Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases

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Abstract Barley LTP1 belongs to a large family of plant proteins termed non-specific lipid transfer proteins. The in vivo function of these proteins is unknown, but it has been suggested that they are involved in responses towards stresses such as pathogens, drought, heat, cold and salt. Also, the proteins have been suggested as transporters of monomers for cutin synthesis. We have analysed the stability of LTP1 towards denaturant, heat and proteases and found it to be a highly stable protein, which apparently does not denature at temperatures up to 100°C. This high stability may be important for the biological function of LTP1.

Key words: Non-specific lipid transfer protein; Barley; Protein stability; Protease digestion

1. Introduction

A group of proteins termed non-specific lipid transfer proteins (ns-LTPs) is found in plants. These proteins were originally identified by their ability to catalyse the transfer of lipids between membranes in vitro [1]. However, the original suggestion that they would act as intracellular transporters of lipids between organelles was contrasted by the finding that ns-LTPs are synthesised with functional N-terminal signal peptides [2] and are found extracellularly. Other functions have been ascribed to ns-LTPs, including transport of cutin monomers [3] and involvement in flowering [4]. Also, ns-LTPs have been suggested to be important in several types of plant stress responses. These include responses towards pathogens [5], drought [6] and temperature changes [7,8]. In spite of these implications it is not clear which specific role ns-LTPs play [9,10].

ns-LTPs are small proteins of about 90 residues with high values of pI (>9). The three-dimensional structures of ns-LTPs from several plant species are known and they all consist of four α-helices held together by four conserved disulphide bonds [11]. Fruit ns-LTPs have been identified as allergens [12], and it has been suggested that this is in part due to a high stability [13]. Barley and wheat ns-LTPs survive exposure to proteases during germination [14]. Also, since ns-LTPs may in general be involved in stress responses, it appears that high stability might be important for the biological function. We therefore decided to study the thermodynamic and proteolytic stability of an aleurone-specific barley ns-LTP, LTP1 [15,16]. In addition to its potential roles in the cell, the stability of LTP1 is also of technological importance [17]. Here we report on our experiments using denaturant- and heat-induced unfolding as well as resistance towards proteolytic digestion. Our findings show that LTP1 is a highly stable protein. To our knowledge, this is the first thermodynamic analysis of the stability of a plant ns-LTP.

2. Materials and methods

2.1. Materials

Barley LTP1 was purified essentially as described [18]. More than 98% of the protein contained the modification described earlier [19]. Foam-LTP1 and folded LTP1 purified from beer [17] were kind gifts from P. Vaag. *N*-Acetyl-tyrosine amide was from Bachem. Thermolysin was from Fluka. Pepsin was from Merck. The protease substrate I-CPY [20] was produced by denaturing yeast carboxypeptidase Y (purified as described previously [21]) for 3.5 h at room temperature in 7 M urea, 90 mM sodium phosphate pH 8 and removing the urea on a NAP-5 column (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris–HCl pH 7.6.

2.2. Denaturant-induced unfolding

Denaturant-induced unfolding was studied by measuring the fluorescence on a Perkin-Elmer LS 50B luminescence spectrophotometer ($\lambda_{ex} = 275$ nm, $\lambda_{em} = 303$ nm) of 2 μM solutions of LTP1 in 30 mM buffer (potassium citrate pH 3.2, potassium succinate pH 5.1, potassium phosphate pH 7.2 or Tris–HCl pH 8.5) and between 0 and 8.5 M guanidine hydrochloride (GuaHCl). The solutions were allowed to equilibrate overnight at 25°C. Data were analysed by non-linear least-squares regression to the equation from [22] using KaleidaGraph (Synergy Software).

2.3. Heat-induced unfolding

For measuring the temperature dependence of LTP1 fluorescence, solutions containing between 2.5 and 10 μM LTP1 in 0.1 M buffer (potassium citrate pH 3.2, potassium succinate pH 5.1 or potassium phosphate pH 7.2) were thermostated to temperatures between 25 and 90°C by coupling the cuvette holder to an external water bath. For comparison, we measured the fluorescence from a 8 μM solution of *N*-acetyl-tyrosine amide.

