DISSOCIATION OF CHITOSOMES FROM *Mucor rouxii* BY BRIJ 36T

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RESUMEN

La incubación de quitosomas parcialmente purificados a partir de células levaduriformes de *M. rouxii* con el detergente no iónico Brij 36T liberó una población de partículas más pequeñas con actividad de quitina sintetasa las cuales se purificaron por cromatografía de exclusión molecular en una columna de Bio-Gel A-0.5m. La microscopía electrónica de especímenes teñidos negativamente de las fracciones más activas de la columna reveló la presencia de partículas isodiamétricas con diámetro de 10-17 nm y con la habilidad de sintetizar fibrillas de quitina cortas y gruesas después de incubarse con el sustrato y los activadores de la quitina sintetasa. La actividad de la quitina sintetasa disociada dependió de la presencia de fosfatidilinositol y, como en el caso de los quitosomas nativos, de la activación por una proteasa exógena. Sobre la base de las características morfológicas y bioquímicas de estas partículas se propone que serían el equivalente de las subunidades 16S liberadas a partir de quitosomas purificados por tratamiento con digitonina.

SUMMARY

Incubation of partially purified chitosomes from yeast cells of *M. rouxii* with the non-ionic detergent Brij 36T released a population of smaller particles with chitin synthetase activity which were further purified by molecular weight exclusion chromatography in a Bio-Gel A-0.5m column. Electron microscopy of negatively stained samples of most active fractions eluting from the column revealed the presence of isodiametric particles, 10-17 nm in diameter, with the ability to synthesize short and thick

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chitin fibrils following incubation with substrate and activators of chitin synthetase. Activity of dissociated chitin synthetase was dependent on the presence of phosphatidylinositol and, as that of native chitosomes, on activation by an exogenous protease. On the basis of the morphological and biochemical characteristics of these particles it is proposed that they are equivalent to the 16S subunits released from purified chitosomes following incubation with digitonin.

INTRODUCTION

Chitin, a linear homopolymer consisting of repeating N-acetylglucosamine units joined to each other by ß(1-4) linkages, is a major structural component of fungal cell walls (Bartnicki-García, 1968; Cabib and Shematek, 1981). Chitin synthetase (UDP-N-acetyl-D-glucosamine: chitin 4-ß-N-acetylglucosaminyl transferase, EC 2.4.1.16), the enzyme responsible for chitin biosynthesis, has been claimed to be confined to two major locations in fungal cells: chitosomes (Bracker *et al.*, 1976; Bartnicki-García and Bracker, 1984) and plasma membrane (Durán *et al.*, 1975; Kang *et al.*, 1985). Chitosomes have been considered as the cytoplasmic conveyors of zymogenic chitin synthetase which transport the enzyme to the cell surface where chitin synthesis takes place (Bartnicki-García *et al.*, 1978). Originally identified in the cytoplasm of yeast cells of *M. rouxii* (Bracker *et al.*, 1976; Ruiz-Herrera *et al.*, 1977), chitosomes were later shown to be present in several other fungi representative of different taxonomic groups (Bartnicki-García *et al.*, 1978). Most of chitin synthetase activity in *M. rouxii* is recovered in these microvesicles which measure 40-70 nm in diameter and are delimited by a biological unit membrane with a double-track appearance (Bartnicki-García, 1980). Chitosomes from yeast cells of *M. rouxii* have a bouyant density of 1.14-1.15 g cm^{-3} (Ruiz-Herrera *et al.*, 1984) and an unusual chemical composition. Accordingly, they are made up of protein and lipid in a 2:1 ratio, contain about equal amounts of neutral (55%) and polar (45%) lipids and sterols and sterol esters constitute the most abundant fraction of neutral lipids (Hernández *et al.*, 1981). The polypeptide composition of purified chitosomes has been determined by SDS-PAGE and silver staining. Ten prominent bands were detected in the range of 16 to 55 kDa and the one of 55 kDa was the most conspicuous (Flores-Martínez *et al.*, 1990).

