β-N-ACETYLGLUCOSAMINIDASE HYDROLYSIS OF LIPOPOLYSACCHARIDE FROM *XANTHOMONAS MALVACEARUM* IN THE COTTON PLANT

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***β-N*-Acetylglucosaminidase obtained from cotton seedlings liberted *N*-acetylglucosamine from lipopolysaccharide of *Xanthomonas malvacearum,* the pathogen of bacterial blight disease in cotton plants. Three days after bacterial inoculation the enzyme activity had decreased in the disease-resistant Im 216 cotyledon, but not in the disease-susceptible Acala 44 cotyledon. However, the decrease in the enzyme activity was accompanied by a decline in fresh weight of cotyledons. These results suggest that no relationship exists between *β-N*-acetylglucosaminidase activity and resistance to bacterial blight disease in cotton plants.**

INTRODUCTION

A gram-negative bacterium, *Xanthomonas malvacearum* (E. F. Sm.) Dows., causes bacterial blight disease in many cultivars of cotton plants. However, the cultivar Im 216 is resistant to all known races of *X. malvacearum* (1). Earlier observations indicated that injection of high doses of *X. malvacearum* inoculum into the Im 216 cultivar resulted in a hypersensitive response in inoculated cotyledonary tissue (2, 3) similar to the one observed by Goodman, *et al*. (4) in tobacco plants. More detailed study showed that host cells responded locally to the pathogen in the immune cotton plant (5).

Some workers believe that hypersensitive response results from immobilization of the pathogen cell in the incompatible host plant by binding of bacterial lipopolysaccharide, which contains *N*-acetylglucosamine, to glycoprotein of host cell wall (6). Cotyledonary tissue of cotton plants undergoing hypersensitive response contains high levels of *N*-acetylglucosamine-hydrolyzing enzyme (7). Despite this tissue's ability to release *N*-acetylglucosamine from polysaccharide, the effect of inoculation on *β-N*-acetylglucosaminidase activity has not been studied. In the present study, it was attempted to determine whether *β-N*-acetylglucosaminidase from cotton plants could react with lipopolysaccharide of the pathogen and how the enzyme activity could respond to inoculation of the cotyledons of the plants.

MATERIALS AND METHODS

Plant Materials

Seeds of upland cotton, *Gossypium hirsutum* L., lines Im 216 and Acala 44, were planted in 30-cm pots containing autoclaved clay-vermiculite-peat mixture (1: 1: 1, v/v) in a greenhouse with a 16-hr photoperiod. The temperature was 32 C during the day and 24 C at night. The seedlings emerged within 6 days. At day 20, when the first foliage leaf appeared, the cotyledons were inoculated with *X. malvacearum.*

Purified *β-N*-acetylglucosaminidase was prepared from cotton seedlings (Im 216 cultivar) according to the method described by Yi (7).

Crude extract was extracted from cotyledons of Im 216 and Acala 44 cotton plants. Cotyledons were frozen immediately after harvest and homogenized with distilled water by using mortar and pestle. The insoluble material, which had no enzyme activity, was discarded by centrifugation.

Enzyme Assay

The crude extract was assayed for enzyme spectrophotometrically using *p*-nitrophenyl *β* -*N*-acetylglucosaminide as substrate (7). One unit of enzyme activity is defined as the amount of enzyme that released 1 micromole of *p*-nitrophenol per min.

When lipopolysaccharide was used as substrate, the reaction mixture in a volume of 0.5 m*l* contained 0.5 to 2.5 mg of lipopolysaccharide in suspension and 1 unit of

purified enzyme in 0.1 *M* Na acetate buffer, pH 5.0. The mixture was incubated at 25 C for 3 hr, and *N*-acetylglucosamine released by the enzyme was determined after terminating the reaction by heating at 100 C for 10 min.

Extraction of Lipopolysaccharide

Race 1 *X. malvacearum* was prepared and grown according to the method described by Cason, *et al.* (2). Lipopolysaccharide was extracted from *X. malvacearum* by the water-phenol method described by Luderitz, *et al.* (8). About 2 g of dried bacteria was treated with 90% phenol at 65 C for 15 min, the aqueous phase was dialyzed and centrifuged at 80,000 × g for 6 hr, and the sediment was suspended in water. The operation was re-repeated 3 times with centrifuging at 105,000 × g for 3 hr each. The final sediment (50 mg of lipopolysaccharide on a dry weight basis) was taken up in a minimum amount of water.

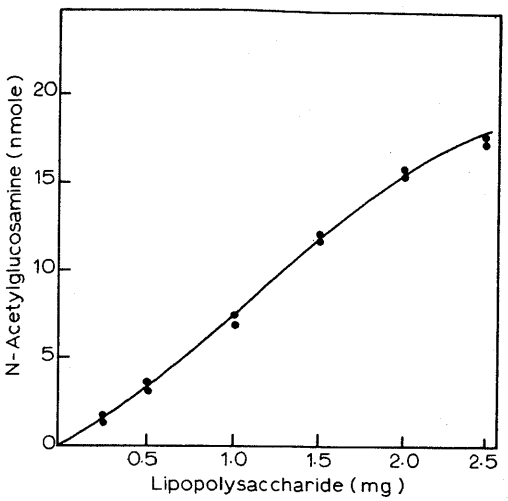

FIGURE 1. *β*-*N*-Acetylglucosaminidase hydrolysis of *X. malvacearum* lipopolysaccharide. The reaction mixture contained 1 unit of enzyme and was incubated at 25 C for 3 hr. In all cases, the blank contained lipopolysaccharide and heat-denatured enzyme.

