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Inhibition of lipoxygenase activity in lentil protoplasts by monoclonal antibodies introduced into the cells via electroporation

Mauro MACCARRONE, Gerrit A. VELDINK and Johannes F. G. VLIEGENTHART

Bijvoet Center for Biomolecular Research, Department of Bio-organic Chemistry, Utrecht University, Utrecht, The Netherlands

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The isolation of lentil protoplasts and the transfer of anti-lipoxygenase monoclonal antibodies into plant protoplasts by electroporation is reported. The dependence of the efficiency of monoclonal antibody incorporation on the field strength is shown as well. The transferred immunoglobulins retained their functional and structural integrity and were able to inhibit the intracellular target enzyme, with a linear relationship between inhibition of lipoxygenase activity and amount of incorporated monoclonal antibody. Moreover, the inhibition of lipoxygenase activity was well correlated with the increase of protoplast viability.

Plasma membranes form a permeability barrier against a variety of substances, including macromolecules like nucleic acids and proteins. In order to reversibly permeabilize cell membranes [1] and introduce non-permeant molecules into the cell, electroporation was used. This technique has found various applications (for a review see [2]), the most widespread being gene transfer into mammalian [3], plant [4] and bacterial [5] cells. More recently, electroporation has been used to transfer DNA/protein complexes [6], viruses [7], restriction endonucleases [8] and other proteins [9] into living cells. Finally, this technique has been successfully used to introduce antibodies into yeast [10] and mammalian [11–14] cells, leading to the possibility of carrying out biochemical and physiological studies on intact, living systems.

In the present investigation, the feasibility of transferring anti-lipoxygenase monoclonal antibodies into lentil (*Lens culinaris*) protoplasts was investigated, with the aim of gaining a better understanding of the physiological role of lipoxygenase.

Lipoxygenases comprise a class of non-heme-iron-containing dioxygenases, which oxidize unsaturated fatty acids with a 1*Z*,4*Z*-pentadiene system to conjugated hydroperoxy fatty acids. Lipoxygenases are widely distributed in nature among plants and animals (for reviews on plant lipoxygenases see [15–17]). Among others, roles in plant senescence [18] and in wound responses [19] have been proposed, suggesting a possible involvement of lipoxygenases in the control of plant-cell viability through the control of membrane integrity.

In this paper, we report the successful introduction of anti-lipoxygenase monoclonal antibodies into lentil protoplasts and the resulting inhibition of the target enzyme. Evidence for the improvement of cell viability as a result of inhibition of lipoxygenase activity is presented as well.

Correspondence to G. A. Veldink, Bijvoet Center for Biomolecular Research, Department of Bio-organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands

Abbreviation. GAM-AP, goat anti-mouse IgG conjugated with alkaline phosphatase.

Enzymes. Lipoxygenase (EC 1.13.11.12); cellulase (EC 3.2.1.4); pectinase (EC 3.2.1.15); pectolyase, mixture of endopolygalacturonase (EC 3.2.1.15) and endopectinlyase (EC 4.2.2.3).

MATERIALS AND METHODS

Materials

Chemicals were of the purest analytical grade. Methanol (Merck, Darmstadt, FRG) was of HPLC quality. Nitrocellulose filters (0.45 μm) were purchased from Bio-Rad (Richmond, CA, USA); octadecyl solid-phase extraction columns (6 ml) were from Baker (Deventer, The Netherlands). All media for plant culture and protoplast isolation were from Sigma (St. Louis, MO, USA). Soybean (*Glycine max*) lipoxygenase was purified according to [20, 21]. Lentil (*L. culinaris*) lipoxygenase, partially purified from lentil seedling homogenates by $(NH_4)_2SO_4$ precipitation followed by DEAE-Sephadex chromatography [22], was a kind gift from Dr. M. P. Hilbers (University of Rome 'Tor Vergata'). Cellulose from *Trichoderma viride*, pectinase from *Rhizopus sp.* and pectolyase from *Aspergillus japonicus* were from Fluka (Buchs, Switzerland). Anti-lipoxygenase monoclonal antibodies were produced at CIVO-TNO (Zeist, The Netherlands) using soybean lipoxygenase and a single hybridoma clone. They were purified according to [23]. Non-immune mouse serum was purchased from Nordic Immunology (Tilburg, The Netherlands) and goat anti-mouse IgG conjugated with alkaline phosphatase (GAM-AP) were from Bio-Rad (Richmond, CA, USA).

Protein determination

Protein concentration was determined according to [24], using bovine serum albumin as a standard.

Inhibition of soybean lipoxygenase and lentil lipoxygenase activities by monoclonal antibodies

The ability of monoclonal antibodies to inhibit soybean lipoxygenase and lentil lipoxygenase activities was determined

as described [22]. Soybean lipoxygenase (5 μg/ml) or lentil lipoxygenase (20 μg/ml) were incubated with different amounts of monoclonal antibodies (concentration range 0–300 μg/ml) in 0.1 M sodium phosphate, pH 6.8. After 1 h at 25°C, lipoxygenase activity was measured polarographically at 25°C in a solution of 1.8 mM linoleic acid in air-saturated 0.1 M sodium phosphate, pH 6.8. Measurements were performed with a CB1 oxygen monitor (Hansatech Ltd, Kings Linn, UK). Controls were by incubating soybean lipoxygenase or lentil lipoxygenase with 300 μg/ml non-immune mouse serum. Specific activity was expressed as μmol O_2 consumed · min^{-1} · (mg protein)$^{-1}$. To check whether the electroporation conditions could impair the ability of monoclonal antibodies to inhibit lipoxygenase activity, kinetic experiments were performed with monoclonal antibodies previously subjected to an electric pulse, delivered by a Gene Pulser apparatus (Bio-Rad) set at 125 μF and 1000 V/cm, in 0.4 cm electroporation cuvettes.

