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# Hydrolysis of polyesters by serine proteases

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**Abstract**

The substrate specificity of α-chymotrypsin and other serine proteases, trypsin, elastase, proteinase K and subtilisin, towards hydrolysis of various polyesters was examined using poly(L-lactide) (PLA), poly(β-hydroxybutyrate) (PHB), poly(ethylene succinate) (PES), poly(ethylene adipate) (PEA), poly(butylene succinate) (PBS), poly(butylene succinate-co-adipate) (PBS/A), poly[oligo(tetramethylene succinate)-*co*-(tetramethylane carbonate)] (PBS/C), and poly(ε-caprolactone) (PCL). α-Chymotrypsin could degrade PLA and PEA with a lower activity on PBS/A. Proteinase K and subtilisin degraded almost all substrates other than PHB. Trypsin and elastase had similar substrate specificities to α-chymotrypsin.

## Introduction

Various polymers comprised of lactic, glycolic, hydroxyl butyric or α-amino acids as building blocks are being widely used in biomedical applications, such as drug-releasing implants, bioabsorbable sutures, and surgical prostheses (Chandy & Sharma 1991). Aliphatic polyesters are considered to be biodegradable plastics owing to their susceptibilities to enzymes and microorganisms (Tokiwa *et al.* 1976, Tokiwa & Suzuki 1977a, Williams 1981, Pranamuda *et al.* 1997, 1999, Jarerat *et al.* 2002). To learn more about the characteristic degradation of polyesters and to design other novel biodegradable plastics, a study of the specificity of hydrolytic enzymes on these polymers is necessary.

α-Chymotrypsin, a serine protease, is confined almost entirely to animals when it is synthesized in the pancreas (Coan *et al.* 1971). As α-chymotrypsin is a mammalian enzyme, its ability to degrade biomedical materials made of polyesters in the human body is of obvious interest. The role of this enzyme in degrading polyesters is still unclear and the enzymatic degradation of polyesters by α-chymotrypsin has attracted little attention (Chandy & Sharma 1991).

In this study, α-chymotrypsin was purified and characterized with respect to its activity against poly(L-lactide) (PLA) and other polyesters, and compared with other serine proteases, trypsin, elastase, proteinase K and subtilisin.

## Materials and methods

### *Materials*

Poly(L-lactide) (PLA: Lacty 1012, $M_n = 1.9 \times 10^5$) was obtained from Shimadzu Co. Ltd., Japan. Other polymer samples used were: poly(β-hydroxybutyrate) (PHB: $M_n = 2.1 \times 10^5$) and poly [oligo(tetramethylene succinate)-*co*-(tetramethylane carbonate)] (PBS/C: $M_n = 1.9 \times 10^5$) with a carbonate content of 10.1 mol % from Mitsubishi Gas Chemical, poly(ethylene succinate) (PES, $M_n = 5.9 \times 10^4$) from Nippon Shokubai Co. Ltd., poly(butylene succinate) (PBS: Bionelle 1020,

$M_n = 4.8 \times 10^4$) and poly(butylene succinate-co-adipate) (PBS/A: Bionelle 3020, $M_n = 2.0 \times 10^4$) from Showa High Polymer Co., poly(ethylene adipate) (PEA: $M_n = 1 \times 10^3$) from DIC Co., and poly(ε-caprolactone) (PCL: Tone P-767, $M_n = 6.7 \times 10^4$) from Union Carbide Corporation. The cellulose paper used was No. 2 filter paper from Advantec Toyo.

Commercially available bovine α-chymotrypsins (see Table 1) were from Sigma Chemical Co. (USA), Merck kGaA (Germany), Wako Pure Chemical Industries Ltd. (Japan), Milan Panic Biochemicals Inc. (Germany), Nacalai Tesque Inc. (Japan), Worthington Biochemical Co. (USA) and Biogenesis (UK). Other serine proteases used were proteinase K (chromatographically purified, ICN Biomedicals Inc.), trypsin and subtilisin (Sigma), and elastase (Wako Pure Chemical Industries Ltd.). Each enzyme was dissolved in a 0.1 M phosphate buffer (pH 7.0), and used after appropriate dilution.

