A Thesis

for the Degree of Master of Science

IMPROVEMENT OF PROTOPLAST VITALITY OF ONION (*Allium cepa* L.) AND SOYBEAN (*Glycine max* Merr.)

by

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DEPARTMENT OF AGRICULTURAL CHEMISTRY

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

1989. 12.

양파 및 大豆의 原形質體 活性 增進 方法

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吳 成 國

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(Supervised by Professor Zang-Kual U.)

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Contents

要 約

양파 (*Allium cepa*) 細胞에서 分離한 原形質體의 vitality를 cytoplasmic streaming 과 urea permeability 로 測定하고 FDA 法에 의해 viability 를 測定한 結果와 比較했다. 한편, 大豆 (*Glycine max*) 葉에서는 原形質體의 活性을 增進시키기 위해서 vibratome 을 利用하여 single cell 分離條件을 確立하고 이로부터 分離한 原形質體의 vitality 에 關해 檢討했다.

1. 양파에서는 2 % cellulase 와 1 % macerozyme 을 處理했을 때 가장 좋은 原形質體 收率 (7 x 10^4 protoplasts/g.fresh weight) 을 얻었는데 生存率은 酵素 濃度가 增加할수록 減少했다.
2. Cellulase 를 濃度別 (1 %, 2 %, 3 %, 4 %) 로 處理한 細胞의 cytoplasmic streaming 速度는 對照區에서 6.5 um/sec (100 %) 로 3 時間 동안 변하지 않았고 1 % cellulase 에서는 vitality 가 76.9 % 그리고 4 % cellulase 에서는 43.1 % 로 그 差異가 33.8 % (76.9 - 43.1) 이었는데 이 값은 FDA 로 測定해서 얻은 6.8 % 보다 差異 더 크기때문에 細密한 vitality 를 測定할 수 있다고 생각된다.

   2 % cellulase 만을 處理 (70 %) 했을 때 보다 BSA 또는 DTT 를 함께 處理했을 때 vitality 는 각각 89.2 %, 93.8 % 로 增加했고, 한편 NaN_3 나 KCN 을 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 permeability 를 測定한 結果 酵素濃度가 增加할 수록 urea permeability 가 增加했다.

   BSA 또는 DTT 를 處理했을 때 對照區에 比해서 urea permeability 가 減少했다. 以上의 結果로 보아 cellulase 處理에 의한 膜損傷은 urea

permeability 의 受動的 吸收를 增加시키는 것으로 생각된다.

4. Vibratome 으로 大豆잎을 잘라서 single cell 을 얻은 結果 1.5×10^7 cells/g.fresh weight 의 收率을 얻었다.

5. 葉組織에서 직접얻은 原形質體보다 single cell 에서 얻은 原形質體의 生存率이 더 컷는데 이것은 處理한 pectolyase 의 水準 (0.1, 0.3, 0.5 %) 에 關係없이 같은 傾向이었다.

   Single cell 의 경우 收率은 0.3 % pectolyase 에서 4.7×10^5 protoplasts/g.cells 이었고 葉組織의 경우는 0.5 % pectolyase 에서 1.7×10^5 protoplasts/g.fresh weight 이었다.

6. Pectolyase 의 濃度를 single cell 에서는 0.3 %, 葉組織에서는 0.5 % 로 固定하고 酵素處理 時間別로 原形質體의 收率과 生存率을 調査한 結果 single cell 은 4 時間 (4.7×10^5 protoplasts/g.cells) 그리고 葉組織은 10 時間 (1.7×10^5 protoplasts/g.fresh weight) 에서 가장 좋은 收率을 얻었다.

I. Introduction

The viable protoplasts 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 (Drawert 1968), cytoplasmic streaming (Kamiya 1959), plasmolysis (Collander 1959), 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 (Osterhout 1914) 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 (*Allium cepa*), and to examine the possibility of improving soybean (*Glycine max*) protoplast viability by the single

cell mothod using vibratome. Effects of enzyme concentrations, incubation times, BSA and DTT were also discussed.

II. Materials and Methods

Exp.I. Comparison of vitality measurement methods

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

1. 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 X 0.5 mm were prepared on the inner surface. The scale was then infiltrated three times with 100 ml 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 um), epidermal cells of 200 mg fresh weight were floated on 5 ml enzyme solution. The enzyme mixture was incubated for 3 hours at 25 °C 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 um. The protoplasts passed through the filter, whereas the cell aggregates did not. The filterate was

transferred to a centrifuge tube and it was spinned at 50xg for 5 min. The debris in the supernatant were carefully removed with a pipette. The remainder was centrifuged at 50xg for 5 min. and suspended in the

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	10 mM
HEPES[c]	10 mM
Sorbitol	0.6 M
pH	5.8

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

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 50xg for 5 min. in order to separate the contaminated debris. The washing process was repeated three times.

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 %.

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

Salts used	Concentration (㎎/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 : 0.6 M sorbitol solution containing the above salt mixture.

Sucrose CPW : 0.6 M sucrose solution containing the above salt mixture.

Urea CPW : 0.6 M urea solution containing the above salt mixture.

pH 5.8 in all cases

After 5 ㎩in. at room temperature the protoplasts were examined for fluorescence using the Inverted Microscope (Nikon Diaphot - TMD). The exciter filter BG12 (Transmission : 330 - 500 ㎚) and the barrier filter (Transmission : > 460 ㎚) were used. Protoplast

viability (%) was calculated as follows ;

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

2. 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 Chemica Co., Belgium)

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

3) Effects of NaN_3 and KCN

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

3. 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 ml 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.

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

Ks = urea permeability constant in cm/sec

d_o = 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).

Exp.2. Vitality improvement by mechanical isolation of single cell

Soybean seeds (*Glycine max*, M. BAEKCHUN) were obtained from Cheju Provincial Rural Development. The seed surface was steriled for 15 min. in 0.5 % sodium hypochlorite containing a few drops of Tween 20 and completely rinsed in tap water. Soybean seeds were germinated and

cultured in pots containing sand and subirrigated with 1/4 MS salts solution (pH 6.5). The cultivation conditions were a 16-h photoperiod (25 °C in day and 16 °C in night), 60 - 70 RH., and 2500 - 3000 lux of fluorescent light (General Electric Powergroove) with additional incandescent lamps.

The first trifoliolate leaf of 4 to 5 week-old plant was selected for experiments.

The soybean leaf was rinsed with tap water and the midribs were removed. Leaves were cut with a razor blade (1 X 3 cm). The pieces were placed in a stryofoam block (1 X 1 cm), mounted on a vibrating microtome (Vibratome, Series 1000, American Scientific Product) and cross - sectioned using a razor blade. The sections were 50 - 100 um thick and transferred into a petri dish (diameter : 5 cm) containing 2 ml of tap water. After transferring it into a 10 ml test tube and adding 3 ml of tap water, the mixture was shaken on a Vortex Mixer (Model : C - MF) for 10 seconds at top speed three times. The mixture was filtered through a 70 um nylon cloth. The filterate was centrifuged at 50xg for 5 min. and suspended with the sucrose CPW solution. After centrifugation at 50xg for 5 min., single cells were collected at the bottom of centrifuge tube.

1. Effects of pectolyase on protoplast yield and viability

The sliced leaves (2 mm X 2 mm wide strips) and single cells mechanically isolated by the vibratome were floated on the different enzyme solutions combined with pectolyase Y - 23 (0.1, 0.3, 0.5 %)

and 2 % cellulase Onozuka RS (Table 3).

After 4 or 10 hours respectively, the enzyme mixtures were sieved through a 70 um nylon cloth and the filterates were collected. The protoplasts were then washed and purified as Exp.1 - 1). The protoplast yield was counted using a heamocytometer and viability count was made using the FDA technique of Larkin.

Table 3. Composition of enzyme solution for isolating *Glycine max* mesophyll protoplasts.

Constituent	Concentration
Cellulase Onozuka RS[a]	2 %
Pectolyase[b]	0.3 %* , 0.5 %**
Calcium chloride	10 mM
HEPES	10 mM
Sorbitol	0.6 M
pH	5.8

* : for single cell
** : for sliced leaf tissue
a : Yakult Honsha Co., Ltd.Japan
b : Sigma Chemical Co., U.S.A.

2. Effects of incubation time on protoplast yield and viability

Isolated single cells and sliced fresh leaves were incubated in the enzyme solutions for 2, 4, 6, 8, 10, and 12 hours. After isolating protoplasts, yield and viability of protoplasts were determined using a heamocytometer and FDA technique.

