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The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*

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Abstract. Partially and fully deacetylated chitosan fragments and oligomers were compared for their potency to elicit formation of the 1.3-β-glucan callose in suspension-cultured cells and protoplasts of *Catharanthus roseus* (line 385). Chitosan oligomers induced little callose formation, while callose synthesis increased with the degree of polymerization of chitosan up to several thousand corresponding to a molecular mass near 10^6 Da. At a comparable degree of polymerization, partially N-acetylated chitosan fragments were less effective. Colloidal chitin and chitin oligomers induced only trace callose synthesis in protoplasts. These results indicate that the primary interaction involved the amino groups of chitosan and numerous negative charges at the surface of the plasma membrane with spacing in the nanometer range and occurring regularly over micrometer stretches. Charged phospholipid head-groups may fulfill these requirements. The resulting alteration of membrane fluidity may lead to the changes in ion transport known to be associated with the induction of callose formation.

Key words: Calcium (callose induction) – Callose induction (chitosan) – *Catharanthus* (callose induction) – Chitosan (N-acetylation, oligomers) – Plasma membrane (callose induction)

Introduction

The activity in vitro of the plasma-membrane-located 1.3-β-glucan synthase is strictly dependent on Ca^{2+}, in the presence of polyamino compounds and-or Mg^{2+} with half-maximal activity attained below 1 μM Ca^{2+} (Kauss 1987a, b; Fink et al. 1987; Hayashi et al. 1987). This regulatory property has led to the suggestion that in the course of defense reactions against pathogens or after mechanical injury, a transient increase in cytoplasmic Ca^{2+} concentration might – at least in part – represent the intracellular signal for activation of 1.3-β-glucan synthase. As a result, a localized callose deposition onto the adjacent inner cell-wall surface would occur in order to seal or tighten the wall. To provide experimental evidence in favour of this working hypothesis, we have been searching for procedures which efficiently allow the induction of callose formation in suspension cells. Certain amphipathic compounds (digitonin, acylcarnitine, Polymyxin B, Echinocandin B) and polycations (chitosan, poly-L-ornithine, poly-L-lysine) are suitable, although their effectiveness varies considerably (Kauss 1987a; Kauss 1990; Waldmann et al. 1988).

Associated with the induction of callose synthesis is an efflux of K^+, an external alkalinization and a net Ca^{2+} uptake into the cells. Evidence is accumulating that these changes in ion-transport rates are not simply caused by the formation of nonspecific "pores" but are more specific and represent part of a general signal-transduction system in the plasma membrane (Kauss 1987a; Kauss 1990; Waldmann et al. 1988). Although Ca^{2+} might be one of the second messengers involved, many details of the system remain to be elucidated. Its function might, however, be of a more general interest in plant cell physiology as treatments inducing callose deposition also lead to other changes in cell metabolism, e.g. the formation of the phytoalexin glyceolline in soybean cell suspensions (Köhle et al. 1984; Kauss 1987a) and of furanocoumarins in parsley cell suspensions (data not shown).

Abbreviations: DP = degree of polymerization; FDA = fluorescein diacetate; PE = pachyman equivalents

It was already obvious from the differences in chemical compositions of the above elicitors that their initial interaction with the cells might be non-specific with regard to their chemical structure. It was also observed that high-molecular-weight poly-L-lysine was more effective than low-molecular-weight poly-L-lysine (Köhle et al. 1985). Although this polycation is a poor callose inducer compared with chitosan, it has been predicted that with polycations in general long molecules might be more efficient callose elicitors. To gain a better insight into the primary steps of callose induction, we describe here the influence of the various physical parameters of chitosan on its ability to induce the production of callose.

Material and methods

Suspension cells. Catharanthus roseus (L). G. Don, line 385, was grown as described by Waldmann et al. (1988) for 5 d (shortly before the end of logarithmic phase). The cells were washed on a funnel with and suspended in 10 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol/2-(N-morpholino)ethanesulfonic acid (Bistris/Mes) buffer, pH 5.7, containing 3% (w/v) sucrose and 4% (v/v) of the growth medium; cell density was 6 g (fresh weight, FW) per 100 ml. In such a suspension the concentration of free Ca^{2+} ($[Ca^{2+}]$) was 60–80 µM, as determined with an ion-selective electrode (Waldmann et al. 1988). Alternatively, undiluted growth medium was used to wash and suspend the cells.

Aliquots of 5 ml of the cell suspension (300 mg FW, about $1.5 \cdot 10^6$ cells) were added to 0.5 ml of water containing the respective chitosan sample and rolled in 25-ml glass vials for 3 h as described by Waldmann et al. (1988). Two parallel samples were run for any condition investigated. The vials were filled with about 20 ml of ethanol, allowed to stand for 1 h or overnight, and the cells harvested by suction through a 2.5-cm GF/A glass microfibre filter (Whatman, Maidstone, UK). After a further washing with ethanol the cells and filter were allowed to dry at room temperature, immersed in 5 ml of 1 N NaOH and homogenized together in a ground-glass homogenizer (Braun, Melsungen, FRG). The homogenate was centrifuged and two aliquots (200 µl) used to determine their callose content by spectrofluorometry with aniline blue (Kauss 1989). The 1.3-β-glucan pachyman was used to establish calibration curves, and the callose content expressed as pachyman equivalents (PE), which represent only a relative measure as the fluorescence efficiency depends on the degree of polymerization (DP) of the 1.3-β-glucan. The absolute values for the callose formed might be severalfold higher than those given in PE (Köhle et al. 1985).

