Biochem. J. (1973) **131**, 799–807
Printed in Great Britain

Studies on the Coagulant Enzyme from *Agkistrodon rhodostoma* Venom

ISOLATION AND SOME PROPERTIES OF THE ENZYME

By M. W. C. HATTON*
Twyford Laboratories Ltd., Twyford Abbey Road, London N.W.10, U.K.

(*Received* 1 *December* 1972)

1. Arvin, a commercial preparation of the coagulant activity from the venom of *Agkistrodon rhodostoma*, is shown to contain a non-coagulant caseinolytic fraction. 2. A method is described for the purification of the coagulant enzyme free from any detectable contaminating protein. 3. The coagulant enzyme is identified as a glycoprotein which probably consists of a single polypeptide chain containing approx. 29% by weight of carbohydrate. Amino acid and carbohydrate analyses are reported and the *N*- and *C*-terminal amino acid residues identified. 4. Electrophoresis on polyacrylamide gel reveals the polymorphic nature of the glycoprotein. Five forms of the enzyme are observed. 5. The coagulant action is correlated with an arginine esterase activity and kinetic properties are studied with both arginine and lysine esters as substrates. The inhibitory nature of guanidine and arginine toward the esterase activity is reported.

Esnouf & Tunnah (1967) reported a method for the extraction of a purified coagulant fraction from the venom of the Malayan pit viper (*Agkistrodon rhodostoma* Boie). The fraction, Arvin,† has been used clinically as a defibrinating agent (Bell *et al.*, 1968; Sharp *et al.*, 1968) and investigated for its proteolytic action on human fibrinogen *in vitro* (Ewart *et al.*, 1969, 1970; Holleman & Coen, 1970). Arvin released several small peptides from the *N*-terminal ends of the α(A) and β(B) chains of fibrinogen. After further purification the coagulant enzyme of *A. rhodostoma* venom cleaved only fibrinopeptides A, AP and AY from the α(A) chain of fibrinogen. Thus it was postulated that a contaminating proteolytic enzyme was present in Arvin (Ewart *et al.*, 1970).

A method is described in the present paper for the purification of the coagulant enzyme in Arvin, and this has been adapted for the preparation of the enzyme direct from the venom. A proteolytic fraction, free from coagulant activity, is shown to be present in Arvin.

The homogeneity of the coagulant enzyme and analytical results are reported, together with a preliminary investigation of the enzymic action on several synthetic amino acid esters. A comparison of the coagulant enzyme with the more widely known properties of thrombin is made where possible.

Experimental

Fractionation of the crude venom on TEAE-cellulose

The chromatographic method has been described by Esnouf & Tunnah (1967); TEAE-cellulose was supplied by Serva (Heidelberg, Germany). Approximately 70% of the coagulant activity of the crude venom was eluted as a broad peak, which was pooled. This fraction is called peak 6.

Isolation of the coagulant enzyme from peak 6 on Amberlite IRC-50 resin

Amberlite IRC-50 (analytical grade; BDH Chemicals Ltd., Poole, Dorset, U.K.) was equilibrated with 0.04M-Tris–phosphate buffer, pH 6.2.

The eluted peak 6 material was added continuously to a column of the resin, until coagulant activity began to pass through the column. The resin was then washed with 0.04M-Tris–phosphate buffer, pH 6.2, until the E_{280} of the eluate had reached a low plateau ($E_{280} = 0.01$) and a protein analysis (Lowry *et al.*, 1951) indicated that no protein was passing through. The coagulant enzyme was eluted from the column in a volume of approx. 100ml by 0.2M-Tris–HCl, pH 8.5, containing 3% (w/v) NaCl.

Isolation of the coagulant enzyme from Arvin on Amberlite IRC-50

Arvin (400 units/ml) was obtained from Mr. G. W. Tunnah (Twyford Laboratories Ltd.). Approximately 10ml of the preparation was desalted into 0.05M-sodium phosphate buffer, pH 6.5, and the enzyme solution was run slowly through a small column (8cm × 1cm) of Amberlite IRC-50, which had been equilibrated with 0.05M-sodium phosphate buffer, pH6.5. After the adsorption step, the column was washed with 0.05M-phosphate buffer, pH 6.5, until the E_{280} of the eluate was 0.01. The coagulant enzyme was then eluted by 0.3M-sodium phosphate buffer, pH 8.0, as a sharp peak identified by clotting activity and protein determination.

