CHITOSAN HEPTAMER ALTERS DNA, INDUCES DEFENSE GENES IN PLANTS AND INDUCES THE ACCUMULATION OF GENE p53 PRODUCT IN ANIMAL CELLS

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Abstract

We have reported extensively on chitosan's gene activation potential in plants and antifungal action against plant pathogens (1-10). The chitosan heptamer (4) is the optimum sized oligomer for both functions. We now report that the chitosan heptamer action is associated with DNA fragmentation in cells of the treated tissue (11,12). Chitosan heptamer has the ability in the presence of Cu^{++} to cleave DNA strands (13). Chitosan heptamer can activate pea defense gene promoters linked with reporter genes when transformed into tobacco, suggesting that it is the promoter sequence, more than species specific membrane receptors or cytoplasmic signals in the tobacco, that are involved in the gene regulation. In animal systems, the protein product of gene p53, a tumor-suppressing factor, accumulates following induced cleavage of nuclear DNA (14) and correspondingly, chitosan and a single strand nicking fungal DNase induce p53 product accumulation in mouse mammary cells (12). Because of the overlapping action of DNA-specific treatments such as chitosan, actinomycin D, mitomycin C, topoisomerase inhibitors and UV radiation, we propose that chitosan's gene activation potential is directed towards DNA conformational changes. We will discuss strategies for using chitosan to both increase disease resistance in plants and suppress tumor development in animals.

Keywords: p53, chitosan heptamer, disease resistance, gene activation, anti-tumor

Materials and methods

Preparation and treatment of pea endocarps

Pea plants (*Pisum sativum* J. cv. Alcan) were grown in a greenhouse. The endocarp surfaces of separated halves of immature pea pods (less than 1.5 cm long) were inoculated with one of the following treatments: sterile distilled H_2O, *F. solani* f. sp. *phaseoli (Fsph)* or *F. solani* f. sp. *pisi* macroconidia, *Fsph* DNase, chitosan heptamer (chitoheptaose heptahydrochloride, WAKO Pure Chemical Industries, Ltd., Osaka, Japan). The inoculum or treatment consisted of 100 μl of solution per 0.5 g of pod tissue. At the end of the incubation period, pods were washed twice in 10 ml sterile water to remove residual treatment solutions. The *Fsph* DNase was purified via 55-80% ammonium sulfate fractionation, isoelectric focusing and to near homogeneity through a (1x18 cm) Sephadex G-75 column (16).

Agarose plugs containing liquid N_2-ground pea endocarp tissue were suspended in 1.0 ml of nuclear buffer containing 0.15 M NaCl, 2 mM KH_2PO_4/KOH (pH-6.4), 1 mM EGTA and 5 mM $MgCl_2$ (17) with 2 mg/ml proteinase K and immediately mixed with 50 μl 1% low melting point agarose/1% pulse field grade agarose (prewarmed to 50 C). The agarose trapped DNA was CHEF gel separated. Low molecular weight DNA that had leaked out of the agarose during proteinase K digestion was separated by standard 1.2% agarose gels.

Three tobacco transgenic lines, 206D-17, S01 and CG8, were used to detect elicitor induction of the PR promoter-driven GUS (glucuronidase) reporter gene (18,19). Line 206d contains a chimeric gene fusion of the pea gene 206d promoter (10) with the GUS gene (Culley, D., unpublished). Line S01 contains a chimeric gene fusion of the *Arabidopsis* PAL1 promoter with a GUS gene and line CG8 contains a chimeric gene fusion of the bean chalcone synthetase gene promoter with a GUS gene (19,20,21). Fifty μl of a solution containing a designated treatment, such as H_2O, chitosan heptamer, *Fsph*-DNase or *Fsph* spores, was forced into the leaf intercellular space. The leaf area around the penetration site was harvested with a no. 6 cork bore and the disk was immediately immersed in liquid N_2 and GUS assayed (18).

Similarly, tobacco leaves were treated with *Fsph* DNase or chitosan to observe the DNA cleavage associated with these two elicitors. The entire contents of the harvested leaf disk were ground in liquid N_2 and used for DNA cleavage analysis following the procedures described for the pea tissue.

Chitosan treatment of mouse tumor cells

Mouse tumor cells were from an anchorage independent WAZ-2T cell line (+SA) derived from an adenocarcinoma that developed spontaneously from hyperplastic BALB/c mouse mammary tissue (22). A preneoplastic cell line, CL-S1, was also from BALB/c mouse. Mouse cells (2×10^5) were treated in 1 ml of DME medium supplemented with 10% bovine calf serum. At the completion of the treatment period the cells were harvested, pelleted in a microfuge tube, and frozen immediately in liquid N_2 to prevent DNA digestion. The pellet was thawed in the presence of 10 μl proteinase K (2 mg ml^{-1}) in the buffer and the high MW DNA was fractionated on a CHEF gel as above and the low MW DNA fractionated by electrophoresis on a 1.2 % agarose gel.

A closely related but non-tumorigenic mammalian cell line, CL-S1 (23), derived directly from hyperplastic tissue, was treated as indicated above. The harvested cells were immersed immediately into the electrophoresis loading buffer, electrophoresed in 10% acrylamide gel, and transblotted to Immobilon P (Millipore) membrane. The electrophoresed and transblotted proteins were western-analyzed for p53 protein accumulation utilizing the following primary monoclonal antisera: Pab 421 (Calbiochem), DO-1 (no. 6) (Neo-Markers, Fremont, CA), Pab 240 (Novocastra Laboratories Ltd., Newcastle, U.K.). The secondary antibody was sheep anti-mouse conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL) and was detected using the Amersham enhanced chemiluminescence (ECL) protocol and hyperfilm-ECL.

Results and discussion

We have previously reported that chitosan is an effective inducer of disease resistance responses in plants (1,5,7). Although we observed 1) that chitosan (especially oligomers of chitosan of at least 7 sugar units in size) can activate plant defense genes (4), 2) that chitosan can enter the nucleus of the pea cell (16) and 3) has a high affinity for cellular DNA (1), the model for its mode of action remains tentative. Its proposed action on DNA in pea plants is consistent with that of a number of documented DNA-specific treatments including mitomycin C, actinomycin D, chromomycin A_3, UV_{260}, UV_{366}-activated psoralens, basic nuclear proteins, base analogs and most recently, DNase.

Our preliminary results indicate that chitosan (Table 1) can also induce the accumulation of gene p53 product in animal cells (12). The p53 protein is an important tumor-suppressing factor. Interestingly the p53 accumulation is stimulated by essentially the same group of DNA-interacting factors listed above (Klosterman S., unpublished), suggesting there are similarities in the modes by which all of these gene activators function. Within 2.5 h following the treatment of pea endocarp with chitosan, *Fsph* DNase or *F. solani* spores, the cellular DNA becomes measurably fragmented (Fig. 1). Within 3 h these same treatments increase the mRNA homologous with several defense genes (5,7,9,10).

Table 1. Chitosan can increase the tumor suppressing gene p53 protein in preneoplastic mouse mammalian cells.

Treatment	Cell Line	Induction Period	p53 Induced
PBS	CL-S1	6 hours	No
		18 hours	No
Chitosan 960 µg/ml	CL-S1	6 hours	Yes
		18 hours	Yes
PBS	-SA	18 hours	No
Chitosan 960 µg/ml	-SA	18 hours	No
PBS	+SA	18 hours	No
Chitosan 960 µg/ml	+SA	18 hours	No

Mouse cell lines: CL-S1=non-tumorigenic breast cells, -SA=somewhat tumorigenic, +SA=tumorigenic, PBS=Phosphate buffered saline.
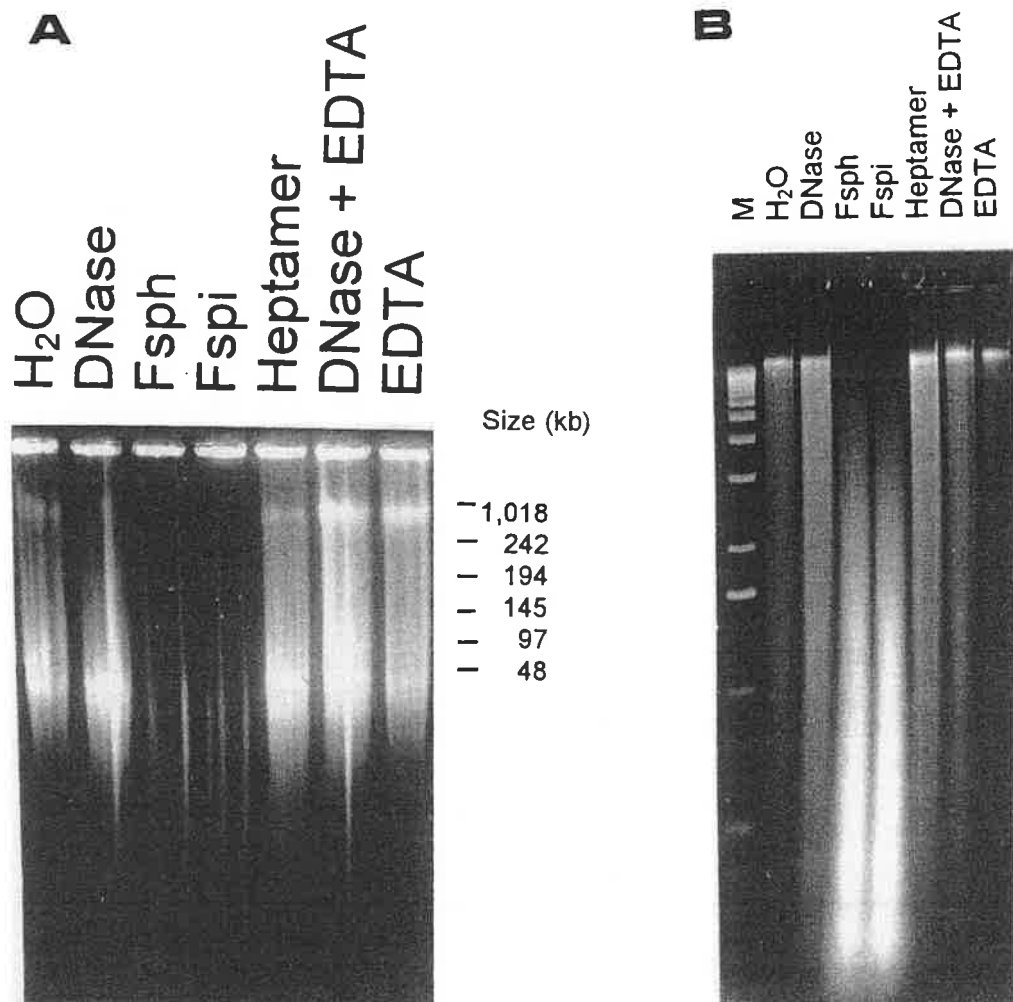
Fig. 1. CHEF gel electrophoresis of "agarose trapped" high molecular weight DNA (A) and conventional agarose gel electrophoresis of low molecular weight, fragmented DNA (total diffusate from CHEF gel plugs) (B) from pea endocarps treated for 2.5 h with: H_2O (lane 1), *Fsph* DNase (32 units ml^{-1}) (lane 2), *Fsph* spores (1 x 10^7) (lane 3), *F. solani* f. sp. *pisi* (*Fspi)* spores (1 x 10^7 ml^{-1}) (lane 4), chitosan heptamer (1 mg ml^{-1}) (lane 5), DNase (2 units μl^{-1}) plus 10 mM EDTA (lane 6), 10 mM EDTA alone (lane 7). Lambda ladder PFG marker bars in (A) and M:BRL 1 kb ladder DNA in (B).

Following the transfer of defense gene promoters from pea, arabidopsis or bean to tobacco, (all linked with the glucuronidase or "GUS" reporter gene) the chitosan inducer remains capable of activating the promoter (11). Again extensive DNA degradation occurs in tobacco tissue within 2 h following treatment (not shown), this is followed by an increase in catalytic activity of the glucuronidase protein product of the transvected GUS gene within 3 h (Table 2). Chitosan can mimic the action of a DNase released by *Fusarium solani* f. sp. *phaseoli* (*Fsph* DNase) on the same defense gene promoters even though the promoters are separated from their inherent transcription factors, receptors, etc. of their inherent plant species. Thus chitosan, like *Fsph* DNase, can function to enhance DNA fragmentation.

Table 2. Induction of PR promoter driven GUS expression 3 h following treatment with the chitosan heptamer administered to transgenic tobacco lines SO1 and CG8. Treatments ($50\mu l$) were introduced into intercellular space through a pin point opening.

Treatment	Conc μl^{-1}	PAL-promoted GUS activity - line SO1	CHS-promoted GUS activity - CG8
		Fold of control	Fold of control
H_2O		1.0	1.0
Chitosan Heptamer	0.5 μg	2.09 ± 0.44	1.65 ± 0.10
	0.25 μg	1.02 ± 0.11	1.24 ± 0.03
	0.12 μg	1.34 ± 0.50	1.27 ± 0.34
	0.06 μg	1.25 ± 0.15	1.53 ± 0.03
	0.03 μg	2.50 ± 1.11	2.65 ± 0.14
	0.01 μg	2.23 ± 0.94	1.32 ± 0.08
Fsph spores	6.7 x 10^3	1.32 ± 0.48	1.51 ± 0.67

The mode by which p53 protein accumulates in animal cells following DNA damage is not totally resolved, however considerable consensus exists that it is a broken DNA strand that provides the site of p53 protein recognition (14,24). The p53 protein undergoing conformational change ultimately becomes stable to degradation and the accumulated p53 product subsequently influences the regulation of an extensive number of interacting proteins.

We observed that chitosan, DNase and actinomycin D treatments to preneoplastic mouse breast cells are associated with the accumulation of p53 gene product (Klosterman, S.,unpublished). Actinomycin D consistently causes the greatest accumulation of p53 product and likewise is the most effective in altering the conformation of cellular DNA (Fig. 2). One role of the p53 protein in animal cells is in DNA repair (26), however to date there is no known function reported for an analogous DNA damage-induced plant gene.
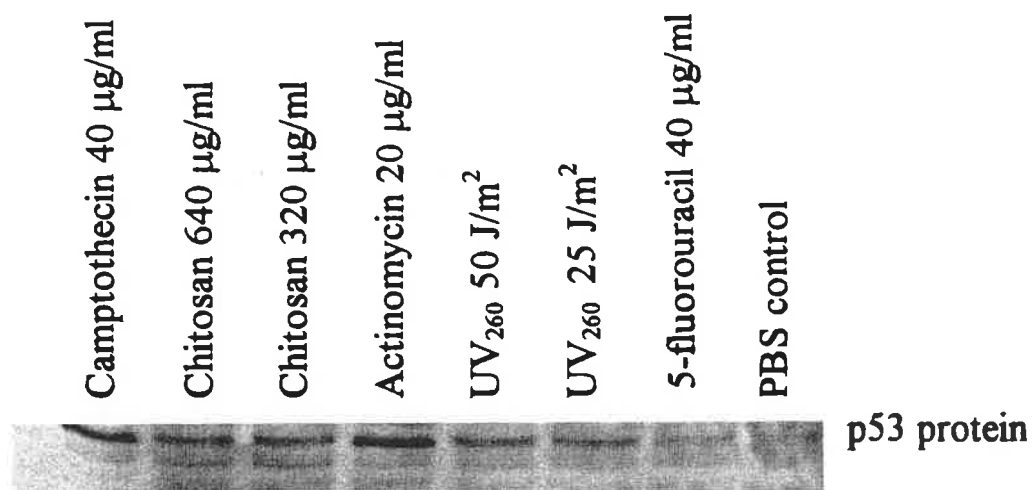
Fig. 2. (A) The preneoplastic mammary cell line CL-S1 was treated 3 h with the "DNA-damaging compounds": Camptothecin, chitosan, actinomycin D, UV_{260} and 5-fluorouracil. The p53 sized protein bands reacting in the western blot with monoclonal anti-p53 antisera are indicated by "p53 protein".

