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Production, Purification and Optimization of Protease by *Fusarium solani* under Solid State Fermentation and Isolation of Protease Inhibitor Protein from *Rumex vesicarius* L

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Alkaline protease enzyme was produced under solid state fermentation by *Fusarium solani*. Various agro-wastes were evaluated to check their potential utilization in the solid state fermentation for protease production. In this connection, the results showed that rice straw was the best agro-waste. The best enzyme activity was achieved at 7th day of incubation, pH 8, temperature 45 °C, inoculum's size 200 µl/100 ml, agitation speed at 140 rpm, and KNO_3 as nitrogen source. The molecular weight of the obtained purified enzyme is 30 KDa. A protease inhibitor protein was isolated and purified from *Rumex vesicarius* L. The molecular weight of the inhibitor protein is 46 KDa and it composes of 17 amino acids. The minimum inhibitory concentration was found at 20 µl of the inhibitor per 40 µl of the enzyme. The obtained results showed that the inhibitor can withstand the heat at 70°C for 30 min, and pH (5-9) for 72 h at 4°C without loss of activity but it cannot be activated at low acidity less than pH 5 and very high temperature more than 70°C. This study will serve to increase the understanding of factors that control the production and inhibition of protease enzyme which may help in many industrial aspects.

Key words: Protease, Enzyme inhibitor, Protein purification, Fermentation, Rice straw.

Proteases are large group of enzymes that play many roles in nature. They are the most important industrial and commercial enzymes and account about 60% of the total enzyme market in the world and approximately 40% of the total worldwide enzyme sale[1]. Proteases are used in many industrial aspects; detergents manufacture[2], food and leather industries, meat processing, cheese making, and they also have medical and pharmaceutical applications[3].

The filamentous fungi can utilize wide variety of substrates as nutrients and elaborate a wide variety of proteolytic enzymes. Various microbes have the ability to produce proteases enzymes, which strictly catalyze the total hydrolysis of proteins[4]. Several genera of fungi including *Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminearum, Penicillium griseofulvin, Scedosporium apiosermum* are reported to produce proteases[5].

Although, proteases are indispensable to the life as mentioned above, they may be potentially damaging when overexpressed or present in higher concentrations. For this reason the activities of these enzymes need to be strictly regulated and controlled[6]. One important control mechanism involves interaction of the active enzymes with proteins that inhibit their activities. These inhibitors form less active or fully inactive complexes with their cognate enzymes, and are called protease inhibitors (PIs). Protease inhibitors are commonly found in the plants and called plant protease inhibitors (PPIs), which are small proteins

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stored in the tissues, such as tubers, seeds, and aerial parts of plants[7]. Plant protease inhibitors are considered one of the defense mechanisms in plants. They are induced in plants in response to injury or attack by pathogens[8].

This work was aimed to produce protease enzyme by using solid state fermentation, optimize the fermentation conditions and isolate and characterize a protease inhibitor from *Rumex vesicarius* L extract.

MATERIALS AND METHODS

Isolation of fungal isolates and screening for protease production

The fungal strains *F. solani*, *F. graminearum, Rhizoctonia solani, Macrophomina phaseolina,* and *Alternaria alternata* were isolated originally from different naturally diseased plants collected from different agricultural fields in Saudi Arabia. The isolated fungi were grown on potato dextrose agar (PDA) (Difco, USA) plates and incubated at 28°C for 4 to 6 days. Purification of the resulting isolates was done using the hyphal tip or single spore techniques to obtain them in pure cultures; the detected isolates were then transferred into slant of PDA and kept at 4°C for further studies. Pure cultures of the isolated fungi were identified according to the cultural properties, morphological and microscopical characteristics of each fungus[9].

Isolated fungi were screened for protease enzyme production on skim milk agar medium. Each fungal isolate disc was placed in a separate plate at the center; all plates were incubated at 25°C for 3 days, after incubation period the clear zone was measured.

Collection and screening of fermentation sludge

Various agro-wastes (wheat bran, rice bran, rice straw, and barley bran) were collected in dried form from different farms. Each of which was used singly as a sole carbon source. Erlenmeyer flasks (250 ml) containing 10 gm of different agro-wast and 10 ml of salt solution (g/l): KNO_3 2.0, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1.0, $ZnSO_4.7H_2O$ 0.437, $FeSO_4.7H_2O$ 1.116, $MnSO_4.7H_2O$ 0.203, at pH 7.2 were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension (the most active isolate), and incubated in an incubator shaker (140 rpm) at 30°C for 7 days. The proteolytic activity in each medium was qualitatively measured by using clear zone method on the surface of skim milk agar medium.

