2006; Kiefer, Arnold, Künzli, Bordoli, & Schwede, 2009; Peitsch 1995) (Fig. 4). The crystal structure of LTP from maize (*Zea mays*) (PDB ID: 1M2M_A) was used as a template due to the high degree of sequence identity of 63% for the modelled residue of the sequence range from 29 to 118 (Han et al., 2001). Here, amino acids 1–27 were not considered when computing overall sequence coverage since this is the signal peptide being predicted using SignalP 3.0 Server with a probability of 99.9% (Bendtsen, Nielsen, von Heijne, & Søren Brunak, 2004; Nielsen, Engelbrecht, Brunak, & von Heijne, 1997; Nielsen & Krogh, 1998) (Fig. 5), which is strongly supported by mass spectrometric peptide identification data, as no peptides were detectable from this region by mass spectrometry.

The four disulfide-bridges are conserved between species and may be important to stabilise the structure of the small LTP.

High sequence identity of LTP from *Vitis* species is also observed with other LTPs known as food allergens, e.g. peach, apricot, cherry and wheat (Suppl. Fig. 1). This indicates similar structures and therefore similar properties concerning the triggering of allergic reactions. However, to what extent LTP from wine can act as an allergen remains to be investigated in clinical trials.

To investigate whether the secondary structure of LTP is affected by the vinification process, the purified LTP, W6, from wine and G4 from grapes, were compared using circular dichroism spectrometry (CD). Both spectra show typical alpha helical curves characterised by two small minima at 208 and 222 nm and an intersection of the baseline at 200 nm (Fig. 6). This observation is in agreement with CD spectra of LTP from peach, cherry, hazelnut,

	Mapping of the detected peptides	AAO33394 Coverage	AAQ96338 Coverage
G4	* 20 * 40 * 60 AAO33394 : MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLRGGAVPAGCCSGIK : 60 AAQ96338 : MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLRGGAVPPACCSGIK : 60 MGSSGAVKLACVMVICMV AAPA VEA TCGQVASALSPCISYLRGGAVP CCSGIK * 80 * 100 * AAO33394 : SLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCSKVT : 119 AAQ96338 : SLNSSAKTTADRQAACKCLKNFSSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCSKVT : 119 SLNS AKTT DRQAACKCLK FSS VSGIN LASGLPGKCGVSVPYKISPSTDC KVT	62.0%	30.4%
W3	* 20 * 40 * 60 AAO33394 : MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLRGGAVPAGCCSGIK : 60 AAQ96338 : MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLRGGAVPPACCSGIK : 60 MGSSGAVKLACVMVICMV AAPA VEA TCGQVASALSPCISYLRGGAVP CCSGIK * 80 * 100 * AAO33394 : SLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCSKVT : 119 AAQ96338 : SLNSSAKTTADRQAACKCLKNFSSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCSKVT : 119 SLNS AKTT DRQAACKCLK FSS VSGIN LASGLPGKCGVSVPYKISPSTDC KVT	63.0%	18.5%
W4	* 20 * 40 * 60 AAO33394 : MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLRGGAVPAGCCSGIK : 60 AAQ96338 : MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLRGGAVPPACCSGIK : 60 MGSSGAVKLACVMVICMV AAPA VEA TCGQVASALSPCISYLRGGAVP CCSGIK * 80 * 100 * AAO33394 : SLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCSKVT : 119 AAQ96338 : SLNSSAKTTADRQAACKCLKNFSSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCSKVT : 119 SLNS AKTT DRQAACKCLK FSS VSGIN LASGLPGKCGVSVPYKISPSTDC KVT	54.4%	9.8%
W5	* 20 * 40 * 60 AAO33394 : MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLRGGAVPAGCCSGIK : 60 AAQ96338 : MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLRGGAVPPACCSGIK : 60 MGSSGAVKLACVMVICMV AAPA VEA TCGQVASALSPCISYLRGGAVP CCSGIK * 80 * 100 * AAO33394 : SLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCSKVT : 119 AAQ96338 : SLNSSAKTTADRQAACKCLKNFSSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCSKVT : 119 SLNS AKTT DRQAACKCLK FSS VSGIN LASGLPGKCGVSVPYKISPSTDC KVT	70.7%	34.8%
W6	* 20 * 40 * 60 AAO33394 : MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLRGGAVPAGCCSGIK : 60 AAQ96338 : MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLRGGAVPPACCSGIK : 60 MGSSGAVKLACVMVICMV AAPA VEA TCGQVASALSPCISYLRGGAVP CCSGIK * 80 * 100 * AAO33394 : SLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCSKVT : 119 AAQ96338 : SLNSSAKTTADRQAACKCLKNFSSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCSKVT : 119 SLNS AKTT DRQAACKCLK FSS VSGIN LASGLPGKCGVSVPYKISPSTDC KVT	77.2%	34.8%

Fig. 2. Mapping of the detected mass spectrometry peptides with the LTP sequence of isoform 4 from *Vitis vinifera* (GenBank ID: AAO33394) and the LTP sequence from LTP form *Vitis aestivalis* (GenBank ID: AAQ96338). The detected peptide regions are highlighted in grey. The identical amino acids of these sequences are shown below the alignment in the third line. Protein sequence coverages were calculated, excluding the signal peptide.

