Journal of Microbiology (2014) Vol. 52, No. 4, pp. 284–291
DOI 10.1007/s12275-014-4070-8
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Functional Characterization of Extracellular Chitinase Encoded by the Yl*CTS1* Gene in a Dimorphic Yeast *Yarrowia lipolytica*

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(Received Feb 3, 2014 / Revised Feb 7, 2014 / Accepted Feb 7, 2014)

The hemiascomycetes yeast *Yarrowia lipolytica* is a dimorphic yeast with alternating yeast and mycelia forms. Bioinformatic analysis revealed the presence of three putative chitinase genes, Yl*CTS1*, Yl*CTS2*, and Yl*CTS3*, in the *Y. lipolytica* genome. Here, we demonstrated that the protein of Yl*CTS1* (YlCts1p), which contains an N-terminal secretion signal peptide, a long C-terminal Ser/Thr-rich domain, and a chitin-binding domain, is a homologue to *Saccharomyces cerevisiae* chitinase 1 (ScCts1p). Deletion of Yl*CTS1* remarkably reduced extracellular endochitinase activity in the culture supernatant of *Y. lipolytica* and enhanced cell aggregation, suggesting a role of YlCts1p in cell separation as ScCts1p does in *S. cerevisiae*. However, loss of YlCts1p function did not affect hyphal formation induced by fetal bovine serum addition. The mass of YlCts1p was dramatically decreased by jack bean α-mannosidase digestion but not by PNGase F treatment, indicating that YlCts1p is modified only by *O*-mannosylation without *N*-glycosylation. Moreover, the *O*-glycan profile of YlCts1p was identical to that of total cell wall mannoproteins, supporting the notion that YlCts1p can be used as a good model for studying *O*-glycosylation in this dimorphic yeast.

***Keywords*:** Chitinase, *Yarrowia lipolytica*, Yl*CTS1*, *O*-mannosylation

Introduction

Chitinase (EC 3.2.1.14) can cleave the β-1,4-linkage between N-acetyl-D-glucosamine (GlcNAc) residues in the linear homopolymer chitin, which is an essential component of yeast and fungal cell walls. Chitinases are grouped into one of two families, 18 or 19 of glycoside hydrolases (GH) on the basis of amino acid sequence similarity (Hartl *et al*., 2012). Family 18 GHs are primarily consist of most fungal chitinases, which are grouped two subclasses, “plant-type” and “bacterial-type’ family 18 chitinases. In the case of the model yeast *Saccharomyces cerevisiae*, it possesses two chitinase-encoding genes: Sc*CTS1* coding for the plant-type chitinase and Sc*CTS2* for the bacterial-type chitinase. In *S. cerevisiae,* Cts1p and Cts2p function independently in bud separation and spore formation, respectively (Kuranda and Robbins, 1991; King and Butler, 1998). *S. cerevisiae* Cts1p (ScCts1p) is the only endochitinase expressed during vegetative growth; *S. cerevisiae* strains lacking this enzyme display incomplete cell separation and show pseudohypha-like growth (King and Butler, 1998). ScCts1p is composed of several domains observed in plant-type chitinases including an N-terminal signal peptide, a catalytic domain, a serine/threonine-rich region and C-terminal chitin-binding domain (Hurtado-Guerrero and van Aalten, 2007). Because of the high degree of *O*-glycosylation at its serine/threonine-rich region without *N*-glycosylation, ScCts1p has been used as a model protein for analyzing *O*-glycosylation patterns in *S. cerevisiae* (Gentzsch and Tanner, 1996, 1997) as well as in the heterologous host *Schizosaccharomyces pombe* (Tanaka *et al*., 2005). Moreover, altered electrophoresis pattern of extracellular chitinase of *Hansenula polymorpha* was also used to study genes involved in *O*-mannosylation in this methylotrophic yeast (Kim *et al*., 2006, 2013).

The hemiascomycetes yeast *Yarrowia lipolytica* is a dimorphic yeast with alternating yeast and mycelia forms that has ability to grow on n-paraffins and produce high amounts of organic acid (Tsugawa *et al*., 1969). This species has been widely used in industrial applications such as a host strain producing citric acid, peach flavor, and single cell protein production (Beckerich *et al*., 1998). More recently, *Y. lipolytica* has been emerged as a potential host for producing heterologous proteins due to its capacity to secrete high levels of extracellular proteins such as alkaline extracellular protease, lipase, and RNase (Madzak *et al*., 2004). This yeast is admittedly nonpathogenic, and several processes based on *Y. lipolytica* have been classified as “generally recognized as safe (GRAS)” (Groenewald *et al*., 2014).

