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# Two-step hydrolyses of a polymeric drug under a model system

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Model polymeric drugs were synthesized by using 6-*O*-carboxymethyl-chitin as a biodegradable carrier and several peptides as spacer. The release of drug (chromogenic compound) was not observed by chymotrypsin-catalyzed hydrolysis until the proper size of oligomeric drug (prodrug) was produced predominantly by lysozymic hydrolysis. The amino-acid composition of the spacer and the spacer length were found to be preliminary regulation factors for two-step hydrolysis of the polymeric drug.

## INTRODUCTION

6-*O*-Carboxymethyl-chitin (CM-chitin), derived from chitin (poly $\beta$-1,4-linked 2-deoxy-2-acetamido-D-glucopyranose), is known to be highly biodegradable, mainly owing to its high susceptibility for lysozyme (Tokura *et al.*, 1983*a*). It has been found that CM-chitin and its lysozymic hydrolysate showed little immunogenicity, including the CM-chitin-derivative-bearing hypnotic reagent through a butylamine spacer (Tokura *et al.*, 1990). The CM-chitin hydrolysate was also found to be metabolized rather smoothly except in the intestine system, when $^{14}$C-labeled CM-chitin at the acetamido group and $^{125}$I-labeled CM-chitin by means of the Bolton–Hunter reagent were injected intravenously or subcutaneously into mice (unpublished data). The biodegradability and low toxicity of CM-chitin would be great advantages for the carrier of sustained release in a drug-delivery system. Calcium ion was found to chelate with CM-chitin and the chelate surface was also favored to adsorb several neutral amino acids, especially phenylalanine (Phe). The several peptides containing Phe were investigated and found to be adsorbed tightly to CM-chitin in the presence of a $Ca^{2+}$ ion. The adsorbed peptides were not bared to the proteolytic hydrolysis in the absence of lysozyme (unpublished data). CM-chitin was also reported to entrap chemotherapeutic reagents in the presence of an $Fe^{3+}$ ion, and the sustained release of reagent was observed when lysozyme reduced the molecular size of CM-chitin by a great extent (Watanabe *et al.*, 1990) (one-step release of drug). However, the molecular-weight distribution of lysozymic hydrolysates and the dependence of drug-holding ability on the molecular weight of CM-chitin have not yet been shown.

In the present study, covalently drug-pendanted CM-chitin was synthesized, in which process the drug was coupled through an enzyme-susceptible bond. In these conjugates, it is expected that the drug will be released through several hydrolysis processes, since oligomerization of CM-chitin by lysozymic hydrolysis is the first step, and the release of the parent active drug is followed by the cleavage of spacer–drug linkage with proteolytic hydrolysis as a second step. Thus, release of the drug from the CM-chitin–drug conjugate will be achieved through the above dual process: 'two-step release of drug', shown in Scheme 1.

To confirm this proposal, chromophore-terminating peptides were coupled with CM-chitin as a model for a CM-chitin–drug conjugate, and the release profiles of drugs from each of these conjugates were investigated by applying lysozymic hydrolysis as a first factor and

(1) **POLYMER**–spacer–**DRUG** $\xrightarrow{E_1}$ **OLIGOMER**–spacer–**DRUG**

(2) **OLIGOMER**–spacer–**DRUG** $\xrightarrow{E_2}$ **OLIGOMER**–spacer + **DRUG**

**Scheme 1.** Two-step release of drug.

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**Table 1.**

| Side chains conjugated to CM-chitin | Abbreviations |
|---|---|
| -phenylalanine *p*-nitroanilide | CMch-Phe-pNA |
| -glycylphenylalanine *p*-nitroanilide | CMch-Gly-Phe-pNA |
| -alanylphenylalanine *p*-nitroanilide | CMch-Ala-Phe-pNA |
| -4-amino-butyrylphenylalanine *p*-nitroanilide | CMch-Abu-Phe-pNA |

α-chymotryptic hydrolysis as a second factor of release. The spacers to couple the model drug with CM-chitin were as shown in Table 1.

