

Methodology article

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## Radiolabeling of lipo-chitooligosaccharides using the NodH sulfotransferase: a two-step enzymatic procedure

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### Abstract

**Background:** The NodH sulfotransferase from *Sinorhizobium meliloti* has been used to radiolabel lipochitooligosaccharidic (LCO) Nod factor signals with <sup>35</sup>S from inorganic sulfate in a two-step enzymatic procedure. The first step involved the production of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a sulphate donor, using enzymes contained in a yeast extract, and the second step used the NodH enzyme. However with this established procedure, only a low incorporation of the initial inorganic sulfate into the Nod factors was obtained (about 7% after purification of the labeled compounds). The aim of this work was to optimize the radiolabelling of Nod factors with <sup>35</sup>S.

**Results:** The limiting step has been shown to be the sulfation of ATP and its subsequent conversion into PAPS (first step), the sulfate donor for the NodH sulfotransferase activity (second step). By the addition of GTP to the reaction mixture and by manipulating the [ATP]/[Mg<sup>2+</sup>] ratio the yield of PAPS has been increased from 13% to 80%. Using the radiolabeled PAPS we have shown that the efficiency of sulfate transfer to LCOs, by the recombinant *S. meliloti* NodH sulfotransferase is strongly influenced by the length of the oligosaccharide chain. Variations in the substitutions on the non-reducing sugar, including the structure of the fatty acyl chain, had little effect and Nod factors from the heterologous bacterium *Rhizobium tropici* could be sulfated by NodH from *S. meliloti*.

**Conclusions:** By characterizing the two steps we have optimized the procedure to radiolabel biologically-important, lipo-chitooligosaccharide (LCO) Nod factors to a specific radioactivity of about 800 Ci.mmol<sup>-1</sup> with an incorporation of 60% of the initial inorganic sulfate. The two-step sulfation procedure may be used to radiolabel a variety of related LCO molecules.

### Background

Sulfated carbohydrates play important signaling and adhesion roles in a large variety of organisms including

animals, bacteria and plants [1-5]. Often the structural requirements for active molecules are very stringent thus preventing their chemical derivatization to produce

probes with which to analyze their biological functioning. Radiolabeling with  $^{35}\text{S}$ , using an appropriate carbohydrate sulfotransferase, thus remains one of the few ways in which high specific activity probes may be obtained.

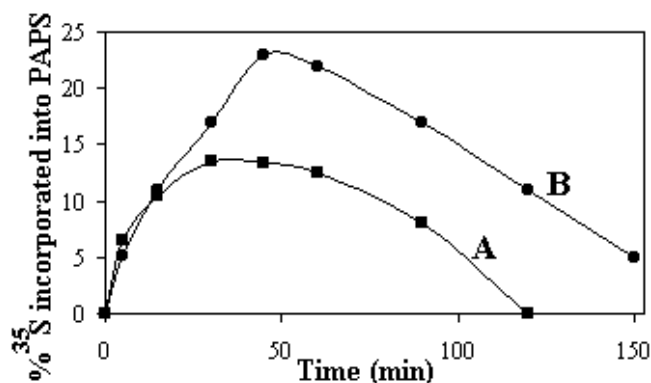
In the agronomically and environmentally important legume-*Rhizobium* symbioses, specificity between appropriate plant and bacterial partners is determined on the bacterial side by the production of lipo-chitooligosaccharide (LCO) Nod factors. For *Sinorhizobium meliloti*, sulfation of its Nod factors is essential for interacting with its host plants: *nodH* *S. meliloti* mutants, which produce non-sulfated factors, are unable to elicit reactions on *Medicago* [1]. The sulfation of Nod factors by *S. meliloti* is dependent on the *nodPQ* and *nodH* genes. The *nodPQ* genes encode ATP sulfurylase [6] which together with an APS kinase produces PAPS which is the sulfate donor for the NodH sulfotransferase [7].