Conclusion

The results discussed are consistent with the explanation that chitosan's action is associated with DNA damage. However, because of the high concentration of positive charges within the chitosan molecule, it is unlikely that negatively charged molecules along its cell entry route remain unaffected. An earlier electron microscope study indicated that both the plant membrane and cell wall are also changed (25) following chitosan treatment. The knowledge that chitosan can activate a promoter in a foreign cell environment suggests practical applications related to gene activation. Chitosan can be utilized to activate genes in plants that are engineered with chitosan-inducible promoters via simple external treatments. Agriculture applications aimed at improving disease resistance in plants (Fig. 3) and controlling the production of pharmaceutically important proteins, also in plants, are underway in our laboratory.
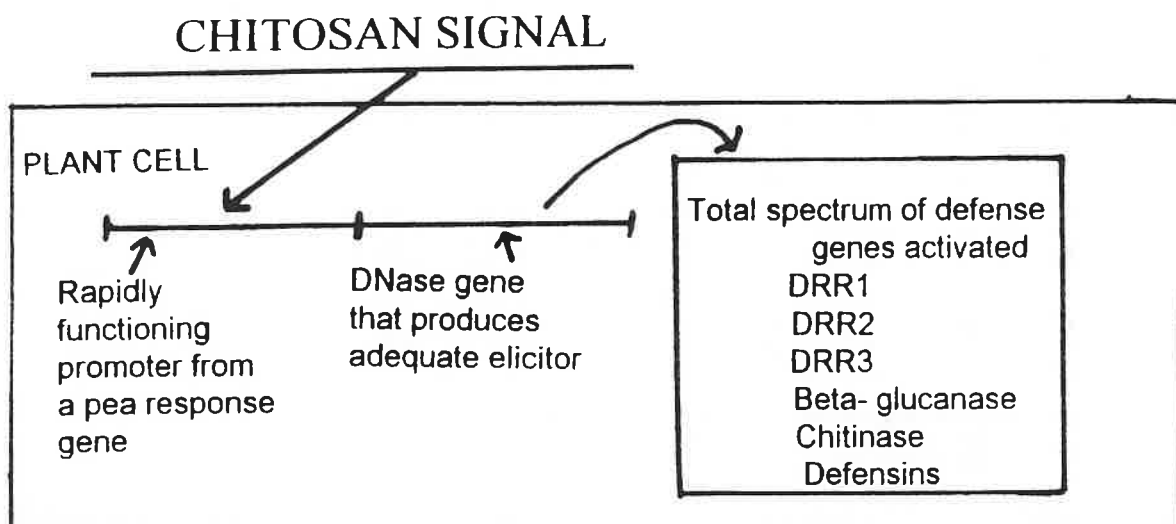
CHITOSAN SIGNAL

PLANT CELL

Rapidly
functioning
promoter from
a pea response
gene

DNase gene
that produces
adequate elicitor

Total spectrum of defense
genes activated
DRR1
DRR2
DRR3
Beta- glucanase
Chitinase
Defensins

Fig. 3. *Fsph* DNase has been shown to induce a disease resistance response that renders pea tissue immune to the pea pathogen, *F. solani* f. sp. *pisi*. We propose transforming potato plants with a gene construction containing a chitosan-inducible promoter from pea linked with the *Fsph* DNase structural gene. The expression of the DNase gene should provide an internal signal to activate the total spectrum of defense genes in the plant host. The promoter activating the DNase gene expression responds to external applications of chitosan.

Further, we propose that because chitosan can activate the plant defense gene promoters as they reside in foreign cells, that a future role of chitosan may exist as a strategic component of suppressed tumor growth. In preneoplastic cells that still possess a wild type p53 gene, chitosan can cause an accumulation of p53 product capable of cell cycle control and DNA repair (26). To confront cancer cells with a mutated p53 gene, chitosan could function following transformation of these cells in a gene replacement therapy (27). The gene replacement construction would contain a chitosan-inducible promoter, linked with a wild-type gene p53 and thus afford an external elicitor (chitosan) control of p53 product accumulation .

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Chitin-protein complex system in insects

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Abstract

Insect chitin occurs as a glycoprotein microfibril in the cuticle reinforcing the exoskeleton. During the early part of the larval instar, chitin is assemled in the endocuticular region and during the latter part, in response to the moulting hormone signal, the cuticle along with the chitin is degraded. In addition to being a glycoprotein, the protein component is cross linked with DOPA and its derivatives resulting in the sclerotization of the cuticle. The protein precursor for the chitin microfibril appears to be present in the epidermis from the beginning of the instar whereas the glycosylase appears during the early part of the stadium in the hemolymph and enters the epidermal cell at a later stage. The pre-pupal moulting fluid and and hemolymph have glycoproteins some of which may be invovled in the assembly or dissolution of the chitin microfibril. A spruce budworm specific DDC probe was synthesized to locate the stage at which sclerotization occurs.

Keywords: (5 -10) spruce budworm, glycosylation, dopadecarboxylase, RT-PCR, RH-5992, moulting fluid, ultrastructure, N-acetylglucosamine, chitin, sclerotization.

Materials and methods

Insects: The spruce budworm larvae were reared on a meridic diet after the method of Grisdale (1970) at 22°C, 70%RH and a photoperiod of 18 h light and 6 h darkness. Moulting larvae showing head capsule slippage as a white band were harvested and allowed to molt (Retnakaran 1980). Newly molted larvae with white untanned heads were designated as 0 h and staged from this time.

Ultrastructure: For RH-5992 (a non-steroidal ecdysone agonist) treatment, precisely staged larvae were force fed 1µg in 1µl as an aqueous suspension. Both treated and untreated larvae were fixed by injecting ice cold 5% aqueous glutaraldehyde in 0.05 M cacodylate buffer containing 0.01 M $CaCl_2$ and 2% sucrose at pH 7.3. The mesothoracic tergite was dissected 10 min later and further fixed in ice cold glutaraldehyde fixative, washed and fixed in OsO_4. The tissue was stained in 2%uranyl acetate, dehydrated and embedded in Araldite. Thin sections were examined and photographed in a Jeol 1200 Ex ll TEM operated at 80kV (for details see Retnakaran et al 1996A).

Glycosylase activity: The glycosylation precursor protein was prepared by collecting 8,<24 h old sixth instar larvae, dissecting the integument (epidermis and cuticle), rinsed in ddH_2O, homogenized in 1 ml of 0.0625M Tris buffer pH 6.8 containing 0.1M Phenylmethylsulfonyl fluoride (PMSF), centrifuged and the supernatant was used. Control protein was prepared similarly from >48 h old sixths. Moulting fluid was collected from pre-pupae showing anterior shrinkage by tearing the apolysed old cuticle, drawing the fluid into a glass capillary needle without damaging the new cuticle and mixing with equal volume of Tris-PMSF. Hemolymph was collected from < 24 h larvae and pre-pupae in equal volume of Tris-PMSF. Five µl of aqueous ^{14}C- N-acetyl glucosamine (NAGA) containing 1 µCi

of radioactivity was mixed with 100 μl of precursor or control protein, 50 μl of moulting fluid or <24 h hemolymph or pre-pupal hemolymph (Total 155 μl/reaction). The reaction mixture was incubated at 30°C for 4 h after which the reaction was stopped by adding 5 ml of ice cold 0.1% trichloracetic acid (TCA) and the protein was allowed to precipiate for > 4 h at 4°C. The protein was collected on a glass fiber filter in a vacuum filter unit. The reaction tube was rinsed with TCA several times, transferred on to the filter and washed. The glass fiber filter was dried, placed in a scintillation vial containing 10 ml of scintillation cocktail and the DPM was counted in a Beckman counter.

Protein gels: The moulting fluid and hemolymph from different stages were collected into 2x loading dye (bromphenol blue in SDS, glycerol, tris and β-mercaptoethanol) and the proteins were separated on 12% tris-glycine SDS-polyacrylamide resolving gel with a 5% polyacrylamide stacking gel. The running buffer was 25 mM tris, 250 mM glycine with 0.1% SDS at pH 8.3 and the gel was run at 50v until the dye moved into the resolving gel after which it was increased to 100v for the duration of the run which was around 12h. The gel was stained for 2h with coomassie brilliant blue R250 and destained in 35%methanol containing 7% acetic acid. For glycoprotein staining the gel was oxidized for 1h in 1%periodic acid made in 3% acetic acid, washed thoroughly in water, stained with Schiff's reagent and stored in 1% sodium metabisulfite (Clarke 1964).
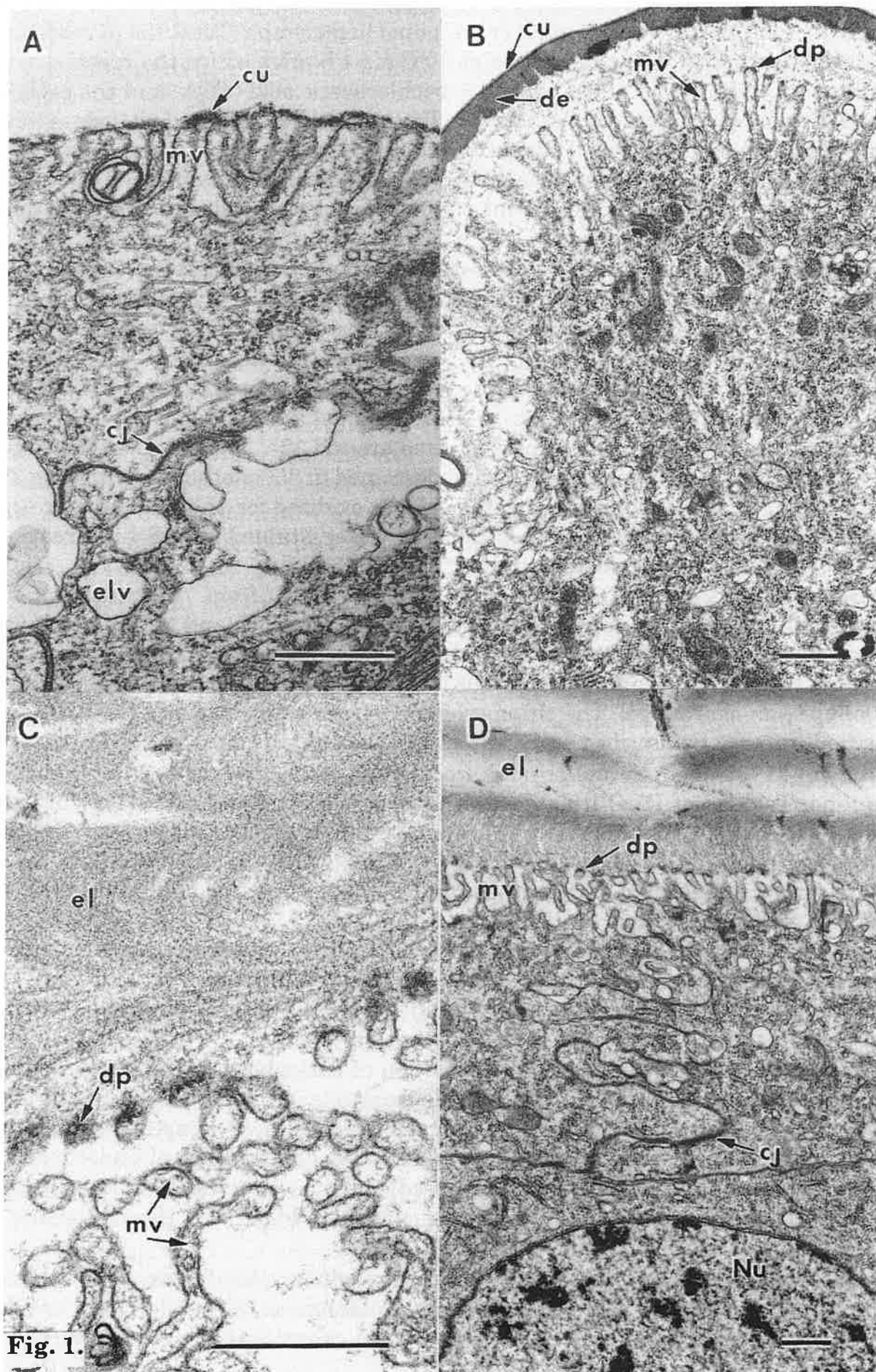
Dopadecarboxylase (DDC) probe: Total RNA from the integument (epidermis+cuticle with no internal organs) of moulting 5th and "white head" 6th instar larvae were extracted with guanidine isothiocyanate-phenol-chloroform method (Chomczyski and Sacchi, 1987) and the quality checked on a 1.3% formaldehyde-agarose gel. The chromosomal DNA was digested and the RNA was purified, reverse transcribed and the resulting cDNA was used in a PCR protocol (Saiki et al 1988). Degenerate primers were designed on the basis of the common sequence regions of DDC from several insects (Fang et al 1997). The forward primer, 5'GC(C/T) TG(C/T) AT(C/T) GG(A/T) TT(C/T) AC(C/T) TGG AT3' and the reverse primer, 5'GG(G/T) AT(T/C) TGC CA(G/A) CG(A/G) TA(A/G) TC3' were synthesized in a Beckman oligosynthesizer. Details are described in Palli et al 1996.
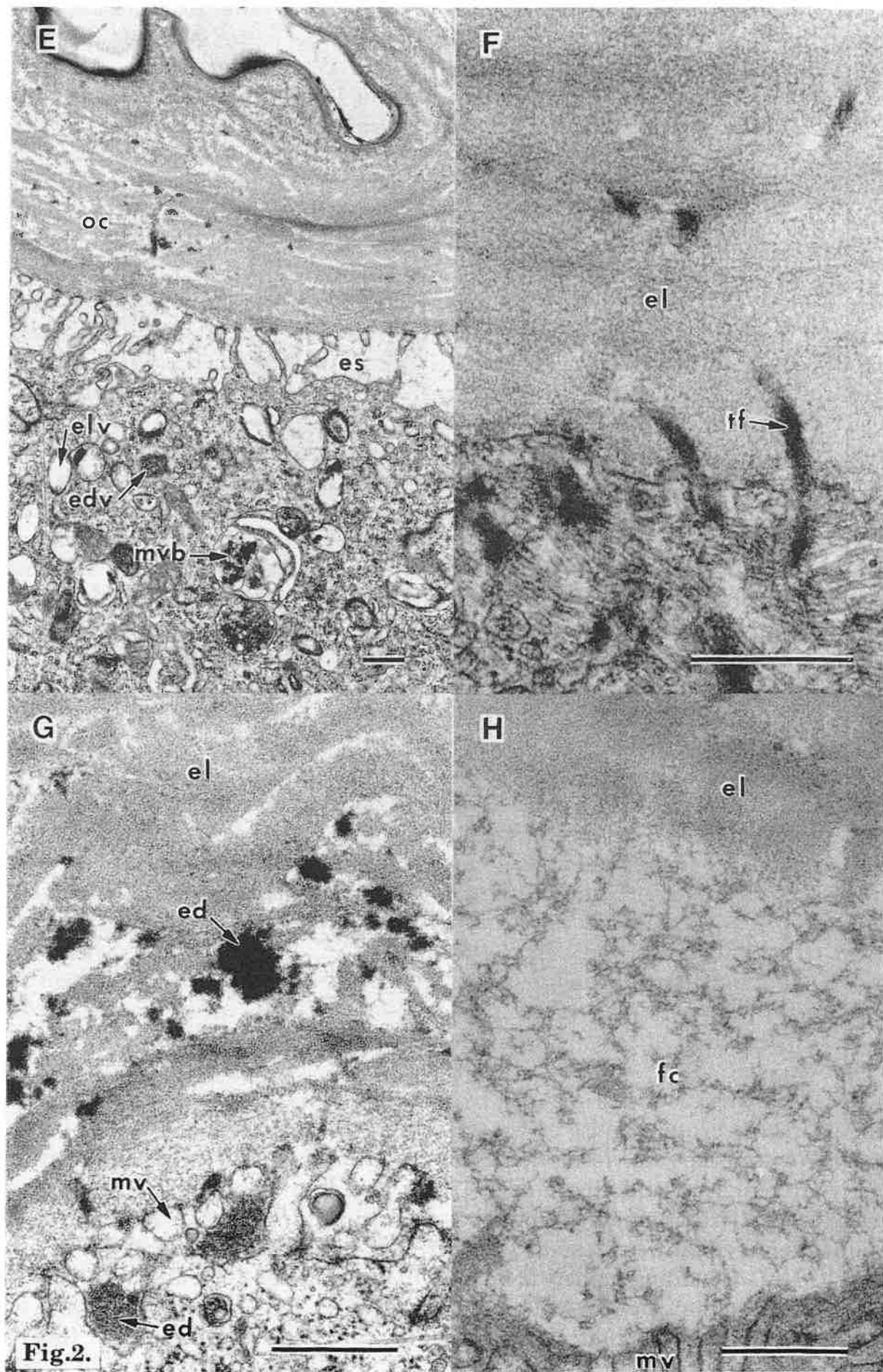
Results and discussion

The β 1-4 linked N-acetylglucosamine polymer, chitin, is the main component of arthropod exoskeletons and is widely distributed among varied groups of inveretebrate phyla as well as fungi (Cabib 1987). The arthropod or to be more

Fig.1. Assembly of chitin fibrils.A. Deposition of cuticulin; B.Formation of dense epicuticle; C.Streaming chitin microfibrils from tips of microvilli.D.Fully formed cuticle; Legend: Bar = O.5μm; cj-cell junction; cu-cuticulin; de-dense epicuticle; dp-dense plaque; ed-ecdysial droplet; edv-electron dense vesicle; el-endocuticular lamella; elv-electron lucent vesicle; es-ecdysial space; fc-fibrous cuticle; mv-microvilli; mvb-multivesicular body; nu-nucleus; oc-old cuticle; tf-tonofibrilla.

