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PROLINE IS A PROTEIN SOLUBILIZING SOLUTE

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Summary. The effect of proline on the prevention of trichloroacetic acid (TCA)-induced protein precipitation is studied. It is found that proline at high concentrations (>4.0 M) completely prevents TCA-induced precipitation of hen egg white lysozyme. Other osmolytes such as ethylene glycol, glycerol and sucrose fail to prevent the TCA-induced precipitation of lysozyme. Viscosity and 1-anilino-8-naphthalene sulphonic acid binding experiments suggest that proline at high concentration forms an ordered supramolecular assembly. Proline is shown to increase the solubility of protein due to formation of such higher order assemblies. A model of the supra-molecular assembly of proline is proposed and a possible *in vivo* role of the increased levels of proline under water stress is discussed.

Key words: Osmolyte, protein aggregation, supramolecular assembly

Plant cells are known to accumulate the imino acid proline in the cytoplasm under water stress conditions (1-3). The accumulation of proline has been speculated to be a compensatory mechanism for better plant survival under drought conditions. In the recent years, the accumulation of proline in plants and other microorganisms is a subject of intensive research. Recently, there have been reports indicating that increased levels of proline could have a osmoregulatory role during water stress conditions (6-8). However, the exact reason(s) for the enhanced levels of proline is still an open question.

In the present communication, we study the effect of proline on the solubility of hen egg white lysozyme. Our results clearly demonstrate that proline at high concentrations forms higher order aggregates and the formation of such supramolecular assemblies is postulated to be responsible for the protein solubilizing effect(s) of proline.

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MATERIALS AND METHODS

Hen egg white lysozyme and 1-anilino-8-naphthalene sulfonic acids were obtained from Merck Co., Germany. L-Proline was purchased from Lancaster, England; Glycerol was obtained from Fischer Scientific, USA; Ethylene glycol was procured from Aldrich Chemical Co., USA. All other chemicals used in this study were of high quality analytical grade. All solutions were made in Milli 'Q' water at 25° ± 5°C.

Protein solubility assay: The protein (hen egg white lysozyme), dissolved in various concentration(s) of proline (upto 6 M), was precipitated by the addition of 5% (w/v) 2,2,2-tricholoroacetic acid (TCA). The resultant solutions were shaken vigorously and the amount of precipitate formed was monitored by measuring the percentage transmittance of the resultant solution(s) at 600 nm. Control experiments with ethylene glycol, glycerol and sucrose were also performed under identical conditions in the absence of proline. The final concentration of the protein in all the experiments was 1mg/ml.

Viscosity: All viscosity measurements were carried out on a manual Ostwald viscometer, at room temperature (25° ± 3°C). The experiment was repeated several times and the viscosity values obtained were averaged.

ANS-binding studies: To appropriate concentrations of proline, 250 μM of 1-anilino-8-naphthalene sulfonic acid (ANS) was added and the fluorescence spectra were recorded between 450 nm and 600 nm using an excitation wavelength of 400 nm. All spectra were corrected for the blank.

Temperature effect: 1mg of hen egg white lysozyme was dissolved in 1 ml of a solution containing 6 M proline. The solutions were incubated in a water bath maintained at various temperatures ranging from 25° C to 70° C. 5% (w/v) solution of TCA was added to precipitate the protein. The protein precipitation was immediately monitored by measurement of transmittance of the solution at 600 nm.

RESULTS AND DISCUSSION

Proline, when accumulated in the cell cytoplasm in high levels under water stress conditions, in our opinion, has a protein solubilizing role in addition to its osmoregulatory function. Hence, in this paper, we embarked on studying the protein solubilizing effect(s) of proline.

2,2,2-Tricholoacetic acid (TCA) is known to precipitate proteins due to the 'salting out' effect (9-11). Thus, TCA-induced protein precipitation serves as an ideal assay to study the protein solubilizing effects of proline and various other osmolytes. Figure 1 shows the effect(s) of proline and other osmolytes on the TCA-induced precipitation of hen egg white lysozyme. It can be observed from Fig. 1 that the solution remained turbid with only about 10% light transmittance upto 2 M proline concentration. This implies that proline in this concentration range (upto 2 M) has no significant effect on the TCA-induced precipitation of lysozyme. However, further increase in the proline concentration (beyond 3 M), there is a steep increase in the light transmittance of the solution. There is almost 100% light transmittance beyond a proline concentration of 4.0 M, suggesting that proline in this concentration (> 4.0 M) completely prevents the protein precipitation by TCA (Fig. 1).

