ADVANCES IN CHITIN & CHITOSAN

V.2.

INFLUENCE OF POLYMERIZATION DEGREES OF GLUCOSAMINE OLIGOMERS ON D-GLYCANASE INDUCTION IN SUSPENSION CELLS AND PROTOPLASTS OF *RUBUS*

YVETTE LIENART, CATHERINE GAUTIER
Centre de Recherches sur les Macromolécules Végétales, CNRS, B.P. 53 X, 38041 Grenoble cedex, France

ALAIN DOMARD
Université Claude Bernard, Laboratoire d'Etudes des Matériaux plastiques et Biomatériaux, 69622 Villeurbanne cedex, France

ABSTRACT

Oligo- or polyglucosamine were compared for their potency to elicit laminarinase activity in suspension of *Rubus* protoplasts (cells). The induced reactions were followed as a function of polymerization degree of elicitors ; kinetic and qualitative features of the responses were reported. The results clearly indicated that the plasmalemma was involved in the primary event of D-glycanase induction, and two behaviours were exhibited from elicitor activity. The first is a specific recognition of oligomers with the DP 4 as the most active inducer, and therefore should be the consequence of a specific interaction of the elicitor with a lectin-like receptor associated with plasmalemma. The second which increases with the chain length corresponds to non specific interactions between chitosan and plasma membranes.

INTRODUCTION

Chitosan exhibits biological properties crucial for resistance in plant-pathogen interactions. The processes includes accumulation of pisatin and of lignin, formation of callose, and increases in D-glycanase and phenylalanine ammonialyase activivies (1-3). Among the induced responses, specific gene activation appears to play a central role (4). The activation of plant defense genes in the nucleus requires the elicitor recognition at the plant-cell surface, and subsequent transduction of a signal to the nucleus (5).

Up to now, little is known about the perception by the cell of glucosamine signal. The aim of the paper is to give

information about the mechanisms underlying the recognition of oligo-,or polyglucosamine by *Rubus* suspended cells. Protoplasts and a series of glucosamine were used for these purposes; thus the elicitor of laminarinase activity can act directly on the plasma membrane, simplifying studies on putative elicitor receptors.

Here, the response of protoplasts (or cells) to elicitation treatment for 1 h was studied by recording the variations in the activities of laminarinase as a function of polymerization degree (DP) of polyglucosamine. We were also interested in dose-response relationships as well as in the timing dependency of elicitor induced reactions.

MATERIALS AND METHODS

Materials

Oligo-, or polyglucosamine were used as inducers of laminarinase activity. Low-molecular weight inducers were prepared by hydrolysis of fully deacetylated chitosan, then using exclusion chromatography step on Biogel P_4 (for oligomers with DP 2 to 15) and on Biogel P_{60} (for oligomers with DP 20 to 60) (6). Higher molecular weights correspond to various fully deacetylated samples characterized by their viscosity-average degree of polymerization (7). All glucosamine oligomers were also characterized by assignment of the ^{13}C nmr signals.

Suspensions of cultured *Rubus fruticosus* L. originally derived from cambial explants from twigs were grown as in (8).

Protoplast preparation

Protoplasts were prepared from exponentially growing cells. 40 g of 19-day-old cells were incubated overnight at room temperature in 300 ml of the growth medium supplemented with 0.56 M mannitol. 0.25% (w/v) Caylase 345 and 0.010% (w/v) Caylase M3. The released protoplasts were passed through a 100 µm mesh nylon net and washed twice before centrifugation (500 g, 5 min.). They were then suspended in buffer, pH 4.8,(25 mM Bistris/HCl, 0.56 M mannitol, 0.06 M sucrose, 1 mM KCl, 1 mM $CaCl_2$) supplemented with 6% (w/v) Ficoll 400 before being purified using Ficoll gradient step. A Ficoll gradient was prepared over the 5 ml protoplast suspension by layering in succession 5 ml of buffer in 20% (w/v) Ficoll and 5 ml of buffer in 12.5% (w/v) Ficoll. This discontinuous gradient was centrifuged at 800 g for 30 min at 4°C. The purified protoplasts were obtained at the 12.5-20% Ficoll interface and were isolated by centrifugation at 500 g for 5 min. The protoplasts were washed with the same buffer without Ficoll and then resuspended in buffer elicitation.

Elicitation experiments

2.10^6 protoplasts (cells) were suspended in 25 ml Bis Tris/HCl buffer for 0-120 min on a roller mixer, with 0-0.45 mM of oligo- or polyglucosamine elicitor. Protoplasts (cells) were then harvested at indicated times, and washed twice with elicitation

buffer without sucrose before they were subjected to D-glycanase extraction.

