γ-Irradiation Influence on the Structure and Properties of Calcium Caseinate–Whey Protein Isolate Based Films. Part 1. Radiation Effect On the Structure of Proteins Gels and Films

Krystyna Cieśla,*,† Stephane Salmieri,‡ and Monique Lacroix‡

Institute of Nuclear Chemistry and Technology, ul. Dorodna 16, 03-195 Warszawa, Poland, and
Canadian Irradiation Center, Research Laboratories in Sciences Applied to Food, INRS-Institute
Armand Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec H7V 1B7, Canada

Brookfield viscosimetry, Fourier transform infrared spectroscopy, transmission electron microscopy (TEM), and measurements of the texture strength of gels formed with $CaCl_2$ and the mechanical and barrier properties of the film were applied in studies of gel formation and structural and mechanical properties of gels and films prepared using calcium caseinate (CC)−whey protein isolate (WPI)−glycerol (1:1:1), control, and irradiated with ^{60}Co γ rays using a 32 kGy dose. The irradiated gels have appeared to be more "fine-stranded" as compared to the more "particulate" control gels and lead to the formation of more rigid films with improved mechanical strength and barrier properties. This results from cross-linking and the modification of protein conformations were induced by irradiation, in particular the increase in the β-sheet and β-strand contents. Structural modifications taking place in CC−WPI composition are related to modifications taking place separately in CC and WPI. Improvement of the properties of the films after irradiation corresponds to the increased density of the cross-linked material because no change in the porosity of the films was observed by TEM.

KEYWORDS: Edible films; milk proteins; calcium caseinate; whey protein isolate; γ-irradiation; cross-linking; gelation; conformation; FTIR; viscosity; texture strength; calcium effect

INTRODUCTION

In recent years, the interest increased in substituting the nondegradable plastics using materials from renewable resources based on proteins, polysaccharides, and lipids. Using such materials for packaging improves economic efficiency and secures no contribution to environmental pollution at a low cost of biodegradability. In particular, the application of edible films and coatings (*1*−*9*) as an effective barrier against gas, moisture, and liquid migration has appeared the appropriate way for prolongation of the shelf-life of ready-to-eat food and for the increase of its quality. Accordingly, these materials should reveal appropriate mechanical and barrier resistance. Therefore, the search for new materials is still continued in purpose to improve biopolymer structure and consequently the functional properties of edible packaging.

Proteins are known to have good film-forming capabilities. The resulting films have, however, rather moderate functional properties. Irradiation with γ rays has appeared to be an effective method enabling us to improve mechanical and barrier properties of films prepared using calcium and sodium caseinates alone

or in composition with globular proteins (*10*−*21*). It occurs owing to the formation of cross-links between protein chains via the disulfide bridge mechanism (*10*−*18*, *22*) [shown by bityrosine production (*11*)]. It was found that using the mixed composition containing calcium caseinate (CC) with whey protein isolate (WPI) and plasticizer (polyol) accompanied by the appropriate combining of irradiation and heating enabled us to produce films with higher tensile strength and lower permeability for water vapor than those prepared using each protein alone (*17*). It is worth mentioning that both of those proteins are susceptible to form disulfide bridges with the other proteins (*23*, *24*). Accordingly, it can be deduced that conformational modifications induced in proteins by irradiation and/or heating are affected as a result of the specific interaction between CC, WPI, and polyol occurring at particular steps of film formation. The necessity has, therefore, appeared to carry out detailed studies dealing with the modification of protein conformations in the mixed CC−WPI−polyol system and to relate the results to the changes taking place in each protein−polyol system separately.

It is well-known that the film-forming ability depends upon the capability of proteins to form gels, the intermediate products obtained during film formation from solutions. Properties of all solutions, gels, and final films, however, depend upon the tertiary structure of proteins. Accordingly, good knowledge of the processes and products resulting from the particular steps

* To whom correspondence should be addressed: Institute of Nuclear Chemistry and Technology, ul. Dorodna 16, 03-195 Warszawa, Poland. Telephone: (48) (22) 5041106. Fax: (48) (22) 8111917. E-mail: kciesla@orange.ichtj.waw.pl.
† Institute of Nuclear Chemistry and Technology.
‡ University of Quebec.

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of preparation (physical treatment of solutions, gelling, and hardening of gels) together with the recognition of the radiation effect are expected to help in further optimization of the preparation conditions, leading to films with improved functional properties.

It has been discovered that both irradiation and heating of WPI with the addition of gelatin and cellulose xanthate induce the modification of the protein conformation consisting of an increase in the β-sheet content at the cost of α helices (*14*). Apart from our preliminary studies (*25*), the conformational changes occurring after γ irradiation and heating conducted in the same film preparation procedure were, however, not recognized yet.

Our group also showed a relationship between the formation of cross-links caused by irradiation of caseinate and caseinate–polyol compositions with doses up to 64 kGy and the increase in the strength of gels formed with CaCl$_2$ (*11, 13*). Although other factors such as the compatibility of components might influence gel strength (at the same pH), it seems that a change in coordination resulting because of conformational modification is of major importance. It regards the fact that the interaction with metallic ions depend upon the structure of the proteins as the binding of metallic ions occurs between two adjacent functional groups (carboxylic and phosphate) of different polypeptide chains (*11, 26*) and is more possible when two protein chains occupy the parallel positions. Therefore, binding of calcium ions is more possible when β sheets or strands are present. Preferred binding of neighboring polypeptide chains contribute to a formation of a denser network and thus stronger gels (*11, 13*). It seems worth mentioning, moreover, that the compatibility/incompatibility factor depends upon structural properties of components in the mixed system. Accordingly, a change in the gel strength appears to be a good indicator of the occurring structural modifications.

At present, properties of gels formed using the nonirradiated and irradiated CC–WPI compositions were examined as well as gel-formation processes taking place upon heating and cooling. Irradiation was carried out using a 32 kGy dose because of its high efficiency in the formation of cross-links not accompanied by a noticeable degradation (*10–12*). Differences observed between the route of gelation of the nonirradiated and irradiated proteins were related to the conformational changes regarding irradiation followed by heating. Moreover, these results were related to the microstructure of the films obtained as a final product and to their mechanical resistance and barrier properties.

A detailed study of protein conformations was carried out using Fourier transform infrared (FTIR) spectroscopy and completed by the studies of the texture of gels formed after CaCl$_2$ addition. Conformational modification taking place under radiation treatment in CC–WPI composition and recorded using both methods is related to those occurring separately in CC and WPI and, consequently, to the interaction between both components in the mixed system.

MATERIALS AND METHODS

Materials and Film Preparation. CC (produced by New Zealand Milk Product, Inc.) (containing 91.8% of total protein mass), WPI (produced by BiPro Davisco) (containing 97.8% of total protein mass), and chemical-grade glycerol were used. Water solutions were prepared containing 3.75 wt % of CC, 3.75 wt % of WPI, and 3.75 wt % of glycerol (weight ratio equal to 1:1:1 and total protein content equal to 7.5 wt %). These solutions were degassed and irradiated under an inert gas atmosphere with ^{60}Co γ rays. Irradiations were performed with a dose of 32 kGy, applying a mean dose rate of 7 Gys^{-1} in GAMMA-

CELL 220 placed at the Canadian Irradiation Centre. The reference nonirradiated solutions were also prepared.