The capacity for glycosylation, which is similar to that of mammalian systems, potentially makes this yeast an excellent host for producing therapeutic glycoproteins. We previously showed that *Y. lipolytica N*-glycans are composed of neutral oligosaccharides as major species, whereas acidic oligosaccharides containing mannose phosphate residues are present as minor species (Song *et al*., 2007; Park *et al*., 2011). Basic information regarding the *N*-glycan structure and its biosynthetic pathway in *Y. lipolytica* has been applied in the development of the glyco-engineered *Y. lipolytica* strains that can produce human-like *N*-glycans. Human-compatible *N*-glycans can be produced by introducing a mannose trimming capacity through the expression of heterologous α-1,2-mannosidase in the *Y. lipolytica* mutant strains, in which the

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Yl*OCH1* and Yl*MPO1* genes were deleted to prevent yeast-specific processing of *N*-glycans (De Pourcq *et al.*, 2012; Moon *et al.*, 2013). Moreover, *Y. lipolytica* strains have been glyco-engineerd to generate *N*-glycans with enhanced mannose-6-phosphate modification to increase the targeting efficiency of recombinant glycoproteins to the lysosome (Tiels *et al.*, 2012). In contrast to these intensive studies and engineering efforts to manipulate the *N*-glycosylation pathway of *Y. lipolytica*, limited information is available regarding *O*-glycosylation pathway of *Y. lipolytica.* To develop a *Y. lipolytica* model glycoprotein for *O*-glycan profile analysis, we have identified a ScCts1p homologue in *Y. lipolytica* and analyzed its functional and biochemical characteristics.

Materials and Methods

Strains and medium

The *Y. lipolytica* SMS397A strain (*MATA ade2 ura3 xpr2*) was used as a parental strain for Yl*CTS1* gene disruption. The disruption of Yl*CTS1* was carried out by using a PCR-based gene disruption method as previously described (Song *et al.*, 2007). Each 5′- or 3′-DNA fragment (300-base pair [bp] or 326-bp) of the Yl*CTS1* gene was amplified using the primer sets, YlCTS1_NF and YlCTS1_NR (5′-ttggagctt cgctcatgtct-3′ and 5′-gctagcggtaccagatcttgaacaccttttgcccag ac-3′) or YlCTS1_CF and YlCTS1_CR (5′-agatctggtaccgcta gcctgctgctgacaagccctctt-3′ and 5′-ctacttgagcatagcaaagac-3′), respectively. A fusion DNA fragment of the 5′- and 3′-DNA fragments was obtained using the primers YlCTS1_NF and YlCTS1_CR, and inserted into pDrive PCR cloning vector (Qiagen, Germany). Next, the *Bgl*II/*Bam*HI-treated *tc-URA3-tc* DNA cassette (Song *et al.*, 2007) was inserted into the linker *Bgl*II site of the pDrive-fusion DNA vector, resulting in a Yl*CTS1* gene disruption cassette. The disruption cassette was cut with *Bam*HI/*Hin*dIII, and introduced into *Y. lipolytica* cells using the one-step transformation method (Chen *et al.*, 1997). Yl*CTS1* gene disruption (Yl*cts1*Δ) strains were selected on SC-URA medium (0.67% [w/v] yeast nitrogen base without amino acids, 2% [w/v] glucose, uracil drop-out amino acid mixture, including all of the required amino acids) at 28°C. The correct deletion was confirmed by PCR using the Yl*CTS1* gene-specific primer sets, YlCTS1_NCF and YlCTS1_NCR (5′-cgtttccactcttttacatccg-3′ and 5′-aacagacgcatcggggtagg-3′) and YlCTS1_CCF and YlCTS1_CCR (5′-tccaggacaaacagaagccc-3′ and 5′-gtagaaggaatagtgga gagg-3′).

Yeast strains were grown routinely in YPD (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] glucose) or synthetic minimal medium (SD, 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with required amino acids). The *Y. lipolytica och1Δ* strain (*MATA ade1 ura3 xpr2 och1::tc*, Song *et al.* (2007)) was used as a control strain for phenotypic analysis in the presence of cell wall synthesis inhibitors, which was conducted on YPD solid medium containing 10 μg/ml calcofluor white (CFW; Sigma, USA), 50 μg/ml Congo red (CR; Sigma), 7.5 mM sodium orthovanadate (Van; Sigma), or 40 μg/ml hygromycin B (Hyg B; Sigma). To induce filamentous growth in *Y. lipolytica*, SSE (0.67% YNB w/o amino acids, 1× [30 mg/L adenine, 90 mg/L leucine, 30 mg/L tryptophan, 30 mg/L uracil], 50 mM citrate buffer [pH 6], 10% fetal bovine serum [Sigma]) medium was used. *S. cerevisiae* L3262 (*MAT**a** ura3-52 leu2-3,112 his4-34*, Park *et al.* (2007)) was used as a control strain for chitinase isolation. *Escherichia coli* DH5α strain was used for DNA manipulation.

Endochitinase activity assays

To measure chitinase activity in culture supernatant, the culture supernatant of yeast cells incubated in YPD was obtained by centrifugation, and filtrated with 0.45-μm membrane (Millipore, USA). The filtrated cell-free supernatant was used for endochitinase activity assay by measuring the release of 4-methyl umbelliferone (4-MU) from 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside hydrate ($[GN]_3$, Sigma), which is a general substrate for endochitinase activity assay. The reaction mixture (100 μl) was composed of $[GN]_3$ (2 μg) and cell-free supernatant in 20 mM ammonium buffer (pH 5.0), and incubated for 60 min at 28°C. Then, 100 μl stop solution (0.5 M glycine-NaOH, pH 10.4) was added, and the fluorescence was measured using spectrofluorometer RF 5310PC (Shimadzu, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The fluorescence intensity of cell-free supernatant was expressed in arbitrary units, which were obtained by subtracting the fluorescence value for YPD medium as a blank.

