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New Non-Destructive Method for the Analysis of Domains' Distribution in Proteins and Biomatrices. Nature of Domains

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Abstract A new non-destructive method to analyze rapidly the domain structure of proteins (lysozyme, chymotrypsinogen-A, ovalbumin, albumin), protein solutions (water and buffer systems), and biomatrices containing proteins (hen eggs) has been developed. The gravitational mass spectra of lysozyme and chymotrypsinogen-A obtained from their solutions in buffer and water are given, the signals of domains, coils, sub micellar and micellar structures are discussed. Domains, coils and coils of protein associates are concluded to be emerged compelled at the expense of energy clusters (EC) in stationary gravitational waves of the space, in accordance with EC only certain domains are able to exist.

Keywords Proteins, Domains, Distribution

1. Introduction

It is known that macromolecule coils are inhomogeneous usually explained by hydrogen or donor-acceptor bonds between the functional groups of polymers that form clusters or domains. Clear understanding of the thermodynamics of such processes is still lacking in the literature. There is however, the basic idea developed by the authors that the Earth penetrating gravitational white noises stabilize organized structures[1]. According to this idea the energy clusters (EC) in the gravitational noises promote the formation of molecular clusters that means the last ones take the energy for their existence from EC[2, 3].

The dynamics of changes in the coils' heterogeneity in proteins is an important part to understand the transformation of conformations under the influence of various environmental factors. For this understanding especially, in applied biochemistry a new non-destructive analysis method is needed. On the other hand, a serious problem in modern biochemistry is the lack of a fast and accessible method to investigate in vivo and in real time. It refers to the structures of proteins and their associates and to the cluster formation in biological liquids furthermore to solvated clusters of acids/bases, salt ion pairs, water clusters as well as to their interaction with surroundings. Understanding the full range of these processes as an unified, will allow a new level of problem solving in applied biophysics and biochemistry.

It is therefore the aim of the present work to use the gravitational mass spectrometry for the analysis of mass heterogeneities in proteins and protein containing biomatrices.

2. Material and Methods

In this work, on the example of simple proteins and their solutions in distilled water we tried to show the possibilities of the GMS-method (earlier called as FNS-method)[2-5] for the analysis of the heterogeneity in proteins. The investigation objects were chicken eggs, lysozyme solution (14 kDa, Sigma) in sodium phosphate buffer (2 mg in 1.4 ml of buffer: pH = 7.5, 137 mmol NaCl, 2.7 mmol KCl, 12 mmol $Na_2HPO_4 \cdot 2H_2O$, 1.2 mmol KH_2PO_4) and chymotrypsinogen-A solution (Sigma, 25,651 Da) 12 mmol/l. The GMS sensor is placed directly into the sample. The electrophoresis of proteins denatured at 368 K proceeds in the PAA-gel (polyacrylamide gel, 12 wt. % SDS buffer) at 20 μA and 290 K. The unreduced egg white is diluted in SDS (1:10, 5 min. 368 K) and 9 μl of which is used for the electrophoresis. The same procedure is made for the egg yolk, but with a buffer dilution of 1:50 and a sample volume of 4.5 μl. The molecular weights of denatured proteins are analyzed in 12 wt. % PAA-gel.

Measurement method is described in[3]. Before the measurement, the samples are stored for a week under exclusion of light and mechanical fields. The GMS sensor is

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directly placed inside the sample (10 ml). Here the sample was placed in a special earthed, metal box that was isolated from energy flows (heat, noise, mechanical and light)[2]. The negative signal in the GMS (*-f*) represents the energy release of a collapsed cluster leading to its higher density and crystallization. On the other side the positive signal (*f*) means the energy absorption at the interaction of the shock wave with the expanded cluster and its melting. The values of collapsed clusters are therefore described with minus and that of expanded one with plus. More information, see in[2]. M_{GMS} – average molecular mass of all clusters in Daltons (Da), $M_{GMS}=\sum|f|\cdot m)$, m – cluster mass (Da), f – energetical part of a cluster in ensemble of clusters, *-f* part of collapsed clusters, D_c – sum of all collapsed clusters and N – number of cluster kinds in ensemble, p – shock wave pressure.

3. Results and Discussion

In the Figure 1 the GMS-spectrum of lysozyme is given.

