J. Biochem. 2009;146(1)43–49
doi:10.1093/jb/mvp045

Re-Evaluation of M-LAO, L-Amino Acid Oxidase, from the Venom of *Gloydius blomhoffi* as an Anticoagulant Protein

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Received February 9, 2009; accepted February 26, 2009; published online March 11, 2009

Many anticoagulant proteins have been found from snake venoms. Recently, L-amino acid oxidase (LAO) from the venom of *Gloydius blomhoffi*, M-LAO, was reported to inhibit coagulation factor IX; however, the mechanism of its anticoagulant activity is still unclear. Here, we re-evaluated the anticoagulant activity of M-LAO. We first purified M-LAO from the venom of *G. blomhoffi*, and examined the effect of LAO inhibitors and the hydrogen peroxide scavenger, catalase, on the anticoagulant activity of M-LAO. We found that the isolated M-LAO fraction prolongs the APTT, PT and fibrinogen clotting time and cleaves the Aα-chain of fibrinogen. LAO inhibitors or catalase did not inhibit these effects. Detailed analysis revealed that the M-LAO fraction contained a small amount of 39-kDa metalloproteinase. The prolongation of clotting time and degradation of fibrinogen were inhibited by a metalloproteinase inhibitor. Therefore, we concluded that the anticoagulant activity of the M-LAO fraction was caused by the 39-kDa metalloproteinase.

Key words: anticoagulant, L-amino acid oxidase, blood coagulation, factor IX, snake venom.

Abbreviations: APTT, activated partial thromboplastin time; LAO, L-amino acid oxidase; IX/X-bp, blood coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*; PT, prothrombin time.

Blood coagulation is initiated when the wall of a blood vessel is damaged (*1*). Tissue factor binds to the activated form of factor VII (factor VIIa) and forms the factor VIIa-tissue factor complex, which activates factor IX to activated factor IX (factor IXa). Factor IXa forms the Xase complex with activated factor VIII and phospholipid in the presence of calcium ions and magnesium ions (*2*, *3*), and the complex converts factor X into its active form. Activated factor X (factor Xa) forms a prothrombinase complex with activated factor V and phospholipid in the presence of calcium ions, which converts prothrombin into α-thrombin. Finally, fibrinogen, a key protein for blood clotting, is converted into fibrin by α-thrombin. Fibrinogen consists of three non-identical chains, Aα, Bβ and γ, and is cleaved at the N-terminus of Aα- and Bβ-chains when fibrinogen is converted into fibrin by α-thrombin (*4*, *5*). Fibrin is finally cross-linked by factor XIII to form a stable clot.

Some snake venoms contain several anticoagulant proteins such as C-type lectin-like proteins, phospholipase A_2s, metalloproteinases and serine proteases (*6*, *7*). Enzymatic proteins such as phospholipase A_2, metalloproteinase and serine protease degrade blood coagulation factors or co-factors which participate in blood coagulation (*6*, *8*). For example, snake venom phospholipase A_2s are thought to exhibit anticoagulant activity by disrupting phospholipid membranes (*6*, *8*). Metalloproteinases and serine proteases are known to cleave the Aα-chain or Bβ-chain of fibrinogen causing a failure to form fibrin clots (*9*). On the other hand, non-enzymatic proteins, such as C-type lectin-like proteins, directly bind to blood coagulation factors (*6*, *8*). For example, IX/X-bp, a C-type lectin-like protein, binds to the Gla domain of factor IX and factor X. The Gla domain, which is highly coordinated with calcium and magnesium ions (*10*), is the binding site for phospholipids. Therefore, IX/X-bp exhibits anticoagulant activity through inhibiting the formation of the complex between factor IX or factor X and the phospholipid membrane (*11*).

L-Amino acid oxidase (LAO) is a dimeric glycosylated flavoenzyme that catalyses the oxidative deamination of an L-amino acid substrate to an α-keto acid along with the production of ammonia and hydrogen peroxide (*12*). LAO is one of the major components in snake venoms and has various biological activities, including the induction of apoptosis, oedema and haemolysis, and effects on platelet function (*12*). Most of these toxicities are inhibited by catalase, a scavenger for hydrogen peroxide, indicating that hydrogen peroxide is responsible for those activities. M-LAO, an LAO from the venom of *Gloydius blomhoffi* (formerly *Agkistrodon halys blomhoffi*; nomenclature of venomous snakes in this study is referred to the NCBI Entrez Taxonomy Browser.), has been recently reported to induce platelet aggregation and exhibit anticoagulant activity (*13*, *14*). LAO was reported to be a new member of the anticoagulant proteins (*6*, *8*, *14*). Interestingly, M-LAO was also shown to prolong activated partial thromboplastin time (APTT), but not prothrombin time (PT), and detailed analyses showed that it drastically inhibits the pro-coagulant activity of

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factor IX. Although previous studies have mentioned that hydrogen peroxide produced by LAO enzymatic activity might be involved in the anticoagulant activity of M-LAO, the molecular mechanism for the inhibition of factor IX by M-LAO is still unclear. In this study, we have attempted to clarify the molecular mechanism of anticoagulant activity of M-LAO. A preliminary report of this work appeared at the 30th Congress of the Japanese Society on Thrombosis and Hemostasis (*15*).

