J. Enzyme Inhibition, 1992, Vol. 6, pp. 175–180
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SHORT COMMUNICATION

PURIFICATION AND CHARACTERIZATION OF INVERTASE INHIBITORS FROM *DIOSCOREA ROTUNDATA* TUBER

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(*Received 29 July 1992*)

Three invertase inhibitors (A), (B) and (C) from *Dioscorea rotundata* tuber were resolved on DEAE-cellulose ion exchanger. Two of the inhibitors, (B) and (C), were proteins and homogenous on polyacrylamide gels M_r 21,000 ± 85 and 26,982 ± 40/36,307 ± 50 respectively. The inhibitors (B) and (C) were inactivated at 60°C and had activity-pH optima at 5.2 and 6.4 respectively. (B) and (C) were non competitive inhibitors of invertase from yam and other sources.

KEY WORDS: Invertase inhibitors, *Dioscorea rotundata* tuber.

INTRODUCTION

The evidence of an invertase inhibitor in potato tuber was first observed by Schimmer and Rachel[1] and subsequently confirmed by Pressey.[2] In the course of our work to purify the enzyme from yam (*Dioscorea rotundata*) tubers, we observed a consistent decrease in its activity despite improved protocol on purification. We thus assumed the presence of an endogenous inhibitor.[1]

Previously, the existence of a trypsin inhibitor from *Dioscorea alata* has been reported.[3] We report here the purification and characterization of three invertase inhibitors from *Dioscorea rotundata* tuber.

MATERIALS AND METHODS

Materials

Yam tubers: Freshly harvested yam tubers (*Dioscorea rotundata*) were obtained from the University farm (A.B.U. Nigeria).

All reagents used were of analytical grade and purchased from Sigma Company.

Extraction of Invertase

Peeled yam tuber (500 g) was homogenized in a blender at low speed, to minimize inactivation of the inhibitor, and the homogenate was transfered onto 300 ml of phosphate buffer (0.025 M, pH 7.4) and filtered by suction. The filtrate was rinsed with about

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2.5 ml of 1 M Na_2SO_3 and 2 g polyvinylpyrolidine and centrifuged at 5000 *g* for 10 min. The recovered supernatant was assayed for invertase and invertase inhibitor activities.

The supernatant was adjusted to pH 2.5 with HCl (0.1 M) and incubated for 60 min at 30°C to inactivate invertase, dissociate the invertase inhibitor complex, and recover inhibitory activity. The preparation was then centrifuged at 8000 *g* for 10 min. The clear supernatant was adjusted to pH4.0 and $(NH_4)_2SO_4$ saturation increased to 60%. The resultant precipitate was collected by centrifugation at 8000 *g* for 10 min and the pellet suspended in acetate (0.05 M) buffer pH 6.0 and dialysed overnight at 4°C against the same buffer.

About 6.0 ml of the dialysate was loaded on a pre-equilibrated DEAE-cellulose column (2.5 × 40 cm) and the column eluted by a linear gradient (0.01–0.22 M NaCl) using acetate buffer at a flow rate of 0.5 ml/min. Sixty fractions of 5 ml each were collected and assayed for invertase inhibitor and protein at 280 nm.

Active fractions were concentrated with $(NH_4)_2SO_4$ and dialysed overnight. The concentrate was used as a source of inhibitor.

Invertase Activity

The invertase enzyme was assayed as described by Colowick and Kaplan.[4] Protein concentrations were determined by the method of Lowry *et al.*[5]

Invertase Inhibition

The inhibitor activity was assayed at each purification step by incubating 0.1 ml of the inhibitor solution with 4.7 units of invertase for 60 min at 30°C in 2.0 ml acetate (0.1 M) buffer pH 4.7 and 2.0 ml sucrose (0.3 M). The reaction was terminated after 10 min by the addition of 3,5-dinitrosalicylate (DNS). The free enzyme was assayed by the previously described method. One unit of the inhibitor activity was defined as that amount of inhibitor that inhibits 1 unit of the enzyme.

Kinetic Analysis

Duplicate standard solutions (0.05–0.3 M) sucrose of 4.0 ml each were incubated with 0.2 ml of the inhibitor solutions for 10 min at 30°C. The reaction was commenced by the addition of invertase (4.7 units), terminated by the addition of DNS and the residual activity determined.

pH-Activity Profiles

The optimum pH for the inhibitor activity profile was determined using citrate and phosphate buffers over the range pH 2.6–8.4 using 0.5 ml of the inhibitor solutions (50 mg/ml).

Temperature Studies

Duplicate samples (0.5 ml) of the inhibitors (50 mg/ml) were separately incubated over the temperature range 25°C–50°C for 10 min and subsequently assayed for residual inhibitor activity. The thermostability of the inhibitors for different periods at 60°C was also determined.

