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ISOLATION, PURIFICATION AND CHARACTERISATION OF PROTEASES FROM VEGETABLE WASTE

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ABSTRACT

Proteolytic enzymes are multifunctional enzymes of commercial importance. The aim of the present investigation was to isolate proteases from vegetable wastes such as cauliflower and elephant yam. The enzyme was extracted from vegetable wastes using Tris-HCl buffer, purified by ammonium sulphate precipitation and dialysis method. Purified enzymes were characterised by determining its specific activity, optimum temperature, optimum pH, effect of inhibitor and metal salts on its activity. Molecular weight of the enzyme was determined using SDS-PAGE analysis. The results depict that the proteases isolated from vegetable waste were acidic proteases and metal salts had enhanced the activity of the enzyme. The present investigation suggests that proteases isolated from vegetable wastes exhibited high enzymatic activity and consequently may alleviate the demand for proteases commercially.

INTRODUCTION

Proteases (EC 3.4.21-24 and 99) are enzymes that catalyse the hydrolysis of proteins [1]. Protease forms a large group of enzymes belonging to the class of hydrolases, ubiquitous in nature and performs a major role with respect to their applications in both physiological and commercial fields. These enzymes are widely distributed nearly in all plants, animals and microorganisms. In higher organisms about 2% of the genes codes are formed by these enzymes. Traditionally the proteinases have been regarded as degradative enzymes which are capable of cleaving protein foods. They liberate small peptides and amino acids needed by the body. Also they participate in the turnover of cellular protein. Indeed, this is one of the best characteristic of the proteinases, such as the mammalian digestive enzymes trypsin, chymotrypsin, and pepsin and the lysosome enzymes cathepsin B and cathepsin D. Proteolytic enzymes have the ability to carry out selective modification of proteins by limited cleavage such as activation of zymogenic forms of enzymes, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes. These properties add considerable interest to an already important group of enzymes. Additionally proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention because several clinical studies are indicating their applications in oncology, inflammatory conditions, blood rheology control and immune regulation. These are also used in crucial biological processes such as regulation of metabolism, enzyme modification, photogenecity, complement system, apoptosis pathways, invertebrate prophenoloxidase activating cascade etc. Furthermore, a study of proteolytic enzymes is valued because of their importance as reagents in laboratory, clinical, and industrial processes. Proteinases from both microbial and non-microbial sources, are extensively used in the food industry (baking, brewing, cheese manufacturing, meat tenderizing), in the tanning industry, and in the manufacture of biological detergents. Thus, there is an increasing interest in the proteinases and peptidases of both eukaryotic and prokaryotic microorganisms [2]. Proteases execute a large variety of pharmaceutical functions; particularly their involvement in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in an attempt to exploit their physiological and biotechnological applications. The present estimated value of the worldwide sales of industrial enzymes is $1billion. Hydrolytic enzymes contribute 75 % of total enzyme sale

including protease, carbohydrase and lipase. Proteases represent one of the three largest groups of industrial enzymes and account for about 59% of the total worldwide sales of enzymes. Proteases are widely distributed in each part of biological source. Due to this, it belongs to one of the subtype of digestive enzyme. Plant kingdom occupies the topmost rank (43.85 %) for finding proteases, followed by bacteria (18.09 %), fungi (15.08 %), animals (11.15 %), algae (7.42 %) and viruses (4.41 %). Isolated proteases only contribute 27 to 67 % of biological origin irrespective of either animal, microbial or plant proteases while remaining proteases are not well studied [3]. Plant proteases are virtually present in every part of plants viz., root, stem, leaf, flower, fruit, seed, gum and latex. Proteolytic enzymes from plant sources have received special attention because of their broad substrate specificity as well as activity in wide range of pH, temperature, and in the presence of organic compounds as well as other additives [4]. For the present investigation, vegetable wastes of cauliflower and elephant yam were used. The vegetable wastes were processed for the isolation of protease. The enzyme thus isolated was purified using dialysis and precipitation techniques. Characterization of the enzyme was carried out by determining its optimum temperature, optimum pH, effect of inhibitors, and effect of metal salts on its proteolytic activity. The molecular weight of the protease was determined using SDS-PAGE.

MATERIALS & METHODS

Plant Materials

The Vegetable waste was collected from the college mess. The outer green covering of Cauliflower (*Brassica oleracea var. botrytis*) and the outermost brown coloured layer of elephant yam (*Amorphophallus paeoniifolius*) were taken separately for the study. The wastes were well washed with water to remove the dirt.

