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STIMULATION OF MAIZE INVERTASE ACTIVITY FOLLOWING INFECTION BY *USTILAGO MAYDIS*

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Abstract—The effect of *U. maydis* infection on the invertase activity of maize leaves has been studied. Infection causes a specific stimulation of an acid invertase in soluble and pellet fractions of homogenized tissue. Invertase activity is stimulated within one day after infection, and is maintained at a high level until 10 days after infection, in contrast to the progressive decline in activity with age, in healthy leaves. Analyses of soluble extracts by gel electrophoresis suggest that the invertase showing this increase is derived from the host and not the pathogen.

INTRODUCTION

A common feature of biotrophic fungal infections [1] of higher plants is an increased translocation of photosynthetic assimilates into infected plant parts which is often accompanied by accumulation of one or more host carbohydrates [2–4]. Recent work has shown that infection of maize by the corn smut, *Ustilago maydis*, stimulates assimilate movement into, and accumulation of soluble sugars, and starch, in infected tissues [5, 6]. Furthermore, changes in the relative concentrations of sucrose, glucose and fructose in infected leaves suggest that sucrose hydrolysis is greatly increased following infection by *U. maydis* [5]. The present paper describes direct measurements of invertase activity (EC 3.2.1.26) in smutted maize leaves.

Increased invertase activities have been reported in infected leaves of a few plants infected by biotrophic fungi [4, 7] and the physiological role of invertase in infected tissue has been considered [4]. However, no biochemical characterization of the enzyme(s) stimulated following infection has so far been attempted, and, in particular, it has not been determined whether the enzyme(s) stimulated is of host or fungal origin. We have now attempted to answer this question, since it is fundamental both to a discussion of the mechanism of stimulation of the enzyme, and to its physiological role, including assimilate movement, in infected tissue. We present here evidence for the stimulation of an invertase of *host* origin following infection of maize by *U. maydis*.

RESULTS

Invertase activity in soluble and pellet fractions of homogenized leaf tissue

The effect of infection on invertase activity was investigated by direct measurement of soluble and pellet fractions of homogenized leaf tissue. In healthy and infected tissue the pH optima of both soluble and pellet invertase activities was *ca* 5 and the pH curves revealed one form of enzyme only. Enzyme activity was not affected by the presence of insoluble PVP (final concentration 5% w/v) during extraction of tissues.

Tables 1 and 2 show changes in soluble and pellet invertase activities during infection. Each value is the mean of two replicate assays and the general pattern of changes following inoculation was similar on 3 separate occasions although the absolute values varied.

The relative proportions of invertase activity in pellet and soluble fractions did not change greatly after infection. In both healthy and infected tissues the pellet invertase fraction constituted a minor component of the total activity. Infection caused an increase both in invertase activity/g fr. wt and in the sp. act. of the enzyme. The sp. act. of both soluble and pellet invertase in infected tissue were *ca* twice that for healthy tissue one day after inoculation. Thereafter the soluble invertase activity remained in infected issue, whilst that in healthy tissue declined sharply until it was less than 5% of the infected tissue activity at 10 days after inoculation (Table 1). The sp. act. of pellet invertase in infected tissue increased gradually throughout this period (Table 2). Table 2 also shows a comparison of invertase activity of ethyl acetate treated leaf discs [4] with that of soluble and pellet fractions derived from the same tissue, at 3 different stages of infection. Invertase activity in tissue discs was *ca* equal to that of *pellet* invertase in both healthy and infected tissues at all times i.e. $<30\%$ of total invertase in homogenized tissue. These results with discs are included to demonstrate that this method may give a very unreliable estimate of total invertase activity. Therefore, it clearly should not be used as the sole basis for theoretical arguments concerning invertase activity following infection or other physiological transitions.

Table 1. Changes in activities of soluble invertase preparations from healthy and infected tissues following inoculation

Time after inoculation (days)	Healthy (H)		Infected (I)		I/H
	*Activity/ g fr. wt	*Activity/ mg protein	*Activity g fr. wt	*Activity mg protein	sp. act.
1	362	201	520	478	2
2	166	153	452	537	4
3	105	667	410	186	3
4	91	77	452	346	5
6	79	32	675	192	6
7	55	38	314	341	9
8	44	11	603	170	16
9	82	29	510	454	16
10	55	16	575	336	21

* Invertase activity expressed as μmol glucose equivalents released into 5 ml medium per hr.

