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Deglycosylation of cellulosomal enzyme enhances cellulosome assembly in *Saccharomyces cerevisiae*

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ABSTRACT

We have estimated the effects of hyper-mannosylation of dockerin-type cellulase on cellulosome assembly by using *Saccharomyces cerevisiae* and 44 protein glycosylation mutants, because the heterologous protein displayed on yeast is assumed to be modified by yeast-specific hyper-mannosylation. First, we constructed the yeast strain CtminiCipA, which displays a heterologous scaffolding protein (miniCipA from *Clostridium thermocellum*) on its cell surface, and glycosylation mutants secreting a dockerin-type cellulase (Cel8Aenz-Cel48Sdoc: a fusion protein of the catalytic domain of *C. thermocellum* Cel8A and the dockerin domain of *C. thermocellum* Cel48S). Next, minicellulosomes were assembled by mixing the CtminiCipA strain and the dockerin-type cellulase secreted by each glycosylation mutant. By using an endoglucanase assay and flow cytometric analysis, we showed that some glycosylation mutants enhanced cellulosome assembly; in particular, disruption of glycosylation genes located in the endoplasmic reticulum showed intense enhancement. These findings suggest that inhibition of the core complex or precursor formation in protein glycosylation enhances cellulosome assembly, meaning that absence of glycosylation is more important for cellulosome assembly than reducing the size of the glycochain.

1. Introduction

In recent years, a wide variety of renewable energy sources have been offered as alternatives to petroleum. Bioethanol, one such renewable energy, has become widely available, but the large-scale production of bioethanol produced from sugar cane or corn can lead to food crisis. Thus, cellulosic ethanol is being developed robustly over the world because it does not compete with food production. The production of cellulosic ethanol requires complex processes, including pretreatment of the biomass, saccharification, fermentation and distillation. Reducing the total cost of the process is absolute prerequisite for the commercial viability of cellulosic ethanol. To this end, consolidated bio-processing (CBP) has been suggested. CBP is a process using one type of microbe that simultaneously produces saccharolytic enzymes, hydrolyzes the pretreated biomass, and ferments pentose sugars and hexose sugars, thereby condensing four steps into a single step in the process configuration and drastically reducing the total cost (Lynd et al., 2002). We have tried to give fermentative *yeast Saccharomyces cerevisiae* saccharolytic ability, which is an element function for CBP technologies.

In order to achieve highly efficient saccharification in yeast, extensive studies have aimed at displaying saccharolytic enzymes on the yeast by using cell-surface engineering. Yeast strains displaying saccharolytic enzymes will permit saccharification of cellulose on the nearby cell surface, leading to an acceleration of sugar uptake and fermentation, to inhibition of the dissipation of the secreted saccharolytic enzymes, and to facilitation of recycling of the saccharolytic enzymes together with the fermentative yeast.

We are interested in the cellulosome as the cellulase source displayed on the yeast cell surface. The cellulosome is a multienzyme complex, produced by several cellulolytic bacteria, in which several saccharolytic enzymes are assembled on a scaffolding protein on the cell surface; thus, various enzymes act to hydrolyze lignocellulose in high density, leading to efficient saccharification by a proximity and concerted effect (Fierobe, 2001). The scaffolding protein contains several 'cohesin' domains that interact with 'dockerin' domains in saccharolytic enzymes. The dissociation constant between cohesins and dockerins is 10^{-9}–10^{-12} M, and this strong association is a key factor of functional cellulosome assembly (Fierobe et al., 1999, 2001; Mechaly et al., 2001; Jindou et al., 2004).

In recent years, there have been several reports regarding yeast strains that produce the cellulosome. For example, Ito displayed a fusion protein comprising cohesin derived from *Clostridium cellulosolvens* conjugated to the Z domain of protein A derived from

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Staphylococcus aureus on the yeast cell surface, and then combined it with a fusion protein comprising dockerin derived from *Clostridium cellulovorans* or the Fc domain of human immunoglobulin G conjugated to saccharolytic enzyme, and then assembled a complex similar to the cellulosome on the yeast cell surface (Ito et al., 2009). We were the first to demonstrate that the cellulosome can be produced in yeast by displaying the cellulose-binding domain and seven cohesin domains of CipA derived from *Clostridium thermocellum* and by combining them with cellulosomal enzymes on the yeast cell surface (Kohda et al., 2008, US Patent application US 2009/0035811). Subsequently, various studies about cellulosomes produced in yeast have been reported from several research institutions moving toward realization of the CBP process by using yeast (Tsai et al., 2009, 2010; Lilly et al., 2009; Wen et al., 2010).

S. cerevisiae has no saccharolytic enzymes, let alone cellulosomes. Thus, the introduction of foreign genes from bacteria or fungi is necessary for simultaneous saccharification and fermentation by yeast. An important issue is that yeast cannot secrete large amounts of heterologous proteins; however, genes have been identified in *S. cerevisiae* that can enhance heterologous protein secretion. In addition, we previously identified genes responsible for enhancement of heterologous protein production by means of systematic genetic transformation with the yeast deletion strain collection (Kitagawa et al., 2010). Another issue is the post-translational modification of protein. It is highly possible that these heterologous enzymes may be modified by yeast-specific hyper-mannosylation, a process that does not occur in bacteria or fungi. Hyper-mannosylation will appear to affect the activities or the interactions of these proteins. If the hyper-mannosylated cellulosomes produced by yeast cannot fulfill their original functions, then it will be necessary to reverse the mannosylation of these proteins in order to recover functionalities.

