Abiotic Stress Tolerance Processes in Rice *(Oryza sativa* L.): Studies on the Role of Abscisic acid and Proline-Biomacromolecular interactions

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I declare that no part of this thesis was earlier submitted for the award of research degree or diploma of any University or Institute.

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Declaration

I hereby declare that the work presented in this thesis entitled **"** *Abiotic stress tolerance processes in rice (Oryza sativa L) : Studies on the role of abscisic acid and proline-biomacromolecular interaction"* **has been carried out by me under the** supervision of **Prof. Arjula Ramachandra Reddy,** Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this has not been submitted for **a** degree or Diploma of any other University or Institute. All assistance and help received during the course of the investigation have been duly acknowledged.

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1. INTRODUCTION

Plant productivity is adversely affected by a variety of abiotic stress factors such as salinity, cold, drought, floods, metal toxicity and high temperature (Boyer, 1982). Continued breeding exercises in the past few decades resulted in the enhanced genetic potential and performance of crop plants under such conditions (Acevado and Fereres, 1993). However, much more is desired to be achieved in ensuring a sustainable food production with the available land and water resources. Encroachment of cultivable land by the exploding urban populations has particularly posed a major threat to crop production, necessitating the development of stress tolerant cultivars suitable for the hitherto unused saline and drought-prone soils.

One of the main hurdles in reaching such a goal has been the limited knowledge on stress tolerance process, known to be governed by many diverse genes. Such Quantitative Trait Loci (QTL) have been uncovered by recent techniques like Restriction Fragment Length Polymorphism (RFLP) and Randomly Amplified Polymorphic DNA markers (RAPD). However, such techniques merely identify the DNA fragments associated with stress tolerance traits, the knowledge of which is of limited utility without deciphering the physiological and molecular basis of marker-phenotype relationship. Further, the new advancements in genetic engineering can not be applied to the development of abiotic stress tolerant crops without the identification of key molecules/components that confer tolerance to diverse stress conditions. For instance, the available recombinant DNA methods and the transgenic plant production technology can identify and transfer key genetic elements across the species and generic barrier, which could be exploited for the production of stress tolerant crops. To this end, extensive studies by a large number of groups are now beginning to focus and identify the central components that operate in divergent stress conditions.

Abscisic acid (ABA) is one such key component that attracted quite an attention in the recent past. Initially thought to be involved in the leaf abscission and stomatal movement, it was soon recognized to play a central role in many of the developmental events in a plant's life cycle. ABA has been convincingly proved to mediate various developmental and physiological processes that affect the agronomic performance of crop plants including the embryomorphogenesis, seed protein synthesis, seed maturation and

onset of dormancy. Most importantly, ABA has been implicated in many of the fundamental abiotic stress responses both at transcriptional and post-transcriptional level (Chandler and Robertson, 1994). ABA levels are known to increase in maturing embryos just before the onset of desiccation tolerance, as a programmed developmental event and in the vegetative tissues during abiotic stresses, resulting in the activation of similar set of genes and pathways (Skriver and Mundy, 1990).

Interestingly, the vegetative tissues in response to ABA and abiotic stress conditions start to mimic some of the molecular and biochemical events exclusive to the maturing embryo, probably by recruiting a similar molecular machinery for the induction of related sets of genes. Identification of similar ABA responsive elements (ABREs) in the regulatory regions of many ABA/stress-induced genes confirms such a possibility. These gene products were presumed to be of paramount importance in conferring desiccation tolerance to plants. The widespread utilization of a conserved stress responsive machinery controlled by a central component like ABA, therefore, supports the existence of common candidates controlling complex traits such as stress tolerance in diverse plants. Hectic efforts are on by various groups to identify the signal transduction cascade starting from the signal perception to the transcriptional activation of stress responsive genes mediated by ABA. Apart from the induction of such stress responsive proteins whose functions are still speculatory, ABA is also widely known to activate a set of anti-oxidant and anti-stress enzymes. Therefore, unraveling the role of ABA would certainly pave the way for a better understanding of stress tolerance process that could eventually contribute to crop improvement.

In cereal crops, exogenous ABA application has been found to mediate several biochemical events involving the induction of many gene families (Chandler and Robertson, 1994). Particularly, ABA was found to induce several proteins belonging to the *Rab* family, *lea* family and other gene products like SalT and dehydrins in rice (Skriver and Mundy, 1990; Moons *et ai,* 1995; Claes *et al.,* 1990; Caplan *et al.,* 1990). Further, many of the ABA responsive enzymes were found to be involved in the anti-oxidative functions in the cells as the active free radical species pose a major threat to the cellular components under abiotic stress condition. However, there were not many attempts to

understand the role of ABA in the salinity and chilling tolerance processes in the rice plant. In rice, ABA has been found to induce many stress responsive enzymes like superoxide dismutase and ascorbate peroxidase (Karunasree, 1998). Further, two polypeptides of molecular weight 23 kDa and 15 kDa were found responsive to ABA and other abiotic stresses in rice (Rao *et a!.,* 1993; Rao, 1993; Reddy, *et ah,* 1993; Karunasree, 1992). A striking feature of all these polypeptides is their high boiling stability which indicates their high hydrophilic nature that has been a characteristic property of many stress responsive proteins reported earlier. Further characterization of these proteins and an analysis of their possible role could lead to a better understanding of stress tolerance processes in rice.

Accumulation of free proline in the cytoplasm is another widespread event observed in crop plants in response to abiotic stresses and ABA application. Intra-cellular hyper-accumulation of osmolytes is governed by traits that are evolutionarily conserved spanning across the prokaryotes and eukaryotes, presumably conferring tolerance to the respective organisms. Many mutants were isolated in plants and bacteria that accumulate high amounts of the osmolytes and withstand the abiotic stress conditions (Dix, 1993; Csonka, 1989; 1981). Although plant species differ in their sensitivity and response to unfavorable growth conditions like decrease in water potential, all plants are inherently capable of adjusting to such stresses with an increase in concentration of some osmolytc or the other in the cytoplasm.

Osmolytes seem to perform diverse functions such as osmorcgulation, osmoprotection, carbon and nitrogen storage, protection of cellular structures and lipid bilayers (by scavenging active oxygen). Further, thc term "compatible solutes" aptly describes their non-intcrfere with the biological functions of the cell even at their highest accumulated concentrations. Osmolytes are also known to stabilize the proteins and membrane structures under diverse stress conditions, which have been widely described. There were many reports suggesting the protective role of osmolytes under stress conditions (Xin and Lee, 1993; Le Rudulier *et al.,* 1984). However, the biophysical and molecular basis of protection offered by osmolytes is very scantily studied, particularly with reference to proline.

The present work was undertaken to explore the biochemical and biophysical basis of the role of ABA and proline in stress tolerance process in rice. Rice, being one of the most important crops of the developing world, was selected as a model plant to carry **out** these studies. About 60% of the world population consume rice as their staple food with most of its production and consumption being restricted to developing world. Further, the low yield of rice production in India that are mainly attributed to the divergent geo-climatic conditions, require the development of efficient stress adaptable cultivars. Attempts were made here to analyze the biochemical basis of stress tolerance processes with a focus on stress responsive proteins induced by ABA using SDS-PAGE and Western analysis. A relatively easier and reproducible screening method was established that makes use of the etiolated seedlings to score the tolerance mediated by ABA. Further, changes in *in vitro* phosphorylation status of proteins upon the application of ABA was investigated in the present work that could possibly lead to the identification of components involved in the signal transduction pathway mediated by ABA.

The work also includes *in vitro* assays that were necessary to analyze the biophysical interaction of osmolytes proline and betaine, with major biomacromolecules such as proteins and DNA. These *in vitro* studies were primarily undertaken to mimic the sub-cellular interactions between osmoprotectants and biomacromolecules whose association may be transient, but vital due to the abundance of osmolytes under the stress adapted conditions. The structural and functional analysis of the interaction could eventually lead to the establishment of vital clues in understanding stress tolerance processes in the cells. Further, the implications of *in vitro* assays performed do not just confine to rice plant but to plants in general as they tend to explore the basis of stress adaptation *in vivo.*

Objectives of this thesis are

- To study the major biochemical changes in rice in response to ABA application and correlate such changes with salinity and chilling tolerance processes
- To standardize a rapid and reliable system to screen the rice seedlings for salinity and chilling tolerance processes mediated by ABA.

- To analyze the role of stress/ABA responsive proteins in salinity and chilling tolerance processes in rice using various inhibitors.
- To understand proline-protein interactions *in vitro* to analyze the protective role of proline under various stress conditions
- To study the proline/betaine-DNA interactions to unravel the mechanisms of tolerance mediates by these osmolytes under salinity stress conditions.

2. LITERATURE REVIEW

The role of ABA in plant metabolism has been subjected to considerable research in the past with much of the work concerned with its involvement in the control of dormancy, leaf abscission and stomatal movement (Walton, 1980; Zeevaart and Creelman, 1988). The focus now shifted, however, to its possible role in many of the abiotic stress responses in a wide range of plant species. The importance of this phytohormone is so well recognized now that there is no other molecule as widely studied and implicated in stress tolerance processes as ABA. Many diverse families of genes were discovered that are induced by ABA under a wide range of developmental and physiological condition in a plant cell (Skriver and Mundy, 1990). However, not all these gene products may be directly involved in the stress responsive processes. For a better crop management under diverse stress conditions, it could be profitable to identify and manipulate thc stress responsive events mediated by ABA. To this end, many attempts were made to understand the role of ABA under diverse stress conditions with a focus on the signal transduction events that are involved in its operation. Knowledge on the biosynthesis of ABA is, however, a pre-requisite to understand its intricate role in developmental and stress responses.

2.1 ABA biosynthetic pathway in plants:

ABA is known to exist in the plant cells both as free and conjugated forms with its biosynthesis reported as a highly conserved process from primitive Bryophytes to Angiosperms (Parry *et a!.,* 1991). ABA is synthesized both in a developmentally regulated fashion and in response to changes in the osmotic balance in plant cells, leading to specific molecular and biochemical events. However, unlike the environmental stress-driven process, its developmentally regulated biosynthesis is rather known to be operative in an exclusive stage specific manner, especially during the late embryogenesis periods. The knowledge on its genetic and developmental regulation is, therefore, very essential to understand the sub-cellular events that are associated with its role in a plant cells under diverse physiological and developmental conditions.

The preliminary understanding on the role of ABA in these cellular events, however, was more dependent on monitoring its endogenous levels and correlating it with the effects that were observed with its exogenous application. Soon, investigations were

begun to study in depth the genetic factors that regulate its biosynthesis, **eventually leading to the** isolation **of ABA** biosynthetic mutants from various crop plants. These mutants **were** truly instrumental in providing the much needed clues to asses **the role of ABA in** stress responsive processes.

*2.1.1 ABA **biosynthetic** mutants:*

Some of the best characterized mutants in the ABA biosynthesis include the *Arabidopsis aba,* tomato *flacca, sitiens* and *notabilis*, potato *droopy,* tobacco *aba1*, pea *wilty* and maize *viviparous (vp).* The abnormal phenotypes of these mutants could clearly help in establishing the events that are associated with its deficiency in biosynthesis. Apart from their utility in providing the information on missing links in ABA biosynthesis, these mutants further helped in gaining an insight in to the ABA signal transduction events in **a** plant cell. The cumulative information gathered from different mutants of various crops could broadly help in deciphering various aspects of ABA biosynthesis in plants in general, as this pathway and its constituents were largely found to be conserved across **the** plant kingdom. Perhaps, the conserved identity of this pathway established across the plant kingdom makes it all the more important to understand its role in stress tolerance process in plants.

ABA belongs to an exclusive class of compounds known as sesquiterpenes with mevalonic acid as the early precursor molecule in its biosynthesis. Mevalonatc eventually gets converted to farmesyl pyrophosphate which, after many intermediate steps involving several enzymes, is converted in to a 15 carbon compound xanthoxin. The xanthoxin is finally converted to ABA that is transported or conjugated. Biosynthesis of ABA, therefore, is a process controlled by various enzymes regulated in a coordinate fashion and is extensively reviewed (Parry *et al.*, 1991). However, molecular basis of the regulatory gene action in ABA biosynthesis is yet to be clearly understood.

Among the biosynthetic *viviparous* mutants of maize, *vp2, vp5, vp7, vp9* represent blocks in the early stages of carotenoid biosynthesis (Moore and Smith, 1985; Neill *et al,* 1986) while the *aba]* mutant of *Arabidopsis* was found to be deficient in **the** epoxidation reaction, converting zeaxanthin to antheraxanthin (Rock and Zeevaart, 1991). The *droopy* mutant of potato (Duckham *et al,* 1989), *flacca* and *sitiens* mutants of tomato (Taylor *et*

al, 1988), *aba/ckr1* mutant of tobacco (Rousselin *et al.* 1992) represent blocks in the final stages of ABA biosynthesis, including the oxidation of ABA aldehyde to ABA. The *nar2a* mutant in barley, however, comprises a special case in the ABA biosynthesis where it is not directly related to the deficiency in any of the enzymes but to a defect in the molybdenum co-factor that is essential for the oxidation of ABA aldehyde to ABA (Walker-Simmons *et al,* 1989). The wealth of data available on the ABA biosynthetic mutants could lead to the identification of enzymes and their respective genes that are involved in its biosynthesis.

2.1.2 ABA insensitive mutants:

Apart from the above ABA biosynthetic mutants, there exist another class of mutants that were isolated as ABA-insensitive. The insensitive mutants are very characteristic that they can germinate and grow in the inhibitory concentrations of ABA. This very special feature was exploited while isolating the first ever insensitive mutant in *Arabidopsis* (Koornneef *et al,* 1984). These mutants offer unique tools to further dissect the molecular events that are associated with ABA action in cells, especially those involving the stress responsive events. The ABA insensitive mutants though produce and accumulate ABA abundantly in the cells, can not respond to ABA both intra-cellularly and to the exogenous ABA application. They can not, therefore, perform functions expected of the phytohormone. These mutants cither lack the components of signal transductory chain mediated by ABA or accessory elements required for the ABA action in the signal transduction. Some of the components that are lacking in the signal transductory events could include regulatory kinases and phosphatases that are the routine signal chain participants for any phytohormone-mediated action in cells. These mutants can not respond to stress stimulus and therefore, serve as the best models to understand the cascade of events associated with its action.

The *abi1, abi2, abi3* mutants of *Arabidopsis thaliana* constitute the well studied ABA-insensitive ones (non-responsive mutants) (Koornreef *et al.,* 1984). Similarly, the *abi4* and *abi5* constitute the seed specific ABA-non responsive mutants (Finkclstein, 1994). The *vp1* mutant of *Zea mays* is among the best characterized seed specific mutant (McCarty *et al,* 1991) while the *cool* mutant in *Hordeum vulgare* is an ABA-insensitive

mutant found specific to the guard cell function of ABA, involved in the stomatal closure (Raskin and Ladymann, 1988). The physiological role of ABA in the water relations and transportation was clearly established in these mutants.

As the multiple functions of ABA in a plant cell were being established **with the** accumulation of the data from the ABA mutants, it was natural that some of the mutants resemble each other both phenotypically and physiologically. Among such known mutants of ABA, *Arabidopsis abi3* and maize *vpl* mutants share some common phenotypes, mainly discernible in seeds (McCarty, *et al,* 1991). The *abi3* gene is transiently expressed beyond seed germination but is confined to the tissues of embryonic origin like cotyledons and hypocotyl (Finkelstein and Somerville, 1990). Further, the *vpl* mutant of maize was shown to inhibit the anthocyanin biosynthetic pathway in aleurone and embryo tissues indicating a tight regulation of two widely unrelated biosynthetic pathways *viz.*, phenyl propanoid pathway and the carotenoids pathway (Dooner, 1985). The anthocyanin biosynthctic pathway is also associated with the abiotic stress responses with an involved stress or ABA signal transduction. In this context, it is worthy to recall that many of the structural and regulatory genes of the maize anthocyanin biosynthetic pathway are up-regulated by abiotic stresses such as cold and metal toxicity (Christie *et al,* 1994). Similar to *vpl,* the developing *abi3* mutants fail to become dormant in spite of the presence of ABA in the cytoplasm. This particular feature is somewhat reminiscent of that was reported from a ABA-deficient mutant *aba* (Finkelstein, 1994).

2.2 Stress responses **in ABA mutants: Possible clues to stress tolerance:**

ABA is known to be responsible for triggering stomatal closure under conditions of water deficiency with well documented support from clectrophysiological studies (Walton, 1980). This effect, however, could be best explained using ABA-deficient mutants that were found to wilt and loose water in excised aerial parts, suggesting the defective stomatal regulation (Neill and Morgan, 1985; Raskin and Ladymann, 1988; Quarrie, 1982). Further, the use of ABA mutants was well appreciated in proving **the** possible role of ABA in stress responses where the basic physiological and biochemical changes were closely monitored. For instance, the *aba1* mutant *of Arabidopsis* does not develop freezing tolerance and is impaired in the production of characteristic root

structures in response to progressive drought (Heino *et al,* 1990; Gilmour and Thomashow, 1991). In tomato, mutants with reduced levels of endogenous ABA have been studied in detail to provide support for the role of ABA in regulation of gene expression. These genes in wild type plants were known to be induced by the environmental stresses involving loss of water that is in association with high internal ABA concentrations and application of ABA. However, in *flacca* mutants, the situation is strikingly different that the stress responsive transcripts are not induced under environmental stress conditions indicating the inability of these mutants to synthesize ABA internally in response to stress signal. However, upon the exogenous application of ABA, these mutants accumulate such transcripts indicating a direct involvement of ABA in the regulation of stress-responsive genes (Tal and Nevo, 1973; Taylor *et al,* 1988). The genotype-phenotype relationships in these mutants could therefore help in dissecting the molecular events and identify the regulatory elements involved in such a process.

These mutants were also instrumental in deciphering the developmental, stage-specific and the organ specific gene expression. For instance, several genes arc known to be ABA regulated followed by dehydration stress in vegetative tissues, yet the same or related genes do not seem to require the presence of ABA for their expression in seeds. These genes therefore seem to be functioning both in ABA dependent and independent manner in the same plant depending on the developmental event it is associated with. For instance, *vp* (viviparous) mutant of maize *(vp2, vp5, vp7)* shows low levels of ABA in embryo during seed development and dehydration conditions, yet the expression of stress responsive proteins (like RAB 17, RAB 28) during embryogenesis does not seem to be strongly correlated with the levels of ABA (Pla *et al,* 1989; 1991). However, in contrast, the expression of genes like *Em* seem to be under the control of ABA both during the embryogenesis and in response to stress in maize (Bostock and Quatrano, 1992).

In ABA deficient *sitiens* mutant of tomato (Tal and Nevo, 1973) and *droopy* mutant of potato (Quarrie, 1982), normal induction of proteinase inhibitor II (PI-II) was reported in response to wounding that was otherwise present in wild type plants. However, upon the application of exogenous ABA, the induction of this protein could be restored on par with that of wound response in wild type. This clearly indicates that the ABA

signaling is required for the induction of specific gene products like PI-II under wounding response (Pena-Cortes *et al,* 1989). However, the developmental regulation of this gene is independent of ABA signaling in some parts of the plant as this gene product could be expressed in tubers of tomato and flowers of tomato and potato both in wild type and mutants (Pena-Cortes, 1991). Thus, within a plant, there could be diverse signaling events for the induction of stress responsive genes that are both ABA dependent and independent.

Similarly, ABA could be having dual control on the transcriptional and translational regulation of the same gene. For instance, the *osmotin* gene in tobacco is very strongly regulated by ABA at the transcriptional level as evident from the abundant accumulation of mRNA. However, ABA has no effect in accumulating the osmotin protein either in leaves or in cells (La Rosa *et al,* 1992). This indicates that there could exist different levels of expression of genes by ABA. The *cis* and *trans* acting elements could be dictating these events in the ABA-regulated gene expression. Among such *cis* acting elements, the ABRE (ABA responsive element) is a 11-mer sequence sufficient to confer ABA responsiveness to a gene (Skriver *et al,* 1991; Gulitinan *et al,* 1990).

2.3 Role of ABA inhibitors:

ABA inhibitors were also instrumental in deciphering the ABA signaling events in plants. Their use could broadly give clues somewhat analogous to ABA biosynthetic and insensitive mutants, firmly establishing the role of ABA in various physiological and biochemical events. Fluoridone is one of the widely used chemical to inhibit the carotenoids biosynthesis and in turn ABA, resulting into the lowered expression of ABA responsive genes in spite of creating the required environmental conditions for their expression. Particularly, in the experiments manipulating the endogenous concentration of ABA, fluoridone has successfully been used to prevent the gene action under abiotic stress conditions (Cammue *et al,* 1989; Hatzopoulos *et al,* 1990; Hughes and Galau, 1991; Nordin *et al.,* 1991; Bartels, *et al,* 1991).

2.4 Stress/ABA responsive proteins in plants:

Biochemical, biophysical and molecular changes in response to abiotic stresses in a plant cell were under the focus for many years with attempts to establish their probable involvement in stress tolerance. Synthesis or inhibition of a set of proteins in response to

ABA and abiotic stress conditions remains one of the widely studied biochemical events in a large number of plant species. In fact, synthesis of stress responsive proteins has been found to be an event conserved across all the living organisms from prokaryotes to eukaryotes. Most often, the induced proteins have been found to get encoded from related gene families cutting across the evolutionary tree. For instance, the HSP 104 kDa protein from yeast finds its immunological homologue in rice, induced in response to ABA and other abiotic stresses (Singla *et al,* 1997). In this context, the gene sequences available from the data bases serve the best purpose to identify the sequence homologies among the stress responsive proteins among all the living organisms. The following described are a set of proteins that are stress responsive in plants with an ABA involvement in majority of these cases.

2.4.1 RAB family of genes:

These are the exclusive class of genes responsive to abscisic acid, reported both from monocots and dicots that are also induced in response to seed desiccation and different abiotic stress conditions. The intricate relationship between the appearance of these proteins with desiccation tolerance in seeds and also during the abiotic stress conditions in vegetative tissues strongly points out the probable recruitment of these gene product for the stress tolerance processes. The RAB 21 was first isolated by Mundy and Chua (1988) and was found to be a glycine-rich cytosolic protein with a molecular weight of 16.5 kDa. It was very extensively studied and was found to accumulate in response to stress conditions in roots, embryos, leaves and suspension cells. The primary sequence of this protein with a duplicated domain structures of A and B peptide repeats is conserved across other stress responsive proteins (Dure *et al,* 1989). The RAB proteins in rice are encoded by at least four tightly linked gene loci spanning about 30 kb region in the rice genome (Mundy and Chua, 1988). Moreover, the four proteins encoded by these genes are highly homologous (65-92%) and share conserved immunological identity.

The mRNAs of four *rab* genes are primarily expressed in immature embryos indicating their role in the embryogenesis. The RAB proteins have been predicted to confer tolerance to seeds or leaves under stress conditions recruited with the ABA mediation. The vegetative tissues, upon the application of ABA, start to behave like a

developing embryo by recruiting the desiccation/ABA responsive machinery. It may be noted that the induction of *rab* is not possible without the ABA involvement either in the developmental program or during abiotic stress adaptations. The sequence comparisons of promoter regions of known *rab* genes revealed that there exist two conserved sequence motifs. While one such motif has 80 % homology to transcription modulating SP1 binding site (Briggs *et al,* 1986), the other was similar to the cyclic-AMP responsive element (Deutsch *et al,* 1988). These observations indicate the probable regulation of *rab* genes through known signal transduction events.

In attempts to study the regulation of the *rab* under embryogenesis and tissue specific expression, *rab*-16gene promoter was fused to GUS reporter gene. Expression of this gene was found to be initiated 10 days after pollination and increased progressively upto 25 days after pollination that coincides with the desiccation-tolerant phase in the embryogenesis. Incidentally, this phase is also in correlation with the enhanced accumulation of ABA in the developmental program of the embryo (Skriver and Mundy, 1990).

A homologous gene of cotton *lea*-D-34 was identified in maize, named *rab*-28 that responds to ABA treatments and encodes a of 28 kDa (Baker *et al,* 1988). In the proximal region of its promoter, a conserved ABA responsive element was identified that was earlier reported from other plant genes as well. The RAB 28 m-RNA has been shown to be ABA responsive in embryos as well as in young leaves. However, RAB 28 transcripts do not accumulate to a significant level during early embryogenesis that could be induced upon ABA application. Thus, the developmental and ABA responses of *rab* genes is independently regulated (Baker *et al,* 1988).

In the water stressed wheat roots, a 15 kDa RAB-15 was identified by King *et al,* (1986). This protein shows significant homology with that of members of RAB family, dehydrins, LEA proteins, from rice (Mundy and Chua, 1988), maize (Vilardell *el al,* 1990), barley (Close *et al,* 1989), *Craterostigma plantagineum*, (Piatkowiski et al. 1990) and cotton, (Baker *et al,* 1988, Dure *et al,* 1989). Similarly, Rao *et al,* (1993) reported the induction of two ABA responsive proteins in rice with apparent molecular weights of 15 and 23 kDa. While the former is a general stress responsive protein in rice (Karunasree,

1992), the later is induced in response to water stress and ABA treatment. Further, this 23 kDa protein was found to be immunologically related to RAB 16 protein and could be induced in the suspension cells of rice in response to osmotic stress and ABA treatment (Reddy *et al.,* 1993).

2.4.2 LEA proteins are ABA responsive and are induced under stress conditions:

The period of desiccation is a developmentally programmed event in the embryogenesis of angiosperms. Embryonic cells of a developing seed are adapted to tolerate severe desiccation with the probable involvement of some exclusive biochemical and molecular events associated with it. Molecular basis of such a tolerance process has been extensively investigated with an emphasis on transcriptional and translational events. Recently, much attention has been paid on a group of genes called *lea* (Late Embryogenesis Abundant) in a number of cereal plants. The *lea* genes seem to have similar inductory machinery that is operated among ABA responsive genes (Finklestein and Crouch, 1986; Dure *et al,* 1989). These proteins also accumulate in vegetative tissues in response to abiotic stresses with a concurrent accumulation of ABA in cytoplasm. The LEA proteins are strongly hydrophilic and glycine rich and represent diverse groups of distinct proteins encoded by a relatively small multi-gene families (Dure *et al,* 1989). They are grouped in to three different classes based on their amino acid homologies that have been variously predicted to perform functions in desiccation tolerance process. When excised immature embryos were treated with ABA or subjected to an osmotic stress, or simply desiccated, many transcripts of *lea* genes are accumulated. Further, these are rapidly degraded upon the withdrawal of stress condition in plant cells denoting a possible protective function for which these were recruited.

The predicted protective function of ABA responsive proteins both in the developmental cascade and in the vegetative tissues in response to abiotic stress condition is based more on circumstantial evidence than any direct proof (Dure *et al,* 1989). Some of them probably act as storage proteins (Finklestein *et al,* 1985; Bray and Beachy, 1985) whereas others are know to function as lectins or enzyme inhibitors (Skriver and Mundy, 1990). Although not much progress is made in elucidating LEA protein function in plants, significant advances have been made in understanding the ABA regulation of *lea* gene

expression in monocots. Search for ABA responsive elements (ABRE) in the promoters of these genes revealed the presence of several putative ABRE in rice and wheat (Guiltinan *et al,* 1990). An active protein in the nuclear extract that interacts with the ABREs from 5' regulatory region of the wheat *Em* gene was identified. The gene product was shown to be a DNA binding protein that interacts specifically with the 8 base pair sequence (CACGTGGC) in the ABRE (Gulitinan *et al,* 1990).

The gene product of the *viviparous 1 (vp1)* gene in maize was shown to activate transcription of *Em* gene in transformed protoplasts (McCarty *et al,* 1991). This experiment further proves the existence of conserved transcription machinery that operates in many a stress and ABA responsive situations cutting across the generic barrier. However, the transduction pathway of ABA and its suggested connection to other *lea*-inducing stress factors remains unclear with many events yet to be unearthed. This can be best explained in cases where the induction of a few LEA proteins takes place irrespective of the accumulation of ABA in cytoplasm. For instance, in cotton embryos during normal development and in cultured wheat embryos, there was no clear correlation between endogenous ABA levels and LEA m-RNA abundance (Hughes and Galau, 1991). The role of ABA in the *vp* mutant embryos of maize that are capable of synthesizing at least some of the LEA proteins and ABA responsive mRNA is still unsolved (Pla *et al,* 1989).

Based on their regulation and aminoacid sequences, the LEA proteins were predicted to play an important role in desiccation tolerance as osmoprotective proteins or simply as desiccation-damage-repair proteins (Dure *et al,* 1989). One of the recent experiments to attribute the stress tolerance role to LEA protein comes from the transgenic approach where the Lea III group protein, HVA 1 from *Hordeum vulgare)* was constitutively expressed using the rice actin-1 promoter in rice suspension cells. Transgenic seedlings were found to show increased tolerance to salinity and drought stress and showed lower injury (Xu *et al,* 1996).

2.4.3 Early methionine labeled (Em) proteins:

The *Em* proteins were first identified in dry wheat embryos and were found to accumulate during late embryogenesis and during application to exogenous ABA (Cummings and Lane, 1979; Grzelczak *et al,* 1985). The *Em* gene encodes a LEA class of

proteins in wheat and rice (Cummings, 1984; Williamson and Quatrano, 1988). The *Em* gene was molecularly characterized (Cummings, 1984; Litts *et al,* 1987; 1992) and its ABRE has been identified (Gulitinan *et al,* 1990; Marcotte *et al,* 1988; 1989).

2.4.4 Accumulation of Dehydrinsin response to ABA:

Dehydrins are a class of immunologically related proteins whose abundance in seedlings increases during gradual non-lethal dehydration conditions (Close *et al,* 1989). These proteins are homologous to a rice protein induced by ABA and salt (Mundy and Chua, 1988) and to a cotton embryo protein (Baker *et al,* 1988). Dehydrins, like many other ABA/stress responsive proteins, display a high degree of heat stability. Moreover, these are highly glycine rich, hydrophilic and cystine and tryptophan free. Dehydrins were found to contain conserved linear repeat sequences showing an immunological relatedness to other stress responsive proteins from cereals (Close and Lammers, 1993). For instance, the barley dehydrin cDNA identifies its homologous RNA in dehydrating wheat and other grass seedlings. These results indicate the similarity of dehydrin proteins and mRNA sequences in different plants including wheat and barley.

2.4.5 Cold stress responsive proteins-ABA mediation:

Several cold regulated proteins have been isolated in diverse plants that have common characteristic features. Gilmour and co-workers (1992) have isolated one cDNA designated as *Cor 47* cDNA that was found to encode a 47 kDa hydrophilic polypeptide showing sequence homology with group II LEA protein (Dure *et al,* 1989). Similarly, a *Cor 66* gene has been characterized to be encoding a 66 kDa alanine rich hydrophilic polypeptide. Lin and Thomashow (1992 a) have isolated a cold responsive *Cor 15* cDNA that encodes a boiling stable protein. This protein was found to stabilize a labile enzyme LDH-M4 from the freeze-thaw stress conditions many times more efficient than any a standard protein like BSA (Lin and Thomashow, 1992 b)

A novel gene called *Kin 1* was shown to be inducible during cold stress in *Arabidopsis thaliana* seedlings (Kurkela and Frank, 1990). *Kin 1* was also induced by water stress and ABA that has been shown to be a common mediator for osmotic stress responses and cold acclimation in higher plants. In *Arabidopsis,* low temperature induced *Lit 140* gene (Nordin *et al,* 1991) was also found to be induced by water stress and ABA.

Expression of this gene was found to be independently signaled in cold stress, ABA and water stress conditions. Thus, the cold regulated gene expression is mediated both by ABA dependent and independent pathways.

2.4.6 Desiccation Responsive proteins: ABA inducibility:

Some of the best characterized desiccation responsive genes and gene products have been isolated and characterized in the African resurrection plant *Craterostigma plantagineum*. This plant is unique in the sense that it can withstand the water loss of as much as 5-10% but can be revived back to normal conditions in a miraculous amount of time. The transcripts and their corresponding genes have been isolated and well characterized recently. Interestingly, these desiccation responsive genes have also been found to be responsive to exogenous ABA application denoting the possible involvement of ABA responsive machinery in the induction of these genes in these plants. Five different cDNAs were extensively characterized from this plant from the desiccation stage and were predicted to encode proteins of molecular mass ranging between 16-34 kDa (Piatkowski *et al.,* 1990). Three of these proteins showed extreme hydrophilic characters and have been of unusual amino acid composition. The sequence homologies of pcC 27-04 and pcC 6-19 displayed partial homology to desiccation related genes expressed in the embryos and dehydrated seedlings of several plants and to that of cotton *lea* genes (Baker *et al.,* 1988).

Attempts to engineer desiccation tolerance in tobacco plants through constitutive expression of predictably the best suited group II LEA protein, however, did not result in the improved tolerance (Iturriaga *et al,* 1992). This may be explained that any single protein component may not exclusively lead to tolerance as stress tolerance is a complex phenomenon.

2.4.7 Salinity responsive proteins:

Significant progress has been made in identifying different classes of salinity responsive proteins from a wide range of plant species. Since their accumulation in the plant cell coincides with the adverse salinity stress conditions, these proteins are predicted to play a role in conferring salinity tolerance to these organisms. However, among the induced proteins, there have been both ABA dependent and independent modes of

induction. Biochemical and molecular studies from various groups of plants revealed the salinity responsive induction of diverse sets of proteins in Citrus (Ben-Hayyim *et al,* 1989), rice (Rani and Reddy, 1995; Rani, 1996), (Shirata and Takahashi, 1990), sugarcane (Ramgopal and Carr, 1991), tobacco (Singh *et al,* 1985), tomato (King *et al,* 1986), alfalfa, (Winicov *et al,* 1989), barley (Ramgopal, 1988), maize (Ramgopal, 1986), wheat (Gullick and Dvorak., 1987). These diverse osmotic or salinity stress responsive proteins that have been identified can be classified in to two classes, namely, thaumatin-related and germin related polypeptides.

2.5 Role of ABA in stress-hardening:

Since the application of ABA is known to harden plants against diverse stress conditions, ABA induced transcriptional and translational machinery is assumed to play a direct role in such a hardening process. Though such results throw open an array of indirect evidences in support of ABA mediated biochemical and molecular machinery, not many studies are conducted to prove the direct involvement of ABA in the stress tolerance process. It is, therefore, worthwhile to pursue the correlative study to gather more information in support of the role of these gene products in tolerance. However, both the positive and negatively regulated gene products could play a role in the stress tolerance processes as ABA is known to both up-regulate and down regulate gene expression.

