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Use of γ-irradiation to produce films from whey, casein and soya proteins: structure and functionals characteristics

M. Lacroix[a],*, T.C. Le[a,b], B. Ouattara[a], H. Yu[a], M. Letendre[a], S.F. Sabato[c], M.A. Mateescu[b], G. Patterson[b]

[a] *INRS-Institut Armand-Frappier, Canadian Irradiation Center, 531 Boulevard des Prairies, Building 22, Laval, Quebec, Canada H7V-1B7*
[b] *Université du Québec à Montréal, Biochemistry-Chemistry department C.P. 8888, Succ.A., Montréal, Quebec, Canada H3C-3P8*
[c] *Departamento de Aplicações Nucleares, IPEN-CNEN/SP, Travessa R. 400, 05508-900 São Paulo, SP, Brazil*

Abstract

γ-irradiation and thermal treatments have been used to produce sterilized cross-linked films. Formulations containing variable concentrations of calcium caseinate and whey proteins (whey protein isolate (WPI) and commercial whey protein concentrate) or mixture of soya protein isolate (SPI) with WPI was investigated on the physico-chemical properties of these films. Results showed that the mechanical properties of cross-linked films improved significantly the puncture strength for all types of films. Size-exclusion chromatography showed for no cross-linked proteins, a molecular mass of around 40 kDa. The soluble fractions of the cross-linked proteins molecular distributions were between 600 and 3800 kDa. γ-irradiation seems to modify to a certain extent the conformation of proteins which will adopt structures more ordered and more stable, as suggested by X-ray diffraction analysis. Microstructure observations showed that the mechanical characteristics of these films are closely related to their microscopic structure. Water vapor permeability of films based on SPI was also significantly decreased when irradiated. Microbial resistance was also evaluated for cross-linked films. Results showed that the level of biodegradation of cross-linked films was 36% after 60 d of fermentation in the presence of *Pseudomonas aeruginosa*. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The use of plastic for packaging has extensively grown in the few years. According to *Green plan* (1992), the inert and non-biodegradable plastic materials represent approximately 30% of municipal solid waste. In this context, biodegradable films can be a source for saving energy and an important issue for the environment protection. Gontard et al. (1994) classed three groups of biodegradable polymers: (a) blends of synthetic polymers (i.e. polyethylene) with natural polymers; (b) bacterial polyesters (polyhydroxyalcanoates) and (c) natural polymers more or less modified (polysaccharides or proteins). Considering the low cost of the raw material and the beneficial impact on the environment, natural polymers group became the subject of intensive studies for the development of biodegradable film. The aim of this study was to develop and to characterize cross-linked protein and biodegradable films produced by γ-irradiation.

*Corresponding author. Tel.: +1-450-687-5010x4489; fax: +1-450-687-5792.

E-mail address: monique.lacroix@inrs-iaf.uquebec.ca (M. Lacroix).

2. Experimental

2.1. Preparation of film-forming solution

Aqueous solution of 5% protein (whey protein isolate (WPI), whey protein concentrate (WPC) or soya protein isolate (SPI)) containing 2.5% glycerol and 0.25% carboxymethylcellulose (CMC), low viscosity (Sigma, St-Louis, MO) was heated at 80°C for 30 min, cooled at the room temperature (20±1°C) for 1–2 h; then degassed under vacuum as previously described (Brault et al., 1997) and irradiated using a ^{60}Co source irradiator (γ-Cell 220) (MDS Nordion, Kanata, Ont., Canada) at the Canadian Irradiation Center (Laval, Que., Canada) at doses from 4 to 128 kGy. Films prepared with SPI were also mixed with poly(vinyl alcohol) (PVA) before irradiation and compared without PVA addition (Aldrich Chemical Ltd. Montreal, Que., Canada). Optichromic and Gammachrome dosimeters were used to validate the dose distribution throughout the samples (Lacroix et al., 1992). Films were cast by applying 5 ml of the solution evenly onto Petri dishes (Fisher Scientific, Montreal, Que., Canada) and allowed to dry overnight (20±1°C).

2.2. Physico-chemical analysis

Puncture tests were carried out using a Stevens LFRA Texture Analyzer Model TA/1000 (NY, USA), as described by Gontard et al. (1992).

2.3. Water vapor permeability (WVP) tests

WVP tests were conducted using a modified ASTM procedure (Gontard et al., 1992). WVP of the film was calculated as follows:

WVP (g mm/m^2 24 h mm Hg) = 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 (hr); P_2–P_1 is the difference in vapor pressure across the film = 9.819 mm Hg (20°C).

