# *In vitro* enzymatic biodegradation of amino acid based poly(ester amide)s biomaterials

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A systematic *in vitro* biodegradation study of regular poly(ester amide)s (PEAs) composed of naturally occurring hydrophobic α-amino acids, fatty diols and dicarboxylic acids was carried out in the presence of hydrolases like trypsin, α-chymotrypsin, and lipase. An automatic potentiometric titration method was used to examine the biodegradation property of the PEAs. Spontaneous immobilization (absorption) of the enzymes onto the PEAs films surfaces was observed. The surface immobilized enzyme not only accelerated the erosion of the PEAs but also was able to catalyze the hydrolysis of both low-molecular-weight (ATEE) and high-molecular-weight (protein) external substrates. It was found that the enzyme surface absorption process is reversible by nature. A kinetic method for a quantitative determination of the enzyme desorbed from the film surface was developed. The enzymes could also be impregnated into the PEAs to make them ''self-destructive'' at a target rate. A comparison of the PEAs' *in vitro* biodegradation data with polylactide (PDLLA) showed that PEAs exhibited a far more tendency toward enzyme catalyzed biodegradation than PDLLA.

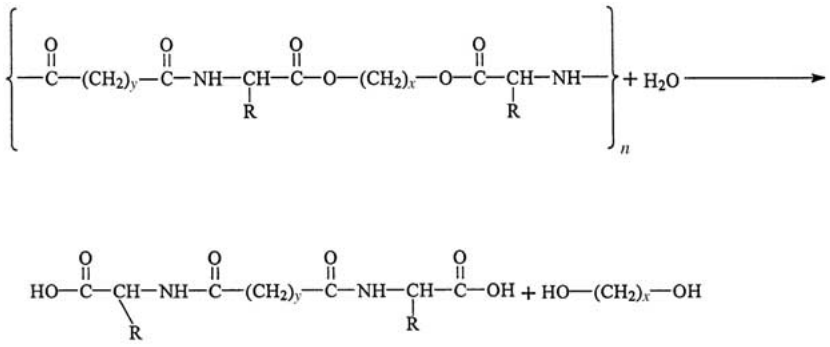

## Introduction

Recently, we have published the synthesis of new novel regular poly(ester amide)s (PEAs) entirely composed of naturally occurring and nontoxic building blocks like hydrophobic α-amino acids, fatty diacids and diols [1]. Those PEAs are one of the representatives of AA-BB type amino acid based bioanalogous heterochain polymers containing various types of linkages in the backbones and prepared mainly by solution polycondensation under mild conditions without the usage of any toxic catalyst [2–5]. The synthetic possibilities of this approach are virtually unlimited. This diversity in synthesis routes permits us to design polymers having a wide range of material properties at a reduced cost. In addition, a wide range of material properties could be achieved by varying three components in the building blocks of the macromolecular backbone during synthesis: α-amino acid, diol, and dicarboxylic acid. This additional advantage would make this class of heterochain biodegradable PEA polymers the prominent candidates for our pursuing of better biomaterials.

In our previously reported study [1], new active polycondensation reaction that proceeded smoothly under mild conditions in common organic solvents has been used to synthesize high molecular weight ($M_w$ up to 167 000) PEAs having good film forming properties. The PEAs obtained appear to be promising as a new class of biodegradable biomaterials for various biomedical applications. Because of the biodegradation nature of these PEAs, a preliminary *in vitro* biodegradation (hydrolysis) of the PEAs films catalyzed by α-chymotrypsin was also carried out using a potentiometric titration method for the purpose of assessing the effect of the building blocks of these new polymers on their biodegradation properties. The findings of that study were complicated by the observed film contraction of some PEAs.

