1 *Original Research Article*
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3 # Characterization of a Novel Thrombin-like
4 Enzyme, Globlase from the Venom of *Gloydius*
5 *blomhoffii* (Japanese Mamushi)
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10 ## ABSTRACT
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Aims: To elucidate the coagulation mechanisms of a novel clotting factor isolated from *Gloydius blomhoffii* venom, its hydrolytic activity on various substrates were examined. Furthermore the primary structure was determined and compared with the other snake venom components.

Methodology: A thrombin-like enzyme was isolated from the crude venom of *G. blomhoffii* by DE52 Cellulose and CM52 Cellulose column chromatography. Enzyme activity was measured by using synthetic substrates (arginine esters, MCA-substrates and 3-(Acyloxy)-4-nitrobenzoic acid). Effect on fibrinogen was detected with bovine and human fibrinogen. Isoelectric point and molecular mass were measured by polyacrylamide gel electrophoresis and MALDI-TOF-MS. Amino acid sequence was decided with a protein sequencer by analyzing enzymatically cleaved peptides.

Results: A clotting factor was found to be homologous as indicated by a single band on SDS-PAGE, and the final preparation was named as globlase. Molecular mass of this enzyme was determined to be 13,876.36 Da and the isoelectric point was 8.8. Globlase showed arginine ester hydrolytic activity, and specificity for substrates of thrombin. Proteolytic activity and phospholipase A_2 (PLA_2) activity were not detected. Complete amino acid sequence analysis indicated that the primary structure of globlase is similar to PLA_2. However the aspartic acid which exists in the active site of PLA_2 was found to be substituted by glutamine.

Conclusion: It was shown in our current investigation that globlase is a novel thrombin-like enzyme isolated from *G. blomhoffii* venom. It was revealed that this enzyme had structure unlike the serine-protease such as the other thrombin-like enzymes.

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13 *Keywords: Gloydius blomhoffii, thrombin-like enzyme, primary structure, phospholipase A_2.*
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15 ## 1. INTRODUCTION
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17 Serine-proteases which affect blood coagulation system are widely distributed in the venoms of Viperidae [1-3]. Various
18 thrombin-like proteases have been purified from Viperidae snake venoms, which catalyze the cleavage of specific bond(s)
19 in the fibrinogen molecule [4-9]. In our previous study, the detailed process of fibrin clot formation by a thrombin-like
20 enzyme, bilineobin, from *Agkistrodon bilineatus* venom was reported [8,10].
21
22 *Gloydius blomhoffii* (Japanese Mamushi, which was formerly classified in genus *Agkistrodon*), widely distribute in Japan
23 and it has been reported that 1,000 cases of bites occur annually [11]. Thrombin-like enzyme and arginine ester hydrolase
24 are reported as the components of *G. blomhoffii* venom [12]. However, little change in prothrombin time (PT), activated
25 partial thromboplastin time (APTT), or fibrinogen levels is observed even in severe cases [13]. Though the only thrombin-
26 like enzyme purified from *G. blomhoffii* venom is halystase, this enzyme does not induce clot formation from fibrinogen.
27 Therefore, we examined the existence of the other thrombin-like enzyme in this venom.
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2. MATERIAL AND METHODS

2.1 Materials

Lyophilized crude venom of *G. blomhoffii* was purchased from Japan Snake Institute (Gunma, Japan). DE52- and CM52-Cellulose were the products of Whatman Laboratory (England). Tosyl-L-arginine methyl ester (TAME) and other arginine esters, methylcoumarylamide (MCA) substrates were obtained from the Peptide Institute, Inc. (Osaka, Japan). Fibrinogen (bovine, human and other animal species), hide powder azure, azocollagen, *p*-amidinophenyl methanesulfonyl fluoride (*p*-APMSF), *N*-bromosuccinimide (NBS), benzamidine hydrochloride and glycoprotein detection kit were purchased from Sigma-Aldrich. Chymotrypsin, endoproteinese Lys-C and endoproteinese Arg-C were from Roche Diagnostics. Other chemicals used were analytical grade from commercial sources.

