Isolation and Characterization of Chitosanase Enzyme from Different Parts of Some Higher Plants

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Abstract: Isolation and characterization of a chitosanase enzyme with high activity from different parts of some higher plants were studied. Different vegetative plant parts (leaves, fruit peels and dried seeds) representing nine families were screened in order to select the best source for extraction of the chitosanase enzyme. Results of screening experiments indicated that the enzymatic activity levels varied not only according to differences in plant species, but also to their morphological parts. In general, pepper, cabbage and purslane were the best leaves of chitosanases extractions and green bean was the best peels of chitosanase extraction while green bean, cabbage and purslane seeds were the best seeds of chitosanases extractions in this study. Results showed that leaves have high chitosanase activity more than seeds by three times. Seeds have high chitosanase activity more than peels of fruits by two times. Green bean peels, pepper leaves and opuntia peels were chosen for further study because of their high chitosanase activity. The activities of these three sources were measured by two methods. The two sources of chitosanase namely pepper leaves (C_1) and opuntia peels (C_2) were chosen for further studies. The optimal chitosanase activities of C_1 and C_2 enzymes on chitosan were obtained in 0.05 M acetate buffer, pH 5.8 and 5.4 at 40°C and 60°C, respectively. The isolated C_1 and C_2 chitosanase enzymes were stable on storage for more than three months at -20°C. Chitosanase C_1 and C_2 were stable for 60 minutes at 50°C and 60°C, respectively. The yields of the crude chitosanases C_1 and C_2 with optimum conditions were 47.616 and 59.146 U/g dry tissue, respectively.
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Key words: chitosanase - chitosan - pepper leaves – opuntia peels – physicochemical properties.

1. Introduction

Chitosanases (EC. 3.2.1.132) represent a class of hydrolytic enzymes acting on chitosan. Chitosan (a polymer of β-1, 4-D-glucosamine) is thought to be absent in plants but it occurs in the cell walls of fungi (**Araki and Ito, 1975**).

Chitosanases from various sources show different hydrolytic action patterns, which are dependent on the degree of polymerization and of acetylation of the substrates. Most of the chitosanases from various sources reported so far were apparently endo-acting in nature, liberating predominantly mixture of dimers, trimers and oligomers from chitosan. Endochitosanase hydrolyze oligomers of glucosamine larger than chitopentaose, and chitosan with 62-100% DDA (degree of deacetylation); but exochitosanase act on chitosan and release glucosamine. Hydrolysis of chitosan with 99% DDA by endochitosanase release chitobiose, chitotriose and chitotetraose as the major products (**Sun *et al.*, 2006** and **Chen *et al.*, 2005)**.

Most of chitosanases occur in a variety of microorganisms, including bacteria and fungi **(Chen *et al.*, 2005; Jung *et al.*, 2006** and **Wang *et al.*, 2008b).** Chitosanases are also found in vegetative parts and seeds of some higher plants **(Ouakfaoui and Asselin, 1992a).** They 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**. Osswald *et al.* (1994)** isolated chitosanase enzyme from sweet orange callus tissue.

Chitosanase produced chitooligosaccharides from chitosan, ranging mainly from chitotriose to chitohexose **(Sikorski *et al.*, 2006).** By controlling the reaction time and by monitoring the reaction products with gel filtration high - performance liquid chromatography (HPLC), chitooligosaccharides with a desired oligosaccharide content and composition were obtained after endohydrolysis steps **(Choi *et al.*, 2004)**. Chain length and the degree of deacetylation are considered the most important factors influencing the biological activities of chitooligosaccharides **(Matheson *et al.*, 1984).** The complete enzymatic hydrolysis of chitosan to free glucosamine is catalyzed by exo-β-D-glucosaminidase (EC. 3.2.1.165) **(Côté *et al.*, 2006)**. This enzyme was also designated as an exochitosanase. D-glucosamine which is the monomer of chitosan, have many biological, nutritional and pharmaceutical effects such as membrane stabilizing activity, liver protective effect, antioxidant activity and

immunostimulating properties **(Liu Wanshun *et al*., 2006 and 2007).**

Chitosanases are generally believed to play an important defense role against invading pathogens because of their potential to hydrolyse fungal wall polysaccharides **(Ouakfaoui and Asselin, 1992b).** Chitosanases have been extensively studied because of their hydrolytic effect on chitosan **(Sikorski *et al*., 2006)** and its derivatives (glycol chitosan, carboxymethyl chitosan and colloidal chitosan) **(Dumas-Gaudot *et al*., 1992).** Low and high molecular weight chitooligosaccharides perform potent antimicrobial, antitumor and immunopotentiating activities **(Matheson *et al*., 1984).** Also chitosanase show applications in various fields, such as biomedical, pharmaceutical, agricultural, biotechnological and in food industry **(Tokoro *et al*., 1988; Tsai *et al*., 2000** and **Qin *et al*., 2005).**

