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Evaluation of the influence of chromium and zinc ions on the collagenolysis process in solutions by the method of dynamic light scattering

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Using the method of dynamic light scattering, it was found that the rate of collagen cleavage under the influence of bacterial collagenase decreases by almost 4 times when protein molecules are doped with Cr^{3+} ions, and when the enzyme activator of Zn^{2+} ions is added, it increases by 1.3 times. Based on the experimental data, the reaction rate constants k_1 were calculated. It was found that when collagen molecules are tanned with a chromium salt, it becomes more resistant to degradation in solutions containing zinc ions than in solutions with the addition of calcium ions, as we described in earlier works.

Keywords: collagen type 1, bacterial collagenase, chromium chloride, zinc chloride, dynamic light scattering method, translational diffusion coefficient, hydrodynamic radius, reaction rate constant, collagenolysis, Clostridium histolyticum.

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Introduction

Study of the process of collagen protein and collagenase enzyme interaction is of the utmost interest for modern science, since the study of collagenolysis mechanism is the basis for development of drugs and various diseases treatment methods.

Violation of collagen synthesis in an organism can result in collagen fibers spreading and be the cause of various diseases (fibrosis of liver, lungs, mammary glands, Dupuytren's contracture, etc.) [1–4]. In many cases the use of injectable form of collagenase or medications based on it can help in obstructive collagen degradation [5]. The most common is a bacterial collagenase, obtained from *Clostridium histolyticum* (CHC) (gas bacillus) [6–8].

At certain diseases, on the contrary, slowing down the process of collagen degradation can be important. For instance, for glaucoma treatment the collagen implant, regulating the pressure inside an eyeball, is often used. Chromium fibers are used to prevent its fast resolution, since chromium strengthens protein molecules and prevents from its cleavage [9]. Also, insufficient synthesis of collagen can result in protein malnutrition in organism and contribute to such diseases as vessels thinning and rupture, arthrosis, osteochondrosis, etc. [10].

Thus, the study of collagen and collagenase interaction is the important task. At the same time it is necessary to study the factors that both accelerate this process and slowing it down. For these purposes the various enzyme activators and inhibitors, as well as trivalent chromium salts, that are tanning agents and stabilizers of collagen fibrils in organism, are used [11–14].

Chrome tanning of collagen molecules results in formation of cross bonds („links") between molecular chains of protein structure and its spatial configuration strengthening. As a result the collagen resistance to enzymes impact increases [15].

Chrome tanning of protein and collagenolysis process were studied by methods of atomic force microscopy and electron microscopy [16–21]. Results of the experiments showed that Cr^{3+} ions increase resistance of collagen matrix and can result in molecular structures (protein complexes) formation. Preliminary study results showed that Cr^{3+} ions can result in aggregation of collagen fibrils and its quaternary structure formation. However, these methods have significant disadvantages: dried collagen fibrils are used for their application and the long-term samples preparation is required.

Efficient spectral and optical method of collagen and collagenase molecules interaction study in solutions is the method of dynamic light scattering. It allows to study Brownian motion of macromolecules in solutions, while defining the translational diffusion coefficient and hydrodynamic radius of particles or their complexes. Using photonic correlation spectroscopy the method of collagen protein cleavage process monitoring in a real-time mode under conditions, close to physiological, can be developed.

This technique will allow to control the collagenolysis behavior, appearing at diseases, related to excessive/deficient content of collagen fibers in living organisms. By changing the environment parameters (temperature, pH, etc.) and us-

ing the activators/inhibitors or tanning agents, the processes, occurring at various pathologies *in vitro*, can be simulated.

Earlier in our works [22,23] it was observed, that chromium ions impact on collagen protein results in slowing the collagenolysis process down, while with adding of calcium chloride activator to doped collagen the rate increases in 1.5 times.

Since the bacterial collagenase is activated with calcium and zinc ions, but activation mechanism acts differently (Zn^{2+} is responsible for activation of enzyme amido group, while Ca^{2+} — for formation of molecule tertiary structure [24]), it is interesting to obtain the experimental data on collagenolysis behavior in protein solutions (at chromium tanning with salt) with Zn^{2+} ions adding and to compare these data with the results, observed earlier [22,23].

