

Different Structural Behaviors Evidenced in Thaumatin-Like Proteins: A Spectroscopic Study

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Abstract Three proteins belonging to the thaumatin-like proteins family were compared in this study from a structural point of view: zeamatin, a new recently isolated PR-5 from *Cassia didymobotrya* and the commercial sweet-thaumatococcus. The former two proteins possess antifungal activities while commercial thaumatin is well known to be a natural sweetener. Intrinsic fluorescence studies have evidenced that the three proteins behave differently in unfolding experiments showing different structural rigidity. All the three proteins are more stable at slight acidic buffers, but sweet-thaumatococcus has a major tendency to destructure itself. Similar observations were made from circular dichroism studies where a structural dependence relationship from the pH and the solvent used confirmed a hierarchic scale of stability for the three proteins. These structural differences should be considered to be significant for a functional role.

Keywords Unfolding · Intrinsic fluorescence · Circular dichroism · Thaumatin-like protein

List of abbreviations

PRP	Pathogenesis related protein
TLPs	Thaumatin-like proteins
Gdn/HCl	Guanidine hydrochloride
CHES	Cyclohexylaminoethanesulfonic acid
Tris	Tris (hydroxymethyl)aminomethane
Bis-Tris	Bis (2-hydroxyethyl)imino-tris(hydroxymethyl)aminomethane
CD	Circular dichroism
TFE	2,2,2-Trifluoroethanol
MWR, θ	Molar mean residue ellipticity
CdTLP	<i>Cassia didymobotrya</i> Thaumatin-like protein
ATR/FT-IR	Attenuated Total Reflectance—Fourier Transform Infra Red
MALDI-ToF	Matrix-assisted laser desorption ionization-time of flight
SDS–PAGE	Sodium dodecylsulfate–polyacrilamide gel electrophoresis
Trp	Tryptophane
Phe	Phenylalanine
Tyr	Tyrosine

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1 Introduction

Plants protect themselves from pathogen attacks by producing proteins and peptides with antimicrobial properties [1]. These proteins, known as pathogenesis-related (PR) proteins, ubiquitous in monocots and dicots, are classified into seventeen families on the basis of their structural similarity, serologic, immunologic and functional relationship [2]. The PR-5 group consists of proteins that share primary sequence similarity with thaumatin, a

sweet-tasting protein isolated from *Thaumatococcus daniellii* [3]. For this reason they are named thaumatin-like proteins (TLPs). The structural architecture of TLPs is composed of three domains: domain I, the compact core of the molecule, consists of a flattened β -sandwich formed by two β -sheets of 5 and 6 β -strands respectively; domain II contains an extended α -helix associated with three shorter α -helices and domain III consists of two short β -strands linked to an extended loop. The structure of the TLPs is stabilized by eight disulphide bridges well conserved in all the TLPs [4–8]. Although the specific function of many PR-5 proteins in plants is unknown, elucidation of antifungal, glycan-binding and allergenic properties has boosted the knowledge of their three-dimensional structure. Until now few of the PR-5 proteins including sweet-thaumatococcal (1RQW) [4], tobacco osmotin (1AUN and 1PCV) [9], Maize permatin (1DU5) [6], and banana TLP [10], have been fully characterized from a structural point of view. The antifungal properties are particularly interesting, because some of the PR-5 proteins are also active against human pathogenic fungi. The exact mechanism of the antifungal activity is still not clear. It has been proposed that the presence of an electronegative cleft formed by the closure of domain I and II, could be responsible for the interaction between the protein and a cationic charged receptor placed on plasma membrane of the fungi. This interaction would cause a change in the pH gradient across plasma membranes with a consequent increase in permeabilization and leakage of the cell content [11, 12].

In this study we compare the structural features of two antifungal TLPs, zeamatin and a recently isolated PR-5 from *Cassia didymobotrya* [13], with that of commercial sweet thaumatin as a function of pH and solvent conditions, in order to evaluate structural differences which could help in elucidate their structure-activity relationship.

2 Materials and Methods

2.1 Proteins and Reagents

Thaumatococcal was purchased from Sigma and further purified by cationic exchange chromatography on a High-Trap 5 mL, Q-sepharose column (Pharmacia) equilibrated in sodium acetate buffer (pH 6.0, 25 mM). The column was developed with a linear gradient of NaCl (0–0.8 M). CdTLP was isolated and purified from *Cassia didymobotrya* (Fres.) cell cultures as described [13]. Zeamatin was purified as reported [14] from seeds of *Zea mays* acquired at a local market. Guanidine hydrochloride (Gdn/HCl) was purchased from Fluka (Buchs, Switzerland) and used without further purification. Other chemicals were from Sigma.

