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Enzymatic degradation of biostatic materials based on polylactide

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Abstract. The paper presents the research results for enzymatic degradation of biodegradable materials in *proteinase K*. Polylactide and its composites containing a biostatic substance in the form of s*ulphanilic acid salt PHMG* were tested as part of the research project. Three different concentrations of the biostatic substance were used: 0.2%, 0.6% and 1.0% of the mass. The research results of differential scanning calorimetry (DSC) for the tested materials were compared both before and after the enzymatic degradation. Photographs obtained from a scanning electron microscope (SEM) and the analysis of the foil physical mass loss confirmed that these materials were susceptible to enzymes.

Key words: enzymatic degradation, *proteinase K*, polylactide, PHMG.

1. Introduction

The natural environment is more and more often monitored for the presence of plants, microorganisms and lichen in certain areas, including degraded areas and areas polluted by the industry (Adamska et al., 2015). There is also a relationship between those data and environmental pollution caused by polymer waste. It is due to the growing global interest in environmental protection that the polylactide (PLA) is becoming more and more valuable as a polymer material used in both scientific research and in the packaging industry. Due to its properties, PLA can be used as a material in various applications. The major areas of its application include objects of common use and medical equipment. The materials of common use include containers and packaging foil used in farming and gardening. The medical equipment includes, among others, bioresorable implants, surgical thread, medical clothing as well as sanitary materials and dressings. Capsules used for slow release of medicines were one of the first areas where PLA was applied (Lin et al., 2007). PLA is a completely biodegradable polymer in the conditions of industrial composting and it is completely assimilable by living organisms (Duda, 2003; Duda & Penczek, 2003; Schlegel, 2003).

Polyhexamethylene guanidine (PHMG) and its derivatives is one of the bactericidal substances which can be applied with polymer materials. PHMG, both in the form of a free base and salt of inorganic acids, has very good water-soluble properties and it shows germicidal as well as disinfecting properties. PHMG derivatives have been applied as disinfectants, antiseptics as well as biocides giving bacteriostatic or biocidal properties to various polymer materials (Vointseva et al., 2006).

Proteinase K (E.C.3.4.21.64) is a serine protease belonging to the hydrolase class and it is synthesized by *Engyodontium album* cells (synonym: *Tritirachium album*) ATCC 22625 (Hildebrandt et al., 2008; http://www.mycobank.org). This fungus is a potential organism used in biodegradation due to its possibility to produce and secrete degrading enzymes (Jeyakumar et al., 2013). The

research topics dealt with in this paper is a continuation of the work on bacteriostatic and germicidal materials (Richert, 2017a, b).

The aim of this paper is to determine the impact of a biostatic additive in the form of a polyhexamethylene guanidine derivative (*i.e. sulphanilic acid salt PHMG*) on the loss of mass, change of crystallinity and the surface structure of biostatic foil under the influence of an enzyme *solution containing proteinase K.*

2. Material and methods

The research focused on two-component biodegradable foil made of 2003 D type polylactide (PLA) (NatureWorks®, USA) and a biostatic substance in the form of *sulphanilic acid salt PHMG* prepared at the Branch of Dyes and Organic Products in Zgierz (currently Institute of Leather Industry in Łódź) (Górecki et al., 2011; Wyrębska et al., 2012).

Before enzymatic degradation, polylactide samples were marked with the symbol B and the composites containing *sulphanilic acid salt PHMG* were marked with the symbol BS (Table 1).

Table 1. Marking and content of individual samples

Sample	Content of sulphanilic acid salt PHMG (%)
B	-
BS02	0.2
BS06	0.6
BS1	1.0

After the process of enzymatic degradation, samples of the foil were marked with the same symbols with the additional symbol "2e."

