EFFECT OF GRISEOFLUVIN ON MYCELIAL GROWTH OF *COCHLIOBOLUS MIYABEANUS*

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Effects of griseofluvin (GRF) on fungal growth was examined. The content of GRF which inhibits 50% of mycelial growth of an ascomycetes fungus, *Cochliobolus miyabeanus* (ATCC-38724), was 1.5 μg/ml. Cell wall constituents of mycelia of this fungus growth in the presence of GRF at ED_{50} concentration were examined. No changes in the β-glucan synthesis were found while the frequency of branched chain linkages of N-acetylgalactosamine in the chitin-like substance was reduced to half compared to the normal mycelia. Effects of GRF on the activities of intracellular β-glucanase and N-acetylglucosaminidase were examined. β-Glucanase activity was repressed by GRF while N-acetylglucosaminidase activity was not. Conuersion of N-acetylglucosamine to N-acetylgalactosamine, on the other hand, was found to be repressed by GRF. It was assumed from the results of this study that GRF could produce morphologically abnormal mycelia by changing the cell wall structure to a slight degree.

Griseofluvin (GRF) is known as a curling factor which causes shortening of fungal conidia and irregular thickening, folding and over-branching of fungal mycelia. It was tested in an animal experiment in 1959 by GENTLES[1], and then applied to clinics by WILLIAMS as a special remedy for rebellious trichophytosis. Despite the clinical usefulness of GRF, its action mechanism is not so entirely studied yet. This report deals with the results of the action mechanism of GRF.

MATERIALS AND METHODS

1. **Microorganism.** An ascomycetous fungi, *Cochliobolus miyabeanus* (ATCC 38724), was subcultured on potato sucrose agar medium and growing mycelia were used in this experiment.

2. **Griseofluvin.** Griseofluvin (GRF, delievered from Sankyo Inc.) was dissolved in dimethyl sulfoxide (DMSO) prior to addition to culture medium. final concentration of DMSO in the medium (adjusted to 1%) showed no effect on mycelial growth.

3. **Measurement of medium inhibitory dose for mycelial growth.** A part of mycelia (3×3 mm) was cut off from the edge of fungal colony, which had been grown on potato agar plate, inoculated to potato sucrose liquid medium containing various amounts of GRF (0.6, 0.8, 1.2, 1.4, 1.5 and 1.6 μg/ml) and cultured on a shaker at 28°C for 72 hrs. The growing mycelia were harvested by centrifugation and dried at 80°C. The degree of growth was determined by measuring the dry weight [(GRF-treated mycelial weight÷control mycelial weight)×100(%)].

4. **Effect of GRF on the growth of colonies.** The tip of mycelia was inoculated on the potato agar containing 1.5 μg/ml (ED_{50} value for mycelial growth), cultured statically at 28°C and diameters of the colonies were measured after 48, 96 and 144 hrs of incubation.

5. **Chemical composition of mycelia.** The fungi was cultured using a medium with or without GRF (1.5 μg/ml) at 28°C for 96 and 144 hrs by shaking method. Growing mycelia were collected, washed, freez-dried and then powder was used for the chemical analyses. The sample was separated into fractions of carbohydrate, sterol, fatty acid ester, protein, phospholipid, fatty acid and nucleic acids (DNA and RNA) according to the previous paper (Fig. 1)[2]. Quantitative analysis of each fraction was performed by the following method using standard substance as indicated in parenthesis : carbohydrate, anthrone method (glucose) ;

Fig. 1 Fractionation of cellular components in mycelia of *Cochliobolus miyabeanus*