The reference solutions as well as the solutions previously submitted to irradiation were dissolved to 5% of the total protein content (2.5 wt % of CC, 2.5 wt % of WPI, and 2.5% of glycerol) and heated at 90 °C for 30 min. Water content was then filled up. Aliquots (7.5 mL) of the resulting final solutions were pipetted onto plexiglass Petri plates and allow to dry for 3 days in air at room temperature. The films were then peeled and conditioned for 48 h in the control atmosphere at 56% humidity (in the desiccator over saturated NaBr solution) before further examination.

Film thickness was measured using a Digimatic Indicator (Mitutoyo, Japan) at 9 positions around each film. The thickness of each film was calculated as the average value. These values were in the range of 60–80 ($\pm$2 μm).

FTIR Spectroscopy. The total reflectance method was applied using the Perkin-Elmer Spectrum One spectrophotometer equipped with Universal ATR Sampling Accessory with a diamond crystal. Spectra (100 scans) were recorded in the region of 650–4000 nm. Measurements were done for four films of each composition, and the average spectrum was then calculated and analyzed. The method based on analysis of the second derivative of the amide I band (with the peak at ca. 1630 nm) was applied in purpose to examine the protein conformation (*27–33*).

Binding of Calcium Ions and Gel-Fracture Strength Measurements. Gels were formed following a procedure of Sakamoto et al. (*35*), modified by Ressounay et al. (*13*). A quantity of 0.4 mL of 5% protein and 0.125% CaCl$_2$ solution was poured into a well of the microtest plate having dimensions of 7 mm in diameter and 11 mm in height. Wells were sealed and placed into a water bath for 4 h at 90 °C. The solution was allowed to cool at room temperature and stored overnight at 4 °C. The gel-fracture strength was measured using a 3 mm diameter cylindrical probe, which was moved through the gel at a constant speed of 2 mm s^{-1}. The force–deformation curves were recorded using a Stevens LFRA Texture Analyser model TA/100 (NY).

Studies of Rheological Properties. Viscosity measurements were carried out applying Brookfield viscometer type LVDV-II+ (Brookfield Engineering Laboratories, Inc., MA) with the close tube system enabling us to create a circulating water bath by connecting the water jacket to the bath inlet and outlet ports. The ULA spindle was used in measurements as well as the Wingather V2.1 program for data collection and calculation. Two 7.5% solutions of each composition were examined (the first-type experiment). Three types of experiments were performed for 5% solutions.

The first-type experiments were carried out at ambient temperature applying a shear rate equal to 112.3, 61.2, and 30.6 s^{-1} (corresponding to the rotation speed of 100, 50, and 25 routes/min, respectively). This range of shear rate was selected in regard to a linear dependence of a shear stress upon a shear rate accompanied by good reproducity of the data (*25*). Consequently, almost constant viscosity values were determined in this shear stress range. Viscosities were calculated as average values determined for the four separately prepared solutions of each composition at the three applied shear rate values (altogether, 12 measurements were done).

The second-type experiments were carried out for the fresh 5 wt % solutions placed in a viscometer and heated rapidly to the required temperature equal to 40, 50, 60, 70, 80, and 90 °C.

The third-type experiments were carried out on dynamic heating and cooling in the range of 25–90 °C. In the last case, the solution was heated in a water bath at 90 °C. Still hot solution was placed (after complexion of water) into the viscometer kept at 90 °C and equilibrated at 90 °C for 3 min before cooling. Viscosity was recorded in each run at several selected temperatures. All of the measurements were done applying a shear rate equal to 30.6 s^{-1} (25 routes/min). Four repetitions of the experiments were done. Heating and cooling were realized with average rates of 2.4 and 1.9 °C min^{-1}, respectively, in the total range of temperature. All of the presented data were obtained under conditions characterized by strictly the same time–temperature dependence. Slightly higher values of viscosity were obtained directly after the third type experiments than after the experiments followed by water complexion because of difficulties with the total avoiding water loss.

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Table 1. Characteristic Frequencies and Assignments for Amide I Band Components (1700–1600 nm) (*27–33*)[a]

mean frequency (nm)	assignment
1618	β-structure (β-strands)
1625	β-structure (β-strands)
1630–1637	β-structure (β-sheets)
1639–1647	unordered (aperiodic)
1652	α-helix
two bands at ca. 1660	unordered cross-linked phase (turns or undefined)
1667	turns
1674	β-structure (β-sheets) and/or turns
1679–1680	turns or β-structure
1683	β-structure and/or turns
1688	turns (or β-structure)
1695 and 1699	turns

[a] Band at 1683 nm accompanied to that at 1618 indicates that β sheets are in antiparallel position (*32, 33*). Band at 1688 nm is attributed to protein folding (*33*). Band at 1612 nm is connected to the presence of amino acid residue (*33*).

Transmission Electron Microscopy (TEM). Dry films were first immersed in a solution of 2.5% glutaraldehyde in a cacodylate buffer, washed, and postfixed in 1.3% osmium tetroxide in colidine buffer (*12*). Samples were then dehydrated in acetone (25, 50, 75, and 100%) before embedding in a SPURR resin. Polymerization of the resin proceed at 60 °C for 24 h. Sections were made with an ultramicrotome (LKB 2128 Ultratome) using a diamond knife and transferred on Formvar-carbon-coated grids. Sections were stained at 20 min with uranyl acetate (5% in 50% ethanol) and 5 min with lead citrate. Grids were observed by a Hitachi 7100 transmission electron microscope operated at an accelerating voltage of 75 keV. The photos were taken for the typical bulk area of the films. Observations were conducted for two films of each composition.

Water Vapor Permeability (WVP) Tests. WVP tests were conducted gravimetrically using an ASTM procedure (*35*) modified by Gontard et al. (*2*). The test films were mechanically sealed to Vapometer cells (NO 68-1, Kalamazoo Paper Chemicals) containing 100 g of anhydrous calcium chloride. Tests were performed in the Shellab 9010L humidity chamber at a temperature of 30 °C and relative humidity of 56% during 24 h. WVP was calculated accordingly to the following formula:

$$\text{WVP} = Wd/A(P_2 - P_1)$$

where *W* is the weigh gain of the cup (in grams) within 24 h, *d* is the film thickness (mm), *A* is the area of exposed film (31.67×10^{-4} m^2), and $P_2 - P_1$ is the difference in vapor pressure across the film equal to 17.82 mmHg.

Mechanical Properties. Puncture tests were carried out using a Stevens LFRA Texture Analyser model TA/100 (NY) accordingly to the method described by Gontard et al. (*2*) and Brault et al. (*10*). A cylindrical probe (2 mm in diameter) was moved perpendicularly to the film surface at a constant speed (1 mm s^{-1}) 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.

