양파의 原形質體 活性 增進 方法

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Improvement of Protoplast Vitality of Onion Epidermal Cells (*Allium cepa* L.)

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要 約

양파(*Allium cepa*) 細胞에서 分離한 原形質體의 vitality를 cytoplasmic streaming과 urea permeability로 測定하고 FDA法에 의해 viability를 測定한 結果와 比較했다. 한편, 大豆(*Glycine max*) 葉에서 原形質體의 活性을 增進시키기 위해서 vibratome을 利用하여 single cell 分離條件을 確立하고 이로부터 分離한 原形質體의 vitality에 關해 檢討했다.

1. 양파에서는 2% cellulase와 1% macerozyme을 處理했을 때 가장 좋은 原形質體 收率(7 $\times 10^4$ protoplasts/g.fresh weight)을 얻었는데 生存率은 酵素 濃度가 增加할수록 減少했다.
2. Cellulase를 濃度別(1%, 2%, 3%, 4%)로 處理한 細胞의 cytoplasmic streaming速度는 對照區에서 6.5μm/sec(100%)로 3時間 동안 변하지 않았고 1% cellulase에서는 vitality가 76.9% 그리고 4% cellulase에서는 43.1%로 그 差異가 33.8%(76.9~43.1)이었는데 이 값은 FDA로 測定해서 얻은 6.8%보다 差異가 더 크기 때문에 細密한 vitality를 測定할 수 있다고 생각된다. 2% cellulase만을 處理(70%)했을 때 보다 BSA 또는 DTT를 함께 處理했을 때 vitality는 각각 89.2%, 93.8%로 增加했고, 한편 NaN_3나 KCN을 處理하면 37.9%, 31.8%로 減少했다.
3. Cellulase 濃度別(1%, 2%, 3%, 4%)로 處理해서 얻은 原形質體의 urea permeability를 測定한 結果 酵素濃度가 增加할수록 urea permeability가 增加했다. BSA 또는 DTT를 處理했을 때 對照區에 比해서 urea permeability가 減少했다. 以上의 結果로 보아 cellulase 處理에 의한 膜損傷은 urea permeability의 수동적 흡수를 증가시키는 것으로 생각된다.

Introduction

The viable protoplats are essentially needed for protoplast culture, protoplast fusion and direct transfer of genes to protoplasts.

Various techniques used for measuring protoplast viability are vital staining (palta et al. 1978), cytoplasmic streaming (Kamiya and Kuroda, 1973), plasmolysis (Stadelmann, 1966), electric conductivity (Dexter *et al.* 1932), active transport properties and TTC method (Kuhn and Terchel 1941). These assays have tested only an individual function or a few specific functions of the cells.

Stadelmann and Kinzel(1972) reported that vital staining leads to alterations in the physiology of the cell and can not distingish between injured and uninjured protoplasts since injured protoplasts may also accumulate stains in the vacuole depending upon the extend of the injury (Palta *et al.* 1978). There is some possibility that organic solvent of staining solution alters lipid portions of plasma membrane. However, cytoplasmic streaming (Kamiya 1973) and urea permeability were used as a sensitive and precise indicator for detecting protoplast vitality.

The purpose of this experiment was to compare FDA method with cytoplasmic streaming method in terms of cell vitality measurement using onion epidermal cells (*Allium cepa*).

Materials and Methods

1. Plant material :

Organically grown onions (*Allium cepa* L.) were purchased from the market and stored at 4℃ in the dark.

2. Protoplast isolation

The upper and the lower quarters of the *Allium cepa* were discarded and the middle portions were used for the experiments. The second or third from the outermost fresh scale was selected and several incisions of approximately 0.5×0.5㎜ were prepared on the inner surface. The scale was then infiltrated three times with 100㎖ tap water. Paired adjacent sections of the inner epidermis were peeled off and floated on the enzyme solution. Cellulase (1, 2, 3 and 4%) and macerozyme 1% were combined to make enzyme solutions (Table 1).

After filtering enzyme solution with an ultra membrane filter(pore size : 0.45㎛), epidermal cells of 200㎎ fresh weight were floated on 5㎖ enzyme solution. The enzyme mixture was incubated for 3 hrs at 25℃ in the dark. After incubation, the unaffected cell aggregates were removed by filtering through nylon cloth, the mesh size of which ranged from 100 ~150㎛. The protoplasts passed through

Table 1. Composition of the enzyme solution for isolating epidermal protoplasts of *Allium cepa* bulb scale.

Constituent	Concentration
Cellulase Onozuka R-10[a]	2%
Macerozyme R-10[b]	1%
Calcium chloride	10mM
HEPES[c]	10mM
Sorbitol	0.6M
pH	5.8

a : Yakult Honsha Co., Ltd. Japan
b : Yakult Pharmaceutical Industry Co., Ltd. Japan
c : N-2-Hydroxyethyperazine-N'-2-ethanesulfonic acid

the filter, whereas the cell aggregates did not. The filterate was transferred to a centrifuge tube and it was spinned at 50 x g for 5 min. The debris in the supernatant were carefully removed with a pipette. The remainder was centrifuged at 50 x g for 5 min. and suspended in the sucrose CPW solution (Table 2). The protoplasts were floated on the surface of the sucrose CPW solution while the remaining cells and debris were at the bottom of the tube. The protoplasts removed with a pipette were resuspended in the sorbitol CPW solution (Table 2), centrifuged at 50 x g for 5 min. in order to separate the contaminated debris. The washing process was repeated three times.

Table 2. Cell and protoplast washing (CPW) solutions.

Salts used	Concentration (mg/l)
KH_2PO_4	22.7
KNO_3	101.0
$CaCl_2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
$CuSO_4.5H_2O$	0.025

Sorbitol CPW : 0.6M sorbitol solution containing the above salt mixture.
Sucrose CPW : 0.6M sucrose solution containing the above salt mixture.
Urea CPW : 0.6M urea solution containing the above salt mixture.
pH 5.8 in all cases

The pellet of protoplast was transferred into a measured volume containing sorbitol CPW solution and counted using the heamocytometer (L : 1 ㎜, W : 1㎜, D : 0.1㎜, American Optical, U.S.A.).

Fluorescein diacetate (FDA, Sigma Chemical Co., U.S.A.) was stored in aceton stock solution (5㎎/㎖) at 4℃. It was added to the protoplast suspension to give a final concentration of 0.01% (Widholm 1972).