Optimization of cultural conditions for maximum protease production

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution at pH 7.2 were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension, and incubated in an incubator shaker (140 rpm) at 30°C for different time intervals of incubation (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days). The proteolytic activity was checked at each medium by using clear zone method on the surface of skim milk agar plates[10].

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution which mentioned above at pH 7.2 were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension, and incubated in an incubator shaker (140 rpm) for 7 days at different incubation temperature (20, 25, 30, 35, 40, 45, 50, 55 and 60°C). The proteolytic activity was checked at each medium by using clear zone method on the surface of skim milk agar plates [11].

Effect of pH

Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution were used. The tested pH values (5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) were adjusted by using a proper phosphate buffer. The media were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension, and incubated in an incubator shaker (140 rpm) at 45°C for 7 days. The proteolytic activity was checked at each medium by using clear zone method on the surface of skim milk agar plates[11].

Effect of nitrogen source

Seven nitrogenous compounds (potassium nitrate, casein, peptone, yeast extract, malt extract, beef extract and urea) were tested as substitute nitrogenous sources. Nitrogenous source of salt solution was substituted with one of the tested sources (containing the same quantity of nitrogen). Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution at pH 8 were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension, and incubated in an incubator shaker (140 rpm) for 7 days at 45°C. The proteolytic activity was checked

at each medium by using clear zone method on the surface of skim milk agar plates[10].

Effect of agitation speed

Nine Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution at pH 8 were autoclaved at 121°C for 30 min, and then inoculated by 200 µl of *F. solani* suspension, and incubated in an incubator shaker at different agitation speeds (100, 120, 140, 160, and 180 rpm) for 7 days at 45°C. The proteolytic activity was checked at each medium by using clear zone method on the surface of skim milk agar plates[10].

Effect of inoculum size

Ten Erlenmeyer flasks (250 ml) containing 10 gm of rice straw and 10 ml of salt solution at pH 8 were autoclaved at 121°C for 30 min, and then inoculated by different inoculum sizes (80 - 220 µl) of *F. solani* spore suspension. The flasks were incubated in an incubator shaker (140 rpm) for 7 days at 45°C. The proteolytic activity was checked at each medium by using clear zone method on the surface of skim milk agar plates[10].

Production of protease enzyme

Solid state fermentation was carried out in 250 ml conical flask contains 10 gm of rice straw with 10 ml of salt solution at pH 8 and autoclaved at 121°C for 30 min. After sterilization, the flasks were inoculated with 200 µl of spore solution (10^3 spores / 200 µl) and incubated at 45°C for 7 days in an incubator shaker at 140 rpm. At the end of fermentation, filtrate was eluted with 100 ml of distilled water by shaking for 2 hrs. The filtrate was then centrifuged at 10,000 g for 10 min at room temperature. The supernatant was used as a crude enzyme extract.

Protease assay

Protease activity was assayed according to Anson's method. The buffer-enzyme solution was prepared by addition of 1 ml of culture filtrate on 1 ml of phosphate buffer at pH 8 in a 100 ml conical flask. One ml of the substrate (2% casein pH 7) was added to the buffer-enzyme solution and incubated at 37°C for 10 minutes in a water bath. At the end of 10 minutes, 10 ml of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was filtered and 5 ml of the filtrate were added to 10 ml of 0.5N NaOH solution and 3 ml of folin ciocalteu reagent (one ml diluted with 2 ml of distilled water). Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate.