The force−deformation curves were recorded. The puncture strength was related to the film thickness measured directly in the point (*21*).

Viscoelastic properties were evaluated using the force−relaxation curves. The same procedure was applied, but the probe was stopped and maintained at 3 mm deformation. The film was then allowed to relax. A relaxation coefficient *Y* was calculated accordingly to the equation

$$Y = (F_0 - F_1)/F_0$$

where F_0 and F_1 are the forces recorded initially and after 1 min of relaxation, respectively. A low relaxation (viscoelasticity) coefficient indicates high film elasticity.

Statistical Analysis. Analysis of variance and Duncan multiple-range tests with $p \leq 0.05$ were used to analyze statistically the results. The SAS statistical package was applied (SAS Institute, Cary, NC). The Student *t* test was used and paired comparison. Differences between means were considered significant when $p \leq 0.05$. Altogether, nine repetitions of gel-texture measurements were performed for each composition for three independently prepared solutions. A total of 12 viscosity values were analyzed for solutions. For each film, two replicates were tested of several samples each. A total of ca. 20 repetitions for each composition were considered for tests of WVP, puncture strength, and puncture deformation, and a total of ca. 10 repetitions were considered for the viscoelasticity coefficient.

RESULTS AND DISCUSSION

Protein Conformations in the Nonirradiated and Irradiated Protein Solutions. The change in protein conformation because of irradiation results in the shift of the maximum intensity of the amide I band in the FTIR pattern to the higher values (1639−1640, 1629−1631, and 1631−1633, in the case of the films prepared from pure CC, pure WPI, and CC−WPI compositions, respectively. Second derivatives of FTIR spectra (amide I bands) recorded for particular compositions are conformed in **Figures 1−5**. Assignments of the characteristic components (*27−33*) are specified in **Table 1**, and a detailed interpretation is shown below. In all of the cases, the presence of the component at 1683 nm simultaneously with that at 1618 nm suggested that intermolecular β-sheets are in antiparallel position (*32, 33*). **Table 2** shows major conclusions concerning the irradiation influence on structural properties of CC, WPI, and CC−WPI compositions.

Very soft gels were formed after heating the CC−WPI or WPI solutions in the absence of calcium ions, and therefore, no measurements were possible for their texture strength. The addition of CaCl$_2$ induces the formation of gels strong enough to perform such measurements (results accompanied by statistical analysis are reported in **Table 3**). Gels formed after irradiation were stronger than those nonirradiated as well in the cases of CC, WPI, and CC−WPI compositions. These differences were significant in terms of statistical analysis ($p \leq 0.05$) (shown as well by Duncan and Student *t* test). Significant

Table 2. Major Conclusions Arising From FTIR Spectroscopy Dealing with the Irradiation Effect on Proteins

number	protein composition	conclusion
1	CC	an increase in the total content of the β-structure with an increase in the content of β-strands; a decrease in the contents of α-helices, the aperiodic phase, and the unordered cross-linked phase
2	WPI	rearrangement of the β-structure followed by an increase of the highly cross-linked β-strand content; some modification of the aperiodic phase
3	CC–WPI	rearrangement of the β-structure followed by an increase of highly cross-linked β-strand content similar to that observed for WPI; additionally, an increase in the content of aggregates (β-strands) and turns accompanied by higher folding; a possible decrease in α-helices content

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Table 3. Fracture Strength (in Newtons) Determined for Gels Prepared after the Addition of CaCl$_2$ to the Solutions Containing CC, WPI, or CC–WPI[a]

number	dose (kGy)	CC	WPI	CC–WPI	
				experimental	calculated[b]
1	0	16.7 ± 2.0 a	189.7 ± 10.4 c	48.0 ± 4.7 b	103.2
2	32	120.9 ± 7.0 A[c]	293.3 ± 2.0 B[c]	413.6 ± 30.5 C[c]	207.1
	increase after irradiation	7.2-fold	1.5-fold	8.6-fold	

[a] The values in each row assigned by a different small letter or a different capital letter differ significantly ($p \leq 0.05$). [b] Calculated as the average value of the means determined separately for CC and WPI. [c] The irradiated samples differ significantly from the nonirradiated samples ($p \leq 0.05$).
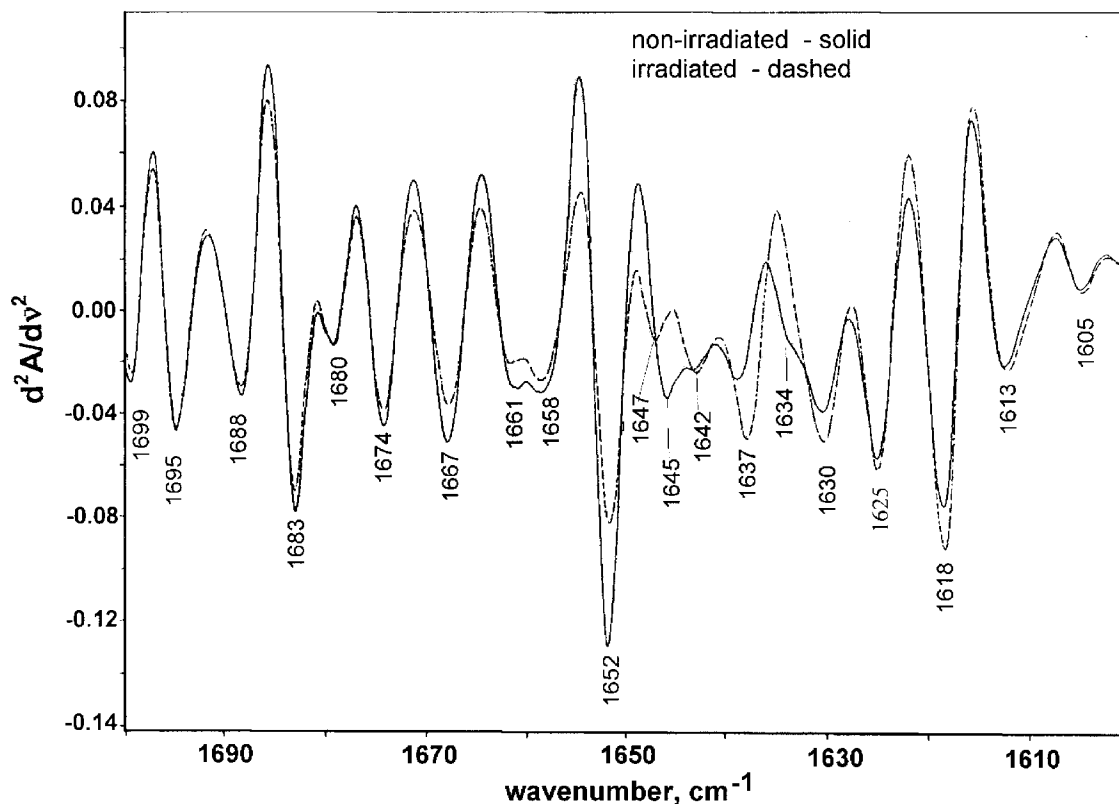
Figure 1. Comparison of FTIR spectra recorded for the nonirradiated and irradiated CC films.

differences ($p \leq 0.05$) were found also between the nonirradiated CC, WPI, and CC–WPI gels and between the irradiated CC, WPI, and CC–WPI gels.