The NodH sulfotransferase has been shown to radiolabel non-sulfated Nod factors and chitin fragments with  $^{35}\text{S}$ , providing  $^{35}\text{S}$ -PAPS is supplied as the sulfate donor [7,8]. Moreover this enzyme has been shown to have a broad substrate specificity and thus may be used to sulfate other important carbohydrates [9]. However the efficiency of the radiolabeling has been very variable hence preventing a wider adoption of this method to produce specific probes. We have shown that yeast (*Saccharomyces cerevisiae*) extracts, which contain sufficient amounts of ATP sulfurylase and APS kinase, can be used to catalyze the formation of ( $^{35}\text{S}$ -PAPS) from inorganic [ $^{35}\text{S}$ ]-sulfate to a high specific radioactivity. The recombinant NodH sulfotransferase of *S. meliloti*, produced by expression in *E. coli*, may then be used in a second step to transfer the labeled sulfate of PAPS onto non-sulfated Nod factors. However, with this established procedure, only a low incorporation of the initial inorganic sulfate into the Nod factors was obtained (about 7% after purification of the labeled compounds). By characterizing the enzymatic activities of the two steps, PAPS synthesis was found to be the limiting step for a high yield incorporation. By optimizing the conditions, about 60% of the supplied inorganic sulfate was recovered in  $^{35}\text{S}$ -Nod factors, with a high specific radioactivity ( $800\text{ Ci}\cdot\text{mmol}^{-1}$ ) suitable for binding studies. Moreover, we have shown that although the efficiency of the NodH reaction yield depends on the structure of the LCO, this procedure may be useful for radiolabeling Nod factors from other *rhizobia* and indeed other carbohydrates.

## Results

### The formation of PAPS is transient

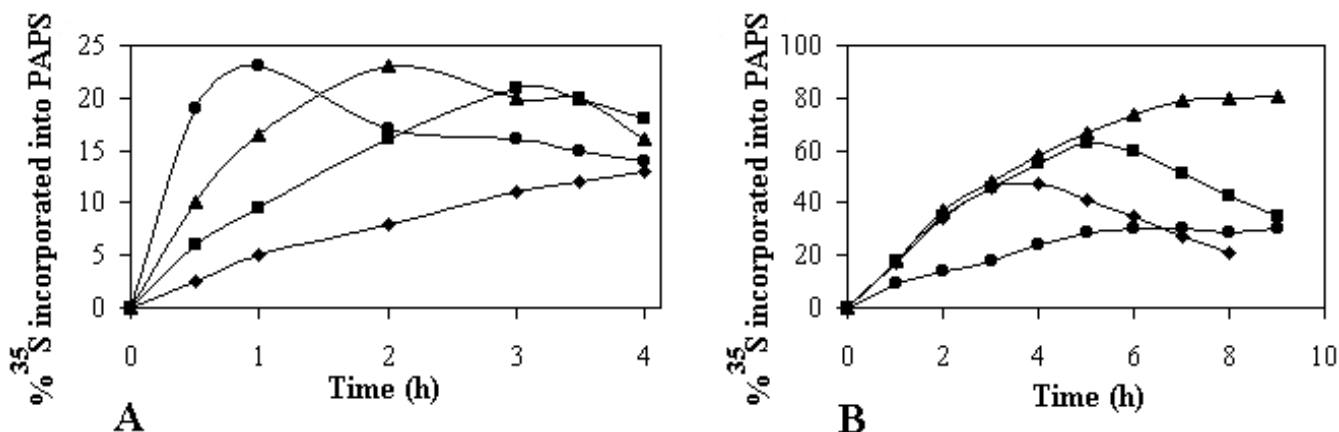
A procedure that allows the formation of [ $^{35}\text{S}$ ]PAPS using  $\text{Na}_2\text{}^{35}\text{SO}_4$  and Mg-ATP as substrates and yeast extract as a source of enzymes has been previously described [8].



