Bangladesh J. Bot. **47**(1): 9-15, 2018 (March)

HISTOCHEMISTRY AND EXTRACTION OF *CRASSULA OVATA* (MILL.) DRUCE POLYSACCHARIDES

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Keywords: *Crassula ovata*, Polysaccharide, Histochemistry, PAS reaction

Abstract

The microstructure of the leaves and stem of *Crassula ovata* (Mill.) Druce belonging to Crassulaceae was studied. Samples were processed for light microscopy, and the storage site of polysaccharide was determined by histochemical method with PAS reaction. The results showed that *Crassula ovata* leaves were composed of epidermal and mesophyll cells. The structure of stems included epidermis, cortex and vascular. PAS reaction showed that polysaccharides mainly store in the stem cortex cells and leaf mesophyll tissue. Orthogonal design was used to optimize experimental conditions for crude polysaccharides from *Crassula ovata* leaves. The optimum extraction conditions were as follows: ratio of material to liquid 1:10, extraction time 120 min, extraction temperature 60ºC, ethanol concentration 70%, ethanol precipitation for 30 min.

Introduction

Crassulaceae plants are perennial succulent herbs, mainly distributed in the northern hemisphere. Most Crassulaceae plants bloom in summer and autumn, flowers are small and lush. The leaves of Crassulaceae plants showed typical drought resistance characteristics with the fleshy leaves (Sendl *et al*. 1993, De Melo *et al*. 2009, Cavero *et al*. 2013). *Crassula ovata* (Mill.) Druce belongs to Crassulaceae and located in southern Africa. It is a perennial succulent plant which contains polysaccharides in the vegetative organs. In the previous study, the workers on Crassulaceae focused on drought resistance and the extraction, purification, component analysis and functional study of Crassulaceae polysaccharides. A number of studies had shown that polysaccharides have multiple biological activities, such as anti-tumor, immune, anticoagulant, hypoglycemic and antiviral activity (Sendl *et al*. 1993, De Melo *et al*. 2009, Rashan *et al*. 2011, Cavero *et al*. 2013, Xing *et al*. 2013). Currently, the method of extraction of polysaccharides is solvent extraction, enzymatic extraction, ultrasound-assisted extraction, microwave-assisted method, pressure extraction method, etc. Usually, a variety of methods combined with each other is used to achieve a better extraction effect (Rashan *et al*. 2011, Niu *et al*. 2015, Gao *et al*. 2017).

The anatomical structure of vegetative organs of *Crassula ovata* was studied in order to reveal the anatomical structure of stem and leaf of *Crassula ovata*, and the location of polysaccharides in vegetative organs was identified by histochemical method. At the same time, *Crassula ovata* polysaccharide was extracted by water extraction and alcohol precipitation, and the optimum extraction process was obtained. The aim of this study was to study the storage positions of polysaccharides in stems and leaves, and provide the theoretical basis for the development and utilization of *Crassula ovata* polysaccharides.

Materials and Methods

Fresh stem and leaf samples of *Crassula ovata* (Mill.) Druce were collected in June 2016. The leaves and stems of *Crassula ovata* were divided into 3 - 5 mm^3 and fixed with FAA solution for 24 hrs at room temperature (25ºC), then dehydrated in an ethanol series and embedded in paraffin.

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The material was sectioned using a rotary microtome (Leica 2235) and the sections (6 - 8 μm thick) were stained with Safranin-Fast green double staining. For histochemical assays, fresh material was sectioned using frozen slicing machine (Leica CM 1950). Sections (15 μm thick) were treated with the Schiff reagents for total insoluble polysaccharides (Kumari *et al*. 2009, Rodrigues *et al*. 2011). All the specimens were examined and documented with a light microscope (Fsx-100, Olympus).

The content of polysaccharides in the leaves of the *Crassula ovata* was determined by anthrone-sulfuric acid method. Glucose was used as a standard and the absorbance was measured at a wavelength of 626 nm. The absorbance A as the ordinate, the amount of glucose as the abscissa linear regression calculation, the glucose standard curve regression equation as: $y = 7.075x + 0.0009$ $R^2 = 0.9939$.