Plant material and protoplast isolation

Lentil (*L. culinaris*) seeds were sterilized in 70% (by vol.) ethanol for 1 min and 10% commercial bleach (NaOCl, 5% solution (mass/vol.) in water) for 15 min, then rinsed in sterile tap water. To germinate, seeds were transferred into culture jars containing Murashige and Skoog medium [25], with 2% (mass/vol.) sucrose and 0.8% (mass/vol.) agar. Seeds were grown at 25°C, in the dark, for 6 d, then leaves were excised from the seedlings, cut into small pieces (< 1 mm) and plasmolysed for 4 h in CPW medium [26], containing 13% (mass/vol.) mannitol and 2% (mass/vol.) $CaCl_2$.

Subsequently, the plasmolysis medium was replaced by the enzyme mixture consisting of CPW medium, 13% (mass/vol.) mannitol, 4% (mass/vol.), cellulase, 3% (mass/vol.) pectinase and 0.1% (mass/vol.) pectolyase, pH 5.6. After incubation for 16 h at 25°C in the dark, the crude protoplast preparation was filtered through two stainless-steel sieves (140 μm and 63 μm mesh, respectively), in order to remove undigested material. The filtrate was centrifuged for 5 min at 700 rpm in a Sigma 302K centrifuge, and the pellet was washed twice with CPW medium containing 9% (mass/vol.) mannitol. Finally, lentil protoplasts were resuspended in 10 mM Hepes, 50 mM NaCl, 5 mM $CaCl_2 \cdot 2\,H_2O$ and 0.4 M mannitol, pH 7.2 (solution A), counted in a Fuchs-Rosenthal chamber and brought to a final concentration of 1.5×10^6 lentil protoplasts/ml. This suspension was used for the electroporation experiments.

Electroporation and transfer of antibodies into lentil protoplasts

Aliquots of the lentil protoplasts suspension (0.7 ml, i.e. 10^6 lentil protoplasts/test) were placed in sterile disposable electroporation cuvettes of path length 0.4 cm (Bio-Rad), with or without monoclonal antibodies (250 μg/test). The final volume was adjusted to 0.8 ml with solution A. Samples were mixed by gentle inversion and electroporated at a capacitance of 125 μF and a resistance of 12 Ω and different field strengths, ranging over 0–1000 V/cm. A gene pulser apparatus, equipped with a capacitance extender device (Bio-Rad) was used to generate and deliver exponentially decaying pulses, with an average time constant (τ) of 1.5 ± -0.2 ms. The same conditions were applied in control experiments, performed by electroporating lentil protoplasts in the presence of non-immune mouse serum instead of monoclonal antibodies.

After electroporation, lentil protoplasts were allowed to remain in the cuvette for 5 min at room temperature. They were then washed twice in solution A by centrifugation for 5 min at 700 rpm, to remove non-incorporated antibodies. Each sample was then resuspended in 1 ml solution A and transferred to a petri dish and kept for 4 h at 25°C in the dark. Subsequently, lentil protoplasts were subjected to viability determinations, using the fluorescein-diacetate-staining procedure [27], or were centrifuged at 700 rpm for 5 min, resuspended in 0.1 M sodium phosphate, pH 6.8 and 0.1% (by vol.) Tween 20 and disrupted by two cycles of rapid freezing (−80°C) and thawing (25°C). The cell extracts were brought to a protein concentration of 1 mg/ml with resuspension buffer and aliquots were used for protein assay, enzyme activity and immunochemical determinations.

Lipoxygenase assay in lentil protoplasts

The activity of lentil protoplast lipoxygenase was measured using aliquots of lentil protoplasts extracts (300 μl/test). 13-Hydroperoxyoctadecadienoic acid, biosynthesized as reported [28], was added to each aliquot (5 nM final concentration) in order to eliminate the induction period necessary for the enzyme activity [17]. Samples were incubated for 1 h at room temperature in 10 ml 100 μM linoleic acid in 0.1 M sodium phosphate, pH 6.8, saturated with O_2. Incubations were stopped by bringing the solutions to pH 3.5 with 2 M HCl. The reaction products were then extracted with octadecyl solid-phase extraction columns as previously described [29], and their concentration was determined spectrophotometrically by recording the absorbance values of the hydroperoxides (hydroperoxyoctadecadienoic acid) at 234 nm, in a 8450A diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA). Calculations were made using a molar absorption coefficient of 25000 $^{-1} \cdot cm^{-1}$ for hydroperoxyoctadecadienoic acid at 234 nm [17]. Lentil protoplast lipoxygenase specific activity was expressed as pmol hydroperoxyoctadecadienoic acid formed · h^{-1} · (mg protein)$^{-1}$. Controls were made by assaying lentil protoplast lipoxygenase activity of both samples, boiled for 5 min and cell extracts incubated without linoleic acid.

