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 20 July 2017

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

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

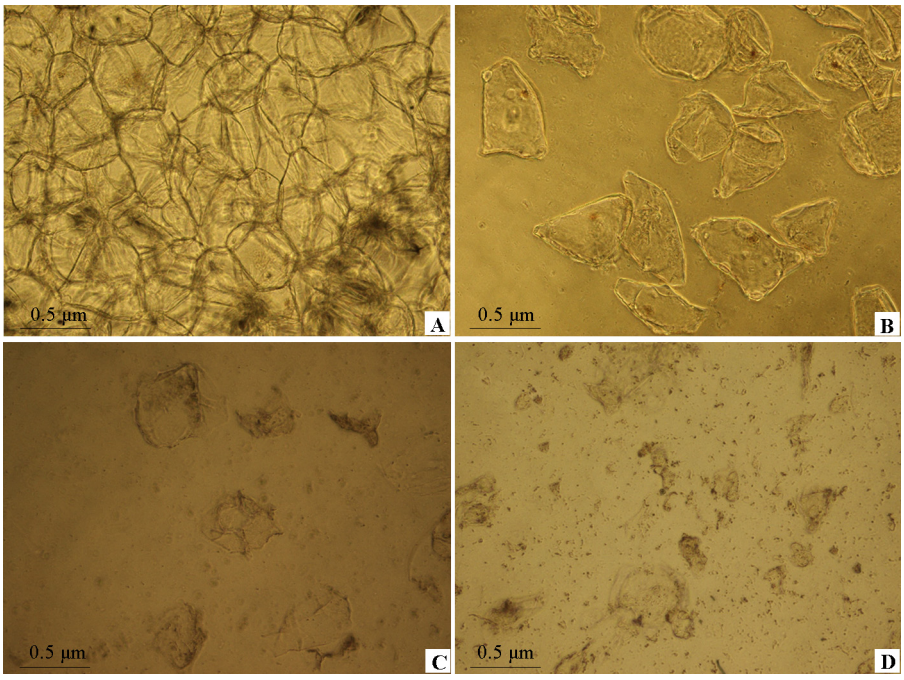

Fig. 1 Separation of single cells from apple fruit pulp

(A) Pulp without enzymatic treatment. Pulp cells were closely connected to each other without separation; (B) Single cells were obtained by enzyme liquid III for 0.5 h; (C) The pulp was treated with enzyme solution III for 1 h. Flesh cells were not complete and showed signs of rupture; (D) The pulp was treated with enzyme solution III for 2 h. Flesh cells were completely ruptured.

in the enzyme liquid and the treatment time was 0.5 h, lots of complete single cells were obtained (Table 2, Fig. 1, B). When the treatment time was 1 h or 2 h, there is a lot of debris (Table 2, Fig. 1, C, D). Therefore, the enzyme liquid III was best for separating the single cells of apple flesh, and the optimum enzymolysis time was 0.5 h (Table 2, Fig. 1).

VBL has a strong affinity to the cellulose of the plant cell wall. Under UV-light, fluorescence can be used to detect the integrity of cells (Huang and Yan, 1980). The single cells in apple flesh were separated by enzyme liquid III, then dyed by VBL. Obvious fluorescence was seen on the cell wall (Fig. 2, A), indicating that the single cells extracted from flesh were intact.

