J. Serb. chem. Soc. 65(2)123-130(2000)
JSCS-2733

UDC 547.962.3:534.321.9:66.084.8
Original scientific paper

Determination of the critical molar mass of ovalbumin oligomers degraded by ultrasound

DRAGANA FILIPOVIĆ, MARIJA RADOJČIĆ and BRATOLJUB H. MILOSAVLJEVIĆ

Vinča Institute of Nuclear Sciences, P.O.Box 522, YU-11001 Belgrade, Yugoslavia

(Received 26 May, revised 28 September 1999)

An experimental method has been developed which enables the determination of the critical molar mass (*Mmc*) of ovalbumin oligomers degraded by ultrasound of known frequency. To test the validity of the *Mmc* postulate, a series of ovalbumin oligomers was prepared by the radiolytic cross-linking of 1% solutions of ovalbumin monomer dissolved in 50 mM Na/K-phosphate buffer pH 7.0 saturated with N_2O. Under these conditions, irradiation with 5 kGy from a ^{60}Co source, yielded ovalbumin dimers, trimers, tetramers, and higher order oligomers. On the basis of the results obtained with the ovalbumin oligomers, it was concluded that for ultrasound of 23 kHz frequency and 5mm amplitude, the *Mmc* was 274000 – 14000 g/mol. Our results confirmed that the two postulates in the chemistry of polymer degradation by ultrasound are valid when ovalbumin oligomers are used as substrates, *i.e.,* (1) that the higher the molar mass of the original macromolecule, the faster is its degradation rate, and (2) that a lower molar mass limit (*LMmL*) exists below which the macromolecules are resistent to further degradation.

Keywords: ovalbumin, ultrasound, critical molar mass.

Biological studies have discovered numerous changes at different levels of cell organization induced by ultrasound treatment, but the mechanisms by which these changes were produced are poorly understood. Thus, for example, transmission electron microscopy showed that ultrasound treatment could produce local disruption of tissues, as well as holes of micrometer diameter in cells.[1] *In vitro* cell systems showed that cell lysis and cell death may also occur after ultrasonic treatment.[1] This might be due to either heat damage or to cavitation events. Ultrasound treatment may also cause disassembly of microtubules[2] and disruption of cell organelles, such as mitochondria and lysosomes.[3] It can also induce more subtle changes such as the alteration of cell membrane permeability to Na^+, K^+ and Ca^{2+}.[4] Further successful implementation of ultrasound presumes a more precise knowledge about the boundary between biohazards and beneficial effects of ultrasound treatment, *i.e.,* about the molecular mechanisms by which the biological effects of ultrasound are achieved. For example, it is not clear what kind of structural changes to cellular proteins may occur during therapeutic ultrasound treatment, *i.e.*,

when ultrasound with frequency of 20–100 kHz is used. The chemistry of ultrasound effects on organic polymers in solution postulated the existence of a critical molar mass (*Mmc*), termed lower molecular mass limit (*LMmL*). According to this postulate, polymers whose *Mm* is below the *LMmL* remain unaffected by ultrasound treatment under the chosen set of conditions. To test the validity of both postulates it was necessary to have a substrate consisting of a set of macromolecules with molar masses that cover the range of *LMmL*. However, such a substrate is difficult to isolate intact from natural sources. Consequently, it was necessary to artificially synthesize a set of macromolecules with defined molar masses. In this paper we present the results of the determination of the *LMmL* for ultrasound of 23 kHz frequency and 5 μm amplitude using a set of artificially produced ovalbumin oligomers with defined molar masses.

