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INFLUENCE OF LYSOZYME ON CHANGES IN SIZE OF CASEIN MICELLES IN MILK

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Key words: lysozyme, casein micelles, cow's milk, ultracentrifugation, light scattering.

The influence of lysozyme on changes in the dimensions of casein micelles in cow's milk was studied. Their size was determined by the fractionation of milk, applying the centrifugation technique as well as by measurements of light scattering. The dimensions of micelles were also determined with the use of an electron microscope. The conducted studies showed that the addition of lysozyme to milk caused certain modifications of casein properties and an increase in the dimensions of its micelles. The obtained results may be useful in an explanation of the method and suitability of milk for modification by this enzyme e.g. in production of baby food and in cheese manufacture.

INTRODUCTION

Studies of the dimensions of casein micelles in cow's milk have revealed a wide range of their sizes which, according to many research workers, can be connected with the physicochemical changes of their properties [1, 2, 11]. It is also commonly considered that there is a quite close relationship between the distribution in size of these micelles and the stability of the colloidal system of casein [10, 11]. This in turn, leads to the assumption that the factors which cause changes in the size of micelles may also cause changes in casein's stability. These statements became the basis for the present studies on the influence of lysozyme upon the changes in casein micelles in cow's milk.

MATERIAL AND METHODS

In the experiments, 5-times crystallized lysozyme of hen egg white, produced by Serva company, was used.

The size of casein micelles was determined by three different methods:

— indirectly by fractionation of micelles by the centrifugation technique,

— by measurements of light scattering,
— directly by means of an electron microscope.

A. FRACTIONATION OF CASEIN MICELLES BY THE METHOD OF MILK CENTRIFUGATION

The experiment was performed with bulk fresh milk and the applied additives of lysozyme were: 0,5; 1.0 and 2.0 mg/cm^3. Casein micelles were centrifuged in an ultraseparator Beckman L-565, with the use of rotor Ti 70, applying three different values of acceleration: 10×10^3 g(fraction I — big micelles), 47×10^3 g(fraction II — medium micelles and $10^7 \times 10^3$ g/fraction III — small micelles). The centrifugation conditions were selected on the basis of the experiments of Saito [14] and in the obtained sediments, the following values were determined: total nitrogen compounds content [4] calcium and phosphorus content [4] values of isoelectric points of the separated fractions [14].

B. MEASUREMENTS OF LIGHT SCATTERING IN SOLUTIONS OF MILK MODIFIED BY LYSOZYME

The experiment was conducted on the basis of a relationship between the scattering of light by the colloidal particles and their size [57]. The phenomenon of scattering was examined by two methods:
— measurement of intensity of light scattered under 90° — I_{90} with the use of a specially constructed device,
— measurement of attenuation of the light beam passing through the diffusing solution, i.e. measurement of turbidity (τ), (Spectrophotometer Opton PMQ MM 12 a).

The samples of milk used in the experiment were prepared on the basis of a method given by Holt (8) and milk was diluted with an artificially prepared salt solution according to Jonnes and Koops [9]. In the studies, two additives of lysozyme: 1.0 and 2.0 mg/cm^3, were used.

C. DETERMINATION OF DIMENSIONS OF CASEIN MICELLES, USING AN ELECTRON MICROSCOPE

In this part of the experiment the microscopic technique for determination of micelles sizes was applied according to the method suggested by Schmidt et al [15]. On the surface of 10 photographs the diameters of micelles with a division into 12 size classes, were measured. The percentage share of micelles in the particular size classes was calculated in relation to the total amount of micelles visible in the photograph. Curves of micelle distribution were evaluated statistically by Smirnov test [3].

RESULTS AND DISCUSION

The effect of lysozyme on casein of cow's milk changes some of its properties, with a simultaneous lowering of the stability of this protein [12, 13]. Changes in hydration and volume of casein micelles, affected by lysozyme have led to the assumption that there is a partial aggregation of casein micelles and induced us to determine their dimensions in milk treated with this enzyme.

Characteristics of micellar fractions obtained from the ultracentrifugation of milk showed that the distribution of micellar dimensions measured by the amount of protein in the particular fractions for samples with lysozyme and for control samples, was similar. The quantities of protein in subsequent fractions separated from the enzyme-modified milk were, however, higher in comparison with the corresponding fractions of the control samples (Tab. 1).

