Pergamon

PII: S0031-9422(98)00204-0

Phytochemistry, Vol. 49, No. 3, pp. 651–655, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

PARTIAL PURIFICATION AND CHARACTERISATION OF SUGARCANE NEUTRAL INVERTASE

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(*Received in revised form* 2 *February* 1998)

Key Word Index—*Saccharum officinarum*; Gromicieae sugarcane; β-D-fructofuranosidase; invertase; neutral invertase; sucrose.

Abstract—Sugarcane neutral invertase (SNI) has been partially purified from mature sugarcane stem tissue to remove any potential competing activity. The enzyme is non-glycosylated and exhibits catalytic activity as a monomer, dimer and tetramer, most of the activity elutes as a monomer of native M_r ca 60 k. The enzyme displays typical hyperbolic saturation kinetics for Suc hydrolysis. It has a K_m of 9.8 mM for Suc and a pH optimum of 7.2. An Arrhenius plot shows the energy of activation of the enzyme for Suc to be 62.5 kJ mol^{-1} below 30° and −11.6 kJ mol^{-1} above 30°. SNI is inhibited by its products, with Fru being a more effective inhibitor than Glc. SNI is significantly inhibited by $HgCl_2$, $AgNO_3$, $ZnCl_2$, $CuSO_4$ and $CoCl_2$ but not by $CaCl_2$, $MgCl_2$ or $MnCl_2$. SNI showed no significant hydrolysis of cellobiose or trehalose.

INTRODUCTION

Neutral invertase (β-fructofuranosidase, EC 3.2.1.26) catalyses the hydrolysis of terminal non-reducing β-fructofuranoside residues in β-D-fructofuranosides like Suc. Neutral invertases have been neglected by biochemists in comparison to the acid invertases [1, 2]. The enzyme has however since been purified and characterised from at least four sources [3–6]. Sugarcane invertases are found in a number of isoforms, namely: neutral invertase; vacuolar acid invertase; cell-wall bound acid invertase and an apoplastic soluble acid invertase, for review see ref. [7, 8]. Previously SNI has been shown to be associated with mature stem tissue and sugarcane soluble acid invertase with immature, actively growing tissue [9–11]. A similar tissue distribution has been reported for other species, for review see ref. [1, 2].

A cycle of synthesis and degradation of Suc has been reported in sugarcane cell suspension cultures [12] and in the sugarcane stem [13–15]. Based on previously reported activites of SNI [9–11, 16, 17] the possibility exists that the enzyme is involved in Suc turnover in sugarcane stem tissue.

There has been no study to comprehensively characterise SNI or investigate possible mechanisms for fine control. Previous studies on SNI utilised dialysed crude extracts [18] or activity eluted after $(NH_4)_2SO_4$ precipitation from sugarcane crusher juice and subsequent size exclusion chromatography [19]. Here we report the characterisation of SNI.

RESULTS

Purification of neutral invertase

SNI was purified to a point of no competing β-fructofuranosidase activity (verified by assay at pH 5.5 and 7.2) by sequential $(NH_4)_2SO_4$ precipitation and anion exchange chromatography. A typical purification produced a 25 fold purification and 81% yield (Table 1). The majority of neutral invertase activity was precipitated at 20–60% $(NH_4)_2SO_4$ saturation while obtaining a reasonable degree of purification (Table 1). The ppt. was resuspended and desalted which resulted in a dilution of the activity but no significant loss. Neutral invertase activity eluted from the anion exchange column at *ca* 300 mM NaCl in the linear gradient. Desalting and concentration by ultrafiltration resulted in no significant loss of activity.

Kinetic properties

SNI displays a monophasic pH profile (Fig. 1) with a pH optimum of 7.2, showing half maximal activity at pH 6.4 and 8.2.

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Table 1. Purification table of a typical purification of neutral invertase from mature stem tissue.

Step	Total Protein(mg)	Total Activity (nkat)	Specific Activity (nkat mg protein^{-1}1)	Purification (fold)	Yield (%)
Crude extract	329	89.9	0.273	1	100
$(NH_4)_2SO_4$ ppt.	49.0	86.1	1.76	6	96
Anion exchange chromatography	10.5	73.2	6.97	25	81

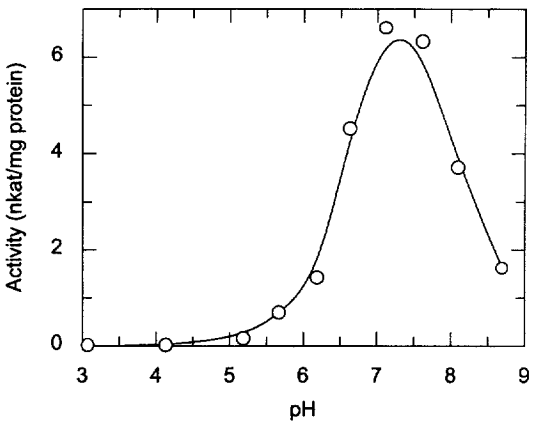

Fig. 1. The effect of pH on the maximum catalytic activity of sugarcane neutral invertase from stem tissue.

