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ACID CELL WALL INVERTASES IN *CONVOLVULUS* CALLUS

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Abstract—Evidence is provided that cell walls isolated from *Convolvulus* callus contain two forms of acid invertase. They differ considerably in temperature sensitivity and MW and can only partly be extracted from the cell walls by salt solutions. The extracted invertases can be separated from each other by gel filtration. During incubation of callus fragments in nutrient solution the activities of both forms increase; in the presence of the amino acid analogue thienylalanine the activities are still further enhanced.

INTRODUCTION

IN SOME micro-organisms evidence has been obtained for the occurrence of multiple forms of acid invertase in the cell wall.[1-3] We decided, therefore, to investigate if this could also be the case with the acid cell wall invertase in *Convolvulus* callus.[4,5]

RESULTS

Increase of wall-bound invertase activity upon subculturing

It was previously shown[4,5] that the activity of acid invertase in the cell walls of *Convolvulus* callus increased considerably upon subculturing. Addition of actinomycin D, cycloheximide or the amino acid analogue thienylalanine did not prevent this rise, but on the contrary resulted in a higher level of invertase. The duration of the lag phase did not change in the presence of thienylalanine and the stimulating action of this amino acid analogue was visible at the end of the lag phase. The increase of invertase activity ceased at about the same time in the control and treated tissues.

Thermal inactivation of wall-bound invertase

The possibility that the acid invertase in the cell wall indeed occurred in multiple forms, was investigated by following its thermal inactivation in the course of time. The thermal inactivation curves so obtained could not be transformed into straight lines by plotting them semi-logarithmically. Initially, a rapid decline in invertase activity occurred and this was followed by a gradual, linear decay of the activity. Because graphic analysis[6] did not indicate the existence of more than two invertase components, the following function was

[1] METZENBERG, R. L. (1964) *Biochim. Biophys. Acta* **89,** 291.
[2] ARNOLD, W. N. (1969) *Biochim. Biophys. Acta* **178,** 347.
[3] NAGASAKI, S., YAMAMOTO, S. and KUBO, H. (1970) *Res. Rep. Kochi Univ.* **19,** 153.
[4] KLIS, F. M. and HAK, A. (1972) *Physiol. Plant.* **26,** 364.
[5] KLIS, F. M., DALHUIZEN, R. and SOL, C. (1974) *Phytochemistry* **13,** 55.
[6] RIGGS, D. S. (1963) *The Mathematical Approach to Physiological Problems*, Ch. 6, Williams & Wilkins, Baltimore.

fitted to the experimental data: $I_t = I_0^S.(\text{exp: } -k_i^S.t) + I_0^L.(\text{exp: } -k_i^L.t)$, where I_t is the wall-bound invertase activity at time t; I_0^S the relatively thermostable invertase activity at time zero; I_0^L the relatively thermolabile invertase activity at time zero; k_i^S the inactivation constant of the stable component and k_i^L the inactivation constant of the labile component. The precise values of these parameters were obtained by means of the iterative procedure "damped newton";[7] intitial estimates of the four parameters were provided for by graphic analysis.

Upon transfer of callus fragments to fresh nutrient medium the activities of both the relatively stable and the relatively labile component were found to increase (Table 1). When thienylalanine was added to the incubation solution the activities of both components were further enhanced. In all cases the activity of the stable component increased less than the labile one. Furthermore, the inactivation constants seem to be positively correlated with the invertase activity.

TABLE 1. THERMAL INACTIVATION OF WALL-BOUND INVERTASE AT 30

Treatment	I_0^S* (nmol.hr^{-1}.mg^{-1})	I_0^L (nmol.hr^{-1}.mg^{-1})	k_i^S (hr^{-1})	k_i^L (hr^{-1})
Before incubation†	136 ± 14·5	92 ± 15·0	−0·014 ± 0·0075	−0·50 ± 0·14
After incubation in nutrient solution	214 ± 22·9	178 ± 24·3	−0·024 ± 0·0086	−0·60 ± 0·157
+0·8 mM D,L-β-2-thienylalanine	310 ± 9·0	295 ± 10·9	−0·036 ± 0·0030	−1·10 ± 0·085

* The values of the parameters were computed by means of the procedure "damped newton".[7] I_0^S: the relatively thermostable component of the invertase activity at time zero; I_0^L: the relatively thermolabile component; k_i^S: the thermal inactivation constant of the relatively thermostable component; k_i^L: the thermal inactivation constant of the relatively labile component. Enzyme activities have been expressed as nmol of sucrose hydrolyzed per hr and per mg dry wt of cell wall material.

