BIO-ACTIVITY MATRICES FOR PARTIALLY ACETYLATED CHITOSAN OLIGOMERS

Bruno M. Moerschbacher and Nour Eddine El Gueddari

Department of Plant Biochemistry and Biotechnology, Westfälische Wilhelms Universität Münster,

Hindenburgplatz 55, 48143 Münster, Germany

Abstract

Partially acetylated chitosan polymers exhibit a number of biological activities, including anti-microbial activities, elicitor activities inducing disease resistance in plants, and diverse stimulatory or inhibitory activities towards a number of human cell types. We have generated and characterized a number of DA- and DP-series of chitosan polymers having constant DP but varying DAs, or constant DA and varying DPs, respectively. Using these highly purified and well characterized chitosans, we have shown that their biological activities are greatly dependent on their physico-chemical properties, most notably their degree of acetylation (DA). In contrast, the degree of polymerization (DP) of the polymers used had less influence on the biological activities. We have visualized the relationship between DA and DP of chitosan polymers on the one hand, and the antimicrobial or elicitor activities on the other hand, in the form of bio-activity matrices. The anti-microbial activities of the chitosan polymers increased with decreasing DA and with decreasing DP so that small, fully de-acetylated polymers were most effective. In contrast, the elicitor activities of partially acetylated chitosan polymers greatly depended on the plant species and the induced resistance response investigated; in general, chitosans with intermediate DAs were most effective. In order to establish more detailed structure-function relationships, we then began to work with oligomers. We generated, purified, and characterized a series of oligomers with a DA of 40 % and ranging in DP from 1 to >19. When these oligomers were analyzed for their elicitor activities towards suspension-cultured wheat cells, oligomers with a DP above 4 were shown to elicit a rapid oxidative burst. We have also prepared a mixture of fully de-acetylated chitosan oligomers with DP ranging from 3 to 9, and this mixture was then partially re-acetylated to yield a series of oligomer mixtures ranging in DA from 0 to 90 %. When these mixtures were tested for their elicitor activities in suspension cultured cells of different plant species, their elicitor activity increased with increasing DA, fully acetylated chitin oligomers always being the most active. In contrast, the antimicrobial activities of chitosan oligomers were weak, only the largest ones significantly inhibited fungal growth.

Introduction

Chitosan, one of the most interesting biopolymers, is commercially produced from chitin, one of the world's most widely available renewable resources. Chitosan is the only naturally occurring polycationic polymer, with potential applications in material sciences, in food sciences, in biotechnology, in cosmetics, in agriculture, in pharmaceutics, and in medicine [1]. In addition to being an all-natural, fully biodegradable. and non-toxic material, chitosan has a number of further, highly appreciated properties, such as being non-allergenic, non-immunogenic, and highly bio-compatible [2]. Its main drawback is its relatively high price, compared to oil-based products. This situation, however, may quickly change, with the international price for chitosan coming down dramatically over the past few years due to the entrance into market of Chinese chitin and chitosan

producing companies, and with the simultaneous rise in oil prices due to shortage of reserves and production. These changes will first positively affect the high price sector of chitosan applications in the life sciences. However, it is here that chitosan has another weakness, namely the poor reproducibility of results obtained when chitosan is applied to complex biological systems.

In the realm of the life sciences, chitosan has been proposed to have a future as an alternative plant disease protectant in agriculture [3], as an antiseptic and scar-free healing promoting dressing for large scale wounds in medicine [4], as a nanoscale carrier for drugs, genes, and vaccines that is easily absorbed by human cells in pharmaceutics [5], etc. As indicated, many reports exist about the surprising and almost miraculous results obtained with chitosan applications in these fields, but these are rarely followed by more detailed mechanistic studies and have so far let to only a very few commercially successful products. The obstacle to market in all these cases appears to be a rather poor reproducibility of results: sometimes, plants become resistant to microbial disease upon treatment with chitosan, and sometimes, difficult wounds heal below a chitosan dressing without leaving any visible scars – but only sometimes, and not always.

We have argued in the past that one reason for the lack of reliability of chitosan-based products in the life sciences may lie in subtle batch-to-batch differences between charges of commercially available chitosans [6]. Today, even the best characterised chitosans available in the market are usually described only concerning their average degree of acetylation and their average degree of polymerisation, their ash content and the absence of contaminating bacteria, in some instances also indicating the polydispersity index. While these parameters may adequately describe the chitosans for applications in material and food sciences, in biotechnology and in cosmetics where the physico-chemical properties of chitosans are all that matters, they are most likely insufficient to predict with certainty the biological activities of chitosans. Here, we have argued that in addition to the above criteria, the distribution of the acetyl groups along the linear backbone of the chitosan molecules may be of crucial importance in defining the interactions with the biological systems [6]. We assume that in many cases, the chitosan polymers applied are first modified by enzymes produced by the cells or tissues to be converted into their biologically active forms. Such modifications will include partial hydrolysis and, possibly, partial de-N-acetylation, but it might also include reaction with other molecules such as proteins. Clearly, the rate of modification as well as the products obtained will depend crucially on the nanoscale or molecular details of the chitosans applied.

Consider lysozyme, the enzyme thought to be of major significance in chitosan breakdown in the human body [7], that seems to strongly prefer three consecutive acetylated N-acetyl-glucosamine (GlcNAc or A) units in its substrate to be able to bind and cleave [8]. The frequency with which this AAA epitope will occur in a given chitosan will not only depend on that chitosan's degree of acetylation (DA), where chances will increase with increasing DA. It will also depend on the distribution of the acetyl groups in the chitosan polymer. As an example, consider three different chitosans, all of them having a DA of 50 % and a DP of 100 (Fig. 1): The AAA epitope will occur with a frequency of 0.5^3 = ca. 0.12 if the acetyl groups are distributed randomly, i.e. an average of twelve preferred cleavage sites will exist in the polymer. But its frequency will be much higher if the acetyl groups are distributed in blocks of e.g. ca. ten consecutive A-units interspersed by blocks of a random number of non-acetylated glucosamine (GlcN or D) units; and it will be zero in a chitosan with alternate A and D units.

