Polymer Degradation and Stability 158 (2018) 31–39

Contents lists available at ScienceDirect

Polymer Degradation and Stability

journal homepage: www.elsevier.com/locate/polydegstab

Thermal stability of bacteriocin nisin in polylactide-based films

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ARTICLE INFO

Article history:
Received 10 January 2018
Received in revised form
25 June 2018
Accepted 23 October 2018
Available online 26 October 2018

Keywords:
Nisin
Polylactide
Polyethylene glycol
Thermal stability
Biodegradable polymer
Antibacterial
Mass spectroscopy

ABSTRACT

This work investigates the thermal stability of bacteriocin nisin in polylactide (PLA) and polylactide/polyethylene glycol (PLA/PEG) blends at temperatures of 90 °C–180 °C. The samples were prepared by solvent cast technique and characterized according to their mechanical and thermal properties. Research on the thermal stability of nisin in the PLA and PLA/PEG systems was carried out by exposing the given films to various temperatures (90 °C, 120 °C, 160 °C, and 180 °C) for a duration of up to 48 h. Assessment of the antibacterial activity of the samples was carried out by the agar diffusion method against *Micrococcus luteus*, while structural analysis involved the use of high-performance liquid chromatography with mass detection. Structural changes in the polymer matrix were evaluated by gel permeation chromatography and scanning electron microscopy. The results showed that nisin retained almost 70% of its antimicrobial activity in the PLA matrix, even after treatment at 160 °C for 15 min. The presence of PEG significantly enhanced the degradation of nisin above 120 °C.

1. Introduction

The study of incorporating antimicrobial agents into polymers has become an important aspect within active and intelligent packaging research [1–3]. Recent decades have seen a rise in scientific and industrial interest in naturally occurring antimicrobial compounds, especially in food packaging, cosmetics, and biomedical applications [4]. Such beneficial compounds for antimicrobial modification include bacteriocins. These natural substances are antimicrobial peptides produced by bacteria, and they are well-known for exhibiting excellent antibacterial properties even at low concentrations [5–7]. A notable example of a bacteriocin that has been studied and deployed is nisin [6,8,9]. This peptide is produced by *Lactococcus lactis* subs. *Lactis*, which is recognized as a biologically safe food preservative by the FAO/WHO organizations [7,9].

Nisin has been used to confer antimicrobial activity to synthetic materials (e.g. polyethylene (PE) [10–14], ethylene vinyl acetate (EVA) [15–17], polyvinyl chloride (PVC) [18], polyvinyl alcohol (PVA) [19], and poly(butylene adipate-*co*-terepthalate (PBAT) [20]) and biopolymers (e.g. cellulose [21,22], chitosan [23], and protein-based polymers [24,25]).

Current demand for bio-based and compostable plastics makes polylactide (PLA) one of the most promising materials for providing interesting alternatives to conventional petrol-based plastics for packaging applications. However, ensuring the safety of foodstuffs predetermines the need for antibacterial modification of said biodegradable PLA. Indeed, this matter has been reflected in a few works published in the literature [26–30].

In this context, biodegradable polymers imbued with nisin are generally obtained by carrying out the solvent casting technique [19,22–26] or via surface modification [21,27,31].

In this context, biodegradable polymers imbued with nisin are generally obtained by carrying out the solvent casting technique (primarily water-soluble polymers, e.g. PVA [19], cellulose [21,22], chitosan [23], and protein-based polymers [24,25]) or via surface modification (e.g. soaking of the PLA and PLA/pectin samples [27] or poly(lactic-co-glycolic acid) (PLGA) matrix in a nisin solution [31]).

From an industrial point of view, it is preferable to obtain active polymer films by applying standard melt blending technological process, due to the possibility of producing large volumes of material in a single step without the use of harmful organic solvents.

Only a few studies have investigated formulating nisin-incorporated biodegradable films by means of melt blending processes (namely films based on PBAT [20] and plasticized PLA [20,28,29]). This is primarily because bacteriocins as proteins are sensitive to various conditions (temperature, pH, water activity,

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https://doi.org/10.1016/j.polymdegradstab.2018.10.019

etc.), in addition to which deterioration in antimicrobial activity can occur during extrusion at high temperature and high shear rates, hence also under high pressure [32].

According to the literature, the maximal temperature at which nisin can retain its bioactivity is 120 °C [15]. Nevertheless, Scaffaro et al. reported that no reduction in antibacterial activity was observed at even higher processing temperatures (140 °C and 160 °C) in the EVA-based material prepared therein, probably as a consequence of the very short residence time inside the extruder (60–90 s) [16].

The processing temperature of polymers appears to be an important factor affecting the chemical stability of nisin, related to loss of efficacy against food spoilage microorganisms. In connection with this, improvement in the stability of nisin in a complex with polyethylene glycol (PEG) has been described by Holcapkova et al. [33]. This finding complements the fact that PEG is deployed as plasticizer for PLA [34].

With this in mind, the objective of this study was to assess the influence of high temperature (in the range of 90 °C–180 °C) on the antibacterial activity of nisin by evaluating the effectiveness of PLA and PLA/PEG based blend films. Antibacterial stability was investigated using the agar diffusion method against the *Micrococcus luteus* bacterial strain, and the content of nisin was determined by high-performance liquid chromatography with mass detection. Moreover, the authors also evaluated structural changes in the prepared samples using gel permeation chromatography and scanning electron microscopy.

