Purification and characterization of a novel chitinase–lysozyme, of another chitinase, both hydrolysing *Rhizobium meliloti* Nod factors, and of a pathogenesis-related protein from *Medicago sativa* roots

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The symbiosis between *Rhizobium meliloti* and *Medicago sativa* (Leguminosae) involves the interaction of lipochito-oligosaccharides (Nod factors) excreted by bacteria with specific proteins of the host plant. The cleavage of Nod factors can be used as an enzymic assay to identify novel hydrolytic enzymes. Here a soluble extract of 3-day-old roots was fractionated by anion exchange, affinity chromatography, gel filtration and native electrophoresis. Two acidic chitinases (pI 4.6–5.4), CHIT24 and CHIT36, designated in accordance with their molecular mass in kDa, were separated. CHIT24 cleaves all tested Nod factors to produce lipotrisaccharides with the preference NodRm-V(S) > NodRm-IV > NodRm-IV(S) ⩾ NodRm-IV(Ac,S); it also hydrolyses colloidal ^{3}H-chitin and has lysozyme activity. The kinetics of Nod factor degradation by CHIT24 depends on substrate structural parameters, namely the length of the oligosaccharide chain and sulphation (S) at the reducing end, but not much on acetylation (Ac) at the non-reducing end. The 25-residue N-terminal sequence of CHIT24 has no similarity with known chitinases or lysozymes, indicating that it is a novel type of hydrolase. CHIT36 also hydrolyses NodRm-V(S) into NodRm-III, but it is inactive towards NodRm-IV(S) and NodRm-IV(Ac,S) formed by *R. meliloti*. Finally, a 17 kDa protein, P17, was co-purified with CHIT24. It neither degrades Nod factors nor exhibits lysozyme activity and shows complete identity, at the 15-residue N-terminal sequence, with a class 10 pathogenesis-related protein, PR-10.

INTRODUCTION

Plants have extremely sensitive chemoperception systems for signal substances derived from micro-organisms. Among the microbial substances that plants can sense at threshold concentrations of 1–100 pM are (lipo)oligosaccharides, (glyco)peptides and lipophilic substances such as ergosterol [1]. These molecules could have an important role in development, pathogen resistance and symbiosis [1,2]. Some soil bacteria, among them *Rhizobium meliloti*, interact with leguminous plants such as *Medicago sativa* (alfalfa or lucerne) to form N_2-fixing nodules. The roots secrete flavonoids that induce the transcription of bacterial *nod* genes involved in the synthesis of specific lipochito-oligosaccharides called Nod factors (Figure 1, top panel). They are β-1,4-linked trimers (NodRm-III) to pentamers (NodRm-V) of *N*-acetyl-D-glucosamine, with a fatty acid (2-*trans*, 9-*cis* hexadecenoic acid, $C_{16:2}$, in *R. meliloti*) replacing the N-acetyl group on their non-reducing end [3,4]; Nod factors of *R. meliloti* can also bear a sulphate group on the reducing end and be O-acetylated on the non-reducing end [5–7].

Plant chitinases and lysozymes have been described as having a role in defence reactions against pathogens [8–10]. They hydrolyse β-1,4 linkages of chitin, a major component of most fungal cell walls, acting as endochitinases and forming chito-oligosaccharides. Some chitinases also show lysozyme activity, hydrolysing the peptidoglycan of bacterial cell walls. It has been proposed that in root nodules, chitinases might protect the symbiotically infected zone from external pathogens or protect the root system from rhizobia [11].

Rhizobial Nod factors have been shown to be cleaved and inactivated by specific chitinases and related enzymes [12–17]. Nod factors are more resistant to hydrolysis by chitinases than unmodified chito-oligosaccharides, and different Nod factors show different degrees of stability against hydrolytic degradation *in vitro* and *in vivo* [12–15]. By using a panel of *R. meliloti* Nod factors, several hydrolases with different cleavage preferences ('fingerprints') including six *Medicago* activities have been identified ([14,15,17], and M. Schultze, C. Staehelin, F. Brunner, I. Genetet, M. Legrand, B. Fritig, E. Kondorosi and A. Kondorosi, unpublished work].

We have therefore attempted to characterize these *Medicago* activities in more detail. This paper describes the isolation and purification of two chitinases and of another protein, devoid of this activity, from *M. sativa* roots. One of the purified proteins represents a new chitinase–lysozyme type.

MATERIALS AND METHODS

Nod factors

Purification of tetrameric and sulphated NodRm-IV($C_{16:2}$,S), O-acetylated NodRm-IV($C_{16:2}$,Ac,S), pentameric NodRm-V($C_{16:2}$,S) and desulphated NodRm-IV($C_{16:2}$) has been described previously [7,17].

Plant material and growth conditions

Seeds (150 g) of *M. sativa* cv. Sitel (Tourneur, Montauban, France) were surface-sterilized [14] and germinated for 24 h in the dark at 24 °C. The seedlings were then dispersed over a plastic mesh that covered 15 cm × 15 cm plastic Petri dishes

Abbreviations used: CHIT24, CHIT36 (after their molecular masses in kDa), chitinases (EC 3.2.1.14) hydrolysing Nod factors or having lysozyme (EC 3.2.1.17) properties; NodRm, 'nodulation factors' from the symbiotic soil bacterium *Rhizobium meliloti* that are lipochito-oligosaccharides (number of *N*-acetyl-β-D-glucosamine indicated by a roman numeral) that bear a N-acylated unsaturated fatty acid ($C_{16:2}$), are modified by a sulphate group (S) at the reducing end and may be O-acetylated (Ac) at the non-reducing end; P17, a class 10 'pathogenesis-related protein' of 17 kDa; PR, pathogenesis-related protein.

