Mycoparasitism of the chitinolytic antarctic strain *Verticillium* cfr. *lecanii* A3 against *Mucor plumbeus*

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The hyphomycete *Verticillium* cfr. *lecanii*, isolated from Continental Antarctica showed mycoparasitism when grown (either at 5 and 25 °C) in dual cultures with *Mucor plumbeus*, a mold involved in refrigerated food spoilage. The fungus-fungus interaction was investigated by observations under the scanning electron microscope. Mycoparasitism increased progressively during cultivation: in the initial phases, *V.* cfr. *lecanii* mycelium took contact with *M. plumbeus* coiling around it and exerting a certain mechanical pressure. Afterward, *M. plumbeus* mycelium began to show characteristic signs of hyphal degradation, spore deflating and loss of turgor. In the advanced phases of mycoparasitism all its structures were invaded by the mycelium of *V.* cfr. *lecanii* that penetrated deeply into the host hyphae, sporangia and sporangiospores.

Introduction

The antarctic strain *Verticillium* cfr. *lecanii* A3 is characterized by a strong chitinolytic activity in media containing chitin as the sole carbon source (Fenice *et al.*, 1996). The extreme environment, where this fungus was isolated, can be colonized only by highly adapted organisms (Fenice *et al.*, 1997). Thus, the characteristics of this organism may be interesting for potential applications in specific biotechnology fields i.e. treatments of chitin-rich wastes at low temperature and/or biocontrol of phytopatogens in cold environments. In this context, the antifungal action of *Verticillium* cfr. *lecanii* (A3) against the spoiling agent of refrigerated foods, *Mucor plumbeus* has been investigated.

Materials and Methods

Chemicals: Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) were from Oxoid (Unipath Ltd.,U.K.). All other chemicals were of analytical grade.

Microrganisms: Verticillium cfr. lecanii A3 is stocked in the culture collection of the Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Viterbo, Italy. *Mucor plumbeus* was from the culture collection of the Bioscience Department, Nestlè Research Center (Lausanne, CH). During the study, cultures were maintained on MEA at 4-6 °C and subcultured every month.

Dual cultures: Petri dishes (10 cm diameter) filled with PDA were inoculated (punctiform inocula, 4 cm apart) with both *V.* cfr. *lecanii* and *M. plumbeus* and incubated at 5 and 25 °C. Mycelial structures in the contact zone of the two colonies were observed under the scanning electron microscope (SEM).

Scanning electron microscopy: Samples cut with a sharp lancet were treated as reported by Onofri *et al.* (1980). Jeol 5002 JSM (Jeol, Japan) scanning electron microscope was used.
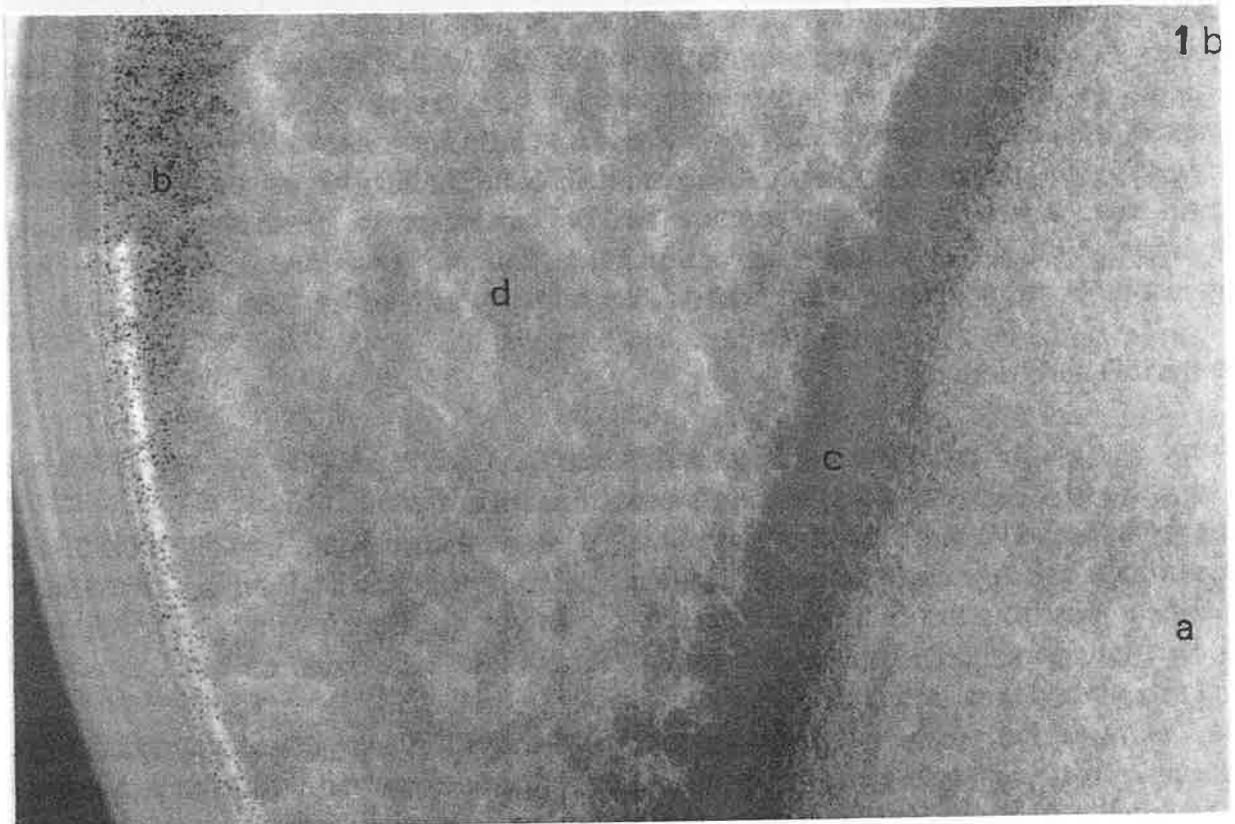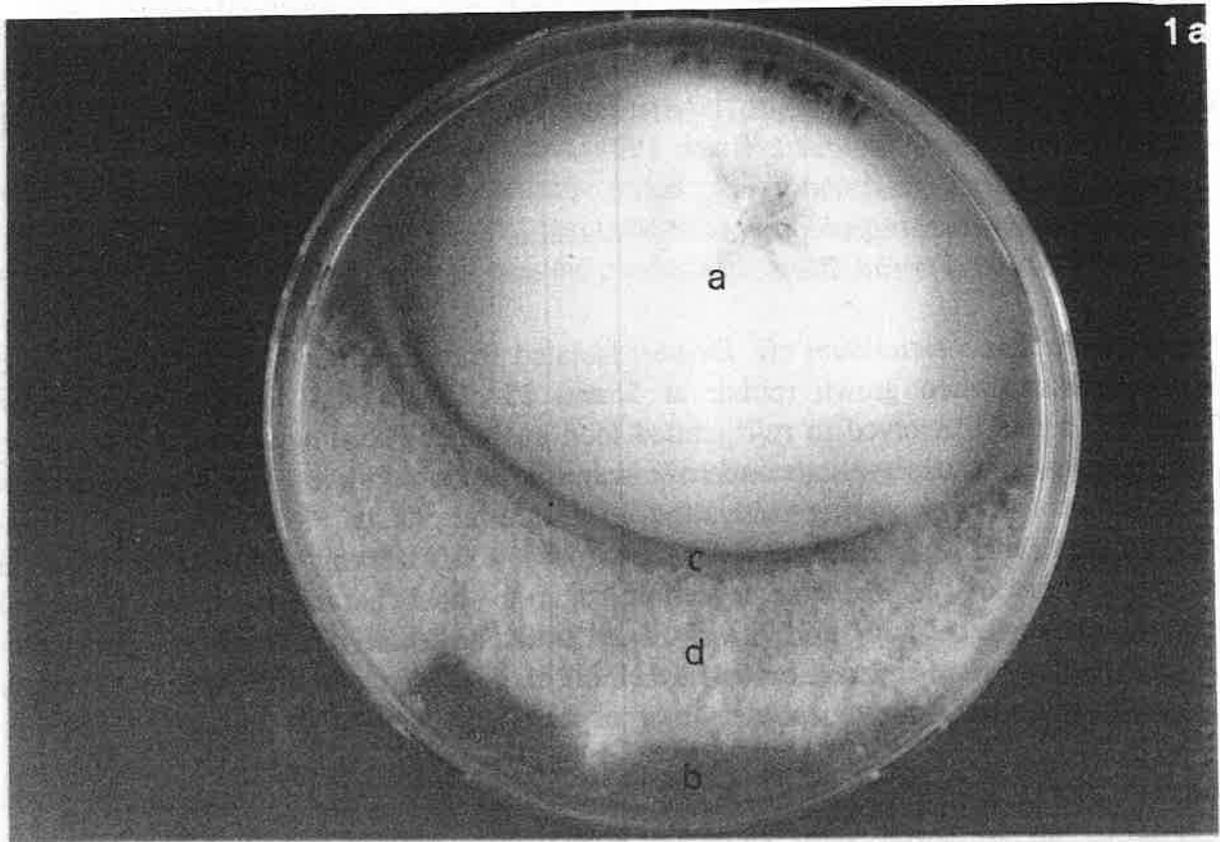
Figure 1. Dual culture of *Verticillium* cfr. *lecanii* and *Mucor plumbeus*:
1-a: (a) white colony, *V.* cfr. *lecanii*; (b) grey colony, *M. plumbeus*; (c) contact zone between the two microorganisms; (d) overgrowth of *V.* cfr. *lecanii* on *M. plumbeus*; 1-b: (detail of 1-a).
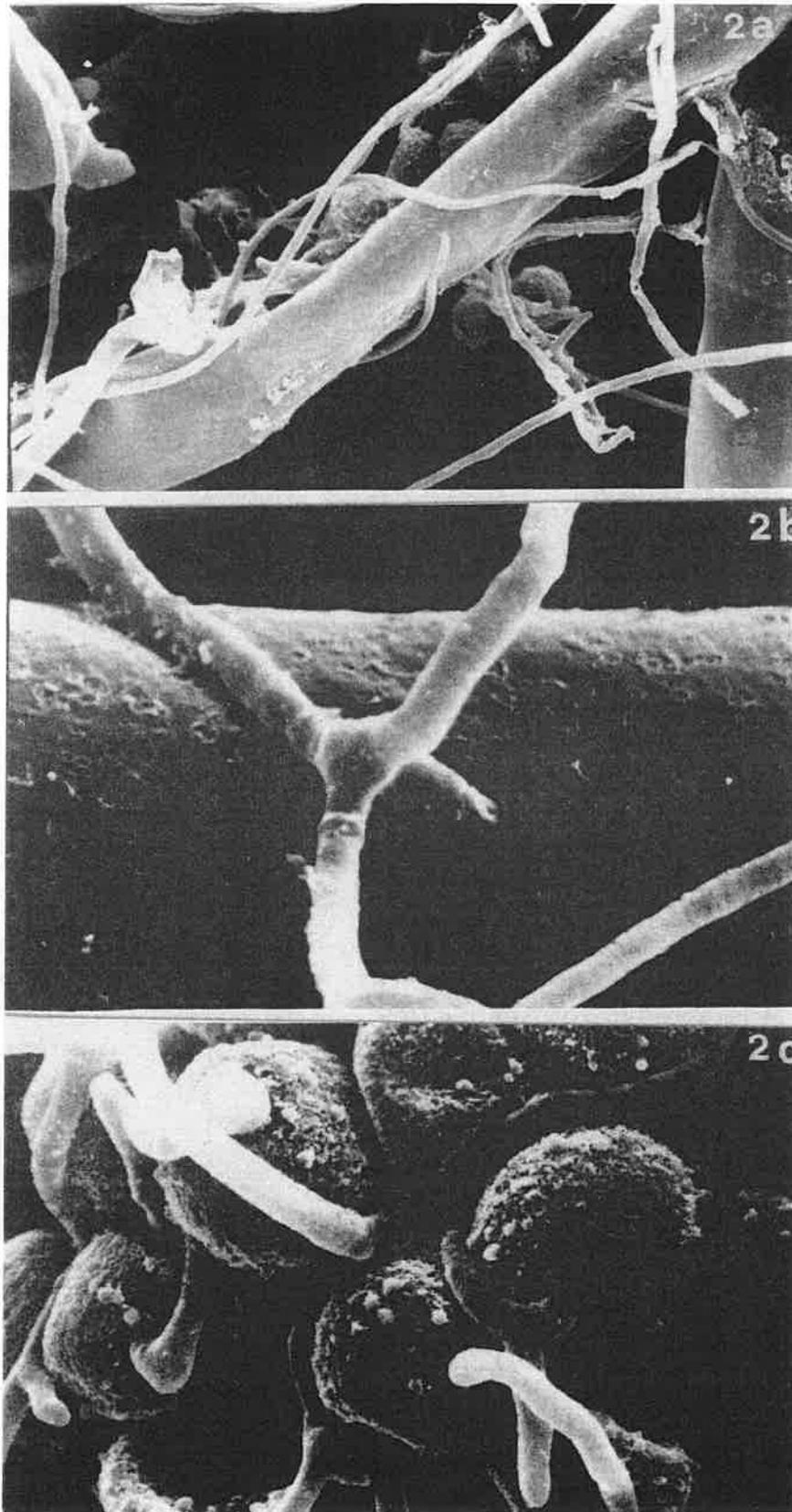
Figure 2. Dual culture of *Verticillium* cfr. *lecanii* (thin hyphae) and *Mucor plumbeus* (thick hyphae), SEM observations; **2-a:** initial phase of mycoparasitism: *V.* cfr. *lecanii* begins coiling around the mycelium of *M. plumbeus* and to exert a mechanical pressure in the contact points; **2-b:** *V.* cfr. *lecanii* penetrates into the host mycelium; **2-c:** penetration of conidia; **2-d/e:** degradation of *M. plumbeus* sporangia.

Figure 2. Dual culture of *Verticillium* cfr. *lecanii* (thin hyphae) and *Mucor plumbeus* (thick hyphae), SEM observations; **2-a:** initial phase of mycoparasitism: *V.* cfr. *lecanii* begins coiling around the mycelium of *M. plumbeus* and to exert a mechanical pressure in the contact points; **2-b:** *V.* cfr. *lecanii* penetrates into the host mycelium; **2-c:** penetration of conidia; **2-d/e:**degradation of *M. plumbeus* sporangia.

Results and discussion

Verticillium cfr. *lecanii* A3 showed clear inhibitory effects on *Mucor plumbeus*: figure 1a shows the appearance of a dual culture of *V.* cfr. *lecanii* (upper) and *M. plumbeus* (lower) after 15 days of incubation at 25 °C. An area of lysis of the mycelium of *M. plumbeus* was present in the contact zone between the two microorganisms (plate center). Aerial mycelium of *V.* cfr. *lecanii* (white) overgrew, almost completely, *M. plumbeus* (light grey mycelium with black spots of sporangia) (fig. 1b).

SEM observations (fig 2-a/h).
Verticillium cfr. *lecanii* established a close contact with *M. plumbeus* surrounding it (fig.2-a) and penetrating into its hyphae (fig 2-b) and sporangiospores (fig 2-c). The mycoparasitism increased during the fungal interaction; *M. plumbeus* mycelium showed characteristic structure degradation (fig.2-d), spore deflating and loss of turgor (fig. 2-e). Same results were obtained at 5°C after 30 days of incubation (data not shown).

Conclusion

Although mycoparasitism is an intriguing aspect of fungal physiology, some typical features, such as hyphal coiling around host mycelium and invasion of host structures, are common in this fungus-fungus interaction. Benhamou and Chet (1993) reported on *T. harzianum* mycoparasitism against *R. solani*: the first coiled around the host mycelium causing collapse and loss of turgor. The same was found in dual cultures of *M. plumbeus* and *V.* cfr. *lecanii* A3 that very likely could be proposed as a new mycoparasite strain. However, further studies are necessary in order to understand the role of the chitinolytic enzymes of *V.* cfr. *lecanii* A3 in its mycoparasitism, and to test its antifungal action against other fungal strains.

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Antimicrobial activity by fractionated chitosan oligomers

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Abstract

The chitosan oligomer of weight average molecular weight (Mw) less than 2,200 was hard to suppress microbial growth and fluorescence was observed inside of bacteria cell. The chitosan oligomer of Mw around 5,500 suppressed the microbial growth dependently on the concentration. On turbidity measurement, almost complete suppression of microbial growth was observed when the oligomer of Mw of around 9,300 was added and fluorescence was observed only outskirts of bacterial cell wall. Minimal inhibitory concentration (MIC) of chitosan oligomer was less than 0.004% and 0.032% for Mw of 9,300 and 5,500, respectively.

Introduction

As chitosan was reported to show an antimicromial activity (1-3), various fractionated chitosan oligomers were prepared to investigate the molecular weight dependence on the antimicrobial activity against several bacteria, e.g., *Escherichia coli*, *Staphylococcus aureus* etc.

Materials and methods

Chitosan: Chitosan of 98% deacetylated was prepared by repeated autoclave treatement in 40%(w/v) sodium hydroxide aqueous solution for 1hr. The degree of deacetylation was estimated both by IR and elemental analysis as reported previously(4).

Depolymelization of chitosan by nitrous acid: Chitosan oligomers were prepared by nitrous acid degradation(5). Briefly,

chitosan was dissolved in 10% acetic acid aqueous solution to prepare chitosan dope. 10% sodium nitrite aqueous solution, 0.3 mole equivalent per amino group of chitosan, was added portionmisely into the chitosan dope at 4 °C and stirred for 2 hrs. Then pH of the reaction mixture was adjusted to 6.0 with sodium hydroxyde aqueous solution and sodium borohydrate powder corresponding 2 mole equivalent per sodium nitrite used, was added slowly to the reaction mixture at 4 °C. The reaction mixture was neutralized with acetic acid followed by the removal of insoluble materials with filtration after stirring overnight at room temperature.

Table 1. Molecular weight and distribution of molecular weight of fractionated chitosan oligomers

Fraction	Mn	Mv	Mw	Mz	Mw / Mn	Mz / Mw
1	9900	10500	10500	11400	1.07	1.08
2	9200	9400	9300	9600	1.02	1.03
3	7200	7900	8000	9300	1.10	1.18
4	7200	7300	7300	7300	1.02	1.02
5	5700	6200	6200	7300	1.09	1.17
6	4900	5000	5500	5300	1.03	1.04
7	3300	4100	4100	7900	1.26	1.92
8	3300	3400	3400	3600	1.04	1.03
9	2000	2400	2200	3500	1.19	1.46

Mn: number average molecular weight
Mv: viscosity average molecular weight
Mw: weight average molecular weight
Mz: z average molecular weight

Fractionation of chitosan oligomers: The fractionation of chitosan oligomers produced were performed by precipitating with methanol, dialysis, ultrafiltration and gel filtration, respectively. The molecular weight distributions of fractionated chitosan oligomers are shown in Table 1 derived from liquid chromatographic analysis.

