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Invertase activity associated with the walls of *Solanum tuberosum* tubers

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Abstract

Three fractions with invertase activity (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) were isolated from mature *Solanum tuberosum* tubers: acid soluble invertase, invertase I and invertase II. The first two invertases were purified until electrophoretic homogeneity. They are made by two subunits with an apparent M_r value of 35 000 and their optimal pH is 4.5. Invertase I was eluted from cell walls with ionic strength while invertase II remained tightly bound to cell walls after this treatment. This invertase was solubilized by enzymatic cell wall degradation (solubilized invertase II). Their K_ms are 28, 20, 133 and 128 mM for acid soluble invertase, invertase I, invertase II and solubilized invertase II, respectively. Glucose is a non-competitive inhibitor of invertase activities and fructose produces a two site competitive inhibition with interaction between the sites. Bovine serum albumin produces activation of the acid soluble invertase and invertase I while a similar inhibition by lectins and endogenous proteinaceous inhibitor from mature *S. tuberosum* tubers was found. Invertase II (tightly bound to the cell walls) shows a different inhibition pattern. The test for reassociation of the acid soluble invertase or invertase I on cell wall, free of invertase activity, caused the reappearance of all invertase forms with their respective solubilization characteristics and molecular and kinetic properties. The invertase elution pattern, the recovery of cell wall firmly bound invertase and the coincidence in the immunological recognition, suggest that all three invertases may be originated from the same enzyme. The difference in some properties of invertase II and solubilized invertase II from the other two enzymes would be a consequence of the enzyme microenvironment in the cell wall or the result of its wall binding.

Keywords: *Solanum tuberosum*; Solanaceae; Potato; *β*-D-Fructofuranoside fructohydrolase; Invertase; Cell wall; Location

1. Introduction

Many studies have been devoted to the purification and characterization of the acid β-D-fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26, optimum pH at the range of 3.5–5.4) from *Solanum tuberosum* tubers (Pressey, 1966; Sasaki, Tadokoro, & Suzuki, 1971; Anderson & Ewing, 1978; Bracho & Whitaker, 1990; Isla, Vattuone, & Sampietro, 1991; Burch et al. 1992). This enzyme catalyses the irreversible cleavage of sucrose to glucose and fructose. For many years the activity of the acid soluble invertase from potato tubers has been thought to be regulated by an endogenous proteinaceous inhibitor (Pressey, 1966, 1967). Recent papers have shown that the endogenous proteinaceous inhibitor and the soluble acid invertase from diverse origin are located in different cell compartments (Isla, Leal, Vattuone, & Sampietro, 1992; Weill, Krausgrill, Schuster, Rausch, 1994). Thus, the soluble acid invertase is vacuolar (symplasmic location), while the proteinaceous inhibitor is located in the cell wall (apoplastic location). As both products are located in non-contiguous compartments, interaction between them appears to be difficult. However, an interaction between the endogenous proteinaceous

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inhibitor and apoplastic invertases is possible. Extracellular invertases from many sources have been described (Singh & Knox, 1984; Faye, Monatassim, & Ghorbel, 1986; Getz, 1991; Ranwala, Iwanami, & Masuda, 1991; Ranwala, Suematsu, & Masuda, 1992). Very often a wall location was attributed to the acid invertases because the invertase activity is present in the wall fraction after tissue homogenization. Extracellular invertases were also described in *Daucus carota* cell suspension cultures where it was demonstrated that polyclonal antibodies raised against the glycosylated enzyme did not cross-react with the chemically deglycosylated invertase (Laurière, Laurière, Sturm, Faye, & Chrispeel, 1988) suggesting that the glycosidic moiety of the invertase has immunogenic properties. However, polyclonal antibodies raised against the vacuolar invertase from *Beta vulgaris* recognize both the enzymatically and the chemically deglycosylated wall invertase from tobacco crown gall cells (Weill & Rausch, 1990). The *N*-terminal sequence of a wall invertase and a soluble invertase from *Daucus carota* had no similarity though some internal sequences were 50–70% identical (Unger, Hofsteenge, & Sturm, 1992). In all cases, the invertase activity that was eluted from walls with high ionic strength was considered as a wall enzyme.

The purpose of this paper is the study of the interrelationships between different invertase activities in plant tissues that are still confusing and ill-understood. This study uses a homogeneous starting material, mature potato tubers, and demonstrates that the freely soluble, NaCl-wall dissociable and tightly wall bound invertases are all derived from the same enzyme. A new experimental design was applied. The catalytic, regulatory and immunological properties of the various invertase forms were established and compared with those of the soluble invertase.