Chitosomes, which exhibit a molecular weight higher than 5 x 10^3 kDa by gel filtration chromatography and a sedimentation coefficient of 105S, have been dissociated with digitonin into active subunits with a molecular weight of about 500 kDa and a sedimentation coefficient of 16S (Ruiz-Herrera *et al.*, 1980; Hanseler *et al.*, 1983). The subunits retained the zymogenic character of undissociated chitosomes and synthesized a product characterized as α-chitin by x-ray diffractometry. Later, Lending

et al. (1991) developed an efficient method to extract 16S chitin synthetase subunits from *M. rouxii* by percolation of a large mass of mycelial walls with digitonin. The enzyme was further purified by density gradient centrifugation and HPLC. SDS-PAGE analysis of these 16S particles revealed seven polypeptides, four of which (21, 23, 33 and 39 kDa), closely correlated with chitin synthetase activity.

This paper deals with an alternative method for the dissociation of *M. rouxii* chitosomes with the non-ionic detergent Brij 36T (polyoxyethylene-10-lauryl ether) into active subunits which exhibit properties comparable to those of 16S particles released by digitonin.

MATERIALS AND METHODS

Organism and culture conditions. *Mucor rouxii*, strain IM-80 (ATCC 24905) from our stock collection was used for this study. To propagate the organism in the yeast-like form, 2-liter Erlenmeyer flasks containing 650 ml of YPG medium (Bartnicki-García and Nickerson, 1962) were inoculated with spores (5×10^5 per ml) obtained as described by Ruiz-Herrera and Bartnicki-García (1976). The flasks were shaken under an atmosphere of N_2:CO_2 (7:3, by vol) at 120 rpm for 13 h at 28°C.

Preparation of cell-free extracts. The cultures were filtered through a coarse sintered glass filter until the volume was reduced to 20 ml. Sufficient amounts of 1 M KH_2PO_4/NaOH buffer, pH 6.5, 1 M $MgCl_2$ and 50% sucrose were added to obtain final concentrations of 50 mM, 10 mM and 10%, respectively. Cells were mixed with an equal volume of glass beads (0.45-0.50 mm in diameter) and broken in a MSK cell homogenizer (Braun) for 45-60 sec while cooling with a stream of CO_2. The homogenate was centrifuged at 1000 x g for 5 min to remove cell walls and unbroken cells and the supernatant was further centrifuged at 54,000 x g for 45 min. The supernatant fraction was carefully aspirated with a Pasteur pipette excluding a floating lipid layer as much as possible, and used as the starting material for the purification of chitosomes.

Purification of chitosomes by gel filtration. About 15 ml of the 54,000 x g supernatant was subjected to gel filtration chromatography in a column (1.5 x 32 cm) of Bio-Gel A-5m equilibrated with 50 mM KH_2PO_4/NaOH buffer, pH 6.5, containing 10 mM $MgCl_2$ (PMB). The sample was eluted with the same buffer and most turbid fractions emerging with the void volume (Vo) were pooled (10-12 ml). The concentration of contaminating ribosomes in the Vo eluate was calculated from its absorbance at 254 nm by the method of Rich (1967) and were digested by incubation of sample with ribonuclease (40 μg per mg ribosomes) for 30 min at 30°C. Precipitated ribonucleoproteins were removed by

centrifugation at 12,000 x g for 17 min and the clear supernatant was concentrated to about 2-3 ml with Aquacide IIA.

Purification of chitosomes by density gradient centrifugation. The Aquacide-concentrated sample was layered on the top of a 36 ml linear-log sucrose gradient (Brakke and van Pelt, 1970) which was centrifuged at 81,500 x g for 2.5 h and then fractionated from the top into one-ml aliquots with an ISCO model 183 fractionator. Absorbance at 280-310 nm was continuously monitored and activity of chitin synthetase was measured in the fractions as described below. Most active fractions were pooled and either used immediately or frozen at -70°C. Since the profile of enzyme activity after DGC in these conditions was essentially similar to that previously described (Martínez-Cadena *et al.*, 1987) it is not shown under Results.