In-gel endochitinase activity assay using cell-free supernatant was done as described below. The cell-free supernatant from 500 ml saturated culture was prepared by centrifugation (3,000 × g, 10 min, 4°C) and filtration with membrane filter (0.45 μm). Then, the supernatant was washed with equal volume of PBS buffer, and concentrated about 10 ml volume by using TFF system equipped with Pellicon XL Filter (Biomax 10K, Millipore), and further reduced to 1 ml volume with Amicon Ultra (Ultracel 10K, Millipore). Aliquot of the concentrate (100 μl) were stored at -70°C until used. Proteins were quantified by spectrophotometer (NanoDrop, Thermoscientific). Then, the samples were mixed with non-reducing sample loading buffer (45 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 1% [w/v] SDS, 0.01% [v/v] bromophenol blue), and run on 8% SDS-polyacrylamide gel at 4°C. After running, the gel was incubated with 50 mM sodium acetate solution (pH 5.0) plus 1% Triton X-100 for 60 min at 4°C to remove SDS in the gel. The gel was further washed in 50 mM sodium acetate solution (pH 5.0) without 1% Triton X-100 for 30 min at 4°C twice. After overlaying with 1% agarose gel (1% [w/v] agarose, 50 mM sodium acetate, pH 5.0) plus $[GN]_3$ (100 μg) and incubating for 60 min at 30°C in dark condition, fluorescent signal was detected by UV transilluminator at 305 nm wavelength.

Purification of chitin-binding proteins

The filtrated cell-free supernatant of yeast cells cultivated in YPD was added to pre-washed chitin-beads (0.5 ml, New England Biolab, USA) packed within Econo-Pac® column (10 ml, Bio-Rad, USA), and the mixture was slowly incubated with rotation for 2 h at room temperature. After removing the clear supernatant, the residual beads were washed with 10-volume PBS buffer. Then, the beads were mixed with

5 ml detaching solution (45 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 1% [w/v] SDS, 5% [v/v] β-mercaptoethanol), and boiled for 10 min. The flow-through was collected, exchanged with PBS, and concentrated up to 100 μl volume by using Amicon 50K and Microcon 10K (Millipore). Twenty-microliter purified supernatant was separated by SDS-PAGE on 6% Tris-glycine polyacrylamide gel, and the gel was stained by using glycoprotein staining kit (Thermo Fisher Scientific Inc., USA).

Microscope analysis of cellular morphology

To observe cellular morphology, yeast cells growing in YPD medium for 24 h were washed in sterile water, and examined by confocal microscope (LSM 510 META, Carl Zeiss GmbH, Germany) using a diode laser (405 nm) and META photomultiplier tube detector (polychromatic 32-channel detector). The images were taken using the LSM 510 META software. The hyphal formation of *Y. lipolytica* was induced by cultivation in SSE medium as previously described (Bal *et al.*, 2013). *Y. lipolytica* cells cultivated in SD medium with at 28°C for 24 h were incubated in fresh SD medium with initial OD_{600} 0.7 for 12 h and stored at 4°C for 12 h. Subsequently, cells were washed with sterile distilled water and incubated in fresh SD or SSE media at 28°C for 6 h. Then cell morphology was observed with Axio Scope.A1 microscope equipped with an AxioCam MRm (Carl Zeiss).

