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Kinetics of the Enzymatic Hydrolysis of Chitosan Films

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Abstract—The kinetics of the enzymatic hydrolysis of chitosan film samples under the action of the enzyme hyaluronidase was studied. It was found that, although a monolithic film sample was subjected to hydrolysis, the enzymolysis of chitosan in this case obeyed the same laws as the enzymatic hydrolysis of chitosan in solution at small substrate concentrations.

Keywords: chitosan, enzymatic hydrolysis, chitosan film
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INTRODUCTION

Chitin and chitosan are natural biopolymers, and the process of their synthesis and, undoubtedly, destruction are related to enzymatic transformations [1–3]. Biodegradability to substances common for the body is an important advantage of chitosan; its compatibility with the body tissues, bacteriostatic activity, and good sorption capacity for wound effluent are among the many other advantages. The set of these factors is responsible for the fact that film materials based on chitosan are of great interest for their use as protective materials in the treatment of surgical, burn, and persistent wounds. The biodegradability of chitosan is important from at least two viewpoints. First, this makes it possible to exclude an extremely painful procedure of rebandage; second, the products of chitosan hydrolysis exhibit a biological activity higher than that of chitosan by an order of magnitude [4].

The biodegradation of chitosan occurs as a process of the enzymatic hydrolysis of macrochains at the β-glycosidic bonds. Obviously, chitinases and chitosanases are the most suitable enzymes for performing the process of enzymatic hydrolysis; they lead to the production of oligosaccharides with degrees of polymerization of 2–5. However, under the conditions of the medical application of chitosan materials, their biodegradation occurs under the action of nonspecific enzymes because both chitinases and chitosanases are absent from the human body. Indeed, the enzymes of human body fluids (for example, lysosomal enzymes or hyaluronidase, which occurs on the wound surface) exhibit catalytic activity toward chitosan [5–8].

In this case, the form of material (for example, solution, gel, or film), is responsible for the kinetics of this process because, from a topochemical point of view, there is a fundamental difference between enzyme accessibility to interactions with polymer units in solution and their accessibility in a monolithic sample. A study of the rate laws of enzymatic hydrolysis finally leads to the prediction of a polymer lifetime under appropriate operating conditions [8]. Although the rate laws of the biodegradation of chitosan in solution have been thoroughly studied [6, 7, 9–12], the question of the enzymatic hydrolysis of the monolithic (film) samples is still an open question, which motivated us to perform this study.

EXPERIMENTAL

A sample of chitosan from ZAO Bioprogress (Shchelkovo, Russia) with the molecular weight $M = 113000$ was used in this study. Hyaluronidase (Liraza) from ZAO Mikrogen (Moscow, Russia) was used as an enzyme preparation. The enzyme preparation content was 3×10^{-3} g in all of the experiments. A 1% acetic acid solution was used as a solvent. In the process of enzymatic hydrolysis, the concentration of chitosan in solution (C_{hyd}) was varied from 0.15 to 5 g/dL. The volume of a chitosan solution taken for the enzymatic hydrolysis was 10 mL.

In the experimental determination of the intrinsic viscosity of chitosan in the course of enzymatic hydrolysis $[\eta]_t$, a solution with the concentration C_{hyd} [g/dL] in acetic acid to which an enzyme preparation solution was added was exposed for a specified time at a temperature of 36°C; thereafter, the process of enzymatic hydrolysis was stopped by boiling the initial solution for 30 min in a water bath. Then, a solution with the concentration $C_{hyd} = 0.15$ g/dL for the determination of intrinsic viscosity was prepared from a solution with the initial concentration C_{hyd} by dilution.

The intrinsic viscosities of both the initial chitosan in a solution of acetic acid $[\eta]_0$ and chitosan after an exposure to the enzyme preparation solution (subjected to enzymatic hydrolysis) were determined with

