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*Full Length Research Paper*

# Alkaline protease from senesced leaves of invasive weed *Lantana camara*

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**Protease from senesced leaves of the weed *Lantana camara* was purified in a 2-step procedure involving ammonium sulfate precipitation and Sephadex G-250 gel permeation chromatography. The Sephadex-G-250 fraction of senesced leaves of *Lantana camara* showed 28.31 fold with a yield of 6.19%. The enzyme was shown to have a low molecular weight of 43 kda by SDS-PAGE. It was strongly activated by metal ions such as $Cu^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Co^{2+}$ and $Mn^{2+}$. It remained active at 60°C, pH 10.5 even after 1 h of incubation when casein was used as substrate. The compatibility of the enzyme was studied with commercial and local detergents, 60% activity of the enzyme was retained even after 1 h of incubation at pH 10.0. The easy availability of the senesced leaves of this common weed makes it a cheaper enzyme source and potential additive in detergents.**

**Key words:** *Lantana camara*, senescenced leaves, alkaline protease, thermostability.

## INTRODUCTION

Proteases cover the 60% of total enzyme market and amongst the most valuable commercial enzyme. Alkaline proteases hold a great potential for application in the detergent and leather industries (Kumar and Takagi, 1999; Oberoi et al., 2001) due to the increasing trend to develop environmentally friendly technologies. Plants, animals and microbes are the main sources for protease production. The preferred sources of proteases are microbes because of their rapid growth, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties (Rao et al., 1998). All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains (Harwood and Cranenburgh, 2008). Application of fungal alkaline proteases is advantageous because of the ease of downstream processing to prepare a microbe-free enzyme at low cost production (Tsomides and Goldberg, 1969; Sharma et al., 2006).

A new thermostable serine proteases named 'wrightin' from the latex of the plant *Wrightia tinctoria* (Tomar et al., 2008), 'Carnein' from the latex of the weed *Ipomoea carnea* spp. fistulosa (Morning glory) (Patel et al., 2007) and 'Milin' from the latex of *Euphorbia milii*, was purified (Yadav et al., 2006) and have potential applications in food and other biotechnology industries. Endoproteases were also isolated from alfalfa, oat and barley senesced leaves which are involved in the process of protein degradation during foliar senescence (Nieri et al., 1998; Miller and Huffaker, 1981; Drivdahl and Thimann, 1977, 1978). Senescing leaves show dominance of proteolytic enzymes belonging to four major classes, which are common in mammals, plants and microbes. Senescence-induced protein breakdown has been well reported in many plant systems, and it results in availability of transportable nitrogen (Smart et al., 1995; Buchanan-Wollaston and Ainsworth, 1997; Griffith et al., 1997). This is associated with several fold increase in activity of protease and is characterized by the degradation and loss of proteins (Dungey and Davies, 1982; Yoshida and Minamikawa, 1996). Hortensteiner and Feller (Hortensteiner and Feller, 2002) indicated that proteolysis of chloroplast proteins begins in an early phase of senescence and the degradation pathways for chlorophylls and chloroplast proteins are partially interconnected, hence the senesced leaves are the major source of protease as compared to the green leaves.

As only few reports are available on the use of plant

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proteases in detergent industry, therefore there is a growing need to exploit plant proteases for commercial application in detergent industry. It is desirable to have new proteases with novel properties from different sources. *L. camara* is one of the ten most invasive noxious weeds in the world. The present study describes the easy protocol of production and potential application of caseinolytic, thermostable alkaline protease by utilizing the senesced leaves of common weed *L. camara.*

## MATERIALS AND METHODS

### Plant material

Senesced leaves of *L. camara* growing in and around the University area in Noida Uttar Pradesh India were collected in forenoon and weighed after washing and rinsing with distilled water.