After 5 min. at room temperature the protoplasts were examined for fluorescence using the Inverted Microscope (Nikon Diaphot-TMD). The exciter filter BG12 (Transmission : 330~500nm) and the barrier filter (Transmission : > 460nm) were used. Protoplast viability(%) was calculated as follows;

$$\frac{\text{no. of fluorescing protoplasts}}{\text{total no. of protoplasts}} \times 100$$

3. Cytoplasmic streaming rate measurement

1) Effects of cellulase concentrations

The speed of cytoplasmic streaming was measured by recording the movement of small particles in *Allium cepa* bulb scale cells under microscope for three hours (30, 60, 120 and 180 min.).

2) Effects of bovine serum albumine (BSA, Sigma Chemical Co., U. S. A.) and dithiothreitol (DTT, Janssen Chemical Co., Belgium)

Allium cepa bulb scale cells were floated on the 2% cellulase solution containing 0.05% BSA or 5mM DTT. And streaming rate was measured at 20min. intervals for 80 min.

3) Effects of NaN_3 and KCN

Allium cepa bulb scale cells were floated on the 2% cellulase solution containing 10mM NaN_3 or 1mM KCN. And the rate was measured at 10 min. intervals for 60 min.

4. Urea permeability measurement

1) Effects of cellulase concentrations

After complete protoplast isolation at each cellulase concentration, protoplasts were washed three times in sorbitol CPW solution and transferred into the falcon petri dish containing 3 ㎖ urea CPW solution (Table 2). Length of protoplast was recorded under microscope at 5 min. intervals for 30 min. Urea permeability (Ks) was calculated using the following equation.

$$Ks = \frac{1}{8} \frac{d_2^4 - d_1^4}{d_0^3 (t_2 - t_1)} \text{ (cm/sec)}$$

Ks = urea permeability constant in cm/sec

d_0 = initial diameter of protoplast

d_1 = diameter of protoplast at t_1

d_2 = diameter of protoplast at t_2

t_1 = time of the measurement.

t_2=time of the measurement.

2) Effects of BSA and DTT

After protoplast isolation in the 2% cellulase solution containing 0.05% BSA or 5 mM DTT, urea permeability was measured in the same way as 3-1).

Results and Discussion

1. Protoplast isolation

Protoplast viability and yield of bulb tissue under the four different enzyme treatments were shown in Fig.1.

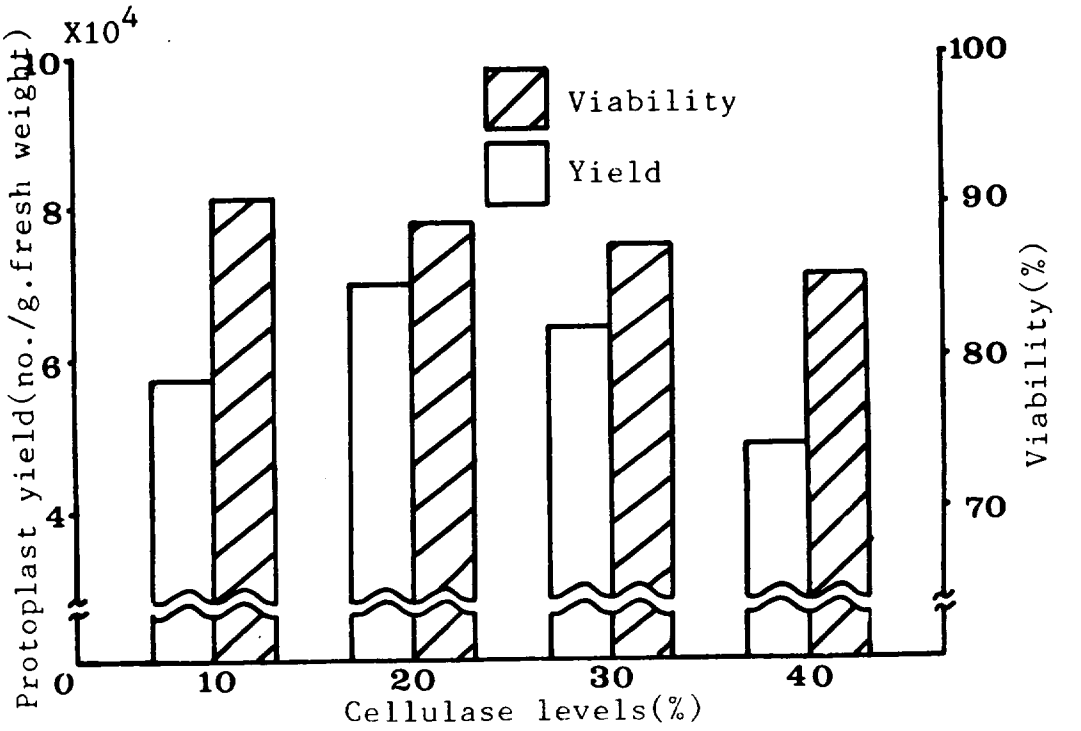

Fig. 1 Changes of protoplast viability and yield according to cellulase levels in the epidermal cells of *Allium cepa* bulb scale.

Highest protoplast yield was obtained (7×10^4 protoplasts/g. fresh weight) at the 2% cellulase concentration. The protoplast yield was decreased at higher cellulase concentration, presumably because of more toxic effects of cellulase. Also, protoplast viability was highest(90.7%), at 1% cellulase. Also, protoplast viability was highest(90.7%) at 1% cellulase, and as cellulase concentration increased, protoplast viability decreased.

2. Cytoplasmic streaming rate measurement

1) Effects of cellulase concentrations

The average speed of cytoplasmic streaming measured with *Allium cepa* bulb scale epidermal cells was 6.5 μm/sec in control and did not change for 3 hours (Fig 2). Higher cellulase concentration decreased streaming rate much more as time elapsed.

2) Effects of BSA and DTT

Fig.3 shows relationships between BSA or DTT treatment and vitality. Protoplast vitality was increased by treating BSA or DTT at each cellulase concentration. In 1% cellulase solution containing BSA or DTT, streaming rate was increased. BSA and DTT was more effective and increased streaming rate by 8.5~14.0 μ m/sec in 4% cellulase solution.

