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PHYTOCHROME CONTROL OF β-FRUCTOSIDASE ACTIVITY IN RADISH

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Key Word Index—*Raphanus sativus*; Cruciferae; radish; invertase regulation; phytochrome; cell-wall enzyme.

Abstract—Soluble and cell-wall fractions from radish seedlings show β-fructosidase activity. Light (via phytochrome) induces a transfer of the enzyme from the cytosol to the cell-wall in roots and hypocotyls. Light also increases β-fructosidase activity in hypocotyls. Cycloheximide prevents the increase of β-fructosidase activity but has no effect on the transfer.

INTRODUCTION

In higher plants sucrose hydrolysis (invertase activity) depends on: (1) β-D fructofuranoside fructohydrolase (β-fructosidase; β-FFase; E.C. 3.2.1.26); (2) α-D glucoside glucohydrolase (α-glucosidase E.C. 3.2.1.20), and (3) sucrose phosphorylase (E.C. 2.4.1.7).

Two different types of β-FFase occur in plants; the so-called acidic and neutral (or alkaline) invertases. These two invertases differ in their optimum pH and in their specificity towards the substrates: the acidic enzyme may hydrolyze sucrose and raffinose whereas the neutral one is inactive on raffinose [1].

Multiple forms of acidic β-FFase have been distinguished by their MW, PI, K_m and thermostability [2–6]. Invertases are glycoproteins and the different forms of the enzyme may correspond to the presence of more or less carbohydrate [3,7]. However, Wray and Brice [8] noticed a lack of prosthetic groups. β-FFases often sediment with cell-wall material. These forms are called insoluble, external or cell-wall invertases. Neutral β-FFase is often present in the supernatant fractions only [9,10] but the acidic enzyme generally occurs in both cell-wall and soluble fractions of the crude extracts [11,12].

Although invertase activity can be measured in the insoluble fraction it is by no means certain that the enzyme is really located in the cell-wall *in vivo*. All of the activity, or part of it, can be released from the cell-wall fraction by washing with NaCl, borate, polyethylene glycol and non ionic detergent solutions [8,13–15]. Thus, invertase may be firmly bound (covalently) or associated with a cell-wall component by H-binding or salt linkages. According to Hawker [13], the insoluble invertase from grapes is an artifact of extraction.

Invertases, mainly cell-wall enzymes, seem to play an important role in sucrose metabolism [12] and cell-wall bound invertase activity can fluctuate according to the development of the tissues [5,16–18]. The apparent synthesis of invertase is increased by wounding [19], hormones [20,21] glucose and sucrose levels [22]. Anand and Galston [23] have shown that one of the P_{fr} (active form of phytochrome) effects on buds of etiolated peas is the promotion of sucrose hydrolysis probably by an increase in β-FFase activity.

In this paper the results of our studies on the phytochrome control of β-FFase activity in radish seedlings are described.

RESULTS AND DISCUSSION

Distribution of invertase (β-FFase) in etiolated and far-red irradiated seedlings

Table 1 shows that soluble and insoluble invertases occur in the hypocotyls and roots of 48 hr dark-grown seedlings (48 hr darkness after sowing). On the other hand invertase activity can hardly be detected in both the fractions from cotyledons.

Table 1. Distribution of β-fructosidase activity in the different parts of radish seedlings in darkness and in far-red light (FR). Measurements were performed 48 hr after sowing

	β-FFase activity (in relative units)					
	Cell-wall fraction		Soluble fraction		Total	
	Darkness	FR	Darkness	FR	Darkness	FR
Cotyledons	0.04 ± 0.02	0.05 ± 0.02	0.08 ± 0.02	0.99 ± 0.06	0.12	1.04
Hypocotyl	0.88 ± 0.05	2.56 ± 0.11	3.22 ± 0.09	2.68 ± 0.08	4.10	5.24
Root	0.39 ± 0.04	0.61 ± 0.06	1.24 ± 0.08	0.98 ± 0.06	1.63	1.59