EXPERIMENTAL

Purified ovalbumin (Sigma, grade VI) was dissolved in 50 mM Na/K-phosphate buffer pH 7.0 and saturated with N_2O by passing the gas over the sample for 3 h. Then the sample was irradiated with ^{60}Co γ-rays at a dose rate of 51.5 Gy min^{-1}, as determined by Fricke dosimetry, and subsequently treated for 50 s with 23 kHz and 5 μm amplitude ultrasound from a Soniprep 150. The ultrasound treatment was repeated six times (300 s total duration) with an intermediate air saturation for 60 min. Both the ultrasound treated and the control samples were first boiled for 2 min at 100 °C in an equal volume of 125 mM Tris-HCl pH 6.8 containing 2% SDS and 5% β-mercaptoethanol and then separated on a 3% acrylamide – 0.5% agarose composite gel. The protein bands were stained by Coomassie Brilliant Blue (Serva R 250), destained with a series of methanol-acetic acid solutions and scanned by a high precision laser densitometer Pharmacia-LKB UltraScan-XL. Under the above staining conditions, the absorbance of the protein bound Coomassie stain is linearly proportional to the ovalbumin concentration. The calibration was performed using thyroglobulin (330 000 g/mol), ferritin (220 000 g/mol), ovalbumin (43500 g/mol), ovalbumin dimer and ovalbumin oligomers as molar mass standards. The viscosity of 1% ovalbumin solution in 50 mM Na/K-phosphate buffer pH 7.0, was measured at 20.0 °C using an Ostwald viscometer. The control and ultrasound treated samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE). The quantification of protein products obtained after ultrasound treatment was performed by comparison of the respective integral area of the control and the treated sample, using an Origin 4.0 PC software package.

RESULTS AND DISCUSSION

To test the validity of the lower molar mass (*LMmL*) postulate, a series of ovalbumin oligomers was prepared by the radiolytic cross-linking of a 1% ovalbumin monomer solution in 50 mM Na/K-phosphate buffer pH 7.0 saturated with N_2O. Under these conditions, the irradiation of the sample with 5 kGy from a ^{60}Co source, yielded ovalbumin dimers, trimers, tetramers, and higher order oligomers. The obtained set of ovalbumin oligomers represents and attractive substrate for testing the ultrasound postulate about the existence of a *LMmL* for several reasons. Thus, in contrast to the more native proteinaceous substrates, (*e.g.*, ribosome, nucleosomes, proteasomes) whose extraction and purification from living cells is tedious and produces only small quantities of the final preparate, ovalbumin oligomers can be obtained in large quantities necessary for the ultrasound treatment experiments.

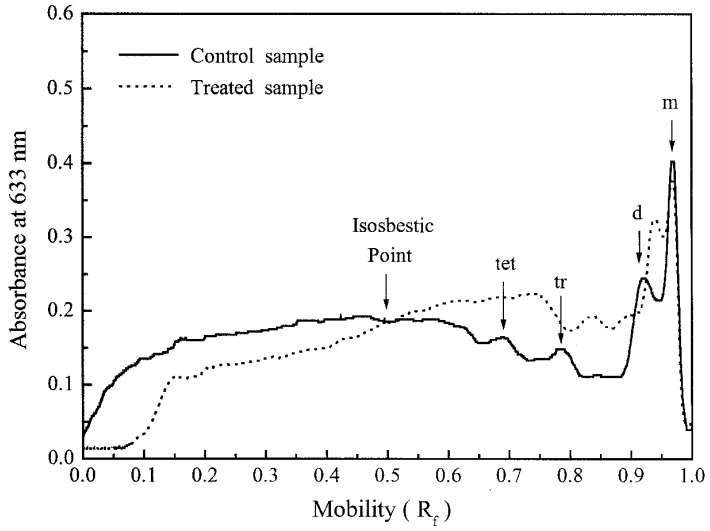

Fig. 1. Absorbance at 633 nm of electrophoretically separated ovalbumin samples *versus* the corresponding mobility (R_f) values. Arrows indicate the isosbestic point, and the position of the ovalbumin monomer m), dimer d), trimer t), and tetramer tet).

Also, their degradation by ultrasound is easily followed using composite gel electrophoresis, and, due to the small differences in molar masses of adjacent ovalbumin oligomers, the experimental error of the *LMmL* determination is less than 10%. The molar mass of the cross-linked ovalbumin products, prepared under the described conditions ranges from monomer, with a molar mass of 43 500 g/mol, to oligomers whose molar mass is approximately 10^6 g/mol, as determined by electrophoresis on the composite 3% acrylamide – 0.5% agarose gel. The irradiation dose of 5 kGy was chosen, as it generated ovalbumin oligomers whose molar masses covered the wide range which was also expected to include the *LMmL* (Fig. 1, control sample). The sample of ovalbumin oligomers was treated for 300 s with ultrasound of 23 kHz frequency and 5 mm amplitude (Fig. 1, treated sample). Then, both the treated and the control sample were separated by electrophoresis on a compoosite 3% acrylamide – 0.5% agarose gel. The protein bands were visualized by Commasie Brilliant Blue staining and their absorbance at 633 nm was determined by scanning densitometer.

