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Hydrolytic degradation of biopolymer systems based on poly-3-hydroxybutyrate. Kinetic and structural aspects

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SUMMARY

The aim of the present work is to record and compare long-term kinetic curves of the hydrolytic degradation of polymer systems: bacterial poly-3-hydroxybutyrate (POB), its copolymer with hydroxyvalerate, and also a composite mixture of POB and polylactide (PLA). To monitor the degree of hydrolytic degradation, use was made of the total weight loss of the specimen and the change in the viscosity-average molecular weight (MW). Atom force microscopy was used to assess the surface state of POB films. It was shown that the rate of hydrolytic degradation depends on the incubation medium (the nature of the buffer), temperature, the chemical composition of the biopolymer, and its molecular weight. Atom force microscopy confirmed that, along with volumetric processes of POB hydrolysis, surface hydrolysis of the polymer also occurs.

INTRODUCTION

Bacterial polyhydroxyalkanoates (POAs) and their main representative poly-3-hydroxybutyrate (POB) constitute a competitive alternative to traditional synthetic polymers such as polypropylene, polyethylene, polyesters, and so on. These polymers are generally non-toxic, the raw materials needed for their production are renewable and do not depend on the production of hydrocarbons, and, most importantly, the products of their decomposition (carbon dioxide, water, and, for POB, hydroxybutyric acid) do not adversely affect the human body or the ecology [1–3]. Being compatible with the environment [4], they are used as alternative packaging materials that, after use, decompose in the ground or in aqueous suspension [5, 6].

The use of POB copolymers, mainly in the form of copolymers of hydroxybutyrate and hydroxyvalerate, enables the service characteristics of highly crystalline POB, such as its brittleness and rigidity, to be improved and the processing temperature to be lowered. Furthermore, these copolymers of POB with 3-hydroxyvalerate [7], with 3-hydroxyhexanoate [8], with 3-hydroxyoctanoate, etc., themselves possess improved thermal and mechanical properties, and, what is more, expand the range of structural and medical materials/products.

To predict the state of POB and its derivatives in the human body or in the bacterial medium of soil, it is necessary to study the kinetics and mechanism of their hydrolytic degradation. As research of this kind has been going on for less than 25 years, problems of the degradation of amorphous and crystalline regions of polymers are a fair way from being finally resolved. Moreover, kinetic investigations of degradation, especially with long periods of exposure (incubation), are encountered extremely rarely [10]. Therefore, the aim of the present work is to record and compare the long-term kinetic curves of degradation of POB, its copolymer with

valerate, and also a composite of POB with polylactide (PLA). For comparison, similar kinetic curves have been investigated for pure PLA, enabling breakdown rates to be compared for the biopolymers most commonly used at the present time. Furthermore, considerable attention is paid to the influence of the initial MW of polymeric materials on their degradation rate.

METHOD

The following reagents were used in the study: polylactides (Fluka, MW = 42.4 and 388.7 kDa), 1-substituted sodium phosphate (Na_2PO_4, KhimMed, MW = 121), Tris-HCl ($C_4H_{11}NO_3$, MW = 121.1, Serva, Germany), sodium azide (NaN_3, Sigma-Aldrich, USA), chloroform (trichloromethane $CHCl_3$, "EKOS-1" CJSC, Russia), hydrochloric acid (HCl, "OSCh 20-4" (very pure), KhimMed, Russia), and sodium hydroxide (NaOH, chemically pure, KhimMed, Russia).

In the biochemical synthesis of POB, use was made of a highly productive producer strain of POB *Azotobacter chroococcum 7*B capable of synthesising up to 80% POB (in terms of the dry weight of cells). Collection strains of *Azotobacter* were maintained on Ashby's medium. To achieve high cell productivity, *Azotobacter* culture was grown in Burk's medium under conditions of an excess content of carbon source in the medium [11, 12]. The isolation and purification of the polymer from *Azotobacter chroococcum* biomass were described in references [12] and [13]. The POB content in the cells was determined by Zevenhuisen's method [14].

The initial molecular weights of the polymers and their changes were determined by viscometry. The viscosity of the POB solution in chloroform was measured at 30°C. The molecular weight was calculated by the Mark–Houwink–Kuhn equation using the following power-law equation with reduced numerical coefficients [15]:

$$[\eta] = 7.7 \times 10^{-5} \times M^{0.82}$$

where $[\eta]$ is the reduced viscosity of the POB solution, and M is the viscosity-average molecular weight of the biopolymer.

