Isolation and characterisation of invertase inhibitor from sweet potato storage roots

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Abstract

BACKGROUND: Plant invertases play important roles in sucrose metabolism. Cell wall invertase has been reported to participate in phloem loading and unloading. Soluble invertases are involved in hexose level regulation in mature tissues and in utilisation of stored sucrose within vacuoles. Invertase inhibitory proteins have been described as one of the possible components for invertase activity regulation in some plant species.

RESULTS: In this work an invertase inhibitor (ITI) coding sequence was cloned by differential display from sweet potato (SP) storage roots. SPITI codes for a protein of 192 amino acids with a predicted molecular mass of 20 624 Da containing a 20-amino-acid signal peptide and four cysteines. Computer analysis of the deduced amino acid sequences of the conserved domain revealed that the protein belonged to the plant invertase/pectin methylesterase inhibitor. Both the corresponding mRNA and protein levels were found to be highest in storage roots, followed by veins. Recombinant SPITI protein from the storage root cDNA clone overproduced in *Escherichia coli* (M15) was purified by affinity chromatography. This protein effectively inhibited the invertase activity in a dose-dependent manner. The results presented in the Lineweaver-Burk plots indicated that the invertase inhibitor displayed a mode of competitive inhibition towards the invertase tested, with a K_i of 3.82×10^{-6} mol L^{-1}.

CONCLUSION: These results suggested that SPITI is a novel member of the ITI family in plants. SPITI genes of sweet potato storage roots display differential gene expression patterns, which may be associated with sucrose metabolism to cope with particular developmental requirements.

Keywords: sweet potato; invertase inhibitor; gene expression; recombinant protein

INTRODUCTION

Plant invertases (β-D-fructofuranosidase, EC 3.2.1.26) catalyse the hydrolytic cleavage of sucrose, which is the major transport form of carbohydrates in higher plants. Sucrose is exported from the source tissues (mature leaves) via the phloem to the different sink tissues (roots, stem, reproductive organs and vegetative storage organs).[1] The large changes in invertase activity are associated with environmental stimuli such as wounding and pathogenic infection,[2–4] gravity[5] and drought.[6] The expression of invertase genes and invertase activity level appear to be regulated by the hexose pool in plant tissues, as they are inhibited by the reaction products glucose and fructose.[7]

The presence of proteins that inhibit invertases in plant tissues suggests the possible mechanism for the regulation of invertases at the enzyme level. The first evidence for an endogenous invertase inhibitor was obtained through analysis of invertase kinetics in potato.[8] Since then, invertase inhibitors with molecular masses ranging from 17 to 22.9 kDa have been found in red beet, sugar beet, sweet potato,[9–11] maize endosperm,[12] yam,[13] tomato fruit[14] and potato.[15] Potato tubers actually contain a small amount of invertase, but its activity is inhibited by high levels of invertase inhibitor present in the tubers. Thus invertase in potatoes is subject to both synthesis and degradation as in other plant tissues; in addition, the actual invertase activity in the tubers is also determined by the balance between the enzyme and its inhibitor.

Invertase inhibitors have been known for a long time[13–15] but have only recently been cloned[16] and characterised.[17,18] The physiological role of the cell wall invertase inhibitor (Nt-inh1), the first cloned

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plant invertase inhibitor (ITI), has been investigated in tobacco suspension-cultured cells.[16] Here we report the cloning of the first SPITI cDNA from sweet potato (SP). We present the characterisation of this invertase inhibitor and demonstrate its inhibitory activity against invertase activity *in vitro* using recombinant SPITI. The comparison of the sequence of our data with those published confirms the existence of a novel class of invertase inhibitor proteins with moderate but significant sequence conservation, acting as inhibitors of an important sugar metabolism enzyme, invertase, and probably involved in various steps of plant development.