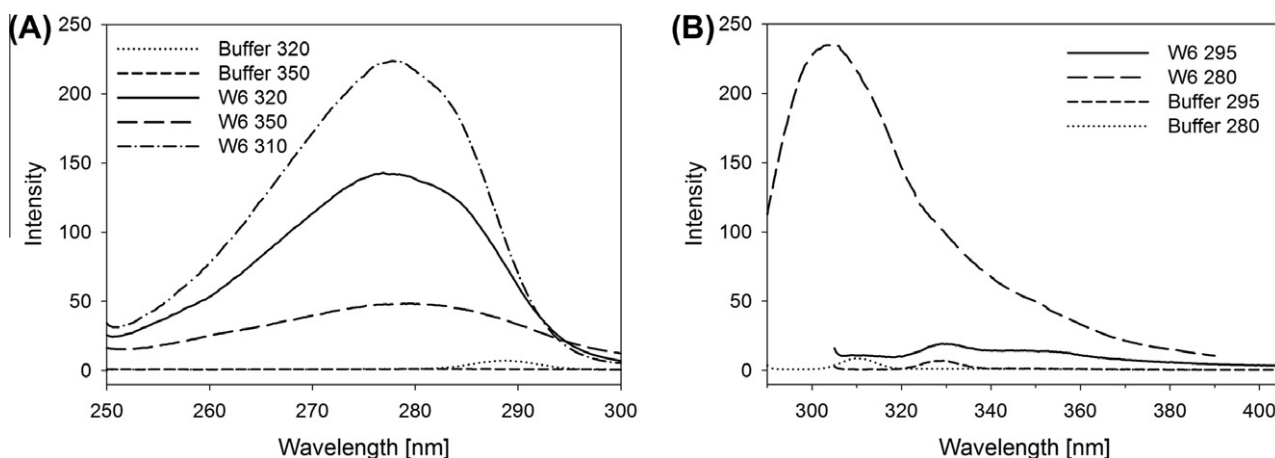


Fig. 3. Fluorescence excitation (A) and emission (B) spectra of purified LTP from wine (fraction W6). The fluorescence emission wavelengths were set to 310, 320 and 350 nm for monitoring the fluorescence excitation spectra between 200 and 300 nm (A). The same spectral features are observed for the other samples, W3, W4, W5 and G6. A fluorescence emission spectrum, with a maximum at 308 nm, was obtained for W6 when it was excited at 280 nm. Excitation at 295 nm (typical for tryptophan fluorescence) does not give a spectrum with a peak above 320 nm.

celery, green bean and mugwort pollen (Gadermaier et al., 2009, 2010; Hartz, Lauer, & Moncin, 2010; Mari & Ferreira 2009; Zocca-

telli et al., 2010) which are all characterised by an alpha helical structure. Our CD spectra do not show major differences between

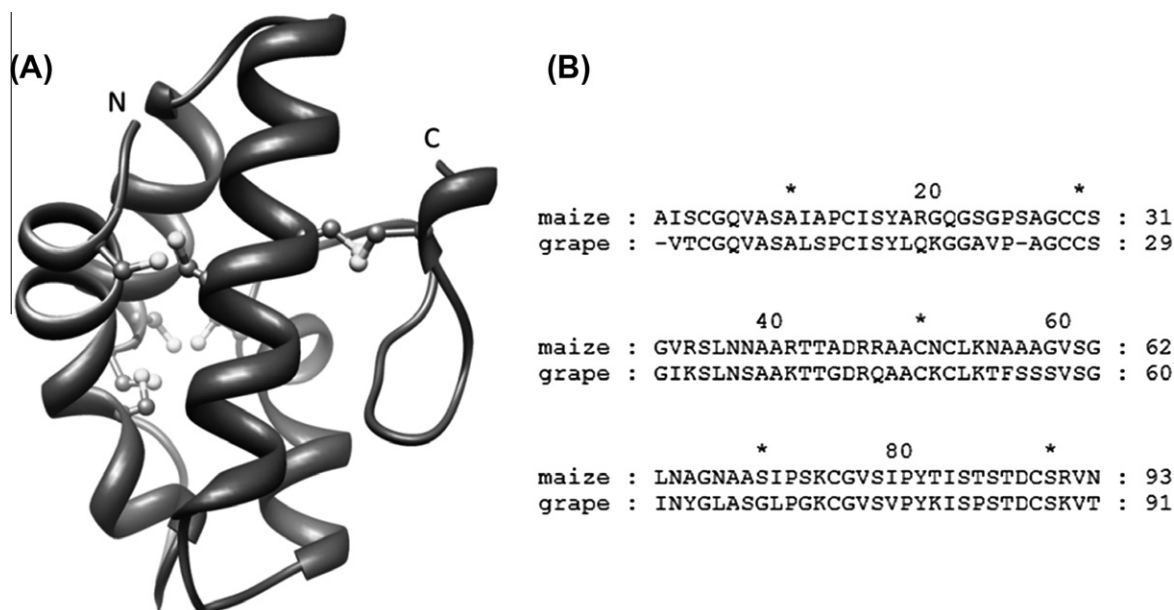


Fig. 4. Structure and sequence of LTP. (A) Homology model of LTP isoform 4 with maize (PDB ID: 1MZM_A) as template, using Swiss model. The eight cysteine residues forming the four disulfide-bridges are shown in stick and balls. (B) Amino acid sequence alignment of LTP isoform 4 from wine with maize, performed with ClustalX. The sequence identity amounts to 63%. For visualisation, UCSF Chimera was used (Pettersen et al., 2004).

