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INTRODUCTION OF FOREIGN DNA INTO *Chlorella saccharophila* BY ELECTROPORATION

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SUMMARY

Plasmid, pBI221, was introduced into protoplasts of *Chlorella saccharophila* c-211-1a prepared from the cells in the stationary phase by electroporation. Transient expression of the introduced plasmid was observed under a field strength of between 600 and 900 V/cm, and a pulse duration of around 400 ms, where high membrane permeability to 70-kDa FITC-dextran was ascertained.

INTRODUCTION

The unicellular, green alga chlorella has been regarded as a potential diet source due to its high protein content and various physiologically active substances. However, its cell wall is too rigid to be digested easily and its constitution of amino acid is somewhat unbalanced for human nutrition (Takeuchi, 1973). These characteristics should be modified before using it for a foodstuff. Genetic modification could be a method for improving its nutritive content.

Electroporation is often used for direct gene transfer. Many species of various cell types have been transformed either transiently or stably by electroporation. However, the mechanism of electroporation is not fully understood and its proper operational conditions need to be determined for individual cells (Chang et al., 1992).

In the present paper, the conditions for efficient protoplast formation of *C. saccharophila* c-211-1a and introduction of exogenous DNA, pBI 221, into the protoplast by electroporation were examined.

MATERIALS AND METHODS

Chlorella strain and protoplast preparation

C.saccharophila c-211-1a used because of efficient formation of its protoplast (Yamada and Sakaguchi, 1982; Göbel and Aach, 1985), was grown in a jar-fermentor containing 600

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ml Lorenzen medium at 28 °C, 400 rpm and 5 vvm aeration rate of air containing 5% CO_2. The fermentor was illuminated by fluorescent lamps with 8000 lux.

Harvested cells were suspended in 25 mM phosphate buffer containing 0.3 M sorbitol, 0.3 M mannitol and a mixture of polysaccharide-degrading enzymes [1% cellulase Onozuka RS and 1% Macerozyme R10 (Yakult Honsha Co. Ltd.)], pH 6.0. The enzyme concentrations were determined by checking both the efficiency of protoplast formation and cell's viability. The suspension (ca 10^8 cells/ml) was incubated for 20 h at 28 °C on a rotary shaker at 80rpm. Protoplast formation was confirmed by Calcofluor White ST staining (Yamada and Sakaguchi, 1982). Frequency of protoplast formation was determined by counting the stained cells using a haemocytometer.

Electroporation

The protoplasts used for electroporation were prepared from the cells harvested under the stationary growth-phase. The protoplasts were washed 3 times with buffer for electropolation [25 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM $CaCl_2$, 0.27 M sorbitol, and 0.27 M mannitol] by centrifuging at 180 × g for 3 min and resuspended in the same buffer at a cell concentration of 1×10^6 cells/ml. The plasmid and carrier DNA (salmon sperm) solutions were added to the above mentioned solution at the final concentration of 10 and 50 μg/ml, respectively. The plasmid pBI221 used coded ß-glucuronidase as a reporter gene under the control of Cauliflower Mosaic Virus 35S promoter. After incubation for 30 min at room temperature without shaking, 1 ml of suspension was transferred into the electroporation chamber consisting of two flat aluminum electrodes separated by an average distance of 0.4 cm.

The electroporation was performed by Cell Porator (BRL Life Technologies Inc.) with electrical field strengths of 300, 600 and 900 V/cm. The parameter, pulse duration, used in electroporation was determined by the following equation.

$$\text{Pulse duration} = \text{Capacitance/Conductivity} \quad (1)$$

The conductivity of the electroporated solution was 4.96 mS, and the pulse duration applied to the suspension was estimated to be 12, 65 and 400 ms at the respective capacitances of 60, 330 and 1980 μF. After 1 h incubation at room temperature without shaking, the electroporated suspension, 0.8 ml, was transferred into the regeneration medium of 2 ml (Lorenzen medium supplemented with 20% (w/v) sucrose) and cultivated at 28 °C with shaking of 80 rpm in the dark. Transient expression of introduced pBI221 was assayed after 48 h.

To examine the permeability of electroporated protoplast membrane, 0.1 mM 70-kDa FITC-dextran (Sigma) was added to the protoplast solution in place of DNA. After incubation of electroporated protoplasts for 1 h, protoplasts were washed 3 times with 10 mM phosphate buffer containing 0.3 M sorbitol and 0.3 M mannitol to remove the residual FITC-dextran by centrifuging at 180 × g for 3 min. The cells stained by the uptaken FITC-dextran were counted by EPICS ELITE™ flow cytometer (Coulter Co. Ltd.).

Transient Assay of GUS Activity

The GUS activity was measured using MUG (4-methylumbelliferyl-ß-glucuronide) as a substrate by the fluorometric assay (Jefferson et al., 1987). To suppress the endogenouss GUS-like activity of chlorella, 5% methanol was added to the reaction solution (Kosugi et al., 1990).