The main objectives of this work were: study of zinc chloride collagenase activator influence on collagen, predoped with trivalent chromium salt, in solutions, calculation of reaction rate constant and the observed data comparing.

1. Dynamic light scattering method

Dynamic scattering method allows to define the translational diffusion coefficient D_t of particles in solutions, by analyzing the specific time of the scattering light intensity fluctuations [25]. Translational mobility of molecules in solutions is characterized with coefficient D_t.

Translational coefficient of particles diffusion is proportional to light scattering intensity fluctuations decay rate. Decay rate is derived from the time-dependent correlation function of light scattering intensity.

In case of polydisperse solution with various size of particles the photo current spectrum is a continuous set (integral) of Lorenz curves with various half-width. Consequently, it is necessary to solve the reverse spectral task as an integral equation with Lorenz core to obtain the particles size distribution (diffusion coefficients):

$$g^{(2)}(t) = [g^{(1)}(t)]^2 + 1\xi(t), \tag{1}$$

$$g^{(1)}(t) = \int_0^\infty P(\Gamma)e^{-\Gamma t}d\Gamma, \tag{2}$$

where $\xi(t)$ — error related to stochastic nature of signal, $P(\Gamma)$ — distribution of decay rates (reverse relaxation times), Γ — half-width of scattering light spectrum, $g^{(1)}(t)$ — normalized correlation function of the first order $g^{(2)}(t)$ — normalized correlation function of the second order.

Value of Γ can be calculated the following way:

$$\Gamma = D_t q^2; \quad q = \frac{4\pi n}{\lambda}\sin\frac{\theta}{2}; \quad D_t = \frac{kT}{6\pi\eta R_H}, \tag{3}$$

where D_t — translational diffusion coefficient, q — scattering vector, n — refraction index, λ — scattering light wave length, Θ — scattering angle, k — Boltzmann's constant, η — solvent dynamic viscosity, T — absolute solution temperature, R_H — hydrodynamic radius.

Equation (1), known as Ziegert equation, allows to calculate the normalized correlation function of the first order $g^{(1)}(t)$ from the normalized correlation function of the second order $g^{(2)}(t)$, accumulated by correlator during experiment. Integral equation (2) is the basis for data processing in photonic correlation spectroscopy [25].

2. Examined samples and their preparation

Collagen — the main protein element of skin, bones, tendons, cartilages, blood vessels and teeth [26]. In this work the collagen of the I type from calf skin, that is a 0.1% (1 mg/ml) solution of collagen in 0.1 M vinegar acid, is studied [27].

Destruction of collagen fibers is performed by active forms of oxygen and/or by enzymic means (hydrolytically). Native collagen is not hydrolyzed with regular peptide hydrolases. The main enzyme of its catabolism is collagenase, that cleaves the peptide bonds in certain sections of spiralized protein areas.

Bacterial collagenase is synthesized by some microorganisms, for instance, *Clostridium Histolyticum* (CHC) releases collagenase, that cleaves the peptide collagen chain in more than 200 sections. This enzyme hydrolyzes the following bond in molecules: X-X-Gly-Pro-U- between the links X and Gly-Pro-proline [28–30]. The appearing enzymes of the collagen are soluble in water, at body temperature they spontaneously denaturize and become available for action of other proteolytic enzymes.

In our experiments we used a collagenase powder, made from bacterium *Clostridium Histolyticum* of IA type [31].

The following concentrations of examined substances were used in the work:

- collagen concentration 0.05 mg/ml;
- collagenase concentration 0.3 mg/ml;
- zinc chloride, chromium chloride, sodium chloride (ionic strength $\mu = 10^{-3}$ mol/l).

Temperature in a cuvet with the examined solution was maintained on a level of 30°C using thermostat, integrated into the device. Averaging of values, presented in fig. 1–4, was made using 4–5 measurements. The presented spreads of values mean standard deviation from the average.

