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Improved Colorimetric Determination of Cell Wall Chitin in Wood Decay Fungi

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The Svennerholm modification of the Elson-Morgan method for glucosamine analysis was evaluated for its applicability to the rapid determination of chitin in wood decay fungi. The evaluation included extent of chromogen interference, sensitivity, color stability, and hydrolysis conditions for maximum release of glucosamine from fungal cell walls. With our further modification, the Svennerholm method was shown to be suitable for rapid quantitative determination of fungal chitin without chromatographic separation of hydrolysate chromogens.

Hexosamines of biological materials have often been determined by the Elson-Morgan (8), Morgan-Elson (16), and deamination (7, 10, 21) methods. The Elson-Morgan method involves condensation of pyrrole derivatives (6, 13) with *N,N*-dimethyl-*p*-aminobenzaldehyde to form chromogens. The deamination method calls for the elimination of amine from glucosamine hydrochloride with nitrous acid to give 2,5-anhydro sugars, which then condense with indole (7), pyrrole (10), or benzothiazolone hydrazone hydrochloride (21) to form chromogens. Because of the complicated nature of the reactions and the composition of biological hydrolysates, many modifications to the Elson-Morgan and Morgan-Elson methods have been made to improve sensitivity (2, 9, 17) and overcome difficulties arising from color interference by neutral monosaccharides and amino acids (1, 17, 19) and instability of the color compounds (2, 19). These and other methods have been used to test for chitin in fungal cell walls.

We sought a simple and fast method to determine the chitin content of the cell walls of several wood decay fungi. Roseman's modification (3) of the Morgan-Elson method was rejected because it required extensive solvent purification of the fungal cell walls and probably loss of some chitin (18). The deamination method as modified by Tsuji (21) and used by Braid and Line (5) and the ninhydrin method used by Gurusiddaiah et al. (12) were rejected because they required time-consuming chromatographic separation of hydrolysate components. The Good (11), Enghofer (9), and Benson (1) procedures, which use the Morgan-Elson method without solvent purification of cell wall material, did not form any chromogens in our experiments, probably due to interference by neutral carbohydrates and amino acids in the hydrolysates. The Boas (4) modification of the Elson-Morgan method, used by Swift (20) to detect chitinous fungi in decayed wood, formed unstable chromogens for us. Svennerholm (19) modified the Blix (2) procedure of the Elson-Morgan method to quantify hexosamines of nervous tissues, but this method specifies elimination of interfering chromogens in the hydrolysates by ion-exchange separation or by dichromatic readings. Our preliminary efforts to determine the chitin content of wood decay fungi by this method, but without chromatographic separation, showed that absorbance by interfering chromogens in the hydrolysates was very small. To further test the applicability of our modified procedure to wood decay fungi, we evaluated the extent of chromogen interference, sensitivity, color stability, and hydrolysis conditions for maximum release of glucosamine from fungal cell walls.

MATERIALS AND METHODS

Thoroughly ruptured and water-washed cell walls of 13 species of wood decay fungi were chemically analyzed for chitin. The common brown-rot fungus *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. (Madison 617) was used to study chromogen interference in cell wall hydrolysates and the hydrolysis time needed for maximum yield of chitin. At least 4 mg of dry cell wall material from four replicate cultures of each fungal species was refluxed at 100°C for 4 h with 6 N hydrochloric acid. The ratio of cell wall to acid was 2 mg of wall to 1 ml of acid. After cooling to room temperature, hydrolysates were filtered, and two 0.2- to 1-ml samples of filtrate were withdrawn and evaporated to dryness at 45 to 50°C under reduced pressure. The dry hydrolysates were then redissolved in distilled water to make a solution containing 5 to 15 μg of glucosamine hydrochloride per ml of solution. Chromogen development of 1 ml samples of this dilute solution followed Svennerholm's procedure, except

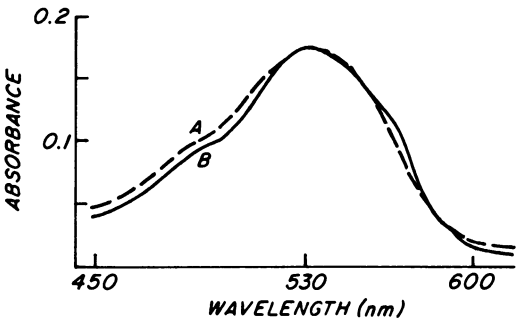

FIG. 1. Visible light absorption curves of the following reaction products, determined by the modified Svennerholm method. A, Glucosamine hydrochloride; B, cell wall hydrolysates (hydrolysates of cell walls after 4 h of reflux with 6 N hydrochloric acid).

