Cereal Chemistry

Vol. 55 March-April 1978 No. 2

GLUCOSAMINE ANALYSIS OF FUNGUS-INFECTED WHEAT AS A METHOD TO DETERMINE THE EFFECT OF ANTIFUNGAL COMPOUNDS IN GRAIN PRESERVATION

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ABSTRACT Cereal Chem 55(2): 121-126

The extent of fungal invasion of wheat grains inoculated with three storage fungi, *viz.*, *Aspergillus candidus, A. glaucus,* and *Penicillium* sp., was measured on the basis of quantitative estimation of the glucosamine in the fungal chitin. In healthy wheat grains, the amount of material responding to glucosamine reaction was about 123 μg/g grain. In grains inoculated with different storage fungi, the glucosamine content increased linearly with storage time in all cases but showed considerable differences when stored under identical conditions. Treatment with some volatile aldehydes, esters, and an acid caused effective preservation of fungi-inoculated wheat against deterioration, as evident from the glucosamine content.

The extent of fungal infection of cereals during storage or in commercial transactions is conventionally tested by determining the percentage of grains that give rise to fungal growth when placed on a suitable agar medium. However, determination of glucosamine content as a measure of fungal chitin in such an inspection for fungal contamination may have great promise, since the assay procedure is much more rapid than the usual plating method; also, the chemical method is considered to be more sensitive and needs few samples for analysis. The method is based on alkaline depolymerization and deacetylation of chitin (a linear polymer of β1,4 linked N-acetyl-glucosamine) to glucosamine which on deamination yields aldehydes that are colorimetrically measured. Although glucosamine is present in different seeds as glycoproteins, it does not interfere with the determination of total glucosamine values since the amount present is known to be characteristic of the species (1).

With this in mind, an investigation was undertaken to study changes in the glucosamine content as a measure of fungal chitin in wheat grains inoculated with different storage fungi and incubated for different periods of time. The method was also employed to evaluate the efficacy of certain volatile organic compounds (heptanal, citral, 2-nonenal, allyl caproate, ethyl salicylate, butyl propionate, and propionic acid) in preventing fungal deterioration of grain.

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0009-0352/78/000016$03.00/0

These compounds have been shown to be effective in preventing fungal growth in agar culture by Maruzzella *et al.* (2), Stevens *et al.* (3), Cole *et al.* (4), Norrman (5), Nandi (6), and in grains by Nandi and Fries (7).

MATERIALS AND METHODS

Wheat grains (100 g) with an initial moisture of about 21.5% were inoculated by thorough mixing with conidial suspensions (approximately 3×10^4 conidia/100 g grain) of *Aspergillus candidus* Link, *A. glaucus* Link, and *Penicillium* sp. Three replicates of each series of inoculated grains were incubated at 30° C and 90% RH. Samples of infected grains were taken out at intervals of 7 days up to a period of 35 days. Uninoculated healthy grains kept under identical conditions served as the control. From each set, about 20 grains, after surface sterilization with 1% sodium hypochlorite for 2 min, were placed on Difco malt agar (2%) containing 10% NaCl and incubated for 7 days so that the extent of infection in the grains could be correlated with the result of the glucosamine assays. Parallel samples of inoculated grains were used for glucosamine estimation. To ensure that the control grains were mycelia-free, only those grains which did not show any fungus when incubated for 7 days on malt agar were selected for glucosamine estimation. This, however, did not exclude the possibility of dead mycelium being present in the grains.

The method of chitin estimation used by Ride and Drysdale (8) was followed with certain modifications. The grains, dried at 104° C to constant weight, ground to a flour of 40 mesh using a Stein mill, were kept in closed bottles until used. The flour (100 mg) taken into glass centrifuge tubes (15 ml) was washed first with 5 ml of acetone and then with distilled water. The residue was treated with 3 ml concentrated KOH and heated at 130° C for 1 hr with periodic agitation. It was then cooled and mixed with 5 ml ice-cold 70% ethanol to allow precipitation of the glucosamine. The mixture was covered with 1 ml Celite suspension (5%) in 70% ethanol and kept in ice for 15 to 20 min. It was then centrifuged (8000 rpm) for 10 min and the residue washed first with ice-cold 40% ethanol and then at least three times with ice-cold distilled water. The final glucosamine residue was suspended in 1.5 ml distilled water and kept for assay.

