JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Glucosamine and *N*-Acetyl Glucosamine in Fungal Cell Walls

AKRAM ZAMANI,*,†,‡ AZAM JEIHANIPOUR,†,‡ LARS EDEBO,§ CLAES NIKLASSON,‡ AND MOHAMMAD J. TAHERZADEH†

School of Engineering, University of Borås, Borås, Sweden, Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden, and Department of Clinical Bacteriology, University of Gothenburg, Göteborg, Sweden

A new method was developed to determine glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) in materials containing chitin and chitosan, such as fungal cell walls. It is based on two steps of hydrolysis with (i) concentrated sulfuric acid at low temperature and (ii) dilute sulfuric acid at high temperature, followed by one-step degradation with nitrous acid. In this process, chitin and chitosan are converted into anhydromannose and acetic acid. Anhydromannose represents the sum of GlcN and GlcNAc, whereas acetic acid is a marker for GlcNAc only. The method showed recovery of 90.1% of chitin and 85.7−92.4% of chitosan from commercial preparations. Furthermore, alkali insoluble material (AIM) from biomass of three strains of zygomycetes, *Rhizopus oryzae*, *Mucor indicus*, and *Rhizomucor pusillus*, was analyzed by this method. The glucosamine contents of AIM from *R. oryzae* and *M. indicus* were almost constant (41.7 ± 2.2% and 42.0 ± 1.7%, respectively), while in *R. pusillus*, it decreased from 40.0 to 30.0% during cultivation from 1 to 6 days. The GlcNAc content of AIM from *R. oryzae* and *R. pusillus* increased from 24.9 to 31.0% and from 36.3 to 50.8%, respectively, in 6 days, while it remained almost constant during the cultivation of *M. indicus* (23.5 ± 0.8%).

KEYWORDS: Alkali-insoluble material (AIM); chitin; chitosan; fungal cell wall; glucosamine (GlcN); *N*-acetyl glucosamine (GlcNAc); nitrous acid; sulfuric acid

INTRODUCTION

Glucosamine (GlcN) and its acetylated form, *N*-acetyl glucosamine (GlcNAc), are the building units of the biopolymers chitin and chitosan. GlcN is the dominating monomer in chitosan, while chitin contains more GlcNAc than GlcN. Chitin is a characteristic component of the exoskeleton of the crustaceans and fungi and is crucial for their shape. Furthermore, chitosan, which is the deacetylated derivative of chitin, is found in the cell wall of zygomycetes (*1*). Both chitin and chitosan have GlcN and GlcNAc monomers that are connected together by glycosidic bonds. At higher degrees of deacetylation, the polymer is named chitosan. Chitin and chitosan are usually found in association with other materials such as minerals in crustacean shells and proteins and glucans in fungal cell walls. The determination of GlcN and GlcNAc content has great importance for the estimation of purity and composition of these polymers (*2*). For measuring GlcN and GlcNAc contents, chitin- and chitosan-containing samples are usually hydrolyzed to cleave the glycosidic bonds between the monomers and to remove the acetyl group from the GlcNAc residues to produce GlcN.

Among different hydrolysis methods of chitin and chitosan, hydrochloric acid hydrolysis has been preferred. The hydrolysis is usually performed by 2.5−10 M hydrochloric acid at 80−140 °C for 1−23 h (*2*−*7*). The recovery of GlcN in this process depends on the crystallinity of the chitin and chitosan materials and reaction conditions (i.e., acid concentration, time, and temperature). High crystallinity of the materials reduces access of the acid to the polymer chains, resulting in lower hydrolysis yield. In this case, very long hydrolysis time is necessary to break the chains. However, prolonged hydrolysis under harsh conditions of reaction leads to degradation of the liberated monomers (*6*). We checked this method in our preliminary experiments, which did not end up with complete hydrolysis of commercial preparations of chitin and chitosan, and a considerable amount of samples was retained at the end of hydrolysis process (data not shown). Under such conditions, HCl might not be the best acid for the hydrolysis process.

Nitrous acid has often been used for the detection of chitosan. This acid causes depolymerization and deamination of chitosan and converts it to anhydromannose (*8*−*10*). However, nitrous acid is not able to cleave the bonds between GlcN and GlcNAc

* To whom correspondence should be addressed. Tel: +46-33-4354360. Fax: +46-33-4354008. E-mail: akram.zamani@hb.se.
† University of Borås.
‡ Chalmers University of Technology.
§ University of Gothenburg.