The fresh leaves were dried and crushed and 2.0 g powder was weighed, placed in a 100 ml beaker. Water was added at a ratio of 1 : 5 - 1 : 25, and extracted 30 - 150 min at 30 - 70 constant temperature water bath. After cooling, the solution was precipitated with 50 - 90% ethanol solution for 30 - 150 min, centrifuged, and the precipitate was dissolved in distilled water to 100 ml. The absorbance was measured at the wavelength of 626 nm, the polysaccharide content was calculated according to the regression equation, and the extraction rate was calculated as:

$$\text{Extraction rate (\%)} = \frac{\text{Xmg} \times 100\ \text{ml}}{0.2\ \text{ml} \times 2000\ \text{mg}} \times 100\%$$

X: The polysaccharide content in the sample (mg).

The effects of various factors on the extraction rate of polysaccharides from *Crassula ovata* were studied by single factor experiment with the ratio of material to liquid, extraction time, water bath temperature, ethanol concentration and ethanol precipitation time, and the best level of each factor was selected.

On the basis of single factor experiments, the extraction rate of polysaccharides from *Crassula ovata* was taken as the index, the orthogonal test was used to optimize the extraction process of polysaccharides from *Crassula ovata*. The ratios of material to liquid, extraction time and water bath temperature were selected as single factors. The $L_9(3^4)$ orthogonal optimization experiment was carried out on the basis of single factor experiments. The setting of each single factor is presented Table 1.

Table 1. Factors and levels of orthogonal test.

Level	Water bath temperature (ºC)	Extraction time (min)	Ratio of material to liquid
1	50	90	1:5
2	60	120	1:10
3	70	150	1:15

Results and Discussion

The microstructure of *Crassula ovata* (Mill.) Druce leaves were composed of upper and lower epidermis, mesophyll and vascular bundles. The upper and lower epidermis had only one layer of cells, arranged closely, with thick outer wall and stratum corneum. Epidermal cells do not have chloroplasts. The shape of mesophyll cells was irregular and of loose arrangement (Fig. 1).

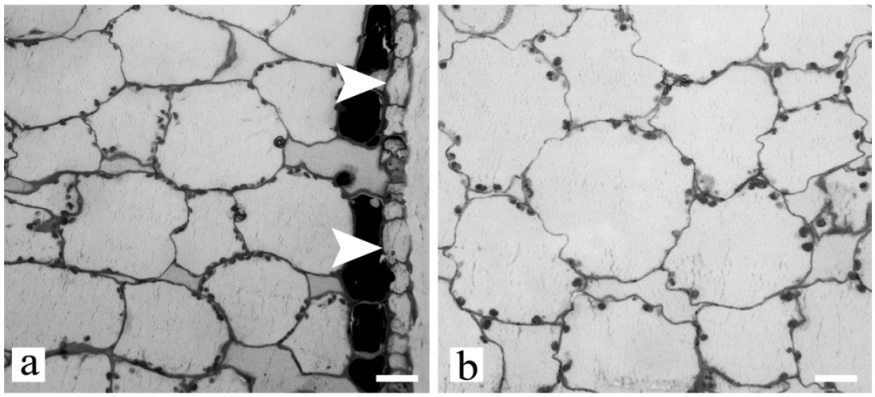

Fig. 1. The microstructure of *Crassula ovata* (Mill.) Druce leaves, a: cells of the epidermis (shown by arrow, Bar =100 μm), b: mesophyll cells (Bar =100 μm).

The stems microstructure of the *Crassula ovata* includes the epidermis, cortex and the vascular. The epidermis consists of a layer of cells in a square shape, arranged closely. Cortex exists in the epidermis and vascular bundles, composed of dozens of layers of parenchyma cells (Fig. 2).

