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# Interaction between chitosomes and concanavalin A

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**Abstract**

Chitosomes, small secretory vesicles with *S*-value of ca 100 that act as conveyors of proteolytically-activatable chitin synthase (ChS) to its site of action at the cell surface of fungi, display an unusually high affinity for concanavalin A (ConA), if present in a compacted milieu. The outstandingly strong bonding between the lectin and 100 S-ChS established under this condition in all probability results from a superposition of four types of interaction: 'classical' lectin recognition, hydrophobic complexation, extended lectin-receptor bridging, and lectin-mediated liposome/liposome aggregation. The following three-step-procedure for the purification of ChS, together with the result of lipo-selective affinity chromatography (AC) of the enzyme on heparin, allowed the identification of a single 60-kDa polypeptide as a UDPGlcNAc transferase with defining enzymic properties of ChS: (i) isolation of gradient-purified chitosomes according to a standard procedure; (ii) ConA-AC of chitosomes to remove non-binding as well as other contaminating proteins that interact with the lectin only at its saccharide binding site; (iii) selective desorption of ChS with digitonin, methyl mannoside or NaCl following dilution of the chitosome-loaded ConA-gel. The results support the notion of a glycoconjugate nature of ChS, provide a means of obtaining homogeneous preparations of ChS for structural and mechanistic analyses as well as for biotechnological applications, throw light on some hitherto unexplained findings encountered using the Scarborough method to label the plasma membrane with ConA, and weaken the experimental basis for the firmly entrenched tenet of the plasma membrane as the only locus operandi of ChS. 

*Keywords:* Chitin synthase (EC 2.4.1.16); Chitosomes; Purification; Concanavalin A; Digitonin; *Mucor rouxii*

## 1. Introduction

Chitosomes are microvesicles (40–70 nm; *S*-value of ca 100) acting as conveyors of chitin synthase (ChS) to its site of action at the cell surface (Bartnicki-Garcia, 1989). They can be considered complex natural analogues of small liposomes (for definition, see Lasic (1993)). Thus, upon treatment with the detergent digitonin (DIG), they dissociate into subparticles [ca 16 *S*; (Ruiz-Herrera, Bartnicki-Garcia & Bracker, 1980; Hänseler, Nyhlén & Rast, 1983a)], and DIG depletion leads to their reconstitution to enzymically active vesicles [ca 120–130 *S*, i.e. 'heavy' chitosomes (Hänseler et al., 1983a; Rodewald, 1990)], the process proceeding via disk/cup-like intermediates (Hänseler et al., 1983a) as required by the concept of lipid vesicle formation by detergent removal (Lasic, 1993; Lasch, 1995). The kinetic and regulatory properties of the DIG-solubilized enzyme and reconstituted chitosomes are basically the same as those of native chitosomes [for these, see (Hänseler et al., 1983b; Cabib, 1987; Horsch, Mayer & Rast, 1996)], but none of the ChS preparations described hitherto can be considered with confidence to represent the pure enzyme (see Discussion for Refs.).

Concanavalin A (ConA) is the most widely used lectin both in cell biology and structural analysis of glycoproteins (Lis & Sharon, 1984). In biochemical mycology, ConA has served as the tool of choice to label the plasma membrane (Scarborough, 1975), especially to establish the locus operandi of ChS (Giménez & Martinez, 1989; Duran, Bowers & Cabib, 1975; Vermeulen, Raeven & Wessels, 1979; Kang, Au-

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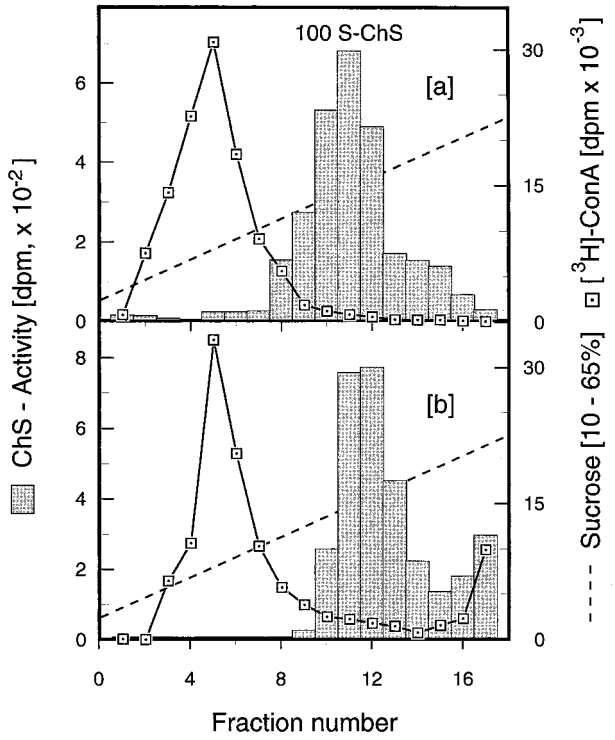


Fig. 1. Prevention of a stable interaction between chitosomal chitin synthase (100 *S*-ChS) and free ConA by sucrose. The samples subjected to rate-zonal centrifugation were: (a) gradient-purified chitosomes (peak fraction ex density gradient; in ca 28% sucrose, supplemented with [$^3$H]ConA); (b) same as (a), but freed from sucrose. For further details, refer to Experimental.

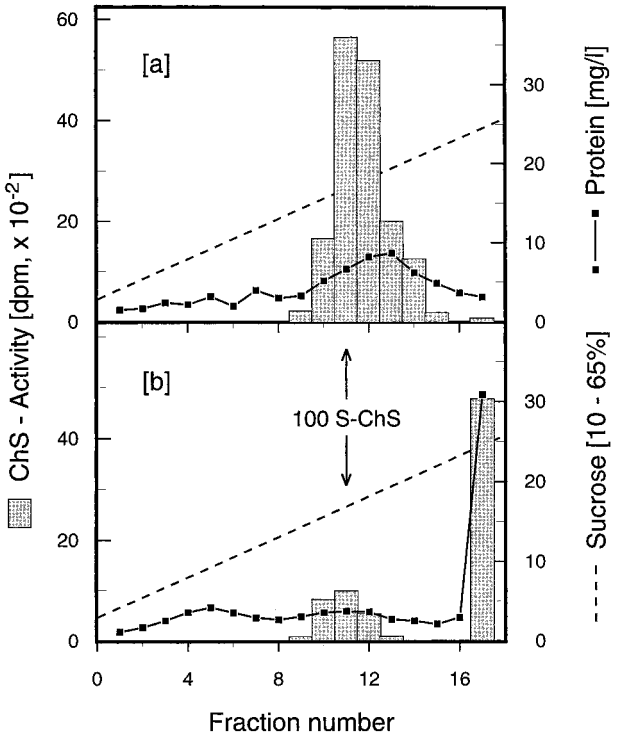


Fig. 2. Interaction between chitosomes and a ConA-gel under batch conditions: (a) control (gradient-purified chitosomes, as in Fig. 1a); (b) as (a), but incubated in the presence of ConA coupled to an agarose gel. Following centrifugation into a sucrose density gradient, about half of the chitosome pool is present in bound form. The experiment was repeated twice. For further details, refer to Experimental.

Young & Cabib, 1985; Cabib, Bowers & Roberts, 1983). Considering the principles of the secretory pathways for enzymes with site of action at the cell surface (Balch, 1989), non-interaction of chitosomes with ConA, as reported (Bartnicki-Garcia, Bartnicki & Sentandreu, 1985; Giménez & Martinez, 1989), would appear rather unusual. All surface-located enzymes appear to be glycoproteins, regardless of whether plasma membrane-bound or secreted on to the protoplast (Dwek, Edge, Harvey & Wormald, 1993; Lis & Sharon, 1993). Moreover, 100 *S*- as well as 16 *S*-ChS can be affinity-purified with lentil lectin (Merz, Horsch, Schaller & Rast, 1993; Merz, 1997), which displays the same basic carbohydrate binding specificity as ConA (α-Man > α-Glc > GlcNAc) but is somewhat more exacting (additionally α-Fuc (Goldstein & Poretz, 1986)). Further, there are several potential glycosylation sites in the coding sequence of ChS genes (Bulawa et al., 1986; Yarden & Yanofsky, 1991). Finally, purification of DIG-solubilized ChS by ConA affinity chromatography has been described (Machida & Saito, 1993).