2.2 Fluorescence Studies

Fluorescence emission spectra ($\text{Ex}^\lambda = 280 \text{ nm}$) were recorded at 25 °C in a 1 cm quartz cell, using a Spex (Edison, USA) Fluoromax spectrofluorimeter as described [15, 16]. Proteins were dissolved in a buffering solution of 10 mmol/L cyclohexylaminoethanesulfonic acid (CHES) ($\delta\text{pKa}/\delta\text{T} = 0.029$), 5-mmol/L tris (hydroxymethyl)aminomethane (Tris) ($\delta\text{pKa}/\delta\text{T} = -0.028$) and 5 mmol/L bis (2-hydroxyethyl)imino-tris(hydroxymethyl)aminomethane (bisTris) ($\delta\text{pKa}/\delta\text{T} = 0.0$). The composition of the buffer ensured minimal perturbations in the pH and ionic strength of the solution over the studied temperature and pH range [17]. Calcium binding to proteins, temperature and guanidine hydrochloride effect on the conformation of the macromolecules were studied as described [17].

The thermodynamic approach used to describe the unfolding reaction of each protein was based on a two-state model, where N is the native state, U is the unfolded state of the molecule and K_{un} is the unfolding equilibrium constant [18]; the free energy change for unfolding can be described by the following equation:

$$\Delta G_{\text{un}}^0 = -RT \ln K_{\text{un}} = \Delta H_{\text{un}}^0 - T\Delta S_{\text{un}}^0 \quad (1)$$

ΔH_{un}^0 and ΔS_{un}^0 are the enthalpy and entropy changes respectively for the unfolding transition. If the protein unfolding is induced by the addition of a chemical denaturant $[d]$, the following linear relationship is generally accepted to describe the thermodynamics of the system [19, 20]:

$$\Delta G_{\text{un}}^0 = \Delta G_{0,\text{un}}^0 - m[d] \quad (2)$$

where $\Delta G_{0,\text{un}}^0$ is the free energy change for the unfolding reaction at the reference condition in the absence of denaturant and m is an empiric parameter indicating the denaturant susceptibility (i.e. $m = -\delta G_{\text{un}}^0/\delta[d]$, the dependence of δG_{un}^0 on denaturant concentration). Intrinsic protein fluorescence change versus guanidine concentration is described by the following equation [18]:

$$F = \frac{F_{\text{ON}} + s_{\text{N}}[d] + (F_{\text{OU}}[d])e^{(-\Delta G_{0,\text{un}}^0 + m[d])/RT}}{1 + e^{(-\Delta G_{0,\text{un}}^0 + m[d])/RT}} \quad (3)$$

In this equation F_{ON} and F_{OU} are the intensities of native and unfolded states in the absence of denaturant, s_{N} and s_{U} are the baseline slopes for the native and unfolded regions. An useful parameter linked to the stability of the protein is the $\text{Gdn}_{50\%}^{\text{F}}$ defined as the ratio of $\Delta G_{0,\text{un}}^0$ and m indicating the denaturant concentration inducing the 50% of fluorescence intensity change (e.g. the mid-point of the curve) [21].

The fluorescence peak change as a function of guanidine concentration was fitted using a phenomenological equation to calculate the guanidine concentration inducing the 50% effect on the peak shift:

$$F_{\text{peak}} = a + \frac{(b - a)}{1 + \left(\frac{c}{[\text{Gdn}]}\right)^d} \quad (4)$$

In this equation c represents the guanidine concentration ($\text{Gdn}_{50\%}^P$) at the mid-point of the curve and a , b and d are fitting parameters.

2.3 Circular Dichroism Measurements

Far-UV circular dichroism (CD) spectra of thaumatin, *CdTLP* and zeamatin were collected at 25 °C using a Jasco-600 spectrophotometer with cuvettes having a 0.1 cm path length. All experiments were performed in a 20 mM phosphate buffer (pH 5.5 and 7.5) and in phosphate buffer with trifluoroethanol (TFE) mixture 30% at the same pHs. Protein concentrations were of 0.22 mg/mL for zeamatin, 0.30 mg/mL for *CdTLP* and 0.28 mg/mL for commercial thaumatin. The spectra were registered from 200 to 250 nm at a scan speed of 20 nm/min with a time response of 4 sec, and a data pitch of 0.1 nm. All the spectra were baseline-corrected. The molar mean residue ellipticity [MWR, θ] was expressed in degrees $\text{cm}^2 \text{dmol}^{-1}$, and calculated as $[\theta] = \theta_{\text{obs}} \text{MWR}/(10 l c)$, where θ_{obs} is the observed ellipticity in degrees; MWR, the mean residue molar weight of the protein (107.07, 107.14 and 107.26 for zeamatin, *CdTLP* and thaumatin, respectively); l , the optical path length in centimetres; and c , the protein concentration in grams per millilitres.