† Incubation lasted 24 hr.

Thermal inactivation of solubilized invertase

Analysis of thermal inactivation curves of untreated cell walls, cell walls washed in 1 M KCl, and dialyzed washing solution, respectively, showed (Table 2) that both the relatively thermostable and the relatively thermolabile invertase component went into solution. However, the labile component was released to a much higher extent (27·5/39·7 = 69%)

TABLE 2. THERMAL INACTIVATION OF INVERTASE AT 40

Source of invertase	I_0^S† (nmol.hr^{-1}.mg^{-1})	I_0^L (nmol.hr^{-1}.mg^{-1})	k_i^S (hr^{-1})	k_i^L (hr^{-1})
Untreated cell walls	25·1 ± 1·96	43·8 ± 3·5	−0·0288 ± 0·00146	−5·6 ± 0·96
Cell walls washed in 1 M KCl*	18·7 ± 0·50	12·2 ± 0·86	−0·0315 ± 0·00050	−4·9 ± 0·73
Dialyzed washing solution*	11·1 ± 1·04	27·5 ± 1·29	−0·44 ± 0·076	−9·1 ± 0·91

* Cell walls were stirred in 1 M KCl for 15 min and subsequently filtrated. The filtrate was dialyzed against 20 mM KCl. The residue was washed 3× with double-dist. H_2O.

† The headings of the columns are explained in Table 1. The values of the parameters were computed by means of the procedure "damped newton".

[7] KOK, J. (1972) *Loshladige Rocks*, 3.4.20. Mathematical Centre; 49, 2e Boerhaavestraat, Amsterdam.

than the stable one (11·1/29·8 = 37%). Both components became more sensitive to thermal inactivation when dissolved, especially the stable one.

Gel filtration of solubilized invertase

The first peak (S-1) voided the Sephadex G-100 column (peak at fraction 18), while the second one (S-2) was considerably retarded (peak at fraction 38) and the proportion S-1:S-2 was about 1:5. The thermal inactivation curves of both S-1 and S-2 were straight lines when plotted semi-logarithmically. This means that both peaks were homogenous, at least by the criterion of temperature sensitivity and in so far as different temperature sensitivities can be established in this way. S-1 appeared to be more thermostable than S-2; the thermal inactivation constants of S-1 and S-2 respectively amounted to $-3{\cdot}1 \pm 0{\cdot}34\ hr^{-1}$ and $-8{\cdot}9 \pm 0{\cdot}41\ hr^{-1}$ (determined at 40°).

DISCUSSION

From the shape of the thermal inactivation curves of both wall-bound invertase and solubilized invertase the existence of two invertase components differing in temperature sensitivity was inferred. When solubilized invertase was chromatographed on Sephadex G-100 two invertase components could indeed be isolated. The first component eluted was the most thermostable one. It was excluded from the gel and, therefore, was supposed to have a MW higher than 150000. The second component, which was more temperature sensitive, was considerably retarded on the gel. Its MW was estimated to be about 15000.[8] Gel filtration of the thermolabile component did not alter its inactivation constant ($-9{\cdot}1 \pm 0{\cdot}91\ hr^{-1}$ before gel filtration, and $-8{\cdot}9 \pm 0{\cdot}41\ hr^{-1}$ after gel filtration; determined at 40°); however, gel filtration of the thermostable component lead to a considerable change in the inactivation component ($-0{\cdot}44 \pm 0{\cdot}076\ hr^{-1}$ before gel filtration, and $-3{\cdot}1 \pm 0{\cdot}34\ hr^{-1}$ after gel filtration; determined at 40°).

