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Carboxyethylester-polyrotaxanes as a new calcium chelating polymer: synthesis, calcium binding and mechanism of trypsin inhibition

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Abstract

A carboxyethylester-polyrotaxane was synthesized as a novel calcium chelating polymer in the field of oral drug delivery and characterized in terms of mechanism of trypsin inhibition. Here, carboxyethylester (CEE) groups are introduced to all the primary hydroxyl groups in α-cyclodextrins (α-CDs), which are threaded onto a poly(ethylene glycol) chain capped with bulky end-groups (polyrotaxane). The solubility of the CEE-polyrotaxane in physiological conditions increased with pH, indicating ionization-related solubility similar to conventional polyacrylates. The ability of calcium (Ca^{2+}) chelation was found to increase in the order of poly(acrylic acid) (PAA) the CEE-polyrotaxane ≫ CEE-α-CD, suggesting that the increased density of carboxyl groups enhances the Ca^{2+} chelating ability. The activity of trypsin was inhibited by these compounds in the same order of the calcium chelation. However, the inhibitory effect of CEE-polyrotaxane was reduced by adding excess Ca^{2+} without precipitation that was observed in the presence of PAA. Such the reduced inhibition and precipitation by CEE-α-CD was not observed. Therefore, the inhibitory effect of CEE-polyrotaxane is due to Ca^{2+} chelation from trypsin without non-specific interaction.

Keywords: Trypsin inhibition; Polyrotaxane; Supramolecular structure; Calcium chelation; Carboxyl groups

1. Introduction

Polyacrylate-based resins (Carbomer and Polycarbophil) have been extensively studied as safe transmucosal penetration enhancers of protein and peptide drugs (Madsen and Peppas, 1999). High percentage of carboxyl groups in those polyacrylate resins allows water swelling in response to pH, mucoadhesion due to hydrogen bonding between the neutralized carboxyl groups and mucous glycoprotein, and chelation of divalent cations such as Ca^{2+} and Zn^{2+} (Singla et al., 2000). Especially, complexation of Ca^{2+} in gastrointestinal (GI) tract is a major role for the oral delivery of protein drugs. This provides an explanation for several biological effects such as dis-

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ruption of epithelial layer (Arturson and Magunsson, 1990), resulting in opening tight junction (Kriwet and Kissel, 1996). Further, several research groups have reported that the complexation of Ca^{2+} from proteolytic enzymes such as trypsin and chymotrypsin inhibits the proteolytic activity in the lumen (Lueßen et al., 1995; Akiyama et al., 1996). Thus, the complexation of Ca^{2+} by polyacrylate formulations is advantageous for protein drugs to prevent degradation and to enhance the penetration through epithelial cells in GI tract. In 1999, however, Walker et al. suggest that direct interaction between the polyacrylates and enzymes is major reason to inactivate the proteolytic enzymes (Walker et al., 1999). This report indicates that intermolecular interactions such as hydrogen bonding and electrostatic interactions by many carboxyl groups play an important role to the biological effects rather than the Ca^{2+} chelation. The Ca^{2+} chelation and the non-specific interactions are attractive characteristics, but there were no regulation of the Ca^{2+} chelation and the physical interaction with the biological components in GI tract. Thus, new material architectures should be designed for the regulation of Ca^{2+} chelation and physical interactions, which may affect the mechanism of mucoadhesion, inhibition of the proteases and the drug permeation.