Purification of protease enzyme

The crude extract was supplemented with different concentrations of saturated ammonium sulphate ranged between 10% and 90% to precipitate protease enzyme. The proteolytic activity was checked at each fraction of ammonium sulphate precipitation. The active fractions were pooled and then dialyzed against 50 mM phosphate buffer (pH 7) for 24 hours at 4°C. These dialyzed active fractions were loaded onto a DEAE-cellulose chromatographic column equilibrated with 50mM phosphate buffer at pH 7. The enzyme was eluted with a linear salt concentration gradient (NaCl, 0.1 - 0.4 M) to activate the ion exchange potentiality of column. Fifty fractions were collected; each one contained 5 ml/ 20 minute. The proteolytic activity was checked at each fraction. The sequential active fractions were collected and then further dialyzed against 50 mM phosphate buffer (pH 7) for 24 hours at 4°C. The active fractions were pooled and loaded onto sephadex G-200 column chromatography equilibrated with 50mM phosphate buffer at pH 7. The active purified enzymatic protein was separated by using SDS-PAGE electrophoresis to determine the molecular weight. The total protein content was estimated at each fraction by using the method of Lowry *et al.,*[12].

DEAE–Cellulose column chromatography

One hundred grams of DEAE–cellulose were placed in 1 liter conical flask and then washed by three volumes of distilled water and 1N HCl until pH becomes at 6.5. The resin was then washed several times with 0.5M NaOH until no more color was removed, and then washed with distilled water until it was free from alkalinity[13]. The washed resin was suspended in about three volumes of phosphate buffer at pH 7.0 and left for overnight to be settled. The clear supernatant was discarded, and the precipitate was used for column packing. The dialyzed enzyme solution was passed through ion-exchange column chromatography (Whatman DE52: 1.5×100 cm) equilibrated with phosphate buffer at pH 7.0. The resin was supplemented with linear NaCl gradient (0.1 to 0.4 M) for ion exchange activation.

Gel filtration column chromatography

Ten grams of sephadex G-200 were dissolved in 400 ml phosphate buffer at pH 7, and

then boiled in water bath for 6 hrs, then cooled at 50 °C, and packed in column (4.5×50 cm). The resin was equilibrated with phosphate buffer at pH 7. Elution was done by the same buffer at a flow rate of 10 ml/25min[14].

Isolation of protease inhibitor protein

Leaves of *Rumex vesicarius* L were collected and homogenized with 3 volumes (w/v) of 0.02M phosphate buffer at pH 7.6. The mixture was stirred at 5°C for 30 minutes and then set aside for 90 min. The mixture was washed with five volumes of cold acetone (5°C) and diethyl ether with stirring for 20 minutes, and then filtered under suction to obtain the clear filtrate. The filtrate was air dried and stored at 4 °C under desiccation. This was designated as the crude extract. The crude extract was subjected to heat treatment at 90 °C for 5 min, cooled to room temperature and centrifuged at 10,000 g for 20 min. The supernatant was designated as the heat treated fraction. The proteolytic activity inhibition was checked by using antagonistic test; 50 µl of crude extract was mixed with 100 µl of purified protease enzyme, this mixture was inoculated in agar wells of skim milk agar medium, and then incubated at 45 °C for 5 – 7 days. Protease inhibitor containing fraction (60 ml) was supplemented with 23.4 grams of solid ammonium sulphate equivalent to 60 % saturation at 4 °C, and then left overnight. The precipitate was collected by centrifugation at 10,000 g for 20 min, dissolved in 0.02M phosphate buffer at pH 7.6, and then dialyzed against the same buffer for 16 h and further centrifuged at 10,000 g for 20 min. The clear supernatant (ammonium sulphate fraction) was pooled and subjected to purification processing.

Amino acids analysis

Amino acids were analyzed by using HPLC (Spectra-Physics Analytical, Inc. A0099- 600 with spectra focus optical scanning detector and spectra system UV 2000 detector and ultra sphere C_{18} Beckman column). The analysis was carried out using a gradient of Pico-Tag solvent A & B at 40°C and flow rate 1 ml/min. Detection of the separated Pico-Tag amino acids at 254 nm wavelength. Before injecting of the sample, the illustrated was calibrated by two injections of the standards[15].

Minimum inhibitory concentration (MIC) of the inhibitor

The purified protease enzyme (20 µl) was supplemented with different concentrations gradient of the inhibitor protein; ranged from 5 µl to 40 µl. These mixtures were incubated at 45°C for 6 hrs at pH 8. The anti-proteolytic activity of an inhibitor was checked at each reaction compared with positive control of protease enzyme. MIC of the inhibitor was determined as the lowest concentration of the inhibitor at which the proteolytic activity disappeared.