TABLE I
Purificatin profile of invertase inhibitor conducted at 4°C

Purification step	Total activity μmol/min	Specific μmol/min/mg	Yield (%)	Purification (fold)
Crude extract	2400	0.81	100	1.0
Low pH precipitation	1700	2.38	64	2.94
50% $(NH_4)_2SO_4$ precipitation	1400	3.70	53	4.51
60% $(NH_4)_2SO_4$ precipitation	1100	6.50	42	8.02
DEAE-cellulose chromatography				
Peak A	100	–	4	–
Peak B	710	95	28	117.2
Peak C	210	83.0	8	101.2

Molecular Weight

The sub-unit molecular weights of the inhibitors were determined by SDS-polyacrylamide gel electrophoresis as described by Weber *et al.*[6]

RESULTS AND DISCUSSION

The purification profile for the invertase inhibitor is shown in Table I. Acidification of the crude extract to pH 2.5 resulted in the complete inactivation of invertase. However the inhibitor was not completely precipitated at this pH. On passing the

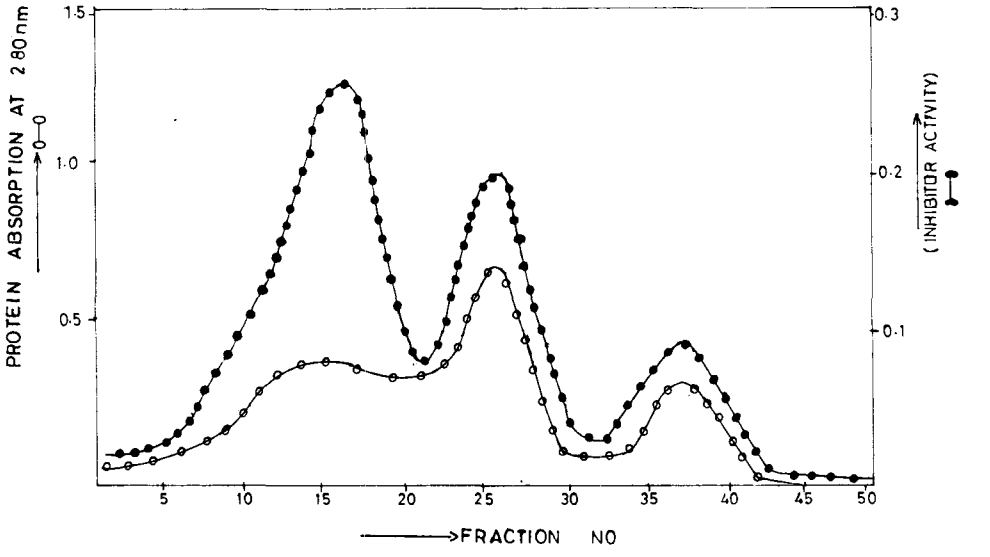

FIGURE 1 Elution profile of invertase inhibitors on DEAE-cellulose column (2.0 × 40 cm) at a flow rate of 2.0 ml/min and 4°C.

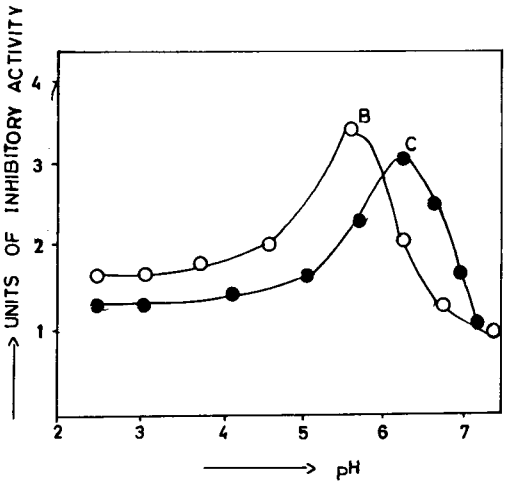

FIGURE 2 The pH-activity profiles of invertase inhibitors at 30°C.

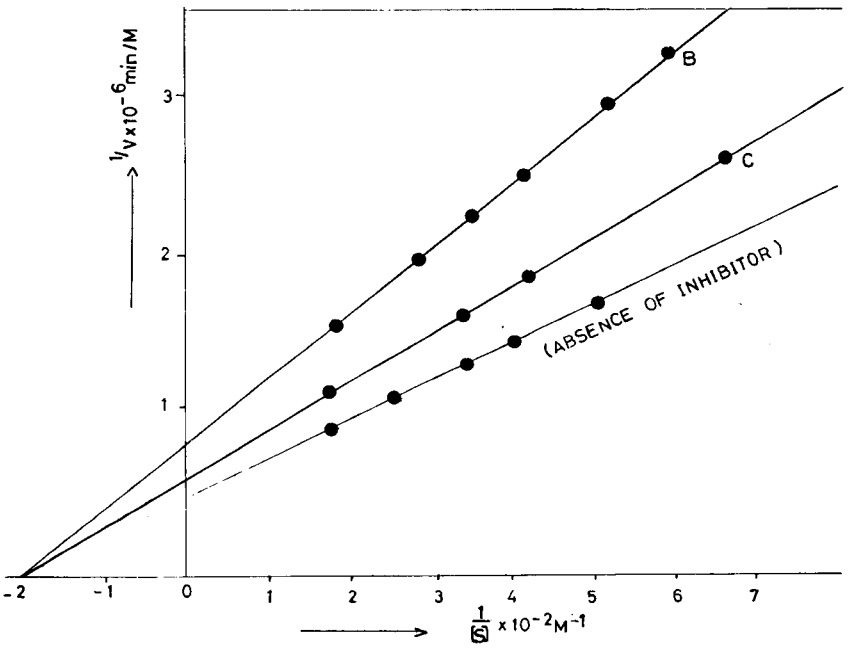

FIGURE 3 Lineweaver–Burk plots showing non competitive inhibition by (B) and (C) of isolated yam tuber invertase at 30°C.