CC, Effect of Irradiation. A decrease in the amount of the α-helix phase accompanied by an increase in the total amount of the well-ordered cross-linked β phase was observed after irradiation in the case of the CC films (**Figure 1**). It is shown by a decrease in the intensity of the band at 1652 nm corresponding to the α-helix, with a simultaneous increase in the intensities of the bands at 1637 and 1630 nm corresponding to β-sheets, respectively. It might also be concluded, on the basis of the split in the band at 1645 nm into two bands at 1642 and 1647 nm, that transformation takes place in the aperiodic phase (random coil). A simultaneous decrease in the intensity of the band at 1667 nm (turns) and of two bands around 1660 nm showed a decrease in the amount of an unordered cross-linked phase (turns or undefined) (*27*, *33*). Changes in relative intensities of the bands at 1630, 1634, and 1637 nm corresponding to β-sheets suggested reorganization of this phase, while the increase in the amount of strongly bonded and fine-ordered β-strands (attributed to intermolecular hydrogen-bonded β-sheets) might be stated on the basis of the increased intensity of the band at 1618 nm.

Gels prepared after the addition of calcium salt to the nonirradiated CC solutions were rather soft (**Table 3**). This is connected to a relatively small content of the β-structure in CC (the relative intensities of the appropriate components in FTIR spectra confirms that) and to the fact that heating alone is relatively not very efficient in the formation of cross-links in CC. On the other hand, such a low gel strength resulted probably because of the susceptibility of CC to the formation of the micellar structure. The strength of gels increased, however, a number of times after irradiation because of the efficient cross-linking. This has confirmed the conclusion arising from FTIR data and concerning the increased amount of the β-structure (especially β-strands) present in the irradiated as compared to the nonirradiated proteins. Cross-linking occurring in the processed CC was described in details in previous papers of our group (*10*−*14*, *22*). Therefore, heating at applied conditions induces a 3−4-fold increase in the apparent molecular weight, while a 10-fold increase was detected after irradiation with a 32 kGy dose (shift from 200 to 2000 kDa) (*12*).

WPI, Effect of Irradiation. Rearrangement of the β-sheets after irradiation, particularly an increase of the amount of β-strands, might be concluded also on the basis of FTIR data in the case of films prepared from pure WPI (**Figure 2**). It was

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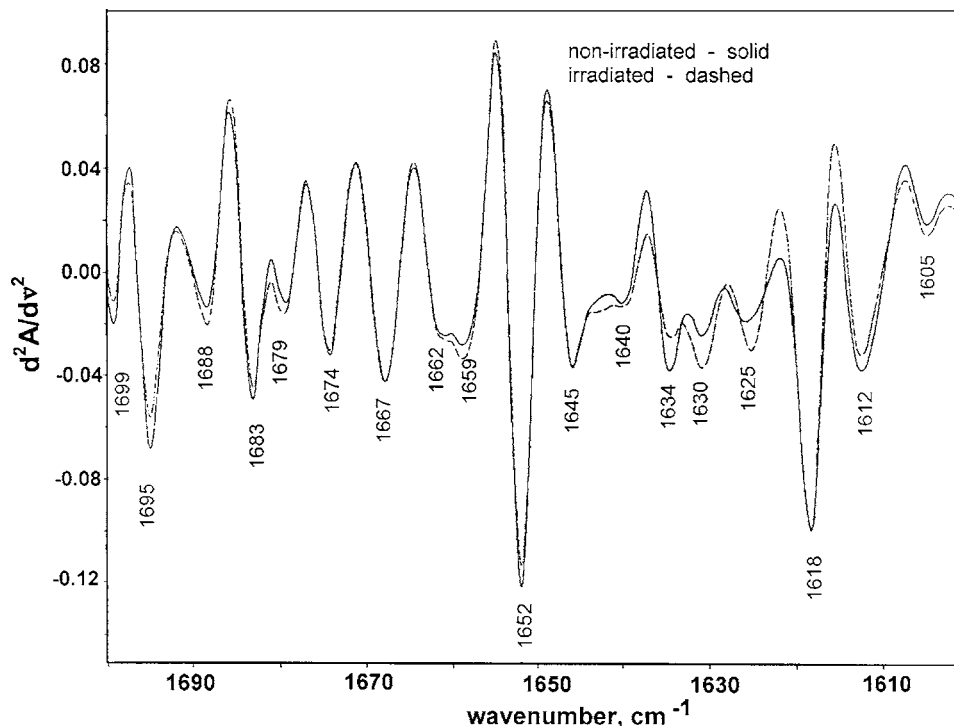
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Figure 2. Comparison of FTIR spectra recorded for the nonirradiated and irradiated WPI films.

shown respectively by a decreased intensity of the bands at 1634 nm and the higher intensity of the band at 1630 nm as well as the increased intensity of the band at 1625 nm probably corresponding to highly cross-linked β-strands. No differences between the intensity of the band at 1618 nm in FTIR patterns of the irradiated and nonirradiated samples show that protein aggregation taking place on heating was not influenced by the former irradiation. Small changes might be, however, supposed in the aperiodic α-helix phase on the basis of differences observed between the pattern region from ca. 1639 to 1643 nm. An important change in the amount of α-helices after irradiation could not be concluded on the basis of FTIR spectra (band at 1652 nm). This result corresponds well to the relatively small content of α-helices, while β-structure constituted a typical conformation of β-lactoglobulin, the predominant component of whey globulins.

Very strong gels were obtained after the addition of CaCl$_2$ to the nonirradiated WPI solutions (**Table 3**). These gels were ca. 5 times stronger than those prepared using nonirradiated CC because of the efficient cross-linking resulting after heating [molecular weight increased from ca. 40 to ca. 2000 kDa (*12*)]. This can be related to the higher content of the cross-linked β strands and the smaller content of α-helices present after heating in WPI as compared to CC (shown by the differences in the relative intensity of the bands at 1618 and 1652 nm in FTIR patterns) (**Figures 1**, **2**, and **4**). The increase in gel strength after irradiation corresponded to the increase in the content of the cross-linked β-strands detected by FTIR. The irradiation effect was, however, clearly smaller than in the case of CC. This correlates well to the less effective cross-linking induced by irradiation in WPI as compared to CC [described in the former paper (*12*)]. In fact, because of the low cross-link density achieved after irradiation, heating is an indispensable stage of WPI film formation. This confirmed the conclusion arising from FTIR spectroscopy pointing out the relatively smaller irradiation influence on the total content of β-structure in WPI as compared to CC.

