Irradiation Dose and Calcium Effect on the Mechanical Properties of Cross-Linked Caseinate Films

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The effect of irradiation dose on the formation of cross-links in calcium caseinate solutions was investigated. The formation of bityrosine was found to increase with the irradiation dose for the basic formulation containing calcium caseinate, carboxymethylcellulose, and glycerol. Addition of $CaCl_2$ to the basic formulation increased the fluorescence signal of bityrosine for all irradiation doses suggesting that $CaCl_2$ can have an indirect effect on the formation of bityrosine by shortening molecular distances between polypeptides, thus making easier the formation of bityrosine. Furthermore, gels were formed for solutions containing $CaCl_2$ at irradiation doses $\geq$16 kGy. The formation of gels was found to be accountable for the increased mechanical strength in the corresponding films. A maximum gel strength was obtained at an irradiation dose of 64 kGy for all formulations, suggesting a maximum cross-linking density. Such a feature was also noted when poly(ethylene glycol), mannitol, and sorbitol were added to the formulations. Although bityrosine cross-links increase with irradiation dose, some damage can occur in another part of the protein structure, accounting for the overall loss of puncture strength at doses >64 kGy.

Keywords: *Gamma-irradiation; edible films; caseinate; milk protein; cross-linking*

INTRODUCTION

The need for improving the shelf life and quality of foods, combined with the urgency to reduce waste generated by synthetic nonbiodegradable packaging, has resulted in an increasing interest for biodegradable or edible materials (Chen, 1995). Natural polymers or polymers derived from natural monomers, like food proteins, offer the greatest opportunities since their biodegradability and environmental compatibility are assured (Krochta and De Mulder-Johnston, 1997).

Many food proteins such as corn zein, wheat gluten, soy protein isolate, whey protein isolate, and caseins have been formulated into edible films or coatings. Unfortunately, the highly hydrophilic nature of these proteins limits their ability to provide desired edible film functions. However, different options have been investigated in order to improve the barrier properties of these films. Addition of fatty acids or beeswax to the formulations have been reported (Gontard et al., 1994). Another approach developed for improving the mechanical properties of these materials is protein cross-linking. Transglutaminase has been widely used for cross-linking many food proteins (Chobert et al., 1996; Yildirim and Hettiarachchy, 1997) including caseins (Ikura et al., 1980; Motoki et al., 1987). Glutaraldehyde has also been used for cross-linking casein, creating a matrix for controlled drug release applications (Latha and Jayakrishnan, 1994; Lenaerts et al., 1991). Unfortunately, the use of enzymes or chemicals is generally costly, which limits their application on a larger scale.

Other techniques have been developed in order to improve the mechanical properties of these films at lower costs. Banerjee et al. (1996) have reported a work in which they have demonstrated that the resistance to puncture of whey protein concentrate and sodium caseinate films could be improved by ultrasound treatment. More recently, Brault et al. (1997) have used γ-irradiation to produce free-standing sterilized cross-linked caseinate films. Their work showed that these films were more resistant to puncture and moisture. It should be noted that their work was limited to irradiation doses $\leq$20 kGy.

γ-Irradiation is slowly becoming accepted in the food industry as a means of improving the shelf life of various fruits and vegetables and eliminating bacterial contamination in meats (Pszczola, 1997). Furthermore, γ-irradiation generates sterile biomaterials which could be used in pharmaceutical or biomedical applications. In a recent work, Mezgheni et al. (1998) have demonstrated that the biodegradability of calcium caseinate films can be controlled by adjusting the irradiation dose, i.e., the number of cross-links. When two different types of films were compared, the degradation of the film containing the highest number of cross-links was delayed 8 days beyond that of the film containing the lowest number of cross-links. This feature is of particular interest since one of the goals concerning the development of biodegradable or edible films is achieving controlled lifetime.

Based on the previous results of Brault et al. (1997), our assumption was that higher irradiation doses in combination with calcium ions could further increase the mechanical strength of the protein films by forming a dense network. Moreover, the addition of platicizers was assumed to enhance the mechanical properties of these films. This paper reports the effect of γ-irradiation and calcium ions on the mechanical properties of protein films based with calcium caseinate. Irradiated solutions were monitored by fluorescence in order to

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ensure that cross-links were generated. Such a step was necessary before conclusions could be drawn from the mechanical test results.

