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Production of Novel Antitumor Chitooligosaccharides by using Purified Chitosanases from *Capsicum annuum* Leaves

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Abstract: Purification of three chitosanases (A_1, A_2 and A_3) from *Capsicum annuum* (pepper) leaves extract was done with different techniques giving three isoenzymes named A_1, A_2 and A_3 with specific activities of 107.3±6.9, 57.8±0.1 and 81.1±0.0 U/mg protein, respectively. The enzymes were purified about 66.2, 35 and 49 fold by three chromatographic steps in Sephadex G-100, diethylaminoethylcellulose (DEAE-cellulose) and Sephadex G-200. The pure enzymes A_1, A_2 and A_3 gave single band by sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE) for each enzyme with molecular weights of 26.2, 59 and 94 KDa, respectively. While the molecular masses of the purified enzymes A_1, A_2 and A_3 were found to be 23, 33 and 34 KDa, respectively by gel filtration technique. They were stable below 50°C for 60 min with maximum activity at pH range of 5.0-5.2 and 45°C. The three enzymes A_1, A_2 and A_3 were highly specific to hydrolyze chitosan exhibiting K_m value of 3.18, 3.9 and 3.7 mg/ml and V_{max} of 30.9, 19.03 and 31.8 U/mg, respectively. The three enzymes could also degraded chitin substrate with lower efficiency. Inhibition of the three chitosanases A_1, A_2 and A_3 was observed with $CoCl_2$, $NiCl_2$, Hg SO_4, $AgNO_3$, $FeCl_2$, $CuSO_4$ and $ZnSO_4$ with different percentages. They were also inhibited by cysteine, EDTA, iodoacetate, D-L-dithiotheritol and sodium azide. High amount of glucosamine (14.6 U) was produced by hydrolysis of chitosan by A_2 compared with A_1 and A_3 (5.3 and 0.0 U, respectively) as determined by acetyl acetone method (AcAc). This indicates that A_2 is exo type and A_1 and A_3 are endo type. These results were confirmed by the appearance of glucosamine in chitosan degraded product by A_2 as identified by TLC and also by HPLC analysis (718.4 mg/100g of sample). The purified chitosanase A_1 was rich in aspartic acid, glutamic acid and histidine. Chitosanase A_2 was rich in aspartic acid, glutamic acid and lysine while chitosanase A_3 was rich in aspartic acid, glutamic, leucine and tyrosine. The three isoenzymes degraded chitosan to chitooligosaccharides (COS) with antitumor activity. COS-A_1 was the most efficient as inhibitor of HEP-G2 (hepatocellular carcinoma cell line) with IC_{50} of 17.8 µg/well; COS-A_3 was superior in suppressing HCT-116 (colon carcinoma cell line) with IC_{50} of 8.62 µg/well. But the cytotoxicity of all COS showed nearly the same results against MCF7 (breast carcinoma cell line).

Key words: Chitosanase - chitosan – chitooligosaccharides - pepper leaves – purification - physicochemical properties - antitumor activity.

INTRODUCTION

Chitosanases (EC. 3. 2. 1. 132) represent class of hydrolytic enzymes acting on chitosan (a polymer of β-1-4-D-glucosamine). It produces chitooligosaccharides from chitosan, ranging mainly from chitotriose to chitohexose (Sikorski *et al*., 2006). Two types of chitosanase are endo- and exo- type cleavage of chitosan. Exochitosanase released a single glucosamine residue from chitosan and glucosamine oligomers. The endochitosanase had a useful reactivity and a high specific activity for producing functional chitooligosaccharides with high degree of polymerization (Chen *et al*., 2005).

Chitosanases occur in a variety of microorganisms, including bacteria and fungi (Wang *et al*., 2008b, Lin *et al.,* 2009, Struszczyk *et al*., 2009 and Cristiane *et al*., 2010). Chitosanases are also found in vegetative parts and seeds of some higher plants (Ouakfaoui and Asselin, 1992a). The same authors reported that *Triticum aestivum* and *Hordeum vulgare* seeds are the best monocotyledons sources of chitosanases while *Pisum sativum* seeds and *Cucumis sativus* fruits are the best dicotyledons sources.

Chitosan and its oligosaccharides, which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries. The biological activities of chitosan and chitooligosaccharides include hypocholesterolemic, antimicrobial, immunostimulating, antitumor and anticancer effects, accelerating calcium and iron absorption, anti-inflammatory, antioxidant and Angiotensin-I-converting enzyme (ACE) inhibitor activities. They effects are correlated with their structures and physicochemical properties (Tsai *et al*., 2000, Qin *et al.,* 2005 and Xia *et al.,* 2010).

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This study aimed at isolation, purification and characterization of a chitosanase enzyme from pepper leaves. Preparation of chitooligosaccharides by the enzymatic hydrolysis of chitosan and studying their antitumor activity are among the aims of the present study.

MATERIALS AND METHODS

Chemicals:

Chitosan, glucosamine, Sephadex G-100, Sephadex G-200, Diethyl aminoethyl cellulose (DEAE-cellulose), molecular weight markers and all resins and reagent for electrophoresis were obtained from Sigma Chemicals Co., St. Louis, USA and from Pharmacia Fine Chemicals, Sweden. Other chemicals were of analytical grade.

Preparation of the Crude Enzymes:

Healthy fresh pepper leaves (*Capsicum annuum*) were collected and cleaned thoroughly with water. They were sliced into small parts and homogenized in Braun multimix Mx 32 with distilled water at 5°C. The resulting homogenate was filtrated through cheese cloth and dialyzed against distilled water for 48 hrs at 5°C. The resulting dialyzates were centrifuged (13,000 xg, 5°C) for 15 mins and the supernatant was used as the crude enzymes (Fig. 1).

Preparation of Soluble Chitosan:

Ten grams of chitosan with 95% degree of deacetylation (DDA) powder was suspended in 400 ml distilled water and dissolved while being stirred in 5ml concentrated acetic acid. This solution was made with up to 1 L of water, and the pH was adjusted by using 1N NaOH (Choi *et al*., 2004).

2.2.3. Enzyme Assay:

Chitosan was used as the substrate in the chitosanase assay. Chitosanase activity was determined by quantitative estimation of the reducing sugar produced from chitosan. The reaction mixture contained 0.9 ml of 1% soluble chitosan (in 0.05 M sodium acetate buffer, pH 5.8), adequate amount of enzyme solution and 1 ml of 0.05 M sodium acetate buffer, pH 5.8. The reaction mixture was incubated at 50°C for 60 min. the reducing sugar formed in the supernatant was estimated spetrophotometrically by using the modified dinitrosalicyclic acid (DNS) method (Miller, 1959). The amount of D-glucosamine released was also determined by using the specific method of Rondle-Morgan (acetyl acetone method) for D-glucosamine (Rondle, 1955).

