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β-GALACTOSIDASE FROM WATERMELON (*Citrullus lanatus*) SEEDLINGS: PARTIAL PURIFICATION AND PROPERTIES.

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ABSTRACT

β-Galactosidase from watermelon (*Citrullus lanatus*) seedlings was partially purified. Extract was fractionated in two steps with ammonium sulfate at 30 & 80% saturations. Ammonium sulfate precipitated enzyme yielded 60% recovery and two folds purification of the enzyme. Further, the enzyme was subjected to CM-Cellulose and Sephadex G-100 chromatography to obtain 24-folds purification. The specific activity was increased to from 1.5 U to 36 U per milligram of protein. The overall yield was 13.2%. The enzyme had optimum pH 4.8 and pH stability between pH 4.0 and 7.0 was observed. Optimum temperature was 50°C and temperature stability was also shown up to 50°C. Thereafter enzyme activity fell and inactivated completely at 70°C. The K_m and V_{max} were found to be 0.2 mM and 34U$min^{-1}mg^{-1}$of protein, respectively. β-mercaptoethanol exhibited very small activation at moderate concentration, but the inhibition was displayed at high concentrations. Heavy metal ions such as Al^{+3}, Ag^{+1}, Hg^{+2}, etc. inhibited the enzyme strongly. The inhibition by heavy metals and SH-reacting reagents suggests that cystein was necessary for enzyme activity. On the addition of β-mercaptoethanol to pre-incubated enzyme with metal ions, the inhibition was relieved. The β-Galactosidase activity was increased in the presence of alcohols. This activation reflected glycosyltransferase activity.

Key Words: β-Galactosidase; watermelon; glycosyltransferase activity; purification; characterization.

INTRODUCTION

Beta-galactosidases (E.C. 3.2.1.23) are found in plants, animals and microorganisms. It catalyzes the hydrolysis of glycosidic bond in lactose, polysacharides or in other macromolecules such as glycoproteins or glycolipids to produce β-galactose (Prasanna *et al*., 2005). β-galactosidases have many applications in food and pharmaceutical industry, (Kaur *et al.,* 2006; Stahl *et al*., 2007; Jokar and Karbassi, 2009; Patil *et al*., 2011). Since the *lac* operon has got importance in the regulation of gene expression, the role of β-galactosidase is being used in genetic engineering and recombinant DNA technology (Asraf and Gunasekaran, 2010).

Beside its hydrolytic activity, it also possesses transgalactosidase activity when the concentration of lactose is very high and galacto-oligo-saccharides formation of take place. Such reaction occurs in bacteria and fungi (Dumortier *et al*., 1994; Yoon *et al*., 1996). A number of seeds are found to contain β-galactosidase (El-Tanboly, 2001) which is involved in the mobilization of galactose containing oligosaccharides reserves in the seeds through hydrolyzing β-galactosyl linkages. When seed begin to germinate, the level of β-galactosidase activity was increased and decreesed the reserves of glycoproteins, oligsaccharides and starch which serve as source of energy. β-Galactosidases appear to function in response to environmental stresses (Melo *et al*., 1994; Gomes-Filho *et al*., 1996; Enéas-Filho *et al.*, 2001) e.g. salt stress, water deficit and lactose deficiency and many changes in specific isoforms are involved. Some studies show that in germinating seeds, oligo and polysaccharides deficiencies can cause an increase in β-galactosidase activity and reserved food of organic macromolecules of sugars, for examples, xyloglucan, galctolipids and glycoproteins etc., are hydrolysed to release terminal galactosyl residues for growth (Eneas-Filho *et al.*, 1995; Alcantara *et al*., 1999; David *et al*., 2000). Several attempts are made to purify β-galactosidases in order to find out their exact biological roles and functions. Only few of these have been purified to large extent e.g., from mango fruit pulp, peach, kidney beans, maize and mung bean seedlings (Esen 1992; Li *et al*., 2001; Biswas *et al*., 2003; Lee *et al*., 2003; Prasanna *et al*., 2005). The present work aimed to partially purification and determines the biochemical properties of β-galactosidase extracted from watermelon seedlings.

MATERIALS AND METHODS

The seeds of watermelon (*Citrullus lanatus*) were collected from Ratta Kulachi Research

Station, Dera Ismail Khan. *p*-nitrophenyl-β-D-galactoside (PNPG) were purchased from Sigma-Aldrich Chem. Co., PCMB and iodoacetate from obtained from BDH.