Potentiometric titration is a facile and fast method to assess the tendency of polymers to hydrolytic degradation, especially for the polymers which contain labile ester linkages that provide rather high rates of chain scission of the polymeric backbones with the release of sufficient amounts of –COOH groups after hydrolysis as shown in Scheme 1. The –COOH end groups of the backbone fragments could be neutralized automatically by an alkaline solution at predetermined pH (end point). Thus, the consumption of the alkaline solution represents the kinetic curve of biodegradation. Potentiometric titration permits us to observe some details of enzyme-catalyzed hydrolysis of polymers, e.g. some lag-period (presumably due to the enzyme absorption onto the film surface) was observed for α-chymotrypsin catalyzed hydrolysis of 4-L-Phe-4 [4].

Potentiometric titration has an advantage over a



*Scheme 1* Hydrolysis of poly(ester amide)s.

gravimetric method in ranking the biodegradability of various polymers. This is because gravimetric measurements at an early initial stage of biodegradation (the first 1–3 h, the time normally used for short-term assessment) are complicated by water absorption, particularly for those polymers having high water affinity [6].

In the present paper, we adopted a modified experimental procedures of α-chymotrypsin catalyzed *in vitro* hydrolysis of PEAs to eliminate the undesirable complication of film contraction during biodegradation study and expanded the scope of the *in vitro* biodegradation study by (a) incorporating hydrolase enzymes other than α-chymotrypsin, (b) investigating enzymes' spontaneous surface-immobilization processes in more details, and (c) enzymes impregnation in the PEAs films for the purpose of developing self-biodegradable biocomposites.

## Materials and methods

### Polymers

The PEAs (III) were prepared by solution polycondensation of di-*p*-toluenesulfonic acid salts of bis(α-amino acid), α, ω-alkylene diesters (I) with active diesters of dicarboxylic acids (II) as described in our previous reported study [1], according to the Scheme 2. The same designations used in our previous publication [1] were used here to label the regular PEAs synthesized, *y*-(*)-AA-*x*, where *y* is the number of methylene groups in dicarboxylic acid residue; *x* is the number of methylene groups in diol residue; (*) is configuration of α-amino acid; AA is the α-amino acids, Phe for phenylalanine (R = $CH_2Ph$) and Leu for leucine (R = $CH_2CH(CH_3)_2$).

For example, PEA 4-L-Phe-6 represents a regular poly(ester amide) of the structure above based on L-phenylalanine amino acid, adipic acid $(y = 4)$ and hexanediol-1,6 $(x = 6)$.

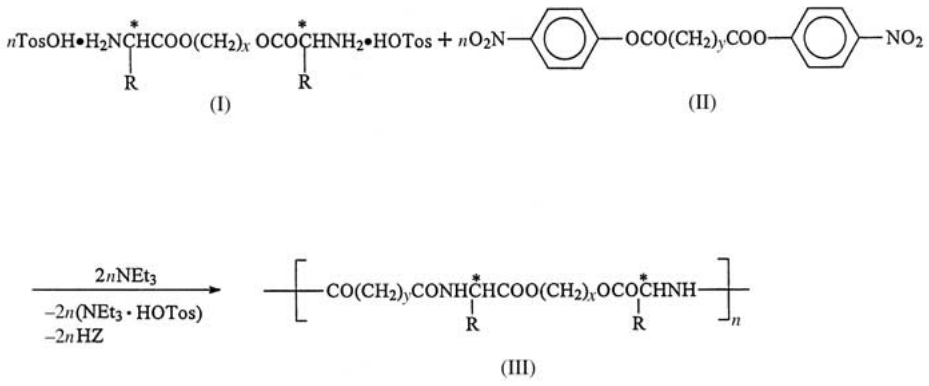
PEA films were cast from chloroform solution (at a concentration 100 mg of PEA in 1 ml of chloroform) onto the smooth glass Petri-dishes hydrophobized by dichlorodimethylsilane (from Aldrich) and the solvent was allowed to evaporate completely at room temperature. The films were additionally dried at 65 °C up to constant weight before use. These dried PEA films were cut into spherical disc form of diameter 4 cm and 400–500 mg each. Poly(D,L) lactide obtained from Boehringer Ingelheim were cast into films the same way as PEA films and were used as the control for comparing biodegradation property.

