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Properties of Invertase from *Fusarium oxysporum*

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Invertase purified from mycelium of *Fusarium oxysporum* showed optimal activity at a PH range of 5.0 – 5.8 and temperatures of 45°C and 55°C with sucrose and raffinose as substrate, respectively. The optimum *p*H of the enzyme was in the range of 5.0-5.8. Depending upon the temperature of the assay system, the K_m for sucrose and raffinose varied from 1.5 to 7.1 and 2.7 to 7.1 mM, respectively, in comparison to 10mM K_m with stachyose at 45°C. V_{max} of the enzyme was in the order of sucrose > raffinose > stachyose. Thermal stability of the enzyme was not enhanced by polyhydric alcohols. The energy of activation with sucrose and raffinose was 48.7 and 66.0 KJ/mole, respectively. High concentrations of sucrose and raffinose gave protection to invertase against thermal denaturation. The enzyme was non-competitively inhibited by $HgCl_2$ (K_i 16-30 μM). This inhibition was reversed by cysteine which also activated the enzyme. A significant increase in secretion of extracellular invertase was observed on addition of inulin to 3-day old growing fungus.

Key Words: Invertase, Deuteromycetes, Sucrose metabolism

Introduction

In plants infected by fungi, it appears that the major soluble carbohydrate released by the host cells at the host parasite interface is sucrose and this sucrose is hydrolyzed prior to uptake by the fungus (Manners & Gay 1983). Though a number of workers have detected large increase in the activity of acid invertase during the development of fungal infection in several plant species (Long et al. 1975, Greenland & Lewis 1983, Mitchell et al. 1978), it is not clear whether the increased activity is of host or fungal origin. One approach to this problem requires the characterization and comparison of invertase from normal plant, infected plant and laboratory cultured pathogen. *Fusarium oxysporum* is an important plant pathogen and causes wilt in a number of plants. When this fungus was grown on sucrose-containing medium, two sucrose-utilizing enzymes, namely sucrose fructosyl transferase which catalyzes the formation of difructosyl glucose and glucose by fructose transfer from one sucrose molecule to another sucrose molecule and invertase which hydrolyses sucrose were separated by using DEAE cellulose column chromatography (Gupta & Bhatia 1980, 1982). Oligosaccharides like di-, tri- and tetra-fructosyl glucose were detected in the medium (Gupta & Bhatia 1980). These short-chained glucofructans act as the transitory carbohydrate reserves which are hydrolysed by invertase to meet the energy demands during later stages of fungal growth (Gupta & Bhatia 1980). This is analogous to the transitory accumulation of fructans during early growth and a subsequent hydrolysis during later stages in some plants (Bhatia et al. 1974, Gupta et al. 1985, Gupta et al. 1986). Although invertases from fungi belonging to ascomycetes (Dickerson 1972, Gascon et al. 1968, Metzenberg 1963, Olutiola & Cole 1980) and

phycomycetes (West et al. 1980) have been studied. Information on the properties of this enzyme from fungi belonging to basidiomycetes and deuteromycetes is scanty. *F. oxysporum* which belongs to class deuteromycetes was, therefore, selected for the present study.

Materials and Methods

Medium employed for fungal growth: The culture of *Fusarium oxysporum* (NCIM 1072), obtained from the National Chemical Laboratory, Pune, was maintained on agar slants by transfer at 14-day intervals. The fungus was grown in the liquid medium containing (g/l) sucrose, 30; $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01 plus 1ml/l each of the solutions of trace elements, vitamins and $ZnSO_4$ (4.4%). Vitamin solution contained (mg/l), thiamine hydrochloride, 100; pyridoxine hydrochloride, 50; calcium pantothenate, 200; *p*-amino benzoic acid, 50; nicotinamide, 200; myoinositol, 400; riboflavin, 50. The trace element solution contained (mg/l) boric acid, 100; ammonium molybdate, 500; cupric sulphate, 800; manganese chloride, 150. The *p*H of this aqueous medium was adjusted to 5.5. Procedure for sterilization of the medium and inoculation was the same as described earlier (Gupta & Bhatia 1980, Gupta et al. 1988).

Extraction and purification of invertase: Mycelium collected after 12 days of fungal growth, was crushed to a fine paste and invertase was extracted, isolated and purified by ethanol precipitation and DEAE Cellulose Chromatography as described earlier (Gupta & Bhatia 1982). Following this chromatographic technique, the sucrose sucrose fructosyl transferase was eluted in the first protein peak with 0.1M sodium acetate buffer (*p*H 5.0) whereas invertase was eluted in the second peak with buffer containing 0.25M NaCl. Properties of second peak are described in this paper. Protein content was determined by Folin phenol reagent (Lowry et al. 1951).