The aim of this study is to isolate, determine and characterize of chitosanase enzymes in some plants from different families

2. Materials and Methods

Experimental plants:

The plant materials used in this study consisted of samples of different vegetative plant parts (leaves and fruits peels) and dried seeds. They are leaves of clover, purslane, artichoke, cabbage, pepper and basil, peels of melon, papaya, water melon, green beans and opuntia fruits and dried seeds of green beans, pepper, cabbage and purslane. They represent nine families namely *Fabaceae, Cucurbitaceae, Portulaceae, Asteraceae, Caricaceae, Cactaceae, Brassicaceae, Solanaceae and Lamiaceae.*

Buffers:

Buffers were prepared according to **Gomori (1955)** and the final pH was checked by pH meter Hanna Instruments, Italy.

Extraction of the chitosanase enzyme from:

a- Dry seeds.

Dry seeds were crushed in Braun multimix MX 32 to be in a powder form. The dry powder was mixed with distilled water for 24 hours at 4°C. The extracts were centrifuged (13,000 xg at 5°C) for 15 minutes and the supernatants were dialyzed against distilled water for 48 hours at 4°C.The dialyzates were centrifuged again (13,000 xg at 5°C) for 15 minutes, and the supernatants were used as the crude enzymes.

b- Leaves and fruits peels.

The healthy fresh leaves and fruits peels of the selected plants were collected and cleaned thoroughly with water. These parts were sliced into small parts and homogenized in Braun multimix Mx 32 with certain extracting solution as distilled water at 5°C. The resulting homogenate was filtrated through cheese cloth and dialyzed against distilled water for 48 hours at 5°C.The resulting dialyzates were centrifuged (13,000 xg at 5°C) for 15 minutes and the supernatant was used as the crude enzymes.

Preparation of chitosan substrate:

The chitosan substrate for the production of chitosan oligosaccharides and low molecular weight chitosan was prepared as follow: ten grams of chitosan with 95% DDA powder was suspended in 400 ml distilled water and dissolved while being stirred with 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)**.

Enzyme assay:

Chitosan was used as the substrate in the chitosanase assay. Chitosanase activity was determined by quantitative estimation of the reducing sugars 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 minutes. The reducing sugars formed in the supernatant were estimated spectrophotometrically by using the modified dinitrosalicyclic acid (DNS) method (**Miller, 1959**), using glucosamine hydrochloride as standard. 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**). One unit of chitosanase was defined as the amount of enzyme that could liberate l nmol of reducing sugar as glucosamine per minute under the conditions described above. The activity of chitosanase values of samples were average values of three repeated measurements.

Physicochemical Properties of the purified chitosanase

Effect of the pH on the enzymes activity:

Small aliquots of the prepared enzymes were assayed with two buffering agents, namely 0.05 M acetate (pH 4.5-5.8) and 0.05 M phosphate (pH 6.0-7.5), for recording pH profile under the standard assay conditions.

Effect of the temperature on enzyme activity and stability

a- Activity of the enzymes with different temperatures

The maximum activities of the prepared enzymes were determined at different incubation temperatures ranged from 30-70°C.

b- Thermostability

Small aliquots of the prepared enzymes were preheated at different temperatures; 30, 40, 50, 60 and 70°C for varying time intervals; 30 and 60 minutes. The remaining enzyme activity was then assayed using the standard assay conditions.

Activity of the enzymes at different reaction times.

The prepared enzymes were incubated with the substrate for different time intervals up to 180 minutes, then the enzyme activity were estimated.

Effect of the different enzyme concentrations:

The enzyme activity was estimated at different concentrations of the chitosanase enzyme per reaction mixture. Then, the products were estimated and its amount was calculated per reaction mixture (µg / reaction mixture). Relation between enzyme concentrations and reaction products was plotted.