2. Results and discussion

2.1. Solubilization of wall associated enzymes

A comparative study on the eluting capacity of the ionic strength and pH changes on wall associated invertases was made. Wall preparation was treated with 20 mM buffers ranging from pH 3.5 to 8.5 for 5 min. After centrifugation the supernatant was assayed for invertase activity (Fig. 1). The maximal release of invertase activity (60%) from walls was produced from pH 6 to 7 (20 mM NaPi buffer). Similar results were obtained with carrot roots (Ricardo & ap Rees, 1970). An improvement in the release of invertase activity was obtained with ionic strength (1 M NaCl, pH 6.0) that produced 70% of invertase solubilization from walls. The solubilized enzyme is called invertase I throughout this paper. A residual wall invertase activity (invertase II) remained in the wall, as occurs in *Lilium* pollen (Singh & Knox, 1984), and could not be removed by simple treatments [buffers of different compositions, ionic strength (2 M NaCl), cation complexing agents (0.5% w/v EDTA), ionic detergents (0.1% sodium deoxycholate) or freezing and thawing]. Thus, the well known acid soluble invertase from potato tubers is apparently accompanied by two wall associated invertases.

2.2. Enzyme purification

Acid soluble invertase and invertase I were purified to electrophoretic homogeneity (Table 1). A similar pattern with only one invertase activity peak was obtained after gel filtration for both enzymes. Acid soluble invertase eluted from the column between 75 and 90 ml and invertase I between 72 and 87 ml. Also both enzymes were eluted at the same ionic strength (0.35 M NaPi, pH 5) from adsorption columns of calcium phosphate (brushite).

2.3. Enzyme properties

Acid soluble invertase and invertase I show similar pH curves, with an optimum at 4.5 (Fig. 1(A)). Instead, the residue of walls after acid soluble invertase separation shows two different optimum pH, one at 4.5 and the other at 6.5 (Fig. 1(B)). However, after solubilization of invertase I only one optimum pH was found (pH 6.5).

Soluble enzymes (acid soluble invertase and invertase I), alone or mixed, show the same apparent M_r (72 kDa) after gel filtration (not shown). Only one active band was detected after native electrophoresis (not shown). On electrophoresis under denaturing conditions they produce a band with the same subunit apparent M_r (35 kDa) (not shown). According to these results both enzymes are composed by two subunits, as was reported before for the acid soluble invertase from *S. tuberosum* tubers (Bracho & Whitaker, 1990). A different behavior was found for the acid soluble invertase from *S. tuberosum* leaves which has a similar apparent M_r but was reported as a monomeric enzyme (Burch et al., 1992). This enzyme attacks α-glucosides and this is not a property reported for invertases from other plant sources. Table 2 shows the K_m of the acid soluble invertase, invertase I, II and solubilized invertase II. The K_m is similar for acid soluble invertase and invertase I. Invertase II and solubilized invertase II show a similar K_m, but differing from the first two enzymes. In each case sucrose was the preferred substrate, but the affinity for raffinose and stachyose was lower. All preparations