10.1021/jf801478j CCC: $40.75
Published on Web 08/27/2008

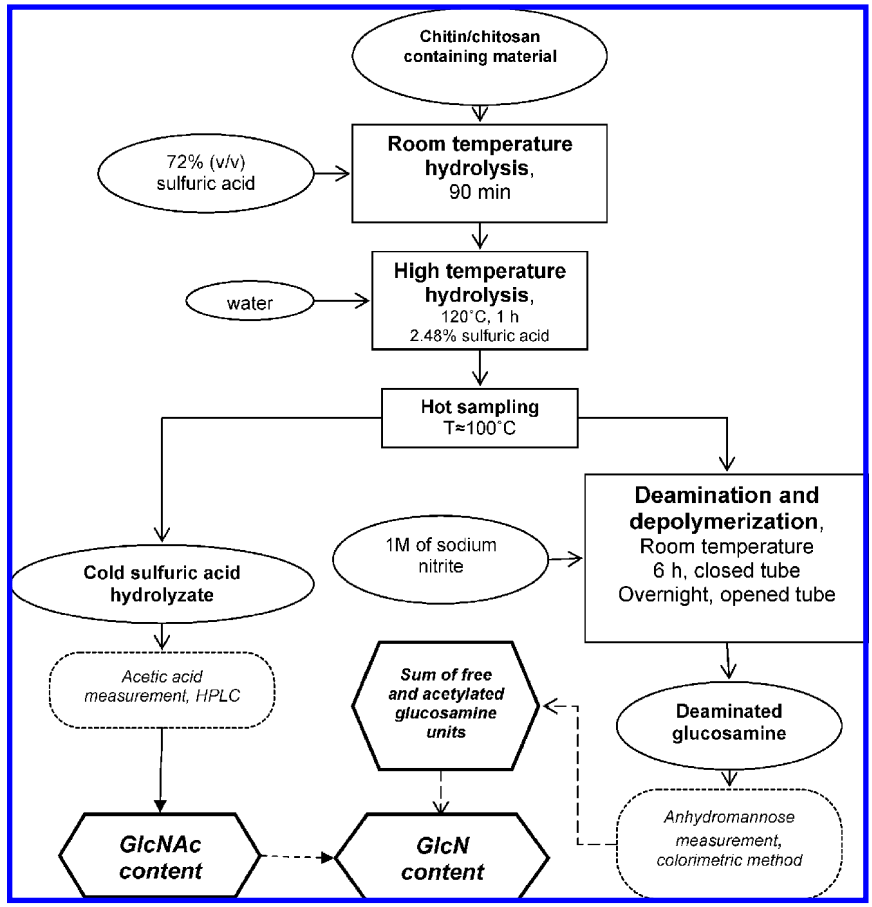

Figure 1. Procedure for the determination of GlcN and GlcNAc in chitin- and chitosan-containing materials.

residues and depolymerization of chitosan stops when it reaches GlcN−GlcNAc bonds (*10*). Consequently, complete depolymerization of chitin and chitosan chains is only possible when the chains are fully deacetylated prior to treatment with nitrous acid.

Complete deacetylation of chitin and chitosan can be performed by high concentrations of sulfuric acid (2.4−7.2 M) in the presence of oxalic acid at high temperatures (110−155 °C). This process is used for measuring the degree of deacetylation of pure chitin and chitosan (*11, 12*). In preliminary experiments with this process, we found that GlcN was largely destroyed under such harsh conditions. Thus, this method is not useful for simultaneous determination of GlcN and GlcNAc (data not shown). On the other hand, sulfuric acid is widely used for the hydrolysis of cellulosic materials and analysis of total sugars in impure samples (*13*). Usually, a two-step sulfuric acid hydrolysis employing concentrated sulfuric acid hydrolysis at low temperature followed by dilute acid hydrolysis at high temperature has been used for analyses of lignocelluloses. The first step loosens the fibrils and initiates depolymerization that proceeds during the second step with release of a high yield of sugars, which can be quantified by, for example, high-performance liquid chromatography (HPLC) (*13*). In preliminary experiments, we tried this process on pure chitin and chitosan. It resulted in complete deacetylation and became the basis of the current work.

