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Physicochemical Properties and Bacterial Resistance of Biodegradable Milk Protein Films Containing Agar and Pectin

M. LETENDRE,[†] G. D'APRANO,[‡] M. LACROIX,*[,†] S. SALMIERI,[†] AND D. ST-GELAIS[§]

Canadian Irradiation Centre (CIC)-Research Laboratories in Sciences Applied to Food, INRS-Institut Armand-Frappier, 531 boulevard Des Prairies, Laval, Québec H7V 1B7, Canada, and Centre de Recherche et de Développement sur les Aliments, Agriculture et Agro-alimentaire Canada, 3600 boulevard Cassavant ouest, Saint-Hyacinthe, Québec J2S 8E3, Canada

Free-standing sterilized edible films based on milk proteins, namely calcium caseinate and whey protein isolate, and polysaccharides, namely pectin and agar, were developed. Cross-linking of the proteins was achieved by the combination of thermal and radiative treatments. Autoclaving pectin and agar prior to their addition to the protein solutions generated films with an improved ($P \leq 0.05$) puncture strength. The presence of proteins and pectin−agar in the film formulation enhanced ($P \leq 0.05$) the moisture barrier of the films by 18%. A strain of *Streptococcus thermophilus* was used to assess the biodegradability behavior of the cross-linked films. Microbiological counts and soluble nitrogen analysis confirmed the biodegradability property of the milk protein films containing autoclaved pectin and agar.

KEYWORDS: Milk proteins; pectin; agar; mechanical properties; water permeability; biodegradability, edible films

INTRODUCTION

The protection of the environment has become a major concern over the past few decades. This has resulted in an increasing interest in biodegradable or edible materials (*1−5*). Natural polymers derived from natural monomers, such as food proteins, offer the greatest opportunities, since their biodegradability and environmental compatibility are assured (*5*). Milk proteins, such as whey proteins and caseinates, have been formulated into coatings and films, owing to their effectiveness in enhancing the shelf life by serving as selective barriers combined with their environmental compatibility and nutritional value (*4−13*).

Milk protein based films exhibit poor water vapor barrier properties due to their hydrophilic nature (*1, 2, 5*). However, cross-linking of milk proteins by means of physical treatments, such as thermal treatment and γ-irradiation, was found to be effective toward the improvement of the barrier properties, as well as the mechanical properties (*3, 8, 14*). Similarly, the combination of both thermal and γ-irradiation processing treatments to protein blend (*11, 13*) and composite films was responsible for cross-linking the proteins (via formation of bityrosine bridges and the modification of the protein structure) and improving the film's mechanical properties and stability (*8, 11−13*). More recently, the addition of carboxymethylcellulose (CMC), a polysaccharide, to two different protein blends resulted in an improvement of both mechanical and barrier properties of the films (*13, 15*).

Pectins and agar are two polysaccharides that have several applications in human foods (*16, 17*). Although pectin is a poor moisture barrier, pectin coatings were reported to retard water loss from food by acting as a sacrificing agent (*5*). Moreover, pectin forms a gel in the presence of calcium ions and can, thus, be used to develop edible films (*17, 18*). Impermeable coatings can be obtained by cross-linking agar following thermal treatments (*19*). It is believed that the presence of pectin and agar in a protein formulation could improve the moisture barrier of the resulting films. Therefore, the benefits of pectin and agar in a blend of milk proteins consisting of calcium caseinate (CC) and whey protein isolate (WPI) on the mechanical and barrier properties of the resulting films were investigated. Biodegradable films could be used to pack some dairy products such as mozzarella cheese. *Streptococcus thermophilus* is one of the thermophilic lactic bacteria starters used to produce this cheese. The protein film used to pack cheese must resist to lactic acid bacteria. In the present study, we have also investigated the microbial resistance of films to *Streptococcus thermophilus*.

MATERIALS AND METHODS

Materials. Calcium caseinate (CC) containing 91.8% proteins, 3.8% ash, 0.7% fat, and 0.1% lactose was provided by New Zealand Milk Products Inc. (Santa Rosa, CA). Whey protein isolate (WPI) containing

* To whom correspondence should be addressed. Fax: +1-450-687-5792. E-mail: Monique.Lacroix@inrs-iaf.uquebec.ca.
[†] INRS-Institut Armand-Frappier.
[‡] Current address: Héma-Québec, 4045 Côte-Vertu, Saint-Laurent (Québec) H4R 2W7, Canada.
[§] Centre de Recherche et de Développement sur les Aliments, Agriculture et Agro-alimentaire Canada.