Antimicrobial activity: A chemically defined medium, arranged Vogel-Bonner medium containing 1% (w/v) glucose, was used as culture medium because of little aggregation with chitosan or chitosan oligomers.

The medium contained chitosan oligomer which was used for turbidity and viable cell count measurement was prepared as follows; 2.5% (w/v) chitosan oligomers in culture medium, and 10%

(w/v) glucose aqueous solution were prepared. Then, chitosan oligomer solutions of various concentration were filtrated by sterilized disposable filter(0.45μm of pore size). 40ml of test medium contained 0.01, 0.05, 0.1, and 0.5% (w/v) chitosan oligomer (Fraction 2 and 9) and 1% (w/v) glucose were prepared by addition of the filtrated chitosan oligomer solution and the steriled glucose aqueous solution. The medium without chitosan oligomer was used as control medium and subculture medium.

The antimicrobial activity of chitosan oligomers was assessed by three different methods. The first method was the turbidity measurement of culture medium suspended with bacteria and the second was the viable cell counts and the third was minimal inhibitory concentration (MIC). The estimation of antimicrobial activity was performed for 5 strains, such as *Escherichia coli*, Enterotoxigenic *E.coli* (ETEC), *Bacillus cereus*, *Salmonella typhimurium* and *Staphylococcus aureus*.

Turbidity measurement: 0.8ml of subcultured cell suspension was inoculated to 40ml of test medium and cultured for 24 hours at 30 °C. During culture, 2ml of culture medium was drawn up to measure the turbidity at 610 nm.

Viable cell count measurement: Subcultured cell suspension was inoculated and cultured for 24 hours at 30 °C. During cultivation, 0.1ml of cell suspension was drawn up and diluted 10 to 10^7-fold with a physiological saline. Diluted suspension was poured in to the sterWled sharles and mixed by nutrient agar medium. After the medium solidified, plates were incubated for more than 24 hours at 30°C. Viable cell count were determined by counting colonies formed after incubation.

Minimal Inhibitory Concentration: The medium containing 0.5%(w/v) of several molecular weight chitosan oligomers were prepared and 2-fold serial dilutions were prepared in the same medium. Subcultured cell suspension was inoculated and cultured for 24 hours at 30 °C. The minimal concentration which suppressed bacterial growth was determined as MIC.

Preparation of FITC labelled chitosan oligomers: 0.2g of chitosan oligomer, 50% of deacetylation both for Mw 9,300 and 2,200, was dissolved in 20 ml 50% aqueous ethanol adjusted to pH 9.0 by dilute sodium hydroxide. 0.08mg of FITC was added to the

oligomer solution at ice cold temperature and reacted under stirring over night. FITC-chitosan oligomer was precipitated by excess ethanol and recovered with centrifugation followed by extensive rinse with ethanol, water and then dried in vacuume.

Fluorescence microscopy: 4ml of inoculum were added to test medium contained 0.01% (w/v) FITC-labelled chitosan oligomers, and then it was cultured at 30 °C with shaking for 24 hrs. Centrifugal precipitate was washed with 0.85% steriled physiological saline extensively by centrifugation. Fluorescence of the cell suspension in 0.85% steriled physiological saline was observed both by fluorescence microscope and confocal laser scanning microscope following to mixing of saline solution with 80% glycerol aqueous solution contained 0.02% (w/v) sodium azide

Results and discussion

The fractionation of chitosan oligomers was achieved successfully as shown in Table 1. Antimicrobial activities of chitosan oligomers, Fraction 2 (Mw:9,300) and 9 (Mw:2,200), were estimated by turbidity and viable cell count measurement together with MIC measurement.

On the results of turbidity and viable cell count measurement, significant growth inhibitions were observed for *E.coli*, Enterotoxigenic *E.coli* and *Staphylococcus aureus* with Fraction 2 even at low concentration as shown in Table 2 and 3. Only a slight chitosan effect was shown for *Salmonella typhimurium* and *Bacillus cereus* as (data not shown). When Fraction 9 was applied, a slight acceleration of growth was observed for *E.coli* and *St.aureus* in turbidity measurement.

MIC for *E.coli* was also decreased as the molecular weight of chitosan oligomers decreased and oligomers less than 5,500 of Mw hardly inhibited the bacterial growth as shown in Table 4.

The fluorescence due to FITC bound chitosan oligomer of fraction 2 was observed only outskirt of the cell wall of E.coli after cultivation in the medium contained FITC labelled chitosan oligomer as shown in Figure 1, whereas an accumulation of fluorescence was observed only inside of bacterial cells with use of FITC labelled chitosan oligomer in Fraction 9 as shown Figure 2.

Table 2. Antimicrobial activity of chitosan oligomers for each bacteria calculated by turbidity.

Strain	Mw	Time (h)	concentration of chitosan oligomer (w/v)			
			0.01%	0.05%	0.10%	0.05%
E.coli	9300	0	0.00	-0.11	-0.22	-0.11
		4	0.75	0.75	0.75	0.85
		8	0.91	0.91	0.91	0.94
		12	0.91	0.90	0.90	0.93
	2200	0	-0.14	-0.14	0.00	0.00
		4	0.02	0.11	-0.19	0.17
		8	-0.43	-0.44	-0.30	-0.29
		12	-0.31	-0.45	-0.49	-0.52
		24	-1.00	-0.94	-1.03	-1.31
St.aureus	9300	0	-0.17	-0.33	-0.17	-0.08
		4	0.84	0.70	0.68	0.68
		8	1.00	0.93	0.92	0.92
		12	1.00	0.97	0.96	0.95
	2200	0	0.00	0.00	0.09	0.18
		4	-0.09	-0.14	-0.14	-0.14
		8	-0.11	-0.15	-0.17	-0.30
		12	0.04	-0.05	-0.07	-0.19
		24	0.15	0.09	-0.18	-0.34
Entero-toxigenic E.coli	9300	0	-0.30	-0.20	-0.20	0.20
		4	0.55	0.63	0.74	0.77
		8	0.89	0.91	0.93	0.93
		12	0.89	0.91	0.93	0.93

Numerical values were calculated from following equation:

1- Abs (O.D.610nm) sample/ Abs (O.D.610nm) control

Table 3. Antimicrobial activity of chitosan oligomers for each bacteria calculated by viable cell count.

Strain	Mw	Time (h)	concentration of chitosan oligomer (w/v)			
			0.01%	0.05%	0.10%	0.05%
E.coli	9300	0	0.66	0.37	0.50	0.05
		4	1.00	1.00	1.00	0.97
		8	1.00	1.00	1.00	0.99
		12	1.00	1.00	1.00	1.00
	2200	0	-0.17	-0.20	-0.37	-0.33
		4	0.80	1.00	1.00	1.00
		8	1.00	1.00	1.00	1.00
		12	0.95	1.00	1.00	1.00
		24	0.92	0.98	0.99	0.97
St.aureus	9300	0	0.64	0.64	0.64	0.60
		4	1.00	1.00	1.00	1.00
		8	1.00	1.00	1.00	1.00
		12	1.00	1.00	1.00	1.00
	2200	0	0.06	-1.39	-0.22	-0.29
		4	-0.27	-1.16	-0.28	-0.80
		8	0.13	0.09	-0.13	-0.11
		12	0.29	-0.05	-0.43	-0.68
		24	-2.42	-1.91	-9.83	-11.20
Entero-toxigenic E.coli	9300	0	-0.06	-0.01	-0.12	-0.35
		4	1.00	1.00	1.00	0.97
		8	1.00	1.00	1.00	1.00
		12	1.00	1.00	1.00	1.00

Numerical values were calculated from following equation:

1- (viable cell count) sample/ (vaiable cell count) control
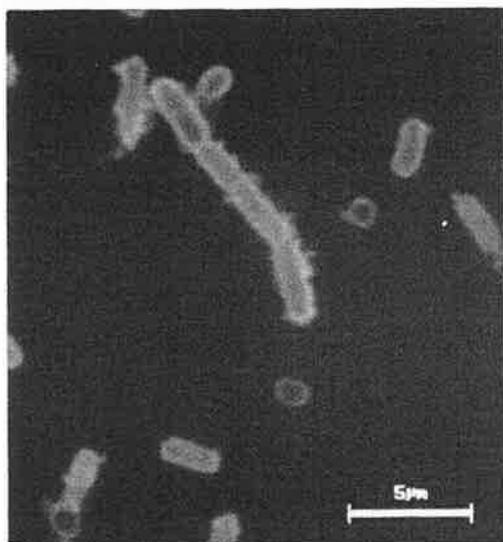
Figure 1. Fluorescence micrographs of *E.coli* attached FITC labelled chitosan oligomer (Fraction 2, Mw: 9300)
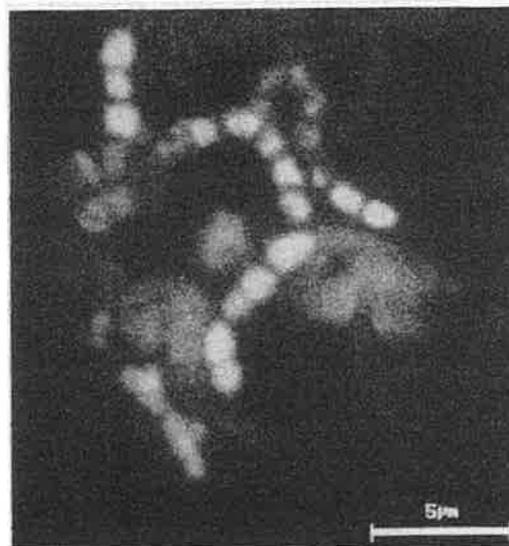

Figure 2. Fluorescence micrographs of *E.coli* attached FITC labelled chitosan oligomer (Fraction 9, Mw: 2200)

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Conclusion

It was elucidated that species specificity was found on the antimicrobial activity by chitosan oligomers with molecular weight dependently through turbidity and viable cell count measurements.

Though molecular weight dependent antimicrobial activities by chitosan oligomers were confirmed for *E.coli* and *St.aureus*, these growth inhibitions were suppressed with decreasing of molecular weight. The growth inhibition was canceled when Mw of chitosan oligomer was lowered than around 5,000 and rather enhancement of cell growth was observed.

On the fluorescent study, it was assumed that the permeation of nutrition through cell wall was inhibited by attaching of high molecular weight chitosan oligomers around the bacterial cell wall and that low molecular weight chitosan oligomers permeated into cell to metabolize it as one of nutritions.

Table 4. Minimal inhibitory concentration (MIC) of chitosan oligomers to *Escherichia coli*

Fraction	Mw	MIC (%)
1	10500	0.004
2	9300	0.004
3	8000	0.008
4	7300	0.008
5	6200	0.016
6	5500	0.032
7	4100	NE
8	3400	NE
9	2200	NE

NE: no effect at 0.5%

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Effect of chitosan adsorption on the cell surface on genetic transformation of bacterial and animal cells by electroporation

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ABSTRACT

The effect of chitosan bound to the cell surface on gene transfer into a bacterium, *Bacillus subtilis* ISW1214 cell by electroporation was investigated. About 90 % of chitosan in cell suspension was bound to the cell surface before or after electroporation. However chitin and sodium alginate were less bound to the cell surface. Gene transfer into *B. subtilis* in buffer containing chitosan was done by electroporation. Using chitosan with molecular weight of 1034, gene transfer efficiency reached a maximum value of 8.5 x 10° cells per μg DNA, and it was 4.5 times of that without chitosan. The chitosan with molecular weight more than 1034 resulted in the steep drop of the gene transfer efficiency.

DNA transformation of a mammalian, Chinese Hamuster overy CHO-H1 cell was also investigated. The chitosan with a low molecular weight of 1034 was effective in gene transfer into CHO-K1 cells and gene transfer efficiency was 2.0 times of that without chitosan, in the same manner of that of *B. subtilis* ISW1214.

This support that, using chitosan with a low molecular weight of 1034, the localization of DNA based on chitosan-DNA ion complex around the cell surface is effective in gene transfer into cells.

Keywords: Chitosan, Gene transfer, transformation, adsorption, electroporation, DNA, *Bacillus subtilis*, mammalian CHO-K1 cells.

Materials and Methods

Strain and plasmid DNA. A Bacterium, *Bacillus subtilis* ISW1214 strain (*hsr*M1, *leu*A8, *met*B5) was purchased from Takara Shuzo Co., Ltd. and subcultured in Pen medium (Difco antibiotic No. 3) at 37°C. Pen agar medium containing antibiotic was used for the selection medium for transformants. A mammalian cell, Chinese Humuster overy cell was obtained from Riken Gene Bank, Japan and incubated in ES medium (Nissui Seiyaku Co., Ltd, Japan) containing 10 % fetal bovine serum. Eagle's minimal essential medium containing

geneticin was used for the selection medium for transformants. As vectors for *B. subtilis*, plasmid pUB110 (4.5 kbp) was obtained from Dr. Y. Yoneda (Nippon Gene Co., Ltd.) and for CHO-K1 cells, plasmid pSV2-*neo* from Dr. Y. Watanabe (Hokuriku Seiyaku Co., Ltd.). Three chitosans with molecular weights of 1034(n=5 ~ 6), 2500(n=13~14), and 8140(n=40~ 50) were purchased from Pias Co., Ltd. and a chitosan with a molecular weight of 380000(n=*ca.* 2000) gifted from Katakura Tikkarin Co., Ltd. Other reagents were purchased from Wako Pure Chemical Industries, Ltd.

Electroporation procedure. [1~4] Electroporation was done by the method of Kusaoke *et al.* *B. subtilis* ISW1214 cells were suspended in suspension buffer at a density of about 1.0×10^{10} ~ 6.0×10^{10} cells/ml and DNA (4~5 μg/ml) and chitosan (1~10 mM per glucosamin residue) added to this mixture. Twenty μl of the cell suspension were placed in a chamber, and the electric field pulse of a decayed wave was applied as a single pulse at the electric field strength of 7 kV/cm and at the condenser capacitance of 10 μF. After the electric treatment, the cells were placed on Pen agar medium, incubated at 37℃ for 24 to 48 h, and the number of regenerated colonies was counted as transformants.

CHO-K1 cells at the density of 10^7 were suspended in EP buffer containing plasmid pSV2-neo (10 μg/ml) and chitosan (0.2 ~2.0 mg/ml). Twenty μl of the cell suspemsion were placed in a chamber, and the electric filed puse of a squared wave was applied as a single pulse at the electric field strength of 2.5 kV/cm and at the pulse duration of 500 μsec. After the electric treatment, the cells were incubated for 60 min on ice and incubated for expression of the *neo* gene in ES medium and 10% fetal bovine serum for 2 days at 37℃ in 5% CO_2. The medium was replaced with fresh Eagle's minimal essential medium containing geneticin and incubated for 10 ~12 days at 37℃ in 5 % CO_2. The number of transformed cells was determined as transformants.

Electroporation apparatus. Electroporation was carried out using SOMATOIC HYBRIDIZER SSH-1 (Shimadzu, Japan) as a high voltage generator with a squared wave or ELECTRIC GENE TRANSFER EQUIPMENT GTE-10 (Shimadzu) as a high voltage generator with a decayed wave.

Results and discussion
Adsorption of chitosan to B. subtilis cell surfaces. [5]

Gene transfer into *B. subtilis* cells in buffer containing chitosan and DNA was done by electroporation. Binding contents of chitosan to cells were investigated, as shown in Fig. 1. About 90 % of chitosan between 1034 and 380000 in molecular weight was highly bound to

cell surfaces in the concentration of chitosan up to 10 mM per D-glucoamine residue in cell suspension. Binding contents of D-glucosamine hydrochloride, N-acetyl-D-glucosamine, chitin and sodium alginate were lower values than that of chitosan.
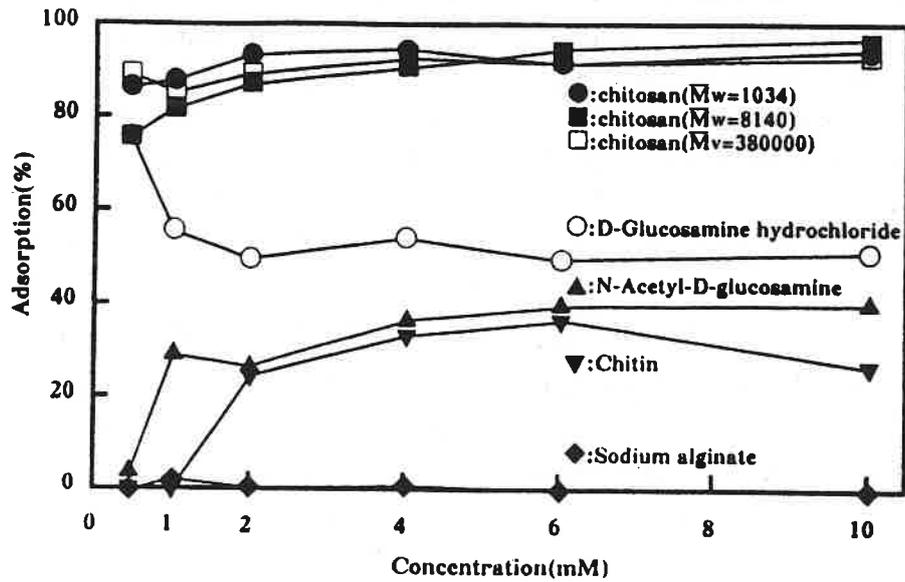

Fig. 1 Binding contents of chitosan, chitin, and sodium alginate to *B. subtilis* cells at various concentrations after electroporation.