Even though the substrate specificity of lysozyme is more complex than assumed above, let us assume for the sake of clarity of the argument that lysozyme would indeed cleave only -AAA- to yield -AA and A-. The first molecule would be degraded into medium sized, partially acetylated oligomers. The A-blocks in the second molecule would be degraded mainly to fully acetylated monomers to trimers [9] while the D-blocks would end up as oligomers of D-units typically flanked by two A-units at the reducing and one A-unit at the non-reducing end [8]. The third chitosan polymer would remain unchanged even after prolonged exposure to lysozyme. Thus, not only the rate of degradation will depend on the pattern of acetylation, but so will the nature of the products

generated. Clearly, these differences will deeply influence the biological activities of the parent chitosan polymers in a tissue where lysozyme is present.

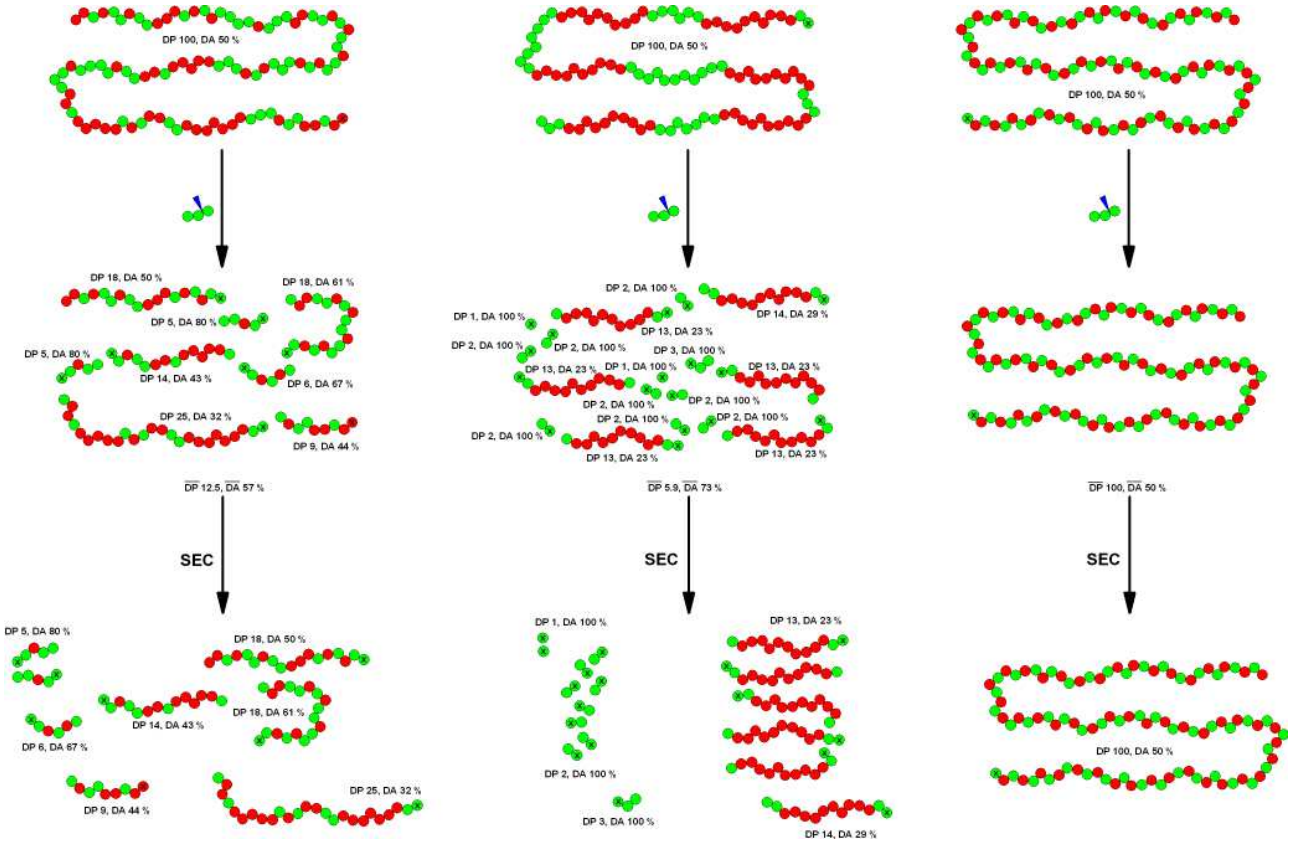

Fig. 1: Digestion of three different chitosan with identical degree of polymerisation and identical degree of acetylation, but differing in their pattern of acetylation (left: random; middle: blockwise; right: regular) by the same chitinase leads to highly different products (red circle: GlcN; green circle: GlcNAc; circle with cross: reducing end).

This conclusion holds irrespective of whether it is the chitosan polymers that exert an effect on the tissue via a physico-chemical interaction with cell surfaces e.g. leading to a perturbation of the plasma membrane integrity, or via molecular recognition of specific chitosan oligomers by surface receptors on the target cells. In the first case, enzymic degradation might destroy the biologically active polymers while in the second case, the biologically active oligomers might only be produced by the action of the enzyme.

A detailed understanding of structure-function relationships on the molecular or nano-scale level is, thus, required to improve the reliability of biological effects of chitosans. For this, appropriate tools to generate chitosan polymers and oligomers with defined and known structure and to analyze their physico-chemical properties are needed. In addition, appropriate bio-assays to quantify the biological activities of chitosans towards micro-organisms, plant and animal/human cells need to be established (Fig. 2). The CARAPAX project had convened a consortium of European researchers from different fields of interest and expertise, to try and critically test our hypothesis of a more sophisticated dependency of the biological activities of chitosans on their physico-chemical properties than previously assumed. The project was designed as a proof-of-principle study of our concept, aiming at improving the reliability of chitosan polymers as novel plant disease protectants in agriculture. Based on the positive and promising outcome of this project, the follow-up project NANOBIOSACCHARIDES is currently underway in which we are trying to apply the knowledge gained during the CARAPAX project on medical and pharmacological applications of well defined chitosans, including polymers and oligomers.

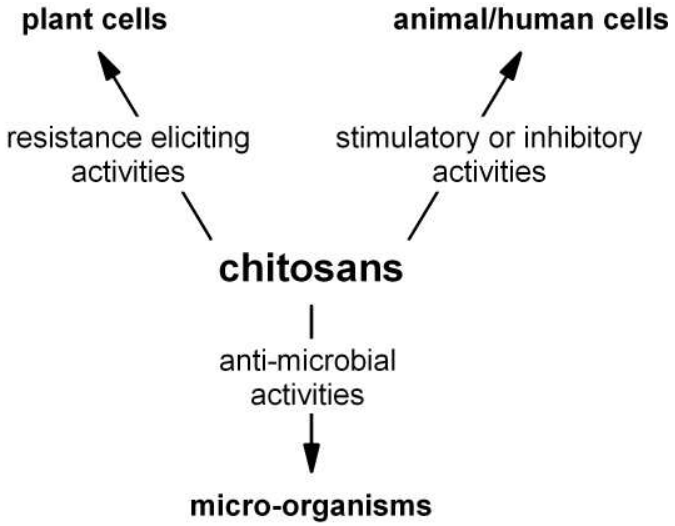

Fig. 2: Chitosans possess different biological activities towards plant, animal and human cells, and micro-organisms.