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94.5% proteins, 2.15% ash, and 3.27% lactose was prepared at the Centre de Recherche et de Développement sur les Aliments (St-Hyacinthe, Québec, Canada). Total protein, fat, and ash contents of calcium caseinate and whey protein isolate were determined in duplicate. Fat content was obtained by the Mojonnier extraction procedure. Total protein was measured by using the macro-Kjeldahl method and ash by heating the sample to 550 °C overnight in a muffle furnace as described previously (*20*). WPI was produced from permeate obtained by tangential membrane microfiltration (MF pilot cross-flow unit). Fresh skim milk was microfiltered 3-fold at 50 °C using a pilot cross-flow MF unit equipped with a Membralox membrane (total membrane surface 1.4 m^2) as described previously (*20*). The proteins contained in the permeate were concentrated 25-fold at 50 °C by ultrafiltration (UF pilot unit, equipped with a Romicon membrane (WO Burn, MA) PM 10, total surface area 1.3 m^2). The concentrate was diafiltered 5-fold by constant addition of water and freeze-dried before use in order to obtain WPI. Low-viscosity carboxymethylcellulose sodium salt (CMC), glycerol (99.5%), and agar were purchased from Sigma Chemicals Co. (St. Louis, MO). Pectin (Certo) was supplied by Kraft Canada (Toronto, Ontario, Canada). Acetonitrile (99.95%) was obtained from Anachemia Chemicals (Montreal, Québec, Canada).

Film Formation. The formulations are based on 5% w/w total protein, 2.5% glycerol, and 0.25% CMC, as previously reported (*21*). Moreover, different polysaccharides were added: a pectin–agar mixture at total concentrations of 0–0.3%. The components were solubilized in distilled water, with stirring, and the solutions were heated at 90 °C for 30 min. The solution was then degassed under vacuum to remove dissolved air and flushed with nitrogen (*8*). Irradiation of the solution was carried out at the Canadian Irradiation Centre (CIC; Laval, Québec, Canada) at a dose of 32 kGy and a mean dose rate of 17.33 kGy/h, using a UC-15 ^{60}Co underwater calibrator unit (MDS-Nordion International Inc., Kanata, Ontario, Canada). Films were then cast by pipetting 5 mL of the solution onto smooth-rimmed 8.5 cm (i.d.) Petri dishes that were sitting on a leveled surface. Solutions were spread evenly and allowed to dry overnight at room temperature (20 ± 2 °C) in a environmental chamber (45–50% RH). Dried films could be peeled intact from the casting surface.

Film Thickness Measurements. Film thickness was measured using a Digimatic Indicator (Mitutoyo, Tokyo, Japan) at five random positions around the film, by slowly reducing the micrometer gap until the first indication of contact.

Mechanical Properties. Puncture tests were carried out using a Stevens LFRA Model TA/1000 Texture Analyzer (Stevens, NY), as described previously (*22*). Film samples (8.5 cm in diameter) were equilibrated in a desiccator at 56% RH with sodium bromide saturated solution. Then film samples were placed in the middle of two acrylic plates with holes of 3.2 cm diameter and the holder was held tightly by two screws. A cylindrical probe (2 mm in diameter) was moved perpendicularly at the film surface at a constant speed (1 mm/s) until it passed through the film. Strength and deformation values at the puncture point were used to determine the hardness and deformation capacity of the film. Puncture strength was calculated by multiplying the reading value (grams) by the gravitational force; to avoid any thickness variations, the calculated puncture strength value was divided by the thickness of the film and was expressed in N/mm. The force–deformation curves were recorded, and the results were expressed in mm.

Water Vapor Permeability. The water vapor permeability (WVP) of films was determined gravimetrically at 30 °C using a modified ASTM 1983 procedure (*23*). The films were mechanically sealed to Vapometer cells (No. 68-1; Kalamazoo Paper Chemicals) containing anhydrous sodium chloride (0% RH, 0 mmHg of water vapor pressure), and the cells were placed in a Shellab 9010L humidity chamber (Sheldon Manufacturing Inc.) maintained at 56% RH (17.82 mmHg of water vapor pressure). The glass permeation cells were 6.35 cm (i.d.), 8.9 cm (o.d.), and 5.10 cm (height), with an exposed area of 31.67 cm^2. The water vapor transferred through the film and absorbed by the desiccant was determined from the weight gain of the cell. Steady-state conditions were assumed to be reached when the change in weight became constant over time (~6 h), and the weights were recorded over 24 h for all samples. The assemblies were weighed initially and at 6, 24, and 48 h intervals. Changes in weight of the permeation cell were recorded to the nearest 0.0001 g. The water vapor permeability was determined as follows (*24–26*): $WVP = wx/AT(p_2 - p_1)$ (g mm/(m^2 day mmHg)), where w is the weight gain of the cup over the time (T = 24 h), x is the thickness (mm), A is the area of exposed film (m^2), and $p_2 - p_1$ is the vapor pressure across the film (17.82 mmHg).