Laminarinase assay

D-Glycanases were solubilized in 50 mM Tris-HCl buffer (pH 7.2) containing 1 M NaCl by homogenizing the protoplasts (or cells) on ice with a Polytron at full speed 15 times for 45 s. The enzyme extracts were dialyzed using ultrafiltration units equipped with a molecular weight cut-off value of 10000 (Ultrafree^{TM}, Millipore). Laminarinase (EC 3.2.1.6) was assayed as follows : 62.5 µg of laminarin were incubated with 10 µg enzyme extract (based on Bradford protein determination (9)) in 200 µl sodium acetate buffer (0.1 M, pH 5.0). The mixture was incubated at 40°C for 180 min and the enzyme reaction was stopped by heating at 100°C for 5 min. The enzyme activity was expressed in µmol reducing sugar-equivalents per mg protein and per hour ; the amount of reducing sugars end groups was measured according to Somogyi (10). For each sample, 9 enzyme assays were at least performed from 3 independent elicitation experiments and kinetic curves were drawn. The slope of the linear-regression fits of the data gave the enzyme activity. Blanks were carried out for each sample as detailed in controls. Enzyme activation was expressed as R which is the ratio of kinetic slopes obtained from treated versus control protoplasts (cells).

Controls

Laminarinase activity was checked in two controls (= non treated protoplasts (cells)) carried out concomitantly during a set of experiments. Enzyme extracts used for laminarinase activity are free of reducing sugars as shown by Somogyi-Nelson tests, and blanks from enzyme assays without the laminarin substrate were also performed.

The viability of protoplasts and cells during treatment was followed by withdrawing 200 µl aliquots from the protoplast or cell suspensions at various times. The aliquots were then mixed with 20 µl of 1% (w/v) Evan's blue indicator. The percentage of protoplasts or cells surviving the treatment is given by the number of unstained protoplasts or cells per 100 counted. The density of living protoplasts or cells was evaluated from 10 µl aliquots from this suspension placed in the counting chamber where a total of 2000 protoplasts or cells were counted.

RESULTS

Influence of DP of chitosan on the elicitation of laminarinase activity

Rubus protoplasts (cells) were incubated for 30 min in presence of 0.3 mM glucosamine elicitor with DP within 3 to 3850. The results reported in figure 1 were obtained from 9 enzyme assays through 3 independent sets of elicitation experiments ; the viability of the protoplasts (cells) had been checked and remained up to 85 (90%) for both the control and the assay after

2 h. The observations made in cells (curve b) with regard to the influence of DP were confirmed and largely amplified in protoplasts (curve a). Among glucosamine oligomers with DP $\leq$ 9, the oligomer of DP 4 exhibited the highest inducer activity ; it promoted a 3.6-fold activation in protoplasts and 1.6-fold only in cells. Polyglucosamine of DP > 9 exhibited a high inducing potency which increased up to DP 850 ; the decrease of induced response beyond this DP was observed both in protoplasts and in cells.

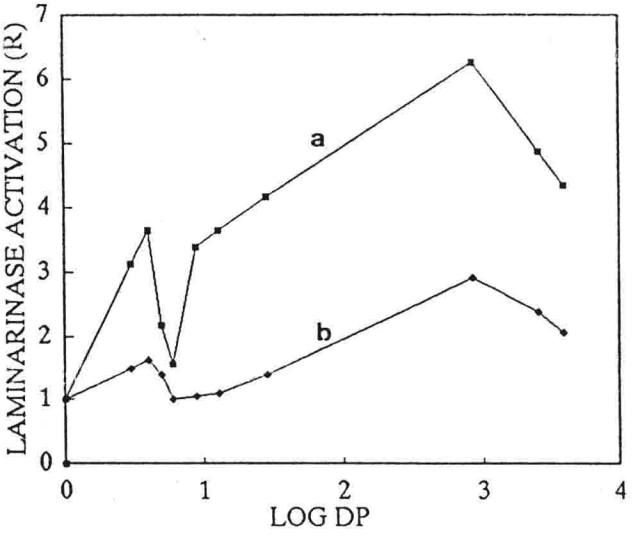

Figure 1. Influence of DP of chitosan of laminarinase activity in protoplasts (a) and in cells (b).

Dose-response of laminarinase activation

For the dose-dependence experiments, protoplasts (cells) were assayed for laminarinase after 30 min of elicitation treatment. The action of increasing amounts of glucosamine oligomer of DP 4 and of polyglucosamine of DP 850 were reported in figures 2A and 2B respectively ; the response of protoplasts and of cells shown in curves a and b respectively were each deduced from 12 enzyme assays carried out through 4 independent sets of elicitation experiments. We observed that the laminarinase activation in protoplasts was strongly dependent on the amounts of elicitor added. The protoplasts exhibited a bell-shape curve response to glucosamine oligomer of DP 4 (Fig. 2A, curve a) while the action of polyglucosamine of DP 850 (Fig. 2B, curve a) resulted in a plateau response beyond 31 µM. In contrast, the cells did not significantly respond to the increase in elicitor dose (Fig. 2A and B, curves b).