The exact role of these gene products is still speculatory without any direct evidence to unequivocally prove the involvement of these gene products in stress tolerance. Lack of knowledge on their biochemical or physiological roles makes it difficult to ascribe any direct functional role for these proteins in tolerance studies. The major activity in this analysis has been to compare the sequence homologies between the stress/ABA responsive proteins with that of the wealth of sequence data that is available from plants, animals and bacteria. A classic example for this has been the identification of dehydrin-like protein in cyanobacteria (Close and Lammers, 1993) and a protein involved in salinity stress tolerance in yeast, homologous to salt stress and ABA responsive protein in plants (Gaxiola *et al,* 1992). However, caution must be exercised to assign the functional identity of stress responsive proteins on the basis of sequence homologies as the structural-functional relatedness could often be misleading.

2.6 The role models in plant stress research:

Most of these studies to assign the role to stress responsive proteins have been mainly correlative where the positive correlation between the induction of these proteins and the attainment of tolerance have been deemed to be worth considered. In such efforts, the mutants and inhibitor studies are quite handy. The natural and induced mutants of bacteria and plants that withstand the stress conditions constitute the best suited research materials to study their characteristic features (both biochemical and physiological) under various stress conditions. Similarly, the wild relatives of plant and crop species many a time posses extraordinary traits for stress tolerance. For instance, a wild relative of rice, *Porterasia coractata* exhibits abnormal levels of salinity tolerance uncharacteristic of rice. The halophytes similarly have some characteristic stress tolerance adaptations that give them growth advantage under adverse stress condition. A couple of classic examples of plants that strikingly show abnormal levels of tolerance to stress conditions are the African resurrection plant *Craterostigma plantagineum* and the halo-tolerant ice plant *Mesembryanthemum crystallium,* whose salient attributes in response to desiccation and salinity tolerance have been studied in detail (Bohnert and Jensen, 1996; Bartels and Nelson, 1994). Those transcripts and proteins exclusively appearing during the stressful phase with ABA mediation in these plants could constitute the candidate tools to improve stress tolerance in commercial crop varieties (Piatkowisky *et al.,* 1990; Bohnert *et ai,* 1995).

2.7 Role of osmolytes **in** stress tolerance processes:

Accumulation of osmolytes in response to various environmental stress conditions has been observed to be an evolutionarily conserved feature in organisms ranging from prokaryotes to higher plants. Drought and salinity are the two most important environmental factors that cause osmotic stress and thus accumulate these osmolytes to counteract the osmotic imbalance in the cytoplasm (Yancey *et a/.,* 1982). In the salinity stress, such an accumulation becomes all the more necessary as the excess of ions in the external medium not only cause severe osmotic stress but inflict severe damage to the cellular components. Plants have a special mechanism to exclude these ions in to vacuoles, to counteract which, plant cells accumulate compatible osmolytes in the cytoplasm that do

not interfere with the cell metabolism. Desiccation of the cytoplasm is, therefore, prevented by the accumulation of these osmolytes that preserve the cell integrity from disintegration. There are many well documented evidences, where these osmolytes have been found to revive the growth of the organisms under severe stress conditions. For instance, the accumulation of proline and betaine in some somaclonal mutants of plants is known to impart salinity tolerance in them (Dix, 1993, Kirti *et al.*, 1991). Similar results exist where the accumulated osmolytes have revived the growth of prokaryotes under severe stress conditions (Csonka, 1989; 1981; Galinski, 1993).

The solutes that accumulate in response to the osmotic stress, as a rule, should be very highly soluble so that they can create water-like (hydrophilic) environment in the cytoplasm. Some of the very well studied osmolytes that have been widely implicated in stress tolerance are iminoacids (proline), quartenary ammonium compounds (betaine [N,N,N trimethyl glycine]), sugar alcohols (mannitol, sorbitol, pinitol, ononitol), methylated quaternary ammonium salts, reducing sugars and glycerol (Jefferies, 1981). These were known to be non-toxic and were widely reported to accumulate in cytoplasm.

Proline among aminoacids and betaine among quartenary ammonium compounds are the chief organic solutes that seem to accumulate under water stress conditions in a wide range of organisms. Among polyols, mannitol among acyclic and pinitol among cyclic sugar alcohols are well characterized in various organisms. These were known to accumulate in halophytes, glycophytes and microorganisms (Stewart and Lee, 1974; Trieschel, 1975; Storey and Wyn-Jones, 1978 a, 1978 b; Wyn-Jones and Storey, 1981).

2.7.1 The sugar alcohols-Role in stress tolerance:

Many prokaryotic organisms are inherently capable of synthesizing sugar alcohols intra-cellularly. The polyols can be grouped in to straight chain compounds (mannitol and sorbitol) and cyclic compounds (*myo*-inositol and its related substances). There is a large body of evidence to suggest the probable recruitment of these osmolytes by the stress tolerance machinery under diverse stress conditions in organisms as wide as prokaryotes, yeast, marine algae, invertebrates and higher animals. Particularly, the excretory organs of higher animals are known to utilize the sugar alcohols as the osmotic compounds. Polyols are known to act both as osmoregulators and osmoprotectants. Alternatively, these are also

known to act as free radical scavengers under abiotic stress conditions. The interaction of polyols with biomacromolecules like proteins and lipid membranes is well studied to prove their probable role in stress tolerance processes (Bohnert *et al,* 1995). Further, these could be acting as the major store of carbon and nitrogen under the stressed conditions that could subsequently be converted to simpler metabolites for the production of energy. Due to their importance in the tolerance processes, the carbon is diverted to osmolyte biosynthesis in many organisms (Bohnert and Jenson, 1996; Yancey *et al,* 1982).

Inositols comprise another group of sugar alcohols predicted to perform the osmoregulatory functions in the cytoplasm. In the ice plant *Mesembryanthemum crystallium*, the production of D-ononitol and subsequently D-pinitol from *myo*-inositol is under strict environmental regulation. Cold stress and salinity stress are known to induce the *imt* gene that in turn leads the pathway to produce the osmolytes (Vernon and Bohnert, 1992 a and b). In fact, accumulation of pinitol in the cytoplasm and chloroplasts of stressed plants is known to reach concentration as high as 700 mM (Adams *et al,* 1992). The regulation of *ino1* gene was similarly reported to be under the influence of environmental stress conditions that utilizes the glucose-6-phosphate as a substrate for the production *myo*-inositol biosynthesis (Bohnert *et al,* 1995). Recently, improved tolerance to drought stress was achieved by manipulating the fructan accumulation in tobacco (Pilon-Smith *et al.*, 1995).

The cyclic alcohols, pinitol and ononitol were similarly reported to be accumulated in a variety of species where their accumulation was often correlated with salinity tolerance process (Paul and Cockburn, 1989). In efforts to hyper-accumulate ononitol in the cytoplasm, transgenic plants were developed where the expression of *imt* was manipulated under the control of constitutive promoters. The resulting transgenic plants were found to accumulate ononitol in the cytoplasm that showed improved tolerance to salinity stress (Vernon *et al,* 1993; Sheveleva *et al,* 1997). Ononitol was also identified in the nodules of *Pisum sativum* and *Glycine max* as a stress adaptive molecule (Streeter, 1985).

Sorbitol has been attributed an osmoregulatory function in the epithelial cells of renal inner medulla where urine is being concentrated (Gracia-Perez *et al,* 1989;

Moriyama, *et al,* 1989). Interestingly, the addition of sorbitol *in vitro* to the restriction enzyme Pst I was found to confer protection to the enzyme against stress conditions (Colaco *et al,* 1992). Sorbitol is synthesized from glucose by the enzyme aldose reductase. Bartels and co-workers (1991) have reported an aldose reductase related protein from barley during the late embryogenesis, which is also responsive to abiotic stress conditions. Its modulation by abscisic acid and abiotic stress conditions in the vegetative tissues and its developmental regulation in the desiccation-tolerant phase of embryogenesis supports the possible implications of sorbitol biosynthesis in the desiccation tolerance processes in plant cells (Bartels *et al,* 1991). Manipulation of sorbitol biosynthesis in the cytoplasm of plant cells could be a profitable strategy in the future to develop stress tolerant crop plants.

In an attempt to understand the possible role of mannitol in the salinity tolerance process, transgenic plants were generated where the *E. coli* gene *mtlD* gene, encoding mannitol-1- phosphate dehydrogenase, was constitutively expressed under the influence of cauliflower mosaic virus 35 S promoter. The resulting transgenic plants were found to accumulate 6 μmol/g fresh weight that could represent 60 mol m^{-3} or more if compartmentalized. In the presence of inhibitory concentrations of salt (250 mM), the transgenic plants accumulating mannitol have been found to exhibit improved tolerance as reflected in their better height gain, less fresh weight loss and more new leaf and root production (Tarczynski *et al,* 1993). Presumably, the accumulated mannitol could be protecting membranes and organelles from desiccation stress. The organellar targeting of the osmolytes could have wider implications for a stable organellar functions under stress. For instance, increased tolerance to oxidative stress was attained by targeting mannitol biosynthesis to chloroplasts (Shen *et al,* 1997 a). Mannitol was, in fact, found to protect biomacromolecules against oxidation by hydroxyl radicals (Shen *et al,* 1997 b).

2.7.2 Betaine: Another promising osmolyte:

Characteristically found abundantly in the members of Chenopodiaceae, glycine betaine (also called betaine) occupies a prominent role among the known osmolytes. The accumulated glycine betaine levels were often correlated with salt tolerance in the

members of Poaceae, with the tolerant genera *Spartina* and *Sistichlis* accumulating predominantly higher quantities, moderately tolerant genera accumulating the intermediate levels. The sensitive among this family were found to accumulate the least quantities of betaine intra-cellularly, indicating the positive correlation between the stress tolerance and the betaine accumulation (Rhodes *et al,* 1989). There are also genetic evidences that suggest the role of glycine betaine in the salinity stress tolerance processes in barley and maize (Gurmeet and Hanson, 1986; Rhodes *et al,* 1989). Clear differences in adjustment to osmotic stress have been observed among isogenic lines of barley accumulating different amounts of betaine where the tolerant have been found to accumulate higher amounts of betaine. Salinity stress is known to induce both the enzymes responsible for betaine biosynthesis in plants and bacteria (Smith *et al,* 1993; Mercer *et al,* 1993; Weretilnyk and Hanson, 1989; 1990). *Synechococcus* PCC 7942 cells transformed with *E. Coli bet* gene, producing glycine betaine from choline, were found to acquire resistance to salinity stress (Nomura *et al,* 1995).

Betaine has been implicated in sustenance of major physiological functions under stress conditions by stabilizing various components involved in these processes. For instance, Papageorgiou and Murata (1995) have reported an unusually strong stabilizing effect of betaine on the structure and function of the oxygen evolving photosystem II complex. In cyanobacterial thylakoid membranes, glycine betaine was found to enhance and stabilize the oxygen evolution and ATP synthesis (Mamedov *et al,* 1991). Similarly, betaine was found to stabilize the association of extrinsic proteins with the photosynthetic oxygen evolving complex (Murata *et al,* 1992). Mohanty and co-workers (1993) have similarly reported the stabilization of Mn-cluster of the oxygen evolving complex by glycine betaine that could have vital implications in the restoration of physiological functions under stress conditions. In an interesting experiment, the stabilization of oxygen evolution complex and primary electron transport reactions in photosystem II were found to be stabilized by betaine and sucrose under heat stress (Allakhverdiev *et al,* 1996). Recently, betaine was closely implicated in cold stress tolerance process in the cyanobactcria *Synechococcus* species PCC 7942 (Deshnium *et al,* 1997). Manipulation of

betaine over-accumulation was reported to enhance the cold tolerance in the transgenic *Arabidopsis* plants (Hayashi *et al,* 1997)

2.7.3 Proline- A compatible imioacid:

Among the major osmolytes accumulated in organisms under stress conditions, proline is widely studied and implicated in stress tolerance processes. Proline accumulation has been found to be a widely observed phenomenon reported from prokaryotes, eukaryotes and marine invertebrates (Delauney and Verma, 1993). It is widely reported to accumulate in the leaves subjected to environmental stresses specially during drought, salinity and cold stresses. The accumulated proline in some halophytes and halo-bacteria was reported to contribute about 20% of the total dry weight, accounting for upto 1.0 M internal concentration (Stewart and Lee, 1974; Le Rudulier *et al,* 1984). Such an accumulation has often been found to be a resultant of the *de nova* synthesis and partly due to the impaired oxidation under environmentally unfriendly conditions. It was also known to accumulate in response to UV radiation and protect the plants from lipid peroxidation that is harmful to the plant life (Saradhi *et al,* 1995). Proline was predicted to protect proteins and membranes from damage by inactivating hydroxyl radicals or other highly reactive chemical species that accumulate as a resultant of the inhibition of electron-transfer processes (Smirnoff and Cumbes, 1989).

Proline has also been reported to form long lived adducts with the free hydroxial radicals and thus prevent the damage inflicted by these reactive species (Floyd and Nagy, 1984). Proline thus performs multidimensional functions in organisms as a rescue molecule that is aptly accumulated under unfavorable conditions. Proline, therefore, is predicted to increase the resistance of plants to various abiotic stress conditions (Aspinall and Paleg, 1981; Venekamp *et al,* 1989; Alia and Pardhasaradhi, 1991).

Accumulation of proline in plants occurs through glutamic acid conversion (Delauney and Verma, 1993). However, its hyper-accumulation could not only result from its elevated biosynthesis in response to abiotic stress conditions but also in response to the impaired degradation (Greenway and Setter, 1979). In barley leaves, proline accumulation was found to primarily result from glutamate conversion. On the other hand, water stress is known to inhibit proline oxidation, also resulting in its hyper accumulation (Bogess *et*

al, 1976). However, high amounts of carbon and nitrogen reserves were found to be necessary for its hyper accumulation under stress conditions. Relevant reports suggest that proline accumulation in the cytoplasm could be due to the escalated intracellular pH (Venekamp, 1989). Recently, proline accumulation was reported to increase more under stress conditions in light grown barley and wheat seedlings than in the etiolated ones suggesting that proline has a bi-functional role in the adaptation to high salt, as an osmoregulant in light and as a substrate for dark respiration to supply energy for the compartmentation of ions into the vacuole in dark conditions (Sanada, *et al,* 1995). The loss of feed back inhibition of γ-glutamyl kinase was shown to accumulate several hundred folds of proline accumulation that was predicted to be responsible for the growth of bacterial cells under severe salinity stress conditions (Csonka 1989; 1981; Dandekar and Uratsu, 1988).

Proline accumulation has been closely associated with the drought and salinity tolerance processes giving a scope to further dissect the events in its role in tolerance processes. However, there are reports to suggest proline accumulation to be a mere reflectance of stress sensitivity. Bhaskaran *et al,* (1985) from their studies related to proline accumulation in diverse stress tolerant varieties of sorghum, reported that accumulation of proline is not consistent with stress tolerance. Similarly, Ferriera *et al,* (1979) ascribe proline accumulation to be a mere symptom of damage suffered by plants during stress periods. Stress tolerance being multi-character in nature, therefore, can not expectedly be controlled by a single character like proline accumulation. In contrast, Singh *et al,* (1972; 1973 a; b and c) advocated the advantage of proline accumulation to be in favor of plants survival under stress conditions.

The mere correlation between stress tolerance and the accumulation of adaptive compounds may not completely explain the possible role of these compounds. Kramer (1983) aptly cautions that such a con-elation should be made with only a deeper understanding of their biomacromolecular interactions as a proof of their involvement rather than a mere coincidence of their presence under diverse stress conditions. The protective effects of proline during stress adaptation are supported by the facts that proline

is a compatible solute and stabilize macromolecules like lipids, proteins, organelles and cells from severe damage (Pollard and Jones, 1979; Paleg *et al,* 1984; Nash *et al,* 1982).

Many proline accumulating mutants in plants and bacteria have been isolated that were both natural and induced (Dix, 1993; Csonka, 1981; 1989). One of the most striking feature of these mutants whether of plant or bacterial origin is their tolerance to salinity stress conditions (Kirti *et al,* 1991; Glaasker, *et al,* 1996; Meury, 1988). Recent efforts to over express the A -5 pyrroline carboxylate synthetase for enhanced proline accumulation were found to result in an increase of 18 fold proline in the cytoplasm of transgenic tobacco plants. These plants when tested for salinity and drought tolerance showed improved tolerance to these stress conditions (Kishorc *et al,* 1995). Thus, proline seems to be helping the plants to withstand the adverse conditions.

Interestingly, proline and many osmoprotectants were reported to preserve structural and functional integrity of biological membranes during freezing and dehydration. Frozen or dehydrated biological membranes or liposomes were reported to be stabilized by proline and trehalose (Rudolf and Crowe, 1985; Crowe *et al,* 1983). While analyzing such a protection, proline was proposed to involve an interaction by intercalating between phospholipid head groups (Rudolf *et al,* 1986). Heber *et al,* (1973) reported that proline at 100 mM is capable of preventing frecze-induced inactivation of membrane activities. Proline prevents the oxidation of unsaturated vegetable oils (Ahmed *et al,* 1983). Proline is likely to act as anti-oxidant to counteract the chilling induced free radicals.

Proline was also found to preserve the plant cells from severe chilling stress conditions (Xin and Li, 1993). It was proposed that proline could confer chilling tolerance to free cells by having interactions with the prevalent biomacromolecules in the cells it is accumulated. A direct protection offered to the biomolecules could be vital as the stress events completely disrupt the structural integrity of cellular constituents. Maize cells were reported to be protected by proline from the chilling stress (Songstad *et al,* 1990).

The biochemical and biophysical basis of proline's interaction with major biomacromolecules is however very poorly understood. More efforts in this direction

could lead to a clearer understanding of the role of these osmolytes in the stress tolerance processes.

3. MATERIALS AMD METHODS

Abbreviations

A_{260}	Absorbance at 260 nm
ABA	Abscisic acid
abi	ABA insensitive mutant
ABRE	ABA Responsive Elements
APS	Ammonium persulphate
ATP	Adenosine triphosphate
CD	Circular dichroism
cor	Cold Responsive gene
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
g	Centrifugal force
GA	Gibberellic acid
GuHCl	Guanidine hydrochloride
HSP	Heat Shock Protein
I%	% of injury
kDa	Kilodaltons
LDH-M4	Lactate dehydrogenase-M4 isozyme
LEA	Late Embryogenesis Abundant
MDH	Malate dehydrogenase
mw	Molecular Weights
mRNA	Messenger RNA
NAD^+	Nicotinamide Adenine Dinucleotide
NaCl	Sodium chloride
nm	Nanometers
OD	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
PEG	Poly ethylene glycol
PMSF	Phenyl methyl sulphonyl fluoride
RAB	Responsive to ABA
rpm	Revolutions per minute
RNase A	Ribonuclease A
SDS	Sodium Dodecyl Sulphate
ssbp	Single Strand DNA Binding Protein
TBS	Tris Buffer Saline
TAE	Tris/Acetic acid/EDTA buffer
TE	Tris/EDTA Buffer
TEMED	N,N,N,N (tetramethyl) amino methyl ethylenediamine
Tris	Tris (hydroxymethyl) amino methane
Tm	Melting temperature
	Microgram
	Microlitre
μCi	Microcurie

3.1 Chemicals used:

Abscisic acid, acrylamide, agarose, L-alanine, ammonium persulphate, butanol, calf thymus DNA, 4-chloro-1-napthol, EDTA, ethidium bromide, D-glucose, glycerol, glycine betaine, glycine, guanidine hydrochloride, horse-radish peroxidase-anti-immunoglobin conjugate (HRPO-Conjugate), L-hydroxy proline, L-leucine, β-mercaptoethanol, methylene-bis-acrylamide, L-proline, protein molecular weight standards (bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lactalbumin), NADH, NADPH, sodium chloride, sodium lauryl sulphate, sodium pyruvate, spermidine, sucrose, TEMED, Tris, L-valine and all the proteins used for the structural stabilization study were purchased from Sigma Chemicals Co. (USA). The *E. coli* single stranded DNA binding protein (ssb protein), *X* Phage DNA, pBR 322 plasmid, pUC 18 plasmid and topoisomerase I, were procured from Bangalore genei, India. DNase I was procured from Boehringer and Mannheim and the S1 nuclease from Pharmacia Biotech, Sweden.

Acetone, bromophenol blue, coomassie brilliant blue, ethanol, glacial acetic acid, methanol, ninhydrin, orthophosphoric acid, polyethylene glycol, ponceau-S, sulphosalicylic acid, trichloroacetic acid were of analytical grade purchased from local companies

3.2 Rice stocks;

Indica cultivars of rice (*Oryza sativa* L.), differing in their relative tolerance to field drought and cold stress, were used in the present investigation. These varieties were previously classified by rice breeders based on their field performance in multi-locational and multi-seasonal yield trials and were procured from the Directorate of Rice Research, Rajendra Nagar, Hyderabad-500030 (Technology for rice production, Directorate of Rice Research, Hyderabad-30, 1990). Further, these varieties were selfed for a couple of generations before being used as experimental material. Hamsa is a drought and salt-sensitive medium-maturing variety while K39 is a cold tolerant variety. Apart from the above cultivars that were used for a detailed biochemical analysis at the seedling stage, mature leaves of seven cultivars were used in the electrolyte leakage tests under salinity

and osmotic stress conditions. The varieties used are Purpleputtu (PU), Rasi (RA), Prasanna (PR), Pokkali (PO), Hamsa (HA), Annada (AN) and Tulasi (TU). Among these, Purpleputtu is a purple sensitive variety, Annada, Tulasi, Prasanna and Raasi are drought tolerant rice lines widely differing in their tolerance levels as classified by the rice breeders. Pokkali is a known salt tolerant variety and is routinely used as a salt tolerant check in the tolerance screening by breeders.

3.3.1 Rice seedling culture:

Rice seeds were imbibed overnight in tap water in clean glass beakers, surface sterilized by treatment with 5% sodium hypochlorite (v/v) for five minutes and were thoroughly washed with sterile water. Seeds were then germinated upon moistened filter papers under dark conditions. Average temperatures during the seedling culture was 27.0 ^{o}C ±1.0 °C. After growing them for six days in dark, these seedlings were treated with 100 uM of ABA or 100 uM GA3, or the inhibitors (25 µM each of cycloheximide, or actinomysin-D) for three days in the dark conditions while the control seedlings continued to receive only the tap water. These seedlings were exposed to different stress conditions later for the stress tolerance analysis.

3.3.2 Stress treatments:

Nine day old control and ABA treated etiolated seedlings were exposed to cold stress by growing them at 4.0 ^{0}C in a dark cold chamber for five days. The salt stress was administered by growing them in dark in the presence of 200 mM sodium chloride solution for three days. The unstressed control seedlings continued to grow in dark. These stressed seedlings along with their unstressed controls were later brought to normal light conditions and were grown in the 1/2 strength Hoagland solution to calculate the percentage of survival. Those seedlings that turn green under the light conditions were treated as survivors of the stress treatment while their inability to turn green with a further drying has been counted as dead. In each treatment, number of seedlings survived among the total stressed seedlings was taken to calculate the percentage of survival. Each of these experiments were repeated at least five times and their averages were taken as the final result.

Shoot and root proteins from control and ABA (along with the inhibitors) treated seedlings were extracted from the nine day old rice seedlings for SDS-PAGE, immuno-blotting, aldose reductase assay and *in vitro* phosphorylation analysis.

3.3.3 Application of inhibitors of transcription and translation :

Classical inhibitors of transcription and translation were employed to understand the precise mode of action of ABA in conferring the chilling and salinity tolerance in rice. Along with 100 μM of ABA, 25 uM each of cycloheximide or actinomycin D were added to rice seedlings to inhibit the transcription or translation mediated by ABA. Such an application of inhibitors has been a standard practice to inhibit the *de nova* synthesis of proteins mediated by gene activators.

3.4.1 Estimation of electrolyte leakage during the rehydration of PEG and NaCl-stressed mature leaf segments:

Cell membranes are the primary targets of stress and the maintenance of membrane integrity and permeability under stress is an important component of stress tolerance. The relative electrolyte leakage from leaf discs exposed to osmotic and salinity stress was measured to quantify dehydration tolerance in rice seedlings. An experimental protocol to evaluate rice lines for their membrane tolerance to osmotic stress caused by PEG and NaCl was designed. The measurement of electrolyte efflux during rehydration under osmotic stress caused by PEG 40% (approximately equal to -3.0 Mpa) and 200 mM NaCl was carried out with seven rice varieties using a conductometer and classified them based on the ability to maintain the membrane integrity. Membrane damage was expressed in terms of injury index (I%). The influence of light on the kinetics of electrolyte efflux during rehydration was also studied by recording the electrolyte leachates from leaf segments for a period of 12 hours under light. The injury index was calculated as per the procedure of Vasquez-Tello *et al.,* (1990).

Leaf segments of seven cultivars of 45 day old plants from the field were brought in glass beakers containing tap water. Smaller leaf segments of 200 mg fresh weight were incubated in distilled water (control), PEG 40 % and NaCl (200 mM) for 4 hours and then rehydrated for 12 hours. This assay was performed primarily to understand the coiTelation

between the electrolyte leachate and membrane integrity with the established characteristic field tolerance of these cultivars.

3.4.2 Measurement of electrolyte leachates upon freezing stress:

The measurement of electrolyte leachate upon the stress treatment has been a well established indicator of stress membrane integrity and in turn, a better survivability index (Tan and Blake, 1993). ABA at 100 µM concentration was applied to K39 seedlings for 72 hours before the exposure to different sub-zero temperatures for 30 minutes. These seedlings (including the controls) were gradually brought back to room temperatures and subsequently were grown under normal condition for two days in 1/2 strength Hoagland solution. All those seedlings that turn green have been considered as the survived ones. Soon after the stress treatment, the shoots of these seedlings were tested for the electrolyte leachate by measuring the conductivity of these samples. The total leachate was measured from the samples after boiling them at 120 C for 20 minutes.

3.5 Determination of pro line content:

Proline content of rice seedlings was estimated by the method of Bates, *et al.*, (1973). One gram of fresh shoot tissue was ground with 20 mL of 3% sulphosalicylic acid (w/v) in a clean mortar. The homogenate was centrifuged to get a clear supernatant. Ninhydrin reagent and glacial acetic acid (2 mL each) were added to 2 mL of the supernatant. The mixture was boiled for one hour in a water bath. The reaction was terminated by dipping these test tubes in an ice bath. The reaction mixture was later extracted with 4 mL of toluene after mixing vigorously on a cyclomixture for 30 seconds. The chromophore (toluene) was aspirated from the aqueous phase and its absorbance was measured at 520 nm on a Hitachi spectrophotometer against the toluene background. Proline concentrations in the sample were determined by a standard curve calibrated with increasing concentrations of proline standard and were expressed in terms of microgram proline per gram fresh tissue weight.

Acid ninhydrin reagent:

Ninhydrin (1.25 gm) was added to a 50 mL reagent solution containing 30 mL glacial acetic acid, 8 mL of orthophosphoric acid and 12 mL of double distilled water. It was stored in an amber colored bottle until use.

3.6 Extraction of rice proteins:

Total proteins from rice seedlings were extracted as per Rao *et al.*, (1993), with minor modifications. Tissue were finely ground in the liquid nitrogen and extracted with buffer (0.5g/mL) containing Tris-HCl (pH 8.0), 0.4% SDS, 20 mM EDTA, 2 mM PMSF and 5% mercaptoethanol by mixing and boiling for five minutes. The extract was centrifuged at 12000 g for ten minutes and the proteins in the supernatant were precipitated with equal volumes of 20% trichloroacetic acid at 4.0 °C. The resulting pellet was washed at least five times with cold absolute methanol containing 0.4 M ammonium acetate and vacuum dried. The pellet was suspended in a known quantity of sterile double distilled water and was stored in freezer till further use.

3.7. Protein estimation:

Protein content was estimated according to Lowry et, al., (1951) method with minor modifications. Reagent A is 4% sodium carbonate in 0.2N sodium hydroxide. Reagent B is 1% cupric sulphate, C is 2% sodium potassium tartrate and D is 1 N Follins reagent. The working reagent is a mixture of A, B and C in a ratio of 23:1:1. One mL of working reagent was added to equal volume of diluted protein sample (990 µL of water with 10 µL of the protein solution) and allowed to stand for 15 min. at the room temperature. Then, 0.2 mL of reagent D was added and mixed thoroughly on a cyclomixer. After incubating for 30 min., the absorbance was recorded at 750 nm. Bovine serum albumin was used as a protein standard to prepare the standard curve.

3.8 SDS-PAGE electrophoresis:

SDS-PAGE was performed according to Sigma Technical Bulletin MWS-877L based on Laemmli (1970). The following buffers and solutions were prepared to polymerize the gels and to perform the SDS-PAGE.

A Acrylamide solution: 28 gm of acrylamide and 0.74 gm of methylene-bis-acrylamide were dissolved in double distilled water and the final volume were adjusted to 100 mL.

B Separating gel buffer (pH 8.9) *:* 36.9 gm of Tris in 100 mL of double distilled water (pH adjusted with HC1).

C. Stacking gel buffer (pH 6.8) : 5.98 gm of Tris in 100 mL of double distilled water (pH adjusted with HC1).

D. 1% ***SDS solution:*** 1.0 gm of SDS in 100 mL of double distilled water.

E. 10% Ammonium persulphate solution: **100** mg **of APS in 1** mL **of water (Prepared** freshly).

F. Reservoir buffer (pH 8.3) *:* 6.025 gm Tris, 14.4 gm glycine and 1.0 gm of SDS in 1000 mL distilled water (No adjustment in the pH is required).

G. Sample buffer (pH *6.7):* 0.062M Tris (pH 6.7), 10% glycerol, 2% SDS, 5% p-mercaptoethanol and 0.001% bromophenol blue.

Fifteen percent separating gels were prepared (Total volume: 30 mL) by mixing 16 mL of solution A, 4.5 mL of solution B, 3 mL of solution D and 6.45 mL of double distilled water and finally polymerized by adding 30 µL of TEMED and 50 µL of solution E. 5% Stacking gel (Total volume 5 mL) were prepared by mixing 0.9 mL of solution A, 2 mL of solution C and D and 1.5 mL of distilled water and polymerized by adding 10 µL of TEMED and 40 µL of solution E. The gels were fixed in the fixative containing acetic acid and methanol and were stained with coomassie brilliant blue.

3.9 In vitro phosphorylation studies:

The *in vitro* phosphorylation studies were undertaken to know if ABA can mediate the phosphorylation of any stress responsive proteins. The six day old rice seedlings were treated with 100 µM of ABA, 100 µM of GA3 and the cycloheximide, actinomycin D. The shoot and root proteins were extracted in the cold non-denaturing conditions, in to 50 mM Tris-HCl buffer (pH 7.6). The protein was estimated by the Lowry method as described earlier. Fqual amount of protein samples were aliquoted into buffer containing 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM $CaCl_2$ and were incubated in 30.0 °C for three minutes. Finally, 4 µCi of radio-active P^{32} was added and incubated at 30.0 C for 30

seconds. The reaction was terminated by adding equal volumes of 2X sample buffer and 1 mM EGTA.

The samples were run on a 12.5% SDS-PAGE and silver stained. The gel was finally dried on a gel drier and same was exposed to the X-ray film in a lead cassette that was developed after 72 hours. The markers were marked on the film by superimposing the dried gel on the developed film.

3.10 Western blotting:

The total proteins separated on SDS-PAGE gels were clectrophoretically and irreversibly transferred onto nitrocellulose filters (Hoeffer Scientifics, USA) using the Bio-Rad western transfer apparatus at 70 mA for three hours according to the manufacturers instructions. The transfer of the proteins onto the membrane were confirmed by staining it with the ponceau-S solution and the molecular weight markers were marked using a ball pen. The nitrocellulose paper was later washed twice with TBS and blocked with 5% BSA. The membrane was washed again with TBS and incubated with the respective antibodies for 2 hours. The non-specifically bound antibodies were removed by washing thrice with TBS. The blot was then incubatcd with the HRPO-anti-Ig G conjugate for one hour and washed twice in TBS. The blot then was incubated in the stain-substrate solution containing 4 chloro-1-napthol to which 50 µL of H_2O_2 was added to stain the bands denoting the presence of the antigen-primary antibody-secondary antibody-HRPO conjugate. Photographs were taken of these blots for a permanent record.

Solutions :

1. ***Electroblotting*** *buffer (pH 8.0):* 20 mM Tris/150 mM glycine buffer in 4:1 combination with methanol.

2. ***Ponceau-S*** *solution:* 0.5% Ponceau-S, 1% glacial acetic acid in 100 ml of distilled water.

3. Blocking buffer: 5% fat free milk powder in TBS.

4. TBS (pH 8.0): Tris and sodium chloride.

5. *Primary antibodies:* Anti-15 kDa antibody (1:200 dilution), anti-23 kDa antibody (1:200 dilution), anti-18 kDa (pcC 6-19-encoded protein) antibody (1:150 dilution), RAB

antibody 1: 200 dilution), anti-34 kDa (pG 22-69 protein) antibody **(1:150** dilution) in the blocking solution.

6. Secondary antibody: HRPO-anti-Ig G conjugate in the blocking buffer in 1:2000 ratio.

7. *Substrate solution:* 200 mg of 4-chloro-1-napthol in 2 mL of methanol.

8. 5% Hydrogen peroxide solution: 5 mL of H_2O_2 made-up to 100 mL with distilled water.

3.11 Assay of aldose reductase enzyme:

One gram of rice shoot tissue was homogenized under liquid nitrogen with 10 mL extraction buffer (20 mM potassium phosphate buffer (pH 7.5), 5 mM mercaptoethanol, 0.5 mM EDTA). The thawed homogenate was centrifuged at 12000g for 20 min. The supernatant was saturated with ammonium sulphate to 40%. After centrifugation, the ammonium sulphate concentration was raised to 70% saturation in the supernatant. The proteins precipitated by ammonium sulphate were dissolved in a small amount of extraction buffer that was dialyzed and aliquots were assayed for enzyme activity. All steps were performed at 0-4 °C. Decrease in the concentration of NADPH at 340 nm for the first five minutes of the reaction was measured as the enzyme activity at room temperature. Assay mixture contained 100 mM sodium phosphate buffer (pH 6.9), 0.15 mM NADPH and 10 mM DL glyceraldehyde (Sigma) as substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 u mol of NADPH per minute under the conditions mentioned above. The protein concentrations were determined using the Bio-Rad protein assay kit as per the manufacturers instructions.

3.12 Protein denaturation studies:

Different proteins with a wide range of known conformati on a 1 status were studied for their structural and functional stability in the presence and absencc of various additives under three different denaturation methods, namely, Freeze-thaw cycles, heat denaturations and chemical denaturations by GuHCl.

