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Production of Plant Proteinase from Jack Fruit (*Artocarpus integrifolis*) as a Source of Dairy Enzyme I. Isolation, Partial Purification and Some Properties

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Abstract: The aim of the present work was to search for a novel plant proteinase enzyme from Jack fruit (*Artocarpus integrifolis*) as a source of dairy enzymes that would be natural products which can be easily extracted at relatively low cost and no legal barriers. This enzyme was subjected to a purification scheme composed of ammonium sulfate fractionation followed by gel filtration on G-100 Sephadex column. The enzyme was purified 2.70-fold with a total yield of 23.77% of the original activity. There were relationship between temperature and incubation time, the enzyme activity increase was observed up to 55°C for 60 min reaction time and still constant thereafter. Proteinase was active over a broad temperature range retained about 37.4 and 24.9% of temperature activity at 35 and 80°C for 5 and 60 min. An energy of activation of 9.98 KJ mole⁻¹ for the enzyme activity was derived from the Arrhenius plot of initial velocity (V_o) across a temperature ranging from 40 to 55°C. The optimum pH was pH 7.5. The rate of thermal inactivation proceeded more rapidly at pH 7.0 and 8.0, when heating at 50°C for 60 min the enzyme activity lost about 95 and 92% its activity, respectively. Michaelis-constant of (K_m) values of 2.0 mg ml⁻¹ and a maximum initial velocity (V_{max}) of 0.75 μ moles mg⁻¹ when casein used as a substrate. A Molecular weight (MW) determination of ~22 kDa was estimated by gel filtration methods using a Sephadex G-100. Cu²⁺, K²⁺, Fe²⁺ and Zn²⁺ strongly inhibited the enzyme. However, Ca⁺⁺ slightly stimulated. EDTA, sodium azide, Sodium citrate and urea among the chemical reagents inhibited the proteinase activity.

Key words: Proteinase, purification, enzyme, enzyme characteristics, jack fruit (*Artocarpus integrifolis*)

Introduction

The plant proteinases and some microbial proteinases belong to the group contains sulfhydryl enzymes (SH), whose activity depends on the presence of one or more SH groups at the active side. Oxidizing agents, alkylating agents and heavy metal ions inhibit (Hartley, 1960). The proteinases of many plant as papain, chymopapain, carican and endoprotease papaya (cysteine protease) have been isolated and purified to homogeneity from commercially papaya (*Carica papaya* L.), proliferous top of pineapple (*Ananas comosus* L.), (*Bromelia plumieri*) and Cardosin B (*Cynara cardunculus*) (Azarkan *et al.*, 1996; Goodenough and Owen, 1987; Maksimenko *et al.*, 1990; Montes *et al.*, 1990 and Zimacheve *et al.*, 1994) and as well as in a large number of microorganisms (White and White, 1997). Among these are *Pseudomonas spp.* (Fernandez *et al.*, 1999; Koka and Weimer, 2000; Matta *et al.*, 1994 and Stepaniak *et al.*, 1982), Basidiomycetes fungi (Venables and Watkinson, 1989), Alkaline proteisase from actinomycetes and bacteria (Dolidze *et al.*, 1982; Gupta *et al.*, 2002) and proteinse from Rhizopus stolonifer, Rhizopus oryzae and Absidia corymbifera (Seong-11 *et al.*, 2002). Thiol proteinases from thermophilic fungus *Humicola lanuginosa* (Shenolikar and Stevenson, 1982). Plant proteinases are

interesting in food and medical usage's because they are natural products which can be easily extracted by aqueous infusion, no legal barriers and low cost (Silva and Malcata, 1999). These enzymes cause development of gelation and off flavor in milk, reducing its shelf life, and may be responsible for softening of curd and yield losses during cheese manufacture (Fairbairn and Law, 1986; Mottar, 1989; White and Marshall, 1973). Proteinases produced by psychrotrophic bacteria can withstand pasteurization (72°C for 15 sec), and treatment at ultrahigh temperatures (138°C for 2 sec), with important implications on the quality of milk and heat-treated dairy products (Cousin, 1982). Jack fruit (*Artocarpus integrifolis*) which planted recently in Egypt. Whereas, from preliminary experiments, it found several important enzymes in dairy field such as proteinase, β -galactosidase (Ismail *et al.*, 1999). The purpose of the present work was initiated to determine proteinase was produced within Jack fruit (*Artocarpus integrifolis*). To achieve this objective, proteinase was isolated, purified and characterized.

