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Chitin synthetase activity is bound to chitosomes and to the plasma membrane in protoplasts of *Saccharomyces cerevisiae*

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The sub-cellular distribution of chitin synthetase was studied in homogenates of *Saccharomyces cerevisiae* protoplasts. Use of a mild disruption method minimized rupture of vacuoles and ensuing contamination of subcellular fractions by vacuolar proteinases. After fractionation of whole or partially purified homogenates through an isopycnic sucrose gradient chitin synthetase activity was found to be distributed between two distinct particulate fractions with different buoyant density and particle diameter. When whole homogenates were used, about 52% of the chitin synthetase loaded was localized in a microvesicular population identified as chitosomes (diameter 40–110 nm; buoyant density (d) = 1.146 g/cm^3). Another vesicular population containing 26% of the activity was identified as plasma membrane vesicles because of its large mean diameter (260 nm), its high buoyant density (d = 1.203 g/cm^3) and by the presence of the vanadate-sensitive ATPase activity. Moreover, after surface labeling of protoplasts with ^{3}H-concanavalin A, the label cosedimented with the presumed plasma membrane vesicles. There was a negligible cross-contamination of the chitosome fraction by yeast plasma membrane markers. In both the plasma membrane and the chitosome fractions, the chitin synthetase was stable and essentially zymogenic. Activation of the chitosome fraction produces microfibrils 100–250 nm in length. Our results support the idea that chitosomes do not originate by plasma membrane vesiculation but are defined sub-cellular organelles containing most of the chitin synthetase in protoplasts of *Saccharomyces cerevisiae*.

Introduction

The localization of chitin synthetase in actively growing cells of yeast has been the matter of conflicting reports. A number of studies [1–5] describe a preferential location in microvesicles (40–80 nm in diameter) called chitosomes which have a buoyant density of (d) = 1.14–1.16 g/cm^3 in a sucrose isopycnic gradient [6,7]. Contrary to this, the bulk of the chitin synthetase has been claimed to be attached to the plasma membrane [8,9]. Although these differences may be in fact due to differences in strain and growth conditions, unequivocal assignment of chitin synthetase to organelles is difficult for a number of reasons: (a) harsh methods of cell disruption may produce small artefactual membrane vesicles difficult to differentiate from pre-existing, physiologically important vesicles, (b) pure preparations of cell organelles are difficult to obtain without cross-contamination, (c) hydrolytic and proteolytic enzymes contained in the vacuolar compartment are

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liberated when cells are broken by the usual methods, and may interfere either with the zymogenicity or the stability of the chitin synthetase bound to the sub-cellular fractions.

Most of these difficulties can now be overcome using mild disruption conditions as those described for the isolation of the fragile yeast vacuoles [10,11]. Indeed, a mild disruption procedure has been found to provide both a cytoplasmic and a well preserved mitochondrial fraction with minimal contamination by vacuolar proteinases [12]. Here we describe the subcellular distribution of the chitin synthetase in *Saccharomyces cerevisiae* homogenates after disrupting protoplasts in conditions minimizing organelle rupture and hence, cross-contamination.

Materials and Methods

Chemicals. Uridine diphospho-*N*-acetyl-D-[U-^{14}C]glucosamine ammonium salt (247 mCi/mmol) and [*N-acetyl*-^{3}H] concanavalin A (47.6 Ci/mmol) were obtained from Amersham/Searle. [γ-^{32}P]ATP (> 5000 Ci/mmol) was purchased from New England Nuclear. Sodium deoxycholate was product No. 6504 from Merck. Crystallized α-chymotrypsin (No. 17160) was obtained from SERVA. Sodium orthovanadate, NADPH, sucrose (gradient quality), *N*-benzoyl-L-tyrosine-*p*-nitroanilide, *p*-nitrophenyl α-D-glucoside and buffer substances were from Sigma Chemical Co. Sepharose 2B was from Pharmacia. All other chemicals were of the highest purity available.

Yeast strain and growth conditions. Saccharomyces cerevisiae 1022 (ETH, Zurich; ATCC 32167), kindly supplied by Dr. A. Wiemken, was grown aerobically at 28°C in G-21 medium [13] and harvested at the late exponential phase of growth.

