

Salt-stress Induced Alterations in Protein Profile and Protease Activity in the Mangrove *Bruguiera parviflora*

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Two-month-old seedlings of *Bruguiera parviflora* were treated with varying levels of NaCl (100, 200 and 400 mM) under hydroponic culture. Total proteins were extracted from leaves of control and NaCl treated plants after 7, 14, 30 and 45 d of treatment and analysed by SDS-PAGE. As visualized from SDS-PAGE, the intensity of several protein bands of molecular weight 17, 23, 32, 33 and 34 kDa decreased as a result of NaCl treatment. The degree of decrease of these protein bands seemed to be roughly proportional to the external NaCl concentration. The most obvious change concerned a 23 kDa-polypeptide (SSP-23), which disappeared after 45 d treatment in 400 mM NaCl. Moreover, the SSP-23 protein, which disappeared in *B. parviflora* under salinity stress, reappeared when these salinized seedlings were desalinized. These observations suggest the possible involvement of these polypeptides for osmotic adjustment under salt stress. NaCl stress also caused an increase in the activity of both acid and alkaline protease. The increasing activity of proteases functions as a signal of salt stress in *B. parviflora*, which induces the reduction of protein level.

Key words: Hydroponic, Polypeptide, Protease

Introduction

Mangroves are constituent plants of tropical inter-tidal forest community. They include woody trees and shrubs, which flourish in the inhospitable zone between land and sea along the tropical coastline of the globe. True mangroves occur only in mangrove habitat and their existence is rare elsewhere. Mangroves are taxonomically diverse; true mangroves include about 54 species in 20 genera belonging to 16 families (Das *et al.*, 2002). The most striking feature of mangrove is their ability to tolerate NaCl up to sea water concentration (~ 500 mM). Mangroves are divided into two distinct groups on the basis of their salt management strategies. One is “secretors” having salt glands or salt hairs for excretion of excess salt and the other is “non-secretors” lacking such morphological features. *B. parviflora* is included in the latter group

that grows in a range of salinities from brackish water up to two times concentrated sea water. Although non-secretors including *B. parviflora* exclude 99% of the salt in surrounding sea water by ultrafiltration (Scholander, 1968), but high level of sodium in xylem sap of *Bruguiera gymnorrhiza* have been detected (Takemura *et al.*, 2000). Due to high salt concentrations in the growth medium halophytes such as mangroves are confronted with the problem of maintaining turgor pressure and protecting their metabolism from high inorganic ion concentration (Flowers *et al.*, 1977). This is accomplished by the accumulation of inorganic ions within the vacuole and/or by the synthesis of inorganic compounds (Hasegawa *et al.*, 2000). Mangroves have developed physiological adaptations to their environment such as salt glands, leaf succulence and ultrafiltration by roots (Tomlinson, 1986). Furthermore, the possible function of hypocotyl as an additional filter to retain salt from the shoot is considered for the mangrove *B. gymnorrhiza* (Werner and Stelzer, 1990). *B. parviflora*, in particular, is not provided with salt glands, but it

Abbreviations: kDa, kilodalton; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MW, apparent molecular weight.

keeps the xylem sap essentially free of NaCl by ultrafiltration in the membranes of root cells (Scholander, 1968; Mallery and Teas, 1984). In *B. parviflora*, it is suggested that sugar, proline and polyphenol act as compatible solutes to regulate water potential (Parida *et al.*, 2002). In addition to synthesis of osmolytic compounds, specific proteins and translatable mRNA induced and increased by salt stress have been reported (Hurkman *et al.*, 1989; Bray, 1993; Swire-Clark and Marcotte, 1999; Xu *et al.*, 2001). Reports on salt stress effects on proteins in mangroves are scant (Sugihara *et al.*, 2000). In the work presented here, we have investigated the changes in protein level and protease activity of the non-secretor mangrove *B. parviflora* under NaCl stress and identified certain polypeptides, which decrease in the stressed plants. This is the first report on SDS-PAGE analysis of proteins from the leaves of salinized and desalinized *Bruguiera* plants showing considerable quantitative and qualitative changes.