Protoplasts. One culture of cell suspension (75 ml, 12–15 g FW), grown as above, was washed in 25 mM of the above Bistris/Mes buffer, pH 5.7, containing 2 mM $CaCl_2$ and 0.4 M mannitol. The cells were suspended in 50 ml of the same buffer containing 10% (w/v) of the enzyme mixture Rohament CT (Röhm GmbH, Darmstadt, FRG). After 2 h of slow shaking at room temperature, the protoplasts were sedimented ($300 \cdot g$, 5 min) and resuspended in 20 ml of the above buffer containing 10% (w/v) Ficoll. Portions of 3 ml were pippeted into 12-ml centrifuge tubes, overlayed by 3 ml of the above buffer and centrifuged ($500 \cdot g$, 30 min, 4° C) in a Minifuge 2 (Heraeus Christ, Osterode, FRG) equipped with a swinging-bucket rotor. The intact protoplasts floating at the interphase were collected, diluted about fourfold with the above buffer, sedimented and resuspended in 30 ml of 25 mM Bistris/Mes buffer, pH 5.7, containing 0.4 M sucrose and 0.2 mM or 2 mM $CaCl_2$, as indicated with the experiments. The use of sucrose as an osmoticum in the final protoplast suspension had the advantage that the protoplasts did less sediment during the subsequent incubation and that, in contrast to mannitol, the osmoticum was fully soluble in 80% ethanol.

Protoplasts were counted in a Fuchs-Rosenthal chamber and diluted to give a suspension of $2 \cdot 10^5 \cdot ml^{-1}$. Aliquots of 2 ml were pipetted into 15-ml round-bottomed centrifuge tubes that contained the chitosan samples in 100 µl of water. The tubes were placed on a gyratory shaker, incubated for 3 h and approx. 10 ml of ethanol were then added to stop any reactions. After centrifugation ($300 \cdot g$, 10 min), the pellet was drained and solubilized in 0.5 ml 1 N NaOH (80° C, 10 min) and two 200-µl aliquots were used for callose determination (Waldmann et al. 1988).

Staining. Aliquots of 100 µl of the cell suspension were withdrawn after the indicated incubation time and mixed with 2 µl of a 0.5% (w/v) solution of FDA (fluorescein diacetate) in acetone. About 5 min later, a 10-µl aliquot was placed in the counting chamber. A total of approx. 100 cells were counted under phase-contrast conditions. Then the illumination was changed to fluorescence conditions and the number of those cells that showed considerable accumulation of fluorescein was recounted. This was performed twice with each batch and the values averaged. The percentage of cells rated FDA-positive after the chitosan treatment was calculated by subtraction of the number of negative cells in the controls. The FDA staining was assessed under a Zeiss (Oberkochen, FRG) epifluorescence microscope equipped with filter set 9 (excitation 450–490 nm, color splitter 510 nm, secondary filter 520 nm). For staining of callose (Kauss 1989) 10 µl of a 0.1% (w/v) solution of aniline blue WS (Merck, Darmstadt, FRG) in 1 M glycine-NaOH buffer, pH 9.5, were added to 100 µl of the cell suspension. The samples were observed with filter set 18 (excitation 390–420 nm, color splitter 425 nm, secondary filter 450 nm). The FDA and callose staining can be performed on the same sample.

Chitosan preparations. The commercial material used to purify sample C (see Table 1) was solubilized ($2\ mg \cdot ml^{-1}$) by stirring in 6% (v/v) acetic acid, centrifuged ($45000 \cdot g$, 10 min) to remove solids and precipitated by neutralization with 1 N NaOH. This procedure was repeated once, the chitosan pellet washed three times with water and freeze dried. Samples H and I were purified by a similar procedure, as described by Domard (1987). Because the chitosan precipitation was brought about by shifting the pH value the final chitosan preparations contained little Ca^{2+}. This was verified by adding stock solutions corresponding to 100 µg of chitosan to 5 ml of the cell suspension buffer. No increase in $[Ca^{2+}]$ was observed.

Partial cleavage of chitosan by nitrous acid was performed according to Muzzarelli (1977) with the following modifications. Chitosan ($20\ mg \cdot ml^{-1}$) was solubilized in 6% (v/v) acetic acid and placed on a magnetic stirrer. The amounts of $NaNO_2$ indicated in Table 1 were solubilized in 2.5 ml of water and added dropwise over 30 min. After a further stirring for about 10 min, 2.5 ml of water per 5 ml chitosan solution were added to decrease the viscosity, and the solution was brought to a pH of 7.0–8.0 with 1 N NaOH. The suspension was left overnight at 4° C and the chitosan collected by centrifugation, washed three to four times with water and freeze dried.