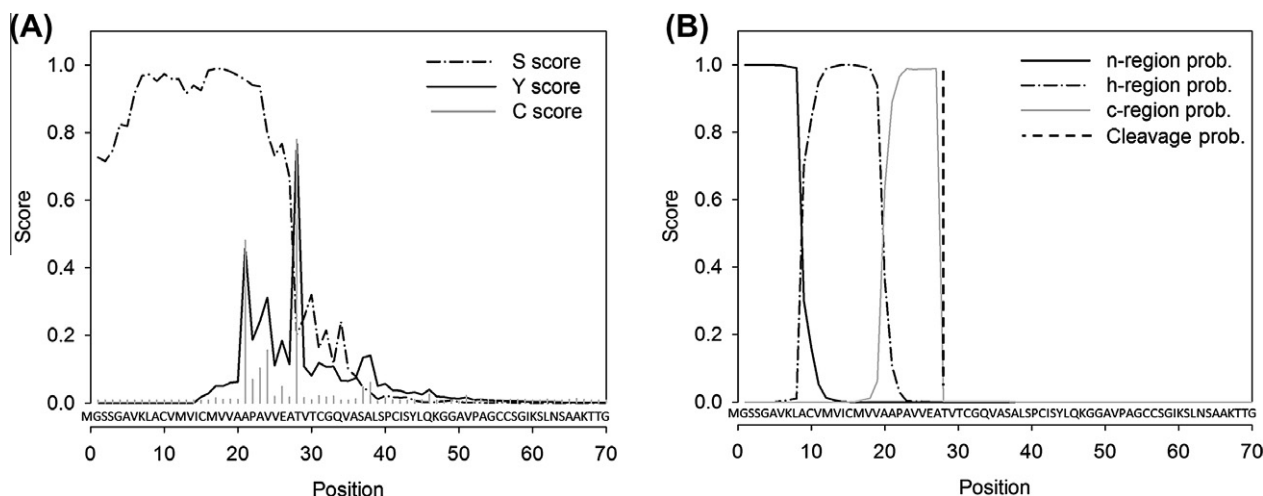


Fig. 5. Identification of the amino acids 1–27 as signal peptide based on SignalP 3.0 Server. The SignalP-NN result (A) and the SignalP-HMM result (B) show the most likely cleavage side between amino acids 27 and 28, with a probability of 99.9% for LTP from *Vitis vinifera*, which also applies for LTP from *Vitis aestivalis*.

LTP from grape and wine. Thus, we conclude that the LTP from grape survives the vinification process, including the long-term exposure to alcohol and the acidic conditions, without major structural changes.

Another important point results from mass spectrometry, which reveals that, in all cases, the C-terminus, as well as the mature N-terminus (after removal of the signal peptide), can be completely identified (Fig. 2), indicating that no proteolytic trimming occurs during vinification.

LTP elutes in multiple peaks from cation-exchange chromatography, indicating differences in net charge of the LTP subfractions. While we could exclude the possibility that this is due to trimming by exopeptidases, this phenomenon is probably the result of deamidation occurring at glutamine or asparagine residues. Indeed, we were able to detect a tryptic peptide (81–100), indicative of a deamidation occurring at position N91, which could be favoured by the acidic conditions found in wine.

4. Conclusion

LTP is an acknowledged allergen in plants. However, up to now, it is not known if the wine-making process and the acidic and alcoholic environment in wine cause structural changes, which could result in an altered allergen response.

Here, we present a purification protocol facilitating the isolation of LTP from Dornfelder red wine and grapes. Identities of the proteins were confirmed by mass spectrometry (LC-MS/MS). Circular dichroism and mass spectrometry revealed the integrity of the structures of LTP from grape and wine, as well as no change in the fold and no proteolytic digestion. According to our data, we conclude that, after the vinification process, the acidic conditions in wine do not cause notable structural changes of LTP. The structural stability indicates that the LTP from wine can also act as an allergen, as well as LTP from grape. Thus, further clinical trials will reveal the allergenic potential of LTP from grapes and wine, in

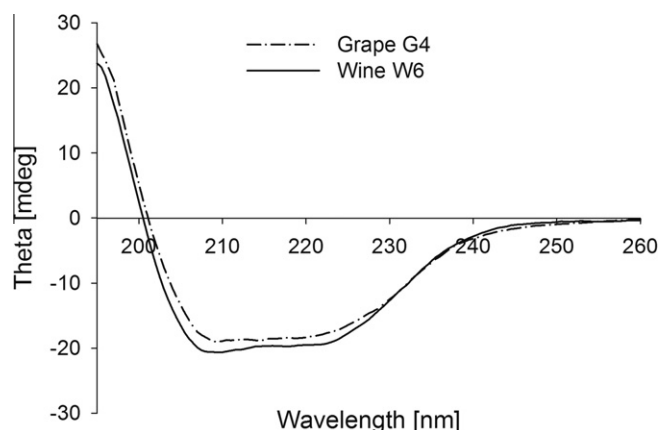


Fig. 6. CD spectra of purified LTP from wine and grape. Wine: fraction W6 (0.52 mg/ml), grape: fraction G4 (0.48 mg/ml).

general, and the influence of minor structural changes and observed deamidations, in particular.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.09.113>.

