Technical Advance

Rapid Colorimetric Quantification of Lipo-chitooligosaccharides from *Mesorhizobium loti* and *Sinorhizobium meliloti*

Joachim Goedhart,¹ Jean-Jacques Bono,² Theodorus W. J. Gadella, Jr.¹

¹Laboratory for Molecular Biology, MicroSpectroscopy Center Wageningen, Department of Plant Sciences, Wageningen University, Dreijenlaan 3, 6703 HA, Wageningen, The Netherlands; ²Signaux et Messages Cellulaires chez les Végétaux, UMR CNRS-UPS 5546, Pôle de Biotechnologie Végétale, 24 chemin de Borde Rouge, BP 17 Auzeville, 31326 Castanet-Tolosan, France

Submitted 29 October 2001. Accepted 23 April 2002.

Nod factors are lipids with a chitinlike headgroup produced by gram-negative *Rhizobium* bacteria. These lipo-chitooligosaccharides (LCOs) are essential signaling molecules for accomplishing symbiosis between the bacteria and roots of legume plants. Despite their important role in the *Rhizobium*-legume interaction, no fast and sensitive Nod factor quantification methods exist. Here, we report two different quantification methods. The first is based on the enzymatic hydrolysis of Nod factors to release N-acetylglucosamine (GlcNAc), which can subsequently be quantified. It is shown that the degrading enzyme, glusulase, releases exactly two GlcNAc units per pentameric nodulation factor from *Mesorhizobium loti* factor, allowing quantification of LCOs from *Mesorhizobium loti*. The second method is based on a specific type of Nod factors that are sulfated on the reducing GlcNAc, allowing quantification analogous to the quantification of sulfolipids. Here, a two-phase extraction method is used in the presence of methylene blue, which specifically forms an ion pair with sulfated lipids. The blue ion pair partitions into the organic phase, after which the methylene blue signal can be quantified. To enable Nod factor quantification with this method, the organic phase was modified and the partitioning was evaluated using fluorescent and radiolabeled sulfated Nod factors. It is shown that sulfated LCOs can be quantified with this method, using sodium dodecyl sulfate for calibration. Both methods allow Nod factor quantification in parallel enabling a fast and easy detection of nanomole quantities of Nod factors. Accurate Nod factor quantification will be crucial for characterization and cross-comparison of the affinity for Nod factors of newly identified Nod factor binding proteins or putative Nod factor receptors.

Additional keywords: BODIPY, chitinase, β -glucuronidase, Reissig.

Nod factors are lipo-chitooligosaccharides (LCOs) secreted by rhizobia and are essential to accomplish symbiosis with legumes

(Long 1996). The exact structure of the Nod factor differs for each *Rhizobium* species, but the basic structure comprises a chitin backbone of three to five β -1,4-linked N-acetylglucosamine (GlcNAc) residues to which a fatty acyl chain is attached at the nonreducing terminal sugar (Fig. 1). Nod factors, either produced by rhizobia or chemically synthesized, can elicit responses at picomolar concentrations on a compatible host plant (Gadella et al. 1997; Heidstra et al. 1994; Lerouge et al. 1990; Spaink et al. 1991).

Quantification of Nod factors is usually done by measuring absorbance of the unsaturated acyl chain (Etzler et al. 1999) or by comparison to standards on reversed phase-high performance liquid chromatography (RP-HPLC) (Gressent et al. 1999). However, purified Nod factors generally consist of a mixture of differently absorbing compounds due to different unsaturations of the acyl chain, each requiring an individual standard. Furthermore, potential UV-absorbing impurities (such as fatty acids, lipopolysaccharides (LPS), or low concentrations of aromatic compounds) can overestimate the real amount of Nod factors, whose extinction coefficient is very low [$\epsilon_{210} \approx 3,000 \text{ M}^{-1} \text{ cm}^{-1}$ for LCO-V(C18:1, S)]. Reliable and fast methods to quantify both purified and synthesized LCOs will be of great help for both basic research and biotechnological applications. For example, the precise knowledge of the concentration of Nod factors is important in experiments aiming to determine the affinity constants of Nod factor-binding proteins (Etzler et al. 1999; Gressent et al. 1999), the measurement of dose-response curves using different bioassays (Felle et al. 1995; Staehelin et al. 1994a), or the preparation of Nod factor solutions deliverable to plants in agronomic trials. A promising method to quantify Nod factors is reported by Staehelin and associates (1994a). In this approach, Nod factors are treated with hydrolyzing enzymes from snail gut (glusulase), releasing GlcNAc monomers (Boller and Mauch 1988) that can be quantified colorimetrically by Reissig's method (Reissig et al. 1955).

Unfortunately, no Nod factor-independent standard was used for calibrating the method, since known amounts of Nod factors from *Rhizobium* sp. NGR (pA28) were used for calibration. In this study, we modified the previously described approach (Staehelin et al. 1994a) to allow quantification without a Nod factor standard. In addition, we analyzed the breakdown products of the *Mesorhizobium loti* Nod factors after treatment with glusulase, showing that the enzyme quantitatively digests these Nod factors.

