

Influence of Activators and Inhibitors on the Collagenase with Collagen Interaction Monitored by Dynamic Light Scattering in Solutions

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Abstract. Real-time digestion of type I collagen molecules by bacterial collagenase from *Clostridium histolyticum* has been monitored using Dynamic Light Scattering method. Time dependencies for translation diffusion coefficient (D_t) and hydrodynamic radius (R_H) were obtained for pure “collagen + collagenase” Tris–HCl buffer solution at different temperatures and for solutions with added CaCl_2 , ZnCl_2 and MgCl_2 and EDTA. It was shown that digestion of type I collagen molecules by bacterial collagenase is the first-order reaction. Reaction rate coefficients were calculated. © 2021 Journal of Biomedical Photonics & Engineering.

Keywords: dynamic light scattering; type I collagen; translation diffusion coefficient; collagenase from *Clostridium histolyticum*; first-order reaction; activators; inhibitors; reaction rate coefficient.

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1 Introduction

Enzyme-based drugs are commonly used in different fields of modern medicine: in surgery, gynecology, otolaryngology, ophthalmology, as well as in dermatology and cosmetology. Bacterial collagenase (for example, from *Clostridium histolyticum*) is one of them. The main collagenase feature is its ability to digest key protein of the animal extracellular matrix – collagen [1].

Violation of the collagen catabolism leads to fibrosis of organs and tissues. Increasing of collagen degradation rate occurs in case of autoimmune diseases (rheumatoid arthritis, lupus, etc.) because of excess collagenase synthesis as a result of immune response [2].

Clostridium histolyticum collagenases G and H (ColG and ColH) can easily digest collagens, regardless of their types and sizes [3]. It breaks down multiple bonds in collagen triple helices as well as peptide bonds [4].

Bacterial collagenase from *Clostridium histolyticum* is extensively used as a clinical tool in the nonsurgical treatment of Dupuytren’s disease [4, 5] in eye’s diseases treatment, for enzymatic debridement, for accelerated resorption of catgut sutures.

Structural changes of collagen fibrils caused by collagenase are well studied by microscopy, and the

degradation rate of protein is determined [6, 7]. Results obtained with microscopy clearly demonstrate the process of collagen fibers digestion and allow one to evaluate the rate of collagen fibril diameter changing. However, these results do not agree well with the results obtained in conditions close to physiological [6, 7].

Optical methods, like Dynamic Light Scattering (DLS), enable us to investigate collagen and collagenase molecules solutions in conditions close to physiological. By changing the solution’s parameters (pH, temperature, solvent type) and adding of collagenase activators and inhibitors we can simulate the processes in living organisms. In our work, we studied the influence of temperature, activators (CaCl_2 and ZnCl_2) and inhibitors (MgCl_2 and EDTA) on collagenase activity. Since collagen by collagenase digestion in solutions takes on average 60 min, DLS enables us to monitor the dynamics of this process in real time [8].

2 Materials

2.1 Collagen

Collagen is the key component of extracellular matrix, making up from 25% to 35% of the whole-body protein

content. Although different types of collagen exist, they all are composed of molecules containing three polypeptide chains arranged in a triple helical conformation [6].

Over 90% of the collagen in the human body is type I collagen. It is a component of skin, bone, tendon and other fibrous connective tissues [9]. Molecular mass of collagen is approximately 300 kDa, average length of molecule is 300 nm [10].

In normal healthy tissues collagen molecules are resistant to most proteases attacks. Only specialized enzymes like collagenases can attack the collagen molecule.

2.2 Collagenase

There are two types of collagenase: tissue and bacterial. Tissue collagenases split collagen in its native triple helical conformation at a specific site, yielding fragments representing 3/4 and 1/4 lengths of the collagen molecule. After fragmentation the pieces tend to uncoil into random polypeptides and are more susceptible to attack by other protease [11].

Bacterial collagenases attack many sites along the helix. Collagenases from *Clostridium histolyticum* degrades the helical regions in native collagen preferentially at the X-Gly bond in the sequence Pro-X-Gly-Pro where X is most frequently a neutral amino acid [12]. This bond in synthetic peptide substrates may also be split. Bacterial collagenase cleaves the peptide bond of collagen in more than 200 places [11].

