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Protein aggregation in aqueous casein solution; effect of irradiation, dose level, concentration, storage, and additives (carbohydrate and lipid)

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With 4 figures and 3 tables

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From the vast amount of research efforts dealing with various aspects of radiation effects on foods and food components (11, 18, 5, 12, 19, 8, 9, 6, 13, 15, 17, 20), it is apparent up to now that much remains to be studied in depth, much may have to be added or corrected about radiation-induced physico-chemical changes in foods. A great many reactions that take place when foodstuffs are subjected to ionizing radiation are still not fully understood. The better understanding of some of the radiation-induced changes in pure proteins as such or in mixture with other food constituents could yield much data which could be meaningfully extrapolated to intact foods and consequently could help to improve the assessment of the wholesomeness of irradiated foods.

It was the purpose of our investigations to elucidate some of the changes in the chemical structure of a pure protein (casein), irradiated as such or with added carbohydrate and/or lipid. The effect of subsequent storage of the irradiated solutions has been also examined. The formation of protein aggregates was studied by gel filtration technique. The application of thin-layer gel filtration, its speed and adaptability to very small samples facilitated the measurements of the extent of aggregation which occurred in protein molecules after irradiation.

Materials and methods

Casein soluble in alkali (Merck, Darmstadt, FRG), trehalose (Serva, Heidelberg, FRG) and commercial sunflower (Thomy, Karlsruhe, FRG) were the materials used in the investigations.

Preparation of casein solutions

Casein solutions were made in the required concentration in 0.1 N NaOH and adjusted to pH 7.0 using dilute solutions of HCl or NaOH. Tween-20 was used to emulsify sunflower oil before being added to casein solutions. Trehalose was added after being dissolved in phosphate buffer (pH 7.2).

Irradiation process

The casein solutions as such or mixed with carbohydrate or lipid were irradiated in air-sealed glass ampoules surrounded with ice water during irradiation. ^{60}Co-Gammacell 220 (Atomic Energy of Canada, Ltd) was used at a dose rate of about 330 krad/hr.

Thin-layer gel chromatography

Gel chromatography (14) was performed to indicate the changes in protein structure. The thin-layer plates (20 × 20 cm) were coated with sephadex G-200 Superfine (Pharmacia, Uppsala, Sweden) dissolved in 0.1 N NaOH using the equipment delivered by Desaga GmbH (Heidelberg, FRG), the slit of the spreader being adjusted to 0.5 mm. The samples were applied on the gel using volumes of 10 µl. The plates were placed in a special chamber and a wick of filter paper ensured the contact between the gel and the buffer reservoir which containted 0.1 N NaOH. The plates were inclined at an angle which gave a liquid flow through the gel with about 1 cm travelling distance in about 1 hour for myoglobin. 5 cm were considered sufficient for good separation of the protein samples.

Protein detection

The print technique (14) was used to identify the protein patterns. The paper prints (SS 2040b, Schleiher & Schüll, Dassel, FRG) were stained with Amidoblack 10B (Serva, Heidelberg, FRG). The prints after being dried in air were evaluated densitometrically in remission (SD 3000, Schöeffel Instrument Corp., Westwood, N.J., U.S.A.), and the monomeric and aggregated fractions were calculated.

Results and discussion

The radiation-induced aggregation in aqueous solutions of purified proteins has indicated a close relation between the amounts of aggregates