Samples of the foil with the dimensions of 15 x 15 x 0.8 mm were used for the tests in solutions of *proteinase K* enzymes. The tests were done according to the research method described by Nagata et al. (1996). The reactions of enzymatic degradation were performed in an enzymatic solution consisting of 10 cm^3 of 0.1M Tris-HCl buffer with pH 8, 2 mg of the enzyme being tested and 2 mg of sodium azide (a factor inhibiting bacterial growth and development of fungi). The tested samples were immersed in a solution with the constant temperature of 37°C maintained during the whole test process.

In order to define the possible impact of the liquid environment of the reaction on the loss of sample mass, measurements of control samples were taken. Those samples were immersed in the same solution as the solution prepared for the degradation in enzyme solutions, but with one exception. The solution did not contain enzymes.

After two months of incubation, the measurements of mass loss were taken with the application of weight method. The samples taken from the solutions were washed with distilled water and dried.

The loss of mass (Δm) was calculated according to formula (1) and expressed as a percentage:

$$\Delta m=\{(m_s - m_f)/m_s\}\cdot 100\ [\%] \quad (1)$$

where:
m_s – the initial mass of the samples,
m_f – the mass of samples after enzymatic degradation.

After the process of enzymatic degradation, tests were also performed to determine the changes in the surface properties of the foil used with the application of scanning electron microscopy (SEM). The Hitachi SU8010 scanning microscope (Hitachi, Japan) was used in those tests.

The content of the crystalline phase (χ) (crystallinity) was determined thanks to scanning tests of DSC differential calorimetry performed in accordance with the PN EN ISO 11357 (2002) standard and thanks to calculations according to formula (2):

$$\chi = \{(H_m - H_c)/H)\}\cdot 100\ [\%] \quad (2)$$

where:
H_c – enthalpy of cold crystallization determined during DSC tests,
H – enthalpy of melting for a given polymer containing 100% of the crystalline phase (it was assumed that for the polylactide the enthalpy value of a completely crystallized PLA was equal to 96 J/g).

3. Results

Figure 1 shows the results of the loss of mass of foil samples B-2e, B02-2e, B06-2e and B1-2e exposed to the action of *proteinase K*.

The figure shows that the biggest mass loss in the samples was observed after a maximum period of incubation for samples B and BS1 in the solution of *proteinase K*, i.e. after two months of enzymatic degradation. The sample foil made of pure polylactide underwent the fastest process of degradation. For samples B, BS02, BS06 and BS1 the loss of mass after two months' incubation in the enzyme solution amounted to 42%, 5.2%, 6.6% and 7.9% respectively.

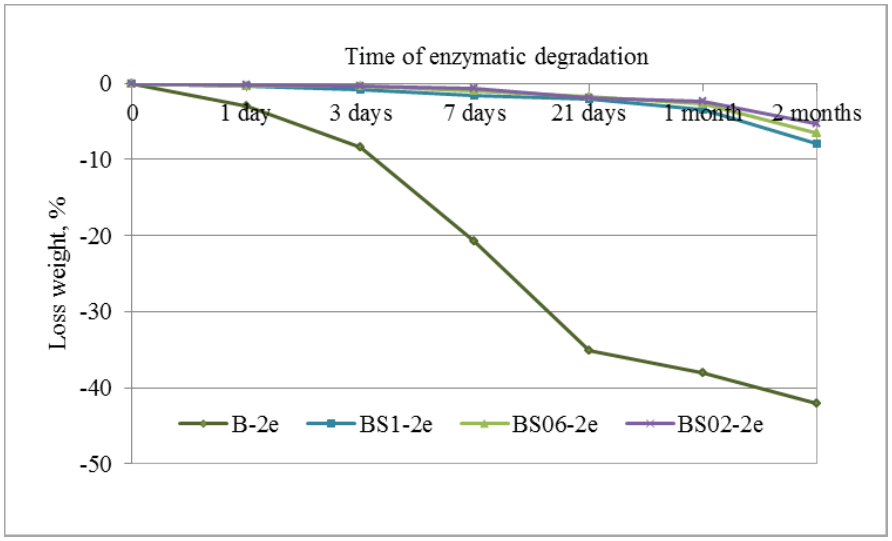

Figure 1. Losses of mass in foil B, BS02, BS06 and BS1 after two months' incubation in an enzymatic solution containing *proteinase K*

Table 2 shows the results of scanning tests of DSC differential calorimetry, the value of cold crystallization enthalpy (ΔH_c), enthalpy of melting (ΔH_m) and degree of crystallization (χ).

