Evaluation of Elicitor- and High-Pressure-Induced Enzymatic Browning Utilizing Potato (*Solanum tuberosum*) Suspension Cultures as a Model System for Plant Tissues

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Potato (*Solanum tuberosum*) suspension cultures were used as a model to obtain basic information regarding the occurrence of enzymatic browning reactions. The cultures were subjected to chitosan, an established stress factor, at concentrations from 2.5 to 100 μg/mL to examine stress reactions and to pressure treatments from 100 to 400 MPa for 10 min at 25 °C to monitor the impact of minimal processing on plant systems. The release of hydrogen peroxide from chitosan or high-pressure-treated potato cells, which constitutes an initial stress response, was correlated with activities of phenylalanine ammonia-lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) as well as with polyphenol production in cell free extracts. Chitosan-induced browning was related to PAL induction and corresponded to the concentration of phenols within the cells. Sensitivity of plant cells to these stress factors was affected by the amount of PO present in the cells. High PO levels in the potato cells resulted in a fast degradation of hydrogen peroxide and reduced PAL induction after chitosan treatment. Different degrees (reversible, irreversible) and locations (tonoplast, plasma membrane) of permeabilization of cell membranes led to polyphenol production, which was correlated to reaction rates of PPO and increased pressure treatments. Pressures higher than 150 MPa resulted in irreversible permeabilization of cell membranes and in loss of compartmentalization in the cells.

Keywords: *Plant cell cultures; stress response; phenylalanine ammonia-lyase; peroxidase; polyphenol oxidase; elicitation; high-pressure treatment; membrane permeabilization*

INTRODUCTION

Browning of damaged potato tissue is due to the activity of the enzyme polyphenol oxidase (PPO), which converts a variety of phenolic substrates to conjugated quinones, forming polyphenols (Matheis, 1987a,b). This browning is the result of processing-induced physical damage to the plant cells, in which naturally occurring monophenolic compounds interact with atmospheric oxygen and endogenous PPO enzymes are hydroxylated to *o*-diphenols and oxidized to *o*-quinones. These quinones may condense and react nonenzymatically to produce colored polymers and pigments.

High shear stress could lead to cell wall fracture, while high strain may cause excessive cell wall deformation. Both events can result in cell membrane damage thought to initiate enzymatic responses. Whether browning necessitates cell wall fracture, cell membrane damage, or cell membrane deformation is an important and mostly unresolved issue (McGarry et al., 1996).

Both the degree of compartmentalization within the plant cells and how decompartmentation takes place during stress reactions are particularly relevant. PPO, for example is believed to be located in plastid membranes (Vaughn and Duke, 1984) including the amyloplast, while phenols are assumed to be located in the vacuole (Matheis, 1987b). Browning reactions are dependent on the degree of enzyme/substrate interactions and on the integrity of the membranes separating them. Spychalla and Desborough (1990) showed that the integrity of cell membranes deteriorated during damage or storage of potatoes (stress), contributing to increased reaction between PPO and its substrates.

Phenolic compounds, together with the activity of PPO, are responsible for some phenomena affecting the color, taste, and nutritional value during the processing of plant products (Lattanzio et al., 1994). Food preservation procedures aimed to prevent postharvest losses and/or to retain quality factors of plant products commonly inactivate the cells and the deteriorative enzymes. There is, however, great interest in developing gentle preservation methods for foods to retain the freshness characteristics and extend the shelf life. Consequently, such minimal processing techniques aim to retain the cell viability (King and Bolin, 1989) which may lead to stress responses of the viable cells.

To reduce postharvest quality losses of plant products on a cellular level, a variety of factors such as knowledge of stress physiology, plant biochemistry, and the utilization of controllable model systems have to be considered. For example, the fact that high pressure induced hydrogen peroxide generation in cell cultures (Schreck et al., 1996) illustrates the importance of collecting more information on stress responses in plant cells and on the mechanisms induced by minimal processing of plant foods.

