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NOTE

Enhanced Secretion of Cell Wall Bound Enolase into Culture Medium by the *soo1-1* Mutation of *Saccharomyces cerevisiae*

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In order to identify the protein(s) secreted into culture medium by the *soo1-1/ret1-1* mutation of *Saccharomyces cerevisiae*, proteins from the culture medium of cells grown at permissive (28°C) and non-permissive temperatures (37°C), were analyzed. Comparison of protein bands separated by SDS-PAGE identified a prominent band of 47-kDa band from a mutant grown at 37°C. N-terminal amino acid sequencing of this 47-kDa protein showed high identity with enolases 1 and 2. Western blot analysis revealed that most of the cell wall-bound enolase was released into the culture medium of the mutant grown at 37°C, some of which were separated as those with lower molecular weights. Our results, presented here, indicate the impairment of cell wall enolase biogenesis and assembly by the *soo1-1/ret1-1* mutation of *S. cerevisiae*.

Key words: cell wall proteins, enolase, *Saccharomyces cerevisiae*, secretion, *soo1-1*

The cell wall of *Saccharomyces cerevisiae* is a dynamic structure, which can provide the cell with protection against external stress. It makes up 15 to 30% of the dry weight of the cell, and is composed mostly of mannoproteins, which are extensively *O*- and *N*-glycosylated fibrous β-1,3-glucan. It is also branched β-1,6-glucan, which links the other components of the wall together. An important minor component is chitin, which contributes to the insolubility of the fiber (Lipke and Ovalle, 1998; Klis *et al.*, 2002; Lee *et al.*, 1999; 2002).

The mannoproteins can be categorized into three groups, depending on the method of extraction: reducing agent-extractable mannoproteins, glucanase-extractable mannoproteins, and sodium dodecyl sulfate (SDS)-extractable mannoproteins (Bowen and Wheals, 2004; Edwards *et al.*, 1999). The glucanase-extractable mannoproteins feature an N-terminal secretion signal sequence, a serine/threonine sequence for glycosylation, and a C-terminal glycosylphosphatidylinositol (GPI) attachment site. The SDS-extractable proteins are not covalently linked to structural polysaccharides, but may be disulphide-linked to other proteins in the cell wall (Shimoi *et al.*, 1998; Edwards *et al.*, 1999; Moukadiri and Zueco, 2001; Bowen and Wheals, 2004).

Enolase (2-phosphoglycerate dehydratase) is one of the most abundant enzymes in the cytosol, and is a cell wall protein which may arrive at the cell wall via a currently unknown pathway in *S. cerevisiae* and *Candida albicans*. The exact function of enolase in the cell wall is still unclear, but it is possible that enolase may have an alternative function, as do other glycolytic enzymes, most notably phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, and alcohol dehydrogenase (Angiolella *et al.*, 1996; Edwards *et al.*, 1999; Pardo *et al.*, 2000).

The secretory pathway is heavily involved in the biosynthesis of cell wall components and in their transport to the cell wall. Transport between compartments of the endomembrane system involves three known vesicle coat complexes: clathrin, COPI and COPII. Clathrin functions in endocytosis and in biosynthetic transport from the *trans*-Golgi network to the endosomes. COPII vesicles carry newly synthesized proteins from the ER to the Golgi, and consist of the small GTPase Sar1p, and two protein complexes, Sec23/24p and Sec13/31p. Whereas COPI vesicles function as a counterpart to COPII, and are involved in the retrieval of proteins from the Golgi back to the ER, and contain the small GTPase Arf1p, and a heptameric protein complex called coatomer, a stable cytosolic complex comprising seven equimolar subunits, α-, β-, β'-, γ-, δ-, ε-, and ζ-COP (Le Borgne and Hoflack, 1998; Rothman *et al.*, 1996; Schekman and Orci, 1996; Lee *et al.*, 1999; 2002).

