Journal of Experimental Botany, Vol. 42, No. 240, pp. 931–934, July 1991

Degradation of Chitotetraose to Chitobiose in the Axenic Rape Rhizosphere

PETER SUNDIN[1,3], STEFAN OLSSON[2] and GÖRAN ODHAM[1]

[1] *Chemical Ecology and* [2] *Microbial Ecology, Department of Ecology, Lund University, Helgonavägen 5, S-223 62 Lund, Sweden*

Received 1 November 1990; Accepted 4 February 1991

ABSTRACT

In the presence of intact roots of 2-week-old axenic rape seedlings, added *N,N′,N″,N‴*-tetraacetylchitotetraose was degraded to *N,N′*-diacetylchitobiose during 48 h of incubation. Only minute amounts of monomer (*N*-acetyglucosamine) were formed from added chitotetraose or chitobiose. The addition of a purified chitin suspension caused no measurable formation of degradation products.

The formation of chitobiose from added chitotetraose was linear, suggesting the action of a constitutive enzyme during the incubation period.

Key words: *Brassica napus* (L.) cv. Brink, rhizosphere, *N,N′*-diacetylchitobiose, *N,N′,N″,N‴*-tetraacetylchitotetraose.

INTRODUCTION

Chitinases have been found in plants of many angiosperm families (Boller, 1986), and in a gymnosperm (Sauter and Hager, 1989), suggesting their ubiquitous occurrence. Polymer-linked hexosamines present in the secondary walls of several plants, however, do not merit as intrinsic substrates (Benhamou and Asselin, 1989). Instead, the role of plant chitinases may be in the defence of plants against fungi. Constitutive levels of chitinases are often found in plant tissues and additional activity may be induced by various stimuli (Boller, 1986).

Most plant chitinases are endochitinases (Roberts and Selitrennikoff, 1988), and degrade chitin to di- and trimer units upon complete hydrolysis. The turnip lysozyme, described by Fleming (1922), has also been found to possess endochitinolytic activity. Its main hydrolysis products when acting upon chitopentaose were chitotriose and chitobiose, with no production of chitotetraose (Bernier, van Leemputten, Horisberger, Bush, and Jollès, 1971).

Using axenic rape plants, *Brassica napus* (L.) cv. Brink, an experiment was performed to test if chitinolytic activity is present in the rhizosphere of intact plants. A suspension of purified chitin or a solution of *N,N′,N″,N‴*-tetraacetylchitotetraose were provided as substrates in parallel treatments. Chitotetraose could only be expected to be degraded to the dimer, *N,N′*-diacetylchitobiose, when acted upon by an endochitinase. In a separate experiment, *N,N′*-diacetylchitobiose was used as the substrate, to account for possible exochitinase or chitobiase activity.

MATERIALS AND METHODS

Chemicals

Purified chitin and *N*-acetyl glucosamine were obtained from the Sigma Chemical Company (St. Louis, MO., USA). *N,N′*-diacetylchitobiose was from Carbohydrates International AB (Arlöv, Sweden). *N,N′,N″*-triacetylchitotriose and *N,N′,N″,N‴*-tetraacetylchitotetraose were obtained from BioCarb Chemicals (Lund, Sweden). The purity of the commercial preparations were verified by HPLC.

Microcosms

Sterile glass microcosms, each of a total volume of 35 cm^3 were prepared as previously described (Sundin, Valeur, Olsson, and Odham, 1990). The microcosm system consisted of an inner tube with about 6·0 cm^3 coarse sand (particle diameter 2–4 mm) as the rooting medium. The inner tube communicated with an outer tube through a small bottom aperture covered with glass wool below the sand. The whole arrangement was heated to 450 °C overnight, then covered with a plastic lid to allow gas exchange, and autoclaved. Five cm^3 autoclaved mineral nutrient solution was added to each microcosm system, whereupon they were planted with axenic rape seedlings from surface-sterilized seeds (10% H_2O_2, 30 min). Unplanted microcosms served as controls.

[3] To whom correspondence should be addressed.

