[原著] Exposure of threony 1 residue at position 10 in fibrinopeptide A is essential to the recognition by habutobin

Exposure of threonyl residue at position 10 in fibrinopeptide A is essential to the recognition by habutobin

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ABSTRACT

We have reported that habutobin acted only on rabbit fibrinogen by cleaving the Arg^{16}-Gly^{17} bond in the Aα chain. We have also demonstrated that habutobin recognized threonine (Thr) at position 10 from the COOH-end of the rabbit fibrinopeptide A (FPA). Since Thr has been revealed to also exist at position 10 in chicken FPA, we postulated that habutobin may recognize chicken Thr, thereby converting it into fibrin gel. We investigated whether habutobin acted on chicken fibrinogen to form fibrin gel. Habutobin clotted chicken fibrinogen more slowly than it did rabbit fibrinogen. It clotted the chicken fibrinogen with a resultant release of three peptides (peak I, II and X peptides), whereas thrombin clotted the chicken fibrinogen with a resultant release of two peptides (peak 1 and 2 peptides). The elution times of the peak I and II peptides were identical to those of peak 1 and 2 peptides. The peak 2 fibrinopeptide was identified as the FPA of chicken fibrinogen. A computer-assisted structural analysis of the rabbit FPA demonstrated that the Thr is located at the top of the N-terminal cluster, whereas the Thr in the chicken FPA exists in a linear and flexible peptide. Since the Thr on the chicken FPA seemed to fluctuate in the peptide, recognition of the Thr by habutobin might be unstable and ineffective. In conclusion, the exposure of Thr at position 10 in the FPA molecule is essential for habutobin-specific recognition of chicken fibrinogen. *Ryukyu Med. J., 23(1, 2) 25～30, 2004*

Key words: habutobin, threonyl residue, rabbit FPA, chicken fibrinogen, molecular recognition

INTRODUCTION

We previously reported that habutobin converts only rabbit fibrinogen to fibrin gel with a resultant release of fibrinopeptide A (FPA) following the cleavage of the Arg^{16}-Gly^{17} bond in the Aα chain of the rabbit fibrinogen[1-3]. We also reported that the residue Thr^{7} in the rabbit FPA sequence was critical for the specific action of habutobin on rabbit fibrinogen employing oligopeptides containing partial sequences of rabbit FPA[4]. This suggested that habutobin recognizes the Thr^{7} in the FPA sequence of the rabbit Aα chain and subsequently cleaves the Arg^{16}-Gly^{17} bond.

The residue Thr^{7} in rabbit FPA is located at position 10 of the FPA sequence (where the position of each amino acid residue was numbered from the C-terminal Arg^{1} according to the viewpoint of Blombäck and collaborators)[5-7]. Among all the sequences of vertebra FPA reported, the Thr residue at position 10 was found only in chicken and rabbit FPA[6-8]. Therefore, we postulated that habutobin would recognize the residue Thr at position 10 in chicken FPA and that habutobin could convert chicken fibrinogen to fibrin gel with the release of FPA.

In this study, we investigated the action of habutobin on chicken fibrinogen. In addition, we

Table 1 Clotting action of habutobin on rabbit, chicken and bovine fibrinogens

Origin of fibrinogen	Fibrin forming time (min)	
	Habutobin	Bovine thrombin
Rabbit	2.5	2.5
Chicken	60	73
Bovine	> 90	3

analyzed the molecular structures of the rabbit and chicken FPAs in order to understand the mechanism of habutobin recognition of Thr based on conformational characteristics of the residue Thr at position 10 in each FPA molecule.

MATERIALS and METHODS

Reagents

Habutobin was purified according to the method of Kosugi *et al*[1]. The rabbit and chicken fibrinogens were purified by the technique of Doolittle *et al*[9]. The rabbit FPA was purified as described previously[10]. Bovine fibrinogen was purchased from Miles Inc. (Kankakee, IL, USA) and bovine thrombin was purchased from Mochida Pharmaceutical Co. (Tokyo, Japan). Acetonitrile and trifluoroacetic acid (TFA), both of HPLC grade, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Unless otherwise stated, all other reagents employed were analytical-grade chemicals.