Enzyme Extraction

100 g of vegetable wastes were weighed and homogenised using mixer grinder. The homogenised material was mixed with 500 ml of extraction buffer (0.05M Tris HCl, pH 6). The sample was centrifuged at 12,000 rpm at a temperature of 5 ^{0}C for 20 minutes. After centrifugation the supernatant was collected in a separate beaker and the beakers were marked as C (for cauliflower sample) and E (for elephant yam sample)

Enzyme Partial Purification

For ammonium sulphate precipitation, the supernatant obtained from enzyme extraction was added to ammonium sulphate (60%) to precipitate the total protein at 4ºC. After 1 h, the precipitate was recovered by centrifugation (30,000 rpm for 45 min), dissolved in Tris buffer, and dialysed twice (for 16 h the first time and 4 h the second time) against Tris buffer. Enzyme subjected to ammonium sulphate precipitation and dialysis was further used for enzymatic assay.

Enzymatic Assay

Protease activity was determined by measuring the release of trichloroacetic-acid soluble peptides from 1 % casein in sodium acetate buffer (pH 5.5) at 55ºC for 30 min. One unit enzyme activity was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg protein. Protein was measured using bovine serum albumin as the standard [5].

Molecular Weight Determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10 % acrylamide gel [6]. Proteins were stained with Coomasie brilliant blue R-250 (Sigma). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting of serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; lysozyme, 14.4 kDa.

Characterisation of the Enzyme

Optimal pH of Enzyme Activity

Optimum pH of protease enzymes isolated from the wastes of cauliflower and elephant yam were determined by using 1 % casein as a substrate dissolved in different pH of sodium acetate buffer (1.0-5.5), sodium phosphate buffer (pH 5.6-7.0) and Tris-HCl buffer (pH 7.0-10) at 55 ºC.

Optimal Temperature for Enzyme Activity

Optimum temperature of protease enzymes isolated from the wastes of cauliflower and elephant yam were determined by using 1 % casein as a substrate dissolved in sodium acetate buffer (pH 5.5) and incubated at varied temperatures ranging from 10-100 ºC.

Effect of Inhibitors on Enzyme Activity

The substrate inhibitor [ethylenediaminetetraacetic acid (EDTA) and dimethyl sulphonium bromide (DMSB)] was added to the protease enzymes isolated from the wastes of cauliflower and elephant yam at a concentration of 1 mM before analysing the enzyme activity. The enzyme activity was then determined by using 1% casein as a substrate dissolved in sodium acetate buffer (pH 5.5) at 55ºC.

Effect of Metal Salts on Enzyme Activity

The metal salt ($CaCl^2$, $MgCl^2$, $MnCl^2$) at a concentration of 1 mM was was added to the purified protease isolated from the wastes of cauliflower and elephant yam before analysing the enzyme activity. The enzyme activity was then determined by using 1% casein as a substrate dissolved in sodium acetate buffer (pH 5.5) at 55ºC.

RESULTS

The vegetable wastes [outer green covering of Cauliflower (*Brassica oleracea var. botrytis*) and the outermost brown coloured layer of elephant yam (*Amorphophallus paeoniifolius*)] were taken separately, homogenised and proteases were extracted using Tris-HCl buffer.

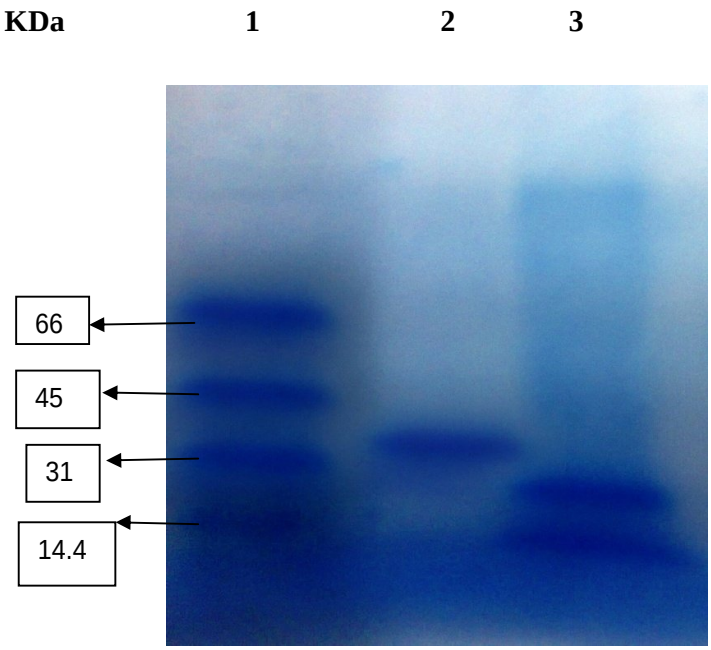
Table 1: Enzyme activity of proteases isolated from cauliflower and elephant yam

