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ORIGINAL PAPER

Purification and characterization of a salinity induced alkaline protease from isolated spinach chloroplasts

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Abstract In cynobacteria and higher plants, salinity is known to inhibit the activity of several enzymes involved in photosynthesis and hence decreases the overall photosynthetic rate. This gave us an impetus to search for a protease, which may be involved in the turnover of non-functional enzymes produced under salinity stress. Taking the possible changes in pH gradient of the chloroplast under consideration, we have tried to identify a protease, which is induced under salinity and characterized it as an alkaline protease using spinach (*Spinacia oleracea*) leaves as a model system. The HIC-HPLC purified homogeneous alkaline serine protease from the isolated spinach chloroplasts had two subunits of molecular weight 63 and 32 kDa. The enzyme was maximally active at pH 8.5 and 50°C. The enzyme showed the property to hydrolyze the synthetic substrate like azocaesin and had sufficient proteolytic activity in gelatin bound native PAGE. The enzyme activity was also dependent upon the presence of divalent cations and reduced environment. The active site residues were identified and the homogeneous alkaline serine protease had cysteine, lysine and tryptophan residues at its active site.

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Keywords *Spinacia oleracea* · Enzyme characterization · Serine protease · Salt stress · Thioisoindole · OPA

Abbreviations

β-ME	β-Mercaptoethanol
Chl	Chlorophyll
DMHNB	Dimethyl-(2-hydroxy 5-nitro-benzyl) sulphonium bromide
E-64	*trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)-butane
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetra acetic acid
EDTA	Ethylene diamine tetra acetic acid
OPA	Ortho-pthalaldehyde
HEPES	*N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulphonic acid
LBTI	Lima Bean trypsin inhibitor
MOPS	2-(*N*-Morpholino) L propanesulphonic acid
PMSF	Phenylmethylsulphonyl fluoride
RT	Room temperature (25 ± 1°C)
STI	Soybean trypsin inhibitor
TLCK	L-1-Chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride
TPCK	L-1-Chloro-3-(4-tosylamido)-4-phenyl-2-butanone
U_{ENZ}	Unit of enzyme activity

Introduction

In agriculture related to crop productivity, salinity is treated as detrimental to plant growth as it adversely affects its metabolism and leads to the accumulation of

non-functional proteins (Allakhverdiev et al. 2000; Suleyman et al. 2002). In this regard, proteases could play an important role in the removal of such proteins (Andersson and Aro 1997). Besides this, recent studies also show that proteases have a significant role in the cellular regulation which is achieved by maintaining the correct level of regulatory proteins (Adam 2000; Mark 2001).

In order to study these proteases in plant cell, chloroplast is an important organellar system. It has a dynamic environment inside where proteases are involved in the processing of precursor proteins, degradation of the incomplete proteins lacking co-factors and the removal of damaged proteins (Adam 1996, 2000). This ability of the chloroplast to degrade proteins has made it a source of the several proteases, some of which have already been isolated and well characterized (Viestra 1996; Kuwabara and Hashimoto 1990). These proteases are found to be localized within the different compartments such as stroma and thylakoid lumen (Schmidt and Mishldnd 1983; Nair and Ramaswamy 2004). All the identified and cloned chloroplast proteases such as Clp, Deg and FtsH are homologues of the bacterial proteases, best characterized in *E. coli* (Lindahl et al. 1996; Shanklin et al. 1995). The presence of the bacterial homologue gives further evidence that chloroplast proteases are the key factor of cellular regulation as in bacteria, their involvement as a regulator is well known (Oren et al. 1996). This fact is also supported from the previous studies. For instance, a chloroplast targeted protease (FtsH6) has been shown to be involved in the degradation of light harvesting complex of photosystem II during the high light acclimation and senescence in *Arabidopsis thaliana* (Zelisko et al. 2005). Similarly, thylakoid lumen protease (Deg1) is found to be involved in the repair of photosystem II from photoinhibition (Kapri-Pardes et al. 2007).

In the present work, we have made an attempt to purify the protease from the isolated chloroplasts that is activated under the direct salt stress in senescing spinach leaves. Spinach is used as a model system because of the ease in promoting the natural senescence of the detached leaves. In our studies we have shown that in response to salinity, the level of protease activity in chloroplasts increased 2–3 times as compared to control. Purification of these proteolytic enzymes is needed to assess their involvement both in the general process of leaf protein degradation, specific proteolysis of the photosynthetic components and to understand their role both in the process and regulation of networks involved in senescence.

Materials and methods

Plant material and experimental treatments

Spinacia oleracea leaves were washed thoroughly and kept in Petri plates as two separate sets. One set of leaves (control) was overlaid with distilled water so as to just submerge them. The second set of leaves (salinity stressed) was overlaid with 1 M NaCl solution in the same way. The Petri plates were incubated in a microprocessor-controlled environmental test chamber on a 12 h light–dark cycle at 25°C and 70 ± 5% humidity. The day on which the sets were incubated was taken as day 1 of treatment. Both the sets were allowed to senesce naturally for 96 h.

Standardization of the conditions for maximal activation of alkaline protease during senescence

The enzyme activity was assayed over different time periods of NaCl treatment starting at 0–120 h at an interval of 24 h. At the optimum time, the enzyme activity was assayed using a range of NaCl concentrations from 0 (control), 0.177, 0.5, 0.75 to 1 M. The optimum NaCl treatment was then given at different temperatures (4, 15, 25 and 37 ± 1°C) and the enzyme activity was determined at the end of 96 h. The NaCl-treated samples were also incubated either in the presence of continuous light illumination of 500 μmol photons m^{-2} s^{-1} or in the dark and enzyme activity was determined at the end of 96 h.

Enzyme purification

Purification of the alkaline protease was carried out by alcohol precipitation of chloroplasts, their re-extraction in phosphate buffer followed by ammonium sulphate fractionation and hydrophobic-interaction chromatography (HIC). All operations were performed at 0–4°C, unless otherwise stated.