2.2 Isolation and Characterization of Globlase

Crude venom (500 mg) was applied to a DE52 Cellulose column (1.5×45 cm) equilibrated with 5 mM sodium acetate buffer, pH 7.2. The adsorbed fraction was eluted with the same buffer containing 0.5 M NaCl. The fraction possessing fibrinogen clotting activity was collected and applied to a CM52 Cellulose column (1.5×45 cm) equilibrated with 5 mM sodium acetate buffer, pH 7.2. Elution was performed with a linear gradient from 5 mM to 0.5 M sodium acetate in a total volume of 600 ml. The homogeneity of the purified sample was confirmed by SDS-PAGE and reversed phase HPLC (Develosil ODS-HG-5 column, 4.6×250 mm). The molecular weight of final preparation was estimated by MALDI-TOF-MS, and the isoelectric point was determined with isoelectric-focusing polyacrylamide gel electrophoresis. The detection of sugar moieties of glycoproteins was performed with the modified Periodic Acid-Schiff (PAS) methods according to the instruction of suppliers.

2.3 Enzyme Activity and Other Assays

Arginine ester hydrolytic activity was assayed using tosyl-L-arginine methyl ester (TAME), benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-arginine methyl ester (BAME) as substrates. The quantity of substrate hydrolyzed was determined by the hydroxamate method of Roberts [14]. One unit was defined as the quantity of protein which hydrolyzed 1 μmol of substrate per minute. Methylcoumarylamide (MCA) substrates which were designed for various serine-proteinases were also used to measure the enzymatic activity of globlase. Release of 7-amino-4-methylcoumarin was determined fluorometrically at 380 nm (λex) and 460 nm (λem) according to the method of Morita et al. [15].

Indirect hemolysis of rabbit erythrocytes was employed to determine phospholipase A_2 (PLA_2) activity [16]. 3-(Acyloxy)-4-nitrobenzoic acid was synthesized to measure PLA_2 activity [17], and the enzyme assay was performed by the method of Holzer and Mackessy [18]. Proteolytic activity was measured by using casein, hide powder azure and azocollagen as the substrate [19-21].

In vitro clotting activity was assayed with fibrinogen of various animal species. Enzyme solution (0.1 ml) was added to 0.9 ml of fibrinogen solution (0.2% in saline). The reaction mixture was incubated at 37°C and the time interval necessary for the first appearance of fibrin clot was recorded. The analysis of digested fibrinogen fragments were performed as follows. The mixture of fibrinogen and globlase were incubated at 37°C, and the reaction was terminated with 20 % trichloroacetic acid. The supernatant was collected by centrifugation (10,000 r.p.m. for 15 min) and separated by Develosil ODS-HG-5 column (4.6×250 mm). Identification of peptides was carried out by protein sequencer described in 2.4.

2.4 MALDI-TOF-MS and Amino Acid Sequence Analysis

Purified sample by reversed-phase HPLC was lyophilized, and then dissolved in 0.1 % trifluoroacetic acid. The solution was mixed with sinapinic acid, and the molecular mass was measured by MALDI-TOF system (Autoflex speed, Bruker Daltonics).

Reduced and carboxymethylated sample [22] was enzymatically cleaved by chymotrypsin, endoproteinese Lys-C and endoproteinese Arg-C according to the reaction condition of suppliers manual. The amino acid sequence of digested fragments were separated by reversed-phase HPLC and analyzed by Applied Biosystems 491 protein sequencer and Model 120A PTH analyzer.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Globlase

3.1.1 Isolation of Globlase

Crude venom was applied to a DE52 Cellulose column (Fig. 1A). Clotting and TAME hydrolytic activities were found in fractions 1 and 2. Fraction 1 was further purified by a CM-Cellulose column (Fig. 1B), and the enzyme activity was found between the fraction number 160 and 170. This fraction was homogeneous with various tests such as SDS-PAGE (Fig. 1B-insert), isoelectric focusing and reversed-phase HPLC. This protein was named as “Globlase” (a thrombin-like enzyme from *G. blomhoffii*). By this procedure, 10.8 mg of purified preparation was obtained from 500 mg of crude venom.