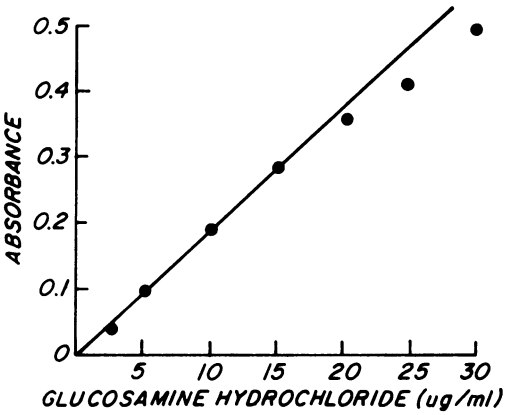

FIG. 2. Relationship of absorbance at 530 nm to concentration of glucosamine hydrochloride. Each value is the mean of four determinations.

that chromatographic separation of hydrolysate was dispensed with, one-half the recommended volume of reagents and solvents was used, and colorimetric measurements were performed after 30 min rather than 1 h of color development.

The following procedure was adopted. The dilute hydrolysate solution (1 ml) was added to 0.25 ml of 4% acetylacetone (4% acetylacetone in 1.25 N sodium carbonate [vol/vol]) and heated at 90°C for 1 h in a test tube covered with a Teflon-lined screwcap. After cooling, 2 ml of ethanol was added, with shaking to dissolve precipitates. Next 0.25 ml of Ehrlich reagent (1.6 g of *N*-*N*-dimethyl-*p*-aminobenzaldehyde in a 30:30 ml mixture of ethanol and concentrated HCl) was added. The color formed in the solution was measured in a 1-cm cuvette at 530 nm with a Bausch & Lomb Spectronic 600 spectrophotometer. Duplicate determinations were performed for each hydrolysate. The glucosamine hydrochloride content of cell wall hydrolysates was measured by comparing the absorbance of glucosamine hydrochloride with that of a standard. The chitin content of the cell walls was then calculated as the 1,4-anhydro-*N*-acetyl-2-deoxy-D-glucopyranose equivalent, with an adjustment of 7% for loss due to hydrolysis (see Fig. 3).

RESULTS AND DISCUSSION

The visible light absorption curve of the cell wall hydrolysates of *G. trabeum* determined by our modified Svennerholm method (Fig. 1) was similar to that of the glucosamine hydrochloride standard. This indicated that color interference at 530 nm by other substances in the hydrolysates was small. To verify this conclusion, we mixed monomers with a molar ratio comparable to the polymers found in some fungal cell walls (14, 15) with a known quantitiy of glucosamine hydrochloride and then analyzed the mixture. The results (Table 1) indicated that a negligible interference (0.54%) at 530 nm appeared in the presence of glucose and amino acids. Similar results have been reported by others (1, 2, 8) when glucose and amino acids in the hydrolysates are only several times in excess of the glucosamine hydrochloride. Because the chromogen interference in the hydrolysates was not significant, a direct determination of chitin in fungi was carried out without chromatographic separation, and correction for interference was omitted. Tissue with a much higher ratio of sugar and/or amino acids to glucosamine than that which occurs in fungi might require chromatographic separation.

The glucosamine hydrochloride concentration

TABLE 1. Comparison of glucosamine hydrochloride standard with glucosamine hydrochloride plus a mixture of monomers prepared in a molar ratio comparable to the polymers found in fungal cell walls

Solution (μg/ml)	Mean absorbance[a] (530 nm)	Glucosamine hydrochloride equivalent (μg/ml)	Interference (%)	Coefficient of variation (%)
Glucosamine hydrochloride (9.2)	0.183	9.20	0	1.36
Glucosamine hydrochloride (9.2) plus monomers[b]	0.184	9.25	0.54	1.41

[a] Each value is the mean of four determinations.

[b] The monomers added were the following (μg/ml): glucose (7.4), aspartic acid (0.27), threonine (0.35), serine (0.54), glycine (0.22), and alanine (0.22).