Analytical Methods

The *N*-acetylglucosamine released during enzymatic hydrolysis of lipopolysaccharide was determined by the method of Ressig, *et al.* (9). For the calibration, *N*-acetylglucosamine was used as the standard. Descending paper chromatography of the hexosamine was done on Whatman No. 1 paper in a solvent system of ethyl acetate-acetic acid-formic acid-water (18:3:1:4), according to the method of Elbein *et al.* (10) and after 16 hr development, hexosamine was located with periodate-permanganate stain (11). Polyacrylamide gel electrophoresis was conducted in the system described by Davis (12), using a Tris-glycine buffer, pH 8.3. Gels were stained for protein with Coomassie Blue and destained with 12.5% trichloroacetic acid. Gels also were stained with 4-methylumbelliferyl *β*-D-*N*-acetylglucosaminide to detect enzyme activity after electrophoresis (13). Protein was determined by the method of Lowry, *et al.* (14). Reducing sugar content was determined by the method of Nelson (15).

RESULTS

Lipopolysaccharide reacted with *β*-*N*-acetylglucosaminidase to produce *N*-acetylglucosamine by enzymatic hydrolysis as shown in Fig. 1. One unit of *β*-*N*-acetylglucosaminidase could release 7 nmole of *N*-acetylglucosamine from 1 mg of lipopolysaccharide in 3 hr. The total amount of *N*-acetylglucosamine in 1 mg of lipopolysaccharide that could be determined by the Ressig, *et al.* method (9) was about 10.3 nmoles.

A part of the reaction mixture was taken to identify the products of the enzyme reaction. The product released from lipopolysaccharide by *β*-*N*-acetylglucosaminidase was analyzed by paper chromatography (Fig. 2). This was also compared with the product of enzymatic hydrolysis of the *β* - (1→ 4) -linked *N*-acetylglucosamine polymer, chitin. The enzymatic products from chitin and lipopolysaccharide had R_f's identical to *N*-acetylglucosamine used as the standard. Some fast-moving spots were not identified. The identity of *N*-acetylglucosamine was confirmed by spotting the standard compound overlapping with lipopolysaccharide hydrolysate. The data clearly show that *β*-*N*-acetylglucosaminidase obtained from the cotton plant can react with lipopolysaccharide of the pathogen.

Cotton plants, disease-susceptible Acala 44 and disease-resistant Im 216 cultivars, were inoculated according to the method described by Cason, *et al*. (2). The bacterial suspension (10^7 cells per m*l* of water) was injected into one cotyledon of each cotton plant. The other cotyledon of the same plant was left without inoculation

to serve as the control. After determined times (Table 1), both cotyledons of each plant were harvested. The experiment was run twice. The enzyme activity, protein content by the Lowry method (14) and total reducing sugar content by Nelson's method (15) of 3 cotyledons were determined and compared with those of cotyledons without inoculation. One representative of the experiments is presented in Table 1. The other experiment showed the same result.

In the early stages after inoculation, fresh weight of inoculated tissues was less than that of control tissue, probably due to damage caused by injection. As the hypersensitive response, including necrosis, progressed, the fresh weight of inoculated Im 216 tended to decrease over a 3-day, while the initial loss in fresh weight was recovered in Acala 44 cotyledons.

When enzyme activity was measured 4 hr after inoculation, inoculated cotyledons contained less β -*N*-acetylglucosaminidase activity than control cotyledons of Acala 44 and Im 216 plants. This decrease in enzyme activity may also be caused by mechanical damage during injection. The total β *N*-acetylglucosaminidase activity per cotyledon decreased steadily in inoculated Im 216 cotyledons during the next 3 days. In inoculated Acala 44 cotyledons, the initial loss in enzyme activity during the first 4 hr was recovered so that the level was the same as in the controls. However, enzyme activity on the basis of fresh weight remained the same throughout 3 days in both treatments of Acala 44 and Im 216 cotyledons.

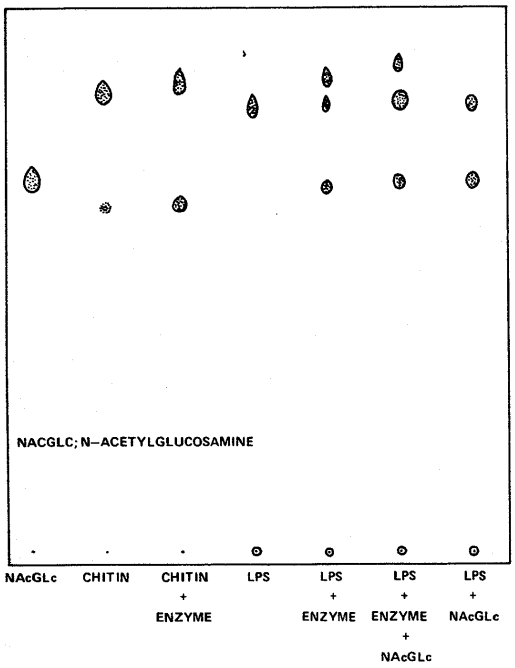

FIGURE 2. Paper chromatogram of end products of lipopolysaccharide degradation by β-*N*-acetylglucosaminindase.