Chloroplast isolation

Chloroplasts were isolated from spinach leaves according to the method of Izawa and Good 1968 and finally suspended in 1 ml of suspension medium [MOPS-NaOH (20 mM; pH 7.2) and NaCl (20 mM)]. Isolated chloroplasts were purified on 10–80% linear percoll density-gradient following the method described by Mullet and Chua 1983. The chloroplast suspension was layered onto a pre-cooled percoll density-gradient and centrifuged at 8,700*g* for 6 min at 4°C. The purified layer of chloroplast was carefully removed and the percoll was diluted with 5–10 volumes of

ice-cold dilution buffer [HEPES—NaOH (50 mM; pH 7.5), sorbitol (330 mM), EDTA (2 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM) and ascorbate (5 mM; freshly added)]. The diluted chloroplast suspension was centrifuged at 4,400*g* for 10 min at 4°C and pellet was suspended in PS II preparation medium [HEPES-NaOH (20 mM; pH 7.5), sucrose (400 mM), NaCl (15 mM), $MgCl_2$ (5 mM) and BSA (1 mg ml^{-1})]. The chl content in the chloroplast preparation was determined in 80% (v/v) acetone filtrates spectrophotometrically (Ramaswamy 1979).

Preparation of crude enzyme extract

Alcohol precipitation was carried out using a modification of the method described by Nair and Ramaswamy 1991. Chilled ethanol (90 ml) was added to 10 ml of chloroplast suspension and the protein was allowed to precipitate. This was followed by centrifugation at 27,000*g* for 30 min. The supernatant was discarded and precipitate was once again washed with chilled ethanol and re-centrifuged at the same speed. The precipitate was air dried for 48–72 h and the dried alcohol precipitate was re-extracted in extraction buffer containing 50 mM phosphate buffer (pH 7.4) and 200 mM NaCl by slow stirring for 30 min. It was then centrifuged at 27,000*g* for 30 min. The supernatant was collected and used as a crude enzyme for further purification.

Purification of alkaline protease

The crude enzyme was fractionated using ammonium sulphate on a weight to volume basis according to the modification of a method described by Nair and Ramaswamy (1991). To 10 ml of the crude enzyme, 1.76 g finely powdered ammonium sulphate was slowly added with constant stirring for 30 min at low speed and later centrifuged at 27,000*g* for 30 min. The precipitate was dissolved in 1 ml of 50 mM phosphate buffer (pH 7.4) and designated as the fraction I (0–30%). The supernatant containing 30% ammonium sulphate was further fractionated and fractions II (30–60%) and III (60–100%) were collected according to the fractionation on a weight to volume basis of 100% ammonium sulphate saturation scheme. All the fractions and the final supernatant arising from fraction III were assayed for alkaline protease activity.

The 0–30% ammonium sulphate fraction-I was purified on a HIC PH-814 column in a Waters HPLC System (Waters Corp., USA). Gradient of buffer-A [phosphate buffer (50 mM; pH 8.0) and ammonium sulphate (10%)] and buffer B [phosphate buffer (50 mM; pH 8.0)] was used as per the following gradient scheme: 0–5 min, 100% buffer A; 6–15 min, 75% buffer A + 25% buffer B; 16–30 min, 50% buffer A + 50% buffer B; 31–35 min, 25% buffer A + 75% buffer B and 36–60 min, 100% buffer B. The gradient was pumped at a flow rate of 1 ml min^{-1}. The protein sample (100 µl) was injected with the help of Waters 717plus autosampler and monitored from injection time (0 min) till 65 min through a Waters 996 photodiode array detector over 275–285 nm range. The entire operation was controlled by Waters Millenium 32 software. One millilitre fractions from the HIC column were collected through a Pharmacia Frac-100 fraction collector and assayed for the alkaline protease activity. The active fraction was concentrated and 8 µg protein was run on both SDS and native gel to know their protein profile. Protein concentration of all the samples was determined (Bradford 1976).

Gelatin zymography

Gelatin zymography of control and NaCl samples were performed (Sarath et al. 1989) by using purified protein samples. Native PAGE was performed at 4°C on a 12% separating gel. The 12% polyacrylamide separating gel was impregnated with gelatin (5 mg ml^{-1}) as the protein substrate. After the run, gels were rinsed thoroughly with distilled water and incubated in 100 mM borate buffer (pH 8.4) at 50°C for 3 h. Following incubation, the gels were fixed with a solution (v/v/v) of 50% methanol, 10% glacial acetic acid and 5% glycerol for 1 h and later stained using coomasie brilliant blue. Clear marked zone against the blue background was observed as the in situ enzyme activity.

Alkaline protease assay

Alkaline protease activity was assayed by using a modification of azocasein digestion assay (Casano et al. 1989). The reaction mixture of 1 ml contained 5 mg ml^{-1} azocasein, 100 mM borate buffer (pH 8.4) and 100 µg of purified enzyme. After incubation for 3 h at 50°C, the reaction was terminated by the addition of 1 ml of ice-cold 10% (w/v) TCA and the tubes were chilled on ice for 30 min. The reaction mix was filtered and absorbance of the filtrate was read at 340 nm. One unit of alkaline protease activity (U_{ALK}) was defined as an increase of 0.01 U in absorbance mg $protein^{-1}$ h^{-1} at 340 nm.

Characterization of the purified enzyme

Optimum pH of the alkaline protease

The optimum pH of the enzyme reaction was determined using buffers of different pH. The buffers used were acetate (pH 4.6 and 5.6), phosphate (pH 6.4 and 7.4), borate (pH 8.4) and glycine-NaOH (pH 9.4 and 10.4).

Temperature optimum of the alkaline protease

The optimum temperature of the enzyme reaction was determined by incubating the reaction mixture at different temperatures: 10, 23, 37, 50 and 60°C.

Substrate specificity

The specificity of the enzyme towards different dye-labelled substrates such as azocasein, azoalbumin, azocollagen and elastin-orcein and non-labelled substrates such as casein, BSA, gelatin and γ-globulin was assayed. The only difference was that for azocollagen and elastin–orcein, the absorbance of the TCA-filtrate was read spectrophotometrically at 520 nm (Sherekar et al. 1997) and 590 nm (Thakore and Harikumar 1993), respectively, while in the case of other non-labelled substrates, the TCA filtrate was used for the determination of acid-soluble amino acids by modified Folin–Lowry method (Miller 1959).

