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PHYSICOCHEMICAL PROPERTIES OF α- AND β-FIBRINOGENASES OF *TRIMERESURUS MUCROSQUAMATUS* VENOM

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Summary

α- and β-Fibrinogenases (EC 3.4.21.5) were purified from *Trimeresurus mucrosquamatus* venom by the technique of recycling chromatography. Both enzymes were single polypeptide chains and homogeneous as judged by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and ultracentrifugation. The sedimentation constants of α- and β-fibrinogenases were 2.52 and 3.04 respectively. The molecular weight of α-fibrinogenase was 21 500—23 400, and that of β-fibrinogenase was 25 000—26 000. The contents of proline, glycine and tryptophan were higher in β-fibrinogenase than in α-fibrinogenase. The isoelectric points of α- and β-fibrinogenases were pH 8.1 and 5.7 respectively. The optimal pH of α-fibrinogenase was about 7.4 and that of β-fibrinogenase was around 8.5. The activity of α-fibrinogenase was completely destroyed after 30 min at 60°C, pH 5.6, 7.4 and 9.0, while that of β-fibrinogenase was not significantly affected by the same treatment. Both enzymes showed proteolytic activities toward fibrinogen and casein, but were devoid of phospholipase A, alkaline phosphomonoesterase and phosphodiesterase activities of the crude venom. The tosyl-L-arginine methylester esterase activity of β-fibrinogenase was about 17 times that of the crude venom, while α-fibrinogenase was completely devoid of this activity. The fibrinogenolytic activity of α-fibrinogenase was markedly inhibited by EDTA and cysteine, while that of β-fibrinogenase was inhibited markedly by phenylmethane sulfonylfluoride and slightly by tosyl-L-lysine chloromethylketone and cysteine.

Introduction

Fibrinogenolysis and fibrinolysis are characteristics of many snake venoms, especially in Crotalidae [1—3]. However, their enzymatic actions on fibrinogen and fibrin are not well studied, and the physicochemical properties of these

TABLE I

SOME PHYSICAL PROPERTIES OF α- AND β-FIBRINOGENASES OF *T. MUCROSQUAMATUS* VENOM

	α-Fibrinogenase	β-Fibrinogenase
Sedimentation constant	2.52	3.04
Molecular weight		
gel filtration	21 500	25 000
SDS-polyacrylamide gel electrophoresis	22 400	26 000
Amino acid analysis	23 362	25 534
$E^{1\%}_{1\,cm}$ at 286 nm	9.36	18.78

active principles are also not known in their purified forms. Recently, we have isolated two fibrinogenolytic enzymes from the venom of *Trimeresurus mucrosquamatus* [4]. α-Fibrinogenase (EC 3.4.21.5) attacks the α(A) chain, while β-fibrinogenase attacks the β(B) chain of fibrinogen molecule. In this paper, we present some physicochemical properties of these two fibrinogenolytic enzymes and also compare their enzymatic properties with trypsin.

Materials and Methods

Materials. Venom, fibrinogen and thrombin were prepared as previously reported [4]. α-Casein, tosyl-L-arginine methylester, L-α-phosphatidyl choline, sodium bis-*p*-nitrophenyl phosphate, disodium *p*-nitrophenyl phosphate, cysteine, *N*-ethylmaleimide, tosyl-L-lysine chloromethylketone (TosLysCH_2Cl), phenylmethane sulfonylfluoride, ε-aminocaproic acid, soybean trypsin inhibitor, ethylenediamine tetraacetic acid (EDTA) and trypsin were purchased from Sigma Chemical Co. Trasylol was purchased from FBA Pharmaceuticals Inc. Other chemical reagents were analytical grade from Wako Pure Chemical Industries Ltd.