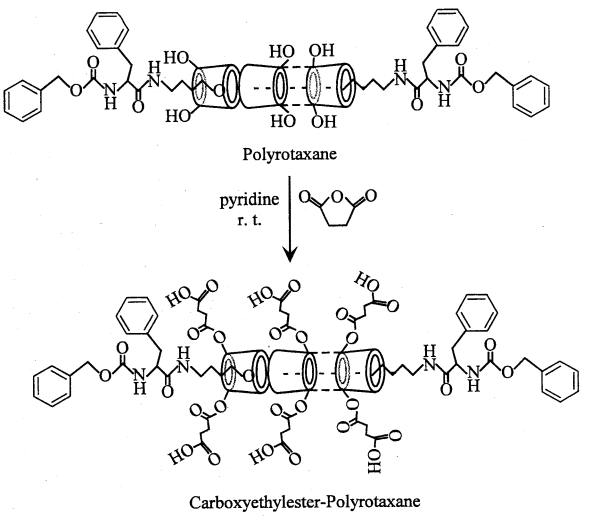
We have studied new biodegradable polymers with unique supramolecular structure for drug delivery systems (Ooya and Yui, 1999) and scaffolds in the fields of tissue engineering (Watanabe et al., 1998; Ichi et al., 2001). Our designed polymers are based on a molecular assembly of α-cyclodextrins (α-CDs), which are threaded onto a poly(ethylene glycol) (PEG) chain capped with bulky end-groups (polyrotaxane) (Harada et al., 1992). The attractive characteristics of the polyrotaxanes involve the design of the bulky end-groups in the polyrotaxane. For example, α-CDs in a pheneylalanylglycilglycine (PheGlyGly)-terminated polyrotaxane was dissociated due to hydrolysis between Phe-Gly bonds by the action of aminopeptidase N that is expressed in intestinal and kidney brush border membranes and other mucosal surfaces (Ooya et al., 2000, 2001a). Further, we developed the polyrotaxanes as multivalent ligands by means of the chemical modification of many hydroxyl groups of α-CDs in the polyrotaxanes (Ooya et al., 2001b; Yui et al., 2002, Ooya and Yui, 2002).

Based on the chemical modification technique, we designed carboxyl group-introduced polyrotaxanes as a novel Ca^{2+} chelating polymer. Controlling the density of carboxyl groups is expected by changing the number of carboxyl groups and/or release of carboxyl-introduced α-CDs by the terminal degradation of the polyrotaxanes, which may lead to a change in the degree of Ca^{2+} chelation (Nieuwenhizen et al., 1985). The objective of this study is to clarify the effect of polyrotaxane structure with carboxyl groups on the Ca^{2+} chelation and physical interactions with biomacromoleules. Trypsin was chosen as a model biomacromolecule in GI environments because the enzyme could be used to analyze both Ca^{2+} chelation and physicochemical interactions. CEE-polyrotaxanes with different number of CEE groups were synthesized and characterized in terms of controlling the number of CEE groups, solubility in aqueous media at various pH and stability of ester linkage of CEE. In vitro Ca^{2+} chelating study was carried out to determine the strength of Ca^{2+} chelation. Finally, trypsin inhibition was examined to discuss the Ca^{2+} chelation and physical interactions with trypsin in comparison with poly(acrylic acid) (PAA) and CEE-introduced α-CD (CEE-α-CD).

2. Materials and methods

2.1. Materials

α-CD was purchased from Bio-Research Corporation of Yokohama (Yokohama, Japan). α-(3-Aminopropyl)-ω-(3-aminopropyl) polyoxyethylene (PEO-BA: $M_n = 4000$) was kindly supplied by Sanyo Chemical Co. (Kyoto, Japan). Benzyloxycarbonyl-L-phenylalanine (Z-L-Phe) was purchased from Kokusan Chemical Co., Ltd (Tokyo, Japan). Succinic anhydride, PAA ($\bar{M}_w = 25000$) and calcium chloride were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). 2-[*N*-



Scheme 1.

morpholono] ethane-sulfonic acid (MES) was purchased form Nacalai Tesque, Inc.(Kyoto, Japan). Trypsin (EC 3.4.21.4, Type IX from porcine pancreas), *N*-α-benzoyl-L-arginine ethylester (BAEE), and *N*-α-benzoyl-L-argnine (BA) were purchased from Sigma Chemical Co. (St. Lous, MO). Other chemicals used were of highest purity available.