Surface images of POB films were obtained by atomic force microscopy. To investigate POB films, use was made of a Solver PRO-M atomic force microscope (Zelenograd, Russia). A piece of film measuring ~2 × 2 mm^2 was fastened in a holder by two-sided scotch tape. Scanning was conducted in a semi-contact regime using NSG01 cantilevers (typical rigidity 5.1 N/m); the scanning frequency was 1–3 Hz and the frame size ranged from 3 × 3 to 20 × 20 μm^2.

To study hydrolysis *in vitro*, a series of POB films were produced, with a thickness of 40 μm and a diameter of 30 mm, and with different MWs (169 kDa (nominally 150 kDa), 349.286 kDa (nominally 300 kDa), 500.365 kDa (nominally 450 kDa), and 950.14 kDa (nominally 1000 kDa)), and a copolymer of 3-hydroxybutyrate with 3-hydroxyvalerate (POVB) with MW = 1056.212 kDa. In addition, a study was made of the degradation *in vitro* of films of polylactides (PLAs) of 40 μm thickness and different MWs (40 and 400 kDa). Furthermore, a blend of POB with PLA was obtained. High-molecular-weight POB (MW = 1000 kDa) and low-molecular-weight PLA (MW = 40 kDa) in a polymer ratio 1:1 (wt:wt) were dissolved together in chloroform, after which composite films were obtained. Films of composite and polymer specimens were prepared by evaporation of the solvent (chloroform) on a glass substrate. The films weighed 50–70 mg. The degree of weight loss of the polymer as a result of degradation was determined gravimetrically on an AL-64 balance (max = 60 g, measurement error d = 0.1 mg, ACCULAB, USA).

To measure the hydrolytic degradation of POB, PLA, and POBV films and the POBV composite, they were incubated in a phosphate buffer (PB) solution (pH = 7.4) and a Tris buffer solution (pH = 7.7) at 37°C and in a phosphate buffer solution (PB) (pH = 7.4) at 70°C in a thermostat (ES 1/80 SPU, Russia) for 83 days. The pH was monitored using a pH meter (Orion 420+, Thermo Electron Corporation, USA). To measure the weight of the polymer, films were taken out of the buffer solution every 3 days and dried. The films were placed in a thermostat for 1 h at a temperature of 70°C and then weighed on a balance (measurement error d = 0.1 mg). A quantity of 2 g/L of sodium azide (NaN_3) was added to the buffer solution to inhibit the growth of microorganisms and prevent their contribution to biodegradation. The buffer *in vitro* was replaced every 3 days in experiments with phosphate buffer [16].

For each measurement (film weight and polymer MW), four films were used, for which measurements were conducted under identical conditions. The standard deviation of measurements did not exceed 2%. Data for one of the four measurements, that closest to the average value, are presented on the graphs and diagrams.

RESULTS AND DISCUSSION

Kinetics of the hydrolytic degradation of POB and its derivatives

Earlier we reported that the degradation of POB and its derivatives in the body of an animal occurs as a result of a combination of hydrolytic and enzymatic degradation, with change in the weight of the specimen and in its physicochemical properties [1]. In this work, the contribution of hydrolytic degradation was studied in two

different aqueous media: phosphate buffer (below, PB), pH = 7.4, and Tris buffer, pH = 7.7, both at 37°C for 3 months. Analysis of kinetic curves (**Figure 1**) indicates that the rates of weight loss of low-molecular-weight PLA (MW = 40 kDa), most susceptible to degradation, and also of POB with a relatively low MW (150 kDa) differ appreciably in different media. In Tris, their degree of degradation is slightly lower than in PB. For the remaining specimens these differences were not found. This is possibly also connected with the fact that changes in their weight are fairly small and lie within the range of measurement error. Furthermore, from **Figure 1** it can be seen that specimens with a high MW are more resistant to hydrolytic degradation in these aqueous media by comparison with specimens with lower MW. In fact, the weight loss of low-molecular-weight POB (MW = 150 kDa) occurs more rapidly, and the residual weight reaches 87.4% of the initial weight, and 94–98% for high-molecular-weight POB (300, 450, and 1000 kDa) over a period of 84 days (see the insert in **Figure 1** (**Figure 1B**)).

