Functional characterization of recombinant batroxobin, a snake venom thrombin-like enzyme, expressed from *Pichia pastoris*

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Abstract A thrombin-like enzyme of *Bothrops atrox moojeni* venom, batroxobin, specifically cleaves fibrinogen α chain, resulting in the formation of non-crosslinked fibrin clots. The cDNA encoding batroxobin was cloned, expressed in *Pichia pastoris* and the molecular function of purified recombinant protein was also characterized. The recombinant batroxobin had an apparent molecular weight of 33 kDa by SDS–PAGE analysis and biochemical activities similar to those of native batroxobin. The purified recombinant protein strongly converted fibrinogen into fibrin clot in vitro, and shortened bleeding time and whole blood coagulation time in vivo. However, it did not make any considerable alterations on other blood coagulation factors. Several lines of experimental evidence in this study suggest that the recombinant batroxobin is a potent pro-coagulant agent.

Keywords: Fibrin clot; Fibrinogen; Recombinant batroxobin; Pro-coagulant; Thrombin-like enzyme

1. Introduction

Snake venoms contain various components which affect the mammalian hemostatic system as either pro-coagulants or anti-coagulants [1–3]. These components interact with diverse proteins of the blood coagulation cascade and the fibrinolytic pathway [2,3]. Generally, they are classified into several groups as follows: (i) fibrinogen clotting enzymes; (ii) fibrino(geno)lytic enzymes; (iii) plasminogen activators; (iv) prothrombin activators; (v) factor V, X activators, (vi) hemorrhagins; and (vii) platelet aggregation inhibitors [3]. Thrombin-like enzymes, which are serine proteases with fibrinogen clotting activity, have been identified and cloned from various snake venoms [4,5]. Batroxobin (EC 3.4.21.29) was known to be a thrombin-like enzyme from *Bothrops atrox moojeni* venom and its cDNA was also cloned [6,7]. In contrast to thrombin, which converts fibrinogen into fibrin by cleavage of fibrinogen A and B chains, batroxobin only splits off fibrinopeptide A [6,7]. Additionally, thrombin activates various blood coagulation proteins such as factors V, VIII, and XIII as well as platelets, endothelial cells, and smooth muscle cells which are closely related to hemostatic system, whereas batroxobin has no direct effects on these factors or cells [1]. The enzyme has a defibrinogenating effect and is currently being used clinically for the treatment of various thrombotic diseases including deep vein thrombosis, myocardial infarction, pulmonary embolism, and acute ischemic stroke [8–10].

Batroxobin contains 231 amino acids and 12 cysteine residues which are involved in six disulfide bonds. There are two putative glycosylation sites in the polypeptide chain of batroxobin [6,7,11]. Although recombinant thrombin-like enzymes of various snake venoms have been expressed in *Escherichia coli*, these recombinant proteins usually formed insoluble inclusion bodies [12–15]. It has proven difficult to refold inclusion bodies of thrombin-like enzymes as functionally active and soluble proteins [16,17].

In the present study, we report the cloning, expression and functional characterization of recombinant batroxobin, a snake venom thrombin-like enzyme, from *Pichia pastoris*. This is the first report demonstrating that the recombinant yeast-produced batroxobin might be developed as a clinical pro-coagulant agent for the treatment of thrombotic diseases.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, molecular weight marker, and *Pichia* Expression Kit were obtained from Invitrogen (Carlsbad, CA). Human fibrinogen, thrombin, biotin, H-D-Ile-Phe-Lys-*p*-nitroanilide, *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide, and *N*-tosyl-Gly-Pro-Arg-*p*-nitroanilide were from Sigma Chemical Co. (St. Louis, MO). H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302), and H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266) were from Chromogenix (Milano, Italy). Heparin–Sepharose and Phenyl–Sepharose were products of Amersham Biosciences (Uppsala, Sweden). Yeast nitrogen base was obtained from Becton Dickinson

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Abbreviations: APTT, activated partial thromboplastin time; PAS, periodic acid-Schiff's reagent; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PT, prothrombin time; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TT, thrombin time

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(Sparks, MD). Native batroxobin was from Pentapharm (Basel, Switzerland).