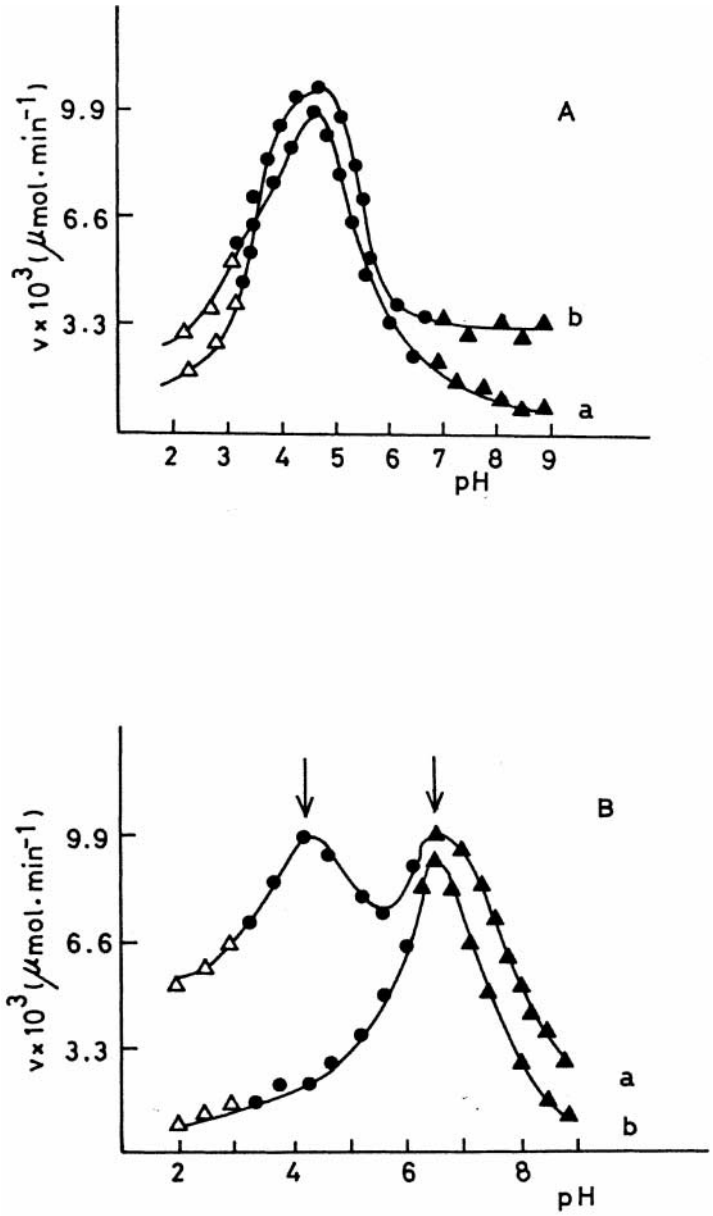

Fig. 1. Effect of pH on invertase activities. (A) Acid soluble invertase (b) and invertase I (a). (B) Fraction C before (a) and after (b) treatment with buffer B. The buffers used were: 0.2 M sodium acetate buffer pH 3–6, – △ –; 0.2 M sodium phosphate buffer pH 6–7, – ● – and 0.2 M sodium barbital buffer pH 7–9.5, – ▲ –.

did not hydrolyse α-glucosides such as trehalose, maltose, melezitose or turanose and α-lactose.

2.4. Effect of reaction products on invertase activities

Glucose produces a classical non-competitive inhibition of the same magnitude on the acid soluble invertase and invertase I activities. However, a higher and similar K_i was found for invertase II and solubilized invertase II. Fructose produces a two site competitive inhibition with interaction between the sites on each invertase. Here again the K_i for the insoluble form of the invertase (invertase II) and for the solubilized invertase II was twice the value obtained for the other two invertases. Acid soluble invertase and invertase I have the same K_i (Table 3). Moreover, the reaction products exhibited the same type and magnitude of inhibition on all the invertase activities after association experiments between soluble invertase and wall free of invertase.

2.5. Effect of proteins, lectins and the endogenous proteinaceous inhibitor on invertase activities

Table 4 shows the effect of proteins and lectins on invertase activities. BSA (bovine serum albumin) pro-

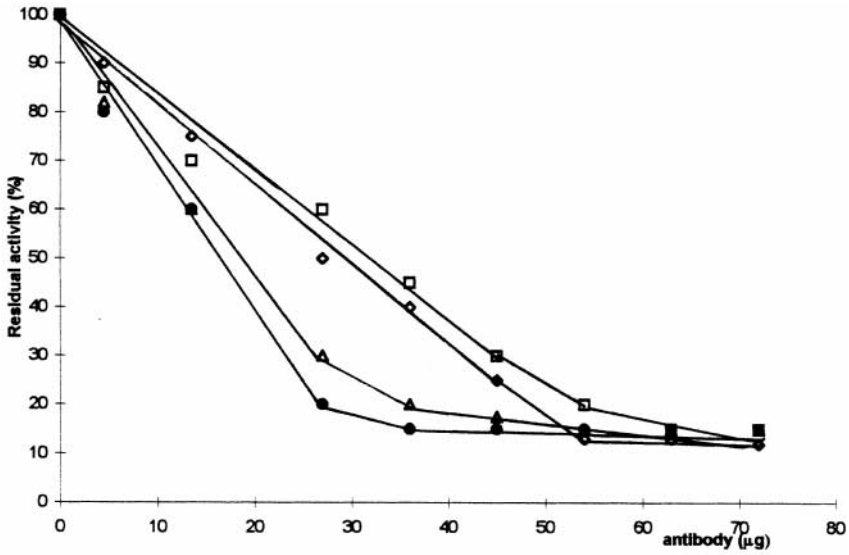

Fig. 2. Effects of the antibody raised against soluble acid invertase on the soluble acid invertase, – ● –; invertase I, – △ –; invertase II, – ◇ – and eluted invertase II, – □ –, activities.

duces an activation of the two soluble invertases and several lectins were inhibitors of all enzymes. The invertase associated with walls shows a different inhibition pattern. The endogenous proteinaceous inhibitor from *S. tuberosum* tubers was inhibitor of all enzymes.