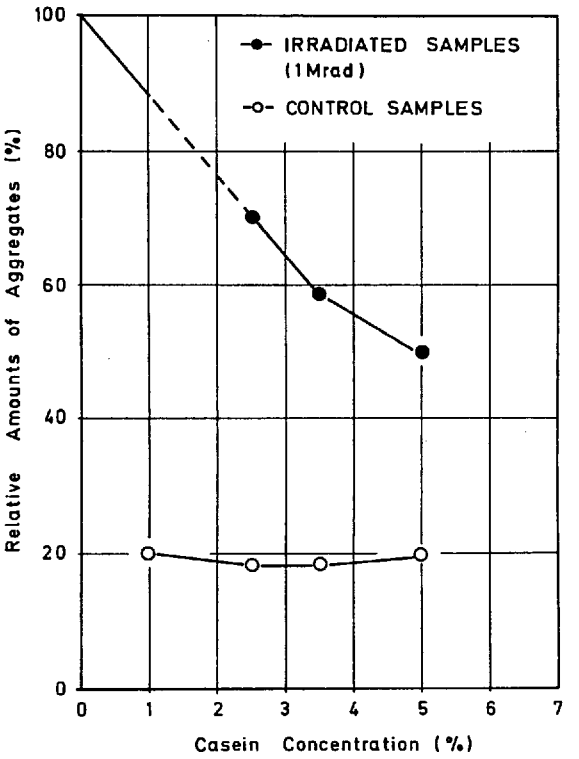

Fig. 1. Relation between casein concentration and relative aggregation at constant radiation dose level, 1 Mrad.

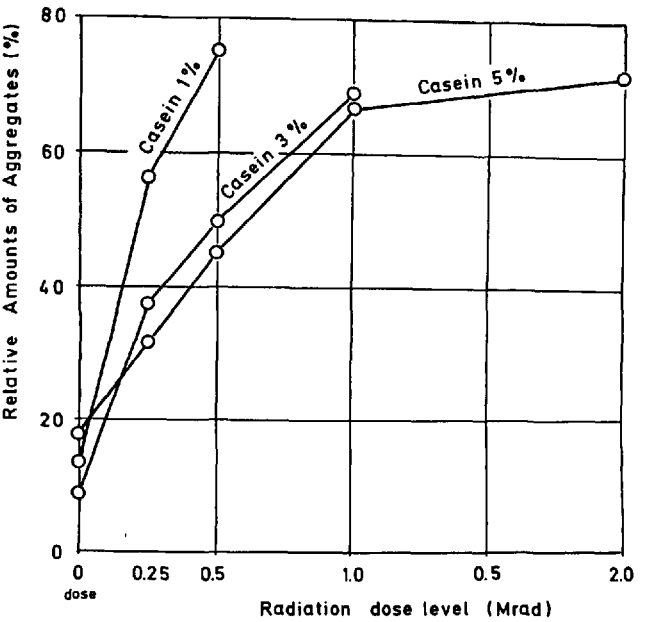

Fig. 2. Protein aggregation in different casein solutions irradiated at different dose levels.

and protein concentration (10, 2, 3). When aqueous solutions of casein were irradiated at 1 Mrad and the samples then subjected to gel chromatography, it was found that the radiation-induced casein aggregation is dependent on protein concentration (fig. 1). When these investigations were carried out using different radiation dose levels (0.25, 0.50, 1.0 and 2.0 Mrad) with different casein concentrations (1, 3, and 5%), the results shown in figure 2 revealed an associate increase in protein aggregation with the increase in radiation dose level. The solution of 1% casein acquired a curdy appearance and eventual coagulation took place at 1 Mrad (hence gel filtration of the casein solution at this stage was not carried out. It was probable that protein denaturation proceeded stepwise with gradual increase in insoluble aggregates; thus the further increase in radiation dose caused the further association between the protein molecules. It is worthy to mention that when casein concentrations of 1, 2.5, 3.5, and 5% were examined with radiation dose levels of 1 Mrad and 2 Mrad, the gel chromatography revealed in figures 3 and 4 indicated that the higher dose of 2 Mrad significantly altered the protein structure. Visible insoluble precipitates occurred in the solution of 1% concentration while complete gel formation occurred in the solutions of 2.5 and 3.5% concentration. This dose level of 2 Mrad, however, did not cause visible aggregates in the casein solution of 5% concentration. It seemed possible that the changes in the solutions of 1, 2.5 and 3.5% concentrations were due to alterations in the covalent structure of the protein molecules resulting from radiation-induced polymerization. At the higher protein

