The Proteolytic Action of Arvin on Human Fibrinogen

By M. R. EWART, M. W. C. HATTON, J. M. BASFORD AND K. S. DODGSON

Department of Biochemistry, University College, Cardiff CF1 1XL, U.K., and Twyford Laboratories Ltd., Twyford Abbey Road, London, N.W. 10, U.K.

(Received 13 March 1970)

1. Human fibrinogen was subjected to proteolysis by enzyme preparations (clinical Arvin and IRC-50 Arvin) from the venom of *Agkistrodon rhodostoma*. 2. IRC-50 Arvin releases three peptides from fibrinogen, and these were identified as fibrinopeptides AP, AY and A. 3. The less purified 'clinical' Arvin releases, in addition to fibrinopeptides AP, AY and A, small amounts of two heptapeptides derived from fibrinopeptides AP and A, probably because it contains another enzyme as well as Arvin. 4. No fibrinopeptide B is released by either Arvin preparation. 5. Thus, although Arvin is known to differ from 'reptilase' from *Bothrops jararaca* in that it does not activate the enzyme that cross-links fibrin (fibrin-stabilizing factor), it is identical with reptilase with respect to the peptides that it liberates from fibrinogen.

Arvin is a purified proteolytic enzyme fraction isolated from the venom of *Agkistrodon rhodostoma*, the Malayan pit viper (Esnouf & Tunnah, 1967). The preparation can be used clinically to produce controlled defibrination in patients requiring anticoagulant therapy. Unlike the usual anticoagulants it acts directly on fibrinogen, and plasma concentrations of fibrinogen can be decreased virtually to zero without apparently affecting other plasma clotting factors (Bell, Pitney & Goodwin, 1968; Sharp, Warren, Paxton & Allington, 1968; Bell, Bolton & Pitney, 1968).

When purified thrombin acts on human fibrinogen *in vitro*, four small soluble peptides (designated fibrinopeptides A, AP, AY and B; see Blombäck, Blombäck, Edman & Hessel, 1966) are released and polymerization of the modified fibrinogen molecule then leads to the formation of a fibrin gel. The action of Arvin on fibrinogen also leads to the production of a fibrin gel, but in this case the gel has very different mechanical properties and is readily dispersed.

In view of the considerable clinical potential of Arvin the elucidation of the precise nature of its action on fibrinogen is desirable. The present paper describes the nature of the peptides released from fibrinogen by two Arvin preparations that differ in their degree of purification. A preliminary account of this work has already been made (Ewart, Hatton, Basford & Dodgson, 1969).

MATERIALS AND METHODS

Human fibrinogen. Partially purified human fibrinogen (95% clottable) was obtained from Kabi Pharmaceuticals

Ltd., London W.5, U.K., and further purified (Straughn & Wagner, 1967) until 99–100% clottable.

Bovine thrombin. A commercial preparation (Parke-Davis Ltd., Hounslow, Middx., U.K.) was purified to a specific activity greater than 300 N.I.H. units/mg (see Magnusson, 1965).

Arvin. Preparations of specific activity equivalent to 1140 N.I.H. units/mg (thrombin) as supplied for clinical use (Twyford Laboratories Ltd.) were generally employed (this material is referred to below as 'clinical Arvin'). For some experiments a more highly purified preparation (1970 units/mg), obtained by ion-exchange chromatography of clinical Arvin on the carboxylic resin Amberlite IRC-50 ('IRC-50 Arvin'; Twyford Laboratories Ltd.) was employed.

Clotting of fibrinogen. A 1% solution of purified fibrinogen in 0.3M-ammonium acetate adjusted to pH7.0 with acetic acid was dialysed for 20–40 h against frequent changes of the same buffer before the addition of sufficient purified thrombin or Arvin to give a concentration of 1–2 units/ml. The whole was allowed to stand at 25°C for 6 h when the clot was broken up and the fluid separated on a Buchner funnel. The broken clot was washed thoroughly with water, and the clot supernatant and washings were combined and freeze-dried immediately.