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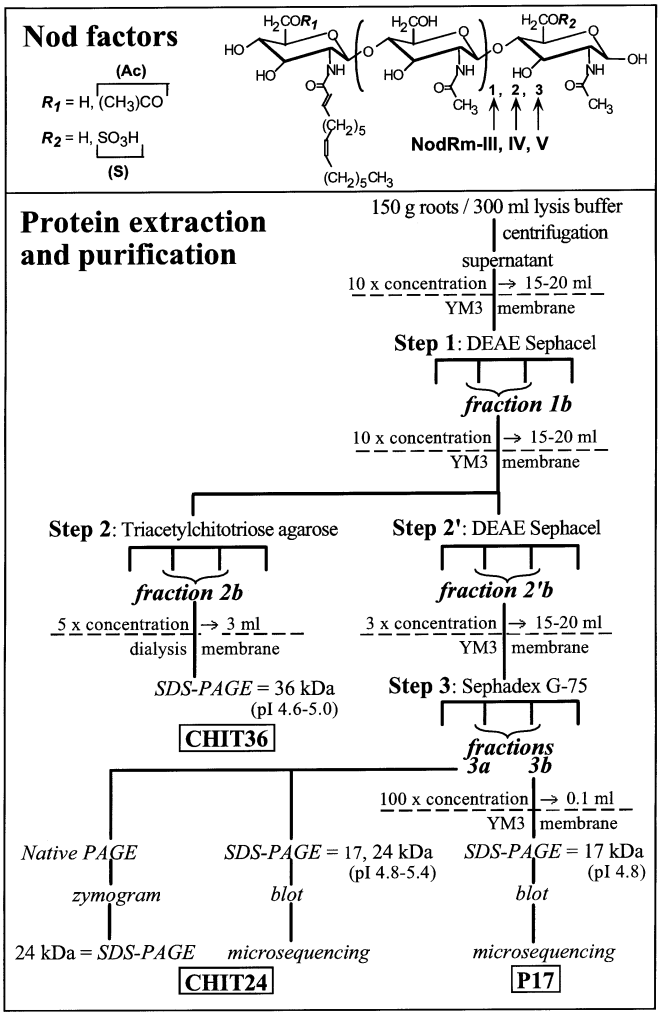

Figure 1 Purification steps of a soluble protein extract from roots of 3-day-old seedlings of *M. sativa* (see the text)

Top panel: structure of Nod factors used in this work, each hexagon representing an *N*-acetylglucosamine monomer; substitutions R_1: $(CH_3)CO$ = acetyl group (Ac) on non-reducing end, R_2: SO_3H = sulphate group (S) on reducing end. Bottom panel: diagram of protein extraction and purification procedure.

filled with 25 ml of sterile Jensen medium [14,18]; they were then grown sterile in the dark at 24 °C.

Soluble protein extract from roots

After 3 days of growth, 150 g of roots on the mesh were shaved over 300 ml of ice-cold extraction buffer and blended for 15 min. The buffer at pH 7.0 contained 20 mM Bistris, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 % (w/v) glycerol, 1.5 % (w/v) polyvinylpyrrolidone (PVP40), 4 μM sodium cacodylate and protease inhibitors [1 mM PMSF, 1 mM pepstatin, 10 mM leupeptin and 10 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (Boehringer Mannheim, Mannheim, Germany)]. The extract was centrifuged at 4 °C for 15 min at 3000 ***g*** and the supernatant again for 15 min at 15000 ***g***.

Purification of plant chitinases (Figure 1)

The protein content of all eluates was estimated by absorbance at 280 nm. Several fractions were then pooled and each group was subjected to activity tests by incubation with Nod factors.

Step 1: anion-exchange chromatography. The soluble protein extract was concentrated and equilibrated with 20 mM Bistris buffer, pH 7.0, to a volume of 15–20 ml by ultrafiltration on a YM3 Diaflo membrane (Amicon-Grace, Beverly, MA, U.S.A.). The concentrate was loaded on a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) anion-exchange column (1.6 cm × 25 cm). The column was washed with the same buffer; basic proteins in the flow-through were not further analysed. Acidic proteins were then eluted with the same buffer, first alone then with a 0–0.5 M NaCl linear gradient at a flow rate of 24 ml/h. The acidic fraction, fraction 1b, that hydrolysed Nod factors was concentrated by ultrafiltration and used for steps 2 and 2′.

Step 2: affinity chromatography. A 1 cm × 4 cm column was filled with 3 ml of *N*,*N*′,*N*″-triacetylchitotriose immobilized on agarose beads (Sigma, St. Louis, MO, U.S.A.) and washed with 20 ml of 0.1 M NaCl. One-tenth (2 ml) of fraction 1b was added and incubated for 30 min at 4 °C. The matrix was then rinsed with 11 ml of 25 mM Bistris/50 mM NaCl buffer (pH 7.0) and the retained proteins were eluted by the same buffer containing 50 mg/ml *N*-acetyl-D-glucosamine. The eluates were collected and assessed by SDS/PAGE, then pooled, dialysed against reaction buffer and tested for Nod factor degradation as described below.