The aim of this work was to develop a simple and rapid method for measuring the GlcN and GlcNAc constituents of fungal cell walls. Pure commercial reference materials mainly from crustaceous sources (chitin, chitosan, GlcN, and GlcNAc) were first tested under different conditions. Then, the condition giving the highest recovery was chosen for measuring GlcN and GlcNAc in impure samples. As examples of the application, alkali insoluble residues from biomass of three strains of zygomycetes *Rhizopus oryzae*, *Mucor indicus*, and *Rhizomucor pusillus*, were characterized by the new method.

MATERIALS AND METHODS

Materials. Our new method was first tested on seven commercial preparations of chitin, chitosan, and amino sugars. Pure chemicals including three commercial shellfish chitosans (Aldrich) of (i) low molecular weight with 20 cP viscosity (1% solution in 1% acetic acid), (ii) medium molecular weight with 200 cP viscosity and (iii) high molecular weight with 800 cP viscosity, and (iv) chitosan from crab shells (Sigma), (v) chitin from crab shells (Sigma), (vi) GlcNAc (Calbiochem), and (vii) GlcN hydrochloride (Fluka) were used as reference materials.

Measurement of GlcNAc and GlcN Content. The procedure containing a two-step hydrolysis by sulfuric acid followed by one-step nitrous acid depolymerization (**Figure 1**) was applied to all of the reference materials. Sulfuric acid hydrolysis was carried out according to laboratory analytical procedure developed by the National Renewable Energy Laboratory (NREL) for lignocelluloses with some modifications (*13*). Samples of 10 mg chitin-/chitosan-containing materials were placed in 15 mL screw cap centrifuge tubes, and 0.3 mL of 72% (v/v) sulfuric acid was added. The suspensions were then mixed every 15 min with a glass bar for 90 min at room temperature. At the end of this step, all solid materials were dissolved in the concentrated sulfuric acid. Shorter mixing times and smaller volumes of sulfuric acid resulted in incomplete dissolution of chitin and lower recovery of this material. Then, 8.4 mL of water was added to each tube, and they were then closed tightly and placed in an autoclave at 121 °C for 1 h for the hydrolysis with the diluted sulfuric acid. At the end of this hydrolysis step, two samples of each 0.5 mL (named A and B) were taken from each tube, while the solutions were still hot around 100 °C. It was necessary to take the samples at high temperature, since chitosan

precipitates by cooling to room temperature, which results in a nonhomogeneous solution (*14*). After the samples were cooled to room temperature, 0.5 mL of 1 M $NaNO_2$ or 0.5 mL of water was added to samples A and B, respectively. All tubes were closed tightly, mixed, and left for 6 h at room temperature. They were then opened and left overnight under the hood to complete the depolymerization−deamination reaction and to remove the NO_2 that arose as a byproduct in the reaction mixture of sample A. A shorter time for both closed and opened door steps (6 h and overnight, respectively) resulted in lower recovery of reference materials (data not shown). At the end of this step, both chitin and chitosan were converted to anhydromannose, which was quantified by the colorimetric method presented by Plassard et al. (*15*) with minor modifications. Briefly, the excess nitrous acid was inactivated by the addition of 0.5 mL of ammonium sulfamate (12 wt %) to samples A and B and mixing for 4 min. Then, 0.5 mL of 0.5% MBTH (3-methyl-2-benzothiozolone-hydrazone-hydrochloride) was added, and the tubes were left for 1 h without mixing. It was followed by addition of 0.5 mL of 0.5% $FeCl_3$ and mixing. MBTH and $FeCl_3$ create a blue color complex with anhydromannose. After 1 h, both A and B were diluted 100 times with water, and the absorbance of solution A was measured at 650 nm against solution B (without $NaNO_2$). A solution of pure GlcN hydrochloride in 2.48% (v/v) sulfuric acid (8.4 mL of water mixed with 0.3 mL of 72% sulfuric acid) was used as a standard, and the concentration of GlcN in samples was measured. Because the samples were taken at high temperature and the measurements were performed at room temperature, the difference between the density of 2.48% sulfuric acid at room temperature and 95 °C (1.027 and 1.003 g/mL, respectively) was used to correct the calculations.

For measuring the GlcNAc, the concentration of acetic acid in cold sulfuric acid hydrolyzates was measured by HPLC with an ion exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA) at 60 °C with 0.6 mL/min eluent of 5 mM sulfuric acid with UV−vis detector (Waters 2486, Waters, MA). Moles of acetic acid in each sample were considered to be equal to moles of GlcNAc.