Effect of pH on the stability of the inhibitor

The inhibitor protein (2.9 µg) was mixed with 0.1 ml water and 0.1 ml of different solutions or buffers, and then incubated for 72 h at 4°C. Phosphate buffer was used at different values of pH ranged between 1 and 10. Therefore, 0.1 ml from each treatment was pipette and injected in agar well of skim milk agar medium incorporated with protease enzyme to assay the anti-proteolytic activity.

Effect of temperature on the stability of the inhibition

The inhibitor protein (1.45 µg) was mixed with 5 ml of water and subjected to heat treatment at different degrees (20 to 100°C) for 30 minutes. The residual inhibitor activity was assayed against protease enzyme activity by using clear zone method.

RESULTS

Isolation of fungal isolates and screening for protease production

Five isolates of pathogenic fungi (*F. solani*, *F. graminearum, R. solani, M. phaseolina,* and *A. alternata*) were isolated and purified originally from different naturally diseased plants collected from different agricultural fields in Saudi Arabia.). The isolated fungi were screened for protease enzyme production on skim milk agar medium. The highest proteolytic activity was observed around the disc of fungal isolate F-5, which was identified as *F. solani* (Data not shown).

Optimization of cultural conditions for maximum protease production

Optimization of culture conditions is essential to get high yield of protease enzyme. Hence, the present study described the optimization of culture conditions for the production of protease enzyme by *F. solani*.

Table 1. Optimum cultural conditions for maximum proteolytic activity and biomass of *F. solani*

Parameter	Value/Matter	Mean value of clear zone (mm)	Mycelium dry weight (mg/ml)
Inoculum size (µl)	80	10	0.54
	100	15	0.65
	120	20	0.80
	140	30	0.91
	160	35	1.04
	180	45	1.41
	200	50	1.78
	220	40	1.24
Incubation period (day)	1.0	20	0.73
	2.0	25	0.84
	3.0	30	0.94
	4.0	35	1.31
	5.0	43	1.75
	6.0	48	1.82
	7.0	52	1.95
	8.0	45	1.41
pH	5.0	15	1.02
	5.5	20	1.20
	6.0	27	1.44
	6.5	33	1.62
	7.0	40	1.71
	7.5	45	1.91
	8.0	50	2.11
	8.5	43	1.85
Incubation temperature (°C)	15	20	1.39
	20	20	1.51
	25	25	1.77
	30	30	1.87
	35	40	1.92
	40	45	2.11
	45	55	2.24
	50	50	2.04
Agitation speed (rpm)	100	40	2.54
	120	50	2.77
	140	55	3.12
	160	45	2.81
	180	35	2.51
Nitrogen source	KNO_3	50	1.97
	Yeast extract	45	1.54
	Malt extract	35	1.47
	Beef extract	30	1.33
	Peptone	40	1.24
	Casein	30	1.18
	Urea	25	1.01
Agro wastes(carbon source)	Wheat bran	30	2.37
	Rice bran	45	2.10
	Rice straw	50	2.44
	Barley bran	40	2.24

On the economic feasibility of fermentation, four agro-wastes (wheat bran, rice bran, rice straw and barley bran) were tested as carbon sources in solid state fermentation. Rice straw was the best agro-waste at which the highest proteolytic activity was observed (Table 1).

Data presented in Table (1) show the proteolytic activity and biomass of *F. solani* during 7 days of incubation period. The obtained results indicated that proteolytic activity and fungal biomass was initially observed at the first day of incubation and gradually increased with the incubation duration and reached its maximum level at 7th day of incubation. Values of the two parameters were found to decline as the incubation period further extended.

Influence of different temperatures (15-50 °C) on the protease activity and fungal biomass is shown in Table (1). Increasing temperature up to 45°C led to an increase in both investigated parameters. Above the optimum temperature the increase in temperature was accompanied by a decrease in the protease activity and a decrease in the fungal biomass.

Different initial pH values (5-8.5) were tested to determine the optimum pH value. The proteolytic activity varied as the medium pH changed between 5 and 8.5. Increasing the medium pH led to an increase in the proteolytic activity up to a certain limit above which any increase in the pH value was accompanied by a decrease in the proteolytic activity. The maximum protease activity and fungal biomass were observed at pH 8 (Table 1).