Electrophoresis and Western blotting

Proteins were separated on 10% SDS/PAGE under reducing conditions [30], performed in a Mini Protean II apparatus (Bio-Rad) with 0.75 mm spacer arms. The electrophoretic runs were stopped as soon as the bromophenol blue dye reached the bottom of the gel, then electroblotting onto nitrocellulose filters was carried out in a Mini Trans Blot apparatus (Bio-Rad), following a published procedure [31].

Blots containing lentil seedlings homogenate (25 μg protein/lane) and fractions of lentil lipoxygenase partially purified by $(NH_4)_2SO_4$ precipitation (15 μg/lane) and DEAE-Sephadex chromatography (8 μg/lane) were reacted with monoclonal antibodies (diluted 1000-fold) as primary antibody, and with GAM-AP (1:3000) as second antibody [32].

The heavy chains of monoclonal antibodies incorporated into lentil protoplasts were detected according to [32], by reacting with the second antibody (GAM-AP, diluted 3000-fold) blots containing cell extracts (100 μl/lane) of lentil protoplasts electroporated in the presence of monoclonal antibodies.

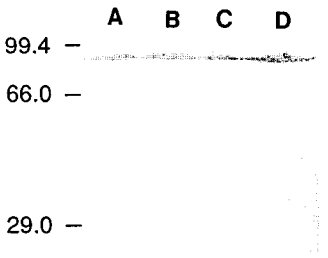

Fig. 1. **Western-blot analysis of lentil lipoxygenase.** Fractions of lentil lipoxygenase from different purification steps were analysed by SDS/PAGE and Western blot. Lentil seedling homogenate (25 μg; lane A) and partially purified lentil lipoxygenase from $(NH_4)_2SO_4$ precipitation (15 μg; lane B) and DEAE-Sephadex chromatography (8 μg; lane C) were reacted with anti-(soybean lipoxygenase) monoclonal antibodies. Purified soybean lipoxygenase (2 μg; lane D) was run as a control. Molecular-mass markers are: phosphorylase *b*, 97.4 kDa, bovine serum albumin, 66 kDa, carbonic anhydrase, 29 kDa. They are shown on the left-hand side.

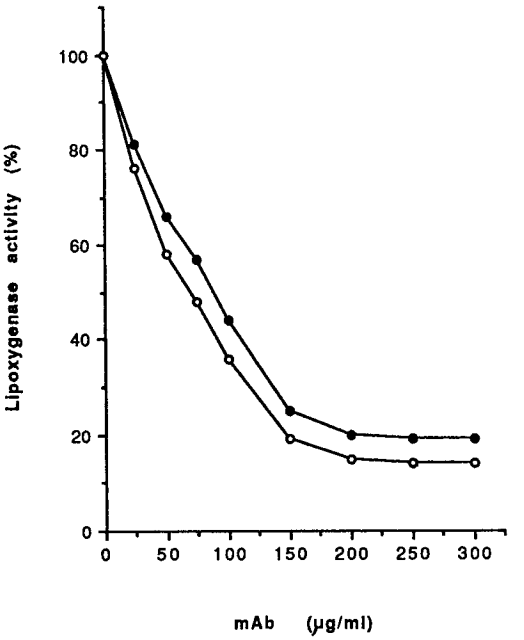

Fig. 2. Inhibition of lentil lipoxygenase by anti-(soybean lipoxygenase) monoclonal antibodies. Anti-(soybean lipoxygenase) monoclonal antibodies were able to inhibit both soybean lipoxygenase (○) and lentil lipoxygenase (●) activities, when incubated with the enzymes. Lipoxygenase-specific activities are expressed as a percentage of the control values, i.e. soybean lipoxygenase and lentil lipoxygenase incubated with non-immune mouse [6 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ (soybean lipoxygenase) and 1 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ (lentil lipoxygenase)].

Detection and quantitation of the incorporated antibody by ELISA

For detection and quantitation of the incorporated antibody, an enzyme-linked immunoadsorbent assay (ELISA) was performed, as previously described [33]. Lentil protoplast extracts (100 μl/well) were used to coat a polystyrene 96-well Nunc-ImmunoPlate (Gibco, Breda, The Netherlands) and were reacted for 1 h at room temperature with GAM-AP (diluted 3000-fold). The substrate for the alkaline phosphatase reaction was *p*-nitrophenylphosphate (0.5 mg/ml) in 0.5 M diethanolamine buffer, pH 9.8. After 30 min at room temperature the color development was stopped by adding 30 μl/well 3 M NaOH, then the plates were read at 405 nm in a model 2550 EIA Reader (Bio-Rad). The absorbance values of lentil protoplast extracts, corrected for the value of the sample incubated with monoclonal antibodies but without being given an electric pulse ($A_{405} = 0.05$), were used to quantitate the amount of monoclonal antibody incorporated into lentil protoplasts. A_{405} values for the unknown samples were compared with a calibration curve, drawn after subjecting different amounts of monoclonal antibodies (in the range of 0.00–0.25 μg/well) to the ELISA test. Controls included wells coated with different amounts of bovine serum albumin. Each data point reported in this paper is the mean of three independent determinations (SD < 5%).

