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# An Invertase Inhibitory Protein From *Pteris deflexa* Link Fronds

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**Plant invertases play important roles in sucrose metabolism. Cell wall invertase was reported to participate in phloem loading and unloading. Soluble invertases would be involved in hexose level regulation in mature tissues and in stored sucrose utilization within vacuoles. Invertase inhibitory proteins were described as one of the possible mechanisms for invertase activity regulation in some plant species; nevertheless, these proteins were found only in sink tissues, suggesting that this mechanism would not be relevant in the sucrose turnover of leaves. This report describes the purification of invertase from *Pteris deflexa* fronds and the occurrence of an invertase inhibitory protein in this fern organ, as well as its purification and invertase–inhibitor interactions. The Mr of the invertase and of its inhibitory protein were 90,000 and 18,000, respectively. SDS-PAGE in the presence of 2-mercaptoetanol gave two subunits for the enzyme (Mr=66,000 and 30,000) and only one for the inhibitor. The inhibitor protein is a glycoprotein (12% w/w of neutral sugars) that did not show agglutinating activity like some others, and also showed a high heat stability at pH 5.0. The optimum pH of invertase activity is 5.0, while invertase inhibitory protein caused maximal inhibition at the same pH value. Invertase–inhibitor complex formation occurs in an immediate manner and a protease activity was discarded. The inhibition is non-competitive (Ki=1.5 × $10^{-6}$ M) without interactions among the binding sites. The complex is slightly dissociable and sucrose was able to partially reduce the inhibitory effect. Up to the present, invertase inhibitory proteins have been found solely in heterotrophic tissues. In this work we demonstrate that this protein is also present in an autotrophic tissue of a lower vascular plant.**

*Keywords*: *Pteris deflexa*; Ptheridophyta; Filicatae; Invertase inhibitor protein; β-D-fructofuranoside fructohydrolase

## INTRODUCTION

Acid β-fructofuranosidases (invertases) hydrolyse sucrose into glucose and fructose. They are of ubiquitous occurrence in the plant kingdom. Most higher plants contain several forms of this

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enzyme that can be distinguished by their pH optima, isoelectric points, solubilities and subcellular locations.

The function of the different β-fructofuranosidases is not entirely clear. It was proposed that cell wall invertases play an important role in phloem unloading[1] and in stress response.[2] Furthermore, it was suggested that soluble invertases participate in the regulation of the hexose level in mature tissues[3] and in the utilization of stored sucrose within vacuoles.[4]

Potential mechanisms for the control of sucrose breakdown by invertases has been described both in source (producers and exporters of sugars) and sink (importers and users of sugars) tissues. It has been reported that the activity of various invertases may increase after ethylene treatment of plant tissues.[5] The extracellular invertase activities are stimulated by auxin and giberellic acid[6–8] suggesting the regulation of sink strength via hormonal control. Furthermore, invertase levels are enhanced by environmental stimuli such as wounding and pathogenic infection.[2]

We demonstrated an entirely different regulatory mechanism based on invertase activity inhibition by the enzyme reaction products (glucose and fructose).[9] In mature leaves of species where fructose is not immediately exported from the vacuole after hydrolysis, this could be an effective regulatory system.[10] Enzyme inhibition by fructose is also observed in invertases from sink or storage tissues, where they could play a similar function.[11,12]

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 inhibitory proteins have only been reported in heterotrophic organs (sink tissues) and cell cultures[13–20] suggesting that this would not be a significant mechanism in the regulation of sucrose turnover in leaves. Some molecular studies on plant soluble invertases and their role on sucrose metabolism in sink tissues have discarded the possibility of an inhibitor-mediated enzyme regulation. We have demonstrated that the soluble acid invertase proteinaceous inhibitor and the soluble acid invertase from *Solanum tuberosum* tubers are located in different cellular compartments.[21] The soluble acid invertase from *S. tuberosum* tubers and most of the protoplast sucrose are vacuolar, while the proteinaceous inhibitor is located in the cell wall. Consequently, a regulation of the activity of the soluble acid invertase (vacuolar) by the proteinaceous inhibitor is not possible.[21] Working with isolated vacuoles and with an analogical *in vitro* system we have also shown the regulatory role of the invertase reaction products on the vacuolar invertase activity.[22] However, Weil *et al*.[20] have reported that the invertase inhibitor expressed in *Agrobacterium tumefaciens* transformed tobacco cells shows several characteristics which appear to indicate a role for an *in vivo* regulation of cell wall invertases.