Table 2. Precipitation of protease enzyme by saturated ammonium sulfate

Fraction	Clear zone (mm)	Total protease content (u)	Total protein content (mg)	Specific activity (u/mg)	Fold purification	% yield
Control	50.0	10,000	180	55.5	1	100
10%	0.0	0.0	5.2	0.0	0.0	0.0
20%	0.0	0.0	5.7	0.0	0.0	0.0
30%	0.0	0.0	6.1	0.0	0.0	0.0
40%	40.0	8,000	8.2	975.6	17.5	80
50%	35.0	7,000	8.8	795.4	14.3	70
60%	0.0	0.0	7.5	0.0	0.0	0.0
70%	0.0	0.0	7.1	0.0	0.0	0.0
80%	0.0	0.0	6.4	0.0	0.0	0.0
90%	0.0	0.0	5.5	0.0	0.0	0.0

One unit of enzyme is the amount required to hydrolyze one micromole of substrate

Table 3. Precipitation of protease inhibitory protein by saturated ammonium sulfate

Fraction	Inhibition zone (mm)	Total protease inhibitory protein content (u)	Total protein content (mg)	Specific activity (u/mg)	Fold purification	% yield
Control	40.0	8,000	120	66.6	1	100
10%	0.0	0.0	1.38	0.0	0.0	0.0
20%	0.0	0.0	1.45	0.0	0.0	0.0
30%	0.0	0.0	1.57	0.0	0.0	0.0
40%	0.0	0.0	1.68	0.0	0.0	0.0
50%	0.0	0.0	1.73	0.0	0.0	0.0
60%	0.0	0.0	1.87	0.0	0.0	0.0
70%	30.0	6,000	2.14	2803.7	42	75
80%	0.0	0.0	2.01	0.0	0.0	0.0
90%	0.0	0.0	1.74	0.0	0.0	0.0

One unit of enzyme inhibitor is the amount required to inhibit one micromole of enzyme

Seven nitrogenous compounds (potassium nitrate, casein, peptone, yeast extract, malt extract, beef extract and urea) were tested as substitute nitrogenous sources. Potassium nitrate was the best nitrogen source for protease production, followed by yeast extract, while urea was the poorest nitrogen source for supporting protease production (Table 1).

Effect of different agitation speeds (100, 120, 140, 160, and 180 rpm) on the protease activity and fungal biomass was investigated (Table 1). Increasing the agitation speeds led to an increase in the proteolytic activity up to the optimum point (140 rpm) at which the maximum activity was recorded. Above this point, any increase in the agitation speed was accompanied by a decrease in the proteolytic activity.

Results presented in Table (1) show the proteolytic activity and biomass of *F. solani* at wide range of inoculum size (80 - 220 µl) of fungal suspension. The proteolytic activity was observed at 80 µl, and then increased gradually until 200 µl at which the maximum proteolytic activity and biomass was obtained, and then declined again.

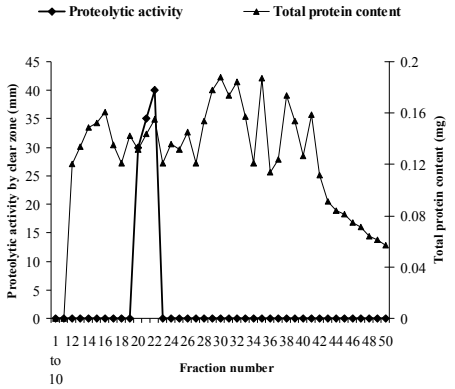

Fig. 1(a). Purification of protease enyzme by ion-exchange column chromatography

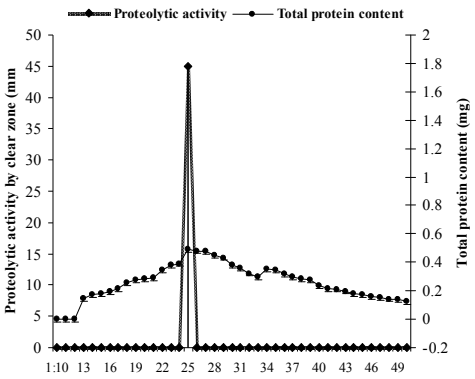

Fig. 1(b). Purification of protease enyzme by gel filtration column chromatography

Production and purification of protease enzyme

Protease production by *F. solani* was carried out under solid state fermentation. The enzyme was precipitated by using different percentages of saturated ammonium sulfate (10 – 90%). Results obtained showed that the enzyme was precipitated at 40 - 50% saturated ammonium sulfate (Table 2). The precipitated fraction at 40% has 8,000 units of protease and 8.2 mg total protein content, so it has 17.5 protease folds and 80% of protease enzyme could be precipitated. The other active fraction of saturated ammonium sulfate at 50% has 7,000 units of protease and 8.8 mg total protein content, so it has 14.3 protease folds and 70% of protease enzyme could be precipitated. Consequently, the highest amount of protease enzyme was precipitated at 40% followed by 50% of saturated ammonium sulfate.