Preparation, labeling and lysis of protoplasts. Protoplasts were prepared and purified according to the method of Schwencke et al. [12]. A suspension of $1 \cdot 10^9$ protoplasts/ml in 0.6 M sorbitol, 10 mM $MgCl_2$, 20 mM Mops (Tris), pH 7.0 (buffer A) was added of 0.25 μCi ^{3}H-concanavalin A (dissolved in buffer A). After 10 min at 30°C, protoplasts were collected by centrifugation (swinging bucket rotor) at $3500 \times g$ for 5 min and washed twice with ice-cold buffer A. The pellet was carefully resuspended in 1 M sorbitol, 25 mM Triethylamine (TEA) (HCl) (pH 8.0), 1 mM EDTA and kept 10 min on ice. The protoplasts suspension was diluted 1:4 with pre-warmed (30°C) disrupting buffer: 0.36 M sorbitol, 25 mM TEA (HCl) (pH 8.0), 50 mM glucose, 2.5 mM K_2CO_3 and incubated 10 min at 30°C. Rupture of protoplasts and vacuole liberation was assessed by phase-contrast microscopy. Contamination by soluble vacuolar proteinases was determined in a $100000 \times g$ supernatant of the lysate by measuring carboxypeptidase Y activity as in Ref. 12. Values of 10 to 15% were usually found with reference to the total activity in the whole homogenate. Higher values (about 27% were found in a $500 \times g \times 15$ min supernatant (Table I) indicating that low density vacuolar vesicles are still present in the low speed supernatant.

Isopycnic sucrose gradient distribution of chitin synthetase. The protoplast lysate was centrifuged at $500 \times g$ for 15 min (swinging bucket rotor) to remove vacuoles, intact protoplasts and debris. The supernatant was applied to a Sepharose 2B column (15 × 5 cm) and eluted with 0.3 M sucrose, 20 mM Mops (Tris) (pH 7.0). Zymogenic chitin synthetase eluted in the void volume. Void-volume fractions were pooled, sucrose was added to adjust the concentration to 20% (w/w) and used in conjunction with a 55% sucrose solution to construct a 20 to 55% (w/w) linear sucrose gradient (36 ml). The gradients were centrifuged in a Beckman SW-28 rotor at $95000 \times g$ (rav) for 22 h. Fractions of 1 ml were collected from the bottom upwards by means of a peristaltic pump. In alternative experiments the whole lysate was adjusted to 20% sucrose and used to prepare a 20 to 55% (w/w) linear sucrose gradient and the gradient centrifuged in similar conditions as described above.

Enzymatic assays. Chitin synthetase was assayed according to Ruiz-Herrera and Bartnicki-Garcia [14] but using 60 μg/ml of α-chymotrypsin to activate the zymogenic chitin synthetase. ATPase was assayed essentially as previously described [15], using 0.1 M ATP added to [γ-^{32}P]ATP to a final specific activity of 2.95 μCi/mol. Liberated [^{32}P]P_i was determined as described in Ref. 16 by scintillation counting after extraction into isobutanol/ cyclohexane/ acetone/ water-saturated ammonium molybdate (750:750:15:1,

v/v). Carboxypeptidase Y activity was measured as described in Ref. 17 except for the addition of 0.5% deoxycholate. α-Glucosidase was assayed as in Ref. 18.

Other methods. Protein was determined according to Bradford [19] using crystalline bovine serum albumin as standard. The standard and all samples were previously digested with 1 M NaOH for 30 min and the NaOH neutralized.

Results

Cell disruption and chitin synthetase assay

The modification of the original method for mild protoplast disruption [12] was necessary because the DEAE-Dextran was found to inhibit the activity of the plasma membrane-bound vanadate-sensitive Mg^{2+}-ATPase. With the revised method (without DEAE-Dextran) about 60–70% of the protoplasts were disrupted while vacuolar integrity was well preserved as indicated by the low contamination of the $500 \times g \times 15$ min supernatant by the vacuolar carboxypeptidase Y (Table I). Sub-cellular fractionation using DEAE-Dextran as in the original disruption method [12], did not alter the distribution of chitin synthetase described here. However, the activity of the vanadate-sensitive Mg^{2+}-ATPase activity bound to the plasma membrane fraction was much lower (not shown). Activation by partial proteolysis was required in order to measure chitin synthetase activity. Only 5 to 10% chitin synthetase activity was found without proteolytic activation in the $500 \times g \times 15$ min pellet while the activity in the $500 \times g$ supernatant was 95–98% zymogenic. Activation by α-chymotrypsin, in the optimal conditions used here, gave more reproducible results than those obtained by trypsin activation. Addition of chymostatin after activation by α-chymotrypsin did not significantly enhanced the specific activity of the chitin synthetase.