2.6. Reassociation of the acid soluble invertase and invertase I to walls

According to the effects of lectins, BSA, the apparent M_r and subunit determinations, the optimum pH, the catalytic and regulatory properties, acid soluble invertase and invertase I appear to be the same enzyme. Thus, the localization of invertase I in the cell wall may be a technical artifact, yet could be produced during or after tissue disruption. To demonstrate this assumption a test for the reassociation of acid soluble invertase to the walls was developed (Table 5). Crude homogenate was used as control. Acid soluble invertase was added to the heat inactivated walls. The mixture was separated by centrifugation. The recovery of acid soluble invertase in the supernatant was 71.5%. This value is similar to the recovery of acid soluble invertase after tissue disruption. The two optimum pH values (4.5 and 6.7) reappeared in the wall. Walls were treated with 1 M NaCl, centrifuged, washed and inver-

Table 1
Purification of the acid soluble invertase and invertase I from *Solanum tuberosum* tubers

Procedure	Invertase	Enzyme units (total)	Total proteins (mg)	Specific activity (units/mg protein)	Purification factor
Homogenization	acid soluble	1000	1250	0.8	1
	invertase I	318	353	0.9	1
Centrifugation at 27 000*g*	acid soluble	600	1034	0.6	0.7
	invertase I	210	344	0.6	0.8
100% Saturation with $(NH_4)_2SO_4$	acid soluble	570	792	0.7	0.9
	invertase I	180	265	0.7	0.7
Centrifugation at 27 000*g*	acid soluble	530	730	0.7	0.9
	invertase I	172	215	0.8	0.8
Gel filtration Sephadex G-100	acid soluble	320	5.82	55.0	68.6
	invertase I	90	3.10	29.0	16.9
Adsorption (brushite)	acid soluble	208	1.30	160.0	200.0
	invertase I	62	0.5	124.0	72.5

Table 2
Substrate specificity of *Solanum tuberosum* tuber invertases

Substrate	Acid soluble invertase, K_m (mM)	Invertase I, K_m (mM)	Invertase II, K_m (mM)	Solubilized invertase II, K_m (mM)
Sucrose	28 ± 1.1	20 ± 1.0	133 ± 3.5	128 ± 2.5
Raffinose	37 ± 1.0	42 ± 1.2	187 ± 2.6	148 ± 4.2
Stachyose	41 ± 1.2	58 ± 1.3	230 ± 4.4	ND

ND: non-determined.Values ($n = 10$) are means $\pm$ SE.

Table 3
Effect of the reaction products on the activity of acid soluble invertase, invertase I, invertase II and solubilized invertase II

Enzyme	Inhibition type			
	glucose	K_i (mM)	fructose	K_i (mM)
Acid soluble invertase	non-competitive	1.00 ± 0.02	competitive	0.18 ± 0.02
Invertase I	non-competitive	0.98 ± 0.02	competitive	0.20 ± 0.02
Invertase II	non-competitive	1.80 ± 0.03	competitive	0.58 ± 0.07
Solubilized invertase II	non-competitive	1.50 ± 0.03	competitive	0.53 ± 0.08

Values ($n = 10$) are means $\pm$ SE.

tase activity was assayed in the pellet and in the supernatant. The same elution pattern was found in the control and in the reassociation experiment. Thus, invertase I and II occurred again in the walls. The reassociation experiment was carried out with invertase I obtaining identical results. Consequently, the vacuolar enzyme structure is able to produce, per se, all the enzyme species present in the wall.

