Substrate Specificity and Kinetic Studies of Nodulation Protein NodL of *Rhizobium leguminosarum*†

Guido V. Bloemberg,‡ Ron M. Lagas,§ Steven van Leeuwen,§ Gijs A. Van der Marel,§ Jacques H. Van Boom,§ Ben J. J. Lugtenberg,‡ and Herman P. Spaink*,‡

Institute of Molecular Plant Sciences, Leiden University Wassenaarseweg 64, 2333 AL Leiden, The Netherlands, and Gorlaeus Laboratories, Leiden University, Department of Organic Chemistry, Postbox 9502, 2300 RA Leiden, The Netherlands

Received April 3, 1995; Revised Manuscript Received July 25, 1995⊗

ABSTRACT: All lipo-chitin oligosaccharides identified from *Rhizobium leguminosarum* carry an *O*-acetyl moiety on C6 of the nonreducing terminal *N*-acetylglucosamine residue. Previously, we have shown that purified NodL protein, using acetyl-CoA as acetyl donor, *in vitro* acetylates *N*-acetylglucosamine, chitin oligosaccharides, and lipo-chitin oligosaccharides. In this paper, the enzymatic properties and substrate specificity of NodL protein were analyzed, using a spectrophotometric assay to quantify NodL transacetylating activity. NodL functions optimally under alkaline conditions. Transacetylating activity has a broad temperature optimum between 28 and 42 °C. NodL protein is stable for at least 15 min up to 48 °C. Glucosamine, chitosan oligosaccharides, terminally de-*N*-acetylated chitin derivatives, and cellopentaose were identified as acetyl-accepting substrates for NodL protein. Quantitative substrate specificity studies show that chitin derivatives with a free amino group on the nonreducing terminal residue are the preferred substrates of the NodL protein. Our results strongly indicate that the nonreducing terminally de-*N*-acetylated chitin oligosaccharides produced by the NodC and NodB enzymes are the *in vivo* acetyl-accepting substrates for NodL protein.

The symbiotic interaction between rhizobial bacteria and leguminous plants results in the formation of nitrogen-fixing root nodules. Host specificity of nodulation is determined by signal exchange between the plant, and the bacterium. In response to flavonoid signal molecules secreted by the plant the rhizobial *nod* or *nol* genes are transcribed [for a review, see Schlaman *et al.* (1992)]. Many of the *nod* gene products mediate the synthesis of secreted lipo-chitin oligosaccharides (LCOs). Purified LCOs can evoke various responses in the host plant roots, which are indistinguishable from those observed during several stages of the nodule formation process [for reviews, see Fisher and Long (1992) and Dénarié and Cullimore (1993)].

The *nodA*, *nodB*, and *nodC* gene products are minimally required for the production of LCOs. NodC protein has sequence homology with chitin synthases (Bulawa *et al.*, 1991; Atkinson *et al.*, 1992; Debelle *et al.*, 1992) and is involved in the production of β-1,4-linked *N*-acetylglucosamine oligomers (Spaink *et al.*, 1993, 1994; Geremia *et al.*, 1994). The *nodB* gene product shows homology with a chitin deacetylase (Kafetzoupoulos *et al.*, 1993), and *in vitro* experiments have demonstrated that the *nodB* gene product is able to remove the *N*-acetyl moiety from the nonreducing terminal residue of chitin oligosaccharides (John *et al.*, 1993). Recently, pentameric and tetrameric forms of terminally de-*N*-acetylated chitin oligosaccharides have been identified as being produced *in vitro* and *in vivo* by the combined presence of the NodB and NodC proteins (Spaink *et al.*, 1994), which led to the designation of these molecules as NodBC intermediates. The NodA protein is assumed to transfer an acyl moiety from a donor to de-*N*-acetylated chitin oligosaccharides, resulting in a basic LCO molecule (Röhrig *et al.*, 1994; Atkinson *et al.*, 1994). Various biovar-specific modifications in LCO structure, which are determined by other *nod* genes, determine the biological activity of LCOs on the host plant. For *Rhizobium leguminosarum* bv. *viciae*, it has been shown that the presence of an *O*-acetyl group on the 6-OH group of the nonreducing terminal residue of LCOs is required for preinfection thread formation and nodule meristem formation (Spaink *et al.*, 1991; van Brussel *et al.*, 1992). Studies of mutants revealed that the *nodL* gene product is required for the presence of this *O*-acetyl group (Spaink *et al.*, 1991). Recently, we have shown that purified NodL protein acetylates LCOs, chitin oligosaccharides, and *N*-acetylglucosamine *in vitro*, using acetyl-CoA as the acetyl donor (Bloemberg *et al.*, 1994). In addition, we showed by structural analyses that NodL transfers this acetyl moiety only to the 6-OH group of the nonreducing terminal residue (Bloemberg *et al.*, 1994).

In this paper, the characteristics and substrate specificity of the transacetylase NodL protein are described in more detail. Our results show that a free amino group on the nonreducing terminal residue of oligosaccharide acceptors is preferred by the NodL protein. On the basis of these and other results a model for *in vivo* LCO synthesis is proposed.

† G.V.B. was supported by financial aid from the Netherlands Organization for Chemical Research; H.P.S. was supported by the Royal Netherlands Academy of Art and Sciences, a Pionier Grant from the NWO, and a contract from the European Community (BIO2-CT93-0400, DG12 SSMA).

* Address for correspondence: H. P. Spaink, Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands. Phone: 071-275055. FAX: 071-275088. E-mail: SBY3HS@rulsfb.leidenuniv.nl.

‡ Institute of Molecular Plant Sciences.

§ Gorlaeus Laboratories.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1995.

0006-2960/95/0434-12712$09.00/0

Table 1: Large Scale Purification of NodL Protein[a]

purification step	volume (mL)	total protein (mg)	total activity (U)	specific activity (U/mg)	pur fac[b]	recovery (%)
soluble protein	11.0	115.2	30.0	0.26	1	100
$(NH_4)_2SO_4$ precipitation	2.9	69.6	27.9	0.40	1.5	93.0
Blue B	29.5	0.41	6.3	15.37	59.1	21.0
MonoQ	5.9	0.09	1.45	16.11	62.0	4.8

[a] Purification data are given for 1 L of induced BL21(DE3) cells containing pMP3401. [b] Purification factor.

EXPERIMENTAL PROCEDURES

Purification of NodL Protein. NodL protein was isolated from *Escherichia coli* strain Bl21(DE3) (Studier *et al.*, 1990) harboring pMP3401 (Bloemberg *et al.*, 1994). pMP3401 contains the *nodL* gene of *R. leguminosarum* bv. *viciae* under the control of the T7 promoter. BL21(DE3) containing pMP3401 was grown in Luria-Bertani (LB) medium supplemented with kanamycin to a final concentration of 0.86 mM. To activate the T7-polymerase promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration of 2.5 mM) was added to a growing culture with an OD_{260} of 0.4. NodL protein was purified to homogeneity from cell cultures induced for 16 h, using a purification procedure consisting of French pressure cell lysis, ultracentrifugation, ammonium sulfate precipitation, Blue B affinity chromatography, and MonoQ anion exchange chromatography, as described previously (Bloemberg *et al.*, 1994). After each purification step, protein samples were dialysed for 16 h against 10 mM Tris-HCl, pH 8.0, at 4 °C and protein concentrations were determined according to a modified Lowry quantification method (Dulley & Grieve, 1975) (see Table 1). Purified NodL protein was stored at −20 °C.