MATERIALS AND METHODS

Reagents. Calcium caseinate (Alanate 380, 91.8% (w/w) protein) was provided by New Zealand Milk Product Inc. (Santa Rosa, CA). Poly(ethylene glycol) (PEG, MW = 8000) and carboxymethylcellulose sodium salt (CMC, low viscosity) were obtained from Sigma Chemicals (St.Louis, MO). Glycerol (99.5%, reagent grade), D-sorbitol pure, and D-mannitol (USP) were purchased from American Chemicals Ltd. Calcium chloride ($CaCl_2$, laboratory reagent) was obtained from BDH Chemicals (Montreal, Quebec, Canada). All products were used as received without further purification.

Film Formation Method. All formulations were based with 5% (w/w) calcium caseinate, 2.5% (w/w) glycerol, and 0.25% (w/w) CMC. They were solubilized in distilled water, under stirring. $CaCl_2$ (0.125% (w/w)) and desired amounts of PEG (0.5% (w/w)), sorbitol, or mannitol (2.5% (w/w)) were added, and the solutions were degassed under vacuum to remove dissolved air. Irradiation of the solutions at doses of 0, 8,16, 32, 64, 96, and 128 kGy was done according to the method previously described by Brault et al. (1997), in a Gammacell 220 irradiator (MDS-Nordion, Kanata, ON, Canada) at the Canadian Irradiation Center (Laval, PQ, Canada). Films were then cast by pipetting 5 mL of the solution onto smooth rimmed 8.5 cm internal diameter Petri dishes sitting on a leveled surface. Solutions were spread evenly and allowed to dry overnight at room temperature (20 ± 2 °C). Dried films were peeled intact from the casting surface.

Film Thickness Measurements. Film thickness was measured using a Digimatic Indicator (Mitutoyo Ltd., Tokyo, Japan) at five random positions around the film. Depending on the formulation and irradiation dose, the average film thickness was in the range of (27−64) ± 2 μm.

Mechanical Properties of Films. Puncture tests were carried out using a Stevens LFRA Texture Analyzer Model TA/1000 (Scarsdale, NY), as described previously by Gontard et al. (1992). Films were equilibrated for 48 h in a desiccator containing a saturated NaBr solution ensuring a 56% relative humidity atmosphere. A cylindrical probe (2 mm diameter) was moved perpendicularly at the film surface at a constant speed (1 mm/s) until it passed through the film. Strength and deformation values at the puncture point were used to determine hardness and the deformation capacity of the film. To avoid any thickness variation, the puncture strength values were divided by the thickness of the film.

Gel Formation and Gel Fracture Strength Measurements. Gels were formed following a modified procedure of Sakamoto et al. (1994). For gel fracture strength analysis, 1.25 mL of the protein solution was poured into a well with care to avoid entrapment of air bubbles. Each well was 7 mm in diameter and 45 mm in height. Wells were sealed and placed into a water bath for 4 h at 90 °C. The solution was then allowed to cool at room temperature and stored overnight at 4 °C. 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. The force−deformation curves were recorded.

Fluorescence Measurements. The formation of bityrosine was measured using a Spectrophotometer 2070 (Varian, Sunnyvale, CA) according to the method previously reported by Davies (1987).

Statistical Analysis. Analysis of variance and Duncan multiple-range tests with $p \leq 0.05$ were employed to analyze all results statistically. For each measurement, three replicates of five films were tested. The Student *t*-test was utilized at the time of the analysis of the variance and paired-comparison with $p \leq 0.05$ (Snedecor and Cochran, 1978).

RESULTS AND DISCUSSION

Preliminary Results. Preliminary tests were conducted in order to determine the concentration of the different components in the formulations. On the basis of the previous work of Brault et al. (1997), a concentration in calcium caseinate of 5% (w/w) and 2.5% (w/w) glycerol was chosen. Three concentrations of CMC were tested (0.25, 0.5, and 0.75% (w/w)). Films containing 0.5 and 0.75% (w/w) CMC were clearly heterogeneous, so a concentration in CMC of 0.25% (w/w) was chosen. The addition of CMC in the formulations improved the films by making them less sticky and more resistant to handling. Films containing a sorbitol concentration of 5% (w/w) were too sticky and easily tore apart. For mannitol, a similar concentration yielded highly rigid films with white residues. At 2.5% (w/w) for both plasticizers (sorbitol and mannitol), films were homogeneous, clear with good mechanical properties. For PEG, a smaller concentration (0.5% (w/w)) was added since films cast from solutions containing ≥1% (w/w) PEG were clearly heterogeneous. A concentration in $CaCl_2$ of 0.125% (w/w) was chosen in order to study the bonding effect of the salt.