3. Experimental results

3.1. Concentration dependencies of solutions of collagen and collagenase

For the purpose of selection of the optimum ratio of molecules of protein and enzyme for dynamic light scattering method the concentration dependencies of translational diffusion coefficient D_t (fig. 1, *a*) and hydrodynamic

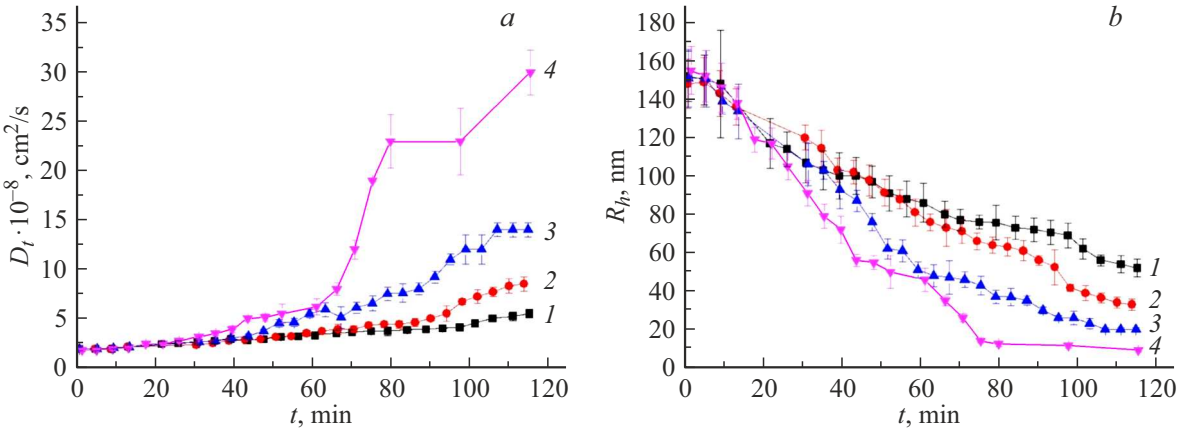

Figure 1. Time dependencies of translational diffusion coefficient D_t (a) and hydrodynamic radius R_h (b) of collagen molecules in solutions at introduction of various collagenase concentrations (mg/ml): *1* — 0.1, *2* — 0.2, *3* — 0.3, *4* — 0.4 mg/ml.

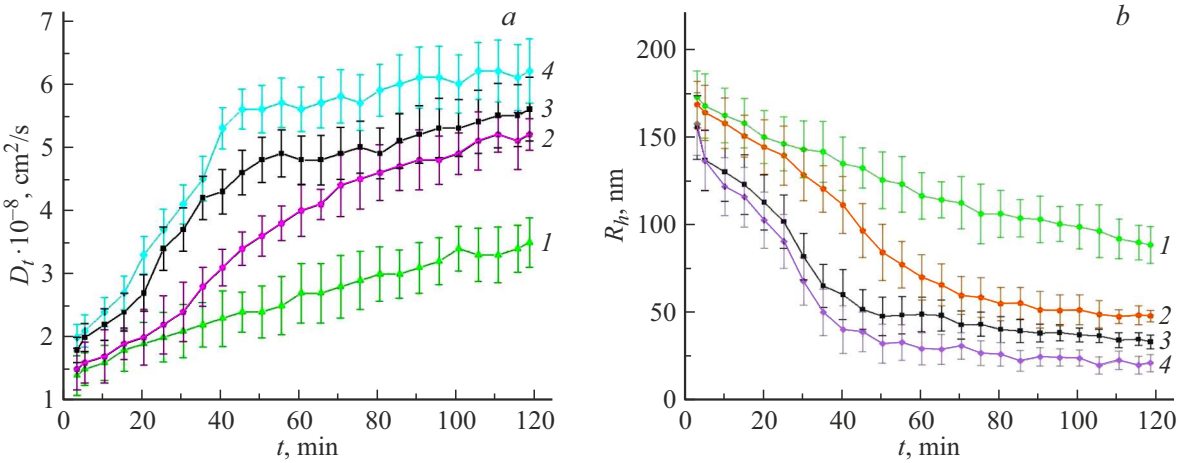

Figure 2. Time dependencies of variation of translational diffusion coefficient D_t (a) and hydrodynamic radius R_h (b) in solutions of collagen and collagenase: *1* — collagen, doped with ions of Cr^{3+}; *2* — collagen, doped with ions of Cr^{3+} in presence of $ZnCl_2$; *3* — pure solution; *4* — in presence $ZnCl_2$.

radius R_h (fig. 1, b) in collagen solutions with introduction of various amount of collagenase were measured.

