Isolation of Protoplast from Leaves of Castor (*Ricinus communis* L.)

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**Abstract.** In the present study, the young leaves of castor were used as materials to obtain protoplast, and the effects of enzymatic hydrolysis system on the yield and quality of protoplast were analyzed detailedly. The results shown that the treatment of the young leaves of castor with 1.5% Cellulase RS and 0.75% Macerozyme R-10 for 80 min could gain the maximum amount of protoplast. In addition, the activity of protoplast was observed with 1% Evans blue staining, and most of the protoplast (76.03%) from castor leaves had physical vitality. It lays a foundation for further studies on plant breeding and functional genes in castor.

1. Introduction

Castor (*Ricinus communis* L.) is one of top ten oil crops in the world. The seeds of castor contain high oil content (about 50%). Those oil can be use as raw material for producing industrial products, such as surfactant, fatty acid glyceride, stabilizers and plasticizers and elastomers, etc., so castor is a high economic value crop. However, there are few reports on basic research in castor, such as the protoplast isolation.

The term of protoplast is coined by Hanstein in 1880 and refers to the part of the naked substance that the plant cell, which removes the cell wall, is surrounded by the plasma membrane. The protoplast has its own genetic information and can be cultured under suitable conditions to regenerate an individual similar to its parent [1, 2]. The isolated protoplast has a wide range of uses, not only can overcome the incompatibility of distant hybridization and hybrid sterility by the way of protoplast fusion [1, 2], but also it is an important medium for genetic engineering operations, especially for the transient expression of genes, so scientists are paying more and more attention to the technique of protoplast isolation [1, 3].

Now the study on the release of castor protoplast has not been reported. In the present study, it hopes to establish and optimize the system of castor protoplast isolation, and providing the necessary basis for further research on breeding and functional genes in castor.

2. Materials and Methods

2.1. Plant Material

Castor seeds named Fen-Bi 10 were provided by the Economic Crop Research Institute, Shanxi Academy of Agricultural Sciences, Fenyang city, Shanxi province.

Castor seeds were surface-sterilized with 75% (v/v) ethanol for 60 s after removing the outer seed coat, soaked in 2% (v/v) sodium hypochlorite (NaClO) for 20 min and finally rinsed 3 times in sterile
distilled water. The embryos were obtained from the sterile seeds and inoculated on Murashige and Skoog salt (MS) medium [4] at 25 °C, under lighting with a period of 12/8 h (light/darkness) for 20 days to acquire perfectly expanded in vitro leaves.

2.2. Preparation of the Filterable Device
The nylon membrane with the diameter of 8.47 μm was clipped into 10×10 cm pieces, and aligned 2 pieces of the cut nylon film to place them on a tea leak, whereafter, covered with another same tea leak. In the end, the protoplast filterable device was gained.

2.3. Protoplast Isolation
Protoplast isolation was carried out on the basis of the reported protocols with some modification [5-7]. The fully expanded in vitro leaves were cut into 2 mm filament with sharp blades. The cut leaf materials of 0.4 ± 0.05 g (for each treatment) was transferred into W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES, pH 5.8) quickly for 10 min in the dark. After removing the W5 solution, the cut leaf materials were placed instantly into 20 mL of the enzymatic hydrolyzate (0.5 M mannitol, 10 mM MES at pH 5.7, 10 mM CaCl2 and 0.1% BSA) contained various combinations of Cellulase RS (Cellulase derived from Rhizopus sp.; RS) and Macerozyme R-10 (Macerozyme derived from Rhizopus sp. No. 10; R-10) (0.75% RS and 0.6% R-10, 1.5% RS and 0.75% R-10, 3% RS and 1.5% R-10) (Yakult, Japan) for different periods (20, 40, 60, 80, 100 and 120 min) in the dark (25 °C) with soft shaking (50 rpm).

After enzymatic hydrolysis process, the digested leaf materials were filtered with the help of the above-mentioned filterable device, collected the protoplast at 700 rpm centrifugation for 5 min and suspended the precipitate in 20 mL W5 solution. The protoplasts were purified in W5 solution for twice by resuspension and centrifugation (700 rpm for 5 min), and then the protoplasts were soft suspended with 2 mL of W5 solution and placed into 2 mL round bottom centrifuge tubes. After centrifugation (700 rpm for 10 min), the supernatant was removed, and finally the protoplasts were resuspended with 1 mL of MMG solution (0.5 M mannitol, 2 mM MES and 15 mM MgCl2, pH 5.8).

The yield of protoplasts were calculated under a microscope equipped with a hemocytometer. The activity of protoplasts was surveyed with 1% Evans blue staining [8] and counted as follows: yield of protoplasts (g/gFW) = (number of protoplasts in 5 large squares×1000×dilution factor) / fresh weight of material; activity of protoplasts (%) = (number of unstained protoplasts / total number of protoplasts) × 100%.

3. Results

3.1. Influence of Enzyme Concentration on the Yield of Protoplasts
The results revealed that the influences of enzyme concentration on the yield of protoplasts isolation from castor leaves were obvious (Table 1 and Figure 1A). When the enzyme concentration was at 1.5% RS and 0.75% R-10, the highest yield (1.18×10^6 protoplast/gFW) of protoplast isolation from castor leaves were gained consummately.