Portions of inoculated cotyledons showing severe desiccation caused by inoculation

TABLE 1. *Fresh weight and β-N-acetylglucosaminidase activity in normal and inoculated cotyledons.*

Inoculation time (hr)	Cultivar	Treatment	Fresh weight (g/cotyledon)	Protein content (mg/cotyledon)	Enzyme activity (unit/cotyledon) $\times 10^{-3}$
4	Acala 44	Control	0.575	2.8	107
	Acala 44	Inoculated	0.455	2.4	80
	Im 216	Control	0.566	3.6	105
	Im 216	Inoculated	0.542	3.6	102
12	Acala 44	Control	0.515	2.7	118
	Acala 44	Inoculated	0.402	2.3	79
	Im 216	Control	0.510	3.0	110
	Im 216	Inoculated	0.502	3.0	103
24	Acala 44	Control	0.725	4.4	160
	Acala 44	Inoculated	0.610	4.0	145
	Im 216	Control	0.453	2.9	107
	Im 216	Inoculated	0.339	2.7	79
72	Acala 44	Control	0.585	3.1	145
	Acala 44	Inoculated	0.581	4.2	147
	Im 216	Control	0.520	2.7	113
	Im 216	Inoculated	0.405	2.1	87

or senescence did not have any enzyme activity.

The protein content per cotyledon did not change significantly after treatment of Acala 44 cotyledons, but in inoculated Im 216 cotyledons, it decreased steadily to the same degree as the enzyme activity (Table 1).

The reducing sugar content was not changed significantly by inoculation, even though there were some variations between samples.

To clarify the contribution of *β-N*-acetylglucosaminidase by the pathogen, the growth medium, nutrient broth, was examined for glycosidase activity by using *p*-nitrophenyl glycosides. No activities of *β-N*-acetylglucosaminidase, arabanase, galactosidases, and pectinase were detected.

The maximum activity of *β-N*-acetylglucosaminidase extracted from normal as well as inoculated cotyledons was at pH 5.6, regardless of cultivar, when determined by using *p*-nitrophenyl *β-N*-acetylglucosaminide of 0.1 *M* sodium phosphate-citrate buffer at various pH values. After polyacrylamide gel electrophoresis of crude extracts, staining with 4-methylumbelliferyl *β-N*-acetylglucosaminide revealed that enzymes from healthy and inoculated Acala 44 and Im 216 cotyledons had the same mobility on electrophoresis. These facts indicate that the two cultivars tested contain the same enzyme and that the blight disease does not alter the type of *β*-N-acetylglucosaminidase present in cotyledonary tissues.

DISCUSSION

The findings in the present study show that *β-N*-acetylglucosaminidase can release a substantial amount of *N*-acetylglucosamine from the lipopolysaccharide of the pathogen. Since the sugar composition of lipopolysaccharide of *X. malvacearum* has not been determined yet, the fraction of *N*-acetylglucosamine releasible by this enzyme could not be estimated. Less than 1% of lipopolysaccharide appears as *N*-acetylglucosamine, which is significantly lower than reported percentage of the hexosamine in various lipopolysaccharides (8, 16). All the *N*-acetylglucosamine residues of lipopolysaccharide are not released by the enzyme owing to the structural complexity, as indicated by Hase and Ritschel (16).

The pathogen, *X. malvacearum*, does not secrete *N*-acetylglucosamine-releasing enzyme into the growth medium. The enzyme activity in the inoculated plant probably originated from the enzyme in the host plant, as *β-N*-acetylglucosaminidase from inoculated plants had the same optimal pH and mobility on electrophoresis as the enzyme from non-inoculated plants.

It is interesting to note that *β-N*-acetylglucosaminidase content is correlated with the change in fresh weight of cotyledon. Since desiccated tissue of inoculated Im 216 cotyledon, or Acala 44 cotyledon succumbed to senescence, does not have enzyme activity, the total activity in inoculated Im 216 cotyledon probably accounts for the enzyme activity of whole cotyledon minus that lost by the desiccated portion. Essenberg, *et al*. (5) observed that the immune cotton (Im 216) tissues responded locally and independently to colonies of the pathogen. Thus, it is strongly suspected that loss of *β-N*-acetylglucosaminidase activity also may be a local response to *X. malvacearum*.

Since this enzyme was present in similar amounts and with the same electrophoretic mobility in healthy cotyledons of susceptible and immune cotton lines and since the measurable activity of the enzyme did not increase in immune cotyledons after inoculation, nor did new isozymes appear, it is unlikely that the enzyme functions in resistance to bacterial blight.

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