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INVERTASE PROTEINACEOUS INHIBITOR OF *CYPHOMANDRA BETACEA* SENDT FRUITS

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This work describes a new invertase proteinaceous inhibitor from *Cyphomandra betacea* Sendt. (tomate de árbol) fruits. The proteinaceous inhibitor was isolated and purified from a cell wall preparation. The pH stability, kinetics of the inhibition of the *C. betacea* invertase, inhibition of several higher plant invertases and lectin nature of the inhibitor were studied. The inhibitor structure involves a single polypeptide (Mr = 19000), as shown by gel filtration and SDS-PAGE determinations. N-terminal aminoacid sequence was determined. The properties and some structural features of the inhibitor are compared with the proteinaceous inhibitors from several plant species (*Beta vulgaris* L., *Ipomoea batatas* L. and *Lycopersicon esculentum* Mill.). All these inhibitors share lectinic properties, some common epitopes, some aminoacid sequences and a certain lack of specificity towards invertases of different species, genera and even plant family. In consequence, the inhibitors appear to belong to the same lectin family. It is now known that some lectins are part of the defence mechanism of higher plants against fungi and bacteria and this is a probable role of the proteinaceous inhibitors.

Keywords: *Cyphomandra betacea* Sendt.; Solanaceae; Invertase proteinaceous inhibitors; β-D-fructofuranoside fructohydrolase

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INTRODUCTION

Invertases, the enzymes responsible of the hydrolysis of sucrose to glucose and fructose are widely distributed in higher plants. They often occur as multiple forms differing in pH optima, isoelectric points and subcellular localization in many tissues. Some invertases may be modulated by the hexose pool in plant tissues because their activity is inhibited by the reaction products, glucose and fructose.[1] Invertase levels are also enhanced by environmental stimuli such as wounding and pathogenic infection,[2] indicating that some of the changes in invertase activity are due to synthesis and destruction of the enzyme.

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 isolated and characterized from potato[3,4] and maize endosperm.[5] Invertase proteinaceous inhibitors were also isolated from red beet, sugar beet and sweet potato roots,[6] *Dioscorea rotundata* tuber,[7] wilting carnation petals[8] and *Nicotiana tabacum* L. crown-gall cells.[9,10]

We have demonstrated that the soluble acid invertase proteinaceous inhibitor and the soluble acid invertase from *Solanum tuberosum* L. tubers are located in different cellular compartments. 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.[11] Working with vacuoles and with an analogous *in vitro* system we have also shown the regulatory role of the reaction products.[12] However, Weil *et al.* [9] have reported that the invertase inhibitor expressed in tobacco cells transformed with *Agrobacterium tumefaciens* shows several characteristics which appears to indicate a role for an *in vivo* regulation of cell wall invertases.

The present paper describes the purification and properties of an invertase proteinaceous inhibitor from *Cyphomandra betacea* Sendt. fruits (tomate de árbol). Its properties and activities are compared with those of *Beta vulgaris* L., *Lycopersicon esculentum* Mill., *Ipomoea batatas* L. and *Solanum tuberosum* L. Finally a new role is proposed for the putative proteinaceous inhibitors of plant invertases.

MATERIALS AND METHODS

Plant Material

Edible *B. vulgaris* L. and *I. batatas* L. roots and *L. esculentum* Mill. fruits were bought in the market. *S. tuberosum* L. tubers var. Kennebec were a kind gift from the Estación Experimental Agroindustrial Obispo Colombres. *C. betacea* Sendt. plants were cultivated in the garden at San Miguel de Tucumán. Ripe fruits of *C. betacea* were collected and frozen at −20°C until use.

Reagents

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

Proteinaceous Inhibitor Purifications

The proteinaceous inhibitors from *S. tuberosum* tubers and *L. esculentum* fruits were extracted and purified according to Isla *et al.*[12] and Pressey,[15] respectively. *B. vulgaris* and *I. batatas* root proteinaceous inhibitors were prepared as described by Pressey.[6] The proteinaceous inhibitor from *C. betacea* Sendt. fruits was prepared as follows: ripe fruits (1 kg) were homogenized in 1 l of cold water. The preparation was adjusted to pH 3 by addition of 2 M HCl, stirred during 15 min and centrifuged at 15,000 × g for 15 min. The insoluble fraction (cell wall) was washed with 1.5 l of cold water at pH 3. Then, it was suspended in 200 ml of 0.25 M NaCl, the pH was adjusted to 1.7 and centrifuged at 15,000 × g for 15 min. The supernatant was adjusted to pH 4.75 and concentrated to 97 ml by tangential ultrafiltration. The extract was applied to a 13 × 1.5 cm DEAE-Sepharose CL-4B column equilibrated with 10 mM sodium phosphate adjusted to pH 5.5 (solution A), washed with the same solution and eluted with a linear gradient of NaCl (0–1 M, 50–50 ml) in solution A. Aliquots of the inhibitor solution were chromatographed on a 23 × 2.5 cm column of Sephadex G-75 equilibrated and eluted with 0.2 M NaCl. Fractions of 2.5 ml were collected. Protein was detected by absorbance at 280 nm. Aglutination was checked with human red blood cells of groups A, B and O from healthy donors. The inhibitory effect on invertase activity was assayed at each purification step. Pure inhibitor preparation was kept at −20°C until use.

