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Purification and Characterization of Invertase Enzyme from Sugarcane

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Abstract: Sugarcane invertase was purified from mature sugarcane stem tissue to an electrophoretically homogenous state, by successive ion-exchange chromatography on DEAE-Cellulose and CM-Cellulose column chromatography. The molecular weight of purified invertase enzyme was determined 218 kDa on SDS-Polyacrylamide slab gel electrophoresis. When the enzyme was characterized, it was found that the sugarcane invertase is glycoprotein in nature and it contained 7.29% sugar. The height enzyme activity was found at pH 7.2 and 60°C temperature. The K_m value of the enzyme was 8.0 mM. NaCl, $MnCl_2$ increased invertase activity remarkably and KCl increased slightly while $MgCl_2$, $CaCl_2$ increased moderately. $HgCl_2$ has completely and $FeCl_2$ showed strong inhibitory effects on enzyme activity while EDTA and acetic acid have remarkable inhibitory effects on invertase activity. $CuCl_2$, $ZnCl_2$ and $CdCl_2$ decreased invertase activity moderately. $AgNO_3$, $AlCl_3$, Glucose and Tris showed less inhibitory effects on invertase activity.

Key words: Sugarcane, purification, characterization, invertase, activity

INTRODUCTION

Sugarcane (*Saccharum officinarum*) belongs to the genus *Saccharum* in the family Gramineae. Sugarcane is only the sugar producing crop in Bangladesh and plays very important role in national economy. Sucrose accumulation is infect a highly sensitive response to discrete enzyme behavior. Sucrose, commonly known as table sugar, is a disaccharide composed of an alpha-D-glucose molecule and a β -D-fructose molecule linked by an alpha-1, 4-glycosidic bond. When this bond is hydrolyzed by invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) or sucrose synthase (UDP glucose: D-fructose 2-D-glucosyltransferase, EC 2.4.1.13) an equimolar mixture of glucose and fructose is generated. This mixture of monosaccharides is called invert sugar. In spite of the potential importance of invertase in the accumulation of reducing sugars, it has not been examined in sugarcane even though it has been detected in several other plant tissues[1-6]. Plant invertases are classified as acid, neutral and alkaline invertases on the basis of their pH-optima. The two enzymes can co-exist in the same tissue and they are especially active in tissues undergoing rapid growth and development[7]. They are also classified as soluble and bound invertase on the basis of their intracellular localization[8]. Invertase is mainly used in the food (confectionery) industry where fructose is preferred over sucrose because it is sweeter and does not crystallize as easily. This study describes the purification and characterization of invertase enzyme from variety Isd-28 of sugarcane.

MATERIALS AND METHODS

Sugarcane variety of Isd-28 was collected from Bangladesh Sugarcane Research Institute, Ishurdi, Pabna, Bangladesh. Invertase was purified from sugarcane juice. All operations were carried out at 4°C. DEAE-Cellulose and CM-Cellulose were purchased from Sigma Chemical Co. U S A. All other reagents used were of analytical grade.

Purification of invertase: The mature sugarcane stem tissue (200 gm) was cut into small pieces and grind in a mortar with pestle in cold 0.1M Phosphate buffer (pH 7.2) and finally crushed into past using a homogenizer. The suspension was then filtered through few layers of cheese cloth. The filtrate was collected and clarified further by centrifugation at 8,000 rpm for 15 mins at 4°C. The supernatant was concentrated to about 1/8th of the original volume by commercial sucrose. It was then dialyzed against 0.1M Phosphate buffer (pH 7.2) for 24 h

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and centrifuged again at 10,000 rpm for 8 min to remove the insoluble materials present. The clear supernatant obtained was used as crude enzyme extract.

Crude enzyme extract was loaded onto the DEAE-Cellulose column (1.5X20 cm) which was previously equilibrated with 0.1M Phosphate buffer (pH 7.2). The separation of protein from DEAE-Cellulose column was achieved by linear (0-0.5 M) NaCl gradient. Three milliliters fraction of elute were collected by an automatic fraction collector at the flow rate of 30 ml h^{-1}. Invertase activity and protein concentration of all these fractions were measured.