*Preparation of polyester-coated paper*

The polyester-coated paper used for investigating enzyme activity was prepared by penetration of polyester into cellulose paper. Pieces of cellulose paper No. 2 (Advantec Toyo; 10 × 10 mm, 12 mg, thickness ca. 240 μm) were immersed one at a time in a polyester solution (1 g polyester pellets dissolved in 100 ml chloroform) for 10 s, air-dried for 30 s, and dried at 30 °C in a vacuum oven for 1 d. Each piece of the cellulose paper was coated with approximately 0.6 mg polyester (thickness ca. 245 μm). The polyester coated papers obtained were thicker than the cellulose paper by ca. 5 μm. For enzymatic degradation, eight pieces of coated paper containing 4.8 mg polyester were used as substrates.

*Enzymatic activity of α-chymotrypsin*

Protease activity was measured by the following method: enzyme (0.5 ml) was added to 2.5 ml 2 % (w/v) bovine serum albumin (BSA) and incubated at 37 °C for 10 min. The reaction was terminated by adding 5 ml of 0.3 M trichloroacetic acid. After 20 min, the mixture solution was filtered through a 0.2 μm membrane. Two ml of filtrate was reacted with 5 ml $Na_2CO_3$ (60 g $l^{-1}$), and 1 ml phenol reagent (1 M) at 37 °C for 30 min. The liberated L-tyrosine was determined spectrophotometrically at 660 nm (Tsuchida *et al.* 1986). One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol equivalent of L-tyrosine $min^{-1}$. α-Chymotrypsin activity was measured by hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE, Sigma) as a substrate and was calculated as units per mg protein.

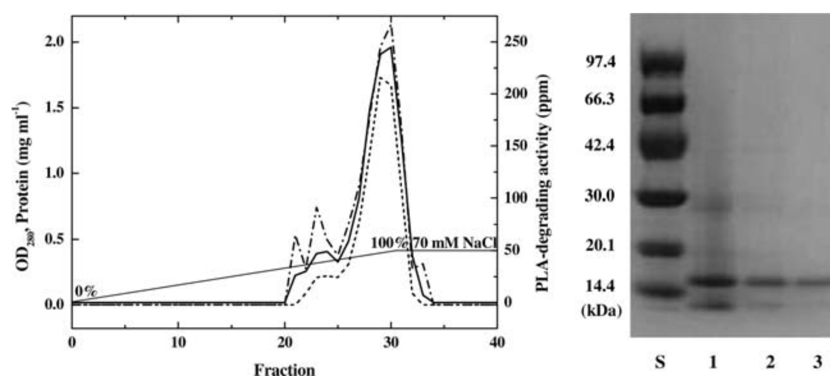


*Fig. 1.* Elution profiles, OD 280 nm (······), protein content (—·—) and PLA-degrading activity (—), from CM (carboxymethyl) Sepharose column chromatography, and SDS-polyacrylamide gel electrophoresis of the purified α-chymotrypsin. The commercial enzyme (Sigma Type II, 10 mg $ml^{-1}$) was applied to a CM Fast Flow HiPrep 16/60 column for anion exchange chromatography. Further purification was continued with size-exclusion chromatography using a Superdex 75 HiLoad 16/60 column. A single peak of protein was obtained of $M_w$ 22.5 kDa. The dialyzed enzyme (10 μg, lane 1), CM Flow Fast (10 μg, lane 2) and Superdex 75 purified enzyme (10 μg, lane 3) was applied on to 12.5 % SDS-PAGE after treatment with 2-mercaptoethanol (100 °C, 3 min) with a marker protein mixture (Amersham Pharmacia Biotech, UK, lane S). The purified α-chymotrypsin exhibited a single band with $M_w$ of about 15 kDa.

*Lipase activity of enzyme*

Lipase activity was determined by a modified procedure based on the method of Tokiwa & Suzuki (1977b). One unit (U) of enzyme activity was defined as the amount of enzyme necessary to produce 1 $\mu$mol free fatty acid $min^{-1}$.

