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Mechanics and surface ultrastructure changes of poly(3-hydroxybutyrate) films during enzymatic degradation in pancreatic lipase solution

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ABSTRACT

A study of *in vitro* biodegradation of poly(3-hydroxybutyrate) (PHB) in model conditions was performed. The porcine pancreatic lipase solutions at different concentrations in the two buffer systems (phosphate-buffered saline (PBS) and simulating body fluid (SBF)) were chosen as model biodegradation media. At first, optimal concentration of pancreatic lipase (0.25 mg/ml in PBS) was determined: in these media the decomposition of PHB films realized faster according to the data of gravimetric analysis. Differential scanning calorimetry showed an increase in the crystallinity of the samples (from 49% to 59%) after enzymatic degradation. These data are confirmed by method of nanoindentation, where the increase of the Young's modulus during the degradation (from 1.37 GPa to 4.4 GPa) was shown. This is due to the crystallization of the amorphous polymer component, and its decomposition and dissolving. During biodegradation three types of polymer ultrastructure changes were observed on the surfaces of the films: appearance of new lamellae, disappearance of lamellae and disintegration of lamellae into shorter fragments.

KEYWORDS

Biopolymers; biodegradable polymers; mechanical properties; nanoindentation; PHA; PHB

Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polymers that are formed as naturally occurring storage polyesters by a wide range of microorganisms [1]. The mechanical properties of polyhydroxyalkanoates are similar to those of polyolefins such as polyethylene and polypropylene. However, due to a number of unique properties such as biodegradability, biological inertness, and compatibility with the tissues of mammals, they have a perspective for use in medical or pharmaceutical fields.

PHAs are completely decomposed into carbon dioxide and water by natural microbial mineralization. They are thermoplastic polymers, resistant to UV radiation, water-resistant and have low hygroscopic properties. The use of biodegradable polymers in the packaging and

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food industry solves the problem of waste packaging by minimizing the harmful impact on the environment.

The development of medicine promotes active use of polymers as a basis for variety of implantable medical devices: stents and sutures in surgery, artificial tissues and organs in transplantation, materials for dentistry, scaffolds for tissue engineering, and drug forms in the pharmaceutical industry.

Poly-3-hydroxybutyrate (PHB) and its copolymers are the most well-known and widely used representatives of polyhydroxyalkanoates (PHAs). Due to complex physical, chemical and biological properties PHB can be used to manufacture resorbable sutures, surgical plates, osteoprostheses and implants with the possibility of gradual replacement of connective tissue of the body [2]. Due to its technological, mechanical and physico-chemical characteristics the polymer exceeds traditional implants of polyesters such as polyglycolides and polylactides. Physical and chemical indicators of PHB (minimal shrinkage, low Young's modulus) provide optimal technological characteristics necessary for the creation of resorbable sutures [1–3].

To predict the state of the PHB and its composites with other polymers in the human body, it is necessary to study the kinetics and mechanism of their biodegradation. Despite the fact that the history of such studies already extends back for 25 years, the problems of degradation of amorphous and crystalline components of the polymer are not well understood, especially there is not enough information on the change of the mechanical properties of these polymers and nanostructures during biodegradation.

There is a number of works that discuss the problem of biodegradable polyhydroxyalkanoates and, in particular, PHB. In these works the enzymes of prokaryotes: PHB-depolymerase of strain *Ralstonia pickettii* [4], and depolymerase of strain *Alcaligenes faecalis T1* are used as an enzyme that breaks down the polymer [5]. However, since PHB is a material for biomedical applications, it is important to study its decomposition under the influence of eukaryotic enzymes, such as pancreatic lipase.

Pancreatic lipase, an enzyme secreted by the pancreas, which function is the hydrolysis of triglycerides to glycerol and fatty acids. Pancreatic lipase has a molecular weight of 48 kDa and an optimum catalytic activity at pH = 8. It is important to note that this enzyme is present not only in the pancreatic secret, but also (at various concentrations) in almost all mammalian and human tissues. The lipase enzyme is one which is supposedly responsible for the degradation of PHB in the body when it is implanted in various laboratory animals and tissues of human. The lipase's mechanism of action is to attack the PHB ester bond followed by cleavage of 3-hydroxybutyrate oligomers. Therefore, the aim of the present work is to track changes of physico-chemical and morphological properties of poly(3-hydroxybutyrate) in the presence of porcine pancreatic lipase [2, 6, 7].

Therefore, the aim of the present work is to record changes of physico-chemical and morphological properties of poly (3-hydroxybutyrate) by porcine pancreatic lipase.

