Determination of Cellular Carbohydrates in Peanut Fungal Pathogens and Baker's Yeast by Capillary Electrophoresis and Electrochromatography

Minquan Zhang,[†] Hassan A. Melouk,[‡] Kelly Chenault,[‡] and Ziad El Rassi*[,†]

Department of Chemistry, and Agricultural Research Service, U.S. Department of Agriculture, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, Oklahoma 74078

In this work, the quantitation of cellular carbohydrates, namely chitin and glucan, in peanut fungal pathogens and baker's yeast was carried out by capillary electrophoresis (CE) and capillary electrochromatography (CEC). The chitin and glucan of the fungi were hydrolyzed by the enzymes chitinase and glucanase, respectively, to their corresponding sugar monomers *N*-acetylglucosamine (GlcNAc) and glucose (Glc). These two monosaccharides were then tagged with 6-aminoquinoline (6-AQ) to allow their separation and detection in CE and CEC. The 6-AQ derivatives of GlcNAc and Glc formed the basis for the determination by CE and CEC of chitin and glucan in peanut fungi and baker's yeast. Several parameters affecting the separation of the 6-AQ derivatives of GlcNAc and Glc, including the separation voltage and the composition of the running electrolyte, were investigated. Under the optimized separation conditions, the contents of cellular carbohydrates including *N*-acetylglucosamine, chitin, glucose, and glucan in some fungi, such as *Sclerotinia minor*, *Sclerotium rolfsii*, and baker's yeast, were successfully determined. The method described here allowed the assessment of genetic differences in *Sclerotium rolfsii* isolates from various locations.

Keywords: *Capillary electrophoresis; capillary electrochromatography; cellular carbohydrates; Sclerotinia minor; Sclerotium rolfsii; baker's yeast; fungal pathogens*

INTRODUCTION

Peanut is attacked by several pathogens that adversely affect its growth and productivity. Two of the most important fungal pathogens are *Sclerotinia minor* and *Sclerotium rolfsii* (*1*). Genetic resistant is an attractive alternative to chemical management of these pathogens. Cell walls of many fungi contain chitin and glucan (*2*). Chitinase and glucanase genes are being introduced in peanut via microprojectile bombardment to produce transgenic peanut lines with elevated activity of these hydrolase enzymes for possible action against cell walls of fungal pathogens (*3*). Therefore, accurate determination of concentration of chitin and glucan in the cell wall of these fungal pathogens will assist in a better interpretation of disease reaction by these fungi on transgenic peanut plants.

Capillary electrophoresis (CE), and more recently capillary electrochromatography (CEC), have been proven useful in carbohydrate analysis. For recent reviews on carbohydrate analysis by CE and CEC, see refs *4*−*7*. These two microcolumn separation techniques offer unsurpassed separation efficiency and resolution, and they require minute amounts of samples and reagents. However, to the best of our knowledge, the potentials of CE and CEC methodologies in the analysis of cellular carbohydrates in fungal pathogens have not yet been demonstrated. This report is concerned with the development of CE and CEC methods for the determination of glucan and chitin content of two peanut fungal pathogens (e.g., *Sclerotinia minor* and *Sclerotium rolfsii*) and the comparison of their carbohydrate content with that of baker's yeast. Baker's yeast is known for its glucan content (*8*). In addition, and similar to *Sclerotinia minor*, baker's yeast is a member of the Class *Ascomycete*, a fact that makes baker's yeast a reference material to which one can compare the carbohydrate content of the peanut fungal pathogens under investigation.

Glucose (Glc) and *N*-acetylglucosamine (GlcNAc) are the constituent monosaccharides of the two polysaccharides glucan and chitin (*2*), respectively, which are the cellular carbohydrates of fungal pathogens. Therefore, these two monosaccharides can form the basis for the determination of chitin and glucan contents of the fungal pathogens under investigation by CE and CEC. In this regard, the two monosaccharides Glc and GlcNAc were first derivatized with 6-aminoquinoline (6-AQ) to impart the two monosaccharides with the charge necessary for CE, the hydrophobicity needed for CEC, and the chromophore required for the sensitive detection by UV (also fluorescence). The optimal conditions for the separation of the 6-AQ derivatives of Glc and GlcNAc by CE and CEC were then established.