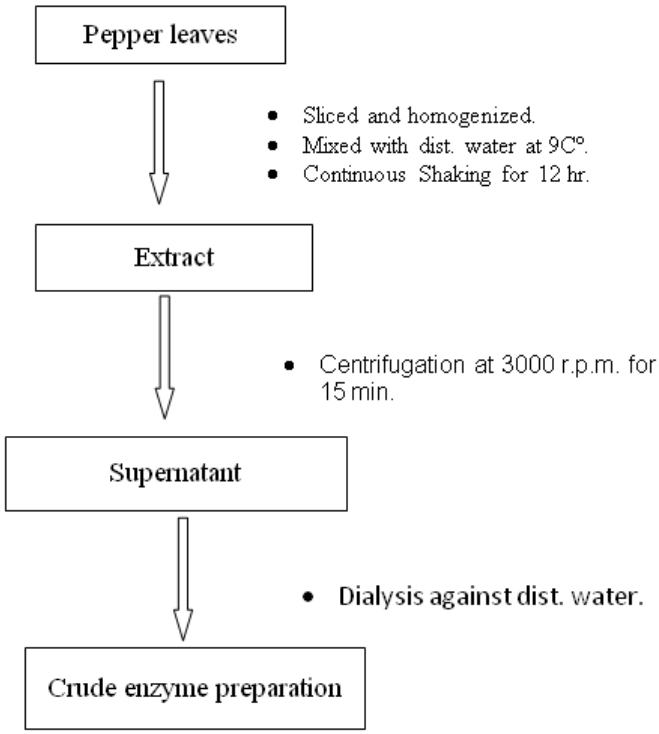

Fig. 1: Schematic presentation of preparation crude enzyme.

One unit of chitosanase was defined as the amount of enzyme that could liberate l μmole of reducing sugar (chitooligosaccharides or/and glucosamine) per min under the conditions described above. The Specific activity of chitosanase is defined as the units per milligram protein.

The activity of chitosanase values of samples were average values of three repeated measurements.

Protein Determination:

Protein was measured according to Lowry *et al*. (1951) method using a bovine serum albumin as a standard. During chromatographic separation, concentration of protein in the fractions was monitored by measuring their absorbance at 280 nm using a Shimadzu UV-2401, UV-VIS recording spectrophotometer.

Purification of the Crude Chitosanase:

Fractional precipitation with acetone for chitosanase revealed unsuitability as precipitating agent due to the poor yield obtained relative to the crude enzyme (data not show). The crude enzyme was precipitated by using ammonium sulfate with different concentrations (0-20, 20-40, 40-60 and 60-80% saturation) of saturation according to the method of Green and Hughes (1955). Each fraction was obtained by centrifugation at 13,000g and 4°C for 15 min. The resulting precipitates were dissolved in appropriate amount of distilled water and dialyzed exhaustively against distilled water for 2 days at 4°C to get rid of the excess of ammonium sulfate. Un-dissolved protein was removed by centrifugation before enzyme assay. Enzyme activity and protein content were determined in each fraction.

Gel filtration by using Sephadex G-100:

The dialyzed ammonium sulfate fraction with high chitosanase activity was concentrated by lyophilization and then applied to Sephadex G-100 column (1.2 × 50 cm) previously equilibrated with 0.01M acetate, pH 5.8. The protein was eluted with the same buffer at a flow rate of 15 ml/hr. The most active fractions were collected and concentrated by lyophilization.

Ion exchange using DEAE-cellulose:

The concentrated active fraction was applied directly on the top of the column (2 x 18 cm) of pre-activated DEAE-cellulose equilibrated with 0.01M acetate, pH 5.8. Elution was carried out using the same buffer at a flow rate of 30ml/h, with a linear gradient of NaCl (0.1-0.5M). Fractions of 5ml were collected at the elution rate 30 ml/hr. The eluted fractions were dialyzed against water for 48 h at 4 °C and monitored at 280 nm for protein and assayed for enzyme activity and protein content. The most chitosanase active fractions were pooled and concentrated by lyophilization.

Gel filtration by using Sephadex G-200:

The concentrated dialyzed active fractions of enzyme from DEAE-cellulose column were separately loaded to the top of Sephadex G-200 the column (1 x 37cm) previously equilibrated with 0.1M acetate, pH 5.8. The protein was eluted with the same buffer at a flow rate of 15 ml/hr.

Molecular Weight Determination:

1) Gel Filtration Chromatography:

The molecular weights was also determined by gel filtration technique using Sephadex G-200 column (1x37 cm) as described by Andrews (1964). The column was calibrated with Lysozyme (14,300), Trypsin from soybean (21,500), carbonic anhydrase (30,000), trypsin from porcine pancrease (33,000) and bovine serum albumin (69,000) as standard protein.

2) Sodium Dodecylsulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE):

It was done under denaturing conditions according to the method of Laemmli (1970).

Physicochemical Properties of the Purified Chitosanase:

Effect of Different pH's:

Small aliquots of the purified enzymes were assayed with two buffering agents, namely 0.1 M acetate (pH 4.5-5.8) and 0.1 M phosphate (pH 6.0-7.5) to record pH profiles under the standard assay conditions.

Effect of Temperature on Activity and Stability of the Pure Enzyme:

The maximum activities of the purified enzymes were determined at different incubation temperatures ranged from 30 to 70°C. Thermal stability was studied by preheating the purified enzymes at various temperatures (30, 40, 50, 60 and 70°C) for varying time intervals (30 and 60 min). The remaining enzyme activity was then assayed using the standard assay conditions.

Determination of Michaelis' Constant (K_m) and Maximum Velocity (V_{max}):

The K_m value and V_{max} were determined according to the method of Lineweaver and Burk (1934), using colloidal chitosan as substrate.

Determination of Substrate Specificity:

The activity of the purified chitosanases enzymes on several substrates (e.g. chitosan, chitin, xylan and CM-cellulose) was tested. All substrates were used in concentration of 1%.

Effect of Different Salts and Reagents on Enzyme Activity:

The effect of different salts on the purified chitosanase activity were investigated using $CaCl_2$, $HgCl_2$, AgCl, $CoCl_2$ $MgCl_2$, NaCl, $ZnSO_4$, $MnSO_4$, K_2SO_4, $CuSO_4$, $AgNO_3$, $FeSO_4$ and Hg $(NO_3)_2$ at concentration of 0.1 mM per reaction mixture. The enzyme was pre-incubated with each salt for 30 min. at room temperature and then the residual activity was measured. The activity of the enzyme without any salt was taken as 100%.