Active fractions from DEAE-Cellulose chromatography was collected and dialyzed against the 0.1M Phosphate buffer (pH 7.2) for 20 hs. The dialyzed sample was centrifuged and supernatant was collected and loaded onto the CM-Cellulose column (1x17 cm) pre-equilibrated with 0.1M Phosphate buffer (pH 7.2). The separation was achieved by linear (0-0.5 M) NaCl gradient. Three milliliters fraction of elute were collected by an automatic fraction collector at the flow rate of 20 ml h^{-1}. Invertase activity and protein concentration of all these fractions were measured.

Protein concentration was determined following the method of Lowry *et al.*[9]. Invertase activity was assayed as described by the method of Mahadevan and Sridhar[10], using sucrose as substrate. The liberated reducing sugars was estimated by dinitrosalisylic acid method[11]. Purity of the enzyme containing fraction at each step was monitored by SDS-Polyacrylamide slab gel electrophoresis described by Laemmli[12].

Characterization of invertase: Molecular weight of purified sugarcane invertase was determined by SDS-Polyacrylamide slab gel electrophoresis according to the method of Laemmli[12] (Fig. 3). Catalase (MW=240,000), β-amylase (MW=200,000), β-galactosidase (MW=116,000), Egg albumin (MW=45,000) and Trypsin (MW=20,000) were used as reference proteins. The molecular weight of the purified invertase was determined from a standard curve (Fig. 4). This standard curve was constructed by plotting the log of molecular weight of standard proteins against their electrophoretic mobility.

Activity of invertase at different pH values (2-10) was measured at 37°C following the procedure as described by Mahadevan and Sridhar[10]. Sucrose solution (2.5%) was made in the above mentioned buffer of different pH and were used as substrates for invertase. Optimum temperature of invertase activity was also measured at different temperature (10-90°C) using 0.1 M phosphate buffer (pH 7.2) following procedure as described by Mahadevan and Sridhar[10].

Test of glycoprotein and estimation of sugar in invertase was conducted by the method of Dubois *et al.*[13]. Effects of metallic salts on invertase activity were determined in presence of various concentrations of metallic salts and compounds following procedure as described by Mahadevan and Sridhar[10].

RESULTS AND DISCUSSION

Purification of invertase enzyme: Invertase from sugarcane juice was successively purified by DEAE-Cellulose column chromatography and CM-Cellulose column chromatography. Fig. 1 shows the elution profile of ion-exchange chromatography of crude enzyme extract on DEAE-Cellulose column. It was found that the crude enzyme extract from sugarcane stem was eluted into one major peak (F-1) and one minor peak (F-2). The major fraction F-1 was eluted by the buffer contained the invertase activity while minor fraction eluted by the buffer with continuous gradient of NaCl had no invertase activity. The fraction F-1 pooled separately and their purity was checked by the SDS-Polyacrylamide slab gel electrophoresis (Fig. 3). F-1 fraction gives more than one band indicating that it contains some other proteins (Fig. 3). Then protein of F-1 fraction dialyzed against 0.1 M phosphate buffer (pH 7.2) for overnight and charged onto CM-Cellulose column previously equilibrated with same buffer for further purification.

The proteins of the F-1 fraction were eluted with a gradient of NaCl (0-0.5 M) in the same buffer. The elution profile of F-1 fraction is shown in Fig. 2. The chromatography yielded only one peak (F-1a), which was eluted by the buffer and contained invertase activity. The active fraction F-1a, from CM-cellulose chromatography