CC$-$WPI, Effect of Irradiation and Effects of Mixing on Nonirradiated and Irradiated Proteins. For CC$-$WPI compositions, the similar conclusions concerning the irradiation effect might be drawn out as for pure WPI (**Figure 3**) on the basis of FTIR data. Additionally, in regard to CC participation, some increase in the aggregates (intermolecular β-strands) might be concluded after irradiation on the basis of a somewhat higher intensity of the band at 1618 nm. Higher intensities of several bands present in the range from 1660 to 1690 nm (*27*, *33*) might suggest, moreover, that the amount of the cross-linked turns was higher after than before irradiation, opposite to the cases of pure CC or pure WPI. In particular, the higher intensity of the band at 1688 nm indicated that folding is higher in the radiation cross-linked proteins than in the control ones despite the unfolding taking place upon heating (*33*).

Gels prepared using the nonirradiated CC$-$WPI composition and CaCl$_2$ have revealed the intermediate properties to those prepared using CC or WPI alone. Gel strength was, however, considerably lower than the average value calculated for both proteins (**Table 1**) and therefore did not correspond to the participation of the particular proteins in the mixed system but seemed similar rather to the strength of CC gels. This result could be attributed to the specific interaction between CC and WPI via the disulfide bridge mechanism (*23*, *24*) resulting in an aggregation of whey proteins with caseinate micelles (*24*) and suggested, furthermore, that the interaction with CC has an negative effect upon binding calcium ions by WPI units. It can be supposed that the appropriate functional groups present in WPI chains capable of connecting calcium are less available in CC$-$WPI than in the pure WPI system. Assuming that direct dependence occurs between the content of the β-structure and the bound calcium ions (*27*, *30*) and comparing the gel strength data obtained separately for CC and WPI, a conclusion might be drawn that the content of a well-ordered β-structure is smaller in the mixed system than it could be expected, summarizing the participation of both components. An opposite conclusion originated, however, based on FTIR data (**Figure 4**). It is
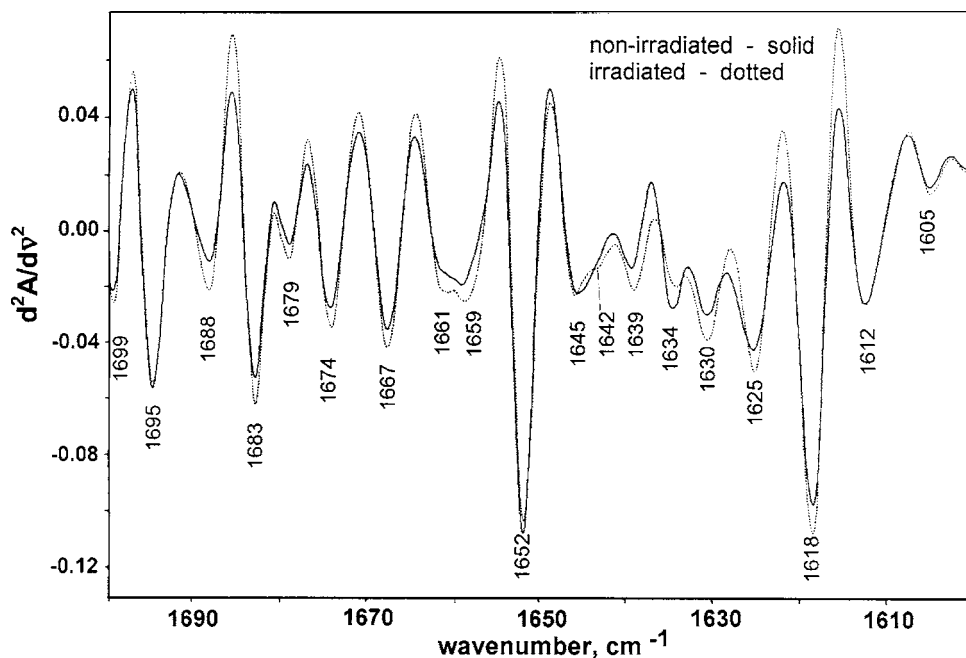
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Figure 3. Comparison of FTIR spectra recorded for the nonirradiated and irradiated CC–WPI (1:1) films.
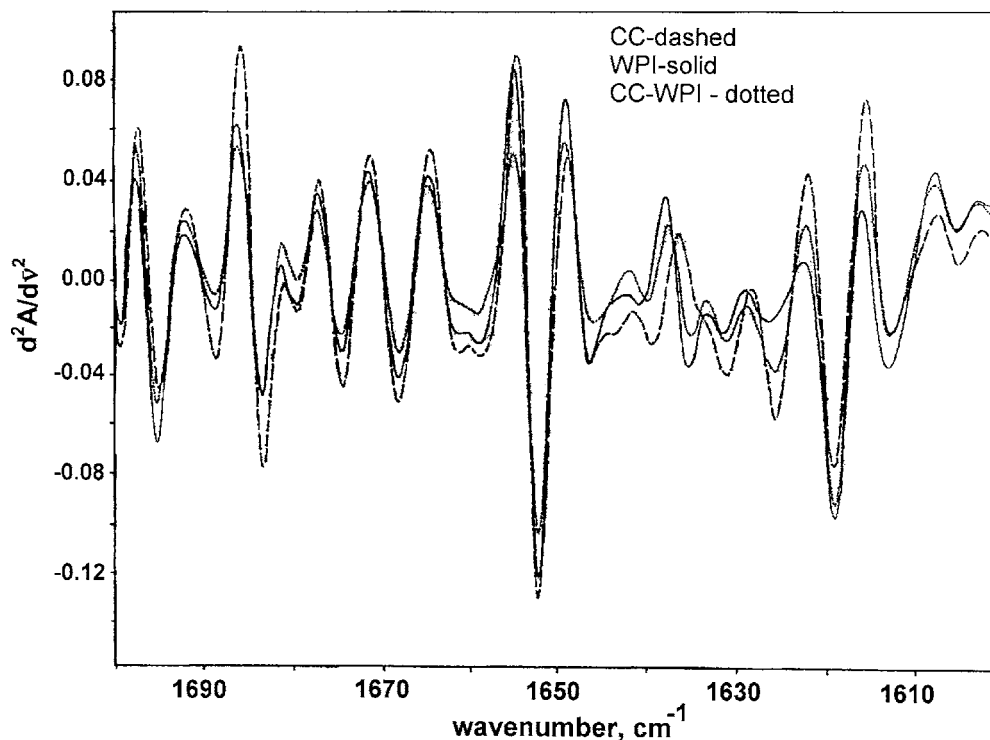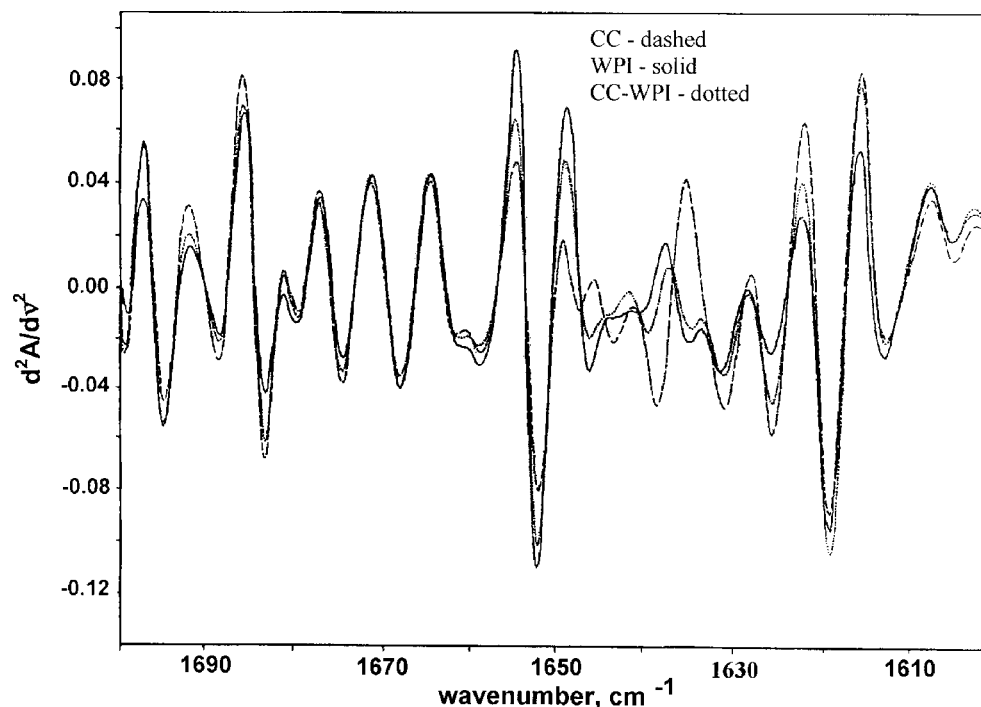

