EVIDENCE FOR A BURIED LOCATION OF NIGERAN IN THE CELL WALL OF *ASPERGILLUS NIGER*

K. K. Tung and J. H. Nordin

Department of Biochemistry
University of Massachusetts
Amherst, Massachusetts 01002

Received July 6, 1967

Nigeran, an unbranched polysaccharide associated with fungi of the *Aspergillus* species consists of alternating α(1→3) and α(1→4) linkages between D glucopyranose units (Barker *et al.* 1957). It has not been clearly resolved whether or not this polysaccharide is a constituent of the cell wall (Reese and Mandels 1964, Johnston 1965). Nigeran can be extracted from mycelia by heating an aqueous suspension to 100° and quickly filtering. The polysaccharide, soluble in hot water, precipitates when the filtrate cools to room temperature.

Since it is insoluble in water it is not known whether the polymer is intimately involved in the architecture of the wall or merely isolated along with wall material by virtue of this insolubility. We wish to present direct evidence indicating nigeran is in fact a constituent of the cell wall of *A. niger* and is located in such a position as to be practically inaccessible to attack by a hydrolytic enzyme (mycodextranase) specific for this polysaccharide.

Materials and Methods

Aspergillus niger QM326 was grown from spore inoculum in surface culture at room temperature. The medium consisted of the

following in grams per liter: sucrose 46, tartaric acid 2.7 $(NH_4)_2NO_3$ 2.6, $(NH_4)_2SO_4$ 0.17, K_2CO_3 0.47, Mg CO_3 0.27 $(NH_4)H_2PO_4$ 0.4, $FeSO_4 \cdot 7H_2O$ 0.047 and $ZnSO_4$ 0.047. Just prior to sporulation the mycelium was harvested and washed and cell walls prepared by a sequential process of homogenization, sonication and the use of a French pressure cell. Following exhaustive washing of the walls at 2-5° they were freeze dried. Examination of the product by light and electron microscopy indicated complete breakage of the hyphae.

Carbohydrate was measured by the phenol sulfuric acid method (Dubois et al. 1956). Descending paper chromatography was done using Whatman No. 1 paper in the following solvent systems: A. 1-butanol, pyridine, water 6:4:3 v/v and B. isopropanol, acetic acid, water 54:8:18 v/v. Detection of components on chromatograms was accomplished using alkaline silver nitrate (Trevelyan et al. 1950). The tetrasaccharide standard was a gift from Dr. E. T. Reese, U. S. Army Natick Laboratories.

Identification of the polysaccharide liberated in the present study as nigeran was based on its (a) unusual solubility properties in water as a function of temperature; (b) characteristic hydrolytic products from mycodextranase action; (c) optical rotation $[\alpha]_D$+ 222° (c, 0.5, NaOH)(Barker et al. 1957); and (d) the fact that glucose is the sole product of its total acid hydrolysis.

Mycodextranase was produced according to the method of Reese and Mandels (1964) and purified about 30 fold prior to use (Tung and Nordin unpublished). The only products of the hydrolysis of nigeran by this enzyme are nigerose (O-α-D glucopyranosyl (1→3)D glucose) and a tetrasaccharide (tetramer) consisting of two nigerose units linked together by an α(1→4) bond[1] (Reese and

[1] This structure has not been proven unequivocally.

Mandels 1964). Our enzyme was also found to give these two products exclusively. It is possible to assay for enzymatic attack in a highly specific manner by measuring the amount of tetramer or dimer produced from wall preparations. Preliminary experiments clearly demonstrated that the ratio of tetrasaccharide to disaccharide (about one) is identical in heated and unheated walls. Thus only the quantity of tetramer present was determined since it has more total carbohydrate per mole of compound.

Results

The chromatographic similarity of the hydrolysis products in comparison with authentic nigerose and tetrasaccharide (Table I) indicates clearly they are the same with either nigeran or cell wall as substrate.

Table 1

Chromatographic Mobilities of Compounds Released by Mycodestranase from Nigeran and A. niger Cell Walls

Compound	R_{glc} (a) Solvent A	R_{glc} Solvent B
Glucose	1.00	1.00
Nigerose	0.74	.70
Tetrasaccharide	0.32	.33
Fast component from Nigeran	0.74	.70
Slow component from Nigeran	0.33	.30
Fast component from cell wall	0.73	.70
Slow component from cell wall	0.32	.33

(a) Mobility of the compound relative to D glucose

A four fold difference in the amount of nigeran hydrolyzed in heated and unheated walls (Table II) is strong evidence for this polymer being an integral component of the wall. If part of the tetramer detected is from exogenous nigeran carried through preparation of the walls, one would expect that preincubation with enzyme should eliminate or decrease the amount

liberated from the unheated walls during the regular incubation.