### Enzymes, their immobilization, impregnation and measurements

The enzymes used in the present study included α-chymotrypsin (from Fluka), trypsin (from Sigma) and lipase (from Wako Pure Chemicals). Our activity measurements of α-chymotrypsin and lipase showed 400–420 ATEE (acetyl tyrosine ethyl ester, from Sigma) units per mg of α-chymotrypsin and 56–60 triacetin units per mg of lipase, while trypsin had activity 1000–2000 BAEE units per mg solid.

In the study of the enzymatic effect on PEA biodegradation *in vitro*, three different means to introduce enzymes into the system were used, and they were: enzymes in aqueous media of 0.1 N NaCl solution, enzymes immobilized spontaneously (absorbed) onto PEA film surface, and enzymes impregnated within PEA films.

The enzyme media were prepared by dissolving 4 mg



*Scheme 2* Synthesis of poly(ester amide)s.

of an enzyme (α-chymotrypsin, lipase, or trypsin) in 10 ml of 0.1 N NaCl.

The enzyme surface immobilization onto the polymeric films was carried out according to the procedures described in our previous studies [1, 4] in that enzymes would spontaneously immobilize onto polymer films during *in vitro* enzyme catalyzed hydrolysis. In the present work, the enzyme immobilization was carried out under standardized conditions – by immersing PEA film discs in the solution of 4 mg of enzyme in 10 ml of 0.1 N NaCl solutions at pH 7.4 and 37 °C for 70 min; the films were then removed from the enzyme solution and washed for 4–5 times by distilled water.

For impregnating an enzyme into the polymeric films, a fixed amount of fine enzyme powders was added to a fixed volume of a chloroform solution of the polymer, the suspension was thoroughly homogenized and cast as described in the Polymers section above. Then, the same film discs of 4 cm diameter and 400–500 mg each were punched out. Based on the procedures, each PEA film discs contained ca. 4 mg of the enzyme.

### *In vitro* biodegradation study

The enzyme catalyzed *in vitro* hydrolysis experiments were carried out according to the procedures described in our previous studies [1, 4]. The ''non-contractive'' PEAs film discs were used without backing, but the PEA films of the same diameter that showed contraction during biodegradation (e.g. Leucine containing PEAs) were placed onto Teflon plates of the same diameter using silicon high vacuum grease (from Baxter Healthcare Corporation) to prohibit the film from contraction during biodegradation study in 0.1 N NaCl solution. To make sure that only the outer surface of the films are subjected to the biodegradation (in other words no enzyme solution penetrates under the films), a powdery dye (methylene blue) was placed under the films. No staining of the enzyme solution by the dye was observed, an indication no enzyme solution penetrated into the back surface of the films.

The film discs were put into a glass vessel containing a solution of 4 mg of an enzyme (α-chymotrypsin, lipase, or trypsin) in 10 ml of 0.1 N NaCl solution under constant stirring. Titration was carried out by a Radiometer RTS-822 automatic titrator at 37 °C and physiological pH 7.4 using 0.1 N NaOH solution as a titrant. In this method, the propensity of PEAs towards biodegradation was estimated by the amount of NaOH (in μmol) consumed per 70 min (one cycle of Radiometer RTS-822 titrator) to neutralize the acidic carboxylic groups released from the hydrolysis of the ester linkages in PEAs. These same procedures were used to study the catalytic action of either surface immobilized or impregnated enzymes on PEA film discs.

