ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 8, Issue, 6, pp. 17330-17334, June, 2017

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

PURIFICATION AND CHARACTERIZATION OF INVERTASE FROM THE MIDGUT OF FIFTH INSTAR LARVAE OF *ANTHEREAE MYLITTA DRURY (DABA TV)*

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DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0332

ARTICLE INFO

Article History:

Received 20th March, 2017
Received in revised form 29th April, 2017
Accepted 30th May, 2017
Published online 28th June, 2017

Key Words:

Anthereaemylittadrury, invertase, midgut, purification, characterization.

ABSTRACT

Invertase obtained from fifth instar larvae of *Anthereaemylittadrury*was purified by 45-85% $(NH_4)_2SO_4$ fractionation followed by dialysis and DEAE column. The enzyme was purified to 15.32 folds with recovery of 20.1% by DEAE-column chromatography and the molecularweight estimated was 66kDa by SDS-PAGE. Fifth instar larvae of *Anthereaemylittadrury (Daba TV)* contains high level of invertase at pH 10, temperature 40°C, using sucrose as substrate. Invertase has a Vmax value of 0.938 µmol/min/mg and Km value of 7.68 µmol/min/mg at temperature 40°C and pH 10.The increase in chemical concentration of $CaCl_2$ and EDTA stimulated theinvertase activity whereas NaCl, Urea, $MgCl_2$ and $ZnCl_2$ inhibited the activity.

INTRODUCTION

Digestion is a phase of insect physiology on which little research has been performed, despite the economic importance of the food of insects and the fact that the most important control measures involve the action of digestive juices on poisons taken into the digestive tract (Ghoshal *et al.*, 2001). Information on digestive enzymes is of fundamental importance in understanding digestive processes, food and feeding habits, host selection, insect resistance etc., Studies on enzyme catalysis explains insect physiology and also helps to develop new management strategies (Bandani *et al*., 2001; Maqbool *et al*., 2001). Fore gut and mid gut forms a seat for enzymes like trypsin, invertase, lipase, maltases (Muhammad Akhtar, 1982). Invertases (β-fructofuranosidase, EC3.2.1.26) also termed fructosidase, saccharase, or sucrose are glycosidehydrolases (EC 3.2.1) that catalyze the cleavage of sucrose (β-D-glucopyranosyl-S Dfructofuranoside)into monosaccharides, glucose and fructose (Henrissat andBairoch, 1993;Sturm and Tang,1999; Naumff, 2001). Invertase thus appears to be a particularly important enzyme for plants and animals. Given this general importance, a surprisingly limited number of studies have tried to quantify invertase activity in ants (Ricks and Vinson, 1972) or other animals (Martinez del Rio, 1990; Zeng Cohen, 2000). This might be due to the particular methodological problems arising from the quantification of invertase in animals whose carbohydrate metabolism is highly active. A better understanding of enzyme catalysis is essential in order to develop methods of insect control (Bandani *et al*., 2001; Maqbool *et al*., 2001). The purpose of the present study is to identify and characterized invertasefrom midgut of fifth instar larvae of *Anthereaemylittadrury (Daba TV)* for understanding digestive physiology. This understanding will lead to new management strategies for improving its feeding efficiency and in turn to improve commercial characters.

Anthereaemylitta Drury a lepidopteran insect of the Saturniidae family produces tasar silk of commercial importance. The insect have 44 ecoraces distributed throughout India. Present work has taken up to purify invertase from the midgut of *Anthereaemylittadrury (Daba T.V)* and to understand its activityunder different pH, temperatures and chemical compounds exposure and also to determine the molecular weight of purified invertase.

