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Full Length Article

Purification and Biochemical Characterization of a Pathogenesis Related Endochitinase *Arachis hypogaea*

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Abstract

Plants protect themselves by producing phytoalexins and pathogenesis related (PR) proteins in response to different physical and biological stresses as well as against pathological attack. In the present study, chitinase, a PR protein, from peanut seeds was purified to homogeneity by ammonium sulfate precipitation, gel filtration chromatography on Sephadex G-75 column and ion exchange chromatography on Q-Sepharose column. Chitinase was identified as monomeric protein with molecular mass of 30 kDa by SDS-PAGE. Chitinase activity was also evaluated on chitin agar plates showing distinct zones. The optimum pH and temperature of purified chitinase activity were found to be 5.2 and 35°C, respectively. The enzyme was stable within broad pH range of 3.6 to 7.6 and up to 55°C temperature. Amongst the various substrates, acid swollen chitin was found to be the best substrate for chitinase when used at the concentration of 1% exhibiting its high specificity in catalyzing glycosidic bonds between N-acetylglucosamine residues. Chitinase activity of the purified enzyme was inhibited by Hg^{2+} and Ag^{2+} at 1 mM concentration and enhanced by Ca^{2+}, Mg^{2+}. The increase in the presence of 2-mercaptomethanol demonstrates the existence of sulfhydryl groups in enzyme active site. The enzyme exhibited a strong inhibitory action towards a variety of fungal species including *Fusarium oxysporum*, *Trichoderma reesei*, *Aspergillus flavus* and *A. niger*. Purified chitinase showed fungicidal properties against *A. flavus* on white bread and therefore, could be applied in bakery products to inhibit the formation and growth of fungal colonies.

Keywords: Antifungal activity; Chitinase; Characterization; Purification; *Aspergillus flavus*

Introduction

Plants respond to pathogenic attack, wounding, exposure to heavy metals, salinity, cold and ultraviolet rays through different defense mechanisms. These defense mechanisms include: strengthening of the cell wall through lignin, suberin and callose deposition as well as by the synthesis of phytoalexins which are toxic to bacteria and fungi by producing Pathogenesis-Related (PR) proteins like chitinases, β-1,3-glucanases, and thaumatin-like proteins (Bowles, 1990).

Chitinases (EC 3.2.1.14) belong to families 18 and 19 of the glycoside hydrolases (Henrissat, 1991). They catalyze the hydrolysis of the β-1, 4-linkages between N-acetylglucosamine residues of chitin. Chitin is the second most abundant polymer in nature after cellulose (Tharanathan and Kittur, 2003). Chitinases have seven classes (I-VII) (Neuhaus, 1999). However, most chitinases present in leguminous plants belong to class I-IV, because information about other three classes is not known yet (Wang *et al.*, 2009). Class I chitinases are basic and consist of N-terminal cysteine rich chitin binding domain. Class II chitinases are acidic and lack the chitin-binding domain but however are similar to class I chitinases. Class III chitinases have no similarity with class I or class II chitinases. Class IV chitinases are also not similar with class I chitinases but it is smaller in size due to four deletions (Shinshi *et al.*, 1990).

Depending on cleavage pattern, chitinolytic enzymes are divided into two categories that are: N-acetylglucosaminidases and Chitinases. N-acetylglucosaminidases (EC 3.2.1.5.2) cause the release of terminal, non-reducing N-acetylglucosamine (GlcNAc) residues from chitin. They have the highest affinity for the dimer N, N-diacetylchitobiose $(GlcNAc)_2$ and convert it to two monomers. Chitinases are further divided into endochitinases and exochitinases. Endochitinases degrade chitin from any point along the polymer chain forming random-sized length products, while exochitinases cleave $(GlcNAc)_2$ from the non-reducing end of the chain (Seidl, 2008).

To date, different types of chitinases have been purified and characterized from different plants like sugar beet (El Sayed *et al*., 2000), soya bean (Gijzen *et al.*, 2001), pineapple (Taira *et al.*, 2005), pomegranate (Yang *et al.*, 2011), and persimmon (Zhang *et al.*, 2013).

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Plant chitinases and glucanases often act together to increase antifungal activity by inhibiting the growth of many pathogenic fungi (Busam *et al.*, 1997). Antifungal effect of chitinases have been extensively studied in many plants including mung bean (Ye and Ng, 2005), lima bean (Wang *et al*., 2008), cranberry beans (Wang *et al.*, 2009), tea leaves (Nisha *et al.*, 2011) and fava beans (Wang *et al*., 2012). Different types of cell wall degrading chitinases and glucanases have been successfully transferred to crops such as silver birch (Pappinen *et al*., 2002) and grapevine (Yamamoto *et al.*, 2000). The objective of the present investigation as the purification and characterization of a chitinase from peanut seeds and its antifungal activity towards some pathogenic fungi.