Invertase assay: The reaction mixture consisting of 100 μmoles of sodium acetate buffer (*p*H 5.0), 200 μl enzyme and 100 μmoles of sucrose or raffinose in a total volume of 1 ml was incubated at optimum temperature (45°c with sucrose and 55°c with raffinose) for 60 min. One unit of invertase was taken as the quantity of enzyme which produced 1 μmole of reducing sugar/min at optimum temperature. Reducing sugars released upon enzymatic hydrolysis were estimated by using alkaline copper tartrate and arsenomolybdate reagents (Nelson 1944). In preliminary assays, the linear reaction rate with respect to substrate concentrations time and temperature were pre-determined.

Results and Discussion

Effect of Hg^{2+} and cysteine on invertase activity: A non-competitive inhibition of invertase by $HgCl_2$ (K_i 16 to 30 μM) was observed (figure 1). With as low as with 2 μM $HgCl_2$ in the assay system, the inhibition was approximately 20% and with 2 mM $HgCl_2$ the enzyme was completely inhibited (table 1). This inhibition indicates that a sulphydryl group is essential for invertase activity. Invertase inhibition with $HgCl_2$ varies greatly with the enzyme source. Invertase from the wheat and oat seedling is completely inhibited with 2 μM $HgCl_2$ (Krishnan et al. 1985, Pressey & Avants 1980). However, with 1 mM $HgCl_2$, lilly pollen invertase is inhibited only to 42% (Singh & Knox 1984). Pressey (1981) observed that 5 μM $HgCl_2$ alone inhibited the avena invertase by 60% but in combination with 40 μM cysteine this inhibition was increased to over 90%. Possibly Hg^{2+} reacts with SH groups of both invertase and cysteine to form E-S-Hg-S-R complex which is less active than E-S-Hg^+ complex (Pressey 1981). In our study, the increasing concentration of cysteine in the reaction mixture gradually relieved invertase inhibition by Hg^{2+} (table 1). At cysteine concentrations of even in the presence of 40 μM and above, 10 μM $HgCl_2$, the enzyme was activated (table 1). Whereas the release of inhibition by cysteine is possibly due to change of E-S-Hg^+ complex to E-SH form, further activation of invertase by this amino acid may be due to attainment of a more favourable enzyme conformation. Activation of invertase in the presence of cysteine alone was more as compared with that due to cysteine + $HgCl_2$ was much more (table 1).

Substrate specificity and optimum pH: Invertase from *Fusarium oxysporum*, grown in sucrose containing medium, cleaved fructose from sucrose, raffinose and stachyose but did not act on inulin (β 2→ 1 linked polymer of fructose). However, when this fungus was grown in fructan-containing medium, then its β-fructosidase did hydrolyse both sucrose and inulin (Gupta et al. 1988, Gupta et al. 1989). V_{max} at 45°C was in

Table 1 *Effect of Hg^{2+} and cysteine on invertase activity*

Concentration (μM) in reaction mixture		Enzyme activity
Mercuric chloride	Cysteine	(%)
0	0	100.0
2	0	78.6
5	0	68.5
10	0	67.1
20	0	64.2
200	0	21.4
2000	0	0.0
10	10	88.8
10	20	93.6
10	40	102.2
10	100	106.6
10	200	113.3
0	100	131.1
0	200	135.5

Table 2 *Michaelis constants and relative maximum velocities at 45°C for invertase with sucrose, raffinose and stachyose*

Substrate	K_m (mM)	V_{max}
Sucrose	7.1	1.00
Raffinose	7.1	0.72
Stachyose	10.0	0.56

Table 4 *Effect of sucrose and raffinose on protection of invertase against thermal denaturation*

Heat treatment in presence of sugars	Enzyme activity (%)
No Sugar	35.1
Sucrose	48.2
Raffinose	56.5

Invertase was heated with and without 50 mM sucrose or raffinose at 55°C for 30 min, made sugar-free by passing through sephades G-25 column and its activity determined with sucrose at 45°C

Table 5 *Time course effect of temperature on invertase activity*

Temperature (°C)	Enzyme activity (%) at different times (minutes)			
	15	30	45	60
45	93.7	92.2	92.2	90.6
55	54.7	37.5	11.1	6.2
65	3.1	0	0	0

