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Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases

(chemoperception/chito-oligosaccharides/signaling/symbiosis)

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ABSTRACT **The bacterial genera *Rhizobium* and *Bradyrhizobium*, nitrogen-fixing symbionts of legumes, secrete specific lipo-chitooligosaccharides that induce the formation of nodules on their host plants. When preparations of such nodulation-inducing factors (Nod factors) were added to suspension-cultured tomato cells, a rapid and transient alkalinization of the culture medium occurred. Lipo-oligosaccharide preparations from *Rhizobium* or *Bradyrhizobium* treated with flavonoids, known inducers of Nod factor synthesis, were up to 100 times more potent in inducing alkalinization than the ones from untreated bacteria. The activity was absent from preparations of the mutant strain *Rhizobium* sp. NGR234Δ*nodABC*, unable to produce any Nod factors. Preparations of Nod factors from various bacteria as well as individual, highly purified Nod factors from *Rhizobium* sp. NGR(pA28) induced alkalinization in the tomato cell cultures at nanomolar concentrations. This demonstrates that Nod factors can be perceived by tomato, a nonhost of rhizobia. Using the alkalinization response as a sensitive bioassay, Nod factors were found to be inactivated by plant chitinases. Root chitinases purified from different legumes differed in their potential to inactivate differently substituted Nod factors produced by *Rhizobium* sp. NGR(pA28). This indicates that the specificity of the bacterium–host plant interaction may be due, at least in part, to differential inactivation of Nod factors by root chitinases.**

The infection of legume roots by *Rhizobium* or *Bradyrhizobium* results in the formation of root nodules in which the bacteria may fix nitrogen (1, 2). During the infection process, flavonoids secreted by the plant induce the transcription of bacterial genes that are required for nodulation, the *nod* genes (3). The products of these *nod* genes, in turn, are involved in the synthesis of the so-called nodulation factors (Nod factors), a family of signal molecules consisting of lipo-chitooligosaccharides—i.e., β-1,4-linked oligomers of *N*-acetylglucosamine with a fatty acid moiety on the nonreducing end (3–6). When applied to host plants, these Nod factors induce rapid membrane depolarization (7), root-hair deformation (3–5), expression of early nodulin genes (5, 6), and mitosis in the root cortex (4–6) and thus appear to play a key role in the establishment of the nodule symbiosis. It is generally thought that a given host plant has specific receptors for specific Nod factors, accepting a bacterium as a symbiont only if it recognizes the Nod factors secreted by the latter (3–6).

Recognition of Nod factors by host plants can be seen as one variation on the theme of perception of microbial signals by plants. Another variation is the recognition of molecules derived from microbial pathogens, so-called elicitors, which cause induction of a defense response (8, 9). Suspension-cultured plant cells are widely used to study elicitor recognition and signal transduction (8, 9). In these model systems, alkalinization of the growth medium is a particularly early reaction to various elicitor preparations and can be used as an early marker for elicitor perception (9–13). Here we report that Nod factors elicit a similar alkalinization response in suspension-cultured cells of tomato, a nonhost of rhizobia, and that the alkalinization-inducing activity of variously substituted Nod factors is differentially inactivated by legume chitinases.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Rhizobium* sp. NGR(pA28) was grown as described (14). *Rhizobium* sp. NGR234 (14), *Rhizobium* sp. NGRΔ*nodABC* (14), *Rhizobium leguminosarum* biovar (bv.) *viciae* RBL5560 (15), *Bradyrhizobium japonicum* 61-A-101 (16), *Rhizobium meliloti* 102F34 (17), and *Rhizobium phaseoli* RCR3622 (from P. R. Hirsch, Rothamsted Experimental Station, Harpenden, U.K.) were grown at 27°C on a rotary shaker at 130 rpm in a minimal medium (14) with the following carbon and nitrogen sources: *R.* sp. NGR234, *R.* sp. NGRΔ*nodABC*, and *R. meliloti*, 0.12 M sodium succinate and 1 mM ammonium chloride; *R. leguminosarum* and *R. phaseoli*, 8 mM mannitol and 5 mM KNO_3; *B. japonicum*, 50 mM glycerol, 1 mM mannitol, and 5 mM KNO_3. Growth media of *R. leguminosarum*, *R. phaseoli*, and *B. japonicum* contained, in addition, 2 mg of yeast extract per ml, pretreated as follows to eliminate inducers of the alkalinization response: the yeast extract (0.1 g/ml) was incubated with 0.1 mg of chitinase per ml from *Serratia marcescens* (Sigma) in 100 mM phosphate (K^+) buffer at pH 6.0, autoclaved, centrifuged, and partitioned several times against fresh 1-butanol (1:1 ratio).