Materials and Methods

Protein Extraction

100 grams of peanut seeds (*Arachis hypogaea* L), purchased from a local market, were ground to powder and soaked in 300 mL of 60 mM acetic acid for one hour on ice. The mixture was centrifuged (SiGMA® Laborzentrifugen) at 8,000 rpm for 30 min. pH of the supernatant was adjusted to 7.6 and placed at 4°C for overnight and again centrifuged at 8,000 rpm for 30 min. The supernatant was designated as crude extract for further investigations.

Chitinase Activity Assay

Chitinase activity was checked by using 1% acid swollen chitin (0.5 g chitin soaked in 50 mM sodium acetate buffer, pH 5.2 containing 0.02% sodium azide for 2 h). The reaction mixture was prepared by adding 0.5 mL acid swollen chitin and 0.5 mL suitably diluted enzyme in a test tube following incubation at 35°C for 30 min. The mixture was centrifuged at 4,000 rpm for 10 min. The reducing sugars released in supernatant were measured by DNS method (Miller, 1959). Blank contained 0.5 ml of 50 mM sodium acetate buffer (pH 5.2) instead of enzyme. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol N-acetyl-D-glucosamine from acid swollen chitin per minute under the standard assay conditions.

Zymogram for Chitinase Activity

15% polyacrylamide gel electrophoresis was carried out under native condition by using the procedure described by Davis (1964). 0.5% acid swollen chitin was used as substrate for chitinase in the zymogram technique. The gel was stained with 0.1% Congo red dye for 30 min following destaining in 2M NaCl solution.

Total Protein Estimation

The protein concentration was estimated by using Bradford method with bovine serum albumin (Sigma Chemicals Co., USA) as the standard (Bradford, 1976).

Purification of Chitinase

Ammonium sulfate precipitation: The crude chitinase extract was adjusted to 80% saturation with ammonium sulfate. The precipitate was harvested by centrifugation at 8,000 rpm for 20 min. Pellet was suspended in 50 mM sodium acetate buffer (pH 5.2) and stored at 4ºC. The chitinase activity and protein concentration of the solution was determined as described above.

Gel Filtration Chromatography

Ammonium sulfate precipitated sample was dialyzed against 50 mM sodium acetate buffer (pH 5.2) at 4ºC overnight with four changes at three hours interval. Dialyzed sample was lyophilized and loaded on Sephadex G-75 (Pharmacia Fine Chemicals, Sweden) column (0.25 x 30 cm) that was pre-equilibrated with the 50 mM sodium acetate buffer (pH 5.2). The protein elution was done with the same buffer at a flow rate of 1 mL/min. Total 60 fractions (2 mL each) were collected. Chitinase activity and protein concentration of each fraction was determined.

Q-Sepharose Chromatography

Fractions displaying chitinase activity were pooled and subjected to ultrafiltration at 4ºC in an Amicon ultrafiltration cell fitted with 10,000 MW cutoff membrane (PTGC 043 Millipore Co., USA) under 50 pounds per square inch nitrogen pressure. The concentrated enzyme sample was fractionated on Q-Sepharose (Sigma Chemical Co. USA) column (1.6 x 10 cm) equilibrated with Tris-HCl buffer (pH 8.5) at natural flow rate. The bound proteins were eluted by a linear gradient of 0 to 1.5M NaCl in the same buffer. 1 mL fractions were collected by using a fraction collector Frac-100 (Pharmacia Fine Chemicals) and assayed for protein and chitinase activity.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Homogeneity and molecular mass of the purified chitinase was estimated by (SDS-PAGE) (BIO-RAD Laboratories Inc., USA) performed by the method described by Laemmli (1970). Samples were mixed 1:4 with sample buffer and boiled for 10 min. Gel containing standard protein size markers (Invitrogen) and samples after electrophoresis, was stained in 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma), 30% (v/v) methanol and 10% (v/v) glacial acetic acid solution and destained in a solution containing 30% (v/v) methanol and 10% glacial acetic acid in distilled water. The molecular mass of purified antifungal protein was determined by comparison of its electrophoretic mobility with those of unstained protein ladder.

Characterization of Purified Chitinase

Effect of pH on activity and stability: The effect of pH on the activity of purified chitinase was estimated by maintaining the pH of assay mixtures using 1% acid swollen chitin as a substrate. Mcllvaine buffer was used for pH range 2.8 to 7.6 and Clark and Lubs buffer for pH range 8.4 to 10.

pH stability of purified chitinase was determined by incubating enzyme in buffers of different pH ranging 2.8 to 10 at room temperature for one hour without substrate. The residual activity was then determined under the standard assay conditions.

