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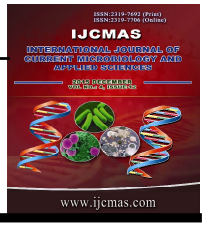

Original Research Article

Purification, Thermostability and Chemical Modification of Pullulanase from Sunflower

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Keywords

Pullulanase, Sunflower, Purification, Thermo-stability,

A B S T R A C T

Pullulanase (EC 3. 2. 1. 41) activity was measured in sunflower (*Helianthus annus* L.) cotyledons. The enzyme was purified from sunflower cotyledons with specific activity of 580 units mg^{-1} protein. The enzyme was purified to homogeneity with single band. The optimal pH 6.0 and the optimal temperature was 45ºC. Sarcosine offered pullulanase appreciable thermostability at 60ºC. The enzyme was modified by various concentrations of dansyl chloride (DnsCl), N-bromosuccinimide (NBS) and N-acetylimidazole (NAI). The modification resulted in an inhibition of enzyme activity with the three tested compounds. The inhibition reveals the presence of lysyl, tryptophenyl and tyrosyl residues. Inclusion of pullulan as a substrate during the incubation offered the enzyme a protection against inactivation.

Introduction

Starch is the major form of carbon reserve polysaccharide being synthesized in plants in cellular organelles called plastide. Starch is a complex structure composed of two types of glucose polymer. The first is amylose (15-25%), comprising largely unbranched α-1,4 linked glucan chains. The second is amylopectin, which is branched glucan polymer constitute about 75-85% of the starch granule mass, and produced by formation of α-1,6 linkages between adjoining straight glucan chains (Tetlow, 2006; Wang *et al.*, 2011).

Pullulan is a fungal exopolysaccharide produced from starch by *Aureobasidium pullulans* (Rekha & Sharma, 2007). The regular alternation of α-(1,4) and α-(1,6) bonds results in enhanced solubility and structural flexibility (Seo *et al.*, 2004). The pullulan molecule characterized by a molecular mass ranges from 1,500 to 810,000 KDa **(**Doman-Pytka & Bardowski, 2004).

Pullulan is used extensively in food industry. It is a slow digesting macromolecule which is odorless and tasteless therefore it is used as a low-calorie food additive providing bulk and texture. Pullulan is characterized by good moisture retention, oxygen barrier property and it inhibits fungal growth. These characters make it suitable material for food preservation (Rekha & Sharma, 2007).

Pullulan is used as a pharmaceutical bulking agent in Japan. Pullulan was investigated for its biomedical applications in tissue engineering, targeted drug and gene delivery, wound healing as well as in diagnostic imaging using quantum dots (Rekha & Sharma, 2007).

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) is a debranching enzyme cleaves certain α-1,6 glucosidic linkage in branched substrates, α-limit dextrin, β-limit dextrin, and pullulan, but it can debranch amylopectin to a lesser extent than polyglucans and form amylase-like substances (Li *et al.*, 1992; Kuroiwa *et al.*, 2005; Yamasaki, 2008). Pullulanase is also known as limit dextrinase, α-dextrin endo-1,6-α-glucosidase, and amylopectin 6-glucanohydrolase (Purich & Allison, 2002).

It has been reported that pullulanase in plants is necessary for starch degradation with α-β-amylases and may be necessary for starch synthesis during ripening of starch, since it plays an essential role in determining the fine structure of amylopectin molecule (Mouille *et al.*, 1996; Nakamura *et al.*, 1996; 1997; Kubo *et al.*, 1999).

Pullulanases have been reported in seeds of monocotyledonous plants such as rice **(**Nakamura *et al.*, 1996; Yamasaki *et al.*, 2008), barley (Maeda & Nikuni, 1978), maize (Doehlert & Knuston, 1991), sugar-beet (Li *et al.*, 1992), spinach (Schindler *et al.*, 2001) and millet (Yamasaki *et al.*, 2007). However, there is a little information on pullulanase from seeds of dicotyledonous plants, (Shain & Mayer, 1968).