Fig.2. Dissolution of old cuticle and breakdown of chitin microfibrils. E. Epidermal cell pulling away from cuticle (apolysis); F. Detachment of muscles from cuticle; G.Exocytosis of ecdysial droplet; H. Dissolution of endocuticular lamella (chitin microfibrils). For legend see Fig.1.
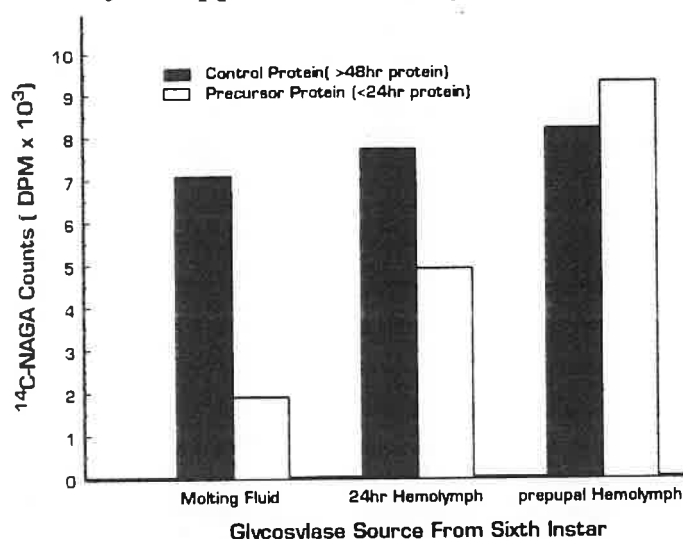
Fig. 1.

Fig.2.

specific, the insect chitin biosynthetic pathway appears to differ from that of fungi in the ultimate polymerising step involving the enzyme, chitin synthase (Mulisch et al 1993; Retnakaran 1995; Retnakaran and Oberlander 1993). We have previously reported on the chitin synthetic profile, lectin binding, and hormonal regulation in the spruce budworm, *Choristoneura fumiferana* (Retnakaran 1995; Retnakaran et al 1995, 1996A). In this paper we describe the ultrastructural assembly and dissolution, the putative glycosylation site, the developmental protein profile and finally synthesis of a DDC probe for locating time of sclerotization.

A.) Ultrastructure: We examined two aspects, assembly and dissolution of the chitin-protein complex during the last larval(6th) instar in the spruce budworm. The assembly begins with the formation of a protective epicuticle which is resistant to degradation by the enzymes in the moulting fluid (Fig 1A). A second protective layer, dense epicuticle is secreted below the cuticulin layer (Fig 1B). We were able to dramatically show both these structures by examining 1 day old sixth instar larvae treated with RH-5992 (Retnakaran et al 1996B). Next we see the secretion coming out of the tips of the microvilli in the form of swirls that form the endocuticular lamellae which is made of chitin microfibrils (Fig 1C). All these features occur during the first day of the last instar larva and by 2 days the cuticle is fully synthesized (Fig 1D). It has been shown that chitin is covalently attached to specific cuticular proteins to form the microfibrils (Schaefer et al 1987). Whether the chitin and protein precursors are bound inside the epidermal cell and secreted through the tips of the microvilli as a chito-protein composite or are secreted as separate strands that are bound together outside the cell has not been resolved. The dissolution of the cuticle occurs at the latter half of the larval stage. As soon as the hormonal signal for initiating the moulting process is received , the epidermal cells pull away from the cuticle above, a process called apolysis creating the ecdysial space. The surface of the epidermal cells takes the form of a lawn covered with active microvilli (Fig 2A). The muscle attachments (tonofibrillae) anchored in the cuticle are detached and as a result the larva at this stage becomes quiescent (Fig 2B). Ecdysial or moulting fluid made in the epidermal vesicles are exocytosed from between the microvilli and they enter into the ecdysial space (Fig 2C). The ecdysial fluid contains endo and exochitinases as well as proteases necessary for digesting the old cuticle(Reynolds and Samuels 1996; Zen et al 1996). The old cuticle is digested and remnants of it can be seen as fibrous material (Fig 2D). The old cuticle consisting of the cuticulin layer, dense epicuticle and distal part of the undigested endocuticle is cast off at ecdysis when the larva enters the next instar.

B.) Glycosylation: Since we know that chitin is in reality a glycoprotein made up of a NAGA polymer bound to specific protein or proteins, we tested several sources of the precursor protein and a glycosylase enzyme source for ^{14}C-NAGA binding activity. We used the same technique that we had used earlier for chitin synthesis activity (Retnakaran and Oberlander 1993). Three possible enzyme sources for glycosylase activity namely prepupal moulting fluid, <24 h hemolymph and prepupal hemolymph were tested. Two different sources for precursor protein for chitin(^{14}C-NAGA) binding namely ,24 h protein designated as precursor protein and >48h protein designated as control protein were tested. Regardless of the source for all three glycosylases, the control protein showed the same level of activity (Fig 3).

When the precursor protein was used the the prepupal hemolymph had the highest activity. It appears that the glycosylase is secreted into the hemolymph possibly from the fat body or hemocytes and transported into the epidermis after 48h. The control protein was taken from the prepupal stage from the epidermis which already had the glycosylase and therefore there was no need for an external source of glycosylase. The precursor protein was taken from <24h old epidermis which did not contain any glycosylase. Moulting fluid as expected did not have any glycosylase activity. It has been demonstrated earlier that this process can be inhibited by the glycosylase inhibitor, tunicamycin (Horst and Walker 1993; Quesada-Allue1982;Retnakaran 1995).

Fig.3. Glycosylation of precursor protein.

C.) Protein source: The moulting fluid and hemolymph protein profiles are shown in Fig 4. The pre-pupal protein profile stained with coomassie blue (4A) shows the
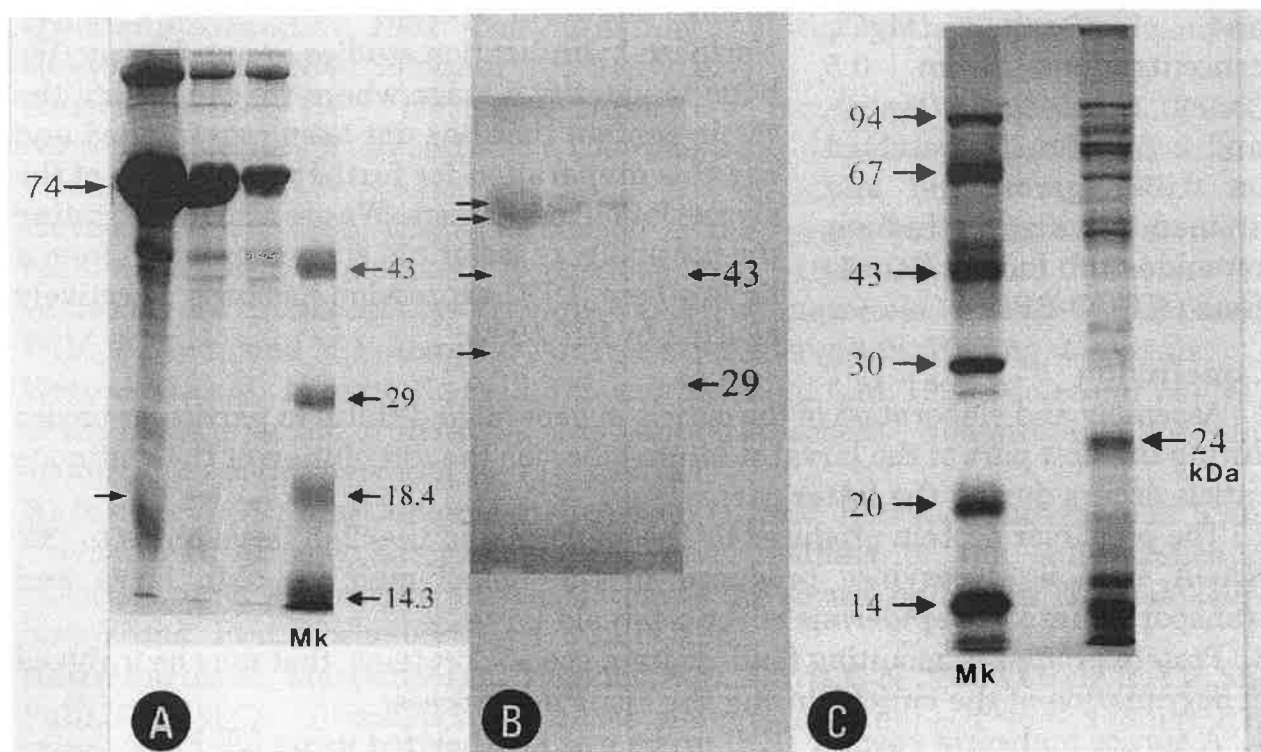

Fig.4. Hemolymph and moulting fluid protein profiles. A. Pre-pupal hemolymph protein stained with coomassie blue and B. with PAS (periodic acid Schiff's): C. Prepupal moulting fluid stained with coomassie blue.

two large proteins,72 and 74 kDa that also stain with Schiff's (4B) indicating that they are glycosylated. These are most likely storage proteins not involved in

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glycosylation. The pre-pupal moulting fluid shows several non-hemolymph proteins especially the 24 kDa protein (4C). Since the moulting fluid has the degrading enzymes, it is likely that it may be either a chitinase or a protease.

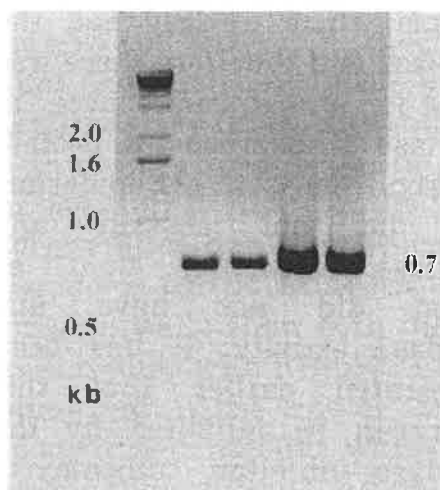**D.) Dopadecarboxylase**: DDC is one of the earliest enzymes that was demonstrated to be under the control of the moulting hormone, ecdysone and has been extensively studied (Hiruma et al 1995). DDC is resposible for the formation of DOPA which is responsible for cross linking the proteins in the cuticle. In addition to chitin being bound to specific proteins (primary stabilization) there is cross linking with DOPA and its derivatives which is described as sclerotization (secondary stabilization) (Anderson et al 1995). Studies with RH-5992, a non-steroidal, stable ecdysone agonist has shown that DDC which is normally expressed after the rise and fall of the ecdysone peak is not expressed when treated with this agonist which is not cleared from the epidermal cells (Retnakaran et al 1995; Retnakaran et al 1997). In order to determine the developmental expression of DDC so that the cross linking stage can be identified we have prepared a spruce budworm specific DDC probe (Fig. 5). Northern hybridization studies are underway. We hope to identify a stage where we can isolate the chitin protein that has not been cross linked and use this preparation for further elucidation of the glycosylation mechanism. We are also investigating the possibility of isolating such a protein from a larva where DDC expression has been selectively blocked by RH-5992.

Fig.5. PCR amplification of DNA using DDC primers and varying $MgCl_2$ concentrations from 0.5 (lane2), 1 (lane3), 1.5 (lane4), and 2 µm (lane5); resolved on 0.8% agarose gel and stained with ethidium bromide. 1kb ladder (lane1) from GIBCO-BRL.

Conclusion

1.) Assembly and elaboration of the cuticle in general and chitin in particular occurs during the first part of the larval stadium whereas the dissolution of the chitinous cuticle occurs during the latter part.

2.) The precursor protein produced by the epidermis in the<24h larva binds to ^{14}C-NAGA with a glycosylase produced in the hemolymph in <24h larva and transported into the epidermis of the >48h old larva.

3.) Protein profiles of moulting fluid contain specific proteins that may be involved in degradation of the cuticle during the moulting process.

4.) A spruce budworm specific DDC probe was synthesized using RT-PCR.

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Acknowledgements

ELEVATION OF BLOOD TRANSFERRIN LEVEL IN MICE ADMINISTERED INTRAPERITONEALLY WITH A PARTIALLY DEGRADED CHITIN

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Abstract:

1. Oral administration of chitin or partially acid–degraded (PD) chitin to mice resulted in increase of number of splenic phagocytes in weak extents.
2. It was also observed that decrease of number of splenic T–cells due to ageing could be restored by the same treatment.
3. Upon *i. p.* administration of PD–chitin to mice, induction of apo–transferrin in the serum was observed by means of *Candida albicans* as the target microbe *in vitro*.

Keywords: Chitin, Partially acid–degraded chitin, *Candida albicans*, Phagocytes, T–cells, Transferrin

Introduction

Since early 1980s, we have been conducting a series of immunological investigations of chitin and N–acetylchitooligosaccharides. During this study, we found that *i. p.* administration of chitin to mice resulted in growth–inhibition against *i. p.* transplantation of Ehrlich adenocarcinoma or of sarcoma 180 ascites tumor. We also found that the same treatment enable to mice the resistance to anti–microbial effects against challenge of pathogenic microbe such as *Staphylococcus aureus* and *Candida albicans*.[1,2] Furthermore, N–acetylchitohexaose (NACOS–6), the water–soluble lower homologue of chitin, was found to display a significant growth–inhibition against transplanted solid tumors in mice, sarcoma 180,[3] Meth A,[4] and Lewis lung carcinoma.[5] NACOS–6 was also effective to challenge of pathogenic microbes, *Listeria monocytogenes*[6] and *C. albicans*[7] in mice. The results of mechanism analyses of NACOS–6 revealed that a variety of cytokines, Interleukin 1(IL–1), Interleukin 2 (IL–2), macrophage–activating factor (MAF), and so on could be produced upon *i. p.* administration of NACOS–6. These findings well agree with those of *in vivo* antitumor and antimicrobial assays.

However, no finding has been published on the immunological effect of *p. o.* administration of chitin. Therefore, we conducted a series of immunological investigations on mice fed chitin or partially degraded (PD)–chitin admixed to a normal feed for a long term,

Additionally, the induction of growth–inhibitory factor to *C. albicans* upon *i. p.* administration of chitin will be reported.

Materials and methods

1. Chitin and partially acid−degraded(PD) chitin

   Chitin (snow crab) and PD−chitin, molecular masses of $>10^6$ and $4-5 \times 10^3$, respectively, were the products of Yaizu Suisankagaku Industry Co. Ltd., Yaizu, Japan.

   The pulverized chitin and PD−chitin, each 200 mesh, were washed with dichloromethane in order to remove bacterial endotoxin until the color intensity of the washing reached to the value of 5μ g/g. Pulverized chitin and PD−chitin were separately admixed with finely pulverized CE−2 solid feed supplied by Nippon Clea, Tokyo, Japan, in 2 w/w%.

2. Animals

   Female ICR strain SPF mice of 6−week age and 29−30 week−age supplied by Nippon Clea were used. For the long−term feeding study, 29−30 week age mice (abbreviated as O−mice), each 25 per group, were fed with feed containing chitin or PD−chitin *ed libitum* for 50 weeks. For the *i.p* .administration experiment, BALB/c, male mice of 5−week, Nippon SLC, Tokyo Japan, were used. PD−Chitin was suspended in a phosphate−buffered saline without Ca^{2+} and Mg^{2+} (PBS (−)) in 5mg/ml, and 0.2ml of the suspension was administered *i.p.* at each 3 day−interval.

3. Fungus

   The *C. albicans* NIH A−207 strain was obtained from Institute of Fermentation, Osaka, and was cultivated in Sabouraud liquid medium at 27°C for 30 h. The resultant yeast form cells were used for the growth−inhibition assay of this fungus as 1×10^1 cells/ml in RPMI−1640.

4. Sera

   Blood were obtained from mice by heart punctuation, and were centrifuged at 5,000 rpm for 5 min to obtain the sera. Blood of PD−chitin−administered mice were collected at 24 h after the final administration.

5. Biochemical assays For the assay of GOT, GPT and LDH

   Sera of chitin and PD−chitin−administered mice were used. GOT and GPT were determined by UV method, and LDH was assayed by Wroblewsk−La Due method.