Effect of Temperature on Activity and Stability

The optimal temperature for purified chitinase was determined at different temperatures in the range of 20°C to 80°C by incubating a known amount of chitinase activity with 1% acid swollen chitin as a substrate in optimized buffer.

Temperature stability profile for purified chitinase was obtained by preincubating enzyme in the buffer of optimized pH at various temperatures ranging 10 to 80°C for one hour prior to the addition of substrate and determining the residual activity under standard assay conditions.

Substrate Specificity

The substrate specificity of the purified chitinase was investigated by incubating the enzyme with different concentrations (0.5–1.5%) of acid swollen chitin, colloidal chitin, chitotriose, glycol chitin, carboxymethyl cellulose (CMC), and chitibiose as substrate in 50 mM sodium acetate buffer (pH 5.2). Acid swollen chitin was taken as control to measure the relative activity for each substrate.

Activation and Inhibition Studies

The enzyme was pre incubated with a 1 mM concentration of different metals ($MgCl_2$, $CaCl_2$, $CuSO_4$, $MnCl_2$, $AgNO_3$, $HgCl_2$ and β-mercaptoethanol) at room temperature. The residual chitinase activity was measured under the standard assay conditions after one hour.

Chitinase Diffusion Assay

Well plate method was used to make wells (4 mm diameters) on chitin agar plates containing 4% agar and 1% acid swollen chitin in 50 mM acetate buffer (pH 5.2). Each well was loaded with different amounts of chitinase activity (1–3 U) while control well contained only 50 mM sodium acetate buffer (pH 5.2). The plates were incubated at 37°C for 24 h. Zones of chitinase activity were observed after staining and destaining with 0.1% congo red dye and 2M NaCl, respectively.

Antifungal Activity of Chitinase

The mycelium of *A. fumigatus, A. flavus, F. oxysporum, T. reesei* were inoculated separately in the middle of the petri plates containing 2% malt extract agar media and incubated at 35°C. After 2 days when colony diameter was 3–4 cm, sterile filter paper discs (0.5 cm in diameter) soaked in chitinase solution containing different concentrations (30, 60, 90 µg) were placed on plates at a distance of about 0.5 cm away from the edge of the growing colonies. The discs soaked in buffer without chitinase served as control. The plates were further incubated at 35°C until the mycelial growth encircled the peripheral disc containing the control and had produced crescents of inhibition around the discs loaded with chitinase antifungal protein (Wong and Ng, 2005).

Chitinase as Food Preservative

Antifungal potential of chitinase against bread mold was evaluated with fresh bread pieces according to the method described by Hans *et al.* (2010). Fresh white bread obtained from local market was sliced approximately into 30 mm pieces. The fungal spores were spread on the bread and chitinase solution applied on the bread samples. The bread pieces without enzyme were used as controls. The pieces packed in plastic bags were incubated at 25°C up to seven days.

Results

Purification of the Chitinase

The summary of purification of chitinase is presented in Table 1. Ammonium sulfate (80%) precipitated sample after dialysis and lyophilization was subjected to Sephadex G-75 column that was pre-equilibrated with 50 mM sodium acetate buffer (pH 5.2). Chitinase activity was separated as a single peak as shown in Fig. 1. Active fractions were pooled and further purified by Q-Sepharose column chromatography. Bound proteins were eluted with a gradient of NaCl (0 to 1.5M) in Tris-HCl buffer of pH 8.5 (Fig. 2). The purified enzyme preparation had a specific activity of 101 U/mg of protein, while the final percentage recovery was 23.8.

Chitinase catalyzes the cleavage of chitin as it diffuses through the gel, leaving a light, unstained circular zone around the well, because the congo red dye binds only to undigested chitin. A plate clearing assay for chitinases (Zarei *et al*., 2012) was used to evaluate the activity of purified chitinase producing distinct zones of chitin hydrolysis as shown in Fig. 3. This chitin plate clearing test seems to be semi-quantitative since diameter of the hydrolysis zone increased with increasing enzyme concentration.

Table 1: Purification scheme of chitinase from *Arachis hypogaea* L. seeds

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	380	45	8.4	1	100
Ammonium sulphate precipitation	305.6	32.5	9.4	1.1	80.4
After Gel Filtration Chromatography	205	6.0	34.1	4.0	53.9
After Ion Exchange Chromatography	90.5	0.9	101	12.02	23.8

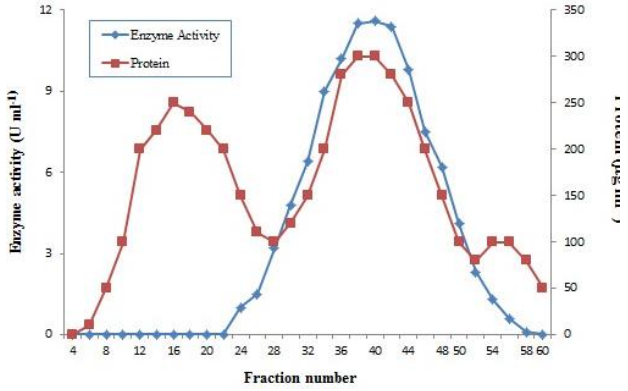

Fig. 1: Gel filtration through chromatographyon Sephadex G-75 column of chitinase from peanut