Effect of the different substrate concentrations on the enzyme activity:

The effect of different substrate concentrations were estimated by incubating different substrate concentrations (1 to 15 mg/reaction mixture) with the same amount of the enzyme concentrations. Then the enzymatic activity was plotted against substrate concentration.

3. Results and Discussion:

Screening for production of chitosanase enzyme from plant tissues.

Chitosanases have been isolated, identified and estimated by the amount of reducing sugar. The aqueous extracts of four seeds, five peels of fruits and six leaves of different plants were incubated separately with colloidal chitosan in order to assess the chitosanase activity.

Screening study focused firstly on sources that have high enzymatic activity for producing glucosamine. Table (1) represents the results of screening experiments for the existence of chitosanase activity in different vegetative parts (leaves and peels of fruits) and dried seeds.

The results indicated that the highest chitosanase activities were estimated in peels of fruits of green bean and opuntia and leaves of pepper, purslane and cabbage extracts (Table 1). The activity increased as chitosanase activity found in extracts of leaves of pepper, purslane and cabbage and peels of fruits of green bean (8.42, 3.622, 2.173 and 6.29 U/g dry tissue, respectively) than that found in the corresponding seeds extracts (0.2497, 1.954, 2.687 and 3.43 U/g dry tissue). On the other hand, the differences in the chitosanase activity found in cabbage leaves and seeds extracts were not significantly higher or lower. Generally, it was observed that leaves and fruits peels have high chitosanase activity more than seeds of the same plant in comparison with the activity by U/g dry tissue (Table 1).

Table (2) represents the results of screening experiments for three sources with high enzymatic activities of chitosanase as determined by the acetyl acetone and the dinitrosalicylic acid methods.

Chitosanases from pepper leaves and peels of opuntia and green bean fruits have high activity for producing of chitooligosaccharides from chitosan that determined by dinitrosalicyclic acid method (Table 2).

Results showed that chitosanase stability of green bean peels decreased by saving it at 4°C for two months, in contrary chitosanases from opuntia fruit peels and pepper leaves when saved at 4°C for more than three months conserve with its activity. Thus, the enzymatic activities were varied not only with the species of the tested plants, but also according to the morphology of the examined parts.

Opuntia fruit peels and pepper leaves were chosen for further study of the optimum assay conditions of their crude enzymes C_1 and C_2, respectively.

The results are to great extent in accordance to those found by **Ouakfaoui and Asselin (1992b)** and **Osswald *et al*. (1994)**. They found that chitosanase occur constitutively in certain organs and tissues of plants and it was isolated from seeds, leaves and stems of bean, pea, tomato, cucumber, barley and maize leaves. Also they found that chitosanase activity of leaves of plants have high chitosanase activity more than chitosanase activity of seeds.

Assessment of the optimum assay conditions for the crude chitosanases isolated from pepper leaves (C_1) and opuntia fruits peel (C_2).

The optimum assay conditions for the crude chitosanases C1 and C2 such as temperature, pH, thermal stability, time of reaction and effect of different concentrations of substrate and enzyme were studied. Chitosanases activities were estimated by the amount of reducing sugar released by (**Miller, 1959)** and the amount of glucosamine released by (**Rondle, 1955)** when incubated with chitosan.

Table (1): Chitosanase activities of water extracts from seeds and vegetative parts of some plants by using acetyl acetone method

Name of plants	Family	Chitosanase activity				
		Leaves		Peels		Seeds
		(U/g) Wet tissue	(U/g) Dry tissue	(U/g) Wet tissue	(U/g) Dry tissue	(U/g) Dry tissue
Pepper (*Capsicum annuum*)	*Solanaceae*	1.519	8.42	ND	ND	0.2497
Cabbage (*Brassica oleraceae*)	*Brassicaceae*	0.4198	2.173	ND	ND	2.687
Purslane *(Portulaca oleracea)*	*Portulaceae*	0.41	3.622	ND	ND	1.954
Artichoke (*Cynara scolymus*)	*Asteraceae*	0.011	ND	ND	ND	ND
Clover (*Trifolium repens*)	*Fabaceae*	0.0058	ND	ND	ND	ND
Basil (*Ocimum basilicum*)	*Lamiaceae*	0.00032	ND	ND	ND	ND
Green bean (*Phaseolus vulgaris*)	*Fabaceae*	ND	ND	0.8496	6.29	3.43
Opuntia (*Opuntia ficus*)	*Cactaceae*	ND	ND	0.0558	0.6805	ND
Melon (*Benincasa hispida*)	*Cucurbitaceae*	ND	ND	0.0055	ND	ND
Water melon (*Citrullus lanatus*)	*Cucurbitaceae*	ND	ND	0.0033	ND	ND
Papaya (*Carica papaya*)	*Caricaceae*	ND	ND	0.00232	ND	ND

