Journal of Enzyme Inhibition and Medicinal Chemistry, 2002 Vol. 17 (2), pp. 123–130

Proteinaceous Inhibitor Versus Fructose as Modulators of *Pteris deflexa* Invertase Activity

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(Received 16 January 2002)

An acid invertase from the fern *Pteris deflexa* Link was purified and the effect of reaction products on enzyme activity was studied. Fructose and glucose were competitive and non-competitive inhibitors of the enzyme, respectively. Since proteins suppressed glucose and fructose inhibition of the enzyme, an invertase modulation by reaction products is unlikely; nevertheless, an invertase proteinaceous inhibitor previously reported could have a role in this respect. The purified enzyme was an heterodimer M_r 90,000 Daltons composed of subunits of 66,000 and 30,000 Daltons. The enzyme had β-fructofuranosidase activity and hydrolyzed mainly sucrose but also raffinose and stachyose, with K_m of 3.22, 10.80 and 38.50 mM, respectively. Invertase activity with an optimum pH at 5.0 was present in almost every leaf fern tissue. Pinnas (sporophyll leaflets) had the higher enzyme levels. Invertase histochemical and immunochemical localization studies showed the enzyme mainly in phloem cells. Epidermis, collenchyma and parenchyma cells also showed invertase protein.

Keywords: *Pteris deflexa* Link; Soluble acid invertase; β-D-fructofuranoside fructohydrolase; Fructose; Proteinaceous inhibitor

INTRODUCTION

Carbohydrate partitioning between photosynthetically active source tissues and photosynthetically less active or inactive sink tissues, such as roots, flowers and fruits is essential for growth and development in higher plants. The long-distance transport of photosynthesis products, mostly in the form of sucrose, occurs in the phloem and is driven by differences in solute concentrations and osmotic potentials.[1] Since the removal of sucrose steepens the gradient and thus enhances the flow toward sinks, enzymes involved in immediate sucrose metabolism are expected to be important both for phloem unloading and for the import of sucrose into sink organs.[2] Provision of carbon and energy is only achieved following cleavage of the disaccharide, in which invertase (EC 3.2.1.26), by directly hydrolyzing sucrose to glucose and fructose is believed to play a key role.[3] Invertase enzymes are classified according to their pH optima (acid and alkaline) and cellular localization (apoplastic or vacuolar and cytoplasmic). Those enzymes associated with the cell wall are considered to be pivotal to numerous metabolic processes including the control of product partitioning.[4]

Some potential mechanisms for the control of sucrose breakdown in sink and source tissues by invertase has been described. We characterized a regulatory mechanism based on activity inhibition by reaction products. In mature leaves of species where fructose is not exported immediately from the vacuole after hydrolysis, this could be an effective regulatory system.[5] Fructose inhibition without suppression by proteins is also observed in enzymes from sink or storage tissues, where it could serve to a similar function.[6–9]

Studies of the invertase from *Equisetum giganteum* showed a hysteretic behavior, suggesting that

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/14756360290026450

activity control mechanism depending on pH may be operating *in vivo*.[10] The occurrence of proteins that inhibit invertase activities has been reported as another possible mechanism for the activity regulation of these enzymes in some plant species. Invertase proteinaceous inhibitors have been reported only in heterotrophic organs (sink tissues) and cell cultures[11–18] suggesting that this would not be a significant element in the regulation of sucrose turnover in leaves. In a previous work, we have reported the presence of soluble acid invertase and an inhibitory protein of it in *Pteris deflexa* fronds.[19] This protein shows several characteristics which appears to indicate a role for the *in vivo* regulation of acid invertase from *Pteris deflexa* Link fronds. The aim of the present work was to analyze the effect of the reaction products, glucose and fructose, on invertase activity and to evaluate the relevance of this effect versus the inhibitory protein effect as a regulatory mechanism of the enzyme *in vivo*. For that purpose the molecular and kinetic properties and the cellular location of the fern fronds acid invertase were studied in order to compare them with invertases possessing known regulatory mechanism of enzyme activity.