### Purification of protease

Senesced leaves were washed with distilled water and blotted dry were weighed, homogenized with mortar and pestle in three volumes (w/v) 10 mM Tris-chloride buffer (pH 8.0) followed by filteration with three layered cheese cloth. This crude enzyme preparation was precipitated with 0-30% ammonium sulphate at 4°C, chilled for 30 min at same temperature then centrifuged for 10 min (4°C) at 10,000 rpm. The pellet was suspended in minimum volume of 10 mM Tris-Cl pH 8 and dialyzed against 6 liters Tris–chloride buffer (10 mM, pH 8.0) at 4°C. The protein pellet obtained after saturation with 0-30% ammonium sulphate was dissolved in 10 mM Tris-HCl buffer and loaded onto a column of Sephadex G-250 (2 × 24 cm) equilibrated with Tris-HCl buffer, pH 8.0. The one ml fractions were collected and checked for protein and protease activity. From the activity profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity (11-20 ml fraction of one ml each) coinciding with a single protein peak (11-20 ml fraction of one ml each). All the fractions with high protease activities were pooled, dialyzed, and concentrated by lyophilization and used for further studies.

### Determination of protease activity

Protein was estimated by Bradford method and protease activity was assayed with casein, albumin and gelatin as substrate, absorbance was taken at 660 nm. Protease activity was assayed by a modified method of Tsuchida et al. (Tsuchida et al., 1986) by using casein as substrate. The reaction mixture, containing 100 µl of 2 mg/ml (w/v) casein in 1 M glycine-NaOH buffer, pH 10.5 and enzyme, was incubated at 45°C for 30 min. Reaction was terminated by the addition of an equal volume of 10% (w/v) chilled trichloroacetic acid and reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The white coloured precipitate was filtered through Whatman no.1 filter paper and acid soluble product in the filterate was neutralized by 5 ml of 0.5 M $Na_2CO_3$ solution. The colour developed after adding 0.5 ml of 3-fold-diluted Folin–Ciocalteau reagent was measured at 660 nm. One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the above assay conditions.

### Hydrolysis of protein substrates

Protease activity with various protein substrates including casein, egg albumin, and gelatin (2 mg/ml) was assayed by mixing 2 µg of the enzyme and 200 µL of assay buffer containing the protein substrates. After incubation at 45°C for 30 min, the reaction was stopped by adding 200 µL of 10% of chilled trichloroacetic acid (TCA) (w/v) and allowed to stand in ice for 15 min. The undigested proteins were removed by filtration or centrifugation at 10,000 rpm for 5 min and amino acid released was assayed as described in previously.

### Effect of pH on enzyme activity

The protease activity of the purified enzyme was measured at different pH values (3.6 – 11). The pH was adjusted using the following buffers (0.2 M) of acetate (pH 2.0- 4.0), phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 45°C for 30 min and the activity of the enzyme was measured.

### Effect of temperature on enzyme activity and stability

The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30, 40, 50, 60 and 70°C at different pH ranges (pH 3.6, pH 5.0, pH 10.0, pH 11.0) were studied. The activity of the enzyme was measured.

### Effect of various metal ions on protease activity

The effects of metal ions (e.g., $Ca^{2+}$, $Mg^{2+}$, $Co^{+2}$, $Fe^{+3}$, $Mn^{2+}$ , $Zn^{2+}$, $Hg^{2+}$, and $Cu^{2+}$ [10 mM]) were investigated by adding them to the reaction mixture and pre-incubated for 30 min at 45°C pH 10.0. The activity of the enzyme was measured.

### Effect of protease inhibitors and chelators on enzyme activity

The effect of various protease inhibitors (10 mM) such as serine inhibitors; phenylmethylsulphonyl fluoride [PMSF], 3,4-dichloroisocoumarin(3,4-DCI) and 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF), cysteine inhibitors; E-64 and iodoacetic acid; aspartic protease pepstatin and metalloprotease 1,10-phenanthroline were determined by pre-incubation with the enzyme solution for 30 min at 45°C. The relative protease activity was measured as in 2.3.

### SDS-PAGE and native page analysis

SDS-PAGE was performed on a slab gel containing 10% ( w/v) polyacrylamide by the method of Laemmli (Laemmli, 1971). Native PAGE was performed on 7% (w/v) polyacrylamide gel.