Table 1. Results of enzyme concentration on the yield of protoplast

| Enzyme (%) | Protoplast yield (×10^6/#/g FW) | Total unbroken protoplasts (×10^6/#/g FW) | Integrated rate (%)*** |
|------------|---------------------------------|------------------------------------------|-----------------------|
| RS R-10    |                                 |                                          |                       |
| 0.75 0.6   | 0.76±0.11b**                    | 0.54±0.08b                               | 71.61±4.31b           |
| 1.5 0.75    | 1.18±0.05a                      | 0.96±0.05a                               | 81.52±3.28a           |
| 3 1.5       | 0.45±0.09c                      | 0.21±0.12c                               | 48.85±3.03c           |

*Data in the table above were obtained on the condition of 20 days of leaf materials, 2 mm filament of leaves, 80 min of enzymolysis and 700 rpm of centrifugation.
**Data in the same column followed by different letters were significantly different at p ≤ 5% level as determined by Duncan’s multiple range test.
***Integrated rate (%) = (Number of intact protoplasts/ Total number of protoplasts)×100%.
3.2. Effects of Enzymolysis Time on the Yield of Protoplasts

It’s clear demonstrated (Table 2 and Figure 1A) that the influences of enzymolysis time on the yield of protoplast isolation from castor leaves were all prominent. Enzymolysis for 80 min was the most suitable time, in which the highest yield of protoplast \((1.18 \times 10^6\) protoplasts/gFW) and the largest integrated rate \((83.05\%\) were received subsequently.

| Enzymolysis time (min) | Protoplast yield \((\times 10^6\)#/g FW) | Total unbroken protoplasts \((\times 10^6\)#/g FW) | Integrated rate (%) |
|------------------------|----------------------------------------|-----------------------------------------------|-------------------|
| 20                     | 0.25                                   | 0.12                                          | 48.00             |
| 40                     | 0.81                                   | 0.426                                         | 52.78             |
| 60                     | 1.09                                   | 0.86                                          | 78.90             |
| 80                     | 1.18                                   | 0.98                                          | 83.05             |
| 100                    | 0.72                                   | 0.41                                          | 56.94             |
| 120                    | 0.65                                   | 0.293                                         | 45.24             |

*Data in the table above were obtained on the condition of 20 days of leaf materials, 1.5% RS and 0.75% R-10, 2 mm filament of leaves and 700 rpm of centrifugation.

![Figure 1. Determination of yield and vigor of protoplast from castor leaves](image)

Figure 1. Determination of yield and vigor of protoplast from castor leaves

(A) Castor protoplasts were gained from the optimized experimental protocol, at the conditions of 20 days of leaf materials, 2 mm filament of leaves, 1.5% RS and 0.75% R-10, 80 min of enzymolysis and 700 rpm of centrifugation; (B) vitality testing of castor protoplast (only dead cells stained with Evans blue). (Bar = 50 μm)

3.3. Determination of Vigour of Protoplast from Castor Leaves

| Number of observed fields | Total number of protoplast | Number of stained protoplast | Number of unstained protoplast | Protoplast viability (%) |
|---------------------------|---------------------------|------------------------------|-------------------------------|-------------------------|
| 1                         | 51                        | 8                            | 43                            | 84.30                   |
| 2                         | 48                        | 15                           | 33                            | 68.75                   |
| 3                         | 52                        | 11                           | 41                            | 78.85                   |
| 4                         | 57                        | 17                           | 40                            | 70.18                   |
| 5                         | 50                        | 10                           | 40                            | 80.00                   |
| 6                         | 54                        | 14                           | 40                            | 74.07                   |

The average protoplast viability (%) 76.03

*Data in the table above were obtained on the condition of 20 days of leaf materials, 2 mm filament of leaves, 80 min of enzymolysis and 700 rpm of centrifugation.
The vigor of protoplast were confirmed by 1% Evans blue staining [8]. The results shown that the number of stained and unstained protoplast were observed randomly in 6 different fields under the optimal enzymatic hydrolysis system, and calculated the protoplast activity (Table 3 and Figure 1B). It could be seen that most of the protoplast from castor leaves had highly vitality, and the average protoplast viability was 76.03% (Table 3 and Figure 1B).

4. Discussion
Some results have shown that the concentration and combination of enzyme have a significant effect on the isolation of protoplasts [5-7]. The results of this study indicated that protoplast from the young leaves of castor could be obtained via enzymolysis with appropriate enzyme combination and concentration. The cellulose, hemicellulose and pectin in the cell wall were removed by enzymatic hydrolysis with those enzymes inducing, so that the protoplasts were released freely and come out into separate individuals. Further studies found that the enzymolysis time also had a large effect on the yield of protoplast [8], the results shown that the longer the initial enzymolysis time proceeded, the more protoplast of castor gained. When the enzymolysis time was at 80 min, the maximum yield of protoplast was obtained subsequently. However, if the enzymolysis time continued to prolong, the protoplast yield would not be increased. It could be because the enzyme itself in the enzymatic hydrolysate would also cause some damage to protoplast due to the long time of enzymolysis. And then the plasma membrane of the protoplast might be ruptured, resulting in a gradual decreased in the number of castor protoplast.

In addition, the yield and viability of protoplast are strongly influenced by pH and osmotic pressure in the enzymatic hydrolysate [2, 9]. What’s more, differences in material sources and pretreatment can also affect the free effects of protoplast. The yield of protoplast is increased by adding certain hormones into the enzymatic hydrolysate [1, 10]. Therefore, in order to obtain a large number of viable protoplast, it is necessary to comprehensively consider various influencing factors, which will be further explored on the protoplast isolation of castor.

5. Conclusion
The protoplasts of young castor leaves were freed by a combination of 1.5% Cellulase RS and 0.75% Macerozyme R-10 for 80 min. The results shown that the enzymatic hydrolysate could hydrolyze the cell wall in young castor leaves to obtain protoplast successfully. Furthermore, the enzymolysis time on yield and vigor of castor protoplast were analyzed subsequently.

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