Microbial resistance of caseinate films crosslinked by gamma irradiation

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The need to improve the shelf life and quality of foods, combined with the urgency to reduce waste generated by synthetic non-biodegradable packaging, have resulted in an increasing interest in biodegradable or edible materials (Chen, 1995). The challenge in the development of such materials lies in achieving controlled lifetime together with compatibility with foods and the environment. Natural polymers or polymers derived from natural monomers such as food proteins offer the greatest opportunities, since their biodegradability and environmental compatibility are assured (Krochta & de Mulder-Johnston, 1997).

Milk proteins such as caseins have been formulated into coatings and films for improving food quality (Baker *et al*. 1994). Unfortunately, the highly hydrophilic nature of these proteins limits the possibility of producing films with the required properties. Gamma irradiation, which induces the formation of crosslinks in the protein structure, is known to improve the mechanical properties and water resistance of such materials (Brault *et al*. 1997; Mezgheni *et al*. 1998*a*; Ressouany *et al*. 1998), and can also sterilize materials for possible biomedical applications (Kaetsu, 1995).

Gamma irradiation is slowly becoming accepted in the food industry as a means of safely improving the shelf life of various fruits and vegetables and eliminating bacterial contamination in meats (Pszczola, 1997). All these features (sterility, increased mechanical strength, controlled life time) make gamma irradiation a promising technique for the production of commercial biodegradable films.

In the present study, we have investigated the microbial resistance of calcium caseinate films irradiated at 4 or 64 kGy. A minimum dose of 4 kGy was required to produce complete sterility in the films without inducing many crosslinks in the protein. A dose of 64 kGy was used to induce maximum crosslinking density (Ressouany *et al*. 1998). The rate of modification of the films was evaluated using three methods. Firstly, microbiological counts were performed on films incubated with a strain of *Pseudomonas aeruginosa*. This strain was chosen because it produces a wide range of proteinases and is often implicated in the process of food spoilage. These results were compared with those obtained for soluble N. Since conversion to CO_2 is one of the standard test procedures to assess biodegradability (El-Din Sharabi & Bartha, 1993), we also analysed the CO_2 produced by the biodegradation of the

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crosslinked films using a modified version of a standard test procedure (American Society for Testing Materials, 1992). This measures the aerobic degradation of plastic materials inoculated with municipal sewage sludge. We have replaced the sludge by a concentrated bacterial strain (*Ps. aeruginosa*) and compared these results with the two previous methods. This last method was used to measure the resistance of the crosslinked films to bacterial degradation.

MATERIALS AND METHODS

Materials

Calcium caseinate (Alanate 380) was provided by New Zealand Milk Products (Santa Rosa, CA 95403, USA). *Ps. aeruginosa* (ATCC 15442) and glycerol (99·5% reagent grade) were obtained from A & C American Chemicals Ltd (Montréal, Québec, Canada H4S 1L2). Carboxymethylcellulose sodium salt (CMC, low viscosity), was purchased from Sigma Chemicals (St Louis, MO 63178–9916, USA). Nutrient Broth and Nutrient Agar were obtained from Becton Dickinson (Cockeysville, MD 21030, USA). All products were used as received without further purification.

Film formation method

Calcium caseinate (50 g/l), glycerol (25 ml/l), $CaCl_2.12H_2O$ (1·25 g/l) and CMC (2·5 g/l) were dissolved in distilled water with stirring. Solutions were then poured into a test tube and flushed with a flow of inert gas for 15 min following the procedure reported by Brault *et al.* (1997). Test tubes were irradiated using a ^{60}Co source (Gammacell 220; MDS Nordion, Kanata, Ontario, Canada K2K 1X8) at a mean dose rate of 2·18 kGy/h for total irradiation doses of 4 and 64 kGy. Films were then cast by pipetting 10 ml of the solution on to smooth-rimmed 85 mm i.d. polymethacrylate (Plexiglas) plates (Fisher Scientific, Montréal, Québec, Canada H3C 2X3) and allowing them to dry overnight under a Sterilguard biological hood at 20 ± 2 °C. Dried circular films could be peeled intact from the casting surface. Bacterial control tests confirmed that the films were sterile.

Microbial resistance measurements

Before inoculation, *Ps. aeruginosa* was subcultured twice in Nutrient Broth with incubation at 37 ± 1 °C for 18 h. For each biodegradability experiment, culture (1 ml) was diluted in 9 ml sterile saline solution (8·5 g NaCl/l) and washed three times by centrifugation at 880 ***g*** and 4 ± 1 °C for 20 min (Model RC-5C automatic superspeed refrigerated centrifuge; Du Pont, Wilmington, DE 19898, USA) to remove all traces of nutrient. Portions (3 ml) of the final dilution (10^9 cfu/ml) were used to inoculate the solutions. To provide an accurate measurement of the bacterial growth due to the degradation of the films, three solutions were compared. The first solution, 297 ml standard stock solution (American Society for Testing Materials, 1992), was inoculated with 3 ml saline solution containing bacterial cells in order to follow the growth of the strain in absence of the films. Two more solutions were analysed, each containing 297 ml standard stock solution, 3 ml saline solution containing bacterial cells and four caseinate films of one type (4 or 64 kGy). All flasks were incubated at 37 ± 1 °C with shaking (130 rev./min) for 60 d. They were sampled and plated in duplicate on Nutrient Agar. Bacterial counts were carried out after incubation at 37 °C for 24 h.