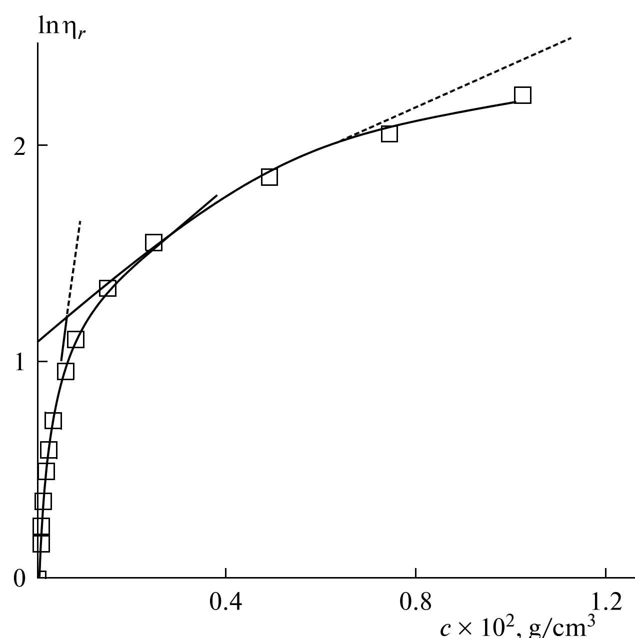


Fig. 1. Dependence of relative viscosity on the chitosan content of solution in the semilogarithmic coordinates.

the aid of an Ubbelohde viscometer using a procedure proposed by Baranov et al. [13], which makes it possible to eliminate the effect of polyelectrolyte swelling in the determination of viscosity [14]:

$$[\eta]^* \equiv \partial \ln \eta_r / \partial c,$$

where η_r is the relative viscosity of chitosan, and c is the concentration of chitosan in solution. The slopes of tangents to the plots of the relative viscosity versus polymer concentration in solution (Fig. 1) correspond to current intrinsic viscosity values. At $c \to 0$, the value of $[\eta]^*$ corresponds to the initial slope of the plot of $\ln\eta_r$ versus c and coincides with the intrinsic viscosity of the polymer in solution $[\eta]$.

For the solution of chitosan in 1% acetic acid used in this work, the initial intrinsic viscosity of the sample that was not subjected to enzymatic hydrolysis was $[\eta]_0 = 7.8$ dL/g. The experimental error was no higher than 3% at a confidence coefficient of 0.95 and five replicate experiments.

The time dependence of the decrease of intrinsic viscosity was linear for all of the used chitosan concentrations at short hydrolysis times (30–40 min). In this section, the initial rate of enzymatic hydrolysis V_0 was determined; it was calculated from the formula [15]

$$V_0 = \frac{C_{\text{hyd}} K^{1/\alpha}([\eta]_t^{-1/\alpha} - [\eta]_0^{-1/\alpha})}{t}, \qquad (1)$$

where t is the time of hydrolysis, min, and K and α are constants in the Mark–Kuhn–Houwink equation.

For determining the constants K and α, which are necessary for the calculation of the initial rate of enzymatic hydrolysis according to Eq. (1) in 1% acetic acid, the initial chitosan sample was fractionated into 10 fractions in a range of molecular weights from 20 000 to 150000 amu. The absolute values of the molecular weights of chitosan fractions were determined by a combination of the methods of high-speed sedimentation and viscometry. The found constants in the Mark–Kuhn–Houwink equation for the test solution of chitosan in 1% acetic acid are $\alpha = 1.02$ and $K = 5.57 \times 10^{-5}$.

The chitosan films were prepared by coating the glass surface in a Petri dish with a 2% solution of chitosan. For studying the process of enzymatic hydrolysis, specimens with the linear dimensions a and b were cut from a film with the use of templates; the specimens measured 0.5 × 0.5, 0.5 × 1.0, 1.0 × 1.0, and 1.0 × 1.5 cm. The films thickness was kept constant and equal to 100 μm. The accurate volume of a film sample was calculated based on the weight and density of a film. The density of a chitosan film sample obtained from 1% acetic acid was determined by pycnometry. It was $\rho = (1.37 \pm 0.03)$ g/cm^3 for the test sample of chitosan.

For conducting an experiment to simulate the enzymatic hydrolysis of chitosan on the wound surface, a chitosan film sample was placed on a support moistened with a solution of the enzyme preparation in 1% acetic acid and exposed at a constant temperature (36°C) for a specified time. The volume of the enzyme preparation solution (0.05 mL) was chosen based on the condition that only one side of the film came into contact with the enzyme solution. After the exposure, the process of enzymatic hydrolysis was stopped by enzyme deactivation upon boiling for 30 min in a water bath. Then, the film was dissolved in 1% acetic acid, and the current intrinsic viscosity of the polymer $[\eta]_t$ was determined using a procedure analogous to that described above for the solutions of chitosan. The surface structure of the films was evaluated by laser scanning microscopy on an LSM-5-Exciter instrument (Carl Zeiss, Germany).