Chemicals. Saccharides used in this study were as follows: *N*-acetylglucosamine (Sigma), *N,N'*-diacetylchitobiose (Sigma), *N,N',N''*-triacetylchitotriose (Sigma), *N,N',N'',N'''*-tetraacetylchitotetraose (Seikagaku), *N,N',N'',N''',N''''*-pentaacetylchitopentaose (Seikagaku), *N,N',N'',N''',N'''',N'''''*-hexaacetylchitohexaose (Seikagaku) (for brevity, the latter five compounds are referred to in the text as chitinbiose, chitintriose, chitintetraose, chitinpentaose, and chitinhexaose, respectively), glucosamine (Sigma), chitosanbiose (Seikagaku), chitosantriose (Seikagaku), chitosantetraose (Seikagaku), chitosanpentaose (Seikagaku), cellopentaose (Sigma), and maltopentaose (Sigma).

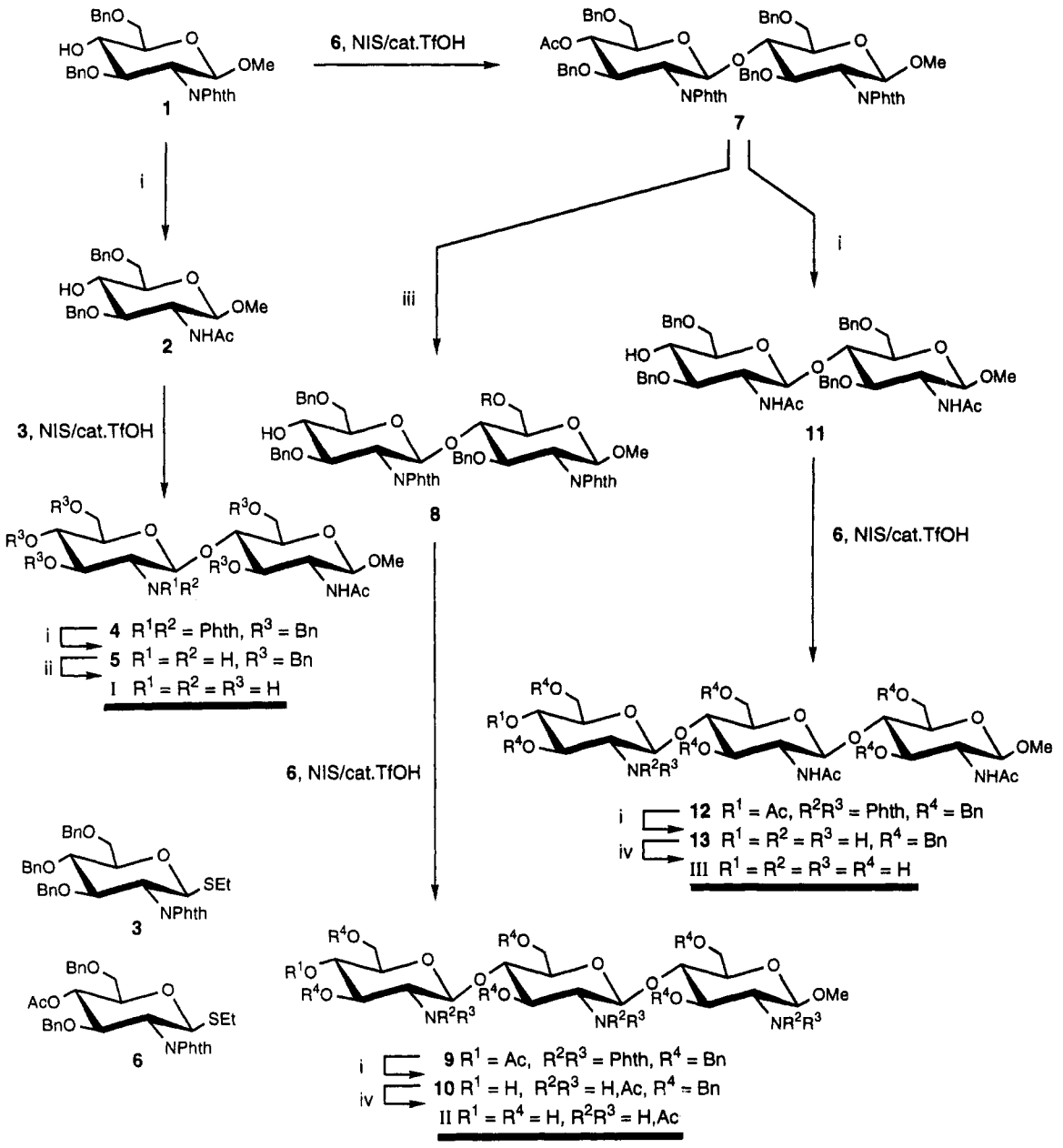
Analysis of Radio-Labeled Reaction Products. In order to demonstrate *in vitro*-acetylated reaction products, 100 nmol of various saccharides were incubated with 1.4 μg of NodL protein and 50 nCi (0.8 nmol) of [1-^{14}C]acetyl-CoA (Amersham) in 10 mM phosphate buffer, pH 7.5, in a total volume of 25 μL for 3 h at 28 °C. In the case of *N*-acetylglucosamine (Sigma), 1 μmol was used for labeling. For de-*O*-acetylation, samples were taken up in 400 μL of 1:1 methanol:concentrated NH_4OH, incubated for 18 h at room temperature, and subsequently dried under vacuum and redissolved in water. ^{14}C-labeled *N*-acetylglucosamine and glucosamine were obtained from Amersham. ^{14}C-labeled chitin fragments were obtained by methods as described by Kamst *et al.* in press. Reaction samples were chromatographed on NH_2 thin layer chromatography (TLC) plates (Merck) using a mobile phase of acetonitrile:water (68:32, v:v). Radioactivity on TLC plates was detected with a PhosphorImaging system from Molecular Dynamics, using the Image Quant software.