MATERIAL AND METHODS

Plant Material

Pteris deflexa Link plants were collected in Horco Molle, Tucumán, Argentina and frozen at −20°C until use.

Reagents

All chemicals used were of analytical grade.

Invertase Extraction

Samples (2 g) of different sporophyte organs (rhizomes, fronds) from *Pteris deflexa* were powdered in liquid nitrogen with a cold morter and pestle. Homogenates were prepared by extracting the powder with 6 ml of 50 mM Na acetate buffer, pH 4.0 containing 500 mM NaCl and 1 mM 2-mercaptoethanol (buffer A) over 1 h at 4°C with shaking. Extracts were centrifuged at 21,000*g* for 15 min. The clarified supernatant (Fraction I) was considered as the soluble fraction.

Invertase Purification of Mature Fronds

Mature fronds of sporophytes from *Pteris deflexa* (200 g) were cut into small pieces and homogenized in 400 ml of buffer A. The homogenate was filtered through two layers of gauze and centrifuged at 21,000*g* for 15 min. Then, solid ammonium sulphate was added to the supernatant. The precipitate obtained between 0 and 90% was collected by centrifugation, resuspended in 10 mM Na phosphate buffer, pH 6.0 containing 1 mM 2-mercaptoethanol (buffer B) and dialyzed against the same buffer for 1 h. Then, it was applied onto a Sephadex G-75 column (30 × 1.7 cm) equilibrated with buffer B. The column was eluted with the same buffer. From this procedure two protein peaks were obtained: one with invertase activity, that was excluded (Peak I), and other with invertase inhibitory activity (Peak II).

Invertase Purification

Peak I fractions were pooled and further purified by gel filtration on a Sephadex G-150 column (2.5 × 35 cm) equilibrated and eluted with 10 mM Na acetate buffer, pH 5.5 containing 1 mM 2-mercaptoetanol (buffer C). Fractions with invertase activity were pooled and subjected to adsorption chromatography through a 1.2 × 7.0 cm brushite column equilibrated with 10 mM sodium phosphate adjusted to pH 5.5 containing 1 mM 2-mercaptoethanol. Proteins were eluted with a 10–750 mM sodium phosphate gradient adjusted to pH 5.5. Just a peak of invertase activity coincident with a protein peak was obtained. The active fractions were then dialyzed against buffer C. By this procedure invertase was purified until electrophoretic homogeneity (0.056 E.U./ml).

Proteinaceous Inhibitor Purification

The Peak II fractions eluted from the Sephadex G-75 column with invertase inhibitory activity were pooled and further purified by adsorption chromatography through a 1.2 × 3.0 cm brushite column equilibrated with 10 mM sodium phosphate adjusted to pH 5.5 containing 1 mM 2-mercaptoethanol. Proteins were eluted with 25 ml of 750 mM sodium phosphate pH 5.5 containing 1 mM 2-mercaptoethanol. The eluted fractions were dialyzed against buffer C during 30 min and assayed for invertase inhibitory activity. The active fractions were pooled and concentrated by lyophilization. Invertase inhibitor (0.04 mg/ml) was kept at −20°C until used.

M_r Determination

The M_r of the enzyme was determined by the method of Andrews.[20] Invertase was filtered through a 2.5 × 35 cm of Sephadex G-150 column. The column was equilibrated and eluted with 10 mM Na acetate buffer, pH 5.5 containing 1 mM 2-mercaptoethanol. The M_r markers used were alkaline phosphatase

(100,000), BSA (66,000), carbonic anhydrase (29,000) and cytochrome c (12,400 Daltons).

Protein samples (2 μg) were treated and analyzed by electrophoresis according to Laemmli.[21] Electrophoresis was carried out at 80 V for 2 h. Molecular weight markers, BSA (66,000), ovoalbumin (45,000), pepsin (34,000), β-lactoglobulin (18,400) and lysozyme (14,300 Daltons) were used as standard. Proteins were visualized by the silver nitrate method.[22]

Polyacrylamide Gel Electrophoresis

Native PAGE was performed according to Ornstein[23] and Davis.[24] Invertase activity was revealed with 2,3,5-triphenyltetrazolium chloride.[25]

Chemical Methods

Proteins were determined with BSA as standard[26] and they were monitored spectrophotometrically at 280 nm in column fractions. Total neutral carbohydrate content was determined by the method of Dubois[27] with glucose as standard.