Table 1. Average degree of polymerization and N-acetylation as well as mode of preparation of the chitosan samples used

Type	N-Ac[a] [%]	MW[b] kD	DP_v[c]	Origin
A	25	1666	7567	[e]
B	23.8	960	4360	[e]
C	22.3	753	3420	Sigma (crab shell)
D	22	322	1460	from C (0.5 mg)[d]
E	21.2	115	520	from C (1.1 mg)
F	21	42	190	from C (2.1 mg)
G	21	18	82	from C (4.3 mg)
H	0	552	2500	[f]
I	0	238	1070	[f]
J	0	120	545	from I (1.0 mg)
K	0	61	275	from I (2.1 mg)
L	0	20	90	from I (4.2 mg)
M	0	–	30	gel perm. chrom.
N	0	–	14	gel perm. chrom.
O	0	–	8	gel perm. chrom.
P	0	–	4	gel perm. chrom.

[a] Degree of N-acetylation from i.r. measurements
[b] Average MW of the acetate form derived from viscosity measurement
[c] Average DP for A–L calculated from viscosity average MW, for M–O from gel permeation chromatography (see *Material and methods*)
[d] Numbers in parenthesis give the mg of $NaNO_2$ added to 100 mg of chitosan in order to decrease the average DP (see *Material and methods*)
[e] Commercial samples from Protan, purified according to Domard 1987
[f] Domard and Rinaudo 1983; Samples H and I have the same origin, but I was not protected by thiophenol during deacetylation

To prepare the oligomer fractions (M–P of Table 1), fully deacetylated chitosan (Domard and Rinaudo 1983) was partially hydrolyzed in HCl (12 N, 72° C), and chromatographed on Biogel in a pH-4.2 ammonium acetate-acetic acid buffer. The oligomers with DP < 15 were pure species; beyond 15, they correspond to fractions with very low polydispersity (< 1.01). In the latter case, the value of the average DP was calculated from a standardization curve established with the elution volumes of the first fifteen oligomers.

The average molecular weight of the chitosan fragments prepared by nitrous-acid cleavage and of the oligomers was determined by viscosity measurement in a 0.2 M acetic acid, 0.1 M sodium acetate buffer (pH 4.3), by means of the Robert's viscosity law (Roberts and Domszy 1982). The degree of N-acetylation was determined by infrared measurements (Domard 1987). Colloidal chitin and chitin oligomers were prepared according to Kauss and Bauch (1988).

As the various chitosan preparations differed in counterions, water content and degree of acetylation, they were all standardized to the same polyglucosamine content before use in the callose-induction experiments. Samples (2 mg · ml^{-1}) were solubilized in 20 mM acetic acid, freeze dried and re-solubilized in the same volume of water. Their glucosamine content was determined using the colorimetric assay according to Ride and Drysdale (1972) and 0.5 ml of a 1:200-diluted chitosan solution. A standard curve with 0 to 10 µg of glucosamine · HCl per 0.5 ml was run in parallel and the polyglucosamine content of the sample calculated on the basis that 1 µg of glucosamine · HCl corresponds to 0.747 µg of polyglucosamine. All chitosan solutions were diluted to give stock solutions containing 1 mg polyglucosamine per ml.

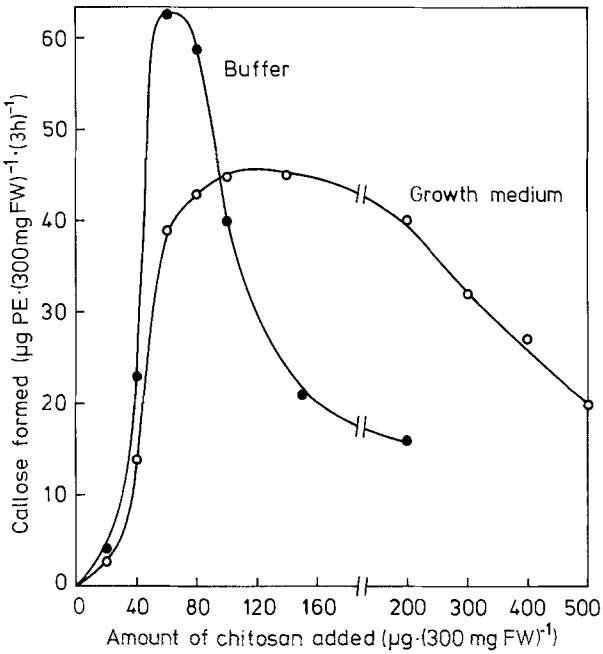

Fig. 1. Dose-response curves for chitosan-induced callose formation in *Catharanthus roseus* cells suspended either in growth medium (○) or suspension buffer (●). Chitosan C (DP 3420, 22.3% N-Ac) was used

Results

Cleavage of a commercial preparation of partially N-acetylated chitosan and a fully deacetylated chitosan (polyglucosamine) with different but limiting amounts of nitrous acid resulted in two series of polymers with decreasing DP (Table 1). These preparations, complemented with some commercial chitosans exhibiting a higher DP (A, B) and with fully deacetylated oligomers (M–P), were used as inducers of callose synthesis.