Inhibitor studies

The mechanistic class to which the protease belongs was determined by inhibitor studies with various types of protease inhibitors (Rawlings and Barrett 1994). In these studies, the substrate was added after the enzyme was pre-incubated with the inhibitor at 25 ± 1°C for 30 min. The inhibitors used for the assay were 100 μM pepstatin (in methanol), 100 μM E-64 (in DMSO), 2 mM PMSF (in ethanol; prepared fresh), 1 mM EDTA, 1 mM EGTA, 100 μg ml^{-1} STI, 100 μM LBTI, 100 μM TLCK, 100 μM TPCK, 10 mM benzamidine-HCl and 500 μM 1,10-phenanthroline (in ethanol). Appropriate carrier blanks were kept to account for inhibition due to the solvents used for dissolving the inhibitors. The reaction mixture without the inhibitor served as a control.

Metal ion susceptibility

Different metals were used to study the effect of metallic ions on the activity of the enzyme. Here, the substrate was added after the enzyme was pre-incubated with the metallic salt solution (1 mM) at 25 ± 1°C for 30 min. The metallic salt solutions used were $CaCl_2.2H_2O$ (Ca^{2+}); $MgCl_2.6H_2O$ (Mg^{2+}); $MnCl_2.4H_2O$ (Mn^{2+}); $ZnCl_2$ (Zn^{2+}); HgCl2 (Hg^{2+}); $PbCl_2$ (Pb^{2+}) and AgCl (Ag^{+}).

Effect of amino acid modifiers on the enzyme activity

Chemical modifiers such as DMHNB, pyridoxal phosphate and iodoacetate specific for tryptophan, lysine and tyrosine, respectively, were used. To see their effect, the enzyme was pre-incubated with the modifiers for 30 min at 25 ± 1°C and enzyme activity was measured.

Effect of the oxidized/reduced environment on the enzyme activity

$Glutathione_{(ox)}$ and $glutathione_{(red)}$ were used to determine the nature of the active site. The enzymes were incubated with $glutathione_{(ox)}$ and $glutathione_{(red)}$ for 30 min at 25 ± 1°C and then enzyme activity was measured.

Determination of the active site residues

OPA has been used as a fluorescent probe for the determination of active site (Janave et al. 1999). First, the disulfide bond of the native protein were reduced by the treatment with β-ME (200 mM) under the N_2 atmosphere at 0–4°C for 2 h. Excess of the β-ME was removed by passing the reduced protein through sephadex G-25 column equilibrated with HEPES buffer [HEPES (10 mM; pH 7.5) and NaCl (20 mM)]. The reduced protein fractions collected from the column were concentrated and used for preparing the thioisoindole derivative of the protein. The reduced enzymes were allowed to react with 0.2 M OPA at 2–4°C under N_2 atmosphere to form the thioisoindole derivative. The unreacted OPA was separated by gel filtration on a sephadex G-25 column and the fraction containing the thioisoindole derivative were used to study the fluorescence properties. The 100 μg ml^{-1} of various protein samples (native, reduced and OPA adduct) in the HEPES buffer were subjected for the analysis of fluorescence spectra.

Results

Studies on the activation of the alkaline protease

The time course studies showed that initially up to 48 h, the alkaline protease activity was at the basal level in chloroplast of control as well as salt stressed leaves. Thereafter it increased till 96 h and then decreased at 120 h. However, the activity from chloroplast of salt-stressed spinach leaves was higher than that of control at each time interval. The maximum activity was obtained at 96 h, where it was 6.56-fold higher as compared to control (Fig. 1). The effect of different NaCl concentrations was studied on the activation of alkaline protease and 1 M NaCl treatment was found to increase the activity by 6.37-fold as compared to control (Fig. 2a). Temperature had a positive effect on the activation of alkaline protease. As the growth temperature increased from 4 to 37°C, the enzyme activity increased to 17.81 times (Fig. 2b). Presence of light also

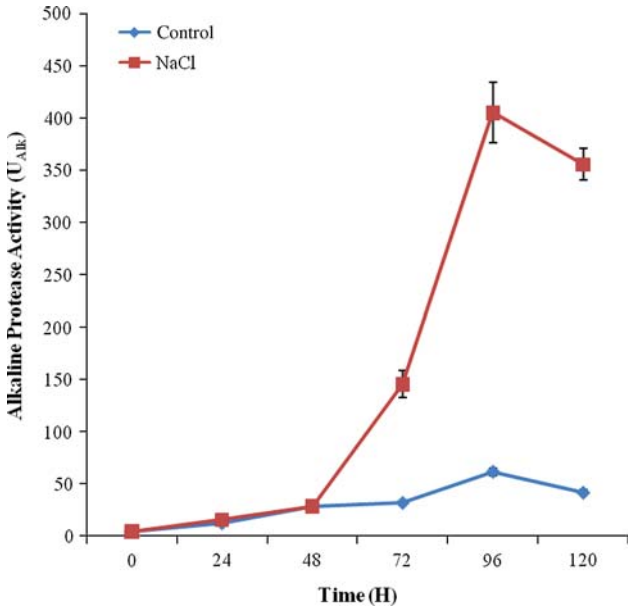


Fig. 1 Time course study of the alkaline protease activity isolated from spinach chloroplasts. Graph represents the temporal regulation of the alkaline protease activity in the 1 M NaCl-stressed leaves as compared to control. Values presented are mean ± SE of three independent sets of experiments

activated the enzyme by 15.82-fold as compared to that of dark incubated samples (Fig. 2c).

Purification of the alkaline protease

The final yield of the protease after purification was found to be 17.53%. The specific activity increased to 3.48- and 16-fold after ammonium sulphate fractionation and HIC-HPLC purification, respectively (Table 1). In HPLC when the gradient of buffer A and buffer B was used with the flow rate of 1 ml min^{-1}, the retention time for alkaline protease was found to be 8 min (Fig. 3). The SDS-PAGE analysis of the purified protein showed two bands at 63 and 32 kDa position while the native gel showed a single band of 95 kDa (Fig. 4).

Gelatin zymography

Gelatin zymography of purified alkaline protease obtained from control and salt-stressed spinach leaves showed clear zones of proteolytic activity against a blue background indicating the presence of alkaline-type gelatin degrading protease activity. The zone of proteolytic activity from salt-stressed chloroplast was larger as compared to control chloroplasts (Fig. 5).

Properties of the alkaline protease

pH optimum of the alkaline protease

The alkaline protease was active over a broad range of pH from 4.4 to 10.4. But the optimal activity was seen at pH 8.4 (Fig. 6a).

