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CHEMICAL PHYSICS OF POLYMER MATERIALS

On the Possibility of Regulating the Rate of Enzymatic Destruction of Chitosan in an Acetic Acid Solution

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Abstract—The enzymatic destruction of chitosan in an acetic acid solution in the presence of sodium chloride and sulfate was considered. It was shown that the addition of these low-molecular electrolytes is accompanied by a tightening of the macromolecular coil, as indicated by a decrease in the α constant in the Mark–Kuhn–Houwink equation and the intrinsic viscosity of chitosan. As a consequence, the chitosan units become less accessible for interaction with the enzyme, and the kinetic parameters of the process (Michaelis constant K_M and the maximum rate of enzymatic destruction V_{max}) change accordingly. This suggests that the resistance of this polymer to the enzyme action can be enhanced by introducing low-molecular electrolyte salts in the solution or film coating to suppress the polyelectrolyte swelling of chitosan.

Keywords: chitosan, enzymatic destruction, kinetics

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INTRODUCTION

The macromolecular reactions are generally associated with a number of specific effects [1]. Among them is the conformation effect, which can change the reaction rate dozen-fold. It was studied in detail, for example, for thermal [2, 3] and oxidative destruction of polymers [4, 5]. One of these effects are also enzymatic reactions involving polymers [6].

Obviously, the effects of the conformational state of the polymer on the rate of enzymatic reaction can be used to regulate the enzymatic stability of polymer materials. For example, earlier we studied the enzymatic destruction of chitosan acetate (ChA) under the action of certain enzyme preparations in solution and in films obtained from acetic acid solutions [7, 8]. Our study showed that it is necessary to increase the stability of ChA against the action of enzymes because the film materials based on this polymer quickly lose integrity on the wound surface as a consequence of enzymatic hydrolysis. In view of the polyelectrolyte nature of ChA, it is reasonable to think that the stability of this polymer against the action of enzymes can be increased by introducing low-molecular electrolyte (LE) salts in the solution or film coating formed from the solution, which suppress polyelectrolyte swelling of ChA and the corresponding compression of the polymer coil. Therefore, the goal of this study was to investigate the enzymatic destruction of ChA in solution in the presence of low-molecular salts, namely, sodium chloride and sulfate and determine the kinetic parameters of the process. As a model enzyme, we used hyaluronidase, an enzyme capable of breaking the β-glycoside bond and present on the wound surface.

EXPERIMENTAL

The object of this study was chitosan (Bioprogress, Shchelkovo, Russia) with a molecular mass M_{SD} = 113000. The enzyme preparation was hyaluronidase (trade name Lidaza, Microgen, Moscow, Russia). As a solvent, 1% acetic acid (chemically pure grade) was used; the low-molecular electrolytes were sodium chloride and sodium sulfate (chemically pure).

The volume of the ChA solution taken for enzymatic destruction was 10 mL. The content of the enzyme preparation in the 1% acetic acid solution was 1 mg.

The ChA concentration in the solution during the enzymatic destruction ($C_{e.d}$) was varied from 0.1 to 5.0 g/dL. The low-molecular electrolytes (LEs) dissolved in a small amount of water were added to the ChA solution in a mole ratio of ChA : LE = 1 : 0.2 and 1 : 0.4 for Na_2SO_4 and 1 : 0.6 and 1 : 1.2 for NaCl. The ChA : LE molar ratio was chosen such that the ionic strength of the solution corresponded to the value of the latter in the previously considered ChA : $AM(SO_4)_2$ system with a molar ratio of components of 1 : 0.05 and 1 : 0.1 [8].