Identification of the Hydrolysate:

Chitooligosaccharides were qualitatively and quantitatively identified by two methods:

1) Thin layer chromatography (TLC) was applied using aluminium sheet of silica layer (qualitative method). The production of chitooligosaccharides was accomplished through enzymatic hydrolysis using the ammonium sulphate fraction (20-60%) and the three purified chitosanases A_1, A_2 and A_3. The reactions mixture (0.9 ml chitosan solution, 0.7 ml enzyme and 0.7 ml of acetate buffer) was incubated at 45°C for 48 hrs. The reaction was stopped by immersing the tube in boiling water for 10 min then the hydrolysates were analyzed by TLC using a solvent system of n-propanol-30% ammonia water (2:1). Sugar spots on the plates were visualized by spraying 0.1% ninhydrin into n-butanol –saturated water and by baking them in an oven at 110 °C for 10 min.

2) High-performance liquid chromatography (HPLC) and a Shim-Pack CLC-NH2 column (Shimadzu Co., Japan) were used. Oligomers analysis was carried out using acetonitrile (60%) as a mobile phase at a flow rate of 0.8 ml/min and a refractive index detector. The glucosamine and chitobiose peaks were quantified using a standard curve (1–10 mg/ml) in accordance to Liang *et al*. (2007), but the other chitooligomers were detected qualitatively.

Biological Activity of the Prepared Chitooligosaccharides:

I*n vitro* potential cytotoxic activities against some tumor cell line were performed in the National Cancer Institute using method of Skehan *et al*. (1990) using sulforhadamine-B (SRA) assay. The prepared chitooligosaccharides from chitosan by enzymatic hydrolysis with the purified enzymes (A_1, A_2 and A_3) were lyophilized. One mg of each lyophilized powders was dissolved in 0.1 ml of DMSO and the volume completed to 1 ml with distilled water. HEP-G2 (hepatocellular carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT-116 (colon carcinoma cell line) were plated separately in 10^4 cells/well for 24 hrs before treatment with the chitooligosaccharides to allow attachment of cell to the plate. Different concentrations of the compound under test (0, 1, 2.5, 5 and 10 µg/well) were applied. Triplicates were prepared for each individual dose. Monolayer cells were incubated for 48 hr at 37°C in atmosphere of 5% CO_2, after 48 hr cells were fixed, washed stained with sulforhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and the chitooligosaccharides concentration was plotted to get the survival curve of each tumor cell line for the specified product of enzyme.

The effective dose required to inhibit cell growth by 50% (IC_{50} µg/well) was determined. Doxorubicin was used as positive control.

Calculation:

The percentage of cell survival was calculated as follows:

Survival fraction = O.D. (treated cells)/ O.D. (control cells).

The IC50 values (the concentrations required to produce 50 % inhibition of cell growth).

Data are expressed as the mean ± standard error (SE) from at least three experiments. For purification steps, each value is the average of seven batches.

RESULTS AND DISCUSSION

In recent years vegetable wastes can serve as substrate for the production of useful products (chemicals and enzymes). Pepper leaves was chosen as the target plant tissue to be studied as it is rich in chitosanase activity (EL-Sayed, *et al.,* 2011). Pepper leaves are considered as a good economic source, because of its availability as an agriculture waste. Similarly, chitosanase was isolated from seeds, leaves and stems of bean, pea, tomatoes, cucumber, barley and maize and the leaves were found to have higher chitosanase activity than seeds

(Ouakfaoui and Asselin, 1992b and Osswald *et al*., 1994).

Partial purification of the crude enzyme was carried out by fractional precipitation using ammonium sulfate fractionation as an initial step of purification, about 41.6 % of the total enzyme activity was recovered in the fraction of 20-60% saturation (2.55fold purification). The ammonium sulfate fraction (20-60% saturation) was applied on Sephadex G-100 column. Two peaks having high chitosanase activity was obtained. Peak I fraction have 34.6 and 1.8 U/mg protein with a yield of about 19.26 and 42.25 % as determined by DNS and ACAC methods, respectively. Peak II have 24.05 U/mg proteins with a yield of about 14.85% as determined by DNS method. Each peak of the two enzymes I and II was applied separately to DEAE-cellulose column (2×18cm). The results of further purification of these fractions by DEAE-cellulose column revealed that a large part of the contaminating proteins was removed. Three chitosanases isoenzymes were eluted from the ion exchange. The first one was eluted at 0.01M sodium acetate buffer fraction having 374.4U with a yield of about 6.87% and purification fold of 47.3, the second one was eluted at 0.1 NaCl in acetate buffer having 174.4U with a yield of about 4.0 % and purification fold of 5.2 and the third one was eluted at 0.1 0.01M sodium acetate buffer having 140.4 U with a yield of about 2.58%. Further purification of the three pooled active fractions was concentrated by lyophilization and applied separately on Sephadex G–200 column. The data in figure (2-a,b,c) reveal that a single peak having high chitosanase activity for each enzyme which also indicates its purity and homogeneity in the final preparation. Data of purification was summarized in table (1) and fig. (3).

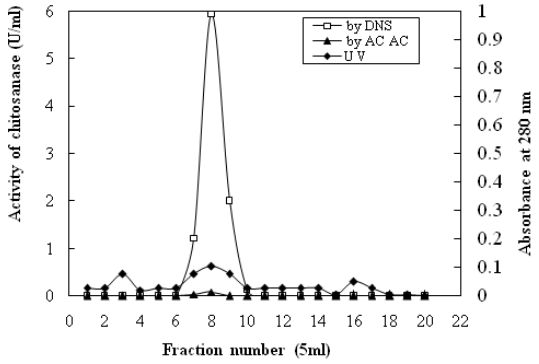

Fig. 2a: Elution profile of chitosanase A1 on Sephadex G-200, using 0.01 M acetate buffer, pH 5.8.

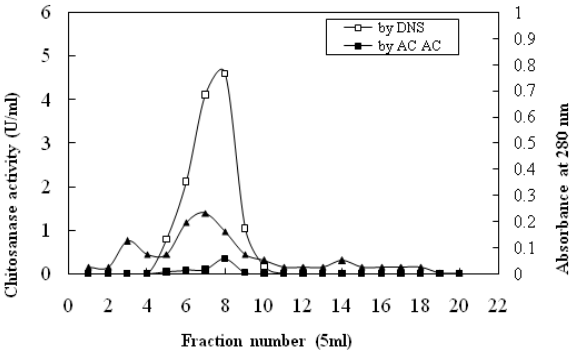

Fig. 2b: Elution profile of chitosanase A2 on Sephadex G-200, using 0.01 M acetate buffer, pH 5.8.