Desalting by gel filtration. Freeze-dried clot supernatants were dissolved in 12 ml of 0.1M-pyridine and desalted by passing through a column (940 mm × 26 mm) of Sephadex G-25 (medium grain). Elution was with 0.1M-pyridine. All material eluted before the salt peak was bulked and freeze-dried.

High-voltage electrophoresis. Peptides were separated on Whatman 3MM paper with 0.25M pyridine–acetic acid buffer, pH4.2 (prepared by adding acetic acid to aqueous pyridine so that the final concentration of the latter was 0.25M; other pyridine–acetic acid buffers described below were prepared in an analogous manner). A potential gradient of 5.3 V/mm for 2 h was employed and peptides

were detected with ninhydrin reagent or the chlorine-starch-KI reagent of Rydon & Smith (1952). Arginine-containing peptides were detected with the Sakaguchi reagent (Roche, Thoi & Hatt, 1954) and phosphate-containing peptides with molybdate reagent after hydrolysis in 0.5M-NaOH (Bandurski & Axelrod, 1951).

Dowex 50 chromatography. Peptides present in the clot supernatants were separated by chromatography on columns of the sulphonic resin Dowex 50 (X2; 200–400 mesh) at 25°C. The procedure finally adopted differs appreciably from that of Blombäck *et al.* (1966) for fibrinopeptides liberated by thrombin, particularly with regard to the buffer systems. Two such systems were employed. With system A, material was loaded on to the column (300mm×9mm) in 0.2M-pyridine-acetic acid buffer, pH2.5, and elution was carried out with 100ml of the same buffer, followed by 120ml of a 240ml linear gradient of pyridine-acetic acid buffer (ranging from 0.2M to 1.0M-pyridine and from pH2.5 to 5.0). Finally 100ml of 8.5M-pyridine-acetic acid buffer, pH5.6, was passed through the column. With system B, material was loaded on to the column (680mm×9mm) in 0.2M-pyridine-acetic acid buffer, pH3.1, and elution was carried out with 140ml of the same buffer.

Samples of the eluate fractions (4ml fractions were usually collected) were tested with the ninhydrin reagent after alkaline hydrolysis (Hirs, Moore & Stein, 1956).

Amino acid analysis. Peptides (approx. 0.1μmol) were hydrolysed with 6M-HCl (1ml) at 110°C in sealed evacuated tubes for 24h and the hydrolysates were then evaporated to dryness under reduced pressure. Amino acid analyses were performed with a Technicon AutoAnalyzer, modified for fast analyses with a 230mm column and a

stepwise elution programmer (Locarte Co. Ltd., London S.W.7, U.K.).

N-Terminal amino acid analysis. The DNS-chloride method (Gray & Hartley, 1963) was used and the DNS-amino acids were separated on polyamide plates (Cheng-Chin Trading Co., Taiwan; see Woods & Wang, 1967).

C-Terminal amino acid analysis. The procedure of Ambler (1967) was employed. Enzymic digestion was with di-isopropyl phosphorofluoridate-treated carboxypeptidase B [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] and liberated amino acids were determined with the Technicon Autoanalyzer.

RESULTS

Separation and identification of fibrinopeptides released from fibrinogen by thrombin. The action of thrombin on human fibrinogen leads to the release of four fibrinopeptides, designated A, AP, AY and B (Blombäck *et al.* 1966). A further peptide (believed to be peptide B lacking the C-terminal arginine) is also generally present in clot supernatants (B. Blombäck, personal communication). These various peptides were separated and identified in the present study in order to provide a basis for comparison with those released by Arvin. The Dowex 50 separation method employed differed appreciably from that described by Blombäck *et al.* (1966), particularly with regard to buffer systems.

