Preliminary

Expression of *Acetobacter xylinum* Cellulose Synthase in Insect Cells

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Introduction

Cellulose is the most abundant biopolymer on earth, and much effort has been devoted to reveal its biosynthetic mechanism[1]. Recently structural analysis of membrane proteins, which are functionally important, has become an active area in structural biology and also in cellulose filed[2]. For such approaches, large quantities as well as high concentration of proteins are needed. Structural analysis by x-ray crystallography or electron crystallography requires, for example, several milligrams of functional protein in concentrated solutions. However, membrane proteins often exist in their native membranes at low abundance. Therefore, strong overexpression, along with fine purification systems; is needed to attain a high concentration of the protein of interest. In this study, therefore, a recombinant bacterial cellulose synthase that catalyses glucosyl transfer (CesA) was expressed in the insect cells, and the conditions for large-scale production and purification were investigated.

Expression of recombinant cellulose synthase in insect cells

The catalytic subunit gene of *Acetobacter xylinum* cellulose synthase operon[3] (*AxCesA*) (*Acetobacter xylinum* BPR2001, Genebank: AB010645[4]) was amplified by PCR. The PCR product was ligated into BamH I and Hind III-cleaved pBlueBacHis2B (Invitorogen) and introduced into baculovirus Bac-N-Blue according to the manufacturer's protocol (Invitorogen). Recombinant baculovirus were then infected to Sf9 insect cells. The expression of CesA protein in insect cells was confirmed by molecular size, which is supposed to be about 86 kDa, in Western blotting. In this protein expression system, the recombinant proteins include His6-tag, which could later be utilized for affinity purification.

Optimization of infection time was examined by timecourse experiment, where the insect cells were harvested every 12 or 24 hrs after infection and the relative amount of protein was analyzed by Western blotting.

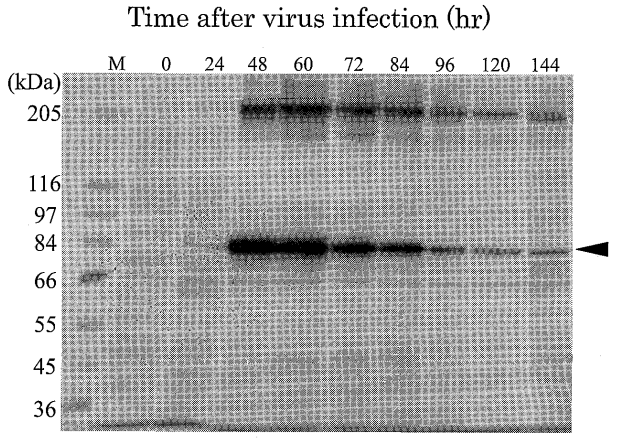

Fig. 1. The result of timecourse experiment. Recombinant CesA (arrow head) was most expressed after 48–60 hrs from baculovirus infection.

The target protein was found to be most expressed in 48–60 hrs (Fig. 1) and thus we used this optimal infection time throughout this study.

Localization of expressed protein in membrane fraction

The membrane fractions of virus-infected insect cells

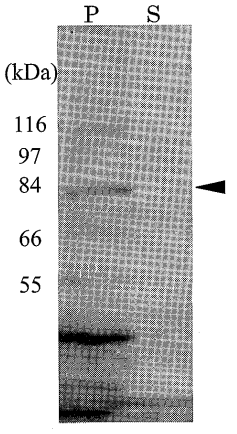

Fig. 2. Western blotting of membrane fraction (P) and supernatant (S) samples. The expressed recombinant CesA (arrow head) was localized in membrane fraction.

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were separated by ultracentrifugation. The precipitant and the supernatant were then analyzed by Western blotting. The recombinant CesA (86 kDa) was found only in the membrane fraction, not in the supernatant (Fig. 2) as we expected, because CesA has been known as a membrane-integrated protein. Therefore, it is likely that recombinant CesA was folded properly and not denatured.

Detergent extraction

In order to find the optimal condition for solubilizing CesA from membrane fraction, a variety of detergents known to be relatively mild was tested. Seven detergents, *n*-dodecyl-β-D-maltoside (DDM), *n*-decyl-β-D-maltoside (DM), *n*-Octyl-β-D-glucoside (OG), *n*-octyl-β-D-maltoside (OM), CHAPS, Triton X-100, MEGA-10 were investigated.

Overall, DM, DDM and CHAPS were relatively successful, while OG and MEGA-10 hardly solubilized the protein of interest. In the subsequent course of experiment, for example, in the crystallization experiment, dialysis would be necessary, so the detergent of simple structure seemed to have advantage in such steps. In view of this point, DM and DDM, which have linear structures (Fig. 3), were employed for the solubilization of gene product.

Compared with DDM, DM was superior in solubilizing more recombinant CesA from the membrane fraction. Therefore, DM in detergent extraction step was routined

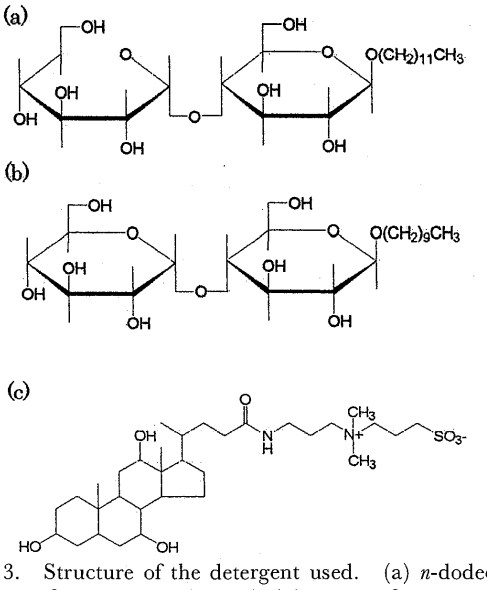

Fig. 3. Structure of the detergent used. (a) *n*-dodecyl-β-D-maltoside (DDM), (b) *n*-decyl-β-D-maltoside (DM), (c) CHAPS.

prior to the His-tag affinity purification.

Mechanical homogenization of membrane fraction before detergent extraction by passing the suspension go and back in a needle-tipped syringe was found extremely effective. Without this process, the amount of solubilized CesA was quite small, remaining most part of target protein in membrane fraction. In addition, solubilization of once-frozen membrane fraction was never better than the sample without freezing. It was true of all types of detergents investigated in this study.

In some reports, pretreatments were applied before detergent extraction to improve the quality of protein purification[5]. The aim of this treatment is to remove unfavorable materials to some extent while conserving the protein of interest. In this study, the pretreatment of the membrane fraction with Na_2CO_3, NaOH, HCl, and urea were examined. In result, HCl and urea were found unsuitable at all, failing in selective removing of unnecessary materials. Among them, the alkali treatments were relatively successful and between the two alkalis used, Na_2CO_3 turned out to be better, remaining more target protein in membrane fraction.

However, these pretreatments are known to denature the target protein, and therefore the Na_2CO_3 treatment would be worthwhile in case sufficient purity would not achieved by the other strategies.

His-tag purification

For His-tag purification, the elution buffer, ranging 10 mM–20 mM imidazole adjusted at pH 7.0–pH 8.0 were investigated. The best elution buffer was a solution containing 20 mM imidazole at pH 7.0, eluting the protein of interest with less background. The amount of the recombinant CesA obtained is, however, still quite small and further examination of purification conditions is needed to produce sufficient amount of protein of the interest for structural analysis such as crystallography.

References

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