Table 2. Results of DSC test

Sample	ΔH_c^{PLA} (J/g)	ΔH_m^{PLA} (J/g)	χ (%)
B	22.5	26.1	3.5
B-2e	12.8	16.2	3.5
BS02	12.7	13.0	0.3
BS06	14.8	18.9	4.3
BS1	19.5	21.5	2.1
BS02-2e	16.8	21.3	4.7
BS06-2e	20.3	23.1	2.9
BS1-2e	22.2	27.9	6.0

The data presented in Table 2 show that the biostatic additive in the form of s*ulphanilic acid salt PHMG* lowered the ΔH_c value of the foil marked with the symbols BS02, BS06 and BS1 by 42.8%, 33.3% and 12.2 % in relations to the reference sample B. The additive also lowered the ΔH_m value for the same samples by 50.2%, 27.6% and 17.6% – in relation to the control sample B. After the processes of degradation in *proteinase K*, the value of cold crystallization enthalpy and enthalpy of melting were higher than the value of the control sample B-2e. For the sample containing the highest concentration of s*ulphanilic acid salt PHMG* (BS1-2e), the increase amounted to 73.4% (for ΔH_c) and 72.2 % (for ΔH_m). On the other hand, the highest change in the degree of crystallization was observed for foil BS1-2e. The increase amounted to about 71.4% in relation to the control sample B-2e.

The surface tests for samples B, BS02 and BS06 started with SEM pictures of those polymers before the process of enzymatic degradation. Figure 2 shows only the surface picture of the primary polylactide (B) because the surface pictures for samples BS02 and BS06 did not differ.

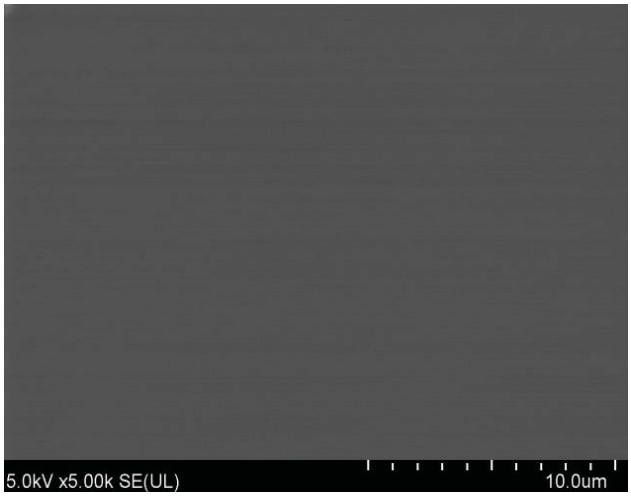

Figure 2. Surface picture of foil B before enzymatic degradation (magnification 5,000 times)

Figure 2 shows that the surfaces of the tested samples (before enzymatic degradation) were homogeneous, uniform and smooth and there were no losses in the shape of pore and gaps.

Figure 3 shows the changes in the geometric structure of the surface of the materials B-2e, BS02-2e, BS06-2e and BS1-2e after two months of degradation in *proteinase K* solution.

Figure 3 shows that the biggest changes occurred in sample B-2e, while the changes in samples BS02-2e, BS06-2e and BS1-2e were smaller. Sample B-2e included a significant number of pores with the size of up to 1μm. On the other hand, the size of the pores caused by a solution of *proteinase K* in the foil containing s*ulphanilic acid salt PHMG* varied from about 0.1 to 0.3 μm (Fig. 3b-d). However, the number of such pores was smaller than in the reference sample (Fig. 3a). The differences in the surface of individual pores were connected with the number, size and depth of the created pores.

