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Evidence for a Phospholipid Requirement of Chitin Synthase in *Schizophyllum commune*

Cornelis A. Vermeulen* and Joseph G. H. Wessels

Department of Developmental Plant Biology, Biological Center, University of Groningen, Haren, The Netherlands

Abstract. Evidence for a lipid dependence of membrane-associated chitin synthase in *Schizophyllum commune* is based on the following observations: Arrhenius plots of the temperature dependence of this enzyme showed deflections from linearity that are characteristic for lipid-affected membrane-bound enzymes. The activity of chitin synthase dissociated by digitonin decreased at increasing digitonin/protein ratios and could be restored by addition of egg lecithin. After further delipification by sucrose gradient centrifugation, enzyme activity progressively decreased, banded at higher densities, and was less effectively restored by lecithin. The activity of dissociated chitin synthase was also restored by soybean phosphatidylcholine and low concentrations of phosphatidylinositol and phosphatidylserine. At higher concentrations, phosphatidylinositol and phosphatidylserine were inhibitory. Lysophosphatidylcholine and phosphatidylethanolamine were slightly stimulatory, whereas no effect resulting from ergosterol was observed.

Chitin, present in the cell walls of most filamentous fungi, is synthesized by chitin synthase (EC 2.4.1.16) which is, at least partly, associated with the plasma membrane in *Saccharomyces cerevisiae* [5], *Candida albicans* [1], and the basidiomycete *Schizophyllum commune* [29]. The chitin synthesized *in vitro* by protoplast membranes from *S. commune* and *S. cerevisiae* remains associated with the membranes [7,29], which supports the view that chitin synthesis is a vectorial process carried out by a membrane-associated chitin synthase with the substrate being available at the cytoplasmic site of the membrane and the product being laid down on the outer surface. The alternative explanation of a lipid intermediate transporting the precursor over the membrane is not supported by experimental data. Therefore, it is likely that the enzyme operates as an integral membrane protein, possibly spanning the lipid bilayer.

Integral membrane proteins often require phospholipids for their functional activities (for reviews see [25,28]); in some cases a specificity towards phospholipid head groups [22] or acyl chain length [15] has been reported. Recently the involvement of the lipid environment in chitin synthase activity was suggested for *S. cerevisiae* [6] and *C. albicans* [19].

The purpose of this study was to investigate the effect of delipification on chitin synthase activity and the influence of changing the phospholipid environment on the activity of the enzyme.

Materials and Methods

Culture conditions. Basidiospores collected from dikaryotic fruiting mycelium of *S. commune* obtained by mating strains 1-40 (CBS 344.81) and 1-50 (CBS 342.81) were inoculated at a concentration of 7×10^7 ml^{-1} in minimal medium and grown for 45 h at 25°C as described before [29].

Isolation of mixed membrane fractions. Mycelium was harvested by centrifugation, washed once with 0.01 M tris/HCl, pH 7.5, containing 2 mM EDTA and 0.6 mM of the protease inhibitor phenylmethylsulfonylfluoride (PMSF) and broken in an X-press (Biotec, Sweden). After thawing, unbroken hyphae and cell wall fragments were removed by centrifugation at $350 \times g$ for 15 min. The supernatant was centrifuged at $40{,}000 \times g$ for 1 h at 4°C to obtain a membrane pellet that was resuspended in incubation buffer (0.01 M tris/HCl containing 10 mM $MgCl_2$, pH 7.5), frozen in liquid nitrogen, and stored at −80°C.

Chitin synthase assay. Thawed mixed membrane fractions (MMF) were incubated for 10 min at 25°C with trypsin at concentrations depending on the amount of protein in the MMF; proteolytic activation was stopped by addition of soybean trypsin inhibitor at a concentration of twice that of trypsin. The assay system contained 50 μl of enzyme source and 25 μl of substrate (6 mM UDP-[U-^{14}C] GlcNAc with a specific activity of 0.08 μCi μMol^{-1} and 60 mM GlcNAc in incubation buffer) and was incubated for 1 h at 25°C before terminating the reaction by

* To whom reprint requests should be addressed.