MATERIALS AND METHODS

Instruments. A P/ACE 5010 capillary electrophoresis system from Beckman Instrument Inc. (Fullerton, CA) equipped with a UV detector and a data handling system comprising an IBM personal computer and Gold software was used for the CE and CEC studies. A Shandon column packer from Keystone Scientific (Bellefonte, PA) was employed for the CEC capillary column packing.

* To whom correspondence should be addressed. Tel: 405-744-5931. Fax: 405-744-6007. E-mail: zelrassi@biochem.okstate.edu.

[†] Department of Chemistry.

[‡] Agricultural Research Service.

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Chemicals and Reagents. Nucleosil silica with a 5-μm average particle diameter, 120 Å average pore diameter, and 200 m^2/g specific surface area was purchased from Alltech Associates Inc. (Deerfield, IL). This silica was used for in-house preparation of the octadecyl-silica (ODS) stationary phase (*9*, *10*). Glass microfiber filter papers were from Whatman Inc. (Clifton, NJ). HPLC grade acetonitrile, methanol, and 2-propanol were from Baxter (McGaw Park, IL). Sodium phosphate monobasic and dibasic and glacial acetic acid were obtained from Mallinckrodt (Paris, KY). Sodium cyanoborohydride, 6-aminoquinoline (6-AQ), and D-(+)-glucose (Glc) were purchased from Aldrich (Milwaukee, WI). *N*-Acetyl-D-glucosamine (GlcNAc), chitin, methanesulfonic acid (MS), glucan, dithiothreitol, and sodium borohydride were obtained from Sigma (St. Louis, MO). Chitinase from *Streptomyces griseus* was also obtained from Sigma. Also, chitinase and glucanase were extracted from peanut leaf tissue. Citric acid was from Fisher (Fair Lawn, NJ). Mycelial fungal preparations were provided by Dr. H. Melouk, USDA-ARS, Department of Entomology and Plant Pathology, Oklahoma State University. Baker's yeast was from Burns Philip Food, Inc. (Fenton, MO).

Capillary Columns and Column Packing. Fused-silica capillary columns of 50 and 100 μm i.d. and 365 μm o.d. were from Polymicro Technology (Phoenix, AZ), and were used for CE and CEC separations, respectively. A slurry packing technique was used to prepare the CEC column as previously reported (*11*−*14*). Acetone was used to prepare the suspension of the ODS stationary phase, and 2-propanol was used as the packing solvent. A moderately strong sintered porous frit of 0.5−1.0 mm in length was made at the outlet end of the fused-silica capillary by first tapping the capillary end into bare 5-μm silica moistened with deionized water, and then by heating the capillary tip over a Bunsen burner for about 1 min. Then the capillary column was packed with the stationary phase at 3000 psi for a period of 1 h. The packed column thus obtained was flushed with deionized water for approximately 30 min to 1 h and cut to the desired length. The inlet retaining frit was made in the same way as the outlet frit, followed by flushing and preconditioning the column with the degassed mobile phase for about 30 min. At last the column was mounted into a Beckman capillary cartridge. In our work, whole packed capillary columns were produced with a detection window at 6.5 cm from the outlet end of the column. The detection window was made before packing the column by burning off the polyimide coating of the capillary with a thermal wire stripper. Whole column packing provides an even conductance and a uniform electroosmotic flow velocity across the column, thus resulting in much reduced bubble formation (*13*−*16*).

Capillary Electrophoresis and Electrochromatography Preconditioning. For CE, the new fused-silica capillary columns were flushed successively with 0.10 M NaOH, deionized water, and running buffer. The capillary column was allowed to equilibrate with running buffer for 20 min before each injection, and after each run the running buffer was renewed. For CEC, the capillary column was first equilibrated with the mobile phase at 2 kV for about 1 h until the detector output was constant before the experiment started. The mobile phase was renewed after several runs to ensure reproducible separations.

Colloidal Chitin Extraction from Chitin Powder, Fungi, and Baker's Yeast. Chitin powder (0.1000 g) from crab shell was dissolved into 6 mL of 56% aqueous methanesulfonic acid (MS) for about 30 min below 10 °C with occasional stirring (*17*). The solution was filtered through glass microfiber filter paper, and the filtrate was added dropwise into 60 mL of deionized ice−water under vigorous stirring. The precipitates were washed with deionized water 2 times, and collected by centrifugation for 20 min every time, then transferred into a culture dish, and dried overnight in open air in the laboratory hood to yield colloidal chitin−methanesulfonic acid (colloidal chitin−MS). This yielded 0.1070 g of colloidal chitin−MS.