Pullulanase is used in detergent industry for producing household detergent. Commercial laundry detergent contain enzyme that can catalyze the degradation of starch-containing stain and improve cleaning by hydrolyzing the starchy glue that bind other dirt and stain to fabric. A mixed amylase and pullulanase enzyme system can accomplish a more complete degradation of starch (Aehle, 2004).

Pullulanase is used in textile industry during textile processing for desizing of cotton fabric to strengthen the yarn and prevent breakage in the weaving process (Aehle, 2004).

The biocatalytic process economics can be enhanced by enzyme reuse and the improvement in enzyme stability afforded by immobilization. The ability to retain or recover enzymes also allows biocatalyst separation from product, thereby permitting continuous processes, and prevents carry-through of protein or activity to subsequent process steps (Polizzi *et al.*, 2007).

The enzyme performance can be improved by immobilization under optimal process reaction conditions (e.g. elevated temperatures, acidity, alkalinity, and organic solvents), a requirement that has often retarded enzyme application in industrial chemical synthesis (Bommarius & Riebel, 2004; Brady & Jordaan, 2009).

Materials and Method

Plant Material

The experimental plant used in this investigation was *Helianthus annus* L. (sunflower, family: Compositae). Pure strains of seeds were obtained from Egyptian Ministry of Agriculture. Seeds were germinated according to El-Shora (2002) and Pullulanase was extracted from cotyledons by the method of Sharma *et al.* (2004).

Enzyme Purification

The crude extract obtained in the preparation mentioned above was treated with solid ammonium sulphate (90% saturation). Dissolve the precipitate in 25 mM sodium acetate buffer (pH 5.3). The enzyme solution was dialysed against 25 mM Tris/HCl buffer (pH 8.0), and the dialysate was applied to a DEAE- cellulofine column (2 × 18 cm).

After the column was washed with 25 mM Tris/HCl buffer (pH 8), a linear gradient of 0-1 NaCl in the buffer was applied. The fractions were measured to determine pullulanase activity.

Assay of Pullulanase Activity

The reaction mixture of pullulanase contained starch as substrate and 0.5 ml enzyme solution in 50 mM sodium acetate buffer (pH 6), and the mixture was incubated at 37 °C for 30 min. After incubation the reaction was stopped by boiling the mixture for 5 min. The liberated maltotriose was determined at 620 nm. One unit (U) of pullulanase is the amount of enzyme that liberates 1 µmole of product per min under assay conditions.

Estimation of Reducing Sugars

The amount of maltotriose formed was measured according to the method of Somogyi (1952).

Determination of the Optimal pH for Pullulanase Activity

The optimal pH for pullulanase activity was determined over a range from 3 – 10. Sodium acetate / acetic (pH 3.0 – 5.0), potassium phosphate buffer (pH 6.0 – 7.0), Tris-HCl buffer (8.0 – 9.0) and sodium bicarbonate buffer (pH 10.0). From the obtained results a graph of enzyme activity against pH was plotted, and the optimum pH for pullulanase was subsequently determined.

Effect of Temperature on Pullulanase Activity

The reaction mixture of pullulanase was incubated at various temperatures 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C. The optimum temperature was determined from the graph of the enzyme activity against temperature. The pullulanase activity was determined by the method described previously.

Estimation of Kinetic Parameters

The Michaelis-Menten's constant (K_m) and the maximum velocity (V_{max}) were estimated by studying the effect of substrate concentration on enzyme activity. The pullulanase activity was determined at various substrate concentrations (S). The Lineweaver-Burk plot [(1/v) against (1/S)], where v is the reaction velocity was then constructed. The K_m and V_{max} were determined for pullulanase.

Immobilization of Pullulanase on Ca-alginate

Sodium alginate solution in (3% w/v) 50 mM sodium phosphate buffer (pH 8.0) was prepared by warming at 50 °C. After cooling down to room temperature, 2 ml of the enzyme stock solution was mixed with 8 ml of sodium alginate solution. The mixture was taken into a syringe, and beads were formed by dropping the solution into 1 M calcium chloride solution with gentle stirring at 4 °C for 2 h. The formed beads were recovered by filtration and thoroughly washed with distilled water. The beads were dried using filter paper (whatman no. 1)

followed by exposure to the open air for 1 h before use (Ertan *et al.,* 2007). The filtered calcium chloride solution was collected for enzyme activity determination.

