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UTILIZATION OF PLANT PROTEINASE FROM JACK FRUIT (*Artocarpus integrifolis*) TO ACCELERATION OF RAS CHEESE SLURRY.

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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 photolytic enzymes to acceleration of Ras cheese slurry. Plant proteinase 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 relationships 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 rang 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 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% respectively. Michaelis-constant of (K_m) values of 2.0 mg/ml and a maximum initial velocity (V_{max}) of 0.75 µ moles/mg when casein used as a substrate. Molecular weight (MW) determination of ~22 k Da was estimated by gel filtration methods using a Sephadex G-100. Cu^{2+}, K^{2+}, Fe^{2+} and Zn^{2+} strongly inhibited the enzyme. However, Ca^{++} slightly stimulated. EDTA, sodium azide, Sodium citrate and urea among the chemical reagents inhibited the proteinase activity.

Crud extracted proteinase was used to accelerate Ras cheese slurry ripening with concentration of 1 and 2 ml/100 g curd. Slurries were incubated at 37°C for 7 days. The results indicated that the ripening indices of slurries (SN/TN, tyrosine and tryptophane) gradually increased as rate of enzyme increased and as ripening period progressed. Also, flavour of all slurries gradually improved during incubation period. At the end of incubation period slurry with 2 ml/100g curd had a high flavour scoring.

Keywords: Proteinase; Purification; Enzyme characteristics; Jack fruit, *Ras cheese slurry*, acceleration

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. 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); 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); Stepaniak *et al.*, (1982)), Basidiomycetes fungi (Venables and Watkinson, (1989)), Alkaline proteinases of actinomycetes and Thiol proteinases from thermophilic fungus *Humicola lanuginosa* (Dolidze *et al.*, (1983)). 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); White and Marshall, (1973)). Proteinases produced by psychrotrophic bacteria can withstand pasteurization (72^{o}C for 15 s), and treatment at ultrahigh temperatures (138^{o}C for 2 s), with important implications on the quality of milk and heat-treated dairy products (Cousin *et al.*, (1982)). Jack fruit (*Artocarpus integrifolis)* which planted recently in Egypt, was found as a new source of several important enzymes in dairy field such as proteinase, B-galactosidase (Ismail *et al.*, (1999)). The purpose of this study aimed to the extraction and characterized of protease enzyme from Jack fruit. The enzyme was used to accelerate of Ras cheese slurry .

MATERIALS AND METHODS

Materials

Jak fruit (Artocarpus integrifolis):

Jak fruit was obtained from the Horticulture Institute, Agric. Res. Centre, Ministry of Agriculture, Cairo, Egypt.

Extraction of enzyme

Extraction of enzyme was prepared according to the method of (El-Tanboly, 2001)

Purification of crude enzymes

Three steps for achieve the purification of enzymes as following:

1- Precipitation with ammonium sulfate

Ammonium sulfate precipitation was carried out according to (Colowick and 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.

2-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).

3- Affinity chromatography and proteinase purification

Five milliliters 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 cm^2) 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 and analyzed 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 according to (Hindazlotnik *et al.*, 1983) 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 colorimetrically at 650 nm according to (Ohnistti 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 (Gomeri, 1955). Moreover, final pH was checked using pH-meter 646 with glass electrodes, Ingold, Knick, Germany.

Purified proteinase properties:

a- 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.

b-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.

c-Thermal and pH stability

Aliquots of the enzyme in 0.05 M phosphate buffer at pH values 7.0 t0 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.

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

Ammonium sulfate saturation (%)	Volume of fraction (ml)	Proteinase activity (Unit /ml)	Total activity (Units)	Protein content (mg/ml)	Specific activity (Unit /mg)	Recovery (%)	Purification factor
Initial extract	100	10.654	1065.4	0.207	51.47	100	1.0
0 - 10 % ppt.	5	6.869	34.345	0.645	10.65	3.22	0.21
10 - 20 % ppt.	5	5.473	27.365	0.405	13.51	2.57	0.26
20 - 30 % ppt.	5	5.969	29.845	0.525	11.36	2.80	0.22
30 - 40 % ppt	5	11.042	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	7.924	39.62	0.072	110.05	3.72	2.14
60 - 70 % ppt.	5	2.401	12.01	0.159	15.10	1.13	0.29
70 - 80 % ppt.	5	0.417	2.085	0.274	1.52	0.20	0.03
80 - 90 % ppt.	5	1.067	5.335	0.316	3.38	0.50	0.07

Table (2) Purification of proteinase from Jack fruit (*Artocarpus integrifolis*).

Ammonium sulfate saturation (%)	Volume of fraction (ml)	Proteinase activity (Unit /ml)	Total activity (Units)	Protein content (mg/ml)	Specific activity (Unit /mg)	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

Michaelis–Menten constant

In this study, Stock solution of 1% casein were diluted with 0.05 M sodium phosphate buffer pH 7.5 to give 0.25-10 mg/ml 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 straight line equation (Lineweaver and Burk, 1934) .