Enzyme Assay

The reaction mixture consisted of 10 μl of enzyme (0.56×10^{-3} E.U.), 10 μl 0.6 M sucrose, 40 μl 0.2 M Na acetate buffer pH 5.0 and distilled water to a final volume of 100 μl. Incubations were performed at 37°C for 15 min and the reactions were stopped by adding the Cu alkaline reagent.[28] Reducing power was measured by the method of Nelson.[29]

One unit of invertase activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of sucrose per min at 37°C and pH 5.0.

Effect of the Reaction Products

The incubation mixtures consisted of 10 μl enzyme, 10 μl 0.05–0.60 M sucrose, 10 μl 0.6–1.8 M fructose or glucose, 40 μl 0.2 M Na acetate buffer pH 5.0 and distilled water to a final volume of 100 μl. Incubations were made at 37°C. Reducing power determinations in presence of glucose and fructose were performed by the methods of Jorgensen[30] and Avigad,[31] respectively.

Effect of Proteinaceous Inhibitor

A constant amount of invertase (10 μl) with varying amounts of inhibitor (3–18 μl) in 0.1 ml incubation mixture containing 80 mM Na acetate buffer, pH 5.0 and 60 mM of sucrose was incubated at 37°C for 15 min and the remaining invertase activity measured as described above. Mixtures that contained no inhibitor were used as enzyme activity controls. One unit of inhibitor was defined as the amount of inhibitory protein that reduced one invertase unit activity to 50%, at pH 5.0 and 37°C.

Histochemical Localization of Invertase

Thin sections from petiole, petiole branches, pinnas (sporophyll leaflets) and rhizomes were free hand cut. Slices were fixed in 4% formalin (pH 7.0) for 3 min at 4°C and rinsed in water at least 10 times throughout a period of 1 h to remove endogenous sugars. Sections were incubated at room temperature in 0.38 M Na phosphate buffer pH 6.0 containing 0.96 mg/ml nitroblue tetrazolium, 0.56 mg/ml phenazine methosulfate, 25 units/ml glucose oxidase and 100 mg/ml sucrose.[32] Control slices were incubated in the same mixture without sucrose. Then, the sectioned tissues were rinsed in water and photographed under light microscopy.

Immunohistochemical Studies

Immunological localization of invertase in fresh tissue slices and tissue prints was conducted. Pinnas, petiole and petiole branches samples were free hand sliced into thin sections and washed with distilled water for 10 min. The tissue printing technique used was a modification of that described by Kingston-Smith and Pollock.[33] Small pieces of the different fern tissues were fixed in 3% agarose and tissue prints were then obtained from longitudinal and transversal-cut pinnas, petiole and petiole branches. Immediately after cutting, the exposed tissue were printed on nitrocellulose membranes (0.45 μm) that had been previously soaked in 0.2 M $CaCl_2$ for 15 min and air dried. Total proteins in the tissue prints were stained with amido black (1%, w/v) in 7% acetic acid for 15 min and then the membranes were de-stained in 7% acetic acid until a clear image was detectable.

The enzymes from fresh tissue and tissue prints were visualized using a specific antiserum. Samples were first incubated for 1 h with 10% (w/v) bovine serum albumin in Tris-buffered saline (TBS) (20 mM tris (hydroxymethyl) aminomethane HCl, pH 7.4 with 0.137 M NaCl) at room temperature with shaking. They were then incubated with antibodies raised against acid soluble invertase (1:1000 diluted invertase antiserum in TBS containing 5% (w/v) BSA) from *Solanum tuberosum* tubers followed by washing, and incubation (30°C, 2 h) with anti-rabbit immunoglobulin conjugate with alkaline phosphatase (Sigma Immunochemicals). After washing, phosphatase activity was visualized by incubating in 100 mM Tris–HCl (pH 9.5), 50 mg/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 340 mg/l nitroblue tetrazolium (NBT), 100 mM NaCl, 0.1% of 50 mM $MgCl_2$ (30°C, 15 min). Specificity of the

TABLE I Specific activities and E.U. per fresh weight in different fern tissues

Tissue	E.U./g of tissue	E.U./mg of protein
Rhizome	0.30	0.25
Petiole	0.54	0.38
Petiole branches	0.66	0.47
Pinnas	1.62	0.27

labeling patterns obtained with the invertase antiserum was assessed by a control test conducted with tissue slices and prints with preimmune serum incubation, instead with anti-invertase antiserum.