Effect of Sarcosine on Pullulanase Stability at 60ºC.

Pullulanase solutions were buffered at pH 7.0 with 30 mM MOPS buffer in the presence of various concentrations of sarconine (Takano et al., 2004).

Chemical Modification

Effect of Dansyl Chloride (DnsCl) on Pullulanase Activity

Stock solutions of DnsCl were freshly prepared in acetonitrile . 0.2 µmol of the enzyme was incubated for 1h at 4ºC with various concentrations of DnsCl (0 - 40 mM) in 100 mM Tris (pH 8). Additionally, the time course of modification was determined by incubating the enzyme with 5 mM DnsCl in 100 mM Tris (pH 8). At designated time intervals (0-60 min), the reaction was terminated by the addition of 30 mM lysine and 20 mM β-mercaptoethanol (Chen, 1968; Du *et al.,* 1998; Kosoy *et al.,* 2004).

Effect of N-bromosuccinamide (NBS) on Pullulanase Activity

Tryptophane groups of pullulanase were modified according to Williams (1975) using NBS. The modification was carried out by adding NBS (1 mM) aliquots to 5 mL of pullulanase enzyme in phosphate buffer (15 mM, pH 6.3). Additions were made slowly as the solution was being stirred on a magnetic stirrer. This procedure minimized formation of turbidity in the solution. After 1 hr of stirring at 28±2ºC, the modified enzyme was dialysed against distilled water for 24 hr. The modified enzyme was purified by preparative poly acrylamide gel electrophoresis and stored at 4ºC (Kumar *et al.,* 2014).

Effect of N-acetylimidazole (NAI) on Pullulanase Activity

The enzyme solution (0.2 µmol in 0.1 ml water) was mixed with 2.4 ml of 0.02 M or 0.2 M or 0.2 M sodium phosphate buffer, pH (7.5), or 0.02 M or 0.04 M sodium borate buffer, pH (7.5). To this was added 0.5 ml of the same buffer solution containing a desired amount of NAI. NAI solution was prepared just before use to avoid the degradation of the reagent. The reaction was allowed to proceed at room temperature for 60 to 80 min. At intervals 5-ml aliquots were removed and diluted with water to determine the enzyme activity. The absorbance of the reaction mixture was measured at 278 nm against the blank solution containing the buffer and the reagent. The reaction was terminated by the addition of several drops of 1 N HCl and the mixture was passed through a column (1.8 × 28 Cm) of Sephadex G-25 equilibrated with water. Modified tyrosine residues were determined spectrophotometrically (Riordan *et al.,* 1966; Kasai *et al.,* 1977). All the data in the present investigation are expressed as mean ± SE obtained from the measurements.

Results and Discussion

Effect of Sarcosine on Pullulanase Stability at 60°C

The influence of sarcosine on pullulanase activity was tested at 10 mM. The enzyme was incubated with sarcosine for 6 days and the enzyme activity was measured daily as relative activity of the control value. The results in (Fig. 1) demonstrated that the enzyme activity declined gradually throughout 6 days. However, in presence of

sarcosine the decline was lower than that in its absence.

Effect of Dansyl Chloride (DnsCl) on Pullulanase Activity

The effect of dansyl chloride on pullulanase activity was studied at various time intervals (10, 20, 30, 40, 50 and 60 min). The activity was tested at 1, 2, 3, 4 and 5 mM. The results shown in (Fig. 2) indicate that by increasing the concentration of dansyl chloride there was continuous decrease in the enzyme activity. Also, the rate of enzyme reduction by the dansyl chloride concentration was dependent on time. It was observed that at both 4 and 5 mM the enzyme activity at 50 and 60 min was completely abolished.

From the results in the previous experiment $t_{0.5}$ was calculated at various tested concentrations, then plotting the log concentration versus $t_{0.5}$ (min). The results in (Fig. 3) show that straight line was obtained with slope value of 1.9.