Preparation of Nod Factors. Lipo-oligosaccharides were extracted 24 h (*Rhizobium* strains) or 48 h (*B. japonicum*) after the addition of appropriate flavonoids to exponentially growing bacteria, using published procedures (14, 15, 18). Briefly, cultures were centrifuged at 27,000 × *g* for 20 min, and the supernatant was extracted with 0.25 volume of 1-butanol by shaking for 2 min. The 1-butanol phase was evaporated, and the residue was taken up in water and extracted with an equal volume of ethyl acetate. The aqueous phase was loaded onto C_{18} SepPac cartridges (Millipore). After washing with water, the adsorbed material containing lipo-oligosaccharides was eluted with methanol and dried in a Speed-vac evaporator. The residue was dissolved in water containing 0.01% 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate (Fluka), filtered through a 0.22-μm Ultrafree-MC filter (Millipore), and stored at −20°C. Individual

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Abbreviation: chitotetraose, *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose.

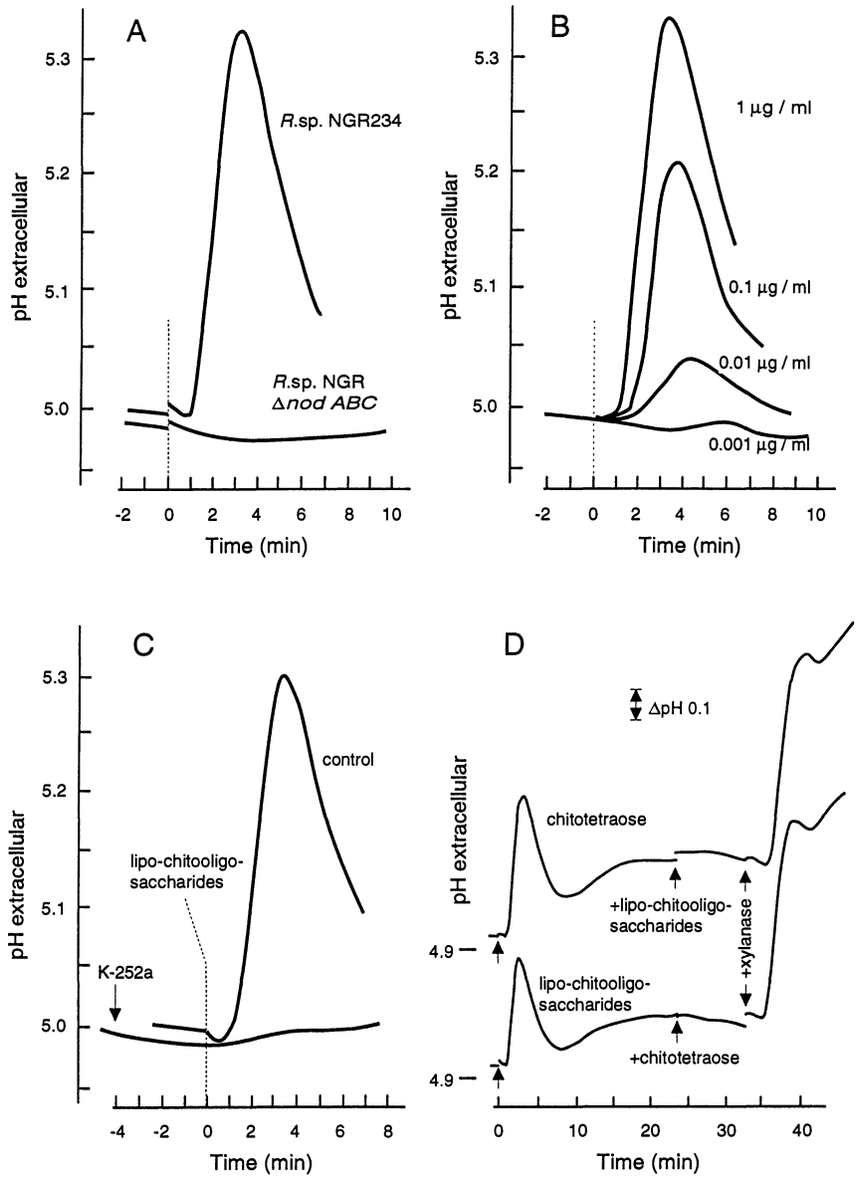

FIG. 1. Alkalinization of the culture medium of tomato cells in response to preparations of Nod factors. (*A*) Comparison of the response to equivalent amounts of lipo-oligosaccharide preparations from *R.* sp. NGR234 and the mutant strain *R.* sp. NGR*ΔnodABC*. (*B*) Response to various concentrations of the Nod factor preparation from *R.* sp. NGR234 added at zero time. (*C*) Effect of K-252a. K-252a (1 μM) was added 4 min before the Nod factor preparation from *R.* sp. NGR234 (corresponding to 100 nM lipo-chitooligosaccharides). Control, cells treated with the Nod factor preparation only. (*D*) Refractory behavior of the tomato cells. The pH of the growth medium was continually registered in two separate samples. Arrows, addition of 1 nM chitotetraose, of the Nod factor preparation from *R.* sp. NGR234 (corresponding to 100 nM lipo-chitooligosaccharides), or of 10 μg of xylanase per ml.

