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A hysteretic invertase from *Equisetum giganteum* L

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Abstract

The invertase from *Equisetum giganteum* L., a lower vascular sporophytic plant, was purified to chromatographic and electrophoretic homogeneity. The enzyme appears to be a pentamer, M_r 91,000, formed by identical subunits (M_r 18,000). An isoelectric point of 4.5 was found for the protein. The optimum pH was about 4.5 and the preferred substrate is sucrose, $K_m = 10.4$ mM. Glucose and fructose are classical non-competitive ($K_i = 120$ mM) and competitive ($K_i = 96$ mM) inhibitors, respectively. Proteins which behave as activators of the enzyme suppress the inhibitory action of the reaction products. The activation energy of the hydrolytic reaction is 18,000 cal/mol. The outstanding property of the invertase is a hysteretic behavior when the pH changes from 3.05 to 4.5. The lag time is independent of the enzyme concentration suggesting that slow conformational changes are induced by pH variation and not by different polymerization states.

Keywords: *Equisetum giganteum* L; Equisetaceae; β-fructofuranoside fructohydrolase; Hysteresis; Invertase

1. Introduction

Most plants produce sucrose as a result of the photosynthetic process, and sucrose is the main form of photosynthate transport. Consequently, as sucrose occupies a central role in plant metabolism, many studies about its metabolism and transport have been undertaken, some of them involving sucrose utilization by plants. Invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) is a relevant enzyme of sucrose degradation. It was shown that sucrose and invertase are within the vacuoles (Isla, Leal, Vattuone & Sampietro, 1992) and a recent work has found a similar vacuolar and in vitro pattern of sucrose hydrolysis in a higher plant (Isla, Vattuone & Sampietro, 1998). Thus, the vacuole has an important role in sucrose utilization of the plant. Two types of acid invertases were essentially described (Sampietro, 1995): those inhibited by fructose in a simple competitive way and those in which fructose behaves as a two site competitive inhibitor with site interaction. In the present report the invertase from *Equisetum giganteum* L., a lower vascular plant used medicinally (diuretic, hemostatic, vulnerary, etc.) in Argentina, was characterized for the first time. Studies of the molecular and kinetic characteristics of the invertase showed simple competitive inhibition by fructose, but a pH-dependent lag period, suggesting a hysteretic behavior of the enzyme.

2. Results

2.1. Enzyme purification

The invertase from *E. giganteum* L. was purified through precipitation with solid ammonium sulfate, gel size exclusion chromatography, and adsorption chromatography on a brushite column, resulting in a 555-fold enzyme purification (Table 1). The purified enzyme was homogeneous as determined by DEAE-Sephadex A-50, Sephadex G-150 and Sephadex G-100 column chromatography, and by polyacrylamide gel electrophoresis (PAGE). In all of the chromatographic

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Table 1
Purification of the soluble acid invertase from *Equisetum giganteum* L

Purification step	Vol. (ml)	EU[a]	Total protein (mg)	Specific activity	Purification
Supernatant of centrifugation (27,100 × *g*)	380	576	3200	0.18	1.00
$(NH_4)_2 SO_4$ sat.	15	475	500	0.95	5.28
Centrifugation (27,100 × *g*)	14.5	428	428	1.0	5.55
Sephadex G-150	120	303	9.2	33.0	183.33
Brushite	20	77	0.7	100	555.55

[a] EU, enzyme units.

and electrophoretic systems the enzyme behaved as a single peak with an activity band coincident with the protein band (data not shown).

2.2. Molecular properties

The M_r determined by gel filtration was about 91,000; however electrophoretic M_r determinations of the invertase in denaturing conditions gave a single protein band with an M_r of 18,000 (Fig. 1), suggesting that the invertase is a pentameric complex. Total neutral sugar determinations showed that the invertase is a glycoprotein with 14% sugars.

