April 2009 *Volume 3 Issue 2*

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 3(2), 2009 [63-69]

Isolation and characterization of protease from the skin of mature papaya (*Carica papaya* L.)

Niranjan Kumar Sana*[1], Md.Rezaul Karim[1], Bidhan Chandra Sarkar[1], Goutam Kumar Sarker[1], Md.Entazul Huque[2], Ranajit Kumar Shaha[1]

[1]Department of Biochemistry and Molecular Biology, The University of Rajshahi, Rajshahi-6205, (BANGLADESH)

[2]Department of Chemistry, The University of Rajshahi, Rajshahi-6205, (BANGLADESH)

Tel : 750041-750049/4109; Fax: 88-0721-750064

E-mail : nksana2003@yahoo.com

Received: 6th January, 2009 ; Accepted: 11th January, 2009

ABSTRACT

A proteolytic enzyme was purified from the skin of papaya (*Carica papaya* L.) using gel filtration on Sephadex G-100 followed by ion exchange chromatography on DEAE-cellulose to the homogeneous state as confirmed by slab gel electrophoresis. The molecular weight of the purified enzyme was estimated to be 25 kDa and 24 kDa by gel filtration chromatography and SDS-PAGE, respectively. The purified enzyme showed maximum activity at pH 8.0 and at temperature 44^0C. The enzyme was able to hydrolyze casein, azoalbumin, azocasein, hemoglobin and gelatin with high specific activity but keratin and collagen was found to be not degraded. Metallic ions Mg^{2+}, Mn^{2+}, Cd^{2+} and Li^{+} have no effect on proteolytic activities but EDTA, urea, Ca^{2+} and Zn^{2+} have an activator effect. Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+} and Ag^{+} have strong inhibitory effect on proteolytic activities. The K_m value of the enzyme was found to be 45 μM for casein as substrate.

KEYWORDS

Papaya (*Carica papaya* L.);
Protease;
Casein;
Purification;
Characterization.

INTRODUCTION

The papaya (Carica papaya) is an important fruit of tropical and subtropical regions of the world. It is a native tropical fruit of America and was introduced in India and Bangladesh in the 16th century. Papaya is one of the cheapest but most nutritious fruit available round the year in Bangladesh. It is an excellent source of vitamin A and C[1]. Nutritionally, papaya is a good source of calcium, iron, potassium, magnesium and sodium[2]. It also contains small amount of carbohydrate, protein and fat. It contains a proteolytic enzyme which sooths the stomach and aids in digestion. The proteases are useful in various ways and their applications are increasing at a fantastic rate. Protease enzyme is very important to play some physiological role during seed maturation and renascence of fruits[3]. They are capable of degrading proteins. Some have hydrolytic functions to degrade polypeptides and proteins for digestive and nutrition purpose. Proteases of subtilisin group are used

FULL PAPER

in the treatment of burns and wounds. Oral administration of proteases produces an anti-inflammatory response in burn patients and speeds up the process of healing[4]. The growth and development of pulse bettle insects require very especially metabolic processes, which involve the action of specialized proteases and peptides[5]. The proteloytic enzymes present in these insects are responsible for the rapid destruction of the pulses[6]. It has also a great importance in medicinal and industrial purpose. Proteases are used in the degumming of silk goods, in the manufacture of liquid glue, in the preparation of cosmetics, detergents, in the meat tenderization, in the preparation of cheese, in medicine preparation and in agriculture as growth promoters[7,8]. Extensive studies have been performed on the purification and characterization of protease from different origins such as from the plant tissues[9], seeds[10], latex of Ervatamia coronaria[11], Ipomoea carnea spp.[12], Vasconcellea spp.[13], Euphorbia milii[14] and Wrightia tinctoria[15] and microorganism Chryseobacterium taeanense TKU001[16].

Till now no detail studies about the proteolytic enzyme from papaya skin was found in the literature. There is an attempt for purification of protease enzyme from the skin of mature papaya. In this experiment, we used one of the varieties of papaya (Bari papaya-1) due to it . s availability in Rajshahi Zone, for enzyme purification and characterization.