MATERIALS AND METHODS

Insect collection and sample preparation

Fifth instar larvae of *Anthereaemylittadrury (Daba TV)* were collected from forest patches of Jakaram, Warangal district. 50 healthy larvae were used to dissect the midgut in ice-cold buffer (6 µMNaCl). A 10 % (w/v) homogenate of the midgut tissue of fifth instar larvae was prepared using mortar and

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pestle with 1 ml of universal buffer. The homogenate was transferred to 2 ml centrifuge tubes and centrifuged at 10,000 rpm for 15 min. The supernatant was pooled and stored at -20 °C for subsequent purification steps and invertaseassay.

Invertase activity

Assay was carried out according to (Nelson, 1994). Midgut extract was mixed with 2ml toluene which arrests the enzyme activity. After 30min, sample mixed with 10 ml of 0.2M glycine (pH 7.2) containing 20mM sucrose held at 20°C for 24h and absorbance was recorded at 340nm. For control midgut extract was placed in boiling water bath for 30 min to destroy enzyme activity and then cooled. One unit of enzyme release one nanomol of glucose per minute using sucrose as substrate. Standard curve was used to measure the specific activity of enzyme (Figure 1).

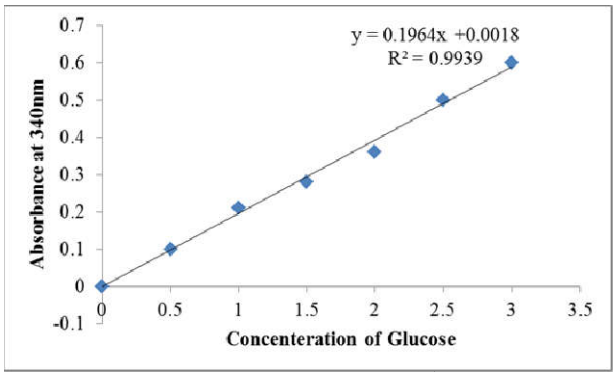

Fig. 1 Standard calibration curve for the determination glucose released by invertase

Purification of enzyme

Purification of midgutinvertase of fifth instar larvae was performed in three steps as described by (Uma *et al*., 2012).

Ammonium sulphate treatment

Midgut samples were first subjected to ammonium sulphate precipitation by 40 and 80% fractions. The precipitated fractions were centrifuged at 15000rpm for 15min and supernatant of each fraction was suspended in 1ml universal buffer (pH 10).

Dialysis

Ammonium sulphate precipitated fractions were dialysed 100mM Tris phosphate buffer (pH 7.5) for 24 hours at 4 C.

DEAE separation

Dialysed filtrate was loaded onto a DEAE-cellulose chromatographic column (25 cm x 2.6 cm) equilibrated with Tris-Hcl buffer, 100mM, pH 7.5.

The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0.4 M) in the same buffer and 5.0 ml fractions were collected at a flow rate of 20 ml per hour.

Kinetic parameters

Each assay was carried out with thirty microliter of diluted enzyme. Final concentrations of substrate were 20,30,40,50 and 60mM. MichaelisMenten constant (Km) and the maximal velocity (Vmax) were estimated by Sigmaplot software version 11. Km and Vmax are the means of ±SE of three replicates.

Effect of various compounds on enzyme activity

To test the effect of various compounds on the invertase activity of fifth instar larva final active fractions from DEAE separation were used. Different concentrations of sodium chloride (10,20,30,40m Mrespectively), Urea (10,20,30 and 40m Mrespectively), calciumchloride (10,20,30and40m Mrespectively), magnesiumchloride (10,20,30a nd40 Mmrespectively), zincchloride (10,20,30,40m Mrespectively) and EDTA (1.0,2.0,3.0.and4.0mMrespectively)were used for enzyme assay. The compounds were added to assay mixture and activity was measured after 45 minutes.

Effect of Temperature on enzyme activity

Temperature effect was determined by incubating final active fractions obtained from DEAE column at 20, 25, 30, 35, 37, 38 40, 45, 50, 55, 60, and 65°C for 24 hours followed by enzyme assay.

Effect of pH on enzyme activity

pH effect on invertaseactivity was measured using final active fractions of DEAE column. Optimal pH for activity was estimated using universal buffer with pH set at 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13.

