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Microscopic visualization of Pulsed Electric Field induced changes on plant cellular level

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ABSTRACT

The effects of Pulsed Electric Fields (PEF) on protoplasts from cultured tobacco cells (*Nicotiana tabacum* b.y.-2) in comparison to the changes on cultured plant cells with cell walls were visualised in order to study the direct impact of PEF on cell components and to clarify the influence of the cell wall on electroporation. Optical microscopic analyses were carried out and images were recorded during PEF treatment. Results showed higher sensitivity of protoplasts to electric fields related to cells with a cell wall. Protoplasts sizes were measured before and after different treatment intensities and protoplasts shrinkage was used as an indicator for cell rupture. It could be demonstrated that cell volume decrease is influenced by PEF intensity, initial cell size, cell orientation in the electric field and nucleus position.

Industrial relevance: Since the beginning of the 20th century the relevance of Pulsed Electric Fields (PEF) technology in food- and biotechnology has increased substantially. However, the mechanism of membrane permeabilization and the PEF induced changes in cell structure remain poorly understood, diminishing the optimal use in food industry. In this study the direct effects of PEF on cultured plant cell material and influencing factors of the degree of membrane disintegration were visualized and identified. The development of new methods to examine cell vitality shall help to convert the basic knowledge into effective processes.

1. Introduction

During the 1990's, industrial interest in developing gentle food technologies, to replace the currently common thermal processing, has increased substantially. The non-thermal application of Pulsed Electric Fields (PEF) is counted among these emerging processes and received considerable relevance in bio- and food technology. It involves the exposure of biological cell material to short repeated pulses of a high voltage with the result of pore formation in cell membrane leading to membrane permeabilization and cell rupture. The benefit of this approach is an important aspect of process and product development because it is aimed to protect quality food attributes, such as sensory quality and nutrition value, as well as to control the microbial safety with minimal or no changes during processing. Besides the use of non thermal technologies to inactivate microorganisms through mechanical destruction of cellular structure (Ho & Mittal, 2000; Wouters, Alvarez, Angersbach & Knorr, 2001; Heinz, Alvarez, Angersbach & Knorr, 2001) the main field of interest is the treatment of plant cell material, for cell membrane disruption leading to increased membrane permeability and to improved mass transfer of inner liquid and cell components (e.g. health related plant metabolites, pigments) from the intracellular vacuoles. Processes such as drying, osmotic dehydration and extraction are facilitated by PEF treatment (Angersbach, Heinz & Knorr, 1998; Ade-Omowaye, Angersbach, Taiwo & Knorr, 2001). The application of osmotic dehydration can be used as a pre-treatment to conventional drying, or freezing for the enhancement of diffusion characteristics with simultaneous maintenance of fruit product attributes and the reduction of energy consumption. Prior PEF treatment intensifies the desired effect due to the improvement of water and solution mass transfer into and out of the tissue. Rastogi, Eshtiaghi and Knorr (1999) investigated the impact of PEF on the dehydration characteristics of carrots and found out that PEF processing of carrot cubes caused a lowering of moisture content during osmotic dehydration. Further successful results have been gained concerning apple slices (Taiwo, Angersbach & Knorr, 2002), mango (Tedjo, Taiwo, Eshtiaghi and Knorr, 2002), and bell peppers (Ade-Omowaye, Rastogi, Angersbach & Knorr, 2002).

Several studies reported on the gentle recovery of sensitive vacuole components such as flavours and dyestuffs in different plant food and on the increase of extraction yield after PEF processing (Eshtiaghi & Knorr, 1999; Bouzrara & Vorobiev, 2000; Bazhal, Lebovka & Vorobiev, 2001; Guderjan, Toepfl, Angersbach & Knorr, 2005). These low energetic cost and short treatment time applications offer alternative possibilities to thermal processes and therefore to optimize process control on food sector. Additionally, the employment of mild heat can be used to intensify the desired target and

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therefore to obtain synergistic effects with the combination of both treatments (Schilling et al., 2008). Another benefit of PEF processing lies in the treatment with mild conditions to induce the production of secondary metabolites with the maintenance of cell viability. The stimulation caused by PEF can be used to target influence the plant for the generation of health related compounds as a stress response of the electric field (Guderjan et al., 2005).

The most accepted theory about the permeabilization mechanism is conceived to be related to electrocompression of the cell membrane (Zimmermann, 1986). Due to the electric field, accumulation and attraction of oppositely charged ions on both sites of the non conductive cell membrane occur, causing membrane thickness reduction. With further increase in the transmembrane potential, as a consequence of the increased electric field, a critical value is reached and membrane compression is intensified leading to the formation of pores and the loss of semi-permeability in the cell membrane. This pore formation can be temporary (reversible) or irreversible (permanent), depending on treatment intensity and sample composition (Zimmermann, 1986). Reversible pore formation takes place when the external electric field is removed while the critical membrane potential is reached and the generated pores are small related to the membrane surface. Characteristic for the processing with mild PEF conditions is that the cell retains its viability in contrast to high energy input treatment (for plant cells, $E \geq 1$ kV/cm), which results in the loss of cell vitality. The effectiveness of PEF technology to permeable cell membranes depends on several factors which can be classified in technical and chemical process conditions as well as in biological product characteristics. The technical factors include PEF process parameters such as electric field intensity, treatment time, pulse shape and applied energy, whereas the electric field intensity has been described as the most relevant factor defining membrane rupture by pulsed electric fields (Hamilton & Sale, 1967; Hülsheger, Potel & Niemann, 1981; Schoenbach, Joshi, Stark, Dobbs & Beebe, 2000; Zhang, Monsalve-González, Barbosa-Cánovas & Swanson, 1994; Tatebe, Muraji, Fujii & Berg, 1995). The chemical and physical characteristics of treated products have also an important impact on the efficiency of PEF. Further product parameters are the composition of treated media, including pH, temperature and especially ionic strength, which is responsible for the conductivity of treated media (Jayaram, Castle & Margaritis, 1993; Vega-Mercado, Pothakamury, Chang, Barbosa-Cánovas & Swanson, 1996). Biological characteristics such as species, size, shape or physiological state influence the degree of membrane permeabilization additionally. Therefore, small microorganism cells were found to be less sensitive against the external electric field, whereas membrane disintegration of larger plant cells occurs in markedly higher percentage by applying same PEF treatment conditions (Sale & Hamilton, 1967; Zhang, Monsalve-González et al., 1994).

