Bioelectrochemistry 112 (2016) 153–157

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Investigation of the metabolic consequences of impregnating spinach leaves with trehalose and applying a pulsed electric field

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ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form 10 February 2016
Accepted 21 February 2016
Available online 24 February 2016

Keywords:
Vacuum impregnation
Pulsed electric field
Spinach leaves

ABSTRACT

The impregnation of leafy vegetables with cryoprotectants using a combination of vacuum impregnation (VI) and pulsed electric fields (PEF) has been proposed by our research group as a method of improving their freezing tolerance and consequently their general quality after thawing. In this study, we have investigated the metabolic consequences of the combination of these unit operations on spinach. The vacuum impregnated spinach leaves showed a drastic decrease in the porosity of the extracellular space. However, at maximum weight gain, randomly located air pockets remained, which may account for oxygen-consuming pathways in the cells being active after VI. The metabolic activity of the impregnated leaves showed a drastic increase that was further enhanced by the application of PEF to the impregnated tissue. Impregnating the leaves with trehalose by VI led to a significant accumulation of trehalose-6-phosphate (T6P), however, this was not further enhanced by PEF. It is suggested that the accumulation of T6P in the leaves may increase metabolic activity, and increase tissue resistance to abiotic stress.

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1. Introduction

The impregnation of leafy vegetables with cryoprotectants using a combination of vacuum impregnation (VI) and pulsed electric fields (PEF) has been proposed by our research group as a method of improving their freezing tolerance [15]. However, achieving a uniform distribution of the cryoprotectant in the highly heterogeneous structure of the leaves is challenging. Furthermore, the mechanisms involved in freezing tolerance are not yet fully known. It is thus important to broaden our knowledge on these two aspects for process optimization, and possible application of the technology to a wider range of plant tissues.

Complete removal of the air from the spongy mesophyll and intercellular spaces of the leaves and its replacement with the solution containing the cryoprotectant (e.g., an isotonic solution of trehalose) can only be achieved by optimizing the VI parameters [5]. Subsequent application of PEF should lead to homogeneous electroporation of the different cells distributed in the different tissues through the cross section of the leaf, ensuring that the cryoprotectant is located in both the intra- and extracellular spaces [15]. The technological challenges of electroporation have been investigated through mathematical modelling by Dymek et al. [2] using both fresh and impregnated spinach leaves as model systems. The discrepancies found between the model and experimental data were attributed to air remaining in the structure of the leaves after VI [19]. Total replacement of the air by the solution may not be possible, as recently speculated by Panarese et al. [14], who showed that oxygen-dependent metabolic processes such as photorespiration still took place in the impregnated spinach leaf after impregnation.

To the best of our knowledge, no other studies have been carried out to investigate the metabolic responses of plant tissues to a combination of VI and PEF. Remaining air in the spinach tissue after VI is believed to cause an increase in metabolic activity [14]. Interestingly, the catabolism of the impregnating sugars taken up by the cells through the corresponding cell membrane transporters and/or endocytosis has also been described [14,20]. The metabolic consequences of a possible increase in the concentration of sugars in the intracellular space and/or stress responses due to electroporation after impregnation remain to be explored.

In this study, we evaluated the efficiency of the VI process in spinach leaves by visualizing any remaining air in their structure with X-ray

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http://dx.doi.org/10.1016/j.bioelechem.2016.02.006
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microtomography. We then used isothermal calorimetry to investigate the effects on the metabolic activity of the leaves caused by the combination of VI and PEF. These measurements were complemented with the analysis of trehalose-6-phosphate (T6P) as a marker of increased trehalose concentration in the cells, and a possible signalling molecule for metabolic response to abiotic stress [3,7,8,10,23].

2. Material and methods

2.1. Raw material and sample preparation

Baby spinach leaves (*Spinacia oleracea*) were grown in a greenhouse at a temperature of 21 °C with a photoperiod of 14 h. Seeds were planted 1.5 cm deep in the soil, in trays with 1.5 cm between the plants in a row and 3 cm between the rows. The plants were watered daily. Leaves 6.0 ± 0.5 cm long and 2.5 ± 0.3 cm wide were harvested in the morning (always at the same time of the day). Leaves from the centre of the plant, which were not shaded by any other leaves, were chosen. The leaves were transported to the lab packed in plastic bags. They were then rinsed with deionized water, and samples 3 cm long and 8 mm wide were immediately cut from the central part of the leaf parallel to the main vein (but not including the vein) with a scalpel. These samples were used for the calorimetric measurements.

The leaves used in the X-ray microtomography experiments were collected from a greenhouse of a local grower (Mechelen, Belgium) 1 h after harvesting, and stored at 4 °C in sealed plastic bags. Leaves of 5.5 ± 0.5 cm length were selected and used in the experiments within 3 days of harvesting.

2.2. X-ray microtomography

A square sample measuring 5 mm × 5 mm was cut close to the main vein half-way along the main vein's length. The sample was removed from the leaf just before the X-ray scan. Three replicate samples were prepared and analysed.