### Compatibility with detergents

The compatibility of protease with local laundry detergents was studied in the presence of 10 mM $CoCl_2$. Detergents used were Henko (Henkel Spic, India); Surf, Surf Excel, Tide, Rin (Hindustan Lever Ltd, India); and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% w/v) and incubated with protease for 30 min at 60°C, and the specific activity was determined. This was then compared with the control samples without detergents.

## RESULTS

The crude enzyme preparation isolated from senesced

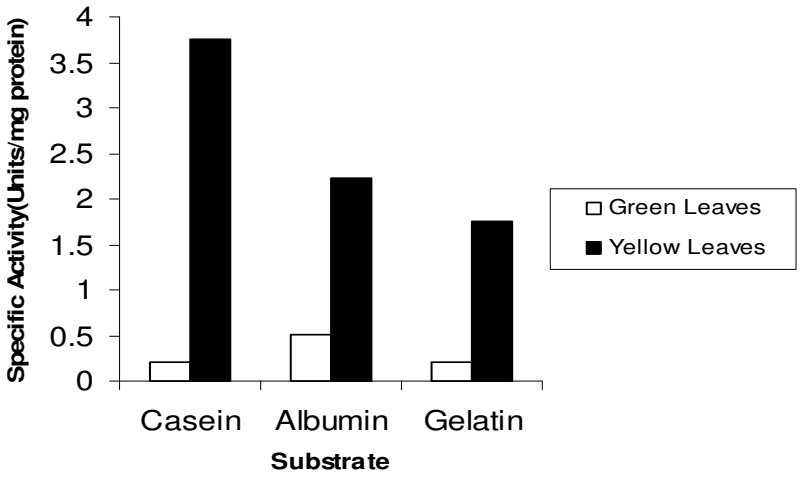


**Figure 1.** The figure denotes 10 fold increased activity of protease in senesced leaves of *Lantana camara* over green leaves using casein as a substrate.

