ABSTRACTS (PH D THESIS)

Study of bacterial cellulose synthase by recombinant protein

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Functional analysis of cellulose synthase was conducted with recombinant protein. Given its easiness, bacterial cellulose synthase is used in this study. First of all, cellulose-synthesizing activity was successfully reconstituted in living cell of *Escherichia coli* by expressing the minimal required subunits CesA and CesB of *Gluconacetobacter*. Then this system, named as "CESEC (cellulose-synthesizing *E. coli*)" was used for checking enzymatic activity of point-mutants, for which site-directed mutagenesis was designed based on the structural model of bacterial cellulose synthase published in 2013 [1]. The obtained data were consistent with this model and previous data, and furthermore spotlighted the pivotal role of sulfur–arene interaction by cysteine in FFCGS motif and surrounding aromatic residues. However, the product of CESEC was a non-native structure of cellulose II, indicating that the reconstituted activity was incomplete or partially denatured. It is then absolutely required to find missing factor(s) for the native cellulose synthase activity. CESEC will be one of the tools useful for identifying such factors by reconstitution approach in following researches.

Introduction

In general, recombinant protein allows variable experiments to be conducted like site-directed mutagenesis and large scale production of protein. It is therefore a useful tool to analyze protein. Cellulose synthase is enzyme of hetero-subunit complex in cell membrane. Given high difficulty to handle membrane proteins with complex associated however, fewer studies have been conducted for cellulose synthase with recombinant protein, and then our understanding of cellulose biosynthesis is limited yet. In this thesis, recombinant cellulose synthase was intensively used for exploring its enzymatic mechanism.

Bacterial model was selected in this thesis given its convenience to use. First, cellulose-synthesizing activity was successfully reconstituted in living cell of *Escherichia coli* by conventional method of heterologous expression of protein. This system, which we named as "CESEC (cellulose-synthesizing *E. coli*), was then demonstrated to be useful tool for the study of cellulose synthase: effect of point-mutation to the activity was surveyed in order to explore catalytic reaction at the level of amino acid residue.

Reconstitution of cellulose-synthesizing activity in *E. coli* [2]

Expression vector of bacterial cellulose synthase was constructed by cloning *cesA* and *cesB* genes of *Gluconacetobacter xylinus* JCM9730, which are the minimally required subunits of bacterial cellulose synthase. These two genes are in a same gene cluster, and then introduced into the vector as it is, together with the upstream sequence of *cesA* gene (Shine-Dalgarno sequence or ribosomal binding site). In addition, c-di-GMP, a small cyclic nucleotide, is another essential factor for cellulose synthesis in bacteria. For producing c-di-GMP in the cell, expression vector of DGC (diguanylate cyclase or c-di-GMP synthase) was constructed by inserting the gene into pBAD33 vector, which can be maintained together with pQE. These two plasmid DNA were then introduced into an *E .coli* strain XL1-Blue (Figure 1) and then transformant by carrying both plasmids were selected by antibiotics.

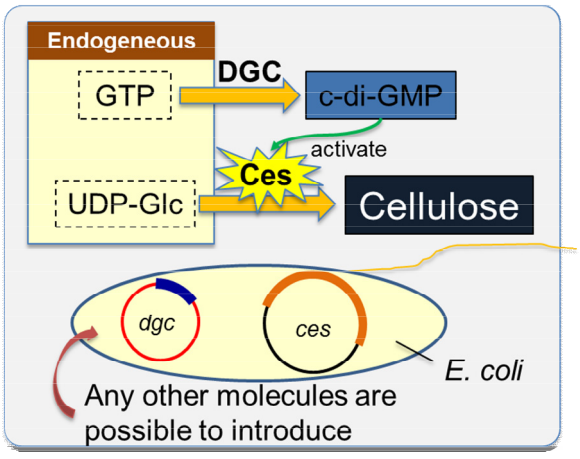

Figure 1. A schematic diagram of CESEC, cellulose-synthesizing E. coli

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It was shown by western blotting that this *E. coli* transformant expresses CesA and CesB proteins in cell membrane correctly. As well, by LC/MS analysis, production of c-di-GMP in the cell was confirmed specifically when DGC was expressed. Finally it was shown that cellulose is produced by this *E. coli* transformant only when CesA, CesB and DGC protein was expressed, reconfirming that two proteins of CesA/ CesB and a nucleotide c-di-GMP are the required factors for cellulose biosynthesis in bacteria.

Analysis of the product in dried state demonstrated that synthesized cellulose was not in the native structure of cellulose I, but crystallized into cellulose II. However GPC analysis showed that weight-averaged molecular weight of the produced cellulose is as high as 700. These indicate that the reconstituted cellulose synthase still keeps the polymerizing activity but lacks crystallization (microfibril formation) mechanism. The reason for this partial denaturation has to be clarified for understanding the machinery to synthesize cellulose as microfibril with crystallographic polymorph I.

Functional analysis of CesA protein with site-directed mutagenesis [3]

The *E. coli* transformant established above uses conventional plasmid DNA for expressing cellulose synthase protein. It is accordingly easy to introduce any mutations to cellulose synthase for testing its effect to enzymatic activity. Then, several point mutants of CesA was prepared by site-directed mutagenesis for counting their cellulose-synthesizing activity to explore roles of amino acid residues in catalysis.

Mutation was designed on sequence similarity and structural model that was reported in 2013 [1]. A significant reduction of activity by mutation was observed when mutation matters (like s mutation of catalytic residue Asp333 to Asn), while an unexpected reduction of activity was found for a mild mutation of cysteine in FFCGS motif, which is well conserved in bacterial type CesA protein. Structural model in 2013 [1] spotlighted the “main chain” carbonyl of this cysteine as one of the keys to locate the molecular end of growing cellulose, which is the acceptor of glucosyltransfer reaction. Given this hypothesis, one can suppose that “side chain” of this cysteine could be replaced by other side chain unless the mutation is deteriorative. However, in addition to deteriorative mutations like valine or phenylalanine, mild mutation to alanine or even serine abolished cellulose-synthesizing activity. This indicates side chain thiol has an active role in catalysis. Probably sulfur–arene interaction between the side chain thiol and surrounding aromatic residues (Phe291, Tyr292, and Phe306) plays a role for cellulose-polymerizing reaction.

Concluding remarks

Cellulose synthesis is not only a chemical reaction to elongate glucan chain by successive transferring reaction, but also a physical reaction to assemble polymer chains into a supemolecular structure – cellulose microfibril. Cellulose-synthesizing activity reconstituted into *E. coli* in this study, CESEC, is a useful tool for studying biochemistry and biophysics of cellulose synthase to understand the mechanism of cellulose biosynthesis in molecular and supermolecular level.

Acknowledgements

LC/MS analysis was done with DASH system (CER and RISH, Kyoto University). Electron microscopic observation was done with ADAM system (RISH, Kyoto University).

References

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