Human fibrinogen (1.0–1.5g portions) was clotted with thrombin for 6h and the peptides present in the

Fig. 1. Separation of the peptides released from fibrinogen by thrombin. Desalted freeze-dried clot supernatant was separated on a column (300mm×9mm) of Dowex 50 (X2; 200–400 mesh) by using an elution scheme of increasing pH and pyridine concentration (system A). Peptides were detected by reaction with ninhydrin after alkaline hydrolysis (○). The pH of the eluate fractions (4ml) is also shown (Δ). The limits and designations of the pooled fractions taken for further study are indicated.

desalted clot supernatant were separated on columns of Dowex 50 with system *A* (Fig. 1). Fractions 6–16 were pooled, freeze-dried and rechromatographed with system *B* (Fig. 2). The following fractions from these two chromatographic runs were combined: from the system *A* run fractions 2–4 (designated fraction T-I), 40–43 (fraction T-IV) and 44–50 (fraction T-V), and from the system *B* run fractions 18–24 (fraction T-II) and 26–35 (fraction T-III). The various pooled fractions were then freeze-dried.

The fractions, with one exception, gave single bands on high-voltage electrophoresis. Mobilities

towards the anode were as follows: fraction T-II, 69mm; fraction T-III, 33mm; fraction T-IV, 33mm; fraction T-V, 20mm. Trace amounts of a band with mobility of 33mm could be detected in fraction T-V, reflecting the overlap of fraction T-IV with this fraction.

Amino acid and *N*-terminal amino acid analyses (Table 1), electrophoretic mobility and staining reactions for arginine and phosphate established that fractions T-II, T-III, T-IV and T-V were fibrinopeptides AP, B, AY and A respectively. The properties of fraction T-I indicate that it is fibrinopeptide B lacking its *C*-terminal arginine.

Separation and identification of the fibrinopeptides released from fibrinogen by clinical Arvin. Fibrinogen (1.0–1.5g portions) was clotted with clinical Arvin for 6h and the desalted clot supernatants were subjected to Dowex 50 chromatography with system *A* (Fig. 3). The following fractions were combined and freeze-dried: 3–4 (designated fraction A-I), 5–6 (fraction A-II), 7–16 (fraction A-III), 18–23 (fraction A-IV), 41–44 (fraction A-V), 45–51 (fraction A-VI) and 60–68 (fraction A-VII).

On high-voltage electrophoresis the pooled fractions, with two exceptions, gave single bands that moved towards the anode with the following mobilities: fraction A-I, 184mm; fraction A-II, 150mm; fraction A-III, 69mm; fraction A-IV, 108mm; fraction A-V, 33mm; fraction A-VI, 20mm. Trace amounts of a band with a mobility of 33mm were detected in fraction A-VI, reflecting the overlap of fraction A-V with this fraction. Fraction A-VII moved towards the cathode but tended to streak on electrophoresis and was not homogeneous.

Amino acid and *N*-terminal and *C*-terminal

Fig. 2. Separation of the component peptides of fractions 6–16 (Fig. 1). These fractions were separated on a column (680mm×9mm) of Dowex 50 (X2; 200–400 mesh) by elution with 0.2M-pyridine-acetic acid buffer, pH3.1 (system *B*). Peptides were detected by reaction with ninhydrin after alkaline hydrolysis (○). The limits and designations of the pooled fractions taken for further study are indicated.

Table 1. *Amino acid analyses and staining reactions of peptide fractions liberated from fibrinogen by thrombin*

(a) Amino acid analyses

Amino acid	Peptide fraction ...	T-I	T-II	T-III	T-IV	T-V
Asp		2.9	2.1	3.0	1.8*	2.0
Ser		0.9	0.7	0.9	1.1	0.9
Glu		3.1	2.2	3.0	2.2	2.0
Gly		2.1	4.8	2.0	4.8	5.2
Ala		1.0	1.9*	1.0	1.0	1.8*
Val		1.1	1.1	1.1	1.1	1.0
Leu		—	0.9	—	1.0	1.1
Phe		1.7	1.0	1.9	1.1	1.0
Arg		—	0.9	0.9	0.9	1.0

(b) Staining reactions

Stain	Peptide fraction ...	T-I	T-II	T-III	T-IV	T-V
Ninhydrin		Weak	+	Weak	+	+
Phosphate		—	+	—	—	—
Sakaguchi		—	+	+	+	+

* *N*-Terminal amino acid (none detected for fractions T-I and T-III).

