Glycoconj J (2006) 23:687–692
DOI 10.1007/s10719-006-9018-8

Chemoenzymatic synthesis of stable isotope labeled UDP-*N*-[^{2}H]-acetyl-glucosamine and [^{2}H]-acetyl-chitooligosaccharides

Hubert F. Becker · Annie Thellend · Annie Piffeteau · Anne Vidal-Cros

Received: 7 April 2006 / Revised: 5 September 2006 / Accepted: 6 September 2006 / Published online: 23 November 2006
© Springer Science + Business Media, LLC 2006

Abstract Labeled UDP-GlcNAc and chitooligosaccharides should be powerful tools for studies of *N*-acetylglucosaminyltransferase such as chitin synthases. We describe here a rapid, inexpensive and a common strategie for the chemoenzymatic synthesis of uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine and the chemical preparation of *N*-[^{2}H]-acetyl chitooligosaccharides (from 2 to 5 mers). Deuterated UDP-GlcNAc analogue was tested as chitin synthase substrate and it exhibited an incorporation level in chitin as the natural substrate. Deuterium labeling of carbohydrates present different advantages: it is a stable isotope and allows glycosyltransferase mechanism studies by a mass spectrometry approach.

Keywords UDP-*N*-acetyl-glucosamine · Chitooligosaccharides · Chemoenzymatic synthesis · *N*-acetylation · Uridylation

Introduction

It is well known that carbohydrate units, in oligosaccharides and in glycoconjugates (glycoproteins and glycolipids), present a crucial role in various biological processes and their synthesis is essential for a better understanding of glycosylation in biology [1–3].

Glycosylation reactions are assured by glycosyltranferases (GTs). These enzymes transfer the sugar moiety from an activated sugar nucleotide to an acceptor. Chitin synthase, a processive glycosyltransferase, represent a membranous isoenzyme family [4], which catalyses the multiple transfer of GlcNAc units from UDP-*N*-acetylglucosamine (UDP-GlcNAc) to the nonreducing end of the growing chitin chain [5]. Chitin polymer is an essential component of filamentous fungi cell wall [6] and is absent in plants and mammals. Therefore, chitin synthase has been recognized as a promising target for new antifungal drugs. It was shown that alteration of chitin content in several mutants affects fungus growth, development [7] or virulence in the case of pathogenic fungi [8]. In order to study, by a mass spectrometry approach, enzyme mechanism and particularly chitin synthase mechanism, structure-function of glycoproteins or cell wall biosynthesis, it is of main importance to have in hand, not only, labeled chitooligosaccharides but also the labeled substrate. In this paper, we wish to describe the chemoenzymatic synthesis of uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine and the chemical preparation of *N*-[^{2}H]-acetyl chitooligosaccharides (Fig. 1). By these methods GlcNAc-tracers were obtained rapidly and in an inexpensive manner. In order to control whether deuterium does not affect substrate recognition by chitin synthase, uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine was tested by the standard radiometric enzymatic test.

H. F. Becker (✉) · A. Thellend · A. Piffeteau · A. Vidal-Cros
Synthèse, Structure et Fonction de Molécules Bioactives
UMR7613, Université Pierre et Marie Curie, Tour 44-45,
3ème étage, 4 place Jussieu, 75252 Paris, France
e-mail: hbecker@moka.ccr.jussieu.fr

Materials and methods

Abbreviations used UDP-GlcNAc, UDP-*N*-acetylglucosamine; GlcNAc, *N*-acetylglucosamine; DPs, degree of polymerization, GlmU, bifunctional enzyme with both acetyltransferase and uridyltransferase activities; TLC, Thin Layer Chromatography.

Positive Negative ESI mass spectra were performed on a triple quadrupole mass spectrometer (Quattro I Micromass).