2.4. Structural analysis

2.4.1. Size-exclusion chromatography

Size-exclusion chromatography was performed on the soluble protein fraction using Varian Vista 5500 HPLC coupled with a Varian Auto Sampler model 9090 (Vachon et al., 2000). 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 Canada Ltd.) followed by Ultra Hydrogel 2000 and 500 (7.5 mm × 30 cm) analytical columns (Waters Ltd., Mississauga, Ont., Canada) were used for the molecular weight determination of the control and cross-linked protein samples.

2.4.2. Isothermal calorimetry

The measurements were obtained with a calorimeter C80 (Setaram, France) in an isothermal mode (heats of swelling) as described by Le Tien et al., 2000. A known weight of dried sample (30 mg) was introduced in a home-made thin glass bulb and sealed under vacuum. The bulb was placed with the water within 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 going through the stopper of the cell. Due to the vacuum in the bulb, water fills the entireglass bulb and interacts with the sample. The value of $\Delta H_{experimental}$ after integration of the heat flow change is the sum of three contributions:

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

The two last terms can be measured by blank experiments and by subtracting their value (about −150 to −200 mJ) from $\Delta H_{experimental}$, the $\Delta H_{interaction}$ is obtained.

2.4.3. FTIR spectroscopic analysis

FTIR spectra were recorded using a BOMEM Hartman & Braun (Bomem, Inc., Que., Canada) equipped with DTGS detector (Deuterated triglycine sulfate). Spectra were analyzed using the BOMEM GRAMS software (Ver. 1.51). The biofilms were placed in the BOMEM cell for scanning spectral region (4000–500 cm^{-1}) and 50 scans were recorded with a 1 cm^{-1} resolution. The second derivatives of spectra which narrow the broad Amide I band related to the different protein chain conformations, were equally analyzed (Byler and Susi, 1988).

2.4.4. X-ray diffraction

The diffraction pattern of whey protein films was recorded by Siemens D-5000 diffractometer with cobalt cathode operating in reflectance mode at wavelength $\lambda = 1.79019$ Å (30,000 V and 16 mA).

2.4.5. Transmission electron microscopy (TEM)

TEM observation was done on dry films according to Vachon et al., 2000.

2.4.6. Biodegradation

Biodegradation was evaluated by the analysis of the percentage of nitrogen from films converted to soluble N content as a function of time for 4 and 64 kGy films as described by Ressouany et al. (1998).

2.4.7. Statistical analysis

Analysis of variance and Ducan multiple-range tests with $p \leqslant 0.05$ were used to analyze the results statistically. For puncture strength and biodegradability measurements, three replicates of seven films were tested. For WVP measurements, three replicates of three films were tested. The Student t-test was used and paired-comparison with $p \leqslant 0.05$ (Snedecor and Cochran, 1978).

3. Results and discussion

3.1. Mechanical properties of calcium caseinate–whey protein films

Fig. 1 shows the puncture strength variations of films cast from solutions containing different WPI–calcium caseinate ratios. For instance, a protein ratio of 50–50 corresponds to 2.5% WPI protein and 2.5% calcium caseinate protein. Addition of WPI in the formulations did not significantly affect ($p > 0.05$) the puncture strength of the films up to a WPI–calcium caseinate ratio of 50–50 representing a puncture strength around 0.1 N/m. γ-irradiation significantly increased ($p \leqslant 0.05$) the mechanical properties of the films by inducing cross-links between protein chains (Ressouany et al., 1998). For instance, for films based only on calcium caseinate (0–100), γ-irradiation increased the puncture strength by more than 35%. For the films containing an equal WPI–caseinate ratio (50–50), cross-linking increased by 20%. The high puncture strength of films containing 50% WPI, comparable with pure calcium caseinate, suggests other favorable interactions than intermolecular bonding between WPI and calcium caseinate.