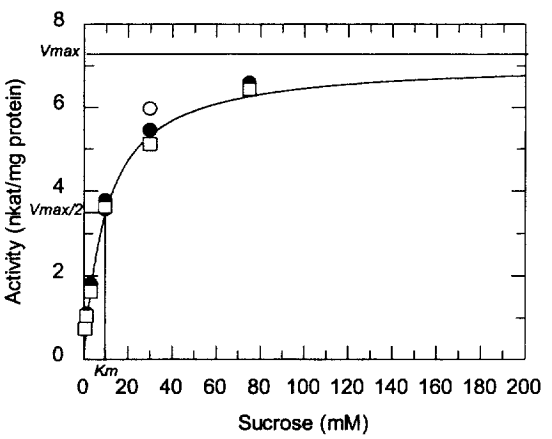

Fig. 2. Substrate saturation curve for sugarcane neutral invertase at pH 7.2. The curve fit was made to the average of three independent determinations of K_m and V_{max}.

SNI displays typical hyperbolic saturation kinetics ($h=1$) with Suc as substrate (Fig. 2). The K_m and V_{max} of SNI for Suc are 9.8 ± 0.37 mM and 7.32 ± 0.092 nkat.mg protein^{-1} respectively. An Arrhenius plot for SNI shows that it has an energy of activation for Suc of 62.5 kJ mol^{-1} below 30°. A transition occurs above 30°, resulting in an energy of activation of -11.6 kJ mol^{-1}. SNI shows no significant hydrolysis of cellobiose (Glc-β-1,4-Glc) or trehalose (Glc-α-1,1-α-Glc) at 100 mM.

Table 2. Inhibition of sugarcane neutral invertase by Tris and various metabolites.

Compound	Inhibition (%)
[1]Tris	85 ± 5.3
[1]Fru	37 ± 4.1
[1]Glc	27 ± 7.4
[2]Glc+Fru	14 ± 5.3
[3]PEP	1 ± 1.3
[3]Citrate	2 ± 1.5
[3]MgATP	0 ± 0.68
[3]MgADP	2.1 ± 3.5
[3]MgAMP	0.84 ± 1.7

± = s.d. of n = 3, [1] 10 mM, [2] 5 mM of each hexose, [3] 1 mM

Table 3. Inhibition of sugarcane neutral invertase by metal ions.

Compound (1 mM)	Inhibition (%)
$HgCl_2$	100 ± 7.3
$ZnCl_2$	99 ± 2.4
$AgNO_3$	98 ± 2.1
$CuSO_4$	97 ± 2.6
$CoCl_2$	32 ± 1.8
$CaCl_2$	0 ± 7.0
$MgCl_2$	0 ± 1.7
$MnCl_2$	0 ± 2.5

± = s.d. of n = 3

Inhibition

Tris significantly inhibits SNI at 10 mM (Table 2). SNI is inhibited by its products Fru and Glc at 10 mM, Fru is the more effective inhibitor of SNI Table 2). PEP, citrate, MgATP, MgADP or MgAMP do not significantly inhibit SNI at or near physiological concentrations (Table 2).

Inhibition by a number of metal ions at 1 mM is observed (Table 3). Almost complete inhibition is found with $HgCl_2$, $ZnCl_2$, $AgNO_3$ and $CuSO_4$. SNI is

partially inhibited by $CoCl_2$ while $CaCl_2$, $MgCl_2$ and $MnCl_2$ do not cause any significant inhibition.

Molecular properties and glycosylation state

Size exclusion chromatography resulted in peaks of SNI activity at *ca* 60 kDa, 120 kDa and 240 kDa, suggesting monomeric, dimeric and tetrameric forms of the enzyme. The majority of the activity eluted as the 60 kDa form. The SNI is probably non-glycosylated as it was not bound by Concanavalin A.

DISCUSSION

SNI is catalytically active in 240 kDa, 120 kDa and 60 kDa forms. Different forms of SNI have been reported previously [19]. The enzyme was found to elute from a gel filtration column at 160 kDa, 66 kDa, 35 kDa and 15 kDa, suggesting a monomer of M_r 15 k aggregating to form a dimer, tetramer and decamer. Most of the activity did, however, elute as the 66 kDa form. Smaller M_r forms of SNI found previously [19] could be hydrolysis products of a *ca* 66 kDa monomer. Evidence from other sources supports this hypothesis; the monomeric form of other neutral invertases are *ca* 57–65 kDa, being homotetramers of native M_r ca 238–260 k [3, 4, 6], or an octamer of 456 k [5].