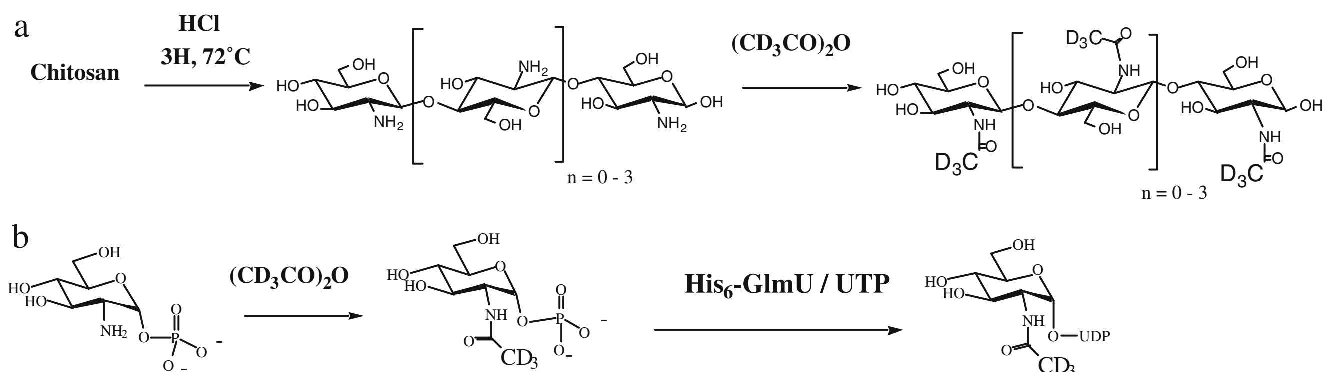

Fig. 1 Pathway for (a) chemical preparation of *N*-[^{2}H]-acetyl chitooligosaccharides (length from 2 to 5 GlcNAc units) and (b) chemoenzymatic synthesis of uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine

^{1}H NMR spectra were recorded on a Bruker-Avance spectrometer 500 MHz.

Preparation of *N*-[^{2}H]-acetyl-glucosamine-1-phosphate

Glucosamine-1-phosphate (1.2 mg) (Sigma-Aldrich) was dissolved in 40 μl of water/methanol (3/1 v/v), and 14 equivalents of D_6-acetic anhydride $(CD_3CO)_2O$ (Sigma-Aldrich) were added. The solution was stirred for 1 h at room temperature and dried under reduced pressure. The dried sample was resuspended in 40 μl of water/methanol (3/1 v/v), and five equivalents of D_6-acetic anhydride were added for an additional hour. Progress of the reaction was followed by TLC in the same conditions as above. Final reaction mixture was dried and coevaporated four times with 200 μl of methanol until total disappearance of acetic anhydride. The product was purified on a graphitized carbon HPLC column, (100×3 mm, Hypercarb, Thermo-Quest) equilibrated in water–0.05% trifluoroacetic acid (TFA) with a flow rate of 0.5 ml/min and UV detection at 210 nm. The gradient was as follows for eluent A (water–0.05% TFA) and B (acetonitrile–0.05% TFA): 0–5 min, 0% B; 5–20 min, 0–15% B. The larger peak containing, 1.1 mg of *N*-[^{2}H]-acetyl-glucosamine-1-phosphate, was recovered and lyophilized with a 68% yield. Negative ESI mass spectrum was obtained using a triple quadrupole mass spectrometer. The expected compound $(C_8H_{13}D_3N_1O_3P_1)$ was observed at $[M\text{-}H]^-$ ion m/z 303.

Overexpression and purification of the His_6-GlmU protein

pFp3 plasmid, containing *E. coli* GlmU gene bearing an N-terminal His_6-tagged form, was a gift from Pr Mengin-Lecreulx from University of Paris-Sud, Orsay, France [9]. Recombinant His_6-GlmU was overexpressed in E. coli BL21(DE3) cells and purified to homogeneity by Ni-NTA agarose affinity chromatography as described before [10]. His_6-GlmU fractions were at least 90% pure as estimated by SDS-PAGE and 11 mg of protein was recovered from 500 ml culture (Luria-Bertani medium). Protein concentration was determined by the Bradford method, with bovine serum albumin (BSA) as standard [11].

Preparation of uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine

The uridylyl transfer was achieved enzymatically from *N*-[^{2}H]-acetyl-glucosamine-1-phosphate, UTP and purified recombinant His_6-GlmU protein. The reaction mixture (500 μl), was inbubated for 18 h at 37°C, and contained: Tris-HCl pH 8.2 50 mM, UTP 25 mM, $MgCl_2$ 3 mM, *N*-[^{2}H]-acetyl-glucosamine-1-phosphate 2.5 mM (0.45 mg) and recombinant *E. coli* His_6-GlmU (50 μg). The mixture was extracted with 500 μl phenol/chloroform 50/50 saturated with Tris-HCl pH=8 and then with 500 μl chloroform. Product purification was performed on a graphitized carbon HPLC column, (100×3 mm, Hypercarb, ThermoQuest) in isocratic conditions: eluent A 93% (ammonium formate 45 mM pH 4.0) eluent B 7% acetonitrile. The flow rate was 0.5 ml/min and UV detection was performed at 210 nm. The material was injected in small amounts (100 μg), and expected fractions were collected and lyophilized. Uridine-5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine (0.75 mg) was recovered and stocked at −20°C in a 100 mM solution in water. Yield: 90%. This compound $(C_{17}H_{24}D_3N_3O_{17}P_2)$ was mainly characterized in the negative ESI mass spectrum by the doubly charged ion $[M\text{-}2H]^{2-}$ m/z 304. The deprotonated molecules $[M\text{-}H]^-$ m/z 609 and $[M\text{-}2H\text{+}Na]^-$ m/z 631 were also observed. ^{1}H NMR assigments were obtained from the analysis of 1 D ^{1}H spectra and 2D homonuclear COSY and TOCSY spectra, for D_2O solutions. All chemicals shifts are reported as δ values (ppm) relative to sodium 2,2-dimethylsilapentane-5-sulfonate (DSS). ^{1}H NMR (500 MHz): δ=7.95 (d, 8 Hz, 1 H, H6), 5.98 (d, 4 Hz, 1 H, H1′), 5.96 (d, 8 Hz, 1 H, H5), 5.51 (dd, 7.3 Hz, 3.2 Hz, 1 H, H1”), 3.99 (dt, 10.5 Hz, 3 Hz,

