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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 4 (5): 564-568, 2001

Purification and Characterization of Raphanin, A Neutral Protease, from *Raphanus sativus* Leaves

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Abstract: A neutral protease of 64000 Dalton molecular weight, named raphanin, has been isolated in a homogeneous state from leaves of *Raphanus sativus* (Radish). The enzyme was purified by a sequence of fractional precipitation with ammonium sulfate, adsorption on hydroxylapatite chromatography, followed by gel filtration chromatography on two successive columns of Sephadex G-120 and Sephadex G-200. The casein activity of the enzyme was tested in several steps of its purification and an increase of almost 168 fold was obtained. Raphanin has been purified to apparent homogeneity, as tested by gel filtration on Sephadex G-200 and showed a single protein band on polyacrylamide gel electrophoresis. Raphanin is active over a broad range of temperature (30-60°C) and has an optimum activity at 50°C. It has a maximum activity at pH 6.5-7.0 up to 120 min., when casein was used as substrate. Raphanin is highly heat stable for 60 min at 30°C with 23% loss of its activity only. The apparent Michael's constants towards casein, haemoglobin, fibrin and collagen are 0.26, 0.44, 0.57 and 1.25% respectively. The enzyme was activated by Pb^{+2} and Cu^{+2} (46-55%) and inhibited by Hg^{+2}, Ag^{+1} and EDTA (38-82%), but only moderately activated by Zn^{+2}, p-chloromercuribenzoate and 2-mercaptoethanol (6-11%).

Key words: Raphanus sativus leaves, cruciferous family, protease, raphanin, purification and characterization.

Introduction

Proteases are important enzymes of plant metabolism and are instrumental in regulating senescence progress (Lauriere, 1983). They are responsible for the degradation of the proteins. Proteolytic enzymes are used extensively in industrial and medical applications (Ward, 1985). Many useful proteases were isolated and characterized from microorganisms(El-Aassar,1995). Higher plants play an important role as a source of many useful enzymes (El-Sayed *et al.*, 1995). Protease activities also have been detected in many higher plant species including seeds and flowers of *Onopordum turcicum* (Tamer, 1993), seeds of *Enterolobium contortisiliqum* and Soybean (Morita *et al.*, 1996) and germinated seeds of *Psophocarpus tetragonlobus* (Usha and Singh,1996). A number of proteases from latex of *Peditanthus tithymaloides*, *Euphorbia Pulcherrima*, *Carica papaya*, *Artocarpus heterophyllus* and *Ervatamia coronaria* have been isolated and their properties extensively were investigated (Prasad and Virupaksha, 1990). Proteases were also purified and characterized from oat, wheat Flag, *Phaseolus vulgaris*, *Onopordum turcicum*, *Spinacia oleracea*, maize, and *Petroselinum crispum* leaves (Jiang *et al.*, 1999). Therefore, the present study was conducted to isolate and purify the protease enzyme from *Raphanus sativus* leaves (radish leaves). Some properties of this enzyme were also studied. Following the common practice, we have given the enzyme the trivial name raphanin.

Materials and Methods

Raphanus sativus roots and leaves (radish) and *Brassica oleracea* (cabbage) leaves, family *Crucifereae*, were purchased from a local market during the winter season.

Preparation of the crude extract: Fresh healthy radish leaves and roots and cabbage leaves were washed thoroughly with distilled water, cut into small pieces and ground with distilled water and sand in a mortar at 4°C. Filtration through cheesecloth and centrifugation at 13000xg, at 4°C were conducted. The supernatant was collected and dialyzed against distilled water for 48 h at 4°C and then used as the crude enzyme solution.