Proteases	**Total Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg Protein)**
Cauliflower	9.5	94.91	0.10
Elephant Yam	15	117.36	0.13

The enzyme extracted was then purified by ammonium sulphate precipitation. The precipitated enzyme was further purified by dialysis. The dialysed enzyme was used for the assay of its protease activity. Proteases isolated from elephant yam showed a higher specific

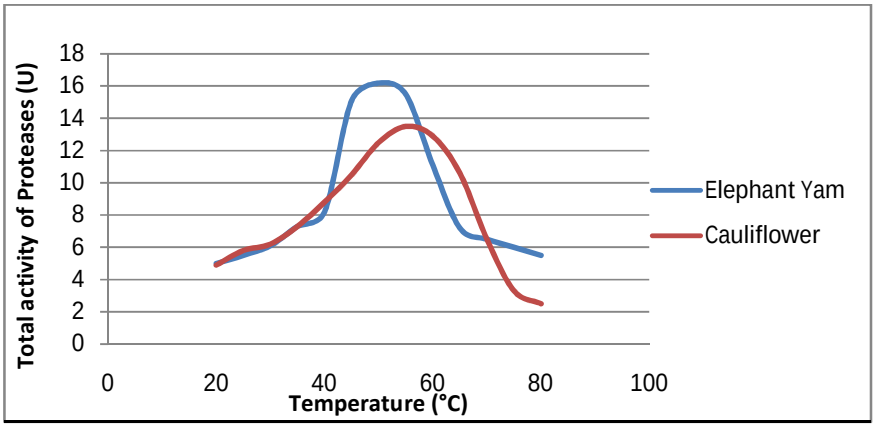
activity than proteases isolated from cauliflower extracts (Table 1). Molecular weight of the enzyme was determined by SDS-PAGE (Fig.1). Proteases isolated from cauliflower depicted a single band of molecular weight around 34 KDa, whereas proteases isolated from elephant yam showed two bands. Molecular weight of one band from elephant yam was found to be 14.4 KDa and that of the other band was around 29 KDa, thus proteases from elephant yam were a dimer comprising of two subunits.

Figure1. SDS-PAGE of proteases isolated from cauliflower and elephant yam. Lane 1: molecular weight of standard protein markers bovine serum albumin 66 KDa; ovalbumin 45 KDa; trypsin inhibitor 31 KDa; lysozyme 14.4 kDa, Lane 2: Cauliflower protease, Lane 3: Elephant yam protease.



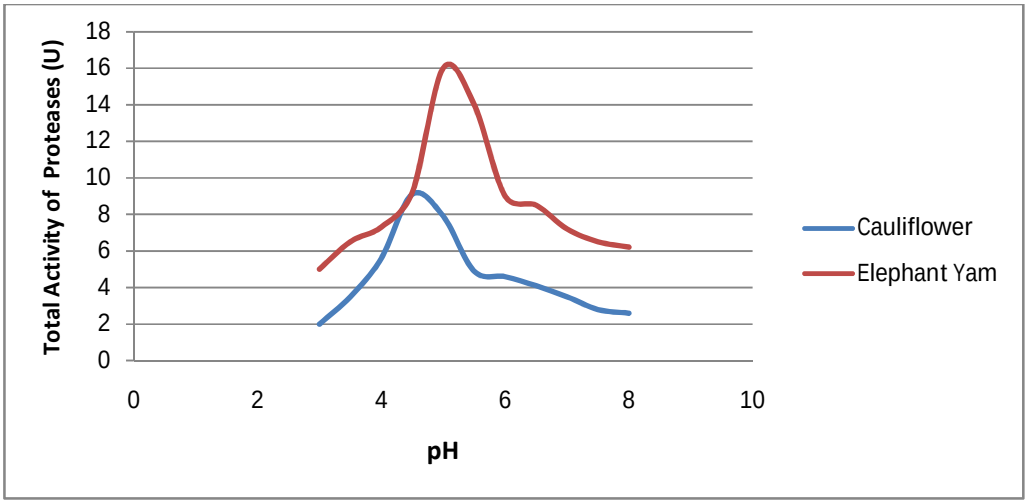
The optimum temperature for protease activity was assayed at different temperature from 10-100 ºC using casein as a substrate dissolved in sodium acetate buffer (pH 5.5). The optimum temperature of protease activity was found to be 50°C for elephant yam and 55°C for cauliflower (Fig. 2).