Materials and Methods

Plant materials and culture conditions

Propagules of *Bruguiera parviflora* (Roxb.) Wt. & Arn. ex Griff. were collected from Bhitarkanika mangrove forest of Orissa, India (latitude 20°4' to 20°8' N and longitude 86°45' to 87°50' E). Seedlings were raised in the greenhouse with non-saline and non-brackish water under photosynthetic active radiance (PAR) of 1220–1236 $\mu\text{mole} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Two months old healthy seedlings were selected for hydroponic culture in Hoagland's nutrient medium, pH 6.0 (Hoagland and Arnon, 1940) and salinized with four levels of NaCl (0, 100, 200 and 400 mM). The cultures were aerated continuously with an air bubbler. The hydroponic cultures were maintained in a culture room under a 14 h photoperiod at light intensities of 300 $\mu\text{mole} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with 22 ± 2 °C room temperature and 80% relative humidity (RH). The culture medium was changed with fresh medium periodically (once in 7 d). The fourth pair of leaves from the top of the shoot was harvested at 0, 7, 14, 21, 30 and 45 d intervals from different treatments besides the control (without NaCl) for extraction and estimation of protein and measurement of protease activity.

Extraction and estimation of total leaf protein

Total leaf protein was extracted by the acetone-TCA (trichloroacetic acid) precipitation method of Damerval *et al.* (1986). Leaf tissues (0.5 g) were homogenized in 10% ice-cold TCA using a pre-chilled mortar and pestle and incubated overnight at 4 °C. The homogenates were centrifuged at $10,000 \times g$ for 10 min. The pellet was washed with 100% acetone in order to remove the pigments. The pigment free pellet was successively washed with 80% ethanol, ethanol/chloroform (3:1 v/v), ethanol/diethyl ether (3:1 v/v) and finally with diethyl ether to remove phenolic compounds. The washed pellet was suspended in a known volume of 0.1 N NaOH for estimation of protein following the method of Lowry *et al.* (1951). Proteins in the unknown samples were estimated at 750 nm using de-fatted bovine serum albumin (fraction V) as standard and expressed per g dry weight basis.

Analysis of protein profile of leaf by SDS-PAGE

Leaf samples were harvested from control and NaCl treated plants after 7, 14, 30 and 45 d of treatment for analysis of soluble protein by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Samples (0.5 g) were homogenized with 2 ml of a buffer containing 50 mM Tris(hydroxymethyl) aminomethane (Tris)-Glycine (pH 8.3), 0.5 M sucrose, 50 mM EDTA, 0.1 M KCl, 2 mM PMSF and 0.1% (v/v) 2-mercaptoethanol in a chilled pestle and mortar at 4 °C. The homogenate was centrifuged in a refrigerated centrifuge (Sigma, 2K15, Germany) at $14,000 \times g$ for 10 min. Protein concentration in the supernatant samples was estimated according to the method of Bradford (1976). The supernatants were stored in small aliquots at -85 °C for SDS-PAGE.

Supernatant samples (40 μg protein) were mixed with equal volumes of solubilizing buffer [62.5 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95 °C, cooled on ice before loading on 12.5% polyacrylamide slab gels.

Gels were made according to Laemmli (1970). A 12.5% separating gel containing 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and $0.4 \mu\text{l} \cdot \text{ml}^{-1}$ TEMED was used for resolving the polypeptides whereas a 4% stacking gel containing 125 mM Tris-HCl, pH 6.8,

0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and $0.5 \mu\text{l} \cdot \text{ml}^{-1}$ TEMED was used to concentrate (stack) the polypeptides. The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3. Electrophoresis was accomplished at 35 mA for 4 h using a Bio-Rad, Protein II xi electrophoresis system.

The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h and destained with 50% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. The gels were photographed and scanned using a densitometer (GS-710, Bio-Rad, USA) and analyzed with Quantity one software from Bio-Rad.

The protein molecular weight marker from Bangalore Genei, India was used. The standard proteins were as follows: phosphorylase b (97.4 kDa); bovine serum albumin (66.0 kDa); ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa); soybean trypsin inhibitor (20.1 kDa); lysozyme (14.3 kDa).

Activities of acid and alkaline protease assay

The activity of acid protease was measured following the procedure of Waters *et al.* (1982). The enzyme was extracted by homogenizing 0.5 g leaf tissue in 5 ml of 50 mM sodium acetate buffer, pH 4.0, using a chilled pestle and mortar at 4 °C. The homogenate was centrifuged at $20,000 \times g$ for 10 min at 4 °C and the supernatant was used as source of enzyme. Enzyme was assayed by pre-incubating 0.2 ml of enzyme extract and 0.8 ml of buffer at 50 °C for 10 min. The reaction was started by adding 1 ml of 1% (w/v) hemoglobin solution in 0.075 N HCl. After 2 h of incubation, the reaction was terminated by adding 2 ml of 10% (w/v) TCA. The blank was prepared similarly by first inactivating the enzyme by adding TCA. The reaction mixture was centrifuged at $10,000 \times g$ for 10 min. The amount of tyrosine (μg) liberated by the activity of enzyme in the supernatant and the protein content in the enzyme extract were estimated following the method of Lowry *et al.* (1951).