Chitin synthetase assay. Unless otherwise stated, the standard assay mixture contained 0.5 mM UDP-[^{14}C]GlcNAc (0.2 Ci per mol), 20 mM GlcNAc, 0.2 mM ATP, 10 mM $MgCl_2$, 200 μg rennilase (an acid protease from *Mucor miehei*), 25 μg of PI (in the case of the Brij-dissociated samples only), 20-30 μl of the enzyme fraction and 50 mM KH_2PO_4/NaOH buffer, pH 6.5, in a final volume of 50 μl. After 60 min (undissociated chitosomes) or 3-4 h (Brij-dissociated samples) of incubation at room temperature, the enzyme reaction was stopped by the addition of 25-50 μl glacial acetic acid. It should be mentioned that when the activity of Brij-dissociated chitosomes was measured, the action of rennilase was stopped after the first hour of incubation by adding 10 μl (10 μg) of pepstatin A. Chitin synthesis was then let to proceed for another 2-3 h. Radioactivity incorporated into chitin was determined by a filtration method as described by Ruiz-Herrera and Bartnicki-García (1976). Enzyme activity was expressed as nmoles of GlcNAc incorporated into chitin in one min. Unless otherwise stated, specific activity was referred to one mg protein.

Dissociation of chitosomes by Brij 36T. Aliquots of the enzyme fraction purified by DGC were incubated for 30 min at 30°C with increasing concentrations of the detergent, usually in the range of 0 to 0.025%. After this period, chitin synthetase activity was measured in the absence (activity A) and in the presence (activity P) of PI as described above. The most efficient concentration of Brij 36T was that yielding dissociated samples with the highest activity P/activity A ratio. This concentration routinely corresponded to 0.0125% for most chitosome batches (see also below).

Separation of Brij-dissociated chitin synthetase by gel filtration chromatography. Routinely 1.5 ml of a chitosome preparation purified by DGC were incubated for 30 min at room temperature with an equal volume of 0.025% Brij 36T in PMB. Immediately after this period, the mixture was subjected to gel

filtration in a column (1 x 63 cm) of Bio-Gel A-0.5m equilibrated with PMB containing 0.0125% Brij 36T. The sample was eluted with the same buffer and one-ml fractions were collected which were chilled as soon as they emerged from the column. Chitin synthetase activity was measured and most active fractions were pooled and used immediately for some of the experiments described below.

Synthesis of chitin microfibrils *in vitro*. To this purpose, the volume of the standard incubation mixture for chitin synthesis was scaled up 10-fold. After 4 h of incubation at room temperature the mixture was centrifuged for 10 min in an Eppendorf centrifuge. The pellet was recovered, thoroughly washed with PMB and resuspended in 50 μl of the latter for electron microscopy.

Electron microscopy. Samples of Brij-dissociated chitosomes or chitin microfibrils synthesized *in vitro* were negatively stained with uranyl acetate as described by Ruiz-Herrera *et al.* (1980). Specimens were examined and photographed with a Jeol JEM-100S electron microscope.

Abbreviations. UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; GlcNAc, N-acetylglucosamine; PI, L- -phosphatidylinositol; DOC, sodium deoxycholate; DGC, density gradient centrifugation.

Chemicals. Bio-Gels A-5m and A-0.5m were from Bio-Rad (Richmond, CA). Brij 36T, Nonidet P-40, DOC, Tritón X-100, pancreatic ribonuclease (type II-A), ATP, UDP-GlcNAc, GlcNAc and L- -phosphatidylinositol (crude, from soybean) were purchased from Sigma Chemical Company (St. Louis, MO). Rennilase was from Novo Enzyme Corporation (Mamaroneck, MY) and pepstatin A was obtained from Peptide Institute Inc. (Japan). Aquacide IIA was from Calbiochem (La Jolla, CA). Ultrapure density grade sucrose was obtained from Schwarz-Mann (Orangeburg, NY). Uridine diphosphate N-acetyl-D-[^{14}C]glucosamine was obtained from ICN (Irvine, CA). All other chemicals were of the highest purity commercially available.

RESULTS

In preliminary experiments carried out to discriminate among several detergents, crude preparations of chitin synthetase such as Aquacide-concentrated samples were incubated for 30 min at room temperature with increasing concentrations of either Nonidet P-40, DOC, Brij 36T or Tritón X-100 and enzyme activity was then measured in the absence and in the presence of PI. As shown in Table 1, incubation of enzyme fractions with these agents resulted in a dramatic loss of chitin synthetase activity as compared with that of undissociated samples. Except for

Table 1. Effect of detergents on crude chitin synthetase from *M. rouxii*. Effect of PI on activity of dissociated samples.