Microbial Resistance Measurements. To evaluate the percentage of N from protein films converted to soluble N as a function of time, films were inoculated with *S. thermophilus.* Before the inoculation, *S. thermophilus* (ST8301, CRDA, Saint-Hyacinthe, Québec, Canada) was subcultured twice in Nutrient Broth M17 (Difco Laboratories, Detroit, MI) and incubated each time for 24 h at 42 °C. For each biodegradability experiment, 1 mL of nutrient broth containing the bacteria was diluted in 9 mL of sterile saline solution and washed by centrifugation at 880*g* and 4 °C for 20 min (Model RC-5C automatic superspeed refrigerated centrifuge; DuPont, Wilmington, DE) at 4 ± 1 °C. This step was repeated three times in order to remove all traces of nutrient. Three milliliters of the final dilution (10^9 cfu/mL) was used to inoculate the solution. To accurately measure the bacterial growth due to the degradation of the films, five solutions were compared. Solution A contained 3 mL of bacterial culture diluted in 300 mL of a permeate solution culture with 15 sterile films irradiated at 4 kGy. Solution B contained the same volume of permeate solution and bacterial culture with 15 sterile films irradiated at 32 kGy. Solution C only contained the bacterial culture in the permeate solution. Solutions D and E only contained the 15 films irradiated at 4 and 32 kGy, respectively, in the permeate solution. The permeate solution consisting of ultrafiltrated skim milk contained 19 g/L of β-glycerophosphate buffer (Sigma, St. Louis, MO). The permeate was sterilized at 121 °C for 15 min. All flasks were incubated at 42 ± 1 °C with shaking (130 rpm) for 73 h. Each film (65 ± 2 ìm thick) was placed in the permeate solution with care to avoid contamination. To accurately measure the bacterial growth due to the degradation of the film measurement, samples (1 mL) were diluted in 9 mL of 0.85% sterile saline solution and plated 3-fold on Nutrient Agar M17. Bacterial counts were carried out after incubation at 42 °C for 48 h. Sampling of the solutions was performed at 0, 1, 2, 3, 6, 24, 48, and 72 h.

Soluble Nitrogen Analysis. Soluble nitrogen analysis was done using a LECO FP-428 apparatus (St. Joseph, MI). Typically, 15 mL of permeate solution containing 15 sterile films was sampled from each flask and centrifuged at 27000*g* for 20 min at 4 °C. The soluble fraction was sampled, frozen at −80 °C for 5 h, and then lyophilized. Dried samples (0.1 g) were encapsulated in aluminum foil and analyzed for N. Four samples were compared: two from solutions contained the films only (irradiated at 4 or 32 kGy) and two from solutions contained the same films inoculated with bacteria. To obtain the net variation in 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 *S. thermophilus* (C − A and D − B).

Statistical Analysis. Analysis of variance and Duncan multiple-range tests with $P \leq 0.05$ were applied to all results statistically. Analysis of variance was carried out with the GLM procedure of the SAS statistical package (SAS Institute, Cary, NC), and at the least significant difference a test was used to differentiate treatments. Differences between means were considered significant when $P \leq 0.05$. The Student t test was also used for WVP measurements to evaluate differences between irradiated and nonirradiated films and paired comparison with $P \leq 0.05$ (*27*). Experiments for mechanical properties and microbial resistance measurements were of randomized completed block design. For mechanical properties of films at different pectin and agar concentrations, a 5 × 6 randomized completed block was used: the experiment was replicated five times, and six concentrations of a pectin–agar mixture were evaluated. For the effect of some physical treatments on pectin–agar on the mechanical properties of films, a 5 × 3 randomized completed block was used: the experiment was replicated five times, and three pretreatments were done on pectin–agar solution (no treatment, irradiation, and autoclaving) before the irradiation of the final formulation. Values were compared with irradiated and nonirradiated formulations without pectin–agar. For microbial resistance measurements, a 3 × 5 × 8 randomized completed block was used: the

Table 1. Mechanical Properties of Calcium Caseinate and Whey Protein Isolate Based Films Containing a 1:1 Pectin–Agar Mixture, at Different Concentrations[a]

mechanical properties	pectin and agar concn (%) 0	0.05	0.1	0.15	0.2	0.3
puncture strength (N/μm)	0.08 ± 0.01[a]	0.07 ± 0.01[a]	0.09 ± 0.01[a]	0.06 ± 0.01[a]	0.07 ± 0.01[a]	0.06 ± 0.002[a]
puncture deformation (mm)	4.08 ± 0.43[a]	3.64 ± 0.34[a]	3.87 ± 0.39[a]	3.86 ± 0.31[a]	3.74 ± 0.19[a]	3.66 ± 0.28[a]

[a] Means followed by the same letter for each raw data point are not significantly different ($p > 0.05$). $n = 10$.

Table 2. Effect of Physical Treatments on the Puncture Strength of Calcium Caseinate and Whey Protein Isolate Based Films Containing a Pectin–Agar Mixture at a Concentration of 0.1% Each[a]

protein soln	physical treatments undergone by pectin and agar	puncture strength (N/μm)
irradiated	irradiated	0.11 ± 0.01[a]
irradiated	autoclaved	0.17 ± 0.01[b]
irradiated	none	0.05 ± 0.01[c]
nonirradiated	–	0.05 ± 0.01[c]
irradiated	–	0.10 ± 0.01[d]

[a] Means followed by different letters in the same column are significantly different ($P \leq 0.05$). A dash (–) indicates that pectin and agar were not added to the protein solution. $n = 10$.

experiment was replicated five times, five different solutions were tested (4 kGy films in the presence of bacteria, 32 kGy films in the presence of bacteria, bacteria only, 4 kGy films only, 32 kGy films only), and eight fermentation times were evaluated (0, 1, 2, 3, 6, 24, 48, 72 h).

RESULTS AND DISCUSSION

Film Thickness Measurements. Depending on the formulation the average film thickness was in the range of 45−65 ± 2 μm.