Cell Viability

Viability of electroporated cells was assayed by trypan blue dye exclusion method after gentle sonication at 20M Hz for 5 s.

Flow Cytometric Assay

The stained cells with FITC-dextran were assayed by the EPICS ELITE™ flow cytometer. Relative number of stained cells was calculated from the histogram of fluorescence intensity of FITC at 525 nm.

RESULTS

Frequency of protoplast formation by the enzymatic degradation of cell wall was significantly affected by the growth phase of cells as shown in Fig.1. It decreased from ca.40% at the early growth-phase to the lowest (ca 5%) in the midst of linear growth-phase, then increased to the highest (more than 80%) during the stationary phase. Therefore, in the electroporation experiment, the protoplasts were prepared from the cells harvested under the stationary phase.

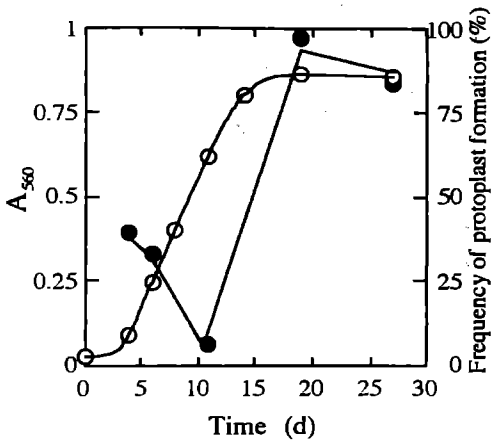

Fig. 1 Relation between growth phase and frequency of protoplast formation (The data were average of 3 samples)
○ A_{560}
● Frequency of protoplast formation

The permeability of the cell membrane to 70-kDa FITC-dextran was increased with the applied electrical field strength and pulse duration as shown in Fig.2. Under the pulse duration of 400 ms, about 30, 55 and 75% cells were stained with FITC-dextran

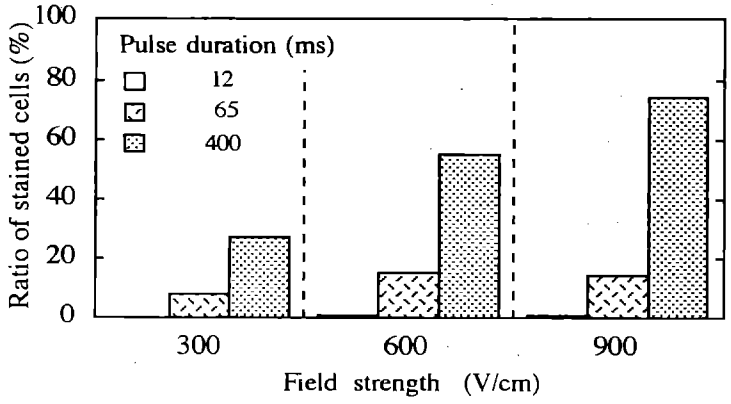

Fig. 2 Effect of field strength and pulse duration on the ratio of cells stained with 70-kDa FITC-dextran

at the respective field strengths of 300, 600 and 900 V/cm, while the stained cells were less than 20% under the shorter pulse duration.

GUS activities, introduced by plasmid DNA pBI221, are shown in Fig.3. The relationships between the detected GUS activity against the operational conditions were similar to those of the permeability of protoplasts to 70-kDa FITC-dextran shown in Fig.2.

DISCUSSION

The dependency of the protoplast formation frequency on the growth phase, shown

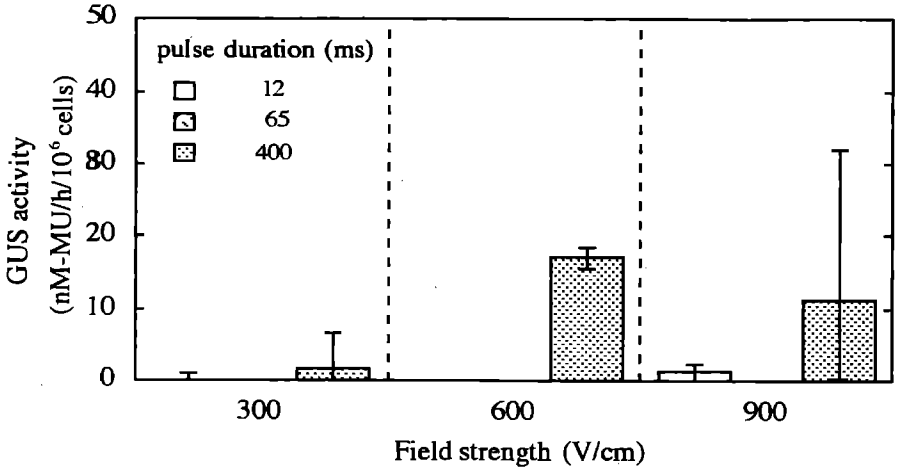

Fig. 3 Effect of field strength and pulse duration on transient GUS expression (The data were average of 3 samples)