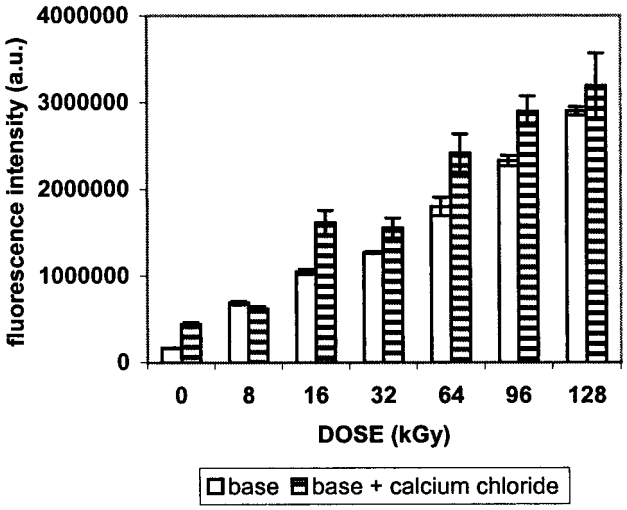

Figure 1. Effect of $CaCl_2$ and irradiation dose on bityrosine fluorescence (arbitrary units).

Formation of Bityrosine. As expected, irradiation of the protein solutions resulted in the formation of bityrosine, as suggested by the fluorescence analysis (Figure 1). Bityrosine is a covalently bound biphenol, produced by reaction of two tyrosyl radicals or a tyrosyl radical plus a tyrosine molecule (Prütz et al., 1983). The mechanisms and the experimental conditions leading to the formation of bityrosine have been extensively studied (Davies, 1987; Davies et al., 1987). It can be seen in Figure 1 that the fluorescence signal of bityrosine increased with the irradiation dose, resulting in a higher number of cross-links between tyrosine units. Brault et al. (1997) have previously reported a work in which they have shown that free-standing cross-linked caseinate films were generated using γ-irradiation. Furthermore, they studied the effect of protein concentration on bityrosine formation and concluded that a higher number of cross-links were produced at an optimal calcium caseinate concentration of 5% (w/w). However, their work was limited to irradiation doses ≤20 kGy. Our results showed that the bityrosine fluorescence continues to increase for higher doses, suggesting, at first glance, an increase in the number of cross-links. For the basic formulation, the bityrosine signal was 20 times more intense at 128 kGy than at 0 kGy.

Table 1. Comparative Data on the Effect of γ-Irradiation on the Puncture Strength, Gel Strength, and Distance to Puncture[a]

	0% $CaCl_2$		0.125% $CaCl_2$		
	puncture strength	distance to puncture	gel strength[b]	puncture strength	distance to puncture
base, %	+(38 ± 3)	+(19 ± 2)	+(76 ± 3)	+(58 ± 5)	no increase
optimal dose, kGy	64	8	64	64	
PEG, %	+(43 ± 4)	+(33 ± 5)	+(85 ± 2)	+(92 ± 5)	+(21 ± 4)
optimal dose, kGy	64	16	64	32	32
mannitol, %	+(17 ± 1)	+(26 ± 4)	+(99 ± 3)	+(36 ± 5)	no increase
optimal dose, kGy	64	16	64	64	
sorbitol, %	+(8 ± 1)	+(16 ± 1)	+(81 ± 2)	+(83 ± 5)	+(13 ± 1)
optimal dose, kGy	64	16	64	16	16

[a] Results at the optimal irradiation doses were compared with the unirradiated solutions. [b] Relative increases in gel strengths were calculated by comparing gel strengths at 64 kGy to gel strengths at 16 kGy, the minimal dose at which gels were formed.