Figure 4. Comparison of FTIR spectra recorded for nonirradiated CC (- - -), WPI (—), and CC–WPI (•••) films.

because the relative intensities of the bands that might be attributed to β-sheets (1630 and 1634 nm) and β-strands (1625 and 1618 nm) were higher regarding the intensities of the bands corresponding to α helices (1652 nm) and turns (1600−1690 nm) in the case of CC−WPI than in the case of both WPI and CC. Moreover, heating alone has appeared efficient in the formation of cross-links in the CC−WPI system (*12*). In particular, an important participation of the protein fraction with ca. 50-fold higher mass than that determined for the control mixed protein solution resulted after heating at the same conditions apart to the non-cross-linked or intramolecularly cross-linked proteins (*12*). Therefore, a small gel strength is probably connected rather to the specific structure of the mixed

system and compatibility of both proteins than to the total amount of β phase.

In contrast, the irradiated CC−WPI compositions have revealed an amazing high strength of gels formed with $CaCl_2$. Gel strength values (**Table 1**) were not only higher than the average value calculated, taking into account the strength of separately prepared CC and WPI gels, but also larger than the one determined for WPI and similar to the sum of both values obtained for CC and WPI (414.2 N). The increase in gel strength after irradiation was larger than those found separately for CC and WPI. This result can be attributed to the incompatibility of the separately cross-linked CC and separately cross-linked WPI chains but also to the creation of a huge amount of cross-links

Figure 5. Comparison of FTIR spectra recorded for irradiated CC (- - -), WPI (—), and CC–WPI (•••) films.

between CC and WPI macromolecules leading to a highly ordered β conformation. The high efficiency of irradiation (32 kGy) combined with heating (90 °C, 30 min) in cross-link formation was proven by a 60-fold increase in the molecular weight (*12*) and might support that supposition. Because of the specific interaction of caseinate with whey proteins, the resulting structure is probably able to embed more calcium than summarized proteins irradiated separately. This result corresponded well to the higher relative intensities of the bands attributed to β-sheets and β-strands against intensities of the bands attributed to α-helices and the aperiodic phase observed in the case of the irradiated CC−WPI as compared to the irradiated WPI (**Figure 5**). The opposite effect was noticed, however, in the case of the irradiated CC and might be connected to the fact that a large part of CC exists in the monomeric form as can be deduced on the basis of the high intensities of the bands at 1630 and 1637 nm.

The question has appeared if a meaningful increase in β-strands and the moderate increase in total β-sheet content after the CC−WPI irradiation (shown by FTIR spectroscopy) could be responsible for the especially large increase in capability to join calcium ions. It might be considered if the functional groups placed in turns might participate in such connections apart to those present in the β phase. The cross-linked turns content increased after CC−WPI irradiation. However, incompatibility of the particular components still should be taken into account.

Viscosity of the Solutions and Gels. Properties of the control and irradiated solution, before and after heating at 90 °C for 30 min, are shown in **Table 4**. Differences between mean viscosity values determined for the irradiated and nonirradiated 5% solutions (heated and nonheated) were found to be significant in terms of statistical analysis ($p \leq 0.05$) (both Duncan and Student t test). A significant effect of heating was shown by the significant differences ($p \leq 0.05$) between means determined before and after heating the irradiated and nonirradiated solutions. It seems worth mentioning that β-lactoglobulin is known to form the "fine-stranded" gels at the applied pH range.

The increase in solution viscosity after irradiation (**Table 2**) was caused by an increase in molecular mass connected to

Table 4. Properties of the CC–WPI Solutions as Prepared, after Irradiation and/or Heating[a]

| dose (kGy)	pH of 7.5% solution	viscosity (cP)		
		7.5% solution	5% solution	5% solution, heated at 90 °C for 30 min
0	6.62 ± 0.02	3.60 ± 0.10	2.11 ± 0.01 a	2.56 ± 0.07 c[c]
32	6.76 ± 0.02	4.37 ± 0.10	2.56 ± 0.09 c[b]	2.35 ± 0.04 b[b,c]

[a] Statistical analysis was performed for viscosity data obtained for 5% solutions. Means followed by different letters are significantly different ($p \leq 0.05$). [b] The mean showed a significant effect of irradiation observed before and after heating. [c] The mean indicates a significant effect of heating on both nonirradiated and irradiated solutions.

proteins cross-linking (reported above). An increase in the viscosity of the control solution after heating at 90 °C regards gel creation probably occurring toward the association of protein particles (*33*). In contrast, a decrease in the viscosity was observed after heating the irradiated solution. This phenomenon is connected probably to the formation of gels that have revealed rather a "fine-stranded" structure. It is known that such "fine-stranded" gels are more elastic (less viscous) than the "particulate" gels. It can be stated therefore that the presence after irradiation of the better ordered protein structure, in particular the increased amount of the highly ordered β-strands, enables the more "fine-stranded" (better ordered) gels to be obtained as compared to the more "particulate" gels formed using the nonirradiated solutions.