Differential scanning calorimetry (DSC) was carried out in an MCS Differential Scanning Calorimeter using 2.7 mg ml^{-1} LTP1 in 50 mM potassium phosphate buffer pH 7.2. The scan rate was 1 K min^{-1}. A blank scan with buffer in both calorimeter cells was subtracted in order to correct for differences between the cells.

For analysing thermolysin resistance, either 7 μg LTP1 or 3 μg of the control substrate I-CPY was incubated in 0.5 ml of 20 mM $CaCl_2$, 1 mM $ZnCl_2$, 50 mM Tris pH 7.6 at 70°C in the presence or absence of 5 μg thermolysin. Reaction mixtures were overlaid with oil to prevent evaporation. To ensure that digestion only occurred at

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Abbreviations: DSC, differential scanning calorimetry; GuaHCl, guanidine hydrochloride; ns-LTP, non-specific lipid transfer protein

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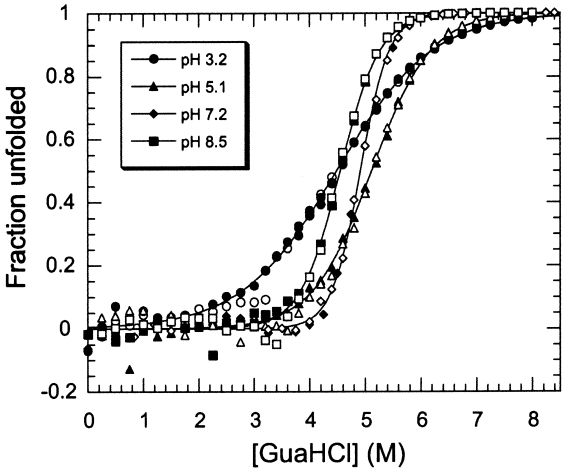

Fig. 1. Denaturant-induced unfolding of LTP1 at different values of pH. Tyrosine fluorescence from LTP1 was measured at several concentrations of GuaHCl at 25°C. The results have been normalised according to a two-state model to show the fraction of unfolded protein. Closed symbols are from denaturation experiments while open symbols represent renaturation.

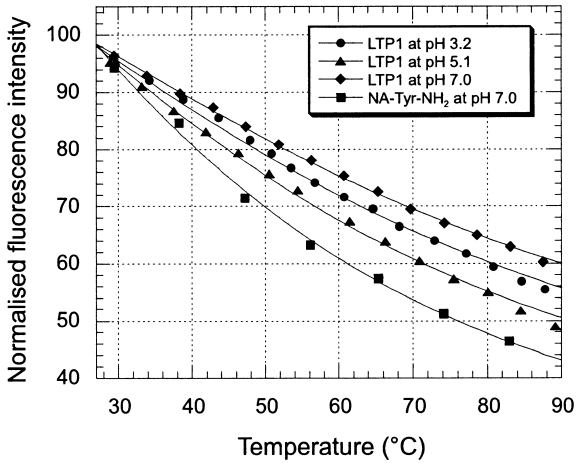

Fig. 2. Fluorescence from LTP1 as a function of temperature compared to that from *N*-acetyl-tyrosine amide (NA-Tyr-NH_2). Fluorescence intensities have been normalised. Solid lines represent non-linear fits to an equation from [27].

70°C, the reactions were preincubated for 5 min at this temperature before thermolysin was added. To check whether thermolysin itself was inactivated during these 30 min incubations, a reaction with thermolysin only was incubated for 30 min at 70°C before I-CPY was added. Digestions were quenched by precipitation with trichloroacetic acid. Samples were analysed by 16% SDS–PAGE [23].

2.4. Pepsin digestion

Pepsin digestions were carried out in 20 μl of 10 mM HCl. 3 μg LTP1, foam-LTP1, native LTP1 purified from beer or I-CPY were incubated in the presence or absence of 1 μg pepsin and analysed by 16% SDS–PAGE [23].

3. Results

3.1. Determination of protein stability

Among the available techniques for determining a protein's thermodynamic stability, the most commonly used are DSC [24] and denaturant-induced unfolding [25]. By perturbing the protein's stability by either heat or addition of denaturant, these methods allow the detection of protein unfolding at equilibrium. In contrast, protease sensitivity can provide information concerning transient unfolding of proteins, thereby complementing equilibrium techniques. We have studied the stability of LTP1 by all of these techniques.

