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γ-Irradiation Influence on the Structure and Properties of Calcium Caseinate−Whey Protein Isolate Based Films. Part 2. Influence of Polysaccharide Addition and Radiation Treatment on the Structure and Functional Properties of the Films

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The influence of γ-irradiation (32 kGy) followed by the addition of polysaccharides (potato starch, soluble potato starch, and sodium alginate) and heating on the properties of the films based on calcium caseinate (CC)−whey proteins isolate (WPI) and the gels formed with $CaCl_2$ was evaluated. Radiation induced an improvement of the mechanical and barrier properties of all films. The polysaccharides' effect on the irradiated and non-irradiated CC−WPI gels could be predicted as the sum of their separate effects on CC and on WPI, apart from the alginate interaction with the irradiated CC−WPI. The better properties of the films achieved after admixing polysaccharides to the formerly irradiated protein solution correspond to the smaller strength of gels. Properties of the films and gels prepared using the irradiated proteins and alginate differed depending on whether alginate was admixed before or after irradiation. Results were related to the protein structure, interaction with polysaccharides, and the film's microstructure.

KEYWORDS: Edible films; milk proteins; calcium caseinate; whey protein isolate; sodium alginate; starch; γ-irradiation; cross-linking; conformation; gel structure; mechanical properties; water vapor permeability; transmission electron microscopy; TEM

INTRODUCTION

Edible films based on proteins have revealed appropriate mechanical resistance but rather unsatisfactory barrier properties connected to the proteins' hydrophilicity. The addition of polysaccharide [carboxymethylcellulose (CMC), cellulose xanthate, pectin, agar, starch, or alginate] to the films' composition at the level of 5 wt % of the total protein mass enables the production of films characterized by improved barrier properties and high mechanical resistance (*1*−*6*). γ-Irradiation carried out before or after admixing of polysaccharide to the proteins solution induces further improvement of these films. It was found, for example, that the addition of CMC to proteins before irradiation enhances their radiation-induced aggregation (*7*). Differences were detected, however, in the effect of various polysaccharides on the films' properties.

Part 1 of the present study (*8*) concerned the processes taking place in calcium caseinate (CC)−whey protein isolate (WPI)−glycerol compositions (1:1:1) under the influence of irradiation and heating. It was found that radiation-induced modifications of protein conformation result in the formation of better structured gels and films with improved functional properties. These results were confronted with the more effective cross-linking achieved after irradiation of the mixed formulations as compared to the separately irradiated components. The objectives of the current study (part 2) are to recognize the interactions taking place in a CC−WPI−glycerol−polysaccharide system (1:1:1:0.1) (CC−WPI), non-irradiated and irradiated using the same dose of 32 kGy, in relation to those occurring in partial systems, CC−glycerol−polysaccharide (2:1) (CC) and WPI−glycerol−polysaccharide (2:1) (WPI) systems, and to relate structural properties of proteins to the functional properties and microstructure of the final films. This concerns three polysaccharides differing in properties. Potato starch (PS) is a common and cheap biopolymer composed of large and branched neutral macromolecules. Soluble potato starch (SPS) is a degradation product. Sodium alginate forms linear chains essentially shorter than those of unmodified starch and electrically charged due to the presence of carboxyl groups. Our studies focused on the influence of polysaccharides introduced to the protein solution after irradiation. However, alternatively, the effect of sodium alginate irradiated together with protein composition was recognized. The good properties of the films containing alginate and specific interactions discovered between the irradiated proteins and alginate indicate the suitability for further optimization of the preparation procedure.

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MATERIALS AND METHODS

Materials and Film Preparation. CC (produced by New Zealand Milk Product Inc.), WPI (BiPro by Davisco), and chemical grade glycerol were used (*8*). The structural properties of CC and WPI studied by means of FTIR spectroscopy were described in ref *8*. Sodium alginate (low viscosity, A2158), PS) S4251), and SPS (S2004) were all Sigma products.

Irradiations were carried out in an inert gas atmosphere for the protein−glycerol solutions (at a weight ratio of 2:1; 7.5% of total protein content) using a dose of 32 kGy at a dose rate of 7 Gy s^{-1} (details in ref *8*). Apart from the pure protein compositions containing CC, WPI, or CC−WPI (1:1), the following compositions were irradiated: CC−alginate−glycerol, WPI−alginate−glycerol, and CC−WPI−alginate−glycerol, all characterized by a weight ratio of total proteins/polysaccharide equal to 1:0.1.

Films were prepared and conditioned by applying the procedure described in ref *8* after dissolving the solution to 5% of the total proteins content. The films were prepared, however, containing an addition of polysaccharides apart from these made using the protein solutions alone. In such cases the final solutions contained 2.5 wt % of CC, 2.5 wt % of WPI, 0.25 wt % of polysaccharide, and 2.5 wt % of glycerol. The polysaccharide suspensions or solutions (containing 0.9 wt % of the selected polysaccharide) were pregelatinized by heating for 30 min at 121 °C under a pressure of 124 kPa (in the autoclave). These pregelatinized solutions were mixed with the 7.5% of protein solutions (irradiated or non-irradiated), dissolved to 5% of the total protein content, and then subjected to the final heating at 90 °C for 30 min. The thickness of the films (after conditioning for 48 h at 56% humidity) was in the range of 60−80 (±2) μm.

Methods. Examination of the films' properties using FTIR spectroscopy and transmission electron microscopy (TEM), measurements of film thickness, and tests for water vapor permeability (WVP), puncture strength, deformation on puncture, and viscoelasticity coefficient as well as complementary viscosity measurements were carried out applying the conditions described in detail in ref *8*. TEM photographs were taken for the bulk area of the films placed in the holder in such a way that the surface of the film was parallel to the top of the picture. A Stevens LFRA model TA/100 (NY) texture analyzer was used for the studies of mechanical properties of the films and for gel strength measurements.

The force−deformation curves were used for the evaluation of hardness and deformation capacity of the films (*8*, *9*). A cylindrical probe (2 mm in diameter) was moved perpendicularly to the film surface at a constant speed (1 mm^{-1}) until it passed through the film. Strength and deformation values at the puncture point were then determined and related to the thickness of the film. The relaxation (viscoelastiicity) coeffecient was evaluated using force−relaxation curves (*9*). The same procedure was applied, but the probe was stopped at 3 mm deformation and the film was allowed to relax.

WVP tests were conducted gravimetrically (*8*, *10*). The test films were mechanically sealed to Vapometer cells containing 100 g of anhydrous calcium chloride and were placed in the humidity chamber at 30 °C and a relative humidity of 56% for 24 h. WVP was calculated according to the formula

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

where W is the weight gain of the cup (g), 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.