Table 2. β-FFase activity in cell-wall and soluble fractions. Etiolated (dark) or far-red (FR) irradiated hypocotyls (72 hr dark or 72 hr FR) were homogenized at different pH (citrate–Pi buffer 0.05 M). The activity was estimated at pH 5

pH buffer	β-FFase activity					
	Cell-wall		Soluble		Total	
	FR	Dark	FR	Dark	FR	Dark
4	0.65 ± 0.03	0.59 ± 0.03	1.04 ± 0.06	1.22 ± 0.07	1.69	1.81
5	2.11 ± 0.05	1.42 ± 0.05	1.60 ± 0.08	1.95 ± 0.06	3.73	3.37
6	1.56 ± 0.03	0.71 ± 0.05	2.10 ± 0.11	2.56 ± 0.09	3.66	3.27
7	1.09 ± 0.04	0.51 ± 0.04	2.52 ± 0.12	2.83 ± 0.11	3.61	3.34

In hypocotyls or roots from 48 hr far-red irradiated seedlings (48 hr under far-red after sowing) a lower activity occurs in the soluble fraction and a higher activity in the cell-wall fraction. The increase of invertase activity detected in the cotyledons only concerns the soluble fraction. Cell-wall β-FFase activity is negligible. The crude extracts from the cotyledons or from the rest of the etiolated and far-red irradiated seedlings are able to hydrolyze sucrose and raffinose but do not show any activity towards trehalose, maltose and α-methyl-glucose indicating a lack of α-glucosidase. In the conditions of the enzyme assay no sucrose phosphorylase activity can be detected. In other words the invertase activity in radish seedlings is only due to β-FFase.

Influence of method of extraction and of treatment of the insoluble fraction on the cell-wall β-FFase level

In the first experiment, the rest of the seedlings after excision of cotyledons were homogenized in 0.05 M citrate–phosphate buffer of different pH. As indicated in Table 2 no significant change occurs in the total activity with regard to pH in a range from 5 to 7. On the other hand, at pH 4 the activity is lower, this result suggests that a degradation of the β-FFase occurs at low pH.

Above pH 5 the level of β-FFase activity is lower in the cell-wall fraction. Nevertheless at pH 7 a large part of the activity is still present in the insoluble fraction. In addition, it can be seen that the effect of far-red light in comparison to the dark control, in for example, higher activity in the cell-wall fraction and lower activity in the soluble fraction, is measurable over the pH range used.

A second series of experiments was carried out using other extraction media. The presence of borate (Table 4), which dissociates invertase-tannin complexes formed during extraction [13], does not change the distribution of β-FFase in comparison with a citrate–phosphate buffer pH 7. The maximum β-Fase activity in cell-wall preparations was obtained by using the glycerol polyethylene glycol mixture [24]. Polyethylene glycol is effective in preventing enzyme-tannin or phenol complex formation [13]; glycerol is well-known to stabilize proteins [5].

Another set of experiments concerned the release of β-FFase from cell-wall fraction prepared, as described in Experimental, under a variety of conditions. Treatment with citrate–phosphate buffer at pH values above 5 leads to a partial release of β-FFase activity (Table 3). Washing with M NaCl does not modify the results obtained by changing the pH (maximum release 26%). A maximum of about 50% release of β-FFase from cell-wall was achieved by treatment with a citrate–phosphate buffer containing 1–5% Tween 20 or Triton X-100. The data suggest that β-FFase occurs in the insoluble fraction of the crude extracts in two forms: (1) covalently bound, (2) ionically bound invertase. The ionically bound β-FFase is considered an artefact of extraction [12–14]. However, Ueda *et al.* [15] observed that all the insoluble β-FFase activity (50% of the total activity) can be fully solubilized with NaCl from the cell-wall prepared from cultured *Daucus carota* cells. On the other hand, the formation of protoplasts by treatment with cellulases and pectinases leads to a loss of 50% of the total invertase activity of the cells. In other words, the ionically bound β-FFase appears as an external invertase *in vivo*. If radish cotyledons were ground, no ionically nor covalently

Table 3. Influence of extraction buffer on the distribution of β-FFase activity in the cell-wall and soluble fraction. The activity was determined in citrate phosphate buffer 0.05 pH 5. Etiolated and far-red irradiated hypocotyls (48 hr) were used