Each sample was placed on a polystyrene foam mounting stage with the adaxial side of the leaf in contact with the stage. A layer of Parafilm® was gently wrapped around the lower surface of the sample and the stage to avoid sample dehydration, and to keep the sample in a vertical position during the scan. Samples were scanned 15 to 60 min after the end of VI.

The samples were scanned using a SkyScan1172 high-resolution X-ray micro-CT system (Bruker, microCT, Kontich, Belgium), operating at 60 kV and 167 μA. The experimental conditions were optimized to obtain high-quality radiographic projection images, while considering the contrast and resolution, as well as manageable scanning times (15 min per sample). The X-ray shadow projections from each angular views obtained with a pixel size of 4.9 μm were captured on a 2000 × 1048 CCD X-ray camera and averaged from 3 frames as the object rotates on a high precision stage with 0.4° rotation step up to a rotation angle of 180°. Cross section images (slices) were obtained using NRecon 1.6.2.0 (Bruker microCT, Kontich, Belgium) tomography reconstruction software. Contrast of the images was enhanced using beam hardening correction, ring artefact reduction and smoothing values of 35%, 8 and 2 respectively. To standardize the greyscale range of the 8-bit bitmap output images, the linear attenuation coefficient range (dynamic range) was set to 0–0.122. This resulted in a 3D stack of 950 virtual sections, each consisting of 1244 × 344 pixel images with an isotropic voxel size of 4.9^3 μm^3.

A segmentation procedure was applied to separate solid voxels from gas filled spaces or voids. The most straightforward technique to segment a grey scale CT image is to define a global threshold value located at an obvious and deep valley in the histogram of grey level frequencies. Voxels with a grey value lower and higher than that threshold value are considered to be background or air spaces, such as pores and object (tissue) respectively [6]. A global threshold of 49 was decided from the Otsu threshold of different datasets. This was checked visually and applied to all the datasets recorded. The segmentation procedure was implemented in CTAn 1.9.1.0 (Bruker, microCT, Kontich, Belgium). In order to exclude the damaged cells as a result of the cutting of the sample, the image datasets were virtually cropped, removing about 100 pixels (0.5 mm) from each cut edge, as shown in Fig. 1. The porosity of the leaf was quantified as the % total volume of pores with respect to the total volume of the sample.

2.3. Vacuum impregnation

The concentration of the trehalose solution isotonic with the leaves was experimentally determined by immersing the leaves in a series of solutions of different concentrations. The concentration leading to neither weight loss nor weight gain, namely 11% trehalose, was used. Based on preliminary experiments to establish the maximum weight gain and to avoid visible damage to the spinach tissue, an impregnation protocol with a minimum absolute pressure of 15 kPa was chosen. Samples cut from the spinach leaves were immersed in the trehalose solution and placed in a vacuum chamber connected to a vacuum controller (S.I.A., Bologna, Italy) and a vacuum pump. The pressure was decreased to 15 kPa over 3.5 min, after which it was kept constant at 15 kPa for 1 min, and subsequently increased to atmospheric pressure over 4.5 min. This cycle was carried out twice.

2.4. Pulsed electric field treatment

The parameters providing reversible electroporation have been determined in preliminary experiments with fluorescence microscopy [1], using propidium iodide as an electroporation indicator and fluorescein diacetate as a cell viability indicator. The parameters were optimized for untreated leaves and for leaves previously vacuum impregnated with trehalose.

A PEF was applied to the sample inside the calorimetric ampoule by equipping the ampoule with two stainless steel electrodes separated by 0.5 cm. The leaf was placed between, and parallel to, the electrodes, as shown in Fig. 2. Deionized water (18 mL), adjusted with NaCl to a conductivity of 600 μS/cm, was injected into the ampoule with a syringe before the PEF treatment was applied. The electrodes were then connected to the pulse generator (Arc Aroma Pure, Lund, Sweden). Fifty, square, monopolar pulses of 250 μs duration and 200 V amplitude, with an

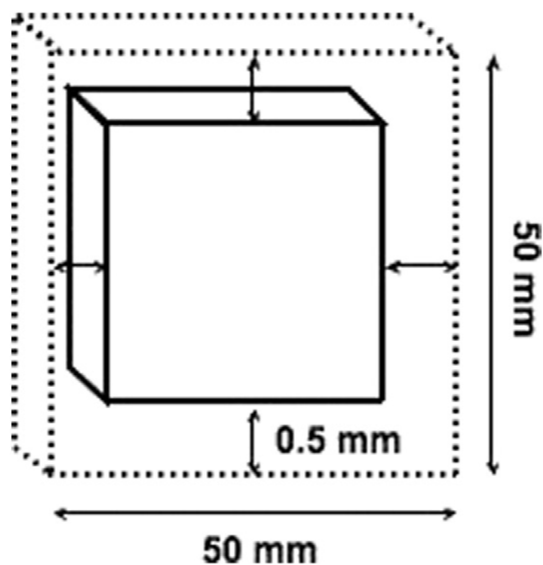

Fig. 1. Trimming operation in the samples. To exclude cells possibly affected by sample preparation, a portion of 0.5 mm was virtually cropped from the sample boundaries for every dataset prior to image analysis.

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