### *Desorption of the surface immobilized enzyme*

For this type of study, the PEA (4-L-Phe-4) with surface immobilized α-chymotrypsin was used. The film was placed into the Radiometer RTS-822 titrator cell and the hydrolysis was recorded for 70 min the same way as described above. Then, the glass vessel was removed from the measuring cell of the titrator and the film inside the glass vessel (vessel # 1) was removed. The solution in the glass vessel #1 was used to test the presence of enzyme that was desorbed from the enzyme surface immobilized onto the PEA discs. In order to determine whether any desorbed enzyme would be surface immobilized onto the electrodes of the titrator, the electrodes of the measuring cell were thoroughly washed with distilled water and 10 ml of a fresh 0.1 N NaCl solution in a new glass vessel (vessel # 2) was adjusted to pH 8.0 (the optimal pH for α-chymotrypsin sensitivity). A α-chymotrypsin specific substrate, ATEE, was added to the solution in the glass vessel #2 to check the absence (or presence) of the α-chymotrypsin absorbed onto the electrodes. Any absence of ATEE cleavage would indicate the lack of α-chymotrypsin absorption onto electrodes of the titrator. After confirming the lack of α-chymotrypsin absorption onto electrodes, the solution in the glass vessel #1 that contained the enzyme desorbed from the PEA disc film was pH adjusted to 8.0, followed by the addition of 0.2 ml of ATEE solution in acetonitrile (stock solution: 1 g in 10 ml). The hydrolysis kinetics of ATEE by the desorbed α-chymotrypsin were then recorded under the same conditions as above.

### *Calibration curve of enzyme concentration vs. enzyme catalyzed ATEE hydrolysis rate* $v_0$

A stock solution of α-chymotrypsin (1 mg of enzyme in 1 ml of 0.1 N NaCl) was diluted by 0.1 N NaCl solution to a predetermined concentration, and 10 ml of this solution was added to the titrator vessel. The vessel was placed within the titrator cell and the temperature was allowed to reach 37 °C before 0.2 ml of ATEE solution in acetonitrile (stock solution: 1 g in 10 ml) was added to the vessel. The hydrolysis kinetics of ATEE were recorded the same way described previously, except that optimum pH 8.0 for α-chymotrypsin was used in these calibration experiments. Three parallel experiments (i.e. using three stock-solutions of the enzyme) were done for each experimental point. In this experiment, no experimental error greater than 15% was observed, which we considered rather well for enzymatic processes.

## Results and discussion

### Enzymes in solution

In our previous publication [1] a preliminary *in vitro* biodegradation (hydrolysis) of the PEAs films catalyzed by α-chymotrypsin was carried out for the purpose of assessing the effect of the building blocks of these new polymers on their biodegradation properties. However, in some cases, especially those polymers based on α-amino acids with fatty lateral substituents (e.g. leucine based PEAs 4-L-Leu-4, 4-L-Leu-6, etc., having $–CH_2CH(CH_3)_2$ group as a lateral substituent) exhibited contraction of the films during biodegradation experiment. In the present paper, we modified the previous experimental procedures for the purpose of eliminating the unknown effect of film contraction during biodegradation.

TABLE I Biodegradation (α-chymotrypsinolysis) of ''non-contractive'' and ''contractive'' poly(ester-amide)s by potentiometric Titration

| PEA | NaOH consumption in μmol for 70 min* | |
|---|---|---|
| | Films free of backing** | Films adhered to the backing |
| 4-L-Phe-4 | 8.0 ± 0.4 | 7.2 ± 0.5 |
| 4-L-Phe-6 | 7.0 ± 0.3 | 7.4 ± 1.6 |
| 4-L-Leu-4 | 5.2 ± 2.8 | 7.1 ± 0.2 |
| 4-L-Leu-6 | 1.8 ± 0.7 | 3.9 ± 0.4 |

*The results of three parallel runs. Enzyme concentration in solution (4 mg/10 ml). **The initial values of NaOH consumptions were divided by factor 2 for reducing to the one surface for comparing with the films having backing, i.e. one active surface.

As shown in Table I, it appears that an increase in NaOH consumption was observed for the contractive polymers (i.e. Leucine-based PEAs) when their contraction was inhibited by adhesion of the films onto Teflon backing. The standard deviation for the contractive films free of backing was the highest because of their inconsistent surface area due to contraction. The inhibition of the film contraction, however, had very little effect on those film samples that are inherently non-contractive (Phe-based PEAs) as evident in the NaOH consumption data in Table I. Therefore, the results obtained suggest that the films' contraction can indeed lead to a lower biodegradation rate.