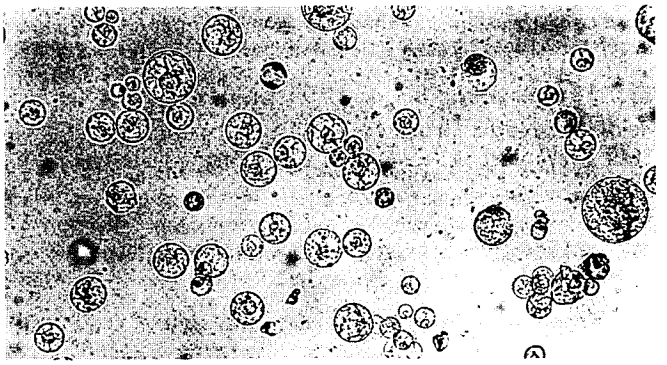

Fig. 3. Isolation of lentil protoplasts. Light micrographs (250×) of protoplasts isolated from lentil leaves.

RESULTS

Ability of monoclonal antibodies to recognize and inhibit lentil lipoxygenase

The anti-(soybean lipoxygenase) monoclonal antibodies are able to cross-react with lentil lipoxygenase, as shown by Western-blot analysis performed on lentil homogenate and partially purified lentil lipoxygenase fractions (Fig. 1).

The inhibitory capacity of monoclonal antibodies towards lipoxygenases was found to be approximately equal for lentil and soybean lipoxygenase (Fig. 2).

In order to verify whether the electroporation conditions could impair the inhibitory ability of monoclonal antibodies, experiments were carried out by incubating lipoxygenases with monoclonal antibodies previously subjected to an electric pulse of 1000 V/cm. The same profiles as shown in Fig. 2 were obtained, indicating that electroporation did not in any way damage the antibody's functionality. No decrease in soybean lipoxygenase and lentil lipoxygenase activities was found upon incubation with non-immune mouse serum instead of monoclonal antibodies.

Protoplast isolation

The effect of varying several parameters in the isolation procedure was investigated. The yield and viability of lentil protoplasts were affected by the duration of the plasmolysis step, and the duration and composition of the digestive mixture. The protocol described here was the most satisfactory one, resulting in a population of intact protoplasts (average diameter 35 μm), largely free from cell debris (Fig. 3). The