The activity of alkaline protease was measured following the procedure of Anwar and Saleemuddin (2000) with a slight modification. The enzyme was extracted by homogenizing 0.5 g leaf tissue in 5 ml of 50 mM sodium phosphate buffer, pH 8.0, using a chilled pestle and mortar at 4 °C. The ho-

mogenate was centrifuged at $20,000 \times g$ for 10 min at 4 °C and the supernatant was used as source of enzyme. The enzyme was assayed by pre-incubating 0.2 ml of enzyme extract and 0.8 ml of buffer at 50 °C for 10 min. The reaction was started by adding 1 ml of 1% (w/v) casein substrate solution prepared in 50 mM sodium phosphate buffer, pH 8.0. After 2 h of incubation, the reaction was terminated by adding 2 ml of 10% (w/v) TCA. The blank was prepared similarly by first inactivating the enzyme by adding TCA. The reaction mixture was centrifuged at $10,000 \times g$ for 10 min. The amount of tyrosine (μg) liberated by the activity of enzyme in the supernatant and the protein content in the enzyme extract were estimated following the method of Lowry *et al.* (1951).

Specific activity of acid protease and alkaline protease was expressed as μmoles of tyrosine liberated per hour per mg protein. One unit of enzyme activity is defined as the amount of enzyme necessary to liberate $1 \mu\text{mole}$ of tyrosine per hour.

Results

Changes in protein content

The total soluble protein content of leaves decreased in *B. parviflora* as a result of NaCl treatment, but without any noticeable changes in untreated controls (Fig. 1). The maximum decrease was noticed in 400 mM NaCl treatment in which protein content decreased from $228.14 \text{ mg} \cdot \text{g}^{-1}$ dry wt to $107.74 \text{ mg} \cdot \text{g}^{-1}$ dry wt with respect to control after 45 d of treatment. The salt induced decline in soluble protein contents continued up to 14 d of treatment and then remained stable (Fig. 1).

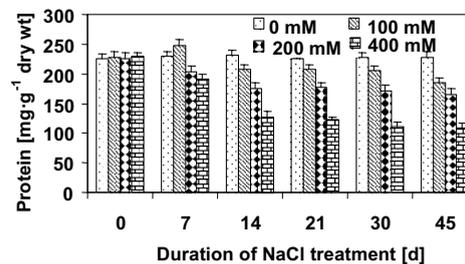


Fig. 1. Effects of NaCl (0 to 400 mM) on total protein levels in leaves of *B. parviflora* measured as a function of the days of NaCl treatment. The values are mean \pm S. E.

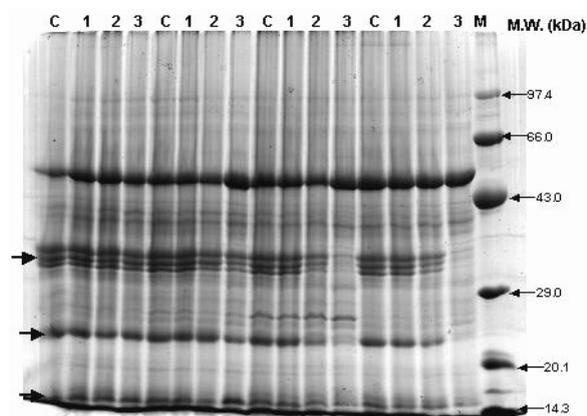


Fig. 2. Effects of NaCl (0 to 400 mM) on polypeptide patterns of total protein from leaf as analyzed on SDS-PAGE. Lanes C, 1, 2 and 3 from left to right represent proteins extracted from control, 100, 200 and 400 mM NaCl treated plants after 7, 14, 30 and 45 d of treatment, respectively, and lane M represents the molecular weight marker. The bold arrows on the left indicate the polypeptides, which showed major changes upon salt stress.