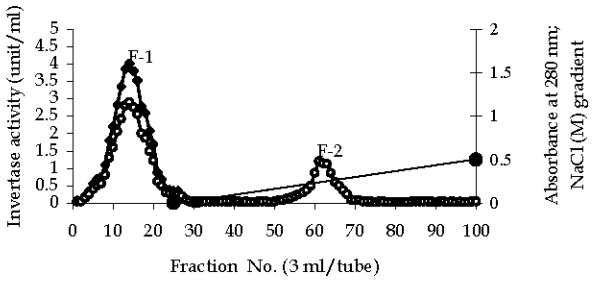

Fig. 1: Elution profile of DEAE-Cellulose ion-exchange column chromatography at 4°C. Invertase was eluted with a linear, 0-0.5 M NaCl gradient. Protein concentration was measured by absorption at 280 nm. Tube No. 1-25 showed the first peak (F-1) and Tube No. 50-70 showed the second peak (F-2). Enzyme activity shows Tube No. 1-25 (F-1). Symbols: (○), OD at 280 nm; (◆), Enzyme activity and (●) NaCl gradient

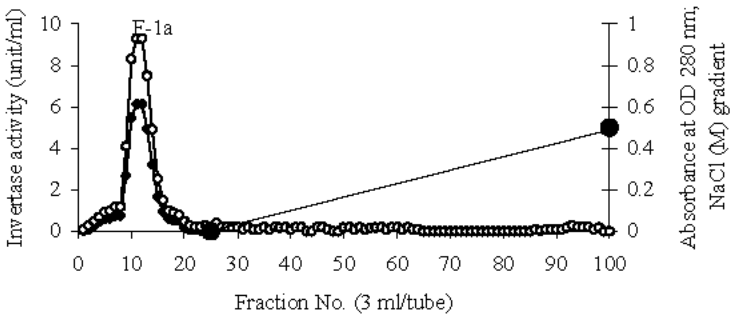

Fig. 2: Elution profile of CM-Cellulose ion-exchange column chromatography at 4°C. Protein concentration was measured by absorption at 280 nm. Tube No. 1-20 show only one peak (F-1a) and also shows enzyme activity. Symbols: (○), OD at 280 nm; (◆), Enzyme activity and (●) NaCl gradient

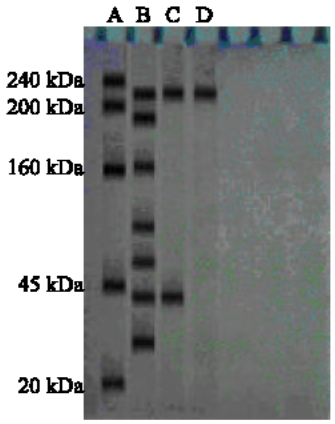

Fig. 3: Molecular weight determination by SDS-Polyacrylamide slab gel electrophoresis. Lane A, Molecular mass markers: (From top) Catalase (240 kDa), ß-amylase (200 kDa), ß-galactosidase (116 kDa), Egg albumin (45 kDa) and Trypsin (20 kDa); Lane B, Crude enzyme extract; Lane C, Fraction from DEAE- Cellulose column; Lane D, Fraction from CM-Cellulose column

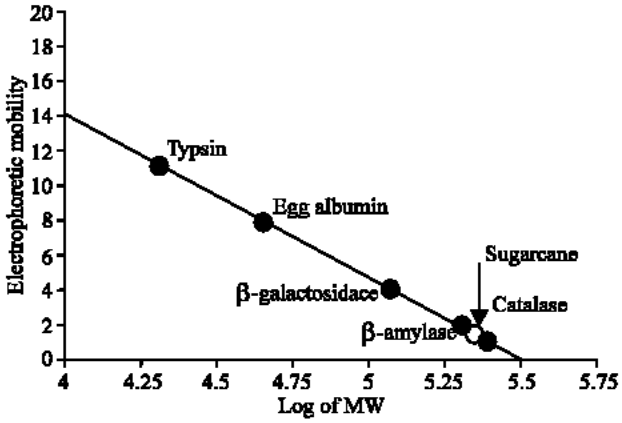

Fig. 4: Standard curve for the determination of molecular weight of enzyme by SDS-Polyacrylamide slab gel electrophoresis

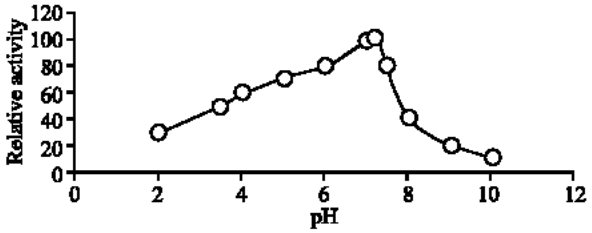

Fig. 5: Effects of pH on activity of sugarcane invertase enzyme

give only one band in the SDS polyacrylamide slab gel electrophoresis (Fig. 3). So this fraction might contain pure enzyme.