Soluble nitrogen analysis

Soluble N was analysed using a Leco FP-428 combustion oven apparatus (Leco Corporation, St Joseph, MI 49085-2396, USA). Typically, 5 ml standard stock solution containing four caseinate films was sampled from each flask and an equal amount of trichloroacetic acid (100 g/l) added to precipitate the high molecular mass (> 15 kDa) protein fraction. The solution was centrifuged at 27 000 ***g*** and 4 ± 1 °C for 20 min. The soluble fraction was then neutralized with dilute NaOH and lyophilized. Dried samples (0·1 g) were encapsulated in tinfoil and analysed for N. Four samples were compared: two from solutions contained the films only (irradiated at 4 or 64 kGy) and two from solutions contained the same films inoculated with bacteria. To obtain net N released by bacteria, values for N released in media containing films without bacteria were subtracted from values for N released in media inoculated with *Ps. aeruginosa* in the presence of films. Values were also corrected for mineral N present in the media and for N released by bacteria in media without films.

Carbon dioxide analysis

We also attempted to quantify the biodegradation of highly crosslinked protein films (irradiated at 64 kGy) by analysing the amount of CO_2 released. The CO_2 analysis was adapted from the standard test procedure of American Society for Testing Materials (1992), which involves incubating the films and bacteria in a C-free medium. Air was purged by bubbling through flasks containing 700 ml 10 M-NaOH and 700 ml 12·5 mM-$Ba(OH)_2$, then flushed through a 0·2 μm filter connected to a 1 l flask to prevent liquid carryover, and dispensed to four bioreactors containing a standard stock solution. Bioreactors were tested that contained the films alone, and the films inoculated with bacteria. The CO_2 produced by biodegradation was entrapped in another flask containing 100 ml 12·5 mM-$Ba(OH)_2$ and precipitated as $BaCO_3$. The remaining $Ba(OH)_2$ was determined by titration with 0·05 M-HCl in the presence of phenolphthalein and subtracted from the initial level to evaluate the extent of biodegradation.

Statistical analysis

Analysis of variance and Duncan's multiple range tests with $P \leqslant 0{\cdot}05$ were applied to all results. Student's t test and paired comparison with $P \leqslant 0{\cdot}05$ (Snedecor & Cochran, 1967) were also used. For bacterial count and soluble N, duplicate measurements were carried out on each of three replicates. For the CO_2 experiment, three separate flasks containing four films in the presence of bacteria were tested simultaneously. A fourth flask contained only films in the sterile stock solution.

RESULTS

Bacterial counts

Fig. 1 shows variations in the *Ps. aeruginosa* population over 60 d. When the films irradiated at 4 and 64 kGy were exposed to the bacteria, in both cases there was a significant decrease ($P \leqslant 0{\cdot}05$) between inoculation (day 0) and the first day of testing. Then, as degradation progressed, the bacterial population increased significantly ($P \leqslant 0{\cdot}05$). A maximum bacterial population was reached on day 5 for the 4 kGy films, but not until day 30 for the 64 kGy films. Visual observations confirmed that the 4 kGy films readily dissolved within 24 h while the 64 kGy films remained solid for > 2 weeks.

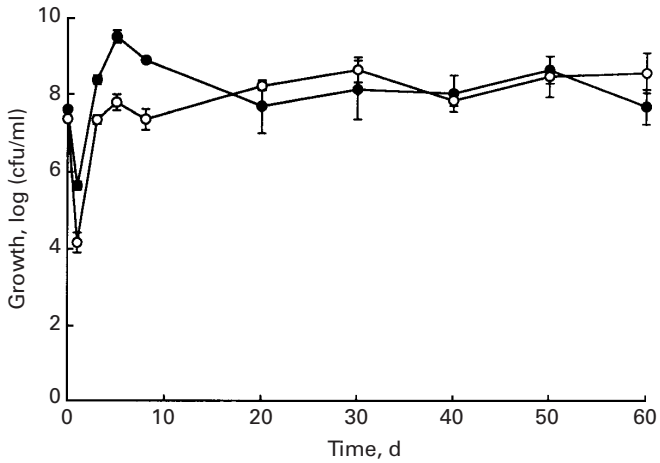

Fig. 1. Growth of *Pseudomonas aeruginosa* in sterile standard stock solutions containing calcium caseinate films irradiated at ●, 4 or ○, 64 kGy. Values are means of three separate experiments with SD indicated by vertical bars. Values were corrected for growth of bacteria in the absence of films.