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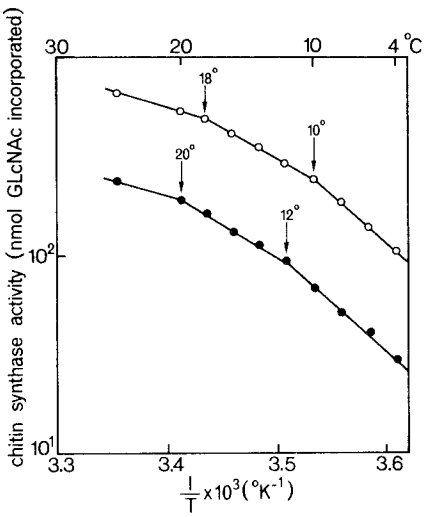

Fig. 1. Temperature dependence of chitin synthase of *Schizophyllum commune*. ●: membrane fraction (10,000 × *g* for 1 h) not activated with trypsin; ○: high speed fraction in the 40,000 × *g* supernatant sedimenting after centrifugation at 300,000 × *g* for 1 h, which was activated with 10 μg ml^{-1} trypsin. No glycerol was used in this experiment.

addition of 100 μl 2 M NaOH. The precipitate collected on Whatman GF/A glass fiber discs was subsequently washed with 0.5 M NaOH, a mixture (80:20, v/v) of 1 M acetic acid and 96% ethanol, and finally with ethanol. The filters were dried and the radioactivity was measured as described before [29].

Preparation of dissociated chitin synthase and phospholipid suspensions. Membrane preparations were incubated with 1 vol of 2% digitonin in incubation buffer with or without glycerol to a final concentration as indicated under Results and vortexed several times during a 1 h incubation period at room temperature. Centrifugation at 220,000 × *g* for 1 h at room temperature in a Beckmann SW50.1 rotor allowed the separation of dissociated chitin synthase present in the supernatant and insoluble material in the pellet, which was resuspended in incubation buffer with or without glycerol as indicated under Results. All samples taken at different times during the dissociation procedure were kept at room temperature until the final step and then simultaneously assayed for chitin synthase activity.

Phospholipids were resuspended in incubation buffer containing 1% digitonin and 22.5% glycerol and were incubated with the enzyme preparations during 30 min at room temperature before addition of enzyme substrate. Egg lecithin was suspended in incubation buffer without digitonin and glycerol.

Density gradient centrifugation. Samples (2 ml) of digitonin-dissociated chitin synthase were layered on 8.5 ml linear 20–60% sucrose gradients in incubation buffer with 10% glycerol and centrifuged at 260,000 × *g* for 40 h at 4°C in a Beckmann SW41.Ti rotor. Fractions (0.8 ml) were collected and assayed for enzyme activity.

Chemicals. Most chemicals were obtained from Sigma Chemical Co., with the exception of trypsin inhibitor (Boerhinger) and egg lecithin (BDH). UDP-[U-^{14}C] GlcNAc (323 μCi μMol^{-1}) was purchased from the Radiochemical Centre, Amersham. Protein was determined after trichloroacetic acid precipitation according to Lowry [18]. Digitonin was processed according to Bridges [2].

Table 1. Effect of digitonin/protein ratio on dissociation and activity of trypsin (100 μg ml^{-1}) activated MMF (4 mg ml^{-1} protein)-associated chitin synthase of *Schizophyllum commune* incubated with 1% digitonin in incubation buffer with 10% glycerol.[a]

	Activity relative to MMF-associated activity (%) digitonin/protein ratio			
	7.5	10	15	22.5
MMF+ digitonin	46.7 (85.0)	36.4 (89.7)	36.4 (99.1)	23.4 (101.9)
Dissociated enzyme	38.4 (64.5)	29.0 (70.1)	22.4 (71.0)	17.8 (72.9)
Insoluble enzyme	15.9 (22.4)	21.5 (15.9)	10.3 (15.0)	9.3 (16.8)

[a] Figures in parentheses refer to activities determined in the presence of 10 mg ml^{-1} egg lecithin.