Molecular mass of collagenase is 79 kDa. Hydrodynamic radius is 4 nm according to the data obtained by dynamic light scattering method [5].

2.3 Enzymes activators and inhibitors

Enzyme activators are molecules or ions that bind to enzymes and increase their activity. Collagenases from *Clostridium histolyticum* are activated by ions Ca^{2+} , Zn^{2+} [13, 14]. Calcium ions support the conformation of the enzyme which is required for binding to the protein peptide chain. This conformation makes the active site available for the reaction. Zn^{2+} ions also are activators of collagenase, but its effect on the enzyme activity is much lower than that of Ca^{2+} . Zn^{2+} ions are located in the active site of the enzyme. Enzyme inhibitors are molecules that binds to an enzyme and decreases its activity. Collagenases from *Clostridium histolyticum* are inhibited by ions Mg^{2+} , EDTA, 1,10-phenanthroline, dipyrityl disulfide, 8-hydroxyquinoline [15, 16].

3 Experimental method

3.1 Dynamic Light Scattering

The DLS method enables one to determine the translation diffusion coefficient of particles in solutions by analyzing characteristic time of scattered light intensity fluctuations [17].

Translation diffusion coefficient of the particles is proportional to the decay rate of light scattering intensity fluctuations. The decay rate is obtained from the time-dependent correlation function of the scattered light intensity.

In the case of poly-dispersed solution where the particle sizes are different, the spectrum of the photocurrent is a continuous set (integral) of Lorentz curves with different half-widths. Consequently, to find the size distribution of particles (diffusion coefficients), it is necessary to solve the inverse spectral problem in the form of an integral equation with a Lorentzian kernel:

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

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

where $g^{(1)}(t)$ – is the normalized autocorrelation function of the signal, $\zeta(t)$ – is the error associated with the stochastic nature of the signal itself [17]. If we neglect the influence of constant experimental noise $\zeta(t)$, Eq. (1) (Siegert relation) will allow us to calculate $g^{(1)}(t)$ in terms of $g^{(2)}(t)$, accumulated by the correlator during the experiment.

Integral Eq. (2) forms the basic principle of data processing in the photon correlation spectroscopy technique. This equation for $P(\Gamma)$ is a Fredholm integral first-order equation, known in mathematics as an ill-posed problem, that is a problem with a fundamentally absent algorithm for finding an exact solution. In this regard, various approximate solution methods are being developed, many of which give very good results.

In the experiments, the results were processed using the *DYNALS* software, in which the search for an approximate solution (4) is carried out using the regularization method of A. N. Tikhonov for integral equations [18].

Γ can be calculated as described below:

$$\begin{aligned} \Gamma &= D_t q^2, \\ q &= \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}, \\ D_t &= \frac{kT}{6\pi\eta R_H}, \end{aligned} \quad (3)$$

where D_t is the translation diffusion coefficient, q is the scattering vector, n is the refraction index, λ is the scattered light wave length, θ is the scattering angle, k is the Boltzmann constant, η is the dynamic viscosity of the solvent, T is the absolute temperature of the solution and R_H is the hydrodynamic radius.

3.2 Samples preparation

Collagen solutions were prepared by dissolving collagen type I from calf skin produced by Sigma Aldrich in 10 mM Tris-HCl (pH 7.5) buffer. Bacterial collagenase

from *Clostridium histolyticum* type IA by Sigma Aldrich was added in collagen solution. Concentration of collagen was 0.1 mg/ml, concentration of collagenase was 0.2 mg/ml (i.e 8 molecules of collagenase per 1 molecule of collagen).

As activators we have used salts CaCl_2 , ZnCl_2 and as inhibitors – salt MgCl_2 and EDTA. Ionic strengths were 0.03 mol/L in all solutions.

The samples were processed in the ultrasonic bath with the frequency 3 kHz during 1 min.