MATERIALS AND METHODS

Materials

One of the varieties of papaya (Carica Papaya L.), Bari papaya-1 was collected from a local horticulture of Rajshahi and Bangladesh Agriculture research Institute (BARI), Ishwardi, Pabna, Bangladesh. The papayas were stored in a deep freeze (-100C) for further experimental purpose. BSA, SDS/PAGE-chemicals and Sephadex G-100 were purchased from Sigma Chemicals Ltd., USA. Standard proteins, DEAE-cellulose were purchased from Pharmacia Fine Chemicals Ltd., Sweden. All other chemicals used were of analytical grade.

Preparation of crude enzyme extract

The skin of green papaya (300 gm) was weighted, cut into small pieces and ground in a pre-cooled mortar with a pestle and homogenized with 50 ml of cold 5 mM phosphate buffer (pH 7.0) using a homogenizer. The suspension was then filtered through few layers of cheesecloth in a cold room. The filtrate was collected and clarified further by centrifugation in a refrigerated centrifuge at 6000 rpm for 15 minutes at 4^0C. The clear supernatant was collected and saturated to 100% by adding solid ammonium sulfate with gentle stirring. The precipitate was collected by centrifugation at 6000 rpm for 10 minutes at 4^0C. Then the precipitate was dissolved in minimum volume of pre-cooled distilled water, and dialyzed against distilled water for 12 hours and against 5mM phosphate buffer, pH 7.0 for overnight at 4^0C. It was again centrifuged at 6000 rpm for 10 minutes to remove any insoluble material and the clear supernatant was used as crude enzyme extract.

Measurement of protease activity

The protease activity was measured following the method of Kunitz[17]. The milk protein casein was used as a substrate. The activity is determined by detecting the release of amino acid, tyrosine by protease. The amount of tyrosine released was calculated from the standard curve constructed with tyrosine. The activity was expressed as the amount of tyrosine in μ mole produced per minute at 450C. The specific activity was expressed as the number of enzyme unit per mg of protein per unit time.

Purification of enzyme

Gel filtration column chromatography

The crude extract after dialysis with 5 mM phosphate buffer at pH 7.0, 4^0C was loaded onto a Sephadex G-100 column (2.5×120 cm) previously equilibrated with the same buffer. The column was eluted with 5 mM phosphate buffer, pH 7.0, at a flow rate of 1.0 ml min^{-1}. Absorbance of each fraction at 280 nm, protease activities and protein concentration were measured and the active fractions were collected.

DEAE-cellulose column chromatography

The enzymatically active protein fractions after gel filtration were collected and dialyzed against 5mM phosphate buffer, pH 7.8 for overnight and then applied to a DEAE-cellulose column (32×1.0 cm, flow rate 25 ml

h-1) previously equilibrated with 5mM phosphate buffer, pH 7.8 and eluted with the same buffer containing a linear gradient of NaCl (100-500 mM). Absorbance at 280 nm, protein concentration and protease activities were determined and the active fractions were collected.

Molecular weight determination of protease

(a) The molecular weight of the purified protease was determined by gel filtration on Sephadex G-100 column (150×3.0 cm) equilibrated with 5mM phosphate buffer, pH 7.0, following the established procedure[18]. Trypsin inhibitor (12 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), phosphorylase-b (97 kDa) and β-galactosidase(116 kDa) were used as marker proteins[19].

(b) Electrophoresis : SDS-PAGE was performed according to the method of Laemmli[20] on a Bio-rad mini electrophoresis system. The standard proteins used were β-lactoglobulin (18.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa) and phosphorylase-b(97.4 kDa). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

Optimum pH of the enzyme

To study the effect of pH on enzyme activity, the enzyme solutions (0.6%) were dialyzed against 5mM buffer of different pH (pH 2.0-3.0, CH_3COONa-HCl; pH 4.0 - 5.0, CH_3COONa-CH_3COOH; pH 5.5-8.0, NaH_2PO_4-Na_2HPO_4; pH 8.5-9.0, $Na_2B_4O_7$ - HCl; pH 9.5, Na_2B_4O7 Na_2CO_3) for 24 hours with frequent change of buffers. After necessary adjustment of pH by adding 0.1N HCl or 0.1N NaOH, the enzyme activities were assayed using casein as substrate.

