J. Serb. Chem. Soc. *70* (11) 1255–1262 (2005) UDC 54–78:637.413:577.112
JSCS–3365 *Original scientific paper*

Gamma-radiation induced damage of proteins in the thick fraction of egg white

MARIJA VUČKOVIĆ[#], MARIJA B. RADOJČIĆ and BRATOLJUB H. MILOSAVLJEVIĆ[*]

Vinča Institute of Nuclear Sciences, P. O. Box 522, 11001 Belgrade, Serbia and Montenegro (e-mail:bmilosav@nd.edu)

(Received 1 February 2005)

Abstract: The thick fraction of egg white saturated with either N_2O or Ar was irradiated in the dose range 1.5–45 kGy at ^{60}Co gamma source. The gel structure decomposition and other processes accompanied with changes in protein molecular mass were followed by Sephadex G-200 exclusion chromatography, denaturing SDS-polyacrylamide gel electrophoresis, viscosity and turbidity measurements. The complex behaviour of viscosity was observed in the N_2O saturated sample (where the hydrated electron was converted into the OH radical); the initial abrupt decrease that gradually slows down reaching the minimum at 12 kGy (η_{min} = 2.7 mPa s) followed by the slow rise was measured. The Ar saturated sample ($[e_{aq}^-] \approx [OH]$) showed both the significantly faster initial decrease and lower viscosity minimum (η_{min} = 2.2 mPa s). The combined Sephadex G-200 exclusion chromatography and denaturing SDS-polyacrylamide gel electrophoresis data revealed that the three-dimensional egg white (hydrated) gel structure was (efficiently) decomposed even in the N_2O saturated sample. The protein scission was detected in the entire dose range studied, while the protein agglomeration is not noticed at low doses (around 1.5 kGy); however, it dominates at higher doses. In the highest dose region studied, the loss of structure in SDS-PAGE chromatograms indicates that the agglomerates are formed from protein fragments rather than from intact proteins. The continuous linear increase in turbidity was measured. The results obtained indicate that ionizing radiation causes the breakdown of the protein network of the thick fraction of egg white *via* the reduction of S–S bridges by the hydrated electron and the protein fragmentation due to the direct action of ionizing radiation. The protein agglomeration is initiated by the reaction of the OH radical; its inefficiency at low doses is attributed to the glucose antioxidant properties and radical immobility.

Keywords: radiation, irradiation, protein, egg white, ovalbumin, ovomucin.

INTRODUCTION

The quantitative and qualitative data on the radiolytic behaviour of moderately complex protein-containing structures may be of great prospective help for better un-

* Corresponding author: Present address: University of Notre Dame, 336 Radiation Laboratory, Notre Dame, IN 46556-5674 USA. e-mail:bmilosav@nd.edu

doi: 10.2298/JSC0511255V

derstanding of the biological effects of radiation *in vivo*.[1] One such convenient model system is the protein hydrogel of the thick fraction of egg white, with the network structure similar to the structure of mammalian mucous tissues.[2,3] Hen egg white consists of an outer and inner thin albumen (egg white) separated by a firm or thick albumen hydrogel network.[4] The thin fraction of egg white was shown to be a true solution of ovalbumin, conalbumin, ovoglobulin and other egg white proteins.[5] The proteins are present in the form of monomers (about 40%) or as protein agglomerates/conglomerates (about 60 %).[5] In addition to the proteins of the thin fraction, the thick fraction of egg white also contains a significant amount (4 %) of highly glycosylated, hydrated protein ovomucin.[6,7] This protein, together with ovomucoid, forms a protein network of the thick egg white fraction which is held by S–S bridges.[5] The network encompasses ovalbumin, conalbumin and ovoglobulin monomers or agglomerates/conglomerates.[5] It was shown previously that the ^{60}Co gamma ray irradiation of the thin fraction of egg white prepared in N_2O saturated solutions led predominantly to protein agglomeration.[8] However, a small amount of protein fragments was also observed.[8] This was opposite to the postulates of radiation chemistry on the radiolytic behaviour of diluted proteins solutions under anaerobic conditions.[9–12] This radiolytic behaviour of the thin fraction of egg white was interpreted as a consequence of its more complex composition[8] compared to the single protein solution.[12] In that view, radiolytic behaviour of the thick fraction of egg white and its hydrogel network might illustrate even closer the complex radiolytic behaviour of higher order protein structures occurring *in vivo*. In this paper we report the results of the detailed study on the mechanism of radiolysis of the thick fraction of egg white.