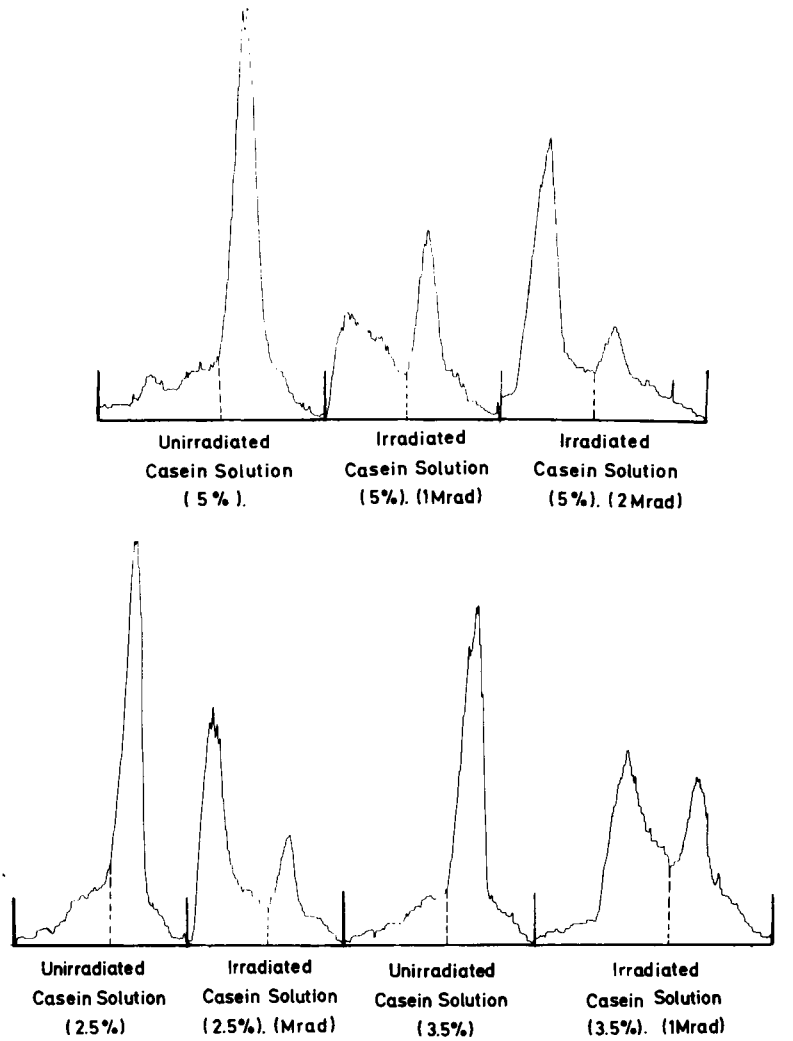

Fig. 3. Densitograms of unirradiated and irradiated casein solutions.

concentration of 5%, the amount and the insolubilizing action of radiation-induced primary water radicals per each protein molecule was limited and consequently large amounts of protein molecules and soluble aggregates escaped from insolubilization and damage.

The aggregation-concentration dependence was also observed in previous studies with horseradish peroxidase, ribonuclease and myoglobin, ovalbumin and serum albumin (10, 1, 2, 3, 4). The extent of radiation-induced aggregation increased with the decrease in protein concentration. Our conclusion was explained as follows: At constant radiation dose, specific amounts of primary water species were formed. In case of low