Invertase was incubated alone at different temperatures for time ranging upto 60 minutes before measuring its activity at 45°C. % activity is expressed on the basis of a control in which no preincubation was done

Table 3 *Effect of high temperatures on invertase activity with varying sucrose and raffinose concentrations*

Sucrose/raffinose (mM)	μmoles of reducing sugar formed/hr			Activity at 65°C calculated as % of that at optimum temp.	
	45°C	55°C	65°C	With sucrose	With raffinose
2.5	1.4	1.3	0.0	—	14.8
	(1.14)*	(1.62)	(0.24)		
5.0	2.4	2.0	0.04	1.6	17.2
	(1.62)	(2.20)	(0.34)		
10.0	3.2	3.0	0.12	3.7	18.2
	(2.00)	(2.86)	(0.52)		
25.0	4.2	3.1	0.37	8.8	37.8
	(2.78)	(3.54)	(1.34)		
50.0	4.3	3.7	1.11	25.8	42.4
	(3.26)	(3.82)	(1.62)		
100.0	4.8	3.8	1.40	29.1	54.5
	(3.82)	(4.40)	(2.40)		

* Values in parentheses represent invertase activity with raffinose

Table 6 *Effect of addition of inulin to the metabolising* ***Fusarium oxysporum*** *on extracellular invertase production*

Carbon source (3%)	Sugar added after 3 days	Invertase units/ml of medium after growth (days)			
		3	6	9	12
Inulin	nil	0.10	0.30	0.30	0.40
Sucrose	nil	0.02	0.02	0.05	0.15
Sucrose	Inulin	0.02	0.21	2.20	1.20

Fusarium oxysporum was grown either on inulin or sucrose containing medium. After 3 days of growth, 3% inulin was adde to the fungus growing on sucrose. Medium collected after centrifugation was used for measuring invertase activity

the order of sucrose > raffinose > stachyose (table 3). Optimum *p*H range (5.0 to 5.8) of this enzyme was typical for an acid invertase

Effect of temperature on K_m energy of activation and thermostability of invertase: Using sucrose and raffinose as the substrates, the effect of increasing temperature on K_m and V_{max} of invertase was studied. With sucrose, both K_m and V_{max} increased till 45°C (figure 2). Depending upon the temperature of the assay mixture, the K_m of the enzyme with sucrose was between 1.5 and 7.1 mM. The K_m values of invertase from *Neurospora, Aspergillus flavus, Phytophthora megasperma* and yeast have been reported to be between 1.3 and 26 mM (Metzenberg 1963, Neumann & Lampen 1969, Olutiola & Cole 1980, West et al. 1980). K_m of invertase with raffinose also varied from 2.7 to 7.1 mM (figure 3) depending upon the temperature of the assay system. Optimum temperatures with raffinose and sucrose are 55°C, and 45°C rerspectively. At 45°C the Km with raffinose and sucrose was 7.1 mM (table 2). However, the invertase from radish seedlings and wheat has a higher K_m for raffinose than for sucrose (Faye et al. 1981, Krishnan et al. 1985). The energy of activation of the enzyme was 66 KJ/mole with raffinose against 48.7 Kj/mole with sucrose. This energy of activation is comparable with the energy of activation (45.3 KJ/mole sucrose) of invertase from *Neurospora* (Metzenberg 1963). A low rate of fructose cleavage from raffinose may be a reflection of high activation energy of invertase with this trisaccharide.

Though the invertase activity at 2.5 to 100 mM sucrose substantially dropped at 65°C as compared to activity of the enzyme at 45° and 55°C, nevertheless

