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Original Research

Assessment of Polylactide Foil Degradation as a Result of Filamentous Fungi Activity

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Abstract

Degree of poly (lactic acid) (PLA) foil degradation by the filamentous fungi i.e. *Aspergillus ustus, A. sydowii, A. fumigatus, Paecilomyces lilicanus* and *Penicillium verrucosum* has been investigated. A degradation process has been conducted in dynamic conditions at 30°C for 10 days in a medium containing 0.1% of foil as carbon source. To activate the enzymes involved in the poly (lactic acid) foil degradation, triple passaging on the medium has been conducted. Each passage lasted 10 days. After passages, increase of the mycelium biomass, its enzymatic activity and structural foil changes have been investigated using scanning electron microscopy (SEM) and differential scanning calorimetry (DSC). Strain passaging has resulted in the activation of the enzymes of the esterase group responsible for foil degradation, expressing itself in the mycelium biomass increase. *Penicillium verrucosum* and *Aspergillus ustus* appeared to be the most active strains. Structural and thermal changes of the material have been demonstrated. Filamentous fungi have a wide range of enzymes of the esterase group which, following initial activation, may actively participate in PLA foil degradation.

Keywords: biodegradation, biodegradable polymers, poly(lactic acid), DSC, SEM

Introduction

In recent years there has been an increase in the interest in poly (lactic acid) as packaging material due to its functional properties similar to the widely used polyethylene, but also due to its greater susceptibility to biodegradation. Application of the poly (lactic acid) foil might resolve the problem of packaging waste disposal. However, the degree and rate of biological polylactate degradation depends on the polymer chemical structure (molecular mass), crystalline phase portion (the greater it is, the harder degradable the polymer is), proper surface and hydrophilicity as well as external factors, i.e. pH or temperature [1]. It is believed that microorganisms and their enzymes are mainly responsible for PLA degradation in natural conditions. Literature includes numerous reports on degradation of this polyester, depending on both physical-chemical [2] and microbiological factors [3, 4, 5, 6]. Also, poly (lactic acid) foil degradation with ready enzymes, i.e. proteinase K or lipase [7, 8], has been dealt with. Liu et al. examined the level of polymer degradation (i.e. PLA and poly-ε-caprolactone) with the application of proteinase K and *Pseudomonas* lipase. He discovered varying specifity of determined enzymes in the degradation process of the foils examined [9]. Among known microorganisms capable of degrading PLA, there has been distinguished the thermophilic *Amycolatopsis* sp. strain or thermophilic *Geobacillus* and *Brevibacillus* bac-

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teria strains [10-13]. On the other hand, less attention in current research has been paid to filamentous fungi. Still, they constitute an interesting array of biological materials as they are provided with a wide range of hydrolytic enzymes involved in the processes of mineralization and degradation of organic complex compounds. To date, it has only been demonstrated that filamentous fungi strains are capable of degrading PLA oligomers and lactic acid [5, 6]. Jarerat et al. demonstrated that the *Tritirachium album* strain is capable of degrading PLA but only in the presence of 0.1% of gelatine [14]. The role of filamentous fungi in the poly (lactic acid) foil degradation is not well known.

The research objective of this paper was to assess poly (lactic acid) foil degradation with the involvement of filamentous fungi. The structural and thermal properties of the material have been investigated. Moreover, the effect of initial passaging of filamentous fungi strains in a medium containing 0.1% of foil on the polylactide degradation has been examined.

Materials and Methods

Microorganisms

Five strains of fungi, i.e. *Aspergillus ustus, Aspergillus sydowii, Aspergillus fumigatus, Paecilomyces lilicanus* and *Penicillium verrucosum* were used in this study. Fungi were isolated from the foil surface in the process of its degradation in composted soil conducted during earlier research [15, 16].

Material

The subject of the research was commercial poly (lactic acid) foil provided by the Chair of the Food Packaging and Biopolymers Department of the University of Agriculture in Szczecin. Tested material was made by European Company and the PLA resource was supplied by Nature Works LLC (USA). According to the manufacturer's dates the poly (lactic acid) foil is regarded as biodegradable material. The main aim of this study was the confirmation of susceptibility to the biological degradation of examined foil. Fig. 1 shows the chemical structure of poly (lactic acid). The material consisted of three layers of polylactide, each of a different cristallinity degree, of total thickness of 30μm. Prior to the degradation process, the foil was sterilized by means of immersing for 1 minute in a 70% solution of ethyl alcohol and then each side was exposed to UV rays for a period of 15 minutes.