Changes in polypeptide pattern of leaf under salinization and desalinization

Total proteins were extracted from leaves of control and NaCl treated plants after 7, 14, 30 and 45 d of treatment and analysed by SDS-PAGE. As visualized from SDS-PAGE intensity of several protein bands of molecular weight 17, 23, 32, 33 and 34 kDa decreased as a result of NaCl treatment (Fig. 2). The degree of decrease of these protein bands seemed to be roughly proportional to the external NaCl concentration (Table I). The dependence of the dissociation of the protein on the length of exposure of plants to salt is shown in Fig. 2. Plants were transferred to a medium containing 100 to 400 mM NaCl and incubated for 45 d. At the end of 7, 14, 30 and 45 d of incubation period, the protein extracted from leaf was analyzed by SDS-PAGE and Coomassie blue staining

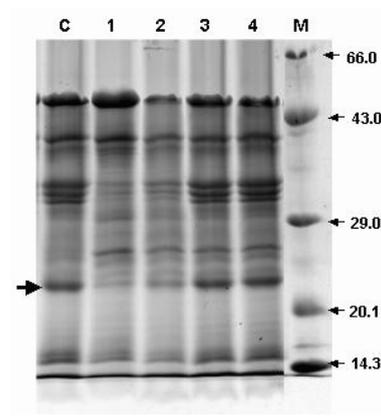


Fig. 3. Changes in protein profile in leaves of *B. parviflora* after 7, 14 and 30 d recovery from salt stress. Lanes C and 1 represent proteins extracted from control and 400 mM NaCl treated (45 d) leaves. Lanes 2, 3 and 4 represent proteins extracted from leaves after 7, 14 and 30 d of desalinization, respectively. Lane M represents the molecular weight marker. The bold arrow on the left indicates the 23 kDa polypeptide, which reappears after desalinization.

(Fig. 2). Analysis of the gel has shown that the decrease in the amount of protein band is days dependent. When plants were grown on 400 mM NaCl decrease of these protein bands was detectable after 2 weeks and more prominent decrease was noticed after 45 d of treatment. After 45 d in 400 mM NaCl, the protein band of molecular weight 23 kDa almost disappeared (Fig. 2). Appearance and disappearance of these polypeptides are reversible during salinization and desalinization in *B. parviflora*. When the plants treated with 400 mM NaCl for 45 d are transferred to 0 mM NaCl and kept at this concentration for 45 d, the 23 kDa-polypeptide which disappeared due to NaCl treatment appeared again (Fig. 3). The degree of reappearance of this polypeptide is also days dependent (Fig. 3).

NaCl [mM]	Average density					
	49 kDa	34 kDa	33 kDa	32 kDa	23 kDa	17 kDa
0	0.93	0.75	0.72	0.71	0.62	0.42
100	0.92	0.70	0.68	0.65	0.53	0.38
200	0.89	0.50	0.42	0.40	0.38	0.24
400	0.87	0.42	0.22	0.20	0.16	0.19

Table I. Changes in average density of major bands by NaCl stress after 45 d of treatment as analyzed from the densitometric scanning of the gel.

Activities of acid protease and alkaline protease

Since the acclimation of *B. parviflora* to high salt concentration involve not only loss of pigments (Parida *et al.*, 2002), but also appreciable reduction in the total leaf protein content. Therefore, it appears that the adaptation to high salt may involve hydrolysis of some proteins. We monitored proteolytic activity of leaf extracts obtained from control and salt treated plants after 7, 14, 21, 30 and 45 d of salt incubation. In Fig. 4 (A & B), it is evident that there is no significant change in acid and alkaline protease activity in control sets, the initial protease activity and activity of proteases after 45 d were nearly same. However, salt exposure to *B. parviflora* roots caused increase in proteolytic activity (both acid and alkaline protease). The extent of increase after 45 d of incubation in 400 mM NaCl was much higher for acid protease (ACP) than for alkaline protease (ALP). The increased activities of proteases are accompanied by a concomitant decrease in protein content (Fig. 1).

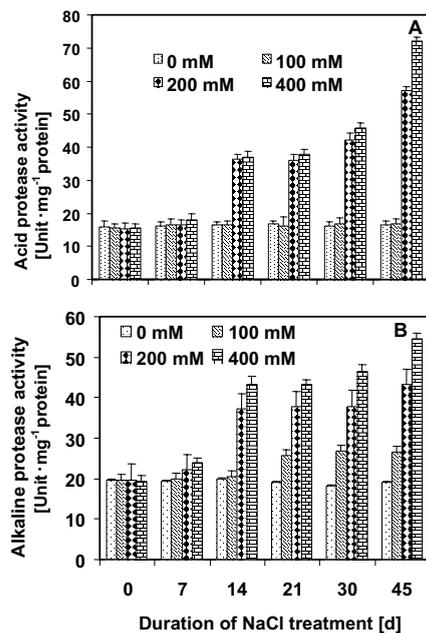


Fig. 4. Effects of NaCl (0 to 400 mM) on (A) acid protease and (B) alkaline protease activities in leaves of *B. parviflora* measured as a function of the days of NaCl treatment. The values are mean \pm S. E.