All proteinaceous inhibitors were prepared to electrophoretic homogeneity.

Soluble Acid Invertase Purification

Soluble acid invertases from *S. tuberosum* tubers var Kennebec, *Ricinus communis* L. and *Carica papaya* L. leaves and *Oryza sativa* L. var. Blue Bell plantlets were prepared according to Isla *et al.*,[17] Prado *et al.*,[18] Rojo *et al.*,[19] and Isla *et al.*,[20] respectively. Invertase from *Equisetum giganteum* L. was prepared as follows: The frozen plants without roots (500 g) were cut into pieces (1.5 cm long.) and homogenized in 250 ml of 50 mM sodium phosphate buffer, pH 7, containing 50 mM NaCl, 5 mM $MnSO_4$ and 1 mM 2-mercaptoethanol. The homogenate was filtered through two layers of gauze and centrifuged at 21,000 × g for 15 min. The supernatant fluid was saturated with solid ammonium sulphate. The pellet was resuspended in 10 ml of 10 mM sodium acetate buffer, pH 4.5, with 50 mM NaCl and 1 mM 2-mercaptoethanol (Buffer A) and dialyzed against the same buffer for 2 h. The extract was filtered through a 2.5 × 45 cm Sephadex G-150 column. Fractions with invertase activity were pooled and concentrated five times by lyophilization. The invertase was adsorbed in a 3 × 10 cm brushite column and eluted with a linear gradient of sodium phosphate solution (0.01–0.5 M, 50–50 ml) adjusted to pH 5.5. Fractions of 2.5 ml were collected and those with invertase activity were pooled. The preparation was filtered through a 1 × 5 cm Bio-Gel P-6 column equilibrated and eluted with Buffer A. Fractions with invertase activity were pooled, concentrated by lyophilization and kept at −20°C until use.

Invertase from *C. betacea* ripe fruits was obtained as follows: ripe fruits (250 g) were cut into pieces (1 × 1.5 cm) and homogenized in 250 ml of 100 mM sodium phosphate buffer, pH 7.5, containing 200 mM NaCl and 1 mM 2-mercaptoethanol. 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 20 and 50% saturation was collected by centrifugation, resuspended in 10 mM sodium acetate buffer, pH 4.5, containing 1 mM 2-mercaptoethanol (Buffer B) and dialyzed against the same buffer for 2 h. It was then applied to a Con A-Sepharose column (5 × 1 cm) equilibrated with Con A buffer (100 mM sodium citrate buffer, pH 6, with 1 M NaCl, 1 mM $MgCl_2$ and 1 mM $MnCl_2$) without α-methylmannopyranoside. The column was washed with the same buffer and eluted with Con A buffer with 250 mM α-methylmannopyranoside. The active fractions were pooled and further purified by gel filtration on a Sephadex G-150 column (2.5 × 45 cm) equilibrated and eluted with 10 mM sodium acetate buffer, pH 4.5. Fractions with invertase activity were pooled and concentrated by lyophilization. All invertases were purified to electrophoretic homogeneity.

Enzyme Assays

Incubation mixtures contained 20 µl of enzyme, 20 µl 0.6 M sucrose, 40 µl 0.2 M sodium acetate buffer pH 4.5 or 5.5 and distilled H_2O or variable amounts of invertase inhibitors (4–12 µg) in a final vol. of 200 µl. Incubations were performed at 37°C and the reactions were stopped by the Cu alkaline reagent.[21] Reducing power was measured by the method of Nelson.[22]

One unit of invertase activity is defined as the amount of enzyme that catalyses the release of 1 µmol of reducing groups per min. at 37°C.

