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Characterization of Chitin Synthase 2 of *Saccharomyces cerevisiae* II: Both Full Size and Processed Enzymes Are Active for Chitin Synthesis

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When chitin synthase 2 of *Saccharomyces cerevisiae* was overexpressed in yeast cells using *GAL1* promoter, deletion of the N-terminal 193 amino acids significantly increased the level of the protein without affecting its characteristics. We partially purified N-terminally truncated chitin synthase 2 by product entrapment and ion exchange column chromatography, and found that it was active even without trypsin treatment when appropriate divalent cations were present in the reaction mixture. This chitin synthase activity was independent of the N-terminal 193 amino acid truncation, because partially purified full length enzyme also exhibited the activity without trypsin treatment in the presence of appropriate cations. Furthermore, the molecular weights of these two forms of chitin synthase 2 were coincident with those estimated from the deduced amino acid sequence, and most of the chitin synthase 2 in the yeast membrane was present as an unprocessed form, as judged from its molecular weight. Treatment of either full length or truncated enzyme with trypsin, however, further increased the enzyme activity by four to fivefold, and produced a 35 kDa polypeptide that specifically reacted with monoclonal antibody raised against the region containing the putative active site of chitin synthase 2. Thus, it appears that predominant native (unprocessed) chitin synthase 2 is active, but the 35 kDa region encompassing the active site is sufficient for the catalytic activity.

Key words: chitin synthase, product entrapment, *Saccharomyces cerevisiae*, zymogen.

Chitin is widely distributed in the cell wall of fungi, and is synthesized by chitin synthases. The content of chitin in the cell wall varies from species to species, and is also regulated by the morphological transition from yeast to mycelial form (*1*). Three types of chitin synthases, chitin synthases 1, 2, and 3 are present in *Saccharomyces cerevisiae*, and they are encoded by the three different genes designated chitin synthase genes 1 (*CHS1*), 2 (*CHS2*), and 3 (*CHS3*), respectively (*2-6*).

Among these three chitin synthases, chitin synthase 1 predominantly exists in yeast cells, but it is not required for the growth of yeast cells, because disruption of the *CHS1* gene does not result in any significant defect in cell growth, viability or morphology (*2*). Inactivation of either one of *CHS2* or *CHS3* is also viable, but simultaneous disruption of the *CHS2* and the *CHS3* genes is lethal (*7*), suggesting that the physiological importance of chitin synthases is not simply determined by the amount of enzyme present in cells. In fact, functional distinction of each chitin synthase has been demonstrated. Chitin synthase 1 is involved in the repair of damaged chitin (*8, 9*), chitin synthase 2 plays important roles in primary septum formation (*7*), and chitin synthase 3 is required for all other chitin syntheses, including the formation of glucan-chitin linkage (*7, 10, 11*).

There are also some differences in the characteristics of the enzymes. Firstly, cation dependence is not the same among three chitin synthases. Chitin synthase 1 and chitin synthase 3 have a preference for Mg^{2+}, whereas chitin synthase 2 shows the highest activity when assayed with Co^{2+} *in vitro* (*12*). Secondly, the zymogenic property of chitin synthase 3 is not simple. Several proteinases including trypsin and chymotrypsin activate chitin synthase 1 as well as chitin synthase 2 *in vitro* (*12, 13*), while simple treatment of chitin synthase 3 with proteinase inactivates the enzyme. Chitin synthase 3 requires the substrate during the proteinase treatment to be activated (*14*). Thirdly, overexpression of either *CHS1* or *CHS2* in yeast cells results in an increase in the activity (*2, 15*), whereas that of *CHS3* does not (*5, 16, 17*). This implies that chitin synthase 3 interacts with a regulatory component which is indispensable for the activity.

Previously, we have demonstrated that *S. cerevisiae* chitin synthase 2 contained a region (con1) that is highly conserved among all chitin synthases, and that two short amino acid stretches found in this region, ^{561}Glu-^{562}Asp-^{563}Arg and ^{601}Gln-^{602}Arg-^{603}Arg-^{604}Arg-^{605}Trp, are completely conserved in all types of chitin synthases and in proteins harboring β-1,4-glycosyltransferase activities, such as NodC proteins of *Rhizobium* bacterias, DG42 protein of *Xenopus laevis*, hyaluronan synthase of *Streptococcus pyogenes*, and cellulose synthase of *Acetobacter xylinum* (*15*). Mutational analyses of these sequences revealed that they function as a putative active site of the

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Abbreviations: GlcNAc, *N*-acetylglucosamine; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; SDS, sodium dodecyl sulfate.

enzyme and are presumably involved in the catalytic reaction (*15*).

In order to gain further insights into chitin synthases, we have partially purified chitin synthase 2 that was overexpressed in yeast cells. The partially purified enzyme retained the zymogenic property, but proteolytic cleavage was not essential for the activity when appropriate divalent cations were present. Thus, both unprocessed and processed forms of chitin synthases are capable of synthesizing chitin.