Temperature optimum of the alkaline protease

Alkaline protease was found to be active over a range of temperatures starting from 10°C onwards. The optimum temperature was found to be 50°C. The enzyme activity was found to increase with temperature. Even at 60°C the enzyme lost only 50% of its activity as compared to that at 50°C (Fig. 6b).

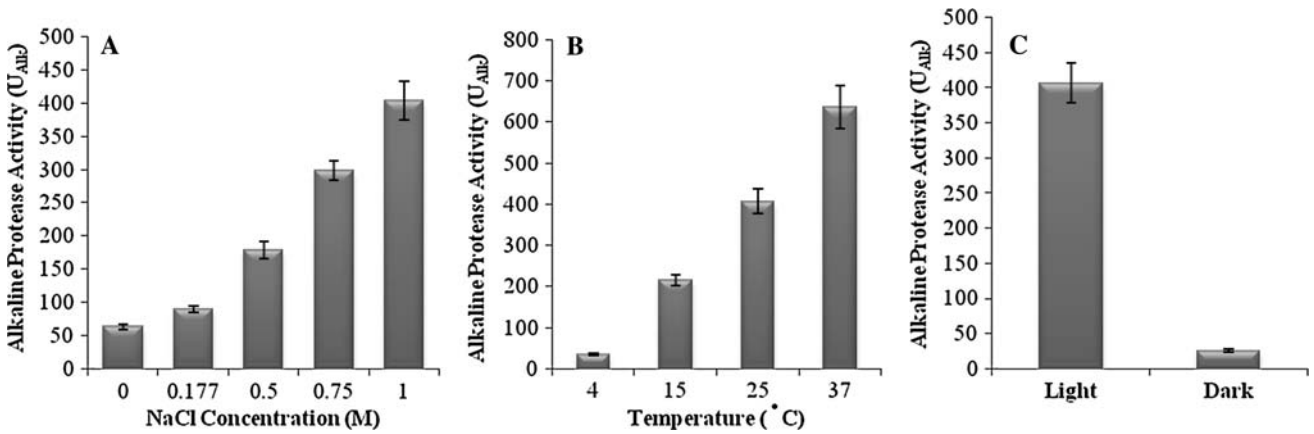


Fig. 2 Effect of various treatments on the activation of alkaline protease. Alkaline protease activity was monitored after incubating the spinach leaves with different concentrations of NaCl (**a**); different temperatures (**b**) and light and dark conditions (**c**). Values presented are mean ± SE of three independent sets of experiments

Table 1 Purification table of alkaline protease obtained from chloroplasts of salt-stressed spinach leaves

No.	Step	Activity (U_{ALK})	Protein (mg ml^{-1})	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield or recovery (%)
Designation		*A*	*P*	*V*	TA	TP	SA	FP	*Y* or *R*
Formula for calculation					$A \times V$	$P \times V$	(TA)step/(TP)crude	(SA)step/(SA)crude	(TA)step/(TA)crude
1	Crude enzyme	320.38	0.617	10	3203.80	6.17	519.25	1	100
2	0–30% Ammonium sulphate fraction (fraction I)	684.58	0.378	2	1369.16	0.756	1811.05	3.48	42.73
3	HIC-HPLC (fraction H)	561.76	0.068	1	561.76	0.068	8261.17	15.90	17.53

Alkaline protease activity was assayed by the azocasein digesting activity. One unit (U_{ALK}) was defined as an increase of 0.01 U in absorbance mg $protein^{-1}$ h^{-1} at 340 nm. Designation denotes various notations used to represent the columns. Formula for calculation shows the way to arrive at the value for each column in the sequential events of purification till the last stage of recovery of the enzyme

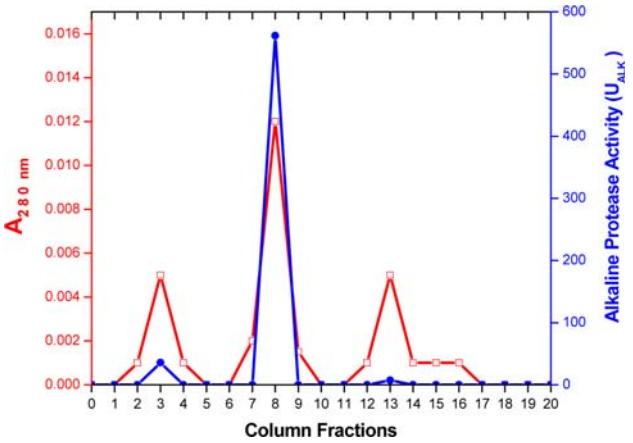


Fig. 3 Elution profile of the ammonium sulphate fraction-I from HIC-HPLC column. 100 µl of the 0–30% ammonium sulphate fraction was loaded onto HIC column and eluted at a flow rate of 1 ml min^{-1}. Absorbance (A_{280}) of the eluted column fractions were monitored spectrophotometrically and assayed for alkaline protease activity. One unit of alkaline protease activity (U_{ALK}) was defined as an increase of 0.01 U in absorbance mg $protein^{-1}$ h^{-1} at 340 nm

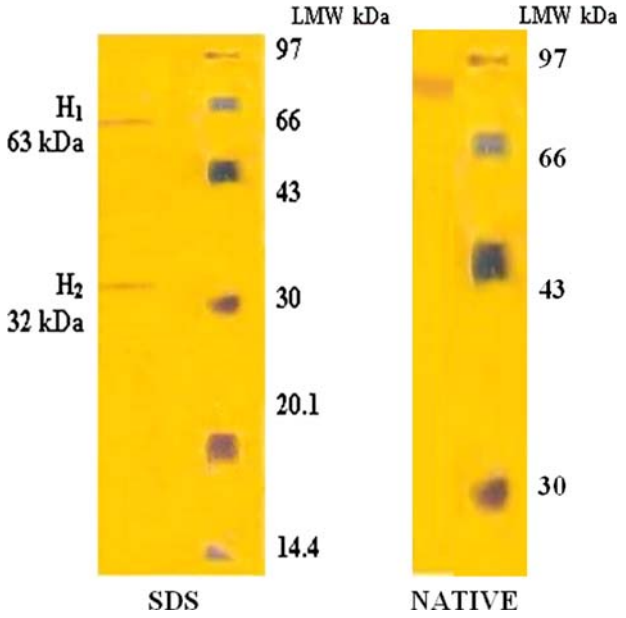
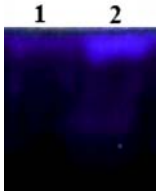


