

A molecular basis for the endo- β 1,3-glucanase activity of the thaumatin-like proteins from edible fruits

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This paper is dedicated to the memory of Prof. J. Agneray and Dr. A. Verbert.

Abstract

Fruit-specific thaumatin-like proteins were isolated from cherry, apple and banana, and their enzymatic and antifungal activities compared. Both the apple and cherry possess a moderate endo- β 1,3-glucanase activity but are devoid of antifungal activity. In contrast, the banana thaumatin-like protein inhibits the *in vitro* hyphal growth of *Verticillium albo-atrum* but is virtually devoid of endo- β 1,3-glucanase activity. Both structural and molecular modeling studies showed that all three thaumatin-like proteins possess an extended electronegatively charged cleft at their surface, which is believed to be a prerequisite for endo- β 1,3-glucanase activity. Docking experiments showed that the positioning of linear (1,3)- β -D-glucans in the cleft of the apple and cherry proteins allows an interaction with the glutamic acid residues that are responsible for the hydrolytic cleavage of the glucan. Due to a different positioning in the cleft of the banana thaumatin-like protein, the linear β -glucans cannot properly interact with the catalytic glutamic acid residues and as a result the protein possesses no enzymatic activity. The possible function of the fruit-specific thaumatin-like proteins is discussed in view of the observed biological activities and structural features.

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1. Introduction

Many plants express proteins that are structurally and evolutionarily related to thaumatin but possess no sweet-tasting properties. Most of these so called thaumatin-like proteins (TLP) are believed to be involved in plant defense because their synthesis is induced or strongly enhanced upon challenge of the plant by pathogens or exposition to abiotic

stress factors (e.g. drought). According to their inducible expression, these TLP are considered pathogenesis-related proteins and classified as class 5 or PR-5 proteins [1,2]. Classical examples of PR-5 proteins are osmotin from tobacco [3] and osmotin-like proteins from many other plant species. In contrast to these inducible PR-5 proteins, some TLP are developmentally regulated. For example, several TLP have been identified that specifically accumulate during the final stages of fruit ripening in banana, elderberry, sweet cherry, grape and apple [4].

During the last two decades a lot of attention has been drawn to the unraveling of the physiological role of TLP. After the discovery of the *in vitro* antifungal activity of osmotin and some other PR-5 intensive efforts were undertaken to corroborate the role of TLP in the plant's defense against pathogenic or nonpathogenic fungi [5–8]. Since experiments with transgenic potato [9] and rice [10] plants clearly indicated that the ectopic expression of osmotin or osmotin-like proteins resulted in an enhanced resistance

Abbreviations: Ban-TLP, banana (*Musa acuminata*) thaumatin-like protein; CD, circular dichroism; Mal-TLP, apple (*Malus domestica*) thaumatin-like protein; Pru-TLP, cherry (*Prunus avium*) thaumatin-like protein; TLP, thaumatin-like protein.

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against various phytopathogenic fungi one can reasonably assume that at least some PR-5 act also in planta as antifungal proteins. At present, the underlying mechanism of this antifungal activity is still not fully understood. It has been suggested previously that osmotins exerts a membrane-permeabilizing activity [11,12], which relies on the presence of an acidic cleft in these proteins [13]. It is questionable, however, whether there is a direct relationship between the antifungal properties of TLP and the presence of an acidic cleft because there are PR-5 that contain an electronegative cleft but nevertheless are devoid of antifungal activity [14]. A few years ago the search for the role and working mechanism of TLP was refueled after the unexpected observation that some TLP bind to (1,3)- β -D-glucans [15] and, in addition, definitely exhibit a more or less pronounced endo- β 1,3-glucanase activity [16]. Though there is no doubt that the acidic cleft of TLP plays an essential role in both the binding and subsequent hydrolysis of (1,3)- β -D-glucans, the exact mechanisms of the binding and catalytic activity remain unclear. Most probably, even subtle differences in sequences and structure may cause huge differences in (1,3)- β -D-glucan binding and cleaving activity. It is interesting to note in this context that of two closely related PR-5 proteins occurring in germinated barley (*Hordeum vulgare*) grains only one (HvPR5c) binds (1,3)- β -D-glucans whereas the other (HvPR5b) does not [17]. Though the physiological consequences of the in situ degradation of (1,3)- β -D-glucans by PR-5 are not yet fully understood, it is believed that reaction products of their enzymatic activity (i.e. β -glucan oligomers of various degree of polymerization) act as elicitors and induce various defense responses of the plants. It has been demonstrated, for example, that (1,3)- β -D-glucan oligomers with a degree of polymerization >4 induce chitinase activity in a suspension culture of rice (*Oryza sativa*) cells [18], and that linear (1,3)- β -D-glucans (laminaropentaose) elicit defense responses in tobacco [19]. The role of the endo- β 1,3-glucanase activity of TLP must not necessarily be restricted to the generation of elicitors but may be extended to a specific degradation of cell wall (1,3)- β -D-glucans [20]. It has been suggested, indeed, that TLP that accumulate in large quantities in ripening fruits contribute to the softening of the pulp or flesh of e.g. banana, grape and cherry [21,22].