In this study, we have evaluated the effects of hyper-mannosylation on cellulosome assembly. Because dockerin-type cellulase has predicted glycosylation sites of both *N*-type and *O*-type, we aimed to remove or reduce the hyper-mannosylation of dockerin-type cellulase by means of yeast protein glycosylation mutants.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strain BY4741 (*MAT*a *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) was used as the parent strain. Forty glycosylation mutants of the haploid *MAT*a non-essential deletion collection (Openbiosystems) were used in the screening. YPD (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and synthetic dextrose (SD) medium (6.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose, and supplemented amino acids) were used for the selection of transformants.

2.2. Plasmids

A single signal peptide of glucoamylase from *Rhizopus oryzae* was used for the secretion of all proteins. *S. cerevisiae aga1* (*Aga1*) (systematic name YNR044 W) was constructed in a plasmid harboring the upstream sequence of the *AAP1* gene and the *His3* marker gene, resulting in pAH-HOR7p-Aga1 (Fig. 1A). Part of *C. thermocellum cipA* (*CtCipA*) (DDBJ/EMBL/GenBank Accession No. L08665) (1678–2109 bp) was constructed in a plasmid harboring the upstream sequence of the *ADH3* gene, V5 epitope, *aga2* gene and *Leu2* marker gene, resulting pDL-HOR7p-CtminiCipA-V5-Aga2 (Fig. 1B). The catalytic domain of *C. thermocellum cel8A* (DDBJ/EMBL/GenBank Accession No. K03088) (397–1548 bp) and the dockerin domain of *C. thermocellum Cel48S* (DDBJ/EMBL/GenBank Accession No. L06942) (2238–2453 bp) were conjugated and constructed in the pRS436GAP vector (DDBJ/EMBL/GenBank Accession No. AB304802) harboring the 2-micron replication origin, a His_6 epitope and the *URA3* marker gene, resulting in pRS436GAP-His-Cel8Aenz-Cel48Sdoc (Fig. 1C).

2.3. Yeast transformation

Both the pAH-HOR7p-Aga1 and the pDL-HOR7p-CtminiCipA-V5-aga2 plasmids were linearized with Sse8387I restriction enzyme (Takarabio, Shiga, Japan), and integrated into the BY4741 chromosome, resulting in a recombinant yeast CtminiCipA strain. The pRS436GAP-His-Cel8Aenz-Cel48Sdoc plasmid was transformed into BY4741 and each of 44 protein glycosylation mutants. All yeast transformations were performed by a Frozen-EZ Yeast transformation II Kit (ZYMO RESEARCH, CA, USA).

2.4. Mini cellulosome assembly

All of the transformants were plated onto each selective medium and incubated for 24 h at 30 °C. A colony of CtminiCipA was transferred to YPD liquid medium and grown for 24 h at 30 °C. Next, 100-μl aliquots of the culture were centrifuged, the supernatants were removed, and the yeast cell pellets were washed twice with phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, and 0.24 g/l KH_2PO_4). BY4741 and the 44 protein glycosylation mutants harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc were transferred to 1 ml of SD-ura + 2% casamino acid liquid medium and grown for 24 h at 30 °C simultaneously. Each culture supernatant was harvested by centrifugation and resuspended with the CtminiCipA cell pellets. To assemble the minicellulosomes, the suspensions were incubated for 12 h at 4 °C.

2.5. Flow cytometric analysis

Next, 32-μl aliquots of the minicellulosome-assembled yeast suspensions were harvested and washed twice with PBS. The cells were stained with the V5 staining solution (8 μg/ml of Anti-V5-FITC Ab [Invitrogen, CA, USA], and 1 mg/ml of bovine serum albumin [BSA; Sigma, MO, USA] in PBS) to detect scaffolding protein on the yeast-cell surface, or with the His staining solution (8 μg/ml of Anti-His_6-FITC Ab [AbD Serotec, UK], and 1 mg/ml of BSA in PBS) to detect the dockerin protein assembled with the scaffolding protein for 30 min on ice. The stained yeast was washed twice with PBS, resuspended with 250 μl of PBS, and then analyzed by flow cytometry.

2.6. Enzyme assay

The endoglucanase activity of the protein glycosylation mutants were evaluated using carboxymethyl cellulase (CMC) as a substrate. The yeast cells with assembled minicellulosomes were harvested by centrifugation and washed twice by PBS. The washed cells were resuspended in 1 ml of substrate solution (100 g/l CMC, 50 mM sodium acetate (pH 5)) at OD_{600} = 1.0 and incubated for 1 h at 60 °C. Next, 5-μl aliquots of each substrate solution were transferred to 100 μl of TZ buffer (Jue and Lipke, 1985), and then subjected to a color reaction for 3 min at 100 °C. The reducing sugars were then evaluated by the absorbance value at 660 nm.

2.7. Purification of dockerin-type cellulases

Protein glycosylation mutants harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc were grown at 30 °C for 24 h. The His-Cel8Aenz-Cel48Sdoc proteins secreted into each culture