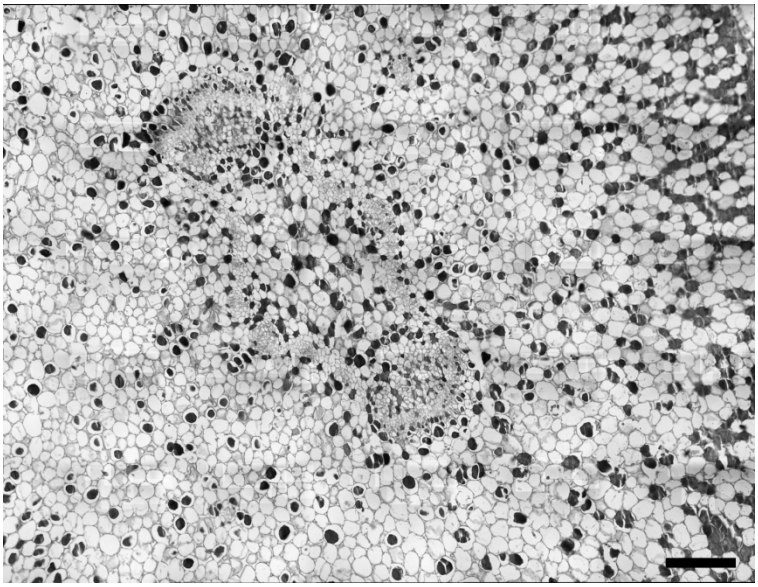

Fig. 2. The microstructure of *Crassula ovata* (Mill.) Druce stem (Bar = 50 μm).

The polysaccharide was localized by Schiff's reagent (PAS), the cells will be stained in purple if the polysaccharides exist in. The results showed that there was no change in the epidermal cells of the leaves after the PAS test, indicating that there was no polysaccharide distribution in the epidermal cells. The polysaccharides mainly distributed in mesophyll cells, histochemistry test showed that this part was stained in purple, the stain effect was significant (Fig. 3a).

The polysaccharides of the stems and the leaves of the *Crassula ovata* were chemically localized by paraffin section, frozen section method and histochemical method with Schiff reagent (PAS). The results showed that there were no polysaccharides in the epidermal cells of the stem, and the polysaccharides in the parenchyma cells were the main storage and accumulation sites, those cells were stained in purple and the staining effect was remarkable. There was no polysaccharide distribution in the vessel elements and sieve tube elements (Fig. 3b).

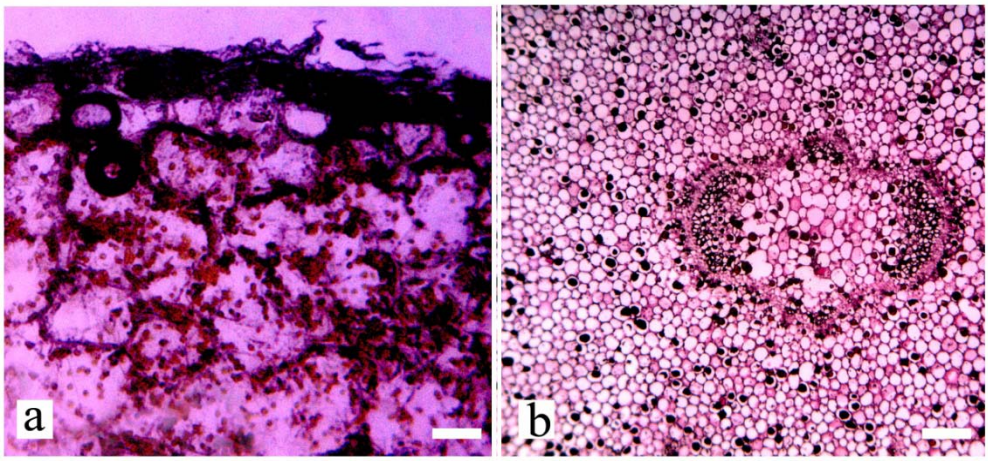

Fig. 3. Histochemical test (PAS) for polysaccharides, a: leaves (bar = 100 μm), b: stem (Bar = 50 μm).

The chemical composition which produced in plants can be divided into two categories: primary metabolites and secondary metabolites. Primary metabolites are carbohydrates, proteins and lipids. Secondary metabolites are substances produced by plants during energy metabolisms, such as glycosides, flavonoids, alkaloids, volatile oils, and resins. The previous studies had reported that the primary metabolites and secondary metabolites in these plants have some medicinal effects, to explore the storage of polysaccharides in plants and to study its medicinal value plays a vital role for human health (Jia *et al*. 2016).

Metabolites in plants have their specific synthesis sites, transport routes and storage sites. In different groups of plants, because of their genetic characteristics and internal structure of the different characteristics, the synthesis of metabolites and the storage location are different. Such as the distribution of polysaccharides in different plants. The contents of polysaccharides in tomato anthers increase and decrease regularly with the development of anthers and the location of the polysaccharides is constantly changing (Yun and Huiqiao 2015). The polysaccharides in the exocarp and the endocarp of *Lycium barbarum* fruits showed a tendency to increase with maturity (Zhao *et al*. 2015).