Figure 1 also shows the influence of the MW of PLA on the kinetics of its weight loss under the influence of hydrolytic degradation. Kinetic curves of these specimens with MW = 40 and 400 kDa practically coincide and are not dependent on MW. By the end of the third month of exposure of specimens in PB, their residual weight amounted to 86–87% of the initial weight. Comparison of the curves of weight loss for films of similar-MW biopolymers PLA and POB (MW = 400 and 450 kDa respectively) showed that polylactides lose weight more rapidly than similar POB specimens. This confirms known published data on the higher degradation rate of PLA compared with POB [1, 8, 17].

Similarly, under the same conditions, i.e. in phosphate buffer at 37°C, pH = 7.4, over a period of 3 months, a study was made of the hydrolytic degradation of films of POBV copolymer and of a composite of high-molecular-weight POB (1000 kDa) with low-molecular-weight PLA (40 kDa) (**Figure 1**). The degradation of POBV proceeds fairly slowly; there is no weight loss over a period of roughly 60 days, in the initial period the film loses 1% of its weight, and after 70 days an appreciable gradual fall in weight begins. From **Figure 1** it can also be seen that a composite of POB and PLA degrades at roughly the same rate as the high-molecular-weight POB in its composition, whereas the low-molecular-weight PLA hydrolyses at a considerably higher rate.

In order to clarify the effect of temperature on the degree of hydrolysis of POB, and to intensify the process, a higher temperature (70°C) and a phosphate buffer were chosen. As expected, the acceleration of hydrolysis under these conditions was extremely marked, as shown in **Figure 2**. Thus, over the course of 45 days, films of PLA were transformed into a finely dispersed powder, and its

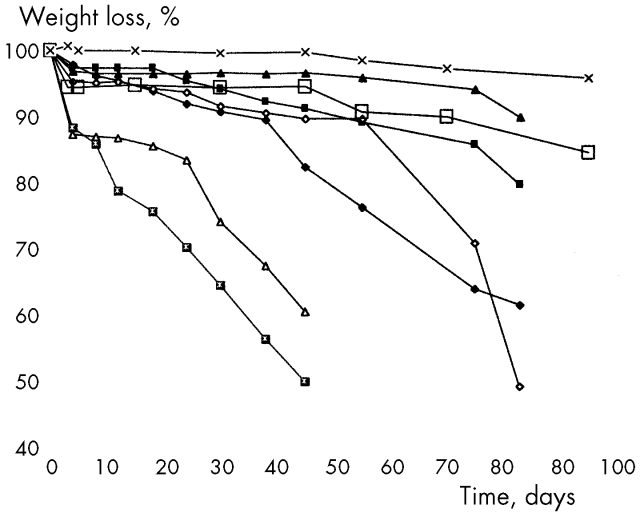

Figure 2. *Kinetic curves of hydrolysis of films of PHB, PLA, and PHV and the composite in PB at 70°C, pH = 7.4*