Figure 6 presents the viscosity values of the irradiated and control solutions as determined at the selected temperatures reached after rapid heating (the second-type experiment). These values were lower when temperatures were higher up to 70 °C for the control and up to 80 °C for the irradiated solutions. A possible dissociation of dimers into monomers [pointed out to be the first step of the fine-stranded gel formation (*33*)] can reveal a similar effect because of an increase in the molecular mobility. A more essential and faster preliminary fall in viscosity observed for the irradiated samples than for the control ones

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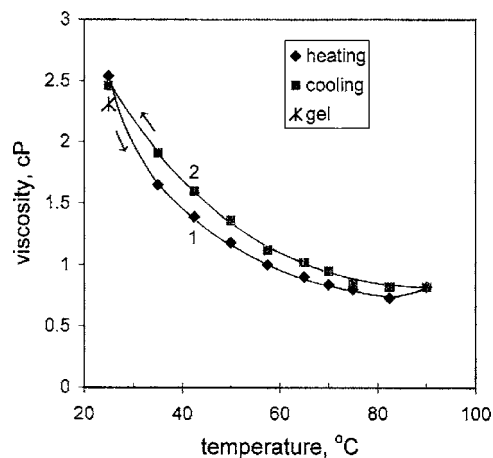*J. Agric. Food Chem.,* Vol. 54, No. 17, 2006 **6381**
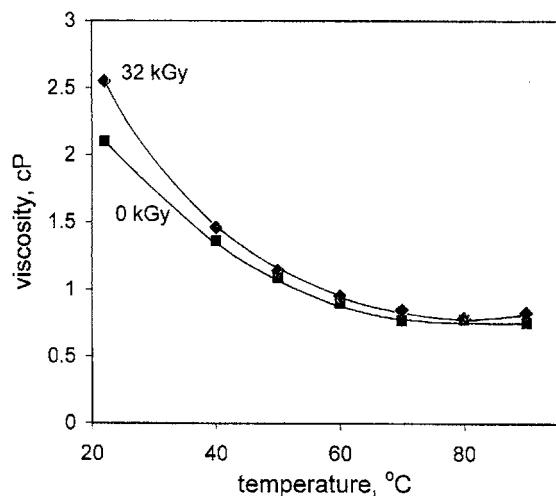
Figure 6. Comparison of the dependence of viscosity of the control and the irradiated solutions determined after fast heating to the selected temperatures in the range of 25–90 °C (statical conditions).
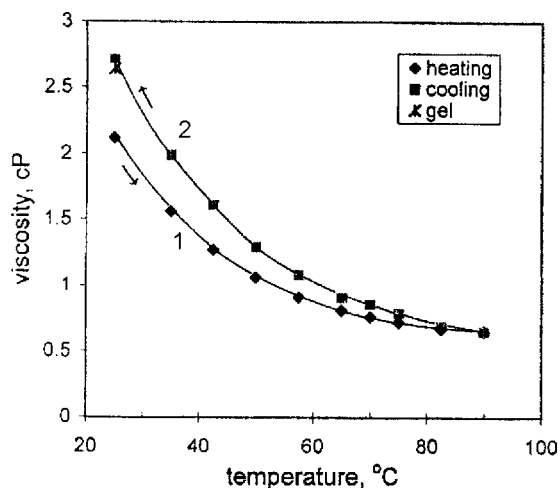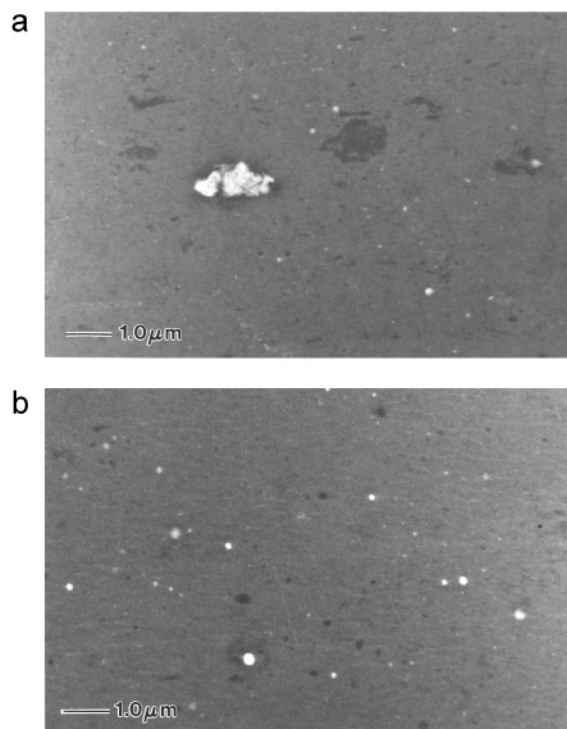

Figure 7. Viscosity determined at selected temperatures for the control CC–WPI solutions during dynamic heating with an average rate of 2.4 °C min^{-1} (curve 1) and dynamic cooling with an average rate of 1.9 °C min^{-1} (curve 2). Viscosity is also shown for 5 wt % gel obtained after heating at 90 °C for 30 min.

Figure 8. Viscosity determined at selected temperatures for the irradiated CC–WPI solutions during dynamic heating with an average rate of 2.4 °C min^{-1} (curve 1) and dynamic cooling with an average rate of 1.9 °C min^{-1} (curve 2). Viscosity is also shown for 5 wt % gel obtained after heating at 90 °C for 30 min.

Figure 9. TEM images of CC–WPI films (a) nonirradiated and (b) irradiated (32 kGy).

can be related to the more intensive dissociation of the larger amount of dimers. This corresponds to the increased after irradiation relative intensity of the band at 1625 nm (β-strand) in the FTIR pattern versus that at 1634 nm (β-sheet) (**Figure 3**), reported to be an indicator of the larger amount of dimers (*33*). As a result, the viscosity values determined for both samples became closed already at 40 °C, although those of irradiated samples were still slightly higher at all temperature ranges than those of the control ones. The viscosity curve stabilizes at a higher temperature when the formation of the gel network took place.

The similar temperature–viscosity curves were recorded for both samples upon dynamic heating (the third-type experiment) (**Figures 7** and **8**), with the exception that some increase in viscosity of the irradiated sample was noticed in the high-temperature region (**Figure 8**). It is presumably connected to the effective cross-linking of the proteins taking place at high temperature, in particular, to the formation of a gel network toward protein–protein aggregation (*36*, *37*), and can be related to the increase of the aggregation component caused by

irradiation (band at 1618 nm in the FTIR pattern). Such an increase in viscosity was not observed after rapid heating (**Figure 6**). It should be pointed out, however, that more cross-links resulted probably on the dynamic than on rapid heating because the sample was kept longer at high temperature. Such a high temperature increase in viscosity was also not noticed for the control sample heated under dynamic conditions (**Figure 7**). It can be concluded, therefore, that cross-link formation upon further heating is more effective after the former irradiation as compared to the nonirradiated proteins.