The enzyme is active in a pH range between 3 and 8 and has its optimum at about 4.5 (data not shown); the pI of the invertase is 4.5. The reaction velocity is linear during 60 min at 37° under the conditions described in Section 4. Sucrose, raffinose and stachyose hydrolysis exhibited simple Michaelis–Menten kinetics, with K_m values of 10.4, 26 and 43 mM, and V_{max} values of 65, 72 and 80 μmole/min, respectively. Thus,

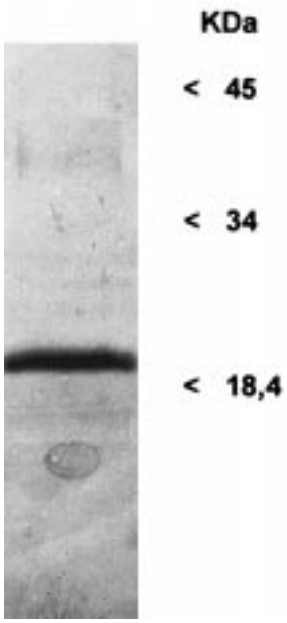

Fig. 1. Subunit M_r determination after SDS-PAGE. The arrows show the marker positions.

the enzyme is more efficient with sucrose as a substrate. Melibiose, melezitose, cellobiose, turanose, α-methylfructoside, β-methylfructoside, inulin and levan are not hydrolyzed by the enzyme (data not shown).

2.3. Effect of the reaction products

The reaction products glucose and fructose were inhibitors of the enzyme. The kinetics of fructose inhibition corresponds to a simple competitive inhibition (Fig. 2A and insert), $K_i = 96$ mM. Glucose is a classical non-competitive inhibitor (Fig. 2B and insert), $K_i = 120$ mM.

2.4. Determination of the activation energy

The activity of the enzyme was determined between 10 and 50°C, and the activation energy was calculated to be 18,000 cal/mol. Arrhenius plot was a straight line (not shown).

2.5. Effect of proteins and lectins

The invertase is activated by bovine serum albumin, ovalbumin, alkaline phosphatase, urease and trypsinogen (Fig. 3). The enzyme activation by proteins depends on the nature of each protein. Thus, higher activation is produced by bovine serum albumin (183%) and lower by urease (42%). The saturation point is independent of the molar concentration for each protein.

When a mixture of invertase and urease was chromatographed on a Sepharose CL-4B column equilibrated and eluted with Buffer A containing 7.0×10^{-7} M urease, a single protein peak, containing urease and invertase activity was obtained (Fig. 4A). This result corroborates the formation of a complex between urease and invertase with an apparent M_r of 569,500. In the absence of urease a single peak of protein and invertase activity was obtained (Fig. 4B).

Lectins were also activators of the invertase (Fig. 5); 95% activation was observed with Con A, 147% with *L. culinaris* and 160% with ricin. *L. culinaris* lectin and Con A are specific for glucose and mannose