The rate of hydrolysis was applied to the discussion of the possibility of sustained release and control of the release rate of the drug.

## EXPERIMENTAL

### Materials

Chitin was prepared from queen-crab shell according to the method of Hackman (1954) and powdered to less than 60 mesh before use.

Amino-acid derivatives purchased were as follows: α-*N*-benzyloxycarbonylphenylalanine (Z-Phe), *t*-butyloxycarbonylglycine (Boc-Gly), and *t*-butyloxycarbonylalanine (Boc-Ala) from the Peptide Institute Inc., and 4-amino-butyric acid (Abu) from Seikagaku Kogyo Co. Ltd. 1,3-Dicyclohexylcarbodiimide (DCC) and 4 M hydrogen chloride/1,4-dioxane were supplied by Kokusan Chemical Works Ltd. *p*-Nitroaniline and 25% hydrogen bromide/acetic acid were purchased from Wako Pure Chemical Industries Ltd. Sephadex G-50 was purchased from Pharmacia, Inc. α-Chymotrypsin (360 units/mg by a spectrophotometric method using *N*-benzoyl-L-tyrosine ethyl ester as described by Hummel (1959)) and egg-white lysozyme (50 000 units/mg by the *M. luteus*-cell method as described by Parry *et al.* (1965)) were purchased from Sigma Chemical Co. and Seikagaku Kogyo Co. Ltd, respectively. These materials were used without further purification, if not otherwise stated.

### Preparation of CM-chitin

CM-chitin was prepared according to the improved method of Trujillo as reported previously (Tokura *et al.*, 1983*b*). The degree of substitution (DS) was estimated to be 0·5 for conjugation and 0·8 for lysozymic hydrolysis of CM-chitin by potentiometric titration as described previously (Tokura *et al.*, 1983*b*).

### Preparation of peptides with chromophore (pNA)

Reactions and purities of each product were monitored by thin-layer chromatography (TLC) on precoated plates of Silica Gel 60 (E. Merck, Darmstadt, Germany), developed with an upper layer of *n*-butanol:acetic acid:water (4:1:5) and chloroform:methanol:acetic acid (95:5:5). The peptides were purified until one spot of the product was obtained on TLC.

