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SHORT COMMUNICATIONS

# Enzymatic Degradation of Sequential Polymers Containing a Tripeptide Sequence L-Alanine–L-Leucine–γ-Ethyl L-Glutamate and an α-Hydroxy Acid L-Lactic Acid or α-Amino Acid L-Alanine

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The sequential polydepsipeptides containing a definite sequence of α-amino acids and α-hydroxy acids are applicable to a wide range of biomedical material systems, for example surgical sutures and drug delivery systems.[1-4] Especially, our interest in this field is to obtain sequential polymers having good biocompatibility and biodegradability. The biodegradable mechanism of sequential polydepsipeptides may be expected to proceed by competition between protease action and esterase action, because it has both the peptide bond (–NHCO–) and ester bond (–COO–) in the main chain. For this purpose, a sequential polydepsipeptide of three α-amino acids such as L-alanine (Ala), L-leucine (Leu), and γ-ethyl L-glutamate (Glu(OEt)) and one α-hydroxy acid such as L-lactic acid (Lac), poly(Ala–Leu–Glu(OEt)–Lac), was synthesized by polycondensation of tetradepsipeptide active ester. At the same time, the synthesis of a sequential polypeptide poly(Ala–Leu–Glu(OEt)–Ala), which consists only of peptide bond (–NHCO–), was carried out. The difference in chemical structure between poly(Ala–Leu–Glu(OEt)–Lac) and poly(Ala–Leu–Glu(OEt)–Ala) is related to the difference in bond-structure, *e.g.*, ester bond for Glu(OEt)–Lac chain and peptide bond for Glu(OEt)–Ala chain, respectively. In this case, a structural formula of Lac is expressed by HO–($CH_3$)CH–COOH. If the OH group containing in Lac is converted into an $NH_2$ group, the compound obtained corresponds to Ala, $NH_2$–($CH_3$)CH–COOH. The preliminary study makes it necessary to evaluate the capability of the new polymers, especially biodegradability. In this case, the sequential polymers were degraded by three kinds of enzymes, lipase as an esterase, papain as an endopeptidase, in order to learn the differences in enzymatic degradation between two polymers. The *in vivo* degradation was attempted by implanting the polymers sub-

cutaneously in the back of rats, for the comparison with the above enzymatic degradation.

## EXPERIMENTAL

A sequential polydepsipeptide poly(Ala–Leu–Glu(OEt)–Lac) has the structural formula given in Figure 1a and was synthesized accoriding to the schematic diagram in Figure 2. *N*-Protected monomer was prepared by a solution method for peptide synthesis involving depsipeptide formation with 2-nitrophenylsulphenyl (Nps) *N*-carboxy α-amino acid anhydride (NCA) and peptide chain elongation by the so called "back up" procedure. Thus, Lac was allowed to react with Nps–Glu(OEt)–NCA to give a depsipeptide free acid Nps–Glu(OEt)–Lac–OH, which was esterified with pentachlorophenol (HOPcp) and dicyclohexylcarbodiimide (DCC) to give an active ester Nps–Glu(OEt)–Lac–OPcp. The Nps-protecting group of the active ester was removed by hydrochloric acid (HCl) to give a depsipeptide active ester hydrochloride HCl·H–Glu(OEt)–Lac–OPcp, which was treated with Nps–Leu in the presence of isobutyl chlorocarbonate (IBC) to yield a tridepsipeptide active ester Nps–Leu–Glu (OEt)–Lac–OPcp. The tridepsipeptide was further allowed to react with Nps–Ala by the back up procedure to give a *N*-protected monomer Nps–Ala–Leu–Glu(OEt)–Lac–OPcp. The monomer obtained was highly purified by repeated recrstallization from tetrahydrofuran (THF), *e.g.*, mp = 190—192°C (decomposition), $Rf = 0.56$ (ethyl acetate–benzene, 1 : 1), $[\alpha]_D = -62.0°$ ($c = 1$, THF), $C_{31}H_{35}N_4O_{10}SCl_5$ (calculated value C = 44.7%, H = 4.2%, N = 6.7%; found value C 44.6%, H = 4.4%, N = 6.7%). The Nps-groups of Nps–Ala–Leu–Glu(OEt)–Lac–OPcp were removed by HCl and the resulting active ester hydrochloride was dissolved at a high concentration in dimethylsulfoxide (DMSO). The polymerization was started by the addition of triethylamine (TEA) into the mono-

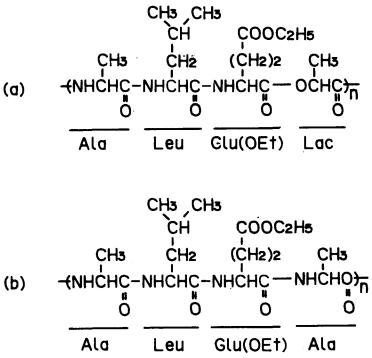


**Figure 1.** Structural formulas of sequential polymers. Kind of polymer: (a) poly(Ala–Leu–Glu(OEt)–Lac); (b) poly(Ala–Leu–Glu(OEt)–Ala).

