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Isolation, Purification and some Properties of Protease from *Calotropis procera* (AIT) Latex

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Abstract
Protease has been purified from *Calotropis procera* (AIT) latex 40.5 fold with overall yield of 21.0% using Ammonium sulphate fractionation, DEAE-sephadex, gel filtration and hydroxyapetite chromatography. The enzyme moved homogenously as a single band in polyacrilimide gel. It has optimum temperature of 60^{0}C and optimum pH of 6.5. The pH stability and thermal stability studies showed the enzyme is stable at pH 4-10 with temperature stable up to 70^{0}C for 2 hours. The enzyme was inhibited by iodoacetate and mercuric chloride but activated by mercaptoethanol, EDTA and cysteine.The result obtained from this work confirmed that this enzyme belongs to the cysteine proteases. The broad pH and temperature stability of the enzyme would make it an excellent enzyme for food and pharmaceutical industries.

INTRODUCTION

Proteases are a single class of enzymes that occupy a pivotal position with respect to their application both physiologically and commercially (Rao *et al.*, 1998). A number of industrial and physiological processes involves breakdown of proteins. Proteolytic enzymes catalyse the cleavage of amide or peptide bond between two amino acids in polypeptides, and closely related compounds.

Proteolytic enzymes of plant origin have received special attention in the field of medicine and industry due to their unique property of being active at very wide range of temperature and pH. Proteases have been isolated from lattices, fruits and seed and most of them belong to cysteine family (Boller, 1986). Proteases of plants play major role in intercellular and extracellular processes such as development and ripening of fruits (Brady, 1985). The latex of *Calotropis gigantea* has been shown to contain cysteine protease (Abraham and Joshi, 1979; Pal and Sinha, 1980).

Calotropis procera (Sodom apple), a shrub that is widely distributed in West, and East Africa and other parts of the tropics (O'Connor, 1993), is a member of plant family *asclepiadiace*. The green puffy parts in edible fruits are obliquely ovoid in shape when matured and have a thick spongy pericarp. Apart from being used as a milk coagulant in traditional cheese making (O'Connor, 1993; Ogundiwin and Oke,1983), extract from the parts of the plant were reported to be used for dehairing of hides and treatment of various diseases (Burkill,1994). This application is linked to presence of proteases in Sodom apples. This paper reports on the isolation, purification and some properties of thiol activated protease from *Calotropis procera* latex.

MATERIALS AND METHODS

Materials

Latex of *Calotropis procera* plant, collected from Zaria environs in the early morning hours in glass containers was used as the enzyme source. It was identified by the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria.

Methods

Preparation of crude Enzymes

The latex was obtained by snipping off the terminal buds, few at a time and using sharp blade to cut the stem. About 200ml of latex weighing 200g was centrifuged at 13,000 X g for 20 min to remove insoluble materials as described by (Roger and Laurence (1993). The supernatant was kept at –4^{0}C for further purification.

Ammonium Sulphate fractionation

The proteases in crude extract were precipitated using 30 – 85% ammonium sulphate and solution kept for 24 hours. The resulting precipitate was dialysed against 0.1M sodium phosphate buffer at pH 7.5. The dialysate was centrifuge to remove minor insoluble materials (Alan, 1994)

Ion Exchange Chromatography

Dialysate from Ammonium sulphate precipitate was placed on the DEAE – sephadex column (1

X 15cm) pre-equilibrated with 0.1M sodium phosphate buffer pH 7.5. The enzyme was eluted with linear gradient of 0.1 – 0.5M NaCl in the same buffer. 3ml fractions were collected at flow rate of 0.5ml/min. (Aworh *et al., 1994*)

Gel Filtration Chromatography

The active fraction from DEAE sephadex column was concentrated and applied to sephadex G. 75 column (1 X 15cm) pre-equilibrated with 0.1M sodium phosphate buffer pH 7.5. 5ml fractions were collected at flow rate of 3ml/min.

Hydroxyapatite Chromatography

The active fractions from gel filtration were pooled together and concentrated and then applied to hydroxyapatite micro column pre-equilibrated with 0.1M sodium phosphate buffer and the enzyme eluted by 0.1 – 1.0M NaCl gradient in the same buffer. 1.0ml fraction was collected at flow rate of 1ml/14hour. The active peak was used for physicochemical studies (Abraham and Joshi, 1979).

Protease assay

Proteolytic activity was measured by the method of Arnon (1970) as described by Testuya et al (1993) using Hammestain casein as substrate. The reaction medium (2.0ml) is made up of 0.1M cysteine – EDTA – phosphate buffer (pH 7.5), 1.0ml enzyme sample, and 0.5% casein. After incubation at 40^0C for 30 min, the reaction was terminated by addition of 3ml of 5% TCA and the resultant precipitation was removed by centrifugation at 13,000 X g for 20 min. Blanks were prepared for each sample in similar manner except for the addition of TCA to enzyme solution before mixing it with casein.