Binding of Calcium Ions and Gel Fracture Strength Measurements. A modified procedure of Sakamoto et al. was applied (*4*, *11*) (details shown in ref *8*). The pregelatinized polysaccharide solutions were prepared by autoclaving at 121 °C (at 124 kPa) for 30 min and introduced to the protein solution at ambient temperature before admixing of $CaCl_2$. A quantity of 0.4 mL of the solution containing 5% proteins, 0.25% polysaccharide, and 0.125% $CaCl_2$ was poured into a well of the microtest plate (7 mm in diameter and 11 mm in height), sealed, and heated for 4 h at 90 °C. The solution was allowed to cool at ambient temperature and stored overnight at 4 °C. A cylindrical probe (3 mm in diameter) was moved through the gel with a constant speed of 2 mm min^{-1}. The gel fraction strength was calculated on the basis of the force−deformation curves.

To describe the effect of polysaccharide addition on gel strength, the incompatibility coefficient (IC) was defined. IC was calculated for the particular protein−polysaccharide composition as a ratio of the mean value of the strength of the gel obtained after the addition of the selected polysaccharide to the mean value of gel strength obtained in the case of those proteins' composition without the polysaccharide addition.

Complementary viscosity measurements were carried out using a Brookfield viscometer (*8*) for the gels obtained by heating of the 0.9 wt % polysaccharide solution/suspension (in the autoclave; 121 °C, 124 kPA, 30 min), cooling to the temperature of 40 or 25 °C, and making up water loss. Measurements were performed for 0.9 wt % polysaccharide gels (corresponding to the polysaccharide solutions used for the protein−polysaccharide film preparation) and for those obtained after mixing of the autoclaved solution with $CaCl_2$ (final concentration of 0.25 wt % of polysaccharide and 0.125 wt % of $CaCl_2$, the same as applied for gel fracture strength measurements with the difference that gels do not contain proteins). Simultaneously, the control (not autoclaved) samples were measured with the same composition. The rotation speed was in the range corresponding to the linear dependence of shear rate on shear stress, and the viscosity values were determined at that, securing the highest percentage of torque (75−80% in each case). Three viscosity measurements were performed for each composition.

Statistical Analysis. The analysis of variance and Duncan's multiple-range tests with $p \leq 0.05$ were used to analyze the results statistically. The Student t test and a paired comparison with $p \leq 0.05$ were used (details shown in ref *8*). For the gel texture strength measurements nine repetitions were done for each composition using three independent solutions. Two sets of films were prepared for each composition using the separately prepared solutions. A total of about 20 repetitions for each film composition were considered for tests of WVP, puncture strength, and deformation at puncture, and total of about 10 repetitions were considered for the viscoelasticity coefficient.

RESULTS AND DISCUSSION

FTIR Spectroscopy of Protein−Polysaccharide Compositions. The addition of PS, SPS, and alginate to the final CC−WPI solutions did not influence the observation of the differences between protein conformation in irradiated and non-irradiated species using FTIR. This shows that the presence of polysaccharide does not affect the amount and ordering of β-conformations in the protein phase (details of observations done for pure proteins were reported in ref *8*).

The maximum intensity of the amide I band was shifted after irradiation from 1631 to 1632 nm, in both cases when PS and SPS have been introduced to the final CC−WPI solution. In the case when alginate was admixed to the final solution, the maximum intensity was shifted after irradiation from 1630 to 1631 nm. The shift from 1630 to 1633 nm was observed after irradiation carried out for CC−WPI−alginate composition.

Effect of Polysaccharide Addition to the Irradiated and Non-irradiated Compositions on Fracture of Gels Formed with $CaCl_2$. Two factors determine properties of gels formed with $CaCl_2$. The conformation of proteins existing before admixing of $CaCl_2$ and their capability for calcium binding as well as the structure of the resulting protein gel fraction is the first one (*12*, *13*). The presence of calcium ions modifies gel structure. For example, whey proteins form particulate gels at high ionic strength, whereas the gels formed using pure proteins are rather fine-stranded at the same pH (about 7, near the 6.6−6.8 found for the present compositions) (*14*, *15*). The second factor depends on the proteins' and polysaccharides' interactions in solutions (compatibility) (*15*−*18*). Weak gels are formed in the case of high incompatibility of the two components, whereas reinforced gels result in the case of compatible pairs. This factor,

Table 1. Fracture Strength (Newtons, N) Determined for Gels Prepared after Addition of Calcium to the Solutions Containing CC–WPI, CC–WPI–PS, CC–WPI–SPS, and CC–WPI–Al[a]

		CC		WPI		CC-WPI	
no.	polysaccharide additive	0 kGy	32 kGy	0 kGy	32 kGy	0 kGy	32 kGy
1	none	16.7 ± 2b	120.9 ± 7.0a*	189.7 ± 10.4b	293.3 ± 2.0c*	48.0 ± 4.7b	413.6 ± 30.5c*
2	potato starch	10.2 ± 2a	146.5 ± 9.2bc*	183.0 ± 11.5b	196.2 ± 13.9a	42.7 ± 5.0a	363.6 ± 21.6b*
3	soluble starch	12.8 ± 2a	162.0 ± 15.8c*	46.3 ± 1.7a	201.1 ± 17.8a*	46.2 ± 2.6b	475.5 ± 26.7d*
4	sodium alginate	no[b]	127.8 ± 13.2ab*	231.0 ± 6.2c	192.3 ± 15.4a*	48.2 ± 3.0b	224.3 ± 8.4a*
5	sodium alginate[c]	no	306.9 ± 18.7d*	258.4 ± 18.8d	236.2 ± 23.1b*	70.6 ± 5.5c	431.6 ± 19.8c*

[a] There is no significant difference ($p > 0.5$) between the values in the same column assigned the same letter. Means followed by different letters in each column are significantly different ($p \leq 0.05$). An asterisk indicates that means obtained for the irradiated gels differ significantly ($p \leq 0.05$) from the means obtained for the non-irradiated gels. All of the irradiated samples differ significantly from the non-irradiated reference samples. [b] Gels were not obtained (heterogeneous suspension). [c] Sodium alginate was introduced to the protein solutions before irradiation.

although depending itself on protein conformation in the presence of calcium ions, might modify the final structure of protein gel and thus influence its calcium binding capacity and the resulting compatibility of the structure of protein−calcium and polysaccharide−calcium gel networks.

Therefore, small gel strength can be connected both to the incompatibility between proteins toward polysaccharide in the presence of calcium and to the diminished capability of the examined proteins to join calcium. To the contrary, the compatibility of two gel networks and the high capability to join calcium both result in a high strength of gels.