Material and Methods

Generation and characterization of partially acetylated chitosan polymers

Chitosan polymers of different DP and DA were produced by the groups of Prof. Dr. Alain Domard, University Claude Bernard Lyon 1, France, and Prof. Dr. Kjell M. Vårum, University of Trondheim, Norway, as indicated in Table 1. DP series of chitosan polymers were generated by partial acid hydrolysis or by partial nitrous acid deamination of a high molecular weight chitosan polymer of a given DA. DA series of chitosan polymers were generated by partial homogeneous de-N-acetylation of a highly acetylated chitin polymer of a given DP, or by partial re-N-acetylation of a fully de-N-acetylated polyglucosamine polymer of a given DP. Analytical methods used to determine DA and DP of the different polymers used in this study are indicated in Table 1.

Table 1: DP and DA series of partially N-acetylated chitosan polymers used in this study

DP	DA	group	methods	references
190	1	Vårum	partial homogeneous de-	[10]
320	15		acetylation	[11]
68	35		partial nitrous acid de-	[12]
121	49		amination	[13]
210	60		intrinsic viscosity	[14]
224	69		^{13}C-NMR, ^{1}H-NMR	
1100	1	Vårum	partial homogeneous de-	[10]
1100	15		acetylation	[11]
550	35		intrinsic viscosity	[12]
540	49		^{13}C-NMR, ^{1}H-NMR	[14]
970	60			
2580	9.8	Domard	complete heterogeneous de-	[15]
2608	29.4		acetylation	[16]
2528	40,0		partial re-acetylation	[17]
2518	49.5		GPC, ^{1}H-NMR	
2447	66.0			
3300	1.5	Domard	intrinsic viscosity	[18]
3400	7.7		IR-spectroscopy	[19]
3200	15.0			
3390	20.0			
3335	24.5			

935	7.2	Domard	intrinsic viscosity	[18]
1635	7.5		IR-spectroscopy	[19]
3400	7.5			
7790	7.5			
200	0	Domard	complete heterogeneous de-	[15]
503	0		acetylation	[16]
591	0		partial HCl hydrolysis	[17]
807	0		GPC, ^{1}H-NMR	
1955	0			
2059	0			
2317	0			
2354	0			
2402	0			
2483	0			
2532	0			

Generation and characterization of partially acetylated chitosan oligomers

Fully N-acetylated GlcNAc oligomers and fully de-N-acetylated GlcN oligomers of different DP were produced by the group of Prof. Dr. Alain Domard, as described previously [14]. A DP series of partially N-acetylated chitosan oligomers was prepared in collaboration with the group of Prof. Dr. Kjell M. Vårum, by partial acid hydrolysis of a chitosan polymer with a DP of 962 and a DA of 42 %, followed by size exclusion chromatography [20]. A DA series of mixtures of chitosan oligomers with a DP ranging from 3 to 9 were produced by the group of Prof. Dr. Alain Domard, by partial re-N-acetylation of a mixture of fully de-N-acetylated GlcN oligomers [21]. Analytical methods used to determine DA and DP of the different polymers used in this study are indicated in Table 2.

Table 2: DP and DA series of GlcN, GlcNAc, and chitosan oligomers used in this study

DP	**DA**	**group**	**methods**	**references**
2 to 7	0	Domard	partial HCl hydrolysis	[22]
			GPC, ^{13}C-NMR, FAB-MS	[23]
2 to 6	100	Domard	partial HCl hydrolysis	[24]
			GPC, ^{13}C-NMR, FAB-MS	[23]
2 to 10	100	Domard	partial HF solvolysis	[25]
			GPC, ^{13}C-NMR, FAB-MS	[23]
2	53	Vårum	partial HCl hydrolysis	[10]
3	51	Moersch-	GPC, ^{1}H-NMR,	[11]
4	46	bacher	MALDI-MS	[12]
5	42			[20]
6	43			
7	42			
8	43			
9	37			
10	38			
11	38			
12	40			
13-15	36			
16-19	34			
>19	35			
3-9	0	Domard	complete de-acetylation	
3-9	24		partial HCl hydrolysis	

3-9 3-9 3-9 3-9	41 60 78 91		partial re-acetylation	

Determination of anti-microbial activities

Anti-microbial activities of chitosan polymers and oligomers were tested in a microtitre plate-based bio-assay [26]. A range of bacteria and fungi belonging to widely different taxonomic groups were used, as indicated in Table 3. Chitosan polymers were dissolved in 20-40 mM acetic acid followed by freeze drying, then redissolved in sterile distilled water (leaf assay) or in assay medium (cell assay). Growth of the bacteria and fungi was quantified by measuring the turbidity of the culture media over a period of 24 hours or seven days, respectively. The Minimum Inhibitory Concentration (MIC) of a given chitosan against a given micro-organism was determined as the lowest concentration of that chitosan completely inhibiting growth of that micro-organism.

Table 3: Bacteria and fungi used in the bio-assay for anti-microbial activities of chitosans

species	**taxonomic group**	**medium**	**pH**	**references**
Bacillus cereus	gram-positive	LB	7	
Bacillus megaterium	gram-positive			
Escherichia coli	gram-negative			
Staphylococcus aureus	gram-negative			
Xanthomonas fragariae	gram-negative	Wilbrink-N	5, 7,8	[27]
Rhizopus stolonifer	zygomycete	complete	5.8	[28]
Alternaria alternata	ascomycete			
Botrytis cinerea	ascomycete			
Fusarium graminearum	ascomycete			
Fusarium solani	ascomycete			
Ustilago maydis	basidiomycete	potato dextrose liquid	5.6	
Phytophthora infestans	oomycete	Henninger	5.2	[29]

Determination of elicitor activities

Elicitor activities of chitosan oligomers and polymers were visualised and quantified .using a number of different assays, performed either on intact wheat leaves [30] or on suspension-cultured wheat cells [31], as indicated in Table 4. Chitosan polymers were dissolved in 20-40 mM acetic acid followed by freeze drying, then redissolved in sterile distilled water (leaf assay) or in assay medium (cell assay).