Effect of chitosan on genetic trnsformation of B. subtilis.[5)]

Gene transfer into *B. subtilis* in cell suspension containing 10 mM of chitosan with molecular weight of 1034 resulted in a maximum value of 8.5 x 10^4 cells/ μ g DNA in efficiency and it was 4.5 times of that without chitosan(Fig.2). The chitosan with the molecular weight more than 8140 resulted in the steep drop of the gene transferefficiency with the increase of the concentration of chitosan.
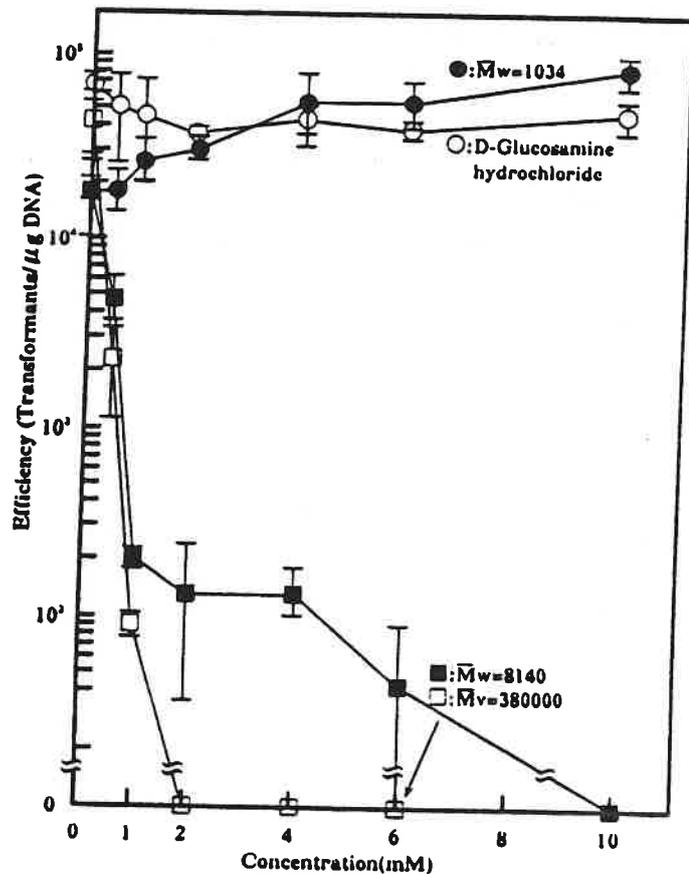

Fig.2 Effect of molecular weight of chitosn on gene transfer efficiency at varivarious chitosan concentrations.

Chitosan, N-acetylgluco-samine, and sodium algin-ate were not effective in gene transfer into *B. subtilis* (Fig. 3).

Ito *et al.* [6] reported that DNA transformation of Yeast was improved 2 times by using spermine as a polyamine. However our experimental results, seve-ral other polyamines except for chitosan were not effective in the gene transfer efficiency (data not shwon). Gene transfer using spermine resulted in a rapid drop of efficiency.
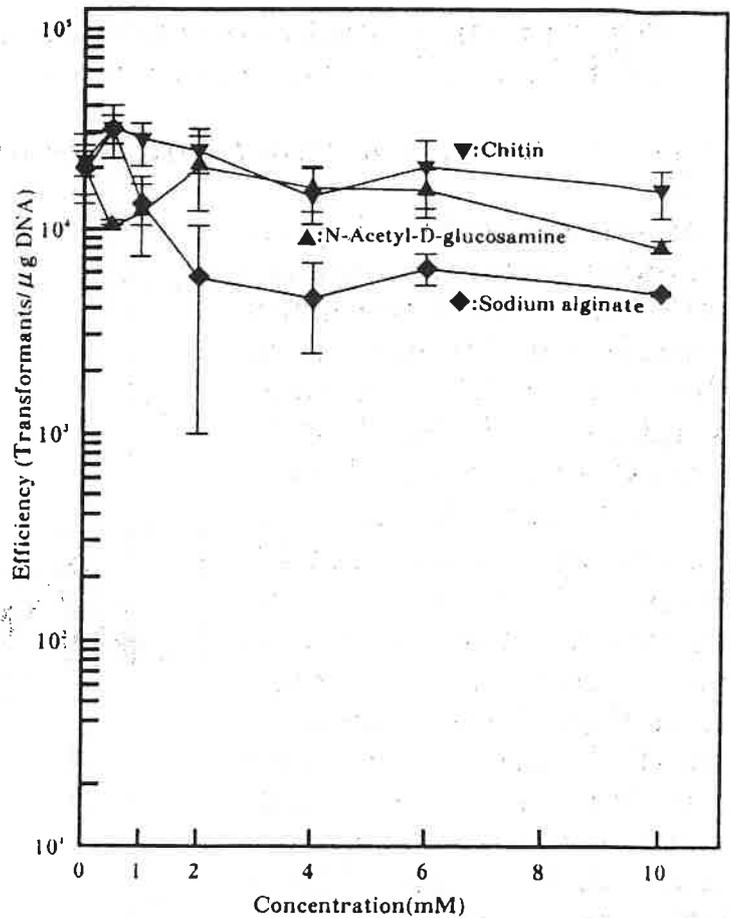
Fig. 3 Effect of chitin and sodium alginate on gene transfer efficiency at various concentrations. DNA concentration was 5.8 μg/ml.

Adsorption of chitosan to mammalian CHO-K1 cells.

As shown in Fig. 4, about 80 % of chitosan between 1034 and 380000 in molecular weight was effectively bound to cell surfaces at the concentrations of chitosan up to 2 mg/ml in cell suspension. Adsorptions of chitosan, sodium alginate, and N-acetyl-D-glucosamine were lower values than that of chitosan, in the same manner with *B. subtilis.*

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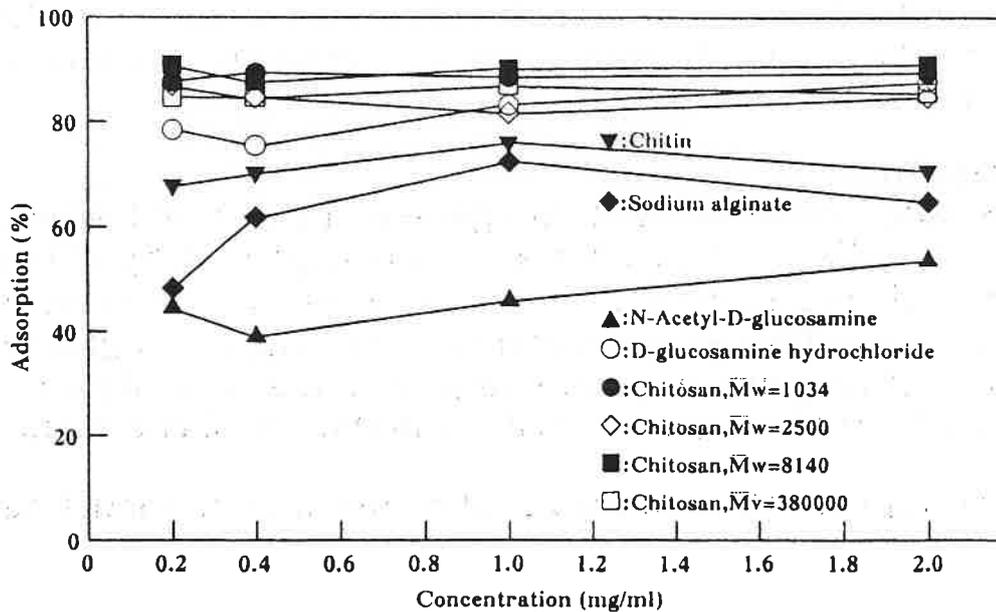
Fig. 4 Binding contents of chitosan, chitin, and sodium alginate to CHO-K1 cells at various concentrations after electroporation.

Effect of chitosan on genetic transforamtion of mammalian CHOK-1 cells.

As shwon in Fig. 5, gene transfer into mammalian cells in cell suspension containing 2.0 mg/ml of chitosan with molecular weight of 1034 resulted in a maximum value of 5.8 x 10^2 cells/μg DNA in efficiency and it was about 2.0 times of that without chitosan. The chitosan with molecular weigh more than 2500 resulted in the slight drop of the gene transfer efficiency with increasing the concentration of chitosan. These data were the same as shown in *B. subtilis.*
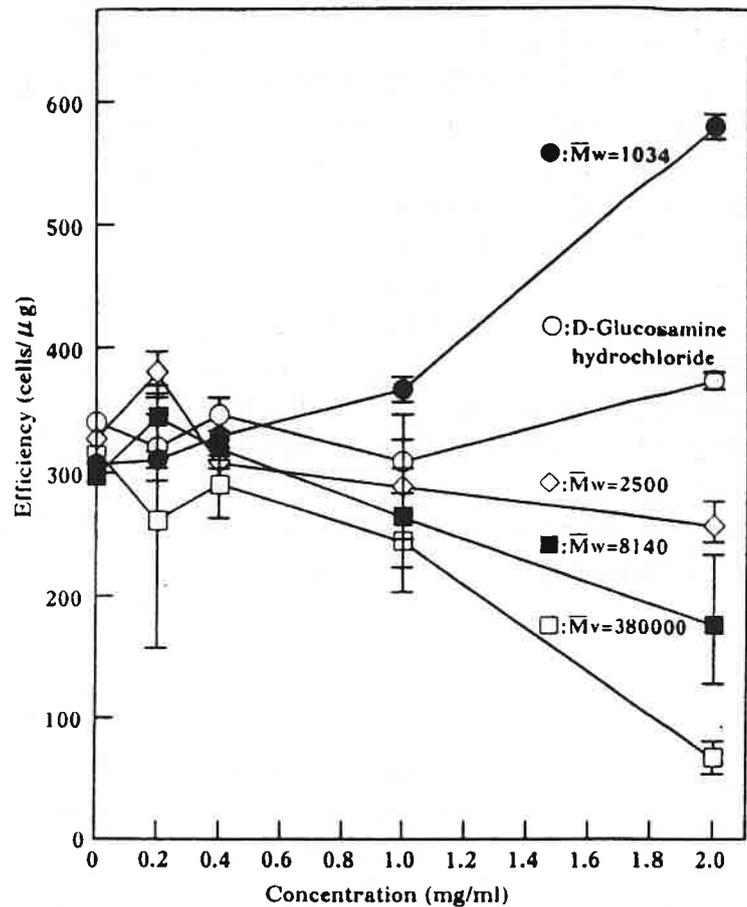
Fig. 5 Effect of molecular weight of chitosn on gene transfer efficiency at various chiotan concentrations.

Chitin, N-acetyl-D-glucosamine, and sodium alginate were not effective in gene transfer into mammalian cells, in the same manner with *B. subtilis* (data not shwon).

Several other polyamine except for chitosan were also not effective in gene transfer in mammalian cells, in the same manner with *B. subtilis* (data not shown).

Conclusion

In this study, we found that the gene transfer into bacterial and mammalian cells by electroporation was improved by containing chitosan with the molecular weight of 1034 (n=4 ~ 5) in buffer. Chitosans except for the molecular of 1034, chitins, D-glucoamine, N- acetyl-D-glucosamine, sodium alginate, and several polyamines were not effective in gene transfer into bacterial and mammalian cells.

We here suppose the intermolecular-interaction model between

chitosan and DNA on the cell surface, as shown Fig. 6. With forming the complex between chitosan with a short molecular chain and DNA, The DNA density aound the cells based on a short molecular chain (the molecular weight of 1034) of chitosan increases and subsequently the gene transfer efficiency increases. However, with forming the complex between chitosan with a large molecular chain and DNA, the DNA density around the cells based on a long molecular chain (the molecular weight more than 1034) of chiosan decreases and subsequently gene transfer efficiency decreases. On the other hand, with not forming sodium alginate-DNA complex, sodium alginate does not affect the gene transfer efficiency.

;molecular chain of chitosan
D⁺ ;DNA

$\sim$;molecular chain of sodium alginate
D⁻ ;DNA
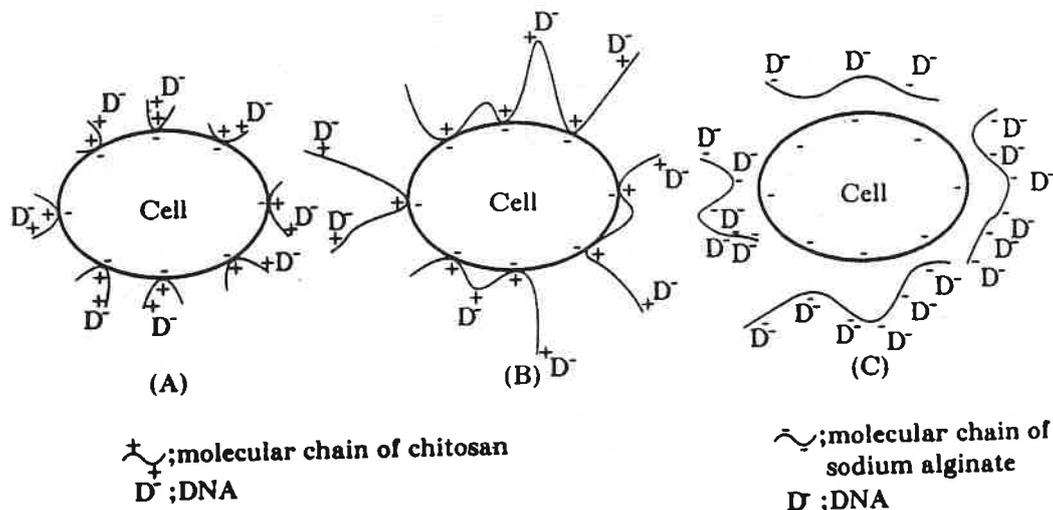
Fig. 6　The model of the intermolecular-interaction between chitosan and DNA on the cell surface. (A); chitosan with molecular weight of 1034, (B); chitosan with molecular weight more than 1034, (C); sodium alginate.

Electroporation is bradly applicable to gene transfer in bacterial, animal, and plant cells. The use of chitosan with low molecular weight of 1034 in electroporation-buffer will be greatly available for efficient DNA transformation of bacterial and animal cells.

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SPECIFICITY IN ENZYMATIC AND CHEMICAL DEGRADATION OF CHITOSANS

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Abstract

Chitosans may be considered as a family of linear binary copolymers of $(1 \rightarrow 4)$ linked 2-acetamido-2-deoxy-β-D-glucopyranose (**A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose (**D**-unit). Fully water-soluble chitosans with fraction of **A**-units (F_A) from 0 to 0.7 and with known random (Bernoullian) distribution of the monomer units can be prepared.

The specificity of chitosan-degrading enzymes has traditionally been studied by extensive enzymatic degradation of the polysaccharide and subsequent isolation and characterization of the oligomers. We recently reported on a new and simpler method for determining the specificity of enzymatic hydrolysis of partially N-acetylated chitosans, using high-field carbon n.m.r.-spectroscopy to determine the identity of the new reducing and non-reducing ends and the variation in their nearest neighbours. Thus, the site-specificity of up to four subsites on the chitosan-depolymerizing enzyme may be determined. For lysozyme, the results suggest a high specificity for **A**-units in lysozyme's subsites C, D and E (where cleavage occur between sugar units bound to subsites D and E), while there is no specificity in subsite F. A *Bacillus* chitosanase showed the same high specificity towards **D**-units as lysozyme towards **A**-units.

The specificity with respect to the cleavage of the four different glycosidic linkages in chitosans (**A-A**, **A-D**, **D-A** and **D-D**) has been studied for nitrous acid depolymerization and acid hydrolysis. The nitrous acid depolymerization reaction mechanism is specific in the sense that HONO attacks the amino group of **D**-units, with subsequent cleavage of the following glycosidic linkage, and together with determination of the identity of the new non-reducing ends it was concluded that only the **D-A** and **D-D**-linkages are cleaved by nitrous acid depolymerization, and that they are cleaved with about equal rates. It has, however, been reported that the rate of the depolymerization increased with increasing F_A of the chitosan, which was explained by the reaction mechanism.

The depolymerization of chitosans by acid hydrolysis was found to be specific in the sense that the protons attack the glycosidic oxygen following the non-charged **A**-units, as the rate of formation of new reducing ends with **A**-units is at least one order of magnitude higher compared to **D**-units, as determined from proton NMR-spectroscopy. From determination of the identity of the new non-reducing ends from carbon NMR-spectroscopy, it was concluded that the rates of acid hydrolysis of the glycosidic linkages in chitosans are of the order **A-A** $\approx$ **A-D** $\gg$ **D-A** $\approx$ **D-D**. Initial acid degradation rates (r) of a series of chitosans were shown to increase somewhat more than in proportion to F_A, and the interpretation of these results with respect to specificity are in line with the NMR-spectroscopy results.

Introduction

Chitosans can be enzymatically degraded by e.g. chitinases, chitosanases and lysozyme. In addition to enzymatic degradation, chitosans may also be depolymerized by various chemical degradation mechanisms (e.g. acid hydrolysis, nitrous acid and oxidative-reductive depolymerization). It has not been our intention to present a comprehensive review of the litterature on enzymatic and chemical degradation of chitosans, but rather to focus on some of the recent results where selected degradation mechanisms, both enzymatically (lysozyme and a chitosanase) and chemically catalysed reactions (nitrous acid depolymerization and acid hydrolysis), have been studied in dilute aqueous solutions in order to determine possible specificities in the reactions. By specificity, we refer to the identity of the sugar units adjacent to the linkage which is cleaved, which for chemically catalyzed reactions in dilute solution usually includes one or two sugar units, whereas for enzymatically catalysed reactions it will in principal include the number of sugar residues recognized by the active site of the enzyme. Previous results on degradation of chitin and chitosan have been reviewed by Roberts (1992)[1].

Materials and methods

Acid hydrolysis with subsequent analysis by NMR-spectroscopy - The chitosan used was a fully acid-soluble chitosan in the hydrochloride salt form with $F_A=0.49$ (determined by proton NMR spectroscopy[20]) and an intrinsic viscosity of 1200 ml/g (pH 4.5 and ionic strength 0.1 (M)). Extensive depolymerization of the chitosan by acid hydrolysis for subsequent analysis by NMR-spectroscopy was performed in concentrated (11 M) HCl at 40° C in order to minimize the rate of hydrolysis of the acetamido group relative to the glycosidic linkage[2].

Rates of acid hydrolysis of chitosans with different F_A-values - The experiments were typically performed in relatively dilute (0.4M) HCl at 60° C by measuring the decrease in viscosity as a function of time in the previously described viscosity assay[3]. Initial rates of acid degradation were corrected for differences in chain stiffness as previously described[4], using the exponents of the MHKS-equation at different F_A-values described previously[5] (see also"Solution properties of chitosan", this volume). The initial rates of acid hydrolysis of a chitosan with $F_A=0.62$ were found to increase in direct proportion to increasing concentration of H^+ from 0.01 to 0.8M, confirming that the chitosan is actually degraded by acid hydrolysis. In the viscosity assay very low degrees of scission, α-α_n (number of chain cleavages divided by the total number of glycosidic linkages) are measured, implying that any hydrolysis of the acetamido groups will result in insignificant changes in the F_A-values during the depolymerization reaction. Details of the acid hydrolysis experiments will be reported elsewhere.