Table 1. Content of protein in fractions of casein micelles obtained by ultracentrifugation of lysozyme-modified milk

Kind of sample	% of protein in relation to the total content of casein in milk (2.46 g/100 g of milk)			
	fraction			soluble casein
	I	II	III	
Control	36.4	52.9	5.9	8.9
With the addition of lysozyme:				
0.5 mg/cm^3	36.0	52.6	6.2	5.0
1.0	38.9	53.6	6.7	4.5
2.0	39.8	54.2	6.7	1.5

In conformity with changes in the quantities of protein in the particular fractions, the level of calcium and phosphorus in the separated sediments of proteins was also changed. Differences in the content of these components between the control samples and those with lysozyme appeared only in case of its highest addition (2.0 mg/cm^3). The calcium to phosphorus ration in the separated fractions did not reveal any distinct tendencies to change (Tab. 2).

It is know that the particular fractions of casein (α, β, $\varkappa$) reveal various values of the isoelectric points from 3.7 to 4.9 pH [1, 10]. It is also know that the share of

Table 2. Content of calcium and phosphorus in fractions of casein obtained by ultracentrifugation of lysozyme-modified milk

Kind of sample	Content of calcium mg/100 g milk			Content of phosphorus mg/100 g milk		
	fractions			fractions		
	I	II	III	I	II	III
Control	43.39	53.29	8.13	22.44	27.86	2.66
With the addition of lysozyme (mg/cm^3):						
0.5	43.21	51.55	8.32	22.44	27.86	3.01
1.0	49.23	52.91	9.30	22.19	29.32	2.92
2.0	49.77	54.65	8.65	22.03	29.48	3.11

the particular casein fractions is not constant. It changes with its size. On the basis of these relationships, values of the isoelectric point of the obtained fractions were determined. In the samples with lysozyme, isoelectric point of subsequent micellar fractions was: 4.60; 4.60 and 4.12 pH and in the control samples: 4.60; 4.42 and 4.12 pH respectively. The addition of the enzyme caused a certain displacement of the isoelectric point only in the case of medium-size micelles (fraction II). These changes as well as differences in the chemical composition of micellar fractions separated from lysozyme-modified and non-modified milk, may have a certain relationship with the effect of this enzyme on the size of casein micelles in milk.

In the further part of the experiment, measurements of light scattering through the lysozyme-modified milk solutions (1.0 and 2.0 mg/cm^3) were performed.

Appearance of casein micelles with differentiated dimensions from 20 to 450 nm in milk make the univocal choice of the light wavelength difficult. On the basis of preliminary measurements in the experiment covered by the present work, the wavelength of dispersed light $\lambda = 700$ nm was accepted.

The results of the measurement of light's intensity (I_{90}) and the turbidity coefficient (τ) for milk samples with the addition of lysozyme and for control samples showed that independently on the casein concentration, the solutions of lysozyme-treated milk were the centres which dispersed light more strongly (Fig. 1, 2). At a determinated concentration of casein both the value of dispersed light intensity and the turbidity coefficient increased with a growing lysozyme addition. Both characteristics of dispersed light are directly proportional to lysozyme in the number of dispersed particles (N) and the square of their volume (V):

$$I_{90} \sim \tau \sim NV^2$$

This indicates that the addition of lysozyme to milk caused an increase in the number of dispersing particles, or an increase in their volume, or both at the same time.

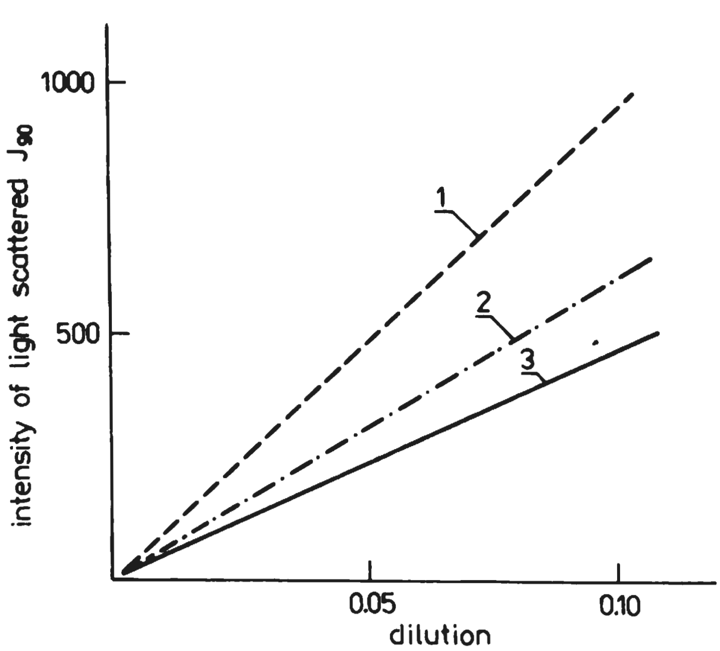

Fig. 1. Changes of scattered-light intensity in milk modified by lysozyme; 1 — sample with lysozyme added 2 mg/cm^3, 2 — sample with lysozyme added 1 mg/cm^3, 3 — control sample