Plant cell cultures offer many advantages for examining correlations regarding enzymatic browning reactions because they are homeostatic and homogeneous. They are also comparable to plant cells in plant tissue with respect to endogenous substrates and enzymes as well as compartmentation by biomembranes. Plant cell cultures offer the possibility to study the impact of stress factors or unit operations on plant foods at the cellular level without interference from stress- or wound-related responses that occur in diced plant tissues after cutting or slicing (Knorr, 1994) or due to microbial contamina-

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tion. The use of cell suspension cultures provides a tool to focus on those physiological and biochemical processes that are directly caused by the stress factors applied.

It was the aim of this paper to demonstrate the effectiveness of plant cell cultures of potato (*Solanum tuberosum*) as a model system to compare biological and physical stress and damage applied by chitosan or high-pressure treatment on a controllable cellular level without stress and wound-response interference usually encountered when one works with cut/sliced/diced plant tissues.

MATERIAL & METHODS

Cells and Culture Conditions. *S. tuberosum* cell cultures were maintained in 200-ml flasks, in the dark, at 24 ± 0.2 °C, under continuous shaking at 100 ± 1 rpm. Approximately 5 g of collected cell cultures was used as inoculum in 60 mL of medium (Murashige and Skoog, 1962). The MS-medium was supplemented with 1 mg L^{-1} 2,4-dihydroxybenzoic acid and 20 g L^{-1} sucrose. The pH was adjusted to 5.8 with KOH. *S. tuberosum* cv. Bintje was initiated from potato tubers and established in suspension in 1994; *S. tuberosum* cv. Desiree culture was kindly supplied by L. Willmitzer, Max Planck Institut für Molekulare Pflanzenphysiologie, Golm, Germany.

Viability Tests. The determination of cell viability was performed by staining the cells with 0.025% Evans Blue solution after microscopic counting and evaluating the dead cells (Baker and Mock, 1994). The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by reductase was determined after incubation of 100 mg of cells (fresh weight) with 2 mL of 0.1% TTC solution and extracted with ethanol as reported by Towill and Mazur (1974). Cell growth was monitored after 14 days of incubation by obtaining fresh weights after filtration through a Buchner funnel.

Stress Determination. Spectrofluorometric determination of hydrogen peroxide production was performed by reduction of pyranine (Apostol et al., 1989) in culture medium (2 μg/mL) and was measured several times after stress induction (0, 10, 20, 30, 45, and 60 min after chitosan treatment; 0, 1, 2, 4, 6, and 24 h after high-pressure treatment).

Enzyme Extractions and Activity Assays. *Phenylalanine Ammonia-lyase (PAL).* Two grams of potato cells (fresh weight) and 3 mL of 0.5 M borax−borate buffer, pH 8.8, were homogenized three times with an Ultra-turrax for 30 s at 23 000 rpm. The cell homogenate was centrifuged 15 min at 25000*g*. Enzyme extraction was carried out on ice. The supernatant was filtered (5 μm) and used in enzyme assay. Five hundred microliters of the extract was added to 1900 μL of borax−borate buffer and 600 μL of 0.1 M L-phenylalanine solution and incubated at 37 °C. Change of absorbance at 290 nm between 15 and 75 min after enzyme addition was used to calculate PAL activity (Seitz et al., 1985).

Peroxidase (PO) and Polyphenol Oxidase (PPO). One gram of potato cells (fresh weight) was homogenized with 0.1 M phosphate buffer, pH 6.5, for 30 s. Cell homogenate was centrifuged 10 min at 25000g. Extraction procedures were carried out during 30 min in ice water. Enzyme extracts were diluted with buffer and used in peroxidase or PPO assay.

PO activity was determined in 5 mM hydrogen peroxide solution, 10 mM pyrogallol solution, and 50 mM phosphate buffer, pH 6.5, after addition of 100 μL of enzyme extract in a total reaction volume of 1000 μL. Enzyme assay was performed at 25 °C for 60 s and the absorbance measured at 420 nm.