SNI displays a monophasic pH profile, showing that there is no significant competing soluble acid invertase activity. The pH optimum of SNI is 7.2, which is consistent with that reported previously [18]. The pH optima of neutral and alkaline invertases purified from other sources vary from pH 6.8–8.0 [3–6]. SNI is not bound by concanavalin A, suggesting that it is not glycosylated. Previously it has been reported that SNI is glycosylated [19]. Other neutral invertases have been shown to probably be non-glycosylated [3–5]. In conjunction with a near neutral pH optimum this suggests that SNI has a cytosolic localisation.

The K_m of SNI for Suc is 9.8 ± 0.37 mM. The K_m of SNI for Suc has previously been reported to be 25 mM [18] and 0.32 mM [19]. The K_m for Suc of other neutral invertases are *ca* 10–20 mM [3–6]. Mature sugarcane stem tissue contains high levels of SNI [9–11, 16, 17]. The variation in calculated K_m values for Suc will not significantly affect the fact that the enzyme will be in substrate saturating conditions, based on the symplastic Suc concentrations in the sugarcane stem of 110–616 mM [20]. SNI is therefore likely to be partially responsible for the cycle of degradation and synthesis of Suc that has been found in sugarcane suspension cells [12], in the immature sugarcane stem [14] and in sugarcane tissue discs [13, 15]. Tris is a potent inhibitor of SNI has been found previously [18]. Inhibition by Tris is also a characteristic of other neutral invertases [3–6].

Suc hydrolysis by SNI obeys hyperbolic saturation kinetics, as would be expected from a single substrate enzyme catalysed reaction obeying simple Michaelis–Menten kinetics. SNI did not significantly hydrolyse cellobiose (Glc-β-1,4-Glc) or trehalose (Glc-α-1,1-α-Glc), indicating that it is a true β-fructofuranosidase. The energy of activation of SNI for Suc below 30° is the energy of activation for the enzyme-catalysed reaction in which temperature-dependent effects on the enzyme are negligible. The negative energy of activation observed above 30° reflects the significant contribution of temperature-dependent effects on the SNI which result in a decreased rate of the reaction [21]. A sugarcane leaf-sheath soluble acid invertase undergoes a similar transition at 30° [22]. This was postulated to be a mechanism for the control of *in vivo* enzyme activity when the temperature in the field exceeds 30°, which occurs in mid-summer.

SNI is almost completely inhibited by Hg^{2+}, Zn^{2+}, Ag^{+} and Cu^{2+} at 1 mM. Complete inhibition by Hg^{2+} is consistent with some reports on neutral invertases [3, 4] but not with [5] in which neither neutral nor alkaline carrot invertase were inhibited. This suggests a distinct difference between these enzymes due to the probable requirement of a reduced sulphydryl group in the neutral invertases inhibited by Hg^{2+}. Marked differences in the metal ion inhibition profiles of the neutral invertases suggests differences in structure related to their catalytic activity.

SNI is inhibited by its products Fru and Glc. At 10 mM Fru is 1.4 times more effective than Glc at inhibiting SNI. Product inhibition of neutral invertase has been demonstrated in other species [4–6]. Product inhibition of SNI provides a mechanism for the fine control of hexose production and Suc cycling mediated by SNI in the cytosol. Product inhibition of SNI would be significant at the symplastic hexose concentrations of internode 2 (Fru (55 mM), Glc (62 mM)) and internode 10 (Fru (11 mM), Glc (7 mM)), but not at the symplastic hexose concentrations of internode 20 (Fru (2 mM), Glc (0 mM)) and greater [20]. It must be noted that the symplast represents both the cytoplasm and vacuole, knowledge of cytosolic hexose concentrations in the sugarcane stem would enable a more accurate prediction of the effect of product inhibition on SNI *in vivo*.

MgATP, ADP and AMP did not effect any significant inhibition of SNI, unlike MgATP inhibiting *Daucus carota* L. neutral invertase [5].

SNI has been shown to be positively correlated with hexose levels in the sugarcane stem [10]. Invertase may be relatively more important in comparison to sucrose synthase (EC 2.4.1.13) as an enhancer of signals to sugar responsive genes, due to the production of two hexoses [23]. This is particularly relevant in the case of SNI due to the repression of soluble acid invertase expression by Glc [24–27], which is a product of invertase and not sucrose synthase.

SNI displays many of the characteristics common to other neutral invertases. Differences in metal ion inhibition profiles between neutral invertases indicate differences at the structural level. The involvement of SNI in Suc turnover will require the quantification of flux in sugarcane stem tissue. Sugarcane neutral

invertase may also play a key role in the control of hexose concentrations in the cytosol of sugarcane stem cells, thus effecting control over the expression of sugar responsive genes. The exact role of SNI remains unknown.