Viscosity of the control solutions increases very fast upon cooling (**Figure 7**). Viscosity determined at the same temperature for the control sample was also clearly higher at dynamic cooling than at dynamic heating. As a result, the clearly higher values were also obtained after cooling the samples to room

Table 5. Confrontation of the Barrier and Mechanical Properties of the CC–WPI Films with the Properties of the Gel Solutions Used for Preparation and Fracture Strength of Gels Formed with CaCl$_2$[a]

| number | dose (kGy) | properties of the films | | | | fracture strength of gels formed with CaCl$_2$ (N) | viscosity of 5% solution heated at 90 °C for 30 min (cP) |
		tensile strength (N mm^{-1})	WVP ($\times 10$ mm/m^2 d mmHg)	deformation at puncture (mm)	viscoelasticity coefficient		
1	0	53.9 ± 2.6 a	16.86 ± 1.01 a	4.46 ± 0.29 a	0.524 ± 0.01 a	48.0 ± 4.7 a	2.56 ± 0.07 a
2	32	77.4 ± 2.6 b	11.49 ± 0.96 b	4.07 ± 0.35 b	0.561 ± 0.01 b	413.6 ± 30.5 b	2.35 ± 0.04 b

[a] The values followed by different letters in each column are significantly different ($p \leq 0.05$).

temperature. In contrast, the viscosity values recorded at the selected temperature upon cooling the irradiated solutions were more closed to those obtained upon heating (**Figure 8**). These results corresponds well to the smaller viscosity values determined at ambient temperature for the irradiated and afterward heated solutions as compared to those that were only irradiated and to the higher viscosity values of the control solutions after heating. Viscosity of the irradiated solution was higher upon cooling in the temperature range up to ca. 50 °C than the viscosity of the control solution but lower in the range of lower temperatures (**Figures 7** and **8**) (*25*).

Discussion of the Relation between the Radiation Effect on Gel Solutions and Gels Formed with CaCl$_2$. It can be noticed that although gels obtained using the irradiated solutions have revealed more 'fine-stranded' structure than those obtained using the control ones, it appeared otherwise after the addition of CaCl$_2$. It is shown by the higher texture strength of the gels formed using the irradiated solutions as compared to the nonirradiated ones. These results correspond well to those showing a decrease in the amount of β-strands occurring in some proteins because of the presence of calcium (*27*, *30*). It was deduced, therefore, that calcium ions are embedded in β structure, which loses as a result of its regularity (*27*). Accordingly, the gels obtained after irradiation, which contain a higher amount of the regular β-structure, are capable of joining more calcium ions and are more affected than those obtained from control solutions. As a result, these gels have appeared more "particulate". The present results can be explained also in terms of the gel-formation mechanism. It is believed that "fine-stranded" gels are formed toward the association of dimers to monomers followed by monomer aggregation, while in the case of "particulate" gels, direct aggregation of dimers takes place (*33*). However, aggregation induced by CaCl$_2$ occurs faster than dissociation; thus, "particulate" gels are formed (*23*, *38*). When more dimers occur in the irradiated than the nonirradiated samples, as can be deduced on the basis of FTIR data (β-strand content), the resulting gels are more "particulate".

Microstructure of the CC−WPI Films and Their Barrier and Mechanical Properties. The dense structure with small pores (showed by white points) was observed in the cases of both control and irradiated films (parts **a** and **b** of **Figure 9**). The irradiated film was, however, more homogeneous. Some bigger pores, inclusions, and big spots (probably representing material with decreased density) were observed only in the control films. The porosities of the irradiated and nonirradiated films were similar, with that difference that the irradiated film contained slightly more pores but with smaller dimensions (and no big pores) as compared to the nonirradiated one.

Films obtained from the irradiated solution are essentially stronger and constitute a better barrier for water vapor than those nonirradiated. This is shown by a higher tensile strength and lower WVP (**Table 5**). This is accompanied by a decrease in film elasticity after irradiation, shown by the lower values of

deformation upon puncture and the higher values of the viscoelasticity coefficient determined for the irradiated films as compared to the nonirradiated ones (**Table 5**). The differences between the mean values of all of the parameters determined for the control and irradiated films were found to be significant in terms of the statistical analysis ($p \leq 0.05$) (both Duncan and Student *t* test).

It can be noticed that the essential improvement of the mechanical strength and barrier properties of the films does not correspond to an adequate decrease in film porosity. It can be thus stated that the modification of these properties results rather owing to the modification of the internal structure of the protein matrix, in particular, to the denser and more regular packing of the protein macromolecules obtained after evaporation of water from the more fine-stranded gels. The increase in regularity of the protein packing leads to a formation of more crystalline and denser films, as shown by their increased strength and rigidity and smaller WVP. This corresponds to the increased homogeneity of the films after irradiation (**Figure 9**).

Concluding Remarks. The conformational changes occurring in milk proteins in regard to radiation-induced cross-linking results in the formation after the further heating of the films with the higher content of the well-ordered β-structure, in particular of strongly bounded β-strands. In particular, an increase of the total β-structure content as well as the aggregates (β-strands) upon the cost of α-helices and the aperiodic phase was found in the case of CC, while rather a modification of the β-structure with an increase of β-strands was noticed in the case of WPI. The modification of CC−WPI consists upon reconstruction of a β-structure similar to that observed for WPI, accompanied by some increase in the content of aggregates (β-strands) and turns with a possible decrease in α-helices.

An essentially higher efficiency of heating in the formation of a cross-linked β-structure in the control WPI as compared to the control CC was confirmed by differences in the efficiency of calcium binding deduced from gel-texture data. In contrast, CC gels have appeared more sensitive than the WPI gels to radiation treatment and the CC−WPI gels have appeared more sensitive to radiation treatment than gels formed using either CC or WPI separately.

Strong interaction between two components in the control CC−WPI solution resulted in the formation of films with a higher content of β-structure (in relation to the other conformations) as compared to those observed in films formed using each component separately. A predominant influence of CC on the properties of the control CC−WPI resulted, however, in a smaller strength of gels formed with CaCl$_2$ than expected, considering the contribution of both components. Simultaneously, a higher gel strength observed in the case of the irradiated CC−WPI gels than separately in the case of CC and WPI gels corresponds to a higher content of strongly bounded β-structure found in the CC−WPI films only as compared to the WPI films.

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Viscosity of the irradiated solutions was higher before heating as compared to the control one, owing to their larger molecular mass. The larger amount of dimers (confirmed by FTIR studies) and the higher efficiency of dissociation of dimers into monomers followed by aggregation can be stated on the basis of viscosity changes during heating of the irradiated solutions as compared to the nonirradiated ones. The creation of a well-ordered protein structure leads to the formation of gels with the improved "fine-stranded" structure, as compared to the more "particulate" gels formed using the nonirradiated control solutions. More homogeneous films characterized by the better ordered structure were formed after further loss of water by those irradiated gels as compared to the nonirradiated ones. Accordingly, the irradiated films had better barrier properties and higher mechanical strength than the nonirradiated ones.

The irradiated gels appeared more "particulate" than the control ones in the presence of calcium salt, although more "fine-stranded" when calcium salt was absent.

ABBREVIATIONS USED

CC, calcium caseinate; WPI, whey protein isolate; WVP, water vapor permeability.

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