The dose-response curves for callose induction depend on the medium used to suspend the cells (Fig. 1). In buffer which contains a low concentration of ions the cells are slightly more sensitive than in growth medium, but the declining part of the dose-response curve is found at lower doses. Using a particular chitosan preparation, we observed variability among different batches of cells, although the cultures were all nominally 5 d old. The concentration at which maximal apparent induction occurred with cells in buffer varied e.g. between about 60 and 100 µg chitosan per 300 mg of cells. This variability caused some problems

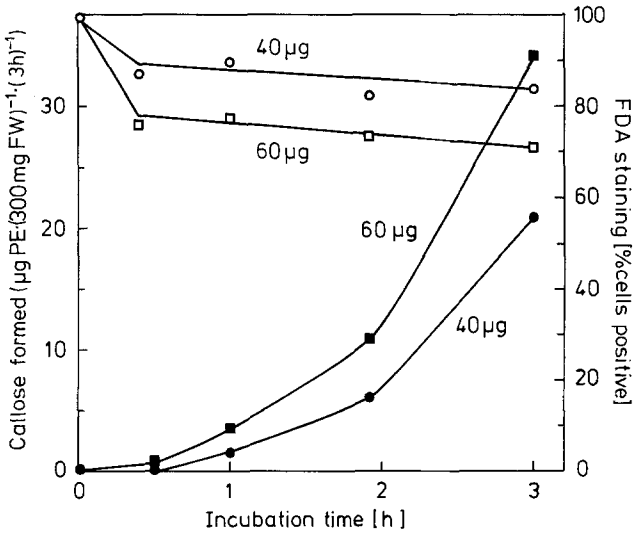

Fig. 2. Time course of chitosan-induced callose formation (●, ■) and percentage of *Catharanthus* cells staining with FDA (○, □). The cells were suspended in growth medium. Chitosan C (DP 3420, 22.3% N-Ac) was used at 40 µg (●, ○) or 60 µg (■, □) per 300 mg cell FW

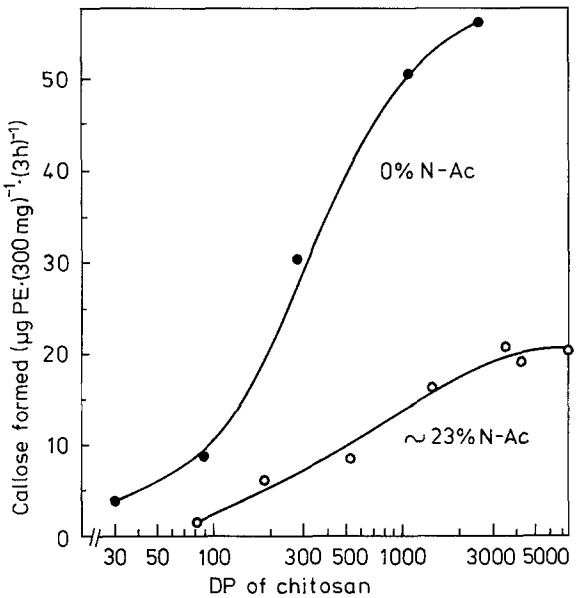

Fig. 3. Influence of DP and degree of N-acetylation of chitosan on the induction of callose formation in *C. roseus* cells suspended in growth medium. A series (Table 1) of fully deacetylated chitosans (●) was compared with another series exhibiting between 21 and 25% N-acetylation (○). Each batch of 300 mg (FW) cells received 40 µg of chitosan, standardized as polyglucosamine. If weight is taken as a basis, the amounts of 21 to 25% N-acetylated chitosan would be 1.26- to 1.31-fold higher, respectively, than with the 0% N-Ac preparation

when chitosans of a very different DP were compared (see last two lines of Table 2). In contrast, when cells were suspended in growth medium the maximal values attained were smaller compared with buffer and the working range was seemingly broader.

At a chitosan concentration of 80 µg (Fig. 1), where nearoptimal callose formation occurred in growth medium, about one-half of the cells were rated FDA-negative after 3 h of incubation. At higher chitosan concentrations, the percentage of FDA-negative cells was higher. The rating procedure was subjective; we often observed minor fluorescent regions in cells considered to be FDA-negative when compared with the majority of the cells exhibiting a bright and unevenly distributed fluorescence. Whether those cells rated FDA-negative were dead is questionable. Many of them showed callose staining but we could not distinguish whether this polymer was formed in the period before or after the cells became FDA-negative. It could well be that the membrane perturbation caused by chitosan allowed an efflux of the fluorescein liberated in the cytoplasm from FDA. Alternatively, the uptake of FDA through the lipid phase of the membrane could have been decreased, as suggested to occur in tobacco suspension cells undergoing bacteria-induced hypersensitive reaction (Keppler et al. 1988).