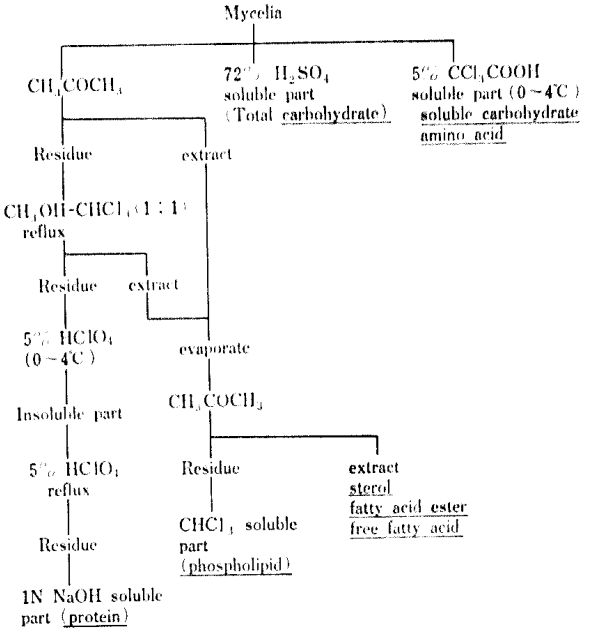
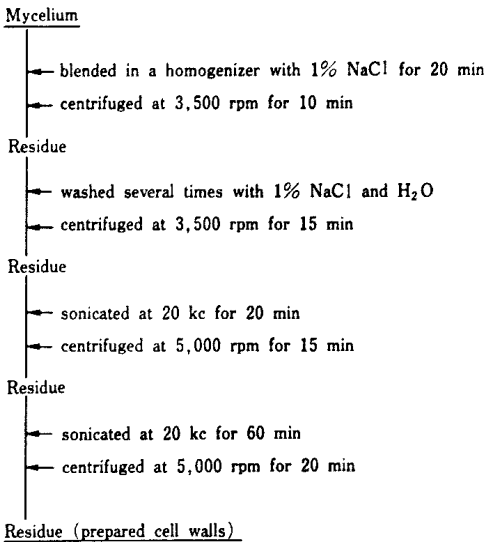


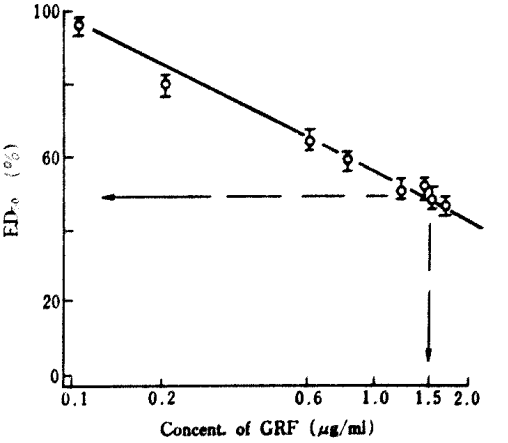
Fig. 2 Preparation of cell wall from mycelia by ultrasonic method



sterol, Sobel's method (ergosterol) ; fatty acid ester, hydroxamic acid method (palmitic and methyl) ; protein, Lowry's method (bovine serum albumin) ; phospholipid, Nakamura's method after heat-degradation with 60% perchloric acid (lecithin) ; fatty acid, titration of ethanol solution of the sample with 0.01 N NaOH using phenolphthalein as indicator ; DNA, indole method (salmon sperm DNA) ; and RNA, orcinol method (yeast-RNA).

6. **Effect of GRF on the chemical composition of cell wall polysaccharides.** The strain was cultured in the presence or absence of GRF and growing mycelia were harvested and washed by centrifugation. After grinding the mycelia, cell walls were obtained by ultrasonic treatment (20 kc, 20 min) according to the method shown in Fig. 2. β-Glucan and chitin-like substance[3),4)] were extracted from the cell wall after confirming the absence of contamination of cell membrane and cytoplasmic substances by I_2KI reaction and lactophenol cotton blue staining[5)]. Namely, the cell wall was degraded by reflux with 4 N acetic acid at 120°C for 12 hrs, ethanol (final 80%) was added to the soluble fraction and precipitate was collected as glucan. The acetic acid-insoluble fraction was also collected as chitin-like substance. These polysaccharides thus obtained were methylated by Hakomori's method as described in the previous paper[3)] and identified by GLC. Galactosamine and glucosamine in the chitin-like substance were measured by Ludowing-Bermannam method[6)] and Blix method[6)], respectively.