Experimental section

Materials and methods

Following reagents were used: disodium hydrogenphosphate (Na_2HPO_4, KHIMMED (Russia)), monopotassium dihydrogenphosphate (KH_2PO_4, KHIMMED), potassium chloride (KCl, KHIMMED), sodium chloride (NaCl, KHIMMED), sodium bicarbonate ($NaHCO_3$), dipotassium phosphate (K_2HPO_4), magnesium chloride ($MgCl_2$), hydrochloric acid (HCl, KHIMMED), sodium hydroxide (NaOH, KHIMMED), chloroform

(trichloromethane $CHCl_3$, EKOS-1 (Russia)), sodium azide (NaN_3, DIA-M (Russia)), porcine pancreatic lipase (Sigma-Aldrich, USA).

A high-yield-producing strain *Azotobacter chroococcum 7B* was used as PHB producer, which is capable of synthesizing PHB up to 80% by dry weight of cells. Collectible Azotobacter strains were maintained in Ashby medium. To achieve high productivity rate of cells they were grown in Burke medium under conditions of excess carbon source. Isolation and purification of the polymer from the biomass of *Azotobacter chroococcum 7B* is described in [8].

For the biodegradation study in vitro, a series of 50 μm thick films of 90 mm diameter was made from PHB, molecular weights of 82 and 826 kDa were used. The films are model objects of study, and are used in many studies of polymer degradation [9–14]. They were prepared by casting from a chloroform solution on the bottom of a glass Petri dish. Films (weight 410 mg) were cut into 3 × 1 cm size pieces. Changes in mass of films during biodegradation were determined gravimetrically on the AL-64 scales (Max = 60 g, d = 0,1 mg, ACCULAB, USA).

The pancreatic lipase was chosen as the enzyme for degradation. Lipase is a non-specific esterase, which is used in a number of studies on the degradation of the polymer [7, 8]. Films of PHB were incubated in solutions of different concentrations: 0.1 mg/mL, 0.25 mg/mL, 1 mg/mL, in two different buffers: Phosphate buffered saline (PBS, pH = 7,4) and a buffer simulating blood plasma (SBF, pH = 7,4) at 37°C in a thermostat for 30 days [9]. Control of pH was performed using a pH-meter (Orion 420+, Thermo Electron Corporation, USA). For weight measurement the films were removed from the solution and were dried, then weighed on scales. Sodium azide (NaN_3) was added to the solution at a concentration of 1 g/l to inhibit the growth of microorganisms and prevent their contribution to the biodegradation. Solutions renewal was conducted twice a week [2].

Images of the surface of PHB films were obtained by atomic force microscopy (AFM). The research was conducted on a microscope Solver PRO-M of NT-MDT company (Russia, Moscow) with a field of view of 100×100×7 microns. Scanning was performed in semi-contact mode in air. ETALON silicon cantilevers were used (radius of tip—10 nm, stiffness—3.5–12 N/m) and NSG 11 (radius 10 nm, stiffness—2.5–22.5 N/m). An optical system combined with AFM was used to select the scan area. AFM images were processed using the Nova ("Nanotechnology MDT") and FemtoScan Online ("Center of Advanced Technologies") software.

Study of the mechanical properties was carried out by method of instrumented nanoindentation according to the requirements of ISO 14577 using scanning nano-hardness tester NanoScan-4D (FSBI TISNCM, Troitsk, Moscow, Russia) [15, 16]. Nanoindentation was performed on the smooth side of the film. The films (~2×2 mm^2) were fixed in phenylsalicylate. The loading was performed in the linear force application mode, the peak load on the sample was 5 mN. Both loading and unloading time was 30 s, with 5 s hold at the peak load. Average penetration depth of the probe was 1 μm. Mechanical properties were averaged over six measurements

Results

Some studies of biodegradation of PHB used bacterial enzymes [17, 18], however, both enzyme and environment of incubation affect polymer degradation [19]. Therefore, it is a major challenge to select an environment to degradation of the polymer. For the comparison of the biodegradation rate of polymer, two buffer solutions were selected: phosphate-buffered saline (PBS) buffer and simulating blood plasma (SBF), and various concentrations of pancreatic lipase in these solutions. All solutions had pH of 7.4. Polymer films were stored at 37°C