*N-Terminal sequence analysis*

*N*-Terminal amino acid sequence analysis of purified α-chymotrypsin was performed in a Procise cLC Sequencer, Model 492 cLC (Applied Biosystems). Quantitative identification of individual amino acids was performed with the use of the amino acid analyzer after hydrolysis of phenylthiohydantoin (PTH)-amino acids.

*Enzymatic degradation of polyesters*

Polyester-coated papers were used as substrates for studying the polyester-degrading activity of enzymes. Enzymatic degradation was carried out in a 50 ml vial using 5 mg substrate suspended in 3 ml of 0.1 M phosphate buffer (pH 7.0) containing 1 ml of 0.05% (w/v) octyl-glucopyranoside and 1 ml enzyme solution (1 mg $ml^{-1}$). The enzyme solution was prepared in 0.1 M phosphate buffer (pH 7.0). In the substrate and the enzyme controls, the enzyme and the substrate were respectively omitted from the mixture. The reaction mixture was incubated on a rotary shaker (100 rpm) at 37 °C for 14 h. After incubation, the solution was filtered through a 0.2 $\mu$m filter membrane for the measurement of water-soluble total organic carbon (TOC) concentration with a TOC-5000A analyzer (Shimadzu Co. Ltd., Japan).

Enzyme activity on succinyl-(L-alanyl-L-alanyl-L-alanine)-*p*-nitroanilide (Suc-$(Ala)_3$-*p*NA) (Sigma), was followed by measuring the concentration of *p*-nitroaniline (*p*-NA) liberated. No significant increase in TOC was observed after incubation of the cellulose paper (without polyester coating) with all serine protease preparations.

*Table 1.* Activities of various commercial α-chymotrypsin preparations from bovine pancreas[a].

| Supplier* | Protease activity U[b] mg protein$^{-1}$ | α-Chymotrypsin activity U[c] mg protein$^{-1}$ | PLA-degrading activity U[d] mg protein$^{-1}$ |
|---|---|---|---|
| Sigma Type I | 20 | 40 | 130 |
| Sigma Type II | 43 | 51 | 210 |
| Merk | 43 | 55 | 220 |
| Wako | 43 | 51 | 250 |
| Milan Panic Biochemicals | 24 | 48 | 170 |
| Nacalai Tesque | 30 | 49 | 170 |
| Worthington Biochemical | 23 | 49 | 130 |
| Biogenesis | 23 | 48 | 150 |

*See materials and methods for addresses.
[a]The protein content of each α-chymotrypsin used for testing the enzyme activity was 1 mg $ml^{-1}$. Protein content of the enzymes was determined using the Lowry method with BSA as the standard.
[b]1 unit (U) = $\mu$mol of L-tyrosine $min^{-1}$.
[c]1 unit (U) = $\mu$mol of BTEE $min^{-1}$.
[d]1 unit (U) = 1 mg/l of water-soluble total organic carbon (TOC) released: the reaction mixture was composed of 4.8 mg (8 pieces of coated paper) as PLA in coated paper, 3 ml of 0.1 of M phosphate buffer (pH 7), 1 ml of 0.05% (w/v) octyl glucopyranoside, and 1 ml enzyme (1 mg $ml^{-1}$).

*Table 2.* Purification of α-chymotrypsin[a].

| Purification step | Volume (ml) | Total protein (mg) | Specific activity (u[b] $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Dialyzed enzyme | 20 | 161.3 | 0.1 | 100 | 1 |
| Carboxymethyl Fast Flow | 10 | 21.4 | 0.8 | 106 | 8 |
| Superdex 75 | 5 | 1.6 | 1 | 10 | 10 |

[a]Sigma Type II.
[b]One unit is defined as the amount of enzyme which formed 1 $\mu$mol L-lactic acid $min^{-1}$ divided by total protein.