To the best of our knowledge, there are no available reports about invertase activity in ferns. These plants do not belong to phanerogamous but share some common features with higher plants. The purpose of this work was the study of the inhibitory protein of the soluble acid invertase from *Pteris deflexa* leaves. Its possible function in the plant is discussed.

## MATERIALS 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 Purification

Soluble acid invertases from *S. tuberosum* tubers var Kennebec, *Ricinus communis* and *Carica papaya* leaves, *Oryza sativa* var Blue Bell plantlets, *Equisetum giganteum* L stem and lateral branches and *Cyphomandra betacea* Sendt fruits were prepared according to the literature[23–28], respectively. Invertases from *Schizophyllum comune* and *Pycnosporus sanguineus* (xylophagous fungi) were prepared according to the literature.[29,30]

### Invertase From *P. Deflexa* (Procedure II)

Mature fronds of the sporophyte from *P. deflexa* (200 g) were cut into small pieces and homogenized in 400 ml of 50 mM Na acetate buffer, pH 4.0, containing 500 mM NaCl and 1 mM 2-mercaptoethanol (buffer A). The homogenate was filtered through two layers of gauze, and centrifuged at 21,000 × *g* for 15 min. Solid ammonium sulphate was added to the supernatant. The precipitate obtained between 0 and 90% of saturation 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 to a Sephadex G-75 column (1.7 × 30 cm) equilibrated with buffer B. The column was eluted with the same buffer. The active fractions from the column void volume 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-mercaptoethanol (buffer C). Fractions with invertase activity were pooled and subjected to adsorption chromatography through a (1.2 × 7 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 (50–50 ml). Just a peak of invertase activity coincident with a protein peak was obtained. The active fractions were then dialyzed against buffer C. The purified invertase preparation was adjusted to 5.6 EU/ml.

### Invertase Inhibitory Protein Purification

Procedure II was applied for protein extraction and concentration. Proteins were fractionated on a Sephadex G-75 column (1.7 × 30 cm) equilibrated with buffer B. The column was eluted with the same buffer. Fractions with invertase inhibitory activity were pooled and further purified by adsorption chromatography through a (1.2 × 3 cm) brushite column equilibrated with 10 mM sodium phosphate adjusted to pH 5.5, containing 1 mM 2-mercaptoethanol. Proteins were eluted with 50 ml of 750 mM sodium phosphate containing 1 mM 2-mercaptoethanol. After dialysis against buffer C the active fractions were concentrated by lyophilization. The purified invertase inhibitory preparation was adjusted to 0.04 mg of protein/ml and stored at −20°C until use.

### Mr Determinations

The Mr of the proteins was determined by the method of Andrews[31]. The inhibitory protein and invertase were filtered through a 1.2 × 30 and 2.5 × 35 cm column of Sephadex G-75 and G-150, respectively. The columns were equilibrated and eluted with 10 mM Na OAc buffer, pH 5.5, containing 1 mM 2-mercaptoethanol. The Mr markers used were: alkaline phosphatase (100,000), BSA (66,000), carbonic anhydrase (29,000) and cytochrome c (12,400).

### Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to Laemmli.[32] Two μg of protein/lane was used. BSA (66,000), ovoalbumin (45,000), pepsin (34,000), β-lactoglobulin (18,400) and lysozyme (14,300) were used as standard. Gels were

stained by the method of silver nitrate for proteins.[33]

### Agglutination Assays

Human blood from healthy donors was collected in 10 mM EDTA. Erythrocytes (type A, O and B) were washed three times with 0.15 M NaCl (pH 7) and adjusted to 5% (w/v). The agglutination assays were carried out in small glass tubes in a final volume of 100 μl containing 50 μl of inhibitor preparation, 25 μl of 5% suspension of red blood cells and 25 μl of 0.15 M NaCl. Titres were recorded visually after 60 min at room temperature. Titer was defined as the reciprocal of the highest dilution showing detectable agglutination in the assay conditions.

### Chemical Methods

Proteins were determined with BSA as standard.[34] Proteins in the column fractions were monitored spectrophotometrically at 280 nm. Total neutral carbohydrate content was determined by the method of Dubois[35] with glucose as standard.

### Rate Measurements

Rate determinations were performed in a reaction mixture consisting of $5.6 \times 10^{-3}$ EU of enzyme, 10 μl 0.6 M sucrose, 40 μl 0.2 M NaOAc buffer, pH 5.0, and distilled $H_2O$ or variable amounts of invertase inhibitor (3–18 μl) in a final vol. of 100 μl. Incubations were performed at 37°C for 15 min and reactions were stopped by the Cu alkaline reagent.[36] Reducing power was measured by the method of Nelson.[37] Mixtures that contained no inhibitor were used as control.

One unit of invertase activity is defined as the enzyme amount that catalyses the hydrolysis of 1 μmol of sucrose per min at 37°C and pH 5.0. One inhibitor unit is defined as the amount of inhibitory protein necessary to reduce to 50% the activity of one invertase unit at pH 5.0 and 37°C.

### Effect of PH on the Invertase and on the Inhibitor–invertase Complex Activities

The invertase and the inhibitor–invertase complex activities were determined in the range pH 3–8. The reaction mixtures contained 10 μl ($5.6 \times 10^{-3}$ EU) invertase obtained by procedure II and 80 mM buffers of various pHs in a final vol. of 100 μl. The activity of the inhibitor–invertase complex was assayed after addition of 7.5 μl of inhibitor to the same incubation mixture. The buffers used were: 0.2 M glycine–HCl (pHs 3.0–4.0), 0.2 M sodium acetate (pHs 4.5–6.0) and 0.2 M sodium phosphate (pH 6.5–8.0). Reactions were initiated by addition of 10 μl of 0.6 M sucrose and incubated for 15 min at 37°C.

### Binding of Invertase–inhibitor in Absence or Presence of Substrate

The experiments were performed at pH 5.0 in 80 mM sodium acetate buffer and at 37°C. Invertase ($5.6 \times 10^{-3}$ EU) and inhibitor (7.5 μl) were preincubated at 4°C in the absence or presence of sucrose (final concentration 2.1 mM) for 15 min. After preincubation the sucrose concentration was adjusted to 60 mM and the residual invertase activity was determined as described.

## RESULTS AND DISCUSSION

### Occurrence of an Invertase Inhibitory Protein in *P. Deflexa* Fronds

Acid invertase extraction was performed by two methods: Procedure I with low ionic strength and slightly alkaline pH (50 mM sodium phosphate buffer, pH 7.5) and procedure II in which high ionic strength and acidic pH (50 mM sodium acetate buffer, pH 4.0, 500 mM NaCl)