This report deals with a detailed analysis of biological activities of the fruit-specific TLP from banana, cherry and apple, and an analysis of the structural features underlying these biological activities with the emphasis on the molecular mechanism responsible for the endo- β 1,3-glucanase activity of some of these proteins.

2. Materials and methods

2.1. Isolation of thaumatin-like proteins

Banana (*Musa acuminata*) thaumatin-like protein (Ban-TLP) and banana endo- β 1,3-glucanase (Ban-Gluc) were iso-

lated from the pulp of ripe fruits, as previously described [23,24].

Apple (*Malus domestica*) thaumatin-like protein (Mal-TLP) [25] was isolated from ripe apples (*Malus domestica*, cv. Jonagold). Apples (5 kg) were peeled, chopped in pieces and homogenized in 10 l of 50 mM acetic acid using a Waring blender. The homogenate was poured through a sieve, adjusted to pH 3.0 with 1 M acetic acid, centrifuged at 9000 *g* for 15 min and the supernatant filtered through filter paper (Whatmann 3MM). The cleared extract was loaded onto a column (5 cm $\times$ 5 cm, $\sim$ 100 ml bed volume) of S Fast Flow (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM acetic acid. After loading, the column was washed with 2 l of a 20 mM solution of acetic acid and the bound proteins eluted in a single step with 400 ml of 50 mM Tris-HCl (pH 7.8). The partially purified protein fraction was adjusted to pH 3.0 with 1 M acetic acid, centrifuged (9000 *g*, 15 min) and loaded onto a column (2.5 cm $\times$ 10 cm; $\sim$ 50 ml bed volume) of S Fast Flow equilibrated with 20 mM acetic acid. After loading the proteins, the column was washed with 500 ml of 20 mM Na-formate buffer (pH 3.8) and the proteins eluted with a linear gradient of increasing NaCl concentration (500 ml, 0.0–0.5 M) in formate buffer. An analysis by SDS-PAGE of the collected fractions indicated that the major peak eluting at a NaCl concentration of $\sim$ 0.1 M contained virtually exclusively a single polypeptide of approximately 20 kDa. Peak fractions were pooled, diluted with three volumes of 20 mM acetic acid and loaded on a column (1.5 cm $\times$ 10 cm, $\sim$ 20 ml bed volume) of S Fast Flow equilibrated with 20 mM acetic acid. The bound proteins were eluted with 10 ml of 0.1 M Tris-HCl (pH 7.8) containing 0.2 M NaCl and loaded onto a column (2.6 cm $\times$ 70 cm; $\sim$ 350 ml bed volume) of Sephacryl 100 equilibrated with 20 mM Tris-HCl (pH 7.8) containing 0.2 M NaCl. The peak eluting with an apparent molecular mass around 20 kDa was collected, dialyzed against appropriate buffers and stored in small aliquots at -20°C until use. Analysis by SDS-PAGE confirmed that the purified protein consisted exclusively of a single 20-kDa polypeptide. Approximately 10 mg of pure Mal-TLP was obtained starting from 5 kg of apples.

Cherry (*Prunus avium*) thaumatin-like protein (Pru-TLP) [21] was isolated from ripe cherries (*Prunus avium*, cv. Bruine biggareau). Cherries (5 kg) were squeezed through cheesecloth. The sap was collected, diluted with an equal volume of distilled water and adjusted to pH 3.0. The diluted sap was centrifuged (9000 *g*, 10 min) and the supernatant filtered through filter paper (Whatmann 3MM). A partially purified Pru-TLP fraction was prepared and further purified as described above for Mal-TLP. The Pru-TLP fraction obtained after the second ion-exchange chromatography step was neutralized, adjusted to 1 M ammonium sulfate and loaded onto a column (2.5 cm $\times$ 5 cm, $\sim$ 25 ml bed volume) of Phenyl Sepharose (Amersham Biosciences, Uppsala, Sweden) equilibrated with 1 M ammonium sulfate. After loading, the column was washed with 100 ml of 1 M ammonium sulfate and the Pru-TLP eluted with distilled water. This

hydrophobic interactions chromatography step was included to get rid of the anthocyanins (that remained on the Phenyl Sepharose column). The Pru-TLP fraction eluted from the Phenyl Sepharose column was dialyzed against 20 mM acetic acid, concentrated on a small column of S Fast Flow and subjected to gel filtration as described above for the Mal-TLP. Analysis by SDS-PAGE confirmed that the purified protein consisted exclusively of a single 20-kDa polypeptide. A yield of 75 mg of pure Pru-TLP was obtained from 5 kg of cherries.

2.2. Circular dichroism spectroscopy

The far-UV circular dichroism (CD) spectra of TLP in the presence of (1,3)- β -D-glucan (Pachyman) was measured at room temperature with a spectropolarimeter Jobin & Yvon CD6 (Division d'Instrumentation S.A., Longjumeau, France) using 0.1 cm path length quartz cuvettes. A concentration of TLP of 150 $\mu\text{g ml}^{-1}$ in 50 mM acetate-buffer, 100 mM NaCl (pH 5.0) was used for the CD measurements. The proportion of β -strand and α -helical segments was estimated from the CD spectra [26].