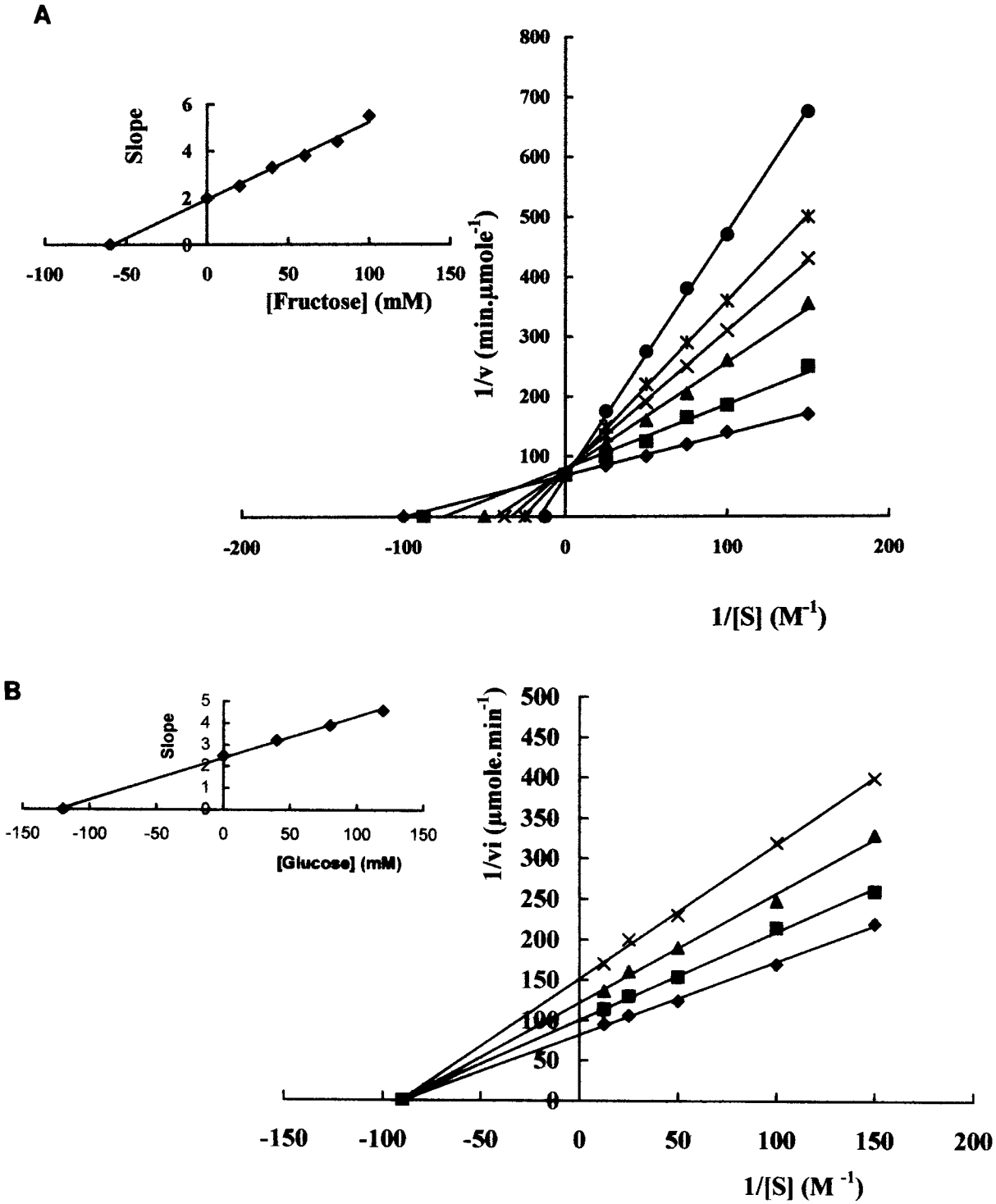

Fig. 2. (A) Lineweaver–Burk plot of the effect of fructose on invertase activity and replot of slope vs fructose concentration: control without inhibitor (♦); 20 mM (■); 40 mM (▲); 60 mM (×); 80 mM (☆) and 100 mM (●) fructose. (B) Lineweaver–Burk plot of the effect of glucose on invertase activity and replot of slope vs glucose concentration: control (♦); 40 mM (■); 80 mM (▲) and 120 mM (☆) glucose.

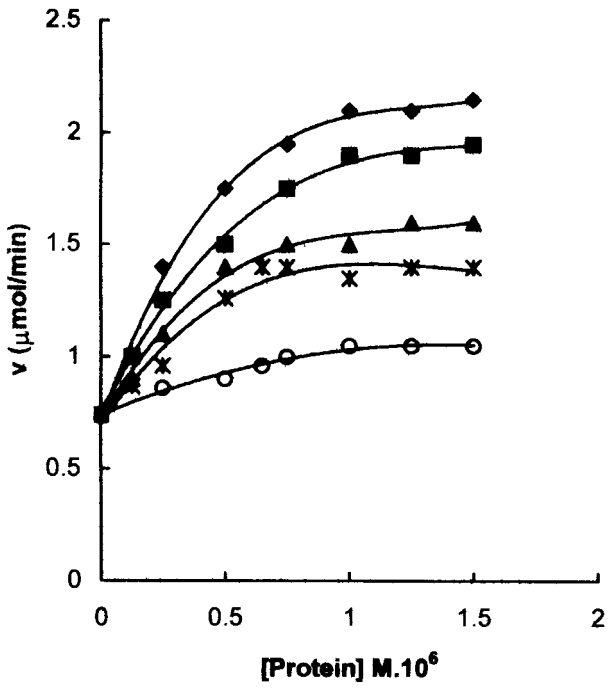

Fig. 3. Activation of *E. giganteum* invertase by proteins: ovalbumin (■); alkaline phosphatase (▲); trypsinogen (×); urease (○) and bovine serum albumin (◆).