Optimum temperature of the enzyme

In order to determine the optimum temperature, the enzyme solutions (0.5%) in 5mM phosphate buffer, pH 7.0, were incubated at various temperatures ranging from 100C -90^{0}C for 15 minutes in a temperature controlled water bath and the enzyme activities were assayed.

Substrate specificity

To determine the substrate specificity of the enzyme casein, azoalbumin, azocasein and hemoglobin were used as substrate during the assay and the proteolytic activities were measured following the method of Kunitz[17].

Influence of metal ions and chemical reagents

The effects of metal ions and chemical reagents on the enzyme activity were examined by incubating the enzyme solution at room temperature in the presence of different ion or compound for 5 minutes and aliquots were withdrawn and assayed under standard reaction conditions (pH 7.0, Temperature 37^{0}C).

Measurement of Km of enzyme

Michaelis constant (Km) was determined by the assay of enzyme activity for various concentration of the substrate (casein) at definite interval. Initial velocities of respective substrate concentration were calculated. Km was calculated from Lineweaver-Burk double reciprocal plot[21].

Protein assay

Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280 nm. The amount of protein was estimated by the published method of Lowry et al.[22], using BSA as a standard substrate.

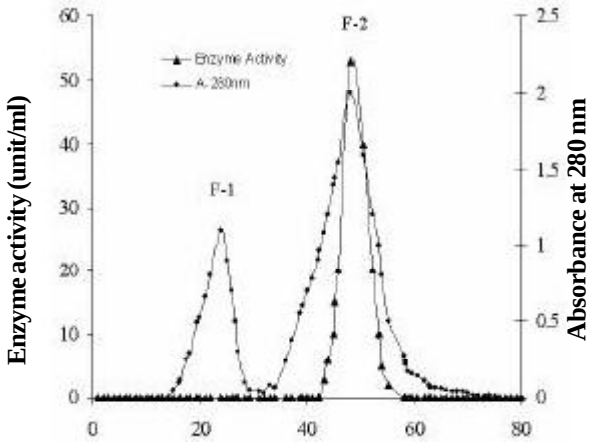

Figure 1: Gel-filtration pattern of crude extract of the skin of mature papaya on Shephadex G-100 column chromatography (2.5×120cm). The column was pre-equilibrated with5mM sodium phosphate buffer, pH 7.0 and the column was eluted with same buffer. The flow rate of the column was 1ml/min. Symbols: (•) absorbance at 280nm, (▲) enzyme activity

FULL PAPER

RESULTS AND DISCUSSION

Purification of protease

In gel filtration column, the crude protein extract from the skin of papaya was eluted as two main peaks namely F-1 and F-2 (figure 1). It was found that only F-2 fraction showed the proteolytic activity while the F-1 peak showed no activity. Hence F-1 fraction was not used for further investigation. The fraction F-2 containing the proteolytic activity was pooled separately and the purity was checked by the slab gel electrophoresis. As shown in the figure (figure 3), F-2 fraction gave more than one band indicating that F-2 fraction contained more than one protein.

The active fraction F-2 from gel filtration when applied to DEAE-cellulose column, were separated into three peaks F-2a, F-2b and F-2c (figure 2). The enzymatic activity of all these fractions were investigated and it was found that the fraction F-2a contained proteolytic activity while the fraction F-2b and F-2c possessed no proteolytic activity. The fraction contained protease activity pooled separately and was found homogenous on slab gel electrophoresis and showed single protein band (figure 3) indicating that the enzyme was in pure form.

The data on purification of protease from the skin of papaya are presented in TABLE 1. The purity of the enzyme from papaya increased 39.71 fold with an overall yield of 16.17% with specific activity of 87.75 unit/mg. The decrease in yield may be due to denaturation of the enzyme during purification or to some other unknown reasons.