PPO activity was measured in 1000 μL of 0.1 M catechol solution, 1950 μL of 0.1 M phosphate buffer pH 6.5, and 50 μL of enzyme solution. Enzyme activity was determined at 25 °C for 60 s and the absorbance measured at 420 nm.

One unit of enzyme activity represents one increment increase in absorbance per minute at these assay conditions (Asaka and Hayashi, 1991).

Protein content in the enzyme extracts was determined according to the Bradford (1976) method with bovine serum albumin as a standard.

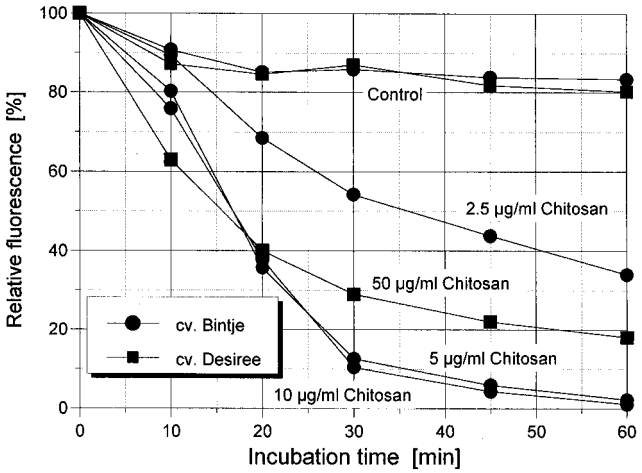

Figure 1. Stress response of two *Solanum tuberosum* cell varieties after treatment with chitosan elicitor determined by fluorescence reduction of pyranine dye.

Polyphenol Measurements. Polyphenol concentration was measured in cell-free extracts at 420 nm directly after enzyme extraction and several times during 4 h of incubation at 24 °C. Increase in absorption at 420 nm was regarded to be proportional to the polyphenol concentration in cell-free extracts. Immediate determination of polyphenol content at 420 nm in extracts after treatment gave also information on phenol release from the cultures. Increase in reaction rates was determined by the slope (*b*-value) in cell-free extracts immediately and 24 h after treatments as well as in culture medium after pressure treatment.

Chitosan Treatment. Water soluble chitosan hydroglutamate (BLV, Pronova a.s, Drammen, Norway) was used as an elicitor (Dörnenburg and Knorr, 1994) in the culture medium at several concentrations (2.5−100 μg/mL). Experiments were carried out with a cell inoculum of 10%. Stress was determined directly after treatment (0−60 min). Enzyme activities were measured 24 h after chitosan addition and incubation of cultures.

Hydrostatic Pressure Treatment. Cells were sealed in sterile bags (Whirl Pak, Nasco, Hamburg, Germany) and placed into additional polyethylene bags. Pressure treatment (100−400 MPa) was performed at room temperature for 10 min using a laboratory pressure unit (Diekers, Krefeld, Germany). Stress was determined 1−24 h after treatment and enzyme activities were measured directly after treatment in cell-free extracts and culture medium and after 24 h of cell incubation in cell-free extracts.

RESULTS AND DISCUSSION

Chitosan-Induced Stress Responses. Treatment of suspension cultures of potato *S. tuberosum* (cv. Bintje and Desiree) with chitosan, an elicitor-active polysaccharide and stress factor (Dörnenburg and Knorr, 1994), resulted in immediate production of hydrogen peroxide which was dependent on chitosan concentration. *S. tuberosum* cv. Bintje cultures seemed to be more sensitive to chitosan treatment than cv. Desiree cultures as indicated by a higher stress response at lower chitosan concentrations (Figure 1). Concentrations of chitosan below 50 μg/mL did not initiate hydrogen peroxide synthesis in Desiree cells. Chitosan concentrations above 10 μg/mL, however, resulted in fast reduction of fluorescence after treatment in Bintje cells. Reduction in cell viability was observed at 50 μg of chitosan/mL of medium for Bintje cultures and at 100 μg for Desiree cultures (data not shown). This led to a decrease of polyphenol production in the cell extracts (Figure 2).