EXPERIMENTAL

The thick fraction of fresh Brown Leghorn (*Gallus gallus*) egg white was isolated by Buchner funnel filtration. Samples were saturated with either N_2O or Ar for 3 h, sealed in ampoules and irradiated at ^{60}Co gamma source. The dose rate was 51.5 Gy/min by Fricke dosimetry. Sample viscosity was measured using an Ostwald viscometer at 20.0 °C ($n = 5$). The absorbance spectra were measured from 190 nm to 900 nm using a Perkin Elmer Lambda 5 UV-VIS spectrophotometer. The analysis of radiation-induced protein damage was performed by gel filtration of Sephadex G-200 chromatography columns and by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.[13] Sephadex G-200 column (76 cm height; 2.5 cm diameter; total volume ≈ 373 ml) was packed in 0.1 M sodium phosphate elution buffer (pH 7.4). The column was calibrated by a Blue Dextran 2000 (DB_{2000}, Pharmacia-1328, molar mass M_m = 2,000,000 g/mol, detected at OD 600 nm) for void volume (V_o), and cytochrome *c* (cyt *c*, Sigma C-7752, M_m = 13,370 g/mol, Stokes radius R_s = 1.79 nm, detected at OD = 528 nm) for total elution volume (V_t). The samples (400 μl) were applied to the column, eluted in 0.6 ml fractions and proteins were followed spectrophotometrically by absorption at 280 nm. For 10% SDS-PAGE samples were dissolved in 0.1 M Tris-HCl pH 6.8 containing 4 % SDS, 5 % β-mercaptoethanol, 6 M urea, 20 % glycerol and 0.1 % brom-phenol-blue (1:1 = vol: vol), boiled for 2 min at 100 °C and separated for 120 min at 100 V. The gel calibration was performed using chicken muscle myosin heavy chain (M_m = 223,000 g/mol) and ovalbumin (M_m = 42,650 g/mol) as standards. A linear relationship between log M_m and protein mobility was used to estimate M_m of egg white proteins. Proteins were stained by 0.125 % Coomassie Brilliant Blue, and scanned by a Pharmacia-LKB UltraScan-XL laser densitometer. The quantification was performed by comparison of the respective integral area of control and treated sample, with experimental error < 8 %.

RESULTS AND DISCUSSION

The thick fraction of egg white was saturated with N_2O and irradiated with increasing doses of gamma rays from ^{60}Co source. Under these conditions the dominant radiation-produced reactive species is the OH radical (G ≅ 6), and most of the protein damage is generated by its action. Radiation-induced damage of proteins was analysed by Sephadex G-200 gel exclusion chromatography (Fig. 1), followed

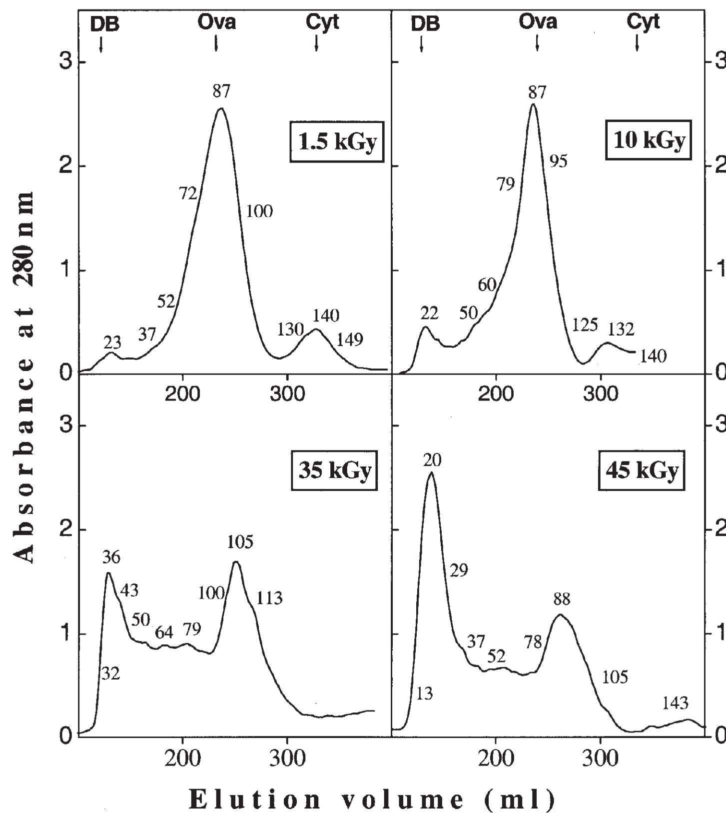


Fig. 1. Sephadex G-200 chromatography of proteins of the irradiated N_2O saturated thick fraction of egg white. Fractions taken for SDS-PAGE analysis are indicated by numbers; DB - marker of V_0; Ova-, Cyt- R_s markers.

