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CHARACTERIZATION AND INHIBITION OF INVERTASES IN SUGAR CANE JUICE*

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Key Word Index—*Saccharum officinarum*; *Gramineae*; sugar cane; invertase inhibition; sucrose inversion; isoenzymes.

Abstract—$(NH_4)_2SO_4$ fractionation followed by Sephadex G-200 chromatography of sugar cane juice gave an acid invertase with MW of 380000 and 23.5% carbohydrate and a neutral invertase with MW of 66000 and 22% carbohydrate. For acid invertase, K_m is 2.8 mM and V_{max} is 2.7 μmol sucrose hydrolysed/hr/mg protein. For neutral invertase, K_m and V_{max} are 0.32 mM and 2.8 μmol hydrolysed/hr/mg protein, respectively. Inhibition of both invertases by either lauryl sulfate or metasilicate is not competitive.

INTRODUCTION

The control of sucrose inversion in harvested sugar cane and in milled juice has been a challenge to chemists and sugar technologists. Present methods of inversion control include spraying of harvested cane with water and use of bactericides during processing, as well as prompt harvesting and milling of mature cane [1]. Another approach is through inhibition of invertase (β-fructofuranosidase, EC 3.2.1.26).

At least two invertases are present in sugar cane on the basis of pH optima and cellular localization and their physiological functions have been elucidated [2]. Two soluble isoenzymes have been identified with pH optima of *ca* 5.3 and 7.0 [3–5] and their K_m values on sucrose were determined [3]. For the acid enzyme, researches on purification and substrate specificities also have been conducted [6–8]. Some invertase inhibitors have been studied including lead, mercury, arsenic, tungsten [4], Tris [3] and sodium metasilicate [8]. The last compound completely inhibited purified invertase at 2–3 mM inhibitor concentration and also reduced the activity of several hydrolytic and oxidative enzymes in sugar cane [9]. However, there has not been any determination of the MW of invertases nor kinetic inhibition studies on the isoenzymes.

The present study deals with the purification of sugar cane invertase and determination of MWs and carbohydrate contents of the acid and neutral isoenzymes. Values of K_m and V_{max} were calculated as well as constants for inhibition by lauryl sulfate and metasilicate.

RESULTS AND DISCUSSION

The filtered sugar cane juice has sp. act's of 0.53 and 0.37 units/mg protein at pH 5.3 and pH 7, respectively. One unit of invertase activity is defined as that amount of enzyme which hydrolyses one μmol of sucrose/hr at 37°. After $(NH_4)_2SO_4$ fractionation the sp. act. of each invertase was increased 6-fold. Sephadex G-200 chromatography resulted in an overall 73-fold purification for each invertase. The ratio of the sp. act. of acid and neutral invertases was equal to 1.4 for the three enzyme preparations. Alexander [7] has reported 15-fold and 80-fold increases in acid invertase activity after $(NH_4)_2SO_4$ precipitation and Sephadex gel filtration, respectively. The elution profile of invertase on Sephadex G-200 is shown in Fig. 1. Two major peaks were obtained, namely A_1 and N_1, which exhibited acid and neutral invertase activities, respectively. A_1 also showed activity at pH 7 which was about half that at pH 5.3, while N_1 had activity at pH 7 of *ca* 25% that at neutral pH. A_1 corresponds to a narrow protein band while N_1, together with smaller neutral invertase peaks, correspond to a broad protein band.

Acid invertase A_1 has a MW of 380000 while the neutral invertase fractions have MW of 66000 (peak N_1),

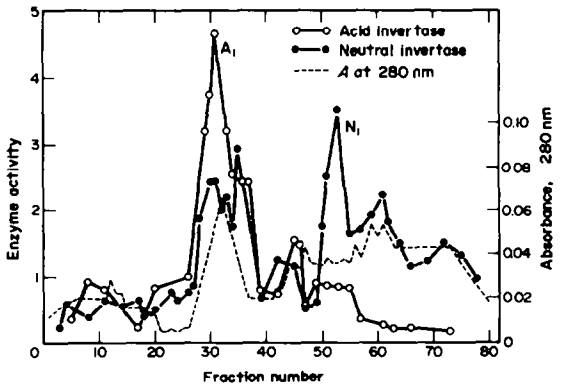

Fig. 1. Elution diagram of sugar cane invertase on Sephadex G-200 gel chromatography.

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35000 and 15000. Two small peaks, with almost coincident maxima in acid and neutral invertase activities, were also obtained whose MWs are 125000 and 160000. These MW values have estimated uncertainty of 15%. It can be seen in Fig. 1 that peak A_1 has a shoulder. Furthermore, the neutral invertase activity corresponding to this peak showed more than one maximum. Although the presence of more than one enzyme fraction cannot be discounted, it is more probable that A_1 corresponds to a single protein molecule. This is supported by the fact that the MW of the neutral invertase maxima in band A_1 are the same within experimental error.

The neutral invertase peaks within the broad protein band have MWs which are related to one another by whole numbers, within experimental error. This implies an aggregation phenomenon such that a monomer with MW of 15000 could form a dimer, tetramer and decamer with MWs of 35000, 66000 and 160000, respectively. A similar aggregation phenomenon of sugar cane invertase was reported by Maretzki and Alexander [6] when the ionic strength of the medium was increased. An association–dissociation equilibrium also applies to yeast invertase whose MW is 270000 [10]. The acid and neutral invertase fractions A_1 and N_1 were found to contain 23.5 and 22.0% carbohydrate, respectively, and suggests that these isoenzymes are glycoproteins. A similar observation applies to yeast external invertase which contains 50% carbohydrate [10–12] and *Neurospora crassa* invertase which has 14% carbohydrate [13].