Protein determination

Concentration of protein was measured using Bovine serum albumin as standard (Bradford, 1976).

Polyacrylamide Gel Electrophoresis (PAGE)

Molecular mass of invertase was carried out using 2.5 % and 6.5% polyacrylamide for the stacking and resolving gels (Parish and Marchalonis1970). The gel was stained with 1.5 % (w/v) Coomassie Brilliant Blue G-250.Later gels were de stained in a solution containing 5% (V/V) acetic acid and 20% methanol (V/V).

Statistical analysis

Each assay was replicated 3 times. Values were expressed as mean ±SE.

Table 1 Purification of invertase from the midgut of fifth instar larvae of *Anthereaemylittadrury* larvae

Purification step	Total protein (mg)	Total invertase Activity µmol/min	Specific Activity µmol/min/mg	Yield (%)	Purification (fold)
Crude extract	0.852±0.016	0.418±0.018	0.491±0.08	100	1
$(NH_4)_2SO_4$ (0-40%)	0.456±0.014	0.314±0.016	0.688±0.062	75.12	1.4
$(NH_4)_2SO_4$ (40-60%)	0.205±0.008	0.215±0.015	1.048±0.052	51.43	2.13
Dialysis	0.156±0.004	0.202±0.014	1.294±0.064	48.32	3.78
DEAE Column	0.09±0.004	0.084±0.015	7.523±0.075	20.1	15.32

RESULTS

The enzymes in insect's life cycle play central role in different biological processes such as digestive mechanism, defense mechanism, locomotion, feeding methods, temperature control, growth, development etc.

Present results showed that invertase is present in the midgut of fifth instar larvae of *Anthereaemylittadrury*. Table 1 depicts purification steps of mid gut sample of fifth instar larvae. Invertase obtained from the midgut of fifth instar larvae was purified by 45-85% $(NH_4)_2SO_4$ fractionation followed by dialysis and DEAE. Protein concentration has reduced by 76% from crude extract to final Ammoniumsulphate extraction step. The Standard curve was used to measure specific activity of invertase (Fig 1). Final invertase purification reported a recovery of 15.32%final purified enzyme with a specific activity of 0.49 of crude extract which has increased to7.52 µmol/min/mg in DEAE Column chromatography. The enhancement was explained as being due to the gradual liberation of the sugar from the ester by the action of a slowly acting esterase.

Table 2 shows that several chemicals affect the activity of invertase in midgut. With the increase in chemical concentration of $CaCl_2$ and EDTA, invertase activity found increased but decreased in presence of NaCl, Urea, MgCl2 and $ZnCl_2$.

Table 2 Activity of midgutinvertase of fifth instar larvae of *Anthereaemylittadrury* towards various compounds

Type of compounds	Concenteration (mmol/l)	Midgutinvertase activity in fifth instar larvae µmol/min/mg
NaCl	10	90.28±2.6
	20	65.25±2.4
	30	25.55±1.6
	40	6.85±1.4
EDTA	1.0	43.25±3.4
	1.5	84.24±4.2
	2.0	128.46±8.4
	2.5	242.45±10.6
UREA	10	115.86±5.8
	20	98.65±5.6
	30	72.86±4.5
	40	2.54±0.8
$CaCl_2$	10	58.46±3.42
	20	78.25±4.5
	30	85.65±7.8
	40	125.42±12.6
$MgCl_2$	10	252.45±14.6
	20	195.25±12.2
	30	112.32±6.8
	40	38.42±4.5
$ZnCl_2$	10	212.25±10.5
	20	168.86±8.5
	30	98.54±9.5
	40	28.65±4.8

Fig 2 shows that optimum temperature range for midgutinvertase of fifth instar larvae was 37°C -45°C whereas maximum activity was recorded at 40°C.Extreme temperatures will disrupt the hydrogen bonds that hold the enzyme in its three dimensional structure finally denaturing the proteins (Donggiun *et al*., 2011).