Protection of Pullulanase activity by Substrate against Inactivation by Dansyl Chloride (DnsCl)

The protective effect of pullulanase substrate on the inactivation of the enzyme by DnsCl was tested at 5 mM DnsCl in presence of 5 and 10 mM of substrate. The results in (Fig. 4) show that in presence of DnsCl alone the retained activity was 14% whereas in presence of 5 mM substrate the retained activity was 53.6% and in presence of 10 mM substrate 74.8% of the enzyme was retained.

Effect of N-bromosuccinimide (NBS) on pullulanase activity

The effect of NBS on pullulanase activity at various concentrations was tested at 1, 2, 3, 4 and 5 mM. At each concentration the enzyme activity was tested at various time intervals 10, 20, 30, 40, 50 and 60 min. The results shown in (Fig. 5) show that the enzyme activity declined at each concentration tested, Also, the decline in the activity was dependent on both the concentration and the time of incubation. It was noticed that at the higher concentrations 4 and 5 mM the enzyme activity was completely abolished after 40, 50 and 60 min of incubation.

The results of the previous experiment were used to calculate $t_{0.5}$ and log concentration. Plotting the values of $t_{0.5}$ versus log concentration (Fig. 6) resulted in straight line with slope of 1.18.

Protection of Pullulanase Activity by Substrate against Inactivation by N-bromosuccinimide (NBS)

The protection of pullulanase inactivation by NBS was studied using pullulanase substrate at both 5 and 10 mM. The results in (Fig. 7) indicate that 23.8% of the enzyme was retained in presence of NBS, but substrate at 5 mM offered protection for the enzyme by 42%, however, higher protection (79.3%) was obtained in presence of 10 mM.

Effect of N-acetylimidazole (NAI) on Pullulanase Activity

The effect of NAI was tested on pullulanase activity at 1, 2, 3, 4 and 5 mM. The enzyme activity was determined through 60 min. The results in (Fig. 8) show that NAI inactivated the enzyme activity at all tested concentrations. The inactivation of each concentration was dependent on the incubation time of the enzyme with NAI. It was noticed that at 4 mM the enzyme activity was completely abolished whereas

at 30, 40, 50 and 60 min the activity was abolished completely at 5 mM.

The results in the previous experiment were used for calculation of $t_{0.5}$ min followed by plotting the values of $t_{0.5}$ against log conc. The results of the present experiment showed a straight line with slope value of 1.1512 in (Fig. 9).

Protection of Pullulanase Activity by Substrate against Inactivation by N-acetylimidazole (NAI)

The protection of pullulanase inactivation by the substrate was tested at both 5 mM of NAI. The results (Fig. 10) reveals the substrate at 5 and 10 mM offered 14.7% and 23.8% activity compared with 11.6% without substrate.

Pullulanase was purified from sunflower cotyledon and characterized. The purification data in Table 1 show that a high purification factor for this enzyme was reached and describes the purification to homogeneity. The purification gave a pure enzyme with a single band on PAGE indicating the homogeneity of the obtained pullulanase.

The specific activity of the obtained pure pullulanase from sunflower cotyledons was 580 units mg^{-1} protein. However, the specific activity from *Clostridium thermohydrosulfuricum* is 481 units mg^{-1} protein (Saha *et al.,* 1988).

The optimum pH of free pullulanase from sunflower cotyledons was 6.0. This pH value of free pullulanase was in harmony with the reported value for the enzyme from rice endosperm (Yamasaki *et al.,* 2008). the enzyme was stable in a pH range of 5.5-8.0. The optimum temperature for the enzyme activity was 45ºC. These results are in harmony with that reported from rice endosperm and *Bacillus cereus* (Yamasaki *et al.,* 2008; Ling *et al.,* 2009).

The short half-life of the native enzyme forced investigators to modify the enzyme, with increased half-life and storage stability. Immobilization onto solid supports facilitates recovery and reuse of the enzyme. In addition, increased enzyme stability can be achieved by both immobilization and chemical modification techniques (Kumar *et al.,* 2014).