* Present address: Department of Pathology, McMaster University, Hamilton, Ontario, Canada.

† Arvin is the registered trade mark of Twyford Laboratories Ltd.

Measurement of coagulant activity

To 0.1 ml of reconstituted freeze-dried human plasma, 0.1 ml of 0.01 M-Tris–HCl buffer, pH 7.2 (containing 0.87% NaCl), and 0.1 ml of 0.025 M-$CaCl_2$ were added with mixing. The solution was incubated at 37°C for 10s and 0.1 ml of enzyme solution was added. The time taken for clot formation to occur from the moment of addition of the enzyme was measured with a stop-watch. The coagulant activity was determined from a standard clotting curve obtained by using a standard Arvin solution kindly supplied by Mr. G. W. Tunnah. One unit of coagulant activity is approximately equivalent to one N.I.H. thrombin unit. Specific coagulant activity is defined as the number of units contained in 1 ml of enzyme when $E_{280}^{1\,cm} = 1.00$.

Purification of thrombin

Bovine thrombin was obtained from Parke–Davis Ltd. (Hounslow, Middlesex, U.K.). It was found that further purification was necessary, for which the method of Rasmussen (1955) was used. The purified enzyme had an activity in the range 1200–1500 N.I.H. units/mg of protein and was stored frozen at −30°C.

Measurement of esterase activity with N-α-benzoyl-L-arginine ethyl ester as substrate

The method given by Rick (1963*c*) was adapted as follows. A volume (3 ml) of 0.625 mM-*N*-α-benzoyl-L-arginine ethyl ester buffered at pH 8.5 with 0.1 M-Tris–HCl was incubated with 0.1 ml of enzyme solution at 25°C, and the linear change of absorbance at 253 nm was measured over 5 min. Activity was expressed as μmol of ester hydrolysed/min. For the measurement of optimum pH, the ester solution was prepared in 0.1 M buffers of acetate, phosphate and Tris–HCl, the substrate solutions ranging from pH 5.5 to 9.9. K_m and K_i were determined by the method described by Lineweaver & Burk (1934) by using statistical regression analysis.

Correlation of esterase and coagulant activity during inhibition

A 3 ml sample of the coagulant enzyme, containing 53 units/ml in 0.1 M-Tris–phosphate buffer, pH 7.2, was incubated at 37°C with 0.2 ml of 0.1 M-phenylmethanesulphonyl fluoride in propan-2-ol. A further 3 ml of coagulant enzyme containing 0.2 ml of propan-2-ol was incubated as a control. Clotting and arginine esterase activities were measured for both samples at frequent intervals during the incubation period of 30 min.

Determination of protein

The absorbance of column effluent fractions was measured at 280 nm. In addition the method of Lowry *et al.* (1951) was used where stated. For the nitrogen determinations on the purified enzyme the method described by Humphries (1956) was used.

Assay of caseinolytic activity

This was done by the procedure given for trypsin by Rick (1963*b*) using Hammarsten casein (BDH Ltd.). The unit of caseinolytic activity was calculated by dividing E_{280} for the substrate supernatant after proteolysis by E_{280} for the enzyme solution used. For Arvin the reaction with casein was found to be first order for enzyme solutions up to $E_{280} = 0.1$.

Assay for haemoglobinolytic activity

A solution of 2% haemoglobin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) in 6 M-urea was prepared in the manner described by Rick (1963*a*). The method of assay was the same as that described for measurement of caseinolytic activity.

Haemorrhagic factor activity

This was kindly assayed by Miss J. Elliot (Twyford Laboratories, Ltd.) by the method of Esnouf & Tunnah (1967).

Chromatography on Sephadex G-100

Concentrated samples of peak 6 (volume 2.5 ml, containing approx. 10 mg of protein) were subjected to chromatography on a column (63.5 cm × 2.5 cm) of Sephadex G-100 and eluted with 0.04 M-Tris–HCl buffer, pH 8.5.