Strengths of gels prepared using the irradiated protein and protein−polysaccharide solution differ significantly in terms of the statistical analysis ($p \leq 0.05$; both Duncan and Student *t* test; data shown in **Table 1**) from these of non-irradiated gels, with the exception of non-irradiated and irradiated gels prepared using WPI−PS composition ($p > 0.05$). Gels composed of CC−polysaccharide and CC−WPI−polysaccharide prepared using the irradiated protein solutions were significantly stronger ($p \leq 0.05$) than the non-irradiated ones, independent of the admixed polysaccharide. The irradiation influence on WPI−polysaccharide composition depends upon the polysaccharide used [increase (SPS; ($p \leq 0.05$)), decrease (alginate; ($p \leq 0.05$)), or no change in strength occur (PS; ($p > 0.05$)]. Significant differences ($p \leq 0.05$) were also detected between the gels prepared using the irradiated protein−polysaccharide composition and those prepared using the irradiated proteins alone, with the exception of the CC−alginate system ($p > 0.05$). Significantly stronger gels ($p \leq 0.05$) were formed when both starch polysaccharides were added into the irradiated CC and CC−WPI solutions, as compared to the protein alone, whereas the addition of alginate into the irradiated CC−WPI and the addition of all the polysaccharides into the irradiated WPI induce a significant decrease in the gel strength ($p \leq 0.05$). A specific effect of particular polysaccharides on the strength of gels prepared using the non-irradiated proteins, depending on both protein and polysaccharide, was also confirmed by statistical analysis results [gel strength values were significantly higher after polysaccharide addition ($p \leq 0.05$; WPI−alginate) or significantly smaller ($p \leq 0.05$; CC−PS, CC−SPS, CC−WPI−PS), or a lack of significant difference occurred ($p > 0.05$; CC−WPI−SPS, CC−WPI−alginate, WPI-PS), or nonhomogeneity appeared (CC−alginate)]. Details of the effects of irradiation and polysaccharide addition on gel strength are shown and discussed in terms of protein−polysaccharide interaction in the following sections.

CC Gels. Gels containing the non-irradiated CC and the addition of PS or SPS were weaker ($p \leq 0.05$) than the gels prepared using the CC alone (**Tables 1** and **2**). On the contrary, addition of both starch polysaccharides to the irradiated CC compositions led to the formation of stronger gels. The effect is larger in the case of soluble (SPS) than in the case of insoluble starch (PS). This result can be related to the compatibility of the CC and starch in the presence of $CaCl_2$. On the other hand, that might mean that the entrapment of the cross-linked protein chains into starch gel networks results in some increase in the amount of the functional groups capable of joining calcium, whereas the opposite effect occurs in the case of the control CC. Furthermore, with the assumption that the amount of bound calcium is the higher when β-structure content is higher, the conclusion can be drawn that the presence of starch gel network induces some increase in the β-structure of the irradiated caseinates. The relatively small change in β-structure that can be concluded on the basis of the small increase in gel strength [up to 1.2 (PS) and 1.3 (SPS) of that existing in absence of starch] might be undetectable by FTIR spectroscopy.

Table 2. IC Values Calculated for the Particular Protein–Polysaccharide Compositions as a Ratio of the Mean Value of the Strength of Protein–Polysaccharide Gel to the Mean Value of the Strength of the Protein Gel[a]

	protein	polysaccharide				IC (alginate*)/
no.	composition	PS	SPS	alginate	alginate*	IC (alginate)
		Non-irradiated				
1	CC	0.6	0.8	nd[b]	nd	nc[c]
2	WPI	1.0	0.2	1.2	1.4	1.1
3	CC–WPI (exptl)	0.9	1.0	1.0	1.5	1.5
4	*CC–WPI (calcd)*	*0.8*	*0.5*	*nc*	*nc*	*nc*
		Irradiated				
5	CC	1.2	1.3	1.1	2.5	2.4
6	WPI	0.7	0.7	0.7	0.8	1.2
7	CC–WPI (exptl)	0.9	1.1	0.5	1.0	1.9
8	*CC–WPI (calcd)*	*0.9*	*1.0*	*0.9*	*1.6*	*1.8*

[a] In the case of the CC–WPI compositions (non-irradiated and irradiated) the values calculated on the basis of the contribution of each protein are presented (average of the values found for CC and for WPI), apart from the experimental values. [b] Not determined (heterogeneous suspension). [c] Not calculated (missing experimental value).

Precipitation occurs after the addition of alginate to the non-irradiated CC in the presence of $CaCl_2$. This salting-out effect indicates the limited potential of the alginate to interact with proteins (*16*, *17*). Addition of alginate to the irradiated CC does not influence significantly the further appearance and strength of the gels. The formation of homogeneous gels has proved, however, a stronger interaction with alginate, as compared to the non-irradiated CC.

Differences between gels prepared using the irradiated and the non-irradiated compositions were even larger after the addition of PS and SPS as compared to the pure CC (**Table 1**). The increase in the gel strength related to protein irradiation

reached values of about 14.4- and 12.6-fold after the addition of PS and SPS, respectively, whereas a 7.2-fold increase was found in the case of proteins alone. Differences between the products formed with $CaCl_2$ due to interaction of the non-irradiated and the irradiated proteins with alginate are obvious.

WPI Gels. Differences were noticed between the effect of particular polysaccharides on the gels prepared using the non-irradiated WPI solutions (**Tables 1** and **2**). PS does not affect the strength of gels, whereas the presence of alginate induces some increase in this parameter. It can be attributed to the compatibility between both gel fractions as well as to the increase in the content of a well-ordered β-structure (probably occurring due to the effect of entrapment in the alginate gel network). The presence of SPS induces, on the contrary, an anomalous decrease in the gel strength. Although the reason for such a decrease in the gel strength is not clear, it presumably involves the formation of soluble complexes between proteins and small SPS molecules (*18*).

Addition of all the polysaccharides to the irradiated WPI induces a decrease in the strength of gels, as compared to the proteins alone. Very close values were obtained independently whether PS, SPS, or alginate was applied. These strength values were close, actually, to the value obtained for gels containing the non-irradiated WPI alone. The possible reasons for such a decrease in gel strength are considered in the section concerning CC−WPI gels.

Differences in the effects of particular polysaccharides on the strength of gels formed using the control and the irradiated WPI solutions led to differences in the observed irradiation effect on the final gel strength. A relatively small increase in gel strength regarding irradiation ($p \leq 0.05$) was noticed for the gels formed after the addition of PS. On the other hand, the strength of gels formed after the addition of alginate was smaller in the case of irradiated than in the case of the non-irradiated proteins (with a mean value of about 0.8 of that determined for the non-irradiated species). This is due to the opposite effect of the addition of sodium alginate to the irradiated and non-irradiated WPI. Essentially larger differences between the irradiated and non-irradiated WPI gels containing SPS addition are related, instead, to the anomalously small strength of the gels formed using non-irradiated WPI−SPS.

CC−WPI Gels. Gels formed after the addition of SPS and alginate to the non-irradiated CC−WPI solutions revealed strengths very similar to those containing proteins alone ($p > 0.05$) (**Table 1**). Gels containing PS were, however, somewhat weaker ($p \leq 0.05$).