One tyrosine unit is defined as the amount of enzyme that liberated 1 µg of tyrosine under the standard assay conditions

Protein determination

This was carried out according to procedure of Lowry et al (1951) using Bovine albumin as standard.

Effect of pH on Enzyme activity

The effect of pH on the activity of purified enzyme was investigated using casein substrate. The buffers used were 0.1M sodium acetate (pH 2 – 5), 0.1M sodium phosphate (pH 6 – 8) and 0.1M tris buffer (pH 9 – 12). The purified enzyme was equilibrated in 0.5ml of buffer at a given pH for 15min and enzyme was added to substrate at the same pH and assayed as described above.

pH Stability study

Stability of enzyme at different pH was studied by incubating the enzyme at different pH in the range (2 – 12) for 12 hours and the residual activity was measured at pH 7.5.

Effect of Temperature on Enzyme activity

The effect of temperature on the activity of enzyme was studied using casein as substrate. The enzyme was incubated at desired temperature in range of 10 – 80^0C for 15 min at pH 7.5 and an aliquot was used for the activity measurement at same temperature.

Thermal stability study

To determine the effect of heating on enzyme, samples were held for 20, 30, 40, 50, 60, 70, 90, 100, 110, and 120 min at 40^0, 50^0, 60^0, 70^0, and 80^0C and the residual activity measured.

Effect of various compounds on Protease activity

Effect of various compounds on protease activity were monitored using some specific activators and inhibitors and metals.

Electrophoretic study

Electrophoresis was carried out in 12.5% polyacrylamide gel which was overlaid with 4% stacking gel.

RESULT AND DISCUSSION

A protease was purified from *Calotropis procera* latex in five steps. Table 1 is a summary of the purification steps showing that about 40 fold purification was obtained. The increase in specific activity and purification observed could be attributed to loss in the inhibition of the enzyme when passed through the anionic exchanger. Most of the protease eluted from NaCl gradient indicating the protease may contain hydrophobic side chains. The elution of the enzyme from hydroxyapetite column at 0.4M NaCl concentrations further explains that the enzyme contains some amount of anionic amino acids.

Figure 1 is the effect of pH on activity of protease while figure 2 is the effect of pH on stability of protease. The pH optimum of the enzyme is 6.5 with casein as substrate. At this pH the enzyme can maintain correct ionic conformation for proper alignment of substrate in the active site. The same pH of plant protease have been obtained by earlier workers (Abraham and Joshi, 1979) The pH stability shows that the enzyme was relatively stable; at least 80% of the activity

Table 1: Purification table of Protease

	Total Protein (mg)	Total Activity (Tyrosine unit/min)	Specific Activity (Unit/mg)	Purification Fold	Recovery (%)
Crude	159.0	788.64	4.96	1	100
Ammonium sulphate precipitation (30 – 85%)	94.35	552	5.85	1.18	70
DEAE –Sephadex	25.70	386.40	15.03	3.03	49
Gel-filtration	16.67	291.80	17.5	3.52	37
Hydroxyapatite	1.82	165.60	200.87	40.50	21