3 Hz, 1 H, H2''), 3.81 (t, 8.5 Hz, 8.5 Hz, 1 H, H3''), 3.54 (t, 9.8 Hz, 1 H, H4''), 3.92 (ddd, 10.3 Hz, 4 Hz, 2 Hz, 1 H, H5''), 3.86 (dd, 12.4 Hz, 2.2 Hz, 1 H, H6''), 3.80 (dd, 12.4 Hz, 4 Hz, 1 H, H6''), 4.38 (m, 1 H, H2′), 4.36 (m, 1 H, H3′), 4.36 (m, 1 H, H4′), 4.24 (ddd, 3.4 Hz, 4.6 Hz, 11.8 Hz, 1 H, H5′), 4.18 (ddd, 3 Hz, 5.3 Hz, 11.8 Hz, 1 H, H5′).

Chitin synthase assay

Chitin synthase assays were performed in a standard 25 µl volume containing 50 mM Tris-HCl pH 7.4, 4.5 nCi UDP [U−^{14}C]- GlcNAc, 40 mM GlcNAc, 0.2% digitonine, 5 mM Mg(OAc)$_2$, 0.5 µg trypsin, 120 µg protein and as substrate 1 mM UDP-GlcNAc or 1 mM uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine. Incubations were performed at 30°C for 60 min and terminated by adding 1 ml of 10% trichloroacetic acid. After filtration through a glass fiber filter (GF/C Whatman) and washing with 3:7 AcOH-EtOH, the discs were dried and radioactivity measured in a liquid scintillation counter (LKB 1214 RackBeta).

Preparation of *N*-[^{2}H]-acetyl chitooligosaccharides

Chitosan (50 mg) (Sigma-Aldrich) was dissolved in 5 ml HCl 36% and acid hydrolysis performed for 3 h at 72°C. Reaction mixtures were diluted with 50 ml water and neutralized with Dowex 2X8-200 resin (hydroxyl charged). After filtration, the solution was lyophilized. Chitosan hydrolysate was then specifically *N*-reacetylated [12] by incubating of 30 mg lyophilisate mixture with 0.63 mmol of D$_6$-acetic anhydride (CD$_3$CO)$_2$O (Sigma-Aldrich) in 600 µl methanol/water (1/5) for 20 h at room temperature. Solvents were removed by reduced pressure on a speed-vac, and the pellet resuspended in 200 µl methanol. The solution was neutralized with 4M NaOH (30 µl), and the supernatant was evaporated again.

The dried sample was resuspended in water before purification on a Bio-gel P2 (45–90 µM mesh, Biorad) column (1.6 cm×200 cm). The flow rate was 0.2 ml/min and the column was warmed at 55°C during the separation. Deuterated chitooses were eluted with water and monitored at 210 nm. The recovered fractions were dried by lyophilization and finally resuspended in water. Labeled oligosaccharides (GlcNAc)$_n$ of various lengths were obtained; n=1 (0.5 mg), n=2 (1.9 mg), n=3 (4 mg), n=4 (3.3 mg) and n=5 (1.5 mg). Determination of deuterated chitooses length was performed using thin layer chromatography (TLC) on silica gel plates with propan-1-ol/water/ammonia 36% (70/30/1.5) as eluent and compared with commercial chitooses markers. Products, labeled (GlcNAc)$_n$, were further characterized by positive ESI mass spectra: n=1, [MNa]$^+$ m/z 247, [MK]$^+$ m/z 263; n=2 [MNa]$^+$ m/z 453.2, [MK]$^+$ m/z 469.2; n=3, [MNa]$^+$ m/z 659.4, [MK]$^+$ m/z 675.4; n=4, [MNa]$^+$ m/z 865.5, [MK]$^+$ m/z 881.5; n=5 [MNa]$^+$ m/z 1,071.5, [MK]$^+$ m/z 1,087.5.