Enzymatic degradation

Traditionally the specificity of enzymes hydrolyzing chitosans has been studied by extensive enzymatic degradation of a partially N-acetylated chitosan and subsequent isolation and characterization of the oligomers. Using this method together with determining lysozyme degradation rates of defined oligosaccharides, Amano and Ito[6] studied the specificity of lysozyme towards chitosan, and found a very high specificity towards **A**-units relative to **D**-units in lysozyme's subsites C and E, and also a relatively high specificity in subsite D, where the cleavage occur between sugar units bound to subsite D and E. Izume *et al.*[7] characterized the specificity of a chitosanase from *Bacillus* species No. 7-M by identifying the oligomers after extensive hydrolysis of a partially N-acetylated chitosan, and they concluded that the chitosanase was absolutely specifictowards cleaving the **D-D**-glycosidic linkage. The specificity of a number of chitosan-degrading enzymes has been reported[8-11], and it has been proposed to classify chitosanases according to their specificity with respect to the cleavage of the three different glycosidic linkages containing **D**-units in partially N-acetylated chitosans (**A-D**, **D-A** and **D-D**)[8],

although additional classes may be added as the specificity will in principal include the number of sugar residues recognized by the active site of the chitosanase[12].

We recently reported on a new method to determine the specificity of enzymes hydrolysing chitosans[12,13], using lysozyme and the *Bacillus* species No. 7-M chitosanase as model enzymes with different specificities. The relative lysozyme degradation rates of chitosans were modelled assuming an Arrhenius-type relation for the relative rate constants, where the apparent activation energy was assumed to consist of additive contributions from **D**-units and **A**-units within the polymer chain interacting with subsites A-F of lysozyme[13]. High-field ^{13}C NMR-spectroscopy was used to determine the identity of the new reducing and non-reducing ends and the variation in their nearest neighbours. Thus, the site-specificity of up to four subsites on the chitosan-hydrolyzing enzyme may be determined. The results have been schematically illustrated in Figure 1, where the sixteen different tetrade sequences in a partially *N*-acetylated chitosan are shown and positioned in the active site of the enzyme in such a way that the tetrasaccharide is hydrolysed in the middle (indicated by an arrow). The specificity of lysozyme and the *Bacillus* chitosanase are indicated by the preferential hydrolysis of only two of the sixteen tetrade sequences.
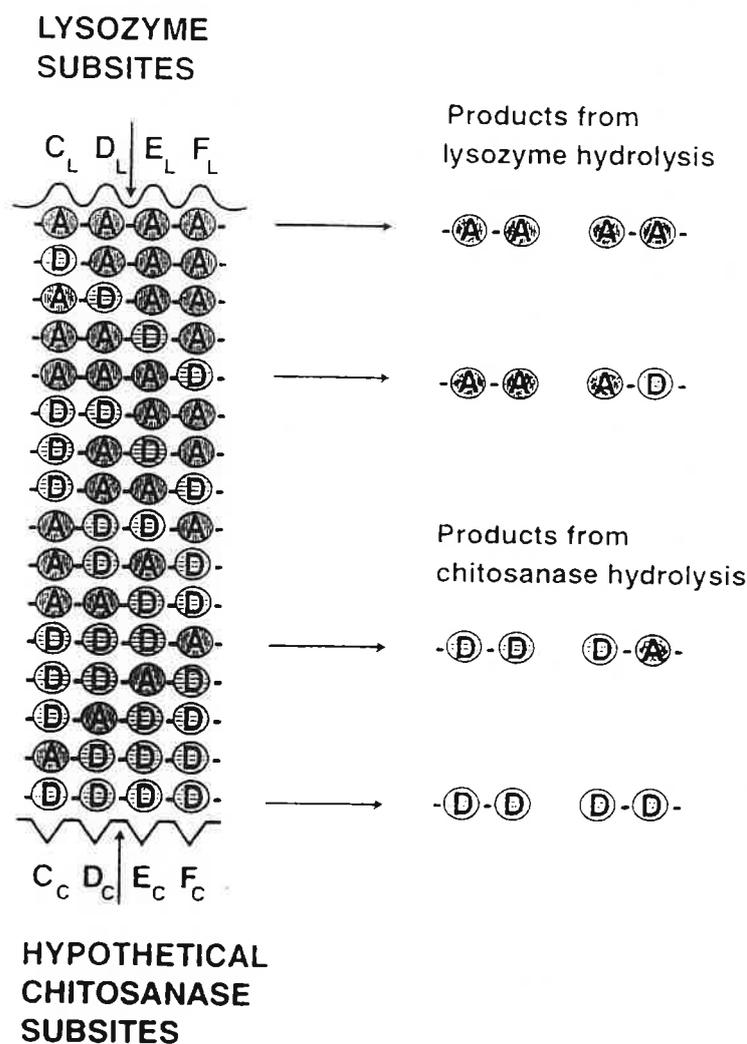
Figure 1. A schematic illustration of the specificity of hydrolysis of lysozyme and *Bacillus* chitosanase towards the sixteen different tetrade sequences in chitosan. Other sequences than indicated by the arrows on the figure may also be cleaved, but at a much lower rate. The tetrade sequences are positioned in the active site of the enzyme in such a way that the tetrades are hydrolysed at the arrow.

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These specificity results on lysozyme are also in accordance with specificity information obtained from the interpretation of lysozyme degradation rates correlated to the chemical composition of chitosans, where it was concluded that hexameric substrates containing three-four or more **A**-units contribute mostly to lysozyme degradation rates of chitosans[3], taking into account that the existence of two additional subsites (A and B) on lysozyme has been identified, which may also show some specificity towards **A**- relative to **D**-units.

Enzymes degrading chitosans may show a high specificity towards degradation of partially *N*-acetylated chitosans, as exemplified herein by lysozyme and a *Bacillus* chitosanase. By enzymatic degradation of well-characterised chitosans (i.e. F_A and sequence of **A**- and **D**-units), it is possible to obtain informations on the specificity of chitosan-degrading enzymes. Such informations may be crucial in order to elucidate biological effects of partially *N*-acetylated chitosans and chitosan oligomers, e. g. induction of chitinases/chitosanases in plants infected by pathogens containing chitin/chitosan in their cell walls. However, in addition to the specificity of the plant's chitin/chitosan-degrading enzymes, the chemical composition (including the sequence of **A**- and **D**-units) of the chitosan from the pathogen will determine the relative amounts of the different partially *N*-acetylated oligomers which are formed upon enzymatic depolymerization of the chitosan. Thus, knowledge on both enzyme specificities and the detailed chemical composition of chitosans will be highly relevant in order to understand the mechanisms behind the biological effects of chitosans or chitosan oligomers in e.g. different plant-pathogen systems.

Chemical degradation

The nitrous acid depolymerization reaction mechanism has been found to be specific in the sense that HONO attacks the amino group of **D**-units, with subsequent cleavage of the following glycosidic linkage. Depending on the rate of cleavage of the glycosidic linkages **D-A** or **D-D**, the new non-reducing ends may be **A**-units or **D**-units. By determining the identity and the relative amounts of the new non-reducing ends (by ^{13}C nmr-spectroscopy) of nitrous acid depolymerized chitosans, it was concluded that the **D-A** and **D-D**-linkages in partially *N*-acetylated chitosans were cleaved with about equal rate by nitrous acid depolymerization[14].

The kinetics of the nitrous acid depolymerization reaction has been studied in detail in a series of papers by Allan and Peyron[15-17]. They found that the rate of the depolymerization increased with increasing F_A of the chitosan, although the concentration of **D**-units decreases with increasing F_A. This was explained by decreasing pK_A-values of the **D**-units with decreasing F_A which decreased the reactivity of the **D**-units towards the positively charged nitrous acidium ion, which was interpreted as the reactive substance[16]. Thus, the reaction mechanism could be equally important as the specificity in order to explain the kinetics of a depolymerization reaction.

The rate of acid hydrolysis of the four different glycosidic linkages in chitosans would be expected to be different, as the activation energies for acid hydrolysis of the glycosidic linkage (E_a) of model compunds as methyl-2-acetamido-2-deoxy-β-D-glucopyranose and methyl-2-amino-2-deoxy-β-D-glucopyranose have been determined to 28.4 and 36.0 kcal/molK, respectively[18]. The corresponding acid degradation rates of the two glycosidic linkages would differ by more than three orders of magnitude, which have been attributed to the proximity of the positively charged amino-group of methyl-2-amino-2-deoxy-β-D-glucopyranose to the glycosidic linkage. The rate of acid hydrolysis of the glycosidic linkages in a chitosan with F_A=0.49 was tested. We first determined the identity of the new reducing ends, and Figure 2 shows the anomer-proton region of the acid hydrolysed chitosan which was depolymerized to a number-average degree of polymerization of 3.5, where the resonance of the new reducing ends are clearly dominated by **A**-units, as the resonance of the α-anomer of H-1 from an **A**-unit is found at 5.15 ppm while the resonance of the α-anomer of H-1 of a **D**-unit is found at 5.43 ppm[19]. Thus, the rates of acid hydrolysis of the **D-D** and **D-A** glycosidic linkages can be neglected relative to the
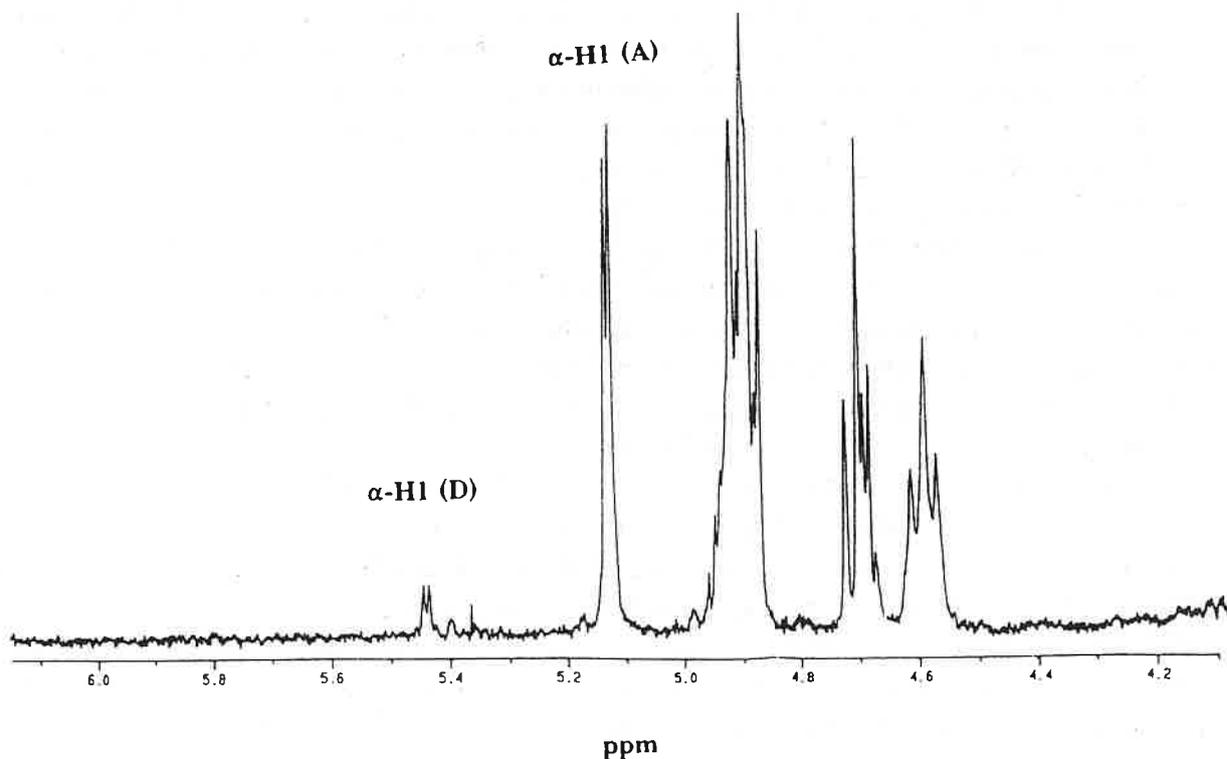
Figure 2. Anomer-proton region of the acid hydrolysed chitosan which was depolymerized to a number-average degree of polymerization of 3.5.

hydrolysis rates of the **A-A** and **A-D** linkages. In order to get information on any possible specificity of the acid hydrolysis of the **A-A** relative to the **A-D** glycosidic linkages, we determined the identity of the new non-reducing ends. Figure 3 shows part of the ^{13}C nmr-spectrum (carbon 4 and 5) of the the acid hydrolysed chitosan (F_A=0.49), showing the new non-reducing ends at 78.9 ppm (**D**-units) and 78.5 ppm (**A**-units). The ratio between the intensity of non-reducing **D**-units and non-reducing **A**-units was 0.96, and as the substrate concentrations of the four different glycosidic linkages in a chitosan with F_A=0.49 and a random distribution of **A**- and **D**-units are close to equal, it follows that the rate of acid hydrolysis of the **A-A** and **A-D** glycosidic linkages are also close to to equal.

We also measured the initial rates of acid degradation of chitosans (r) of a series of chitosans with different chemical composition (F_A),using a viscosity assay to determine the r-values [3]. The r-values increased systematically with increasing F_A, and the results are presented in Figure 4 as a double logarithmic plot of r (relative to a rate of 1 of the chitosan with F_A=0.16) versus F_A. The linearity in the plot with a slope of the line of 1.3 shows that the initial rates of acid degradation of chitosans increase somewhat more than in proportion to F_A. In order to compare the acid degradation data with the specificity results from NMR, we need to express the rate of acid degradation as a function of F_A. The initial rate of acid degradation of chitosans (r) can be related to the rate of cleavage of the four different glycosidic linkages as follows:

$$r = k_{AA} \cdot F_{AA} + k_{AD} \cdot F_{AD} + k_{DA} \cdot F_{DA} + k_{DD} \cdot F_{DD} \qquad (1)$$

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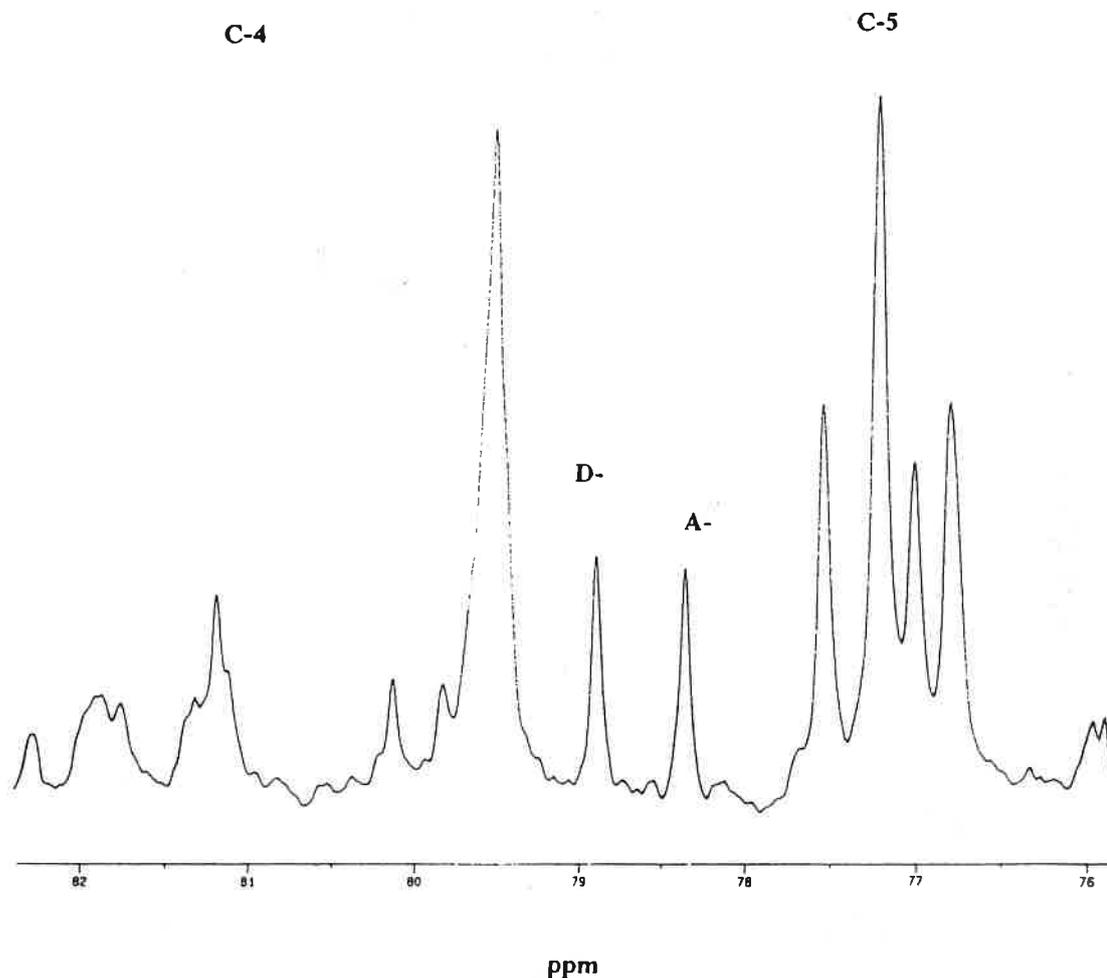
Figure 3. Part of the ^{13}C nmr-spectrum (carbon 4 and 5) of the the acid hydrolysed chitosan (F_A=0.49).