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Results of the mass spectrometry from samples G1-2 and W1-2.

Fraction	Band	Database	ID	Description	Score	% P	Peptide		mW	pl	Average		% Modified	% Peptides with Missed Cleavage Sites
							Matches	Coverage			Mass Error	Mass Error	Peptides	
G1	1	GenBank	AAB65776.1	class IV endochitinase Vitis vinifera	10.2	35.9	4	16.5	27224.4	5.3	2.1	2.2	50.0	50.0
	1	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	9.9	28.2	3	11.1	20108.0	4.3	1.7	1.7	100.0	0.0
	1	GenBank	CAN66515.1	hypothetical protein Vitis vinifera	8.9	10.3	3	9.3	23864.8	4.4	1.7	1.7	100.0	0.0
	1	NCBI Reference Sequence	XP_002273601.1	PREDICTED hypothetical protein Vitis vinifera	10.6	55.8	2	5.5	43449.7	6.5	17.7	17.9	100.0	0.0
	1	GenBank	CBI24659.3	unnamed protein product Vitis vinifera	10.4	44.2	2	5.2	46337.3	7.7	17.7	17.9	100.0	0.0
G2	1	GenBank	AAB65776.1	class IV endochitinase Vitis vinifera	10.2	34.9	3	16.1	27224.4	5.3	1.2	1.2	66.7	0.0
W1	1	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	9.7	21.9	2	11.1	20108.0	4.3	0.5	0.5	100.0	0.0
	2	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	10.1	33.5	3	11.6	20108.0	4.3	11.4	12.4	100.0	0.0
	2	GenBank	CAN68052.1	hypothetical protein Vitis vinifera	10.3	39.7	2	10.5	22444.0	7.3	15.7	19.2	100.0	0.0
	3	GenBank	CAA71883.1	osmotin like protein Vitis vinifera	10.1	33.2	6	24.4	23858.8	4.4	6.0	8.2	100.0	0.0
	3	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	5.1	0.2	5	17.9	20108.0	4.3	6.8	8.9	100.0	0.0
	3	NCBI Reference Sequence	XP_002282964.1	PREDICTED hypothetical protein Vitis vinifera	2.4	0.0	5	15.1	23842.8	4.5	6.8	8.9	100.0	0.0
	3	NCBI Reference Sequence	XP_002276431.1	PREDICTED hypothetical protein Vitis vinifera	-3.7	0.0	3	9.3	25290.7	4.9	9.1	11.1	100.0	0.0
	4	GenBank	CAA71883.1	osmotin like protein Vitis vinifera	10.1	33.3	7	24.4	23858.8	4.4	6.8	8.0	100.0	0.0
	4	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	11.0	78.8	6	17.9	20108.0	4.3	7.2	8.5	100.0	0.0
	4	NCBI Reference Sequence	XP_002276431.1	PREDICTED hypothetical protein Vitis vinifera	-4.3	0.0	3	9.3	25290.7	4.9	9.8	11.0	100.0	0.0
	4	GenBank	AAB65776.1	class IV endochitinase Vitis vinifera	10.2	34.9	3	16.1	27224.4	5.3	1.9	1.9	66.7	0.0
	4	GenBank	AAQ10093.1	class IV chitinase Vitis vinifera	10.1	32.6	3	15.9	27510.6	5.3	1.9	1.9	66.7	0.0
	4	NCBI Reference Sequence	XP_002275386.1	PREDICTED class IV chitinase Vitis vinifera	10.1	32.6	3	15.9	27510.6	5.3	1.9	1.9	66.7	0.0
	4	GenBank	CAN74833.1	hypothetical protein Vitis vinifera	9.6	20.6	2	9.8	23960.2	8.1	4.5	4.9	100.0	0.0
	4	NCBI Reference Sequence	XP_002283064.1	PREDICTED hypothetical protein Vitis vinifera	9.6	19.9	2	9.8	24220.3	8.0	4.5	4.9	100.0	0.0
	4	NCBI Reference Sequence	XP_002283042.1	PREDICTED hypothetical protein Vitis vinifera	9.6	19.9	2	9.8	24139.2	7.7	4.5	4.9	100.0	0.0
	4	GenBank	CAN80707.1	hypothetical protein Vitis vinifera	9.6	19.9	2	9.8	24139.2	7.7	4.5	4.9	100.0	0.0
	4	GenBank	CAN76134.1	hypothetical protein Vitis vinifera	9.6	19.9	2	9.8	24217.6	8.0	4.5	4.9	100.0	0.0
	5	GenBank	AAB65776.1	class IV endochitinase Vitis vinifera	10.3	40.8	9	31.0	27224.4	5.3	3.6	5.6	66.7	22.2
	5	GenBank	ABD64681.1	thaumatin like protein Vitis vinifera	10.5	50.0	8	24.4	23847.8	4.5	4.9	8.7	100.0	25.0
	5	NCBI Reference Sequence	XP_002282964.1	PREDICTED hypothetical protein Vitis vinifera	10.5	50.0	8	24.4	23842.8	4.5	4.9	8.7	100.0	25.0
	5	GenBank	CAA71883.1	osmotin like protein Vitis vinifera	10.1	33.3	7	24.4	23858.8	4.4	5.6	9.3	100.0	0.0
	5	GenBank	AAB65777.1	class IV endochitinase Vitis vinifera	-3.8	0.0	6	23.5	27519.6	5.3	2.7	3.7	50.0	33.3
	5	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	11.0	84.1	5	17.9	20108.0	4.3	7.2	11.0	100.0	0.0
	5	GenBank	CAN65933.1	hypothetical protein Vitis vinifera	-4.2	0.0	4	13.3	23929.1	7.4	1.1	1.2	100.0	50.0
	5	NCBI Reference Sequence	XP_002276431.1	PREDICTED hypothetical protein Vitis vinifera	-3.3	0.0	3	9.3	25290.7	4.9	8.7	13.4	100.0	0.0
	5	NCBI Reference Sequence	XP_002273601.1	PREDICTED hypothetical protein Vitis vinifera	10.7	61.4	2	5.5	43449.7	6.5	20.7	20.8	100.0	0.0
	5	GenBank	CAN74833.1	hypothetical protein Vitis vinifera	9.6	20.4	2	9.8	23960.2	8.1	1.8	1.8	100.0	0.0
	6	GenBank	ABD64681.1	thaumatin like protein Vitis vinifera	10.5	50.0	7	23.1	23847.8	4.5	9.1	10.7	100.0	0.0
	6	GenBank	CAA71883.1	osmotin like protein Vitis vinifera	10.1	33.3	6	24.4	23858.8	4.4	6.4	8.5	100.0	0.0
	6	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	11.0	84.1	5	17.9	20108.0	4.3	7.5	9.3	100.0	0.0
	6	NCBI Reference Sequence	XP_002276431.1	PREDICTED hypothetical protein Vitis vinifera	-3.3	0.0	3	9.3	25290.7	4.9	10.2	11.6	100.0	0.0
	6	GenBank	AAB65776.1	class IV endochitinase Vitis vinifera	10.2	34.9	3	16.1	27224.4	5.3	1.4	2.0	66.7	0.0
	6	GenBank	CAN65933.1	hypothetical protein Vitis vinifera	-4.2	0.0	3	12.0	23929.1	7.4	9.6	11.2	100.0	0.0
	6	NCBI Reference Sequence	XP_002273601.1	PREDICTED hypothetical protein Vitis vinifera	10.6	55.7	2	5.5	43449.7	6.5	19.7	19.7	100.0	0.0
	6	GenBank	CAN74833.1	hypothetical protein Vitis vinifera	9.6	20.4	2	9.8	23960.2	8.1	2.7	2.7	100.0	0.0
	7	GenBank	AAC33732.1	PR 4 type protein Vitis vinifera	11.2	100.0	2	24.5	15218.1	5.4	2.3	2.9	100.0	0.0
	7	GenBank	CAB85636.1	putative thaumatin like protein Vitis vinifera	9.7	21.9	2	11.1	20108.0	4.3	1.3	1.3	100.0	0.0
W2	8	UniProtKB/Swiss-Prot	P47180.1	Polygalacturonase Short PG AltName Full PGase SM AltName Full Pectinas	10.7	50.0	15.0	47.9	37263.0	8.2	2.8	3.7	40.0	2.0
	9	UniProtKB/Swiss-Prot	P47180.1	Polygalacturonase Short PG AltName Full PGase SM AltName Full Pectinas	10.7	50.0	18.0	59.8	37263.0	8.2	3.6	4.5	44.4	2.0
	9	NCBI Reference Sequence	XP_002284738.1	PREDICTED probable histone H2A variant 3 Vitis vinifera	9.5	15.0	2.0	11.9	14264.0	10.9	4.7	5.1		