Purification of α- and β-fibrinogenases. The venom of *T. mucrosquamatus* was fractionated by CM-Sephadex column chromatography as previously reported procedure [4] into 20 fractions. The α-fibrinogenase (Fr. 13) and β-fibrinogenase (Fr. 8), were further purified by recycling chromatography with an LKB 4900A Automatic ReCychrom Apparatus according to the techniques suggested by the manufacturer. A Sephadex G-75 (40—120 μm) column with a bed volumn of 150 ml was used. The elution was carried out with 0.01 M ammonium bicarbonate (pH 7.0).

Sedimentation study. This was carried out in a Spinco Model E Analytical Ultracentrifuge, at protein concentrations of 14.4 mg/ml (α-fibrinogenase) and 16.7 mg/ml (β-fibrinogenase) in 0.01 M KCl. The rotor speed was 59 780 rev./min. Photographs were taken at various times after the rotor had attained correct speed.

Estimation of molecular weight. The molecular weights of the enzymes were measured by: (1) gel filtration on Sephadex G-75 column according to the method of Andrews [5], (2) SDS polyacrylamide gel electrophoresis according to the method of Weber and Osborn [6] and (3) amino acid analysis.

Extinction coefficient. Protein solution was prepared by dissolving 0.2—0.5

mg of protein per ml of 0.1 M sodium hydroxide. The value of $E^{1\%}_{1cm}$ was calculated from the maximal absorbance, in the range between 270 and 290 nm with a 1-cm cell [7].

Amino acid analysis. This was carried out on a Beckman Model M121 analyzer. The 22-h hydrolysis sample was used at the basis for calculations, because of no significant difference from the 48- and 72 h results. Tryptophan was determined by the spectrofluorometric method of Duggan and Udenfriend [8].

Estimation of carbohydrate. This was estimated by the method of Dubois et al. [9] using dextrose as standard. Amino sugars were not measured.

Microzone electrophoresis. This was performed as described previously [10].

Fibrinogenolytic activities. The method of Ware et al. [11] was used.

Enzymatic activities. Caseinolytic activity was determined as previously described by us [4]. Esterase activity was measured by the method of Hestrin [12] modified by Habermann [13]. Phospholipase A activity was determined by the method of Brown and Bowles [14]. Alkaline phosphomonoesterase and phosphodiesterase were measured by the method of Suzuki and Iwanaga [15] as modified by Lo et al. [16].

Effects of enzyme inhibitors on fibrinogenolytic activities. 0.25 ml of α-(8 μg/ml) or β-fibrinogenase (80 μg/ml) was incubated with 0.25 ml of enzyme inhibitors (pH 7.4) at 37°C for 30 min, and then 0.5 ml of bovine fibrinogen (2%) was added. After another 30 min, 0.2 ml of this mixture was withdrawn and the clottable fibrinogen was assayed. Effects of the inhibitors were presented as percent inhibition of the fibrinogen-destruction activities of these fibrinogenolytic enzymes.

Results

Purification of α- and β-fibrinogenases. α- and β-Fibrinogenases were purified by means of recycling chromatography on Sephadex G-75 column. After 3 times passing through the column, the α- and β-fibrinogenases were found to be homogeneous as judged by SDS polyacrylamide gel electrophoresis in the presence or absence of β-mercapthoethanol.

Physical properties of α- and β-fibrinogenases. A single symmetrical boundary at 2.52 (α-fibrinogenase) and 3.04 (β-fibrinogenase) S. was obtained by ultracentrifugation (Figs. 1 and 2). The molecular weights of α-and β-fibrinogenases estimated by gel filtration were 21 500 and 25 000, by SDS-polyacrylamide gel electrophoresis 22 400 and 26 000 and by amino acid analysis 23 362 and 25 534, respectively. The maximal absorbance of both enzymes was at wavelength 286 nm. The extinction coefficients at this wavelength were calculated to be 9.36 for α-fibrogenase and 18.78 for β-fibrinogenase (Table I).