(Entlicher, Tichá, Kostir & Kokourek, 1969; Goldstein, Hollerman & Merric, 1965), while ricin is specific for galactose and N-acetylgalactosamine (Nicolson, Blaustein & Etzler, 1974). The differences in sugar specificities of the activating lectin suggest a complex sugar composition of invertase. Proteins suppress the inhibitory effect of the reaction products (data not shown).

2.6. Effect of pH on invertase activity

Fig. 6 shows the invertase activity when the enzyme is preincubated at 4°C for 30 min at pH 3.05 and 4.5 and then incubated at pH 4.5 for different times. The preincubated enzyme at pH 3.05 showed a lag period of about 10 min before a constant velocity was achieved. It is clear that the enzyme changes from a less active to a more active form at pH 4.5.

When enzyme pre-incubated at pH 3.05 was put into acetate buffer, pH 4.5, and the time before adding the substrate was varied, the lag period was abolished after 10 min of incubation. Otherwise, the lag period diminishes with increasing concentrations of substrate in the assay (Fig. 7). This behavior is typical of hysteretic enzymes (Frieden, 1979; Ricard & Cornish-Bowden, 1987; Valero & Garcia-Carmona, 1991) and can be attributed to a conformational change or to a change in the degree of enzyme polymerization.

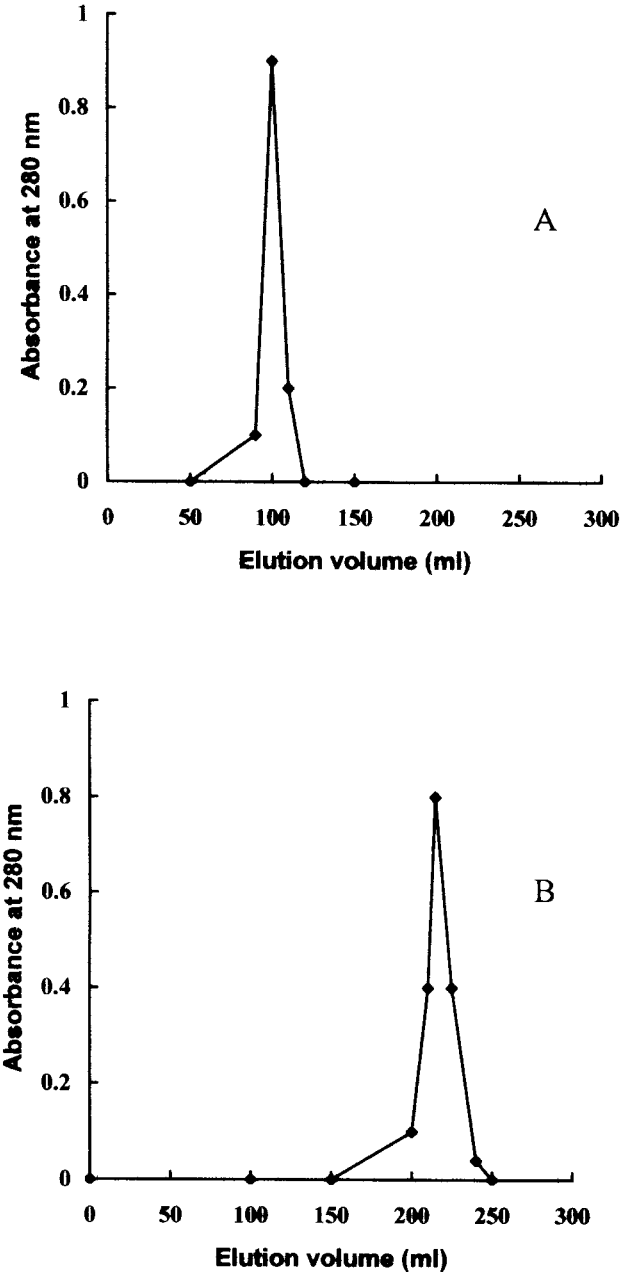

Fig. 4. (A) Gel filtration chromatography of the complex invertase-urease; the column was equilibrated and eluted with Buffer A added with 7.0×10^{-7} M urease. (B) Gel filtration chromatography of purified acid soluble invertase.