III. Results and discussion

Exp.1. Comparison of vitality measuring methods

1. Protoplast isolation

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

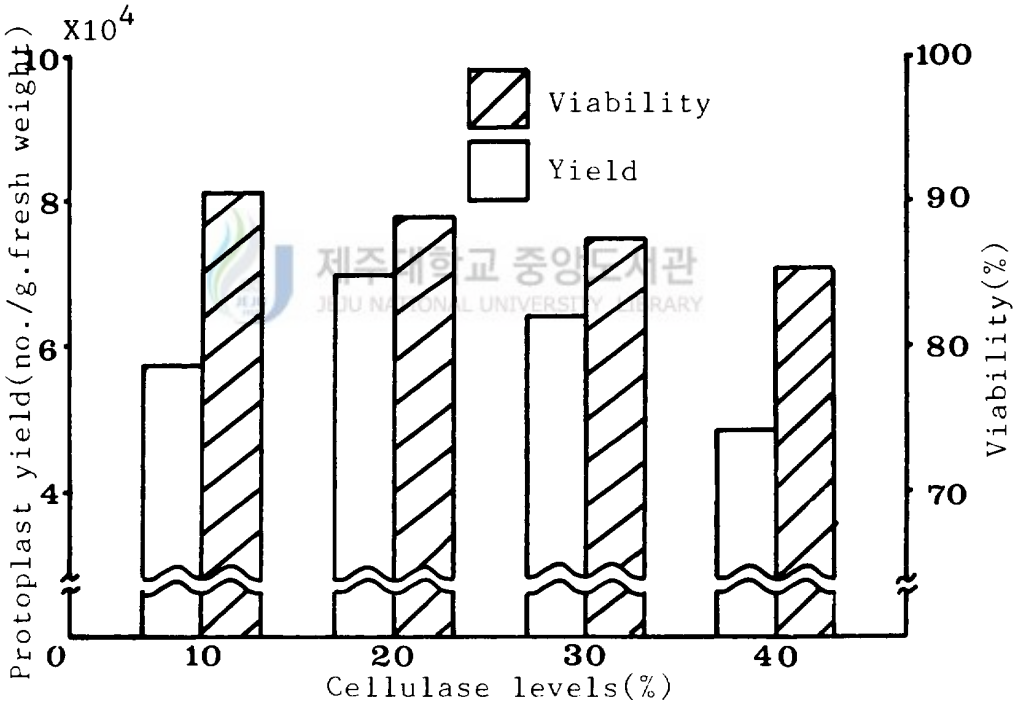

Fig.1. Changes of protoplast viability and yield according to cellulase levels in the epidermal cells of *Allium cepa* bulb scale.

Highest protoplast yield was obtained (7 x 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, and as cellulase concentration increased, protoplast viability decreased.

2. Cytoplasmic streaming rate measurement

1) Effects of cellulase concentrations

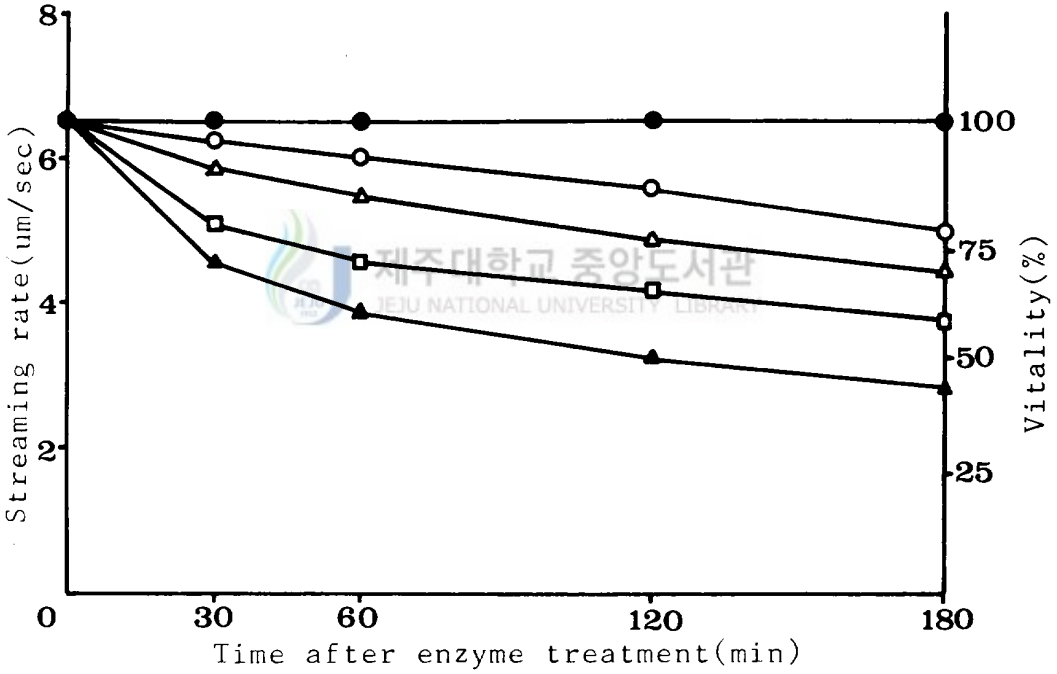

Fig.2. Effects of cellulase levels on cytoplasmic streaming in the epidermal cells of *Allium cepa* bulb scale (●—● : control, ○—○ : 1 % cellulase, △—△ : 2 % cellulase, □—□ : 3 % cellulase, ▲—▲ : 4 % cellulase).

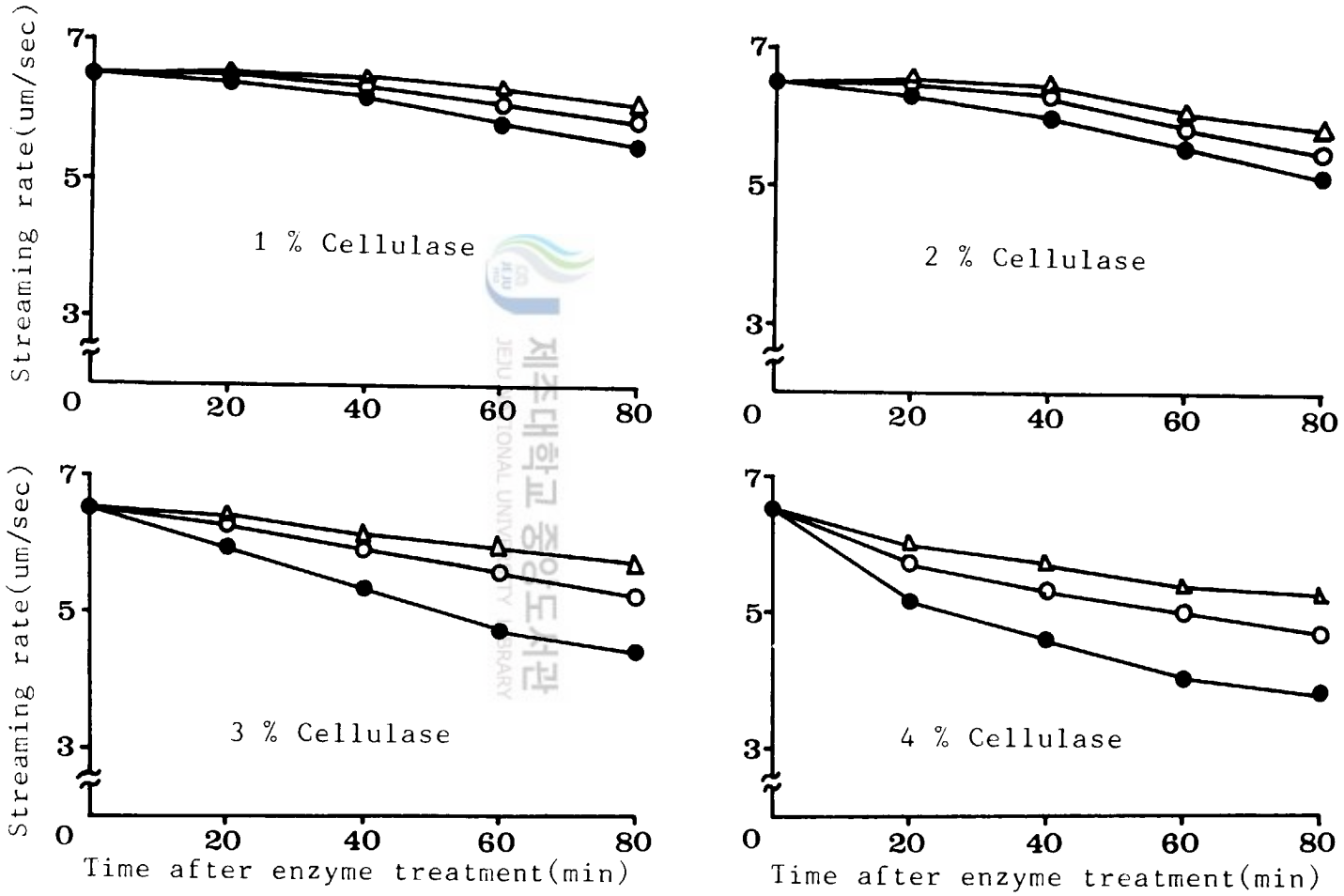

Fig.3. Effects of BSA and DTT on cytoplasmic streaming in epidermal cells of *Allium cepa* bulb scale (●—● : control, △—△: 5 mM DTT, ○—○ : 0.05 % BSA).