*Phenylalanine p-nitroanilide hydrobromide (HBr·H-Phe-pNA)*

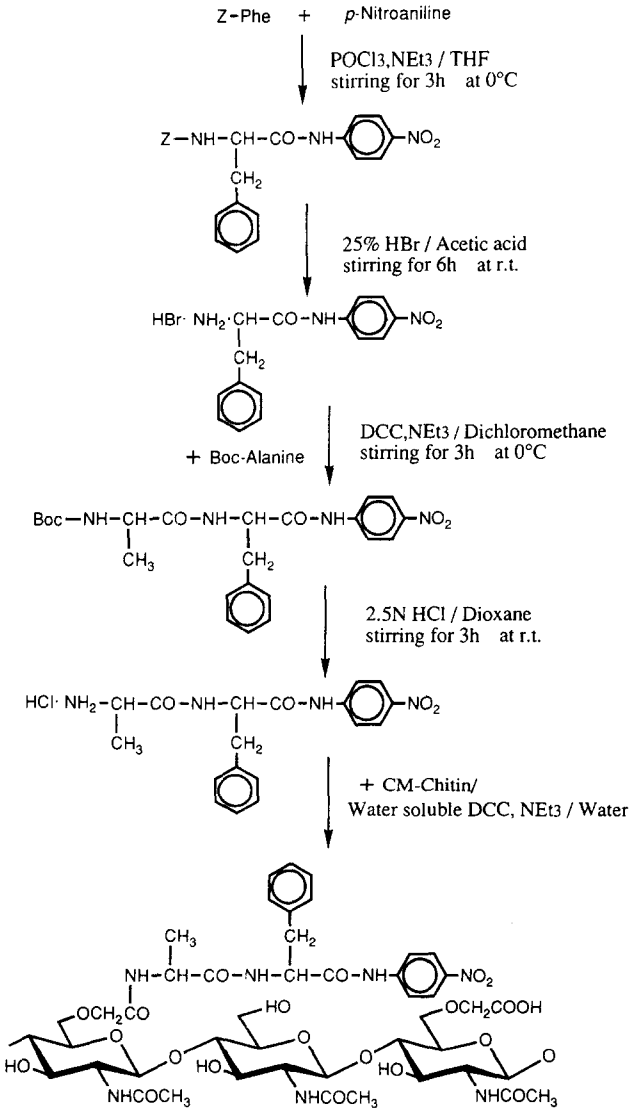
*p*-Nitroaniline (0·27 g (2 mmol)) and Z-Phe (1·8 g (6 mmol)) were dissolved in 5 ml of tetrahydrofuran (THF), and phosphorous oxychloride (4 mmol) and triethylamine ($NEt_3$, 4 mmol) were then added to the mixture at −15°C. The reaction mixture was stirred for 3 h at 0°C and then for 12 h at room temperature. The solution was evaporated to dryness, and the residue was dissolved in ethyl acetate, this being followed by washing with water and 5% aqueous sodium bicarbonate. After drying on $Na_2SO_4$, Z-Phe-pNA was crystallized by the addition of *n*-hexane; yield 83·3%. A removal of the Z group from Z-Phe-pNA (1·6 mmol) was achieved in 15 ml of 25% HBr/$CH_3COOH$ with stirring for 12 h at room temperature. HBr·H-Phe-pNA was precipitated by the addition of diethyl ether (yield 91·5%).

*Alanylphenylalanine p-nitroanilide hydrochloride (HCl·H-Ala-Phe-pNA)*

The HBr·H-Phe-pNA obtained above was condensed to *t*-butyloxycarbonylalanine (Boc-Ala) by the dicyclohexylcarbodiimide (DCC) method in dichloromethane. Boc-Ala (1·3 mmol) and HBr·H-Phe-pNA (0·8 mmol) were mixed in 5 ml of dichloromethane and then $NEt_3$ (2 mmol) and DCC (1·3 mmol) were added at 0°C. The reaction mixture was stirred for 2 h at 0°C and then for 10 h at room temperature. After the evaporation, the residue was dissolved in ethyl acetate, and dicyclohexylurea was removed by filtration. The organic layer was washed with 5% aqueous citric acid and 5% aqueous sodium bicarbonate and then dried on $Na_2SO_4$. The peptide was crystallized by *n*-hexane (yield 95·1%). The Boc group of the peptide (0·7 mmol) was removed in 4 ml of 2·5 M HCl/dioxane at room temperature. The peptide hydrochloride was precipitated by ether (yield 98·2%).

*Glycylphenylalanine p-nitroanilide hydrochloride (HCl·H-Gly-Phe-pNA) and 4-amino-butyrylphenylalanine p-nitroanilide hydrochloride (HCl·H-Abu-Phe-pNA)*

These products were obtained by employing the same procedure as for HCl·H-Ala-Phe-pNA. The yields of




**Scheme 2.** Synthetic route of CMch-Ala-Phe-pNA.

the coupling and deprotection were as follows: 93·4% and 91·5% for Gly-peptide, 92·8% and 97·9% for Abu-peptide, respectively.

## Preparation of polymeric substrates

Polymeric substrates were prepared by the coupling of peptides with CM-chitin by using morpho CDI (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluenesulfonate) in deionized water at 4°C. After 24 h of the coupling reaction, the reaction mixture was precipitated in acetone and then washed with methyl alcohol to remove impurities. The precipitates were dissolved in deionized water and lyophilized. The amount of bound peptides with chromophore was estimated spectrophotometrically ($\lambda_{max}$ 320 nm; in $H_2O$). Results are shown in Table 2.