P: Probability

Fraction	GenBank ID													
G4	Name	Score	% Probability	Peptide Matches	Coverage	mW	pI	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame	
	AAO33394.1	3.98	98.73	7	47.90	11677.79	8.63	V. vinifera	3.02	4.52	71.43	100		
		Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
		728.36	3	2182.07	2182.08	0.01	3.36	262.09	41.28	28	47	(A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)	
		640.34	2	1278.66	1278.66	0.00	-0.57	70.71	63.64	37	47	(S)ALSPCISYLQK(G)	Carbamidomethyl C (5)	
		971.49	2	1940.97	1940.98	0.01	6.48	319.61	92.66	81	100	(K)TFSSSVSGINYGASGLPGK(C)		
		648.00	3	1940.97	1940.98	0.01	5.41	284.80	61.47	81	100	(K)TFSSSVSGINYGASGLPGK(C)		
		971.50	2	1940.98	1940.98	0.00	0.13	62.15	64.22	81	100	(K)TFSSSVSGINYGASGLPGK(C)		
		847.44	2	1692.87	1692.87	0.00	-0.50	94.38	75.26	83	100	(F)SSSVSGINYGASGLPGK(C)		
		455.22	2	908.43	908.44	0.01	10.62	183.97	94.59	101	108	(K)CGVSVYPK(I)	Carbamidomethyl C (1)	
		497.73	2	993.45	993.44	0.00	-1.47	189.43	72.09	109	117	(K)ISPSTDCSK(V)	Carbamidomethyl C (7)	
		397.67	2	793.33	793.33	0.00	-0.62	103.49	93.55	111	117	(S)PSTDCSK(V)	Carbamidomethyl C (5)	
		Name	Score	% Probability	Peptide Matches	Coverage	mW	pI	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
		AAQ96338.1	3.99	100.01	3	23.53	11756.84	8.64	LTP V. aestivalis	3.79	6.14	100	100	
		Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
		640.34	2	1278.66	1278.66	0.00	-0.57	70.71	63.64	37	47	(S)ALSPCISYLQK(G)	Carbamidomethyl C (5)	
		455.22	2	908.43	908.44	0.01	10.62	183.97	94.59	101	108	(K)CGVSVYPK(I)	Carbamidomethyl C (1)	
		504.74	2.00	1007.46	1007.46	0.00	0.18	161.87	58.14	109	117	(K)ISPSTDCSK(V)	Carbamidomethyl C (7)	