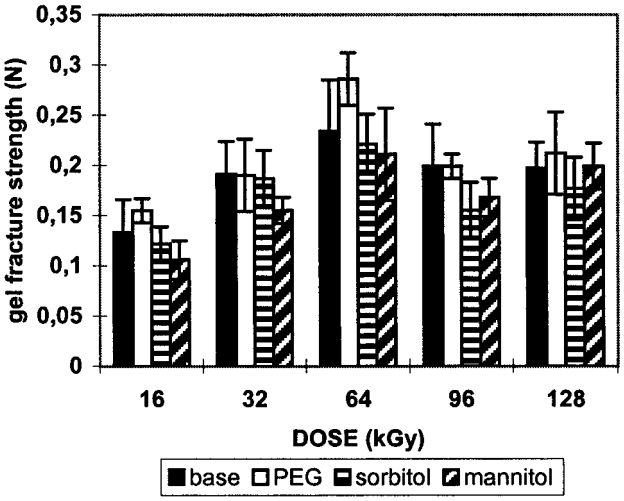

Figure 2. Plasticizer and irradiation dose effect on gel fracture strength for formulations containing $CaCl_2$.

When calcium chloride was added to the basic formulation, a significant increase ($p \leq 0.05$) in the fluorescence signal was noted at 16, 32, 64, and 96 kGy as well as for the unirradiated formulation. At 0 kGy, the fluorescence intensity signal was almost three times more important than for the corresponding solution without $CaCl_2$. At an irradiation dose of 128 kGy, the salt-containing formulation still exhibited a fluorescence intensity 10% more important than the basic formulation. These results generally suggest that the addition of calcium ions enhanced the formation of bityrosine. It is hypothesized that $CaCl_2$ could have an indirect effect by forming salt bridges between adjacent protein molecules or particles (Hongsprabhas and Barbut, 1997), thus reducing the molecular distances, making the formation of bityrosine easier.

Formation of Gels. In the absence of calcium ions, gels were not formed. Homogeneous gels were formed for solutions containing $CaCl_2$ and irradiated at doses ≥16 kGy. Figure 2 shows the plasticizer effect on the variation of gel fracture strength as a function of the irradiation dose for formulations containing $CaCl_2$. The fracture strength of gels with or without plasticizer increased significantly ($p \leq 0.05$) with the irradiation dose followed by a significant decrease ($p \leq 0.05$) at doses ≥96 kGy. It can be seen that a maximum gel strength was obtained for all formulations at an irradiation dose of 64 kGy. In the previous section, bityrosine results showed that a maximum fluorescence intensity was not reached at 128 kGy (Figure 1). Competing interactions at the molecular level could explain why gel strength decreases at higher doses. Although the number of cross-links between tyrosine amino acids increases with the irradiation dose, some degradation can occur in another part of the protein structure, thus resulting in an overall loss of mechanical strength. It has been reported that proteins irradiated at very high doses were more susceptible to molecular damage. γ-irradiation interacts with a protein molecule and may cause a permanent damage in the form of covalent-bond breaks or conformational changes (Garrisson, 1987). On the basis of the results shown in Figure 2, it is assumed that cross-linking mechanisms are predominant for doses ≤ 64 kGy while other bond-breaking mechanisms would prevail at higher doses. In light of these results, bityrosine measurements should be carefully assessed and rationalized since this method fails to detect other mechanisms generated by γ-irradiation.

When formulations containing plasticizers are compared with the basic formulation at different doses, it can be seen that plasticizers had very little effect on gel fracture strength results. Furthermore, irradiation at the optimal dose of 64 kGy increased the gel strength of the basic formulation by 76% when compared to the gel strength of the same formulation at 16 kGy. For formulations containing PEG, mannitol, and sorbitol, the gel strength increased by 85, 99, and 81%, respectively, between 16 and 64 kGy (Table 1). It was assumed that plasticizers would have an effect on the gel fracture strength, either by increasing electrostatic bonds or disrupting the gel network. These results suggest that the plasticizers do not intervene in the gel matrix. Such a feature could be due to a microscopic phase separation. Manoj et al. (1996) recently reported that the maltodextrin−sodium caseinate gel system separated into a macroscopic bilayer at higher temperatures. In was suggested that the structure of the composite gel formed at refrigerator temperature resulted in a continuous network penetrated by discontinuous inclusions. Further investigation, like differential scanning calorimetry (DSC) analysis or other rheological methods, could be carried out in order to determine if the our gels are also characterized by a microscopic phase separation.