MATERIALS AND METHODS

Materials—Lyophilized venom of *G. blomhoffi* was gifted from Dr. Sadaaki Iwanaga, and purchased from the Japan Snake Institute. Catalase from human erythrocytes and bovine fibrinogen were purchased from Sigma-Aldrich. Horseradish peroxidase was purchased from Zymed laboratories. The LAO inhibitor, *o*-aminobenzoic acid and the metalloproteinase inhibitor, *o*-phenanthroline, were purchased from Wako Pure Chemical Industries. Human control plasma was purchased from Eisai. Dade Actin reagent and Thromborel S reagent were purchased from Dade Behring.

LAO Purification—Purification of M-LAO was performed by using essentially equal chromatographic steps (*13*). Lyophilized venom measuring 200 mg was dissolved in 20 mM imidazole-HCl buffer (pH 6.0). Insoluble material was removed by centrifugation at 15,000 r.p.m. for 15 min at 4°C. The supernatant was applied onto a Hiload Superdex 200 prep grade column (ϕ 2.6 × 60 cm). Fractions, which exhibited LAO activity, were collected and applied onto a Hiload SP-Sepharose High Performance column (ϕ 1.6 × 10 cm) which was equilibrated with the same buffer. Fractions with LAO activity were collected, dialysed against 50 mM Tris–HCl buffer (pH 8.0), and applied onto a Hiload Q-Sepharose High Performance column (ϕ 1.6 × 10 cm) equilibrated with the same buffer. Bound proteins were eluted by a linear gradient of the same buffer containing 1 M NaCl. Fractions with LAO activity were collected, dialysed against 5 mM sodium phosphate buffer (pH 6.8), and applied onto a hydroxyapatite column, CHT2-I, which was equilibrated with the same buffer. Bound proteins were eluted by a linear gradient of 250 mM sodium phosphate buffer (pH 6.8). M-LAO fractions were eluted at a concentration of ~100 mM phosphate ion. After dialysis with 50 mM Tris-buffered saline (pH 7.4), the M-LAO fraction was used for assays.

Assay for LAO Enzymatic Activity—Fifty microlitres of eluted fractions was incubated with 50 mM Tris-buffered saline (pH 7.4) containing 2 mM DL-norleucine for 10 min at 37°C. After the incubation, 50 µl of the reaction mixture [0.5 M citrate buffer (pH 5.5) containing 1 mg/ml *o*-phenylenediamine and 0.1 µg/ml peroxidase] was added. LAO activity was determined by the production of hydrogen peroxide detected by the optical density at 405 nm.

Assays for APTT, PT and Fibrinogen Clotting Time—APTT, PT and fibrinogen clotting time assays were used to evaluate the anticoagulant activity of the M-LAO fraction. First, 40 µl of human standard plasma and 40 µl of the M-LAO fraction were incubated for 30 min at 37°C. Subsequently, for APTT, 40 µl of Dade Actin reagent was added to the mixture and incubated for 2 min. After the incubation, 40 µl of 20 mM $CaCl_2$ was added and the clotting time was recorded. For PT, after incubation of human standard plasma and the M-LAO fraction, 80 µl of Thromborel S reagent was added to the mixture and the clotting time was recorded. For fibrinogen clotting time, 1.7 mg/ml fibrinogen, 3 mM $CaCl_2$ and the M-LAO fraction were mixed and incubated for 30 min at 37°C. After incubation, 1 U/ml thrombin was added and clotting time was recorded using an Amelung coagulometer. Inhibitors, such as *o*-aminobenzoic acid and *o*-phenanthroline, and catalase were added at final concentrations of 7.5 mM, 3 mM and 100 U/ml, respectively.

Surface Plasmon Resonance Analysis—Surface plasmon resonance analysis was performed using a Biacore 3000. Isolated factor IX diluted with 10 mM sodium acetate (pH 4.5) was immobilized on a CM5 sensor chip. A measure of 10 mM HEPES-buffered saline (pH 7.4), containing 0.005% Surfactant P-20 and 5 mM $CaCl_2$, was used for the elution buffer. Analytes were diluted with elution buffer and injected into the system at a flow rate of 20 µl/min.

Analysis for Degradation of Fibrinogen—A mixture of 1 mg/ml fibrinogen and 1.25 µM M-LAO fraction were incubated for 30 min at 37°C. Subsequently, the mixture was electrophoresed on 12.5% SDS–polyacrylamide gel. Inhibitors, such as *o*-aminobenzoic acid and *o*-phenanthroline, and catalase were added at concentrations of 12 mM, 3 mM and 100 U/ml, respectively.