Purification of raphanin enzyme: Unless otherwise stated, all purification steps were performed at 4°C and centrifugation at 13 000xg. The crude enzyme solution from radish leaves was precipitated by ammonium sulfate at different concentrations (30, 80 and 100% w/v). The active fraction with high protease activity was chosen and was mixed with hydroxylapatite in 0.001M phosphate buffer, pH 6.0 in a ratio of 2:1 and the suspension was allowed to stand for 15 min, then centrifuged. The enzyme was eluted with the same buffer at different molarities in the range of 0.001-0.2 M. The active fraction was dialysed against water and passed through two successive columns of Sephadex G-120 (0.8x36cm) and G-200 (1.3x27cm) pre-equilibrated with 0.01M phosphate buffer, pH 7.0. The protein was eluted with the same buffer at a flow rate of 25 ml/h.

Disc electrophoresis: The peak with the enzymatic activity was checked for purity using 7.5% polyacrylamide gel electrophoresis (3mA, 3h, Tris-HCl buffer pH 8.2; Davis, 1964) and stained with amidoblack dye. The purified enzyme thus obtained was used for physicochemical analysis.

Assay of proteolytic activity: Proteinase activity was determined according to the method carried out by Abdel-Fatah and Khella (1995) with some modifications. This method is based on the caseinolytic action in which casein was used as substrate. The standard reaction mixture contained 0.1M phosphate buffer of pH 7.0 and 1.0% (w/v) casein and an appropriate amount of enzyme solution in a final volume of 0.3ml. It was incubated for 90 min at 50°C. The reaction was stopped by 12% trichloroacetic acid followed by measurement of the digested protein by folin-ciocalteau phenol reagent, and referred to a standard curve for tyrosine.
One unit of proteinase activity was defined as the amount of the enzyme which liberates the digest products (not precipitated with 12% trichloroacetic acid) and give the folin colour equivalent to one μmole of tyrosine per minute's reaction at 50 °C .
Protease activity was expressed either as the amount of μmole released tyrosine per g of leaf fresh weight, or as μmole released tyrosine liberated per mg protein extracted.

Protein determination: Protein was estimated either at 280 and 260 nm or according to the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Molecular weight determination: The molecular weight of the enzyme was determined by Sephadex G-200 gel filtration through a column (1.3x27cm) equilibrated with 0.01M phosphate buffer, pH 7.0 (Adrews, 1964). Bovine serum

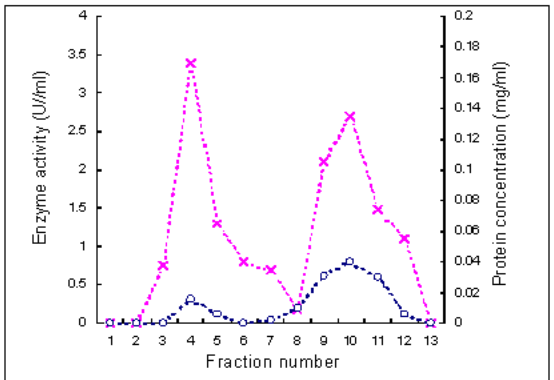

Fig. 1:Typical elution profile for the chromatography of raphanin on Sephadex G-120, o----o protein and x--x protease activity

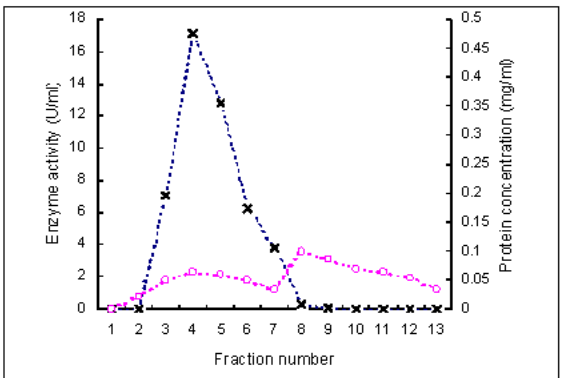

Fig. 2: Typical elution profile of raphanin enzyme on Sephadex G-200 column. o---o protein and x---x protease activity

Fig. 3: Electrophoretic behaviours of the purified raphanin on plyacrylamid gel disc electrophoresis