## Enzymatic cleavage of polymeric substrates

*α-Chymotryptic hydrolysis of polymeric substrates in the presence of lysozyme*

Polymeric substrates were pre-incubated in the mixture of 50 mM Tris-HCl buffer, pH 7·4 and at 25°C, containing 50 mM $CaCl_2$, and then 2·8 ml of the solution was added to a sample cuvette. To the cuvette, 0·1 ml of lysozyme (E1) stock solution and 0·1 ml of α-chymotrypsin (E2) stock solution were added to be [E1] = 20 mg/ml, [E2] = 100 mg/ml, and the enzyme reaction was monitored by absorbance at 320 nm.

*Lysozymic hydrolysis of CM-chitin and gel-permeation chromatography*

CM-chitin was applied for the hydrolytic reaction with lysozyme to investigate a molecular-weight distribution of lysozymic hydrolysate by gel-permeation chromatography (GPC). A 500-ml volume of 0·1% (w/v) CM-chitin (DS = 0·8) phosphate-buffer solution (pH 6·2, M/15) was hydrolyzed at 37°C by 2500 units/ml of egg-white lysozyme, which was a similar level to that of human tear. A part of the reaction mixture was drawn to apply for GPC at 7, 24, 72, and 96 h, respectively. Aqueous trichloroacetic acid (20%) was added to 5% of the final concentration to precipitate the protein fraction by centrifugation. The supernatant was concentrated under reduced pressure at 30°C and precipitated in acetone. The CM-chitin oligomer collected by centrifugation was redissolved in deionized water and applied for GPC by using Sephadex G-50. The molecular weight of CM-chitin oligomer was estimated both by the titration of reducing end to calculate the number of molecules (Imoto & Yagashita, 1971) and by the HCl-indole method to give the total *N*-acetylglucosamine (GlcNAc) residue (Ohno *et al.*, 1985). The molecular weight (mol.wt) of the CM-chitin oligomer was calculated by applying the following equation:

**Table 2. The amount of model drugs incorporated into CM-chitin**

| Abbreviation | Amount of model drug (nmol/mg polymer) | Chromophore-bearing residue / Total residue of CM-chitin |
|---|---|---|
| CMch-Phe-pNA | 763 | 0·22 |
| CMch-Gly-Phe-pNA | 398 | 0·13 |
| CMch-Ala-Phe-pNA | 213 | 0·07 |
| CMch-Abu-Phe-pNA | 103 | 0·03 |

$$\text{mol.wt} = \frac{\text{Number of GlcNAc residues}}{\text{Number of molecular chains}} \times \text{RW}$$

where RW is the residual molecular weight estimated from the degree of substitution of CM-chitin.

*Lysozymic hydrolysis of polymeric substrates*

Degradation of polymeric substrates by lysozyme-catalyzed hydrolysis was monitored by titration of the reducing group of liberated sugar. Polymeric substrates were pre-incubated in the mixture of 50 mM Tris-HCl buffer, at pH 7·4 and 25°C, containing 50 mM $CaCl_2$, and then the lysozyme stock solution was added to give [E1] = 10 mg/ml. After a certain time, 3 ml of the reaction mixture was taken and boiled for 30 min to inactivate the enzyme in a test tube. A 1·5 ml volume of each oligomeric-substrate solution was mixed with 2 ml of color-reagent solution and incubated in boiling water for 15 min. After cooling, the absorbance at 420 nm was measured. The number-average molecular weight of polymeric substrates ($M_n$) was calculated from the increment of the reducing power in the reaction mixture.

*α-Chymotryptic hydrolysis of oligomeric substrates*

The oligomeric-substrate solutions obtained above were pre-incubated at 25°C, and then α-chymotrypsin (E2) stock solution was added to give [E2] = 0·5 mg/ml, and release of the drug was followed by absorbance at 320 nm.

