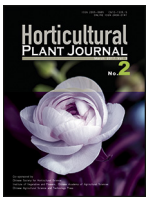
March 2017. Horticultural Plant Journal, 3 (2): 47–52.

Horticultural Plant Journal

Available online at www.sciencedirect.com
The journal's homepage: http://www.journals.elsevier.com/horticultural-plant-journal



# A Rapid Method for Isolating Single Cells from Apple Flesh

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Received 17 October 2016; Received in revised form 17 January 2017; Accepted 16 February 2017

Available online

A B S T R A C T

The identification, separation and analysis of individual living cells can be used to analyze the heterogeneity and operation mechanism of living systems. The study of fruit development is based on the extraction of active single cells. In this study, we investigated the effects of different enzymes and enzymatic hydrolysis times on the extraction of single cells from the 'Fuji' apple (*Malus × domestica* Borkh. 'Fuji'). The results showed that the extraction of single cells in apple flesh was a suitable method when 0.1% macerozyme was used and the enzymolysis time was 0.5 h. Fluorescent brightening agent VBL staining showed that the cell wall was intact, while fluorescein diacetate FDA and azo dye Evans blue staining indicated that the extracted single cells were active. The extracted single cells could be further used as materials for protoplast extraction.

*Keywords*: apple; flesh; single cells; enzyme liquid concentration; enzymolysis time

## 1. Introduction

Cells are the basic unit of life. Specific single cell behavior, individual differences and heterogeneity among cells play very important and decisive roles in critical life processes, such as embryonic development, cell differentiation, and the occurrence and development of diseases (Tang, 2004). Moreover, because single cell techniques do not depend on the cultivation and proliferation of cells, they have the potential for application in the fields of biological development, biological energy, climate change, disease diagnosis, food safety, and agricultural ecology. Gawad et al. (2016) studied microbial dark matter by using genome sequencing of single cells and evaluated the pathogenic role of the genetic mosaic in multicellular organisms. In the plant community, we can lay the foundation for improvement of plant varieties that can multiply via sexual propagation by isolating sperm cells to culture *in vitro*. By separating the guard cell protoplasts, it is possible to study the physiological and biochemical characteristics of guard cells and their relationship with stomatal function. van Beijnum et al. (2008) identified specific expression genes in tissue by isolating cells from vascular and phloem tissue. Haigler et al. (2012) studied the process of cell wall and cellulose formation using cotton fiber single cells, which can help improve the quality of cotton fiber in production. Current studies of gene expression are based on a group of cells, which cannot correctly reflect the comprehensive and actual information describing complex biological systems. Most importantly, they seriously conceal the behavior of independent individual and random phenomena in life. Such gene expressions are actually the average of the cell specific expression of each individual cell. However, gene expression analysis of individual cells can provide a new mechanism for the development of single cell hidden among group of cells (Narsinh et al., 2011; Bloch and Yalovsky, 2013). There are various ion channels such as the $Ca^{2+}$, $K^{+}$, and $Na^{+}$ channels on the membranes of single cells that regulate the flow of ions into and out of the cell; therefore, the patch clamp technique can be used to explore the characteristics of ion channels in single cells. Information provided by such analyses is beneficial to further investigation of numerous physiological mechanisms of biological signal transduction, cell nutrition and stress resistance. Li et al. (2008) revealed the key factors responsible for the reversal of ion flow in the same channel by applying the patch clamp technique to investigate the $K^{+}$ channel of single cells and to identify the transform mechanism controlling the flow through the $K^{+}$ channel. It has also been reported that high concentrations of $Ca^{2+}$

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Peer review under responsibility of Chinese Society for Horticultural Science (CSHS) and Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS)

http://dx.doi.org/10.1016/j.hpj.2017.07.005



Please cite this article in press as: GUAN Yaqin, and QU Haiyong, A Rapid Method for Isolating Single Cells from Apple Flesh, Horticultural Plant Journal (2017), doi: 10.1016/j.hpj.2017.07.005

influence the structure and texture of fruit, which could delay softening and senescence, thereby prolonging shelf life (Ciccarese et al., 2013; Ngamchuachit et al., 2014).

The development of fruit is the process by which the cell population grows, which consists of single cell pulp. This process directly determines the flavor and quality of fruit, length of storage time and degree of disease occurrence. Therefore, it is the basis to separate active single cells from apple fruit. In this study, we investigated a method for extracting single cells of apple flesh, and used mature 'Fuji' apples as the test material.

## 2. Materials and methods

### 2.1. Materials

Mature Fuji (*Malus × domestica* Borkh. 'Fuji') apples were collected from the teaching and practicing orchard of Qingdao Agriculture University (Late October 2016) and then stored at 4 °C for future use.

### 2.2. Equipment

A fluorescence microscope (LEICA, DM 2500, Leitz, Germany), laser scanning confocal microscope (LEICA, TCS SP5, Leitz, Germany), pulp refiner, low-speed centrifuge, constant temperature shaker and magnetic stirrer were used.