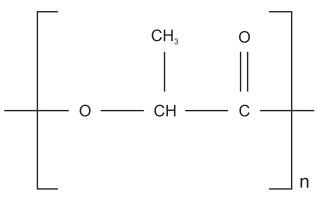

Fig. 1. Chemical structure of poly (lactic acid).

Biodegradation Process

The PLA foil degradation was conducted in dynamic conditions (200 rev/min) for 10 days at 30°C. Sterile foil fragments in the amount of 0.1% were placed in a flask containing 25 ml of a mineral medium. A basal medium was composed of yeast extract (1g/l); $MgSO_4 \cdot 7H_2O$ (1g/l); $(NH_4)_2SO_4$, (1g/l); KH_2PO_4 (1g/l); $NaNO_3$ (1g/l); distilled water, 1l. The initial pH was 5.6 ± 0.2. Medium was inoculated with a suspension of appropriate mould strains in the amount of 10% of the inoculum (density of conidia was 10^6/ ml). A mineral medium with 0.1% of glucose was the control culture. After 10 days of cultivation, the mycelium grown was used as inoculum to inoculate another medium containing polylactide foil.

Assessment of Biodegradation Process

After the degradation process, the following have been examined:

- *mould increase* based on the measurement of the mycelium biomass obtained after the 1st, 2nd and 3rd passage in the control culture and that containing 0.1% of foil. Mycelium biomass after cultivation was filtered through Buchner funnel and dried until solid at 105°C. Each test was repeated three times. Mycelium biomass increase was expressed as an average result of the three repetitions, allowing for standard test deviation.
- *enzymatic filamentous fungi capabilities* in post-cultivation liquid by means of Api-ZYM tests by bioMerieux. Enzyme activity was evaluated on the basis of colour intensity of the sample, at a grade of 0–5, according to the manufacturer's scheme. Tests given grade 0 were regarded as negative reaction (lack of enzyme) and 1, 2, 3, 4, 5 were intermediate reactions depending on the level of intensity (2, 3, 4, 5 were regard as positive).

Enzymes determined in the Api-ZYM test are respectively:

1. alkaline phospatase
2. esterase
3. esterase lipase
4. lipase
5. leucine arylamidase
6. valine arylamidase
7. cystine arylamidase
8. trypsin
9. α-chymotrypsin
10. acid phosphatase
11. naphthol-AS-BI -phosphohydrolase
12. α-galactosiasde
13. β-galactosiadase

14. β-glucuronidase
15. α-glucosidase
16. β-glucosiadase
17. N-acetyl-β-glucosaminidase
18. α-mannosiadase
19. α-fucosidase

– *changes of material surface structure and thermal properties* – foil structural changes were conducted using scanning electron microscopy (Hitachi 3000N) (SEM). Before analysis the material was covered with a thin layer of gold. Changes of the material thermal properties were examined using differential scanning calorimetry (DSC) on a MICRO DSC 111 apparatus (Setaram, France). Foil samples weighing 14.5 mg were heated in steel cells within temperatures from 20 to 200°C at the rate of 5°C/min. in an atmosphere of pure nitrogen. An empty DSC pan was used as a reference. DSC apparatus was calibrated with indium. Characteristics of the foil thermal properties included determination of the glass transition temperature, melting temperature and melting enthalpy expressed in J/g of the sample.