Fig. 8 shows measures of the lag period as a function of enzyme concentration. Even with a 5-fold enzyme increase the lag period was not modified. According to these experiments the rate changes are not due to different polymerization states but to a conformational change of the protein.

For comparative purposes *Ricinus communis* invertase (Prado, Vattuone, Fleischmacher & Sampietro, 1985) was purified and assayed for hysteretic behavior under similar pH conditions. *R. communis* invertase

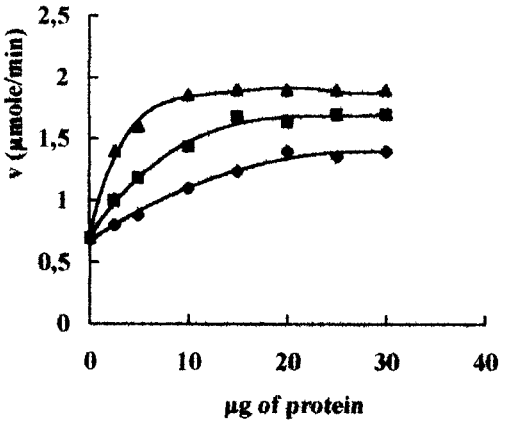

Fig. 5. Lectin effect on the activity of the *E giganteum invertase*: Ricin (▲), *Lens culinaris* lectin (■) and Concanavalin A (◆).

was not affected by preincubations at acid pH (data not shown).

3. Discussion

Invertases from higher plants are currently the subject of many studies. However, studies on pteridophytes are very limited. Pteridophytes are lower vascular plants that differ from higher vascular plants essentially in their reproduction by spores. These plants have life cycles with alternation of free gametophytic (haploid) and sporophytic (diploid) phases. A South American plant of the order Equisetales, *Equisetum giganteum* L. (horsetail), was selected for our studies. Invertase was purified to electrophoretic and chromatographic homogeneity from the aerial parts (sporophyte) of *E. giganteum*. The enzyme works as a soluble acid invertase. Consequently, the vacuole is the most likely intracellular location for the enzyme. In *Solanum tuberosum* (Isla et al., 1998), *R. communis* (Prado et al., 1985), sweet clover (Boudet, Canut & Alibert, 1981), sugar beet (Leigh, Rees, Fuller & Banfield, 1979) and barley leaves (Wagner & Wiemken, 1986) soluble acid invertase was found to be