MATERIALS AND METHODS

Chemicals

Tris and electrophoretic reagents were purchased from E Merck Inc. (Darmstadt, Germany). Seeblue prestained markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), including myosin (250 kDa), phosphorylase (148 kDa), bovine serum albumin (BSA, 98 kDa), glutamate dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa), were from Invitrogen (Groningen, The Netherlands). β-Actin was obtained from ABcam Inc. (Cambridge, MA, USA). Coomassie brilliant blue G-250, acid invertase from baker's yeast (*Saccharomyces cerevisiae*) and other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Plant materials

Fresh storage roots of sweet potato (*Ipomoea batatas* (L.) Lam. 'Tainong 57') were purchased from a local market. After cleaning with water, the roots were placed in a thermostatted (28 °C) growth chamber and sprayed with water twice a day. Sprouted plants were cultivated in the greenhouse to collect sprouts of storage roots, sprouted storage roots, veins and fully expanded green leaves, together with storage roots, for experiments.

Polymerase chain reaction (PCR)-based subtractive hybridisation and rapid amplification of cDNA ends (RACE) PCR

Total RNA was isolated separately from the storage roots and sprouts of roots of sweet potato according to the method of Sambrook *et al*.[19] Then mRNA was purified with a purification kit (Promega, Madison, WI, USA) and used for differentially expressed first-strand cDNA synthesis with a PCR-based subtractive hybridisation kit (Clontech, Palo Alto, CA) following the protocol supplied by the manufacturer. The double-strand cDNAs of the storage roots were subtracted by the sprouts of roots, then ligated to the pGEM-T easy vector for *Escherichia coli* DH5α competent cell transformation. Recombinant plasmids were isolated for DNA sequencing using an ABI PRIZM 337 DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data were analysed using the Genetics Computer Group (GCG) program (Accelrys, San Diego, CA). A full-length cDNA clone was obtained by performing 5′ and 3′ RACE with the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. A gene-specific primer (5′-CTCAT CTCAT CTCAT CATCA TATCC TCC-3′) was designed for the 5′ and 3′ RACE reaction.

Expression of invertase inhibitor in *Escherichia coli*

Invertase inhibitor (ITI) was expressed in *E. coli*. The coding sequence was amplified from ITI cDNA using an oligonucleotide (5′-GCA TGC TGC AAG ATG AAG AGT TTA TTC-3′) with a *Sph* I site (underlined) at the putative initial Met residue and an oligonucleotide (5′-GAT AAG CTT TTT CAT AAC AGC A-3′) with a *Hind* III site. The PCR fragment was subcloned in pGEM-T easy vector. The plasmid was then digested with *Sph* I and *Hind* III and subcloned in pQE32 expression vector (QIAexpress expression system, Qiagen, Hilden, Germany). The resulting plasmid, termed pQE-ITI, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-NTA columns (Qiagen) according to the manufacturer's instructions.

RNA isolation and northern blot analysis

Total RNA was extracted from different tissues of sweet potato with a TRIzol reagent kit (Invitrogen) according to the manufacturer's instructions. For northern blotting, 10 μg samples of total RNA isolated from storage roots, sprouts, sprouted roots, veins, fully expanded green leaves and flowers were applied to a formaldehyde denaturing gel, then transferred to an Amersham Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) after electrophoresis, according to Sambrook *et al*.[19] The filter was hybridised sequentially with α-^{32}P-labelled invertase inhibitor full-length cDNA. The procedures for hybridisation and autoradiography were according to Sambrook *et al*.[19] Visualisation of hybridisation bands was carried out using X-ray film (Eastman Kodak, Rochester NY, USA).

Phylogenic analysis of SPITI

Amino acid sequence alignment of SPITI after GCG/Pileup comparison was used for phylogenic tree construction. The distances among entries were calculated with the neighbour-joining (NJ) method.[20] The internal support was evaluated by bootstrap analyses. In parsimony analysis, each of 100 bootstrap replicates was analysed with the heuristic search option invoking one random addition replicate each, and

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not invoking the retention of multiple parsimonious trees. The phylogenic tree was drawn using NJ plot and redrawn by the graphic software of CLUSTALX 1.81.[21]

Protein staining of ITI on 10% SDS-PAGE gels

Samples were mixed with sample buffer, namely 60 mmol L^{-1} Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol and 0.1% bromophenol blue with 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining.[22]