The puncture strength of irradiated soy protein isolate film (γS) was 0.043 N/m, being 37% higher than the unirradiated one (S) (0.032 N/m) (Table 1). The value
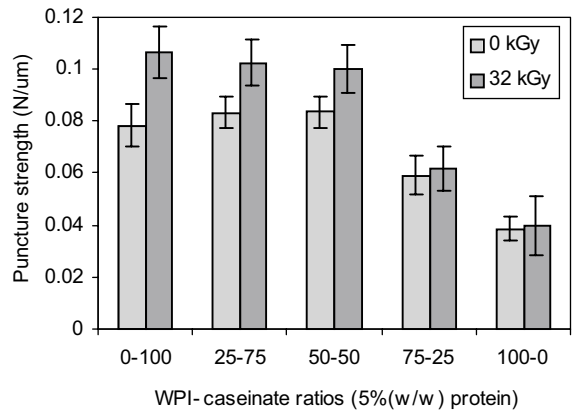

Fig. 1. Puncture strength of unirradiated and irradiated (32 kGy) WPI–calcium caseinate films. Ratios express the proportion in WPI or calcium caseinate for a formulation based on 5% w/w total solution. For instance, the formulation 25–75 represents 1.25 g WPI protein and 3.75 g calcium caseinate protein per 100 g solution.

for irradiated soy protein isolate with CMC film (γS1) was 0.052 N/m in relation to correspondent unirradiated one (S1) (0.049 N/m), representing an increment of 7%. The puncture strength of irradiated soy protein isolate with CMC and PVA (γS2) was 0.059 N/m and was 12% higher than the correspondent unirradiated one (S2) (0.053 N/m). Irradiated soy protein isolate film with CMC and PVA (γS2) presented the highest puncture strength value among all treatments. In fact, PVA is a cross-linkable polymer.

3.2. Water vapor permeability

Results of WVP show that heating or γ-irradiation treatment reduced significantly ($p \leqslant 0.05$) the WVP. Lowest WVP were obtained with caseinate–WPI (25:75) formulation with permeability values of 2.07

Table 1
Puncture strength of protein-based edible films

Films	Composition (ratio)	Puncture strength (N/mm)	
		Non-irradiated	Irradiated (32 kGy)
S	SPI:Gly (2:1)	31.53 ± 2.34^{b}	43.30 ± 2.75^{b}*
S1	SPI:Gly:CMC (20:10:1)	48.63 ± 2.67^{d}	52.20 ± 2.52^{d}*
S2	SPI:Gly:CMC:PVA (20:10:1:2)	52.49 ± 2.25^{e}	59.00 ± 3.10^{d}*
SW	SPI:WPI:Gly (1:1:1)	28.60 ± 2.40^{a}	40.32 ± 2.87^{a}*
SW1	SPI:WPI:Gly:CMC (10:10:10:1)	32.79 ± 2.83^{b}	41.27 ± 3.52^{ab}*
SW2	SPI:WPI:Gly:CMC:PVA (10:10:10:1:2)	37.48 ± 1.64^{c}	46.07 ± 3.56^{c}*

S = films based on SPI; SW = films based on mixture of SPI and WPI; SPI = soy protein isolate; WPI = whey protein isolate; Gly = glycerol; CMC = carboxymethylcellulose; PVA = polyvinyl alcohol. Means followed by different letters in each column are significantly different ($p \leqslant 0.05$). Means followed by asterisk in each row are significantly different ($p \leqslant 0.05$).

and 1.38 g.mm/m^{2}d mm Hg for unirradiated and irradiated samples, respectively. Values of WVP range between 2.90 and 3.16 g mm/m^{2}d mm Hg in the non-irradiated soya-based films. In the presence of whey proteins and CMC, soya-based films had a WVP of 2.68 g mm/m^{2}d mm Hg. The contribution of the irradiation treatment was found to be significant only in formulation based on SPI, CMC and glycerol. The WVP went from 3.16 to 2.03 g mm/m^{2}d mm Hg, representing a decrease of 36% (data not shown).

3.3. Size-exclusion chromatography

Heating calcium caseinate at 90°C for 30 min increased the molecular weight from 3 to 4-fold (data not shown). However, when the protein was submitted to γ-irradiation at a dose of 32 kGy, cross-linking occurred and the molecular weight distribution peak shifted to higher molecular weights. Our observations indicate that the cross-linking of caseinate more efficient by irradiation and the cross-linking of whey proteins is more efficient by heating. Based on the