2.2. Synthesis of carboxyethylester-polyrotaxanes

This compound was synthesized by a modified method of Tanaka et al. (Tanaka et al., 1994). The synthetic route of CEE-polyrotaxane (132CEE-α/E4-PHE-Z) is shown in Scheme 1. A polyrotaxane, in which many α-CDs are threaded onto the PEO-BA capped with Z-L-Phe, was prepared according to our method (Ooya and Yui, 1997). The obtained polyrotaxane (the number of α-CDs: 30 determined by ^{1}H-NMR measurements) (6.03×10^{-6} mol) and succuinic anhydride (3.26×10^{-3} mol) were dissolved in dry pyridine and stirred at room temperature. The reaction mixture was poured into excess ether and washed with ether three times. The precipitate was collected by centrifuging and dried in vacuo to give the CEE-polyrotaxanes (CEE-α/E4-PHE-Zs). A CEE-α-CD was synthesized in a similar manner of CEE-polyrotaxanes. Table 1 summarizes the synthetic conditions and the results. The degree of substitution of CEE groups in the polyrotaxane was estimated from the ratio of the methylene peak of CEE (2.28 ppm) and C(1)H of α-CD (4.88 ppm) on the ^{1}H-NMR spectra.

2.2.1. CEE-polyrotaxanes

^{1}H-NMR (D_2O + NaOD, ppm): δ 7.35–7.18 (aromatics of Z-L-Phe), 4.88 (C(1)H of α-CD), 4.00–3.30 (C(3)H, C(5)H, C(6)H, C(4)H and C(2)H of α-CD), 3.58 (CH_2 of PEG), 2.28 (CH_2 of CEE).

2.2.2. CEE-α-CD

^{1}H-NMR (D_2O + NaOD, ppm): δ 4.88 (C(1)H of α-CD), 4.50–3.20 (C(3)H, C(5)H, C(6)H, C(4)H and C(2)H of α-CD), 3.58 (CH_2 of PEG), 2.50–2.00 (CH_2 of CEE).

2.3. Solubility in a buffer at various pH

The solubility of 132CEE-α/E4-PHE-Z and 6CEE-α-CD (Table 1) in phosphate bufferd saline (PBS) at various pH was determined by phenol–

Table 1
Synthesis of carboxyethylester-polyrotaxanes and carboxyethylester-α-CD

Sample code[a]	Reaction time[b]	# α-CD/polyrotaxane[b]	# CEE/α-CD[c]	# CEE/PRX[c]
33CEE-α/E4-PHE-Z	2	22	2	33
68CEE-α/E4-PHE-Z	6	22	3	68
132CEE-α/E4-PHE-Z	24	22	6	132
6CEE-α-CD	1	–	6	–

[a] CEE-α/E4-PHE-Z: carboxyethylester-polyrotaxane; M_n of PEG = 4000. CEE-α-CD: carboxyethylester-α-cyclodextrin.
[b] Feed ratio of succinic anhydride and hydroxyl group in α-CD was 1.0.
[c] Calculated from the ^{1}H-NMR spectra.

H_2SO_4 assay. The excess weight of 132CEE-α/E4-PHE-Z and 6CEE-α-CD was suspended in a 0.5 M PBS. The pH was adjusted by adding 5 M NaOH solution. The phenol–H_2SO_4 assay was followed by the previous method (Watanabe et al., 1998). From the results of glucose content, the concentration of 132CEE-α/E4-PHE-Z was calculated with the number of α-CDs in the polyrotaxane.

2.4. Calcium binding assay

132CEE-α/E4-PHE-Z ($0–1.29 \times 10^{-4}$ mol) or 6CEE-α-CD ($0–3.23 \times 10^{-3}$ mol) were dissolved in 50 mM MES aqueous solution adjusted with 1 M KOH to pH 6.7 (MES/KOH buffer, pH 6.7) containing 13 mM calcium chloride, and stirred for 2 h at room temperature. The concentration of unbound Ca^{2+} ($[Ca^{2+}]_{free}$) was determined using a Ca^{2+}-sensitive electrode (HORIBA, Japan). The concentration of chelated Ca^{2+} ($[Ca^{2+}]_{bind}$) was calculated by the following equation:

$$[Ca^{2+}]_{bind} = [Ca^{2+}]_{total} - [Ca^{2+}]_{free}$$

where $[Ca^{2+}]_{total}$ is the total concentration of Ca^{2+}.

2.5. Trypsin inhibition assay

The following samples dissolved in the MES/KOH buffer, pH 6.7 were prepared for trypsin inhibition assay: (a) 0.18% (w/v) PAA, (b) 0.75% (w/v) 132CEE-α/E4-PHE-Z and (c) 0.66% (w/v) 6CEE-α-CDs. In this condition, the concentration of the carboxyl groups was adjusted to 25 mM. The MES/KOH buffer was used as control.