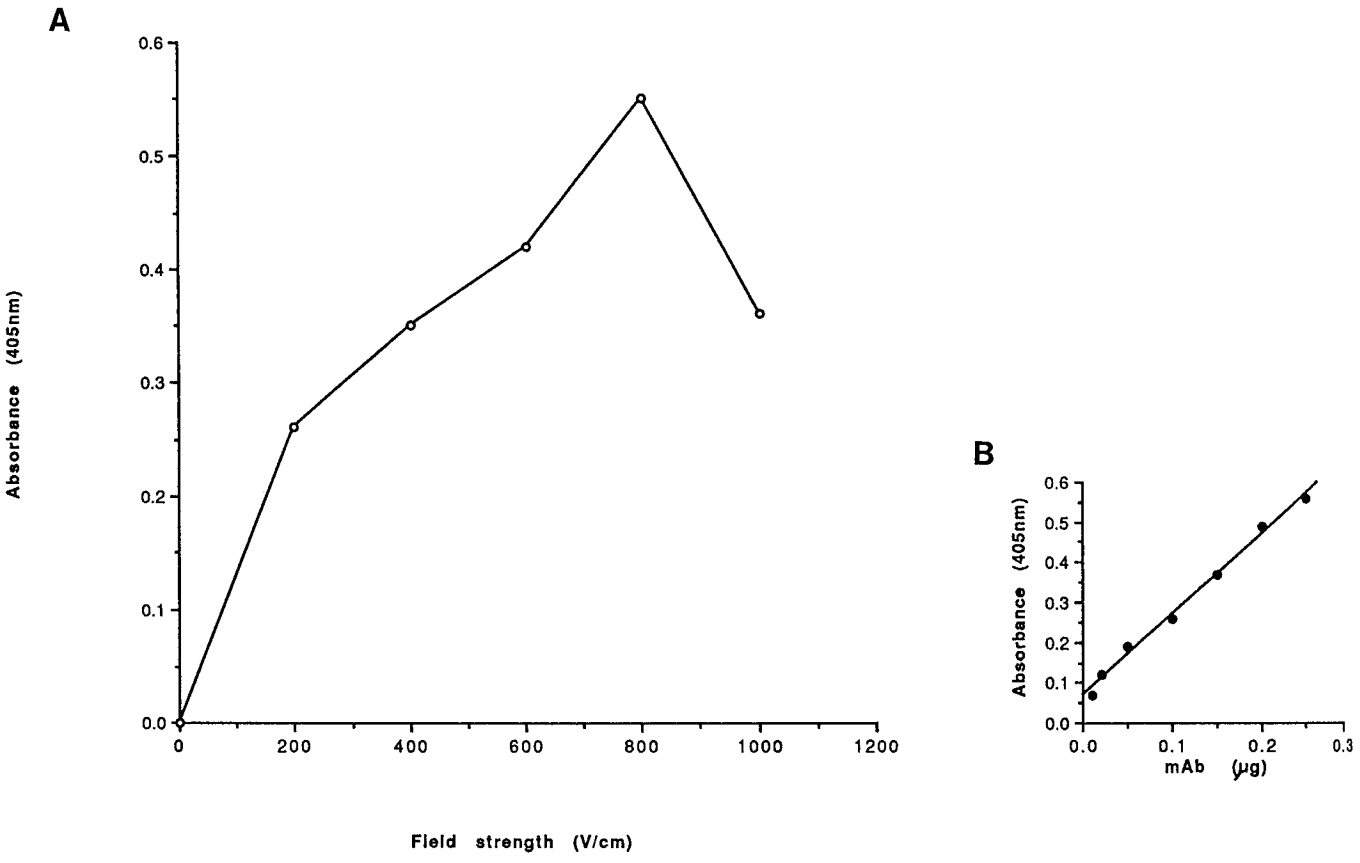

Fig. 4. Quantitation of incorporated antibodies by ELISA. (A) The amount of monoclonal antibodies transferred into lentil protoplasts was evaluated by the ELISA test. The absorbance values of the samples were corrected for the value ($A_{405} = 0.05$) of the sample incubated in the presence of monoclonal antibodies without electroporation (field strength 0 V/cm). 100 µg each cell extract were loaded/well. (B) Calibration curve, drawn by subjecting different amounts of monoclonal antibodies to the ELISA test, in the concentration range 0.00–0.25 µg/well.

yield of isolated protoplasts was approximately 2.0×10^6 lentil protoplasts/g leaves and lentil protoplasts were 90% viable.

Detection and quantitation of incorporated monoclonal antibodies

Cell extracts of lentil protoplasts, subjected to electroporation at different field strengths (in the range 0–1000 V/cm), in the presence of monoclonal antibodies, were analysed by ELISA. This test indicated that the transfer efficiency into the cells of monoclonal antibodies was dependent on the voltage applied, i.e. monoclonal antibody incorporation was found to increase with field strength in the range 0–800 V/cm (Fig. 4). Additionally, ELISA allowed a quantitation of the incorporated antibodies by relating the absorbance values of the unknown samples to the calibration curve (Fig. 4, inset). This procedure enabled us to calculate that, using 250 µg monoclonal antibody for electroporation of 10^6 lentil protoplasts, the amount of incorporated antibodies ranged over 0.5–1.0 µg, with field strengths ranging over 200–800 V/cm. This represents a maximal incorporation of 1.0 pg monoclonal antibody/lentil protoplast (2.4 µg monoclonal antibody/mg protein).

Immunoglobulins transferred into lentil protoplasts at 800 V/cm field strength had structurally intact heavy chains, as demonstrated by Western-blot analysis of lentil protoplasts extracts (Fig. 5). Therefore, the electric shock due to electroporation appeared not to damage the integrity of the antibody. This finding was corroborated by SDS/PAGE analysis of monoclonal antibodies subjected (alone) to an electric pulse of 1000 V/cm; no differences were found between treated and untreated immunoglobulins (data not shown).

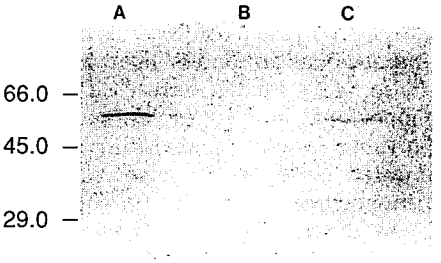

Fig. 5. Western-blot analysis of lentil protoplasts electroporated in the presence of monoclonal antibodies. Monoclonal antibodies (2 µg; lane A) and two cell extracts (100 µg/lane) of lentil protoplasts incubated in the presence of monoclonal antibodies, without electroporation (lane B) or with an electric pulse of 800 V/cm (lane C), were analysed by SDS/PAGE and Western blotting. After electroporation and incorporation into lentil protoplasts, the heavy chains of the transferred monoclonal antibodies showed the same electrophoretic mobility as the control immunoglobulins (compare lanes A and C). In the absence of an electric pulse, no incorporaton of antibodies could be detected in lentil protoplasts extracts (lane B). Molecular-mass markers bovine serum albumin (66 kDa), ovalbumin (54 kDa) and carbonic anhydrase (29 kDa) are shown on the left-hand side.

