Chromatographic Estimation of Fungal Mass in Plant Materials

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ABSTRACT

The determination of glucosamine from fungal chitin following acid hydrolysis, ion-exchange chromatography, and reaction with ninhydrin, provides a simple rapid method to estimate the quantity of fungi in plant tissue and fungal cultures. The use of chromatography and ninhydrin circumvents some disadvantages of other colorimetric methods and allows use of the automatic amino acid analyzer.

Phytopathology 65:1032-1034

Additional key words: fungal growth.

Studies of the role of fungi in the parasitic and saphrophytic degradation plant tissues often require an estimation of the amount of fungal material present in plant materials. However, the separation of fungi from infected plants or plant material in which fungi are growing is a difficult, and often impossible, task. Since most fungi have cell walls containing chitin which is a polymer of acetyl glucosamine (15), chemical methods for estimating the total mass of fungi in plant materials expressed as glucosamine have been attempted (1, 2, 6, 8, 9, 13). Most glucosamine determinations have used various modifications of the colorimetric method of Elson and Morgan (5) which is not specific and extremely sensitive to a number of variables as described by Boas (3). On the other hand, Eastoe (4) has succeeded in separating and identifying glucosamine and galactosamine on a column of Dowex 50 using the ninhydrin colorimetric method which was originally described by Moore and Stein (7) for amino acid analysis. We now report the quantitative determination of amino sugars by using the automatic amino acid analyzer (10) which is simpler, more rapid, and more accurate than manual methods.

Chitin exists in complex systems and is not found in a pure state. It is generally accepted that chitin is always linked to protein. The naturally occurring complex was found to contain a glycoprotein from which protein was removed to yield chitin (14). Acid hydrolysis is used to release both glucosamine and amino acids. Traditionally, protein has been hydrolyzed in a sealed glass tube either in vacuum or under an atmosphere of nitrogen. Several modifications of the hydrolysis apparatus have been described which are usually limited to micro or semimicro samples. Since the amount of glucosamine from fungal tissue is relatively small, a rather large sample must be hydrolyzed. We describe a simplified hydrolysis apparatus and the automatic quantitation of amino sugars with special reference to the assessment of fungal activity in the saprophytic attack upon plant tissues. The

application of a similar method to infected plants will appear elsewhere (11).

The hydrolysis apparatus consisted of a round-bottom flask having 24/40 ground glass joints with a groove for a Nitrile Buna-N O-ring (2.58 × 15.30 × 20.45 mm, Federal Mogul Corp., Chicago, Illinois) and a stopcock with a Teflon plug (Scientific Glass Apparatus Co., Bloomfield, New Jersey). The size of the round-bottom flask can be varied with the amount of sample used. The acid resistant O-ring was normally replaced after being used two or three times or when excessive "swelling" was noticed. This prevented the joint from sticking. This apparatus is reusable, easy to seal, chemically inert, safe to handle, and holds a vacuum well. The apparatus has been in use in this laboratory for over 10 years, and proved to be very satisfactory and inexpensive for acid hydrolysis of plant and animal tissues for amino acid analysis.

The tissue sample was weighed into the round-bottom flask and the appropriate amount of 6 N hydrochloric acid added. After the tissue was soaked for 1 hour, nitrogen gas was bubbled through the contents for 4 minutes from a Pasteur pipette to sweep out oxygen. The stopcock assembly, previously flushed with nitrogen gas, was inserted in the flask as the pipette was withdrawn, and the stopcock then closed. The flask was evacuated with a water aspirator before the stopcock was closed and it was heated in an autoclave at 110 C.

After hydrolysis, the flask and sample were cooled to room temperature before being opened. The excess hydrochloric acid was removed by rotary evaporation from the same flask, thereby avoiding possible error due to an additional transfer. The residue was taken up in 0.2 M sodium citrate buffer of pH 2.2, suction filtered through Whatman No. 50 filter paper to remove most of the humic substance, and made up to an appropriate volume.

An aliquot was applied to the short column of the amino acid analyzer and eluted with 0.35 M sodium citrate buffer (pH 5.28) as described by Spackman et al. (10) for basic amino acids. The temperature was 53 C and the buffer flow rate was 45 ml per hour. Glucosamine and galactosamine were eluted between the aromatic acids and lysine. Increasing the column height over 18 cm gave sufficient resolution of the amino sugars to allow quantitation of both glucosamine and galactosamine.

It was necessary to determine the best hydrolysis time, since amino sugars are somewhat unstable under the acid hydrolytic conditions (3, 12). Hydrolysis of 10 mg of pulverized crab-shell chitin (Calbiochem) in 10 ml 6 N hydrochloric acid at 110 C yielded maximum glucosamine levels after 4 hours. After heating 5 μmole glucosamine hydrochloride in 25 ml 6 N hydrochloric acid for various lengths of time (1, 2, 4, and 6 hours), the recovery of glucosamine was 100, 96, 88, and 80%, respectively. In the presence of the mycelium of Lentinus edodes (Berk.) Sing. and sawdust, the recovery of added glucosamine was 87, 100, 79, and 76%, respectively. Under the same conditions, a culture of L. edodes grown in a medium containing much sawdust also yielded maximum glucosamine at 2 hours. Thus, the best hydrolysis time for maximum glucosamine recovery represents a balance between glucosamine liberation and destruction, and is about 2-4 hours at 110 C.

Once the optimum conditions for hydrolysis were determined, various samples were hydrolyzed in 6 N hydrochloric acid at 110 C for 2 hours and then analyzed for glucosamine. The standard curves for glucosamine from different samples showed a linear relationship between the amount of added glucosamine hydrochloride and recovered glucosamine up to 10 μmole. A linear relationship was found with increasing amounts of crab-shell chitin up to 20 mg, and with L. edodes mycelium (grown either in liquid medium or in sawdust) up to 1,000 mg. This is illustrated in Fig. 1.

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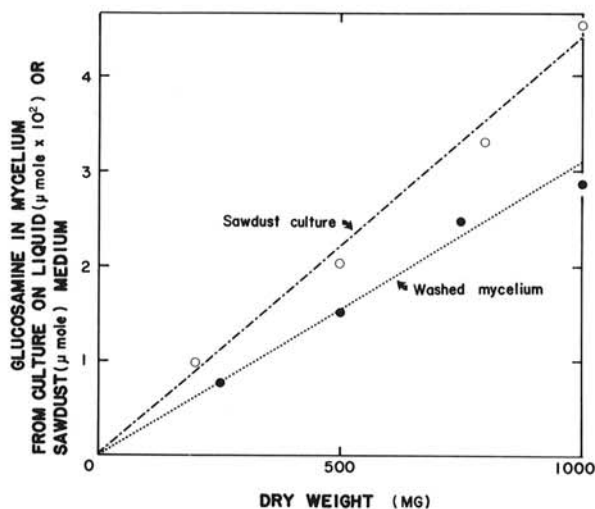9. RIDE, J. P., and R. B. DRYSDALE. 1972. A rapid method

Fig. 1. Glucosamine analysis technique for estimating hyphae of Lentinus edodes. Relationship between the recovered glucosamine and the dry weight of washed mycelium or sawdust cultures of L. edodes hydrolyzed and analyzed.

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