2.4 ATR-FT-IR Measurements and Analysis of Data

ATR/FT-IR spectra were recorded on a *Spectrum One* (Perkin-Elmer) spectrophotometer equipped with an ATR accessory with a ZnSe reflection element. Spectra were recorded after 40 scans at a 1 cm^{-1} of resolution. Before protein analysis, an open beam background spectrum of clean crystal was recorded. Subsequently the buffer solution spectrum was recorded followed by the protein spectrum measurement. The spectra of buffers were hence subtracted from the protein ones. During measurements performed at 25 °C, the crystal was continually flushed with nitrogen to eliminate residual water vapors. Proteins were dissolved in 25 mM phosphate buffer and 2 μL of each solution were deposited onto the ATR accessory.

Spectra were recorded in the range between 2500 and 1100 cm^{-1} .

Data analysis was made with Perkin-Elmer *Spectrum One* software. Amide I analysis of each protein was performed with Peak Fit 4.12 (Sea Solve Software, Inc., San Jose, CA) program. ATR FT/IR spectra were used to obtain a second derivative. A 17% smoothing process, employing the Savitzky-Golay algorithm, was performed and the resulting peaks were used as a reference for the subsequent peak fitting analysis performed with the same program. A linear baseline was employed and Gaussian peaks were produced after an iterative adjustment of data until the SSE statistical parameter was under 1×10^{-4} , indicating a good fitting analysis. The resulting peak areas of Amide I were used to determine the contribution of each secondary structure motif.

3 Results and Discussion

Zeamatin was purified from seeds to homogeneity following the procedure reported from Wilson et al. [22], *CdTLP* was purified from the medium of a suspension cell culture of *Cassia didymobotrya* as described [13], while commercial thaumatin was further purified by passage on a cationic exchange column. Each protein was analyzed to assess its purity by MALDI-ToF mass analysis and SDS-PAGE (data not shown).

Intrinsic Fluorescence studies were performed in order to follow the performance of zeamatin, *CdTLP* and thaumatin during folding and unfolding experiments, in presence of guanidine hydrochloride as unfolding inducer.

In Table 1 are reported, for each protein, the number of aromatic residues (Trp, Phe, Tyr), the fluorescence emission peaks at 280 nm of the folded and unfolded species, the percentage of fluorescence variation upon unfolding transition and the $\text{Gdn}_{50\%}^F$ and $\text{Gdn}_{50\%}^P$, calculated according to Eqs. 3 and 4, respectively, as function of the pH.

Fluorescence emission spectra ($\text{Ex}^\lambda = 280 \text{ nm}$) of folded (close symbols) and unfolded (open symbols) proteins at pH 7.5 are reported in Fig. 1a–c, respectively. Proteins, similar in aromatic aminoacid composition, showed differences both in the fluorescence maximum emission peak and in the intensity variation upon unfolding transition. A similar behavior was observed when the excitation wavelength was set at 260 or 295 nm (data not shown).

Figure 2a, b shows protein fluorescence intensity and maximum emission peak as a function of guanidine hydrochloride concentration at $\text{Ex}^\lambda = 280 \text{ nm}$. The fluorescence intensity increases upon denaturation, an uncommon but not rare behavior observed in immunoglobulin-like domains [15].

Table 1 In this table are reported, for each proteins, the number of aromatic residues (Trp, Phe, Tyr), the fluorescence emission peaks at 280 nm of the folded and unfolded species, the percentage of fluorescence variation upon unfolding transition and the $Gdn_{50\%}^F$ and $Gdn_{50\%}^P$, calculated according to Eqs. 3 and 4, respectively, as function of the pH