3.2. Denaturant-induced unfolding

Denaturant-induced unfolding was studied by measuring fluorescence from the three tyrosines in LTP1 at increasing concentrations of GuaHCl. Normalised fluorescence intensities at four pH values between 3.2 and 8.5 are shown in Fig. 1. Coincidence between denaturation and renaturation experiments showed that the unfolding reaction is reversible. Before normalisation, data were analysed according to a two-state model using the linear extrapolation method [26] by non-linear least-squares regression [22]. This analysis results in the estimation of three interrelated thermodynamic parameters: D_{50} (the denaturant concentration at which half of the protein is unfolded), the *m* value (the dependence of protein stability on denaturant concentration) and $\Delta_r G_{H2O}$ (the unfolding Gibbs' energy extrapolated to zero concentration of denaturant). These parameters are presented in Table 1.

Table 1
Thermodynamic parameters of denaturant-induced LTP1 unfolding at 25°C

pH	D_{50} (M)	*m* value (kJ mol^{-1} M^{-1})	$\Delta_r G_{H2O}$ (kJ mol^{-1})
pH 3.2	4.5 ± 0.2	3.0 ± 0.4	13 ± 2
pH 5.1	5.1 ± 0.1	4.9 ± 0.6	25 ± 3
pH 7.2	4.92 ± 0.04	9 ± 1	44 ± 6
pH 8.5	4.55 ± 0.05	7.3 ± 0.8	33 ± 4

3.3. Heat-induced unfolding

Heat stability was studied in several different ways. Measurement of LTP1 fluorescence as a function of temperature (20–90°C) at pH 3, 5 and 7 gave curves, which were similar to those of *N*-acetyl-tyrosine amide (Fig. 2). All curves could be fitted to an equation expressing the temperature dependence of fluorescence intensity [27]. The fact that the temperature effect on LTP1 fluorescence is similar to that of a low-molecular weight model compound suggests that no major structural change takes place in this temperature range. This result is

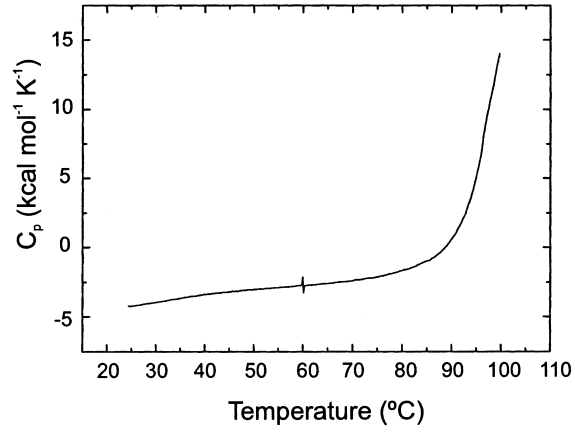

Fig. 3. DSC curve for LTP1 at pH 7.2. The small spike at 60°C is electronic noise.

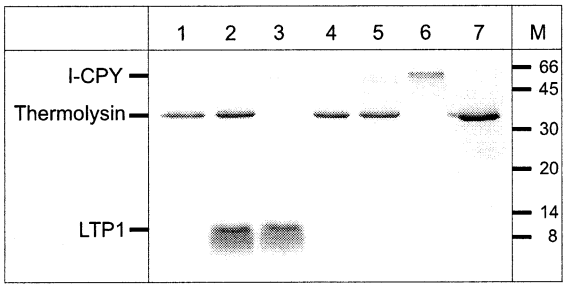

Fig. 4. LTP1 is not sensitive to thermolysin digestion at 70°C for 30 min as detected by SDS–PAGE. Lane 1, thermolysin. Lane 2, thermolysin and LTP1. Lane 3, LTP1. Lane 4, thermolysin and a partially refolded form of yeast carboxypeptidase Y termed I-CPY [20]. Lane 5, thermolysin incubated for 60 min at 70°C with I-CPY added after 30 min. Lane 6, I-CPY. Lane 7, unheated thermolysin. M: The numbers to the right indicate molecular weights (in thousands) of marker proteins. All reaction mixtures except those corresponding to lanes 5 and 7 were incubated for 30 min. Lanes 4 and 5 serve as a positive control for thermolysin activity.

supported by DSC (Fig. 3), which shows a melting temperature well above 100°C. Finally, we used protease sensitivity as a criterion for unfolding. Fig. 4 shows that LTP1 is resistant towards proteolysis at 70°C with the thermostable protease thermolysin [28].