Chemical properties of α- and β-fibrinogenases. The amino acid analysis of the purified α- and β-fibrinogenases was given in Table II. The number of residues per molecule was expressed as nearest integer. It was found to be composed of 18 common amino acids. The carbohydrate contents were about 2% in both enzymes. If the carbohydrate moiety were glucose, the molar ratio of carbohydrate moiety to enzyme was 2—3 residues per molecule calculated from their molecular weights. This indicates that these two enzymes are glycopro-

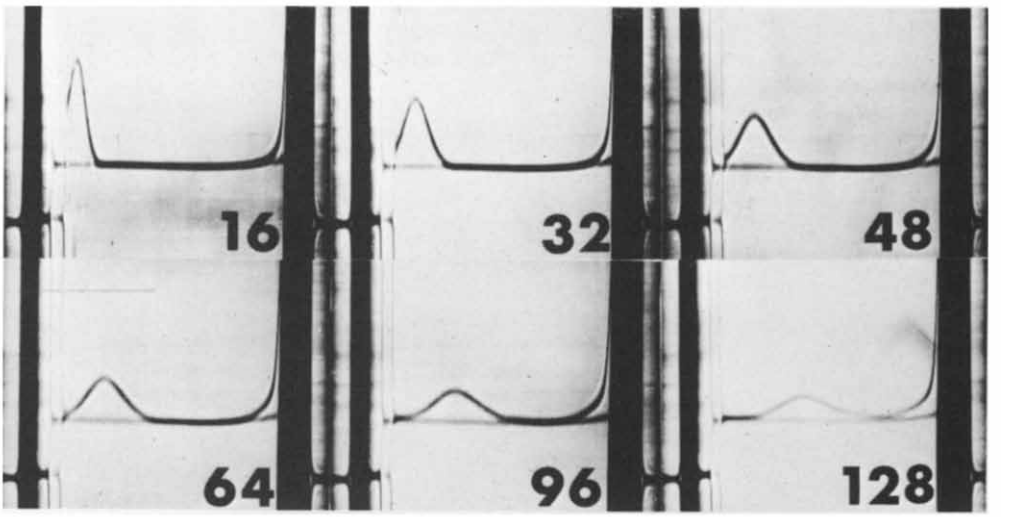

Fig. 1. Schlieren patterns of α-fibrinogenase of *T. mucrosquamatus* venom obtained with Spinco analytical ultracentrifuge with rotor speed at 59 780 rev./min. Sedimentation from left to right. The figures on chart refer to time in min when photograph was taken.

teins. The isoelectric points were determined by electrophoresis on cellulose acetate membrane to be pH 8.1 (α-fibrinogenase) and pH 5.7 (β-fibrinogenase) in citrate/phosphate buffer.

Optimal pH values of α- and β-fibrinogenases. The optimal pH value of α-fibrinogenase was 7.4, while that of β-fibrinogenase was around 8.5.