The strength of gels prepared using the irradiated CC−WPI solutions was essentially higher than that of the gels prepared using control solutions, as well after the addition of all the polysaccharides as containing proteins alone. It can be noticed, however, that the addition of alginate or PS to the irradiated protein solutions led to the formation of the gels characterized by significantly smaller strength ($p \leq 0.05$) than that determined for the gels prepared without polysaccharide addition (**Tables 1** and **2**). The most probable reason for that result is the incompatibility between two gel fractions formed with calcium ions participation: that containing strongly cross-linked proteins and that containing PS or alginate. It can be supposed, in fact, that the cross-linked protein network revealed a diminished accessibility for long polysaccharide chains, in particular alginate reinforced with calcium (egg-box structure) (*19*). Considering the possible weakening of the interaction between calcium ions and the functional groups in irradiated proteins chains and the decreased possibility to embed calcium ions in protein networks showing decreased β-structure content, it can be noticed that the hypothesis of conformational changes in proteins after the addition of polysaccharides was not confirmed by FTIR spectroscopy. Actually, a relatively small decrease in the amount of the β-structure that can be concluded on the basis of gel strength data due to PS addition might not be detected by FTIR. However, the essential change in the conformation that might be deduced regarding the addition of the alginate was expected to influence FTIR spectra. It can be deduced, therefore, that another factor influences gel strength in the presence of alginate. It is possible that the functional groups in proteins capable of binding calcium are blocked because of the stiffening of the protein conformation due to entrapment in the surrounding polysaccharide network. A similar consideration can be related to the influence of polysaccharides on the irradiated WPI.

In contrast, the presence of SPS led to the formation of stronger gels ($p \leq 0.05$) than those produced using irradiated proteins alone (**Tables 1** and **2**). That synergistic effect points to the compatibility between the irradiated proteins and relatively short SPS molecules in solutions. It can be supposed, simultaneously, that the SPS rather promotes binding of calcium ions to the irradiated protein chains, likewise in the case of the irradiated CC alone.

The incompatibility coefficients (IC) were compared for the particular protein−polysaccharide compositions (**Table 2**) to analyze the effect of PS, SPS, and alginate on the strength of CC−WPI gels in relation to their effect on CC gels and WPI gels.

The IC value determined for the non-irradiated CC−WPI mixed with PS was intermediate within the values calculated separately for control CC−PS and for the control WPI−PS gels and near their mean. It can be deduced therefore that the influence of the PS on the CC−WPI composition corresponds to the sum of its separate interactions with the CC and with the WPI (with a possible stronger effect with the WPI). The small strength of the CC−WPI gels (**Table 1**) followed by any effect of alginate addition differs from the effects of alginate on pure WPI gels as well as from the appearance of heterogeneous suspensions in the case of CC−alginate composition. It should be considered, however, that only a negligible or no increase in the gel strength attributed to WPI participation can be expected in both cases due to the strong effect of the CC on the properties of CC−WPI gels, resulting in a decreased susceptibility of WPI segments for the calcium binding (*8*). The lack of a significant effect of the SPS addition on the strength of the control CC−WPI gels is similar to the rather small decrease in the strength of CC gels caused by SPS, but differs essentially from the extremely significant SPS effect on the WPI gels. It can be noticed, furthermore, that the strengths of CC−WPI−SPS gels were similar to strengths of WPI−SPS gels.

The effects of the PS or SPS addition on the irradiated CC−WPI correspond to the sum of their effects on CC and WPI. This was shown by the IC values corresponding to the mean values calculated separately for the CC and for the WPI (with a possible shift toward the CC in the case of SPS addition) (**Table 2**). On the contrary, the interaction of alginate with the CC−WPI composition is not equivalent to the participation of the two proteins in the mixed system. In this case the IC value was out of range of the two values calculated separately for the irradiated CC and the irradiated WPI and considerably smaller than their mean. The interaction of alginate with mixed protein gels seems in fact to be similar (but probably stronger) to its interaction rather with the WPI than with the CC. A decrease in the strength of gels prepared using the irradiated CC−WPI

Table 3. Viscosity Values (Centipoise, cP) Determined for the Polysaccharide Gel/Solutions (Suspensions)[a]

		0.9% of polysaccharide			0.25% of polysaccharide + 0.125% of $CaCl_2$		
no.	treatment	PS	SPS	sodium alginate	PS	SPS	sodium alginate
1	control	1.18 ± 0.16[b] aA	1.29 ± 0.03[b] aA	20.60 ± 0.26 cB	1.31 ± 0.04[b] aA	1.26 ± 0.02[b] aA	1136.20 ± 5.97 cC
	autoclaved (40 °C)	15.16 ± 1.02 bC	1.18 ± 0.11 aA	6.16 ± 0.12 aB	1.29 ± 0.04 aA	1.24 ± 0.03 aA	930.19 ± 7.76 aD
2	autoclaved (25 °C)	16.05 ± 1.03 bC	1.21 ± 0.15 aA	6.75 ± 0.08 bB	1.34± 0.04 aA	1.26 ± 0.02 aA	971.76 ± 3.91 bD

[a] Autoclaving was performed for a 0.9% solution/suspension of polysaccharide, and the autoclaved solution was then cooled to 40 or 25 °C. Measurements were carried out for the liquids containing 0.9 wt % of polysaccharide or after mixing of the autoclaved/control solution with $CaCl_2$ (final concentration of 0.25 wt % of polysaccharide and 0.125 wt % of $CaCl_2$). Means followed by the same lower case letter in each column (representing the liquids with the same formulation but subjected to various treatments) are not significantly different ($p > 0.05$). Means followed by the same capital letter in each row (representing the liquids with different formulations but subjected to the same treatment) are not significantly different ($p > 0.05$). [b] Heterogeneous suspension.

due to the addition of alginate compared with a decrease in the gel strength observed for the irradiated WPI gels and no significant effect noticed in the case of the irradiated CC shows it.

Polysaccharide Effect on Protein Gel Properties in Terms of the Gelation Route. Gels obtained using the irradiated CC−WPI with the addition of polysaccharide revealed strength increasing depending on the addition in the following sequence: alginate < PS < SPS. The same sequence was found for the CC (irradiated)−polysaccharide composition. Differences between the strengths of gels prepared using the WPI (irradiated) with the addition of particular polysaccharides were not significant ($p > 0.05$), although a similar sequence was found for mean values.

It is known that polysaccharides containing carboxyl groups and the linear polysaccharides (such as alginate) are less compatible toward proteins than the neutral species and the branched polysaccharides (starch) (*17*) and thus form softer gels. The reinforced gels result due to the formation of joint phases or in such cases when one of these components acts as a filler in the gel network of the second component. The melted starch granules are incorporated thus into a network of the protein gels (*17*, *18*, *20*−*22*). The continuous gel phase is also formed as a result of the macromolecules leaching from the granules. On the contrary, alginate easily forms a separate gel network surrounding the protein network (*23*). Although two separate phases originate in both cases, it can be supposed that the intermediate phase containing both components arises on the border. A high incompatibility of the protein−polysaccharide pair might be thus attributed to the distinct phase separation, when the blurred phase border might result in the case of the intermediate incompatibility.