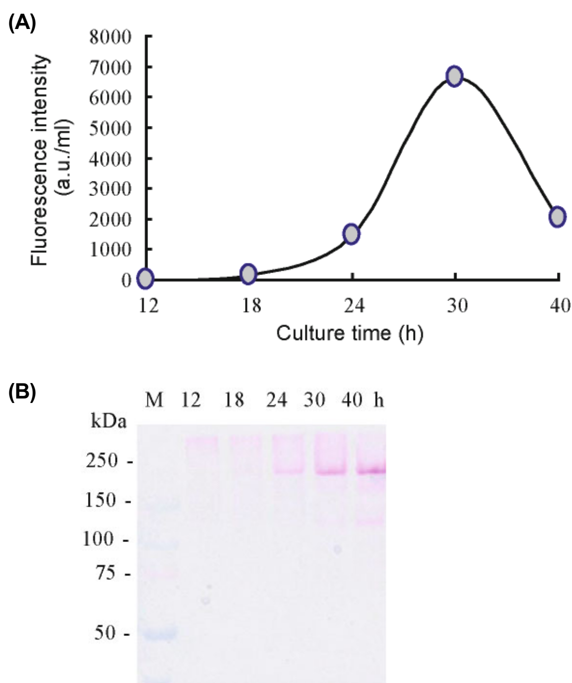

Fig. 1. Extracellular endochitinase activity in cell-free culture supernatant of *Y. lipolytica*. (A) Time-course profile of endochitinase activity. Concentrated cell-free supernatants of *Y. lipolytica* wild-type strain were mixed with $[GN]_3$ in 20 mM ammonium acetate (pH 5.0), and incubated for 1 h at 28°C. Next, stop solution (0.5 M glycine-NaOH, pH 10.4) was added, and fluorescence was measured. Fluorescence intensity was expressed in arbitrary units (a.u), which was obtained by subtracting the fluorescence value for YPD as a blank. $[GN]_3$: 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside. (B) Purification of putative chitin-bound proteins. Concentrated cell-free supernatants were incubated with chitin-beads for 2 h at room temperature. Equal volumes of the purified samples were separated on a 6% Tris-glycine polyacrylamide gel, which was stained using glycoprotein staining kit (Thermo Fisher Scientific Inc.). M, Precision plus protein dual color standard (Bio-Rad).

Glycan profiling analysis

O-Glycans from concanavalin A (Con A)-purified cell wall mannoproteins (YlCWP, 20 μg) and the purified YlCts1p (60 μg) were isolated by hydrazinolysis, and labeled with 2-aminobenzoic acid (AA) as previously described (Park *et al.*, 2011). AA-Glycans were analyzed by using normal phase (NP)-HPLC equipped with TSK-80 amide column (Tosoh, Japan) and fluorescence detector (Waters 2475, USA). The column was equilibrated with a solution comprising 80% solvent A (100% acetonitrile, Burdick and Jackson, USA) and 20% solvent B (50 mM ammonium formate, pH 4.4, Sigma). After sample injection, the proportion of solvent B was increased linearly up to 50% for 30 min. AA-Glycans was detected by fluorescence (excitation and emission wavelengths of 320 and 400 nm, respectively). To identify sugar residues and glycosidic linkages on *O*-glycans, AA-glycans were reacted with jack bean α-mannosidase (JBM, Sigma) for 24 h at 37°C and JBM-treated glycans were purified by using Amicon® Ultra (10K, Millipore).

Results

Extracellular endochitinases activity in *Y. lipolytica* culture supernatants

ScCts1p is an extracellular endochitinase, which is a secreted glycoprotein modified with intensive *O*-mannosylation. To examine the presence of such extracellular chitinases in *Y. lipolytica*, we first investigated endochitinase activity in cell-free culture supernatant of wild-type *Y. lipolytica* SMS397A strain. The culture supernatant of yeast cells incubated in YPD medium was obtained at various time points and used in an endochitinase activity assay by measuring the release of 4-methyl umbelliferone (4-MU) from 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside hydrate ($[GN]_3$). Figure 1A shows the extracellular chitinase activity of *Y. lipolytica* over culture time, which clearly displayed the increased endochitinase activity in culture supernatant as the yeast cells reached the stationary phase. We further attempted to purify chitin-bead-bound proteins from the cell-free supernatant by using pre-washed chitin-beads to investigate the possible presence of a chitin-binding domain in *Y. lipolytica* extracellular chitinases. Purified chitin-bead-bound proteins were

Table 1. Amino acids sequence identity between *Saccharomyces* and *Yarrowia* chitinases

	YlCts1p	YlCts2p	YlCts3p	ScCts2p
ScCts1p	47.50	11.62	42.77	7.81
YlCts1p		11.23	44.81	8.98
YlCts2p			12.50	35.25
YlCts3p				8.05

[a] Abbreviations used: Sc, *S. cerevisiae*; Yl, *Y. lipolytica*
[b] GenBank accession nos. for *S. cerevisiae* Cts1p and Cts2p are NP_013388.1 and NP_010659.1, respectively.

separated by SDS-PAGE, and then detected by using glycoprotein staining kit. As shown in Fig. 1B, some protein bands, particularly around 250 kDa, showed strong signals with increased intensity over cultivation. This indicates that the chitin-bound proteins of *Y. lipolytica*, which are expected to be primarily composed of extracellular chitinases, are glycoproteins. A discrepancy between the chitinase activity of culture supernatant and the amount of chitin-bead-bound proteins was observed at 40 h. The decreased chitinase activity at 40 h might be partly due to accumulation of uni-

Fig. 2. Comparative analysis of YlCts1p with other GH 18 fungal chitinases. (A) Representative domains of *S. cerevisiae* and *Y. lipolytica* chitinases. SS, signal sequence; CD, catalytic domain; S/T, serine/threonine-rich domain; ChBD, chitin-binding domain. N-terminal signals were predicted using SignalP V3 (http://www.cbs.dtu.dk/services/SignalP). The functional domains were searched by SMART (http://smart.embl-heidelberg.de/). (B) Amino sequence alignment of yeast Cts1 proteins with endochitinase activity. Several yeast homologs to ScCts1p, belonging to Class *Saccharomycetes,* were compared with YlCts1p at the level of amino acid sequences by using Clustal W (http://www.genome.jp/tools/clustalw/). Signal peptides and the cleavage locus were marked as quadrangular boxes and an arrow, respectively. Two consensus motifs, SxGG (substrate-binding site) and DxxDxDxE (catalytic site) in family 18 chitinases, are indicated by dotted box I and II. Conserved six-cysteine residues in type 2 ChBD are indicated by triangles. ScCts1p (GenBank accession no. NP_013388.1), ScCts2p (NP_010659.1), KlCts1p (KLLA0C04730p, XP_452410), CaCht3p (XP_719348).

dentified inhibitory metabolites in culture supernatants at the late stationary phase of *Y. lipolytica* cultivation.