*Table 3.* Hydrolysis of various polyesters by purified α-chymotrypsin (see Table 2) and other serine proteases.

| Substrate | Unit of specific activity | Mammalian protease | | | | Microbial protease | |
|---|---|---|---|---|---|---|---|
| | | Purified α-chymotrypsin[a] | Commercial α-chymotrypsin[b] | Trypsin[b] | Elastase[b] | Proteinase K[b] | Subtilisin[b] |
| BSA[c] | μmol L-tyrosine $min^{-1}$ $mg^{-1}$ protein | 60 | 30 | 30 | 20 | 40 | 10 |
| Suc-$(Ala)_3$-*p*NA | μmol *p*NA $min^{-1}$ $\mu g^{-1}$ protein | 0.02 | 0.01 | 0.12 | 40 | 2.77 | 2.85 |
| Olive oil | μmol fatty acid formed $min^{-1}$ $mg^{-1}$ enzyme | 0 | 0 | 0 | 0 | 1.7 | 0.6 |
| PLA | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 79 | 50 | 20 | 31 | 179 | 89 |
| PHB | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 0 | 0 | 0 | 0 | 2 | 0 |
| PES | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 0 | 0 | 0 | 0 | 23 | 0 |
| PEA | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 21 | 23 | 3 | 0 | 91 | 49 |
| PBS | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 0 | 0 | 0 | 0 | 11 | 6 |
| PBS/A | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 1 | 1 | 0 | 0 | 24 | 13 |
| PBS/C | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 0 | 0 | 0 | 0 | 15 | 7 |
| PCL | μg TOC[d] $h^{-1}$ $\mu g^{-1}$ protein | 0 | 0 | 0 | 0 | 55 | 12 |

[a] Purified α-chymotrypsin solution (see Table 2) with a protein concentration of 64 μg $ml^{-1}$.
[b] A 0.01% (w/v) enzyme solution of protein concentration 1 mg $ml^{-1}$. Commercial α-chymotrypsin is Sigma Type II.
[c] Bovine serum albumin.
[d] Water-soluble total organic carbon.

## Results and discussion

### *PLA degradation by various α-chymotrypsin preparations*

The commercial α-chymotrypsins were screened for their ability to degrade PLA by using coated cellulose paper (Table 1). All degraded PLA. The activities of protease (BSA as a substrate) and α-chymotrypsin (BTEE as a substrate) showed similar tendency to PLA-degrading activity. PLA-degrading ability of α-chymotrypsin is useful in the field of medical application because α-chymotrypsin is active in the human body.

### *Purification of α-chymotrypsin*

Of the eight commercial α-chymotrypsins, the most active three preparations were selected for purification (data not shown). Although these preparations had a commercially high purity and each company states that its product does not contain any other protease (as set out in its catalogue), there remains a possibility that these α-chymotrypsins contain other enzymes as impurities. Thus, the α-chymotrypsin (Sigma Type II) was further purified to homogeneity (Figure 1). Table 2 summarizes the results of the purification. The α-chymotrypsin was purified 10-fold with a specific activity of 1 U mg $protein^{-1}$ and a recovery of 10% (only one fraction of a main peak was collected).

### *N-Terminal sequence*

Mammalian chymotrypsins such as bovine chymotrypsin A have the amino terminal sequences A and B chain (Cohen *et al.* 1981, Asgeirsson & Bjarnason 1991). In this study, the N-terminal sequence of the first 10 amino acids in purified α-chymotrypsin was read in the B chain (Ile-Val-Asn-Gly-Glu-Glu-Ala-Val-Pro-Gly), whose sequence is presented above and four amino acids of A and C chains, Gly-Val-Pro-Ala and Ala-Asn-Thr-Pro, respectively.

### *Specificity of the purified α-chymotrypsin and other serine proteases*

The purified α-chymotrypsin showed the highest protease activity when BSA was employed as substrate (Table 3). Its degrading activity against

*Table 4.* Effect of various inhibitors and surfactant on PLA degradation by serine proteases.