Table 1 showed the data on purification steps of invertase from sugarcane juice. The specific activity of the enzyme was found to be increased in each step. Although the yield was low, the purification was achieved about 18 folds for sugarcane invertase. The decrease in yield may be due to denaturation of the enzyme during lengthy purification procedures or for some other reasons. Porntaveewat *et al.*[14] and Konno *et al.*[15] found the recovery about 19.5% from Muscat Baily grapes and about 14.1% from the tomato fruit, respectively.

Characterization of invertase: The purified enzyme was a glycoprotein in nature, as it gave yellow-orange color with the phenol sulphuric acid test[16]. The percent of sugar present in the glycoprotein was calculated from the standard graph of glucose and it was found that the sugarcane invertase contained 7.29% sugar. Geracinmo *et al.*[17] reported that the purified potato invertase was a glycoprotein with 10.9% carbohydrate. Other

Table 1: Summary of purification of invertase from sugarcane

Steps	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (Units mg^{-1})	Yield (%)	Purification fold
Crude	162	145.80	417.60	2.86	100.00	1.00
DEAE	39	1.95	86.66	44.44	20.75	15.52
CM	21	0.59	29.87	50.79	7.15	17.73

Table 2: Effects of various metallic salts and chemicals at concentration 0.005 M on invertase activities

Test salts/chemicals	Relative activity (%)
None	100.00
$MgCl_2$	115.00
KCl	110.82
NaCl	120.00
$MnCl_2$	120.00
$CaCl_2$	114.24
$HgCl_2$	1.02
$CuCl_2$	30.00
$FeCl_2$	20.25
$ZnCl_2$	68.27
$CdCl_2$	55.26
$AgNO_3$	80.00
$AlCl_3$	78.00
EDTA	52.74
Glucose	76.00
Acetic acid	45.30
Tris	89.00

investigators using similar methods have also reported plant invertase as glycoprotein[18,19]. Molecular weight of purified invertase was determined 218 kDa in sugarcane variety Isd-28. Similar result (207 kDa) was reported by Sikder[21] in the sugarcane. Vorster and Botha[22] found that sugarcane neutral invertase is a homotetramer with a native molecular weight of 240 kDa. They also stated that sugarcane neutral invertase was electrically active in 240 kDa, 120 kDa and 60 kDa. Molecular weight of cherry fruit invertase was reported 400 kDa by Krishan and Pueppke[2]. Singer and Knox[23] observed an apparent molecular weight of 450 kDa for lily pollen invertase. Krishan *et al.*[19] found that acidic invertase from wheat coleoptiles has an apparent molecular weight of only 158 kDa. Chungliang *et al.*[24] reported that molecular weight of alkaline invertase from etiolated rice seedling shoots was 240 kDa. Wang[25] found that extracellular invertase form yeast culture (Saccharomyces *cerevisiae* or *Saccharomyces carlsbergensis*.) had a molecular weight of 270 kDa.

The purified enzyme gave maximum activity at pH 7.2 (Fig. 7). It also found that the activities were found to be decrease gradually in the acidic pH but rapidly in the alkaline pH. These observations indicate that the purified enzyme was relatively stable in the acidic to neutral pH region than that of alkaline region. Vorster and Botha[22] reported that the optimum pH of sugarcane neutral invertase was 7.2, which is identical to the present investigate. The similar result was also reported by Hatch *et al.*[26]. Chungliang *at el.*[24] reported that the optimum pH of alkaline invertase from etiolated rice seedling shoots was 7.0.