2.3. Endo- β 1,3-glucanase activity

The endo- β 1,3-glucanase activity of TLP was assayed using carboxy-methylated Pachyman (CM-Pachyman, Megazyme, Wicklow, Ireland) as a substrate. Proteins (20 μg) were incubated at 37 °C for 3 d in 0.3 ml of 0.5% (w/v) CM-Pachyman in 50 mM sodium acetate, 100 mM NaCl containing 0.05% (w/v) sodium azide (pH 4.0 and 5.0) or 20 mM potassium phosphate buffer, 100 mM NaCl containing 0.05% (w/v) sodium azide (pH 6.0). The enzymatic activity was measured against substrate blanks (devoid of TLP and similarly incubated) by adding 50 μl of the samples to 4.0 ml of 0.1% (w/v) of tetrazolium blue in 50 mM NaOH, 0.5 M sodium potassium tartrate and heating in a boiling water bath for 3 min [27]. After cooling for 3 min in running tap water, the quantity of liberated D-glucose equivalents was estimated by measuring the absorbance at 660 nm. Measurements were performed in triplicate. Results are expressed as nkcat mg^{-1} of protein (1 kat corresponds to the formation of 1 mol of D-glucose equivalent per second).

The V_m and K_m were estimated from measurements performed on 100 μl samples of Mal-TLP and Pru-TLP (50 μl of increasing concentrations) after 30, 60, 120 and 180 min of incubation at 37 °C in CM-Pachyman solutions (500 μl) of concentrations ranging from 0.1% to 0.5% (w/v) in 50 mM sodium acetate, 100 mM NaCl containing 0.05% (w/v) sodium azide (pH 4.0 and 5.0). All the measurements were performed in triplicate.

2.4. Antifungal activity in vitro

The antifungal activity of TLP was evaluated by agar diffusion assays. A series of different pathogenic or non-pathogenic fungi (*Aspergillus niger*, *Botrytis cinerea*, *Can-*

didia albicans, *Fusarium gramineum*, *Penicillium commune*, *Phytophthora infestans*, *Trichoderma viride* and *Verticillium albo-atrum*) were grown on Potato Dextrose Agar (PDA) in Petri dishes. Hyphal growth inhibition was measured by placing sterile filter paper disks containing either buffer (50 mM sodium acetate (pH 5.0) containing 100 mM NaCl) (control) or test TLP (30, 60, 90 or 120 μg in the same buffer) adjacent to the colony margin and allowing the fungal growth to proceed for several days at room temperature. The absence of growth over the disks and/or the occurrence of an inhibition zone surrounding the disks were used as an assessment of growth inhibition.

2.5. Molecular modeling and docking experiments

The amino acid sequences of TLP were aligned using CLUSTAL-X [28]. The alignment was manually modified on the basis of the HCA plots [29] to delineate the structurally conserved regions (SCR) common to the proteins. The HCA plots were generated using drawhca (<http://www.lmcp.jussieu.fr/~soyer/www-hca/hca-form.html>).

The three-dimensional models of Mal-TLP and Pru-TLP were built by homology modeling on a Silicon Graphics Octane station from the available X-ray coordinates of thaumatin (RCSB PDB code 1THV) [30], the PR-5 thaumatin-like of tobacco (RCSB PDB code 1AUN) [31] and the crystal structure of Ban-TLP (A. Roussel, unpublished), using Modeller 4.0 [32] and the suite of programs InsightII, Homology and Discover3 (Accelrys, San Diego, CA, USA). Steric conflicts resulting from the replacement or the deletion of some residues in the TLP were corrected during the model building procedure using the rotamer library [33] and the search algorithm implemented in the Homology program [34] to maintain proper side-chain orientation. Energy minimization and relaxation of the loop regions were performed during the model building by several cycles of steepest descent and conjugate gradient using the cvff forcefield of Discover3. The program TurboFrodo (Bio-Graphics, Marseille, France) was used to optimize the geometry of the loop regions. A global minimization of the final models was carried out by simulated annealing and a few cycles of conjugate gradient. The stereo-chemical assessment of the three-dimensional models was checked with PROCHECK [35] and overall G -factors were calculated.

Using the atomic coordinates of the modeled TLP, the partial charges of the atoms have been assigned with the GRID program [36]. The program MEAD [37] was then used to calculate the terms W_{ij} (ionizable group's interaction within the protein), $\Delta\Delta G_{\text{Born}}$ (Born solvation energy) and $\Delta\Delta G_{\text{Back}}$ (background charge interactions), representing the electrostatic energy contribution of ionizable groups. The inner and outer dielectric constants applied to the protein and the solvent were fixed at 4.0 and 80.0, respectively, and the calculations were performed keeping a ionic strength of 0.15 M. From the $pK_{1/2}$ values (pH at which a site is half-protonated, $\theta = 0.5$) and the ionization state energies calculated for each ionizable site, new atomic coordinate files

Table 1

Specific activities measured after 3 d of incubation for the fruit PR-5 proteins at pH 4.0 and 5.0, using CM-Pachyman as a substrate