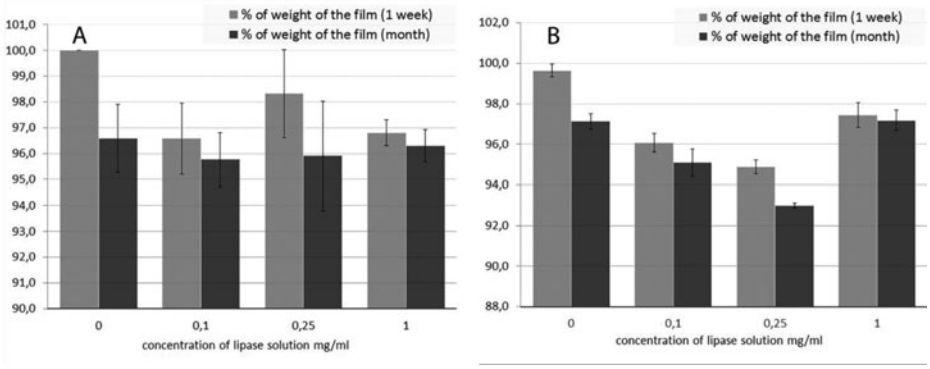

Figure 1. Diagrams of changes in weight of films during degradation in SBF (a) and PBS (b) buffers depending on the concentration of pancreatic lipase for a week and for a month.

for a month. Figure 1 shows the change in weight of PHB films after 1 week and month of biodegradation by pancreatic lipase.

The diagrams show that both of the buffer solutions contribute to polymer degradation, but a solution of lipase in phosphate-buffered saline has a greater effect on the degradation of the polymer. Lipase solution with a concentration of 0.25 mg/ml had the greatest influence on the polymer film, which weight decreased by 7% from the initial during a month. At concentrations of 0.1 mg/mL and 1 mg/mL of lipase solution average weight loss of films was around 4.7% per month in PBS and 4% in a SBF buffer. Effect of lipase concentrations can be explained with the fact that at 1 mg/mL substrate inhibition occurs, a process similar to that described for PHB-depolymerase. At high enzyme concentration degradation is reduced because the access to enzyme catalytic domain for the polymer molecules is blocked [18]. At a concentration of 0.1 mg/mL, not all the polymer molecules at the film surface are occupied by the enzyme, therefore the rate of degradation does not reach its peak. Concentration of 0.25 mg/mL corresponds to a concentration of pancreatic lipase in human body [20] and seems to be optimal for carrying out experiments on the degradation of PHB.

By PHB is a semicrystalline polymer. The degree of crystallinity affects the rate of enzymatic degradation of the polymer and its affinity for animal tissues [21]: enzymatic degradation rate of amorphous component is about 20 times faster than the crystalline. Differential scanning calorimetry data for the degree of crystallinity of PHB were prepared before and after lipase treatment (Table 1).

A control sample had a degree of crystallinity of 49%. The crystallinity of the sample, which was subjected to biodegradation for 1 week in the lipase solution was 57%, and after a month—59%.

With increment of the degree of crystallinity the Young's modulus (stiffness) of the sample also increases. The study of sample stiffness was conducted by the scanning nano-hardness tester NanoScan-4D (Table 1). The force of 1, 3, and 5 mN was applied to the sample. The

Table 1. The degree of crystallinity and a Young's modulus of the sample before and after biodegradation.

Sample	T_m °C, beginning	T_m °C, peak	T_c °C, peak	H_m, J/g	Crystallinity, %	Young's modulus, GPa
Before the enzymatic treatment	155	161	59	72	49%	1.37
1 week in the lipase solution	159	168	62	84	57%	3.47
1 month in the solution	153	161	54	87	59%	4.4

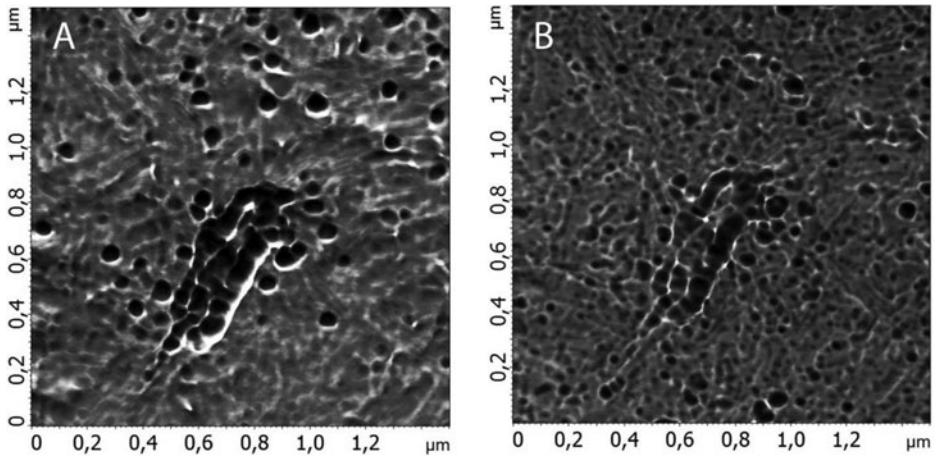

Figure 2. The phase images of PHB film before (A) and after (B) hydrolysis in lipase solution. Incubation time—3 weeks, the temperature—37°C.