**Figure 1**

**Time course of PAPS formation.** The reaction mixture containing  $26\ \mu\text{M Na}_2\text{SO}_4$  ( $10\ \mu\text{Ci}$  of labeled sulfate) was incubated at  $37^\circ\text{C}$  in  $20\ \text{mM Tris-HCl}$  buffer pH 8.5,  $40\ \text{mM MgCl}_2$ ,  $4\ \text{mM ATP}$ . The reaction was started by addition of  $30\ \mu\text{l}$  of yeast extract, either (A) without GTP (■) or (B) in the presence of  $10\ \text{mM GTP}$  (●).

Under the set experimental conditions ( $4\ \text{mM ATP}$ ,  $40\ \text{mM MgCl}_2$ ,  $30\ \mu\text{l}$  yeast extract in  $20\ \text{mM Tris}$  buffer pH 8.5) we have found that PAPS synthesis is transient with about 13% of the initial sulfate being incorporated by 30 min but then with it being degraded by 120 min (Fig. 1A). If the yeast extract was concentrated two-fold by ultrafiltration, the initial velocity of the reaction increased, and the same maximum was reached within 15 min, but the product was degraded twice as fast (data not show). These data indicate that the PAPS loss was not due to chemical or physical degradation, but that it was enzymatically degraded. Additions to the reaction mixture of 50 I.U. of inorganic pyrophosphatase or 100 I.U. of pyruvate kinase and phosphoenolpyruvate (which catalyses the conversion of ADP into ATP), in order to eliminate inorganic pyrophosphate (PPi) or ADP which are potent inhibitors of ATP sulfurylase and APS kinase respectively [9], were unable to improve the final yield. Moreover, preincubation of the yeast extract at  $37^\circ\text{C}$  during 30 min before addition of ATP,  $\text{Mg}^{2+}$  and labeled inorganic sulfate did not change the time course of the reaction, showing that the PAPS synthesis enzymes were not heat inactivated or inhibited by chemicals contaminating the yeast extract. In contrast, if the preincubation was done in the presence of ATP and  $\text{Mg}^{2+}$ , the subsequent addition of sulfate did not lead to the formation of PAPS. This observation suggests that the yield of PAPS might be improved by altering the composition of the reaction mixture.

**Figure 2**

**Influence of MgCl<sub>2</sub> and ATP concentrations on PAPS synthesis.** 26 μM Na<sub>2</sub>SO<sub>4</sub> (10 μCi of labeled sulfate) was incubated at 37°C in 20 mM Tris-HCl buffer pH 8.5, 5 mM GTP and supplemented with (A): 2 mM ATP, and variable concentrations of MgCl<sub>2</sub>: (◆) 1 mM; (■) 2 mM; (▲) 4 mM; (●) 8 mM or (B): 4 mM MgCl<sub>2</sub> and variable concentrations of ATP: (◆) 4 mM; (■) 8 mM; (▲) 16 mM; (●) 32 mM. The reactions were started by addition of 30 μl of yeast extract.

#### **Addition of GTP to the reaction mixture increases PAPS formation**

Figure 1B shows the incorporation of labeled sulfate into PAPS when 10 mM GTP was added to the reaction mixture. The reaction proceeded with the same initial velocity, but over a more extended period allowing the formation of 20% of labeled PAPS after a 45 min incubation. However, GTP did not inhibit the degradation reaction and the PAPS had virtually disappeared within 150 min. Similar results were obtained over a range of GTP concentrations (from 1 to 10 mM). Consequently, the standard reaction mixture was systematically supplemented with 5 mM GTP.