2.2. Construction of expression plasmid for batroxobin

The full-length cDNA of batroxobin (GenBank Accession No. J02684) was kindly donated by Dr. Yamashina [7]. The cDNA encoding batroxobin for yeast expression was cloned by polymerase chain reaction (PCR) with the N-terminal primer 5′-CTCGAGA-AAAGAGTCATTGGAGGTGATG-3′ and the C-terminal primer 5′-TTCACGGGCTCGCAGTTTTATTTCCTGCAATAATGCTC-3′. The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced. The amplified cDNA of batroxobin was transferred into *P. pastoris* expression vector, pPIC9 (Invitrogen). The pPIC9-batroxobin vector was digested with *Sal*I (NEB, Beverly, MA) restriction enzyme. This linearized plasmid was transformed into electro-competent GS115 cells (Gene Pulser, Bio-Rad, Hercules, CA). Methanol utilization ability of the His^+ pPIC9-batroxobin transformants was analyzed by replica selection on minimal dextrose and on minimal methanol plate. Finally, His^+ and Mut^+ colony was selected for batroxobin expression.

2.3. Expression of recombinant batroxobin by fermentation

Fermentation was performed using a BIOSTAT® B fermenter (B. Braun Biotech International, Melsungen, Germany) equipped with software control (MFCS/Win 2.0) of temperature (30 °C), agitation (1000 rpm), pH, anti-foam addition, supplemental oxygen addition, and feeding rate (glycerol and methanol). Stock cells were grown in 200 ml of yeast nitrogen base media for 24 h. The cultured cells were transferred to a fermenter containing 1.5 liter of basal salt-glycerol media (H_3PO_4, 27 ml/l; $CaSO_4 \cdot 2H_2O$, 0.9 g/l; K_2SO_4, 18 g/l; $MgSO_4 \cdot 7H_2O$; 15 g/l, KOH, 4.13 g/l; and glycerol, 40 g/l) supplemented with 4.4 ml/l trace mineral solution. The pH of culture media was maintained at 5.0 with 12.5% (w/v) NH_4OH during fermentation. Growth in batch mode was continued until the dissolved oxygen concentration increased as a result of carbon source limitation (20 h). A 50% glycerol containing 12 ml/l trace mineral solution and supplemental oxygen was automatically administered to maintain the dissolved oxygen concentration above 40% for 10 h. After carbon source starvation (30 min) for complete glycerol consumption, methanol induction was initiated. At the initiation of methanol feeding, feed rate was lowered without oxygen supply (5 h) for adaptation of cells to methanol. Methanol feeding (100% methanol containing 12 ml/l trace mineral solution) was controlled by dissolved oxygen concentration (above 40%). During the production phase (72 h), newly expressed batroxobin was monitored by SDS–PAGE analysis.

2.4. Purification and characterization of recombinant batroxobin

The batroxobin-expressed supernatant was collected by centrifugation. Ammonium sulfate was added to the supernatant to reach 2 M and then was then loaded to a phenyl–Sepharose column followed by elution with a gradient of 2–0 M ammonium sulfate. Recombinant batroxobin fractions were further purified by heparin–Sepharose affinity chromatography. The recombinant protein was assayed by a chromogenic synthetic substrate throughout the purification procedure. Purified recombinant protein was analyzed by SDS–PAGE. In addition, N-terminal and internal amino acid sequences of recombinant batroxobin were analyzed by automated Edman degradation procedure and by MS/MS spectroscopy, respectively. The glycosylation of recombinant and native batroxobins was examined by PAS staining. To deglycosylate the proteins, denatured batroxobins were incubated with N-glycosidase F (Roche Applied Science, Mannheim, Germany) for 24 h at 37 °C in 20 mM sodium phosphate (pH 7.5). Deglycosylated batroxobins were analyzed by SDS–PAGE. Western blotting was performed by using an anti-rabbit serum which was obtained from native batroxobin-immunized rabbit. The immune complex was visualized using chemiluminescence (Amersham Biosciences).

2.5. Zymographic and fibrinogen clotting assay

Zymographic assay was performed as previously described [18,19]. Recombinant and native batroxobins were electrophoresed on SDS–PAGE under non-reducing conditions. To remove SDS, the gel was soaked in an excess volume of 2.5% Triton X-100 for 20 min and then washed four times with 20 mM Tris–HCl (pH 7.5) for 10 min. The gel was then overlaid on a fibrinogen (0.7%, w/v)-agarose (0.5%, w/v) plate and incubated at 37 °C. Fibrinogen clotting assay was measured as previously described [20]. After recombinant and native batroxobins were added to 0.5 ml of human fibrinogen (0.5 mg/ml) in 20 mM Tris–HCl (pH 7.5), clotting was monitored by changes in turbidity at 405 nm using a DU650 spectrophotometer (Beckman Coulter, Inc. Chaska, MN).