MATERIALS AND METHODS

Plasmids and Yeast Strains—*S. cerevisiae* strain, RRA400-1U (MAT*a leu2 trp1 chs1Δ::URA3 chs3Δ::HIS3*), in which the endogenous *CHS1* and *CHS3* genes had been disrupted, was described in a previous paper (*15*). YpLCS2 was constructed by ligating *S. cerevisiae CHS2* gene at the *Xba*1 cleavage site of YpLX that harbors *GAL1* promoter, *GAPDH* terminator and the *LEU2* gene as a selectable marker (*15*). YpLCS2Δ193 was the same as YpLCS2 except that the part of coding region encoding the N-terminal 193 amino acids of the *CHS2* gene was deleted. Thus, expression of *CHS2* or *CHS2Δ193* was under the control of *GAL1* promoter. Both of the full length *CHS2* gene and the Δ193*CHS2* gene were obtained by PCR. Primers used for PCR were $^{5'}$GACTCTAGAATGACGAGAAACCCG$^{3'}$ and $^{5'}$CCTCTAGATTAGCCCTTTTTGTGGAA$^{3'}$ for the full length *CHS2* gene, and $^{5'}$GGTCTAGAATGGACACTTTCAATGAAACA$^{3'}$ and $^{5'}$CCTCTAGATTAGCCCTTTTTGTGGAA$^{3'}$ for the Δ193*CHS2* gene. Cells of RRA400-1U transformed with YpLCS2 or YpLCS2Δ193 were designated as RRA-CHS2 or RRA-CHS2Δ193, respectively, and were cultured in YND medium (0.7% yeast nitrogen base, 5% ammonium sulfate, and 2% dextrose) supplemented with required amino acids. To induce *CHS2* expression, cells of RRA-CHS2 or RRA-CHS2Δ193 were cultured in YND medium until OD_{600} reached 1.0, washed twice with H_2O, and further cultured in YNG medium (0.7% yeast nitrogen base, 5% ammonium sulfate, 2% galactose) for 12 h. Introduction of DNA into yeast cells was carried out by electroporation as described (*18*).

Extraction and Purification of Chs2p—Cells of RRA-CHS2 or RRA-CHS2Δ193 which were cultured in galactose medium were harvested, washed twice with 20 mM Tris-HCl (pH 7.5), suspended in 5 ml per g yeast of buffer A containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.25 mM PMSF, 2 μg/ml chymostatin, 1.5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml antipain, and then lysed with glass beads (0.25 mm in diameter) using a Braun homogenizer. Cell debris was removed by low-speed centrifugation at 2,000×*g* for 2 min, then the total membrane was sedimented at 100,000×*g* for 1 h at 4°C, washed once with 20 mM Tris-HCl (pH 7.5), and suspended in 1.5 ml per g yeast cells of 20 mM Tris-HCl (pH 7.5) containing 33% glycerol. In order to solubilize Chs2p, digitonin [final 1 (w/v)%] and phosphatidylserine (final 0.25 mg/ml) were added to the membrane, and the mixture was incubated at 30°C for 30 min with vigorous shaking. After centrifugation at 100,000×*g* for 1 h at 4°C, supernatants were collected and used as the solubilized enzyme.

Product entrapment was carried out according to the method of Duran and Cabib (*13*) with some modifications. To the digitonin-solubilized enzyme, GlcNAc (final 4 mM), UDP-GlcNAc (final 6 mM), and $MnCl_2$ (final 6 mM) were added, and the mixture was incubated at 30°C for 30 min. The chitin-enzyme complexes were sedimented by low-speed centrifugation (3,000×*g* for 5 min), and washed once with a buffer containing 20 mM Tris-HCl (pH 7.5), 30% glycerol, 0.1% digitonin, 0.02% phosphatidylserine, 4 mM GlcNAc, 6 mM UDP-GlcNAc, and 6 mM $MnCl_2$. Then the enzyme was released from the chitin by suspending the chitin-enzyme complexes in buffer B containing 20 mM Tris-HCl (pH 7.5), 30% glycerol, 0.1% digitonin, and 0.02% phosphatidylserine followed by centrifugation at 80,000×*g* for 5 min at 4°C. After extraction of the enzyme five times, the supernatants of all the centrifugations were combined. Both N-terminally truncated and full length Chs2p were purified by repeating the product entrapment twice, and were used for experiments or subjected to further purification by Mono Q column chromatography.

In some experiments, 1 mg protein of the total membrane of RRA-CHS2Δ193 suspended in 100 μl of a buffer containing 20 mM Tris-HCl (pH 7.5) and 33% glycerol was incubated with 0.2, 2, or 5 μg/ml trypsin at 30°C for 15 min. Trypsin treatment was terminated by adding PMSF to give a final concentration of 0.1 mM, and the resulting trypsin-treated membrane (total 120 μl) was fractionated by ultracentrifugation at 100,000×*g* for 60 min at 4°C. Precipitates were then re-suspended in 120 μl of the same buffer [20 mM Tris-HCl (pH 7.5) and 33% glycerol], and 3 μl aliquots of the S100 and P100 fractions were assayed for chitin synthase activity as mentioned below.

Mono Q Column Chromatography—Fifty milligram protein (3 ml) of Chs2Δ193p partially purified by product entrapment was applied to a Mono Q HR5/5 column equilibrated with buffer B containing 25 mM NaCl. After extensive washing with the same buffer, Chs2Δ193p was eluted with buffer B containing a 25-500 mM linear gradient of NaCl at a flow rate of 1 ml/min. One milliliter of each fraction was assayed, and fractions containing chitin synthase 2 activity were combined and used for the experiments.

Assay of Chitin Synthase—Chitin synthase activity was determined by the method of Sburlati and Cabib (*12*) with some modifications. Unless otherwise specified, chitin synthase assay was carried out in a standard 50 μl reaction mixture containing 30 mM Tris-HCl (pH 7.0), 5 mM $MnCl_2$, 32 mM GlcNAc, 0.1 mM [^{3}H]UDP-GlcNAc (specific activity, 95,880 dpm/nmol), and appropriate amounts of proteins at 30°C for 60 min. The reaction was terminated by adding 10% TCA, and radioactivity incorporated into acid-insoluble chitin fractions was counted in a toluene-based liquid scintillator. In some cases, the enzyme was treated with the indicated amounts of proteinases at 30°C for 15 min followed by the addition of proteinase inhibitors to prevent further digestion.

