Development of Biodegradable Films from Whey Proteins by Cross-Linking and Entrapment in Cellulose

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When cross-linked by heating or by γ-irradiation and entrapped in cellulose, whey proteins can generate insoluble biofilms with good mechanical properties and high resistance to attack by proteolytic enzymes. Interchain cross-linking of proteins generated an increase in the puncture strength, and a decrease in water vapor permeability. Gelatin was added in film formulation as a stabilizer to improve the puncture strength and film appearance. The structure of the biofilms was also analyzed. SDS−PAGE revealed that heating and γ-irradiation produce an increase of the molecular weight of the cross-linked protein. Size exclusion chromatography showed a molecular mass of 40 kDa for un-cross-linked whey proteins, whereas for the soluble fractions of the cross-linked proteins, molecular distributions were between 600 and 3800 kDa for the heated proteins and between 1000 and 2000 kDa for γ-irradiated proteins. No major alteration of the structural conformation of the proteins was observed by FTIR for biofilms obtained after heat treatment, whereas γ-irradiation induced some modifications in the protein structure. X-ray diffraction analysis suggests that cross-linking by γ-irradiation seems to modify the conformation of proteins, which became more ordered and more stable.

Keywords: *Whey proteins; films; biodegradable; entrapment; cross-linking; heating; γ-irradiation; proteolysis; rheological properties*

INTRODUCTION

The use of plastic for packaging has grown extensively in recent years. According to Hunt et al. (1990), inert and non-biodegradable plastic materials represent ~30% of municipal solid waste. In this context, biodegradable films can be a source of saving of energy and an important issue for environmental protection.

Gontard (1994) classified biodegradable polymers into three groups of (a) blends of synthetic polymers (i.e., polyethylene) with natural polymers, (b) bacterial polyesters (polyhydroxyalkanoates), and (c) natural polymers more or less modified (polysaccharides or proteins). Considering the low cost of raw material and the beneficial impact on the environment, the natural polymers became the subject of intensive studies for the development of biodegradable films.

Numerous studies demonstrated that whey proteins have interesting nutritional value and possess several functional properties important for biofilm formation. Whey proteins are byproducts of cheese manufacture and contain two major protein types, α-lactalbumin and β-lactoglobulin. A huge amount (~20×10^6 t) is produced per year, much of which is never used (Kinsella and Whitehead, 1989). Formerly, this fluid whey was discharged into a river, which represents a risk for the environment.

Methods to produce films have been described in several papers. Generally, a process of proteins cross-linking is necessary to obtain a flexible, easy to handle film. McHugh et al. (1994) produced whey proteins isolate (WPI) films by heating protein solutions to between 75 and 100 °C. Heat was essential for the formation of the intermolecular disulfide bonds. Brault et al. (1997) used γ-irradiation to produce cross-linked caseinate films. It was demonstrated that γ-irradiation produces bityrosine bridges between protein chains (Davies et al., 1987; Prütz et al., 1983). Several enzymes are known to be involved in protein cross-linking. Mahmoud and Savello (1992) and Motoki et al. (1987) used tranglutaminase to produce films. Tranglutaminase (γ-glutamyltransferase, EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the formation of covalent ϵ-(γ-glutamyl)lysyl cross-links. Major advantages of the γ-irradiation process are the following: the method is less expensive than using enzymes and allows the formation of insoluble and sterilizable films. Physical or enzymatic methods allow formation of protein films with good mechanical properties (Motoki et al., 1987). However, these films are soluble and sensitive to humidity, limiting their area of application.

Composite films of proteins and lipids were also formulated with the aim to decrease their solubility. Although interesting, protein−lipid films are often difficult to obtain. For example, bilayer film formation requires the use of solvents or high temperatures, making production more costly. Furthermore, separa-

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tion of the layers may occur with time. For films cast from aqueous, lipid emulsion solutions, the process is complex, and the incorporation of lipids is limited.

The aim of this study was to develop and to characterize whey protein films made by chemical and physical techniques. The methodology is essentially based on films cross-linked by heating and γ-irradiation followed by the inclusion of proteins in a cellulose matrix. The structure of these films was examined by Fourier transform infrared analysis (FTIR) and X-ray diffraction analysis.

MATERIALS AND METHODS

Materials. *Reagents.* Whey protein concentrate (WPC; 76.27% protein, 5.79% fatty acids, and 14.22% lactose) powder was from Les Fromages Saputo Ltée (St-Hyacinthe, PQ, Canada). Whey protein isolate (WPI; 90.57% protein), from the Food Research Center of Agriculture and Agri-food Canada, was produced from the permeate obtained by tangential membrane microfiltration (MF). 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 previously described (St-Gelais et al., 1995). The proteins contained in the permeate were concentrated 25-fold at 50 °C by ultrafiltration (UF) using a 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 to obtain WPI. Glycerol (99.5% reagent grade) was from American Chemicals Ltd. (Montreal, PQ, Canada). Cellulose, gelatin, and acetic acid were from Sigma Chemical Co. (St. Louis, MO).

Cellulose Xanthate Preparation. A solution of 4% cellulose dissolved in 18% aqueous NaOH at 20 °C (alkali cellulose) was converted to cellulose xanthate by the addition of up to 1.3−1.6% carbon disulfide (CS_2). After stirring (2−3 h), the excess of CS_2 was evacuated under vacuum, and the solution was incubated for 2−3 days at 15−20 °C (Champetier and Monnerie, 1969).

Film Formation. *(a) Preparation of Film-Forming Solution by Heating.* Solutions containing 5% whey protein (WPI or WPC) and 2.5% glycerol were heated at 80 °C for 30 min. The solution was cooled at room temperature (20 ± 1 °C) during 1−2 h, and then 0.25% cellulose xanthate and up to 1% gelatin were added, always under stirring. Indeed, the reason for cellulose addition resides in its role as a matrix, whereas gelatin, at concentrations <1%, can play the role of a stabilizing agent, which prevents films from breaking or swelling in contact with the water and improves film appearance. Films were cast by applying 5 mL of the solution evenly onto Petri (8.5 cm diameter) dishes (Fisher Scientific, Montreal, PQ, Canada) and allowed to dry overnight (20 ± 1 °C).

(b) Preparation of Film-Forming Solution by γ-Irradiation. Solutions containing the same components as for the heating procedure described above (5% whey protein containing 2.5% glycerol) were degassed under vacuum to remove dissolved air and flushed under an inert atmosphere, as previously described (Brault et al., 1997). Solutions were transferred to amber glass bottles, sealed with Parafilm, and irradiated using a ^{60}Co source irradiator (γ-cell 220) (MDS Nordion, Kanata, ON, Canada) at the Canadian Irradiation Center (Laval, PQ, Canada) at a dose of 32 kGy. Then, 0.25% cellulose xanthate and up to 1% gelatin were added under stirring, at 20 °C. Films were cast by applying 5 mL of the solution evenly onto Petri dishes and allowed to dry overnight, as mentioned previously.

