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LOCALIZATION OF INVERTASE ACTIVITIES IN *RICINUS COMMUNIS* LEAVES

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Key Word Index—*Ricinus communis*; Euphorbiaceae; vacuole; invertase; cell wall.

Abstract—Leaf tissue from *Ricinus communis* possesses cell wall and soluble invertases. These activities may be distinguished on the basis of their optimum pH and K_m and the action of various inhibitors. *Ca* 84% of the soluble invertase was found in vacuolar preparations.

INTRODUCTION

Acid invertases in higher plants occur as soluble and cell wall bound enzymes [1, 2]. Cell wall invertases in sugar cane are thought to participate in the translocation of sucrose from the apoplasm to the symplasm. This interpretation is based on the recovery of symmetrically radiolabeled sucrose after cell uptake of the asymmetrically labelled sugar [3, 4]. *Canna indica* [5] and corn radicle [6] behave like sugar cane, thus, the same role for invertase may be expected. However, these plants appear to be exceptional because there is evidence that *Lycopersicon esculentum* [7], bean endocarp [8], tobacco leaves [9], *Ricinus communis* [10], potato tuber [11], tissue cultures of carrot [12], *Pisum sativum* [13], *Beta vulgaris* [14] and cotton [15] absorb sucrose without inversion. More recently, it was proposed that these enzymes could function as a tissue valve in phloem unloading [16].

Soluble acid invertases are assumed to be vacuolar enzymes which participate in the intracellular metabolism of sucrose. The recent development of vacuole isolation has led to the localization of soluble acid invertases in these organelles in beetroot [17] and cultured tobacco cells [18]. Moreover, sucrose was demonstrated in vacuoles from *Beta vulgaris* [17], *Hippeastrum*, *Tulipa* [19] and *Ricinus communis* endosperm [20] and a new role for fructose, a reaction product acting as an invertase modulator in sugar cane leaf sheaths, was described [21]. However the localization of invertases in most plants is still unknown. This paper is an attempt to determine whether *R. communis* leaves possess such cell wall and vacuolar invertases.

RESULTS

Effect of pH on cell wall activity

Figure 1 shows a typical pH activity curve obtained with cell wall preparations from *R. communis* leaves. Two pH optima were observed (*ca* pH 3.7 and 5) and these were treated as independent isoenzymes throughout this work.

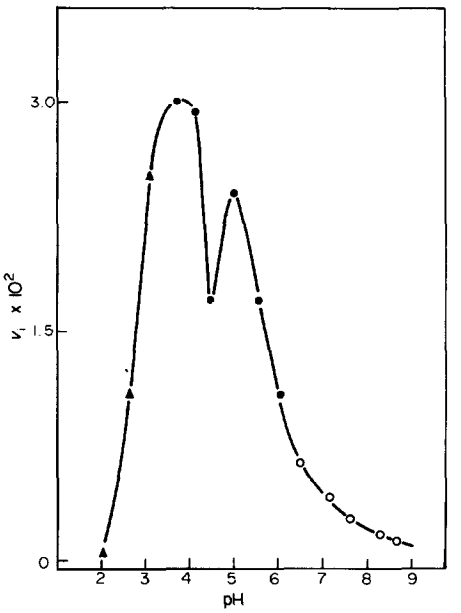

Fig. 1. Effect of pH on cell wall invertase activity. The buffers used were: (▲) 0.2 M glycine–HCl (●) 0.2 M NaOAc and (○) 0.2 M NaPi.

Attempts to solubilize enzymes

Cell wall preparations were made with 50 mM buffer (pH 7.5) to avoid fixation of intracellular enzymes to the cell wall [22–26]. Table 1 shows the effect of washing the cell walls with buffers reported as good eluting agents for cell wall enzymes. Only minor fractions of the cell wall activity at pH 3.7 were eluted by buffer 3. Buffer 4 produces a slight elution of the activity at pH 5. Tween 60 [27, 28] did not remove these activities from the cell wall. Similar lack of effect was observed by homogenizing the tissue in the presence of 0.8 mg casein per mg dry tissue.

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Table 1. Attempts to release invertases from the cell wall

	Total enzyme units			
	pH 3.7		pH 5	
Buffer*	Cell wall	Solubilized enzyme	Cell wall	Solubilized enzyme
1	36.9	3.19	32.4	0.64
2	40.3	2.96	30.6	0.64
3	26.0	5.92	21.5	1.28
4	24.0	3.16	15.8	1.89

*The composition of the buffers and the procedures are described in the Results.