Antibody Generation—Polyclonal antibody raised against a part of Chs2p (from amino acid position 192 to 624) has already been described (*15*). For the generation of monoclonal antibody, a region of Chs2p that is highly conserved among all chitin synthases (designated con1, from amino acid position 490 to 607) was expressed in *E. coli* as a fusion protein with glutathione-*S* transferase (GST) (*19*). Insoluble GST-con1 fusion protein was fractionated on SDS-polyacrylamide gels, excised from the gels and injected into

mice. A hybridoma line producing a monoclonal antibody against con1 was cloned and IgG fractions containing anti-con1 monoclonal antibody were collected from the culture media of the hybridoma and purified by protein A-Sepharose column chromatography (*20*).

Western Blotting—An indicated amount of the total membrane or partially purified Chs2p was fractionated on SDS-polyacrylamide gels, transferred to a PVDF membrane electrophoretically (*20*), incubated with anti-Chs2p polyclonal or monoclonal antibody and then horseradish peroxidase conjugated with anti-rabbit or anti-mouse IgG, and visualized with cyclic diacrylhydrazides (ECL detection kit, Amersham) followed by exposure to X-ray film.

Northern Blotting—Exponentially growing cells of RRA-CHS2 and RRA-CHS2⊿193 were suspended in a buffer containing 100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5), and 0.2% SDS, and lysed by homogenization with glass beads (*21*). The total RNA was then extracted with phenol followed by precipitation with ethanol, and polyA$^+$ RNA was isolated with Oligotex dT_{30} (*22*). Twenty micrograms of polyA$^+$ RNA was fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with radiolabeled probes and visualized by autoradiography (*23*). A 2.4 kb *Xho*I fragment of YpLCS2 (for *CHS2*) and a 1.0 kb *Pst*I/*Xba*I fragment of pSV2-γ-actin (for actin) were radiolabeled by the random priming method with [α-^{32}P]dCTP (*23*) and used as probes. Hybridization and washing of filters were carried out under stringent conditions (50% formamide at 42°C for hybridization and 0.1×SSC at 60°C for washing).

RESULTS

Chitin synthase 2 is known to be a zymogen. In order to establish the mechanism of activation, we have purified and characterized the enzyme. Since chitin synthase 2 could not be expressed in bacterial cells or insect cells in an active form, we chose *S. cerevisiae* cells as a host for its overexpression. When Chs2p was overexpressed using galactose promoter in cells lacking both *CHS1* and *CHS3*, the protein level was not particularly high as judged by Western blotting. During the course of experiments using deletion mutants of Chs2p, we found that the deletion of the N-terminal 193 amino acids increased the enzyme activity by two to threefold, while deletion of 230 amino acids or more nearly completely inactivated the enzyme (Fig. 1). This increase in the activity of Chs2⊿193p was due to the increase in its protein level, because the amount of Chs2-⊿193 in the total membrane was also two- to threefold higher than that of intact (full length) Chs2p (Fig. 2). However, this N-terminal deletion of Chs2p did not affect its mRNA level (Fig. 2), suggesting that the increase in the protein level of Chs2p caused by deleting the N-terminal 193 amino acids was not a consequence of changes in transcriptional processes, but might be due to enhancement of the translational efficiency.

N-Terminally truncated Chs2p (Chs2⊿193p) was extracted from the total membrane of RRA-CHS2⊿193 cells with digitonin, and was purified by product entrapment. During the course of experiments, we found that digitonin-extracted Chs2⊿193p produced chitin fibers in the presence of $MnCl_2$ when UDP-GlcNAc was added to the enzyme, and that this chitin synthesis was completely independent of trypsin treatment of the enzyme. By repeating the product entrapment, an 88 kDa protein that specifically reacted

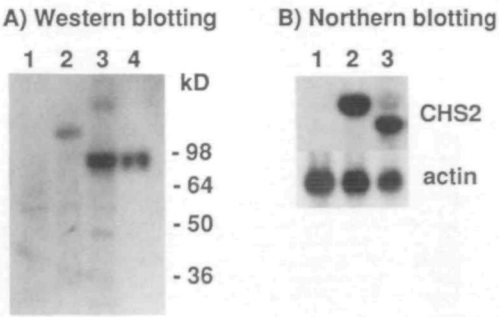

Fig. 2. **Increased level of chitin synthase 2 expression by deletion of N-terminal 193 amino acids.** (A) One milligram protein of the total membrane was prepared from RRA400-1U cells that had been transfected with YpLX (lane 1), YpLCS2 (lane 2), or YpLCS2⊿193 (lane 3), separated on a 10% SDS-polyacrylamide gel, and hybridized with anti-Chs2p polyclonal antibody. In lane 4 of panel A, 1 μg protein of Chs2⊿193p-containing fraction of the second product entrapment was fractionated. (B) Twenty micrograms of polyA$^+$ RNA was extracted from RRA400-1U cells that had been transfected with YpLX (lane 1), YpLCS2 (lane 2), or YpLCS2⊿193 (lane 3), fractionated on an agarose gel, and examined by Northern blotting using the *CHS2* gene or the actin gene as probes. For details, see "MATERIALS AND METHODS."