RESULTS

Invertase Presence in Different Fern Tissues

In a previous work[19] we have reported that in Na phosphate buffer, pH 7.5, a soluble acid invertase (pH optimum, 5.0) co-eluted with an inhibitory protein, while at pH 4.0 free invertase could be separated. The same yield of the enzyme was obtained by using Na acetate 50 mM pH 4.0 buffer with and without ionic strength. Specific activities and E.U. per fresh weight in different fern tissues (rhizome, petiole, petiole branches and pinnas) were estimated (Table I). Acid invertase activity in mature sporophyte of *P. deflexa* was found to a greater extent in fronds than in rhizome, while in fronds the higher invertase activity was localized in pinnas; nevertheless, higher specific activities were found in petiole and its branches. Electrophoresis of all preparations on non-denaturing-polyacrilamide gels resulted in the invertase activity profiles shown in Figure 1. Two invertase isoforms were found in every analyzed tissue, although both isoforms were demonstrated in lower quantity in rhizomes than in pinnas.

Sephadex G-75 chromatography of both rhizome and pinnas preparations yielded two peaks with invertase activity (Figure 2). In pinnas, the 22.4 ml eluted fraction accounts for about 85% of total activity, while in rhizomes the fraction that eluted at 42.0 ml was more abundant (65% of total activity).

Histochemical and Immunohistochemical Localization of Soluble Acid Invertase

Longitudinal and transversal sections of petiole and petiole branches and pinna were used for localization studies of invertase at cellular level. Histochemical techniques showed a strong intracellular staining in phloem veins and in subepidermis, epidermis and parenchyma cells (colour plates available from Dr M.I. Isla on request). *In situ* immunolocalization of the invertase protein showed a tissue specific localization, mainly in vascular bundles. The enzyme was also present in epidermis, subepidermic collenchyma and parenchyma cells. Invertase was also visualized in vascular bundles of pinnas cross cut by immunolocalization in tissue prints.

Fronds' Invertase and Invertase Inhibitor Purification

The invertase and the inhibitory protein were extracted from fronds with Na acetate buffer pH 4.0 containing 500 mM NaCl. The homogenate was concentrated with solid ammonium sulphate until 90% saturation, dialyzed and chromatographed by Sephadex G-75. Under these conditions free invertase and inhibitor could be separately obtained. The enzyme was found in the void volume while the

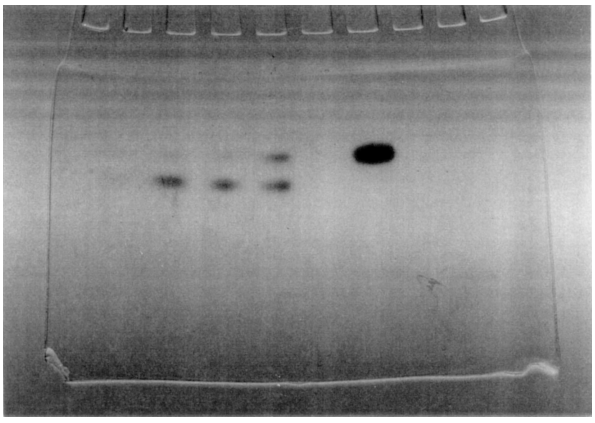

FIGURE 1 Native PAGE of extracts from different fern tissues. Analyzed samples were, from left: rhizome, petiolule, petiole and pinna. The right line corresponds to purified enzyme. The technique was performed according to Ornstein and Davis' method and invertase activity was revealed with 2,3,5-triphenyltetrazolium chloride.