The average speed of cytoplasmic streaming measured with *Allium cepa* bulb scale epidermal cells was 6.5 um/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

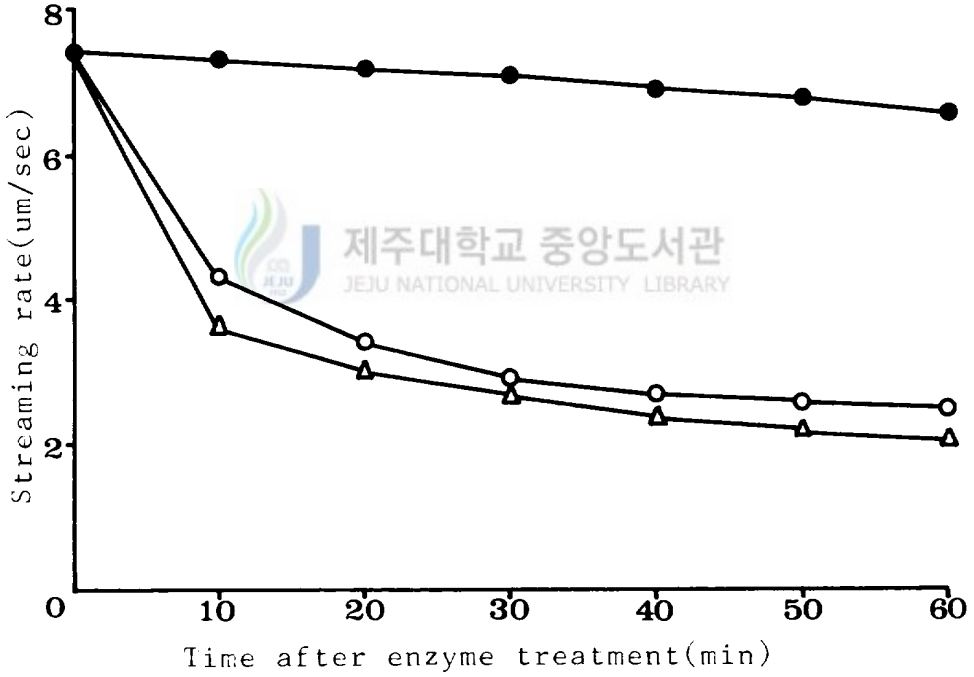

Fig.4. Effects of NaN_3 and KCN on cytoplasmic streaming in the epidermal cells of *Allium cepa* bulb scale (●—● : control, O—O : 10 ml NaN_3, Δ—Δ : 1 mM KCN).

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 um/sec in 4 % cellulase solution.

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 solution. 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).

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 initial 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

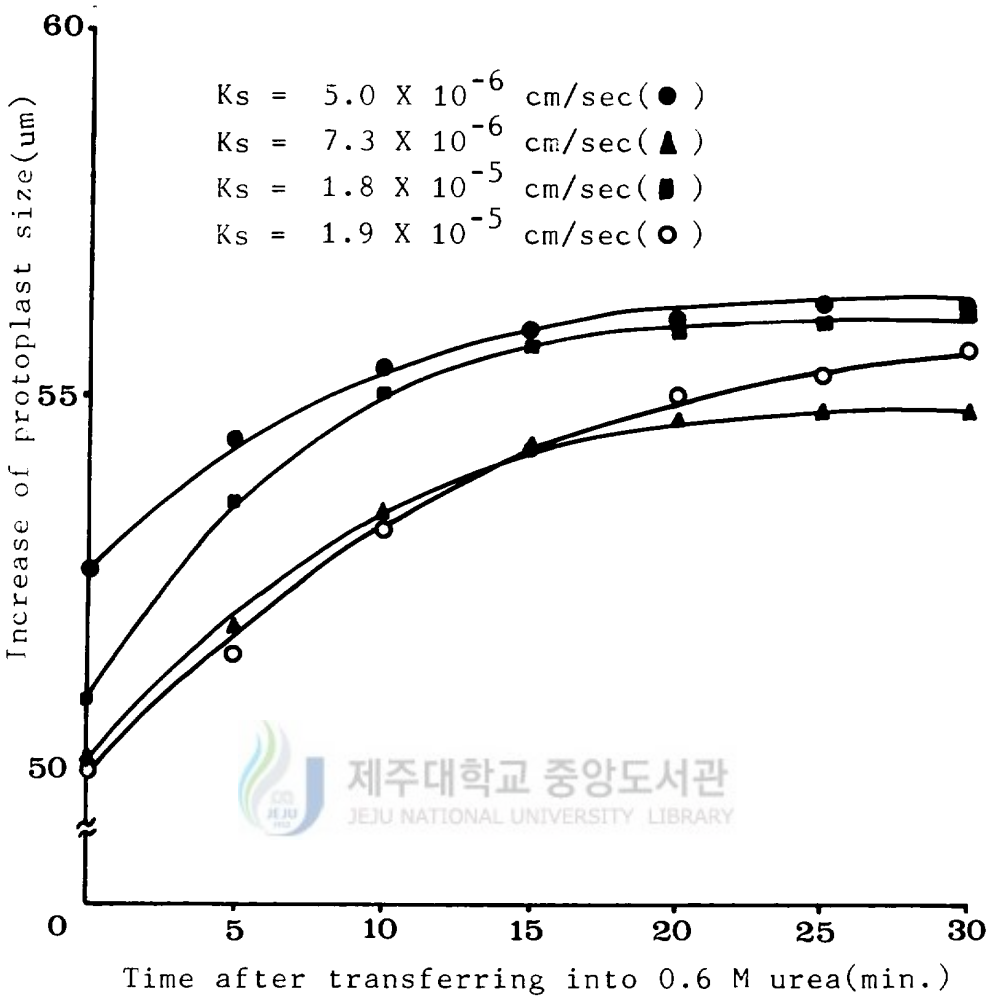

Fig.5. Changes of protoplast size increased by urea permeability differences of *Allium cepa* bulb scale epidermal protoplasts treated with 1 % cellulase (●—●), 2 % cellulase (△—△), 3 % cellulase (□—□), 4 % cellulase (○—○).

protoplast membrane was damaged by protease-type cellulase and membrane damage increased urea permeability.

2) Effects of BSA and DTT

Branton (1971) reported that BSA protected protoplast membrane from the damage which occurred from the action of protease-type enzyme.