Fig. 4 PAGE profile of alkaline protease activity purified on a HIC-HPLC column. *Left and right panels* showed the electrophoresis pattern on SDS and Native gel, respectively. SDS gel showed two bands of 32 and 63 kDa (H_1 and H_2) while native gel showed only single band of 95 kDa (H_0). Electrophoresis was done with 8 µg of protein on 12% native and SDS gels. Gels were stained by silver staining method

Fig. 5 Gelatin zymography of alkaline protease. Samples were run on a gelatin (5 mg ml^{-1}) incorporated native PAGE (12%). *Lanes 1* and *2* represent the purified samples (8 µg) from control and salinity treated samples



Substrate specificity

Among the non-labelled substrates, casein was found to be most preferable substrate. Performance of other substrate was in the following order, BSA > gelatin > γ-globulin, whereas, among the dye-labelled substrates the most preferred substrate was azocasein followed by azoalbumin and azocollagen. Since no appreciable activity was found with azocollagen and elastin-orcein, bars corresponding to their activity were not shown in the graph (Fig. 7).

Inhibitor studies

PMSF was maximally effective in inhibiting the activity of alkaline protease. Its pre-incubation led to 86.03% inhibition, whereas other serine class protease inhibitors such as STI, LBTI, TLCK, TPCK and benzamidine-HCl were unable to effectively inhibit the enzyme (Table 2).

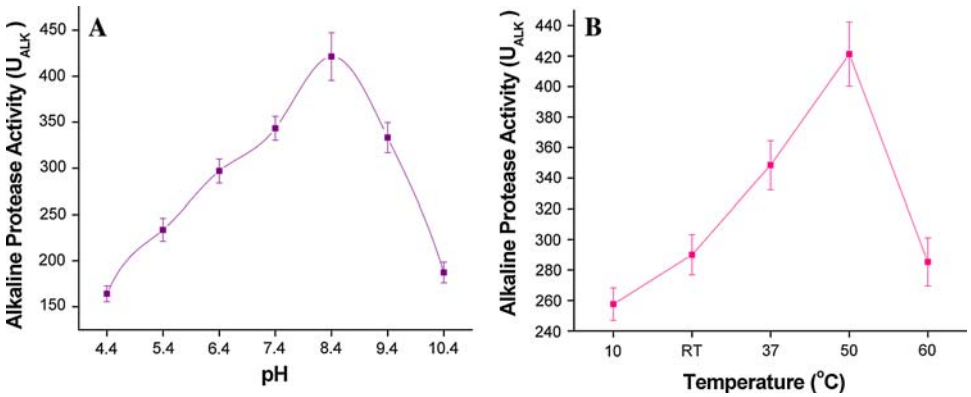


Fig. 6 pH (**a**) and temperature (**b**) profile of the alkaline protease. Alkaline protease activity was monitored under different pH and temperature conditions. Values presented are mean ± SE of three independent set of experiments

EDTA and EGTA inhibited the enzyme by 79.67 and 78.56%, respectively. Other class-specific inhibitors like pepstatin (aspartate proteases), E-64 (cysteine proteases) and 1,10-phenanthroline (metalloproteases) were actually found to activate the enzyme by 40, 44 and 15.75, respectively.

Metal ion susceptibility

Of the different metal ions tested Ca^{2+}, Mg^{2+} and Mn^{2+} were found to increase the alkaline protease activity by 22.18, 6.16 and 12.85%, respectively (Table 3). Zn^{2+} was found to strongly inhibit the enzyme activity (77.03%), while Hg^{2+}, Pb^{2+} and Ag^{1+} were also found to inhibit the alkaline protease activity by 71.84, 55.30 and 70.72%, respectively.

Table 2 Effect of different protease inhibitors on alkaline protease activity

Inhibitors	Final concentration in assay	Activity retained (%)
Control (no inhibitor)	NIL	100
Pepstatin	100 μM	140
E-64	100 μM	144
PMSF	2 mM	14.33
EDTA	1 mM	20.33
EGTA	1 mM	21.44
STI	100 μg ml^{-1}	97.93
LBTI	100 μM	92.35
TLCK	100 μM	93.33
TPCK	100 μM	95.38
Benzamidine–HCl	10 mM	94
1,10-Phenanthroline	500 μM	115.75

The enzyme was pre-incubated with the inhibitors at 25°C ± 1 for 30 min before addition of the substrate except in case of PMSF where the incubation time was 1 h. Percentage retention of activity was calculated from mean values ± SE of three independent set of experiments

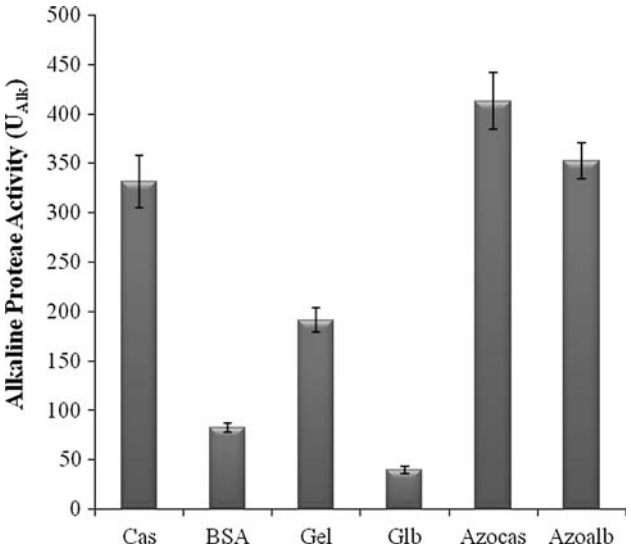


Fig. 7 Substrate specificity of the alkaline protease. Alkaline protease activity was assayed by using the different substrates such as casein (*Cas*), bovine serum albumin (*BSA*), gelatin (*Gel*); γ-globulin (*Glb*), azocasein (*Azocas*) and azoalbumin (*Azoalb*). Values presented are mean ± SE of three independent set of experiments

Susceptibity towards the chemical modifiers of the amino acids

The activity of alkaline protease was found to be sensitive towards all the chemical modifiers used. DMHNB gave complete loss of activity while pyridoxal phosphate and iodoacetate, decreased the activity by 70 and 85%, respectively as compared to control (Table 4).