Mechanical Properties of Films. To study the effect of $CaCl_2$ on the mechanical properties of the films, the puncture strength of films with or without calcium chloride was determined. Figure 3 shows the variation in puncture strength for films cast from the basic formulation (without $CaCl_2$) in combination with different plasticizers. Similarly to what was previously observed for gel fracture strength analysis, a maximum puncture strength was obtained for all films (except sorbitol) at an irradiation dose of 64 kGy. That optimal irradiation dose has yielded the highest number of cross-links without inducing protein degradation. At that

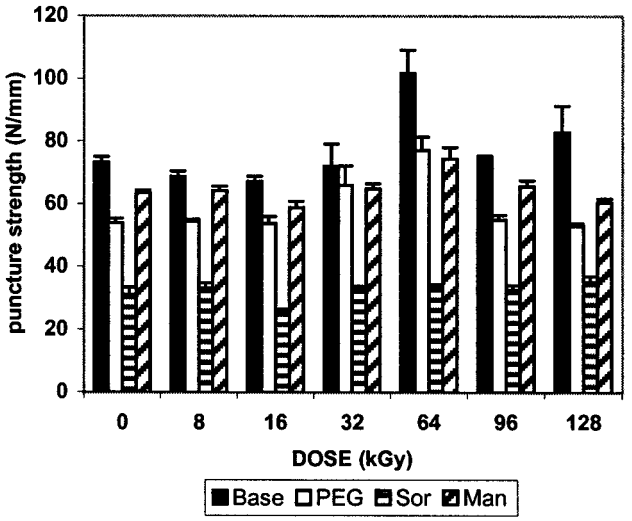

Figure 3. Plasticizer and irradiation dose effect on the puncture strength of films.

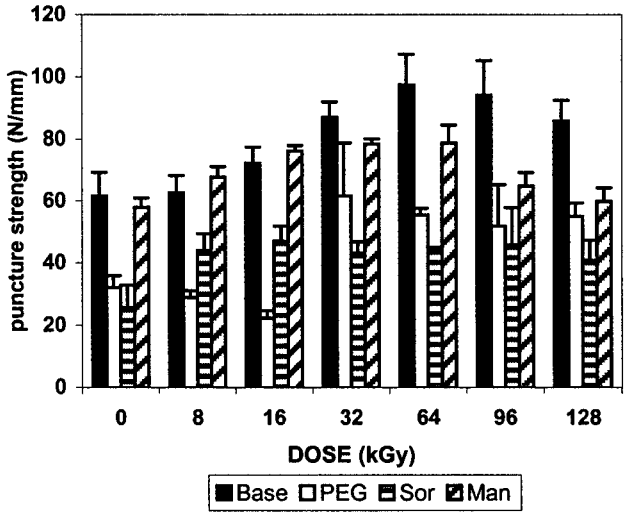

Figure 4. Plasticizer and irradiation dose effect on the puncture strength of films containing $CaCl_2$.

particular dose, the puncture strength of the basic formulation improved by 38% when compared to the unirradiated solution. Similarly, the puncture strength of films containing PEG and mannitol increased by 43 and 17%, respectively (Table 1). For each irradiation dose, results showed that all the plasticizers significantly reduced ($p \leq 0.05$) the puncture strength of the films when compared with the basic formulation (Figure 3). For the unirradiated films, PEG, mannitol, and sorbitol decreased the puncture strength by 26, 13, and 58%, respectively. At an optimal irradiation dose of 64 kGy, films containing sorbitol were three times weaker than films cast from the basic formulation at that same dose. The plasticizing effect of mannitol is quite different from its isomer, sorbitol. This could be explained by the greater solubility of sorbitol which could retain more water in the films.

