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Production and chitinase-binding ability of lipo-chitopentaose nodulation factor

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Abstract
The penta-*N*-acetyl-chitopentaose **2** has been prepared by using recombinant *E. coli* strains harboring the *nodC* gene (encoding chitooligosaccharide synthase) from *Azorhizobium caulinodans*. Then, the deacetylase NodB removed the *N*-acetyl moiety from the nonreducing terminus of **2** to give tetra-*N*-acetyl-chitopentaose **3**. *N*-Acylation of **3** with stearyl chloride was performed in DMF containing water and provided the corresponding lipo-chitopentaose nodulation factor **4**. A binding chitinase assay indicated that **4** was much more stable than **3**.

Keywords: *Lipo-chitopentaose nodulation factor, production, chemo-enzymatic method, binding chitinase assay*

Introduction

Rhizobia are symbiotic soil bacteria that fix nitrogen in legume root nodules, through a reciprocal exchange of signal molecules. Roots of host plants secrete specific flavonoid type molecules that induce the transcription of bacterial *nod* genes that are involved in the synthesis of extracellular lipochitooligosaccharide nodulation factors (NFs). NFs signal back to the plant and set in motion the program of nodule organogenesis leading to the formation of a novel organ (the root nodule) in which the bacteria fix atmospheric nitrogen [1]. These lipochitooligosaccharide NFs represent a large family of lipochitooligosaccharides consisting of a chitin fragment (three-five *N*-acetyl-D-glucosamine units) with an *N*-acyl chain attached to the nonreducing unit and a variety of additional substituents [2]. The variations are characteristic of each bacterial strain and define the high degree of specificity in the legume-rhizobia interaction. These lipochitooligosaccharide NFs were, therefore, ideal targets for the demonstration of progress achieved in oligosaccharide synthesis by organic chemists during the last decade [3]. However, these synthetic methods are complicated and lengthy. In this paper, we describe the production of lipo-chitopentaose nodulation factor **4** (Scheme 1) by chemo-enzymatic method, and assay the inactivating ability to chitinase of **4**.

Materials and methods

General

The size exclusion chromatography was performed on Biogel P4.Optical rotation was determined at 25°C with a Perkin-Elmer Model 241-Mc automatic polarimeter. ^{13}C and ^{1}H NMR spectra were recorded using a Bruker DPX-300 spectrometer at 75 and 300 MHz, respectively. Mass spectrum was recorded with a VG PLATFORM mass spectrometer using the ESI mode. The chitinase from *Serratia marcescens* and trace mineral solution were purchased from Sigma company.

Bacterial strains, plasmids and growth conditions

The recombinant *Escherichia coli* strains co-expressing the *nodBC* genes (encoding the chitooligosaccharide

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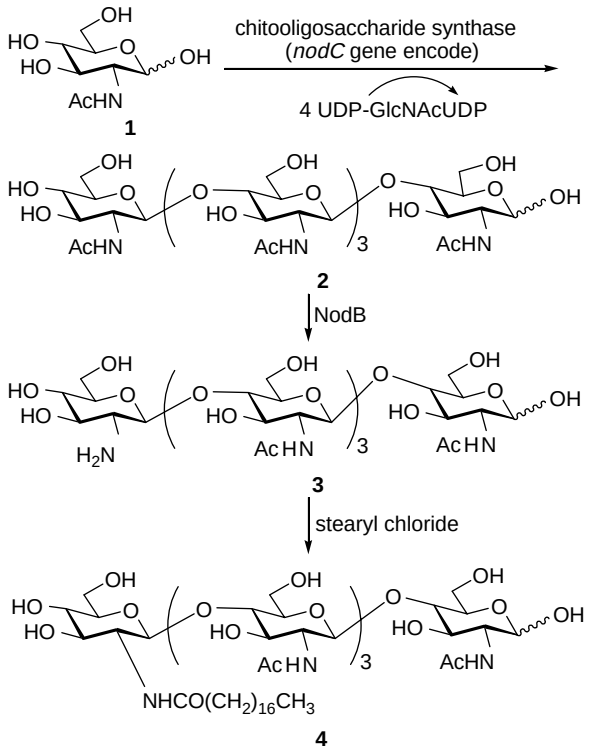