Distribution of chitin synthetase, vanadate-sensitive Mg^{2+}-ATPase and ^{3}H-concanavalin A label in sub-cellular fractions obtained from ^{3}H-concanavalin labeled yeast protoplasts

As shown in Table I, a substantial amount of chitin synthetase activity remains in the $500 \times g \times 15$ min supernatant after protoplast lysis and coelutes with the vanadate sensitive Mg^{2+}-ATPase and the ^{3}H-concanavalin A label in the void volume after filtration through Sepharose 2B. Soluble vacuolar proteinases are largely entrapped in vacuoles and hence sedimented in the $500 \times g \times 15$ min pellet as indicated by the carboxypeptidase Y activity (Table I). Soluble vacuolar proteolytic activities remaining in the $500 \times g \times 15$ min supernatant were completely separated from the particulate fraction in the Sepharose 2B step. Also, α-glucosidase (a cytoplasmic marker) was undetectable in the particulate fraction eluting in the void volume of the Sepharose 2B column.

TABLE I

DISTRIBUTION OF ENZYMATIC ACTIVITIES AND ^{3}H-CONCANAVALIN A IN SUBCELLULAR FRACTIONS AFTER DISRUPTION OF *S. CEREVISIAE* PROTOPLASTS

Protoplasts were labeled with ^{3}H-concanavalin A and washed before disruption (see Methods for details). n.d., not detected.

	Total protein (mg)	Total enzyme activity (μmol/min)				^{3}H-concanavalin A (total cpm)
		chitin synthetase	vanadate-sensitive Mg^{2+}-ATPase	α-glucosidase	carboxypeptidase Y	
Whole lysate	189.7	48.2	641	351	79.9	318546
$500 \times g$ pellet [a]	57.6	27.0	595	121	54.8	161784
$500 \times g$ supernatant	117.7	21.9	417	196	22.0	74942
Sepharose 2B void volume eluate	12.4	6.6	624	n.d.	n.d.	38000

[a] This pellet ($500 \times g \times 15$ min) contains a few cells, intact protoplasts (about 35 to 40% of the initial amount) as well as agglomerates of permeabilized but otherwise non disrupted protoplasts.