(c) Film Insolubilization (Entrapment). After 24 h, dried films obtained by heating or by γ-irradiation were peeled and treated in baths with ethanol/acetic acid (5:1, v/v) solution for 15 min. Ethanol helps to fix proteins in the films and the acid to regenerate insoluble cellulose from soluble cellulose xanthate, rendering thus the film insoluble. To remove the excess acid, films were repeatedly rinsed in baths of ethanol/water (1:1, v/v) followed by ethanol/glycerol/water (4:1:5, v/v/v). The rationale for the addition of the glycerol at the second washing was related to its role as stabilizer. Films were then reconditioned in a desiccator containing a saturated NaBr solution, ensuring 56% relative humidity (RH) at room temperature (20 °C), for at least 48 h (Gontard et al., 1992).

Characterisation of Films. *(a) Solubility Test.* The test consists of determining the differences between the initial dry weight (IDW) and the dry weight after treatment (DWT) of two types of biofilms (obtained by cross-linking whey proteins and entrapment in the cellulose matrix) in water. The rationale behind each of the two treatments was to determine the amount of matrix material of the films lost by solubilization in water in drastic conditions (boiling for 30 min) followed by long-term exposure (24 h) at 20−37 °C. The average dry weight of the biofilms was determined for each of the 14 samples by drying them in an oven at 45 °C until constant weight was achieved (7 days). Two different treatments were performed. In one, seven biofilms were dropped in flasks with 100 mL of boiling water for 30 min; the flasks were then removed from the heat and the biofilms kept in water (room temperature) for another 24 h. For the other treatment, seven biofilms were incubated in a flask at 37 °C for the same period of time (with 0.02% of sodium azide added in the water to prevent microorganisms growth) (Gontard, 1992). After 24 h, the solid biofilms were removed and dried in an oven as previously described. Results are expressed as the yield of recovery (YR), which was calculated using the following equation:

$$\text{yield of recovery (insoluble matter, \%)} = (\text{DWT/IDW}) \times 100$$

(b) Film Thickness Measurements. Film thickness was measured using a Mitutoyo Digimatic Indicator (Mitutoyo, Tokyo, Japan) at five random positions around the film. The average film thickness was in the range of 50−60 μm and depended upon the formulation.

Rheological Analysis. Puncture tests were carried out using a Stevens LFRA texture analyzer model TA/1000 (Scarsdale, NY), as described by Gontard et al. (1992). A cylindrical probe (0.2 cm diameter) was moved perpendicularly at the film surface at a constant speed (1 mm/s) until it passed through the film. Strength values at the puncture point were used to calculate hardness and deformation capacity of the film. In order to avoid any variation related to thickness, the puncture strength values were normalized to the thickness of the film (Peleg, 1979).

Viscoelastic properties were evaluated using relaxation curves. The same puncture-test procedure as described before was used, but the probe was stopped and maintained at 3 mm deformation. The Young's modulus, $Y(1\ \text{min}) = (\text{IF} - \text{FF})/\text{IF}$, where IF and FF (Peleg, 1979) were, respectively, forces recorded initially (IF) and finally (FF) for relaxation after 1 min. A low relaxation coefficient ($Y \rightarrow 0$) indicates high film elasticity, whereas a high coefficient ($Y \rightarrow 1$) indicates high film plasticity.

(c) WVP Tests. WVP tests were conducted using a modified ASTM procedure (Gontard et al., 1992). The film was sealed on a glass permeation cup containing 5.0 g of phosphorus pentoxide (0% RH). For each film, the cup was stored at 20 °C in desiccator with saturated NaBr solution (56% RH). After steady state conditions were reached, the cups were weighed at 24 h and WVP of the film was calculated as

$$\text{WVP (g·mm/m}^2\text{·24 h·mmHg)} = Wx/AT(P_2 - P_1)$$

where W is the weight gain of the cup (g), x is the film thickness (mm), A is the area of exposed film (m^2), T is the time of gain (h), and $P_2 - P_1$ is the difference of vapor pressure across the film [$P_2 - P_1 = 9.819$ mmHg (20 °C)].

Stability and Biodegradability Tests. Stability of films to proteolysis was done using trypsin, and biodegradability of films was evaluated using pancreatin.

(a) Stability of Films at Proteolysis by Trypsin. Biofilms were incubated in the trypsin solution, and the protein residues were quantified as they were liberated into the medium in 1 day intervals. The enzyme solution contained 0.05% trypsin from porcine pancreas (type II-S, 3550 enzyme units/mg of solid, Sigma Chemical Co., St. Louis, MO) in 20 mM Tris (pH 7.5). Biofilms were cut into small pieces of 2.6 cm diameter (corresponding to 25 mg of protein), and each piece was incubated in 5 mL of trypsin solution at room temperature (23 °C). One enzyme unit was considered to be the amount of enzyme able to hydrolyze 1 μmol of benzoyl-L-arginine ethyl ester (BAEE) in 1 min. The Bradford (1976) method was used to determine soluble protein.

The yield of recovery was calculated as

$$\text{YR (\%)} = [(\text{QPF} - \text{QPL})/\text{QPF}] \times 100$$

where QPF is the quantity of protein in the film and QPL is the quantity of soluble proteins liberated in the medium (quantity of total proteins in medium − quantity of enzyme).

(b) Biodegradability of Films. Biofilms were incubated in the pancreatin solution, and their weight loss was determined as a function of time (15 min interval). The enzyme solution contained 1% pancreatin (Fisher Scientific Co., Fair Lawn, NJ) in 50 mM potassium phosphate buffer (pH 7.5). Each film of 8.5 cm diameter (500 mg of protein) was incubated in 50 mL of enzyme solution at room temperature (23 °C).

Structural Analysis. For analysis by electrophoresis or size exclusion chromatography, the samples studied were represented by the soluble fraction only of the film-forming solution containing 0.5% proteins (control and treated). For isothermal calorimetry, FTIR, and X-ray diffraction analysis, samples were biofilms containing 5.0% proteins, 2.5% glycerol, and 0.25% cellulose.

(a) Electrophoretic Analysis. Molecular mass characteristics of the control, heated (30 min at 80 °C), and γ-irradiated (32 kGy) WPC and WPI were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to the method of Laemmli (1970) using a Mini-PROTEAN II cell (Bio-Rad Laboratories, Hercules, CA). Acrylamide stacking gel (4%) and separating gel (12%) were used. Gels were stained with 0.1% Coomassie brilliant blue R-250 for 30 min in 10:40:50 acetic acid/methanol/water (v/v/v) and destained in the same solvent system without dye.