Experiments have been carried out using the photon-correlation spectrometer Photocor-Complex with diode laser (wavelength of 647 nm, power 25 mW) [19]. Photocor-Complex spectrometer is equipped with built-in thermostat which allows one to control sample temperature.

4 Results and Discussions

4.1 Temperature dependencies

Using the DLS method we have experimentally obtained translation diffusion coefficient (D_t) on time dependencies of scattering particles in “collagen + collagenase” Tris-HCl buffer solution at different temperatures (Fig. 1).

The uniform increase in the mobility of scattering particles over time was observed at 22 °C and 30 °C. The maximum growth of D_t (and speed) is observed during the first 30 min, then the increase is not so rapid. At 40 °C average mobility of scattered particles is increasing threefold during first 30 min and then D_t on time dependence reaches saturation.

Since $T = 40$ °C is not optimal for collagen (protein can denature), all further experiments were carried out at $T = 30$ °C (as the most optimal temperature).

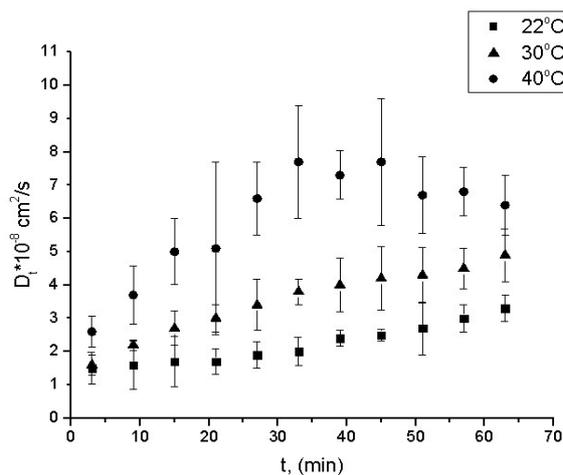


Fig. 1 Time dependencies of translation diffusion coefficient (D_t) on of scattering particles in “collagen + collagenase” Tris-HCl buffer solution at different temperatures.

Using the DYNALS package of programs evaluated values of hydrodynamic radius R_H has been calculated

from D_t and then $\ln R_H(t)$ dependencies were obtained (Fig. 2).

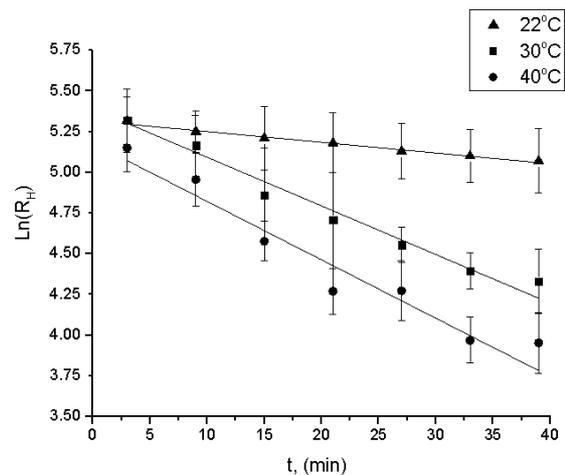


Fig. 2 The $\ln R_H$ dependencies on time at different temperatures, where R_H is hydrodynamic radius of scattering particles in “collagen + collagenase” Tris-HCl buffer solution.

In the first approximation, we can assume that concentration of collagen molecules in solution linearly depends on radius. In this case concentration of collagen molecules (C) exponentially decreases with time. Such character of $C(t)$ dependence corresponds to the first-order reaction [20].

The equations describing first-order reaction kinetics are given below. C_0 is initial concentration, k_1 is the reaction rate coefficient in units 1/time.

$$\ln C = \ln C_0 - k_1 \tau, \quad (6)$$

$$k_1 = -\tan \alpha. \quad (7)$$

Plotting $\ln(C)$ with respect to time for a first-order gives us a straight line with the slope of the line equal to $-k_1$ (Fig. 2).

In the Table 1 values of k_1 (min^{-1}) for different temperatures are presented. STD k_1 was calculated using a graphing program based on the least squares method.

Table 1 Values of k_1 (min^{-1}).