where k_{AA}, k_{AD}, k_{DA}, and k_{DD} are the rate constants for cleavage of the four different glycosidic linkages **A-A, A-D, D-A** and **D-D**, respectively, and F_{AA}, F_{AD}, F_{DA} and F_{DD} are the fractional content of the diads **AA, AD, DA** and **DD**, respectively. Since the results from our NMR-studies suggested that $k_{AA} \approx k_{AD} >> k_{DA} \approx k_{DD}$, equation 1 can be simplified to:

$$r \approx k_{AA} \cdot F_{AA} + k_{AA} \cdot F_{AD} \qquad (2)$$

Since the chitosan has a Bernoullian (random) distribution of **A** and **D**-units [20,21], implying that $F_{AA} = F_A^2$ and $F_{AD} = F_A \cdot F_D$, we get:

$$r \approx k_{AA} \cdot F_A (F_A + F_D) \qquad (3)$$

As $F_A + F_D = 1$, r should increase in direct proportion to F_A, with the above assumptions. The experimental acid degradation data presented as as a function of F_A in Figure 4 are essentially

in line with our NMR-results. However, as the r-values increase somewhat more than in proportion to F_A, it suggests that k_{AA} may be slightly larger than k_{AD}.
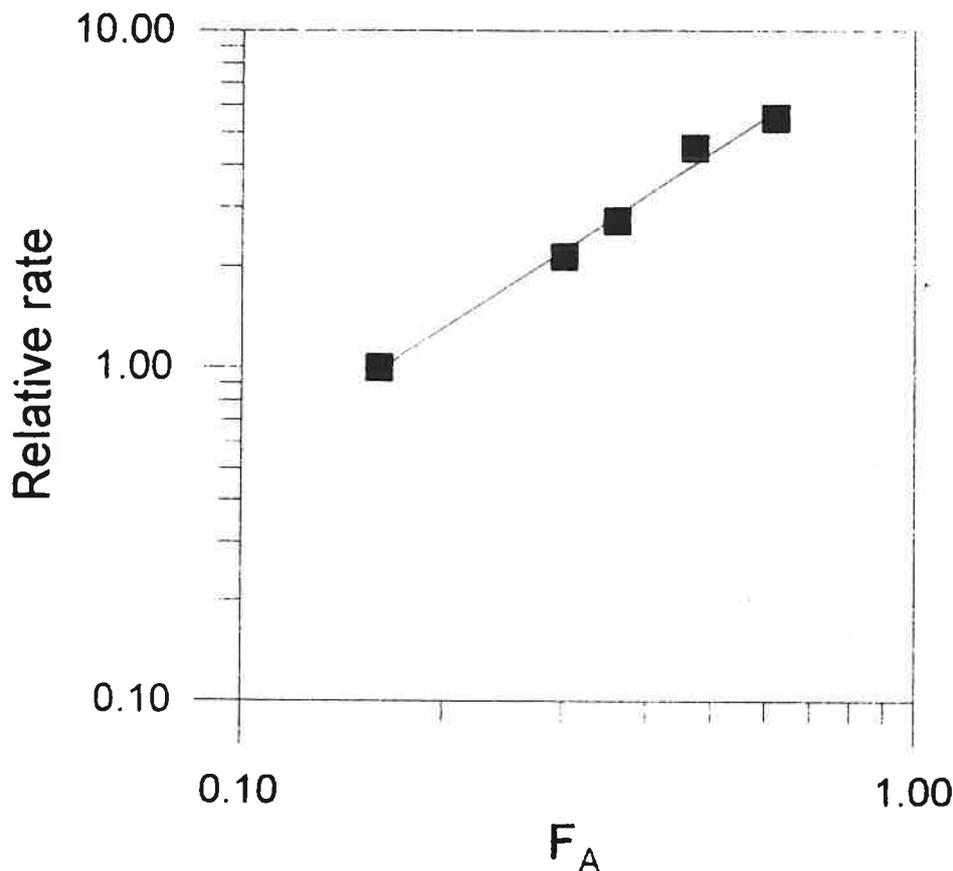
Figure 4. Double logarithmic plot of initial acid degradation rates (relative to a rate of 1 of the chitosan with F_A=0.16) versus F_A of the chitosans. Rates were determined in the previously described viscosity assay [3] at a chitosan concentration of 1.5 mg/ml in 0.4M HCl at 60° C.

Chitosans may be used in a wide variety of applications, and the molecular size is one of the fundamental parameters characterizing the macromolecule, and both controlled methods for chitosan depolymerization as well as knowledge on how to prevent chitosan depolymerization may prove to be essential in successfull applications of the polysaccharide. Thus, knowledge on the degradation mechanisms and possible specificities in the degradation reactions will be of interest to the scientific community, the chitosan industry and their customers.

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Structural Basis of Chitin Hydrolysis in Bacteria

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Introduction

Chitin, the homopolymer of *N*-acetyl-D-glucosamine is not only the major constituent of the fungal cell wall and the arthropod exosceleton but also an important nutrient source of carbon and nitrogen for bacteria. Based on the currently available biochemical, genetical and structural information we have started to understand the interaction of bacteria chitin degrading enzymes with their insoluble substrate as well as the mode of their enzymatic action. Recent protein structure data have shed light on the enzymatic mechanism of the action of bacterial chitin hydrolyzing enzymes Chitinase A, Chitobiase and Chitosanase at the molecular level. The currently available information is going to be summarized in this paper

Chitin degradation in bacteria

The last five years the number of chitin hydrolyzing - modifying proteins - genes has been dramatically increased. However, the number of published bacteria chitin hydrolyzing enzymes are by far lower compared to plant and fungal chitin hydrolyzing/modifying enzymes.

Chitin degradation by bacteria has two alternative routes as shown in the scheme adopted from Muzzarelli's article (1993). From the enzymes presented in the scheme of Fig. 1, several enzymes have been isolated and for three of them we have extensive biochemical, genetical and structural analysis i.e Chitinase A, Chitobiase and Chitosanase. Although chitin deacetylase has been isolated and characterized from various organisms and the

modification pattern of chitin to chitosan is also well studied, there is not information about the structure of this protein and its enzymatic mechanism.

CHITIN

Chitinase   Chitin deacetylase

$(GlcNAc)_{2-6}$ Chitosan

N-acetyl-glucosaminidase   Chitosanase

GlcNAc $(GlcNAc)_2$

Deacetylase   Glucosaminidase

GlcN
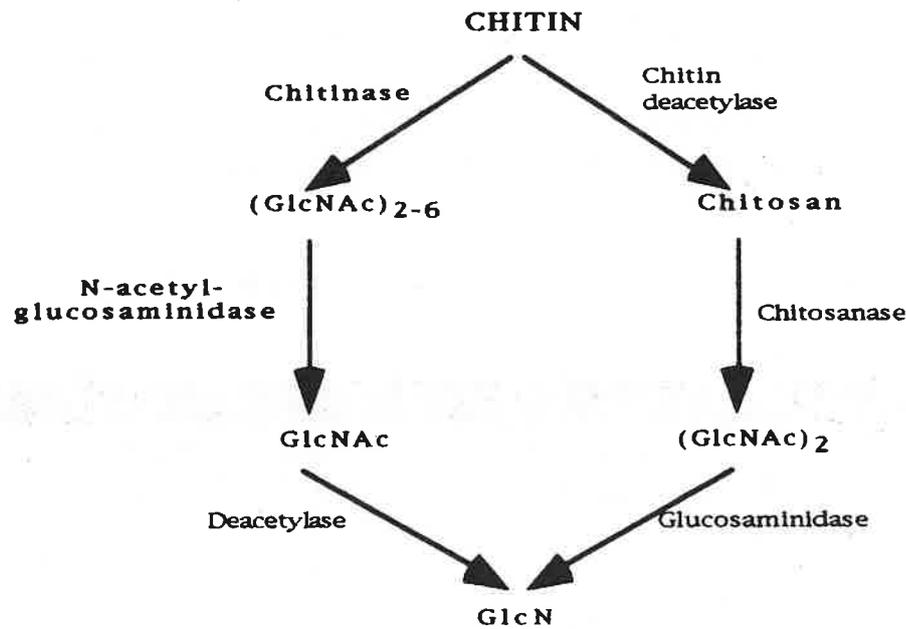
Figure 1

Alternative routes in the enzymatic degradation of chitin.

Primary structure of bacteria chitin degrading

Computer search in the current available data bases, results in a substantial number of new chitin hydrolyzing/modifying genes.

This paper focused on the bacteria genes and presents them in the following tables.

Table I presents the bacteria chitobiases, while table II summarizes bacteria chitosanases and table III bacteria chitinases

Organism	AC	Reference
Vibrio Harveyi	P13670	Soto-Gil, R.W. and Zyskind, J.W., PNAS 90, 6751 (1993)
Alteromonas sp.	I39596	Tsujibo, H., Fujimoto K., Tanno, H., Miyamoto K., Imada, C., Okami, Y., Inamori, Y. Gene 146, 111-115, (1994)
Vibrio parahaemolyticus	PH0889	Zhu B.C.R., Lo J.Y., Li Y.T., Li S.C., Jaynes J.M., Gildemeister O.S., Laine R.A., Ou, C.Y. J. Biochem. 112, 163-167 (1992)
Serratia marcescens	L43594	Tews, I., Vincentelli, R., Vorgias C.E. Gene 170, 63-67 (1996)

Table I
Compilation of known bacteria chitobiases

Organism	AC	Reference
Bacillus Circulans.	P33673	Ando A., Noguchi K., Yanagi M., Shinoyama H., Kagawa Y., Hirata H., Yabuki M., Fujii T. J. Gen. Appl. Microbiol. 38, 135-144 (1992)
Nocardioides sp. (Strain N106).	P48846	Masson J.Y., Boucher I., Neugebauer W.A., Ramotkar D.,Brzezinski R. (unpublished)
Streptomyces sp. (Strain N174).	P33665	Boucher I., Dupuy A., Vidal P., Neugebauer W.A., Brzezinski R.; Appl. Microbiol. Biotechnol. 38, 188-193 (1992)
Bacillus subtilis	BSU93875	Sorokin A., Bolotin A., Purnelle B., Hilbert H., Lauber J., Duesterhoeft A., Ehrlich S.D. (unpublished)

Table II
Compilation of known bacteria chitosanases

Organism	AC	Reference
Aeromonas caviae	U09139	J. Bacteriol. 177:4187-4189(1995).
Amycolatopsis methanolica	U31277	J. Bacteriol. 178:149-155(1996).
Alteromonas sp.	D13762	J. Bacteriol. 175:176-181(1993).
Aeromonas sp.	D63139-42	Biochim. Biophys. Acta 1305:44-48(1996).
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Ewingella americana	X90562;	Unpublished
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Streptomyces thermoviolaceus	D14536	Gene 134:113-117(1993).
Vibrio harveyi	U81496	Unpublished

Table III
Compilation of known bacteria chitinases

178

3D-structure of bacteria chitin degrading enzymes

1. Chitinases A from *Serratia marcescens*

The X-ray structure of Chitinase A from the chitinolytic bacterium *Serratia marcescens* has been solved by multiple isomorphous replacement (MIR) and refined at 2.3 Å resolution resulting in a crystallographic R-factor of 16.2% (1,2). The structure of Chitinase A consists of three domains (Fig. 2). The amino-terminal domain (aa 24-137) which consists only of β-strands, connects through a hinge region (residues 138-158) to the main $(\alpha\beta)_8$-barrel domain (residues 159-442) and (517-563). The third domain has an α+β fold and is formed by an insertion in the barrel motif (residues 443-516).
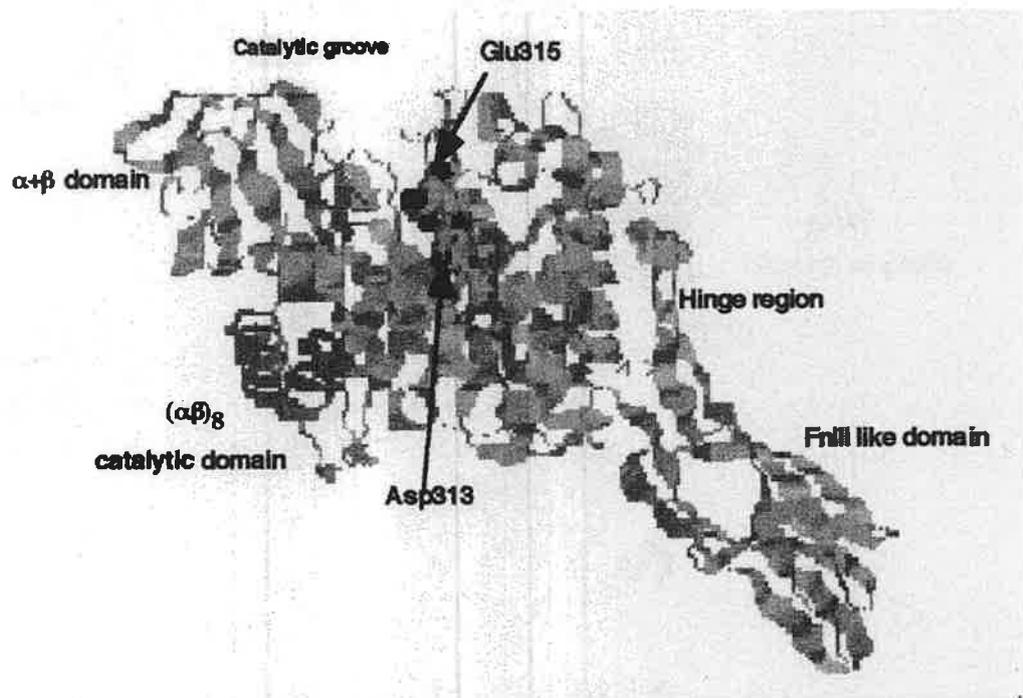
Figure 2

Ribbon diagram illustrating the structure of Chitinase A, its three domains and the groove of the active site where the catalytic amino acid Glu315 is located.

2. Chitobiase from *Serratia marcescens*

The 3D structure of chitobiase was solved by MIR and refined to a resolution of 1.9 Å. Chitobiase has an eight stranded $(\alpha\beta)_8$-barrel structure (domain III, see fig. 3) with three additional domains. The N-terminal domain I, 154 aa, comprises two β-sheets. Domain II, 122 aa, has $\alpha+\beta$ topology. Domain III, 483 aa, an $(\alpha\beta)_8$-barrel motif, is the core of the structure, around which the other domains are organised. The active site is on the C-terminal end of the barrel from where long loops interacting with domain I. The eight β-strands are surrounded by seven α-helices (3, 4).
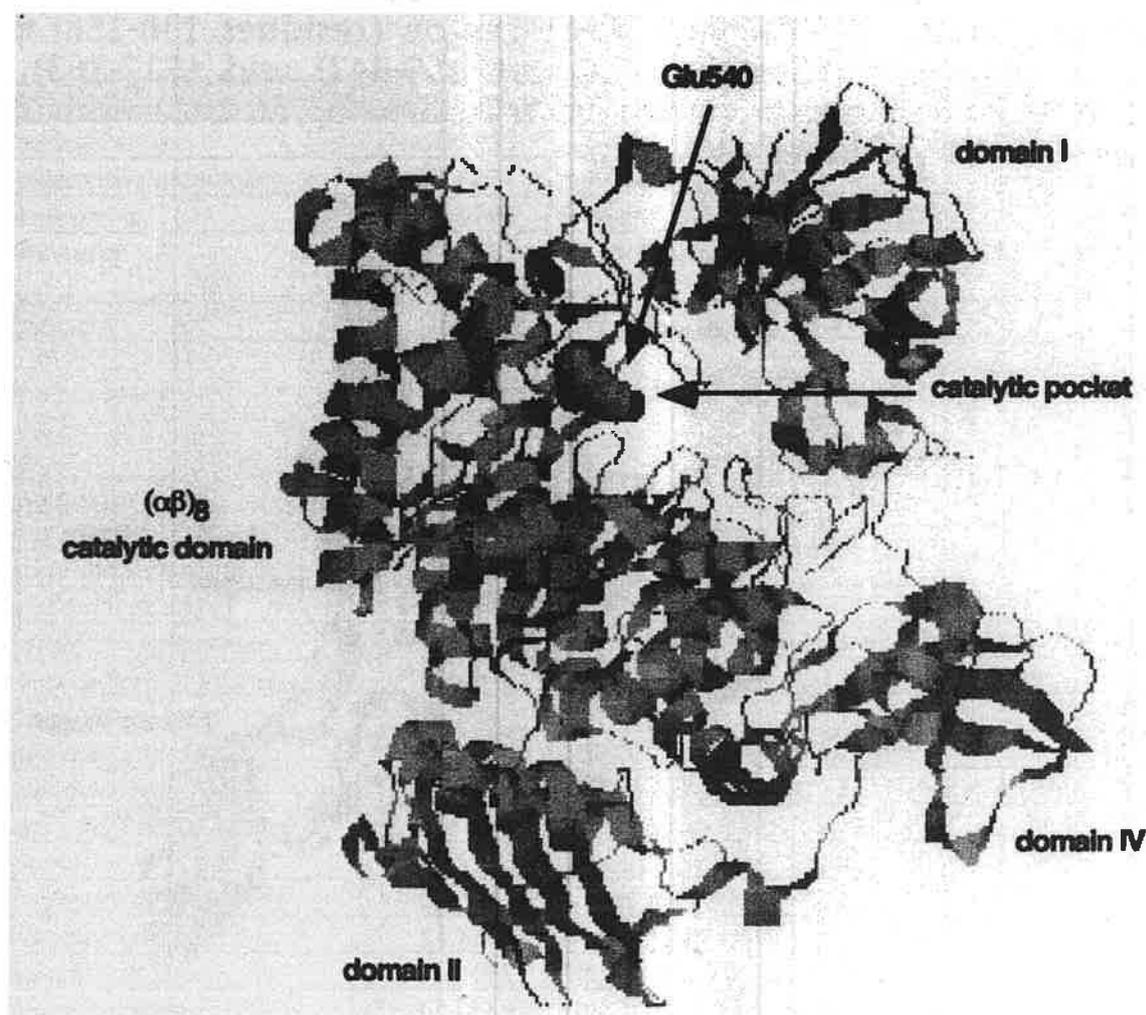
Figure 3.

Schematic diagram illustrating the structure of chitobiase and its domains. The amico acid residue responsible for catalysis Glu 540, is in space filling.

Several deviations from the classical $(\alpha\beta)_8$-barrel motif have been identified. Domain IV, as domain I, comprises two β-sheets, but

has only 67 aa. Overall dimensions of the protein are roughly 90 x 80 x 60 Å.