Identification of Yl*CTS1* encoding an extracellular chitinase in *Y. lipolytica*

In order to identify the putative gene(s) coding for extracellular *Y. lipolytica* chitinases, we searched for *Y. lipolytica* homologs to *S. cerevisiae CTS1* (Sc*CTS1*) and *CTS2* (Sc*CTS2*) in the *Yarrowia* genome database (http://cbi.labri.fr/Genolevures/elt/YALI/). Three open reading frames, YALI0D22396g, YALI0F04532g, and YALI0A01870g, were identified as candidate genes coding for potential chitinases and referred to as Yl*CTS1*, Yl*CTS2*, and Yl*CTS3*, respectively. A SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/) analysis of each of the *Y. lipolytica* putative chitinase proteins revealed that YlCts1p (979 amino acids), YlCts2p (383 amino acids) and YlCts3p (343 amino acids) are endochitinases (EC 3.2.1.14), with each containing a conserved Glyco_Hydo_18 domain (Pfam00704). The two *Y. lipolytica* homologs YlCts1p and YlCts3p show a higher level of amino acid sequence identity to ScCts1p (47.5% and 42.77%, respectively) compared to ScCts2p. On the other hand, YlCts2p has a catalytic domain similar to that of ScCts2p and thus shows much closer identity to ScCts2p (35%) compared to ScCts1p (Table 1).

Interestingly, only YlCts1p was predicted to contain several motifs conserved in fungal extracellular chitinases, including an N-terminal secretion signal peptide, a long C-terminal Ser/Thr-rich domain, and a chitin-binding domain (Fig. 2A). The other two *Y. lipolytica* homologs YlCts2p and YlCts3p do not contain N-terminal signal sequence, suggesting that they are intracellular proteins. It is notable that, compared with ScCts1p (562 amino acids), YlCts1p (979 amino acids) appears to be much larger because of the presence of a longer Ser/Thr-rich domain. Comparison of amino acid sequences of several yeast homologs and ScCts1p, which belong to Class *Saccharomycetes*, with that of YlCts1p showed that YlCts1p also contains two consensus motifs, including SxGG (substrate-binding site) and DxxDxDxE (catalytic site), which are observed in family 18 chitinases (Synstad *et al.*, 2004). In addition, the six-cysteine residues conserved in type 2 ChBD (Colussi *et al.*, 2005) were also present in YlCts1p (Fig. 2B). Considering the presence of an N-terminal secretion signal peptide, a Ser/Thr-rich domain, and a chitin-binding domain in YlCts1p, it is highly speculated that the extracellular chitinase of *Y. lipolytica* observed in Fig. 1 would be the product of Yl*CTS1*.

Construction and characterization of Yl*cts1* deletion mutant strains

To confirm that YlCts1p is responsible for the chitinase activity observed in the culture supernatants of *Y. lipolytica*, we constructed a *Y. lipolytica* mutant strain in which the Yl*CTS1* gene was deleted (Yl*cts1*Δ) and analyzed extracellular chitinase activity (Fig. 3). Deletion of Yl*CTS1* dramatically reduced extracellular endochitinase activity strongly supporting that the Yl*CTS1* gene is the primary factor responsible for extracellular chitinase activity (Fig. 3A). Additionally, we performed an in-gel endochitinase activity assay (Fig. 3B) by using the cell-free supernatants, as described previously (Tronsmo and Harman, 1993; Liau and Lin, 2008). Notably, strong fluorescent signals at ~250 kDa and ~125 kDa were detected in the samples of wild-type *Y. lipolytica* and *S. cerevisiae*, respectively, whereas no signal was observed for the Yl*cts1*Δ sample. This clearly demonstrates that extracellular chitinase of *Y. lipolytica* is indeed encoded by Yl*CTS1*.

To investigate the physiological function(s) of Yl*CTS1*, we further examined any apparent change in the growth of the Yl*cts1*Δ strain in the presence of cell wall synthesis inhibitors, including calcofluor white (CFW) and Congo red (CR). The Yl*och1*Δ strain, which was shown to display defective growth in the presence of cell wall disturbing reagents (Song *et al.*, 2007), is used as a control strain for the phenotypic analysis. As shown in Fig. 4A, compared to the wild-type strain, the Yl*cts1*Δ strain exhibited slightly retarded growth on YPD but displayed comparable sensitivity to CFW and CR, which function by binding to chitin polymer and thus inhibiting the assembly enzymes that link chitin to β1,3- and β1,6-glucans in yeast cell walls (Ram and Klis, 2006). Overall, the Yl*cts1*Δ strain did not show apparent defective phenotypes in the presence of sodium orthovanadate (Van),