The intrinsic viscosity of ChA [η] in the acetic acid solution was determined in a Ubbelohde viscosimeter at a temperature of (25 ± 0.1)°C by Baranov's method

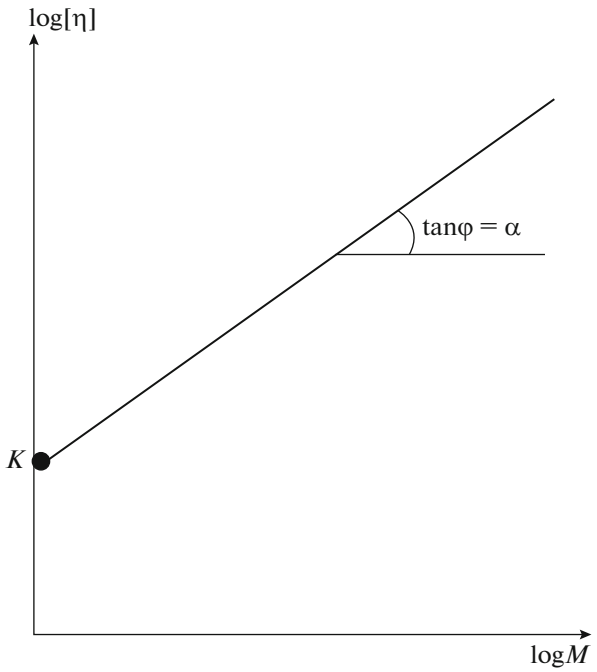

Fig. 1. On the determination of constants in the Mark–Kuhn–Houwink equation.

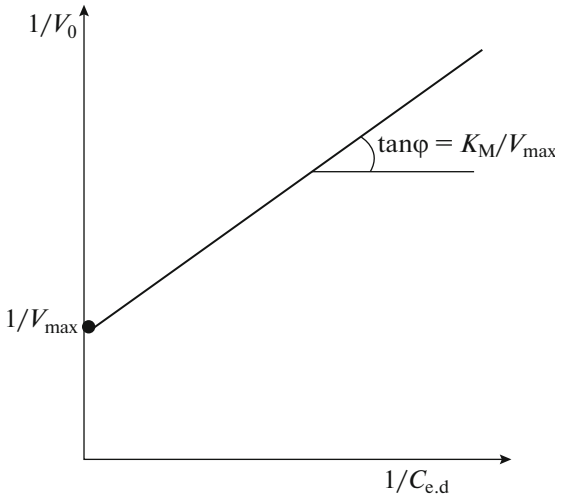

Fig. 2. Lineweaver–Burk plot of the dependence of the initial rate of the enzymatic reaction on the concentration of the substrate (ChA in our case) in double reciprocal coordinates.

from the slope on the initial section of the curve of $\ln\eta_r$ versus the ChA concentration c in solution [9]. In the experiments on determination of the intrinsic viscosity of ChA during enzymatic destruction, $[\eta]_t$, the polymer solution in acetic acid with an addition of the enzyme preparation solution was kept for a certain time at a temperature of (36 ± 0.1)°C, after which the process was stopped by boiling the starting solution for 30 min on a water bath. Then the solution with the initial concentration $C_{e.d}$ was diluted in order to prepare a solution with a concentration $c = 0.10$ g/dL to determine the characteristic viscosity $[\eta]_t$. At short destruction times, the curves of intrinsic viscosity versus the time of storage with the enzyme preparation solution were linear. The $[\eta]_t$ value required for calculating the initial rate of enzymatic destruction V_0 [10] was determined on this section.

The value of V_0 was calculated by the equation [11]:

$$V_0 = C_{e.d}K^{1/\alpha}\frac{[\eta]_t^{-1/\alpha} - [\eta]_0^{-1/\alpha}}{t}, \quad (1)$$

where t is the destruction time, min; and K and α are the constants in the Mark–Kuhn–Houwink equation:

$$[\eta] = KM^{\alpha}. \quad (2)$$

To determine the K and α constants required for calculating the initial rate of enzymatic destruction by Eq. (1) in 1% acetic acid, the starting ChA sample was fractionated into 10 fractions with molecular masses from 20000 to 150000 Da. For the obtained fractions, the absolute values of molecular masses were determined by combining high-rate sedimentation and diffusion methods and intrinsic viscosities. This made it possible to determine graphically the K parameter (by cutting off a segment on the ordinate axis) and α (as the slope of the straight line) in (2) by means of double logarithmic transformation (Fig. 1). The Michaelis constant K_M and the maximum rate of enzymatic destruction V_{max} of ChA solutions were determined by the Lineweaver–Burk graphic method (Fig. 2).