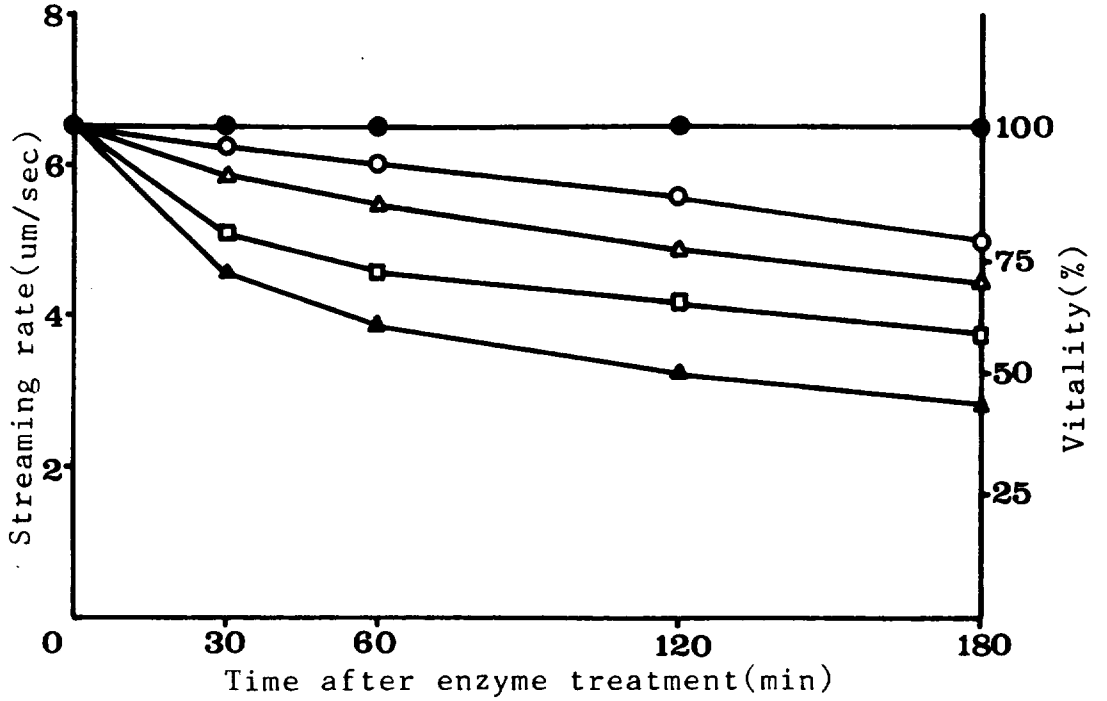

Fig. 2 Effects of cellulase levels on cytoplasmic streaming in the epidermal cells of *Allium cepa* bulb scale (●—● : control, ○—○ : 1% cellulase, △—△ : 2% cellulase, □—□ : 3% cellulase, ▲—▲ : 4% cellulase).

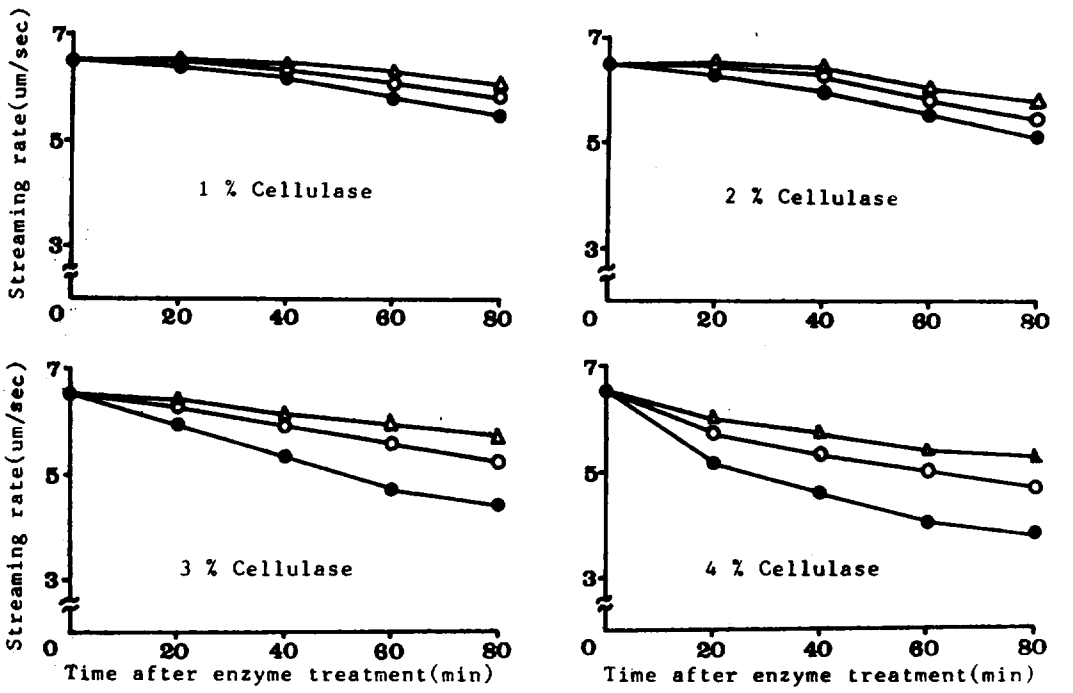

Fig. 3 Effects of BSA and DTT on cytoplasmic streaming in epidermal cells of *Allium cepa* bulb scale (●—● : control, △—△ : 5 mM DTT, ○—○ : 0.05% BSA).

3) Effects of NaN_3 and KCN

The treatment of NaN_3 or KCN for 60 min. almost stopped cytoplasmic streaming in 1 hour. This inhibition was fully reversible and cytoplasmic streaming resumed in 5 min. when water replaced the inhibitor soluton. In treated cells, streaming rate decreased to a half within 10 min. (Fig.4).

These effects of NaN_3 and KCN on cytoplasmic streaming were presumably due to ATP depletion (Hayashi 1960), acidification (Tazawa and Shimmen 1982) and/or free calcium (Williamson 1975).