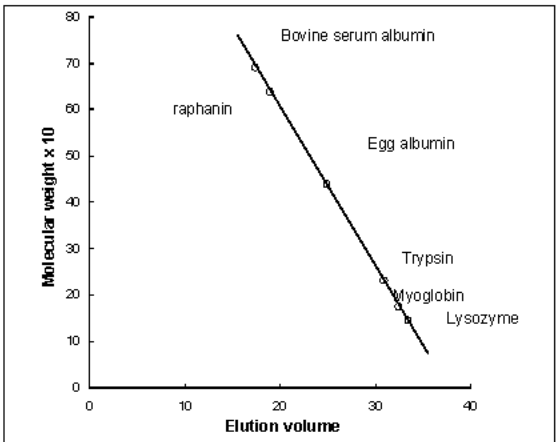

Fig. 4: Determintion of molecular weight of raphanin by gel filtration on Sephadex G-200 column

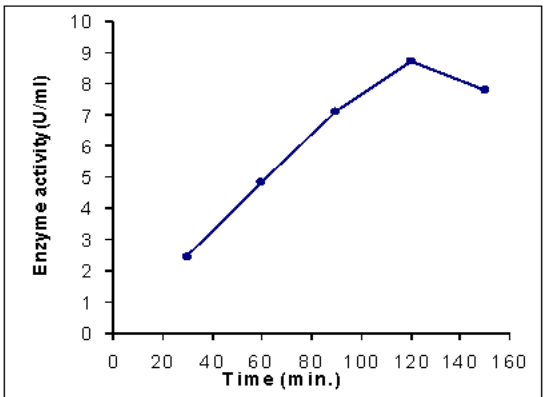

Fig. 5: Hydrolysis rate of casein by raphanin

albumin (69000), egg albumin (44000), trypsin (23000), myoglobin (17200) and lysozyme (14200) were used as standards.

Enzyme properties .

Optimum pH for enzyme activity: For the optimum pH of the proteolytic activity, the pure enzyme solution was reacted with casein solution (1.0% w/v) in various pH values (5.0 – 7.5) at 50°C for 90 min.

Effect of temperature on enzyme activity and stability: The influence of different temperatures on proteolytic activity for the pure enzyme was determined by holding the reaction mixture at various temperature (30-60°C) for 90 min during standard enzyme assay. Thermal stability was determined by holding the enzyme solution at various temperature's (30, 40, 50, 60 and 70°C) for varying time intervals (15, 30, 45 and 60 min.). The remaining activity was measured under standard assay conditions.