3. Chitosanase from *Streptomyces* N174

The 2.4 A X-ray crystal structure of chitosanase, a protein with chitosan endo-hydrolase activity isolated from *Streptomyces* N174, was previously published (5). The structure was solved using phases acquired by SIRAS from a two-site methyl mercury derivative combined with solvent flattening and non-crystallographic two-fold symmetry averaging, and refined to an R-factor of 18.5%. The mostly α-helical fold reveals a structural core (fig. 4) shared with several classes of lysozyme and barley endochitinase, in spite of a lack of shared sequence.
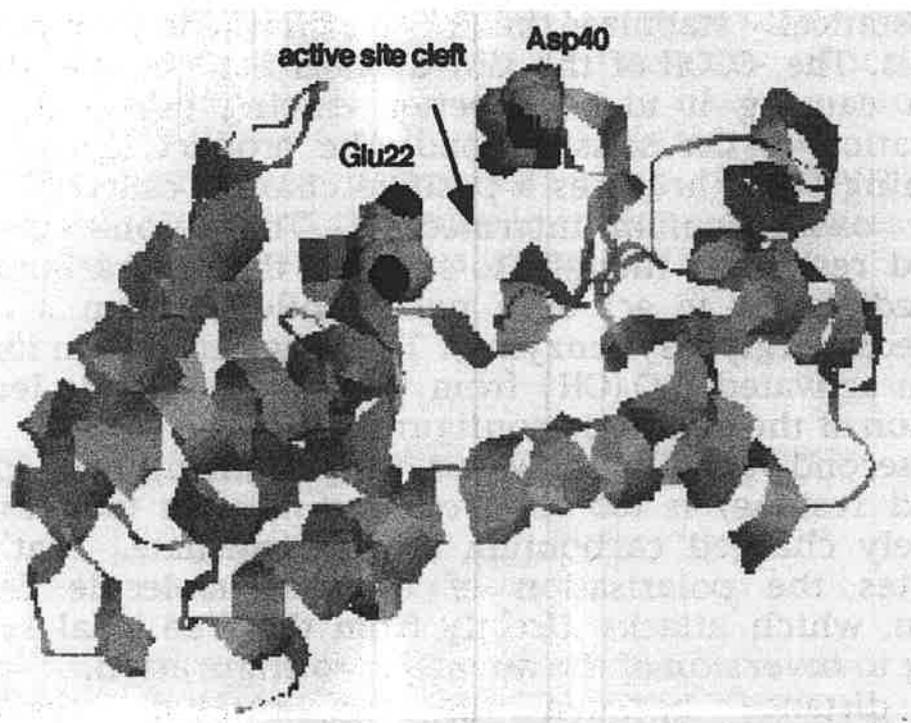
Figure 4.

Schematic diagram illustrating the structure of chitosanase. The amico acid residues responsible for catalysis, Glu22 and Asp40 are in space filling and located in both site of the active site cleft.

Based on the structural similarity with lysozyme and barley endochitinase, the authors postulate a putative active site, an acid/base mechanism of catalysis as well as the mode of substrate recognition. It appears that Glu22 acts as an acid and Asp40 serves as a general base to activate a water molecule for an SN_2 attack on the glycosidic bond. A series of amino-acid side chains and backbone carbonyl groups may bind the polycationic chitosan substrate in a deep electronegative binding cleft.

Current knowledge on the catalytic mechanism and stereochemistry of chitin hydrolysis

Enzymatic hydrolysis of the glycosidic bond takes place *via* general acid-base catalysis that requires two critical residues: a proton donor and a nucleophile base. This hydrolysis can give rise to either an overall retention or an inversion of anomeric configuration.

There are two variations of the classical acid-base catalysis mechanism which is common for glycosyl hydrolases.

1. The first variation involves a protonated acidic residue as proton (H^+) donor which is the -COOH group of a Glu residue and a second negatively charged amino acid residue which electrostatically stabilizes the positive charge at C_1 fromed while catalysis. The -COOH of the Glu donates a H^+ to the glycosidic oxygen causing it to be a better leaving group due to the polarisation of the scissile bond. The product leaves and the remaining sugar acquires a positive charge, called *carbonium ion* or *oxocarbonium* intermediate. The second negatively charged residue is thought to stabilise this oxocarbonium ion intermediate, or to act as a nucleophile to form a covalent intermediate (glycosyl-enzyme). Then the carbonium ion reacts with an activated H_2O (OH^-) from the equatorial side leading to *retention* of the anomeric configuration of the C1.

2. In the second variation, however, the stabilising base (negative charged residue) is too far from the C_1 atom to stabilize the positively charged carbonium ion intermediate. That residue facilitates the polarisation of a water molecule from the solution, which attacks directly from the free axial side, thus leading to *inversion* of the anomeric configuration.

The distance between the two catalytic residues is characteristic of the mode and stereochemistry of the reaction mechanism: 4.8 - 5.3 Å is typical for hydrolysis with retention of anomeric configuration (via a double displacement mechanism), and roughly 9 - 9.6 Å for inversion (single displacement), where an additional water is positioned between the anomeric carbon and the second protein carboxylate.

Biochemical experiments have clearly shown that Chitinase A and Chitobiase and several other bacterial chitin hydrolyzing enzymes are retaining enzymes (6,7) A structurally conserved Glu has been assigned to be the proton donor in acid-catalysis (i.e Glu315 in the Chitinase A, and Glu540 in Chitobiase). In the strucutre of Chitinase A and Chitobiase, no negatively charged residue can be unambiguously assigned to stabilise the carbonium ion intermediate. Therefore, the positive charge at C_1 is proposed to

be stabilised at its axial side through the O7 carbonyl oxygen of the N-acetyl group, which has a partial negative charge. In this way a water molecule can attack C1 from the equatorial side, resulting in retention of the anomeric configuration. This kind of stabilisation is in contrast to many other retaining glycosyl hydrolases, however it has been prosoped few decades ago by Koshland (7, 8, 9) and called *substrate assisted catalysis or anchimeric assistance of the N-acetyl group*. The current crystallographic and biochemical data provide strong evidence for this mechanism (10, 11, 12).

The role of FnIII domain on the interaction of chitinase with chitin

The 3D structure of Chitinase A has revealed that the N-terminus of the protein forms a domain which has an immunoglobulin-like fold. This domain is likely to facilitate the interaction of Chitinase A with its insoluble substrate.

Sequence searches performed using the BLASTP suite of programs has intentified this domain in three chitinases and three viral putative chitinase genes, as shown in fig. 5.

```
          ..26.29..33...38.......   50................66.69 .........
          ..*..*...*....*............*...................*..*
Chia_Serma AAPGKPTIAWGNTKFAIVEVDQAAT AYNNLVKVKN AADVSVSWNLWNGDAGTGPKIL   57
Chit_Aerca ---A----GS-P--------N---S ---Q--T-HKDC-P---T----S--V-QTA-V-   58
Chit_Altso ---ST--LD-QPQQYSF---NVDCLGS-KQ--KAKD VV-ISIK--A-S-SG-DNY-VY   58
Chit_Npvac -L--T-V-D-ADRNY-L--INYE-- --E--IKPKE QV--Q----V-N--I-DIAYV-   57
Chit_Npvcf -L--T-V-D-ADRNY-L-KINSD-- --E--IQRND HVS-Q----V-N..........   47
Chit_Npvbm -I--T-V-D-AERNY-L-KINYE-- --E--IKLKE QV-..................   38
                  + + +  *   *  +      *  +           *  *

Chia_Serma LNGKEAWSG  PSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDGSH  114
Chit_Aerca -DV--V---  -AS AA-------T-----------------L--KK-LM--------  114
Chit_Altso FDDLLVNQ-SL-AGTK--VVQ-PYT-S--H-LYLE--EGTV-AR-AGK---I-----A-  117
Chit_Npvac FDEQQV-K-  DAE  -KR-TI--LVS-QFN-R-K--NE-G-SV--PVLV-------G-  112
Chit_Npvcf ..........................................................
Chit_Npvbm ..........................................................
```

Figure 5.

Alignment of the sequences with the FnIII motif. Chia_Serma is for *Serratia marcescens* chitinase; Chit_Aerca is for *Aeromonas caviae*; Chit_Altso for *Alteromonas* sp. Chit_Npvac, Chit_Npvcf and Chit_Npvbm are for the putative chitinases from *Autographica californica*, *Bombyx mori* and *Choristoncura fumiferana* nuclear polyhedrosis viruses, respectively.

In two bacteria *Streptomyces* and one *Bacillus* sp. the same domain has also been identified and is located either either at the

end, the middle or the start of the molecule. Table IV summarizes the occurence of the FnIII domain in various bacteria chitinases.

Current available information does not support the direct involvement of the FnIII domain to the binding of chitinases to chitin (13,14). However, it is beleived that this domain is participating in the efficient degradation of chitin polymers by various bacteria chitinases.

Organism	Gene	Position
Serratia marcescens	CHIA_SERMA	Start of the molecule
Alteromonas sp.	CHIT_ALTO	Start of the molecule
Aeromonas caviae	U09139	Start of the molecule
Bacillus circulans	CHI1_BACCI	End of the molecule
Bacillus circulans	CHID_BACCI	Start of the molecule
Streptomyces lividans	CHIT_STRLI	Middle of the molecule
Streptomyces plicatus	CHIT_STRPL	Start of the molecule
Streptomyces olivaceoviridis	CHIX_STROI	Start of the molecule
Autographa caliphornica Nuclear Polyhedrosis Virus	CHIT_NPVAC	Start of the molecule
Bombyx mori Nuclear Polyhedrosis Virus	U12688	Start of the molecule
Choristoneura fumiferana Nuclear Polyhedrosis Virus	M97906	Start of the molecule

Table IV.

Occurence and location of FnIII-like domain in bacteria chitinases and viral putative chitinases.

PDB	Protein
1ctn	Chitinase a
1fnf	fibronectin
1bec	14.3.D t cell antigen receptor M
2hft	human tissue factor
8fab-B	Fab fragment from human immunoglobulin
1clc	endoglucanase

Table V.

Fold similarity of the FnIII-like domain from Chitinase A to various other domains or structures in the PDB.
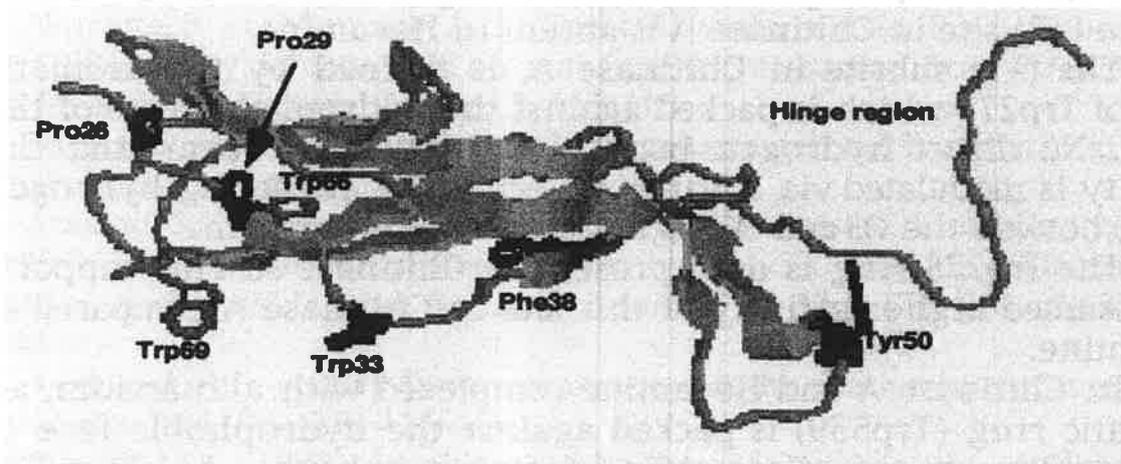
Figure 6.

Schematic diagram illustrating the structure of FnIII domain of *Serratia marcescens* Chitinase A. Higly conserved amino acid residues indicated on Fig. 5 are presented on the 3D-model of this domain, (aa numbering is according the 1ctn PDB file).

Structural data on carbohydrate binding:
How would chitin substrate binds on the enzyme?

The modes of binding of a carbohydrate substrate on its hydrolase is difficult to be established directly by X-ray crystallography since the substrate is rapidly degraded. The first insight was provided by the X-ray structure of a(NAG)₃ (a strong competitive inhibitor of lysozyme) with lysozyme. The results of this study were used as the initial model for protein-carbohydarte interactions. The first protein-carbohydrate review appeared in the middle of '80s. Since then, the number of well refined proteins with bound carbohydrates is constantly increasing.

From the elucidation of the structure of Hevamine and Chitinase A with (NAG)₃ and (NAG)₂ oligomers, respectively and allosamidine, several subsites have been defined for the binding of the sugar substrate on the cleft (groove) around the catalytic site.

The (+2) subsite in Chitinase A is mainly defined by the stacking of the aromatic residue Phe396 against the hydrophobic face of the sugar. Two direct hydrogen bonds are present: (i) between the carboxylate oxygen of Asp391 and the N2 of the N-acetyl group of the sugar and (ii) between the Nz atom of Lys320

and O8 atom of the sugar. The Phe396 ring which is characteristic for the (+2) site in Chitinase A is absent in Hevamine.

The (+1) subsite in Chitinase A, is defined by the aromatic ring of Trp275 which is packed against the hydrophobic face of the sugar. No direct hydrogen bonds exist with this sugar and the affinity is modulated via 3 water molecules by mediating hydrogen bonds betwen the O3 and O6 sugar atoms and the protein.

The Trp275 ring is only present in Chitinase A. This supports the assumed higher affinity of this site in Chitinase A compared to Hevamine.

In Chitinase A and Hevamine complexed with allosamidin, an aromatic ring (Trp539) is packed against the hydrophobic face of the double ring of allosamidin, defining subsite (-1). This Trp residue is present in all three enzymes. The interactions of the oxazoline group in the Chitinase A-allosamidin complex are similar to the ones described for the complex with Hevamine.

The second sugar in subsite (-2) forms hydrogen bonds to the carboxyl oxygens of Glu540 and Glu473 with the O7 and N2 atoms of the N-acetyl group respectively. The O6 atom of the sugar hydrogen bonds with the main chain nitrogen of Thr276.

The (-3) subsite is defined in Chitinase A with Trp167, which also packs almost perfectly against the sugar ring as modelled in Hevamine, where that Trp is absent. The two direct hydrogen bonds in Hevamine are disrupted and presumably substituted by the hydrophobic interaction.

The sugar in the (-4) subsite of Chitinase A binds in a different manner than in Hevamine. While getting further from the active site it is expected that the mode of binding is not well conserved and this sugar ring could adopt a different position.

Further experiments are required to fully understand this complex protein-sugar interaction.

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ENZYMATIC DEACETYLATION OF CHITIN

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Abstract

Chitin deacetylases are involved, either in the formation of the cell wall (2, 6) in combination with chitin synthases as in *Mucor rouxii,* or in the deacetylation of chitin oligosaccharides following the action of an endochitinase on cell walls during autolysis (1). In the plant pathogen *Colletotrichum lindemuthianum* the involvement of this enzyme in plant-pathogen interactions has also been proposed (12). The isolation of the first CDA encoding gene from the fungus *M. rouxii* revealed a remarkable sequence similarity to the rhizobial nodB proteins which suggested functional homology of these evolutionary distant proteins (9). It also allowed the identification and molecular characterization of two sporulation specific genes, *CDA1* and *CDA2*, the products of which account for the total chitin deacetylase activity in *S.cerevisiae* (4). Mutational analysis of these two genes revealed their contribution to spore wall resistance. Genetic analysis showed that the action of the corresponding proteins is redundant. In order to investigate differences between the two enzymes we focused on their purification to homogenity and we established a sensitive method which will help us to characterize not only the reaction products of these two but also those of the other available chitin deacetylases.

Materials and methods

Purification and characterization of *M. rouxii* and *C.lindemuthianum* CDAs. As described in references 7, 11 and 12.

Cloning of *M.rouxii* CDA gene. As described in reference 9.

Cloning of *S.cerevisiae* CDA2 gene. As described in reference 4.

Strains and growth media . The strain used for the purification of CDA2 was the diploid CEN PK 75B (MATa/ MATα, leu2/leu2, cda1/cda1) derivative of CEN PK 7B (MATa, leu2, his3, cda1) and CEN PK 5B (MATa, leu2, trp1, cda1). For synchronous sporulation cells were grown in sporulation medium (YEPA) to a density of 5x 10^7cells/ml, washed and resuspended in 1% potassium acetate at half density. The efficiency of sporulation was 80% after 36 hours.

Gene disruptions in the yeast genome. The plasmid constuction used to disrupt the CDA2 gene from the genome of CEN PK 7B and CEN PK 5B stains is described in reference 4

Growth of S. cerevisiae. Yeast cells grown in sporulation medium at a density of 5x 10^7cells/ml, were washed with water and resuspended in 1%potassium acetate for 24

hours. Sporulating cells were harvested by centrifugation at 5000xg for 10min. ~3 gr of sporulating cells (wet weight) per liter was obtained.

Enzyme purification. Frozen sporulating cells (~ 40gr) and 40gr of glass beads were added to 35ml of 50mM Tris-Cl , pH 8.0/ 0.2mM (buffer A) phenylmethylsulfonyl fluoride and homogenized by vortexing. After the extraction was completed, glassbeads were settled and removed, and the extract was centifuged for 1h30´ at 20000xg at 4°C. The supernatant was applied onto a Q Sepharose fast flow column previously equilibrated with 50mM Tris-Cl , pH 8.0 (buffer B). The column was washed with buffer B and subsequently developed with a linear gradient of NaCl (200 ml; 0-0.75M) in buffer B at a flow rate of 60ml/hr. Fractions (5ml) containing enzyme activity were pooled and dialysed against 25mM sodium formate buffer.(pH 4.0;buffer C). The column was washed with buffer C and subsequently developed with a linear gradient of NaCl (100ml; 0-1M) in buffer C at a flow rate of 50ml/hr. Fractions containing enzymatic activity were pooled and dialysed against 50mM Tris-Cl/ 0.3M NaCl and applied to a S200 Sephacryl gel filtration column previously equilibrated with the same buffer. Fractions (2.5ml) containing enzyme activity were pooled and dialysed against 25mM sodium formate buffer.(pH 4.0;buffer C). Pooled fractions were applied onto MonoS FPLC column equilibrated with buffer C. The column was washed with buffer C and subsequently developed with a linear gradient of NaCl (30ml; 0-1M) in buffer C at a flow rate of 1ml/min. Fractions containing enzymatic activity were pooled and stored at -20°C

Enzyme activity assays. As described in reference 8

Polyacrylamide Gel Electrophoresis. As described by Laemmli (10)

Protein determination. Protein content was determined by the method of Brandford (3) with bovine serum albumin as a standard.

Precolumn labeling of reducing carbohydrades. According to reference 7

HPLC analysis. Labeled chitin and chitosan oligomers were analysed in an CHO-C18 column (Perkin-Elmer) with a flow rate of 0.4ml/min under a 4500psi pressure. The mobile phase consisted of a mixture of acetonitile and acetate buffer pH 5.5 (19,81). Labeled oligosaccharides were detected at 245nm.