The present study was performed to investigate the apparently exclusive behaviour of chitosomes towards ConA and, thus, possibly also to gain a new means of purification of ChS. Preliminary accounts of the work presented here have been published (Merz, Horsch, Ruffner & Rast, 1996; Merz, 1997).

## 2. Results

### *2.1. Binding of chitosomes to ConA in batch vs column operation*

No binding occurred between 'standard' chitosomes (peak fraction of the sucrose density gradient centrifugation step) and ConA, as judged from the lack of co-migration of ConA and ChS upon centrifugation of chitosomes previously presented with the lectin in its free form (Fig. 1a). Removal of sucrose, which is a competitor at the saccharide binding site of ConA (Goldstein & Poretz, 1986), prior to incubation did allow some ligand/receptor coupling, but only to a minor extent (Fig. 1b). Batch incubation of chitosomes with ConA-coupled gels (Sepharose 4B, macroporous acrylic beads), however, resulted in a good interaction between the two (Fig. 2; Merz, 1997). This was clearly lectin-mediated, as no binding at all occurred with the bare gel matrices. In fact, the ConA-gel/chitosome

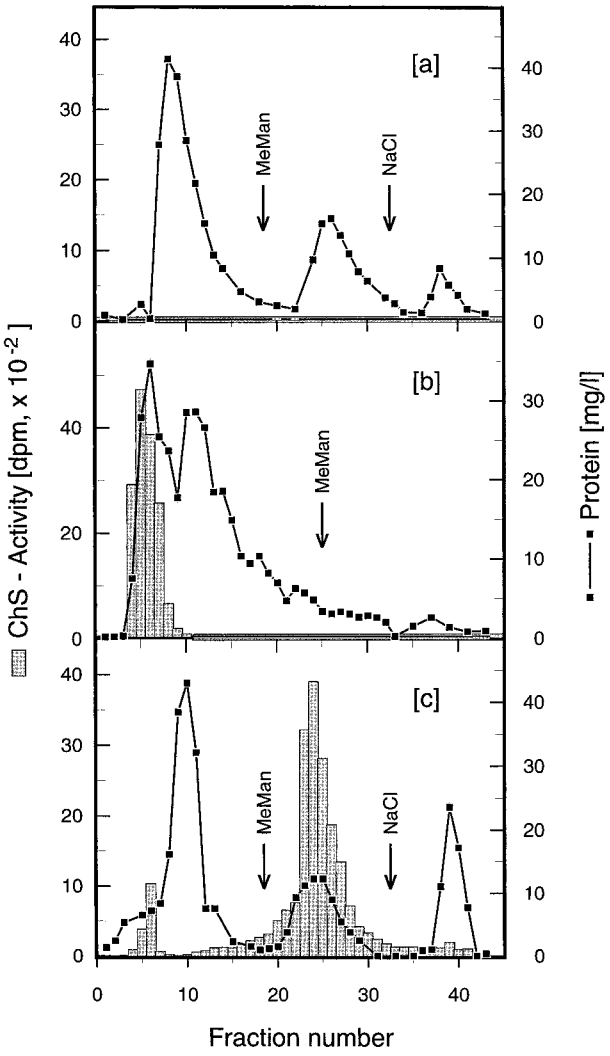


Fig. 3. Strong binding of chitosomes to ConA-gel packed in column. (a) A preparation of chitosomal ChS (100 *S*-ChS; peak fraction of sucrose density gradient; freed from sucrose by gel filtration) was transferred on to a ConA-Sepharose column. Following washing with buffer, elution was performed with MeMan and NaCl (for details, see Experimental). None of the desorbed proteins displayed ChS activity. (b) Prevention of chitosome binding to the ConA affinity column by pre-equilibrating with sucrose (10%). (c) 'Normal' lectin binding of chitosomes upon pre-equilibration of the column with DIG (0.1%). All experiments were repeated twice, with basically the same results.

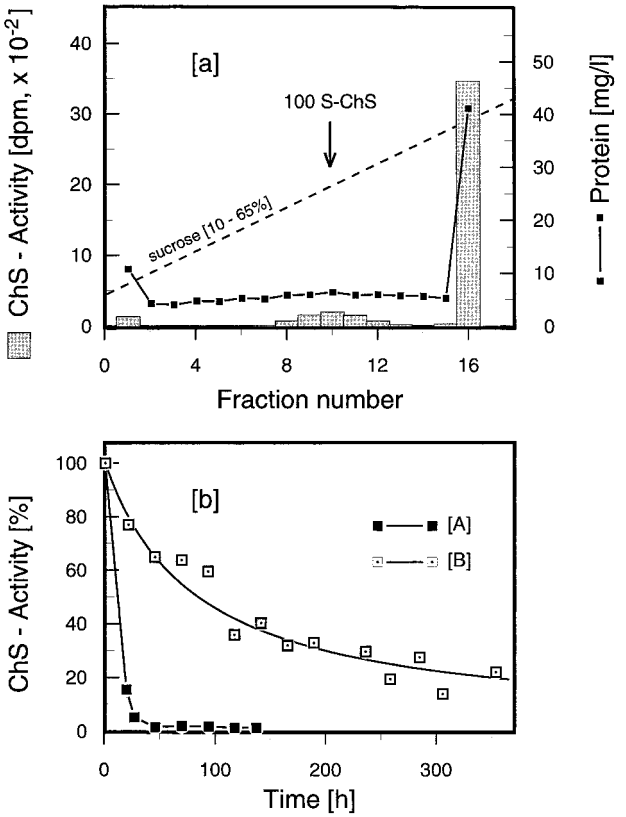


Fig. 4. Establishment of a stable interaction between chitosomes and ConA-gel in a column. (a) A sample (1 ml) of chitosome-loaded ConA-gel (obtained after subjecting a preparation of gradient-purified 100 *S*-ChS to conditions of conventional AC: see Fig. 3a and Experimental) was centrifuged into a sucrose density gradient. Most of the ChS remained firmly bound to the ConA-gel sedimenting to the bottom of the tube. The arrow indicates the position of non-bound 100 *S*-ChS. (b) Increase in chitosome stability upon immobilization on ConA-gel. Samples: (A) ChS ex 5–50% density gradient (control); (B), same preparation as used in (a). 100% ChS activity corresponds to 20,157 dpm $h^{-1}$ for (A) and 3968 dpm $h^{-1}$ for (B). For (a) as well as for (b), representative data are shown from one out of three experiments.

complex proved to be stable enough to be only partly interfered with by centrifugation into a high-sucrose density gradient (compare Fig. 2 with Fig. 1).