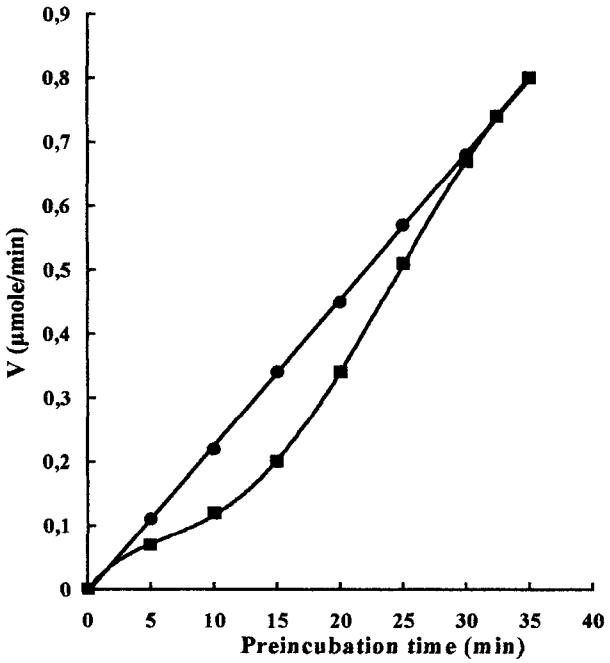

Fig. 6. Activity of the acid soluble invertase preincubated at pH 3.05 (◆) and at pH 4.5 (●). Invertase was preincubated at pH 3.05 and at pH 4.5 at 4°C for 30 min. Then, the pH was adjusted to pH 4.5. Invertase activity was determined as described in Section 4.

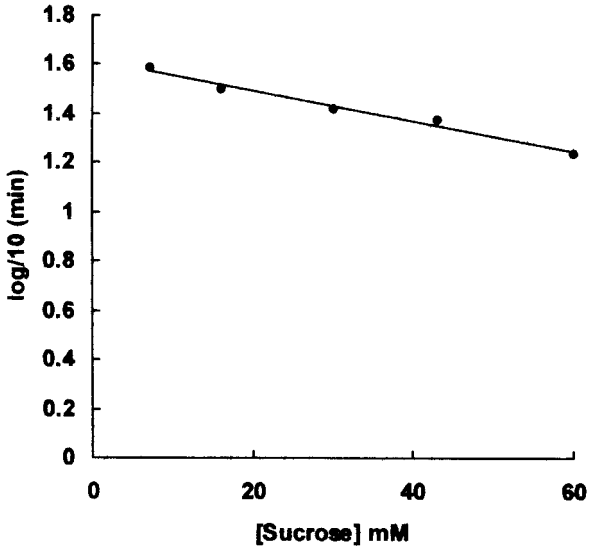

Fig. 7. Effect of substrate concentration on the lag period produced when invertase is preincubated at pH 3.05 and the activity assayed at pH 4.5.

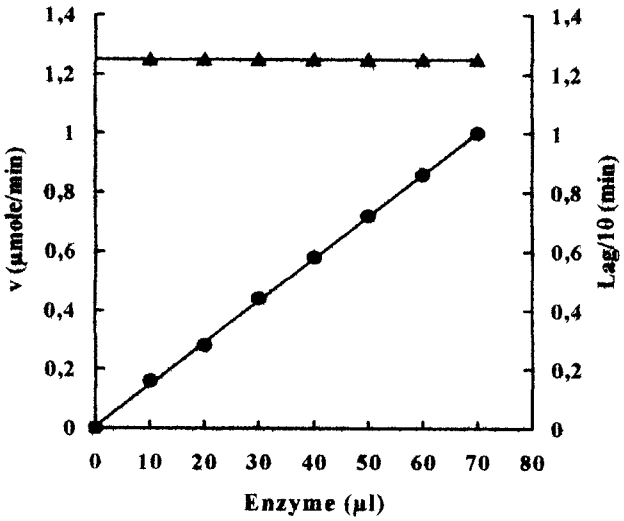

Fig. 8. Effect of enzyme concentration on the reaction rate (●) and on the lag period (▲). Enzyme assays were performed at pH 4.5.

present in isolated vacuoles. According to M_r determinations of the native protein by gel filtration chromatography and in denaturing gel electrophoresis conditions, the enzyme appears to be a pentameric complex made of identical subunits. The enzyme forms complexes with proteins and lectins and these complexes are more active than the original non-complexed invertase. The activated enzyme exists as a dimeric heterologous form composed of one molecule of enzyme and one molecule of the added protein, as demonstrated by M_r determinations of the complex invertase-urease. Both enzymes of the complex show their respective enzyme activity. This behavior has some similarities with the soluble acid invertase from *R. communis*. In this case the activated complex is made by two subunits of the heptameric invertase and one molecule of the activator (Prado et al., 1985). When urease was used as activator it also retained the activity in the heterologous trimer. Soluble invertases from *Carica papaya* fruits (Lopez, Vattuone & Sampietro, 1988), *Solanum tuberosum* tubers (Sampietro, Vattuone & Prado, 1980), sugar cane leaf sheaths (Isla, Vattuone & Sampietro, 1991), *Tropaeolum majus* (Isla et al., 1988) and *R. communis* (Prado et al., 1985) leaves have been also reported to be activated by proteins.