Protein	Concentration		pH 4.0		pH 5.0	
	($\mu\text{g ml}^{-1}$)	(μM)	Activity (nkat mg^{-1})	Number of cleaved glycosidic linkages	Activity (nkat mg^{-1})	Number of cleaved glycosidic linkages
Pru-TLP	5.0	0.214	3.0 ± 1.2	4.2 ± 1.7	1.9 ± 0.6	2.6 ± 0.8
Mal-TLP	5.0	0.215	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
Ban-TLP	50.0	2.355	0.03 ± 0.0	0.04 ± 0.0	0.03 ± 0.01	0.03 ± 0.01
Ban-Gluc	0.15	0.004	9.3 ± 1.5	20.3 ± 3.3	6.2 ± 0.9	13.5 ± 2.0

of the modeled TLP corresponding to different pH (4.0, 5.0, 6.0 and 7.0) values were generated. Electrostatic potentials at different pH were calculated with DelPhi [38] and displayed on the surface of the modeled TLP with GRASP [39].

The anchoring of (1,3)- β -D-glucans into the cleft of the TLP was performed with GRID [36]. Coordinates of β -D-glucose, β -D-laminaribiose, -triose and -tetraose were taken from the SWEET2 data bank (<http://www.dkfz-heidelberg.de/spec/sweetdb/>). Higher polymerized β -D-glucans were built from the NMR coordinates of a (1,3)- β -D-hexaglucon [40]. The optimized docking of the smaller ligands into the cleft was used as a reference for the docking of higher polymerized (1,3)- β -D-glycans. The geometry of the docked oligosaccharides satisfying the known energy map of the (1,3)- β -linkage was optimized by several steps of conjugate gradient and simulated annealing using the DOCKING program of InsightII. The docked models exhibiting the best superposition with the smaller saccharides and displaying the lowest binding energy were analyzed.

3. Results

3.1. Purification of fruit-specific thaumatin-like proteins from banana, cherry and apple

The fruit-specific TLP from apple, cherry and banana were purified to homogeneity using a combination of selective extraction and conventional protein purification techniques. Overall yields were approximately 2, 75 and 100 mg pure protein per kg of apple, cherry juice and banana pulp, respectively. All three proteins yielded a single band of approximately 20 kDa upon SDS-PAGE (results not shown).

3.2. Antifungal activity

To check whether Ban-TLP, Mal-TLP and Pru-TLP exhibit the same biological activities as some previously described TLP or PR-5, the possible antifungal activity and endo- β 1,3-glucanase activity of the three fruit-specific proteins was studied in some detail. Growth inhibition assays revealed that Ban-TLP exhibited a weak antifungal activity against *V. albo-atrum* whereas both Mal-TLP and Pru-TLP were apparently devoid of in vitro antifungal activity.

3.3. Endo- β 1,3-glucanase activity

Determination of the endo- β 1,3-glucanase activity using CM-Pachyman as substrate indicated that Pru-TLP and Mal-

TLP exhibit a reasonable enzymatic activity at pH 4.0 and 5.0, respectively (Table 1). Under the same conditions, Ban-TLP exhibited only a very weak enzymatic activity whereas Ban-Gluc, the genuine endo- β 1,3-glucanase from banana pulp, which was included as a positive control [25], showed an activity well superior to that of Pru-TLP and Mal-TLP (Table 1).

The experimentally determined K_m values for Pru-TLP were 27.2 ± 6.8 and $28.6 \pm 2.5 \mu\text{mol l}^{-1}$ at pH 4.0 and 5.0, respectively. Pru-TLP had a V_m value of $1.02 \pm 0.09 \mu\text{mol l}^{-1} \text{ s}^{-1}$ at pH 4.0 (as determined at a concentration of $80 \mu\text{g ml}^{-1}$) and of $0.49 \pm 0.02 \mu\text{mol l}^{-1} \text{ s}^{-1}$ at pH 5.0 (as determined at a concentration of $80 \mu\text{g ml}^{-1}$) and $120 \mu\text{g ml}^{-1}$ (Table 2). Mal-TLP exhibited a considerably higher K_m value ($70.3 \pm 25.78 \mu\text{mol l}^{-1}$ at pH 4.0) than Pru-TLP when tested under the same conditions and, in addition showed a lower V_m value ($0.57 \pm 0.09 \mu\text{mol l}^{-1} \text{ s}^{-1}$ as determined at a concentration of $120 \mu\text{g ml}^{-1}$ at pH 4.0) (Table 2). It can be concluded, therefore, that Pru-TLP and, to a lesser extent, Mal-TLP, possess an endo- β 1,3-glucanase activity whereas Ban-TLP is virtually devoid of such activity. It should be emphasized, however, that the observed endo- β 1,3-glucanase activity of Pru-TLP and Mal-TLP is by far lower than that of Ban-Gluc. To verify the specificity of the endo- β 1,3-glucanase activity, the enzymatic activity of Pru-TLP and Mal-TLP was also checked with other substrates. No hydrolytic cleavage could be detected at various pH values using (1,4)- α -D-glucans (starch), (1,4)- β -D-glucans (cellulose) or poly(1,4)- β -N-acetyl-D-glucosamine (chitin, chitosan) as substrates indicating that Pru-TLP and Mal-TLP specifically act on (1,3)- β -D-glucans.