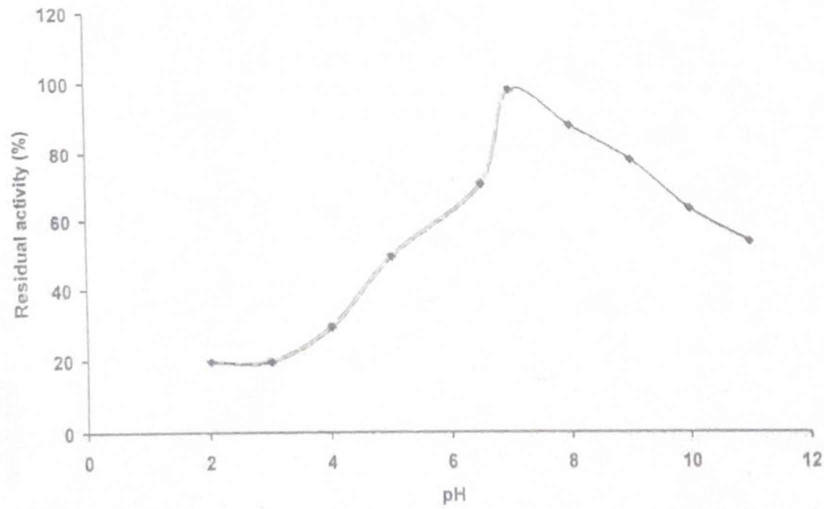

Fig. 1: Effect of pH on Activity of Protease

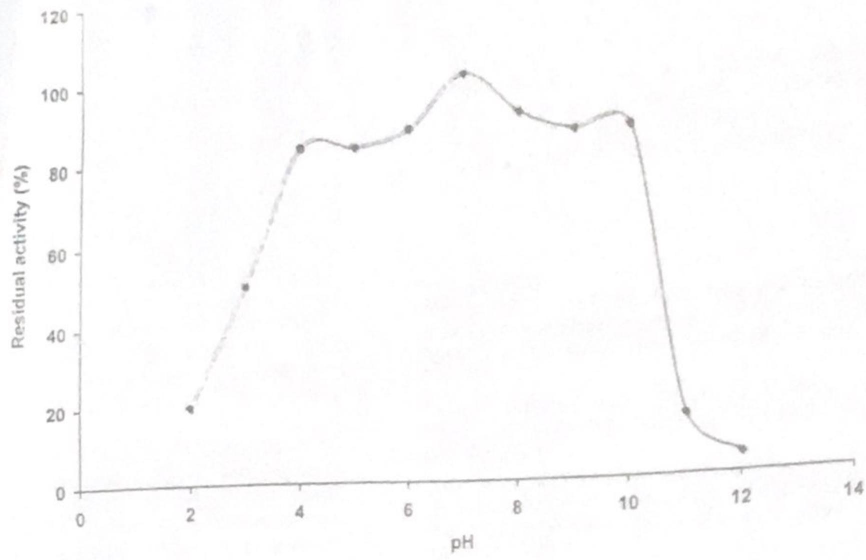

Fig. 2 Effect of pH on Stability of Protease

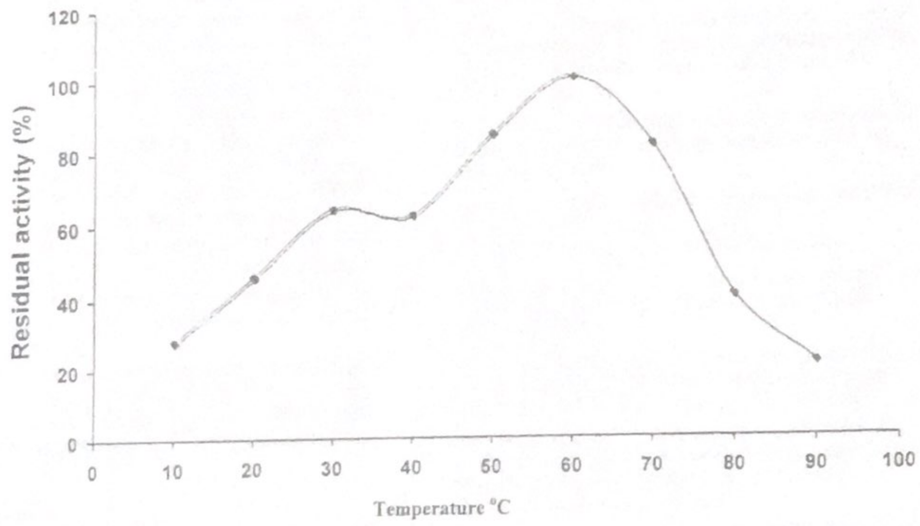

Fig. 3: Effect of Temperature on the Activity of Protease

remaining from pH range of 4 to10 (fig. 2).The result was also in good agreement with pH stability reported for plant proteases(Murachi, 1976).

The enzyme had broad optimum activity at 60°C and was stable between 40°C and 70°C with at least 70% of activity remaining for two hours (figs. 3 and 4). The thermal stability can be useful during production of enzyme as heat may be used to destroy contaminant enzyme activity (Treva *et. al.*, 1990). The broad pH and temperature stability of the enzyme would make it an excellent enzyme for food and pharmaceutical industries.

The enzyme was completely inactivated by incubation with 1.0mM iodoacetate and mercuric iodide and was activated by mercaptoethanol, cysteine and EDTA (Table 2). Inhibition of the enzyme by thiol specific inhibitors, iodoacetic acid and mercuric chloride was clear indication of the presence of sulphydryl (-SH) group at the active site of the enzyme. Heavy metals like mercury generally block or oxidize the active site and thus make the (-SH) group unavailable for catalytic action of the enzyme. Similar observations have been reported for most plant enzymes (Aworh *et. al.*, 1994). Activation of enzyme by mercaptoethanol and cysteine-HCl was made possible by disulphide bond breakage and formation of free –SH group which is needed for catalysis. A similar observation by this reducing agent was also reported for papain (Kimmel and Smith, 1954), Ficin (Englund *et. al.*, 1968), Calotropain (Pal and Sinba, 1980) and Procerin (Vikash and Jagannadhan, 2003). These observations with thiol as specific inhibitor and Mercaptoethanol as activator confirm that this enzyme belong to the cysteine proteases. The absence of inhibition of the enzyme by EDTA excludes the possibility that metal ions is involved in the catalytic site.

The enzyme migrated homogenously as a single band in SDS polyacilymide gel (fig. 5 and 6). The Molecular weight could not be determined because the protein markers were not available at the time.

CONCLUSION

Calotropis procera latex is a good source of protease. The broad pH and temperature stability of the enzyme would make it an excellent enzyme for food and pharmaceutical industry. Further work should be done on purification, characterization and immobilization of the enzyme and also biotechnological application.

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