Pure GlcN and GlcNAc were treated in the same way as chitin and chitosan, and their recoveries were calculated according to the following equations:

$$R_{\mathrm{GlcNAc}} = \frac{C_{2,\mathrm{GlcNAc}}}{C_{1,\mathrm{GlcNAc}}} \quad (1)$$

$$R_{\mathrm{GlcN}} = \frac{C_{2,\mathrm{GlcN}}}{C_{1,\mathrm{GlcN}}} \quad (2)$$

where R_{GlcNAc} is the recovery of GlcNAc (mol/mol), R_{GlcN} is the recovery of GlcN (mol/mol), $C_{1,\mathrm{GlcNAc}}$ and $C_{1,\mathrm{GlcN}}$ are known respective concentrations of GlcNAc and GlcN before hydrolysis, and $C_{2,\mathrm{GlcNAc}}$ and $C_{2,\mathrm{GlcN}}$ are their concentrations after hydrolysis as measured by HPLC and spectrophotometric methods, respectively.

GlcNAc and GlcN yields as well as recovery of the reference materials were calculated according to the following formulas:

$$Y_{\mathrm{GlcNAc}} = \frac{m_{\mathrm{Ac}} \times 203}{R_{\mathrm{GlcNAc}} \times W} \quad (3)$$

$$Y_{\mathrm{GlcN}} = \frac{\dfrac{m_{\mathrm{GlcN}}}{R_{\mathrm{GlcN}}} - \dfrac{m_{\mathrm{Ac}}}{R_{\mathrm{GlcNAc}}}}{W} \times 161 \quad (4)$$

$$R = Y_{\mathrm{GlcNAc}} + Y_{\mathrm{GlcN}} \quad (5)$$

where Y_{GlcNAc} is the GlcNAc yield (g/g), Y_{GlcN} is the GlcN yield (g/g), and R is the total recovery from the chitin and chitosan reference materials in the form of GlcNAc and GlcN (g/g). W is the sample weight (g), m_{Ac} is moles of acetic acid, and m_{GlcN} is total moles of GlcN. R, Y_{GlcNAc}, and Y_{GlcN} of the reference materials are presented in **Table 1**. The same equations were used for measurement of GlcN and GlcNAc in the fungal materials.

Cultivation of Fungi and Preparation of Alkali Insoluble Material (AIM). *R. oryzae* CCUG 28958, *M. indicus* CCUG 22424, and *R. pusillus* CCUG 11292 were obtained from Culture Collection University of Gothenburg (CCUG, Göteborg, Sweden). These fungi were maintained on agar slants containing (g/L): D-glucose (40), soy peptone (10), and agar (20), at pH 5.5 by incubating them at 32 °C for 5 days.

Cultivations were performed in 200 mL working volumes in 500 mL cotton-plugged Erlenmeyer flasks in a shaker incubator at 32 °C and 150 rpm. The medium contained (g/L): $(NH_4)_2SO_4$ (7.5), KH_2PO_4 (3.5), $MgSO_4 \cdot 7H_2O$ (0.75), $CaCl_2 \cdot 2H_2O$ (1.0), yeast extract (5), and glucose (50). It was autoclaved at 121 °C for 20 min. The pH was adjusted to 5.5 ± 0.2 at the beginning of the cultivation. The medium was inoculated with 1 mL of suspension containing 5×10^7 spores/mL. At the end of the desired fermentation period, mycelia were harvested by filtration, washed three times with water, and dried at 50 °C. Cell wall material was prepared according to the method presented by Synowiecki et al. (*5*) with some modifications. Dry mycelia of each strain were treated with 30 mL of 0.5 M sodium hydroxide per g of dry mycelia at 90 °C overnight. The AIM was separated by centrifugation (10 min, 4000*g*) and washed 10 times with distilled water, dried at 50 °C, weighed, and stored. All experiments were performed at least in duplicate, and results are presented as averages.

Table 1. Yields of GlcN (Y_{GlcN}) and GlcNAc (Y_{GlcNAc}) as well as Total Recovery (R) of Various Chitin and Chitosan Reference Material

substrate	R[a]	Y_{GlcNAc}[b]	Y_{GlcN}[c]
chitosan, high MW	0.871 ± 0.019	0.209 ± 0.006	0.662 ± 0.014
chitosan, medium MW	0.924 ± 0.061	0.198 ± 0.010	0.726 ± 0.058
chitosan, low MW	0.864 ± 0.015	0.086 ± 0.003	0.778 ± 0.012
chitosan[d]	0.857 ± 0.057	0.114 ± 0.003	0.743 ± 0.056
chitin	0.901 ± 0.058	0.709 ± 0.037	0.192 ± 0.095

[a] Recovery of reference materials in the form of GlcN and GlcNAc (g/g substrate). [b] GlcNAc yield of reference materials (g GlcNAc/g substrate). [c] GlcN yield of reference materials (g GlcN/g substrate). [d] This chitosan was provided as flakes, from the supplier.