The total β-glycosidase activity of hyaluronidase was determined by the ferricyanide method based on the determination of the concentration of reducing sugars [12]. As a unit of activity, we took the amount of the enzyme preparation formed by 1 μmol of reducing sugars within 1 min of the hydrolysis of 50 mg of filter paper at room temperature.

RESULTS AND DISCUSSION

The intrinsic viscosity of a polymer in a solution can easily and reliably be determined by viscosimetry. A decrease in this characteristic can be used to evaluate the enzymatic destruction. However, in order to pass from the rate of decrease in the intrinsic viscosity to the rate of reduction of the molecular mass of the polymer, which actually suggests biodestruction, it is necessary to know the relationship between the intrinsic viscosity and the molecular mass of the polymer (i.e., the K and α constants in the Mark–Kuhn–Houwink equation). For the ChA sample in the 1% acetic acid solution under study, $K = 0.56$ and $\alpha = 1.02$ [13] (Table 1). The determination of the constants for the chitosan–acetic acid system in (2) made it possible to calculate by (1) the initial rates of enzymatic destruction and then the Michaelis constants and the maximum rates of enzymatic destruction (Table 1) from the

Table 1. Intrinsic viscosities of chitosan acetate and constants in the Mark–Kuhn–Houwink equation determined for the 1% acetic acid solutions of chitosan in the presence of low-molecular electrolytes

LE	ChA : LE, mol/mol	[η], dL/g	$K \times 10^4$	α	K_M, g/dL	$V_{max} \times 10^6$, g/(dL min)
–	–	7.80	0.56	1.020	3.37	0.50
$AM(SO_4)_2$	1 : 0.05	5.45	2.28	0.865	4.16	0.37
	1 : 0.10	5.28	3.19	0.832	4.46	0.31
NaCl	1 : 0.60	5.43	2.36	0.863	4.12	0.38
	1 : 1.20	5.29	3.27	0.833	4.40	0.33
Na_2SO_4	1 : 0.20	5.49	2.28	0.867	4.20	0.41
	1 : 0.40	5.27	2.36	0.835	4.51	0.36

change in the intrinsic viscosity of the polymer resulting from the cleavage of the ChA macrochains in the interaction of ChA with the enzyme preparation.

As ChA is a polyelectrolyte capable of changing the ionic strength of the solution in the presence of low-molecular electrolytes, its conformational state is substantially altered. This is evidenced by regular changes in K and α in the Mark–Kuhn–Houwink equation, as well as the decrease in the characteristic viscosity of ChA. For example, for the previously studied system ChA–amikacin sulfate (antibiotic of the aminoglycoside series), which is a low-molecular electrolyte, it was shown that the addition of amikacin to an acetic acid solution of chitosan [8] was accompanied by compression of the macromolecular coil, as indicated by a decrease in the α constant and the intrinsic viscosity of chitosan (Table 1). This, in turn, led to a corresponding decrease in the maximum rate of enzymatic destruction and a slight increase in the Michaelis constant due to the decreased accessibility of ChA units for interaction with the enzyme [8].

Since amikacin is a low-molecular salt that is highly soluble in water and completely dissociates into ions, in this sense it differs little from any other low-molecular salt. Indeed, the addition of low-molecular electrolytes, for example, sodium chloride and sulfate, to the ChA solution leads to a similar effect, namely, to a decrease in the α parameter and the characteristic viscosity of the polymer and hence to a decrease in the size of macromolecular coils. Importantly, the addition of sodium sulfate and chloride leads not only to a change in the conformational state of ChA similar to that caused by amikacin, but also to the corresponding changes in the kinetic parameters of enzymatic destruction.