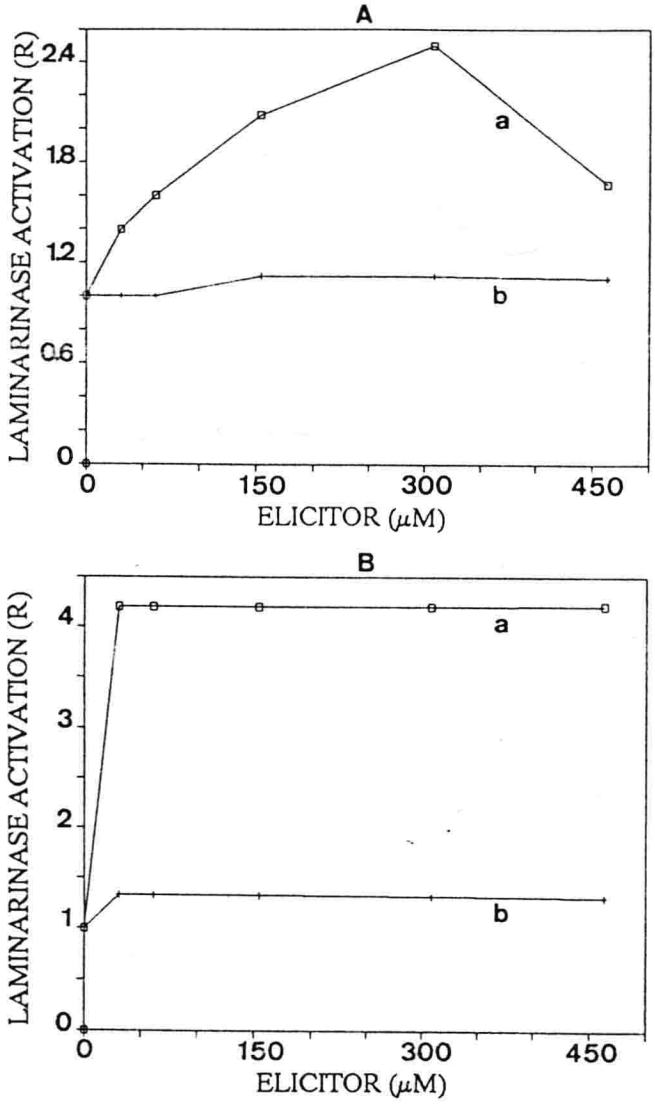

Figure 2. Laminarinase activation induced by glucosamine oligomer of DP 4 (A) or by DP 850 polyglucosamine (B) in protoplasts (curves a) and in cells (curves b).

Laminarinase induction kinetics

The kinetics of laminarinase were measured in protoplasts and in cells to directly compare the responses to oligomer of DP 4 and to DP 850 polyglucosamine ; the time-courses were presented in

figure 3A and B respectively. The results were obtained from 9

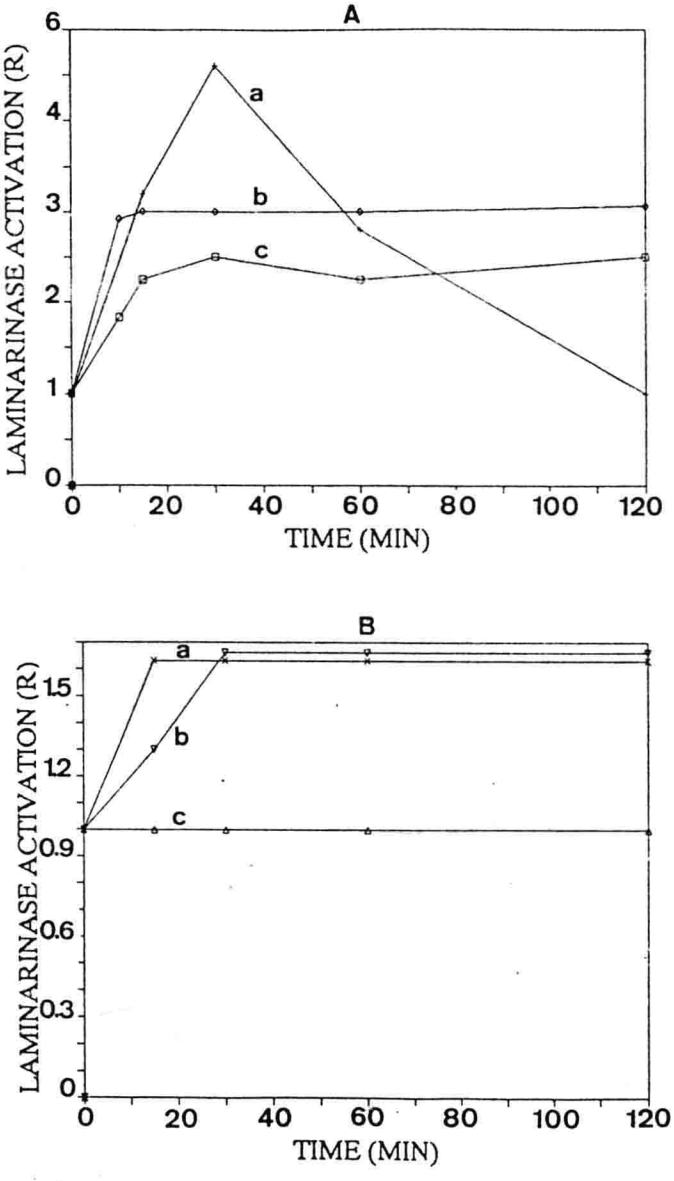

Figure 3. Time course of laminarinase activation in protoplasts (A) and in cells (B) elicited in presence of 0.3 mM (curves a) or 61 µM (curves c) glucosamine oligomer of DP 4, and in presence of 61 µM polyglucosamine of DP 850 (curves b).