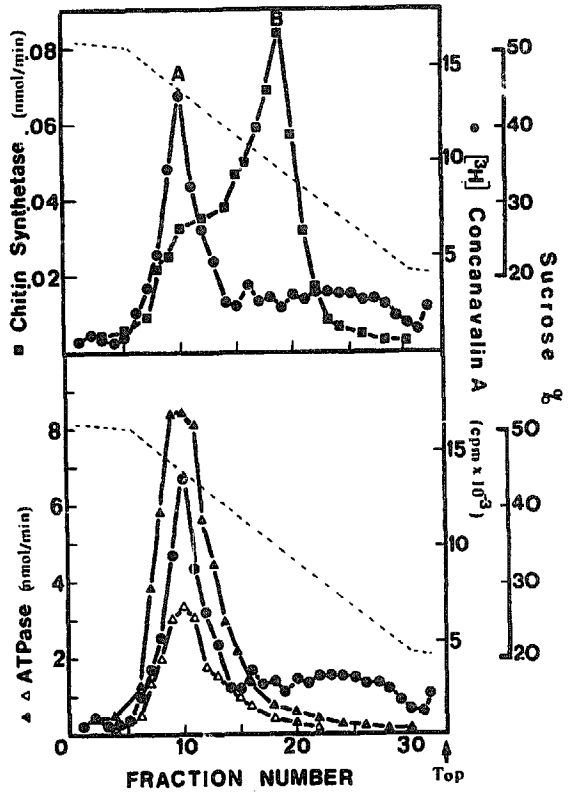

Fig. 1. Isopycnic sucrose gradient centrifugation of a partially purified vesicular fraction. The void volume eluate of the Sepharose 2B column obtained as detailed under Materials and Methods was used to construct a 20 to 55% (w/w) linear sucrose gradient. Tubes were centrifuged at 95000 × *g* (rav) for 24 h. Top panel: ●, ^{3}H-concanavalin A; ■, chitin synthetase activity. Bottom panel: ●, ^{3}H-concanavalin A; ▲, Mg^{2+}-ATPase activity; △, Mg^{2+}-ATPase activity assayed in the presence of 50 μM sodium vanadate.

Distribution of chitin synthetase after sucrose gradient centrifugation of a partially purified fraction

In four separate experiments when the void volume of the Sepharose 2B column was subjected to isopycnic sucrose gradient centrifugation (20–55%), two peaks of chitin synthetase activity were reproducibly obtained (Fig. 1). The main peak (peak B) contained microvesicles having the same size distribution (40–110 nm; median diameter: 75 nm), electron microscopic appearance (Fig. 3B)and buoyant density (1.1463 g/cm^3) described for chitosomes [1,2,7]. The second peak (peak A) consisted of larger vesicles (190–350 nm; median diameter 260 nm), smooth appearance (Figs. 3A$_1$ and 3A$_2$) and higher buoyant density (1.203 g/cm^3). Vesicles in peak A appear to correspond to plasma-membrane particles as indicated by their buoyant density [24] and by the fact that the vanadate-sensitive Mg^{2+}-ATPase [15,27] and the ^{3}H-concanavalin A label [28,29] co-sediment with them (Fig. 1, low panel). Both peaks of chitin synthetase activity were highly zymogenic i.e. without proteolytic activation less than 2% of the total chitin synthetase activity could be measured in the chitosomal fraction and less than 5% in the plasma membrane fraction. It is also interesting to note that α-glucosidase (a cytoplasmic marker), carboxypeptidase Y, proteinase B (soluble vacuolar markers [12]), and α-mannosidase (a marker for vacuolar membranes [20]) were not detected in the sucrose gradient (data not shown), indicating that there was no cross-contamination by either soluble vacuolar proteinases, entraped soluble cytoplasmic enzymes or vacuolar membranes.

Distribution and quantification of chitin synthetase after sucrose gradient centrifugation of a whole protoplasts lysate

Selective loses of chitosomal and/or the plasma membrane fraction may occur when using the semi-purified Sepharose 2B void volume as a starting material. Therefore we decided to use whole protoplasts lysates to construct the sucrose gradient. In order to facilitate the identification of vesicles originating from the yeast plasma membrane, protoplasts were labeled with ^{3}H-concanavalin A before disruption. The resulting whole

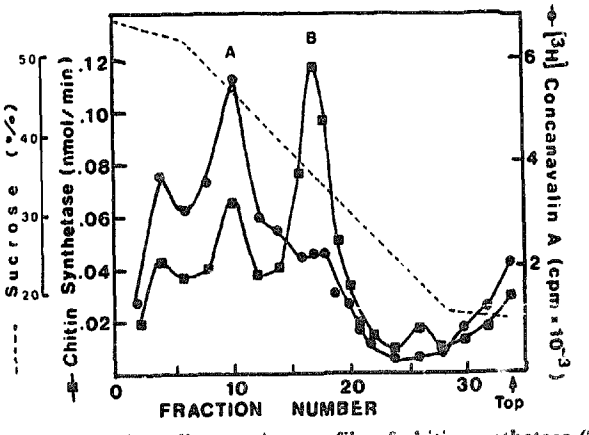

Fig. 2. Isopycnic sedimentation profile of chitin synthetase (■) and ^{3}H-concanavalin A (●) from a whole protoplast lysate. Protoplasts were lysed as described in Materials and Methods and the whole homogenate used to build a linear sucrose gradient (20 to 55%; w/w) and centrifuged at 95000 × *g* (rav) for 24 h.