Table 2

V_m values (expressed as $\mu\text{mol l}^{-1} \text{ s}^{-1}$) calculated at pH 4.0 and 5.0 for different concentrations of PR-5 proteins, using CM-Pachyman as a substrate

Protein	Concentration		pH 4.0	pH 5.0
	($\mu\text{g ml}^{-1}$)	(μM)		
Pru-TLP	10.0	0.43	0.12 ± 0.01	
	20.0	0.86	0.31 ± 0.03	
	40.0	1.72	0.58 ± 0.04	0.12 ± 0.01
	80.0	3.44	1.02 ± 0.09	0.33 ± 0.01
	100.0	4.29		0.35 ± 0.02
	120.0	5.14		0.49 ± 0.02
Mal-TLP	80.0	3.45	0.36 ± 0.09	
	120.0	5.17	0.57 ± 0.09	

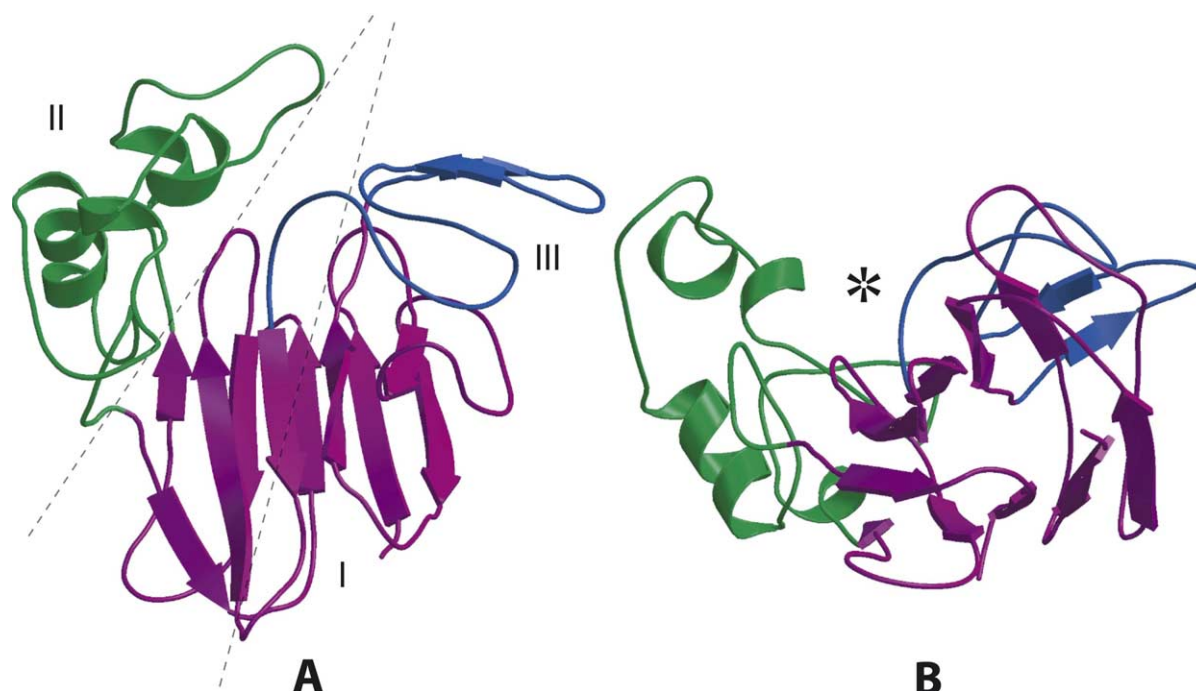


Fig. 1. Ribbon diagram showing the modeled three-dimensional fold of Pru-TLP. (A) front face showing the organization in three domains: I (magenta), II (green) and III (blue) of Pru-TLP. Dashed lines delineate the acidic cleft located in the core domain I. (B) lateral view showing the size of the acidic cleft (*) and the β -sandwich organization of the core domain I (magenta).

3.4. Structural analysis of the thaumatin-like proteins from banana, cherry and apple

Sequence comparisons revealed that Ban-TLP, Pru-TLP and Mal-TLP share a high sequence identity/similarity with thaumatin (1THV) and the tobacco PR-5 (1AUN). As could be expected on the basis of this high sequence identities/similarities, the modeled structures of all these proteins exhibit a very similar three-dimensional fold. They typically comprise three distinct domains: (1) an N-terminal core domain (I) built up of two bundles of antiparallel strands of β -sheet interconnected by loops organized in a flattened β -sandwich, (2) a C-terminal domain (II) consisting of an α -helix associated with three shorter helical segments and (3) a third domain (III) comprising two β -strands linked by a short loop, prolonged by a more extended loop (Fig. 1). Domains I and II delineate a central cleft which contains predominantly hydrophilic residues. Eight conserved disulfide bridges contribute to the stability of the PR-5 proteins. The validity of the three-dimensional models of Mal-TLP and Pru-TLP was assessed by a Ramachandran plot analysis. Approximately 75% of the residues of all modeled loops occur in the core region of the plot (vs. 82% for thaumatin 1THV). It should also be mentioned here that a very similar three-dimensional fold was reported for zeamatin, a PR-5 protein from maize [13]. CD-analysis of Ban-TLP, Pru-TLP and Mal-TLP indicated a predominance of β -sheet structures and confirmed the structural predictions made on the basis of molecular modeling. Addition of CM-Pachyman to the solutions of Ban-TLP, Pru-TLP and Mal-TLP did not alter the spectra (result not shown), indicating that no substantial

conformational change occurs upon binding of (1,3)- β -D-glucans to the TLP.