Results and Discussion

Assessment of Mould Growth in Control Medium and Medium Containing Foil as a Source of Carbon

All strains after the 3rd passage obtained a higher mould biomass than after the 1st passage, both in the control medium containing 0.1% of glucose and the medium with foil (Fig. 2). The greatest biomass increase was recorded in the case of *Aspergillus sydowii* and *Paecilomyces lilicanus* strains, where the mycelium mass after the 3rd passage increased by 37 and 48%, respectively, similarly to the control medium with glucose. Biomass of the *Aspergillus fumigatus* after the 3rd passage increased by 16% as compared to the biomass after the 1st passage. In the *Aspergillus ustus* and *Penicillium verrucosum* cultures, the volume of biomass after the 3rd passage was

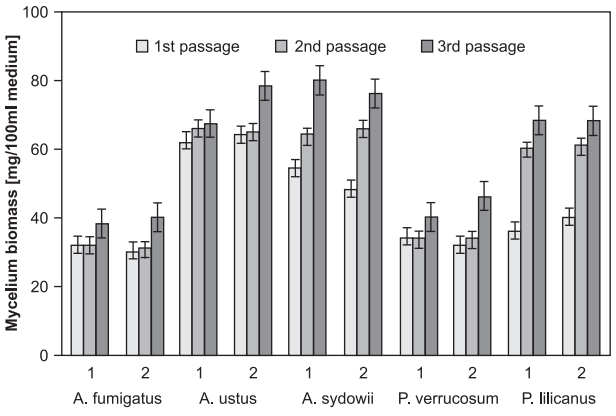

Fig. 2. Mycelium biomass after 1st, 2nd and 3rd passage 1 – control medium, 2 – medium containing 0.1% of foil.

15% higher in the medium with foil than in the control medium. Results of these experiments indicate that the addition of foil in the amount of 0.1% induced mycelium growth similarly to glucose, which unambiguously indicates that the material examined can be used as a source of carbon and can be degraded. Similar results were observed in study with the strains of actinomycete [3, 17]. Ikura et al. investigated the degradation of PLA foil in medium with *Amycolaptopsis* strains. In the culture medium with foil maximum bacterial growth was 0.27 at OD_{660}, while in the control medium without foil it was only 0.062 at OD_{660} [3]. The study with actinomycete, *Kibdelosporangium aridum,* showed that PLA foil can influence the growth of microorganisms. The researcher demonstrated that dry cell after cultivation was higher (53 mg per 100ml medium) in the basal medium with PLA foil than in the medium without foil (42 mg per 100ml medium) [17].

Assessment of Filamentous Fungi Enzymatic Capabilities

Assessment of the enzymatic activity indicated that the passaging impacted on the change of enzymatic profile only in the case of *Penicillium verrucosum* and *Aspergillus fumigatus* culture (Table 1). Additional activity of alkaline phosphatase, esterase, β-glucuronidase and N-acetyl-β-glucosoaminidase (*Penicillium verrrucosum*) and α-mannosidase (*Aspregillus fumigatus*) was demonstrated after the 3rd passage in the medium containing foil.

The *Aspergillus ustus* strain in the medium with foil additionally produced 5 enzymes, i.e. alkaline phosphatase, esterase, esterase lipase, valine arylimidase and β-galactosidase, whereas the *Penicillium verrucosum* strain additionally produced esterase, β-glucuronidase and N-acetyl-β-glucosoaminidase. These results correlate with the obtained greater mycelium biomass of these strains in the medium containing foil than in the medium containing glucose, which proves that they are involved in the degradation process of the material examined. Analysis the profile of the enzymes of the remaining strains did not show the influence of passing on the extracellular hydrolytic enzyme production characteristics.

Involvement of the enzymes in the poly (lactic acid) degradation has been examined many times. Most frequently, activity of proteinase K and lipases degrading poly (lactic acid), oligomers of the polyester as well as PLA foils has been examined [5, 7, 14, 18]. In this study, the enzyme activity which was determined following cultivation on the substrate containing polylactide foil comes from the group of enzymes hydrolyzing ester bonds, which is particularly important in the case of poly (lactic acid) degradation. They included, above all, esterase, acid phosphatase and esterase lipase. Strains producing these enzymes in the presence of the PLA material will be active in the process of its degradation. It ought to be pointed out that esterase and acid phosphatase in the case of *Aspergillus ustus* were active in the 1st, 2nd and 3rd pas-

Table 1. Enzymatic profiles of filamentous fungi after 1st, 2nd and 3rd passage in the medium containing 0.1% of foil and in the control medium 1 – 19 – enzymes determined (see in Materials and Methods), I – 1st passage in medium with foil, II – 2nd passage in medium with foil, III – 3rd passage in medium with foil, K – 3rd passage in control medium.