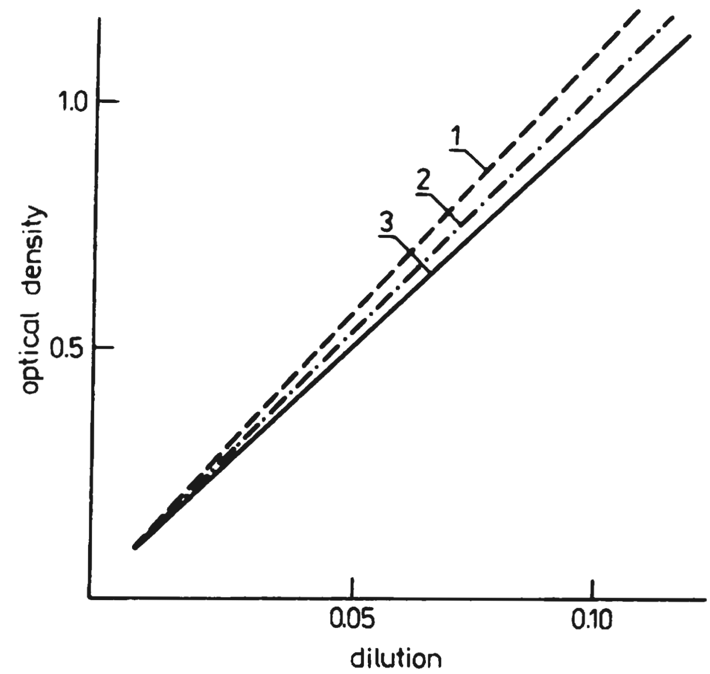

Fig. 2. Changes of turbidity coefficient in milk modified by lysozyme; 1 — sample with lysozyme added 2 mg/cm^3, 2 — sample with lysozyme added 1 mh/cm^3, 3 — control sample

The results of fractionation of micelles and measurement of light scattering showed only tendencies of changes in size of casein micelles affected by lysozyme. These studies were supplemented with a microscopic observation of enzyme-treated preparation of milk. The microscopic photographs pointed to the presence in milk of micelles of differentiated size from 30 to 350 nm (photo 1, Fig. 3). Addition of lysozyme to milk caused a change in dimensions of casein micelles. Statistical analysis of the distribution of these sizes showed differences in the course of distribution of lysozyme-treated samples in comparison with the control samples (Fig. 3). The decrease in percent share of small micelles with a diameter below 60 nm was stated on to the advantage of an increase in amount of micelles with larger dimensions (Tab. 3, photos 1, 2, 3).