Step 2′: second anion-exchange chromatography. The remaining nine-tenths of fraction 1b were applied to a 2.6 cm × 25 cm column of DEAE-Sephacel and eluted with 20 mM Bistris buffer, pH 7.0, with a linear gradient of NaCl from 0.1 to 0.4 M at a flow rate of 15 ml/h. Fraction 2′b, which had hydrolytic properties, was collected and concentrated by ultrafiltration to a volume of 15–20 ml.

Step 3: gel-filtration chromatography. The concentrated fraction 2′b was equilibrated in 25 mM Bistris/0.2 M NaCl buffer (pH 7.0). The 2.6 cm × 75 cm column of Sephadex G-75 (Pharmacia) was precalibrated with the following molecular mass markers: aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (31 kDa) and BSA (66 kDa). Samples were eluted at 4 ml/h. Fractions degrading Nod factors were combined (fraction 3a), concentrated and equilibrated by ultrafiltration with the activity-test buffer described below.

Native PAGE and zymogram

Non-denaturing, i.e. detergent-free, PAGE (native PAGE) allows the determination of enzymic activity directly on gels (zymogram). The acidic proteins were separated at pH 8.9 with a 10 % (w/v) polyacrylamide gel containing 0.04 % glycol-chitin as substrate [19]. The gel was incubated at 37 °C for 3 h then stained with 0.01 % Calcofluor White M2R (Sigma) in 100 mM sodium acetate buffer, pH 5.0, washed several times with distilled water and detected by UV illumination (Image Master VDS; Pharmacia). The lytic zone, appearing as a dark band on a bluish fluorescing background, was dissected and incubated for 10 min at 60 °C with SDS/PAGE sample buffer ([20], pp. 133–144).

SDS/PAGE

Protein-denaturing SDS/PAGE was performed on slab gels with a 4–18 % (w/v) polyacrylamide gradient ([20], pp. 133–144). Standard markers (Bio-Rad, Hercules, CA, U.S.A.) were phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and hen egg white lysozyme (14.4 kDa). The gels were either stained with Coomassie Brilliant Blue R-250 or silver nitrate ([20], pp. 133–144).

Isoelectric focusing

Isoelectric focusing was performed on a Pharmacia Multiphor system in 5% (w/v) polyacrylamide gels, 0.5 mm thick, containing 5% (v/v) carrier ampholyte at pH 3.5–9.5 [21].

Nod factor degradation assays

Laboratory-prepared Nod factors (10 μM) were incubated as described in [15] at 37 °C for 15–36 h with protein fractions in 100 μl buffer, pH 5.0, containing 20 mM sodium acetate, 0.5% (v/v) DMSO, 10 μg/ml acetylated BSA (New England Biolabs, Beverly, MA, U.S.A.). The products were extracted with an equal volume of distilled butan-1-ol and dried under reduced pressure. Nod factors and acylated cleavage products were fractionated on reverse-phase HPLC with 35% (v/v) acetonitrile with 40 mM ammonium acetate as the mobile phase [14]. Degradation products were quantified by the integration of peak areas. The kinetics of release of the acylated product (NodRm-III) against substrate concentration of NodRm-IV(S) was analysed with the Marquardt algorithm (Prism 2.0; GraphPad, San Diego, CA, U.S.A.).

Chitinase assay with colloidal ^{3}H-chitin

Chitinase activity was measured with colloidal ^{3}H-chitin. The assay is based on the liberation of soluble chito-oligosaccharides from insoluble chitin [22]. The reaction mixture (125 μl) contained the protein preparation, 20 mM sodium acetate, pH 5, 50 mM NaCl, 0.5% (v/v) DMSO and 50 μl of ^{3}H-chitin (125000 d.p.m.), kindly provided by Professor Thomas Boller (University of Basel, Basel, Switzerland). Samples were incubated at 37 °C and the reaction was stopped by the addition of 125 μl of trichloroacetic acid (10%, w/v). After centrifugation (4000 ***g*** for 15 min), 75 μl of the supernatant was analysed for radioactivity. One pkatal unit, for a given protein concentration, is the activity that catalyses the release of soluble chito-oligosaccharides corresponding to 1 pmol of *N*-acetyl-D-glucosamine in 1 s.

Lysozyme assay

The lysozyme assay was done at 37 °C. The reaction mixture (0.5 ml) contained 20 mM sodium acetate, pH 5, 10 mM NaCl, 0.5% (v/v) DMSO and 45 μg of freeze-dried cells of *Micrococcus lysodeikticus* (Sigma). The reaction was stopped by the addition of 0.5 ml 0.5 M Na_2CO_3 and the decrease in A_{645} was recorded photometrically.

Protein microsequencing

Proteins were electroblotted and the N-terminal amino acid sequences of proteins were determined by the automated Edman degradation method (model 610A sequencer; Applied Biosystems, Foster City, CA, U.S.A.). To validate the results the top and bottom parts of the blotted bands were excised separately and used for analyses.