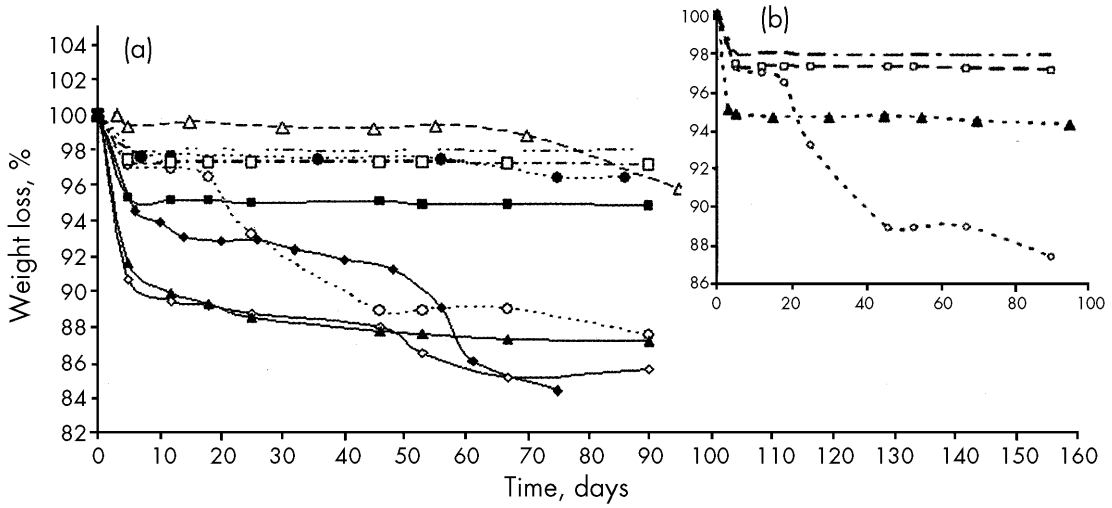

Figure 1. *(A) Kinetic curves of degradation of polymers in a phosphate buffer (PB) and a Tris buffer in vitro at 37°C. (B) Kinetic curves of hydrolysis of POB films in a phosphate buffer (PB) (pH = 7.4) at 37°C*

weight loss for a specimen with MW = 40 kDa was up to 50%, and for a specimen with MW = 400 kDa up to 40%. After 83 days incubation in buffer, POB with a low MW (150 kDa) lost 51% of the original film weight and was greatly fragmented. After 83 days incubation, POB films of greater molecular weight (300, 450, and 1070 kDa) lost less weight: 20, 10, and 15% respectively. It is interesting that, for composite films, the weight loss within 83 days was up to 50%, whereas films of POBV with increase in temperature were extremely resistant and, by the end of 95 days, lost only 4% of the initial polymer weight. Here it must be noted that, during biosynthesis, the introduction of 3-hydroxyvalerate units into the POB macromolecule leads to two processes having opposite effects on the sorption of water. On the other hand, there is an increase in the hydrophobic nature of the chain, i.e. a decrease in the ratio of the number of ester groups to the hydrocarbon groups (methyl, ethyl, and methylene), whereas the degree of crystallinity in such a polymer falls from 70 wt% to roughly 30 wt%. Each of these processes has an opposite effect on the water sorption capacity of the copolymer. In the general case, becoming hydrophobic lowers the water content of the polymer, but a fall in the degree of crystallinity increases it [18]. Therefore, the higher hydrolysis resistance of the copolymer seems to be due to the predominant influence of the POBV polymer chain becoming hydrophobic.

Change in the molecular weight of POB and POBV

In the study of hydrolytic degradation, we assessed the change in the molecular weight of POB and POBV *in vitro*. Specimens incubated in a phosphate buffer at 70°C underwent considerable degradation (**Figure 3**). Thus, after incubation for 83 days, the reduction in MW of polymer films amounted to 82.25% (from 169 to 30 kDa) and to 91% (from 500 to 45 kDa). Reduction in MW occurred after only 45 days, and then the fall in MW slowed down; thus, a polymer with an initial MW of 349 kDa had MW = 39.6 kDa after 45 days, with a further fall in MW to 35.2 kDa by 83 days incubation. For comparison, we will show that the same polymer in a phosphate buffer at 37°C has an MW of 287 and 266 kDa after 45 days and 83 days incubation respectively. At 83 days incubation of POB of 300 kDa molecular weight in phosphate and Tris buffer, the MW amounted to 266 and 265 kDa respectively.

Surface analysis of BPGB films by atom force microscopy

The morphology, structure, and surface roughness of POB films subjected to the action of different corrosive media (phosphate buffer, NaOH solution) were investigated by atom force microscopy (AFM). In **Figure 4**, a specimen of POB with MW = 150 kDa was chosen as the control, produced by casting from its solution in chloroform on to a glass substrate. This film production method presupposes a possible difference in the structure/morphology of the surface facing the glass and the surface facing the air. In fact, as can be seen from **Figure 4**, one of the sides, facing the air, is rough (**Figure 4A**), with many pores of 500–700 nm depth. The surface is covered with interlaced protrusions of 200–400 nm width and 1–2.5 μm length, which are possibly crystalline regions. The reverse side of the film, facing the glass (**Figure 4B**), has a less prominent morphology, which is characterised by a pore depth of less than 100 nm. At large magnification (not shown here), stacks of parallel positioned structures of ~100 nm width and 500–800 nm length can be seen in some places.