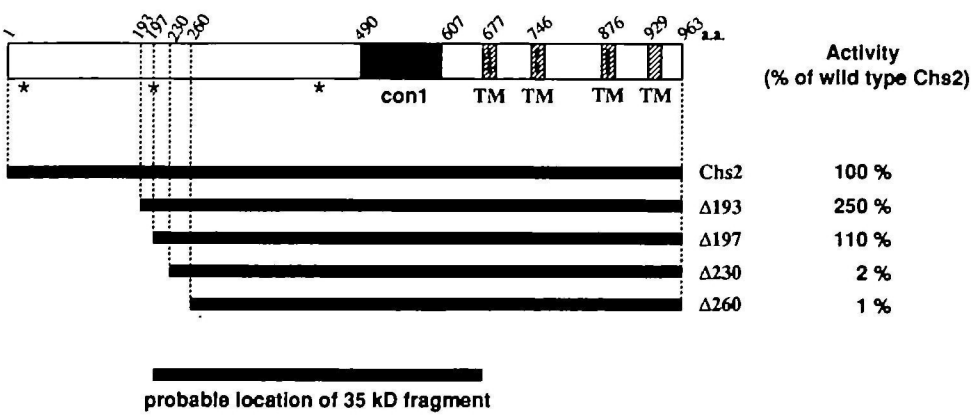

Fig. 1. **Structure and deletion mutants of Chs2p.** The structure of Chs2p is illustrated together with the chitin synthase activity of the deletion mutants and the positions of amino acids from the N-terminal end. The region that contains the putative active site of the enzyme and that was used for generating monoclonal antibody is indicated as con1. TM and star indicate transmembrane domains and potential *N*-glycosylation sites, respectively. The possible location of the 35 kDa fragment of Chs2p that was produced by the trypsin treatment is shown at the bottom.

with both polyclonal and monoclonal antibodies raised against Chs2p was highly enriched (Fig. 3A), demonstrating that the 88 kDa protein was indeed Chs2⊿193p. The molecular weight of partially purified Chs2⊿193p (88 kDa) coincided with that estimated from its deduced amino acid sequence (88.5 kDa), and there was no other protein that reacted with anti-Chs2p antibodies (Fig. 3A). Thus, partially purified Chs2⊿193p remained in the unprocessed form and it was not degraded by proteinases. Unexpectedly, neither endoglycosidase H nor endoglycosidase F affected the molecular weight of partially purified Chs2⊿193p, whereas that of invertase was clearly reduced by the same treatment (data not shown), suggesting that chitin synthase 2 was not a highly glycosylated protein in spite of its membrane localization.

The Chs2⊿193p partially purified by product entrapment was further purified by Mono Q ion exchange column chromatography. As shown in Fig. 3, B and C, chitin synthase activity was eluted with a linear gradient of NaCl, and the elution profiles of chitin synthase activity and the 88 kDa protein that specifically reacted with anti-Chs2p antibody were identical. The above result also confirmed that the 88 kDa protein was active Chs2⊿193p. Although further purification inactivated the enzyme, Chs2⊿193p was purified over 300-fold with recovery of approximately 20% of total chitin synthase activity present in the membrane by digitonin extraction, product entrapment and Mono Q column chromatography.

As mentioned earlier in this paper, partially purified Chs2⊿193p exhibited chitin synthase activity in the presence of Mn^{2+} without trypsin treatment. Since Co^{2+} was reported to be the preferred cation for Chs2p (*12*), we also examined the effects of Co^{2+}, Mg^{2+}, and Mn^{2+} on the enzyme activity. Among the three cations, Mn^{2+} was found to be the most effective cation for partially purified Chs2⊿193p (Fig. 4A). Activity of Chs2⊿193p was completely dependent on cations; without any cation, the partially purified enzyme did not show any chitin synthase activity. More importantly, the size of Chs2⊿193p was unaffected by the addition of divalent cations; it remained 88 kDa in size even in the presence of cations (Fig. 5). However, partial digestion of Chs2⊿193p with trypsin increased the enzyme activity by several-fold as reported previously (Fig. 4A) (*12*). Divalent cations were also required for the activity of trypsin-treated Chs2⊿193p; no activity was detected in the absence of cation even when Chs2⊿193p was treated with trypsin. Further, trypsin treatment of the enzyme decreased the size of Chs2⊿193p from 88 to 75 kDa and smaller (Fig. 5). These results clearly demonstrated that partially purified Chs2⊿193p retained the zymogenic property, but is partially activated by some divalent cations without proteolytic cleavage, at least as far as could be detected in terms of changes of mobility on SDS-polyacrylamide gel electrophoresis. The same result was obtained with full length Chs2p that was also partially purified by product entrapment. Activity of