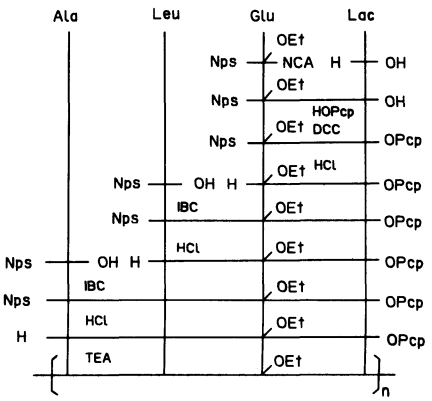


**Figure 2.** Synthetic scheme of a sequential polydepsipeptide poly(Ala–Leu–Glu(OEt)–Lac).

mer solution with vigorous stirring. The reaction was allowed to continue for 2 days at 30°C. The polymer was isolated by diluting the polymerization system with methanol and reprecipitated from dichloroacetic acid–diethyl ether. The sequential polydepsipeptide thus obtained had a specific viscosity $\eta_{sp}/c = 0.14\,\mathrm{dl\,g^{-1}}$ ($c = 0.5\,\mathrm{g\,dl^{-1}}$, 37°C) in dichloroacetic acid.

A sequential polypeptide poly(Ala–Leu–Glu(OEt)–Ala), which has the structural formula given in Figure 1b, was synthesized by polycondensation of a tetrapeptide *N*-hydroxysuccinimide (HONSu) active ester, Nps–Leu–Glu(OEt)–Ala–Ala–)–ONSu, as a monomer.[5,6] This monomer was recrystallized from THF, *e.g.*, mp = 178—180°C (decom-

position), $Rf=0.68$ (THF–benzene–methanol, 4:4:1), $[\alpha]_D=-91.9°$ ($c=0.5$, THF), $C_{29}H_{40}N_6O_{11}S$ (calculated value C=51.2%, H=5.9%, N=12.4%; found value C=51.3%, H=6.0%, N=12.2%). After the Nps-group of Nps–Leu–Glu(OEt)–Ala–Ala–ONSu was removed by HCl, the polymerization was carried out in DMSO at 30°C for 2 days using TEA as an initiator. The specific viscosity of a sequential polypeptide poly(Ala–Leu–Glu(OEt)–Ala) determined in dichloroacetic acid at 37°C was found to be $\eta_{sp}/c=0.25\,\mathrm{dl\,g^{-1}}$ ($c=0.5\,\mathrm{g\,dl^{-1}}$).

These polymers were previously treated under a pressure of $200\,\mathrm{kg\,cm^{-2}}$ for 3 minutes at 75—100°C, called a melt-pressing procedure,[7] to obtain the needle-type polymer matrix with high density and rigidity. The final size of the melt-pressed polymer was 1.5 mm in diameter and 4 mm long.

The *in vitro* degradation of sequential polymers was evaluated by the action of enzymes, for example, lipase from Porcine pancreas (pH 7.0) as an esterase, papain from Papaya latex (pH 6.2) as an endopeptidase, and carboxypeptidase A from Bovine pancreas (pH 7.5) as an exopeptidase (purchased from Sigma Chemical Company). Thus, the melt-pressed polymer was first immersed into a flask filled with 30 ml of buffer solutions containing 0.1% (w/v) enzymes. Then, the flask was shaken at a rate of 30 times a minute at 37°C (5 needles/flask, 5 flasks/group). The enzyme solution was exchanged with fresh medium at 24-hour intervals. The polymers enzymatically degraded were collected, lyophilized, and weighed. The degree of *in vitro* degradation (%), $100\,(D_a-D_b)/D_a$, was estimated from the ratio of weight of polymer degraded by enzyme for required period ($D_b$) and weight of polymer before enzymatic degradation ($D_a$). For comparison, the *in vivo* degradation was checked. In this case, the melt-pressed polymer was implanted subcutaneously in the back of male adult Wistar strain rats ($425\pm25$ g), *e.g.*, 5 needles/rat and 5 rats/group. As 5th day, 10th day, 20th day, 30th day, 40th day, and 50th day from start of the implantation, the animals were sacrified. The implants were excised from the sacrificed animals, lyophilized after removal of surrounding tissue, and weighed. The degree of *in vivo* degradation was estimated from the decrease in weight of polymer in analogy with *in vitro*.