Results

Temperature dependence. The activity of many membrane-bound enzymes is affected by physical modifications in the lipid bilayer as in the case of thermal phase transition from the liquid to the solid crystalline state occurring at a certain temperature (T_c). This temperature dependence is expressed as a break in the Arrhenius plot of log activity versus 1/T (see for review [25]), which is often close to T_c [16]. The temperature dependence of the active form of chitin synthase in a 10,000 × *g* for 1 h membrane fraction not subjected to trypsin activation, and of a trypsin-activated (10 μg ml^{-1}) high-speed fraction (40,000 × *g* for 1 h supernatant pelleted after centrifugation at 300,000 × *g* for 1 h) were determined as shown in Fig. 1. The membrane-bound active form of chitin synthase shows deflections from linearity at 12°C and 20°C. Similar breaks at 10°C and 18% were observed for the trypsin-activated chitin synthase in the high-speed fraction.

Detergent effect on activity and dissociation of chitin synthase. Initial experiments indicated that a number of detergents (SDS, Nonidett P40, and Triton X100) severely inhibited enzyme activity already at low concentrations. The residual activity was restricted to the insoluble fraction. Digitonin, Octyl-β-D-glucoside, and, to a minor extent, Tween 80 appeared to dissociate chitin synthase, although in the latter two cases considerable inactivation occurred. With digitonin, inactivation of chitin synthase was dependent on the digitonin/protein ratio and could be restored by preincubation with egg lecithin (Table 1). As the ratio increased from 7.5 to

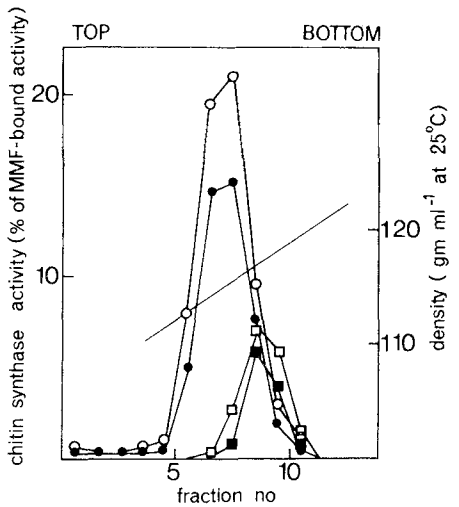

Fig. 2. Sucrose gradient centrifugation of digitonin-dissociated chitin synthase of *Schizophyllum commune*-derived MMF. Depicted are the enzyme distributions in gradients loaded with fractions dissociated at digitonin/protein ratios of 22.5 (□) and 7.5 (○), taken from the experiment described in Table 1. Open symbols refer to activities determined in the presence of 10 mg ml^{-1} egg lecithin; closed symbols refer to activities determined in the absence of lecithin.

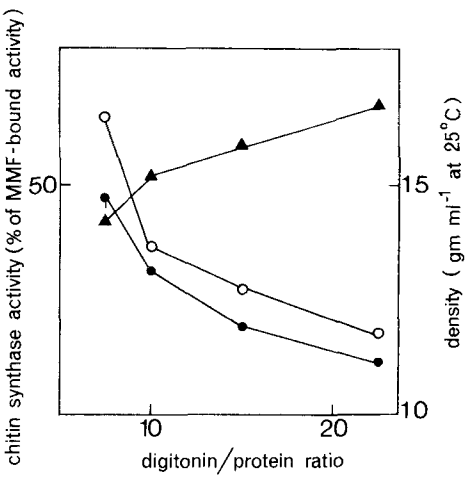

Fig. 3. Activity and banding pattern of dissociated chitin synthase of *Schizophyllum commune* after gradient centrifugation. The recoveries of enzyme activities after the gradient centrifugation described in Fig. 2 were determined in the presence (○) or absence (●) of 10 mg ml^{-1} egg lecithin. The mean positions of the chitin synthase distributions are also indicated (▲).