Proteins dissolved in 10 mM Tris-HCl buffer (pH 7.5) were frozen in liquid nitrogen for 60 seconds and thawed at room temperature for five minutes. Such cycles were repeated several times until the protein underwent structural change as evidenced

from the precipitation formed in its solution. Proline or other additives were added in different concentrations to check the protection conferred by it under the freeze-thaw stress.

For heat denaturation studies, enzymes were heat incubated at 50.0 °C temperature for various time intervals ranging from 1-3 hours to register the structural alteration. Proline was added in various concentrations to check the structural stability conferred by it. Aliquots were used for the enzyme assay to correlate the functional integrity of the enzyme.

Similarly, GuHCl (0.5 M) was added to the enzyme in the presence of various concentrations of proline to register the structural and functional stability conferred by it. Aliquots were used for the fluorescence as well as enzyme activity studies.

3.13 PEG precipitation assay:

The PEG precipitation of enzymes in the presence of different osmolytes were conducted based on the procedures of Paleg *et al.*, (1984). The enzymes LDH M_4 and MDH at a concentration of 100 μg/mL each were independently added to different concentrations of PEG-6000 to check the precipitating ability of PEG in a concentration dependent manner. Further, enzymes were added to 15% PEG in the presence of different osmolytes (at 1.0 M concentrations each) as this concentration of PEG was found to precipitate the proteins completely. These were left on ice for an hour. The eppendorf tubes were then centrifuged at 5000 rpm and were allowed to drain in cold room until all the supernatant got decanted. The precipitant was resuspended in 10 mM Tris buffer (pH 7.5). Aliquots from it were used for the enzyme assay.

3.14 Fluorescence spectral studies:

The intrinsic fluorescence of tryptophane was monitored to record its contribution to the emission intensity at an excitation wavelength 295 nm using Hitachi Spectrofluorimeter. The relative fluorescence and *X* emission max were determined under different denaturing conditions in the presence and absence of proline and certain other osmolytes have been recorded. The fluorescence was recorded at the room temperature, with a band pass of 5 nm.

3.15 Circular dichroic spectral studies:

CD spectra of the enzyme LDH-M4 were recorded with Jasco 20 Spectropolarimeter at room temperature. Sample solutions contained enzyme (3.74 μM) dissolved in 10 mM Tris buffer (pH 7.5). The CD values were computed for molar elipticities (G) using the method of Rao and Kumar (1991). Each spectrum is an average of three scans . The spectra could not be recorded in the presence of high concentrations of proline as it interferes with the CD spectrum of the enzyme at such concentrations. Therefore, the CD spectra were recorded with 10 mM proline that was later corrected for its contribution to the CD signal of the enzyme.

3.16 LDH assay:

The formation of NAD was measured spectrophotometrically at 340 nm. The assay was followed essentially as described by Tamiya *et ah,* (1985). The assay mixture contained 80 mM Tris-HCl (pH 8.5), 150 μM NADH, 5 mM pyruvic acid and the assay was run at the room temperature. The decrease in OD at 340 nm was measured for the first four minutes of the reaction as a mark of enzyme activity.

3.17 MDH assay:

The formation of L-Malate and NAD in the reaction mix was measured spectrophotometrically at 340 nm. The assay was followed according to the procedure of Yoshida, (1969). The assay mix contained 83 mM of Tris (pH 8.8), 160 μM NADH, 5 mM oxaloacetic acid and the assay was run at room temperature. The decrease in OD at 340 nm was recorded during the first 4 minutes of the enzyme assay.

3.18 Solvent-accessibility calculations:

After the experimental data on the stabilization of fifteen protein in the presence and absence of proline was obtained, studies were made to understand the conformational features of each of these proteins through the available Brookhaven Protein Data Bank (PDB). All the selected proteins have already been well resolved (at < 2.0 A resolution) through X-ray crystallography and were selected from the PDB for further analysis. The existing VAX version of standard public domain software was used to analyze the solvent accessibility calculations.

Solvent accessibility contact area of each amino acid of the selected individual proteins was calculated using the PSA program that in turn was based on the method of Lee and Richards (1971) with the probe radius 1.4 A. These calculations were carried out for amino acid side chains, main chains, polar side chains, non-polar side chains and total atoms. The proportional share of total accessible hydrophobicity of each protein was arrived at by calculating the percentage of non-polar side chain accessibility from the total side chain accessibility. The main chain component was avoided as it is not considered to be accessible for an interaction.

3.19 DNA melting studies:

DNA melting studies were conducted in a buffer (1 mL) containing 10 mM Tris-HC1 (pH 7.5) and 2 mM EDTA and the indicated concentrations of salt and additives. Calf thymus DNA (1.0 A_{260}) inthe above buffer, with or without the additives, was taken in a 1 cm path teflon-stoppered quartz cell and incubated at the initial assay temperature for 5 min. DNA melting profile was monitored in a Hitachi spectrophotometer attached to a temperature programmer KPC-6 and temperature controller SPR-7. Both the sample and reference cells were heated together at a rate of 1.0 C per minute and the net absorbance was recorded after every 1.0 °C increase. The Tm of DNA was determined graphically from the transition mid-point of the absorbance versus temperature profile.

3.20 DNase I sensitivity assay:

The sensitivity of DNA to DNase I digestion was studied spectrophotometrically by measuring an increase in absorbance at 260 nm in a Hitachi spectrophotometer in the presence of different concentrations of proline. DNase I (1 μg) was added to double stranded calf thymus DNA (1.0 A_{260}) in a buffer (1 mL) containing 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. The enzyme was diluted to required concentration in 10 mM Tris-HCl (pH 7.8) and 50% V/V glycerol. DNase I sensitivity of DNA was also analyzed by agarose gel electrophoresis. 1 μg each of λ phage DNA, or pUC 18 DNA or calf thymus DNA in the DNase I buffer was incubated at 37° C for ten minutes with 25 ng of DNase I in the presence of different concentrations of proline and the digestion products were separated on a 0.8% agarose gel. The DNase I sensitivity of

calf thymus DNA was also compared in various concentrations of proline, betaine and sucrose.

3.21 S1 nuclease sensitivity assay:

The S1 nuclease reaction mixture (30 μL) contained calf thymus DNA (0.5 μg), buffer {5 mM sodium acetate (pH 4.7), 15 mM sodium chloride, 0.1 mM $ZnCl_2$} and proline in different concentrations. The DNA samples in the presence of increasing concentrations of proline were heated at 65.0 C for five minutes and quickly chilled on ice. The reaction was started by adding S1 nuclease (1 Unit) and incubated at 37^0 C for 15 min. The digestion was stopped by adding EDTA and SDS to a final concentration of 50 mM and 1% respectively and the S1 nuclease products were separated on a 0.8% agarose gel.

3.22 Single strand DNA binding protein (ssbp) gel shift assay:

The *X* phage DNA (0.5 ug) in 30 μL buffer containing 10 mM Tris-HCl (pH 8.1), 1 mM EDTA and 20 mM sodium chloride, was heated at 65.0 °C in the presence or absence of 3.0 M proline for 5 minutes and quickly chilled on ice. Increasing concentrations of *ssb protein* was added and after incubation at room temperature for five minutes, the samples were electrophoresed on a 0.7% agarose gel at 25 Volts.

3.23 Displacement of DNA-bound ethidium bromide by proline:

Ethidium bromide (0.4 μg) in the buffer (10 mM Tris-HCl (pH 7.5) and 50 mM sodium chloride) was excited at 480 nm and the emission was recorded between 500-660 nm in a Hitachi spectrofluorimeter. Later, calf thymus DNA (0.5 ug) was added to record the enhancement in the fluorescence emission intensity. Similarly, the emission spectra were recorded with the addition of increasing concentrations of proline to the above reaction mixture after incubating it at room temperature for 15 min.

3.24 Topoisomesrase I assay:

pBR 322 plasmid DNA at a concentration of 500 ng was added to the 10 X assay buffer (2 μL) and 1 uL of Topoisomerase I (2 units). This volume was made up to 20 uL of distilled water such that the concentration of the assay buffer reaches 1 X. It was allowed to incubate at 30.0 °C for 15-20 min. The topoisomerase I activity was arrested by

adding 10% SDS and 1μL of 250 mM EDTA. Loading buffer (3 μL) was added to this mixture that was loaded to the agarose gel and run for 8 hours at 25 Volts.

3.25 Ruthenium complex displacement study:

Ruthenium drug [Ru (Phen)2(dppz) Cl_2] at a concentration of 10 μM was added to calf thymus DNA (1 mg/mL) that was excited at 440 nm to record the fluorescence emission intensity. Addition of proline at 3.0 M was checked for its effect on the fluorescence emission intensity of the drug-DNA complex. The ruthenium complex itself does not show any fluorescence in the aqueous environment that is enhanced several folds with the addition of DNA (Friedeman *et al.*, 1990).

4. RESULTS

4.1.1 Effect of exogenous ABA application on salinity and chilling tolerance:

The present investigation is aimed at understanding the possible role of ABA in salinity and chilling tolerance in rice seedlings.

Six day old etiolated K39 (a cold tolerant cultivar) and Hamsa (a cold sensitive cultivar) seedlings were treated with 100 μM ABA for 72 hours and subsequently exposed to chilling and salinity stress conditions. In order to characterize the mode of action of ABA in imparting tolerance, actinomycin D and cycloheximide (transcriptional and translational inhibitors, respectively) were added to the seedlings along with ABA that arrest the synthesis of any new transcripts or proteins mediated by ABA. Gibberellic acid, a phytohormone involved in promoting the growth, was also added to these seedlings individually and also in combination with ABA, before screening them for tolerance. In fact, treatments with gibberellic acid constitute an additional control.

Application of ABA to young rice seedlings significantly improved their tolerance to chilling and salinity stress *{Table: 4.1.1*). Both the varieties tested were protected by ABA from the stresses with an almost equal efficiency (upto 80-90%) in terms of percent survival *(Figure: 4.1.1).* The addition of actinomycin D and cycloheximide along with ABA, however, significantly diminished the protection mediated by ABA in both cultivars, indicating the requirement of *de novo* synthesized proteins in stress tolerance process mediated by ABA. While the control and GA3 (100 μM) treated seedlings perished upon the stress treatment, a significant increment in their tolerance was observed when ABA (100 μM) was added along with GA3 *(Table: 4.1.1).*

4.1.2 Effect of exogenous ABA application on membrane damage upon freezing stress:

Since the application of ABA was found to significantly improve the chilling and salinity tolerance in young seedlings, an attempt was made to analyze membrane integrity by measuring the electrolyte leachates upon stress. Pre-treatment of seedlings with 100 μM ABA resulted in an improved survival by 40% and 32% after an exposure to 0 °C -2 °C respectively *(Table: 4.1.2*). A reduction in electrolyte leachates was correspondingly associated with their improved survival *(Figure: 4.1.2 and Table: 4.1.2).* The differences between ABA treated and control seedlings were indeed significant in terms of the magnitude of electrolytes leached upon an exposure to freezing temperatures *(Figure:*

4.1.2). ABA presumably confers tolerance by maintaining the membrane integrity under severe stress conditions. However, none of the seedlings survived the freezing stress beyond -2 °C, irrespective of the application of ABA denoting the restricted utility of ABA-induced machinery in imparting stress tolerance beyond such a critical temperature.

4.1.3 Injury index in rice cultivars in response to salinity and osmotic stress:

As the reduced electrolyte leachates was associated with an improved stress tolerance in seedlings, we attempted to score the stress injury in a range of rice cultivars that differ in their tolerance response. Leaf discs from mature leaves of rice cultivars grown in the field were exposed to salinity and dehydration stress by treating them with 200 mM sodium chloride and 40% PEG respectively. Electrolyte leachates from these discs were measured in rehydrating conditions both in light and dark conditions for 12 hours. The injury index was calculated as described by Vasquez-Tello *el al.,* (1990).

Upon exposure to salinity stress, Prasanna and Pokkali, which are known for their high degree of field tolerance to salinity stress, displayed low injury in dark (about 30%) while Raasi showed a medium injury index (about 35%). The leaf discs of salt sensitive cultivars Hamsa, Annada and Tulasi displayed a high injury of above 35% *(Figure: 4.1.3A).* When leaf discs were rehydrated in the presence of light, the stress sensitive Hamsa exhibited the maximum injury index (about 40%) while the tolerant cultivars Prasanna and Pokkali significantly displayed a low injury index (about 25%). It may be noted here that stress tolerance/sensitivity used in this context are relative terms described by the rice breeders that were based on the yield performances of these varieties under multi-locational trials (Technology for Rice Production, DRR, 1990). The results obtained in this study are therefore in broad agreement with their known behavior in the field, as the sensitive cultivars showed significantly higher injury than the tolerant varieties in the given experimental conditions.

In a similar analysis of dehydration effect (in response to PEG application), Raasi showed a high injury (18%), while drought tolerant Annada displayed the lowest injury (4%). In fact, leaf discs of Annada displayed an injury index four times lower than the sensitive variety (*Figure: 4.1.3 B).* The other varieties studied displayed intermediate range of injury in tune with their known difference in field tolerance. Electrolyte leachate

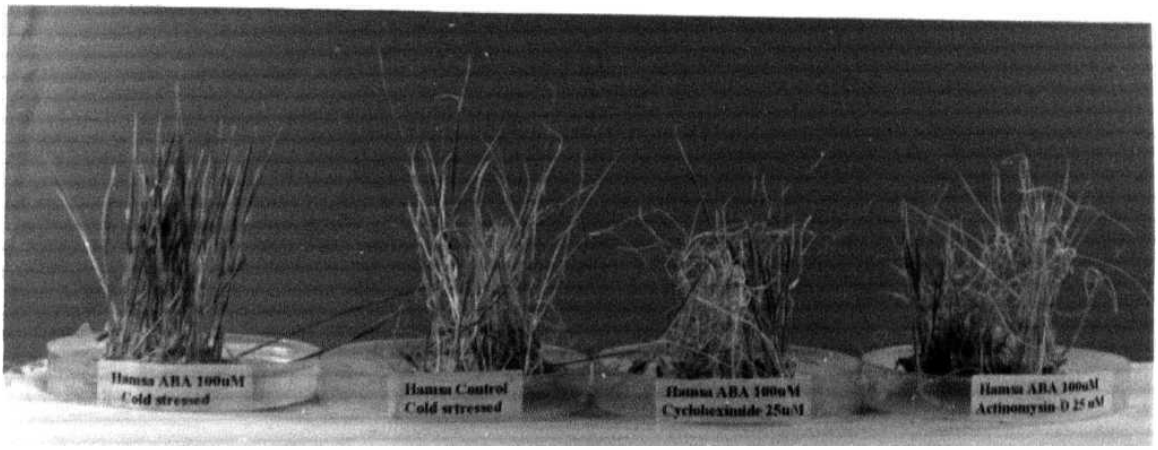


Figure 4.1.1.A. Rice seedlings (cv. Hamsa) exhibiting improved cold tolerance upon ABA (100 μM) pre-treatment.

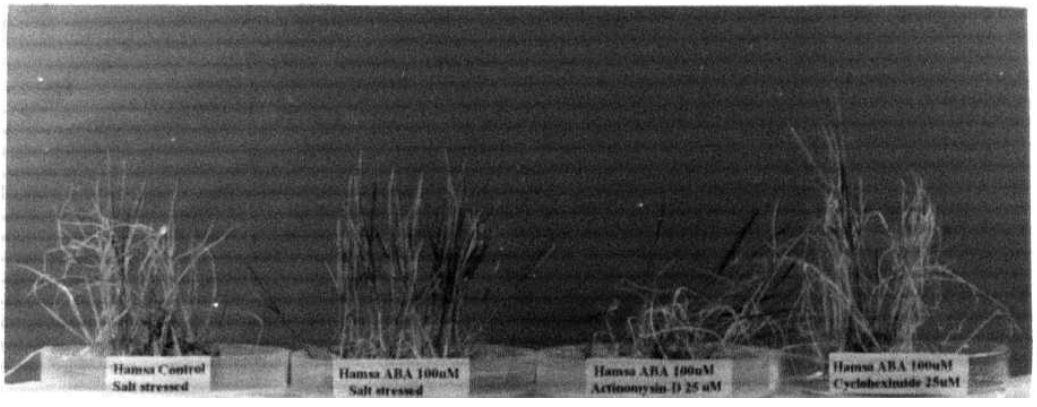


Figure 4.1.1.B. Rice seedlings (cv. Hamsa) exhibiting improved salinity tolerance upon ABA (100 μM) pre-treatment.

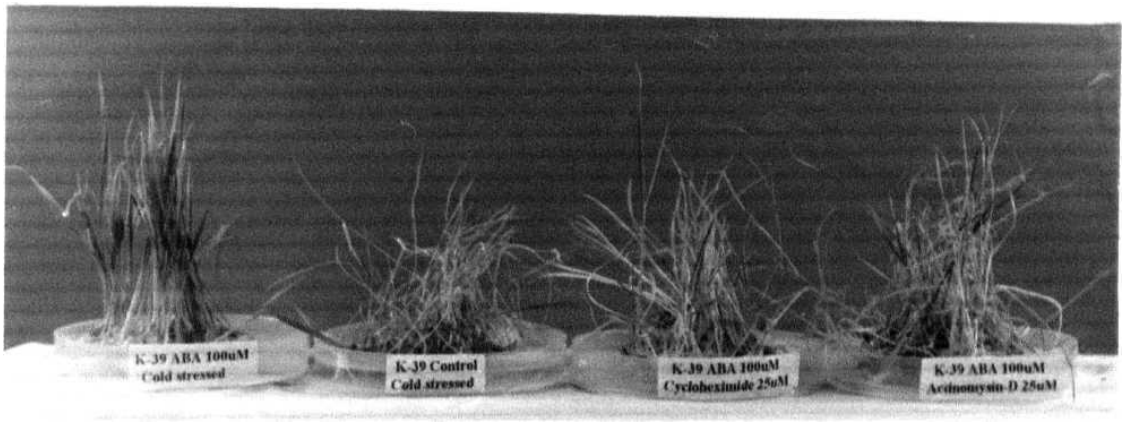


Figure 4.1.1.C. Rice seedlings (cv. K39) exhibiting improved cold tolerance upon ABA (100 μM) pre-treatment.

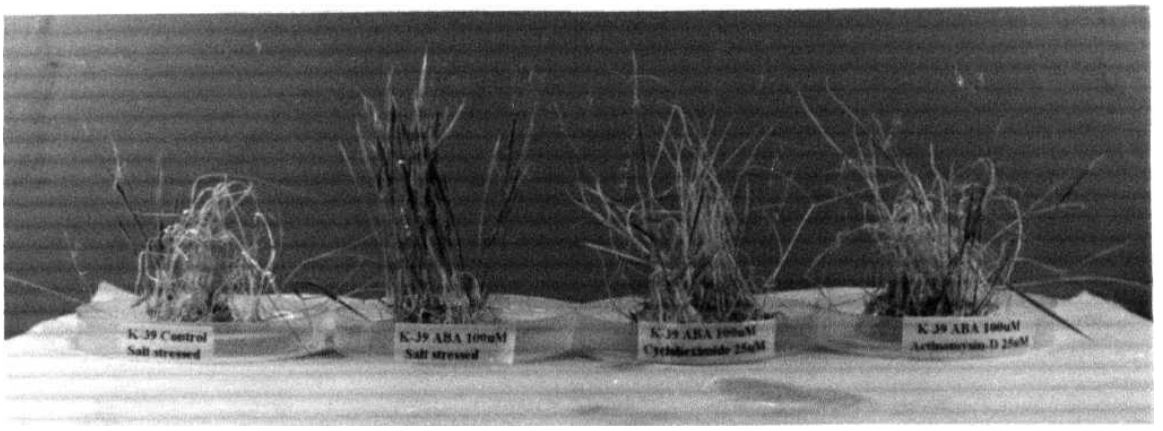


Figure 4.1.1.D. Rice seedlings (cv. K39) exhibiting improved salinity tolerance upon ABA (100 μM) pre-treatment.

Treatment	Percentage survival			
	Cold		Salinity	
	K39	Hamsa	K39	Hamsa
Control (Stressed)	5	3	4	4
100 μM ABA	85	78	89	76
100 μM GA3	3	0	0	3
ABA + GA3 (100 μM each)	77	73	85	77
100 μMABA + 25 μM actinomycin D	7	3	8	4
100 μM ABA+ 25 μM cycloheximide	5	0	10	0
Control (Unstressed)	100	100	100	100

Table 4.1.1 ABA-mediated salinity and cold tolerance response in rice seedlings:

Temp (^{0}C)	Percentage survival	
	Control	ABA
0.0	10	40
-2.0	0	32
-4.0	0	0
-6.0	0	0
-8.0	0	0

Table 4.1.2 ABA-mediated freezing tolerance in rice seedlings (cv. K39):

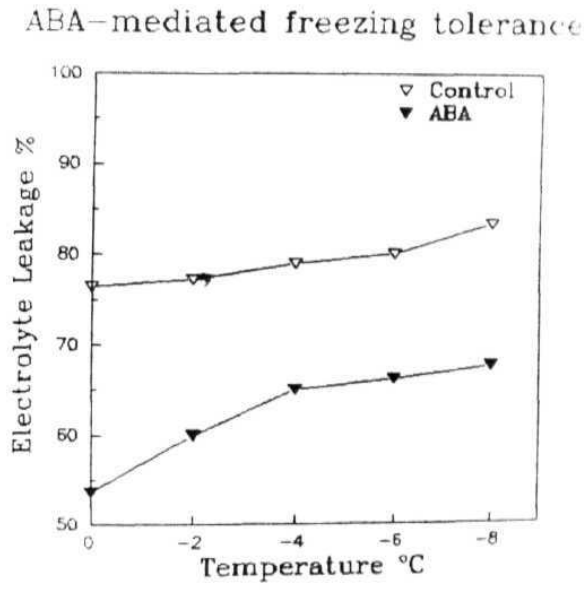


Figure 4.1.2 Electrolyte leakage upon exposure to freezing stress in eontrol and ABA (100 μM) treated rice (cv. K39) seedlings.

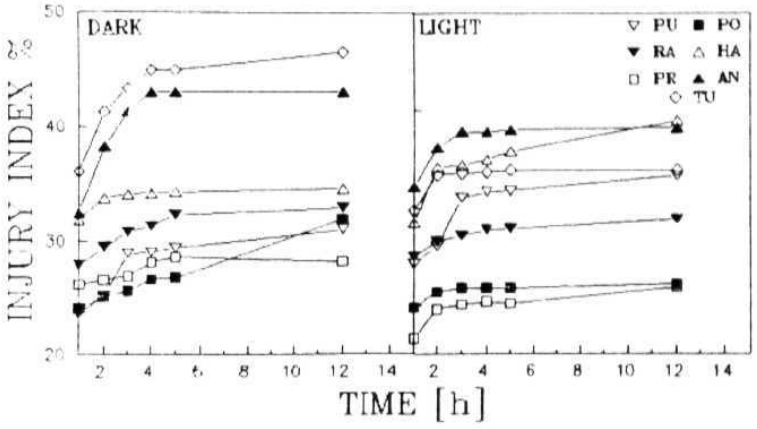


Figure 4.1.3.A. Kinetics of electrolyte release during 12 hours of rehydration in leaf segments of rice upon exposure to salinity stress (200 mM NaCI).

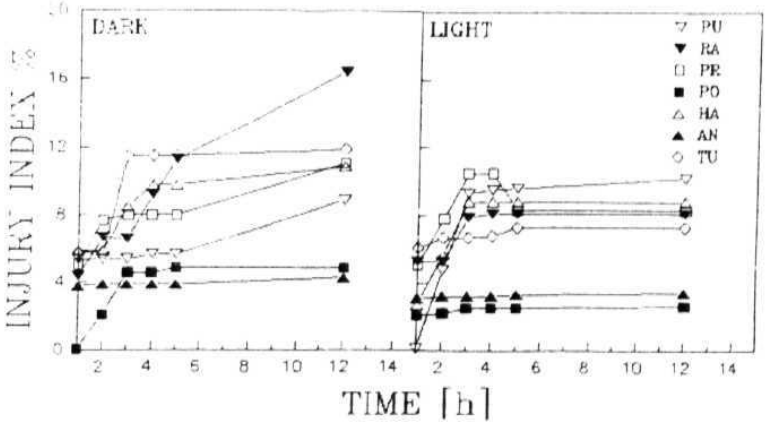


Figure 4.1.3.B. Kinetics of electrolyte release during 12 hours of rehydration in leaf segments of rice upon exposure to osmotic stress (PEG 40%).

measured in the presence of light showed a similar trend where the tolerant cultivars Annada and Pokkali showed the least injury (3%), while the sensitive varieties Purpleputtu and Hamsa displayed the maximum injury index (12%). The salinity stress inflicted 2-4 times higher injury on the leaf discs when compared to osmotic stress mediated by PEG treatment.

4.1.4 Effect of ABA on proline accumulation in rice cultivars:

Rice seedlings were analyzed for proline accumulation in shoots after an exposure to 100 μM of ABA, osmotic (20% PEG) and salinity (200 mM NaCl) stresses. All the three tested cultivars (Hamsa, K39 and Akashi) accumulated considerable amount of proline in response to stress. The cold tolerant K39 and drought tolerant Akashi, however, accumulated significantly higher amounts of proline in response to ABA than Hamsa, a relatively stress sensitive cultivar *{Figure: 4.1.4).* Such a trend was observed with salinity and osmotic stress conditions as well, where Hamsa accumulated relatively lower amount of proline in shoots than the other cultivars *(Figure: 4.1.4).* Salinity stress was found to induce a higher accumulation of proline when compared to other treatments.

4.1.5 Effect of ABA on the induction of polypeptides:

Apart from the quantification of proline in rice seedlings in response to ABA, changes in the induction pattern of polypeptides were investigated as the *de nova* synthesis of stress/ABA responsive polypeptides comprises one of the major biochemical events associated with ABA action in a plant cell. Rice seedlings of K39 were found to accumulate several proteins in shoots upon treatment with 100 |iM ABA. Equal quantities of total shoot proteins (75 μg) from control and ABA treated seedlings were loaded on to 15% SDS-PAGE gel. Some of the ABA-induced proteins observed prominently were 8 kDa, 15 kDa, 16 kDa, 18 kDa, 20 kDa, 23 kDa and 34 kDa, among others *(Figure: 4.1.5, Lane: 3).* These induced proteins were further characterized through Western analysis, using the antibodies raised against some of the stress/ABA responsive proteins from rice and other plants. Since the addition of 100 μM ABA with GA3 significantly restored the tolerance response in seedlings that was absent upon a treatment with GA3 alone *(Table: 4.1.1),* a comparison was felt necessary between these situations in the induction pattern of

polypeptides. Further, the application of cycloheximide along with ABA was found to effectively prevent the onset of tolerance mediated by the latter *{Table: 4.1.1*), requiring a further analysis of these seedlings to identify the major changes in the induction pattern of these polypeptides under the above situations.

4.1.5.1 The 15** kDa osmotic stress responsive protein is induced in response to **ABA:

Induction of a 15 kDa polypeptide was observed prominently in K39 seedlings upon the application of 100 uM ABA *(Figure: 4.1.6).* Antibodies raised against a 15 kDa osmotic stress responsive polypeptide from rice (Karunasree *et al.*, 1992) were used to monitor its induction pattern upon treatment with increasing concentration of ABA. The Western analysis revealed that ABA at 20 uM and above induced detectable levels of this protein in shoots *{Figure: 4.1.6 A Lane:* 7-5). Western analysis was also performed to probe the induction pattern of this polypeptide in response to ABA, both individually and in combination with cycloheximide and gibberellic acid. Application of ABA (100 μM) prominently induced this protein in shoots *{Figure: 4.1.6 B, Lane: 2)* which, however, could be inhibited upon co-addition with cycloheximide *{Lane: 3).* Similarly, GA3 treatment did not induce detectable levels of this polypeptide *(Lane: 4)* that could otherwise be restored upon its co-addition with ABA *(Figure: 4.1.6 B, Lane: 5).*

*4.1.5.2 Accumulation **of RAB**s in correlation with the stress tolerance:*

Western analysis employing the anti-RAB-antibodies revealed a gradual accumulation of RAB protein in rice shoots in response to increasing concentration of ABA *(Figure: 4.1.7, Lanes 1-4).* Nearly, a two fold increase in its induction was observed at 100 μM ABA. Again, the application of GA3 failed to accumulate the RAB protein in seedlings *(Lanes 6)* that could otherwise be induced when ABA was added along with GA3 *(Lane:* 7). The prominent appearance of RAB was therefore in correlation with an enhanced stress tolerance observed in rice seedlings.

***4.1.5.3** Effect of ABA on the induction of desiccation responsive **pcC** 6-19 encoded protein in rice:*

A 18 kDa protein, immunologically related to a desiccation responsive protein encoded by pcC-6-19 sequence from *Craterostigma plantagineum,* was prominently detected in rice in response to ABA. Equal quantities of shoot proteins (75 ug) were

loaded on to the SDS-PAGE that was subsequently processed for an immunoblotting using the anti-pcC-6-19 antibodies. Western analysis revealed a progressive accumulation of this protein in response to increasing concentration of ABA (*Figure: 4.1.8, Lanes 1-7*). Interestingly, this protein is also induced in response to osmotic stress mediated by 20% PEG treatment *(Lane: 9).* The induction pattern of this protein was checked in shoots of the seedlings that were screened for stress tolerance *(Figure: 4.1.8 B).* The protein could be prominently detected in the seedlings treated with 100 µM ABA (*Lane: 2)* and also in seedlings where ABA was co-added with GA3 *(Lane: 5).* The application of cycloheximide with ABA, however, prevented the induction of this protein *(Lane: 3).*

4.1.5.4 *Effect of ABA on the induction of 23* ***kDa*** *polypeptide:*

The induction pattern of 23 kDa polypeptide in response to ABA was probed using the antibodies raised against this osmotic-stress responsive polypeptide from rice (Rao *el al.*, 1993). This protein is one of the most prominently induced in rice seedlings exposed to increasing concentrations of ABA *(Figure: 4.1.9 A, Lanes 3-7).* Further, the protein was also found to be induced in response to osmotic stress mediated by PEG treatment *(Lane: 9).* The application of cycloheximide along with ABA inhibited its *de nova* synthesis mediated by ABA *(Figure: 4.1.9 B, Lane: 3).* The co-addition of ABA with GA3 resulted in its prominent induction in seedlings *(Lane: 5)* that exhibited improved stress tolerance.

4.1.5.5 ***Al*** *dose reductase-related protein is responsive to ABA and osmotic stresses in rice seedlings:*

A 34 kDa aldose reductase related protein was detected in rice seedlings in response to ABA application. It was identified using antibodies raised against a similar ABA modulated protein encoded by an aldose reductase related cDNA pG 22-69 from barley embryos (Bartels, 1991). Aldose reductase is an enzyme involved in the biosynthesis of sorbitol, a prominent stress-induced osmolyte. Western analysis revealed a prominent induction of this protein in the seedlings of K39 variety upon treatment with 100 µM ABA *(Figure: 4.1.10 A, Lane: 2).* Although GA3 failed to induce this protein in seedlings *(Lanes: 5)* its induction could be restored by its co-addition of ABA *(Lane: 6).*

The shoot proteins that were analyzed for the induction of this protein were also assayed for the aldose reductase activity. Enhanced enzyme activity was observed only in

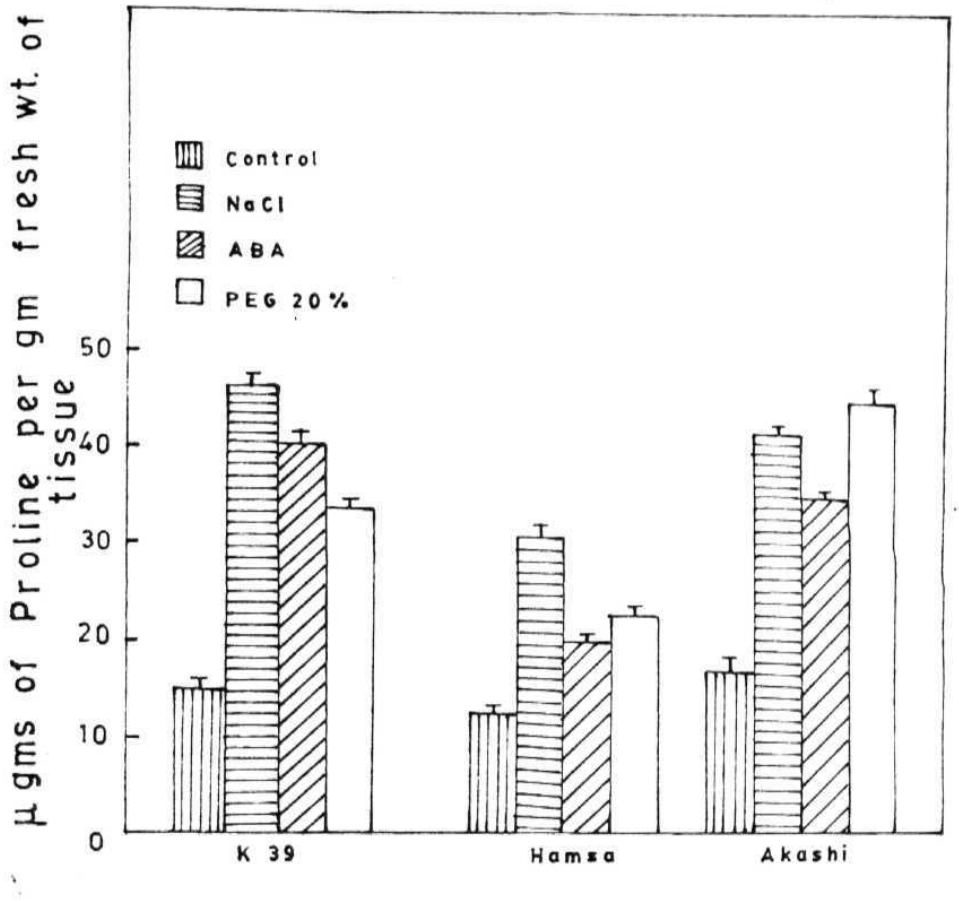
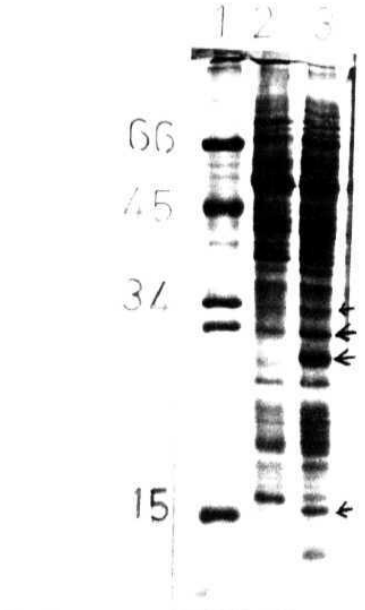
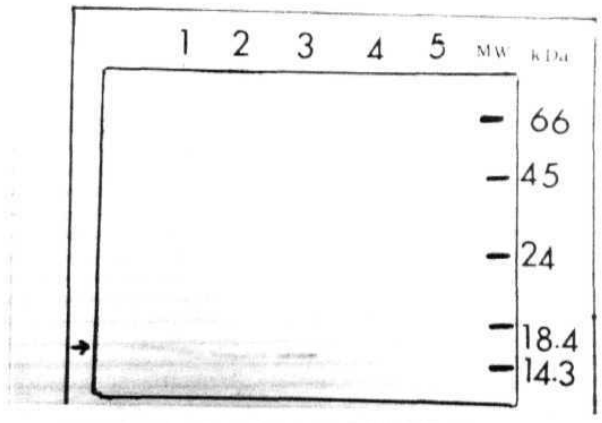


Figure 4.1.4 Accumulation of free proline in different rice cultivars in response to salinity (200 mM NaCI), ABA (100 µM), and osmotic (20% PEG) stresses.