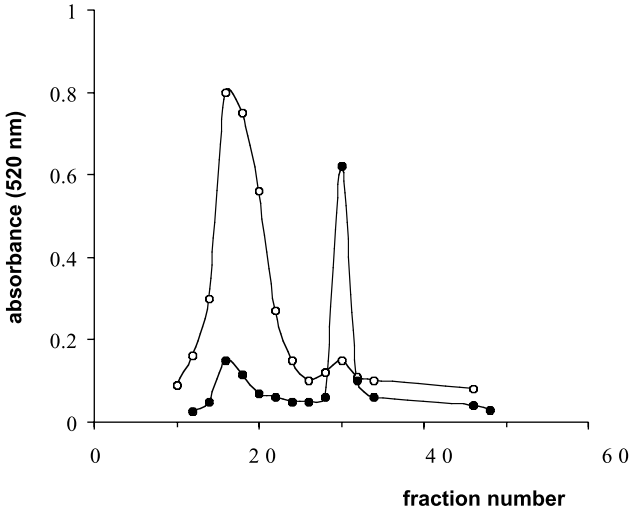

FIGURE 2 Sephadex G-75 chromatography of crude extract. Fractions of 1.4 ml were collected. Each fraction was analyzed for invertase according to the procedures described in "Materials and methods section". (○–○) pinnas; (●–●) rhizome.

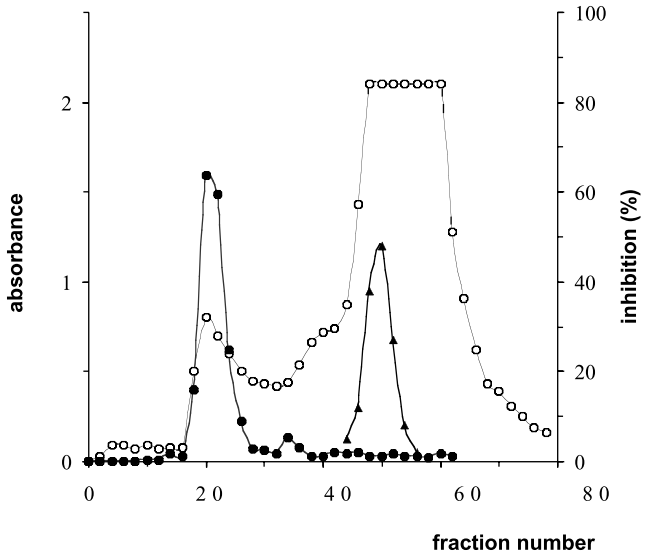

FIGURE 3 Sephadex G-75 chromatography of crude extract. Fractions of 1.4 ml were collected. Each fraction was analyzed for invertase (•–•) and invertase inhibitory (▲–▲) activities according to the procedures described in "materials and methods". Proteins were estimated by absorbance at 280 nm (○–○).

protein with inhibitory activity eluted at 70 ml (Figure 3). The fractions with invertase activity were pooled, subjected to gel filtration followed by adsorption chromatography. Just a peak of invertase activity coincident with a protein peak was obtained.

The inhibitor was further purified by adsorption chromatography. With this procedure invertase inhibitor and invertase were purified until electrophoretic homogeneity.

Enzyme Molecular Properties

The M_r of invertase was determined to be 90,000 Daltons by gel filtration; The enzyme, under denaturing conditions was dissociated in two subunits of MW 66 and 30 KDa (not shown), suggesting a dimeric complex.

The enzyme was active in a pH range between 3.0 and 8.0 and its optimum was at about 5.0. The reaction rate was linear at least during 40 min at 37°C.