3.5. Structural features responsible for the endo- β 1,3-glucanase activity

As shown in Fig. 4, the cleft located between domains I and II is highly electronegative in all three fruit-specific TLP. It should be emphasized, however, that the acidic character of this cleft depends on the pH (Fig. 2). At pH 5, which is the optimal pH value for the endo- β (1,3)-glucanase activity of both Mal-TLP and Pru-TLP, the clefts display a less widespread electronegativity which coincides with the location of the side-chains of some acidic residues (Asp and Glu) protruding from the surface of the cleft. Docking experiments performed with extended (1,3)- β -D-glucan chains revealed that those glycan chains, which exhibit the lowest binding energies lie down at different locations on the surface of the TLP (Fig. 3). Hydrophobic interactions of the glucose units with apolar and aromatic residues protruding from the surface of the cleft mainly account for these different docking locations. Thereby, the core region of the (1,3)- β -D-glycans occupies the center of the acidic cleft whereas two β -D-glucose units usually occupy two hydrophobic plots occurring on the surface of the cleft. The rest of the glycan chain is positioned either inside or outside the cleft. Similar positions of docked (1,3)- β -D-glycans have been reported on the surface of the modeled barley PR-5 protein HvPR5c [17]. In all the modeled locations, the core region of the (1,3)- β -D-glycan inserts between two acidic residues (Asp⁹² and Glu¹⁰⁵ in Pru-TLP). However, these residues are unlikely to act as