Spectrophotometric Assay for Transacetylating Activity. Transacetylating activity of NodL protein was spectrophotometrically analyzed using the method of Alpers *et al.* (1965). This method is based on a disulfide interchange reaction between CoA, liberated from acetyl-CoA after the transfer of the acetyl group to the acceptor substrate, and dithiobis(2-nitrobenzoic acid) reagent (NBT) (Ellman, 1959). The appearance of the reaction product thionitrobenzoic acid is followed spectrophotometrically at 415 nm. Unless indicated otherwise, reaction mixtures had a final volume of 75 μL and contained 125 nmol of acetyl-CoA (Sigma) and 500 nmol of NBT (Fluka) in 67 mM Tris-HCl, pH 8.0. Furthermore, each reaction mixture contained an amount of purified NodL protein and a variable amount of a saccharide. Reactions were performed at 28 °C, and OD_{415} was measured in flat bottom microtitration plates (Greiner) using a microplate reader (Model 3550, BioRad). To avoid evaporation, 40 uL of mineral oil was added to each reaction mixture.

Determination of NodL Enzyme Characteristics. In order to determine the influence of pH on the transacetylating activity of NodL protein, reaction mixtures containing 125 nmol of acetyl-CoA, 20 ng of NodL protein, and 25 nmol of chitosanpentaose (Seikagaku, Corp.) were incubated for 15 min in final volumes of 50 μL in 50 mM phosphate buffer, pH 5.7, 6.2, 6.7, and 7.3, in 50 nM Tris-HCl buffer, pH 7, 7.45, 8.0, 8.3, and 9.0, and in 50 mM glycine buffer, pH 9.1, 9.6, and 10.1, respectively. Subsequently, the reaction mixtures were rapidly cooled in ice−water, and the amount of free CoA was determined spectrophotometrically after addition of 500 nmol of NBT in 25 μL of 100 mM Tris-HCl, pH 8.0. The influence of the Tris concentration on NodL protein activity was determined by the testing of standard reaction mixtures, containing 20 ng of NodL protein and 25 nmol of chitosanpentaose, in final Tris-HCl (pH 8.0) concentrations of 0.067, 0.134, 0.201, 0.268, 0.335, and 0.402 M, respectively.

The influence of temperature on the stability of NodL protein activity was determined after preincubating 20 ng of NodL protein for a period of 15 min at 4, 16, 25, 28, 37, 43, 48, 56, and 62 °C. The activity of each preincubated NodL sample was tested in a standard reaction mixture containing 25 nmol of chitosanpentaose. After an incubation period of 15 min at 28 °C the amounts of free CoA were determined. The temperature dependence of NodL protein activity was determined by the incubation of standard reaction mixtures containing 20 ng of NodL protein and 25 nmol of chitosanpentaose for 15 min at the temperatures listed above.

The influence of mono- and divalent metal ions on the activity of NodL protein was determined by supplementing standard reaction mixtures with $CaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, NaCl, or KCl to a final concentration of 10 mM. The influence of chelators on NodL protein activity was tested



Reagents and conditions: i. a) $H_2NNH_2.H_2O$, EtOH, 90°C b) MeOH, Ac_2O; ii. $Pd(OH)_2$/C, EtOH, 90°C; iii. $Pd(OH)_2$/C, EtOH, H_2O, HOAc; iv. MeOH, *t*-BuOK; v. Pd/C, H_2, *i*-PrOH, H_2O, HOAc.

FIGURE 1: Organic synthesis of 1-methyl *N'*-acetylchitobiose (**I**), 1-methyl *N,N',N''*-triacetylchitotriose (**II**), and 1-methyl *N',N''*-diacetylchitotriose (**III**). For details, see Experimental Procedures.

by addition of EDTA to a final concentration of 5 mM in a standard reaction mixture.

Chemical Synthesis and NMR Spectroscopy of De-N-acetylated Chitin Derivatives. The assembly of the requisite 1-methyl *N'*-acetylchitobiose (**I**), 1-methyl *N,N',N''*-triacetylchitotriose (**II**), and 1-methyl *N',N''*-diacetylchitotriose (**III**) was readily accomplished, as shown in Figure 1, by *N*-iodosuccinimide-(NIS) and catalytic triflic acid-(TfOH) mediated (Veeneman *et al.*, 1990) elongation of the terminal acceptor **1** (Alais & David, 1990) and **2** with appropriate donors **3** and **6** (Srivastava & Hindsgaul, 1991). Thus, hydrazinolysis of **1** and subsequent condensation with **3** gave the fully protected dimer **4**. Hydrazinolysis of **4** followed by hydrogenolysis of **5** afforded homogeneous 1-methyl *N'*-acetylchitobiose (**I**). In addition, glycosylation of **1** with donor **6** yielded the fully protected dimer **7**, deacetylation of which afforded the partially protected dimer **8**. NIS–TfOH-assisted glycosylation of the latter with donor **6** gave, after hydrazinolysis of **9** followed by selective *N*-acetylation and then hydrogenolysis of **10**, homogeneous 1-methyl *N,N',N''*-triacetylchitotriose (**II**). On the other hand, hydrazinolysis of dimer **7**, and subsequent selective acetylation of the free amino groups, resulted in the isolation of the partially protected dimer **11**. Elongation of dimer **11** with acceptor **6** gave the fully protected trimer **12**. Hydrazinolysis followed by hydrogenolysis of **13** led to homogeneous 1-methyl *N',N''*-diacetylchitotriose (**III**).

Experimental Details. Methyl 3,6-Di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (2). Compound **1** (312 mg) dissolved in ethanol (96%, 30 mL) was heated at 80 °C after hydrazine·monohydrate (0.5 mL) was added. After 16 h, the reaction mixture was concentrated *in vacuo* and the residue was dissolved in MeOH (20 mL), and at 0 °C, acetic anhydride (1.0 mL) was added. After stirring for 16 h at 20 °C, the reaction mixture was concentrated and coevaporated with toluene (5 × 5 mL). Purification of the residual oil by silica gel column chromatography (MeOH:Et_2O, 0:100 to 10:90, v:v) afforded **2** (180 mg, 70%). $^{13}C\{^1H\}$-NMR ($CDCl_3$): δ 23.4 (CH_3 NHAc), 54.5 (CH_3 Me), 56.1 (C-2), 68.7, 74.5, 75.2 (C-6, 2 × CH_2 benzyl), 75.2, 74.6, 77.2 (C-3, 4, 5), 100.5 (C-1), 127.3−128.4 (CH_{arom} benzyl), 137.9, 138.3 (2 × Cq benzyl), 170.6 (C=O NHAc).