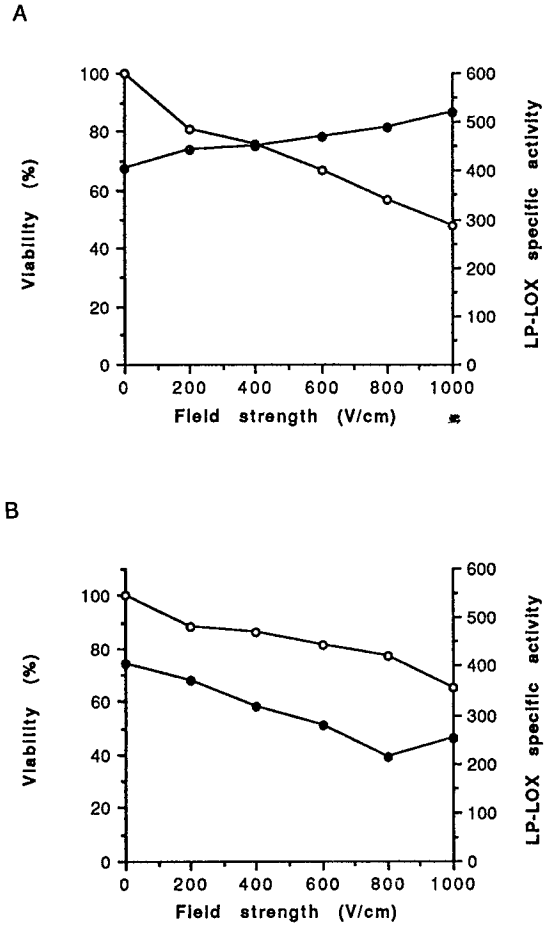

Fig. 6. Effect of electroporated monoclonal antibodies on lentil protoplast lipoxygenase activity and lentil protoplast viability. The values of lentil protoplast lipoxygenase specific activity (●) and lentil protoplast viability (○) were monitored in electroporation experiments at different field strengths. The monoclonal antibodies were absent (A) or present (B) during the electroporation. Lentil protoplast lipoxygenase (LP-LOX) specific activity is expressed as pmol HPOD · min^{-1} · mg^{-1} and lentil protoplast viability is expressed as a percentage of the non-electroporated sample.

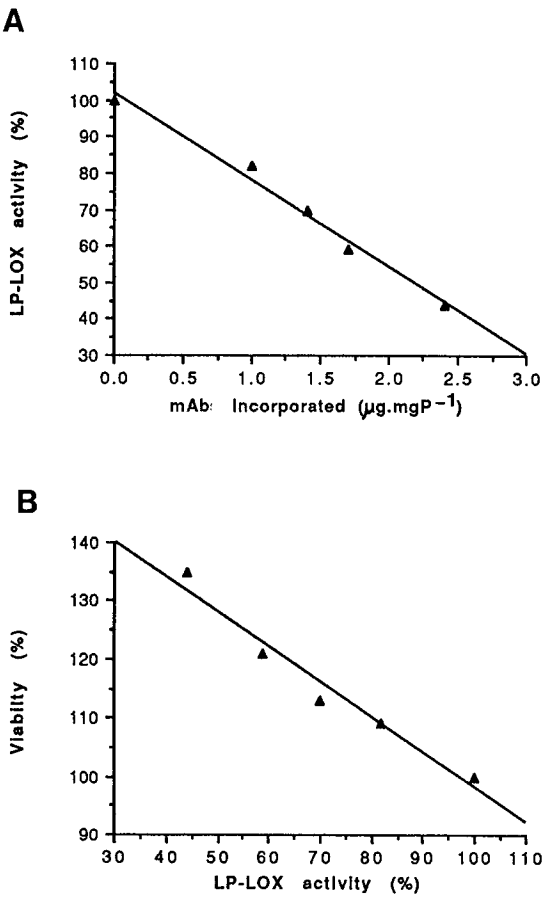

Fig. 7. Dependence of lipoxygenase activity on the incorporation of monoclonal antibodies and dependence of lentil protoplast viability on lentil protoplast lipoxygenase activity. (A) Linear relationship between lentil protoplast lipoxygenase inhibition and incorporation of monoclonal antibodies into lentil protoplasts. (B) Decrease of lentil protoplast viability at increasing values of lentil protoplast lipoxygenase activity. The values of lentil protoplast lipoxygenase activity and lentil protoplast viability are those recorded in samples electroporated in the presence of monoclonal antibodies and are expressed as a percentage of the corresponding values of samples electroporated in the absence of antibodies. Points in Fig. 7A correspond to field strengths of 0, 200, 400, 600 and 800 V/cm, respectively. In Fig. 7B, the lentil protoplast lipoxygenase activity values of Fig. 7A are related to the viability values recorded at the same field strengths.

Effects of monoclonal antibody incorporation on lentil protoplast lipoxygenase activity and lentil protoplast viability

The electroporation of lentil protoplasts at increasing field strengths caused an increase in lentil protoplast lipoxygenase specific activity and a decrease in lentil protoplasts viability (Fig. 6A). When electroporation experiments were performed in the presence of monoclonal antibodies, an opposite trend in the lentil protoplast lipoxygenase profile was observed, i.e. a linear decrease in the specific activity, in the range 0 – 800 V/cm (Fig. 6B). In the presence of monoclonal antibodies, the decrease in viability at increasing field strengths was less severe (Fig. 6B).

In order to better correlate the decrease in lentil protoplast lipoxygenase activity with the incorporation of antibodies, lentil protoplast lipoxygenase specific activities of samples electroporated with monoclonal antibodies in the range 0 – 800 V/cm (Fig. 6B) were expressed as percentages of the values of the corresponding samples electroporated without monoclonal antibodies (Fig. 6A). A linear relationship was found between inhibition of lentil protoplast lipoxygenase and incorporation of monoclonal antibodies at the different field strengths (Fig. 7A). In particular, the incorporation of the highest amount of immunoglobulins (2.4 µg/mg at 800 V/cm) decreased lentil protoplast lipoxygenase activity to 44% of the reference value. Moreover, by expressing the viability of lentil protoplasts, electroporated with monoclonal antibodies in the range 0 – 800 V/cm (Fig. 6B), as percentages of the values of the corresponding samples electroporated without antibodies (Fig. 6A), a direct correlation was found between increase in the viability and the extent of lentil protoplast lipoxygenase inhibition, at the different field strengths (Fig. 7B). Higher values of lentil protoplast lipoxygenase inhibition corresponded to higher values of viability, with a maximum for both lentil protoplast lipoxygenase inhibition (56%) and lentil protoplasts viability (135%) upon electroporation at 800 V/cm. Controls, performed by electroporating lentil protoplasts with non-immune mouse serum instead of monoclonal antibodies, yielded the same profiles of lentil protoplast lipoxygenase activity and lentil protoplast viability as the samples electroporated in the absence of antibodies.