(b) Size Exclusion Chromatography Analysis. Size exclusion chromatography was performed on the soluble protein using a Varian Vista 5500 HPLC coupled with a Varian autosampler model 9090. Detection of the protein fractions was done using a standard UV detector set at 280 nm. Supelco Progel TSK PWH (7.5 mm × 7.5 cm) and TSK GMPW (7.5 mm × 30 cm) guard columns (Supelco, Sigma Aldrich PQ, Canada Ltd.) followed by Ultra Hydrogel 2000 and 500 (7.5 mm × 30 cm) analytical columns (Waters Ltd., Mississauga, ON, Canada) were used for the molecular weight determination of the control and cross-linked protein samples. The total molecular weight exclusion limit was 25×10^6 Da based on linear poly(ethylene glycol) (PEG) mass. The eluant (80% v/v aqueous and 20% v/v acetonitrile) was flushed through the columns at a flow rate of 0.8 mL/min. The aqueous component of the eluant was 0.02 M Tris buffer (pH 8.0) containing 0.1 M NaCl. The molecular weight calibration curve was established using series of protein molecular weight markers (Sigma, MW-GF-1000) ranging from 2×10^6 to 29×10^3 Da. All soluble protein solutions (0.5% w/v) were filtered through a 0.45 μm nylon membrane (Nalge Co., Rochester, NY) prior to injection.

(c) Isothermal Calorimetry. Measurements were obtained with a calorimeter Setaram C80 (Lyon, France) in an isothermal mode (heats of swelling). A known weight of dried sample (30 mg) was introduced in a homemade thin glass bulb and sealed under vacuum. The bulb was placed in water into a cell equipped with Teflon joints to prevent water evaporation, and then the cell was placed into the calorimeter. After thermal equilibrium, the bulb was broken by pushing gently, from the top of the calorimeter, a stem through the stopper of the cell. Due to the vacuum in the bulb, water filled the entire glass bulb and interacted with the sample. The value of ΔH_{exptl} after integration of the heat flow change is the sum of three contributions:

$$\Delta H_{\text{exptl}} = \Delta H_{\text{interaction}} + \Delta H_{\text{glass-breaking}} + \Delta H_{\text{vaporization}}$$

The two last terms can be measured by control experiments. By subtracting their value (about −150 to −200 mJ) from ΔH_{exptl}, $\Delta H_{\text{interaction}}$ is obtained (Le Lay and Delmas, 1998).

(d) FTIR Spectroscopic Analysis. FTIR spectra were recorded using a BOMEM Hartman & Braun (Bomem, Inc., Québec, PQ, Canada) equipped with deuterated triglycine sulfate (DTGS) detector. Spectra were analyzed using the BOMEM GRAMS software (ver. 1.51). The biofilms were placed in the BOMEM cell for the scanning spectral region (4000−500 cm^{-1}), and 50 scans were recorded with a 1 cm^{-1} resolution. Also studied were the second spectra derivatives, which narrowed the broad amide I band related to the different protein chain conformations (Byler, 1988).

(e) X-ray Diffraction. The diffraction pattern of whey protein films was recorded by a Siemens D-5000 diffractometer with cobalt cathode operating in reflectance mode at wavelength $\lambda = 1.79019$ Å.

Statistical Analysis. Analysis of variance and Duncan multiple-range tests with $p \leq 0.05$ were used to analyze the statistics of the results. For solubility, puncture strength, and biodegradability measurements, three replicates of seven films were analyzed. For viscoelasticity and WVP measurements, three replicates of three films were analyzed. The Student t test was stat paired-comparison with $p \leq 0.05$ (Snedecor and Cochran, 1978).

RESULTS AND DISCUSSION

The results presented here show that there are differences between films obtained from concentrated whey proteins (WPC) and those obtained from isolated whey proteins (WPI). These two preparations differ in terms of their composition: WPC contains 76% whey proteins, 5.8% fatty acids, and 14.2% lactose, whereas WPI (obtained by microfiltration) contains 91% whey proteins. All data refer to WPC or WPI films entrapped in cellulose but submitted to various treatments. Gelatin is added after the cross-linking reaction as a stabilizing agent. No significant effect ($p > 0.05$) on the solubilization, WVP, and biodegradability of films was observed for gelatin concentrations <1%. Only an increase of ~4.0% puncture strength was noted in all cases.

Solubility Tests. Water (in) solubility is one of the most important properties. The potential of application of these biofilms is higher if the film is more resistant to water. Table 1 presents the insoluble fraction of films obtained after entrapment in cellulose of cross-linked WPC and WPI. Results show that heating or γ-irradiation produced biofilms that were less soluble. The best yield of recovery was obtained for γ-irradiated WPI films. Over 98% of recovery was observed for WPI films treated at 100 °C/30 min and >99% recovery for WPI films treated at 37 °C/24 h. No statistical differences ($p > 0.05$) were found between WPI and WPC heated or γ-irradiated films for solubility test at 37 °C, whereas the solubility test at 100 °C/30 min showed significant differences between WPC and WPI films ($p \leq 0.05$). The higher solubility of WPC films may be due to the presence of other components (i.e., fatty acids) in the film composition that could prevent hydrogen bonding of cellulose and then hinder entrapment of proteins. Another possibility could be that lactose (present in WPC), when dissolved, may make the films more porous. The same observation was noted by Vachon et al. (2000).

Table 1. Insoluble Fraction of WPC and WPI Films Evaluated as Yield of Recovery, Following Different Treatments

		yield of recovery[a] (%)	
solubility test	type of film	WPC	WPI
30 min/boiling water and 24 h/23 °C	control (without treatment)	$67.8 \pm 4.7^{a,1}$	$94.5 \pm 2.3^{c,2}$
	heated (80 °C/30 min)	$77.1 \pm 4.1^{b,1}$	$97.7 \pm 2.0^{d,2}$
	irradiated (32 kGy)	$79.3 \pm 3.0^{b,1}$	$98.6 \pm 1.3^{d,2}$
24 h/37 °C	control (without treatment)	$92.8 \pm 1.4^{c,1}$	$97.8 \pm 0.9^{d,2}$
	heated (80 °C/30 min)	$97.8 \pm 1.1^{d,1}$	$99.0 \pm 0.8^{d,1}$
	irradiated (32 kGy)	$96.6 \pm 1.5^{d,1}$	$98.7 \pm 0.9^{d,1}$

[a] Means followed by the same number in each row are not significantly different at the 5% level. Means followed by the same letter in each column are not significantly different at the 5% level. For each measurement, three replicates on seven films types were analyzed.