Enzyme Specificity

Sucrose, raffinose and stachyose hydrolysis by the enzyme exhibited simple Michaelis–Menten kinetics with K_m values of 3.22 ± 0.08, 10.8 ± 0.05 and 38.5 ± 0.04 mM ($n = 5$) and V_{max} values of 0.037, 0.021 and 0.025 mmol/min, respectively. According to the first-order rate constant values (k), the enzyme is more efficient with sucrose as substrate. Approximately 1.1% of the sucrose, 0.19% of the raffinose and 0.06% of stachyose present at any time are converted to product per unit of time. Melibiose, melezitose, cellobiose, trehalose, maltose, turanose, α-methyl-fructoside, β-methylfructoside, inulin and *Zymomonas mobilis* levan were not hydrolyzed by the enzyme. According to these results, the enzyme hydrolyzed only sucrose and those oligosaccharides with terminal fructose residues. Chromatograms of 0.1 and 0.6 M sucrose hydrolyzed by 0.056 E.U., at 37°C for 4 h showed only fructose, glucose and sucrose. Since no oligosaccharide was observed,

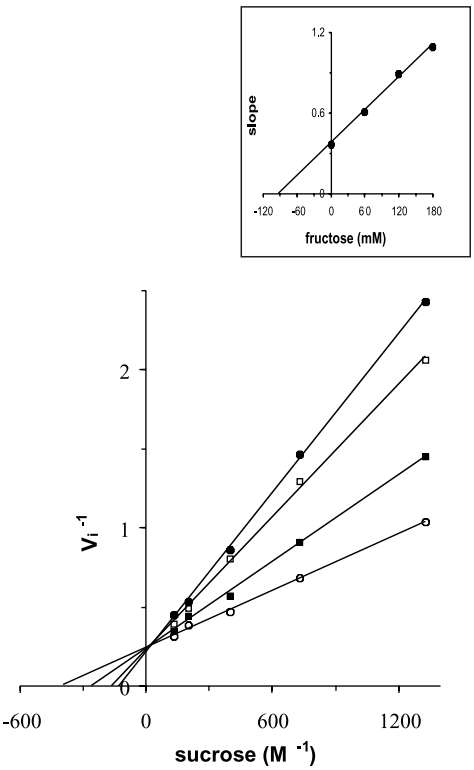

FIGURE 4 Lineweaver-Burk plot of the effect of fructose on invertase activity and replot of slope vs. fructose concentration: control without inhibitor (○–○); 60 mM (■–■), 120 mM (□–□) and 180 mM (•–•) fructose. Replot of slope against fructose concentration (Inset).

transglycosylation reactions were excluded. These results suggested that the enzyme was a β-fructofuranosidase (EC 3.2.1.26).

Effect of the Reaction Products

Figure 4 shows the effect of fructose on the initial velocity of sucrose hydrolysis by *P. deflexa* invertase. The plot corresponds to a competitive type of inhibition. Inset is the replot of slopes *versus* fructose concentration confirming competitive inhibition with a K_i value of 90 mM. Glucose was a non-competitive inhibitor with a K_i value of 120 mM (Figure 5).

Effect of Proteins

Soluble invertases from *Carica papaya* fruits,[7] *Solanum tuberosum* tubers,[6] sugar cane leaf sheaths,[8] *Tropaeolum majus*[34] and *Ricinus communis*[35] leaves and *Equisetum giganteum* stems and lateral branches[10] have been reported to be activated by proteins. Our studies on *P. deflexa* invertase revealed that only some proteins, such as BSA and ovoalbumine, were able to produce activating effects (*c.a.* 30%). Assays with other proteins (alkaline phosphatase, urease, trypsinogen, trypsin, glucosidase) did not show effects on the activity of *P. deflexa* invertase. However, BSA suppressed fructose and glucose inhibitory effects. This fact infers the absence, *in vitro* and probably *in vivo*, of an invertase functional regulation by the reaction products.