Scheme 1. Production of lipo-chitopentaose nodulation factor **4** with stearyl group at the non-reducing terminus.

synthase and the chitooligosaccharide *N*-deacetylase) are from *Azorhizobium caulinodans* [4]. Routine cultures were grown in LB medium (5). Cell density cultures were carried out as previously described [4] in 10 L bioreactors containing an initial culture volume of 7 L, respectively. The culture medium was slightly modified and had the following composition: *N*-acetyl D-glucosamine **1** (1 g/L), glycerol (15 g/L), $NH_4H_2PO_4$ (7 g/L), KH_2PO_4 (7 g/L), $MgSO_4{\cdot}7H_2O$ (1 g/L), thiamine·HCl (4.5 mg/L), trace mineral solution (7.5 mL/L), citric acid (0.5 g/L), KOH (2 g/L). $MgSO_4$ was added from a concentrated solution that was autoclaved separately. Thiamine was sterilized by filtration. The trace mineral stock solution contained: nitrilotriacetate (70 mmol/L, pH 6.5), ferric citrate (7.5 g/L), $MnCl_2{\cdot}4H_2O$ (1.3 g/L), $CoCl_2{\cdot}6H_2O$ (0.21 g/L), $CuCl_2{\cdot}2H_2O$ (0.13 g/L), H_3BO_3 (0.25 g/L), $ZnSO_4{\cdot}7H_2O$ (1.2 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.15 g/L). For the cultivation, the medium was supplemented with leucine (1 g/L). The antibiotic ampicillin was used to ensure maintenance of the plasmid and was prepared in concentrated stock solution as described by Sambrook et al. [5]. Its final concentration was 50 mg/L for ampicillin. Unless otherwise indicated, the feeding solution contained: glycerol (450 g/L), $MgSO_4{\cdot}7H_2O$ (12 g/L), trace mineral solution (25 mL/L).

Cell density cultures were inoculated at 2% (v/v) with a culture grown in LB medium. Throughout the cultivation, the dissolved oxygen was maintained at 20% of air saturation by manually increasing the air flow rate and automatically adjusting the stirrer speed, the pH was regulated at 6.8 by automatic addition of aqueous NH_3 (15% w/v), and the temperature was maintained at 34°C. After consumption of the initial glycerol, indicated by a sudden increase in the dissolved oxygen level, the feeding was started with an initial flow rate of 9 mL/h/L. After 5 h of cultivation, the feeding rate was lowered to 4.8 mL/h/L and kept constant until the end of the culture.

Purification of compound 3

After being harvested by centrifugation (20 min at 12000 × *g*), bacterial cells were resuspended in a volume of distilled water equal to that of the original culture medium and disrupted by boiling for 45 min. After cooling, 10 μL concentrated HCl was added. Cell debris and precipitated proteins were eliminated by centrifugation (30 min at 12000 × *g*) and the 1 L supernatant was mixed with an equal quantity (125 g) of activated charcoal (Norit) and Celite. The slurry was filtered on Whatman no. 4 paper and washed thoroughly with distilled water to remove the salts. The adsorbed oligosaccharides were eluted with 1.3 L of 55% (v/v) aqueous ethanol. This last fraction was loaded on to a 100 × 1-cm column of Biogel P4. The flow through fraction was recovered and lyophilized. The 1.8 g **3** was obtained in 51% yield.