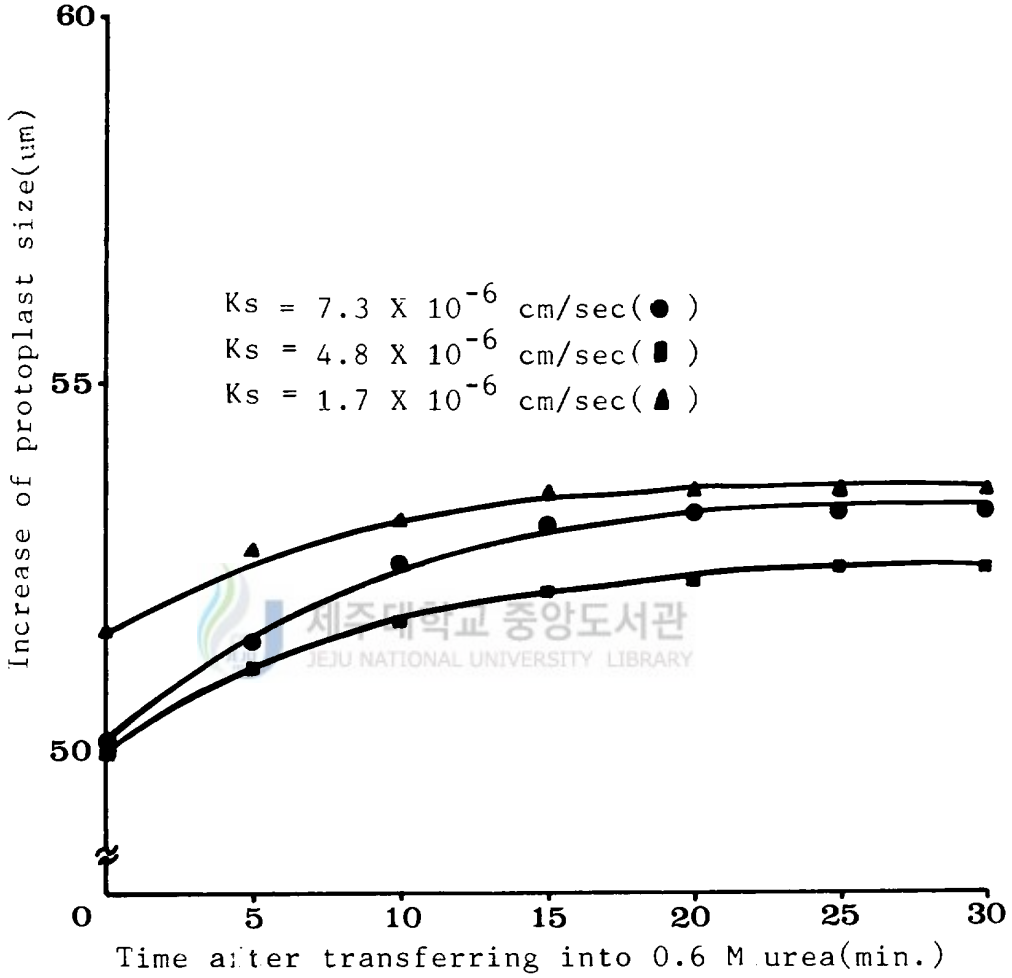

Fig.6. Changes of protoplast size increased by urea permeability differences of *Allium cepa* bulb scale epidermal protoplasts treated with 2 % cellulase (●—●), 2 % cellulase containing 0.05 % BSA (■—■), 2 % cellulase containing 5 mM DTT (▲—▲).

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

As Branton reported, BSA and DTT protected membrane from the damage by protease-type enzyme and increased vitality of protoplasts.

Exp.2. Vitality improvement by mechanical single cell isolation

1. Effects of pectolyase on protoplast yield and viability

The results of the enzyme application to the trifoliolate leaf were remarkable. The numbers of protoplasts released per gram of the trifoliolate leaf under three different enzyme treatments were shown in Fig.8. Protoplast viability was shown in Fig.7.

At each concentration of pectolyase, the protoplasts isolated from single cells showed higher viability than that from the leaf. And as pectolyase concentration increased, viability was decreased and highest at 0.1 % pectolyase (94.5 %). Single cells gave higher protoplast yield than sliced leaf tissues. When 0.3 % pectolyase was treated, protoplast yield isolated single cells was highest (4.7 x

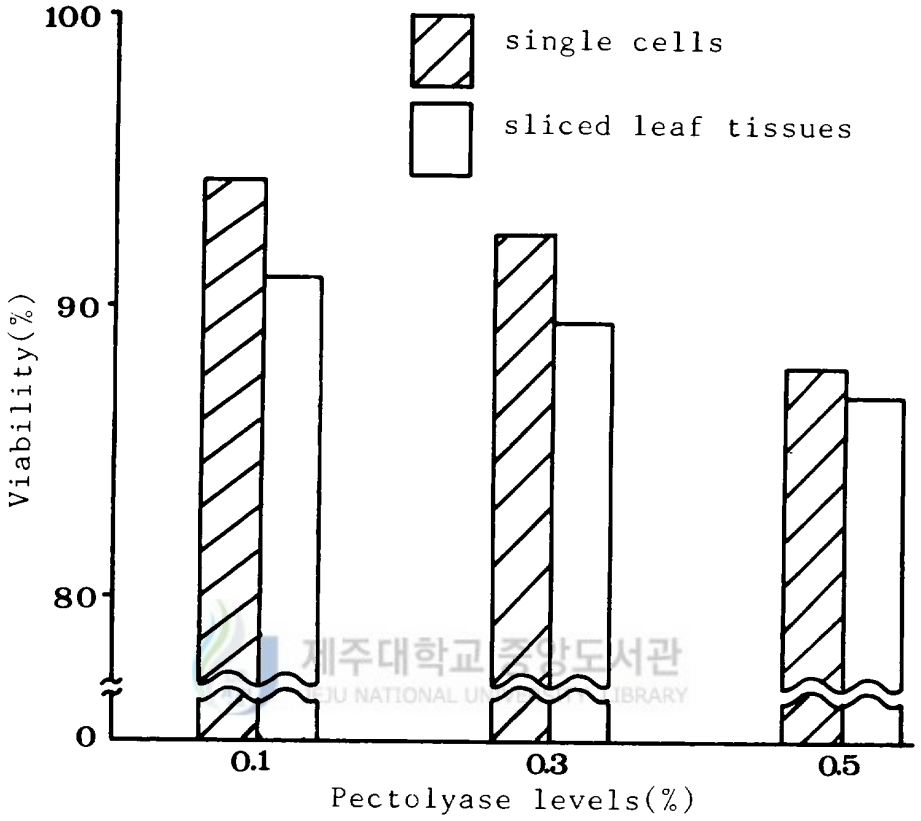

Fig.7. Comparison of protoplast viability when the different levels of pectolyase were treated to the single cells mechanically isolated by vibratome and to the sliced leaf tissues directly.

10^5 protoplasts/g.cells).

Fowke (1985) reported that the pectinase dissolved the middle lamella and in this way separated the cell from each other.

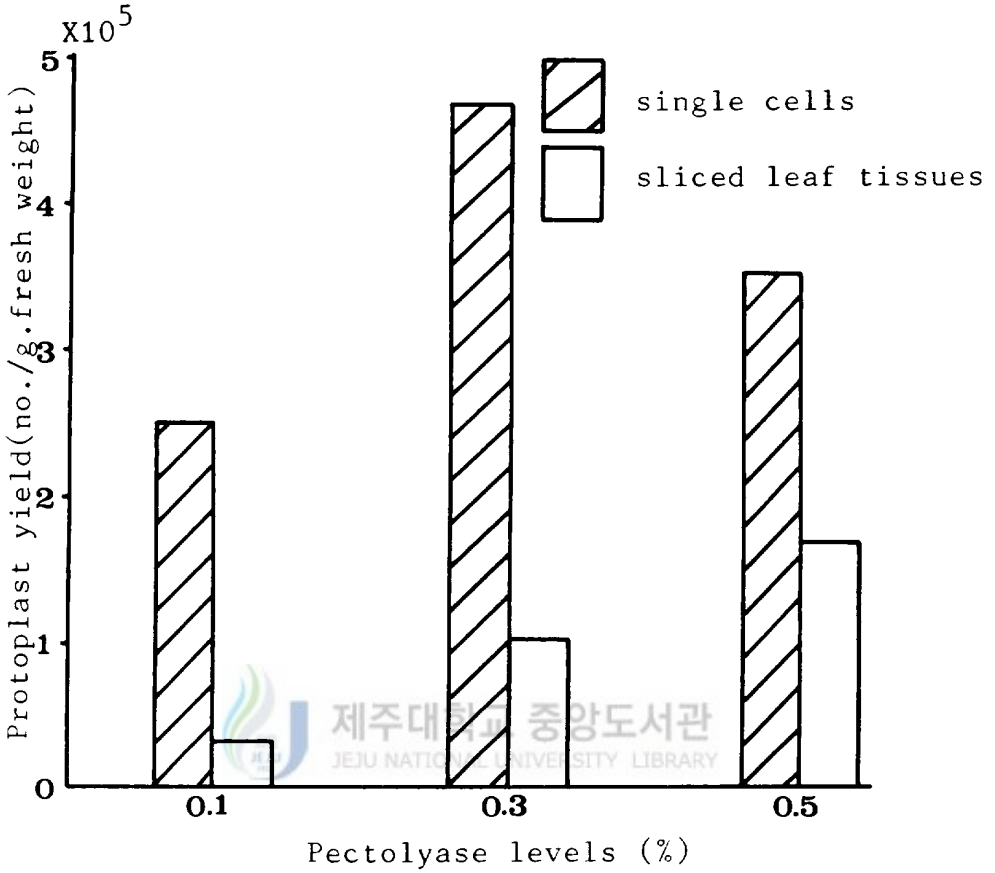

Fig.8. Comparison of protoplast yield when the different levels of pectolyase were treated to the single cells mechanically isolated by vibratome and to the sliced leaf tissues directly.

Especially, pectolyase was effective in releasing protoplasts of soybean leaves when mixed with cellulase RS.