Clotting action of habutobin on rabbit, chicken and bovine fibrinogens

20 μl of habutobin (1000 μg/ml) or bovine thrombin (4.5 units/ml) was mixed with 250 μl of 1% chicken, rabbit and bovine fibrinogens, and fibrin forming times were measured as described previously[1].

High-performance liquid chromatography (HPLC)

The fibrinopeptides in the supernatant of fibrin clots were quantified employing a Hitachi 6210/6010 high-performance liquid chromatograph (HPLC) equipped with a Model AS-4000 sample injector, a Model L-4000 UV-detector and a Model L-2500 integrator, all from Hitachi Ltd. (Tokyo, Japan). A 0.05% TFA-acetonitrile solvent system was employed with a flow rate of 0.5 ml/min on a reverse-phase C4 column (Cosmosil 5C4-AR-300, 4.6 mm i.d. x 150 mm), from Nacalai Tesque, Inc. (Kyoto, Japan), as described previously[4].

HPLC analysis of fibrinopeptides released from chicken fibrinogen

150 μl of 1% rabbit and chicken fibrinogens were mixed with 15 μl of habutobin (300 μg/ml) or bovine thrombin (4.5 units/ml), and the mixture was then incubated at 37℃ for desired periods. Following the incubation, 165 μl of cold ethanol was added to the reactant mixtures and agitated vigorously. They were then left to stand for 2 hr in ice. After centrifugation at 15,000 rpm for 30 min at 4 ℃, 200 μl of supernatants were collected. Fibrinopeptides in 50 μl of each sample were quantified employing a C4 reverse-phase HPLC with 0-40% acetonitrile gradient for 40 min.

Effects of rabbit FPA on the release of chicken fibrinopeptides by habutobin

A 5 μl of purified habutobin (300 μg/ml) was mixed with 10 μl of purified rabbit FPA (from 0 to 1000 μM). The mixture was then incubated at 37℃ for 60 min. 150 μl of the chicken fibrinogen (1 % or 0.5-2 %) was added to the reactant mixture, and then incubated at 37° C for 3 hr. A 165 μl of cold ethanol was then added and agitated vigorously. After 2 hr incubation on ice, the reactant mixture was centrifuged at 15,000 rpm for 30 min, and 50 μl of supernatant was employed for analysis by HPLC as described above.

Imaging molecular structures of chicken and rabbit FPA

On the basis of amino acid sequences of the rabbit FPA[3] and the chicken FPA[8], the structures of the chicken and rabbit FPAs were imaged by means of computer graphics using the CS Chem Office® software (Cambridge Soft Co., Cambridge, MA, USA)[11].