2.6. Measurement of plasma clotting time in vitro

Rat plasma was obtained from whole blood by centrifugation. Recombinant and native batroxobins were treated in the plasma, and then plasma clotting time was measured by using an Automated Coagulation Laboratory (ACL) 100 Instrument (Instrumentation Laboratory Company, Milano, Italy).

2.7. Substrate specificity of batroxobin and thrombin

To investigate substrate specificity of recombinant batroxobin, native batroxobin, and thrombin, various synthetic substrates (100 μM) were incubated with thrombin (60 nM) or each batroxobin (120 nM) in 0.2 ml of 20 mM Tris–HCl (pH 7.5). The hydrolytic reaction was monitored at 405 nm by the formation of *p*-nitroaniline and μKat value was calculated as described previously [21]. The chromogenic substrates examined were: H-D-Ile-Phe-Lys-*p*-nitroanilide, *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide, *N*-tosyl-Gly-Pro-Arg-*p*-nitroanilide, H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302), and H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266).

2.8. Measurements of bleeding time and whole blood coagulation time

The bleeding time was measured as previously described [22]. Sprague–Dawley rats ($n = 5$, 250–300 g) were pretreated by the intravenous injection of recombinant and native batroxobins (1 NIH unit/kg) in phosphate-buffered saline (PBS). After 1 h, the tail of rats was transfected at 5 mm from the tip and 1.5 cm of distal portion was vertically immersed in PBS. The whole blood coagulation time was measured by modified method in the previous report [23]. Rats ($n = 5$) were pretreated by intravenous injection of recombinant and native batroxobins (2 NIH unit/kg) in PBS. After 1 h, the coagulation time of 0.5 ml citrated whole blood containing 10 mM $CaCl_2$ was measured.

2.9. Measurements of coagulation parameters

The plasma clotting times, including activated thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and fibrinogen concentration, were measured as described previously [22,24]. Rats ($n = 5$) were pretreated by the intravenous injection of recombinant and native batroxobins (0.1 NIH unit/kg) in PBS. After 2.5 h, the batroxobin-treated plasma was obtained from rat citrated whole blood by centrifugation. Then, coagulation parameters were measured by using an ACL 100 Instrument.

3. Results and discussion

3.1. Expression and purification of recombinant batroxobin

Analysis of cDNA sequence encoding batroxobin revealed that batroxobin is composed of 231 amino acids and 12 cysteines, which might be involved in six disulfide bonds (Fig. 1A). A great number of disulfide bonds in the polypeptide chains are known to be significant obstacles to refold the recombinant proteins as active forms from insoluble inclusion bodies in *E. coli* [16,17]. It has also proven difficult to obtain recombinant thrombin-like enzymes as functionally active proteins from *E. coli*-expression system. In this work, however, recombinant batroxobin was successfully expressed as a secretary recombinant protein in *P. pastoris* by methanol induction (Fig. 2A, lane 1) and easily purified from the culture media by means of chromatographic fractionations (data not shown). Initially, recombinant proteins in the cultured media of *P. pastoris* containing 2 M ammonium sulfate were