Ground fungi powder (1.000 g) was treated in the same way as the chitin powder except that 60 mL of aqueous methanesulfonic was used for dissolving the fungi, and 600 mL of deionized ice−water was employed for precipitating the product. This resulted in 0.2400 g of colloidal chitin−MS.

Ground baker's yeast (1.000 g) was also treated in the same way as for fungi to extract the chitin. This yielded 0.1432 g of colloidal chitin−MS.

Glucan Extraction from Glucan, Fungi, and Baker's Yeast. Glucan (0.0100 g) was extracted with 3% w/w sodium hydroxide and treated with acetate buffer using the method of Bacon et al. (*8*). First, the glucan was crumbled into 1 mL of 3% (w/w) NaOH at 75−80 °C, and the mixture was stirred for 1 h. The suspension was poured into 3 mL of deionized water at room temperature and stirred for a further 1 h, and then centrifuged for 20 min. Two further extractions were made in a similar way, beginning with suspension of the residue in 2 mL of 3% NaOH. Second, the resulting residue was suspended in 2 mL of water, and the pH was adjusted to 7.0 with acetic acid. To this suspension, 0.6 mL of 0.5 M sodium acetate buffer, pH 5.0, was added, the pH was adjusted to 5.0, and the suspension was stirred for 3 h at 75 °C. Third, the supernatant was discarded and the residue was washed twice by centrifugation with 2 mL of water and then suspended in 1 mL of water. To this suspension 0.002 g of sodium borohydride was added, and the mixture was stirred for 16 h at 22 °C. Fourth, the wet residue was extracted two times at 75 °C with 1 mL of 3% NaOH containing 0.001 g sodium borohydride. The total volume of extracted glucan solution from glucan was 2.0 mL.

Freeze-dried fungal mycelia (0.1000 g) and baker's yeast (0.1000 g) were extracted in the same way as above, and the total volumes of extracted glucan solution from fungi and baker's yeast were 7.0 mL each.

Enzymatic Hydrolysis of Chitin and the Chitin from Fungi and Baker's Yeast with Chitinase. An amount of 0.0200 g of the above colloidal chitin−MS substrate suspended in 2.0 mL of buffer (50 mM citric acid adjusted to pH 6.6 with 100 mM Na_2HPO_4) was mixed with 1.2 mg of chitinase from *Streptomyces griseus* (1.07 U/mg) dissolved in 4.0 mL of buffer (0.30 mg of chitinase/mL of above buffer), and incubated in a test vial at 37 °C with occasional shaking for 1 hr.

The colloidal chitin−MS substrates extracted from fungi and from baker's yeast were enzymatically hydrolyzed in the same way as that from the chitin powder.

Enzymatic Hydrolysis of Glucan and the Glucan from Fungi and Baker's Yeast with Glucanase. First, the enzyme for hydrolyzing glucan, the glucan from fungi, and from baker's yeast was extracted by the following method (*18*): grind 0.5 g of fresh peanut leaf tissue in extraction buffer (100 mM NaOAc with 2 mM dithiothreitol, adjusted to pH 5.0 with acetic acid); collect extract by centrifugation at 2500 rpm for 15 min. Then, per reaction, mix 1.0 mL of peanut leaf tissue extract, 0.5 mL of reaction buffer (100 mM NaOAc, pH 5.0), and 1.0 mL of substrate from 2.0 mL of extracted glucan solution or 1.0 mL of substrate from 7.0 mL of extracted glucan solution from fungi and from baker's yeast. Incubate the mixture at 37 °C for 60 min, stop the reactions by the addition of 50 μL of 2.0 M HCl, incubate on ice for 30 min, and centrifuge at 2500 rpm for 10 min.