Effect of heat treatment on α- and β-fibrinogenases. As shown in Table III,

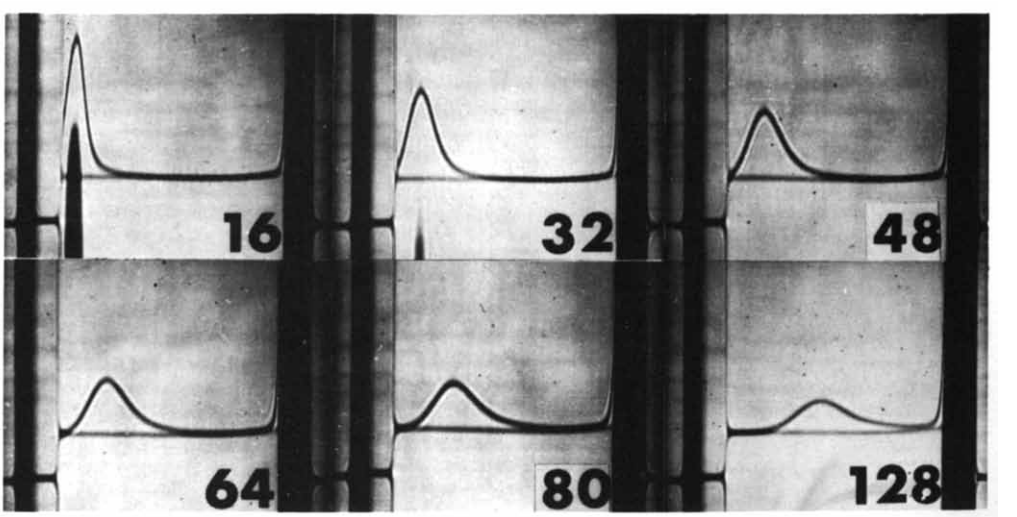

Fig. 2. Schlieren patterns of β-fibrinogenase of *T. mucrosquamatus* venom obtained with a Spinco analytical ultracentrifuge with rotor speed at 59 780 rev./min. Sedimentation from left to right. The figures on chart refer to time in min when photograph was taken.

TABLE II

AMINO ACID COMPOSITIONS OF α- AND β-FIBRINOGENASES OF *T. MUCROSQUAMATUS* VENOM (RESIDUES/MOLECULE)

Amino acid	α-Fibrinogenase	β-Fibrinogenase
Lysine	15	11
Histidine	7	5
Arginine	6	7
Aspartic acid	28	26
Threonine	14	12
Serine	14	13
Glutamic acid	21	15
Proline	7	18
Glycine	9	24
Alanine	9	12
Half-cystine	6	9
Valine	16	16
Methionine	6	3
Isoleucine	9	11
Leucine	17	19
Tyrosine	6	8
Phenylalanine	5	6
Tryptophan	8	14
Total	203	229

fibrinogenolytic activity of α-fibrinogenase was completely destroyed at 60°C, pH 5.6 and 7.4, and only 9% residual activity was detected at pH 9.0. On the other hand, β-fibrinogenase could not be significantly affected at pH 5.6, 7.4 and 9.0 after the same treatment.

Enzymatic properties of α- and β-fibrinogenases. The enzymatic properties of purified α- and β-fibrinogenases were compared with that of the crude venom. As shown in Table IV, α-fibrinogenase possessed the strongest fibrinogenolytic and caseinolytic activities. Concerning the esterase activity, β-fibrinogenase was 17 times higher than that of the crude venom, while α-fibrinogenase was completely devoid of this activity. Both α- and β-fibrinogenases were devoid of phospholipase A, phosphodiesterase and alkaline phosphomonoesterase activities which were found in the crude venom. Tosyl-L-arginine methylester

TABLE III

THE EFFECTS OF HEAT TREATMENT ON THE FIBRINOGENOLYTIC ACTIVITIES OF α- AND β-FIBRINOGENASES

Both enzymes were in a concentration of 1 mg/ml and heated at 60°C, pH 5.6, 7.4 and 9.0 for 30 min. After proper dilution with imidazole saline (pH 7.4), the remaining fibrinogenolytic activities were assayed and the residual activities were calculated as percentage of initial values (means of 3 determinations were presented)

	Residual fibrinogenolytic activity (%) at pH:		
	5.6	7.4	9.0
α-Fibrinogenase	0	0	9
β-Fibrinogenase	95	100	100

TABLE IV

COMPARISON OF THE ENZYMATIC ACTIVITIES OF THE PURIFIED α- AND β-FIBRINOGENASE AND THE CRUDE VENOM OF *T. MUCROSQUAMATUS*

	Crude	α-Fibrinogenase	β-Fibrinogenase
Fibrinogenolysis (mg/min per mg protein)	24 ± 3	120 ± 14	20 ± 5
Caseinolysis (μmol/min per mg protein)	0.09 ± 0.01	0.10 ± 0.02	0.03 ± 0.01
TAMe Esterase (μmol/min per mg protein)	87 ± 22	0	1500 ± 210
Phospholipase A (% hemolysis, 0.1 mg/ml)	32 ± 7	0	0
Phosphodiesterase (*A* 440 nm × 1000/min per mg protein)	24 ± 4	0	0
Alkaline phosphomonoesterase (*A* 440 nm × 1000/min per mg protein)	27 ± 8	0	0

esterase activity of β-fibrinogenase (2 μg/ml) was inhibited by phenylmethane sulfonyl fluoride, TosLysCH_2Cl and cysteine at concentrations of $2.5 \cdot 10^{-3}$ M. The percent inhibitions were 33.7 ± 11.4 for phenylmethane sulfonyl fluoride ($n = 5$), 26.7 ± 10.3 for TosLysCH_2Cl ($n = 6$) and 24.5 ± 6.6 for cysteine ($n = 3$).