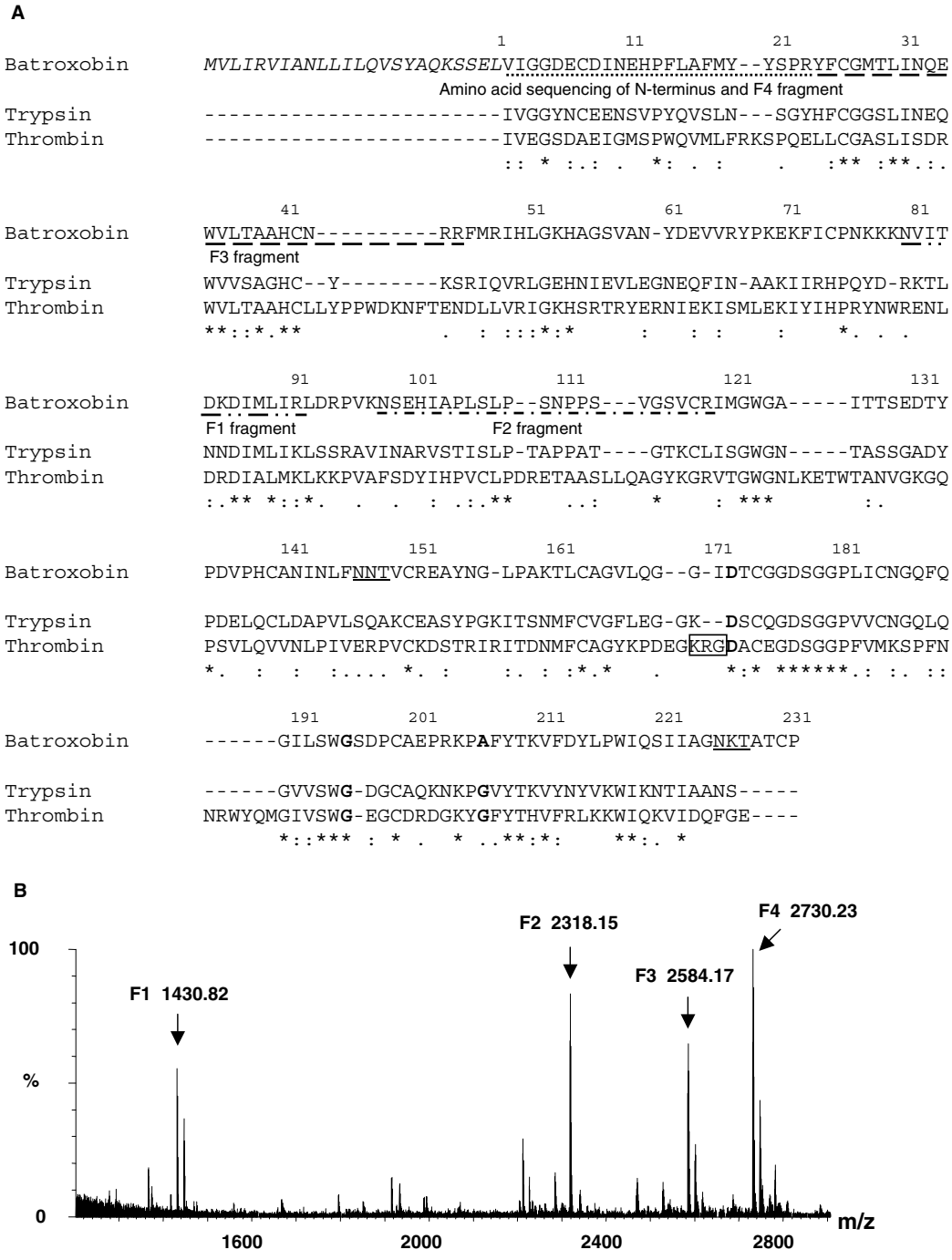

Fig. 1. Deduced amino acid sequence of batroxobin. (A) The deduced amino acid sequence from the cDNA of batroxobin (GenBank Accession No. J02684) is denoted by one-letter symbols and aligned with those of other serine proteases. The zymogen peptide is shown in italics. Two putative glycosylation sites are underlined. Amino acid sequences determined by Edman degradation and MS/MS spectroscopy are underlined with dashes. The primary (S1), secondary (S2) and tertiary (S3) specificity sites of each protease are shown in bold-faced type. The amino acids of S1 loop in thrombin are boxed. (B) MS spectrum of trypsinized-recombinant batroxobin is shown. The internal amino acid sequences of several peptide fragments (F1–F4) were analyzed and the observed mass of each peptide fragment is also shown.

fractionated by a phenyl–Sepharose chromatography with a linear gradient of ammonium sulfate. Recombinant batroxobin fractions were pooled and dialyzed against 20 mM Tris–HCl (pH 7.0) buffer, and were then further purified by using a heparin–Sepharose affinity column with a linear gradient of 0–0.5 M NaCl.

While native batroxobin migrated as a molecular weight of 37 kDa, purified recombinant batroxobin migrated as an apparent molecular weight of 33 kDa in SDS–PAGE (Fig. 2A, lanes 3 and 4). Such a difference in protein mobility may be due to the distinct glycosylation system between yeast and snake. The carbohydrate content of thrombin-like enzymes varies