TABLE II
Inhibition by the isolated inhibitors (B) and (C) of intertases from other sources

Source of invertase	Inhibition constant (K_i)			
	Yeast	*Neurospora crassa*	Potato tuber	Pig liver
Inhibitor	(K_i)	(K_i)	(K_i)	(K_i)
B	2.2×10^{-2} M	5×10^{-6} M	0.5×10^{-2} m	2.8×10^{-7} M
C	1.82×10^{-2} M	2×10^{-2} M	1.1×10^{-2} M	1.18×10^{-5} M

crude extract through the ion exchanger, three peaks exhibiting inhibitory activity were observed (Figure 1).

Fractions from these peaks were separately concentrated on Sephadex G-25 and subjected to electrophoresis on SDS polyacrylamide gels. While protein bands were observed for peaks (B) and (C), no such band was observed for peak (A) despite repeated electrophoretic runs on concentrated samples. It would appear that there are two proteinacious and one non protein inhibitor of invertase in *Dioscorea rotundata* yam species. The pH-inhibitor activity profiles for the two proteinacious inhibitors (B) and (C) showed optima at pH 5.2 and 6.4 respectively (Figure 2).

Previously, it has been shown that invertase has a pH-optima within the range 4.6–6.4.[7] It would seem that the maximal interaction between the enzyme and its inhibitor is physiologically favoured. Akazawa and Okamoto[9] have reported that the conversion of sucrose to starch in potatoes does not play a significant role in this plant storage organ during dormancy, but a break down of sucrose, which is a more common mechanism, is actively regulated at pH 4.7 by proteinacious inhibitor.

Data obtained from initial velocity studies was used to determine the type of inhibition (Figure 3). Both inhibitors exhibited non competitive inhibition patterns. The isolated inhibitors were tested on invertases isolated from yeast, neurospora, potato and pig liver. The inhibition binding constants determined from double

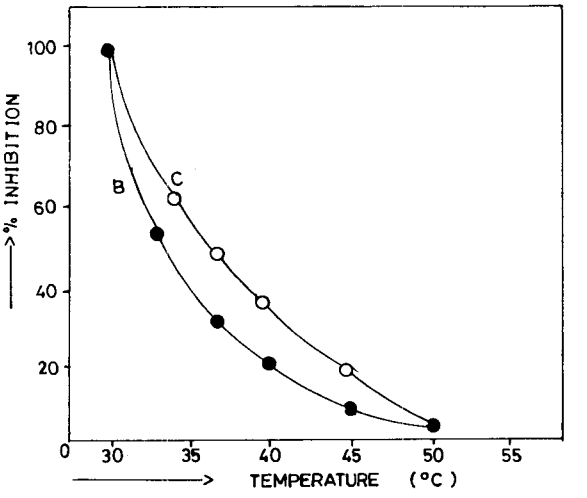

FIGURE 4 Plot of percentage inhibition as a function of temperature for inhibitors (B) and (C).

reciprocal plots of initial velocity data were comparatively lower on pig liver and neurospora invertases than on invertases from other sources. The patterns on inhibition were non competitive in all cases (Table II). This observation makes it imperative to analyse the levels of these inhibitors in yam peels frequently used in feed formulation.

Thermal stability studies conducted at different temperatures showed a progressive decrease in inhibitory activity (Figure 4). Both inhibitors lost their activity completely at 60°C after 15 min. The first order deactivation rate constants for inhibitors (B) and (C) at 50°C were $0.045\,min^{-1}$ and $0.056\,min^{1}$ corresponding to half lives ($t_{1/2}$) of 15.38 min and 8.02 min respectively. It is unlikely from this data that these inhibitors will have any effect in the human system since yams are usually boiled before consumption. However it is not the same case with yam peels used in animal feed. This further justifies the need for the analysis of yam peels.

Bands indicating the presence of the inhibitors were developed after SDS-PAGE. The inhibitor (B) had a single band corresponding to a molecular weight of 21,000 $\pm$ 50. The inhibitor (C) gave two bands corresponding to molecular weights of 26,982 $\pm$ 112 and 36,307 $\pm$ 50. This implies that the latter inhibitor could exist in a dimeric form with a minor and major polypeptide. The estimated molecular weight for potato invertase inhibitor is 17,000[7] and that from sugar beet is about 20,000.[9] The variation in inhibitor molecular weight could arise owing to the source and possibly the time required for isolation.

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