Corresponding author: T. W. J. Gadella Jr.; Telephone +31 20 525 6259; Fax: +31 20 525 6271; E-mail: Gadella@science.uva.nl

Current Address for J. Goedhart and T. W. J. Gadella, Jr.: Laboratory for Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 316, 1098 SM Amsterdam, The Netherlands.

Subsequently, a novel approach is described for quantification of sulfated Nod factors, based on the quantification of sulfated lipids by a method originally developed by Kean (1968) that was improved by Radin (1984). In this assay, comprising a biphasic liquid system with an aqueous layer and an apolar organic layer, sulfated lipids form an ion pair with a cationic dye, after which the complex enters the organic phase. The sulfolipid can be quantified by measuring the absorbance of the cationic dye in the organic phase. Using a novel sulfated fluorescent Nod factor derivative, we optimized the partitioning of Nod factors into the organic phase. Determination of the partitioning efficiency and the color yield of the ion pair allows the quantification of Nod factors from *Sinorhizobium meliloti* that have a sulfate group on the reducing end of the chitin backbone (Lerouge et al. 1990).

RESULTS

Hydrolysis of LCOs by glucusase.

The first method to quantify Nod factors is based on the quantification of GlcNAc monomers enzymatically released from the chitin backbone of the Nod factors by treatment with glucusase. To investigate conditions under which the glucusase completely hydrolyses chitooligosaccharides, we measured the release of GlcNAc from tetra-*N*-acetyl-chitotetraose [(GlcNAc)₄] or nodulation factors from *Mesorhizobium loti* (NodRlo) during incubation with snail juice containing this enzyme. (GlcNAc)₄ itself was not detected by the Reissig assay (15 nmol (GlcNAc)₄ gives an optical density at 585 nm (OD₅₈₅) of less than 0.01). When 10 to 25 µl of glucusase was used, (GlcNAc)₄ was completely hydrolyzed to GlcNAc monomers within 1 h (data not shown), which agrees with a previous observation (Boller and Mauch

1988). Subsequently, (GlcNAc)₄ was used to prepare a calibration standard by incubation with enzyme and subsequently measuring the amount of released GlcNAc using the Reissig method. The OD₅₈₅ was linearly dependent on the amount of (GlcNAc)₄, indicating that this approach can be used for calibration (Fig. 2, inset). The calibration curve was characterized by a slope of 44 OD units per µmol of (GlcNAc)₄.

Identical conditions were used to check whether the enzyme was also able to release GlcNAc units from the Nod factors. The release of GlcNAc from NodRlo was measured at different time points to ensure complete digestion by the enzyme (Fig. 2). At time point 0, almost no absorbance was measured. Apparently LCOs, like GlcNAc polymers (Domard and Vasseur 1991), are hardly detected by the method of Reissig. After a rapid increase of free GlcNAc within the first half hour, the amount of GlcNAc reached a constant level. The results indicate that GlcNAc can be released from NodRlo and that all accessible GlcNAc monomers are released enzymatically from the Nod factor within 1 h.

Analysis of the products produced by glucusase.

It is well established that glucusase hydrolyzes chitooligosaccharides completely into GlcNAc monomers (Boller and Mauch 1988; Cabib and Bowers 1971). On the other hand, plant chitinases break the oligosaccharide backbone of Nod factors down to tri- or dimers, but not to monomers (Staehelin et al. 1994b). For our assay, it was essential to correlate the amount of GlcNAc removed from NodRlo by glucusase to the amount of unreacted NodRlo and the breakdown products containing the fatty acyl chain and the nonreducing end. Therefore, we analyzed Nod factor breakdown products produced by the action of glucusase by RP-HPLC.

RP-HPLC profiles of NodRlo incubated with glucusase for 0, 5, and 60 min are shown in Figure 3. At time point 0, the major component in the HPLC profile has a retention time (Rt) of approximately 6 min and represents intact NodRlo, which is a mixture of NodRlo-V(C18:1, NMe, Carb, AcFuc) and NodRlo-V(C18:0, NMe, Carb, AcFuc) (Lopez-Lara et al. 1995). After 5 min incubation with glucusase, the peak at Rt of 6 min decreases and two new peaks arise at Rt of 2.3 and 8.9 min. The peak at Rt of 2.3 min is due to a component of the enzyme preparation (data not shown), whereas the peak at Rt of 8.9 min represents a NodRlo breakdown product. After 60 min incubation, the peak corresponding to the intact Nod factors is almost completely absent, whereas the peak at Rt of 8.9 min is still present and is increased. The time scale of NodRlo breakdown is compatible with the kinetics of the release of GlcNAc monomers and shows that the saturation of the curve after 30 min (Fig. 2) indeed reflects complete digestion of NodRlo.

To obtain data on the exact composition of the more hydrophobic breakdown product (Rt of 8.9 min.), we performed a mass spectrometric analysis. The spectrum contains a main pseudomolecular ion at m/z 907.3. Calculation of the molecular weight of expected products, i.e., a shorter LCO carrying four, three, or two GlcNAc units, gives 1,110, 907, and 703, respectively. Thus, the main pseudomolecular ion at m/z 907.3 is consistent with a Nod factor with three GlcNAc units: NodRlo-III(C18:1/C18:0, NMe, Carb), implicating that glucusase removes two GlcNAc units per NodRlo. In the case of NodRlo, one of the two released GlcNAc residues still bears the AcFuc substitution at C6. However, from the structures of the chromogens that are formed during the Reissig assay (Roden et al. 1997), it can be inferred that GlcNAc substituted at C6 will be detected similar to unsubstituted GlcNAc.