Enzyme assay: β-Galactosidase activity was determined as described by Biswas (1987). The assay was performed at 50°C in 1 mL assay medium containing 0.1 M acetate buffer, pH 5.0 and 0.25 mM PNPG as substrate. The small amount of enzyme was added to initiate the reaction for 30 min and then, the hydrolytic reaction was stopped with 2 ml of 0.1 M NaOH. The absorbance of the liberated *p*-nitrophenol was recorded at *400 nm using UV-Visible Spectrophotomerer (JASCO, Japan) Model V-630*. One unit of β-galactosidase activity is defined as the amount of enzyme required to release of 1 μmol of *p*-nitrophenol min^{-1} under these conditions. Specific activity is expressed as number of enzyme units per milligram protein. Optimal pH was determined over pH range of 3.0-9.0 using 0.1 M acetate buffer (pH 3.8-6.0), 0.1 M barbital buffer (6.0-6.7) and 0.1 M Tris-HCl buffer (7.0-9.0) and optimal temperature was determined over temperature range from 30°C to 80°C was measured under above conditions. The effects of metal ions and other reagents such as alcohols at various concentrations on the activity were determined as described above. Temperature stability was determined by incubating the enzymes at temperatures from 40°C to 80°C for 10 min. In the same way, pH stability was assessed by incubating the enzyme in different buffers for 30 min. The aliquots were drawn and remaining activities were assessed under standard assay method. The kinetic parameters were determined against PNPG as substrate. The enzyme activities were determined against substrate concentrations of 0.05 mM to 5 mM. Double reciprocal plot of 1/v versus 1/S was constructed to evaluate K_m and V_{max}.

Effects of reducing agents on enzyme stability: Dithiothreitol (DTT) or β-mercaptoethanol was added to the enzyme at 0-2000 mM concentrations. Pre-incubation was performed at 50°C for period of 10 min. The enzyme activity in each sample was assayed as per routine procedure.

Glycosyltransferase activity: To study the glycolsyltransferase activity, the hydrolysis of PNPG was carried out under above conditions in the presence of various alcohols. For *p*-nitrophenol measurement, the hydrolytic reaction was quenched by adding NaOH. The absorbance of the released *p*-nitrophenolate ions was monitored spectrophotometrically at A_{400}. The amount was calculated from *p*-nitrophenol standard curve. For galactose (reducing sugar) determination, a method of Bernfield (1955**)** was followed. To 20mL of 5% DNS (3,5-dinitrosalicylic acid) solution dissolved in 2 M NaOH, 30 g of sodium potassium tartarate was added with constant stirring. The whole content was diluted to 100 mL. The enzyme assay medium contained 900 μL of acetate buffer, containing 0.25 mM PNPG, 50% alcohol and 100 μL enzyme. Two blanks were prepared in which enzyme and alcohol were replaced by water simultaneously. The mixtures were incubated for same period of 30 min and same temperature of 50°C. Then 1.0 mL of DNS solution was added, followed by boiling for 15 min. The intensity of the color developed was determined spectrophotometrically at 540 nm. The concentration of galactose was calculated from standard curve of galactose. Galactose transferred to alcohols was the difference between nitrophenol released and galactose produced.

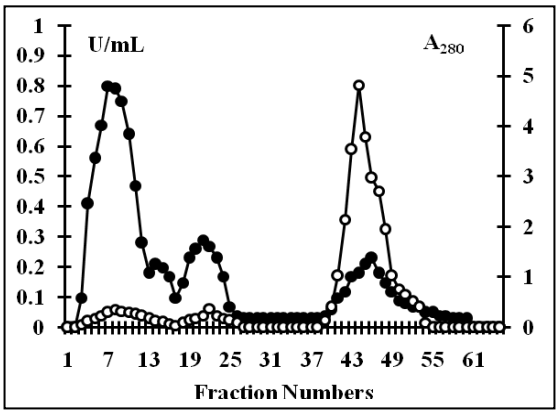
Protein determination: Protein concentration was estimated from absorbance at 280 nm for column fractions or by Lowry *et al.,* (1951**)** with slight modification using serum albumin as standard.