Production of polyclonal antibody and western blot hybridisation

Expressed SPITI protein was cut from the 10% polyacrylamide gel and eluted, mixed with an appropriate amount of pH 7.5 phosphate buffer saline (PBS) containing 0.1% SDS. The eluted proteins were precipitated with acetone containing 10% trichloroacetic acid (TCA) at −20 °C for 2 h. After centrifugation at 13 000 × *g* for 20 min the pellet was washed with acetone twice and then dried at room temperature. The acetone powder was redissolved in a small amount of PBS containing 0.1% SDS and used as antigen for subcutaneous injections of rabbit to prepare the first antigens (Taiwan Bio-Pharm Inc., Taipei, Taiwan). The second antigen (goat against rabbit Fc portion of Ig) was a product of Sigma Chemical Co. Polyclonal antibodies obtained from rabbit antiserum were utilised for western blot hybridisation to study the gene expression of SPITI in different organs of sweet potato.

Protein extraction and electroblotting analysis of invertase inhibitor

All steps were carried out at 4–8 °C. Sweet potato storage roots, sprouts, sprouted roots, veins and fully expanded green leaves were cleaned, air dried and homogenised with 4 volumes (v/w) of 50 mmol L^{-1} Tris-HCl buffer (pH 7.5) in a Polytron homogeniser (Lucerne, Switzerland). The homogenate was filtered through two layers of cheesecloth and then centrifuged in a Sorvall RC-2B (DuPont Instruments-Sorvall, Wilmington, Del, USA) with an SS-34 rotor at 10 000 × *g* for 20 min. The protein concentration in the supernatant was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA). The supernatant was saved for electroblotting. The crude extract was subjected to 15% SDS-PAGE according to Laemmli.[23] After electrophoresis, gels were equilibrated in transfer buffer (25 mmol L^{-1} Tris-HCl, pH 8.3, 150 mmol L^{-1} glycine and 100 g L^{-1} methanol). The separated proteins were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) in transfer buffer at pH 8.3 for 1 h at 100 V. Membranes were blocked for 2 h at room temperature in 5% non-fat dry milk powder and then incubated with polyclonal antibodies as the primary antibodies against SPITI. After incubation, membranes were washed in Tris-buffer saline with 0.5 g L^{-1} Tween (TBST) three times (10 min each), then incubated with anti-rabbit alkaline phosphatase-conjugated antibody, washed in TBST three times (10 min each) and developed using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma Chemical Co.). The second antigen (goat against rabbit Fc portion of Ig) was a product of Sigma Chemical Co.

Measurement of invertase and invertase inhibitor activities

Invertase activity was determined by measuring reducing sugars formed from sucrose hydrolysis using the Somogyi method as described previously.[11] The reaction was carried out at 37 °C in 0.1 mol L^{-1} sodium acetate buffer (pH 5). The amount of reducing sugars produced was measured colorimetrically at 520 nm, 10 min after the reaction. Inhibitor activity was measured by pre-incubating the acid invertase (6 units) for 30 min with varying amounts of inhibitor in 0.12 mL of 0.1 mol L^{-1} acetate buffer (pH 5) at 37 °C, then 0.24 mL of 0.4 mol L^{-1} sucrose (final concentration 0.267 mol L^{-1}) was added and the remaining invertase activity was measured as described above. Invertase incubation mixture without added inhibitor served as a control. One unit of inhibitor was defined as the amount of inhibitor required to inhibit acid invertase activity by 50%.

Statistical analysis

Means of triplicates were calculated. Student's *t* test was used for comparison between two treatments. Some data (reported as % of control value) were expressed as mean ± standard error. A difference was considered to be statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and nucleotide sequence of an invertase inhibitor cDNA clone from sweet potato storage roots

A SPITI cDNA clone of sweet potato storage roots was isolated by differential display. We have completed the sequencing of the clone, which was named *SPITI* (GenBank Accession Number AF529166). The open reading frame of this cDNA encodes a pro-protein of 192 amino acids with a predicted molecular mass of 20 624 Da (pI 4.47). A comparison of the deduced amino acid sequence of SPITI with precursor proteins indicates 58% identity to the *Nicotiana tabacum* ITI sequence. The SPITI sequence was different from other ITI sequences in signal peptide portions. Figure 1A shows a multiple alignment of SPITI protein and other homologous plant precursor ITI proteins available in the GenBank. Computer analysis of the deduced amino acid sequences of the conserved domain revealed that the protein belonged to the plant invertase/pectin methylesterase inhibitor. This domain