Results and discussion

UDP-*N*-[^{2}H]-acetyl-glucosamine preparation

Chemical and enzymatic syntheses of UDP-*N*-acetyl-glucosamine [13, 14] have been previously described. Although few of them are applicable for the gram-scale preparation of UDP-GlcNAc, these approaches lead mainly to unlabeled compounds. Leiting et al. have proposed an efficient synthesis of radiolabeled UDP-*N*-[1-^{14}C]-GlcNAc based on enzymatic conversion of [1-^{14}C]-acetate into UDP-*N*-[1-^{14}C]-GlcNAc [15]. In our research program, we wanted to develop a new method for the synthesis of UDP-GlcNAc bearing an isotope tag in order to have a suitable tool for mass spectrometry studies. Quite recently [16], the synthesis of tetradeuterium labeled pyridylamino monosaccharides has been described and these derivatized sugars were used as internal standards in mass spectrometry quantification. These compounds are not suited for our studies since the anomeric position is modified and is devoided of the essential UDP motif.

We proposed a new chemoenzymatic way for the synthesis of UDP-*N*-[^{2}H]-acetyl-glucosamine from glucosamine-1-phospate (Fig. 1b). This approach implies a first chemical step followed by an enzymatic step with the GlmU protein, as described for UDP-*N*-[^{14}C]-acetyl-glucosamine preparation. In our case, only the uridyltransferase activity of recombinant GlmU [9] was necessary. Indeed GlmU is a bifunctional protein having uridyltransferase and *N*-acetyltransferase activities organized in two separate domains in the N- and C-terminus of the protein, respectively. Exhibition of GlmU *N*-acetyltransferase activity requires the trimer organization of the protein, whereas this trimerization is clearly not essential for expression of the uridyltransferase activity of GlmU [10]. Therefore, chemical acetylation seemed much easier than the enzymatic method and chemical acetylation conditions could be extended to labeled oligosaccharides preparation (see further).

Chemical *N*-acetylation of glucosamine-1-phosphate was performed first by two successive additions of D$_6$-acetic anhydride on glucosamine-1-phosphate, in order to introduce deuterium labeling on the acetyl position of UDP-*N*-acetyl-glucosamine. Excess of D$_6$-acetic anhydride and acetic acid formed were eliminated by few coevaporations with methanol, to prevent *O*-acetylation of glucosamine-1-phosphate. Traces of doubly acetylated glucosamine-1-phosphate were observed and easily separated by HPLC

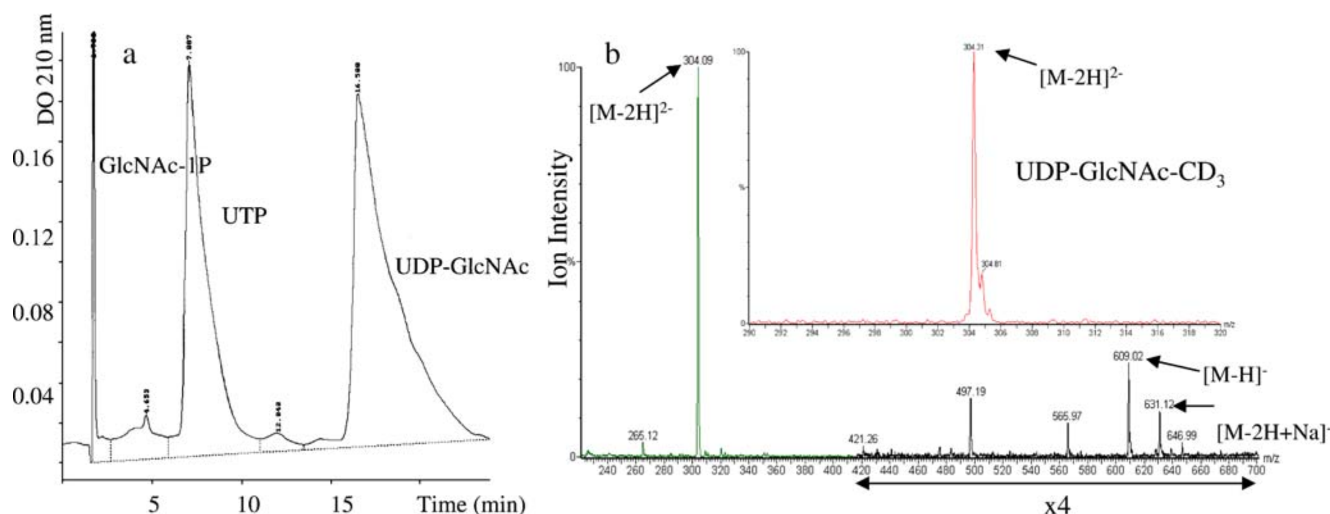

Fig. 2 Uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine: (a) HPLC chromatogram on an Hypercarb column of a mixture containing GlcNAc-1P, UTP and UDP-GlcNAc, in isocratic conditions (ammonium formate buffer 45 mM pH 4.0/acetonitrile 93/7). The product UDP-GlcNAc exhibited a high retention time in these conditions, adapted for purification. (b) Negative ESI mass spectra of uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine (UDP-GlcNAc-CD_3) (cone voltage 20 V). Upper insert corresponds to an higher resolution measurement of the 290-320 m/z region. The presence of the ions $[M\text{-}H]^-$ m/z 609 and $[M\text{-}2H]^{2-}$ m/z 304 revealed total [^{2}H]-acetylation