Biodegradation data of the PEA 4-L-Phe-4 sample for three hydrolases (α-chymotrypsin, trypsin and lipase), are given in Fig. 1. Since it was virtually impossible to use the enzymes with equal catalytic activity, we adopted an equal enzyme concentration (4 mg in 10 ml) for all three enzymes (with inherent activity given in Materials and methods above) and hydrolysis experiments carried out under the same conditions described previously. The data in Fig. 1 show that, in both enzyme solution (lines 1–3) and surface immobilized enzyme (lines 1′–3′) cases, lipase (lines 1 and 1′) had the highest activity toward PEA 4-L-Phe-4 followed by α-chymotrypsin (lines 2 and 2′) and non-specific trypsin (lines 3 and 3′). The latter two proteases had rather similar enzymatic activity (in enzyme solution case), even though trypsin is not a specific enzyme for the substrate used.

When comparing with those PEAs, poly(DL-lactic acid) (PDLLA), one of the most popular biodegradable polyesters, showed significantly lower tendency toward both pure and enzyme-catalyzed hydrolyses than PEAs (lines 4–6). Lipase did not show any enzymatic hydrolysis toward PDLLA, although α-chymotrypsin exhibited a slight enzymatic catalyzed hydrolysis than pure buffer. The observed relatively higher α-chymotrypsin reactivity toward PDLLA is consistent with Chu *et al*. and Williams studies [7, 8]. In their separated studies, both Chu *et al*. and Williams *et al*. demonstrated that α-chymotrypsin slightly accelerated the hydrolytic degradation of linear polyesters like polyglycolide.

## Surface immobilized enzymes

We found previously [1, 4] that strong spontaneous α-chymotrypsin immobilization (surface adsorption from aqueous enzyme solutions) onto the PEA film surface took place, and this immobilized enzyme caused PEAs to biodegrade at a relatively higher rate. In this study, we examined the same possibility of surface immobilization for lipase and trypsin on PEA, and found that these enzymes were indeed immobilized onto the PEA film surface spontaneously (Fig. 1, lines 1′ and 3′). It appears that the surface immobilized lipase and α-chymotrypsin (lines 1′ & 2′) had nearly similar high activity toward the hydrophobic surface of 4-L-Phe-4, while in enzyme in solution case, lipase (Fig. 1, line 1) showed higher hydrolytic activity toward 4-L-Phe-4 than α-chymotrypsin (Fig. 1, line 2). The surface immobilized non-specific enzyme (trypsin) had the lowest activity among the 3 enzymes immobilized (Fig. 1, line 3′). Therefore, the data in Fig. 1 showed that all the three enzymes used could be immobilized spontaneously onto the PEAs' surface.