6. Distribution analysis of immunocompetent cells.

   Spleens from mice were homogenized and washed with Tris−HCl buffer pH 7.2, to remove erythrocyte. Then the cell−distribution of the remained cells were analyzed. FITC−anti−Mac−1 antibody, FITC−anti−mouse Thy−1 antibody and FITC−anti mouse I−A^d antibody were obtained from Nippon Becton Dickinson, Tokyo, Japan. For the determination of Mac−1−, Thy−1− and I−A^d −positive cells in the splenic cell homogenate, each 4μ l of the corresponding antibody solution was added to the splenic cell suspension, 1ml (1×10^6 cells/ml), and the mixture was left in an ice−bath for 30 min. The mixture was then analyzed for the distribution of the cells by means of a FACScan (Nippon Becton−Dickinson).

7. Growth−inhibition assay of *C. albicans*

   Viable yeast form cells of *C. albicans* NIH A−207 strain cells (1×10^4 cells/ml in RPMI−1640), 100μ l and mouse serum, 100μ l, were mixed and incubated at

37°C for 12 h under 5% CO_2 containing air. After completion of cultivation, Alamar blue (Alamar Biosciences, Inc., CA, USA), 10μ l, was added, and the mixture was further incubated at 37°C for 3 h. Change of optical densities between 540 and 620nm, correlating with reducing activity of viable microbial cells, was determined spectrophotometrically.

8. Restoration of inhibited growth of *C. albicans* by anti–transferrin antibody or ferric sulfate

    C. albicans cell suspensions (1×10^4 cells/ml), 1 ml and each 50 μ l of anti–mouse transferrin antibody (30μ g/ml) or of ferric sulfate (20μ g/ml) were mixed and incubated at 37°C for 12 h. Number of the viable *C. albicans* cells was enumerated by the Alamar blue method.

9. Serum transferrin assay

    This was conducted by an ELISA method in accordance with the description by Watanabe *et al.*[8]

10. Determination of total iron–binding capacity (TIBC) and unsaturated iron–binding capacity (UIBC) of mouse sera

    The TIBC and UIBC in the serum from PD–chitin administered mice were measured by TIBC and UIBC Microtest "Daiichi" (Daiichi Radioisotope Labs., Ltd., Tokyo, Japan).

Results

1. Observation on long–term feeding mice with chitin– or PD–chitin–admixed diet

    Difference between the amounts of consumed diets admixed with chitin or PD–chitin was not significant. Body weights of mice administered *p.o.* with chitin or PD–chitin accessed to the maxima during 19 and 29 weeks from start of this study, respectively. Thereafter, gradual decrease of body weights were seen, although any significant difference was not observed between mice of 3 groups. In terms of survival ratios, it was found that mice fed with normal diet only was 60.5%, and those of chitin and PD–chitin–administered groups were 63.5 and 51.0%, respectively.

2. Biochemical assay of serum enzymes, GOT, GPT and LDH

    For the purpose of confirmation of effect of long–term *p.o.* administration of chitin or PD–chitin on mice, assays of transaminase (GOT and GPT) and lactic dehydrogenase (LDH) in mouse sera were conducted as the parameters of liver function. The results indicated that any significant difference was not observed between activities of GOT, GPT and LDH in sera from mice of chitin– and PD–chitin–administered groups.

3. Distribution assay of immunocompetent cells

    Phagocytes from the splenic cells in chitin and PD–chitin–administered groups showed a significant increase of Mac–1–positive cells compared with that of young (6 –week age) group of mice (Fig 1). It is therefore possible to state that both chitin and PD–chitin are able to display an induction effect of phagocytic cells, although their intensities are quite low. Although number of Thy–1–positive cells in aged mice was smaller than that of 6–week old young mice in significant extent, a marked restoration of number of the cells has been observed by the administration of chitin or

PD–chitin (Fig. 2). From these findings, chitin and PD–chitin were found to display an accelerating effect of induction of T cell from spleen cells. On the other hand, chitin and PD–chitin were not effective to induce B cells (I–A^d positive cells), i. e., number of B cells in spleens from 6–week old and old groups was not different from those of the corresponding administered groups with chitin and PD–chitin, respectively (data not shown).

4. Effect of chitin and PD–chitin on induction of anti– *C. albicans* growth factor in mouse serum

Fig. 1. Number of Mac–1 cells in mice administered with chitin or PD–chitin *via* p. o. route

Fig. 2. Number of Thy–1 cells in mice administered with chitin or PD–chitin *via* p. o. route

Determination of anti– *C. albicans* growth factor provided the following findings; *p. o.* administration of both chitin and PD–chitin with the normal diet, CE–2, were effective in borderline extents. Furthermore, an attempt was made on *i. p.* administration of chitin and PD–chitin in order to determine difference of induction effects upon change of the administration route (Fig. 3). These results indicate that transferrin, an iron–binding protein, is the chemical entity of the anti– *C. albicans* growth factor.

Fig. 3. Restoration of *C. albicans*–growth by serum from mice administered with PD–chitin *via i. p.* route.
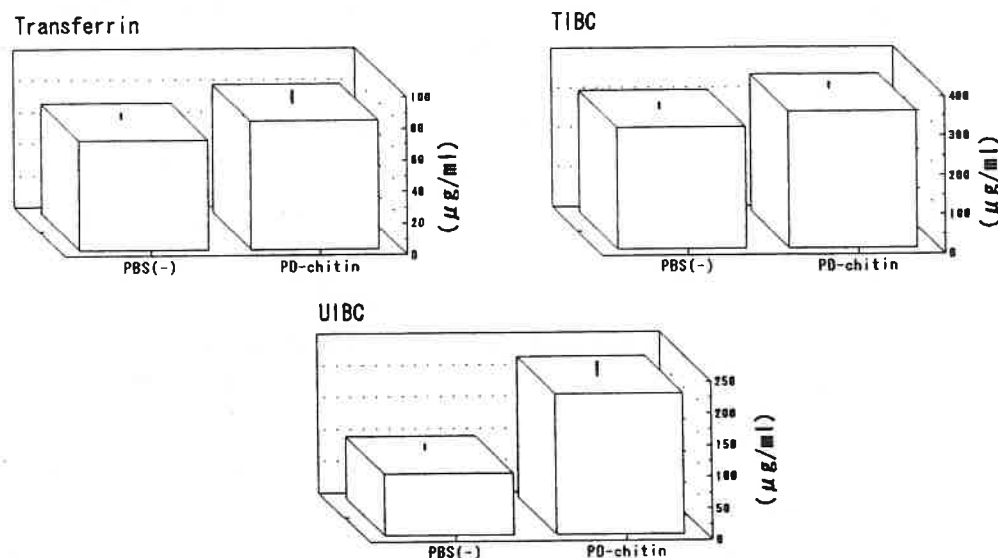

Fig. 4. Amounts of transferrin as TIBC and UIBC in sera from mice administered with PD–chitin *via i. p.* route

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5. Characterization of anti– *C. albicans* growth factor as apo–transferrin

It was shown by Shiraishi and Arai [9] that apo–transferrin is able to display a growth–inhibitory effect against *C. albicans* as well as the other microbes. In order to obtain confirmatory evidences on the correlation between *i. p.* administration of PD–chitin and apo–transferrin response in serum, determination of serum transferrin as TIBC and UIBC values have been conducted. Transferrin level and TIBC value in sera of PD–chitin–administered mice were not significantly different from those of the control group mice (PBS(–)) as shown in Fig. 4. On the other hand, UIBC value of PD–chitin administered mice was higher than that of the control group, PBS(–)–administered group, thus providing an evidence that administration of chitin is responsible for the induction of apo–transferrin.

Discussion

In the present study, it was found that mice fed with chitin or PD–chitin had not cause loss of body weight and life span. The fact that the serum enzymes, GOT, GPT and LDH values were not changed after feeding of chitin– or PD–chitin–containing diet indicate that the influences of both chitin and PD–chitin on mammals with adverse effect are practically low. The results of immunocyte analyses demonstrated that, upon feeding with chitin or with PD–chitin, increase of number of monocyte · granulocyte series cells has been confirmed. It is therefore presumable that activation of the non–specific infection defense mechanism upon feeding chitin or PD–chitin took place. Additionally, upon long–term feeding experiment, decrease of number of T cells has been observed (Fig. 2), this fact can be interpreted to be lowering of thymus function due to aging. However, number of T cells of PD–chitin–administered group was maintained at almost the same level of that of the young age mice. These findings indicate that decrease of differentiation and developmental functions of thymus might be restored by feeding chitin and PD–chitin. Administration of chitin or PD–chitin in mice *via p. o.* route resulted in anti– *C. albicans*–growth factor, which could not be identified to be a known factor, transferrin. In order to induce the same factor in large amounts in blood of mice, PD–chitin was administered *i. p.* to young mice.

It was confirmed that the anti– *C. albicans*–growth factor has been induced in blood of mice of young age group (Fig. 3). Because this activity underwent inhibition entirely with anti–mouse transferrin antibody, participation of transferrin to this activity became evident (Fig. 3). Another fact that ferric sulfate could restore the inhibited growth of *C. albicans* provides supporting a evidence for the participation of transferrin as reported by Shiratani and Arai [9]. Furthermore, in determination of serum transferrin and TIBC values, any significant difference was not observed between PD–chitin and PBS(–)– administered groups. On UIBC values, however, a significant elevation of PD–chitin–administered group was confirmed, providing a supporting evidence for the presence of large amounts of apo–transferrin.

From the above findings, it was revealed that induction of apo–transferrin takes place in mouse blood, and this iron–binding protein displays as a *C. albicans*–growth inhibitor.

As the future objective, application studies of water-soluble chitin derivatives which can pass through payer plate allocating on the small intestine should be conducted.

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CHANGES IN CHITIN AND GLYCOCONJUGATES DURING PREECDYSIAL DEGRADATION OF THE OLD CUTICLE OF *Carcinus maenas* (CRUSTACEA, DECAPODA) AS REVEALED BY LECTIN PROBES.

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Abstract

Semithin and ultrathin sections of the branchiostegite cuticle of *C. maenas* at different preecdysial stages were probed with five lectins binding either to chitin (WGA, LEA) or to different sugar residues (Con A, SBA, Jac). The results show that the degradation or "gelification" of the membranous layer during preecdysis is accompanied by the appearance of glycosylated residues. They suggest that these glycosylated residues are discharged by the epidermis in the form of ecdysial droplets. Since the "gelified" membranous layer of decapod crustaceans forms from the inner cuticular lamellae and contains glycosylated compounds and digestion-resistant chitin-protein fibres, this gel layer might be regarded as an ecdysial membrane comparable to that identified in insects

Keywords: chitin, glycoconjugates, cuticle, decapod crustaceans, moulting, degradation, ecdysial droplets, ecdysial membrane.

Introduction

In decapod crustaceans as in other arthropods, the inner layers of the old exoskeleton (i.e. the principal layer and membranous layer) undergo, before exuviation, partial digestion by moulting fluid enzymes, mainly proteinases and chitinases (Roer and Dillaman, 1993; Goffinet and Jeuniaux, 1994). Compère *et al.* (1997b) recently demonstrated in *Carcinus maenas* and *Macropipus puber* that the first ultrastructural signs of degradation occur in the membranous layer with the epidermal discharge of ecdysial droplets similar to those identified in insects (Locke and Krishnan, 1973; Gnatzy and Romer, 1984) and of exolysosomes that appear as structures peculiar to decapods (Compère *et al.*, 1997a). While in the principal layer digestion of the chitin-protein microfibres is complete, so that fibres are no longer ultrastructurally observable, the degraded membranous layer subsists as a digestion-resistant fibrous network.

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Jeuniaux (1959, 1963), Jeuniaux *et al.* (1986), and Goffinet and Jeuniaux (1994) interpreted the appearance of the hygroscopic gel macroscopically observable on the inner side of exuviae as resulting from the direct transformation of chitin and proteins of the membranous layer into a peculiar insoluble, digestion-resistant, hygroscopic glycoprotein complex.

To test this hypothesis, we have investigated the chemical changes occurring in the inner cuticle layers of decapod sclerites during preecdysial degradation. To do this we have probed sections of the branchiostegite integument of *C. maenas* at different preecdysial stages with different lectins binding to chitin or to different sugar residues.

Materials and Methods

Biological material and tissue preparation

Atlantic shore crabs (*Carcinus maenas* L.) were collected at the Wimereux Marine Station (Nord-Pas de Calais, France) and kept in the "Aquarium Dubuisson" in Liège.

Pieces of integument were excised from branchiostegites of individuals in anecdysis (stage C_4) and at preecdysial moulting stages D_0, D_1', D_1'', D_1''' and D_2 as determined according to Drach and Tchernigovtzeff (1967). They were fixed by immersion for 2 h at 20°C in 2.5% glutaraldehyde either in diluted sea water (7/10, pH 7.4) or in 0.1 M Na-cacodylate (pH 7.4) with sucrose, and decalcified for 3-4 days at 4°C in 0.2 M EDTA (pH 8.0). The pieces were then rinsed in distilled water, dehydrated in an ethanol series, and embedded in epoxide resin according to the routine procedure or in the hydrophilic, acrylic resin "Unicryl" (British BioCell Int.)(Scala *et al.*, 1992).