RESULTS

Purification of LAO from the Venom of G. blomhoffi—In order to elucidate its anticoagulant mechanism, we purified M-LAO from the crude venom of *G. blomhoffi* by four successive chromatographic steps according to the previous method (*13*): gel-filtration, cation-exchange, anion-exchange and hydroxyapatite chromatography (see MATERIALS AND METHODS section and Supplementary Fig.1). LAO-containing fractions were traced through LAO enzymatic activity and the absorbance at 450 nm. Finally, 2 mg of 60-kDa protein, which possessed LAO enzymatic activity, was obtained from 200 mg of crude venom. As the N-terminal twenty amino acids of the 60-kDa protein were identical to that of M-LAO (Fig. 1), we concluded that this 60-kDa protein was M-LAO.

Analyses of the Anticoagulant Activity of the M-LAO Fraction—We analysed the anticoagulant activity of the M-LAO fraction. Sakurai *et al*. (*14*) have previously shown that M-LAO prolongs APTT, but not PT, and that the anticoagulant activity of M-LAO is induced by specific inhibition of factor IX. We first evaluated the anticoagulant activity of the M-LAO fraction by measuring APTT. As demonstrated in the previous study (*14*), the M-LAO fraction prolonged APTT (Fig. 2). Several biological activities of LAOs, such as activation or inhibition of platelet aggregation and induction of apoptosis, are inhibited by the hydrogen peroxide scavenger, catalase (*13*, *16*, *17*), indicating that hydrogen

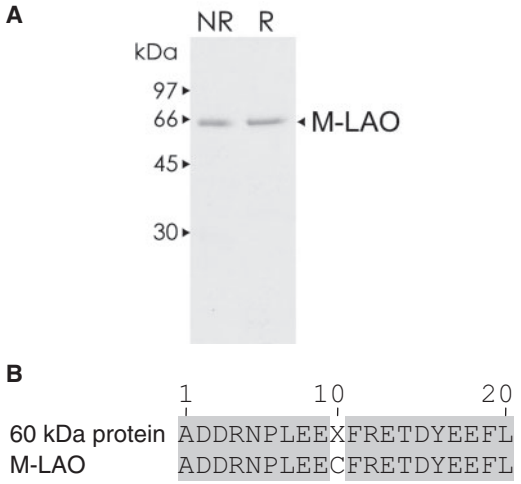

Fig. 1. **Isolation and identification of M-LAO from the venom of *G. blomhoffi*.** (A) One microgram of M-LAO fraction was electrophoresed on a 15% SDS–polyacrylamide gel. NR, non-reduced condition; R, reduced condition. (B) Analysis for N-terminal amino acid sequence of 60-kDa protein. The N-terminal 20 amino acids of the 60-kDa protein were identical to those of M-LAO except for the 10th cysteine residue.

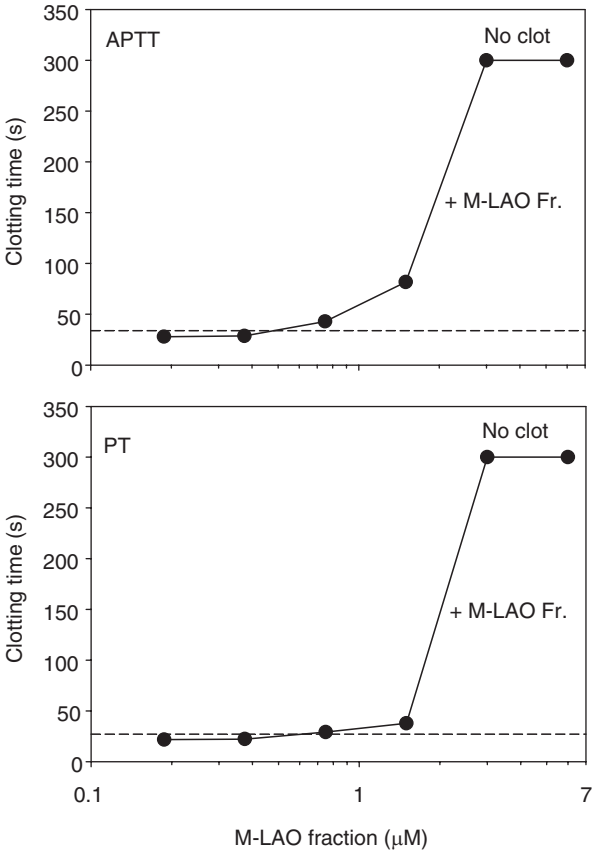

Fig. 2. **Anticoagulant activity of M-LAO fraction.** The M-LAO fraction was incubated with human standard plasma. Control data is represented as dashed lines. Anticoagulant activity of the M-LAO fraction was observed at a concentration of 1.5 μM. M-LAO fraction of 2 μM or above completely inhibited clot formation.

peroxide participates in the biological activity of LAOs. We next examined whether the prolongation of clotting time is induced by hydrogen peroxide generated by LAO. We found that catalase does not inhibit prolongation of APTT (Fig. 3), indicating that hydrogen peroxide does not take part in the anticoagulant activity of the M-LAO fraction. We next examined the binding ability of M-LAO to factor IX in the presence of calcium and magnesium ions using Biacore. We immobilized factor IX on a CM5 sensor chip. Although the factor IX- and X-binding protein from Habu snake, IX/X-bp, could bind to immobilized factor IX, no specific binding was observed on the M-LAO fraction (Supplementary Fig. 2). These results indicate that the mechanism for the anticoagulant activity of M-LAO fraction is not due to binding to factor IX.