Nod factors from the overproducing strain *R.* sp. NGR(pA28) were isolated and purified by HPLC as described (14).

Quantification of Lipo-Chitooligosaccharides. Lipo-chitooligosaccharides were quantified by measuring *N*-acetylglucosamine after enzymatic hydrolysis, as described for chito-oligosaccharides (19): preparations were treated with 25 mg of dialysed snail gut enzyme per ml (cytohelicase from IBF, Villeneuve-la-Garenne, France) in 50 mM phosphate (K^+) buffer at pH 6.5, 37°C, for 24 h. The *N*-acetylglucosamine released was determined colorimetrically. Known amounts of purified Nod factors from *R.* sp. NGR(pA28), digested in the same manner, were used as standards.

Assay and Purification of Root Chitinases. Chitinase was assayed with [^{3}H]chitin as substrate (20), defining 1 nkat as the amount releasing 1 nmol of *N*-acetylglucosamine equivalents per s at infinite dilution (19). Chitinases were purified from cowpea (*Vigna unguiculata* L. Walp.) cv. Red Caloona (Rawling Seeds, Orpington, Kent, U.K.), from bean (*Phaseolus vulgaris* L.) cv. Saxa (Samen Wyss, Zuchwil, Switzerland), and from pea (*Pisum sativum* L.) cv. Dunkelgrüne Markerbse (Ditzler AG, Möhlin, Switzerland) after exposing plants (3 weeks old) to 10 nl of ethylene per ml for 48 h (20). Plant roots were ground in liquid nitrogen and extracted with 100 mM phosphate (K^+) buffer at pH 7.0 (1 ml/g of fresh weight). The extract was centrifuged (18,000 × *g*, 15 min) and precipitated with ammonium sulfate (80% saturation). The redissolved precipitate was loaded onto a column of regenerated chitin (21). Chitin-binding proteins were eluted with 100 mM acetic acid, dialyzed, lyophilized, redissolved in 10 mM phosphate (Na^+) buffer at pH 6.0 containing 140 mM NaCl, and chromatographed in the same buffer on a Superose12 column (Pharmacia). Fractions with chitinase activity were pooled, dialyzed, subjected to electrophoresis on a $NaDodSO_4$/10% (wt/wt) polyacrylamide gel (22), and stained with Coomassie brilliant blue R 250.