Effects of enzyme inhibitors on the fibrinogenolytic activities of α- and β-fibrinogenases. Table V showed the effects of some enzyme inhibitors on the fibrinogenolytic activities of α- and β-fibrinogenases, comparing with trypsin. It was clearly shown that the fibrinogenolytic activity of α-fibrinogenase was markedly inhibited by cysteine and EDTA. Effect of β-fibrinogenase was inhibited markedly by phenylmethane sulfonyl fluoride and slightly by TosLys-CH_2Cl and cysteine. Effect of trypsin was completely inhibited by phenylmethane sulfonyl fluoride, trasylol, TosLysCH_2Cl and soybean trypsin inhibitor.

TABLE V

EFFECTS OF SOME ENZYME INHIBITORS ON THE FIBRINOGENOLYTIC ACTIVITIES OF α- AND β-FIBRINOGENASES AND TRYPSIN

Each experiment was performed in triplicate. Percent inhibitions were presented as means ± S.E. Final concentrations: ε-aminocaproic acid (EACA), 10^{-2} M; Trasylol $5 \cdot 10^{-5}$ M; Ethylenediamine tetraacetic acid (EDTA), $2.5 \cdot 10^{-4}$ M; Soybean trypsin inhibitor (SBTI), 10^{-4} g/ml; Cysteine, *N*-ethylmaleimide (NEM), tosyl-L-lysine chlormethylketone (TLCK) and phenylmethanesulfonylfluoride (PMSF); $2.5 \cdot 10^{-3}$ M; α-fibrinogenase, 2 μg/ml; β-fibrinogenase, 20 μg/ml; trypsin, 1 μg/ml; fibrinogen 10 mg/ml.

	α-Fibrinogenase	β-Fibrinogenase	Trypsin
EACA	−6 ± 23	0 ± 6	10 ± 9
Trasylol	−30 ± 4	−18 ± 12	94 ± 8
EDTA	85 ± 13	0 ± 6	8 ± 7
SBTI	14 ± 8	−14 ± 8	95 ± 7
Cysteine	93 ± 8	26 ± 5	24 ± 10
NEM	−6 ± 4	11 ± 8	5 ± 10
TLCK	7 ± 9	37 ± 6	97 ± 3
PMSF	7 ± 12	82 ± 11	110 ± 5

Discussion

The pathogenesis of hypofibrinogenemia in vivo induced by snake venoms may be classified into two groups. Snake venoms containing thrombin-like enzymes can decrease fibrinogen level by converting fibrinogen to fibrin-like substance which does not form a solid clot but which is rapidly broken down in vivo by fibrinolytic system (defibrination syndrome) [17,18]. Other snake venoms decrease plasma fibrinogen primarily by destroying fibrinogen directly [1,19] or indirectly through the activation of plasminogen [20].

Studies of fibrinogen-fibrin transformation and the fibrinolytic system may contribute not only to understanding of the blood coagulation mechanisms but also to development of new therapeutic agents for the treatment of thrombosis. Thrombin-like enzymes of snake venoms, especially Arvin from *Agkistrodon rhodostoma*, have been extensively studied, and to date, these appear to be very valuable as antithrombic agents for human use [21,22]. However, fibrinogenolytic or fibrinolytic activities of snake venoms have not been well studied. We isolated and purified two enzymes possessing fibrinogenolytic activity from the venom of *T. mucrosquamatus*.