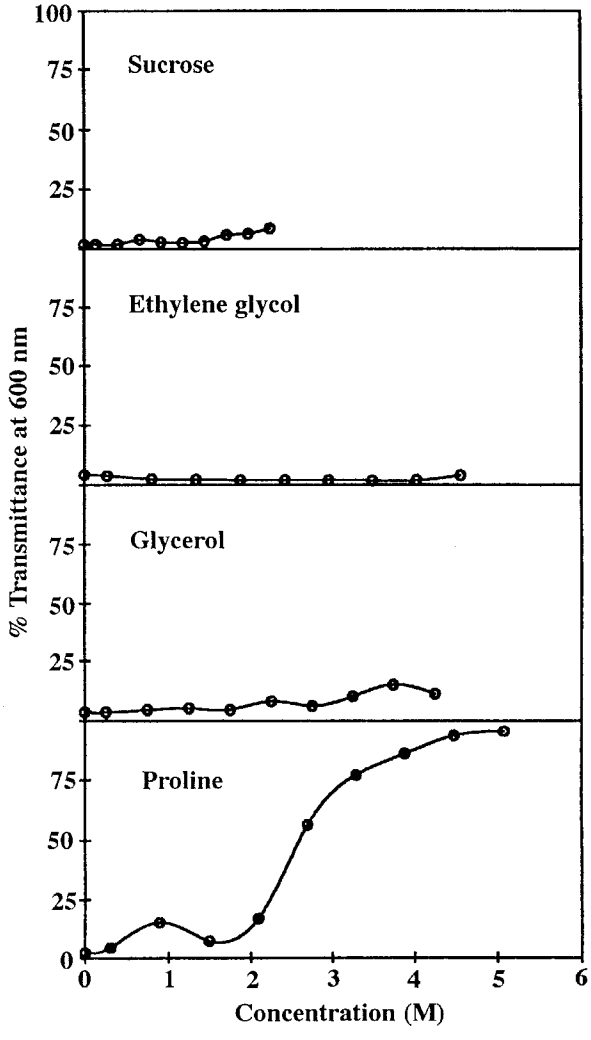

Fig. 1. The solubility profile of the effect of various osmolytes on the TCA-induced precipitation of lysozyme. The concentration of the protein used for precipitation was 1 mg/ml. The protein precipitation was carried out using 5% (w/v) TCA.

Sucrose, ethylene glycol and glycerol are known to be good osmolytes (12-14). Hence, we decided to compare the protein solubilizing effect(s) of these compounds with proline. It is obvious from Fig. 1 that all the three compounds used were unsuccessful in preventing the TCA-induced precipitation of lysozyme. The percent transmittance of the solutions was less than 10% even at higher concentrations of these compounds. From these results it is apparant that among the osmolytes used, proline is special with respect to its ability to dissolve the TCA-induced precipitate in lysozyme.

We investigated the structural basis for the protein solubilization activity of proline at high concentrations, using viscosity and fluorescence measurements.

Figure 2 shows the change(s) in relative viscosity as a function of increasing concentrations of proline and glycerol. The change in viscosity is not significant upto a proline concentration of 3.5 M. Interestingly, beyond 3.5 M the relative viscosity value is found to exponentially increase with the increase in proline concentration. Glycerol, on the other hand, does not show such steep increase in its relative viscosity (Fig. 2). As expected, the viscosity of glycerol increases steadily with increase in concentration. The anamalous increase in the relative viscosity value as observed for proline (at high concentrations), is not expected for solutes in their monomeric state. the asymptotic rise in viscosity of the solution is reminiscent of solutes which show a tendency to form higher order aggregates (15). As the viscosity profile of proline shows an exponential behaviour, it is possible, that at higher concentrations, proline forms a supramolecular assembly. Formation of such multimeric assemblies by proline could account for its protein solubilizing effect(s) observed at high concentrations.

It is important to probe into the nature of the aggregate(s) formed by proline at high concentrations. ANS is an useful probe to monitor the formation of ordered hydrophobic surfaces in the supramolecular assembly of proline (16). Figure 3 shows that the emission maxima of ANS blue-shifted by about 25 nm from 524 to 499 nm in the proline concentration range of 0 - 4.5 M. Such significant blue shifts in the emission maxima could be attributed to the formation of hydrophobic pockets in the supramolecular assembly of proline. The emission intensity of ANS, which is yet another polarity sensitive property, shows a 7-fold increase in the range of proline concentration used (0 - 4.5 M). It is interesting to note that