2. Effects of incubation time on protoplast yield and viability

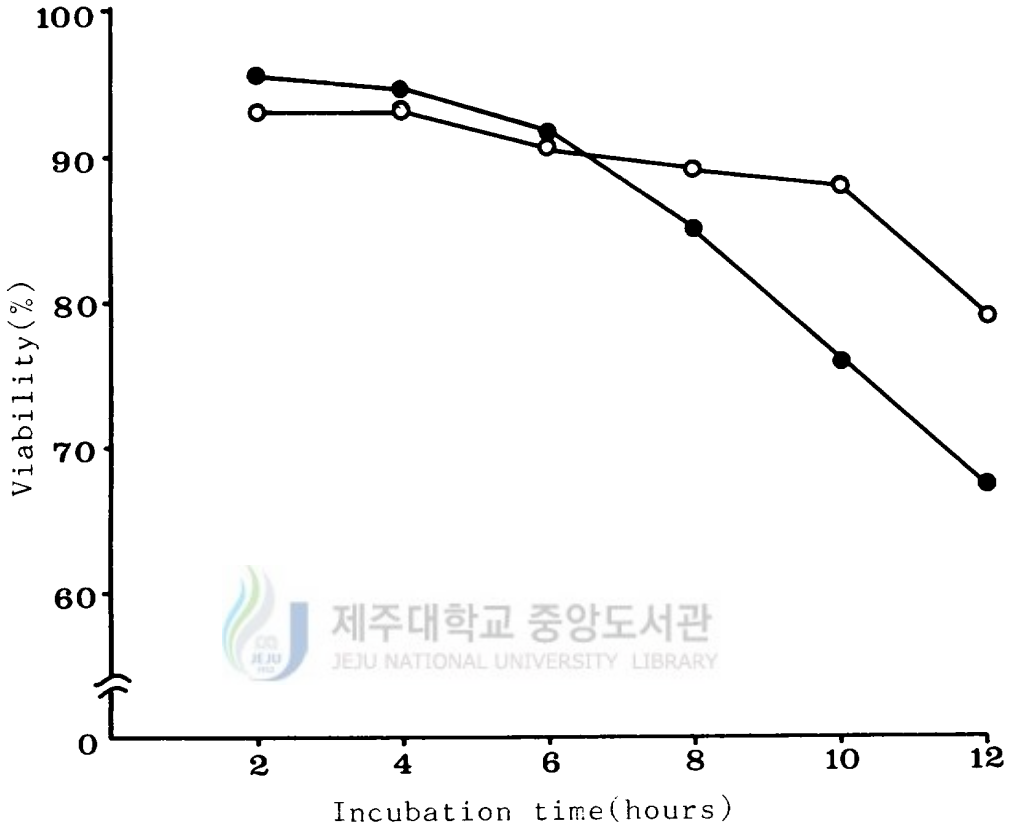

Fig.9. Changes of protoplast viability according to incubation time when single cells (●—●) were treated with 0.3 % pectolyase and sliced leaf tissues (O—O) with 0.5 % pectolyase.

The relationship between the protoplast viabilities from single cell and from leaf tissue was shown in Fig.9. Protoplast viability was highest at 2 hours (95.5 %). As time elapsed, viability decreased.

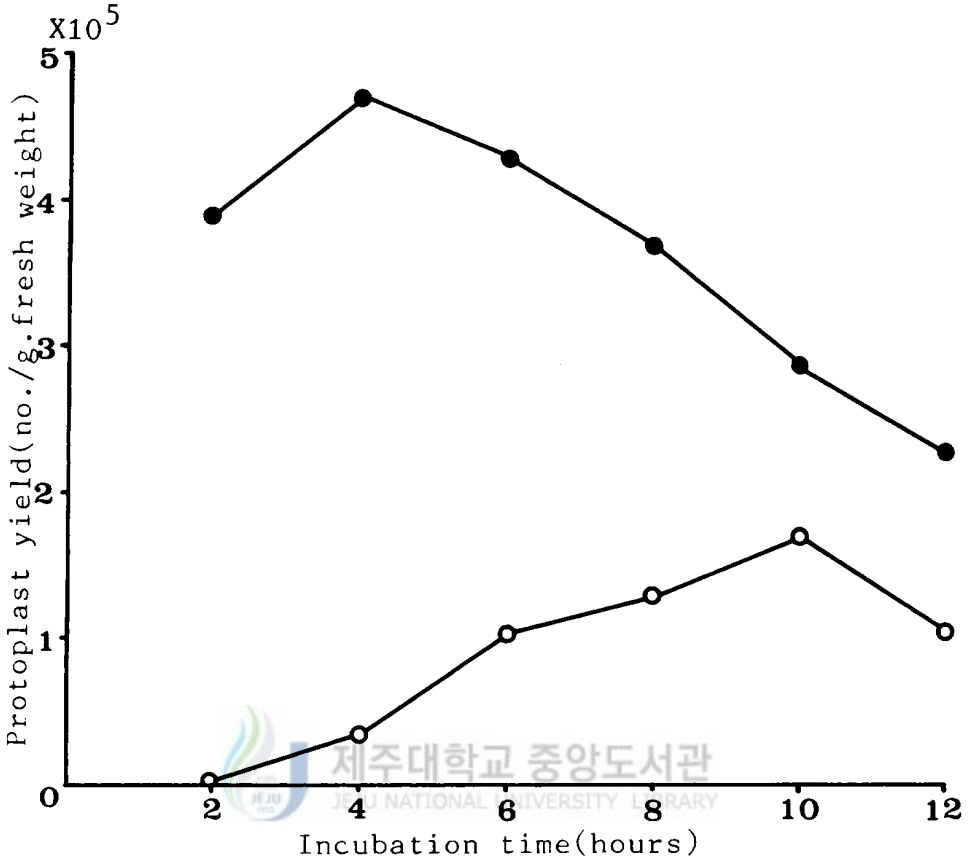

Fig.10. Changes of protoplast yield according to incubation time when the single cells (●—●) were treated with 0.3 % pectolyase and the sliced leaf tissues (O—O) 0.5 % pectolyase.

And in the protoplast isolated from single cell, viability was rapidly decreased after 4 hours. The protoplasts directly isolated from leaf tissue gave the highest viability value (93 %). Viability was slowly decreased after 2 hours and rapidly after 10 hours.

Fig.10 shows the relationships between incubation time and

protoplast yield. The yield of protoplasts isolated from single cells was 4.7 x 10^5 protoplasts/g.cells and from leaf tissue 1.7 x 10^5 protoplasts/g.fresh weight at 10 hours.

Summary

To investigate vitality of protoplasts isolated from onion (*Allium cepa*) bulb cells, cytoplasmic streaming, urea permeability and FDA staining method were compared. Also, whether single 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 x 10^4 protoplasts/g.fresh weight) at 2 % cellulase and 1 % macerozyme. And viability decreased as cellulase concentrations increased.
2. The cytoplasmic streaming rate was 6.2 um/sec in the control cell and did not change for 3 hours.
   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 X 10^{-6} or 1.9 X 10^{-5} cm/sec, and urea permeability increased as cellulase concentrations increased.
   When BSA or DTT was treated, urea permeability was 4.8 X 10^{-6} or 1.7 X 10^{-6} cm/sec, respectively.
4. Single cell yield isolated from soybean leaf tissue by vibratome was 1.5 x 10^7 cells/g.fresh weight.
5. Viability of protoplast isolated from single cells was higher than that from sliced leaf tissue at each pectolyase level.

Protoplast yield from single cells was highest (4.7 x 10^5 protoplasts/g.cells) at 0.3 % pectolyase while protoplast yield from sliced leaf tissues was highest (1.7 x 10^5 protoplasts/g.fresh weight) at 0.5 % pectolyase.

6. Optimum incubation times for the highest protoplast yield were 4 hours in case of single cells and 10 hours in sliced leaf tissues.

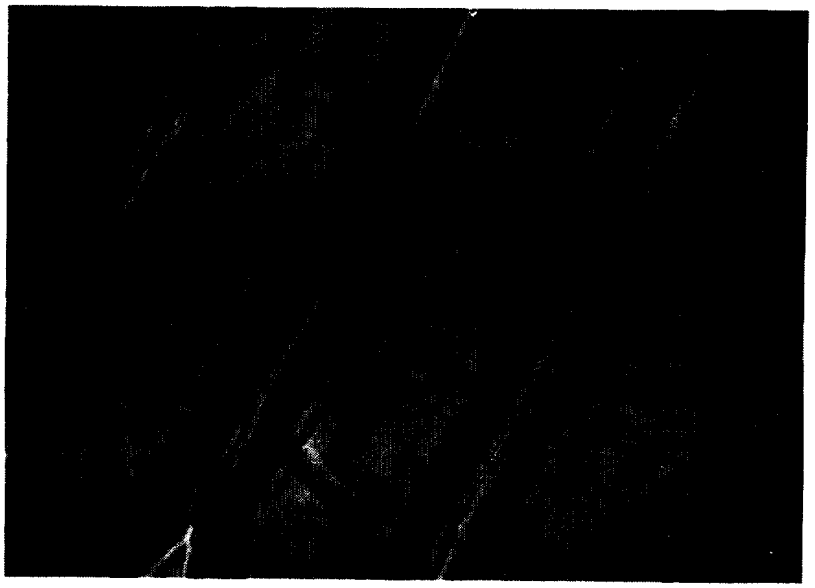

Plate 1. Cytoplasmic streaming on the epidermal cell of *Allium cepa* bulb scale. (MF : microfilament, PA : particles, CW : cell wall).