Detergent	%	Chitin synthetase activity (nmoles GlcNAc/min/mg protein) Without PI	With PI
None (control)		3.6	3.6
Nonidet P-40	0.05	0.09	0.08
	0.10	0.04	0.00
	0.30	0.06	0.03
DOC	0.05	0.44	0.32
	0.10	0.20	0.04
	0.30	0.05	0.07
Brij 36T	0.05	0.11	3.42
	0.10	0.07	2.47
	0.30	0.05	0.08
Triton X-100	0.05	0.14	0.17
	0.10	0.04	0.01
	0.30	0.01	0.10

Note: Some detergent was present during the assay of chitin synthetase activity at a concentration which depended on the amount used for dissociation.

Table 2. Dissociation of chitosomal chitin synthetase by Brij 36T and effect of PI on the activity of dissociated samples.

Brij 36T (%)	Chitin synthetase activity (nmoles GlcNAc/min/mg protein) Without PI(A)	With PI (B)	B/A ratio
None (control)	29.0	27.6	0.95
0.0015	25.4	31.3	1.2
0.0031	20.1	25.4	1.3
0.0062	10.0	29.5	3.0
0.0125	1.8	20.1	11.0
0.0250	4.3	12.6	2.9

samples treated with Brij 36T, activity could not be restored by the addition of PI to the assay mixture. In the presence of this phospholipid, samples treated with 0.05% Brij 36T showed an activity comparable to that of the control. Higher concentrations of this detergent led to an irreversible loss of enzyme activity. It is also apparent from the same Table that PI did not affect the activity of undissociated samples. In a further experiment, the optimum concentration of Brij 36T for the dissociation of DGC-purified chitosomes was found at 0.0125% (Table 2). Again, chitosomes were unaffected by PI. Since the detergent/protein ratio seems to be critical for an efficient chitin synthetase dissociation, this relationship, which varied between 0.66 and 0.96, had to be determined for every chitosome batch.

The elution pattern of dissociated chitin synthetase from a column of Bio-Gel A-0.5m is shown in Fig. 1. Most of the activity emerged in the form of a sharp, symmetrical peak between fractions 20 and 25 (but see below). A minor peak with a lower gel filtration rate was also detected in fractions 26 through 29. This latter was not further explored.

Using most active fractions (pool 22-23) from the Bio-Gel A-0.5m column as the source of dissociated chitosomes, the effect of variable concentrations of PI on activity was determined. As shown in Fig. 2A, the optimum concentration for restoring chitin synthetase activity was 0.10 mg of PI per ml. The enzyme was nearly unresponsive to higher concentrations of the phospholipid up to 0.20 mg per ml. The zymogenic character of dissociated chitin synthetase was also investigated. To this purpose, activity was measured as a function of increasing concentrations of rennilase. A 3-fold increase in enzyme activity was observed at the optimum concentration of 1 mg rennilase per ml (Fig. 2B).

It has been shown that 16S chitin synthetase particles synthesize α-chitin microfibrils following incubation with substrate and activators (Ruiz-Herrera *et al.*, 1980; Hanseler *et al.*, 1983). This prompted us to run similar experiments in this study. Accordingly, samples of pool 22-23 were negatively stained with uranyl acetate before and after incubation for chitin synthesis as described in Materials and Methods. Electron microscopy of non incubated samples revealed a population of isodiametric particles, 10-17 nm in diameter, morphologically similar to 16S subunits (Fig. 3A). These particles catalyzed the formation of an abundant mesh of short and thick chitin microfibrils indistinguishable from those made by 16S subunits (Fig. 3B).

Determination of the molecular weight of Brij-dissociated chitin synthetase by molecular exclusion chromatography in Bio-Gel A-0.5m has been unsuccessful. Its elution pattern was unfortunately inconsistent in the sense that it varied from batch to batch. In other words, in some experiments the enzyme emerged,