The calibration standard prepared by hydrolysis of (GlcNAc)₄ (Fig. 2, inset) is characterized by a slope of 46 OD units per µmol (GlcNAc)₄ from which the absorbance of hydro-

Fig. 1. Structures of the lipo-chitooligosaccharides (LCOs) used in this study. **A**, The chitinlike headgroup with decorations R₁ to R₄ and different degrees of GlcNAc polymerization referred to as trimers ($n = 1$), tetramers ($n = 2$), and pentamers ($n = 3$). **B**, The NodRlo factors are a mixture of LCOs having either a cis-vaccenic acid (C18:1) or stearic acid (C18:0) acyl chain and various substitutions, including a 4-*O*-acetylfucose (AcFuc) residue on the terminal reducing sugar. Sulfated Nod factors carry a ³⁵S radiolabeled sulfate group at R₄ and have **C**, the natural occurring C16:1Δ9 acyl chain at R₁, or they carry a nonlabeled sulfate group at R₄ and a fluorescent BODIPY FL-C₁₆ fatty acid at position R₁.

lyzed NodRlo is derived: 23 OD units per μmol . Now, the amount of the NodRlo can be quantified after hydrolysis with glucusase and subsequent determination of GlcNAc. For example, the final absorbance of the hydrolyzed NodRlo sample shown in Figure 2 minus background was 0.14, which is 6.1 nmol NodRlo.

Partitioning of sulfated LCOs in a two-phase solvent system.

The second method to quantify Nod factors colorimetrically is based on the specific decoration of Nod factors produced by *Sinorhizobium meliloti* that have a sulfate group. Sulfated lipids can be quantified by a method developed by Kean (1968) that was improved by Radin (1984). In this method, a two-phase solvent system is used: an aqueous phase in which a cationic dye is dissolved (methylene blue or azure A) and an apolar phase consisting of organic solvents that excludes the cationic dye. The principle of the assay is that the sulfated lipid forms an ion-pair with the cationic dye, after which the resulting complex partitions into the organic phase. Hence, the staining of the organic upper phase with the cationic dye is proportional to the amount of sulfated lipid. This amount can be easily quantified by measuring the absorbance of the cationic dye.

We started to use the conditions published by Radin (1984), which use a relative apolar upper phase (isooctane/chloroform/isoamylalcohol/isopropanol (vol/vol/vol/vol) 3:1:1:1). Unfortunately, under these conditions, no sulfated Nod factors could be detected in the upper phase, probably because the solvent polarity of the upper phase was too low (data not shown). Hence, we tried to increase the polarity of the upper organic phase in order to accomplish partitioning of LCOs to this phase. However, the organic upper phase cannot be too polar, as it should exclude uncomplexed methylene blue. As butanol is used to isolate Nod factors from aqueous medium (Lerouge et al. 1990; Spaink et al. 1991), our first effort was to try butanol as the organic phase. Unfortunately, even in the absence of sulfated lipids, methylene blue partitioned extensively into the butanol layer (data not shown). To achieve intermediate polarity of the upper phase, hep-

tane was included at different percentages, and partitioning of methylene blue was examined in the absence of sulfated lipids. As shown in Figure 4, increasing the amount of heptane reduced the amount of methylene blue in the organic phase. From this data, it can be inferred that the methylene blue background in the organic phase is sufficiently low when at least 60% of heptane is included in the upper phase, whereas the presence of 50% heptane is still usable but gives rise to a relative high background absorbance (around 0.1).

To investigate whether, under these new conditions, LCOs could partition into the upper phase, we used fluorescent sulfated LCOs and quantified their partitioning efficiencies. As expected, increasing percentages of heptane reduced the amount of Nod factor in the organic phase (Fig. 4). With 40% heptane in the upper phase, all Nod factors were present in the organic phase, whereas less than half of the fluorescence was observed when the heptane percentage was increased to 60%. Hence, the most favorable situation (50% heptane present in the upper phase) is still far from optimal; the background is significant (around 0.1) and only 75% of the Nod factors are in the upper phase.

In order to increase the partitioning of Nod factor into the upper phase, we attempted to increase the hydrophobicity of the Nod factors by peracetylation with acetic anhydride. Thin-layer chromatographic (TLC) analysis on silica-coated plates with chloroform and methanol (4:1, vol/vol) as the mobile phase showed a single spot with $R_f = 0.45$ for the peracetylated LCO-IV(BODIPY FL- C_{16} , S) and $R_f = 0.0$ for the unreacted LCO (data not shown), clearly indicating the increased hydrophobicity of the LCO after peracetylation.

The partitioning experiments at different percentages of heptane were repeated with the acetylated LCO. As can be inferred from Figure 4, the peracetylated LCO-IV partitioned well into the organic upper phase, up to a heptane percentage of 60%.