Figure 1 represents the absorbance at 633 nm of the control and ultrasound treated ovalbumin oligomer samples *versus* their mobility (R_f values). It shows monomers whose mobility was 0.97, dimers whose mobility was 0.92, trimers whose mobility was 0.78, tetramers whose mobility was 0.69 and pentamers and other oligomers whose mobility was in the range from zero to 0.63. The cross-section between the control and the ultrasound treated sample presents the mobility of the oligomer whose mass concentration remained constant during the ultrasound treatment. This point is termed the isosbestic point (*iso*, in Greek, means equal; and

sbestos means extinguished; *i.e.*, the point where the absorption of the two light-absorbing forms is equal). The experimental results presented in Fig. 1. were repeated several times ($n = 12$) in order to obtain a stastically significant value for R_f. Thus, the calculated R_f value of the isosbestic point was 0.496–0.020. The experimental error of these measurements was less than 5%, which indicated that the method was reproductive.

To determine the critical molar mass (*Mmc*) it was necessary to calibrate the composite 3% acrylamide – 0.5% agarose gel. For this purpose standards of known molar mass, thyroglobulin (330 000 g/mol) and ferritin (220 000 g/mol), were used. The linear dependence of the logarithm of molar mass (log *Mm*) of the standard proteins *versus* the corresponding R_f values was obtained:

$$\log Mm = -1.076\, R_f + 2.971 \text{ (correlation coefficient} = 0.99)$$

From this equation the critical molar mass (*Mmc*), which was 274 000–14000 g/mol for the ultrasound of 23 kHz frequency and 5 mm amplitude, was calculated. Thus, the ultrasound used in our experiments affected only ovalbumin oligomers whose R_f was less than 0.496, *i.e.*, those with molar mass above 274 000 g/mol. This molar mass value was the lower molar mass limit (*LMmL*) below which the macromolecules resisted further degradation.

This conclusion was further corroborated by the results of viscosity measurements. Namely, it is known from the polymer cross-linking literature, that the viscosity of a polymer increases with increasing average molar mass, although the mass concentration of a polymer remains constant.[5] In our expeirments the viscosity of the control,

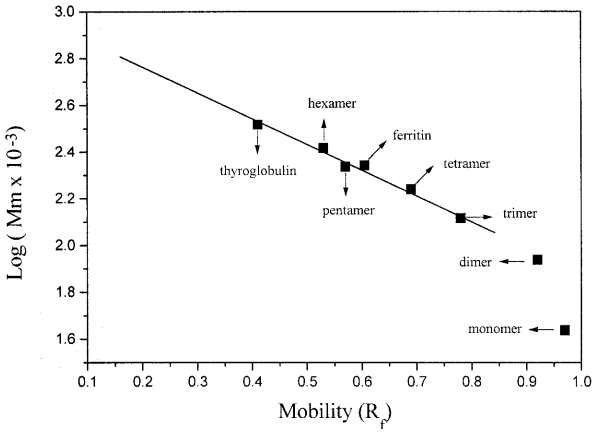

Fig. 2. The dependence of the logarithm of the molar mass (log *Mm*) on the mobility values (R_f) for each of the ovalbumin *n*-mers, and for the marker proteins: thyroglobulin and ferritin. The molar masses are given in thousands. The ovalbumin monomer and dimer lie outside the curve, as linearity holds only for the medium part of the gel.