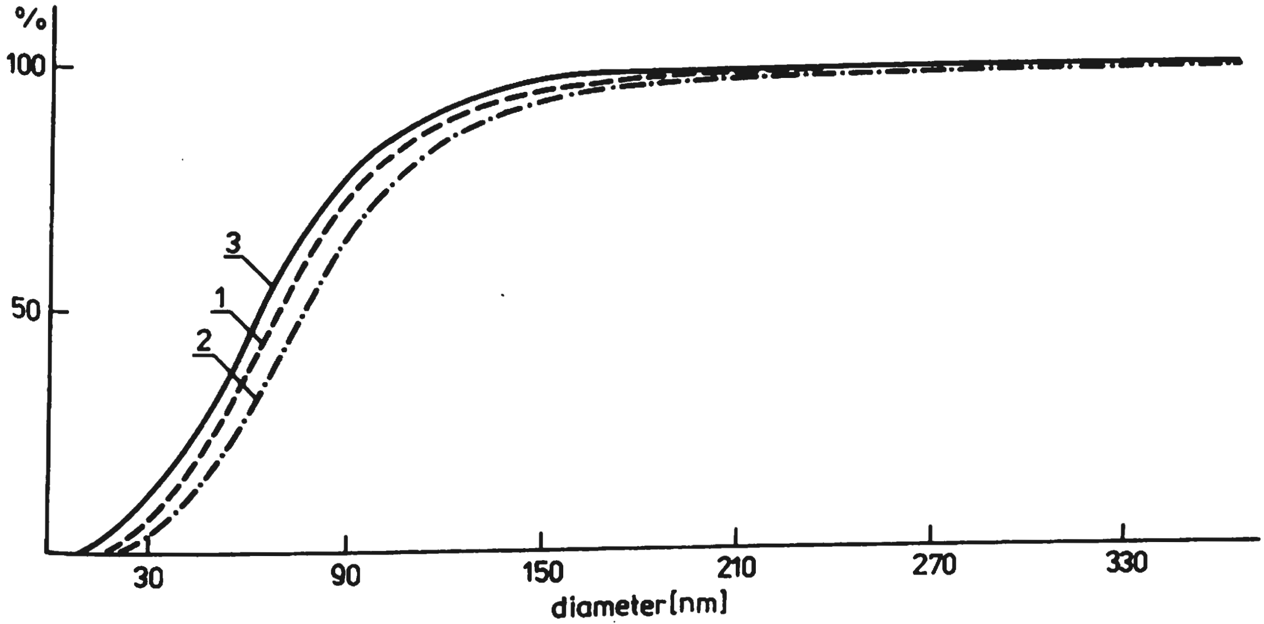

Fig. 3. Size distribution of micelles in milk modified by lysozyme; 1 — sample with lysozyme added 1 mg/cm^3, 2 — sample with lysozyme added 2 mg/cm^3, 3 — control sample

The observations seem to point out that lysozyme caused an increase in dimensions of micelles in milk, which complies with the results of our earlier studies [12, 13]. It is difficult, however, to state univocally whether this enzyme affects

Table 3. Distribution of dimensions of casein micelles in lysozyme-modified milk

No. of class	Kind of sample size (nm)	Control	With the addition of lysozyme (mg/cm^3)	
			1.0	2.0
		% of the total number of micelles in milk		
1	<30	5.69	1.80	0.94
2	30-60	29.10	23.38	17.67
3	60-90	36.93	39.66	35.90
4	90-120	18.18	22.26	27.54
5	120-150	6.34	6.48	9.68
6	150-180	1.86	3.28	4.32
7	180-210	0.65	1.01	1.79
8	210-240	0.56	0.79	0.84
9	240-270	0.28	0.45	0.47
10	270-300	0.46	0.34	0.37
11	300-350	0.00	0.11	0.28
12	350	0.09	0.56	0.18