Synthesis of 4

Sodium hydrogencarbonate (2 equiv) and a solution of stearyl chloride in THF (0.82 mol/L, 1 equiv) were added to a solution of **3** (17 μmol) in DMF/water (2.5:1, 350 μL). The reaction mixture was warmed to 60°C, then more acid chloride solution (5 equiv) and sodium hydrogencarbonate (6 equiv) were added in six portions over a period of 18 h. Flash chromatography with ethyl acetate/methanol/water (7:2:1) as the eluent and deposition of the reaction residue at the top of the column in dichloromethane/methanol (5:1) gave **4** (47%). Solid. $[\alpha]_D$ −8 (*c* 0.5, H_2O). ^{1}H NMR (300 MHz, D_2O): δ 5.06 (d, 0.7H, H-1α), 4.40 (m, 4H, H-1^{II-V}), 4.35 (d, 0.3H, H-1β), 3.88–3.30 (m, 30H), 2.20 (m, 2H, $COCH_2$), 1.95, 1.91 (s, 12H, $COCH_3$), 1.33 [m, 30H, $(CH_2)_{15}$], 1.00 (t, 3H, CH_3). ^{13}C NMR (75 MHz, D_2O): δ 175.57, 175.42, 175.30 (C=O), 102.42, 102.25 (C-1^{II-V}), 95.80 (C-1β), 91.43 (C-1α), 80.63, 80.17, 79.95, 76.90, 75.51, 74.42, 73.46, 73.04, 71.00, 70.68, 70.24, 61.51, 60.96, 56.58, 56.01, 54.63 (C-2^{I-V}, C-3^{I-V}, C-4^{I-V}, C-5^{I-V}, C-6a^{I-V}, C-6b^{I-V}), 34.35 ($COCH_2$), 33.28–27.00 [$(CH_2)_{15}$], 23.09, 22.88, 14.72 (CH_3). ESI-MS: *m*/*z* 1280 $[M+Na]^+$.

Assay for chitinase activity

The chitinase activity was measured by determining the amount of reducing sugar equivalents released

on incubation of the enzyme with the substrate *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide under the condition stated [6–7]. Assays were terminated by heating to 100°C, and the release of reducing sugars was shown to be linear with respect to time and amount of enzyme used. The chitinase (40 μg/mL), BSA (0.8 mg/mL), glycerol 10% (v/v), and **3** (or **4**, 20 mmol/L) were incubated at 18°C in 40 mmol/L sodium acetate buffer at pH5.0. The residual activity was determined at 100 min intervals.

Results and discussion

Production of lipo-chitopentaose nodulation factor

The carbohydrate segment, the tetra-*N*-acetyl-chitopentaose **3** (Scheme 1), was prepared by cultivating, at high cell density, recombinant *Escherichia coli* strains co-expressing the *nodBC* genes (encoding the chitooligosaccharide synthase and the chitooligosaccharide *N*-deacetylase) from *Azorhizobium caulinodans* [4]. Glycerol was provided as the carbon source.

After centrifugation of the culture broth, chitooligosaccharide was recovered exclusively in the pellet containing the bacterial cells. After disruption of the cells by boiling, cell debris was removed by centrifugation and the chitooligosaccharide was purified by activated charcoal adsorption and aqueous ethanol (55%, v/v) elution. The crude product was further purified by size exclusion chromatography on Biogel P4, and **3** was obtained in 51% yield. In our system, the exclusive formation of pentamer is probably due to the fact that the synthesis was carried out in growing *E. coli* cells in which the physiological pool of UDP-GlcNAc is maintained at a high level.

N-Acylation of **3** with stearyl chloride was performed in DMF containing water and provided the corresponding lipo-chitopentaose nodulation factor **4** in 47% yield. The water was required for solubility.

Binding chitinase assay

In principle, the chemical stability of lipochitooligosaccharide NFs should influence the biological activity of the lipochitooligosaccharide mixtures secreted by rhizobia. Therefore, we also assayed the inactivating ability of **3** and **4** towards the chitinase from *Serratia marcescens*. The chitinase activity was measured by determining the amount of reducing sugar equivalents released on incubation of the enzyme with the substrate *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide under the condition stated [6–7]. The inhibition constants (k_i) found for **3** and **4** were 13.9 μmol/L and 55.3 μmol/L, respectively, so that, **4** is much more stable than **3**. This indicates that the *N*-acyl chain at the non-reducing terminus of lipochitooligosaccharide NFs dramatically increases the stability of these lipochitooligosaccharide NFs.

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