The invertase activity is inhibited in a simple non-competitive way by glucose and competitively by fructose. As the protein-activated enzyme is not inhibited by the reaction products, it appears improbable that these effectors could exert control on invertase activity in vivo. Most of these properties suggest this enzyme is similar to the *R. communis* invertase (Prado et al., 1985). The vacuolar invertase from *R. communis* is an acid soluble enzyme, inhibited in vitro by glucose and fructose in a classical non-competitive and competitive fashion, respectively. *R. communis* invertase is activated by proteins, with suppression of the inhibitory effect of the reaction products. Fructose inhibition is also observed in enzymes from sink or storage tissue, where it could be part of an effective regulatory system of the short-term balancing of sucrose breakdown (Lopez et al., 1988; Sampietro et al., 1980).

E. giganteum invertase appears to be a hysteretic enzyme as suggested by the lag period occurring after pH changes from 3.05 to 4.5. Since the lag time is independent of the enzyme concentration, a change in the polymerization degree of the enzyme is not likely.

The possibility of enzyme inactivation was ruled out by enzyme preincubation during varying time lengths under the assay conditions. Otherwise, the lag period observed at pH 3.05 decreased when sucrose concentration was increased in the incubation medium. We were not able to detect hysteretic properties in the invertase from *R. communis*, and hysteresis seems not to be a common behavior among invertases. To our knowledge the invertase from *E. giganteum* is the first reported hysteretic invertase. This suggests that the described control mechanism for invertase activity may be operating in vivo.

4. Experimental

4.1. Materials

Fresh *E. giganteum* L. plants were provided by the Botanical Institute Miguel Lillo, Tucumán, Argentina. All chemicals used were of analytical grade.

4.2. Enzyme purification

Stems and lateral branches (100 g) of *E. giganteum* L. (sporophyte) were cut into small pieces, suspended in 250 ml of 50 mM sodium phosphate buffer, pH 7, containing 1 mM 2-mercaptoethanol and 50 mM sodium chloride, and were homogenized in a blender. The homogenate was filtered through two layers of cheese cloth. The filtrate was centrifuged at $27{,}000 \times g$ for 10 min. Then, the supernatant was saturated with solid ammonium sulfate and centrifuged at $27{,}000 \times g$ for 10 min. The pellet was resuspended in 10 ml of 10 mM sodium acetate buffer, pH 4.5, containing 50 mM sodium chloride and 1 mM 2-mercaptoethanol (buffer A). The solution was then dialyzed against buffer A. After clarification by centrifugation the solution was filtered through a 2.5×40 cm Sephadex G-150 column equilibrated and eluted with buffer A. An invertase peak eluting at 110 ml was pooled and subjected to adsorption chromatography through a 2×11 cm brushite column equilibrated with 10 mM sodium phosphate adjusted to pH 5. Proteins were eluted with a 0.01–0.5 M sodium phosphate gradient (50 ml–50 ml) adjusted to pH 5. Just a peak of invertase activity coincident with a protein peak was obtained. This preparation showed a single peak on ion exchange chromatography on DEAE-Sephadex A-50 and rechromatography on Sephadex G-100 and Sephadex G-150.

4.3. Invertase assays

Reaction mixtures consisted of 10 μl of 0.6 M sucrose, 40 μl of 0.2 M sodium acetate buffer, pH 4.5, and 20 μl of acid soluble invertase, in a final volume of 100 μl. The mixtures were incubated at 37°C for 30 min and the reactions were stopped with the alkaline reagent of Somogyi (1945). The increase of reducing sugars was determined by the method of Nelson (1944).

4.4. Enzyme units

One enzyme unit was defined as the enzyme amount that produces 1 μmol of product in one min at 37°C and pH 4.5.