Figure2. Effect of temperature on proteases isolated from cauliflower and elephant yam.



The optimum pH for protease activity was assayed at different pH from 1-10 using casein as a substrate at 55 ºC. The optimum pH of protease activity was found to be 5 for elephant yam and 4.5 for cauliflower (Fig. 3).

Figure2. Effect of pH on proteases isolated from cauliflower and elephant yam.



The effect of two inhibitors on protease activity isolated from cauliflower and elephant yam is shown in Table 2. In both the samples (proteases isolated from cauliflower and elephant yam) dimethyl sulphonium bromide (DMSB) inhibited the enzyme more. The EDTA was found to be a weak inhibitor when compared to DMSB.

Table 2. Effect of inhibitors on proteases isolated from cauliflower and elephant yam.

Inhibitors	Cauliflower protease (Total activity U)	Elephant Yam Protease (Total activity U)
EDTA	5.4	7.6
Dimethyl Sulphonium Bromide	4.8	5.8

The protease activity was enhanced with an addition of Mn^{2+}, $Ca^{2+}$and $^{Mg2+}$ resulting in an increased enzyme activity of proteases isolated from cauliflower and elephant yam. The metal salts served as an enhancer of protease enzyme activity. Of the three metal salts, Mn^{2+} had increased the activity of proteases isolated from cauliflower and elephant yam considerably (Table 3).

Table 3. Effect of metal salts on proteases isolated from cauliflower and elephant yam.

Metal Salts	Cauliflower protease (Total activity U)	Elephant Yam Protease (Total activity U)
$CaCl_2$	10.4	16.8
$MgCl_2$	10.9	17.4
$MnCl_2$	11.3	17.9

DISCUSSION

Proteases are a unique class of enzymes; they have immense physiological as well as commercial importance. They possess both degradative and synthetic properties. They occur ubiquitously in plants, animals and microbes. Crude preparation of the enzyme has a wide specificity due to the presence of various proteinase and peptidase isozymes. The activity of the enzyme depends on the plant source, and the methods used in its extraction and purification [7]. Proteases from cauliflower and elephant yam were purified by ammonium sulphate precipitation and dialysis, in accordance with the present study proteases were isolated from *Cynara cardunculus* [8] using ammonium sulphate precipitation. Specific activity of proteases from elephant yam was found to be more than of cauliflower suggesting that

ammonium sulphate precipitation had concentrated the enzyme; a similar observation was made on the specific activity of protease isolated from mature coconut endosperm [9] after ammonium sulphate precipitation. The SDS-PAGE analysis of proteases from cauliflower showed a single band specifying that it is a monomeric protein, this in agreement with the studies on proteases isolated from soyabean [10]. Proteases from elephant yam showed two bands in SDS-PAGE analysis suggesting that it is a dimeric protein, a similar report was made on proteases isolated from the plant *Cynara cardunculus* [11] on SDS-PAGE analysis. Proteases isolated from cauliflower and elephant yam exhibited maximum activity at 55 °C and 50 °C temperature and 4.5 and 5 pH, suggesting that the proteases are acidic proteases; this is in agreement with the study on proteases isolated from the ripe fruit of *Solanum granuloso-leprosum* [12]. The activity of the protease enzyme isolated from cauliflower and elephant yam was decreased in the presence of inhibitors, this is in accordance with the study on proteases from horsegra [13]. The protease activity isolated from cauliflower and elephant yam was enhanced with an addition of Mn^{2+}, Ca^{2+} and Mg^{2+} suggesting that metal ions had a capability to protect enzyme against denaturation and maintained its active conformation, a similar report was observed in proteases isolated from *Bacillus megaterium* [14].

CONCLUSION

The results of the present investigation indicate that the proteases isolated from cauliflower and elephant yam is acidic proteases. The specific activity of proteases isolated from elephant yam is higher than that of proteases isolated from cauliflower. Proteases isolated from cauliflower and elephant yam exhibited maximum activity at 55 °C and 50 °C temperature and 4.5 and 5 pH. The activity of the protease enzyme isolated from cauliflower and elephant yam was decreased in the presence of inhibitors where as the activity of the enzymes was enhanced with an addition of Mn^{2+}, Ca^{2+} and Mg^{2+} salt. Thus, these proteases may turn out to be an efficient choice for the pharmaceutical, medicinal, food and biotechnology industry.

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