This cultivation method allows collection of the culture solution with minimal risk of damage to the roots, since the inner tube may be raised with the roots undisturbed, leaving the culture solution in the outer tube.

Substrate preparation

After 13 d the microcosms were supplemented with a sterile filtered substrate solution (Gelman Acrodisc 4192, 0·22 μm) of purified chitin, *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose or *N*,*N*′-diacetylchitobiose. The chitin was initially dispersed in deionized water or mineral nutrient solution by sonication. The recovery of chitin after sterile filtration was measured by drying and weighing the filtrate of the water dispersion and was found to be 6% by weight.

Substrate addition

Eleven days after planting 0·5 mg chitobiose, dissolved in 100 mm^3 mineral nutrient solution, was added to each of two axenic rape microcosms and two sterile control microcosms. Thirteen days after planting, 2·5 mg chitotetraose, dissolved in 150 mm^3 mineral nutrient solution, was added to each of 12 axenic rape microcosms and three sterile control microcosms. Thirteen days after planting, 0·25 mg of purified chitin (equal to 6% of the amount dispersed before filtration), dispersed in 200 mm^3 mineral nutrient solution, was added to each of 12 axenic rape microcosms and three sterile control microcosms.

Sterility test

At sampling, 100 mm^3 of each culture solution was aseptically transferred to autoclaved plastic dishes containing 0·3% Tryptone Soy Broth (Difco) and 1·5% Bacto-Agar (Difco). The dishes were checked for bacterial growth after 4 d at ambient temperature in the dark.

Sampling

Replicates of four axenic rape microcosms and one sterile control microcosm of the chitin and chitotetraose treatments were sampled at 4, 24 and 48 h after substrate addition. The mineral nutrient solutions containing substrate and products were collected. The shoot lengths were measured, and the plants were separated into roots and shoots, lyophilized and weighed. The two axenic rape microcosms, together with the two sterile control microcosms of the chitobiose treatment, were sampled at 96 h after substrate addition.

Chemical analysis

In the experiment where chitin or chitotetraose was used as the substrate, the neutral solutes of the culture solutions were purified on a disposable column solid phase extraction system (J.T. Baker, Phillipsburg, N.I., USA), consisting of a quaternary amine cation exchange column, an aromatic sulphonic acid anion exchange column and an octadecyl (C18) column in series. The column stacks were rinsed with 5·0 cm^3 methanol and 5·0 cm^3 deionized water before passage of each sample, followed by a rinse with 0·5 cm^3 deionized water which was added to the sample. The recovery of chitotetraose from the extraction system was 98 ± 2% (mean ± s.d., $n = 3$). The *N*-acetylated amino sugars of the purified samples were separated on a 20 cm × 0·4 cm (inner diameter) Spherisorb NH_2 column using acetonitrile/water (63:37) as the mobile phase. The column was fitted into a Varian 5000 liquid chromatograph equipped with a 10 mm^3 loop injector and a UV detector at 195 nm. The detector signal was recorded on a Hewlett Packard 3390A Reporting Integrator. Dose-response curves of *N*-acetyl glucosamine, and the di-, tri-, and tetramers, were constructed to verify the linearity of the method in the concentration range being studied. Detection limits were about 200 ng cm^{-3}.

In the experiment where *N*,*N*′-diacetylchitobiose was used as the substrate the culture solutions were centrifuged and 500 mm^3 aliquots were removed from each clear supernatant. The aliquots were lyophilized and dried over P_2O_5. The dry aliquots were then silylated according to Sweeley, Bently, Makita, and Wells (1963), the reagents were evaporated and the derivatives were finally dissolved in 200 mm^3 heptane. The silylated compounds were separated on a 4·0 m × 0·2 mm (inner diameter) fused silica capillary column (SGE International Pty. Ltd., Australia) coated with immobilized SE-54 (Alltech Ass., USA). The column was fitted into a Varian 3700 gas chromatograph, equipped with a split/splitless injector and a flame ionization detector, in conjunction with a Hewlett Packard 3390A Reporting Integrator. Hydrogen was used as the carrier gas at a flow rate of 2·0 cm^3 min^{-1}, and nitrogen served as the make-up gas. The injector temperature was 250 °C, the detector temperature was 280 °C and the oven temperature was programmed from 150 °C to 250 °C at a rate of 20 °C min^{-1}. The split valve was opened 40 s after injection.