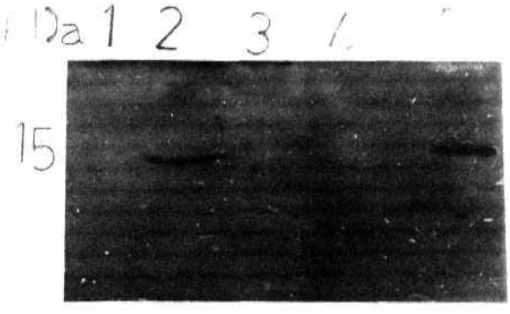
SDS-PAGE profile of rice (cv K39) proteins.

Figure 4.1.5 SDS-PAGE (15%) profile of shoot proteins from control and ABA treated (100 μM) rice seedlings. 75 μg of protein were loaded into each slot. Lanes (1) Protein standard markers, (2) control, (3) 100 μM ABA.



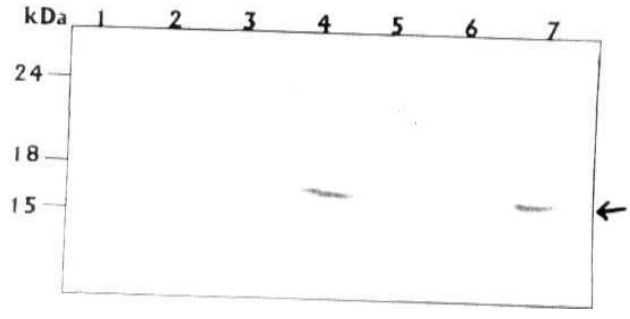
Immunoblot of rice (cv K39) shoot proteins probed with anti-15 kDa antibody.

Figure 4.1.6.A. Western analysis of the induction of 15 kDa polypeptide in rice shoots in response to different concentrations of ABA. Lanes (1) 100 μM ABA (2) 60 uM ABA, (3) 20 uM ABA, (4) 10 μM ABA, (5) control. Equal quantity of sample (75 μg) was loaded into each slot.



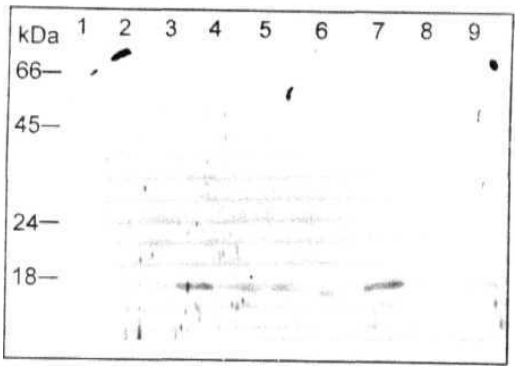
Immunoblot of rice (cv K39) shoot proteins probed with anti-15 kDa antibody.

Figure 4.6.1.B. Western analysis of the induction of 15 kDa polypeptide in rice shoots in response to ABA alone and in combination with GA3 and cycloheximide. Lanes (1) control, (2)100 μM ABA, (3) 100 uM ABA + 25 μM cycloheximide, (4) 100 μM GA3, (5) 100 μM ABA + 100 μM GA3. Equal quantity of sample (75 μg) was loaded into each slot.



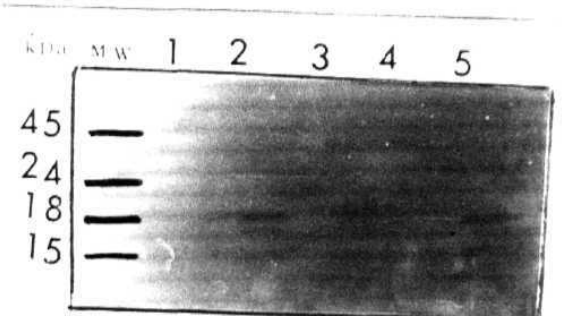
Immunoblot of rice (cv K39) shoot proteins probed with anti-*RAB 16* antibody.

Figure 4.1.7 Western analysis of the induction of RAB protein in rice shoots in response to increasing concentration of ABA. (1) 10 μM ABA, (2) 20 μM ABA, (3) 60 μM ABA, (4) 100 μM ABA, (5) control, (6) 100 μM GA3, (7) 100 μM ABA +100 μM ABA. Equal quantity of sample (75 μg) was loaded into each slot.



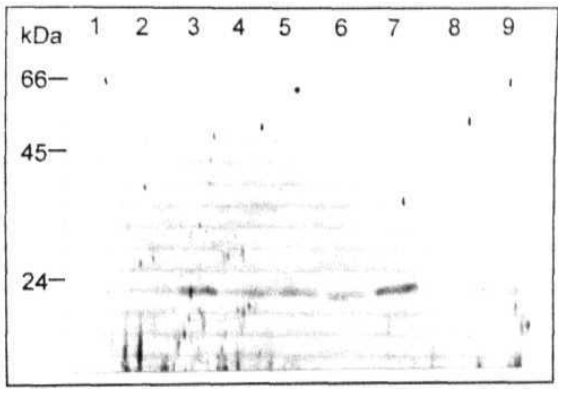
Immunoblot of rice (cv K39) shoot proteins probed with anti-pcC *6-19* antibodies.

Figure 4.1.8.A. Western analysis of the induction of 18 kDa protein in rice shoots in response to increasing concentration of ABA. (1) 10 μM, (2) 20 μM, (3) 40 μM, (4) 50 μM, (5) 60 μM, (6) 80 μM (7) 100 μM (8) control (9) 20% PEG. Equal quantity of sample (75 μg) was loaded into each slot.



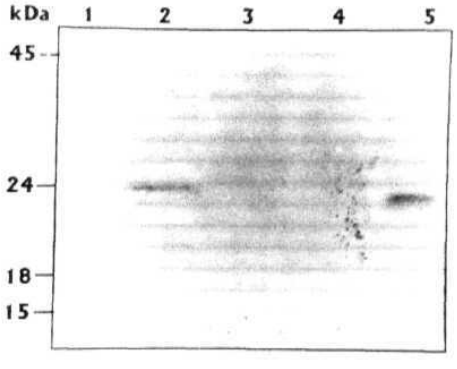
Immunoblot of rice (cv K39) shoot proteins probed with anti-pcC *6-19* antibodies.

Figure 4.1.8.B. Western analysis of the induction of 18 kDa protein in rice shoots in response to ABA alone and in combination with GA3 and cycloheximide. Lanes (1) control (2) 100 μM ABA, (3) 100 μM ABA + 25 μM cycloheximide, (4) 100 μM GA3, (5) 100 μM ABA + 100 μM GA3. Equal quantity of sample (75 μg) was loaded into each slot.



Immunoblot of rice (cv K39) shoot proteins probed with anti-23 kDa antibodies.

Figure **4.1.9.A.** Western analysis of the induction of 23 kDa protein in rice shoots in response to increasing concentration of ABA. (1) 10 μM, (2) 20 μM, (3) 40 μM, (4) 50 μM, (5) 60 μM, (6) 80 μM (7) 100 μM (8) control (9) 20% PEG. Equal quantity of sample (75 μg) was loaded into each slot.



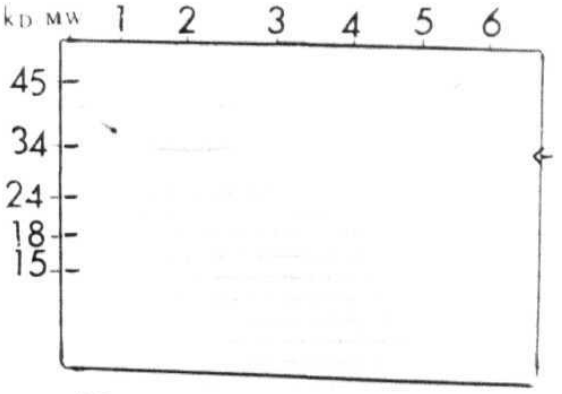
Immunoblot of rice (cv **K39)** shoot proteins probed **with anti-23 kDa** antibodies.

Figure **4.1.9.B.** Western analysis of the induction of 23 kDa protein in rice shoots in response to ABA alone and in combination with GA3 and **cycloheximide.** Lanes (1) control, (2) 100 μM ABA, (3) 100 μM ABA + 25 μM **cycloheximide,** (4) 100 μM GA3, (5) 100 μM ABA + 100 μM GA3. Equal quantity of sample (75 μg) was loaded into each slot.

the seedlings where the protein could be detected immunologically *(Figure: 4.1.10 B).* The co-addition of cycloheximide and actinomycin D with ABA effectively prevented the induction of this protein in shoots *(Figure: 4.1.10 A, Lane: 3* and *4)* and in turn its activity *(Figure: 4.1.10 B).* Such an observation confirms the *de nova* synthesis of aldose reductase-related protein in rice seedlings mediated by ABA. Further, the accumulation of this protein was observed in a concentration dependent manner in response to ABA *(Figure: 4.1.10 C).* Osmotic stress mediated by PEG prominently induced this protein in shoots, denoting its stress responsiveness mediated by changes in the water status *(Figure: 4.1.10 C, Lane:* 7). In essence, the induction of an aldose reductase-related protein was in fine correlation with an improved stress tolerance in the seedlings.

4.1.6 ABA-mediated changes in the in vitro phosphorylation of polypeptides:

Six day old rice seedlings (cv. K39) were treated with ABA for 72 hours and the total proteins were extracted in the native form. These proteins were tested for *in vitro* phosphorylation, labeling them with radio-labeled ^{32}P. The results revealed that ABA induces the *in vitro* phosphorylation of a 55 kDa protein in roots that was not observed in control and GA3 treated roots. However, the shoot proteins did not reveal any detectable changes in the phosphorylation status of proteins *(Figure: 4.1.11).*



Immunoblot of rice (cv K39) shoot proteins probed with anti-*pG22-69* antibodies.

Figure 4.1.10.A. Western analysis of the induction of aldose reductase related protein in rice shoots in response to ABA alone and in combination with GA3, cycloheximide and actinomycin D. Lanes (1) control (2) 100 μM ABA, (3) 100 μM ABA + 25 μM cycloheximide, (4) 100 μM ABA + 25 μM actinomycin I) (5) 100 μM GA3, (6) 100 μM ABA + 100 μM GA3. Equal quantity of sample (75 μg) was loaded in to each slot.

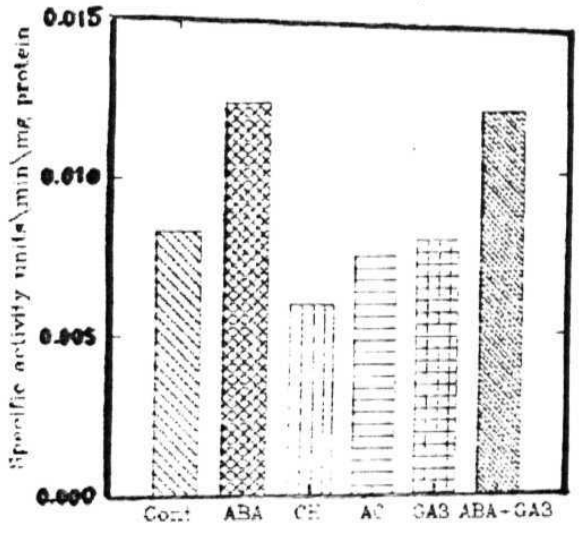
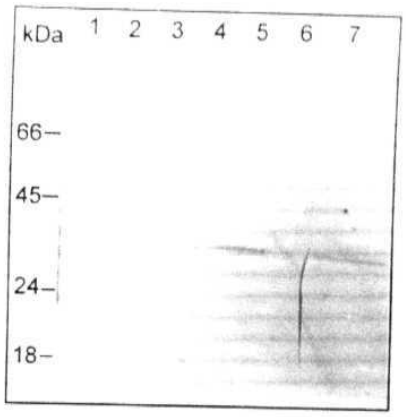


Figure 4.1.10.B. Aldose reductase activity in the shoots of rice seedlings (cv. K39) in response to ABA alone and in combination with GA3 and the inhibitors.



Immunoblot of rice (cv K39) shoot proteins probed with anti-*pG22-69* antibodies.

Figure 4.1.10.C. Western analysis of the induction of aldosc reductase related protein in rice shoots in response to increasing concentration of ABA. (1) 10 μM, (2) 20 μM, (3) 40 μM, (4) 80 μM, (5) 100 μM, (6) control, (7) 20% PEG. Equal quantity of sample (75 fig) was loaded in to each slot.

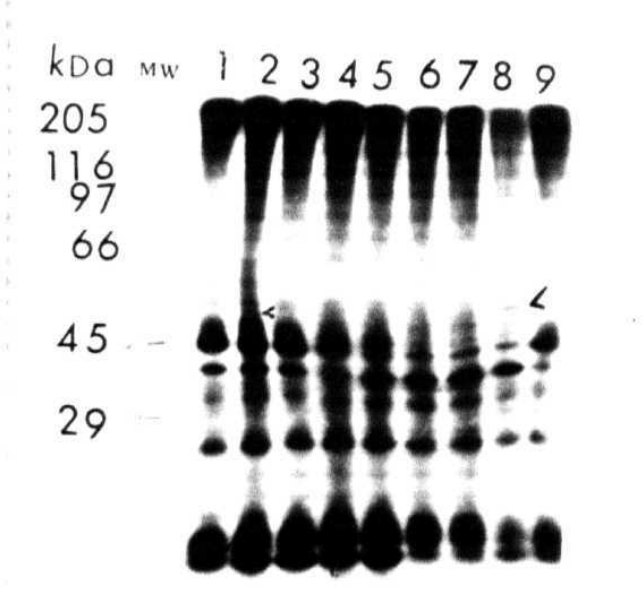


Figure 4.1.11 Autoradiogram depicting the *in vitro* phosphorylated proteins extracted from the shoots and root of rice (cv. K39) seedlings treated with ABA and GA3. Equal quantities of proteins were loaded on to the SDS-PAGE (15%) which was dried and exposed to the X-ray film.

4.2. Proline-protein interactions: Protection of structural and functional integrity of proteins:

Proline-protein interactions were studied in order to get an insight into the mode of action by which proline confers protection to the target proteins. A variety of proteins were subjected to different stress conditions in the presence and absence of proline and other osmolytes and the corresponding structural and functional integrity of the proteins were monitored through spectral studies.

4.2.1 Effect of proline and other osmolytes on protein precipitation by PEG:

Effect of osmolytes in ameliorating the denaturing influence of PEG was tested. A gradual increase in precipitation of malate dehydrogenase (MDH) and M4-lactate dehydrogenase (LDH-M4) was observed upon an addition of poly ethylene glycol (6000), with a near complete precipitation observed at 15% PEG (*Table: 4 2 1).* Different osmolytes (1.0 M each) were added to the reaction mix in the presence of 15% PEG to compare their efficiency in conferring protection to the enzymes. Addition of proline to the enzymes prevented upto 80% of their precipitation. In contrast, hydroxy proline, was much less efficient in protecting the enzymes. Sucrose and glycerol offeree! protection of about 65%, where as glycine was found to prevent the precipitation of these enzymes to the extent of 60% *(Table: 4.2.2)*

4.2.2 Effect of proline on the protection of enzymes from chemical denaturation, freeze-thaw and heat stresses:

Fifteen enzymes of varied conformational status were analyzed for their stability *in vitro* in the presence of proline and other osmolytes under diverse stress conditions such as heat, freeze-thaw and chemical denaturation (by exposure to GuHCl). These included dehydrogenases, kinases, proteases, nucleases, peroxidases among others. Lactate dehydrogenasc-M4 *(EC 1.1.1.27* from rabbit muscle), malate dehydrogenase *(EC 1.1.1.37* from bovine pancreas) and glyceraldehyde-3-phosphate dehydrogenase *(EC 1.2.1.12* from rabbit muscle) were selected for a further analysis among the dehydrogenases. Fluorescence emission of trvptophan was measured to monitor the structural integrity of these proteins as any structural alterations at secondary or tertiary level are clearly reflected in changes in the fluorescence emission intensity of these moieties.

Percentage of PEG 6000	% precipitation of MDH	% precipitation of LDH-M4
0	0.09	0.06
5	30.06	37.73
7.5	36.27	36.40
10	42.86	39.10
12.5	68.69	63.20
15	94.54	95.00
20	95.64	94.56
30	94.94	96.33

Table 4.2.1 Effect of PEG 6000 on the precipitation of MDH and LDH-M4:

Concentration of PEG/Osmolyte	% precipitation of MDH	% Precipitation of LDH-M4
15% PEG alone	94.54	95.00
No PEG	0.090	0.060
15% PEG + proline	15.70	12.60
15% PEG + hydroxy proline	42.00	25.70
15 % PEG + glycine	52.00	39.30
15 % PEG + sucrose	34.00	28.00'
15% PEG + glycerol	29.09	20.40

Table 4.2.2 Effect of different osmolytes on PEG-induced precipitation of MDH and LDH-M4 (All osmolytes are of 1 M concentration each).

4.2.2.1 Protection of dehydrogenases:

Fluorescence emission spectra of LDH-M4 freeze-thawed *in vitro* with different osmolytes (1 M concentration each) revealed that proline exhibited a strong stabilizing effect on the enzyme's structure compared to other osmolytes tested *(Figure: 4.2.1).* The fluorescence emission intensity of the proline-protected enzyme was closer to the unstressed control, reflecting the protection of its secondary structure. Hydroxy proline was found to be less effective in conferring protection than its structural analogue, proline. Valine was the least efficient in offering freeze-thaw protection to the enzyme *(Figure: 4 2.1).* Since proline was far superior than other osmolytes tested in conferring protection to LDH-M4, its cryoprotective ability was studied at increasing concentrations. Proline was effective in offering protection to the enzyme's secondary structure at concentrations as low as 10 mM reaching saturation at 250 mM and above (*Figure: 4.2.2).* The corresponding enzyme activities measured revealed a concentration dependent protection of the enzyme activity (inset).

In order to investigate the effectiveness of proline in conferring thermo-stability to the labile LD1I-M4, the fluorescence emission spectra of the enzyme were recorded after an exposure to increasing temperatures in the presence and absence of 1.0 M proline. The data revealed that an addition of proline at 1.0 M concentration could considerably stabilize the structural integrity of the enzyme at elevated temperatures *(Figure: 4.2.3).* The inset correspondingly showed a clear protection of enzyme activity in the presence of proline at various temperatures. More importantly, the effect of proline in maintaining the structural and functional integrity of the enzyme was evident even at the highest temperature tested, i.e., 47.0 C *(Figure: 4.2.3).*

Guanidine hydrochloride is known to dissociate the tetrameric form of LDH into inactive monomers (Appella and Market, 1961; Ma and Tsou, 1991). An attempt was made to study the effect of proline in protecting the enzyme from such a chemical denaturation. The fluorescence spectral data revealed a concentration dependent protection of LDH-M4 by proline under chemically denaturing conditions *(Figure: 4.2.4).* Such a protection by proline was in agreement with the enzyme activity study (inset) that is both

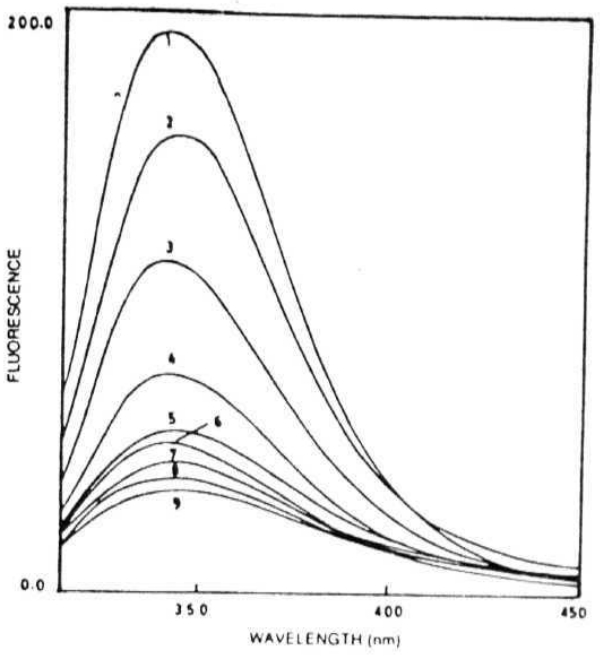


Figure 4.2.1 Fluorescence emission spectra of LDH-M4 (1) control, (2-8) freeze-thawed in the presence of 1.0 M each of (2) proline, (3) sucrose, (4) mannitol, (5) glycine (6) hydroxy proline, (7) glucose, (8) valine and (9) freeze-thawed enzyme.

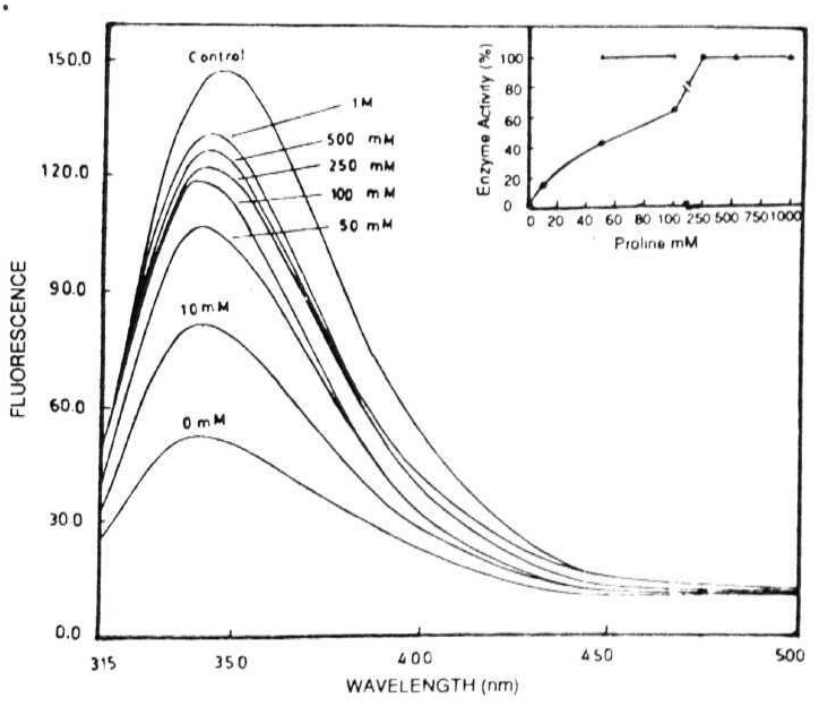


Figure 4.2.2 Fluorescence emission spectra of LDH-M4 control and freeze-thawed enzyme in the presence of increasing concentration of proline as indicated. Enzyme activity is shown in the inset, control (A) and freeze-thawed (•) with proline.

the structure and function of the tetrameric form were evidently stabilized under the denaturing conditions of GuHCl *(Fig. 4.2.4).*

Circular dichroic spectra were recorded for LDH-M4 in the presence and absence of proline after exposing the enzyme to freeze-thaw cycles to estimate the α-helical content of a protein, which in turn reflects its structural integrity under stress conditions. The CD spectra of the native and proline-protected enzymes show typical characteristics of a protein with good helical content *(Figure: 4.2.5)* The native enzyme showed minima at 212 and 222 nm, while the proline-protected enzyme, under the freeze-thaw conditions, showed a minima at 210 and 225 nm. However, the freeze-thawed enzyme in the absence of proline showed a single minimum from 215 nm to 224 nm indicating the disturbed secondary structure. The α-helicity of the proline-treated enzyme was found to be protected when compared to the denatured form. These results arc in agreement with that of fluorescence data that revealed the protection of the secondary structure by proline under severe freeze-thaw conditions.

Since the LDH-M4 was found to be effectively stabilized by proline, a detailed structural analysis was conducted from the X-ray crystallographic data obtained from the Brookhaven Protein Data Bank (PDB). It was evident from these studies that LDH-M4 has quite a number of helices accessible to the solvent that can interact with the osmolytes *(Figure: 4.2.6).* However, the X-ray resolved structure of dog fish M4-LDH was used to explore the structural details of the interaction as the rabbit LDH-M4 structure is not available in the PDB (Zapetero, *el a!.,* 1987). Nevertheless, the clog fish M4 LDH exhibits more than 85% sequence homology to that of rabbit M4-LDH with 95% residues found similar in the regions of helices and strands. For all practical purposes, the structure of rabbit LDH M4 was assumed to be broadly similar to the dog fish LDH-M4. The tryptophan residues were clearly marked in the structure whose positions are located on different helices *(Figure: 4.2.6).* The fluorescence emission from these evenly positioned tryptophane residues in LDH-M4 reflect its structural integrity.

Malate dehydrogenase (MDH) from bovine pancreas was tested by similar analysis under different stressful conditions. Proline, in a concentration dependent manner,

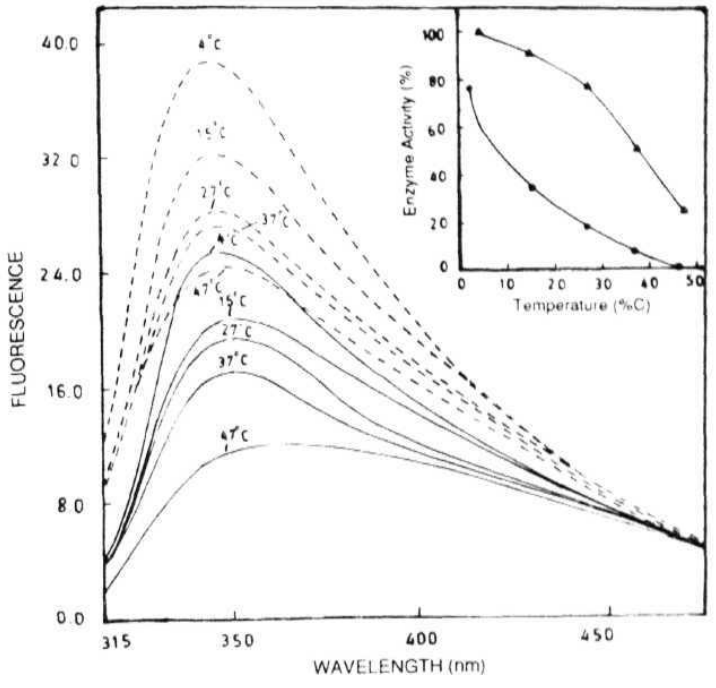


Figure 4.2.3 Fluorescence emission spectra of heat denatured LDH-M4 in the absence (—) and presence (——) of proline (1.0 M). Enzyme activities shown in the inset w ith (A) and without (•) proline.

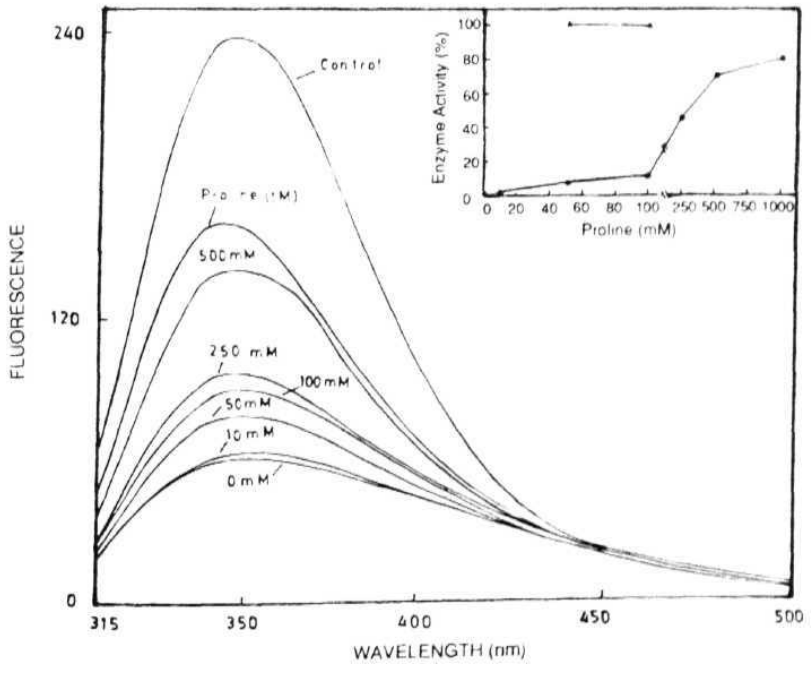


Figure 4.2.4 Fluorescence emission spectra of LDH-M4 control (without GuHCl and proline) and GuHCI (0.5 M) denatured enzyme in the presence of increasing concentration of proline as indicated. The inset shows the enzyme activity in the presence (•) and absence of GuHCl(▲).

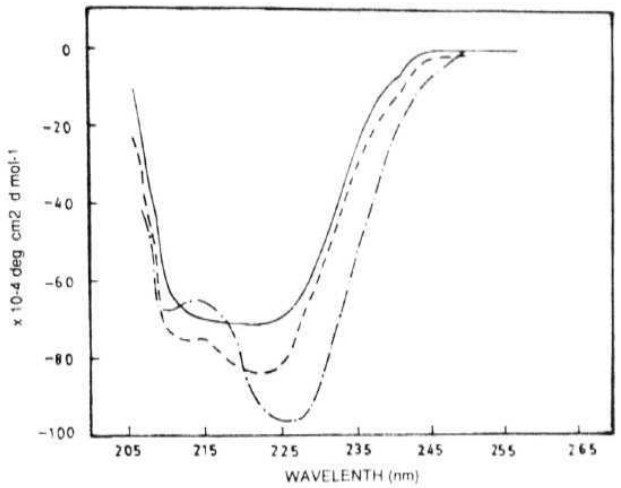


Figure 4.2.5 Circular dichroic spectra of native LDH-M4 (- - - -), freeze-thawed enzyme (—) and freeze-thawed enzyme in the presence of 10 mM proline(-.-.-.-.).

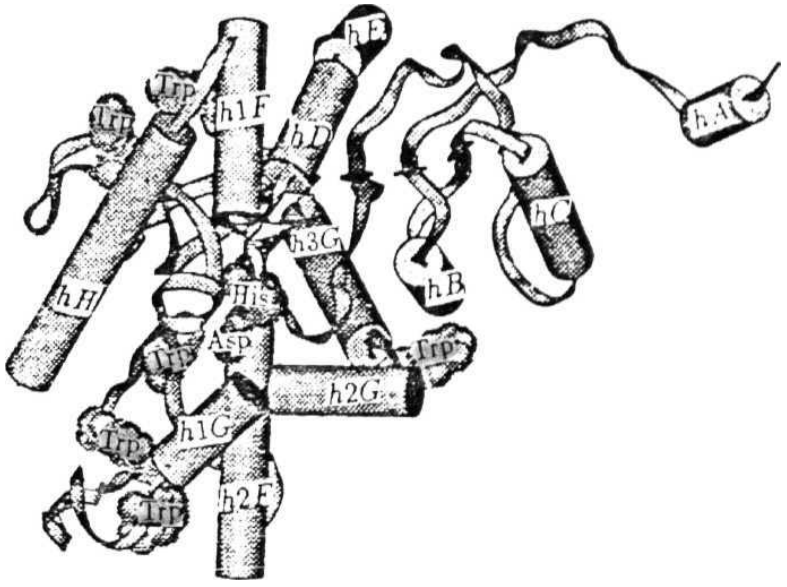


Figure 4.2.6 Molecular model of LDH-M4 depicting the solvent-accessible helices. All tryptophan residues are marked whose fluorescence emission intensity was monitored .

conferred protection to the enzyme under freeze-thaw stress as evident from the fluorescence emission spectra *(Figure: 4.2.7 A).* The inset shows protection of the enzyme activity with increasing proline concentrations where the enzyme activity curve reached a saturation point at 1.0 M proline *(Figure: 4.2.7 A Inset).* A comparative analysis of effect of hydroxy proline and proline revealed that proline is more effective in offering the protection to the enzyme from the freeze-thaw stress condition *(Figure: 4.2.7 B).* Further, proline was found to protect both the structural and functional status of MDH from heat stress and chemical denaturation. The fluorescence emission intensities of the enzyme recorded after an exposure to 0.5 M GuHCl *(Figure: 4.2.8 A)* and heat-stress *(Figure: 4.2.8 B)* have revealed a stabilizing effect by proline in a concentration dependent manner. The insets show a clear protection of the enzyme activity by proline under denaturing conditions. Further, there was a complete protection of enzyme activity at 2.0 M concentration of proline, denoting the total compatibility even at high concentrations of the osmolyte.

Proline, in a concentration dependent manner, was also found to stabilize holo-D-glyceraldehyde-3-phosphate dehydrogenase *(EC 1.2.1.12* from *Bascillus stereothermophillus)* under heat stress condition *(Figure: 4.2.9 B).* As evident from the fluorescence emission spectra, proline was found to be far more effective than its structural analogue, hydroxy proline *(Figure: 4.2.9 A).* The control enzyme heat stressed without any additives, however, exhibited loss of structural integrity.