was used. In procedure I the homogenate was concentrated with solid ammonium sulphate until 90% of saturation, dialyzed and chromatographed through Sephadex G-150. A loss of invertase activity was observed when the eluted fractions were stored at −20°C. The effect of freezing on the invertase activity was evaluated by invertase activity determinations after the extract was fractionated and stored at −20°C for different times. An increasing activity loss was observed when the invertase activity was assayed after increasing storage times. These findings suggested the occurrence of an invertase–inhibitor complex in the enzyme preparation. In fact, the recovery of invertase activity was about 60% after a 20-fold dilution of the active fractions as soon as they were eluted from the column (Fig. 1). Furthermore, increasing enzyme concentration (2.5–12.5 μg of protein in 100 μl of final volume) in the assays produced no proportional increase in the reaction rate (Fig. 2). Still more, a substantial loss of invertase activity (44%) was observed when heat inactivated (7 min at 100°C) partially purified invertase preparation (procedure I) was added to the assays (Fig. 3). An attempt at separation of both compounds was made by adsorption chromatography on Brushite. An electrophoretically pure invertase preparation free from the inhibitory protein was obtained, but the latter protein could not be recovered. A new procedure for inhibitor and invertase isolation was outlined.

### Invertase and Invertase Inhibitor Purification

The application of high ionic strength and acid pH to the tissue homogenization allowed the purification of an invertase and an invertase inhibitory protein from *P. deflexa* fronds. In this procedure (procedure II), the homogenate was concentrated with solid ammonium sulphate until 90% saturation, dialysed and chromatographed through Sephadex G-75. The invertase activity in the preparation did not undergo activity increase by dilution. Otherwise, a straight line was obtained with increasing enzyme concentration in the assays (not shown). Invertase was found in the void volume of the Sephadex G-75 column while the protein with inhibitory activity was included in the column and eluted at 70 ml (Fig. 4). The void volume fractions with invertase activity were pooled and subjected to gel filtration and to

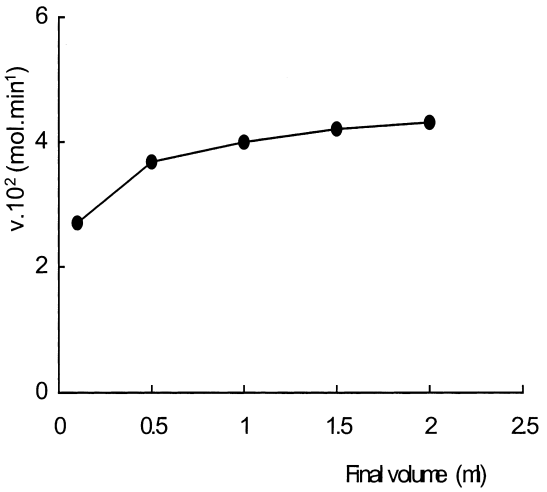


FIGURE 1 Effect of dilution on enzyme activity. Reaction mixtures consisting of 60 mM sucrose, 80 mM NaOAc buffer, pH 5.0, 10 μl of enzyme preparation ($1.6 \times 10^{-3}$ UE) in variable final volumes (0.1–2 ml) were incubated at 37°C for 15 min.

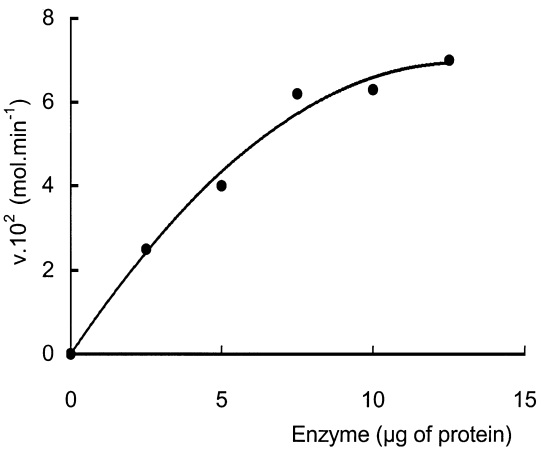


FIGURE 2 Effect of enzyme concentration on the reaction rate. Reaction mixtures consisting of 60 mM sucrose, 80 mM NaOAc buffer, pH 5.0 and variable amounts of enzyme (2.5–12.5 μg of protein) in a final volume of 100 μl were incubated at 37°C for 15 min.