DISCUSSION

In order to inhibit lipoxygenase activity by transferring antibodies against the target enzyme into intact, viable plant cells, experiments were carried out to ascertain the ability of anti-(soybean lipoxygenase) monoclonal antibodies to cross-react and inhibit lentil lipoxygenase (Figs 1 and 2). Lentil seedlings were chosen because they could be grown in a short time, and reproducibly yielded good amounts of intact, healthy protoplasts (Fig. 3). In these cells, inhibition of lipoxygenase by monoclonal antibodies was observed *in vivo*.

Electroporation was used to introduce monoclonal antibodies into lentil protoplasts because of the many advantages of this technique over other established methods (e.g. microinjection, addition of organic-solvent fixatives or osmotic permeabilization [11, 12, 23]). Pilot experiments had to be performed in order to find optimal values for various parameters (solution-A resistance, capacitance, field-strength range and cuvette geometry) and to render the method suitable for antibody incorporation into plant protoplasts with high percentages of viability, an issue not yet addressed in the literature. Results reported here indicate a more efficient transfer of monoclonal antibodies into lentil protoplasts at higher field strengths, with a maximum at 800 V/cm (Fig. 4), in line with the already-known dependence of nucleic-acids transfer efficiency on the field strength reported for mammalian [34], plant [35, 36] and bacterial [37] cells. Below 200 V/cm, incorporation of monoclonal antibodies was found to be poor. The electroporation procedure, performed under the conditions described above, does not destroy the integrity of the antibodies, in fact Western-blot analysis of cell extracts of lentil protoplasts electroporated in the presence of monoclonal antibodies showed intact immunoglobulin heavy chains (Fig. 5). Since lentil protoplasts were disrupted 4 h after electroporation, this finding also indicates that no proteolytic degradation of incorporated monoclonal antibodies occurred inside the lentil protoplasts during the 4 h following the electric shock. This is in agreement with previous work on the fate of incorporated antibodies in mammalian cells [11, 13, 23].

Reports published so far on the effects of electroporated immunoglobulins on cell functioning showed modifications of cell growth [10] and metabolism [11], which could be indirectly correlated to the effective interaction of the transferred antibodies with the specific intracellular target. In this investigation, the possibility of assaying lipoxygenase activity in lentil protoplasts electroporated with or without antibodies was used to obtain direct evidence of the specific inhibitory effect of the incorporated monoclonal antibodies on the target enzyme. Our results clearly indicate inhibition of lentil protoplast lipoxygenase upon transfer of anti-(soybean lipoxygenase) antibodies into lentil protoplasts (Fig. 6A and B). Moreover, the inhibition of the target enzyme was found to be a linear function of the amount of transferred immunoglobulins (Fig. 7A). Interestingly, a significant improvement of lentil protoplast viability was observed upon electroporation in the presence of monoclonal antibodies (Fig. 6A and B) which was well-correlated with the inhibition of lentil protoplast lipoxygenase activity (Fig. 7B). These results indicate that the effect of the antibody on cell viability was mediated by the interaction with the target enzyme, suggesting a key role for lipoxygenase in the control of cellular integrity. When cells were electroporated without monoclonal antibodies, an increase in lentil protoplast lipoxygenase activity was observed at increasing field strengths, with a parallel decrease of cell viability (Fig. 6A). Cellular damage due to the electric shock was less severe when electroporation experiments were performed in the presence of antibodies able to specifically inhibit lentil protoplast lipoxygenase (Fig. 6B). Therefore, it can be concluded that the damaging effect of electroporation on the cells was, at least in part, mediated by the increase in lipoxygenase activity. These findings are in good agreement with previous observations, indicating an improvement of protoplast viability when lipoxygenase activity was inhibited by means of *n*-propylgallate [38–40]. Moreover, they can be explained by taking into account that lipoxygenases may generate highly reactive species, like free radicals and alkyl hydroperoxides [17], which could eventually decrease protoplast viability. Mammalian lipoxygenases have been shown to be involved in initiating the breakdown of reticulocyte mitochondrial membranes [41] and the production of highly potent lipid mediators like leukotrienes and lipoxins [42]. Although fundamentally different, some kind of analogy with the mammalian system may exist in plants, e.g. a role for lipoxygenase in response to wounding, infection or the formation of regulatory molecules like jasmonic and traumatic acids (for a review, see [16]).

In conclusion, we report for the first time, the application of electroporation to the transfer of antibodies into plant cells and present direct evidence for the inhibitory effect of the immunoglobulins on the target enzyme, lipoxygenase. Our results, besides showing the suitability of electroporation for macromolecular incorporation into living plant cells, give clues for a better understanding of the role of lipoxygenases in plants, indicating the involvement of these enzymes in the control of cell viability.