According to Fig. 3, as in the case of the introduction of amikacin in the ChA solution, the introduction of the sodium sulfate and chloride low-molecular electrolytes affects not only the intrinsic viscosity of the starting chitosan (not subjected to destruction), but also the rate of its decrease during storage with the enzyme preparation solution. Figure 3 (curves *1–5*) shows the decrease in the intrinsic viscosity of the 1% ChA solutions in the absence and presence of sodium sulfate and chloride. A comparison of these curves suggests that the introduction of low-molecular electrolytes in the ChA solution leads to a considerably less significant decrease in the intrinsic viscosity under the action of enzyme than in the absence of these salts. Also note that the kinetic curves of the decrease in the intrinsic viscosity for ChA in the presence of LE (sodium chloride and sulfate, as well as amikacin sulfate) actually coincide.

The observed dependences of the initial rate of enzymatic destruction of ChA in the presence of low-molecular electrolyte salts (Fig. 4) on the substrate (chitosan) concentration, as well as in the case of a solution of individual ChA [7, 8, 10] and a solution of ChA in the presence of $AM(SO_4)_2$, can be described in terms of the Michaelis–Menten scheme.

The Michaelis constants K_M and the maximum rates of enzymatic destruction V_{max} of ChA solutions determined using the Lineweaver–Burk plot are presented in Table 1. Note that the K_M and V_{max} values are close for systems with close ionic strengths of solution.

Importantly, the decrease in the rate of enzymatic hydrolysis of ChA in the presence of sodium sulfate and chloride (as well as in the case of the enzymatic hydrolysis of ChA in the presence of amikacin considered earlier) is observed along with the absence of their influence on the total glycosidase and endoglycosidase activity of the enzyme preparation, as evidenced by the direct determination of enzyme activity (Table 2).

CONCLUSIONS

Thus, the obtained data indicate that the addition of a low-molecular electrolyte to the ChA solution leads to a decrease in the α and [η] parameters in all cases; i.e., it is accompanied by a decrease in the size of the macromolecular coil. In this case, the greater the ChA : LE molar ratio, the greater the decrease in