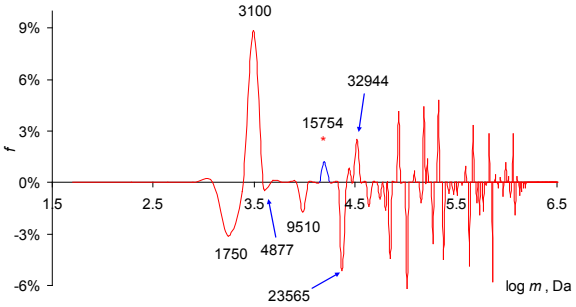
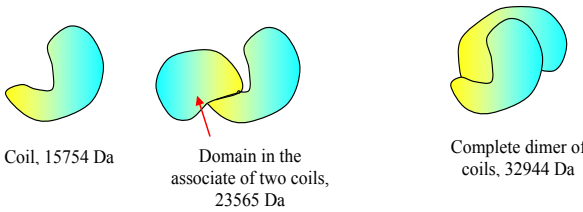


Figure 1. General GMS-spectrum of lysozyme. GMS is obtained by subtracting the spectra of the solvent clusters from those of the lysozyme solution clusters (mass ensembles up to 3.2 MDa). The Zubow constant amounts to $6.2\cdot10^{-15}$ N/m. 293 K. * - lysozyme coil signal. Scanning time - 30 s

Table 1. Cluster masses in the GMS-spectrum of a lysozyme solution. The spectra were obtained by subtracting the spectra of the clusters in the solvent from those of the clusters in the lysozyme solution (mass ensembles up to 16.3 kDa).

Oscillator kind 293 K	Mass part of cluster %	*m*, Da	Cluster form
domain	up to 21	1750	collapsed
"-"	up to 3	2393	"-"
"-"	up to 60	3100	expanded
"-"	up to 2	3938	collapsed
"-"	up to 1	4877	expanded
"-"	up to 12	9510	collapsed
coil		15754	expanded

As visible in Figure 1, the GMS subtraction spectrum reveals 3 main signals at 1750, 3100 and 9510 Daltons (domains in the lysozyme coil), where the coil itself oscillates as a weak expanded cluster with the mass of 15,754 Da. The signals of larger masses than the mass of the coil are ascribed to coil associates, sub- micellar and micellar structures that are formed by coils in the solution. For example, the cluster with the mass of 32,944 Da belongs to the lysozyme dimer consisting of two coils and 4.4 wt. % water. Water seems to be there, where the coils contact each other. The cluster signal with the mass of 23,565 Da being 1.5 times higher the mass of the lysozyme coil itself represents an unique cluster that appears in all molecular systems[6, 7]. It is possible that this oscillator is formed compulsorily between one coil, the two domains of 3,100 and 4,877 Da and 9 water molecules (bound water). Such an oscillator is shown in the scheme below as a part of an associated coil; it differs from the complete dimer only by the conformation.



According to a computer simulation on the base of NMR data lysozyme coils are found to consist of at least 3 areas of mass concentrations (60, 20 and 10 %, Figure 2)[8]. This agrees well with GMS, which analyzed 3 types of large oscillating masses with weight fractions of 60, 21 and 12 % (Table 1). The practical coincidence of these values has encouraged the authors to develop the method for use in biochemistry on. With the GMS-method a fast and direct determination of cluster/domain masses in Daltons is possible.

The effect of pressure on the coil structure of lysozyme investigated by Rafaee[8] can be discussed in terms of a changed quality of white noises. Strengthening the noise background (translation into colored noise) as the result of increased pressure should lead to changes in organized systems such as protein coils too.