When calcium chloride is added to the basic formulation (Figure 4) and compared to the previous results (Figure 3), it can be seen that $CaCl_2$ increased the puncture strength for films irradiated at doses ≥16 kGy; i.e., gels were formed. For irradiation doses of 0 and 8 kGy, the puncture strength of the films cast from the salt-containing formulation was significantly lower ($p \leq 0.05$) than that of the corresponding salt-free formulation. However, for irradiation doses ≥16 kGy, i.e., an increasing number of cross-links, a shift in puncture strength is observed as salt-containing films exhibit a puncture strength significantly ($p \leq 0.05$) greater than that of the salt-free formulations. An exception was noted for the 64 kGy dose where comparable puncture strength values were obtained for salt-free and salt-containing formulations alike. When mannitol and sorbitol were added to the formulations, a similar behavior was observed. For instance, at 64 kGy, calcium chloride increased by 32% the puncture strength of the films cast from the formulations containing sorbitol. For mannitol, the addition of calcium chloride was most noticeable at irradiation doses of 16 and 32 kGy where it improved the puncture strength by 31 and 21%, respectively. PEG formulations exhibited a much different puncture strength behavior when $CaCl_2$ was added to the solutions. For doses ≥32 kGy, all films exhibited similar puncture strength values to those of the salt-free solutions except for the 64 kGy dose which had a puncture strength value significantly ($p \leq 0.05$) lower than that of the salt-free solution. Moreover, at low irradiation doses (8 and 16 kGy), PEG formulations exhibited an even lower puncture strength value when combined with $CaCl_2$. Such a feature could be due to the long-chain structure of PEG which could directly inhibit the formation of salt bridges and electrostatic bonds.

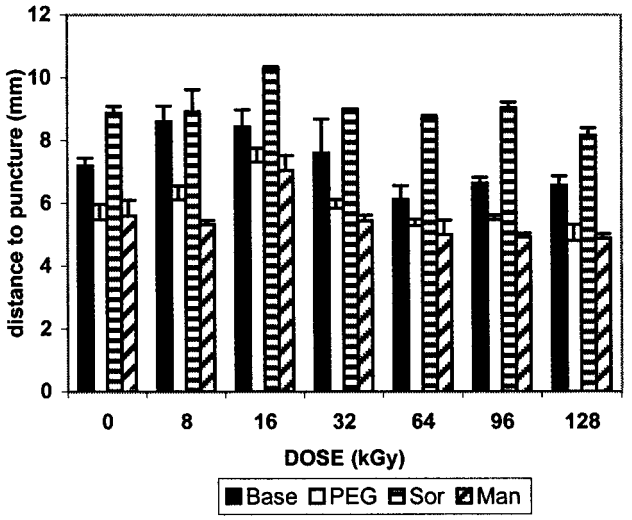

Figure 5. Plasticizer and irradiation dose effect on the distance to puncture.

The premise for adding $CaCl_2$ to the solutions was that it would improve the cohesion of the material due to the formation of electrostatic bonds. Not only was it shown that $CaCl_2$ increased significantly puncture strength for the plasticizer-free formulation at irradiation doses ≥16 kGy but also that the improved mechanical properties were due to the formation of gels. The synergistic effect of $CaCl_2$ and a critical number of cross-links produced by γ-irradiation allowed the protein system to cross the percolation threshold resulting in a fractal like structure that is homogeneous at the macroscopic level and heterogeneous at the microscopic level (de Kruif et al., 1995). It is the formation of that large-scale network that seems most likely accountable for the increased mechanical strength.

Plasticizers also affect the distance to puncture. Figure 5 shows the plasticizer and irradiation dose effect on the distance to puncture for different formulations.

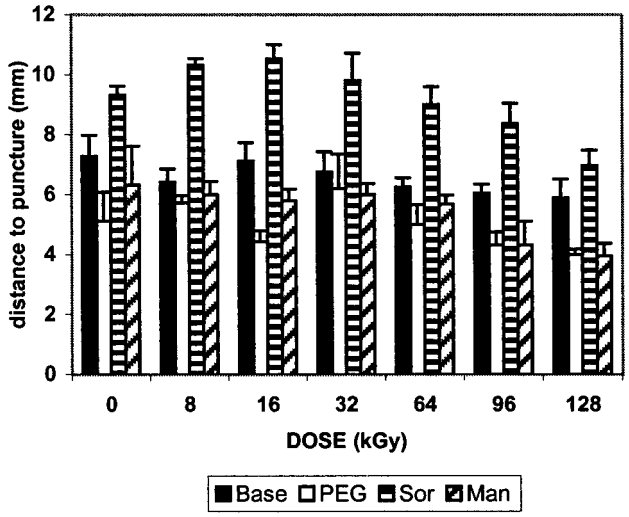

Figure 6. Plasticizer and irradiation dose effect on the distance to puncture for films containing $CaCl_2$.