4.2.2.2 Protection of Kinases:

The protective role of proline was tested by analyzing the structural alterations of labile enzyme phosphofructokinase (PFK) *(EC 2.7.1.11* from *Bascillus stereothermophillus)* in the presence and absence of proline and its hydroxylated analogue. Addition of proline in increasing concentrations was found to confer protection to PFK from chemical denaturation (0.5M GuHCl) and heat stress *(Figure: 4.2.10 A and B).* In both these cases, fluorescence emission intensity of the enzyme protected by proline was closer to that was recorded for the control unstressed enzyme, denoting the stabilized secondary structure of the enzyme. In a comparative analysis with its hydroxylated analogue, proline was found to be more efficient in conferring heat-stability than hydroxy

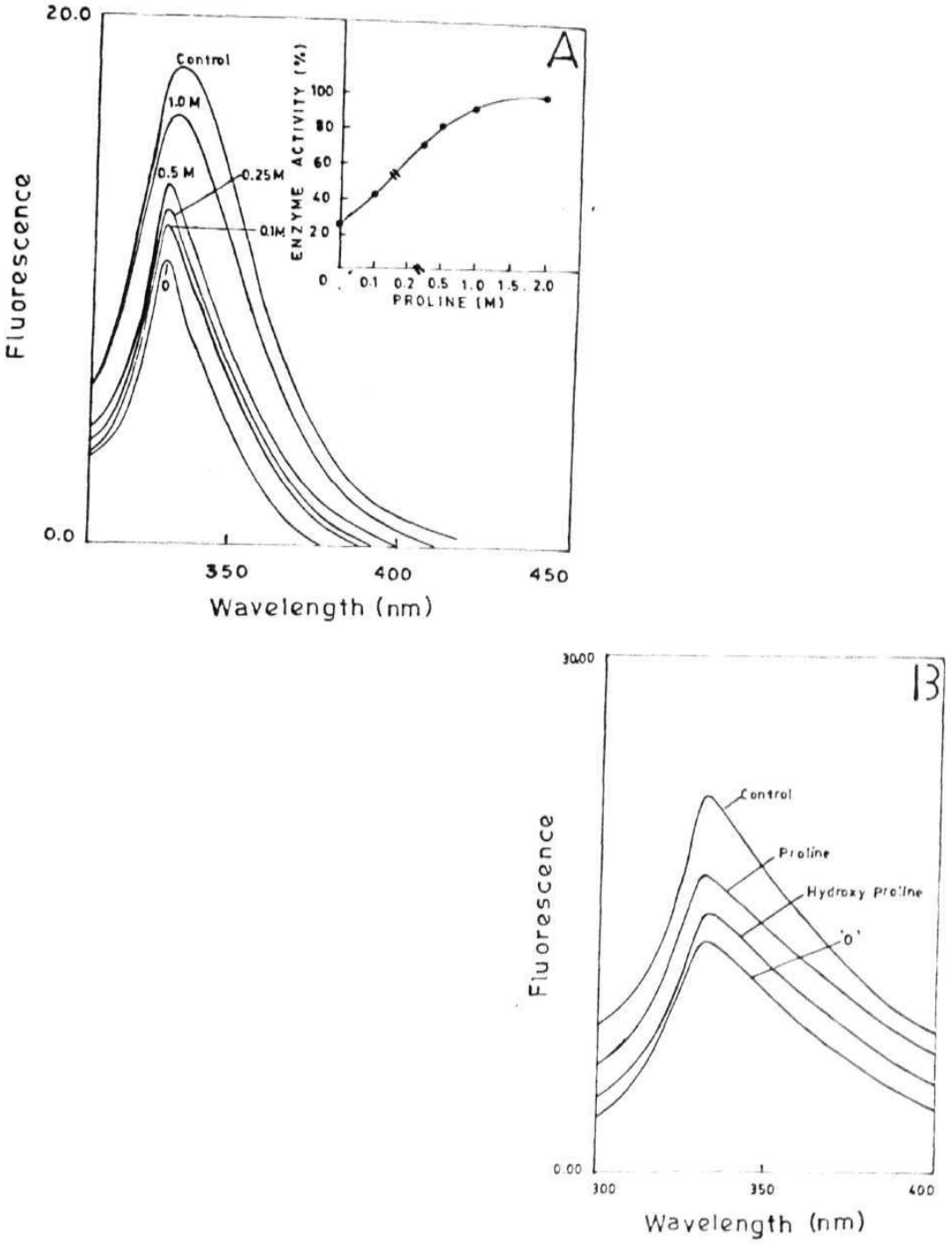


Figure 4.2.7 Fluorescence emission spectra of malate dehydrogenase (EC 1.1.1.37) (A) freeze-thaw stressed in the presence of increasing concentration of proline, Inset shows the corresponding enzyme activity, (B) freeze-thaw stressed in the presence of proline and hydroxy proline (1 M each)

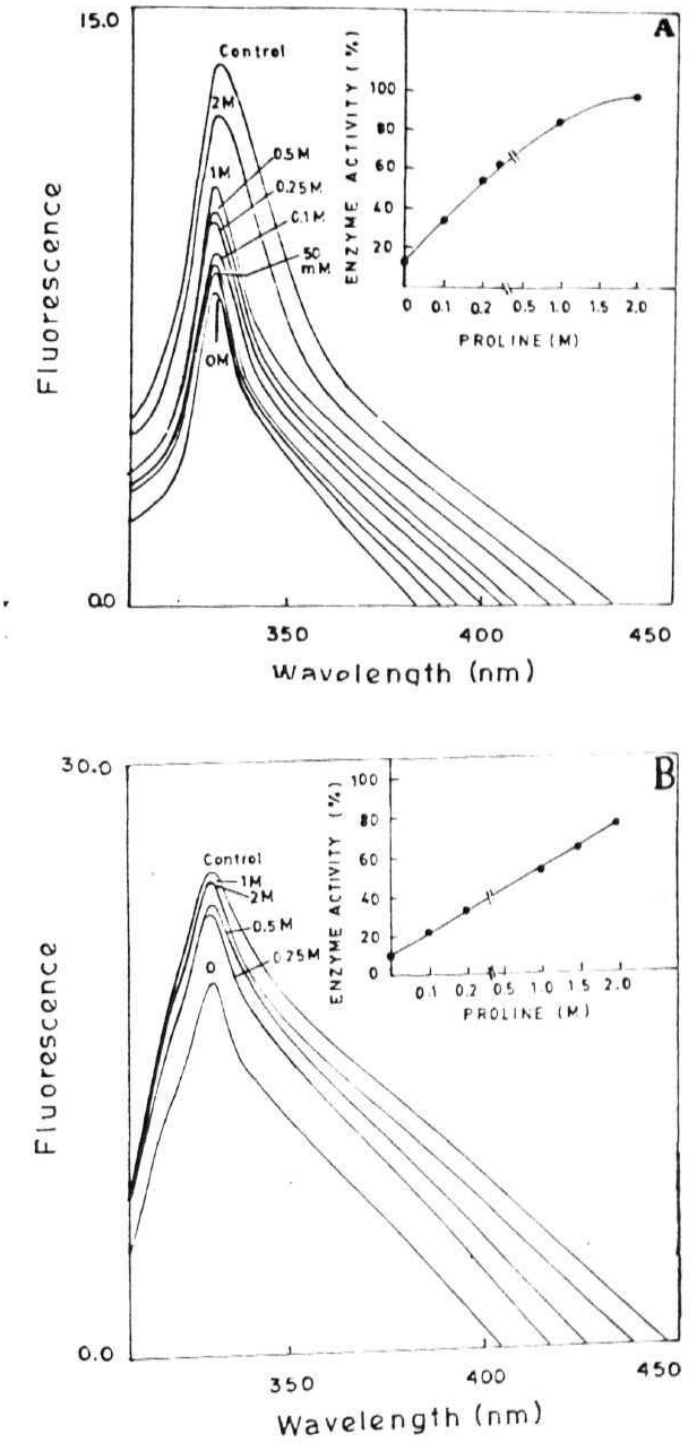


Figure 4.2.8 Fluorescence emission spectra of malate dehydrogenase (EC 1.1.1.37) (A) chemically (GuHCl) denatured (B) heat stressed (50 ^{0}C) in the presence of increasing concentration of proline. Insets show the corresponding enzyme activity.

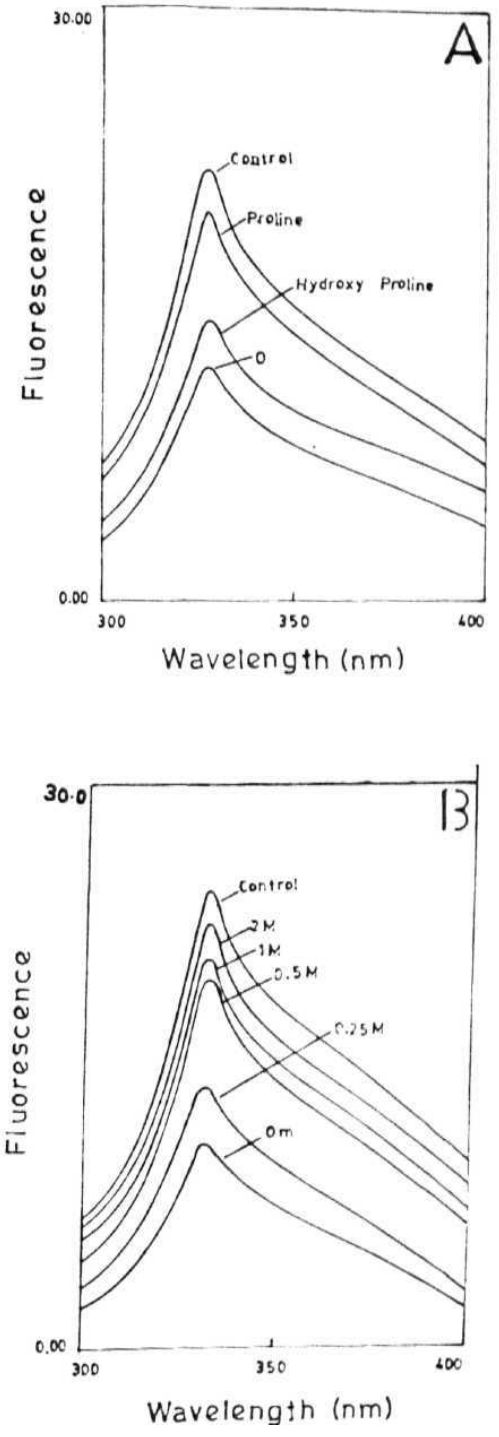


Figure 4.2.9 Fluorescence emission spectra of holo-D-glycerladehyde3-phosphate dehydrogenase (EC 1.2.1.12) heat stressed (50 C) (A) in the presence of proline and hydroxy proline (1 M each) and (B) in the presence of increasing concentration of proline.

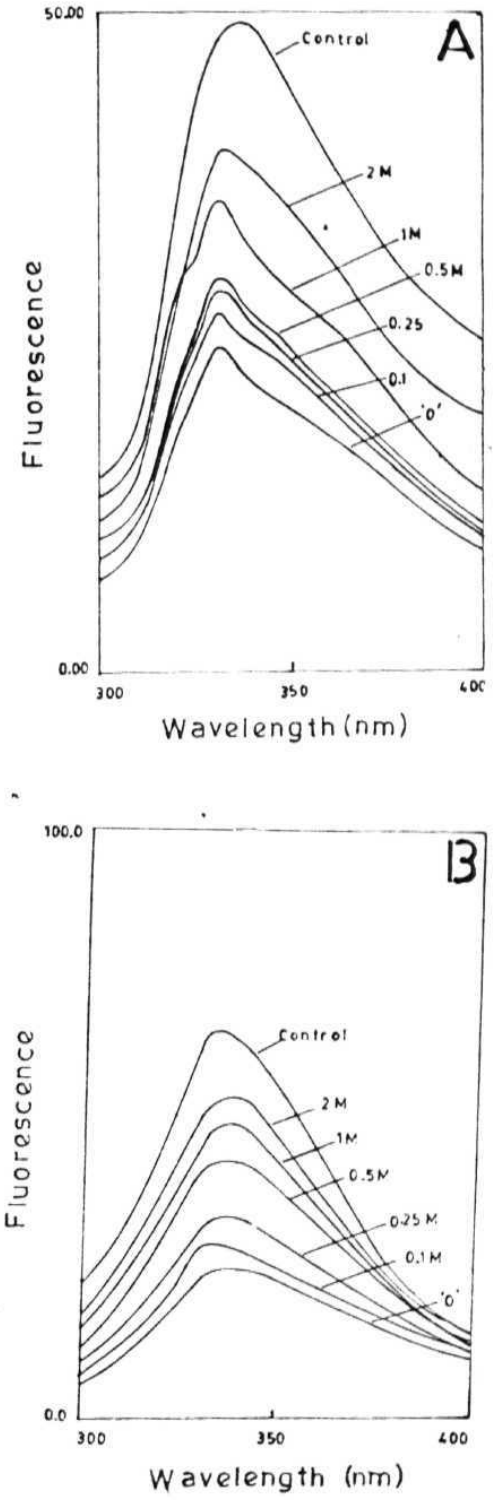


Figure 4.2.10 Fluorescence emission spectra of phosphofructo kinase (EC 2.7.1.11) (A) chemically denatured (GuHCl) and (B) heat stressed (50 °C) in the presence of increasing concentration of proline.

proline as the proline-protected enzyme displayed a higher fluorescence emission intensity, closer to the native unstressed enzyme *(Figure: 4.2.11 B).* The control enzyme heat-stressed without any additives, however, showed the least fluorescence emission, reflecting a considerable loss of secondary structure. The results presented here, however, comprise an extension of a previous observation (Carpenter *et al.,* 1986) and explore a rationale behind such an observation through structural analysis.

Apart from phosphofructokinase from *Bascillus sterothermophillus,* another kinase (phosphoglycerate kinase) from this organism was chosen for its interaction with osmolytes under stress. As evident from the fluorescence emission spectra, proline was found to be more efficient in offering protection to phosphoglycerate kinase *(EC 2.7.2.3; Bascillus stereothermophillus)* from the heat stress than hydroxy proline *(Figure: 4.2.11 A).* The control enzyme, heat stressed without any additives however, showed the loss of structural integrity as reflected in the reduced fluorescence emission.

4.2.2.3 Protection of nuclease:

The restriction endonucleases are some of the well known labile proteins that are prone to denaturation by milder stress conditions. The effect of proline was studied to understand its possible role in conferring structural stability to these en/ymes under stress conditions. Proline was found to confer a high degree of protection to the nucleuses from the stress conditions when compared with its structural analogue, hydroxy proline. Among the exonuclease, DNase I *(EC 3.1.21.1,* from bovine pancreas), RNase A *(EC 3.1.27.5* from bovine pancreas) and RNase H *(EC 3.1.26.4* from *E. coli)* were included in the analysis.

Proline was found to confer a considerably higher degree of protection to RNase H *(Figure: 4.2.12 A),* DNase I *(Figure: 4.2.12 B),* RNase A *(Figure: 4.2.12 C)* as reflected in the retention of their fluorescence emission intensities under stress. In fact, the emission intensities of the proline-protected enzymes were closer to the unstressed controls, indicating the near complete retention of their structural integrity under stress The protection of RNase A evident from these fluorescence spectral analyses *(Figure: 4.2.12 C)* is in agreement with the results obtained by Srinivas and Balasubramanian (1995).

Further, among the endonucleases, EcoR V *(EC 3.1.21.4* from *E. coli*), Bam H I *(EC 3.1.21.4* **from** *Bascillus amyloliquifaciens*) **and EcoR I** ***(EC 3.1.21.4* from *E. Coli*)** were selected for their interaction with proline. The results revealed a considerable level of protection offered by proline. While the heat stress was fund to diminish the fluorescence emission of these enzymes, addition of proline prior to the stress treatment has considerably retained the emission intensity of the enzymes, indicating the protection of structural integrity of these enzymes under stress conditions (*Figure: 4 2 13 A, B* and C) These results are encouraging as the restriction endonucleases are prone to denaturation and their *in vitro* stabilization could be useful in practical applications.

4.2.2.4 Protection of proteases:

A couple of proteases namely, a-chymotrypsin *(EC 3.4.21.1* from bovine pancreas) and proteinase K *(EC 3.4.21.14* from bovine pancreas) were tested for their interaction with proline under heat stress conditions. The fluorescence emission data revealed a loss of emission intensity in the absence of additives that was otherwise restored by proline and hydroxy proline. Proline was. however, more effective than hydroxy proline and sucrose in conferring protection to a-chymotrypsin *(Figure: 4.2.14* ***A).***

Proteinase K was also found to be. protected by proline from thermal denaturation. Interestingly, the addition of hydroxy proline sensitized the enzyme compared to the control without any additives *(Figure: 4.2.14 B).*

4.2.2.5 Protection of peroixidase and triose phosphateisomerase:

Fluorescence emission analysis, revealed that proline protects the target enzyme triose phosphate isomerase *(EC 5.3.1.1* from rabbit muscle) in a concentration dependent manner *(4.2.15 C).* In contrast, hydroxy proline was found to be ineffective in imparting protection to peroxidase *(EC. 1.11.1.7* from *A. ramosus*) and triose phosphate isomerase when compared to proline *(Figure: 4.2. 15 A* and *B).*

4.2.3 Analysis of the solvent accessible regions of the target proteins involved in interaction with proline:

As proline was found to confer a high degree of protection to proteins of different structural and functional characteristics, an attempt was made to understand the fine

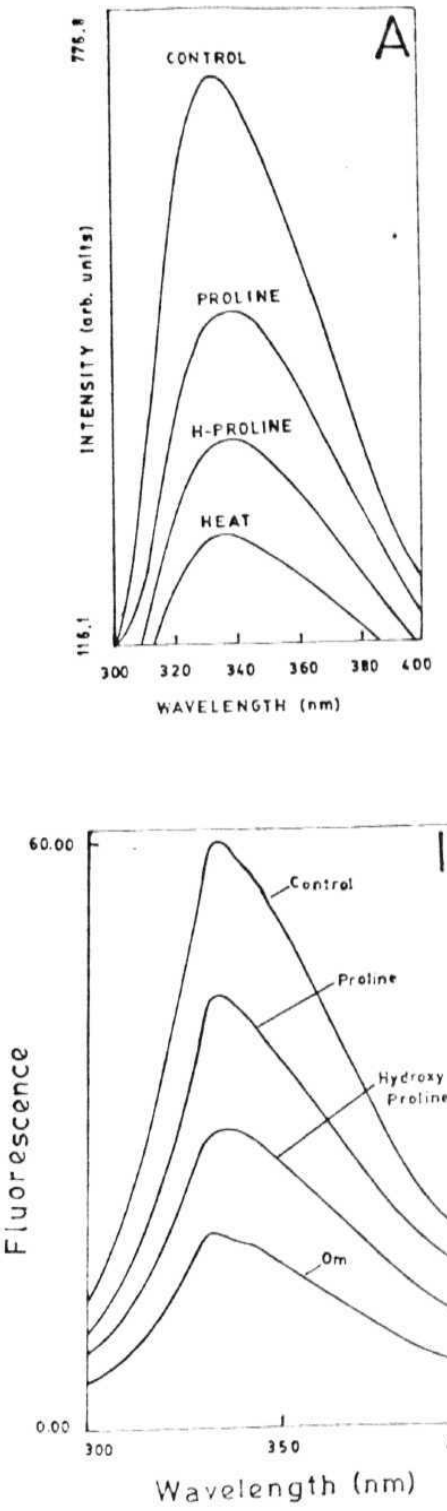


Figure 4.2.11 Fluorescence emission spectra of (A) phosphoglycerate kinase (EC 2.7.2.3) heat stressed (50 °C) and (B) phosphofructo kinase (EC 2.7.1.11) freeze-thaw stressed in the presence of proline and hydroxy proline (1 M).

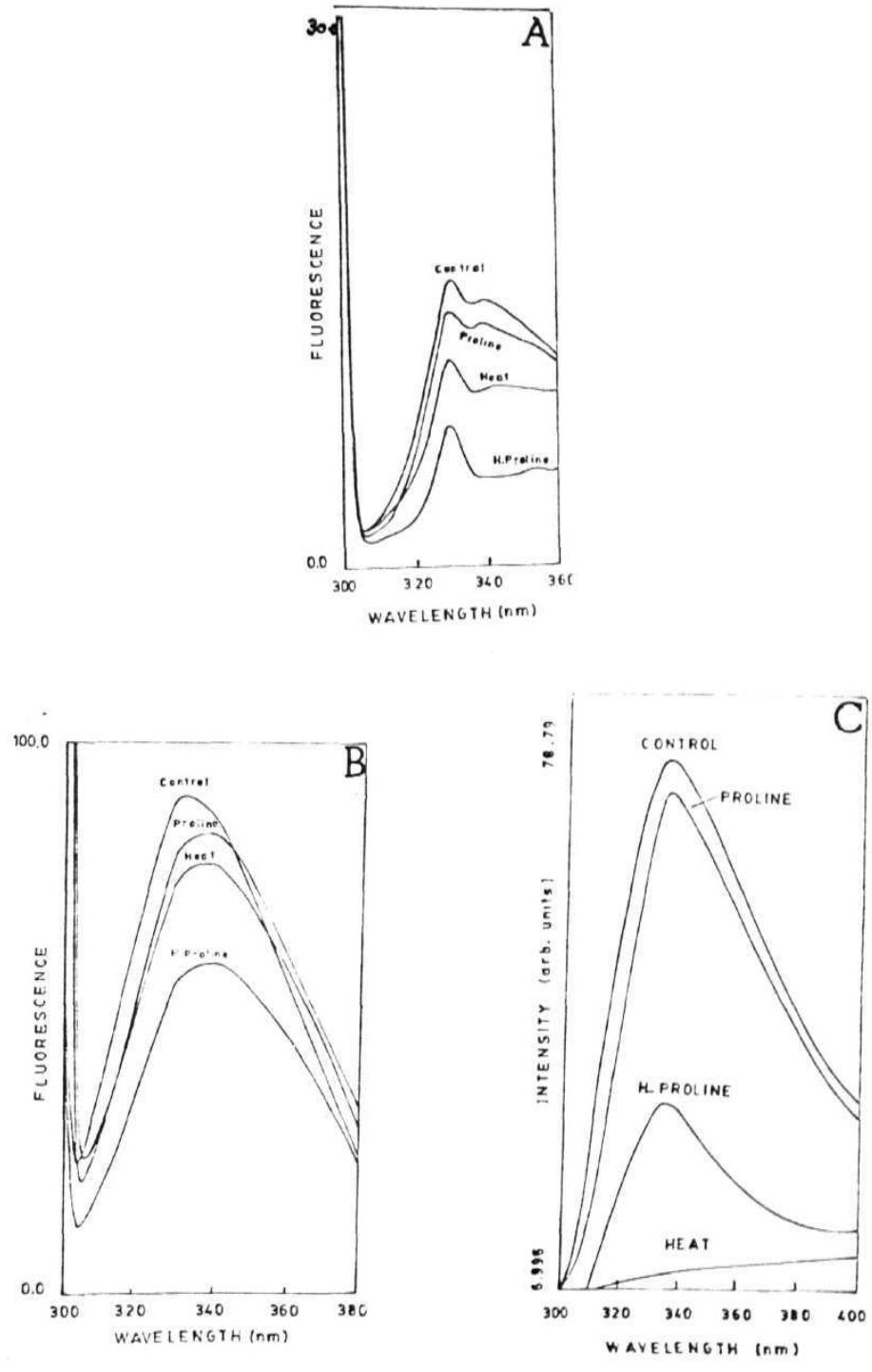


Figure 4.2.12 Fluorescence emission spectra of (A) RNase H, (EC 3.1.26.4) (B) DNase I (EC 3.1.21.1) and (C) RNase A (EC 3.1.27.5) heat stressed (50 ^{0}C) in the presence of proline and hydroxy proline (1M).

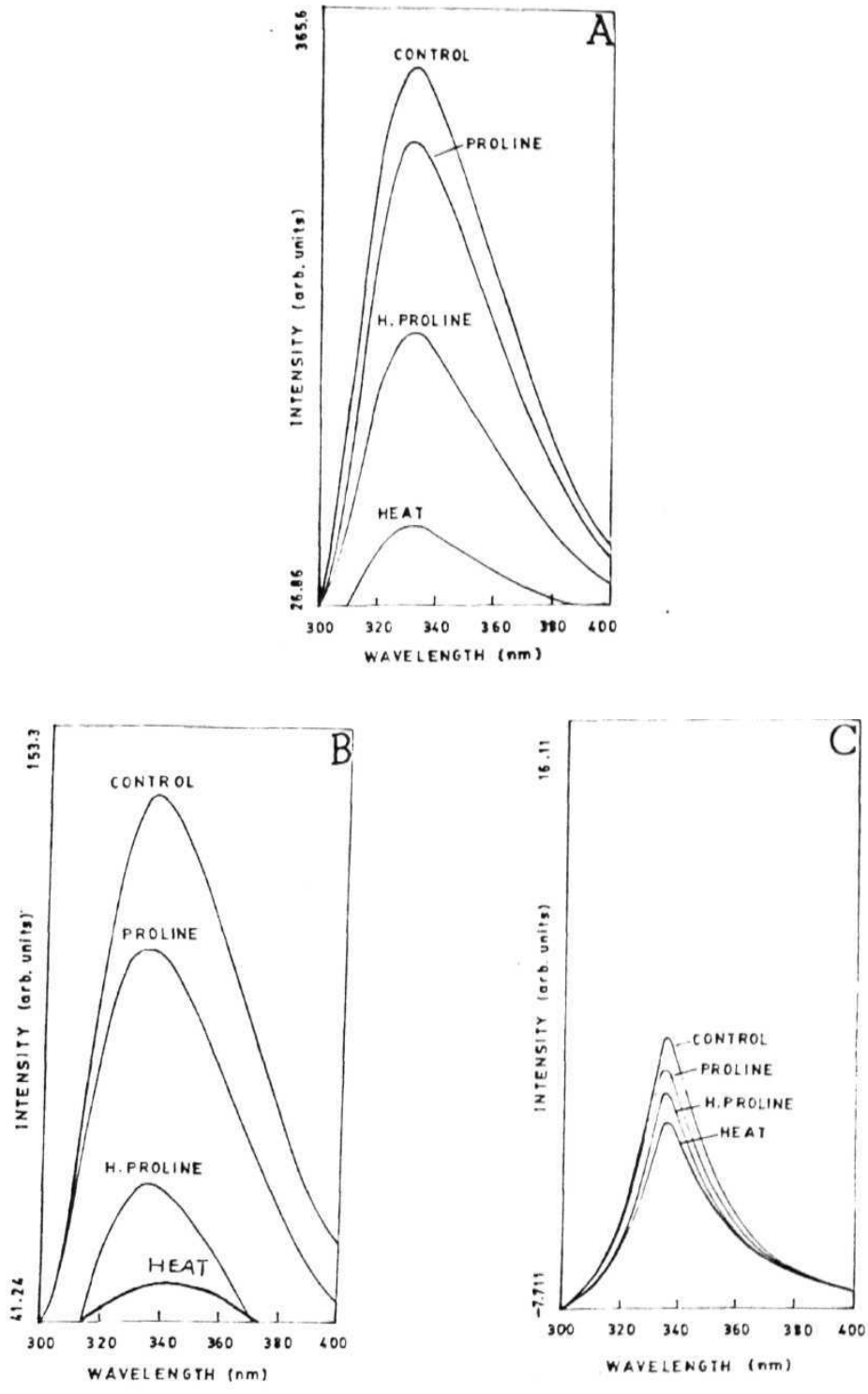


Figure 4.2.13 Fluorescence emission spectra of (A) Eco RV (EC 3.1.21.4) (B) Bam HI (EC 3.1.21.4) and (C) Eco RI (EC 3.1.21.4) heat stressed (50 ^{0}C) in the presence of proline and hydroxy proline (1 M).

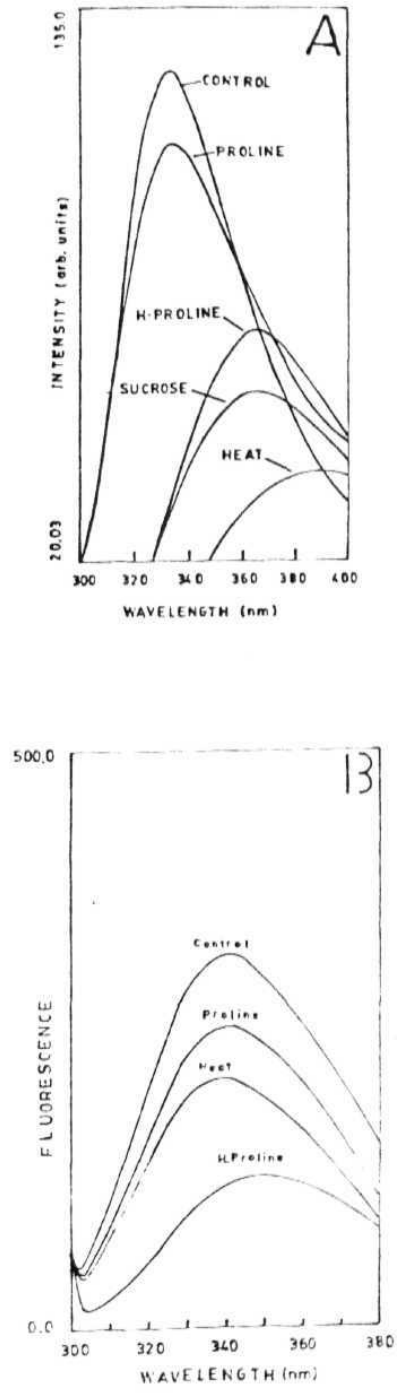


Figure 4.2.14 Fluorescence emission spectra of (A) α-chymotrypsin (EC 3.4.21.1) and (B) proteinase K (EC 3.4.21.14) heat stressed (50 ^{0}C) in the presence of proline and hydroxy proline (1 M).

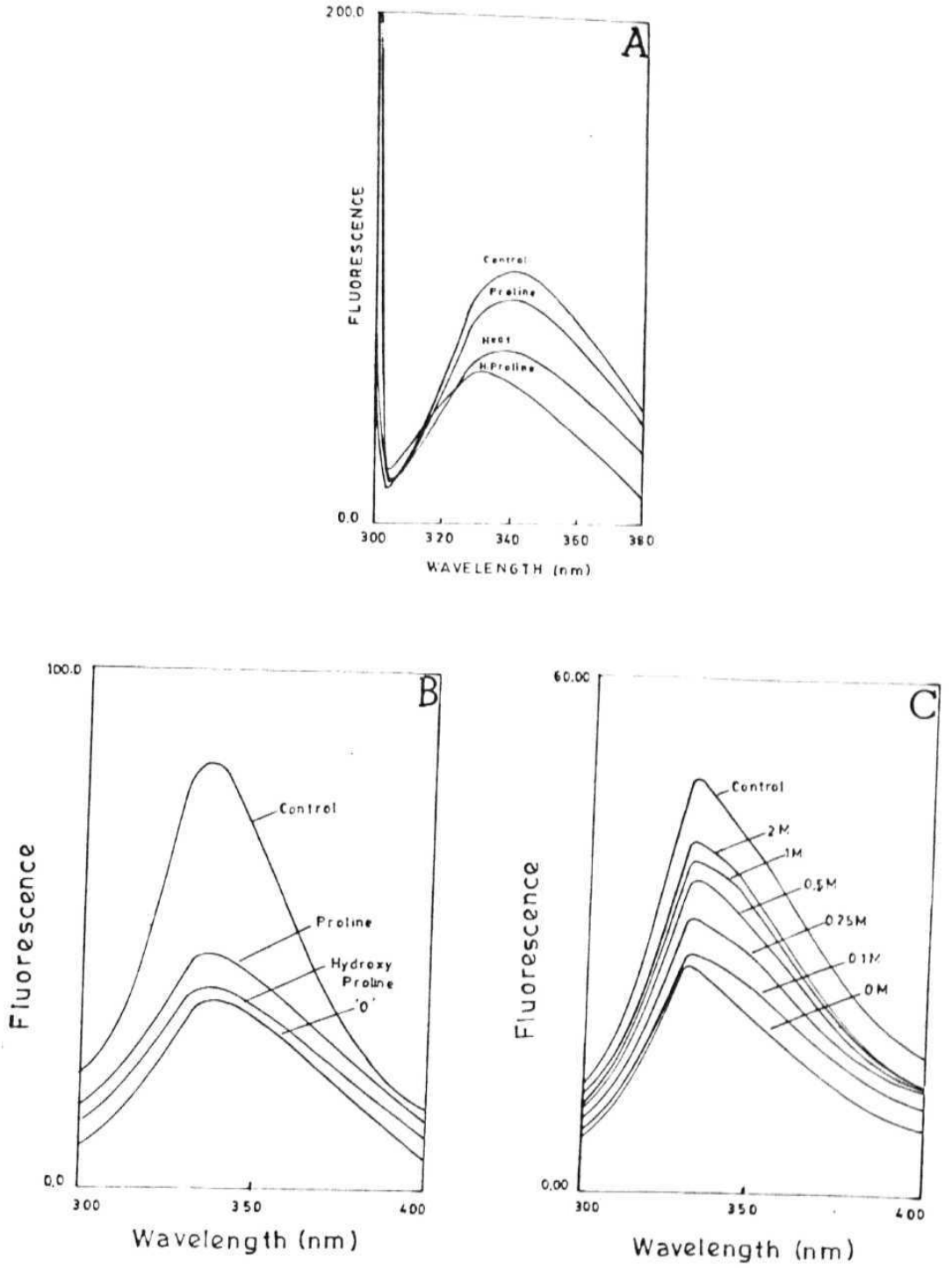


Figure 4.2.15 Fluorescence emission spectra of (A) peroxidase (EC 1.11.1.7), (B) triose phosphate isomerase (EC 5.3.1.1) heat stressed (50 ^{0}C) in the presence of proline and hydroxy proline (1M) and (C) triose phosphate isomerase (EC 5.3.1.1) heat stressed (50 C) in the presence of increasing concentration of proline.

structural interactions between proline and proteins. From the Brookhaven Protein Data Bank (PDB), the percentage accessibility of the hydrophobic regions of target proteins that could be accessible for an interaction with additives has been analyzed. The results reveal that all those proteins whose structures were evidently stabilized by proline have large hydrophobic areas accessible to solvents that can form an interaction with the hydrophobic backbone of the proline-multimeric structure (see Discussion). Among the proteins tested, the range of such an accessibility varied from 64 to 75 % that is stabilized under stressed conditions by proline *{Table: 4.2.3)*

No	Enzyme Code	EC No.	Enzyme	Source	Total atom. access.	Nonpolar side chain access.	Polar side chain access.	Total side chain acc.	Total main chain acc.	% hydro-phobicity
1	LDHM4	1.1.1.27	M4 Lactate dehydrogenase	Dog fish	4693.08	3001.03	1134.08	4135.10	557.9	*72.5*
2	4MDH	1.1.1.37	Malate dehydrogenase	Porcine heart	7818.58	4825.37	2045.71	6871.01	947.64	*70.2*
3	1GD1	1.2.1.12	Gl. 3-Phosphate dehydrogenase	*B. stereother mophilus*	4464.51	2717.08	1179.60	3896.72	567.70	*69.72*
4	3PFK	2.7.1.11	Phosphofructo kinase	*B. stereother mophilus*	3959.64	2364.47	1106.27	3470.77	488.92	*68.12*
5	1PHP	2.7.2.3	3-Ph. Glycerate kinase	*B. stereother mophilus*	4927.17	2833.61	1483.445	4317.07	610.14	*65.63*
6	2PRK	3.4.21.14	Proteinase K	*T. album limber*	2995.75	1762.38	757.34	2519.72	475.89	*69.94*
7	5CHA	3.4.21.1	α-Chymotrypsin	Cow	5505.06	3202.32	1384.35	4586.66	918.41	*69.81*
8	1SSC	3.1.27.5	RNase A	Bovine pancreas	1999.15	1200.05	506.26	1706.24	292.90	*70.33*
9	2RN2	3.1.26.4	RNase H	*E.coli*	2594.59	1495.64	755.79	2251.36	343.20	*66.43*
10	3DNI	3.1.21.1	DNase I	Bovine pancreas	3141.69	1726.04	954.31	2680.38	461.30	*64.39*
11	1ERI	3.1.21.4	EcoR I	*E.coli*	3802.47	2312.73	1005.83	3318.53	483.86	*69.69*
12	1RVE	3.1.21.4	EcoR V	*E.coli*	7004.60	4043.93	2095.90	6139.75	864.86	*65.86*
13	1BAM	3.1.21.4	BamH I	*B.amyloliq uifaciens*	2882.55	1585.97	973.57	2559.55	323.04	*61.97*
14	1HTI	5.3.1.1	Troise Phosphate isomerase	Rabbit muscle	5551.65	3447.09	1343.04	4789.99	761.57	*71.96*
15	1ARP	1.11.1.7	Peroxidase	*A.ramosus*	3814.17	2438.74	779.51	3218.19	595.96	*75.77*

Table 4.2.3 The percentage of solvent accessible hydrophobicity of the target proteins that were stabilized by proline under diverse stress conditions.