In the present study the in vitro evaluation of midgutinvertase indicated that enzyme activity increased steadily from pH 2 to 10 in fifth instar larvae (Fig 3). The optimum pH for invertase was in the range of 6-10.Invertase has maximum activity at pH 10 and after this threshold level, activity got decreased with the increase of pH. More than 50% activity was decreased below pH 4 and beyond pH 10.

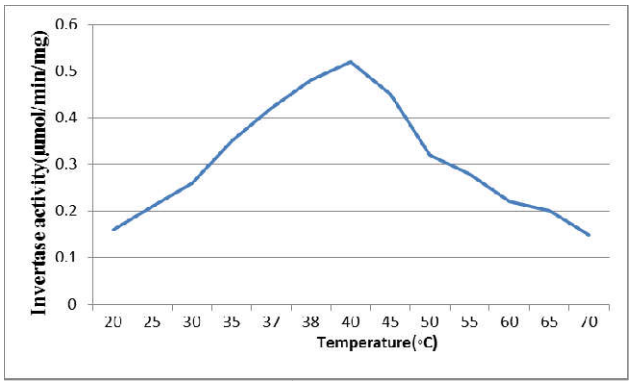

Fig. 2 Temperature effect on midgutinvertaseactivity of fifth instar larvae of *Anthereaemylittadrury*

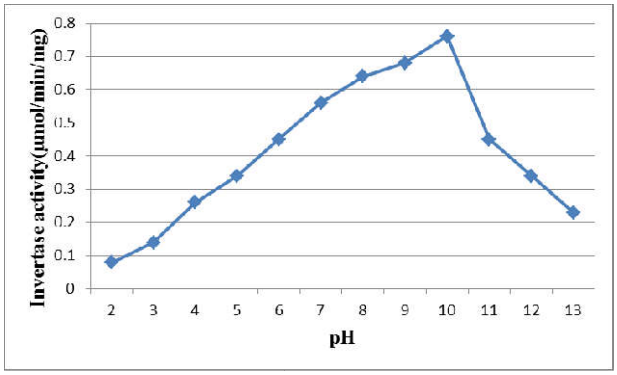

Fig. 3pH effect on midgutinvertase activity of fifth instar larvae of *Anthereaemylittadrury*

It can be seen from Fig 4 the kinetic parameters for purified invertase activity were determined using sucrose as the substrate. The values of Km and Vmax were calculated by Linewaver Burk plot. A Lineweaver-Burk plot of the enzyme affinity for sucrose gave a straight line plot from which the Km as 7.68 µmol/min/mg and Vmax was 0.938 µmol/min/mg The values were similar to that obtained with the invertase from *Rhodortorulaglutinis* (Rubi *et al*., 2002).

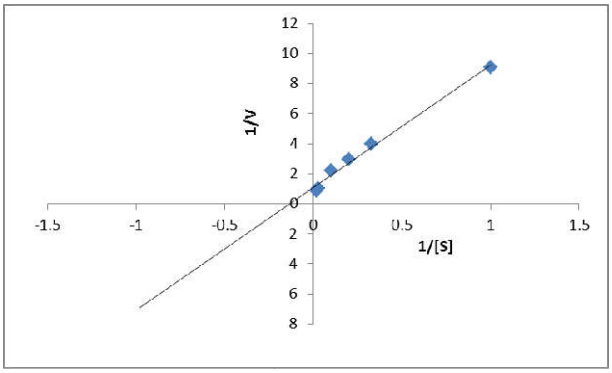

Fig. 4 Line Weaver -Burk plot(Vmax and Km) of invertase extracted from fifth instar *Anthereaemylittadrury* larvae

In the present study final enzyme fraction obtained from DEAE column was subjected to SDS-PAGE of which invertase showed a molecular weight of 66kDa. The molecular weight was compared with the high range protein marker (Fig 5).