Characterization of protease

Determination of molecular weight

The molecular weight of purified protease (F-2a) as determined by comparing their elution volume on Sephadex G-100 were found to be 25 kDa. The molecular weight of the papaya protease obtained in this investigation is in reasonable agreement with the molecular weight estimated for protease from other sources. Ma et al.,[23] purified a protease from marine yeast with molecular weight of 32 kDa, Turkiewicz et al.[24], purified a serine protease from Euphausia superba Dana

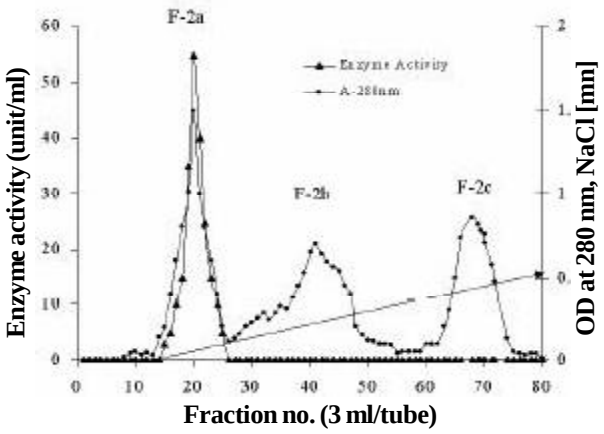

Figure 2: DEAE-cellulose column chromatography of papaya protease from F-2 fraction obtained from gel filtration. The column (32×1.0 cm) was pre-equilibrated with 5mM phosphate buffer, pH 7.8 and was eluted with the same buffer. The column was then eluted with a linear gradient of NaCl (0.1 to 0.5 M) in the same buffer. The flow rate of the column was 25 ml/h. Symbols: (●) absorbance at 280 nm, (▲) enzyme activity and (▸) NaCl gradient

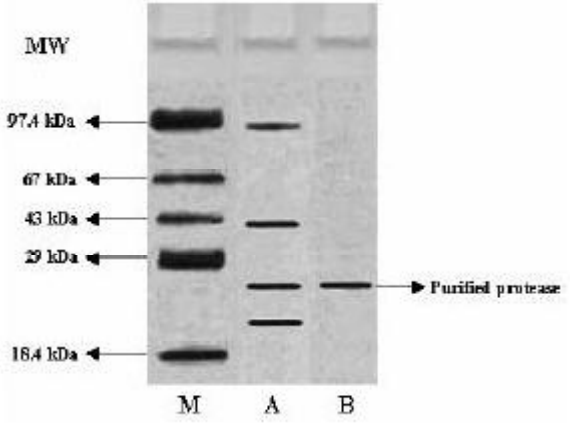

Figure 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified protease and marker proteins for the determination of purity and molecular weight of the enzyme; Lane-A: After gel filtration column, Lane-B: After DEAE-cellulose column, Lane-M: Marker protein solution containing Phosphorylase B (97.4 kDa), Bovin Serum Albumin (67.0 kDa), Ovalbumin (43.0 kDa), Carbonic anhydrase (29.0 kDa), □β-lactoglobulin (18.4 kDa).

TABLE 1: Summary of papaya protease purification

Steps of purification	Total protein (mg)	Total activity (unit/mg)	Specific activity (unit/mg}	Yield (%)	Purification folds
Crude extract	460	1015	2.21	100	1
Ammonium sulfate saturated	370	890	2.41	87.68	1.09
Gel-filtration chromatography	10.12	294.19	29.07	28.98	13.46
DEAE-cellulose fraction	1.87	164.09	87.75	16.17	39.71

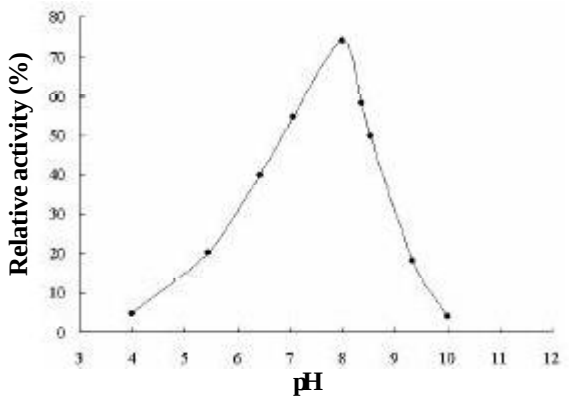

Figure 4: Effect of pH on the activity of protease from the skin of mature papaya