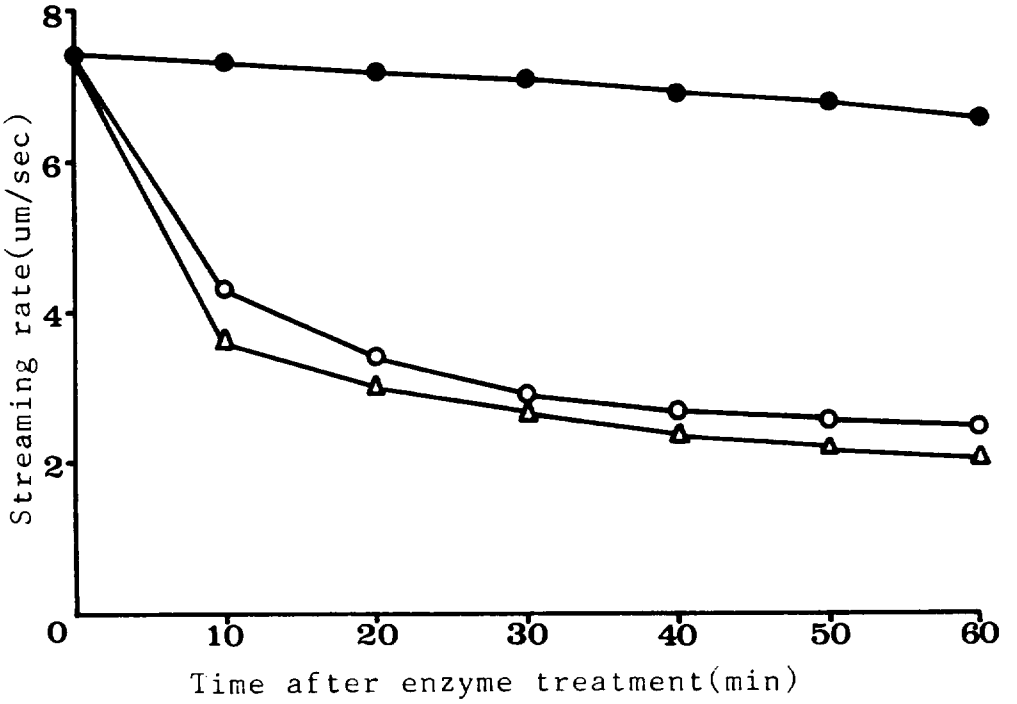

Fig. 4 Effects of NaN_3 and KCN on cytoplasmic streaming in the epidermal cells of *Allium cepa* bulb scale (●—● : control, ○—○ : 10㎖ NaN_3, △—△ : 1 mM , KCN).

3. Urea permeability measurement

1) Effects of cellulase concentrations

As expected, plasmolysis itself was not affected with the cellulase pretreatment. However, significant increase in urea permeability was detected with the pretreatment of each cellulase concentration (Fig.5).

Length of protoplast increased by 12.8 % of the inital size in 4% cellulase solution. 20 min. after transferring into urea, protoplast size was not almost increased. By the pretreatment of each cellulase concentration, length of protoplast was more increased. It seemed that protoplast membrane was damaged by protease-type cellulase and membrane damage increased urea permeability.

2) Effects of BSA and DTT

Herth and Meyer(1977) reported that BSA protected protoplast membrane from

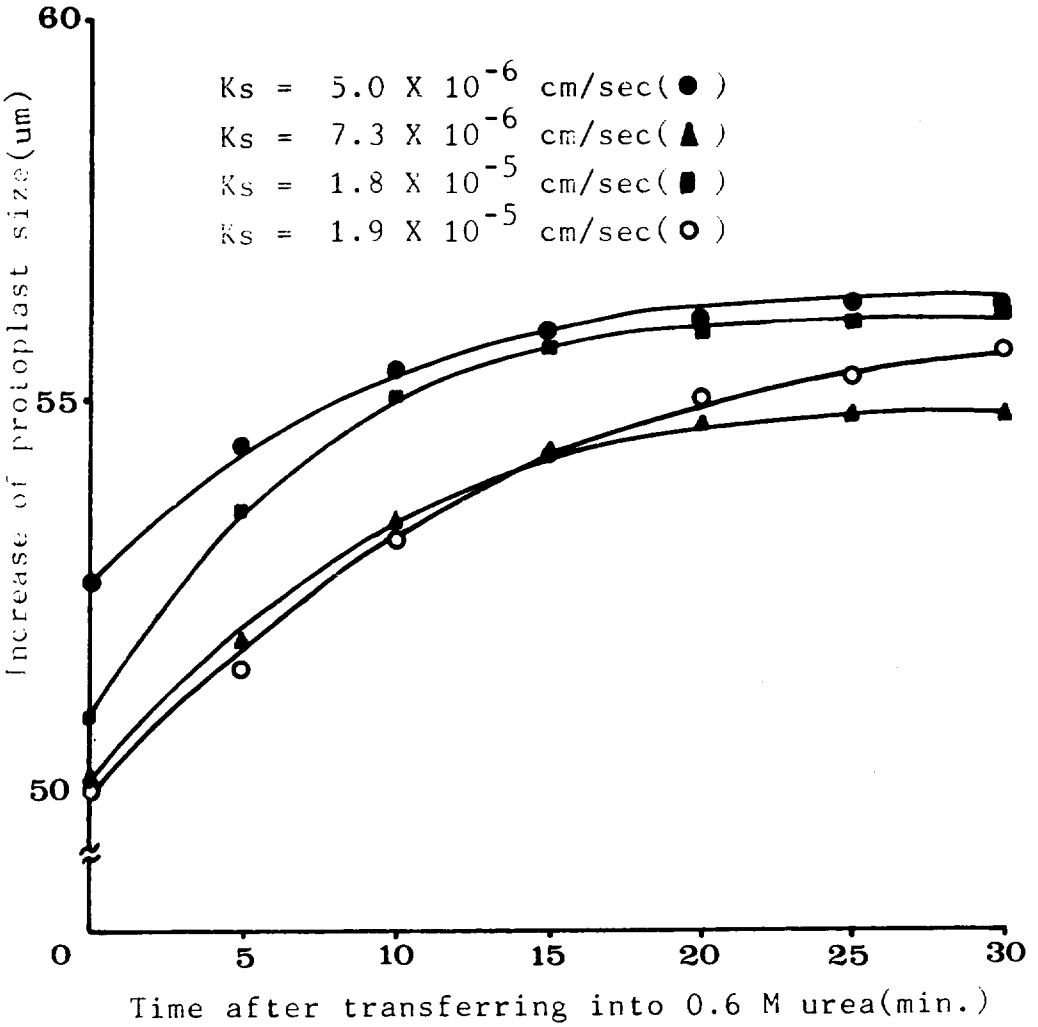

Fig. 5 Changes of protoplast size increased by urea permeability differences of *Allium cepa* bulb scale epidermal protoplasts treated 1% cellulase (●—●), 2% cellulase (△—△), 3% cellulase (□—□), 4% cellulase (○—○).

the damage which occurred from the action of protease-type enzyme.

DTT at low concentrations also altered the protease activity of cellulase. At 0.1 to 0.3 mM, DTT inhibited 10% to 20% by acting as a competitive substrate (Pilet 1985).