For the basic formulation, puncture deformation values were significantly higher ($p \leq 0.05$) for irradiation doses of 8, 16, and 32 kGy when compared to the unirradiated films. However, for films irradiated at doses >32 kGy, the distance to puncture values were all significantly lower ($p \leq 0.05$) than that of the 0 kGy formulation. In fact, the 64 kGy formulation had the lowest distance to puncture value of all which could confirm again that the highest number of cross-links (i.e. increased rigidity) was reached at that particular irradiation dose. When plasticizers were added to the formulations, results showed that PEG and mannitol significantly ($p \leq 0.05$) decreased the distance to puncture for all irradiation doses when compared to the basic formulation. Sorbitol, however, had an opposite effect and significantly ($p \leq 0.05$) increased the distance to puncture for all irradiation doses. The maximum distance to puncture values for plasticized fomulations were noted at an irradiation dose of 16 kGy. At that particular dose, PEG and mannitol decreased the distance to puncture by 13 and 16.5%, respectively, while sorbitol increased the distance to puncture by more than 20%. Figure 6 shows the plasticizer and irradiation dose effect on the distance to puncture for formulations containing $CaCl_2$. It can be seen that the distance to puncture values of the sorbitol-containing films still increased with the irradiation dose up to 16 kGy followed by a decrease at higher irradiation doses. The addition of calcium chloride to the sorbitol-containing formulation had little effect on the overall distance to puncture values. For the basic formulation, $CaCl_2$ generally decreased the distance to puncture. However, when PEG, sorbitol, and mannitol were added, that effect was less noticeable.

It was expected that the addition of plasticizers would increase the distance to puncture for all formulations by reducing the internal binding and internal forces thereby increasing molecular spacing. Sorbitol is the only plasticizer that had such an effect on the films. Mannitol, on the other hand, significantly reduced ($p \leq 0.05$) the distance to puncture for all irradiation doses. Previous results also showed that mannitol did not improve the puncture strength of these films. In short, mannitol films can be described as weaker than the basic formulation as shown by the lower puncture strength and smaller distance to puncture values. Sorbitol also affected the films by decreasing the puncture strength but significantly increased ($p \leq 0.05$) the distance to puncture values. Therefore, the loss of film rigidity was compensated for a gain in elasticity. Due to its greater water solubility, sorbitol could retain more water in the films accounting for such differences between the two isomers (sorbitol and mannitol). Finally, addition of PEG was most detrimental to the films. Similarly to mannitol, PEG decreased the distance to puncture and puncture strength values for all irradiation doses. That effect was even more pronounced when $CaCl_2$ was added since, in some cases, the puncture strength of the PEG-containing films was reduced by 50% compared to the basic formulation. Unlike mannitol, PEG is a long-chain polymer which can disrupt and inhibit the formation of electrostatic bonds.

CONCLUSION

This report showed the effect of γ-irradiation and $CaCl_2$ on the mechanical properties of cross-linked calcium caseinate films. $CaCl_2$ induced an increase in the fluorescence signal for all irradiation doses and even for the 0 kGy solution. This was explained in terms of salt bridges and electrostatic bonds that could reduce molecular distances making the formation of bityrosine easier. Furthermore, addition of salt combined with irradiation at doses ≥16 kGy resulted in the formation of homogeneous gels. These large-scale networks directly increased the mechanical properties of the corresponding films. The maximum gel fracture strength was obtained for all formulations at 64 kGy while previous bityrosine measurements showed a gradual increase with irradiation dose. It seems that bityrosine measurements fail to detect other mechanisms generated by γ-irradiation (degradation, bond cleavage) which could explain mechanical strength loss at high doses. Other more appropriate techniques, like gel filtration chromatography or swelling experiments, should be considered for evaluating the molecular weight and cross-linking density of these materials.

Addition of plasticizers to the film formulations generally decreased the puncture strength values. Sorbitol had the greatest plasticizing effect and increased the distance to puncture of the films for all irradiation doses while mannitol decreased the distance to puncture. Such a feature could be due to the greater solubility of sorbitol, which seems more effective than its isomer in retaining water in the films.

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