The inactivation constants of both components of wall-bound invertase activity seem to be positively correlated with the invertase activity in the cell wall (Table 1). This can be explained by assuming that the temperature sensitivity of invertase depends on the number of intermolecular bonds between invertase and cell wall. The fact that solubilized invertase is much more thermolabile than wall-bound invertase supports this interpretation. A comparable phenomenon was observed by Monsand and Durand,[9] who improved the thermostability of yeast invertase by fixing it to bentonite. Similar results were obtained in *Streptococcus faecalis* demonstrating that wall-bound autolysin could better resist thermal inactivation than when dissolved.[10] Furthermore, if we assume that at a higher invertase level in the cell wall the average number of intermolecular bonds between invertase molecules and the cell wall will decrease, then the invertase will become more sensitive to thermal inactivation.

Wall-bound invertase activity was partially released from the cell walls in concentrated salt solutions (Table 2); this fraction of the invertase is apparently ionically linked to the cell wall. Comparable results were obtained for invertase activity in cell walls isolated from *Helianthus tuberosus*.[11] That does not necessarily mean that the remainder is covalently bound to the cell wall. It is conceivable that part of the invertase molecules cannot leave

[8] Determann, H. (1969) *Gel Chromatography*, 2nd edn. Springer, Berlin.
[9] Monsand, P. and Durand, G. (1971) *FEBS Letters* **16**, 39.
[10] Shockman, G. D. and Cheney, M. C. (1969) *J. Bacteriol.* **98**, 1199.
[11] Little, G. and Edelman, J. (1973) *Phytochemistry* **12**, 67.

the cell wall because they are physically confined within it. This supposition agrees with the observation that the relatively thermolabile and lighter invertase form is released from the cell wall to a much higher extent than the relatively thermostable and heavier form (Table 2).

Upon transferring callus fragments to nutrient solution the wall-bound invertase activity increases. In the presence of actinomycin D, cycloheximide or thienylalanine the invertase activity is still further enhanced.[4,5] It was previously[4] argued that this increase was due to synthesis *de novo* and not to activation of an inactive form of invertase. Thus, wall-bound invertase activity had a relatively short half-life (4·4 hr, as estimated from its time course).[4] This seems to exclude activation, because that would imply an improbably large pool of inactive invertase, in fact, a supply sufficient for 72 hr. This argument also seems to hold for the relatively thermolabile form of acid invertase (Table 1) even if the thermal degradation constant as determined *in vitro* is too high an estimate of its value *in vivo*. However, it clearly does not serve for the more thermostable form.

EXPERIMENTAL

Incubation experiments. Tissue cultures,[12] 4–8 weeks old, were dissected into fragments weighing *ca* 200 mg each. These were incubated under sterile conditions in culture tubes (16 cm × 0·21 mm i.d.), each containing 4 fragments in 6 ml of liquid nutrient medium. The tubes were slowly rotated (2 rpm) in the dark at 30° until sampling.

Cell wall preparations. The tissue was taken up in 20 mM mercaptoethanol/20 mM K phosphate buffer (pH 7·2; 5 ml/g callus) and homogenized for 1 min. Cytoplasmic constituents were removed with 2 × -dist. H_2O by centrifuging at 270 *g* (5 ×). The residue was taken up in a double vol. of 2 × -distilled H_2O and stored at −20°. All operations were carried out at 0–4°.

Measurement of invertase activity. Wall-bound invertase activity was determined according to Klis and Hak.[4] Soluble invertase activity was assayed in a reaction mixture of 0·4 ml containing 50 mM phthalic acid/KOH (pH 4·5), 50 mM sucrose and enzyme soln at 30°. Incubation was ended by adding a 2 × vol. of ice-cold glucose oxidase/peroxidase/*o*-dianisidine reagent (pH 7).[13] Subsequently, the liberated glucose was determined in a second incubation period of 30 min at 38°, which was terminated by adding 1·6 ml 5 M HCl. The absorbance was measured at 540 nm. The slow hydrolysis of sucrose occurring during the 2nd incubation period was corrected for by omitting the first incubation period and by adding the reagent directly to a reaction mixture of equal composition.

[12] KLIS, F. M. (1971) *Physiol. Plant.* **25,** 253.
[13] LLOYD, J. B. and WHELAN, W. J. (1969) *Anal. Biochem.* **30,** 467.