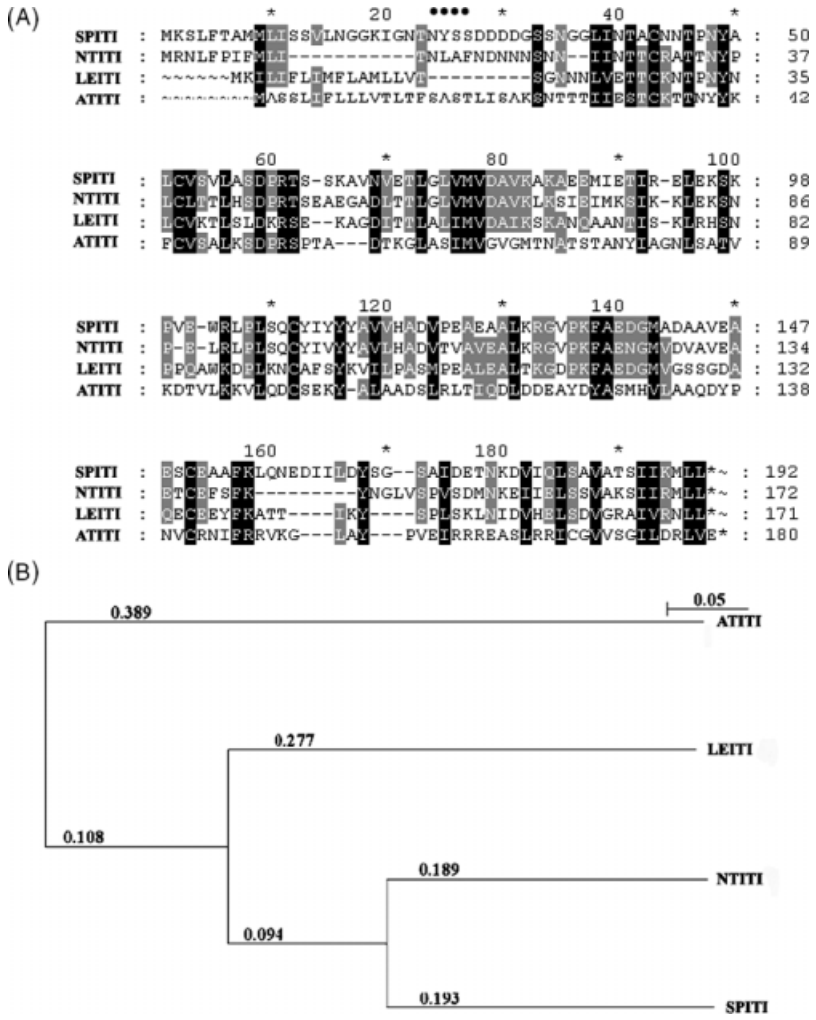

Figure 1. Multiple alignments of plant invertase inhibitor proteins. (A) The sequences are from sweet potato ITI (SPITI, AF529166), *Nicotiana tabacum* ITI (NTITI, AY594179), *Lycopersicon esculentum* ITI (LEITI, AJ010943) and *Arabidopsis thaliana* ITI (ATITI, AA050542). The consensus N-glycosylation site motif is marked with black dots. The proteins were aligned using the GCG program. Black shading indicates the same amino acid at that position among all sequences. Grey shading shows those amino acids with similar side chain properties. The numbers above all sequences stand for the positions of the amino acids within individual proteins corresponding to the numbering system of *A. thaliana* ITI (ATITI, AA050542), which is the longest among all sequences shown. The numbers at the right-hand end of each line stand for the cumulative total number of amino acids in each line of each preproprotein sequence. (B) Phylogenetic analysis of invertase inhibitors based on their amino acid sequences.