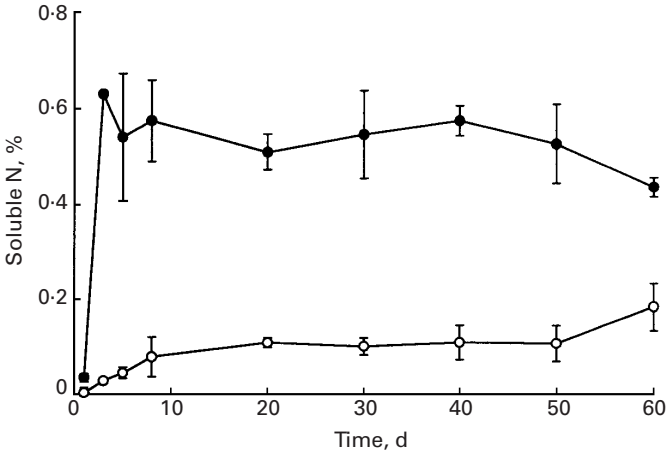

Fig. 2. Nitrogen from calcium caseinate films converted to soluble N by *Pseudomonas aeruginosa* in standard stock solutions. Films were irradiated at ●, 4 or ○, 64 kGy. Values are means of three separate experiments with SD indicated by vertical bars. Values were corrected for N in absence of bacteria, bacterial metabolic N and mineral N.

Soluble nitrogen analysis

Fig. 2 shows the percentage of N from calcium caseinate films converted to soluble N as a function of time for the 4 and 64 kGy films. When the films irradiated at 4 kGy were incubated with the bacteria, there was a significant ($P \leqslant 0{\cdot}05$) increase in soluble N on day 3. Thereafter, soluble N decreased slightly, but remained $> 0{\cdot}5\%$ until day 50. Consistent with the results for bacterial counts, the 4 kGy films were degraded more rapidly.

When the 64 kGy films were subjected to the same treatment, the N converted to soluble N was $< 0{\cdot}1\%$ until day 50. The overall increase for the 64 kGy films over the entire experimental period was much less than for the 4 kGy films. On day 60, the net soluble N released into the media containing films treated at 4 and 64 kGy was 86 and 36% respectively, confirming the effectiveness of crosslinking.

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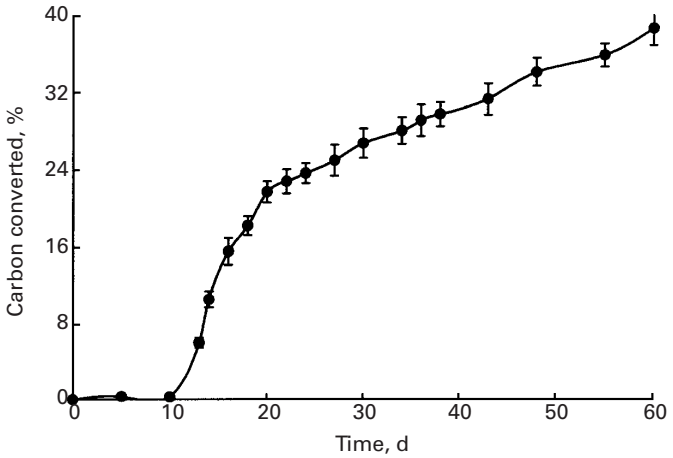

Fig. 3. Carbon from calcium caseinate films irradiated at 64 kGy converted to CO_2 by *Pseudomonas aeruginosa* in standard stock solutions. Values are means of three separate experiments with SD indicated by vertical bars.

Carbon dioxide analysis

Fig. 3 shows the percentage of C converted to CO_2 by biodegradation of the films irradiated at 64 kGy. On average, four dry films generated a theoretical amount of 0·693 g organic C yielding a theoretical production of 2·54 g CO_2. Results did not vary significantly between days 1 and 10, when only a very small increase in CO_2 was observed. The first significant increase was on day 13, and this continued until day 60, when calculation gave a biodegradation of $\sim 40\,\%$.

DISCUSSION

The biodegradability of calcium caseinate films observed when these films were tested for their resistance to microbial degradation experiments was significantly different for the two irradiated films (4 *v*. 64 kGy). Gamma irradiation, which induces the formation of crosslinks in the protein structure, was a determining factor that affected the bacterial proteolysis resistance of these films. Our previous work (Lacroix *et al*. 1998) has shown that the number of crosslinks is related to irradiation dose. It has also been demonstrated that the formation of crosslinks through irradiation increases the mechanical strength of calcium caseinate films (Mezgheni *et al*. 1998*a*; Ressouany *et al*. 1998). However, the materials included in films have a considerable impact on their resistance to microbiological degradation. The overall biodegradability of films prepared with calcium caseinate and polyethylene glycol and irradiated at 4 and 64 kGy was similar for both doses (Mezgheni *et al*. 1998*b*), but the period of degradation was 8 d longer for the 64 kGy films. In the present experiment, the overall biodegradation pattern was significantly different for the 4 and 64 kGy films. With the latter, degradation increased markedly on day 3, and was progressive over 60 d. Preliminary gel permeation chromatography results have indicated that CMC made an important contribution to the crosslinking reactions.

Further investigations on the chemical analysis and on oxygen and water vapour permeability of these films are in progress. These promising films may find application as microencapsulating agents for flavours and drugs, in coating of fruits, vegetables and cheese, and in food packaging.

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