After precipitation of protease enzyme, it was subjected to purification through three successive stages; dialysis, ion-exchange, and gel filtration. The two active fractions of saturated ammonium sulfate (40 and 50%) were dialyzed to eliminate all impurities and ions from enzyme emulsion and passed through ion-exchange column chromatography compacted with DEAE-cellulose. Fifty fractions were obtained at rate 5 ml/20 min. Proteolytic activity and total protein

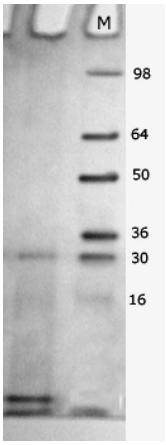

Fig. 2. SDS-PAGE analysis of purified protease enzyme

content were measured at each fraction. The proteolytic activity was observed only in three successive fractions (20-21-22) (Fig. 1a). These fractions were dialyzed and then passed through gel filtration column chromatography compacted with Sephadex G-200. Fifty fractions were obtained at rate 5 ml/20 min. Proteolytic activity and total protein content were measured at each fraction. The proteolytic activity was observed in only one fraction[25] (Fig. 1b). The molecular weight of purified enzymatic protein was determined by using SDS-PAGE, which showed a single band at approximately 30KDa (Fig. 2).

Isolation and purification of protease inhibitor protein

The inhibition activity of a crude extract of *R. vesicarius* leaves was investigated against the produced protease enzyme using antagonistic test on skim milk agar medium. The obtained result of this test revealed that the proteolytic activity disappeared completely.

The protease inhibitory protein was precipitated at 70% saturated ammonium sulfate. The total protease inhibitory protein and total protein content were assayed in each fraction. Thereby, specific activity, fold purification, and yield percentage were calculated (Table 3). The active fraction was dialyzed and then passed through ion-exchange column chromatography followed by gel filtration column chromatography.

At ion-exchange column chromatography, the inhibited proteolytic activity was observed in only two successive fractions[35-36] as shown in (Fig. 3a). These fractions were pooled, dialyzed and then passed through sephadex G-200 column chromatography. The inhibited proteolytic activity was observed in only one fraction[45] as shown in (Fig. 3b). The molecular weight of purified enzymatic protein was determined by using SDS-PAGE, which showed a single band at approximately 46 KDa. The protease inhibitor protein was analyzed by HPLC to determine the amino acids composition (Fig. 4). This inhibitory protein has 17 amino acids; aspartic acid, glutamic acid, serine, glycine, histidine, arginine, therionine, alanine, praline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine, and lysine.

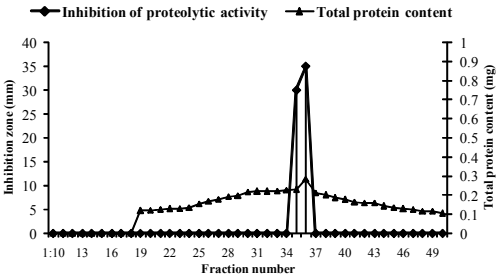

Fig. 3(a). Purification of protease enyzme inhibitory protein by ion-exchange column chromatography

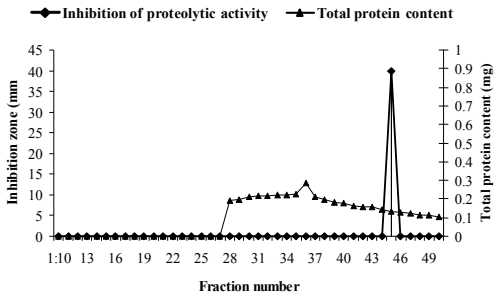

Fig. 3(b). Purification of protease enyzme inhibitory protein by gel filtration column chromatography