2. Experimental

2.1. Materials and chemicals

Polylactic acid (PLA) 2002D was purchased from NatureWorks®Ingeo™ (USA). Two types of polyethylene glycol (PEG) of different molecular weight (M_w ~1000 and 6000 g mol^{-1}) were employed. PEG1000, PEG6000, and nisin standard from *Lactococcus lactis* (2.5%, balance sodium chloride and denatured milk solids) were purchased from Sigma-Aldrich (USA). Chloroform and sodium chloride (NaCl) were supplied by IPL (Czech Republic). The medium required for antimicrobial testing, Mueller Hinton agar, was bought from HiMedia (India). The bacterial strain *Micrococcus luteus* (CCM 1569) was obtained from the Czech Collection of Microorganisms, Masaryk University in Brno, Czech Republic. LC-MS grade acetonitrile and water were supplied by Biosolve (Netherlands), LC-MS grade formic acid was purchased from Sigma-Aldrich (Germany). HPLC grade tetrahydrofuran (THF) was sourced from Carl Roth (Germany), and the stabilizing agent butylated hydroxytoluene (BHT) was bought from Sigma-Aldrich (Russia).

2.2. Preparation of samples

Blend films of PLA, PLA/nisin, PLA/PEG1000/nisin, and PLA/PEG6000/nisin were prepared by the solvent casting technique. The PLA, or a mixture of PLA and PEG (20 wt %), was dissolved in chloroform under stirring for approximately 2 h at 40 °C. Then nisin standard at the amount of 6 wt % (0.15 wt % pure nisin) was added, and the solution was stirred for another 25 min. Following this, the mixture was poured into a glass dish that had previously been cleaned with ethanol. The resultant samples were placed under a chemical fume hood for 3 days in order to evaporate the solvent. Afterward, the matrix was dried under vacuum conditions for 24 h at 65 °C to eliminate the remaining solvent. The thickness of the prepared blend films equaled 214 ± 6 μm.

Concentration of the PEG plasticizer was fixed to 20 wt % based on the previous study of Mohaparta et al. who investigated blends of PLA/PEG 6000 systems within the PEG concentration range 10–30 wt % [35]. The PEG content of 20 wt % is optimal from the viewpoint of both mechanical properties and morphological structure of the resulting blend where PEG forms droplets (bacteriocin reservoirs) dispersed in the continuous phase of the PLA matrix. The nisin content 0.15 wt %) in the blends considers antimicrobial activity of the nisin and physical form of the additive that is stabilized in a mixture with sodium chloride.

2.3. Scanning electron microscopy (SEM)

Micrographs of the samples were taken on a scanning electron microscope, model Vega II LMU (Tescan, Czech Republic), after applying a thin coating of gold/palladium by a sputter coater of type SC 7640 (Quorum Technologies, UK). The electron accelerating voltage was set to 5 kV.

Prior to testing, thermally treated and untreated specimens were cryofractured, and the morphological arrangements in the samples were highlighted by washing the water-soluble PEG portion out of the matrix into demineralized water at 60 °C for 18 h.

2.4. Differential scanning calorimetry (DCS)

Thermal analyses were carried out on a DSC1 differential scanning calorimeter (Mettler Toledo, Belgium) under a nitrogen atmosphere. The temperature ramp (initial heating phase) comprised the range −35 °C to 200 °C (at a heating rate of 10 °C min^{-1}), followed by annealing at 200 °C for 1 min. Subsequently, a cooling scan was run to −35 °C (10 °C min^{-1}). After maintaining this temperature for 2 min, another heating cycle was conducted under the same conditions. Glass transition temperature (T_g) was gauged from the inflection point of the DSC curve from the second heating thermal cycle. Melting temperature (T_m) was reported as the peak value of the melting endotherm from the first heating cycle.

2.5. Mechanical properties

Tensile experiments were carried out according to the ASTM D882-12 standard test method known as the “Standard Test Method for Tensile Properties of Thin Plastic Sheeting”, using an M350-5CT tensile-testing device (Tensometric, UK) [36]. The initial length of samples was 75 mm, while the width measured 5 mm and the thickness 214 ± 6 μm. The deformation rate was set to 50 mm min^{-1}. Average values for Young's modulus and tensile strength were calculated from the stress-strain dependencies of seven specimens.

2.6. Gel permeation chromatography (GPC)

GPC analysis was conducted by using the PL-GPC 220 chromatographic system (Agilent, Santa Clara, USA), equipped with a dual detection system (refractive index detector and viscometric detector). The samples were dissolved in THF (2–3 mg mL^{-1}) stabilized with BHT (125 ppm) and filtered using a syringe filter (0.45 μm). Separation was carried out on a series of gel-mixed bed columns (Polymer Laboratories Ltd., Amherst, UK) as follows: 1 × PL gel-mixed-A bed column (300 × 7.8 mm, 20 μm), 1 × PL gel-mixed-B bed column (300 × 7.8 mm, 10 μm), and 1 × PL gel-mixed-D bed column (300 × 7.8 mm, 5 μm); the mobile phase contained the THF stabilized with BHT (125 ppm) at 40 °C. The flow rate of the mobile phase was set to 1.0 mL min^{-1} and injection volume equaled 100 μL. The GPC system was calibrated with polystyrene standards for molecular weight within the range 580 and 6,000,000 g mol^{-1} (Polymer Laboratories Ltd., UK). The mass average molar mass (M_w), number average molar mass (M_n), and

polydispersity index ($Đ = M_w/M_n$) of the tested samples were determined from peaks corresponding to the polymer fraction according to the universal calibration method. All data processing was carried out using Cirrus software.