Table 4: Bio-assays for elicitor activity of chitosans in wheat leaves and cells

parameter	**assay**	**method**	**references**
necrosis	leaf	visual	[14]
phenylalanine ammonium lyase	leaf	spectrophotometric	[32]
peroxidase	leaf / cells	spectrophotometric	
chitinase	leaf / cells	spectrophotometric	[20]
ß-1,3-glucanase	leaf / cells	spectrophotometric	
hydrogen peroxide	cells	luminometric	[31]

Results and Discussion

Anti-microbial activities of partially acetylated chitosan polymers

Using a microtitre plate-based bio-assay, we determined the anti-microbial activities of partially acetylated chitosan polymers against a range of gram-positive and gram-negative bacteria,

ascomycete and basidiomycete fungi, and oomycetes. When the three DA-series of chitosan polymers (DAs ranging from ca. 0 to 70 %), having constant DP of ca. 100, 1000, and 3500, were used, all chitosans exhibited anti-microbial activities against most micro-organisms. Different chitosans differed in their anti-microbial activities towards a given micro-organism. The efficiency consistently increased with decreasing DA so that chitosans with the lowest DA were most active. Also, different micro-organisms differed in their sensitivity towards a given chitosan. In some few cases, no significant inhibitory activity or even a stimulatory activity was observed with lower concentrations of chitosans. Invariably in these cases, the micro-organism was shown to secret chitosanolytic enzymes into the culture medium, explaining the failure of chitosan to inhibit growth of these organisms.

When a DP series of chitosan polymers (DPs ranging from 20 to 3700) having a constant DA of 0 % were investigated, the anti-microbial activities increased with decreasing DP. Small polymers with DPs between 20 and 200 exhibited the highest anti-microbial activities in all cases studied.

When the bio-assays were performed at different pH values in the range tolerated by the micro-organism under investigation (e.g. pH 5-7), the anti-microbial activity of the chitosans increased with decreasing pH. The differences observed between different chitosans as described above were observed irrespective of the pH of the medium.

Anti-microbial activities of partially acetylated chitosan oligomers

Preliminary bio-assays using chitosan samples with a low average DP seemed to indicate that partially acetylated chitosan oligomers might also possess strong anti-microbial activities. On closer inspection, however, it turned out that the anti-microbial activities of these samples decreased with increasing level of purification, indicating the possibility that contaminating polymers might in fact be responsible for the anti-microbial effects observed. We therefore used a DP series of partially acetylated chitosan oligomers with a constant DA of ca. 40 % and ranging in DP from 2 to >19 in further experiments. Only weak anti-microbial activities were seen using these highly purified and well characterized chitosan oligomers, and their efficiency increased with increasing DP.

A bio-activity matrix for the anti-microbial activities of chitin and chitosans

The results obtained in the bio-assays to quantify the anti-microbial activities of chitosan polymers and oligomers can be summarized in a bio-activity matrix as shown in Fig. 3. The best efficiency was seen with small, fully de-acetylated chitosan polymers (DA 0 %, DP 20-200). These results together with the pH dependency of the effect strongly suggest that the anti-microbial activities of chitosans are dependent mainly on the cationic nature of this bio-material. Artificial cations such as poly-L-lysines are well known for their cytotoxicity which is much stronger than that of chitosans [33]. The rather weak toxicity of chitosan is probably due to the characteristically and unusually low pKs of the amino group in chitosan – this possibly explaining why chitosan is the only known naturally occuring polycationic biopolymer. Interestingly, chitosans do not appear to have significant cytotoxic effects towards plant and animal or human cells [1-5]. The reason for the comparatively higher sensitivity of bacterial and fungal (including oomycete) cells remains unresolved at present. It is, however, interesting to note that certain small, partially polycationic and partially hydrophobic peptides, the defensins produced by animal and plant cells, exhibit anti-microbial activities against bacteria and fungi, but are not cytotoxic to plant and animal cells [34-36].