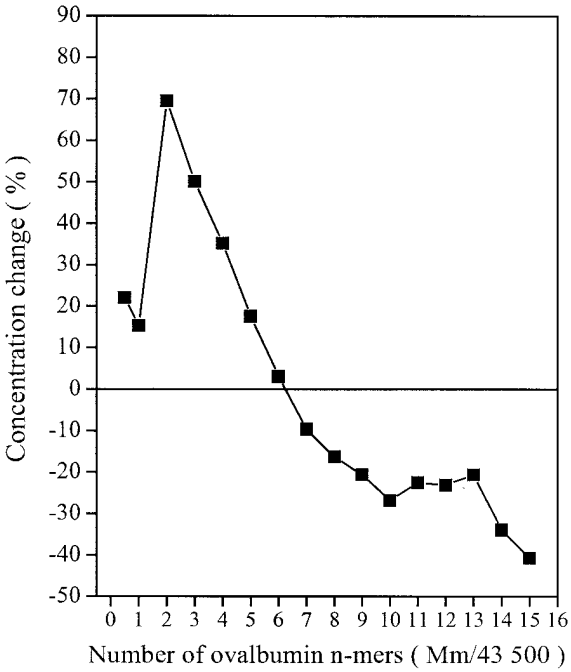

Fig. 3. The percent changes in protein concentration for each of the ovalbumin *n*-mers (molar mass of ovalbumin is 43 500 g/mol).

nonirradiated 1% ovalbumin monomer solution was 1.17 mPa s, while the viscosity of the 5 kGy irradiated ovalbumin sample was slightly higher, *i.e.*, 1.45 mPa s. This small, but persistent difference suggested that the ionizing radiation induced cross-linking of the ovalbumin, *i.e.*, the formation of ovalbumin oligomers might have affected the sample viscosity. However, after ultrasound treatment of the ovalbumin oligomers,

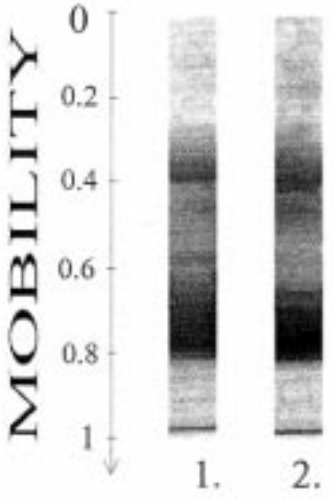

Fig. 4. The SDS-PAGE profiles of: 1. intact ovalbumin monomer and 2. ovalbumin monomer treated for 300 s by ultrasound of 23 kHz frequency and 5 mm amplitude.

the viscosity dropped to 1.34 mPa s. This value was slightly smaller compared to the viscosity of the radiolytically cross-linked ovalbumin, and, taken together with the result of electrophoretic separation shown in Fig. 1, suggested an alteration in the average molar mass of the ovalbumin oligomers after ultrasound treatment.

TABLE I. Molar masses and percent changes in the concentration of ovalbumin *n*-mers before and after treatment by ultrasound of 23 kHz frequency and 5 mm amplitude. The R_f values for the ovalbumin monomer and dimer are labelled by asterix as they are experimental values taken from Fig. 1

Ovalbumin *n*-mere	Molar mass $Mm \cdot 10^{-3}$	log $Mm \cdot 10^{-3}$	Mobility R_f	Control sample/%	Treated sample/%	Treated-Control/%
Monomer	43.5	1.64	0.97*	7.76	8.94	1.18
Dimer	87	1.94	0.92*	4.45	7.54	3.09
Trimer	130.5	2.12	0.78	6.63	9.96	3.33
Tetramer	174	2.24	0.69	8.82	11.93	3.11
Pentamer	217.5	2.34	0.57	10.91	12.82	1.91
Hexamer	261	2.42	0.53	6.98	7.19	0.21
Heptamer	304.5	2.48	0.45	5.64	5.11	–0.53
Octamer	348	2.54	0.39	5.98	5.01	–0.97
Nonamer	391.5	2.59	0.35	6.76	5.37	–1.39
Decamer	435.4	2.64	0.31	4.41	3.23	–1.18
11-mer	478.5	2.68	0.27	3.99	3.09	– 0.90
12-mer	522	2.72	0.24	3.39	2.61	– 0.78
13-mer	565.5	2.75	0.21	3.09	2.45	– 0.64
14-mer	609	2.79	0.18	3.03	2.00	–1.03
Oligomer ‡ 15 mer	800	2.90	0–0.18	2.70	1.60	–1.14
Fragments	10	1	0.92–1	2.36	2.89	0.53
Ovalbumin	43.5	1.64	0.97			
Ferritin	220	2.34	0.61			
Thyroglob.	330	2.52	0.41			