Effect of the oxidized and reduced environment

The pre-treatment of enzyme with glutathione$_{(red)}$ increased its activity by 30% as compared to control while the incubation with the glutathione$_{(ox)}$ completely inhibited its activity (Table 5).

Table 3 Effect of different metal ions on alkaline protease activity

Metal ions	Activity retained (%)
Control (no metal ion)	100
Ca^{2+}	122.18
Mg^{2+}	106.16
Mn^{2+}	112.85
Zn^{2+}	22.97
Hg^{2+}	28.16
Pb^{2+}	44.70
Ag^{1+}	29.28

The enzyme was pre-incubated with 1 mM metal ions at 25°C ± 1 for 30 min before addition of the substrate. Percentage retention of activity was calculated from mean values ± SE of three independent sets of experiments

Table 4 Effect of different amino acid modifiers on alkaline protease activity

Modifier	Final concentration in assay (mM)	Activity retained (%)
Control (no inhibitor)	NIL	100
DMHNB	5	NIL
Pyridoxal phosphate	5	29.41
Iodoacetate	5	15.44

The enzyme was pre-incubated with the modifiers at 25°C ± 1 for 30 min before addition of the substrate. Percentage retention of activity was calculated from mean values ± SE of three independent set of experiments

Table 5 Effect of various activators on alkaline protease activity

Activator	Final concentration in assay (mM)	Activity retained (%)
Control (no activator)	NIL	100
$Glutathione_{(red)}$	5	130
Glutathione(ox)	5	NIL

The enzyme was pre-incubated with the activators at 25°C ± 1 for 30 min before addition of the substrate. Percentage retention of activity was calculated from mean values ± SE of three independent set of experiments

Fluorescence spectra of oxidized, reduced and OPA bound enzymes

The oxidized (native) enzyme, when excited at 240 nm, gave the emission at 300 and 336 nm (Fig. 8), which was the characteristic of the tyrosine and tryptophan (Teale 1960; Teale and Weber 1957). The reduced form of enzyme also gave similar spectra but with reduced emission at 300 and 336 nm (Fig. 9). In OPA-bound enzyme a small peak at 418 nm was observed along with the decrease in tyrosine and tryptophan emission (Fig. 10).

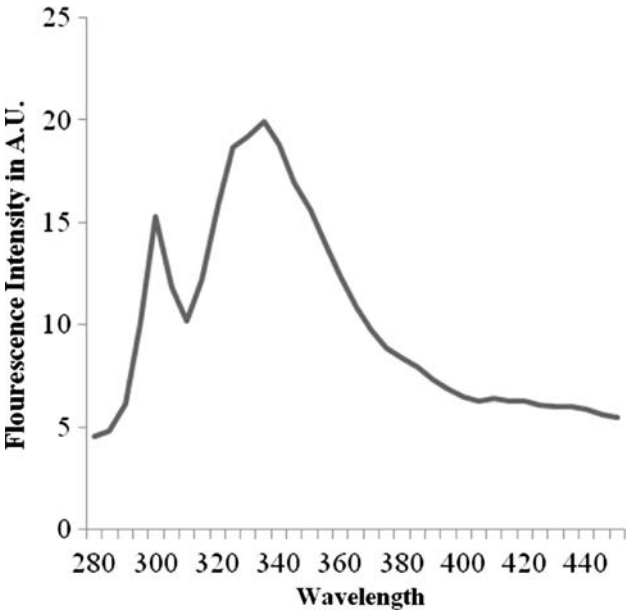


Fig. 8 Emission fluorescence of the oxidized (native) enzyme. Emission spectrum of the native enzyme was recorded upon excitation at 240 nm. The fluorescence intensity is expressed in arbitrary unit

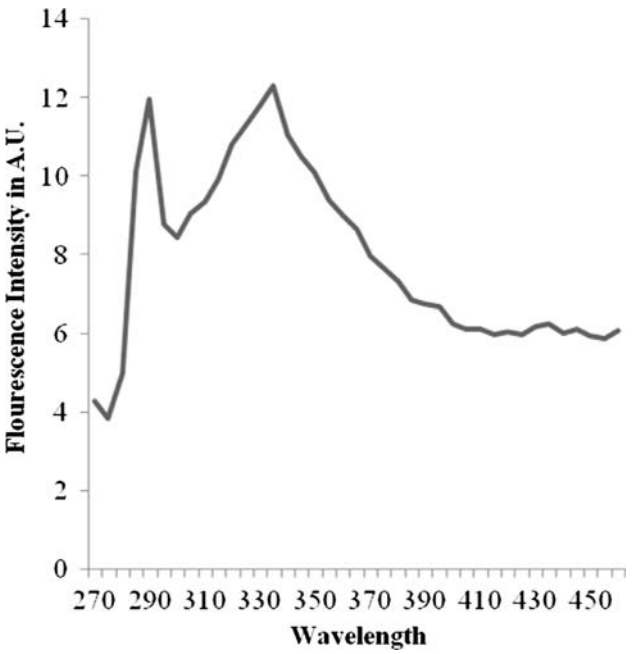


Fig. 9 Fluorescence spectra of the reduced enzyme. Emission spectrum of the reduced enzyme was recorded upon excitation at 240 nm. The fluorescence intensity is expressed in arbitrary unit