Statistics

The measured parameters were normally distributed according to the Kolmogorov–Smirnov one-sample test. The linearities of substrate consumption and product appearance with time were verified by linear regression and testing the significance of slope. Differences in total plant dry weights were tested by a two-way analysis of variance (Sokal and Rohlf, 1981).

RESULTS

All microcosms were uncontaminated by microorganisms at sampling, as determined by the sterility tests. The added chitotetraose and chitobiose preparations contained no impurities, but the chitin dispersion caused a detectable HPLC peak with retention time between that of the monomer and the dimer.

In the axenic rape microcosms supplemented with chitotetraose, the presence of the dimer was detected 4 h after addition, increasing linearly over the next 44 h (Fig. 1). The monomer and trimer forms were also detected, although in considerably smaller amounts. Simultaneously, chitotetraose decreased linearly during the 48 h of incubation. In the sterile control microcosms (without plants) the amounts of chitotetraose remained intact.

The recoveries of chitotetraose and the molar equivalents of its degradation products from the microcosms were 82 ± 9% (mean ± s.d., $n = 15$), independent of the presence of a plant, suggesting no significant loss of the chitotetraose degradation products in the presence of a plant. The ratio between chitotetraose and *N*-acetylglucosamine formed was 3·5 ± 0·6. Deviation from the expected value of 3·0 could possibly be due to some uptake of the monomer by the plants.

In the axenic rape microcosms supplemented with chitin, no degradation products could be detected.

In the axenic rape microcosms supplemented with

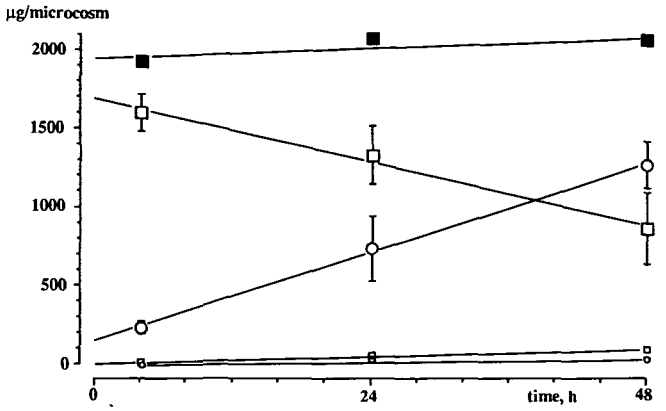

FIG. 1. Total amounts of *N*-acetylglucosamine (○), *N,N'*-diacetylchitobiose (○), *N,N',N''*-triacetylchitotriose (□) and *N,N',N'',N'''*-tetraacetylchitotetraose (□) in sterile control microcosms (filled symbols) and in axenic rape microcosms (open symbols) supplemented with 2·5 mg *N,N',N'',N'''*-tetraacetylchitotetraose at day 13 after planting, 4 h before the first sampling (second sampling 24 h and third sampling 48 h after substrate addition). Filled points represent one replicate, open points represent means with standard deviations of four replicates. The correlations with time of the measured amounts in the axenic rape microcosms are; monomer: $r^2=0{\cdot}836$, $t=7{\cdot}147$, $P<0{\cdot}0001$; dimer: $r^2=0{\cdot}915$, $t=10{\cdot}365$, $P<0{\cdot}0001$; trimer: $r^2=0{\cdot}911$, $t=10{\cdot}116$, $P<0{\cdot}0001$; and tetramer: $r^2=0{\cdot}777$, $t=5{\cdot}908$, $P<0{\cdot}0001$.