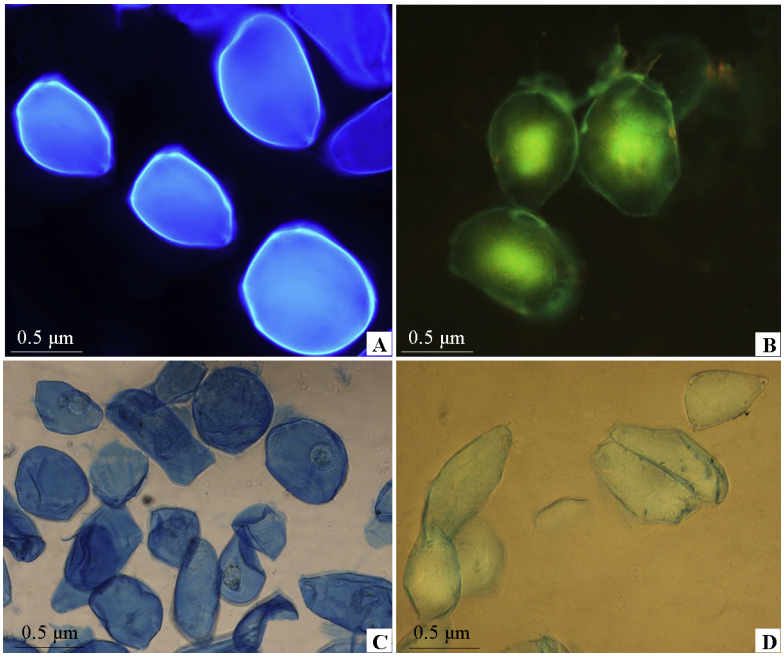

Fig. 2 Assay of integrity and activity of extracted flesh single cells

(A) Detection of cell wall integrity by VBL fluorescent dyes; (B) Detection of the activity of single cells by FDA staining; (C) Evans blue stained single cells after heat treatment (100 °C) for 1 h; (D) Evans blue stained single cells without heat treatment.

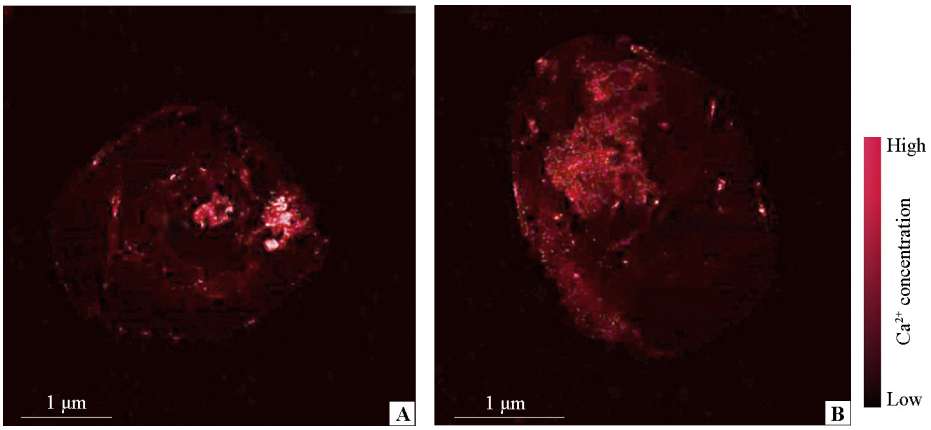

Fig. 3 The distribution of Ca^{2+} in single cells

(A) Ca^{2+} is distributed around the nucleus of single cells; (B) Ca^{2+} distribution in single cells from the circumference to the central part.

FDA is commonly used to identify the cell activity of animals and plants (Yang, 1986). When the active cells have an intact cell membrane, the fluorescein generated by hydrolyzed FDA will accumulate in the cells, resulting in green fluorescence under blue light. However, dead cells have lost their selective permeability; therefore, the fluorescein will not accumulate in these cells and there will be no fluorescence (Saruyama et al., 2013). After single-cell staining by FDA, the whole cell showed green fluorescence, indicating that the single cells extracted from apple flesh were viable (Fig. 2, B). Cells were incubated at 100 °C for 30 min, then stained with Evans blue (Fig. 2, C). On the other hand, unheated flesh single cells cannot be infected with Evans Blue (Fig. 2, D), further demonstrating that the extracted flesh single cells are active.

Fluo-4 AM is an intracellular Ca^{2+} fluorescent labeled probe that can detect the concentration and distribution of Ca^{2+} in cells. After the fluorescent probe was esterified, it crossed the plasma membrane into the cytoplasm and was hydrolyzed by non-specific esterase to generate a hydrophilic product, which combined with Ca^{2+}. The Ca^{2+} distribution was uneven in the flesh cells, being found mainly in the nuclei and vesicles (Fig. 3, A, B).

Table 2 Statistical analysis of the effects of different enzymes and different treatment time on the extraction of single cells and enzymolysis time

Enzymolysis time/h	Enzyme liquid	Number of single cell/(n · mL^{-1})	Number of cell debris/(n · mL^{-1})
0.5	I	133.33 ± 33.33 d	1 566.67 ± 202.76 a
	II	466.67 ± 120.19 bc	1 133.33 ± 120.18 ab
	III	1 133.33 ± 218.58 a	166.67 ± 66.67 c
1.0	I	33.33 ± 33.33 d	1 833.33 ± 240.37 ab
	II	200.00 ± 115.47 cd	1 600.00 ± 680.69 ab
	III	566.67 ± 120.19 b	900.00 ± 152.75 bc
2.0	I	33.33 ± 33.33 d	1 500.00 ± 173.21 ab
	II	33.33 ± 33.33 d	2 000.00 ± 208.17 a
	III	233.33 ± 33.33 cd	1 033.33 ± 145.30 bc

Note: Different lowercase letters represent significant differences at 0.05 level.