Methyl 4-O-(3,4,6-Tri-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (4). To a mixture of acceptor **2** (162 mg), donor **3** (320 mg), and powdered 4 Å molecular sieves in 1,2-dichloroethane (DCE, 7 mL) at 0 °C (N_2 atmosphere) was added 5.1 mL of a solution containing *N*-iodosuccinimide (230 mg) and trifluoromethanesulfonic acid (10 μL) in DCE:Et_2O (1:1, 10 mL). After 3 h, the reaction mixture was neutralized with Et_3N, diluted with EtOAc, and filtered over Celite. The filtrate was washed with 10% $Na_2S_2O_3$, 10% $NaHCO_3$, and H_2O, dried ($MgSO_4$), and concentrated. The resulting oil was purified by silica gel column chromatography (MeOH:Et_2O, 15:85, v:v) to afford **4** (129 mg, 34%). $^{13}C\{^1H\}$-NMR ($CDCl_3$): δ 23.3 (CH_3 NHAc), 52.9 (CH_3 Me), 56.1, 56.4 (C-2, 2′), 68.1, 68.9, 72.6, 73.3, 74.7 (C-6, 6′, 5 × CH_2 benzyl), 73.9, 74.5, 74.8, 77.5, 78.8, 79.4 (C-3, 3′, 4, 4′, 5, 5′), 96.8, 101.2 (C-1, 1′), 123.2−133.8 (CH_{arom} benzyl, Phth), 131.4 (Cq Phth), 137.9−138.5 (5 × Cq benzyl), 169.9 (3 × C=O Phth, NHAc).

Methyl 4-O-(3,4,6-Tri-O-benzyl-2-deoxy-2-amino-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (5). Hydrazine·monohydrate (223 μL) was added to a solution of **4** (129 mg) in ethanol (96%, 10 mL). The reaction mixture was heated for 25 h at 80 °C. After concentration *in vacuo*, the residue purified by silica gel column chromatography (MeOH:Et_2O, 0:100 to 10:90, v:v) afforded **5** (59 mg, 53%). $^{13}C\{^1H\}$-NMR ($CDCl_3$): δ 23.4 (CH_3 NHAc), 54.5 (CH_3 Me), 56.4, 57.6 (C-2, 2′), 68.6, 68.8, 73.1, 73.2, 74.5, 75.2 (C-6, 6′, 5 × CH_2 benzyl), 74.8, 75.0, 74.8, 75.9, 77.4, 78.4, 84.8 (C-3, 3′, 4, 4′, 5, 5′), 100.9, 103.2 (C-1, 1′), 127.3−128.4 (CH_{arom} benzyl), 137.9−138.8 (5 × Cq benzyl), 170.6 (C=O NHAc).

1-Methyl N′-Acetylchitobiose (I). $Pd(OH)_2/C$ (28 mg) was added to a solution of **5** (59 mg) in EtOH:H_2O (40:1, v:v, 4.1 mL) and HOAc (10 μL), and the mixture was shaken under an atmosphere of hydrogen. After 48 h at 20 °C, the reaction mixture was filtered, concentrated, and purified by LH-20 column chromatography (MeOH:H_2O, 4:1, v:v) and additionally Fractogel HW 40 S column chromatography (0.15 M TEAB in MeOH:H_2O, 10:90, v:v) to furnish **I** (15 mg, 56%). $^{13}C\{^1H\}$-NMR (D_2O): δ 22.7 (2 × CH_3 NHAc), 55.7 (CH_3 Me), 57.2, 57.6 (C-2, 2′), 60.8, 61.2 (C-6, 6′), 70.1, 75.2, 75.2, 76.1, 76.6, 79.0 (C-3, 3′, 4, 4′, 5, 5′) 102.4, 103.2 (C-1, 1′), 175.2 (2 × C=O NHAc).

Methyl 4-O-(4-O-Acetyl-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (7). To a mixture of acceptor **1** (450 mg), donor **6** (650 mg), and powdered 4 Å molecular sieves in DCE (10 mL) at 20 °C (N_2 atmosphere) was added 12.0 mL of a solution containing N-iodosuccinimide (900 mg) and trifluoromethanesulfonic acid (39 μL) in DCE:Et_2O (1:1, 40 mL). After 1 h, the reaction mixture was neutralized with Et_3N, diluted with CH_2Cl_2, and filtered over Celite. The filtrate was washed with 10% $Na_2S_2O_3$, 10% $NaHCO_3$, and H_2O, dried ($MgSO_4$), and concentrated. The crude product was purified by silica gel column chromatography (EtOAc:light petroleum, 30:70 to 50:50, v:v) to afford **7** (800 mg, 88%). $^{13}C\{^1H\}$-NMR ($CDCl_3$): δ 20.7 (CH_3 acetyl), 55.4 (CH_3 Me), 56.1, 56.2 (C-2, 2′), 67.9, 69.2, 72.5, 73.4, 73.8, 74.3 (C-6, 6′, 4 × CH_2 benzyl), 72.5, 73.2, 74.3, 76.0, 76.7 (C-3, 3′, 4, 4′, 5, 5′), 96.4, 98.8 (C-1, 1′), 123.0−133.9 (CH_{arom} benzyl, Phth), 131.5 (Cq Phth), 137.6−138.4 (5 × Cq benzyl), 169.5 (5 × C=O Phth, Ac).

Methyl 4-O-(3,6-Di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (8). Compound **7** (230 mg) was dissolved in dry MeOH (5 mL), and *t*-BuOK was added to adjust the pH to 10. After stirring for 2 h at 20 °C, the reaction mixture was neutralized with Dowex 50 XW4 resin (H^+ form), filtered, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc:light petroleum, 0:100 to 40:60, v:v) to yield **8** (170 mg, 78%).

Methyl 4-O-[4-O-(4-O-Acetyl-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl]-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (9). Acceptor **8** (170 mg) and donor **6** (125 mg) were dissolved in DCE (5 mL) and treated under conditions identical to those described for the preparation of **7**, using 23 mL of the NIS−TfOH mixture. Purification of the resulting oil by silica gel column chromatography (EtOAc:light petroleum, 30:70 to 50:50, v:v) afforded **9** (140 mg, 61%).

Methyl 4-O-[4-O-(3,6-Di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (10). Trimer **9** (120 mg) was treated in a manner identical to that used for the preparation of dimer **5**. The crude product was purified by silica gel column chromatography (MeOH:toluene, 10:90, v:v) and LH-20 column chromatography (MeOH:CH_2Cl_2, 1:2, v:v) and afforded pure **10** (73 mg, 76%)

1-Methyl N′,N″-Triacetylchitotriose (II). Compound **10** (50 mg) was dissolved in *i*-PrOH:H_2O (4:1, v:v, 4.0 mL) and HOAc (0.18 mL). The mixture was shaken with Pd/C (10%) (25 mg) under an atmosphere of hydrogen for 16 h. Workup and purification were identical to those used for the preparation of compound **I** to give pure **II** (13 mg, 51%).$^{13}C\{^1H\}$-NMR (D_2O): δ 22.9, 23.0 (3 × CH_3 NHAc), 55.7−58.2 (C-2, 2′, 2″, Me), 60.8−61.4 (C-6, 6′, 6″), 70.6, 73.0, 74.3, 75.3, 75.4, 75.6, 76.7, 80.2 (C-3, 3′, 3″, 4, 4′, 4″, 5, 5′, 5″), 102.2, 102.3, 102.7 (C-1, 1′, 1‴), 175.4, 175.5 (3 × C=O NHAc).