Effect of substrate concentration on the cell wall activities

The activities at pH 3.7 and 5 have K_m of $(1.32 \pm 0.02) \times 10^{-2}$ M and $(9.65 \pm 0.95) \times 10^{-3}$ M, respectively. Both correspond to a simple case of the Michaelis–Menten equation. The reaction rates were constant for at least 20 min.

Effect of various chemicals

Table 2 shows the effect of some chemicals on the activity of the cell walls at pH 3.7 and 5.

Soluble invertase

A soluble invertase, accounting for 84.9% of the total leaf invertase activity, was found (Table 3). Consequently, a comparative study of protoplast and vacuole preparation was undertaken in order to establish the localization of the soluble invertase. Acid lipase, α-glucosidase, β-galactosidase, acid protease, succinic dehydrogenase, $(Mg^{2+} + K^+)$-ATPase at pH 6.5 and 9, chlorophyll and protein were measured (Table 4). Phosphatase activities were measured using *p*-nitrophenylphosphate and β-glycerophosphate because there is evidence that different phosphatases may be involved in the hydrolysis of these substrates [29]. In *R. communis*, the *p*-nitrophenylphosphatase was strongly activated in the vacuolar preparation, while the β-glycerophosphatase activity behaves as a different enzyme. An inhibitor of the *p*-nitrophenylphosphatase was probably removed during vacuolar preparation. Contamination of vacuoles with other cellular organelles in the low gravity field used is unlikely. This was confirmed by the lack of succinic dehydrogenase and chlorophyll in the vacuolar preparation. Contamination with protein components of the digesting cellulase and hemicellulase is improbable because of the extensive washing. Microscopic examination of the vacuolar suspension showed a homogeneous, protoplast-free preparation. The number of protoplasts and vacuoles obtained were 105×10^6 and 60×10^6, respectively. Consequently, a vacuolar yield of 57% was attained. The percent yield of each marker corrected for the vacuolar yield is shown in Table 4. According to these results invertase and β-glycerophosphatase activities are vacuolar. Most of the α-glucosidase activity is also found in the vacuole, but *ca* 33% of this activity appears to be exovacuolar. The activities of $(Mg^{2+} + K^+)$-ATPase were measured at pH 6.5 and 9. The vacuolar yield of these activities was *ca* 100% but these enzymes are known to be activated in vacuolar preparations [30]. According to our results, lipase, protease and probably β-galactosidase cannot be vacuolar enzymes. Sucrose, fructose and glucose were also present in high yield in the vacuolar preparation.

Table 2. Effect of chemicals on the activity of cell wall invertases at the different optimum pHs

		% Activity	
Effector	Concentration (mM)	pH 3.7	pH 5
Ca^{2+}	5	70.6	95.3
Mg^{2+}	10	84.9	94.8
Co^{2+}	1	91.3	112.8
Hg^{2+}	1	30.9	47.7
Tris	4	88.0	111.3
Tris	50	55.0	82.3
Urea	200	64.0	91.9
EDTA	10	79.0	104.0
Heptamolybdate	5	100.0	46.5
δ-Gluconolactone	100	45.0	63.6

Each reaction contained 0.018 enzyme units at pH 3.7 and 0.012 enzyme units at pH 5. 100 mM sucrose was used.

Properties of the soluble enzyme

The optimum pH of the soluble invertase was 3.5. A K_m of 8.6×10^{-3} M was determined and the kinetics cor-

Table 3. Distribution of invertase activity between the cell wall and soluble fractions

Enzyme preparation	Total E.U.	Total protein (g)	Specific activity (E.U./mg protein)
Homogenate	378	8.63	0.044
Cell wall	42	6.90	0.006
Soluble invertase	321	1.72	0.187

Table 4. Determination of enzyme activities, sugars, protein and chlorophyll in protoplasts and vacuoles