Table (2): Chitosanase activities of water extract by using two methods (dinitrosalicylic acid and acetyl acetone).

Plants	Part used	Chitosanase activity by	
		DNS (U/g dry tissue)	Acetyl acetone (U/g dry tissue)
Green bean	Peels of fruits	67.78	6.29
Pepper	Leaves	47.616	8.42
opuntia	Peels of fruits	59.146	0.6805

Effect of the different pHs on enzymatic activity:

The effect of 0.05 M acetate buffer at different pH values ranging from 4.2 to 7.0 pH are shown in figures (1-2). The results indicated that the chitosanase C_1 has optimum activity at pH 5.8 by the dinitrosalicyclic acid method and 5.4 by the acetyl acetone method (Figure 1). But the chitosanase C_2 has optimum activity at pH 5.4 by the two methods (dinitrosalicyclic acid and acetyl acetone methods) (Figure 2). Similarly, **Wang *et al*. (2008a)** reported that the optimum pH for chitosanase activity isolated from *Gongronella* Sp. JG was pH 5.6. But **Osswald *et al*., 1994** reported that chitosanase isoforms isolated from *Citrus sinensis* have optimum pH at 5.0.

Effect of the temperature on enzymatic activity:

The reaction rate of the crude chitosanase C_1 was measured at various temperatures (30-70°C).

Figure (3) showed that chitosanase C_1 has optimum assay temperature at 40°C by the dinitrosalicyclic acid and acetyl acetone methods. Chitosanase C_1 activity is quite stable up to 50°C and at higher temperatures the reaction rate declined slightly till reaches 30% at 70°C. Figure (4) indicated that chitosanase C_2 is very stable and has high activity at high temperature. It was showed that chitosanase C_2 has optimum assay temperature at 60°C by the dinitrosalicyclic acid and acetyl acetone methods then the activity slightly decrease with increasing the temperature to 70°C (only 5 % decrease of its activity). Similarly, the optimum temperature for the chitosanase isolated from *Citrus sinensis* was 60°C (**Osswald *et al.*, 1994**).

Effect of the incubation times:

The effect of the incubation time on the crude extract of chitosanase C_1 activity was studied up to 180 minutes (Figure 5). It was indicated that, chitosanase C_1 activity increased as the incubation time increased up to 180 minutes. There is linear relationship between C_1 activity and incubation time up to 180 minutes. Chitosanase C_2 activity increased with increasing the incubation time of reaction up to 120 minutes (Figure 6).

Effect of the different substrate concentrations on enzyme activity:

Linear relationship was observed between colloidal chitosan concentration and the activity of chitosanases C_1 and C_2, as shown in figures (7-8). The activity of chitosanase enzymes from C1 and C2 increase with increasing the concentration of chitosan up to 13 mg/reaction mixture for both enzymes.

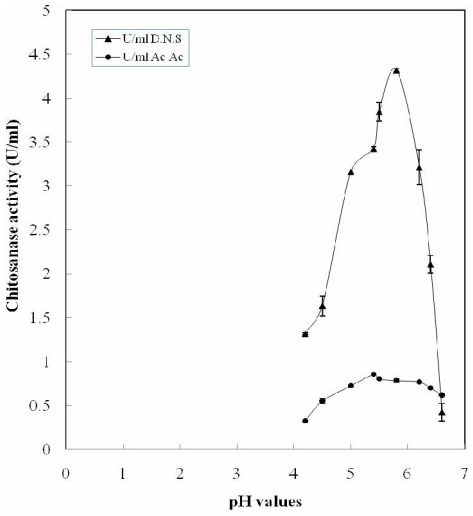


Figure (1): The effect of pH on the crude chitosanase activity (C_1).