Lectin binding assays

Lectin binding assays were performed on ultrathin and semithin sections according to an indirect procedure described previously (Compère, 1996; Compère *et al.*, 1996), using biotinylated lectins as primary markers secondarily revealed with 10-nm-gold-conjugated streptavidin-albumin complex (Au-STV, Sigma-4275). The lectins used were WGA (Wheat Germ aggl., Sigma L-5142) and LEA (*Lycopersicon esculentum* aggl. or Tomato lectin, Sigma L-9389) for detection of N-acetyl-D-glucosamine (GlcNAc) oligomers, Con A (concanavalin A, Sigma C-2272) for detection of α-mannose residues (α-Man), SBA (Soy bean aggl., Sigma L-3395) for terminal N-acetyl-D-galactosamine residues (GalNAc), and Jac (Jacalin, Vector B-1155) for galactosyl (β-1,3)N-acetyl-D-galactosamine structures (Gal[β-1,3]GalNAc) in O-glycosidically linked oligosaccharides.
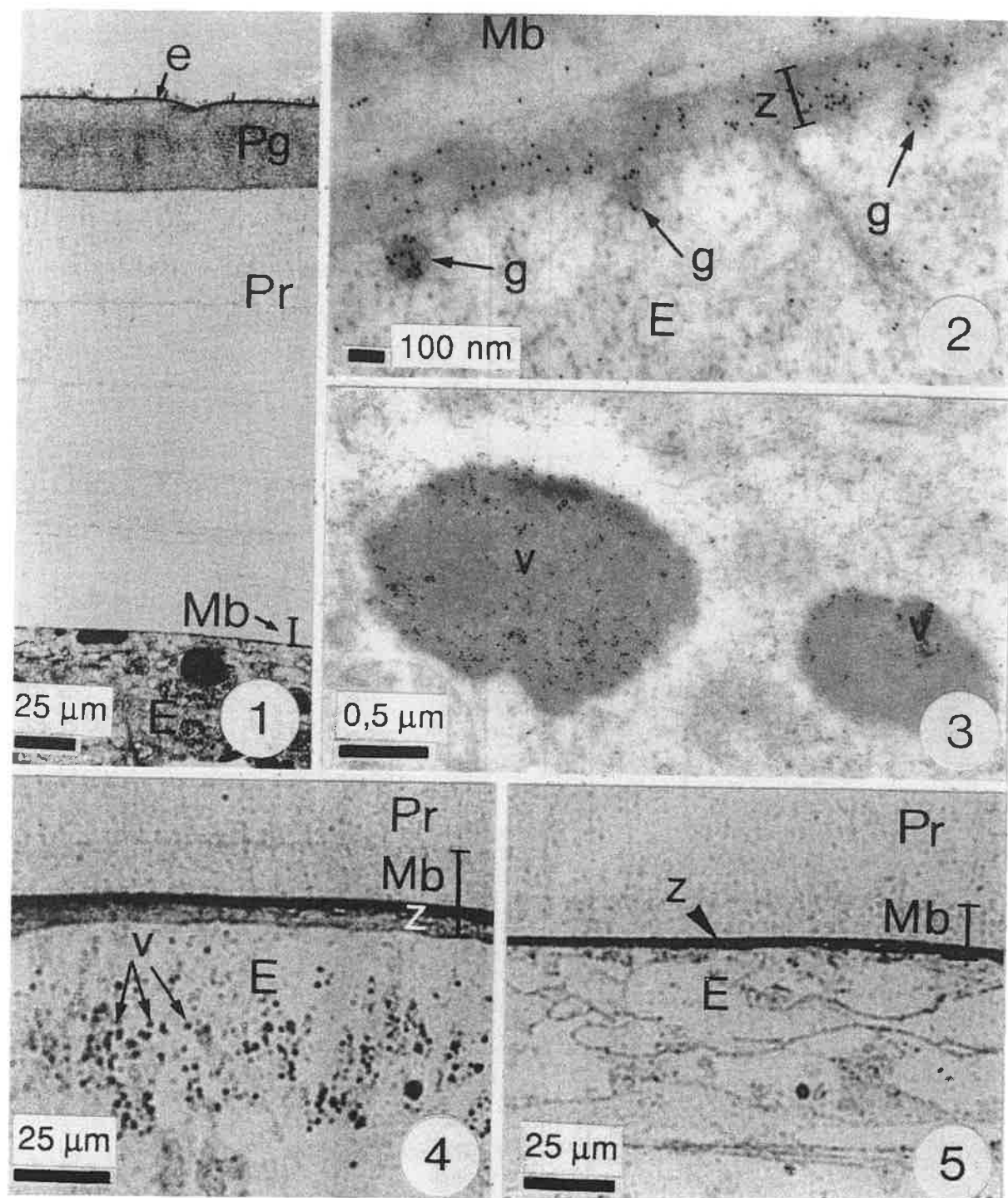
The sections were first blocked with 0.5% fish gelatine in buffer and incubated for 1-2 h at 20°C in the lectin-containing medium [0.01 M Na-phosphate or HEPES (for Jac only) containing 0.15 M NaCl, 0.1% fish gelatine, 0.05% Tween 20, and 0.05% NaN3. Lectin concentration, pH of the buffer, and cofactors were respectively 100 µg/ml, pH 7.4, 1 mM CaCl2, and 1 mM MgCl2 for WGA, 50 g/ml, pH 7.3 for LEA, 40 µg/ml, pH 6.8, 0.1 mM CaCl2, 0.1 mM MnCl2 for Con A, 100 µg/ml, pH 6.8 for SBA, and 50 µg/ml, pH 7.5, 0.1 mM CaCl2 for Jac. Thereafter, the sections were rinsed, air dried, blocked again, and incubated for 2 h at 20°C with Au-STV (1/100) in PBS-gelatine-Tween (pH 7.5), then rinsed and air dried again. The following specificity controls were performed: no incubation with lectin; addition of inhibitory sugars to the lectin incubation media (GlcNAc, chitotriose, α-Man, GalNAc); enzyme treatment (mannosidase). Before TEM observation, the labelled ultrathin sections were stained with uranyl acetate. For light microscopy, the gold label on the semithin sections was revealed by silver enhancement (IntenSE M kit, Amersham RPN 491)
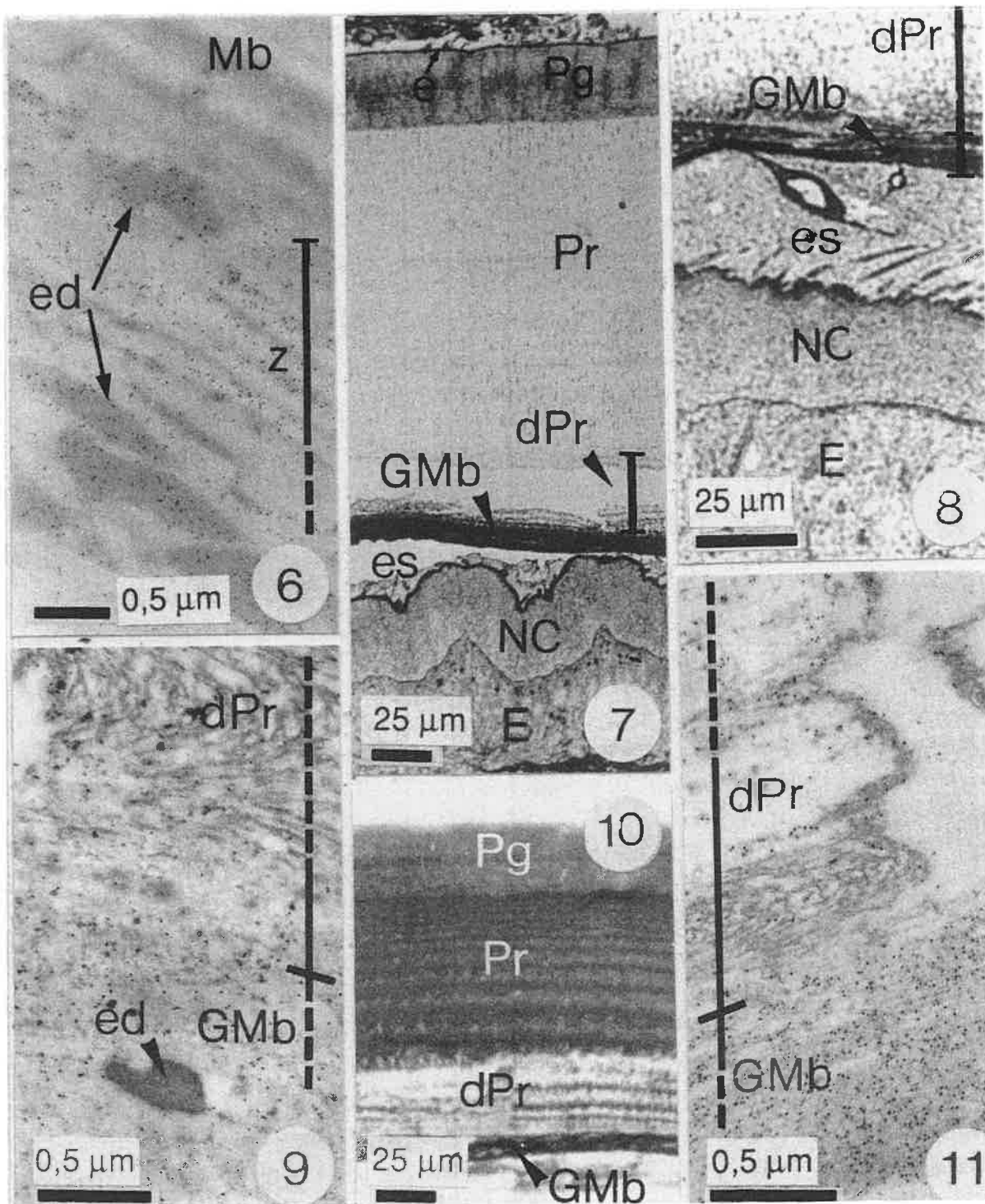
Results and discussion

The results presented in Figures 1 to 9 show that degradation or "gelification" of the membranous layer of decapod crustaceans during preecdysis is accompanied by the appearance of glycosylated compounds. These compounds mainly bear oligosaccharidic structures rich in α-Man, terminal GalNAc, and/or Gal(β-1,3)GalNAc, as they are recognised by Con A, SBA and Jac respectively. The intense reaction of Jac suggests the presence of O-linked oligosaccharides. The results also suggest that glycosylated compounds are discharged by the epidermis at early preecdysis by way of ecdysial droplets. The latter progressively release their glycosylated contents in the membranous layer undergoing degradation, which becomes "gelified".

At the onset of preecdysis, the most obvious change in the lectin affinity of the cuticle layers is that the degraded lamellae of the membranous layer strongly react with Con A, SBA, and Jac (Figs 4, 5, 7 and 8), while the intact part of the layer remains negative as in the anecdysial cuticle (Fig. 1). From stage D_0, the positive zone increases in thickness as the degradation front progresses so that, from late preecdysis to ecdysis, the whole degraded membranous layer is intensely labelled and contrasts with the principal layer which remains unlabelled even in its degraded part (Figs 7 and 8).

In the epidermis, the labelling patterns obtained with Con A, SBA, and Jac reveal the organelles involved in secretion of ecdysial droplets during stages D_0 and D_1' (Figs 2, 3 and 4). These are large vacuoles in the basal part of the epidermal cells, smaller ones in the apical part, and electron-dense secretion granules that are seen to discharge their labelled content at the apical cell surface, thus giving rise to ecdysial droplets as previously described by

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Figs 1 to 5. Con A (Figs 1, 2, 3 and 5) and SBA (Fig. 4) labellings of Unicryl (Fig. 1) and epoxide resin sections (Figs 2 to 5) of the branchiostegite of *C. maenas* in anecdysis or early preecdysis. **Fig. 1.** Semithin section of the anecdysial integument. **Figs 2 and 3.** Ultrathin sections of the epidermal cells at the stage D0 showing apical secretion granules (g) and electron-dense vacuoles (v) involved in the discharge of ecdysial droplets. **Figs 4 and 5.** Semithin sections of the epidermis and inner cuticular layers at stages D1' (Fig. 4) and D1" (Fig. 5). e, epicuticle; E, epidermis; Mb, membranous layer or its intact part; Pg, pigmented layer; Pr, principal layer; v, vacuoles in epidermal cells; z, degraded zone of the membranous layer.

Figs 6 to 11. Unicryl (Figs 7, 8, 11) and epoxide resin (Figs 6, 9, 10) sections of the branchiostegite of *C. maenas* at D1''' (Fig. 6) and late D2 stages (Figs 7 to 11) after labelling with lectins . **Fig. 6.** SBA; ecdysial droplets (ed) in the degraded zone of the membranous layer (z). **Fig. 7 and 8.** Semithin sections showing the "gelified" membranous layer (GMb) and the new cuticle (NC). **Fig. 7.** Con A. **Fig. 8.** Jac. **Fig. 9.** Con A; ecdysial droplet remnant (ed) in the "gelified" membranous layer (GMb). **Fig. 10.** WGA; semithin section of the old cuticle. **Fig. 11.** LEA; ultrathin section of the"gelified" membranous layer (GMb). dPr, degraded zone of the principal layer; e, epicuticle; E, epidermis; Mb, membranous layer or its intact part; Pg, pigmented layer; Pr, principal layer.

Compère *et al.* (1997b). All these labellings are absent in anecdysis (Fig. 1) and disappear in $D_1"$ (Fig. 5). During later stages, the ecdysial droplets seem to gradually release their glycosylated contents as they progress into the membranous layer which they intersperse. At early preecdysial stages D_0 and D_1, the freshly secreted ecdysial droplets are more intensely labelled than the membranous layer undergoing degradation (Fig. 6). The reverse is true in late preecdysis: the digestion-resistant fibres of the membranous layer bear numerous gold particles while the ecdysial droplet remnants are no longer labelled by any of the lectins used (Fig. 9).

These observations agree with the histochemical data of Skinner (1962), showing the appearance of P.A.S.-positive material on the inner side of the old cuticle of *Gecarcinus lateralis* during preecdysis, and of Locke and Krishnan (1973), demonstrating that insect ecdysial droplets discharge P.A.S.-positive material. They also confirm the biochemical data of Kumari and Skinner (1995), revealing an increase in the proportion of glycoprotein in degraded-membranous-layer extracts.

Our results, however, show that the P.A.S.-positive material observed by Skinner (1962) does not correspond, as suggested by this author, with an additional layer secreted by the epidermis. Our data further strongly suggest that the glycosylated residues in the degraded membranous layer arise neither by unmasking of pre-existing compounds, as proposed by Kumari and Skinner (1995), nor through degradation of chitin, as proposed by Renaud (1949), nor through transformation of the chitin-protein complex as suggested by Jeuniaux (1959, 1963), Jeuniaux *et al.* (1986), and Goffinet and Jeuniaux (1994), notwithstanding the validity of the latter authors' proposal that formation of a hygroscopic gel results from the presence of glycoconjugates. Labelling of ecdysial droplets and secretion products in epidermal cells proves, rather, that glycosylated residues are discharged by the epidermis and impregnate a pre-existing layer, the membranous layer.

On the other hand, since the gelified membranous layer of decapod crustaceans appears P.A.S.-positive, contains glycosylated residues, derives from the inner lamellae of the endocuticle, and is resistant to moulting-fluid enzymes, it might be regarded as an ecdysial membrane comparable to that identified in insects (Passoneau and Williams, 1953; Malek, 1958; Delachambre, 1967; Gnatzy and Romer, 1984). Digestion-resistant chitin-protein fibres in the degraded membranous layer have been observed ultrastructurally until ecdysis by Compère *et al.* (1997b), and the persistence of chitin is confirmed by the high affinity of these fibres for chitin-binding lectins (Figs 10 and 11). This contrasts with the degraded part of the principal layer, where the fibres are broken down and chitin is hydrolysed.

It is also worth noting that the inner endocuticle layers of articular membranes undergo changes in ultrastructural appearance identical to those of the gelified membranous layer of sclerites: persistence of digestion-resistant fibres and their affinity for lectins. An affinity for lectins is also encountered

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during preecdysis in the innermost endocuticle layer of the inner branchiostegite. These observations suggest that the presence of an ecdysial membrane in decapods is not limited to the sclerites.

In conclusion, the ecdysial membrane can no longer be viewed as a structure of unknown role exclusively encountered in moulting insects. Our data support the view that it results from glycosylation of the inner cuticular lamellae, this process yielding a lubricant gel facilitating the exuviation of arthropods.

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POSSIBLE INVOLVEMENT OF CHITINOUS EXTRACELLULAR FIBRILS ON THE SEXUAL DEVELOPMENT IN THE ZYGOMYCETE *PHYCOMYCES BLAKESLEEANUS*

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Abstract: The zygomycete *Phycomyces blakesleeanus*, with the two mating types (+) and (-), displays a morphologically dynamic sexual reaction. The physical contact of two sexual organs is essential for the progress and completion of mating. Electron microscopic observation revealed, that the two progametangia of the opposite mating types were connected by extracellular fibrils. In in-vivo staining experiments, the fibrils stained specifically with Calcofluor White, Congo Red, Evans Blue and fluorescein labelled wheat germ agglutinin, suggesting that they contain chitinous materials. Most of the stained progametangia failed to develop to further stages, even after the dyes were removed. Cultivation at low (4°C) or high (25°C) temperature or under strong blue light as well as treatment with cycloheximide resulted in underdevelopment or damage of the fibrils. Also, the fibrils developed poorly when mutants affected in the carotene biosynthesis pathway were mated.

These results imply, that the extracellular fibrils are a necessary structural feature in the mating response of *Phycomyces*. They may be involved in the orientation of the two progametangial cells and also serve to strengthen the structural bond between the progametangia.

Keywords: chitin, extracellular fibrils, *Phycomyces*, sexual development, Zygomycetes.

In a previous paper (2), we suggested that extracellular fibrils connecting the progametangia play a crucial role in the mating process of *Phycomyces*. In the present study, the function of these fibrils was further investigated by checking their development in incomplete mating responses. By use of histochemical techniques, we also gathered preliminary information on the composition of these extracellular structures.

Materials and methods

1) Strains, growth conditions, and mating

The minus-standard wild type strain NRRL1555 and the plus-standard wild type strain A56 (1) of *Phycomyces blakesleeanus* were grown vegetatively on PDAYC-medium at 20°C under continuous overhead illumination with white light (2). For mating, small mycelium slices were cut from the (+) and the (-) strain precultures and inoculated 4 cm apart on Petri-dishes (ø 6 cm) containing 10 ml glucose-glutamate agar medium (SI; 3). The dishes were incubated at 17°C for 20 days in a humidified dark box.

2) Electron microscopy

The sexual organs, which formed during the ongoing mating process, were isolated together with a 5 x 5 mm agar block and fixed with 3% glutaraldehyde followed by dehydration in a graded acetone series (4). Then the samples were dried in a critical-point-dryer (JCPD-5, JEOL) under liquid CO_2 and coated with Pt/Pd prior to observation with a Scanning Electron Microscope (JSM-T330A, JEOL, operated at 5 kV). For comparison, specimen were alternatively fixed with 3% glutaraldehyde, postfixed with 1% OsO_4, dehydrated in a graded ethyl alcohol series and dried after replacement of the alcohol by a graded isoamyl acetate series.

3) In-vivo staining

The sexual organs were stained with 0.25% Calcofluor White (CFW), 10^{-3} M Congo Red (CGR), and 10^{-4} M Evans Blue (EVB), respectively, which are widely used dyes for staining cell walls of plants and algae (5). The staining results of CFW and EVB were observed using an epifluorescence microscope (YF-EFD2, Nikon; 365 nm excitation filter), or a laser-scanning confocal microscope system (Meridian Instruments, excitation wavelength 540 nm). Some specimen were also stained with two kinds of fluorescein-isothiocyanate-labelled lectins, 10^{-5} % wheat germ agglutinin (FITC-WGA) or 10^{-5} % concanavalin A (FITC-ConA) for fluorescence-microscopy (excitation filter 450 nm).