Strain	Passage	Enzyme														
		1	2	3	5	6	10	11	12	13	14	15	16	17	18	19
A. fumigatus	I	4	3	3	3	0	4	3	3	0	0	0	3	0	0	0
	II	5	3	1	2	0	3	2	2	0	0	0	5	0	1	0
	III	4	3	1	2	0	4	3	2	0	0	0	4	0	1	0
	K	4	3	1	4	0	3	3	3	0	0	0	4	0	2	0
A. ustus	I	5	3	4	5	3	3	5	5	4	0	0	5	5	0	0
	II	5	3	4	4	3	3	4	5	4	0	0	4	4	0	0
	III	5	3	4	5	3	3	5	5	4	0	0	5	4	0	0
	K	0	0	0	4	0	4	4	4	0	0	0	0	0	0	0
A. sydowii	I	5	3	3	2	1	4	3	4	2	0	0	5	4	0	0
	II	4	3	4	2	1	3	2	0	0	0	0	5	0	0	0
	III	4	2	2	2	0	2	0	0	0	0	0	4	0	0	0
	K	4	3	4	2	1	4	3	3	2	0	0	5	3	0	0
P. verrucosum	I	3	0	0	3	0	2	2	3	1	0	2	4	0	0	3
	II	0	0	1	3	0	0	0	3	1	1	0	0	0	0	3
	III	2	2	1	3	0	1	2	3	1	1	1	4	4	0	3
	K	1	0	1	3	0	4	3	3	2	0	4	4	0	0	3
P. lilicanus	I	5	3	4	5	1	1	5	4	0	0	0	0	2	0	0
	II	5	3	4	4	3	1	5	3	0	0	0	0	2	0	0
	III	5	3	5	5	3	2	5	4	0	0	0	0	3	0	0
	K	5	4	5	5	3	2	4	5	2	0	0	0	5	5	0

sages, whereas in the case of *Penicillium verrucosum* the activity of these enzymes was only determined in the 3rd passage. Fukuzaki et al., who examined the impact of esterases, mainly *Rhizopus delemer*, on PLA degradation, demonstrated that these enzymes accelerated the process [18]. In Sakai's study it was also demonstrated that esterase from the termophilic strain of *Bacillus smithia* was capable of degrading poly (lactic acid) [19]. Krasowska et al. confirmed that enzymatic hydrolysis plays the main role in the polyester degradation process [20]. The studies quoted as well as the results obtained induce the conclusion that activity of esterases has a particular importance in the degradation of poly (lactic acid) materials.

Analysis of Changes of Material Surface Structure and Changes of Thermal Properties

The surface changes of foil were observed with SEM analysis. It has been determined that the foil surfaces after the 1st passage with the involvement of all the strains have not been changed as compared to the foil surface before degradation.

The structural changes of the material after incubation with strains of *Aspergillus fumigatus* and *Aspergillus sydowii* were not discovered. Fig. 3 shows a SEM image of the foil following cultivation with the *Aspergillus fumigatus* after the 1st and 3rd passage. At this stage of research no changes to the foil structure can be seen, despite the mycelium biomass

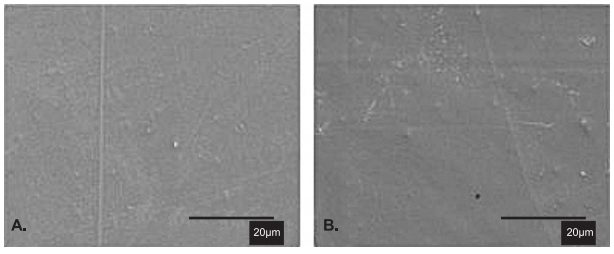

Fig. 3. Scanning electron micrographs of structure of the polylactide foil's surface after 10 days of incubation with *Aspergillus fumigatus* A – after 1st passage, B – after 3rd passage.

increase of the strains as well as the enzymes produced by them of esterase groups after the 3rd passage (Fig. 2, Table 1) indicate the possibility of material degradation by these strains. The foil changes occurred after the 3rd passage in the culture with *Aspergillus ustus* (Fig. 4) and *Penicillium verrucosum* (Fig. 5). On the foil surface the losses (Fig. 4) and fogging (Fig. 5) were observed. This result suggests that the strains of fungi can degrade the PLA material. Jarerat et al. indicated that in liquid medium with actinomycete, *Kibdelosporangium aridum*, a large number of small pits in PLA foil occurred, while on the solid medium only grooves were observed [17]. These results show that there is the rapid diffusion of secreted enzymes in the liquid medium and the quick degradation of material appears.