One unit of inhibitor is defined as the amount of proteinaceous inhibitor that reduces to 50% the activity of 2 EU (enzyme units) at pH 4.5 and 37°C.

Mr Determination

The Mr of the protein was determined by the method of Andrews[23] using a 1.2 × 90 cm column of Sephadex G-75 equilibrated and eluted with 10 mM sodium acetate buffer, pH 4.5 or 5.5. The Mr markers used were BSA (66,000), carbonic anhydrase (29,000) and cytochrome c (12,400).

SDS-PAGE

Protein samples (2 µg) were treated and analyzed by electrophoresis as described by Laemmli.[23] BSA (66,000), ovalbumin (45,000), pepsin (34,000), β-lactoglobulin (18,400) and lysozyme (14,300) were used as standards.

Aminoacid Sequence Analysis

C. betacea inhibitor (5 µg) was subject to SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA; Immobilon, pore size 0.45 µm) at 100 Volt. during 1 h and then 20 min at 150 Volt. The membrane was stained with Coomassie Brillant Blue R-250 and the inhibitor band was cut out of the membrane. The N-terminal amino acid sequence was determined on an Applied Biosystem Model 476 A protein sequencer equipped with an on-line analysis system.

Western Blot

All invertase inhibitors (2 µg) were separated by SDS-PAGE on a 15% gel and then transferred to a nitrocellulose membrane. The filter was then treated with antibodies raised against purified invertase inhibitor (1/1600

diluted inhibitor antiserum in TBS) from *S. tuberosum* tubers. Cross-reacting bands were identified using anti-rabbit immunoglobulin conjugate labeled with alkaline phosphatase (Sigma Immunochemicals) and staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Agglutination Assays

Human blood was collected in 10 mM EDTA. The erythrocytes (type A, O and B) were washed 3 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 preparations, 25 µl of a 5% suspension of red blood cells and 25 µl of 0.15 M NaCl. Titres were recorded visually after 60 min at room temperature. The titre is defined as the reciprocal of the highest dilution giving visible aggregation. One hemagglutination unit (HAU) is the minimum amount of lectin able to produce a visible aggregation of red cells.

The carbohydrate-binding specificity of the *C. betacea* proteinaceous inhibitor was determined by inhibition assay. A constant amount of lectin (5 µg) in 50 µl and 25 µl of red blood cells were added to each tube containing 25 µl of different sugar amounts between 0.01–0.1M. Controls were carried out.

Chemical Methods

Protein was determined with BSA as standard.[25] Protein in column fractions was monitored spectrophotometrically at 280 nm. Total neutral carbohydrate content was determined by the method of Dubois *et al.*[14] with glucose as standard.

RESULTS AND DISCUSSION

Purification of the Invertase Proteinaceous Inhibitor from *C. betacea* Fruits

The occurrence of an invertase proteinaceous inhibitor in crude extracts of *C. betacea* fruits, an old Inka crop, was determined for the first time in this work. The extraction and purification of the invertase proteinaceous inhibitor was accomplished taking advantage of the fact that the inhibitor is stable at

pH 1.7 while the soluble acid invertase is labile in these conditions, as described for the potato inhibitor.[13] The invertase inactivation by acid treatment was complete at pH 1.7 for fruit extracts. This treatment followed by chromatography on DEAE Sepharose CL-4B yielded a solution of invertase proteinaceous inhibitor free of invertase. Gel filtration of the inhibitor solution on Sephadex G-75 removed other associated proteins. This procedure was required for complete purification of the invertase proteinaceous inhibitor. About 5,000 inhibitor units. Kg^{-1} of pericarp tissue were found in ripe fruits. The purified inhibitor had 315.7 HAU/mg of protein (Table I).

Properties of *C. betacea* Invertase Proteinaceous Inhibitor

The Mr of the invertase inhibitor was determined to be 19,000 Daltons by gel filtration on Sephadex G-100 (not shown) and is coincident with the Mr determined by SDS-PAGE (Figure 1). In consequence the structure of the native protein is monomeric. Structural carbohydrates were not detected in the inhibitor[14] as is the case for all the reported proteinaceous inhibitors.[15]

The proteinaceous inhibitor produced erythrocyte agglutination and consequently may be defined as a lectin. Its agglutinating capacity was not inhibited by sugars such as D-galactosamine, α and β-methyl galactoside,