Unexpectedly, we also found that the M-LAO fraction prolonged PT (Fig. 2) and that catalase did not inhibit the prolongation of PT as well as APTT (Fig. 3). These results suggest that the prolongation of clotting time is due to inhibition of coagulation factors downstream of factor IX, such as factor X, prothrombin and fibrinogen. We examined the specificity of M-LAO for factor IX. The M-LAO fraction was pre-incubated with isolated factor IX or factor IX-deficient plasma. After the incubation, factor IX-deficient plasma or isolated factor IX was added to the mixture, and their clotting times were measured (Fig. 4). As a result, the M-LAO fraction prolonged APTT when it was pre-incubated with factor IX-deficient plasma, indicating that the M-LAO fraction inhibits the component in factor IX-deficient plasma. On the other hand, no difference in the prolongation of clotting time was observed between the M-LAO fraction and the control when the M-LAO fraction was pre-incubated with isolated factor IX. These results indicate that the anticoagulant activity of the M-LAO fraction is not specific for factor IX, and is induced through inhibition of factors downstream of factor IX. Taken together, we concluded that the mechanism for the anticoagulant activity of the M-LAO fraction is neither hydrogen peroxide nor binding to factor IX, and considered that the M-LAO fraction exhibits anticoagulant activity through inhibiting factors downstream of factor IX.

The M-LAO Fraction Degrades Fibrinogen—We next tested whether the M-LAO fraction affects fibrinogen-clotting time. As shown in Fig. 5A, the M-LAO fraction prolonged fibrinogen-clotting time in a concentration-dependent manner, indicating that it might target fibrinogen. We further examined the molecular alteration of fibrinogen by the addition of the M-LAO fraction using SDS–PAGE. SDS–PAGE analysis revealed that the M-LAO fraction cleaves the Aα-chain of fibrinogen (Fig. 5B, lane 2). Although the LAO inhibitor, *o*-aminobenzoic acid, slightly inhibited the degradation of fibrinogen (Fig. 5B, lane 4), another LAO inhibitor, *m*-chlorobenzoate, did not inhibit the degradation of fibrinogen (Supplementary Fig. 3, lane 4). In addition,

catalase did not inhibit the fibrinogenolytic activity of the M-LAO fraction (Fig. 5B, lane 3). These results indicate that the fibrinogenolytic activity of the M-LAO fraction is independent of either LAO enzymatic activity or generated hydrogen peroxide. Since many snake venoms contain fibrinogenolytic metalloproteinases, we examined whether the fibrinogenolytic activity of the M-LAO fraction is caused by fibrinogenolytic metalloproteinase. We found that *o*-phenanthroline, a metalloproteinase inhibitor, completely inhibits the degradation of fibrinogen by the M-LAO fraction (Fig. 5B, lane 5). Furthermore, *o*-phenanthroline neutralized the prolongation of APTT (Fig. 5C, closed square), indicating that the degradation of fibrinogen is caused by a certain metalloproteinase. We next examined whether the M-LAO fraction contains metalloproteinase. SDS–PAGE analysis revealed that the M-LAO fraction contained a small amount of a protein with a molecular mass of 39 kDa (Fig. 6A). The N-terminal amino acid sequence of the 39-kDa protein was homologous to that of metalloproteinase from *Gloydius blomhoffi brevicaudus*, which is a very closely related species of *G. blomhoffi* (Fig. 6B). These results suggest that the 39-kDa metalloproteinase in the M-LAO fraction cleaves the Aα-chain of fibrinogen, and that the anticoagulant effect of the M-LAO fraction is due to this proteinase, and not M-LAO.

We further fractionated M-LAO and the 39-kDa metalloproteinase by hydroxyl apatite chromatography, and examined their effects on APTT, PT, and fibrinogen molecules (Fig. 7). As shown in Fig. 7A, fraction numbers 98 and 99, that scarcely contained 39-kDa metalloproteinase, did not prolong either APTT or PT. Moreover, the degradation of fibrinogen and the prolongation of APTT and PT were dependent on the concentration of the 39-kDa metalloproteinase. These results indicate that the anticoagulant effect of the M-LAO fraction is induced by the 39-kDa metalloproteinase, and not M-LAO.