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Our recent reports show that the *soo1-1/ret1-1* mutation in Soo1p/α-COP is responsible for the thermo-sensitive osmo-fragile phenotype. Thus α-COP (Soo1p) may play a crucial role in the recognition and translocation of proteins such as β-1,3-glucan synthase complex and cell wall protein, and may also contribute to the maintenance of osmotic integrity in cells (Lee *et al.*, 1999; 2002). In this study, we investigated cell wall proteins, which are secreted into the culture medium at high levels by the *soo1-1/ret1-1* mutation. We also revisited the issue of the cell wall defect in the α-COP mutant of the budding yeast, *S. cerevisiae*.

S. cerevisiae strain LP0353 (*MATα ura3-52 lys2-801 soo1-1 bgs2 gal2*) and LP0353RS1 (same in genetic background with LP0353 except the *SOO1* replacement) (Lee *et al.*, 2002) were employed in this study. The yeast strains were grown in YEPD (1% Bacto-yeast extract, 2% Bacto-peptone and 2% dextrose) or synthetic complete medium, SC (0.67% Bacto-yeast nitrogen base without amino acid, 20 mM amino acids and 2% dextrose). D-Sorbitol was added at a concentration of 1.2 M as an osmotic stabilizer in all cases. Cells were grown at either 28°C or 37°C, with shaking at 220 rpm untill exponential phase.

In order to detect copiously secreted proteins, culture media were harvested by centrifugation at 22,000 × g for 15 min, and the proteins in the supernatant were concentrated using the Rapid-Con™ Protein Concentration Kit (Elpis-biotech Co., Korea). Proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were visualized by staining with a silver stain kit (Elpis-biotech Co., Korea) or electroblotted on a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech Co., USA). Protein from the PVDF membrane was partially sequenced via Edman degradation. Protein was identified from the partial peptide sequences using the *Saccharomyces cerevisiae* Genome Database.

Cell walls were prepared as previously described (Kitagaki *et al.*, 1997; Shimoi *et al.*, 1998). In brief, exponential cells were harvested from 100 ml culture by centrifugation, washed, and then disrupted using a Minibeadbeater (Biospec Co., USA). The glass beads were removed by decantation, and the cell walls were pelleted by centrifugation and washed with 5 M LiCl. Equal amounts of cell wall fraction were suspended in 0.2 ml of SDS-extraction buffer (50 mM Tris-HCl pH 8.0 containing 2% SDS, 100 mM EDTA, and 40 mM dithiothreitol) and then heated at 100°C for 10 min, in order to prepare non-covalently bound proteins and proteins bound by disulfide bridges. After removing the cell debris by centrifugation, 30 μl each of the SDS-extractable cell wall fractions were used for SDS-PAGE and Western analysis.

Fusion proteins containing the N-terminal GST tag and amino acid residues 251~437 of Eno1p, or amino acid residues 1~178 of Gpi16p were expressed in *Escherichia coli* or purified by GST column, respectively. Approximately 50 μg each of the fusion proteins was injected into mice, and antisera were obtained after four injections of the antigens, in both cases. For Western analysis, proteins from the culture media and SDS-extractable cell wall fractions were separated by SDS-PAGE on 10% gel. After transfer of the separated proteins to a PVDF membrane, membranes were incubated overnight with mouse polyclonal antibody against enolase or Gpi16p at a concentration of 1 : 1000 in 1% non-fat milk/PBS, respectively. An 1 : 5000 dilution of peroxidase conjugated goat anti-mouse IgG antibody (Amersham Pharmacia Biotech Co., USA) was used as the secondary antibody, and developed using a Western blot detection kit (Elpis-biotech Co., Korea).

The temperature-sensitive phenotype of the *ret1-1/soo1-1* mutation, which exhibits an amino acid substitution of Asp for Gly227 in α-COP, is osmo-fragile. We previously presented evidence that the *soo1-1* mutation causes defects in post-translational modification and assembly of the yeast cell wall proteins, which may contribute to the maintenance of cell wall integrity (Lee *et al.*, 2002). The osmo-fragile phenotype caused by the cell wall defect could be suppressed by 1.2 M sorbitol as an osmo-stabilizer. All strains were grown in synthetic complete medium to harvest culture soups, instead of YEPD, in order to obviate the argument that YEPD contains proteins from yeast extracts. Sorbitol was also added to all media for the same reason.