2.7. Evaluation of antibacterial activity

The antibacterial activity of the blend films was determined via the agar disk diffusion method [37] with modifications, utilizing *Micrococcus luteus* as the model nisin-sensitive bacterial strain. Briefly, the Mueller Hinton agar plates were inoculated with an aliquot (1 mL) of bacterial culture (10^8 CFU mL^{-1}). Then, samples 8 mm in diameter were placed on the agar plate and incubated at 30 °C for 24 h. After incubation, the diameters of the inhibition zones were determined.

2.8. High-performance liquid chromatography with mass detection (HPLC-QTOFMS)

HPLC analysis was performed on a 1260 Infinity LC system (Agilent Technologies, Santa Clara, USA). Chromatographic separation of the components of the samples was carried out on an Aeris Widepore XB-C8 column (150 mm × 4.6 mm, 3.6 μm) (Phenomenex, Torrance, California, USA) at a flow rate of 1 mL min^{-1} that was maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient was as follows: 0–13.5 min, linear gradient from 5% to 50% B; 14–18.5 min, isocratic at 95% B; 18.5–19 min, linear gradient to 5% B; the postrun lasted 5 min. The total running time was 24 min for each sample; sample injection volume equaled 10 μL.

Detection was performed on a quadrupole Time of Flight mass spectrometer (6530 Q-TOF, Agilent Technologies, Santa Clara, USA) employing an electrospray ion (ESI) source set to positive mode. The mass spectrometer operated under following parameters: capillary voltage 4000 V, nebulizer pressure 40 psig, drying gas 6 L min^{-1}, and gas temperature 300 °C. Mass spectra were acquired over the m/z 100–1000 range at a scan rate of 3 scan s^{-1}. Accurate mass measurements were obtained via a calibrating solution involving the use of internal reference masses (purine ($C_5H_4N_4$) at m/z 121.050873, and HP-0921 [hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at m/z 922.009798). Data were recorded and processed in MassHunter software v. B.05.01 (Agilent Technologies).

Nisin was measured as a $[M+5H]^{5+}$ molecule ion, while the second most abundant signal in the MS spectrum corresponded to a $[M+4H]^{4+}$ molecule ion. In order to heighten the selectivity of the method, fragmentation of the precursor ion employing collision energy at 20 V was carried out.

The stock solution of nisin (200 μg mL^{-1}) was prepared in 0.1% formic acid in water and stored at 4 °C. The working standard solution of 10 μg mL^{-1} was prepared by dilution in 0.1% formic acid in water and subsequently utilized for preparing the solvent calibration standards, which were stored at 4 °C. A new stock solution was made after one week of storage.

Prior to testing, the thermally treated and untreated nisin-incorporated specimens were immersed in 0.1% formic acid in water (~25 mg mL^{-1}). Afterward, the samples were constantly shaken (80 min^{-1}) on an IKA KS 130 basic orbital shaker (IKA, USA) at room temperature for 1 h. The nisin released via this procedure was detected by the HPLC-QTOFMS method as described above. All experiments were carried out in triplicate for each sample type and the results were expressed as concentration of nisin in sample solution (ng mL^{-1}). Then, the released amount of nisin from film tested was calculated (μg g^{-1}).

2.9. Thermal treatment

The PLA, PLA/nisin, and PLA/PEGs/nisin blend films were thermally treated at 90 °C, 120 °C, 160 °C, and 180 °C for various time periods in a universal oven (Memmert UF30, Schwabach, Germany). Afterwards, the influence of the modifiers on antibacterial activity, the nisin content, the molecular weight, and the morphology of the samples before and after such thermal treatment were evaluated using the methods specified above.

The antibacterial activity AA (%) of the investigated samples was calculated as follows:

$$AA = \frac{IZ_T}{IZ_U}\ 100 \qquad (1)$$

where IZ_T and IZ_U represent the diameters of inhibition zones (mm) of the thermally treated and untreated test samples, respectively. The antibacterial activity of thermally untreated films was taken as 100% and the value of thermally treated films was calculated according to Equation (1). The experiment was carried out in four replications for each sample type.

3. Results and discussion

3.1. Material characterization

The degradation of PLA-based products is critically affected by the properties of the input material, its composition, and processing history. Therefore, knowledge of the initial properties of the material under investigatation is necessary to subsequently evaluate data obtained experimentally after thermal treatment.

Fig. 1 shows the morphology of the blend films prepared. As can be seen, the structure of the PLA/nisin sample (Fig. 1 b) closely resembles that of neat PLA (Fig. 1 a). However, obvious changes occurred in the PEG-incorporated blends (Fig. 1 c, d) after leaching the same in demineralized water. These alterations resulted from the incompatibility of the polymer matrices and the subsequent

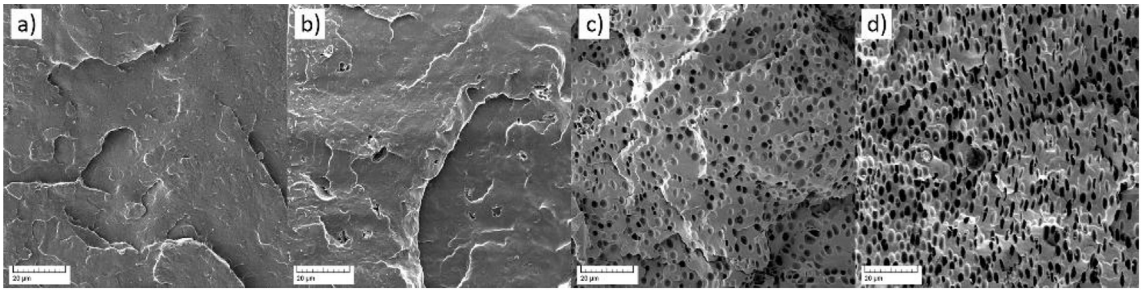

Fig. 1. SEM micrographs of cross-sections of a) PLA, b) PLA/nisin, c) PLA/PEG1000/nisin, and d) PLA/PEG6000/nisin after leaching in demineralized water.