enzyme assays through 3 independent sets of elicitation experiments carried out in presence of 61 µM or 0.3 mM elicitor. Qualitatively, the induction kinetics were similar for both the elicitors except for 0.3 mM glucosamine oligomer of DP 4 which triggered a rapid and transient response in protoplasts (Fig. 3A, curve a). The differences in the absolute levels of laminarinase induction reflected the better capability of protoplasts to be activated. They also revealed the higher elicitor activity of DP 850 polyglucosamine (Fig.3, curves b) compared to the oligomer of DP 4 (Fig.3, curves c). Furthermore the elicitor concentration has effect not only on the extent, but also on the timing of the induced response.

DISCUSSION

The findings from *Rubus* indicate that an elicitation response is retained and amplified in protoplasts as compared with suspended cells. This had been previously reported about the elicitation of coumarin synthesis, of callose formation and of laminarinase activation (11-13). Since the very short period of contact between elicitor and protoplasts required for laminarinase activation is not sufficient for cell-wall regeneration, the plant cell wall itself is not involved in the signal perception. Therefore the elicitor directly interacts wih plasma membrane.

The mechanism of laminarinase activity elicited by the poly(glucosamine) series in *Rubus* protoplasts can be described assuming two processes. The first is highly selective towards the oligomer of DP 4 and should be the consequence of a specific interaction with a receptor site. This site may be the lectin specific for glucosamine oligomers which had been previously isolated from *Rubus* membranes ; the lectin was shown to exhibit the highest activity for the oligomer of DP 4 (14). The second takes place for higher molecular weights, and in this case the biological activity increases linearly with log DP ; a similar behaviour was previously reported from the same series used as callose inducers in protoplasts (12). This is compatible with the fact that the primary event of induced reaction is not by specific binding to receptor but by a more general change in membrane properties which results of non specific interactions certainly of electrostatic kind.

REFERENCES

1. Walker-Simmons, M.L., Hadwiger, M.L. and Ryan, C.A., Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem. Biophys. Res. Commun., 1983, **110**, 194-9.

2. Köhle, H., Jeblick, W., Poten, F., Blaschek, W. and Kauss, H., Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+} dependent process. Plant Physiol., 1985, **77**, 544-51.

3. Liénart, Y., Driguez, H. and Domard, A., Chitosan as elicitor of β-glycanases from *Rubus* cells. In Chitin and Chitosan, ed. G. Skjak-Brack, T. Anthosen, P. Sandford, Elsevier Applied Science Publishers, London, 1989, pp. 225-31.

4. Kendra, D.F., Fristensky, B., Daniels, C.H. and Hadwiger, L.A., Disease resistance response genes in plants ; expression and proposed mechanisms of induction. In Molecular Strategies for Crop Protection, ed. C.A. Ryan, C. Arntzen, A.R. Liss, Inc., pp.13-24.

5. Scheel D. and Parker, J.E., Elicitor recognition and signal transduction in plant defense gene activation. Z. Naturforsch,, 1990,**45**, 569-75.

6. Domard, A. and Cartier, N., Glucosamine oligomers : 1. Preparation and characterization. Int. J. Biol. Macromol., 1989, **11**, 297-302.

7. Domard, A. and Rinaudo, M., Preparation and characterization of fully deacetylated chitosan. Int. J. Biol. Macromol., 1983, **5**, 49-52.

8. Hustache, G., Mollard, A. and Barnoud, F., Culture illimitée d'une souche anergiée de *Rosa glauca* par la technique des suspensions cellulaires. C.R. Acad.Sci. Paris, 1975, **281**, 1381-4.

9. Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principe of protein-dye binding. Anal. Biochem., 1976, **72**, 248-54.

10. Somogyi, M., Notes on sugar determination. J. Biol. Chem., 1952, **195**, 19-23.

11. Dangl, K.D., Hauffe, S., Lipphardt, S., Hahlbrock, K., Scheel, D., Parsley protoplasts retain differential responsiveness to UV light and fungal elicitor. EMBO J., 1987, **6**, 2551-6.

12. Kauss, H., Jeblick, W. and Domard, A., The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. Planta, 1989, **178**, 385-92.

13. Liénart, Y., Gautier, C. and Driguez, H., Silica-bound N-acetylglucosaminyl residues elicit laminarinase activity in *Rubus* protoplasts. Plant Science, 1991, **77**, 41-5.

14. Liénart, Y., Gautier, C. and Domard, A., Isolation from *Rubus* cell-suspension cultures of a lectin specific for glucosamine oligomers. Planta, 1991, **184**, 8-13.