Table 4
Effect of bovine serum albumin and lectins on acid soluble invertase and invertase I and II activities

Effector	Acid soluble invertase	Invertase I	Invertase II
% Activation			
Bovine serum albumin	30	10	0.0
% Inhibition			
Vicia sativa aggln.	17	14	31
Triticum vulgare aggln.	100	89	44
Crotalaria juncea aggln.	80	82	85
Vicia villosa aggln.	0.0	10	
Ulex europaeus aggln.	50	46	87
Ricine	0.0	0.0	15
Concanavalin A	20	20	35
S. tuberosum aggln.	70	65	80
Endogenous inhibitor from *S. tuberosum* tubers	65	65	72

2.7. Effect of antibodies on invertase activities

Antibodies raised against acid soluble invertase produced a similar in vitro inhibitory pattern on acid soluble invertase, invertase I, II and also on solubilized invertase II activities (Fig. 2). These results suggest structural similarities in these enzymes, or that at least

Table 5
Distribution of invertase activity before and after different cell wall treatments

Experiment	Fraction	Recovery of enzyme activity (% E.U.[a])
1	Control[b] (disrupted tissue)	100
2	Acid soluble invertase	72.9
3	Cell wall preparation before 1 M NaCl treatment	27.1
4	Cell wall preparation after 1 M NaCl treatment	5.8
5	Heated cell wall	0.0
6	E.U. of total preparation after the inactivated cell wall were treated with 1.4 E.U.	100
7	E.U. adsorbed on cell wall preparation after experiment 6	28.5
8	E.U. retained on cell wall preparation after 1 M NaCl treatment	6.4

[a]Enzyme units.
[b]Disrupted tissue (1.5 g fresh weight) produced 0.8 g of wet walls.

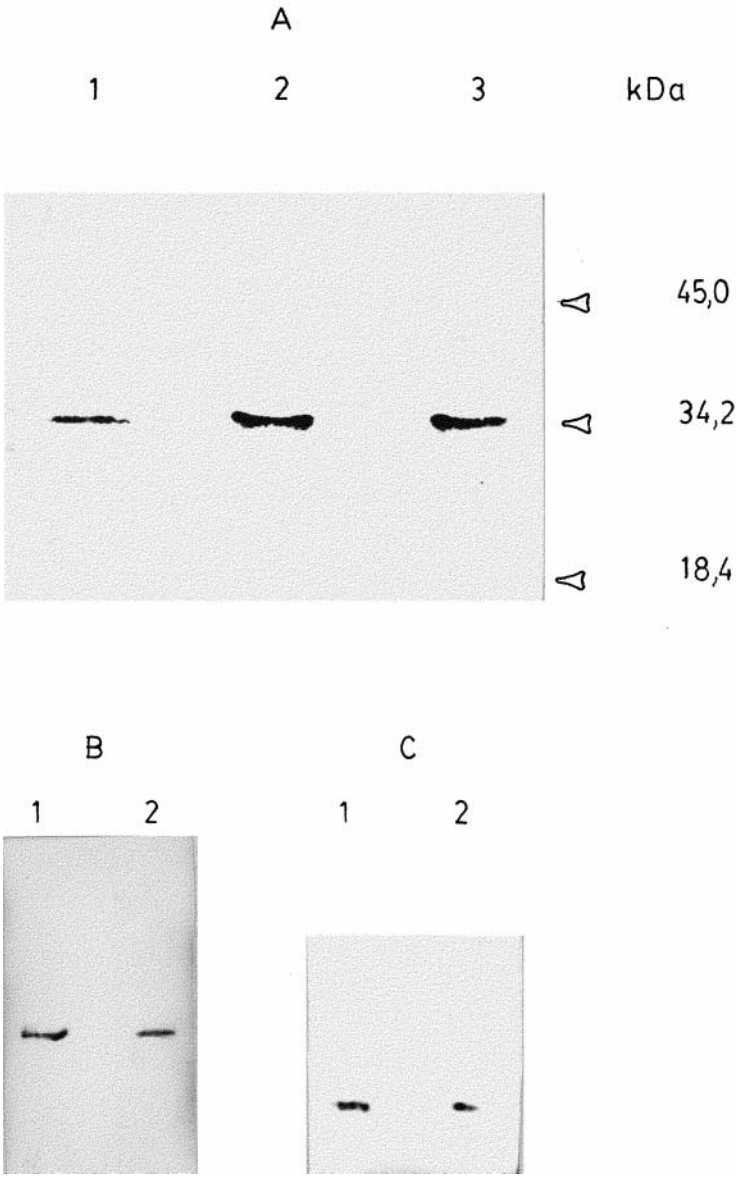

Fig. 3. Immunoblotting of: (A) lane 1: solubilized invertase II after enzymatic wall digestion; lane 2: invertase I and lane 3: acid soluble invertase. The arrows show the position of the M_r markers. (B) Lane 1: deglycosylated acid soluble invertase; lane 2: deglycosylated invertase I. (C) Lane 1: acid soluble invertase; lane 2: deglycosylated acid soluble invertase. Deglycosylation was performed with peptide-*N*-glycosidase F. The deglycosylation process was monitored with the sugar determination by the phenol sulfuric acid method.