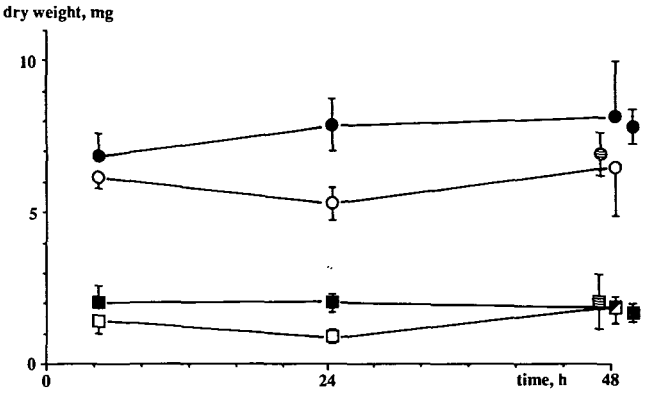

FIG. 2. Dry weights of the shoots (●) and roots (■) of axenic rape seedlings supplemented with *N,N',N'',N'''*-tetraacetylchitotetraose ($n=4$), and the shoots (○) and roots (□) of axenic rape seedlings supplemented with purified chitin ($n=4$), at day 13 after planting in sterile microcosms, 4 h before the first sampling (second sampling 24 h and third sampling 48 h after substrate addition). Dry weights of the shoots (⊜) and roots (▤) of axenic rape seedlings supplemented with *N,N'*-diacetylchitobiose ($n=2$) 4 d before sampling (which was performed 15 d after planting in sterile microcosms, corresponding to 48 h after tetraose or chitin addition above). Dry weights of the shoots (⊛) and roots (▩) of unsupplemented rape seedlings at day 15 after planting in sterile microcosms ($n=8$; Data from Sundin *et al.*, 1990). Points represent means with standard deviations.

chitobiose no degradation was obvious, although small amounts of *N*-acetylglucosamine did appear (11 ± 1 µg per microcosm). No traces of *N*-acetylglucosamine could be detected in the sterile control microcosms.

The substrate-product transformations are summarized in Table 1.

All plants appeared healthy at harvest, with the exception of three plants (of which two were from the chitin treatment) displaying yellow cotyledons at the sampling, 48 h after substrate addition. The dry weights of the plants supplemented with chitotetraose remained constant during the 3 d of samplings (Fig. 2). The total dry weights of the plants supplemented with purified chitin were lower than the tetraose-supplemented plants ($F=11{\cdot}233$, $P<0{\cdot}005$). The differences in dry weights were not reflected in the shoot lengths.

In the experiments where chitobiose was added, the root and shoot dry weights did not differ from those of the tetraose treatment after a culture period of 15 d.

DISCUSSION

In the axenic rape microcosms, added chitin caused no formation of degradation products, whereas added chitotetraose was split into dimers. The incapacity of intact rape roots to degrade the polymer could be due to steric hindrance, or that the tetramer-splitting agent did not act on chitin. Bernier *et al.* (1971), however, showed that the 'turnip lysozyme' degraded chitopentaose into di- and trimers, and that it had a high chitinase activity. If the corresponding enzyme system was active in this study, the results suggest that it is located within the root, where the diffusion of chitin polymers is obstructed, but where the more diffusible chitotetraose molecules become accessible to the hydrolytic activity.

During the incubation, the formation of chitobiose in the axenic rape microcosms supplied with chitotetraose was a linear process (Fig. 1). This indicates that, during this period, constitutive enzymes acted upon the tetramer without any induction of additional activity. Recently, it

TABLE 1. *Degradation of* N,N'-*diacetylchitobiose* (NAG_2) *to* N-*acetylglucosamine* (*NAG*), *and* N,N',N'',N'''-*tetraacetylchitotetraose* (NAG_4) *to* N,N',N''-*triacetylchitotriose* (NAG_3), NAG_2 *and NAG in axenic rape rhizosphere*

Samples were from 15 d old plants, incubated for 96 h with NAG (500 µg, $n=2$) or 48 h with NAG_4 (2500 µg, $n=4$). Mean values of total amount per microcosm are given with standard deviations. Detection limits were about 1 and 2 µg per microcosm with the HPLC and the GC method, respectively.