Extraction and purification of β-galactosidase: Fifty grams of three to five days old grown watermelon seeds were homogenized with 100 mL of acetate buffer. The content was centrifuged at 5000 RPM for 30 min using refrigerated Beckman Centrifuge J-21 with rotor J-14 and supernatant was collected. This was considered as crude extract and to it, ammonium sulfate was added to the level of 30 % saturation. The small amount of precipitate formed was spun down. To the supernatant, ammonium sulfate was added to the level of 80 % saturation to precipitate the proteins. The precipitate was dissolved in 100 mL of buffer and dialyzed against total volume of 1L of same buffer with small changes. The sample was subjected to ion exchange chromatography on CM-Cellulose column (2.7x 27 cm; total vol. 154 mL). The column was extensively washed with buffer to remove unwanted proteins. The bound proteins and β-galactosidase activity were eluted with salt gradient from 0.0 to 0.5 M NaCl in the same buffer (total volume of 300 mL). The most active fractions were pooled, concentrated by ultrafiltration and placed on Sephadex G-100 column (1.8 x85 cm). The column was eluted with the 0.01 M acetate buffer, pH 5.5 containing 0.1M NaCl (Fig. 1).

RESULTS AND DISCUSSION

β-galactosidase purification: Extract was fractionated in two steps with ammonium sulfate at 30 & 80% saturations. Ammonium sulfate precipitated enzyme yielded 60% recovery and two folds purification of the enzyme. When this enzyme

was subjected to CM-Cellulose chromatography, 38% activity was not bound and the remaining bound enzyme was eluted as a highest single peak yielding overall 37.6% recovery. The enzyme had 8-folds purification with specific activity of 12 U per milligram of protein. Sephadex G-100 chromatography resulted in further 3-folds purification (Fig. 1).



A

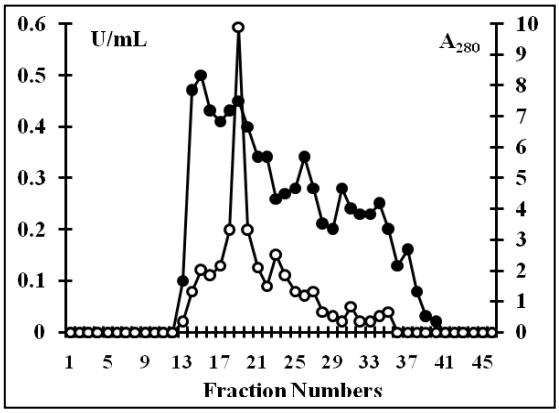


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Fig. 1: Purification of β-galactosidase from watermelon seedlings.
(A) Ionexchange chromatography on CM-Cellulose. Flow rate was 30 mL/h and 10mL fractions were collected.
(B) Gel filtration on Sephadex G-100.
Fractions of 5mL were collected at flow rate of 35mL/h. Ordinates: Protein at A_{280} nm (●—●); β-galactosidase activity (○□□○), U/ml Arrows show start of linear salt gradient.

Table 1: Purification of β- galactosidase from watermelon seedlings

STEPS	Volume (ml)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Recovery %
Extract	100	7.45	745	4.97	497	1.5	1.00	100.00
80% $NH_4)_2SO_4$ Precipitation & Dia;ysis	125	3.57	446.25	1.15	43.75	3.1	2.0	60
CM–Cellulose	10	28	280	2.32	23.2	12.0	8.0	37.6
Sephadex G-00	5.0	19.8	99	0.55	2.75	36	24	13.2

The results from this preliminary purification scheme show that the enzyme was purified 24 times with total yield of 13.2%. The specific activity was increased to 36 U/mg of protein (Table 1). Our results were consistent with β-galactosidase isolated from Durian seeds (El-Tanboly, 2001).

pH and temperature optima and stability: The enzyme was active between pH 4.0 and 5.0 and had optimum pH 4.8 (Fig. 2) which is almost similar to those from other sources such as cowpea (pH 3.0-4.0) (Enéas-Filho *et al.*, 2001), mung bean seedlings (pH 3.6-4.0) (Li *et al.*, 2001) and germinating seeds of *vigna sinensis* (pH 4.5) (Biswas, 1985). The pH stability between pH 4.0 and 7.0 was observed (Fig. 3).