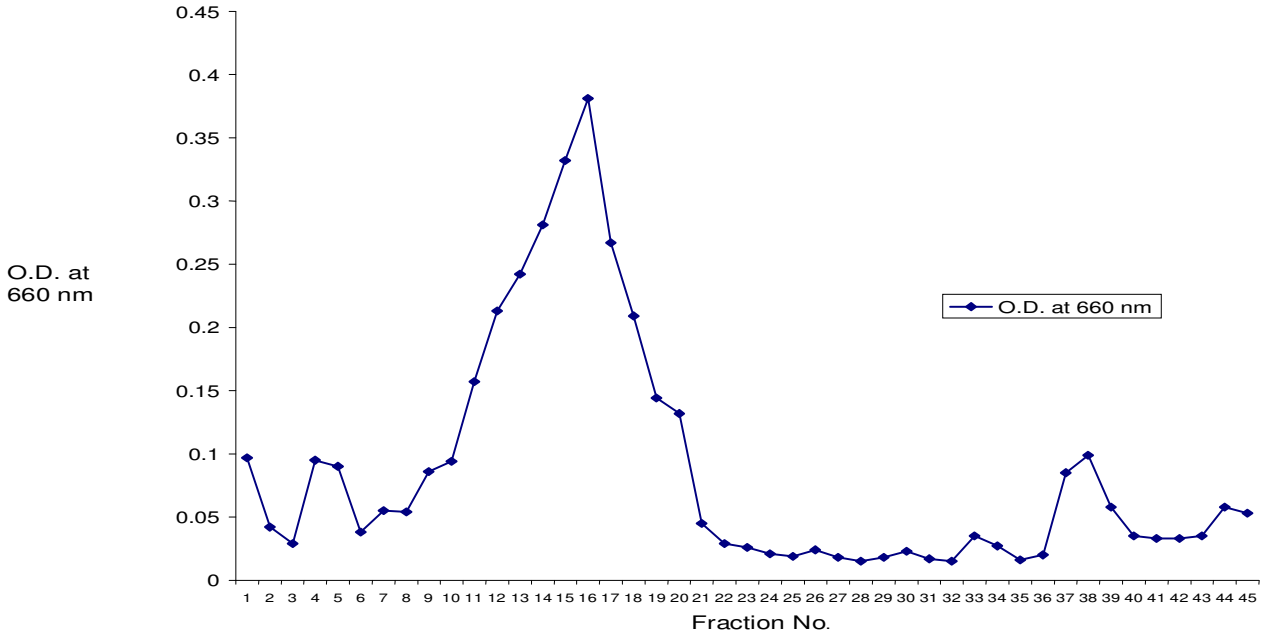


**Figure 2.** Activity profile of protease isolated from senesced leaves of *Lantana camara* by Sephadex G-250.

leaves of *L. camara* showed 10.5 times more protease activity when compared with the crude enzyme preparation from green leaves using casein as substrate (Figure 1). Senesced leaf enzyme preparation also showed increased activity with other substrates like albumin and gelatin. The protein pellet obtained after 30% saturation with ammonium sulphate was dissolved in 0.1 M Tris-HCl buffer and dialyzed against 6 liters Tris–chloride buffer (10 mM, pH 8.0) at 4°C and loaded onto a column of Sephadex G-250 (2 × 24 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The elution profile of gel filtration chromatography is shown in (Figure 2).

From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak. Fractions (11-20) with protease activities were pooled, dialyzed, and concentrated by lyophilization and used for further studiesmentioned below. The summary of purification steps involved for alkaline protease is presented in (Table 1).

**Table 1.** Summary of purification steps of alkaline protease from senesced leaves of *Lantana camara.*

| Purification Step | Total enzyme activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Crude enzyme | 668.864 | 59.579 | 11.22 | 1.0 | 100 |
| $(NH_4)_2SO_4$ precipitation, dialyzed (0-30%) | 156 | 9.84 | 15.85 | 1.4 | 23.32 |
| Sephadex G-250 | 41.35 | .1306 | 317.69 | 28.31 | 6.19 |