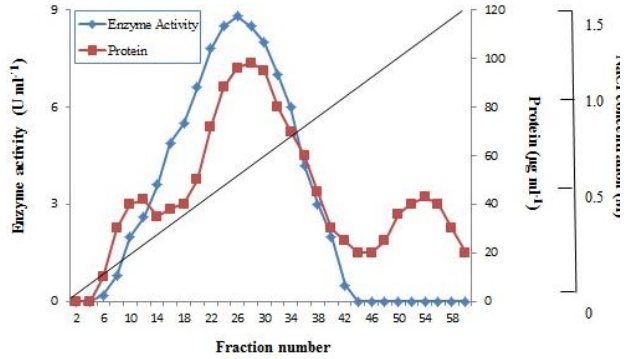

Fig. 2: Q-Sepharose chromatography of chitinase after concentration by ultrafiltration

Characterization of Purified Chitinase

Molecular weight determination: The molecular weight of purified chitinase was appraised to be 30 kDa on SDS-PAGE as shown in Fig. 4A. Chitinase activity was further confirmed under native conditions in 15% (w/v) polyacrylamide gel containing 0.5% acid swollen chitin as substrate following staining with congo red. Chitinolytically active enzyme was visualized as clear band on red background of the gel as shown in Fig. 4B.

Effect of pH and Temperature on Activity and Stability of Purified Chitinase

Purified chitinase was found to be the most active at pH 5.2 as shown in Fig. 5. Purified chitinase was stable in the broad pH range from 3.6 to 7.6 as shown in Fig. 6. Enzyme retained 99% of its activity at pH 4.4 and about 55% at pH 9.2. The purified enzyme was optimally active at 35ºC as shown in Fig. 6.

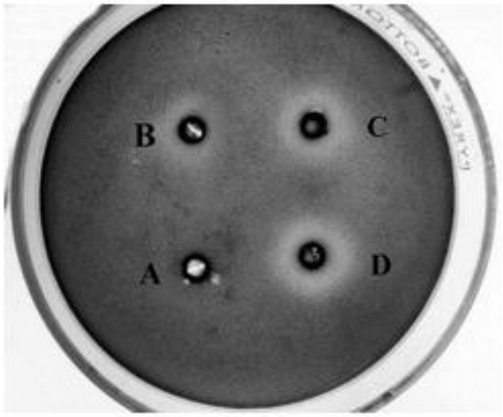

Fig. 3: Holes around the wells in a chitin-agar plate. Wells were loaded with different amounts of chitinase and incubated at 35^{O}C for 24 hours. The plate was stained with congo red and then destained with 1 M Nacl. A: Control; B: 1U of chitinase; C: 2U of chitinase; D: 3U of chitinase

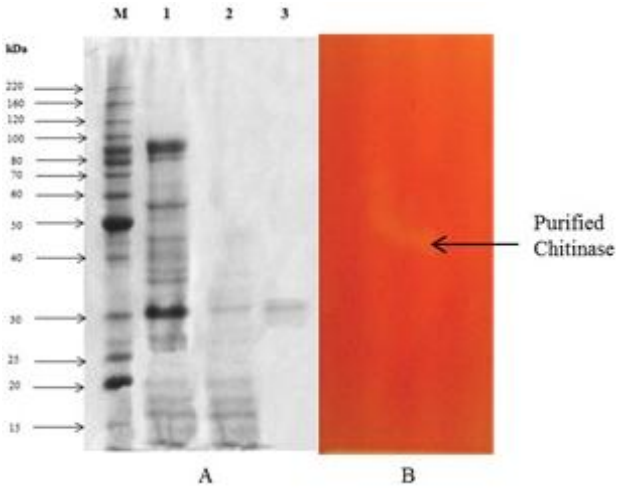

Fig. 4: SDS-PAGE analysis of chitinase from peanut. Lane K: Marker; Lane 1: Crude enzyme; Lane 2: After Gel Filtration Chromatography; Lane 3: After Q-sepharose chromatography. B. Zymography analysis of purified chitinase