Methyl 4-O-(3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-acetamido-β-D-glucopyranoside (11). Hydrazinolysis of dimer **7** (630 mg) under the same conditions as those described for dimer **5** gave, after purification by silica gel column chromatography (MeOH:toluene, 10:90, v:v), pure **11** (311 mg, 63%). $^{13}C\{^1H\}$-NMR ($CDCl_3$): δ 23.1, 23.4 (2 × CH_3 NHAc), 51.3 (CH_3 Me), 54.5, 56.4 (C-2, 2′), 69.6, 70.6, 72.2, 73.4, 73.6 (C-6, 6′, 4 × CH_2 benzyl), 73.3, 74.6, 77.7, 80.5 (C-3, 3′, 4,

4′, 5, 5′), 99.8, 101.6 (C-1, 1′), 127.2−128.4 (CH_{arom} benzyl), 138.4−138.0 (4 × Cq benzyl), 170.3, 170.6 (2 × C=O NHAc).

*Methyl 4-O-[4-O-(3,6-Di-O-benzyl-2-deoxy-2-amino-β-D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-3,6-di-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (**13**).* Glycosylation of acceptor **11** (172 mg) with donor **6** (180 mg) under the same conditions as those described for dimer **7** gave, after purification by silica gel column chromatography (EtOAc:light petroleum, 20:80, v:v), trimer **12**. This resulting trimer was dissolved in EtOH (96%, 15 mL), hydrazine·monohydrate (2.0 mL) was added, and the reaction mixture was heated at 90 °C. After 16 h, the solution was concentrated *in vacuo*, and silica gel column chromatography (MeOH:toluene, 10:90, v:v) afforded **13** (32 mg, 11%).

1-Methyl N′,N″-diacetylchitotriose (III). Hydrogenolysis and subsequent purification of compound **13** (32 mg) under conditions identical to those described for the preparation of **II** afforded **III** (9 mg, 49%). $^{13}C\{^1H\}$-NMR (D_2O): δ 22.8 (2 × CH_3 NHAc), 55.6, 56.2, 56.6, 57.8 (C-2, 2′, 2″, Me), 60.8, 60.9, 61.0 (C-6, 6′, 6″), 70.2, 72.2, 73.0, 73.3, 75.1, 75.2, 77.1, 77.4, 79.9 (C-3, 3′, 3″, 4, 4′, 4″, 5, 5′, 5″), 102.0, 102.5 (C-1, 1′, 1‴), 175.3, 175.4 (2 × C=O NHAc).

Finally, the products I, II, and III were dissolved in 75% acetonitrile:water (v:v) and purified on a nucleosil 120-7 NH_2 HPLC column (Maschery Nagel) using an isocratic elution in 75% acetonitrile in water, giving a recovery of 30% for each.

Quantitative Analysis of Substrate Specificity. For quantitative studies of substrate specificity of NodL protein, standard reaction mixtures containing 100 ng of NodL protein were incubated at 28 °C and the change in OD_{215} was monitored in time. The initial reaction rates of transacetylating activity were determined using 0.3 mM saccharide substrates (V[0.3 mM]). For poor substrates, concentrations were raised to 3 or 33 mM and reactions rates for 0.3 mM were obtained by linear extrapolation. For several acetyl-accepting substrates, a Michaelis−Menten curve was obtained after the initial reaction rates were determined at various saccharide concentrations. Estimates of K_m values were calculated by linear regression from Lineweaver−Burke plots. These estimates were used to calculate K_m values by nonlinear least squares using Statgraphics sofware.

RESULTS

In Vitro Transacetylating Labeling Studies Using [1-^{14}C]-Acetyl-CoA. Previously, we have shown, using TLC and structural analyses, that, *in vitro*, *N*-acetylglucosamine, chitin oligosaccharides, and lipo-chitin oligosaccharides are substrates for the NodL protein (Bloemberg *et al.*, 1994). Chitin oligosaccharides consisting of a maximum of five *N*-acetylglucosamine units were tested and shown to be acetyl-accepting substrates for NodL protein. To test whether the NodL protein can acetylate longer chitin oligosaccharides, chitinhexaose was incubated with purified NodL protein and [1-^{14}C]acetyl-CoA. TLC analysis of the labeled reaction products shows that only one chitinhexaose dependent spot is detected (Figure 2A, lane 7), which migrates slower than the *O*-acetylated form of chitinpentaose (Figure 2A, lane 6), as could be expected from its length. In both lanes 6 and 7, a spot is present in the middle of the TLC plate which is not saccharide dependent and is assumed to be [^{14}C]-acetate (Figure 2A, lane 1). For a further substrate analysis of NodL protein, several other commercially available saccharides were used. TLC analysis of the labeled products of reaction mixtures containing glucosamine, chitosanbiose, chitosantriose, chitosantetraose, and chitosanpentaose shows that in each case a single radiolabeled saccharide-related spot is produced, with different R_f values on TLC, presumably due to differences in their lengths (Figure 2A, lanes 8−12). All reaction products migrate more slowly than the *O*-acetylated