4. Discussion

Isolation of single cells is the premise and foundation of studying the physiological and molecular characteristics of animals and plants. At present, research at the single cell level has attracted increasing attention. The results of such studies have been widely used in single cell PCR amplification, genome sequencing and cell engineering (Chen and Huang, 2005; Tang et al., 2009; Islam et al., 2011; Hashimshony et al., 2012; Yu et al., 2013). Isolation of single plant cells is primarily accomplished by enzymatic hydrolysis. Treatment with cellulose and pectinase destroyed the cell wall and enabled the cells to be separated from each other. Single cells or protoplasts could then be isolated. Zhang et al. (2004) isolated single cells from potato leaves by enzymatic hydrolysis and found that pectinase was a key enzyme in cell separation. However, evaluation of the separation rate of cells revealed that their enzymolysis time was longer than ours (at least 4 h). Too long enzymolysis time will damage the integrity and activity of cells. Many studies have adopted this method to directly extract protoplasts, which can degrade the cell wall. Liao (2010) used cellulose to obtain protoplasts from mesophyll cells of *Arabidopsis*. Additionally, protoplasts of Sainfoin were extracted with cellulase, pectinase and segregation enzyme (Chen et al., 2008).

Cellulose and pectinase can promote degradation of the cell wall, which plays an important role in cell growth and development (Caffall and Mohnen, 2009). Specifically, the cell wall is involved in a variety of metabolic reactions, such as cell differentiation, cell identification and disease resistance response, as well as a series of cell development events such as cell wall function, cell polarity formation, directional transportation of plant hormones, and changes in the polarity of the cytoskeleton and directional distribution and transportation of vesicles connected to each other (Cosgrove, 2005). Cell polarity, which is the basic attribute of an organism, controls information exchange, pattern generation and identification among cells in multicellular living beings (Bloch and Yalovsky, 2013). Therefore, obtaining single cells with intact cell walls is important to investigate the physiological and biochemical functions of cells. McAtee et al. (2009) heated the flesh of apples and pears to boiling while stirring for 0.5 h with a magnetic stirrer. However, the obtained single cells

had lost their activity, and they could only be used to detect the size and shape of flesh cells. In this study, we obtained intact cell walls under the treatment of VBL and found that single cells had remarkable activity through the dyeing of FDA and Evans blue. The principle of enzymolysis treatment is to use the lowest enzyme concentration and the shortest enzymolysis time to obtain many active protoplasts (Xie and Liu, 2004). We have obtained single cells with intact cell, which were less than the time required to gain protoplasts through enzymosis (Mazarei et al., 2008; Zhai et al., 2009). In addition, the single cells extracted from apple flesh were further treated with cellulase to obtain protoplasts. These results indicated that the extracted flesh single cells were not only active, but also that the CPW solution concentration was suitable.

Ca^{2+} is an essential nutrient element for plant growth and development that can protect the cell membrane and delay senescence and deterioration of the fruit postharvest. Moreover, Ca^{2+} acts as the second messenger of the coupling extracellular signal and intracellular physiological and biochemical reaction, which can regulate the growth and development of cells in many stages. The demand for Ca^{2+} uptake is higher than that of other elements for apple. Ca^{2+} is an important component of various organs in apple trees that plays a critical role in growth regulation (Chen and Zhou, 2004). A higher Ca^{2+} concentration will maintain fruit firmness, decrease the respiration rate, increase protein synthesis and inhibit ethylene synthesis, thereby prolonging the postharvest storage time. However, Ca^{2+} deficiency can lead to decreased mechanical strength in fruit or its organs, which shortens the storage period. The cell wall could also not be formed, which influences cell division (Johnston et al., 2002; Manganaris et al., 2007; Akhtar, 2010). Ca^{2+} deficiency may also affect root growth, and ultimately decrease the ability of a root to absorb materials, leading to a series of physiological diseases (Chen and Huang, 1990). Currently, detection of Ca^{2+} in the single cells of flesh is accomplished at the tissue and organ level. Moreover, tested cells are dead after being fixed, and therefore lack viability (Cabanne, 2003). In this study, we isolated single cells and detected their activity, which reflected the dynamic state of Ca^{2+} distribution and signal transduction.

5. Conclusion

In this study, a better method of separating single cells from apple flesh was developed in which samples were treated with 0.1% macerozyme for 0.5 h and then stirred for 40 min. The developed method can obtain living single cells with intact cell wall.

Acknowledgments

We thank all scientists for helping us perfect the manuscript. The work was supported by The National Key Research and Development Program of China (2016YFD0201120) and Shandong Province Fruit System Job Expert (SDAIT-06-05).

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