Fig. 2. Quantification of NodRlo-V with glucusase. After digestion, the amount of GlcNAc was determined by Reissig's method (Reissig et al. 1955). The data shown are the results of two experiments. A monoexponential curve is fit to the average of the two experiments, yielding a halftime of 5 min. The inset shows a calibration curve with a slope of 46 OD units per μmol prepared by glucusase hydrolysis of a known amount of (GlcNAc)₄. The data shown is the average of two measurements.

Fig. 3. Reversed phase-high performance liquid chromatographic analysis of NodRlo-V breakdown products after hydrolysis with glucusase for 0, 5, and 60 min. Due to the treatment with glucusase, the peak at 6 min representing intact NodRlo-V decreases, whereas a new more hydrophobic breakdown product accumulates with a retention time of 9 min.

Hence, peracetylation clearly improved the partitioning conditions, since it allows the assay to be performed at a heptane percentage of 60%, yielding a low background (<0.05) and substantially improved partitioning of more than 90%.

Calibration with SDS as a standard.

To evaluate whether the upper phase consisting of butanol and heptane (2:3) is compatible with quantification of sulfated lipids by the Kean assay, a calibration curve was prepared by using different amounts of sodium dodecyl sulfate (SDS). The absorbance of methylene blue in the organic phase shows a linear dependence on the amount of SDS present (Fig. 5), clearly demonstrating that SDS has a good partitioning and can be used as standard.

Based on the slope of the curve, we calculated that our upper phase had an OD of 51 units per μmol of sulfated lipid, which corresponds to an absorbance coefficient of the SDS-methylene blue complex of $51,000 \text{ M}^{-1} \text{ cm}^{-1}$, assuming complete partitioning of SDS into the upper phase. Using the same volumes but the upper phase composition as described by Radin (1984), we obtained an OD of 65 units per μmol of sulfated lipid at 655 nm (data not shown). Hence, the color yield of the methylene blue-SDS complex is lower in the case of the more polar organic upper phase.

Determination of the color yield of the LCO-methylene blue complex.

As the color yield of a LCO-methylene blue complex is unknown, we took advantage of the availability of a fluorescent derivative of Nod factors to correlate the amount of LCO based on the absorbance of the fluorescent acyl chain with the amount of methylene blue that partitioned into the organic phase. The concentration of acetylated LCO-IV(BODIPY FL- C_{16} , S) and acetylated LCO-V(BODIPY FL- C_{16} , S) was measured by absorbance in methanol, $\epsilon_{505} = 90,000 \text{ cm}^{-1} \text{ M}^{-1}$ (Haugland 1996). Subsequently, a known amount was used in the assay, and the sulfate concentration was determined by absorbance of methylene blue, using SDS as a standard. Based on the SDS calibration, about 90% of the total amount of LCO added was detected in the upper phase for both species (Table 1), which

corresponds to a color yield of 45.9 OD units per μmol LCO. This measured color yield is the product of the partitioning efficiency and the true color yield of the LCO-methylene blue complex. Since the recovery of fluorescent LCOs in the upper phase is known (Fig. 4), it is possible to calculate the true color yield of the complex. According to this quantitative comparison, we could determine the average color yield of the LCO-methylene blue complex in the upper phase, which is 48.1 OD units per μmol (Table 1). This value is slightly lower than that of the SDS-methylene blue complex (about 94%).

Partitioning of natural sulfated Nod factors.

The partitioning experiments with the fluorescent Nod factor show that this method can be used to quantify LCO-IV(BODIPY FL- C_{16} , S). However, our aim was to quantify sulfated Nod factors with natural acyl chains. Hence, the partitioning of natural LCOs into the upper phase containing 60% heptane was determined by using ^{35}S radiolabeled LCOs. The partitioning efficiency of the radiolabeled Nod factors was determined by counting the radioactivity associated with the upper phase and the lower phase in three separate experiments. The recovery of acetylated radiolabeled LCO in the upper phase listed in Table 2 shows that natural LCOs partition slightly less well into the upper phase as compared with the fluorescent LCOs. Furthermore, the partitioning into the upper phase decreases in the order such that LCO-III($\text{C}_{16}:1$, ^{35}S) is greater than LCO-IV($\text{C}_{16}:1$, ^{35}S) which is greater than LCO-V($\text{C}_{16}:1$, ^{35}S) as the overall hydrophobicity of the LCOs decreases due to the increased contribution of the polar oligosaccharidic moiety. Assuming a similar color yield of the LCO-methylene blue complex for fluorescent and natural acyl chain-substituted LCOs, we calculated the effective OD of the upper phase per μmol of added LCO (Table 2). Because of the lower partitioning efficiencies, these numbers are slightly lower than for the fluorescent acyl chain-labeled compounds.

To compare the quantification based on sulfate to the usual quantification of LCOs by dry-weight, we used the partitioning

Fig. 4. Effect of the organic upper phase composition on background absorbance and lipo-chitooligosaccharide partitioning. The volume percentage of heptane is increased at the expense of butanol. $\diamond$ represents absorbance of methylene blue in the upper phase in the absence of sulfated lipid. $\circ$ and $\bullet$ represent recovery of fluorescence in the upper phase using nonacetylated ($\circ$) or acetylated ($\bullet$) LCO-IV(BODIPY FL- C_{16} , S).