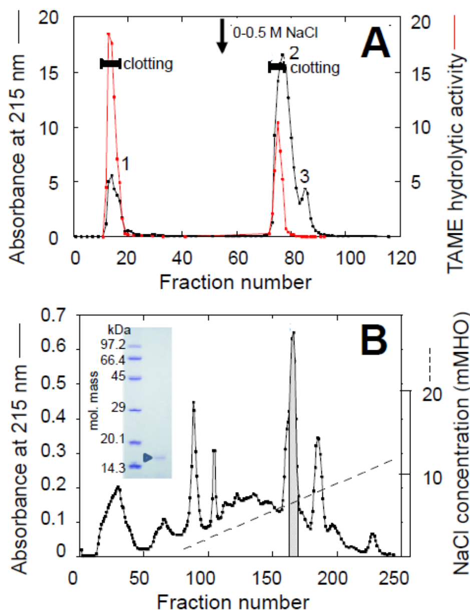

Fig. 1. Isolation of globlase

(A) The first step: Crude venom of G. blomhoffii (500 mg) was applied on a DE52 Cellulose column (1.5×45 cm) equilibrated with 5 mM sodium acetate. The adsorbed fraction was eluted with equilibration buffer containing 0.5 M NaCl.

(B) The second step: Fraction 1 from the first step was applied to a CM52 Cellulose column (1.5×45 cm) equilibrated with 5 mM acetate buffer (pH 7.2). Elution was performed with a linear gradient from 5 mM to 0.5 M sodium acetate buffer (pH 7.2) in a total volume of 600 mL. Fractions of 3.0 mL were collected at a flow rate of 10.8 mL/hr. The fraction possessing TAME hydrolytic activity was indicated by shadowed area. (insert) SDS-PAGE of isolated enzyme.

3.1.2 Biochemical Properties

The molecular mass of globlase measured by MALDI-TOF-MS was 13,876.36 Da with the isoelectric point of 8.8. The modified Periodic Acid-Schiff (PAS) method indicated that this protein did not contain any carbohydrates.

Globlase possessed coagulation activity on bovine and human fibrinogen. Enzyme specificity was measured by synthetic peptides TAME, BAME and BAEE for serine proteinase (Table 1). Among these substrates, TAME was hydrolyzed most actively and the specific activity was 27.04 units/mg of protein. *t*-Butoxycarbonyl-Asp(OBz)-Pro-Arg-MCA which was designed for α-thrombin was the most suitable substrate among various MCA-substrates which participate in blood coagulation. The hydrolyzing activity of globlase against MCA-substrates of factor Xa, activated protein C and plasmin were relatively low. Proteolytic activities on casein, hide powder azure and azocollagen were relatively low or not detected. Neither hydrolytic activity on a synthetic PLA_2 substrate nor indirect hemolytic activity was detected.

TAME hydrolytic activity and clotting activity of globlase (30 μg) were stable to heat treatment. The residual activities after the heat treatment at 100°C for 10 min were 38.8% and 34.7%, respectively. Both activities were inhibited by *p*-APMSF and NBS. However, no inhibition was observed with benzamidine or EDTA.

According to the previous study, it is reported that halystase, a serine proteinase, exists in *G. blomhoffii* venom [9]. This enzyme consists of 238 amino acid residues and N-linked carbohydrates with the molecular mass of 38 kDa. Halystase is structurally similar to thrombin-like snake venom enzymes and cleaves fibrinogen. However, it does not induce fibrin clot formation. Since coagulation factor except halystase is not isolated from *G. blomhoffii* venom, globlase is considered a novel thrombin-like enzyme.