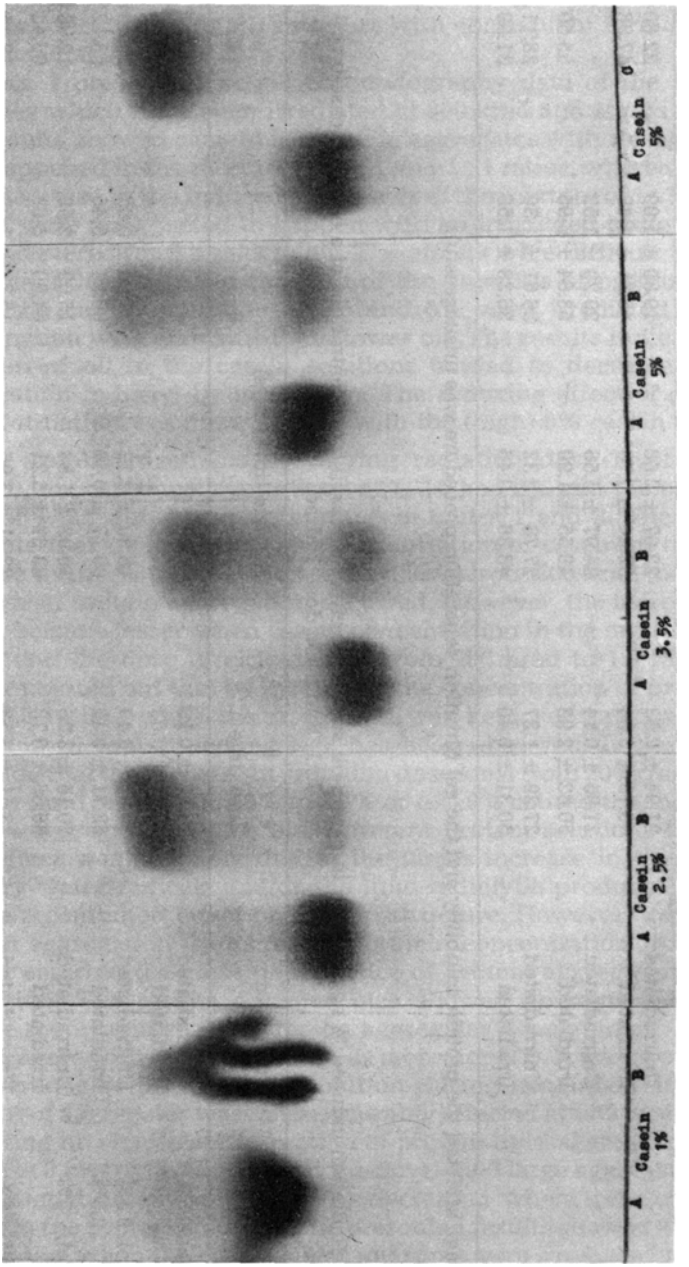

Fig. 4. Radiation-induced aggregation of casein as a function of protein concentration. Thin-layer gel chromatography on Sephadex G-200 Superfine. Protein staining with Amidoblack 10B. The starting line is down. A: unirradiated. B: irradiated at 1 Mrad. C: irradiated at 2 Mrad.

Table 1. Irradiation and subsequent storage effect on the % aggregation of casein in solution mixed with sunflower oil (1%). Effect of varying casein : oil ratios. Casein concentration kept constant at 1%. Radiation dose *200* Krad.

Casein : oil (Ratio)	Treatment	Storage time (days) Zero time	1	8	14
3 : 1	Unirradiated	21.94 ± 2.53	22.07 ± 2.68	19.80 ± 1.09	22.60 ± 3.10
	Irradiated	46.75 ± 0.53	52.24 ± 0.73	46.21 ± 1.49	47.98 ± 0.18
1 : 1	Unirradiated	21.85 ± 0.14	24.07 ± 5.56	20.20 ± 1.52	*
	Irradiated	50.82 ± 2.93	48.55 ± 1.90	53.84 ± 0.95	55.59 ± 0.40
1 : 3	Unirradiated	27.29 ± 1.27	27.78 ± 2.32	26.61 ± 1.30	28.11 ± 1.93
	Irradiated	50.31 ± 0.40	48.86 ± 0.46	36.38 ± 1.56	31.99 ± 2.07

* Storage affected the protein solubility and the gel filtration separation procedure was unsuccessful.

Table 2. Radiation-induced % aggregation in casein-oil solutions, effect of varying concentrations at a constant casein : oil ratio of 1 : 1. Radiation dose 1 Mrad.

Casein : oil (Ratio)	Treatment	Storage time (days) Zero time	1	8	14
Casein sol. 2.5%	Unirradiated	18.82 ± 0.18	–	–	–
	Irradiated	70.57 ± 0.63	–	–	–
Casein sol. 2.5%	Unirradiated	14.31 ± 0.49	16.80 ± 2.12	21.90 ± 0.85	25.46 ± 1.16
+ Oil sol. 2.5%	Irradiated	63.71 ± 0.46	69.77 ± 0.44	66.34 ± 0.45	58.12 ± 0.40
Casein sol. 5%	Unirradiated	20.15 ± 3.26	19.93 ± 1.68	15.09 ± 2.58	12.08 ± 2.99
	Irradiated	66.66 ± 0.78	63.67 ± 2.21	64.90 ± 5.68	66.42 ± 1.77
Casein sol. 5%	Unirradiated	23.51 ± 3.53	22.21 ± 3.96	15.33 ± 1.55	12.44 ± 3.90
+ Oil sol. 5%	Irradiated	56.99 ± 0.62	52.92 ± 2.50	51.58 ± 3.63	54.50 ± 4.34

Each value represents mean ± standard deviation of at least three determinations.

amounts of protein molecules available in solution (dilute solutions), maximum efficiency of utilizing these active water radicals in attack or/ and in reaction with protein molecules occurred, thereby extending their chemical effects on protein structure with consequent formation of large amounts of aggregates.