Fig.6 shows that urea permeability was affected by the pretreatment of 2% cellulase containing BSA or DTT. BSA

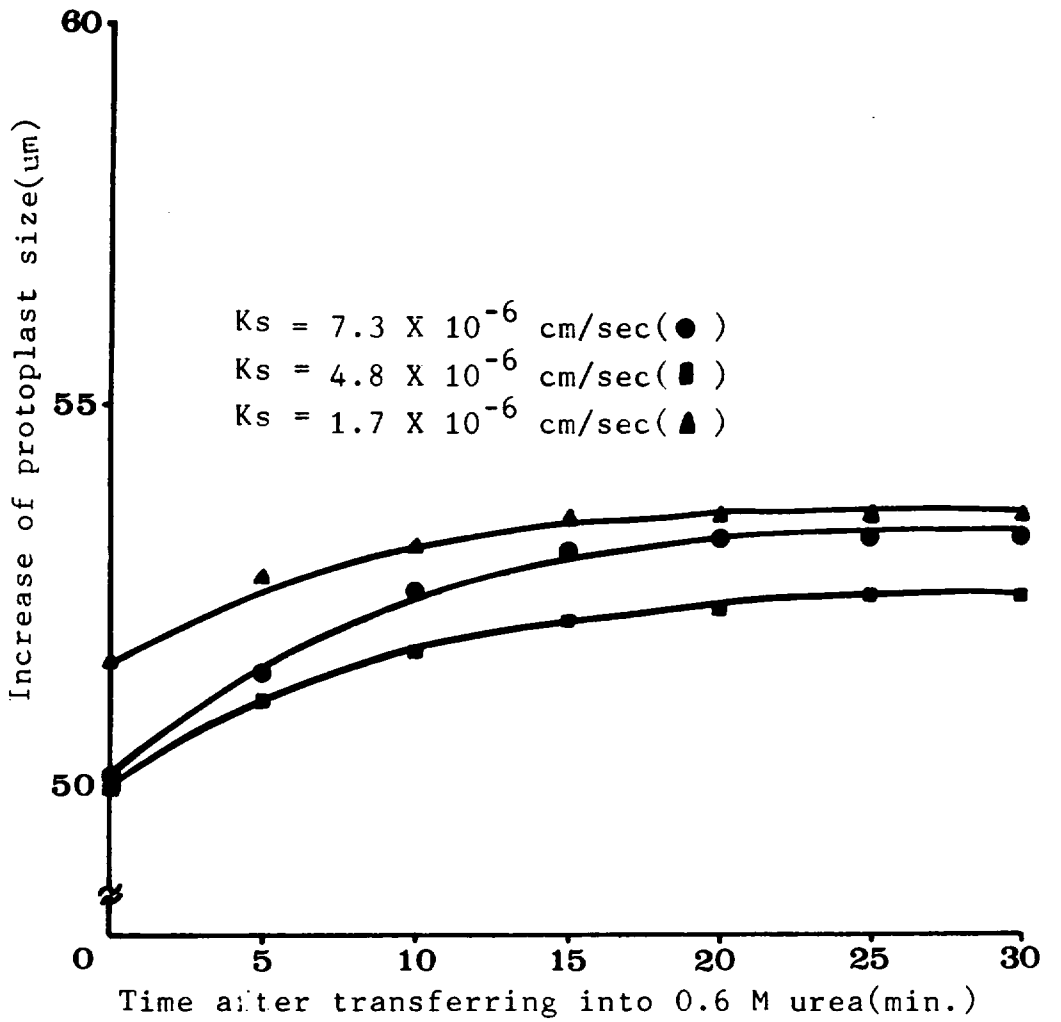

Fig. 6 Changes of protoplast size increased by urea permeability differences of *Allium cepa* bulb scale epidermal protoplasts treated with 2% cellulase (●—●), 2% cellulase containing 0.05% BSA (■—■), 2% celullase containing 5mM DTT (▲—▲).

or DTT decreased urea permeability considerably when they were treated during the permeation process (deplasmolysis). The increase of protoplast stopped at 20 min. after transferring into urea.

As Herth and Meyer(1977) reported, BSA and DTT protected membrane from the damage by protease-type enzyme and increased vitality of protoplasts.

Summary

To investigate vitality of protoplasts isolated from onion (*Allium cepa*) bulb cells, cytoplasmic streaming, urea premeability and FDA staining method were compared. Also, whether singie cells made by vibratome can produce more vitable protoplasts of soybean was examined with different levels of pectolyase and incubation times.

1. Protoplast yield was highest (7.4×10^4 protoplasts/g.fresh weight) at 2% cellulase and 1% macerozyme. And their viability decreased as cellulase concentrations increased.

2. The cytoplasmic streaming rate was 6.2 μm/sec in the control cell and did not change for 3 hrs.

Provided that the vitality of control is 100%, vitality at 1% or 2% cellulase treatment was 76.9% or 43.1%, and at BSA, DTT, NaN_3, or KCN was 82.9%, 93.8%, 37.9% or 31.8% respectively.

3. Urea permeability (Ks) at 1% cellulase or 4% cellulase treatment was 5.0×10^{-6} or 1.9×10^{-5} cm/sec, and urea permeability increased as cellulase concentrations increased.

When BSA or DTT was treated, urea permeability was 4.8×10^{-6} or 1.7×10^{-6} cm/sec, respectively.

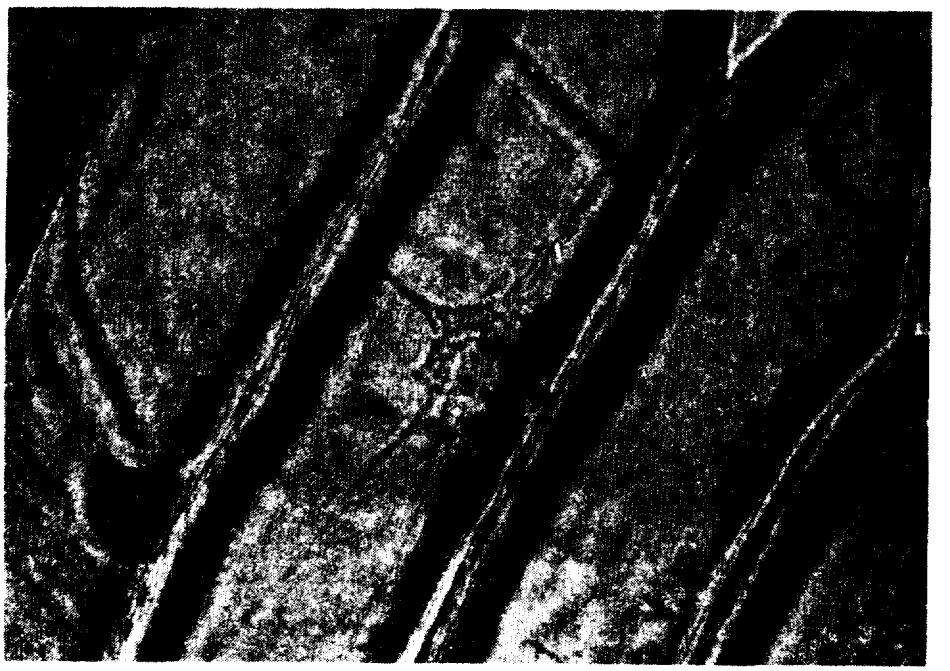

Plate. 1 Cytoplasmic streaming on the *Allium cepa* bulb scale epidermal cell. (MF : Microfilament, PA : Particle, CW : Cell wall).