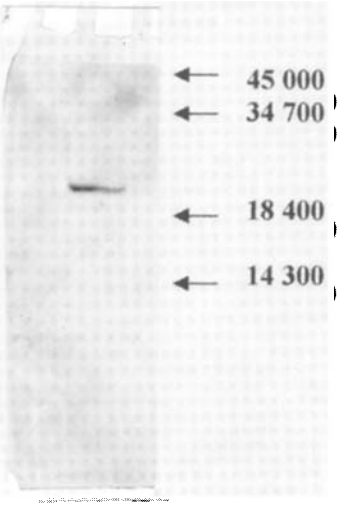

FIGURE 1 Purity of *Cyphomandra betacea* Sendt. inhibitor analyzed by SDS-PAGE. The sample contained 2 µg of protein. The arrows indicate the position of the molecular weight markers (Sigma) used: ovalbumin (45,000), pepsin (34,000), β-lactoglobulin (18,400) and lysozyme (14,300). Proteins were stained with $AgNO_3$.

TABLE I Purification of *Cyphomandra betacea* Sendt. invertase proteinaceous inhibitor from ripe fruits

Fraction	*Total Protein* (mg)	*Total Volume* (ml)	*Total HAU**	*Total Inhibitor Units*	*Specific Activity* (IU/mg *protein*)	*Yield (%)*	*Purification Fold*
Crude extract	4025	875	204800	43065	10.7	100	1
DEAE Sepharose CL 4B	36	100	6400	6250	173.6	14.5	16.22
Sephadex G-75	3.8	75	1200	5000	1315.8	11.61	123

* One hemagglutination unit: the minimum amount of lectin able to produce a visible aggregation of red cells.

D-glucosamine, D-fructose, rhamnose, raffinose, D-xylose, L-arabinose, β-methyl fructoside, stachyose, melezitose, mannose, D-glucose, N-acetylglucosamine, fucose, lactose, sucrose, turanose, N,N′-diacetylchitobiose, chitin oligossacharides and the glycoprotein mucin. However, the proteinaceous inhibitor recognized the oligosaccharide N,N′,N″-triacetylchitotriose and the glycoprotein fetuin. When the *C. betacea* inhibitor preparation was heated for 5 minutes at 100°C, it not only retained its inhibitory action on invertase activity but also its agglutinating capacity as was found for tomato inhibitor.[15] Invertase inhibitors have been proposed as modulators of invertase activities, but if invertase inhibitors are lectins,[16] a different physiological role would be attributable.

The purification and N-terminal sequence of invertase proteinaceous inhibitor from *N. tabacum* cell suspension culture, *L. esculentum* fruit and *Arabidopsis thaliana* have been previously described.[10,15] We have determined the N-terminal sequence of *C. betacea* inhibitor: ISDFNGPGKKLNDD. The comparison of this sequence with those of other proteinaceous invertase inhibitors didn't reveal homology after a data base search (SWISS-PROT 23, PC Gene, Intelligenetics).

Inhibitory Mechanism of Invertase Inhibitor

Addition of the *C. betacea* invertase inhibitor to reaction mixtures containing invertases from different sources (*O. sativa*, *R. communis*, *C. papaya*, *S. tuberosum*, *E. giganteum*, and *C. betacea*) decreased the sucrose hydrolysis rate. The inhibition of *C. betacea* invertase by the inhibitor was pH-dependent, with maximal inhibition about pH 4.75. Firstly, pre-incubation experiments were carried out to check whether the inhibitory action of the inhibitor depended on proteolysis or binding interaction. Pre-incubation of the invertases and inhibitor for periods ranging from 0–30 minutes at 37°C before the addition of the substrate did not increase the inhibition, indicating that the inhibitor was not a protease that destroys the enzyme.

The effect of three inhibitor concentrations on invertase activity is shown in Figure 2. The inhibition is non-competitive with $K_i = 3.3$ mM. The high K_i value obtained indicates that large inhibitor amounts would be required for effective inhibition and a dissociable complex could be expected. The same results were obtained with and without pre-incubation of the mixture proteinaceous inhibitor-enzyme.