Discussion

The total protein content of leaf gradually decreased with increasing concentration of NaCl. This decrease in protein content might be due to the increasing activity of acid and alkaline proteases. As reported earlier, levels of free amino acid increase as a result of salt stress in *B. parviflora* (Parida *et al.*, 2002). Thus, the NaCl treatments on *B. parviflora* at high salt concentration showed an increase in total amino acid pool by decreasing protein content, which reflects the mode of adjustment to salinity stress. Our results showed that NaCl stress causes increased activities of both acid and alkaline proteases, decrease in protein content and increase in free amino acids content in *B. parviflora*. Our results agree with Kennedy and De Fillippis (1999) who reported increased activities of both acid and neutral proteases in *Grevillea ilicifolia* and *G. arenaria* under NaCl stress. Muthukumarasamy *et al.* (2000) also reported an increased protease activity in radish under NaCl stress. These results suggest that decreased protein content in *B. parviflora* might be due to increased activity of both acid and alkaline protease. Proteins are hydrolysed by proteases to release amino acids for storage and/or transport and for osmotic adjustment during NaCl stress in *B. parviflora*. Osmotic adjustment, protection of cellular macromolecules, storage form of nitrogen, maintaining cellular pH, detoxification of the cells, and scavenging of free radicals are proposed functions of free amino acid accumulation.

In *B. parviflora*, the intensity of polypeptides of molecular weight 17, 23, 32, 33 and 34 kDa decreased as a result of salt stress. The most prominent decrease is attributed to a 23 kDa-polypeptide (SSP-23), which disappeared after 45 d treatment in 400 mM NaCl. Moreover, the 23 kDa-polypeptide, which disappeared in *B. parviflora* under salinity stress, reappeared when these salinized seedlings were desalinized. These changes in polypeptides may be due to adaptation of the plant to NaCl. Our results tent to agree with Unni and Rao (2001) who reported that certain outer membrane proteins of molecular weight 22, 38, 40, 42, 62 and 68 kDa markedly decrease in the presence of salt in *Rhizobium*. However, in *Bruguiera gymnorhiza*, the intensity of a 33 kDa-protein with pI 5.2 increased as a result of NaCl treatment (Sugihara *et al.*, 2000). SDS-PAGE analysis of proteins in peanut (*Arachis hypogaea* L.) reveals that plants grown under NaCl show induction (127 and

52 kDa) or repression (260 and 38 kDa) in the synthesis of few polypeptides (Hassanein, 1999). Rangan and Swaminathan (2002) have reported that out of 58 spots detected by 2-D PAGE in *Potresia coarctata*, 35 are affected by salt stress; of these, 26 are increased, and 17 are present only in stressed plants. Our results contrasts with earlier reports of increased accumulation of a 22 kDa-protein, pI of 7.5 by salt stress in radish leaves (Lopez *et al.*, 1994). Yen *et al.* (1997) have reported the accumulation of five polypeptides with estimated molecular masses of 40, 34, 32, 29 and 14 kDa by SDS and 2D-PAGE in callus of *Mesembryanthemum crystallinum* under NaCl stress and these polypeptides have been classified into two distinct groups according to their course of induction: early responsive (40, 34, 29 kDa) and late-responsive (32, 14 kDa) proteins. The disappearance of a 60 kDa-polypeptide in response to salinity is observed in *Prosopsis* (Munoz *et al.*, 1997). NaCl induces accu-

mulation of four polypeptides with molecular mass of 61, 51, 39 and 29 kDa in maize roots (Tamas *et al.*, 2001). The SSP-23 protein of *B. parviflora* is cross-reacted with only anti-SSP-23 antibody, which was confirmed by Western blotting. Further molecular characterization of the SSP-23 protein by N-terminal sequencing and tissue specific localization by immuno gold is now in progress in our laboratory using SSP-23 antibody probe. This will predict us the function of the SSP-23 protein, which can serve as a marker to the degree of stress imposed on the plant.

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