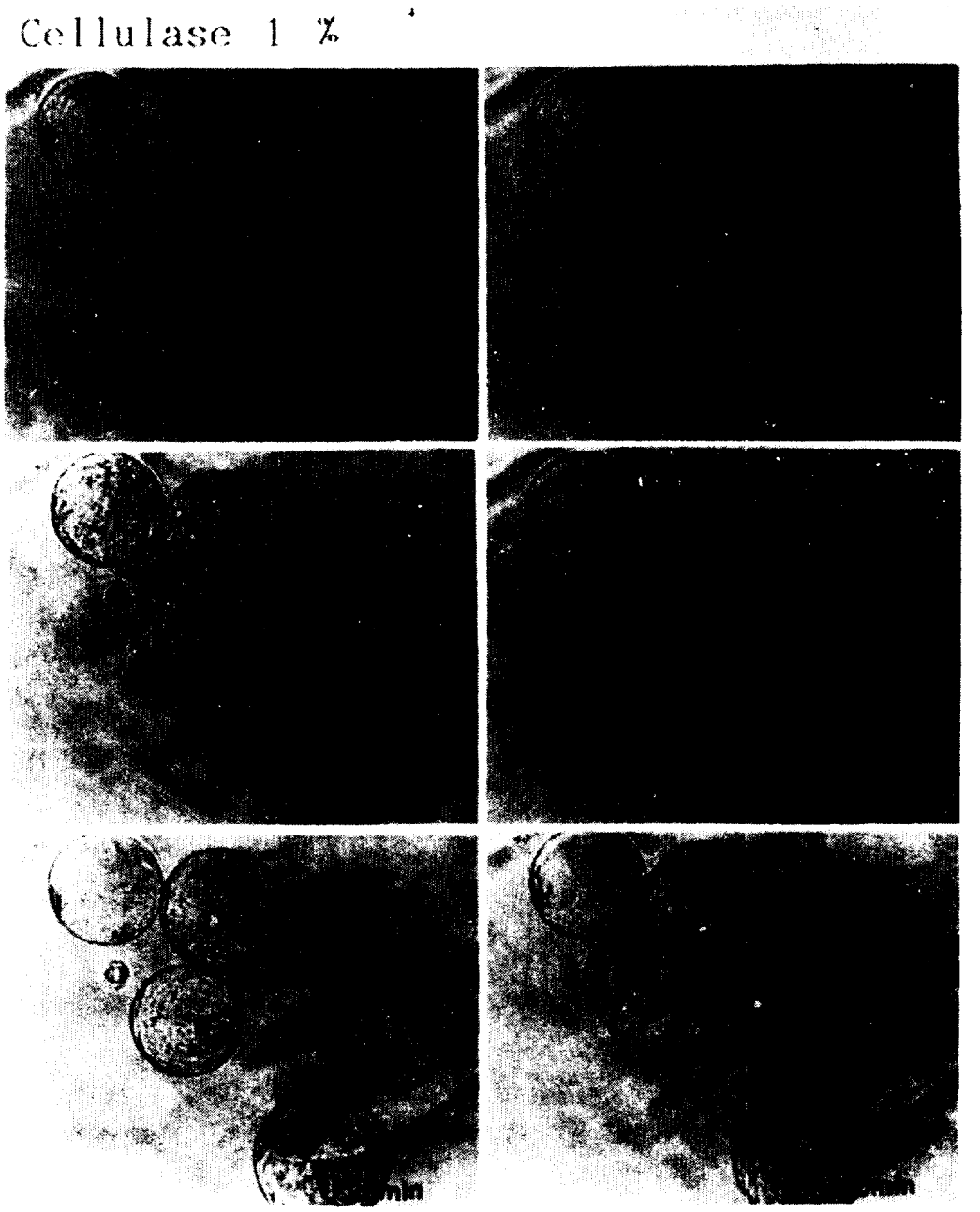

Plate. 2 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 1% cellulase. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

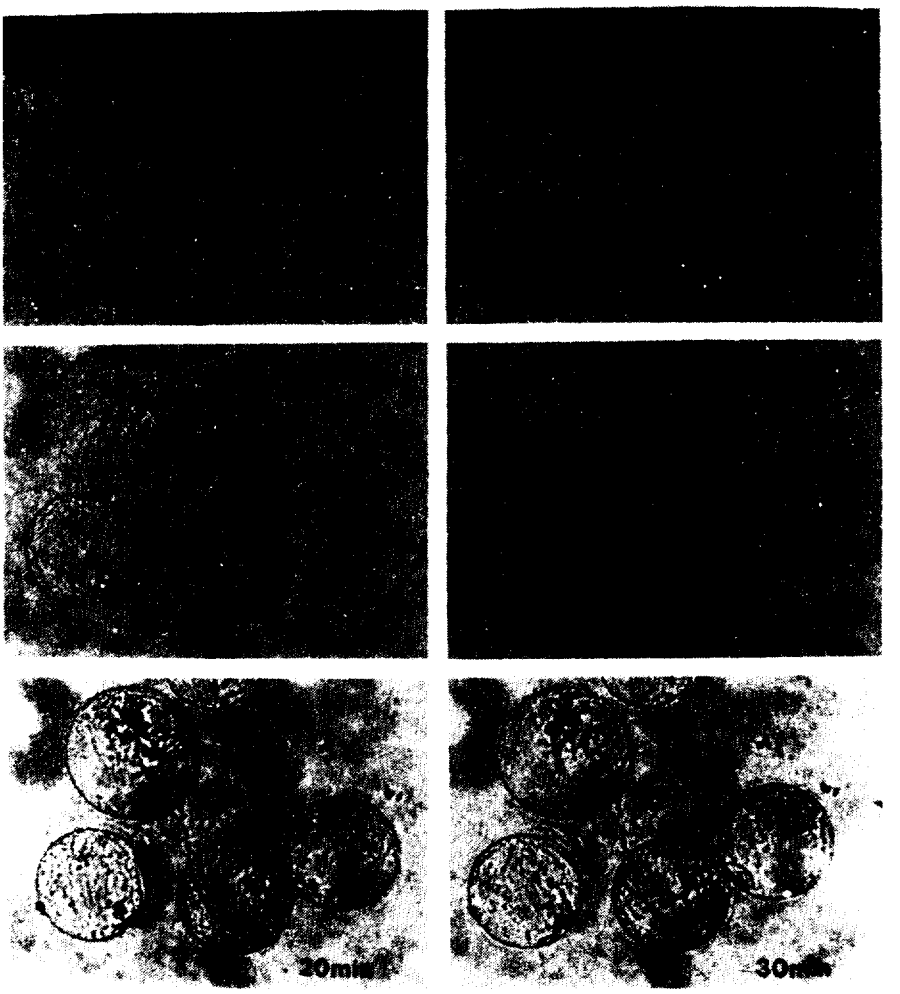

Plate. 3 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 2% cellulase. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