The histochemical method has been widely used to determine the site of chemical constituents in plants, and this method is easy to operate and the results are obvious. In recent years, the localization of chemical constituents in plants, such as saponins, flavonoids and ginsenosides has been studied with this method. According to the anatomical structure of vegetative organs of *Bupleurum chinense*, the storage site of saikosaponin in the vegetative organs of *Bupleurum chinense* was determined by the solution of magnesium acetate in acetic acid as the color reagent, and the storage site of flavonoids also were determined in *Bupleurum chinense* vegetative organs with the magnesium acetate solution in methanol (Liang *et al*. 2013). Ginsenosides were mainly

distributed in the pericarp, phloem and secretory ducts in the roots of *Panax quinquefolium*. In *Panax ginseng* root, ginsenosides were found in the phloem, the parenchyma cells near the xylem vessels and xylem ray cells. *Gynostemma pentaphylla* ginsenosides are mainly distributed in the assimilation tissue of vegetative organs and phloem parenchyma cells, the epidermis and periderm also have a small amount of distribution (Zheng and Li 1986).

Table 2. The orthogonal experimental results of extracting polysaccharide from *Crassula ovata.*

No.	Water bath temperature (ºC)	Extraction time (min)	Ratio of material to liquid	Extraction rate (%)
1	1(50)	1(90)	1(1:5)	1.704
2	1(50)	2(120)	2(1:10)	1.435
3	1(50)	3(150)	3(1:15)	1.463
4	2(60)	1(90)	2(1:10)	1.636
5	2(60)	2(120)	3(1:15)	1.930
6	2(60)	3(150)	1(1:5)	1.509
7	3(70)	1(90)	3(1:15)	1.810
8	3(70)	2(120)	1(1:5)	1.382
9	3(70)	3(150)	2(1:10)	1.619
K_1	1.534	1.717	1.532	
K_2	1.692	1.582	1.563	
K_3	1.604	1.530	1.734	
R	0.158	0.187	0.202	

From the orthogonal Table 2 it is apparent that the optimal extraction rate was 1.930%. The optimum extraction conditions were as follows: ratio of material to liquid 1 : 10, extraction time 120 min, extraction temperature 60ºC, ethanol concentration 70%, ethanol precipitation for 30 min.

Previously, the research on the presence of polysaccharides in plants had also used the histochemical method; polysaccharides can be identified by the PAS reaction. The principle of PAS reaction is that the strong oxidizing agent periodic acid can destroy the carbon chain of the polysaccharide molecules and produce the aldehyde group which is very stable and without any further oxidation. The aldehyde group can fully react with the Schiff reagent and produce the insoluble purple precipitation (Sobin *et al*. 1992, Gallão *et al*. 2013). According to this principle, many research works had been carried out on the presence of polysaccharides in many species of plants. For example, polysaccharides are stored at multiple sites in the rhizomes of *Rheum palmatum* (Zhang and Li 2010). Histochemical localization of polysaccharides in different organs of *Dendrobium officinale* showed that the polysaccharides mainly existed in the parenchyma cells of the stems, the cortical cells of the roots and the mesophyll cells of the leaves (Lv *et al*. 2007). Histochemical localization of polysaccharides in different plants showed that plant polysaccharides are usually found in the parenchyma and cortex of plants, but the amount of the polysaccharides, the time of production and the specific location of the polysaccharides are different due to the different plants. The use of PAS reaction on the roots of *Polygala tenuifolia* polysaccharides histochemical localization showed that the secondary phloem parenchyma cells were stained in purple, indicating that these sites contained polysaccharides (Teng *et al*. 2009).

The presence of polysaccharides in the leaves and stems of *Crassula ovata* was studied by paraffin section, frozen section method and the method of histochemistry (PAS staining). The results showed that there were no polysaccharides distributed in the epidermips of leaves, and the polysaccharides in leaves were mainly distributed in mesophyll cells. No polysaccharides in the epidermal cells of the stems, and the cortical parenchyma were the main accumulation site of the polysaccharides. This study provided a theoretical basis for the localization of polysaccharides in plant cells and further research is needed to find out the function and bioactivity of polysaccharides from *Crassula ovata*.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No.: 31300157, 31401435).

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(Manuscript received on 20 January, 2017; revised on 15 October, 2017)