by SDS-PAGE of the denatured chromatographic fractions (Fig. 2). Due to its high viscosity, the intact thick fraction of egg white was unable to penetrate Sephadex G-200 gel (not shown). In contrast to that, the sample irradiated with 1.5 kGy entered the column and was eluted as a broad peak at the position of ovalbumin (Fig. 1). When analysed on SDS-PAGE these fractions contained conalbumin (M_m = 87,000 g/mol), ovoglobulins G_2 and G_3 and ovalbumin (M_m = 42,650 g/mol) (Fig. 2a). In the fractions 72 and 87 significant yields of the degraded proteins were also present, which were released from the protein aggregates after the denaturation prior to the SDS-PAGE. The degradation could be attributed to the direct effect of

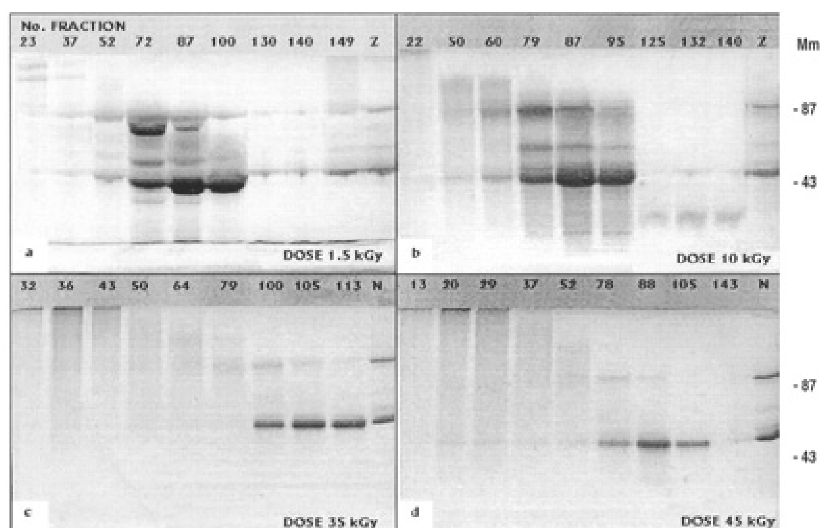


Fig. 2. SDS-PAGE of Sephadex G-200 separated protein fractions: a) 1.5 kGy; b) 10 kGy; c) 35 kGy; d) 45 kGy. Fraction numbers are indicated above gel: N-control; Z-irradiated. M_m (g/mol) of conalbumin and ovalbumin are given in thousands.

radiation since ≈ 10 % of the radiation energy is absorbed directly by the proteins. The fractions eluted with DB_{2000} contained ovomucin ($M_m \approx 3 \times 10^6$ g/mol), the molecular mass of which indicates that it underwent the reduction and was released from the gel structure.[6] Also, those close to cyt *c* contained traces of lysozyme (M_m = 13,930 g/mol). No protein aggregation was observed, which could be attributed to the glucose antioxidant action,[8] since at this particular dose the OH radical concentration produced is more than 20 times lower than that of glucose (which is a natural constituent of egg white). Relatively low mobility of the radicals captured in the gel structure is also expected to lower the agglomeration yield. After irradiation with 10 kGy the chromatographic profile was significantly changed, the protein peak coeluting with DB_{2000} being the most affected one. It contained several higher M_m species that were partially retained on the stacking gel of SDS-PAGE (Figs. 1 and 2b). Apparently, the protein aggregation has progressed a great deal owing to the much higher ratio [OH]/[glucose] and increased radical mobility. The degraded protein concentration was also increased since the ratio direct/indirect effect of radiation remained the same, but the rate slowed down as the target size was decreased. In the cases of radiation doses of 35 and 45 kGy, drastically different protein profile was observed on Sephadex G-200 column. For both doses the protein peak coeluting with ovalbumin was notably shifted towards DB_{2000} (Fig. 1). When analysed by SDS-PAGE these fractions contained high amount of aggregated proteins with M_m > 250,000 g/mol, unable to penetrate resolving PAG (Fig. 2 c,d). Also, the fractions that were eluted closer to cyt *c* contained a notable amount of protein fragments in M_m range 15,000–40,000 g/mol (Fig. 2 c,d).