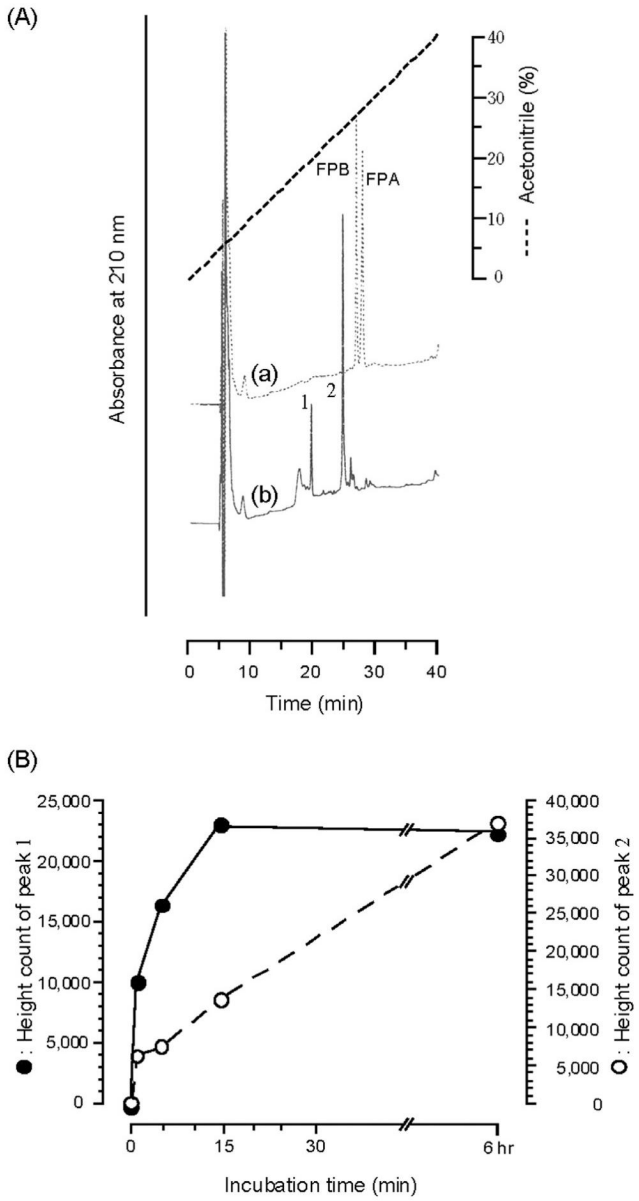


Fig. 1 HPLC analysis of fibrinopeptides released from chicken fibrinogen by bovine thrombin. (A) Chromatogram of HPLC analyzing the release of peptides from the rabbit fibrinogen (a) and the chicken fibrinogen (b). (B) Kinetic analysis of the release of chicken peak 1 peptide (●) and of peak 2 peptide (○). In (B), the amount of each peptide is indicated as the height count of each peak on chromatogram, which was calculated by integrator in HPLC system.

RESULTS

Clotting action of habutobin upon rabbit, chicken and bovine fibrinogens

As shown in Table 1, habutobin and bovine thrombin clotted the rabbit fibrinogen, and both enzymes clotted the chicken fibrinogens more slowly than the rabbit fibrinogen. Habutobin was not able to convert the bovine fibrinogen to fibrin gel, although the bovine thrombin clotted both bovine and rabbit fibrinogen with almost the same activity.

Release of fibrinopeptides by habutobin and bovine thrombin

As shown in Fig. 1A, thrombin released both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from the rabbit fibrinogen. Thrombin also released two peptides from the chicken fibrinogen, as indicated by peak 1 and peak 2 (Fig. 1A). As shown in Fig. 1B, the chicken peak 1 peptide was released earlier than the peak 2 peptide, indicating that the peak 1 peptide was FPA and the peak 2 peptide, FPB. On the other hand, habutobin released FPA preferentially from the rabbit fibrinogen, while it liberated three peptides (I, II and X) from the chicken fibrinogen, of which the former two (I and II) were identical to the thrombin-released peak 1 and peak 2 peptides, respectively (Fig. 2A). As shown in Fig. 2B, the peak I peptide was released earlier than the peak II peptide, and the release of the peak X peptide was much slower than those of the peak I and II peptides.

Inhibition of the release of chicken fibrinopeptides by rabbit FPA

As shown in Fig. 3, release of the chicken peak I peptide was decreased by the addition of rabbit FPA, and the inhibitory effect of rabbit FPA was increased when its concentration was increased. On the contrary, release of the chicken peak II peptide was not remarkably affected by the addition of rabbit FPA.