BIOSYNTHESIS OF CELLULOSE SUSCEPTIBLE FOR CHITINOLYTIC ENZYME BY ACETOBACTER SP

Ryu Ogawa, Manabu Sato, Yoshiaki Miura, Masashi Fujiwara*
Mitsuo Takai* and Seiichi Tokura
Department of Polymer Science, Faculty of Science
*Department of Applied Chemistry, Faculty of Engineering
Hokkaido University, Sapporo 060, Japan

ABSTRACT

The incorporation of N-acetylglucosamine (GlcNAc into bacterial cellulose (BC) was achieved by the use of Acetobacter xylinum strains which repeated the transfer procedures to the GlcNAc liquid medium (containing only GlcNAc as a carbon source) or grown on a GlcNAc solid medium. The incorporation of GlcNAc residue was detected by the amino acid analysis of acid hydrolysate; 4 mol% of GlcNAc was the highest content found in the glucose residue. The GlcNAc incorporated BC(N-AcGBC) was found to be susceptible to not only cellulase, but also chitinase and lysozyme, even at a low GlcNAc content. This was confirmed by turbidimetry and scanning electron microscopy (SEM) observation.

INTRODUCTION

Chitin, a glucosaminoglycan of β-1,4 glycoside, is known to have a very tight crystalline structure owing to the strong hydrogen bonds formed by the acetamido group at the C-2 position of N-acetylglucosamine (GlcNAc) residue. These strong hydrogen bonds have not been studied sufficiently to investigate the crystalline structure, chemical modification and enzymatic hydrolysis of chitin. The present study was conducted in order to examine the substantial hydrogen bonds of chitin by the dispersion of tight hydrogen bonds among hydrogen bonds that are weaker because of glucose (Glc) residues; the chitin crystalline structure is formed by dual hydrogen bonds (1). The bacterium Acetobacter sp is known to produce a cellulose of β-1,4 glycoside linkage at high purity on the surface of a nutrient medium; Acetobacter xylinum is the best known type of Acetobacter sp to produce bacterial cellulose (BC (2). To prepare a mucopolysaccharide consisting of a Glc (as the major component) and GlcNAc (as the minor component) residue as shown in Fig. 1, we incorprated GlcNAc into the BC main chain through A. xylinum only by changing the culture conditions. The BC containing GlcNAc residue was also expected to become a

multifunctional polymer with both cellulosic and chitinous properties. This paper describes the preliminary study of the incorporation of the GlcNAc residue into pellicles by A. xylinum strains and of the chitinolytic enzyme susceptibility of the resulting mucopolysaccharide.

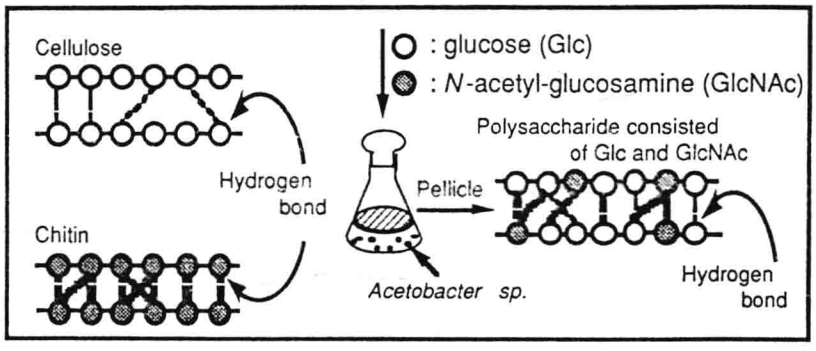

Figure 1. Proposal of hydrogen bond formed in bacterial cellulose by the incorporation of GlcNAc residue.

MATERIAL AND METHODS

Bacterial strains and culture conditions

The medium compositions are shown in Table 1; Scharmm-Hestrin (SH) Glc medium is the general medium for culture of the A. xylinum strains (2). The bacterial strains used for pellicle production are shown in Fig. 2.

Table 1
The components of the mediums
(The pH value of all mediums is 6.0)

Components of medium	Concentration (w/v%)						
	SH Glc med.(a)	SH mixture med.(b)					SH GlcNAc med.(c)
Glc	2.0	1.8	1.4	1.0	0.6	0.2	0.0
GlNAc	0.0	0.2	0.6	1.0	1.4	1.8	2.0
Bacto peptone	0.5						
Yeast extract	0.5						
Disodium hydrogenphosphate	0.27						
Citric acid	0.115						