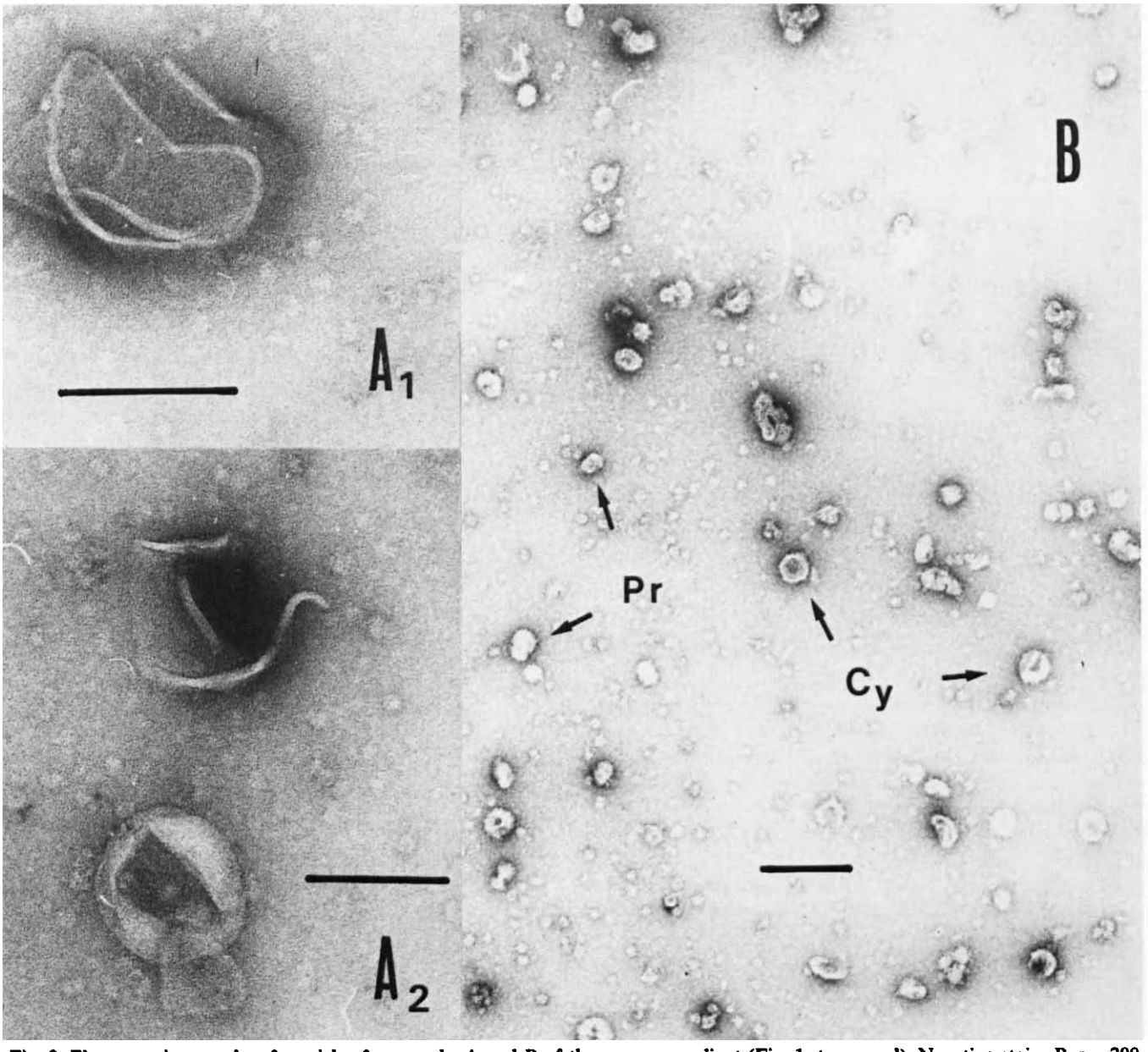

Fig. 3. Electron micrographs of particles from peaks A and B of the sucrose gradient (Fig. 1, top panel). Negative stain. Bar = 200 nm. (A_1 and A_2) plasma-membrane fraction. (B) Chitosome fraction. Proctoid (Pr) and cycloid (cy) forms.