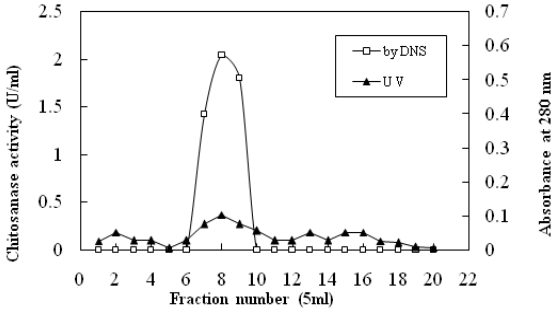

Fig. 2c: Elution profile of chitosanase A3 on Sephadex G-200, using 0.01 M acetate buffer, pH 5.8.

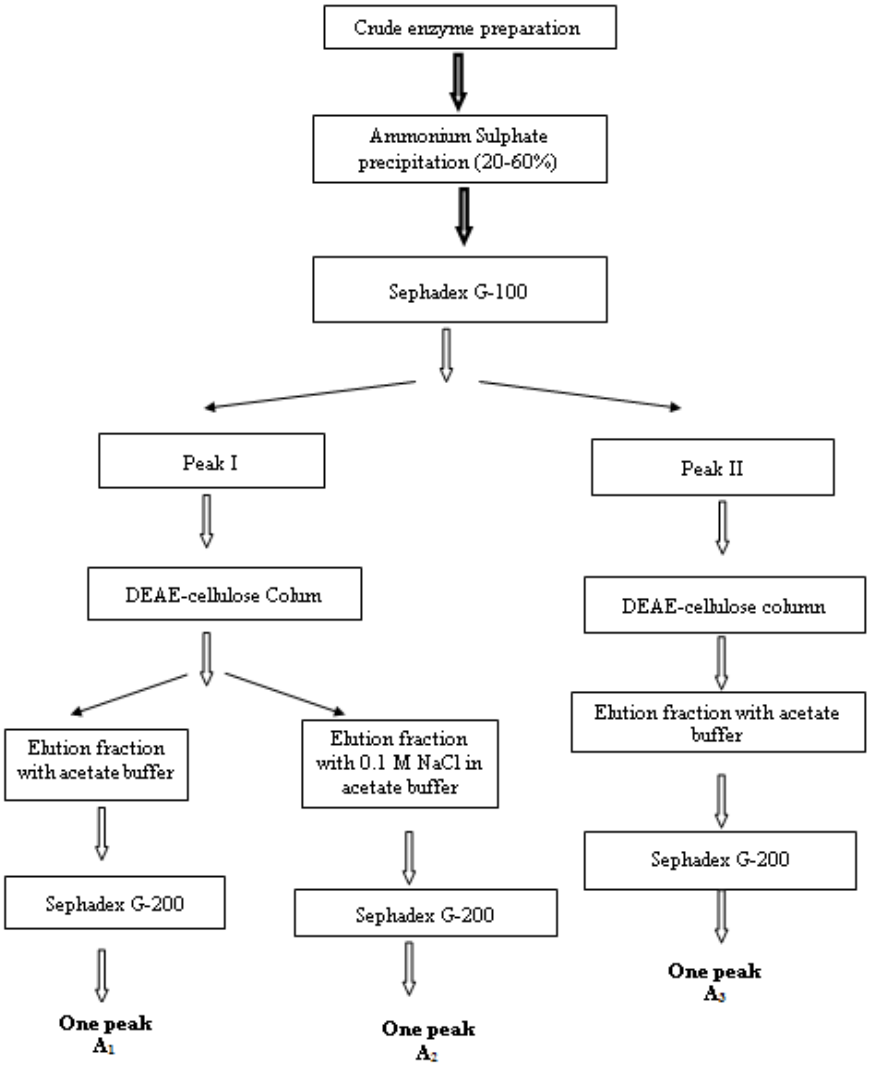

Fig. 3: Schematic presentation of purification enzymes (A_1, A_2 and A_3) from pepper leaves.

High purification folds of the three purified chitosanases A_1, A_2 and A_3 (66.2± 2, 35±0.03 and 49.2± 0.0, respectively) were higher than that of *Bacillus cereus* and *Microbacterium* Sp (8.8, 8 and 25) purification fold (Gao *et al*., 2008 and Sun *et al*., 2006).

The three purified chitosanases (A_1, A_2 and A_3) were isolated from pepper leaves with high recovery (5.6±0.0, 6.2±0.008 and 1.43±0.0 %, respectively). Thus, the total recovery was 12.2% similar to that obtained by Kurakake *et al*. (2000) and Wang *et al*. (2008b). The result of enzyme recovery was much better than those obtained by Gao *et al*. (2008) and Li *et al*. (2008).

The three purified chitosanases (A_1, A_2 and A_3) have high specific activities of 107.3±6.9, 57.8±0.1 and 81.1±0.0 U/mg, respectively. These values were higher than that of chitosanases from *Aspergillus* Sp. with 6.46 U/mg and 18.26 U/mg specific activities, respectively (Chen *et al*., 2005). On the other hand 334 and 800 U/mg specific activities of chitosanase was obtained by Shimosaka *et al*. (1995) which was much higher than that of our results.

Physicochemical Properties of the Purified Enzymes:

Effect of the pH on the Enzymes Activity:

The results in figure (4) demonstrated pHs optimum of A_1, A_2 and A_3 as 5.2, 5 and 5.2, respectively which were close to chitosanase optimum pH isolated from *Gongronella* Sp. and from marine bacterium *Bacillus subtillus* (Wang *et al*., 2008a and Hsu *et al.,* 2012).

Table 1: Steps of purification of chitosanases from Pepper leaves (100 g wet leaves).