Materials and Methods

Jak fruit (*Artocarpus integrifolis*): Jak fruit was obtained from the Horticulture Institute, Agric. Res.

Centre, Ministry of Agriculture, Cairo, Egypt (Fig. 1 and 2).

Extraction of enzyme: For maximum extraction of enzyme was prepared according to the method of (El-Tanboly, 2001) as follows: Two handard grams of fresh Jak fruit (*Artocarpus integrifolis*) seeds were ground in a mixer with 1 l of 0.05 M phosphate buffer (pH 7.5) for 15 min at room temperature, then the mixture was separated by filtration using Whatman filter paper No. 4 to remove the precipitation and the filtrate solution was used as an enzyme extract for purification procedure.

Purification of crude enzymes: Three steps for achievement the purification of enzymes as following

Precipitation with ammonium sulfate: Ammonium sulfate precipitation was carried out according to Kaplan (1955). Ammonium sulfate namely 10, 20, 30, ... and 90% saturation was added to 100 ml of enzyme extract with rapid stirring using a magnetic stirrer at 4°C for 20 min. The formed precipitates were then centrifuged under cooling (4°C) at 4000 rpm for 15 min.

Dissolving and dialyzed of precipitate: Each precipitate was dissolved immediately in 5 ml 0.05 M phosphate buffer pH 7.5, dialyzed against the same buffer using cellulose bags and kept in refrigerator overnight. The resultant fractions were then tested for enzyme activity and protein content to identify the most suitable ammonium sulfate concentration for enzyme precipitation (Table 1).

Affinity chromatography and proteinase purification: Five ml of the dialyzed enzyme solution after ammonium sulfate precipitation (40-60% saturation) was added to the top of the gel bed in the column (45x2.5 cm²) of Sephadex G -100 (Pharmacia, Uppsala, Sweden) with bed volume 250 ml with the same buffer for purification. Fractions of 5 ml were collected at a flow rate of 1ml min⁻¹ and analysed for protein and proteinase activity. Enzyme fractions with high specific activity were pooled, and stored at 4°C and used in subsequent experiments.

Enzyme Assay: Proteinase activity was assayed by using tyrosine as standard as suggested by (Greenberg, 1957). Proteinase activity is expressed in units, where one unit of activity (U) is defined as the amount of enzyme required to release one µg of tyrosine under the standard conditions of pH and temperature employed.

Quantification of protein: Protein concentration of enzyme samples in each step was determined calorimetrically at 650 nm according to (Ohnisti and Barr, 1978) using Folin-Ciocalteu's reagent (Sigma). Bovine serum albumin was used as reference in the preparation of the calibration curve. Protein in column effluents was monitored by measuring the absorbency at 280 nm.

Buffers: All the buffers used in pH measurements were prepared according to (Gomori, 1955). Moreover, final pH was checked using pH-meter 646 with glass electrodes, Ingold, Knick, Germany. Purified proteinase properties.

Effect of temperature and incubation time: This was achieved by incubating the reaction mixture at various temperatures ranging between 35 to 80°C for different times 10, 15, 30, and 60 min, then enzyme activity was assayed at these different temperatures / times to define the optimum incubation temperature and optimum incubation time for proteinase activity. Energy of activation of proteinase was determined from the slope of an Arrhenius plot of activity measurements at temperature above mentioned.

Effect of pH values: Proteinase activity was measured at different pH values ranging from 3.2 - 9.0 to define the optimum enzyme pH with 0.2 N HCl or 0.2 N NaOH where appropriate and then buffered with citrate phosphate (pH 3.2-7.0), phosphate (pH 6.8-8.0) and Tris-HCl (pH 8.0-9.0) buffers. Moreover, final pH was checked using pH-meter 646 with glass electrodes, Ingold, Knick, Germany. Incubation temperature was 37°C for 30 min.

Thermal and pH stability: Aliquots of the enzyme in 0.05 M phosphate buffer at pH values 7.0 to 8.0 were heat treated for 10, 15, 30, and 60 min. in water bath set at different temperatures of 50, 55 and 60°C, followed by rapid cooling. They were all analyzed immediately for residual enzyme activity.

Michaelis-Menten constant: In this study, Stock solution of 1% case in were diluted with 0.05 M sodium phosphate buffer pH 7.5 to give 0.25-10 mg ml⁻¹ final concentration of the substrate in the reaction mixture (Matta *et al.*, 1994). The proteinase activity was determined as previously described under enzyme assay. Calculation of the Michaelis-Menten constant (km) was carried out by double reciprocal plot of (Lineweaver and Burk, 1934) straight line equation.