the three enzymes would be isoforms, sharing conserved polypeptide domains. Identical results were obtained when the same experimental design was applied to deglycosylated acid soluble invertase and deglycosylated invertase I (not shown). This suggests that the *N*-glycosidic invertase moieties, have no immunogenic significance because antibodies raised against native acid soluble invertase interact with native and deglycosylated invertases.

2.8. Immunoblot analysis

Western blot analysis revealed a related structure among the invertases [Fig. 3(A)]. The apparent subunit M_r obtained for solubilized invertase II was 35 000 that is coincident with the subunit M_r of invertase I and acid soluble invertase. The cross reaction of the antibodies raised against native soluble acid invertase with native and deglycosylated acid soluble invertase, and deglycosylated invertase I suggests an identity in the peptidic moiety [Fig. 3(B) and (C)].

Our results cast some doubts about many reports on the occurrence of cell wall invertases based on wall elution patterns. The reassociation experiments show that the basic structure of the acid soluble invertase has, per se, all the necessary features to produce the apparent isoforms found in the cell wall. However, our results cannot be interpreted as disproving the presence of wall

invertases. Accepting the physiological occurrence of cell wall invertases, the results of solubilization procedures must be interpreted with care to distinguish between actual cell wall and soluble isoforms of the invertase.

3. Experimental

3.1. Plant material

Mature *Solanum tuberosum* tubers var. Kennebec maintained for 2 months at 4°C were used. The tubers were obtained from the Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina.

3.2. Chemicals

All chemicals used were of analytical grade.

3.3. Invertase extraction

All procedures were carried out at 4°C and all centrifugations at 27 000*g* for 15 min unless otherwise stated. Peeled tubers of *S. tuberosum* (100 g) were cut in thin slices (2 mm thick). The slices were put in distilled water and maintained in the dark for 3 h. After being filtered the pieces were homogenized in 125 ml of 50 mM NaPi buffer, pH 7.5, containing 1.3 mM 2-mercaptoethanol (buffer A). The crude homogenate was filtered through gauze. The solid material was washed several times with 50 ml of buffer A and it was collected by centrifugation. The liquid fractions were pooled and centrifuged to eliminate fine debris. The supernatant is fraction A. Solids were resuspended in 200 ml of 10 mM NaOAc buffer, pH 6, containing 1.3 mM 2-mercaptoethanol and 1 M NaCl (buffer B) and left standing for 1 h. Then, the suspension was centrifuged. The supernatant is fraction B. The pellet was several times washed first with buffer B and then with buffer B without NaCl. The solid material (fraction C) was suspended in 10 ml of 10 mM NaOAc buffer, pH 6, containing 1.3 mM 2-mercaptoethanol and 0.2 M NaCl (buffer C) and kept at −20°C until use. Proteins from fractions A and B were separately concentrated, by addition of solid $(NH_4)_2SO_4$ (100% saturation), and were sedimented by centrifugation. The corresponding pellets were suspended in 10 ml of buffer C, dialyzed against the same buffer and clarified by centrifugation. The preparations obtained from fraction A and B contain an acid soluble invertase and a solubilized invertase (called invertase I), respectively. Fraction C contains walls (as shown by microscopical examination) with invertase activity (invertase II).

Solubilization of invertase I at various pH values was examined in 20 mM NaOAc buffers from pH 3.5 to 6, in 20 mM NaPi buffers from pH 6 to 7 and in 20 mM sodium barbital buffers from pH 7 to 8.5. The solubilization of invertase I with ionic strength (0–2 M NaCl) was made in 20 mM NaOAc buffer pH 6.0.

3.4. Purification of acid soluble invertase and invertase I

Fraction A (15 mg of protein) and fraction B (12 mg of protein) were filtered through Sephadex G-100 columns (40×2.5 cm), equilibrated and eluted with buffer C adjusted at pH 4.75 (buffer D), in separate runs. Fractions (2.5 ml) containing invertase activity were pooled and chromatographed through a brushite (Tiselius, Hjertén, & Levi, 1956) column (5×2 cm) equilibrated with 10 mM NaPi adjusted to pH 5 containing 1.3 mM 2-mercaptoethanol. The columns were washed with the same soln and were then eluted with 100 ml of a 10 to 500 mM gradient of NaPi adjusted to pH 5 containing 1.3 mM 2-mercaptoethanol. Fractions with invertase activity were pooled and filtered through a Bio-Gel P-6 column (5×1.5 cm) equilibrated and eluted with buffer D. Again, fractions containing invertase activity were pooled, concd and stored at −20°C until use. Thus, two purified invertases were obtained, the acid soluble invertase and invertase I.