Steps of purification	Total enzyme activity (U/ml)		Total protein (mg/ml)	Specific activity (U/mg)		Purification fold		Yield (%)	
	As Glucos-amine	As Reducing sugur		As Reducing sugur	As Glucos-amine	As Reducing sugur	As Glucos-amine	As Reducing sugur	As Glucos-amine
Crude extract	5427.2±36.5	129.7±7.0	3289.4±22.5	1.625±0.0	0.04±0.0	1±0.0	1±0.0	100±0.0	100±0.0
Ammonium sulfate fraction (20-60%)	2263.6±8.5	104.5±6.9	550.85±6.9	4.2±0.0	0.19±0.0	2.55±0.0	4.75±0.0	41.6±0.0	80.5±0.0
Sephadex G-100 column:									
1-Peak I	1044.5±6.9	54.87±0.9	30.17±1.9	34.6±0.0	1.8±0.0	21.25±0.0	45±0.0	19.26±0.09	42.25
2-Peak II	367.85±6.9	0.0	15.27±0.1	24.05±0.0	0.0	14.85±0.08	0.0	6.76±0.0	0.0
DEAE-cellulose column of peak I fraction:									
1-Enzyme eluted with acetate buffer	374.4±6.9	6.25±0.3	4.87±0.0	76.8±6.9	1.25±0.0	47.3±0.0	31.25±0.4	6.87±0.0	4.8±0.0
2-Enzyme eluted with 0.1M NaCl in acetate buffer	174.4±6.9	23.96±6.9	20.35±0.0	8.55±0.9	1.2±0.0	5.2±0.0	29.85±7.0	4.0±0.0	18.5±0.
DEAE-cellulose column of peak II fraction:									
3-Enzyme eluted with acetate buffer	140.4±7.0	0.0	3.37±6.9	41.6±2.9	0.0	25.6±1.0	0.0	2.58±0.0	0.0
Sephadex G-200 column:									
A1	305.05±6.9	5.3±0.0	2.83±0.07	107.3±6.9	1.86±0.0	66.2±2	46.5±0.0	5.6±0.0	4±0.0
A2	341.3±0.0	14.6±0.0	6.0±0.0	57.8±0.1	2.53±0.0	35±0.03	63.3±0.0	6.2±0.008	11.2±0.0
A3	77.9±0.0	0.0	0.96±0.04	81.1±0.0	0.0	49.2±0.0	0.0	1.43±0.0	0.0

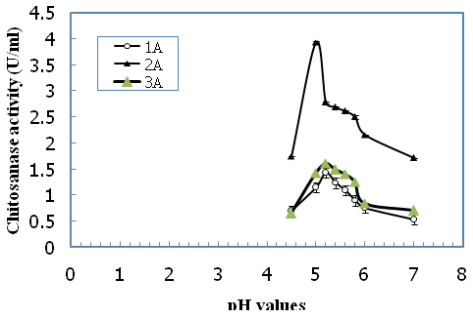

Fig. 4: The effect of pH on chitosanases A_1, A_2 and A_3, activities.

Effect of the Temperature on Enzyme Activity and Stability:

a-Activity of the Enzymes with Different Temperatures:

Based on fig. (5), the three enzymes A_1, A_2 and A_3 showed optimum activity at 45 °C and retained 70-75%, 79% and 89 % of their activity at 37 °C, respectively. At 70 °C A_1, A_2 and A_3 lost 70, 65 and 78% of their activity, respectively. The results were similar to that from *Bacillus* Sp. (Uchida and Ohtakara, 1988 and Chiang *et al*., 2003), but not as high as that found for *Bacillus Cereus*, *Gongronella* Sp., and bamboo shoots (55-60°C) (Jung *et al*., 2005, Wang *et al*., 2008a and Oh *et al.,* 2011).

b- Thermostability:

The thermal stabilities studies of A_1, A_2 and A_3in absence of substrate have indicated that the pure enzymes are completely stable and fully active at 40 °C after 60 min of incubation Fig. (6a, b and c). These results were in the same range of chitosanase illustrated by Jung *et al*. (2005), Gao *et al*. (2008) and Oh *et al*., (2011) while they were lower than that reported by Osswald *et al*. (1994).

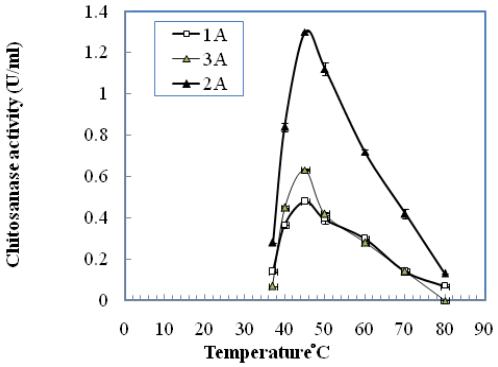

Fig. 5: The effect of temperature on chitosanases A_1, A_2 and A_3, activities.

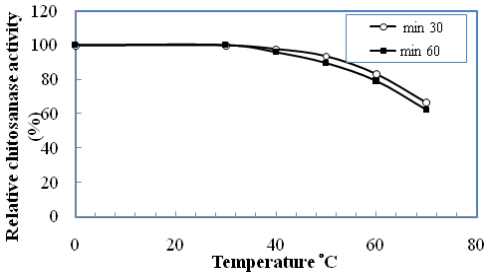

Fig. 6a: The effect of different temperatures on chitosanase A_1 stability at different times.

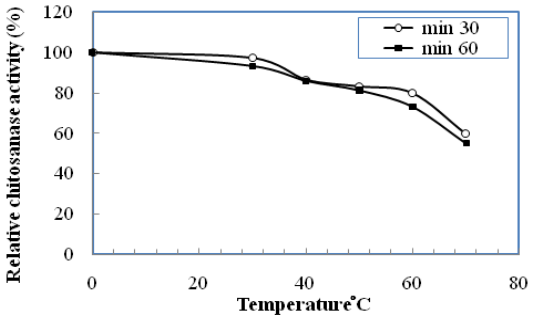

Fig. 6b: The effect of different temperatures on chitosanase A_2 stability at different times .

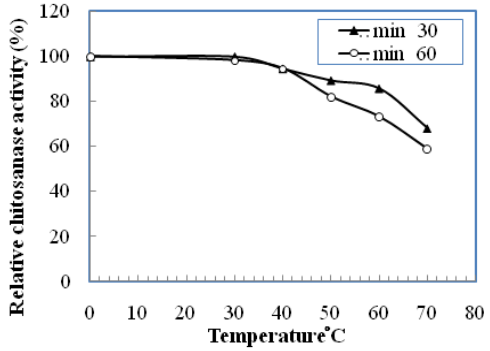

Fig. 6c: The effect of different temperatures on chitosanase A_3 stability at different times.

Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Singlebands for each purified chitosanases (A_1, A_2 and A_3) were performed by using SDS-PAGE method

indicated the high purity of the three isoenzymes (Fig. 7). The molecular weights of the three purified chitosanases (A_1, A_2 and A_3) were 26.2, 59 and 94 kDa, respectively. The molecular weight value of chitosanase purified from marine bacterium was detected as 29 kDa (Oh *et al.,* 2011). The molecular weights of most endo-chitosanases have been reported to range from 20 to 50 kDa by SDS-PAGE, while exo-chitosanases ranged from 94 to 135 kDa (Chen *et al*., 2005).