Molecular weight determination: The molecular weight (MW) of the purified enzyme was estimated by the gel filtration method of (Andrews, 1964) using Sephadex G-100 column (45x2.5 cm²) of under the same conditions;

these were bovine serum albumin (67 KDa), egg albumin (45 KDa), and trypsin inhibitor (8 KDa) were used as standard proteins.

Effect of metal ions and chemical reagents: Metal ions were used as chloride salts (Cu^{2+} , K^{2+} , Fe^{2+} , Ca^{2+} , Zn^{2+} , Na^{2+}) and urea, Ethylenediamine tetracetic acid (EDTA), sodium azide, Sodium citrate) as chemical reagents at a concentration of 1 mM. The remaining activity was measured under standard assay condition, and expressed as percentage of the control without additions.

Results and Discussion

Proteinase purification: The ammonium sulfate precipitated enzyme (40-60%) saturation on dialysis yielded 12.71% recovery and 1.92 -fold purification factor. The fractionation of the purified enzyme on sephadex G-100 gave four protein peaks (Fig. 3) one of which (peak B) showed protease activity. Fractions 12-19 were consequently pooled, desalted by dialysis against the same buffer and stored at 4°C and used in subsequent studies. A typical purification procedure show that the enzyme was purified 2.70-fold with a total yield of 23.77% of the original activity (Table 2).

Effect of temperature and incubation time: Fig. 4 illustrates the relationship between temperature and incubation time, the enzyme activity increase was observed up to 55°C for 60 min reaction time and still constant thereafter, which were considered as a the optimum temperature and optimum time. It is known that temperature increase the reaction velocity and also affects the rate of enzyme destruction, producing a gradual fall

the concentration of active enzyme. Proteinase was active over a broad temperature range retained about 37.4 and 24.9% of temperature activity at 35 and 80°C for 5 and 60 min. Fig. 5 is an Arrhenius plot showing two different slopes with a breakpoint around 55°C. Arrhenius activation energy was 9.98 KJ mol⁻¹ between 40 and 55°C. The obtained results are in agreement with (Zherebtsov and Shcheblykina, 1983; Fairbairn and Law, 1986). On the other hand, Baral *et al.* (1995) reported that the optimum temperature of 40°C for proteinase from *Pseudomonas tolaasii*. The activation energy was estimated to be 82 KJ mol⁻¹.

Effect of pH values: From the results on Fig. 6, the purified proteinase is alkaline with peak activity at pH 7.5. Proteinase was active in a wide pH range, with residual activities of 9.29 and 11.79% at pH 3.2 and 9. The optimum pH is similar to that of plant pathogen, *Pseudomonas tolaasii* (Baral *et al.*, 1995 and Zimacheve *et al.*, 1994) who found that the optimum pH for purified enzyme activity from *Ananas comosus* L. was 9.7.

Thermal and pH stability: The rate of thermal inactivation proceeded more rapidly at pH 7.0 and 8.0 than at pH 7.5. Thus, when heating at 50°C for 60 min the enzyme activity lost about 95, 92 and 83% of its activity at 7.0 and 8.0 than at pH 7.5, respectively (Fig. 7). At 60°C for 10 and 15 min, and pHs 7.0 and 7.5, the enzyme retained 3, 25, 2, 10% of its activity, respectively. In this respect the enzyme is similar to the protease purified from *Pseudomonas* spp. by (Stepaniak *et al.*, 1982; Koka and Weimer, 2000).

Table 1: A preliminary ammonium sulfate fractionation of proteinase from Jack fruit (*Artocarpus integrifolius*)

Ammonium sulfate saturation %	Volume of fraction (ml)	Proteinase activity (Unit mg ⁻¹)	Total activity (Units)	Protein content (mg ml ⁻¹)	Specific activity (Unit mg ⁻¹)	Recovery (%)	Purification factor
Initial extract	100	10.65	1065.4	0.207	51.47	100	1.0
0 - 10 % ppt.	5	6.86	34.34	0.645	10.65	3.22	0.21
10 - 20 % ppt.	5	5.47	27.36	0.405	13.51	2.57	0.26
20 - 30 % ppt.	5	5.96	29.84	0.525	11.36	2.80	0.22
30 - 40 % ppt.	5	11.04	55.21	0.534	20.68	5.18	0.40
40 - 50 % ppt.	5	9.11	45.55	0.202	45.09	4.28	0.88
50 - 60 % ppt.	5	7.92	39.62	0.072	110.05	3.72	2.14
60 - 70 % ppt.	5	2.40	12.01	0.159	15.10	1.13	0.29
70 - 80 % ppt.	5	0.41	2.08	0.274	1.52	0.20	0.03
80 - 90 % ppt.	5	1.06	5.33	0.316	3.38	0.50	0.07