Polyacrylamide-gel electrophoresis

The method of Clarke (1963) was adapted as follows. Gels containing 10% (w/v) acrylamide were prepared in tubes (0.65 cm × 7.5 cm). After polymerization for 1 h at room temperature, the gels were equilibrated with the running buffer, 0.005 M-Tris–glycine, pH 8.5, for 2 h at 2.5 mA/tube. By this means a steady voltage was obtained before application of the samples. After loading with a 20 μl Microcap (Shandon Ltd.), electrophoresis was maintained for either 30 or 60 min with freshly prepared running buffer. The gels were fixed in 20% (w/v) sulphosalicyclic acid for 1 h at 70°C and then stained in 0.25% Coomassie Brilliant Blue (BDH Ltd.) for 20 min at room temperature. With practice it was possible to stain the gel sufficiently for viewing the protein bands, without necessitating a lengthy destaining step. The periodate–Schiff procedure for carbohydrate detection and the Toluidine Blue method for lipids are described by Clarke (1963).

Analysis of the coagulant enzyme

(*a*) *Amino acid analysis.* This was kindly undertaken by Dr. J. M. Basford (University College of South Wales, Cardiff) using a Technicon autoanalyser. The samples, each containing 1.0ml of enzyme (E_{280} = 1.00), were hydrolysed by 5.7M-HCl in sealed glass tubes at 110°C, for 24, 48 and 96h. The values for the acid-labile amino acids, e.g. serine and threonine, were extrapolated to zero hydrolysis time. For those residues only slowly released, e.g. valine and isoleucine, the maximum value obtained is given. Tryptophan was determined spectrophotometrically by the method of Beaven & Holiday (1952).

(*b*) *N-Terminal amino acid analysis.* Samples were labelled with *N*-dimethylaminonaphthalenesulphonyl chloride by a modification of the method of Gray & Hartley (1963*a*,*b*). A solution of the protein in 8M-urea was used to denature the structure sufficiently to allow reaction to take place. The labelled protein was separated from excess of dansyl chloride, urea and other reaction products on a column (23cm × 2.5cm) of Sephadex G-25, by using acetone–0.1M-$NaHCO_3$ (2:3, v/v). The yellow fluorescent tubes of effluent were pooled, dried and 1 ml of 5.7M-HCl was added to each. After hydrolysis in a sealed tube at 110°C for 18–30h the products were examined by polyamide plate chromatography (Woods & Wang, 1967) in the solvent systems (*a*) 1.5% (v/v) formic acid and (*b*) *n*-heptane–butan-1-ol–acetic acid (3:3:1, by vol.).

(*c*) *C-Terminal amino acid analysis.* The procedure described by Ambler (1967) was used. Carboxypeptidase A (di-isopropyl phosphorofluoridate-inhibited) was obtained from Sigma. At intervals during incubation with carboxypeptidase, a sample of the amino acids cleaved from the coagulant enzyme was labelled with dansyl chloride and determined by polyamide plate chromatography.

(*d*) *Carbohydrate analysis.* Total hexoses were determined by the orcinol–sulphuric acid method of Francois *et al.* (1962) with a solution of glucose–galactose (1:1, w/w; 200μg/ml) as a standard. After acid hydrolysis of the enzyme in 4M-HCl at 105°C for 4h in a sealed glass tube, the released hexoses were qualitatively analysed. Descending chromatographs on Whatman no. 1 paper were run in two solvent systems: (A) butan-1-ol–pyridine–0.1M-HCl (5:3:2, by vol.) for 24h and (B) butan-1-ol–ethanol–water (10:1:2, by vol.) for 96h. Samples of glucose, galactose, mannose and fucose were included as standard non-nitrogenous sugars most likely to occur in glycoproteins. The silver nitrate method described by Smith (1969, p. 316) was used to detect the spots.

Hexosamines were assayed by the procedure of Rondle & Morgan (1955) after acid hydrolysis in 4M-HCl for 2 and 4h at 105°C. In addition the hydrolysate was developed in butan-1-ol–pyridine–0.1M-HCl (5:3:2, by vol.) and the products were identified with the Elson–Morgan reagent (Smith, 1969, p. 789) or ninhydrin. Glucosamine and galactosamine were included as standards.