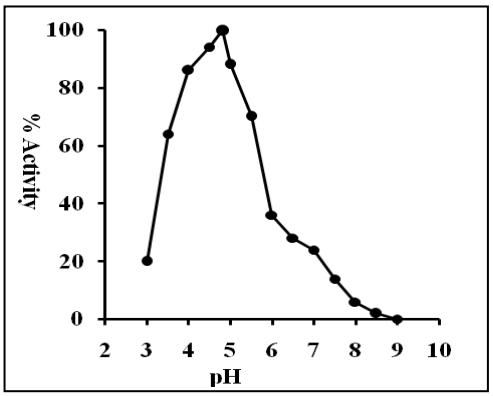


Fig. 2: Optimum pH of β-galactosidase from watermelon seedlings

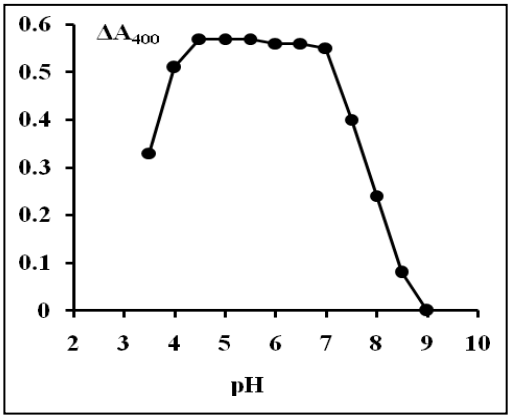


Fig. 3: pH stability of β-galactosidase from watermelon seedlings.

These observations are similar to the results reported on enzyme from seeds of *Tamarindus indica* (Shlini and Siddalinga, 2011). The enzyme exhibited optimum temperature of 50°C (Fig. 4). A rapid decrease in activity was observed thereafter. The same value was also reported in maize (Esen, 1992) and peach (Lee *et al*., 2003). This optimal temperature was higher than for *Bacillus subtilis* (35°C) (Abd El-Kader *et al*., 2012). But it was lower than that of apricot seed of (70°C) (Yossef and El Beltagey, 2014). Temperature stability was shown up to 50°C. Thereafter its activity fell and inactivated completely at 70°C (Fig. 5). The same results were reported for the enzyme from *Vigna sinensis* (Biswas, 1985).

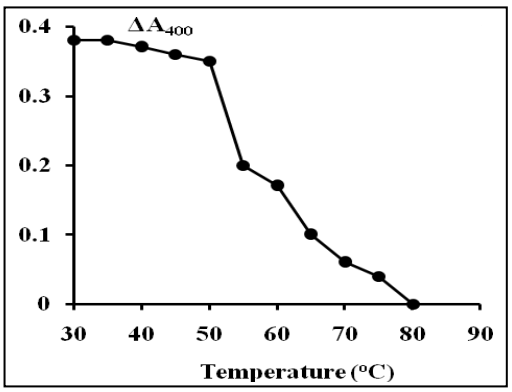


Fig. 5: Temperature stability of β-galactosidase from watermelon seedlings.

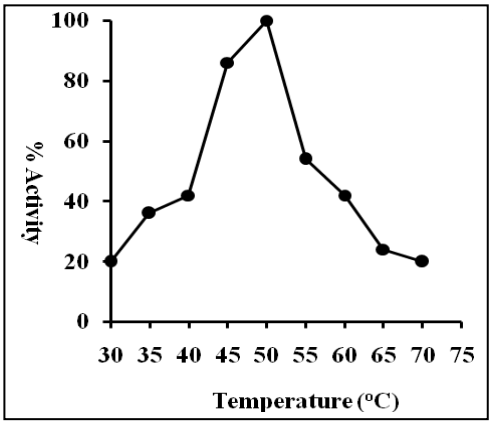


Fig. 4**:** Optimum temperature of β-galactosidase from watermelon seedlings.

Kinetic parameters: Data for the determination of K_m and V_{max} was analyzed using linear regression analysis. K_m and V_{max} were found to be 0.2 mM and 34 $Umin^{-1}mg^{-1}$of protein, respectively.

Effects of metal ions and SH- reacting reagents: Metal ions have shown different effects on the β-galactosidase activity. The enzyme was unaffected by monovalent ions such as Na^+ and K^+ and divalent metal ions, for example Ca^{+2} and Mg^{+2}, … etc, while Zn^{+2} and Co^{+2} inhibited the enzyme. Heavy metal ions such as Al^{+3}, Cu^{+2}, Ag^{+1}, Hg^{+2}, etc. inhibited the enzyme strongly (Table 2).

Table 2: Effect of metal ions/SH-reacting agents on the β-galactosidase activity.