To circumvent the above problems, we employed chitosan concentrations between 20 and 60 µg·300 mg^{-1} FW. Under these conditions only a small percentage of the cells became FDA-negative during the first 30 min, prior to appreciable callose formation (Fig. 2). At a later time the further decrease in the number of cells staining with FDA was comparatively slow, and callose synthesis began with a rate increasing over time up to 3 h (Fig. 2). At this time, callose staining could also be observed at many of the cells rated FDA-negative. In this case it is more likely that the callose was deposited after the cells became FDA-negative, indicating that those cells were not dead but exhibited altered membrane properties, as suggested above. After 3 h and with 40 µg of chitosan, callose formation occurred at an almost constant rate for an additional 3 h, and then gradually decreased (data not shown).

When chitosan preparations of a similar DP but differing in the degree of N-acetylation were compared in experiments similar to Fig. 1, the dose-response-curves were shifted to lower concentrations for the fully deacetylated chitosan fragments. Similarly, increasing the DP caused an increase in callose formation at low concentrations of chitosan, and also raised the maximum values attained (details not shown). When the various chitosan preparations were compared at a given low

Table 2. Callose induction in suspension cells of *Catharanthus* by fully deacetylated chitosan oligomers and fragments of a higher DP. Three experiments performed with different batches of cells are given. In each case, the cells were suspended in induction buffer

Chitosan used		Callose formed (μg PE·(300 mg cells)$^{-1}$·(3 h)$^{-1}$)		
Type	DP	Experiment		
		I[a]	II[a]	III[b]
P	4	0.6	0.6	0
O	8	1.3	2.7	0
N	14	8.8	3.9	0
L	90	59	31	44
K	275	47[c]	24[c]	85

[a] 75 μg chitosan added·(300 mg cells)$^{-1}$
[b] 50 μg chitosan added·(300 mg cells)$^{-1}$
[c] 75 μg of chitosan of DP 275 were presumably already at the declining side of the dose-response curve (compare Fig. 1, buffer)

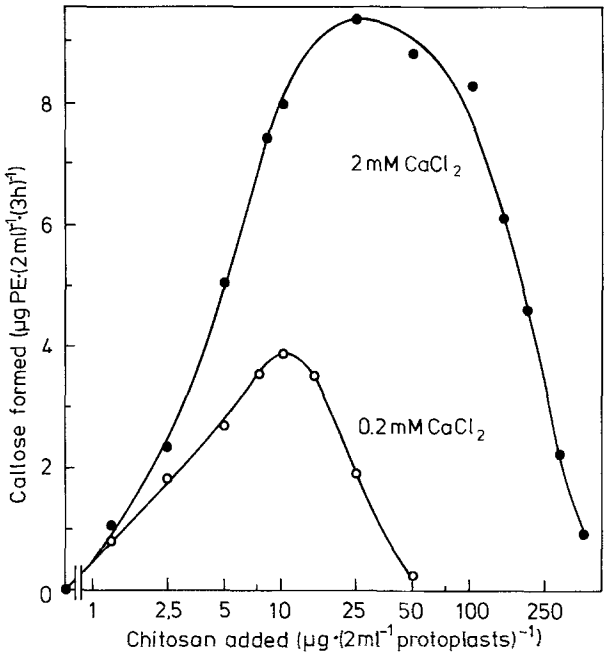

Fig. 4. Dose-response curve for chitosan-induced callose formation by *C. roseus* protoplasts suspended in buffer containing either 2 mM $CaCl_2$ (●) or 0.2 mM $CaCl_2$ (○). Chitosan (DP 3420, 22.3% N-Ac) was used. The concentrations given were standardized to polyglucosamine content and referred to $4 \cdot 10^5$ protoplasts in 2 ml of buffer. Incubation time was 3 h. Within this time, the control protoplasts in 2 mM $CaCl_2$ but without chitosan produced 0.5 μg PE callose. This value was subtracted

concentration, both trends combined to render fully deacetylated chitosan with a high DP the most effective callose elicitor (Fig. 3). It should be noted that the relative position of the curves varied between different experiments. In Fig. 3, where we worked at a low point of the dose-response curve (see Fig. 1, 40 μg), both curves come near to each other in the low-DP range. In contrast, with higher chitosan amounts (e.g. 60 μg), both curves tended to come near to each other in the high-DP range; presumably, as for the highly active fully deacetylated chitosans, the apparent optimum (see Fig. 1) was approached.

In spite of the presumed increase in instability of the cells (Fig. 1), we investigated in some detail the previously published buffer system (Kauss 1987a, b; Köhle et al. 1985; Waldmann et al. 1988). This appeared necessary as current research on the callose-induction mechanism showed that changes in the transport rates of various ions are early and important physiological events. Further examination of these changes with ion-selective electrodes or radioactive tracers requires external ion concentrations to be far lower than present in the growth medium. We, therefore, also performed experiments similar to those shown in Figs. 2 and 3 with cells suspended in buffer, and found similar results. Some of these data are presented in Table 2 which also shows that at the lower end of the DP scale, chitosan oligomers caused only a slight or, in some experiments, no callose induction.