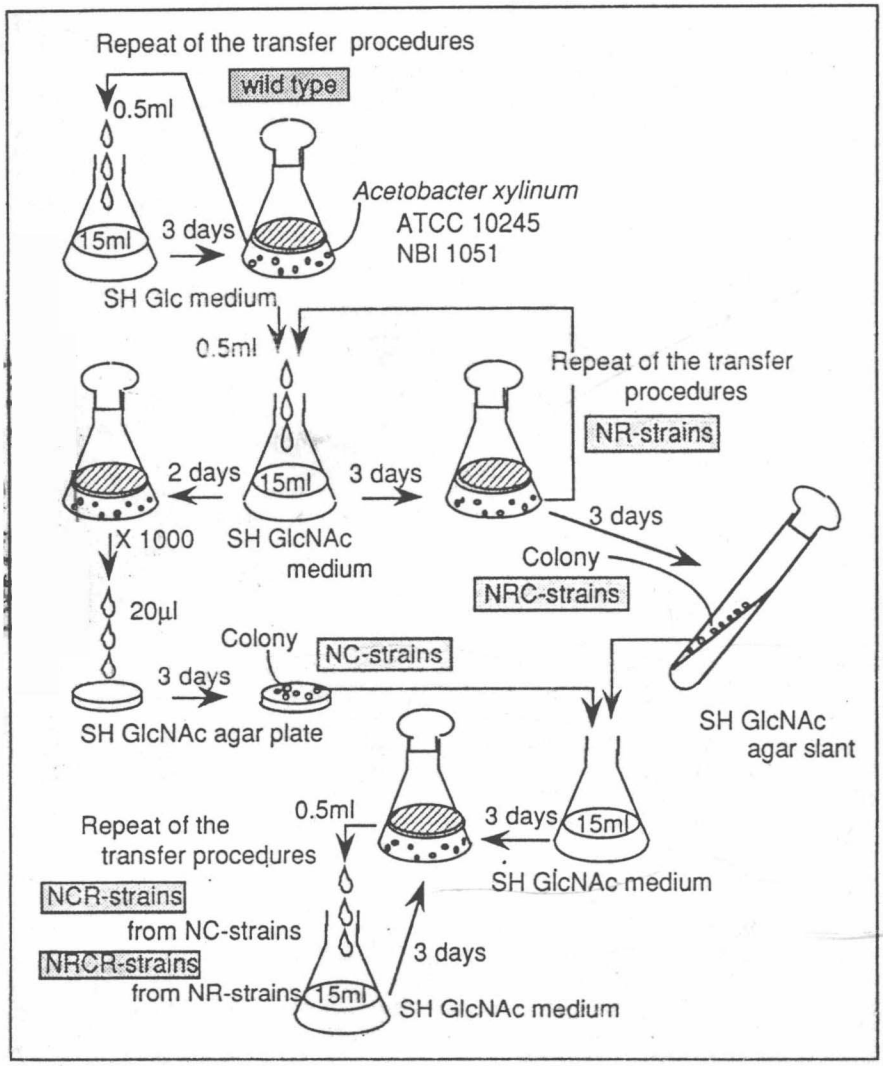

Figure 2. Culture procedure for activation of *A. xylinum* strains. All cultures were incubated statically at 28°C; the medium compositions are shown in Table 1. Strains achieved the respective procedure are represented in ▭.

Pellicle production and purification

0.5 ml of the cultures or a colony (as shown in Fig. 2) were inoculated into 15 ml of SH Clc or mixture medium (Table 1 (a) or (b) and incubated statically at 28°C. The pellicles produced at the

surface of the mediums were harvested after 4-7 days and washed with 2% (w/vP sodium dodecyl sulfate aqueous solution, 1% (w/v) aqueous NaOH, 1% (v/v)AcOH and water, respectively.

Estimation of incorporated GlcNAc in pellicles
The washed pellicles were dried at 105°C on glass or stainless steel plates and then swelled in 85% phosphoric acid as described in the text [3] and dried after extensive rinsing with deionized water. The resulting samples were hydrolyzed with 2N HCl for 12 hr at 100°C under reduced pressure. After the hydrolysis, excess acid was removed by evaporation in vacuo over NaOH. The GlcNAc content in the pellicles was estimated by amino acid analysis of the hydrolysate.

Enzyme assay
Egg white lysozyme (50,000 units/mg), chitinase-GODO (0.6 units/mg, Godo Shusei) and cellulase-Onozuka R-10 (1.5 units as carboxymethyl cellulase activity, Yakult) were purchased from Seikagaku Kogyo Co., Ltd., and applied without further purification. The general BC prepared in SH Glc medium by A. xylinum (wild type) and lyophilized β-chitin from Squid pens were used as standard substrates for each enzyme. Substrate suspensions were prepared by granulating solids with the "Waring 7012S" blender in a micro-container (#8575, at 14,700 rpm for 1 min x 5) at room temperature in a 0.05M acetate buffer (pH 4.5 for cellulase, pH 5.2 for chitinase, pH 6.0 for lysozyme). The optical density of the suspension at 540mm was adjusted to 1.0 by each buffer. Pellicles suspended in the buffer were prepared by a previously described method (4). The enzyme assays were carried out at 37°C, and measured the decrease of turbidity at $O.D._{540mm}$ for 10 min in the case of homogenized substrate. The enzyme assay and the surface observation of the pellicles were carried out by previously described methods (4).