closest to the literature data (1.4 GPa) Young's modulus was found the value obtained from nanoindentation for 3 mN indentation load. The stiffness of freshly prepared films of PHB was 1.37 GPa. After a week of degradation, the stiffness of films increased up to 3.47 GPa, and a month later—up to 4.4 GPa. This suggests that in the first week most of the components of the amorphous polymer is crystallized and then undergoes a process of decomposition.

Figure 2 shows the two phase-images corresponding to PHB films that were before and after incubation in lipase solution. After treatment with a lipase solution PHB film acquired certain features.

To describe the morphological changes induced by enzymatic degradation, the films were studied by AFM. The lamellar structure is easily distinguished by phase imaging, so this method was applied. Three types of morphological changes were observed: the emergence of new lamellar structures, fragmentation of lamellar structures, disappearance of lamellar structures [22].

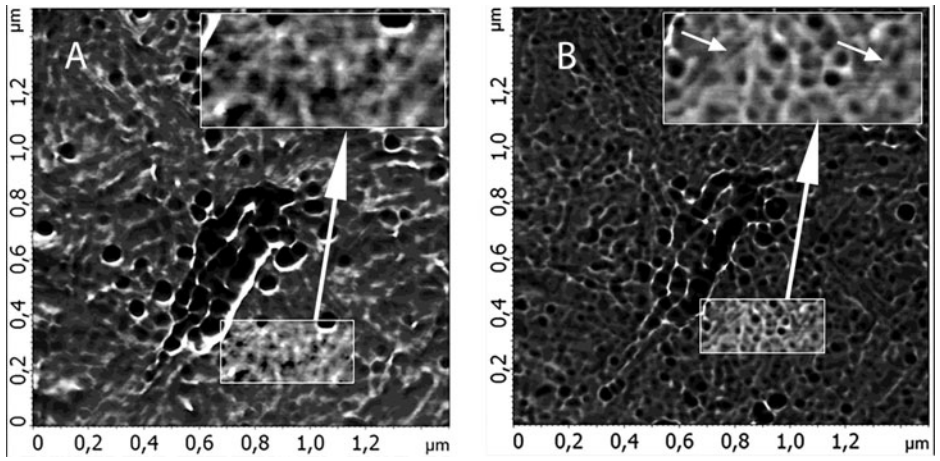

Figure 3. The phase images of PHB film before (A) and after (B) hydrolysis in lipase solution. Arrows indicate the new lamellas.

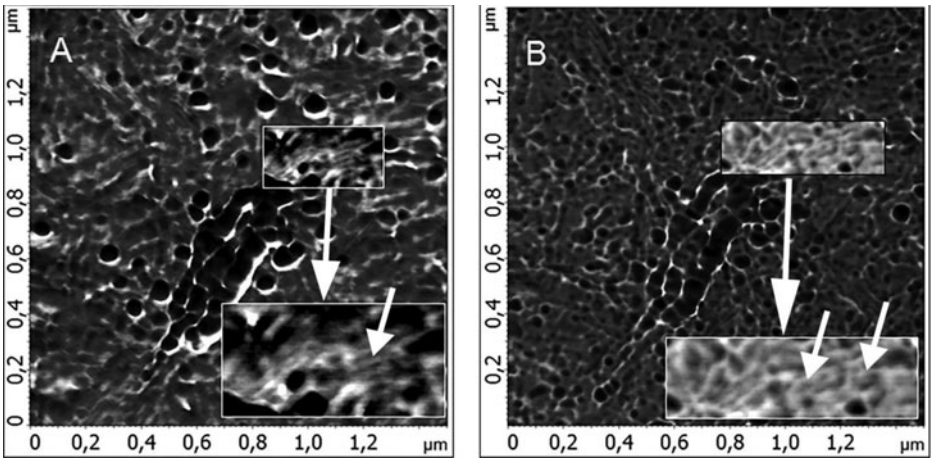

Figure 4. The phase images of PHB film before hydrolysis in lipase solution (A) and after (B). Arrows indicate the fragmentation of lamellae.