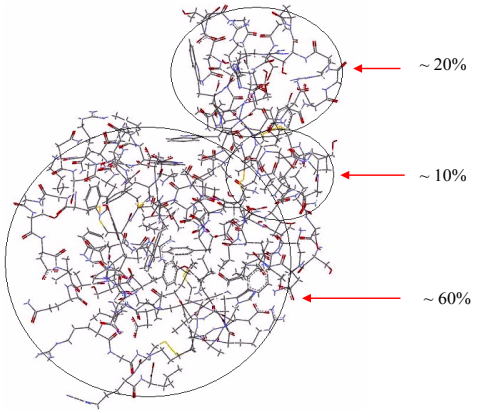


Figure 2. Computer model of a lysozyme coil on the base of NMR data [8], which is used for the comparison with the GMS results (Table 1)

The signal of the lysozyme coil in the GMS spectrum is rather weak and is about three times less than that of the most common dimeric form of this enzyme[9]. In the lysozyme solution at 293 K reveals a strong signal ($\log m = 4.51$) which is attributed to its dimer (expanded cluster, f = 3%). According to calculations based on GMS data the dimer is analyzed to contain 8.9 wt. % water. The molecular mass of the oscillating coil amounts to 15,754 Da, which differs by

11 % of the mass (14 kDa) that is determined by Sigma. This difference has been attributed to solvated water in the crystalline lysozyme (9 wt. %,[10]).

Hence, already in the solution the dimer is to be regarded as a seed crystal. In the lysozyme coils themselves, no water clusters have been found; water seems to be present in an other form in the coils, therefore.

With GMS, it is possible to get an idea on the heterogeneity inside the protein coils at the level of clusters /domains furthermore, to determine the cluster mass fractions as well as the masses of individual clusters themselves.

GMS-spectra of this enzyme can be recorded both in the solid (powder) and dissolved state at all temperatures and pressures. These features facilitate to investigate the mutual cluster transformations (expanded↔collapsed), cluster interactions with surroundings inside the coil and the coil interactions with neighbors. Additionally, signals of coil associates in solutions (dimers, trimers, etc.) can be analyzed, which allows to determine fast the number of coils in these associates. Similar studies are carried out to analyze clusters in the coils of aldolase and chymotrypsinogen-A in PBS-buffer. Here oscillating clusters are found too and the cluster distribution at different temperatures can be calculated. Furthermore, using the method it is possible to get some idea on the state of water included inside the coils, on the degree of clusters' agglomeration in associates and on their stability in solutions (Table 3).

Figure 2 shows the dynamics of the cluster signals in egg white in vivo at heating. Comparing the information provided in Figure 2 with the data obtained by electrophoresis separation of the components in hens eggs (Figure 3 and Table 2), the main egg components can be identified as well as structure and density changes under temperature increasing. Concerning the molecular masses of the ingredients in egg yolk and egg white, there has been found a good match between the GMS and electrophoresis data (Figure 3). This encouraged the authors to propose GMS as a new rapid analysis method (1…30 s) to determine the masses of domains and coils in proteins and to investigate their interaction with the surroundings. On the other hand, comparing the results from GMS and electrophoresis, it is possible to analyze the content of water and salts in the protein coils. In addition, super polymeric structures like dimeric, tetrameric, etc. protein coils that oscillate as a single unit (Table 2) can be recorded. This table shows a comparison of the results obtained by GMS and electrophoresis; it is based on the understanding that mass concentrations (clusters/domains, coils) are formed compulsorily under the influence of white noises[2]. To avoid from anthropogenic noises (energy fields), the samples are kept in a box most isolated from these influences.

As visible the masses of GMS are slightly higher than that of the electrophoresis where these differences are ascribed to included water and possibly, to salt traces. The water concentration in coils is in the range from 2.2 to 10 wt. % (from 57 to 897 molecules), the smaller the mass of the coil the higher the content of water.