Upon attempting ConA affinity chromatography (AC) of chitosomes, no protein with ChS activity could be eluted with 0.25 M MeMan (Fig. 3a). This situation was not changed by increasing either the ionic strength of the buffer (up to 0.5 M NaCl) or the concentration of MeMan (up to 1 M). Although 100 *S*-ChS is inhibited under these conditions (90 and 75%, respectively; Merz, 1991), the residual activity should have been sufficiently high to be detectable in eluate fractions. It was, therefore, concluded that the absence of ChS activity in the eluate was due to tight binding of the chitosomes to the ConA-gel. Indeed, the thoroughly washed and fractionated ConA-gel material obtained following AC of the chitosome preparation (as in Fig. 3a) displayed ChS activity, the bulk of which was located in the upper third of the column (Merz, 1997). The ConA-gel chitosome interaction generated in the column proved to be so strong that it was only slightly weakened (as judged from the release of free chitosomes) by high centrifugal forces (Fig. 4a). Binding of the enzyme to ConA-gel also greatly increased its longevity (Fig. 4b). The possibility can be excluded that the high affinity of chitosomes to the ConA-gel would be due to an interaction with the polysaccharide matrix of the ConA-gel or to a mere

Table 1
Efficacy of various agents tested as desorbents for ConA-Sepharose immobilized ChS (Prepared as described in Methods, paragraph 'Affinity chromatography'). The experiment was repeated twice, with basically the same results

| Compound | | ChS activity[a] | | Effect of desorbent on 100 *S*-ChS activity[b] | | Relative recovery of ChS[c] |
|---|---|---|---|---|---|---|
| (final concentration) | | dpm | | dpm | % | *x* |
| | | (1) | (2) | (3) | (4) | (5) |
| Buffer (control) | | 1132 | 196 | 6020 | 100 | 1 |
| Methyl mannoside (MeMan) | (0.5 M) | 3034 | 786 | 4214 | 70 | 6 |
| NaCl | (0.5 M) | 690 | 246 | 602 | 10 | 13 |
| MeMan | (0.5 M) | | | | | |
| + NaCl | (0.5 M) | 808 | 193 | 542 | 9 | 11 |
| Urea | (0.1 M) | 1545 | 223 | 5358 | 89 | 1 |
| EDTA | (0.1%) | 1934 | 145 | 3371 | 56 | 1 |
| Dioxan | (1%) | 1138 | 203 | 4395 | 73 | 1 |
| Ethylene glycol | (10%) | 1437 | 462 | 4575 | 76 | 3 |
| Tween-20 | (0.1%) | 545 | 90 | 3552 | 59 | 1 |
| Digitonin (DIG)[d] | (0.1%) | 5808 | 4066 | 8067 | 134 | 15 |
| DIG | (0.1%) | | | | | |
| + MeMan | (0.5 M) | 6344 | 5307 | 6321 | 105 | 26 |

[a] Samples (300 µl) of ConA-immobilized ChS from column (Fig. 1a) were mixed with an equal volume of test solution and slowly stirred during 30 min (RT). ChS activity was assessed (1) in the suspension, and (2) in the supernatant (corresponding to the net recovery of ChS activity from the gel) obtained after low-speed centrifugation (Eppendorf 5414 S9, 10 s).
[b] Activity assessed separately with gradient-purified chitosomes.
[c] Calculated from the values given in row (2) and accounting for the effect of the desorbent on the ChS activity of free chitosomes [row (4)]. The factor $x = 1$ refers to buffer as the desorbent.
[d] From SIGMA; formula 'for use in aqueous solutions'.

physical entrapment of the particles, since the same occurred with macroporous acrylic ConA beads, and since ChS could be quantitatively recovered in the void volume upon simple permeation chromatography of the sample through the bare gel matrix (Merz, 1997). The same conclusion can be drawn from the data presented in Fig. 3b, which demonstrates that binding does not occur in the ConA-gel column if this is pre-equilibrated with 10% sucrose (corresponding to the ConA binding inhibitory activity of 12% Glc (Goldstein & Poretz, 1986)). Performing the analogous experiment (Fig. 3a) with the glycolipid DIG changes the type of the chitosome ConA interaction in the column from one of very strong adsorption to that of common lectin chromatography (Fig. 3c). As observed in the case of MeMan and NaCl, neither sucrose nor DIG alone could, however, be used for elution of ChS in ConA-AC once the chitosome ConA-gel complex had been established.

### 2.2. Desorption of chitosomes from ConA-gel

To disrupt the strong chitosome ConA-gel bonding generated in a tightly packed column (Figs. 3 and 4) various measures [(i)–(v)] potentially conducive to desorption were tested.

(i) A decrease in the hydrophobicity of the micro-environment of the chitosome ConA complex [in view of the existence in ConA of hydrophobic binding sites unrelated to the saccharide binding site (Goldstein & Poretz, 1986; Reeke & Becker, 1988)], which could be effected by removal of the ChS-loaded ConA-gel from the column, diluting the sample with buffer and performing desorption under batch conditions; (ii) weakening of the chitosome ConA interaction mediated by the saccharide affinity site (Figs. 1 and 3) through complexation of the metalloprotein with EDTA [the $Mn^{2+}$ and $Ca^{2+}$ constituents of ConA are essential for its saccharide binding activity (Reeke & Becker, 1988)]; (iii) the use of an established agent suitable to desorb proteins (through induced conformational changes) that have been non-specifically attached by hydrogen bonding, such as urea; (iv) application of apolar solutes, to interfere with any non-specific hydrophobic adsorption of chitosomes; and (v) displacement of ChS from the hydrophobic binding site through competition with amphiphiles.

As shown in Table 1, row (5), of all agents tested singly as desorbents of active ChS, DIG performed best. Other surfactants were ineffectual. DIG and MeMan in combination acted slightly synergistically. A total of four desorption steps released ca 98% of the bound ChS activity. Activity testing of ChS samples obtained following desorption with DIG/

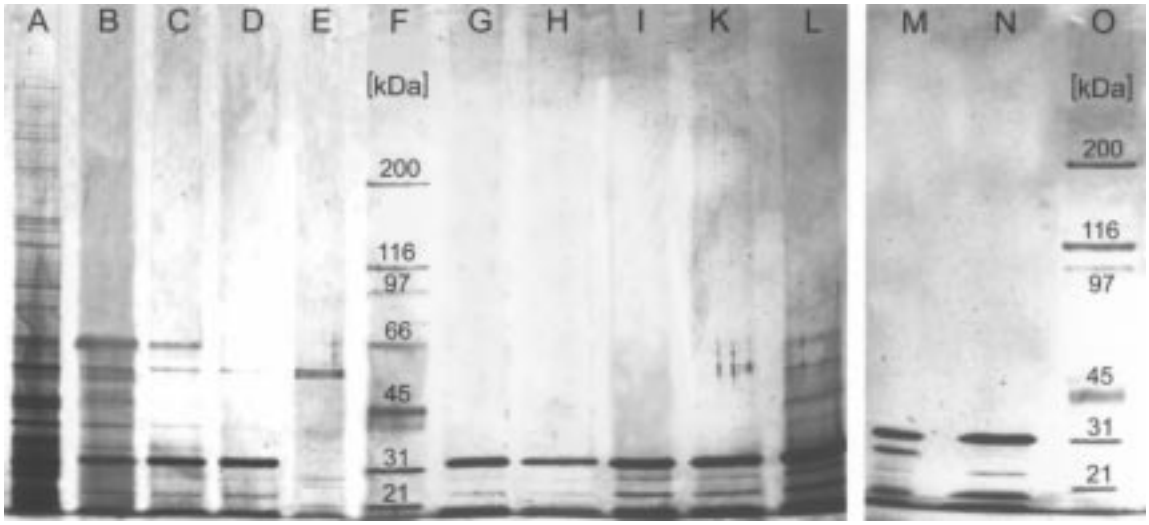


Fig. 5. Ability of various agents to selectively desorb ChS polypeptides from a ConA-gel previously loaded with gradient-purified chitosomes, as assessed by SDS-PAGE. For the isolation of samples, see captions to Figs. 1a and 3a, and Table 1 [row (2), and footnote a; sample size was 40 μl (lanes A–D), and 60 μl (G–L). A, gradient-purified chitosomes; B–D and G–L: ChS obtained following desorption with, respectively, DIG/MeMan (0.1%/0.5 M), MeMan/NaCl (each 0.5 M), NaCl (0.5 M), urea (1 M), EDTA (0.1%), ethylene glycol (10%), dioxan (1%) and Tween-20 (0.1%); E, ChS preparation ex heparin-AC (second minor peak; see Experimental); M, free ConA standard protein; N, zero control of method (see Results); F and O markers. Sample sizes (μl) were 6 (lane A), 40 (lanes B–D), 80 (lane E), and 60 (G–L). For further details, refer to Experimental.