The compatibility between protein and polysaccharide gels is known to depend on the rate of gelation of each component and the strength of their gels (*18*, *24*). Muhrbeck and Eliasson (*18*) have proved that the strength of starch−protein gels could be calculated as a sum of contribution of two components when the speeds of gelation were similar. Gel strengths were, however, considerably diminished in relation to the value calculated using such an "additivity model" when the protein gelation was very slow in comparison to polysaccharide gelation and/or takes place when the polysaccharide gel had been already hardened. The authors attributed this observation to an inhibited penetration of proteins into the strong polysaccharide gels. This is because the formation of the continuous protein network is accompanied by the partial tangling of the unfolded protein molecules with the formerly existing polysaccharide aggregates.

A relatively small content of polysaccharides (polysaccharide/protein ratio was equal to 1:20) was applied in the present experiments; thus, their small contribution on gel properties might be expected. Large differences observed in the majority of cases between the protein gels and the protein−polysaccharide gels result thus rather due to the specific interaction between both components. Because the pregelatinized polysaccharides were applied, the polysaccharide gel network exists at the beginning of the protein gelation. The alginate forms strong gels, in particular in a calcium salt environment (*17*, *19*, *23*), whereas the PS gels are weak (although rather strong in the absence of calcium) (compare **Table 3**). It can be expected, therefore, that the incorporation of proteins into a starch gel network occurred more easily than that into an alginate gel network (besides the first step of the process until Ca^{2+} has penetrated the gel). No obstruction might be expected for the SPS to participate in the formation of the protein gel in each stage of the process (confirmed by the low viscosity of gels in the presence as well as in the absence of $CaCl_2$; **Table 3**). Therefore, the ability of tangling with proteins increases in the following sequence: alginate < PS < SPS. Consequently, it can be expected that the strength of gels will increase in the same sequence. In fact, such a sequence corresponds to that found in the present experiments in the case of the irradiated proteins. Furthermore, differences between the control CC−polysaccharide gels can also be explained on the basis of the above model.

The control CC forms weak gels with $CaCl_2$ (**Table 1**). Therefore, the slow tangling of the control CC with the existing polysaccharide gel network might be expected. Such a limited penetration into the starch gel network led to the formation of weak gels, characterized by an even lower strength than that of gels formed using control CC alone (**Table 1**). Moreover, the tangling of proteins with the stronger alginate gel network is excluded, as shown by the salting-out effect (**Table 1**). The control WPI effectively forms cross-links during heating [the increase in the apparent molecular mass from 40 to about 2000 kDa was found after heating at the same conditions (*5*)], and similarly strong gels were obtained as a result of fast protein gelation taking place in the presence as well as in the absence of a rather small addition of the PS or alginate. Due to the contribution of both components in the non-irradiated CC−WPI gels their properties are slightly influenced by the PS presence, but no significant effect was noticed according to the alginate or SPS interaction with proteins (**Table 1**).

The formation of protein gels during further heating occurs more effectively when the radiation cross-linking was formerly conducted (*8*). Therefore, a more effective tangling of proteins with alginate, PS, and SPS might be expected. Accordingly, the strength of CC gels obtained after polysaccharide addition is similar to or greater than that of the pure CC gels and increases in the predicted sequence (alginate < PS < SPS). It is not clear on the basis of the above model why gels formed by the irradiated WPI after the addition of polysaccharides are weaker than the gels prepared using the WPI alone. The same concerns gels formed using an irradiated CC−WPI composition

Table 4. Mechanical Properties of the Films Prepared Using the Particular Compositions[a]

no.	sample	dose (kGy)	puncture strength (N mm^{-1})	deformation (mm)	viscoelasticity coefficient
	Films Formed Using Proteins Alone or When Polysaccharides Were Introduced to Protein Solution after Irradiation				
1	CC–WPI	0	53.9 ± 2.6 b	4.5 ± 0.3f	0.52 ± 0.01 a
2	CC–WPI	32	77.4 ± 3.2 f	4.1 ± 0.3 cd	0.56 ± 0.01 cd
3	CC–WPI–PS	0	52.3 ± 2.6 b	4.0 ± 0.3 c	0.56 ± 0.01 bc
4	CC–WPI–PS	32	72.0 ± 5.4 e	3.2 ± 0.3 a	nd[b]
5	CC–WPI–SPS	0	41.0 ± 3.4 a	4.4 ± 0.3 ef	0.52 ± 0.01a
6	CC–WPI–SPS	32	52.2 ± 1.9 b	3.7 ± 0.3 b	0.57 ± 0.01 d
7	CC–WPI–alginate	0	60.1 ± 4.1 c	4.0 ± 0.3 cd	0.55 ± 0.01 bc
8	CC–WPI–alginate	32	89.2 ± 4.2 g	3.4 ± 0.2 a	nd
	Films Formed When Alginate Was Introduced to the Protein Solution Prior to Irradiation				
8	CC–WPI–alginate	32	88.9 ± 5.8 g	3.7 ± 0.3 b	nd

[a] Means followed by different letters in each column are significantly different ($p \leq 0.05$). Twenty repetitons were done for puncture strength and deformation tests and 10 repetitions for viscoelasticity tests [b] Not determined according to the performed procedure because the samples were broken in the majority of cases.

with the alginate or PS addition, especially because irradiation is known to induce a more effective cross-linking in the CC−WPI composition than separately in the CC or WPI (*5*, *8*, *25*). In particular, irradiation with the same dose of 32 kGy was found to induce an increase in the apparent molecular mass of CC from about 200 to about 2000 kDa and to affect slightly that of the WPI, whereas a large fraction with a molecular mass at about 10000 kDa appears in the case of the CC−WPI (*5*). It can be noticed, however, that the irradiated WPI and irradiated CC−WPI gels (polysaccharide free) are clearly stronger than the gels prepared using all of the other compositions (**Table 1**). Therefore, the fast hardening of these gels might appear as an obstruction for tangling with polysaccharides, in contrast to the other compositions. It can be supposed, moreover, on the basis of the similarities between the PS and alginate effect on the irradiated CC−WPI and on the irradiated WPI (**Table 2**, discussion in the preceding section) that the inhibition of tangling with polysaccharides is related to the WPI segments rather than the CC segments. On the other hand, the weakening of the irradiated CC−WPI or WPI gels corresponding to the addition of polysaccharides might be connected to the reduced calcium binding in the protein fraction, stiffened according to the entrapment in the polysaccharide network (shown by TEM). It can be supposed, furthermore, that high preservation against the loss of regularity accompanied calcium binding (*12*, *13*), and the stabilization of the well-ordered proteins β-structure corresponds probably to the highly reduced protein−polysaccharide penetration.