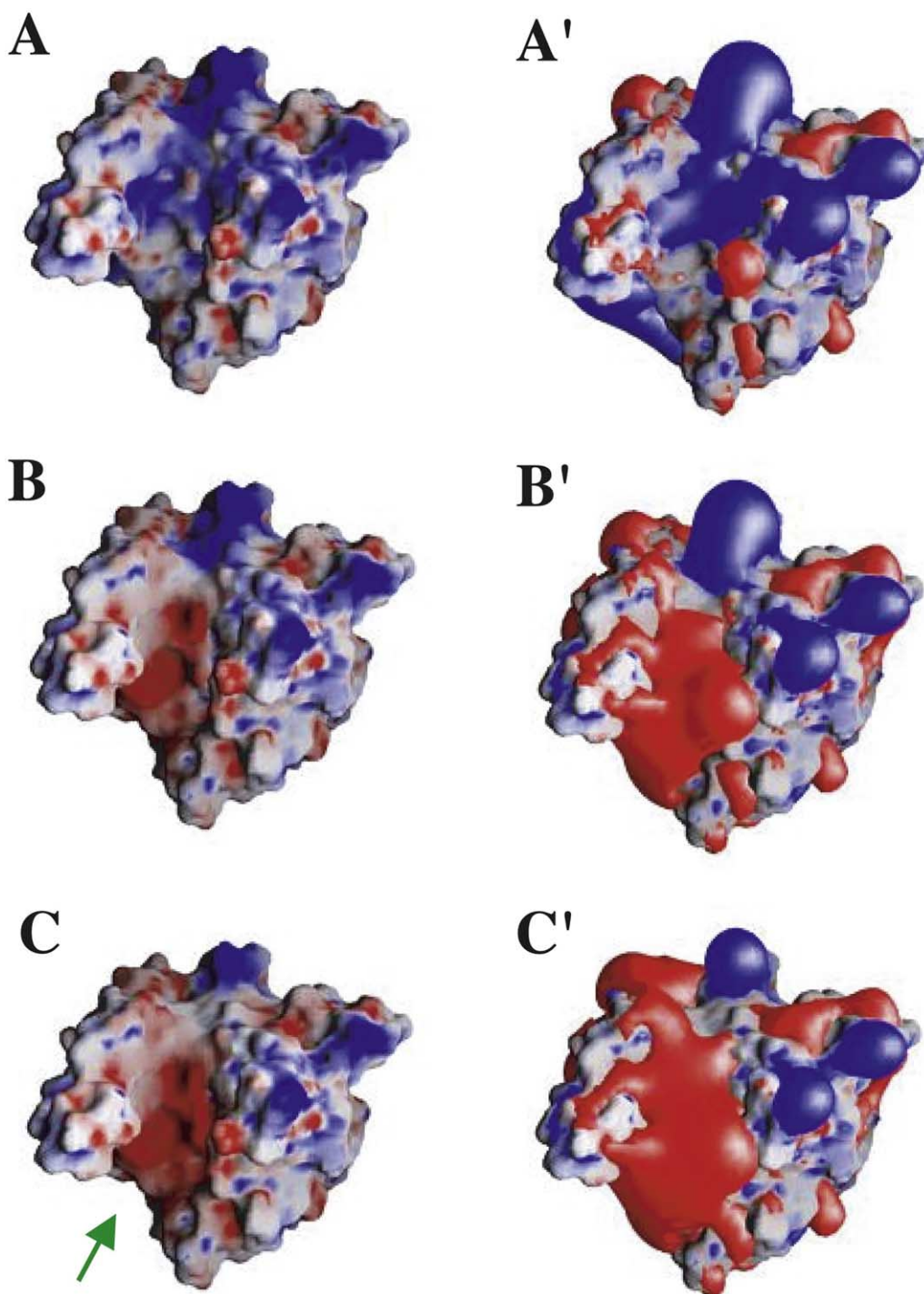


Fig. 2. GRASP [39] representation of the electrostatic surface potentials of the front face of Pru-TLP. Panels A, B and C represent the calculated potentials at pH 5.0, 6.0 and 7.0, respectively. The negative potentials, colored red, and the positive potentials, colored blue, are displayed at -5 kT level and $+5$ kT level (1 $kT = 0.6$ kcal), respectively. Neutral surfaces are white. In panels A', B' and C' the electrostatic contours of Pru-TLP at pH 5.0, 6.0 and 7.0, respectively, are displayed at ± 1 kT .

catalytically active residues since they are separated by a too short distance ($\text{Asp}^{92}\text{O}\delta 2\text{-Glu}^{105}\text{O}\epsilon 2 = 2.92$ Å). Similarly, the distance between Asp^{92} and Glu^{21} (>12.0 Å) prevents these acidic residues to promote the cleavage of a glycosidic bond. The distances measured between other acidic residues of the cleft, which are susceptible to act as nucleophile residues and proton donors, in a retaining ($d \sim 5.5$ Å) or an

inverting ($d = 6.5\text{--}9.5$ Å) mechanism of glycan hydrolysis are 6.77 Å for $\text{Asp}^{92}\text{O}\delta 2\text{-Glu}^{110}\text{O}\epsilon 2$, 7.93 Å for $\text{Glu}^{71}\text{O}\epsilon 2\text{-Glu}^{150}\text{O}\epsilon 2$, and 9.07 Å for $\text{Glu}^{105}\text{O}\epsilon 2\text{-Glu}^{110}\text{O}\epsilon 2$, respectively (Fig. 4). Residues Glu^{71} and Glu^{150} , which are positioned at the top of the acidic cleft and separated from each other by a favorable distance (7.93 Å), could be involved in the cleavage of the (1,3)- β -D-glycan chain that spreads be-

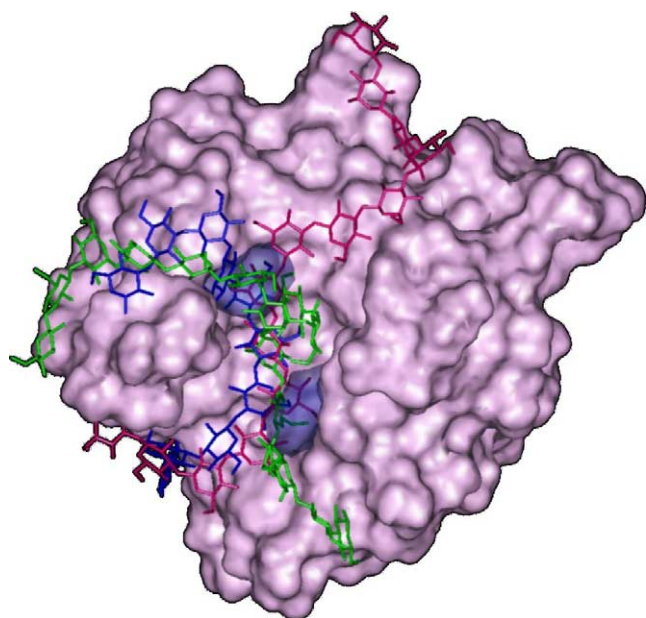


Fig. 3. Docking of $\beta(1,3)$ -D-glucans in the acidic cleft of Pru-TLP. The topology diagram shows different positions (shown in pink, blue and green) of the $\beta(1,3)$ -D-glucans with the lowest calculated binding energies in the cleft of Pru-TLP at pH 5.0. In the GRASP [39] representation the molecular surface of Pru-TLP is colored light purple. The two blue colored spots correspond to hydrophobic cavities occurring on the surface of the cleft.

tween these two residues in a location of lowest binding energy into the cleft.

4. Conclusion

The present study of the biological activities of three different fruit-specific TLP indicate that they have either no (Pru-TLP and Mal-TLP) or a weak (Ban-TLP) antifungal activity against *V. albo-atrum*. In this respect, the fruit-specific TLP clearly differ from the previously described PR-5 proteins with a more pronounced fungistatic activity (like osmotin and zeamatin). Based on the extremely rapid toxic effect of zeamatin and osmotin on some phytopathogenic fungi, the hypothesis has been put forward that antifungal effect of PR-5 proteins relies on a membrane permeabilization mechanism [11]. Although the exact mechanism causing the dramatic increase of the plasma membrane permeability still remains unclear, it has been suggested that polarized negative charges occurring in the cleft of PR-5 proteins enable them to interact with positively charged membrane proteins like ion or water channels and provoke an enhancement of the water flow across the plasma membrane [13]. Though Pru-TLP, Mal-TLP and Ban-TLP also possess a strongly acidic cleft they exhibit either no or a very weak antifungal activity, which implies that the presence of such an acidic cleft in se does not provide a TLP with antifungal properties. In this respect, the results obtained with Pru-TLP, Mal-TLP and Ban-TLP confirm earlier findings done with a