Software-predicted structures of chicken and rabbit FPA

We attempted to image the molecular structures of chicken and rabbit FPAs (Fig. 4). In the predicted structure of rabbit FPA, Val (position 16), Phe (position 9) and Ile (position 8) gathered together and formed a cluster with the acidic regions consisting of two Asp (at positions 7 and 15) and two Glu (at positions 6 and 12). In addition, the Thr at position 10 was seen at the top of the N-terminal cluster. On the other hand, the predicted structure of chicken FPA was a linear and flexible

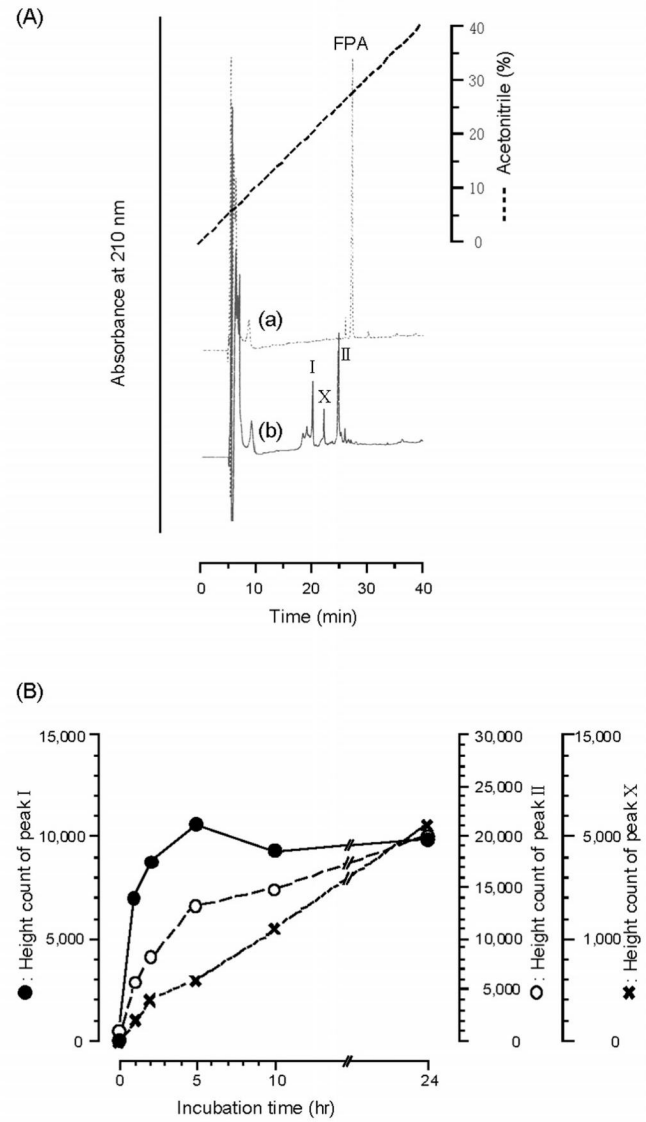


Fig. 2 HPLC analysis of fibrinopeptides released from the chicken fibrinogen by habutobin. (A) Chromatogram of HPLC analyzing habutobin-released peptides from the rabbit fibrinogen (a) and the chicken fibrinogen (b). (B) Time course of peptide release (●: peak I peptide, ○: peak II peptide, and ×: peak X peptide), where the amount of each peptide is indicated as the height count calculated by the integrator in HPLC system.

structure, in which the Thr at position 10 was located at the turning point of the peptide chain. Moreover, this residue lay surrounded with a hydrophobic phenylalanyl residue (position 9) and two alkaline lysyl residues at positions 7 and 12.