Table 1
Certain material properties of PLA-based blend films [39].

Sample	T_{mPEG}[a] (°C)	T_{mPLA}[a] (°C)	T_g[b] (°C)	E[c] (GPa)	σ_B[d] (MPa)
PLA	–	155.0	59.6	2.8 ± 0.2	50 ± 4
PLA/nisin	–	155.2	59.8	3.1 ± 0.2	48 ± 3
PLA/PEG1000/nisin	37.8	152.6	n.d.^{OL}	1.4 ± 0.1	24 ± 2
PLA/PEG6000/nisin	57.6	154.9	n.d.^{OL}	1.6 ± 0.1	27 ± 1

n.d.^{OL} – not detected, overlapped signals.
[a] Melting temperatures related to PEG (T_{mPEG}) and PLA (T_{mPLA}).
[b] Glass transition temperature.
[c] Young's modulus.
[d] Tensile strength.

phase separation. In addition, less occurrence of pore frequency was observed in the case of PEG1000, which functions as regular plasticiser. Low molecular weight PEG is probably able to penetrate PLA chains more easily than the high molecular form. Therefore, PEG1000 was more involved in direct interaction with the PLA amorphous phase than PEG6000. The PLA/PEG system investigated herein possessed a partially immiscible quality, with some mutual mixing of the PEG and PLA phases [38].

As a point of reference, further to the aforementioned morphological properties, detailed characterization of the thermal and mechanical properties of the prepared blend films was previously carried out in an earlier study by the authors [39]. In brief, said thermal properties were evaluated using differential scanning calorimetry (DSC), and tensile experiments were conducted according to the ASTM D882-12 standard test method for tensile properties of thin plastic sheeting. Selected values for the mechanical and thermal properties of the PLA and PLA/PEG samples are illustratively shown in Table 1. DSC curves of these samples can be found as supporting information in Figure S1.

DSC analysis and tensile experiments revealed that adding nisin had no demonstrable effect on the melting temperature (T_m), glass transition temperature (T_g), or on mechanical properties (Young's modulus and tensile strength) in comparison with the PLA blend film. However, after introducing the PEG(s), thermal and mechanical properties were seen to change significantly. Firstly, two types of melting endotherms were detected in the initial heating scan. In the case of PLA/PEG1000/nisin, T_{mPEG} equaled around 38 °C, while the value for PLA/PEG6000/nisin was approximately 58 °C. Such values corresponded with those for the neat components. The presence of T_{mPEG} showed that some of the PEG underwent phase crystallization after the films had been prepared, hence its separation from the PLA matrix. Such behavior suggests a high degree of incompatibility in the investigated systems, which was also visible in the SEM micrographs mentioned above. The glass transition region of the PEG-incorporated samples was incredibly difficult to discern, due to its overlap with the endotherm of melting for PEG.

In the case of mechanical properties evaluation, adding 20 wt % of PEG into the PLA caused significant plasticization, characterized by reduction in Young's modulus and tensile strength, and significantly increased ductility. The effect of plasticization was more intense for samples of PEG at lower (~1000 g mol^{-1}) molecular weight. This finding corresponds to the literature, i.e. that plasticization is improved in line with decrease in the molecular weight of PEG [40].

Table 2
GPC data on PLA-based samples prior to thermal treatment.

Sample	M_w[a] (g mol^{-1})	M_n[b] (g mol^{-1})	$Đ$[c]
PLA	143 000	63 000	2.26
PLA/nisin	136 000	64 000	2.12
PLA/PEG1000/nisin	149 000	66 000	2.27
PLA/PEG6000/nisin	146 000	65 000	2.25

[a] Mass average molar mass.
[b] Number average molar mass.
[c] Polydispersity index.

Evaluating the molecular weight of the PLA-based films required the use of gel permeation chromatography. As shown in Table 2, the values for the prepared blend samples were quite similar to each other (differing by no more than 10%), demonstrating that no significant changes in the molecular structure of the PLA took place during processing. These results had been anticipated, as the solvent casting technique is not as drastic as thermoplastic processing, which brings high temperatures and shear stress levels to bear on the polymer matrix. Hence it was assumed that the casting method would not lead to a reduction in antimicrobial activity, in addition to which the initial extent of nisin concentration was not significantly influenced during preparation of samples.

Previous research had revealed that the hydrophilicity, degradation rate and drug release rate of the PLA/PEG blends were enhanced by PEG [41]. This finding corresponded to the microbiological results herein [39]. The thermally untreated PLA/PEG/nisin films showed slightly larger diameters for the inhibition zone (about 25 mm) compared to the PLA/nisin film (23.4 mm). This value was taken as IZ_U (Equation (1)) for calculating the degree of antibacterial activity (Fig. 2). The presence of PEG affected both the hydrophobic nature of the PLA and the diffusion of nisin from the matrix, thereby boosting the antibacterial effect against the *Micrococcus luteus* bacterial strain.