Protein	Number of Trp	Number of Phe	Number of Tyr	pH	Peak folded (nm)	Peak unfolded (nm)	Fluorescence change upon unfolding (%)	$Gdn_{50\%}^F$ ($\Delta G_{0,un}^F/m$) (mol/L)	$Gdn_{50\%}^P$ (mol/L)
Cd/TLP	1 expected 2	0 expected 7	3 expected 8	5.5	325	353	+238	3.7 ± 0.3	3.3 ± 0.2
				7.5	325	355	+238	3.0 ± 0.3	2.6 ± 0.1
				9.5	327	355	+232	3.1 ± 0.3	2.6 ± 0.2
Thaumat	3	11	8	5.5	337	354	+66	2.1 ± 0.2	1.9 ± 0.2
				7.5	337	355	+78	1.7 ± 0.1	1.5 ± 0.1
				9.5	337	353	+80	1.3 ± 0.1	1.4 ± 0.1
Zeamat	3	12	8	5.5	333	352	+134	2.7 ± 0.2	2.5 ± 0.2
				7.5	333	353	+176	2.2 ± 0.2	2.1 ± 0.2
				9.5	333	353	+191	1.6 ± 0.1	1.7 ± 0.1

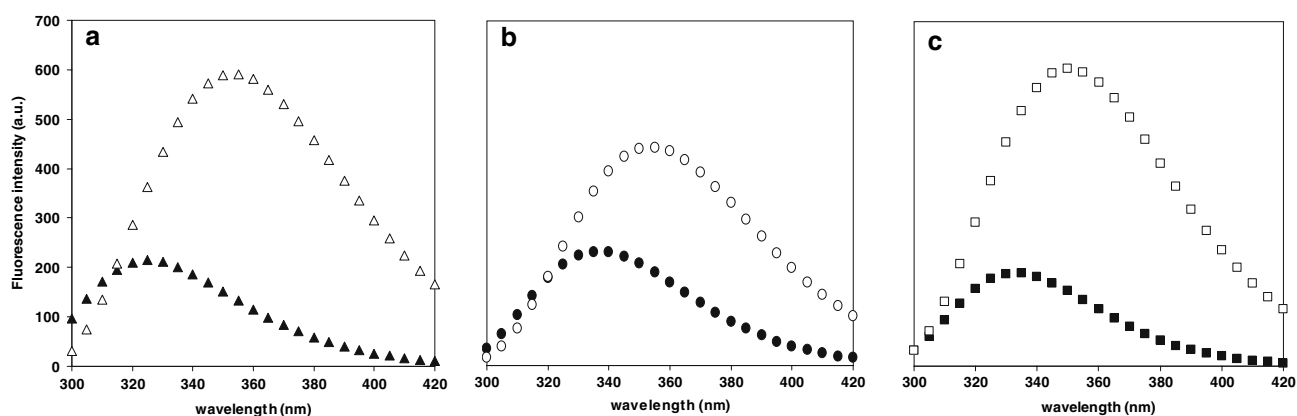


Fig. 1 Fluorescence emission spectra ($Ex^{\lambda} = 280$ nm) of folded/unfolded CdTLP ($\blacktriangle/\triangle$, Fig. a) thaumat ($\bullet/\circ$, Fig. b) and zeamat ($\blacksquare/\square$, Fig. c). Spectra were collected at 25 °C at pH 7.5 in a 1 cm

quartz cell, in a buffer solution mixture of 10 mmol/L CHES, 5 mmol/L TRIS and 5 mmol/L bis-TRIS, in order to ensure minimal perturbation in the pH and ionic strength of the solution

Tryptophan residues, highly sensitive to solvent effects, dominate protein fluorescence. Bluer emitting tryptophan residues are those with the indole ring surrounded by other apolar amino acid side chains and interactions between excited state and polar solvent molecules or other polar functional groups, cause redder fluorescence. Typically, the exposure to solvent of aromatic amino acids leads to a decrease in their fluorescence intensity due to water quenching effect and a red shift of maximum emission wavelength. However, in some proteins, such as immunoglobulin-like domains, the interactions between tryptophan and tyrosine residues in the buried region of the folded molecule, lead to a mutual quenching that is stronger than the quenching effect of the solvent. This leads to determining an increased fluorescence intensity after unfolding process. For CdTLP the fluorescence emission maximum is suggestive of buried emitting tryptophans (325 nm). The

fluorescence change upon unfolding is larger than 200% indicating a strong mutual quenching effect of tryptophan and tyrosine residues and suggesting a native compact structure of the protein. In addition the thermodynamic parameters suggest the CdTLP to be a very stable protein needing high concentration of denaturing agent ($Gdn_{50\%}^F$ and $Gdn_{50\%}^P > 3M$) to induce the unfolding reaction. Completely different consideration can be made for commercial thaumat. Red shifted emission peak (337 nm), fluorescence modification linked to the unfolding reaction of $\approx 70\%$, low $Gdn_{50\%}^F$ and $Gdn_{50\%}^P$ values are all phenomena suggestive of unsteady or partially unfolded protein. An intermediate behavior is observed for zeamat in which a pH-dependent stability relationship is more evident as depicted in Fig. 3 where the curves of folding-unfolding transitions for zeamat at different pHs are reported. Like thaumat, lower pH increases stability of the