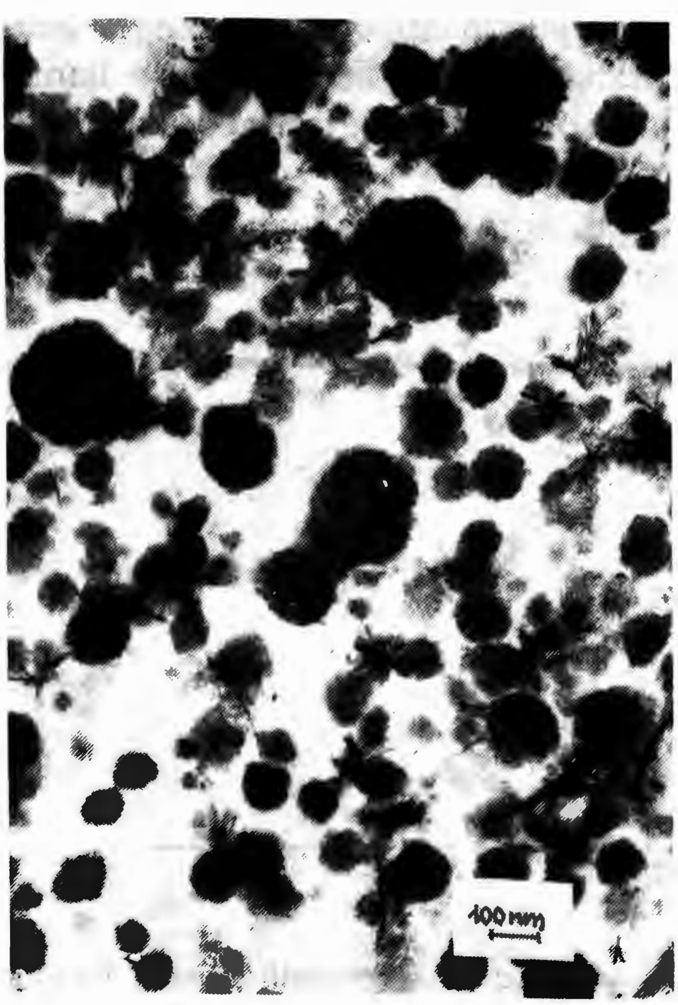

Photo 1. Electronogram of casein micelles of cow milk

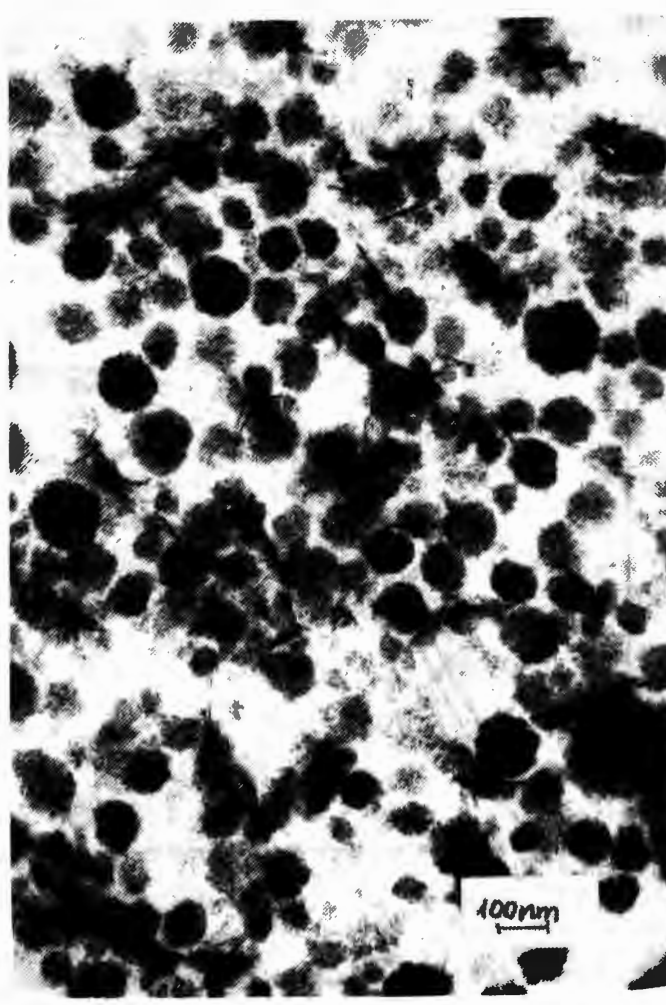

Photo 2. Electronogram of casein micelles of milk treated with lysozyme (1 mg/cm^3)

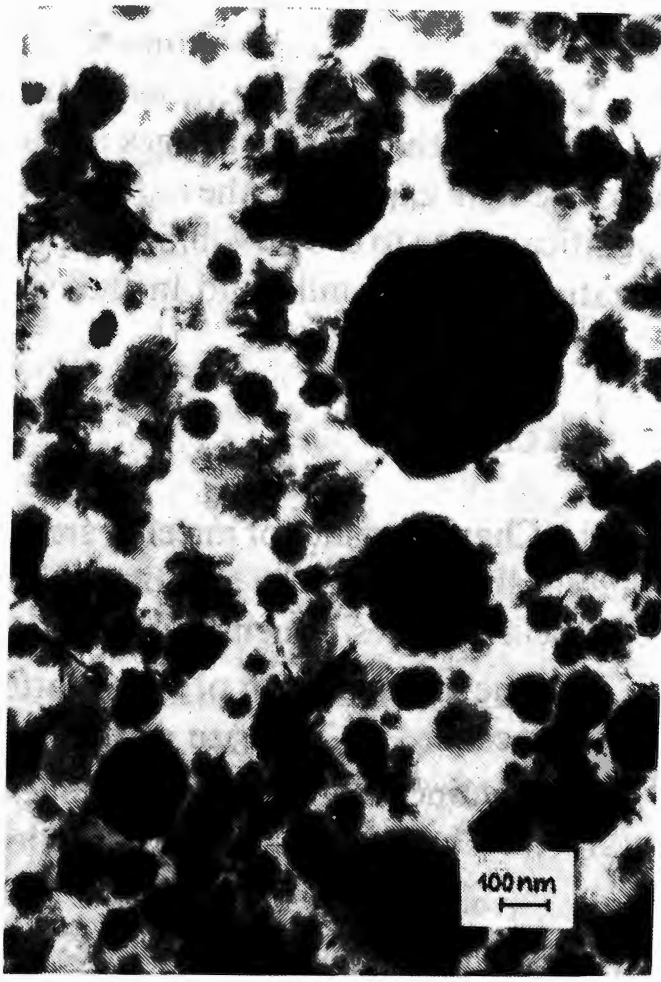

Photo 3. Electronogram of casein micelles of milk treated with lysozyme (2 mg/cm^3)