Table 1. Enzyme activities of globlase

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Substrate	activity
Arginine esters	(units/mg)
TAME (Tos*-L-Arg methyl ester)	27.04
BAME (Bz**-L-Arg methyl ester)	3.43
BAEE (Bz-L-Arg ethyl ester)	2.10
MCA†-substrates (designed for)	AMC†† released (μmol/min/mg)
Boc***-Asp(OBz)-Pro-Arg-MCA (α-thrombin)	8.21
Boc-Val-Pro-Arg-MCA (α-thrombin)	2.71
Boc-Ile-Glu-Gly-Arg-MCA (factor Xa)	0.90
Boc-Leu-Ser-Thr-Arg-MCA (activated protein C)	1.20
Boc-Val-Leu-Lys-MCA (plasmin)	0.75
Proteinase	(units/mg)
Casein	ND
Hide powder azure	
Azocollagen	0.41
Phosphlipase A_2	(units/mg)
3-(Acyloxy)-4-nitrobenzoic acid	ND

148 *Tos: tosyl. **Bz: benzoyl. ***Boc: t-butoxybalbonyl. †MCA: Methylcoumarylamide.
149 ††AMC: aminomethylcoumarin.ND: not detected.
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151 ### 3.1.3 Effect of Globlase on Fibrinogen
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153 Since the final preparation possessed clotting activity, the effect of globlase (32.5 μg) on fibrinogen of various animal
154 species was further tested. The clot formation of bovine fibrinogen was the fastest (30 sec), followed by sheep and human
155 fibrinogen (45 and 70 sec, respectively). Fibrinogen from dog, horse, guinea pig was also coagulated, but their clotting
156 time was relatively slow. Time-dependent degradation of bovine fibrinogen was observed on SDS-PAGE (Fig. 2A). Aα
157 and Bβ chains were immediately hydrolyzed and their bands were shifted below in a few minutes. It seems the γ chain of
158 fibrinogen was resistant to hydrolysis with globlase.
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160 Two major peaks were detected in the elution profile of the supernatant of digested bovine fibrinogen (Fig. 2B). The
161 peptide eluted at 19 min was identified as fibrinopeptide A. However, the peptide obtained in elution time 20-21min was
162 not identified, and fibrinopeptide B was not found. These results indicate that globlase specifically hydrolyze Aα chain of
163 bovine fibrinogen and release fibrinopeptide A. Globlase also hydrolyze Bβ chain, but the digestion does not produce
164 fibrinopeptide B.
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166 Various thrombin-like enzymes have been classified according to their ability to release fibrinopeptides [2]. Similar to
167 thrombin, the enzymes from *Bitis gabonica* and *Bothrops insularis* catalyze fibrinopeptides A and B [23,24]. But most of
168 venom thrombin-like enzymes release either fibrinopeptide A or B. Similar to globlase, reported fibrinopeptide A-releasing
169 enzymes hydrolyze TAME and/or synthetic thrombin substrate. However, they belong to serine-proteinase family and the
170 molecular weights are larger than globlase. Furthermore, most of these enzymes contain carbohydrate chains [2].
171 Therefore, globlase is supposed to be a novel thrombin-like enzyme.
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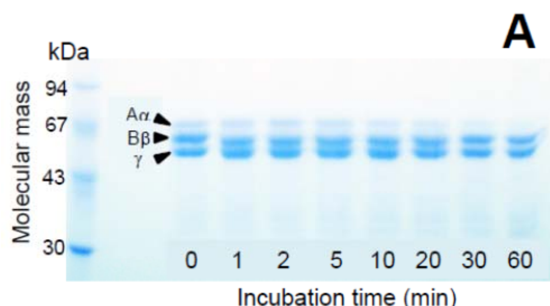