The assay method was based on the deamination of glucosamine to aldehyde with nitrous acid. As such, a set of external standard solutions of glucosamine-HCl in distilled water was prepared to obtain graded concentrations of 100, 75, 50, 25, and 10 μg/ml. These solutions and the water blanks were incorporated into the assay. The glucosamine suspensions or the standard solutions (1.5 ml) were mixed with equal volumes of 5% $NaNO_2$ and 5% $KHSO_4$ and kept for 15 min with periodic shaking. The mixture was finally centrifuged. Two samples (1.5 ml each) of the supernatant were taken in separate tubes and mixed with 0.5 ml $NH_4SO_3NH_2$ (12.5%) for 5 min. One-half milliliter of MBTH (0.5% aqueous solution of 3-methyl-2-benzothiazolone hydrazone hydrochloride) was then added and the mixture was heated in boiling water for 5 min. It was then cooled and mixed with 0.5 ml $FeCl_3$ (0.5%) and allowed to stand for 30 min. The absorbances of the solutions were measured at 650 nm.

The amounts of glucosamine as measure of chitin from infected grains were quantitatively estimated using the standard curve of the graded external glucosamine-HCl solutions.

For glucosamine analysis, samples were also taken from grains inoculated with storage fungi and then treated with different volatile compounds. In such cases, 2 g of grains inoculated with a conidial mixture (approximately 1.1×10^3/g) of the three storage fungi were taken into glass vials, treated for 5 days with 2 μl/g of three aldehydes (heptanal, citral, and 2-nonenal), three esters (allyl caproate, ethyl salicylate, and butyl propionate), and one acid (propionic acid) and then stored for different periods at 30° C and 90% RH. These compounds showed strong grain preservative properties against storage fungi in earlier experiments (Nandi and Fries, 7). Grains which were inoculated but untreated with such compounds were used as the control.

RESULTS

Uninfected wheat grains had about 123 μg of material giving the glucosamine reaction per g of dry grain (Fig. 1). Following infection with the three fungi the

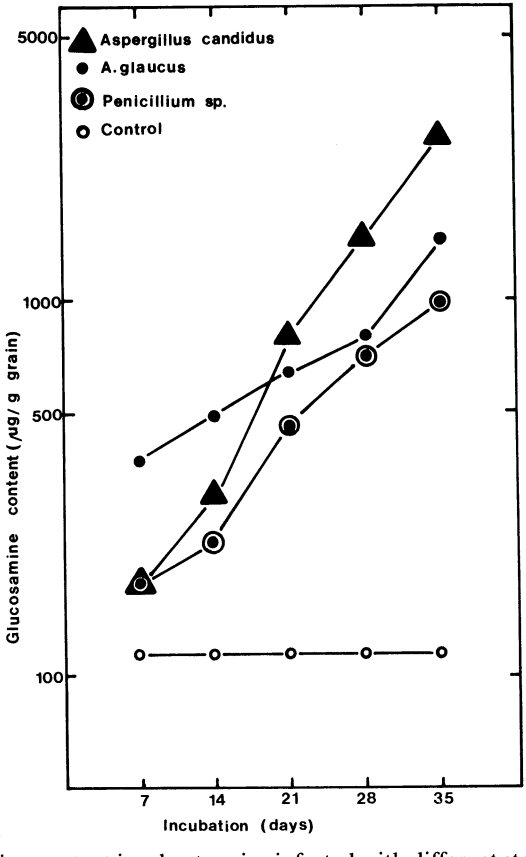

Fig. 1. Glucosamine content in wheat grains infected with different storage fungi. Results are mean values of six samples from three replicates.

glucosamine content increased with storage time. After 35 days' incubation, the highest value was obtained in the grains inoculated with *A. candidus,* followed in descending order by *A. glaucus* and *Penicillium* sp.

In the agar test, more than 70% grains inoculated with the test fungi proved to be infected after 7 days and 100% after 14 days of incubation. It is interesting to note that, after 35 days of incubation, when all the grains were infected, the glucosamine content showed significantly higher values in grains inoculated with *A. candidus* than those with the other two fungi. This could be explained on the basis that much more hyphal growth occurred in individual grains inoculated with *A. candidus* than those with either *A. glaucus* or *Penicillium* sp. Alternatively, the differences noted in glucosamine could have been due to the fact that not all the grains tested were equally infected with fungus.[2]

In the experiment with fungi-inoculated grains which were immediately treated with volatile compounds, glucosamine assay showed that such treatments caused effective preservation. In all treatments, the glucosamine contents of grains after 15 days of storage remained more or less the same as in uninoculated grains (Table I). Even after prolonged storage (30 days), no significant rise in the glucosamine content of the treated grains could be observed. Control grains, inoculated similarly but not treated with volatiles, showed glucosamine values that increased with the length of the storage period.