Protein determination

The protein content was determined by the bicinchoninic acid method [23] with BSA as standard (BCA method; Pierce, Rockford, IL, U.S.A.).

RESULTS

Purification of chitinases

Two acidic chitinases of 36 and 24 kDa were purified by using Nod factors as substrates for enzymic assays. Figure 1 summarizes the purification procedure, and Table 1 indicates the degree of purification and yield at each step. In addition, a 17 kDa protein showing no hydrolytic activity was obtained. After anion-exchange chromatography with a DEAE Sephacel column, which removed considerable amounts of polyphenols and other impurities, Nod factor-degrading activity was present in different fractions called 1a, 1b and 1c (Figure 2A). The pentameric Nod factor NodRm-V(S) was cleaved into NodRm-

Table 1 Yield and Nod factor-cleaving activity of each purification step

See Figures 1 and 2 for purification procedure and description of fractions; activities were measured at 37 °C with pentameric NodRm-V(S) as substrate.

Purification step	Pooled fraction	Pool volume (ml)	Protein content (mg)	Protein yield (%)	NodRm-V(S) hydrolysis (pkatal)	Specific activity (pkatal/mg)
Crude extract	–	150	102	100	–	–
1. Anion exchange	1b	174	29	28.0	290	10
2′. Anion exchange	2′b	48	2.40	2.33	72	30
3. Gel filtration	3a	12	0.027	0.03	21	780
3. Same	3b	16	0.081	0.08	0	0
2. Affinity chromatography	2b	14	0.050	0.05	15	300

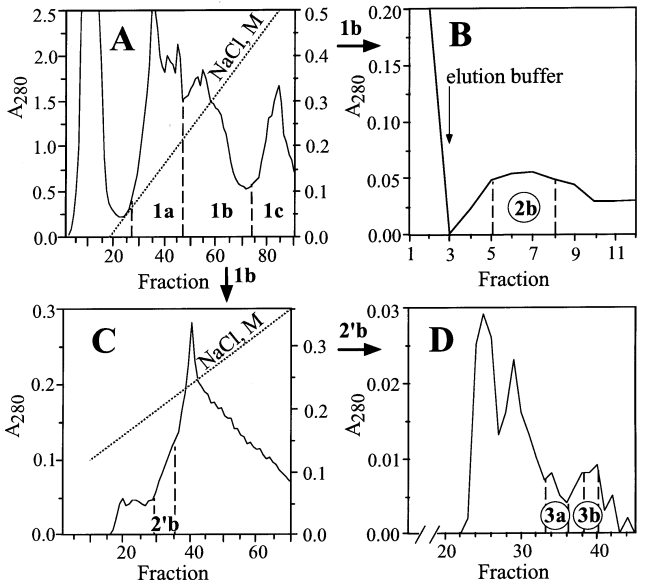

Figure 2 Chromatography of the root protein extract at different steps of purification (see Figure 1 and Table 2)

(**A**) Purification step 1: elution profile from the first DEAE-Sephacel anion-exchange purification step; column size 1.6 cm × 20 cm, flow rate 24 ml/h, fraction size 8 ml, elution with 0–0.5 M linear gradient of NaCl, pH 7.0 (fractions 17–84 and upwards); peak 1a, fractions 17–46; peak 1b, fractions 47–73 pooled; peak 1c, fractions 74–90 pooled. (**B**) Purification step 2: resolution of fraction 1b by affinity chromatography on *N*,*N*′,*N*″-triacetylchitotriose-agarose; column size 1 cm × 4 cm, flow rate 8 ml/h, elution buffer 25 mM Bistris (pH 7.0)/50 mM NaCl containing 50 mg/ml *N*-acetyl-D-glucosamine, fraction size 3.5 ml; peak 2b, fractions 5–8 pooled. (**C**) Purification step 2′: separation of fraction 1b by a second DEAE-Sephacel anion-exchange column; column size 2.6 cm × 25 cm, flow rate 18 ml/h, elution with 0.05–0.4 M linear gradient of NaCl (fractions 10–70), fraction size 6 ml; peak 2′b, fractions 29–35 pooled. (**D**) Purification step 3: resolution of fraction 2′b by gel-filtration chromatography on Sephadex G-75; column size 2.6 cm × 75 cm, flow rate 12 ml/h, fraction size 4 ml; peak 3a, fractions 33–36 pooled; peak 3b, fractions 38–40 pooled. All eluates were monitored for their protein content by absorbance at 280 nm.

Table 2 Characterization of Nod factor-cleaving activity of each purification step with different Nod factors as substrate

See Figures 1 and 2 and Table 1 for a description of fractions. Incubations were made with 10 μM Nod factor at 37 °C. Abbreviation: n.d., not detected.