Fig. 5. Calibration of the partitioning assay using sodium dodecyl sulfate (SDS). After partitioning between the aqueous methylene blue-containing phase and the upper phase consisting of 40% butanol and 60% heptane, the upper phase was isolated, and the absorbance at 655 nm was measured (average and standard deviation of three measurements) and was plotted as a function of the initially added amount of SDS. The slope of the calibration curve is 51 OD units per μmol .

method to quantify a sulfated Nod factor sample with a known dry weight. A triplicate quantification of LCO-V(C18:1, S) yielded 2.34 ± 0.07 nmol. This value was 22% lower than the value determined by dry weight (3.0 nmol). This might reflect an overestimation of the Nod factor quantity in the dry-weight sample, probably due to the presence of salts or water.

Effect of contaminants.

Although the method is developed to quantify HPLC-purified Nod factors, it is relevant to examine the effect of contaminants in the sample of Nod factors. In the original article, a number of contaminants have been checked (Kean 1968). Here, we describe only the effect of a few contaminants that could be relevant for the quantification of Nod factors. The methylene blue absorbance of 0 and 10 nmol of SDS was evaluated in the presence of 10 nmol of nonsulfated NodRlo factors, 40 μ g of stearic acid and 10 μ g of lipopolysaccharide, which were all subjected to acetylation before the partitioning. As can be inferred from Figure 6, a minor increase in absorbance is observed when nonsulfated Nod factors are included, whereas no effect of the acyl chains is observed. In contrast, a strong increase in both background, i.e., 0 nmol of SDS, and the sulfate-containing sample is observed when LPS is present. Thus, LPS interferes with the methylene blue partitioning method, indicating that the method can only be applied to purified samples when Nod factors are obtained from a biological source.

DISCUSSION

Quantification by enzymatically released GlcNAc.

Under the conditions described, complete enzymatic degradation of pentameric to trimeric Nod factors has been obtained, allowing reliable quantification of NodRlo by the release of GlcNAc monomers. The assay is completed within 2 h and can be calibrated with GlcNAc monomers. However, the use of (GlcNAc)₄, which is completely hydrolyzed to GlcNAc monomers by glucosylase, can also be used and appears more convenient, because it can act as a positive control for enzymatic activity.

The absence of tetramers generated from the hydrolysis of NodRlo-V strongly suggests that the enzyme mixture does not remove single GlcNAc units. In agreement with this observation, the enzyme mixture was unable to degrade fluorescent LCO-IV(BODIPY FL-C₁₆) (data not shown). Therefore, glucosylase most likely releases a GlcNAc dimer from NodRlo that is subsequently hydrolyzed to GlcNAc units that can be quantified. The RP-HPLC analysis shows that the enzyme is not able to hydrolyze NodRlo-III.

The release of two GlcNAc units per Nod factor gives rise to increased sensitivity of the assay. Possibly, Nod factors tetramers can be quantified by this assay after including chitinase from *Streptomyces griseus*, which has been shown to degrade fluorescent and natural Nod factor tetramers to dimers (Heidstra et al. 1994; J. Goedhart, unpublished data). Unfortunately, glucosylase cannot remove GlcNAc from a pentameric sulfated LCO (data not shown). This stresses the need for a different quantification method for sulfated Nod factors.

Quantification based on the sulfate group.

Detection based on the sulfate has the advantage that it is sensitive and quick. A minor drawback is that this method can only be applied to sulfated Nod factors, which have to be further modified (peracetylated) to determine concentrations quantitatively. However, when the presence of sulfated Nod factors is to be detected qualitatively, the method can still be applied using an upper phase with 50% heptane, which has the advantage that the LCOs can be recovered.

The fact that approximately 92 to 77% of acetylated natural sulfated LCO is found in the upper phase, depending on the size of the chitin backbone, is due to incomplete partitioning into the upper phase. Despite the incomplete partitioning, Nod factors can be quantified using SDS as a standard, when both the partitioning percentage and the color yield of the complex is taken into account (Table 2).

For sulfated LCOs with other acyl chains, the partitioning efficiencies of the LCO-methylene blue complexes are unknown. Based on our results, we hypothesize that in the case of a higher hydrophobicity of an unknown sulfated LCO as compared with LCO-IV(C16:1, S), one can assume a partitioning efficiency of greater than 84%, yielding a maximal error of 16% in the determination. The presence of large amounts of contaminants can interfere with this assay, indicating that the quantification should be used on purified Nod factors rather than on crude preparations.

CONCLUSION

Accurate quantification of Nod factors is required when one is interested in Nod factor production by chemical or biological processes or when dose dependence response experiments are carried out either in vitro (e.g., for the calculation of the binding thermodynamic constants) or in vivo (for biological activity tests or crops treatments). The main advantages of the colorimetric methods over other analytical methods, such as HPLC analysis, are the use of inexpensive and readily available equipment, the ability to process and analyze multiple samples in parallel, and the quantification independent of a Nod factor standard. Therefore, these assays can be used routinely for quantifying Nod factor fractions during purification of natural or synthetic Nod factors. Furthermore, these approaches can be used as a starting point for developing quantification assays for other Nod factors.