The molecular masses of A_1, A_2 and A_3 were determined also by a gel-filtration method on Sephadex G-200 and were found to be 23, 33 and 34 kDa, respectively (Fig.8). In comparison, Uchida and Ohtakara (1988) indicated that chitosanase molecular weight from *Bacillus* sp is 41 kDa by SDS, and 30 kDa by gel filtration on Sephadex G-100. Wang *et al*. (2008b) indicated that chitosanase molecular weight from *Serratia marcescens* was 21 and 18 kDa by SDS-PAGE and gel filtration.

Effect of the Reaction Time:

The prepared enzymes were incubated with the substrate for different time intervals up to 180 min, then the enzyme activity were estimated. The A_1, A_2 and A_3 activities display linear relationship with incubation times up to 150 mins using colloidal chitosan as the substrate. It could be concluded that excess products with increasing time of reaction may not interfere with the enzyme activities. This property could be considered as suitable one for using these enzymes for long time. These results were in accordance with results of purified chitosanase from *Metarhizium anisopliae* (Cristiane *et al*., 2010).

Effect of Different Enzyme Concentrations:

The enzyme activity was estimated at different concentrations of the purified chitosanase enzyme (µg / reaction mixture). The enzyme activities of the three chitosanases (A_1, A_2 and A_3) were increased by increasing the enzyme concentration up to 54, 267 and 56 µg protein per reaction mixture, respectively.

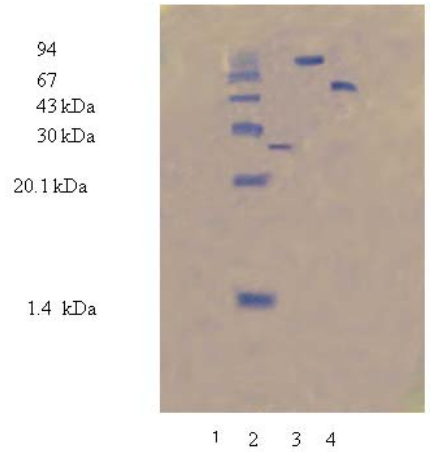

Fig. 7: SDS-PAGE of the three purified chitosanases (A_1, A_2 and A_3) extracted from leaves of pepper plant.
Lane1 Standard proteins, phosphorylase b (molecular mass, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and a lactalbumin (14.4 kDa).
Lane 2 chitosanase A_1
Lane 3 chitosanase A_2
Lane 4 chitosanase A_3

Effect of Different Substrate Concentrations:

Different substrate concentrations were incubated with the same amount of the enzyme concentrations. Linear relationships were observed between colloidal chitosan and the activities of the three chitosanases A_1, A_2 and A_3 up to 5.5, 4.7 and 5.4 mg/reaction mixture. The K_m values of A_1, A_2 and A_3 were estimated to be 3.18, 3.9 and 3.7 mg/ml respectively, while their V_{max} were 30.9, 19.03 and 31.8 U/mg, respectively (Fig. 9-a,b,c). These results are quite similar to K_m of *Bacillus* Sp (Uchida and Ohtakara, 1988). But they are slightly lower than that of *Bacillus Cereus* and *Gongronella* Sp (Gao *et al*., 2008 and Wang *et al*., 2008a).

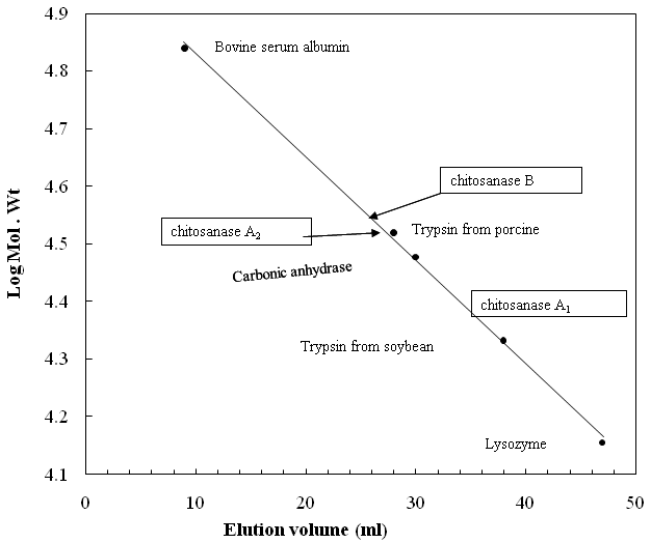

Fig. 8: Determination of molecular weights of chitosanases A_1, A_2 and A_3 by gel filteration on Sephadex G-200 (1× 37cm), with the using of the protein markers.

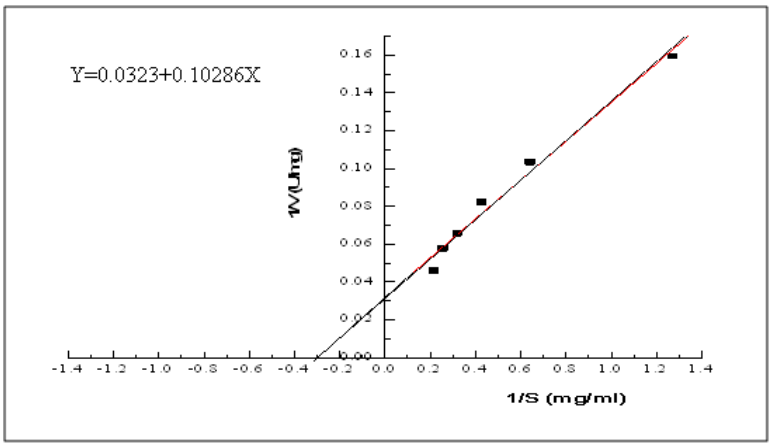

Fig. 9a: Lineweaver-Burk plot for hydrolysis of chitosanase A_1 by colloidal chitosan.

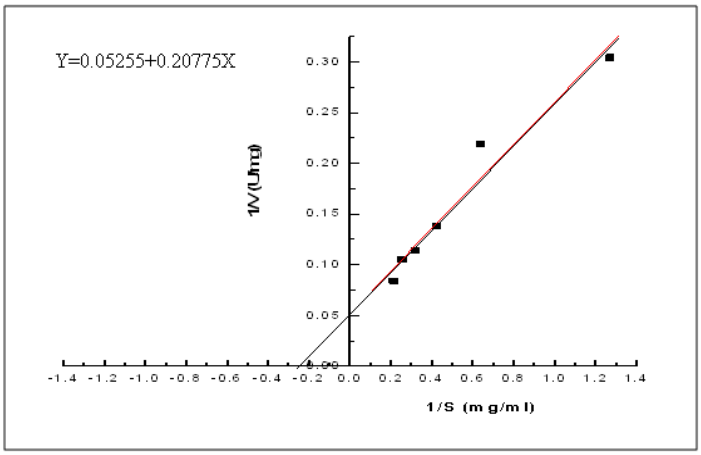

Fig. 9b: Lineweaver-Burk plot for hydrolysis of chitosanase A_2 by colloidal chitosan.