Table 2. Mechanical Properties of Films from Untreated (Control), Heated, and γ-Irradiated Whey Protein (WPC and WPI)

	control[a] (not treated)		heated[a] (80 °C/30 min)		γ-irradiated[a] (32 kGy)	
type of film	WPC	WPI	WPC	WPI	WPC	WPI
puncture strength (N/mm)	$59.84 \pm 2.88^{a,1}$	$65.14 \pm 2.96^{b,2}$	$69.60 \pm 3.16^{b,1}$	$74.55 \pm 3.88^{c,1}$	$70.22 \pm 2.82^{c,1}$	$81.35 \pm 3.62^{d,2}$
puncture deformation (mm)	$5.24 \pm 0.30^{a,1}$	$5.02 \pm 0.29^{a,1}$	$4.41 \pm 0.28^{b,1}$	$4.17 \pm 0.29^{b,1}$	$4.53 \pm 0.30^{b,1}$	$3.84 \pm 0.24^{c,2}$
viscoelasticity coefficient	$0.44 \pm 0.03^{a,1}$	$0.46 \pm 0.02^{a,1}$	$0.51 \pm 0.02^{b,1}$	$0.53 \pm 0.02^{b,1}$	$0.50 \pm 0.03^{b,1}$	$0.53 \pm 0.02^{b,1}$

[a] Means followed by the same letter in each row are not significantly different at the 5% level. For each group (control, heated, and γ-irradiated), means followed by the same number are not significantly different at the 5% level. For puncture strength and puncture deformation measurements, three replicates of seven films types were tested. For viscoelasticity measurement, three replicates of three films were analyzed.

Heating and γ-irradiation treatments induced the cross-linking of proteins and consequently made the films more insoluble. To our knowledge, this is one of the first descriptions of insoluble films based on milk proteins. Cellulose alone, at the concentration of 0.25% used in our preparation, cannot generate film.

Mechanical Properties Tests. *(a) Puncture Strength.* Table 2 presents the puncture strength of WPC and WPI films cross-linked by heating and γ-irradiation, followed by entrapment. Results showed that heating or γ-irradiation increased the puncture strength of films significantly ($p \leq 0.05$). Puncture strength values for γ-irradiated WPC and WPI were, respectively, 70 and 81 N/mm, whereas for heated WPC and WPI the values were, respectively, 70 and 75 N/mm, all of them being higher than those for untreated WPC and WPI (59 and 65 N/mm, respectively). By comparing results between WPC and WPI films, statistical analysis indicated that WPC films obtained by heating and by γ-irradiation were not significantly different ($p > 0.05$) from WPI films obtained by heating. However, γ-irradiation of WPI at a dose of 32 kGy increased the puncture strength of films significantly ($p \leq 0.05$), up to a value of 81 N/mm. This last observation demonstrates that γ-irradiation had a more potent effect on WPI, probably due to the higher proteins content of WPI (90.6% proteins).

(b) Puncture Deformation. Table 2 shows the puncture deformation for control, WPC, and WPI films. Films obtained by heating or by γ-irradiation treatment are significantly less extensible and less elastic compared to control (untreated) films ($p \leq 0.05$). This could be due to the formation of new covalent intermolecular bridges (disulfide or bityrosine) that retain strongly two macromolecular chains of proteins and, consequently, decrease the films' tensile characteristics (compared to the control that was characterized only by weak bonds: hydrogen, ionic, hydrophobic interactions). No statistical differences ($p > 0.05$) were found for puncture deformation between heated (4.17−4.41 mm) or irradiated (3.84−4.53 mm) WPC and WPI films. A similar observation has been already reported for caseinate films by Brault et al. (1997). However, whey proteins when cross-linked by heating or by irradiation and entrapped in cellulose decrease the film extensibility.

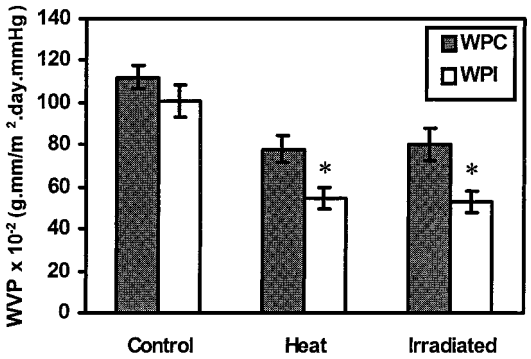

Figure 1. Effect of heating and γ-irradiation on WVP of whey protein films. A single asterisk (*) at the top of a column indicates a significant difference ($p \leq 0.05$) within the same column.

(c) Viscoelasticity Coefficients. Table 2 shows the viscoelasticity coefficients for these films. A low viscoelasticity coefficient means that the material is highly elastic, whereas a high coefficient indicates that the material is more rigid and easily distorted. Viscoelasticity coefficients for heated and γ-irradiated WPC and WPI varied between 0.50 and 0.53, all of them being higher than those of untreated WPC and WPI, 0.44 and 0.46, respectively. There is no significant difference ($p > 0.05$) between film-forming proteins treated by heating or by γ-irradiation. Indeed, formation of cross-links by heating (intermolecular disulfide bonds) or by γ-irradiation (bityrosine bridges) increases the stability of proteins and, therefore, decreases the elasticity of the biofilms.

Water Vapor Permeability. Figure 1 shows that whey proteins cross-linking by heating and γ-irradiation treatment followed by entrapment in cellulose reduced significantly ($p \leq 0.05$) WVP. Best values (lowest WVP) were obtained with WPI (0.53 g·mm/m^2·day·mmHg). This suggests that cross-linking whey proteins decrease the rate of moisture uptake (diminution of hydrophilic character). At the same time, there is no significant difference ($p > 0.05$) between heated and γ-irradiated films nor for WPC and WPI films (despite the presence of important amounts of fatty acids in WPC).