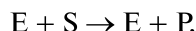
RESULTS AND DISCUSSION

In the study of the enzymatic hydrolysis of chitosan, the determination of the rate of this process is reduced to the determination of changes in the intrinsic viscosity of the polymer with time, which occur as a consequence of the rupture of the macrochains of chitosan upon its interaction with the enzyme preparation. Figure 2 shows the time dependence of changes in the intrinsic viscosity on the degradation of a film sample and chitosan solutions of different concentrations.

As can be seen in Fig. 2, in the case of both a film and solutions of chitosan, an increase in the contact time of chitosan with the enzyme was accompanied by a decrease in intrinsic viscosity, which is indicative of a decrease in the molecular weight of chitosan. In the

both cases, the dependences were linear at the initial stage of the reaction. Note that the shapes of kinetic curves were similar for the films and the solutions, although these reactions are essentially different from a topochemical point of view. On the destruction in solution, in the first approximation, it is possible to consider the equally probable accessibility of any glycosidic linkage in any chain to the reaction with the enzyme. In the film samples, this reaction can occur only with the units arranged on the film surface that contacts with the enzyme solution.

In the case of a solution, the observed dependence of the initial rate of enzymatic hydrolysis, which is calculated from Eq. (1), on the concentration of chitosan in the solution (Fig. 3) are adequately described by the Michaelis–Menten equation. The Michaelis constant K_M was 3.42 g/dL, as determined from the Lineweaver–Burk plot [16]. It is likely that this high value of K_M was due to the fact that hyaluronidase is not an enzyme specific for chitosan, and the pH of a 1% solution of acetic acid is not an optimum pH value for the action of hyaluronidase. The maximum rate of enzymatic hydrolysis V_{max}, which determines the maximum possibility of the formation of the reaction product at a given concentration of the enzyme under conditions of an excess of the substrate, was 1.50×10^{-6} g/dL min. The parameter V_{max}/K_M has the physical meaning of the rate constant of the reaction



$$E + S \rightarrow E + P,$$

where E is the enzyme, S is the substrate, and P refers to the reaction products. This parameter is $V_{max}/K_M = 0.44 \times 10^{-6}$ min^{-1}. The Michaelis–Menten mechanism is reduced to the above reaction scheme at low substrate concentrations. In this case, the dependence of the reaction rate on substrate concentration at the initial stage is approximated by a straight line with the slope V_{max}/K_M.

In the case of films, the situation can be considered analogous because the fraction of surface units accessible to destruction can be very small, as compared with the total number of glycosidic linkages. In the very first approximation, the problem of describing the kinetics of degradation of a film can be represented as the determination of the rate of degradation in solution with a chitosan concentration (g/dL), which corresponds to the concentration of surface units in the volume of the enzyme solution, that is,

$$c_s = m_s / V_{sol}, \quad (2)$$

where m_s is the weight of the monomer units of chitosan on the film surface, g; V_{sol} is the volume of the solution of the enzyme preparation, which contacted with the film, dL.

The following reasoning was used for the estimation of the weight of chitosan monomer units on the film surface. Based on the known weight m_f of a film sample, the number of chitosan monomer units in the entire film volume can be calculated:

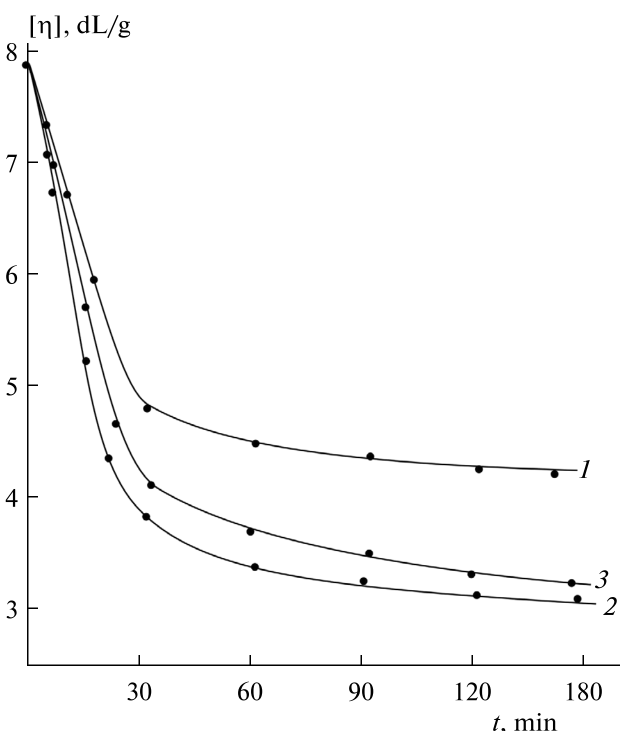


Fig. 2. Dependence of the intrinsic viscosity of chitosan isolated (*1*) from a film sample and (*2*, *3*) from solutions with chitosan concentrations of (*2*) 0.5 and (*3*) 1.0 g/dL on the time of exposure with a solution of the enzyme preparation.

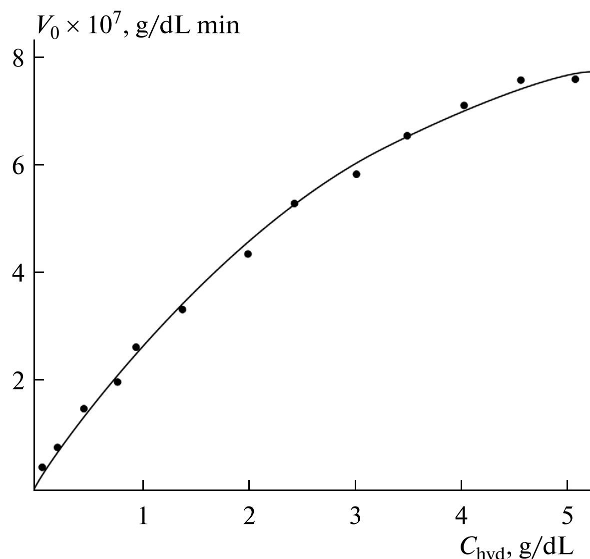


Fig. 3. Dependence of the initial rate of enzymatic hydrolysis of chitosan on its concentration in the solution.

$$n_{unit} = m_f \frac{N_A}{M_{unit}}, \quad (3)$$

where M_{unit} is the molecular weight of a chitosan unit, and N_A is Avogadro's number.

Kinetic parameters of the enzymatic hydrolysis of chitosan film samples

$c_s \times 10^4$, g/dL	$V_0^{*} \times 10^{11}$, g/dL min	$V_0^{**} \times 10^{11}$, g/dL min	$V_0^{***} \times 10^{11}$, g/dL min
1.28	3.1	5.6	5.6
1.41	3.4	6.2	6.1
1.56	3.7	6.8	6.7
1.74	4.2	7.6	7.7

* The value of V_0 was determined from Eq. (1).

** The value of V_0 was determined from Eq. (5).

*** The value of V_0 was determined from Eq. (1) with consideration for surface roughness.

Let us assume that the monomer unit of chitosan is inscribed in a cube with the face d. The volume occupied by a monomer unit in the film volume is $v_{\text{unit}} = V_f/n_{\text{unit}}$, where V_f is the volume of a film sample. Hence, the face size is $d = (v_{\text{unit}})^{1/3}$. Now, we can estimate how many monomer units, each of which occupies the area $s_{\text{unit}} = d^2$, are arranged on a film surface with the area S_f, which contacts with a solution of the enzyme preparation:

$$n_{\text{unit}} = S_f/s_{\text{unit}}. \tag{4}$$

After determining n_{unit}, we can find the unknown value of c_s. Thus, by varying the size of a film specimen, we can obtain a number of films with different concentrations c_s of chitosan units and determine for them the rates of enzymatic hydrolysis V_0 from Eq. (1) (see the table).

On the other hand, based on the fact that the rate constant of glycoside bond cleavage on the surface of a film is equal to that in a polymer solution, the value of V_0 can be calculated as follows:

$$V_0 = \frac{V_{\text{max}}}{K_M} c_s. \tag{5}$$

However, the rates of the enzymatic hydrolysis of film samples calculated from Eqs. (1) and (5) radically differ from each other. The observed discrepancy can be explained with consideration for the fact that the film formed had a rough surface; as a result of this, its real surface area differed from the area calculated as ab.