#### **The concentration of Mg<sup>2+</sup> modulates the velocity of the reaction**

The effect of various concentrations of MgCl<sub>2</sub> (in the presence of 2 mM ATP) on PAPS synthesis is represented in Figure 2A. The results show that increasing the MgCl<sub>2</sub> concentration stimulates the initial velocity of the reaction: <sup>35</sup>SO<sub>4</sub><sup>2-</sup> was incorporated at 0.08% per min into PAPS in the presence of 1 mM MgCl<sub>2</sub>, and the incorporation increased as a function of MgCl<sub>2</sub> concentrations to reach 0.63% per min in the presence of 8 mM MgCl<sub>2</sub>. At these concentrations the formation of PAPS was sustained for several hours, showing that decreasing the Mg<sup>2+</sup> concentration from 40 mM (under the initial conditions) to 1–8 mM strongly modulates the enzymatic activities, favoring the synthesis of PAPS. However, the maximal incorporation of <sup>35</sup>S-sulfate into PAPS was practically constant (20–25%), showing that the magnesium concentration is crit-

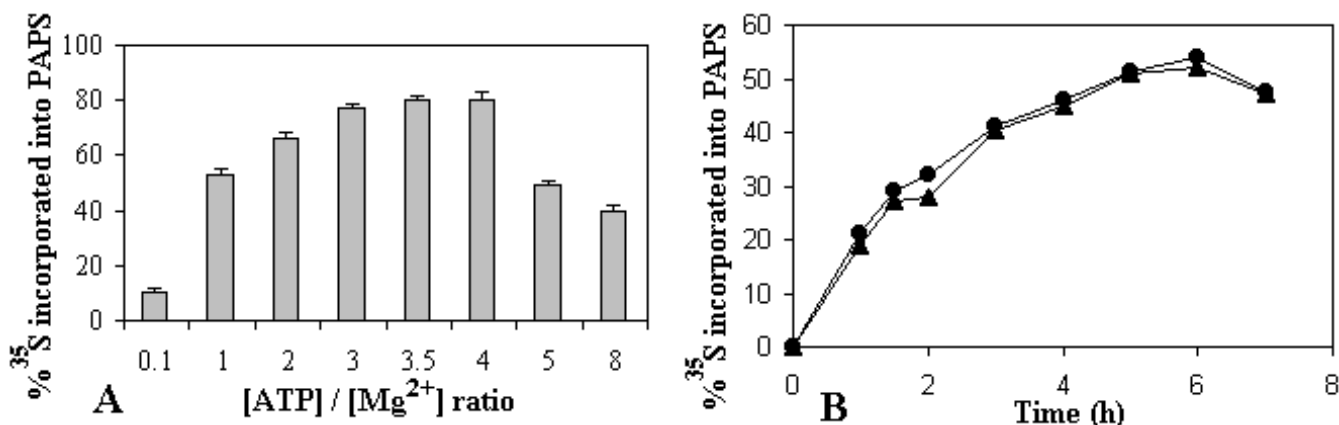
ical for the velocity of the reaction, but not for the amount of PAPS produced.

#### **The concentration of ATP affects the final yield of the reaction**

The influence of the ATP concentration, at a fixed Mg<sup>2+</sup> concentration (4 mM) was studied. The results (Fig. 2B) show that increasing the ATP concentration does not change the initial velocity of the reaction, (with the exception of the highest ATP concentration (32 mM)), but strongly affects the final yield of PAPS. Maximum conversion (80%) was obtained in the presence of 16 mM ATP, whereas lower (4 or 8 mM) or higher (32 mM) concentrations led to a lower PAPS formation.