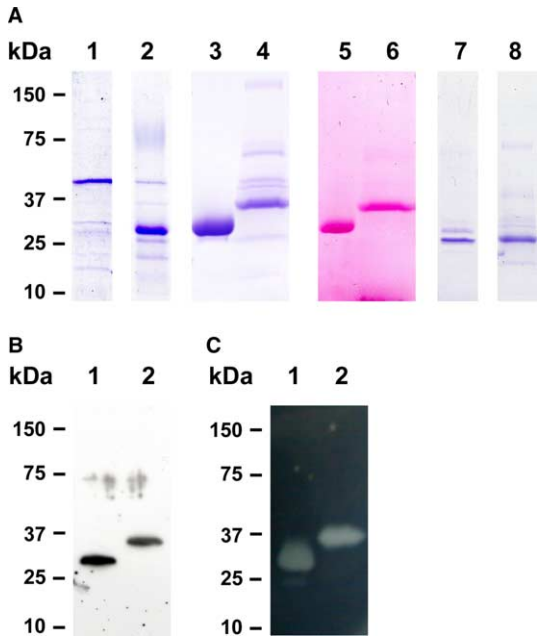

Fig. 2. Characterization of recombinant batroxobin from *P. pastoris*. (A) Recombinant batroxobin was expressed from *P. pastoris* by methanol induction. Culture media induced by methanol for 72 h were precipitated with 20% trichloroacetic acid and then analyzed by a 4–20% gradient SDS–PAGE (lane 1). Recombinant batroxobin fraction of phenyl–Sepharose (lane 2), purified recombinant batroxobin (lane 3), and native batroxobin (lane 4) were also analyzed by the SDS–PAGE. Both batroxobins were stained with PAS reagent (lanes 5 and 6) and deglycosylated batroxobins by N-glycosidase migrated as a molecular weight of 26 kDa (lanes 7 and 8). (B) Recombinant batroxobin (lane 1) was also reacted with anti-native batroxobin serum. (C) Recombinant batroxobin (lane 1) and native batroxobin (lane 2) converted fibrinogen into fibrin, which results in the formation of turbid band on the fibrinogen-agar plate.

also considerably depending on snake species and it is responsible for the different molecular mass of these proteins [3]. PAS staining result indicated that recombinant and native batroxobins are glycoproteins (Fig. 2A, lanes 5 and 6), which have two putative glycosylation sites in the polypeptide chain (Fig. 1A). Native batroxobin is known to be containing about 40 different oligosaccharide subfractions including a unique oligosaccharide pattern of snake venom [11], whereas recombinant batroxobin expressed from *P. pastoris* might be containing the yeast-type carbohydrate structures [25]. When the covalently attached carbohydrate structures were removed from both proteins by *N*-glycosidase treatment, recombinant and native deglycosylated batroxobins migrated as a smaller molecular size corresponding to 26 kDa (Fig. 2A, lanes 7 and 8), which is consistent from that of the calculated molecular weight (25504.3) based on the cDNA-deduced amino acids of batroxobin. The N-terminal and internal amino acid sequences of recombinant batroxobin were also found to be identical to the deduced polypeptide sequence (Fig. 1). Finally, 7 mg of recombinant batroxobin was purified with a yield of 53.2% from 1 liter fermentation media (Table 1). Western blot analysis demonstrated that yeast-derived recombinant batroxobin as well as native protein specifically reacted with anti-native batroxobin rabbit serum (Fig. 2B).

3.2. *In vitro fibrinogen clotting activity of recombinant batroxobin*

The functional activity of recombinant batroxobin was examined by using zymography assay and fibrinogen polymerization assay. Under non-reducing conditions, recombinant and native batroxobins were able to form turbid bands corresponding to their molecular sizes on the fibrinogen-agar plate (Fig. 2C). Additionally, in vitro fibrinogen clotting assay demonstrated that both batroxobins converted fibrinogen into fibrin clot (Fig. 3A). To perform exact and quantitative analysis of batroxobin clotting activity, plasma clotting time was measured with batroxobin-treated plasma in vitro by using an ACL 100 Instrument. Both recombinant and native batroxobins were able to coagulate plasma in a dose-dependent manner, and the activity of purified recombinant batroxobin fraction was more powerful than that of native protein fraction (Fig. 3B). As shown in Fig. 2A, the purity of recombinant batroxobin is much higher than that of native protein and it may be responsible for stronger activity of recombinant protein than native protein fraction.