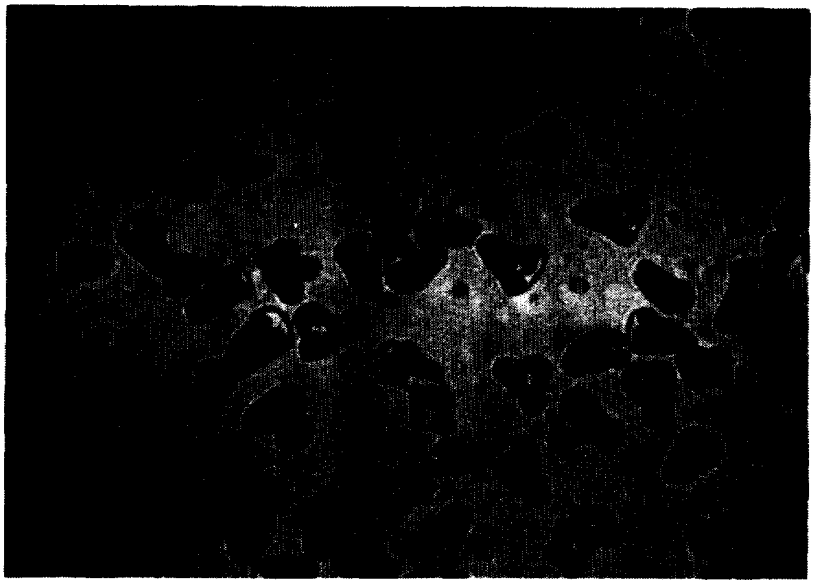

Plate 2. Single cells mechanically isolated from *Glycine max* mesophyll tissues.

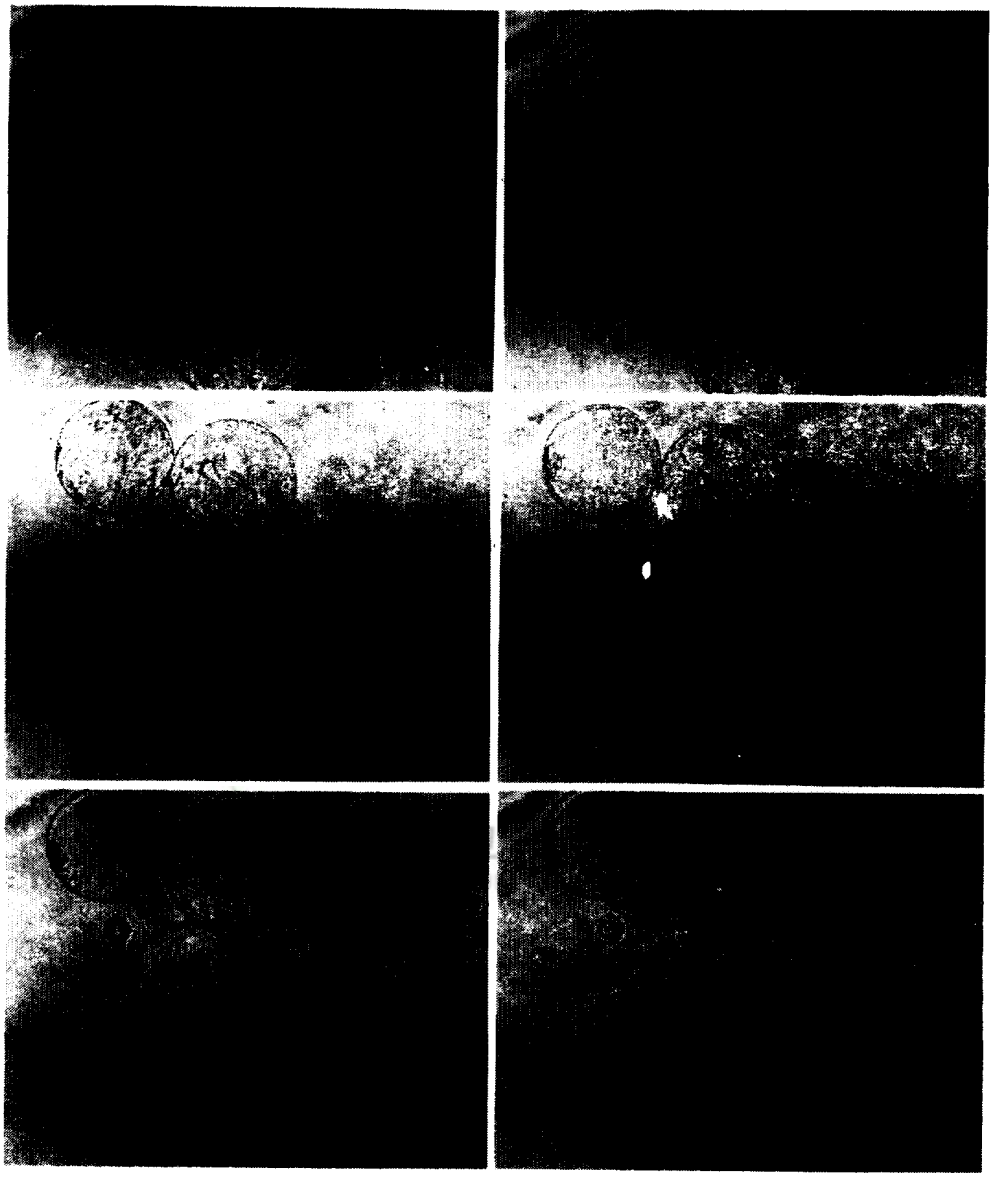

Plate 3. Time course for urea permeability of protoplasts isolated, using 1 % cellulase, from the epidermal cell of *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transferred into urea solution.