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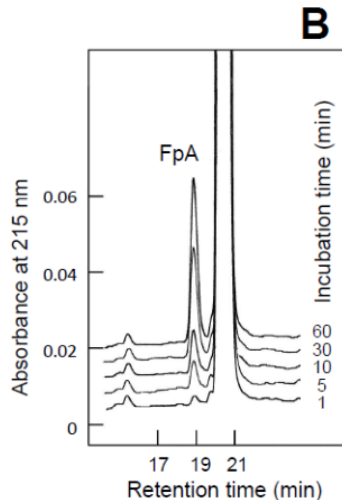

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175 **Fig. 2. Effect of globlase on bovine fibrinogen**
176 *(A) Time-dependent digestion of bovine fibrinogen. Fibrinogen (0.2 % in saline) was incubated with globlase at 37°C for various time*
177 *intervals and analyzed by SDS-PAGE. (B) Elution profile of the digests of fibrinogen by reversed-phase HPLC. Bovine fibrinogen (5*
178 *mg) and globlase (1.63 μg) were incubated at 37°C for indicated time intervals. The reaction was terminated by addition of 20 %*
179 *TCA, and centrifuged (10,000 r.p.m.) for 15 min. The supernatant was separated by Develosil ODS-HG-5 column (4.6×250 mm)*
180 *with a linear gradient from 15 to 35 % acetonitrile in 40 min.*
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182 3.1.4 Amino Acid Sequence of Globlase

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184 The sequence from the amino-terminal to His(47) was identified by the direct analysis of native globlase (Fig 3). The
185 sequence from Gln(48) to Cys(122) were determined by using the results of analysis of digested fragments. From these
186 results, globlase was found to be a single chain protein consisting of 122 amino acid residues. The theoretical molecular
187 weight calculated from the total sequence (Fig. 3) was 13876.97, and it consistent with the result obtained by MALDI-
188 TOF-MS.
189
190 Since the primary structure of globlase seems to homologous with PLA_2, the sequence was compared with PLA_2s from *G.*
191 *blomhoffii* venom [25,26] (Fig. 4). The enzymes from the venom of *G. ussuriensis* and *P. flavoviridis*, which inhabit the
192 near geographical range, were also aligned [27,28]. The sequence of basic and acidic PLA_2s isolated from *G. blomhoffii*
193 venom were apparently different from that of globlase, and the identity of these enzymes was 91.0 % and 60.7 %,
194 respectively. It is interesting that enzyme of *G. ussuriensis,* which inhabit the eastern part of Eurasian continent, is most
195 homologous to globlase. The homology of both enzymes was 97.5 %, and only the difference in three amino acid residues
196 was found. Zhang et al. reported that Gln49-PLA_2 from *G. ussuriensis* venom possess thrombin-like activity [29]. Because
197 the His residue of active site [30] replaced to Gln, this enzyme lacks PLA_2 activity. Furthermore, this enzyme hydrolyzed
198 arginine ester and human fibrinogen, and its clotting activity was equivalent to 1100 NIH thrombin U/mg. Among snake
199 venom PLA_2 analogues reported, enzyme possessing similar characteristics with thrombin-like serine proteinase is only
200 globlase and Gln49-PLA_2. It is interesting that snakes inhabiting the geographically close area have the similar enzyme.
201 Detailed examination of PLA_2 analogues without PLA_2 activity is necessary to find out the enzymes possessing similar
202 characteristics to these two enzymes.
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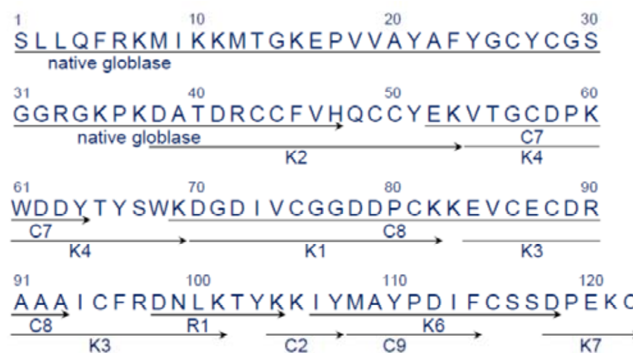