We had tried to analyze proteins released into growth media for both the wild-type and the mutant-type, based on the idea that the *soo1-1* mutant, with its impaired cell wall assembly, could more easily lose the cell wall-bound proteins into growth media than could the wild-type. Total proteins released into 100 ml of growth media of the mutant LP0353 having the *soo1-1* mutation, and the wild-type strain LP0353RS1, were concentrated and separated by SDS-PAGE. Upon silver staining of the PAGE gel, a prominent thick band of 47-kDa was detected from the growth medium of the LP0353 grown at the non-permissive temperature, 37°C (Fig. 1).

In order to identify this 47 kDa protein, a duplicate of the unstained gel was transferred to a PVDF, and was partially sequenced. The peptide sequence n-AxSKVYARSV-c was obtained, where 'x' represents ambiguous residues. A database search revealed that this peptide exhibited a high level of identity with the enolases (2-phosphoglycerate dehydratases), Eno1p and Eno2p, which are indistinguishable from each other due to their high identity (93%) and similarity (96%) with regard to amino acid sequence (Fig. 2).

Previous report demonstrated that enolase was extracted from the cell walls by the high pH/2-mercaptoethanol procedure, which prepares non-covalently attached or soluble cell wall proteins. Localization of enolase in the soluble cell wall protein fractions was also confirmed by Western blotting with an anti-enolase antibody and biotinylation of

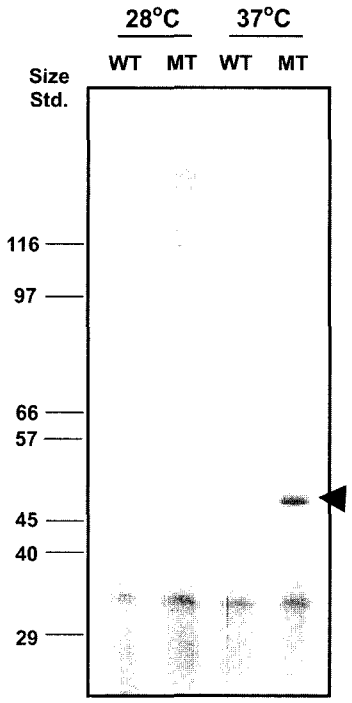

Fig. 1. Isolation of highly secreted proteins into the culture medium of the *soo1-1* mutant grown at non-permissive temperature. Concentrated proteins from 100 ml of culture medium at OD_{600}=1.0 were treated with 60 μl of SDS sample buffer. Thirty μl of the each samples was loaded onto a 12% gel, separated and silver-stained. All strains were grown in the synthetic complete media containing 1.2 M sorbitol, at the indicated temperature. Arrowhead indicates a highly secreted 47-kDa protein band. WT, wild type; MT, *soo1-1* mutant type.

cells (Edwards *et al.*, 1999). Therefore, it was suggested that enolase is, in addition to its location in the cytoplasm, an SDS-extractable cell wall protein, which contains no known secretion signals. Though the function of glycolytic enzymes in the cell walls of yeast remains obscure, enolase has been shown to be associated with glucan in the cell walls of *Candida albicans*, and is a major antigen in systemic candidasis infections (Edwards *et al.*, 1999; Angiolella *et al.*, 1996). Our results, which are consistent with those of previous reports, also suggest the existence of enolase in the cell wall protein fractions.

In order to confirm the localization of enolase in the wild-type cell wall, and enhanced release of cell wall-bound enolase into the culture media by the *soo1-1* mutation, the enolase in the cell walls and culture media of wild-type, and mutant-type strains grown at both permissive and non-permissive temperatures was assessed by Western blotting with an antiserum generated against the amino acid residues 251~437 of Eno1p.