The activities of the phenol-oxidizing enzymes PO (data not shown) and PPO were relatively constant

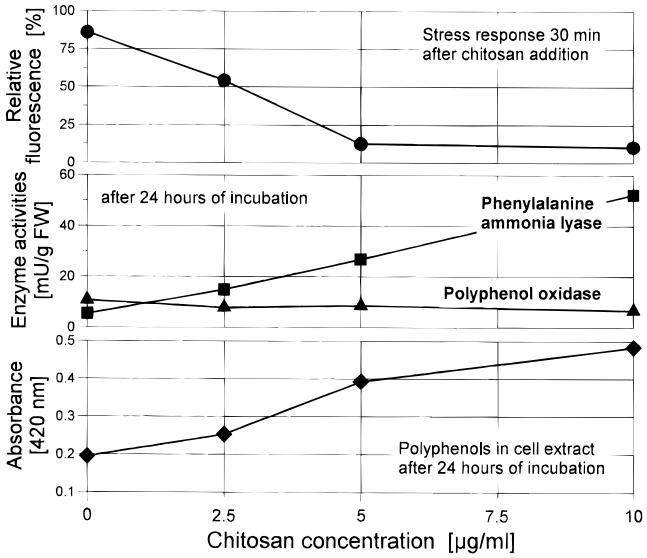

Figure 2. Effect of chitosan concentration on stress responses in *Solanum tuberosum* cv. Bintje cells, enzyme activities and polyphenol content in cell homogenates.

Table 1. Initial Activities of Indicator Enzymes in *S. tuberosum* Cell Varieties (A) and Phenylalanine Ammonia-lyase Activities (PAL) after 24 h of Elicitation with Chitosan (B), (n = 4)[a]

(A) Indicator Enzymes

	activity (munits/g of FW)	
enzymes	Bintje	Desiree
PAL	5.33 ± 0.61	4.99 ± 1.06
PO	155 ± 15	255 ± 28
PPO	42.2 ± 1.3	38.9 ± 0.1

(B) Phenylalanine Ammonia-lyase

chitosan (µg/mL)	PAL activity	
	Bintje	Desiree
2.5	2.8-fold	1.6-fold
5	4.2-fold	1.5-fold
10	4.9-fold	1.4-fold
25	6.5-fold	1.7-fold
50		2.8-fold

[a] PAL, phenylalanine ammonia-lyase; PO, peroxidase; PPO, polyphenoloxidase.

within 24 h after treatment (Figure 2) and were thus not induced. The data suggest that these oxidases cannot be regarded as stress indicators in cultured potato cells or that chitosan is not an active elicitor for the synthesis of these enzymes.

Initial enzyme activities in *S. tuberosum* cv. Bintje and Desiree show the differences between these potato varieties (Table 1A). Higher peroxidase levels in Desiree compared to Bintje might be the reason for the increased resistance of the former cell line to chitosan treatment (Figure 1), as plant survival against potential cytotoxic effects is dependent on the presence of reduced molecules or antioxidative enzymes (Piqueras et al., 1996). These proteins can aid in the prevention of cellular damage resulting from oxidative stress. For example, stress-generated hydrogen peroxide is removed via catalase or peroxidase (Kumar and Knowles, 1993; Piqueras et al., 1996).