4.3 Proline/betaine-DNA interactions: Destabilization of the DNA helix.

The present study explores the possible role of naturally occurring osmolytes such as proline and betaine in salinity tolerance process. Also experiments were conducted to test whether proline counteracts the undesirable effect of NaCl and spermidine on DNA stability.

4.3.1 Effect of proline on the Tm of calf thymus DNA:

The initial studies on osmolyte-DNA interactions *in vitro* included the monitoring of Tm of calf thymus DNA in the presence of different osmolytes including proline and betaine. Any decrease in the Tm value reflects the destabilization of the double helix. Proline was found to significantly lower the melting temperature of calf thymus DNA in a concentration dependent manner *(Figure: 4.3.1).* Such an effect could manifest at concentration as low as 60 mM that became more pronounced with an increase in concentration. However, an appreciable decrease in Tm was observed consistently *(Figure: 4.3.1)* at concentrations ranging from 250 mM to 1 M that were specifically tested as these concentrations are widely known to be biologically relevant (see Discussion).

Further, proline at 4.5 M and above could destabilize the double helix within a sharp range of temperatures denoting the elimination of base pair composition dependence on melting at these concentrations. The sigmoidal curve of the Tm takes a sharper rise with an increase in proline concentrations. These results are reminiscent of that reported for betaine, another salinity induced osmolyte, which could reduce the base pair composition dependence of thermal melting (Rees *et al.*, 1993). Though such an effect could be observed starting from 3.0 M proline concentration, it was found to be more pronounced beyond 4.5 M. For instance, proline, at 5.5 M could reduce the Tm by as much as 25.0 ^{0}C with the melting occurring within a narrow margin of temperatures. Further, proline and betaine (1.0 M each), when added together, were found to have an additive effect by reducing the Tm of calf thymus DNA by 8.0 ^{0}C.

4.3.2 Effect of different solutes on the Tm of calf thymus DNA:

In order to test whether such a destabilizing effect shown by proline and betaine are specific, several other amino acids and sugars were tested as controls. The results

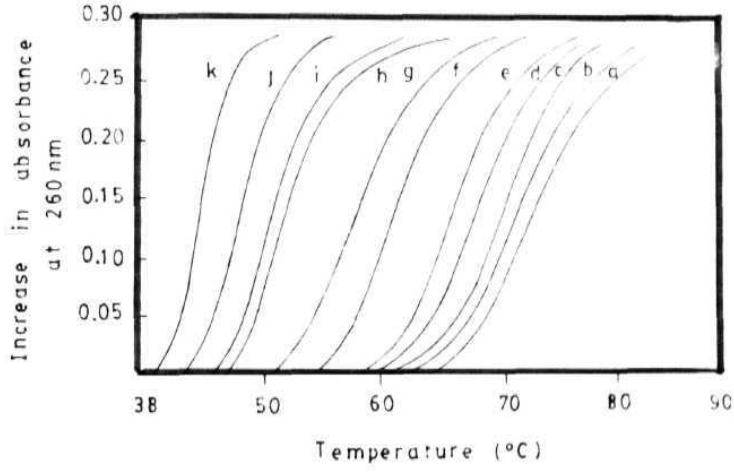


Figure 4.3.1 Effect of increasing concentration of proline on the Tm of calf thymus DNA. (a) control, (b) 0.06 M, (c) 0.25 M, (d) 0.5 M, (e) 1.0 M, (1) 2.0 M, (g) 3.0 M, (h) 4.0 M, (i) 4.5 M, (j) 5.0 M, (k) 5.5 M.

revealed that none of the other osmolytes tested even at high concentrations could induce a similar effect *{Table: 4.3.1).* On the contrary, some amino acids tested showed a strong stabilizing effect on the double helix, while the others remained more or less neutral with no apparent effect on the Tm of the target DNA. Among the osmolytes found to stabilize the double helix, glycine (2.0 M) and its dipeptide, glycyl glycine (0.5 M) were prominent in enhancing the Tm of DNA to 76.0 °C and 82.0 C respectively compared to 71.0 V recorded for the control calf thymus DNA. Serine (2.0 M) too was found to stabilize the DNA with an enhancement in Tm by 8.0 C compared to the control. However, alanine, valine, leucine and sarcosine had no apparent effect on the Tm of DNA. Interestingly, with an addition of methyl group(s) on the glycine structure, alanine, valine and leucine have sequentially lost both the aqueous solubility and the stabilizing effect on DNA. While glycine (2.0 M) was found to stabilize the double helix by 5.0 'C , other aminoacids alanine (1.0 M), valine (0.25 M) and leucine (0.1.0 M) had no such stabilizing effect on Tm ***(Table:*** *4.3.1*).

The methylated form of glycine, sarcosine (N, methyl glycine), at a comparable concentration could not induce a stabilizing effect. On the other hand, the N,N,N, trimethylated form of glycine, betaine, (N,N,N trimethyl glycine), at 2.0 M could reduce the Tm by as much as 8.0 C. Interestingly, the addition of each N-methyl group on the glycine structure could bring about a drastic reduction in the Tm of DNA by 14.0 C observed with betaine. Hydroxy proline, at its maximum aqueous solubility point (2.0 M), could reduce the Tm by 8.0 C. Proline, by virtue of its high aqueous solubility, could reduce the Tm beyond such a solubility point. Similarly, the addition of 1.0 M of glucose did not alter the Tm of calf thymus DNA indicating the lack of destabilizing effect by the sugar *(Table: 4.3.1).* However, the differential aqueous solubility of tested solutes prevented a more accurate comparison between them in their interaction with DNA.

433 Effect of prol in e on salt and spermidine- induced DNA stability:

Since proline was found to reduce the Tm of DNA, an effort was made to test the relaxing effect in negating the presence of sodium chloride and spermidine that are known to greatly stabilize DNA. These studies are significant as the salinity induced-

Concentration of the additive	Tm of DNA $\pm 1.0^0$ C
Control DNA	71.0
+ 1.0 M proline	65.0
+ 2.0 M proline	60.0
+ 2.0 M glycine	76.0
+ 2.0 M serine	79.0
+ 1.0 M alanine	72.0
+ 0.25 M valine	71.0
+ 0.1 M leucine	71.0
+ 2.0 M hydroxy proline	63.0
+ 0.5 M glycyl glycine	82.0
+ 2.0 M sarcosine	72.0
+ 1.0 M glucose	71.0
+ 1.0 M betaine	67.0
+ 1.0 M proline + 1.0 M betaine	63.0
+ 10 mM spermidine	93.0
+ 0.5 M NaCl	96.0
+ 10 mM spermidine + 1.0 M proline	85.0
+ 0.5 M NaCl +1.0 M proline	90.0
+ 0.5 M NaCl + 2.0 M proline	86.0
+ 0.5 M NaCl + 2.0 M glycine	94.0

Table 4.3.1. Effect of proline and other amino acids on the Tm of calf thynius DNA in the presence and absence of additives.

proline/betaine accumulation could have a bearing on the plants survivability by negating the lethal effects of salts on DNA stability as a stress adaptation.

The effect of increasing concentrations of salts and spermidine on the Tm of DNA was tested and the results revealed a proportional increase in Tm of DNA (*Table: 4.3. 1).* For instance, 0.5 M sodium chloride could increase the Tm of DNA by 25.0 °C while 10 mM spermidine was found to increase the Tm of DNA by 22.0 C. Proline and betaine were found to considerably counteract such a stabilizing effect of salts on DNA stability. While betaine was previously reported to reduce the effect of KCl on DNA stability (Rees *et al.*, 1993), 1 M proline was found to reduce the effect of NaCl (0.5M) by 6.0 C and the spermidine (10 mM) by 8.0 C (*Table: 4.3.1).* Proline at 1.0 M was used for interaction studies as it has been found to be biologically relevant in its intracellular accumulation *(see 5.2.4).* On the contrary, the co-addition of glycine (2.0 M) with sodium chloride (0.5 M) did not influence the effect of the latter on DNA indicating the ineffectiveness of glycine in counteracting the salt effect *(Table: 4.3.1).* This control was included to be certain that the observed effect of proline is not due to a mere competitive phenomenon.

4.3.4 Effect of proline on DNase I sensitivity:

The spectrophotometric analysis of DNase I digestion in the presence and absence of proline revealed a progressive protection of DNA from DNase I digestion by proline, particularly, with the effect being evident at 3.0 M proline and above (*Figure: 4.3.2).* This assay that records the absorbance of DNA at 260 nm in the presence of DNase 1 enzyme clearly denoted a near-complete protection of calf thymus DNA by proline above 5.0 M. Such an insensitivity to DNase 1 action was further confirmed by the gel electrophoresis of digested DNA samples of diverse origin in the presence of various concentrations of proline. The *X* phage DNA, pUC 18 plasmid and calf thymus DNA were treated with DNase I and were resolved on agarose gel electrophoresis to visualize the amount of uncut DNA *(Figure: 4.3.3).* All these DNA samples showed a good amount of resistance to DNase I digestion in the presence of proline concentrations above 3.0 M concentrations. The samples from rice and barley genomic DNA were also subjected to a similar digestion

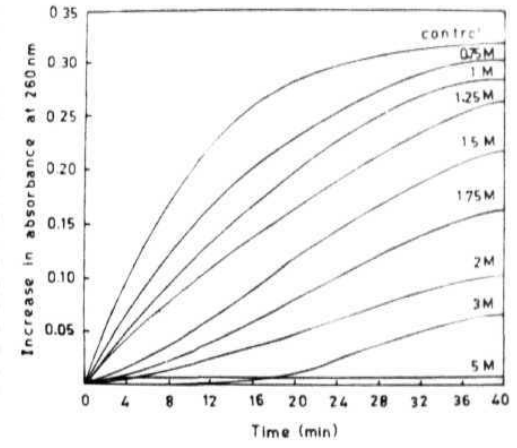


Figure 4.3.2 Spectrophotometric analysis of DNase I sen: ivity of calf thymus DNA in the presence of increasing concentration of proli.e.

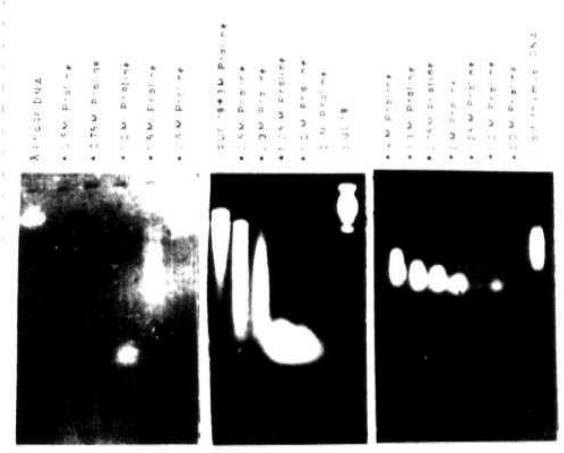


Figure 4.3.3 DNase 1 sensitivity of λ phage DNA, pUC 18, and calf thynius DNA in the presence of different concentrations of proline as analyzed from the agarose gel electrophoresis.

in the presence of increasing concentrations of proline. The results revealed the protection of DNA by proline at 3M and above *(Figure: 4.3.4* and *4.3.5).*

Similarly, the effect of proline (3.0 M) was compared to that of sucrose (3.0 M) in making the *X* phage DNA insensitive to DNase 1 action since it becomes necessary to confirm if any additive, by virtue of its high concentration, does physically deny the enzyme an access. Of the two solutes tested at equal concentration, only proline was found to make the DNA insensitive to the enzyme action that was clearly absent in the presence of sucrose *(Figure: 4.3.6).* Further, an addition of 3.0 M, proline could leave the DNA uncut by the enzyme *(Lane: 2).*

4.3.5 A comparative analysis of effect of proline and betaine on DNase I sensitivity:

A comparison was made between proline and betaine in their ability to make the DNA insensitive to DNase I action, as both osmolytes were proved to reduce the 1 m in a concentration dependent manner. True to their differences observed in the Tm studies *(Table: 4.3.1),* the DNase I assay too reflected a higher amount of resistance in presence of proline compared to betaine *(Figure: 4.3. 7).* For instance, with the addition of 3.0 and 4.0 M concentration each of betaine and proline to the calf thymus DNA, more amount of DNA always remained uncut in presence of proline than in betaine. This effect is either due to a decreased binding of DNase 1 to the DNA or destabilization of the double helix. The former is less likely as proline or betaine do not interfere with the binding properties of proteins that interact with the DNA (see below).

4.3.6 Effect of proline on the sensitivity of DNA to SJ nuclease:

In contrast to the DNase I sensitivity assay, proline at increasing concentrations was found to make the double stranded calf thymus DNA more accessible to S1 nuclease digestion *(Figure: 4.3.8).* The effect was particularly more pronounced beyond 3.0 M, with a near complete digestion observed in the presence of 4.5 M proline. This assay further suggests that the action of S1 nuclease on destabilized DNA (substrate) is not affected by high concentrations of proline. The assay was also performed with the rice genomic DNA, which too showed a similar level of susceptibility to S1 nuclease in the presence of increasing concentrations of proline *(Figure: 4.3.9).*

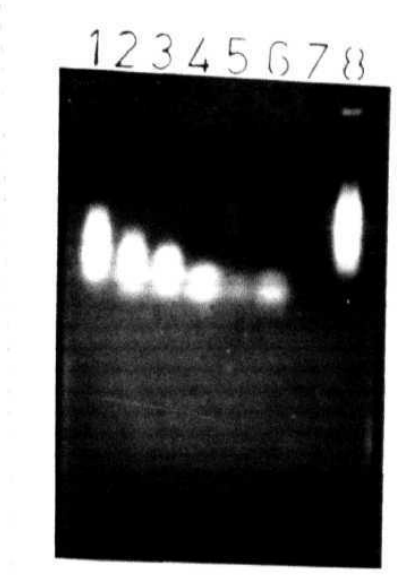


Figure 4.3.4 DNase I sensitivity of rice genomic DNA in the presence of decreasing concentration of proline. Lanes (1) 4.0 M, (2) 3.3 M, (3) 3.0 M, (4) 2.0 M, (5) 1.5 M, (6) 1.0 M, (7) 0.0 M, (8) control DNA.

Figure 4.3.5 DNase I sensitivity of barley genomic DNA in the presence of decreasing concentration of proline. Lanes (1) 3.0 M, (2) 2.5 M, (3) 2.0 M, (4) 1.5 M, (5) 1.0 M, (6) control DNA.

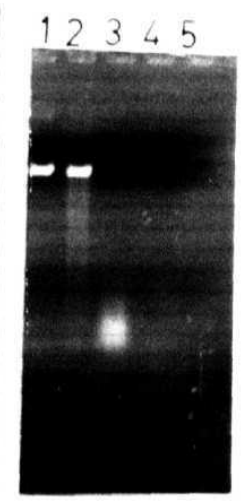


Figure 4.3.6 DNase I sensitivity of *X* phage DNA in the presence of proline and sucrose. Lanes (1) control (2) 3.0 M proline, (3) 2.0 M proline, (4) 3.0 M sucrose, (5) DNA + DNase I.

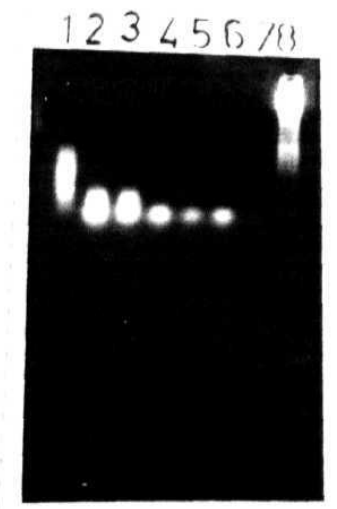


Figure 4.3.7 DNase I sensitivity of calf thymus DNA in the presence of decreasing concentration of proline and betaine. Lanes (1) 4.0 M proline (2) 4.0 M betaine, (3) 3.0 M proline, (4) 3.0 M betaine, (5) 1.5 M proline, (6) 1.5 M betaine, (7) 0.0 M proline, (8) control DNA.

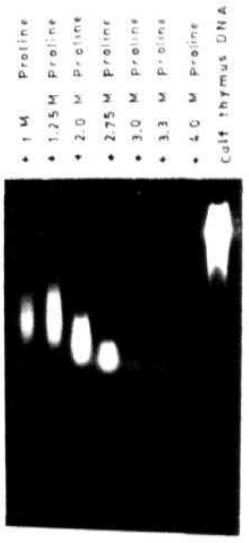


Figure 4.3.8 S1 nuclease sensitivity of calf thymus DNA in the presence of increasing concentration of proline.

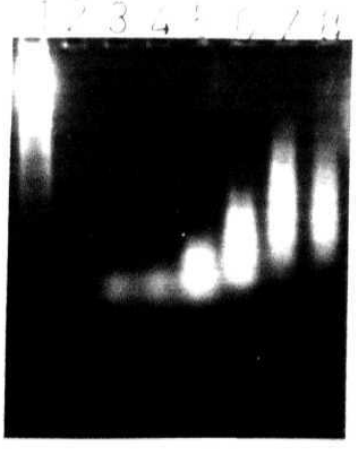


Figure 4.3.9 S1 nuclease sensitivity of rice genomic DNA in the presence of decreasing concentrations of proline. Lanes (1) control, (2) 4.5 M, (3) 4.0 M, (4) 3.3 M, (5) 3.0 M, (6) 2.5 M, (7) 2.0 M, (8) 1.5 M.

4.3.7 Effect of proline on the gel mobility shift assay of λ phage with ssbp:

As the results in the S1 nuclease sensitivity assay clearly showed the destabilization of DNA in presence of proline, it was further deemed essential to reconfirm such a destabilizing effect by the gel mobility shift assay. The results revealed the binding of increasing amounts of ssb protein to λ phage DNA in the presence of 3.0 M proline resulting into the retarded mobility of DNA-protein complex *(Figure: 4.3.10)*. Such a complex formation was clearly absent in the control DNA sample even with an addition of 12 μg of ssbp, suggesting the lack of any destabilization in the absence of proline and, in turn, the complex formation. These results further indicated a significant non-interference of proline in interactions between such proteins and DNA.

4.3.8 Displacement of DNA-bound ethidium bromide by proline:

The fluorescence emission intensity of ethidium bromide is known to be enhanced upon its addition with DNA. The ability of proline on the stability of such a complex was tested and the results revealed that proline is marginally effective in replacing the DNA bound ethidium bromide. Such a feature is expected of the substances that destabilize the double helix and decrease the Tm of DNA. This effect was concentration dependent with the maximum displacement observed at 6.0 M (*Figure: 4.3.11*).

4.3.9 Displacement of DNA bound Ruthenium complex by proline:

Ruthenium complex [Ru $(Phen)_2$ (dppz) Cl_2] is known to interact with DNA double helix resulting into several-fold enhancement in its fluorescence emission in solutions (Friedeman *el ai,* 1990), a property somewhat analogous to that is displayed by ethidium bromide. The effect of proline (3 M) when added to such a complex was tested. The data revealed a considerable reduction in the emission intensity reflecting the destabilization of the helix by proline thereby reducing the affinity of the ruthenium drug with the helix ***{Figure:*** *4.3.12*).

4.3.10 Effect of proline on Topoisomerase I activity:

Since proline or betaine were found to destabilize the double helix, a further analysis was necessary to study the site of interaction. To this end, the activity of topoisomerase I on pBR 322 plasmid DNA was studied in the presence of betaine and

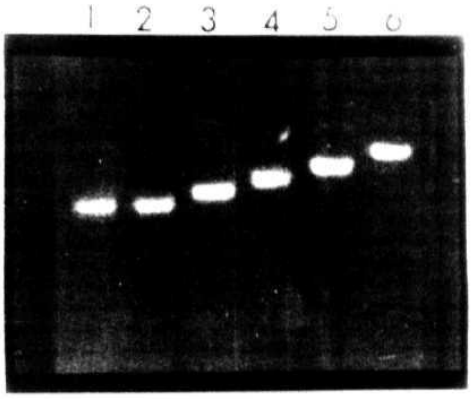


Figure 4.3.10 Gel mobility shift assay of *X* phagc DNA. Lanes (1) *X* phage DNA (2) *X* phage DNA + 12 μg of ssbp, (3-6) *X* phagc DNA in the presence of 3.0 M proline with increasing concentration of ssbp; (3) 3 μg, (4) 6 μg, (5) 9

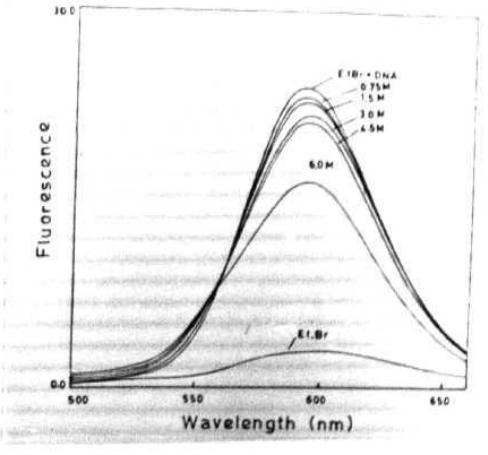


Figure 4.3.11 Fluorescence emission spectra of ethidium bromide. Effect of proline in displacing the DNA-bound ethidium bromide.

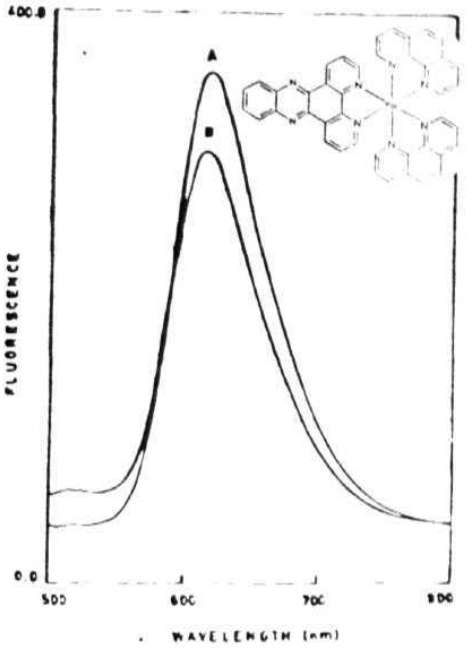


Figure 4.3.12 Fluorescence emission spectra of ruthenium drug complex. Effect of 3.0 M proline in disassociating the DNA-drug complex. (A) UNA + drug, (B) DNA + drug + 3.0 M proline. The drug itself does not show any fluorescence emission. Structure of the ruthenium complex is shown in the inset.

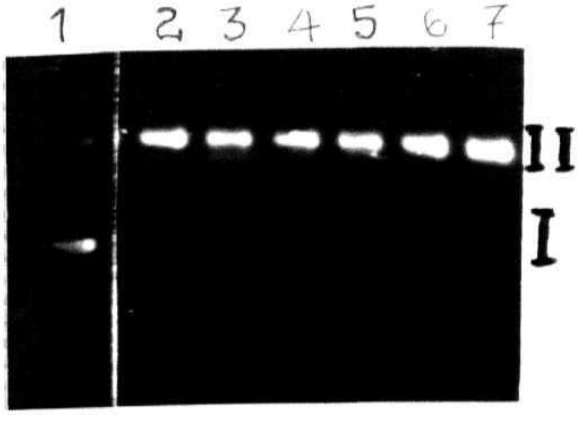


Figure 4.3.13 Topoisomerase I activity on pBR 322 DNA in the presence of increasing concentration of proline and betaine. Lanes (1) pBR 322 plasmid (2) no proline, (3) 2 M proline, (4) 2 M betaine, (5) 3 M proline, (6) 3 M betaine, (7) 5 M proline.

proline as such an assay is known to be a sensitive measure to check any intercalation by compounds. Topoisomerase I does not act upon the supercoiled plasmid if intercalated by any molecule. The results from this assay indicated the absence of any inhibitory effect by proline or betaine on the activity of the enzyme even at high concentration *{Figure: 4.3.13).* Topoisomerase I was able to convert the supercoiled form of pBR 322 plasmid DNA to the relaxed circle form even at the highest concentration of proline tested, i e., 5 M. Such a non-interference of proline in DNA-protein interactions is reminiscent of that reported for betaine (Rees *et al.*, 1993).

5. DISCUSSION

Investigations on abiotic stress response mechanisms in plants have identified two major groups of molecules namely a) ABA and the ABA responsive-proteins/enzymes and b) compatible osmolytes. Cellular events associated with such molecules under diverse stress conditions seem to be widely conserved across plant species. The objective of our investigation is to decipher in rice the role of these two components in stress tolerance processes that could be of utility in the crop improvement. The results are discussed below.

5.1.1 ABA-mediated proline accumulation has a protective role in stress tolerance:

Rice seedlings accumulated considerable amounts of free proline in response to exogenous ABA and abiotic stress conditions such as salinity (NaCl) and osmotic stresses (PEG). Significantly, the stress tolerant cultivars, Akashi and K39, accumulated higher amounts of proline in shoots than the sensitive cultivar, Hamsa *(Fig: 4.1.4)*. The results are in agreement with that reported by Igarashi and co-workers (1997), wherein the tolerant rice cultivars in response to ABA and abiotic stresses were shown to accumulate higher levels of proline than the sensitive ones.

Among cereals, application of ABA is known to induce the accumulation of proline in leaves of barley (Aspinall *et al,* 1973; Rajagopal and Anderson, 1978; Stewart, 1980; Pesci, 1987, 1988) and rice (Chou *et al,* 1991). ABA-induced proline accumulation appears to depend on the activation of genes and/or protein synthesis (Stewart and Voetberg, 1985; Pesci, 1987). In fact, spraying of cucumber plants and maize cells with ABA or its analogue led to an increase in free proline content and in turn, tolerance to chilling stress (Flores *et al,* 1988; Xin and Lee, 1993). Our results are in agreement with the above that the accumulation of proline in response to exogenous ABA in rice seedlings was correlated with their improved tolerance to salinity and chilling stresses *(Table: 4.1.1),* Apart from contributing to the cellular osmotic adjustments, proline seems to be involved in protecting the cellular constituents from stress. Interestingly, proline and its intermediate metabolites were also found to induce certain gene products in rice which are also ABA/stress responsive (Iyer and Caplan, 1998). Thus, ABA and proline are presumably involved either individually or collectively in the induction of gene products that could contribute to stress tolerance in rice.

Extensive genetic analyses have revealed that proline-accumulating plant and bacterial mutants exhibit an improved tolerance to salinity stress (Dix *et al,* 1993; Csonka, 1981, 1989). Further, the genetic modification for increased proline accumulation in tobacco reduced the transgenic plant's sensitivity to salinity stress under tested laboratory conditions (Kishore, *el al.*, 1995). The results suggest the probable role of proline in stress tolerance process which could be used as a marker for stress tolerance in rice. Moreover, proline exhibits some unique features, unlike the other osmolytes that qualify its role as an osmolyte widely compatible with the major biomacromolecules (Sec Discussion 5.2 and 5.3).

5.1. 2 Exogenous ABA offers protection from stress injury:

Exogenous application of ABA to rice seedlings was found to significantly improve their tolerance to chilling and salinity stresses as scored morphologically from the percent survival *(Tables 4.1.1 and 4.1.2).* The protection of rice seedlings mediated by ABA was aptly reflected in their reduced extent of injury *(Fig: 4.1.2).* However, ABA was ineffective beyond -2 °C although significant differences were observed in the magnitude of electrolytes leached between control and ABA-treated seedlings at these temperatures (*Table: 4.1.2).* This was in contrary to the effect observed in a temperate plant *Arabidopsis* where the ABA-induced freezing tolerance was reported at temperatures as low as -10 C (Gilmour and Thomashow, 1991). Presumably, the threshold tolerance response mediated by ABA (possibly through the induced proteins and osmolytes) could be different among plants of temperate and tropical habitat based on their physiological and genetic constitution. The role of proteins in freezing stress tolerance was, in deed, confirmed by the enlianced expression of a synthetic anti-freeze protein in potato which led to reduced electrolyte release at freezing temperatures (Wallis *el al,* 1997). The results presented here, however, constitute the first attempt to probe the chilling tolerance response in rice mediated by ABA *vis*-a`-*vis* the membrane integrity.

5.1.3 Injury index in rice as a stress tolerance indicator:

Stress tolerant cultivars were found to significantly resist the injury by salinity and osmotic stresses compared to the sensitive cultivars as measured by the electrolyte leachate assays. For instance, Pokkali, a cultivar routinely used as a standard check in

screening for salt tolerance, exhibited a two fold higher resistance to the salinity injury than a sensitive variety *(Fig: 4.1.3 A)*. Similarly, the drought tolerant Annada was found to exhibit a better membrane integrity than the sensitive cultivars tested under PEG-mediated osmotic stress *(Fig: 4.1.3 B)*. These results are broadly in tune with our observations that the reduced freezing/chilling injury in rice seedlings upon the ABA treatment could be correlated with their improved survival *(Fig: 4.1.2 & Tables 4.1.2)*. Further, salinity stress inflicted several fold higher injury to the leaf discs than the osmotic stress which is also reflected at the crop level (Technology for Rice Production, WO). The broad variation observed in injury index levels among the seven rice cultivars tested could be attributed to their wide genetic backgrounds. Injury index, as calculated from the electrolyte leachate assays, could be of considerable utility in a large-scale screening of rice varieties.

5.1.4 ABA-induced proteins: Possible role in stress tolerance process:

Accumulation of ABA/stress responsive proteins was analyzed in rice seedlings through SDS-PAGE and Western analysis *(Figures 4.1.5 & 4.1.6-4.1.10)*. An improved chilling and salinity tolerance observed in seedlings treated with ABA with a concomitant induction of ABA/stress responsive proteins indicate their role in stress tolerance process *(Table: 4.1.1)*. Such an observation was confirmed by the application of cycloheximide where the inhibition of these proteins led to a drastic reduction in the tolerance response *(Figures 4.1.6-4.1.10)*. In a related study, maize cells treated with ABA were found to exhibit an improved tolerance to chilling stress which was negated by the application of cycloheximide (Xin and Lee, 1993). Thus, the ABA-responsive *de nova* synthesized proteins seem to be involved in the chilling/salinity tolerance process. The utility of stress responsive proteins was further appreciated from the studies using gibberellic acid as a control which individually failed to induce these proteins in shoots and in turn the stress tolerance *(Table 4.1.1 and Figures 4.1.6-4.1.10)*. However, a clear-cut increment in tolerance coupled with the induction of these proteins in seedlings treated collectively with ABA and GA3 confirmed the role of ABA induced proteins in stress tolerance. These results further suggest that application of GA3 along with ABA does not negate the positive effect by the latter in conferring stress tolerance to rice.

In the ABA-treated seedlings, a prominent induction of five different polypeptides with molecular weights 15, 16, 18, 23 and 34 kDa was observed. Apart from these qualitative differences observed in the protein profiles in response to ABA, their quantitative induction could also be critical in imparting stress tolerance to the seedlings. For instance, an application of 10 µM ABA was reported to be insufficient in bringing about perceptible change in the chilling tolerance response in rice seedlings (Takahashi, *et al,* 1994). Our results are in agreement with the above observation that none of the proteins analyzed here could be induced in detectable levels in rice seedlings (as evident from the immuno-detection) at lower concentrations of ABA *(Figures: 4.1.6-4.1.10).* However, these proteins accumulated to critical concentrations in response to 100 µM ABA (added individually and in combination with GA3) that could contribute to stress tolerance response. Further, an improved salinity and chilling tolerance observed in both the stress tolerant (K39) and sensitive (Hamsa) varieties upon the application of ABA indicates that ABA inducible machinery is present in both the varieties, imparting tolerance upon its activation *{Table 4.1.1).* In contrast. Moons *et al,* (1995) have reported a preferential induction of a group of LEA proteins in response to ABA in tolerant rice variety Pokkali compared to the sensitive T(N)1 variety. Thus. the wide differences in genetic constitution among varieties determine their sensitivity/tolerance which is presumably mediated by ABA and the other stress related factors.