Mechanical Properties. Table 1 illustrates that the addition of both pectin and agar in a formulation containing 1:1 CC:WPI, glycerol, and CMC did not significantly affect ($P \leq 0.05$) either the puncture strength or the puncture deformation, independently of the concentration of these polysaccharides. Owing to these observations, the concentration of the pectin−agar mixture was set at a concentration of 0.1% each for the continuation of the following trials.

Both pectin and agar underwent then different physical treatments before being added to the milk protein formulation, namely heating at high temperature (121 °C, 15 min) and γ-irradiation (32 kGy). The puncture strength of the resulting films was assessed, and the results are summarized in **Table 2**. This investigation pointed out that both thermal and irradiation treatments of pectin and agar have a significant effect ($P \leq 0.05$) on the puncture strength of the resulting films and, thus, the mechanical properties of the films.

The puncture strength of the irradiated films containing untreated polysaccharides is similar to that of the nonirradiated films without pectin and agar (0.05 N/μm). It is assumed that the bulky structures of both polysaccharides might increase the interchain gap, thus weakening the protein network. This assumption might also explain that films with untreated pectin and agar have puncture strength significantly lower ($P \leq 0.05$) than that of the irradiation-based formulation without pectin and agar: 0.05 vs 0.10 N/μm.

When pectin and agar are irradiated at 32 kGy prior to their addition to the irradiation-based formulation containing 1:1 CC:WPI, glycerol, and CMC, a significant increase ($P \leq 0.05$) in the puncture strength, 142%, could be observed: 0.11 vs 0.05 N/μm. Moreover, this puncture strength value is significantly higher ($P \leq 0.05$) than the puncture strength of the irradiated films without pectin and agar, although the difference between these values is 10% (0.11 vs 0.10 N/μm).

When pectin and agar were autoclaved at 121 °C for 15 min, the resulting puncture strength of the resulting films reached the highest value in this investigation. Autoclaving generated a significant increase ($P \leq 0.05$) in the puncture strength (51%) with respect to irradiation, 0.17 vs 0.11 N/μm, whereas a significant difference of 168% was observed with respect to the irradiation-based formulation with nontreated polysaccharides: 0.17 vs 0.05 N/μm. It is worth noting that the autoclaving process allows sterilizing both pectin and agar, which is of paramount importance in the production of edible packaging.

The effect of autoclaving on the puncture strength can be attributed to the fact that high temperatures generate a less ordered structure, making more functional groups available (*17, 19*). Since the polysaccharides are added immediately after the autoclaving process to the protein solution, the fully disordered and dissociated polysaccharide chains might interact favorably with both calcium caseinate and WPI chains. Polysaccharides, such as CMC, methylcellulose, sodium alginate, and pectin, were reported to form charge−charge electrostatic complexes with proteins (*13, 15, 17, 28*−*30*). Moreover, pectin may also form cross-links with proteins under certain conditions (*17*). Therefore, autoclaving enhances protein−polysaccharide pectin−protein, and agar−protein interactions, resulting in a three-dimensional network with improved mechanical properties. A recent isothermal calorimetric investigation has pointed out the interaction of pectin with both of the proteins calcium caseinate and WPI (*31*). A further polysaccharide, CMC, was recently reported to enhance protein aggregation following physical treatments, such as heating or γ-irradiation (*15, 31*).

The opposite is also true, that untreated polysaccharides keep their orderly structure, thus preventing any favorable interactions between their functional groups and those of the milk proteins. Our experimental data confirm the lack of interactions between untreated polysaccharides and the proteins, resulting in poor physical properties.

An impact on the barrier properties was expected from the improvement of the puncture strength values, since aggregation was reported to decrease the diffusivity of the permeant (*32*). Moreover, agar and pectin were reported to improve the moisture barrier under certain conditions (*5, 17, 19*).

Barrier Properties. Figure 1 exhibits the effect of the concentration of autoclaved pectin and agar on the water vapor permeability (WVP) of the untreated milk-based films containing 1:1 CC:WPI, glycerol, and CMC. Upon addition of autoclaved pectin and agar in a 1:1 ratio, values of WVP ranged between 70×10^{-2} and 59×10^{-2} g mm/(m^2 day mmHg). Pectin and agar lowered significantly ($P \leq 0.05$) the WVP of the films, even at a very low concentration of 0.05%. It can be observed that the WVP of the films remained almost stable in the presence of pectin and agar. Nevertheless, the presence of an autoclaved mixture of pectin and agar at a ratio of 0.1% improved the

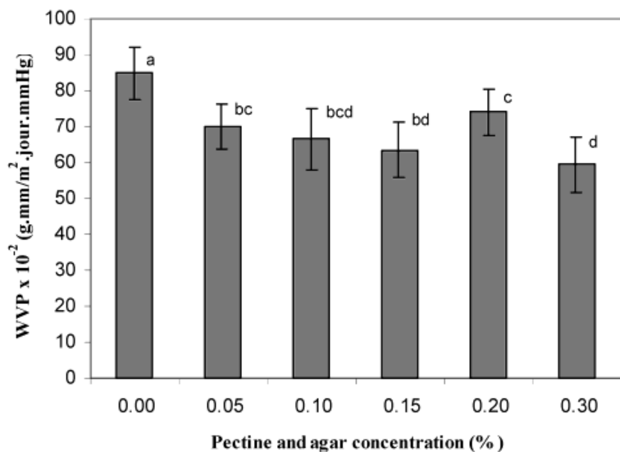

Figure 1. Effect of a 1:1 pectin–agar mixture, at different concentrations, on the WVP of calcium caseinate and whey protein isolate based films.