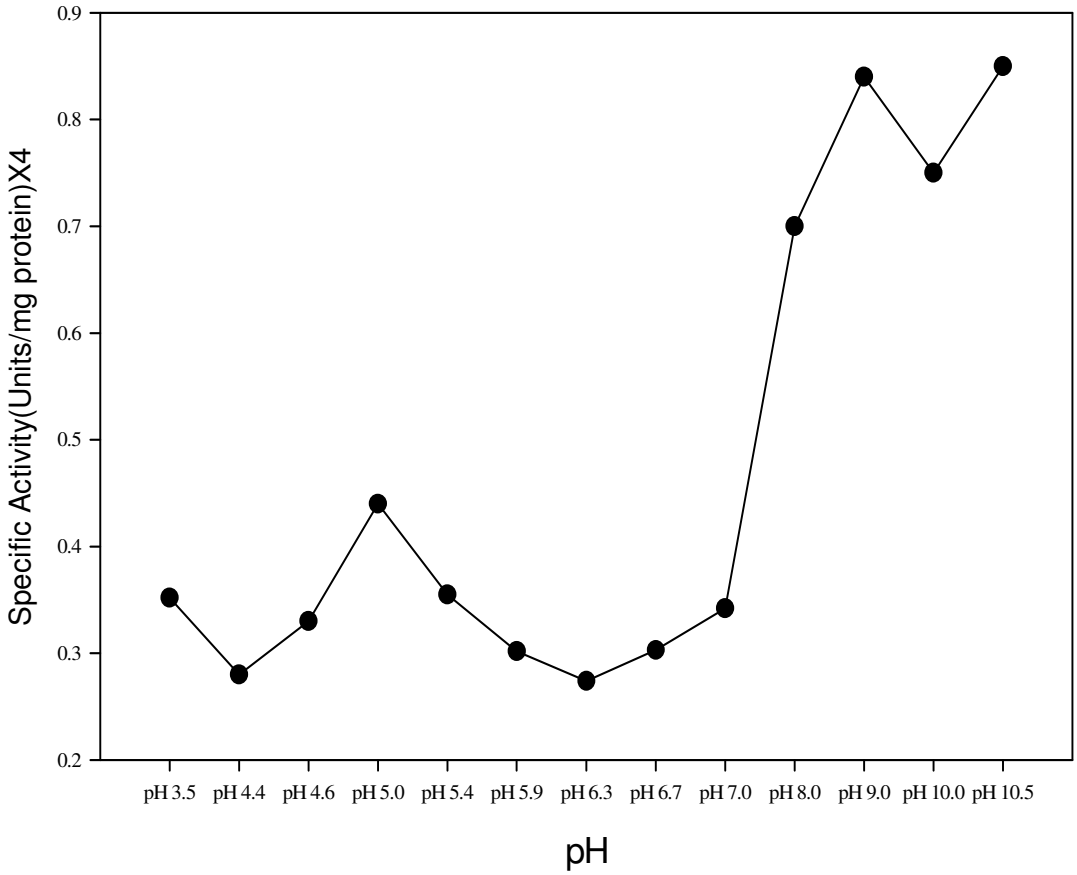


**Figure 3.** Effect of pH on *Lantana camara* alkaline protease activity.

Activity of the enzyme was determined at different pH ranging from 3.6-11. Protease activity was found to be stable in the alkaline range starting from the pH 8-11 at 45°C (Figure 3).

The thermal stability of the enzyme was also tested at different temperatures 30, 40, 50, 60, 70°C and different pH 3.6, 5, 10, 11.0 on incubation for 60 min (Figure 4). The optimum temperature recorded was at 60°C for protease activity and the enzyme was 100% stable at 60°C even after 1 h of incubation. The enzyme activity gradually declined at temperatures beyond 60°C.

The enzyme was strongly activated by metal ions such as $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ (10 mM each). In this study we report that $CoCl_2$ (10 mM) strongly increases the enzyme activity (Figure 5). These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at high temperatures (Pan and Lin, 1991; Steele et al., 1992; Donaghy and Mckay, 1993). This is possible because of the activation by the metal ions. Other metal ions such as $Zn^{2+}$, $Hg^{2+}$, $Fe^{+}$ (10 mM each) did not show any appreciable effect on enzyme activity.

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center (Sigma and Mooser, 1975).