Table 2: Purification of proteinase from Jack fruit (*Artocarpus integrifolius*)

Ammonium sulfate saturation %	Volume of fraction (ml)	Proteinase activity (Unit mg ⁻¹)	Total activity (Units)	Protein content (mg ml ⁻¹)	Specific activity (Unit mg ⁻¹)	Recovery (%)	Purification factor
Initial extract	100	10.654	1065.4	0.207	51.47	100	1.0
40 - 60 % ppt.	10	13.545	135.45	0.137	98.87	12.71	1.92
Gel filtration on Sephadex G-100	65	3.896	253.24	0.028	139.14	23.77	2.70



Fig. 1: Outer shape of Jack fruit (*Artocarpus integrifolius*)



Fig. 2: Horizontal cutting of jack fruit (*Artocarpus integrifolius*)

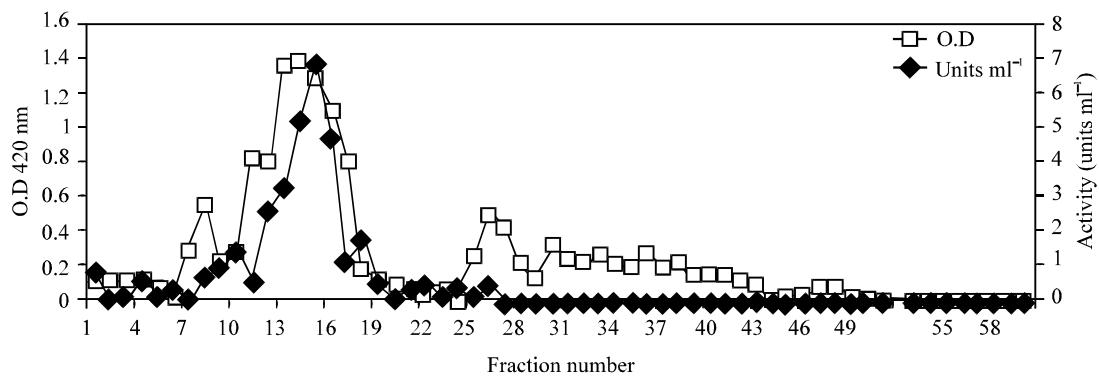


Fig. 3: Purification of proteinase precipitation by ammonium sulfate on Sephadex G-100 from Jack fruit (*Artocarpus integrifolius*)

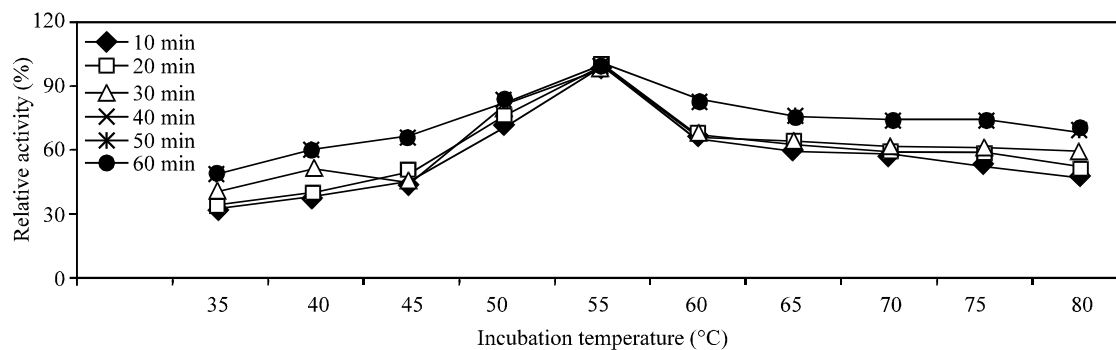


Fig. 4: Effect of temperature and incubation time on purified proteinase activity from Jack fruit (*Artocarpus integrifolius*)