The analysis of legume mutants in the early steps of the rhizobium-legume interaction (Wais et al. 2000; Walker et al. 2000), the identification of lectin-binding proteins (Etzler et al. 1999), and the purification of Nod factor-binding proteins (Bono et al. 1995; Niebel et al. 1997; Gressent et al. 1999)

Table 1. Quantification of sulfated lipo-chitooligosaccharides (LCOs) with fluorescent acyl chains

LCO	Recovery of sulfate ^a (%)	Recovery of LCO ^b (%)	LCO-MB ^c OD/ μ mol
LCO-IV(BODIPY FL-C ₁₆ , S)	89.5	95.9 $\pm$ 2.8	47.6
LCO-V(BODIPY FL-C ₁₆ , S)	91	95.2 $\pm$ 4.4	48.8

^a Recovery of sulfate in the upper phase using the SDS calibration curve and assuming identical color yield.

^b Recovery of Nod factor (average and standard deviation of three measurements) in the upper organic phase as measured by fluorescence.

^c Color yield of the LCO-methylene blue complex calculated by (recovery sulfate/recovery LCO) * 51 OD units per μ mol.

Table 2. Quantification of radiolabeled lipo-chitooligosaccharides (LCOs) with natural acyl chains

LCO	LCO Recovery [%] ^a	LCO-MB ^b OD/ μ mol
LCO-III(C16:1, ³⁵ S)	92.0 ^c	44.3
LCO-IV(C16:1, ³⁵ S)	83.7 $\pm$ 4.2	40.3
LCO-V(C16:1, ³⁵ S)	77.0 $\pm$ 5.3	37.1

^a The recovery of Nod factor (average and SD of three measurements) in the upper organic phase as measured by liquid scintillation counting.

^b The color yield of the LCO-methylene blue complex by correcting the average value (48.1) previously obtained for the recovery.

^c Only one measurement was performed due to the limited amount of material.

have yielded several candidates for the putative Nod factor receptor. Scatchard Nod factor-binding analysis and accurate discrimination of affinity constants for differently decorated Nod factors of these proteins clearly will be essential in order to correlate Nod factor-binding efficiency and bioactivity of the proteins. In this light, fast and accurate determination of Nod factor concentrations in stock solutions is indispensable.

MATERIALS AND METHODS

Materials.

(GlcNAc)₄ was obtained from Seikagaku (Tokyo, Japan), LPS (serotype 055:B5), stearic acid, GlcNAc, p-dimethylaminobenzaldehyde (DMAB), and snail intestinal juice (β -glucuronidase EC 3.2.1.31, catalog number G 7017) were from Sigma (Zwijndrecht, The Netherlands). Heptane (99%) was from Acros ('s-Hertogenbosch, The Netherlands), 4-(dimethylamino)-pyridine (DMAP), 1-butanol p.a., and methylene blue (for microscopy) from Merck (Darmstadt, Germany). SDS was either from Life Technologies (Breda, The Netherlands) or from Biorad (Hercules, CA, U.S.A.). GlcNAc, (GlcNAc)₄, phosphate, and borate stock solutions were stored at -20°C . A DMAB stock solution was prepared as reported (Reissig et al. 1955) and could be stored at 4°C for at least three months without deterioration. Before use, 0.5 ml of snail juice was mixed with 0.5 ml of 20 mM KCl and applied to a Biorad biogel P-6 column. Preequilibration and elution was done at 4°C with 10 mM KCl containing 1 mM EDTA. The first 1.5 ml after the void volume was collected and stored at 4°C . This fraction was used in all experiments.

Nod factors.

The structures of the LCOs used in this study are shown in Figure 1. NodRlo was purified according to Lopez-Lara and associates (1995) with a slight modification. Prepurification on the octadecyl extraction column was done by eluting with 40% (vol/vol) and 60% (vol/vol) acetonitril. Both fractions were further purified by RP-HPLC on a Pharmacia Superpac spherisorb ODS 2 column (5 μm , 4×250 mm). Samples were applied at 20% (vol/vol) acetonitril and eluted with 40% (vol/vol) acetonitril and 60% (vol/vol) H₂O. The elution flow rate was 1

ml per min, and the eluent was monitored at 206 nm. Nod factors radiolabeled with ³⁵S were prepared as described (Gressent et al. 1999). Synthesis of LCO-IV(BODIPY FL-C₁₆, S) and LCO-V(BODIPY FL-C₁₆, S) will be described elsewhere (P. De Medina, S. Bedel, P. Tisnes, and C. Picard, *unpublished data*).

Degradation of chitoooligosaccharides by glusulase.

To 1 to 20 μl of Nod factor or of (GlcNAc)₄ stock solution, 5 μl of 1 M phosphate buffer (pH, 7.1), 25 μl of snail juice, and H₂O to a final volume of 125 μl were added. This was incubated for 1 h at 37°C , unless indicated otherwise.

Identification of breakdown products.