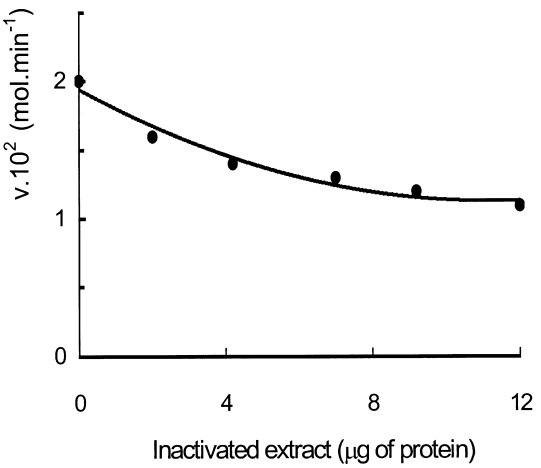


FIGURE 3 Effect of heat inactivated partially purified enzyme preparation on invertase activity. Reaction rates were assayed in mixtures consisting of 60 mM sucrose, 80 mM NaOAc buffer, pH 5.0, 10 μl of purified enzyme preparation ($5.6 \times 10^{-3}$ UE) and variable amounts of heat inactivated (at 100°C for 7 min) partially purified enzyme preparation (0–12 μg) in a final volume of 100 μl. Incubations were performed at 37°C for 15 min.

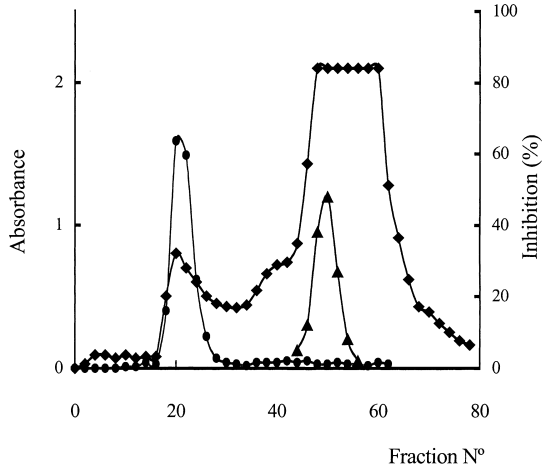


FIGURE 4 Sephadex G-75 cromatography of crude extract. Fractions of 1.4 ml were collected. Each fraction was analysed for invertase (●–●), and invertase inhibitory (▲–▲) activities according to the procedures described in Materials and Methods. Proteins (◆–◆) were estimated by absorbance at 280 nm.

adsorption chromatography. Just a peak with invertase activity coincident with a protein peak was obtained.

The inhibitory protein was further purified by adsorption chromatography up to electrophoretic homogeneity. This procedure allowed the recovery of active acid soluble invertase and invertase inhibitory protein. The Mr of the inhibitor and the invertase were 18,000 and 90,000, respectively, as determined by gel filtration. Otherwise, SDS-PAGE of the inhibitor in the presence of 2-mercaptoethanol gave only one band, Mr=18,000 (not shown). Similar Mr values were found for invertase inhibitors from higher plants.[13–21] Under the same conditions *P. deflexa* invertase was dissociated into two subunits (Mr=66,000 and 30,000) suggesting that it is a heterodimer.

### Invertase–inhibitor Interactions

The optimum pH of the invertase activity is 5.0, and this is the pH in which the inhibitor caused maximal invertase inhibition (Fig. 5). This behavior suggests that an appreciable interaction of invertase–inhibitor occurs around this pH. In order to investigate the complex formation kinetics, the enzyme alone and the enzyme-inhibitor mixture were incubated at 4°C and pH 5.0 for different times before substrate addition. An inhibition increase was not detected (not