3.5. Preparation of solubilized invertase II from fraction C

After thawing, fraction C was washed several times with 1 and 0.2 M and 10 mM NaCl and finally with H_2O. With this procedure, an invertase I free fraction C was obtained. This preparation was resuspended in 50 mM NaOAc buffer, pH 6.5, containing 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4% PEG-6000 and 0.1 mM of a phenylmethylsulfonyl fluoride (PMSF). Half of the preparation was incubated with shaking at 30°C for 3 h as control and the other half was incubated under the same experimental conditions with the addition of 2% (w/v) of cellulase Onozuka (appropriate controls were performed). Then, the preparations were centrifuged, the pellets were discarded and solid $(NH_4)_2SO_4$ was added to the supernatants up to saturation. Solids were gathered by centrifugation, resuspended and dialysed against buffer C. The dialysates were clarified by centrifugation and separately filtered through a Bio-Gel P6 column (5×1 cm), equilibrated and eluted with buffer C.

3.6. Reassociation of the acid soluble invertase and invertase I to the walls

Wall preparation (14 ml containing 0.8 g of cell wall wet wt, fraction C) was heated at 100°C for 5 min. Then, the walls were washed 3 times by suspension in

buffer B and collected by centrifugation. The residue was washed in the same buffer without NaCl, divided in 2 fractions and centrifuged. One fraction was resuspended in 1 ml of the last buffer and used as control and the other fraction was resuspended in 1 ml of the acid soluble invertase preparation (1.4 E.U.) or invertase I (1.4 E.U.) in separate experiments. The mixtures were left to stand at 4°C for 10 min. Afterwards, the walls were separated by centrifugation and washed 3 times with 7 ml of buffer A. The supernatant and washing liquids were pooled. The residue was washed 3 times with buffer B and then with the same buffer without NaCl. Again the washing liquids were pooled. Invertase activity was determined in the different fractions after concn by lyophilization, when necessary. The results are the mean of 5 determinations. The same protocol was used when the reassociation of invertase I was performed.

3.7. Molecular mass measurement

The native M_r of soluble acid invertase and invertase I was measured on a column (40×2.5 cm) packed with Sephadex G-100 (Andrews, 1964) equilibrated with buffer C. The standards used were chymotrypsinogen A (25 000), egg albumin (43 000), BSA (67 000) and alkaline phosphatase (100 000).

3.8. Protein determinations

Proteins were determined according to Lowry, Rosebrough, Farr, and Randall (1951) using BSA as standard and by absorption at 280 nm in the column fractions.

3.9. Sugar determinations

Neutral sugars were determined by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956).

3.10. Enzyme units

One enzyme unit was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min at 37°C and at the optimum pH.

3.11. Enzyme assays

Reaction mixtures consisted of 25 μl of 0.6 M sucrose, 50 μl of 0.2 M NaOAc buffer, pH 4.5 (for acid soluble invertase and invertase I), or pH 6.5 (for invertase II or solubilized invertase II) and 20, 50 or 100 μl of either acid soluble invertase, invertase I, invertase II or solubilized invertase II in a final volume of 250 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).

3.12. Protein purity

Vertical gel slab electrophoresis of proteins was performed by standard techniques to determine subunit M_r and purity. Briefly: 2 μg of protein/lane were heated in 20 μl of loading buffer for 5 min according to the method of Laemmli (1970) or 10 μg of protein/lane for activity determinations. The run was made at 70 V for 2 h and at 90 V for 1 h. Proteins and invertase activity were localized on the gel by $AgNO_3$ (Blum, Beier, & Gross, 1987) and by 2,3,5-triphenyltetrazolium chloride methods (Gabriel & Wang, 1969), respectively. A kit for M_r markers (Dalton Mark VI) from Sigma was used as standard.