purification on graphitized carbon column (data not shown). Pure *N*-[^{2}H]-acetyl-glucosamine-1-phosphate was obtained in good yield and enzymatically converted to UDP-*N*-[^{2}H]-acetyl-glucosamine by the uridylyl transferase activity of GlmU protein. Uridylyltransferase assay described in Materials and Methods, converted *N*-[^{2}H]-acetyl-glucosamine-1-phosphate and UTP to UDP-*N*-[^{2}H]-acetyl-glucosamine with a good activity. To isolate UDP-*N*-[^{2}H]-acetyl-glucosamine from the reaction mixture, we performed organic extraction and HPLC purification on a graphitized carbon HPLC column (Hypercarb) in isocratic conditions (ammonium formate 45 mM pH 4.0, acetonitrile; 93/7), respectively [17]. Adsorption of molecules adsorption on the column is based on hydrophobic interactions and electron donor–acceptor interactions between the graphitized carbon column and compounds to purify. A typical elution chromatogram with a standard mix containing UDP-*N*-acetyl-glucosamine, UTP and *N*-acetyl-glucosamine is presented on Fig. 2a. It is clear that molecules with planar properties (containing UDP group) are more retained than less planar carbohydrate structures. The elution profile of the reaction mixture after one night incubation, revealed two peaks at 4 and 12 min corresponding to UTP and UDP-*N*-[^{2}H]-acetyl-glucosamine elution, respectively (data not shown). All *N*-[^{2}H]-acetyl-glucosamine has been seemingly consumed. We also obtained UDP-*N*-[^{2}H]-acetyl-glucosamine from glucosamine-1-phosphate in two steps with a total yield of 60%. UDP-*N*-[^{2}H]-acetyl-glucosamine was characterized by negative ESI mass spectra and ^{1}H NMR. Total labeling of acetyl group by deuterium was clearly demonstrated and no trace of unlabeled UDP-*N*-acetyl-glucosamine was observed (Fig. 2b).

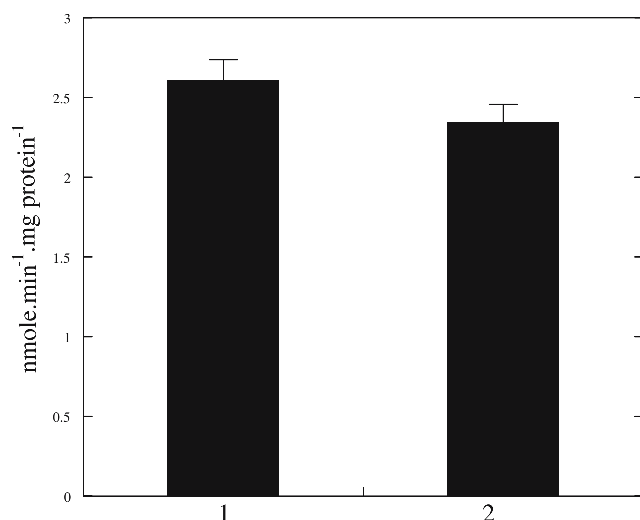

Fig. 3 Uridine 5′-diphospho-*N*-[^{2}H]-acetyl-glucosamine as chitin synthase substrate: The amount of ^{14}C-chitin formed in different chitin synthase test conditions are presented in the form of a histogram. *Black bars* correspond to ^{14}C-chitin formed by chitin synthase in presence of UDP[U-^{14}C]-GlcNAc with UDP-GlcNAc or UDP-*N*-[^{2}H]-GlcNAc. Reproducibility from three experiments is ±2%

Chitin synthase test with UDP-*N*-[^{2}H]-acetyl-glucosamine as substrate

Chitin synthase test was performed using *Saccharomyces cerevisiae* microsomal preparations obtained as previously described [18]. Chitin synthase activity was assayed by measuring the rate of formation of ^{14}C-chitin polymer from UDP-^{14}C-GlcNAc in presence of UDP-GlcNAc or UDP-*N*-[^{2}H]-acetyl-glucosamine. Enzymatic activity required (1) activation of enzyme present as zymogen by addition of trypsin in the assay and (2) addition of essential divalent cation Mg^{2+} to ensure a measure chitin synthase I activity mainly. Thus, UDP-*N*-[^{2}H]-acetyl-glucosamine was tested as chitin synthase substrate. Equivalent amounts of ^{14}C-