inhibits pectin methylesterases (PMEs) and invertases through formation of a non-covalent 1:1 complex. It has been implicated in the regulation of fruit development, carbohydrate metabolism and cell wall extension (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Moreover, the putative cleavage site on the hydrophobic N-terminal signal peptide for targeting to Endoplasmic Reticulum (ER) was predicted between Ile-20 and Gly-21.[24] In addition, one potential site of N-glycosylation, 24NYSS27 (N-[^P]-S/T-[^P], where [^P] indicates non-proline residues), was found. Comparison of the SPITI protein sequence with other ITI-related proteins shows the conservation of four Cys residues as well as other amino acids located throughout the protein. This allows us to infer that in the homologous proteins, whose primary structure was deduced only by cDNA sequencing, four Cys residues are engaged in two disulfide bridges and constitute a common structural motif.[25] Whether the conserved Cys residues are also involved in the regulation of apoplasmic invertase inhibitor from tobacco (INH) activity is not known. The activity of at least some INHs may be reduced by treatment with DTT.[16]

An invertase inhibitor (ITI-R) was purified from sweet potato storage roots. The optimal pH for interaction between ITI-R and acid invertase was 5 and the molecular mass of ITI-R was 22 kDa.[11]

The phylogenic tree of *SPITI* (AF529166) together with amino acid sequences of three other members was constructed. SPITI was similar to other ITIs by comparison (Fig. 1B).

Both invertase inhibitor mRNA and protein levels are developmentally regulated

The presence and amounts of different sweet potato ITI mRNAs were examined in various organs and tissues by northern blot analysis. Figure 2A shows that the ITI probe hybridised to mRNA species of approximately 1 kb. ITI mRNA levels were highest in storage roots, followed by veins and sprouted roots,

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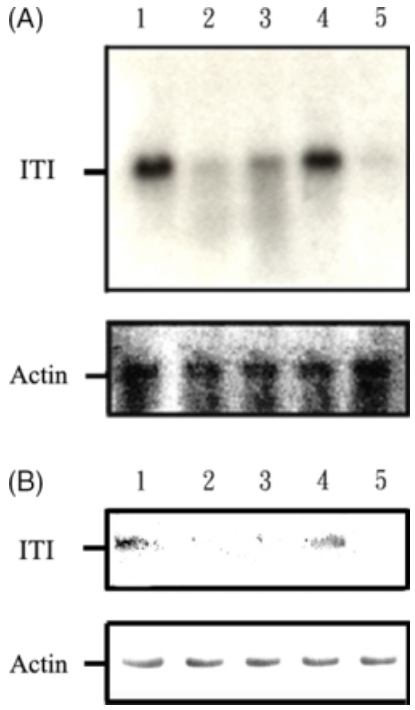

Figure 2. Northern and western blot detections of sweet potato invertase inhibitor. (A) Northern blot analysis. Samples (10 μg) of total RNA were isolated from different organs of sweet potato, and actin (AY905538) was utilised as an internal control of mRNA from sweet potato. Blots were hybridised to α-^{32}P-labelled 3′-specific cDNA probes. (B) Western blot analysis. Crude protein extracts (10 μg) from sweet potato were analysed by 150 g L^{-1} SDS-PAGE, then the gels were transferred onto PVDF membranes that were probed with a 1:1000 (v/v) dilution of rabbit antibodies raised against SPITI using goat-antirabbit alkaline phosphatase as the second antibody. Lanes: 1, storage roots; 2, sprouts of storage roots; 3, sprouted storage roots; 4, veins; 5, fully expanded green leaves. β-Actin was used as a control. The experiments were done twice and a representative one is shown.