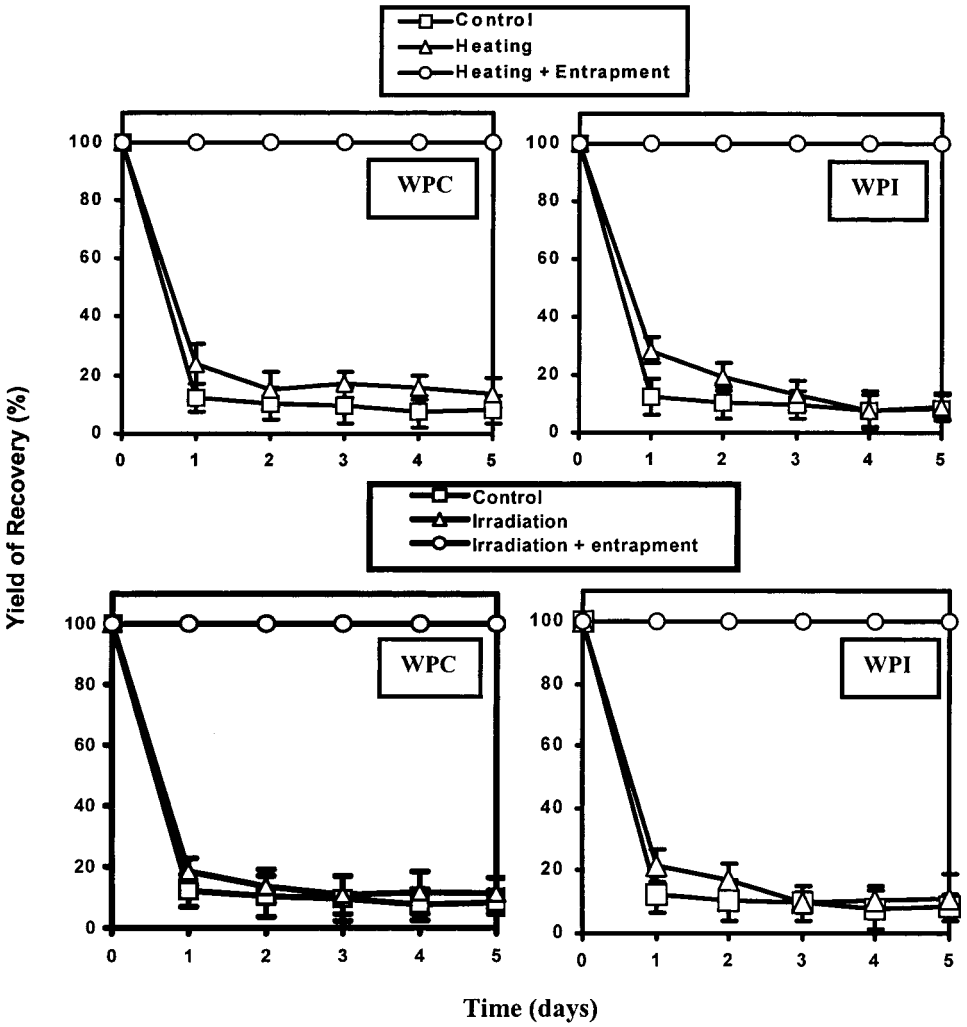

Figure 2. Effect of heating and γ-irradiation with and without entrapment in cellulose on the resistance at trypsin degradation of whey protein (WPC and WPI) films.

Resistance at Proteolysis with Trypsin. The results showed that films obtained by entrapment of heated or irradiated proteins are more stable than control (no entrapment and no treatment) when faced with proteolytic attack by trypsin. No proteolytic degradation at all was found for periods >3 weeks (Figure 2).

Biodegradability. Because it is difficult to evaluate biodegradability from trypsinolysis data, films were incubated in an extremely high proteolytic medium based on pancreatin. Figure 3 shows that the biofilms based on cross-linked proteins are generally biodegradable and that biofilms based on WPC are more stable to proteolysis. The yield of recovery was >30% after 105 min of incubation for irradiated WPC as compared to 0% for irradiated WPI. The essential factor in the higher stability of WPC films to pancreatin attack could be the presence in WPC of other components such as fatty acids, which can hinder the access to the proteolysis site, reducing thus the catalytic activity of the enzymes. The irradiated WPI films are more resistant, whereas WPI films obtained by heating are degraded in the same time period as the control. These results suggest that the method of cross-linking by γ-irradiation affords better stability to proteolysis for short to medium intervals, still allowing the biodegradability at long term. The same behavior was observed by Mezgheni et al. (1998) and Ressouany et al. (1999) in studies of the biodegradability of γ-irradiated calcium caseinate films by a

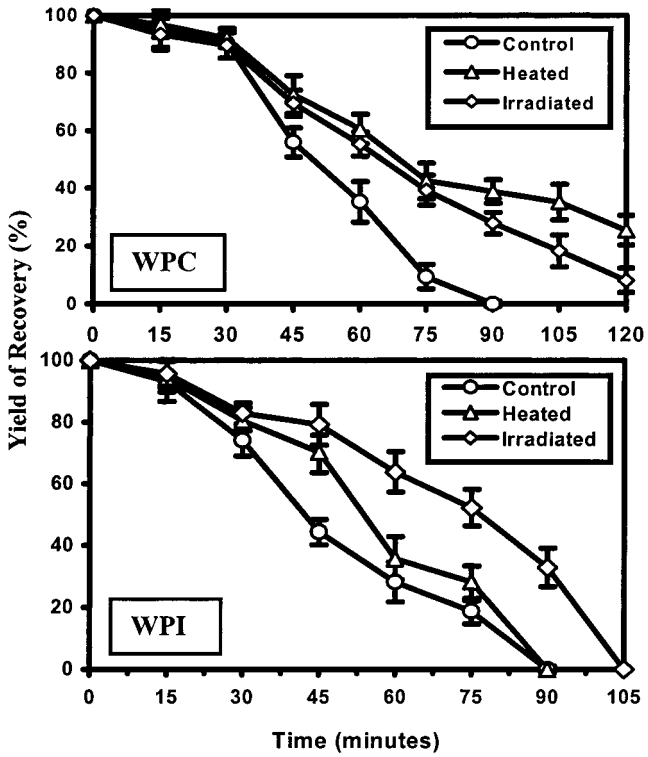

Figure 3. Effect of heating and γ-irradiation on the susceptibility to pancreatin degradation of WPC (a) and WPI (b) films.