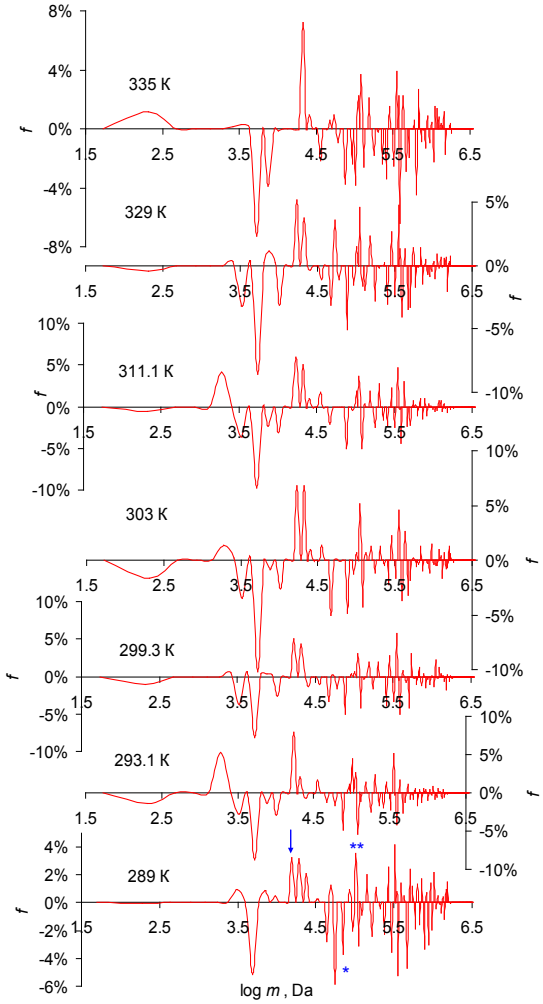


Figure 2. GMS-spectra of egg white biomatrices (in vivo) from free range hens. p> 1 N/m^2, Zubow constant = 6.2 · 10^{-15} N/m, scanning time at each temperature is 30 s, arrow - lysozyme coil, * ovalbumin coil, ** albumin coil

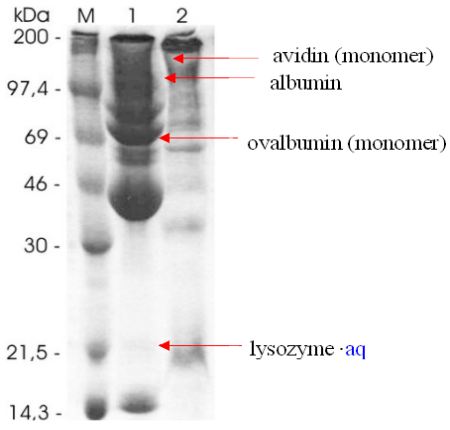


Figure 3. Protein electrophoresis of egg white (1) and egg yolk (2) at 293 K. M - reference substance being a mixture of proteins with known molecular weights ("Rainbow", Amersham Life Sci. Ltd)

The oscillation signals of the mass concentrations (domains) reflecting the albumin coils, show that in egg yolk at

heating, the coils change their form from the expanded (293 K) to collapsed while they are present in the egg white only in the collapsed form in the whole temperature interval.

Regarding the lysozyme coils in the egg white their expanded form reaches a maximum at 293 and 311 K where at 335 K the density of the coils even increases. Since the signal intensity (f) reflects the coils' interaction with the surroundings two variants how the temperature influences the density of the coils are possible:

1. internal changes of the macromolecules' conformations leading to a loosening of the coils (weakening of the cohesive interactions),

2. reinforcement of the adhesive interactions between the coil surfaces with surroundings.

If we assume that the conformation of macromolecules in the coil does not change significantly in a narrow temperature range, the large $f|$ values suggest a decrease in adhesive interactions of cluster masses (coils) with the surroundings and an increase of the cohesive interactions in them. The more the signal intensity in the GMS spectrum the less the interaction between oscillator and the surroundings and the higher its individualization degree.

Collapsed clusters are observed to act as seed crystals of regular, crystalline structures therefore; they could be interesting objects for studies in biochemistry. In Figure 4 is shown how the sum of collapsed clusters in egg yolk changes at heating (12 K/h). The change does not proceed monotonously, the curve is characterized by some extremes for example, the maximum at 305 K may indicate a larger number of them at temperatures being close to the formation of hens embryos.

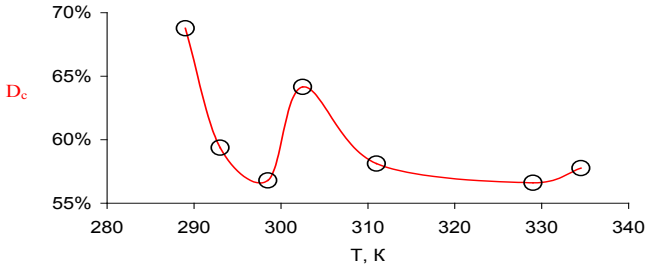


Figure 4. Temperature dependence of the sum of collapsed clusters (Σ |(-f)|) in egg yolk

Thus the transformation of "white noise" in "color noise"[1] by heat flow changes in the cluster structure of egg yolk qualitatively. The data presented in Figure 5 illustrate the nature of such changes.