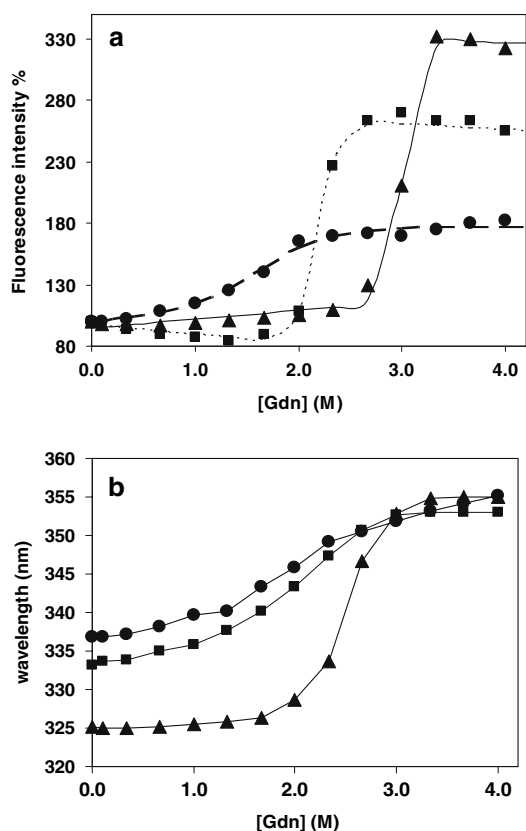


Fig. 2 Fluorescence intensity (a) and maximum emission peak (b) changes as a function of guanidine hydrochloride concentration of CdTLP (▲), thaumatin (●) and zeamatin (■). Spectra were collected at 25 °C at pH 7.5 in a 1 cm quartz cell, in a buffer solution mixture of 10 mmol/L CHES, 5 mmol/L TRIS and 5 mmol/L bis-TRIS, in order to ensure minimal perturbation in the pH and ionic strength of the solution

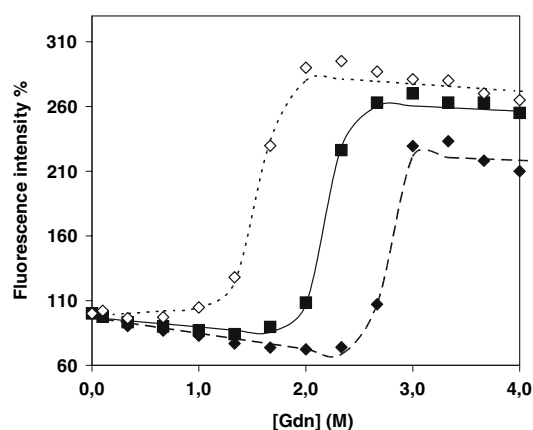


Fig. 3 Zeamatin fluorescence intensity as a function of guanidine hydrochloride concentration at pH 5.5 (◆), 7.5 (■) and 9.5 (◇). Guanidine hydrochloride concentrations ranged from 1.0 to 4.0 M

zeamatin (see Table 1) almost doubling the $Gdn_{50\%}^F$ and $Gdn_{50\%}^P$ for the unfolding reaction in respect to alkaline condition. No differences were found in protein

fluorescence properties as function of calcium concentration (data not shown).

Analysis of the far-UV CD spectra shows different behaviors of the three proteins, depending on the buffer composition and the pH (Figs. 4a,b and 5a,b). The zeamatin spectra at pH 7.5 (Fig. 4a) and 5.5 (Fig. 4b) are characterized by a negative minimum at 210 nm and by a weak band at 229 nm. A similar curve is obtained in TFE at pH 5.5 (Fig. 5b), where the band at 229 becomes weaker, but the overall characteristics of the spectrum remains unaltered. A different observation is made at pH 7.5 in TFE 30% where the negative minimum shifts at 205 nm and a complete lost of the band at 229 nm is accompanied by the formation of a negative broad band around 225 nm suggesting the incipient formation of alpha helical structures (Fig. 5a).