Table 3. Effect of Thermal and Radiative Treatment on the WVP of Calcium Caseinate and Whey Protein Isolate Based Films Containing a Pectin–Agar Mixture at a Concentration of 0.1% Each[a]

biofilm composition (ratio)	WVP ($\times10^{-2}$ g mm/(m^2 day mmHg)) nonirradiated	irradiated
CC:WPI:glycerol:CMC (10:10:10:1)	70.53 ± 2.95^{c}	$58.81 \pm 2.98^{b*}$
CC:WPI:glycerol:CMC:pectin:agar (25:25:25:2.5:1)	66.52 ± 1.22^{ab}	$47.60 \pm 0.93^{a*}$

[a] Means followed by different letters in each column are significantly different ($P \leq 0.05$). Means followed by an asterisk in each row are significantly different ($P \leq 0.05$). $n = 10$.

barrier properties of the films by 24% with respect to the formulation without the polysaccharides: from 85×10^{-2} to 64×10^{-2} g mm/(m^2 day mmHg).

The effect of thermal and radiative treatment of the milk protein solution on the WVP is summarized in **Table 3**. It is obvious that heating the protein solution at 90 °C for 30 min in combination with γ-irradiation at 32 kGy dramatically improved the barrier property to moisture of 16%: from 70×10^{-2} to 59×10^{-2} g mm/(m^2 day mmHg), due to the formation of cross-links (*11*−*13*). Moreover, the addition of autoclaved pectin and agar to the treated protein solution further enhances the moisture barrier by 18%: from 59×10^{-2} to 48×10^{-2} g mm/(m^2 day mmHg), due most probably to the enhancement of aggregation caused by the interactions between the proteins and the polysaccharides (*13*).

The films consisting of 1:1 CC:WPI, glycerol, CMC, pectin, and agar have WVP values 2 orders of magnitude lower with respect to soy protein films obtained in other studies under different compositions and conditions (*13, 33, 34*); the WVP obtained in this study (48×10^{-2} g mm/(m^2 day mmHg)) is the lowest reached so far with caseinate protein films developed in our laboratory (*35*).

Bacterial Counts. Table 4 exhibits the variation of the *S. thermophilus* population over time in the presence and in the absence of the milk-based protein consisting of 1:1 CC:WPI, glycerol, CMC, and an autoclaved 1:1 pectin−agar mixture, irradiated at 4 kGy and at 32 kGy. As expected, no bacterial growth was noted for solutions containing the films only (solutions D and E) throughout the entire experiment (data not shown). This result confirms that films were sterile prior to the inoculation. In solution C, where the inoculum evolves in the absence of nutrients, the population was relatively stable up to 72 h.

Table 4. Variation of the *Streptococcus thermophilus* Population over Time (log CFU/mL)[a]

time (h)	soln A[b]	soln B[b]	soln C[b]
0	7.37 ± 0.27^{a}	7.81 ± 0.01^{a}	7.42 ± 0.03^{a}
1	$7.35 \pm 0.30^{ab(1)}$	$7.60 \pm 0.01^{a(2)}$	$7.29 \pm 0.03^{a(1)}$
2	$7.22 \pm 0.05^{a(1)}$	$7.51 \pm 0.01^{a(2)}$	$7.46 \pm 0.01^{a(1)}$
3	$7.28 \pm 0.13^{a(1)}$	$7.56 \pm 0.01^{a(2)}$	$7.48 \pm 0.03^{a(1)}$
6	$7.63 \pm 0.03^{b(1)}$	$7.58 \pm 0.01^{a(2)}$	$7.96 \pm 0.01^{a(1)}$
24	$9.72 \pm 0.04^{c(2)}$	$9.26 \pm 0.01^{b(2)}$	$8.54 \pm 0.01^{b(1)}$
48	$8.89 \pm 0.24^{d(2)}$	$9.80 \pm 0.01^{c(2)}$	$7.92 \pm 0.01^{a(1)}$
72	$7.41 \pm 0.05^{e(2)}$	$8.54 \pm 0.01^{d(2)}$	$7.93 \pm 0.01^{a(1)}$

[a] Means followed by different letters in the same column are significantly different ($P \leq 0.05$). Means followed by different numbers in the same raw data point are significantly different ($P \leq 0.05$). $n = 15$. [b] Conditions: (solution A) 4 kGy films in the presence of bacteria; (solution B) 32 kGy films in the presence of bacteria; (solution C) bacteria only.

When films irradiated at 4 kGy were exposed to *S. thermophilus*, to ascertain the sterility of the films (solution A), the bacterial population remained relatively stable for the first 3 h with 10^7 CFU/mL. A maximum was observed at 24 h after the inoculation, representing a level of 10^{10} CFU/mL. The number of microorganisms was found to decrease significantly ($P \leq 0.05$) at 48 and 72 h following the inoculation to reach 10^9 and 10^7 CFU/mL, respectively. When films irradiated at 32 kGy (solution B), to generate cross-links through the formation of bityrosine (*8*), were inoculated with *S. thermophilus*, the bacterial population was relatively stable during the first 6 h with 10^8 CFU/mL. The first significant increase of *S. thermophilus* was observed at 24 h. The maximum bacterial population was reached after 48 h with a level of 10^{10} CFU/mL. The population then decreased ($P \leq 0.05$) to 10^8 CFU/mL at 72 h after the inoculation.