Substance	Protoplast	Vacuole	Vacuolar yield (%)	Specific activity: Protoplast (A)	Specific activity: Vacuole (B)	Purity (A/B)
Invertase	0.116 E.U.	0.0554 E.U.	83.8	0.000725	0.00326	4.49
α-Glucosidase	0.0168 E.U.	0.0064 E.U.	67.0	0.000105	0.00038	3.62
β-Galactosidase	0.015 E.U.	0.0021 E.U.	24.0	0.000094	0.000123	1.31
p-Nitrophenylphosphatase	0.005 E.U.	0.0042 E.U.	147.0	0.000031	0.00025	8.06
Acid lipase	450 E.U.	30.00 E.U.	12.0	2.81	1.76	0.628
Acid protease	0.17 E.U.	0.0014 E.U.	1.0	0.00107	0.000082	0.077
Succinic dehydrogenase	35.5 E.U.	0.00 E.U.	0.0	0.222	—	—
β-Glycerophosphatase	0.295 E.U.	0.143 E.U.	85.0	0.0018	0.0084	4.67
$(Mg^{2+} + K^+)$-ATPase, pH 6.5	1.028 E.U.	0.571 E.U.	97.0	0.0064	0.0336	5.25
$(Mg^{2+} + K^+)$-ATPase, pH 9	0.857 E.U.	0.489 E.U.	100.0	0.0051	0.0288	5.33
Sucrose	265 μmol	90.8 μmol	60.0	—	—	—
Fructose	124.9 μmol	28.5 μmol	40.0	—	—	—
Glucose	7.1 μmol	1.6 μmol	39	—	—	—
Protein	160 mg	17.0 mg	19	—	—	—
Chlorophyll	28.807 μg	14.65 μg	0.05	—	—	—

Table 5. Effect of chemicals on the activity of the soluble invertase

Effector	Concentration (mM)	% activity
Ca^{2+}	5	75.3
Mg^{2+}	10	100.0
Co^{2+}	1	72.5
Hg^{2+}	1	14.5
Tris	4	85.5
Tris	50	78.3
Urea	200	65.2
EDTA	10	75.3
Heptamolybdate	5	58.0
δ-Gluconolactone	100	94.2

Each reaction contained 0.064 enzyme units at pH 3.5 and 100 mM sucrose.

respond to a simple case of the Michaelis–Menten equation. Table 5 shows the effect of some chemicals on the soluble invertase.

DISCUSSION

The cell wall from *R. communis* leaves contains a bound invertase activity. This activity is firmly bound to the cell wall, as proved by treatments with buffers of different ionic strength containing EDTA, mercaptoethanol and boric acid. The insolubility of this activity in Tween 60 and the persistence of cell wall bound invertases after homogenization in the presence of high levels of exogenous proteins appears to confirm this assertion. Sugar cane possesses similar enzymes and the organs of some varieties contain cell wall invertases having more than one optimum pH [31]. Further, callus cultures of sugar cane appear to produce a type of cell wall invertase that is not present in the whole plant [32]. One possible explanation [33] was that sugar cane possesses a set of invertases whose activities overlap in the pH curve. Thus, quantitative differences among the set of cell wall invertases would be sufficient to produce a different pH curve. The presence of two optima in the pH curve of *R. communis* cell walls suggests a parallelism with the corresponding invertases from sugar cane. A definitive answer to the problem would need a very different methodological approach. The properties of cell wall activities from *R. communis* differ from those of the soluble invertase. Thus, bound and soluble activities appear to originate from different enzymes. Most of the soluble invertase was present in vacuolar preparation together with a β-glycerophosphatase activity. Reasonable amounts of α-glucosidase, *p*-nitrophenylphosphatase and $(Mg^{2+} + K^+)$-ATPases, measured at pH 9 and 6.5, were found. Thus, these enzymes appear to be normal components of the vacuoles from *R. communis* leaves. Vacuolar preparations showed a high level of sucrose, fructose and glucose. From the fact that sucrose and invertase were found together in the same compartment the occurrence of a regulatory system of the invertase may be expected. According to these results *R. communis* presents a close analogy with sugar cane with respect to invertase localization. However, as *R. communis* cells incorporate sucrose from the apoplasm without previous scission [10] it is difficult, at present, to suggest a physiological role for its cell wall invertases. The hypothesis of cell wall invertases acting as tissue valves in phloem unloading [16] needs additional confirmation. Soluble invertase must have a role in the intracellular utilization of sucrose since both sucrose and invertase are found in the vacuole.

EXPERIMENTAL

Plant material. Young leaves from *R. communis* cultivated in the field were used.

Cell wall preparation. The preparation was performed as described in ref. [31]. *Ca* 200 g of leaves was used.