For the $(NH_4)_2SO_4$-purified invertases, values of K_m and V_{max} which were determined using the methods of Lineweaver and Burk [14], Hofstee [15] and Eisenthal and Cornish-Bowden [16], are given in Table 1. For acid invertase, the 3 methods gave mean values for K_m and V_{max} of 2.8 mM and 2.7 μmol sucrose hydrolysed/hr/mg protein with average deviations from the mean of less than 10%. For neutral invertase, average values of 0.32 mM and 2.8 were obtained for K_m and V_{max}, respectively. The average values are quite close to the results obtained according to the method of Eisenthal and Cornish-Bowden which has been found to be quite accurate [17]. However, the K_m values obtained in the present study are smaller than those reported in the literature [3].

Lauryl sulfate inhibition of acid and neutral invertases gave straight lines when plotted according to the method of Lineweaver and Burk [14]. The lines were parallel with identical slopes within 15% which could indicate uncompetitive inhibition. However, more detailed studies using highly purified enzymes are needed in order to see if a more complicated type of inhibition is involved. Uncompetitive inhibition by lauryl sulfate is somewhat difficult to rationalize in molecular terms since this detergent binds to a variety of proteins and enzymes [18, 19]. In any case, invertase inhibition by lauryl sulfate was clearly *not* competitive.

Table 1. Estimation of K_m and V_{max} of acid and neutral invertases using three methods of fitting the Michaelis–Menten equation

Method	K_m (mM)		V_{max} (μmol sucrose hydrolysed/hr)	
	acid	neutral	acid	neutral
Lineweaver and Burk	2.78	0.32	2.64	2.19
Hofstee	3.25	0.33	2.86	2.85
Eisenthal and Cornish-Bowden	2.60	0.30	2.70	2.86

The inhibition of both invertases by metasilicate was found to be noncompetitive and implies inhibitor binding to both free enzyme and enzyme-substrate complex. The slopes of the straight lines in the Lineweaver–Burk plots greatly exceeded the estimated uncertainty of 20%. The constants for noncompetitive inhibition K_{ii} by metasilicate of the acid and neutral invertases were 0.96 mM and 0.28 mM, respectively. The K_{is} values were 1.22 mM and 0.067 mM, respectively, for the acid and neutral enzymes. K_{ii} and K_{is} are the inhibitor concentrations which double the intercept and slope, respectively, in the Lineweaver–Burk plots [20]. For acid invertase, K_{ii} and K_{is} differ by only 27% which is slightly greater than the experimental error. However, for neutral invertase K_{ii} is *ca* four times K_{is} and implies that metasilicate is much more effective in increasing the slope than the intercept in the double-reciprocal plots. Assuming uncompetitive inhibition by lauryl sulfate, the inhibition constant was calculated to be 0.54 and 12 mM for the acid and neutral enzymes, respectively. These values indicate that lauryl sulfate has a greater affinity for acid invertase than for the neutral enzyme while metasilicate binds to the latter more strongly.

On the basis of the inhibition results of the present study, prospects look bright in the use of lauryl sulfate for inversion control in milled juice and, perhaps, in harvested cane. Previous results from our laboratory [5] show complete inhibition of both invertases in filtered juice by 12 mM lauryl sulfate and 42 mM metasilicate. $(NH_4)_2SO_4$ purification reduced these concentrations to 4 and 20 mM for lauryl sulfate and metasilicate, respectively. Commercial detergent 'Tide' was also inhibitory to cane invertases, especially after enzyme purification.

EXPERIMENTAL

Enzyme purification. Sugar cane crusher juice, pH 4.4, was obtained from the Canlubang Central, Laguna from mature stalks of CAC 57-11, CAC 57-50 and Phil 56-226 cane varieties. It was chilled to 2°, passed through 5 layers of muslin cloth and centrifuged at 3500 rpm for 10 min. $(NH_4)_2SO_4$ addition and centrifugation were done twice in order to obtain the 20–40% $(NH_4)_2SO_4$ ppt. This was dissolved in pH 5.5 buffer (0.1 M Na citrate) and dialyzed against the same buffer for at least 6 hr at 3°. Dialyzate was placed on a Sephadex G-200 column (2.5 × 55 cm) which had been equilibrated with the pH 5.5 buffer and eluted with the buffer to which was added Na azide (final concn, 0.02%) as bactericide. Fractions (3.5 ml) were collected at 28° and analyzed for acid and neutral invertase activities and A at 280 nm. MWs of the invertase isoenzymes were determined using the method of ref. [21] using as reference proteins cytochrome *c*, α-chymotrypsinogen, β-galactosidase, glutamate decarboxylase, aldolase, BSA and ovalbumin. MW values given in refs [21] and [22] were used.

Enzyme assay. Acid and neutral invertase activities were determined at pH 5.3 (0.1 M Na-citrate buffer) and pH 7 (0.2 M Na Pi buffer), respectively, from the amount of reducing sugars produced from sucrose. Reaction mixture contained 1 ml filtered cane juice or dialyzed enzyme, 1 ml 2% sucrose soln, 1.5 ml either pH 5.3 or pH 7 buffer, with or without inhibitor, in a total vol. of 4.5 ml and incubated for 1 hr at 37°. In control runs, sucrose was omitted in the mixture. Sucrose was determined according to ref. [23] and reducing sugars were analysed using the dinitrosalicylate method [24].

Kinetic studies. Initial velocities were determined from acid and neutral invertase assay on the $(NH_4)_2SO_4$-purified enzyme after 0.5, 1, 2, 3 and 4 hr of incubation at 37°. Inhibition studies were done using 1 hr incubation which gave the same initial velocities.

Protein and carbohydrate determination. Soluble protein was analyzed using the procedure of ref. [25] with BSA as standard. The carbohydrate content of the invertase was determined using the method of ref. [26].

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