Effect of additives on enzyme activity: The effect of some

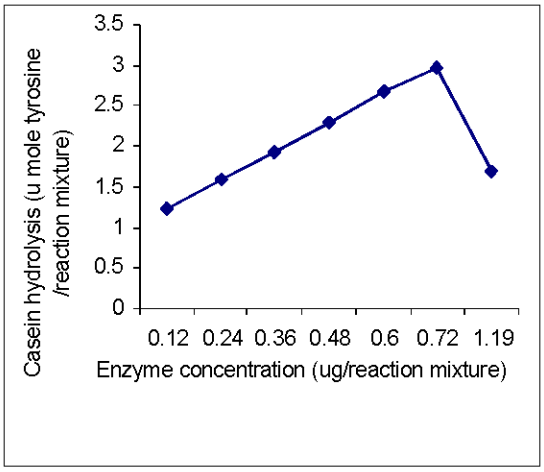

Fig. 6: Effect of raphanin concentration on the rate of casein hydrolysis

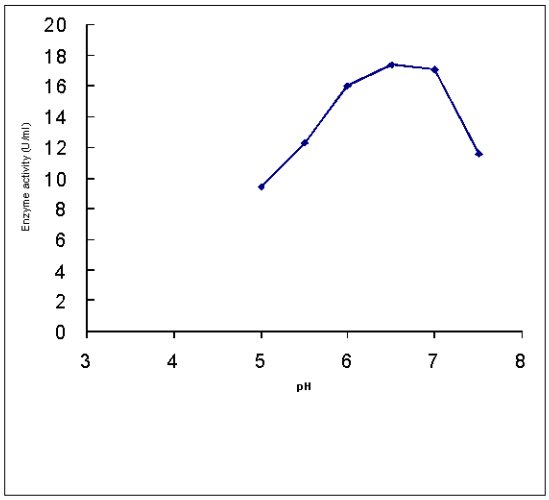

Fig. 7: pH optimum of raphanin activity. The buffers were used: 0.1M acetate buffer (pH5.0); 0.1M citrate-phosphate buffer (pH 5.5-7.0) and 0.1M tris buffer pH (7.5)

metal cations (Cu^{+2}, Hg^{+2}, Ag^{+1}, Pb^{+2} and Zn^{+2}) and some inhibitors (EDTA, p-chloromercuribenzoate., and 2-mercaptoethanol) on enzyme activity was tested by preincubating the pure enzyme with 1.0 mM for each inhibitor and $2x10^{-7}$mM for p- chloromercuribenzoate at 25 °C for 30 min. The residual activity was measured by the caseinolytic assay procedure relative to control. `

Determination of substrate specificity: The proteolytic activity of the purified enzyme was tested using various natural proteins, namely casein, haemoglobin, fibrin and collagen. The substrates were incorporated in the reaction mixture.

Michael's constant: Apparent Michael's constants were determined according to Lineweaver-Burk (1934), using casein, haemoglobin, fibrin and collagen as substrates.

Ultraviolet-absorption measurement: The absrbances of the

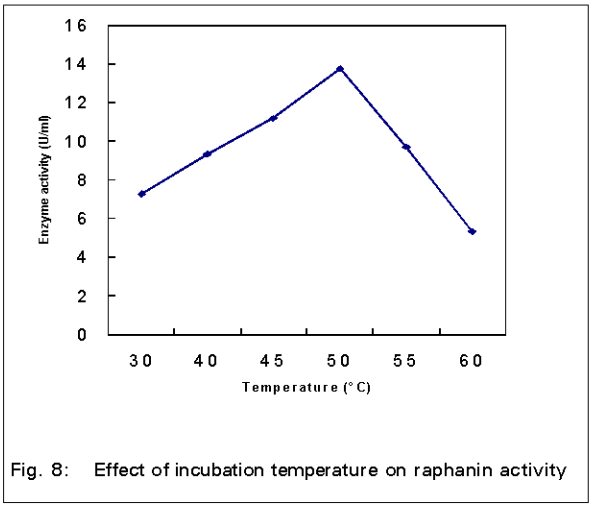

Fig. 8: Effect of incubation temperature on raphanin activity

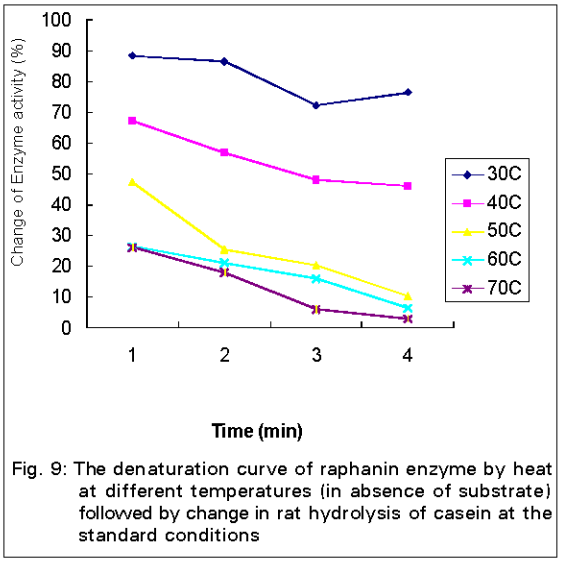

Fig. 9: The denaturation curve of raphanin enzyme by heat at different temperatures (in absence of substrate) followed by change in rat hydrolysis of casein at the standard conditions

pure enzyme preparations were measured in 0.01M phosphate buffer, pH 7.0.