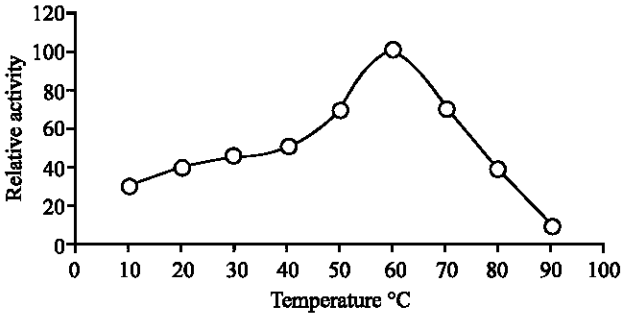

Fig. 6: Effects of temperature on activity of sugarcane invertase enzyme

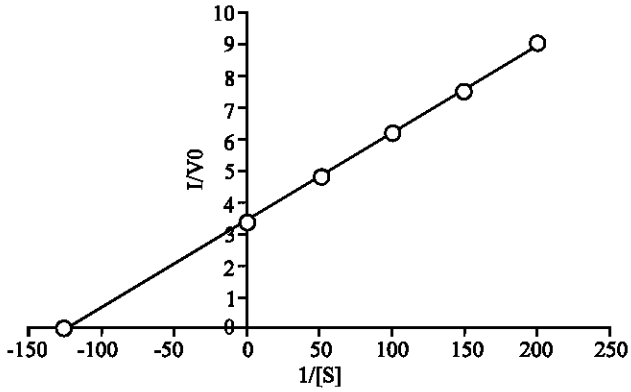

Fig. 7: Line weaver Burk double reciprocal plot for the determination Km Value of purified sugarcane invertase enzyme

The activity of invertase was increased gradually with temperature and the maximum activity was observed at 60°C (Fig. 8). Further, the activities gradually decreased with the rise of temperature and there was a drastic drop in the enzyme activity above 70°C. Nakanishi *et al.*[23] reported that the optimum temperature of wine and grape juice invertase at 75°C. Porntaveewat *et al.*[14] reported the higher optimum temperature of Muscat Baily invertase and it was 80°C. Krishan and Pueppke[2] reported that cherry fruit invertase had an optimum temperature of 60°C.

Invertase actively hydrolyzed sucrose. Kinetic constant, K_m the invertase was calculated from double reciprocal (1/V versus 1/S) Line weaver Burk plots (Fig. 7). The purified enzyme had a K_m value of 8 mM for sucrose Vorster and Botha[22] reported that the K_m value of sugarcane invertase was 9.8±0.37 mM, while Hatch

et al.[26] found that the K_m value of sugarcane invertase was 8 mM using sucrose as substrate which is identical with our result. Vitolo and Yasuda[28] reported that the value of K_m of soluble invertase was 7.4 using sucrose as substrate.

The effects of metallic salts and chemicals on the activity of invertase from sugarcane juice were presented in the Table 2. In this Table about 15, 10, 20, 20 and 14% activity was increased in presence of (0.005 M) $MgCl_2$, KCl, NaCl, $MnCl_2$ and $CaCl_2$, respectively. Nakanishi *et al.*[27] reported that effect of 10^{-2} M of KCl, NaCl, $MnCl_2$, $MgCl_2$ and $CaCl_2$ on the activity of both wine and grape juice invertase enzyme were negligible.

The activity of invertase was almost completely inhibited by 0.005 M $HgCl_2$. Vorster and Botha[28] reported similar inhibitory effects of $HgCl_2$ on the activity of sugarcane neutral invertase. It was also found that 0.005 M $FeCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$, $AlCl_3$, EDTA and $AgNO_3$ reduced activity of invertase approximately 80, 73, 32, 45, 22, 48 and 20%, respectively. Similar results were also noted by Vorster and Botha[28], Nakanishi *et al.*[27], Ishimoto and Nakamura[29] and Lijung *et al.*[20].

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