Our results indicate that the bacterial population started to increase significantly ($P \leq 0.05$) as the degradation of the film progressed. The bacterial population reached a maximum of 10^{10} CFU/mL at 48 h for the 32 kGy films, while a comparable number of microorganisms was already reached at 24 h in the case of the non-cross-linked 4 kGy films. This feature is probably due to a smaller number of cross-links in the case of 4 kGy films. Visual observations confirmed that the dissolution of the 4 kGy films is faster than that of the 32 kGy films.

Soluble Nitrogen Analyses. Figure 2 shows the variation in soluble nitrogen content (N; %) as a function of time for the four solutions investigated. Soluble N concentrations in solution A**,** containing only the 4 kGy films, increased significantly ($P \leq 0.05$) during the first 24 h from 0.19 to 0.31% and then decreased to 0.21% after 72 h. Soluble N concentrations in solution B were more stable during the entire investigation. Indeed, no significant increase or decrease ($P > 0.05$) could be observed between 1 and 72 h. The average soluble N concentration for solution B was 0.19%. Soluble N concentrations were found to be significantly higher ($P \leq 0.05$) in solution A with respect to solution B, indicating that the 4 kGy films solubilized more readily than the 32 kGy films despite the absence of the *S. thermophilus*. Soluble N was not detectable in the solution containing the bacteria only (data not shown).

When the 4 kGy films were incubated with the bacteria (solution C), soluble N content was found to be more important as compared to solution A (4 kGy films alone). A significant increase ($P \leq 0.05$) of soluble nitrogen (186%) was noted at

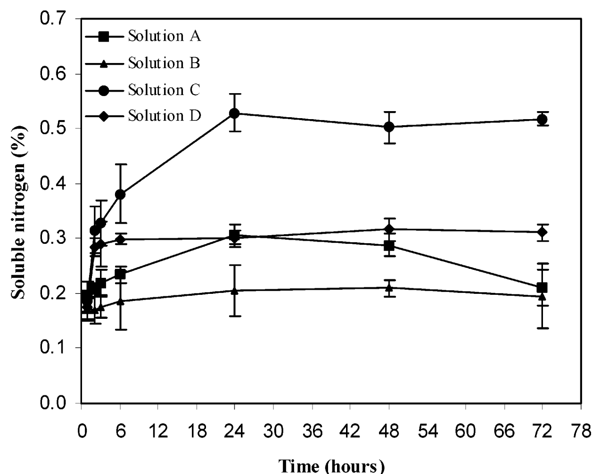

Figure 2. Variation in soluble nitrogen content (%) with time: (solution A) 4 kGy films only; (solution B) 32 kGy films only; (solution C) bacteria + 4 kGy films; (solution D) bacteria + 32 kGy films.

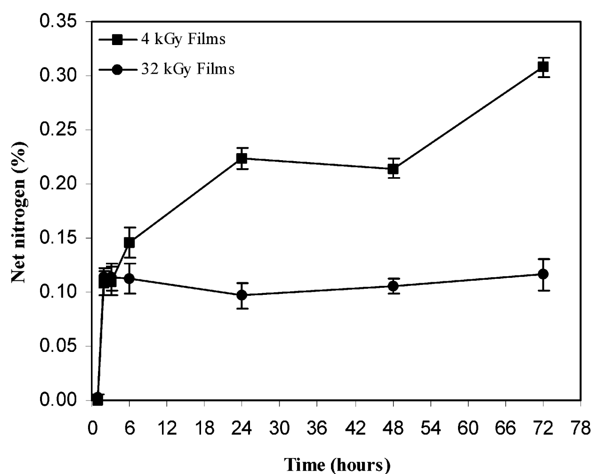

Figure 3. Net variation of soluble nitrogen content (%) due to the bacterial degradation (4 kGy films vs 32 kGy films).

24 h. Following that period, the soluble N remained almost stable until 72 h. The solution containing the 32 kGy films and the bacteria (solution D) show a lower soluble N concentration with respect to solution C (4 kGy and the bacteria) but a higher concentration with respect to solution B, the 32 kGy films without the bacteria (**Figure 2**). From 24 to 72 h the mean contents of soluble N in solution containing the 4 kGy (solution C) and 32 kGy films (solution D) were respectively 0.51 and 0.31%, and these were significantly different ($P \leq 0.05$). The results obtained for solutions C and D confirmed that, in the presence of the bacteria, the 4 kGy films release more soluble N than the 32 kGy films.