protein calibration curve, the molecular weight distribution of the cross-linked soluble calcium caseinate fraction was $\geqslant 2 \times 10^3$ kD, representing an increase greater than 60-fold. Bityrosine is expected to be the major component formed during γ-irradiation due to the strong characteristic fluorescence, other mechanisms for protein cross-linking should also be considered (Davies et al., 1987; Ressouany et al., 1998). Bityrosine is more likely to form between two protein chains (intermolecular bonding) than within a single protein, accounting for the increase in molecular weight. Ressouany et al. (1998) demonstrated that the maximum cross-linking density was obtained at an irradiation dose of 64 kGy for similar calcium caseinate solutions. When soy protein was heated at 90°C for 30 min following γ-irradiation at 32 kGy, cross-linking occurred and the molecular weight peak shifted from 60 to 2000 kDa (data not shown). Similar results were obtained when WPI is mixed with SPI in a 1:1 ratio (data not shown). Based on the protein calibration curve, the molecular weight of the cross-linked soy protein fraction increased more than 33-fold.
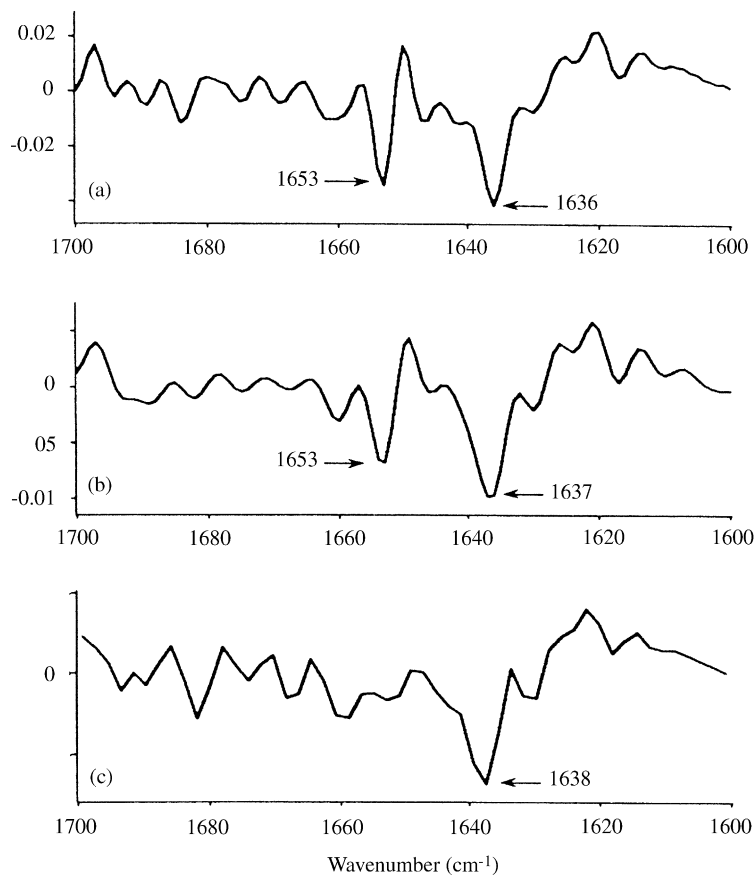

Fig. 2. Second derivative of the FT-IR spectra of whey protein films. (a) Control film; (b) heated film; (c) irradiated film. Assignment of main frequencies (inset) is based on spectral data from 17 proteins according to Byler and Suzi (1988).

3.4. Isothermal calorimetry

The values of the $\Delta H_{interaction}$ with water at 30°C of the samples showed that the unirradiated samples had higher negative values of ΔH than irradiated samples. The ΔH of caseinate, WPI and WPC proteins were, respectively, −88, −65 and −62 J/g. The ΔH of unirradiated caseinate and caseinate–WPI films were, respectively, −27 and −20 J/g as compared with −19 and −13 J/g for the respective cross-linked films. The negative values of the ΔH are associated with the formation of hydrogen bonds between water and proteins at the moment of immersion. The diminution of $\Delta H_{interaction}$ due to the irradiation treatment from −88 to −27 up to −19 to −13 J/g reflects the loss of film–water interaction.

3.5. FT-IR analysis

FT-IR analysis spectra obtained from films were done in two interesting spectral regions: 3600–3000 cm^{-1} and 1700–1600 cm^{-1} (amide I). For the spectral region 3600–3000 cm^{-1}, a strong band was observed at 3293 cm^{-1} (data not shown) almost due to NH stretch of proteins. No major differences were observed for all films. However, a larger band (3300–3600 cm^{-1}) was observed in the untreated films. This phenomenon could be related to the unbounded or "free" HO- groups vibration. These "free" HO- groups could be ascribed