Barrier and Mechanical Properties of CC−WPI Films Prepared after the Addition of Polysaccharides to the Final Solutions. CC−WPI films produced using the irradiated solutions were characterized by a significantly higher strength ($p \leq 0.05$) and an increased rigidity [shown by smaller values of deformation at puncture and higher values of the viscoelasticity coefficient ($p \leq 0.05$)] as compared to the non-irradiated films (**Table 4**). These stronger films had better barrier properties than the non-irradiated ones [shown by their lower WVP values ($p \leq 0.05$) (**Figure 1**)].

Likewise in the case of CC−WPI composition alone, the films prepared using the irradiated solutions were stronger and more rigid also in all of the cases when polysaccharides (PS, SPS, or alginate) were introduced into the final solutions than those

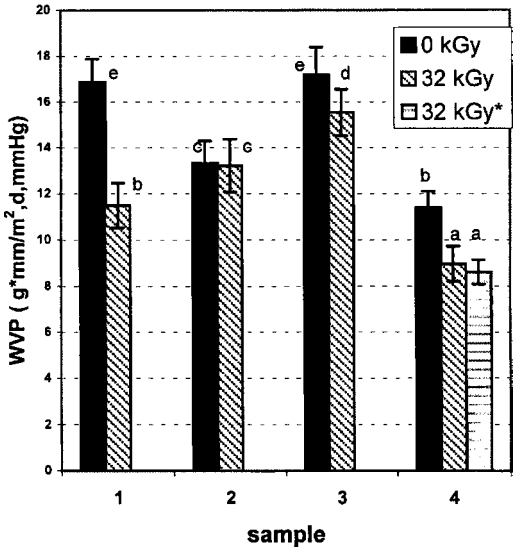

Figure 1. WVP of the films prepared using particular compositions, non-irradiated and irradiated: 1, CC–WPI; 2, CC–WPI–PS; 3, CC–WPI–SPS; 4, CC–WPI–Al. * The solution was irradiated after the addition of sodium alginate. Values assigned different letters are significantly different ($p \leq 0.05$). Twenty repetitions were done for each composition.

prepared using the non-irradiated solutions with the same formulations (**Table 4**). Irradiated films revealed 1.3−1.5 higher strengths than the non-irradiated ones. Barrier properties were better in the case of the irradiated films than in the case of the control ones after the addition of SPS or alginate (**Figure 1**). WVP values of the irradiated films reached 0.8 and 0.9 of those determined for the non-irradiated ones, respectively, in the cases of the CC−WPI−alginate and the CC-WPI−SPS.

Addition of PS to the non-irradiated solution did not influence puncture strength of the resulting films but caused a meaningful improvement of the barrier properties as compared to the films containing only proteins (and significant, $p \leq 0.05$), shown by a lower value of WVP (**Figure 1**). In contrast, addition of PS to the irradiated solution led to the formation of weaker films than those produced using the irradiated protein solution alone and characterized by the higher WVP value ($p \leq 0.05$). This happens despite the puncture strength of the films prepared after the addition of starch to the irradiated solution being significantly higher ($p \leq 0.05$) than that of the films prepared using the non-irradiated solutions with the same composition. The increase in puncture strength was slightly lower after irradiation in the case of the CC−WPI−PS films than in the case of the CC−WPI films. The barrier properties of the films prepared with PS addition were similar, independent of whether the initial solutions were irradiated or not (shown by the similar WVP values, revealing no significant differences $p \leq 0.05$; **Figure 1**).

A significant difference was not detected ($p > 0.05$) between WVP of the films prepared using the non-irradiated CC−WPI solution with the addition of SPS and that of the films prepared using the protein solutions alone (**Figure 1**), although a smaller puncture strength was determined in the case of the films containing SPS (**Table 4**). Addition of SPS to the irradiated protein solution results in the formation of the films characterized by the smaller puncture strength and higher WVP than those of the films produced using proteins solution alone ($p \leq 0.05$; **Table 4**; **Figure 1**). This happens because the radiation-induced increase of the puncture strength and decrease in the WVP value were rather small (although significant, $p \leq 0.05$) in comparison

to that observed in the cases of all the other compositions (**Table 4**; **Figure 1**).

Addition of the alginate to the non-irradiated as well as the irradiated solutions enabled the production of films characterized by better barrier properties and higher puncture strength ($p \leq 0.05$) as compared to films prepared using strictly the same procedure but containing proteins alone or with the addition of both starch polysaccharides. The WVP of the non-irradiated CC−WPI−alginate films was not only essentially lower than the value determined for the non-irradiated CC−WPI films but also comparable ($p > 0.05$) to that of CC−WPI films improved by irradiation (32 kGy dose). In regard to the effect of radiation combined with the proteins' interaction with the alginate, the films obtained by irradiation of protein solution followed by the addition of alginate were characterized by the smallest permeability and the largest resistance to tensile among all of the examined films (**Table 4**; **Figure 1**).

Independent of the admixed polysaccharide, all of the films containing them were more rigid as compared to the respective films prepared using the CC−WPI composition, despite the fact that each polysaccharide affects their barrier properties and puncture strengths differently. It was shown by the significantly smaller values of deformation on puncture and higher viscoelasticity coefficients found for these films as compared to the pure protein films ($p \leq 0.05$; **Table 4**). The only exception constituted the films prepared using the non-irradiated protein solution and SPS addition, showing the same values of both parameters as the non-irradiated films containing proteins alone ($p > 0.05$) and thus the same rigidity. Moreover, the films prepared using the irradiated as well as the non-irradiated proteins have revealed similar rigidity independent of whether PS or alginate was added to their compositions [shown by the lack of significant differences between the viscoelastic parameters ($p > 0.05$)]. Therefore, a deformation of puncture of the films prepared with the addition of PS or alginate was as high as about 0.9 in the cases of the non-irradiated films and as high as 0.8 in the cases of the irradiated films of the values found for the respective pure protein films. Viscoelasticicty coefficients of the non-rradiated films were about 1.07 times higher prior to admixing either the PS or the alginate, whereas these coefficients were not determined for the appropriate irradiated films in regard to their high rigidity (**Table 4**).

Statistical analysis results (**Table 4**, both Duncan and Student *t* test) confirmed that a significant increase ($p \leq 0.05$) in puncture strength of all the films prepared using the protein or protein−polysaccharide composition occurred prior to irradiation. A significant decrease in deformation at puncture and a significant increase in viscoelasticity coefficients (or destruction of the most rigid films during tests) indicate the increase in the films' rigidity connected to irradiation. Significant differences ($p \leq 0.05$) found between the WVP values determined for the irradiated formulations and those of the non-irradiated ones (excluding the CC−WPI−PS composition) ($p > 0.05$) have confirmed an improvement of the barrier properties of the irradiated films (**Figure 1**). The significance or lack of differences (discussed above) between all of the parameters of the films prepared using the compositions containing the CC−WPI (non-irradiated and irradiated) and particular polysaccharides, as compared to the appropriate pure protein system, was also confirmed by statistical analysis ($p \leq 0.05$ or $p > 0.05$; **Table 4**; **Figure 1**).