Metal ions/ SH-reacting reagents	Concentration (mM)	β-Galactosidase (% Activity)
H_2O	0.0	100
NaCl	5.0	97
KCl	5.0	103
$CaCl_2$	5.0	96
$MgCl_2$	5.0	90
$MnCl_2$	5.0	94
Na_2MoO_4	1.0	16
$ZnCl_2$	5.0	37
$CoCl_2$	5.0	35
$HgCl_2$	5.0	13
Cu_2 SO_4	5.0	1.0
NaF	5.0	105
β-mercaptoethanol	5.0	103
PCMB	0.1	3.0
Iodoacetate	10	7.0
Tartrate	5.0	46

The results were expressed as relative percentage of activity respect to the control reaction without ion/reagent added.

The SH-reacting reagents (PCMB and iodo-acetate) had abolished the activity almost completely. The inhibition by heavy metals and SH-reacting reagents suggests that cystein was necessary for enzyme activity. The exposure of enzyme with DTT or β-mercaptoethanol at or below 500 mM concentrations showed maximum activation by 1-7 %. These behaved as reducing agents. At 500-2000 mM concentrations of these, inhibitions were displayed (Table 3). Thus both

reducing agents at lower concentrations may have as stabilizing effect. Table 4 shows the enzyme inhibition with some metal ions in the presence of β-mercaptoethanol. Pre-incubation of enzyme with metal ions (12.5 mM) was carried out in the presence of 20 mM β-mercaptoethanol for 10 min at 37°C. Following incubation, the activity was determined as usual. The same were treated without β-mercaptoethanol to compare the inhibitions. Al^{+++}, Zn^{++}, Hg^{++} and Ag^{+1} showed around 77 %, 80 %, 100 % and 91% inhibitions, respectively, without β-mercaptoethanol. With 20 mM β-mercaptoethanol, the extent of inhibition was decreased. The values of inhibition obtained were 50, 57, 90 and 82%, respectively. The activity recovery was 27, 23, 10 and 9%, respectively. Thus, inhibition with metal ions was reduced by β-mercaptoethanol. Results are illustrated in Table 4.

Table 3: Effect of different concentrations of reducing agents on the enzyme activity.

Concentrations (mM)	DTT Activity (%)	β-mercaptoethanol Activity (%)
0	100	100
10	101	103
20	103	105
50	105	104
100	107	105
200	100	102
500	95	93
1000	90	84
2000	83	78

After pre-incubation of enzyme with reducing agents for 10 min, activity measurements were made.

Table 4: Inhibition by metal ions in the presence or absence of β-mercaptoethanol.

Metal ions (12.5 mM)	without β-mercaptoethanol		with β-mercaptoethanol (20 mM)		Recovery
	Act.	% Act.	Act.	% Act.	(% Act.)
No metal ions (control)	0.420	100	0.44	100	-
Al^{+3}	0.096	23	0.22	50	27
Zn^{+2}	0.084	20	0.189	43	23
Hg^{+2}	0.000	00	0.044	10	10
Ag^{+1}	0.037	09	0.079	18	9

Glycosyltransferase activity: Methanol and ethanol increased the activity by 1.5 folds at a concentration of 70%, while 70% propanol has little effect on the enzyme activity (Table 5). Propanol between concentrations 80 and 100% resulted in the inhibition of the enzyme activity. These activations reflect glycosyltransferase activity that catalyses the transfer of glycosyl group from *p*-nitrophenyl-β-D-galactoside substrate to alcohols (Table 6).

Table 5: Effect of different concentrations of alcohols on β-galactosidase activity.

Alcohols/Concentrations	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
	% activity										
Methanol	100	105	113	133	138	142	148	157	158	ND	156
Ethanol	100	103	105	120	125	130	139	144	138	ND	136
Propanol	100	102	102	104	105	112	113	114	98	ND	97

Assay against water was taken as 100%. ND: not determined

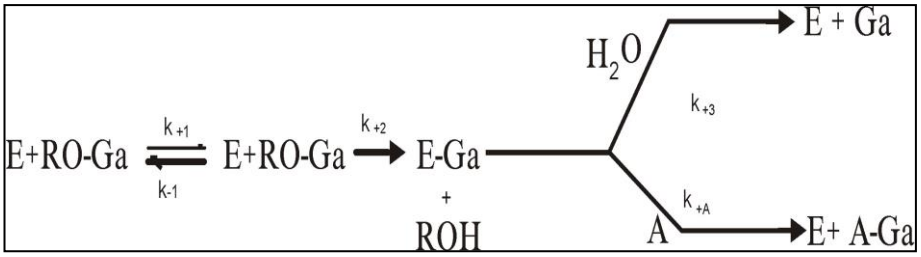
Table 6: β-Galactosidase catalysed galactosyl transfer reaction in the presence of various alcohols as galactose acceptors.