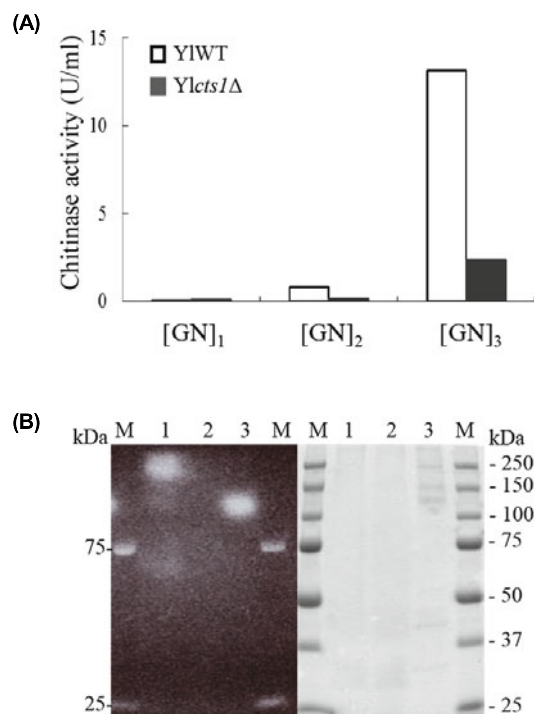

Fig. 3. Functional analysis of the Yl*CTS1* gene. (A) Chitinase activity assay using cell-free culture supernatants. $[GN]_1$, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; $[GN]_2$, 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside; $[GN]_3$, 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside. (B) In-gel assay of chitinase activity. The concentrated cell-free supernatants (containing total 12.5–25 μg proteins) were separated on 8% SDS-polyacrylamide gel, overlaid on 1% agarose gel containing $[GN]_3$ in 50 mM sodium citrate (pH 5.0), and incubated at 30°C under dark condition. Then, the gel was irradiated by UV transilluminator at 305 nm wavelength. Lanes: 1, *Y. lipolytica* SMS397A strain; 2, Yl*cts1*Δ mutant strain; 3, *S. cerevisiae* L3262 strain.

hygromycin B (Hyg B), and SDS, indicating that a loss of a functional YlCst1p does not affect significantly cell wall integrity in *Y. lipolytica*.

It was previously reported that a knockout of the *CTS1* gene in *S. cerevisiae* resulted in a "clumping" phenotype, and that deletion of chitinase 3 (*CHT3*) from a pathogenic yeast *Candida albicans* also generated elongated chains of unseparated cells in the yeast growth phase, resembling the phenotype of *S. cerevisiae cts1* knockouts (Dünkler *et al.*, 2005). We observed that the Yl*cts1*Δ mutant strain showed more increased cell aggregation during yeast growth phase compared to the wild-type strain (Fig. 4B), indicating that extracellular chitinase is also involved in cell separation in *Y. lipolytica* as reported in *S. cerevisiae*. In the respect of hyphal formation, deletion of the Sc*CTS1* gene was shown to rather enhance pseudohyphal growth in *S. cerevisiae* (King and Butler, 1998). In *C. albicans*, the growth on solid media was also found to lead to increased filament formation in *cht1* and *cht2* strains (Dünkler *et al.*, 2005). Thus we further examined whether the function of *Y. lipolytica* Cts1p is also associated with hyphal formation. It was observed that even though the Yl*cts1*Δ mutant strain showed much higher portion of unseparated cell in non-hyphal induction medium (SD) compared with wild-type strain, there is no difference in true hyphal formation in liquid hypha-induction medium containing 10% fetal bovine serum (SSE) (Fig. 4C) or on solid medium between the wild-type and Yl*cts1*Δ mutant strains (data not shown).