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REFERENCES

1. Zimmerman, U. & Vienken, J. (1982) *J. Membr. Biol. 67*, 165–182.
2. Potter, H. (1988) *Anal. Biochem. 174*, 361–373.
3. Neumann, E., Schaefer-Ridder, M., Wang, Y. & Hofschneider, P. H. (1982) *EMBO J. 1*, 841–845.
4. Fromm, M., Taylor, L. P. & Walbot, V. (1985) *Proc. Natl Acad. Sci. USA 82*, 5824–5828.
5. Fiedler, S. & Wirth, R. (1988) *Anal. Biochem. 170*, 38–44.
6. Bertling, W. (1987) *Bioscience Rep. 7*, 107–112.
7. Watts, J. W., King, J. M. & Stacey, N. J. (1987) *Virology 157*, 40–46.
8. Winegar, R. A., Phillips, J. W., Youngblom, J. H. & Morgan, W. F. (1989) *Mutat. Res. 225*, 49–53.
9. Graziadei, L., Burfeind, P. & Bar-Sagi, D. (1991) *Anal. Biochem. 194*, 198–203.
10. Uno, I., Fukami, K., Kato, H., Takenawa, T. & Ishikawa, T. (1988) *Nature 333*, 188–190.
11. Chakrabarti, R., Wylie, D. E. & Schuster, S. M. (1989) *J. Biol. Chem. 264*, 15494–15500.
12. Berglund, D. L. & Starkey, J. R. (1989) *J. Immunol. Methods 125*, 79–87.
13. Raptis, L. & Firth, K. L. (1990) *DNA & Cell Biol. 9*, 615–621.
14. Berglund, D. L. & Starkey, J. R. (1991) *Cytometry 12*, 64–67.

15. Vliegenthart, J. F. G. & Veldink, G. A. (1982) in *Free radicals in biology* (Pryor, W. A., ed.) pp. 29–64, Academic Press, New York.
16. Vick, B. A. & Zimmerman, D. C. (1987) in *The biochemistry of plants. A comprehensive treatise* (Stumpf, P. K. & Conn, E. E., eds) pp. 53–90, Academic Press, New York.
17. Veldink, G. A., Vliegenthart, J. F. G. & Boldingh, J. (1977) *Prog. Chem. Fats other Lipids 15*, 131–166.
18. Vick, B. A. & Zimmerman, D. C. (1983) *Biochem. Biophys. Res. Commun. 111*, 470–477.
19. Zimmerman, D. C. & Coudron, C. A. (1979) *Plant Physiol. 63*, 536–541.
20. Finazzi-Agrò, A., Avigliano, L., Veldink, G. A., Vliegenthart, J. F. G. & Boldingh, J. (1973) *Biochim. Biophys. Acta 326*, 462–470.
21. Galpin, J. R., Tielens, L. G. M., Veldink, G. A., Vliegenthart, J. F. G. & Boldingh, J. (1976) *FEBS Lett. 69*, 179–182.
22. Peterman, T. K. & Siedow, J. N. (1985) *Arch. Biochem. Biophys. 238*, 476–483.
23. Chakrabarti, R., Pfeiffer, N. E., Wylie, D. E. & Schuster, S. M. (1989) *J. Biol. Chem. 264*, 8214–8221.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
25. Murashige, T. & Skoog, F. (1962) *Physiol. Plant. 15*, 473–497.
26. Frearson, E. M., Power, J. B. & Cocking, E. C. (1973) *Dev. Biol. 33*, 130–137.
27. Widholm, J. M. (1972) *Stain Technol. 47*, 189–194.
28. Van Os, C. P. A., Rijke-Schilder, G. P. M. & Vliegenthart, J. F. G. (1979) *Biochim. Biophys. Acta 575*, 479–484.
29. Verhagen, J., Wassink, G. A., Kijne, G. M., Viëtor, R. J. & Bruynzeel, P. L. B. (1986) *J. Chromatogr. 378*, 208–214.
30. Laemmli, U. K. (1970) *Nature 227*, 680–685.
31. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl Acad. Sci. USA 76*, 4350–4354.
32. Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984) *Anal. Biochem. 136*, 175–179.
33. Abdillahi, H. & Poolman, J. T. (1987) *FEMS Microbiol. Lett. 48*, 367–371.
34. Chu, G., Hayakawa, H. & Berg, P. (1987) *Nucleic Acids Res. 15*, 1311–1326.
35. Christou, P., Murphy, J. E. & Swain, W. F. (1987) *Proc. Natl Acad. Sci. USA 84*, 3962–3966.
36. Leon, P., Planckaert, F. & Walvot, V. (1991) *Plant Physiol. 95*, 968–972.
37. Bottger, E. C. (1988) *BioTechniques 6*, 878–880.
38. Saleem, M. & Cutler, A. J. (1987) *J. Plant Physiol. 128*, 479–484.
39. Krens, F. A., Jamar, D., Rouwendal, G. J. A. & Hall, R. D. (1990) *Theor. Appl. Genet. 79*, 390–396.
40. Peterman, T. K. & Siedow, J. N. (1983) *Plant Physiol. 71*, 55–58.
41. Schewe, T., Halangk, W., Hiebsch, C. & Rapoport, S. M. (1975) *FEBS Lett. 60*, 149–152.
42. Samuelsson, B., Dahlén, S.-E., Lindgren, J. Å., Rouzer, C. A., Serhan, C. N. (1987) *Science 237*, 1171–1176.