Preparation of [^{3}H]Chito-Oligosaccharides. Water-soluble [^{3}H]chito-oligosaccharides were prepared from [^{3}H]chitin with 0.5 nkat of purified bean chitinase as described (21).

Assay of the Alkalinization Response. The tomato cell line Msk8 was grown as a fine suspension as described (23). For assays, aliquots of 2 ml, containing ≈0.3–0.5 g of cells, were incubated in open 20-ml vials on a rotatory shaker at 120 cycles per min, and the pH of the culture medium was continually registered (13). The maximal pH increase (ΔpH_{max}), occurring 2–5 min after application of the stimuli, was taken as a measure of activity (13). Where indicated, cells were treated with 1 nM *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose (chitotetraose; BioCarb, Lund, Sweden), 10 μg of xylanase per ml (from *Trichoderma viride*, Fluka), or 1 μM of K-252a (Fluka).

Incubation of Nod Factors with Chitinases. The reaction mixture (150 μl) contained 100 nM purified Nod factors from *R.* sp. NGR(pA28), 1% (vol/vol) dimethyl sulfoxide, 10 mM phosphate (Na^+) buffer at pH 6.0, 10 mM NaCl, 4 mM NaN_3, and 1 nkat of purified chitinase. After incubation at 37°C, samples were loaded onto C_{18} SepPac cartridges (Millipore), eluted with 2 ml of methanol, and dried in a Speed-Vac evaporator. The residue was taken up in 5 μl of dimethyl sulfoxide, diluted with 45 μl water, and used for the assay of the alkalinization response.

RESULTS

Alkalinization Response of Tomato Cells Induced by Nod Factors. As shown previously, suspension-cultured tomato cells have a sensitive perception system for chito-oligosaccharides and react to subnanomolar concentrations of these molecules by a rapid and transient alkalinization of the growth medium (13). To test the reaction of tomato cells to Nod factors, lipo-oligosaccharides were isolated from cultures of the broad host-range rhizobium *R.* sp. NGR234, treated with the flavonoid apigenin to induce the *nod* genes. 1-Butanol extraction followed by batch chromatography on a C_{18} SepPac cartridge yielded about 1 mg (dry weight) of a lipo-oligosaccharide preparation per liter of culture. We added aliquots of this preparation to tomato cells and found that they induce an alkalinization of the growth medium (Fig. 1*A*). Similar amounts of a preparation from flavonoid-treated cultures of the mutant *R.* sp. NGRΔ*nodABC*, a strain unable to produce Nod factors, did not induce alkalinization (Fig. 1*A*), indicating that the response depends on Nod factors. With the preparation from wild-type *R.* sp. NGR234, concentrations as low as 1 ng/ml elicited a measurable alkalinization (Fig. 1*B*). The initial lag phase and the ΔpH_{max} depended on the dose; at higher concentrations, the pH started to increase after about 1 min and reached its peak after 5 min (Fig. 1*B*).

The tomato cells used are obviously highly sensitive to Nod factor preparations, but they are even more sensitive to underivatized, hydrophilic chitin fragments (13). To test for the presence of such hydrophilic factors, the Nod factor preparation of *R.* sp. NGR234 was partitioned again between 1-butanol and water and chromatographed over a C_{18} SepPac cartridge. The alkalinization-inducing activity was mostly found in the butanol phase, was practically completely adsorbed by the C_{18} SepPac material, and could be quantitatively eluted from the cartridge by methanol (Table 1). As a control, a preparation of [^{3}H]chito-oligosaccharides was subjected to the same procedures. In this case, all radioactivity remained in the water phase, and all appeared in the flow-through of the C_{18} SepPac cartridge (Table 1). Thus, the alkalinization-inducing substances in the Nod factor preparations are lipophilic.