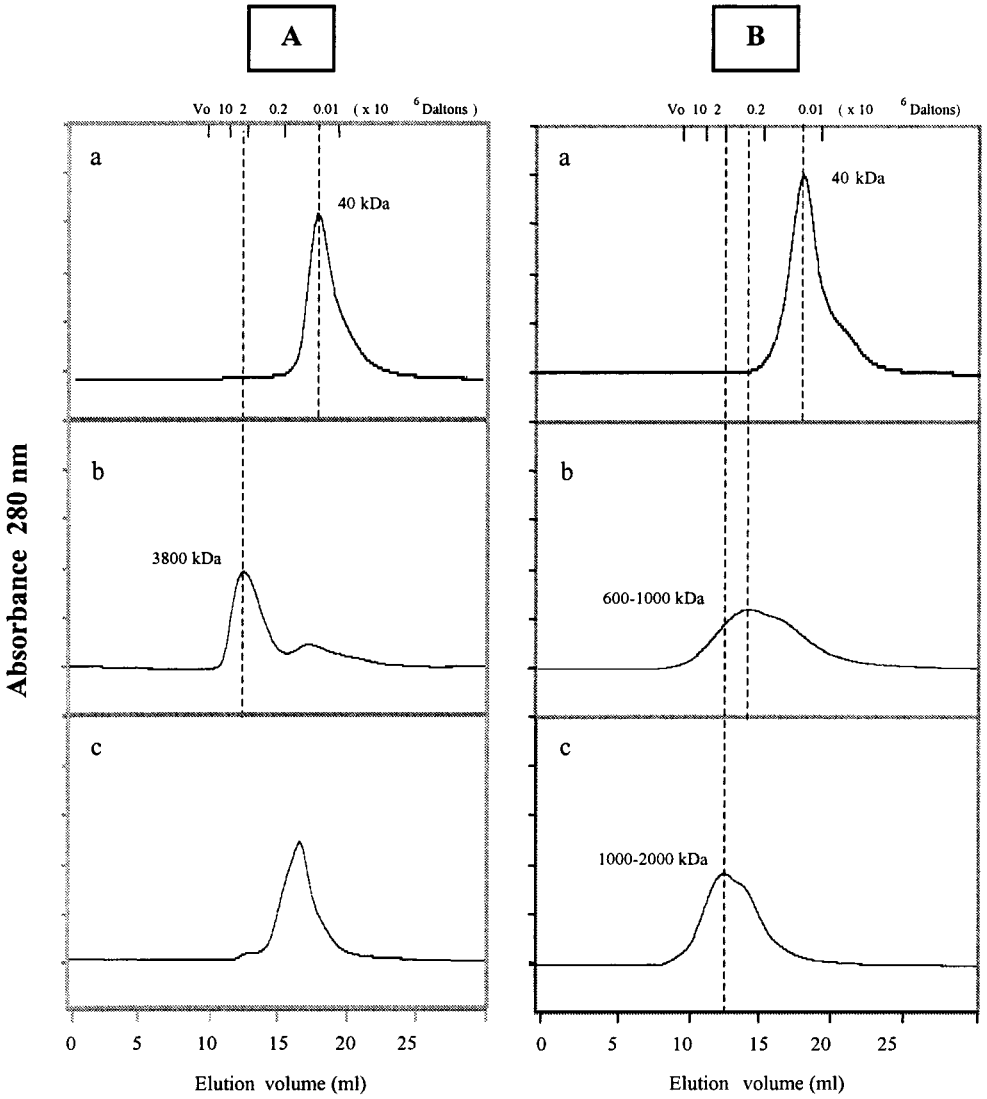

Figure 4. Size exclusion chromatography of control (a), heated (b), and γ-irradiated (c) WPC (A) and WPI (B) soluble fractions.

microbial strain, *Pseudomonas aeruginosa*. According to Ressouany et al. (1999), cross-linked films are resistant to microbial degradation.

Structural Analysis. *(a) Electrophoresis.* SDS−PAGE can give information on changes in the molecular masses of protein in the films. The electrophoretic pattern showed an important accumulation of an immobile protein fraction retained in the stacking gel. This accumulation was observed for both heated and irradiated whey proteins (WPC and WPI), suggesting development of cross-links induced by heating (intermolecular disulfide bonds) and by γ-irradiation (bityrosine bonds). This aggregation appears to be more pronounced in irradiated samples. Therefore, it is assumed that cysteine or aromatic side chain cross-linking may be responsible for the immobility of protein aggregates following heating and γ-irradiation treatments. Protein aggregates in electrophoretic patterns were also noted by Gennadios et al. (1998) in treated soy protein films by UV. However, the determination of the apparent molecular weight of aggregates in this case is impossible by electrophoresis and recourse to size exclusion chromatography was necessary.

(b) Size Exclusion Chromatography. Figure 4 shows the elution curves obtained for native, heated, and irradiated soluble fractions of WPC (A) and WPI (B) film-forming solutions. When the WPC solution was heated for 30 min at 80 °C, it readily underwent cross-linking via formation of disulfide bonds. This solution contained two distinct molecular weight fractions. The apparent molecular weight of the predominant fraction was ~3800 kDa, whereas the smallest fraction (40 kDa) can be ascribed to not cross-linked native protein or intramolecularly cross-linked proteins (Figure 4Ab). Similar results were obtained with heated WPI of apparent molecular weight varying between 600 and 1000 kDa (Figure 4Bb). These results are consistent with those reported by Hoffmann et al. (1997) on the molecular mass distributions of heat-induced β-lactoglobulin, where aggregates with molecular mass up to 4000 kDa were separated. However, when the WPC solution was submitted to γ-irradiation at a dose of 32 kGy to cross-link proteins via bityrosine bridges, very few molecular weight changes (Figure 4Ac) were found. This feature is not surprising considering that whey proteins contain fewer tyrosine residues (Wong et al., 1996). Results obtained for irradiated WPI demonstrated that γ-irradiation induced protein cross-linking and increased the apparent molecular weight to 1000−2000 kDa (Figure 4Bc). The explanation of these differences could be due to the presence in WPC of others components, essentially lactose (14.22%) and lipids

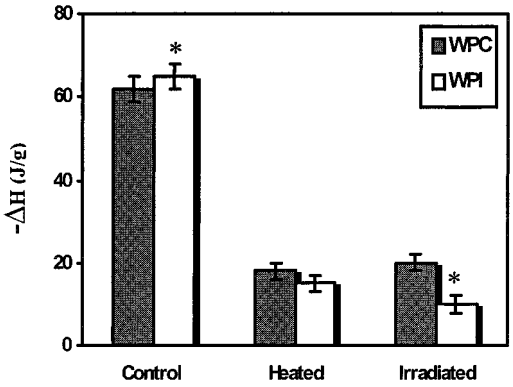

Figure 5. Effect of heating and γ-irradiation on interaction between water and whey protein based films. A single asterisk (*) at the top of a column indicates a significant difference ($p \leq 0.05$) within the same column.

(5.79%). These components, particularly the lipids, may limit the formation of phenoxyl radicals and their dimerization to form bityrosine linkages. However, they have not affected the process of protein cross-linking by heating. Comparison of the aggregation for WPC and WPI reveals that cross-linking by heating is most efficient for WPC and γ-irradiation for WPI.

(c) Isothermal Calorimetry. Figure 5 shows the values of $\Delta H_{\text{interaction}}$ with water at 30 °C of the control and of the treated samples. The highly negative values of ΔH (−65 to −62 J/g) for the control samples reflect the hydrophilic character of the nontreated films. Negative values of ΔH are associated with the formation of hydrogen bonds between water and proteins. The effect of treatments is probably to form hydrogen bonds between the chains of the protein. Consequently, the −OH or other polar groups are no longer available for interacting with water. As a result of cross-linking, protein chains are closer and hydrogen bonding favored. Because the polar groups are involved in chain−chain hydrogen association, they are no more available for hydration, explaining thus the lower solubility. The diminution of $\Delta H_{\text{interaction}}$ due to the treatment from the range of −65 to −62 up to the range of −15 to −10 J/g reflects the loss of film−water interaction. These results reinforce the data obtained by the solubility tests. The decreases in absolute value of $|\Delta H_{\text{interaction}}|$ for both the irradiated and the thermally treated films seem to be similar. Letendre (1999) obtained the same results when caseinate−WPI-based films were treated by heating and γ-irradiation. The range of $\Delta H_{\text{interaction}}$ was from −14 to −12 J/g. Experiments under different conditions (concentration, duration of irradiation, and thermal history) are necessary to compare more closely the effects of the two treatments and also their influences on the hydrophilic character of treated WPC and WPI films.