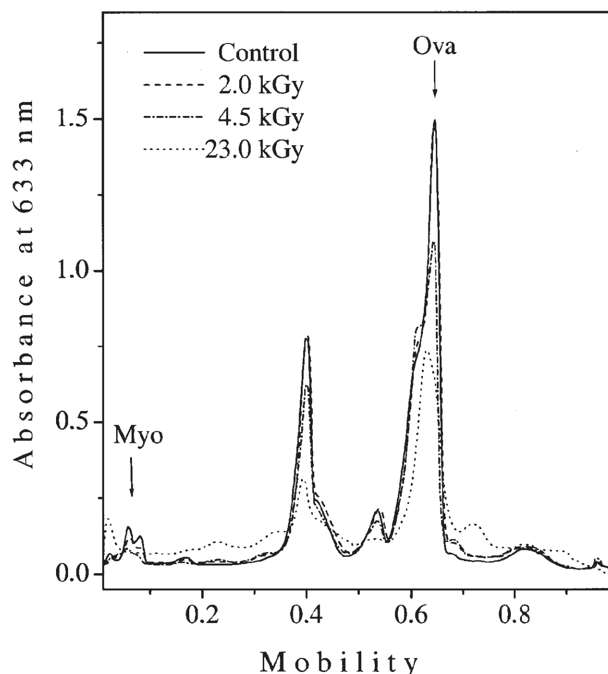


Fig. 3. SDS-PAGE densitograms of proteins of the irradiated N_2O saturated thick fraction of egg white. Positions of M_m markers (Myo, Ova) are indicated by arrows.

In order to quantify protein aggregation and fragmentation, irradiated egg white samples were denatured by 5 % β-mercaptoethanol and 2 % SDS, and directly analysed by SDS-PAGE (Fig. 3). The percentages of protein crosslinking and fragmentation were presented as a function of the radiation dose (Fig. 4). The graph indicated that protein fragmentation was more pronounced at the lower radiation doses up to 10–15 kGy, while protein crosslinking prevailed at the radiation doses above 15 kGy. Figure 4 also shows an onset of several kGy in the crosslinking, which is in an agreement with the Sephadex G-200 gel exclusion chromatography data (Figs. 1 and 2a). Owing to the upper limit 1×10^6 Da, very little can be concluded about the early stages of the gel structure degradation from chromatography data. The molecular mass region studied can be expanded by using viscometry, since our earlier work has established the relation between the solution dynamic viscosity and the solute molecular mass distribution.[14] Figure 5 compares the viscosities of the N_2O and Ar saturated samples. One can observe that the Ar saturated sample viscosity decreases faster than that of the N_2O saturated sample. Since the protein concentrations are the same in both samples, it can be concluded that the average protein agglomerate size is smaller in the Ar sample. This further means that in a sample where the radiolytically produced electrons are not scavenged by N_2O, the gel degradation is faster. This piece of data combined with the previously reported pulse radiolysis data of egg white,[15] suggests that the hydrated electron is responsible for the gel structure decomposition. Figure 5 also shows the turbidity (measured at 384 nm) *vs.* dose relation. Because of the qualitative rule "the smaller the molecules, the less intense the light scattering" we expected an abrupt de-

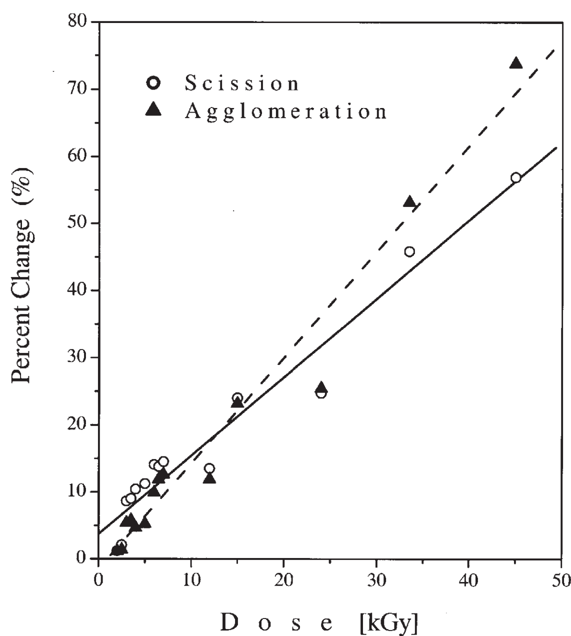


Fig. 4. Dose dependent changes in percent of ^{60}Co gamma radiation-induced protein agglomeration and fragmentation in the N_2O saturated thick fraction of egg white.

crease in the turbidity owing to the rapid degradation of the gel structure. However, the linear increase in turbidity was observed in the entire dose region indicating the complex influence of the egg white radiolysis (*e.g.*, changes in protein conformation accompanied with dehydration, scission, and aggregation) on the light scattering.