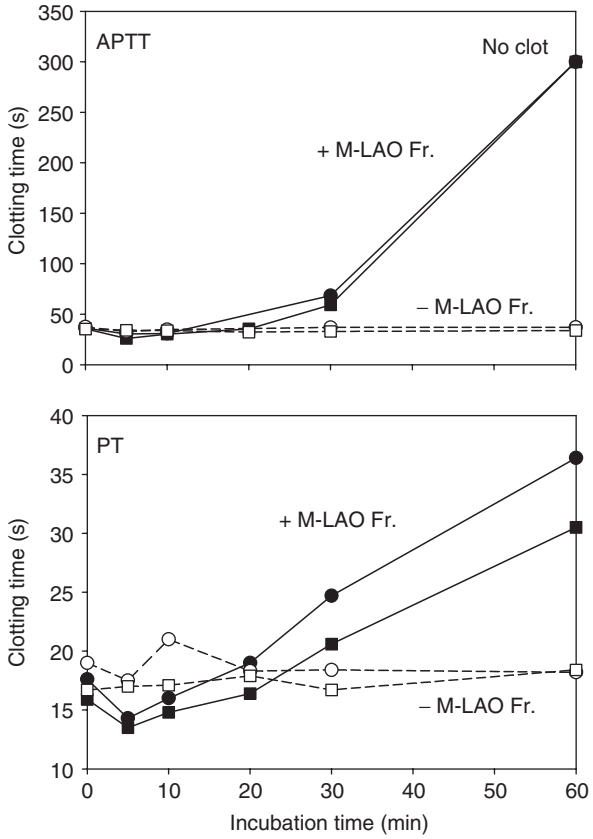

Fig. 3. **Catalase did not affect the anticoagulant activity of the M-LAO fraction.** TBS (pH 7.4) without or with catalase (open circle or open square), or 750 nM of M-LAO fraction without or with catalase (closed circle or closed square) was incubated with human standard plasma for 0–60 min. Catalase itself did not affect clot formation, and did not inhibit the anticoagulant activity of the M-LAO fraction.

DISCUSSION

Enzymatic Activity of LAO and Hydrogen Peroxide—M-LAO from the venom of *G. blomhoffi* (formerly *A. halys blomhoffi*) was characterized as a novel factor IX-specific anticoagulant protein in 2003 (*14*). LAOs have been reported to be a new family of anti-platelet proteins with a mechanism distinct from other established mechanisms (*6*, *8*). Although it has been reported that hydrogen peroxide, a product of the LAO enzymatic

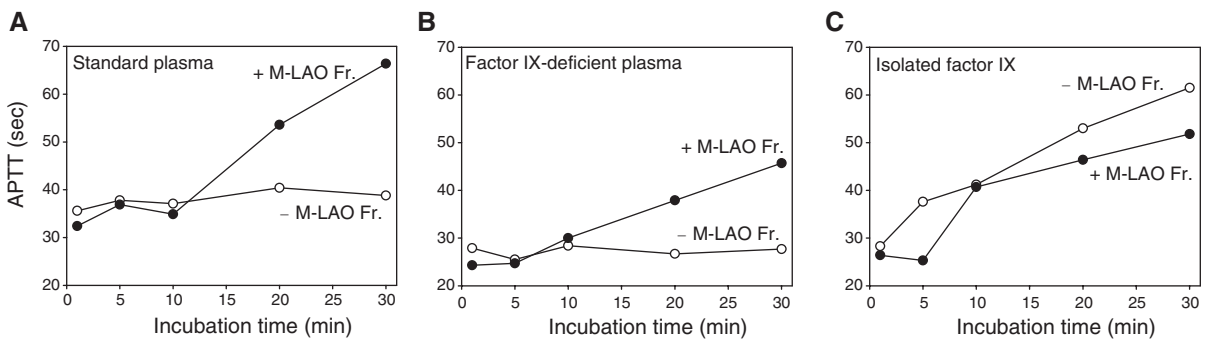

Fig. 4. **Pre-incubation of M-LAO fraction with factor IX-deficient plasma prolonged APTT.** TBS (pH7.4) (open circle) or 1 μM of M-LAO fraction (closed circle) was incubated with human standard plasma (A), factor IX-deficient plasma (B) or isolated factor IX (C) for 1–30 min. The M-LAO fraction prolonged APTT when it was pre-incubated with human standard plasma (A) or factor IX-deficient plasma (B). There was no difference in the prolongation of APTT between the pre-incubation of isolated factor IX with M-LAO fraction or TBS (pH 7.4) (C).