Because of the high binding capacity of cell walls for chitosan (Young and Kauss 1983), chitosan-induced callose synthesis in protoplasts derived from the *C. roseus* suspension-cultured cells was investigated. Compared with cells (Figs. 1, 2), induction in protoplasts occurred even at very low concentrations of chitosan (Fig. 4). Inclusion of 0.2 mM $CaCl_2$ in the suspension-buffer resulted in a steep decline in callose synthesis at elevated chitosan concentrations. In contrast, 2 mM external $CaCl_2$ resulted in higher total amounts of callose, and callose formation declined at higher chitosan doses. Agglutination of the protoplasts by chitosan made impossible an accurate count of those protoplasts surviving low doses of chitosan. At doses above 10 μg a considerable number of protoplasts lysed after 3 h, although aniline-blue staining clearly indicated callose synthesis prior to lysis. At 2 mM $CaCl_2$ and 200 μg of chitosan, essentially all protoplasts lysed, although the data in (Fig. 4) show that some callose formation had occurred before lysis.

The observations made in cells with regard to the influence of DP and degree of N-acetylation of chitosan (Fig. 3) were confirmed in protoplasts.

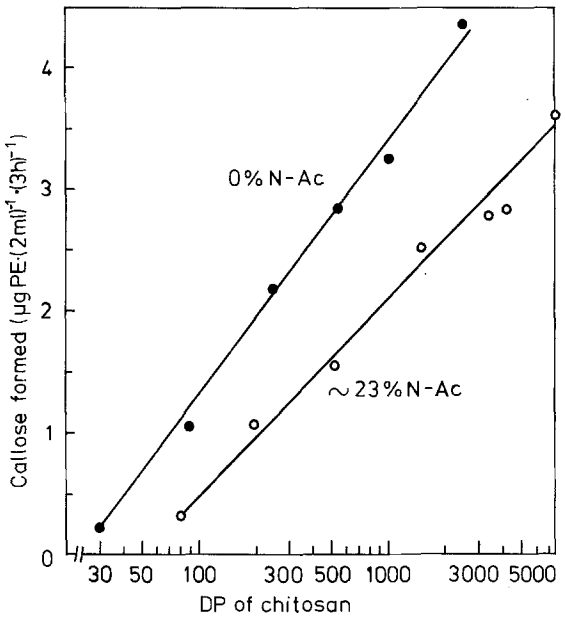

Fig. 5. Influence of DP and degree of N-acetylation of chitosan on callose formation in *C. roseus* protoplasts suspended in buffer containing 2 mM $CaCl_2$. Each batch of $4 \cdot 10^5$ protoplasts in 2 ml of buffer received 5 µg of partially acetylated (○) or fully deacetylated (●) chitosan, standardized as polyglucosamine (see Fig. 3 for remarks). In similar experiments the oligomers O (DP 8) and N (DP 14) induced only traces of callose

Oligomers exhibited a rather low inducing potency, which increased greatly up to the highest DP of fully deacetylated chitosan used. At corresponding DP values, the partially acetylated chitosans were less effective (Fig. 5). In some experiments similar to those shown in Fig. 5, the two curves were not linear at the highest DP values, especially when higher chitosan concentrations were used. For the fully deacetylated chitosans, this might have resulted simply from the fact the apparent optimum of the dose-response curve was reached (Fig. 4) but because of physical and material limitations, apparent optima could not be reestablished for each batch of protoplasts and chitosan used. For the partially N-acetylated chitosans, however, an additional rate-limiting parameter was evident from the following observation. With the same batch of protoplasts as was used for Fig. 5, we also performed an experiment where each sample received a double concentration (10 µg) of chitosan. A maximum of about 3.5 µg PE callose was already reached with partially acetylated chitosans at a DP of about 500, and there was no further increase at higher DP values. In contrast, fully deacetylated chitosans induced twice the amount of callose synthesis (data not shown). The latter indicates that the overall ability of the protoplasts to form callose was higher than reached with the partially acetylated chitosans. It might be that most of the protoplast surface was already covered and free spaces for further binding of the high-DP chitosan molecules became scarce.

At a concentration of 2 mM Ca^{2+} in the protoplast suspension, the same concentration that was used during protoplast preparation, we also observed a small but significant formation of callose without addition of any chitosan (legend of Fig. 4). This might be the first sign of spontaneous cell-wall regeneration which is slow in *C. roseus* protoplasts used here. It is known, however, that in protoplasts from many plants, callose represents the prevailing building material during the early steps of wall regeneration (Franz and Blaschek 1985). It is of interest in this context that the callose induced with chitosan, especially with incubation times greater than 3 h, has a tendency to surround the protoplasts in a shell-like manner, as shown by fluorescence microscopy after staining with aniline blue (results not shown).

With protoplasts and under the conditions described in Fig. 5, we have also assayed the inducing potency of chitin oligomers (DP 5 to 7) and of colloidal chitin. No induction occurred with 5 µg of both materials, and 50 µg induced only about 5% of the callose amounts elicited by 5 µg of chitosan C.