MeMan [see Table 1, row (2); scaled up 8-fold] and ultracentrifugation into a 10–65% sucrose density gradient without DIG yielded about 5% of the total enzyme activity as 16 *S*-ChS, 15% as chitosomes, and the rest as distinctly different heavy particles sedimenting at the bottom (Merz & Rast, unpublished data).

### *2.3. Selectivity of desorbents for ChS from chitosome-loaded ConA-gel*

The SDS-PAGE polypeptide patterns of samples afforded by treatment of ConA-immobilized ChS with the agents listed in Table 1 show two prominent bands, at 32 and 20 kDa, and minor ones at 24 and 28 kDa (Fig. 5, lanes B–D and G–L), besides 1–7 bands in the 60 kDa region. For arguments (i)–(iv), the polypeptides of the 30 kDa area are ConA-contaminants of the ChS-eluates. (i) The reference ConA produces the same four bands (Fig. 5, lane M). In fact, electrophoretic polydispersity of commercial ConA preparations, even of best grades available (as used in the present study), has been reported repeatedly (Sophianopoulos & Sophianopoulos, 1981; Marikar, Zachariah & Basu, 1992; Fountoulakis & Juranville, 1993) and is undoubtedly due to the co-occurrence of the main 32 kDa-protein with some of its posttranslationally-produced precursors (for details see Bowles et al. (1986) and Discussion). (ii) Staining in the low-molecular-weight range of the gels from ChS preparations obtained by other types of lectin-AC was considerably weaker and the corresponding pattern in any case not the same as for ConA (Merz, 1997), and with ChS samples ex heparin-AC staining in the area concerned was even negligible (Fig. 5, lane E). (iii) Removal of the lectin (monomers or dimers) from gel-bound ConA (tetramer at the conditions of ligand coupling, at ca pH 8.0 (Goldstein & Poretz, 1986)) can occur through formation of soluble lectin/receptor aggregates with certain sample components, e.g., hydrophobic glycoproteins (Marikar et al., 1992), or even be caused by eluants themselves (Fountoulakis & Juranville, 1993). (iv) The SDS-pattern of the zero control (sample obtained by performing all steps of the chitosome/ConA-gel interaction protocol followed here, but in the absence of chitosomes) also displayed the prominent 32- and 20-kDa bands (Fig. 5, lane N), the 28 kDa-component (the unglycosylated ConA monomer species) presumably having been removed at the adsorption step.

Therefore, the region of the gels below 32 kDa is to be disregarded with respect to the ChS polypeptide(s) sought, and differences in the remaining part of the patterns observed must be due to differences in selectivity of the various agents tested for disruption of the ChS/ConA interaction. Thus, on the one hand, virtually no protein was released from the ChS/ConA-gel upon treatment with urea (G), EDTA (H), ethylene glycol (I) and dioxan (K); on the other hand, Tween-20 as well as a digitonin(DIG)/MeMan combination (B) afforded a polypeptide pattern with several entities (5–7: L and B). The similarity of the two patterns concerned is probably due to both detergents being soluble amphiphiles with ability to solubilize proteins through formation of micelles. The two agents are dis-

tinctly different, nevertheless, in other relevant properties (Helenius & Simons, 1975). Indeed, only in the presence of DIG (a type B surfactant) was there desorption of ChS polypeptides with retention of enzyme activity (see Table 1), whereas Tween-20 displayed a denaturing action analogous to that of SDS (both type A surfactants). However, the ChS preparation obtained with the DIG/MeMan desorbing agent still contains protein components not intrinsically necessary for ChS activity in vitro, since replacement of the saponin by NaCl reduced the number of possibly relevant bands to 3–4 (Fig. 5, lane C). NaCl alone proved to be the most selective of all desorbents tested in that the ChS isolate afforded only one band, corresponding to a 60 kDa-protein (lane D). The identity of this with a ChS polypeptide carrying the catalytic site was confirmed by a comparison with the SDS-PAGE pattern of an active ChS-preparation produced by heparin-AC of gradient-purified 100 *S*-ChS, which displays the very same band, in addition to a 57 kDa-polypeptide (Fig. 5, lane E).

## 3. Discussion

### *3.1. Nature of the chitosome/ConA interaction*

In contrast to earlier papers stating that chitosomes lack ConA-binding sites (Giménez & Martinez, 1989; Bartnicki-Garcia et al., 1985), interaction between 100 *S*-ChS and the lectin occurs and can even be very strong (Figs. 2, 3, 4a; Table 1). This binding concurs with an increase of the longevity of the enzyme (Fig. 4b), possibly because it stabilizes the ChS polypeptide in its native conformation—analogous to the situation experienced by the lectin ligand upon complexation at the saccharide binding site (Loris, Hamelryck, Bouckaert & Wyns, 1998). The reported non-binding was undoubtedly due to a superposition of three experimental parameters used in those studies, any of which is conducive to the establishment of an association/dissociation equilibrium that is shifted greatly towards the free receptor ligand: (1) binding analyses were performed in the presence of the competitor sucrose (Goldstein & Poretz, 1986; Figs. 1 and 3a,b) and DIG (Fig. 3a,c), (2) the lectin ligand was provided in its free instead of an immobilized form (see Figs. 1 and 2), and (3) attempted complexation occurred under batch conditions in solution.

Thus, there are two major conditions favouring stable complexation between ConA and chitosomes (100 *S*-ChS): some alignment of ConA as achieved through immobilization to an appropriate matrix, and a lowering of the incidence of dissociation events through reducing diffusion, while still providing for sufficient accessibility of the lectin to the macroligand. Both requirements are best met by a ConA column (further advantages of the lectin column procedure over other lectin-based methods for the study of lectin-binding are summarized elsewhere (Lakhtin, 1995)). Because of arguments 1–8 that follow, it is proposed that the unusually tight adsorption of chitosomes to ConA generated under these conditions is due to a superposition of four types of interactions: 'classical' lectin complexation, hydrophobic binding, extensive lectin/receptor bridging, and lectin-mediated liposome-liposome fusion:

1. ConA is known to display various types of nonpolar interactions (Goldstein & Poretz, 1986), of which only two have been investigated to some extent. The first involves a region adjacent to the monosaccharide binding site that contains several subsites for nonpolar bonding and, thus, provides for the higher affinity of ConA for particular oligosaccharides and monosaccharides carrying a hydrophobic aglycon (Loris et al., 1998). The second concerns a hydrophobic region with which nonpolar ligands establish stable interactions independently of any carbohydrate binding (Hardman & Ainsworth, 1973) and is presumably the main hydrophobic core of the ConA monomer (Loris et al., 1998).
2. Pre-equilibration of the ConA-AC column with the competitor sucrose precludes chitosome/ConA interaction (Fig. 3b), and impairment of hydrophobic bonding results in a type of adsorption that can be disrupted by specific carbohydrate competition (Fig. 3c). The possibility can be excluded that this binding would not concern a sugar residue of the receptor, but represent a selective mimicry of carbohydrate by peptide recognition (Scott, Loganathan, Easley, Gong & Goldstein, 1992; Oldenburg, Loganathan, Goldstein, Schultz & Gallop, 1992), since ChS does not display the necessary consensus sequence Tyr-Pro-Tyr. These findings thus suggest that the primary recognition event occurs at the saccharide binding site of the lectin, but is not responsible alone for the unusually strong ConA/chitosome complexation. Nevertheless, the immobilization and ensuing orientation of the macroligand at the carbohydrate binding site of ConA is likely to facilitate subsequent nonpolar interaction in an aqueous environment generally hostile to hydrophobic binding and, thus, to confer this some specificity, even if it were unspecific per se. Tight hydrophobic bonding to ConA that is only partially interfered with by carbohydrate competition has been reported also for certain plasma membrane receptors (Nachbar, Oppenheim & Aull, 1976) and been interpreted similarly, i.e. as a secondary hydrophobic interaction of the lectin with