Transmission Electron Microscopy. Two phases were discovered in the films produced using CC−WPI−alginate composition, both irradiated and non-irradiated (**Figure 2**). The

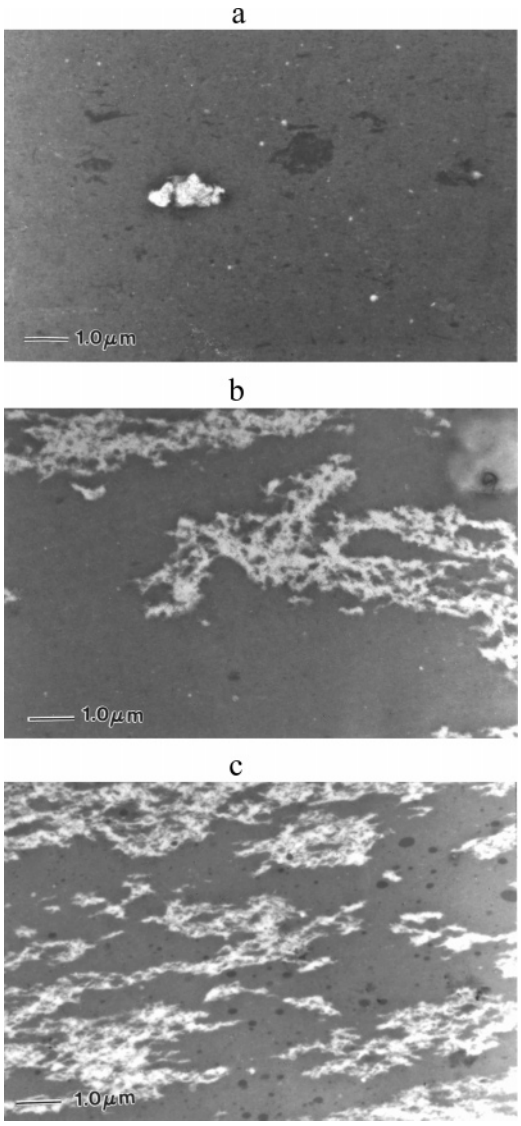

Figure 2. TEM photos of the films prepared using the following compositions: (**a**) non-irradiated CC–WPI; (**b**) CC–WPI (non-irradiated)–alginate; (**c**) CC–WPI (irradiated)–alginate. Alginate was introduced after irradiation. The pictures were made for the film placed in the holder in such a way that the film surface was parallel to the top of the photo.

alginate inclusions (white) are incorporated into the protein matrix and form a stiff skeleton. Comparison of the microstructure of films with their tensile strength and WVP shows that the presence of that strongly bonded chain material is responsible for the increase in the puncture strength of the films produced after the addition of alginate and their better barrier properties as compared to the films produced using proteins alone (**Figure 2a**,**b**; **Table 4**).

TEM images show more homogeneous incorporation of the alginate chains and their better orientation in the films obtained after irradiation of protein solutions as compared to the non-irradiated films (**Figure 2b**,**c**). The essential improvement of barrier properties and the mechanical strength of the films are probably caused as well by the formation after irradiation of the denser protein network alone (*8*) as by the more homogeneous distribution of the alginate chains in the bulk film material.

It is worth pointing out that the homogeneous distribution and a high orientation of the alginate inclusion observed in the irradiated films can be achieved in such a case when the appropriate incorporation of the alginate has occurred in the

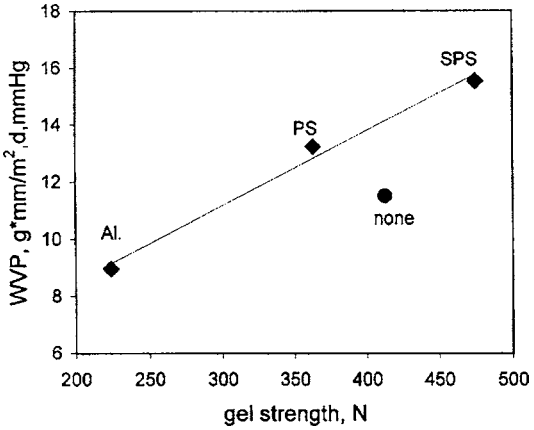

Figure 3. Dependence of the mean values of WVP obtained for the films containing polysaccharides on the mean strength of gels formed after the addition of $CaCl_2$. The point is also shown representing pure CC–WPI composition.

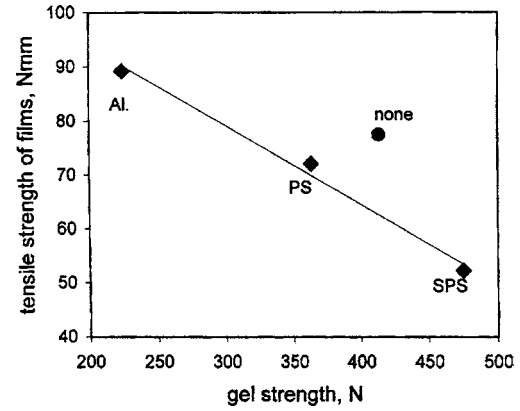

Figure 4. Dependence of the mean values of puncture strength obtained for the films containing polysaccharides on the mean strength of gels formed after the addition of $CaCl_2$. The point is also shown representing pure CC–WPI composition.

two-phase gel formed as the intermediate product (the mechanism of gel formation in the protein−polysaccharide system was discussed in the foregoing section). A homogeneous incorporation of alginate chains probably causes stiffening and thus stabilization of the well-ordered protein β-structure (as was postulated in the previous sections) and consequently the formation of well-structured films (*8*).

Relationship between Properties of the Films and Properties of the Gels Prepared after the Addition of Polysaccharide to the Irradiated CC−WPI Solutions. Films prepared after the addition of the presently used polysaccharides were stronger and had better barrier properties in the cases of the compositions, enabling softer gels to be obtained with Ca^{2+}. Moreover, a linear dependence was found between the average values of gel strength and the average values of puncture strength as well as the average values of gels strength and the WVP (**Figures 3** and **4**). The relationship of this dependence to the consideration concerning gel strength data might lead to a conclusion that the formation of the films characterized by small mechanical strength and high WVP occurs in these cases when the irradiated proteins were compatible toward the polysaccharide in solution. In contrast, high incompatibility results in strong and dense films constituting a good barrier for water vapor. This occurs probably because in the last case protein gels might be created on the formerly existing stiff polysaccharide skeleton.