Table 1 presented the gel chromatography data of the protein-lipid mixtures which have been irradiated at 200 krad and stored till 2 weeks. The results showed a slight increase in aggregates with storage time. This case happened in the mixtures of 3 : 1 and 1 : 1 ratios, whereas there was a large decrease in the induced aggregates of the mixture of 1 :.3 ratio. These results were unexpected to happen with an irradiated protein-lipid mixture and stored for 2 weeks in air. The effects were difficult to explain.

Table 2 demonstrated the data of the amounts of protein aggregates when the casein solutions of 2.5 and 5% were irradiated alone or in combination with emulsified sunflower oil. The results indicated that the addition of oil to the casein solutions tended to decrease the protein aggregation induced by irradiation. The reducing effect of oil on aggregates formation was more evident with the (high) 5% casein solution.

The combined effects of varying radiation dose levels (200 krad, 1 Mrad), the casein concentrations of 1, 2.5 and 5% and the oil concentrations of 1, 2.5 and 5% are presented in tables 1 and 2. The data clearly illustrate that by increasing the concentration of casein in the casein-oil mixture from 1% to 2.5% and the dose level from 200 krad to 1.0 Mrad, an increase in protein aggregation occurred. However, the increase in aggregation became lesser when casein concentration in the mixture increased to 5% and the dose level increased from 200 krad to 1.0 Mrad. Table 2 further pointed out that by increasing the concentration of protein and oil from 2.5 to 5%, while the dose level was kept constant at 1.0 Mrad, a reduction in protein aggregation has been observed. It was possible to conclude that the increase in radiation dose level from 200 krad to 1.0 Mrad and the lipid level from 1.0% to 2.5% or to 5.0% caused the increase in the protein aggregation in the two different protein solutions (2.5 and 5%). This effect was probably due to the larger increase in the amounts of primary water radicals and/or the lipid radiolytic products which could induce a combined effect on protein structure. However, the decrease in protein aggregation by increasing protein concentration from 2.5 to 5% further asserted the close dependence of protein aggregation on its concentration. The results obtained also showed no remarkable effect of storing the irradiated mixtures on aggregates development. The present results agree with those found in this laboratory (7). It was observed that in the presence of fat in casein solution during irradiation at 5 Mrad the amount of aggregates was not measurably affected after 2 weeks of storage indicating no significant formation of protein-lipid aggregates.

Table 3 clearly indicated that the developed large aggregation induced in irradiated casein solution (5%) decreased when trehalose (5%) was added to the protein solution. The presented results showed a reduction in aggregation when the casein-sugar mixtures were irradiated at 1 Mrad and at 2 Mrad. The lowering action of trehalose on the formation of aggregates were possibly through saving the protein molecules from being attacked and denatured by the water-active species. Trehalose acted as a water

Table 3. Radiation-induced aggregation in casein solutions (5%); effect of radiation dose, added carbohydrate and/or Lipid and subsequent storage.