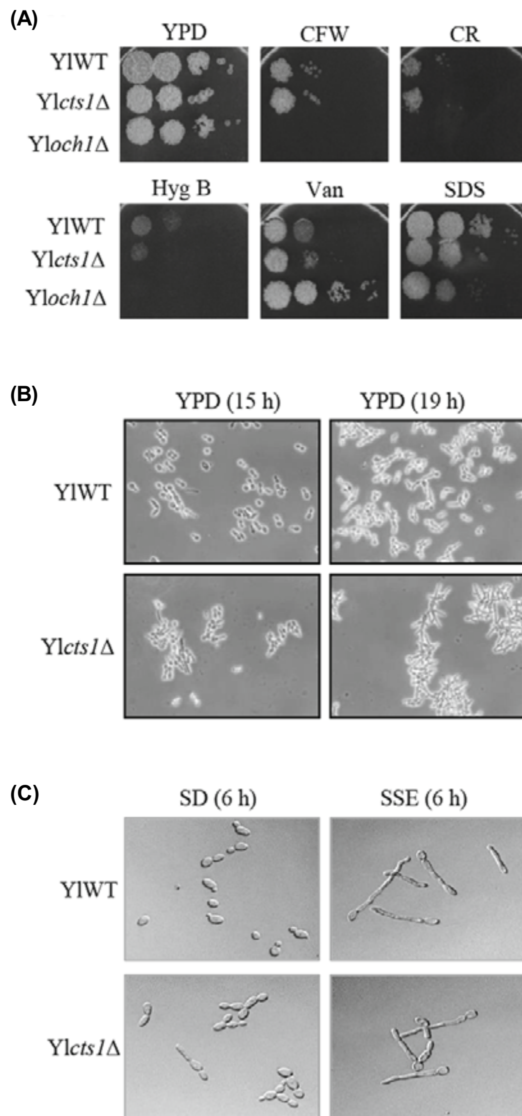

Fig. 4. Phenotypic analyses of the Yl*cts1*Δ mutant strains. (A) Sensitivity of Yl*cts1*Δ mutant strains to cell wall synthesis inhibitors. *Y. lipolytica* wild-type and Yl*cts1*Δ mutant cells were spotted onto YPD medium plates containing 10 mg/ml of CFW, 50 mg/ml of CR, 7.5 mM Van, or 40 mg/ml of Hyg B and incubated for 3 to 4 days at 28°C. (B) Cell morphology of wild-type and Yl*cts1*Δ mutant strains. Yeast cells were cultivated in YPD at 28°C for 24 h and then examined by a confocal microscope (LSM 510 META; Carl Zeiss GmbH) using a diode laser (405 nm) and META photomultiplier tube detector (polychromatic 32-channel detector). The images were taken using the LSM 510 META software. YlWT, *Y. lipolytica* SMS397A; Yl*cts1*Δ, Yl*cts1*Δ mutant strain. (C) Hyphal formation analysis. Strains were incubated in non-hypha induction medium (SD) and hypha induction medium (SSE) at 28°C for 6 h in liquid media and observed microscopically (10 × 40 magnification).

Glycosylation analysis of YlCts1p, a secreted glycoprotein

The presence of a long Ser/Thr-rich domain in YlCts1p strongly suggested that the protein would be subject to heavy *O*-glycosylation. Moreover, the molecular weight (MW) of YlCts1p observed on gel electrophoresis was ~250 kDa, which is much higher than the predicted MW of ~110 kDa, suggesting the presence of intensive post-translational modification of YlCts1p. To investigate the glycosylation pattern of YlCts1p, purified YlCts1p was treated with jack bean α-mannosidase (JBM), which can cleave three types of α-mannose linkages (α-1,2, α-1,3 and α-1,6), and with peptide-*N*-glycosidase F (PNGase F), which can cleave *N*-glycans attached to glycoproteins. As shown in Fig. 5A, the MW of YlCts1p, according to electrophoresis result, was decreased to ~110 kDa after JBM treatment but did not change after PNGase F treatment. This strongly supports that YlCts1p is intensively modified only via *O*-mannosylation by the addition of a large numbers of mannose residues connected through α-linkages.

To compare the *O*-glycan profiles between YlCts1p and total cell wall mannoproteins, *O*-glycans from the Con A-purified cell wall mannoproteins (YlCWP) and the purified YlCts1p were isolated by hydrazinolysis, and labeled with 2-aminobenzoic acid (AA) as previously described (Park *et al.*, 2011). AA-Labeled *O*-glycans were separated by normal phase-high-pressure liquid chromatography (HPLC). As indicated in Fig. 5B, the overall *O*-glycan profile of YlCts1p was nearly identical to that of YlCWP (Fig. 5B, a *vs* b). *O*-Glycan chains attached to YlCts1p were primarily composed of Man2-Man4 (M2-M4), with the M2 peak corresponding to two mannose residues as the major species as previously observed on the *O*-glycome profile of wild-type *Y. lipolytica* (Park *et al.*, 2011). To obtain detailed information regarding sugar residues and their glycosidic linkages on *O*-glycans of YlCts1p, AA-labeled *O*-glycans from YlCts1p were treated with JBM and then analyzed by HPLC. After JBM treatment, all the peaks of YlCts1p *O*-glycans were converted to a single peak M1 consisting of one mannose residue (Fig. 5B,