The differences between the sides become particularly noticeable when the roughness parameters are compared. To describe the surface of specimens, two roughness parameters were calculated:

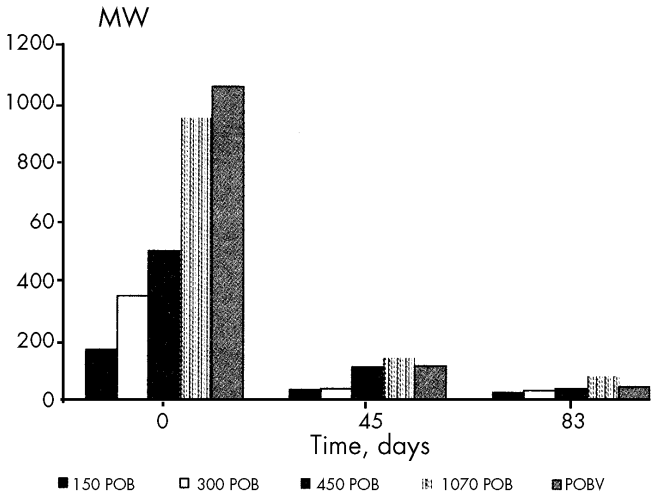

Figure 3. *Change in the MW of PHB and PHV in PB at 70°C and pH = 7.4 as a result of hydrolytic hydrolysis*

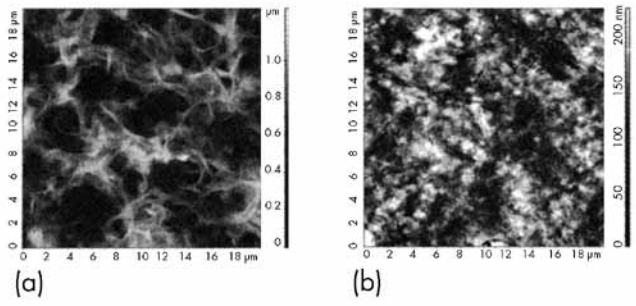

Figure 4. *Rough (A) and smooth (B) surfaces of polyhydroxybutyrate film, used as control surfaces. The film was produced on a glass substrate by evaporation of solvent. The rough side faced the air, the smooth side the glass*

average roughness

$$R_a = \frac{1}{N}\sum_{n-1}^{N}|r_n|$$

rms roughness

$$R_q = \sqrt{\frac{1}{N}\sum_{n-1}^{N}|r_n^2|}$$

These parameters were calculated from three 20 × 20 μm^2 frames, and in each frame there were 512 × 512 points.

Thus, analysis of the roughness of different surfaces of the same film indicates that the average and rms roughnesses of planes facing the air and facing the glass differ roughly by a factor of 10. Differences of this kind are due to the conditions of desorption of the solvent (chloroform) from the POB specimens formed. In the case of evaporation of chloroform from the surface, the flow of solvent into the surrounding air forms additional channels – pores that are fixed as the specimen solidifies and crystallises. At the same time, the morphology of POB on the opposite surface is less prone to the effect of solvent transport and is governed by the surface energetics (surface tension) at the glass–POB boundary. The placing of the POB specimen in buffer solution (Tris) for a prolonged period (83 days) leads to a threefold increase in roughness on the surface previously facing the glass and hardly affects the roughness and morphology of the opposite surface (**Table 1**).

CONCLUSIONS

The data obtained indicate that, along with volumetric processes of hydrolysis of POB, surface hydrolysis of the polymer also occurs. The rate of hydrolytic degradation depends on the incubation medium, temperature, the chemical composition of the biopolymer, and its molecular weight. Comparison of the curves of weight loss for films with similar MWs of the biopolymers PLA and POB under identical conditions showed that polylactides lose weight more rapidly than similar POB specimens.

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Table 1. Roughness parameters of POB films

Specimen	Contact surface	R_a, nm	R_q, nm
Initial	Air	130 ± 10	165 ± 10
Initial	Glass	15 ± 2	20 ± 1
After contact with buffer, 37°C	Air	135 ± 5	166 ± 7
After contact with buffer, 37°C	Glass	46 ± 2	59 ± 1

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