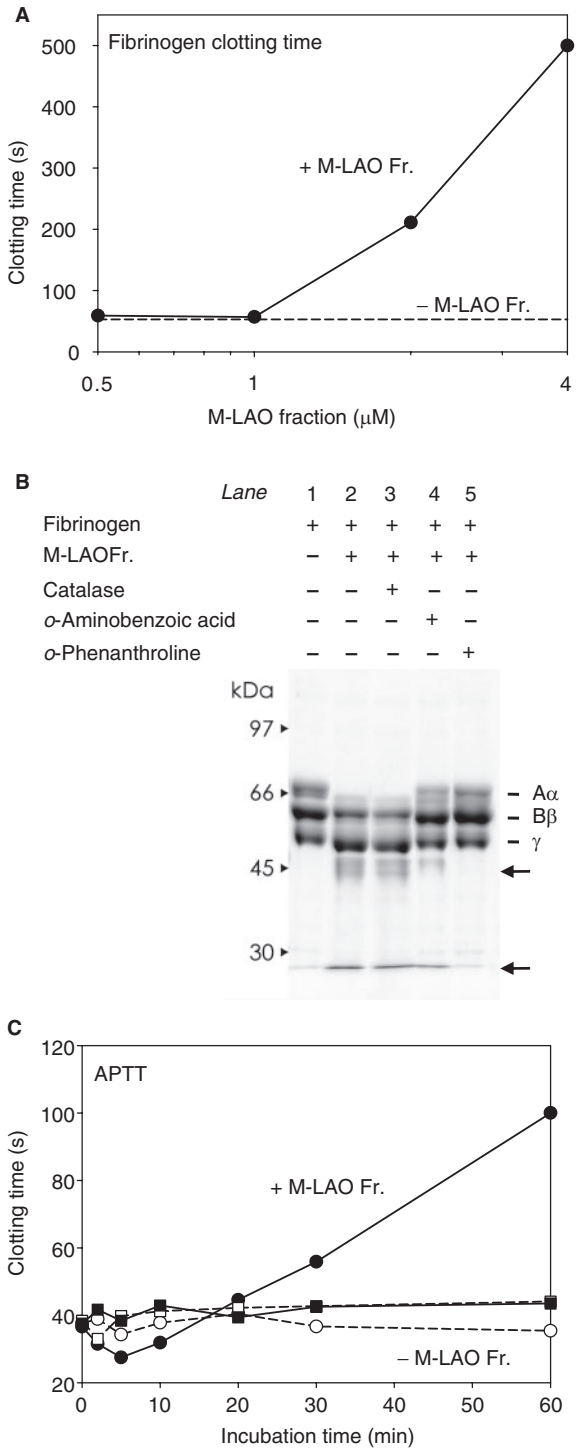

Fig. 5. **Anticoagulant activity of M-LAO fraction is mediated by the degradation of fibrinogen.** (A) The M-LAO fraction prolonged fibrinogen-clotting time. Control data are represented as a dashed line. (B) The M-LAO fraction degraded the Aα-chain of fibrinogen. Degraded products are indicated by arrows. (C) 750 nM of the M-LAO fraction or TBS (pH 7.4) was incubated with human standard plasma in the presence of *o*-phenanthroline for 0–60 min. TBS (pH 7.4) without *o*-phenanthroline (open circle), M-LAO fraction without *o*-phenanthroline (closed circle), TBS (pH 7.4) with *o*-phenanthroline (open square) and M-LAO fraction with *o*-phenanthroline (closed sqaure).

reaction, participates in the anticoagulant activity of M-LAO, the molecular mechanism was still unclear. Here, we had hypothesized that hydrogen peroxide produced by the LAO enzymatic reaction is responsible for anticoagulant activity, because hydrogen peroxide participates in other biological activities of LAOs, such as the induction of apoptosis and effects on platelet aggregation. In this study, we found that catalase does not inhibit the prolongation of APTT and PT (Fig. 3), indicating that hydrogen peroxide is not involved in the anticoagulant activity of the M-LAO fraction. In addition, LAO inhibitors, *o*-aminobenzoic acid and *m*-chlorobenzoic acid, and catalase did not inhibit the degradation of fibrinogen (Fig. 5 and Supplementary Fig. 3), indicating that LAO activity and hydrogen peroxide produced by LAO activity are independent of the fibrinogenolytic activity of the M-LAO fraction. We also found that the degradation of fibrinogen and the prolongation of APTT were inhibited by a metalloproteinase inhibitor, *o*-phenanthroline. As *o*-phenanthroline did not inhibit LAO enzymatic activity (Supplementary Fig. 4), we concluded that the anticoagulant activity of the M-LAO fraction is due to the degradation of fibrinogen by metalloproteinase activity, and not LAO activity or hydrogen peroxide.

Fibrinogenolytic Activity is Mediated by Metalloproteinase but not M-LAO—We focused on fibrinogen instead of factor IX, because both APTT and PT were prolonged by the addition of the M-LAO fraction (Fig. 2). As shown in Fig. 5, the M-LAO fraction prolonged fibrinogen-clotting time and cleaved the Aα-chain of fibrinogen. In addition, a metalloproteinase inhibitor, *o*-phenanthroline, completely inhibited the degradation of fibrinogen and the prolongation of APTT. We found that the M-LAO fraction contains a slight amount of a 39-kDa metalloproteinase which is homologous to the fibrinogenolytic metalloproteinase, Mt-d, from *G. blomhoffi brevicaudus* (Fig. 6). *Gloydius blomhoffi brevicaudus* is a very closely related species of *G. blomhoffi*, and Mt-d has been reported to be a fibrinogenolytic metalloproteinase (*18*). Therefore, it is possible for the 39-kDa metalloproteinase in the M-LAO fraction to exhibit fibrinogenolytic activity. We also demonstrated that the fibrinogenolytic activity and the anticoagulant activity are dependent on the concentration of the 39-kDa metalloproteinase, but not on M-LAO (Fig. 7). Taken together, we concluded that the anticoagulant activity and the fibrinogenolytic activity of the M-LAO fraction are due to the 39-kDa metalloproteinase and not M-LAO.