The 15 kDa polypeptide found responsive to ABA (*Fig 4.1.6)* is a cytosolic, boiling stable and a general stress responsive protein induced in response to salinity, chilling and water stress conditions in rice (Karunasree, 1992). On the other hand, the 23 kDa polypeptide *(Fig: 4.1.9)* was reported as an exclusive water stress responsive polypeptide immunologically related to the RAB family of proteins (Rao *et al,* 1993, Rao, 1993). Rice cell cultures exposed to osmotic stress and ABA also accumulated this polypeptide in abundance indicating its induction both at cellular (Reddy *et al,* 1993) and organismic level *(Fig: 4.1.9).* The stress/ABA mediated induction of these proteins in seedlings coupled with an improved stress tolerance indicates their possible involvement in tolerance process. Employing rice cell cultures as a model, two proteins, a HSP 70 and

ubiquitin, were identified to be associated with water stress adaptation (Borkird *et al.*, 1991). Detection of an ABA responsive 18 kDa protein homologous to the desiccation responsive protein (encoded by pcC 6-19) from *Craterostigma platagineum* (*Fig 4.1.8*) denotes the conserved biochemical responses towards stress tolerance among the monocots and dicots. In fact, *Craterostigma plantagineum* (popularly called African resurrection plant), with an extraordinary ability to withstand the desiccation stress, serves as a role model to analyze the molecular and biochemical events associated with desiccation tolerance (Ingram and Bartels, 1996). The cDNA pcC 6-19 is also homologous to pcC 27-04, another desiccation responsive cDNA which, in turn, is homologous to members of LEA gene family (Piatkowski, 1990). Further, the sequence comparison of pcC 6-19 and pcC 27-04 revealed the presence of characteristic sequence motifs conserved across several plant species which are expressed abundantly in dehydrating embryos and are related to water stress responses (Baker *et al,* 1988; Close *et al,* 1989; Mundy and Chua, 1988; Vilardell *et al,* 1990). Thus, the proteins encoded by pcC 27-04 and pcC 6-19 and, in turn, its immunological homologue identified in rice *(Fig: 4 1.8)* belong to a class of genes present in an array of monocotyledonous and dicotyledonous plants that arc associated with stress tolerance. Such a conservation further indicates that metabolic pathways for desiccation tolerance share some common components in embryos and leaves of resurrection plant and rice.

A 34 kDa aldose reductase-relatcd protein was identified in rice shoots in response to ABA and osmotic stress (PEG mediated) conditions *(Fig: 4.1.10 A and (').* A concomitant induction of aldose reductase activity was observed in these tissues treated with ABA *(Fig: 4.1.10 B).* Aldosc reductase is an enzyme involved in the biosynthesis of sorbitol, a prominent osmolyte that accumulates in various plant species. Detection of an aldose reductase-related protein in response to ABA in shoots coupled with an improved stress tolerance observed in them *(Fig: 4.1.10)* and during the desiccation tolerant phase of embryogenesis (Karunasree, 1998) suggests the utility of sorbitol biosynthetic pathway in stress tolerance in rice seedlings. The possible role of osmolytes in stress tolerance processes is. however, discussed in 5.2 and 5.3.

Two lines of evidence support the possible role of ABA in stress tolerance: one that the ABA treatment at normal growth temperatures can increase the stress tolerance of a wide range of plants (Chen *et al,* 1979; Chen and Gusta, 1983; Mohapatra *et al,* 1988) including rice *{Table: 4.1.1)* and the second that the ABA levels increase in certain plants in response to abiotic stresses (Chen *et al,* 1983; Guy and Haskell, 1988; Guy. 1990; Lalk and Dorffling, 1985). Chen *et al,* (1983) have, therefore, hypothesized that abiotic stresses bring about elevated levels of ABA which, in turn, trigger the synthesis of proteins responsible for stress tolerance. For instance, several cold responsive genes in different plants including rice have also been found to be regulated by ABA (Skriver and Mundy, 1990; Mohapatra *et al,* 1988; Robertson *et al,* 1987). The studies of Gilmour and Thomashow (1991) on freezing tolerance in the ABA deficient *(aha)* and ABA insensitive *(abi)* mutants of *Arabidopsis* (Koornneef *et al,* 1982; 1984) clearly suggest that low temperature and ABA regulate the expression of *cor* genes through independent mechanisms. These studies indicate that ABA mediated gene products are essential in conferring salinity and chilling tolerance to crop plants.

A direct evidence for the role of ABA/stress responsive proteins in salinity and chilling tolerance processes, however, comes from the studies by Xu *et al,* (1996) wherein transgenic rice expressing a group III LEA gene (encoding HVA 1 protein) showed delayed injury in response to salinity and drought stress conditions. The improved tolerance in these seedlings was attributed to the hyper-accumulation of LEA protein. On the contrary, transgenic tobacco accumulating a desiccation responsive group II LEA protein from *Craterostigma plant agineum* did not show any perceptible tolerance to desiccation (Iturriga *et al,* 1992). Therefore, caution must be exercised to attribute exclusive tolerance-conferring property to the stress responsive proteins whose roles could be qualitatively different from each other or interdependent. A strategy involving the co-expression of more than one gene is preferable as stress tolerance is controlled by multiple, interdependent factors than just one. Further, the identification of an *in vitro* phosphorylated 55 kDa polypcptide in ABA treated roots of rice seedlings is significant as such changes were deemed to be an important component in the signal transduction process involving ABA *(Fig: 4.1.11).*

5.2 Proline-protein interactions: Relevance to stress tolerance:

The possible role of osmolyte proline in stress tolerance processes have been investigated. Proline and related osmoprotectants, by virtue of their abundance in the cytoplasm under stress-adapted conditions, have a high probability to interact with major biomacromolecules, particularly, the proteins. To this end, proline-protein interactions *in vitro* were widely studied with a focus on the functional integrity of various target proteins under stress conditions (Nash *et ai,* 1982; Paleg at al., 1981). The results suggest a concentration-dependent protection of activity of enzymes by proline although the biophysical basis of such a protection is poorly understood. An attempt was made here to analyze *in vitro* the proline-protein interactions and to elucidate their functional role under stress.

5.2.1 Proline rescues enzymes from PEG-induced precipitation:

Proline was found to ameliorate the effect of PEG on protein precipitation with an unmatched efficiency compared to other osmolytes tested. Hydroxy proline is particularly less effective than proline (*Table 4.2.2).* In fact, PHG has long been known to precipitate enzymes with various conformational diversities (Poison *et al.,* 1964) with its effect known to depend on factors such as its polymer size, protein molecular weight, effect of pH and ion concentration (Ingham,1977; 1978; Miekka and Ingham, 1978). Interestingly, such a protection was also reported for betaine, another stress induced osmolyte (Paleg *et ai,* 1984). These osmolytes could presumably be exerting a protective effect on protein structure by maintaining the *status quo* in a situation that probably has strong analogies with the consequent biological dehydration.

5.2.2 Proline stabilizes proteins under freeze-thaw, heat and chemical denaturations:

Proline was also found to confer a high degree of protection to a large number of enzymes against physical stresses such as freeze-thaw, heat and chemical denaturations (*Figures: 4.2.1-4.2.15).* The interpretations of the data is discussed in the following sections. The tryptophan fluorescence emission recorded here is a sensitive and ideal parameter to monitor the alteration in a protein's secondary and tertiary structure. In fact, such a method was strongly advocated to analyze the structural alterations of proteins in the presence of osmolytes (Lalonde *et ai,* 1991).

5,2.3 Mechanism of action of proline vis a vis other osmolytes:

Proline was proposed to display anomalous solution properties in water by self associating through alternate stacking of the hydrophobic pyrrolidone ring. Such a multimeric structure is known to possess a hydrophobic backbone and hydrophilic groups on the surface (Schobert and Tschesche. 1978). We envisage that the hydrophobic backbone of a proline-multimcr could favorably interact with hydropohobic residues in its surroundings. Accordingly. the solvent-accessible hydrophobic regions of these enzymes were calculated that were apparently stabilized by proline (*Table 4 2 3).* The proteins, indeed, were found to contain large hydrophobic areas accessible for an interaction with the hydrophobic backbone of the proline-multimers. Such an interaction could possibly explain an ability of proline to increase the solubility of otherwise sparingly soluble proteins (Schobert and Tschesche. 1978). In contrast, hydroxy proline did not protect the enzymes as effectively as proline *(Figures 4.2.1-4.2.15)* indicating that the hydroxyl side chain, due to steric hindrance, disrupts the self-association among proline molecules thus preventing the formation of multimeric structures. Prolinc-mediatcd protection observed in cytochrome C (Taneja and Ahmed 1994) and pyruvate phosphate dikinase (Krall. 1989) under heat stress could be explained by such an interaction.

A concentration dependent protection of different enzymes by proline was observed against freeze-thaw, heat and chemical denaturations. Such a protection assumes significance since the formation of multimeric structures by proline molecules was expected to be enhanced with an increase in its concentration (Schobert and Tschesche, 1978). The solvent-accessible hydrophobic regions of the target proteins are presumably protected more efficiently at higher concentrations of proline providing a better hydrophobic micro-environment than at lower concentrations *(Figures: 4.2.1-4.2.15).* It is well known that such solvent-accessible hydrophobic regions of proteins are comparatively more sensitive to heat and freeze-thaw stresses. which tend to aggregate among themselves. Protein precipitation which usually occurs in response to an external physical disturbance is a result of such a phenomenon. By protecting the hydrophobic regions of the target proteins from these disturbances, proline facilitates their enhanced

stability under stress. On the other hand, the exposed hydrophilic groups of proline-multimers on their surface could form a favorable association with surrounding water.

5.2.4 Proline-A "protein compatible hydrotrope":

Our studies indicate that proline, through its specialized interaction, is superior to its hydroxylated analogue in offering protection to target proteins. The hydrotropic behavior of proline proposed by Srinivas and Balasubramanian (1995) in deed, supports such a proposed interaction between proline and proteins. Hydrotropes are highly hydrophilic solutes capable of dissolving hydrophobic substances in aqueous environment by offering a micro-hydrophobic milieu. Such a hydrotropic behavior arising out of its multimerization was strikingly absent in hydroxy proline due to the steric hindrance explained above. Proline in aqueous solutions Was, in fact, found to solubilize the hydrophobic aromatic probe FDA, unlike hydroxy proline, choline and betaine (Srinivas and Balasubramanian. 1995).

The hydrophilic nature of proline (a solubility of 7 M in water) makes it an ideal hydrotrope with a biological role as it aids in preserving the necessary water content when cells and molecules are water stressed. While a range of other hydrophilic osmolytes can also be substituted for this function, proline with its self aggregating property, with a mild hydrophobic backbone, can provide a host system that can solubilize or sequester hydrophobic molecules. Such a dual property makes the micro-environment of proline system closer or compatible with that of water and thus is far more benign than a range of hydrotrope molecules. It is this specific feature that demarcates proline from its structural analogue hydroxy proline and other osmolytes (including hydrotropes), helps maintain the native conformational features of the target proteins and in turn, keeps them functional. Thus, proline qualifies to be called a "protein-compatible hydrotrope". Furthermore, it was suggested that proline could make a better acceptable solubilizing agent in comparison to detergents and other hydrotropes, which tend to denature proteins and inactivate them biologically (Srinivas *et al.*, 1997). The detergents, though act somewhat analogous to proline, are usually stronger in their solubilizing effect that disturb the hydrophobic core of the proteins unlike proline.

5.2.5 Compatibility paradigm of proline:

In an attempt to probe the compatibility paradigm of proline, activities of LDH and MDH were measured in presence of high concentrations of proline. The results revealed a total compatibility of these enzymes with proline which did not alter the efficiency of the enzyme activities even at high concentrations *(Figures; 4.2.1-4.2.8)*. These results are in accordance with the observations by Wang and Boeln (19%) on LDH-M4. In view of this, it is proposed that the broad compatibility of proline denotes its close association with biomacromolecules under stress.

5,2.6. Proline-Biomacromolecular interactions and the in vivo concentration paradigm:

Since the concentration of proline in organisms is expressed on the basis of total cell water (most of water in cells is associated with biomacromolecules than in free form), without allowance for compartmentation, the amount of water that participates as a solvent could be much lower than anticipated. Therefore, the concentration of proline in a particular location in the cell could be higher than the average values normally reported for the whole cell/tissue. In fact, the accumulation of proline in halophytes and halo-tolerant bacteria has been reported to exceed 1.0 M internal concentration (Stewart and Lee, 1974; Bohnert and Jenson, 1996; Le Rudulier *et al.*, 1984). It can therefore be concluded from our studies that the proline concentration which the LDH-M4 and MDH can tolerate without impairing their function, is considerably higher than the average concentrations found in water stressed organisms (*Figures: 4.2.1-4.2 8)*. Moreover, proline, in view of its amphiphilic nature, presumably has a double advantage of being compatible to both the hydrophobic and hydrophilic proteins.

5.2.7 The preferential hydration phenomena: Relevance to proline:

Stabilization of proteins by osmolytes is interpreted by the preferential hydration phenomenon where the structural components of proteins are excluded from an interaction with the osmolytes (Arakawa and Timasheff, 1982 a. 1982 b). Such a preferential exclusion of proteins in solutions can not alone explain their stability under stress conditions in view of the enormous diversity in target protein structures and the physical properties of their interacting osmolytes. Many reports exist where the protection offered by one osmolyte differed markedly with that of the other (Paleg *et al.*, 1984; Rudolf and

Crowe, 1985). These differences were indeed confirmed in our results *(Results 4.2).* The physico-chemical properties of various naturally occurring osmolytes are different from each other as they include diverse compounds such as amino acids (proline, glycine etc.), sugars (glucose, fructose etc.), polyols (mannitol, sorbitol, pinitol, ononitol) and quartenary ammonium compounds (betaine etc.). Accordingly, their hydrophobicity/hydrophilicity could be varying from each other. Obviously, any single theory can not explain the general stabilization of macromolecules without considering the various physico-chemical properties of the target molecules and the osmolytes involved. Further, if one osmolyte can offer a general protection under diverse stress conditions, accumulation of any osmolyte could have been sufficient for its role *in vivo.* However, of all the available compounds, only a few are widely prevalent and accumulate across the organisms. For instance, valine was found to be much inferior in conferring protection to LDH-M4 than proline *(Fig: 4.2.1).* Though both proline and valine induce preferential hydration of lysozyme, valine is ineffective in conferring protection to the enzyme (Arakawa and Timasheff, 1985). Upon unfolding the target protein, the non-polar side chain of valine can bind to the exposed hydrophobic residues, thus favoring the denatured state of the target protein. It is, therefore, not surprising that strong hydrophobic substances such as valine do not figure prominently in the list of natural osmolytes.

The preferential hydration theory can possibly explain the stabilization of proteins by osmolytes of high hydrophilic nature as the strong hydrophilizers were reported to induce a strong preferential hydration of the target proteins (Arakawa and Timasheff, 1982, a, b). However, the strength of preferential hydration could also depend on the hydrophobic status of the target protein and the osmolyte involved. Thus, a moderately hydrophobic osmolyte can find a favorable interaction with a hydrophobic protein and vice versa. For instance, serine was found to sensitize LDH and protect the membrane bound Ca^{+2}-ATPase. Similarly, proline was found to be a better cryoprotector of LDH than glutamate, while the opposite was found with LADH and Ca -ATPase (Carpenter and Crowe, 1988) Such a disparity among osmolytes tested in conferring protection to the target enzymes was in conformity with our observations *(Figures: 4.2.1-15, Table: 4.2.1)*

It can be argued that cryoprotection mechanisms that apply to water-soluble enzymes may not pertain to membrane-bound hydrophobic proteins.

The interaction of proline with proteins is of particular interest considering its role as a compatible solute in a large number of organisms and as an enzyme protector (a term used to suggest that it stabilizes the native structure of enzymes and does not inhibit enzyme activity). The CD spectral analysis in our studies have, in fact, revealed increased stability of the native structure of LDH-M4 in the presence of proline under freeze-thaw stress *(Fig: 4.2.5).* The protective effect by proline increased with the increasing concentrations in contrast to those observed with hydrophobic substances (Carpenter and Crowe, 1988), a result in conformity with our data *(Results 4.2).* Thus, proline by virtue of its high hydrophilic property could be inducing the preferential hydration of its target proteins similar to other hydrophilic osmolytes. On the other hand, the hydrophobic backbone of proline multimeric structure could be having a favorable interaction with the solvent-accessible hydrophobic residues of proteins.

Apart from the hydrophobic character of osmolytes, the surface tension and net protein surface charge considerations are likely to be of importance (Paleg *el ai,* 1984; Bowles and Somero 1979; Boeln and Fisher, 1969). Further, the osmolyte-biomaromolecular interactions could also be influenced by dielectric constants (Castaneda-Agullo and Del Castillo, 1959 a and b) and water activity (Rand *el ai,* 1993). With the exception of proline and a few osmolytes, it is extremely difficult for a solute in the 2 M range not to alter one or more of these solution properties and thereby alter the kinetic properties of the protein function.

For an osmolyte to provide a selective advantage in biological adaptation to a stress, it should meet two requirements: 1) the osmolyte/osmoprotectant must alleviate the effects of the stress without adversely affecting the stability of the target macromolecule in cells and 2) the presence of the osmolyte can not substantially alter the functional activity of the macromolecules and other cellular components. The latter condition is as important as the former, because osmolyte mediated protection would be of little selective advantage if functional activity was not preserved to maintain the intricate control of the metabolic pathways necessary to sustain life. More importantly, the property of not interfering with

the functional integrity of proteins is the defining characteristic of osmolytes which only can be termed as compatible osmoprotectants. Proline ideally fits in two these requirements in view of its specialized interaction with proteins described above.

5.2.8 Proline-The "mini-chaperone"?

The property of maintaining the structural stability of target proteins by proline is somewhat analogous to one of the characteristic features of molecular chaperones. which are known to stabilize the structural integrity of its target proteins through hydrophobic interaction (Pleham, 1986). Thus, proline can be considered a "mini-chaperone" protecting the target proteins from the stress. It is, however, purely co-incidental that, many of the stress responsive proteins (specially the classes belonging to HSP family) which are co-induced with proline in the cytoplasm do stabilize the target proteins from denaturation by similar hydrophobic interactions (Skriver and Mundy, 1990). Thus, proline and these stress responsive proteins share the property of maintaining the functional integrity of cells fry offering protection to cellular components.

The percentage hydrophobicity of the target enzymes protected by proline range from 61% to 75 % which can possibly have a favorable interaction with the hydrophobic backbone of prolinc-multimers (*Table 4.2.3).* However, the structural stability of a protein is also dictated by various factors such as the disulfide bridges and trans-helical interactions, especially under stress conditions.

*5.3 **The** role of **proline/betaine-DNA** interactions in salinity tolerance process:*

Proline was found to bring down the Tm in a concentration dependent manner *(Fig. 4.3.1),* somewhat similar to another osmolyte, betaine, which was reported to lower the Tm and partly reduce the impact of KCl on DNA stability. While 1 M proline could reduce the Tm of calf thymus DNA by 6.0° C *(Table: 4.3.1)*, betaine at a similar concentration could reduce the Tm of poly (dG-dC) by 5.0 ^{0}C and the bacterial DNA by 4° C (Rees *et al.,* 1993). The results are significant in view of the reported hyper bio-accumulation of these osmolytes under salinity stress. Such an effect was not found with other tested amino acids, of which, glycine, glycyl glycine and serine were, in fact, found to considerably stabilize the DNA. Interestingly, with the addition of methyl group(s) on the glycine structure, alanine, valine and leucine have correspondingly lost both the

aqueous solubility and the stabilizing effect on the DNA. Betaine (N,N,N,-trimethylglycine) was found to be helix destabilizing when compared to glycine and sarcosine (N-methyl glycine) (*Table 4.3.1*). In one such related attempt to test the influence of methyl groups on the potency of osmoprotection, it was demonstrated that, contrary to glycine and sarcosine. compounds of betaine series with trimethyl groups on the nitrogen, were found to ameliorate the effect of high salinity (0.8 M) on the growth of *E. coli* (Le Rudulier, 1984). Similarly, the observed inability of glycine in counteracting the effect of NaCl on DNA (*Table 4.3.1)* could probably be accounted as one of the reasons for its failure to protect *E. coli* from high salinity (0.8 M NaCl) stress (Le Rudulier *et al,* 1984). Though preliminary, these results apparently establish a correlation between the reported capability of these osmolytes to protect the organism from salinity stress with their ability to negate the salt effect on DNA stability.

The antagonistic effect of proline to that of NaCl on DNA stability *in vitro* (*Table 4.3.1)* suggests a similar interaction *in vivo* where proline could counteract the effect of high concentration of salt and cations accumulating under stress conditions. Presumably, DNA surrounded by a high concentration of salts is biologically less active than that is surrounded by both salts and their counteracting osmolytes such as proline and betaine. Moreover, proline and betaine were shown to have an additive effect on DNA stability *{Table 4.3.1)* and when present together could account for effective concentrations *in vivo.* Apart from the suggested effect on DNA, these osmolytes are known to be highly bio-compatible with a proven role in the stabilization of proteins, organelles and cells (Paleg *el al.,* 1981; 1984; Nash *et al.,* 1982; Rudolf *et al,* 1986; Schobert and Tschesche, 1978; Xin and Lee, 1993; Csonka 1989) which can not be ascertained with other amino acids. Interestingly, upon increase in salinity of the growth medium, *Lactobacillusplant arum* cells were found to instantaneously accumulate betaine and proline in preference to alanine as an adaptive measure (Glaasker *et al,* 1996). It is relevant here to note that, contrary to alanine, proline and betaine are compatible osmolytes with an ability to negate the salt effect on DNA. Similarly, of the 150 compounds tested, only proline and betaine series were found to effectively protect *E. coli* from the severe salinity stress suggesting the versatility of these osmolytes in comparison to other solutes (Le Rudulier *et al,* 1984).

In an interesting analysis, Kishore (1988) had observed that an addition of proline and betaine could improve growth of rice cells under the inhibitory concentrations of sodium chloride but not when sarcosine (N-methyl glycine), dimethyl glycine and glycine were added. It could therefore be argued that, unlike their structural analogues mentioned above, only betaine and proline could confer advantage to the organisms by counteracting the effect of salts on DNA stability (*Table: 4.3.1).* Apart from such a protective effect on DNA, these two compounds are biologically compatible which must be an essential feature of any osmoprotectant.

DNA destabilizauon by proline in our study was confirmed by (he observed resistance of DNA to DNase I in thc presence of high concentrations of proline (*Figures: 4.3,2- 4.3.5).* In fact, it is known that the activity of this enzyme on a stable double helix is 5000 times higher than that on a destabilized helix (Travers, 1989). Further, this could not be due to structural changes in thc enzyme induced by proline as there are evidences that proline, even at high concentrations, does not substantially affect the structure and function of proteins (Paleg *et al.*, 1981; 1984). On the other hand, proline was found to confer structural stability to DNase I at higher temperatures *(Fig: 4.2.12 B)*. Results from the S1 nuclease sensitivity assay provided evidence for the destabilization of double helix in the presence of increasing concentrations of proline. Various DNA samples were included in the studies to test the generality of the interaction *(Fig: 4.3.8-4.3.9)*. Increased resistance to DNase I digestion and susceptibility to S1 nuclease in thc presence of increasing proline concentrations suggest that the destabilized DNA structures could exist at physiological temperatures under stress adapted conditions. Results obtained from the gel shift assays were in agreement with the conclusions drawn from the DNase I and S1 nuclease sensitivity assays. The binding of single stranded DNA binding protein to DNA and the subsequent retardation in its mobility *(Fig: 4.3.10)* indicate the destabilization of DNA by proline. The fluorescence emission spectra of DNA-ethidium bromide/ruthenium complex in presence of proline have further confirmed the destabilization of the double helix by proline *(Figures: 4.3.11, 12)* Results obtained from the topoisomerase I assay suggest that proline does not intercalate the double helix *(Fig: 4.3.13)*.

Several studies indicate that both the *in vitro* binding affinities rate binding of certain transcriptional regulatory proteins to their target sites on DNA are extremely sensitive to the electrolyte concentrations of the buffers used (Record Jr. *et al.* 1985). Since DNA at the physiological pH exists as a highly charged anion, it is expected to be surrounded by cations which have a natural binding affinity. Moreover, the salts which accumulate during salinity stress may also unduly stabilize the double helix which could adversely inhibit the DNA function in replication and transcription (Csonka, 1981; 1989). Proline and betaine presumably, play an important role in partially alleviating such an effect. In fact, *E. coli* cells grown at very high salinity conditions (1 M NaCl) were found to actively concentrate glycine betaine as much as 10 times that of the medium (Le Rudulier *et al,* 1984). It was further envisaged that during severe stress conditions in bacteria, cellular constituents may completely be bathed in osmoprotectants that reach concentrations above 1 M and interact with biomacromolecules (Le Rudulier *et al,* 1984). Similarly, the presence of high internal concentrations of betaine under the stress-adapted conditions was found to reverse the effects of salinity mediated osmotic stress on DNA replication and cell division in *E. coli* which supports the role of osmoprotectants in alleviating the stress effects on DNA function (Meury, 1988). Thus, the selective accumulation of these two osmolytes in a wide range of organisms under the salt stress appears to be a conserved adaptive measure rather than a mere coincidence. While such an adaptive value of betainc/proline-DNA interactions can bc envisaged in prokaryotes where a direct access for osmoprotectants to DNA exists, the same can not yet be ascertained with respect to eukaryotes with a distinct nuclear membrane barrier. However, such interactions could logically be possible during certain stages of cell division where the nuclear membrane barrier transiently disappears. Though a direct interaction *in vivo* of proline or betaine with DNA is yet to be established, these osmolytes are the likely biological choices to counteract the effect of accumulated salts on DNA.

6. SUMMARY

The following conclusions are drawn from the present investigation

- Application of exogenous ABA confers a significant degree of tolerance to salinity and chilling stress in rice seedlings. ABA application leads to a better membrane integrity under severe freeze stress and enhances the survivability of rice seedlings.
- Accumulation of free proline in response to exogenous ABA correlates well with an improved salinity and cold tolerance in rice seedlings. The stress tolerant cultivars preferentially accumulated higher amounts of proline in shoots in comparison to sensitive cultivar tested.
- A simple and reliable procedure was developed to test the effect of ABA on stress tolerance using the etiolated rice seedlings as an experimental system.
- Stress tolerant rice cultivars show greater resistance to injury upon exposure to salinity and osmotic stress. Further, salinity stress inflicts a greater damage to the leaf discs than the osmotic stress mediated by PEG. Injury index therefore is a measure to demarcate the stress tolerant cultivars from the sensitive ones.
- Accumulation of different stress responsive polypeptides [15 kDa, RAB, 18 kDa (pcC 6-19, immunologoically related to a desiccation tolerant protein from *Craterostigma plant agineum*), 23 kDa (related to RAB), and 34 kDa (aldose reductase related protein] in response to exogenous ABA correlates well with improved stress tolerance observed in rice seedlings.
- A 34 kDa polypeptide immunologically related to aldose reductase, (an enzyme involved in the sorbitol biosynthesis) is responsive to ABA and osmotic stresses in rice. The activity of aldose reductase is enhanced upon treatment with ABA.
- A 18 kDa polypeptide was identified in rice that is immunologically related to desiccation-responsive polypeptide (encoded by pcC 6-19) from African resurrection plant, denoting the conserved biochemical responses towards stress tolerance process.
- A 55 kDa polypeptide was identified in ABA-treated roots which is phosphorylated *in vitro.*

- Proline stabilizes and maintains the functional status of target proteins somewhat analogous to chaperones through offering hydrophobic micro environment. Maintenance of secondary structure of target proteins was reflected in various spectroscopic studies.
- Proline/betaine destabilize the DNA helix and reduce the effect of salts and spermidine on the helical stability.
- Proline makes the double helix resistant to DNase 1 digestion and sensitive to S1 nuclease digestion.
- Proline or betaine do not intercalate the DNA as evidenced from the topoisomerase 1 assay.
- Localized destabilization of helical structure could be possible in the presence of high internal concentrations of proline/betaine under specialized stress adaptive conditions.
- Betaine and proline could constitute some of the biological choices to counteract the effect of salts and cations on DNA stability.

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1. Chadalavada S.V. Rajendrakumar, Boojala V.B. Reddy & Arjula R. Reddy *(1994).* Proline-protein interactions: Protection of structural and functional integrity of M4-**Lactate Dehydrogenase.** ***Biochemical and Biophysical Research Communications*** **201** (2) 957-963.

2. Chadalavada S.V. Rajendrakumar, B. Karunasree, & Arjula R. Reddy *(1996)* Molecular approaches to abiotic stress tolerance. pp. 112-121. In the ***5th National Rice Biotechnology Network (The Rockefeller Foundation National Grantees Meet),*** ICGEB, New Delhi, Nov-13-16.

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- Abscisic acid mediated salinity and chilling tolerance in rice requires the induction of stress responsive polypeptides
  Chadalavada S.V. Rajendrakumar B. Karunasree & Arjula R. Reddy
  (Under preparation to *Plant Science)*

- Identification of a developmentally regulated and ABA responsive aldose reductase related protein in rice *(Oryza saliva L)*
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Abstracts presented at the Conferences **from the present investigation:**

- **Rajendrakumar,** C.S.V., B.V.B. Reddy & A.R. Reddy (1994). Biochemical and bio physical study of protection of enzyme's structural and functional integrity of proline. **At the *Second Asia Pacific Conference on Agricultural Biotechnology,*** 6-10 **March.** Madras, India.
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- U.R. Rani, B. Karunasree, C.S.V. **Rajendrakumar,** G. Madhuri, K. Joanny, M Padmavathy, S. Jayasheela, K.R.K. Reddy & A.R. Reddy (1994). Studies on Stress **response in Rice.** ***The National Rice Biotechnology Network. Third annual meeting*** 3-5 March. Madras, India.
- **Rajendrakumar C.S.V,** B.V.B. Reddy & A.R. Reddy (1994). Evidence for proline mediated protection of enzyme's structure and function-Can it be related to stress **resistance in plants?** ***Seventh meeting of the International program on rice biotechnology***, 16-20 May. Bali, Indonesia.

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- Karunasree, B., Rajendrakumar, CSV and Arjula R Reddy Identification of a developmentally regulated and ABA responsive aldose reductase related protein in rice *(Oryza sativa* L) accepted in Fourth Asia Pacific Conference on Agricultural Biotechnology to be held in Darwin, Australia, 13-17, July, 1998.

Practical course work/ Workshops attended during the tenure as a doctoral fellow:

1. Attended the practical course work on ''Plant Transformation'' during Nov.-Dec. 1994 held at Int. Center for Genetic Engineering and Biotechnology (ICGEB) conducted by UNIDO at New Delhi
2. Attended the practical course work on "Fluorescence in situ hybridization using non radioactive nucleic acid probes "(FISH) held at the Indian Institute of Science, Bangalore between 22 July to 2 Aug. 1995 (Prof. 11. Sarat Chandra's Lab)
3. Attended the Mahabaleswar seminar seris on modern biology ''Nucleic acid protein interactions" held at TIFR centre Ooty in December 1995
4. Attended the Indo-Japan workshop on ''DNA and protein foot-printing'' held at Centre for Cellular and Molecular Biology, Sponsored by JSPS-DST in March, 1997

Symposia & Conferences attended during the doctoral investigation:

1. Annual Meeting of the Society of Biological Chemists (India) held at Centre for Cellular and Molecular Biology, Hyderabad 1992
2. Attended the Second Asia Pacific Conference on Agricultural Biotechnology held at Madras, India, between 6-10 March-1994
3. International Symposium on Plant Molecular Biology & Biotechnology held at International Centre for Genetic Engineering and Biotechnology in December 1994 at New Delhi. November 1995
4. Pot poury meet of Society of Biological Chemists (Hyderabad chapter) at Centre for Cellular and Molecular Biology Hyderabad March 21 1995
5. II International Crop Science Congress, New Delhi, Nov. 17-23

Membership in professional scientific organizations:

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PROLINE - PROTEIN INTERACTIONS: PROTECTION OF STRUCTURAL AND FUNCTIONAL INTEGRITY OF M_4 LACTATE DEHYDROGENASE

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Summary: A well defined labile isozyme, rabbit muscle M4 - lactate dehydrogenase was denatured under freeze-thaw, heat and GuHCl treatment in the presence and absence of proline, and the corresponding structural changes of the enzyme were monitored through fluorescence and CD spectral studies. The data reveal that proline confers protection to the structural integrity of the enzyme, thereby protecting its activity. This was attributed to its property of forming hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with protein. Unlike other osmolytes, proline is proposed to act on the enzyme stability not only by inducing preferential hydration of proteins but also through the interactions of its multimeric hydrophobic backbone with the solvent-accessible hydrophobic regions of the enzyme.

There have been reports on the protection of enzyme activity, organelle systems and free cells by proline from the deleterious effects of heat, *pH*, salt and chemicals (1-4). In all these cases, a significant concentration dependent protection was observed against a range of stressful conditions. It was reasoned that any proline-induced decrease in enzyme precipitation would reflect a lessening of the thermodynamically unfavourable conditions leading to precipitation, which in turn could result in enhanced stability of an enzyme to remain in the solution (5 - 7). In aqueous solutions, proline forms aggregates by a step-wise stacking and hydrophobic interactions of the pyrrolidine ring, forming multimers which contain a hydrophobic backbone with hydrophilic groups on the surface (8). Thus, proline was proposed to increase the solubility of sparingly soluble proteins by its hydrophobic interaction with the hydrophobic surface residues of the protein. To our knowledge, however, there is no experimental evidence for proline-protein interactions in the maintenance of structural and functional integrity of any enzyme under different stress conditions. To this end, we provide

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here such an evidence by making use of a labile enzyme, M_4 lactate dehydrogenase and show that proline confers protection to its structure and function under severe freeze-thaw, high temperature and chemical denaturation. A possible mechanism involved is discussed.

Materials and Methods

Rabbit muscle L-lactate dehydrogenase-5(M4) isozyrne type VS (EC 1.1.1.27), proline, hydroxy-proline, guanidinium hydrochloride, NADH, sodium pyruvate were purchased from Sigma. All other chemicals used were of analytical grade and purchased locally.

Denaturation studies. The enzyme was denatured by three different methods, namely freeze-thaw cycles, heat denaturation and chemical denaturation by GuHCl (0.5M). $LDH—M_4$ at a concentration of 60 $\mu g/ml$ dissolved in 10 mM tris buffer (pH 7.5) was frozen in liquid nitrogen for 60 seconds and thawed at room temperature for five minutes. Such cycles were repeated for a minimum of three times with or without proline. For a comparative analysis of cryoprotection conferred by different osmolytes, enzyme at a concentration of 60 $\mu g/ml$ dissolved in 10 mM tris buffer (pH 7.5) was freeze-thawed, with different osmolytes (1M each) added separately. Aliquotes were used for fluorescence measurements. For the heat denaturation studies, the enzyme at a concentration of 15$\mu g/ml$ was incubated at different temperatures for 15 minutes with or without proline (1.0 M). These were left at 4^0C for six hours. Aliquots were used for fluorescence measurements as well as enzyme assays. GuHCl (0.5 M) was added to the enzyme at a concentration of 90 $\mu g/ml$ containing different concentrations of proline and incubated for an hour at 27° C. Aliquots from the above samples were taken for fluorescence studies and enzyme assay. The assay was followed essentially as described by Tarniya *et al.*, (1985) (9).