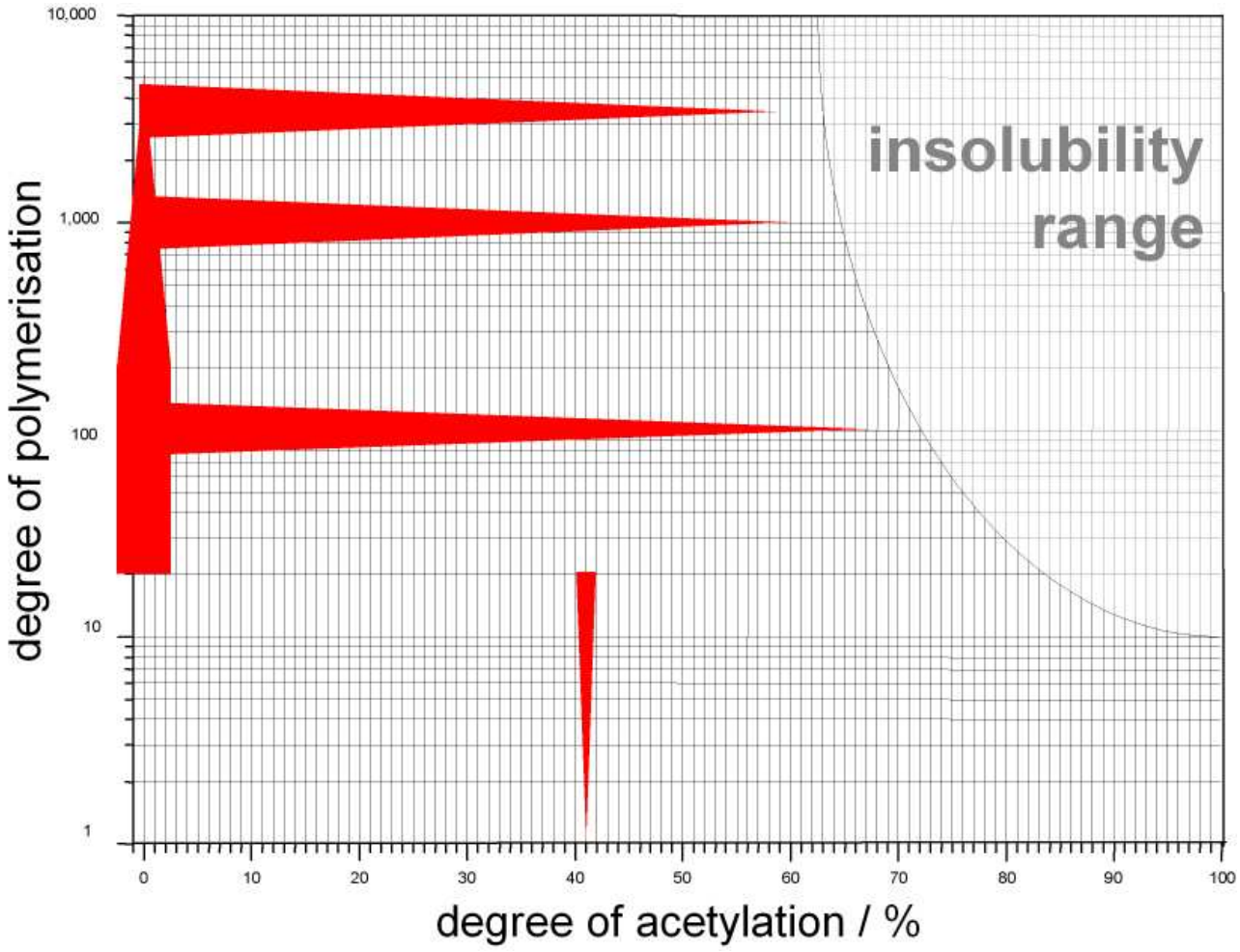

Fig. 3: Bio-activity matrix for the anti-microbial activities of chitin and chitosans against bacteria and fungi. Anti-microbial activities are indicated in red, the width of the area indicating the intensity of the anti-microbial activity. Fully de-N acetylated chitosan polymers with low DPs exhibit the highest anti-microbial activities.

Elicitor activities of partially acetylated chitosan polymers

We have previously shown that the elicitor activity of partially acetylated chitosan polymers (DP ca. 1000, DAs ranging from 1 to 60 %) to induce disease resistance related reactions in intact wheat leaves strongly depends on the DA of the chitosans [14]. Hypersensitive-like programmed cell death was most strongly elicited by chitosans with intermediate DA. The most highly acetylated chitosan studied then (DA 60 %) exhibited the weakest elicitor activity. When chitosans with even higher DAs up to 70 % are used, they are inactive as an elicitor of programmed cell death. When DA series with lower and higher DPs of ca. 100 and ca. 3500, respectively, were used, similar results were obtained as reported in our previous study. Interestingly, different aspects of the induced cellular lignification response involved in programmed cell death [37] were best induced by different chitosans. The activity of the first enzyme of lignin biosynthesis, phenylalanine ammonium-lyase, was strongly induced by all chitosans, with those of high DA (35 to 60 %) being about twice as active as those of low DA (1 to 15 %). The activity of the last enzyme of lignin biosynthesis, peroxidase, was barely induced by low DA chitosans, but elicitation increased strongly with increasing DA.

A similar but slightly different picture emerged in a bio-assay using suspension-cultured wheat cells. Chitosans with very low DA were almost inactive, but peroxidase elicitor activity quickly increased with increasing DA and was almost constant with chitosans of DA 15 to 60 %. Similarly, but distinctly differently, the elicitation of a rapid and transient oxidative burst [31] increased consistently with increasing DA of the chitosan polymers used. A DP series of chitosan polymers

with a constant DA of ca. 7.5 % and ranging in DPs from 1000 to 8000 indicated that the DP of the polymers had a negligible influence on their elicitor activities.

Elicitor activities of partially acetylated chitosan oligomers

Fully de-acetylated GlcN-oligomers (DA 0 %, DPs ranging from 2 to 7) were completely inactive as an elicitor of any aspect of resistance investigated in intact wheat leaves and in suspension-cultured wheat cells. When fully acetylated GlcNAc oligomers (DA 100 %, DPs ranging from 2 to 10) were tested, oligomers of DP 4 and higher exhibited elicitor activities in suspension-cultured cells, and oligomers of DP 7 and higher exhibited elicitor activities in intact leaves, albeit at high concentrations only. Partially acetylated chitosan oligomers (DA ca. 40 %, DPs ranging from 2 to >19) were inactive as an elicitor when injected into healthy wheat leaves, but oligomers of DP 5 and higher exhibited an oxidative burst in suspension-cultured cells.

We next used a series of mixtures of chitosan oligomers, all of them ranging in DP from 3 to 9, but differing in their DA from 0 to 91 %. Oligomer mixtures with low DA (ranging from 0 to 41 %) were weak elicitors of the oxidative burst in suspension-cultured wheat cells, while mixtures with high DA (ranging from 60 to 91 %) were strong elicitors. Preliminary experiments indicate that at higher concentrations, oligomer mixtures with very low DA (0 to 19 %) and with high DA (60 to 91 %) might also strongly elicit peroxidase activities in suspension-cultured wheat cells, while oligomer mixtures with intermediate DA (41 %) were almost inactive in this assay.

A bio-activity matrix for the elicitor activities of chitin and chitosans in wheat

The results obtained in the bio-assays to quantify the elicitor activities of chitosan polymers and oligomers can be summarized in a bio-activity matrix as shown in Fig. 4. The highest elicitor activities were seen with chitosan polymers of intermediate DAs of 15 to 50 %. In contrast, oligomers were most active when they were fully acteylated. These results together with the observation that different aspects of the hypersensitive resistance reaction were best induced by different chitosans suggest that (i) different recognition systems are involved in the stimulatory activity of chitosan oligomers and polymers towards wheat cells and/or that (ii) chitosan polymers become enzymatically degraded into elicitor-active oligomers (Fig. 5). Chitin and chitosan oligomers are most likely acting via a molecular interaction with surface bound receptors in the plant plasma membrane. Receptors for fully acetylated GlcNAc oligomers have been identified in several plant species [38, 39]. It seems reasonable to assume that these receptors can also bind with lower affinity partially acetylated chitosan oligomers, but the presence of additional specific receptors cannot yet be ruled out entirely. The elicitor activities of partially acetylated chitosan polymers might fully or partially be explained by their slow enzymatic degradation via apoplastic plant chitinases. However, a purely electrostatic interaction between the positively charged chitosan polymers and the negatively charged plant surfaces may in addition lead to a receptor-independent stimulation of stress responses in the plant cells [40].