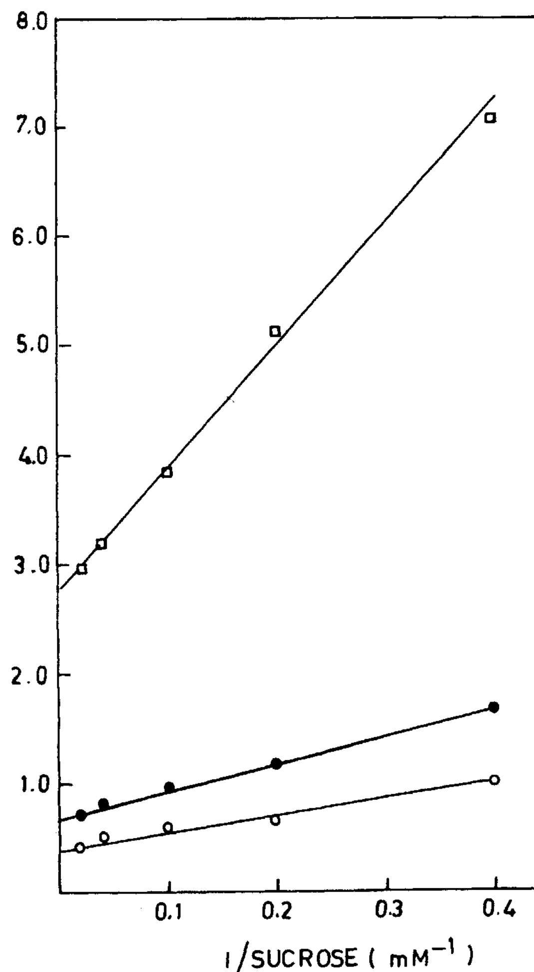

Figure 1 Inhibition of invertase with $HgCl_2$. (o), no $HgCl_2$; (●) 10 μM $HgCl_2$ and (□), 200 μM $HgCl_2$

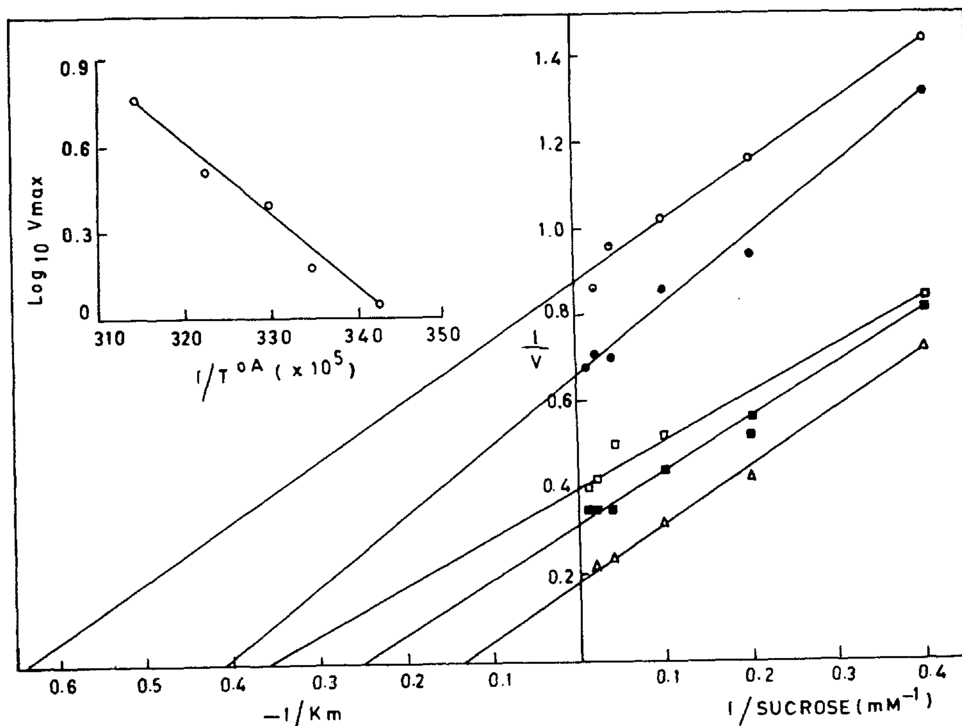

Figure 2 Lineweaver Burk plot showing the effect of temperature on V_{max} and K_m using sucrose as the substrate. (o), 19°; (●) 26°; (□), 30° (■) 37° and (Δ), 45°C. The plot in top left corner shows the relationship between log V_{max} of invertase and reciprocal of the absolute temperature. Velocity was defined as the *μmoles of reducing sugars formed* hr^{-1}

with increase in sucrose concentration from 5.0 to 100 mM, the activity of invertase at 65°C as compared to the activity at its optium temperature (i.e. 45°C) increased from 1.6 to 29.1% (table 3). Obviously, at high substrate concentration the enzyme seems to be present mainly in the form of enzyme-substrate complex and this complex appears to be more stable to heat as compared to free enzyme which mostly exists at very low substrate concentrations. Using raffinose as a substrate, the V_{max} increased till the temperature of the reaction mixture was 55°C. Invertase was heated with and without 50 mM sucrose or raffinose at 55°C for 30 min. These substrate concentrations are much higher than the K_m of invertase and, therefore, the active site of enzyme under these conditions is primarily occupied by the substrate. After the heat treatment, enzyme was made sugar free by passing through a sephadex G-25 column. Invertase which was heated in the absence of sucrose or raffinose showed significantly less activity as compared to activity of invertase heated in the presence of substrate (table 4). These data clearly showed that invertase-substrate complex is thermally more stable as compared to free invertase. Conceivably, the OH groups of the substrate enter into hydrogen bondings with invertase thus stability to enzyme is imparted against higher temperature.