Results

The crude enzyme solutions were extracted with distilled water from some higher plants (cabbage leaves and radish roots and leaves), family *Crucifereae*. The aqueous extracts were incubated with casein dissolve in different pH's (5-7) at 50°C in order to assess their protease activities. The results in Table 1 indicated that aqueous extract of fresh radish leaves contains protease activity towards casein more than in the

Table 1: Proteolytic activity levels in some higher plants.

Plants	Common name	Part use	Protease ativity (U//mg) at pH		
			5	6	7
Raphanus sativus	radish	roots	1	0.53	0.5
Raphanus sativus	radish	leaves	0.8	1.65	1.8
Brassica oleracea	cabbage	leaves	0.97	1.12	0.82

Table 2: Summary of Raphanin purification from 200g fresh green radish leaves.

Purification steps	Total activity (U)	Specific activity (U//mg protein)	Purification time	Yield(%)
Crude extract	360	1.53	1.0	100.0
Ammonium sulfate fraction (30-80%)	212	2.9	1.9	59.0
Hydroxylapatite fraction	130	7.65	5.0	36.0
Gel filtration on Sephadex G-120:				
peakI	79	214.0	139.9	21.9
peak II	24	73.0	47.7	6.7
Gel filtration on Sephadex G-200:	54	257.0	168.0	15.0

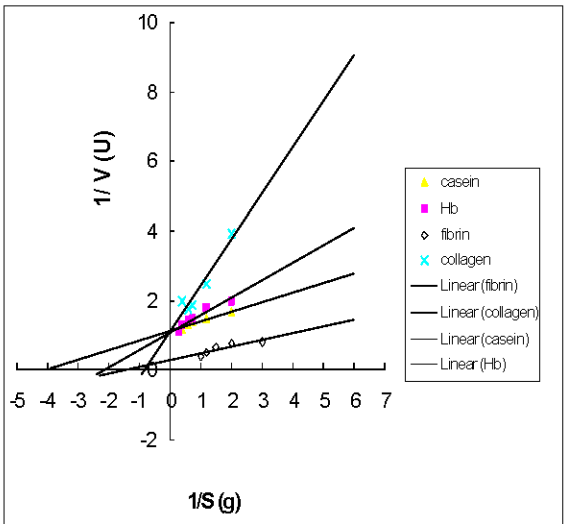

Fig. 10: Lineweaver-Burk plot for some portein hydrolysis by raphanin

Table 3: Effect of some metal cations and some reagents on the raphanin activity

Added cation	Residual activity (% change)
Control	100.0
Cu	155.0
Hg	58.0
Ag	18.0
Pb	146.0
Zn	108.0
EDTA	62.0
P-chloromercuribenzoate	106.0
2-mercapto ethanol	111.0

*Raphanin activity without added of metal ions was taken as 100% activity.

extract of radish roots and cabbage leaves. Thus, aqueous extract of radish leaves was found to be a convenient source and was selected for preparation of pure protease enzyme. Following the common practice, we have given the enzyme the trivial name raphanin.

Purification of enzyme. The aqueous extract of radish leaves was subjected to ammonium sulfate fractionation. Ammonium sulfate of 30-80% saturation were found to be the best active enzyme fraction with 2.9 U/mg protein and 59% yield. The dialyzed enzyme solution of ammonium sulfate fraction was further purified with hydroxylapatite chromatography at different molarities of phosphate buffer (0.001M to 0.2 M), pH 6.0. The protease activity of raphanin was eluted with 0.01M phosphate buffer with specific activity 7.65U/mg protein and 36% yield. The protease eluted from hydroxylapatite chromatography was further purified by Sephadex G-120 column. It was separated into two peaks I and II, having protease activities (Fig. 1). Higher protease activity was shown in the peak I (214U/mg and 21.9 %yield) than in the peak II (73U/mg and 6.7%). Furthermore, purification of peak I on Sephadex G-200 column had to be conducted and gives a single peak of protease activity coinciding exactly with its protein peak (Fig. 2). The final preparation was obtained with specific activity 257U/mg protein, 15% yield and 168 purified times.