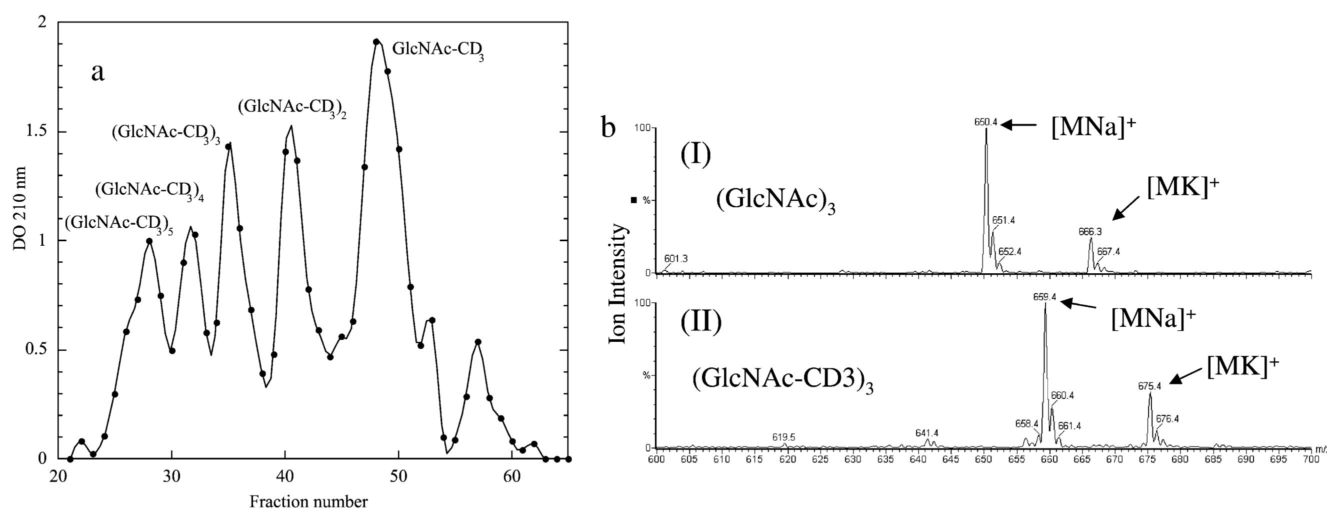

Fig. 4 Labeled Chitooligosaccharides: (a) Gel chromatography separation of *N*-[^{2}H]-acetyl chitooligosaccharides on a Bio-gel P2 column (2.5×200 cm) at 55°C and eluted with water. Each peak content was analyzed by TLC on silica plates (conditions described in materials and methods). Number of GlcNAc units contained in each *N*-[^{2}H]-acetyl chitooligosaccharides was also deduced and indicated on the figure (GlcNAc-CD_3)$_n$. (b) Positive ESI mass spectra of chitotrioligosaccharides (cone voltage 40 V). Mass spectrum of (*I*) the standard *N*,*N*,*N*-triacetyl-trichitooligosaccharides (Glc-NAc)$_3$ and of (*II*) labeled *N*,*N*,*N*-[^{2}H]-triacetyl-trichitooligosaccharides (Glc-NAc-CD_3)$_3$ are represented. The difference (9u) between m/z values of labelled [MNa]$^+$ ion and unlabeled [MNa]$^+$ ion revealed complete and totally regiospecific *N*-[^{2}H]-acetylation of chitotrioses

chitin, 2.5 nmoles min^{-1} mg protein^{-1}, was obtained with either UDP-GlcNAc or UDP-*N*-[^{2}H]-acetyl-glucosamine (Fig. 3); demonstrating that deuterium introduction in UDP-*N*-[^{2}H]-acetyl-glucosamine does not affect the recognition by chitin synthase.

Chitooligosaccharides preparation

Progresses in automation of solid-phase-supported oligosaccharides synthesis led to differently attached carbohydrates [19–21]. However, the application to the β (1-4)-GlcNAc polymer seems more difficult since the 4-OH moiety of a GlcNAc unit is not very nucleophilic, thus rendering this link quite difficult to make [22]. Chemical chitooligosaccharides preparation with degree of polymerization (DPs) from 4 to 10 could be carried out by chitin treatment with anhydrous hydrogen fluoride [23]. Enzymatic approaches have been described for the preparation of *N*-acetylchitooligosaccharides. NodC for example is capable to catalyse the formation of the chitooligosaccharides with DPs from 2 to 5 [24]. Chitosan degradation by chitinase or by an enzyme complex was described to obtain chitooligosaccharides distribution from 3 to 10 GlcNAc units in large scale production [12], [25]. More recently, an improved preparation of chitobiose by continous enzymatic degradation of colloidal chitin in dialysis tubing, with recycling of the chitinase was described [26]. β-*N*-acetylhexosaminidase of *Aspergillus oryzae* was also used to prepare *N*-acetylchitooligosaccharides ((Glc-NAc)n with n=2 to 4) by catalyzing the reaction between *N*-acetyl-D-glucosamine and p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside [27]. In this paper, the method we describe for the preparation of stable isotope labeled *N*-acetylchitooligosaccharides with DPs from 2 to 5 Glc-NAc units (Fig. 1a) allows production of significant amounts of deutered-chitooligosaccharides, useful for glycosyltransferase mechanism or protein–carbohydrate interaction studies by mass spectrometry.