Samples from the incubation mixture were taken and extracted with an equal amount of butanol at indicated time points. The butanol layer was isolated after centrifugation and evaporated, and the residue was dissolved in 20% (vol/vol) acetonitril and H₂O and analyzed by RP-HPLC on a Pharmacia Superpac spherisorb ODS 2 column (5 μm , 4×250 mm). Elution was done with a linear gradient of acetonitril in H₂O from 40 to 80% (vol/vol) in 30 min. Other conditions were as described above. To determine the release of GlcNAc, samples were taken at indicated time points and quickly frozen in liquid nitrogen. Time point 0 was obtained by omitting glusulase.

Reissig method for determination of GlcNAc.

The method of Reissig and associates (1955) was used to quantify the amount of GlcNAc. To 125 μl of the GlcNAc-containing solution was added 25 μl of 0.8 M borate (pH, 9.1). After incubation at 100°C for 3 min, the samples were put on ice. After cooling down, 750 μl of a DMAB solution was added (diluted 10 $\times$ from stock with glacial acetic acid just before use). Subsequently, the samples were incubated at 37°C for 20 min. The samples were cooled on ice, and absorbance at 585 nm was measured against water (care was taken to avoid erratic absorbance readings due to condensation on the cuvettes).

Kean partition assay.

A stock solution of methylene blue was prepared by dissolving 0.2 g of methylene blue in 100 ml of a 12.5 mM sulfuric acid solution. The lower phase consisted of 50 μl methylene blue stock, the proper amount of SDS or Nod factor, and water added to a total volume of 500 μl . Subsequently, 1 ml of organic upper phase was added, and the samples were mixed vigorously by vortexing for 10 s. After centrifugation, the concentration of methylene blue in the upper phase was quantified by measuring absorbance at 655 nm.

Partitioning of fluorescent Nod factors into upper phase.

To 0.25 ml of lower phase (discussed above), 0.5 ml of organic solvent, consisting of different percentages of heptane and butanol, was added. Subsequently, 10 μl of 10 μM fluorescent LCO was added and mixed thoroughly by vortexing. After phase separation by centrifugation (10 s 14,000 g), 100 μl of upper phase was diluted into 900 μl of ethanol containing SDS to remove any methylene blue that could possibly quench Nod factor fluorescence. The OD of the sample was always lower than 0.1. The fluorescence intensity of the solution was measured with a Fluorolog-3 (ISA Inc., Edison, NJ, U.S.A.) by excitation at 480 nm (slit width 4 nm) and 516 nm (slit width 4 nm) emission. The 100% value was obtained by adding an equal amount of fluorescent Nod factor directly to the ethanol solution.

Acetylation of LCOs.

In the case of Nod factor stock solutions in DMSO, DMSO was removed by adding 2 volumes of H₂O and 2 volumes of

Fig. 6. Influence of contaminants on sulfate quantification. Samples without (open bar) and with 10 nmol sodium dodecyl sulfate (closed bar) were taken, and 10 nmol NodRlo, 40 μg stearic acid, or 10 μg lipopolysaccharides were added as a contaminant before partitioning. The absorbance of the upper phase was measured at 655 nm (average and standard deviation of three measurements).

(water-saturated) butanol. After separation and isolation of the butanol layer, the extraction was repeated three times with the same volume of butanol. Subsequently, the collected butanol layers were evaporated.

For efficient acetylation, at least 1 mole equivalent of catalyst (DMAP) is required relative to the amount of free hydroxyl groups (Gupta et al. 1977), which corresponds to 25 µg of DMAP for 20 nmol of LCO. To the dried LCO sample, 10 to 20 µl of freshly prepared solution of 5 mg of DMAP per ml acetic anhydride was added. The yellowish solution was thoroughly mixed to dissolve the LCOs. The mixture was incubated overnight at room temperature. The reaction mixture can either be used directly (the presence of acetic anhydride and DMAP during quantification increases the background absorbance slightly but not the partitioning or the efficiency of ion-pair formation) or can be extracted to remove any DMAP and acetic anhydride. First, 50 µl of methanol was added, and the solution was incubated for at least 15 minutes, after which 100 µl of CHCl₃ and 50 µl of 0.6 M HCl were added. After separation of the layers, the lower layer was washed 2 times with a solution of 100 µl MeOH and 0.6 M HCl (1:1) and then evaporated. Alternatively, an acetic anhydride and pyridine mixture can be used for acetylation, which can then be easily removed by evaporation as described (Tadano-Aritomi and Ishizuka 1983). TLC analysis of the reaction mixture was done with silicagel 60 TLC plates using chloroform and methanol (4:1 vol/vol) as the mobile phase.

Partitioning of radiolabeled Nod factors into upper phase.

First, the radiolabeled Nod factors were acetylated and partitioned as described above. After phase separation, 80% of each phase was transferred to counting vials and the amount of LCO in each layer was determined by liquid scintillation counting.

ACKNOWLEDGMENTS

We are grateful to G. Oudgenoeg (Laboratory for Biochemistry, Wageningen University, The Netherlands) for the liquid chromatography-mass spectrometry measurements. We thank B. M. Gadella (Department of Veterinary Biochemistry, Utrecht University, The Netherlands) for suggesting the Kean assay he uses for quantification of sulfo-galactoceramides for quantifying sulfated LCOs. This work was supported by the Netherlands Organization for Scientific Research-Council of Earth- and Life Sciences (NWO-ALW) (J. G.), by a NWO-Van Gogh French-Netherlands collaborative research grant (J. J. B. and T. W. J. G.), and by the Royal Netherlands Academy of Arts and Sciences (KNAW) (T. W. J. G.).