Although background knowledge and theories about the PEF induced plasmolysis increased (Cruzeiro-Hanson, 1988; Dimitrov, 1984; Sugar & Neumann, 1984), the mechanism of membrane permeabilization and the changes on cell level after PEF processing remain poorly understood, degrading the full potential use of this technology. One problem in the study of biological cells is that the dynamic process of electroporation is extremely rapid. Pore building occurs within 10 ns at sites where the membrane potential reaches/obtain 1 V (Dimitrov & Jain, 1984). This celerity causes difficulties in the visualization of pore formation and the subsequent exchange of intra- and extracellular compounds. Membrane recovery can be happen in a broader range, from 0.1 ms to 2.8 h (Ho & Mittal, 1996). Furthermore, research not only about effects of PEF on cell membranes but also on other cell materials as cell walls is still scarce. Bazhal, Lebovka and Vorobiev (2003) analysed textural parameters of PEF treated apples and reported that the electric field affects not only plasmalemma membranes but also the cell wall integrity. Moreover, the function of the cell wall as a possible protection for the cell against the electric field as well as the interaction between cytoplasm and cell wall during post permeabilization are largely unknown.

In the study undertaken, enzymatic protoplasts (cells without cell wall) have been prepared from a tobacco plant cell culture to create a model system for the analysis of PEF induced membrane changes on cell level. The cells were exposed to a microscope with an integrated PEF unit in order to visualize membrane disintegration not only afterwards but also during the period of PEF processing. To gain better inside of the permeabilization mechanism, specific methods for the indication of cell vitality of plant cell cultures after PEF treatment were developed. Additionally, protoplasts were compared with cell wall cells to analyse the impact of PEF on different cell types and to explain the role of cell wall in electric cell rupture.

2. Materials and methods

2.1. Plant cells and protoplasts

Cultured tobacco cells (*Nicotiana tabacum* L. cv Bright Yellow-2) (Takebe, Otsuki & Aoki, 1968; Mathur & Koncz, 1998; Nagata & Kumagai, 1999) were grown in MS medium (Murashige, 1962) for 7 days at 25 °C in the dark with reciprocatory shaking at 120 rpm.

For protoplast preparation, tobacco cells were vacuum filtered and 2 g fresh weight cells were resuspended in 10 ml solution of isotonic buffer W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glucose, pH 5.7) combined with a mixture of cellulolytic and pectolytic enzymes (0.01 g Rohament Cl, 0.1 g Rohament PL) (AB Enzymes, Darmstadt, Germany) for the residence time of 4 h. After digestion of cell wall components, the obtained spherical protoplasts were washed twice with 0.6 M mannitol. Isolated protoplasts were finally resuspended in 6 ml unbuffered isotonic mannitol solution to perform pulsed electric field treatment (Fig. 1). Buffer was excluded in order to render a low conductivity medium for PEF operation.

Pre-treatment of tobacco cells with cell wall was carried out with vacuum filtration and resuspension of 2 g cells in 6 ml mannitol solution before PEF processing. The measured conductivity of both cell types (protoplasts and cells with cell wall) mixed with mannitol was 3.3 mS/cm.

2.2. Tobacco cells

2.2.1. Staining

For the visualization of cell rupture from cells with cell wall after PEF processing, vital dyes were needed, to penetrate into permeabilized cells and indicate irreversible membrane disintegration. Therefore, freshly prepared solution (0.1%) of the vital dye Phenosafranine (dry content 80%, Sigma-Aldrich, USA) was added to cell suspension in a ratio 1:2 directly before treatment.

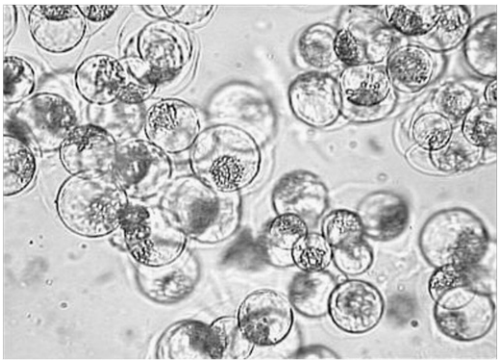

Fig. 1. Isolated protoplasts of seven-day-old *Nicotiana tabacum* cell suspension after enzymatic cell wall degradation.