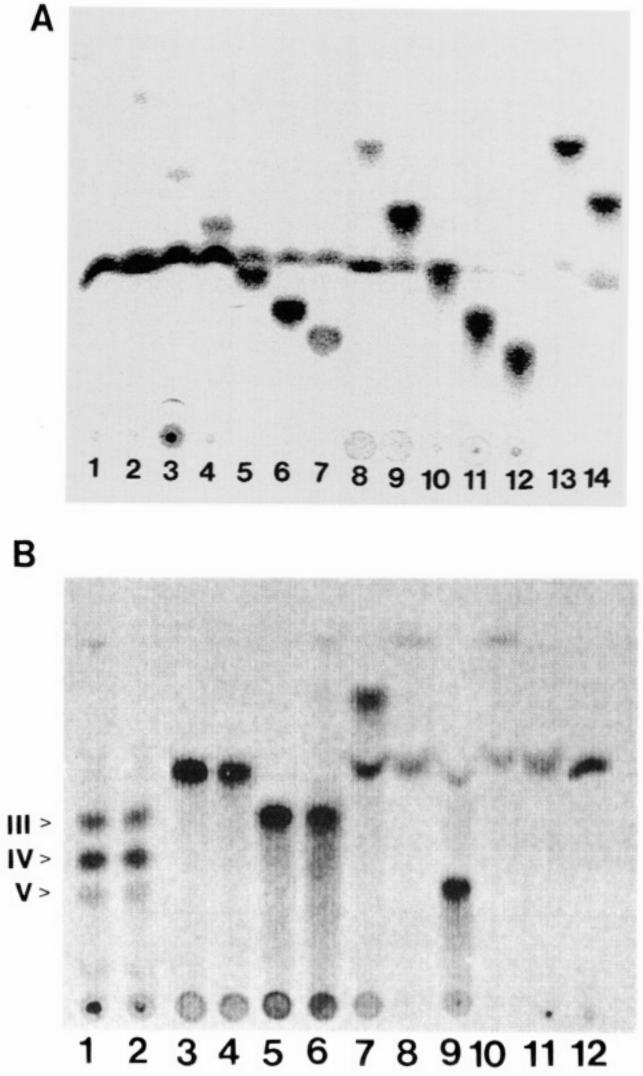


FIGURE 2: Analysis and characterization of reaction products acetylated by NodL protein using NH_2 TLC. A. After incubation, reaction mixtures containing NodL protein, [1-^{14}C]acetyl-CoA, and various mono- or oligosaccharides were analyzed by TLC. Lanes: 1, no saccharides added to the reaction mixture; 2, *N*-acetylglucosamine; 3, chitinbiose; 4, chitintriose; 5, chitintetraose; 6, chitinpentaose; 7, chitinhexaose; 8, glucosamine; 9, chitosanbiose; 10, chitosantriose; 11, chitosantetraose; 12, chitosanpentaose; 13, 1-methyl *N′*-acetylchitobiose; 14, 1-methyl *N′,N″*-diacetylchitotriose. B. Mild alkaline hydrolysis of NodL dependent reaction products or reference compounds. Shown are untreated (uneven lanes) and alkaline-treated samples (even lanes). Lanes: 1 and 2, ^{14}C-labeled chitin fragments; 3 and 4 ^{14}C-labeled N-acetylglucosamine; 5 and 6, ^{14}C-labeled glucosamine; 7−12, reaction products of mixtures containing NodL protein, [1-^{14}C]acetyl-CoA, and glucosamine (7 and 8), chitosanpentaose (9 and 10), and no saccharide added (11 and 12). The migration positions of chitinpentaose (V), chitintetraose (IV), and chitintriose (III) are indicated with arrowheads.

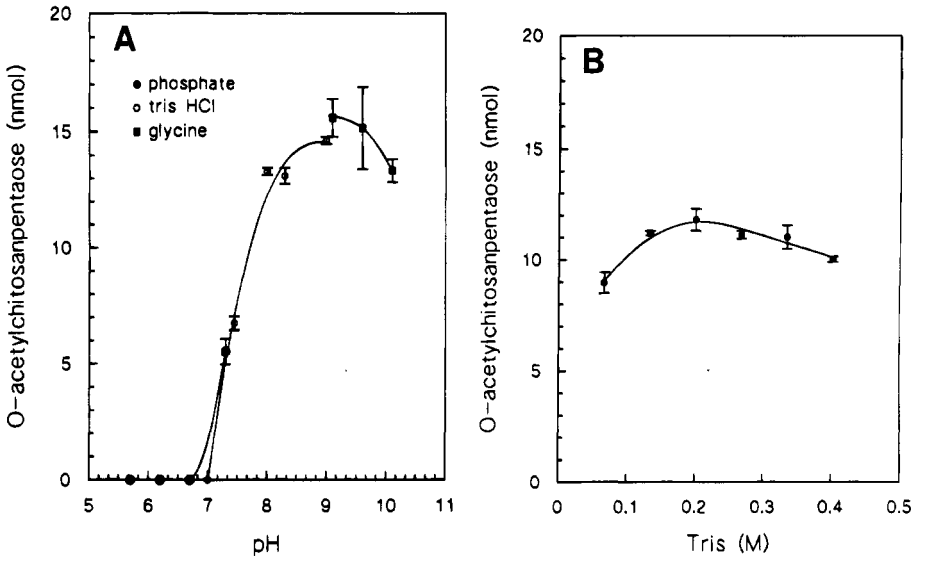


FIGURE 3: Dependence of NodL transacetylating activity on pH and Tris-HCl concentration. A. NodL transacetylating assays were performed in phosphate buffer, Tris-HCl buffer, or glycine buffers at final concentrations of 0.05 M. B. Using 25 nmol of chitinpentaose, reactions were performed under standard conditions in Tris-HCl, pH 8.0, of different molarities, and amounts of acetylated chitosanpentaose were determined 15 min after initiation of the reaction.

chitin oligosaccharides of the same length (Figure 2A, lanes 2−6).

The acetylated reaction product of glucosamine is migrating faster than *N*-acetylglucosamine or glucosamine (Figure 2B, lanes 3, 5, and 7). In addition, it is shown for the reaction products of glucosamine and chitosanpentaose that the ^{14}C-acetyl group is sensitive to mild alkaline hydrolysis (Figure 2B, lanes 7−10), showing that chitosan fragments are *O*-acetylated by the NodL protein. TLC analysis shows that the spots of the chitosan reaction products are more intense than those of the corresponding *O*-acetylated chitin oligosaccharides, indicating that chitosan oligosaccharides are acetylated more efficiently than chitin oligosaccharides. Incubation of NodL with mixtures of chitosanpentaose and NodRlv-V(C18:4) in ratios of 5 nmol:5 nmol and 5 nmol:15 nmol, respectively, yields *O*-acetylated chitosanpentaose as the major product (data not shown). In conclusion, in comparison with lipo-chitin oligosaccharides or chitin oligosaccharides, chitosan oligosaccharides are the preferred substrates of the NodL protein.

Enzymatic Characteristics of NodL Protein. In order to optimize the conditions for transacetylation, the enzymatic properties of the NodL protein were studied in detail. For these studies, chitosanpentaose was chosen as the acetyl-accepting substrate, since it is the best commercially available acetyl-accepting substrate for NodL (Figure 2A). For a quantitative analysis of transacetylating activity, the spectrophotometric assay according to Alpers *et al.* (1965) was optimized as described in Experimental Procedures. Transacetylating activity of NodL protein was determined at various pH values in the range 5.7−10.1 using different kinds of buffering agents. Figure 3A shows that the optimal pH for the NodL protein is approximately 9.0. However, studies for getting indications about the *in vivo* substrates experiments were carried out at pH 8.0 since (i) the activity of NodL protein is still relatively high at pH 8 (Figure 3A) and (ii) pH 8.0 is presumed to be closer to the cytosolic pH than the optimal pH that we determined. The dependence of NodL transacetylating activity on Tris-HCl concentration at pH 8.0 was determined (Figure 3B). Activity shows an optimum at 0.2 M Tris-HCl. NodL protein has a broad temperature optimum, between 28 and 42 °C (Figure 4A). NodL protein appears to be very stable at high temperatures, since incubating NodL protein samples for 15 min at temperatures up to 48 °C, prior to the transacetylating reaction being performed at 28 °C, does not lead to a significant loss of activity (Figure 4B). The effect of the presence of metal ions on NodL protein activity was determined by addition of various mono- and divalent metal ions in concentrations of 10 mM. Addition of K^+, Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} results in transacetylating activities of 112, 104, 90, 83, 57, and 13%, respectively. Addition of 5 mM EDTA does not affect transacetylating activity.

On the basis of these results, we have identified a set of standard conditions for the NodL transacetylation reaction (see Experimental Procedures). We have defined one unit of NodL as an amount of protein which can acetylate 1 μmol of chitosanpentaose in 1 min, under these standard conditions. This unit definition was used to monitor the large scale purification of NodL protein (Table 1), which was used for further analysis of substrate specificity.