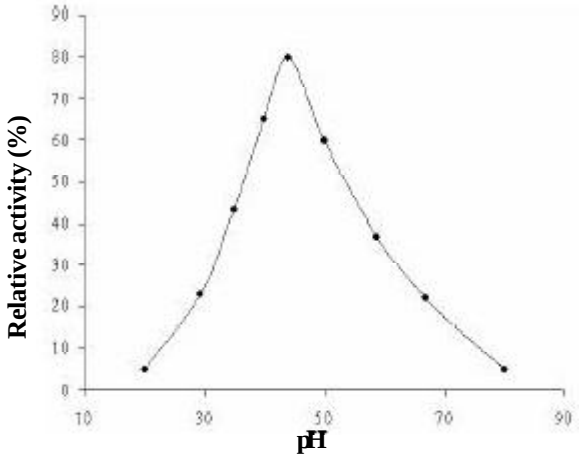

Figure 5: Effect of temperature on the activity of protease from the skin of mature papaya

TABLE 2 : Substrate specificity of protease

Substrate	Relative activity (%)
Casein	100
Azoalbumin	97
Azocasein	92
Hemoglobin	65
Gelatin	55
Keratin	00
Collagen	00

The activity for casein was taken as 100%

(*Antarctic krill*) of molecular weight of 33 kDa and Wang et al.[25], purified a protease from Bacillus cereus with molecular weight of 35 kDa by gel filtration. The molecular weight of the purified protease was also determined by SDS-polyacrylamide slab gel electrophoresis and was found to be 24 kDa (figure 3). The molecular weight of papaya protease is similar to that of proteases from Trichoderma atroviride (24 kDa)[26], latex of Ervatamia coronaria (27.6 kDa)[11] and latex of Calotropis procera (28.8 kDa)[27]. The molecular weight of the enzyme was found to be unchanged in the presence or in the absence of β-mercaptoethanol indicating that the protease contained no subunit.

Effect of pH on the activities of protease

The purified protease gave maximum activity at pH 8.0 (figure 4). From the result it might be concluded that the protease isolated from the skin of mature papaya belong to the category of alkaline protease. Very similar pattern of pH profile have been reported for the proteases from the latex of Euphorbia milli[14], latex of Ervatamia coronaria[11] and Chryseobacterium taeanense [16]. The activity was found to decrease gradually in the acidic pH, but in the alkaline pH it decreased rapidly. Very negligible enzyme activity was observed below pH 3.0 and above pH 10.0. Anwar and Saleemuddin observed the optimum pH 11.0 for protease from larvae of Spilosoma oblique[28], which is higher than the present finding. Patel et al.[12], reported the optimum pH 6.5 for protease from Ipomoea carnea, which is lower than the present finding.

Effect of temperatures on the activities of protease

The effect of temperature on the activity of the protease from the skin of mature papaya was examined in the range of 10-90^{0}C. The optimum activity of the protease was observed at 44^{0}C (figure 5). There was a sharp increase in activity with gradual increase in temperature up to 44^{0}C while the activity gradually decreased with further rise in temperature indicating the loss in active conformation of the enzyme. Very little activity was found at or above 90^{0}C and at or below 10^{0}C. These results are in accordance with those previously published for protease from *Candida Pulcherrima*[29] and protease from *Ustilago maydis*[30]. Tomar et al.[15], observed optimum temperature of 70^{0}C for protease from the latex of the plant Wrightia tinctoria (Roxb.) which is higher than our result.

Substrate specificity

The substrate specificity of the protease was studied using some proteins as substrate and the results are summarized in TABLE 2. The enzyme was able to hydrolyze casein, azoalbumin, azocasein, hemoglobin and gelatin with high specific activity but keratin and collagen were not degraded. This finding is in good agree-

FULL PAPER

TABLE 3 : Effect of various metallic salts and chemicals on the activity of protease

Reagent	Relative activity (%)		
	1mM	3mM	5mM
None	100	100	100
$FeCl_2$	05	04	01
$MgCl_2$	93	85	78
AgCl	07	06	05
LiCl	85	78	55
$MnCl_2$	100	98	91
$CuCl_2$	10	05	03
$ZnCl_2$	101	102	105
$HgCl_2$	01	00	00
$CdCl_2$	64	59	42
$FeCl_3$	09	08	07
$CaCl_2$	110	115	120
EDTA	104	112	116
Urea	102	105	106

ment with those reported by Gotoh et al.[29] and Mercado-Flores et al.[30].