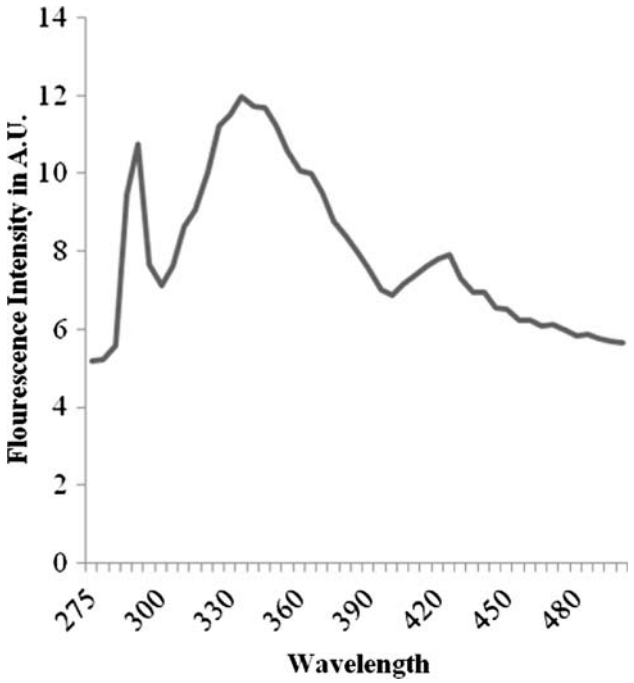


Fig. 10 Fluorescence spectra of the OPA bound enzyme. Emission spectrum of the reduced enzyme was recorded upon excitation at 240 nm. The fluorescence intensity is expressed in arbitrary unit

Discussion

In the plant cell, chloroplast proteases are always in a dynamic equilibrium and their level changes in response to various treatment conditions. Increased activity of these proteases can be explained by several ways such as activation of pre-existing enzymes, de novo synthesis and stimulation of the activity in situ. Apart from this, physiological stresses such as salinity and drought are also known to increase the expression of these proteases (Koizumi et al. 1993).

Studies on chloroplast proteases were initiated to understand their relationship with leaf senescence (Buetow 1997). Here for the first time, we have purified and characterized a salinity induced alkaline protease from isolated spinach chloroplasts. Alkaline protease was isolated from the chloroplast preparation by HIC-HPLC of ammonium sulphate fraction. Since the vacuolar proteases can contaminate such chloroplast preparation, chloroplast prepared from the spinach leaves were purified and made free from vacuoles by percoll density gradient. The SDS and Native PAGE analysis of the purified protein showed that the active enzyme had two subunits of 63 and 32 kDa (Fig. 2). The enzyme has the property to hydrolyse the synthetic substrate like azocaesin and showed the sufficient proteolytic activity in gelatin bound native PAGE (Fig. 3). The gelatin zymography of the control and salt-stressed chloroplast indicated the salinity induced nature of alkaline protease. For the maximal activation of alkaline protease, the spinach leaves were incubated at 25°C under salinity (1 M NaCl) for 96 h. The 25 ± 1°C temperature was chosen during senescence considering the ease of maintaining the growth chamber at 25°C.

The purified enzyme was found to be maximally active at pH 8.5 (Fig. 6a) and 50°C (Fig. 6b). Inhibition of the enzyme by PMSF suggested that it belongs to the serine class of protease. Interestingly its activity was not affected by some common broad spectrum inhibitors like ST1, TLCK etc. (Table 2). Strong inhibition by EDTA and EGTA along with significant stimulation by exogenous Ca^{2+}, Mg^{2+} and Mn^{2+} indicated the requirement of metal ions for the enzyme activity (Table 3). Unlike divalent metal cations, heavy metals like Hg^{+} and Pb^{2+} were found to be inhibitory which suggested that –SH groups are also involved in the enzyme catalysis. In other words we can say that enzyme activity is dependent upon the reduced environment. This was further evidenced with the increase in enzyme activity by the reduced glutathione. Oxidized glutathione completely inhibited the enzyme activity (Table 4).

For understanding the mechanism of enzyme catalysis we tried to identify the active site amino acid residues. Group specific chemical reagents such as DMHNB (Gibson and Svensson 1986; Zhou and Tsou 1985), PLP (Paech and Tolbert 1978; Rippa et al. 1967) and Iodoacetate have been used for tryptophan, lysine and tyrosine, respectively, to show their involvement in the catalytic function of enzyme. The strong inhibition of activity in response to these modifiers showed that the corresponding amino acids are essential for maintaining the proper geometry of active site (Table 4). Further, we have used OPA as an active site directed reagent and fluorescent probe (Paelezewski et al. 1983; Bhagwat and Gopalakrishna 1986). The emission spectra of the oxidized (Fig. 8) and reduced (Fig. 9) enzyme were compared which suggest that there is a conformational change in the reduced form of enzyme which resulted in the decrease of emission fluorescence at 300 and 336 nm. Treatment of the reduced enzyme with OPA leads to almost complete inhibition of enzyme activity which suggested the formation of thioisoindole derivative (data not shown). This is further confirmed by the observed emission at 418 nm in the OPA adduct which is the characteristic of thioisoindole derivative (Fig. 10). The exact fluorescence of thioisoindole derivative depends upon its hydrophobic environment and it ranges from 400–450 nm (Simons et al. 1979). The observed emission at 418 nm of the thioisoindole derivative suggested that the –SH groups involved at active site are confined to a hydrophobic environment. The formation of thioisoindole

derivative which requires free –SH and $–NH_2$ group clearly indicated that cysteine and lysine amino acids are in close proximity to each other. This also explained the inhibition of enzyme activity seen in the presence of PLP (Table 5). In thioisoindole derivative there was a decrease in the emission of tyrosine and tryptophan which indicated that cysteine and lysine amino acids (involved in the formation of thioisoindole derivative) are also associated with tryptophan so that internal energy transfer could occur resulting in the decrease in emission at 300 and 336 nm.

In conclusion, salinity induced alkaline protease has been purified and characterized from the isolated spinach chloroplast. The data obtained in response to specific amino acid modifiers and OPA fluorescent probe showed that cysteine, lysine and tryptophan residues are located at or near the active site geometry of alkaline protease. The present work also leads to an interesting area of investigation about the nature of the signals that initiate and regulate the activation of this protease under salinity stress. It will be interesting to find out the exact role of such proteases under stressed conditions.