4. Discussion

Biodegradable polymer materials are widely used in medicine and pharmacy both as implants and drug carriers. Their biocompatibility and their capability of natural biodegradation in the human body under the influence of enzymes is undoubtedly an advantage of those materials. According to forecasts, polylactide (PLA) can replace or to a large extent exclude traditional non-biodegradable polymers used in the packaging industry (Deng & Hao, 2001; Nampoothiri et al., 2010; Woodruff & Hutmacher, 2010). The processes

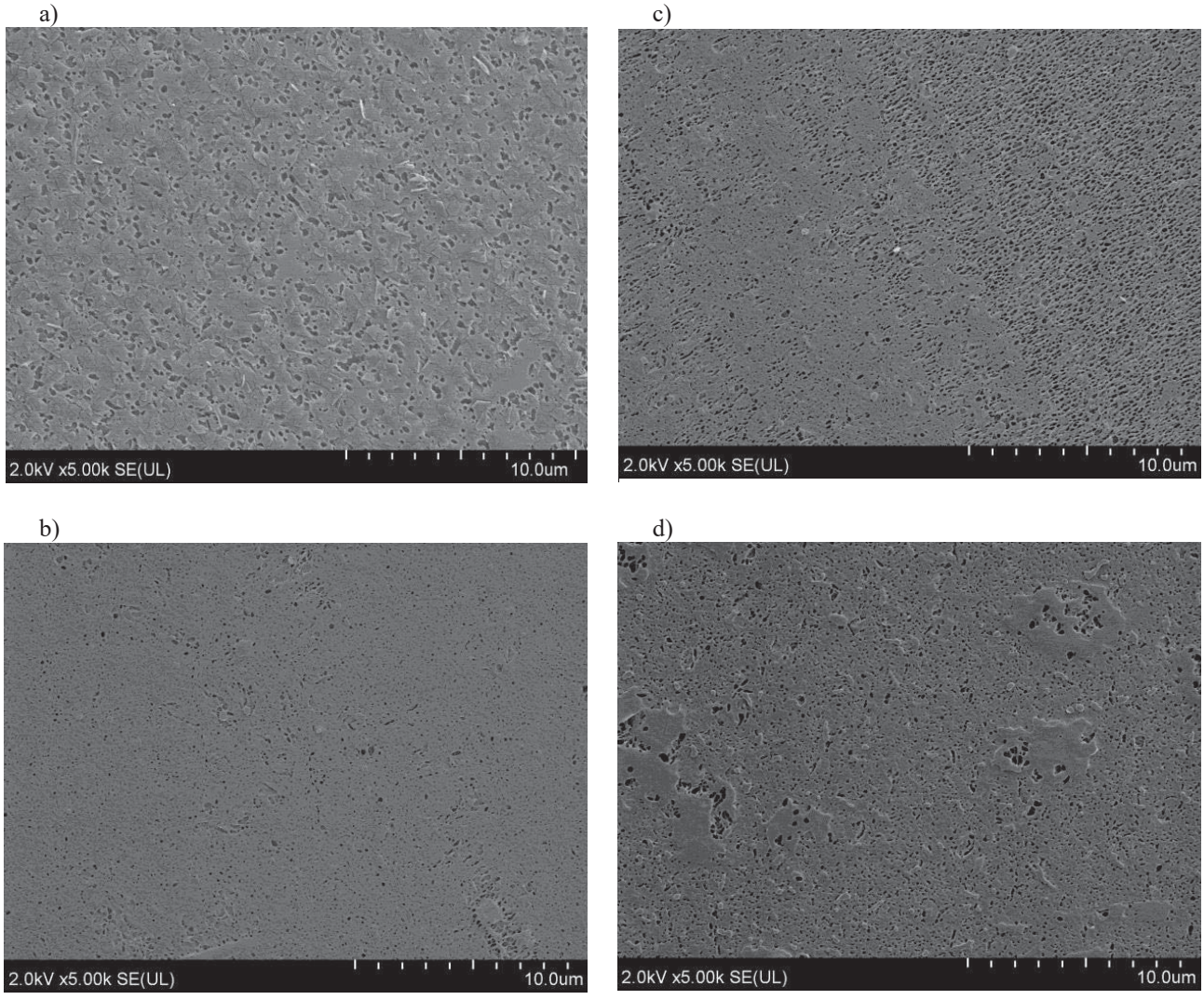

Figure 3. Analysis of the changes in the surface structure of polymer foil a) B-2e, b) BS02-2e, c) BS06-2e and d) BS1-2e after two months of enzymatic degradation with *proteinase K* (magnification 5,000 times)

of enzymatic biodegradation of biodegradable materials, including polylactide, have been put to many tests and their results have been published in many papers (Li et al., 2001; Yew et al., 2005; Li et al., 2007; Peng et al., 2010; Kemme et al., 2011; Zhang et al., 2011; Rodríguez-Contreras et al., 2012, Yang et al., 2012). Unfortunately, a direct comparison of the results of those tests causes problems because of the various conditions in which such tests were taken (e.g. performing the process of degradation in various environments and with the application of various enzymes) and because of the various effects of the impact of enzymes belonging to the same sub-class and produced by various microorganisms at the same time (Manna & Paul, 2000; Chandra & Rustgi, 1998). Tsuji et al. (2006) concluded that the following polymer properties have a significant impact on the process of polymer degradation: chemical structure, average molecular weight, wettability, content of crystalline phase and the size of crystals as well as the content and kind of additional components and the properties of the top layer. The loss in the mass of a given material during enzymatic degradation can be used as a measurement of the degree of its degradation (Richert et al., 2017; Nagata et al., 1996). The authors of many papers on various kinds of degradation also emphasize visual changes occurring on the surface of tested materials, for example, under the influence of enzymes (Richert et al., 2017; Cho et al., 2011; Wada et al., 2007). Richert et al. (2013) proved that the bacteriostatic additive in the form of s*ulphanilic acid salt PHMG* does not decrease the PLA enzymatic degradation and thus it does not inhibit the processes of biodegradation. In addition, they proved that a bacterial biofilm easily develops on the foil containing s*ulphanilic acid salt PHMG*, which suggests that those materials are susceptible to biodegradation, among others in compost (Richert et al., 2013).