4) Inhibitor treatment

Mating was also performed on SI agar containing 10^{-7}-10^{-3} M cycloheximide (CHM). To investigate the effect of the staining dyes on the formation of sexual organs, organs developed in 4-day old cultures were treated for 2 h with the respective dyes, washed with distilled water (DW) and cultivated for another two days in the dark. The treatment was carried out under red safety-light and repeated three times per culture, because sexual development does not occur syncronously in *Phycomyces*.

Results

1) Observation of extracellular fibrils

By scanning electron microscopy of S3 and S4 progametangia (Fig.1, A and B series, respectively), well-developed extracellular fibrils could be observed at the joining sites of the two progametangial cells, thus confirming the findings of O'Donnel et al. (6). The fibrils stretched perpendicular to the joining areas, bridging the gap and connecting the two progametangial cells. In S2-zygophores, however, which are intertwined knots of heavily branched hyphae, only few and weakly developed fibrils were present. Double fixation with glutaraldehyde followed by OsO_4, the hitherto solely employed method for electron microscopic preparations of *Phycomyces*, proved to cause some structural damage on the fibrils of both S3 and S4 progametangia (Fig.1, A3, B3).
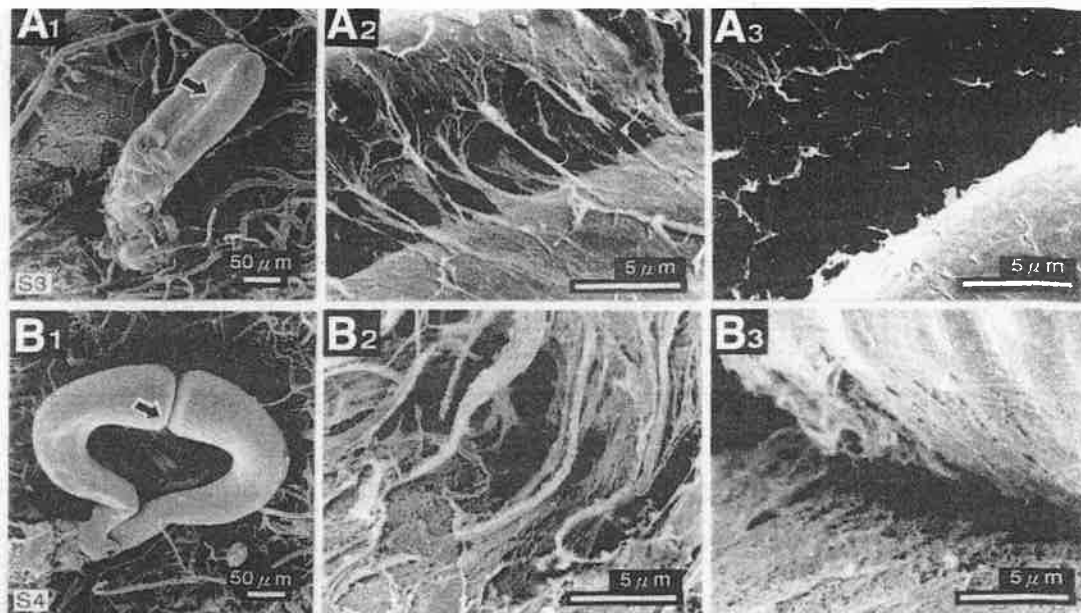
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Fig. 1 SEM micrographs of the sexual organs and extracellular fibrils. Progametangia at S3 (A1) and S4 (B1) at low magnifications, indicating the regions magnified for A2 and B2 , respectively (arrowed). A2 and B2: Well-developed extracellular fibrils at the junction of two enlarged progametangia. The fibrous structures are preserved even after fixation and acetone-dehydration (A2,B2), but damaged after double-fixation and alcohol-dehydration (A3,B3). Bar = 50 μm for A1 and B1; 5 μm for the others.

Fig.2 Microphotographs of the progametangial junctions stained with dyes and fluorescein-labelled lectins. Stained with 0.25% CFW at S3 (A1) and S4 (A2, A3) and with FITC-WGA (B1, B2) and FITC-ConA (C). Bar = 50 μm for A1, A3, B1, C, and 100 μm for A2, B2.

2) In-vivo staining of extracellular fibrils

The fibrils connecting the progametangial cells in S3 and S4 progametangia showed a bright white fluorescence after treatment with CFW (Fig.2, A series) or dark red after treatment with CGR (data not shown). The remaining fibril fragments at the inner surface of the looped S4 progametangia, representing the former joining area of S3, appeared as strongly stained as the intact fibrils at the joining apices of the progametangial cells (Fig.2, A3). In S3- and S4-progametangia stained with EVB, bright

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red fluorescence radiated from the fibrils at the junction, specifically. When stained with FITC-WGA, the fibrils showed a bright yellowish green fluorescence (Fig.2, B). No specific staining could be observed using FITC-ConA (Fig.2, C).

Table 1. Mating reactions of *Phycomyces* treated with CHM or dyes.

Treatment		S2	S3	S4	S5-6	S7-8	Total
CHM	none	366	52	4	26	156	604
	10^{-7}M	384	96	28	36	71	615
	10^{-6}	1909	202	12	4	5	2132
	10^{-5}	2468	28	0	0	0	2496
	10^{-4}	814	0	0	0	0	814
Washing by							
	DW	511	132	49	120	117	929
	CFW	777	55	49	50	42	973
	CGR	466	45	25	27	46	609
	EVB	824	110	76	59	37	1106

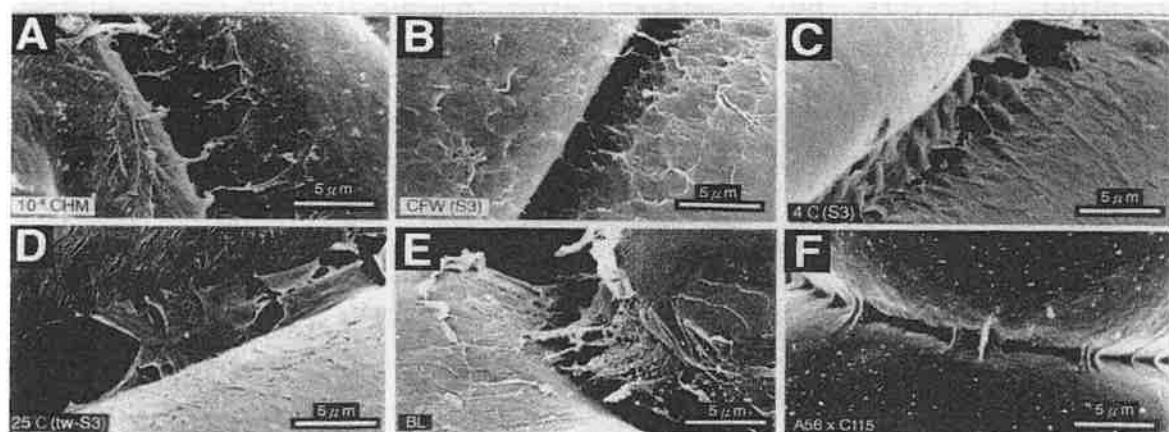The sexual organs are represented as the number per unit area (1 cm^2).

Fig. 3 SEM micrographs of the extracellular fibrils at the junction of S3-progametangia when treated with mating inhibitors or disruptors; 10^{-6}M CHM (A), 0.025% CFW (B), 4°C (C), 25°C (D), blue light (E), mating with ß-carotene-overproducing mutant (F). Bar = 5 μm.

3) Effect of CHM and dyes

Both the mating reaction and the mycelial elongation rate were suppressed by addition of CHM to the medium (Table 1). Mycelial growth was completely inhibited at a CHM concentration of 10^{-3} M, whereas 10^{-6} M CHM proved ineffective on mycelial growth but strongly impeaded the process of the mating reaction at S3. Cessation of the mating process at S2 was found in the presence of 10^{-5}-10^{-4} M CHM. The effects of CFW, CGR, and EVB are also listed in Table 1. Compared to a treatment with DW alone, the development

of the S5-S8 sexual organs significantly decreased when treated with these inhibitors.

SEM micrographs revealed, that the fibrils at the joining sites of S3 progametangia were only weakly developed in the presence of 10^{-6} M CHM in the medium (Fig.3, A). Underdeveloped S2-zygophores, looking like twisted filaments, were often found on media containing 10^{-4} M CHM. Compared to DW-control experiments, treatment with CFW (Fig. 3, B), CGR or EVB caused severe morphological damage to the fibrils.

4) Effects of external factors

When mating was conducted at 4°C or 25°C, the mating process almost always stopped at S4 and no S8 zygospores were formed. A large number of S2 zygophores, however, occured at cultivation at 25°C, probably because of nutritional competition. Also, abnormally twisted pillars in S3 or strongly deformed loops in S4 (failure in loop formation) were found in about 50% of the S3- and 30% of the S4-progametangia. In the 4°C-culture, the number of sexual organs was significanly decreased compared to the standard cultures kept at 17°C. In contrast to fibrils formed at 17°C, those formed at 4°C or 25°C were short and warty (Fig.3, C) or resembling thin film (Fig.3, D), respectively.

Irradiation with green light had no effect on the course of the mating reaction but sexual development did not develop further than S4 in most cases when irradiated continuous-ly with blue light. Blue light, but not green light, also inhibited the formation of extracellular fibrils (Fig.3, E). Besides, twisted pillars in S3 and deformed loops in S4 progametangia were often observed under blue light irradiation.

5) Extracellular fibrils on sexual organs in imperfect mating

Mating between strain A56 (+), wild type, and strain C115 (-), ß-carotene overproducing mutant, was imperfect, resulting in a cessation of development at S3 or S4 and in the formation of twisted progametangia. On these organs, only weak fibrils were formed (Fig.3, F).

Discussion

The single-step fixation procedure, which was originally established for EM preparations of bacterial pili composed of glycoproteins (4), proved also suitable for *Phycomyces* extracellular fibrils. By this procedure, morphologically undamaged fibril structures were maintained. The existence of the extracellular fibrils was confirmed by observations of fresh S3 and S4 progametangia with a Nomarski differential interference microscope (Olympus). Therefore the fibrils are unlikely to be artifacts caused by fixation and/or dehydration procedures.

Because of their staining behavior with polysaccharide-staining dyes, such as CFW, CGR and EVB, we conclude, that the extracellular fibrils of *Phycomyces* are most probably composed of

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polysaccharides including chitin. This conclusion is supported by the strong reaction of the fibrils with FITC-labeled WGA, which is a chitobiose-binding lectin. In *Phycomyces*, chitin and chitosan are the major cell-wall components (7).

The inhibition of fibril development caused by this dyes is probably due to depolymerization effects. Such an inhibition was always followed by disturbance or cessation of the mating process, occuring particularly at S3 and S4. Both the inhibition of fibril development and the interruption of the mating process were also found when *Phycomyces* was mated at high or low temperature, under blue light, in the presence of CHM, or in a combination between a wild type strain and a hypercarotenogenic mutant. Mating inhibition at high temperatures or in the presence of CHM has also been reported for *Ustilago* (8) and *Mucor* (9). CHM may exert a double inhibitory effect on the mating reaction by both disturbing the mating-specific protein synthesis and the development of the extracellular fibrils.

Further studies will be necessary to prove a functional role of the extracellular fibrils as clamps for thightly fixing the two progametangial cells in a specific three-dimensional configuration and consequently allow a close and specific cell-to-cell communication.

Conclusion

We underwent our survey of the mating processes of *Phycomyces blakesleeanus* taking into consideration intercellular communication between the cells of the two mating types. We found several recognizable stage-specific interaction patterns, one of them at the progametangial stages (S3 and S4). The contact of the two progametangia in exactly the right position and configuration seems to be essential for the further development of the sexual organs. The chitinous extracellular fibrils probably play a role in the maintenance of these structural features.

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ULTRASTRUCTURE OF LEAF CELLS TREATED WITH CHITOSAN

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Abstract

Effects of chitosan applied by injection, watering and spraying on the ultrastructure of leaf cells of tobacco, cucumber and bean plants were studied. Chitosan induced various ultrastructure changes in leaf cells such as disorganization of plasmalemma and tonoplast, appearance of small vesicles, the apposition of wall cells and plugging of intercelluar spaces with amorphous and fibrillar substances. At least some of them may be implicated in restricting the fungal infection. However, it is not possible to determine from our data the real role played by these ultrastructural changes in preventing plants from viral and bacterial infection.

Keywords: chitosan, injection, watering, spraying, tobacco, cucumber, bean plants, ultrastructure of leaf cells.

Introduction

Chitosan a natural B-(1,4)-glucosamine can induce a multitude of biological processes in plant tissus, including the stimulation of chitinases, accumulation of phytoalexins, synthesis proteinase inhibitors, increased lignification and callose synthesis (1,2). Chitosan inhibited the growth of phytopathogenic fungi and bacteria (3,4) and induced resistance of plants to fungal, viral, bacterial and viroid infections (5,6,7,8).
The objective of this study was to investigate the ultrastructure leaves cells treated with chitosan in the realation to the induction plants resistance to pathogens.

Materials and Methods

Chitosan solution (0.1%) was prepared by dissolving chitosan in 0.05% acetic acid and adjusting the pH to 5.8 with 0.1N KOH.
For electron microscopy study were used leaves of: Nicotiana tabacum „Xanthi nc", Cucumis sativus L. „Monastyrski", Phaseolus vulgaris „Fana" from the following experiments:
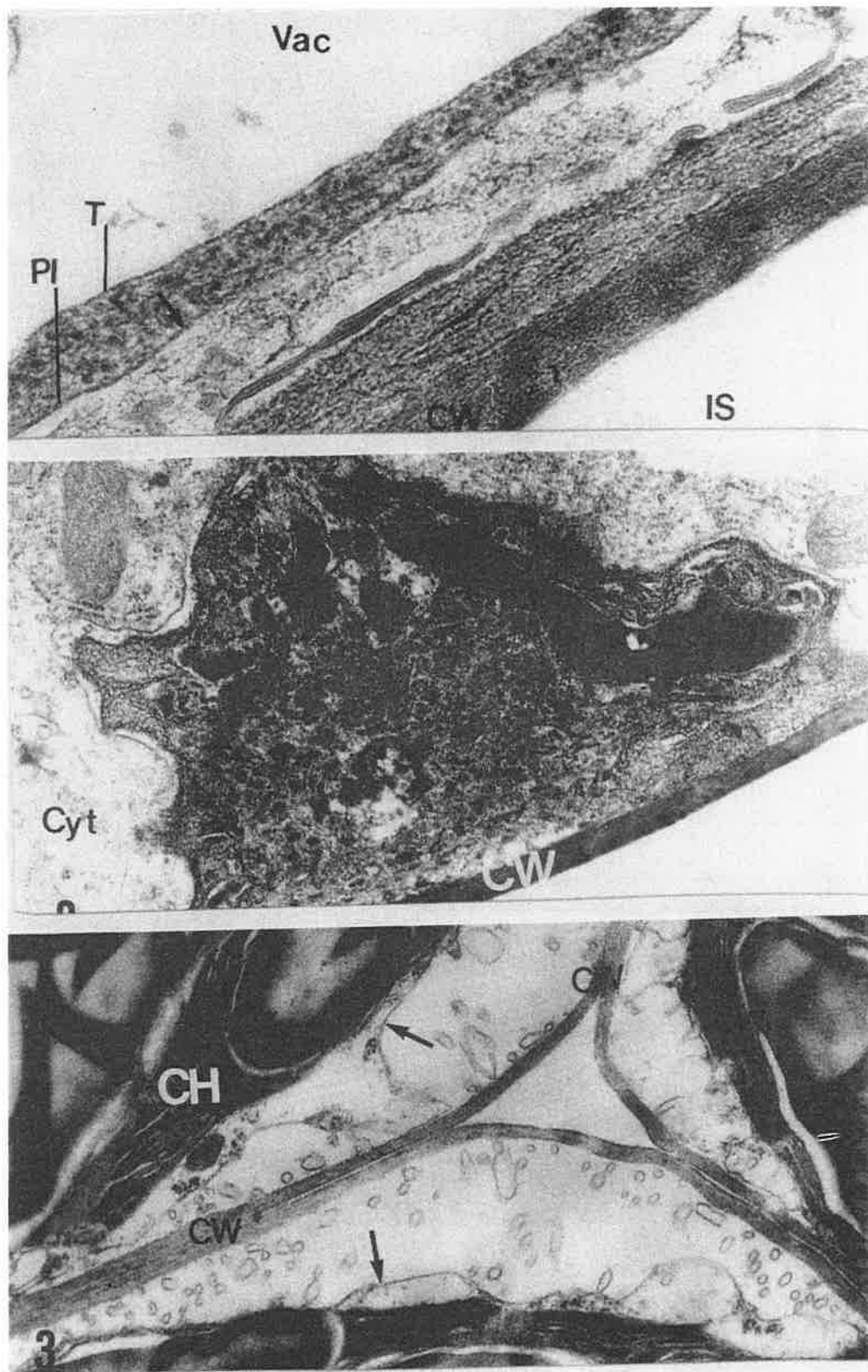1. 0.1% chitosan solution was injected into leaves of tobacco plants and on the third day after injection treated leaves were taken.
2. Cucumber plants were watered with 0.05% chitosan solution and on the fourteenth day from the begining of the experiment first leaves were taken.
3. Bean plants were watered with 0.05% chitosan solution and on the eleventh day from the begining of experiment first and second pair of leaves were taken.
4. First leaves of bean plants were sprayed with 0.1% chitosan solution and 48 hours after that were harvested.
Small pieces of leaves were fixed in 3% glutaraldehyde in 0.1% M phosphate buffer pH 7.2, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol-acetone

series and embedded in Epon 812. Ultrathin sections were cut in Reichert OM-U2 ultramicrotome. They were stained with uranyl acetate and lead citrate and examined in a Philips EM 201 electron microscope.