Figure 5 presents the GMS spectra of egg yolk clusters at heating in the dashed form. As visible egg yolk contains stable super cluster formations, marked by arrows however, there are slight changes in the cluster distribution. With a more prolonged thermostating these differences become more pronounced, which can be applied to understand the beginning of an embryo formation.

The presentation of the spectra in the dashed form promotes the automation of data administration. Strichcodes are a kind of "fingerprint" of a given biological system they clearly show that the distribution of clusters in biomatrices is irregular.

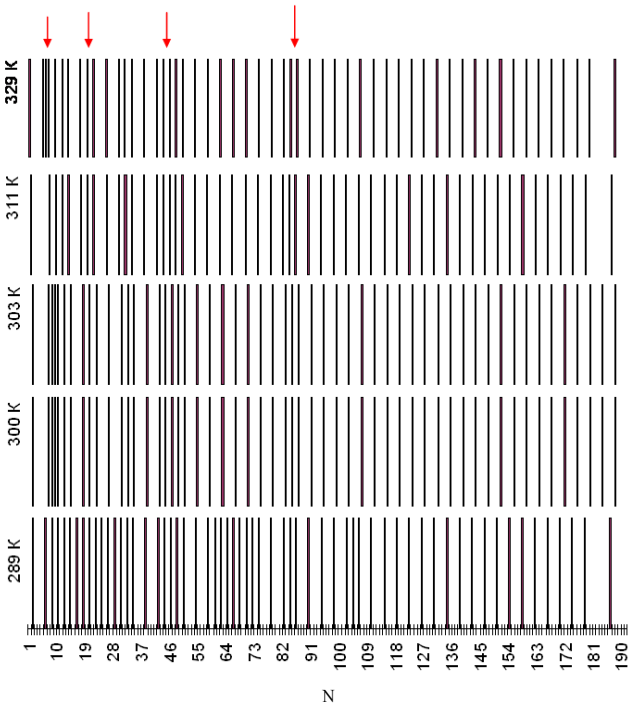


Figure 5. GMS spectra (dashed form) of clusters in egg yolk at heating (12 K/h). Every line corresponds to one cluster regardless of its signal intensity, the distribution of clusters is described by the following equation: log (m) = 0.9214 · ln (N) + 2.3216, where m is the mass of the cluster in Daltons, N - its serial number in the spectrum (see Table 3). Before each measurement, there will be a 30-minute thermostating

Clusters themselves form super cluster structures. Such super cluster structures and their formation dynamics at heating/ageing of biological systems as well as their interaction with physical fields and the effects of chemical substances on them may be an interesting research object for biochemists.

In Figure 6, the GMS-spectrum of chymotrypsinogen-A is given.

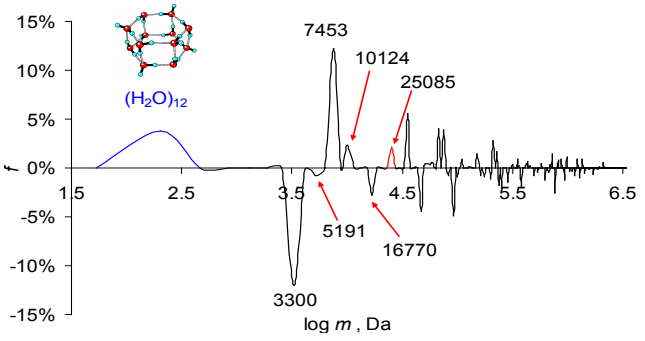


Figure 6. Overview GMS-spectrum of chymotrypsinogen-A. It is obtained by subtracting the water cluster spectrum from the chymotrypsinogen-A solution spectrum (mass ensembles up to 3.2 MDa). Zubow constant = 6.6 10^{-15} N/m, 293 K. The numbers above the signals means the mass of the main domains and of the coil (25,085 Da). The model of the base water cluster has been kindly provided by Professor Lenz. Scanning time is 30 s