mainly to water retained in the control film. It is supposed that amino or HO- groups of not cross-linked proteins are more susceptible to hydrate. When cross-linked, these groups are more involved in hydrogen association and less susceptible to hydration. For the control and heated films, two other strong bands were also noted at 1653 and 1638 cm^{-1} (Fig. 2). These bands most likely result from α-helix and β-sheet conformation, respectively. However, the band at 1653 cm^{-1} was not found for γ-irradiated films. The second derivative was undertaken for a comparative study. The role of second derivative of this broad band (1655–1635 cm^{-1}) consisting of a number of overlapping peaks, is to resolve the broad band into its original components (Byler and Susi, 1988). Similar results were observed with our films and in all cases, by irradiation treatment no bands at 1652 cm^{-1} were found (Fig. 2). Consequently, these results suggest that the γ-irradiation can cross-link proteins and, at the same time, led an alteration of the conformation proteins while the heating did not. These changes could be related to a tendency of the proteins to adopt a more stable structure after cross-linking.

3.6. X-ray diffraction

Results for diffractogram showed that, by increasing the γ-irradiation dose from 48 to 64 kGy, a change in the X-ray diffraction profiles was observed, showing that
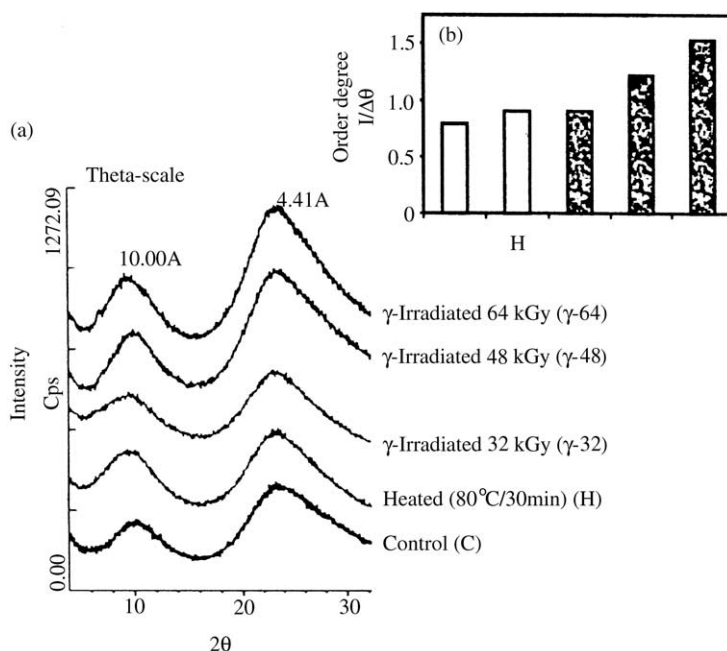

Fig. 3. X-ray diffraction of whey protein films (a) and ordered degree ($I/\Delta\theta$, where I is the intensity of diffraction maxima and $\Delta\theta$ is the width at half-peak maxima) for different treatments (b).

the sharp angle of films increases in function of the degree of the cross-linking (Fig. 3). The γ-irradiation, induced chains cross-linking and a new structure, may be more ordered and more stable. This hypothesis can equally explain the disappearance of the α-helical conformation observed by the FTIR analysis.

3.7. Microstructure observations

Cross-sections of the films observed by transmission electronic microscopy showed that the structure of these films was highly porous. However, the microstructure of the irradiated films is clearly more dense (data not shown). The increased molecular proximity as well as the additional molecular bonds, proved by size exclusion chromatography, directly influence the macroscopic characteristics of the films in terms of the mechanical strength and water resistance.

3.8. Biodegradation

Results of biodegradation demonstrated that, when the films irradiated at 4 kGy were incubated with the bacteria, a significant increase ($P \leqslant 0.05$) of soluble N content was noted on d 3 and reached a value of 0.63%. Following that period, the soluble N decreased slightly, but remained higher than 0.5% until d 50. When the 64 kGy films were incubated in the presence of the bacteria, the percentage of nitrogen converted to soluble N was lower than 0.10% until d 50. On d 60, the net bacterial degradation was, respectively, 86% and 36% for 4 and 64 kGy films, confirming that cross-links produced by γ-irradiation slowed the biodegradation of the material.

4. Conclusion

This report shows that γ-irradiation was efficient for inducing cross-links in proteins edible films. The cross-linking reactions affected moderately the protein structure. Modification of protein conformation could be a result of irradiation treatment, inducing modified structures more ordered and more stable. Combination of radiative and thermal treatments of the films resulted in an increase in the puncture strength of the films and better physico-chemical properties.

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