Commercial sweet thaumatin at pH 5.5 and 7.5 in aqueous buffers (Fig. 4a,b), shows spectra resembling those of zeamatin with some slight variations. The cross-over for thaumatin is at 200 nm and the negative minimum is at 209 nm. The main differences arise from the spectra recorded in TFE at pH 7.5 and 5.5 (Fig. 5a,b). Under these

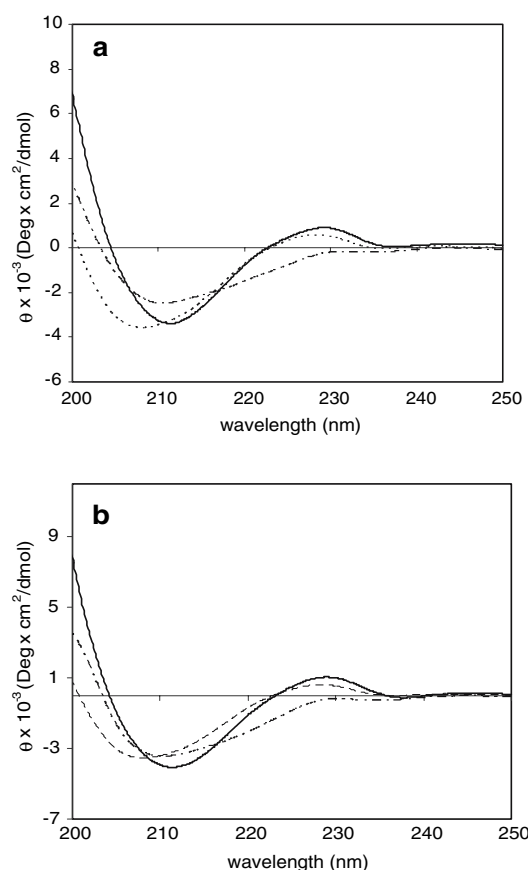


Fig. 4 Far UV-CD spectra of zeamatin (—), thaumatin (---) and CdTLP (---). Spectra were recorded at 25 °C in 20 mM phosphate buffer at pH 7.5 (a) and 5.5 (b) using a 0.1 cm quartz cell

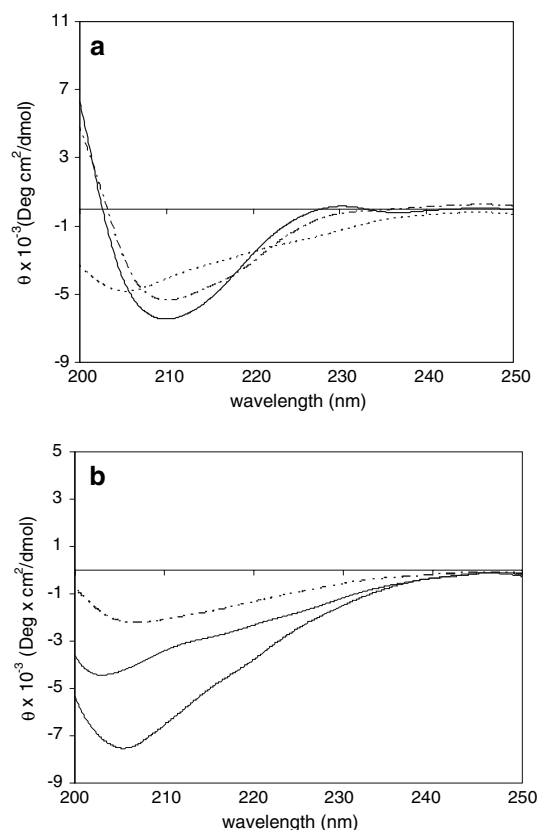


Fig. 5 Far UV-CD spectra of zeamatin (—), thaumatin (---) and *CdTLP* (— · —) in 30% TFE solution (v/v) in 20 mM phosphate buffer at pH 7.5 (**a**) and in 30% TFE solution (v/v) at pH 5.5 (**b**)

conditions the positive band at 229 nm present in aqueous buffer is replaced by a negative broad band around 220 nm while the negative minimum shifts to 200 nm suggests a loss of the β -sheet structures in favor to α -helix and random-coil motifs.