(Table 2) summarizes the results of the purification procedure of raphanin from 200g fresh, green radish leaves. The purification procedure yielded 270U and 1.05mg protein of pure enzyme raphanin from 1Kg fresh green leaves.

Homogeneity: The final enzyme isolated was shown to be homogenous protein on the basis of polyacrylamide gel electrophoresis (Fig. 3). It showed a single protein band, indicating the homogeneity of the enzyme protein.

Enzyme properties: The apparent molecular weight of raphanin was 64000 Dalton on the basis of gel filtration on Sephadex G-200 (Fig. 4).

Hydrolysis of casein by the enzyme preparation was linear with respect to time up to 120 min (Fig. 5). All subsequent experiments were conducted for either 60 or 90 min to place them in the linear portion of the curve.

The rate of reaction was directly proportional to the enzyme concentration up to 0.72 μg/reaction mixture. (Fig. 6). Based on these results, all tests were made using the enzyme concentration in the linear portion.

Results of Fig. 7 illustrated the effect of pH on the protease activity of the pure enzyme. Raphanin exhibited protease activity over the entire range from pH 5.0 to 7.5 with a maximum activity at pH 6.5-7.0 using casein as substrate. Thus, the enzyme was found to be a neutral protease.

In the presence of the substrate, raphanin was active at temperature ranging from 30 to 60°C, with optimum activity at 50 °C Above 50°C , the rate of activity was decreased to about 50% at 60°C (Fig. 8).

Results in Fig. 9 illustrated show the effect of temperature on the protease activity of raphanin in the absence of substrate. The activity of raphanin was stable for 60 min at 30°C with loss 23% of its activity only. It was loss 75% of the activity when kept at 70°C for 15 min.

The pure enzyme raphanin showed various hydrolysis activities against different proteins. It was highly active on casein (100%) and haemoglobin (69%) than on fibrin (24%) and collagen (11%).

The rates of hydrolysis of casein, hemoglobin, fibrin and collagen by raphanin were linear until substrate concentrations were 0.67, 0.83, 1.33 and 1.33% respectively, beyond this range, inhibition occurred.

The apparent K_m values for the enzyme towards casein, haemoglobin, fibrin and collagen were 0.26, 0.44, 0.57 and 1.25% respectively (Fig. 10).

Effects of several divalent metal cations on raphanin activity were examined. Protease activity was significantly inhibited by 1.0 mM EDTA, Hg^{+2} and Ag^{+1} by 38to 82% and moderately activated by $2x10^{-7}$mM p-chloromercuribenzoate (6%) and 1.0 mM 2-mercaptoethanol (11%) (Table 3). It was activated by Pb^{+2} and Cu^{+2} (46-55%).

The purified enzyme showed absorption maximum at 275nm.

No significant absorbance could traced in the visible region indicating the absence of chromofore group. The ratio of absorbency at 280 to 260 nm for raphanin was 1.16.

Discussion

In radish leaves, protease activity have been quantitatively identified, isolated and purified. Their properties were identified. The protease activity was purified by fractional precipitation with ammonium sulfate followed by adsorption on hydroxylapatite and chromatography on two successive columns of Sephadex G-120 and G-200.The purification procedure described here gave neutral, homogenous protease raphanin with high specific activity (257 U/mg protein) and 270U yield from 1.0 Kg fresh radish leaves. The overall purification was 168-fold. Similarly, proteases were purified from seeds, flower and leaves of *Onopordum turcicum* and seeds of *Enterolobium contortisiliqum* with 200 and 300-fold and 46% yields (Silva *et al.*, 1994).