## RESULTS AND DISCUSSION

*α-Chymotryptic hydrolysis of polymeric substrates in the presence of lysozyme*

As shown in Fig. 1, a polymeric conjugate containing a spacer composed of a single amino-acid residue (CMch-Phe-pNA) showed little release of the model drug (*p*-nitroaniline; pNA) within 6 hours of the reaction time when the conjugate was treated with α-chymotrypsin and lysozyme. After 6 hours, however, pNA started to be liberated from the conjugate. It seemed that the susceptibility of polymeric substrates for proteolytic hydrolysis was significantly related to the molecular weight of the polymeric carrier, because the release of pNA observed for CMch-Phe-pNA was regarded as the result of oligomerization of CM-chitin by lysozymic degradation.

The smaller the molecular size of the conjugate, the larger was the amount of pNA liberated, resulting from a reduction of steric hindrance by polymer backbone. These results would suggest the possibility of two-step release of drug and usefulness of CM-chitin as a drug carrier, i.e. drug would be released by proteolytic hydrolysis secondary from the oligomeric conjugate that is the primary product of lysozymic hydrolysis.

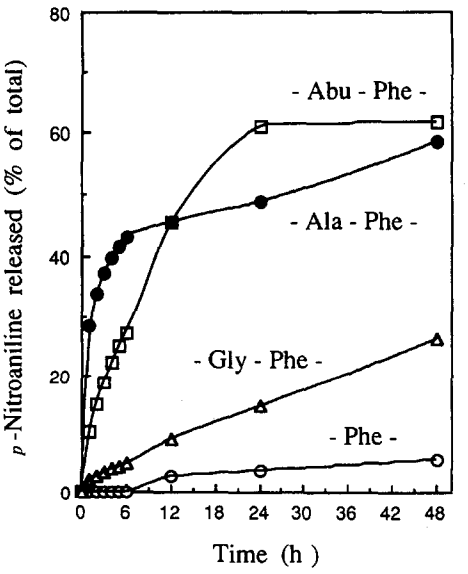



**Fig. 1.** Chymotryptic hydrolysis of polymeric substrates in the presence of lysozyme (5000 units/ml): ○ CMch-Phe-pNA; △ CMch-Gly-Phe-pNA; ● CMch-Ala-Phe-pNA; □ CMch-Abu-Phe-pNA.

Whereas, in conjugates with a spacer composed of two amino acid residues such as CMch-Gly-Phe-pNA, CMch-Ala-Phe-pNA, and CMch-Abu-Phe-pNA, the delay of drug release was not observed; the release profile of the model drug depended on the composition of the spacer. The disappearance of the delay of drug release may suggest the reduction of steric hindrance against proteolytic hydrolysis. As seen in Fig. 1, the rate of drug release was enhanced the longer the methylene chain of the adjacent residue of Phe: -Abu-Phe-pNA > -Gly-Phe-pNA ≫ -Phe-pNA. This may be due to the increment of mobility of the side chain. The higher rate of CMch-Ala-Phe-pNA for chymotryptic hydrolysis than that of other conjugates was regarded as a synergism of its higher susceptibility and the degree of mobility of the side chain for chymotrypsin-catalyzed hydrolysis.

*Lysozymic hydrolysis of CM-chitin*

The time-dependent molecular-weight distribution in the CM-chitin hydrolysate is shown in Fig. 2 following the lysozymic hydrolysis of CM-chitin. It is shown that the molecular-weight-distribution curves of CM-chitin hydrolysates maintain sharpness even in the low-molecular-weight region under experimental conditions. However, in practical conditions, the CM-chitin conjugate may be hydrolyzed from the surface of the conjugate, because CM-chitin tends to coagulate by the loss of water *in vivo* after subcutaneous injection, i.e. at a higher molecular weight, CM-chitin coupled to a drug will coagulate at the site of injection, and release of the drug will therefore result in 'surface release' from the coagulated mass. For example, a hypnotic drug was maintained for more than 100 hours