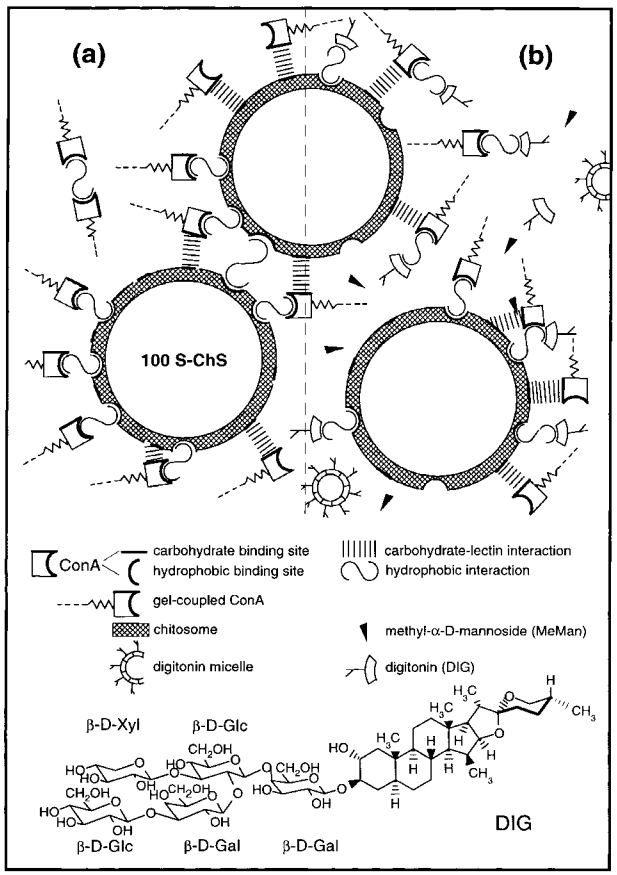


Fig. 6. A graphical summary of the interactions between chitosomes and ConA that provide for the extraordinarily strong adsorption of the receptor to the lectin in a compacted milieu (an inert, macroporous ConA affinity gel in a column, with lectin ligands exhibiting self-aggregation through hydrophobic oligomerization) (a), and a scheme depicting some of the interactions between ConA-bonded chitosomes and solutes that effect desorption of chitin-synthesizing particles upon a decrease of the hydrophobicity of the medium (established through dilution of the chitosome/ConA packing and transferring it to batch conditions) (b). (a) Tight chitosome/ConA bonding is generated by a superposition of four types of interactions: 'classical' lectin binding, hydrophobic complexation independent of carbohydrate recognition, extended lectin-receptor bridging, and lectin-mediated liposome/liposome aggregation. (b) Disruption of the multivalent chitosome/ConA complex by the combined action of a strong amphiphile and a hydrophilic agent (the steroid glycoside DIG and MeMan) by specific competition at the respective binding site of the lectin. Additional interactions, occurring at the supramolecular level, i.e. ConA-mediated vesicle/vesicle fusion and the disintegration of chitosomes by the micelle-forming detergent, are not depicted.

the polypeptide portion of the glycoprotein following attachment of the oligosaccharide moiety of the glycoprotein.

3. Nonpolar binding of 100 *S*-ChS to ConA could also be considered to have the character of a protein/protein complexation between a subsite of the apolar region next to the saccharide binding site of the lectin (see argument 1) and a hydrophobic stretch of the enzyme polypeptide (Yarden & Yanofsky, 1991), or even with an aromatic residue of the presumptive catalytic site itself (Horsch & Sowdhamini, 1996; Merz, Horsch, Nyhlèn & Rast, 1999). Such an interpretation is, however, incompatible with the observed failure of apolar solvents to release the enzyme from the ConA-gel (Table 1 and Fig. 5)—unless one would invoke a recently described unique property of ConA (Figlas, Arias, Fernandez & Alperin, 1997), namely, to become greatly stabilized, through a higher degree of folding upon complexation of an appropriate carbohydrate receptor ligand with the lectin's saccharide binding site (Loris et al., 1998).
4. ConA binds to simple cholesterol- and carbohydrate-free phospholipid bilayers (Van der Bosch & McConnell, 1975; Boldt, Speckart, Richards & Alving, 1977). Inasmuch as it can be inhibited (in part) by MeMan, the reaction is considered specific and involves a hydrophobic interaction of the lectin with the fatty acid partial structure of the phospholipid (Van der Bosch & McConnell, 1975). Since chitosomes are complex natural liposomes (see Introduction) and are rich in non-glycoconjugated glycerophosphatides (Hernandez, Lopez-Romero, Cerbon & Ruiz-Herrera, 1981; Weete, Furter, Hänseler & Rast, 1985), the same must hold for the ConA/chitosome system. Nevertheless, the strong nonpolar complexation occurring between ConA and chitosomes (Fig. 3) cannot be due solely to this phenomenon, because such binding would lack the observed specificity (see arguments 5 and 7 below).
5. The fact that among a whole range of apolar agents tested singly as desorbents only the saponin DIG (for formula, see Fig. 6) performed satisfactorily (Table 1), indicates specificity of the hydrophobic receptor binding reaction sought. Yet, some minor contribution of the carbohydrate side chain of the amphiphile to the disruption of the chitosome/ConA complex cannot be ruled out, since the terminal β-1,3-linked glucosyl residue complies to the stereochemical conditions of a (moderate) competitor and since there occurs a slight inhibition of carbohydrate binding by DIG, as indicated by the small proportion of chitosomes occurring in the void volume upon ConA-AC with a column pre-equilibrated with DIG (Fig. 3c). However, Glc does not act as an eluent (data not shown). Hence, if the oligosaccharide moiety of DIG makes a specific and substantial contribution to its good performance as a desorbent it would probably have to be sought at the level of the quaternary structure of the lectin that can provide for a higher level of specificity (Loris et al., 1998). One may, therefore, assume that it is basically the very hydrophobic aglycone part of the steroid glycoside that acts as the specific competitor at the affinity site responsible for the outstandingly strong ConA-chitosome interaction.

6. The specificity displayed by DIG as a competing ligand at the affinity site responsible for the strong hydrophobic chitosome/ConA complexation (Table 1) is paralleled by the specificity also of the DIG/chitosome interaction that brings about a stimulation of enzyme activity of 100 *S*-ChS (Hänseler et al., 1983b) or a 'solubilization' of membrane-bound ChS with retainment or even enhancement of enzyme activity (Ruiz-Herrera et al., 1980; Hänseler et al., 1983a; Rodewald, 1990; Machida & Saito, 1993; Gooday & DeRousset-Hall, 1975; Duran & Cabib, 1978; Leal-Morales, Gay, Fèvre & Bartnicki-Garcia, 1997), since other surfactants, including various steroid analogues, are ineffectual (Machida & Saito, 1993; Duran & Cabib, 1978).
7. Through oligomerization, ConA becomes a multivalent ligand with orientation of binding sites especially suitable for lateral association with ligands; in the presence of oligosacharides, this results in the formation of highly ordered homogeneous cross-linked lattices (Loris et al., 1998; Brewer & Bhattacharyya, 1988; Drickamer, 1995). Multivalency of carbohydrate ligands also holds for 100 *S*-ChS, as this consists of several, presumably identical 16 *S* subparticles that are glycoconjugated (see Introduction, and (Rodewald, 1990)). Extensive ConA-mediated bridging of chitosomes is undoubtedly particularly favoured in an AC-column (Fig. 3), as opposed to a batch system (Figs. 1 and 2), because the high concentration of the affinity ligand relative to that of the receptor promotes more rapid and quantitative binding (Cummings, 1994). Further, the enormous size difference between the two ligands also is of importance. Thus, complexation between a chitosome and the AC-ligand—even if it were to occur on just one site—generates an immobilized ConA/receptor configuration that exposes many binding sites in a highly ordered array, a situation, which (a) greatly reduces the proportion of 'non-productive' binding events that are caused by the dissipation of the receptor ligand and the ensuing shift of the association-dissociation equilibrium towards the free receptor ligand, and (b) drastically favours extensive three-dimensional cross-linking of the ligands. This finally results in the generation of extended ConA-chitosome networks within the polysaccharide matrix, to which these are 'pinned' at the sites of the primary affinity ligand/receptor coupling.
8. With the size and density of single lectin/chitosome nets increasing and finally merging with each other, the meshes will even extend into the interstices between the AC-gel beads, thus causing the reaction medium to become less polar and greatly compacted. This is a situation where hydrophobic interactions are furthered, not only at the molecular (see arguments 2 and 4) but also at the supramolecular level. Thus, macroligand/macroligand recognition and aggregation will occur, for which 100 *S*-ChS has an innate tendency (Ruiz-Herrera, Bracker & Bartnicki-Garcia, 1984), as have secretory vesicles in general (Lodish et al., 1995). The aggregation between ConA-bridged chitosomes could even take the character of a ConA-mediated liposome/liposome fusion, as described for a simple phospholipid system (Van der Bosch & McConnell, 1975; see also argument 4), and be furthered by the calcium present in the AC-buffer (for the fusigenicity of $Ca^{2+}$; see Lasic, 1993). Not only the gel-coupled but also the free lectin ligand generated under AC-conditions (see Fig. 5 and Results) is likely to contribute considerably to this effect.