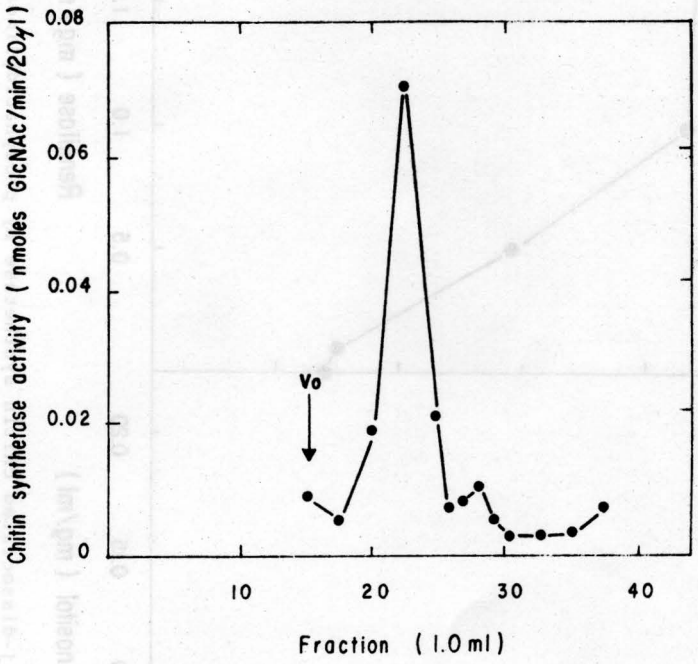

Fig. 1. Elution profile of Brij-dissociated chitin synthetase from a Bio-Gel A-0.5 column. A partially purified chitosome preparation was incubated with Brij 36T in the conditions described in Materials and Methods. The mixture was then subjected to gel filtration in Bio-Gel A-0.5m equilibrated with Brij-containing PMB. Following elution with the same buffer, 1.0 ml fractions were collected in which chitin synthetase was measured in the presence of phosphatidylinositol. It is worth mentioning that in some experiments enzyme activity appeared in the Vo fraction (see text).

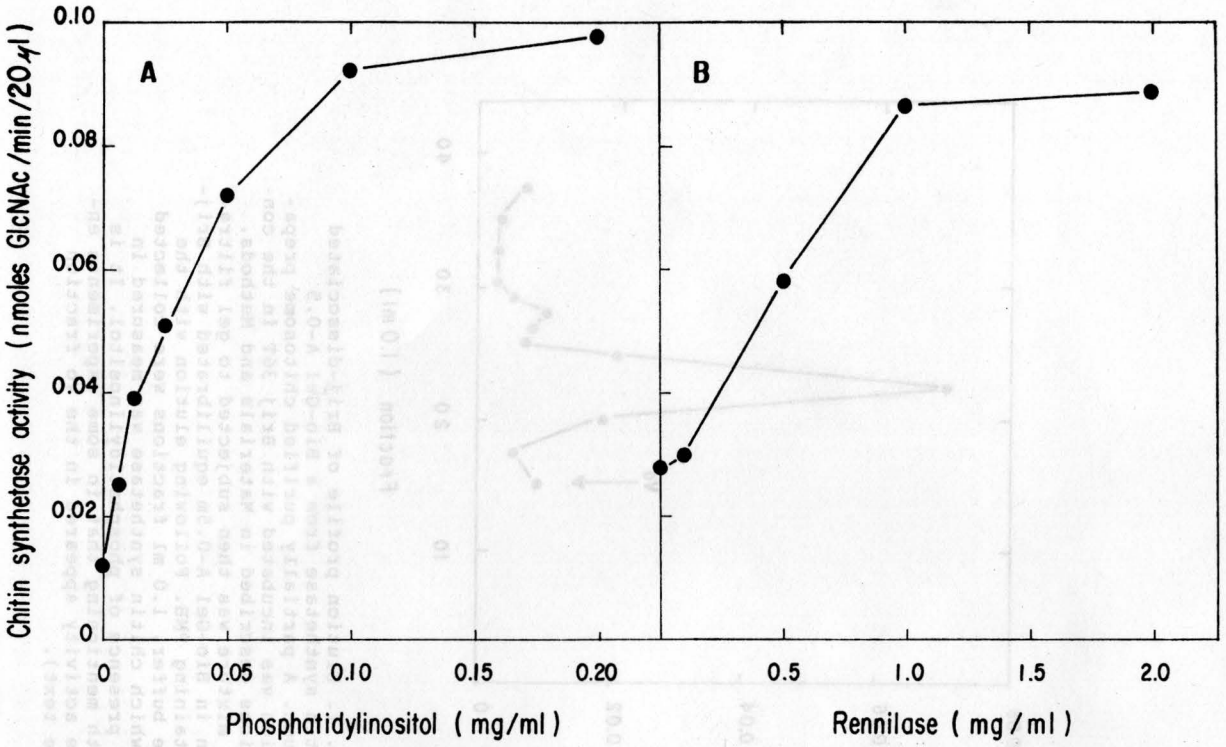

Fig. 2. Activation of Brij-dissociated chitin synthetase by phosphatidylinositol and rennilase. Chitin synthetase activity was measured as described in the text as a function of increasing concentrations of either phosphatidylinositol (A) or rennilase, an acid protease from M. miehei (B).