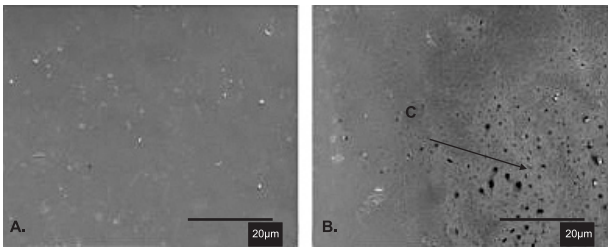

Fig. 4. Scanning electron micrographs of structure of the polylactide foil's surface after 10 days of incubation with *Aspergillus ustus* A – after 1st passage, B – after 3rd passage, C – surface changes.

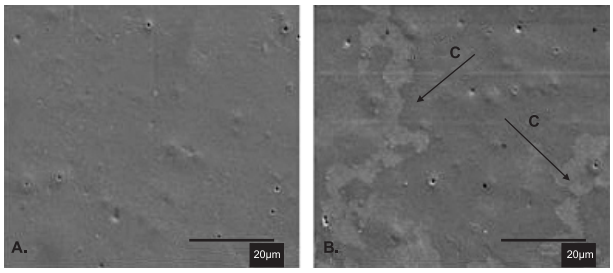

Fig. 5. Scanning electron micrographs of structure of the polylactide foil's surface after 10 days of incubation with *Penicillium verrucosum* A – after 1st passage, B – after 3rd passage, C – surface changes.

Changes of the material thermal properties were analyzed with DSC, in which no changes of the structure under the SEM microscope were discovered (*Aspergillus fumigatus*) and in which structural changes were discovered with the SEM (*Aspergillus ustus* and *Peniciliium verrucosum*). The results of thermal analysis are given in Table 2. We showed the results only after the 1st and 3rd passages, because we wanted to indicate the role of microorganisms in the biodegradation process. We expected to reach the significant changes of materials just after the 3rd passage. The glass transition temperature T_g and the melting temperature T_m of foil before degradation amounted to 62.7°C and 149.9°C, respectively. After the degradation process the melting temperatures after the 1st and 3rd passage insignificantly decreased (slightly over 1°C), whereas the glass transition temperature decreased by 1°C to 8°C. In the case of the *Aspergillus fumigatus* strain, the value of temperatures after the 3rd passage amounted to: T_g = 54.6°C (a decrease by over 8°C) and T_m = 148.9°C (Table 2); in the case of the *Aspergilus ustus* strain: T_g = 61.5°C (a decrease by over 1°C) and T_m = 148.8°C (Fig. 6, Table 2), and in the case of the *Penicillium verrucosum* strain: T_g = 59.3°C (a decrease by over 3°C) and T_m = 149.7°C (Table 2). Glass transition and melting processes enthalpy of foil before degradation

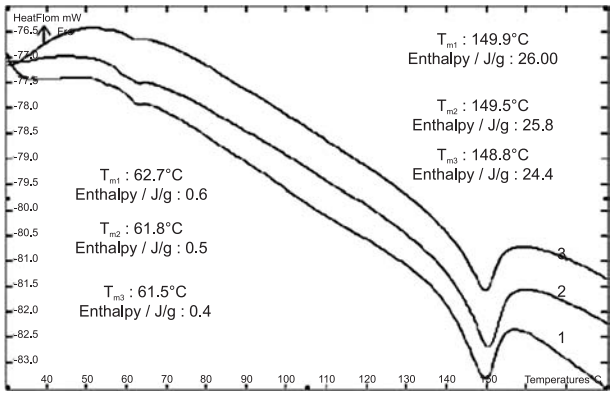

Fig. 6. DSC thermogram of polylactide foil after incubation with *Aspergillus fumigatus* 1 – foil before degradation, 2 – foil after 1st passage, 3 – foil after 3rd passage.

Table 2. Thermal properties of PLA foil before and after degradation.