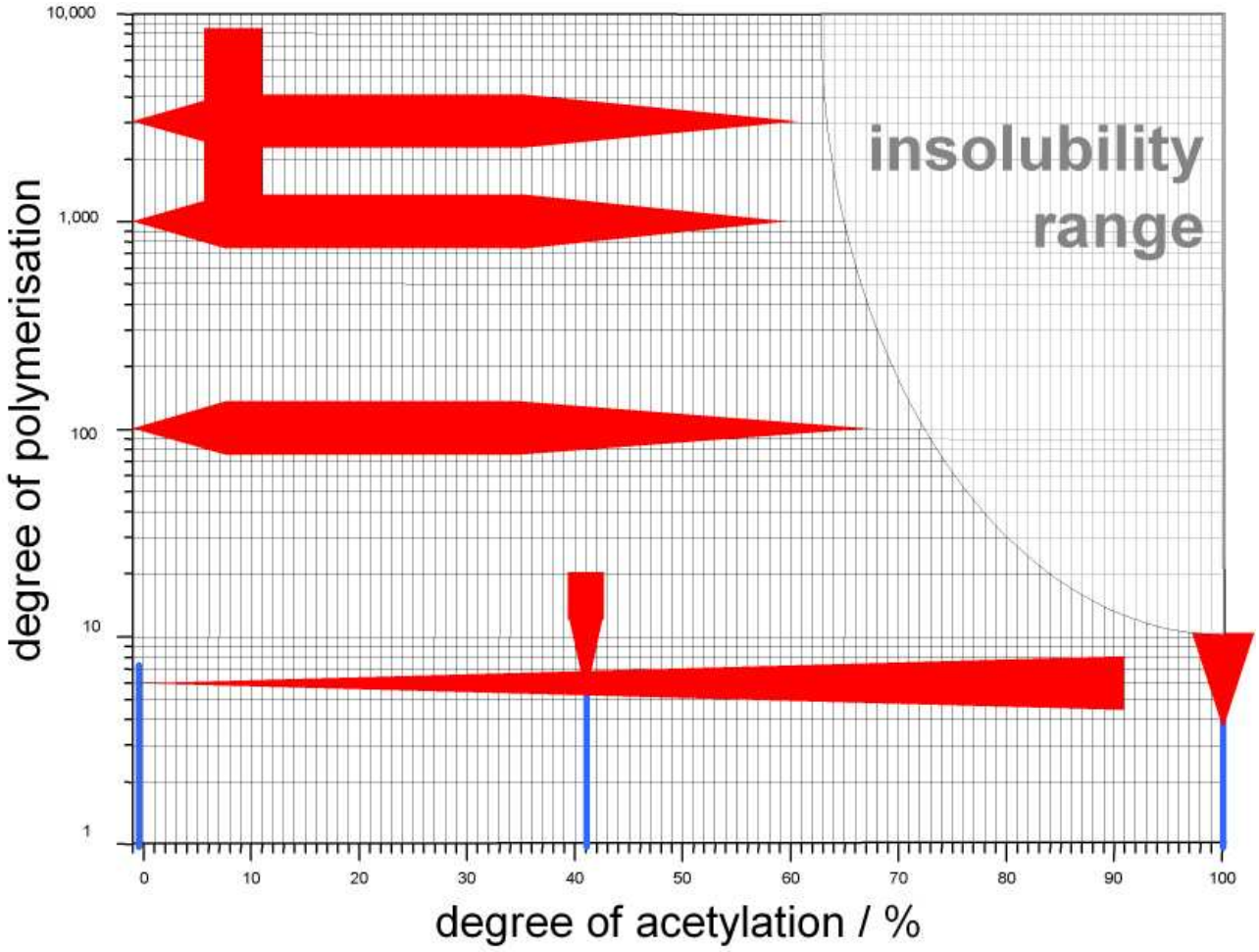

Fig. 4: Bio-activity matrix for the elicitor activities of chitin and chitosans in wheat leaves and cells. Elicitor active chitosans are indicated in red, the width of the area indicating the intensity of the elicitor activity; chitosans lacking elictor activity are indicated as blue lines. Partially acetylated chitosan polymers of intermediate DAs are most active as elicitors of disease resistance reactions in wheat, while the elicitor activity of oligomers increases with increasing DA.