The amount of 1.5 mmol *N*-α-benzoyl-L-arginine ethylester was dissolved in the sample solutions (20 ml). Five milliliter of the different substrate dilutions was used for one degradation experiment. The degradation was started by adding 24.0 IU trypsin/ml to the different preparations at 37 °C (final trypsin concentration: IU). In order to analyze the degradation using high performance liquid chromatography (HPLC), the reaction mixture (50 μl) was sampled at an appropriate time and diluted in 1 ml phosphoric acid (pH 2) to stop trypsin activity. The degradation product (*N*-α-benzoyl-L-arginine, BA) was determined by the HPLC with a reversed-phase column (COSMOSIL 5C18-AR-II, 250×4.5 mm^2; Nacalai Tesque, Inc. Kyoto Japan) at a flow rate 0.75 ml/min. The mobile phase consists of: elute A, 86% (v/v) 10 mM ammonium acetate (pH 4.2) and 14% (v/v) acetonitrile and elute B, 20% (v/v) 10 mM ammonium acetate (pH 4.2) and 80% (v/v) acetonitrile. Gradient elution was performed as follows: 0–8 min: 92%A/8%B, isocratic; 8–10 min: 50%A/50%B, linear gradient; 10–13 min: 50%A/50%B, isocratic. BA was detected at 253 nm. In this condition, the elution peak of BA was found to be 6.35 min.

The degree of tyrpsin inhibition was expressed by the inhibition factor (IF) (Madsen and Peppas, 1999):

$$IF = AUC_{control}/AUC_{polymer}$$

where AUC is the area under BA vs time curve in the absence of any polymer ($AUC_{control}$) and in the presence of polymer ($AUC_{polymer}$).

3. Results and discussion

3.1. Synthesis and characterization of carboxyethylester-polyrotaxanes

CEE groups were introduced by the reaction between hydoxyl groups in the polyrotaxane and succinic anhydride in pyridine. We selected this reaction because the nucleophilic reaction using anhydrides is known to maintain the polyrotaxane structure (Watanabe et al., 1999). From the ^{1}H-NMR spectrum of the recovered samples, all the peaks were attributed to α-CDs, PEG-terminated groups and CEE groups (Fig. 1). Furthermore, single peak of 132CEE-α/E4-PHE-Z was detected and the elution time was much shorter than that of 6CEE-α-CD from GPC analysis (Fig. 2). These results indicate that the polyrotaxane structure was maintained after the chemical modification. As shown in Table 1, the number of CEE groups was controllable by the reaction time. Feed ratio of the hydroxyl groups of α-CDs in the polyrotaxane and succinic anhydride was

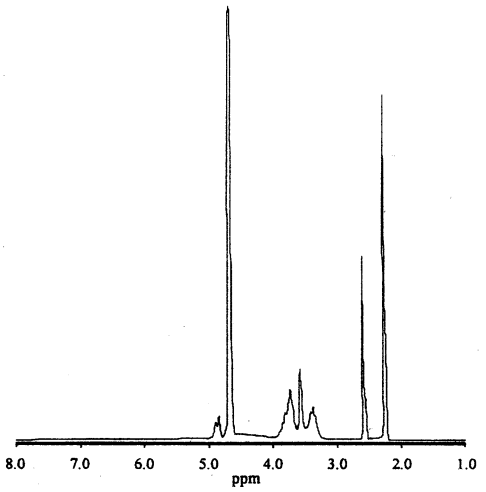


Fig. 1. ^{1}H-NMR spectrum of 132CEE-α/E4-PHE-Z.

not effective to control the number of CEE (data not shown). The number of the CEE groups was maximally approximately 132. This indicates that CEE groups were introduced to all the primary hydroxyl groups of α-CDs in the polyrotaxane. All the primary hydroxyl groups of α-CD in 6CEE-α-CD were also modified (Table 1). The following characterization was carried out using 132CEE-α/E4-PHE-Z and 6CEE-α-CD to estimate the effect of supramolecular structure of the polyrotaxane in terms of solubility at various pH conditions, Ca^{2+} chelation and trypsin inhibition.