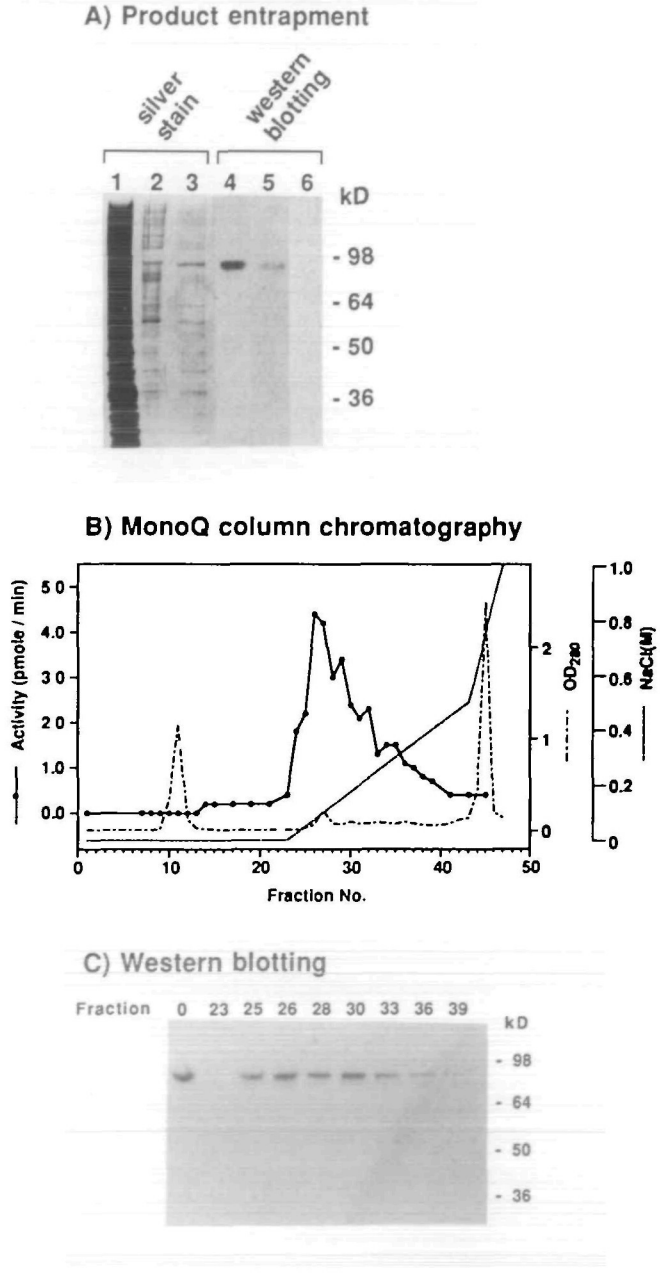

Fig. 3 **Partial purification of chitin synthase 2.** (A) Solubilization from the membrane and product entrapment. Chs2⊿193p that had been extracted from the membrane and purified by the product entrapment was separated on a 10% SDS-polyacrylamide gel and stained with silver (lanes 1-3). Partially purified Chs2⊿193p after the second product entrapment was further analyzed by Western blotting (lanes 4-6) Lane 1: 10 μg protein of the digitonin extract of the total membrane, lane 2: 1 μg protein of the extract of the first product entrapment, lane 3, 1 μg protein of the extract of the second product entrapment, lane 4: 1 μg protein of the extract of the second product entrapment probed with anti-Chs2p polyclonal antibody, lane 5 10 μg protein of the digitonin extract of the total membrane probed with anti-Chs2p monoclonal antibody, lane 6. 1 μg protein of the extract of the second product entrapment probed with control rabbit serum (B) Mono Q column chromatography. Chs2⊿193p that had been partially purified by repeating the product entrapment was further purified by Mono Q column chromatography. Elution profiles of total proteins as determined by OD_{280} and chitin synthase activity are indicated Chitin synthase assay was carried out in the presence of 5 mM $MnCl_2$ without trypsin treatment of the enzyme (C) Western blotting of Mono Q fractions. Twenty microliters of the indicated fraction was separated on 10% SDS-polyacrylamide gels and analyzed by Western blotting using anti-Chs2p polyclonal antibody. Fraction 0 represents samples before fractionation by Mono Q column chromatography. For details, see "MATERIALS AND METHODS."

Chs2p partially purified by product entrapment was 10 times lower than that of Chs2Δ193p (Fig. 3). This was presumably due to the lower concentration of Chs2p in the fraction compared to that of Chs2Δ193p, because Western blotting demonstrated that 10 times more protein of Chs2p fraction was required to get a similar level of Chs2p to that of Chs2Δ193p (Fig. 5). Partially purified Chs2p whose molecular weight was determined as 110 kDa by SDS-