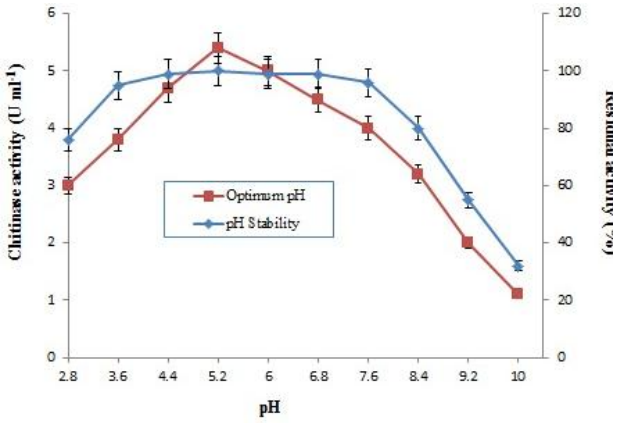

Fig. 5: Optimum pH and pH stability of purified chitinase from peanut. The effect of pH on enzyme activity was determined at 37ºC using 0.05 M of varing buffers. Buffers used were: Mcllvaine (2.8-7.6), Clark and Lubs (8.4-10.0) For pH stability, the enzyme was diluted with different buffers and incubated at room temperature for 1 h and determined the residual activity at 35ºC. The data is representative of the mean of three experiments

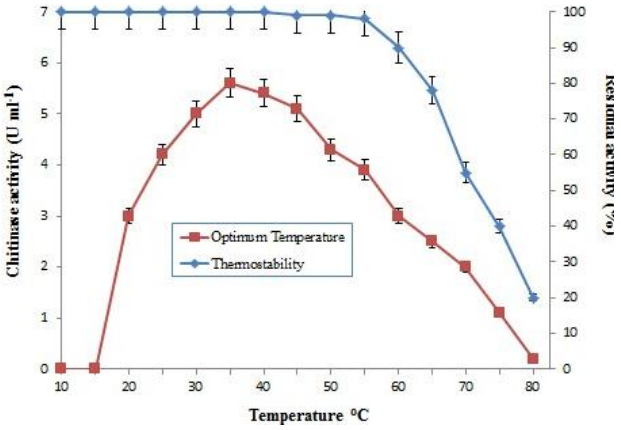

Fig. 6: Optimum temperature and thermal stability of purified chitinase from peanut. The optimal temperature was determined at different temperatures by incubating known amount of activity with 1% chitin. Temperature stability was obtained by preincubation enzyme at various temperatures ranging 10-80ºC for 1 h prioe to the addition of substrate and determining the residual activity. The data is representative of the mean of two experiments

Substrate Specificity of Purified Chitinase

Polymeric substrates having glycosidic bonds were used as substrates for determination of substrate specificity of purified enzyme. Acid swollen chitin was found to be the best substrate at 1% concentration but significant activity was also obtained against colloidal chitin as shown in Fig. 7.

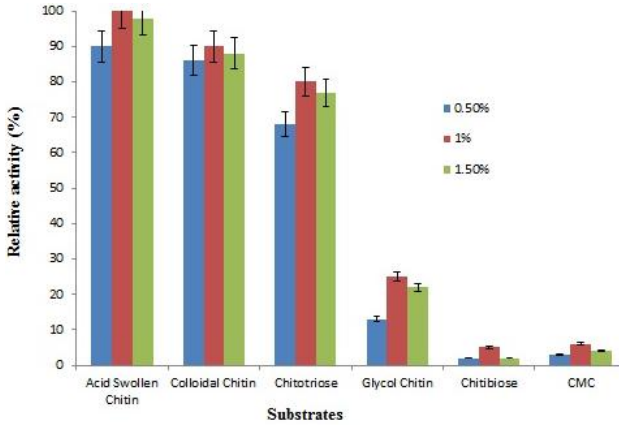

Fig. 7: Effect of dirrerent substrates on purified chitinase activity. Data are presented as mean of three replicates with standard deviations

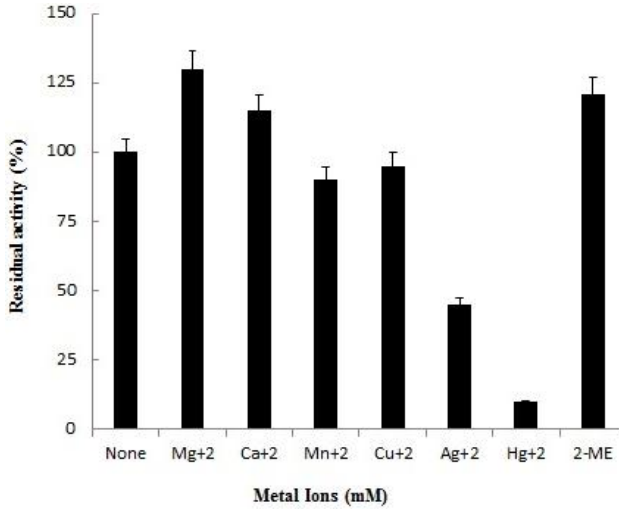

Fig. 8: Effect of metal ions on the activity of purified chitinase. The data is representative of the mean of three experiments

However, glycol chitin and chitotriose were found to be poor substrates demonstrating reduced approach of enzyme to the substrate. Activity of chitinase was very low against carboxymethyl cellulose and chitibiose even when enzyme concentration was increased up to 1.5%.

