Effect of Dextran Derivatives on Arginine Amidase Activities Released from Isolated Rabbit Arteries

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In the present research we examined the levels and types of arginine amidase activities that were released from isolated rabbit arteries treated with heparin or chondroitin sulfate. Heparin accelerated the release of arginine amidase activity from the isolated rabbit ear artery, the induction was not significant; a slight increase in activity was observed in the level of arginine amidase released from isolated rabbit aorta, but no significant difference was observed. On the other hand, it was revealed that the addition of chondroitin sulfate, accelerated this release from isolated rabbit ear artery with 5% significant differences.

After the addition of chondroitin sulfate, the arginine amidase activity released from isolated rabbit arteries was analyzed using various affinity adsorption methods. This analysis confirmed the presence of two types of fibrinolytic enzymes: plasminogen/plasmin activity and plasminogen activators, but no thrombin was detected.

Key words isolated rabbit artery; arginine amidase activity; heparin; chondroitin sulfate; fibrinolytic enzyme

It was previously reported that arginine and lysine amidolytic enzymes are secreted from rabbit arteries, and the physiological functions of those enzymes are under investigation.¹⁾ Forty to fifty percent of the arginine amidase activity consists of trypsin (EC 3.4.21.4)-like enzyme(s) showing affinity to lima bean trypsin inhibitor (LBTI) and aprotinin columns.^{1,2)} Many trypsin-like arginine amidases have important physiological actions as enzymes involved in the blood pressure regulation system, the blood coagulation system and the fibrinolytic system, and in the respective control mechanisms. The kininogenases showing acidic and basic optimum enzyme reactions in canine aorta has been reported by Moshi *et al.*³⁾ but the physiological roles of these enzymes were not elucidated.

A heparin-like substance and thrombomodulin, present in vascular endothelial cells, are thought to be associated with anticoagulation of blood.^{4,5)} Moreover, vascular endothelial cells are thought to produce and secrete tissue plasminogen activator (t-PA, EC 3.4.21.69).⁶⁾ We have already confirmed the release of plasminogen/plasmin (EC 3.4.21.7) from isolated rabbit aorta.¹⁾ The relationship between the fibrinolytic enzymes released from vascular endothelial cells and the dextran derivative substances is now receiving attention due to its possible regulatory role in the vessel wall factor in blood coagulation and dissolution mechanisms.

This paper reports the levels and types of arginine amidase activities released from isolated rabbit arteries treated with heparin and chondroitin sulfate.

MATERIALS AND METHODS

Measurement of Arginine and Lysine Amidolytic Activity D-Valyl-cyclohexylalanyl-L-arginine-*p*-nitroanilide (Val-CHA-Arg-pNA, American Diagnostica Inc., U.S.A.) was used as the substrate for tissue kallikrein (EC 3.4.21.35) for measurement of the arginine amidase activity released from the isolated arteries. *tert*-Butyloxy-carbonyl-L-leucyl-glycyl-L-arginine-*p*-nitroanilide (Boc-Leu-Gly-Arg-pNA, Kabi

Diagnostica Co., Sweden.) was also used as a substrate for plasminogen activators. The amidolytic method described by Amundsen *et al.*⁷⁾ was carried out with slight modifications by us for use of microplate,⁸⁾ and the reaction was performed at 37 °C in 0.05 mol/l Tris-HCl buffer (pH 8.5) containing 0.15 mol/l NaCl on the microplates. The final reaction volume and final substrate concentration were 0.2 ml and 0.5 mmol/l, respectively. Amidolytic activity was expressed in terms of nanomole of substrate hydrolyzed per minute (nmol/min), after measuring the absorbance at 405 nm of *p*-nitroanilide (pNA, molar extinction coefficient = 10000), which was resultantly released by the amidolytic enzyme reaction from the above mentioned tripeptidyl substrate using a microplate reader.

Rabbit Artery Preparation Rabbit artery preparation was carried out by the method using Japanese white rabbits weighing 2 to 3 kg which was described in our recent study.^{1,2)} The aorta preparation was divided in the center, and the heart and diaphragm sides of the aorta preparation were tentatively called the upper portion and lower portion, respectively. After preparation of the arteries, they were washed with Krebs solution as modified by Ishii *et al.*⁹⁾ with bubbled 95% O₂ and 5% CO₂ gas to eliminate of contaminating plasma.