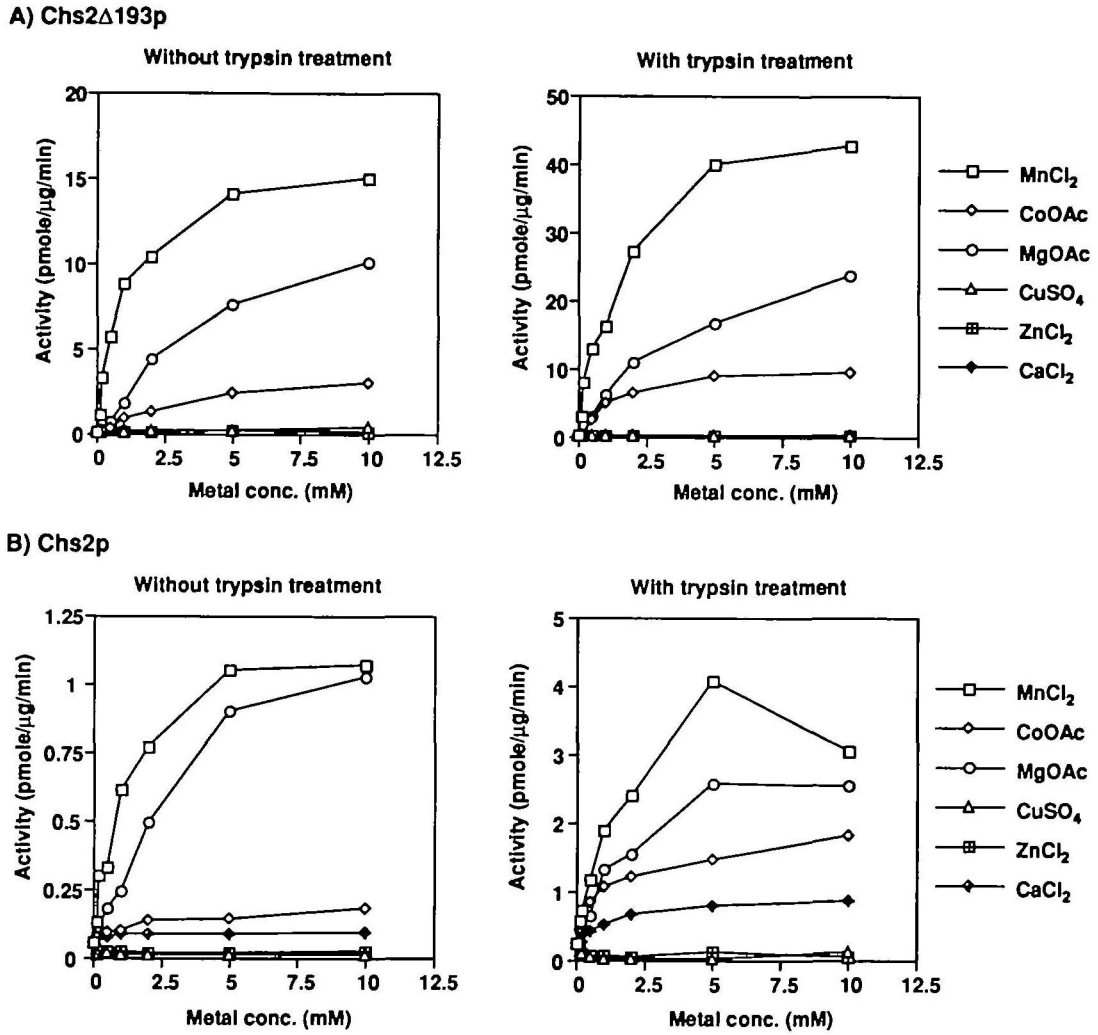

Fig. 4. **Cation dependence of partially purified chitin synthase 2.** One microgram protein of Chs2Δ193p-containing fraction of Mono Q column chromatography and 10 μg protein of Chs2p-containing fraction of the second product entrapment were untreated or treated with 0.2 μg/ml trypsin, and then assayed for chitin synthase activity in the presence of the indicated concentrations of divalent cations. For details, see "MATERIALS AND METHODS."

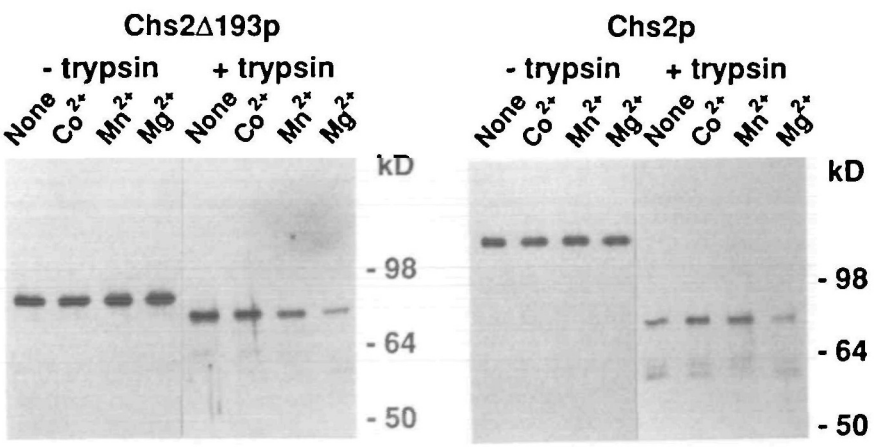

Fig. 5. **Western blotting of chitin synthase 2 in the presence of divalent cations.** Chs2Δ193p and Chs2p that had been untreated or treated with 0.2 μg/ml trypsin in the presence of 5 mM of the indicated divalent cations were separated on 10% SDS-polyacrylamide gels and visualized by Western blotting using anti-Chs2p polyclonal antibody. One microgram protein of Chs2-Δ193p-containing fraction of Mono Q column chromatography and 10 μg protein of Chs2p-containing fraction of the second product entrapment were used for the experiments. For further details, see "MATERIALS AND METHODS."

polyacrylamide gel electrophoresis (this molecular weight was identical with that estimated from the deduced amino acid sequence) also showed significant chitin synthase activity in the presence of Mg^{2+} or Mn^{2+}, even without trypsin treatment. However, partial digestion with trypsin hyper-activated the enzyme and decreased the molecular weight from 110 to 75 kDa and less (Figs. 4B and 5). Thus, cation-dependent, but proteolysis-independent chitin synthase activity was not unique to Chs2Δ193p, and it was a general characteristic of chitin synthase 2. Indeed, we did not find any significant difference in characteristics between partially purified Chs2Δ193p and Chs2p; both showed a zymogenic property, similar K_m value for the substrate, similar K_i value with nikkomycin Z, and the same preference of cations for the activity (Table I).

Since trypsin treatment of either partially purified Chs2Δ193p or Chs2p produced 75 kDa polypeptide and several smaller fragments (Fig. 5), chitin synthase 2 can be

TABLE I. **Characteristics of Chs2p and Chs2Δ193p.** Both intact and N-terminally (193 amino acids) truncated Chs2p were partially purified by product entrapment. K_m and K_i values were determined using trypsin-treated enzymes.