Temperature, °C	k_1	STD k_1
22	0.012	0.001
30	0.031	0.003
40	0.054	0.003

The highest value of k_1 corresponds to 40 °C. However, this temperature is not optimal for collagen. With prolonged thermal action protein may denature, which happened at 90th min of the experiment.

4.2 “Collagen + collagenase” solutions with activators CaCl_2 and ZnCl_2

We have selected the following scheme for investigations of “collagen + collagenase” solutions with activators and inhibitors: experimentally obtain translation diffusion coefficient on time $D_t(t)$ dependence, evaluate values of hydrodynamic radius R_H from D_t , plot $\ln R_H(t)$ dependencies.

The $\ln R_H(t)$ dependencies for pure “collagen + collagenase” Tris-HCl buffer solution and in solutions with added CaCl_2 and ZnCl_2 are presented in Fig. 3. Calculated values of k_1 (min^{-1}) are presented in Table 2.

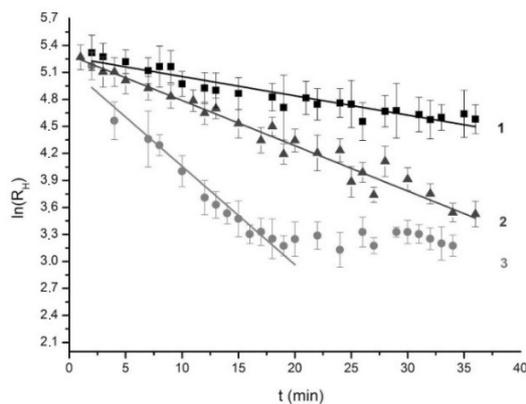


Fig. 3 The $\ln R_H$ time dependencies, where R_H is hydrodynamic radius of scattering particles, in pure “collagen + collagenase” Tris-HCl buffer solution (1) and with added ZnCl_2 (2) and CaCl_2 (3), $T = 30^\circ\text{C}$.

Bacterial collagenase initially contains Zn^{2+} and Ca^{2+} ions. Zn^{2+} is responsible for the activation of amide group and Ca^{2+} for the formation of tertiary structure [21].

When ZnCl_2 is added to “collagen + collagenase” Tris-HCl buffer solution, Zn^{2+} ions may occupy calcium positions in collagenase, but due to the difference in ionic radii ($r_{\text{ion}}(\text{Ca}^{2+}) = 114 \text{ pm}$, $r_{\text{ion}}(\text{Zn}^{2+}) = 88 \text{ pm}$) the effect of activity centers formation is not fully achieved (Fig. 3, curve 2).

When CaCl_2 is added to “collagen + collagenase” Tris-HCl buffer solution Ca^{2+} ions occupy all available positions in collagenase and form maximum number of active centers (Fig. 3, curve 3).

Also, for the solution with CaCl_2 added $\ln R_H(t)$ dependence becomes non-linear after 20 min (Fig. 3, curve 3). We suppose that it corresponds to further digestion of collagen molecule’s pieces by bacterial collagenase. Unlike tissue collagenase which splits collagen in its native triple-helical conformation at a specific site, bacterial collagenase is able also to attack and degrade collagen molecule’s fragments [11].

4.3 “Collagen + collagenase” solutions with inhibitor EDTA

To investigate inhibitor influence on collagen by collagenase digestion, we have added EDTA to

“collagen + collagenase” Tris-HCl buffer solution. During first 30 min D_t value was changed from $(1.5 \pm 0.2) \times 10^{-8} \text{ cm}^2/\text{s}$ to $(2.1 \pm 0.3) \times 10^{-8} \text{ cm}^2/\text{s}$, and during next 30 min from $(2.1 \pm 0.3) \times 10^{-8} \text{ cm}^2/\text{s}$ to $(2.6 \pm 0.4) \times 10^{-8} \text{ cm}^2/\text{s}$.