Chitosan, a partially *N*-deacylated glucosaminoglycan, with higher solubility in water than chitin, was used as starting material. Chitosan was acid hydrolysed at 72°C, reaching the totally amino deprotected oligosaccharides. Without purification and just after neutralization of the mixture, oligosaccharides were specifically *N*-acetylated with deuterated acetic anhydride $(CD_3CO)_2O$ in the presence of water/methanol [12]. Total separation of chitooligosaccharides was performed on a Bio-gel P2 gel filtration column at 55°C with a good resolution (Fig. 4a), and each of the expected deuterated-chitooligosaccharides was obtained. Deuterated-chitooligosaccharides presented the same retention time as natural chitooligosaccharides. Chitooligosaccharides chain length was rapidly determined by TLC and by ESI mass spectrometry to characterize precisely each oligosaccharide. Figure 4b represents the positive mass spectra of *N*,*N*,*N*-[^{2}H]-acetyl-chitotriose and *N*,*N*,*N*-acetyl-chitotriose as reference. One can observe, from the [MNa]$^+$ m/z 659.4 ion for the labeled chitotriose, that *N*-[^{2}H]-acetylation is practically complete and totally regiospecific. The difference between m/z values of labelled [MNa]$^+$ ion and unlabeled [MNa]$^+$ is 9μ. This value is in total agreement with addition of 9 deuterium atoms in *N*,*N*,*N*-[^{2}H]-acetyl-chitotriose.

Conclusion

In this paper, we have developed an isotope tag method for the synthesis of labeled UDP-GlcNAc and labeled chito-oligosaccharides. The first takes advantage of recombinant GlmU to introduce the UDP motif into the deuterated *N*-acetylglucosamine unit, and the second implies acid-hydrolysis of chitosan and an acetylation step leading to deuterated oligosaccharides. The notable advantage of these two approaches is, unlike most traditional chemical synthesis, the use of unprotected sugars to obtain tagged building blocks. This result was reproducible and should be extended to larger scale synthesis. The recognition of UDP-*N*-[^{2}H]-acetyl-glucosamine as substrate by chitin synthase was also clearly demonstrated. These isotopically tagged compounds should be quite useful tools for glycosyltransferase mechanism studies by mass spectrometry.

Acknowledgments We are grateful to Elisabeth Chatel for initial work on UDP-*N*-[^{2}H]-acetyl-glucosamine preparation. We thank Pr Jean-Claude Tabet / Dr Denis Lesage and Pr Olivier Lequin, UMR7613 Université Pierre et Marie Curie, for, respectively, mass spectrometry and NMR product characterizations. We thank Pr Mengin-Lecreulx, Université Paris-Sud France, for providing the plasmid-producing GlmU. We wish to thank Pr Martine Boccara for critical reading of the manuscript.