The alkalinization response of tomato cells to chitin fragments can be blocked by the protein kinase inhibitor K-252a (13), a substance that inhibits various other responses to elicitors as well (10, 24). K-252a also prevented the alkalinization response elicited by addition of the Nod factor preparations (Fig. 1*C*). This suggests that the pH increase is not due to simple buffer effects or to nonspecific membrane leakage but is triggered by a process involving protein kinases.

Table 1. Partitioning of the alkalinization-inducing activity of a Nod factor preparation from *R.* sp. NGR234 and of chito-oligosaccharides during butanol extraction and chromatography on a C_{18} SepPac cartridge

Fraction	Alkalinization-inducing activity, % initial concentration	[^{3}H]Chito-oligosaccharides, cpm
Water phase*	19	2428
1-Butanol phase*	81	15
Flow-through†	1	2556
Methanol eluate†	80	12

*Phases after 1-butanol extraction.
†From a C_{18} SepPac cartridge.

To test if the responses to the Nod factor preparations and to chitin fragments were related, we made use of the refractory behavior of the tomato cells to repeated stimulation: cells stimulated once with chitotetraose are unresponsive to a second stimulation with chitin fragments for up to 6 h but show a full alkalinization response when exposed to a different stimulus, xylanase (13). As shown in Fig. 1*D*, cells treated first with chitotetraose did not respond to a subsequent treatment with the Nod factor preparation and vice versa. In both cases, the cells were still sensitive to xylanase (Fig. 1*D*). This strongly indicates that tomato cells respond to the Nod factor preparation in the same way as to chitin fragments (13).

Dose–response curves were established for individual, highly purified Nod factors from *R.* sp. NGR(pA28), using the ΔpH_{max} as a measure of the response (Fig. 2*A*). Differently substituted Nod factors from *R.* sp. NGR(pA28) were similarly active in our system. Concentrations of <1 nM caused a measurable alkalinization, and doses of ≈10 nM induced the response half-maximally (Fig. 2*A*). Similar dose–response curves were obtained for mixtures of Nod factors

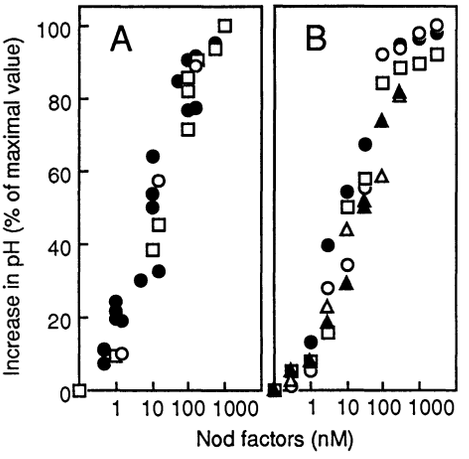

FIG. 2. Alkalinization induced by different concentrations of Nod factors, expressed in percent of the maximal response. (*A*) Dose–response curves for individual, purified Nod factors from *R.* sp. NGR(pA28), NodNGR-V (MeFuc) (○), NodNGR-V (MeFuc, Ac) (□), and NodNGR-V (MeFuc, S) (●). (*B*) Dose–response curves for Nod factor preparations from *R.* sp. NGR234 (●), *R. leguminosarum* bv. *viciae* RBL5560 (○), *R. meliloti* 102F34 (△), *R. phaseoli* RCR3622 (▲), and *B. japonicum* 61-A-101 (□).