Results and discussion

Differentiation of yeast chitin deacetylases. Comparison of the deduced amino acid sequence of *M. rouxii* chitin deacetylase (MrCDA) (9) with the EMBL database revealed the existence of two similar and closely linked ORF's (EMBL accession number u17247) on chromosome XII of S.cerevisiae. The two genes were isolated from a yeast genomic plasmid library and were further studied (4). The two genes were transcribed at the same time during sporulation and gave transcripts of the same size at comparable amounts. CEN PK 75B ΔCDA1 and ΔCDA2 mutant strains exhibit approximately equal activities, with CEN PK 75BΔCDA2 exhibiting a slightly higher activity in that background strain. Finally, the phenotypic behaviour of the two strains is comparable as shown by their fluorescent levels. All these indices suggest that the function of the two, coded by these genes, enzymes is redundant.

However the conservation of two functional enzymes of chitin deacetylase in

S.cerevisiae throught the evolution suggest that a fine difference might happen in the action and/or the localization of the two enzymes. In order to detect such differences we traced the CDA activity in the mutant strains at the same time intervals. The two enzymes are maximumally expressed at different times with CDA1 reaching its peack activity 4 hours earlier than CDA2.

Since the two enzymes are similarly transcribed, their different expression probably reflects differences at the translational or postranslational level. Putative secretion signals are found at both predicted aminoacid sequences of the corresponding genes. However the signal of CDA1 is probably uncleavable while that of CDA2 is probably cleavable. This prediction could reflect a postranslational modification that affects the localization of the two enzymes.

In order to investigate whether is a differential localization of the two enzymes and its correlation whith fine differences of substrate specificities we decided to purify the CDA enzymes, starting from CDA2.

CDA2 partial purification. Sporulating cells of δCDA2 strain were isolated as described in materials and methods after 24 hours in sporulation medium at the time of maximum CDA2 expression. The partial purification of CDA2 was performed as described in Materials and Methods. As tested by SDS-PAGE three bands are detected of 45, 35 and 30 kda. The molecular weight of the 35kda band coincides with the predicted molecular weight of CDA2. Interestingly this preparation is not stained with periodate/Schiff reagent and does not react with an antibody against native glycosylated *M.rouxii* CDA, indicating absent or limited glycosylation of the protein.

Further purification and characterization of the protein is in progress and will give us more information about the biochemical nature of this protein.

Characterization of the oligomers produced by the action of chitin deacetylases . In previous studies we obtained some preliminary results for the action of chitin deacetylase on oligomeric substrates by quantifying the released acetic acid. However this method has two disadvantages, It has low sensitivity and gives no information about the released product. In order to overcome this problems we developed a more sensitive method taking advantage of the action of two enzymes; exo-b-D-glucosaminidase and exo-b-D-N-acetyl-glucosaminidase which specifically hydrolyse glucosamine and N-acetyl-D-glucosamine respectively from the non- reducing end of chitin oligosaccharides. Oligomers are incubated for different time periods with chitin deacetylase.The reaction mixture is then treated separately with each of the above enzymes and labeled with 1-phenyl-3-methylpyrazolone-5(PMP). These products can be easily analysed with HPLC and detected at 245nm. An example of the analysis of standard oligomers of chitin and chitosan labeled with PMP is shown in the figure 1.
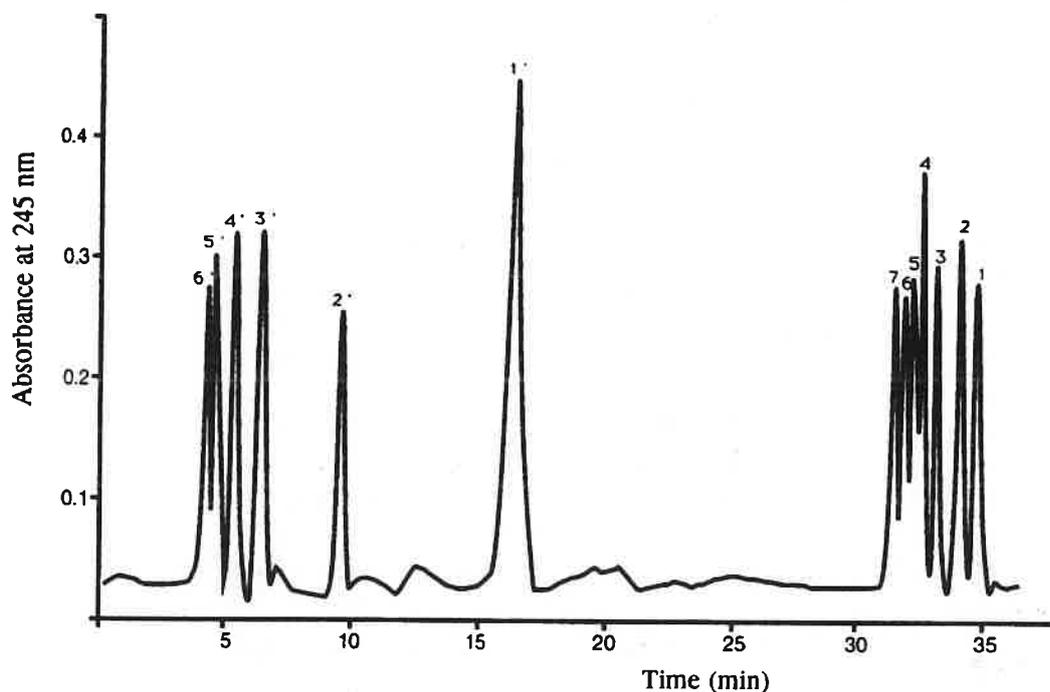
Figure 1. HPLC analysis of PMP labeled chitin and chitosan oligomers in a CHO-C18 column. A mixture of oligomers (1μg each) was labeled with PMP and analysed in a CHO-C18 column. Acetonitrile and buffer of ammonium acetate, pH5.5 (19,81) was used as mobile phase. The mixture contained monomer(1), dimer(2), trimer(3), tetramer(4), pentamer(5), examer(6), and eptamer(7) of N-acetylglucosamine and monomer(1'), dimer(2'), trimer(3'), tetramer(4'), pentamer(5'), and examer(6') of glucosamine.

An example of the method in the case of chitotriose is shown in figure 2.

After treatment with chitin deacetylase the substrates are separated in three parts, The first part is used as control, the second part is treated with exo-b-D-glucosaminidase (Ex) and the last one with exo-b-D-glucosaminidase (E). The reaction products are labeled with PMP and analysed with HPLC. The comparison of the three chromatographs can reveal the structure of the reaction products. We assume in the case of a trimeric substrate that three products are obtained which correspond to three chromatographic peaks. After treatment with exo-b-N-acetyl-D-glucosaminidase the peak corresponding to the trimeric product will be analysed into two new peaks of monomeric and dimeric N-acetyl-D-glucosamine, while the other two initial peaks will remain intact.

On the contrary after treatment with exo-b-D-glucosaminidase the peak of the trimeric substrate will remain intact, while the other two initial peaks will be analysed into new peaks corresponding to free glucosamine, N-acetylglucosamine and chitobiose.

This method has already been applied with success in the analysis of the *Mr*CDA reaction products
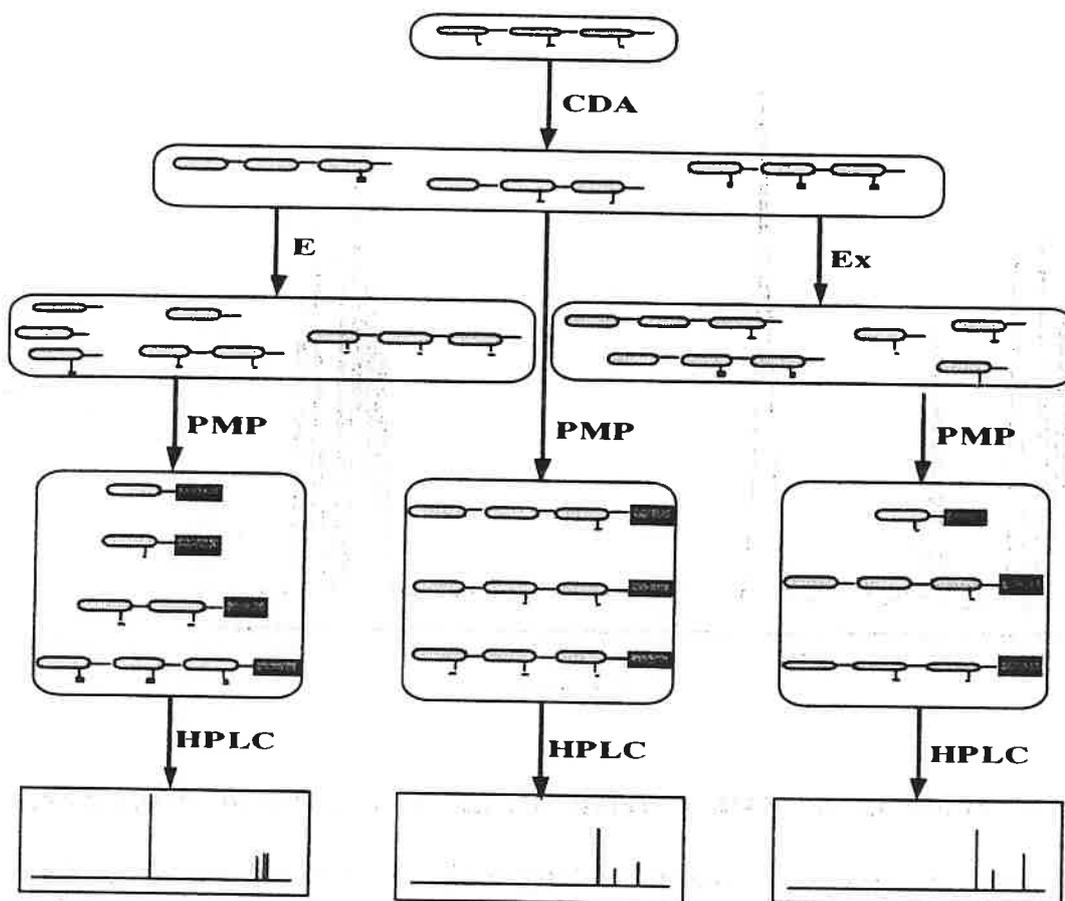
Figure 2. Analysis strategy for the educidation of the structure of deacetylation products produced by the action of chitin deacetylase(CDA) in a trimeric substrate using the system of two exo-glucosidases. E, exo-glucosaminidase, Ex, exo-N-acetylglucosaminidase PMP, 1-fhenylo-3-methylpyrazolone-5

Conclusion

Recent information about chitin deacetylases from different species revealed that although these enzymes have the same catalytic activity and stringent specificity for chitinous substrates, they are involved in many different biological processes depending on the individual organism. Thus in fungi *M.rouxii* and *A.coerula*, CDAs are constitutively expressed enzymes (6, 8), localised in the periplasmic space (2) in order to perform their task in cell wall construction, whereas in the plant pathogen *C. lindemuthianum* and in *A.nidulans* CDAs are secreted enzymes postulated to act on chitin oligomers released by fungal cell walls, in order to promote plant invasion (12) or cell wall degradation(1). In procaryotes, the NodB family of genes encode CDA that participate in the biosynthesis of the Nod factors that promote plant nodulation (9), while putative CDA homologs are found in non-endosymbiotic bacteria (9).

The chitin deacetylase function has been shown to be indispensable for the formation of rigid spore walls(4). Two genes are responsible for this function. In order to investigate possible biochemical and functional differences of the corresponding enzymes we are about to purify one of them, CDA2, and study its substrate specificity. For such a purpose we have developed an accurate method for the determination of chitin deacetylases reaction products.

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MODE OF ACTION OF CHITIN DEACETYLASE FROM *MUCOR ROUXII* ON A FULLY WATER-SOLUBLE, HIGHLY ACETYLATED CHITOSAN.

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ABSTRACT

The mode of action of chitin deacetylase (CDA) from *Mucor rouxii* on fully water-soluble and highly *N*-acetylated chitosan as substrate was investigated. The relative rate of deacetylation of CDA increased linearly with increasing fraction of acetylated units (F_A) on water-soluble partially *N*-acetylated chitosans, indicating that CDA does not preferentially attack any sequence in the chitosan molecule. A highly *N*-acetylated chitosan with F_A of 0.681, with a Bernoullian distribution of acetylated (GlcNAc (A)) and deacetylated (GlcN (D)) units, and a number-average degree of polymerization (DP_n) of 30 was selected as substrate for CDA. This chitosan was enzymatically deacetylated to decreasing F_A-values (F_A of 0.582, 0.400 and 0.188), and the nearest neighbour frequencies were determined by NMR spectroscopy. The transition frequencies (F_{AD} and F_{DA}) determined, were lower than those expected to be derived from a random (Bernoullian) distribution in the further enzymatically deacetylated chitosans, while F_{AA} and F_{DD} were higher compared to a random distribution. The experimental results were compared with model data, assuming an endo-type mechanism with no preferential attack at any sequences in the chitosan chain. The comparison suggested that CDA hydrolysed acetyl-groups according to a multiple attack mechanism, with a degree of multiple attack of at least three. No deacetylation could be detected at the non-reducing end of the three enzymatically deacetylated chitosans.

KEYWORDS

Chitin, chitosan, chitin deacetylase, *Mucor rouxii*, mode of action.

MATERIALS AND METHODS

Chitin was isolated from shrimp shells [1]. The chitosan samples were prepared by homogeneous *N*-deacetylation of chitin [2], or by heterogeneous *N*-deacetylation and extraction of the acid-soluble fraction [3,4]. The characterisation of the chitosans used for the CDA kinetic experiments are given in Table 1. The fraction of *N*-acetylated units (F_A) and the diad frequencies were determined from high-field proton NMR spectroscopy [5] and the intrinsic viscosities ($[\eta]$) of the chitosans were determined as previously described [6]. The chitosan used as substrate in the NMR-experiments was depolymerized by nitrous acid and reduced with $NaBH_4$ as previously described [7,8], to obtain a lower molecular weight chitosan suitable for direct analysis by NMR spectroscopy and enzymatic

deacetylation studies. The fraction of N-acetylated units (F_A) was determined by ^{13}C-NMR spectroscopy [9] to be 0.681. The number-average degree of polymerization (DP_n) was determined from the intensity of the non-reducing end carbon 3 and 5 resonances compared to the internal carbon 3 and 5 resonances [7].

Chitin deacetylase from *Mucor rouxii* has been purified to homogeneity and further characterized employing both conventional and immunoaffinity chromatography [10,11]. The enzyme is an acidic glycoprotein with a molecular weight of ~ 75 KDa with 30% (w/w) carbohydrate content. Further biochemical characterization revealed that the enzyme has a very narrow specificity for chitinous substrates [12]. Homogeneous CDA preparations have also been isolated from *Colletotrichum lindemuthianum* [13], *Absidia coerulea* [14] and *Aspergillus nidulans* [15]. Furthermore, a cDNA to the *Mucor rouxii* mRNA-encoding chitin deacetylase was isolated, characterized and sequenced [16]. Protein sequence comparisons revealed significant similarities of the fungal chitin deacetylase to rhizobial nodB proteins, suggesting functional homology of these evolutionary-distant proteins. Recently, two genes encoding CDA have been cloned from *Saccharomyces cerevisiae* [17].

Enzymatic deacetylation reactions employing purified chitin deacetylase were performed in glutamate buffer pH 4.5 at 50 °C using 0.3% (w/v) of chitosan as substrate. Removal of acetate released, known to be a CDA inhibitor [12], was effected using dialysis membranes (molecular weight cut off of 10.000 Dalton) for the enzymatic reactions. The chitosan substrate was incubated with increasing amounts of purified chitin deacetylase to obtain chitosans with suitable F_A-values. Reactions were stopped by heat inactivation (100°C, 15 min), and the samples were extensively dialysed in deionized water before lyophilization.

Using high-field proton and carbon NMR-spectroscopy, we have recently reported methods [5,9] to determine the nearest-neighbour (diad) frequencies AA, AD, DA and DD of chitosans, a counting procedure which determines the average number of places along the chain where an A-unit is followed by an **A**, an **A** followed by a **D**, and so on. It was found that water-soluble chitosans prepared by both homogeneous and heterogeneous thermochemical deacetylation gave values for the diad and triad frequencies that were consistent with a random arrangement of **D** and A-units [5,9]. This identification of the different sequences in chitosans has recently been used to model the change in the diad and triad frequencies (F_{AA} and F_{AAA}) in lysozyme-depolymerized chitosan [7,18].

The ^{13}C-NMR experiments on the chitosan samples were performed as previously described [9]. As the chitosan substrate was of a low molecular weight (number-average degree of polymerization (DP_n) of 30), no depolymerization was necessary before obtaining the spectra.

The action pattern of CDA on the chitosan substrate was modelled assuming an endo-type mechanism with no preferential attack at alternating sequences, and using the degree of multiple attack as an adjustable parameter. A high degree of multiple attack, e.g., termination of the deacetylation of a sequence when either encountering a chain end or a **D**-unit followed by a new attack by the enzyme, corresponds to the single-chain mechanism [19,20]. Likewise, the limiting case of a multichain attack mechanism, e.g. deacetylation of one unit per attack followed by random attack elsewhere in the chitosan ensemble, is also embodied in the model when the degree of multiple attack is low [19,20]. The calculations were carried out using simulated ensembles of 500 chitosan chains with DP_n of 30, and A-units were introduced according to the experimentally determined Bernoullian distribution to a total F_A of 0.681. Changes in the sequence distributions of the chitosan during the simulated enzymatic deacetylation were determined.

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RESULTS AND DISCUSSION
Kinetics of CDA deacetylating chitosans

The enzymatic kinetics of deacetylation for a series of chitosans with increasing F_A from 0.08 to 0.62 (Table 1) was detected by measuring the time course of release of acetic acid at substrate saturation.

The Michaelis-Menten constant (K_M) for the substrate with $F_A=0.08$ was only 20% lower than K_M for the substrate with $F_A=0.62$. Thus, assuming that the rate of dissociation of the enzyme-substrate complex to enzyme and substrate is low compared to the rate of formation of enzyme and product, the approximately equal K_M-values suggest that the affinities of CDA to the two chitosans are comparable.

The relative rate of deacetylation of CDA as a function of F_A is given in Figure 1, showing a linear increase in rate with increasing F_A. As the distribution of **A** and **D**-units in water-soluble partially N-acetylated chitosan substrates is random, the results indicate that CDA does not preferentially attack any sequence in the chitosan molecule. Such preferential attacks have been shown for pectin esterase [21,22].

TABLE 1.