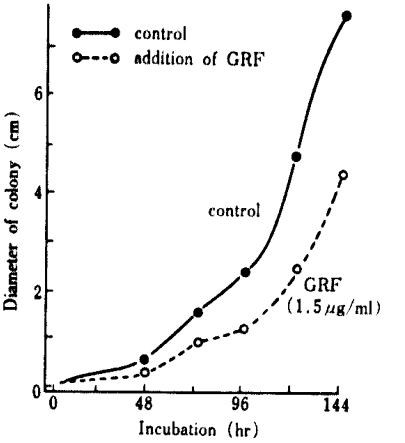
Fig. 3 ED_{50} value of griseofluvin on growth of mycelia



7. **Effect of GRF on the activities of polysaccharide-synthesizing and hydrolyzing enzymes.** The growing mycelia was obtained by shaking culture at 28°C for 48 hrs, enzyme solution were prepared by the procedure shown in Fig. 3. First, the mycelia which had been homogenized in phosphate buffer (0.05 M, pH 7.2) at 0°C were centrifuged at 10,000 rpm for 20 min and the supernatant was collected as crude enzyme preparation. This solution was again centrifuged at 45,000 rpm for 60 min and the obtained supernatant was used as the "soluble enzyme". The pre-

cipitates were dissolved in Tris-HCl buffer (0.01 M, pH 8.2) and used as the "particulate enzyme". Syntheses of β-glucan (at 27°C) and chitin-like substance (at 25°C) were performed using the particulate enzyme solution and the preparation of the reaction mixtures were showed as follows : For glucan-0.5 ml of 0.08 M Tris-HCl buffer (pH 8.2 containing 0.01 M $MgCl_2$ and 0.001 M EDTA), 0.5 mg cell wall glucan, 0.5 ml of 0.08 M UDP-^{14}C-glucose, 0.5 ml of enzyme solution (12 mg protein/ml) and 0.1 ml of 0.08 M UDP-N-acetylglucosamine (with or without). For chitin-like substance-0.5 ml of 0.08 M Tris-HCl buffer (pH 7.5 containing 0.01 M $MgCl_2$ and 0.001 M EDTA), 0.8 mg cell wall chitodextrin, 0.5 ml of 0.08 M UDP-^{14}C-N-acetylglucosamine, 0.5 ml of enzyme solution (10 mg protein/ml) and 0.1 ml of 0.08 M UDP-glucose (with or without). A portion of each reaction mixture was withdrawn at intervals, and these sample were used for quantitative analysis of synthesized polysaccharides. The reaction mixture was mixed with the same volume of $CHCl_3$: MeOH (9 : 1) in order to remove proteins and ethanol (final 80%) was added to this deprotein solution. The obtained precipitates were used as the synthesized glucan. And the same method was used as for chitin-like substances. After the obtained these polysaccharides were hydrolysed with 10% MeOH-HCl in a sealed tube, the amount of glucose was determined by anthrone method and glucosamine in the hydrolyzate was measured by