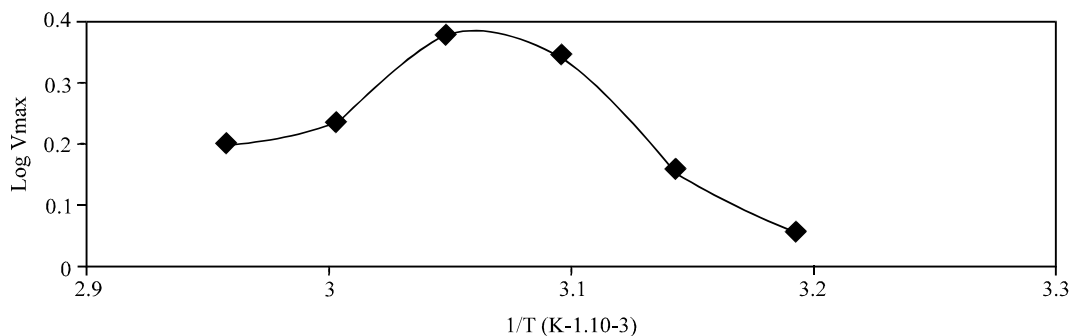


Fig. 5: Arrhenius plot for the casein hydrolysis reaction by purified proteinase from Jack fruit (*Artocarpus integrifolis*)

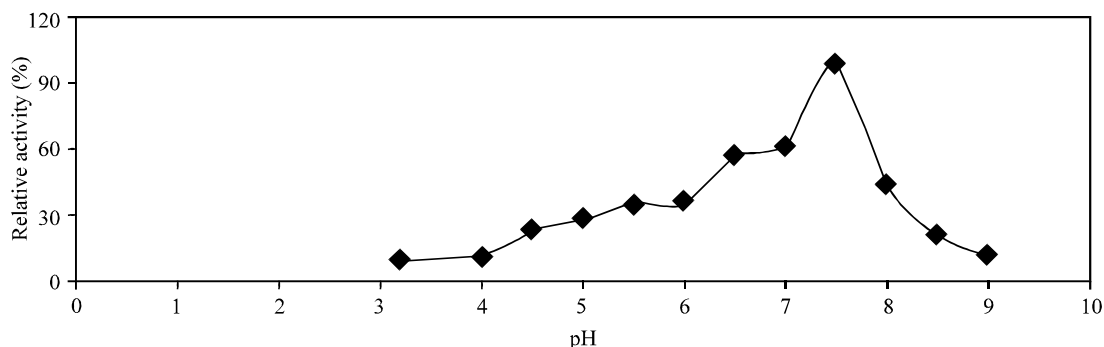


Fig. 6: Effect of pH on activity of the purified proteinase from Jack fruit (*Artocarpus integrifolis*)

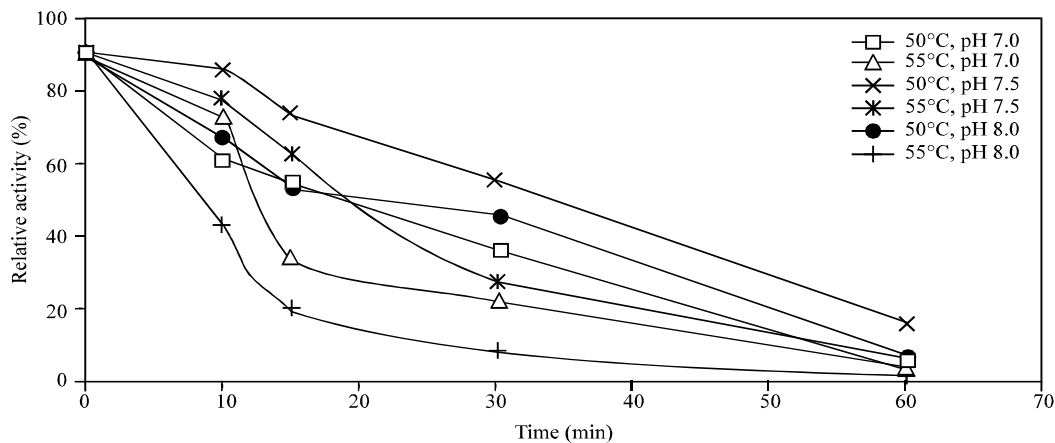


Fig. 7: Effect of thermal and pH stability of purified proteinase activity from Jack fruit (*Artocarpus integrifolis*)