(d) FTIR Analysis. FTIR spectra obtained from the three series of films (control, heated, and γ-irradiated) showed two spectral regions of interest: 3600−3000 and 1700−1600 cm^{-1} (amide I). For the spectral region 3600−3000 cm^{-1}, a strong band was observed at 3293 cm^{-1} (Figure 6a) essentially due to OH and NH stretching mode. Several studies on proteins concerning this spectral region showed that the band of NH stretching mode is generally at 3254 cm^{-1} (Bandekar, 1992). There is therefore a displacement of the band that could be due, in our case, to the presence of other components in the biofilm formulation, especially glycerol and cellulose (with a large amount of hydroxyl groups). No major differences were observed for the treated WPC and WPI films (heated and γ-irradiated), but the treated films differed from the control films, for which a larger band (3300−3600 cm^{-1}) with a shoulder at 3420 cm^{-1} was observed. Thus, only data for WPI are presented in Figures 6 and 7. This broadening of the band in the untreated films could be related with the unbound or "free" −OH group vibration. These free −OH groups could be assigned mainly to water retained in the control films. An explanation can be postulated on the basis of protein cross-linking. In fact, a moderate reduction of the width of the peak can be related to the protein cross-linking, with chains closer favorable to each other higher hydrogen bonding and lower susceptibility to hydration. As mentioned above in the studies of solubility and isothermal calorimetry, cross-linked protein films hydrate less than the control film (nontreated) with a considerable affinity for the water, which could be the cause of the broadening of the band. It is supposed that amino or −OH groups of non-cross-linked proteins can form hydrogen bonds with −OH cellulose matrix and are more susceptible to hydration. However, when cross-linked, these groups are involved more in hydrogen bonding association and are less susceptible to hydration. A relationship between cross-linking degree and the width of the 3000−3600 cm^{-1} band has been described for another polyhydroxylic polymer (Mateescu et al., 1984), poly(vinyl alcohol) (PVA). Furthermore, it is important to note that unbonded −OH groups could be equally due to cellulose present in films (Kondo, 1998).

Whey proteins contain mostly α-lactalbumin and β-lactoglobulin (Creamer et al., 1983; Acharya et al., 1990). These proteins are organized generally in α-helix

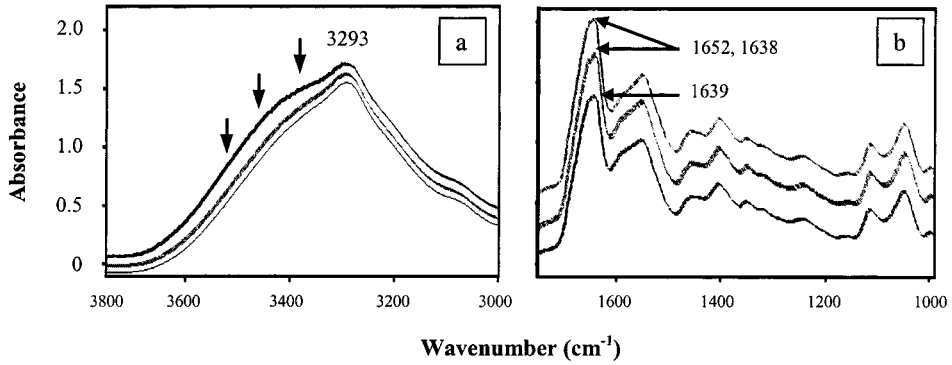

Figure 6. FTIR spectra of whey protein film: (a) spectral regions 3600−3000 cm^{-1}; (b) spectral regions 1700−1000 cm^{-1} (upper curve, control film; middle curve, heated film; lower curve, irradiated film).