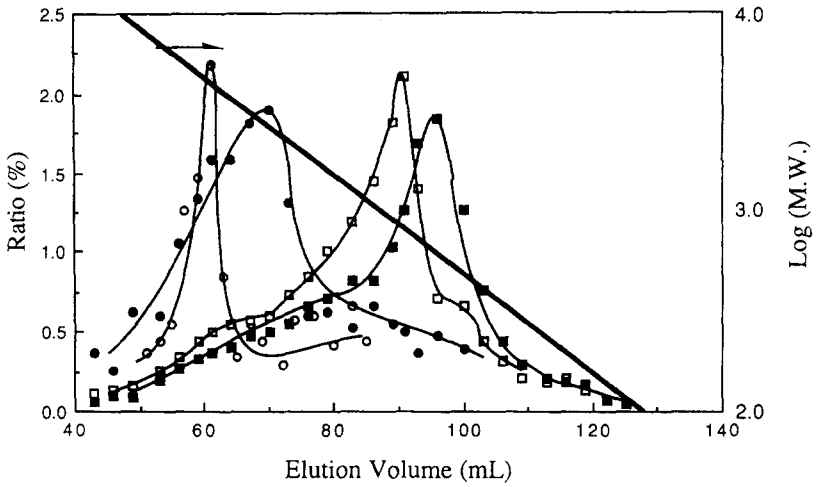



**Fig. 2.** Gel-permeation chromatogram of CM-chitin hydrolysate of lysozymic hydrolysis. ○ for 7 h; ● for 24 h; □ for 72 h; ■ for 96 h. Ratio (%) = (number of chains)/(number of total residues) × 100.

at a fairly high level in blood, when hypnotic-drug-bound CM-chitin was injected subcutaneously into rabbit (Tokura *et al.*, 1990).

*Lysozymic hydrolysis of polymeric substrates*

Lysozymic hydrolyses of polymeric substrates are shown in Fig. 3. This hydrolytic process corresponds to the first step of our proposed process. CM-chitin conjugates attached by peptides with chromophore to a part of the carboxymethyl group of CM-chitin were highly susceptible to lysozyme as well as CM-chitin. Lysozymic hydrolysis of CM-chitin conjugates was increased with an increment of enzyme concentrations. It is expected that a decrease in steric hindrance of CM-chitin backbone and an increase in susceptibility for α-chymotrypsin of the side chain as a second factor of drug release will proceed smoothly, when lysozymic hydrolysis is achieved as the first step predominantly to proteolytic hydrolysis.

*α-Chymotryptic hydrolysis of oligomeric substrates*

Figure 4 shows the dependence of the rate of α-chymotrypsin-catalyzed hydrolysis ($v_0$) on the number-average molecular weight ($M_n$) of oligomeric substrates corresponding to the steric hindrance of CM-chitin backbone. The release of chromophore from CM-chitin conjugate with a spacer of -Ala-Phe- (CMch-Ala-Phe-pNA) was not clearly shown in the molecular-weight range of 4000–50 000, but a significant

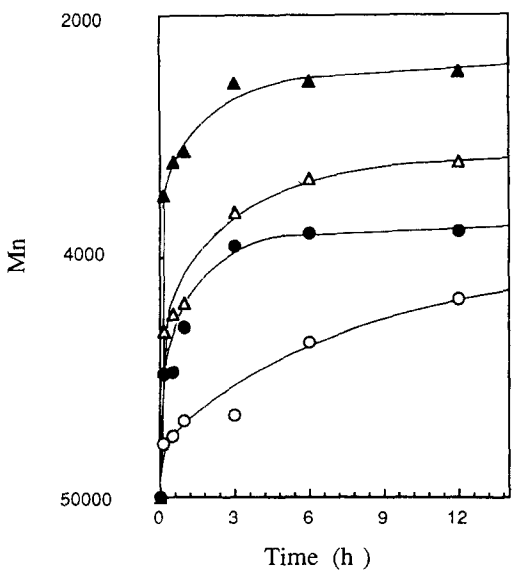



**Fig. 3.** Lysozyme-catalyzed hydrolysis of CMch-Ala-Phe-pNA at various enzyme concentrations: ○ 250 units/ml; ● 500 units/ml; △ 1000 units/ml; ▲ 2500 units/ml.