Table 2. Yield of lipo-chitooligosaccharides from cultures of various flavonoid-induced bacterial strains and induction factor of the alkalinization-inducing activity as compared to preparations from uninduced cultures

Strain	Flavonoid added	Conc., μM	Yield of lipo-chito-oligosaccharides, nmol/liter	Alkalinization-inducing activity (induction factor)
R. sp. NGR234	Apigenin	1	87	10
R. sp. NGRΔ*nodABC*	Apigenin	1	<1	—
R. leguminosarum bv. *viciae*	Naringenin	1	15	85
R. meliloti	Luteolin	10	6	25
R. phaseoli	Naringenin	1	5	6
B. japonicum	Daidzein	1	21	100

The induction factor was determined by comparing dose–response curves for equivalent preparations of lipo-chitooligosaccharides from flavonoid-induced and uninduced bacterial cultures, adjusted to equal volumes per liter of culture medium.

from various *Rhizobium* and *Bradyrhizobium* strains (Fig. 2*B*).

The alkalinization response of tomato cells can be used as a rapid and sensitive bioassay for Nod factors. We used this assay to test the impact of flavonoids, the inducers of *nod* genes, on Nod factor production. An alkalinization response was observed with preparations from untreated as well as from flavonoid-treated bacteria, but, based on dose–response curves, the preparations from the flavonoid-treated bacteria produced up to 100 times more of the stimulating activity than the ones from untreated cultures (Table 2).

Nod Factors as Substrates for Plant Chitinases. Chitinases were purified from the roots of ethylene-treated plants, a treatment that increased chitinase activity in roots [cowpea, 3-fold; bean, 6-fold; pea, 3-fold (data not shown)]. Analysis of the purified chitinases by $NaDodSO_4$/polyacrylamide gel electrophoresis showed single bands with apparent molecular mass values of ≈30 kDa (Fig. 3), similar to the size of ethylene-induced chitinases from bean leaves (20), pea pods (21), and azuki bean leaves (25). Differently substituted Nod factors from *R.* sp. NGR(pA28) were incubated with the purified chitinases, adjusted to equal catalytic activity toward chitin. All chitinases retained at least 50% of the initial activity throughout the incubation period (data not shown). After incubation, samples were chromatographed over a C_{18} SepPac cartridge and the alkalinization-inducing activity of the lipophilic material, determined by establishing dose–response curves, was expressed as a percentage of the initial activity. All chitinases examined were able to reduce the activity of NodNGR-V (MeFuc) in a time-dependent way (Fig. 4*A*). This strongly indicates that this Nod factor is cleaved by the chitinases. Interestingly, substituted Nod factors—namely, NodNGR-V (MeFuc, Ac), which has an additional acetyl group (Fig. 4*B*), and NodNGR-V (MeFuc, S), substituted with a sulfate group (Fig. 4*C*)—were resistant to inactivation by bean or pea chitinases but not by cowpea chitinase. Thus, chitinases from legumes may differ in their capacities to hydrolyze variously substituted Nod factors.

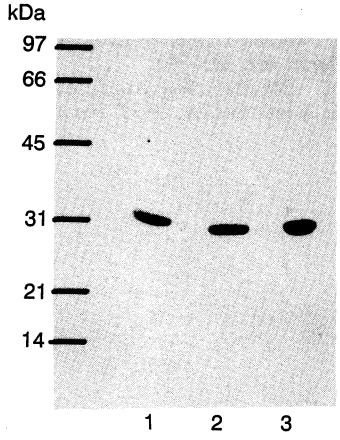

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of chitinases (5 nkat) purified from roots of ethylene-treated cowpea (lane 1), bean (lane 2), and pea (lane 3). Positions of molecular mass markers are indicated.