EXPERIMENTAL

Plant material

Neutral invertase was extracted from field grown mature sugarcane stalks of sugarcane variety NCo376. Stalks were cut in the early morning and used immediately.

Enzyme purification

Mature rindless stem tissue was homogenised in ice cold, degassed extraction buffer (10 mM HEPES pH 7.2, 10 mM DTT, 1.5% (wt/v) prehydrated PVPP, 0.2 mM PMSF, 2 mM benzamidine-HCl) at a ratio of 2:1 (v/wt), and filtered through 2 layers of nylon mesh. Insoluble material was removed by centrifugation at 10 000 *g* for 5 min at 4°. Protein was precipitated with gentle stirring by the addition of ground, solid $(NH_4)_2SO_4$ at 4° to 20% sat and centrifugation at 10 000 *g* for 20 min at 4°. The supernatant was further saturated with solid $(NH_4)_2SO_4$ to 60% and the precipitated protein collected by centrifugation at 10 000 *g*. The 20–60% $(NH_4)_2SO_4$ ppt. containing the majority of the SNI activity was resuspended in a minimal vol. of buffer A (25 mM HEPES, 0.02% $Na(N)_3$, pH 7.2) and desalted on a Sephadex G-25 column equilibrated with buffer A. The sample was applied to a DEAE-Sephacel anion exchange column pre-equilibrated with buffer A. The column was washed with buffer A until the $A_{280\,nm}$ had decreased to zero. Activity was eluted with a linear gradient of 0–500 mM NaCl in buffer A and 5 ml fractions were collected. Protein elution was monitored by $A_{280\,nm}$, fractions containing SNI were pooled. Pooled fractions were desalted and concentrated by ultrafiltration at 4° in buffer A.

Enzyme assays and protein determination

SNI was assayed at 30° in 50 mM HEPES, pH 7.2 and 125 mM Suc in a final vol. of 1 ml. The assay system was the same for the acid invertases except that a 50 mM Na-citrate/Na-Pi buffer at pH 5.5 was used. Reactions were stopped by a 2 min incubation at 90° and were then stored at −80°. Reducing sugars were measured using the method of ref.[28]. All reactions were shown to be linear with time. For neutral invertase kinetic constant determination, an appropriate amount of partially purified SNI was used. Suc concn was varied from 0–100 mM, K_m and V_{max} were determined by non-linear curve fitting. Inhibition by various metabolites and Tris was assessed by including them at various concns, metal ions were at a final concn of 1 mM in the same assay system. In the case of Glc inhibition, excess Glc was removed with glucose oxidase and catalase prior to Fru determination [28]. The combined inhibitory effect of glucose and fructose was determined by including ^{14}C-sucrose in the standard assay and subsequently quantifying the ^{14}C-reducing sugars by HPLC and inline isotope detection. The pH optimum was determined using the same assay conditions with a 25 mM Na-citrate/Na-Pi buffer (pH 4–9). The energy of activation was determined by measuring the rate of the enzyme catalysed reaction at various temp. between 20° and 40° in substrate saturating conditions at pH 7.2. Hydrolysis of cellobiose (Glc-β-1,4-Glc) or trehalose (Glc-α-1,1-α-Glc) by SNI was assayed under standard conditions with the sugars at a concn of 100 mM. Protein concn was determined by the method of ref.[29], using gamma globulin as a standard.

Native M_r determination

Native M_r was determined using a calibrated Biorad Econo Column (1.5 × 50 cm) packed with Sephacryl S300 (Pharmacia) to a bed height of 30 cm and equilibrated with elution buffer (HEPES pH 7.2, 200 mM NaCl, 0.02% $Na(N)_3$). 1 ml of the neutral invertase sample concentrated after the anion exchange purification step was applied to the size exclusion column and eluted at a flow rate of 0.5 ml min^{-1}. 0.6 ml fractions were collected and assayed for SNI. Native M_r was estimated from a standard curve of K_{av} (partition coefficient) *vs* log M_r. Standard proteins were prepared from the Combithek (Boehringer Mannheim) M_r standards kit and protein elution was monitored at 280 nm.

Glycosylation state determination

100 μl of Concanavalin A (Type III ASCL, Sigma) was incubated with 2 mg of the desalted 20–60% $(NH_4)_2SO_4$ fraction for 5 min at 25° and then spun down at 10 000 *g* at 4° for 5 min. An aliquot of the supernatant was removed. Protein and SNI were determined as described previously.

Acknowledgements—We gratefully acknowledge the Foundation for Research Development for financial support.

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