References

Adam Z (1996) Protein stability and degradation in chloroplasts. Plant Mol Biol 32:773. doi:10.1007/BF00020476

Adam Z (2000) Chloroplast proteases: possible regulators of gene expression? Biochimie 82:647–654. doi:10.1016/S0300-9084(00)00612-X

Allakhverdiev S, Sakamoto A, Nishiyama Y, Inaba M, Murata N (2000) Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. Plant Physiol 123:1047–1056. doi:10.1104/pp.123.3.1047

Andersson B, Aro E (1997) Proteolytic activities and proteases of plant chloroplasts. Physiol Plant 10:780–793. doi:10.1111/j.1399-3054.1997.tb00005.x

Bhagwat AS, Gopalakrishna K (1986) *O*-phthalaldehyde as a probe in the active site of ribulose 1,5-bisphosphate carboxylase. Biochim Biophys Acta 873:45–52

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Buetow DE (1997) Plastid proteases. In: Pessarakli M (ed) Handbook of photosynthesis. Marcel Dekker, New York, pp 315–330

Casano LM, Desimone M, Trippi VS (1989) Proteolytic activity at alkaline pH in oat leaves, isolation of an aminopeptidase. Plant Physiol 91:1414–1418

Gibson RM, Svensson B (1986) Chemical modification of barley malt α-amylase 2: involvement of tryptophan and tyrosme residues m enzyme activity. Carlsberg Res Commun 51:295–308. doi:10.1007/BF02907163

Izawa S, Good N (1968) The stoichiometric relation of phosphorylation to electron transport in isolated chloroplast. Biochim Biophys Acta 162:380–391. doi:10.1016/0005-2728(68)90124-2

Janave MT, Ramaswamy NK, Nair PM (1999) Studies on the determination of the active site amino acid residues in glyoxylate synthase from potato tuber chloroplasts. Plant Physiol Biochem 37:121–129. doi:10.1016/S0981-9428(99)80073-4

Kapri-Pardes E, Naveh L, Adam Z (2007) The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in *Arabidopsis*. Plant Cell 19:1039–1047. doi:10.1105/tpc.106.046573

Koizumi M, Yamaguchi-Shinozaki K, Tsuji H, Shinozaki K (1993) Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene 129:175–182. doi:10.1016/0378-1119(93)90266-6

Kuwabara T, Hashimoto Y (1990) Purification of a dithiothreitolsensitive tetrameric protease from spinach PS **II** membranes. Plant Cell Physiol 31:581–589

Lindahl M, Tabak S, Cseke L, Pichersky E, Andersson B, Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. J Biol Chem 271:29329–29334. doi:10.1074/jbc.271.46.29329

Mark E (2001) Proteases and cellular regulation in plants. Curr Opin Plant Biol 4:254–260. doi:10.1016/S1369-5266(00)00169-2

Miller GL (1959) Protein determination for large numbers of samples. Anal Chem 31:964. doi:10.1021/ac60149a611

Mullet JE, Chua N-H (1983) In vitro reconstitution of synthesis, uptake and assembly of cytoplasmically synthesized chloroplast proteins. Methods Enzymol 97:502–509

Nair JS, Ramaswamy NK (2004) Chloroplasts proteases. Biol Plant 48:321–326. doi:10.1023/B:BIOP.0000041081.00086.85

Nair P, Ramaswamy NK (1991) Isolation of 34-kDa protein from spinach chloroplasts having ferrooxidase and H_2O_2-dependent dark O_2 evolution activities. Indian J Biochem Biophys 28:22–29

Oren O, Sarit T, Oded Y, Roni S, Adam Z (1996) Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts. Eur J Biochem 236:932–936. doi:10.1111/j.1432-1033.1996.00932.x

Paech C, Tolbert NE (1978) Active site studies of ribulose-1,5-bisphosphate carboxylase/oxygenase with pyridoxal 5′-phosphate. J Biol Chem 253:7864–7873

Paelezewski K, Hargrave PA, Kochman M (1983) O-Phthalaldehyde, a fluorescence probe of aldolase active site. Eur J Biochem 137:429–435. doi:10.1111/j.1432-1033.1983.tb07846.x

Ramaswamy NK (1979) The novel pathway for the utilization of CO_2 for the synthesis of solanidine in the isolated chloroplast from greening potato tubers. In: Correlation between chlorophyll and solanine synthesis in greening potatoes (Ph. D. Thesis). Univ. Bom, Bombay, pp 63–98

Rawlings ND, Barrett AJ (1994) Families of serine peptidases. Methods Enzymol 244:19–61. doi:10.1016/0076-6879(94)44004-2

Rippa M, Spanio L, Pontremoli S (1967) A specific interaction of pyridoxal 5′-phosphate and 6-phosphogluconic dehydrogenase. Arch Biochem Biophys 118:48–57. doi:10.1016/0003-9861(67)90276-7

Sarath G, Motte RS, Wagner FW (1989) Protease assay methods. In: Beynon RJ, Bond JS (eds) Proteolytic enzymes: a practical approach. IRL Press (at Oxford University Press), Oxford, pp 25–56

Schmidt GW, Mishldnd ML (1983) Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase small subunit in chloroplasts. Proc Natl Acad Sci USA 80:2623–2636. doi:10.1073/pnas.80.9.2632

Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. Plant Cell 7:1713–1722

Sherekar SV, Chander R, Karani MN, Warrier SB, Gore MS, Thomas P (1997) Proteinase activity in Jawala prawns (*Acetes indicus*) at alkaline pH. Fish Technol 34:47–50

Simons JSS, Thompson EB, Johnson DE (1979) Flourescent chemoaffinity labeling. Potential application of a new affinity labeling technique to glucocorticoid receptors. Biochemistry 18:4915–4922. doi:10.1021/bi00589a020

Suleyman IA, Yoshitaka N, Sachio M, Hiroshi Y, Noritoshi I, Yu K, Norio M (2002) Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*1. Plant Physiol 130:1443–1453. doi:10.1104/pp.011114

Teale FWJ (1960) The ultraviolet fluorescence of proteins in neutral solution. Biochem J 76:381–388

Teale FWJ, Weber G (1957) Ultraviolet fluorescence of the aromatic amino acids. Biochem J 65:76–482

Thakore D, Harikumar P (1993) Purification and characterization of an alkaline protease from kidney cortex lysosomes. Indian J Biochem Biophys 30:42–48

Viestra R (1996) Proteolysis in plants; mechanisms and functions. Plant Mol Biol 32:275–302. doi:10.1007/BF00039386

Zelisko A, Garcia-Lorenzo M, Jackowski G, Jansson S, Funk C (2005) AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. Proc Natl Acad Sci USA 102:13699–13704. doi:10.1073/pnas.0503472102

Zhou HM, Tsou CL (1985) An essential tryptophan residue for rabbit muscle creatinine kinase. Biochim Biophys Acta 830:59–63