Inhibition and Activation Studies

The effect of some potential metal ions on chitinase activity was investigated in an assay system containing 1 mM of each chemical. As shown in Fig. 8, the chitinase activity was strongly inhibited by Ag^{+} and almost completely by Hg^{2+}. During present studies, the presence of Mg^{+2} and Ca^{+2} enhanced 30 and 15% chitinase activity, respectively when used at a concentration of 1 mM in the reaction mixtures.

Antifungal Activity of Chitinase

Antifungal assays were performed using major phytopathogenic fungi. The purified chitinase showed antifungal activity by forming a crecent at concentrations 30, 60, and 90 µg against *A. fumigatus, T. reesei, A. flavus,* and *F. oxysporum* as shown in Fig. 9.

Chitinase as Food Preservative

Antifungal potential of chitinase was assessed as a food preservative by applying purified enzyme on bread infected with *A. flavus* as white bread is mainly vulnerable by fungal pathogens, particularly the species of genus *Penicillum, Aspergillus, Fusarium*, and *Rhizopus* (Lavermicocca *et al*., 2003; Guynot *et al*., 2005). The fungal spores and antifungal protein when used in different combinations at a concentration of 60 µg of purified chitinase produced maximal inhibitory effect on the growth of *A. flavus* (Fig. 10).

Discussion

Chitinases are very important enzymes that are distributed in bacteria, fungi, plants and animals. These are produced under normal conditions as well as a result of different environmental and developmental factors in plants. Due to compact structure, enzymes are stable at extreme conditions of pH and temperature. Chitinases are also resistant to proteolytic degradation and remain soluble and stable under such conditions retaining their antifungal activities and surviving ability during food processing (Gorjanovic, 2009).

Enzyme purification is essential for understanding the mode of action and substrate specificity of an enzyme. Product inhibition studies and calculation of Michaelis-Menten constants can be carried out with purified. Many other workers have reported multistep procedure for purification of chitinase. Ye and Ng (2005) purified a chitinase after three chromatographic steps (CM-Sephadex C-50, POROS HS and Sephadex G-75 column chromatography) from mung beans to 3.9 folds and Taira *et al.* (2005) purified chitinase from pineapple leaf after four steps such as chitin affinity column chromatography, Butyl-Toyopearl 650M column chromatography, Resource Q column chromatography, HPLC on Phenyl superpose column.

A plate clearing assay was used to evaluate the activity of purified chitinase producing distinct zones of chitin hydrolysis as shown in Fig. 3. Chitinase catalyzes the cleavage of chitin as it diffuses through the gel, leaving a light, unstained circular zone around the well, because the congo red dye binds only to undigested chitin. During current trials, chitin plate clearing test seemed to be semi-quantitative since diameter of the hydrolysis zone increased with increasing enzyme concentration. Plate assay method was also performed by Zarei *et al.* (2012) to examine the active fungal strains producing chitinases.

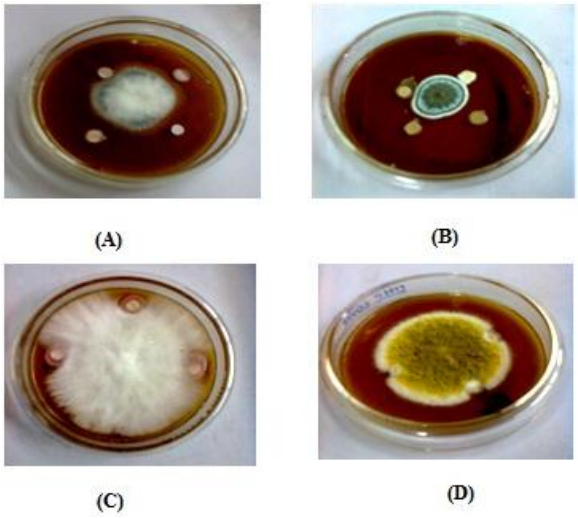

Fig. 9: Antifungal activity against different pathogenic fungi. (A) *A. fumigatus*, (B) *T. reesei*, (C) *A. flavus*, and (D) *F. Oxysporum*