RESULTS

GlcN and GlcNAc Measurements of Reference Materials. The newly developed method shown in **Figure 1** was applied on reference samples of chitin and chitosan, and the GlcN and GlcNAc concentrations as well as total recoveries of them are recorded (**Table 1**). During the initial treatment with 72% sulfuric acid at room temperature for 90 min, all of the materials dissolved, indicating improved access to the polysaccharide chains. After the subsequent treatment with 2.48% sulfuric acid at 121 °C for 1 h, almost all of the GlcNAc residues had been deacetylated. Depolymerization of chitin and chitosan chains might also occur during the treatment with sulfuric acid. After the sulfuric acid treatments, the samples were completely clear in the hot sulfuric acid solution; however, after they were cooled to room temperature, precipitation appeared. Because chitosan is soluble in dilute sulfuric acid only when the solution is hot (*14*), precipitation indicated that depolymerization of chitosan was not complete after the sulfuric acid hydrolyses. Further depolymerization of deacetylated chains of chitin and chitosan was achieved with nitrous acid solution, which produced the deaminated derivative of GlcN (*10*). The recoveries of the reference materials varied between 86 and 92%. During hydrolysis with sulfuric acid, GlcN might be destroyed, and acetic acid librated from the GlcNAc residues might be evaporated. Therefore, pure GlcN and GlcNAc were treated at the same conditions, and their recoveries were calculated according to eqs 1 and 2 (0.920 ± 0.009 and 0.953 ± 0.018, respectively) and were used as correction factors in the eqs 3 and 4. This type of calibration has been developed and accepted for hydrolysis of lignocelluloses with sulfuric acid (*13*). This method was applied to study the chitin and chitosan contents of cell wall preparations (AIM) of three strains of zygomycetes at different growing times.

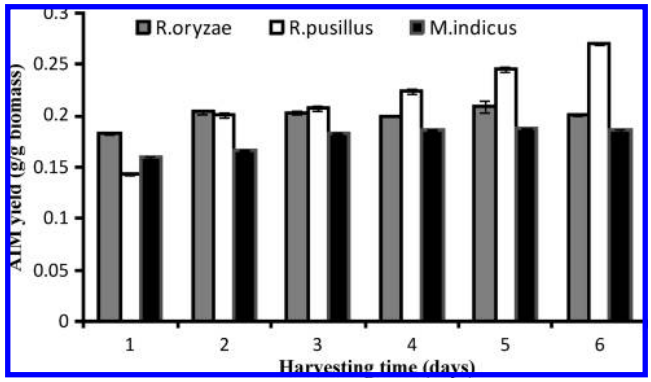

Figure 2. Yield (g/g biomass) of AIM in *R. oryzae* (gray), *R. pusillus* (white), and *M. indicus* (black).

Effect of Cultivation Time on Concentration of AIM. The three strains of zygomycetes *R. oryzae*, *M. indicus*, and *R. pusillus* were cultivated in shake flasks in a semisynthetic medium containing 50 g/L glucose, and their biomasses were harvested at 1 day intervals for 6 days. The AIMs were prepared from the fungal biomasses, and the yields of AIM were measured (**Figure 2**). The concentration of AIM in the biomass of *R. oryzae* was 18.3% of the dry weight of the cells after the first day of cultivation and was then around 20.0% during the following days (**Figure 2**). The AIM of *R. pusillus* was 14.4% of its biomass after the first day and increased with an increase in the cultivation time up to 27.1% of the biomass after 6 days of cultivation (**Figure 2**). The AIM in *M. indicus* was 16.0% of the biomass after the first day and increased to 18.6% within 3 days and then remained almost constant until the sixth day (**Figure 2**).