Quantitative Analysis of NodL Protein Substrate Specificity. In order to get indications about *in vivo* acetyl acceptor substrates for NodL, a quantitative analysis of substrate specificity was carried out using the protocol described in Experimental Procedures. Initial reaction rates of transacetylating activity were determined for various chitin and chitosan oligosaccharides. In addition, pentameric forms of α-1,4-linked glucose (maltopentaose) and β-1,4-linked glucose (cellopentaose) were tested. Initial reaction rates for the substrates (0.3 mM) were compared with the initial reaction rate of the model substrate chitosanpentaose (Table 2). The results show that (i) reaction rates for chitosan oligosaccharides are much higher than those for chitin oligosaccharides, (ii) glucosamine and *N*-acetylglucosamine are much less efficiently acetylated than their oligomeric forms, (iii) no detectable acetylation of maltopentaose was observed, and (iv) cellopentaose is acetylated by NodL protein at a rate which is higher than that for chitinpentaose. To analyze the

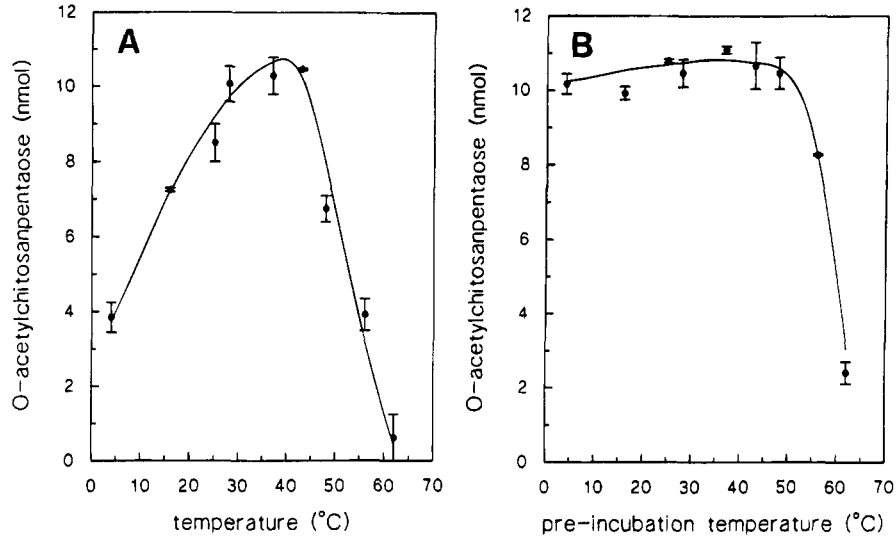


FIGURE 4: Effect of temperature on NodL protein. A. Dependence of the transacetylating activity of NodL protein on temperature. Assays were performed at different temperatures using standard conditions. Amounts of acetylated chitosanpentaose were determined 15 min after initiation of the reaction. B. Influence of temperature on the stability of NodL transacetylating activity. NodL protein was preincubated for 15 min at different temperatures. Transacetylating activity was subsequently determined at 28 °C under standard conditions.

Table 2: Substrate Specificity of NodL Protein

substrate	V[0.3 mM][a] (nmol/min)	relative V[b] (%)	K_m (10^{-3}M)
chitosanpentaose	3.97	100	1.1 ± 0.1
chitosantetraose	2.70	68.1	1.5 ± 0.2
chitosantriose	0.32	8.0	6.0 ± 0.9
chitosanbiose	0.20	5.0	nd[c]
glucosamine	2.10×10^{-3}	5.3×10^{-2}	nd
chitinhexaose	3.97×10^{-3}	1.0×10^{-1}	nd
chitinpentaose	1.59×10^{-2}	4.0×10^{-1}	>500
chitintetraose	9.93×10^{-3}	2.5×10^{-1}	nd
chitintriose	9.13×10^{-5}	2.3×10^{-3}	nd
chitinbiose	8.34×10^{-5}	2.1×10^{-3}	nd
N-acetylglucosamine	2.30×10^{-5}	5.8×10^{-4}	nd
cellopentaose	0.19	4.7	nd
maltopentaose	0	0	
1-methyl *N',N''*-diacetylchitotriose	11.76	296.2	nd
1-methyl *N,N',N''*-triacetylchitotriose	7.54×10^{-5}	1.9×10^{-3}	nd

[a] Initial reaction rates of transacetylating activity determined for saccharides at 0.3 mM. Reaction mixtures with final concentrations of 0.67 mM Tris-HCl, 1.5 mM acetyl-CoA, and 6.0 mM Ellman's reagent contained 100 ng of purified NodL protein, had a final volume of 75 μL, and were incubated at 28 °C. [b] Percentages of initial reaction rates relative to chitosanpentaose. [c] nd = not determined.

substrate affinity of NodL in more detail, K_m values for several acetyl acceptors were determined from Lineweaver–Burke plots using nonlinear regression (Table 2). The results show that the K_m value for chitinpentaose is at least 500-fold higher than the K_m value for chitosanpentaose (Table 2). Furthermore, a comparison of K_m values for chitosanpentaose, chitosantetraose, and chitosantriose shows that an increase in the length of the chitosan fragment leads to a decrease in the K_m value.

Substrate Specificity for Synthetic Terminally De-N-acetylated Chitin Derivatives. Since our results show that for NodL protein chitosan is the preferred substrate, when compared to chitin oligosaccharides and LCO, the NodBC metabolites are good candidates for being *in vivo* acetyl-accepting substrates. Since NodBC metabolites produced by *Rhizobium* strains cannot be purified in large quantities, possibly due to the instability of these compounds (unpublished results), the molecules, 1-methyl *N'*-acetylchitobiose and 1-methyl *N',N''*-diacetylchitotriose, which are structurally similar to NodBC metabolites, were chemically synthesized (Figure 1), and their structures were confirmed by NMR spectroscopy (see Experimental Procedures). The 1-methyl group was added in order to facilitate a better monitoring of products during synthesis and because it is expected to stabilize the product. TLC analysis of NodL reaction mixtures containing the synthetic compounds shows that in all cases only one reaction product is formed (Figure 2A, lanes 13 and 14). Quantitative substrate analysis shows that the reaction rate determined at 0.3 mM for 1-methyl *N',N''*-diacetylchitotriose is 3 times higher than that for chitosanpentaose (Table 2). Due to the limited quantities of the chemically synthesized compounds, we have not been able to calculate an accurate K_m value. 1-Methyl *N,N',N''*-triacetylchitotriose, which was chemically synthesized as a control compound, is not more efficiently acetylated than chitintriose (Table 2)

DISCUSSION

In order to analyze their functions, *in vitro* studies with several Nod proteins have been performed (Schwedock *et al.*, 1990; John *et al.*, 1993; Sutton *et al.*, 1994; Atkinson *et al.*, 1994; Röhrig *et al.*, 1994; Geremia *et al.*, 1994). Previously, we have shown that purified NodL protein *in vitro* catalyses acetyl transfer from acetyl-CoA to the C-6 position of *N*-acetylglucosamine, chitin oligosaccharides, and LCOs (Bloemberg *et al.*, 1994). In this work, substrate specificity and other enzyme characteristics of the NodL protein have been studied in further detail.