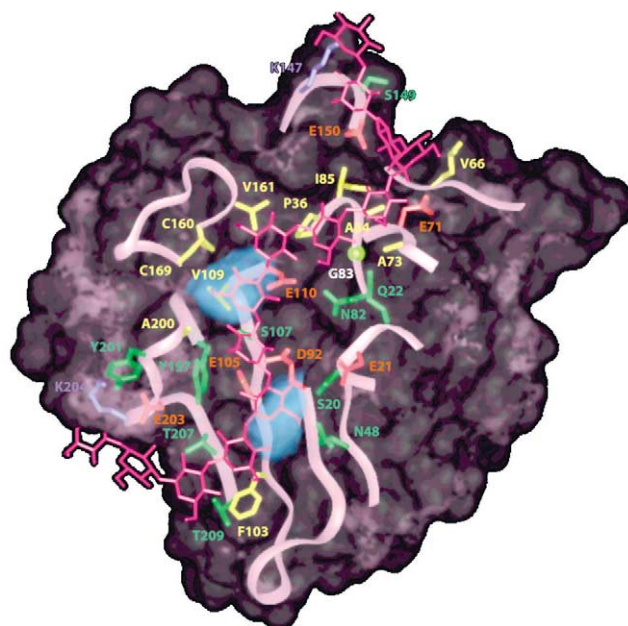


Fig. 4. Diagram showing the amino acid residues of the cleft of Pru-TLP susceptible to interact with the docked (1,3)- β -D-glucan chain (pink) at pH 5.0. Labeled residues colored orange, blue, green and yellow correspond to acidic, basic, aromatic and hydrophobic residues, respectively. The two spots colored light blue correspond to hydrophobic cavities occurring on the surface of the cleft. The molecular surface (colored black) of Pru-TLP was built with GRASP [39].

fruit-specific TLP from elderberry that has no antifungal properties in vitro in spite of the presence of a strongly acidic cleft in its structure [14].

Two of the tested fruit-specific TLP, namely Pru-TLP and Mal-TLP clearly exhibit a reasonably high endo- $\beta(1,3)$ -glucanase activity. Molecular modeling and docking studies showed that the acidic cleft accounts for the weak endo- $\beta(1,3)$ -glucanase activity of these fruit-specific TLP. However, these studies also demonstrated that there are besides the acidic character some structural requirements, the cleft needs to meet in order to acquire $\beta(1,3)$ -glucanase activity. An apparently crucial structural feature of the cleft is the presence of Asp and Glu residues that are correctly positioned to act as proton donor and nucleophile residue in a retaining or inverting mechanism of glucan hydrolysis. The endo- $\beta(1,3)$ -glucanase activity of the TLP also requires a favorable positioning of the (1,3)- β -D-glucans towards the catalytically active acidic residues in order to be cleaved. Both in Pru-TLP and Mal-TLP several more or less favorable locations can be predicted for (1,3)- β -D-glucans inside the cleft, which can explain the endo- $\beta(1,3)$ -glucanase activity of both proteins. A survey of the available amino acid sequences of other TLP and glucan-hydrolases clearly indicates that the location of the catalytically active acidic residues (Glu⁷¹ and Glu¹⁵⁰ in Pru-TLP) at the top of an extended cleft is quite unusual among endoglucanases. The active residues of endoglucanases (family 17 of glucan-hydrolases, E.C. 3.2.1.39) most currently occur in the center of a cleft that extends across the surface of the enzyme [41], and are separated by a distance of ~5–6 Å that favors a retaining mechanism of hydrolytic

cleavage of glycan polymers. It should be emphasized, however, that the enzymatic activity of Pru-TLP and Mal-TLP is very low as compared to that of genuine glucanases. Therefore, the proposed role of TLP in tissue softening (through the degradation of cell wall glycans) during fruit ripening [22] should be interpreted with care. This does not imply that TLP play no role at all in fruit ripening. On the contrary, one can imagine that the generation of short (1,3)- β -D-glucans triggers some defense responses in fruits. Alternatively, the fruit-specific TLP may be attractive proteins with a pleasant taste for fructiferous animals that help to disseminate the seeds [23].

Irrespective of the exact physiological function in fruits, the TLP found in edible fruits are also important in view of food safety because they have been recognized as being potent food allergens susceptible to trigger allergic diseases in sensitive individuals [42,43]. It is important to note in this context that an allergenic IgE-binding epitope has been identified on the surface of the mountain cedar (*Juniperus ashei*) allergen Jun a3, a typical PR-5 protein present in pollen [44]. As shown in the three-dimensional model built for Jun a3, the main IgE-binding epitope coincides with the main α -helix of the second C-terminal domain (II). This domain II delineates the acidic cleft of the TLP and its main α -helix is well conserved in PR-5 proteins and, particularly in fruit-specific TLP (P. Rougé, unpublished results). Especially worthy of mention is, in this respect, the apparent structural relationship which seems to exist between the glycan-binding and allergenic properties in fruit TLP.

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