Substrate	NAG (µg)	NAG_2 (µg)	NAG_3 (µg)	NAG_4 (µg)	Method
NAG_2	11 ± 1	495 ± 33			GC
NAG_4	22 ± 5	1262 ± 151	82 ± 14	853 ± 230	HPLC

came to our attention that at least four isoforms of chitinase are present in rape (Ulla Rasmussen, Risø National Laboratories, Denmark, personal communication). In her study, however, infection with *Phoma lingam* caused about a 3-fold local increase in chitinase activity of cotyledons 48 h after inoculation.

The minute amounts of monomer and trimer formed from chitotetraose suggests the absence of any significant exochitinase or chitobiase activity. This is supported by the results from the chitobiose treatment (Table 1). From unpublished data obtained in an earlier experiment (Sundin *et al.*, 1990) it was concluded that *N*-acetylglucosamine and chito-oligomers are absent from axenic rape rhizospheres that have not been supplemented with chitobiose or chitotetraose.

The addition of chitobiose or chitotetraose to the rhizosphere environments caused no adverse effects on plant dry weight gain. Instead, following a culture period of 15 d, 96 and 48 h after chitobiose or chitotetraose addition, the root and shoot dry weights of the supplemented plants fully agreed with the corresponding weights obtained after 15 d in an earlier experiment, when unsupplemented rape was cultured under similar conditions in identical microcosms (Sundin *et al.*, 1990, also shown in Fig. 2). The addition of chitin, however, caused a reduction in dry weight gain. An explanation could be that the chitin preparation suffered some degree of *N*-deacetylation (Muzzarelli, 1986), which could have disturbed root membrane functions (Leuba and Stossel, 1986).

To sum up, despite the fact that no degradation of chitin was observed, the presence of chitinolytic activity cannot be excluded, since the results may have been caused by the localization of the hydrolytic system in undamaged roots combined with the differences in diffusibility between chitin and chitotetraose.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Natural Science Research Council and the Swedish Council for Forestry and Agricultural Research, which are gratefully acknowledged. We thank Ulla Rasmussen, Risø National Laboratories, Denmark, for valuable discussions.

LITERATURE CITED

BENHAMOU, N., and ASSELIN, A., 1989. Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. *Biology of the Cell*, **67,** 341–50.

BERNIER, I., VAN LEEMPUTTEN, E., HORISBERGER, M., BUSH, D. A., and JOLLÈS, P., 1971. The turnip lysozyme. *FEBS Letters*, **14,** 100–4.

BOLLER, T., 1986. Chitinase: a defense of higher plants against pathogens. In *Chitin in nature and technology*. Eds R. Muzzarelli, C. Jeuniaux and G. W. Gooday. Plenum Press, New York. Pp. 223–30.

FLEMING, A., 1922. On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society of London Series B*, **93,** 306–17.

LEUBA, J. L., and STOSSEL, P., 1986. Chitosan and other polyamines: antifungal activity and interactions with biological membranes. In *Chitin in nature and technology*. Eds R. Muzzarelli, C. Jeuniaux and G. W. Gooday. Plenum Press, New York. Pp. 215–22.

MUZZARELLI, R., 1986. Chemically modified chitosans. In *Chitin in nature and technology*. Eds R. Muzzarelli, C. Jeuniaux and G. W. Gooday. Plenum Press, New York. Pp. 295–320.

ROBERTS, W. K., and SELITRENNIKOFF, C. P., 1988. Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology*, **134,** 169–76.

SAUTER, M., and HAGER, A., 1989. The mycorrhizal fungus *Amanita muscaria* induces chitinase activity in roots and in suspension-cultured cells of its host *Picea abies*. *Planta*, **179,** 61–6.

SOKAL, R. R., and ROHLF, F. J., 1981. *Biometry*, second edition. W. H. Freeman and company, New York.

SUNDIN, P., VALEUR, A., OLSSON, S., and ODHAM, G., 1990. Interactions between bacteria-feeding nematodes and bacteria in the rape rhizosphere: effects on root exudation and distribution of bacteria. *FEMS Microbiology Ecology*, **73,** 13–22.

SWEELEY, C. C., BENTLY, R., MAKITA, M., and WELLS, W. W., 1963. Gas–liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *Journal of the American Chemical Society*, **85,** 677–87.