W3	Name	Score	% Probability	Peptide Matches	Coverage	mW	pl	Description LTP isoform 4	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
	AAO33394.1	3.74	77.87	3	25.21	11677.79	8.63	V. vinifera	2.30	2.64	66.67		100
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
	648.00	3	1940.98	1940.98	0.01	3.84	270.68	67.89	81	100	(K)TFSSSVSGINYGSLGLPGK(C)		
	497.73	2	993.44	993.44	0.00	2.40	193.23	72.09	109	117	(K)ISPSTDCSK(V)	Carbamidomethyl C (7)	
	547.26	2	1092.51	1092.51	0.00	0.67	53.88	79.59	109	118	(K)ISPSTDCSKV(T)	Carbamidomethyl C (7)	
	728.37	3	2182.07	2182.08	0.00	2.01	216.38	39.45	28	47	(A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)	
	455.23	2	908.44	908.44	0.00	0.94	141.46	89.19	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)	
	Peptide	Score	% Probability	Matches	Coverage	mW	pl	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
	AAQ96338.1	3.99	100.01	1	7.56	11756.84	8.64	LTP V. aestivalis	0.85	0.85	100		100
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
	504.74	2	1007.46	1007.46	0.00	-0.85	154.97	58.14	109	117	(K)ISPSTDCTK(V)	Carbamidomethyl C (7)	
455.23	2	908.44	908.44	0.00	0.94	141.46	89.19	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)		

	Name	Score	% Probability	Peptide				Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame	
				Matches	Coverage	mW	pl						Description
W4	AAO33394.1	3.86	88.14	4	25.21	11677.79	8.63	V. vinifera	1.11	1.53	75	100	
		Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications
		971.50	2	1940.99	1940.98	0.00	-0.63	278.51	87.16	81	100	(K)TFSSSVSGINYGSLASGLPGK(C)	
		648.00	3	1940.98	1940.98	0.00	0.44	268.11	66.06	81	100	(K)TFSSSVSGINYGSLASGLPGK(C)	
		497.73	2	993.44	993.44	0.00	-0.06	184.57	72.09	109	117	(K)ISPSTDCK(V)	Carbamidomethyl C (7)
		547.26	2	1092.51	1092.51	0.00	-1.01	68.20	69.39	109	118	(K)ISPSTDCK(V)	Carbamidomethyl C (7)
		397.67	2	793.33	793.33	0.00	-2.85	82.01	87.10	111	117	(S)PSTDCK(V)	Carbamidomethyl C (5)
		728.36	3	2182.07	2182.08	0.01	3.24	194.49	36.70	28	47	(A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)
				Peptide					Average	RMS	% Modified		
		Name	Score	% Probability	Matches	Coverage	mW	pl	Description	Mass Error	Mass Error	Peptides	% Peptides with Missed Cleavage Sites

AAQ96338.1	3.99	100.01	1	7.56	11756.84	8.64	LTP V. aestivalis	4.54	4.54	100	100	
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications
	504.74		2	1007.45	1007.46	0.00	4.54	129.53	58.14	109	117 (K)ISPSTDCTK(V)	Carbamidomethyl C (7)