Fig. 6a represents a diagrammatic summary of the types of interactions between chitosomes and ConA that account for the outstandingly strong bonding of the receptor to ConA-gel under AC-conditions. Theoretically, desorption should follow the reverse order of events leading to adsorption, but since the present system is very heterogeneous with respect to types of bonding, there will be overlapping, and the physical integrity of the multidentate macroligand vesicle is not likely to be preserved. Thus, the dismantling of the three-dimensional chitosome/ConA network overlying points of attachment to the gel should occur first. Indeed, the chitosome/ConA-gel complex cannot be interfered with by lectin binding inhibitors (Fig. 3a) prior to a reduction of the hydrophobicity of the milieu. This measure is not, however, per se sufficient to cause more than just a small de-complexation, even in the presence of a saccharide binding inhibitor (Fig. 4a). The first attack must, therefore, concern the abolition of chitosome aggregation configurations through the action of a detergent, for which purpose DIG—a spirostanol glycoside (Fig. 6)—was found to be suitable (Table 1). Since this is a neutral and 'soft' micelle-forming detergent able to solubilize chitosomal ChS (see Introduction), its interaction with gel-bound chitosomes in all probability leads to the generation of mixed DIG/ChS micelles and, finally, proteoliposomes built of chitosomal phospholipid and sterol [for their identities, see Hernandez et al. (1981), Weete et al. (1985), and Lopez-Romero, Monzon and Ruiz-Herrera (1985)] as well as ChS-protein and DIG. These vesicles may be called 'digitosomes' and mimick a biological membrane (Lasic, 1993; Helenius & Simons, 1975; Martinek, Levashov, Klyachko, Khmelnitski & Berezin, 1986). With the dismantling of supramolecular chitosome/ConA aggregation structures and the breakage of lectin/receptor bridges proceeding, competition at the specific hydrophobic and saccharide binding

sites of gel-coupled ConA gradually becomes more effective (Fig. 6b).

The sum of the following observations (1)–(6) represents strong, although still circumstantial evidence for the hypothesis that the hydrophobic receptor structure of chitosomes for ConA might be ergosterol (E). (1) E is the characteristic membrane sterol of fungi synthesizing chitin (Gooday, 1995). (2) Chitosomes contain a fair proportion of sterol, with E as the major component (Weete et al., 1985; Lopez-Romero et al., 1985; Schmid, 1988). (3) Chitosomes and DIG are competitors for the main hydrophobic binding site of ConA (Fig. 3c; Table 1; Fig. 6). (4) DIG binds hydrophobically to chitosomes (Hänseler et al., 1983b; this study) as well as to 3β-hydroxysterols, the latter interaction resulting in the generation of a very stable steroid/sterol adduct (Akiyama, Takagi, Sankawa, Inari & Saito, 1980; Cenedella, 1982; Nishikawa, Nojima, Akiyama, Sankawa & Inoue, 1984; see also Budavari, 1996). (5) Both chitosomes and DIG are strongly hemolytic (Merz, 1997; Tschesche & Wulff, 1973), the activity of the spirostanol being due to displacement of cholesterol from the erythrocyte membrane. (6) Another excellent E-binding glycolipid with similar micelle-forming ability to DIG and with a definite selective preference for E over cholesterol, namely, the polyene macrolide antibiotic amphotericin B [(AmB); Barwicz & Tancrède, 1997; Bolard, 1986; for earlier Refs. see Hammond, 1977], also tightly binds to chitosomal ChS—as evidenced by its modulatory effect on enzyme activity (Rast & Bartnicki-Garcia, 1981; Rodewald, 1990) and by the co-migration of chitosomes and AmB during gel filtration, with a clear-cut separation of the chitosome/AmB peak containing the bulk of the polyene from that of non-complexed AmB (Rodewald, 1990).

### 3.2. Biochemical implications

#### 3.2.1. Glycoconjugation of ChS

The interaction of 100 *S*-ChS with ConA requiring participation of the lectin's carbohydrate binding site (Fig. 3b,c; arguments 1–3 in Discussion) and the specificity of ConA for complexation only with certain glycoproteins (Goldstein & Poretz, 1986; Reeke & Becker, 1988) clearly suggest that the enzyme is glycosylated (for further supporting arguments, see Introduction and Merz et al. (1999)). Some direct evidence comes from total carbohydrate determinations of purified, although not homogeneous ChS isolates ex lens lectin AC (the binding fraction affords several bands upon SDS-PAGE, besides a diffuse band at 60 kDa; Merz et al., 1993; Schaller, 1992), and from the distinctly resolved positive staining with the PAS-reagent of the 60-kDa-protein on SDS/PAGE of FPLC-purified 16 *S*-ChS ex walls (Rodewald, 1990). A detailed analysis as to the type of *N*-glycoconjugation of the enzyme implied by these results, however, requires the availability of sufficient quantities of homogeneous ChS polypeptide(s).

#### 3.2.2. Purification of ChS and biotechnological applications

The procedure described in this work to purify ChS using selective adsorption of chitosomes to ConA-gel and selective desorption of the enzyme (Fig. 3, Table 1) yields the purest ChS preparations described hitherto, with components identified separately as genuine ChS polypeptides or contaminant proteins (Fig. 5, lanes E and F) regardless of whether obtained from a mixture of membranes and wall fragments (Kang, Elango, Mattia, Au-Young & Robbins, 1984), from unspecified microsomes (Montgomery, Adams & Gooday, 1984), chitosomes (Ruiz-Herrera, Martinez, Casanova, Gil & Sentandreu, 1987; Martinez, Giménez, Bracker & Bartnicki-Garcia, 1989; Rodewald, 1990; Flores-Martinez et al., 1990; Lending, Leal-Morales, Flores-Martinez, Bracker & Bartnicki-Garcia, 1991), or from a plasma membrane fraction (Leal-Morales et al., 1997). The fact that ConA affinity columns display leakage of various ConA species, of which the 32 kDa component is the most prominent (see Results), casts doubt on the claim by Machida and Saito (1993) to have purified ChS to homogeneity, purportedly represented by a single 30 kDa polypeptide, as their isolation protocol involves ConA-AC (in the presence of DIG; analogous to Fig. 3c). Indeed, the amino-terminal sequence of the putative 30 kDa-ChS protein (12 amino acids) was identical with that of the corresponding stretch of ConA (Carrington, Auffret & Hanke, 1985) and clearly distinct from that of ChS. That this polypeptide was glycoconjugated, as is to be expected for ChS, cannot weaken the strength of our argument, because ConA preparations used in preparative biochemical work probably all contain a glycoconjugated precursor form (33 kDa) of the non-glycosylated, mature ConA monomer (28 kDa) studied in physico-chemical investigations (Bowles et al., 1986; Loris et al., 1998). Thus, the ChS activity of the enzyme isolate described in (Machida & Saito, 1993) in all probability must have been due to ChS protein having been present in a concentration below the detection limit of the silver stain method under conditions exhibiting only the main (ConA)component of the eluate. This is also evident from the authors' data on sample sizes applied. Additional, independent support for the likelihood of an artefactual origin of the 30 kDa-protein in the ChS isolate concerned comes from the SDS-PAGE patterns of ChS preparations purified by other methods. Thus, no such band is present in the samples obtained by heparin-AC of chitosomes (Fig. 5, lane E) or by