			Type of product (% of given substrate)			
Fraction	Substrate	Incubation time (h)	Undegraded	NodRm-II	NodRm-III	NodRm-III(Ac)
1a	NodRm-V(S)	24	5	48	47	–
	NodRm-IV(S)	24	14	86	n.d.	–
1b	NodRm-V(S)	24	0	5	95	–
	NodRm-IV(S)	24	14	76	10	–
1c	NodRm-V(S)	24	61	4	35	–
	NodRm-IV(S)	24	83	17	n.d.	–
2′b	NodRm-V(S)	18	21	16	63	–
	NodRm-IV(S)	18	59	29	12	–
2b	NodRm-V(S)	18	69	n.d.	31	–
3a	NodRm-V(S)	15	29	n.d.	71	–
	NodRm-IV(S)	15	88	n.d.	12	–
	NodRm-IV(Ac,S)	15	89	n.d.	n.d.	11

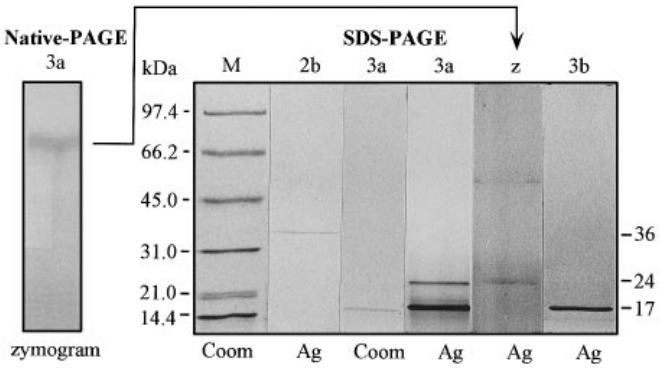

Figure 3 Native PAGE (left panel) and SDS/PAGE (right panel) profiles

The indicated fractions (see Figures 1 and 2) were analysed by SDS/PAGE (right panel); fraction 3a was also separated by non-denaturing PAGE (10% gel) and stained for activity in degrading 0.04% glycol-chitin (left panel) in the gel (zymogram). SDS/PAGE (protein amount in parentheses): lane M, molecular mass standards (2 μg); lane 2b, fraction 2b (0.2 μg) containing purified CHIT36; lanes 3a, fraction 3a (1 μg) containing CHIT24 and P17; lane z, purified CHIT24 (dissected active band of the zymogram); lane 3b, fraction 3b (1.5 μg) containing the purified 17 kDa protein (P17). Bottom of gels: zymogram, Calcofluor White staining; Coom, Coomassie Blue staining; Ag, silver nitrate staining (the upper dark line seen on lane z is an artifact).

II and NodRm-III (Table 2), in the order 1b > 1a ≫ 1c. Fraction 1b also cleaved NodRm-IV(S) to NodRm-III, indicating hydrolysis of the glycosidic bond next to the sulphate group at the reducing end. This cleavage preference, previously identified in a *M. sativa* cell culture [17], has not hitherto been found in plant chitinases and lysozymes. Because of this unique specificity, fraction 1b was retained for the next steps (Table 2).

One-tenth of fraction 1b was used for affinity chromatography with triacetylchitotriose-agarose. A single chitinase was eluted with *N*-acetyl-D-glucosamine as fraction 2b (Figure 2B). The hydrolase was called CHIT36, after its molecular mass of 36 kDa as analysed by SDS/PAGE (Figure 3, lane 2b/Ag). Isoelectric focusing indicated a pI of CHIT36 between 4.6–5.0 (results not shown). Although CHIT36 cleaved the pentameric Nod factor

Table 3 Specific activities of CHIT24 and CHIT36

Aliquots of the partly purified enzyme were incubated for 18 h with 10 μM Nod factor or 150 μl of colloidal chitin (chitinase activity, measured by the formation of lipotrisaccharides or of chito-oligosaccharides respectively) or added for 20 h to *M. lysodeikticus* suspension (lysozyme activity, measured by decrease in D_{645}, which reflects cell lysis). Results are means of three (Nod factors assays) or two (colloidal chitin and lysozyme tests) experiments made at 37 °C. Abbreviation: n.m., not measured.

	Hydrolytic assay (pkatal/μg of protein)	
Substrate	CHIT24 (fraction 3a)	CHIT36 (fraction 2b)
NodRm-IV(Ac,S)	0.08 ± 0.02	0
NodRm-IV(S)	0.09 ± 0.02	0
NodRm-IV	0.42 ± 0.14	n.m.
NodRm-V(S)	0.76 ± 0.05	0.44 ± 0.04
Colloidal ^{3}H-chitin	24.0 ± 5.0	90.8 ± 5.8
	Lysozyme assay (ΔD_{645}/h per μg of protein)	
Substrate	CHIT24 (fraction 3a)	CHIT36 (fraction 2b)
M. lysodeikticus suspension	-5.0 ± 1.5	0

NodRm-V(S), the purified hydrolase was unable to degrade the tetrameric NodRm-IV(S) (Table 3). This clearly shows that CHIT36 was not the enzyme releasing NodRm-III from NodRm-IV(S).

The other part of fraction 1b was subjected to a second anion-exchange chromatography that yielded fraction 2′b (Figure 2C), particularly capable of hydrolysing NodRm-IV(S) to NodRm-III. Therefore fraction 2′b was subjected to further purification by Sephadex G-75 gel filtration. In this final step, one peak, fraction 3a (Figure 2D), degraded NodRm-IV(S) releasing NodRm-III, whereas the neighbouring fraction 3b (Figure 2D) was devoid of Nod factor-cleaving activity.

