Chitosan Immobilization and Permeabilization of *Amaranthus tricolor* Cells

D. Knorr* and R. A. Teutonico

Cultured cells of *Amaranthus tricolor* were entrapped in chitosan gel to determine the polycationic properties of chitosan on plant cell membrane permeability. During 5 days, a maximum of 2.09 ± 1.63 mg of oxalate, a storage product of *A. tricolor* cells, was released from chitosan-immobilized cells per gram fresh weight as compared to 0.17 ± 0.16 mg of oxalate from cells entrapped in alginate gels.

INTRODUCTION

Entrapment is the most widely used immobilization method for cultured plant cells. Advantages for using immobilized cells over freely suspended cells include reuse of the biocatalysts, protection of shear-sensitive cells from mechanical damage, partial protection against microbial contamination, use of high cell densities, availability of a continuous process, and relatively simple process control and metabolite recovery. Entrapment also increases the potential for commercial production of secondary metabolites (Felix et al., 1981; Brodelius and Mosbach, 1982; Shuler et al., 1983). Plant metabolites of commercial interest are often intracellular products that require membrane permeabilization for their release (Brodelius and Nilsson, 1983). Compounds commonly used to permeabilize plant cell membranes include toluene and dimethyl sulfoxide (Lerner et al., 1978; Brodelius and Nilsson, 1983). Use of the polycation chitosan (deacetylated chitin) for immobilization of whole cells (Vorlop and Klein, 1981; Knorr, 1985) and for controlled release of food additives and food processing aids (Rodriquez-Sanchez and Rha, 1981) has been reported. Recently, the interaction between chitosan and plant cell membranes has been examined (Young et al., 1982; Young and Kauss, 1983; Köhle et al., 1984). According to Young et al. (1982), chitosan binds to polygalacturonate, a plant cell wall component, and induces leakage of low molecular weight compounds as well as some proteins (>5000 D). The increase in cell permeability by chitosan may be due to disruption of the intermolecular bonding responsible for maintaining an intact membrane, changes in membrane fluidity, or effects on the components associated with membrane transport (Young and Kauss, 1983). The objective of this study was to examine the function of chitosan as an immobilizing agent and its effect on the release of intracellular plant metabolites, from freely suspended as well as from chitosan gel entrapped cultured cells using vegetable amaranth (*Amaranthus tricolor*) as the plant cell model. Oxalate, a low molecular weight metabolite stored in the plant vacuoles and found at an average concentration of 6.8–40.5 mg/g (dry weight) in cultured *A. tricolor* cells (Teutonico and Knorr, 1985a), was selected as indicator metabolite.

MATERIALS AND METHODS

Cell suspensions of *A. tricolor* were cultured at a density of 0.75 g (fresh weight)/25 mL of medium in half-strength B5 medium (Gamborg et al., 1968). Cultures were incubated in a controlled-environment incubator shaker (Psychrotherm G26, New Brunswick Scientific, Edison, NJ) at 25 ± 1 °C under continuous illumination (approximately 25 400 lx) at 100–105 rpm (Teutonico and Knorr, 1985b). Commercially available chitosan (Bioshell Inc., Albany, OR) was dissolved in 1.6% aqueous ascorbic acid and added to cell suspensions at 5 or 10 mg of chitosan/100 mL of medium. Cultured cells were entrapped in chitosan gels following the procedure of Vorlop and Klein (1981). Ionotropic gelation was performed using 2.4% (w/v) of chitosan dissolved in 1.6% aqueous ascorbic acid (Muzzarelli et al., 1984) as polyelectrolyte and sodium tripolyphosphate (3.0% aqueous solution) as the multivalent counterion. Alginate globules were formed using 3.2% aqueous sodium alginate (Sigma Chemical Co., St. Louis, MO) and 100 mM $CaCl_2$ as polyanion and counterion, respectively (Brodelius et al., 1979). Globules were hardened in their respective counterion solutions for 2 h before being transferred to half-strength B5 medium. Chitosan-entrapped cells were maintained at pH 6.0 by incorporating 20% pH 8 phosphate buffer (AOAC, 1980) into the medium. A pH of 5.8 was obtained for all the other media used.