3.2. Thermal stability evaluation

In its dry state, nisin powder is highly stable under the given recommended storage conditions. Nevertheless, the thermostability of nisin is largely related to the pH levels it is exposed to in solutions and food systems [42]. According to Davies et al. nisin solution was seen to be most stable when exposed to autoclaving at pH 3 (demonstrating less than 5% loss in activity under treatment at 115 °C for 20 min, and 15% loss in activity under treatment at 121 °C for 15 min) [43].

The influence of high temperature on the antibacterial activity of the prepared blend films was evaluated as the dependence of antibacterial activity on the duration of exposure at the tested temperatures (Fig. 2).

Firstly, evaluation was made as to the effect of temperatures below the T_m of PLA (approximately 155 °C, see Table 1) (Fig. 2 a, b). As can be seen, no significant drop in activity was discernible in any of the samples during the heating cycle at 90 °C for two days, or in the samples treated at 120 °C for up to 1 h. This finding is consistent with the literature, where retention has been reported of nisin activity when the same is incorporated into LDPE-based [14] and EVA-based [15,17] films by an extrusion process carried out at 120 °C. However, herein the adverse effect of PEG was observed in films treated above 100 °C for an extended period. After 2 days at 120 °C, PLA/nisin had diminished by only 20% in activity, unlike the PLA/PEG/nisin films that saw a reduction of about 70% in activity.

The second step was to investigate the effect of heat treatment above the T_m of PLA (approaching the processing temperature of PLA) (Fig. 2 c, d). After 5 min at 160 °C, the blend films had lost just over 20% activity. After 1 h at this temperature, almost no antibacterial activity was observed in the PLA/PEG/nisin films, whereas the PLA/nisin samples merely showed reduction by about 50%. As expected, the greatest drops in antibacterial activity occurred in samples treated at 180 °C. After 5 min, the blend films had diminished in antibacterial activity by over 60%. Indeed, the PLA/PEG/nisin films displayed no sign of antibacterial activity after 15 min.

Interestingly, the significant influence of the molecular weight

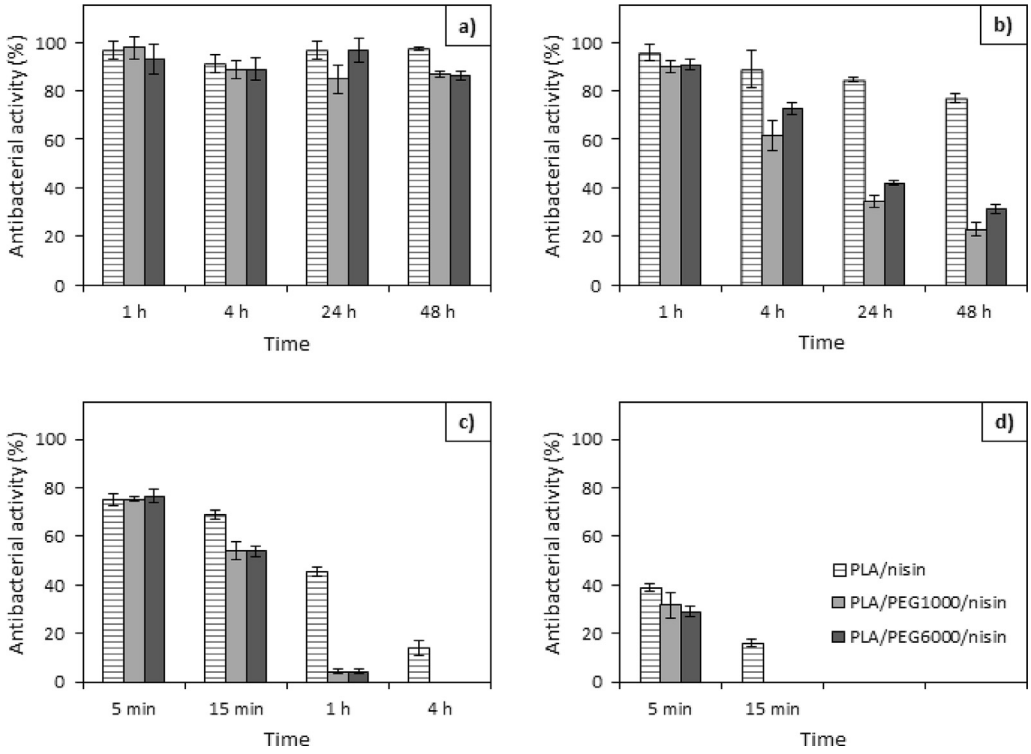

Fig. 2. Antibacterial activity of PLA/nisin and PLA/PEG/nisin blends treated at a) 90 °C, b) 120 °C, c) 160 °C, and d) 180 °C for different durations; antibacterial activity of thermally untreated samples is taken as 100%; data points are average values of four independent analyses.

of PEG on the stability of the prepared films was not observed. Therefore, it was assumed that the presence of PEG itself exerted a positive effect on the stability of nisin, a deduction based on previous research in addition to referencing the potential for formation of nisin-PEG conjugates [33,44,45]. However, as in the previous case, observation was made of the negative effect of PEG on the antibacterial activity of the investigated films. The reason for this could have been the very low T_m of the polyethylene glycols (see Table 1), which are able to interact further in their molten state with the nisin molecule and cause its decomposition, triggering a subsequent loss in antibacterial activity. Moreover, the authors assumed it was likely that the greater proportion of the nisin had been incorporated into the PEG phase as a consequence of its hydrophilic nature.