Enzyme (entrapped)	K_m for substrate	K_i of nikkomycin Z	Zymogenic property	Metal requirement
Chs2p	1.3 mM	6.8 μM	Yes	$Mn^{2+} > Mg^{2+} > Co^{2+}$
Chs2Δ193p	1.4 mM	4.2 μM	Yes	$Mn^{2+} > Mg^{2+} > Co^{2+}$

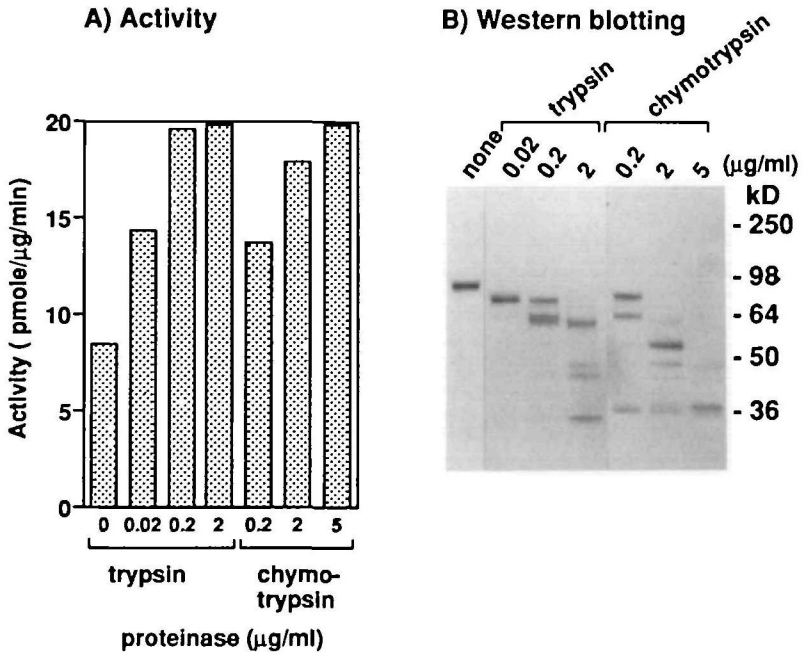

Fig. 6. **Hyper-activation of chitin synthase 2 by trypsin treatment.** One microgram protein of Chs2-Δ193p-containing fraction of Mono Q column chromatography was treated with the indicated concentrations of trypsin or chymotrypsin, and assayed for chitin synthase activity (A) or analyzed by Western blotting using anti-Chs2p monoclonal antibody (B). Chitin synthase assay was carried out in the presence of 5 mM $MnCl_2$. For details, see "MATERIALS AND METHODS."

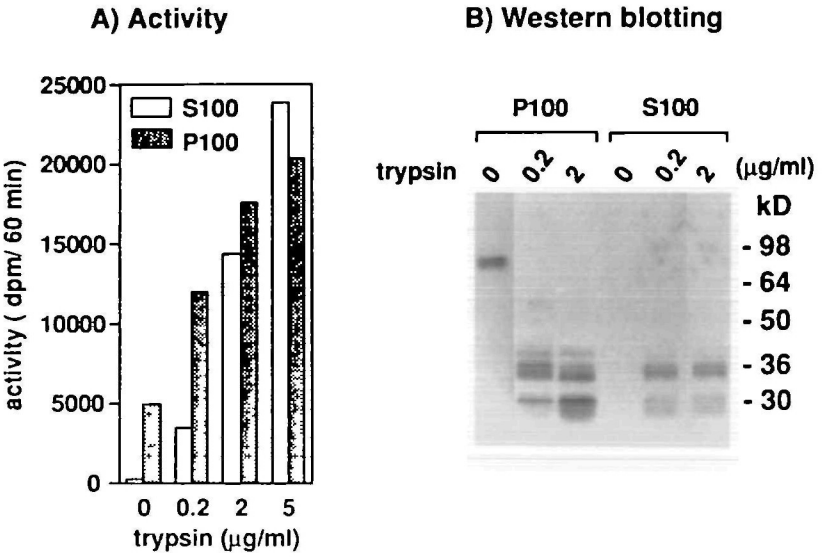

Fig. 7. **Appearance of chitin synthase 2 fragments on trypsin treatment of the total membrane.** One microgram protein of the total membrane of RRA-CHS2Δ193 was treated with the indicated concentrations of trypsin, and fractionated by ultracentrifugation. After suspending the precipitates in the same volume as the supernatants, 3 μl each from the supernatant (S100) and precipitate (P100) fractions was assayed for chitin synthase activity in the presence of 5 mM $MnCl_2$ (A), or analyzed by Western blotting using anti-Chs2p monoclonal antibody (B). For details, see "MATERIALS AND METHODS."

much shorter than 75 kDa polypeptide without loss of the activity. To examine this further, we treated partially purified Chs2Δ193p with various concentrations of trypsin or chymotrypsin. As shown in Fig. 6, treatment of Chs2Δ193p with increased concentrations of trypsin or chymotrypsin enhanced its chitin synthase activity, and this increase in the activity was accompanied with degradation of the 88 kDa Chs2Δ193p. When Chs2Δ193p was treated with a higher concentration of trypsin (2 μg/ml), a 35 kDa polypeptide that still strongly hybridized with anti-Chs2p monoclonal antibody became predominant, yet Chs2Δ193p remained hyper-activated (Fig. 6). Further, this 35 kDa polypeptide also appeared when partially purified full length Chs2p was hyper-activated by trypsin treatment (data not shown).