Fig. 3 shows the solubility of 132CEE-α/E4-PHE-Z and 6CEE-α-CD in PBS at various pH. The solubility of 6CEE-α-CDs and 132CEE-α/E4-PHE-Z increased with pH up to 4 due to the ionization of carboxyl groups. The solubility of

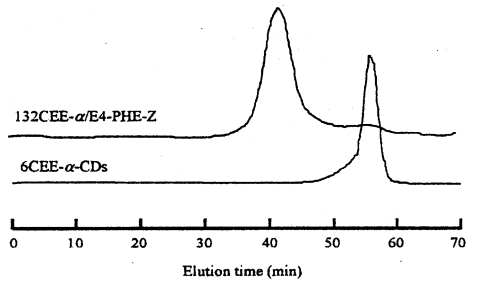


Fig. 2. GPC charts of 132CEE-α/E4-PHE-Z and 6CEE-α-CD.

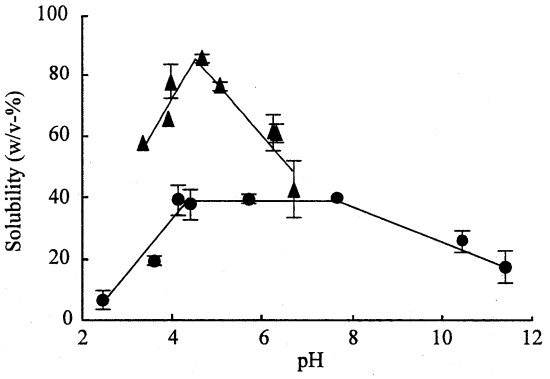


Fig. 3. Solubility in PBS in response to pH: 132CEE-α/E4-PHE-Z (●), 6CEE-α-CDs (▲) (mean ± S.E.M., $n = 3$).

132CEE-α/E4-PHE-Z kept the similar level from pH 4 to 8 and then gradually decreased to 11. This phenomenon may be due to neutralization effect by sodium ion. Since the sodium hydroxide solution was added to the mixture to adjust pH of solution containing 132CEE-α/E4-PHE-Z, concentration of the sodium ion increased with pH. It is suggested that the neutralization of carboxyl groups by such the ion decreases the hydration of 132CEE-α/E4-PHE-Z. Such the effect of the neutralization has been reported in the case of Carbopol (Ünlü et al., 1992). The solubility of 6CEE-α-CD decreased from pH 5, which was different tendency from 132CEE-α/E4-PHE-Z. The decrease in the solubility from pH 5 is presumably due to inclusion complexation between the CEE group and the cavity of α-CD, since the peak attributed to CEE group on the NMR spectrum shifted to down field (data not shown). The solubility of 132CEE-α/E4-PHE-Z was less than that of 6CEE-α-CD, suggesting that hydrogen bonding between unmodified hydroxyl groups of α-CD in 132CEE-α/E4-PHE-Z (secondary hydroxyl group) reduced the solubility. These ester linkage of CEE were found to be stable at pH 6–8 over 2 months (data not shown).

3.2. Effect of the polyrotaxane structure on calcium ion chelating

Fig. 4 shows the bound Ca^{2+} to the 132CEE-α/E4-PHE-Z, PAA and 6CEE-α-CD at pH 6.7 as a

function of CEE concentration per total Ca^{2+} concentration ($[CEE]/[Ca^{2+}]_{total}$). $[Ca^{2+}]_{bind}$ of PAA increased in proportion to $[CEE]/[Ca^{2+}]_{total}$ by the value around 2–3, and then gently increased with $[CEE]/[Ca^{2+}]_{total}$. This result was consistent with the previous report by Kriwet and Kissel (1996). The 132CEE-α/E4-PHE-Z chelated Ca^{2+} with increasing $[CEE]/[Ca^{2+}]_{total}$ up to 90%. This result indicates that Ca^{2+} chelating capacity of 132CEE-α/E4-PHE-Z is similar to or a little bit less than that of PAA. On the other hand, maximum $[Ca^{2+}]_{bind}$ value of 6CEE-α-CD was around 40%. Presumably, the inclusion complexation of CEE groups (Section 3.1) and/or the lower number of CEE in one molecule reduced the capacity. Thus, the calcium chelating was enhanced by the supramolecular structure of the polyrotaxane in relation to increasing the density of CEE groups.