22.5 the enzyme activity decreased by a factor of 2, before and after separation of the dissociated fractions. At all ratios approximately 70–80% of MMF-associated chitin synthase was dissociated as calculated from the activities in the dissociated fractions relative to that in the digitonin-incubated MMF before centrifugation. In the latter fractions, activity was restored to almost the original level if determined after preincubation with egg lecithin. A similar effect was observed in case of the dissociated fractions where the enzyme activity reached a level of 70% of MMF-associated activity, which is comparable to 90–100% of the dissociated chitin synthase.

After sucrose gradient centrifugation of chitin synthase dissociated at different digitonin/protein ratios, enzyme activity was recovered as a single, symmetrical peak which coincided with that of activity determined in the presence of lecithin, indicating that no digitonin-masked activity was left on top of the gradients (Fig. 2). At increasing ratios, the recovery of enzyme activity decreased compared to the activity in the dissociated fractions before gradient centrifugation (Fig. 3). This further inactivation of the enzyme after gradient centrifugation of chitin synthase dissociated at increasing digitonin/protein ratios coincided with a shift in sedimentation to higher sucrose densities (Figs. 2 and 3), indicating progressive delipification. Restoration of enzyme activity by preincubation with lecithin, which was very effective before centrifugation, became less effective after gradient centrifugation.

Effect of various phospholipids on dissociated chitin synthase. The effect of various phospholipids is depicted in Fig. 4. With the exception of ergosterol, all phospholipids tested had a stimulatory effect on the activity of dissociated chitin synthase. Phosphatidylinositol (PI) at low concentrations and soybean phosphatidylcholine (SPC) at a much higher concentration restored the enzyme activity to 60–65% of the original MMF-associated activity, which means that restoration was almost complete since approximately 70% of the chitin synthase was dissociated in this experiment. Phosphatidylserine (PS) was less effective than PI, although the possibility that PS stimulates more effectively at concentrations lower than those tested here was not excluded. Lysophosphatidylcholine (LPC) and phosphatidylethanolamine (PE) only slightly stimulated chitin synthase. Except for SPC at higher concentrations, the stimulatory effect disappeared or inhibition occurred as in the case of PI and PS. Ergosterol (ERG) was ineffective in this experiment.

Discussion

The observed temperature dependence of chitin synthase clearly points to an influence of lipids on

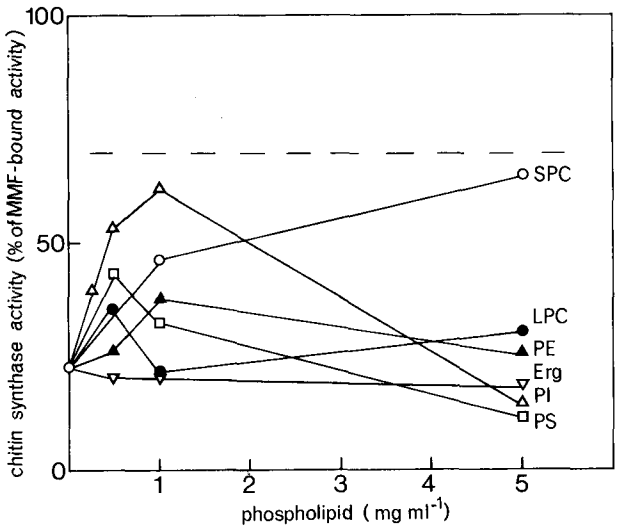

Fig. 4. Effect of phospholipids on chitin synthase of *Schizophyllum commune* after trypsin (100 μg ml^{-1}) activation of MMF (1.8 mg ml^{-1} protein), dissociation and further dilution with 1% digitonin in incubation buffer containing 22.5% glycerol to a digitonin/protein ratio of 110. The broken line indicates the percentage of chitin synthase dissociated from the MMF by digitonin.

the enzyme activity but does not permit an easy interpretation of the significance of the breaks in the Arrhenius plots [26,27]. Also, the different thermal behavior of the nonactivated MMF-associated form of chitin synthase and the trypsin-activated cytoplasmic form does not necessarily point to differences in associated lipids since it cannot be excluded that the two forms react differently towards temperature-mediated changes in their lipid environments.