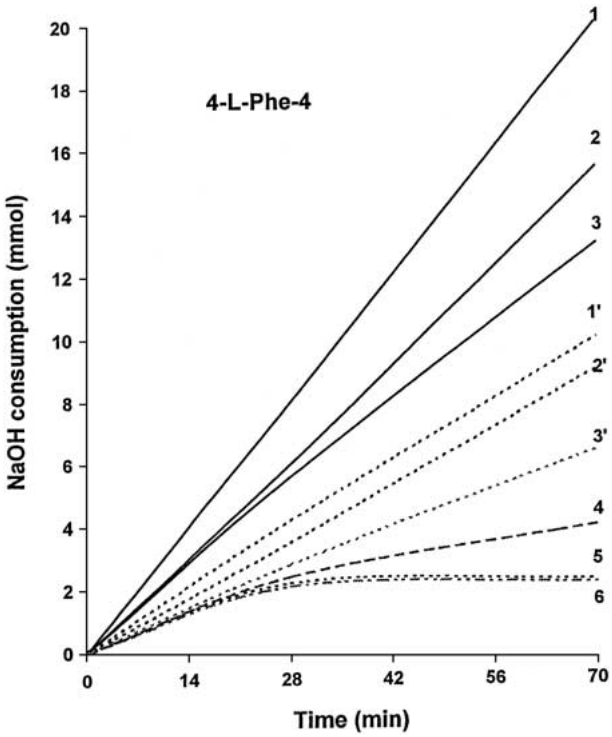



*Figure 1* Hydrolysis kinetics (from potentiometric titration data) of the PEA 4-L-Phe-4 films catalyzed by various enzymes: lipase (1 and 1′), α-chymotrypsin (2 and 2′), and trypsin (3 and 3′). Curves 1–3, enzymes in solution; Curves 1′–3′, surface immobilized enzymes; Curves 4–6 are for PDLLA catalyzed by α-chymotrypsin (4) and lipase (5), and in pure phosphate buffer (6).

This capability of surface immobilization of enzymes onto biomaterials may have some practical applications. In our previous publication [1], we showed that the PEAs composed of α-amino acids with fatty lateral substituents (e.g. L-Leu) had poor or none enzyme surface immobilization capability, whereas those PEAs having the high hydrophobic benzyl side groups (e.g. L-Phenylalanine-based like 4-L-Phe-4) showed considerable enzyme surface immobilization. This suggests that the possibility of the incorporation of L-Phenylalanine into those PEAs having poor or no enzyme surface immobilization capability via physical blend or copolymerization for imparting enzyme spontaneous immobilization capacity onto these polymers. Table II demonstrates such a possibility and shows the hydrolysis

TABLE II The effect of poly(ester-amide) blends on imparting surface immobilization of α-chymotrypsin and α-chymotrypsinlysis of the PEA blends*

| PEA or blend of PEAs (w/w) | NaOH consumption (7μmol) for 70 min | |
|---|---|---|
| | Enzyme in solution (4 mg/10 ml) | Surface immobilized enzyme |
| 4-L-Phe-4 | 16 ± 0.8 | 9.6 |
| 4-L-Phe-4/4-L-leu-4 (75/25) | 16.0 | 5.3 |
| 4-L-Phe-4/4-L-leu-4 (50/50) | 14.7 | 4.4 |
| 4-L-leu-4 | 10.4 ± | 0.7 |

*Phenylalanine and leucine-based PEAs. Potentiometric titration data and same enzyme-catalyzed biodegradation condition as Table I.

data for the polymeric blends 4-L-Phe-4/4-L-Leu-4 at 75/25 and 50/50 (w/w) composition ratio with surface immobilized α-chymotrypsin. The hydrolysis rate of these blends under the surface immobilized enzyme condition were significantly higher (about 6–7 times) than that of the 100% 4-L-Leu-4, and the higher the 4-L-Phe-4 content in the blend was, the higher biodegradation rate was observed. However, these Phe and Leu blended PEAs still had lower hydrolysis rate than the 100% Phe-based PEA in the surface immobilized enzyme case.

This surface immobilized enzyme is able to catalyze the hydrolysis of both external low-molecular-weight substrate like ATEE [1, 4] and high-molecular-weight substrates like proteins (muscular protein of rabbit α-actinin was chosen as a substrate for this experiments). The gel electrophoresis pattern of α-actinin with time in the presence of surface-immobilized α-chymotrypsin shows that the quantity of both fragments at 55 and 30 Kda gradually decreased with time due to the enzyme catalyzed hydrolysis. It is known [9] that α-actinin consists of two subunits and each subunit contains two domains: 70 and 30 Kda which appear at the first stages of the α-chymotrypsin catalyzed hydrolysis. Afterward the surface-immobilized α-chymotrypsin hydrolysis, the domain of 70 Kda quickly hydrolyzes to the fragments of 55 Kda.

The hydrolysis of external substrates could be catalyzed by the enzyme either fixed onto the film surface or desorbed from the surface owing to the equilibrium nature of the enzyme absorption process. However, the surface immobilized enzyme may have difficulty to catalyze the hydrolysis of high-molecular-weight external substrates like α-actinin due to steric hindrance.