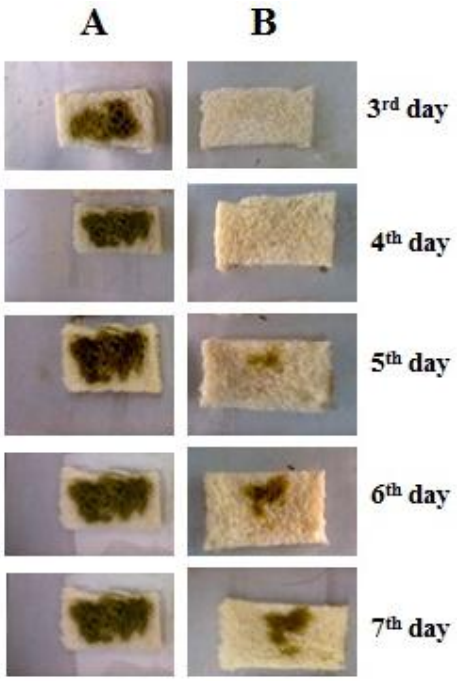

Fig. 10: Effect of antifungal activity of chitinase on growth of *A. flavus* on contaminated white bread. Bread samples were contaminated with *A. flavus* in the presence of antifungal protein (B). Control samples were treated in similar way but without enzyme (A)

Chitinases of plant origin are generally endochitinases and possess molecular masses in the range of 25 to 35 kDa (Sahai and Manocha, 1993). Similar molecular weight chitinases are reported by Patel *et al.* (2010), Yang and Gong (2002), Ye and Ng (2005) from *Ipomoea carnea* latex (30 kDa), *Hydrangea macrophylla* (30 kDa) and *Phaseolus*

mungo seeds (30.8 kDa), respectively. However, low molecular weight chitinase were also purified from *Vicia faba* seeds (25.5kDa) (Wang *et al.*, 2012) and *P. limensis* seeds (28.6 kDa) (Wang *et al.*, 2008). Many researchers have reported higher molecular weight of chitinase contrary to present studies *i.e.*, *Beta vulgaris* (64 kDa) (El sayed *et al.*, 2000), leaf rachises of *Cycas* (40 kDa) (Taira *et al.*, 2009), *Astragalus membranaceus* seed (35 kDa) (Kopparapu *et al.*, 2011) and *Camellia sinensis* leaves (34 kDa) (Nisha *et al.*, 2011).

pH is one of the major factors affecting tertiary and quaternary structure of proteins and enzymes. Since enzymes are amphoteric in nature, therefore possess both acidic and basic amino acids. Based on dissociation constants and pH of the environment, these varying charges on amino acids affect the overall charge along with their peripheral distribution thus influencing the activity, stability and solubility of enzymes (Chaplin and Bucke, 1990). Chitinase purified during present studies showed optimum activity at 5.2, which is in close conformity to the findings of Wang *et al.* (2008) and Santos *et al.* (2004) who purified a chitinase from lima bean and *Adenanthera pavonina* seeds showing maximum activity at pH 5.4 and 5.5, respectively. While chitinase obtained from *Oryza sativa* possessed pH optimum of 5.5–6.5 (Xayphakatsa *et al.*, 2008). On the contrary, purified chitinases, reported by Bansode and Bjekal (2005) and Kuzu *et al*. (2012) exhibited maximum activity at pH 9. Since purified chitinase showed pH optima in the acidic range, therefore this enzyme could possibly be exploited for control of fungal plant pathogens (Moore *et al*., 2004). Some chitinases are reported displaying even broader pH range. Patel *et al*. (2010) purified a chitinase from *Ipomoea carnea* latex that was stable from pH 4.0 to 9.5, while chitinase purified by Kopparapu *et al.* (2011) had been shown to maintain its activity in the pH range of 3.0–9.0.

Temperature is one of the critical constraints that regulate the enzyme activity. The resistance of enzymes to thermal denaturation is due to the presence of hydrophobic interactions, hydrogen bonding and ionic stabilization in polypeptide chains (Voordouw *et al*., 1976). During contemporary trials, 35°C was found to be optimal temperature for chitinase, which is in accordance with the results reported by Molano *et al.* (1979) from wheat grains. Research papers are also present on chitinases that possess high optimum temperatures like *P. mungo* seed chitinase (40–50ºC) (Ye and Ng, 2005), *Oryza sativa* chitinase (35 to 40ºC) (Xayphakatsa *et al*., 2008), *Ipomoea carnea* latex (50ºC) (Patel *et al*., 2010), *Vicia faba* seed chitinase (50ºC) (Wang *et al*., 2012) and *A. membranaceus* seed chitinase (50ºC) (Kopparapu *et al.*, 2011).

Thermostable chitinase could be useful for the chitin industry and biotechnological applications; therefore, the thermostability of purified chitinase was examined at various temperatures. Such studies demonstrated that activity of purified chitinase was preserved up to 55ºC when incubated for one hour (Fig. 6). Earlier reports have also revealed comparable thermal stability of chitinases *i.e.*, peanut seeds below 50ºC (Wang *et al*., 2008). However, Patel *et al*. (2010) have reported even high thermally stable peanut chitinase (up to 80ºC) from *Ipomoea carnea*. Wide-ranging pH and temperature stability is criterion of enzymes for their industrial applications as many biotechnological processes occur at high temperature and at acidic or basic pH values (Antranikian, 2009).