#### **PAPS formation depends on the [ATP]/[Mg<sup>2+</sup>] ratio**

Since the concentrations of ATP and magnesium play a key role in PAPS synthesis by influencing the yield and the velocity of the reaction respectively, the ratio of [ATP]/[Mg<sup>2+</sup>] could be critical for the reaction process. Indeed, the modification of the [ATP]/[Mg<sup>2+</sup>] ratio has a marked effect on PAPS formation and enzymatic behavior (Fig. 3A). Firstly, increasing the [ATP]/[Mg<sup>2+</sup>] ratio from 0.1 (that corresponds to the initial conditions [8]) to 4, increased the yield of PAPS from 13% to 80%. For higher ratio values, PAPS formation was lower. Secondly, only when ratio values were equal or higher than 3 was the PAPS maintained during at least 4 hours after maximal PAPS formation. With molar ratios less than 3, the product was degraded after reaching a maximal value. Both effects were seen according to the ratio value, regardless of the ATP or Mg<sup>2+</sup> concentrations. This effect of the [ATP]/

**Figure 3**

**Effect of the relative concentrations of ATP and MgCl<sub>2</sub> on PAPS synthesis.** Na<sub>2</sub>SO<sub>4</sub> (26 μM containing 10 μCi of labeled sulfate) was incubated at 37°C in 100 mM Tris-HCl buffer pH 8.5, 5 mM GTP (A) at different ratios of [ATP]/[Mg<sup>2+</sup>]. Each bar corresponds to the mean of 2 to 4 experiments, performed at different ATP and Mg<sup>2+</sup> concentrations while keeping the same ratio. (B) at a ratio value of 1, at either 5 mM MgCl<sub>2</sub>, 5 mM ATP (▲) or 20 mM MgCl<sub>2</sub>, 20 mM ATP (●).

[Mg<sup>2+</sup>] ratio is clearly seen in Figure 3B: simultaneously increasing the Mg<sup>2+</sup> and ATP concentrations from 5 to 20 mM (while keeping the ratio at 1) had no effect on either the velocity or the yield of PAPS. Therefore, the [ATP]/[Mg<sup>2+</sup>] ratio rather than their concentrations has the major effect on PAPS production.

#### Labeling of Nod factors

Nod factors are oligomers of 3 to 5 N-acetylglucosamine residues, N-acylated on the non reducing end. The major Nod factor produced by *S. meliloti* (NodSm factors) is a tetrameric chitin oligomer acylated by a C16:2 chain, and with a sulfate group on C6 of the reducing sugar, and an O-acetyl group on C6 of the non-reducing end sugar (NodSm-IV(Ac, S, C16:2)). Non-sulfated Nod factors can be produced by a *nodH* mutant.

In the following experiments the optimized conditions for <sup>35</sup>S-PAPS synthesis was used for radiolabeling this non-sulfated Nod factor. As the aim was to incorporate the maximum amount of the radioactive sulfate into a product with a very high specific radioactivity, the Nod factor was added in excess. Using standard conditions of 2 mCi of labeled inorganic sulfate, about 1 nmol of <sup>35</sup>S-PAPS could now be obtained (8-fold higher than with the initial conditions), and it was found that increasing the concentration of non sulfated Nod factor substrate (to 50 nmol) was optimal for Nod factor labeling (about 50-fold excess). Under these conditions, about 65% of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> was incorporated into NodSm-IV(Ac, <sup>35</sup>S, C16:2), indicating that the yield of sulfate transfer to Nod factors was approximately 80%. After extraction by butanol and purification

by reverse phase HPLC, the overall yield of the radiolabeled Nod factor was 59% of the initial inorganic sulfate. This high amount allowed a direct detection by UV spectrophotometry during elution from the HPLC column. An enzymatically synthesized <sup>35</sup>S-Nod factor displayed an UV spectrum (between 200 and 365 nm) and a retention time identical to that of a pure, authentic chemically-synthesized LCO. The specific radioactivity, determined by measuring both the quantity of Nod factor and its corresponding radioactivity, was 800 Ci.mmol<sup>-1</sup>. Such a figure is consistent with previously obtained data [8], showing that the optimized conditions do not dilute the inorganic (SO<sub>4</sub><sup>2-</sup>) or the organic (PAPS) sulfate sources.