At 64th minute CaCl_2 as an activator was added to “collagen + collagenase + EDTA” solution. In half an hour after that D_t value changed from $(2.6 \pm 0.4) \times 10^{-8} \text{ cm}^2/\text{s}$ to $(10.0 \pm 1.2) \times 10^{-8} \text{ cm}^2/\text{s}$, and in 1 h to $(13.0 \pm 2.1) \times 10^{-8} \text{ cm}^2/\text{s}$ (Fig. 4)

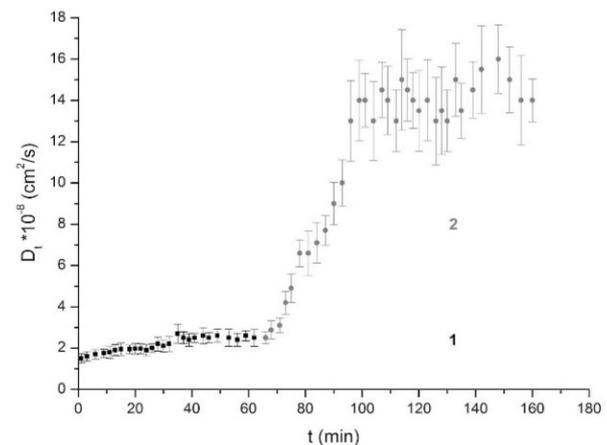


Fig. 4 Translation diffusion coefficient (D_t) on time dependencies of scattering particles in “collagen + collagenase” Tris-HCl buffer solution with added EDTA (1) and EDTA with CaCl_2 (2), $T = 30^\circ\text{C}$.

Corresponding hydrodynamic radius R_H values were calculated and $\ln R_H(t)$ dependencies were obtained for pure “collagen + collagenase” Tris-HCl buffer solution and for solutions with added EDTA and EDTA + CaCl_2 (Fig. 5). Values of k_1 (min^{-1}) are presented in Table 2.

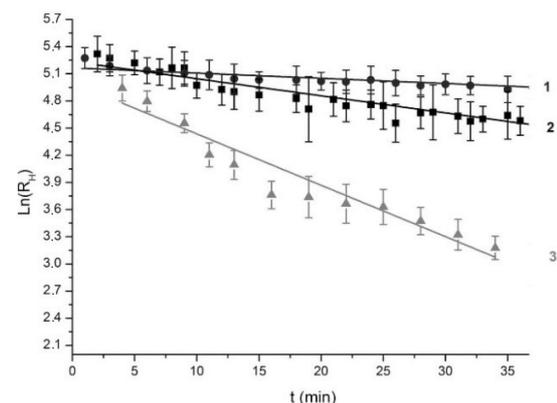


Fig. 5 The $\ln R_h$ time dependencies, where R_h is hydrodynamic radius of scattering particles, in pure “collagen + collagenase” Tris-HCl buffer solution (2) and with added EDTA (1) and EDTA with CaCl_2 (3), $T = 30^\circ\text{C}$.

EDTA molecules bind with calcium ions in solution and block calcium ions access to all available places in collagenase. So collagen by collagenase digestion slows down (Figs. 5, 6). When we add CaCl_2 to

“collagen + collagenase + EDTA” solution, amount of Ca^{2+} ions recovers and collagenase activity restores.

4.4 “Collagen + collagenase” solutions with inhibitor MgCl_2

The same way we have investigated MgCl_2 influence on collagen by collagenase digestion. $D_t(t)$ and $\ln R_H(t)$ dependencies were obtained (Figs. 6, 7).

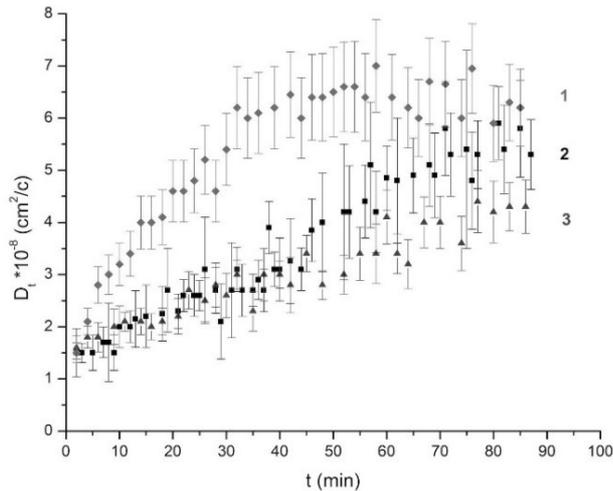


Fig. 6 The time dependencies of translation diffusion coefficient (D_t) of scattering particles in “collagen + collagenase” Tris-HCl buffer solution (2) with added MgCl_2 (3) and with added MgCl_2 and CaCl_2 (1), $T = 30^\circ\text{C}$.