Glycosyl Acceptors	Concentrations (%)	*p*-Nitro-phenol produced (n mol)	Galactose produced (n mol)	Galactose Transferred to alcohols (n mol)	Galactose / *p*-nitrophenol
Water	-	14	12.9	1.1	0.9214(≈1)
Methanol	50	22	12.3	9.7	0.559
Ethanol	50	19.5	12.5	7.0	0.641
Propanol	50	16.8	13	3.8	0.774

Galactose transferred to alcohols was the difference between nitrophenol released and galactose produced.

Methanol seems to be better acceptor of glycosyl group than ethanol. Propanol was the least acceptor of glycosyl group. The presence of methanol, the rate of glycosyl transfer was greatly

increased involving the formation of glcosylated enzyme intermediate. Methanol, ethanol and propanol increased the decomposition of substrate as can be shown by the formation of *p*-nitrophenol (**Table 6**) when compared with water. The galactose produced remained almost constant. Thus, the determination of *p*-nitrophenol and galactose liberated in the reaction with alcohols which compete with water in hydrolytic reaction of glcosylated enzyme covalent intermediate (see scheme) showed the transglycosylation reaction by β-galactosidase as less than 1 ratio of galactose/*p*-nitrophenol was obtained.



Where E represents enzyme, RO-Ga *p*-nitro phenyl β-galactoside, E.RO-Ga enzyme-substrate complex, E-Ga glycosylated enzyme covalent intermediate, ROH *p*-nitrophenol, A the alcohol and A-Ga glycosyl alcohol.

The methanol had ratio of 0.524 which reflected better acceptor of glycosyl group than propanol (0.774) and more transglycosylation reaction was pronounced than ethanol or propanol. On the analogy of phosphotyrosine protein phosphatase (PTPase) from bovine heart (Zhang and Van Etten, 1991), the rate limiting step is not the formation glycosylated enzyme covalent intermediate (reaction with k_{+2}) because alcohol reacts with covalent intermediate and does not increase the formation of *p*-nitrophenol, but the rate limiting step is the hydrolysis of glycosylated enzyme covalent intermediate (reaction with k_{+3} or k_{+A}) because the presence of alcohol increased the formation of *p*-nitrophenol. The level of galactose remained almost unchanged and was equal to level of galactose, produced in the absence of alcohol (13.9 nmol), demonstrating the hydrolysis of a glycosylated enzyme covalent intermediate is rate limiting step. The similar results were also reported by Cirri *et al*., (1993) with substrate, benzoyl phosphate and acceptor, glycerol and enzyme, maltose binding protein - PTPase. To determine the role of tryptophan in enzyme catalysis, β-galactosidase enzyme was pre-incubated for 30 min with tryptophan modifycation reagent, N-bromosuccinimide (NBS) at concentrations ranging from10 to 60 mM. The remaining activity was assayed. The enzyme was inactivated by NBS suggesting that tryptophan plays an important role in the catalytic mechanism. When enzyme was pre-incubated with NBS along with active site protector, galactose (equimolar mixture of NBS and galactose), and the inhibition was relieved. The presence of galactose, NBS could not inhibit the enzyme which proves that tryptophan is the part of the active site (Fig. 6).

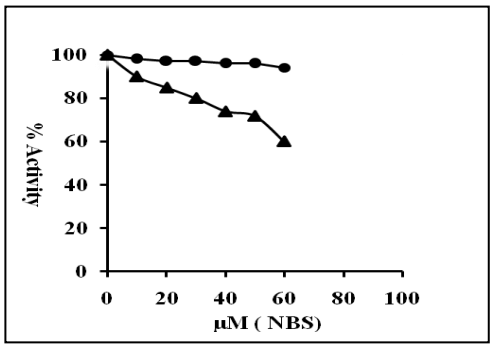


Fig. 6**:** Inactivation with tryptophan modification reagent, N-bromosuccinimide(NBS).
in the presence of galactose (●—●),
in the absence of galactose (▲□□▲)

Conclusions

β-Galactosidase from watermelon seedlings was partially purified and characterized. This enzyme is similar to β-galactosidase isolated from Durian seeds. Beside its hydrolytic activity, it also possesses transgalactosidase activity. Transgalactosylation reaction has been shown in variety of bacterial and fungal species. Cystein and trypotphan are very necessary for catalytic mechanism.

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