Table 2. Changes in activities of pellet invertase preparations, and of tissue discs from healthy and infected tissues following inoculation

Time after Inoculation (days)	Pellet					Tissue discs	
	Healthy (H)		Infected (I)		I/H	*Activity/ g fr. wt	
	*Activity/ g fr. wt	*Activity/ mg protein	*Activity g fr. wt	*Activity/ mg protein	sp. act.	H	I
1	46	66	41	9	2		
2	3	1	24	7	5		
3	8	1	112	11	10		
4	10	1	123	40	40	11	160
6	8	12	139	22	2		
7	13	4	123	47	12	7	109
8	7	2	170	30	14		
9	8	10	103	108	11	7	66
10	6	3	78	46	17		

* Invertase activity expressed as μmol glucose equivalents released into 5 ml medium per hr.

Further characterization of invertase activity in infected tissue

The specific stimulation of invertase activity observed in infected tissue might be due to an activation, or increase in concentration, of host enzyme, of an enzyme present within the fungal hyphae, or of a fungal enzyme secreted into the surrounding host tissue. In order to distinguish between these possibilities the invertase activities of infected and healthy plants, and of intracellular and extracellular extracts derived from haploid sporidial cultures of *U. maydis* were compared. Since both soluble and pellet invertase activities are increased following infection, and since the soluble enzyme is much more active, only soluble extracts were used in these experiments.

Extracts were analysed, without further purification, by gel electrophoresis at pH 9.5 under non-dissociating conditions, and gels were stained specifically for invertase activity [8]. This stain detects glucose, one of the products of sucrose hydrolysis, by oxidation with glucose oxidase, and simultaneous reduction of phenazine methosulphate, and finally of tetrazolium dye, which forms a blue precipitate in the gel. Since only sucrose is supplied as substrate, and all endogenous sugars have been removed by dialysis, glucose is only produced where invertase is present.

Healthy maize leaves contain two soluble invertase activities of different mobilities (Fig. 1a). Infected leaves show a single invertase band on electrophoresis, which co-migrates with the major invertase activity from healthy leaves (Fig. 1a). No further bands were revealed following prolonged incubation in the invertase stain, so that if present, additional invertase species must represent a minute proportion of the total invertase activity of infected leaves. However, infected tissue invertase can be clearly distinguished from both intracellular and extracellular fungal invertases by electrophoresis under these conditions (Fig. 1b). Invertase mobilities, relative to bromophenol blue, in the different extracts were: (mean of at least 3 analyses) healthy, 0.28 and 0.57; infected, 0.28; intracellular fungal, 0.22; extracellular fungal, 0.35.

DISCUSSION

This work has demonstrated the presence of an acid invertase (pH optimum 4.5–5) in both the soluble and pellet fractions obtained following homogenization of healthy and smutted maize leaf tissue. No alkaline invertase (pH optimum 7–7.8) was detected. Since extraction conditions markedly affect association of plant acid invertases with cell wall material [9], the pellet invertase activity described here does not necessarily equate with enzyme bound to cell walls *in vivo*.