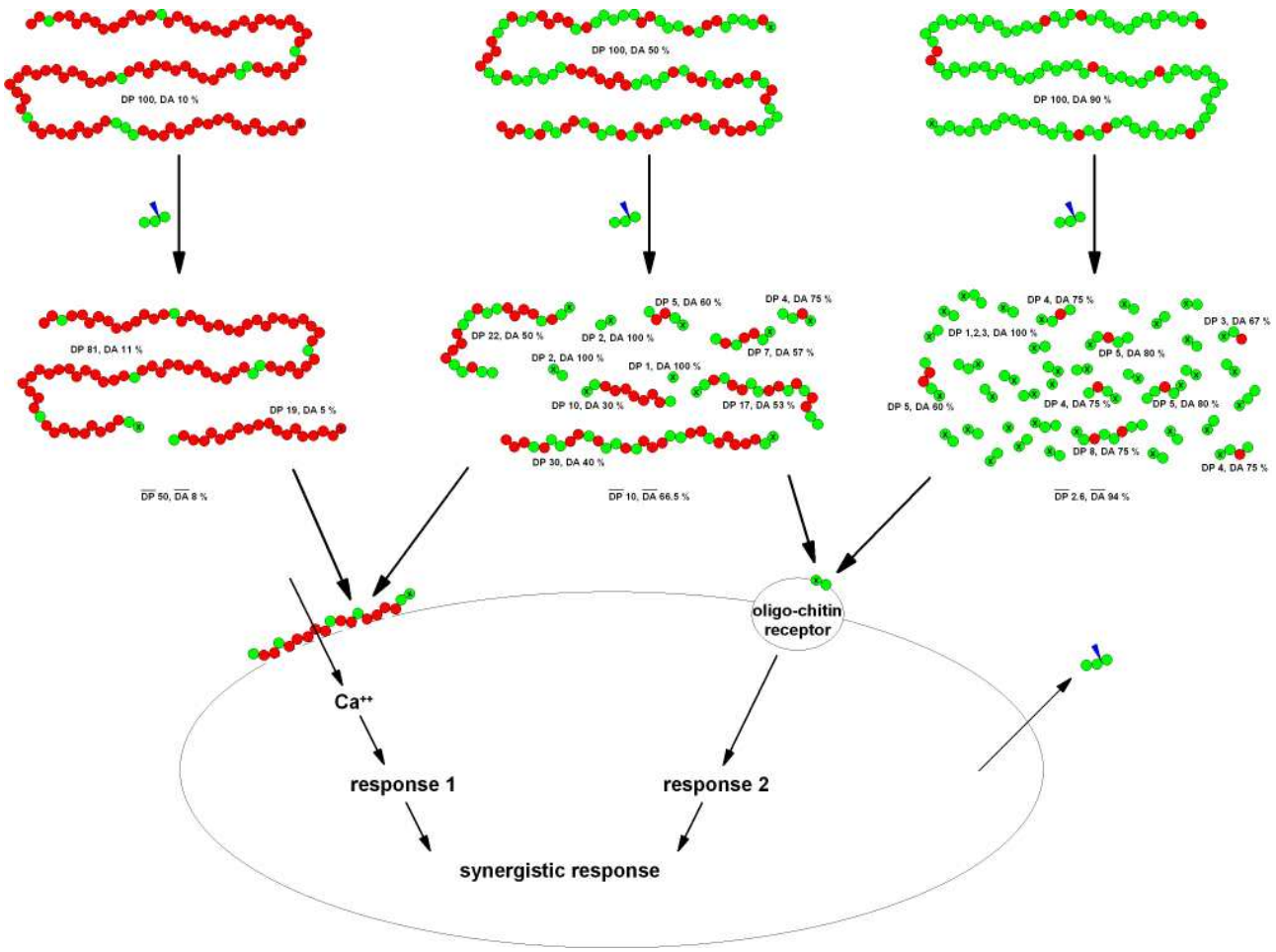

Fig. 5: Chitosans with identical DP but different DAs, all of them with a random distribution of acetyl groups, may be degraded differently by extracellular plant chitinases resulting in different products with different elicitor activities. Highly de-acetylated chitosan polymers may interact physico-chemically with the negatively charged cell surface, inducing stress response 1. Fully acetylated chitin oligomers may be recognized by a cell surface receptor, inducing response 2. Only chitosan with an intermediate DA, thus, may induce both responses, leading to a synergistic effect. If e.g. response 2 were an induction of monolignol biosynthesis and response 1 were the triggering of an oxidative burst, peroxidases may use the hydrogen peroxide produced during the oxidative burst as a co-substrate to form monolignol radicals which would spontaneously polymerize to form lignin. Intracellular lignification could then lead to cell death as a form of the hypersensitive resistance reaction of plants to pathogen attack (red circle: GlcN; green circle: GlcNAc; circle with cross: reducing end).

Domard's universal law of behavior and the bio-activity matrices for chitosans

Domard and his group have recently proposed a universal law of behavior for chitosan polymers in aqueous solutions [41] According to this law, three distinct DA ranges can be identified. Chitosan polymers with low DAs ranging from 0 to ca. 25 % are easily soluble in acidic solutions where they behave like typical polyelectrolytes. Chitosan polymers with high DAs ranging from 50 to ca. 70 % are far less soluble in aqueous solutions, but their solubility is independent of pH, and they typically form nanoparticles. Chitosan polymers in the transition range of DA 25 to 50 % exhibit mixed properties of both polyelectrolytes and nanoparticles. The solution properties of chitosans are surprisingly stable in this transition range.

When the bio-activity matrices for chitosans are viewed in the light of this universal law of behavior for the solution properties of chitosans, new insights into structure-function relationships may be gained (Fig. 6). Clearly, the anti-microbial properties of chitosans correlate with the polyelectrolyte nature of highly de-N-acetylated chitosans. In contrast, the elicitor activities of chitosans in wheat - and possibly other plants - appear to be related to the transition range of intermediate DAs. It remains to be seen whether other biological activities of partially acetylated chitosans, e.g. in the

medical or pharmaceutical field, are dependent on the nanoparticle nature of highly acetylated chitosans. Animal and human cells, in contrast to plant, fungal and bacterial cells, are devoid of a rigid cell wall so that the chitosans can more easily and more directly interact with the membrane surface of these cells. Indeed, chitosan nanoparticles have been shown to possess just the right size, and they appear to be highly susceptible to endocytotic uptake by these cells [42]. We are currently analysing the subcellular fate and trafficking of the endocytotic vesicles containing chitosan nanoparticles in a range of human cells, the enzymic degradation of partially acetylated chitosan polymers into oligomers, and their stimulatory and/or inhibitory activities. These studies aim at setting up detailed bio-activity matrices for biological activities of chitosans towards human cells, as a pre-requisite for the development of knowledge-based, reliable applications for chitosans in the medical and pharmaceutical fields.

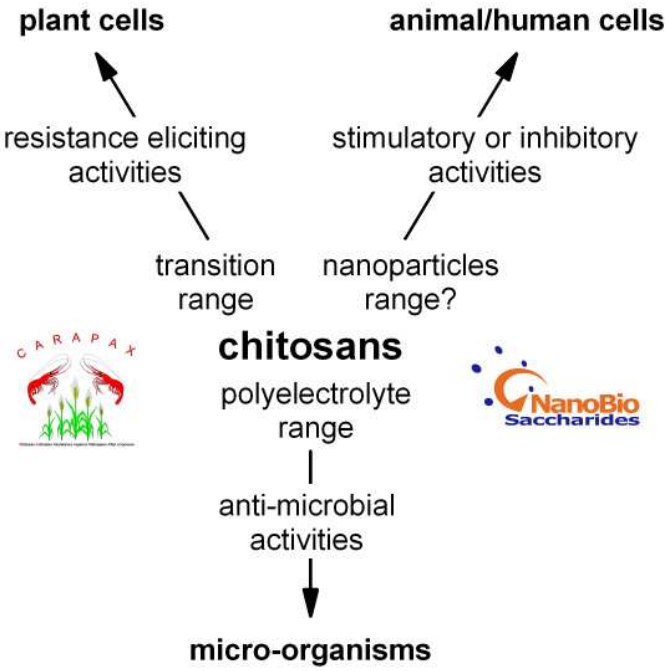

Fig. 6: The CARAPAX project analyzed the bio-activities of chitosans towards plant cells and micro-organisms while the NANOBIOSACCHARIDES project aims at understanding the bio-activities of chitosans towards human cells. The three ranges of solution properties of partially acetylated chitosans described by Domard's universal law of behavior, the polyelectrolyte range of low DA chitosans, the nanoparticles range of high DA chitosans, and the transition range of chitosans with intermediate DA might be reflected in the different bio-activities of these chitosans.