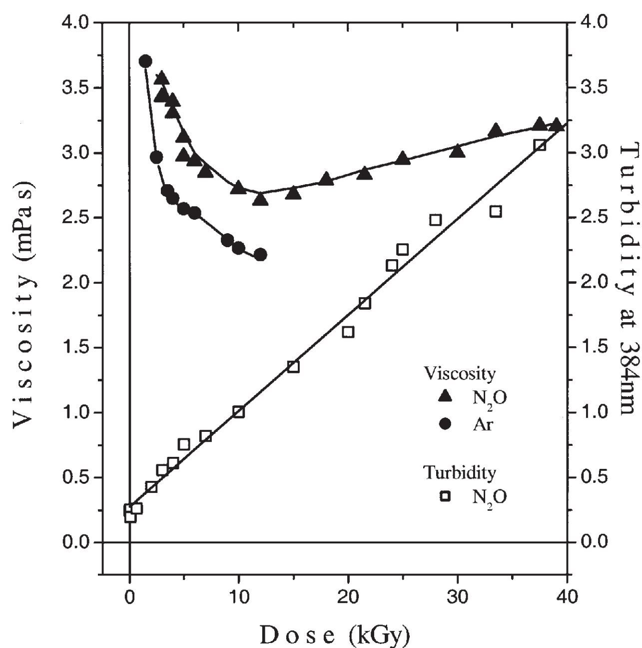
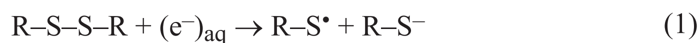


Fig. 5. Dose dependent changes in viscosity (▲), turbidity at 384 nm (□) of the N_2O saturated thick fraction of egg white and viscosity (●) of the Ar saturated sample.

The results obtained (related to the changes in protein molecular mass) can be summarized in the following reaction scheme for the radiolysis of the thick fraction of egg white:



$$R–S–S–R + (e^-)_{aq} \rightarrow R–S^{\bullet} + R–S^- \quad (1)$$

$$\text{Protein} \xrightarrow{\text{High Density Ionizations and Excitatons}} \text{Fragment}_1 + \text{Fragment}_2 \quad (2)$$

The above reactions lead to the gel degradation accompanied with a rapid decrease in the viscosity. From our measurements, we were not able to establish the role of the previously evoked dry electron in the egg white radiolysis. In reactions (3) and (4) protein radicals are formed which further agglomerate either in the grafting reaction (5) or in the crosslinking reaction (6). Reaction (7) explains the glucose antioxidant action.

$$\text{Protein} \xrightarrow{\text{Low Density Ionizations and Excitations}} \text{Protein radical}^{\bullet} \quad (3)$$

$$\text{Protein} + {}^{\bullet}OH \rightarrow \text{Protein radical}^{\bullet} + H_2O \quad (4)$$

$$\text{Protein radical}^{\bullet} + \text{Protein} \rightarrow \text{Dimer}^{\bullet} \quad (5)$$

$$\text{Protein radical}^{\bullet} + \text{Protein radical}^{\bullet} \rightarrow \text{Dimer} \quad (6)$$

$${}^{\bullet}OH + \text{glucose} \rightarrow \text{inactive products} \quad (7)$$

CONCLUSIONS

According to Sephadex G-200, SDS-PAGE, viscosity and turbidity analyses, ^{60}Co gamma ray irradiation of N_2O saturated thick fraction of egg white leads to degradation of its hydrogel and to the aggregation and fragmentation of its constituent proteins. The extent of hydrogel decomposition, protein crosslinking and fragmentation are dose dependent processes. The results obtained indicate that ionizing radiation causes the breakdown of the protein network of the thick fraction of egg white *via* the reduction of S–S bridges by the hydrated electron and the protein fragmentation due to the direct action of ionizing radiation. From our measurements, we were not able to establish the role of the previously evoked dry electron contribution to the S–S bridges reduction. The protein agglomeration is initiated with the reaction of the OH radical; its inefficiency at low doses is attributed to the glucose antioxidant properties and radical immobility.