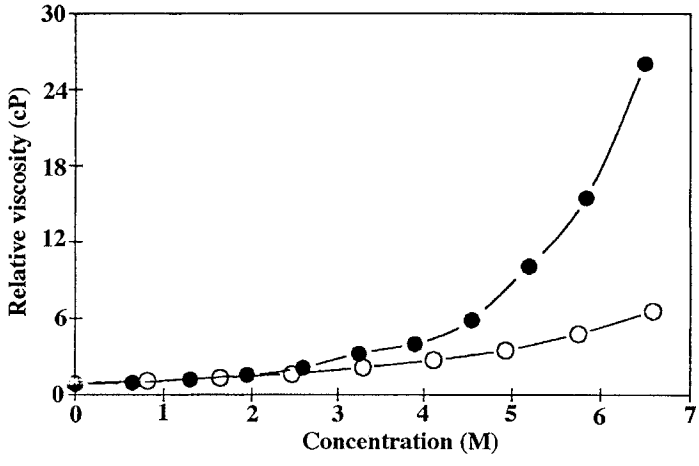

Fig. 2. Relative viscosity changes of proline (●) and glycerol (○) solutions. The relative viscosity values represent a mean of five experimental values obtained under identical conditions (25° ± 5°C).

the increase in the emission intensity is not appreciable upto a proline concentration of 2 M (Fig. 3); but beyond this concentration, the increase in emission is steep. This implies that the supramolecular assembly formation steps-up beyond 2 M proline concentration. Thus, the ANS binding experiments demonstrate that proline forms an ordered supramolecuar assembly and the assembly possess distinct hydrophobic surface(s) conducive for ANS binding.

The organization of any supramolecular assembly should be sensitive to temperature and is subject to collapse at some fixed higher temperature. Thus, if proline were to organize into an supramolecular assembly at higher concentrations, then the organization is also liable to be destroyed at some higher temperature. Under these conditions of disorganisation, proline is expected to behave as a monomer even at its higher concentrations. Thus, it can be argued that if protein solubilizing effect(s) of proline is due to the formation of supramolecular assembly, then proline is not predicted to support the solubilization of TCA-induced protein precipitation in the disorganized monomeric state. It can be seen from Figure 4, that proline at 6 M concentration prevents TCA-induced precipitation of lysozyme upto 55°C and the percentage transmittance of the solution(s) at 600 nm in the temperature range (25° - 55°C) is greater than 90%. However, beyond this temperature (> 55°C) the percentage transmittance decreases drastically and falls to about 25% at 70°C. It is important to note that the percentage transmittance of solutions in the absence of proline (in water), show no variation in the percentage of transmittance. Thus, the results clearly demonstrate that proline exists in a ordered multimeric state at high concentrations and below 55°C and its existence in the supramolecular assembly facilitates it (proline) to solubilize the TCA-induced protein precipitate. Beyond this temperature (55°C) the protein solubilizing effeciency tends to fall due to the collapse of the supramolecular assembly.

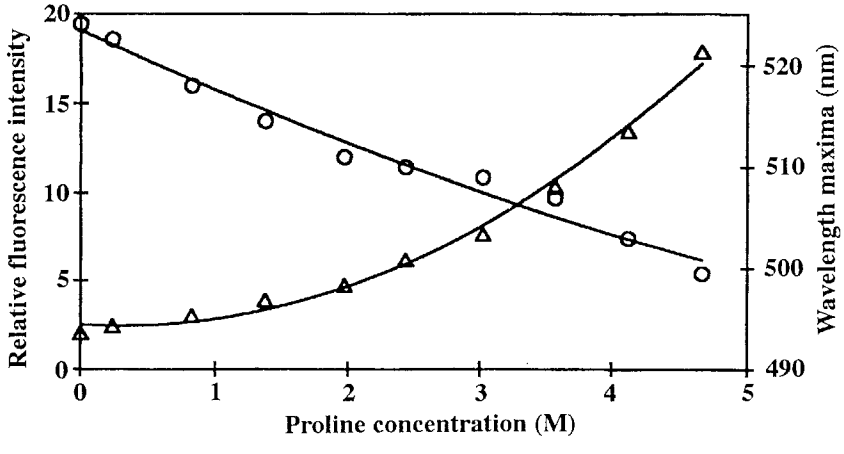

Fig. 3. ANS binding profiles of proline at various concentrations. Δ - relative emission intensity; O-shift in the emission maximum of ANS. All experiments were conducted using an excitation wavelength of 400 nm. The final concentration of ANS used was 250 μM.