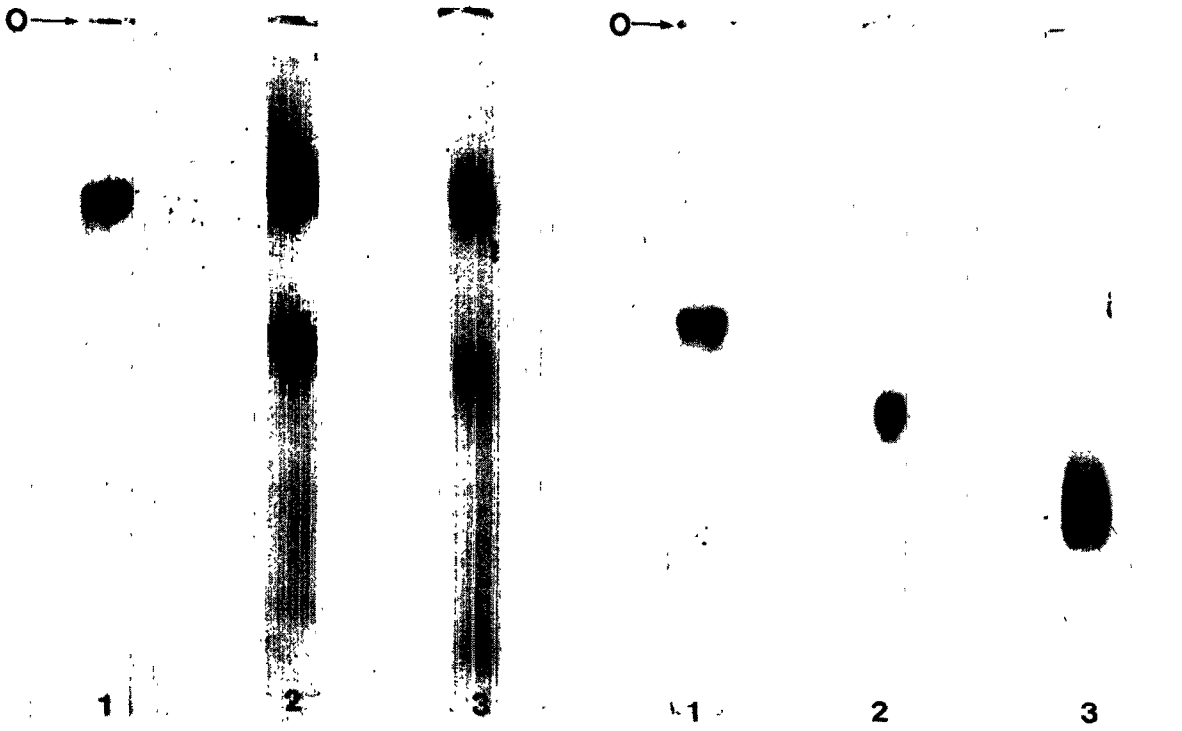

Fig. 1.

Gel electrophoresis of invertase extracts at pH 9.5. Migration from top to bottom, cathode at top. O = sample origin.

a. Gel 1—soluble invertase from infected leaves
Gel 2—soluble invertase from healthy leaves
Gel 3—soluble invertase from healthy leaves
} two independent preparations

b. Gel 1—soluble intracellular invertase of haploid *U. maydis* spordia; Gel 2—soluble invertase from infected leaves; Gel 3—extracellular invertase from haploid *U. maydis* sporidia. Gels in b were run for a longer time than gels in a, and are therefore not directly comparable.

Smut infection causes a specific two fold stimulation of invertase as early as one day after inoculation, coinciding with changes in soluble sugar concentrations [5] but before disease symptoms are evident. In contrast with the decline with age in healthy tissue, invertase activity in smutted leaves remains high until 10 days after inoculation. Clearly, the increase in invertase activity could be due to a stimulation of enzyme derived from the host or from the fungus. Soluble invertase of infected tissue is electrophoretically distinct from both intracellular and extracellular soluble invertase produced by haploid *U. maydis* sporidia in culture. It is possible that one of these fungal enzymes is structurally modified following infection or during extraction from infected tissue, and thus has altered electrophoretic properties, or indeed that the fungus synthesizes a unique form of invertase during its dikaryotic phase, in maize tissue. However, the identical mobility of one of the two healthy tissue invertases and the infected tissue enzyme on electrophoresis does suggest that the enzyme stimulated by infection is a host enzyme. Definitive proof for this conclusion must await detailed purification and characterization of the enzyme. The mechanism of invertase stimulation is unknown but in other systems invertase activity is regulated by specific inhibitors [10], by synthesis and by degradation of the enzyme, through control systems mediated by hormones or carbohydrates, or both [11]. Increases in the levels of IAA have been detected [12] and increases in cytokinin levels implicated [5, 6] in maize tissue infected with *U. maydis*.

High acid invertase activity usually correlates with low activities of alkaline invertase, and of sucrose synthetase, another enzyme which metabolizes sucrose. However, since it has been suggested that sucrose synthetase is involved in utilization of sucrose for polysaccharide synthesis, and in smutted maize tissue starch synthesis, at least, is stimulated [5], the effect of infection on sucrose synthetase activity should be investigated.

High acid invertase activity is characteristic of plant tissues in which there is a requirement for hexose production from stored or recently translocated sucrose [9]. Smutted maize tissue appears to be rapidly growing and dividing [5], and infection of plant tissues with fungal biotrophs usually results in stimulated rates of respiration [13]; a stimulated maize invertase activity would provide the hexoses needed for these processes. Furthermore, stimulation of host invertase activity in smutted tissues could be responsible for accelerated import of assimilate into these tissues either directly by causing faster unloading of sucrose from sieve tubes [14], or indirectly, for example, by supplying energy [15] or by increasing the concentration gradient of sucrose.