Precolumn Derivatization of Carbohydrates. Standard Glc and GlcNAc, as well as those derived from chitin and glucan, were derivatized with 6-AQ via reductive amination as previously reported (*6*, *19*). Briefly, 0.50 M aqueous solution of 6-AQ was prepared and titrated to pH 5.0 with glacial acetic acid. Then 40 mg of sodium cyanoborohydride was added to 1 mL of the 6-AQ solution just before mixing it with a 1-mL aliquot of the hydrolyzed colloidal chitin−MS substrate extracted from chitin or fungi or baker's yeast. The same was also performed on the hydrolyzed extract from glucan or fungi or baker's yeast. The reaction mixture was incubated at 72 °C for 24 h.

For the preparation of 6-AQ-Glc and GlcNAc standard derivatives, 0.25 M aqueous solution of 6-AQ was prepared and titrated to pH 5.0 with glacial acetic acid. Then 40 mg of sodium cyanoborohydride was added to 2 mL of the 6-AQ

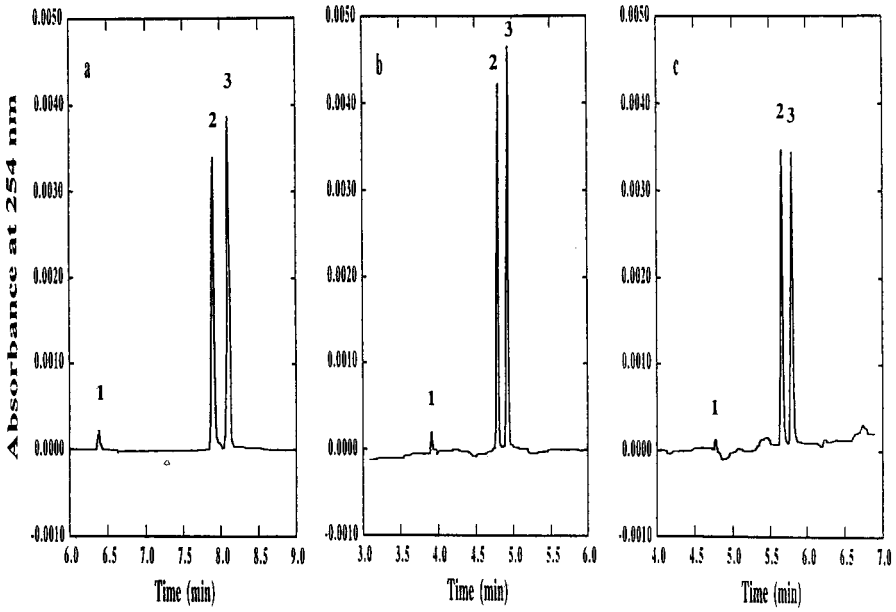

Figure 1. Capillary electropherograms of 6-aminoquinolyl derivatives of glucose and *N*-acetylglucosamine. Conditions: capillary, fused-silica tube, 50 cm (to detector), 57 cm (total length) × 50 μm i.d.; (a) running electrolyte, 100 mM sodium phosphate monobasic, pH 5.0; running voltage, 15 kV; (b) same running electrolyte as in (a) but running voltage is 20 kV; (c) running electrolyte, 100 mM sodium phosphate monobasic containing 50 mM tetrabutylammonium bromide, pH 5.0; running voltage, 20 kV. In all cases, injection pressure was 20 psi for 1 s. Solute order: 1, excess 6-AQ; 2, 6-AQ−Glc; 3, 6-AQ−GlcNAc.

solution just prior to the addition of 0.1106 g of GlcNAc and 0.0991 g of Glc. The reaction mixture was incubated at 72 °C for 24 h.

RESULTS AND DISCUSSION

CE and CEC of 6-Aminoquinoline Derivatives of Standards Glucose and *N*-Acetylglucosamine. As indicated in the Introduction, Glc and GlcNAc are the monosaccharide constituents of glucan and chitin, respectively (*2*). Therefore, it was first necessary to establish the separation conditions of the 6-AQ derivatives of the two monosaccharides by CE and CEC. Figure 1 shows the CE electropherograms of the 6-AQ derivatives of standards Glc and GlcNAc under different separation conditions. Increasing the running voltage from 15 to 20 kV benefited the separation by decreasing the analysis time by more than 41%, as well as by sharpening the peaks (compare Figure 1a and b). In fact, the theoretical plate numbers for 6-AQ−Glc and 6-AQ−GlcNAc increased from 62000 to 83000 and from 79000 to 110000, respectively, when the voltage was increased from 15 to 20 kV. Keeping everything else the same and adding 50 mM tetrabutylammonium bromide to the running electrolyte yielded a marginal increase in resolution at the expense of longer analysis time, compare Figure 1b and c. However, upon adding tetrabutylammonium bromide to the running electrolyte, the theoretical plate numbers for 6-AQ−Glc and 6-AQ−GlcNAc decreased from 83000 to 30000 and from 110000 to 40000, respectively. Therefore, the conditions of Figure 1b seem to be a good compromise as far as the analysis time, resolution, and separation efficiency are concerned.