Casein solution + Additive	Radiation dose (Mrad)	Zero time	Storage time (days) 1	8	14
1. Casein solution (5%)	Control	20.15 ± 3.26	19.93 ± 1.68	15.09 ± 2.58	12.08 ± 2.99
	1.	66.66 ± 0.78	63.67 ± 2.21	64.90 ± 5.68	66.42 ± 1.77
	2.	71.79 ± 2.66	Gelatinized	–	–
2. Casein sol. (5%) + Trehalose (5%)	Control	21.86 ± 2.34	20.20 ± 3.39	15.68 ± 2.70	11.83 ± 2.87
	1.	48.94 ± 2.85	46.86 ± 2.02	47.85 ± 2.73	42.27 ± 3.90
	2.	57.33 ± 1.44	55.16 ± 2.93	56.07 ± 2.97	65.52 ± 2.43
3. Casein sol. (5%) + Trehalose (5%) +Oil sol. (5%)	Control	25.55 ± 3.56	20.01 ± 1.95	13.60 ± 5.47	8.88 ± 3.38
	1.	49.97 ± 2.90	45.04 ± 3.08	42.36 ± 0.53	45.43 ± 3.28
	2.	58.76 ± 0.00	57.58 ± 3.34	59.41 ± 4.87	58.63 ± 1.33
4. Casein sol. (5%) + oil sol. (5%)	Control	23.51 ± 3.53	22.21 ± 3.96	15.33 ± 1.55	12.44 ± 3.90
	1.	56.99 ± 0.62	52.92 ± 2.50	51.58 ± 3.63	54.50 ± 4.34
	2.	Gelatinized	73.48 ± 5.04	68.32 ± 6.63	71.40 ± 4.33

Each value represents mean ± standard deviation of at least three determinations. Control means unirradiated samples.

radical scavenger, especially the ȮH species which are important in the protein-aggregation processes. The amount of aggregation which has been observed was probably due to the protein interaction with the irradiated medium. It is possible to conclude that complexes may have been formed between protein and the carbohydrate radiolytic products such as the carbonyl compounds (16). It was found (16) that in glucose solutions hydrogen abstraction → by ȮH may mainly occur. Previous studies with RNase (15) and other proteins in aqueous solution (12) provided evidence that the presence of carbohydrate caused a reduction in radiation-induced aggregation.

It is interesting to mention that the addition of emulsified sunflower oil at a concentration of 5% to the casein-trehalose solution exerted only an insignificant change in protein aggregation behaviour (table 3). Moreover, the gel chromatography data (table 3) revealed that an induction in protein aggregation occurred when the casein solution in the presence of oil has been irradiated at 1 Mrad, whereas the dose of 2 Mrad caused only an immediate temporary gelatinized state, which has recovered great parts of its solubility one day after irradiation and consequently the gel separation procedure has become possible at this stage. The presented results indicated that the presence of lipid radicals in the irradiated medium with protein molecules decreased to some extent the probability of protein particles of being associated in larger aggregates. This trend was not obvious when the radiation dose level increased to be 2 Mrad. This high dose level (2 Mrad) probably increased the protein denaturation process with consequent increase in the formation of large aggregates in the irradiated medium. The data also showed that the storage periods had practically no clear effect on the formation of further amounts of aggregates. Radiation-induced aggregation in aqueous solutions of myoglobin, ovalbumin and serum albumin had been investigated (4). Moreover, the effect of adding varying amounts of carbohydrates and lipids had been studied (4). They found that the addition of carbohydrates greatly reduced the amount of radiation-induced aggregates, whereas the addition of lipids scarcely diminished the tendency of the irradiated proteins for aggregation. The above-mentioned studies (4) also showed that if both carbohydrates and lipids were included in the irradiated protein solutions, the decrease in aggregation caused by the carbohydrate addition was counteracted by the addition of the lipid; as increasing amounts of lipid were added, the effect of carbohydrate addition became smaller.

The presented experiments have provided some information upon some of the radiation-induced chemical changes in protein solutions as such or in the presence of carbohydrate and/or lipid. However, feeding experiments with animals with the use of varying ratios of protein : lipid : carbohydrate in simulated food models are necessary. Such studies will help in the understanding of the nutrition metabolism and the physiological features of animals maintained under various irradiated feeding-regimes.

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Summary

Radiation-induced aggregation of protein in aqueous solutions was studied. Different concentrations of casein solutions have been irradiated as such or in the presence of carbohydrate and/or lipid. Gel chromatography data indicated a close relation between the amounts of aggregates and protein concentration. Protein aggregation increased with the increase in radiation dose level. The addition of carbohydrate (trehalose) reduced the amount of radiation-induced aggregates, whereas the sole addition of oil caused an induction in protein aggregation when the solution was irradiated at 1 Mrad. However, insignificant changes in protein aggregation were observed when emulsified oil was added to the casein-trehalose solution.

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