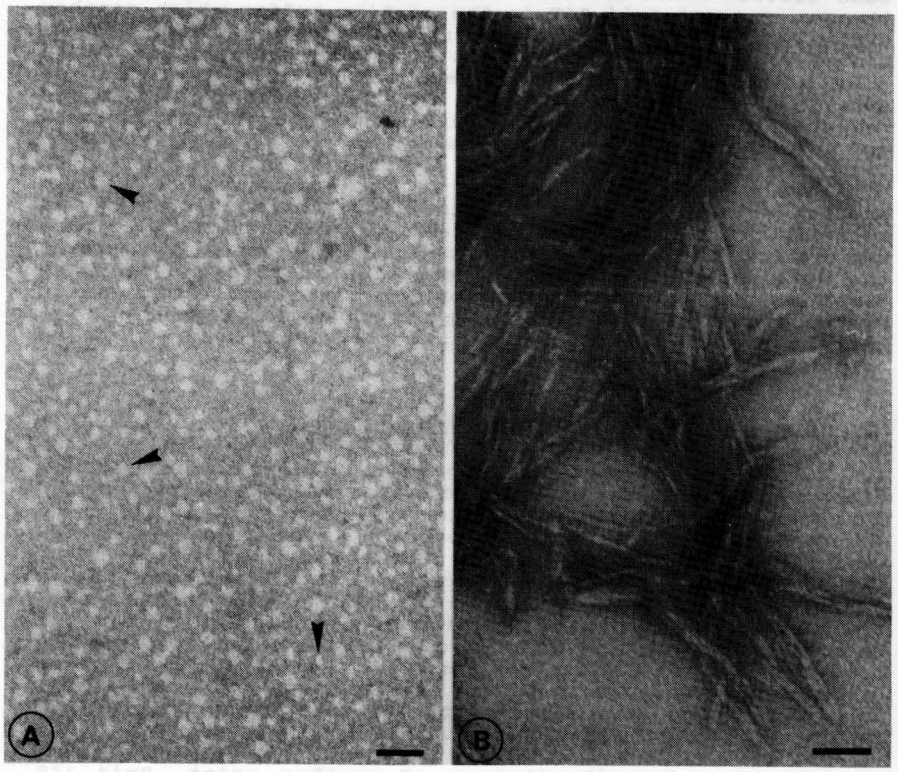

Fig. 3. Electron microscopy of negatively stained preparations of Brij-dissociated chitin synthetase before (A) and after (B) incubation with substrate and activators of the enzyme. Arroheads in A point to chitosomal particles. Notice the short and thick chitin microfibrils in B. Magnification bar, 50 nm.

as shown in Fig. 1, well after the void volume revealing a molecular weight lower than 500 kDa. In one of these runs activity co-eluted with catalase thus indicating a molecular weight of 247 kDa. In other experiments, however, most of activity was recovered in the Vo eluate. Furthermore, catalase and other protein markers such as alcohol dehydrogenase (150 kDa) and ß-amylase (200 kDa) gave also an erratic behaviour under the same elution conditions. We attribute these inconsistencies to the presence of Brij 36T in the eluting buffer which somehow affects the chromatographic properties of these proteins. Enzyme eluted with Brij 36T-free PMB was invariably recovered in the Vo eluate indicating some reaggregation of chitin synthetase subunits. Electron microscopic examination of these samples revealed the presence of structure-less aggregates of variable and undefined morphology (not shown).