	Name	Score	% Probability	Peptide Matches	Coverage	mW	pl	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
	LTP isoform 4												
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
W5	AAO33394.1	3.99	99.92	13	48.74	11677.79	8.63	V. vinifera	4.80	8.94	69.23		100
	728.37	3	2182.07	2182.08	0.00	1.57	320.64	39.45	28	47	(A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)	
	768.91	2	1535.80	1535.80	0.00	-0.32	142.54	91.78	34	47	(Q)VASALSPCISYLQK(G)	Carbamidomethyl C (8)	
	683.86	2	1365.70	1365.70	0.00	-1.25	102.85	78.69	36	47	(A)SALSPCISYLQK(G)	Carbamidomethyl C (6)	
	640.34	2	1278.67	1278.66	0.00	-2.00	142.32	80.00	37	47	(S)ALSPCISYLQK(G)	Carbamidomethyl C (5)	
	376.21	2	750.41	750.43	0.02	29.61	80.26	84.00	42	47	(C)ISYLQK(G)		
	648.01	3	1940.99	1940.98	-0.01	-5.22	336.05	65.14	81	100	(K)TFSSSVGINYGLASGLPGK(C)		
	971.50	2	1940.98	1940.98	0.00	1.01	238.60	89.91	81	100	(K)TFSSSVGINYGLASGLPGK(C)		
	648.00	3	1940.98	1940.98	0.00	0.38	233.18	66.06	81	100	(K)TFSSSVGINYGLASGLPGK(C)		
	847.44	2	1692.87	1692.87	0.00	0.07	220.88	91.75	83	100	(F)SSSVGINYGLASGLPGK(C)		
	481.77	2	961.52	961.52	0.00	2.22	72.84	75.51	91	100	(N)YGLASGLPGK(C)		
	455.23	2	908.43	908.44	0.01	9.07	198.06	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)	
	497.73	2	993.44	993.44	0.00	1.17	81.37	81.40	109	117	(K)ISPSTDCK(V)	Carbamidomethyl C (7)	
	497.73	2	993.44	993.44	0.00	1.35	59.08	76.74	109	117	(K)ISPSTDCK(V)	Carbamidomethyl C (7)	
	547.27	2	1092.52	1092.51	0.00	-2.68	133.23	73.47	109	118	(K)ISPSTDCKV(T)	Carbamidomethyl C (7)	
	441.19	2	880.36	880.36	0.00	-4.23	65.85	81.08	110	117	(I)SPSTDCK(V)	Carbamidomethyl C (6)	
	397.67	2	793.32	793.33	0.00	5.92	121.32	93.55	111	117	(S)PSTDCK(V)	Carbamidomethyl C (5)	
	345.69	2	689.37	689.37	0.00	-1.68	76.98	64.52	61	67	(K)SLNSAAK(T)		
	971.99	2	1941.97	1941.97	0.00	-1.26	28.56	39.45	81	100	(K)TFSSSVGINYGLASGLPGK(C)	Deamidation N (10)	
	760.41	2	1518.80	1518.80	0.00	0.00	13.67	40.00	85	100	(S)SVSGINYGLASGLPGK(C)		
	Name	Score	% Probability	Peptide Matches	Coverage	mW	pl	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
	AAQ96338.1	3.99	100.02	8	26.89	11756.84	8.64	LTP V. aestivalis	7.58	11.66	87.50		100
	768.91	2	1535.80	1535.80	0.00	-0.32	142.54	91.78	34	47	(Q)VASALSPCISYLQK(G)	Carbamidomethyl C (8)	
	683.86	2	1365.70	1365.70	0.00	-1.25	102.85	78.69	36	47	(A)SALSPCISYLQK(G)	Carbamidomethyl C (6)	
	640.34	2	1278.67	1278.66	0.00	-2.00	142.32	80.00	37	47	(S)ALSPCISYLQK(G)	Carbamidomethyl C (5)	
	376.21	2	750.41	750.43	0.02	29.61	80.26	84.00	42	47	(C)ISYLQK(G)		
	455.23	2	908.43	908.44	0.01	9.07	198.06	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)	
	504.73	2	1007.45	1007.46	0.01	8.54	158.35	58.14	109	117	(K)ISPSTDCTK(V)	Carbamidomethyl C (7)	
	554.27	2.00	1106.52	1106.53	0.00	3.97	61.65	75.51	109	118	(K)ISPSTDCTKV(T)	Carbamidomethyl C (7)	
	404.68	2	807.34	807.34	0.00	5.90	63.72	74.19	111	117	(S)PSTDCTK(V)	Carbamidomethyl C (5)	

Name	Score	% Probability	Peptide Matches	Coverage	mW	pl	Description	Average Mass Error	RMS Mass Error	% Modified Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
AAO33394.1	8.88	100	21	55.46	11677.79	8.63	V. vinifera	4.52	7.00	61.90		100
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications
	728.37		3	2182.07	2182.08	0.00	1.57	391.58	36.70	28	47 (A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)
	728.37		3	2182.08	2182.08	0.00	-2.01	370.09	36.70	28	47 (A)TVTCGQVASALSPCISYLQK(G)	Carbamidomethyl C (4), Carbamidomethyl C (14)
	768.91		2	1535.80	1535.80	0.00	0.16	208.83	95.89	34	47 (Q)VASALSPCISYLQK(G)	Carbamidomethyl C (8)
	683.86		2	1365.70	1365.70	0.00	-1.25	167.09	86.89	36	47 (A)SALSPCISYLQK(G)	Carbamidomethyl C (6)
	640.34		2	1278.67	1278.66	0.00	-2.67	214.06	78.18	37	47 (S)ALSPCISYLQK(G)	Carbamidomethyl C (5)
	604.82		2	1207.63	1207.63	0.00	-2.93	33.29	67.35	38	47 (A)LSPCISYLQK(G)	Carbamidomethyl C (4)
	548.28		2	1094.54	1094.54	0.00	-1.34	109.06	93.02	39	47 (L)SPCISYLQK(G)	Carbamidomethyl C (3)
	376.22		2	750.42	750.43	0.01	6.75	77.62	84.00	42	47 (C)ISYLQK(G)	
	345.69		2	689.36	689.37	0.01	13.64	92.24	64.52	61	67 (K)SLNSAAK(T)	
	971.50		2	1940.98	1940.98	0.00	2.45	369.84	92.66	81	100 (K)TFSSSVGINYGLASGLPGK(C)	