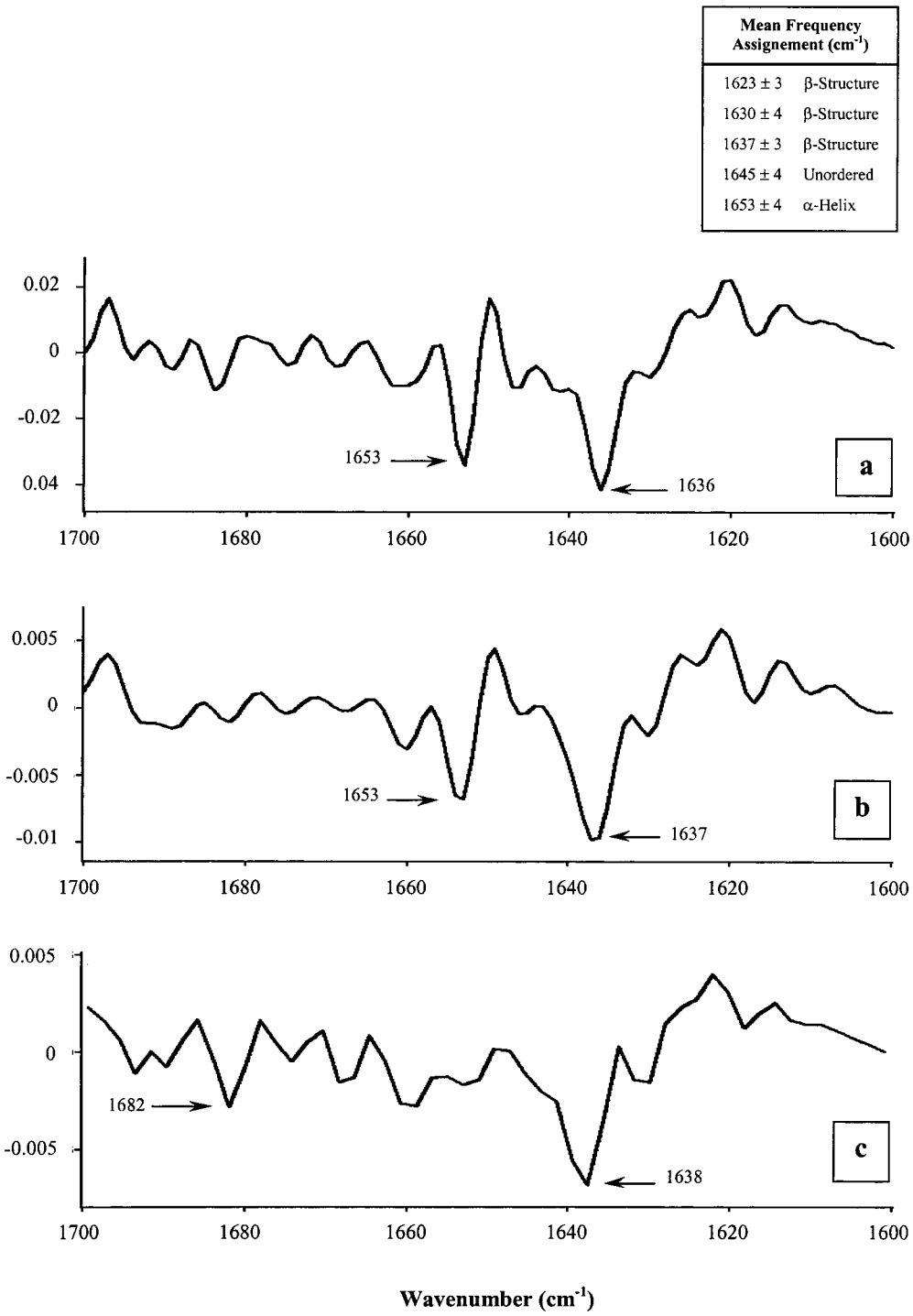

Figure 7. Second derivative of the FTIR spectra of whey protein films: (a) control film; (b) heated film; (c) irradiated film. Assignment of main frequencies (insert) is based on spectral data from 17 proteins (including β-lactoglobulin and α-lactalbumin) according to Byler and Suzi (1988).

(small fraction) and β-sheet structure. Another important fraction consists of random conformations, which can be considered as "unordered" structure. By deconvolution of the amide I, several bands in the 1655−1635 cm^{-1} spectral region were observed (Bandekar, 1992). For the control and heated films, two strong bands was noted at 1653 and 1638 cm^{-1} (Figure 6). These bands most likely result from α-helix and β-sheet conformation, respectively. However, the band at 1653 cm^{-1} was not found for films processed by γ-irradiation. The second derivatives were undertaken to study a number of overlapping peaks, resolved in their components (Byler and Susi, 1988). Similar results were observed with WPC and WPI films, with the mention that in both cases, no bands at 1652 cm^{-1} were found after irradiation, as presented for WPI (Figure 7). Furthermore, γ-irradiation could led to an alteration of the protein conformation, which heating did not. In addition, an increased band intensity at 1682 cm^{-1} was observed that was ascribed to antiparallel β-sheet structure (Subirade et al., 2000). Consequently, these changes could be related to a tendency of the proteins to adopt a more crystalline and stable structure after cross-linking.

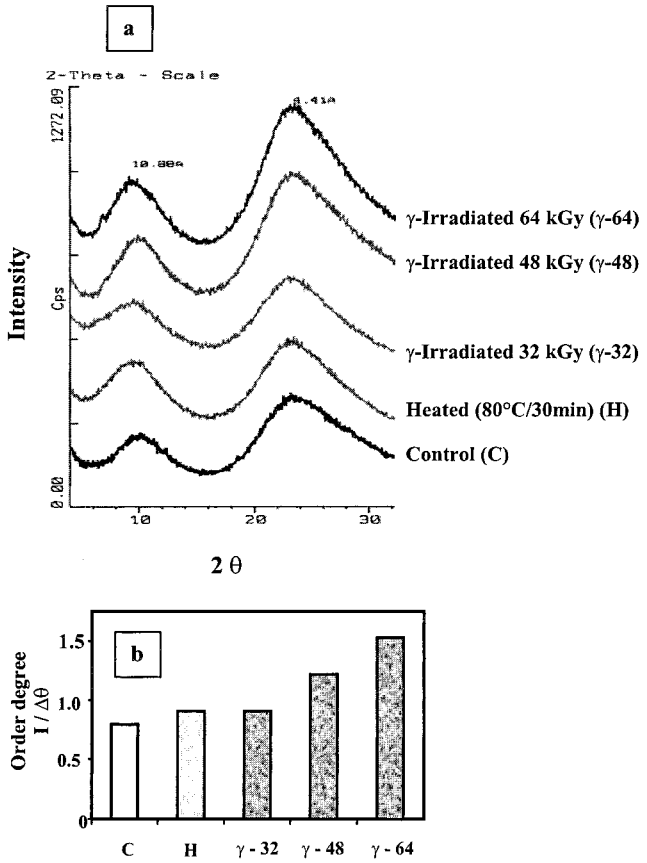

Figure 8. X-ray diffraction of whey protein films (a) and ordered degree ($I/\Delta\theta$, where I is the intensity of diffraction maxima and $\Delta\theta$ is the width at half-peak maxima) for different treatments (b).

(e) X-ray Diffraction. Results for the X-ray diffractogram of WPI films are presented in Figure 8. When the γ-irradiation dose was increased up to 48−64 kGy, a change of the X-ray diffraction profiles was observed (Figure 8a), showing that the maxima became sharper with the increase of the cross-linking degree. The γ-irradiation induced chain cross-linking, generating a more ordered and more stable structure (Figure 8b). This can explain the disappearance of the α-helical conformation observed by the FTIR analysis. Similar X-ray patterns were found for films submitted to different degrees of relative humidity (0, 56, and 100% RH), suggesting that the order of organization of these films is independent of moisture.

Conclusion. The use of cellulose as a matrix for entrapment of cross-linked proteins enabled the preparation of insoluble biofilms with good mechanical properties and increased resistance to enzymatic attack. This entrapment of whey proteins in cellulose seemed to be necessary to obtain insoluble biofilms. The use of gelatin as a stabilizer agent improved film appearance and prevented dehydration phenomena. Heating and γ-irradiation were responsible for cross-linking the proteins and improved the films' mechanical stability and resistance to proteolysis. In addition to covalent cross-linking, hydrogen associations cellulose−cellulose, protein−protein, or cellulose−protein are supposed to contribute to the film stability. Structural analysis of biofilms was conducted to explain, at least in part, the behavior of the whey protein biofilms, particularly the biofilm obtained by γ-irradiation, which exhibits better properties. The γ-irradiation responsible for the cross-linking (via formation of bityrosine bridges) affected the protein structure only moderately. Modification of protein conformation could be a result of this treatment, inducing modified structures more ordered and more stable. Consequently, the potential of application of these biofilms as packaging and wrapping can be of interest for various materials and probably compatible with several types of foods.

ABBREVIATIONS USED

FTIR, Fourier transform-infrared analysis; WPI, whey protein isolate; WPC, whey protein concentrate; WVP, water vapor permeability.

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