lysate was made 20% with solid sucrose and used to obtain a 20–55% (w/w) sucrose gradient. After a single isopycnic centrifugation chitin synthetase distribution was similar to that obtained above for the mixed vesicle population present in the void-volume fraction after Sepharose 2B chromatography. Two main peaks of chitin synthetase were detected (Fig. 2), one (peak A) with a specific gravity of 1.199 g/cm^3 (plasma membrane) and the other (peak B) with specific gravity of 1.146 g/cm^3 (chitosomes). In these conditions, the plasma membrane peak accounted for 26% of the total chitin synthetase activity loaded while 52% of the activity was found in the chitosomes peak. Directly measurable chitin synthetase activity in these fractions (i.e. without proteolytic activation) varied between 0.5 and 1% of the total activity in the chitosomal fraction and between 3 and 5% in the plasma membrane ghost vesicles.

Electron microscopy of vesicles separated by isopycnic sucrose gradient

Further evidence that chitin synthetase activity is in fact bound to two different types of sub-

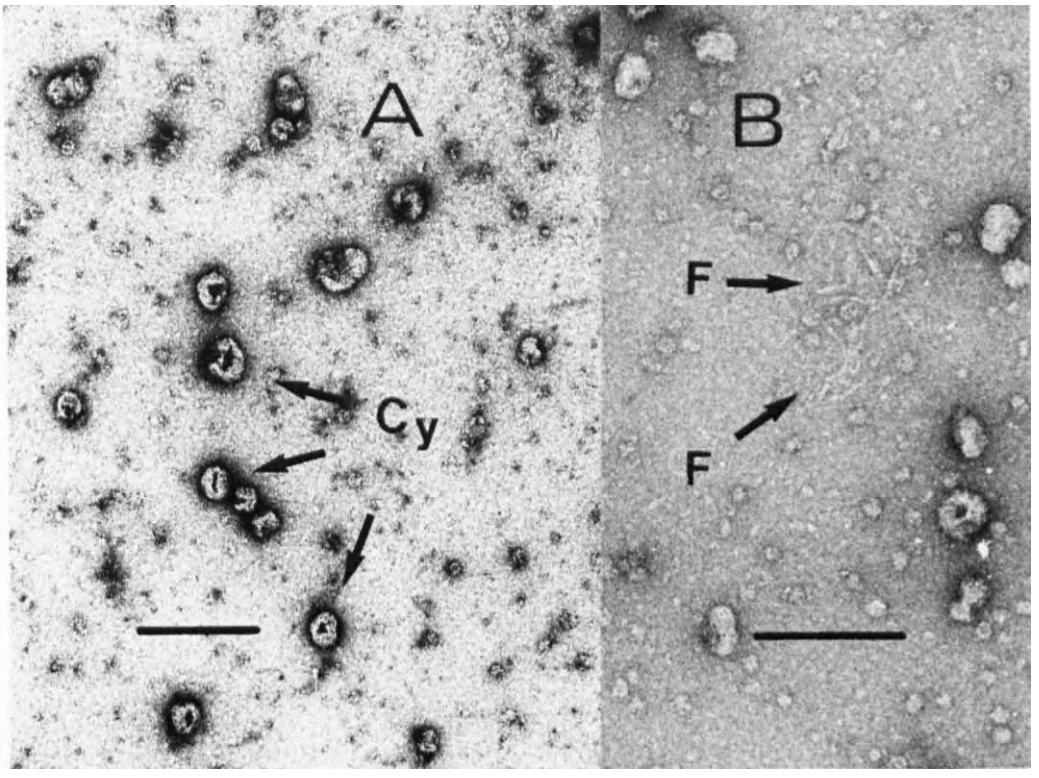

Fig. 4. Electron micrograph of chitosomes after α-chymotrypsin activation. Negatively stain. Bar = 200 nm. (A) General picture showing mostly cycloid (Cy) forms. (B) Microfibrils (F).