Figure 2 is a CEC electrochromatogram of 6-AQ derivatives of standards Glc and GlcNAc. The solutes were well separated under the optimized electrochromatographic conditions using a mobile phase of 60% v/v acetonitrile and 40% v/v of 3.0 mM aqueous sodium phosphate buffer, pH 5.0. CEC seems to provide much higher resolution than CZE (compare Figure 1 to Figure 2). This difference is not surprising, because in CEC not only is the difference in charge-to-mass ratio influencing the solute's migration, but also the difference in the solute's polarity.

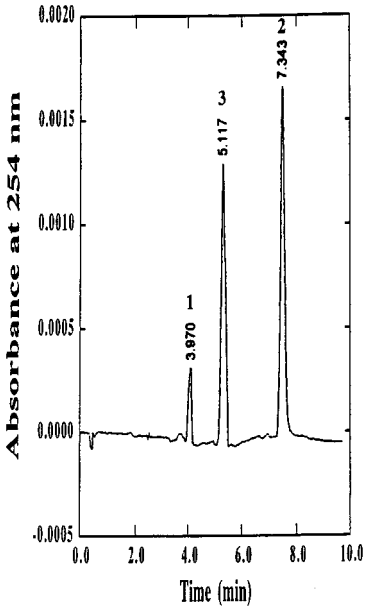

Figure 2. Capillary electrochromatogram of 6-aminoquinolyl derivatives of *N*-acetylglucosamine and glucose. Conditions: capillary, fused-silica tube, 20.5/27 cm × 100 μm i.d., whole packed with 5 μm ODS; mobile phase, 60% v/v acetonitrile and 40% v/v 3 mM sodium phosphate monobasic buffer, pH 5.0; running voltage, 15 kV; injection pressure at 20 psi for 1 s. Solute order: 1, excess 6-AQ; 2, 6-AQ−GlcNAc; 3, 6-AQ−Glc.

As shown in Figures 1 and 2, both CE and CEC are desirable for the analysis of the two 6-AQ sugar derivatives. Obviously, the two separation methods provided different selectivities. In CE, the elution order is 6-AQ−Glc and 6-AQ−GlcNAc, because the latter solute has a lower charge-to-mass-ratio, thus migrating behind 6-AQ−Glc. On the other hand, in CEC the elution order is the

reverse, indicating that 6-AQ−GlcNAc is more hydrophilic than 6-AQ−Glc.

Because CE and CEC seem to complement each other and there is no real advantage for one method over the other, CE was adopted in the rest of this study for the quantitative determination of Glc and GlcNAc in the various extracts. To further confirm the identity of the 6-AQ derivatives derived from real fungi samples, qualitative analyses were performed by both CE and CEC.

For the quantitative aspects of this study, calibration curves for 6-AQ−Glc and 6-AQ−GlcNAc were established by CE. The curves were linear over 2 orders of magnitude (i.e., from 0.0625 mM to 6.25 mM), with $R^2 = 0.99992$ and 0.99976 for 6-AQ−Glc and 6-AQ−GlcNAc, respectively. The detection limit ($S/N = 3$) for derivatized Glc and GlcNAc was about 1.2×10^{-5} M.

CE and CEC of Derivatized Chitin and Glucan Digests. To determine the chitin and glucan content of the fungal pathogens under investigation, standard chitin and glucan were enzymatically digested to liberate their monosaccharide constituents (i.e., GlcNAc and Glc, respectively) by subjecting the chitin and glucan to chitinase (from *Streptomyces griseus* or from peanut leaf tissue) and glucanase (from peanut leaf tissue), respectively. The specificities of chitinase and glucanase toward chitin and glucan, respectively, are well documented in the literature (*2*, *8*, *17*). The digests were then precolumn derivatized with 6-AQ and analyzed by CE and CEC under the same conditions of Figs 1b and 2, respectively. The 6-AQ derivatized digests of the chitin and glucan yielded a single major peak each in both CE and CEC whose migration time (CE) or retention time (CEC) corresponded perfectly to that of the standards 6-AQ−GlcNAc and 6-AQ−Glc.