SDS/PAGE of the fractions (3a and 3b) obtained by gel filtration

Staining of fraction 3a with Coomassie Brilliant Blue R-250 revealed a single 17 kDa protein named P17 (Figure 3, lane 3a). Because of its relative abundance P17 was purified to homogeneity in fraction 3b (Figure 2D), as shown by silver staining (Figure 3, lane 3b). However, fraction 3b, containing P17, neither had chitinase properties (zymogram test with glycol-chitin and hydrolytic measurements with Nod factors) nor exhibited lysozyme activity against *M. lysodeikticus*. From isoelectric focusing studies (results not shown), the pI of fraction 3a was between 4.8 and 5.4; that of fraction 3b corresponding to protein P17 was 4.8. Staining of fraction 3a with silver nitrate revealed two proteins, one of high intensity at 17 kDa (as with Coomassie staining) and one of lower intensity at 24 kDa (Figure 3, lane 3a/Ag). When fraction 3a (10 μg) was analysed as a zymogram by native PAGE with glycol-chitin in the gel as substrate (Figure 3, left lane), one single chitinase-active band was revealed. This band was dissected and re-analysed by SDS/PAGE, revealing one band of approx. 24 kDa (called CHIT24) with silver staining (Figure 3, lane z).

Sephadex G-75 chromatography (results not shown) suggested that the two isolated chitinases (CHIT36, CHIT24) and P17 were

Table 4 N-terminal amino acid sequences of P17 and CHIT24

Top row, N-terminal amino acid sequence of P17 (17 kDa band from fraction 3b); second row, sequence of the putative protein of the PR10 gene from *M. sativa*; bottom row; N-terminal amino acid sequence of CHIT24 (24 kDa band from fraction 3a). Results with an asterisk are from this study.

Name	Element sequenced	N-terminal sequence																									
		1				5					10					15											
P17*	Protein	G	V	F	N	F	E	D	E	T	T	S	I	V	A	P											
		2			5					10					15											
PR10	Gene	G	V	F	N	F	E	D	E	T	T	S	I	V	A	P											
		1				5					10					15					20					25
CHIT24*	Protein	V	Q	P	V	F	D	K	H	G	N	P	L	N	P	S	S	Q	Y	Y	I	L	P	A	P	D

native monomers because they were eluted at positions with molecular masses similar to those determined by denaturing SDS/PAGE.

N-terminal amino acid microsequencing

Purified P17 was separated by SDS/PAGE and electroblotted on a PVDF membrane (Sigma). The blot was stained with Amido Black and one band was excised for N-terminal amino acid sequencing of the first 15 residues. Scanning of a data bank (Blastp + Beauty Search Results) showed various similarities with putative proteins deduced from some genes, especially of the pathogen-related (PR) proteins. There was complete identity with the PR10 gene of *M. sativa* [24,25] and the PR10-1 gene of *Medicago truncatula* (P. Gamas, F. de Billy, J. Cullimore and G. Truchet, unpublished work); no similarity was found with known hydrolases, confirming the above lack of hydrolytic activity.

A 50 μg sample of fraction 3a from purification step 3 (Figure 2D) was separated by SDS/PAGE. One band of 24 kDa, seen by silver staining and corresponding to CHIT24, was sequenced for 25 residues (Table 4, last row). No good match was found with any sequence present in databases. The best was 52 % identity (68 % similarity) over these 25 residues with a 20 kDa α-L-fucosidase from pea stem [26]. Because of its limited amount, CHIT36 could not be sequenced.

Substrate specificity and kinetic properties

Figure 4 shows an HPLC analysis of the acylated products after incubation of different Nod factors with the two isolated chitinases. CHIT36 (fraction 2b) hydrolysed sulphated pentameric NodRm-V(S) to the lipotrisaccharide NodRm-III (Figure 4A); sulphated tetrameric Nod factors were not degraded (results not shown).

CHIT24 (fraction 3a) cleaved sulphated NodRm-V(S) and NodRm-IV(S), as well as desulphated NodRm-IV, to NodRm-III (Figures 4B, 4C and 4D). Interestingly, the hydrolase did not release from NodRm-V(S) the tetrasaccharidic NodRm-IV, indicating that it is an enzyme that forms exclusively lipotrisaccharides. The O-acetylated and sulphated tetramer NodRm-IV(Ac,S) was degraded to acetylated NodRm-III(Ac) (Figure 4E). As shown in Table 3, Nod factors were more susceptible to chitinase hydrolysis with increasing length of oligosaccharide chain, and sulphated NodRm-IV(S) was more resistant to hydrolysis than unsulphated NodRm-IV. The order of degradation by CHIT24 (fraction 3a) was NodRm-V(S) > NodRm-IV > NodRm-IV(S) $\geqslant$ NodRm-IV(Ac,S).

Further studies of CHIT24 and CHIT36 activities were undertaken with colloidal ^{3}H-chitin and *M. lysodeikticus* for chitinase and lysozyme assays respectively (Table 3). Both enzymes cleaved colloidal chitin. The chito-oligosaccharides formed were quantified in relation to their monomer *N*-acetyl-D-glucosamine. Therefore 1 pkatal of ^{3}H-chitin degradation cannot be directly compared with the hydrolysis of Nod factors.

It has been found that hen egg lysozyme is able to cleave the glycosidic bond next to the sulphate group at the reducing end of the Nod factors (M. Schultze, C. Staehelin, F. Brunner, I. Genetet, M. Legrand, B. Fritig, E. Kondorosi and A. Kondorosi, unpublished work). This bond is also accessible to CHIT24. On the basis of these similarities, we expected lysozyme properties for CHIT24. Indeed, CHIT24 had considerable lysozyme activity against *M. lysodeikticus* cells (Table 3). CHIT36 had chitinase activity but no lysozyme activity.