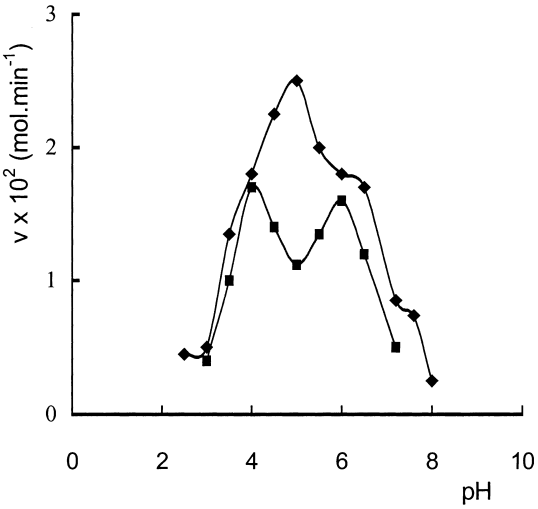


FIGURE 5 Effect of pH on invertase and inhibitor complex activities. Invertase activity with (■–■)and without (◆–◆) inhibitory protein was analysed.

shown). Consequently, the inhibitor was not a protease and the inhibitory action was merely dependent on the interaction of invertase-inhibitor. The effect of sucrose concentration on invertase activity in the presence of variable amounts of inhibitor is shown in Fig. 6. The inhibition is non-competitive (Ki=1.5 × $10^{-6}$ M) without interactions among the binding sites (Fig. 6 inset). The small Ki value obtained suggests the existence of an undissociable or slightly dissociable complex. In order to examine the binding dissociability of the complex, the enzyme concentration was held constant and the amount of added inhibitor was varied. When the reciprocal of the reaction rate was plotted against the added inhibitor (μg), a pronounced curve was obtained (Fig. 7A). Furthermore, a straight line was obtained when the percentage inhibition was plotted against variable inhibitor amounts and a 50% inhibition was reached with 1.6 × $10^{-6}$ M inhibitor concentration (Fig. 7B). These results are consistent with the presence of an undissociable complex. If the complex were highly dissociable, the first method of plotting would have produced a straight line, while with the second method a curve would have been obtained. Figure 8 shows the effect of a constant inhibitor concentration on increasing enzyme amounts. In this case the percentage inhibition decreased with increasing enzyme concentration, and the slope of the curve decreased with increasing enzyme amounts. This finding is also consistent with an undissociable invertase–inhibitor complex. A highly dissociable complex would produce the same percentage inhibition regardless of enzyme concentration. Considering these results, one may conclude that the invertase–inhibitor complex is of an undissociable nature under our experimental conditions. Nevertheless, enzyme concentration experiments carried out with partially purified enzyme from procedure I (Fig. 2) showed a deviation from linearity and the enzyme activity of this enzyme preparation increased after a 20-fold dilution (Fig. 1). In contrast, this behavior was not observed in the experiments carried out with partially purified extracts from procedure II (not shown), which was adequate to separate the inhibitor from the enzyme. We conclude that the invertase–inhibitor complex is slightly dissociable rather than undissociable.

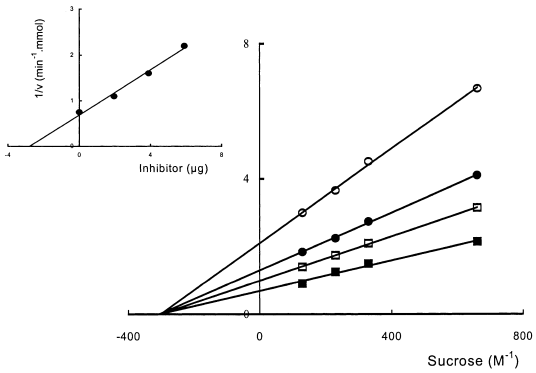


FIGURE 6 Lineweaver–Burk plots for invertase–inhibitor complex activities. Invertase activity was analysed in mixtures that contained variable amounts of inhibitor: 0.19 μg (□–□), 0.39 μg (•–•), 0.59 μg (○–○) and without inhibitor (■–■). Reaction rates were assayed after incubation at 37°C for 15 min. Inset: replot of 1/v as function of inhibitor amount (μg).

