SHORT REPORT

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A New Assay for the Enzymatic Degradation of Polylactic Acid

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ABSTRACT: A new fluorescent assay for the degradation of polylactic acid was developed based on the reaction of the carboxyl groups of lactic acid and its soluble polymers with a fluorescent agent, *o*-phthalaldehyde, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The assay was used to show polylactic acid degradation catalyzed by proteinase K, subtilisin Carlsberg and subtilisin BPN′. The proteolytic degradation was shown to be activated or inhibited by the presence of detergent.

KEYWORDS: Polylactic acid, proteinase K, subtilisin, detergent.

INTRODUCTION

Polylactic acid or polylactide (PLA) is an aliphatic polyester which is an attractive biodegradable plastic for various reasons. PLA is made from lactic acid which can be easily prepared by fermentation of renewable resources such as starch. Moreover, PLA possesses good properties, such as a high melting point (180 °C), transparency, composibility and safety.[1] In addition, PLA has been shown to be useful in certain biomedical applications.[2] Microbial degradation of PLA has been well studied. There have been reports on the degradation of PLA oligomers (molecular weight ~ 1000) by *Fusarium moniliforme* and *Penicillium roquefort*[3] and the degradation of PLA by *Amycolatopsis* sp[4,5] and by *Bacillus brevis*.[6] In addition, enzymatic degradation of low molecular weight PLA (molecular weight ~ 2000) has been shown using esterase-type enzymes such as *Rhizopus delemer* lipase, hog pancreatic lipase, and carboxylic esterase,[7] whereas proteinase K could catalyze the hydrolysis of high molecular weight PLA.[8,9] These and other biodegradative studies of PLA have employed a number of available methods of detection. Total organic carbon, analysis of water-soluble fraction,[10] Fourier transform infrared spectroscopy, gas chromatography-mass spectrometry and scanning electron microscope[11] have been used to accurately measure the PLA degradation. However, these methods require expensive equipments. On the other hand, simpler methods, namely turbidity measurement,[12] pH measurement[13,14] and size-exclusion chromatography[11,14] are not as accurate. To enable us to investigate more fully the enzymatic degradation of PLA, we have developed a new fluorescent assay. This paper reports on the new assay method and its application in the study of the effects of detergents on the proteolytic degradation of PLA.

MATERIALS AND METHODS

Polylactide from Shimadzu, LACTY 1012 (number-average molecular weight = 1.3×10^5), in pellet form, was a gift from Dr. Yutaka Tokiwa of the National Institute of Bioscience and Human Technology, Tsukuba, Japan. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, *o*-phthalaldehyde, *Tritirachium album* proteinase K and *Bacillus licheniformis* subtilisin Carlsberg were from Sigma. Bacterial subtilisin BPN′ was from Fluka.

PLA Film Preparation

Films (each 0.5 mm thick) were prepared by compression molding using a hydraulic press equipped with two heating plates, as described previously.[15] An aliquot (3.5 g) of PLA pellets was placed between two polished metal plates (25×25 cm). The metal plates were inserted between the heating plates. The assembled plates were first maintained at 200 °C for 3 min to melt the pellets. Then, a pressure of 15 tons force/cm^2 was applied for 5 min to form the film. After removing the pressure, the plates were left to cool to room temperature. Then, the film was removed from the plates and cut into square pieces (0.5 × 0.5 cm).

Fluorescent Assay of PLA Degradation

PLA degradation was assayed by measuring the carboxyl groups of lactic acid and its soluble polymers, which were released from a PLA film, by reacting with

o-phthalaldehyde (OPA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The reaction between the carboxyl group and EDC/OPA was adapted from a previously reported method.[16] A piece of PLA film (0.5×0.5 cm) weighing about 13 mg was incubated in 0.5 ml of an enzyme solution (30 units) in 20 mM phosphate buffer, pH 8.0 in a test tube (100×13 mm) at 37 °C in a shaking water bath. At various time intervals, an aliquot of 10 µl of the solution was withdrawn using a micropipette and mixed with 25 µl of EDC solution (5% w/v) which was freshly dissolved in 0.2 M sodium cacodylate-HCl buffer, pH 5.0. The mixture was left at room temperature for 1 h. Then, an aliquot of 10 µl of the mixture was withdrawn and added to 2.5 ml of OPA reagent in a tube (75×12 mm). The resulting solution was mixed thoroughly and the fluorescence was measured using a fluorospectrometer (JASCO FP-777). The excitation wavelength was 340 nm and the emission wavelength was 455 nm. A substrate blank and an enzyme blank were also included in the assay and the fluorescence of the blanks was subtracted from that of the sample.