Effect of the different enzyme concentrations on enzymatic activity:

The results presented in figure (9) indicated that the enzymatic activity of chitosanase C_1 increased with increasing enzyme concentration up to 240 µg protein per reaction mixture. Figure (10) indicated that chitosanase C_2 increased with increasing the enzyme concentration up to 513 µg protein per reaction mixture.

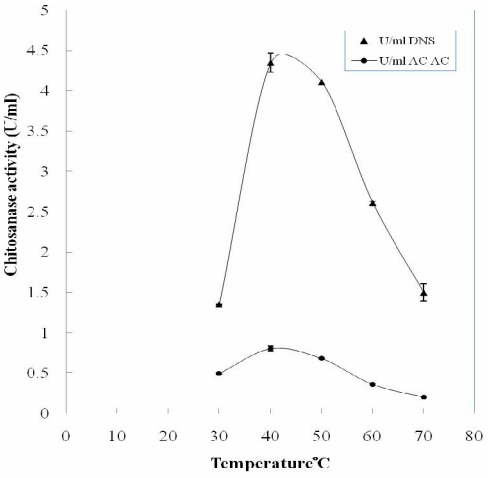
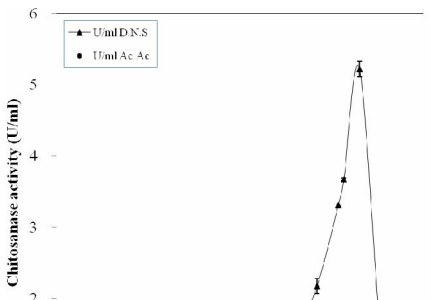


Figure (3): The effect of different temperatures on crude chitosanase activity (C1).



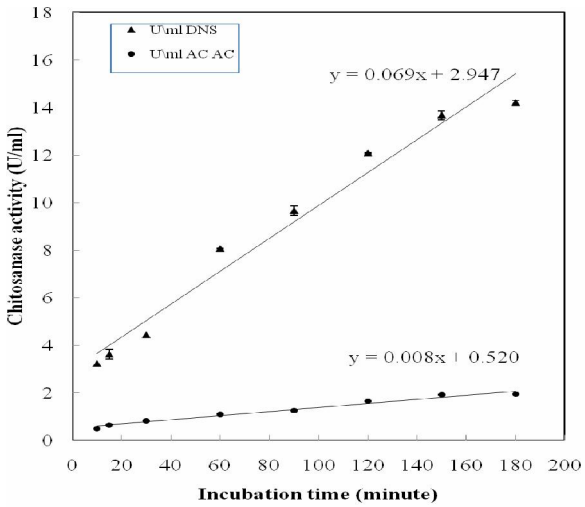


Figure (5): The effect of reaction time on the crude chitosanase activity (C_1).

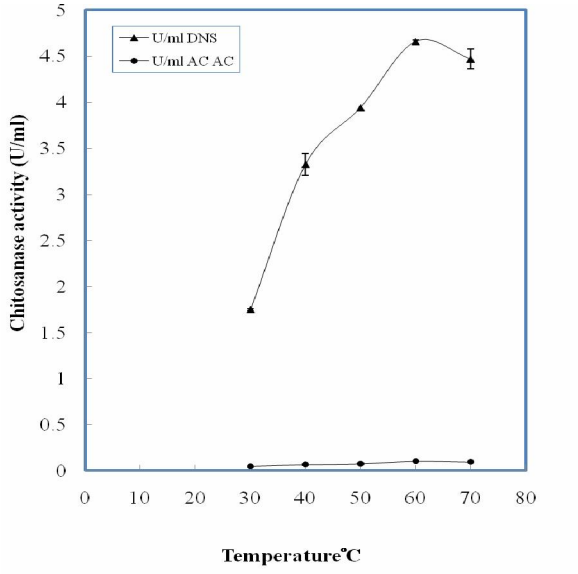


Figure (4): The effect of different temperatures on the crude chitosanase activity (C2).