Table 2. Comparison of GMS (30 s) and electrophoresis (Figure 3) on the example of hen eggs. Regarding GMS the mass concentrations and their signal intensities are taken into account

Electrophoresis	335 K (GMS)		311 K (GMS)		293 K (GMS)		included H_2O	
log m	egg white	egg yolk	egg white	egg yolk	egg white	egg yolk	mass difference*	
lysozyme ·aq	log m	log m	log m	log m	log m	log m	log m	molecules
4.16	4.2	4.2	4.2	4.2	4.2	4.2	3.16	81
	-0.1 %	0.7 %	6 %	2.9 %	7.9 %	-0.1 %		
	ovalbumin·aq							
tetramer	5.27	5.27	5.27	5.27	5.27	5.27	4.21	897
	-0.2 %	-1.2 %	2.7 %	-0.1 %	2.4 %	0.2 %		
monomer	4.64	4.64	4.64	4.64	4.64	4.64	3.01	57
4.63	0.6 %	-2.1 %	-2.1 %	-0.6 %	-1.2 %	0.2 %		
	avidin (sub. oxidase)							
dimer ·aq	5.21	5.21	5.21	5.21	5.21	5.21	3.56	203
5.20	-1.8 %	+0.4 %	-2.5 %	-1.5 %	-2.5 %	5.2 %		
monomer ·aq	4.93	4.93	4.93	4.93	4.93	4.93	3.76	321
4.90	-2.3 %	-0.4 %	-0.5 %	-2.5 %	1.1 %	0.5 %		
	albumin							
4.84	4.85	4.85	4.85	4.85	4.85	4.85	3.12	73
[11]	-3.8 %	-3.4 %	-5.1 %	-4.2 %	-4.9 %	0.5 %		

where: * -difference of masses obtained by GMS and electrophoresis.
(-) – collapsed cluster (+) – expanded cluster.

Table 3. Real signals of domains in chymotrypsinogen-A coils (Sigma, 25,651 Da). Aqueous solution (0.012 mmol/l), 310 K. For the spectra calibration regarding the energy, the formation/destruction energies of known water clusters[5] are used, which have been determined in the same measuring cell. Comment in the text

Part of mass %	*m*, Da	ω, kHz	E, kJ/mol	Oscillators
0.2	2547	6.3	0.004	expanded domain
16	3300	5.5	0.616	"-"
1	4193	4.9	0.019	"-"
15	5191	4.4	-0.396	collapsed domain
0.3	6246	4.0	0.011	expanded domain
47	7453	3.7	-1.626	collapsed domain
0.01	8767	3.4	-0.002	"-"
0.2	10124	3.2	-0.216	"-"
20	16770	2.5	1.687	expanded domain
	25085	2.0	-1.804	collapsed coil

Table 4. Clusters/domains (log m) and oscillation frequencies under the influence of shock waves observed in liquids, polymers, biomolecules (explanation in text)

№, N	Water 293K	Methanol 293 K	*p*-Xylene 293 K	*n*-Hexane 293 K	Agarose gel 3 wt. %	Egg white 293 K	Egg yolk 293 K	Chymotrypsinogen-A 293 K	Amylopectin 295 K	ω, Hz
1	2.30	2.28	2.28	2.28	2.30	2.30	2.30	2.31	2.32	22222
2			3.24	3.24	3.26	3.26			3.28	7353
3	3.39	3.38			3.39			3.41		6289
4	3.51	3.49			3.51	3.51	3.51	3.52	3.53	5525
5	3.61	3.60			3.61			3.62		4902
6	3.70	3.69			3.70	3.70	3.70	3.72	3.73	4405
7	3.78	3.77						3.80		4016
8	3.86	3.85			3.86	3.86	3.86	3.87	3.89	3676
9	3.93	3.92						3.94		3390
10	3.99	3.98			3.99	3.99	3.99	4.01	4.02	3155
11					4.11				4.14	2755
12	4.21	4.20			4.21	4.21	4.21	4.22	4.24	2451
13	4.30		4.29	4.29		4.30	4.30			2203
14	4.39	4.37	4.37	4.37	4.39	4.39	4.39	4.40	4.41	2004
15			4.45	4.45						1838
16	4.53	4.52	4.52	4.52	4.53	4.53	4.53	4.54	4.56	1695
17			4.58	4.58	4.60					1575
18	4.65	4.64	4.64	4.64	4.65	4.65	4.65	4.67	4.68	1471
19	4.71	4.70	4.70	4.70		4.71	4.71	4.70		1377
20	4.76	4.75			4.76	4.76	4.76	4.75	4.74	1297
21		4.80			4.81			4.78	4.79	1225
22								4.83		1192
23	4.86	4.85	4.85	4.85	4.86	4.86	4.86	4.87	4.89	1160
24		4.89	4.89	4.89	4.91			4.92		1103
25	4.95	4.93	4.93	4.93	4.95	4.95	4.95	4.96	4.97	1050
26	4.99	4.97			4.99	4.99	4.99	5.00		1002
27	5.03	5.01	5.01	5.01	5.03	5.03	5.03	5.04	5.05	959
28	5.06					5.06	5.06			919
29	5.10	5.09	5.09		5.10	5.10	5.10	5.11	5.13	882
30		5.15	5.15	5.15						832
31	5.17	5.18			5.17	5.17	5.17	5.18	5.19	817