Dissociation of chitin synthase by digitonin has been reported for *Coprinus cinereus* where it stimulated enzyme activity [8], for *S. cerevisiae* where it was inhibitory [6], and for *Mucor rouxii* [23] and *S. commune* [20] where low concentrations of digitonin were stimulatory but higher concentrations inhibitory. Other detergents such as SDS, Brij 36T, and sodium cholate have been described as being ineffective in dissociating chitin synthase [3,23]. However, the strong inactivation of chitin synthase from *S. commune* by SDS, Nonidett P40, and Triton X100 does not permit conclusions with respect to the dissociation of the enzyme by these detergents.

Dissociation of membranes by detergents results in the formation of protein-lipid-detergent complexes and lipid-detergent mixed micelles, in which the lipid is progressively replaced by detergent at increasing detergent/lipid ratios (for review see [12]). The higher density of the protein-detergent-residual lipid complexes facilitates their separation from the lipid-detergent mixed micelles by gradient centrifugation [30]. Dissociated chitin synthase from membranes of *S. commune* banded after centrifugation on 20–60% sucrose gradients at various densities depending on the digitonin/protein ratios in the samples applied (Fig. 3), indicating that progressive delipification occurred. Sedimentation of digitonin-dissociated chitin synthase at lower densities in 5–20% sucrose gradients have been found in the case of *S. commune* [20] and for chitosomes of *M. rouxii* [24]. Possibly the low densities of these gradients and the presence of digitonin [24] prevented an effective separation of chitin synthase from digitonin and lipids, thus resulting in low density complexes.

Preincubation of digitonin-dissociated chitin synthase of *S. commune* with lecithin increased the activity [20]. Such an effect could be due to removal of inhibitory digitonin from the enzyme, rather than to a direct interaction of lecithin with the enzyme. Removal of digitonin as such does not restore the enzyme activity, as shown by gradient centrifugation of chitin synthase dissociated at high digitonin/protein ratios (Fig. 3). This suggests that after extensive delipification irreversible inactivation of chitin synthase occurred as shown for sarcoplasmic ATPase [10]. In the latter case, reactivation was possible if 20% glycerol was included during delipification [4]. Unfortunately, this procedure was not successful in the case of chitin synthase (unpublished). In the less defined system, that is, in the presence of digitonin and original lipids, reactivation of chitin synthase dissociated with digitonin was to some extent dependent on the phospholipid species used (Fig. 4). PI and PS were particularly effective at low concentrations but inhibitory at higher concentrations, whereas SPC stimulated even at very high concentrations (5 mg ml^{-1}). Durán and Cabib [6] in their study on digitonin-dissociated chitin synthase from *S. cerevisiae* also found PS to be most effective in this respect but found PC to be inhibitory at concentrations above 0.25 mg ml^{-1}. These studies at least suggest that the lipid environment may play a regulatory role in the activity of the enzyme. Such a role is also suggested by studies which show that mutational changes in the PI and PS content of membranes from *C. albicans* [19] and *Neurospora crassa* [9,14] have an effect on the synthesis of chitin. In the study of Pesti and Peberdy [19] the altered phospholipid composition was correlated with sterol deficiency that also might implicate sterols in the regulation of

chitin synthase activity. Inhibition of chitosomal chitin synthase in *M. rouxii* by polyene antibiotics [21] known to interact with sterols [17] as does digitonin [11], also supports a role of sterols in chitin synthase action. On the other hand, in the present study and that of others [6], both using membrane-derived chitin synthase, no effect resulting from ergosterol was observed.

Recently, an analysis of chitosomal lipids showed that these structures contain more sterols compared to total membranes, but did not contain PS [13]. Possibly the transfer of chitin synthase from these inactive chitosomes to the plasma membrane results in activation of the enzyme depending on the local concentration of certain phospholipids, e.g., PS, in this membrane.

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