Discussion

The use of chitosan as an elicitor of defense reactions in plants has physiological significance because this polymer is a component of the wall of pathogenic fungi and the exoskeleton of insects. Besides callose formation, a variety of other physiological changes have been induced with chitosan. Few attempts, however, have been made to characterize the influence of the physical parameters of the chitosan preparations used. Pisatin formation in pea pods was low with chitosan trimers and increased up to heptamers, whereas the evaluation methods used did not distinguish whether preparations of a higher DP showed an equal or greater potency (Kendra and Hadwiger 1984). Monomers to tetramers of fully deacetylated chitosan were inactive in induction of lignification in wounded wheat leaves, but partially or fully deacetylated polymeric chitosans were very active (Barber et al. 1989). However, in the latter system, lignification was also induced by chitin and chitin oligomers which were almost inactive in the callose system described here. Nevertheless, with regard to chitosan, a tendency that induction requires polymers

was evident for both the pea pod and the wheat leaf system. This conclusion is extended in our present contribution on callose synthesis in suspension cultures.

Limited cleavage by variable amounts of nitrous acid resulted in a range of preparations of fully or partially deacetylated chitosans exhibiting different DP values (Table 1). The mode of cleavage results in one anhydromannose residue per split bond at the non-reducing end of every second molecule (Muzarelli 1977). It could be argued that this anhydromannose residue alters the properties of the chitosans sufficiently to render them less efficient as elicitors of callose synthesis. Fortunately, however, with the fully deacetylated chitosans we have low-DP preparations (M to P in Table 1) resulting from acid hydrolysis which do not bear the anhydromannose end. The results with these fragments fall in line with those for the nitrous-acid-cleaved fragments (Table 2, Fig. 5). This justifies the assumption that the anhydromannose residue does not greatly influence the callose-elicitation properties.

Provided that the appropriate amounts of chitosan, established by dose-response curves similar to Fig. 1, are applied, then the effectiveness of chitosan in the induction of callose synthesis increases in cells up to an average DP of about 4000 (Fig. 3). In protoplasts this tendency is even more pronounced. Some further increase can be found at still higher average DP values (Fig. 5) and might indicate that the cell wall is hindering preferentially the longer molecules of the population as a result of pore-size exclusion. Chitosan molecules in solution can be described as rigid straight rods up to DPs of approx. 100. At higher DPs they are only moderately rigid and can be represented by a worm-like model (Pogodina et al. 1986). This presumably is the reason why polymers of such a high molecular weight can penetrate the cell wall. That the cell wall partially protects the cells against chitosan becomes evident from the observation that in protoplasts approx. 1 µg of chitosan elicites a considerable response (Fig. 4) whereas in cells at least 20 µg are required (Fig. 1). Obviously, in cells at this latter concentration the chitosan is preferentially consumed by some event different from that causing callose induction, presumably by ionic binding of chitosan to wall polymers (Young and Kauss 1983). Taken together, the cell wall appears to partly protect the protoplast but is not required for the callose induction.

Although slightly less sensitive, the cells appear to be more stable towards chitosan in undiluted growth medium compared with suspension buffer, which contains comparatively low concentrations of divalent cations (Fig. 1). A similar effect is evident with protoplasts where an increase in the $CaCl_2$ concentration resulted in the decreasing portion of the dose-response curve occurring at a far higher chitosan concentration (Fig. 4). These observations represent another example of the well-known fact that external Ca^{2+} plays a dual role. It not only serves as a pool for that small fraction of Ca^{2+} required to enter cells as a second messenger but also greatly stabilizes cell membranes (see Kauss 1987a for more literature).

Applied at the same and low concentration, the effectiveness of chitosan in cells increases to a DP of 4000 (Fig. 3) and to even greater DP values in protoplasts (Fig. 5). The distance of the repeating units in chitosan in the solid state is 0.52 nm (Ogawa et al. 1984). A DP of 4000, therefore, corresponds to about 2 µm in overall chain length. To explain the increased effectiveness which occurs with the increase in DP, the binding of chitosan to the plasma-membrane surface must take place not at single points but at numerous sites occurring in abundance over long distances. Nevertheless, a 21–25% N-acetylation considerably decreases the efficiency of chitosan (Figs. 3, 5). The partially N-acetylated chitosans used were prepared by alkaline N-deacetylation of chitin. It is likely that the remaining N-acetyl groups are randomly distributed over the length of the molecule. One argument in favour of this assumption is the fact that chitin oligomers are water-insoluble above DP values of 6 to 7. This indicates that if the N-acetyl groups remaining in the chitosans were arranged in blocks of this size, they would render the chitosan insoluble. A random distribution of the N-acetyl groups over the chitosan molecule indicates that the row of amino groups is, at least statistically, interrupted in the 2- to 3-nm range. These N-acetyl residues would be sterically less favorable for the long-range interaction of chitosan with the membrane surface, possibly due to repulsive (hydrophobic) interaction with hydrophilic sites. To be most effective, the points to which binding of chitosan occurs on the plasma membrane must not only be very abundant but also must not be separated by more than a few nanometers. Phospholipids, which occupy an area of 0.4–0.6 nm^2, fulfill these conditions (Jain and Wagner 1980). Our observations on the impact of DP and the degree of N-acetylation of chitosan, therefore, indicate that the amino groups of this polymer may bind by electrostatic or ternary interactions with the negatively charged head-groups of phospholipids exposed on the plasma membrane. A chitosan molecule sitting as a