RESULTS AND DISCUSSION

The formation of colonies
Colonies of NC-strains grown on the SH GlcNAc agar plate showed a configuration distinct from that of the wild-type rough and small colonies (5) on the SH Glc agar plate, which were smaller and brilliant white (Fig. 3-B). The number of colonies on the GlcNAc plate is also fewer than on the Glc plate, especially ATCC 10245 (Fig. 3-a). These observations seem to be related to the lower growth rate on GlcNAc than Glc (data not shown). Since colonies of a similar size as the wild type colonies were observed in the case of the NRC-strain colonies (Fig. 3-c), A. xylinum strains seem to have a relatively high adaptability to the SH GlcNAc medium, and a mutation of A. xylinum does not seem to have been introduced by cultures on the GlcNAc (neither did microscope observation show any morphological distinctions in the NR, NRC-strains and wild type colonies).

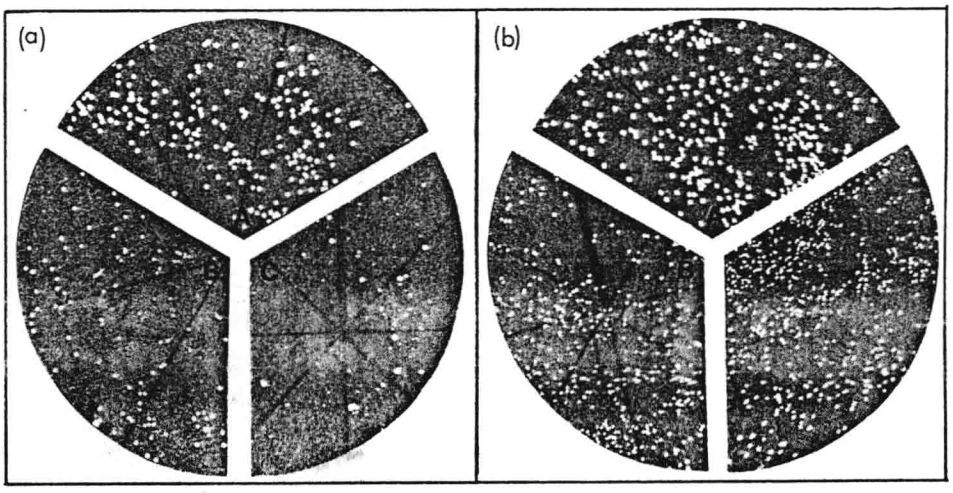

Figure 3. Colonies grown on SH Glc (A) or GlcNAc (B,C) agar plate; (a) : *A. xylinum* ATCC 10245, (b) : NBI 1051. (A) : wild type, (B) : NRC-strains, (C) : NR-strains by 10 times of transfer to SH GlcNAc medium

The profiles of pellical formation of A. xylinum strains

Fig. 4 shows the profiles of pellicle formation on the Glc or GlcNAc medium both by wild type and NR-strains. The thickness of the wild type pellicle (gel-like material; contained a large quantity of water) increased steadily as the culture aged. It is known that the amount of cellulose produced by the culture of the wild type A. xylinum increases exponentially with bacterial growth within 3 days' culture, and then the producibility of cellulose is increased (6). On the other hand, a very thin pellicle was produced in the SH GlcNAc medium, and the yield was approximately 1/10 of the wild type pellicle (the GlcNAc content was ca. 2-4 mol%). This result suggests that a part of the GlcNAc incorporated into cytoplasm is converted to Glc 6-phosphate (Glc-6-P), because it is known that cellulose synthesis start in A. Xylinum from the Glc-6-P, which synthesized through the two alternate pathways (7).

The Incorporation of GlcNAC into Pellicles

The incorporation of GlcNAc into pellicles reached a maximum value (ca. 2 mol%), in the case of the STG-strain, by five transfers to the SH GlcNAc medium and by culture in the SH medium, replacing 0.6% of the Glc with GlcNAc, as shown in Fig. 5. It is worth noting that some GlcNAc is usually present in the general BC produced by the wild type A. xylinum in the SH Glc medium, as shown in Fig. 5. No enzyme, acetamido group, transferase or enzyme picking up GlcNAc as a substrate seems to be present in the cellulose synthetic system. NR-strains were enhanced by the incorporation of GlcNAc up to 4.2 mol% with inoculation on the SH GlcNAc slants (Fig. 5). The GlcNAc content of the pellicles produced by the NC-strains (without