Effect of metal ions and organic compounds

The effect of various metallic salts and chemicals on the activity of purified protease from the skin of mature papaya is presented in TABLE 3. The activity of the enzyme gradually increased with the increase in concentration of calcium. From the result it may be suggested that calcium is needed for maintaining the enzyme molecule in the optimum configuration for maximum activity and stability.

Metallic ions like Mg^{2+}, Mn^{2+}, Cd^{2+} and Li^{+} showed no effect on proteolytic activities while EDTA, urea, Ca^{2+} and Zn^{2+} showed an activator effect. Fe2+, Fe3+, Hg^{2+}, Cu^{2+} and Ag^{+} had strong inhibitory effect on proteolytic activities. The enzyme present in the skin of papaya may thus be a characteristic of cysteine type proteolytic enzyme, specifically for the enhancement of activity by EDTA. Ahmed et al.[31], reported that proteases from the larval gut of *Spodoptera litura*, which were not inhibited by Mg^{2+} and Mn^{2+} while the presence of Fe^{2+} and Hg^{2+} completely inhibited the three enzymes. Ma et al.**[23]**, reported that an alkaline protease from the marine yeast, was inhibited by Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and Co^{2+}. Cazzulo et al.**[32]**, reported that the protease from *Trypansoma cruzi*, was activated by urea. Darby et al.[33] and Abe et al.[34], also reported that the proteolytic activity is increased in the presence of EDTA and Ca^{2+}. These reports are in support of the present results. The increase of enzyme activity in the presence of urea and EDTA is difficult to explain at this stage. The enhancement of enzyme activity observed with different concentrations of urea and EDTA may probably be due to the unfolding effect on casein (substrate) thus making it a better substrate.

Km values of protease

The Km value of protease using casein as substrate was calculated from Lineweaver-Burk plot and found to be 45 μM. The Km value of protease from the latex of the plant Wrightia tinctoria (Roxb.)[15], has been reported to be 50 μM which is very close to our result and from the latex of Cryptolepis buchanani reported to be 10 μM[35] which is lower than the present finding.

ACKNOWLEDGMENTS

The authors wise to thank the research facilities provided by the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh.

REFERENCES

[1] J.A.Duke, A.A.Atchley, B.R.Christie; 'Proximate Analysis, The Handbook of Plant Science in Agriculture', CRC Press, Inc., Boca Raton, FL, **(1984)**.

[2] H.Y.Nakason, R.E.Paull; Papaya, H.Y.Nakason, R.E.Paul; 'Tropical Fruit', CAB International Press, Wallingford. UK, 239-269 **(1998)**.

[3] F.Hashinaga, D.Sawa, S.Itoo; J.Japan Soc.Hort. Sci., **47**, 282-288 **(1978)**.

[4] P.C.Shaw; Br.J.Clin.Prac., **23(1)**, 25-26 **(1969)**.

[5] D.Sikder; Study on growth and development of pulse bettle insects and the action of specialized proteases and peptides. M.Sc.Thesis. Department of Biochemistry, University of Rajshshi, Rajshahi, Bangladesh, **(1992)**.

[6] A.Begum, M.Rahaman, S.Begum; J.Biol.Sci., **2(11)**, 47-51 **(1993)**.

[7] P.F.Fox, P.A.Morrissey, L.J.Vital, V.Simon; 'Industrial and Clinical Enzymology', Pergamon Press, Oxford, 43 **(1980)**.

[8] D.Y.Graham, J.S.Holcenberg, J.Roberts; 'Enzymes as Drugs', Wiley, New York, 331 **(1981)**.

[9] S.K.Dutta, S.U.Dhan, L.N.Nath; Extraction, purification and study of new proteolytic enzymes, Indigenous medicinal plant, International seminar on

medicinal plant, Dhaka, Bangladesh, **(1992)**.