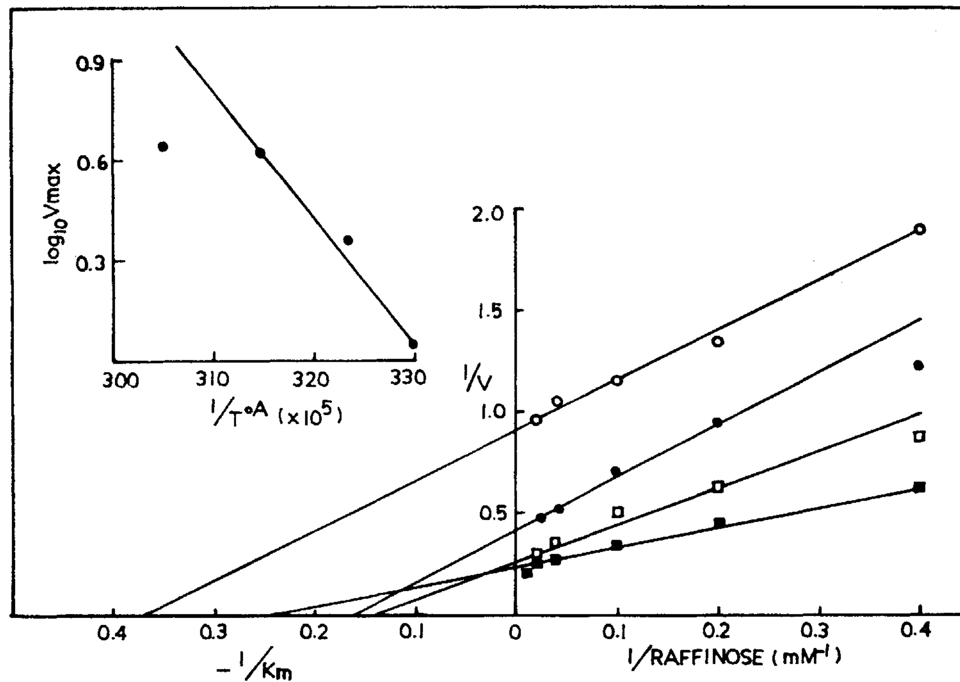

Figure 3 Lineweaver Burk plot showing the effect of temperature on V_{max} and K_m using raffinose as the substrate. (o), 30°; (●), 36°; (□), 45° and (■), 55°C. The plot in top left corner shows the relationship between log V_{max} and reciprocal of absolute temperature. Velocity was defined as the μ moles of reducing sugars formed hr^{-1}

Thermal stability of invertase was further studied by incubating the enzyme at different temperatures for various time intervals (table 5). Preincubation of enzyme for 60 min at 55°C resulted in only 6.2% of activity (table 5) against 80% of its activity when it was assayed without any preincubation (calculated from table 3). The invertase from *Neurospora* has a half life of about 6·7 min at 55°C (Metzenberg 1963) whereas in the present case even after 15 min incubation of invertase at 55°C only 45% denaturation occured (table 5). Apparently, the invertase from *Fusarium oxysporum* is much more stable at higher temperature as compared to invertase from *Neurospora*.

The presence of 10% glycerol or ethylene glycol or sorbitol in the reaction mixture did not protect invertase activity at 55°C (data not given). In this respect, the invertase from *Fusarium oxysporum* differs from those enzymes which are known to be protected by polyhydric alcohols against thermal denaturation (Gerlsma 1968, Gupta et al. 1990).

Secretion of mycelial invertase into the mediu[illegible] *F. oxysporum* upon grown on sucrose secreted on[illegible] negligible amount of invertase into the medium. Whe[illegible] grown on inulin, the maximum level of invertase in th[illegible] medium was 0.4 units/ml after 12 days of growth (tabl[illegible] 6). However, when inulin was added to the medium [illegible] days after of initiation of growth, there was enormous increase in the level of extracellular invertase which i[illegible]creased to 2.2 enzyme units/ml after 9 days of growt[illegible]. Though the reasons for such increased secretion of in[illegible]vertase into the medium cannot be speculated at pre[illegible]sent, however, these findings could be of industrial im portance if similar phenomenon were shown to be operative in other micro-organisms, especially those producing high amounts of invertase.

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