Experimental Protocol Artery preparations were suspended in a water-jacketed organ chamber (10 ml) containing 4.5 ml of modified Krebs solution⁹⁾ at 37 °C with bubbling of 95% O₂ and 5% CO₂ gas. The modified Krebs solution was changed at 30-min intervals over a total period of 120 min and extracted solution containing enzyme activity was used for the assay of Val-CHA-Arg-pNA amidolytic activity. The final concentrations of chondroitin sulfate and heparin were 0.02% (w/v) in arginine amidolytic activity measurement.

Affinity Columns The lysine, antithrombin III and anti-t-PA affinity columns for use in separations of plasminogen/plasmin, thrombin (EC 3.4.21.5) and t-PA were prepared by respective coupling of the above mentioned materials (ligand) and Formyl-Cellulofine gel at pH 7.5 for 8 h, with a

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Fig. 1. Effect of Heparin on the Level of Arginine Amidase Activity Released from Isolated Rabbit Arteries

Val-CHA-Arg-pNA amidolytic activity was expressed in terms of nmol/min/mg of wet artery weight. 1: ear artery; 2: upper part of aorta; 3: lower part of aorta. A: without heparin; B: with heparin.

small amount of sodium borohydride (NaBH_4) as reducing reagent. After the coupling reaction, a small amount of 1 mol/l Tris-HCl buffer at pH 7.5 was added to a suspension of the gel, and this mixture was reacted for 2 h with a small amount of NaBH_4 . The suspended gels coupled with ligand were then packed into columns, and these Cellulofine columns for affinity chromatography were individually washed with large volumes of 0.05 mol/l Tris-HCl buffer at pH 8.5 containing 0.15 mol/l NaCl. These affinity columns equilibrated with the above mentioned buffer were stored in a refrigerator (4°C) until use.

Materials All chemicals, substrates, and other materials used in this investigation including chondroitin sulfate and heparin were of analytical grade and medical products, and were obtained from commercial vendors.

Statistical Analysis Data was shown as mean $\pm$ S.D. and the *t*-tests were carried out using the method of the parametric test with Stat View J 4.5 software for use with a Macintosh computer.

RESULTS

Effect of Heparin on the Level of Arginine Amidase Activity Released from Isolated Rabbit Arteries The level of arginine amidase activity from isolated rabbit arteries with (B) and without (A) addition of heparin was measured (Fig. 1) and the overall results are summarized in Table 1. The aorta was divided into the upper portion (heart side) and lower portion (diaphragm side) for this analysis.

The release of arginine amidase from the ear artery after addition of heparin increased in 4 out of 6 tests conducted, while some decline was observed in the other 2 tests (Fig. 1-1). The average increase in arginine amidase activity after the heparin treatment in the 6 experiments was 3.56 ± 3.66 times, and the ratio of the mean value with heparin *versus* that without heparin (B/A value in Table 1) was 3.96 fold, neither of which was a significant difference (Table 1). The release of arginine amidase activity from aortas increased after the addition of heparin for 5 out of 7 cases in the upper aortas (Fig. 1-2), while 3 out of 7 in the lower aortas (Fig. 1-3). No significant differences were observed in either the upper or lower

Table 1. Effect of Heparin and Chondroitin Sulfate on the Level of Amidolytic Activity Released from Isolated Rabbit Arteries

	Ear artery	Upper part of aorta	Lower part of aorta
Heparin			
Number of experiments (n)	6	7	7
Amidolytic activity			
A: without heparin	0.87 ± 0.27	1.89 ± 1.68	2.59 ± 1.90
B: with heparin	3.45 ± 3.16	2.26 ± 1.13	1.98 ± 1.2
B/A	3.96	1.20	0.76
Average of B/A calculated for each sample	3.56 ± 3.66	1.56 ± 1.10	0.89 ± 0.32
Chondroitin sulfate			
Number of experiments (n)	9	8	8
Amidolytic activity			
A: without chondroitin sulfate	2.49 ± 2.74	2.73 ± 4.37	3.48 ± 5.03
B: with chondroitin sulfate	6.58 ± 7.37	5.21 ± 8.23	3.60 ± 5.33
B/A	2.65	1.90	1.03
Average of B/A calculated for each sample	3.94 ± 0.58	2.63 ± 2.03	1.75 ± 0.99

Val-CHA-Arg-pNA was used as the substrate and the amidolytic activity was expressed in terms of nmol/min/mg of wet artery weight.