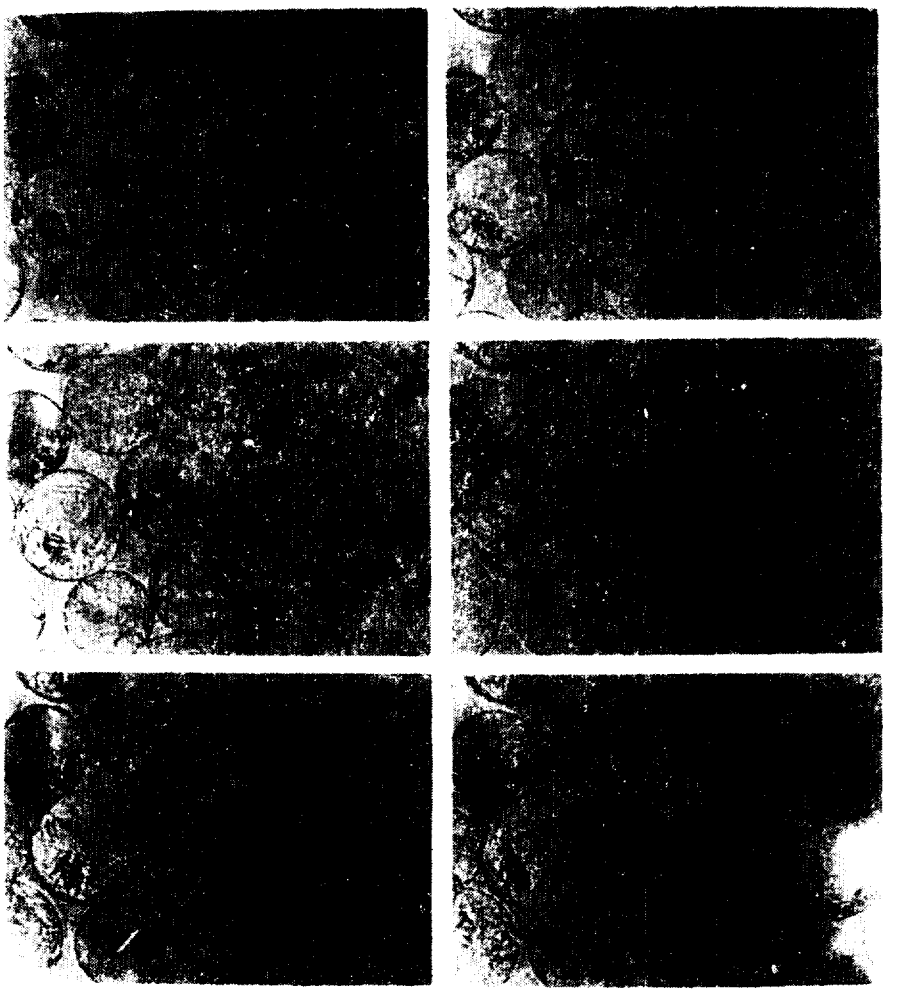

Plate. 4 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 3% cellulase. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

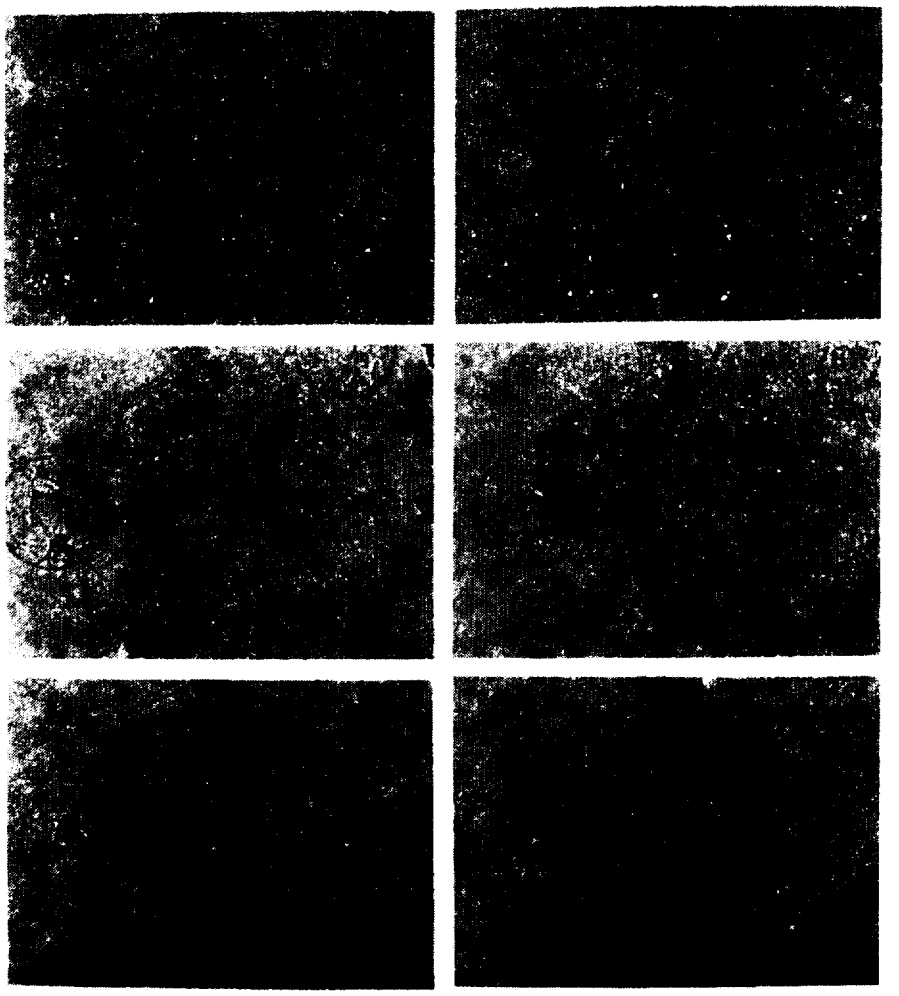

Plate. 5 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 3% cellulase. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