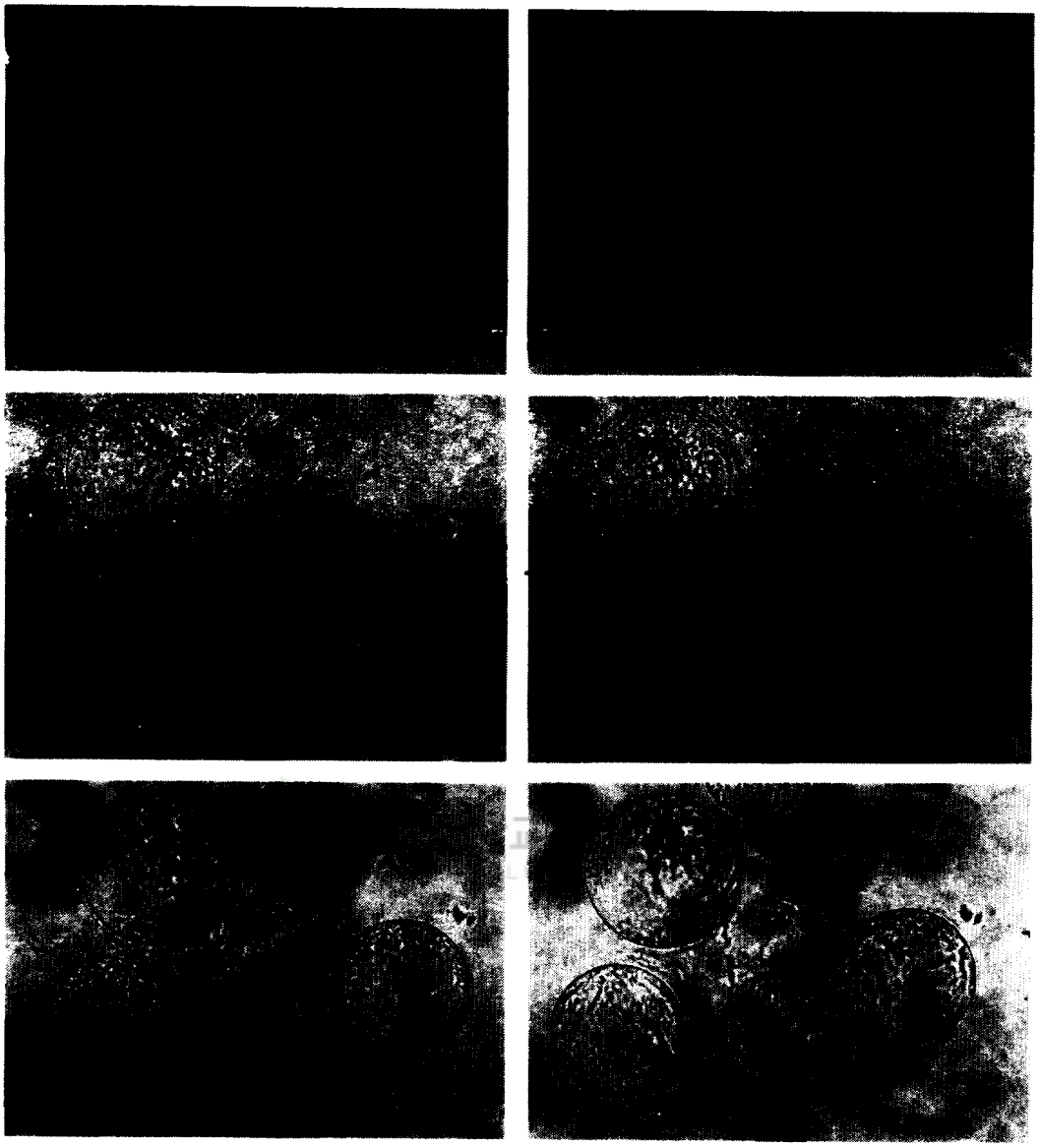

Plate 4. Time course for urea permeability of protoplasts isolated, using 2 % cellulase, from the epidermal cell of *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transferred into urea solution.

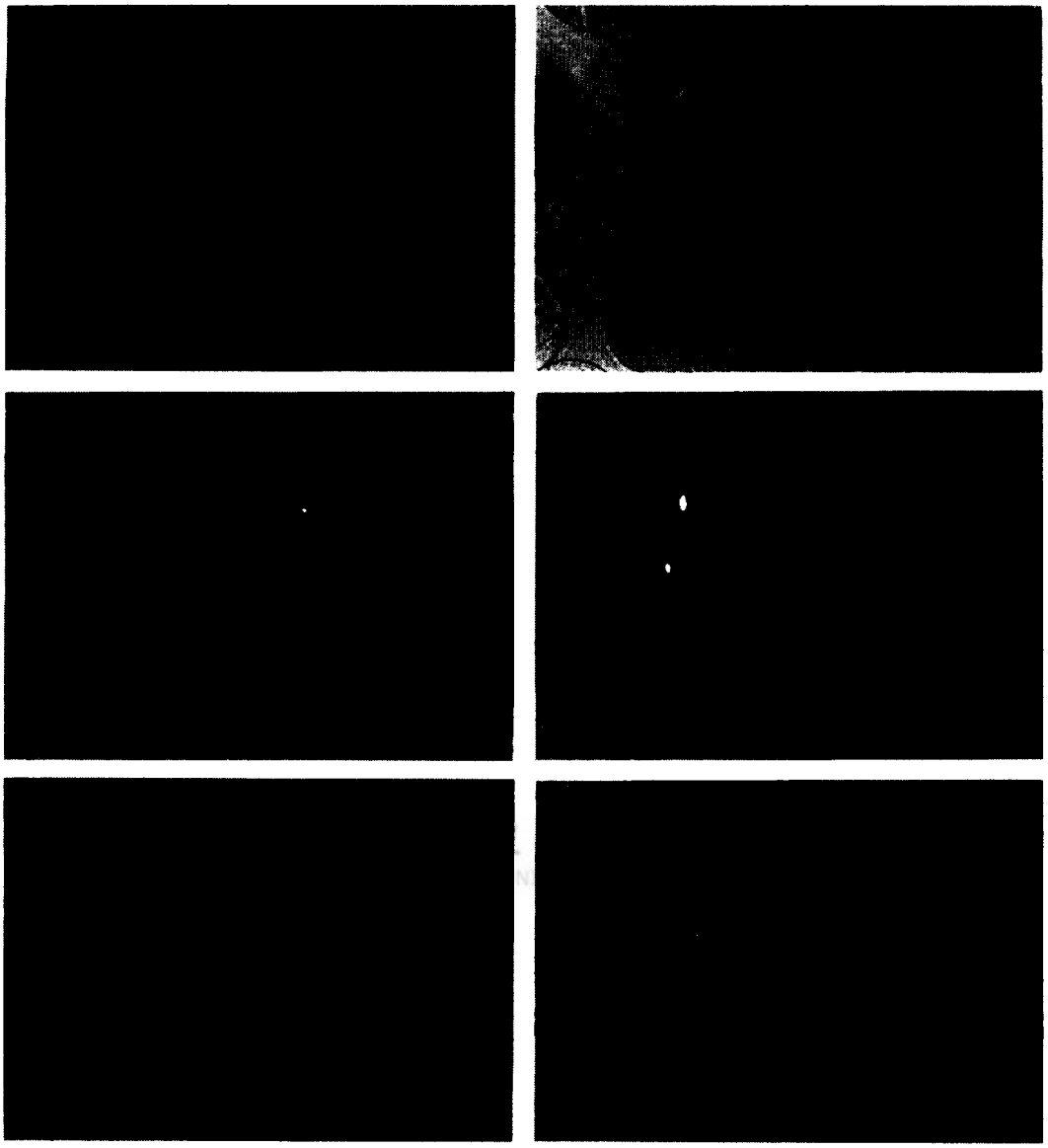

Plate 5. Time course for urea permeability of protoplasts isolated, using 3 % cellulase, from the epidermal cell of *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transferred into urea solution.

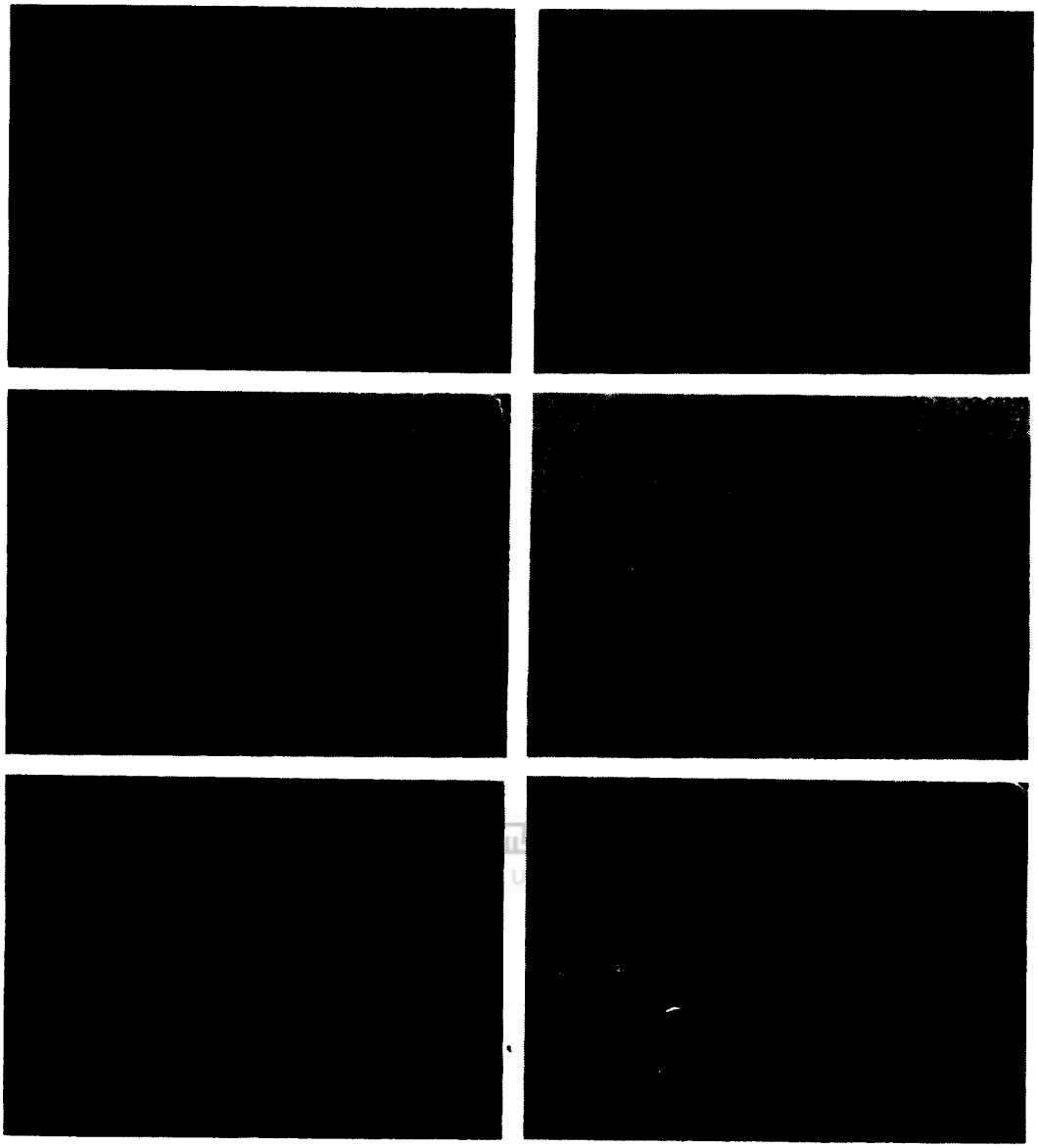

Plate 6. Time course for urea permeability of protoplasts isolated, using 4 % cellulase from the epidermal cell of *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transferred into urea solution.