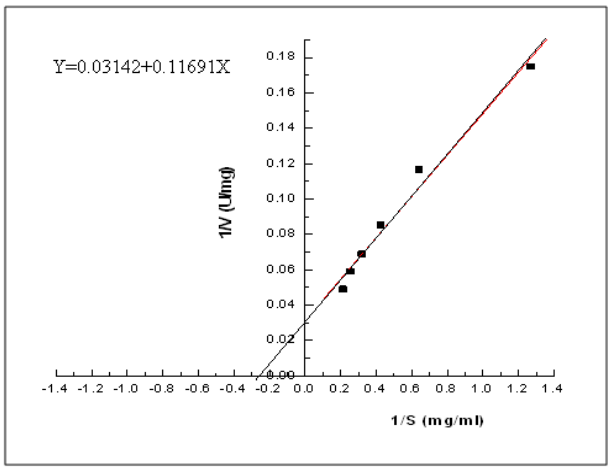

Fig. 9c: Lineweaver-Burk plot for hydrolysis of chitosanase A_3 by colloidal chitosan.

Substrate Specificity:

The three purified chitosanases A_1, A_2 and A_3 are specific for hydrolysis of chitosan, very low hydrolytic effect on chitin and CM-cellulose and no hydrolytic effect on xylan. These results are similar to that of *Gongronella* Sp, *Mucorcircinelloides* and Bamboo shoots (Wang *et al*., 2008a, Struszczyk *et al*., 2009 and Hus *et al*., 2012).

Ultraviolet Absorption Spectrum:

The absorbance behavior of the enzymes was studied at 200-350nm in 0.01M acetate buffer, pH 5.8 using CECIL instruments CE595 U.V spectrophotometer (Inoue *et al*., 1966). The ultraviolet absorbency profiles of A_1, A_2 and A_3 showed maximum absorbence at wave lengths 265, 265 and 270 nm, respectively which showed typical protein absorption spectrum. No significant absorbance could be traced in the visible region indicating the absence of chromophore (Inoue *et al*., 1966). The ratio of absorbance at 280 nm to 260 nm was 1.11 for the three isoenzymes. These indicated that the enzyme showed of no abnormalities (Bucher, 1955).

Activators and Inhibitors:

The effects of various activators and inhibitors at a concentration of 0.1 mM on the three chitosanases are presented in table (2). Only 0.1 mM of NaCl activated the chitosanase A_1 and A_2 by 10.63 and 26.6 %, respectively. The enzymes were inhibited by Hg^{+2}, Cu^{+2}, Ag^{+2}, Fe^{+2} and Cd^{+2} ions.

Amino – Acids Analysis:

Amino acid analysis was carried out by acid hydrolysis (with hydrochloric acid) of proteins (Millipore and Cooperative, 1987). The purified chitosanase A_1 was rich in aspartic acid, glutamic acid and histidine, chitosanase A_2 was rich in aspartic acid, glutamic acid and lysine and chitosanase A_3 was rich in aspartic acid, glutamic, leucine, tyrosine and alanine (Table 3).

A)Enzymatic Production of Chitooligosaccarides:

Hydrolysis of chitosan by the prepared partially purified and purified chitosanases were qualitatively and quantitatively analyzed by two methods.

a) Thin Layer Chromatography (TLC):

The end products of hydrolyzed chitosan were analyzed by using TLC sheet after 48 hrs incubation. Different chitooligosaccharides were detected in the hydrolysates. Glucosamine was detected only in the hydrolysates of chitosanase A_2.

b) High-Performance Liquid Chromatography (HPLC):

Chitooligosaccharides produced by enzymatic degradation of chitosan by the prepared chitosanases (A_1, A_2 and A_3) were subjected, side by side, with standard samples of glucosamine and chitobiose to HPLC technique mentioned previously in the methodology. Identification of the compounds were carried out by running them with standard samples, comparing the eluted peaks for the same typical retention times. The concentrations were calculated depending on the absorption of each peak area. as well as the retention time. The results of HPLC

analysis revealed the presence of glucosamine, chitobiose and chitooligosaccharides in the chitosan degraded products. The HPLC analysis also showed the presence of glucosamine in products produced by chitosanase A_2 with retention time of 5.033 min and showed the presence of chitobiose in products produced by A_1 and A_3 at 4.892 and 4.892 min, respectively. Also HPLC analysis showed the presence of different chitooligosaccharides in products produced by the purified chitosanases with different retention times and different areas. Table (4) shows the levels of glucosamine, chitobiose and chitooligosaccharides in the samples. Glucosamine were detected in chitosanase A_2 products by 718.4 mg/100 g of sample.

Table 2: Effect of some activators and inhibitors on the three chitosanases (A1, A2 and A3) activity under standardized reaction conditions.

Activators and inhibitors (0.1 mM /reaction mixture)	Relative activity of chitosanase A_1 (%)	Relative activity of chitosanase A_2 (%)	Relative activity of chitosanase A_3 (%)
Control	100	100	100
Mn^{+2}	102.12	98.1	103
Co^{+2}	22.34	2.8	81.13
Ni^{+2}	27.66	0.95	71.7
Hg^{+2}	70.21	65.7	94.34
Ag^{+2}	57.44	0.0	0.0
Na^{+1}	110.63	126.6	98.11
Fe^{+2}	74.46	0.0	0.0
Cu^{+2}	76.6	14.3	30.2
Zn^{+2}	21.3	76.2	22.6
Cysteine	0.0	0.0	0.0
EDTA	0.0	0.0	0.0
Iodoacetate	0.0	0.0	0.0
D-L-dithiotheritol	0.0	0.0	0.0
Sodium azide(NaN_3)			

*Three chitosanases activity without adding activators or inhibitors was taken as 100% activation.
*The experiments were performed by using the standard assay conditions described in the text.

Table 3: Amino acid contents of the three purified chitosanases (A_1, A_2 and A_3) from pepper leaves.