Fig. 3. Separation of the peptides released from fibrinogen by clinical Arvin. Desalted freeze-dried clot supernatants were separated on a column (300mm $\times$ 9mm) of Dowex 50 (X2; 200–400 mesh) by using an elution scheme of increasing pH and pyridine concentration (system A). Peptides were detected by reaction with ninhydrin after alkaline hydrolysis (O). The pH of the eluate fractions (4ml) is also shown (Δ). The limits and designations of the pooled fractions taken for further study are indicated.

Table 2. *Amino acid analyses and staining reactions of peptide fractions liberated from fibrinogen by clinical Arvin*

(a) Amino acid analyses

Amino acid	Peptide fraction	...	A-I	A-II	A-III	A-IV	A-V	A-VI
Asp			3.2	1.9	2.0	1.8	1.8*	2.0
Ser			0.6	0.7	0.7	0.9	0.9	0.8
Glu			2.8	1.1	2.1	1.1	2.1	2.0
Gly			2.8	2.0	5.0	2.2	5.0	5.2
Ala			1.1	1.0*	1.9*	0.9*	1.2	1.9*
Val			0.7	—	1.1	—	1.1	1.1
Leu			—	—	0.9	—	1.0	1.1
Phe			1.7	—	1.0	—	0.9	1.0
Arg			—	—	1.0†	—	1.0†	1.0†

(b) Staining reactions

Stain	Peptide fraction	...	A-I	A-II	A-III	A-IV	A-V	A-VI
Ninhydrin			Weak	+	+	+	+	+
Phosphate			—	+	+	—	—	—
Sakaguchi			—	—	+	—	+	+

* *N*-Terminal amino acid (none detected for fraction A-I).

† *C*-Terminal amino acid.

amino acid analyses, staining reactions for arginine and phosphate (Table 2) and chromatographic and electrophoretic mobilities established that fractions A-III, A-V and A-VI were fibrinopeptides AP, AY and A respectively.

The results also suggested that fractions A-II

and A-IV were heptapeptides derived from the *N*-terminal sections of fibrinopeptides AP and A respectively. This was confirmed by the isolation of the same two peptides after incubation of fibrinopeptides AP and A with clinical Arvin under the standard clotting incubation conditions. In each

Fig. 4. Separation of the peptides released from fibrinogen by IRC-50 Arvin. Desalted freeze-dried clot supernatants were separated on a column (300mm × 9mm) of Dowex 50 (X2; 200–400 mesh) by using an elution scheme of increasing pH and pyridine concentration (system A). Peptides were detected by reaction with ninhydrin after alkaline hydrolysis (○). The pH of the eluate fractions (4 ml) is also shown (△). The limits and designations of the pooled fractions taken for further study are indicated.

Table 3. *Amino acid analyses and staining reactions of peptide fractions released from fibrinogen by IRC-50 Arvin*

(a) Amino acid analyses					
Amino acid	Peptide fraction	...	PA-I	PA-II	PA-III
Asp			2.1	1.8*	2.0
Ser			0.6	0.9	0.9
Glu			2.2	2.1	2.0
Gly			5.1	4.9	5.1
Ala			1.9*	1.3	1.9*
Val			1.1	1.1	1.2
Leu			1.0	1.0	1.0
Phe			1.0	1.0	0.9
Arg			0.8†	0.9†	0.9†
(b) Staining reactions					
Stain	Peptide fraction	...	PA-I	PA-II	PA-III
Ninhydrin			+	+	+
Phosphate			+	—	—
Sakaguchi			+	+	+
* <i>N</i> -Terminal amino acid.					
† <i>C</i> -Terminal amino acid.					

case a second degradation product containing arginine was also detected, the chromatographic properties of which fell within the limits exhibited by fraction A-VII (cf. Fig. 3). This product was presumed to be the arginine-containing nonapeptide that would remain after liberation of the *N*-terminal heptapeptide section from either fibrinopeptide

AP or fibrinopeptide A. However, this material has not yet been characterized.