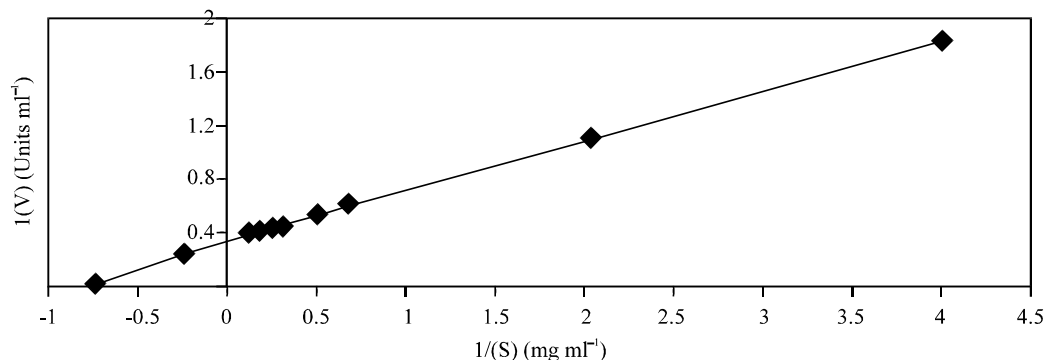


Fig. 8: Line weave-Brk plot of the reaction velocity versus substrate concentration for the purified proteinase from Jack fruit (*Artocarpus integrifolis*)

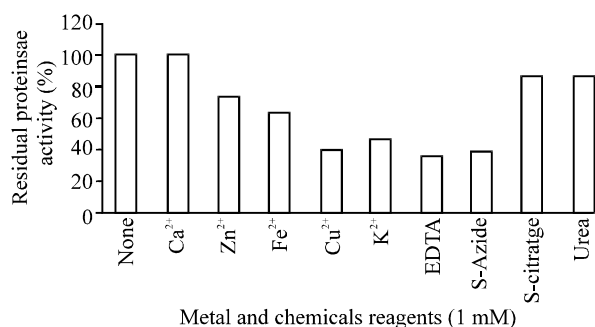


Fig. 9: Effect of metal ions and chemicals reagent on the activity of purified proteinase from Jack fruit (*Artocarpus integrifolius*)

Michaelis–Menten constant: The K_m of purified proteinase was approximately 2.0 mg ml^{-1} and the V_{max} of the reactions was $0.75 \mu \text{ moles mg}^{-1}$ when casein used as a substrate suggesting a wide specificity of enzyme towards different substrates (Fig. 8). Similarly K_m values for proteinase from *Pseudomonas sp.* AFT-36 and *Pseudomonas fluorescens* INIA 745 (Fernandez *et al.*, 1999; Matta *et al.*, 1994 and Stepaniak *et al.*, 1982).

Molecular weight: The purified proteinase was found to be a homogeneous preparation of molecular weight 22 kDa as estimated by gel filtration on a column of Sephadex G-100 using molecular weight standards. Considerable similarity was observed for the molecular weights reported for *Carica papaya* plant 23 kDa from papain and 24 kDa from chymopapain (Goodenough and Owen, 1987). On the other hand it was lower than those reported for other *Pseudomonas* spp. proteinases aspartic proteinase from sunflower seeds (Baral *et al.*, 1995; Matta *et al.*, 1994 ; Stepaniak *et al.*, 1982 and Hyekeong *et al.*, 2001).

Effect of metal ions and chemical reagents: The proteinase activity was inhibited to the extent of 59.46, 52.36, 36.82 and 25%, respectively in the presence of by Cu^{2+} , K^{2+} , Fe^{2+} and Zn^{2+} . However, calcium had a slight stimulating effect (Fig. 9). Stimulating effect of Ca^{2+} on proteinase activity may be attributed to the stabilization of the enzyme by this cation (Mckellar and Cholette, 1985 and Muderriszade *et al.*, 2001). EDTA and sodium azide among the chemical reagents inhibited the proteinase activity by 62.28 % and 60.42%, respectively, Inhibition by EDTA suggest that the enzyme is a metalloproteinase (Mitchell and Marchall, 1989; Stepaniak *et al.*, 1982) while Sodium citrate and urea exhibited 12.5 and 13.85 inhibitions. Inhibitions of proteinase by urea may be attributed to usual denaturation of the enzyme.

General Conclusion: Jack fruit (*Artocarpus integrifolius*) produces a heat-stable proteinase which can be purified to homogeneity by sequential use of ammonium sulfate precipitation and gel filtration chromatography on Sephadex G-100. Plant proteinases are interesting in food usage's such as the curing of cheese, the aging of meat, and production of protein hydrolyzates because they are natural products which can be easily extracted by aqueous infusion, no legal barriers and low cost.

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