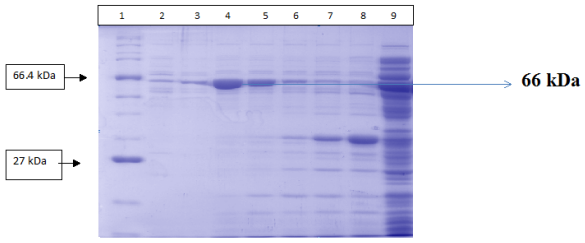

Fig. 5 SDS PAGE showing purified invertase molecular weight as 66 kDa Lane 1-marker, Lane 2,3,4,5-Purified Invertase from DEAE Cloumn, Lane-6,7,8- Ammonium sulphate purified Sample, Lane-9 Crude

DISCUSSION

The present study shows that midgut of fifth instar larvae of *Anthereaemylittadrury* shown invertase activity. Reports concerning lipase characterization have beenobtained from several species of insects. Midgut homogenates of mosquito *Anopheles quadrimaculatus* had shown high concenterations of invertase (Rubio and Navarro, 2006).

In the present work effect of metal ions on invertase activity was recorded. Some metal ions stimulated invertase and some ions inhibited. Maximum invertase activity in *Aspergillusflavus* has been reported with calcium chloride (Uma *et al*., 2010). Ca2+ ions have activatory effects on the lipase and invertase activities of the coleopteron *Callosobruchus* chinensis (Applebaum, 1985). Generally metal ions protect the enzymes against thermal denaturation at extreme high temperatures (Uma *et al*., 2010). EDTA is the stimulating agent for invertase activity in Saccharomyces cerevisiae (Shanker *et al*., 2014).

Present results showed that the maximum pH for invertase is alkaline (around pH 10). The optimum temperature range recorded was 37°C-45°C respectively. The optimum pH and temperature for invertase activity in hemipteron *Chrysomphalusaonidum* are 5.5 and 30 °C respectively (Ishaaya and Swirski, 1970). 37°C is the suitable temperature for invertase activity in *Actinomycetes*s train (Kaur and Sharma, 2005). Maximum invertase activity was recorded at 37°C for invertase in *Lactobacillus reuteri* (Gine, 2010). Purified invertase has an optimal temperature of 45°Cfor catalyzing sucrose (Donggiun, 2011). The optimal pH generally reflects the pH of the environment in which the enzyme normally functions. SuitablepH for invertase activity in *Chilosuppressalis* is 11 and the way in which pH affects reaction rates is by altering the charge state of the substrate or of the active site of the enzyme (Zibaee, 2008). Biological reactions occur faster with increasing temperature up to the point of enzyme denaturation, above which temperature enzyme activity and the rate of the reaction decreases sharply (Zeng and Cohen, 2000; Agblor, 1994; Applebaum, 1985). Current study results also showed that the molecular weight determined for invertase by SDS PAGE is 66kDa. These results are almost similar to the determined molecular mass (69kDa) of invertase from pea seedlings (Donggiun, 2011). When the action of digestive enzymes is inhibited, insect's nutrition is impaired, growth and development are retarded and eventually death occurred due to starvation (Shekari *et al.,* 2008).

CONCLUSION

Purification and characterization of insect digestive enzymes will facilitate to understand the mechanisms and will help to design new and specific strategies for improving its feeding efficiency and also commercial characters.

Acknowledgments

The author Dr.Lakshmi Marepelly is thankful to University Grants Commission, New Delhi for providing financial assistance under Post- Doctoral Fellow for Women (No.F.15-1/2011-12/PDFWM-2011-12-GE-AND-5800 (SA-II).

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How to cite this article:

Lakshmi Marepally.2017, Purification and Characterization of Invertase from the Midgut of Fifth Instar Larvae of Anthereae Mylitta Drury (Daba TV). *Int J Recent Sci Res.* 8(6), pp. 17330-17334. DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0332

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