Acknowledgements

Over the past years, many students and colleagues have contributed to the work reviewed here, that was financially supported by the European Union in the 5th FP research project CARAPAX – Chitosan Activates Resistance Against Pathogens After eXposure, and in the 6th FP research project NANOBIOSACCHARIDES – NANOtechnologies for BIO-inspired polySACCHARIDES, namely: Dr. Peter Vander, Dr. Heiko Bauknecht, Dr. Dianer Yang, Dr. André Luis Wendt dos Santos, Dr. Imke Ortmann, Enio Nazaré de Oliveira jr., Marita Hermann, and Philip Känel from our lab; Prof. Dr. Alain Domard, Dr. Stéphane Trombotto, and Dr. Guillaume Lamarque from the University of Lyon, France; and Prof. Dr. Kjell Morten Vårum and Dr. Åre Kristiansen from the University of Trondheim, Norway.

References

[1] F. Shahidi, R. Abuzaytoun, Adv. Food Nutr. Res., 49(2005) 93-135.

[2] M. N. V. Ravi Kumar, R. A. A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A. J. Domb, Chem. Rev., 104(2004) 6017-6084.

[3] J. Synowiecki, N. A. Al-Khateeb, Crit. Rev. Food Sci. Nutr., 2(2003) 145-71.

[4] C. Shi, Y. Zhu, X. Ran, M. Wang, Y. Su, T. Cheng, J. Surg. Res., 2(2006) 185-92.

[5] M. Ravi Kumar, G. Hellermann, R. F. Lockey, S. S. Mohapatra, Expert Opi. Biol. Ther., 8(2004) 1213-1224.

[6] N. E. El Gueddari, B. M. Moerschbacher, Adv. Chitin Sci. 7(2003) 56-59.

[7] K. M. Vårum, M. M. Myhr, R. J. N. Hjerde, O. Smidsrød, Carbohydr. Res., 299(1997) 99-101.

[8] K. M. Vårum, H. K. Holme, M. Izume, B. T. Stokke, O. Smidsrød, Biochim. Biophys. Acta 1291(1996) 5-15.

[9] K. Amano, E. Ito, Eur. J. Biochem., 85(1978) 97-104.

[10] K. M. Vårum, M. V. Anthonson, H. Grasdalen, O. Smidrød, Carbohydr. Res., 211(1991a) 17-23.

[11] K. M. Vårum, M. V. Anthonson, H. Grasdalen, O. Smidrød, Carbohydr. Res., 217(1991b) 19-27.

[12] M. W. Anthonson, K. M. Vårum, Carbohydr. Polymers, 22(1993) 193-201.

[13] K. M. Vårum, M. H. Ottøy, O. Smidsrød, Carbohydr. Polymers, 25(1994) 65-70.

[14] P. Vander, K. Vårum, A. Domard, N. E. El Gueddari, B. M. Moerschbacher, Plant Physiol., 118(1998) 1353-1359.

[15] G. Lamarque, C. Viton, A. Domard, Biomacromol., 5(2004) 992-1001.

[16] G. Lamarque, C. Viton, A. Domard, Biomacromol., 5(2004) 1899-1907.

[17] G. Lamarque, J. M. Lucas, C. Viton, A. Domard, Biomacromol., 6(2005) 131-142.

[18] M. Miya, R. Iwamoto, S. Yoshikawa, S. Mima, Int. J. Biol. Macromol., 2(1980) 323-324.

[19] G. A. F. Roberts, J. G. Domszy, Int. J. Biol. Macromol., 4(1982) 374-377.

[20] H. Bauknecht, N. E. El Gueddari, B. M. Moerschbacher (in preparation).

[21] A. Wendt dos Santos, P. Känel, I. Ortmann, S. Trombotto, A. Domard, N. E. El Gueddari, B. M. Moerschbacher (in preparation).

[22] A. Domard, N. Cartier, Int. J. Biol. Macromol., 11(1989b) 297-302.

[23] C. Bosso, A. Domard, Org. Mass Spectrom., 27(1992) 799-806.

[24] A. Domard, N. Cartier, Chitin, Chitosan Sources, Chemistry, Biochemistry, Physical Properties and Applications, Elsevier, (1989a) pp 71-86.

[25] C. Bosso, J. Defaye, A. Domard, A. Gadelle, Carbohydr. Res., 156(1986) 57-68.

[26] D. Yang, G. Lamarque, B. M. Moerschbacher, N. E. El Gueddari (in preparation)

[27] H. Koike, Phytopathol., 55(1965) 317-319.

[28] G. Pontecorvo, Adv. Gen., 5(1953) 150-151.

[29] H. Henninger, Phytopathol. Z., 94(1959) 285.

[30] B. M. Moerschbacher, B. E. Flott, U. Noll, H. J. Reisener, Plant Physiol. Biochem. 27(1989) 305-314.

[31] I. Ortmann, G. Sumowski, H,. Bauknecht, B.M. Moerschbacher Physiol. Mol. Plant Pathol., 64(2004) 227-232.

[32] B. M. Moerschbacher, U. M. Noll, B. E. Flott, H. J. Reisener, Physiol. Mol. Plant. Pathol., 33(1988) 33-46.

[33] D. M. L. Morgan, V. L. Larvin, J. D. Pearson, J. Cell Sci., 94(1989) 553-559.

[34] M. S. Castro, W. Fontes, Protein Pept. Lett., 12(2005) 11-16.

[35] M. H. Braff, R. L. Gallo, Curr. Top. Microbiol. Immunol., 306(2006) 91-110.

[36] H. Jenssen, P. Hamill, R. E. Hancock, Clin. Microbiol. Rev., 19(2006) 491-511.

[37] B. M Moerschbacher, U. Noll, L. Gorrichon, H. J. Reisener, Plant Physiol., 93(1990) 465-470.

[38] N. Shibuya, H. Kaku, K. Kuchitsu, M. J. Maliarik, FEBS Lett., 329(1993) 75-78.

[39] K. Baureithel, G. Felix, T. Boller, J. Biol. Chem., 269(1994) 17931-17938.

[40] H. Kauss, W. Jeblick, A. Domard, Planta, 178(1989) 385-392.

[41] P. Sorlier, C. Schatz, A. Montembault, G. Lamarque, C. Viton, A. Domard, Adv. Chitin Sci. 8(2005) 17-25.

[42] I. Behrens, A; Isabel, M. J. Alonso, T. Kissel, Pharma. Res. 19(2002) 1185-1193.