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 cm^2) of under the same conditions. 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 teteracetic 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.

Preparation of Ras cheese slurry

Ras cheese slurry was prepared conventionally as described by Hofi et al.,(1973) which were:

I. as a control
II. with crud extract 1 ml/100g curd
III. with crude extract 2 ml/100 g curd.

All treatments were blended with 4% sterilized NaCl solution, 0.5 % potassium sorbate and incubation of slurry at 37°C for 7 days with daily agitation. Three replicates were made from each treatment, and each observation was the mean of two determinations.

Slurries were analyzed when fresh and after 3,5 and 7 days for chemical composition and flavour evaluation.

Methods of analysis of slurry:

Slurries of all treatments were analyzed for acidity, pH , moisture, total nitrogen and soluble nitrogen according to A.O.A.C.(1990). Soluble tyrosine and tryptophane content were measured according to Voakeleris and Price (1959). Flavour properties (50 points) were carried out for all slurries by staff members of Dairy Lab. National Research Center.

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 Table (2). The fractionation of the purified enzyme on sephadex G-100 gave four protein peaks (Fig. 1) 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

Figure (2) 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 of the concentration of active enzyme. Proteinase was active over a broad temperature rang retained about 37.4 and 24.9% of temperature activity at 35 and 80□C for 5 and 60 min respectively. Figure (3) is an Arrhenius plot showing two different slopes with a breakpoint around 55°C. Arrhenius activation energy was 9.98 KJ/mol 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.

Effect of pH values

From the results on Figure (4), 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); 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 pH 7.0 and 8.0 than at pH 7.5, respectively (Fig. 5). At 60°C for 10 and 15 min, and pH 7.0 and 7.5, the enzyme retained 3%, 25% and 2%, 10% of its activity, respectively. In this respect the enzyme is similar to the protease purified from *Pseudomonas* spp. by Stepaniak and Fox, (1982); Koka and Weimer, (2000).

Michaelis–Menten constant

The K_m of purified proteinase was approximately 2.0 mg/ml and the V_{max} of the reactions was 0.75μ moles/mg when casein used as a substrate suggesting a wide specificity of enzyme towards different substrates (Fig. 6). 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 & Fox, (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 k Da from chymopapain (Goodenough and Owen, 1987). On the other hand it was lower than those reported for other *Pseudomonas* spp. proteinases (Baral *et al.*, (1995); Matta *et al.*, (1994) and Stepaniak & Fox, (1982)).

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 Cu^{2+}, K^{2+}, Fe^{2+} and Zn^{2+}. However, calcium had a slight stimulating effect (Fig. 7). Stimulating effect of Ca^{2+}on proteinase activity may be attributed to the stabilization of the enzyme

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الاستفادة من إنزيم البروتييز النباتي المستخلص من نبات الجاك فروت في إسراع تسوية معلق الجبن الراس

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تم دراسة استخلاص إنزيم البروتييز من مصدر نباتي جديد (نبات الجاك فروت) والاستفادة منه في إسراع تسوية معلق الجبن الراس، ومن مميزات البروييز النباتي انه منتج طبيعي سهل الاستخلاص بتكلفة قليلة ولا توجد موانع للاستفادة منه.

تم استخلاص الإنزيم من نبات الجاك فروت في محلول منضم من الفوسفات وتنقيته بواسطة كبريتات الأمونيوم على عمود الجل Sephadex G-100 حيث أوضحت النتائج ان معامل درجة تنقية الإنزيم ٢,٧ مرة من المستخلص الانزيمي الخام ونسبة استرداد النشاط الكلي ٢٣,٧٧% . كما تم دراسة خواص الإنزيم المنقي وكانت درجة حرارة التحضين المثلي لنشاطه على ٥٥ $^{\circ}$م لمدة ٦٠ دقيقة ،ودرجة الأس الهيدروجيني المثلى ٧,٥ .وبلغ ثابت ميكالس ٢,٠ مل جرام لكل ملي مستخلص بينما الوزن الجزيئي للأنزيم حوالي ٢٢ كيلو دالتون. وكان لكل من النحاس والحديد و الزنك تأثير مثبط شديد على نشاط الأنزيم

وتم إضافة مستخلص الأنزيم لمعلق الجبن الراس بنسبة ١ مل– ٢ مل لكل ١٠٠ جرام خثرة وتحضين الخثرة علي ٣٧ م لمدة ٧ أيام ودراسة تحلل البروتين والنكهة. وقد أظهرت النتائج زيادة في قيم البروتين الذائب والتيروزين والتربتوفان بزيادة نسبة الأنزيم المضاف. كما حصلت النكهه على درجات أعلى بزيادة فترة التحضين عن عينة المقارنة. وكان من النتائج المتحصل عليها أنه يمكن استخدام أنزيم البروتييز النباتي من ثمرة الجاك فروت بنسبة ٢مل لكل ١٠٠ جرام خثرة الجبن الراس للحصول على نكهة جيدة.