DISCUSSION

Nod factors, when added to tomato cell cultures, stimulate a rapid, transient alkalinization of the medium, comparable to the one elicited by chitin fragments (13). This can be used as a simple, sensitive bioassay for the Nod factors of the rhizobia. The low, but detectable pH-stimulating activity of cultures prepared without flavonoids may be due to Nod factors that are produced in the absence of flavonoids (26) or due to unknown lipo-chitooligosaccharides. Based on the identical refractory behavior toward a second stimulation (Fig. 1*D*), tomato cells do not appear to discriminate between Nod factors and underivatized chitin fragments and detect both Nod factors and chito-oligosaccharides with the same perception system, specific for oligomers of *N*-acetylglucosamine. This perception system may function in the recognition of chitin-containing fungi and other chitin-containing organisms in general (13). In contrast, the responses of legume roots to Nod factors, such as root-hair curling, are

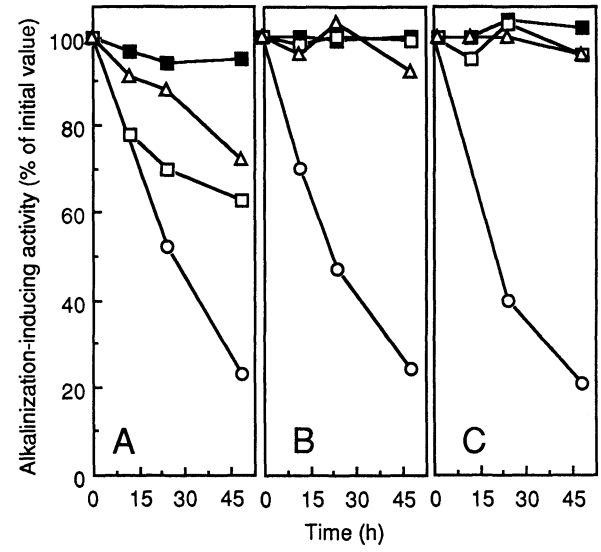

FIG. 4. Sensitivity of Nod factors from *R.* sp. NGR(pA28) to treatment with different chitinases. Alkalinization-inducing activity was measured in preparations of NodNGR-V (MeFuc) (*A*), NodNGR-V (MeFuc, Ac) (*B*), and NodNGR-V (MeFuc, S) (*C*) treated for different lengths of time without chitinase (■) or with 1 nkat of purified root chitinase of cowpea (○), bean (□), or pea (△).

induced only by Nod factors but not by chitotetraose (15). Thus, legumes might have evolved a more selective perception system that also recognizes the substituents of Nod factors, thereby discerning specifically their symbiotic partners. It remains an open question whether plants contain endogenous molecules homologous to chitin fragments or Nod factors that are recognized by related perception systems, as recently postulated on the basis of the activity of a purified Nod factor in carrot embryogenesis (27).

By using the alkalinization bioassay, we were able to show that certain Nod factors are inactivated by plant chitinases. Chitinases are often associated with defense reactions against bacterial or fungal attack (28, 29). In addition, chitinases may have endogenous functions and may be involved in plant somatic embryo development (30), perhaps by releasing endogenous factors related to Nod factors (5, 6, 27).

With respect to the interaction with rhizobia, we have previously found in soybean plants that chitinase accumulates in the cortex of root nodules (31): this chitinase appears at a relatively late stage in the symbiosis and may protect the infected zone from external pathogens in the effective symbiosis or prevent spreading of pathogenic rhizobia in the ineffective symbiosis.

The present data indicate a further possible role for chitinases constitutively present in legume roots—namely, to hydrolyze and inactivate Nod factors. This activity might be important already at an early stage of the infection process. It is particularly intriguing that different legume root chitinases differ in their ability to cleave Nod factors from *R.* sp. NGR(pA28). Thus, the host specificity of rhizobia may reside not only in the postulated specificity of Nod factor receptors but may be due, at least in part, to inactivation of Nod factors by chitinases or other hydrolases, adding a new dimension of complexity to host–symbiont recognition. This possibility has recently been tested in more detail by studying the pattern and kinetics of cleavage of Nod factors involved in host specificity: differently substituted Nod factors from *R. meliloti*, known to differentially induce symbiotic responses in *Medicago* and *Vicia*, respectively (4), were cleaved differentially by the enzymes present in roots of *Medicago* and *Vicia* (32).

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