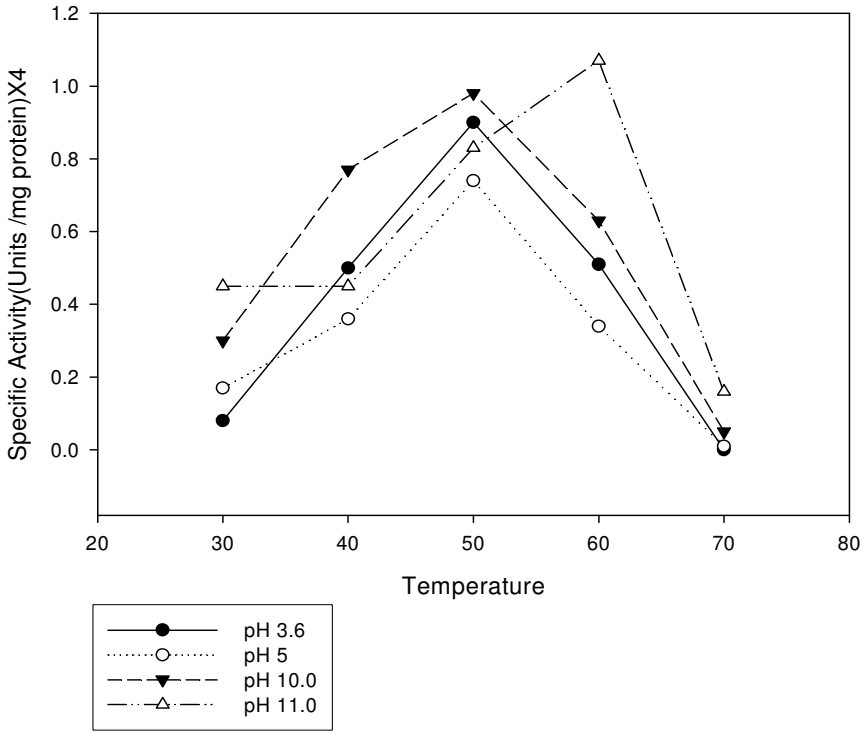


**Figure 4.** Effect of temperature on *Lantana camara* alkaline protease activity.

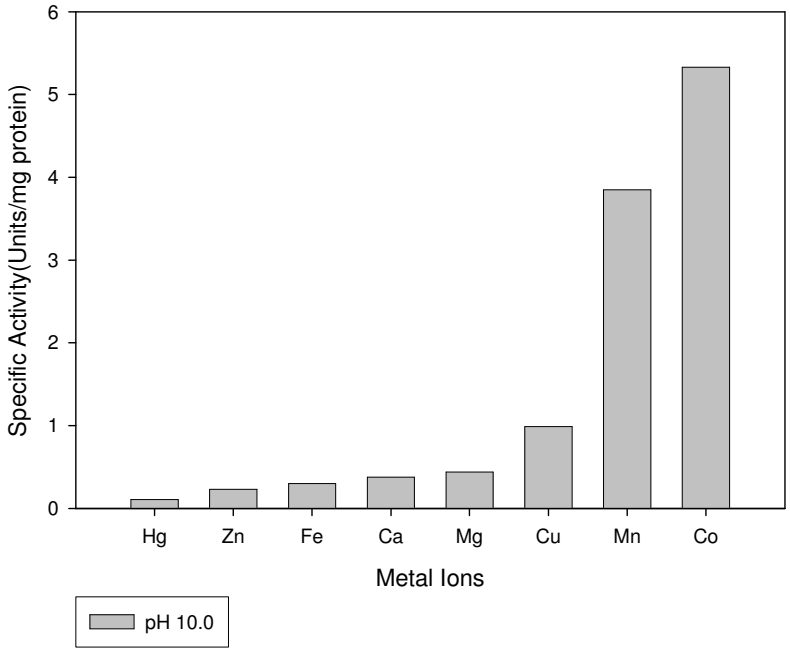


**Figure 5.** Effect of metal ions on *Lantana camara* alkaline protease activity.

The effect of different inhibitors on the enzyme activity of the purified protease was studied (Table 2). E-64 inhibited enzyme activity by 99.6% (at 10 mM concentration); this indicated that it is a cysteine alkaline protease. Slight inhibition was observed with PMSF (37.3%), pepstatin (23.8%) and AEBSF (26.6%). The protease was not

**Table 2.** Effect of protease inhibitors on *Lantana camara* enzyme activity. The enzyme was inhibited 99.6% in presence of E-64 and not inhibited in presence of 3,4-DCI.

| Inhibitors (10 mM) | Relative Inhibition in enzyme activity (%) |
|---|---|
| control | 0 |
| AEBSF | 26.6 |
| PMSF | 37..3 |
| 3,4-DCI | 0 |
| E-64 | 99.6 |
| Iodoacetic Acid | 10.4 |
| Phenanthroline | 5.7 |
| Pepstatin | 23.8 |

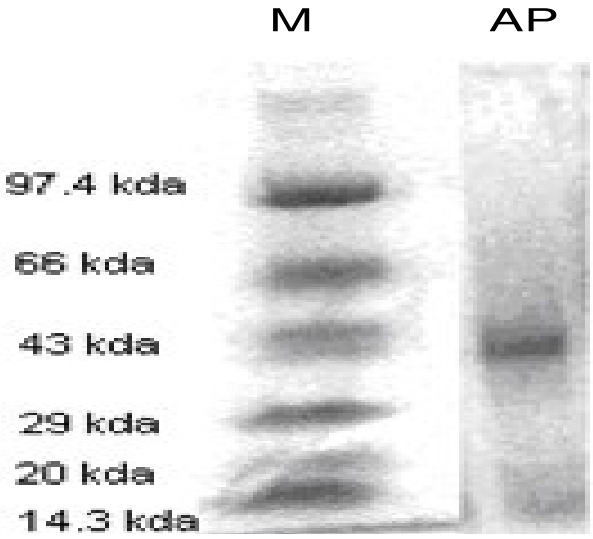


**Figure 6.** Purified alkaline protease (AP) on 10% SDS PAGE showing the monomeric nature of enzyme showed molecular weight of 43 kda. M, molecular weight markers.

inhibited by 3,4-DCI.