Figure 3 exhibits the net percentage of N from protein films converted to soluble N as a function of time for 4 and 32 kGy films. It can be observed that the overall net release of soluble N is different between the 4 kGy and the 32 kGy films. A sharp increase was observed for the 4 kGy films between 1 and 24 h, while a more progressive increase with time occurred with the 32 kGy films. We have previously demonstrated that γ-irradiation of caseinate solutions resulted in a significant increase of bityrosine bridge formation between protein chains, i.e., cross-links, inducing more ordered and more stable structures and increasing the molecular weight of caseinate. Moreover, γ-irradiation of caseinate solutions accounts for the insolubility, an improvement of the mechanical strength, a resistance to proteolysis, and a reduction of the water vapor permeability of films obtained from irradiated solutions (*8, 11, 12, 20*). Such behavior could be related to a higher number of cross-links in the irradiated films. The 4 kGy films generated higher soluble N content than 32 kGy films: 31% vs 12%, respectively. This result is an indication of a higher bacterial activity with non-cross-linked films. Therefore, cross-links generated by γ-irradiation confer a greater resistance toward microbiological degradation. Similar results were recently reported with other milk-based formulations (*9, 10, 12*).

A lack of amino acids or peptides in media may slow or inhibit the growth of *S. thermophilus*. For this reason, *S. thermophilus* is often paired with proteolytic partners as *Lactobacillus delbrueckii* ssp. *bulgaricus* for milk fermentation (*36*). Therefore, *S. thermophilus* is more demanding in terms of population growth with respect to other bacteria, such as *Pseudomonas aeruginosa*. Permeate used in this experiment, as a media for bacterial growth, was obtained after ultrafiltration of skim milk and can be considered as fat and protein free (*37, 38*). Normally, *S. thermophilus* does not grow in the permeate solution due to the lack of nitrogen. The higher population of *S. thermophilus* in the presence of films (**Table 4**) shows that the production of soluble N (**Figure 3**) in the permeate solution may come from the film degradation in order to obtain the nitrogen required for his growth. The level of biodegradation in the films, calculated on the basis of net N released after 72 h, was 12% for the 32 kGy films and 31% for the 4 kGy films.

CONCLUSION

Treatments of both pectin and agar by autoclaving, prior to their being added to protein solutions, generated films with improve puncture strength and better WVP resistance. Films obtained from irradiated solutions also exhibit better mechanical properties. It was assumed that further interactions, namely pectin−protein and agar−protein interactions, occurred when the autoclaved polysaccharides were added to the milk protein solution, accounting for the enhancement of the mechanical properties and the moisture barrier of the resulting films. Besides this benefit, the autoclaving process sterilizes both pectin and agar, thus contributing to the maintenance of the film's sterility.

As expected, cross-linking had a direct impact on the microbial resistance of the milk protein biofilms containing autoclaved pectin and agar. It is believed that these promising films might find application in food packaging.

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LITERATURE CITED

(1) Kester, J. J.; Fennema, O. *Edible films and coatings: A review.* **1986** (December), 47−59.
(2) Peyron, A. L'enrobage et les produits filmogènes: un nouveau mode d'emballage. *Viandes Prod. Carnés* **1991**, *12*, 41−46.
(3) Gennadios, A.; Weller, C. L. Edible films and coatings from soymilk and soy protein. *Cereal Food World* **1991**, *36*, 1004−1009.
(4) Chen, H. Functional properties and applications of edible films made of milk proteins. *J. Dairy Sci.* **1995**, *78*, 2563−2583.
(5) Krochta, J. M.; De Mulder-Johnston, C.; Edible and biodegradable polymer films: challenges and opportunity. *Food Technol.* **1997**, *51*, 61−74.