A previous study showed that M-LAO prolongs APTT at around sub-nanomolar concentrations (*14*), while a 10 times higher concentration of M-LAO was required for anticoagulant activity in our study. In addition, Sakurai *et al*. (*14*) examined the anticoagulant activity of M-LAO with incubation for 1 min. However, we did not observe the prolongation of APTT and PT under the same conditions. Incubation for over 20 min was required to prolong APTT and PT in our study (Supplementary Fig. 5). Furthermore, we could not find any expressed sequence tags coding for another LAO, although we comprehensively examined the gene expression in the

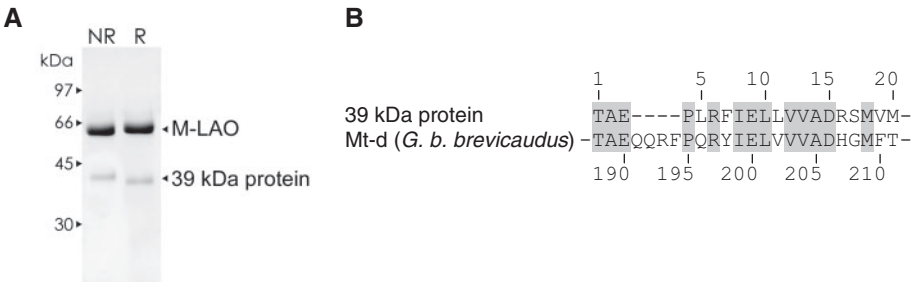

Fig. 6. **Identification of 39-kDa protein in the M-LAO fraction.** (A) M-LAO fraction of 2.6 μg was electrophoresed on a 15% SDS–polyacrylamide gel. NR, non-reduced condition; R, reduced condition. (B) Analysis for N-terminal amino acid sequence of 39-kDa protein. Twenty amino acid residues were sequenced. The N-terminal amino acid residues of 39-kDa protein were homologous to those of Mt-d, a metalloproteinase from *Gloydius blomhoffi brevicaudus*.

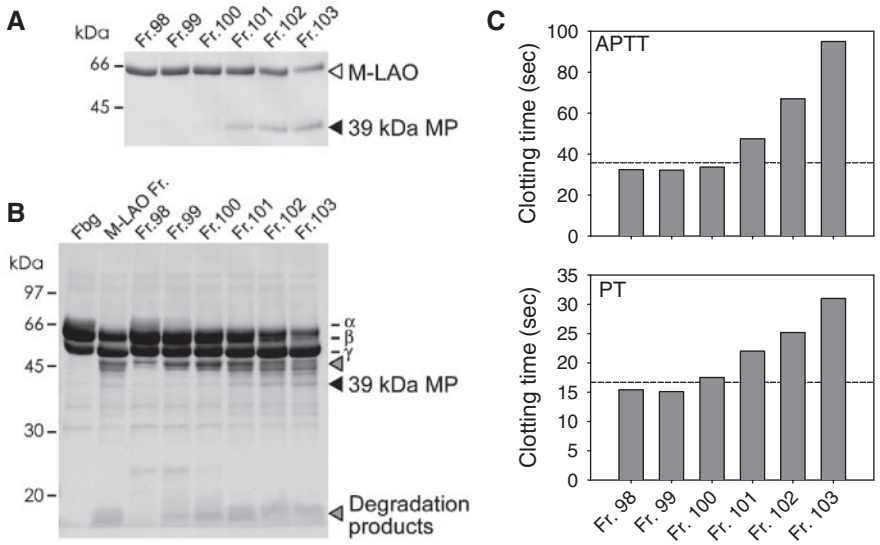

Fig. 7. **Fibrinogenolytic and anticoagulant activity of M-LAO fractions.** (A) SDS–PAGE analysis of further purified M-LAO fractions. A 12.5% SDS–polyacrylamide gel was used. M-LAO and the 39-kDa metalloproteinase (39 kDa MP) are indicated by open arrowhead and closed arrowhead, respectively. (B) Fibrinogenolytic activity of M-LAO fractions. A 12.5% SDS–polyacrylamide gel was used. The 39-kDa metalloproteinase (39 kDa MP) and degradation products of fibrinogen are indicated by closed arrowhead and grey arrowhead, respectively. (C) Anticoagulant activity of M-LAO fractions. M-LAO fractions and human standard plasma were incubated for 30 min at 37°C. Control (TBS, pH 7.4) is shown by dashed lines.

venom gland of *G. blomhoffi* (unpublished data). Thus, we consider that M-LAO purified by us is identical to M-LAO purified by Sakurai *et al*. (*14*). Given these results, we concluded that the difference between the current and previous studies might be due to the amount of 39-kDa metalloproteinase in the purified M-LAO fraction.