The literature states that oxidative thermal degradation of PEG occurs because PEG is susceptible to free radical oxidative attack in the presence of oxygen, this at an elevated temperature exceeding 70 °C, and the resulting PEG radical could initiate the oxidation of other compounds [46]. Based on this information, the hypothesis might be made, based on the findings herein, that an oxidized form of nisin is created, which according to Wilson-Stanford et al., leads to a complete loss in bactericidal activity [47].

One of the objectives of the present study was to confirm the presence of the nisin molecule in the thermally treated samples. For this purpose, the HPLC-QTOFMS method was optimized and validated. For chromatographic separation by HPLC, a reversed phase column was used to separate the peptides. The nisin was eluted for 7.25 min (Fig. 3 a). In order to detect the target analyte, the $[M+5H]^{5+}$ (m/z 671.3163) molecule ion was monitored (fragmentation of the precursor $[M+5H]^{5+}$ ion provided the most abundant product - ion m/z 811.3722). The mass spectra also contained a very abundant ion, m/z 838.8936, which corresponded to the $[M+4H]^{4+}$ molecule ion of nisin (Fig. 4).

The matrix components within the analyzed samples were capable of interfering with the ionization process in the MS source, meaning that the actual concentrations of the target analytes might be underestimated or overestimated. So as to assess these matrix effects, different quantification methods were compared (the method of external calibration and the method of standard addition), and the responses of the nisin were seen not to be affected by the presence of the matrix. To this end, quantification was carried out through an external calibration curve in the range of concentration of 200–10 000 ng mL^{-1}.

The limit of detection (LOD) was determined by repetitive analysis ($n = 5$) of the low levels of solvent standard solutions, and was defined as the concentration of the target analyte for which a signal-to-noise ratio (S/N) of 3 was obtained. The LOD of nisin was 50 ng mL^{-1}.

Employing the HPLC-QTOFMS method, nisin was determined in extracts of the PLA/nisin blend films. The levels of free nisin in the extracts of PEG-incorporated samples were under the limit of detection for the method used. The vast proportion of the nisin probably occurred in the form of nisin-PEG conjugates, as mentioned above. An example of an extracted ion chromatogram for the $[M+5H]^{5+}$ molecule ion of nisin in the actual PLA/nisin sample is shown in Fig. 3 b.

The amount of nisin released from the thermally untreated samples corresponded to 380 ± 40 μg g^{-1} of the PLA/nisin sample. Subsequently, investigation was made as to the influence of thermal treatment exceeding the T_m of PLA exerted on the content of nisin in the PLA/nisin films. After 5 min at 160 °C, the amount of nisin dropped significantly to 110 ± 40 μg g^{-1} PLA/nisin, which means that the content of nisin was diminished by about 70%. In fact, thermal treatment for 5 min at 180 °C led to such a huge decrease in nisin that its concentration in the sample was below the limit of detection of the method used.