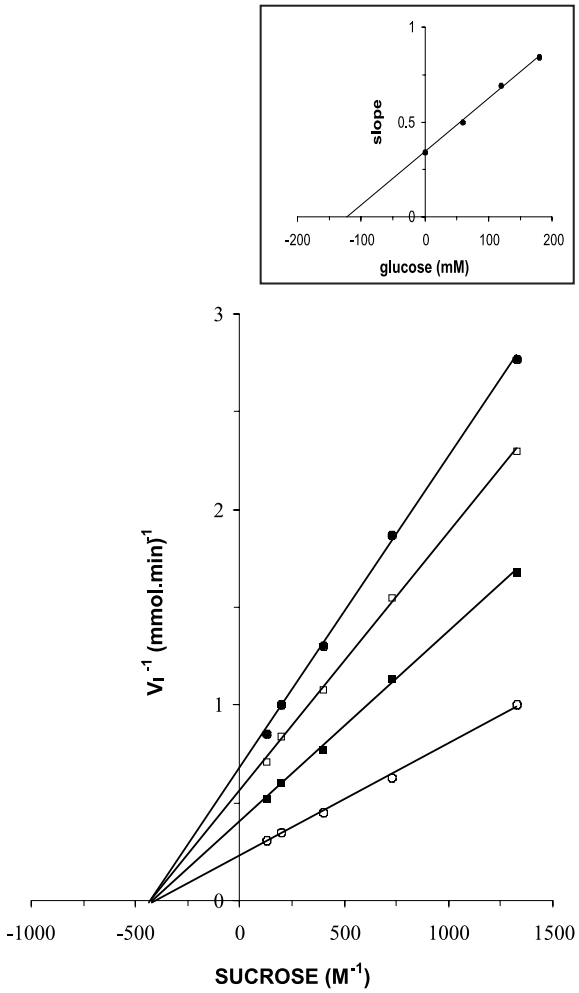

FIGURE 5 Lineweaver-Burk plot of the effect of glucose on invertase activity and replot of slope vs. glucose concentration: control without inhibitor (○–○); 60 mM (■–■), 120 mM (□–□) and 180 mM (•–•) glucose. Replot of slope against glucose concentration (Inset).

Effect of Lectins

Structural sugars content of *P. deflexa* invertase was *c.a.* 30% (w/w). Although the enzyme is a glycoprotein, it was not complexed by lectins. The lectins which bind mannose and glucose (Con A, *Vicia sativa*) and those which bind oligomers of *N*-acetylglucosamine (wheat germ aglutinin, STA) or α-fucose (*Ulex*) were not effectors of the enzyme. This behavior was different from that observed for other acid soluble invertases from higher and lower vascular plants.[6–10,34–35]

Effect of pH on the Invertase Activity

To our knowledge, the invertase from *Equisetum giganteum*[10] is a hysteretic enzyme, and this control mechanism for invertase activity may be operating *in vivo*. The hysteresis does not seem to be a common behavior among invertases. Because of this, the invertase from *P. deflexa*, other lower vascular plant, was assayed to investigate whether a hysteretic behavior existed under acid pH conditions. When the enzyme was preincubated at several pH values and then incubated at the optimum pH for different times, the reaction rates were not modified. This behavior has similarities with soluble acid invertases from higher plants.

pH Stability

The enzyme retained 100% of its activity between pH 5.0 and 7.0 after preincubation at 4 and 37°C during 15 min (not shown).

Determination of the Activation Energy

The activity of the enzyme was determined between 20 and 50°C. The activation energy was calculated to be 13700 cal/mol. An Arrhenius plot gave a straight line graph (not shown).

Effect of Chemicals

Metallic ions were invertase inhibitors, specially Hg^{+2}, Mn^{+2}, Co^{+2}, Ag^{+1}, Cu^{+2} and also *p*-chlor-

omercuribenzoate. Other inhibitors were Ba^{+2} and NH_4^{+1} and anions such as phosphate. Ammonium and pyridoxal or pyridoxal phosphate inhibited the *P. deflexa* invertase.