In conclusion, we tried to elucidate the molecular mechanism for the anticoagulant activity of M-LAO using various inhibitors, such as LAO inhibitor, metalloproteinase inhibitor and hydrogen peroxide scavenger. We demonstrated that the anticoagulant activity of the M-LAO fraction is independent of either LAO enzymatic activity or generated hydrogen peroxide. In addition, we found that the M-LAO fraction exhibits fibrinogenolytic activity which is inhibited by a metalloproteinase inhibitor, and that the fibrinogenolytic activity of the M-LAO fraction is dependent on the concentration of the 39-kDa metalloproteinase in that fraction. Given these results, we concluded that the M-LAO fraction induces anticoagulant activity through the degradation of fibrinogen by a typical fibrinogenolytic 39-kDa metalloproteinase, but not M-LAO itself.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

ACKNOWLEDGEMENTS

We thank to Dr Sadaaki Iwanaga for providing lyophilized venom of *G. blomhoffi*.

FUNDING

Science Research Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan (to T.M.).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Butenas, S. and Mann, K.G. (2002) Blood coagulation. *Biochemistry (Mosc).* **67**, 3–12
2. Sekiya, F., Yamashita, T., Atoda, H., Komiyama, Y., and Morita, T. (1995) Regulation of the tertiary structure and function of coagulation factor IX by magnesium (II) ions. *J. Biol. Chem.* **270**, 14325–14331
3. Sekiya, F., Yoshida, M., Yamashita, T., and Morita, T. (1996) Magnesium(II) is a crucial constituent of the blood coagulation cascade. Potentiation of coagulant activities of factor IX by Mg^{2+} ions. *J. Biol. Chem.* **271**, 8541–8544
4. Mosesson, M.W. (2005) Fibrinogen and fibrin structure and functions. *J. Thromb. Haemost.* **3**, 1894–1904
5. Mosesson, M.W. (2008) Structure and functions of fibrinogen and fibrin in *Recent Advances in Thrombosis and Hemostasis* (Tanaka, K. and Davie, E.W., eds.) pp. 3–26, Springer, Tokyo
6. Yamazaki, Y. and Morita, T. (2007) Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. *Curr. Pharm. Des.* **13**, 2872–2886
7. Yamazaki, Y. and Morita, T. (2008) Snake venoms and other toxic components affecting thrombosis and hemostasis in *Recent Advances in Thrombosis and Hemostasis* (Tanaka, K. and Davie, E.W., eds.) pp. 462–482, Springer, Tokyo
8. Kini, R.M. (2006) Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochem. J.* **397**, 377–387
9. Swenson, S. and Markland, F.S., Jr. (2005) Snake venom fibrin(ogen)olytic enzymes. *Toxicon* **45**, 1021–1039
10. Shikamoto, Y., Morita, T., Fujimoto, Z., and Mizuno, H. (2003) Crystal structure of Mg^{2+}- and Ca^{2+}-bound Gla domain of factor IX complexed with binding protein. *J. Biol. Chem.* **278**, 24090–24094
11. Atoda, H., Yoshida, N., Ishikawa, M., and Morita, T. (1994) Binding properties of the coagulation factor IX/factor X-binding protein isolated from the venom of Trimeresurus flavoviridis. *Eur. J. Biochem.* **224**, 703–708
12. Du, X.Y. and Clemetson, K.J. (2002) Snake venom $_L$-amino acid oxidases. *Toxicon* **40**, 659–665
13. Takatsuka, H., Sakurai, Y., Yoshioka, A., Kokubo, T., Usami, Y., Suzuki, M., Matsui, T., Titani, K., Yagi, H., Matsumoto, M., and Fujimura, Y. (2001) Molecular characterization of $_L$-amino acid oxidase from Agkistrodon halys blomhoffii with special reference to platelet aggregation. *Biochim. Biophys. Acta* **1544**, 267–277
14. Sakurai, Y., Shima, M., Matsumoto, T., Takatsuka, H., Nishiya, K., Kasuda, S., Fujimura, Y., and Yoshioka, A. (2003) Anticoagulant activity of M-LAO, $_L$-amino acid oxidase purified from *Agkistrodon halys blomhoffii*, through selective inhibition of factor IX. *Biochim. Biophys. Acta* **1649**, 51–57
15. Fujisawa, D., Yamazaki, Y., and Morita, T. (2007) Analysis of the mechanism for anticoagulant activity of L-amino acid oxidase. *Jpn J. Thromb. Hemost.* **18**, 459
16. Li, Z.Y., Yu, T.F., and Lian, E.C. (1994) Purification and characterization of $_L$-amino acid oxidase from king cobra (*Ophiophagus hannah*) venom and its effects on human platelet aggregation. *Toxicon* **32**, 1349–1358
17. Torii, S., Yamane, K., Mashima, T., Haga, N., Yamamoto, K., Fox, J.W., Naito, M., and Tsuruo, T. (2000) Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis-inducing factor with $_L$-amino acid oxidase activity. *Biochemistry (Mosc).* **39**, 3197–3205
18. Jeon, O.H. and Kim, D.S. (1999) Molecular cloning and functional characterization of a snake venom metalloprotease. *Eur. J. Biochem.* **263**, 526–533