Artificial Cell Wall for Plant Protoplast. Coating of Plasma Membrane with Hydrophobized Polysaccharides

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Protoplasts of *Daucus carota* were coated by a hydrophobized polysaccharide, such as cholesterol-bearing modified pullulan (CHP) or cholesterol-bearing xyloglucan phosphate (CHXP), that provided an artificial cell wall to the protoplast. The polysaccharide-coating was confirmed directly by a confocal laser microscopy using FITC-labeled polysaccharide derivative. The artificial cell wall certainly increased the viability of the protoplasts even in the presence of cellulase.

Sunamoto and his coworkers have already revealed coating of liposome with hydrophobized polysaccharide brings about physicochemical and biochemical stability of the liposome both *in vitro* and *in vivo*.[1] Considering previous results in stabilization of liposomes, therefore, coating of protoplast with the hydrophobized polysaccharides is expected to stabilize also its plasma membrane and consequently to increase the cell viability. In this communication, we report the effect of treatment of carrot protoplast with a hydrophobized polysaccharide.

CHP-70-1.3, pullulan-70 (Mw 70000) substituted by 1.3 cholesterol groups per 100 anhydroglucoside units, was from Nippon Oil & Fats Ltd. (Tokyo, Japan) and exactly the same as that used in our previous work.[2] Xyloglucan was a kind gift of Honen Oil Ltd.(Tokyo, Japan). Cellulase Onozuka R10 was purchased from Yakult (Tokyo, Japan), and Driselase was from Kyowa (Tokyo, Japan). Fluorescein diacetate and Ficoll 400 were purchased from Sigma (MO, USA). Other chemicals and solvents were from Wako Pure Chemicals Co. Ltd. (Kyoto, Japan) and used without further purification. CHXP (cholesterol-bearing xyloglucan phosphate) was synthesized as follows. Xyloglucan-1500 (MW 1,500,000, 1.6 g) was dissolved under heating in dry DMSO (100 ml) containing pyridine (3 ml). After addition of cholesteryl *N*-(6-isocyanatehexyl) carbamate (0.14 g),[2] the reaction mixture was stirred for 8 h at 100 °C. Pyridine was then removed by distillation under reduced pressure. To the cooled reaction mixture was added pyrophosphoric acid (2.7 g) and formamide (24 ml), and the resulting mixture was stirred for 21 h at room temperature.[3] After pouring the reaction mixture into ethanol (300 ml), the precipitates were separated by filtration, submitted to dialysis against distilled water, and lyophilized to give 1.4 g of CHXP. The degree of substitution of cholesterol and phosphate groups was determined by elemental analysis.[2] It was found that xyloglucan was substituted by 1.8 cholesterol and 12 phosphate groups per 100 monosaccharide units. The product was coded as CHXP(12)-1500-1.8. To directly certify the coating of the protoplast with these hydrophobized polysaccharides (*vide infra*), labeling of fluorescent probe (FITC) to CHP and CHXP was carried out as described.[4] The substitution degree of FITC was determined spectrophotometrically to be 0.7 for CHP and 0.4 for CHXP, respectively, per 100 monosaccharide units.