Generation of hydrogen peroxide is known to be a second messenger in the stress signal cascades (Apostol et al., 1989). Additionally to hydrogen peroxide generation in plant cells and subsequent degradation of

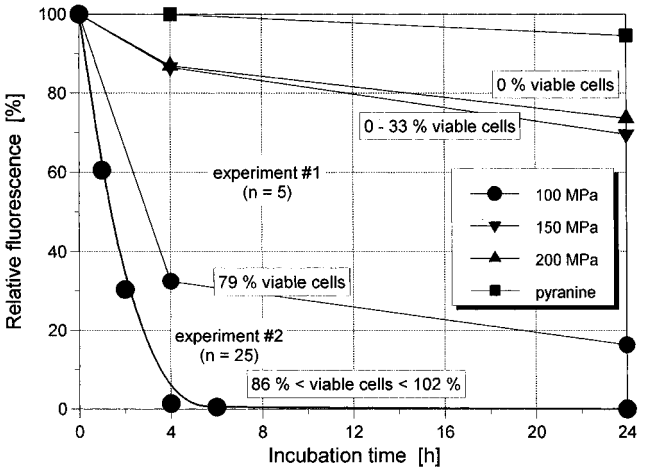

Figure 3. Time dependent fluorescence degradation of pyranine dye after high pressure treatment and viability of *Solanum tuberosum* cv. Desiree cells.

fluorescence after elicitor treatment (Figure 1), there are also long-term responses involving activation of genes responsible for the production of defense-related enzymes, e.g. PAL (Orr et al., 1993; Table 1B). This indicates a connection between the possibility to induce PAL (Table 1B) and the polyphenol concentration in extracts of both potato cell cultures (data not shown), which could correspond to the amount of PO activity present within the potato varieties (Table 1A).

Increase in PAL activity (Table 1B; Figure 2) and constant PPO levels during 24 h of incubation of Bintje cell cultures indicated an increase in phenol synthesis, which led to higher polyphenol production after loss of cell compartmentation in cell extracts depending on chitosan concentration (Figure 2). Homogenization of the plant cells resulted in a loss of cell compartmentation, allowing interactions of phenolic substrates and PPO and resulting in the synthesis of quinones that polymerized to polyphenols.

Pressure-Induced Stress Responses. Yahraus et al. (1995) have shown that the application of pressure results in mechanical forces acting at the cell wall/membrane level, which can also induce the rapid synthesis of hydrogen peroxide. The authors concluded that plant cells can 'sense' a mechanical perturbation at their cell surfaces and do respond. To confirm the ability of potato cells to respond to mechanical stimuli with induction of stress metabolites, cells were subjected to hydrostatic pressure.

High pressure (physical) induced stress seems to follow other ways of signal transduction than biological stress (Low and Merida, 1996) as demonstrated by a lag phase prior to hydrogen peroxide release from pressure-treated plant cell cultures (Schreck et al., 1996). Moderate pressure treatment at 100 MPa resulted in a delayed hydrogen peroxide production, dependent on the viability of treated cells in different experiments, which corresponded from 102 to 79% as compared to untreated potato cultures (Figure 3).

Permeabilization of Membranes by Pressure Treatment. Pressures of 150 MPa gave cell viabilities from 33% to total viability loss in the different experiments, whereas pressure treatment at 200 MPa generally caused loss in reductase activity (Figure 4). We confirmed that induced loss of compartmentation and subsequent release of the content of the vacuoles into the cytoplasma was induced at these pressures (Wille and Knorr, 1997) and assumed that the release of acidic

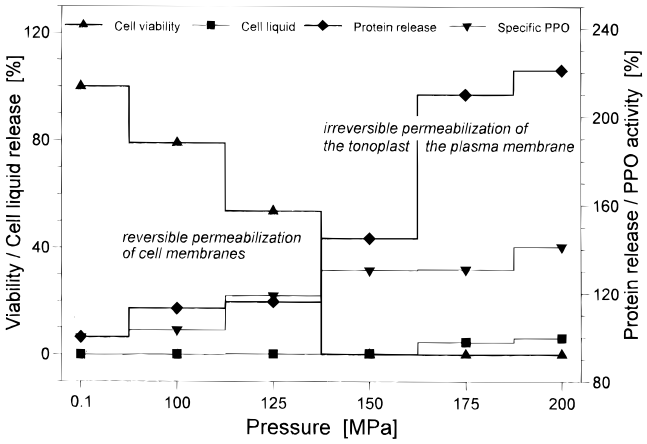

Figure 4. Course of cell parameters characterizing and indicating plant membranes permeabilization in Desiree cultures dependent on pressure treatment.