It is known, that with collagenase amount increase relating to collagen, the collagenolysis reaction rate increases [32]. The following enzyme concentrations were selected in our work: 0.1, 0.2, 0.3, 0.4 mg/ml for monitoring the process dynamics and the following selection of optimum concentration of substance for photonic correlation spectroscopy method. From the observed results we can conclude, that enzyme concentration in solution influences on protein degradation rate (fig. 1), specifically, with increase of collagenase amount in solution the rate of collagen cleavage increases, that corresponds to the results, observed earlier [32].

Collagenase concentration of 0.4 mg/ml results in protein molecules destruction in 80 min (fig. 1, b, curve *4*), at concentrations of 0.1, 0.2 and 0.3 mg/ml the collagen cleavage to 60, 40 and 20 nm occurs respectively within two hours (fig. 1, b, curves *1–3*).

For the further experiments we chose the enzyme concentration $c = 0.3$ mg/ml, since it is optimum for evaluation of kinetic dependencies and collagen degradation reaction time.

3.2. Time dependencies of D_t and R_h in solutions of collagen and collagenase with introduction of ions of Cr^{3+} and Zn^{2+}

Using dynamic light scattering method the time dependencies of variation of translational diffusion coefficient D_t (fig. 2, a) and hydrodynamic radius R_h (fig. 2, b) in solutions of collagen and collagenase with introduction of salts of $CrCl_3$ and $ZnCl_2$ were observed.

Based on experimental data (fig. 2) it is revealed that with introduction of zinc chloride activator (curve *4*) the collagenolysis reaction accelerates, while with introduction of ions of trivalent chromium (curve *1*) — the reaction

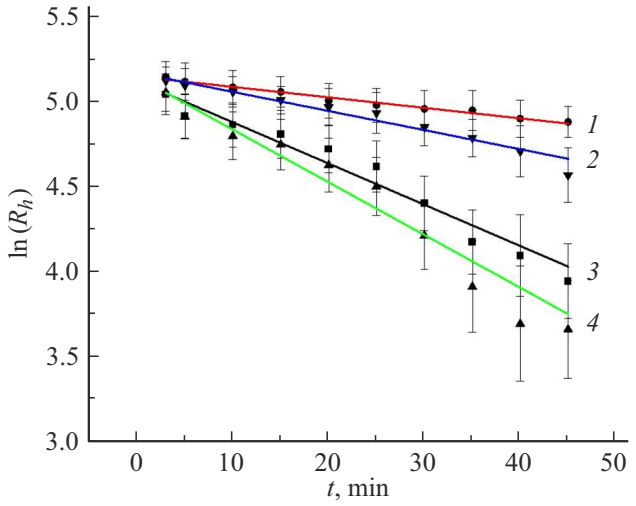

Figure 3. Time dependencies of $\ln R_h(t)$ in solutions of collagen and collagenase: *1* — collagen, doped with ions of Cr^{3+}; *2* — collagen, doped with ions of Cr^{3+} in presence of $ZnCl_2$; *3* — pure solution; *4* — in presence of $ZnCl_2$.

slows down compared with pure solution of collagen and collagenase (curve *3*).

With introduction of zinc chloride activator to collagen (after tanning with ions of Cr^{3+}) (curve *2*) the process of protein cleavage accelerates compared to solution, not containing the activator (curve *1*), however the values of D_t and R_h, observed in pure solution of collagen and collagenase, are still not achieved.

For more illustrative evaluation of the process the reaction rate constants k_1 were calculated for the first 45 min of the reaction based on the observed data [33].

The observed values of k_1 for the examined samples are presented in the table.

With introduction of Zn^{2+} ions to solution of collagen (after tanning with Cr^{3+} ions) and collagenase (fig. 3, curve *2*), the reaction rate increases by a factor of 1.3 compared to solution without introduction of the activator (fig. 3, curve *1*). However, the reaction rate decreases by a factor of 2.9 compared to pure solution of collagen and collagenase (fig. 3, curve *3*).