References

1. Dwek, R.: Glycobiology: Toward understanding the function of sugars. Chem. Rev. **96**, 683–720 (1996)
2. Bertozzi, C.R., Kiessling, L.L.: Chemical glycobiology. Science **291**, 2357–2364 (2001)
3. Zhang, Z., Gildersleeve, J., Yang, Y.Y., Xu, R., Loo, J.A., Uryu, S., Wong, C.H., Schultz, P.G.: A new strategy for the synthesis of glycoproteins. Science **303**, 371–373 (2004)
4. Choquer, M., Boccara, M., Goncalves, I.R., Soulie, M.C., Vidal-Cros, A.: Survey of the Botrytis cinerea chitin synthase multigenic family through the analysis of six euascomycetes genomes. Eur. J. Biochem. **271**, 2153–2164 (2004)
5. Sugiyama, J., Boisset, C., Hashimoto, M., Watanabe, T.: Molecular directionality of beta-chitin biosynthesis. J. Mol. Biol. **286**, 247–255 (1999)
6. Bernard, M., Latge, J.P.: Aspergillus fumigatus cell wall: composition and biosynthesis. Med. Mycol. **39**, 9–17 (2001)
7. Roncero, C.: The genetic complexity of chitin synthesis in fungi. Curr. Genet. **41**, 367–378 Epub 2002 Aug 1 (2002)
8. Soulie, M.C., Piffeteau, A., Choquer, M., Boccara, M., Vidal-Cros, A.: Disruption of Botrytis cinerea class I chitin synthase gene Bcchs1 results in cell wall weakening and reduced virulence. Fungal Genet. Biol. **40**, 38–46 (2003)
9. Pompeo, F., van Heijenoort, J., Mengin-Lecreulx, D.: Probing the role of cysteine residues in glucosamine-1-phosphate acetyltransferase activity of the bifunctional GlmU protein from Escherichia coli: Site-directed mutagenesis and characterization of the mutant enzymes. J. Bacteriol. **180**, 4799–4803 (1998)
10. Pompeo, F., Bourne, Y., van Heijenoort, J., Fassy, F., Mengin-Lecreulx, D.: Dissection of the bifunctional Escherichia coli *N*-acetylglucosamine-1-phosphate uridyltransferase enzyme into autonomously functional domains and evidence that trimerization is absolutely required for glucosamine-1-phosphate acetyltransferase activity and cell growth. J. Biol. Chem. **276**, 3833–3839 Epub 2000 Nov 17 (2001)
11. Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**, 248–254 (1976)
12. Aiba, S.: Preparation of *N*-acetylchitooligosaccharides by hydrolysis of chitosan with chitinase followed by *N*-acetylation. Carbohydr. Res. **265**, 323–328 (1994)
13. Heidlas, J., Lees, W., Pale, P., Whitesides, G.: Gram-scale synthesis of Uridine 5′-Diphospho-*N*-acetylglucosamine: Comparison of Enzymatic and Chemical Routes. J. Org. Chem. **57**, 146–151 (1992)
14. Bulter, T., Elling, L.: Enzymatic synthesis of nucleotide sugars. Glycoconj. J. **16**, 147–159 (1999)
15. Leiting, B., Pryor, K.D., Eveland, S.S., Anderson, M.S.: One-day enzymatic synthesis and purification of UDP-N- [1-14C]acetyl-glucosamine. Anal. Biochem. **256**, 185–191 (1998)
16. Yuan, J., Hashii, N., Kawasaki, N., Itoh, S., Kawanishi, T., Hayakawa, T.: Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography–mass spectrometry. J. Chromatogr. A **1067**, 145–152 (2005)
17. Koizumi, K.: High-performance liquid chromatographic separation of carbohydrates on graphitized carbon columns. J. Chromatogr. A **720**, 119–126 (1996)
18. Orlean, P.: Two chitin synthases in Saccharomyces cerevisiae. J. Biol. Chem. **262**, 5732–5739 (1987)
19. Seeberger, P.H., Haase, W.C.: Solid-phase oligosaccharide synthesis and combinatorial carbohydrate libraries. Chem. Rev. **100**, 4349–4394 (2000)
20. Sears, P., Wong, C.H.: Toward automated synthesis of oligosaccharides and glycoproteins. Science **291**, 2344–2350 (2001)
21. Wu, X., Schmidt, R.R.: Solid-phase synthesis of complex oligosaccharides using a novel capping reagent. J. Org. Chem. **69**, 1853–1857 (2004)
22. Crich, D., Dudkin, V.: Why are the hydroxy groups of partially protected N-acetylglucosamine derivatives such poor glycosyl acceptors, and what can be done about it? A comparative study of the reactivity of *N*-acetyl-, *N*-phthalimido-, and 2-azido-2-deoxy-glucosamine derivatives in glycosylation. 2-Picolinyl ethers as reactivity-enhancing replacements for benzyl ethers. J. Am. Chem. Soc. **123**, 6819–6825 (2001)
23. Bosso, C., Defaye, J., Domard, A., Gadelle, A.: The behavior of chitin towards anhydrous hydrogen fluoride. Preparation of b-(1-4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides. Carbohydr. Res. **156**, 57–68 (1986)
24. Samain, E., Drouillard, S., Heyraud, A., Driguez, H., Geremia, R.A.: Gram-scale synthesis of recombinant chitooligosaccharides in *Escherichia coli*. Carbohydr. Res. **302**, 35–42 (1997)
25. Zhang, H., Du, Y., Yu, X., Mitsutomi, M., Aiba, S.: Preparation of chitooligosaccharides from chitosan by a complex enzyme. Carbohydr. Res. **320**, 257–260 (1999)
26. Matsuoka, K., Matsuzawa, Y., Kusano, K., Terunuma, D., Kuzuhara, H.: An improved preparation of N,N’-diacetylchitobiose by continuous enzymatic degradation of colloidal chitin using dialysis tubing as a convenient separator. Biomacromolecules **1**, 798–800 (2000)
27. Singh, S., Packwood, J., Samuel, C.J., Critchley, P., Crout, D.H.: Glycosidase-catalysed oligosaccharide synthesis: Preparation of *N*-acetylchitooligosaccharides using the beta-*N*-acetylhexosaminidase of Aspergillus oryzae. Carbohydr. Res. **279**, 293–305 (1995)