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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 spectroscopy (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.

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 spectroscopy; LTP, lipid transfer protein; NaCl, sodium chloride; NaN_3, 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 NaN_3 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) glycerin/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 Binninger Analytik, Schwäbich-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,