3.3. Effect of the polyrotaxane structure on inhibitory mechanism of trypsin activity

It is known two hypotheses of trypsin inhibitory mechanism by polyacrylates: one is Ca^{2+} chelation (Lueßen et al., 1995, 1996, 1997) and another is direct interaction with the enzyme (Walker et al., 1999). In order to estimate how 132CEE-α/E4-PHE-Z affects the inhibition of trypsin activity, degradation of BAEE in the presence of 132CEE-α/E4-PHE-Z, PAA and 6CEE-α-CD was examined. Fig. 5 shows the inhibitory effect of these compounds on trypsin activity with or without additional calcium chloride (20 mg/ml). Here, the excess calcium chloride was added just before trypsin incubation to estimate the participation of Ca^{2+} chelation on trypsin inhibition. Without the additional calcium chloride, the hydrolyzed BAEE (*N*-α-benzoyl-arginine, BA) increased with time in the order of 6CEE-α-CD $\gg$ 132CEE-α/E4-PHE-Z > PAA. This order seems to be in inverse proportion to the capacity of Ca^{2+} chelation. When the excess calcium chloride was added before the incubation, the amount of BA with 132CEE-α/E4-PHE-Z and PAA during 60 min increased but not changed with 6CEE-α-CD. These results suggest that Ca^{2+} chelation by 132CEE-α/E4-PHE-Z and PAA is related to the trypsin inhibition. To quantitatively determine the inhibitory effect, IF of the 132CEE-α/E4-PHE-Z, PAA and 6CEE-α-CD during 60 min was calculated (Fig. 6). IF of 132CEE-α/E4-PHE-Z and PAA significantly decreased by the addition of

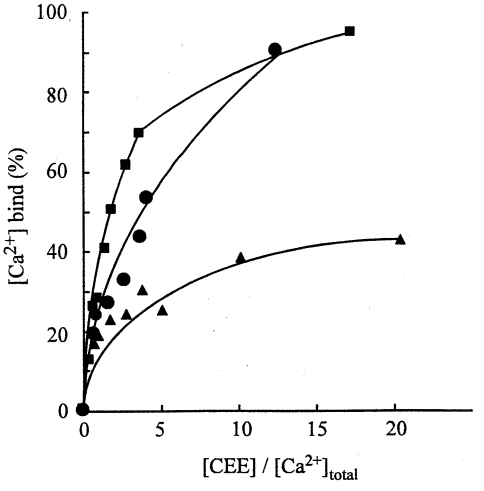


Fig. 4. Ca^{2+} binding ability of 132CEE-α/E4-PHE-Z (●), 6CEE-α-CDs (▲), PAA (■).

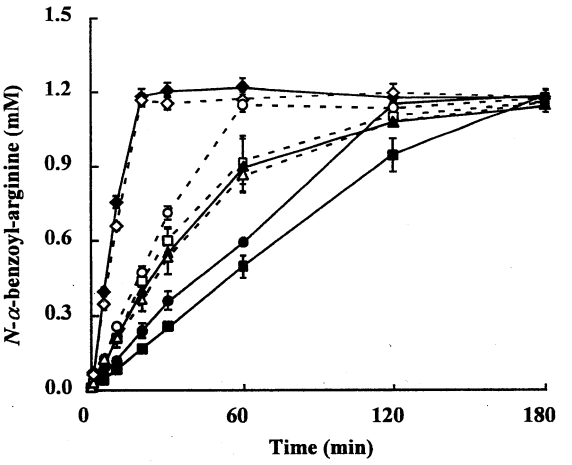


Fig. 5. Formation of BA following incubation of BAEE with trypsin with or without 20% (w/v) $CaCl_2$ (mean ± S.E.M., $n = 3$). (∅, control (only BAEE); ◇, control with $CaCl_2$; ●, 132CEE-α/E4-PHE-Z (25 mM COOH groups); ○, 132CEE-α/E4-PHE-Z (25 mM COOH groups) with $CaCl_2$; ■, PAA (25 mM COOH groups); □, PAA (25 mM COOH groups) with $CaCl_2$; ▲, 6CEE-α-CD (25 mM COOH groups); △, 6CEE-α-CD (25 mM COOH groups) with $CaCl_2$).