3.13. Effect of the reaction products

The reaction mixtures contained 4 to 25 μl of 0.6 M sucrose, 20, 50 or 100 μl of acid soluble invertase, invertase I, invertase II or solubilized invertase II, respectively, 50 μl of 0.2 M NaOAc buffer, pH 4.5, for the acid soluble invertase and invertase I and, pH 6.5 for invertase II and solubilized invertase II. For inhibition studies 4 to 35 μl of 6 M fructose or 5 to 65 μl of 4 M glucose were added. The reaction mixtures were completed to 250 μl with H_2O. Incubations were performed at 37°C for 30 min. Fructose and glucose were determined by the method of fructose dehydrogenase (Prado & Sampietro, 1994) and glucose oxidase (Jörgensen & Andersen, 1973), respectively.

3.14. Antiserum production

Rabbits were injected with the purified acid soluble invertase (200 μg of protein) emulsified in complete Freund's adjuvant. Five booster injections were given with invertase emulsified in incomplete adjuvant. Blood was collected 15 days after the last injection. The antiserum was partially purified from the serum fraction by precipitation with solid $(NH_4)_2SO_4$ (40% satn) and DEAE cellulose. The immunoglobulin-G fractions were collected and used in further experiments.

3.15. Enzyme immunoassay

Crude extract (5 μl of a 1/10 v/v diluted crude extract) or purified acid soluble invertase (5 μl) or invertase I (10 μl) was seeded on 1×1 cm pieces of polyvinylidene difluoride (PVDF). Each membrane was washed with 50 mM Tris–HCl buffer pH 8 with 150 mM NaCl and 0.05% Tween 20 (TBST) sup-

plemented with 2% fat-free milk powder (Molico) for 2 h followed by thorough washings with 50 mM Tris–HCl buffer pH 8 with 150 mM NaCl (TBS). Then, the membranes were incubated with 1/500 diluted invertase antiserum in TBS for 1 h and washed with TBS. Afterwards, they were incubated with anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Immunochemicals) at room temperature for 1 h and washed with TBST and TBS. Then, the membranes were incubated with 1 ml of 100 mM TRIS buffer, pH 9.5, containing 0.1 M NaCl, 5 mM $MgCl_2$ and 40 μg of Na *p*-nitro-phenylphosphate at 37°C for 90 min. Phosphatase activity was measured by readings at 405 nm (Endo, Nakagawa, Ogura, & Sato, 1990). Acid soluble invertase (1 to 5 μg of protein) produced a linear relationship regarding the activity of the immunologically fixed alkaline phosphatase.

3.16. Enzymatic deglycosylation

Deglycosylation with peptide-*N*-glycosidase F (NPGase F: removal of high-mannose and complex glycans, from Sigma) was made as follows: acid soluble invertase and invertase I (0.33 E.U., in separate experiments) contained in 400 μl were incubated at 37°C with 500 mU (15 μl) of NPGase F in 20 mM NaPi buffer, pH 6.8, with 20 mM EDTA, 1 mM NaN_3 and 1 mM PMSF in a final vol. of 800 μl for 20 h. The products were separated by gel filtration through a Bio Gel P6 column (60×2 cm) equilibrated and developed with 10 mM NaOAc buffer, pH 4.75, added with 0.2 M NaCl and 1 mM 2-mercaptoethanol. Fractions of 2 ml were collected. Proteins were monitored by *A* at 280 nm and sugars by the phenol–H_2SO_4 absorbance method. Controls were made incubating invertase in buffer for the same time.

3.17. Immunoblot analysis

Native acid soluble invertase and invertase I and both deglycosylated invertases (3 μg of protein/lane) were subjected to SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (70 V for 2 h and then 90 V for 1 h). Blotted membranes were also analysed as immunoblots by probing with antibodies raised against purified native soluble acid invertase (1/500 diluted invertase antiserum in TBS), 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). The position of the M_r markers was detected with Ponceau S.

3.18. Effect of polyclonal antibodies raised to the acid soluble invertase

In separate experiments 0.05 E.U. of acid soluble invertase, invertase I, invertase II and solubilized invertase II were treated with 5 to 63 μg of soluble acid invertase antibodies expressed as protein content. Reaction mixtures consisted of 30 μl of enzyme, 5 to 65 μl of a dilution (1/500) of antibody soln, 50 μl of 0.2 M NaOAc buffer, pH 4.5 or 6.5 and H_2O up to a final vol. of 225 μl. After 2 min incubation at 4°C 25 μl of 0.6 M sucrose was added and the mixtures were incubated at 37°C for 30 min. Sucrose hydrolysis was followed by Nelson (1944).

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