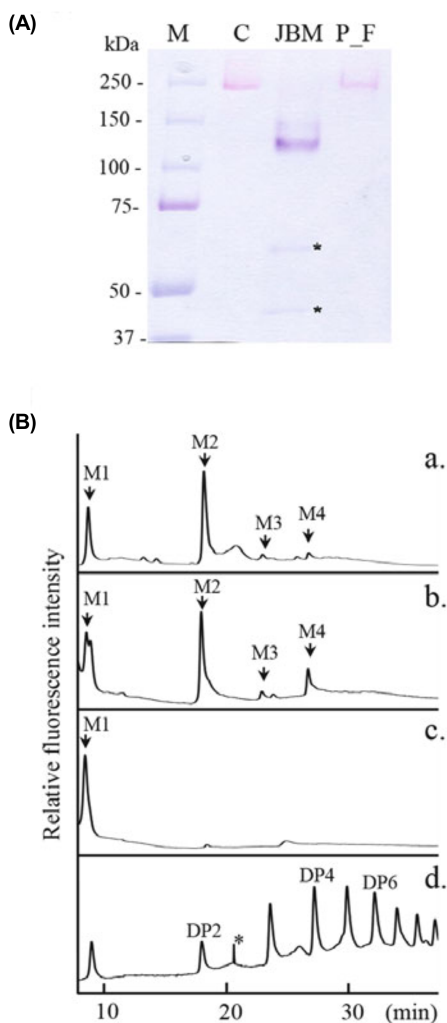

Fig. 5. Glycosylation analysis of YlCts1p. (A) Glycosylation type analysis of YlCts1p. Purified YlCts1p (2 μg) was reacted with JBM (0.12 U) or PNGase F (500 U) for 16 h at 37°C, and separated on 8% Tris-glycine polyacrylamide gel. The proteins were initially detected by using glycoprotein staining kit and then subsequently stained by Coomassie solution. The red-colored protein bands are generated by glycoprotein staining and the blue-colored proteins are shown by Coomassie staining. M, Precision plus protein dual color standard (Bio-Rad); C, purified YlCts1p without enzyme treatment; JBM, YlCts1p treated with JBM; P_F, YlCts1p treated with PNGase F. Asterisks are subunits of JBM. (B) *O*-Glycan profiling by HPLC analysis. *O*-Glycans were labeled with 2-AA and separated on HPLC using TSK-80 amide column. a, *O*-glycans from YlCWP; b, *O*-glycans from YlCts1p; c, *O*-glycans from YlCts1p after JBM treatment; d, dextran ladder (Sigma) as a size marker. M, mannose residue; DP, degree of polymerization, * unidentified peak.

c). This supports that the *O*-glycans of YlCts1p are composed of mannose residues, primarily present in α-linkages; these results are consistent with those shown in Fig. 5A.

Discussion

Chitinases are the enzymes that catalyze the hydrolysis of the β-1,4-glycosidic bonds between GlcNAc monomers in polymers of chitin, an important component of arthropod exoskeletons and the cell walls of fungi (Adams, 2004). Chitinases have been implicated in the maintenance of cell wall plasticity and separation of the mother and daughter cells during vegetative growth in yeast. Genome analysis reveals that yeast species generally possess several chitinase-encoding genes. For example, there are 2 genes for *S. cerevisiae* (Sc*CTS1* and Sc*CTS2*), 4 genes each for the pathogenic yeasts *C. albicans* (*CHT1*, *CHT2 CHT3*, and *CHT4*) (Dünkler *et al.*, 2008) and *Cryptococcus neoformans* (*CHI2*, *CHI21*, *CHI22*, and *CHI4*) (Baker *et al.*, 2009), respectively. Interestingly, *C. albicans* has only one Sc*CTS1* homolog among 4 chitinases, and there is no Sc*CTS1* homolog found in the *C. neoformans* genome. On the other hand, *Ashbya gossypii* has only one chitinase gene coding for a homolog of *S. cerevisiae* Cts2p (Dünkler *et al.*, 2008). Because Sc*CTS1* encodes a plant-type chitinase secreted extracellularly with intensive modification via *O*-mannosylation, ScCts1p and its homologs have been employed as a useful model glycoprotein in analyzing yeast mutant strains having defects in *O*-mannosylation, which is the major *O*-glycosylation pathway in yeast and fungal species.

Over the last few decades, various studies have been conducted to use several yeast and fungal species as secretion hosts that produce therapeutic glycoproteins with human complex type *N*-glycans (Gerngross, 2004). In addition to *N*-glycan engineering, several attempts to reduce yeast-specific *O*-mannosylation were recently made in a methylotrophic yeast *Ogataea minuta* for recombinant antibody production (Kuroda *et al.*, 2008) and in a genetically engineered *S. cerevisiae* strain to produce mammalian mucin-type sugar chains (Amano *et al.*, 2008). The oleaginous yeast *Y. lipolytica* has been also explored as a potential host for producing recombinant proteins particularly based on its high capacity of protein secretion and posttranslational modifications similar to the mammalian system (Madzak *et al.*, 2004; De Pourcq *et al.*, 2012). In the present study, we identified 3 putative chitinase genes (Yl*CTS1*, Yl*CTS2*, and Yl*CTS3*) in the *Y. lipolytica* genome and demonstrated that Yl*CTS1*, a Sc*CTS1* homolog, encodes an extracellular chitinase involved in cell separation of *Y. lipolytica*. We further showed that YlCts1p is subjected to intensive modification via *O*-mannosylation without *N*-glycosylation during secretion in *Y. lipolytica*, proposing YlCts1p as a good model protein in studying *Y. lipolytica O*-glycosylation. Prior to conducting a detailed *O*-glycan profile analysis, defects in *O*-glycosylation in *Y. lipolytica* can be simply observed by alterations in the electrophoresis pattern of YlCts1p. This will be a convenient indicator to provide useful information for developing *Y. lipolytica* host strains without yeast-specific *O*-mannosylation.

Acknowledgements

This work was partly supported by a grant from the Korean Ministry of Rural Development Administration (Next-Generation BioGreen 21 Program No. ADFC2013-PJ009586). Chang Pyo Han was supported by Chung-Ang University Research Scholarship Grants in 2012.

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