The heptapeptides are probably formed after the release of fibrinopeptides AP and A as they were either absent, or present only in traces, in clot supernatants obtained by incubating fibrinogen with clinical Arvin for a period of only 1 h. At that

time the release of the 'A' peptides was essentially complete. No fibrinopeptide B could be detected among the products of the action of clinical Arvin. However, the minor fraction A-I showed electrophoretic, chromatographic and staining properties consistent with it being fibrinopeptide B lacking the C-terminal arginine. The amino acid composition was very similar to that expected for this peptide but with a rather high value for glycine. This may not be significant in view of the fact that only very small quantities of this peptide were available for analysis. Further comment about this fraction is made below.

Action of IRC-50 Arvin on fibrinogen. Portions of fibrinogen (1.0–1.5g) were clotted with IRC-50 Arvin for 6h and the desalted clot supernatants were subjected to Dowex 50 chromatography with system A (Fig. 4). The following fractions were combined and freeze-dried: 7–14 (fraction PA-I), 41–44 (fraction PA-II) and 45–50 (fraction PA-III). On high-voltage electrophoresis the pooled fractions, with one exception, moved as single bands towards the anode with the following mobilities: fraction PA-I, 69mm; fraction PA-II, 33mm; fraction PA-III, 20mm. Trace amounts of a band of mobility 33mm were detected in fraction PA-III, reflecting the overlap of fraction PA-II with this fraction.

Amino acid composition, N-terminal and C-terminal amino acids, staining reactions (Table 3), chromatographic and electrophoretic behaviour established that fractions PA-I, PA-II and PA-III were fibrinopeptides AP, AY and A respectively. Fibrinopeptide B was not present in these clot supernatants. Notable also was the absence of material corresponding to the heptapeptides (fractions A-II and A-IV; Fig. 3) and the basic material (fraction A-VII; Fig. 3) released by clinical Arvin.

DISCUSSION

The four fibrinopeptides released by the action of thrombin have been shown (see Blombäck *et al.* 1966) to possess the amino acid sequences shown in Fig. 5.

These four fibrinopeptides have all been recognized in the thrombin clot supernatants in the pre-

sent work. In each case the liberation of the 'A' peptides and the 'B' peptide is the result of the cleavage of an arginyl-glycyl bond. In contrast, the clotting enzyme of *Agkistrodon rhodostoma*, referred to as IRC-50 Arvin, liberates only the 'A' peptides from fibrinogen. In this respect it appears to resemble reptilase (Blombäck, 1958), a clotting enzyme prepared from the venom of *Bothrops jararaca*. The two snake-venom enzymes thus exhibit a most remarkable specificity towards fibrinogen in that they cleave only the arginyl-glycyl bond linking the 'A' peptides to the remainder of the fibrinogen molecule. Loss of 'A' peptides only presumably leads to the production of a polymer of the type $[(\alpha\beta(B)\gamma)_2]_n$ (International Committee on Haemostasis and Thrombosis, 1967) rather than the $[(\alpha\beta\gamma)_2]_n$ -type fibrin polymer resulting from the action of thrombin.

The action of clinical Arvin on fibrinogen is more complex than that of IRC-50 Arvin and this reflects the presence in the former preparation of a second proteolytic enzyme (M. W. C. Hatton, unpublished work). The action of this enzyme leads to the appearance in clot supernatants of peptides additional to the 'A'-type fibrinopeptides released by the clotting enzyme. These additional peptides are present in relatively small amounts. Evidence has been presented indicating that two of them are the acidic N-terminal heptapeptides derived from fibrinopeptides AP and A, and having the following amino acid sequences respectively:

Ala-Asp-SerP-Gly-Glu-Gly-Asp

and

Ala-Asp-Ser-Gly-Glu-Gly-Asp

The action of clinical Arvin also leads to the appearance of basic material in the clot supernatants. This has not yet been characterized, but probably includes the nonapeptide fragment that would remain after liberation of the N-terminal heptapeptides from fibrinopeptides AP and A.