Strain	Number of passages	Glass transition temperature T_g (°C)	Glass transition enthalpy (J/g)	Melting temperature T_m (°C)	Melting enthalpy (J/g)
foil before degradation		62.7	0.6	149.9	26.0
Aspergillus fumigatus	1st	54.8	0.8	148.6	25.3
	3rd	54.6	0.5	148.9	24.8
Aspergillus ustus	1st	61.8	0.8	149.5	25.7
	3rd	61.5	0.6	148.8	24.3
Penicillium verrucosum	1st	61.6	0.5	149.9	25.8
	3rd	59.3	0.4	149.7	24.4

amounted to 0.6 J/g and 26.0 J/g sample, respectively. The value of glass transition temperature of PLA is compared with T_g = 61.8°C as reported by Cao et al. [21]

Decrease of the glass transition and melting temperatures is observed in the case of polymer degradation, or reduction of its molecular mass, to be more precise. The same correlation was observed by Li examining poly (lactic acid) degradation. He recorded a glass transition temperature change from 56°C to 44°C and melting temperature change from 168°C to 153°C. These changes were relatively higher than the changes presented in this study due to a longer degradation time [22]. Also, Hakkarainen et al. determined that after 4 weeks of degradation in biotic conditions, the poly (lactic acid) melting temperature decreased by 6.5°C as compared to the initial temperature [23].

Analyzing the average value of process enthalpy, i.e. glass transition and melting, it was also observed that the values decreased after the 1st as well as the 3rd passage, subject to the change being greater after the 3rd passage. After the 1st passage of the strain examined, the foil--melting enthalpy value decreased by approx. 0.5 J/g of the sample, whereas after the 3rd passage the enthalpy value was 1.0 to 1.7 J/g lower than the material melting enthalpy value prior to degradation. Melting enthalpy of the foil after the 3rd passage with A*spergillus ustus* and *Penicillium verrrucosum* decreased from 26.0 J/g to approx. 24.3 J/g, whereas after cultivation with *Aspergillus fumigatus* to 24.8 J/g of the sample. These results are similar to the observations described in the study by Cam et al., in which it has been stated that the decrease of the vitrification (glass transition) process enthalpy is associated with selective scattering of amorphous regions. Low vitrification process enthalpy values further indicate a significant degree of sample cristallinity, which may hinder the poly (lactic acid) material biodegradation process [24]. Krasowska et al. examined the degradation process of poly (ε-caprolactone) belonging to the same group of polyesters as poly (lactic acid), based on the changes of the melting enthalpy values, with the DSC. In her study, she has stated that the decrease of enthalpy also indicates amorphous phase degradation and, consequently, crystalline regions, which results in polymer material destruction [25]. Analyzing the experiment results, it was discovered that degradation with the involvement of filamentous fungi used in this study had already been initiated. Additionally, the examination results imply that the A*spergillus ustus* and *Penicillium verrrucosum* fungi, whose mycelium biomass increase after the 3rd passage, was higher in the medium containing foil than in the medium containing glucose, resulting in a higher degree of the examined material amorphous phase degradation (greater change of the melting enthalpy value).

Summary

Among the 5 filamentous fungi strains examined, only *Aspergillus ustus* and *Penicillium verrucosum*, appeared the most active in poly (lactic acid) foil degradation. Activity of the enzymes of the esterase group was induced after the 3rd passage in culture medium with these strains. *Aspergillus ustus* and *Penicillium verrucosum* were used for the PLA foil as a source of carbon. The results showed that the mycelium biomass increased after the 3rd passage, which was 15% higher in the medium containing foil than in the control medium containing glucose. SEM and DSC analyses confirmed structural changes of foil after incubation with the above-mentioned fungi. We have to indicate that the tested material consisted of three PLA layers and SEM analysis provides information only about the structure of foil surface, while DSC analysis evaluates the average results about the properties and structure of the material after degradation. Therefore, in order to obtain comprehensive information of the material degradation, numerous analyses ought to be conducted. In the case of the *Aspergillus fumigatus* strain, clear foil's changes were only demonstrated in DSC study. The results indicate that the material degradation in natural conditions, not fully controlled, may proceed very slowly. Finally, it ought to provide the selected and adapted set of filamentous fungi in order to ensure greater effectiveness of polymer biodegradation.

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