LITERATURE CITED

Boller, T., and Mauch, F. 1988. Colorimetric assay for chitinase. *Methods Enzymol.* 161:430-435.

Bono, J. J., Riond, J., Nicolaou, K. C., Bockovich, N. J., Estevez, V. A., Cullimore, J. V., and Ranjeva, R. 1995. Characterization of a binding site for chemically synthesized lipo- oligosaccharidic NodRm factors in particulate fractions prepared from roots. *Plant J.* 7:253-260.

Cabib, E., and Bowers, B. 1971. Chitin and yeast budding. *J. Biol. Chem.* 246:152-159.

Domard, A., and Vasseur, V. 1991. Non-specificity of a colorimetric method for the estimation of N-acetyl-D-glucosamine. *Int. J. Biol. Macromol.* 13:366-368.

Etzler, M. E., Kalsi, G., Ewing, N. N., Roberts, N. J., Day, R. B., and Murphy, J. B. 1999. A nod factor binding lectin with apyrase activity from legume roots. *Proc. Natl. Acad. Sci. U.S.A.* 96:5856-5861.

Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1995. Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. *Plant J.* 7:939-947.

Gadella, T. W. J., Jr., Vereb, G., Hadri, A.-E., Röhrig, H., Schmidt, J., John, M., Schell, J., and Bisseling, T. 1997. Microspectroscopic imaging of nodulation factor-binding sites on living *Vicia sativa* roots using a novel bioactive fluorescent nodulation factor. *Biophys. J.* 72:1986-1996.

Gressent, F., Drouillard, S., Mantegazza, N., Samain, E., Geremia, R. A., Canut, H., Niebel, A., Driguez, H., Ranjeva, R., Cullimore, J., and Bono, J.-J. 1999. Ligand specificity of a high-affinity binding site for lipo- chitooligosaccharidic Nod factors in *Medicago* cell suspension cultures. *Proc. Natl. Acad. Sci. U.S.A.* 96:4704-4709.

Gupta, C. M., Radhakrishnan, R., and Khorana, H. G. 1977. Glycerophospholipid synthesis: Improved general method and new analogs containing photoactivable groups. *Proc. Natl. Acad. Sci. U.S.A.* 74:4315-4319.

Haugland, R. P. 1996. Handbook of fluorescent probes and research chemicals. Molecular Probes Inc. Eugene, OR, U.S.A.

Heidstra, R., Geurts, R., Franssen, H., Spaik, H., van Kammen, A., and Bisseling, T. 1994. Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* 105:787-797.

Kean, E. L. 1968. Rapid, sensitive spectrophotometric method for quantitative determination of sulfatides. *J. Lipid Res.* 9:319-327.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature.* 344:781-784.

Long, S. R. 1996. *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell.* 8:1885-1898.

Lopez-Lara, I. M., van den Berg, J. D., Thomas-Oates, J. E., Glushka, J., Lugtenberg, B. J., and Spaik, H. P. 1995. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. *Mol. Microbiol.* 15:627-638.

Niebel, A., Bono, J.-J., Ranjeva, R., and Cullimore, J. V. 1997. Identification of a high affinity binding site for lipooligosaccharidic NodRm factors in the microsomal fraction of *Medicago* cell suspension cultures. *Mol. Plant-Microb. Interact.* 10:132-134.

Radin, N. S. 1984. Improved version of the Kean partition assay for cerebroside sulfate. *J. Lipid Res.* 25:651-652.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. 1955. A modified colorimetric method for the estimation of N-acetyl amino sugars. *J. Biol. Chem.* 217:959-966.

Roden, L., Yu, H., Jin, J., Ekborg, G., Estock, A., Krishna, N. R., and Livant, P. 1997. Analysis of the Morgan-Elson chromogens by high-performance liquid chromatography. *Anal. Biochem.* 254:240-248.

Spaik, H. P., Sheely, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity in *Rhizobium*. *Nature.* 354:125-130.

Staehelin, C., Granado, J., Muller, J., Wiemken, A., Mellor, R. B., Felix, G., Regenass, M., Broughton, W. J., and Boller, T. 1994a. Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. *Proc. Natl. Acad. Sci. U.S.A.* 91:2196-2200.

Staehelin, C., Schultze, M., Kondorosi, E., Mellor, R. B., Boller, T., and Kondorosi, A. 1994b. Structural modifications in *Sinorhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. *Plant J.* 5:319-330.

Tadano-Aritomi, K., and Ishizuka, I. 1983. Determination of peracetylated sulfoglycolipids using the azure A method. *J. Lipid Res.* 24:1368-1375.

Wais, R. J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R. V., Cook, D., Gough, C., Denarie, J., and Long, S. R. 2000. Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 97:13407-13412.

Walker, S. A., Viprey, V., and Downie, J. A. 2000. Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by nod factors and chitin oligomers. *Proc. Natl. Acad. Sci. U.S.A.* 97:13413-13418.