The smaller effect of the SPS than the PS on the rigidity of the irradiated films (**Table 4**) corresponds to the high compatibility of proteins with SPS and to their moderate incompatibility with the PS. The highest incompatibility of the alginate and the irradiated proteins results, however, in the films characterized by rigidity not being significantly different ($p \leq 0.05$) from that of the films containing PS. These results are probably connected to the different structures of protein−starch and protein−alginate gels (discussed above).

Properties of Gels and Films Produced Using the Irradiated CC−WPI−Alginate Solutions. Addition of sodium alginate to the CC−WPI solutions and further irradiation led to the formation of stronger gels as compared to the gels obtained in the cases when sodium alginate was introduced after irradiation. (**Table 2** presents ratios of the mean values of gel strength obtained in these two cases for particular protein compositions.) A strong irradiation effect was noticed for the CC−WPI−alginate composition shown by a 6.1-fold increase in the gel strength as compared to a 4.7-fold increase obtained in the case when alginate was introduced to the protein solution after irradiation. This can be related to an essential radiation modification of the CC−alginate composition leading from the suspension to strong gels. On the contrary, a decrease in the gel strength was noticed in the case of the WPI−alginate composition, comparable to that observed in the case when the alginate was introduced to the protein solution after irradiation (the strength of the irradiated gels reached in both cases 0.8 that of the control gels).

The slightly higher value of the gel strength than in the case of pure proteins points to compatibility of the alginate and the CC−WPI composition after the simultaneous irradiation (**Table 1**), in contrast to the incompatibility found in the case when the alginate was introduced to the protein solution after irradiation. This result can be related to the high compatibility between the alginate and the CC after simultaneous irradiation, whereas a moderate incompatibility was found between the alginate and the WPI under the same treatment (similarly as in the case when the alginate was admixed to the final solutions) (**Tables 1** and **2**). The above data suggest that during the simultaneous irradiation the alginate is attached to the protein chains (despite the effect of the solution aging discussed in the next paragraph). This could be explained by the formation of charge−charge electrostatic complexes similar to those formed by proteins with CMC (*7*). It seems, moreover, that such an interaction with the CC chains is probably more effective than the interaction with the WPI chains (**Table 2**). This statement can be attributed to the greater ability of the CC for the creation of cross-links after irradiation, as compared to the WPI (*25*).

The non-irradiated CC−WPI−alginate gels have revealed a greater strength when the initial solutions were prepared simultaneously with the solutions irradiated after the addition of the alginate as compared to the solutions made directly before heating (**Table 1**, points 4 and 5). This result can be attributed to the aging of the alginate gel during further storage and possible binding with proteins. No particular storage effect was noticed in the cases of the control WPI−alginate and CC−alginate.

The interaction of the alginate with a mixed protein composition in the solution does not correspond after simultaneous irradiation to the sum of the contributions of its interaction separately with the CC and with the WPI, similar to the case when alginate was introduced after irradiation. The ratio of the experimental IC value to the calculated one (considering

contribution of the CC and the WPI) was equal to about 0.6 in both cases of gels formed with $CaCl_2$ (**Table 2**).

Statistical analysis of data (**Table 1**) proved that the strengths of gels of the irradiated WPI−alginate and CC−WPI−alginate differ significantly from the non-irradiated ones ($p \leq 0.05$; both Duncan and Student *t* test), whereas the basic quality difference is observed between the homogeneous irradiated CC−alginate gels and nonhomegeneous non-irradiated CC−alginate sample. All of the irradiated as well as non-irradiated gels containing CC, WPI, and CC−WPI differ also significantly ($p \leq 0.05$) from the composition containing the appropriate proteins alone (with the exception of the nonhomogeneous CC−alginate and homogeneous pure CC systems). Moreover, significant differences ($p \leq 0.05$) were detected between all of the compositions irradiated together with the alginate and the appropriate compositions containing alginate admixed after irradiation. Furthermore, the significance of the differences ($p \leq 0.05$) was confirmed between the reference gels containing the non-irradiated CC−WPI−alginate composition prepared in these two different experimental series as well as between the two non-irradiated gel series containing the WPI and the alginate.

A significant difference was not found between both puncture strength and the WVP of the films containing alginate [$p > 0.05$; both Duncan and Student *t* test)] independent of whether the alginate was introduced to the protein solution before or after irradiation (**Table 4**). Significantly ($p \leq 0.05$) more elastic films were, however, obtained in the case when irradiation was carried out for the CC−WPI−alginate solution in relation to the films obtained in the case when the alginate was introduced to the irradiated protein solution. The higher elasticity of these films corresponds probably to the more elastic connections between the stiff polysaccharide skeleton and entrapped proteins network within the intermediate fraction containing simultaneously proteins and the alginate and resulting accordingly in the cross-linking between protein and alginate chains. The radiation-induced degradation of the alginate (*26*, *27*) probably promotes the formation of such connections and consequently increases the films' elasticity, similarly to using low molecular weight starch (SPS) instead of the high molecular weight one (PS) (**Table 4**). These radiation-induced processes probably altogether improve the homogeneity of the alginate distribution in the protein fraction.

Concluding Remarks. The effect of polysaccharide addition on the strength of gels formed in a calcium salt environment using the irradiated (32 kGy dose) and the non-irradiated CC−WPI composition can be expressed as the sum of their effects on the CC gels and on the WPI gels, with the exception of the alginate effect on the irradiated CC−WPI gels. The influence of the polysaccharides on the strength of the CC, WPI, and CC−WPI gels can be explained in terms of the slight modification of protein structure in the presence of polysaccharide and in terms of the compatibility between the protein and polysaccharide gel networks. The presence of the alginate stabilizes probably the β-structure in the irradiated CC−WPI because of the entrapment of cross-linked proteins in the stiff alginate gel network.

The addition of the polysaccharide to the irradiated as well as to the non-irradiated protein solutions induces a modification of the final film properties. The formation of stronger films with the better barrier properties obtained due to the addition of polysaccharide to the irradiated CC−WPI solutions corresponds to the smaller compatibility of the irradiated proteins with polysaccharide in the Ca^{2+} presence, whereas less strong films with deteriorated barrier properties are formed in the case of high compatibility. An improvement of the film properties resulting due to alginate addition is connected to the presence of strongly bonded chain material observed by TEM.

It can be concluded that irradiation of the CC−WPI−sodium alginate composition led to the formation of the cross-linked protein−polysaccharide network. It was proved by the compatibility between alginate and the proteins detected in such a case, in contrast to the incompatibility found in the case when the alginate was introduced to the protein solution after irradiation. The high efficiency of cross-linking with the alginate concerns CC rather than WPI chains.

ABBREVIATIONS USED

WVP, water vapor permeability; WPI, whey protein isolate; CC, calcium caseinate; PS, potato starch; SPS, soluble potato starch; alginate, sodium alginate.

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