To investigate whether the enzyme-inhibitor interaction was reversible or irreversible, the enzyme concentration was held constant and the inhibitor amount was varied (4–12 μg). As shown in Figure 3 a straight line was

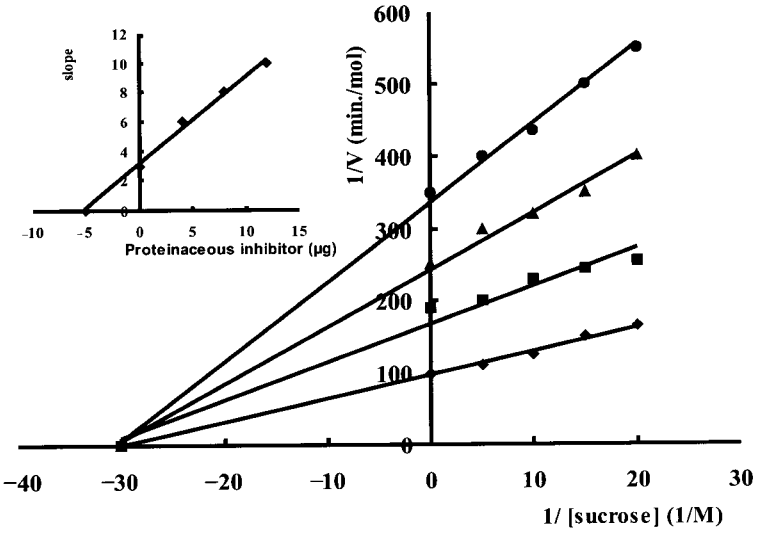

FIGURE 2 Lineweaver-Burk plot of inhibition of soluble acid invertase from *Cyphomandra betacea* Sendt. by the proteinaceous inhibitor from the same plant. Control (-◆-), 4 µg (-■-), 8 µg (-▲-), and 12 µg (-□-) of protein. Inset: replot of slope vs. inhibitor amount (µg).

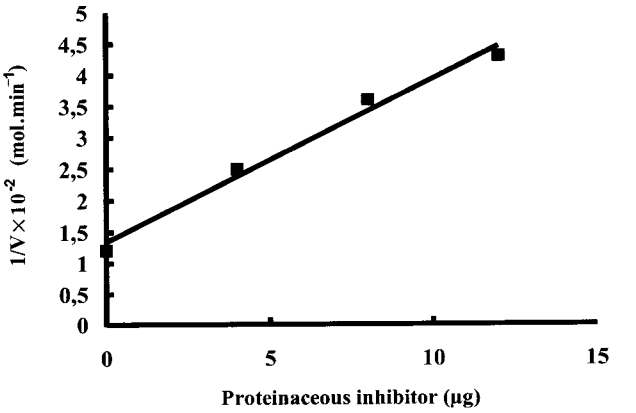

FIGURE 3 Effect of increasing amounts of invertase proteinaceous inhibitor from *Cyphomandra betacea* Sendt. on soluble acid invertase activity from the same source. The experimental conditions are described in Materials and Methods.

obtained when the reciprocal of the reaction rate was plotted against increasing inhibitor amounts. This result is consistent with a dissociable complex. When the enzyme concentration was varied and the inhibitor

concentration and all other components of the incubation mixture were held constant, the reaction rate had a linear relationship with respect to the enzyme concentration. Consequently, the percent inhibition was constant (Figure 4).

Otherwise, when the proteinaceous inhibitor-enzyme complex was incubated at constant pH and substrate concentration in increasing reaction volumes the reaction rates in the control and in the assay remained constant

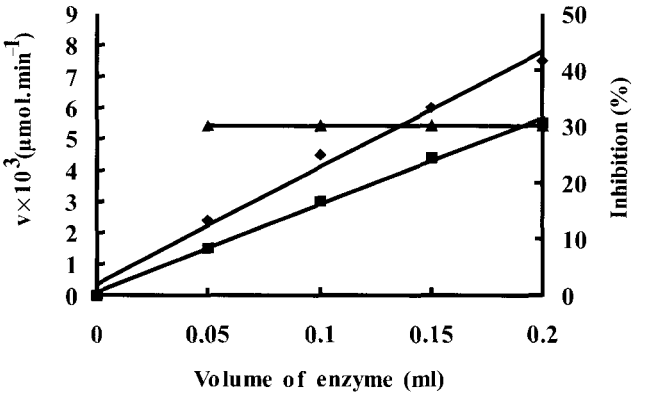

FIGURE 4 Soluble acid invertase activity as a function of increasing amounts of enzyme (up to 4.6 EU) in the presence and absence of *Cyphomandra betacea* Sendt. inhibitor. The final volume of the reaction mixture was 350 µl. All other components were held constant. Reaction rate without inhibitor (-◆-) and with 15 µg of inhibitor (-■-). Percentage inhibition (-▲-).