Pullulanase was stabilized by sarcosine due to the osmolyte effect. Timasheff showed that osmolytes are preferentially excluded from the vicinity of a protein. Because the thermodynamic stability of proteins is determined by the delicate balance between the Gibbs energies of the native and denatured state (Takano *et al.,* 2004).

Several approaches were carried out to improve stabilization of enzymes in soluble form. These includes alteration of primary structure of the enzyme by chemical modification (Bieniarz *et al.,* 1998).

Chemical modification play an important role in probing the mechanism of enzyme activity. This technique can be utilized for identification of those individual amino acid residues responsible for the catalytic properties of the entire protein. In chemical modification experiments, changes in enzymatic specificity have been noted, but often not predicated (Kaiser *et al.,* 1985). Chemical modifications of pullulanase using DnsCl, NBS and NAI have been attempted. This modification of the enzyme to examine long term-operational stability and improved activity and half-life.

Table.1 Purification Procedure of Pullulanase from Sunflower Cotyledons

Stage	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Fold
Crude extract	1017	320	0.3	100	1
Ammonium sulphate (40-80%)	287	315	1.1	98.4	3.6
Phenyl sepharose	38	213	5.6	66.5	18.6
Sephadex G-200	0.2	84	420	26.2	1400
Hydroxyl apatite	0.1	58	580	18.1	1933.3

Fig.1 Effect of Sarcosine on Pullulanase Stability at 60 °C

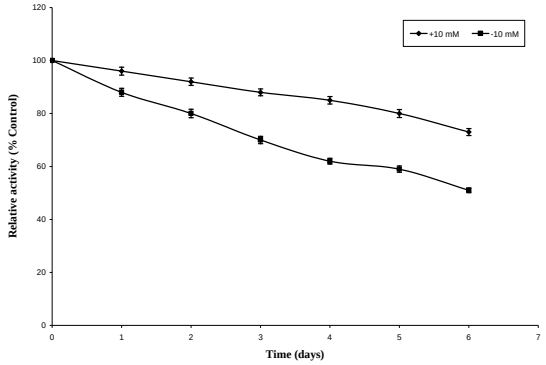


Fig.2 Effect of Dansyl Chloride on Pullulanase Activity

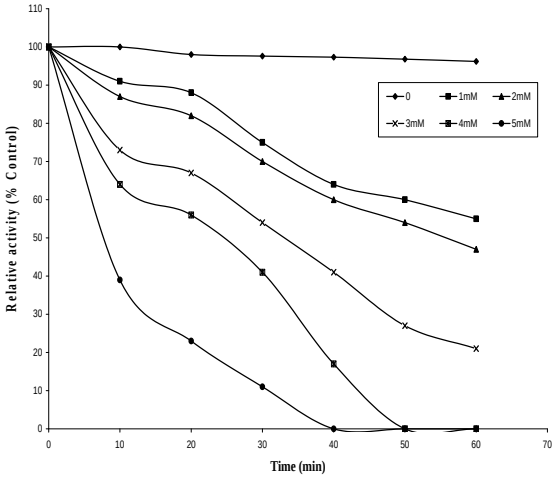


Fig.3 Half-Life of Pullulanase at Different Concentrations of Dansyl Chloride (1-5 mM)

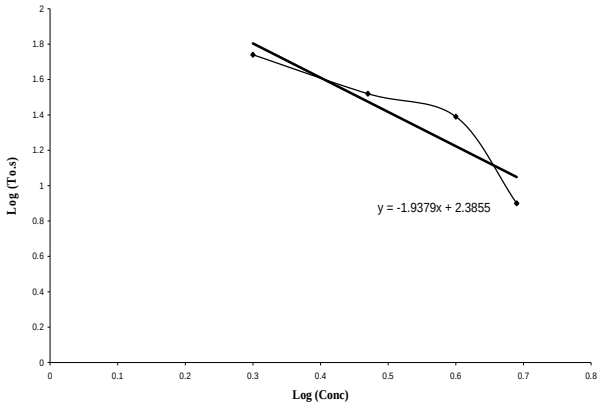


Fig.4 Protection of Pullulanase Activity by Substrate Against Inactivation by Dansyl Chloride