Results and Discussion

1. Injection of tobacco leaves with 0.1% chitosan resulted in very strong ultrastructure changing. In intercellular spaces deposits of fibrillar and granular materials were observed. They often adhered to cell walls. Chitosan stimulated cell walls apposition between plasmolemma and cell wall composed of heterogenous fibers, membranes and amorphous substances. Sometimes two layers of deposits were observed: first under plasmalemma seen as translucent material mixed with osmiophilic one, and second over cell wall seen as electron-opaque material (Fig. 1). Presumably the translucent layer can be a callose deposit. Sometimes deposits are very large and invaginate into cell protoplast (Fig. 2). Moreover in this combination the following others changes were observed:
    -exfoliation of tonoplast
    -ppearance of various lomasomes
    -changes of chloroplast ultrastructure
    -higher concentrations of callose in sieve element
    -appearance of seminecrotic and necrotic cells
2. The watering of cucumber plants with 0.05% chitosan solution induced following ultrastructure changes in leaf cells:
    -very strong vacuolization of palisade and spongy mesophyll cells cytoplasm
    -more frequent appearance of lomasomes in comparison with control
    -frequent invagination of cytoplasm to chloroplast
    -swollen mitochondria with lighter matrix and decreased number of cristae
    -exfoliation of tonoplast was not observed.
3. In the first leaves of bean plants watered with 0.05% chitosan solution were observed following ultrastructure changes:
    -exfoliation of tonoplast
    -convolution of plasmalemma and its separation from cell wall; presence of vesicles in the periplasmic space (Fig.3)
    -appearance of large number of lomasomes (Fig. 4)
    -appearance of small callose deposits in sieve elements
    -appearance of electron-opaque amorphous material on organelles in epidermal cells. Ultrastructure of second untreated leaves cells did not differ very much from control
4. Solution of 0.1% chitosan sprayed on first leaves of bean plants induced following ultrastucture changes:
    -deposition of fibrillar and granular clusters in intercellular space cell walls (Fig. 5).
    -exfoliation of tonoplast (Fig. 6)
    -retraction, convolution and vesiculation of plasmalemma (Fig. 7)
    -frequent appearance of lomasomes
    -aggregation of cytoplasm and cell organelles
    -more frequent appearance of lipids in cytoplasm.
    In untreated bean leaves of the chitosan sprayed plant following ultrastructure changes were observed:
    -small number of lomasomes

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Fig. 1. A cell wall apposition between plasmalemma and cell wall /arrows/ /x70.000/
Fig. 2. Very large cell wall apposition invaginate into cell protoplast /x 26.000/
Abbreviations: Vac-vacuole. T-tonoplast. Pl-plasmalemma. IS-intercelluar space. Cyt-cytoplasma. CW-cell wall.
Fig. 3. Separation of plasmalemma from cell wall. Present of vesicales in the periplasmic space /arrows/ /x 25.000/
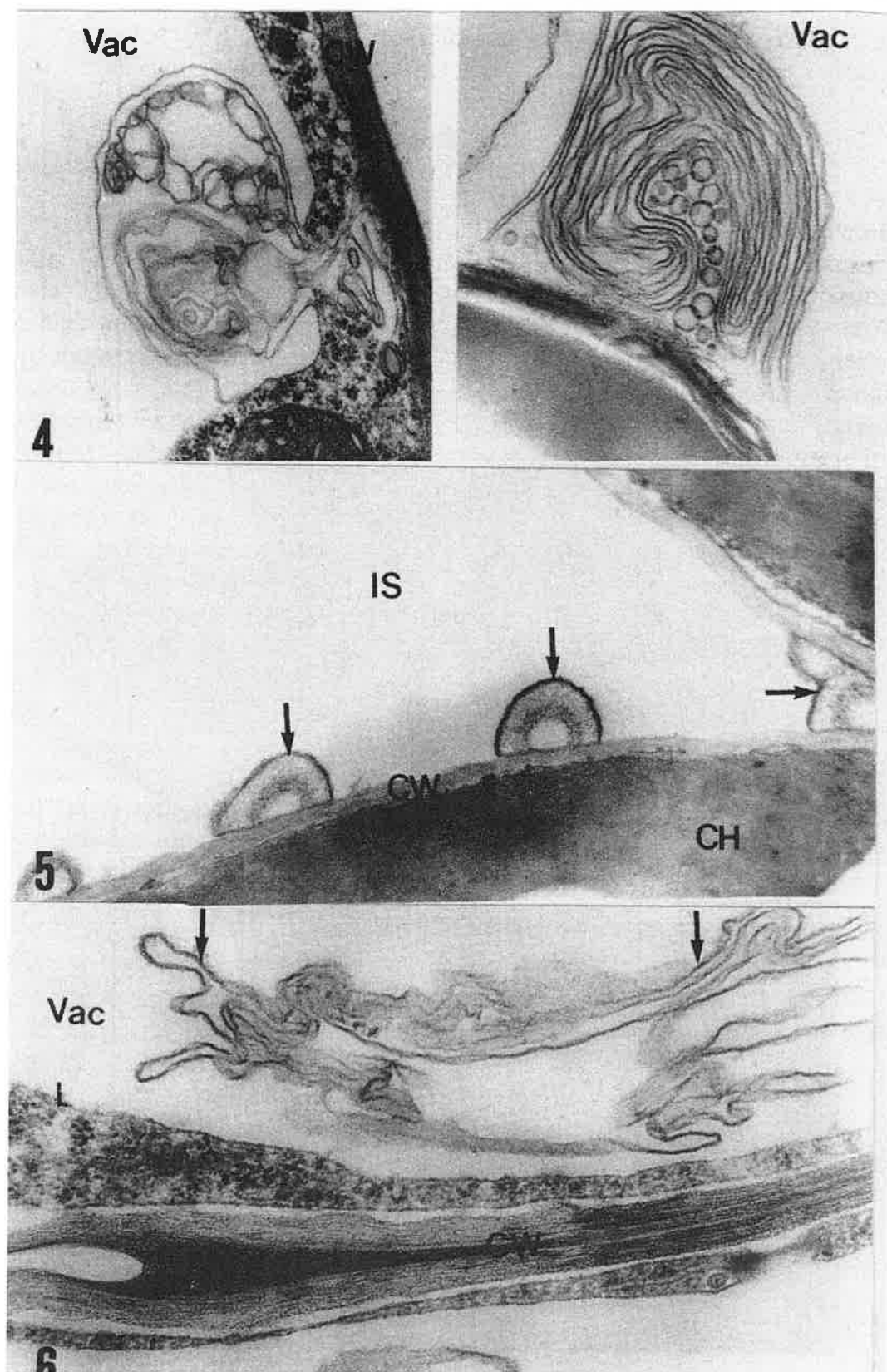
Fig. 4. Protrusion of the lomasomes into cell protoplast /x a-33.000, b-46.000/
Abbreviations: CH-chloroplast, CW-cell wall, Vac-vacuole.

Fig. 5. Deposition of fibrillar and granular clusters in intercellular spaces /arrows/ /x 27.500/

Fig. 6. Exfoliation of tonoplast /arrows/ /x 40.000/
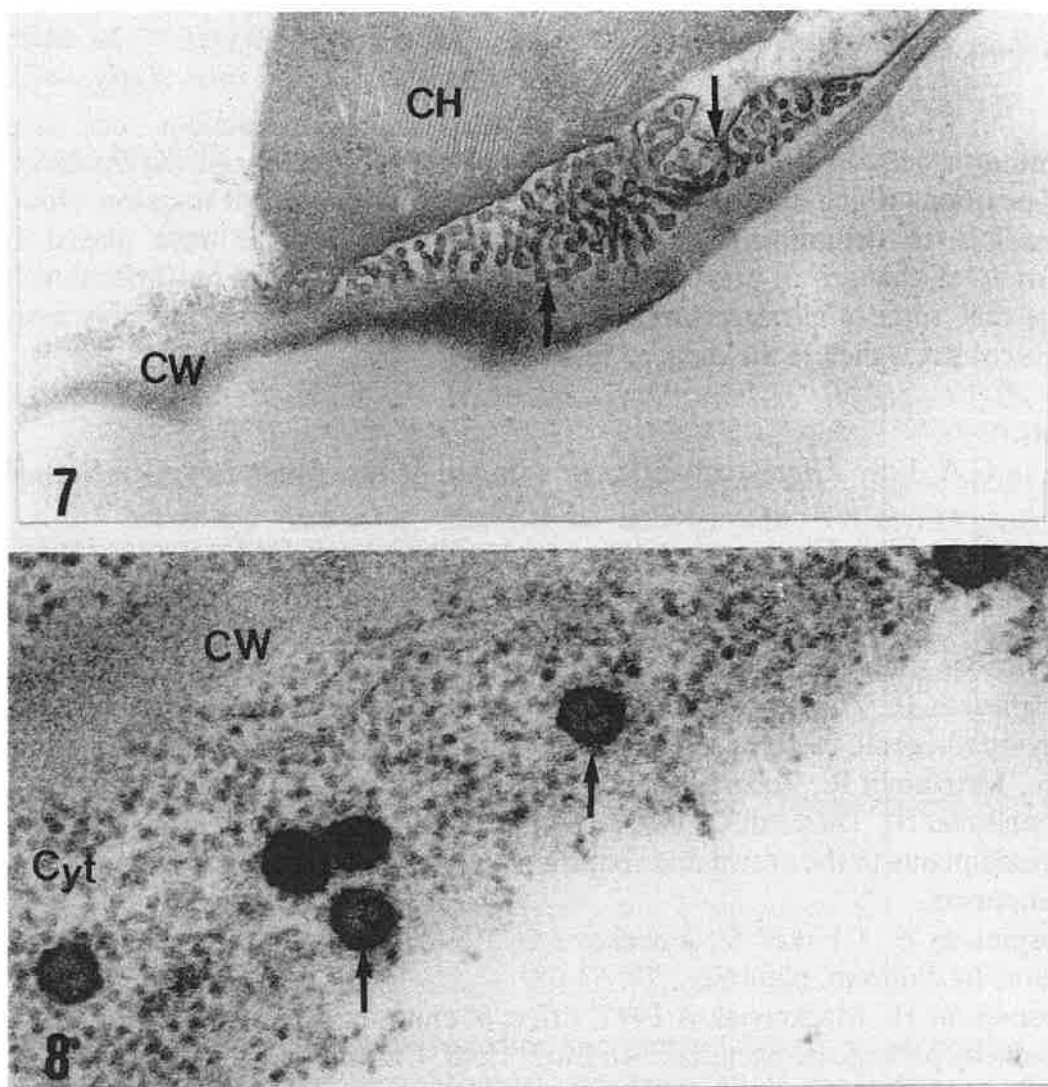Abbreviations: CW-cell wall, IS-intercelluar space, CH-chloroplast, Vac-vacuole

Fig. 7. Retraction. convolution and vesiculation of plasmalemma /arrows/ /x 46.000/

Fig. 8. Small vesicles with very distinct osmiophilic stained membrane in cytoplasm /arrows/ /x 76.000/

Abbreviations: CW-cell wall. CH-chloroplast. Cyt-cytoplasma

-mitochondria with lighter matrix and decreased number of cristae

-appearance of small vesicles with very distinct osmiophilic stained membrane and faintly stained filaments inside (Fig. 8). They were easily noticeable. Politis and Goodman (9) suggests that Golgi dictiosomes and highly dilated endoplasmatic reticulum produce numerous vesicles that migrate towards the plasmalemma. It is possible. that these vesicles also may carry microfibrils. polymer precursor or enzymes each contributing to the formation of wall apposition.

Our results presented here and earlier (10) correspond with results obtained by others for cucumber and tomato plants (11). Chitosan stimulated various ultrastructure changes in plant cells. which type and intensity depended on plant species. the concentration of chitosan and on the mode of its application. Higher concentration of chitosan and long term contact of leaf cells with chitosan induced more distinguished ultrastructure changes. In all our experiments chitosan stimulated disorganization of plasmalemma and tonoplast and appearance of small vesicles. Very important for a pathogen invasion. specially fungal may have the apposition of wall cells and plugging intercelluar space with amorphus and fibrillar substances.

Conclusion

Chitosan induced various mechanical defensive reactions such as plugging intercelluar spaces with amorphous electron opaque substances and the formation of cell wall appositions which may be implicated in restricting the fungal invasion. However it is not possible to determine from our data the extent of the role played by these ultrastructural changes in preventing plants from viral and bacterial infection. It is also possible that some of ultrastructural changes caused by chitosan are probably more related to chemical stress then to the induced resistance.

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Acknowledgements

Production of N-Acetyl-D-Glucosamine and Chitinolytic Enzymes by a Strain of *Verticillium* cfr. *lecanii* (A3) Cultivated in Bench-top Fermentor.

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Abstract

Submerged cultures of *Verticillium* cfr. *lecanii* A3 were carried out either in shaken flasks and in a 2-1 bench-top fermentor. Chitinolytic activity and N-acetyl-D-glucosamine production were followed while different culture conditions, such as temperature, pH, stirrer speed and aeration rate, were varied. Maximum enzyme and aminosugar productions were obtained when the fungus was grown on a medium containing 1.0% of yeast nitrogen base and 1.0% of colloidal chitin under the following culture conditions: initial pH, 5.0, temperature, 28 °C; stirrer speed, 300 rpm; aeration rate, 1.0 v/v/m.

Introduction

Chitinolytic enzymes have been widely studied and some of fungal origin appear of great interest for possible applicative uses (Monreal and Reese, 1968; De la Cruz *et al.*, 1993; Muzzarelli 1993; Fenice *et al.*, 1996). The production of chitinases at industrial level is mainly obtained from *Serratia marcescens* and *Streptomyces griseus;* the only industrial chitinase of fungal origin comes from selected strains of *Trichoderma harzianum*. However, their cost is still to high to make enzymatic chitin degradation and processing of chitin-rich wastes possible and profitable. Aloise (1996) proposed an industrial process to obtain N-acetyl-D-glucosamine (NAG) using the chitinolytic enzyme of *S. marcescens*. So far, however, no process using a fungal strain or its enzymes appears to have been studied. *Verticillium* cfr. *lecanii*, a fungal strain isolated from Continental Antarctica produces chitinolytic enzymes in a wide range of temperatures (Fenice *et al.*, 1997) on raw and colloidal chitin (Fenice *et al.*, 1996).

In this paper we report on the study of the growth conditions for optimal NAG and chitinase production by *Verticillium* cfr. *lecanii* A3, cultivated either in shaken flasks and in a 2-liter bench-top fermentor. The time courses of growth, enzyme and aminosugar productions under the optimal fermentation conditions are shown.

Materials and methods

Chemicals: Chitin (from crab shells), and N-acetyl-D-glucosamine (NAG) were from Sigma (USA); Malt Extract Agar (MEA) was from Oxoid (U.K.); Yeast Nitrogen Base (YNB) was from Difco (USA). All other chemicals were of analytical grade.

Microorganism:Verticillium cfr. *lecanii* A3, is stocked in the culture collection of the Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Viterbo, Italy.

Culture media and growth conditions: The effect of different amounts of chitin on the enzyme production was carried out in the basal medium (BM) of Fenice *et al.*, (1996) modified for its colloidal chitin concentration: 0,2; 0,5; 1,0; 1,5; 2,0 %. All media were sterilized for 20 min at 121 °C.