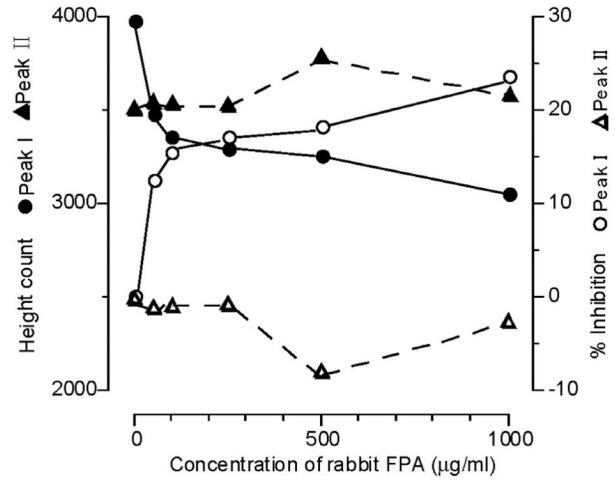
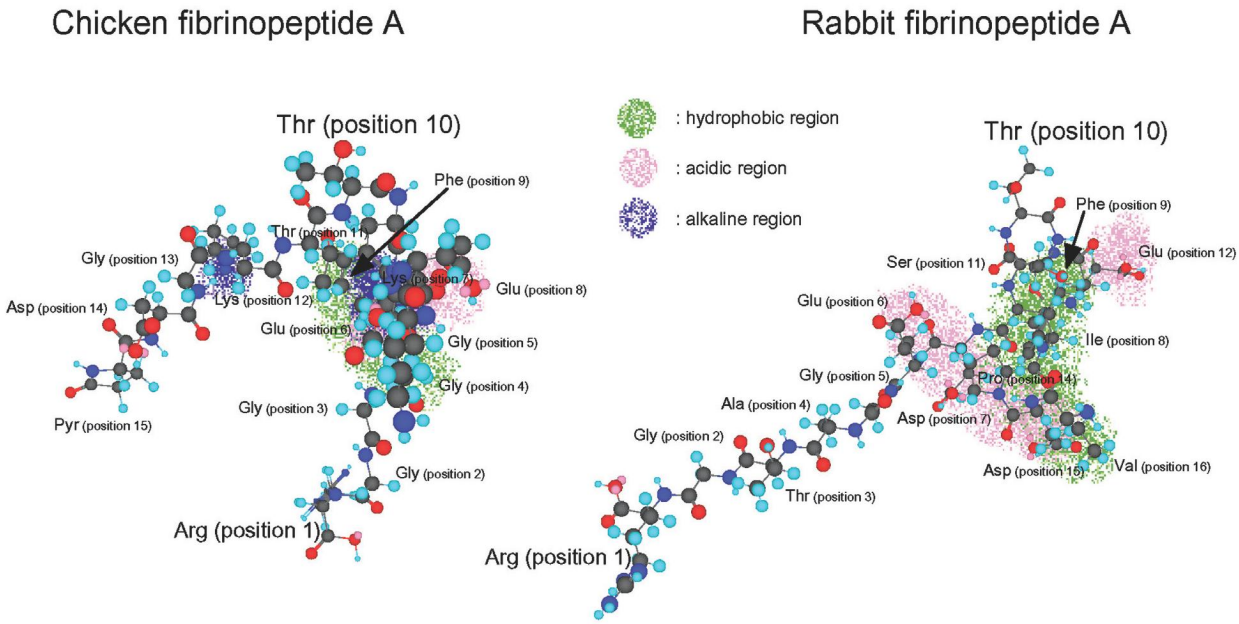


Fig. 3 Inhibitory effect of the rabbit FPA on the release of chicken fibrinopeptides. The amount of peptides released are line-plotted with the function of the concentration of the rabbit FPA (●: peak I peptide, ▲: peak II peptide). Dotted lines represent % inhibition of the release of peptides (○: peak I peptide, △: peak II peptide).