Fig. 4 ED_{50} of growth-inhibition on mycelia of griseofluvin

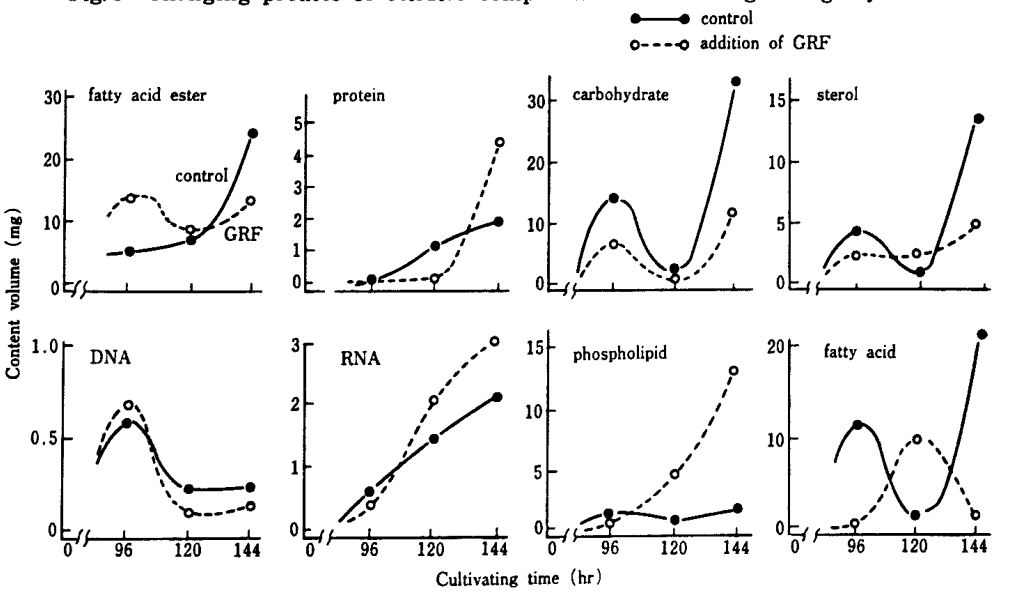


Blix method. β-Glucanase activity in the soluble enzyme solution was measured by mixing 3 ml of the enzyme solution (10 mg protein/ml) with 5 ml of 0.05 M phosphate buffer (pH 7.5) containing cell wall glucan (10 mg) and the mixture was incubated at 37°C. For the measurement of N-acetylglucosaminidase activity, 2 ml of the enzyme solution (15 mg protein/ml) was mixed with 5 ml of 0.1 M phosphate buffer (pH 8.2) containing cell wall chitodextran (5.4 mg)[3] and incubated at 27°C. A portion of the reaction mixture was withdrawn at intervals, proteins in the sample were removed and the amounts of released sugars were measured by the above-mentioned method.

RESULTS AND DISCUSSION

The fungi was grown by shaking-culture at 28°C

Fig. 5 Changing profiles of cellular components content in growing mycelia



for 72 hrs using potato medium containing various concentrations of GRF and the amounts of growing mycelial weights were measured. As shown in Fig. 3, the ED_{50} value of GRF found to be 1.5 μg/ml. Fig. 4 shows the sizes (diameter) of growing colonies on potato agar plate containing 1.5 μg/ml of GRF. Mycelial growth in this case was also a half of the control. The authors have reported[7] that ED_{50} values of amphotericin B (AMPH·B) and cycloheximide (CH) against *C. miyabeanus* were found to be 0.08 and 4.5 μg/ml, respectively. Since GRF showed a strong antifungal activity next to AMPH·B, in addition to its low toxicity compared to AMPH·B and its excellent clinical effect against systemic infections of fungi by oral administration, some studies were performed on GRF in order to elucidate its reaction mechanisms. Changes in the cellular components during growth were first examined using the mycelia which was cultured for 96, 120 and 144 hrs in the presence of 1.5 μg/ml of GRF. As shown in Fig. 5, the contents of total carbohydrate, as well as sterol and fatty acid, reduced to about 50% of that of the control after 96 and 144 hrs cultivation. On the contrary, the contents of phospholipid and protein showed a tendency to increase with the GRF-treatment. PAGET[8] and THYAGARAJAN[9] et al. have reported that GRF inhibited mitosis and nucleic acid metabolism of dermatophytic fungi. In this experiment, however, no remarkable changes in the contents of DNA and RNA were observed by the GRF-treatment.

When the mycelia which was cultured in the presence of GRF were observed by a scanning electron microscope (Hitachi SSM-II), thickening of the cell wall was found in comparison with the normal cells. Therefore, soluble materials were removed from the mycelia by ultrasonic-treatment at 20 kc and the obtained cell wall components were examined. As shown in Table 1, the contents of protein and lipid were increased. Furthermore, remarkable the increase of acetic acid (4 N) soluble materials and the decrease of the insoluble materials were found. Then, the changes in the chemical components of two fractions were evaluated. As described in the previous paper[3,4], the main components in the acetic acid-soluble and insoluble materials are β-glucan and chitin-like substance, respectively. β-Glucan which was isolated from the cell wall of GRF-treated mycelia was methylated by Hakomori's method. The confirmation of the complete methylation was checked on a disappearence of the absorbance at 3,200-3,700 cm^{-1} (specific for OH-groups) by IR spectrum. The methylated sample was hydrolyzed with 5% MeOH-HCl at 120°C for 30 min in a sealed tube by means of a conventional method and then the hydrolysate was desalted for the GLC analysis. As shown in Table 2, the ratio of 2, 3, 4, 6-tetra-O-methyl-, 2, 4, 6-tri-O-methyl- and 2, 4-di-O-methylglucose could not find the hardly change in the presence (1.0 : 3.1 : 1.0) and absence (1.0 : 3.2 : 1.0) of

Table 1 Quantitative analyses of chemical composition in cell wall

Component	Control	GRF-treated
total-N_2	1.30	1.60
protein (N×6.25)	7.60	10.80
insoluble material (4N AcOH)	38.30	52.40
soluble material (4N AcOH)	51.47	32.54
total lipid	0.57	1.99
ash	2.33	2.27