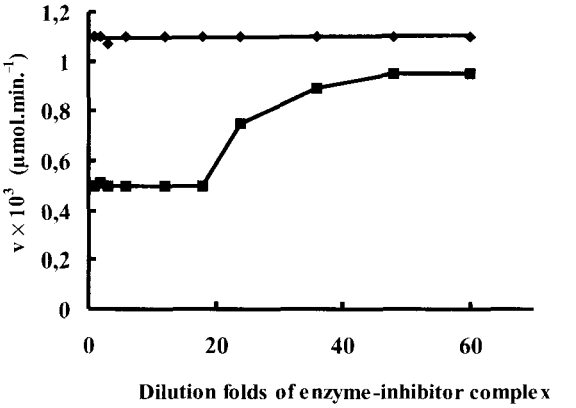

FIGURE 5 Dilution effect on the complex proteinaceous inhibitor-invertase from *Cyphomandra betacea* Sendt. fruit. Invertase with proteinaceous inhibitor (-■-), invertase without proteinaceous inhibitor (-◆-).

until 20 fold dilution. Under these experimental conditions the inhibitor-enzyme complex seems to be a non dissociable complex. For higher dilutions (from 20–60 fold) the complex was dissociated into its components (Figure 5). The enzyme-inhibition interaction was not affected by foaming nor by ionic strength (2–20 mM $CaCl_2$, NaCl, $ZnSO_4$). This suggests that this interaction was not of an ionic nature.

Sucrose (up to 20 mM) did not protect the *C. betacea* invertase against proteinaceous inhibitor inhibition.

Comparison of the *C. betacea* Inhibitor Properties with these of Other Inhibitors

A similar inhibitory effect was found for *S. tuberosum* and *L. esculentum* (Solanaceae), *Ipomoea batatas* (Convolvulaceae) and *B. vulgaris* (Chenopodaceae) proteinaceous inhibitors on different higher plant invertases. As shown in Table II the minimum amount of the proteinaceous inhibitor that produced 50% inhibition was different. Low levels of the proteinaceous inhibitor were required to attain inhibition. These results show that the proteinaceous inhibitors are nonspecific for invertases from different species, genera, and even families of plants.

A test of agglutination was performed for all inhibitors (Table III). The preparation of each proteinaceous inhibitor produced erythrocyte agglutination and consequently these inhibitors may be defined as lectins.

In order to further compare all inhibitors their immunological relationships have been analyzed by western blot analysis (not shown). For this purpose a cross reaction was performed among polyclonal antiserum raised against the proteinaceus inhibitor from *S. tuberosum* tubers and purified

TABLE II Effect of proteinaceous invertase inhibitors from different origins on higher plant soluble invertases

Invertase	*Proteinaceous inhibitors (μg of protein)**				
	B. vulgaris	*L. esculentum*	*C. betacea*	*I. batatas*	*S. tuberosum*
O. sativa	7.2	1.55	2.08	12.43	12.23
R. communis	16	2.29	1.98	18.35	15.3
C. papaya	16	2.13	12.7	17	11.21
C. betacea	4.73	2.86	2.16	18.20	18.8
E. giganteum	3.58	2.01	17.7	15.4	26.70
S. tuberosum	2.85	3.82	2.23	14.3	5.46

* Minimal amount of the proteinaceous inhibitor that produces 50% inhibition (The reaction mixture contained 2 invertase units/ml in 0.2 M sodium acetate buffer, pH 4.75).

TABLE III Agglutination titre of invertase inhibitors

Proteinaceous inhibitors (5 μg of protein)	*Agglutination titre*
Beta vulgaris	2
Cyphomandra betacea	16
Ipomoea batatas	2
Lycopersicon esculentum	8
Solanum tuberosum	32

preparations of invertase proteinaceous inhibitors from other sources.[12] This antiserum cross-reacted with all analyzed proteinaceous inhibitors. The results indicate that all the analyzed polypeptides have at least some similar amino acid sequence and share some common epitopes.

In this work we demonstrated the presence of a new invertase proteinaceous inhibitor in *C. betacea* fruits. Other authors have demonstrated the presence of invertase proteinaceous inhibitors in non-photosynthetic organs, such as tubers, roots, fruits, cell suspension culture. Until now there is no information on the presence of invertase proteinaceous inhibitors in photosynthetic tissues. These findings would suggest that the function of these inhibitors would not be important in the regulation of sucrose turnover in photosynthetic tissue.

The activities of the proteinaceous inhibitors are nonspecific, since the invertase proteinaceous inhibitor from one species can exert its action on invertases from other plant origins. Furthermore, all of them are lectins and share some structural similarities. Consequently, the *C. betacea* inhibitor may belong to a group of homologous proteins and a similar function would be expected for them in plants. At this time their physiological significance is not clear.

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