DISCUSSION

In this study, we found that habutobin clotted chicken fibrinogen with a resultant release of three fibrinopeptides, while bovine thrombin released two fibrinopeptides. It has been reported that thrombin preferentially acts on chicken A α chain rather than on B β chain[12,13]. Therefore, thrombin might release FPA earlier than FPB from chicken fibrinogen. Taken together, the thrombin-released peak 1 and peak 2 peptides liberated from the chicken fibrinogen in this study might be FPA and FPB, respectively. Habutobin on the other hand released three fibrinopeptides from the chicken fibrinogen, of which the peak I and II peptides were identical to the I and II peptides respectively released by thrombin. Thus, it was found that habutobin released a peak I peptide (tentative FPA), a peak II peptide (tentative FPB) and a third peptide of unknown origin. Although only FPA was released from the rabbit fibrinogen by habutobin, both FPA and FPB were released from the chicken fibrinogen. We think that while the rabbit FPA inhibited the action of habutobin on the B β chain of the rabbit fibrinogen, the chicken FPA did not inhibit its action on the B β chain of the chicken fibrinogen.

In this study, we demonstrated that the rabbit



Amino acid sequences of rabbit FPA and chicken FPA

	Position															
	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Rabbit	Val	Asp	Pro	Gly	Glu	Ser	Thr	Phe	Ile	Asp	Glu	Gly	Ala	Thr	Gly	Arg
Chicken		Pyr	Asp	Gly	Lys	Thr	Thr	Phe	Glu	Lys	Glu	Gly	Gly	Gly	Gly	Arg

Fig. 4 Software-predicted structures of chicken and rabbit fibrinopeptides A, which were imaged by means of computer graphics using the ChemOffice® software. The position of each according to the viewpoint of Blombäck and collaborators (Doolittle and Blombäck, 1964; Söderqvist and Blombäck, 1971; Doolittle, 1976). Rabbit FPA sequence was denoted elsewhere (Kinjoh et al., 1997), and the sequence of chicken FPA was determined by Takagi et al.(1978).

FPA inhibited the release of the chicken peak I peptide (tentative FPA) competitively. Thus, it is considered that the chicken peak I peptide (tentative FPA) may be a mimic of the rabbit FPA thus making it possible for habutobin to release this fibrinopeptide from the chicken fibrinogen. The residue Thr at position 10 in the rabbit FPA is the critical residue for the specific action of habutobin, as we demonstrated previously[4]. In addition, the chicken FPA is the only fibrinopeptide, which possess the residue Thr at the same position 10 as rabbit FPA[6-8]. Therefore, we postulated that habutobin would recognize the residue Thr at position 10 in the FPA sequence of the chicken A α chain, and this enzyme could cleave the Arg-Gly bond, thereby releasing the chicken FPA.

Our data demonstrates that the action of habutobin on the chicken fibrinogen is far weaker than on the rabbit fibrinogen. Considering a report that chicken fibrinogen was hardly clotted by any thrombins other than chicken thrombin[14], it is inferred that the conformation of chicken fibrinogen may be different from those of any other species. The conformational peculiarity of the chicken fibrinogen might interfere with the habutobin recognition of Thr thereby causing a delay in the release of FPA. In a computer-imaged molecular structure of the rabbit FPA, the residue Thr at position 10 was seen at the top of the N-terminal cluster constituted by hydrophobic residues and acid residues. In the chicken FPA, on the other hand, the Thr at position 10 existed in a linear and flexible peptide chain so that this residue was not tightly fixed but seemed to fluctuate in the FPA molecule. In addition, the Thr in the chicken FPA was positioned between two alkaline regions formed by Lys. Such an alkaline region was not found in the rabbit FPA. Alkaline environments (charged positively) in the chicken

FPA might interfere with the interaction between habutobin and the weakly negative-charged Thr at position 10. Consequently, the release of FPA from the chicken fibrinogen occurred slower than the release of the rabbit FPA.

In this study, it was found that habutobin not only liberated FPA (peak I peptide), but also FPB (peak II peptide) and a third peptide of unknown origin from the chicken fibrinogen. The mechanism of the release of FPB and the third peptide from the chicken fibrinogen is still unknown. Regarding the mechanism of the FPA release by habutobin, the exposure of Thr residue at position 10 of the FPA molecule could be essential for the specific action of habutobin.

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