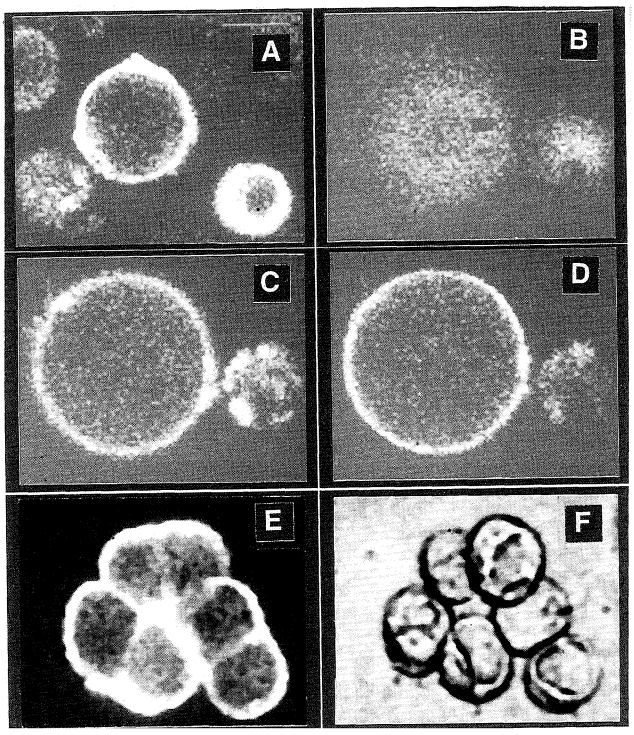

Figure 1. Images of a laser scanning confocal fluorescence microscopy for binding of FITC-CHP (**A-D**) and FITC-CHXP (**E and F**) to carrot protoplasts. Protoplasts (1×10^6 cells/ml) were treated with 0.6 mg/ml FITC-CHP or 0.3 mg/ml FITC-CHXP for 3 h. With **A**, magnification is × 20 and bar indicates 40 μm. With **B-D**, magnification is × 40. They show changes in depth of focus from the top of the protoplasts; **B**, 2 μm; **C**, 8 μm; and **D**, 12 μm. With **E** and **F**, magnification is × 20; **F** is phase contrast of **E**.

Table 1. Effect of hydrophobized polysaccharides on the viability of carrot protoplasts in the presence or absence of cellulase (1 mg/ml) [a]

Days	Cell viability / %					
	I	II	III	IV	V	VI
1	77 ± 5	55 ± 6	82 ± 3	79 ± 7	84 ± 3	89
2	67 ± 4	47 ± 5	78 ± 4	69 ± 6	79 ± 5	65
4	64 ± 4	42 ± 4	73 ± 4	68 ± 4	80 ± 2	-

[a]Experiments were triplicated except run VI.

I, Control without any polysaccharide treatment

II, In the presence of cellulase without polysaccharide coat

III, Coated with CHP-70-1.3

IV, Coated with CHP-70-1.3 in the presence of cellulase

V, Coated with CHXP(12)-1500-1.8

VI, Coated with CHXP(12)-1500-1.8 in the presence of cellulase

Cell suspension of *Daucus carota* cv. Kuroda-gosun was cultured as described in literature.[5] Protoplasts were isolated from 6-day-old cells in protoplast isolation medium (Murashige-Skoog (MS) medium) according to the method already established.[6] Protoplasts (1×10^6 cells/ml) were treated with FITC-labeled and unlabeled CHP (0.6 mg/ml) or CHXP (0.3 mg/ml) and incubated for 3 h at 24 °C under mild shaking in the dark using the same MS-medium. The protoplasts were then washed three times with the culture medium by centrifugation at $50 \times g$ for 4 min. The polysaccharide coating was directly visualized on images of confocal optics (Figure 1) using a confocal laser scanning microscope (Bio-Rad, MRC600, CA, USA) equipped with an argon-ion laser for excitation at 488 nm. This microscopic observation revealed that CHP (Figure 1**A** - **D**) or CHXP (Figure 1**E**) certainly coated only the outermost surface of protoplasts. This is more clearly shown for a larger living cell when the depth of confocus was changed (from **B** to **D**). CHXP without FITC label also was found to coat the plasma membrane, which was determined using Calcofluor White M2R (Sigma) that specifically interacts with β(1-3)glucoside moiety (though images are not shown). We confirmed also that both FITC-labeled pullulan and xyloglucan phosphate without any hydrophobic anchor did not coat the plasma membrane at all. Furthermore, in all the cases, we did not see any fluorescence in the cytosol except natural emission.

We studied also the viability of the protoplast by the Fluorescein Diacetate method[7] under various conditions (Table 1). It was clear that the viability of protoplasts significantly increased by the polysaccharide-coating, especially after the incubation for a long time enough. This effect was more obvious in the medium that contains cellulase (1 mg/ml) (runs II, IV and VI in Table 1).

When the protoplast was treated with pullulan-70 (1.2 mg/ml) carrying no cholesterol anchors, the cell viability was found to be 60 % after 4 days incubation at 24 °C even in the absence of cellulase. In our case, therefore, the hydrophobic anchor substituted to the polysaccharide seems essential to enhance the coating efficiency to the plasma membrane.[4] The slightly better coating efficiency of CHXP than CHP (Table 1) was more obvious when a Ca^{2+}-ionophore (A23187) was present in the culture medium.[8] This might be come from a large structural difference between CHP and CHXP. Studies of protoplast viability and cell division showed the hydrophobized polysaccharide-coated did not affect regeneration of cell wall (data not shown). Hisamatsu and his coworkers have used a water soluble glycan, (1→3)-β-D-glycan (glucopyranan), for protecting yeast protoplast (*S. cerevisiae*), and also found an effect of glucopyranan on the regeneration frequency of yeast protoplast.[9] Of course, we can not apply such the polysaccharide to plant protoplasts.

References and Notes

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