massive block on the membrane clearly will alter the membrane fluidity in the area adjacent to the binding site. Our observations that chemically different substances like digitonin, peptide antibiotics or acylcarnitine are also able to induce callose synthesis are compatible with the idea that the primary event of callose induction is not by specific binding to receptor-like molecules but by a more general change in membrane properties. The further signal transduction in the membrane remains obscure. One early event involved appears to be an alteration of ion-transport properties; possible links between the initial binding and the production of second messengers are discussed in more detail by Kauss (1990).

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References

Barber, M.S., Bertram, R.E., Ride, J.P. (1989) Chitin oligosaccharides elicit lignification in wounded wheat leaves. Physiol. Mol. Plant Pathol. **34**, 3–12

Domard, A. (1987) Determination of N-acetyl content in chitosan samples by c.d. measurements. Int. J. Biol. Macromol. **9**, 333–336

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

Fink, J., Jeblick, W., Blaschek, W., Kauss, H. (1987) Calcium ions and polyamines activate the plasma membrane-located 1,3-β-glucan synthase. Planta **171**, 130–135

Franz, G., Blaschek, W. (1985) Wall regeneration in protoplasts of higher plants. In: The physiological properties of plant protoplasts, pp. 171–183, Pilet, P.E., ed. Springer, Berlin Heidelberg New York

Hayashi, T., Read, S.M., Bussell, J., Thelen, M., Lin, F.-C., Brown Jr., R.M., Delmer, D.P. (1987) UDP-Glucose:(1->3)-β-glucan synthases from mung bean and cotton. Plant Physiol. **83**, 1054–1062

Jain, M.K., Wagner, R.C. (1980) Introduction to biological membranes. John Wiley, New York

Kauss, H. (1987a) Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. **38**, 47–72

Kauss, H. (1987b) Callose Synthese. Regulation durch induzierten Ca^{2+}-Einstrom in Pflanzenzellen. Naturwissenschaften **74**, 275–281

Kauss, H. (1989) Fluorometric measurement of callose and other 1,3-β-glucans. In: Modern methods of plant analysis 10, pp. 127–137, Linskens, H.F., Jackson, J.F., eds. Springer, Berlin Heidelberg New York Tokyo

Kauss, H. (1990) Role of the plasma membrane in host/pathogen interactions. In: The plant plasma membrane – structure, function and molecular biology, Larsson, Ch., Møller, I.M., eds. Springer, Berlin Heidelberg New York Tokyo, (in press)

Kauss, H., Bauch, B. (1988) Chitin deacetylase from *Colletotrichum lindemuthianum*. Methods Enzymol. **161B**, 518–523

Kendra, D.F., Hadwiger, L.A. (1984) Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. Exp. Mycol. **8**, 276–281

Keppler, L.D., Atkinson, M.M., Baker, C.J. (1988) Plasma membrane alteration during bacteria-induced hypersensitive reaction in tobacco suspension cells as monitored by intracellular accumulation of fluorescein. Physiol. Mol. Plant Physiol. **32**, 209–219

Köhle, H., Jeblick, W., Poten, F., Blaschek, W., Kauss, H. (1985) Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. Plant Physiol **77**, 544–551

Köhle H., Young, D.H., Kauss, H. (1984) Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. Plant Sci. Lett. **33**, 221–230

Muzzarelli, R.A.A. (1977) Chitin. Pergamon Press, Oxford

Ogawa, K., Hirano, S., Miyanishi, T., Yvi, T., Watanabe, T. (1984) A new polymorph of chitosan. Macromolecules **17**, 973–975

Pogodina, N.V., Pavlov, G.M., Bushin, S.V., Mel'nikov, A.B., Lysenko, Y.B., Nud'ga, L.A., Masheva, V.N., Marchenko, G.N., Tsvetkov, V.N. (1986) Conformational characteristics of chitosan molecules as demonstrated by diffusion-sedimentation analysis and viscosimetry. Polymer Sci. U.S.S.R. **28**, 251–259

Ride, J.P., Drysdale, R.B. (1972) A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol. **2**, 7–15

Roberts, G.A.F., Domszy, J.G. (1982) Determination of the viscometric constants for chitosan. Int. J. Biol. Macromol. **4**, 374–377

Waldmann, T., Jeblick, W., Kauss, H. (1988) Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. Planta **173**, 88–95

Young, D.H., Kauss, H. (1983) Release of calcium from suspension-cultured *Glycine max* cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. Plant Physiol. **73**, 698–702

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