Amino – acid	Chitosanase A_1 (%)	Chitosanase A_2 (%)	Chitosanase A_3 (%)
Aspartic acid	15.8	23.64	14.04
Threonine	4.04	6.62	3.85
Serine	5.93	8.99	5.96
Glutamic acid	33.63	26.7	15.83
Glycine	0.0	0.0	2.48
Alanine	2.42	2.75	9.99
Valine	0.0	4.8	2.49
Leucine	0.0	7.44	10.02
Tyrosine	5.03	3.97	10.89
Phenylalanine	7.58	5.92	9.47
Histidine	15.41	0.0	4.11
Lysine	7.1	9.15	6.16
Arginine	3.06	0.0	1.02

Chromatographic analysis by TLC and HPLC of the end products released up on incubation of the colloidal chitosan with the three purified chitosanases (A_1, A_2 and A_3) revealed that A_1 and A_3 produced chitobiose, chitotriose, chitotetraose and chitopentaose. Chitosanase A_2 produced large amounts of glucosamine. These results confirmed that the two chitosanases (A_1 and A_3) isoenzymes have endo-type for producing chitooligosaccharides, while chitosanase A_2 have exo-type for producing glucosamine. These results are in agreement with chitosanases from, *Bacillus* sp, *Aspergillus* sp. CJ22-326 and a *Microbacterium* sp (Chen *et al*., 2005, Sun *et al.*, 2006 and Gao *et al*., 2008).

Table 4: Concentrations of enzymatic hydrolysis of chitosan by A_1, A_2 and A_3 as determined by HPLC analysis.

Enzymatic hydrolysis of chitosan by	Concentration of enzymatic hydrolysis of chitosan (mg/100g of sample)		
	Chitooligosaccharides	Chitobiose	Glucosamine
Chitosanase A_1	positive	6.377	N.D
Chitosanase A_2	positive	N.D	718.4
Chitosanase A_3	positive	33.69	N.D

A)Biological Activity of Chitooligosaccharides (COS) Produced by the Prepared Chitosanases (*A_1, A_2 and A_3****):***

In vitro cytotoxicity effect of the products (chitooligosaccharides) resulted from enzymatic degradation of colloidal chitosan by A_1, A_2 and A_3 on the growth of three tumor cell lines was studied using sulforohdamine B

(SRP) assay. The incubation of hepatocellular carcinoma (Hep-G2), colon carcinoma (HCT-116) and breast carcinoma (MCF7) with gradual dose of chitooligosaccharides cause gradual inhibition in the cell growth. The best effect of chitooligosaccharides against Hep-G2 was due to chitooligosaccharides obtained by chitosanase A_1 (COS-A_1) with IC_{50} of 17.8 µg/well and against HCT-116 was due to chitooligosaccharides of chitosanase A_3 (COS-A_3) with IC_{50} of 8.62 µg/well (Table 5 and Fig 11-a ,b and c). It was also found that all the tested chitooligosaccharides give a nearly the same result against MCf7 with IC_{50} 18 µg/well. Shen *et al*. (2009) studied inhibitory effects of chitooligosaccharides on tumor growth and metastasis; they showed that Hep-G2 cells were the most sensitive cancer cells to chitooligosaccharides, as compared with AGS human gastric cancer cells and COLO205 cells. Huang *et al*. (2006) studied the anticancer activities of differently charged chitooligosaccharides derivatives using three cancer cell lines: Hela, Hep-3B and SW480. Karagozlu *et al*. (2010) studied anti-proliferative effect of aminoderivatized chitooligosaccharides on AGS human gastric cancer cells.

Table 5: IC_{50} of chitooligosaccharides (COS) resulted from the degradation of chitosan by A_1, A_2 and A_3.

Enzymes	Chitooligosaccharides (COS)	HEPG2 (IC_{50})	HCT-116 (IC_{50})	MCF7 (IC_{50})
Chitosanase A_1	COS-A_1	17.8	15.9	18.4
Chitosanase A_2	COS-A_2	21.9	14.6	18.5
Chitosanase A_3	COS- A_3	26.1	8.62	18.1

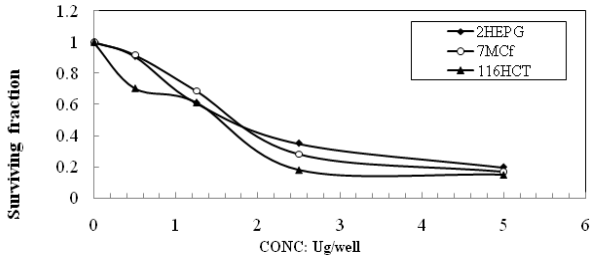

Fig. 10a: Cytotoxic activity of Sephadex G 200 purified chitosanase A_1.

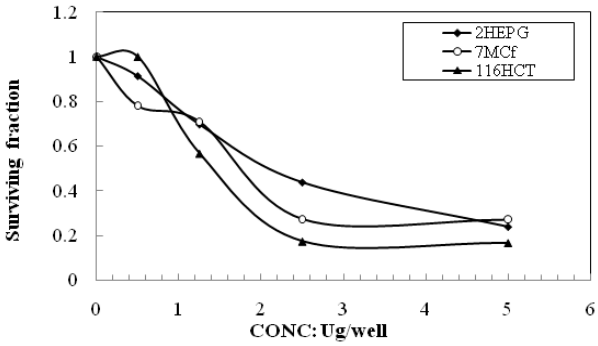

Fig. 10b: Cytotoxic activity of Sephadex G 200 purified chitosanase A_2.

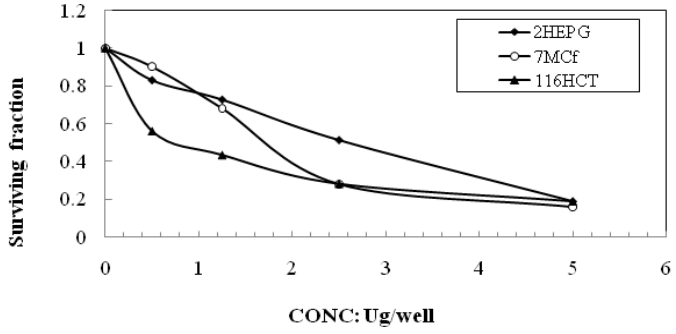

Fig. 10c: Cytotoxic activity of Sephadex G 200 purified chitosanase A_3.

Conclusion:

The present basic study showed that the vegetable waste (pepper leaves) can be considered as a potential substrate for producing good yield of pure chitosanases to be used in industrial applications. Biochemical properties of this pure enzyme revealed that it may be used in the medical field. At the same time, the study participates in solving some of the pollution problems caused by one of the Egyptian agricultural wastes. It is highly recommended to apply the described procedure extraction and purification of chitosanase enzyme on industrial scale. It can be used to prepare novel antitumor COS by enzymatic degradation of chitosan.

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