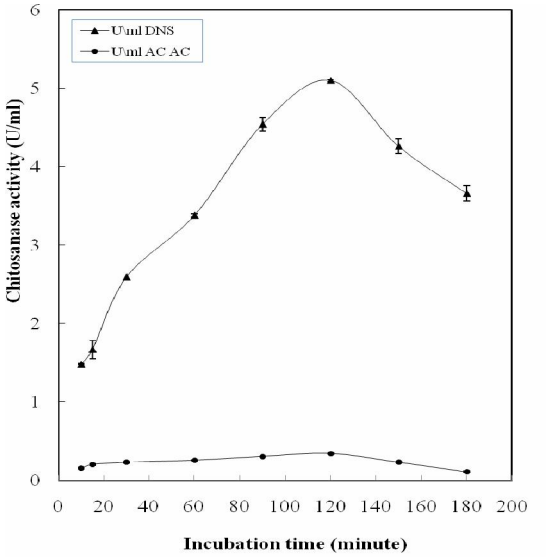


Figure (6): The effect of reaction time on the crude chitosanase activity (C_2).

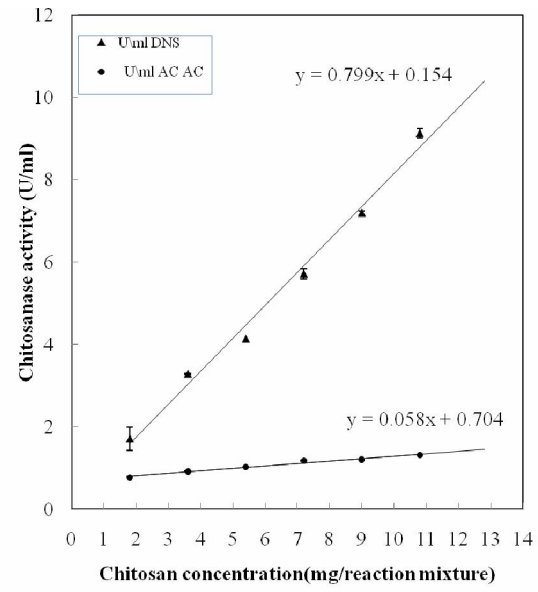


Figure (7): The effect of different substrate concentration on the crude chitosanase activity (C_1).

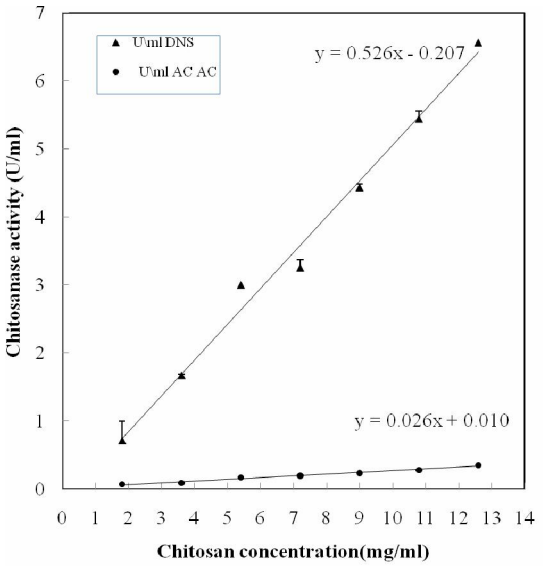


Figure (8): The effect of different substrate concentration on the crude chitosanase activity (C_2).

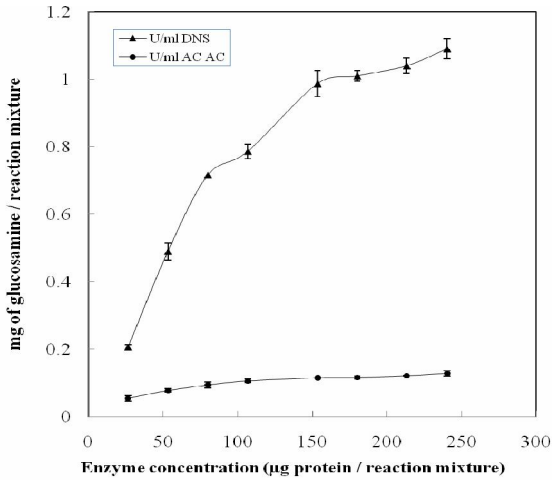


Figure (9): The effect of different concetrations of the crude chitosanase (C_1).