cellular fractions was obtained after negative staining and electron microscopy examination of the two peaks separated by isopycnic sucrose gradient centrifugation. Thus, the microvesicle population which equilibrates at a density of 1.146 g/cm^3 (Fig. 3) shows the same morphological characteristics of those already described for the yeast chitosomes [1,7,21], namely spheroidal particles 40–110 nm in diameter (mean diameter: 75 nm) (Fig. 3B). A characteristic central depression (cycloid form) was found in about 25% of the population while the rest of the particles did not show it (Proctoid form). After chymotrypsin activation no essential changes were observed in the chytosomal population, both cycloid and proctoid forms were seen (Fig. 4A). Microfibrils (100–280 nm long), presumably of chitin, were also observed (Fig. 4B). The vesicular population equilibrating at a buoyant density of $d = 1.203$ g/cm^3 was found to be quite different from chitosomes both in diameter (mean diameter: 260 nm) and appearance since they are seen as collapsed, smooth vesicles resembling plasma membrane ghosts (Figs. $3A_1$ and $3A_2$). No evidence of chitosomes either alone, agglomerated or bound to the large vesicles was found in this fraction. Conversely, the chitosomal fraction, was found to be virtually free of plasma membranes.

Discussion

Chitosomes were originally proposed to convey zymogenic chitin synthetase activity to the sites of chitin synthesis in the plasma membrane [1,2,6]. In spite of the strong collective evidence indicating their physiological reality (reviewed in Ref. 21), their existence as true subcellular structures has been questioned [22]; it was argued that they may be artefactual vesicles derived from the plasmalemma during cell breakage since yeast plasma-membrane has been claimed to contain the bulk of the chitin synthetase activity [8]. However, since chitin synthesis most probably occurs at the plasma membrane, chitosomes should be intrinsically able to recognize and bind to plasma membrane 'in vivo' and probably also 'in vitro' during or after disruption of yeast cells. Thus we believe that the opposite artefact must also be

considered i.e. most of the chitin synthetase activity originally in chitosomes may become bound to plasma membrane fragments during cell breakage by the usual harsh mechanical methods. This would explain claims indicating that most if not all of the chitin synthetase activity is bound to plasma membrane vesicles [8,9]. In an effort to help to resolve the controversy, we isolated chitosomes and plasma membrane fragments after disruption of yeast protoplasts by a modification of a very mild method described by Schwencke et al. [12]. This method preserves vacuolar integrity and minimizes contamination of homogenates by vacuolar proteinases preventing the non-physiological 'activation' of zymogenic chitin synthetase by protease B [23–25]. Using this mild disruption method, chitin synthetase activity is found in two different particulate fractions after isopycnic sucrose gradient centrifugation. One fraction contain collapsed smooth surface vesicles (mean diameter: 260 nm) when examined by electron microscopy negative staining and equilibrate at a buoyant density of $d = 1.203$ g/cm^3. This fraction shows a variety of characteristics that correspond to plasma membrane ghost vesicles. Indeed yeast plasma membrane vesicles has been found to equilibrate between $d = 1.18$–1.23 g/cm^3 [26] and at 1.22 g/cm^3 [27] in a sucrose gradient. Also, the characteristic vanadate-sensitive Mg^{2+}-ATPase of the yeast plasma membrane [15,27] co-sediments with this heavier peak. Moreover, in protoplasts labeled with ^{3}H-concanavalin A which binds strongly to the external surface of the yeast plasmalemma [28,29] the peak of radioactivity co-migrated with the heavier peak of chitin synthetase activity, further indicating its plasmalemmal origin. The main peak of chitin synthetase activity can be identified as chitosomes because of its microvesicular size (mean diameter: 75 nm), its morphological characteristics, the production of microfibrils after partial proteolytic activation and its low buoyant density ($d = 1.146$) [1,2,7,21]. Significantly, this fraction was found to be essentially free of contamination by plasma membrane vesicles as indicated by the presence of only background levels of the ^{3}H-concanavalin A label, by the lack of vanadate sensitive Mg^{2+}-ATPase (Fig. 1 low panel) and by the absence of β-1,3-glucan synthetase (not shown). These results clearly argues against the idea that chitosomes may originate by fragmentation of the yeast plasma membrane.