Table 2. Effect of High Pressure on Enzyme Activities and Polyphenol Production Measured in Culture Medium of Desiree Cells Immediately after Treatment ($n = 2$)[a,b]

pressure (MPa)	PO activity (%)	PPO activity (%)	poly-phenols (%)	PP production reaction rate[c]
0.1	100 ± 3.3	100 ± 1.3	100 ± 1.7	0.010
100	81 ± 1.7	136 ± 2.1	154 ± 2.8	0.036
125	89 ± 2.0	164 ± 1.3	181 ± 1.3	0.042
150	102 ± 0.7	247 ± 1.4	367 ± 3.2	0.067
175	109 ± 1.3	360 ± 2.6	556 ± 9.1	0.006
200	95 ± 2.5	441 ± 2.5	553 ± 5.7	0.009

[a] PO, peroxidase; PPO, polyphenol oxidase; PP, polyphenol. [b] 5−10 min after treatment. [c] [Δ Abs/h].

compounds and lytic enzymes from the vacuoles caused cell death (Dörnenburg and Knorr, 1993).

Additionally to the loss of viability at 150 MPa (Figure 4), increase in protein content in the medium (Figure 4) and the phenol release from the cells and its subsequent polymerization to polyphenols (Table 2) indicated an irreversible permeabilization of the tonoplast surrounding the cell vacuole. Irreversible permeabilization of the plasma membrane occurred during pressurization at 175 MPa as seen by an increase in protein release and in the loss of intracellular liquid into the culture medium (Figure 4) as well as in high polyphenol concentration (Table 2). Release of intracellular liquid after pressure treatment at 175 MPa indicated a decompartmentation of the cells leading to interactions between phenolic substrates located in the vacuoles and oxidizing/polymerizing enzymes, such as PPO. For example, in tomato cells, PPO has been reported to be compartmentalized in the chloroplast and separated from phenolic substrates. Consequently, only after disruption of plant tissue can, oxidation of phenolics by PPO be initiated (Constable et al., 1995).

Pressures between 100 and 200 MPa had no effect on PO activity in the culture medium, whereas PPO activity increased with higher pressure (Table 2; Figure 4). However, as PPO activity increased in the culture medium, the reaction rate of polyphenol synthesis as determined by the slope of regression lines was suppressed by the amount of polyphenols accumulated in the culture medium after pressure treatment at 175 and 200 MPa (Table 2). This indicates a feedback inhibition of PPO (Zawistowski et al., 1991) or the possibility of a substrate-limited enzyme reaction.

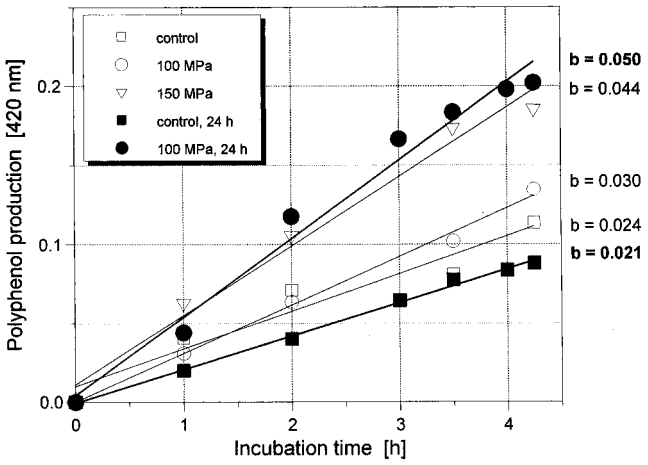

Figure 5. Effect of high-pressure treatment on polyphenol production in phosphate buffer extracts of Desiree cultures homogenized immediately after pressure treatment and after 24 h of incubation.