The spectrum of chymotrypsinogen-A contains the signals of the base water clusters[12, 13] which are as independent kinetic units are included in the protein. The analysis of the main signals of this protein is given in Table 3. Table 3 presents the main characteristics of domains in chymotrypsinogen-A coils, recorded with the highest GMS sensitivity. To obtain the real signals of the enzyme the solvent signals are subtracted from those of the enzyme solution. As visible from the Table 3 the chymotrypsinogen -A coil is not homogeneously. It consists at least of nine domains that are characterized by different densities and formation energies. Investigating the temperature influence on intracoil processes and the coil's conformation both are observed to be relatively stable at domains' level and unstable at microdomains' one (smallest clusters).

Comparing the data of this table with computer protein models (PDB) a satisfactory compliance has taken however, it should be noted that the data of PDB bases on crystalline enzyme structures, that don't really reflect their native state.

The successful application of the GMS-method for the rapid analysis of casein, collagen and proteins in muscle tissue is described in[4, 6].

The sequences of investigated clusters in natural waters[14], distilled water[5], polymers[7, 15, 16], solutions[17, 18], protein coils and polysaccharides (lysozyme, L-arginine, chymotrypsinogen-A, amylopectin, etc.[6]) are characterized by similar masses. Common to all is a series consisting of the cluster masses shown in Table 5 (only 31 members are listed in an ordered sequence). These sequences including lysozyme (Table 1) are satisfactorily described by the following Zubow equations:

$$\log m = (0.80\pm0.13)\cdot\ln N + (2.34\pm 0.12)$$

where N is the order cluster number in the spectrum beginning with the smallest mass of 0.2 kDa (N = 1).

The Zubow equation[19] relating the cluster mass (m) with its oscillation frequency (ω) is the following:

$$m = 10^{11}\cdot\omega^{-2}$$

The applicability of the Zubow equations for all molecular matter compounds points to a common mechanism for the formation of clusters/domains, which get the energy for their organization from energy clusters[2, 6].

4. Conclusions

GMS method allows more accurate, more reliable and faster to determine the mass of protein domains in coils as well as the mass of coils and coil associates additionally, to get an idea on sub micellar and micellar coil structures of proteins in their solutions and biomatrices.

Under anthropogenic influence the white noise is transformed in color one, which change the stabilizing effect of noise on organized systems hence long-term effects of anthropogenic noises in urban environments, factories, transport is assumed to affect at least the super cluster structure of biological systems. In our opinion, there should be a limit of such an overload, above which irreversible changes in biological systems take place e. g. in protein conformations, water clusters, solvated clusters of ion pairs, which means, in the whole biomatrix and as a consequence to accelerate mutagenesis processes.

At the domain formations in proteins and biomatrices hydrogen and donor-acceptor bonds are observed to play a secondary role, only while the energy clusters in stationary gravitation waves play the "first violin", they serve as an energy source for the formation of domains and clusters.

Other gravitational fluctuations (energy cluster state in stationary gravitational waves) e.g. outside of our Galaxy change the domain distribution in proteins, what should bring about new properties of proteins and automatically new life forms.

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