The kinetics of NodRm-IV(S) degradation to NodRm-III by CHIT24 (fraction 3a) is shown in Figure 5: $K_m \approx 50\ \mu$M and $V_{max} \approx 0.5$ pkatal/μg of protein (nmol/s per mg of protein).

DISCUSSION

The molecular masses of plant chitinases and lysozymes are generally in the range 24–36 kDa; the enzymes occur as monomers and are basic or acidic, i.e. have high or low isoelectric points [8–10]. Chitinase isoenzymes differ in their substrate specificities with respect to oligomeric substrates and usually need a chain length of at least three β-1,4-linked *N*-acetyl-D-glucosamine residues. Some chitinases release the *N*-acetyl-D-glucosamine monomer after digestion of chitin; others release chitobiose as the smallest end-product (see, for example, [14,27,28]). Many plant chitinases also possess some lysozyme activity, hydrolysing β-1,4 linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine in the bacterial peptidoglycans (see, for example, [8,28–30]).

In the present study, two chitinolytic enzymes have been purified from *Medicago* roots. Instead of chitin, Nod factors from *R. meliloti* were used as substrate as described for the isolation of another *Medicago* enzyme that forms exclusively lipodisaccharides from Nod factors [15].

An activity hydrolysing the glycosidic bond next to the sulphated *N*-acetyl-D-glucosamine of the tetrameric NodRm-IV(S) had been seen in a *Medicago* cell suspension culture [17]. This substrate preference has not been found in lysozymes and chitinases from plants, where the sulphate group protects the Nod factors against hydrolysis of the bond next to the sulphated *N*-acetyl-D-glucosamine at the reducing end ([14,15,17], and M. Schultze, C. Staehelin, F. Brunner, I. Genetet, M. Legrand, B. Fritig, E. Kondorosi and A. Kondorosi, unpublished work). Therefore CHIT24 appeared as a hitherto unknown enzyme type and we purified the protein by using the release of NodRm-III

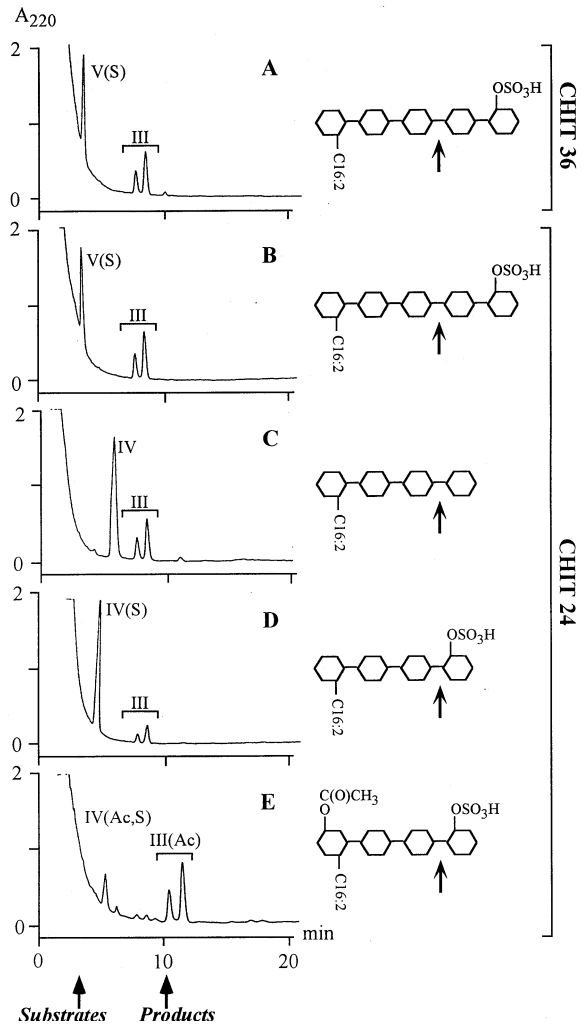

Figure 4 Reverse-phase HPLC of different Nod factors and their acylated degradation products after incubation with CHIT24 and CHIT36

The length of the oligosaccharide chain of Nod factors (roman numerals), the presence of O-acetyl (Ac) and sulphate (S) groups are indicated in chromatograms (left panels) and in the schematic structure of Nod factors (right panels). The cleavage site is shown by an arrow. On the chromatograms, the peak on the left represents the added Nod factor, that on the right the produced lipotrisaccharide [O-acetylated in (**E**)], which was separated into anomers (double peaks). Nod factors (10 μM, in 100 μl of assay buffer) were incubated at 37 °C for 18 h (**A**–**C**), 20 h (**D**) or 36 h (**E**) with CHIT36 (fraction 2b) and CHIT24 (fraction 3a) respectively. (**A**) NodRm-V(S) degraded by CHIT36 (25 ng of protein); (**B**) NodRm-V(S) degraded by CHIT24 (10 ng of protein); (**C**) desulphated NodRm-IV degraded by CHIT24 (10 ng of protein); (**D**) NodRm-IV(S) degraded by CHIT24 (25 ng of protein); (**E**) NodRm-IV(Ac,S) degraded by CHIT24 (50 ng of protein).