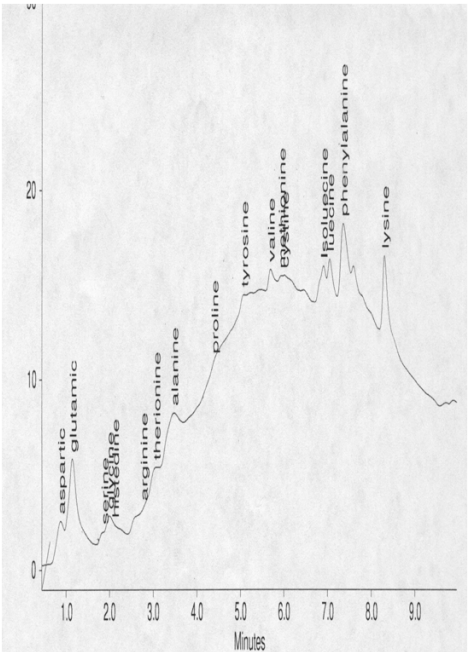

Fig. 4. Amino acids analysis of protease inhibitory protein

Characterization of protease inhibitor protein

The MIC of the inhibitor protein was determined against the purified protease enzyme. The MIC was observed at 20 µl of an inhibitory protein per 40 µl of protease enzyme. Stability of the inhibitor protein was investigated against some physical and chemical treatments. Data obtained revealed that the protease inhibitor protein was stable at temperatures 30 - 70°C for 30 min. However, the inhibition activity disappeared at 20°C and at temperature more than 70°C. The optimum temperature for an inhibitor was observed at 50°C (Fig. 5a). On the other hand, the inhibition activity was investigated along certain range of pH constricted between 4 and 10. The inhibition activity was observed at pH 5 and increased with the increase of the pH value and reached the maximum activity at pH 7 after which the inhibition activity decreased gradually and disappeared at pH 10 (Fig. 5b).

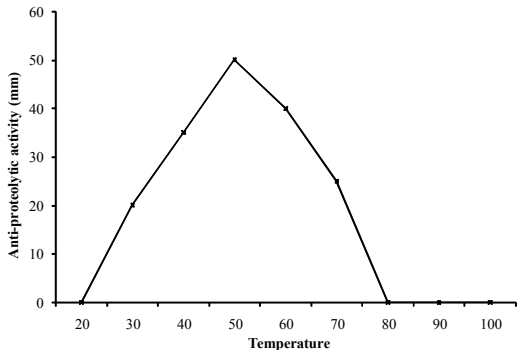

Fig. 5(a). Heat stability of protease inhibitory protein

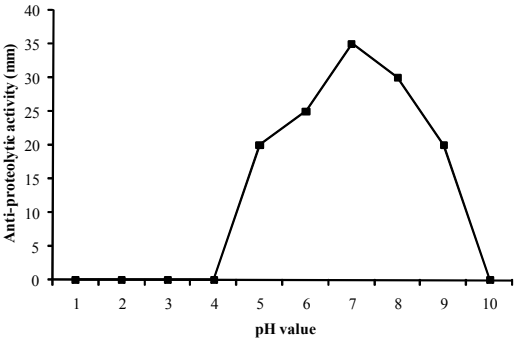

Fig. 5(b). pH stability of protease inhibitory protein

DISCUSSION

Of the seven pathogenic fungi screened to production of protease enzyme, the highest protease activity was recorded for the fungal isolate F-5, which was identified as *F. solani*. This result is in agreement with that of Olivieri *et al*. [16] whom reported the proteolytic activity of *F. solani* and *F. solani* f. sp. *eumartii* as an infection mechanism used in the dry rot disease of potato cultivars.

Extracellular enzymes are important to fungi not only for digestion but also in many instances for the pathogenic process: the enzymes may function in overcoming the natural resistance of the host and degrading the host cell components as well as in providing soluble products that can be absorbed and used as food[17]. The production of extracellular proteases by plant pathogenic fungi is also well documented, and it has been proposed that in some fungus-plant interactions these enzymes may function as pathogenic factors[18].