Characterization of chitosan fractions used in the enzyme kinetics study. The experimental diad frequencies of each chitosan are compared with the calculated diad frequencies of a chitosan with random (Bernoullian) distribution of **A** and **D**-units.

| Sample	F_A	F_{AA}	$F_{AD}=F_{DA}$	F_{DD}	$[\eta]$ (ml/g)
1	0.08	0.02	0.06	0.86	1580
random	0.08	0.01	0.07	0.85	
2	0.15	0.04	0.12	0.73	740
random	0.15	0.02	0.13	0.72	
3	0.35	0.11	0.22	0.44	760
random	0.35	0.12	0.23	0.42	
4	0.51	0.30	0.22	0.26	450
random	0.51	0.26	0.25	0.24	
5	0.62	0.41	0.21	0.17	820
random	0.62	0.38	0.24	0.14	
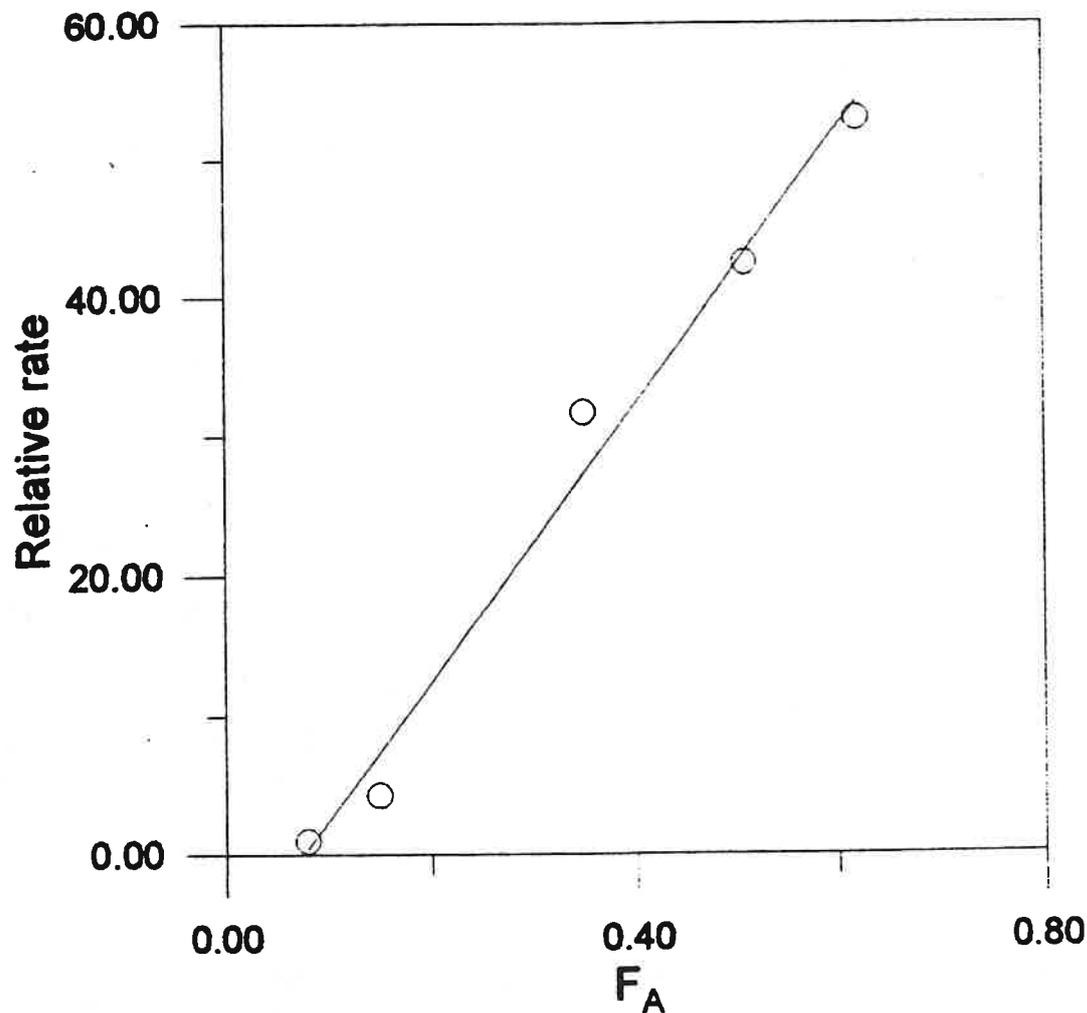
Figure 1.

Initial rate of deacetylation (relative to a rate of 1 of $F_A=0.08$) versus F_A for CDA.

Sequence analysis of the chitosan substrate and the further CDA-deacetylated chitosans

The chitosan selected as substrate for the chitin deacetylase was a highly N-acetylated chitosan ($F_A=0.681$) with a number-average degree of polymerization (DP_n) of 30. The sequential structure of this chitosan corresponded closely to a Bernoullian distribution of the A and D-units (Table 2). Figure 2 shows part of the 125 MHz proton decoupled ^{13}C-NMR-spectrum of the chitosan substrate and the same chitosan with increasing degrees of enzymatic deacetylation (F_A of 0.582, 0.400 and 0.188). Table 2 gives the chemical composition (F_A) and the nearest-neighbour (diad) frequencies for the substrate and the enzymatically N-deacetylated chitosans determined from high-field carbon n.m.r.-spectroscopy, together with the calculated diad frequencies for a Bernoullian distribution.

It is seen that the transition frequencies (F_{AD} and F_{DA}) are lower than expected from a random distribution in the further enzymatically deacetylated chitosans, while F_{AA} and F_{DD} are higher compared to a Bernoullian distribution. Such a change in the nearest-neighbour frequencies in the further enzymatically deacetylated chitosans are qualitatively in agreement with an enzyme operating according to a multiple-attack mechanism.

TABLE 2

Composition (F_A) and diad frequencies of substrate and products of the enzymatic deacetylation determined by ^{13}C-NMR spectroscopy.

	F_A	F_{AA}	F_{AD}	F_{DA}	F_{DD}
Substrate random	0.681±.027 0.681	0.462±.033 0.464	0.212±.010 0.217	0.230±.006 0.217	0.118±.025 0.102
1 random	0.582±.010 0.582	0.361±.012 0.339	0.211±.011 0.243	0.217±.010 0.243	0.208±.010 0.175
2 random	0.400±.017 0.400	0.193±.007 0.16	0.206±.016 0.240	0.207±.016 0.240	0.394±.032 0.360
3 random	0.188±.013 0.188	0.099±.004 0.035	0.085±.014 0.153	0.089±0.01 0.153	0.728±.020 0.659

Comparison of experimental sequence data with model data

Enzymatic action patterns for enzymes that modify in-chain units on a linear binary heteropolysaccharide may be divided into three main types designated *multiple-chain*, *multiple attack*, and *single-chain* mechanism. While the multiple-chain mechanism with no preferred attack will result in a binary heteropolysaccharide with a Bernoullian (random) distribution of the units, the multiple attack and the single-chain mechanism will generate block-copolymer structures. The latter types of enzymatic action patterns have been reported to occur by epimerisation of alginates [23] and de-esterification of pectins [21].

The experimental results were compared with model data, assuming an endo-type mechanism with no preferential attack at alternating sequences, in accordance with the data from the enzyme kinetics. Figure 3 shows the experimentally determined diad fractions versus degree of conversion determined as reduction in F_A for the chitosan substrate and the further enzymatically deacetylated samples. The calculated diad frequencies from the model when chitin deacetylase removes acetyl-groups on the chitosan substrates (F_A = 0.681, DP_n=30), assuming three different enzymatic action patterns (single-chain, multiple attack with degree of multiple attack equal to three, and multichain attack) are shown on the same graph. The comparison of the experimentally determined diad frequencies with the model data shows that the diad frequencies of the further enzymatically deacetylated samples deviate from a Bernoullian distribution, and hence, that the enzymatic mechanism can not be described in terms of multichain attack. The comparison of experimental diad frequencies with the model data suggests that the chitin deacetylase hydrolyses acetyl-groups according to a multiple attack mechanism, with a degree of multiple attack of at least three. It is difficult to quantitatively determine the degree of multiple attack because the block-length distribution in the chitosan substrate (the number-average block length (A-units) of the chitosan substrate is 3.2) represents an experimental constraint in the present sample with respect to the degree of multiple attack. This limitation can in principle be relaxed by using substrates with higher F_A-values, but the insolubility of more highly
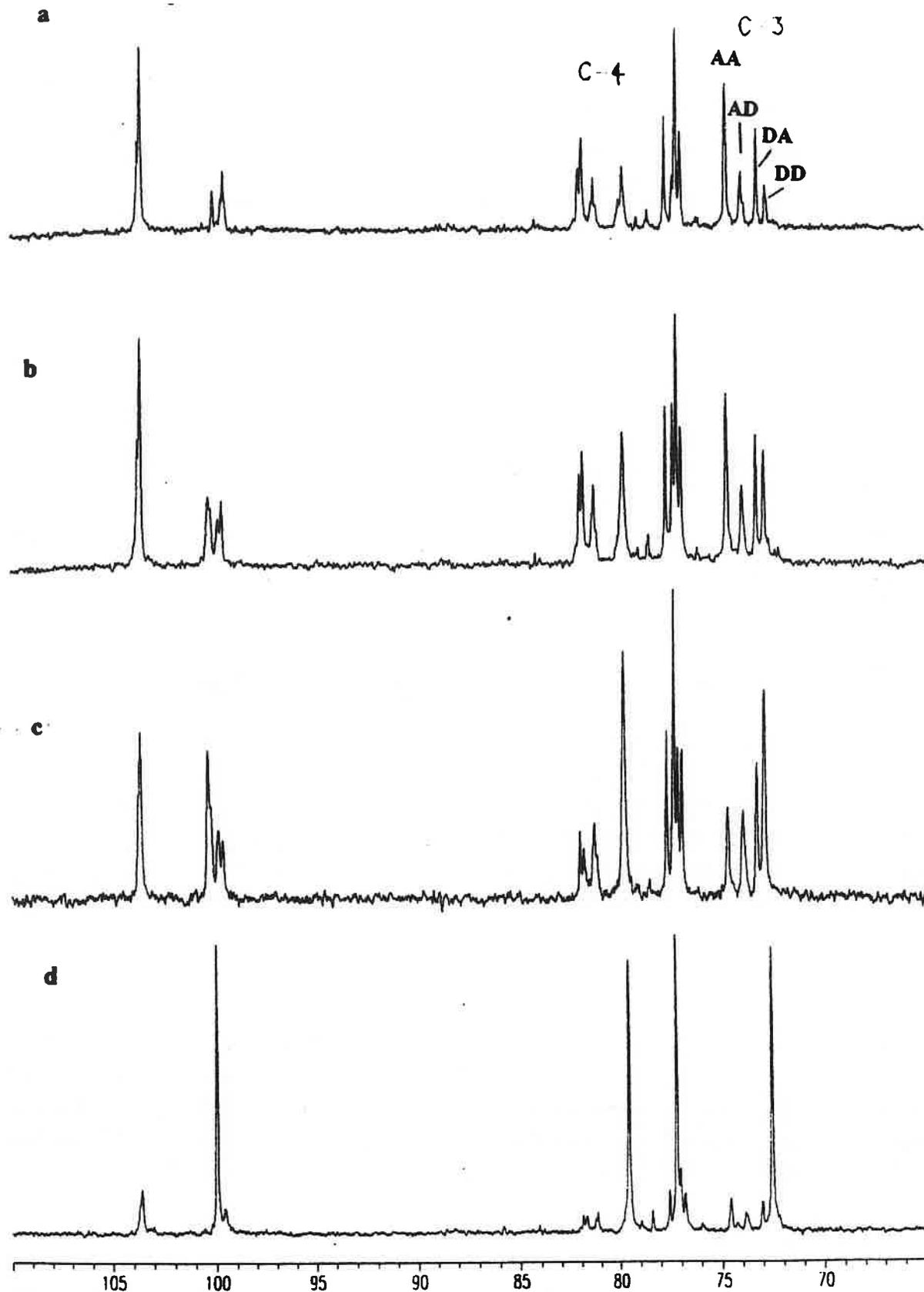
Figure 2.
Part of the ^{13}C-NMR spectrum (125 MHz) of a solution of the chitosan substrate (a) and the chitosans produced by incubating the chitosan substrate with increasing amounts of chitin deacetylase from *Mucor rouxii*: 6 mU of enzyme for 45 minutes (b), 11 mU for 45 minutes (c) and 70 mU for 2 hours (d).

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acetylated substrates in aqueous solvents represents a practical limitation for the CDA-chitosan system. Attempts to use an amorphous chitin as substrate were unsuccessfull, as no acid-soluble chitosan could be isolated and characterised.
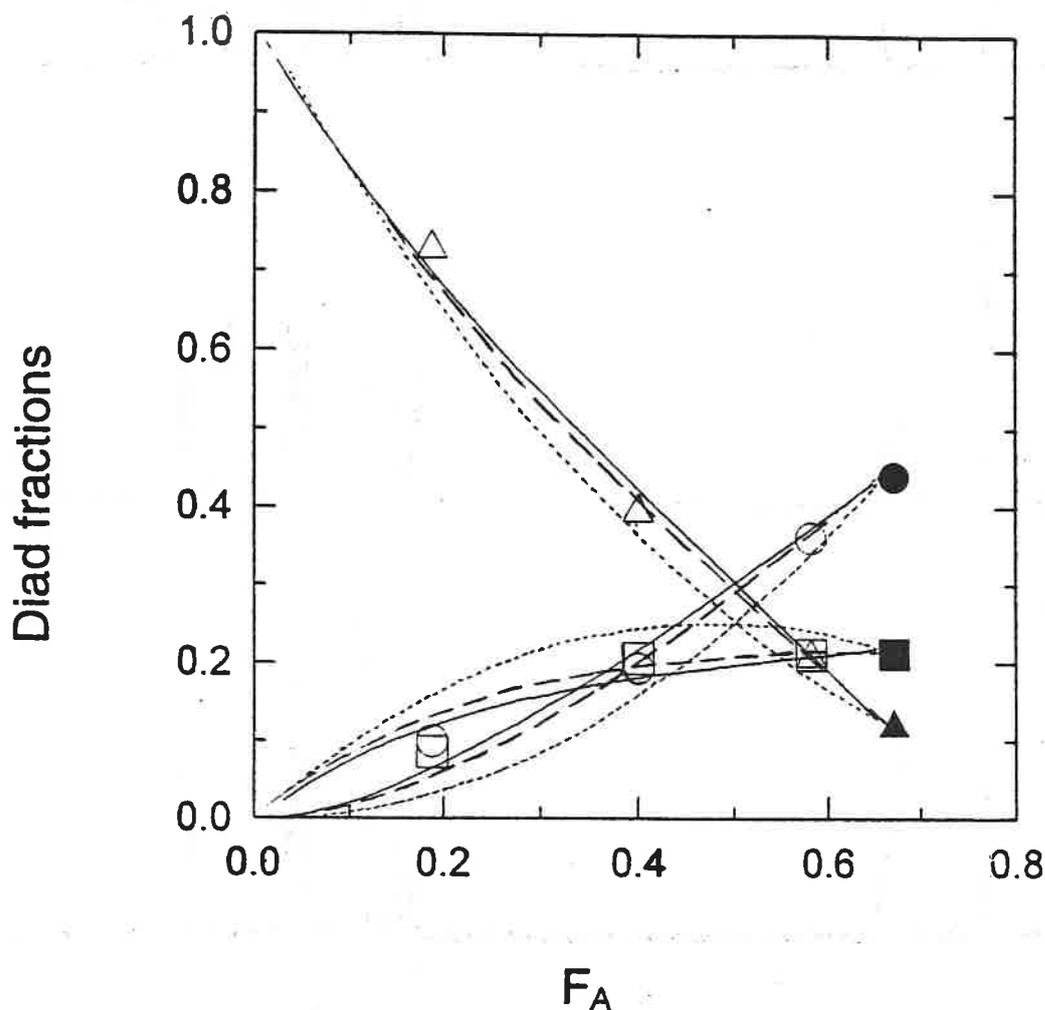

Figure 3.

Experimentally determined diad fractions F_{AA} (o, •), F_{DA} (□,■) and F_{DD} (Δ , ▲) of the chitosan substrate (filled symbols) and samples subjected to chitosan deacetylase (open symbols). Calculated diad fractions (originating from the substrate) for chitosan deacetylase acting of the F_A = 0.681 substrate assuming single-chain attack (_____), multiple attack with degree of multiple attack equals three (__ __ __), and multichain attack (- - - -).

Polarity of CDA

With respect to the polarity of CDA, it is not possible to compare the rate of deacetylation at the reducing and non-reducing end, since the chitosan substrate was prepared by nitrous acid depolymerization and subsequently reduced, thereby creating a 2,5-anhydro-<u>D</u>-mannitol unit at the reducing end. However, no apparent increase in the non-reducing end **D** units (78.9 ppm) relative to **A** units (78.5 ppm) upon enzymatic deacetylation from the substrate with F_A=0.681 (Fig. 2a) to the chitosan with F_A=0.188 (Fig. 2d) can be observed. This result may be explained by i) polarity of CDA towards the reducing end, or ii) CDA cannot deacetylate the non-reducing end unit independently of its polarity.

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CONCLUSION

The mode of action of purified CDA from *M. rouxii* using a highly acetylated but water-soluble chitosan as substrate has been determined. The results show that the diad frequencies of the enzymatically deacetylated chitosan deviate from a Bernoullian distribution, and suggest that CDA from *M. rouxii* deacetylates highly *N*-acetylated chitosan according to a multiple attack mechanism.

The mode of action of the chitin deacetylase from *M. rouxii* suggests that the cell-wall chitosan from this fungus may be a block polymer. Unfortunately, the chitosan isolated from *Mucor rouxii* is highly deacetylated with F_A around 0.1 ([24], Vårum & Martinou, unpublished results), and it is difficult to analyze the distribution of acetyl groups in chitosans with such low F_A-values. It should, however, be noted that both F_A and the distribution of **A** and **D**-units in fungal cell wall chitosan are important for both their biodegradability by chitinases/chitosanases and the identity (DP_n and acetylation pattern) of the resulting oligosaccharides.

Present applications of chitosan are based on relatively highly deacetylated chitosans (F_A less than 0.2) which are produced by thermochemical alkaline deacetylation of chitin. We have here demonstrated that by further enzymatic deacetylation of a water-soluble chitosan, chitosans with a distribution of **A** and **D**-units deviating from random (Bernoullian) distribution may be produced. This opens up the possibility for biotechnological production of chitosans with physical-chemical properties and biological function different from chitosans produced by thermochemical alkaline deacetylation.

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