Table II

Enzymatic Hydrolysis of Nigeran in Cell Wall Preparations

Preincubation time (min.)	Micro moles of tetramer liberated per 10 mg of cell walls	
	Unheated walls (a)	Heated walls (a)
0	.07	.28
0	.07(b)	.27(b)
30	.035	.28
60	.035	.27
120	.035	.27

Cell wall suspensions (10 mg per ml.) in 0.02M citrate phosphate buffer pH 4.5 were preincubated at 30° with 0.1 unit (c) of mycodextranase for the times indicated. They were then washed three times in ice cold buffer and resuspended to the same concentration. Each suspension was divided into two portions, one of which was heated 10 minutes in a boiling water bath and cooled. After equilibration at 40° 1.0 ml suspensions of walls were then incubated 20 minutes with 0.1 unit of enzyme in a total volume of 1.1 ml. (The quantitative values above represent the product released during the second time period.) The reaction was stopped by heat and the water soluble material deionized and chromatographed in solvent B for 36 hours. The areas on the dry chromatogram corresponding to the tetramer were excised and eluted with water and the quantity of tetramer (above blank values) determined by the phenol sulfuric acid method using a glucose standard.

(a) While these walls contain a very low level of reducing power (Nelson 1944) per unit weight prior to enzymatic attack, there is no detectable difference between heated and unheated walls, and neither contain any tetrasaccharide or nigerose as evidenced by paper chromatography.

(b) 0.2 unit of enzyme.

(c) One unit of enzyme will liberate 1.0 micro mole of reducing equivalents expressed as glucose per minute from a 0.4% suspension of nigran at 50° and pH 4.5.

This was found to be the case. In addition, extended preincubation did not reduce the amount of nigeran subsequently liberated from heated walls, indicating a definite inaccessibility of nigeran to the enzyme. Limiting enzyme is not responsible for the failure of unheated walls to yield more nigeran since doubling enzyme concentration does not change the quantity of tetrasaccharide released from them. It also indicates complete digestion of nigeran in the heated walls.

It is probable that the continued release of small amounts of tetrasaccharide occurring with extended preincubation of unheated walls is due to enzymatic attack during the second incubation at a number of restricted or partially exposed sites in the wall. To check this point an experiment was conducted in which walls were carried through enzymic preincubation and then incubated for periods of up to 60 minutes at 40° in the absence of enzyme. Enzyme was again added to these preparations and the reducing power liberated measured (Nelson 1944) after 20 minutes. Only the low basal level of reducing power was present both in walls incubated at 40° for 60 minutes, and those subjected directly to enzymatic attack without additional heating. If a slow extraction of nigeran were occurring at 40° during this period, it would have accumulated and become available for rapid attack by the enzyme during the final incubation.

In control experiments, nigeran added to unheated walls was completely hydrolyzed indicating no heat labile inhibitor is present in unheated walls that would lead to the observed difference. Further, preincubation of walls in 0.5N NaOH for four hours at 0° resulted in release of nigeran as compared to walls treated at an identical ionic strength, but neutral pH. Since nigeran is alkali soluble, this agent extracts the polymer from the wall thereby permitting enzymatic hydrolysis. Thus a method other than heat will suffice to indicate the inaccessibility of the polymer to mycodextranase. This inaccessibility is most likely not due to covalent bonding with another molecule since heat alone frees the polymer for enzymatic attack. There is also a possibility that nigeran may exist in some crystalline array in the wall and that a heat labile molecular arrangement confers resistance to enzymic hydrolysis.

Calculations based on measurements of both nigerose and tetrasaccharide released from heated walls indicate nigeran makes up 7-8% of the weight of these preparations. The only other material liberated from the wall by heat is a water soluble, methanol insoluble fraction, composed of approximately equimolar quantities of glucose and galactose, which makes up about 10-12% of the wall. Investigation of this fraction is now underway since it may be involved in protecting the nigeran from enzymatic attack.

Acknowledgement

This work was supported by a grant (GB 5162) from the National Science Foundation.

REFERENCES

Barker, S. A., Bourne, E. J., O'Mant, D. M. and Stacey, M., J. Chem. Soc., 2448 (1957).
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., Anal. Chem. 28, 350 (1956).
Johnston, I. R., Biochem. J. 96, 65 (1965).
Nelson, N., J. Biol. Chem. 153, 375 (1944).
Reese, E. T. and Mandels, M., Can. J. Microbiol. 10, 103 (1964).
Trevelyan, W. E., Proctor, D. P. and Harrison, J. S., Nature 166, 444 (1950).