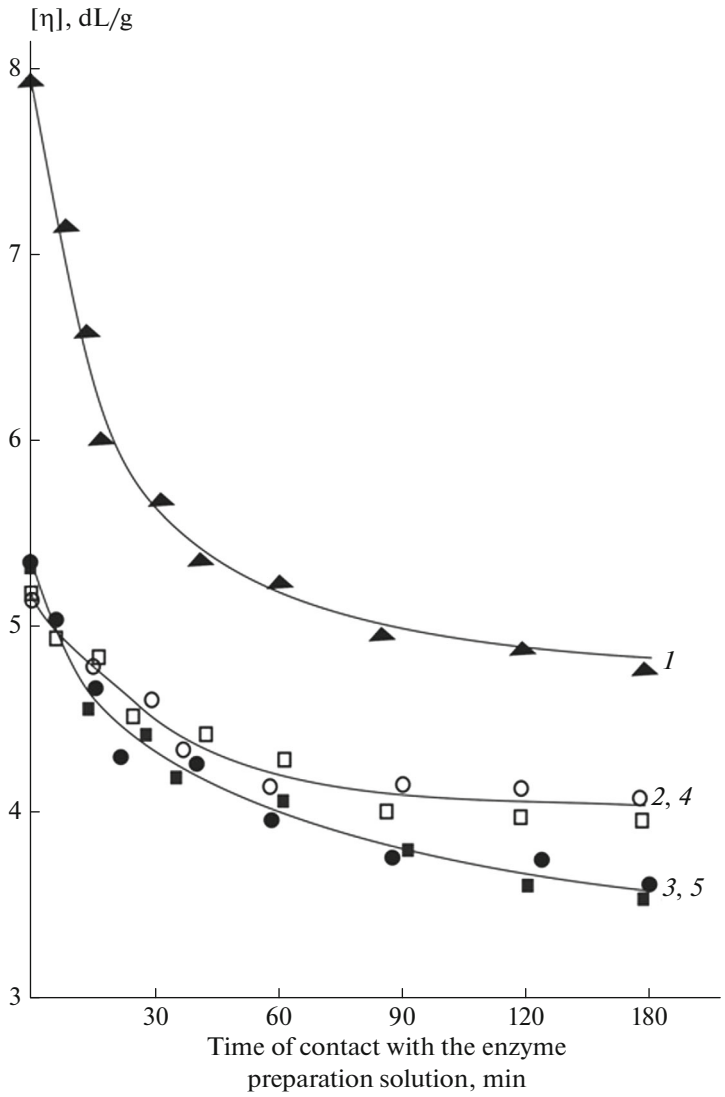

Fig. 3. Dependence of the intrinsic viscosity of ChA isolated from the enzymatically degraded solution with a concentration of 1 wt % (*1*) in the absence and (*2*) presence of (*2*, *3*) sodium sulfate and (*4*, *5*) sodium chloride on the time of contact with the enzyme preparation solution with a concentration 0.1 g/L. The sodium sulfate content was (*2*) 0.4 and (*3*) 0.2 mol; the sodium chloride content was (*4*) 1.2 and (*5*) 0.6 mol per mole of chitosan.

the size of the macromolecular coil compared to its original size. As a consequence of changes in the conformational state of ChA, the accessibility of ChA links for interaction with the enzyme decreases in all instances under study, and a regular decrease in the maximum rate of enzymatic destruction and a slight increase in the Michaelis constant are observed. The simultaneous increase in the Michaelis constant and the decrease in the maximum rate V_{max} indicate that the V_{max}/K_M ratio, which has the physical meaning of the reaction rate constant of the enzymatic destruction of ChA, decreases [14]. This allows us to consider the

Table 2. Total β-glycosidase activity of hyaluronidase

System	Total β-glycosidase activity, units/g
Hyaluronidase solution	70.1 ± 1.4
Hyaluronidase solutionin the presence of 0.05 M $AM(SO_4)_2$	69.3 ± 1.4
Hyaluronidase solution in the presence of 0.20 M Na_2SO_4	71.0 ± 1.4
Hyaluronidase solution in the presence of 0.60 M NaCl	70.2 ± 1.4

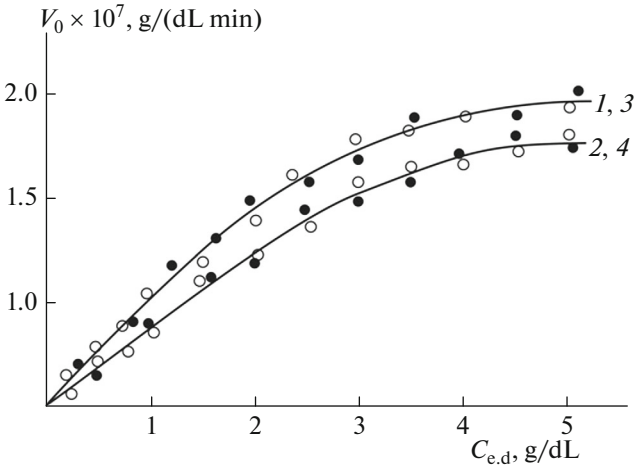

Fig. 4. Dependence of the initial rate of enzymatic hydrolysis of ChA in the presence of sodium sulfate on the ChA concentration in solution. The sodium sulfate content was (*1*) 1.2 and (*2*) 0.6 mol; the sodium chloride content was (*3*) 0.2 and (*4*) 0.4 mol per 1 mol of ChA.

addition of low-molecular electrolyte salts, including those that play the role of pharmaceuticals, as a method for selectively regulating the rate of enzymatic destruction of ChA.

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