excess calcium chloride, and that of 6CEE-α-CD was not changed. Similar result of IF was obtained during 180 min. In the excess calcium condition, the solution of 132CEE-α/E4-PHE-Z and PAA became emulsion and precipitated states, respectively. The precipitation with PAA suggests that all the carboxyl groups in PAA stoichiometrically participate Ca^{2+} chelation (Kriwet and Kissel, 1996), and the concentration of PAA in the solution decreases. On the other hand, the emulsion with 132CEE-α/E4-PHE-Z indicates that non-participated CEE groups exist in the solution, which was supported by the a little less Ca^{2+} chelation than PAA (Fig. 4). Since the PAA is considered to directly interact with trypsin to reduce its activity (Walker et al., 1999), the IF value with PAA and the excess calcium chloride should increase if all the PAA was dissolved under the excess Ca^{2+} condition. Therefore, the mechanism of the trypsin inhibition by 132CEE-α/E4-PHE-Z was Ca^{2+} chelation due to the relatively weak chelation property. The inhibitory effect of the trypsin activity by 6CEE-α-CD was suggested to be caused by the other factors. Presumably, inclusion complexation with aromatic groups in BAEE and/or trypsin was related to the reduced accessability of trypsin to BAEE (Rekharsky and Inoue, 1998).

4. Conclusion

The CEE-polyrotaxane was synthesized and evaluated in terms of mechanism of trypsin inhibition. The density of CEE groups that was introduced to primary hydroxyl groups in α-CD was increased by threading of the CEE-α-CDs onto a PEG chain. This polyrotaxane structure showed Ca^{2+} chelation property that was milder than that of PAA. The obtained trypsin inhibition concludes that the inhibitory effect of CEE-polyrotaxane is due to Ca^{2+} chelation from trypsin without non-specific interaction. This property is considered to be applicable to the other biological effect of Ca^{2+} chelation such as opening tight junction in intestine.

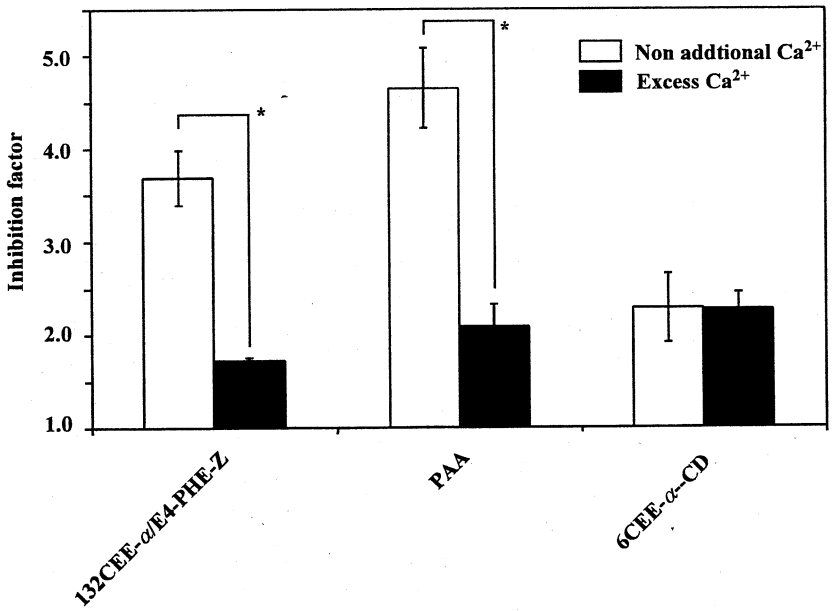


Fig. 6. IF of the 132CEE-α/E4-PHE-Z, PAA and 6CEE-α-CD ($\pm$ S.E.M., $n = 3$). Asterisk indicates significantly different IF at $P < 0.05$ between low calcium concentration and excess calcium concentration, where calculated using a t-test.

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