Proteases are inducible enzymes; secrete only in the presence of their substrates, which hardly affect fermentation process depending on their nature. Therefore, the successful fermentation process depends on the proper choice of an appropriate inducing substrate. These substrates are usually agronomical residues in solid state fermentation to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling[19]. In this connection, four agro-wastes (wheat bran, rice bran, rice straw, and barley bran) were tested. Of the tested substrates, rice straw was found to be the most effective in protease production. Rice straw is an enriched raw material with stored energy in glycosidic bonds of starch, which has large number of carbon atoms and hydrolyzed enzymatically to give considerable amounts of glucose molecules as end product. This result is in accordance with that of Ishtiaq *et al*.[20] that used rice straw as a by-product for the production of protease enzyme using submerged fermentation technique.

Solid state fermentation has many advantages than that of submerged fermentation; including ample productivity, easy use machinery, use of inexpensive substrates, simpler downstream processing, and lower energy requirements[3]. The fermentation medium must be controlled by optimum environmental conditions, which play an important role in the propagation of microbial

biomass and metabolic activity. Among these conditions are the fermentation medium, incubation period, temperature and pH, which reported to have substantial effects on the production of proteases[21]. In this connection, it was found that the incubation for 7 days gave the highest protease production. These results are supported by Chinnasamy *et al*.[22] who reported the maximum protease enzyme production at the 7th day of incubation using *A. flavus*. The incubation period is directly related to production of enzymes and other metabolites to a certain extent. After that, the enzyme production and the growth of the fungus decrease; this can be attributed to the reduced availability of nutrients and the production of toxic metabolites[23].

On the other hand, the highest protease activity was achieved by incubation at 45 ºC. Therefore, protease enzyme is located in the region between mesophilic and thermophilic enzymes. Another study reported that, the protease enzyme had optimum activity at a temperature of 45°C and showed a rapid decrease of activity at 48°C [24]. Incubation temperature is an important factor on which the proteases production is dependent. Although, many proteases are sensitive to higher temperature and may be denatured some others are heat stable, which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature[4].

In this investigation, the produced protease enzyme was designated as an alkaline enzyme, because the highest activity was observed at pH 8. Protease production by microbial strains strongly depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production[5].

Potassium nitrate salt was found to be as the best nitrogen source. This result is in agreement with that of Chinnasamy *et al.,*[22] who reported that, KNO_3 (at 3% concentration) was found to be the most suitable nitrogen sources for protease production. Protease enzyme could be produced under aerobic conditions with hard agitation for good aeration. In this connection, the maximum yield of proteolytic activity was obtained at 140 rpm in incubator shaker, at which abundant oxygen amount was present without foams formation.

Using SDS-PAGE, the molecular weight of the purified protease enzyme was separated at 30 KDa. The same results was recorded by Dobinson *et al.,*[25] who reported that the plant pathogenic fungus *Verticillium dahliae* produced extracellular alkaline protease enzyme, which separated at 30 KDa.

The second aim of this work is the isolation of protease inhibitor protein from the leaves of *R. vesicarius*. The molecular weight of the purified inhibitor protein was 46 KDa. The protease inhibitor protein was found to contain of 17 amino acids at different percentages. Jergensen *et al.,*[26] reported that, inhibition of *Fusarium* protease was carried out by an inhibitory protein, which present in SDS-PAGE at 46 KDa and identified by tryptic peptide mass fingerprinting. Plant serpins (serine protease inhibitors) family has molecular mass of 39 - 46 KDa, with amino acid and nucleotide homology with other well characterized serpins. The majority of serpins inhibit serine proteases, but serpins that inhibit caspases[27] and papain like cysteine proteases[28-30] have also been reported.

Stability of the purified inhibitor protein against some physical and chemical treatments was studied. Protease inhibitory protein was found to has a high heat stability up to 70°C for 30 minutes. Saini[31] reported that, trypsin-protease inhibitors were resistant to dry heat and retained more than 50% activity after heating at 105°C for 15 minutes. Furthermore, the purified inhibitor protein was found to has a high stability towards a wide range of pH (5-9). Another study reported that protease inhibitor protein is stable at pH 2- 12 at 25°C, and is highly specific for chymotrypsin at pH 7-12 [32].

In conclusion, in the present study, it was observed the capability of *F. solani* to produce and secrete protease enzyme. This study will serve to increase the understanding of factors that control the production and inhibition of protease enzyme which may help in many industrial aspects.

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