Chitinase is specific in hydrolyzing the compounds that contain glycosidic linkage. Ikeda *et al*. (2009) have also reported that amongst different insoluble substrates used for substrate specificity studies, colloidal chitin was more effectively digested by chitinase purified from *Pennahia argentatus.*

Generally, the presence of various chemicals results in inhibition or activation of enzymes. Rouvinen *et al.* (1990) suggested that enzyme inhibition might be due to the binding of metal ions to sulfhydryl groups of cysteine residues playing a significant role in maintaining the integral active site structure of enzymes. Xia *et al*. (2001) have described the different prospects of Hg^{2+} impact on enzyme. The ions can bind the sulfhydryl or carboxyl groups of amino acids or interacts with tryptophan residues as such. Furthermore, in other cases metals can lead to the dearth or substitution of indispensable ions in metalloproteins (Van Assche and Clijsters, 1990). Kim *et al.* (2003) have reported inhibitory effect of Ag^{+} and Hg^{2+} on chitinase similar to present studies, purified from *Streptomyces* sp. M-20 when used at 1 mM concentration. The mechanism by which metal ions enhance the reaction is through their binding to the substrates so as to orient them properly for enzymatic reaction. Metal ions may facilitate the reaction through the charge shielding of bound substrates (Villafrance, 1992).

Interestingly, in contrast to our studies, Ca^{2+} and $Mg2^{+}$ partially inhibited *Serratia marcescens* chitinase while Mn^{2+} enhanced enzyme activity at 1 mM concentration (Zarei *et al.*, 2011). Five chitinases purified from different microorganisms, Bansode and Bjekal (2005) reported that Ca^{2+} remarkably increased the chitinase activity as compared to other metal ions used. This study also reported that enzyme activity of purified chitinase was enhanced (21%) in the presence of 2-mercaptomethanol in the assay mixture suggesting the presence of SH- groups in active site of the enzyme. These findings were further established when purified enzyme was found to be inhibited in the presence of divalent ions such as $HgCl_2$ and such metal cations enzyme inhibition revealed the presence of at least one Cys residue in the enzyme active site (Rouvinen *et al*., (1990). Present results also agree with earlier findings reported previously by Kim *et al*. (2003) and Ueno *et al.* (1990).

Fungi are widely distributed in every ecological unit. They have ability to inhabit plants, human and animals as their substrates. The previous studies revealed that fungi are

the major threat to immune-compromised patients and their infection lead to the death of the patient. However, these infections can be suppressed by the development of antifungal that have the ability to prevent such infections (Shoham and Levitz, 2005). Chitinases occupy a distinct place in agricultural biotechnology because their lytic activity inhibit fungal development by degrading chitin and glucan components of cell wall and have proven their potential as antifungal agents (Punja and Zhang, 1993; Jung *et al.*, 2005). Antifungal effect of different plant chitinases has been studied. Kirubakaran and Sakthivel (2007) purified a 35 kDa chitinase exhibiting antifungal activity against *B. cinerea, P. theae, B. oryzae, Alternaria sp., C. lunata and R. solani.* A 28 kDa chitinase purified from maize seeds had the potential to inhibit the growth of *T. reesei, A. solani* and *F. oxysporum* (Huynh *et al*., 1992). Ye and Ng (2005) isolated chitinase from mung beans with antifungal activity against *F. oxysporum, M. arachidicola, P. aphanidermatum and S. rolfsii.*

Besides the advancement in scientific knowledge, food industry is still facing unsolved food spoilage problems due to incursion of molds and spores. The use of conventional chemical preservatives or other physical procedures in food items have their own inherent disadvantages. Hence to evade chemical preservation methods, natural plant extracts could be promising alternative; therefore, to explore new plants materials with antifungal properties have become an interesting research area in food industry (Irkin and Korukluoglu, 2007). The cell wall of the fungi contains mostly chitin along with other polysaccharides and chitinase enzymes break down chitin of fungal cell wall by hydrolyzing its ß-(1, 4) glycosidic linkages, therefore directly prevent germination and growth of molds and fungi. Hans *et al.* (2010) recounted that antifungal activity of oat (*Avena sativa*) seed extracts exhibited its great potential to prevent and repress the spore germination of *P. roqueforti*, when applied on rye bread. Moreover Fig leaves extracts exhibiting both antifungal and antibacterial activities against detrimental microbes in foods have been reported previously (Hu *et al.*, 2007; Balestra *et al*., 2009; Oliveira *et al*., 2009; Aref *et al.*, 2010).

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