TABLE 2. Stability of glucosamine hydrochloride-derived chromogens by the modified Svennerholm method

Time after color development (min)	Absorbance (530 nm) with the following amt of glucosamine hydrochloride (μg/ml):			
	5	10	15	20
5	0.097	0.177	0.270	0.364
10	0.099	0.177	0.282	0.377
15	0.094	0.181	0.283	0.372
20	0.094	0.182	0.283	0.371
30	0.094	0.182	0.283	0.371
40	0.097	0.182	0.284	0.371
60	0.100	0.183	0.286	0.371

was linear with absorbance, i.e., Beer's law applied, between 5 and 15 μg/ml (Fig. 2), with coefficients of variation of 3.03 and 1.35%, respectively. This sensitivity is comparable to that of the latest modification of the Morgan-Elson (9) and deamination (10) methods. The lower limit of 2.5 μg/ml was rejected because the coefficient of variation was 8.2%. The color of glucosamine hydrochloride-derived chromogens formed by the modified Svennerholm procedure (Table 2) remained stable between 20 and 40 min after color development.

Maximum glucosamine release from pure chitin, hydrolyzed in hydrochloric acid at reflux temperature, varied with acid concentrations. With 6 N hydrochloric acid, maximum glucosamine release was reached after 5 h of reflux, with a 93% recovery of glucosamine hydrochloride (Fig. 3). With 2 N hydrochloric acid, maximum release was reached after 12 h of reflux, with a 57% recovery. For fungal cell walls of *G. trabeum* hydrolyzed in 6 N hydrochloric acid,

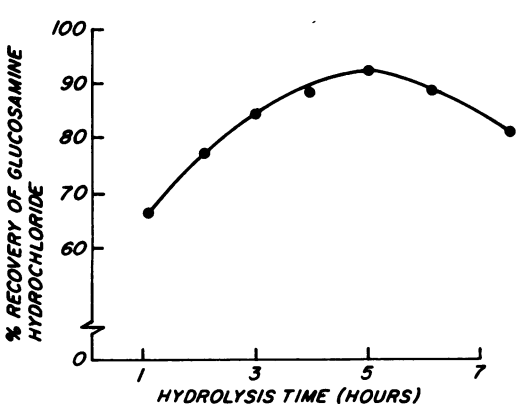

FIG. 3. Hydrolysis time for maximum recovery of glucosamine hydrochloride from pure chitin (150 mg) refluxed at 100°C in 6 N hydrochloric acid (75 ml) for up to 7.5 h. Each value is the mean of two determinations.

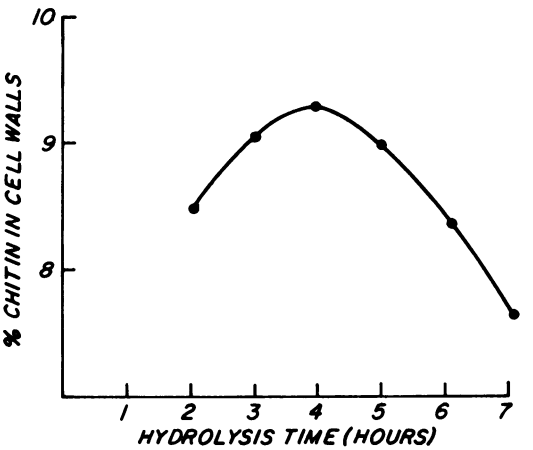

FIG. 4. Hydrolysis time for maximum release of chitin from fungal cell walls (40 mg) refluxed at 100°C in 6 N hydrochloric acid for up to 7 h. Each value is the mean of two determinations.

maximum chitin release from cell walls was reached after 4 h of reflux (Fig. 4).

By this procedure, the mean chitin content of 13 wood decay fungi was shown to be 6.6 to 23.9% of cell wall dry weight (B. R. Johnson and G. C. Chen, Holzforschung, in press). The chitin content of non-wood-decay fungi reportedly ranges from 2.6 to 26.6% (3). *Schizophyllum commune*, one of our 13 wood decay fungi, has been analyzed by others (14) and shown to have a chitin content of 10.8% and an additional 1.7% of nonchitinous glucosamine. Our analysis produced a slightly lower value of 9.6%. Hence, the modified Svennerholm method proved valid in making colorimetric determinations of the cell wall chitin content of wood decay fungi. It seems probable that the method would work equally well with other chitinous fungi.

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