The OPA reagent was prepared according to a published method.[17] Briefly, 1 ml of OPA (10 mg/ml in ethanol) and 1 ml of 2-mercaptoethanol (5 µl/ml in ethanol) were mixed with 60 ml of 50 mM sodium borate buffer, pH 9.5. To calibrate the fluorescent method, an aliquot (10 µl) of lactic acid of various concentrations was used instead of the solution from PLA degradation in the reaction with EDC/OPA described above. Protease activity was determined spectrophotometrically using azoalbumin as substrate as described previously.[17] One unit of protease activity was expressed as the amount of protease required to produce an absorbance change of one under the assay conditions.

Lipase activity was assayed spectrophotometrically using *p*-nitrophenyl-palmitate as substrate according to a previously reported method.[18] One unit of lipase activity was defined as the amount of enzyme which caused the release of 1 µmole *p*-nitrophenol per minute under the assay conditions. Protein concentration was determined by a modified Bradford method.[19]

Results and Discussion

Performance of the Fluorescent Assay of Enzymatic Degradation of PLA

The new fluorescent method was first tested using lactic acid as the carrier of carboxyl groups which reacted with EDC/OPA. Optimization of the reactant concentrations and reaction time was carried out using lactic acid. A linear relationship was established between the fluorescence intensity and the lactic acid concentration up to 26.5 µM. This method was then used to follow the proteolytic degradation of PLA. A linear relationship between the fluorescence intensity and the enzyme concentration was observed for proteinase K, subtilisin Carlsberg and subtilisin BPN′ (Fig 1). An enzyme blank was included to correct for the fluorescence due to carboxyl and amino groups of the enzymes. At the same level of enzyme activity, proteinase K was more active than the subtilisins in catalyzing PLA degradation. The two subtilisins were equally active in promoting this degradation. Furthermore, the fluorescence intensity also increased linearly with the surface area of the PLA film (Fig 2). This was observed using either one piece of PLA film or multiple pieces of the film of the same total area.

Using the new assay, other proteases tested, namely bromelain, papain, carboxypeptidase A, pepsin, trypsin and proteases from *Aspergillus oryzae*, *Bacillus subtilis* (proteinase N), *Bacillus thermoproteolyticus rokko*, *Rhizopus sp.*, *Streptomyces griseus*, were found to be

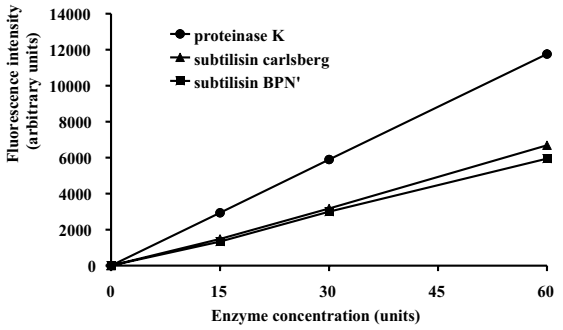

Fig 1. Effect of enzyme concentration on PLA film degradation. After incubation of proteinase K, subtilisin Carlsberg and subtilisin BPN′, and PLA film at 37 °C for 72 h in a shaking water bath, the degradation products released were measured by the fluorescence assay described in the methods.

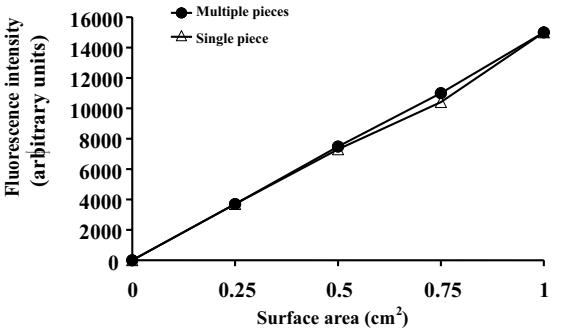

Fig 2. Effect of surface area of PLA film on its degradation. One ml of proteinase K solution (60 units) was incubated with varying numbers of pieces of PLA film (0.5 × 0.5 cm) or a single film piece at 37 °C with a shaking water bath. After incubation of 72 h, degradation products were measured by fluorescent assay.

ineffective in causing PLA degradation. Similarly, lipases from various sources were found to cause no degradation of PLA. The commercial lipases tested were from *Aspergillus niger*, *Candida antarctica, Candida cylindracea, Mucor miehei, Pseudomonas cepacia, Pseudomonas fluorescens, Penicillium roqueforti, Rhizopus arrhizus, Rhizopus niveus,* and hog pancreas. Each protease or lipase was assayed for its activity using a spectrophotometric method and 30 units were used in the test.

Effects of Detergents on the Enzymatic Degradation of PLA

When a detergent was added to the reaction, the degradation of PLA was altered. Depending on the detergent used, the effect can be an inhibition or an activation or both. Among the detergents tested, those which caused an inhibition were sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), cetylpyridinium bromide, hexadecyl-maltoside, Triton X-100, Tween 20, Brij W1 and Tergitol NP-40 (data not shown). On the other hand, detergents causing an activation included heptyl-thioglucoside and octyl-glucoside (Fig 3). Another group of detergents showed an activation at low concentration and an inhibition at higher concentration. These were dodecyl-glucoside, dioctyl sulfosuccinate, and decyl-maltoside (Fig 4). Furthermore, the three proteases used in the study showed similar patterns of inhibition/activation caused by detergent though the extent of the effect varied depending on the enzyme. At the maximal concentration tested, each detergent was found to have no effect on the activity of the three enzymes when assayed using azoalbumin. In addition, each detergent was also found at its maximal test concentration to have no effect on the fluorescence yield of the assay using lactic acid.