Colloidal chitin was prepared as reported by Hankin and Anagnostakis (1975).

Shaken cultures: after inoculation (ca. 0.5 x 10^6 conidia/ml), cultures were incubated on a rotary shaker, 200 rpm, for 9 days at 5, 15, 25, 28, 30, 35°C and initial pH of 4.0,. 4.5, 5.0, 5.5, 6.0. Samples were taken every 12-24 h and, after centrifugation (10,000 g for 10 min). supernatants used as enzyme solutions for the enzyme assay.

Analytical methods: Chitinolytic enzymes activity was determined as previously reported (Fenice et al, 1996).

The amount of NAG was measured spectrophotometrically (190 nm) after elution through a revers phase column (LiChroCART, Merk, D) connected to a HPLC system (Violet, I) using H_2O as mobil phase.

The mycelial growth was measured as reported by Fenice *et al.* (1996)

*Bioreactor and fermentation conditions:*The fermentor used was a 2-liter (total volume) jacketed bench-top reactor (Applikon Dependable Instruments, Schiedam, NL) filled with 1.2 l of BM. The standard condition for fermentations were as follows: inoculum size 0.5 x 10^6 conidia/ml; stirrer speed 400 rpm; aeration 1.0 V/V/m; temperature 28 °C; initial dissolved oxygen 100% of saturation non-controlled; initial pH 5.0, non-controlled; fermentation medium: BM + 0,2 % siliconic antifoam.

Effect of stirrer speed, aeration and pH: The influence of the agitation on the mycelial growth, NAG and enzyme productions was evaluated at 200, 300, 400 and 500 rpm. The effect of aeration was tested at 0.5, 1.0 and 1.5 V/V/m. The effect of maintaining the pH of fermentation constant was carried out at pH 5.0, 6.0, 6.5 and 7.0. starting the control action when the fermentation broth (pH 5.0 at the beginning of fermentation) reached the setpoint value.
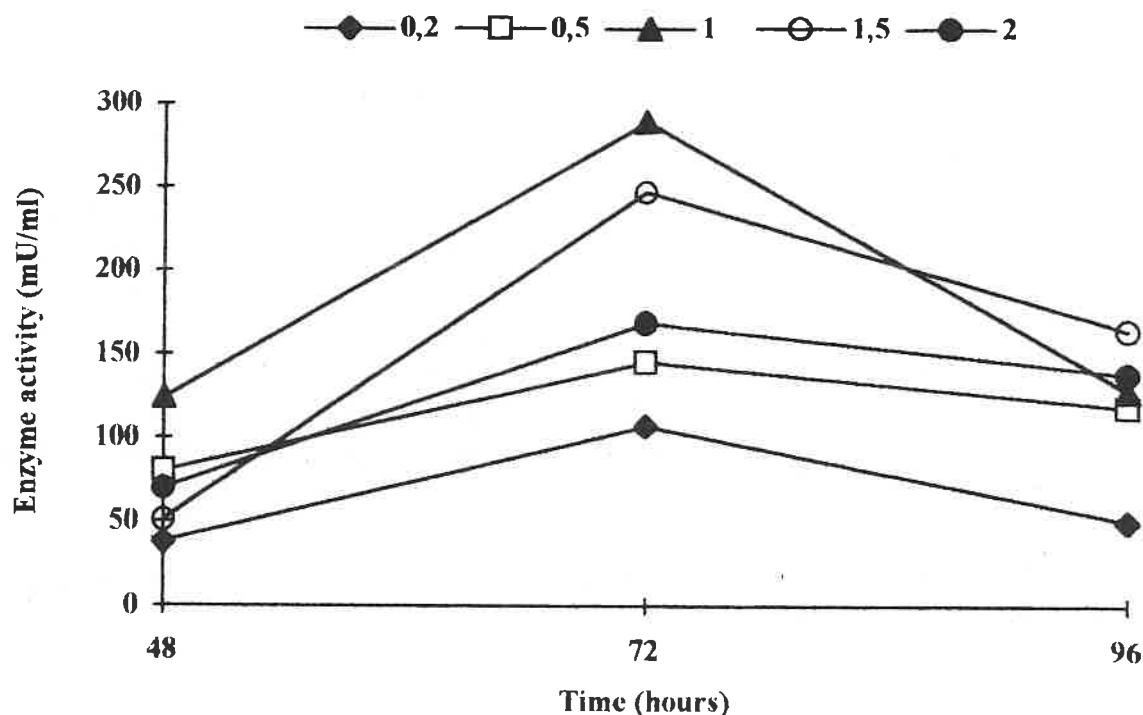

Results and discussion

Shaken cultures

In a previous work (Fenice *et al.*, 1996) the most suitable medium for the cultivation of *Verticillium* cfr. *lecanii* was found to be YNB (1%) and colloidal chitin (1%). Thus, this medium was utilized as basal medium (BM) in this work.
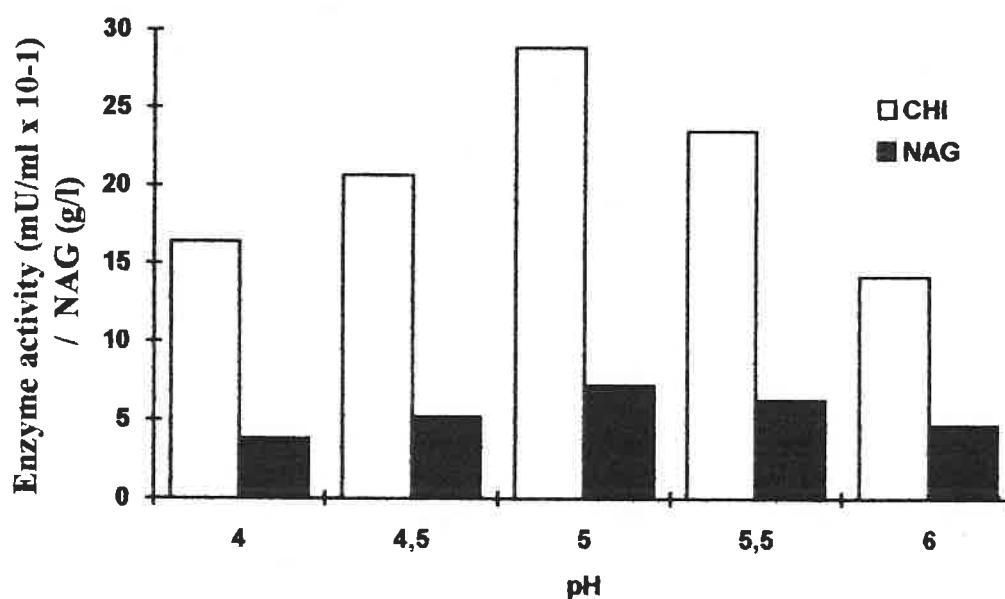
Chitin concentration in the culture medium. Figure 1 shows the chitinolytic activity of *V.* cfr. *lecanii* grown in YNB media supplemented with different amounts of colloidal chitin. The concentration of 1.0 % resulted in the maximum release of enzyme activity (289 mU/ml) while a certain inhibition was observed at higher concentrations. NAG production was maximal at a concentration of 1.5% of colloidal chitin.

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Figure 1. Chitinase production by *Verticillium* cfr. *lecanii* grown on YNB medium supplemented with different amounts of colloidal chitin.

Figure 1 legend: —◆— 0,2 —□— 0,5 —▲— 1 —○— 1,5 —●— 2

pH and temperature. Initial pH 5.0 appeared to be the best either for chitinase and for NAG production (289 mU/ml and 7.2 gr/l, respectively). Fermentations carried out at initial pH 4,0 and 6,0 resulted in strong reduction of the maximum enzyme activity (< 200) mU/ml) (figure 2).

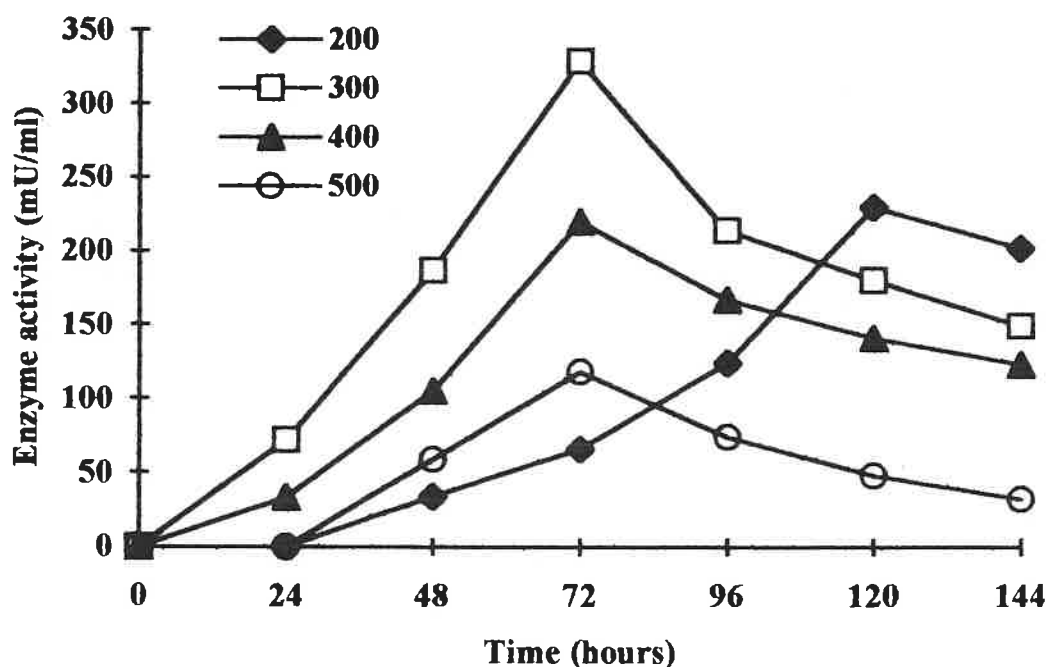**Figure 2.** Maximum chitinase and NAG production at different initial pH fermentation.

As for the temperature, *V.* cfr. *lecanii* grew and released its chitinase in a wide range of temperatures (5-35 °C) (data not shown). Maximum enzyme production was achieved at

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28 °C, over 30 °C enzyme production and growth were markedly reduced, thus confirming previous results (Zucconi *et al.*, 1996; Fenice *et al.*, 1997).

Production in bench-top fermentor.

Effect of stirrer speed. Figure 3 shows the effect of increasing the stirrer speed from 200 to 500 rpm on the chitinase production of *V.* cfr. *lecanii*. At 200 rpm the enzyme release in the cultural broth was slow: maximum production (230 mU/ml) was reached after 120 h of fermentation. Maximum enzyme production was obtained at 300 rpm (329 mU/ml) after 72 h of fermentation. The time course of the chitinase production at 400 and 500 rpm were very similar to that obtained at 300 rpm, but the maximum productions were lower (220 and 118 mU/ml, respectively) probably due to the sensitivity of *V.* cfr. *lecanii* toward to shear stress. NAG maximum production (7.8 gr/l) was obtained at 300 rpm after 96h of fermentation (data not shown). Stirrer speed optimization led to an increase of enzyme production of 43% confirming that this variable is one of the most important fermentation parameter for fungal strains (Milagres and Lacis, 1991).

Figure 3. Time course of chitinase production by *Verticillium* cfr. *lecanii* in bench-top fermentor at different stirrer speed.

Effect of areation. Maximum production of chitinase and NAG were obtained at 1.0 V/V/m (329 mU/ml and 7.8 gr/l of chitinolytic enzyme and NAG, respectively). At 0.5 V/V/m, the productions were definitly lower (198 mU/ml and 4,8 gr/l of chitinase and NAG respectively), a flow rate of 1.5 V/V/m resulted in a slight reduction, only (data not shown).

Effect of pH control. During the fermentation, pH increased progressively from the initial value (pH 5.0) up to ca. 7.5; likely causing enzyme inactivation. Fermentations at

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controlled pH were carried out at 5.0, 6.0, 6.5 and 7.0. Unexpectedly, NAG and chitinase productions were maximal when the pH was let free to rise (data not shown).

Fermentation under optimized conditions. Figure 4 shows the time course of NAG and chitinase production, mycelial growth and dissolved oxygen of a typical *Verticillium* cfr. *lecanii* fermentation under the optimal conditions reported in table 1.
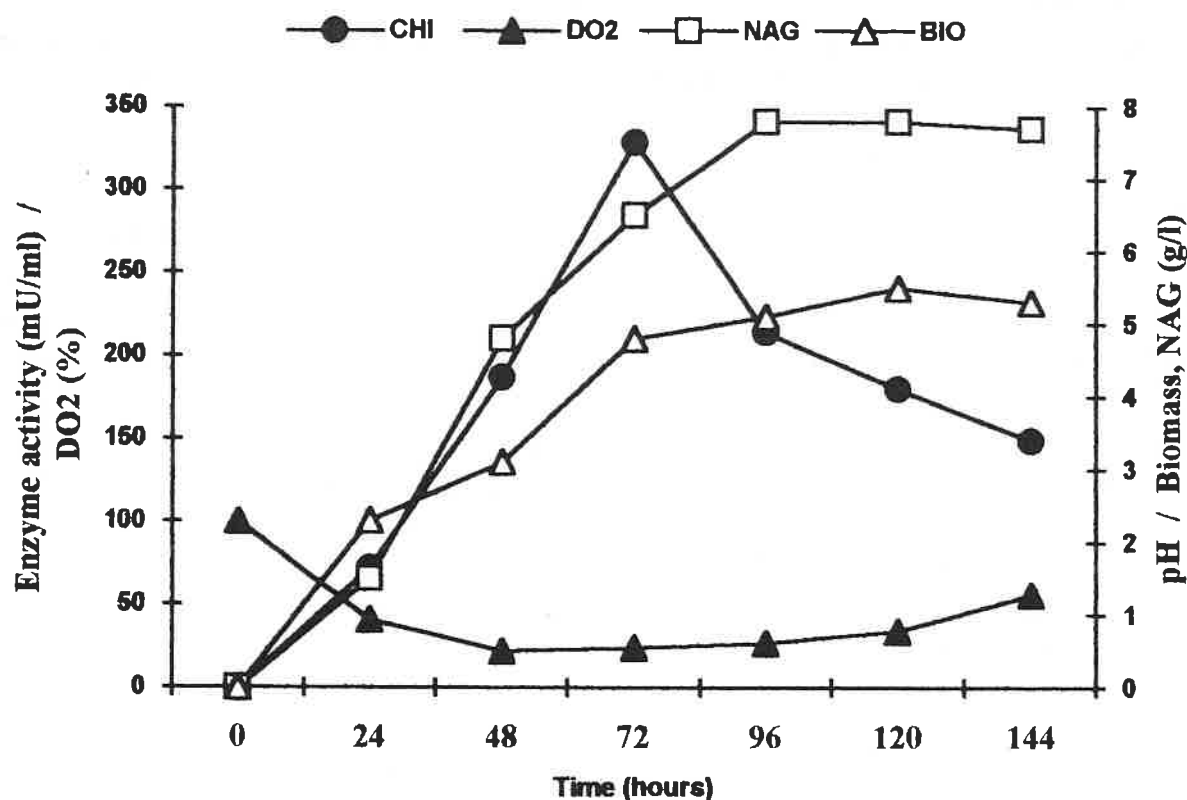
Table 1. Optimal physico-chemical parameters for the cultivation of *Verticillium* cfr. *lecanii* in bench-top fermentor.

Parameter	optimum	range	Experimental
pH	5.0	4.0-7.0	S + F
Temperature (°C)	28	5.0-35.	S
Stirrer speed (r.p.m.)	300	200-500	F
Aeration (V/V/m)	1.0	0.5-1.5	F

Legend: S = shaken flasks; F = bench-top fermentor.

Chitinase and NAG productions increased almost parallelely starting from the 24th h of fermentation and reached their maxima after 72 h and 96 h respectively. After that time the enzyme activity decreased rapidly while NAG concentration remained almost constant for further 48 h.

Figure 4. Time course of growth, dissolved oxygen, chitinase and NAG productions of *Verticillium* cfr. *lecanii* cultivated in bench-top fermentor under optimized conditions.

Conclusion

Optimization at laboratory scale of fermentation parameters is important in view of process scale-up. In this respect, enzymes of fungal origin have been widely studied (Traeger *et al.*, 1991; Milagres and Lacis, 1991 and Petruccioli *at al.*, 1995). On the contrary, the optimization of fungal chitinase production in bench-top fermentors has not been previously reported. In this work, the optimized fermentation of *Verticillium*. cfr. *lecanii*, led to appreciable increase of enzyme production (> 40%).

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