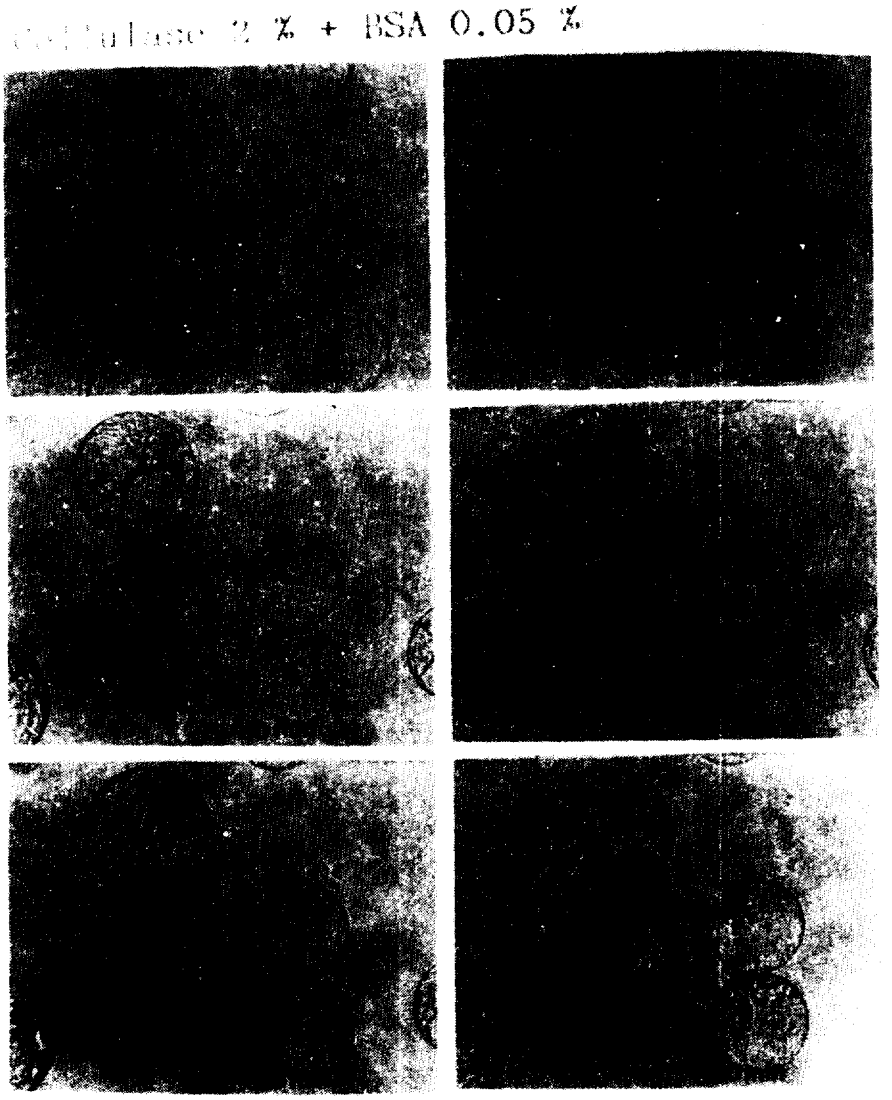

Plate. 6 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 2% cellulase+0.05% BSA. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

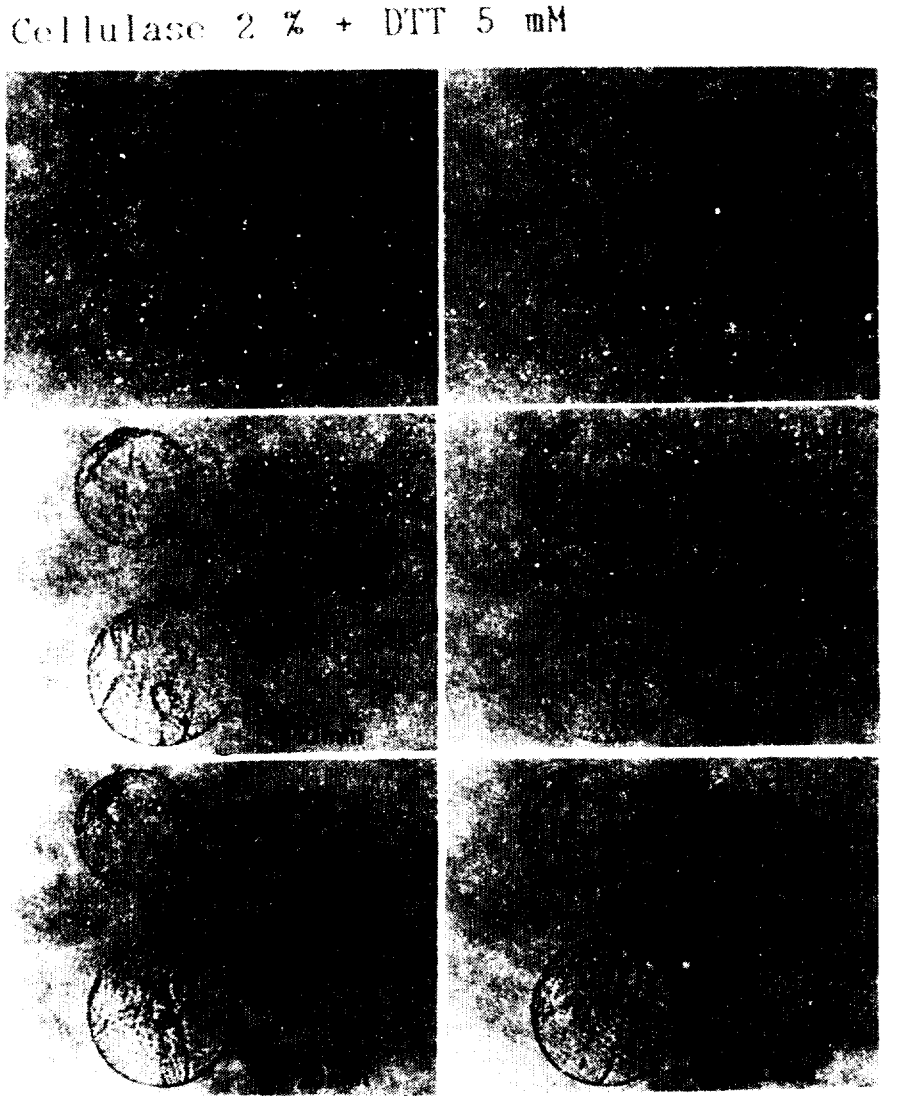

Plate. 7 Time course for urea permeability of protoplasts isolated from *Allium cepa* bulb scale epidermal cell by 2% cellulase+5mM DTT. (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min, (E) 20 min, (F) 30 min after transfer into urea solution.

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