4.5. Polyacrylamide gel electrophoresis

Native PAGE was performed according to Ornstein (1964) and Davis (1964). Invertase activity and proteins were detected by the methods of Grabriel and Wang (1969) and Blum et al. (1987), respectively. SDS-PAGE was performed by the method of Laemmli (1970).

4.6. M_r determinations

Relative molecular weight of the native invertase was determined by the method of Andrews (1964) using a 2.5 × 40 cm column of Sephadex G-150. The molecular markers were: ribonuclease A (13,700), chymotrypsinogen A (25,000), bovine serum albumin (67,000), aldolase (158,000). The relative molecular weight of invertase was also determined by the point of optimal activation method (Sampietro, Prado, Sayago & Vattuone, 1988). Invertase from *E. giganteum* (5 μg) was previously inactivated by heating at 100°C for 2 min in a boiling bath. Electrophoresis was carried out at 80 V for 2 h. Molecular weight markers were: β-lactoglobulin (18,400), pepsin (34,700) and ovalbumin (45,000). The M_r of the complex invertase-urease was determined on a 2.5 × 40 cm column of Sepharose CL-4B equilibrated and eluted with Buffer A containing 7.0×10^{-7} M urease.

4.7. Isoelectric focusing

The isoelectric point was determined by electrophoresis on gel slabs prepared with 5% polyacrylamide, 10% glycerol, 2% ampholites selected to establish a pH gradient from 3 to 10. Gels were pre-run 1 h at 200 V. Electrophoresis was run at 100 V for 3 h. Cathodic and anodic solutions consisted of 20 mM sodium hydroxide and 10 mM phosphoric acid, respectively.

4.8. Effect of pH and temperature on invertase activity

The enzymatic reactions were carried out at 37°C for 30 min in three different buffer systems (50 mM): Na acetate buffer, pH 3–5.5, Na-P_i buffer, pH 6.5–8.5; and Na_2CO_3–$NaHCO_3$ buffer, pH 9–10.5. Reactions were run at the optimum pH and temperatures ranging between 20 and 50°C.

4.9. Kinetics of substrate specificity

Incubation mixtures were composed of 10 μl of 0.05–1 M sucrose or 0.05–0.5 M raffinose or stachyose, 40 μl of 0.2 M sodium acetate buffer, pH 4.5 and 20 μl of enzyme in a final volume of 100 μl. Reducing sugars were determined as previously described.

4.10. Effect of the reaction products

Reaction mixtures contained 10 μl of 0.2–1 M fructose or 0.4–1.2 M glucose, 40 μl of 0.2 M sodium acetate buffer, pH 4.5, 10 μl of 0.05–1 M sucrose and 20 μl of enzyme in a final volume of 100 μl. Protein effect on the invertase inhibition by the reaction products was determined using 20 μl of a BSA solution containing 1 mg/ml of protein. Fructose was determined as described by Prado and Sampietro (1994) and glucose by the method of Jorgensen and Andersen (1973).

4.11. Assays for hysteretic behavior

The preincubation mixture consisted of 200 μl of invertase and 40 μl of 0.01 M sodium acetate buffer, pH 3.05. The mixture was incubated at 4°C for 30 min. Thereafter, 40 μl fractions of the mixture were taken and the pH adjusted to pH 4.5. To these fractions 40 μl of 0.2 M sodium acetate buffer, pH 4.5, 50 μl of distilled water and finally 10 μl of 0.6 M sucrose were added. The reaction was followed as described in Section 4.3. Controls with similar preincubation mixtures were performed at pH 4.5 and pH 3.05 and then were put directly in acetate buffer, pH 4.5 and the time of incubation before adding the substrate was varied. The same methodology was applied to *R. communis* invertase obtained and purified as previously described (Prado et al., 1985).

4.12. Protein and lectin effects

The same procedures as for invertase assays were used, except that 20 μl of protein solution (containing up to 1 mg/ml of protein) was added in a final volume of 100 μl.

4.13. Chemical determinations

Neutral sugars were determined by the method of Dubois et al. (1965) using glucose as standard. Proteins were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard.

Acknowledgements

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