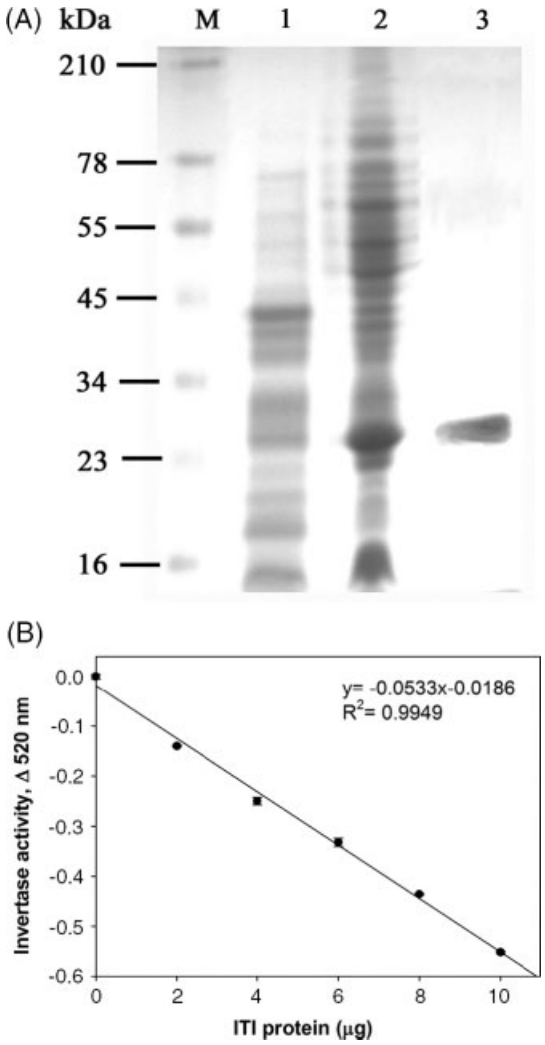

Figure 3. Purified recombinant sweet potato invertase inhibitor. (A) SDS-PAGE analysis. Crude protein extracts (5 μg) from *Escherichia coli* (M15) transformed with pQE32 (lane 1, as a control) or pQE32-SPITI (lane 2) were analysed by 100 g L^{-1} SDS-PAGE, then the gels were stained with Coomassie brilliant blue G-250. Molecular masses of standard proteins are indicated at the left of the figure. His-tagged SPITI was purified by Ni^{2+}-chelated affinity chromatography (lane 3). (B) Invertase inhibitor activity analysis. The experiments were done twice and a representative one is shown. Each absorbance value represents the average of triplicates of different samples analysed.

while they were lowest in sprouts of storage roots and fully expanded green leaves.

Western blot hybridisation using ITI polyclonal antibody from rabbit antiserum was used for the detection of ITI in crude extracts from different sweet potato organs (Fig. 2B). SPITI levels were highest in storage roots, followed by veins, but there was no signal at all in sprouted roots, sprouts of storage roots and fully expanded green leaves.

In maize, invertase inhibitor (ZM-INVINH1) interacts with an apoplastic invertase during early kernel development. ZM-INVINH1 provides a link between invertase activity, known to be important for the control of carbohydrate partitioning, and the regulation of events during early kernel development by showing expression of *ZM-INVINH1* in the ESR (embryo surrounding region), an important and intriguing cluster of cells surrounding the embryo.[26] Sweet potato storage roots actually contain a small amount of invertase, but the activity is inhibited by high levels of invertase inhibitor present. When the storage roots are stored in cold, invertase synthesis proceeds until the enzyme exceeds the inhibitor and then invertase activity becomes evident. Thus, besides regulation at gene expression level, invertase activity in sweet potato storage roots is regulated both by enzyme protein turnover (synthesis and destruction) as in other plant tissues and by the relative amounts of the active enzyme and its inhibitor.[9,11]

Expression of invertase inhibitor in *Escherichia coli*

SDS-PAGE analysis of SPITI crude extracts from transformed *E. coli* (M15) showed high amounts of a polypeptide with the expected molecular mass (~24 kDa) (Fig. 3A). The expressed protein, which contained a signal peptide, was found as a soluble protein in the supernatant (Fig. 3A, lane 2) but was absent in protein extracts obtained from *E. coli* transformed with pQE-32 vector (Fig. 3A, lane 1 as

a control). The expressed protein was highly purified from crude extracts as His-tagged SPITI (Fig. 3A, lane 3).

To determine whether His-tagged SPITI functions as an invertase inhibitor *in vitro*, we carried out invertase inhibitor activity assays using the Somogyi method[11] as described previously. As shown in Fig. 3B, the His-tagged SPITI protein inhibited the invertase activity as compared with the control. These results suggested that the His-tagged SPITI inhibits invertase in a dose-dependent manner.