Buffer	β-FFase activity					
	Cell-wall		Soluble		Total	
	FR	Dark	FR	Dark	FR	Dark
Citrate–Pi 0.05 M pH 5.0	2.46 ± 0.12	0.87 ± 0.06	2.52 ± 0.15	3.38 ± 0.14	4.98	4.35
+ Borate 0.05 M	2.27 ± 0.11	0.79 ± 0.08	2.58 ± 0.14	3.27 ± 0.12	4.96	4.06
Glycerol ethylene glycol (4:1)	2.65 ± 0.16	1.52 ± 0.10	2.27 ± 0.11	2.95 ± 0.15	4.92	4.47

Table 4. Influence of washing on the release of β-FFase from cell-wall 72 hr etiolated or FR irradiated hypocotyls which are homogenized as indicated in the section of material and methods. After 10 min. washing, the suspension was centrifuged at 900 *g* for 20 min and the activities of β-FFase were estimated in the supernatant and pellet. β-FFase activity is expressed as % of the activity of the normal cell-wall preparation

		% β-FFase activity					
		Pellet		Supernatant		Total	
Cell-wall washing by		FR	Dark	FR	Dark	FR	Dark
Citrate–Pi pH	4.0	83.4 ± 0.4	82.6 ± 0.4	0.2 ± 0.1	0.3 ± 0.1	83.6	82.9
	5.0	98.8 ± 0.5	98.1 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	100	98.3
	6.0	75.6 ± 0.4	66.2 ± 0.5	8.5 ± 0.2	12.4 ± 0.3	84.1	78.6
	7.0	72.4 ± 0.5	56.4 ± 0.4	14.1 ± 0.2	18.3 ± 0.2	86.5	74.7
NaCl	1 M	75.1 ± 0.3	66.7 ± 0.4	13.6 ± 0.2	18.1 ± 0.3	88.7	84.8
Citrate–Pi + Tween 20 0.5%		64.3 ± 0.4	45.6 ± 0.5	30.1 ± 0.3	33.7 ± 0.3	94.4	79.3
Citrate–Pi + Triton X-100 0.5%		62.2 ± 0.3	42.4 ± 0.4	27.9 ± 0.3	30.4 ± 0.3	90.1	72.8

bound β-FFase could be detected. Whether or not the ionically bound invertase is an artifact is an open question. However, the increase of insoluble β-FFase activity prepared from far-red irradiated seedling concerns at least the covalently bound enzyme (Tables 2–4) although phytochrome P_{fr} induces the synthesis of phenolic compounds which are assumed to form complexes with enzymes and cell-wall material [13].

As indicated in Table 4, the insoluble β-FFase activity is higher for far-red irradiated than for etiolated seedlings in all the conditions used. We also noticed that the loss of total invertase activity can be neglected (10%) with preparations from far-red irradiated seedlings. However, this loss reaches about 25% in the case of etiolated seedlings. We can assume either: (1) the denaturation of the solubilized β-FFase is easier than that of insoluble enzyme or (2) the β-FFase in the bound form is more active than in solution.

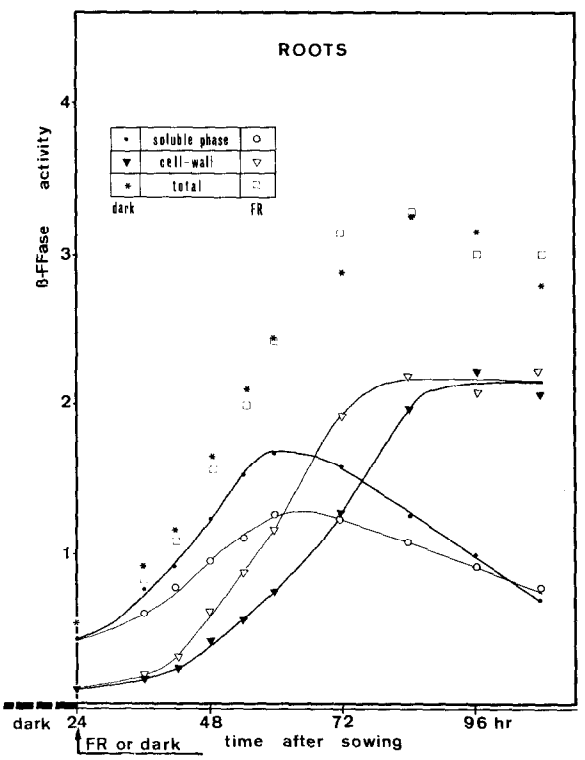

Fig. 1. Time course of total, cell-wall and soluble β-fructosidase activity in root from etiolated and far-red irradiated radish seedlings. Each point represents the mean of 8 to 12 experiments.