Before elucidating whether the process of an enzyme immobilization on PEA substrates is reversible or not, first we need to determine the desorbed enzyme, if any, would hydrolyze an external substrate like ATEE. The NaOH consumption data from the α-chymotrypsin-catalyzed hydrolysis of the ATEE in the medium that was originally used to immobilize α-chymotrypsin onto PEA 4-L-Phe-4 film sample suggested that the enzyme immobilization process under the specified experimental condition was of an equilibrium nature, i.e. spontaneous enzyme adsorption (surface immobilization) onto and desorption from the PEAs films.

The desorbed enzyme concentration (active form at least) could be determined from calibration curve [E] vs. $v_0$, where [E] is the α-chymotrypsin concentration and $v_0$ is an initial rate of the α-chymotrypsin-catalyzed ATEE hydrolysis at the ATEE concentration [S] much higher than [E] ([S] ⩾ [E]). Fig. 2 shows such a calibration curve of α-chymotrypsin. The linear relationship ($R^2 = 0.994$) between [E] and $v_0$ is consistent with the Mikhaelis-Menten kinetics which expect $v_0$ to be linearly dependent on enzyme concentration ([E]) in the solution at [S] ⩾ [E] [10].

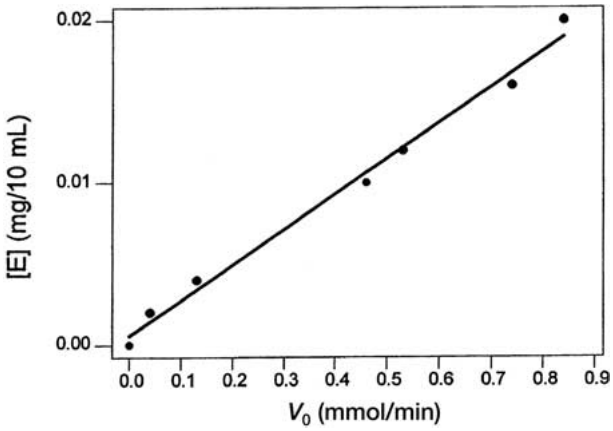



*Figure 2* Calibration curve [E] vs. $V_0$. [E], α-chymotrypsin concentration in 10 ml of 0.1 N NaCl solution; $V_0$, initial rate of ATEE hydrolysis at various [E].

Based on this linear calibration curve (Fig. 2), the amounts of the α-chymotrypsin enzyme (active form) desorbed from the PEA 4-L-Phe-4 film surface for the first 70 min were 0.018 ± 0.002 mg that represented 0.45% of the total amount of enzyme in the starting solution at time 0 (4 mg in 10 ml). A more complete and systematic study of enzyme desorption using kinetic study is in progress.

## Impregnated enzymes

It is known from literature [11–13] that lipase is rather stable in the media of lypophilic solvents. Such stability provided us with a reasonable means to impregnate the enzyme into the PEAs for the purpose of designing so called ''self-destructive'' polymers. In this study, various amounts of lipase and α-chymotrypsin – from 0.5 mg to 10 mg per 1 g of the PEA were impregnated into the 4-L-Phe-4 films. As shown in Fig. 3, the lipase-impregnated PEAs were self-biodegraded and their biodegradation rates depended on the initial enzyme loading. The same 4-L-Phe-4 sample in buffer without lipase, however, showed no NaOH consumption under the same experimental condition.

The dependence of the biodegradation rate on the concentration of the impregnated enzyme was found to