Loss of cell compartments in potato cultures was caused by high-pressure treatment at 150 MPa (Figure 4). Pressure treatment at 100 and 150 MPa resulted in an increased polyphenol production in cell extracts (Figure 5) possibly due to improved substrate or enzyme extraction caused by reversible or irreversible permeabilization of the cell membranes or to pressure activation of PPO.

Pressure-Induced Browning Reactions. The level of PPO activity was not relevant for the concentration of polyphenols produced in the enzyme extracts after 24 h of incubation of pressure-treated potato cells. The decrease of PO activity in the extract to 32.6% resulted in retarded polyphenol production in the untreated culture (Figure 5), whereas PPO activity increased to 120%. Increase of reaction rate of polyphenol production (2.4-fold) in 100 MPa treated cultures could be correlated to the increase in PO activity (2.4-fold). This might be induced by a higher content of phenolic substrates (Kim and Yoo, 1996), synthesized by PAL with high activities of 182% in untreated and 199% in pressure-treated potato cells (data not shown). High-pressure treatment at 100 MPa led to stress reactions in potato cultures from Desiree. Generation of hydrogen peroxide (Figure 3) as well as increase in polyphenol production (Figure 5) indicated the synthesis of stress enzymes and metabolites in plant cell cultures subjected to minimal processing procedures.

Conclusion. Chitosan-induced stress was followed by the induction of PAL, which is known to be rate limiting for the synthesis of phenolics in plant cell cultures. PO and PPO were not induced by chitosan treatment and could not be identified as stress enzymes. A summary of the proposed effects of chitosan or high-pressure treatment is given in Table 3. The intensity of stress reactions, expressed as hydrogen peroxide production, was dependent on the amount of PO in the culture species used, which acts as an antioxidative enzyme. PPO is responsible for the enzymatic browning reaction in potato cell cultures. However, enzymatic browning measured as polyphenol production was not affected by the level of PPO present but was highly affected by the amount of phenols present in the reaction medium. For the formation of polyphenols it was necessary to combine PPO and its substrates due to permeabilization of cellular membranes and decompartmentalization of the cells by homogenization or by application of high-pressure treatments. Browning

Table 3. Proposed Effects of Chitosan or High-Pressure Treatment on Stress Reactions, Enzymes, and Membranes in *S. tuberosum* cv. Desiree Cultures

	chitosan	pressure < 100 MPa	pressure > 125 MPa
stress response			
H_2O_2	induction	induction	no effect
substrates			
oxygen	no effect	no effect	no effect
phenols	induction	induction	no effect
enzymes			
PAL	induction	induction ?	no effect
PO	no effect	induction ?	(in)activation
PPO	no effect	induction ?	activation
compartmentation			
membrane permeability	no effect	reversible	irreversible

occurred when a physical stress sufficient to cause membrane damage was applied to the potato cells containing the required enzymes and substrates capable to produce polyphenols. The degree of browning in cell-free extracts correlated with PAL activity and subsequent phenol concentration in the potato cell cultures.

PAL also catalyzes the first step in the biosynthesis of defense-related phenylpropanoids, by synthesizing antimicrobial metabolites (e.g. phytoalexins). The high level of PO activity in potato cultures of Desiree seems to make them more resistant to stress factors. Observations and results regarding initial peroxidase activity indicated the involvement of this enzyme in cell protection due to its antioxidative activity. High activity of PO in plant cells can become important for the selection of potato varieties used for minimal processing, because of the reduction of deterioration of active oxygen species responsible for the degradation of quality-determining factors.

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