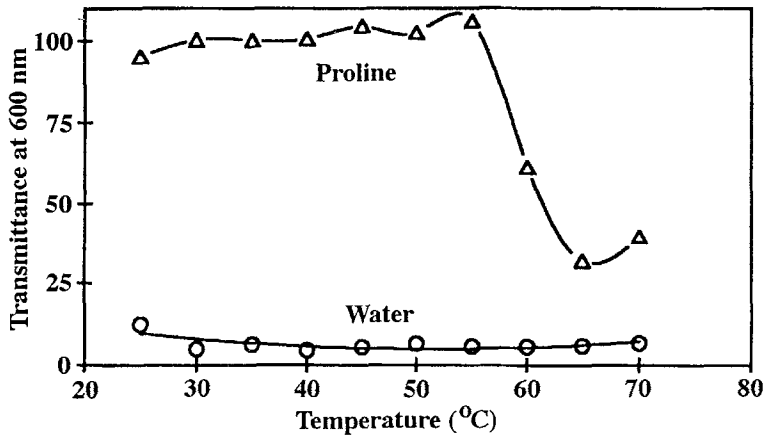

Fig. 4. Solubility profile of TCA-induced precipitate of lysozyme in 6M proline, at various temperatures. The protein precipitation was monitored by measurement of transmittance of the solution at 600 nm.

Proline appears to exist in equilibrium in a monomeric or a multimeric (supramolecular assembly) state depending on the conditions used (Fig. 6). Below 100 mM concentrations, it exists in a monomeric state and does not support protein solubilization. At concentrations greater than 4M and in the temperature range of 25°- 55°C it appears to exist in a multimeric state promoting protein solubilization. The supramolecular assembly of proline formed at high concentrations could also be disorganised into the monomeric state at temperatures beyond 55°C. Under these conditions, proline is unable to support protein solubilization.

We are tempted to propose a model for the supramolecular assembly of proline in consistence with our experimental results (Fig. 6). We believe that proline could form aggregate/supramolecular assembly involving the stacking of pyrrolidine rings one over the other, just as a pile of coins. Such an ordered stacking could bestow an amphiphilic nature to the supramolecular assembly with the imino and the carboxyl groups facing on one side of the assembly providing the polar surface and the methylene groups of the pyrrolidine ring constituting the hydrophobic surface (Fig. 5). The whole supramolecular assembly appears to be stabilized by both the weak hydrophobic interactions among the methylene groups of the various pyrrolidine rings stacked one above the other and also by the intra and inter assembly hydrogen bonding among the imino and carboxyl groups belonging to the same or different proline stack(s). As proline is postulated to form supramolecular amphiphilic assembly, it could effectively provide the necessary polar and non-polar surface(s) for the protein and thereby prevents the self-association/aggregation of protein molecules and which is a cause for the TCA-induced protein precipitation. We believe that *in vivo*, under water stress conditions the increased accumulation of proline could help the proteins in the cells *milieu* to exist in a solubilized state.

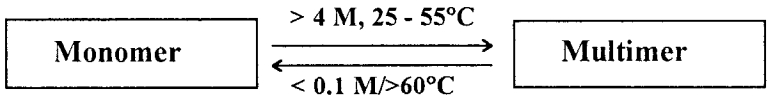

Fig. 5. Schematic diagram of the structural transitions of proline.

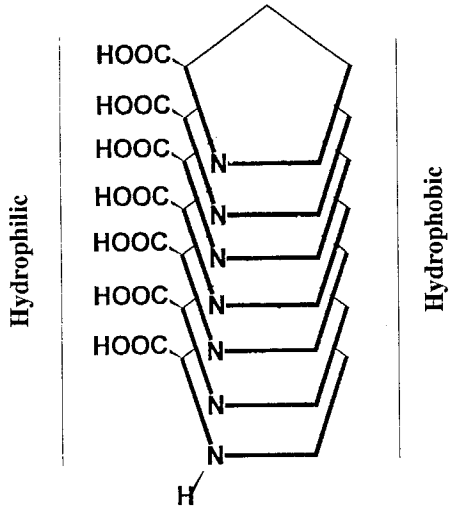

Fig. 6. A model of the supramolecular assembly of proline. For reasons of clarity the imine protons in the stacked pyrrolydine rings of proline has been omitted.

It is important to address the question of the physiological relevance of the high concentrations of proline, wherein the protein solubilizing effects are observed. Proline is considered as a bio-compatible solute (17). Results of numerous studies have demonstrated that under conditions of water and salt stress, plants and microorganisms accumulate very high concentrations of proline (18-20). The cytoplasmic accumulation of proline and other osmolytes are found to exceed 3 mol/Kg of water. Recently, Gzik (21) studying the accumulation of various α-amino acids in sugar-beet plants show that accumulation of proline in these plants under water and salt stress conditions exceed 2M. Interestingly, accumulation of proline or other osmolytes in higher concentrations have been shown to have no interference with the cells' metabolism. Thus, in the context of the results of these studies, we feel that the concentration range (0-4.5 M) of proline used in this study is physiologically meaningful.

Thus, the results presented in this paper clearly demonstrate that under stress conditions, accumulation of proline could not only have an osmoregulatory role in the cytoplasm but also help in the maintainance of the proteins in a solubilized state. Work is in progress to further characterize the supramolecular assembly to proline.

ACKNOWLEDGEMENTS

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