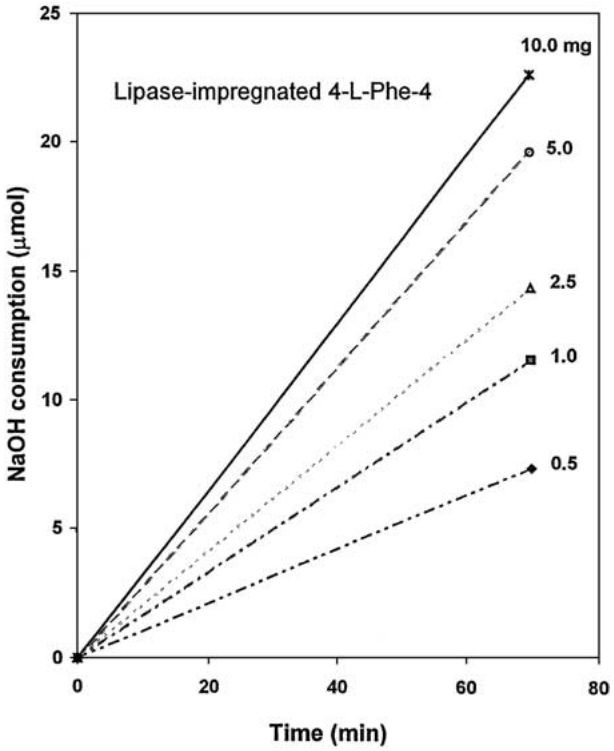



*Figure 3* Kinetics of the hydrolysis of PEA 4-L-Phe-6 films impregnated by various amounts of lipase. No NaOH consumption was detected within the time frame in buffer control without lipase.

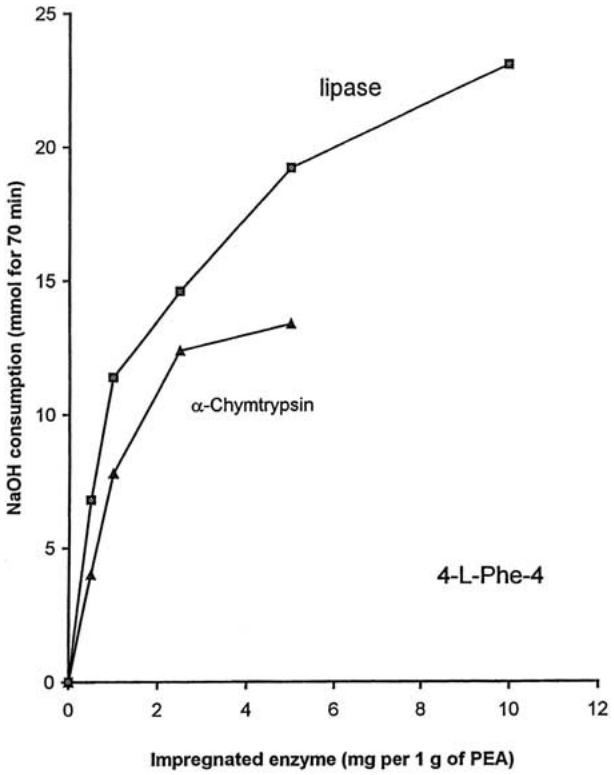



*Figure 4* The amounts of NaOH consumption for 70 min as a function of enzyme loading concentration in the PEA 4-L-Phe-4 films.

become less pronounced at high concentrations of the loaded enzyme, particularly α-chymotrypsin as shown in Fig. 4. Like lipase, α-chymotrypsin was found to be rather stable in chloroform. The α-chymotrypsin-impregnated PEA 4-L-Phe-4 was found to be slightly less prone to the hydrolysis than lipase-containing PEAs (Fig. 4).

The potential biomedical applications of the α-chymotrypsin impregnated PEAs would be in those situations where the release of the proteolytic enzyme into surrounding tissues is desirable, whereas the lipase containing self-degradable PEAs could be promising in those drug control/release devices where intact bioactive peptides or proteins would be released.

## Conclusion

The PEAs synthesized and studied are entirely composed of nontoxic building blocks of naturally occurring essential α-amino acids, fatty diacids and diols. They show a wide range of material properties from strong film forming to hydrophilic elastomers[1]. Hydrolases of various classes, such as proteases (trypsin and α-chymotrypsin), and lipase showed high catalytic activity toward the hydrolysis of the PEAs synthesized; these enzymes remained active either in solutions, surface immobilized or impregnated into the PEAs.

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