in Fig.1, is probably due to the differences in the chemical composition of the cell wall at the various growth phase. Yamada and Sakaguchi (1982) found that the cell wall of *C. saccharophila* C-211 consisted of two major layers, an inner bulky microfibrillar layer and a thin outer monolayer. The outer layer was rigid which would protect the digestion of the inner layer by cellulase. Takeda and Hirokawa (1982, 1984) have demonstrated the changes in cell wall composition throughout the cell cycle of *C. ellipsoidea* and the related strains, including *C. saccharophila*. They showed that the rigid cell wall constituents in the cell wall of *C. ellipsoidea* C-27, which mainly consisted of glucosamine and amino acids, increased significantly during the mitotic phase of the cell cycle and decreased in the other phases, and could not be degraded even by alkali. From these findings, it was considered that the changes of the rigid cell wall components during the cell cultivation might be the reason that resulted in the dependency of protoplast formation on the growth phase in our study.

When the cell is electroporated, pores across the cell membrane called "electropores", will be formed through which large molecules such as exogenous DNA can permeate. A necessary requirement for introducing exogenous DNA into a cell is to increase the permeability of the cell membrane to the target DNA. Permeability of the electroporated cell is usually dependent on the electric field strength and pulse duration (Chernomordik et al., 1987; Tsong, 1991). This dependency was also confirmed from the result shown in Fig.2, where higher permeability of the electroporated cell membrane to FITC-dextran was obtained under higher electric field strength and longer pulse duration. The diameter of electropores has been estimated in the range of 1-10 nm using FITC-dextran (Sowers and Lieber, 1986), while the size of DNA was usually larger (Sukharev et al., 1992). From this difference, Sukharev et al. (1992) proposed the interaction of DNA and electropores by which the larger DNA molecule was capable of permeating through the cell membrane. The size of 70-kDa FITC-dextran was estimated to be 5 nm (Laurent and Granath, 1967) and, under the

electroporating condition at which the fluorescence permeated into *Schizosaccharomyces pombe* cells, a plasmid DNA with the size of 7.9 kb larger than PBI221 (5.7 kb) used in our study was expressed (Hood and Stachow, 1992). Moreover, under ideal conditions, when DNA transfer through a permeable membrane is driven by electrophoretic force only, the amount of DNA taken up by the cell is proportional to the product of the electric field strength and pulse duration (Sukharev et al., 1992). These reports can explain the result shown in Fig.3, where higher GUS activity was obtained under higher electric field strength and longer pulse duration. However, in the case of 900 V/cm, the GUS activity showed somewhat scattered data compared with that at 600 V/cm. This result was ascribed to the cell damage at the higher field strength of 900 V/cm and longer duration of 400 ms. Figure 4 shows the effects of electric field strength and pulse duration on the cell viability. The cell viability at 900 V/cm under 400 msec duration was so low (about 35%) that the damaged cells could not be recovered after pBI221 plasmid permeating.

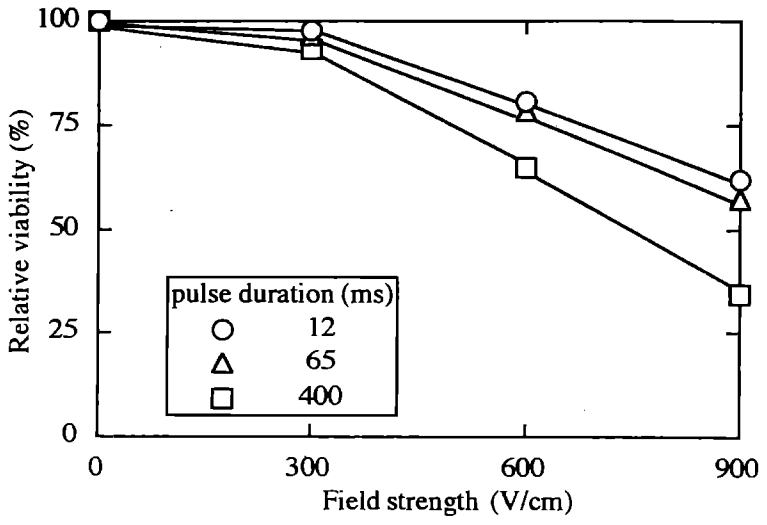

Fig.4 Relative viability of after electroporation to that of unelectroporated cells

From the results in the present study, the conditions needed for foreign gene introduction into *C. saccharophia* c-211-1a by electropolation are suggested as a field strength of between 600 and 900 V/cm and a pulse duration time of around 400 ms. Additional experiments should be carried out further for constructing the stable expression.

CONCLUDING REMARKS

Protoplasts of *C. saccharophila* c-211-1a prepared from the cells harvested under the stationary growth-phase were the most appropriate for introducing foreign gene by electropolation. The optimum electropolation conditions for the transient expression of introduced gene into the cells were a field strength between 600 and 900 V/cm and a pulse duration time around 400 msec.

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