To calculate the molar mass of each of the obtained *n*-mers of ovalbumin, a calibration diagram of log *Mm versus* R_f value was constructed (Fig. 2). The percent change in the concentration of each ovalbumin *n*-mere was calculated before and after ultrasound treatment. The results are shown in Table I and in Fig. 3. It can be seen that all ovalbumin *n*-mers whose molar mass exceeded 274000 g/mol (*i.e.*, 6-mers and above) were significantly degraded by treatment with ultrasound of 23 kHz frequency and 5 mm amplitude. The results shown in Fig. 3 may also be interpreted in such a way that all the macromolecules present are degraded, but that the degradation probability depends on the molar mass. In this case a similar change

in molar mass distribution might be observed. However, the opposite argument was obtained when a 1% ovalbumin monomer solution was treated for 300 s with ultrasound of 23 kHz frequency and 5 mm aplitude, and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 4). As may be observed in Fig. 4, after ultrasound treatment the SDS-PAGE profile corresponding to the treated sample (lane 2) remained the same as the profile of the untreated 1% ovalbumin (lane 1). This result speaks in favour of our determination of the *LMmL*, according to which the value of the molar mass of ovalbumin (43500 g/mol) is well below the critical molar mass of 274000 g/mol, and, therefore, ovalbumin monomer would not be affected by the ultrasound treatment.

Taken together, our results confirm that the two postulates of the chemistry of polymer degradation by ultrasound are valid in the case when ovalbumin oligomers were used as the substrate. Namely, our results clearly show that the higher the molar mass of ovalbumin oligomers, the faster was their degradation, and they also indicate the existence of a lower molar mass limit (*LMmL* = 274000 g/mol) below which the ovalbumin oligomers resist further degradation.

И З В О Д

ОДРЕЂИВАЊЕ КРИТИЧНЕ МОЛАРНЕ МАСЕ ОЛИГОМЕРА ОВАЛБУМИНА ТРЕТИРАНОГ УЛТРАЗВУКОМ

ДРАГАНА ФИЛИПОВИЋ, МАРИЈА РАДОЈЧИЋ И БРАТОЉУБ Х. МИЛОСАВЉЕВИЋ

Институт за нуклеарне науке "Винча", п.пр. 522, 11001 Београд

Развили смо методу која омогућава одређивање критичне моларне масе протеина (*KMm* или *LMmL* од енглеског "lower molecular mass limit") третираних ултразвуком познате фреквенције. Да би проверили претпоставку о постојању *KMm*, направљена је серија олигомера овалбумина радиолитичким умрежавањем 1% овалбумина (макромономера) у 50 mM K/Na-фосфатном пуферу pH 7,0, засићеном N_2O. Озрачавањем узорка дозом од 5 kGy из извора ^{60}Co добијени су димери, тримери, тетрамери и олигомери овалбумина већих моларних маса. На основу резултата добијених на овом супстрату закључили смо да је за ултразвук фреквенције 23 kHz и амплитуде 5 mm критична моларна маса олигомера овалбумина 274 000 – 14 000 g/mol. Наши резултати су потврдили да два постулата о деградацији полимера ултразвуком важе у случају деградације олигомера овалбумина, односно да се: (1) олигомери овалбумина брже деградирају уколико је њихова моларна маса већа, и да (2) постоји критична моларна маса *KMm*, те да олигомери чија је моларна маса мања од *KMm* не подлежу разградњи у току третмана ултразвуком.

(Примљено 26. маја, ревидирано 28. септембра 1999)

REFERENCES

1. G. R. ter Haar, in *Physics in Medicine and Biology, Encyclopedia*, F. T. McAinsh, Ed., Pergamon Press, Oxford, 1986, p. 843
2. J. Adler, O. Necas, I. Hrazidra, *Folia Biologica (Praha)*. 39 (1993) 55
3. M. Dyson, in *Biological Effects of Ultrasound*, W. Nyborg and M. Ziskin, Eds., Churchil Livingston, New York, 1985, p. 121

4. A. J. Mortimer, in *Proceedings of International Symposium on Therapeutic Ultrasound*, A. Mortimer and N. Lee, Eds., Canadian Physiotherapy Association, Winnipeg, 1981, p. 67
5. L. Santra, B. K. Chatterjee, D. Bhaumik, S. C. Roy, *Physical Review A.* **43** (1991) 4296.