Fig. 2. Effect of Chondroitin Sulfate on the Level of Arginine Amidase Activity Released from Isolated Rabbit Arteries

Val-CHA-Arg-pNA amidolytic activity was expressed in terms of nmol/min/mg of wet artery weight. 1: ear artery; 2: upper part of aorta; 3: lower part of aorta. A: without chondroitin sulfate; B: with chondroitin sulfate.

aortas. The average increase in arginine amidase activity, based on the average of experiments, was 1.56 ± 1.10 for the upper aortas and 0.89 ± 0.32 for the lower, and the ratio to the mean values with and without heparin was 1.20 and 0.76, respectively (Table 1). Thus the activity released from the lower aortas was below both the mean values.

Effect of Chondroitin Sulfate on the Level of Arginine Amidase Activity Released from Isolated Rabbit Arteries Figure 2 shows the results of measurement of the effects of chondroitin sulfate on the level of arginine amidase activity released from isolated rabbit arteries, and a summary of the results is also presented in Table 1.

The release of the activity from the ear artery increased markedly after chondroitin sulfate was added, except in 1 case in which no release of Val-CHA-Arg-pNA amidolytic activity was observed; significant differences were found at a 5% level (Fig. 2-1). In the upper aorta, an increase in the arginine amidase activity after addition of chondroitin sulfate was observed in every case (Fig. 2-2). While 6 out of 8 cases

Fig. 3. Profiles of Affinity Adsorption and Elution Using Lysine (A) and Anti Thrombin III (B) Columns

↓, elution with pH 2.0 HCl solution; ●, absorbance at 280 nm.

Fig. 4. Profiles of Affinity Adsorption and Elution Using an Anti-t-PA Affinity Column

↓, elution with pH 2.0 HCl solution; ●, absorbance at 280 nm; ○, Val-Leu-Arg-pNA amidolytic activity; □, Boc-Leu-Gly-Arg-pNA amidolytic activity.

using lower aortas showed an increase in the activity, 2 cases showed some decline (Fig. 2-3). No significant difference was observed between chondroitin sulfate treated and non-treated preparation. The average increase of activity was 3.94 ± 0.58 fold for the ear artery, 2.63 ± 0.03 fold for the upper aortas and 1.75 ± 0.99 fold for the lower aortas. The ratios of the mean value with and without chondroitin sulfate treated in the these arteris (B/A value in Table 1) were 2.65, 1.91 and 1.03 times, respectively.

Analysis of Arginine Amidase Activity Released from Isolated Rabbit Aorta after the Addition of Chondroitin Sulfate To identify the types of arginine amidase activity extracted from isolated rabbit aorta when chondroitin sulfate was added, affinity adsorption analysis was conducted using connected lysine and antithrombin III columns, equilibrated with 0.05 mol/l Tris-HCl buffer at pH 8.5 containing 0.05 mol/l NaCl and 100 international units (IU)/ml of heparin. An anti-t-PA affinity column, equilibrated with 0.05 mol/l Tris-HCl buffer at pH 8.5 containing 0.05 mol/l NaCl, was also used for the analysis. Typical elution profiles of these three chromatographs are respective shown in Fig. 3A (lysine affinity adsorption), Fig. 3B (antithrombin III affinity adsorption) and Fig. 4 (anti-t-PA affinity adsorption). Plasminogen (relative enzyme activity as measured by addition of 25 units of human urokinase, EC 3.4.21.73) and plasmin-like enzyme released from isolated rabbit aorta were adsorbed to a lysine

affinity column, as shown in Fig. 3A. Two plasminogen activators showing Boc-Leu-Gly-Arg-pNA amidolytic activity were also adsorbed to the anti-t-PA affinity column (Fig. 4). No arginine amidolytic activity in the extract of aorta treated with chondroitin sulfate was adsorbed the antithrombin III affinity adsorption column (with 100 IU/ml of heparin solution). Thus, no thrombin (EC 3.4.21.5)-like enzyme was identified from isolated rabbit aorta after treatment with chondroitin sulfate (Fig. 3B).