3.3. *Substrate specificity analysis*

To compare substrate specificity of recombinant, native batroxobin and thrombin, various synthetic chromogenic substrates were reacted with each enzyme. The substrate specificity of recombinant batroxobin was similar to that of native protein (Fig. 3C). Generally, both thrombin and batroxobin preferentially hydrolyze arginine over lysine at the peptide bond cleavage site [26]. However, the substrate specificity of batroxobin was remarkably different with that of thrombin [26]. Although thrombin demonstrated a strong hydrolyzing activity on the substrates, which have proline at the P_2 position, recombinant and native batroxobins showed less effective activities on these substrates. In case of the substrate containing leucine at the P_2 position, such as S-2266, batroxobin had more powerful activity than that of thrombin (Fig. 3C). As demonstrated in Fig. 1A, batroxobin has the primary (S1), secondary (S2) and tertiary (S3) specificity sites (Asp^{172}, Gly^{195}, and Ala^{206}) similar to those of thrombin and trypsin, allowing the large substrate side chain access to the base of the catalytic site of enzyme [27,28]. However, batroxobin primary structure lacks amino acid residue insertions around the active site cleft (S1 loop) in comparison to thrombin (segment Lys-Arg-Gly, boxed amino acids in

Table 1
Purification of recombinant batroxobin from 1 liter of *P. pastoris* culture media

Purification step	Total protein (mg)	Total activity (NIH units)	Specific activity (NIH units/mg)	Purification fold	Purification yield (%)
Culture media	178.75	3430.98	19.19	1	100
Phenyl–Sepharose	61.10	3127.38	51.8	2.67	91.2
Heparin–Sepharose	6.95	1825.28	262.63	13.69	53.2

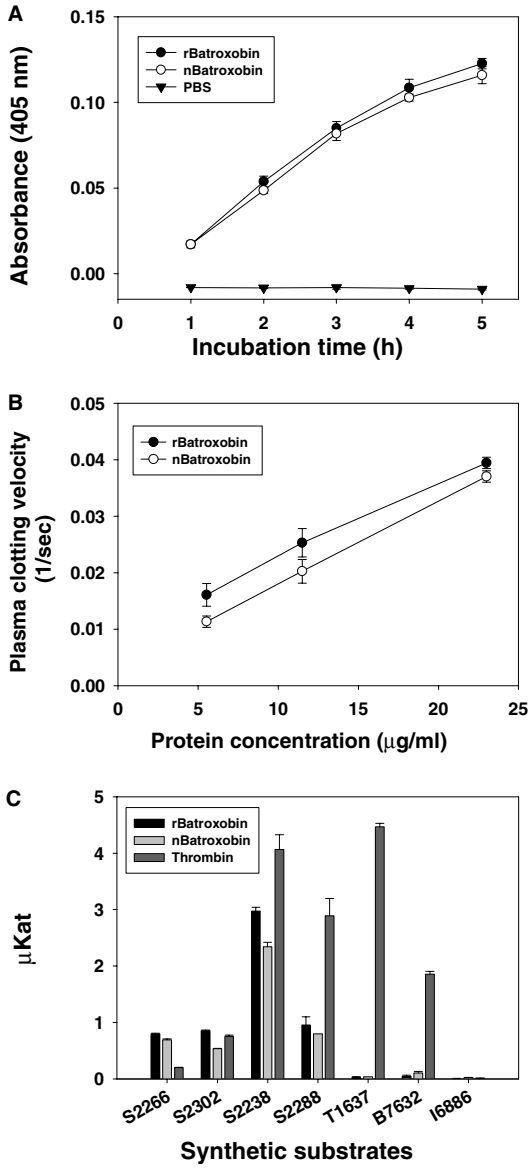

Fig. 3. Fibrinogen clotting activity and substrate specificity of batroxobin. (A) Both recombinant and native batroxobins converted fibrinogen into fibrin clot in vitro. (B) Both batroxobins were also able to clot plasma in a dose-dependent manner. (C) The catalytic activity of thrombin and both batroxobins was measured by using several synthetic substrates, including H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266), H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302), H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), *N*-tosyl-Gly-Pro-Arg-*p*-nitroanilide (T1637, Sigma Cat. No.), *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide (B7632, Sigma), and H-D-Ile-Phe-Lys-*p*-nitroanilide (I6886, Sigma). Data were expressed as means ± S.E.M. from three separate experiments.