electrofocussing of 16 *S*-ChS (Rodewald, 1990). Obviously, the use of antibodies (ABs) raised against the 30 kDa-'ChS'polypeptide (Machida & Saito, 1993) to assess the cellular location of ChS is prone to produce a misleading result. Indeed, although there occurred some AB binding at or near the plasma membrane, most of the immunolabel was present elsewhere, at the inner edge of large vesicles (ca 0.5–1 μm; i.e. vacuoles; Machida, Itoh, Kishida, Higasa & Saito, 1994). This criticism does not discount the possibility that part of a cell's ChS pool resides in the vacuole (Chuang & Schekman, 1996), but this is a location highly unlikely to be the site of action of ChS in a growing cell (see, also, Section 3.3).

To increase the efficiency and possibly also the resolution of ConA-AC for the purification of chitosomal ChS in a one-step operation, a Cu-chelate:ConA sandwich affinity sorbent as described by Anspach and Altmann-Haase (1994) might serve this purpose even better than the procedure described here. In either case, however, co-eluting ConA would have to be eliminated (e.g. by membrane filtration) to yield homogeneous ChS polypeptide(s) suitable for truly reliable investigations into the structure and mode of action of the enzyme. As gradient-purified chitosomes can easily be lyophilized following removal of sucrose (although with a loss of up to 50% of the initial activity at the time of collection of the pool) and are stable for months (Schmid 1988; Hänseler, 1983; Furter, 1985), since binding of 100 *S*-ChS to ConA increases the longevity of the enzyme (Fig. 4b), and because there is 100% recovery of ChS from chitosome-loaded ConA-gel following diluting this with buffer (Merz, 1997), the prospects of thus obtaining bulk amounts of pure ChS in form of dry chitosome/ConA powders suitable for long-term storage are good. Such ChS-products would be particularly advantageous for biotechnological applications, e.g., in high-throughput screening for highly specific inhibitors of the enzyme as well as the rational design of broad-spectrum antifungals having only ChS as the target for use in medicine or crop protection.

### *3.3. Cytological implications*

Knowledge of the exact location of Chs at the cell surface while in action is important not only for practical considerations relating to the bioavailability of inhibitors of the enzyme as potential antifungals and the likelihood of their leading to multidrug resistance of the hosts, but also to answer fundamental questions of fungal developmental cell biology. On Occam's razor, the concepts of the secretory pathway for the transfer of proteins to the cell surface (Lodish et al., 1995) should also hold for ChS vesicles, which carry the enzyme in a latent, proteolytically-activatable form (Ruiz-Herrera, 1992). One of these principles is the fusion of the transport vesicles with the plasma membrane, regardless of whether the proteins are designated to become intrinsic components of this, or to be released on to the protoplast surface. It appears unquestionable, therefore, that there is at any time some ChS associated with the cell membrane of a growing cell. This is held widely to be the only site of chitin synthesis, with the enzyme spanning the plasma membrane thought to bind the substrate at the cytoplasmic face and to feed out the immobile product at the outer face (Cabib, 1987; Gooday, 1995). On the other hand, the possibility has to be considered also that chitosomes dissociate upon fusion with the plasma membrane and that post-translational modification of the 16 *S*-ChS thus set free results in the release of ChS into the periplasmic space and the adjacent wall region (for arguments, see Hänseler et al. (1983a), Horsch et al. (1996) and Horsch, Mayer, Sennhauser and Rast (1997)). The necessity inherent in the second hypothesis for a mechanism effecting the transfer of the ChS substrate on to the protoplast surface, poses no obstacle in principle to its acceptability, since this requirement could conceivably be met either by fusion with the plasma membrane of Golgi-derived vesicles carrying UDPGlcNAc (St-Pierre, Ruetz, Epstein, Gros & Arias, 1994; Abejion, Mandon, Robbins & Hirschberg, 1996; Traynor et al., 1996), or by direct extrusion of the nucleotide from the cytoplasm by an organic solute transporter of the ABC superfamily with broad substrate specificity (Fath & Kolter, 1993; Rea, Li, Lu & Drozdowicz, 1998).

The evidence for the first hypothesis relies heavily on results obtained with the Scarborough procedure (Scarborough, 1975), which consists in surface-labelling the cells with radioactive ConA as a marker for the plasma membrane, lysing them and then fractionating of the homogenate. The major premiss of the method is that only components of the plasma membrane are able to bind ConA and that this remains bound during homogenization, i.e. that there is no displacement of label to internal ConA receptors, e.g. secretory vesicles (Bourett & Howard, 1994), to which chitosomes belong (Bartnicki-Garcia, 1989). This becomes of a major concern if the lectin surface coat, which also serves to stabilize the membrane against vesiculation, is heavy. In case of the quantitative assessment of the cell membrane *vs* chitosome location of ChS there is another possible source of error not accounted for by the Scarborough method, namely, the likely fusion of (some of) the ChS transport vesicles with the plasma membrane particles (see Lodish et al., 1995, and paragraph above), thus artefactually increasing the proportion of the total cellular ChS pool presumably present in the cell membrane in vivo (a view expressed earlier by others (Flores-Martinez & Schwenke, 1988)). Considering these complications, it is not unexpected that the corresponding

values are widely divergent, in as much as they range from 1 to 80% (Duran et al., 1975; Vermeulen et al., 1979; Kang et al., 1985; Flores-Martinez & Schwenke, 1988; Bartnicki-Garcia, Bracker, Lippman & Ruiz-Herrera, 1984; Leal-Morales, Bracker & Bartnicki-Garcia, 1994).

The avidity of chitosomes for ConA and the unusual strength of the complex once established (fair stability under high centrifugal forces even in the presence of the competitor sucrose; non-dissociation by MeMan alone; stability to disruption by EDTA; see Results), together with the fact that free ConA causes bridging, aggregation and even fusion of vesicles (arguments 7 and 8 in Section 3.1), afford an explanation for some of the puzzling observations stated in Duran et al. (1975) and Kang et al. (1985). Thus, upon using ConA in a low concentration (0.5 mg $ml^{-1}$; with some 25% of the protein content of the plasma membrane fraction being accounted for by the lectin) in a lysis solution not containing EDTA (as in the original Scarborough protocol), the bulk of the total ChS activity recovered was associated with the plasma membrane fraction, and ca 20% with apparently non-labelled low-density particles unrelated to this (presumably chitosomes). Using EDTA as the lysing agent, however, more label (up to some 40%) was found in these particles with increasing ConA concentrations (up to 5 mg $ml^{-1}$) applied at the coating step. Moreover, the density and the size distribution of these particles increased considerably. A likely reason for these discrepancies is, thus, that part of the ConA load of the plasma membrane fraction was released through complexation of EDTA with the metal ions of the lectin that are essential to keep this in the locked conformation necessary for carbohydrate binding (Loris et al., 1998). The ConA thus set free would, thence, become bound to chitosomes (analogous to the situation represented by Fig. 2b) and these increasingly become aggregated with each other and even with plasma membrane fragments. A similar caveat for a possibly artefactual nature of supporting experimental data concerns a recent hypothesis, whereupon chitosomes would be endosomes (Chuang & Schekman, 1996). This notwithstanding, in the authors' model of the same year (Ziman, Chuang & Schekman 1996), the alternative view that chitosomes may be Golgi-derived vesicles of the constitutive secretory pathway has been maintained. Furthermore, according to a very recent study (Ziman, Chuang, Tsung, Hamamoto & Schekman, 1998), again involving subcellular fractionation with ConA as a plasma membrane marker, both regulated and secretory pathways are postulated for chitosomes, depending on the ChS species to be surface-targeted.