The appearance of very small amounts of an acidic peptide (believed to be fibrinopeptide B lacking the C-terminal arginine) in clot supernatants from both thrombin and clinical Arvin is rather puzzling, particularly as the latter enzyme does not release fibrinopeptide B. Minute traces of a similar

Fibrinopeptide A	Ala-Asp-Ser -Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg
Fibrinopeptide AP	Ala-Asp-SerP-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg
Fibrinopeptide AY	Asp-Ser -Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg
Fibrinopeptide B	Pyr-Gly -Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg

Fig. 5. Amino acid sequences of fibrinopeptides A, AP, AY and B. SerP represents serine O-phosphate and Pyr represents pyroglutamic acid.

material may also be present in IRC-50 Arvin clot supernatants (see Fig. 4). In our experience the material has been a consistent feature of clot supernatants from both thrombin and clinical Arvin, and Dr B. Blombäck has indicated that he has also frequently noted it in clot supernatants. However, in terms of quantity, the amount present in Arvin clot supernatants is negligible compared with the amounts of the 'A' peptides that are released.

The benign action of Arvin *in vivo* is probably because, unlike thrombin, the enzyme does not activate fibrin-stabilizing factor (Barlow, Holleman & Lorand, 1970). In this respect Arvin differs from reptilase as the latter enzyme has been shown to be capable of activating fibrin-stabilizing factor (Kopeck, Latallo, Stahl & Wegrzynowicz, 1969). Under these circumstances Arvin microclots formed *in vivo* cannot become cross-linked and are likely to be good substrates for the fibrinolytic system. However, the non-release of fibrinopeptide B may also be of importance as it is possible that the $[(\alpha\beta(B)\gamma)_2]_n$ polymer is more susceptible to the action of the fibrinolytic system than the $[(\alpha\beta\gamma)_2]_n$ type. The former polymer consists of very thin filaments (Hall & Slayter, 1959), unlike the latter where relatively thick interconnected fibres are formed.

Note added. Since submission of this paper for publication it has been noted that Holleman & Coen (1970) have published a paper in which they come to similar conclusions to those arrived at in the present paper and in our previous publication (Ewart *et al.* 1969).

M.R.E. is grateful to the Science Research for a CAPS award.

REFERENCES

- Ambler, R. P. (1967). In *Methods in Enzymology*, vol. 11, p. 155. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
- Barlow, G. H., Holleman, W. H. & Lorand, L. (1970). *Res. Comm. Chem. Path. Pharmac.* **1**, 39.
- Bell, W. R., Bolton, G. & Pitney, W. R. (1968). *Br. J. Haemat.* **15**, 589.
- Bell, W. R., Pitney, W. R. & Goodwin, J. F. (1968). *Lancet*, i, 490.
- Blombäck, B. (1958). *Ark. Kemi*, **12**, 321.
- Blombäck, B., Blombäck, M., Edman, P. & Hessel, B. (1966). *Biochim. biophys. Acta*, **115**, 371.
- Esnouf, M. P. & Tunnah, G. W. (1967). *Br. J. Haemat.* **13**, 581.
- Ewart, M. R., Hatton, M., Basford, J. M. & Dodgson, K. S. (1969). *Biochem. J.* **115**, 17P.
- Gray, W. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 59P.
- Hall, C. E. & Slayter, H. S. (1959). *J. biophys. biochem. Cytol.* **5**, 11.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.
- Holleman, W. H. & Coen, L. J. (1970). *Biochim. biophys. Acta*, **200**, 587.
- Kopeck, M., Latallo, Z. S., Stahl, M. & Wegrzynowicz, Z. (1969). *Biochim. biophys. Acta*, **181**, 437.
- Magnusson, S. (1965). *Ark. Kemi*, **24**, 349.
- Roche, J., Thoi, N.-V. & Hatt, J. L. (1954). *Biochim. biophys. Acta*, **14**, 71.
- Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
- Sharp, A. A., Warren, B. A., Paxton, A. M. & Allington, M. J. (1968). *Lancet*, i, 493.
- Straughn, W. & Wagner, R. H. (1967). *Thromb. Diath. haem.* **16**, 198.
- Woods, K. R. & Wang, K.-T. (1967). *Biochim. biophys. Acta*, **133**, 269.