In Western blot analysis of the SDS-extractable cell wall fractions with the anti-enolase antibody, no difference was observed between wild-type and mutant cells grown at permissive temperature; however, almost no enolase was detected in the mutant cells which were grown at non-permissive temperature (Fig. 3A). On the other hand, relatively large amounts of enolase were detected in the culture media of the mutant grown at non-permissive temperature (Fig. 3B, upper panel). Although 1.2 M sorbitol in the culture medium tends to support the growth of the mutant strain at non-permissive temperature, the enhanced

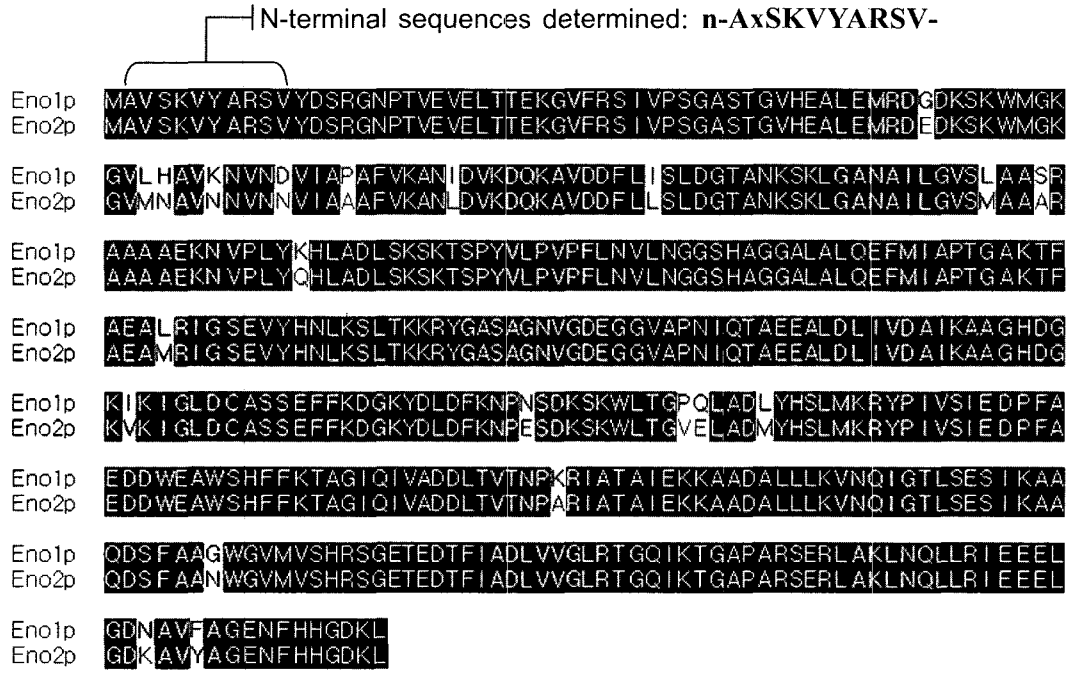

Fig. 2. N-terminal sequences of the 47-kDa protein matched well with those of enolase. N-terminal amino acid sequences determined by the method of Edman-deradation show high identity with enolase gene products, Eno1p and Eno2p. Eno1p and Eno2p of *Saccharomyces cerevisiae* are aligned using BOXSHADE v3.21. Black and grey boxes represent identical and similar amino acids, respectively.