The first effect of decomposition—the emergence of new lamellar structures (Fig. 3). The arrow shows an extended structure, which can be interpreted as a single lamella (Fig. 3A). After treatment with a lipase solution, it becomes more visible, and another lamella appears to the right of it (Fig. 3B). These changes in the morphology of the film explained as the stack of lamellas are coated with a thin layer of an amorphous polymer [7]. Lipase, that had adsorbed on the surface of the film, begins to decompose amorphous polymer, exposing the crystalline structures under them.

The second change in the morphology - the fragmentation of lamellar structures. Figure 5 A shows an image of PHB film before enzymatic hydrolysis. The selected area contains a system of parallel strips, interpreted as a stack of lamellae. After treatment with lipase (Fig. 4) lamellae are fragmented. Before enzymatic degradation lamellae length in the stack was 300 $\pm$ 20 nm, after the degradation the length of the fragments had decreased to 80 $\pm$ 10 nm. The reason for that are probably the defects in the crystal structure. Lipase attacks such defects,

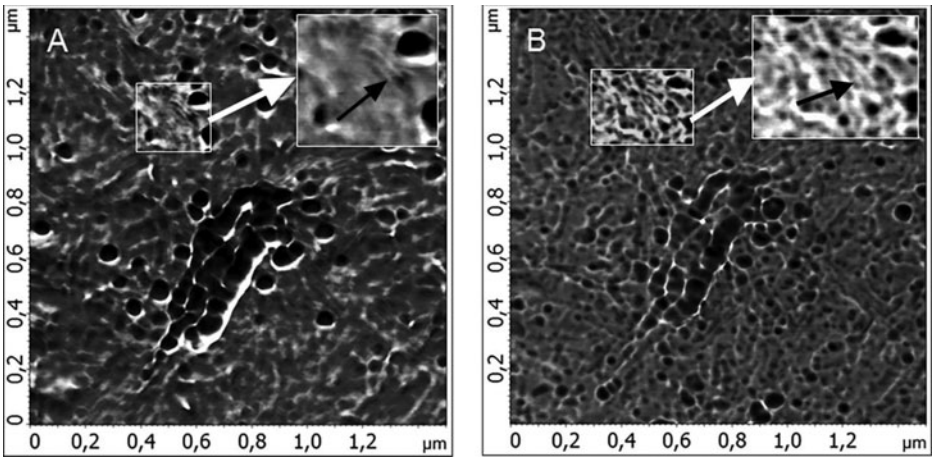

Figure 5. The phase images of PHB film before (A) and after (B) hydrolysis in lipase solution. Arrows indicate the disappearance of lamellar structures. It is important to note that changes in a lamellar structure during the process of degradation, typical of other semicrystalline polymers for example for polymers such as polylactide [24].

which results in the disintegration of lamellae into smaller pieces. A similar phenomenon was observed in the study of the decomposition process of PHB films by bacterial depolymerase of strain *A. faecalis T1* [23].

Third morphological change is the disappearance of lamellar structures. Figure 6 shows the PHB film images before and after lipase treatment. A system of parallel strips structure interpreted by us as a stack of lamellae was found on a PHB film before treatment with lipase. After hydrolysis in lipase solution this structure has changed dramatically: most of the lamellae have disappeared, and the remaining was highly fragmented (Fig. 5). The reason for the disappearance of lamellae is the same, as in the situation with fragmentation of lamellae—there are defects in lamellae, but in this case their number is much greater, allowing the lipase to almost completely destroy lamellae stack during the experiment [23].

Summary

Degradation of PHB was examined by pancreatic lipase in solutions of 0.1, 0.25, and 1 mg/mL in PBS and SBF with pH 7.4 and by lipase solution at a concentration of 0.5 mg/mL in PBS pH 7.4 for one month. It is shown that the degradation of films proceeds faster at a concentration of 0.25 mg/mL in PBS.

Differential scanning calorimetry showed an increase in the crystallinity of the samples after enzymatic degradation. These data are confirmed by nanoindentation, where the degradation was shown to increase the Young's modulus in the process of decomposition. This is due to decomposition and degradation of the amorphous polymer component in the process of erosion.

Atomic force microscopy shows that three major types of morphological changes after PHB films surface treatment with lipase were observed: the appearance of new lamellae and lamellae portion disappearance (fragmented into shorter lamellae portions).

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