GlcN and GlcNAc Content of AIM. The newly developed method for measuring GlcN and GlcNAc was used for the determination of these two components in the AIM of the zygomycetes described above. As shown in **Figure 3**, the GlcN concentration of AIM of *R. oryzae* and *M. indicus* was almost constant during the 6 days of cultivations, while in *R. pusillus*, it decreased slowly. In contrast, the concentration of GlcNAc in AIM increased markedly in *R. pusillus* and somewhat less so in *R. oryzae* (**Figure 3**). For *M. indicus*, the GlcNAc concentration of AIM remained almost constant during the cultivation period (**Figure 3**). Throughout the cultivation period, after 6 days of cultivation, GlcN and GlcNAc made up 69.2 ± 3.6% of AIM of *R. oryzae*, 80.7 ± 2.6% of *R. pusillus*, and 65.5 ± 2.4% of *M. indicus* within 6 days of cultivation (**Figure 3**).

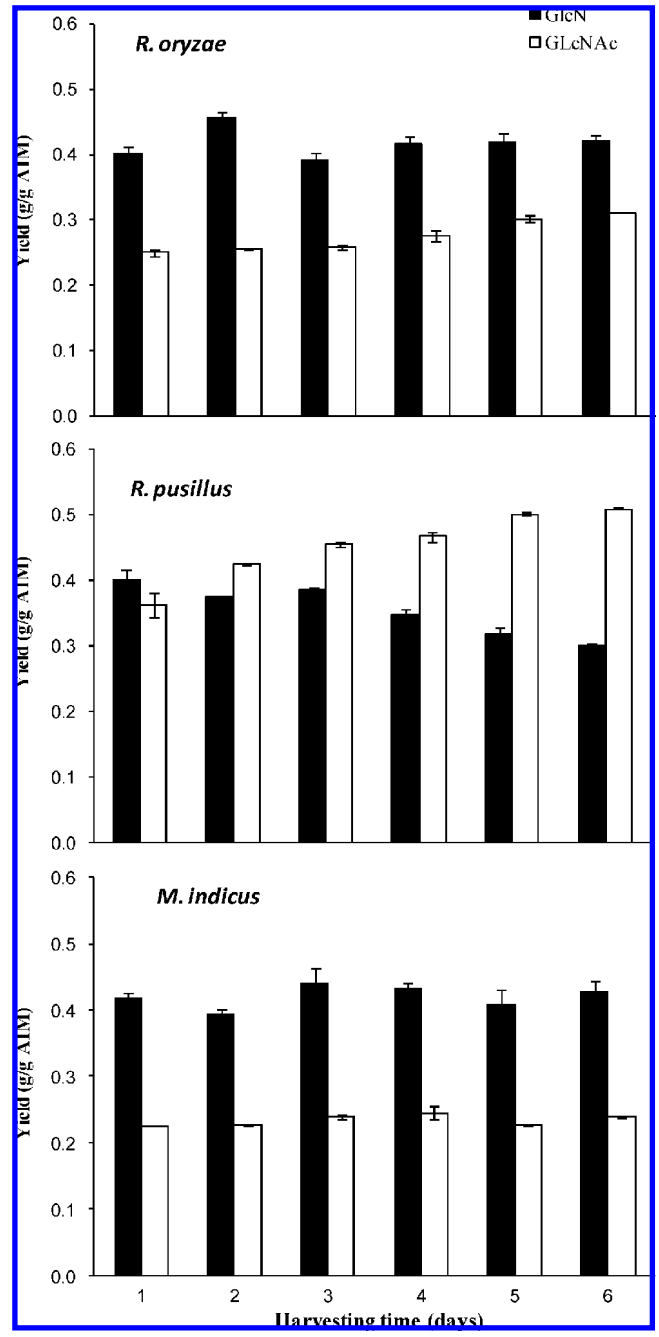

Figure 3. GlcN (black) and GlcNAc (white) concentration of AIM (g/g AIM) in different harvesting times (1−6 days) for *R. oryzae*, *R. pusillus*, and *M. indicus*.

DISCUSSION

In this work, a method for quantifying the GlcN and GlcNAc content of the AIM of zygomycetes biomass and other chitin-/chitosan-containing materials was developed. This method is based on the previous reports on deacetylation of chitin by sulfuric acid (*11, 12*), the temperature-dependent solubility of chitosan in sulfuric acid (*14*), and its unique degradability in nitrous acid solutions, which is not shared with other materials of fungal cell wall such as glucan and mannan (*8, 10*), in combination with principles employed for the hydrolysis of lignocelluloses (*13*).