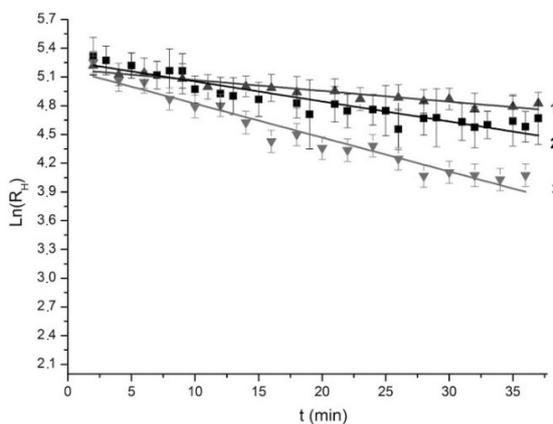


Fig. 7 The $\ln R_h$ time dependencies, where R_h is hydrodynamic radius of scattering particles, in pure “collagen + collagenase” Tris-HCl buffer solution (2) added MgCl_2 (1) and MgCl_2 with CaCl_2 (3), $T = 30^\circ\text{C}$.

According to the Pearson acid base concept Ca^{2+} and Mg^{2+} are hard Lewis acids and Zn^{2+} is intermediate Lewis acid [22].

When MgCl_2 is added to “collagen + collagenase” Tris-HCl buffer solution magnesium mainly aims to replace the zinc in collagenase due to close values of the ionic radii: $r_{\text{ion}}(\text{Mg}^{2+}) = 6 \text{ pm}$, $r_{\text{ion}}(\text{Zn}^{2+}) = 88 \text{ pm}$.

However, Mg^{2+} is hard Lewis acid and it decreases the activity of the amide group in enzyme. This fact leads to the inhibition of protein destruction process (Fig. 3, 5). Calculated values of k_1 (min^{-1}) are presented in Table 2.

When we add CaCl_2 to “collagen + collagenase + MgCl_2 ” solution, collagenase activity partially restores but initial value is not achieved.

5 Conclusions

Presented results allow one to draw a conclusion that digestion of type I collagen molecules by bacterial collagenase is the first-order reaction. Corresponding values of k_1 (min^{-1}) calculated for solutions with activators and inhibitors are given in Table 2.

Table 2 Values of k_1 (min^{-1}).

Solution	k_1	STD k_1
Collagen + Collagenase	0.023	0.002
Collagen + Collagenase + ZnCl_2	0.051	0.004
Collagen + Collagenase + CaCl_2	0.104	0.008
Collagen + Collagenase + MgCl_2	0.011	0.001
Collagen + Collagenase + EDTA	0.006	0.001
Collagen + Collagenase + MgCl_2 + CaCl_2	0.035	0.003
Collagen + Collagenase + EDTA + CaCl_2	0.057	0.004

It was found that the most efficient activator of collagen type I digestion by collagenase is Ca^{2+} and the most efficient inhibitor is EDTA.

It was shown that influence of EDTA and MgCl_2 on collagen type I digestion by collagenase may be reduced by adding CaCl_2 to the solution. Ca^{2+} ions restore collagenase activity.

The obtained results demonstrate that Dynamic Light Scattering method can be used for first-order reaction rate coefficient k_1 determination. The key advantage of Dynamic Light Scattering based k_1 measurement is the possibility of real-time digestion monitoring. By varying temperature, solution formula and collagen-collagenase ratio different physiological states can be simulated for *in vitro* investigation.

Disclosures

All authors declare that there is no conflict of interests in this paper.

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