#### NodH specificity depends on the saccharidic moiety of the LCOs

Different rhizobial species produce different Nod factors with variations in both the chemical substitutions on the sugars and the structure of the acyl chain. These variations are important for host specificity [12]. Moreover, studies on the selectivity of a Nod factor binding site, associated to *Medicago varia* cell suspension cultures, revealed the importance of some structural elements for a high affinity binding [11]. The possibility to label Nod factors from different rhizobial species or LCOs differing by their affinity for the Nod factor binding protein may help to a better knowledge of the perception mechanisms of these compounds. We thus attempted to label a variety of LCOs harboring various decorations, or differing by the length of the oligochitin backbone or the nature of the fatty acid.

**Table 1: NodH specificity towards differently substituted LCOs.**

Substrates	Incorporation of <sup>35</sup> S into LCOs <sup>a</sup>
NodSm-IV(Ac, C16:2Δ2,9)	80 +/- 3.4
NodSm-IV(C16:2Δ2,9)	79 +/- 2.3
LCO-IV(C18:1Δ9)	77 +/- 2.5
LCO-IV(C16:1Δ9)	82 +/- 2.7
LCO-V(C16:1Δ9)	38 +/- 1.2
LCO-III(C16:1Δ9)	19 +/- 0.8
LCO-II(C16:1Δ9)	6 +/- 0.2
LCO-V(C16:1Δ9) reduced <sup>b</sup>	0
NodRt-V(Me, C16:0)	38 +/- 4.3
NodRt-V(Me, C18:1Δ11)	40 +/- 2.7

<sup>a</sup>Values are expressed as percentage of total offered <sup>35</sup>S-PAPS and represent the means +/- S.D. of 3 to 6 experiments. <sup>b</sup>The anomeric carbon was reduced. The nomenclature used for LCOs and Nod factors correspond to that established in [1].

Table 1 reports the percentage of labeled sulfate transferred from PAPS to the various LCOs (added in excess). The results show that NodH recognizes NodSm-IV(Ac, C16:2), NodSm-IV(C16:2), (NodSm-IV(C16:1) and NodSm-IV(C18:1) with the same efficiency, indicating that NodH does not discriminate the fatty acyl chain according to its length and unsaturation degree, or the O-acetyl group on the non reducing end. In contrast, trimeric LCOs were sulfated to a much lower extent than their tetrameric counterparts (respectively 19 and 82%), and the dimer was practically not sulfated by NodH (6%). Pentameric Nod factors were sulfated to 38% efficiency whereas LCO-V(C16:1) in which the anomeric carbon was chemically reduced was not sulfated at all. Interestingly, two Nod factors from a different bacterium *R. tropici* (NodRt-V(Me, C16:0) and NodRt-V(Me, C18:1)) incorporated about 40% of labeled PAPS, demonstrating that NodH does not discriminate the N-methyl group borne by NodRt factors on the C2 of the non reducing end.

These results demonstrate that NodH is highly specific for the sugar backbone length and needs an intact structure of the reducing sugar for transferring the sulfate group from PAPS to the Nod factor.

## Discussion

In this paper, we have shown that improvements in the synthesis of radiolabeled PAPS formation can lead to dramatic improvements in the efficiency of radiolabeling with the NodH sulfotransferase, such that about 60% of the initial inorganic <sup>35</sup>S-sulfate may be incorporated into the LCO end-product with a specific radioactivity of 800 Ci.mmol<sup>-1</sup>. This compares favorably with the yield from the initial conditions where only about 7% of the inorganic sulfate was incorporated [8].

PAPS formation was the limiting step of the sulfation reaction, and changes to the initial procedure enhanced the yield of PAPS formation from 13 to 80%. Firstly, addition of GTP stimulates the reaction, a quite unexpected result because eukaryotic ATP sulfurylases (including yeast) do not exhibit the canonical GTP binding site, exhibited by the prokaryotic GTP-dependent enzymes such as NodQ from *S. meliloti* [13,14]. Moreover, it has been demonstrated that GTP has no effect on the activity of the yeast ATP sulfurylase *in vitro* [15]. Therefore, GTP probably acts in an indirect manner and its mechanism of action remains to be determined.