small and big micelles to the same extent. The characteristics of fractions obtained by the ultracentrifugation of milk show that greater changes concerned micelles with the smallest dimensions and probably also submicelles. This may be due to more effective neutralization of the charge of small micelles be the constant positive charge of the lysozyme. Hence, the greater scattering of light by solutions of lysozyme-modified milk could be the result of partial aggregation of small micelles and submicelles i.e. of an increase in the number of particles with dimensions affecting substantially the effect of scattering. This was also confirmed by the results of microscopic observations. The addition of lysozyme to milk caused an increase in the share of micelles with dimensions from 60 to 120 nm and a decrease in the quantity of micelles with dimensions from 30 to 60 nm.

Changes in size of casein micelles in milk affected by cationic compounds (including lysozyme) have been demonstrated also by other authors [1, 2, 10, 11]. Shoeren et al [16] in their experiments conducted with the use of preparative centrifugation, stated that the amount of casein in the obtained sediments was increased and its content in supernatant was lowered proportionally to the rise in concentration of calcium ions in milk which, in these authors opinion was connected with a shift of equilibrium of β-casein dissociation from micelles and changes in their dimensions.

The microscopic observations conducted by Green and Marshall [6, 7] did not show any significant influence of cations on the sizes of casein micelles. On the other hand, measurements of light scattering showed that cationic compounds caused an increase in average dimensions of micelles.

The results of the present work show that introduction of lysozyme to milk is connected with defined changes in the structure of casein micelles. The data are also useful in explaining the nature of enzyme's effect on casein which seems to be significant due to the possibility of practical utilization of lysozyme in the humanization of cow's milk and in the cheesemaking process.

CONCLUSIONS

1. Characteristics of micellar fractions obtained from the ultracentrifugation of milk showed that the addition of lysozyme caused changes in the distribution of casein micelles dimensions in the milk.

2. The measurement of light scattering showed that the addition of lysozyme to milk caused an increase of the mean size of casein micelles.

3. The increase of casein micelles size in lysozyme-modified milk was also confirmed by microscopic observations.

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WPŁYW LIZOZYMU NA ZMIANY ROZMIARÓW MICELI KAZEINY W MLEKU

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Streszczenie

W badaniach podjęto próbę określenia wpływu dodatku lizozymu (0,5; 1,0 i 2,0 mg/cm^3) na rozkład rozmiarów miceli kazeinowych w mleku. Wielkość miceli określano 3 różnymi metodami:

— pośrednio przez frakcjonowanie miceli metodą wirowania,

— przez pomiary rozproszenia światła,

— bezpośrednio za pomocą mikroskopu elektronowego.

Uzyskane wyniki wykazały, że ilości kazeiny odwirowanej w poszczególnych frakcjach z mleka modyfikowanego lizozymem były wyższe w porównaniu z odpowiadającymi im próbami kontrolnymi. Różnice te, a także charakterystyka fizykochemiczna odwirowanych frakcji wykazały, że lizozym powodował pewne zmiany w rozkładzie miceli kazeinowych w mleku (tab. 1 i 2).

Wyniki pomiarów natężenia światła (I_{90}) oraz współczynnik zmętnienia (τ) roztworów mleka modyfikowanego lizozymem wykazały, że niezależnie od stężenia kazeiny roztwory mleka traktowanego lizozymem stanowiły ośrodki silniej rozpraszające światło (rys. 1 i 2). Wskazuje to pośrednio na zwiększenie rozmiarów cząsteczek kazeiny w mleku z lizozymem.

Wniosek ten potwierdziły również obserwacje mikroskopowe preparatów mleka modyfikowanego lizozymem. Analiza uzyskanych elektronogramów wykazała, że enzym wpłynął na zmianę rozmiarów miceli kazeinowych (zdjęcie 1, 2, 3; tab. 3, rys. 3). Analiza statystyczna rozkładu tych wielkości wykazała różnice w przebiegu dystrybuanty próbek traktowanych lizozymem w porównaniu z próbkami kontrolnymi (rys. 3). Stwierdzono zmniejszenie procentowego udziału miceli małych o średnicy poniżej 60 nm na korzyść wzrostu ilości miceli o rozmiarach większych.