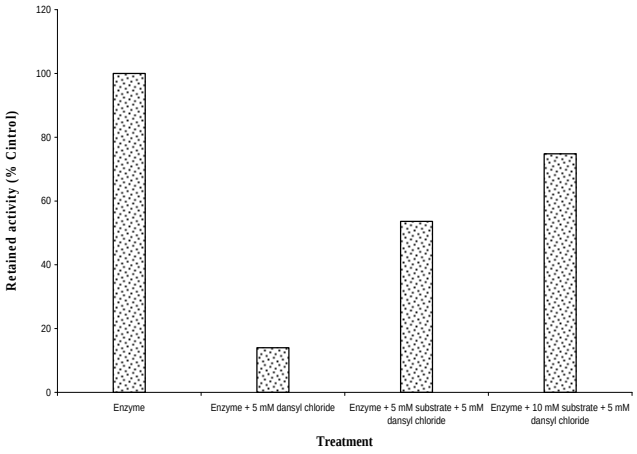


Fig.5 Effect of N-bromosuccinimide on Pullulanase Activity

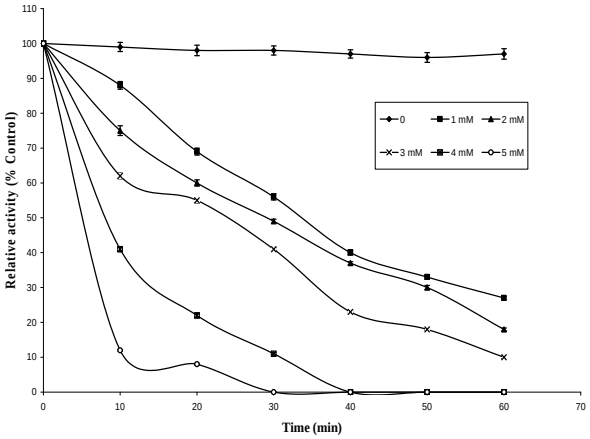


Fig.6 Half-life of Pullulanase at Different Concentrations of N-bromosuccinimide (1-5 mM)

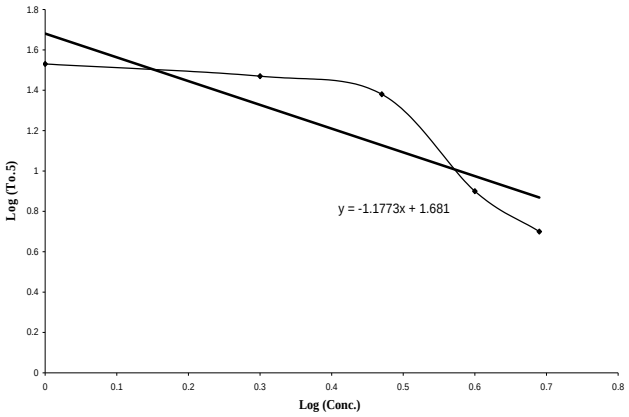


Fig. 7 Protection of Pullulanase Activity by Substrate Against Inactivation by N-bromosuccinimide