Radish leaves are considered as convenient source for preparation protease enzyme. Previously, proteases have been isolated, purified and characterized from soybean seeds, germinated winged-bean seeds, leaves of *Phaseolus vulgaris* and latex of *Ervatamia coronaria*, (Sundd *et al.*, 1998).

Raphanin has a maximum activity at the pH (6.5-7.0), suggesting that it be classified as a neutral proteinase according to the terminology of Hartley (1960). Similarly, neutral proteases were isolated from *Cnidoscolus chayamansa, Ervatamia coronaria* and *Phaseolus vulgaris* leaves (Popovic *et al.*, 1998). However, the isolated proteases from *Pedilanthus tithymaloides*, wheat Flag, maize and Alfalfa leaves were active in acid pH range from 4.8 to 5.5 (Nieri *et al.*, 1998)

The apparent molecular weight of raphanin was 64000 Dalton on the basis of gel filtration on Sephadex G-200 column. Proteases with approximately equal molecular weights (6000 to 74000 Dalton) were purified from *Euphorbia pulcherrima* latex, *Hevea brasiliennsis* latex, *Enterolobium contortisiliqum* seeds and *Petroselinum crispum* leaves (Jiang *et al.*, 1999). In contrast, proteases with lower molecular weight of 19000 to 43000 Dalton were prepared from *Onopordum turcicum* and *Ervatamia coronaria* leaves (Sundd *et al.*, 1998).

Raphanin has optimum activity at 50°C . This value was in agreement with that reported for proteases isolated from seeds, flower and leaves of *Onopordum turcium*, leaves of wheat Flag and latex of *Ervatamia coronaria* (Sundd *et al.*, 1998).

The affinity of raphanin towards natural protein substrates like casein, haemoglobin, fibrin and collagen were tested and their apparent K_m values were calculated. The raphanin had more affinity to casein and haemoglobin more than to fibrin and collagen. Raphanin hydrolyzed casein and haemoglobin similarly to the protease isolated from latex of *Pedilanthus tithymaloides, Cnidoscolus chayamansaleaves*, germinated winged-bean seeds and *Ervatamia coronaria* (Sundd *et al.*, 1998).

Raphanin activity was enhanced by Pb^{+2} and Cu^{+2} (46-55% increase). Thus, it is a metallo-protease. It is inhibited by EDTA (38%), similarly to the acid proteinase of hemp seeds and the neutral protease of Ervatamina coronaria (St Angelo *et al.*, 1970 and Sundd *et al.*, 1998). Such inhibition may be due to the participation of a metal cation (Harper *et al.*, 1977). However, EDTA had no effect on the activities of euphorbin p and l, suggesting that metal cations are not critical to the catalytic activity (Lynn and Clevette-Radford, 1984 a). 2-mercaptoethanol and p-chloromercuribenzoate also did not inhibit Raphanin. Thus, protease was not found to belong to the so-called sulfhydryl-dependent proteases. In this respect it is similar to proteases of *Cannalies sativa and Sorghum* (Garg and Virupaksha, 1970). This is disagreed with the activity of the purified protease isolated from bean, which was reported to belong to the so-called sulfhydryl-dependent proteases (Pike and Briggs, 1972). It was not inhibited by EDTA but was significantly inhibited by 2-mercaptoethanol and p-chloromercuribenzoate.(Yomo and Srinivasan, 1973). So on the basis of the inhibition study, raphanin must be classified as a metallo-protease and not affected by-SH inhibitors.

Thus, a neutral protease raphanin was purified to homogeneity from radish leaves by a simple purification procedure. It is expected that extensive application of the hydrolytic ability of the purified raphanin will be possible in medical and food industries. It may be used in cheese coagulation, bread making, leather industry, and use in medicine in the treatment of gastric bezoars and as blood clotting regulators.

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