Isolation of Silk Degrading Microorganisms and Its Poly(L-lactide) Degradability

Yutaka Tokiwa,* Mariko Konno,† and Haruo Nishida†
National Institute of Bioscience and Human-Technology, 1-1 Higashi, Tsukuba, Ibaraki 305-0046
†*Tsukuba Research Laboratory, Tokuyama Corp., 40 Wadai, Tsukuba, Ibaraki 300-4247*

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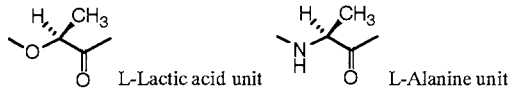
To examine a hypothesis that microorganisms regard poly(L-lactide) (PLLA) as a protein substrate like silk, a silk-utilizing actinomycete, *Amycolatopsis* sp. strain KT-s-9, was isolated. The strain KT-s-9 was confirmed to degrade and utilize silk fibroin rapidly. By the clear zone method, it was determined that the strain KT-s-9 can also degrade PLLA.

Poly(L-lactide) (PLLA) has attracted attention recently due to its biodegradability. Until now studies on PLLA biodegradation have focused mostly on enzymatic[1] or hydrolytic[2] degradation. These studies indicated that PLLA was especially degraded by proteinase K, but not by lipases or PHB depolymerase.

Proteinase K from *Tritirachium album* Limber is an extracellular serine endoproteinase.[3] The above suggests that PLLA is regarded as a protein substrate by microorganisms. This may be based on structural similarity between L-lactic acid unit and L-alanine unit.



Silk consists of fibroin, sericin, a little lipid, carbohydrate, and inorganic constituents. Strydom *et al.*[4] reported that the amino acids sequence of crystalline domain in the silk fibroin is $(Gly\text{-}Ala)_2\text{-}Gly\text{-}Ser\text{-}Gly\text{-}(Ala)_2\text{-}Gly\text{-}[Ser\text{-}Gly\text{-}(Ala\text{-}Gly)_n]_8\text{-}Tyr$, where n is usually 2.

Cocoonase is a proteolytic enzyme produced by silk moths and degrades a sericin component to soften the cocoon. Lucas *et al.*[5] reported that proteolytic enzymes do not readily attack fibrous fibroins because of the close packing of the molecular chains based on the high proportion of amino acids with short side chains. Prasad and Singh[6] reported that storage fungi, *Fusarium moniliforme,* caused a considerable loss in total and buffer-soluble protein content of the tasar cocoons infected. However, no study concerning the microbial degradation of silk fibroin, in particular, its crystalline domain, has been reported.

In this study, to examine a hypothesis that PLLA is regarded as a protein substrate by microorganisms, silk-fibroin degrading microorganisms were isolated from natural environments and their silk degradability, utilizability, and PLLA degradability were investigated.

Mechanical silk powder from *Bombyx mori Linnaeus* was washed with hot water to remove water-soluble components such as sericin, lipid, and carbohydrate. The amino acid composition of the silk powder was evaluated by elemental and amino acid analyses. This gave the elemental composition (wt%): C, 38.40; H, 6.35; N, 14.23; O, 32.17; amino acid composition (mol%): glycine, 44.6; alanine, 29.5; serine, 11.7. These analytical results indicate that the silk powder consists of protein, 75% by weight and inorganic materials such as magnesium phosphate, 25% by weight.

Culturable colony numbers of silk-degrading microorganisms in samples from natural environments were counted by the clear-zone method[8] using the basal medium plates containing 100 ppm yeast extract (YE) and 1000 ppm emulsified silk powder within a 110-day period. Twenty-eight samples were collected from various environments in Kanto, Kansai, and Chugoku regions. Clear-zones were observed around some colonies on the plates of 12 samples. In the samples for which clear-zone formation was observed, the ratio of the clear-zones to the total colonies was generally low (<0.1%).

Some silk-fibroin degrading strains were successfully isolated from the colonies forming clear zones on the plates. All isolates were Actinomycete species. A degrading strain KT-s-9 formed clear-zones in the shortest period (7days) as shown in Figure 1. Strain KT-s-9 was gram-positive, nonmotile and grew in the region of pH 5 - 10 at 20 - 37°C but not at 45°C. Colony morphology was irregular, undulate edge, aerial mycelium, wrinkled matt, and convex. Some standard physiological tests were carried out by conventional methods. As results, it was shown follows; catalase (+), oxidase (-), gelatin hydrolysis (+), VP test (-), MR test (-), nitrate reduction (+/-), pigment production (+, red), O-F test (-), and fatty acid composition (16:0 iso, 38.58%; 15:0 iso, 13.53%; 14:0 iso, 12.11%).

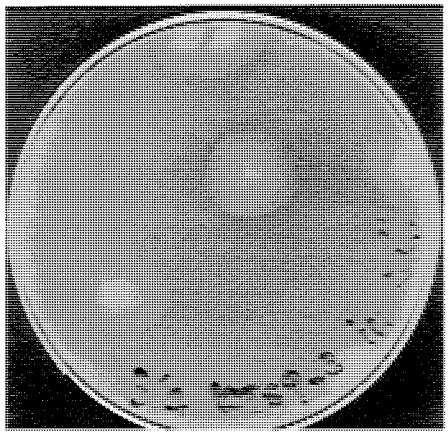


Figure 1. Photograph of strain KT-s-9 colonies on agar plate emulsified with silk powder. The plate was incubated for 7 days.