Table 2 Molar ratio of methylated sugars in cell wall

Compound	Cell wall	
	Normal	GRF-treated
2, 4, 6-tri-O-Me-glucose	3.13	3.21
2, 4-di-O-Me-glucose	1.00	1.00
2, 3, 4, 6-tetra-O-Me-glucose	1.09	1.10

NGS column
Column temp. 180°C

Table 3 Molar ratio of N-acetylglucosamine and N-acetylgalactosamine in cell wall

Treatment	N-acetylglucosamine	N-acetylgalactosamine
control	12.3	1.0
GRF-treated	24.0	1.1

Table 4 Molar ratio of methylated amino sugars in cell wall

Compound	Cell wall	
	Control	GRF-treated
3, 4, 6-tri-O-Me-galactosamine	1.00	1.00
3, 4, 6-tri-O-Me-glucosamine	1.13	0.98
3, 6-di-O-Me-glucosamine	10.10	22.21

NGS column
Column temp. 180°C

GRF. This result indicates that the chemical structure of glucans in the normal and GRF-treated mycelia are the same. In other words, GRF has no effects on the synthesis of β-glucan in the mycelial cell wall. Chitin-like substance which is composed of β-1,4 linked polymer of N-acetylglucosamine with α-linked branches of N-acetylgalactosamine[4] was next examined. The contents of galactosamine in the cell wall hydrolysate from GRF-treated mycelia were determined by Ludowing-Bermannam method. Table 3 shows the results of the quantitative analysis of glucosamine and galactosamine. The ratio of glucosamine content was observed about 2 times by the GRF-treatment. Also, cell walls were treated with lysozyme (0.01 M phosphate buffer, pH 5.4, 37°C, 140 hrs) and the chemical structure of the obtained chitodextrin-like substance was methylated by Hakomori's method. As shown in Table 4, the ratio of branching points to linear linkages reduced to 1/2 time by the GRF-treatment compared to the normal mycelial cell wall. These results indicate that GRF has an effect on the frequency of branched linkages of N-acetylgalactosamine in chitin-like substance of the cell wall. Although an assumption has been proposed on the possible role of GRF on some changes in the cell wall chitin[10], this study clearly revealed its function to change chemical structure of chitin-like substance in the cell wall. In addition, these results also indicate a certain kind of relation-ship between GRF and morphological changes in the GRF-treated mycelia such as the rough-face and thickened cell wall.

The authors reported that β-glucan and chitin-like substance were synthesized from UDP-glucose and UDP-N-acetylglucosamine[3], by the actions of the particulate enzyme obtained from the mycelia of *Cochliobolus miyabeanus*[11]. In the that paper, the authors also described that the hydrolyzed activities to these two polysaccharides were found in the soluble fraction. The effects of GRF, therefore, were examined against the synthesis and hydrolysis of β-glucan and chitin-like substance. As shown in Fig. 6, the amounts of synthesized two substances *in vitro* were not affected by the presence of GRF. On the contrary, as shown in Fig. 7, the β-1,3 glucanase activity in the soluble fraction was inhibited by the addition of GRF, but the activity of N-acetylglucosaminidase was not repressed. In addition, as already has reported[11], an enzyme which converted N-acetylglucosamine to N-acetylgalactosamine was found in the particulate enzyme fraction. Therefore, the amounts of N-acetylgalactosamine formed by the conversion in a reaction mixture were measured at intervals in the presence of GRF. As shown in Fig. 8, the amounts of N-acetylgalactosamine converted from N-acetylglucosamine obviously decreased by the addition of GRF. This result coincides with the previous result that the ratio of N-acetylglucosamine in synthesized chitin-like substance was decreased by GRF (as shown in Table 3).