Time-course of β-FFase activity in roots and hypocotyls from etiolated and far-red irradiated seedlings

Because of the partial loss of activity when one estimates separately the covalently and ionically bound β-FFases, the experiments regarding the time course were carried out by measuring the total cell-wall activity.

After 24 hr in complete darkness (i.e. 24 hr after sowing) the seedlings were separated into two lots: one was maintained in darkness and the second transferred to far-red light. The β-FFase activity was estimated in the cell-wall and soluble fractions separately prepared from roots and hypocotyls. The time courses were estimated between 24 and 108 hr after sowing.

Figure 1 shows that the total β-FFase activity in the root extracts increases and then slowly declines but to the same extent in the light as in the dark. On the other hand, under far-red light a decrease of the 'soluble activity' sets in, this decrease being correlated with an increasing activity in the cell-wall fraction. Therefore after the seedlings are transferred to far-red light—there may be a movement of β-FFase from the cytoplasm to the cell-wall *in vivo*.

The same experiments were performed with hypocotyls. Figure 2 shows that far-red irradiation leads to (a) an increase of the total β-FFase activity and (b) an apparent movement of the enzyme from the cytoplasm to the cell-wall. Copping and Street [11] reported a rapid movement of acid invertase from the cytoplasm to the cell-wall in sycamore cells when they were transferred to a fresh medium.

The photocontrol of this transfer is further supported by results obtained with cycloheximide and actinomycin D. If, 36 hr after sowing, the seedlings are treated with

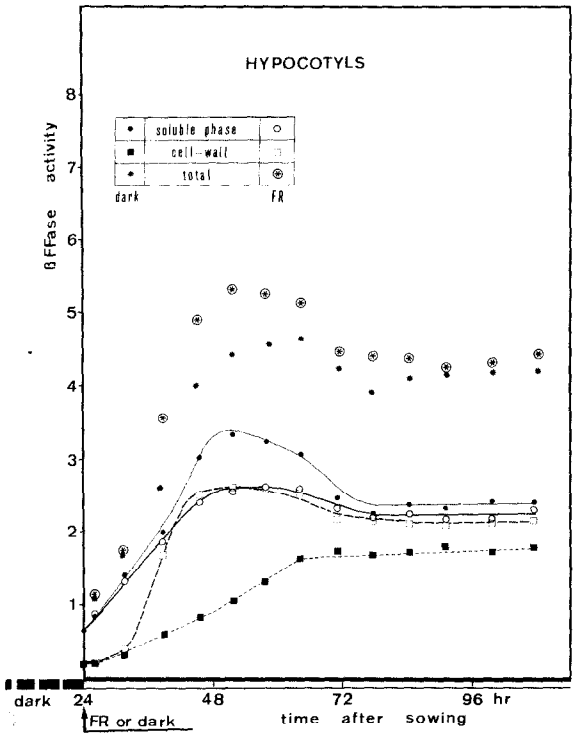

Fig. 2. Time course of total, cell-wall and soluble β-fructosidase activity in hypocotyls from etiolated and far-red irradiated radish seedlings. Each point represents the mean of 8 to 12 experiments.

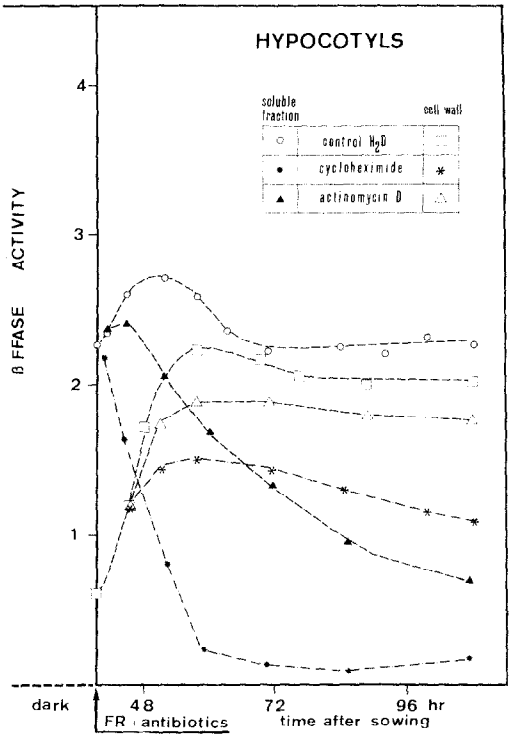

Fig. 3. Effect of actinomycin D and cycloheximide on the β-fructosidase activity in cell-wall and soluble fractions from hypocotyls from far-red irradiated radish seedlings. Far-red light and antibiotic treatment are given 36 hr after sowing.