[10] S.Shinamo, K.Fukushima; Agr.Biol.Chem., **33**, 1236-1243 **(1968)**.

[11] S.Nallamsetty, S.Kundu, M.V.Jagannadham; J.Protein Chem., **22(1)**, 1-13 **(2003)**.

[12] A.K.Patel, A.K.Singh, M.V.Jagannadham; J.Agric. Food Chem., **55(14)**, 5809-18 **(2007)**.

[13] T.Kyndt, E.J.Van Damme, J.Van Beeumen, G.Gheysen; FEBS J., **274(2)**, 451-62 **(2007)**.

[14] L.P.Moro, M.T.Murakami, H.Cabral, A.Vidotto, E.H.Tajara, R.K.Arni, L.Juliano, G.O.Bonilla-Rodriguez; Protein Pept.Lett., **15(7)**, 720-30 **(2008)**.

[15] R.Tomar, R.Kumar, M.V.Jagannadham; J.Agric. Food Chem., **56(4)**, 1479-87 **(2008)**.

[16] S.L.Wang, C.H.Yang, T.W.Liang, Y.H.Yen; Bioresour Technol., **99(9)**, 3700-7 **(2008)**.

[17] M.Kunitz; Journal General Physiology., **30**, 291-310 **(1947)**.

[18] P.Andrews; Biochem.J., **91**, 222 **(1974)**.

[19] K.Weber, M.Osborn; J.Biol.Chem., **244**, 406 **(1966)**.

[20] U.K.Laemmli; Nature., **227**, 680-685 **(1970)**.

[21] T.F.Robyt, D.J.White; 'Biochemical Techniques: Theory and Practical', Waveland Press, Inc., Illinois, USA, 295-296 and 304-305 **(1990)**.

[22] O.H.Lowry, N.J.Rosenbrough, A.L.Farr, R.J. Rendall; Biol.Chem., **183**, 265-275 **(1951)**.

[23] C.Ma, X.Ni, Z.Chi, L.Ma, L.Gao; Mar.Biotechnol. NY, **9(3)**, 343-51 **(2007)**.

[24] M.Turkiewicz, E.Galas, E.Kalinowska, I. Romanowska, M.Ziolinska; Acta Biochem., **33(2)**, 85-99 **(1986)**.

[25] S.L.Wang, C.H.Chao, T.W.Liang, C.C.Chen; Mar. Biotechnol (NY)., Epub ahead of Print, **(2008)**.

[26] L.Kredics, K.Terecskei, Z.Antal, A.Szekeres, L. Hatvani, L.Manczinger, C.Vagvolgyi; Acta Biol. Hung., **59(2)**, 259-68 **(2008)**.

[27] V.K.Dubey, M.V.Jagannadham; Phytochemistry, **62(7)**, 1057-71 **(2003)**.

[28] A.Anwar, M.Saleemuddin; Arch Insect Biochem. Physiol., **51(1)**, 1-12 **(2002)**.

[29] T.Gotoh, K.Kikuchi, K.Kodama, H.Konno, T.Kakuta, T.Koizumi, K.Nojiro; Biosci.Biotechnol. Biochem., **59(3)**, 367-71 **(1995)**.

[30] Y.Mercado-Flores, G.Guerra-Sanchez, L.Villa-Tanaca, C.Hernandez-Rodriguez; Curr.Microbiol., **47(5)**, 408-11 **(2003)**.

[31] Z.Ahmed, M.Saleemuddin, M.Siddik; Insect Biochem., **10**, 667-673 **(1990)**.

[32] J.J.Cazzulo, M.C.Cazzulo Franke, J.Martinez, B.M. de Cazzulo; Biochem.Biophys.Acta, **1037(2)**, 186-91 **(1990)**.

[33] N.J.Darby, D.B.Lackey, D.G.Smith; Eur.J.Biochem., **195(10)**, 65-70 **(1991)**.

[34] M.Abe, S.Arai, M.Fujimaki; Agric.Biol.Chem., **41(5)**, 893-899 **(1977)**.

[35] M.Pande, V.K.Dubey, S.C.Yadav, M.V. Jagannadham; J.Agric.Food Chem., **54(26)**, 10141-10150 **(2006)**.