W6		971.49	2	1940.97	1940.98	0.02	8.74	343.38	92.66	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		648.00	3	1940.99	1940.98	0.00	-1.07	321.81	57.80	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		648.01	3	1940.99	1940.98	-0.01	-5.16	281.57	55.96	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		971.50	2	1940.98	1940.98	0.00	2.14	185.67	84.40	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		648.00	3	1940.98	1940.98	0.01	2.64	147.29	64.22	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		971.50	2	1940.98	1940.98	0.00	2.14	76.70	78.90	81	100	(K)TFSSSVSGINYGASGLPGK(C)	
		971.99	2	1941.97	1941.97	0.00	0.50	201.99	78.90	81	100	(K)TFSSSVSGINYGASGLPGK(C)	Deamidation N (10)
		648.33	3	1941.97	1941.97	0.00	1.45	132.43	62.39	81	100	(K)TFSSSVSGINYGASGLPGK(C)	Deamidation N (10)
		847.44	2	1692.87	1692.87	0.00	0.87	323.80	88.66	83	100	(F)SSSVSGINYGASGLPGK(C)	
		760.41	2	1518.81	1518.80	0.00	-1.53	56.61	70.59	85	100	(S)SVSGINYGASGLPGK(C)	
		716.89	2	1431.77	1431.77	0.00	1.53	171.02	97.47	86	100	(S)VSGINYGASGLPGK(C)	
		481.77	2	961.53	961.52	0.00	-1.90	105.31	75.51	91	100	(N)YGASGLPGK(C)	
		400.24	2	798.46	798.46	0.00	-0.23	82.93	67.44	92	100	(Y)GLASGLPGK(C)	
		455.23	2	908.44	908.44	0.00	1.41	183.19	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)
		455.22	2	908.43	908.44	0.01	9.94	170.87	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)
		497.73	2	993.44	993.44	0.01	7.37	115.04	76.74	109	117	(K)ISPSTDCSK(V)	Carbamidomethyl C (7)
		497.72	2	993.43	993.44	0.01	10.44	106.41	79.07	109	117	(K)ISPSTDCSK(V)	Carbamidomethyl C (7)
		547.25	2	1092.49	1092.51	0.02	21.12	186.51	75.51	109	118	(K)ISPSTDCSKV(T)	Carbamidomethyl C (7)
		597.79	2	1193.56	1193.56	0.00	-1.64	27.04	54.55	109	119	(K)ISPSTDCSKVT(-)	Carbamidomethyl C (7)
		441.19	2	880.36	880.36	0.00	3.05	138.55	94.59	110	117	(I)SPSTDCSK(V)	Carbamidomethyl C (6)
		397.67	2	793.32	793.33	0.01	13.54	129.03	93.55	111	117	(S)PSTDCSK(V)	Carbamidomethyl C (5)
		510.76	2	1019.51	1019.50	-0.01	-7.48	12.60	40.82	63	72	(L)NSAAKTTGDR(Q)	
		666.33	2	1330.65	1330.64	-0.01	-8.16	163.89	68.66	81	93	(K)TFSSSVSGINYL(A)	
Peptide													
Name	Score	% Probability	Matches	Coverage	mW	pl	Description	Average Mass Error	RMS Mass Error	% Modified	Peptides	% Peptides with Missed Cleavage Sites	Reading Frame
AAQ96338.1	3.99	99.98	10	26.89	11756.84	8.64	LTP V. aestivalis	4.61	5.99	90.00	100		
	Submitted Mass	Submitted Charge	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modifications	
	768.91		2	1535.80	1535.80	0.00	0.16	208.83	95.89	34	47	(Q)VASALSPCISYLQK(G)	Carbamidomethyl C (8)
	683.86		2	1365.70	1365.70	0.00	-1.25	167.09	86.89	36	47	(A)SALSPCISYLQK(G)	Carbamidomethyl C (6)
	640.34		2	1278.67	1278.66	0.00	-2.67	214.06	78.18	37	47	(S)ALSPCISYLQK(G)	Carbamidomethyl C (5)
	604.82		2	1207.63	1207.63	0.00	-2.93	33.29	67.35	38	47	(A)LSPCISYLQK(G)	Carbamidomethyl C (4)
	548.28		2	1094.54	1094.54	0.00	-1.34	109.06	93.02	39	47	(L)SPCISYLQK(G)	Carbamidomethyl C (3)
	376.22		2	750.42	750.43	0.01	6.75	77.62	84.00	42	47	(C)ISYLQK(G)	
	455.23		2	908.44	908.44	0.00	1.41	183.19	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)
	455.22		2	908.43	908.44	0.01	9.94	170.87	94.59	101	108	(K)CGVSVPYK(I)	Carbamidomethyl C (1)
	504.73		2	1007.45	1007.46	0.01	9.33	54.03	60.47	109	117	(K)ISPSTDCTK(V)	Carbamidomethyl C (7)
	504.73		2	1007.45	1007.46	0.01	6.91	48.01	76.74	109	117	(K)ISPSTDCTK(V)	Carbamidomethyl C (7)
	554.27		2	1106.53	1106.53	0.00	-3.64	98.94	63.27	109	118	(K)ISPSTDCTKV(T)	Carbamidomethyl C (7)
	404.67		2	807.33	807.34	0.01	13.53	103.89	74.19	111	117	(S)PSTDCTK(V)	Carbamidomethyl C (5)