Strain KT-s-9 was also identified at the genus level by 16S rRNA gene fragment amplification as described by Shida *et al.*[9] The nucleotide sequences determined have been deposited in the DDBJ/EMBL/GenBank data libraries. Calculation of nucleotide substitution rate, construction of a neighbor-joining phylogenetic tree, and a bootstrap analysis with 1,000 replicates for evaluation of phylogenetic tree topology were carried out with a computer using the CLUSTAL W program. The results of analyses based on 16S rRNA gene sequence demonstrated

that strain KT-s-9 is classified as a member of the family *Amycolatopsis*. This is the same genus as that of the PLLA degrading microorganisms reported by Pranamuda *et al.*[7]

Pre-culture of strain KT-s-9 was carried out on a yeast extract-malt extract agar (ISP-2) medium plate at 30°C for 2 days. Five-loopfuls of the colonies on the ISP-2 plate were suspended into 30 ml of sterilized water. The suspension was used as inoculum for time course test.

The time course test of silk powder degradation was carried out in 300 ml Erlenmeyer flasks containing 100 ml of the basal medium, 5 mg of yeast extract, and 100 mg of silk powder. These were inoculated by 0.5 ml aliquot of the cells suspension, and incubated on a reciprocal shaker (120 spm) at 30°C for 32 days. Residual silk powder was recovered by filtration with a 5 μm membrane filter, and dried *in vacuo*. Under SEM observations, most of the cells were found to attach on the filter with biofilm. Filtrate was nearly transparent. A 5 ml aliquot of the filtrate was taken for analysis of total water-soluble organic carbon (TOC). The aliquot was filtered with a 0.2 μm membrane filter for removing free cells. TOC was measured with a Shimadzu TOC-5000 analyzer. Experiments were done in duplicate and good reproducibility was obtained. The mean value for each set of experiments was used as the result. Results are shown in Figure 2.

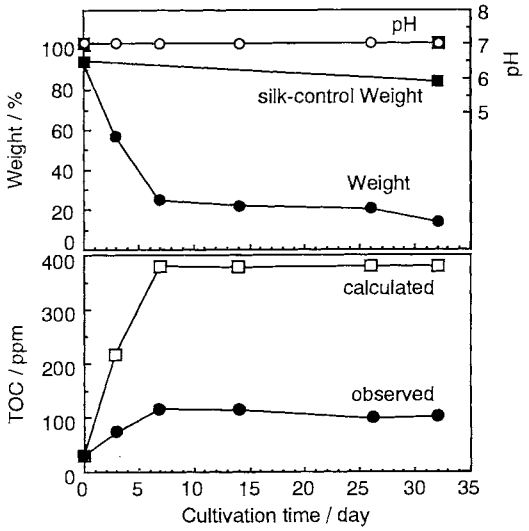


Figure 2. Silk powder degradation by strain KT-s-9.

In Figure 2, the weight of residual silk powder, including the cells with biofilm, decreased promptly to 24% during the initial 7 days. This decrease is reflected in increase of TOC. However, during 7 to 32 days, there was only a small further decrease to 13.9% in weight. The silk powder recovered at 10 days culture was observed by an energy dispersive X-ray spectrometer under SEM with a JEOL JSM-6400F. From the observation, the recovered was estimated to consist mostly of the inorganic components such as magnesium phosphate.

On the other hand, the weight loss of 16% in silk-control culture was considered due to partial secondary dispersion.

Therefore, the protein component (75% content) of the silk powder was entirely degraded during the initial 7 days of culture. From these results, microbial degradation of the silk fibroin by strain KT-s-9 is obvious.

In Figure 2, calculated TOC values,which are evaluated from the silk weight loss values in test cultures and observed TOC values of the cell-control cultures, are shown. Metabolism of silk powder was estimated by comparison of the observed TOC values with the calculated TOC values in test cultures. Values of the metabolism were found to be maintained at high values (67 - 74%) during the culture time. This indicates that strain KT-s-9 metabolized about 70 wt% of amino acid carbons in the silk powder. Therefore, the metabolism of silk fibroin by strain KT-s-9 is distinct and the strain is obviously a silk fibroin-utilizing microorganism.

PLLA degradability of strain KT-s-9 was also examined by the clear zone method[8] with a basal medium-YE plate containing 1000 ppm emulsified PLLA. A 0.1 ml aliquot of the cells suspension of strain KT-s-9 was spread out on the PLLA emulsified plate. The culture was carried out at 30 °C for 37 days. After the colony formation at 1 day culture, the clear zones were formed at 37 days culture as shown in Figure 3. This result indicates that strain KT-s-9 can also degrade PLLA.

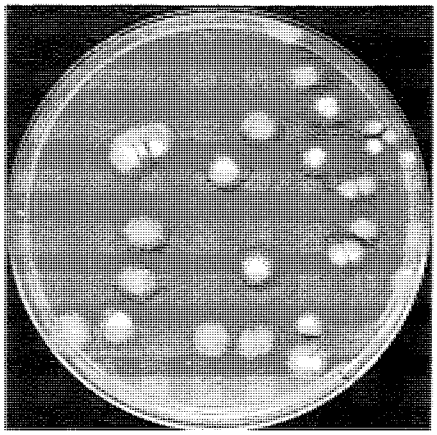


Figure 3. Photograph of strain KT-s-9 colonies on agar plate emulsified with PLLA. The plate was incubated for 37 days.

This could demonstrate that the repeated unit of PLLA is seen as alanine unit or glycine unit of silk fibroin by the silk-utilizing microorganisms. To confirm the hypothesis, it will be necessary to clarify whether a purified silk-degrading enzyme can degrade PLLA. This will be reported in the future.

References and Notes

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