In previous reports, hydrochloric acid was used for the hydrolysis of chitin and chitosan prior to GlcN analysis (*2*−*7*). However, hydrolysis by HCl is usually a time-consuming process to achieve complete depolymerization and high yield. Bosworth and Scott (*3*) reported that the maximum yield of hydrolysis of chitosan with 5.5 N HCl at 102 °C was not reached in less than 23 h. Chen and Chio (*7*) recovered only 63.7% of chitin as GlcN after 60 min of hydrolysis with 2.5 M HCl at 140 °C. Wu et al. (*6*) used 6 M HCl at 110 °C and recovered 93.0% of chitosan after 3 h and only 68.7% of chitin after 12 h as GlcN. Zhu et al. (*2*) got 85.3% recovery of chitin at 110 °C after 4 h. In all of these works, the sum of free and acetylated GlcN was reported. Our new method measures the GlcN and GlcNAc concentrations separately. It is therefore difficult to make a direct comparison between the results of the two methods. The sum of the GlcN and GlcNAc yields from the different chitin and chitosan reference preparations with our new method were 86−92%, indicating high performance of the method for the analysis of materials containing these polymers (**Table 1**).

In earlier methods, GlcN has been quantified by hydrolysis in HCl solutions. However, part of the sample that was not depolymerized by HCl and stayed in the solid form was not included in the measurement (*2, 3, 6, 7*). In the new method, sulfuric acid deacetylates the samples and produces polyglucosamine chains with different degrees of polymerization that are completely soluble in hot dilute sulfuric acid (*14*). Because the samples are taken from the hot hydrolyzates, the chitosan is not precipitated from the solution. In this case, the whole initial sample is considered for the estimation of GlcN content.

Characterization of AIM of cell wall preparations by the new method showed conspicuous differences between the species tested (**Figures 2** and **3**). During the 6 day cultivation period, *R. pusillus* nearly doubled the AIM yield and increased the concentration of GlcNAc in AIM by 40%, whereas the concentration of GlcN in AIM decreased by 25%. In samples taken from cultures of *R. pusillus* after 2 day or longer cultivation periods, GlcNAc dominated, whereas in all remaining samples, GlcN dominated. During the cultivation period, *M. indicus* showed an increase of the AIM yield (**Figure 2**), whereas in *R. oryzae*, there was a slight increase of the GlcNAc yield (**Figure 3**). Taken together, there seems to be an increase of the GlcNAc concentration of the biomass as the cultivation proceeds. However, the differences were great between the species. Whether they are due to dissimilarities with respect to cell wall synthesis or caused indirectly as consequences of differences in carbohydrate metabolism (*16*) needs further investigation. The pathway of chitosan synthesis in the cell wall appears to proceed by tandem action of chitin synthase and chitin deacetylase. The former produces chitin chains, while the latter deacetylates them as soon as they reach a critical length. In the old cells, crystallization of chitin occurs in the cell wall to increase the cell resistance against environmental stresses. This process at the same time decreases the rate of deacetylation of chitin, because chitin deacetylase is not able to deacetylate crystalline chitin (*17*−*20*). Stopping the GlcN production in the fungal cell wall skeleton in old cells in our work might confirm this hypothesis. Among the three strains, the AIM fraction was increased obviously in cultivation of *R. pusillus* during the 6 days (**Figure 2**). This might show the thickening of the cell wall that is accompanied with increasing the GlcNAc content and decreasing the fraction of other components such as GlcN in the AIM.

A method has been developed using two steps of sulfuric acid hydrolysis and one step of nitrous acid treatment to convert chitin and chitosan into anhydromannose and acetic acid with high yield. This procedure has been applied for the estimation of GlcN and GlcNAc in AIMs of fungal cell walls from *M. indicus*, *R. pucillus*, and *R. oryzae* showing great differences between the species and the time of cultivation.

LITERATURE CITED

(1) Rinaudo, M. Chitin and chitosan: Properties and applications. *Prog. Polym. Sci.* **2006**, *31* (7), 603–632.

(2) Zhu, X. L.; Cai, J. B.; Yang, J.; Su, Q. D. Determination of glucosamine in impure chitin samples by high-performance liquid chromatography. *Carbohydr. Res.* **2005**, *340* (10), 1732–1738.

(3) Bosworth, T. R.; Scott, J. E. A specific fluorometric assay for hexosamines in glycosaminoglycans, based on deaminative cleavage with nitrous acid. *Anal. Biochem.* **1994**, *223* (2), 266–273.