Both zeamatin and sweet thaumatin exhibits quite unusual spectra for a protein consisting mainly of β -sheet and β -turn structures, but they are characteristic for the proteins belonging to the TLPs family [23]. *CdTLP* spectra in aqueous buffer both at pH 7.5 and 5.5 are characterized by a negative band around 210 nm and a crossover at 204 nm as observed in zeamatin (Fig. 4a,b). The weak positive band at 225 observed in zeamatin and in sweet thaumatin is instead absent, being replaced by a slight negative signal at 235 nm, resembling better in this case the spectrum of a beta strand rich protein. In TFE, the CD spectrum of *CdTLP* shows, like zeamatin, a pH dependent behavior. At pH 5.5 (Fig. 5b) the curve is similar to that obtained at the same pH in aqueous buffer (Fig. 4b), while a more relaxed structure is observed at pH 7.5 characterized by a shift and a weaker intensity of the band at 210 and a broadening of the negative band around 225 nm, suggestive of an enhancement in alpha helices content (Fig. 5a). From a

functional point of view, a comparison of the three proteins is more intriguing at pH 5.5 in presence of TFE (Fig. 5a). The hydrophobic environment created by this solvent and the acidic pH were chosen to mimic the fungal plasma membrane. Under these conditions zeamatin, in a manner similar to *CdTLP*, maintain the structure observed in aqueous solution. Sweet thaumatin, on the contrary, loses its native structure exhibiting a more relaxed architecture. These data are in good agreement with the fluorescence studies where a similar pH-dependent stability relationship was observed for the three proteins.

In order to get a complete comparison of the structure arrangement of the three proteins, ATR/FT-IR experiments were also performed. The spectroscopic signals in the IR bands derive from $\nu(\text{C}=\text{O})$, $\delta(\text{N}-\text{H})$ and $\nu(\text{C}-\text{N})$ vibrations, while the CD data arise from electronic transitions; these two spectroscopic methods complement each other giving different and independent structural information. In fact, while CD measurements give more accurate estimations of the α -helix content, IR is more sensitive to β -sheets and β -turns structures typical for the class of proteins considered in this study. The results obtained from CD and FT/IR (Fig. 6) analyses show that the three TLPs considered in this study possess very similar secondary structure arrangements. This findings were supported by the following Amide I analysis (Fig. 7a–c). It is generally accepted that a second derivative followed by a deconvolution operation of the Amide I ($1600\text{--}1700\text{ cm}^{-1}$), represents a good description of the contribution of the various secondary structure motifs in a given protein [24, 25]. The data obtained in this study for zeamatin (1DU5) and thaumatin (1RQW) are in good agreement with the crystallographic data deposited in Protein Data Bank for the same proteins, confirming the goodness of this method for these proteins (Tables 2 and 3). At the same time the analysis of *CdTLP* Amide I gave secondary structure motif percentages comparable to thaumatin and zeamatin

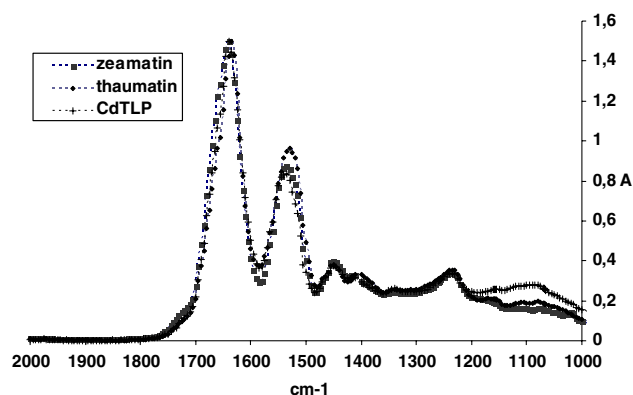


Fig. 6 ATR/FT-IR spectra of zeamatin, thaumatin and *CdTLP* in 25 mM phosphate buffer at pH 7.0