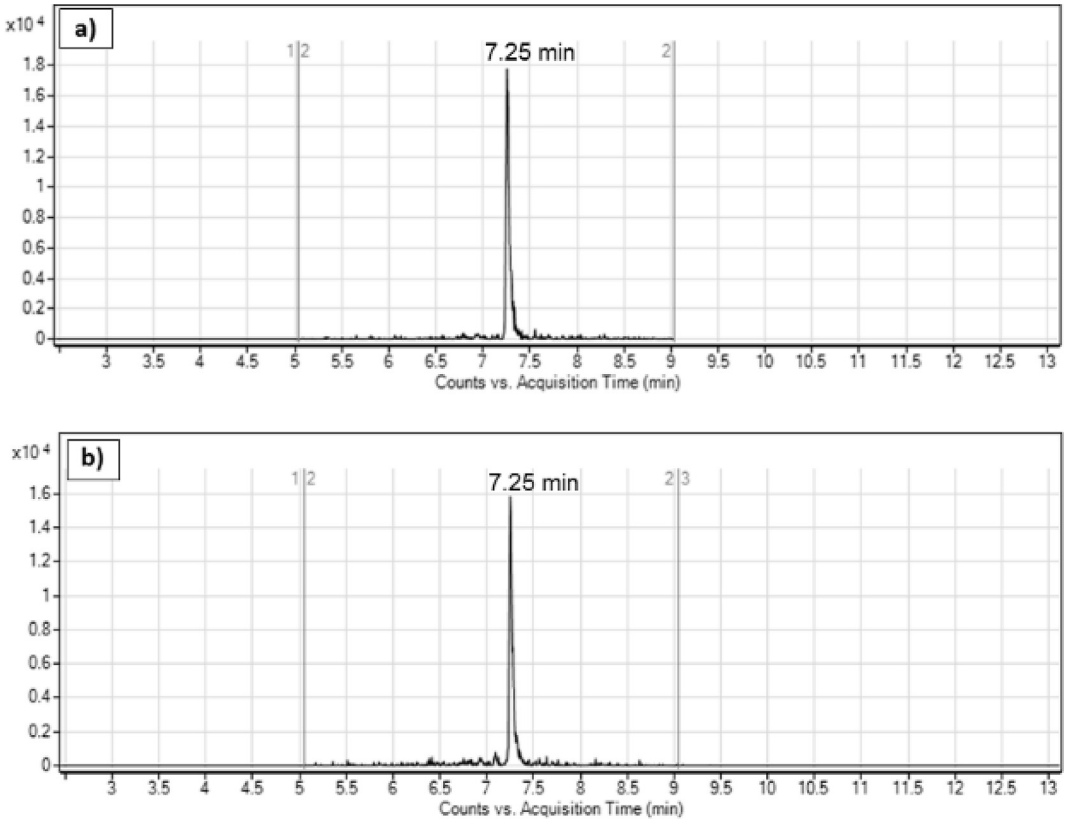

Fig. 3. Extracted ion chromatogram of the $[M+5H]^{5+}$ molecule ion of nisin in a) solvent standard at the concentration of 10 000 ng mL^{-1}, and b) a sample containing 8100 ng mL^{-1} of nisin.

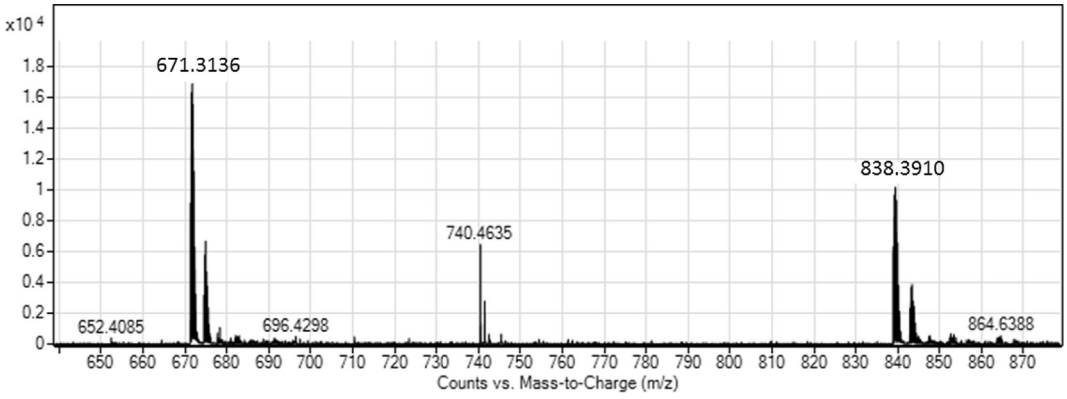

Fig. 4. Identification of nisin in the mass spectrum of the solvent standard at the concentration of 10 000 ng mL^{-1}; *m/z* 671.3136 corresponded to $[M+5H]^{5+}$ (mass error 4.05 ppm), and *m/z* 838.3910 corresponded to $[M+4H]^{4+}$ (mass error 3.08 ppm).

The effect of high temperature on structural changes in the prepared blend films was investigated using gel permeation chromatography and scanning electron microscopy.

As can be seen in Fig. 5, the authors evaluated the dependence of average molar weight and the polydispersity index of the PLA-based samples on the duration of thermal treatment. It was found that no significant effect exerted by the content of nisin was noticeable on the PLA matrix. In fact, the results for the PLA and PLA/nisin samples are almost identical. Moreover, it was found that the influence of high temperature on these samples was not particularly significant, exhibiting just a 20% decrease in molecular weight under treatment at 160 °C, and a 30% reduction in M_w under treatment at 180 °C for 1 h.

However, the inclusion of PEG dramatically reduced the M_w and increased the polydispersity index of PLA in the PLA/PEG/nisin blends. These results indicate that there were more short-chain PLA molecules in the PEG-incorporated blends than in the neat PLA or PLA/nisin blend, respectively. Thus, in the presence of PEG, the authors observed significant degradation in the PLA matrix. The reasons for this probably mirror those described above pertaining to the discussion of the antibacterial stability of the investigated samples.

Furthermore, it was found that the molecular weight of PEG did not significantly affect the degradation behavior of the PLA. The average molecular weights of the PLA/PEG1000/nisin and PLA/PEG6000/nisin blends were almost identical. Only a slight