When the ammonium sulphate precipitation fraction was analyzed on Native Gel (7%) and SDS PAGE (10%) (Figure 6), it showed a single band indicating a homogeneous preparation. The enzyme has a low molecular weight of 43 Kda.

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. The enzyme fraction that showed protease activity showed excellent stability and compatibility in the presence of locally available detergents (Tide, Henko, Surf, Surf Excel, Ariel, and Rin) (Figure 7). The enzyme showed good compatibility with Hi Power Surf at pH10.5 and Henko at pH 8.0. The enzyme remained active at wide pH range along with Rin, Tide, Ariel, Surf excel. 60% enzyme activity was retained in presence of most detergents even after 1 h incubation at 60°C.

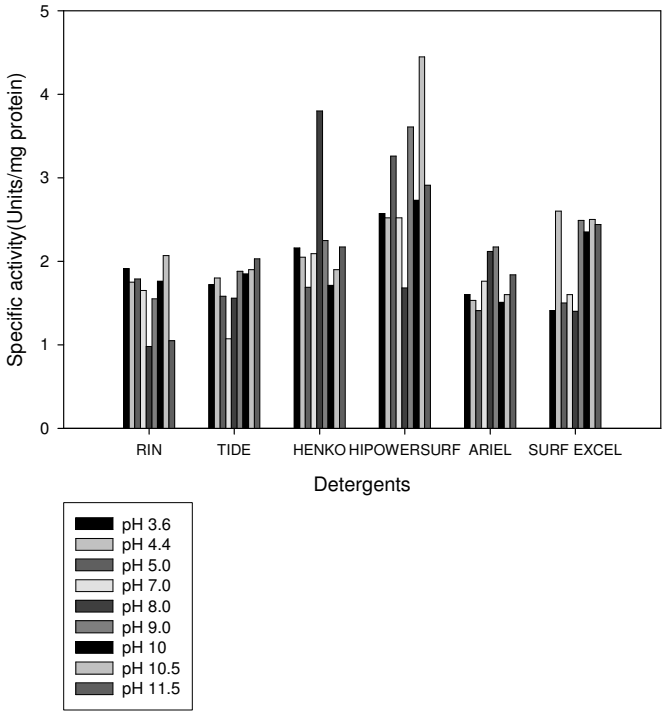


**Figure 7.** Study depicting compatibility of alkaline protease from *L. camara* with commercial detergents at different pH and 60°C.

## DISCUSSION

Natural senescence is associated with several fold increase in activity of protease and is characterized by the degradation and loss of proteins therefore proteases have been a common target of study (Dungey and Davies, 1982; Yoshida and Minamikawa, 1996). Internal factors such as plant hormones, reproduction and cellular differentiation influence senescence (Noodén and Leopold, 1988; Smart, 1994; Thomas and Stoddart, 1980). Degradation of macromolecules is one of the major events that occur during leaf senescence, the process also involves the synthesis of RNA and proteins *de novo* and degradation is remobilized to young and expanding organs of the plants (Buchanan-Wollaston, 1997).

We have developed a two step methodology to purify protease from senesced leaves of *L. camara*, an invasive noxious weed. The enzyme was thermostable at 60°C and retained activity in presence of detergents. The easy availability of the senesced leaves of this common shrub makes it a cheaper enzyme source which is thermostable and makes it a good choice to be an additive in the cheaper detergents already available in the market. Protease's extracts from microorganism are now used in the detergent industries to improve the detergent quality. The application of this protease in food industry is being investigated. In future the inhibitor would be purified and compared with other protease inhibitors using bioinformatics tools and its other uses would be in pharmaceutical industry.

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