DISCUSSION

The amount of material giving glucosamine reaction varies widely in different seeds depending on the quantity of glucosamine and N-acetyl-D-glucosamine, the two main constituents of seed glycoproteins (1,9). For example, Pusztai (1) has shown that the glucosamine content ranges from only traces in barley grains to 51,000 μg/g in clover seeds. Donald and Mirocha[2] reported radical differences in the content of such compounds between uninfected soybean seed (15–25 μg/g) and corn grains (100–120 μg/g).

[2]Unpublished data, W. W. Donald and C. J. Mirocha.

TABLE I
Glucosamine Content in Wheat Inoculated with a Conidial Mixture of *Aspergillus candidus, A. glaucus,* and *Penicillium* sp., Followed by Treatments with Volatiles, and then Stored for Different Periods

Compound	Concentration μl/g	Glucosamine Content[a] 15 days μg/g	30 days μg/g
Heptanal	2	135.7 ± 5.2	137.7 ± 15.0
2-Nonenal	2	142.0 ± 7.6	138.5 ± 8.5
Citral	2	137.7 ± 10.4	136.0 ± 9.0
Allyl caproate	2	132.3 ± 16.7	125.0 ± 15.2
Ethyl salicylate	2	130.7 ± 15.3	128.5 ± 8.5
Butyl propionate	2	130.0 ± 21.7	161.5 ± 21.3
Propionic acid	2	130.0 ± 11.5	143.5 ± 8.5
Control		260.0 ± 10.9	612.5 ± 13.7

[a]Mean of six samples from three replicates ± standard errors.

Wheat grains, free from any infection by microorganisms, showed an average of 123 μg of glucosamine compounds per gram dry grain. Marked differences in the glucosamine content of grains infected with different storage fungi were observed within 7 days under identical environmental conditions. *A. glaucus* showed maximum growth as assessed by glucosamine content in the initial stage. The rate of increase in glucosamine content was maintained more or less uniformly throughout the period of investigation. In grains inoculated with *A. candidus,* the initial rate, although comparatively low, increased steadily until the end of the experimental period. With *Penicillium* sp., the glucosamine content increased slowly in the early phase but, from the third week onward, it was slightly higher and uniform although the contents were much lower than those in the other two cases. However, with all the three fungi, the glucosamine increased linearly with time. These results are comparable with those observed by Donald and Mirocha[2] in moldy corn, but are in contrast to their results with soybean seeds, which showed inhibition of fungal growth and consequently low glucosamine values that resulted in the formation of a plateau in the curve. Longer incubation generally caused more hyphal growth in grains, which seems to account for the increased glucosamine contents. Such increases are shown to differ according to fungal species, possibly depending on their growth rates and amount of mycelium produced.

Inoculated grains treated with volatiles showed no evident increase in glucosamine content. The results confirmed earlier observations (7) of strong antifungal properties of the compounds that caused effective preservation of grains against storage fungi.

The isolation of fungi from grains as observed by plating method and the glucosamine content may not show perfect correlation in all cases. For example, after 14 and 35 days of incubation when 100% grains were infected, the determination of percentage of infected grains as a relative measure of fungal growth seems to be of little value as the amount of glucosamine continued to increase linearly. However, the two methods give different informations—the agar method measuring the number and species of living fungi while the chemical method measuring critically the total amount of hyphae from living as well as dead fungi present in grains.

[2]Unpublished data, W. W. Donald and C. J. Mirocha.

Acknowledgments

I wish to express my indebtedness to Nils Fries, head of the Institute of Physiological Botany, University of Uppsala, for his constant guidance, advice, and stimulating discussions during the period of work. My sincere thanks are due to Docent T. Unestam for his helpful suggestions. Thanks are also due to Nigel Rollison for linguistic revision and to Eva Gustavsson for typing the manuscript. I express my thanks to the Swedish Institute for financial support.

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[Received January 28, 1977. Accepted June 3, 1977]