Furthemore, the substrate, sucrose, protected the invertase activity against the inhibition produced by the inhibitory protein (Fig. 9). This behavior is similar to that found for cell wall invertase and inhibitor obtained from *Agrobacterium tumefaciens* transformed tobacco cells. Such a system was reported to work not only *in vitro* but also *in vivo* assays.[38] Sucrose protection might control invertase inactivation by invertase inhibitor and this fact would be of physiological significance.[20]

## Similarities Among the Properties of *P. Deflexa* and Other Invertase Inhibitory Proteins

Invertases from *Schyzophylum commune*, *O. sativa* and *E. giganteum* were not inhibited by *P. deflexa* invertase inhibitory protein. However, *S. tuberosum* and *Lenzites elegans* invertases lost 47 and 30% of their activities, respectively, when incubations of these enzymes with *P. deflexa*

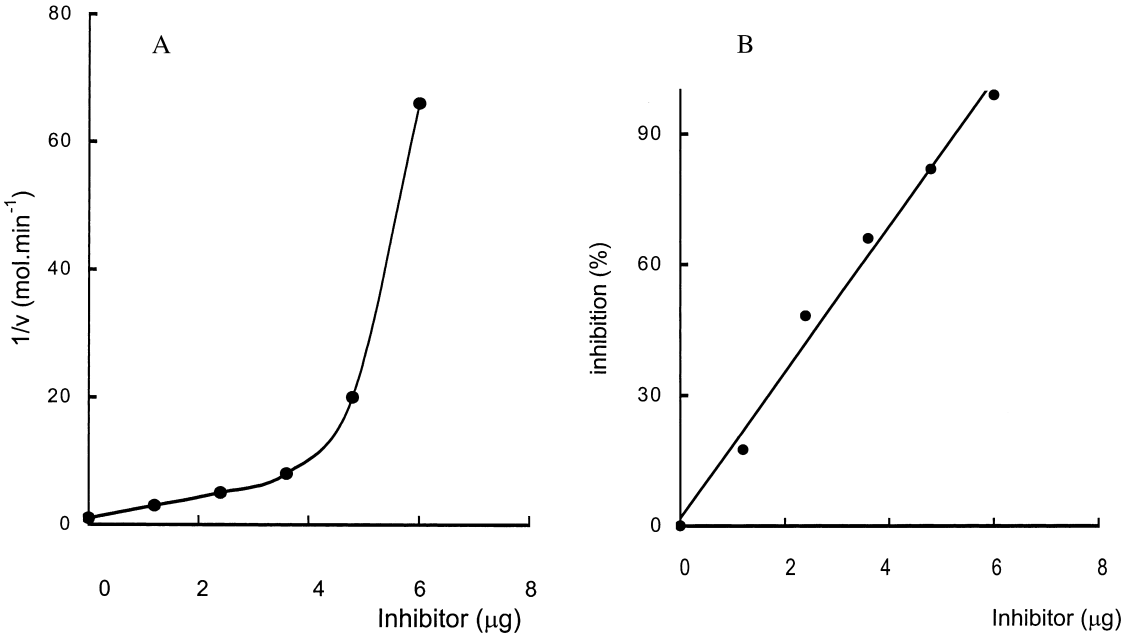


FIGURE 7 Effect of various amounts of inhibitor added to a fixed amount of enzyme. Invertase activity was measured in mixtures containing $5.6 \times 10^{-3}$ UE of invertase, 80 mM NaAcO buffer, pH 5.0, 60 mM sucrose and variable amounts of inhibitor (2–6 μg). Results were plotted as reciprocal of reaction rate (A) and as percentage of inhibition (B).

invertase inhibitory protein were made. A similar inhibitory effect was produced by *S. tuberosum*, *Ipomoea batatas*, *Beta vulgaris*, *C. betacea* inhibitory proteins on different fungi and higher plant invertases[28]. These results suggest that the inhibitors may not be entirely specific for invertases from different tissues, species, genera and families of plant. The invertase inhibitor from *P. deflexa* leaves showed high heat stability