## RESULTS AND DISCUSSION

The degradation mechanism of a sequential polydepsipeptide poly(Ala–Leu–Glu(OEt)–Lac) by the action of enzymes may proceed by competition between protease and esterase, in contrast to only protease for sequential polypeptide poly(Ala–Leu–Glu(OEt)–Ala). Further evidence for competing enzymatic action was sought by using three kinds of enzymes, namely lipase, papain, and carboxypeptidase A. Lipase acts as an enzyme to cut the ester bond. Papain and carboxypeptidase A are classified into proteases, which act as enzymes to cut peptide bonds. However, such an action of cutting is distinct for each. That is, papain acts as an endopeptidase-type enzyme which randomly cuts any peptide bonds existed in the main chain, while carboxypeptidase A as an exopeptidase-type enzyme which regularly acts to cut all peptide bonds from the terminal amino groups or the terminal carboxyl groups. The degree of degradation of sequential polymers by the above enzymes is shown in Fugure 3. The sequential polydepsipeptide is subject to degradation by the action of lipase and the resulting degree of *in vitro* degradation went up 100% at 6th day from the start of the test. The same tendency was observed when papain was used, though the period required for 100% degradation was found to be 7 days. This means that a sequential polydepsipeptide poly(Ala–Leu–Glu(OEt)–Lac) is completely degraded by both esterase-type and endopeptidase-type enzymes and these enzymes have the same degrading ability. In contrast to this result, the degree of degrada-

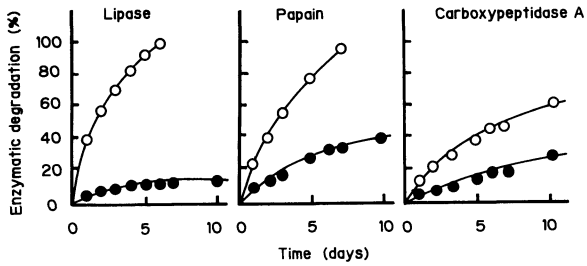


**Figure 3.** *In vitro* degradation of sequential polymers. Kind of polymer: (○) poly(Ala–Leu–Glu(OEt)–Lac); (●) poly(Ala–Leu–Glu(OEt)–Ala).

tion by the action of carboxypeptidase A was only 46% even after 7 days test. It is therefore said that the action of exopeptidase-type enzyme is not so strong for the poly-depsipeptide. In a sequential polypeptide poly(Ala–Leu–Glu(OET)–Ala) which can degrade only by the action of proteases, the degree of *in vitro* degradation was found to be 33% for papain and 17% for carboxypeptidase A at 7th day from start of the test, respectively. This polymer was scarcely degraded by lipase. These data clearly show that an endopeptidase-type enzyme papain has the strongest ability of degradation for the polypeptide. On the other hand, when the sequential polymers used in this study were treated by immersion in buffer solutions without enzymes for 10 days at 37°C, the decrease in weight was within 4% in any system. We wish to propose from this result that the *in vitro* degradation mechanism of sequential polymers mainly proceeds by the action of enzymes.

For comparison, the *in vivo* degradation was attempted by implanting the polymers subcutaneously in the back of rats and is shown in Figure 4. The *in vivo* degradation of sequential polymers showed an increase with the passage of implantation time, but it markedly differed according to the polymer. The degree of *in vivo* degradation of the polydepsipeptide, implanted over a period of 50 weeks, reached 100%, in contrast to 21% for the polypeptide. The action of an enzyme on *in vivo* degradation is not clear at present. However, such a tendency of *in vivo* degradation between two sequential

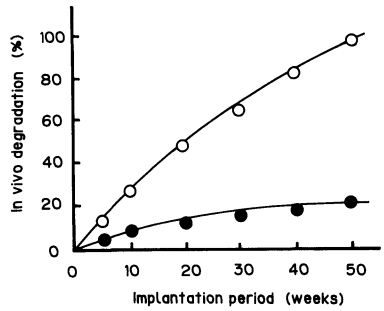


**Figure 4.** *In vivo* degradation of sequential polymers. Kind of polymer: (○) poly(Ala–Leu–Glu(OEt)–Lac); (●) poly(Ala–Leu–Glu(OEt)–Ala).

polymers well corresponds with that of *in vitro* degradation. Therefore, it is reasonable to conclude here that *in vitro* and *in vivo* degradations of sequential polypeptides are controlled by the introduction of α-hydroxy acids, for example L-lactic acid, in other words, by the introduction of ester-bonds in sequential polypeptide chains to convert the polypeptide into sequential polydepsipeptides.

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