| Inhibitor/Surfactant | Concentration | Residual PLA-degrading activity (%)[a] | | | | |
|---|---|---|---|---|---|---|
| | | α-Chymotrypsin[b] | Trypsin | Elastase | Proteinase K | Subtilisin |
| Control[c] (without inhibitor) | | 100 (165) | 100 (90) | 100 (70) | 100 (392) | 100 (250) |
| Aprotinin[d] | 1 (mg $l^{-1}$) | 16 (26) | 9 (8) | 21 (15) | 35 (137) | 26 (65) |
| | 10 (mg $l^{-1}$) | 15 (25) | 7 (6) | 14 (10) | 20 (78) | 24 (60) |
| PMSF[d] | 1 (mM) | 27 (45) | 9 (8) | 26 (18) | 39 (153) | 39 (98) |
| | 10 (mM) | 21 (35) | 9 (8) | 21 (15) | 34 (133) | 32 (80) |
| EDTA-$Na^{2+}$[d] | 1 (mM) | 22 (36) | 2 (2) | 23 (16) | 24 (94) | 23 (58) |
| | 10 (mM) | 19 (31) | 2 (2) | 19 (13) | 22 (86) | 18 (45) |
| Plysurf[e] | 100 (mg $l^{-1}$) | 22 (36) | 26 (23) | 27 (19) | 9 (35) | 0 (0) |

[a]Values in parentheses are the absolute degrading activities (TOC formation, ppm) of each enzyme.
[b]Sigma Type II.
[c]The degrading activity of the enzyme without inhibitor is defined as 100% relative activity.
[d]The inhibitors used were aprotinin (Sigma Chemical Co.), phenylmethylsulfonyl fluoride (PMSF) and EDTA–$Na^{2+}$ (Wako Pure Chemical Industries Ltd.).
[e]Surfactant, the chemical structure of Plysurf A210G (Dai-Ichi Kogyo Seiyaku Co., Ltd.) is $RO-(CH_2CH_2O)_n-P(=O)(OA)(OH)$ R, alkyl group; A, -H or $-(OCH_2CH_2)_n$-OR.

PLA was lower than that of proteinase K and similar to that of subtilisin but higher than that of commercial α-chymotrypsin. All serine proteases showed proteolytic activity against Suc-$(Ala)_3$-*p*NA. Low lipase activity was detected in microbial serine proteases, proteinase K and subtilisin. Purified α-chymotrypsin and commercial α-chymotrypsin degraded only PLA, PEA and a little PBS/A. In contrast, the purified α-chymotrypsin did not degrade PHB, PES, PBS, PBS/C and PCL. PHB could not be degraded by almost serine proteases. PEA was degraded by all serine proteases except for elastase. Although, PHB and other polyesters have the same ester bond but the stereochemical structure of PHB is selective for PHB-depolymerase (Jaeger *et al.* 1995, Takeda *et al.* 1998). PEA was cleaved by lipase, PHB-depolymerase and serine protease (Tokiwa & Suzuki 1977b). These results suggest that degradation of PES, PEA, PBS, PBS/A, PBS/C and PCL by microbial serine proteases is due to their lipase-like activity. Indeed, it has previously been reported that PEA, PBS/C and PCL are degraded by lipases (Tokiwa *et al.* 1976, Tokiwa & Suzuki 1977a, b).

### *Effect of various inhibitors on PLA degradation by serine proteases*

The effect of surfactant and various inhibitors on PLA-degrading activity of serine proteases is summarized in Table 4. Aprotinin, 10 mg $l^{-1}$, and 10 mM EDTA–Na were the most potent. α-Chymotrypsin was considerably less sensitive to inhibition by aprotinin. The specific inhibitor of serine protease, PMSF, also reacts with the serine in the active site, causing strong inhibition of enzyme activity. Although EDTA–Na is a specific inhibitor of metallo-protease, it inhibited the serine proteases. All serine preteases were also inhibited when the surfactant, Plysurf A210G, was added to the reaction mixture. Surprisingly, in case of microbial enzymes, Plysurf caused stronger inhibition than other inhibitors. Plysurf A210G at high concentrations ($>$100 mg $l^{-1}$) does inhibit both the PBSA-degrading and lipase activity but at a low concentration it has no effect (Tokiwa & Suzuki 1978, Uchida *et al.* 2000).

A point of considerable interest regarding the results of this study is that, although PLA, PHB, PBS and PCL fall in the same group of polyester-type plastics, purified α-chymotrypsin and commercial α-chymotrypsin preferentially degraded PLA (Table 3). Additionally, the mammalian enzymes had similar activities in several substrates. It is clear that mammalian enzymes are specific on α-ester bond of PLA.

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