NodL transacetylating activity was analyzed using labeling studies and after the quantitative spectrophotometric assay described by Alpers *et al.* was optimized (1965). Enzymatic properties of the NodL protein have been characterized in order to optimize reaction conditions. The results show that NodL activity has a pH optimum of approximately 9, is stable at temperatures up to 48 °C, and has a temperature optimum around 35 °C (Figures 3 and 4). These properties seem remarkable for a protein that is located in the cytosol (Bloemberg *et al.*, 1994) and that has an optimal growth

temperature of 29 °C. From a practical point of view, these properties facilitate handling and storage of the NodL protein. The reaction conditions finally chosen for studying the NodL protein should be considered to represent a compromise between the activity optima we determined and the presumed physiological conditions. Our optimization of the reaction conditions for NodL is valuable for the production of *O*-acetylated compounds for labeling studies and for obtaining new oligosaccharides in large amounts.

The *in vivo* acetyl-accepting substrate(s) for NodL protein is not known. Chitin oligosaccharides and LCOs could be considered to be putative *in vivo* acceptors for the NodL protein (Bloemberg *et al.*, 1994). Surprisingly, our *in vitro* studies show that NodL protein is also able to *O*-acetylate glucosamine, chitosan oligosaccharides, and cellopentaose (Figure 2A,B, Table 2). The results show that chitosan oligosaccharides are even better substrates for NodL than chitin oligosaccharides. Cellopentaose is also more efficiently acetylated than chitinpentaose, but less efficiently than chitosanpentaose (Table 2). This shows that the presence of an *N*-acetylglucosamine residue at the nonreducing terminus is a more important negative factor than the presence of a glucose residue. NodL protein cannot acetylate maltopentaose in detectable amounts, showing its structural specificity for β-1,4-linked residues. The results also show that NodL has a different specificity for oligosaccharides varying in length. NodL protein shows the highest affinities for tetrameric and pentameric oligosaccharides, which corresponds well with the lengths of the *N*-acetylglucosamine backbones of the major LCOs produced by *Rhizobium*. Quantitative substrate specificity studies for LCOs are extremely difficult, because of their very low solubility in water. However, competition studies using small amounts of substrate and radiolabeled acetyl-CoA show that chitosanpentaose is a much better substrate than LCO.

The conclusion that the NodL protein has a much higher specificity for chitosan oligosaccharides than for chitin oligosaccharides (Table 2) gives indications for its *in vivo* substrates. Recently, pentameric and tetrameric chitin derivatives were identified which lack the *N*-acetyl group on the nonreducing terminal residue, which are produced by means of the NodB (chitin deacetylase) and NodC proteins (chitin synthase) and are therefore referred to as NodBC metabolites (Spaink *et al.*, 1994). These metabolites are assumed to be intermediates in the biosynthesis of LCOs, functioning as acyl acceptors for the NodA protein (Figure 5). *O*-Acetylated NodBC metabolites were produced in the presence of the *nodL* gene, indicating that NodL *in vivo* is able to acetylate the chitin backbone of LCOs before the substitution of the acyl moiety (Spaink *et al.*, 1994). To date, the isolation of milligram quantities of NodBC metabolites for *in vitro* studies has not been successful, possibly due to their instability. As an alternative, chitin derivatives resembling NodBC intermediates in structure, up to a length of three residues, were chemically synthesized (Figure 1). Quantitative analysis shows that 1-methyl *N'*,*N''*-diacetylchitintriose is the compound most efficiently acetylated by NodL protein of all the compounds tested. The additional 1-methyl group located on the reducing terminal residue has little influence on substrate specificity, as can be concluded from a comparison of the reaction rates for 1-methylchitintriose and chitintriose (Table 2). In conclusion, the results show that a free amino group on the nonreducing terminal residue of the oligosaccharide backbone is preferred by the NodL protein. Taken together, the results of Spaink *et al.* (1994) and this report strongly suggest that NodBC intermediates are the *in vivo* acetyl-accepting substrates for NodL rather than chitin oligosaccharides or LCOs, as depicted in the model presented in Figure 5.

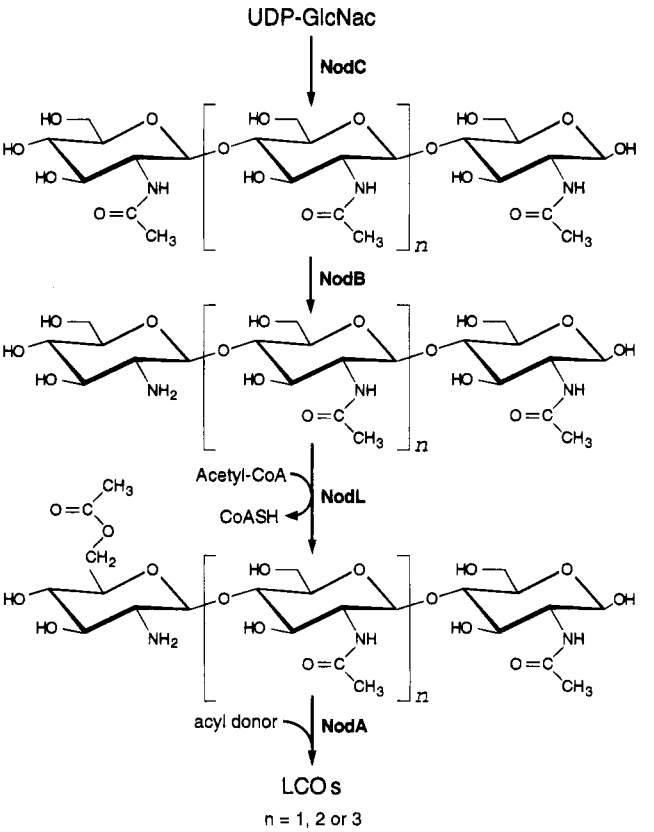


FIGURE 5: Proposed model for the synthesis of lipo-chitin oligosaccharides (LCOs) *in vivo*. The following steps in the biosynthesis of LCOs are indicated: i, NodC functions as a chitin synthase producing chitin oligosaccharides of variable length; ii, NodB de-*N*-acetylates the nonreducing terminal residue; iii, NodL acetylates the NodBC intermediates at the 6-OH position of the nonreducing terminal residue using acetyl-CoA as the acetyl donor; iv, NodA acylates the NodBCL intermediates, transferring an acyl moiety which can be of variable structure.

ACKNOWLEDGMENT

We thank Caroline van Hoek for her help with some of the experiments and J. E. Thomas-Oates for critical reading of the manuscript.

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BI950739Z