3.4. Susceptibility of LTP1 to gastric digestion

During beer production, LTP1 is extracted from malt together with other solutes and withstands subsequent steps of the brewing process, including wort boiling and fermentation. In beer foam, LTP1 is a major proteinaceous substance and is important for the formation of a good head of the beer [17]. About 90% of the LTP1 isolated from beer foam is in a denatured form, termed foam-LTP1, which is formed during wort boiling [29]. The remaining 10% is folded and is more similar to LTP1 isolated directly from barley. Some fruit ns-LTPs are known to be allergenic [30]. Since many food allergens are known to be resistant to pepsin digestion in vitro [31] we examined whether this was the case for LTP1. In Fig. 5 is shown the result of a pepsin digestion of LTP1 purified from barley, foam-LTP1 and folded LTP1 isolated from beer. The results show that whereas native LTP1 is pepsin resistant, most of the LTP1 found in beer is degraded to lower-molecular material by pepsin.

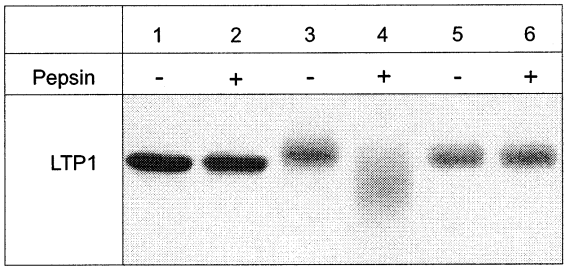

Fig. 5. Pepsin digestion of LTP1 analysed by SDS–PAGE. LTP1 purified from barley (lanes 1 and 2), foam-LTP1 (lanes 3 and 4), and folded LTP1 from beer (lanes 5 and 6) were incubated with or without pepsin at pH 2 for 60 min at 37°C. I-CPY was used as a positive control (not shown). The lower mobility of LTP1 purified from beer is interpreted to be due to carbohydrate adducts formed non-enzymatically during the brewing process [19].

4. Discussion

Denaturant-induced unfolding has been studied by measuring tyrosine fluorescence. Unfolding was shown to be reversible by (A) coincidence of GuaHCl unfolding and refolding curves detected by fluorimetry (Fig. 1) and (B) the ability of LTP1 to regain resistance towards proteolytic digestion with trypsin after refolding (data not shown). Thermodynamic analysis using a two-state model reveals that LTP1 is a highly stable protein, exhibiting maximum stability around neutral pH ($\Delta_r G_{H2O} = 44$ kJ mol^{-1}). This is higher than hen egg-white lysozyme (37 kJ mol^{-1}), RNaseA (31 kJ mol^{-1}) and α-lactalbumin (18 kJ mol^{-1}) under similar conditions [32].

When heat denaturation of LTP1 was attempted, we also found it highly stable. Both fluorimetric and calorimetric results suggest that LTP1 has a melting temperature above 100°C. To complement these thermodynamic experiments we used proteolytic digestion to detect transient unfolding of LTP1. However, even at 70°C, LTP1 resists thermolysin digestion, showing that also according to this criterion LTP1 is highly stable. These results are in agreement with early experiments on thermal inactivation of an ns-LTP from maize [33].

The high stability of barley LTP1 towards proteolysis is important for the brewing process since it is essential to the final product that LTP1 is neither digested by malt nor by yeast proteases. Also, while we found native LTP1 to be completely resistant towards pepsin digestion, foam-LTP1 is at least partially digestible. Some fruit ns-LTPs are allergenic and this fact may be connected to their resistance towards pepsin digestion [13]. Also, urticaria from beer has been reported in rare cases, and it has been suggested that these patients react to LTP1 [34]. Since foam-LTP1 constitutes most of the LTP1 found in beer, it is interesting to note that it is far more pepsin-labile than native LTP1.

We have here shown that LTP1 is a highly stable protein towards a wide variety of hostile conditions. Although this may be an evolutionary coincidence we find it more likely that it is important for its biological function for example during stress response.

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