cycloheximide (28 μg/ml) and placed under continuous far-red irradiation, the soluble β-FFase activity immediately decreases. However, the cell-wall β-FFase activity rises with the control at least for about 12 hr and then remains nearly constant (Fig. 3). After treatment with actinomycin D (50 μg/ml) at the same time (36 hr after sowing) the soluble β-FFase activity increases and after 48 hr decreases. The increase of insoluble β-FFase is only partially prevented after 12 hr by actinomycin D (Fig. 4). These results indicate that the transfer of β-FFase from cytoplasm to cell-wall takes place even if new synthesis of this enzyme is inhibited by cycloheximide and actinomycin D.

Cycloheximide treatment leads to a drop of soluble β-FFase activity to a low level indicating that the enzyme has a relatively short half life, about 12 hr (Fig. 4). The same conclusion is provided from the experiments of Kaufman *et al.* [25], Matsushita and Uritani [19]. The decrease of the soluble invertase only starts 48 hr after the onset of treatment with actinomycin D (Fig. 4). This means that the *m*RNa of β-FFase has a relatively long half-life. As actinomycin D and cycloheximide have no effect on cell-wall invertase activity, this indicates that the insoluble β-FFase is stable.

The effects of these antibiotics raises the question of their specificity on RNA and protein synthesis. Actinomycin D and cycloheximide may inhibit energy transfer and cell membrane permeability independently of their influence on transcription and translation [26]. Klein-Eude [27] has shown that cycloheximide has no effect on permeability and respiratory rate of radish cotyledons. Furthermore these two antibiotics at the same concentrations show different effects on the development of some phytochrome-dependent enzymes in radish seedlings. Cycloheximide represses the decline of phenylalanine ammonialyase activity [28]. It should also be noted that actinomycin D increases the rate of decline of PAL activity [28] and has no effect on the development of amylase activity (Blondel, personal communication).

EXPERIMENTAL

Plant material. Radish (*Raphanus sativus* cv. "Longue rave saumonée") seeds were germinated at 25 ± 0.25° on 4 layers of moist filter paper in 16 × 10 cm plastic boxes. 100 seeds were sown per box. Time zero ($t = 0$) means the time of moistening.

Light source. The standard far-red light source (equivalent to 720 nm monochromatic light) was used as described in ref. [29]. This light, which induces a photostationary state $P_{fr}/P_{total} \simeq 2.5\%$ [30], does not support chlorophyll synthesis [31].

Extraction of soluble and insoluble β-FFases. 40 hypocotyls, roots or cotyledons were normally ground at 2° in a mortar with 15 ml of a 50 mM citrate–Pi buffer pH 5. For the study of the insoluble invertase other isolation medium were used as described in the text. The homogenate was centrifuged at 900 *g* for 15 min. The pellet was washed 2 × and suspended in 5 ml of the extraction buffer. This fraction contains the cell-wall invertase. The supernatant was centrifuged at 15000 *g* for 15 min. The pellet does not show any invertase activity. The supernatant after a Sephadex G25 filtration was used as the soluble fraction.

Enzyme assay. 0.1 ml of the enzyme preparation plus 0.9 ml of substrate (0.1 M sucrose soln in a 50 mM citrate–Pi buffer pH 5 were incubated at 37 ± 0.25° for 30 min. For the determination of the soluble invertase activity the reducing sugar formed was measured according to the method of ref. [32].

The invertase activity of the cell-wall fraction was determined by measuring the glucose formed by the glucose oxidase method [33]. The activity of the enzyme is expressed as μmol of reducing sugar produced per hr and per organ. In this paper the terms decrease or increase of enzyme activity are used without regard for the mechanism of this phenomenon, such as activation, increase of synthesis or decrease of degradation.

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