Thus, the validity of claims about the exact location of ChS in the living cell is doubtful, if based on results obtained with the Scarborough method (at low ConA concentrations used to label the cell membrane, this will not be sufficiently stabilized against disruption and yield small vesicles at the lysis step with similar sedimentation behaviour to chitosomes; at high concentrations, the above-mentioned phenomena will occur). However, two procedures not relying on ConA, namely, (1) separation of chitosomes and plasma membrane vesicles by a combination of isopycnic and velocity sedimentations (Leal-Morales, Bracker & Bartnicki-Garcia, 1988), and (2) application of a preparation of an anti-CHS antibody (ChS2 gene product antibody) to identify the enzyme in cell fractions and localize the enzyme in situ, (Sietsma, Beth Din, Ziv, Sjollema & Yarden, 1996), also yielded conflicting results. Whereas method (1) indicated approximately equal distribution of the enzyme between the plasma membrane and chitosomes, (2) did not display ChS associated with the cell membrane upon fractionation, but labelled the chitosomal membrane. Clearly, the ideal method to approach the problem of the subcellular location of ChS and, perhaps more importantly (see Section 3.2.2), the exact site of its action at the cell surface, remains to be found.

## 4. Experimental

### 4.1. Standard buffer

Unless stated otherwise, 50 mM $KH_2PO_4$/NaOH, pH 6.5, containing 10 mM $MgCl_2$, was used throughout.

### 4.2. Chitin synthase (ChS) assay

The assay mixture contained, in a total volume of 125 μl of buffer, enzyme preparation (50 μl), Rennilase (a crude acid protease, 125 μg), 1 mM UDP-[U-$^{14}$C]GlcNAc (Amersham; 55,000 dpm), and 20 mM GlcNAc; the incubation was at room temperature (RT) and the reaction time was 20–60 min. Chitin deposition was determined by a filtration method (Ruiz-Herrera & Bartnicki-Garcia, 1976). Thus, incorporation of 1000 dpm $h^{-1}$ into chitin corresponds to an enzyme activity of $0.6 \times 10^{-3}$ nkat.

Some adsorption of UDPGlcNAc to the ConA-gel occurs (not, however, to the gel matrix itself: data not shown). This amounted to 610 pmol UDP-GlcNAc per sample, corresponding to $268 \pm 14$ dpm ($N = 4$); and this has been accounted for in the results presented in Figs 2 and 4.

### 4.3. Source and purification of chitosomes

Apart from minor modifications, chitosomal ChS was isolated from log-phase hyphae of *Mucor rouxii*

ATCC 24905 grown in submerged liquid culture, as described previously (Bartnicki-Garcia, Bracker, Reyes & Ruiz-Herrera, 1978; Rast, Horsch, Furter & Gooday, 1991). The homogenate was centrifuged at 4000 *g* (5 min), the supernatant centrifuged again, at 54,000 *g* (45 min; Beckman type 30 rotor), and the supernatant (turbid; ca 30 ml) used for further fractionation. The sample (3 ml) obtained following gel filtration of the enzyme on a column (26 × 380 mm) of BioGel A-5 m (100–200 mesh), ribonuclease treatment and concentration by ultrafiltration (Amicon 202, Diaflo XM-300 A membrane), was loaded on to a convex sucrose density gradient (5–50% (wt/v), 36 ml) and centrifuged (104,000 *g*, 2 h 40 min; Beckman SW-28). Contaminating sucrose was removed from the pool of the combined peak fractions (3 ml) by gel filtration on a column of BioGel A-5 m (10 × 200 mm; flow rate 0.4 ml $min^{-1}$) previously equilibrated with standard buffer.

Separation of free chitosomes from ConA-bound 100 *S*-ChS was by rate-zonal centrifugation in a linear sucrose density gradient (10–65% (wt/v), 8 ml; 199,000 *g*, 18 h; Beckman SW-41 Ti rotor). Fractions of 0.5 ml were collected with a Buchler Auto Densiflow II and assayed for ChS activity and protein (Bradford, 1976; microprocedure) using a microtiter plate reader.

### 4.4. Batch incubation of chitosomes with ConA

ChS samples (0.5 ml; gradient-purified) were incubated either with free [$^3$H]ConA (*N*-acetyl-[$^3$H]ConA, Amersham, 1 μCi, and unlabelled ConA (type V; Sigma) as the carrier, 520 μg $ml^{-1}$) or with ConA-Sepharose 4B (Pharmacia); the final volume was 1 ml, the incubation time 15 min (RT). Separation of components was by linear sucrose density gradient centrifugation (see Section 4.3).

### 4.5. Affinity chromatography (AC)

For ConA-AC the sucrose-free enzyme preparation was transferred on to a column of ConA-Sepharose 4B (10 × 100 mm, graduated; flow rate 0.17 ml $min^{-1}$; 4°C), extensively washed and pre-equilibrated with standard buffer supplemented with 50 mM NaCl and $MnCl_2/CaCl_2$ (1 mM each; hereafter referred to as ConA buffer). The column was eluted with α-D-methyl mannoside (MeMan; 0.25 M) followed by NaCl (0.5 M) and 1 ml fractions collected. Following extensive washing with buffer (to remove NaCl, which inhibits ChS: see Results), buffer (2 ml) was applied on to the top of the column and carefully mixed into the upper 2 ml of the gel filling. The preparation of ConA-Sepharose-immobilized ChS thus obtained (ca 4 ml) served for further experimentation and for purification.

Heparin-AC was carried out under the same general conditions as ConA-AC, but omitting the NaCl/$MnCl_2/CaCl_2$ supplement; desorption was effected with a two-step NaCl-gradient (0.1 and 0.5 M). Besides the major peak (eluting with 0.5 M NaCl, together with the bulk of the protein), there were two minor ChS peaks, in the void volume and in the 0.1 M salt fractions, respectively (details in Merz, 1997).

### 4.6. SDS-PAGE

Electrophoresis was carried out by the Lämmli (1970) procedure (modified). Thus, linear gradient gels containing 3–17% (wt/v) acrylamide were prepared from a stock solution of 32% (wt/v) of acrylamide and 6% (wt/v) of *N,N′*-diallyltartardiamide (Sigma) as the cross-linker. Final concentrations in the gel were 0.38 M Tris-HCl (pH 8.8) and 0.1% SDS. The gel (140 × 110 mm; thickness 1 mm) was polymerized chemically by addition of 0.12% of *N,N,N′,N′*-tetramethyl ethylenediamine and ammonium persulfate. The electrode buffer (pH 8.3) contained 0.25 M Tris, 0.19 M glycine, and 0.1% SDS. The samples (up to 80 μl) contained (final concentrations of) 0.5 mM Tris-HCl (pH 6.8), 0.4 % SDS, 8.4% glycerol, 0.3% 1,4-dithio-DL-threitol, 0.001% ethylenediamine-tetraacetate (EDTA), and 0.02% bromophenol blue. Electrophoresis was carried out at 40 mA until the dye marker reached the bottom of the gel. The proteins were fixed with a solution of EtOH (27%) and formaldehyde (5%) for 1 h, and freed from the fixing solution by rinsing with $H_2O$ overnight. Visualization of proteins was performed by silver staining. $M_r$ values were estimated using a Bio-Rad standard kit.

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