It should be noted that the amount of GlcNAc released from chitin was the same regardless whether the chitin was digested by chitinase from peanut leaf tissue or by chitinase from *Streptomyces griseus*. Thus, a crude chitinase extract from peanut leaves is as effective as the purified chitinase from *Streptomyces griseus.*

Determination of Chitin and Glucan in Fungi by CE and CEC. Using the same methodologies as described in the two sections above, the fungi digests obtained by either chitinase or glucanase hydrolysis were subjected to 6-AQ derivatization followed by CE and CEC analysis. Figures 3a and 4a are the CE electropherogram and CEC electrochromatogram, respectively, of the chitin digest derived from *Sclerotinia minor* fungus. In both cases, one major peak corresponding to the migration time (CE) and retention time (CEC) of 6-AQ−GlcNAc was observed in addition to the reagent peak (i.e., 6-AQ peak). The % 6-AQ−GlcNAc content in the fungus is lower by a factor of ∼4.5 than that in standard chitin. It is interesting to note that the electropherogram and electrochromatogram of Figs 3a and 4a, respectively, do not show matrix interferences, thus demonstrating the selectivity of the 6-AQ derivatization. The 6-AQ derivatized chitin digest derived from the fungus was injected as is (i.e., without sample cleanup) into the capillary column.

Figures 3b and 4b are the CE electropherogram and CEC electrochromatogram, respectively, of the glucan digest derived from the *Sclerotinia minor* fungus. Again, and in both cases, one major peak corresponding to 6-AQ−Glc was observed in addition to the reagent peak.

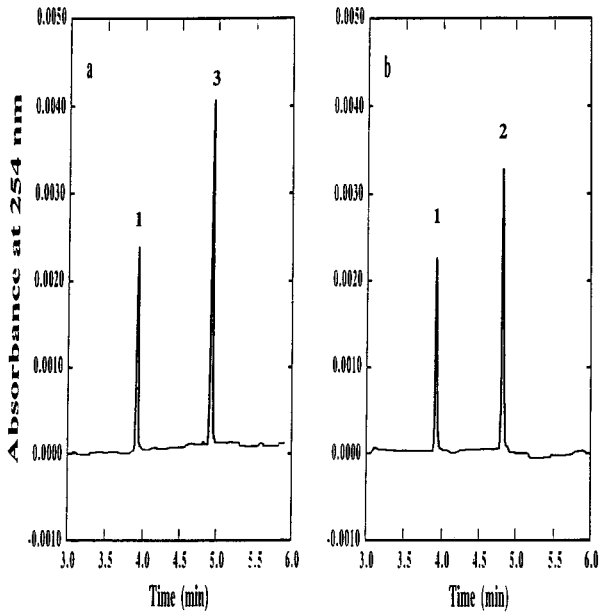

Figure 3. (a) CZE of chitin from *Sclerotinia minor* extracted with methanesulfonic acid, digested with chitinase, and derivatized with 6-AQ; and (b) CZE of glucan from *Sclerotinia minor* extracted with alkali, digested with glucanase from peanut leaf tissu, and derivatized with 6-AQ. Conditions: capillary, fused-silica tube, 50 cm (to detector), 57 cm (total length) × 50 μm i.d.; running electrolyte, 100 mM sodium phosphate monobasic, pH 5.0; running voltage, 20 kV; injection pressure , 20 psi for 1 s. Solute order: 1, excess 6-AQ; 2, 6-AQ−Glc; 3, 6-AQ−GlcNAc.