We have reported previously [4] that the two purified fibrinogenolytic enzymes differ in their mode of actions on the fibrinogen. α-Fibrinogenase digested specifically the α(A) chain of fibrinogen, while β-fibrinogenase digested mainly the β(B) chain. We report in this paper some differences in their physicochemical properties. The molecular weight of β-fibrinogenase is larger than that of α-fibrinogenase, determined either by gel filtration, SDS-polyacrylamide gel electrophoresis or calculated from amino acid analysis. Concerning the amino acid composition, the content of aspartic acid is highest in both enzymes. However, the content of glycine, proline and tryptophan of β-fibrinogenase is much higher than that of α-fibrinogenase. There are also marked differences in the heat stability, optimal pH values and isoelectric points.

Concerning the enzymatic properties, α- and β-fibrinogenases possess proteolytic activity toward casein and fibrinogen, but were devoid of phospholipase A, phosphodiesterase and alkaline phosphomonoesterase activities found in the crude venom. In addition, β-fibrinogenase also possesses strong esterase activity toward tosyl-L-arginine methylester (1500 units/mg protein). The fibrinogenolytic activities of both enzymes are not dependent on plasminogen activation because ϵ-aminocaproic acid and trasylol do not inhibit their actions. Their fibrinogenolytic actions are directly on fibrinogen, and may be trypsin-like or plasmin-like. However, trypsin and plasmin can digest the α(A) and β(B) chains and, on prolonged incubation, also γ-chain of fibrinogen [23,24]. From these results, it is suggested that the enzymatic action of both fibrinogenolytic enzymes on fibrinogen are more specific than those of trypsin and plasmin.

As shown in Table V, EDTA inhibited the fibrinogenolytic activities of α-fibrinogenase. However, the caseinolytic [4] and fibrinogenolytic activities of β-fibrinogenase were not affected significantly by EDTA. The most remarkable difference between both venom enzymes and trypsin was that the fibrinogenolytic activities of neither venom enzymes was affected by concentration of the soybean trypsin inhibitor, which produced complete inhibition of trypsin.

Cysteine inactivated completely the fibrinogenolytic activity of α-fibrinogenase. It seemed that the disulfide bridges were essential for the biological activity of this enzyme. This result may also explain the heat-labile property of this enzyme. The importance of disulfide bridges for fibrinogenolytic activity was also reported in thrombin [25], thrombin-like enzyme [26] and fibrinolytic enzyme [27]. However, β-fibrinogenase, similar to trypsin, is only slightly affected by this reducing agent.

Neither α- nor β-fibrinogenase was inhibited by sulfhydryl group reagent, *N*-ethylmaleimide. This indicated that neither enzyme is a thiol-enzyme such as ficin, bromelain and papain [28].

β-Fibrinogenase, but not α-fibrinogenase, was inhibited by TosLysCH$_2$Cl. TosLysCH$_2$Cl has been used as a histidine-reactive reagent to elucidate the role of this amino acid in the active centers of plasmin [29], trypsin [30], thrombin [25] and thrombin-like enzymes [27,31,32]. The inhibition of β-fibrinogenase by TosLysCH$_2$Cl was less than that of trypsin, therefore, it probably reflected structural difference in the reactive histidine residue of the venom enzyme. But, nevertheless, the inhibitory effect of TosLysCH$_2$Cl suggests the involvement of histidine in fibrinogenolytic activity of β-fibrinogenase.

Sulfonation with phenylmethanesulfonyl fluoride also inhibited the fibrinogenolytic activities of β-fibrinogenase but not α-fibrinogenase. Sulfonation also inhibited trypsin, chymotrypsin [33], thrombin [34] and thrombin-like enzyme [31].

The esterase activity of β-fibrinogenase was also inhibited by phenylmethane sulfonyl fluoride, TosLysCH$_2$Cl and cysteine. This indicates that both esterase and fibrinogenolytic activities may be due to the same active site of this enzyme. However, phenylmethane sulfonyl fluoride caused stronger inhibition in fibrinogenolytic than in esterase activities. This may suggest that some groups in β-fibrinogenase, which are easily sulfonated, are important for the hydrolysis of fibrinogen but not of tosyl-L-arginine methylester.