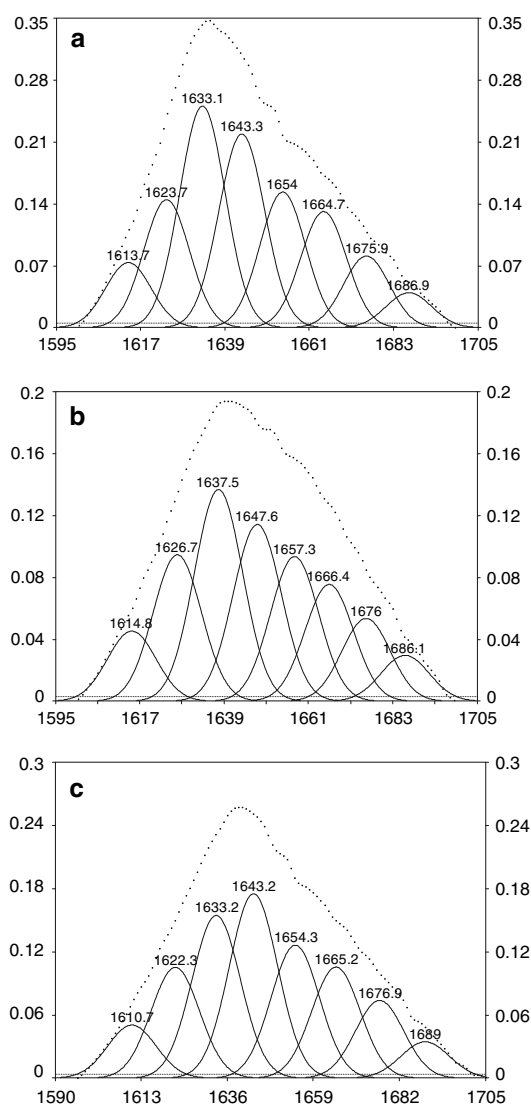


Fig. 7 Amide I ($1600\text{--}1700\text{ cm}^{-1}$) deconvolution of thaumatin (a), zeamatin (b) and CdTLP (c) after curve fitting of the second derivative

Table 2 List of centroids, fractional areas and assignments of the Amide-I bands of thaumatin (PDB: 1RQW)

Centroid	% Area	Assignment	Total contribute (%)	PDB (1DU5)
1615	8	Side chain	8	—
1627	15	β -Sheet	36	34
1637	21	β -Sheet		
1647	18	Unordered	18	—
1657	14	α -Helix	14	15
1666	12	Turns		
1676	8	Turns	24	21
1686	4	Turns		
100			100	

In the last column are listed the percentages for the secondary structure motifs obtained from PDB crystallographic data

Table 3 List of centroids, fractional areas and assignments of the Amide-I bands of zeamatin (PDB: 1DU5)

Centroid	% Area	Assignment	Total contribute (%)	PDB (1RQW)
1614	7	Side chains	7	—
1624	13	β -Sheet	36	36
1633	23	β -Sheet		
1643	20	Unordered	20	—
1654	14	α -Helix	14	15
1665	12	Turns		
1676	7	Turns	23	22
1687	4	Turns		
100			100	

In the last column are listed the percentages for the secondary structure motifs obtained from PDB crystallographic data

(Table 4), confirming the great structural similarity shared among the three TLPs considered as also evidenced by CD data.

TLPs are a class of multifunctional proteins. Some of them are able to permeabilize the plasma membrane and to act as inhibitors of amylases enzymes, other act as glucanase-like enzymes, other like sweet thaumatin, as signal molecule. Their expression is also enhanced by diverse biotic and abiotic stimuli evidencing their implication in more complex metabolic networks. For this reasons the study of the structure-activity relationship of TLPs is crucial in order to understand their functional role.

The data presented in this study focalized on the different ability in expressing an antifungal activity from the three proteins considered, reveal that despite a high structural similarity, zeamatin, thaumatin and CdTLP show in a membrane mimicking environment, very different folding characteristics.

The greater flexibility exhibited by thaumatin is probably important for the signalling function of this proteins as an animal attractor. A flexible structure could be more adaptable to diverse shapes of receptors, favoring the

Table 4 List of centroids, fractional areas and assignments of the Amide-I bands of *Cassia didymobotrya* TLP (P84334)

Centroid	% Area	Assignment	Total contribute (%)
1611	6	Side chains	6
1622	13	β -Sheet	32
1633	19	β -Sheet	
1643	21	Unordered	21
1654	15	α -Helix	15
1665	13	Turns	
1677	9	Turns	26
1689	4	Turns	
100			100

recognition of sweet taste by different animal species. On the other hand a more rigid structure as shown by zeamatin and CdTLP, could be important for the recognition of a specific receptor in different pathogenic species. In fact although all of the known antifungal PR-5 proteins are similar in their overall structures and in the acidic cleft regions, they show different activities and specificities against their target cells, a phenomenon likely due to a specie-specific molecular recognition. Moreover maintenance of a correctly folded structure could be essential in keeping the cleft region in the right position in order to exploit the β -glucanase enzymatic activity proposed for some TLPs [26–29].

The acidic cleft is considered to be a crucial feature for the expression of the antifungal activity of TLPs [8, 12]. The authors propose that the structural arrangements that may occur in the fungal plasma membrane, should be also considered in order to fully comprehend the pathogen–protein interaction mechanisms. To shed light on this problem, more detailed information may be obtained by employing appropriate mutant forms of antifungal TLPs. These studies would be justified in the view of potential applications of these proteins as pest controller in agriculture and in medical field as new antifungal drugs.

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