DISCUSSION

Pteris deflexa is a leptosporangiate fern of the *Pteridaceae* family having big fronds with highly divided lamina (pinna), strong and branched petioles and a short rhizome. The histochemical localization of invertase activity from the fern *P. deflexa* showed the enzyme in almost every leaf tissue. In cross sections of petiole and petiole branches the invertase activity was located within epidermis, subepidermis collenchyma and nearby parenchyma cells. The enzyme staining was very strong in phloem cells (both transversal and longitudinal slices) and no invertase activity was noticed in endodermis nor surrounding parenchyma cell layers. Narrow branches such as petiolules, that lack an endodermis, showed the same invertase location pattern as the petiole. Vascular tissues of petiole and petiole branches consist of divergent central xylem bundles surrounded by phloem (solenostella). These enzyme locations were confirmed by an immunocytochemical staining applied to fresh tissues samples. Nitrocellulose tissue prints of pinna cross sections stained by an immunocytochemical method showed invertase in vascular veins. Pinnas had the biggest amount of extractable enzyme per fresh weight, while petiole, its branches and petiolules had the highest invertase specific activity. This finding is in agreement with Kingston-Smith and Pollock's[33] observations who reported in barley and pea a significant invertase concentration around the veins, contrasting with the reduced staining of total proteins, in immunological localization of the enzyme in nitrocellulose tissue prints. By an immunocytochemical localization study of pea cell-wall invertase, Wu *et al*.[37] found an abundant immunogold particles density in phloem cells. According to the latter finding, pea cell-wall invertase mRNA localization in the phloem tissue was reported by Zhang *et al*..[38] These authors proposed that the physiological significance of the invertase in phloem could be either the maintenance of a steep sucrose concentration gradient between the source and sink regions of a plant or the provision of hexoses for the metabolism of companion cells. Phloem localization of invertase both in pinnas and petiole, petiole branches and petiolules of *P. deflexa* would be consistent with the involvement of the enzyme in sucrose loading and long distance transport. Epidermal occurrence of invertase activity has been reported in barley.[39] The cells of epidermis behave essentially as heterotrophic tissue.[40] The localization of the enzyme within epidermal cells and subepidermal collenchyma of *P. deflexa* fronds would be necessary for sugars storage and metabolism in these tissues.

The invertase purification method applied to fronds allowed the purification of one of the two enzyme isoforms that were present in every assayed tissue. In our preparations the enzyme occurs as an heterodimer of 90,000 Daltons with subunits of 66,000 and 30,000 Daltons, respectively and it was shown to be a glycoprotein with *c.a.* 30% of neutral sugars, as has been found for the acid invertases of higher plants.[3] The enzyme was a β-fructofuranosidase that hydrolyzed mainly sucrose *in vitro*. The reaction products were inhibitors of the enzyme. Fructose produced a classical competitive inhibition, while glucose was a non-competitive inhibitor. Fructose inhibition was also observed in higher plants invertases from sink or storage tissue, where it could be part of an effective regulatory system of the short-term balancing of sucrose breakdown.[8–11] Nevertheless, since both effects were suppressed by proteins, reaction products are unlikely modulators of the invertase activity *in vivo*. This behavior is similar to acid invertase from some higher plants such as *T. majus*[34] and *R. communis*[35] leaves and a vascular cryptogamous plant such as *Equisetum giganteum*.[10] No hysteretic effect was observed by pH changes, like that observed with *E. giganteum* invertase.[10] In a previuos paper[19] we have reported inhibition the inhibition of *P. deflexa* invertase by an endogenous inhibitor of protein nature. Some molecular and kinetic properties of the inhibitor were found to be similar to those of invertase inhibitors from heterotrophic tissues of higher plants. An appreciable invertase–inhibitor interaction has been verified around pH 5.0. The inhibition was non-competitive ($K_i = 1.5 \times 10^{-6}$ M). The invertase–inhibitor complex was partially dissociable under our experimental conditions. Furthermore, the substrate (sucrose) protected the invertase against the inhibition produced by the inhibitory protein. A similar behavior was found for cell wall invertase and invertase inhibitor obtained from *Agrobacterium tumefaciens* tranformed tobacco cells.[36] Sucrose protection might control invertase inhibition, and this fact would be of physiological significance.[17]

On the basis of our results, we conclude that the inhibition produced by the endogenous inhibitor on leaf invertase may be physiologically relevant since glucose and fructose were found unlikely to be *in vivo* modulators of enzyme activity and no hysteretic effect by pH modification could be observed on invertase activity from *P. deflexa* fronds. The same conclusion is suggested by the K_i value of the

endogenous inhibitor which is lower than those of the reaction products.

Acknowledgements

This work was supported by grants from the Secretaria de Ciencia y Técnica from the Universidad Nacional de Tucumán and from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

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