Values of rate constants k_1 for examined solutions

	Solution	k_1, min^{-1}
1	Col_{cr} + collagenase	0.006 ± 0.001
2	Col_{cr} + collagenase + $ZnCl_2$	0.008 ± 0.001
3	Collagen + collagenase	0.023 ± 0.002
4	Collagen + collagenase + $ZnCl_2$	0.051 ± 0.002

Similar measurements were made in the work [23] in presence of calcium chloride activator. From comparison with the previous results we can conclude, that with introduction of calcium ions to collagen solution (after tanning with chromium ions) the reaction is slightly faster ($k_1 = 0.009\,min^{-1}$) than with introduction of zinc ions ($k_1 = 0.008\,min^{-1}$). Thus, collagen, doped with salt of $CrCl_3$, is more resistant to degradation in solutions, containing zinc ions, than in solutions with introduction of calcium ions [23].

3.3. Influence of Cl^- ions on collagenolysis process in solutions

Since in our studies we usually use chlorides of various metals, for evaluation of Cl^- ions influence in collagen cleavage process we calculated time differences of D_t (fig. 4, *a*) and R_h (fig. 4, *b*) for solutions of collagen and collagenase with introduction of sodium chloride.

As seen from the observed diagrams (fig. 4), the dependencies for D_t and R_h in solutions, containing salt of NaCl, are identical to dependencies for pure solutions of collagen and collagenase. Consequently, the ions of Cl^- do

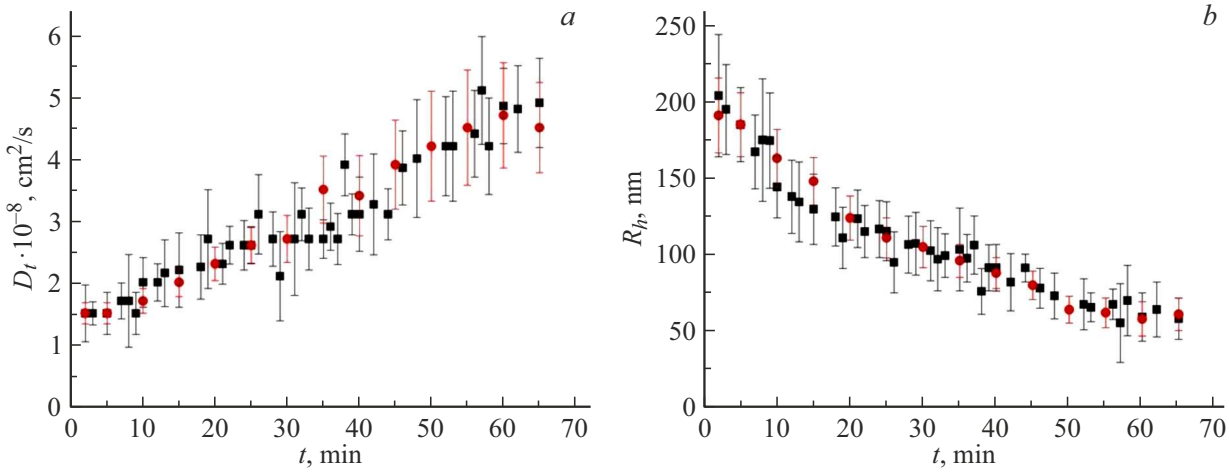

Figure 4. Time dependencies of translational diffusion coefficient D_t (*a* and hydrodynamic radius R_h (*b*) for solutions of collagen and collagenase — pure and with introduction of NaCl.

not have any significant influence on the examined process, thus allowing to use chlorides in experiments.

Conclusion

The performed study of collagenolysis process allowed to define the enzyme concentration, optimum for evaluation of kinetic dependencies, equal to 0.3 mg/ml.

It was revealed, that with introduction of zinc chloride activator to solutions of collagen and collagenase, the protein cleavage process accelerates less, than in presence of calcium chloride — both in pure solutions and solutions, containing chromium ions. With introduction of activators of zinc chloride and calcium chloride to solutions of collagen, doped with chromium salt, protein is subject to degradation in lesser degree compared to collagen without tanning [23].

The observed experimental data can be used for development of technique for collagenolysis process monitoring in real-time mode using photonic correlation spectroscopy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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