The main findings on the effects of GRF obtained from this experiment are itemized as follows : 1) GRF has no effects on the syntheses of β-glucan and chitin-like substance (at least on the synthesis

Fig. 6 Effect of griseofluvin on polysaccharide syntheses

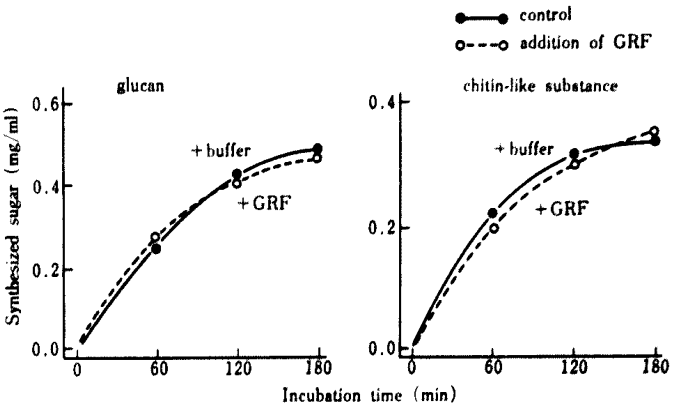


Fig. 7 Liberations of sugar by β-1,3 glucanase or N-acetylglucosaminidase in soluble enzyme fraction from mycelia

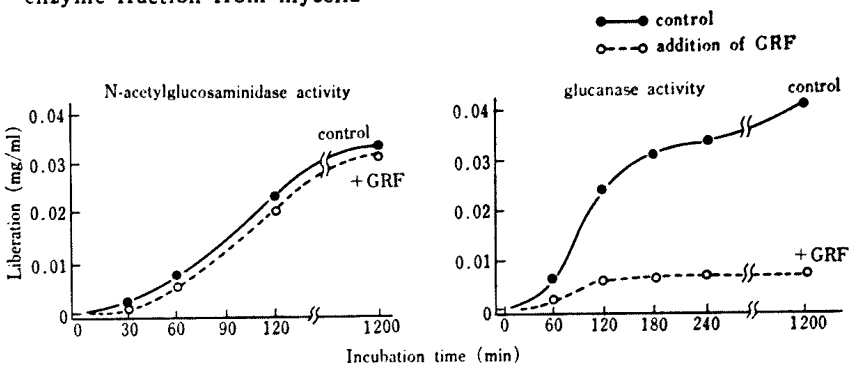
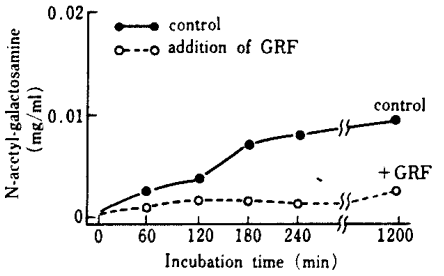


Fig. 8 Convertable activity from N-acetylglucosamine to N-acetylgalactosamine in particulate enzyme from mycelia



of β-1,4 linear chains of N-acetylglucosamine), 2) it has some effects on the conversion of N-acetylglucosamine to N-acetylgalactosamine and branched linkage of N-acetylgalactosamine and 3) it has no effects on the N-acetylglucosaminidase activity, but inhibited the β-1,3 glucanase activity. For the normal growth of cell, proper regulations are required on the synthesis and hydrolysis of constituents of cell wall which is the most outside part of the cell structure. In the presence of GRF, however, thickening of the cell wall occurs since GRF represses hydrolysis of β-glucan, while synthesis of the cell wall polysaccharides proceeds normally, and morphologically abnormal changes like curling also seem to occur when frequency of side chain linkages in the chitin-like substance decreases by the effect of GRF.

Studies on the action mechanisms of GRF will be continued further in detail.

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Griseofluvin の真菌菌糸生育に及ぼす作用について

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抗真菌剤として用いられている griseofluvin(GRF) の真菌生育に及ぼす作用について検討した。*Cochliobolus miyabeanus* (ascomycetes spp.) 菌糸の生育を 50% 抑制するのに必要な GRF は 1.5 μg であったが，この GRF 濃度の添加によって生育した菌体の cell wall 構成成分を調べたところ，glucan 合成には何らの変化も認められなかったのに対し，chitin 様物質では，N-acetylgalactosamine の分枝鎖結合の頻度が正常菌糸の 1/2 に減少する影響が認められた。さらに菌体中に存在する glucanase および N-acetylglucosaminidase 活性に及ぼす GRF の作用を検討したところ glucanase 活性は抑制されたが，N-acetylglucosaminidase 活性には何らの影響もみられなかった。しかし，N-acetylglucosamine から N-acetylgalactosamine への convert は GRF によって抑制されることが認められた。以上の結果，GRF は cell wall 構造に変化を与えることにより，異常形態を生じさせ得ると推定した。