Enzyme kinetics of commercial invertase in presence of inhibitor sweet potato ITI

Figure 4A shows the effect of various concentrations of SPITI in the presence of increasing concentrations of sucrose. Under experimental conditions the linear relationship between initial velocity and substrate (sucrose) concentration holds only when the sucrose concentration is below 50 mmol L^{-1}, no matter whether SPITI is present or not; this is consistent with 'substrate inhibition of invertase' in previous reports.[27–29]

The data were fitted to the Michaelis–Menten equation, which revealed that increasing concentrations of SPITI increased the apparent K_m for the substrate without affecting V_{max}. The Lineweaver–Burk plots for inhibition of invertase by SPITI (0, 2, 4 and 8 μg) in different concentrations of sucrose (0, 25, 50, 100 and 200 mmol L^{-1}) were obtained. The same K_i (3.82×10^{-6} mol L^{-1}) was measured in the presence of various concentrations of invertase, as expected for a simple linear competitive inhibitor (Fig. 4B). In sweet potato, ITI-R inhibited invertase by reversibly binding to it and required a short pre-incubation period for maximal inhibition.[11] This is identical to the behaviour of the sweet potato invertase inhibitor described by Matsushita and Uritani[30] and Wang *et al.*[11] as well as to that of the maize invertase inhibitor.[26] By contrast, potato invertase inhibitor requires a longer pre-incubation time and forms a non-dissociable complex with invertase.[31]

The occurrence of proteins that inhibit invertase activities has been reported as a possible mechanism for the activity regulation of invertase at the enzyme level in some plant species. Invertase inhibitors from *Dioscorea rotundata* tuber were non-competitive inhibitors of invertase.[13] In *Pteris deflexa*, invertase–inhibitor complex formation occurs in an immediate manner and a protease activity was discarded. The inhibition is non-competitive ($K_i = 1.5 \times 10^{-6}$ mol L^{-1}) without interactions among the binding sites. The complex is slightly dissociable and sucrose was able to partially reduce the inhibitory effect.[32]

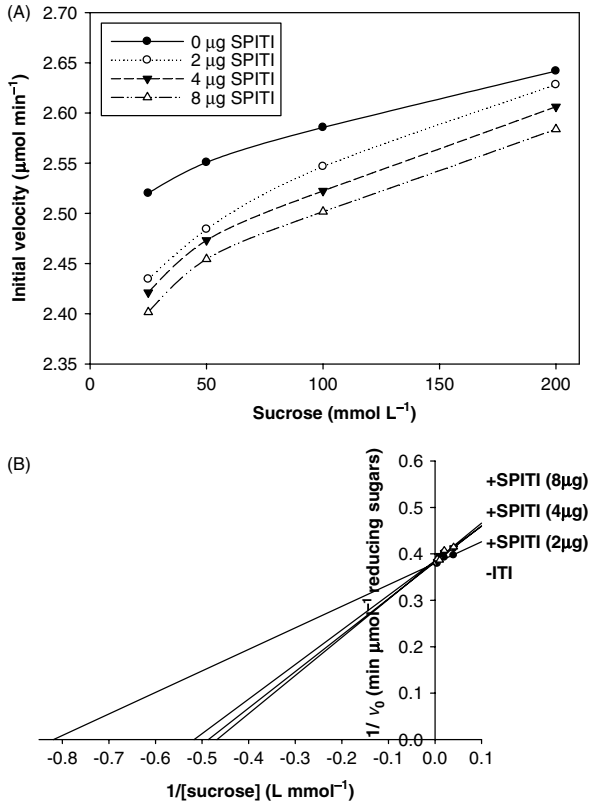

Figure 4. (A) Effect of increasing concentrations of SPITI on apparent K_m for sucrose substrate. (B) Lineweaver–Burk plots for inhibition of invertase by sweet potato invertase inhibitor (SPITI; 0, 2, 4 and 8 μg) in different concentrations of sucrose (0, 25, 50, 100 and 200 mmol L^{-1}). Inhibitor activity was measured by pre-incubating the acid invertase (6 units from yeast) for 30 min with varying amounts of inhibitor.

CONCLUSION

We report here for the first time the isolation of SPITI cDNA and its expression levels in different organs of sweet potato. However, the biochemical effect of SPITI proteins on sucrose metabolism and their physiological role during plant development are still unclear. To identify the physiological function of SPITI and to examine the possible utilisation of SPITI in the food industry, we are now investigating the development of transgenic plants using sense and antisense SPITI cDNAs.

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