DISCUSSION

We have already indicated that isolated rabbit arteries secreted various types of arginine and lysine amidase activities, and approximately 40 to 50% were trypsin-like enzymes with LBTI and aprotinin affinity chromatography columns.^{1,2)} We suspect that these proteinases play roles in the local regulation of blood coagulation and fibrinolysis as well as heat release from rabbit ear arteries by mean of regulating blood flow through them. However, the release of thrombin, a key enzyme of blood coagulation, from rabbit arteries has not been confirmed,¹⁾ so that the role of proteinases in the local blood coagulation system remains unknown at present. In fact, the present study failed to identify any thrombin-like enzymes secreted from isolated rabbit ear arteries treated with chondroitin sulfate (Fig. 4).

Some differences between the amidolytic enzyme activities released from rabbit ear arteries and aorta were detected (Table 1). We believe that differences in the relative activities in these two preparations reflect different proteinase induction effects of heparin and chondroitin sulfate in the ear arteries and aorta. Somewhat different effects of heparin (Fig. 1) and chondroitin sulfate (Fig. 2) on levels of amidolytic enzyme activity released from rabbit arteries were also observed. These results may suggest that the structure of the dextran sulfate derivatives containing sugar content is influenced considerably.

Dermatan sulfate, one type of dextran sulfate, reportedly enhances the release of plasminogen activator from rat hindquarters.⁶⁾ On the other hand, vascular endothelial cells are thought to produce a heparin-like substance and to regulate the blood coagulation process in conjugation with thrombin and/or anti-thrombin III.^{5,10)} Thus, we examined whether dextran sulfates accelerate the secretion of arginine amidase activity from arteries. The present results showed that, while heparin apparently increased the release of arginine amidase activity from some isolated rabbit ear artery preparation, no significant effect was identified (Fig. 1). In contrast, it was revealed the chondroitin sulfate worked to accelerate the above secretion from isolated rabbit arteries (Fig. 2). In particular, the effect was notable for the secretion from ear arteries with significant differences at the 5% level (Fig. 2-1), concerning rat hindquarters and that of Marzia *et al.*⁶⁾ concerning the effects of dermatan sulfate.

Ferdander *et al.*¹¹⁾ reported that the dermatan sulfate increases fibrinolytic activity in various experimental models of thrombosis. The present research confirmed the presence of a plasminogen/plasmin-like enzyme and plasminogen activators in the arginine amidase activity released from isolated rabbit arteries (Fig. 3A and C, respectively), but no thrombin-like enzyme was detected (Fig. 3B). We previously reported that the urokinase had a weak affinity to an anti-t-PA when t-PA was measured by enzyme immunoassay.¹²⁾ In the present study, two forms of plasminogen activator activities were obtained from an anti-t-PA affinity adsorption column (Fig. 4), suggesting that both urokinase type and t-PA type plasminogen activators were secreted from isolated rabbit

arteries. This result also suggests that the latter and former peaks of plasminogen activator-like amidolytic activity from the anti-t-PA affinity adsorption column are t-PA and urokinase, respectively, because the latter eluting activity had stronger affinity to the anti-t-PA column than did the former. Overall evaluation of the present results together with those cited above^{6,12)} clearly suggests that chondroitin sulfate so added acts to accelerate the secretion of plasminogen activators and plasminogen/plasmin from rabbit arteries and thereby activates the fibrinolytic system. The effect of heparin accelerating the release of thrombin^{4,5)} was not confirmed under the present experiment (Fig. 3B). Thus, the addition of heparin does not appear to accelerate the release of arginine amidase activity in the absence of thrombin. We will conduct further research on this topic, together with similar research on dermatan sulfate and anti thrombin III, to clarify their roles in the process.

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