Attempts at solubilization of cell wall invertases. A cell wall preparation was treated successively with the following solns: (1) 0.2 M NaPi buffer, pH 7.5, 1 mM 2-mercaptoethanol (2-ME), 1 M NaCl; (2) 0.2 M NaPi–citrate buffer, pH 8.5, 1 mM 2-ME; (3)

0.2 M NaPi–citrate buffer, pH 8.5, 1 mM 2-ME, 1 M NaCl, 30 mM EDTA; and (4) 0.2 M Na borate buffer, pH 8.5, 1 mM 2-ME. The cell wall suspension was centrifuged at 3020 *g* and resuspended for 30 min in 15 ml of the corresponding buffer. Then the suspension was centrifuged at 3020 *g* and the pellet washed and resuspended in 15 ml 10 mM NaOAc buffer, pH 5.5, 1 mM 2-ME. This treatment was repeated twice. The buffers used for the elutions were dialysed against 10 mM NaOAc buffer, pH 5.5, containing 1 mM 2-ME. Invertase activities were measured in the washed cell walls and in the dialysed eluates.

Assay of cell wall invertases. The reaction medium (100 μl) consisted of 20 μl 2 M sucrose, 50 μl of the cell wall suspension and 30 μl 0.2 M buffer of the desired pH. The reactions were run at 37° for 15 min and stopped by addition of the alkaline copper reagent of ref. [34] and the reducing power was determined by the arsenomolybdate method [35]. Cell wall preparations were kept at −20°.

Invertase unit. 1 unit of enzyme was defined as the amount which hydrolysed 1 μmol substrate per min at 37° at the optimum pH.

Protoplast preparation. Leaf tissue (20 g) was sliced into 10 mm strips. The tissue was floated on a soln of 40 ml 1 M mannitol containing 0.8 g Macerozyme and 1.2 g Cellulase (Onozuka R-10), 0.4 g PVP, 4.5 mg $CaCl_2$ and adjusted to pH 5.6. The mixture was incubated 4 hr at 28° with occasional slight stirring. Digested leaf tissue was filtered through four layers of gauze and the filtrate was centrifuged for 3 min at 100 *g*. The pellet was washed × 8 by suspension in 20 ml fractions of 1 M mannitol. The pellet was resuspended in 20 ml 1 M mannitol. A Neubauer counting chamber was used for counting the suspended protoplasts. A half of the suspension was used as the protoplast preparation and the other half was kept for vacuole preparation.

The protoplast preparation was centrifuged for 3 min at 100 *g* and the pellet was suspended in 14.5 ml 10 mM NaOAc buffer, pH 5.5, containing 1 mM 2-ME (buffer A). This preparation was dialysed against 2 l. buffer A. 15 ml of protoplasts lysate was obtained.

Vacuolar preparation. Half of the protoplast suspension was centrifuged for 3 min at 100 *g*. The pellet was suspended in 14.5 ml 0.82 M mannitol and left to stand for 1 hr. Then the number of vacuoles was counted using a Neubauer counting chamber. The vacuolar suspension was centrifuged for 3 min at 100 *g* and the pellet was resuspended in buffer A and the vacuolar lysate was dialysed against 2 l. buffer A.

Soluble invertase. The supernatant of the cell wall preparation was centrifuged at 27 100 *g* and the supernatant was satd with solid $(NH_4)_2SO_4$. After 30 min the suspension was centrifuged at 27 100 *g* and the ppt was resuspended in 4 ml buffer A and dialysed against the same buffer. The preparation, 6.4 ml, contained 270 invertase units.

Enzyme determinations. Acid lipase [36], acid protease [37], β-galactosidase [38], succinic dehydrogenase [39] and α-glucosidase [35] were measured according to their respective references. Phosphatase activities were measured by using β-glycerophosphate [40] and *p*-nitrophenylphosphate. The liberation of *p*-nitrophenol was measured following ref. [38]. ATPase activities were measured at pH 6.5 and 9 according to ref. [41].

Sugars, protein and chlorophyll determinations. 5 g of tissue was used for sugar estimation following the method of ref. [21]. Protoplast and vacuolar chlorophyll was determined according to ref. [42]. Protein was determined by the method of ref. [43].

Assay of soluble invertase. (A) *Comparative studies of vacuolar and protoplast invertases.* The reaction mixture consisted of 200 μl of extract, 100 μl of 0.2 M NaOAc buffer, pH 3.5, and 100 μl 0.4 M sucrose. After 30 min incubation at 37°, 15 μl of the mixture was taken for reducing power determinations. (B) *Properties of soluble invertase.* The reaction mixture consisted of 10 μl of a 10-fold diluted extract, 20 μl 0.6 M sucrose, 30 μl 0.2 M NaOAc buffer, pH 3.5, and H_2O to a final vol. of 100 μl. The reactions were run for 10 min at 37°. In both cases (A) and (B) the reactions were stopped by the alkaline copper reagent of ref. [35] and the reducing power was determined by the method of ref. [36].

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