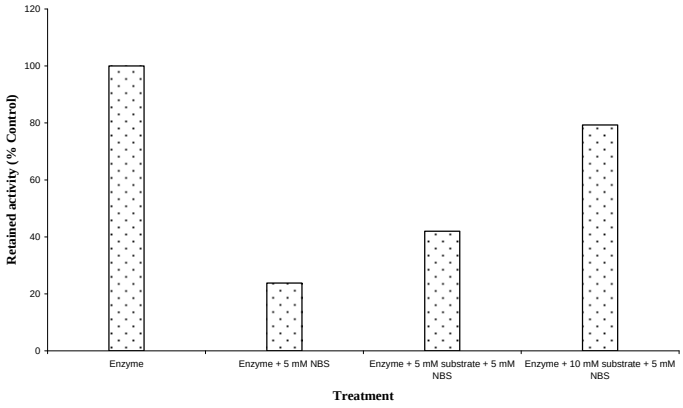


Fig.8 Effect of N-acetylimidazole on Pullulanase Activity

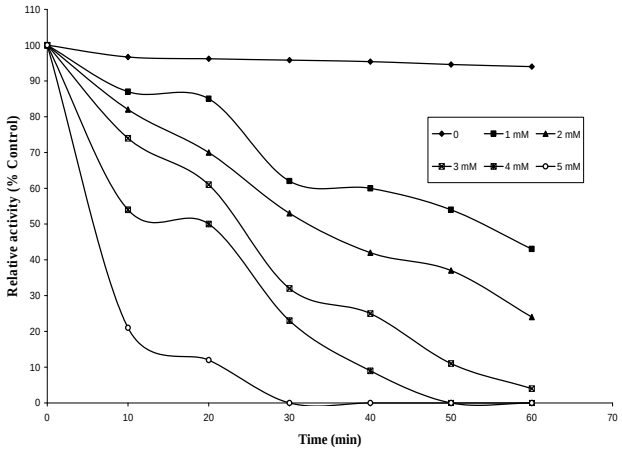


Fig.9 Half-life of Pullulanase at Various Concentrations of N- acetylimidazole (1-5 mM)

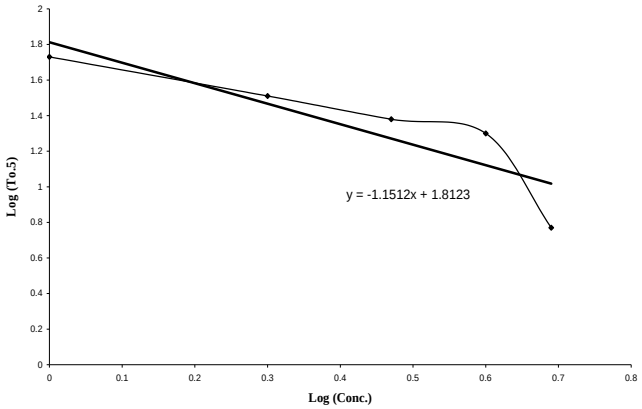
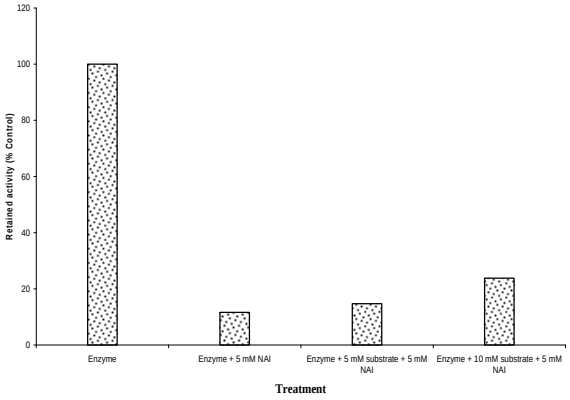


Fig.10 Protection of Pullulanase Activity by Substrate Against Inactivation by N-acetylimidazole



Pullulanase is irreversibly inactivated by relatively low concentrations of DnsCl. Incomplete inactivation was observed due to the destruction of reagent by hydrolysis. The complete inactivation can be correlated with the dansylation of a single lysine residue.

Pullulanase can be inactivated completely by DnsCl, but the substrate of the enzyme protects it from inactivation and reduces the degree of covalent modification up to 74.8% of its original activity (Kosoy *et al.*, 2004).

Strong inhibition on the pullulanase activity by NBS was observed at low concentrations suggesting a crucial involvement of tryptophanyl residues at the active site of the enzyme. The Presence of tryptophanyl residue in the active site of pullulanase played functional role in the enzyme catalysis. These results are in agreement with those of Teng et al. (2006).

Upon treatment with a tyrosine selective agent, NAI, tyrosine residues in pullulanase were acetylated. NAI was demonstrated to be a potent inhibitor with modification occurring at an active site tyrosine residue. NAI modify tyrosine residues by different chemistry yielding products with different characteristics.

All tyrosine residues in the enzyme were modified with NAI with loss of the native conformation. NAI seems to be a useful reagent to investigate the state and/or reactivity of tyrosine residues (Kasai *et al.*, 1977).

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