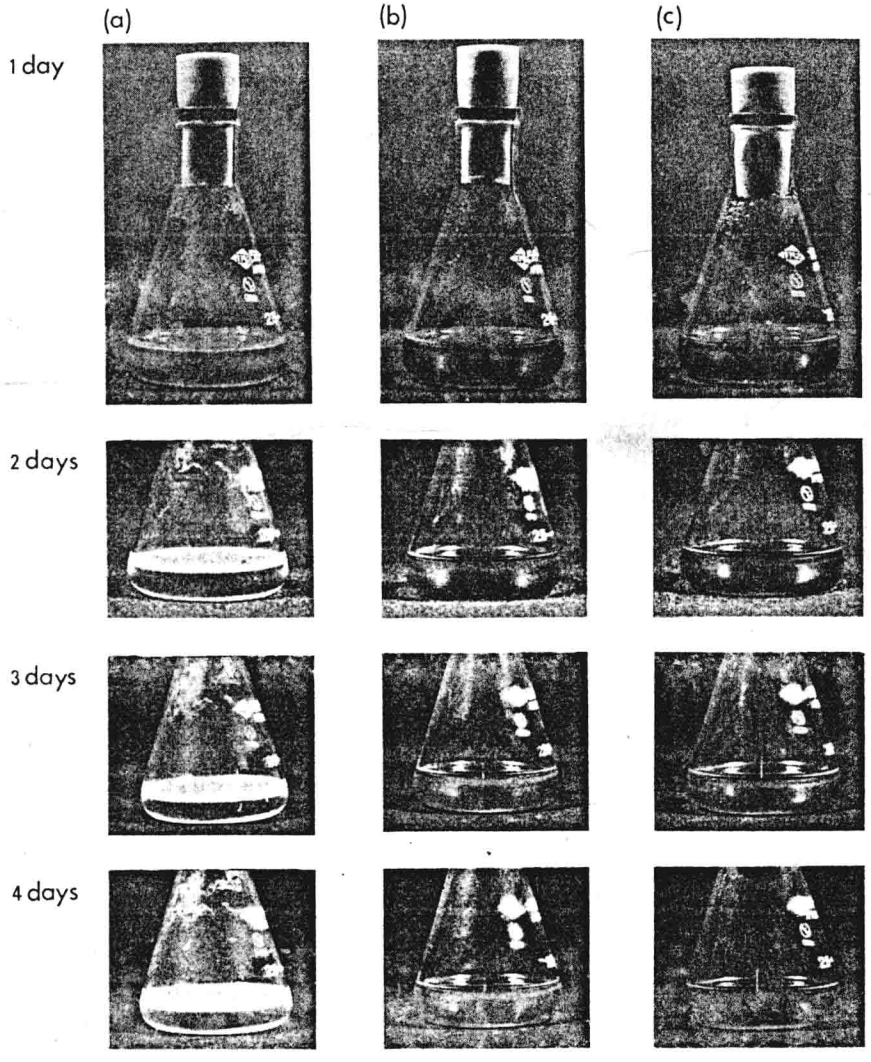

Figure 4. The profiles of the pellicle formation at the surface of the medium with the culture grew older.

(a) : the culture of wild type in SH Glc medium

(b) : the culture of wild type in SH GlcNAc medium

(c) : the culture of NR-strains (by 15 times of transfer to SH GlcNAc medium) in SH GlcNAc medium

serial transfers to the SH GlcNAc medium) was similar to that of pellicles produced by the NR-stains with serial transfers to the Sh GlcNAc medium (ca. 2 mol%). The GlcNAc content of the pellicles produced by the NCR-strains began to decrease by the number of transfers to the SH GlcNAc medium. Such a decrease was also exhibited by the NRCR-strains, and the growth on the SH GlcNAc agar slants was no longer effective in enhancing the incorporation of GlcNAc. The ability to incorporate GlcNAc into the pellicles of A. xylinum strains is shown in Fig. 6. Because a slight increase of GlcNAc content in the pellicles was also observed in the NR-strains in the SH Glc medium (Fig. 5), and because the GlcNAc content of pellicles obtained by the mediums composed of Glc and GlcNAc through the NC0 or NRC-strains were identical (ca. 2 mol% and 4 mol%, respectively), it can be assumed that the activation of a certain enzyme system is brought about by the repeated transfers to the GlcNAc medium and culture on the solid GlcNAc medium. The decrease of the content of GlcNAc in pellicles produced by the NCR-strains and NRCR-strains might suggest the regeneration of cellulose synthase activity, since the reproduction of the pellicles appeared when the pellicle-deficient strains were cultured stationarily (8). At the same time, the yield of pellicles in the SH Glc and mixture medium through NR strains was reduced markedly by the number of transfers to the SH GlcNAc medium despite the enhancement of the GlcNAc content, while the yield did not change so markedly in the strains transferred more than 5 times.

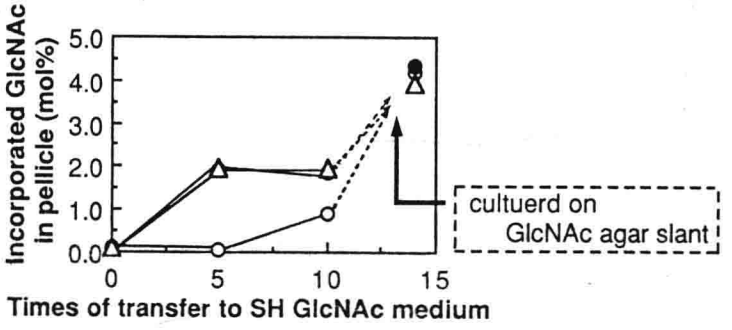

Figure 5. The relationship between times of transfer to SH GlcNAc medium and the amount of incorporated GlcNAc into pellicle (*A. xylinum* ATCC 10245 wild type and NR-strains).
○ : 2% of Glc in medium (SH Glc medium)
● : 1.4% of Glc and 0.6% of GlcNAc in SH mixture medium
△ : 0.6% of Glc and 1.4% of GlcNAc in SH mixture medium