These results suggest that the 35 kDa proteolytic product of Chs2p is one of the major components of the active form of chitin synthase 2. If the 35 kDa fragment of Chs2p produced by trypsin treatment is really an active form of the enzyme, it should also appear when the yeast membrane is treated with trypsin. Therefore, we treated the total membrane prepared from RRA-*CHS2*Δ193 cells with trypsin, and fractionated the products by ultracentrifugation in the absence of detergent. As expected, similar sizes of Chs2Δ193p fragments (35 and 36 kDa) appeared after trypsin treatment of the membrane. Interestingly, about half of the 35 and 36 kDa polypeptides was detected in S100 fraction, whereas fragments of 45 kDa or larger molecular weight remained in the P100 fraction (Fig. 7B). Furthermore, S100 fraction that contained 35 and 36 kDa fragments of Chs2Δ193p was fully active in synthesizing chitin (Fig. 7A), and neither 35 kDa/36 kDa polypeptide nor chitin synthase activity was detected in S100 fractions unless the membrane was treated with trypsin. These results also strongly support the idea that the soluble 35 kDa polypeptide produced by the trypsin treatment of Chs2Δ193p retains the catalytic activity, and is one of the hyper-activated forms of chitin synthase 2.

DISCUSSION

We have overexpressed and purified N-terminally truncated Chs2p. So far as tested, deletion of the N-terminally 193 amino acids did not change any characteristic of the enzyme, and both N-terminally truncated and full length Chs2p showed chitin synthase activity in the presence of Mn^{2+} and Mg^{2+} even without proteolytic cleavage. There was no obvious change in molecular weight of proteins when they were incubated with Mg^{2+} or Mn^{2+}. However, partial digestion of either form of the enzyme with trypsin or chymotrypsin significantly increased the activity, and reduced the molecular weight. Thus, it appears that unprocessed Chs2p (at least Chs2p showing no detectable change in molecular weight on SDS-polyacrylamide gel electrophoresis) is active when appropriate divalent cations are present, but is hyper-activated by proteolytic cleavage.

Treatment of partially purified Chs2Δ193p with higher concentrations of trypsin or chymotrypsin hyper-activated the enzyme, and produced a 35 kDa fragment that reacted with anti-Chs2p monoclonal antibody. Since this monoclonal antibody was raised against con1 region that contains the putative active site of Chs2p (*15*), this 35 kDa fragment of Chs2p should contain the active site of the enzyme. Similar sizes of active polypeptides (35 and 36 kDa) were also produced by the trypsin treatment of the total membrane, and they were found in the soluble S100 fraction. Deletion of more than 230 amino acids from the N-terminal end completely inactivated the enzyme *in vivo*. All these results strongly support the idea that the 35 kDa fragment is one of the hyper-activated forms of chitin synthase 2, and that the 35 kDa fragment corresponds to the region of residues 197 and 676, just before the first transmembrane domain (Fig. 1). We also tried to purify this 35 kDa fragment to address whether or not the 35 kDa fragment of Chs2p alone is sufficient for catalytic activity and to define its amino acid sequence, but without success, due to its instability.

It is still controversial whether or not the hyper-activation of chitin synthase by proteolytic cleavage happens *in vivo*. Most of the Chs2p expressed in yeast cells remained unprocessed, as judged from its molecular weight (110 kDa), and no protein below the 50 kDa size marker was detected by anti-Chs2p antibodies in the yeast total membrane (Fig. 2A). Therefore, it is highly likely that some divalent cations regulate the activity of unprocessed Chs2p to let it function *in vivo*.

Previously, it was demonstrated that Co^{2+} was the most effective cation for the chitin synthase 2 activity, whereas chitin synthase 1 shows a preference for Mg^{2+} (*12*). Partially purified Chs2p in this study, however, was efficiently activated by Mn^{2+} as well as Mg^{2+}, and Co^{2+} only slightly activated the enzyme. Since we also found that chitin synthase 2 activity in the total membrane prepared from cells harboring *CHS2* and *CHS3* was much higher with Co^{2+} than with Mn^{2+} or Mg^{2+}, the above difference in cation dependence may suggest that chitin synthase 2 has a different cation dependence when it co-exists with other chitin synthases.

Machida and Saito (*24*) also purified chitin synthase from *Absidia glauca* without proteolytic activation of the enzyme. The molecular weight of the purified enzyme was 30 kDa, and trypsin treatment converted the zymogen into a 28.5 kDa active form. We do not know if proteolytic cleavage of this protein is essential for enzyme activity, but it is of interest that the molecular weight of *A. glauca* chitin synthase is quite similar to that of *S. cerevisiae* Chs2p fragment produced by trypsin treatment (35 kDa). Further, NodC protein of *Azorhizobium caulinodans* has a molecular weight (46.8 kDa) much smaller than that of yeast chitin synthases, and also possesses *N*-acetylglucosaminyl transferase activity (*25*). Thus, it is conceivable that a small part of yeast chitin synthase is sufficient for catalytic activity, but other parts of the enzyme are needed for other functions, *e.g.*, membrane localization, binding to chitin and export of chitin fibers. In fact, our preliminary experiments revealed that the deletion of the C-terminal region of Chs2p also drastically decreased the enzyme activity of the molecule expressed in yeast cells.

Subcellular localization of yeast chitin synthases is determined by fractionating cell extracts and subsequent electron microscopy. Chs1p and Chs2p of *S. cerevisiae* are detected both in plasma membrane and in unique particles designated chitosomes (*26, 27*). Chs2p possesses 3 potential *N*-glycosylation sites. However, our preliminary experiments demonstrated that endoglycosidase H or endoglycosidase F did not affect the mobility of Chs2p in SDS-

polyacrylamide gel electrophoresis, whereas the size of invertase was remarkably reduced after treatment with these enzymes. This finding raises the possibility that migration of Chs2p into chitosomes or plasma membrane does not require high-mannose type glycosylation of Chs2p and that an unknown secretory pathway that is independent of high-mannose type glycosylation is present for the membrane localization of Chs2p.

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