Acknowledgement. The work was supported by the Grants of Ministry of Science and Environmental Protection.

И З В О Д

ОШТЕЋЕЊА ПРОТЕИНА ГУСТЕ ФРАКЦИЈЕ БЕЛАНЦА ИЗАЗВАНА ГАМА ЗРАЧЕЊЕМ

МАРИЈА ВУЧКОВИЋ, МАРИЈА Б. РАДОЈЧИЋ и БРАТОЉУБ Х. МИЛОСАВЉЕВИЋ

Инсӣиӣуӣ за нуклеарне науке „Винча", Беоӣрад

Квантитативни и квалитативни подаци о радиолизи протеинских структура средње комплексности могу бити од великог потенцијалног значаја за боље разумевање биолошких ефеката јонизујућег зрачења *in vivo*. Један такав модел-систем представља протеински хидрогел густе фракције беланца јајета, чија мрежаста структура наликује структури мукоидних ткива сисара. У овом раду протеински хидрогел густе фракције беланца засићен је у једном случају са N_2O, а у другом са Ar и озрачен дозама од 1,5 до 45 kGy на ^{60}Co извору гама зрачења. Промене молекулске масе протеина хидрогела су анализиране ексклузионом хроматографијом на Sephadex G-200, денатуришућом електрофорезом на SDS-полиакриламидном гелу и мерењем вискозности и турбидности. Резултати мерења су показали да зрачење доводи до разградње мрежасте структуре хидрогела беланца и да доводи до умрежавања и кидања његових протеина. Разградња протеинске мреже густе фракције се дешава у реакцији редукције R–S–S–R мостова хидратисаним електроном и преко кидања протеина које се дешава у директној акцији зрачења, односно када се јонизације и ексцитације велике густине ("blobs" и "tracks") дешавају на молекулима протеина или њиховим агрегатима, а не на молекулима растварача (воде). Умрежавање протеина је иницирано реакцијом протеина са OH радикалом у којој долази до апстракције водоничног атома. Та реакција је инхибирана у области малих доза (≈ 1,5 kGy) услед споре дифузије радикала и антиоксидативног дејства глукозе која је саставни део беланца.

(Примљено 1. фебруара 2005)

REFERENCES

1. E. J. Hall, "Linear Energy Transfer and Relative Biological Effectiveness", in *Radiobiology for the Radiologist*, Eric.J. Hall, Ed., J. B. Lippincott Co., Philadelphia, 1995, pp. 153-164
2. M. C. Rose, W. A. Voter, C. F. Brown, B. Kaufman, *Biochem. J.* **222** (1984) 371
3. J. K. Sheehan, I. Carlstedt, *Biochem. J.* **221** (1984) 499
4. A. L. Romanoff, A. J. Romanoff, "Chemistry of the Nonembryonic Portions of the Egg", in *Biochemistry of the Avian Embryo*, A. L. Romanoff, A. J. Romanoff, Eds., Wiley, New York, 1967, pp. 177–232
5. M. Vučković, M. Radojčić, B. H. Milosavljević, *J. Serb. Chem. Soc.* **65** (2000) 157
6. C. Rabouille, M. A. Aon, D. Thomas, *Arch. Biochem. Biophys.* **270** (1989) 495
7. D. S. Robinson, J. B. Monsey, *Biochem. J.* **121** (1971) 537
8. Lj. Josimović, M. Radojčić, B. H. Milosavljević, *Radiat. Phys. Chem.* **47** (1996) 445
9. K. J. A. Davies, *J. Biol. Chem.* **262** (1987) 9895
10. W. M. Garrison, *Chem. Rev.* **87** (1987) 381
11. O. Yamamoto, "Effect of Radiation on Protein Stability", in *Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation*, T. J. Ahern, M. C. Manning, Eds., Plenum Press, New York, 1992, p. 361
12. Z. Tuce, E. Janata, M. Radojčić, B. H. Milosavljević, *Radiat. Phys. Chem.* **62** (2001) 325
13. U. K. Laemmli, *Nature* **227** (1970) 680
14. J. Kuljanin, G. M. Vujičić, M. Radojčić, B. H. Milosavljević, *J. Polym. Sci., Part. B: Polymer Physics* **38** (2000) 1729
15. B. Cercek, L. Cercek, *Int. J. Radiat. Biol.* **24** (1973) 137.