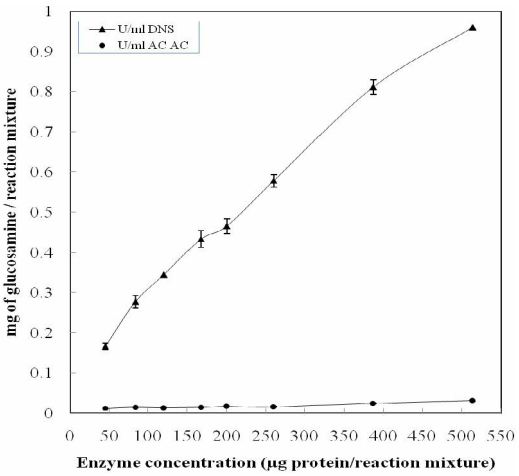


Figure (10): The effect of different concetrations of the crude chitosanase (C_2).

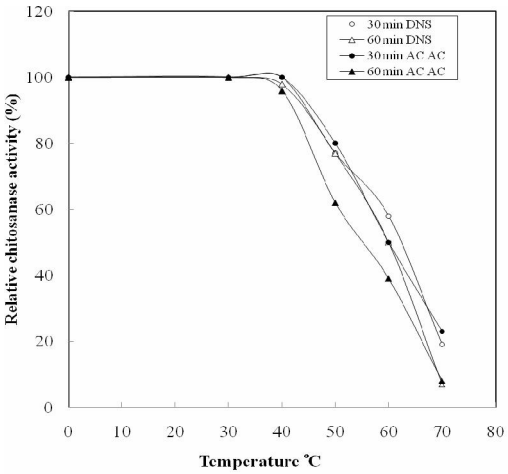


Figure (11): The effect of different temperatures on the crude chitosanase stability (C_1).

Temperature stability of the chitosanase enzymes:

The effect of preincubation of the crude chitosanase enzymes C_1 and C_2 in 0.05 M sodium acetate buffer, pH 5.8 at different temperatures (30-70°C) for 30 and 60 minutes in absence of substrate was carried out. The results illustrated that the activity of the crude enzyme C_1 is stable up to 40°C and the enzyme activity decrease by increasing the temperature (Figure 11). But the activity of the crude enzyme C_2 is stable up to 50°C and then the activity of enzyme highly declined at 60°C by the acetyl acetone method and decrease only by 25% by the dinitrosalicyclic acid method at 70°C (Figure 12). This is in general agreement with the prior results obtained from similar studies with *Streptomyces griseus* chitosanases (Osswald *et al*., 1992).

From these results, it was observed that chitosanase C_1 and C_2 has a high enzyme activity from (50-60°C) by using the dinitrosalicyclic acid method. Chitosanase C_1 has high enzymatic activity more than that of C_2 by using the acetyl acetone method (by eleven times).

The optimum assay conditions that gave the highest chitosanase activity of C_1 was obtained by using 0.05 M sodium acetate buffer, pH 5.8 by the dinitrosalicyclic acid method and pH 5.4 by the acetyl acetone method for 1.5 hours at 40°C.

Also The optimum assay conditions that gave the highest chitosanase activity of C_2 was obtained by using 0.05 M sodium acetate buffer, pH 5.4 by the dinitrosalicyclic acid method and pH 5.4 by the acetyl acetone method for 1.5 hours at 60°C.

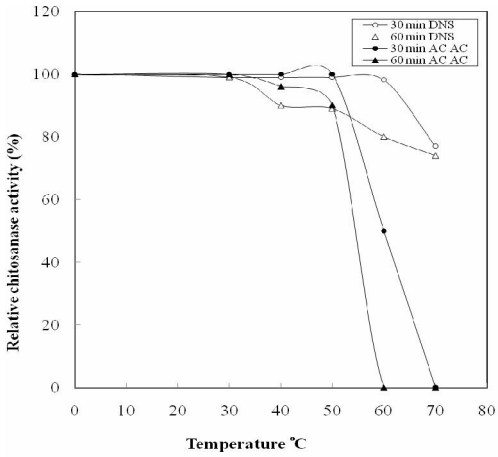


Figure (12): The effect of different temperatures on the crude chitosanase stability (C_2).

Conclusions and Recommendation

From this study, pepper leaves may be the best suitable source for chitosanase extraction with high activity and high heat stability. Moreover, pepper leaves are considered as a very economic source, because of its availability as an agriculture waste.

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