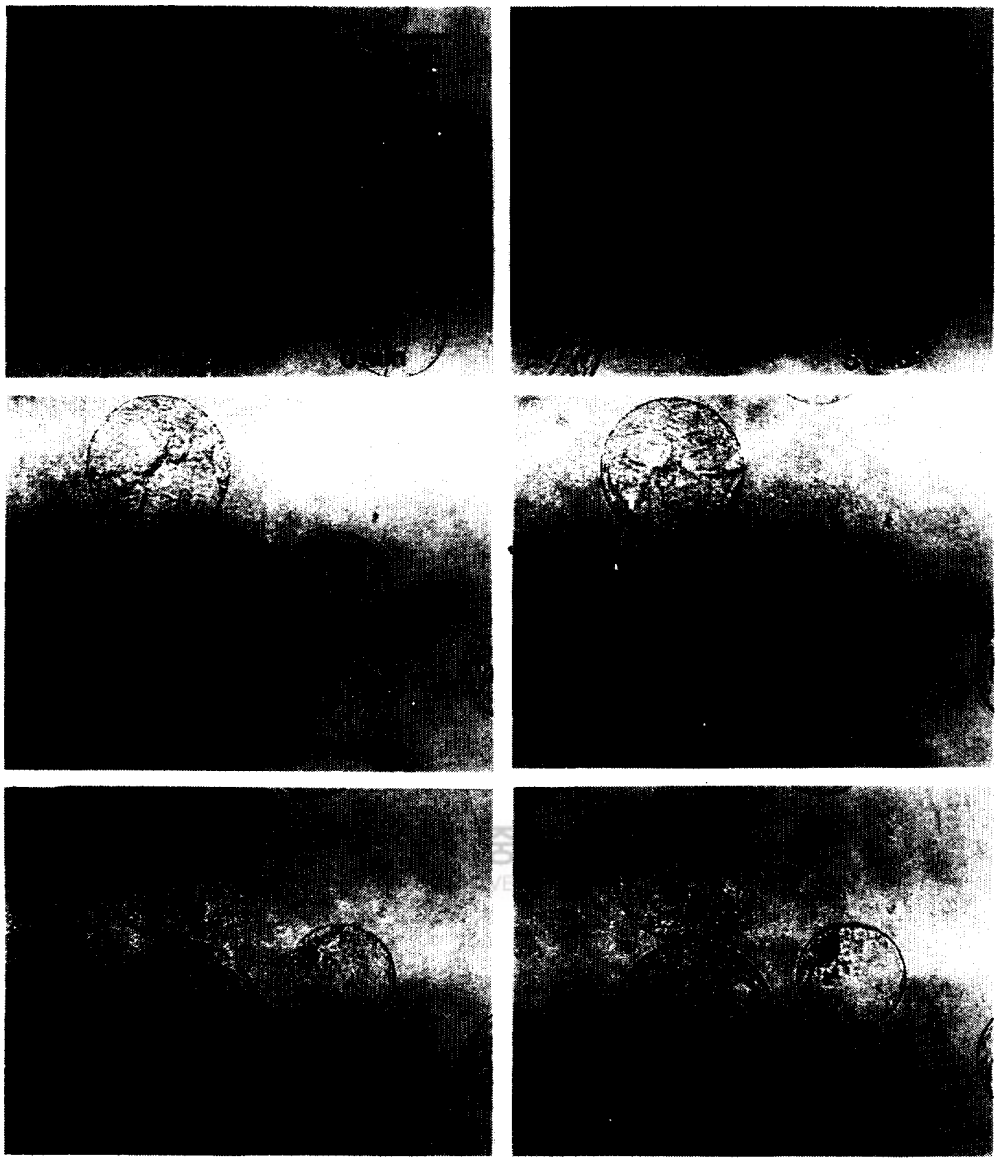

Plate 7. Time course for urea permeability of protoplasts isolated, using 2 % cellulase + 0.05 % BSA from the epidermal cell of *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transferred into urea solution.

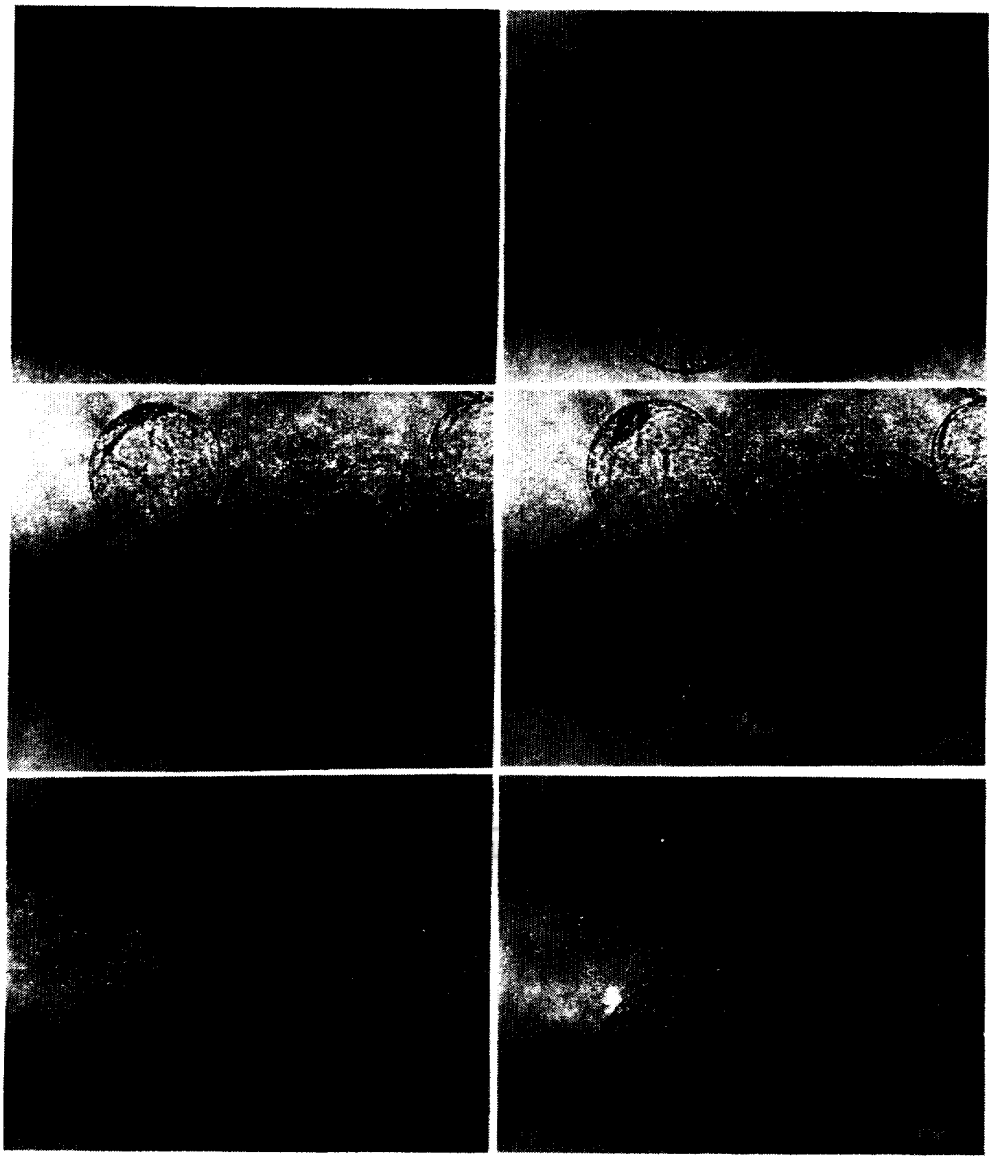

Plate 8. Time course for urea permeability of protoplasts isolated, using 2 % cellulase + 5 mM DTT from the epidermal cell *Allium cepa* bulb scale. (A) 0 min., (B) 5 min., (C) 10 min., (D) 15 min., (E) 20 min., (F) 30 min. after transfer into urea solution.

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