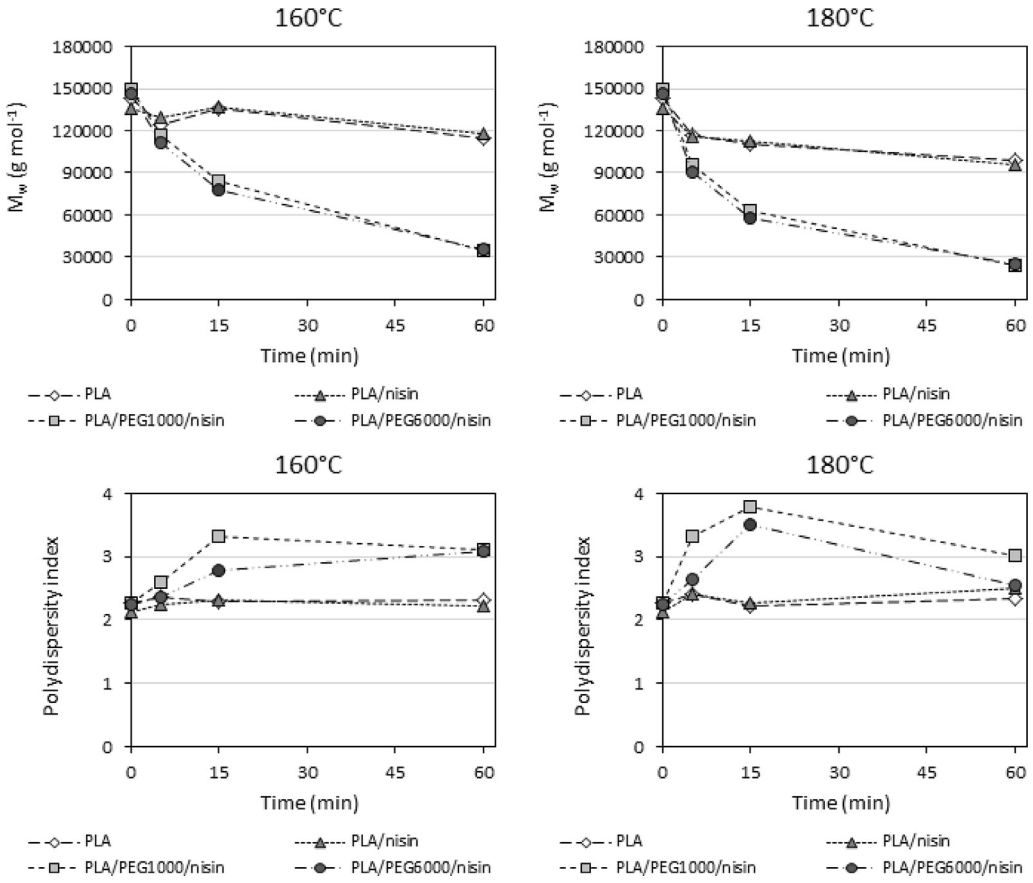

Fig. 5. GPC data on PLA-based blend samples during thermal treatment exceeding the T_m of PLA.

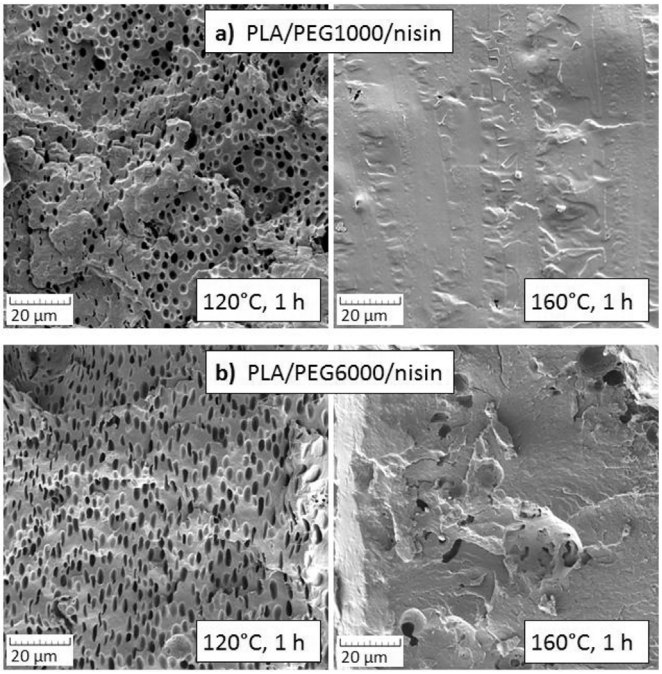

Fig. 6. SEM micrographs of PLA/PEG/nisin samples treated at 120 °C and 160 °C for 1 h.

difference was observed with respect to the results of the polydispersity index, where higher values were discerned in the sample with PEG1000. This finding is consistent with the aforementioned results, i.e. that the larger portion of the PEG phase was involved in the PLA amorphous phase in the case of PEG1000. Therefore, the degradation of the PLA matrix during thermal treatment was slightly greater in the presence of lower molecular weight PEG.

In order to illustrate the influence of thermal treatment above and below the T_m of PLA on the morphology of samples, SEM micrographs of the PEG-incorporated samples were taken after 1 h of treatment at 120 °C and 160 °C (Fig. 6). As can be seen, the porous structure of the samples was retained when treated below the T_m of PLA. However, this typical structure was completely lost after 1 h at 160 °C. Only remnants of the pores were observed for PEG6000. This finding corresponds to GPC results, where slightly greater degradation was observed in the PLA/PEG1000/nisin samples based on the polydispersity index values.

4. Conclusions

The primary aim of this work was to evaluate the thermal stability of bacteriocin nisin in PLA and PLA/PEG blend films. The nisin-incorporated PLA and PLA/PEG films that were cast underwent treatment at various temperatures (90 °C, 120 °C, 160 °C, and 180 °C) for up to 48 h. The results reveal that the PLA-based films displayed antimicrobial properties even when they were thermally treated above the melting temperature of PLA. However, antibacterial activity was reduced by about 25% under treatment at 160 °C for 5 min and by more than 60% under treatment at 180 °C for 5 min.

Despite the fact that PEG improves the stability of nisin in long-term storage, and can effectively modify mechanical properties of PLA, the adverse effect of PEG on the stability of nisin in polymer

films treated above 120 °C was evident.

These results reveal that nisin incorporated in polymer matrix can maintain its antibacterial activity at temperatures relevant to processing window of plastics used for packaging and/or medical devices production.

Acknowledgements

This work was co-financed by the Ministry of Agriculture of the Czech Republic (project no. QJ1310254), and the Ministry of Education, Youth and Sports of the Czech Republic (project no. LO1504). P. Holcapkova is grateful for the support lent by the Internal Grant Agency of Tomas Bata University in Zlin (project no. IGA/CPS/2018/003).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.polymdegradstab.2018.10.019.

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