Fluorescence spectral studies. The intrinsic fluorescence of tryptophan was monitored to record its contribution to the emission intensity of the enzyme at an excitation wave length 295 nm using Hitachi Spectrofluorimeter. The relative fluorescence and $\lambda_{emissionmax}$ were determined under different denaturing conditions in the presence and absence of proline and certain other osmolytes have been recorded (Fig. 1 and 2). The fluorescence was recorded at room temperature, with a band pass of 5 nrn.

Circular Dichroic spectral studies. ("I) spectra were recorded with Jasco 20 Spectropolarimeter at room temperature. Sample solutions contained enzyme (3.74 μM) dissolved in 10 mM tris buffer pH7.5The CD values were computed for molar elipticities *(6)* using the method of Rao and Kumar (1991) (10). Each spectrum is an average of three scans. The spectra could not be recorded in the presence of high concentrations of proline as it interferes with the CD spectrum of enzyme at such concentrations. Therefore, the CD spectra were recorded with 10 mM proline which was later corrected for its contribution to the CD signal of the enzyme,

Results

The fluorescence intensities of the enzyme freeze-thawed with different osmolytes revealed that proline exhibited a stabilizing effect on the enzyme's structure in comparison with the other osmolytes tested (Fig. 1). Proline-mediated cryoprotection of enzyme becomes evident at as low concentrations as 10mM and appeared to saturate at 250 mM (Fig. 2a). Similarly at elevated temperatures, proline (1M) maintains both fluorescence intensity as well as enzymatic activity (Fig. 2b). However, proline was found to be ineffective beyond 45°C. Further we investigated the protection of enzyme by proline under the denaturing influence of GuHCl. GuHCl was known to dissociate the tetrameric form into inactive monomers

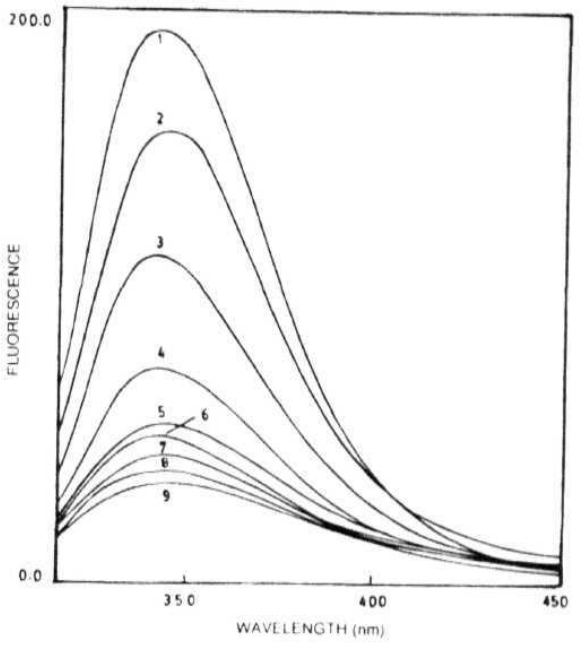


Figure 1. Fluorescence emission spectra of $LDH - M_4$ (1) control, freeze-thawed with 1.0 M each of (2) proline, (3) Sucrose, (4) Mannitol, (5) Glycine, (6) Hydroxy-proline, (7) Glucose, (8) Valine and (9) Freeze-thawed enzyme.

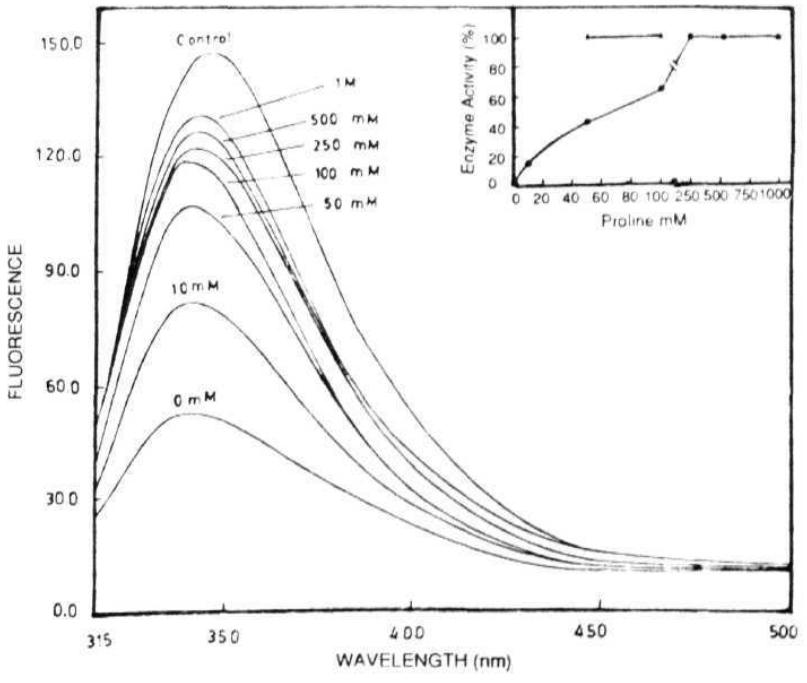


Figure 2A. Fluorescence emission spectra of $LDH - M_4$ control and freeze-thawed with different concentrations of proline as indicated. Enzyme activity shown in the inset, control (▲) and freeze-thawed (●) with proline.

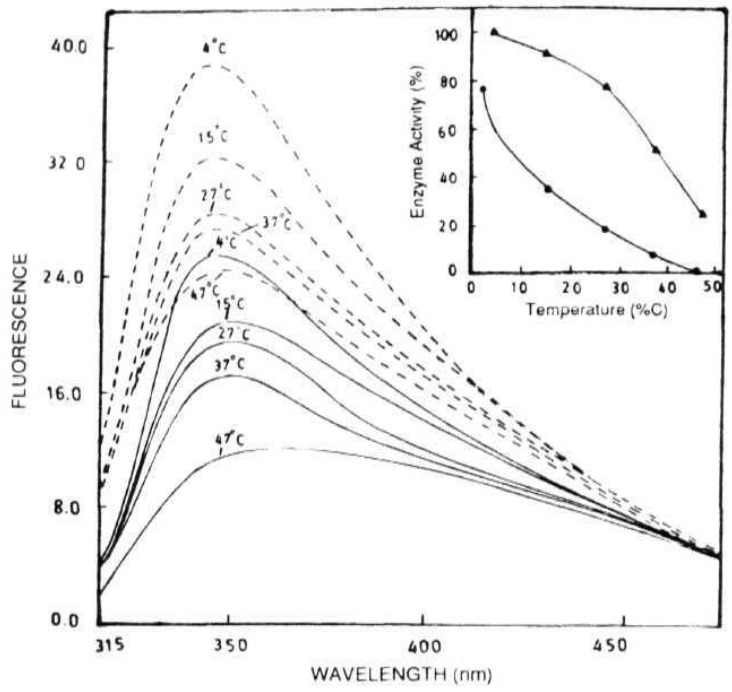
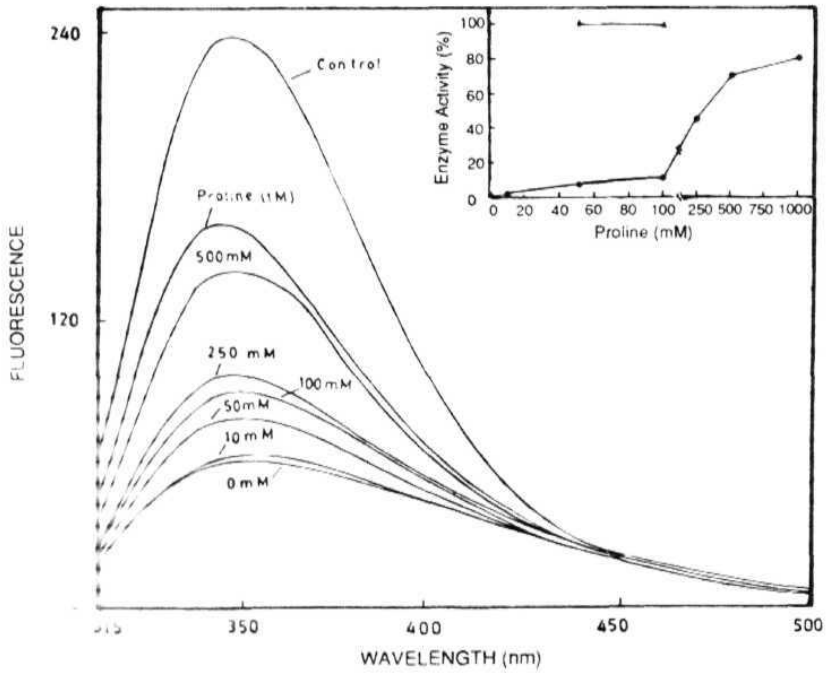


Figure 2B. Fluorescence emission spectra of heat denatured $LDH-M_4$ in the absence (——) and presence (- - - -) of proline (1.0 M). Enzyme activity shown in the inset, with (▲) and without (●) proline.



Fluorescence emission spectra of $LDH-M_4$, control (without GuHCl and [illegible] GuHCl (0.5 M) denatured enzyme with different concentrations of proline [illegible] The inset shows the enzyme activity in the presence (●) and absence of [illegible]

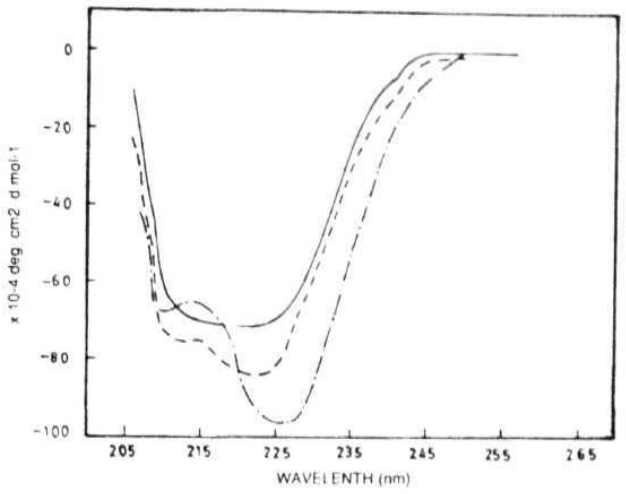


Figure 3. CD spectra of native $LDH - M_4$ (- - - -), freeze-thawed enzyme (——) and freeze-thawed enzyme in the presence of 10 mM proline (— · — · —).

(11,12). The results revealed that proline,in a concentration dependent manner, exhibited a stabilizing effect on the fluorescence intensity of the enzyme. This was also supported by the enzyme activity study, wherein the structure and function of the tetrameric form was evidently stabilized under the denaturing conditions of GuHCl (Fig. 2c).

The CD spectra of the native and proline-protected enzymes show typical characteristics of a protein with good helical content (Fig. 3). The native enzyme showed minima at 212 nm and 222 nm, while the proline protected enzyme, under freeze-thaw, showed a minima at 210 and 225 nm. However, the freeze-thawed enzyme without proline showed a single minimum from 215 run to 224 nm indicating the disturbed secondary structure. The α helicity of the proline-protected enzyme was found to be increased marginally when compared to the native form. On the contrary, the freeze-thawed enzyme exhibited a decrease in α helicity.

Discussion

The stabilization of native protein structure as a result of preferential hydration, induced by certain osmolytes, has been reported previously (13-15). However, such an increase in preferential hydration of a protein can not fully explain its stability under stress. For instance, both proline and valine induce preferential hydration of lysozyme, although valine is ineffective in conferring protection to the enzyme (16). Proline has been implicated in the structural stabilization of a range of enzymes. Present study reveal that proline is a better protective agent of $LDH - M_4$ when compared to other tested amino acids and sugars. This could be attributed to the formation of proline aggregates in aqueous media (8). Thus, proline behaves like a hydrophilic colloid with a hydrophobic backbone thereby inducing

is somewhat analogous to the chaperone function wherein a similar kind of hydrophobic interaction prevents the enzymes from denaturation (21).

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DNA helix destabilization by proline and betaine: possible role in the salinity tolerance process

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Abstract **Evidence is provided for the ability of proline, a salinity induced osmoprotectant, to destabilize the double helix and lower the T_m of DNA in a concentration dependent manner. At the reported salinity-adaptive bio-accumulation of 1 M and above, proline could considerably decrease the T_m and partially counteract the effect of sodium chloride and spermidine on DNA stability. On the contrary, several other amino acids tested did not show any such destabilizing effect on DNA helix. Enhanced susceptibility to S1 nuclease and insensitivity to DNase I in presence of increasing proline concentrations have further suggested a clear destabilization of the double helix. Such an effect is somewhat reminiscent of the interaction between betaine, another salinity induced osmolyte, and DNA resulting in decreased T_m values. These interactions may be significant in view of the abundance of such osmolytes in cells under salinity stress-adapted conditions, with many a bacterial mutant accumulating them exhibiting improved tolerance to salinity.**

Key words: Proline; Betaine; DNA helix; T_m curves; Salinity stress

1. Introduction

Proline and betaine are the two known major osmoprotectants which accumulate in plants, bacteria, algae and marine invertebrates in response to an array of abiotic stresses, most prominent being the salinity stress [1–4]. More often, this accumulation is the result of an adaptive de novo synthesis in cells contributing a major share among osmolytes [5–8]. Such accumulations were found to reach up to 1 M internal concentration in certain halophytes and bacteria, accounting for as much as 10–20% of the dry weight [9–12]. These two osmolytes were also reported as efficient stabilizers of proteins, lipid membranes, organelles and cells under severe stress conditions without being inhibitory to cellular functions [13–19]. Further, genetically engineered hyper-accumulation of proline was reported to confer salinity tolerance in tobacco seedlings under laboratory conditions [20]. Many plant and bacterial mutants accumulating proline and betaine have also been found to exhibit an increased tolerance to salinity stress [11,12,21–24].

We have investigated the interaction of these osmolytes with DNA, since their access, even transiently, to DNA in vivo under the stress adapted conditions can not be ruled out due to their abundance. In fact, betaine was proved recently to considerably destabilize DNA [25]. We report here that proline destabilizes DNA and partially counteracts the effect of sodium chloride and spermidine on the stability of the double helix within the adaptive bio-accumulated concentrations. The present study indicates a possible role of these osmolytes in salinity tolerance process by negating the undesirable effect of NaCl on DNA stability.

2. Materials and methods

L-Proline, hydroxy proline, glycine, alanine, valine, leucine, serine, betaine, D-glucose, sarcosine, calf thymus DNA, Tris, EDTA, spermidine, NaCl, agarose and λ phage DNA were purchased from Sigma (St. Louis, MO, USA). *E. coli* single strand DNA binding protein (ssb protein) and pUC 18 plasmid were procured from Bangalore Genei (Bangalore, India). DNase I was procured from Boehringer-Mannheim (Mannheim, Germany) and the S1 nuclease from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade purchased locally.

2.1. DNA melting studies

DNA melting studies were conducted in a buffer (1 ml) containing 10 mM Tris-HCl (pH 7.5) and 2 mM EDTA and the indicated concentrations of NaCl and additives. Calf thymus DNA (1.0 A_{260}) in the above buffer, with or without the additives, was taken in a 1 cm path teflon-stoppered quartz cell and incubated at the initial assay temperature for 5 min. The increase in absorbance at 260 nm was monitored in a Hitachi spectrophotometer attached to a temperature programmer KPC-6 and temperature controller SPR-7. Both the sample and reference cells were heated together at a rate of 1°C/min, and the net absorbance was recorded after every 1°C increase. The T_m of DNA was determined graphically from the transition mid-point of the absorbance versus temperature profile.

2.2. DNase I sensitivity assay

The sensitivity of DNA to DNase I digestion was studied spectrophotometrically (Hitachi) by measuring the increase in absorbance at 260 nm at 37°C in presence of different concentrations of proline. DNase I (1 μg) was added to double stranded calf thymus DNA (1.0 A_{260}) in a buffer (1 ml) containing 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. The enzyme was diluted to required concentration in 10 mM Tris-HCl (pH 7.8) and 50% (v/v) glycerol. DNase I sensitivity of DNA was also analysed by agarose gel electrophoresis. Calf thymus DNA, λ phage DNA, or pUC 18 DNA (1 μg each) in the DNase I assay buffer (30 μl) was incubated at 37°C for 10 min with 25 ng of DNase I in the presence of different concentrations of proline and the digestion products were separated on a 0.8% agarose gel.

2.3. S1 nuclease sensitivity assay

The S1 nuclease reaction mixture (30 μl) contained calf thymus DNA (0.5 μg), buffer (5 mM sodium acetate (pH 4.7), 15 mM sodium chloride, 0.1 mM $ZnCl_2$) and proline. DNA samples in presence of increasing concentrations of proline were heated at 65°C for five minutes and quickly chilled on ice. Reaction was started by adding S1 nuclease (1 unit) and incubated at 37°C for 15 min. The digestion was stopped by adding EDTA and SDS to a final concentration of 50 mM and 1%, respectively, and the products were separated on a 0.8% agarose gel.

2.4. Single strand binding protein gel shift assay

The λ phage DNA (0.5 μg) in 30 μl buffer containing 10 mM Tris-

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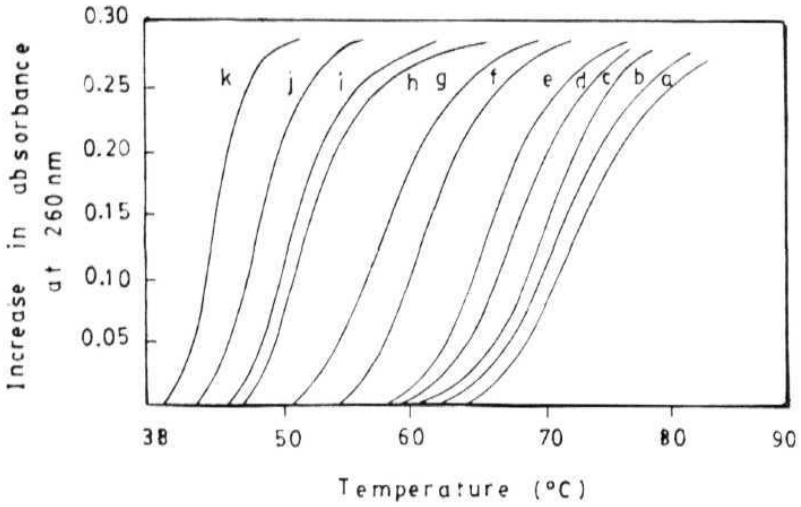

Fig. 1. Effect of increasing concentrations of proline on the T_m of calf thymus DNA: (a) Control DNA (without proline), (b) 0.06 M, (c) 0.25 M, (d) 0.5 M, (e) 1.0 M, (f) 2.0 M, (g) 3.0 M, (h) 4.0 M, (i) 4.5 M, (j) 5.0, (k) 5.5 M.

HCl (pH 8.1), 1 mM EDTA and 20 mM NaCl, was heated at 65°C in the presence or absence of 3.0 M proline for 5 min and quickly chilled on ice. Increasing concentrations of ssb protein was added and after incubation at room temperature for 5 min, the samples were electrophoresed on a 0.7% agarose gel.

2.5. Displacement of DNA bound ethidium bromide by proline

Ethidium bromide (0.4 μg) in the buffer (10 mM Tris-HCl (pH 7.5) and 50 mM NaCl) was excited at 480 nm and the emission was recorded between 500–660 nm in a Hitachi spectrofluorimeter. Later, calf thymus DNA (0.5 μg) was added to it to record the enhancement in fluorescence emission intensity. Similarly, the emission spectra were recorded with the addition of increasing concentrations of proline to the above mixture after incubating at room temperature for 15 min.

3. Results

Destabilization of DNA double helix by proline was analysed by various methods. Proline was found to significantly lower the melting temperature of calf thymus DNA in a concentration dependent manner. Though such an effect found at 60 mM was marginal, an appreciable decrease in T_m was observed consistently (Fig. 1) at concentrations ranging from 250 mM to 1 M, which are widely reported to be biologically relevant [9–12]. In order to know whether the effects shown by proline are specific, several other amino acids were tested as controls. The results reveal (Table 1) that none of the amino acids tested could induce a similar effect even at high concentrations. While glycine, glycyl glycine, and serine were found to significantly stabilize the double helix and increase the T_m, alanine, valine, leucine and sarcosine could not greatly alter the T_m. However, hydroxy proline at its maximum aqueous solubility point (2.0 M), could reduce the T_m by 8°C. Proline, unlike its hydroxylated analogue, with a high aqueous solubility (6.0 M) due to the reported anomalous solution properties [18], was found to destabilize DNA even beyond such a concentration (Fig. 1). However, the differential aqueous solubility of tested solutes prevented an ideal comparison between them in their interaction with DNA.

Proline and betaine (1 M each) were found to have an

Table 1
Effect of proline and other amino acids on the T_m of calf thymus DNA in the presence and absence of additives

Concentration	T_m of DNA ± 1.0°C
DNA	71.0
+1.0 M proline	65.0
+2.0 M proline	[illegible]
+2.0 M glycine	[illegible]
+2.0 M serine	[illegible]
+1.0 M alanine	[illegible]
+0.25 M valine	[illegible]
+0.1 M leucine	[illegible]
+2.0 M hydroxy pro[illegible]	[illegible]
+0.5 M glycyl glycin[illegible]	[illegible]
+2.0 M sarcosine	[illegible]
+1.0 M glucose	[illegible]
+1.0 M betaine	[illegible]
+1.0 M proline+1.0 M [illegible]	[illegible]
+10 mM spermidine	[illegible]
+0.5 M NaCl	[illegible]
+10 mM spermidine [illegible]	[illegible]
+0.5 M NaCl+1.0 M [illegible]	[illegible]
+0.5 M NaCl+2.0 M [illegible]	[illegible]
+0.5 M NaCl+2.0 M [illegible]	[illegible]

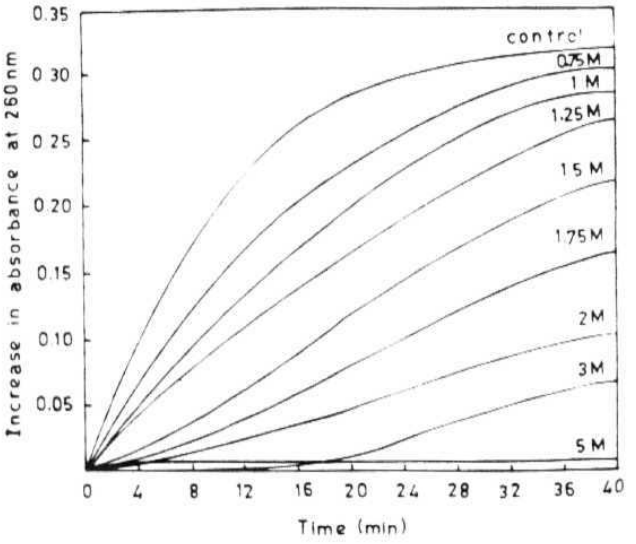

Fig. 2. DNase I sensitivity of calf thymus DNA in the presence of increasing concentrations of proline.

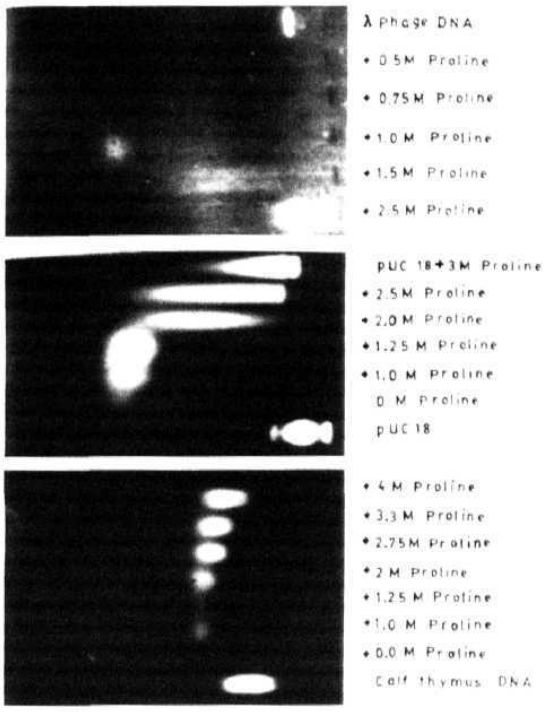

Fig. 3. DNase I sensitivity of λ phage, pUC 18 and calf thymus DNA in the presence of different concentrations of proline.

dditive effect in the reduction of T_m (Table 1). Moreover, roline (1 M) was found to individually reduce the effect of aCl (0.5 M) and spermidine (10 mM) on DNA stability as dicated by the decrease in T_m by 6°C and 8°C, respectively. n the contrary, the co-addition of glycine (2 M) with sodium loride (0.5 M) did not influence the effect of the latter on NA indicating the ineffectiveness of glycine in counteracting e salt effect (Table 1).

The helix destabilization was further confirmed with the DNase I and S1 nuclease sensitivity assays. In the spectrophoometric analysis of DNase I digestion, increased proline conentrations were found to progressively protect the calf thyus DNA against the digestion, with a near complete rotection observed at higher than 3.0 M (Fig. 2). This was urther demonstrated by gel electrophoresis profile of DNase I igested samples of λ phage, plasmid and calf thymus DNA Fig. 3). Rice and barley DNA did show a similar pattern of esistance to DNase I activity in the presence of proline (data ot shown). This effect is either due to a decreased binding of DNase I to DNA or destabilization of the double helix. The ormer is less likely because proline does not affect the binding properties of proteins which interact with DNA (see below). In contrast, proline at increasing concentrations was found to make the double stranded calf thymus DNA more susceptible to S1 nuclease digestion (Fig. 4). In the gel retardation assay, binding of increasing amounts of ssb protein to λ DNA in presence of 3.0 M proline was found to retard the mobility of the DNA-protein complexes which was clearly absent in the control λ DNA with the addition of 12 μg of ssb protein (Fig. 5). These results indicate the non-interference of proline in interactions between such proteins and DNA. Finally, the ability of proline in replacing the ethidium bromide bound to double stranded calf thymus DNA was tested and the fluorescence emission data (Fig. 6) revealed a marginal displacement which is expected of compounds that destabilize the double helix.

4. Discussion

Proline was found to bring down the T_m in a concentration dependent manner (Fig. 1), somewhat similar to betaine which was reported to lower the T_m and partly reduce the impact of KCl on DNA stability [25]. While 1 M proline could reduce the T_m of calf thymus DNA by 6°C (Table 1), betaine at a similar concentration could reduce the T_m of poly (dG-dC) by 5°C and the bacterial DNA by 4°C [25]. The results are significant in view of the reported hyper bio-accumulation of these osmolytes under salinity stress. Such an effect was not found with other tested amino acids, of which, glycine, glycyl glycine and serine were in fact found to considerably stabilize the DNA. Interestingly, with the addition of methyl group(s) on the glycine structure, alanine, valine and leucine have correspondingly lost both the aqueous solubility and the stabilizing effect on the DNA. Similarly, *N,N,N*-trimethylglycine (betaine) was found to be helix destabilizing when compared to glycine and sarcosine (Table 1) [25]. In one such related attempt to test the influence of methyl groups on the potency of osmoprotection, it was demonstrated that, contrary to glycine and sarcosine, compounds of betaine series, with trimethyl groups on the nitrogen were found to ameliorate the effect of high salinity (0.8 M) on the growth of *E. coli* [12]. Similarly, the observed inability of glycine in counteracting the effect of NaCl on DNA (Table 1) could probably be accounted as one of the reasons for its failure to protect *E. coli* from high salinity (0.8 M NaCl) stress [12]. Though preliminary, these results apparently establish a correlation between the reported capability of these osmolytes to protect the organism from salinity stress with their ability to negate the salt effect on DNA stability.

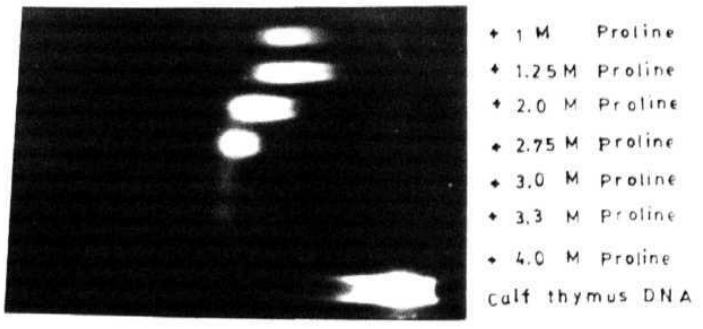

Fig. 4. S1 nuclease sensitivity of calf thymus DNA in the presence of different concentrations of proline.

However, the destabilizing effect shown by hydroxy proline is biologically insignificant as it is not known to accumulate in cells under the stress-adapted conditions. Proline, on the contrary, is a widely reported osmoprotectant, known to stabilize proteins somewhat analogous to chaperones [13] and act as a protein compatible hydrotrope [26]. Further, the antagonistic effect of proline to that of NaCl on DNA stability in vitro possibly suggests a similar interaction in vivo where proline could counteract the effect of high concentration of salt and ations accumulating under stress conditions. Presumably, DNA surrounded by a high concentration of salts is biologically less active than that is surrounded by both salts and their counteracting osmolytes such as proline and betaine. Moreover, proline and betaine were shown to have an additive effect on DNA stability (Table 1), and when present together could account for effective concentrations in vivo. Apart from the suggested effect on DNA, these osmolytes are known to be highly bio-compatible with a proven role in the stabilization of proteins, organelles and cells [13 19] which can not be ascertained with other amino acids. Interestingly, upon increase in salinity of the growth medium, *Lactobacillus plantarum* cells were found to instantaneously accumulate betaine and proline in preference to alanine as an adaptive measure [27]. Similarly, of the 150 compounds tested, only proline and betaine series were found to effectively protect *E. coli* from the severe salinity stress suggesting the versatility of these osmolytes in comparison to other solutes [12].

DNA destabilization by proline in our study was further confirmed by the observed resistance of DNA to DNase I in the presence of high concentrations of proline. In fact, it is known that the activity of this enzyme on a stable double helix is 5000 times higher than that on a destabilized helix [28]. Further, this could not be due to structural changes in the enzyme induced by proline as there are evidences that proline, even at high concentrations, does not substantially affect the structure and function of proteins [13 18]. On the other hand, proline was found to confer structural stability to DNase I at higher temperatures (data not shown). Increased resistance to DNase I digestion and susceptibility to S1 nucle-

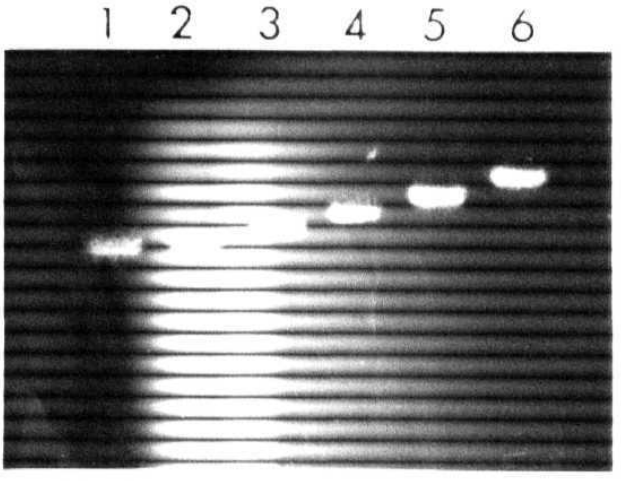

Fig. 5. Gel mobi... ...DNA in the absence and presence of 3.0 M ... ...increasing concentrations of ssb protein. Lane 1, λ pha... ...; lane 2, λ phage DNA+12 μg of ssbp. Lanes 3-6, ...A in the presence of 3.0 M proline with increasing co... ...as follows: 3, 3 μg of ssbp; 4, 6 μg of ssbp; 5, ...of ssbp.

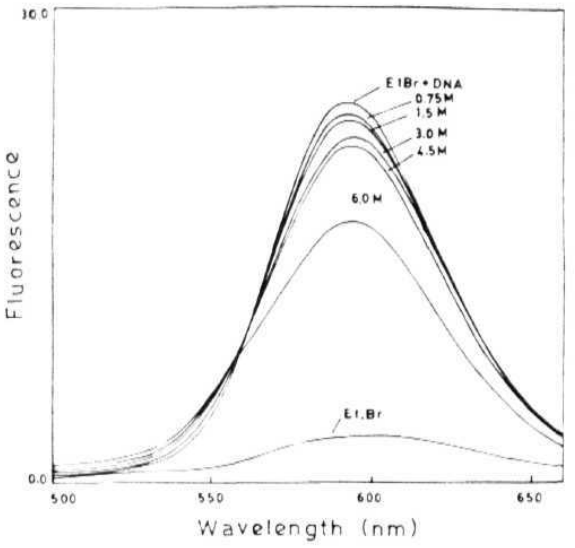

Fig. 6. Fluorescence emission spectra of ethidium bromide in f... and DNA-bound form; effect of proline in displacing the DN... bound ethidium bromide.

ase in the presence of increasing proline concentrations su... gest that the destabilized DNA structures could exist at phy... iological temperatures under stress adapted conditions.

Several studies indicate that both the in vitro binding affi... ities and rate of binding of certain transcriptional regulato... proteins to their target sites on DNA are extremely sensiti... to the electrolyte concentrations of the buffers used [29]. Sin... DNA at physiological pH exists as a highly charged anion, ... is expected to be surrounded by cations which have a natur... binding affinity. Moreover, the salts which accumulate durin... salinity stress may also unduly stabilize the double helix whi... could adversely inhibit the DNA function in replication an... transcription [3]. Presumably, proline and betaine play a... important role in partially alleviating such an effect. In fac... *E. coli* cells grown at very high salinity conditions (1 M NaC... were found to actively concentrate glycine betaine as much ... 10^3 times that of the medium [12]. It was further envisage... that during severe stress conditions in bacteria, cellular co... stituents may completely be bathed in osmoprotectants tha... reach concentrations above 1 M and interact with biomacro... molecules [12]. Similarly, the presence of high internal conce... trations of betaine under the stress-adapted conditions wa... found to reverse the effects of salinity mediated osmotic stre... on DNA replication and cell division in *E. coli* which suppo... the role of osmoprotectants in alleviating the stress effects o... DNA function [30]. Thus, the selective accumulation of thes... two osmolytes in a wide range of organisms under the sa... stress appears to be a conserved adaptive measure rather tha... a mere coincidence. While such an adaptive value of betain... proline-DNA interactions can be envisaged in prokaryot... where a direct access for osmoprotectants to DNA exis... the same can not yet be ascertained with respect to eukaryo... with a distinct nuclear membrane barrier. However, such ... teractions could logically be possible during certain stages ... cell division where the nuclear membrane barrier transien... disappears. Though a direct interaction in vivo of proline ... betaine with DNA is yet to be established, these osmolytes a... the likely biological choices to counteract the effect of acc... mulated salts on DNA.

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