The key to the development of the fluorescent assay was the use of the reaction between a carboxyl group and OPA in the presence of EDC.[16] The reaction has been used to assay carboxyl group-containing compounds such as glucuronic acid, galacturonic acid, gluconic acid, glucono-d-lactone, *n*-acetylneuraminic acid, palmitic acid, succinic acid, glycolic acid, glyceric acid, benzoic acid.[16] This study is the first to use this sensitive fluorescent assay to measure the carboxyl groups released by PLA degradation. The soluble carriers of the carboxyl groups include lactic acid and soluble short-chain polymers of lactic acid. The development of the fluorescent assay mainly involved optimization of the reagent concentrations and optimization of the reaction time so that the relationship between lactic acid concentration and the fluorescence intensity was linear. This study

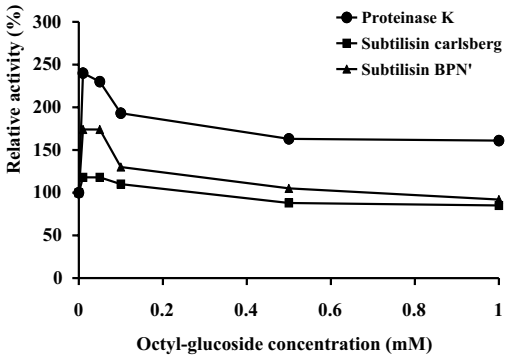

Fig 3. Effect of octyl-glucoside on the proteolytic degradation of PLA. The reaction mixture (500 ml) containing enzyme, PLA film (0.5 × 0.5 cm) and various detergent concentrations was carried out at 37 °C in a shaking water bath for 72 h. The amounts of the enzyme used are as follows: proteinase K, 15 units; subtilisin carlsberg, 30 units; subtilisin BPN′, 15 units.

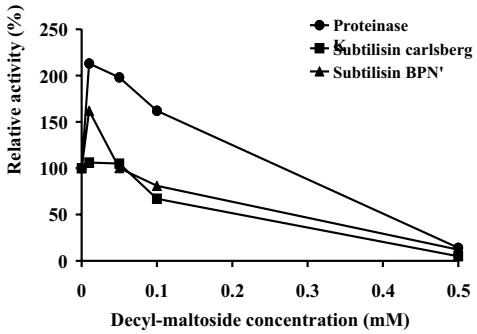

Fig 4. Effect of decyl-maltoside on the proteolytic degradation of PLA. The experimental condition was the same as that described in Fig 3.

demonstrated that the new method is a very simple and convenient assay for the PLA degradation, using only a fluorospectrometer. Fluorescent measurement showed an increasing amount of degradation products with increasing reaction time. The linear increase in the rate of PLA degradation, with enzyme concentration (Fig 1) and with surface area of PLA (Fig 2) further confirm that the new method is valid.

However, there are some limitations in the method. Precaution has to be taken to ensure that other carboxyl carriers, if present, do not cause a high fluorescence (background). In the study of the protease-catalyzed degradation of PLA, the enzyme blank containing a protease (with carboxyl and amino groups) was no more than 10% of the fluorescence intensity of the sample. Another limitation has to do with the fluorescent measurement. It has to be tested that no other reagents in the assay mixture interfere with the fluorescence intensity. In the study on the effects of detergents, it was confirmed that each detergent used did not alter the fluorescence intensity.

Using this convenient assay, proteinase K was found to be a good catalyst for PLA degradation whereas subtilisin Carlsberg and subtilisin BPN′ were not as effective (Fig 1). Several other proteases and lipases were ineffective in catalyzing the PLA degradation. The finding that lipases were unable to cause the breakdown of PLA was similar to that of a previous report.[2] This result suggests that the new assay may be used in screening for new enzymes or microbial sources of PLA degrading enzymes.

The effects of detergents on the proteolytic degradation of the solid substrate, PLA, but not on the protease activity towards a soluble substrate, azoalbumin, indicated that the detergents were interfering with the binding of the enzyme to the solid PLA film. Those that activated the degradation probably enhanced enzyme binding whereas those which caused inhibition, probably blocked the binding. For the detergents that activated the binding at a low concentration but caused inhibition at a higher concentration, a crowding effect at the high detergent concentration may be the explanation. As the actions of detergents are complex, it is not possible to relate their structures to their effects. Nevertheless, these findings show that the degradation of PLA in the soil may be accelerated by adding appropriate detergents.

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