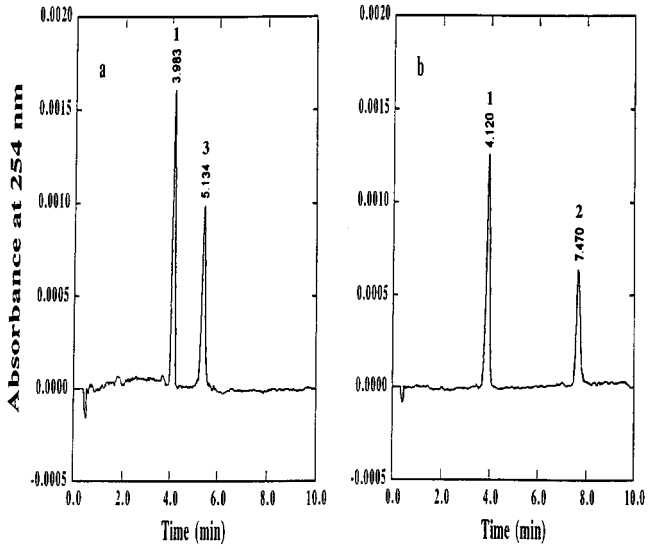

Figure 4. (a) CEC of chitin from *Sclerotinia minor* extracted with methanesulfonic acid, digested with chitinase, and derivatized with 6-AQ; and (b) CEC of glucan from *Sclerotinia minor* extracted with alkali, digested with glucanase from peanut leaf tissue, and derivatized with 6-AQ. Conditions: capillary, fused-silica tube, 20.5/27 cm × 100 μm i.d., whole packed with 5 μm ODS; mobile phase, 60% v/v acetonitrile and 40% v/v 3 mM sodium phosphate monobasic buffer, pH 5.0; running voltage, 15 kV; injection pressure, 20 psi for 1 s. Solute order: 1, 6-AQ, 2, 6-AQ−Glc; 3, 6-AQ−GlcNAc.

The % 6-AQ−Glc content in the fungus is lower by a factor of ∼28 than that in the standard glucan.

As stated earlier, the amount of GlcNAc, and in turn chitin, in the *Sclerotinia minor* fungus was the same regardless of whether the fungus was subjected to hydrolysis by chitinase from peanut leaf tissue or by

Table 1. GlcNAc, Chitin, Glc, and Glucan Content in Some Fungi and Baker's Yeast

fungus	% GlcNAc	% chitin	% glucose	% glucan
baker's yeast (*Saccharomyces spp*)	6.40	13.39	18.21	29.31
Sclerotinia minor	10.78	22.43	2.203	3.545
SR St[a]	7.486	15.54	4.937	7.946
SR FtC[a]	5.837	12.14	6.283	10.11
SR Q[a]	10.41	21.66	2.591	4.170
SR Y[a]	8.150	16.94	3.981	6.406
SR T[a]	15.23	31.67	1.637	2.634

[a] *Sclerotium rolfsii* isolates from various locations. St, isolate from Stillwater, OK; FtC, isolate from Fort Cobb, OK; Q, isolate from Quincy, FL; Y, isolate from Yoakum, TX; T, isolate from Tifton, GA.

chitinase from *Streptomyces griseus*. This indicates that the crude chitinase extract is as useful as the purified chitinase preparation in the digestion of the chitin from the fungus.

From Table 1, it can be seen that the GlcNAc content, and in turn the chitin content, of the fungi is about the same as or higher than that of the baker's yeast, whereas the Glc content, and subsequently the glucan content, of the fungi under study is much lower than that in the baker's yeast. An important observation which can be readily made from Table 1 is the fact that the *Sclerotium rolfsii* isolates from various locations yielded different chitin and glucan contents. *Sclerotium rolfsii* is a genetically diverse fungus, and therefore, differences in glucan and chitin content in the isolates may be attributed to genetic differences (*20*). Furthermore, with the exception of the *Sclerotium rolfsii* isolate from Tifton, GA, the *Sclerotinia minor* fungus has the highest chitin content and the lowest glucan content compared to those of the Sclerotium *rolfsii* fungus from any location. Furthermore, it is interesting to note that within the *Sclerotium rolfsii* isolates from various locations, the isolate with the highest chitin content yielded the lowest glucan content.

CONCLUSIONS

CE and CEC proved very suitable for side-by-side use in the rapid and accurate qualitative and quantitative analyses of chitin and glucan content of two peanut fungal pathogens and baker's yeast. In addition, the study revealed the differences in chitin and glucan content of the *Sclerotium rolfsii* fungus isolates from various locations. These differences are attributed to the genetic diversity of the fungus.

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