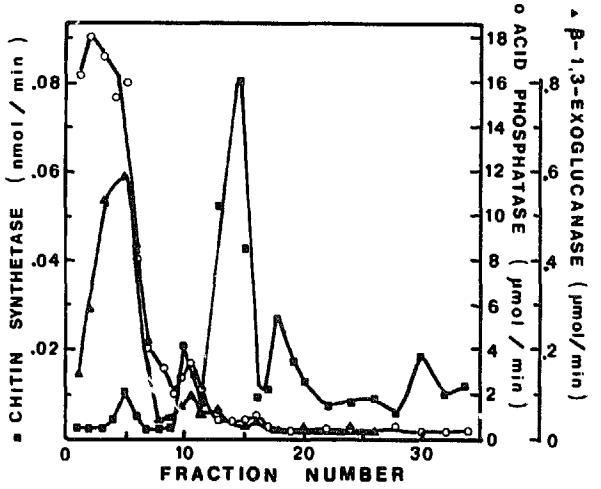

Fig. 5. Linear-log sucrose gradient centrifugation of partially purified vesicular fraction. The void volume eluate of a Sepharose 2B column (see Materials and Methods) was concentrated against Aquacid IIA. 1 ml of concentrate was charged over a gradient prepared accordingly to Brakke and Van Pelt [30] and centrifuged in a SW.28 rotor at 90000 × g (rav) for 2.5 h. 1.1 ml fractions were collected and assayed for chitin synthetase (■), acid phosphatase (○) and β-1,3-exoglucanase (▲).

When protoplast homogenates are subjected to a single isopycnic centrifugation, some other membrane-bound enzymatic activities, i.e. acid phosphatase and β-1,3-exoglucanase can be found in the chitosomal fraction [4]. However, after a linear-log sucrose gradient centrifugation [30] these activities no longer contaminated the chitosomal fraction (Fig. 5). Similar results have been found during the purification of *Candida albicans* chitosomes [5].

Both chitin synthetase fractions were found to be 95 to 99% zymogenic indicating that whatever the mechanism for chitin synthetase activation it does not occur during purification. The absence of contamination by vacuolar proteinases, was an absolute requirement for maintaining zymogenicity. This is in accordance with the reported in vitro artefactual 'activation' of the zymogenic chitin synthetase by vacuolar proteinase B [23–25]. Recent evidence indicates the existence of two chitin synthetases (Chs1, Chs2) encoded by the genes: CHS1 and CHS2 [31,32]. Chitin synthetase 1 (Chs1) has been proposed to correspond to the proteolytically activatable type, while Chs2 has

been proposed either not to be proteolytically activatable [32] or zymogenic [33]. Therefore a clear cut differentiation of the type of chitin synthetase present in chitosomes cannot be established on the basis of zymogenicity. Moreover, the lack of requirement for the proteolytic activation reported for Chs2 [32] is also doubtful because of the probable contamination with vacuolar proteinases of the 'mixed-membrane' preparation used. On the other hand in EDTA-treated 'mixed membrane' preparations Co^{2+} appears to be strongly inhibitory to Chs1 but not to Chs2 [32]. Recent work within purified sub-cellular fractions indicate that the chitosomal chitin synthetase activity and the plasma membrane-bound activity are both strongly inhibited by 3 mM Co^{2+} [37] hence, it appears that Chs1 is presented in both vesicular populations and this may also be the case for Chs2.

The relative distribution of the zymogenic chitin synthetase between the plasmalemma and the chitosomal fraction that we have found here probably does not exactly reflect the 'in vivo' situation because of the possible fusion of plasma membrane and chitosomes which can occur after cell disruption and during the purification. However, the existence of chitosomes as a well defined subcellular organelle, clearly different from artefactual plasma membrane vesicles, is strongly supported by the results presented here, as well as by other different experimental approaches [34,36] and by the collective evidence reviewed in Ref. 21. Our conclusions on the localization of the chitin synthetase are not restricted to protoplasts; similar findings have also been found by Leal-Morales et al. [37] with walled cells of *S. cerevisiae*. The existence of two chitin synthetases and (at least) two clearly established sub-cellular locations produces a variety of interesting questions. In this context, the use of purified plasma membrane fractions (i.e., after concanavalin A loading) and chitosomal fractions rather than ill defined 'mixed membrane' preparations, appears to be imperative if unambiguous answers are to be obtained.

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