(4) Cousin, M. A. Chitin as a measure of mold contamination of agricultural commodities and foods. *J. Food Prot.* **1996**, *59* (1), 7381.

(5) Synowiecki, J.; AlKhateeb, N. Mycelia of *Mucor rouxii* as a source of chitin and chitosan. *Food Chem.* **1997**, *60* (4), 605–610.

(6) Wu, T.; Zivanovic, S.; Draughon, F. A.; Sams, C. E. Chitin and chitosan-value-added products from mushroom waste. *J. Agric. Food Chem.* **2004**, *52* (26), 7905–7910.

(7) Chen, W. L.; Chiou, R. Y. Y. A modified chemical procedure for rapid determination of glucosamine and its application for estimation of mold growth in peanut kernels and koji. *J. Agric. Food Chem.* **1999**, *47* (5), 1999–2004.

(8) Datema, R. W.; Vandenende, H. Hyphal wall of *Mucor mudeco*. 2. Hexosamine containing polymers. *Eur. J. Biochem.* **1977**, *80* (2), 621–626.

(9) Ruiz-Herrera, J. *Fungal Cell Wall: Structure, Synthesis, and Assembly*; CRC Press: Boca Raton, 1992; p 248.

(10) Ng, C. H.; Hein, S.; Ogawa, K.; Chandrkrachang, S.; Stevens, W. F. Distribution of D-glucosamine moieties in heterogeneously deacetylated cuttlefish chitin. *Carbohydr. Polym.* **2007**, *69* (2), 382–390.

(11) Niola, F.; Basora, N.; Chornet, E.; Vidal, P. F. A rapid method for the determination of the degree of *N*-acetylation of chitin-chitosan samples by acid hydrolysis and HPLC. *Carbohydr. Res.* **1993**, *238*, 1–9.

(12) Ng, C. H.; Hein, S.; Chandrkrachang, S.; Stevens, W. F. Evaluation of an improved acid hydrolysis-HPLC assay for the acetyl content in chitin and chitosan. *J. Biomed. Mater. Res. B: Appl. Biomater.* **2006**, *76B* (1), 155–160.

(13) Ruiz, R.; Ehrman, T. Chemical Analysis and Testing Task, determination of carbohydrates in biomass by high performance liquid chromatography. Laboratory Analytical Procedure, NREL LAP-002, Technical Report, office of energy efficiency and renewable energy, a national laboratory of the U.S. Department of energy; 1996.

(14) Zamani, A.; Edebo, L.; Sjostrom, B.; Taherzadeh, M. J. Extraction and precipitation of chitosan from cell wall of zygomycetes fungi by dilute sulfuric acid. *Biomacromolecules* **2007**, *8* (12), 3786–3790.

(15) Plassard, C. S.; Mousain, D. G.; Salsac, L. E. Estimation of mycelial growth of basidiomycetes by means of chitin determination. *Phytochemistry* **1982**, *21* (2), 345–348.

(16) Millati, R.; Edebo, L.; Taherzadeh, M. J. Performance of *Rhizopus, Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and wood hydrolyzates. *Enzyme Microb. Technol.* **2005**, *36* (2−3), 294–300.

(17) Davis, L. L.; Bartnickigarcia, S. Chitosan synthesis by the tandem action of chitin synthetase and chitin deacetylase from *Mucor rouxii*. *Biochemistry* **1984**, *23* (6), 1065–1073.

(18) Kafetzopoulos, D.; Martinou, A.; Bouriotis, V. Bioconversion of chitin to chitosan, purification and characterization of chitin deacetylase from *Mucor rouxii*. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (7), 2564–2568.

(19) Tan, S. C.; Tan, T. K.; Wong, S. M.; Khor, E. The chitosan yield of zygomycetes at their optimum harvesting time. *Carbohydr. Polym.* **1996**, *30* (4), 239–242.

(20) Tsigos, I.; Martinou, A.; Kafetzopoulos, D.; Bouriotis, V. Chitin deacetylases: New, versatile tools in biotechnology. *Trends Biotechnol.* **2000**, *18* (7), 305–312.

Received for review May 12, 2008. Revised manuscript received July 24, 2008. Accepted July 29, 2008. This work was financed by the Swedish Knowledge Foundation (KK-Stiftelsen) in collaboration with Medical Equipment Development AB (Sweden) and Rexcell AB (Sweden).

JF801478J