EXPERIMENTAL

Maize plants (*Zea mays*, var. Inra 200) were inoculated with a mixture of 2 compatible haploid spores of *Ustilago maydis*

(D.C. Corda) 10 days ofter sowing, as described in ref. [6]. Comparable samples of the basal area of the 4th leaf blade of healthy and infected plants were used for anaysis. One day after inoculation this basal area showed no visible signs of infection, a very few chlorotic spots appeared 2 days after inoculation and chlorosis was widespread 4 days after inoculation. Neoplastic growths, or galls, began to develop 5 days after inoculation, and these galls reached their maximum size 2 days later; diploid spores appeared 9 days after inoculation and by 11 days spores were numerous [5, 6, 16].

Measurement of invertase activity. Invertase was fractionated into easily solubilized form, and pellet form, using a modification of the method of ref. [11]. All procedures were carried out at 0–4°. Healthy and infected tissue (0.5 g fr. wt) was ground using a pestle and mortar, without sand, in 10 ml 0.2 M Pi–citrate buffer pH 4.5. The brei was filtered through 4 layers of cheesecloth and centrifuged at 1700 *g* for 5 min. The pellet was resuspended in 5–8 ml extraction buffer. Both the pellet and the supernatant were dialysed against 1 l. H_2O ($\times 3$; 30 min each). After extraction enzyme prepns were either frozen or assayed immediately; freezing did not affect enzyme activity significantly. Enzyme preparation (1 ml) was incubated at 37° with 4 ml of 0.1 M sucrose, 0.1 M Pi–citrate buffer pH 4.5. Aliquots (0.8 ml) were removed after 1 hr and the reaction stopped by deproteinization using the Somogyi method [17]. The reducing sugar content of the deproteinized sample was assayed by the method of ref. [17]. A range of Pi–citrate buffers (pH 3–8) was used to determine pH optimum. Protein was determined by the method ref. [18]. Invertase activity in tissue discs was determined by the method of ref. [4].

Preparation of extracts for electrophoresis. Soluble extracts were prepared, as described above, using 0.5 g of 4th leaf tissue from 20-day-old infected plants, 10 days after inoculation. Similar extracts from healthy plants of the same age had insufficient invertase activity, even after concn, to detect after electrophoresis. Since invertase activity in the 4th leaf of healthy plants is much greater at 11 days of age (1 day after inoculation, see Table 1), 10 g of this tissue was used, instead, as a source of invertase, and extracted as above. Soluble extracts of healthy and infected tissues were dialysed against 19 mM Tris–glycine pH 9.5 (twice, 4 hr each), centrifuged at 15000 *g* for 10 min to remove chloroplasts, and concentrated by ultrafiltration using an Amicon UM52 cell. Soluble fungal invertase extracts were prepared from separate cultures of the two compatible haploid mating types of *U. maydis* [5, 6] grown for 4 days at 25° in complete medium [19] in which glucose was replaced by sucrose (10 g/l.). Sporidia were harvested by centrifugation at 10000 *g* for 15 min. Protein was recovered from the supernatant by ppn with $(NH_4)_2$ SO_4, redissolved in and dialysed against 19 mM Tris–glycine pH 9.5 and finally concd by ultrafiltration This soln was used as the extra cellular fungal invertase extract. The sporidia of both mating types (6 g) were resuspended in 50 mM NaOAc pH 5, and disrupted by sonication. Sporidial fragments were removed by centrifugation (1700 *g*; 5 min), and the supernatant dialysed against 19 mM Tris–glycine pH 9.5, and used without further concn as the soluble intracellular fungal extract.

Electrophoresis of invertase extracts. Invertase extracts were analysed by electrophoresis on 7% polyacrylamide gels with a continuous buffer system, 94 mM Tris–glycine, pH 9.5, as described in ref. [20]. Samples were applied in sucrose, 19 mM Tris–glycine pH 9.5. Bromophenol blue was included as a marker dye, and, within each group, all gels were run until the dye had migrated a similar distance. After electrophoresis, gels were stained for invertase activity as described in ref. [8] except that glucose oxidase was included in the staining reagent at a final conc of 110 units/ml In addition glucose oxidase was incorporated into the acrylamide gel soln, before polymerization, and in the running buffer at final concn of 70 units/ml and 8 units/ml respectively. In some cases calf serum (1 µl) was added to invertase extracts and co-electrophoresed with them as an internal standard. Gels were then stained for protein with coomassie blue. The mobilities of major serum proteins relative to bromophenol blue were identical whatever the source of the invertase extract with which they were co-electrophoresed. Thus differences in the composition of the various extracts should not affect the mobilities of invertases relative to bromophenol blue.

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