This paper shows the results of the research into the loss of mass as well as the changes in the PLA surface structure of the biostatic composites based on polylactide and s*ulphanilic acid salt PHMG* occurring during the degradation of those polymers under the influence of *proteinase K.* The biggest loss of mass occurs in PLA, while the loss in the composites is smaller. It means that it was PLA that underwent the fastest enzymatic degradation in the conditions of the tests. The surface topography pictures for individual pieces of tested foil confirm the results of the research into the loss of mass (Fig. 1). The earlier papers by Richert et al. (2017) confirm that two months' degradation is a sufficient period to find out if the materials being tested show susceptibility to the action of *proteinase K.*

The search for new recycling methods seems to be justified by the need to protect the natural environment. It is also necessary to look for microorganisms capable of settling environments polluted with polymer waste and to use such microorganisms and enzymes in the process of waste utilization (Emadian et al., 2017).

5. Conclusions

The research showed that the tested two-component composite foil containing polylactide and s*ulphanilic acid salt PHMG* were susceptible to action of the enzyme – *proteinase K.*

– The biggest loss of mass was noticed in foil B after two months' incubation in an enzymatic solution.

– The results of the tests for the loss of mass obtained with the application of the weight method provide a good confirmation of the pictures obtained with the SEM method.

– The tests done with the application of the DSC method showed an increase in the crystallinity of composite BS1 during the process of enzymatic degradation in *proteinase K.* This phenomenon was not observed during the degradation of the other BS02 and BS06 materials, which may have been caused by the amount of the PHMG derivative used.

– Degradation of polymer material with a content of microbiological enzymes seems to be justified in the context of ecology and environmental protection.

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