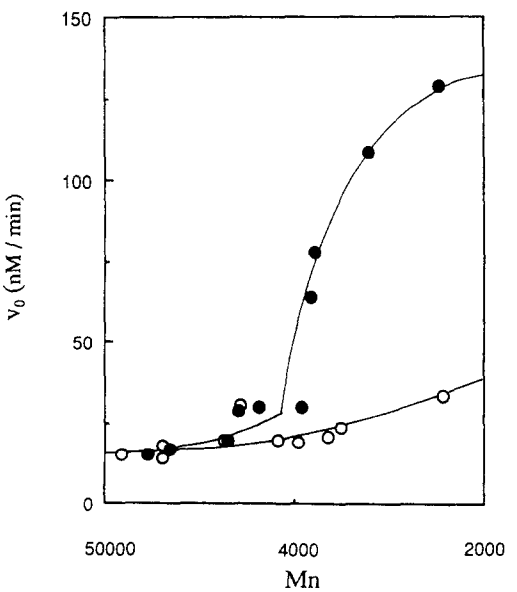



**Fig. 4.** Dependence of the rate of α-chymotryptic hydrolysis ($v_0$) on the number-average molecular weight, $M_n$, of polymeric substrates. ● CMch-Ala-Phe-pNA and ○ CMch-Abu-Phe-pNA.

increase in the rate of α-chymotryptic hydrolysis was observed on the reduction of $M_n$ below 4000. This suggests that the susceptibility of polymeric substrates ($M_n$ = 4000–50 000) for α-chymotrypsin is affected by the molecular conformation of the polymer chain, but the susceptibility of oligomeric substrates ($M_n$ < 4000) for α-chymotrypsin seems to be almost similar to that of low-molecular-weight substrate, because the spacer-chromophore bond is no longer protected by the CM-chitin molecule. On the other hand, there is no significant change in $v_0$ in the $M_n$ range of 2000–50 000 in the case of CM-chitin conjugate with a spacer composed of -Abu-Phe-(CMch-Abu-Phe-pNA). The independence of $v_0$ of CMch-Abu-Phe-pNA on $M_n$ seemed to reflect the lower substrate specificity of the side chain for the chymotryptic hydrolysis, i.e. the smaller the carrier chain, the higher is the value of $V_{max}$ or $k_{cat}/K_m$ for CMch-Ala-Phe-pNA but not for CMch-Abu-Phe-pNA. It is likely that chymotrypsin recognizes the naturally occurring Ala residue more readily than the Abu residue as an adjacent residue to Phe of the cleaved site. The combined results of CMch-Ala-Phe-pNA and CMch-Abu-Phe-pNA provide that the difference in the structure of the spacer also affects the α-chymotryptic hydrolysis in addition to the molecular weight of the drug carrier. In other words, the structure of the spacer is one of the factors to control the rate of drug release. Thus the information obtained would be evaluated as a guide to the release rate of the drug.

## CONCLUSION

A pendant-type polymeric drug was shown to release an active drug through two-step hydrolyses by a dual-enzyme system when biodegradable CM-chitin was applied as the carrier. The main factor to regulate the above hydrolyses is the stabilization of the drug under the physiological conditions that resist the enzymatic attack while the carrier maintains a high molecular weight. The structure of a spacer composed of amino acid residues is also a significant factor to control the presence of a significant variation in the susceptibility for enzyme and the release rate of the drug.

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