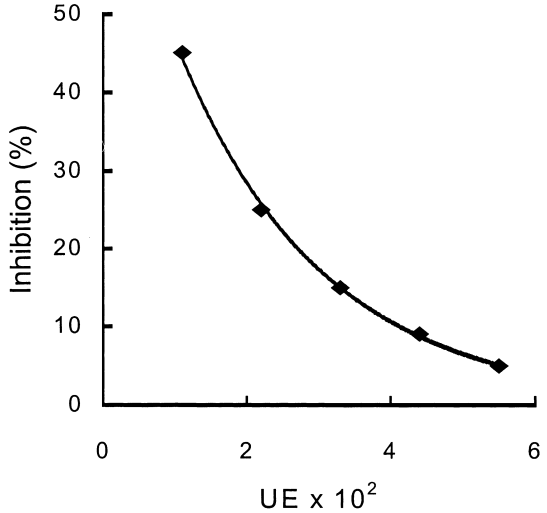


FIGURE 8 Effect of a constant inhibitor concentration on increasing enzyme amounts (■–■).

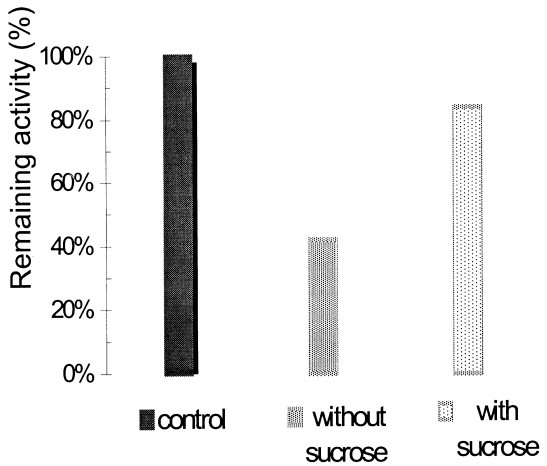


FIGURE 9 Protective effect of sucrose against the invertase inhibition produced by the inhibitory protein. Invertase ($5.6 \times 10^{-3}$ UE) and inhibitor were preincubated at 4°C in the absence or presence of sucrose (final concentration 2.1 mM) for 15 min. After preincubation the sucrose concentration was adjusted to 60 mM and the residual invertase activity was determined as described in Materials and Methods.

at pH 5.0, like that which occurs with other protein inhibitors from heterotrophic tissues. Furthermore, invertase inhibitors from other higher plants have similar molecular weights and monomeric structures.

### Differences Between the Properties of *P. Deflexa* and Other Invertase Inhibitory Proteins

*Pteris deflexa* invertase inhibitory protein was shown to be a glycoprotein (12% w/w of neutral sugars) while others are not. Furthermore, in spite of other known invertase inhibitory proteins that show agglutinating activity, the inhibitor from *P. deflexa* did not have this property.

### Conclusions

In plants, the function of invertase inhibitory proteins is not entirely clear. In some systems it was suggested a specific interaction between invertase and the inhibitory protein of the same tissue location suggesting a role in invertase activity regulation[20]. Up to the present time, invertase inhibitory proteins were found solely in heterotrophic tissues, suggesting that this is not a significant element in the regulation of sucrose turnover in leaves. Considering our results, this conclusion should be reviewed taking into account that the leaf is an organ where this protein can be found, at least in a Pteridophyte, a lower vascular plant. Although the physiological function of this inhibitor is still unknown, a role in leaf invertase activity modulation should not be discarded.

In this work we demonstrated that this inhibitor has some properties that are different from other invertase inhibitory proteins isolated from heterotrophic tissues (tubers, fruit, flowers). This is the first report on the presence of an invertase inhibitory protein in photosynthetic tissues.

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