Figure 4 shows the surface profile of a chitosan film in contact with a support according to scanning laser microscopy data. As a result of roughness and porosity, the surface area of the film that contacted with a glass surface was higher by a factor of 1.8 than the calculated value. Accordingly, the surface concentration of chitosan units accessible to interaction with a solution of

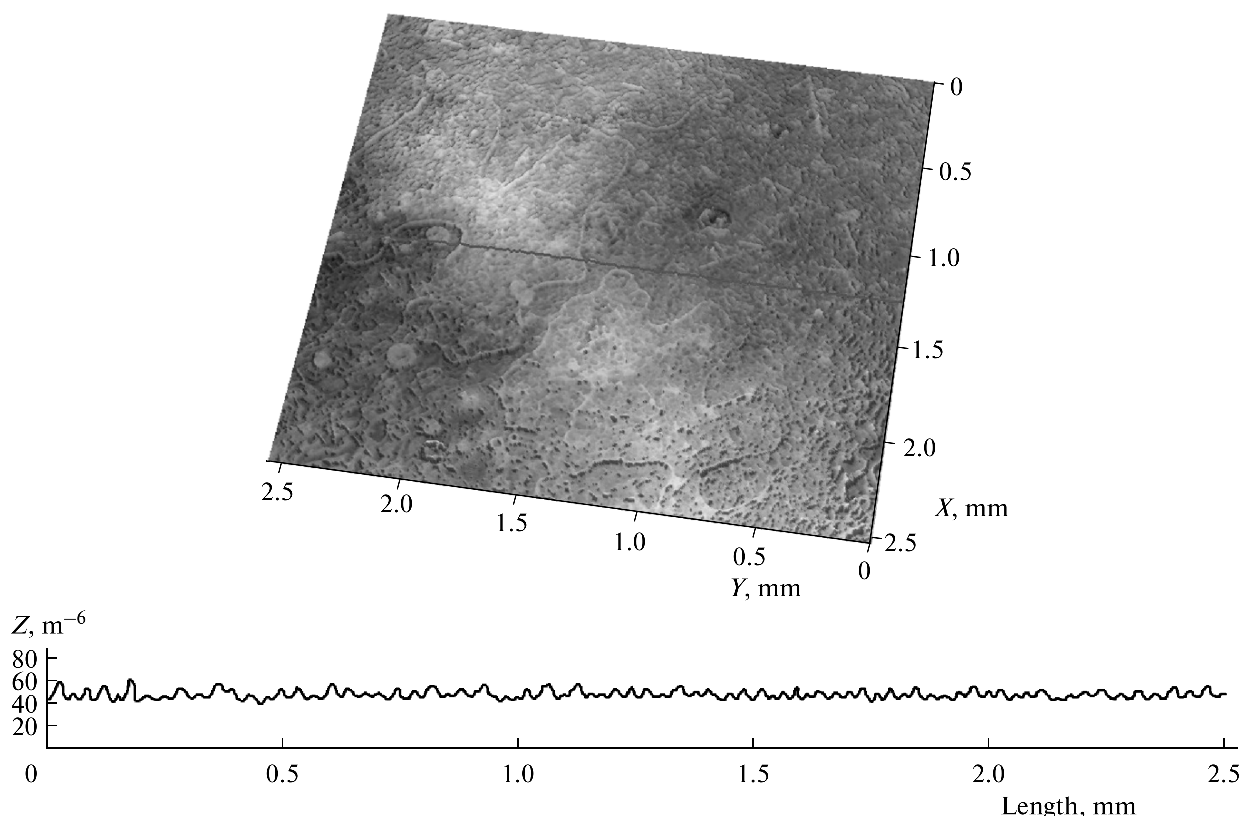


Fig. 4. Micrograph and surface profile of a chitosan film (contacting with a support).

the enzyme preparation was also greater. If we recalculated the surface concentrations of chitosan taking into account surface roughness, the initial rates of enzymatic hydrolysis of the film samples determined from Eqs. (1) and (5) were consistent with each other (see the table).

Thus, it is believed that the hydrolysis of a film obeys the same laws as the hydrolysis in solution at small substrate concentrations, although a monolithic film specimen was subjected to hydrolysis.

CONCLUSIONS

(1) We found that the enzymatic hydrolysis of the film samples prepared from a solution obeys the same rate laws as the hydrolysis of chitosan in solution at small substrate concentrations.

(2) We were the first to determine the kinetic characteristics of the activity of the enzyme hyaluronidase in the enzymatic hydrolysis reaction of chitosan; the parameter having the physical meaning of the rate constant of an enzymatic reaction is $V_{max}/K_M = 0.44 \times 10^{-6}$ min^{-1}.

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