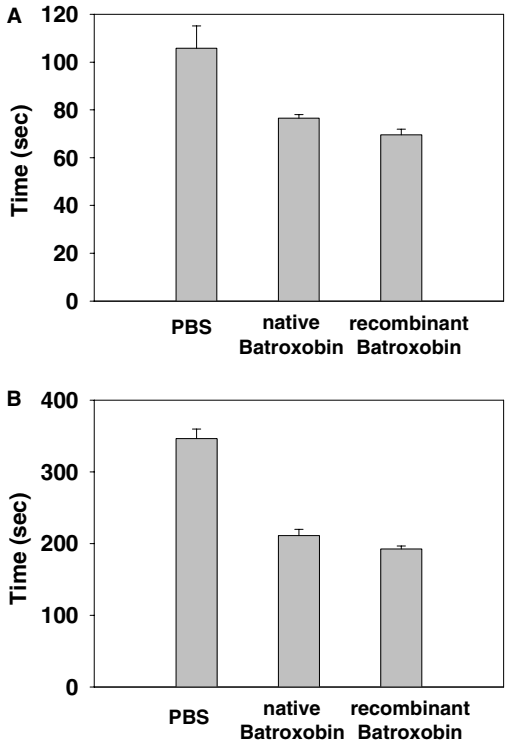

Fig. 4. Effects of batroxobin on in vivo bleeding time and whole blood coagulation time. Both recombinant and native batroxobins reduced rat tail bleeding time (A) and whole blood coagulation time (B). Rats ($n = 5$, 250–300 g) were pretreated by the intravenous injection of recombinant and native batroxobins (1 NIH unit/kg) in PBS. After 1 h, the bleeding time and the whole blood coagulation time were measured as described in Section 2.

Fig. 1A). Residues 175 and 176 have also been recognized as key residues affecting snake protease specificity for the P_2 subsite [29]. These regions of batroxobin are composed of more non-polar and smaller amino acids than thrombin. Such different structural motifs can be responsible for the distinction of substrate specificity between batroxobin and thrombin. Thrombin-like enzymes of venombin A class, including ancrod, batroxobin, and crotalase, are known to cleave the Arg^{16}–Gly^{17} peptide bond in the Aα-chain of fibrinogen leading to the release of fibrinopeptide A and the conversion of fibrinogen into a fibrin clot [3]. However, thrombin cleaves not only the Arg^{16}–Gly^{17} peptide bond in the Aα-chain but also the Arg^{14}–Gly^{15} peptide bond in the Bβ-chain, releasing fibrinopeptide A and fibrinopeptide B from fibrinogen [30]. Specifically, residues 194–196 are known to be an important region in regulating the interaction of thrombin with fibrinogen. The mutation of these residues in thrombin compromises the pro-coagulant activity up to 500-fold [27,31]. While residues Trp^{194} and Gly^{195} are strictly conserved in thrombin and thrombin-like enzymes (Fig. 1A), residue Ser^{196} is widely diverse in thrombin-like enzymes. Mutant studies on these functionally important amino acid residues of batroxobin will be valuable for understanding the different substrate specificity between thrombin and thrombin-like enzymes.

Although most snake venom serine proteases demonstrate significant similarities of the primary structures, they are known to have various and distinct effects on the hemostatic system, for example, fibrino(geno)lysis, fibrinogen clotting, plasminogen activating, and activation of other coagulation factors [1–3]. Even thrombin-like enzymes showed different substrate specificities on fibrinogen according to their classification [3]. The venombin A class hydrolyzes fibrinogen α-chain and releases fibrinopeptide A preferentially, and the venombin AB class releases both fibrinopeptides A and B. The third class of thrombin-like enzyme, venombin B, had preferential hydrolytic activity on fibrinogen β-chain. However, the structure–function relationships related with the molecular structure and substrate specificity of thrombin-like enzymes