### 2.3. Test method

#### 2.3.1. Single cell separation

Cell protoplast wash medium (CPW) was prepared as described by Zhang et al. (2015). Briefly, 101.0 mg · $L^{-1}$ $KNO_3$, 27.2 mg · $L^{-1}$ $KH_2PO_4$, 246.0 mg · $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 1 480.0 mg · $L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.16 mg · $L^{-1}$ KI, 0.025 mg · $L^{-1}$ $CuSO_4 \cdot 5H_2O$ and 0.4 mol · $L^{-1}$ mannitol were mixed and stored at 4 °C until use. Cellulose, pectinase and macerozyme were dissolved in the CPW solution. The enzyme solutions are shown in Table 1.

For extraction, the flesh under the apple skin was cut into 150 small square pieces (0.5 cm side length) with a scalpel, then further cut into slices and placed in 50 mL of enzyme liquid I, II and III (Table 1) and shaken at 70 r · $min^{-1}$ to conduct enzymolysis for 0.5, 1 and 2 h respectively (27 °C, dark conditions). After being allowed to stand for a few minutes, the enzyme liquid was removed by washing with CPW solution three times, and the samples were transferred to a 50 mL flask. Next, samples were stirred for 40 min with a magnetic stirrer, after which they were observed by optical microscope.

#### 2.3.2. VBL to test cell wall integrity

The cell wall staining solution, fluorescent brightening agent VBL [4,4′-Bis (4-anilino-6-hydroxyethy-1-amine-S-triazin-2-ylamino)-2,2′-stilbene], was prepared according to Huang and Yan (1980), then dissolved in 0.5 mol · $L^{-1}$ mannitol solution to a final concentration of 0.1% (w/v), pH 7.0. Finally, solution was stored in the darkness at 4 °C until use.

**Table 1 Composition of enzyme liquid** w/v

| Enzyme liquid | Cellulose/% | Pectinase/% | Macerozyme R-10/% |
|---|---|---|---|
| I | 0.1 | 0.05 | 0.1 |
| II | 0 | 0.05 | 0.1 |
| III | 0 | 0 | 0.1 |

Cell wall staining was accomplished by adding 400 μL of cell suspension solution into a 1.5 mL centrifuge tube, then 20 μL 0.1% VBL was added. Samples were incubated for 5 min in a dark place. Next, samples were washed with CPW solution three times after dyeing to eliminate fluorescence from the background. Finally, single cells were observed with a fluorescence microscope (345 nm excitation laser light and 430 nm emission filter).

#### 2.3.3. Single cell viability assay

Fluorescein diacetate (FDA) is a non-fluorescent compound that releases fluorescein after penetrating living cells, which is resulting in production of green fluorescence under the excitation of blue light (Fan et al., 2013). Briefly, 5 mg · $L^{-1}$ FDA was prepared by dissolving 25 mg FDA in 1 mL acetone. After complete dissolution, 4 mL of this solution was added to 0.65 mol · $L^{-1}$ mannitol solution and stored in the dark at 4 °C.

Cells were stained by adding 500 μL cell suspension solution into 1.5 mL centrifuge tubes, after which FDA dye solution was added to give a final concentration of 0.01% for 5 min at room temperature. Finally, the activity of single cells was detected by fluorescence microscope (493 nm excitation laser light and 510 nm emission filter).

The viability of single cells was examined by Evans blue staining. Briefly, Evans blue dye (Sigma, St. Louis, MO) was dissolved in CPW solution, then we added dye liquor into the solution of single cells, which is up to final concentration of 0.04%. We stained flesh single cells with heat treatment (100 °C) for 1 h and no heat treatment respectively, then incubated for 10 min at room temperature. Evans blue is excluded by living single cells, whereas dead single cells and cell debris are stained a deep blue color.

#### 2.3.4. Measurement of $Ca^{2+}$ in living cells by Fluo-4 AM

Fluo-4 AM is an acetyl methyl ester derivative that can enter cells incubated in its presence. AM can be hydrolyzed by intracellular esterase following entry into cells, after which it forms Fluo-4 via combination with $Ca^{2+}$ and emits fluorescence. Fluo-4 AM solutions were prepared and Fluo-4 AM was loaded into single cells according to our published method (Qu et al., 2016). Next, samples were washed with CPW solution three times to eliminate Fluo-4 AM from outside of the cells after staining for 30 min at room temperature in the dark. Cells were then observed using a Laser scanning confocal microscope (495 nm excitation laser light and 515 nm emission filter).

#### 2.3.5. Data analysis

We used Office Excel 2010 software (Microsoft, USA) for data collection and adopted SPSS 19.0 software (IBM, USA) for multiple comparisons.

## 3. Results

The linkage between cells is mainly composed of cellulose, pectin and polysaccharide in apple flesh. The individual cells in apple flesh cannot be separated without enzyme (Fig. 1, A). In addition, enzyme liquid containing 0.1% cellulose, 0.05% pectinase and 0.1% macerozyme (I) or 0.05% pectinase and 0.1% macerozyme (II) produced few single cells and a large number of fragments (Table 2). However, when only 0.1% macerozyme (III) was present

Please cite this article in press as: GUAN Yaqin, and QU Haiyong, A Rapid Method for Isolating Single Cells from Apple Flesh, Horticultural Plant Journal (2017), doi: 10.1016/j.hpj.2017.07.005