Secondly, the ratio of [ATP]/[Mg<sup>2+</sup>] was found to be crucial in controlling the balance of PAPS synthesis/degradation. The ATP sulfurylase reaction is known to display an unfavorable equilibrium constant in the physiological direction ( $K = 10^{-8}$ ) [16], especially at non saturating SO<sub>4</sub><sup>2-</sup> concentrations. The yeast enzyme displays a very high affinity for PPi which is a substrate in the reverse direction [15], and studies on ATP sulfurylase from *Anabaena cylindrica* [17] and from rat chondrosarcoma [16] have shown that PPi inhibition is Mg<sup>2+</sup> dependent. Thus it could be hypothesized that the [ATP]/[Mg<sup>2+</sup>] ratio may be crucial for making MgATP available for the forward reaction by limiting the formation of MgPPi, the substrate of the backward reaction. However, attempts to eliminate PPi by addition of inorganic pyrophosphatase were unsuccessful, a result also obtained on rat ATP sulfurylase [16], and inconsistent with this hypothesis.

The sulfotransferase activity of the *S. meliloti* NodH protein has been previously demonstrated *in vitro* by different groups [7,8,18]. However, NodH specificity towards different LCO substrates is a matter of debate. One group has demonstrated on chitin oligomers varying from 3 to 6 N-acetylglucosamine residues, that the sugar backbone length does not influence the sulfation process, but the dimeric compound was labeled to a lesser extent [7]. Our results are consistent with the observation of Schultze *et al* [18], that NodH showed a specificity for tetrameric LCOs (the major type of LCOs produced by *S. meliloti*), and did not discriminate the presence of the O-acetyl group on the non reducing end. Moreover, our results indicate that *S. meliloti* NodH has no selectivity either for the length or structure of the fatty acyl chain, nor for a N-methyl group on C2 of the non reducing end sugar. However, a LCO resulting from the reduction of the free anomeric carbon of the reducing sugar was not sulfated. Our data suggest that NodH requires at least three sugars, and that the non-reducing end decorations are not involved in the recognition process. However, the enzyme has a higher affinity for LCOs than for the corresponding chitooligosaccharides [7,18]. Moreover, it has been demonstrated that NodH from *S. meliloti* was active on LCOs bearing a

methyl-fucose residue on C6 of the reducing sugar [19] and it has been shown that this enzyme will sulfate other carbohydrates [9]. Thus, other biologically-important carbohydrates, in addition to Nod factors, may be radiolabeled using this enzyme.

## Conclusions

This paper describes the optimization of a previously published method for radiolabelling LCOs using  $^{35}\text{S}$ . These probes should represent valuable tools for the biochemical characterization of the proteins encoded by the recently cloned genes involved in Nod factor perception [20-22].

Moreover, the simple and inexpensive way to obtain PAPS with both a high yield and a high specific radioactivity may be useful for radiolabeling, with other PAPS-dependent carbohydrate sulfotransferases, biologically important compounds involved in extracellular signaling and adhesion [5].

## Methods

### Biochemicals

All the biochemicals were of analytical grade. Inorganic pyrophosphatase (E.C. 3.6.1.1), pyruvate kinase (E.C. 2.7.1.40), PhosphoEnolPyruvate, GTP, ATP and  $\text{MgCl}_2$  were purchased from Sigma (St. Louis, MO, USA). Radiolabeled carrier-free  $\text{Na}_2^{35}\text{SO}_4$  (1500 Ci.mmol $^{-1}$ ) was from Dupont De Nemours (Wilmington, DE, USA).

Biologically produced non sulfated Nod factors were purified from *Rhizobium* culture supernatants as described [10]. Non-sulfated LCOs were chemically synthesized according to [11], and were kindly provided by Dr. Hugues Driguez (CERMAV, Grenoble, France).