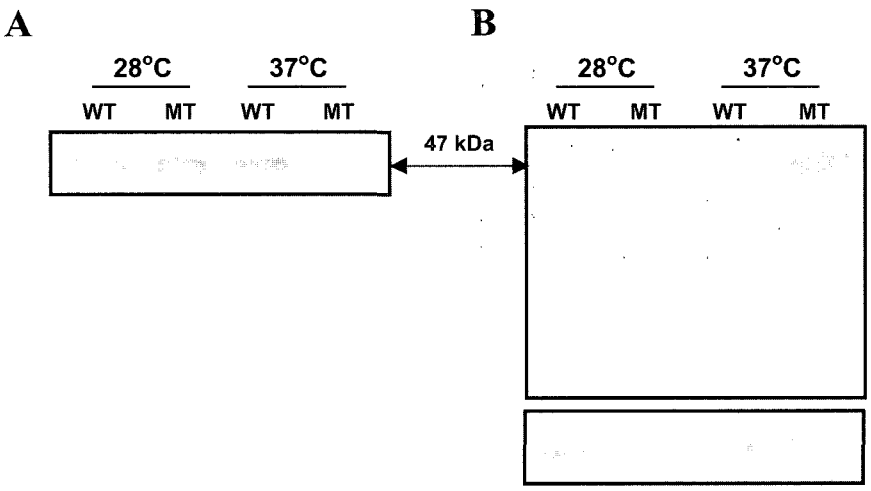

Fig. 3. Western blot analysis of the SDS-extractable cell wall proteins (A) and concentrated medium proteins (B). Fifty mg (wet weight) of cell wall fractions were treated with 200 μl of SDS-extraction buffer. Thirty microliters of the SDS-extractable cell wall fractions were loaded into each lane, separated on 10% SDS-PAGE, electroblotted onto to a PVDF membrane, and analyzed with anti-enolase antibodies (A). Concentrated proteins from 100 ml of culture medium at OD_{600}=0.8 were treated with 60 μl of SDS-sample buffer. Thirty μl of each sample was loaded and separated onto a 10% SDS-PAGE, electroblotted onto to a PVDF membrane, and analyzed with anti-enolase antibody (B, upper panel) or anti-Gpi16p antibody (B, lower panel). All strains were grown in the synthetic complete media containing 1.2 M sorbitol at the indicated temperature. WT, wild type; MT, *soo1-1* mutant type.

release of enolase into the culture medium could be the result of the mutant-cell disintegration. The fact, however, that no considerable changes in Gpi16p, one of the ER-membrane associated proteins, were detected in the concentrated culture media (Fig. 3B lower panel), ruled out the release of enolase by cell disintegration, but supported the enhanced secretion of enolase either by a defect in its integration into cell walls, or by some other unknown secretory mechanism.

These results clearly demonstrated not only the association of enolase with the cell wall, but also enhanced secretion of cell wall-bound enolase into the culture media by the *soo1-1* mutation, which causes impairment in both cell wall biogenesis and integrity (Lee *et al.*, 1999; 2002). It was also noted that the bands detected in the culture medium of the mutant grown at non-permissive temperature showed not only a band of the expected size, 47 kDa, but also several bands of lower molecular weight, which might have been generated by proteolytic cleavage of the 47 kDa protein (Fig. 3B, upper right most panel). In addition, the amount of enolase in the culture media of the mutant grown at permissive temperature was notably larger than that of the wild type suggesting that the *soo1-1* mutation is rather leaky, and could thus affect the secretion of cell wall proteins into the culture medium, even at permissive temperatures. It is also notable that recent analyses of cell wall proteomes revealed enhanced secretion of the cell wall-associated forms of other glycolytic enzymes, such as triose-phosphate isomerase (Tpi1p) and heat shock proteins, such as Ssa2p (Pardo *et al.*, 2002) into the culture medium by the *soo1-1* mutation (unpublished data).

Although the pathways for both transport and function in cell walls remain obscure, our results, presented here, clearly confirm the previous report (Edwards *et al.*, 1999; Pardo *et al.*, 2002) regarding the localization of the glycolytic enzyme, enolase, in the SDS-extractable cell wall proteins. It was also suggested that the assembly into cell walls, as well as the post-translational modifications of the cell wall-associated form of enolase were influenced by the *soo1-1* mutation, like other cell wall proteins (Lee *et al*, 1999; 2002). Furthermore, the abundant secretion of cell wall-bound enolase into growth medium has the potential to be used as a marker for defects in cell wall integrity and biogenesis in *S. cerevisiae.*

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