An outstanding feature of Brij-dissociated chitin synthetase was its extreme unstability, losing virtually all activity after a few hours of standing at either 2-4°C or room temperature (22-24°C). This characteristic has seriously hampered the determination of the sedimentation coefficient of these particles. Thus, centrifugation of dissociated chitosomal chitin synthetase in sucrose density gradients as described by Ruiz-Herrera *et al.* (1980) brought about a total loss of enzyme activity in several experiments.

DISCUSSION

Previous studies dealing with solubilization and/or dissociation of fungal chitin synthetase with detergents indicate that the enzyme can be solubilized following treatment of crude membrane preparations from *Saccharomyces cerevisiae* (Duran and Cabib, 1978) or *Coprinus cinereus* (Gooday and Rousset-Hall, 1975) with digitonin, a non-ionic surfactant of type B according to the classification of Helenius and Simons (1975). This sterol-complexing agent has been also successfully used for the reversible dissociation of chitosomal chitin synthetase from the mycelium of *Agaricus bisporus* (Hanseler *et al.*, 1983) and yeast cells of *M. rouxii* (Ruiz-Herrera *et al.*, 1980) into active small particles which have a sedimentation coefficient of 16S, as determined by centrifugation in sucrose density gradients.

Here we describe the use of another agent, Brij 36T (polyoxyethylene-10-lauryl ether), a non-ionic surfactant of type A (Helenius and Simons, 1975) for the dissociation of chitosomal chitin synthetase from *M. rouxii*. At the optimum detergent/protein ratio of 0.66-0.96, which usually corresponded to a concentration as low as 0.0125%, Brij 36T released a population of particles morphologically similar to 16S subunits. However, diameter of chitosomal subunits released by this detergent (10-17 nm) differed from that of digitonin-released 16S

subunits (7-12 nm, Ruiz-Herrera *et al.*, 1980) and better compared to that of *A. bisporus* particles (10-20 nm; Hanseler *et al.*, 1983). Following incubation with substrate and activators, Brij-released enzyme subunits synthesized a mesh of short and thick chitin microfibrils indistinguishable from those formed by 16S subunits and, as already observed by Ruiz-Herrera *et al.* (1980), very different to the long and slender fibrils synthesized by native chitosomes after short periods of incubation.

In accordance with previous findings by other authors, detergent-dissociated chitin synthetase required a phospholipid for activity. Thus, it has been observed that digitonin-solubilized yeast chitin synthetase is best activated by phosphatidylserine and lysophosphatidylserine (Durán and Cabib, 1978) whereas 16S chitosomal subunits from *M. rouxii* depend on phosphatidylinositol to be active (Lending *et al.*, 1991). Addition of this phospholipid to assay mixtures increased chitin synthetase activity of Brij-dissociated samples about 8-fold (at the saturating concentration of 0.1 mg per ml) with no detectable effect on activity of undissociated chitosomes. The activity-restoring ability of PI clearly depended on the concentration of surfactant used for dissociation; thus, irreversible loss of enzyme activity was observed at concentrations higher than the optimum, most likely due to protein denaturation.

Digitonin-solubilized chitin synthetase from *S. cerevisiae* (Durán and Cabib, 1978) and 16S chitosomal subunits from *M. rouxii* (Ruiz-Herrera *et al.*, 1980) and *A. bisporus* (Hanseler *et al.*, 1983) have shown to retain the zymogenic nature of native enzyme. Brij-dissociated chitin synthetase, as shown here, was also activated by a protease (rennilase) which is in agreement with the idea, first forwarded by Ruiz-Herrera *et al.* (1980), that the zymogenic state most likely resides in the chitin synthetase protein itself and not in some other component or structural characteristic of the chitosome in which the enzyme is confined. The mechanism of dissociation of chitosomes by Brij 36T is not clear but it might be similar to that of digitonin, a 3-ß-hydroxysteroid precipitating agent (Haslam and Kline, 1953), in the sense that it may also depend on the presence of sterols which together with sterol esters represent the major portion of chitosome neutral lipids (Hernández *et al.*, 1981).

The unreliable behaviour of Brij-dissociated chitin synthetase following gel chromatography and its unstability has precluded further analysis of other parameters such as its sedimentation properties in sucrose density gradients, the molecular weight and also more detailed studies on the reversibility of the disaggregation process. Nevertheless, based on the properties examined in this study, it is our belief that the particles released from chitosomes by Brij 36T are equivalent to the 16S subunits released by digitonin.

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