### Enzymatic preparations

A yeast extract containing APS kinase and ATP sulfurylase activities was prepared as described [8]. NodH sulfotransferase activity was extracted from *E. coli* cells expressing the *S. meliloti nodH* gene following the previously published procedure [8], except that the last dialysis step was replaced by molecular filtration through a PD-10 column (Pharmacia). One ml of NodH extract was loaded on the column equilibrated in buffer H (10 mM potassium phosphate, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol (w/v), 1 mM phenylmethanesulphonyl fluoride) and the proteins were eluted in  $5 \times 1$  ml of the same buffer. Fractions 3 to 5, containing macromolecules, were pooled, and the resulting NodH extract was stored at  $-80^\circ\text{C}$  until use. Alternatively the commercially available recombinant protein from Calbiochem has been used.

### Optimization of PAPS synthesis

The reaction mixture (51  $\mu\text{l}$ ) contained  $\text{Na}_2\text{SO}_4$  (26  $\mu\text{M}$ , containing 10  $\mu\text{Ci}$   $^{35}\text{S}$ -labeled sulfate), and various concentrations of GTP,  $\text{MgCl}_2$  and ATP (the ATP was buffered with Tris which was found not to influence the velocity or yield of the reaction). The reaction was started by addition of 30  $\mu\text{l}$  yeast extract (about 5 mg of proteins), and incubated at  $37^\circ\text{C}$ .  $^{35}\text{S}$ -PAPS synthesis was monitored by spotting 1  $\mu\text{l}$  of the reaction mixture onto a PEI-cellulose F t.l.c. plastic plate (Merck) and developing with 1 M NaCl as solvent. Radioactivity was detected on a PhosphoImagerSI (Molecular Dynamics), and results were quantified by using ImageQuantSI (Molecular Dynamics) software.

### Synthesis and purification of [ $^{35}\text{S}$ ]sulfated Nod factors

In the optimized procedure, PAPS synthesis was performed overnight at  $37^\circ\text{C}$ , in 100 mM Tris-HCl buffer pH 8.5 in the presence of 2 mCi  $^{35}\text{S}$  carrier-free sodium sulfate (1500 Ci.mmol $^{-1}$ ); 28 mM ATP; 8 mM  $\text{MgCl}_2$ ; 5 mM GTP and 30  $\mu\text{l}$  of yeast extract in a total volume of 51  $\mu\text{l}$ . The reaction mixture was then boiled for 2 min and centrifuged. The supernatant was transferred to a tube containing approximately 0.5 mM of non sulfated Nod factors (50  $\mu\text{g}$  resuspended in 5  $\mu\text{l}$  DMSO). 30  $\mu\text{l}$  of NodH extract (0.2 mg of protein) was added, and the reaction mixture was incubated for 90 minutes at  $30^\circ\text{C}$ . At this stage, the formation of sulfated products was monitored by spotting 0.1  $\mu\text{l}$  of the reaction mixture onto a silica 60 t.l.c. plates (Merck), and developing with chloroform/methanol/14% ammonium hydroxide (45:45:15 by vol). Nod factors were extracted twice from the reaction mixture by 150  $\mu\text{l}$  butan-1-ol and purified as previously described [11].

### Protein determination

Protein was measured by the Bicinchoninic acid procedure developed by Pierce (Rockford, USA) with bovine serum albumin as the standard.

### List of abbreviations

APS: Adenosine 5'-phosphosulfate. LCO: Lipo-ChitoOligosaccharide. PAPS: 3'-phosphoadenosine 5'-phosphosulfate. PPI: inorganic pyrophosphate.

### Authors' contributions

FG carried out the biochemical studies and drafted the manuscript. JJB conceived the study (aided by JVC and RR), supervised the work and coordinated interactions with the laboratories supplying Nod factors and LCOs. All authors read and approved the final manuscript.

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