The same cell density and incubation conditions were used for the freely suspended cells and for the gel-entrapped cells. Two-milliliter samples of the medium were sampled after 24, 72, and 120 h and analyzed for oxalate concentration after Roughan and Slack (1973) and Teutonico and Knorr (1985b).

RESULTS AND DISCUSSION

Figure 1 presents the release of oxalate from cultured vegetable amaranth (*A. tricolor*) cells subjected to various treatments. Freely suspended cells without chitosan and calcium alginate gel entrapped cells released significantly ($p < 0.01$) lower amounts of oxalate than all three treatments where chitosan was present. No differences in oxalate release were found between chitosan gel entrapped cells and freely suspended cells with 5 mg of chitosan present in 100 mL of the medium. The addition of 10 mg of chitosan in 100 mL of medium resulted in significantly higher oxalate release after 5 days than for the 5 mg/100 mL samples ($p < 0.01$), but no difference was found when compared to chitosan gel entrapped cells. Comparisons of overall means of treatments (Keuls, 1952) revealed significant differences ($p < 0.01$) between the freely suspended cells and all cultures treated with chitosan. Differences between freely suspended cells and calcium alginate entrapped cells were significant ($p < 0.01$) except for day 3.

Oxalate release after 5 days was lowest ($p < 0.01$) from calcium alginate gel entrapped *A. tricolor* cultures, indicating a potential inhibitory effect of calcium ions on the leakage of secondary plant metabolites. A lower release of protein from calcium alginate gel entrapped cultured milkweed cells as compared to chitosan gel entrapped cells was recently observed by Miazga and Knorr (1985). Na-

Biotechnology Group, Department of Food Science, University of Delaware, Newark, Delaware 19716 (D.K.), and Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (R.A.T.).

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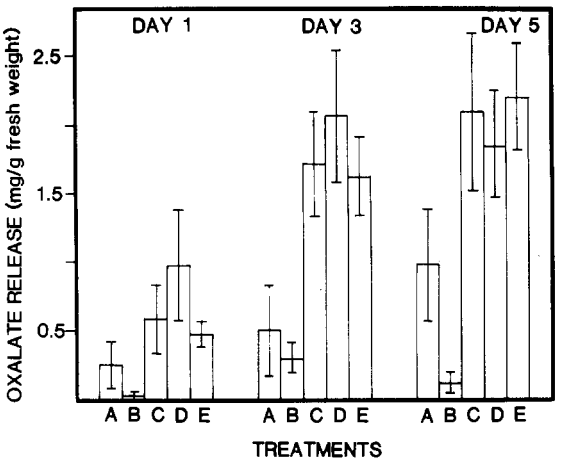

Figure 1. Mean values and standard deviations of oxalate released from cultured *A. tricolor* cells (N = 8) during 5 days of various treatments: A, freely suspended cell cultures; B, calcium alginate gel entrapped cell cultures; C, chitosan gel entrapped cell cultures; D, freely suspended cell cultures with 5 mg of chitosan in 100 mL of medium; E, freely suspended cell cultures with 10 mg of chitosan in 100 mL of medium.

kajima et al. (1985) found similar results when comparing leakage of metabolites from cultured *Lavendula vera* cells entrapped in calcium alginate gels as compared to cells entrapped in agar.

Viability of the cells has not been measured during these experiments. However, viability data after Towill and Mazur (1975) from our laboratory with chitosan entrapped common milkweed (*Asclepias syriaca* L.) cells (Miazga and Knorr, 1984) and data on sustained viability for calcium alginate gel entrapped cells (Brodelius and Nillson, 1983) show that plant cell viability of immobilized plant cells can be maintained under the conditions selected.

In summary, the data presented indicate that chitosan in plant cell media, or when used for ionotropic gelation, resulted in oxalate release that was superior to that from freely suspended and calcium alginate gel entrapped *A. tricolor* cells. This suggests that chitosan, when subjected to ionotropic gelation, can serve concurrently as effective immobilizing and permeabilizing agent for cultured plant cells.

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