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206 **Fig. 3. Determination of amino acid sequence of globlase**
207 *Arrows indicate residues determined by sequence analysis. The following abbreviations were used for the peptides digested by:*
208 *K; endoproteinese Lys-C, C; chymotrypsin, and R; endoproteinese Arg-C.*

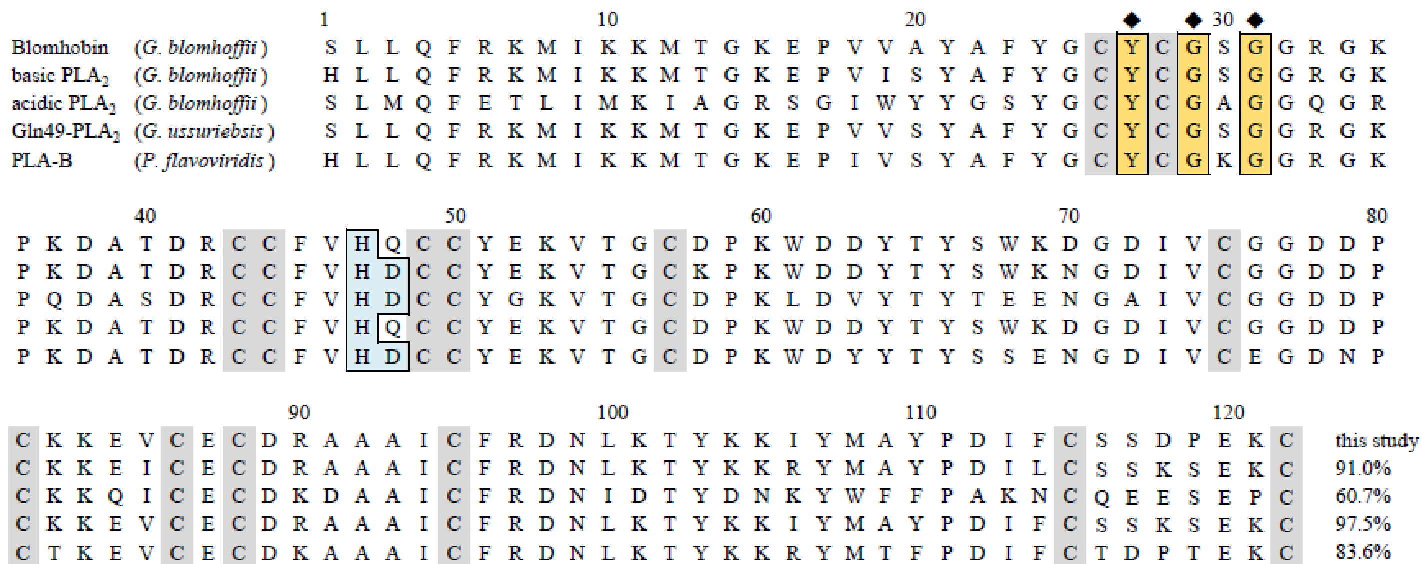

Fig. 4.Comparison of amino acid sequence of globlase and phospholipase A_2s

The protein sequence was aligned based on the position of Cys residues. The important amino acids in the catalytic center of phospholipase A_2 are boxed. The calcium-binding site is indicated with (◆).

4. CONCLUSION

It was shown in our current investigation that globlase is a novel thrombin-like enzyme isolated from *G. blomhoffii* venom. It was revealed that this enzyme had unique structure unlike the other serine-proteases. This result is interesting in studying the process of the evolution of snake enzymes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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