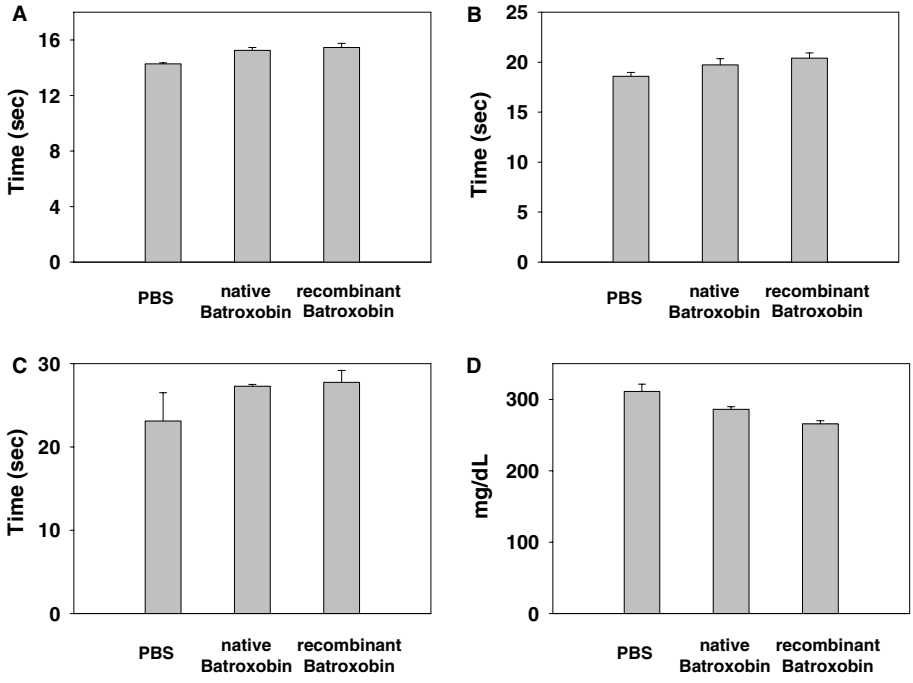

Fig. 5. In vivo effects of batroxobin on coagulation parameters. Effects of both batroxobins on activated partial thromboplastin time, APTT (A), prothrombin time, PT (B), thrombin time, TT (C) and fibrinogen concentration in rat plasma (D) were examined. Rats ($n = 5$) were pretreated by the intravenous injection of recombinant and native batroxobins (0.1 NIH unit/kg) in PBS. After 2.5 h, the batroxobin-treated plasma was obtained from rat citrated whole blood by centrifugation. Then, coagulation parameters were measured by using an ACL 100 Instrument.

were not clearly examined and understood. Site-directed and Ala-scanning mutagenesis studies based on our expression system will be useful tools to investigate the structure–function relationships of thrombin-like enzymes.

3.4. Effects on bleeding time and whole blood coagulation time in vivo

To examine the pro-coagulant activity of the recombinant batroxobin in vivo, bleeding time and whole blood coagulation time were measured by using rats which were administered with recombinant and native batroxobins. As shown in Fig. 4, recombinant and native batroxobins were capable of reducing the tail bleeding time and whole blood coagulation time. Based on these experimental results, it is possible to suggest that recombinant batroxobin is able to form clottable fibrinogen in whole blood and to shorten bleeding time as a pro-coagulant agent in vivo.

3.5. Effects of recombinant batroxobin on coagulation parameters

To investigate whether the recombinant batroxobin has an influence on other blood coagulation factors in vivo, the coagulation parameters, including APTT, PT, and TT, were measured with batroxobin-treated rat plasma. The coagulation parameters were not significantly affected by the treatments of both batroxobins (Fig. 5A–C). However, these parameters tended to slightly decrease. Experimental data of coagulation parameters analysis demonstrated that recombinant batroxobin has no significant influence on other coagulation factors. Snake venom thrombin-like enzymes such as batroxobin, crotalase and ancrod were known to have not only potent pro-coagulant activities but also defibrinogenating activities, which are associated with the reduction of fibrinogen level in plasma by clearing converted fibrinogen and enhancing fibrinolytic activity [9,10,32–34]. Recombinant and native batroxobins also decreased fibrinogen concentration in rat plasma (Fig. 5D), which might be closely correlated with slight reductions of coagulation parameters including APTT, PT, and TT.

In this work, the recombinant batroxobin, a snake venom thrombin-like enzyme of *Bothrops atrox moojeni*, was successfully expressed and purified from *P. pastoris* by high density fermentation. Here, we report that the molecular functions of recombinant batroxobin were also characterized and compared to its native protein. Several lines of experimental results in this study suggest that the recombinant batroxobin has a potent pro-coagulant activity in vitro and in vivo assay systems. Since the coagulation studies on reptilase in the 1950s [35], thrombin-like enzymes have been extensively studied because of their potential therapeutic use in various thrombotic diseases, and some enzymes have been used in the prevention of thrombus formation and in improving the blood circulation by reducing blood viscosity [8–10]. Native batroxobin also has a good therapeutic effect in ischemic reperfused rats and is a good reagent used in quantitative determination of fibrinogen, especially in the plasma patients under heparin treatment [32]. The recombinant batroxobin in this work is the first highly active and pure recombinant snake venom thrombin-like enzyme, which might be developed as a clinical agent in substitute for native protein. In aspects of basic research, the experimental results and data of recombinant batroxobin will make it possible to study structure–function relationships of thrombin-like enzymes as well as other snake venom serine proteases acting on mammalian hemostasis.

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