(6) Baker, R. A.; Baldwin, E. A.; Nisperos-Carriedo, M. O. Edible coatings and films for processed foods. In *Edible coatings and films to improve food quality*; Krochta, J. M., Baldwin, E. A., Nisperos-Carriedo, M. O., Eds.; Technomic Publishing: Lancaster, PA, 1994; pp 89−105.
(7) McHugh, T. H.; Krochta J. M. Milk-protein-based edible films and coating. *Food Technol.* **1994** (January), 97−103.
(8) Brault, D.; D'Aprano, G.; Lacroix, M. Formation of free-standing sterilised edible films from irradiated caseinates. *J. Agric. Food Chem.* **1997**, *45*, 2964−2969.
(9) Mezgheni, E.; Vachon, C.; Lacroix, M. Biodegradability behaviour of cross-linked calcium-caseinates films. *Biotechnol. Prog.* **1998**, *14*, 534−536.
(10) Ressouany, M.; Vachon, C.; Lacroix, M. Microbial resistance of caseinate films cross-linked by γ-irradiation. *J. Dairy Res.* **2000**, *67*, 119−124.
(11) Vachon, C.; Yu, H. L.; Yefsah, R.; Alain, R.; St-Gelais, D.; Lacroix, M. Mechanical and structural properties of milk protein edible films cross-linked by heating and γ-irradiation. *J. Agric. Food Chem.* **2000**, *48*, 3203−3209.
(12) Le Tien, C.; Letendre, M.; Ispas-Szabos, P.; Mateescu, M. A.; Delmas-Patterson, G.; Yu, H. L.; Lacroix, M. Development of biodegradable films from whey proteins by cross-linking and entrapment of cellulose. *J. Agric. Food Chem.* **2000**, *48*, 5566−5575.
(13) Sabato, S. F.; Ouattara, B.; Yu, H.; D'Aprano, G.; Lacroix, M. Mechanical and barrier properties of cross-linked soy and whey protein based films.*J. Agric. Food Chem.* **2001**, *49*, 1397−1403.
(14) Shimada, K.; Cheftel, J. C. Sulfhydryl group disulfide bond interchange during heat induced gelation of whey protein isolate. *J. Agric. Food Chem.* **1989**, *37*, 161−168.
(15) Yu, H.; D'Aprano, G.; Sabato, S. F.; Lacroix, M. Effect of the addition of CMC on the aggregation behaviour of proteins. *J. Agric. Food. Chem.,* submitted for publication.
(16) Bégin, A. L'emballage au naturel (Natural packaging). *Alimentech* **1993**, *6*, 6−7.
(17) Thakur, B. R.; Singh, R. K.; Handa, A. K. Chemsitry and uses of pectin-a review. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 47−73.
(18) Schultz, T. H.; Owens, H. S.; Maclay, W. D. Pectinate films. *J. Colloid Sci.* **1948**, *3*, 53−62.
(19) Bégin, A.; Riad, A.; Lebbar, R. De nouvelles possibilités pour l'agar (New potential for agar). *Alimentech* **1994**, *7*, 10−13.
(20) St-Gelais, D.; Piette, M.; Bélanger, G. Production of cheddar cheese using milk enriched with microfiltered milk retentate, a preliminary study. *Milchwissencschaft* **1995**, *50*, 614−619.
(21) Ressouany, M.; Vachon, C.; Lacroix, M. Irradiation dose and calcium effect on the mechanical properties of cross-linked caseinate films. *J. Agric. Food Chem.* **1998**, *46*, 1618−1623.
(22) Gontard, N.; Guilbert, S.; Cuq, L. L. Edible wheat gluten films: influence of the main process variables on film properties using response surface methodology. *J. Food Sci.* **1992**, *57*, 190−195.
(23) ASTM. *Standard Test Methods for Water Vapour Transmission of Materials*; American Society for Testing and Materials: Philadelphia, PA, 1983; Method 15.09:E96,
(24) Heiss, R. Shelf life determination. *Mod. Packag.* **1958**, *31*, 119.
(25) Karel, M.; Proctor, B. E.; Wiseman, G. Factors affecting water-vapour transfer through food packaging films. *Food Technol.* **1959**, *13*, 69.
(26) Labuza, T. P.; Contreras-Medellin, R. Prediction of moisture protection requirements for foods, *Cereal Foods World* **1981**, *26*, 335.
(27) Snedecor, G. W.; Cochran, W. G. One-way classifications. Analysis of variance. *Statistical methods*; The Iowa State University Press: Ames, IA, 1978.
(28) Imeson, A. P.; Ledward, D. A.; Mitchell, J. R. On the nature of the interaction between some anionic polysaccharide acid proteins. *J. Sci. Food Agric.* **1977**, *28*, 661.
(29) Bernal, V. M.; Smajda, C. H.; Smith, J. L.; Stanley, D. W. Interactions in protein/polysaccharide/calcium gels. *J. Food Sci.* **1987**, *52*, 1121−1125.
(30) Shih, F. F. Interaction of soy isolate with polysaccharide and its effect on film properties. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1281−1285.
(31) Letendre M.; D'Aprano, G.; St-Gelais, D.; Delmas-Patterson, G.; Lacroix, M. Isothermal calorimetry study of calcium caseinate and whey protein isolate edible films cross-linked by heating and γ-irradiation. *J. Agric. Food Chem.* **2002**, *50*, 6053−6057.
(32) Krochta, J. M.; Baldwin, E. A.; Nisperos-Carriedo, M. *Edible coatings and films to improve food quality*, 1st ed.; Technomic Publishing: Lancaster, PA, 1994.
(33) Stuchell, Y. M.; Krochta, J. M.; Enzymatic treatments and thermal effects on edible soy protein films. *J. Food Sci.* **1994**, *59*, 1332−1337.
(34) Jo, K. H.; Park, H. J.; Jung, S. T.; Rhim, J. W.; Weller, C. L. Water vapour permeability and mechanical properties of soy protein films as affected by plasticizer, solvent, pH and salt. *Food Biotechnol.* **1996**, *5*, 243−248.
(35) Ouattara, B.; Le, T. C.; Vachon, C.; Mateescu, M. A.; Lacroix, M. Use of Gamma-irradiation Cross-linking to improve the Water vapor Permeability and the Chemical Stability of Milk Protein Films. *Radiat. Phys. Chem.* **2002**, *63*, 821−825.
(36) Marshall, V. M. Fermented milks and their future trends. 1-Microbiological aspects. A review. *J. Dairy Res.* **1987**, *54*, 559−574.
(37) Tong, P. S.; Barbano, D. M.; Jordan, W. K. Permeate flux during ultrafiltration of whey: Influence of milk coagulant used for cheese manufacture. *J. Dairy Sci.* **1988**, *71*, 2342−2348.
(38) St-Gelais, D.; Hache, S.; Gros-Louis, M. Combined Effects of Temperature, Acidification, and Diafiltration on Composition of Skim Milk Retentate and Permeate. *J. Dairy Sci.* **1992**, *75*, 1167−1172.

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