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References

1 Ouyang, C. (1957) J. Formosan Med. Assoc. 56, 435—448
2 Meaume, J. (1966) Toxicon 4, 25—58
3 Jimenéz-Porras, J.M. (1970) Clin. Toxicol. 3, 389—431
4 Ouyang, C. and Teng, C.M. (1976) Biochim. Biophys. Acta 420, 298—308
5 Andrews, P. (1964) Biochem. J. 91, 222—233
6 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
7 Bencze, W.L. and Schmid, K. (1957) Anal. Chem. 29, 1193—1196
8 Duggan, D.E. and Udenfriend, S. (1956) J. Biol. Chem. 223, 313—319

9 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350—356
10 Ouyang, C., Hong, J.S. and Teng, C.M. (1971) Thromb. Diath. Haemorrh. 26, 224—234
11 Ware, A.J., Guest, H.M. and Seegers, W.H. (1947) Arch. Biochem. Biophys. 3, 231
12 Hestrin, S. (1949) J. Biol. Chem. 180, 249—261
13 Habermann, E. (1959) Arch. Exp. Pathol. Pharmakol. 236, 492—502
14 Brown, J.H. and Bowles, M.E. (1966) Toxicon 3, 205—212
15 Suzuki, T. and Iwanaga, S. (1958) J. Pharm. Soc. Japan 78, 354—361
16 Lo, T.B., Chen, Y.H. and Lee, C.Y. (1966) J. Chinese Chem. Soc. 13, 25—39
17 Ashford, A., Ross, J.W. and Southgate, P. (1968) Lancet 1, 486—489
18 Ouyang, C. and Teng, C.M. (1976) Toxicon 14, 49—54
19 Phillips, L.L., Weiss, H.J. and Christy, N.P. (1973) Thromb. Diath. Haemorrh. 30, 499—508
20 Forbes, C.D., Turpie, A.G.G., McNicol, G.P. and Douglas, A.S. (1966) Scot. Med. J. 11, 168—175
21 Bell, W.R., Pitney, W.R. and Goodwin, J.F. (1968) Lancet 1, 490—493
22 Sharp, A.A., Warren, B.A., Paxton, A.M. and Allington, M.J. (1968) Lancet 493—499
23 Pizzo, S.V., Schwartz, M.L., Hill, R.L. and Mckee, P.A. (1972) J. Biol. Chem. 247, 636—645
24 Blombäck, B., Hessel, B., Iwanaga, S., Reuterby, J. and Blombäck, M. (1972) J. Biol. Chem. 247, 1496—1512
25 Seegers, W.H. (1967) Prothrombin in Enzymology, Thrombosis, Hemophilia, p. 3, Charles C. Thomas, Springfield, Illinois
26 Markland, F.S. and Damus, P.S. (1971) J. Biol. Chem. 246, 6458—6473
27 Boffa, M.C. and Boffa, G.A. (1975) Biochim. Biophys. Acta 344, 275—290
28 Shaw, E. (1970) Physiol. Rev. 50, 244—296
29 Groskopf, W.R., Hsieh, B., Summaria, L. and Robbins, K.C. (1969) J. Biol. Chem. 244, 359—265
30 Shaw, E., Mares-Gula, M. and Cohen, W. (1965) Biochemistry 4, 2219—2224
31 Ouyang, C. and Hong, J.S. (1974) Toxicon 12, 449—453
32 Collins, J.P. and Jones, J.G. (1974) Eur. J. Biochem. 42, 81—87
33 Fahrney, D.E. and Gold, A.M. (1963) J. Am. Chem. Soc. 85, 997
34 Marciniak, E. and Seegers, W.H. (1965) Thromb. Diath. Haemorrh. 15, 633—634