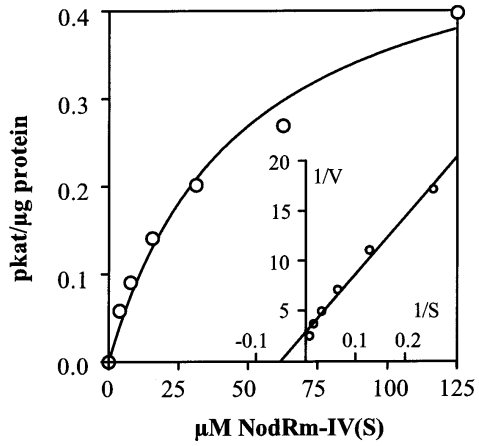

Figure 5 Determination of the Michaelis–Menten constant of CHIT24 for NodRm-IV(S) as substrate

Aliquots of CHIT24 (fraction 3a) were incubated at 37 °C for 18 h. The fitted hyperbola gives a K_m of 47 ± 10 μM and a V_{max} of 0.52 ± 0.05 pkatal/μg of protein, with $r^2 = 0.983$. Inset: the corresponding double-reciprocal plot of degradation rate against substrate concentration.

from NodRm-IV(S) as the target activity. The N-terminal amino acid sequence obtained from the purified CHIT24 protein did not show any similarity to known chitinase–lysozymes, indicating that CHIT24 represents a novel enzyme type.

CHIT24 has a high lysozyme activity. For example, the ratio of lysozyme to chitinase activity was approx. 20-fold that of the chitinase–lysozyme purified by Mauch et al. [28]. CHIT24 has an acidic pI and represents only a small portion of total lysozyme activity in crude extracts of *Medicago* roots (results not shown). Plant enzymes with known lysozyme function are usually strongly basic and are considered to be vacuolar [8,31,32]. Acidic lysozyme–chitinases have been found only in parsley [33] and tobacco [31], but their molecular masses are 30.4 and 28.7 kDa compared with 24 kDa for CHIT24, to which they show no sequence similarity at the N-terminal end.

CHIT24 has a unique cleavage activity towards the sulphated tetrameric Nod factors NodRm-IV(S) and NodRm-IV(Ac,S), releasing lipotrisaccharides. Hen egg white and human lysozymes show this type of cleavage of NodRm-IV(S), but they also hydrolyse NodRm-V(S) to NodRm-IV and then to NodRm-III (M. Schultze, C. Staehelin, F. Brunner, I. Genetet, M. Legrand, B. Fritig, E. Kondorosi and A. Kondorosi, unpublished work), which CHIT24 is unable to do. CHIT24 is a unique 'trimer-forming' hydrolase that releases exclusively lipotrisaccharides from Nod factors, whereas a 'dimer-forming' enzyme from *Medicago* has previously been characterized [15]. Therefore CHIT24 is a new type of plant chitinolytic enzyme.

Besides CHIT24, a 36 kDa chitinase was purified by a triacetylchitotriose-agarose column. CHIT36 hydrolyses the pentameric Nod factor NodRm-V(S) to the lipotrisaccharide, whereas the sulphated tetrameric Nod factor NodRm-IV(S) was resistant to degradation (Table 3). CHIT36 therefore has the same substrate preference ('fingerprint') for Nod factors as the 30 kDa chitinases of *M. sativa* and *Vicia sativa* [14], as well as a number of other purified class I chitinases (M. Schultze, C. Staehelin, F. Brunner, I. Genetet, M. Legrand, B. Fritig, E. Kondorosi and A. Kondorosi, unpublished work). Thus CHIT36 most probably represents an acidic class I chitinase. Although CHIT36 and CHIT24 have chitinase properties and cleave pentameric Nod factors to NodRm-III, they have quite different substrate specificities with respect to sulphated tetrameric Nod factors and bacterial peptidoglycan.

P17, a 17 kDa protein, was co-purified with CHIT24. P17 shows neither chitinase nor lysozyme activity. The N-terminal 15-residue sequence of P17 is identical with the putative pathogenesis-related protein PR-10 from *M. sativa* (Table 4). A sequence similarity has been found between a ginseng RNase and a parsley PR-10, suggesting that PR-10 could be an RNase [34], even though no activity has been demonstrated so far. Our method could serve to purify PR-10 proteins and to test their putative RNase activity.

Conclusion

In the present study two hydrolytic enzymes cleaving specific Nod factors and one pathogenesis-related protein were purified from alfalfa roots. CHIT24, by its sequence and its lysozyme activity, seems novel. The physiological properties of CHIT24 and CHIT36 remain to be established, as they do not contribute to the rapid Nod factor degradation observed in the rhizosphere of *Medicago* [14,15], where their 'fingerprint' of activity is not observed. The same is true of the function of a pathogenesis-related protein P17, which we found to be devoid of activity against Nod factors. At the outset, the purified proteins might be thought to participate in a pathogenesis response that deserves further investigation. The lytic effect of CHIT24 on *M. lysodeikticus* might reflect a specific role of this enzyme against pathogenic bacteria and perhaps in defence reactions against *Rhizobium* as well [11,35,36].

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