The total sialic acid was assayed by the method of Weissbach & Hurwitz (1959) after hydrolysis of the enzyme in 0.05M-H_2SO_4 for 1h at 80°C. The sialic acids present in the hydrolysate were identified by using the thiobarbituric acid procedure (Warren, 1960) after chromotography in butan-1-ol–acetic acid–water (12:3:5, by vol.) and butan-1-ol–pyridine–0.1M-HCl (6:4:3, by vol.) for 24h. Samples of *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid (Sigma) were run as standards.

Action of the coagulant enzyme on lysine esters

(*a*) *Lysine methyl ester* (*Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.*). A solution of the ester (10mg/ml) was prepared in 0.05M-sodium phosphate buffer, pH6.7. Coagulant enzyme (0.2ml, containing 80 units) was added and the whole was incubated at 37°C for 24h. Samples were taken for paper chromatography at 0, 1, 2, 4, 6 and 24h together with samples from a control ester solution without enzyme present. The chromatograms were developed in 2-methylpropan-2-ol–formic acid–water (14:3:3, by vol.) for 24h. After spraying with ninhydrin the spots were measured semi-quantitatively with a Joyce–Loebl Chromoscan apparatus.

(*b*) *N-α-Benzyloxycarbonyl-L-lysine p-nitrophenyl ester* (*Calbiochem Ltd., London W.*1, *U.K.*). A stock solution of the ester (5mg/ml) was prepared in ethanol. A volume (2.8ml) of 0.1M-Tris–phosphate buffer, pH6.5, was placed in a 1cm-light-path glass cuvette with 0.1ml of enzyme solution. After mixing, the cuvette was inserted into the heat-controlled (25°C) cell carriage of a Unicam SP.500 spectrophotometer. Finally 0.1ml of substrate was added, the solutions were mixed and the stop-clock was started. At 30s intervals the E_{400} was read and the reaction allowed to proceed for 5min. Solvolysis of the ester was determined from a solution containing 2.9ml of buffer and 0.1ml of substrate. The molar extinction coefficient of *p*-nitrophenol at pH6.5 was measured as 4000 at 400nm.

Action of the coagulant enzyme on N-α-benzyloxycarbonyl-L-phenylalanine p-nitrophenyl ester

The ester was prepared in ethanol (0.5mg/ml). The method used was essentially that described for *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester except that 0.1M-Tris–HCl, pH8.5, was used to buffer the reaction. The reaction was measured over 2.5min from the addition of substrate. The molar extinction coefficient of *p*-nitrophenol at pH8.5 was measured as 19500 at 400nm.

Results

Proteolytic activity of Arvin on casein and haemoglobin

Preparations of Arvin, bovine thrombin and the crude coagulant fraction from the venom after TEAE-cellulose chromatography (peak 6) were incubated with solutions of 1% casein and 2% denatured haemoglobin at 37°C. Table 1 summarizes the observations for each enzyme preparation and shows the variation of caseinolytic activity obtained for three different batches of Arvin. The proteolytic action of venom peak 6 was further investigated after chromatography on Sephadex G-100. The two protein peaks eluted, Arvin and the haemorrhagic factor (as described by Esnouf & Tunnah, 1967), both possessed caseinolytic activity but, as expected, only the haemorrhagic factor peak had haemoglobinolytic activity. Fig. 1 shows the elution profile obtained. The coagulant action of the Arvin peak did not coincide completely with the caseinolytic activity, which suggests that the two activities were probably not the function of a single enzyme.

Purification of the coagulant activity of Arvin

Fig. 2 shows the elution profile when Arvin was chromatographed on Amberlite IRC-50. The recovery of the coagulant activity was between 85 and 100%. This coagulant enzyme preparation has a specific coagulant activity of 1800–2000 compared with the value of 1200 for Arvin before fractionation. Further fractionation on Amberlite IRC-50 did not significantly improve the specific coagulant activity. The resin procedure for the isolation of the coagulant enzyme was extended for use with peak 6 material. The conductivity and pH of the pooled peak 6 was equivalent to that of 0.04M-Tris–phosphate buffer, pH 6.2. When peak 6 was passed through a column of Amberlite IRC-50 equilibrated to these conditions the coagulant activity was fully adsorbed. Generally 40–50% of the peak 6 protein passed through the column without adsorption and this fraction contained the caseinolytic, haemoglobinolytic and haemorrhagic components but no coagulant activity. The coagulant activity was eluted as a very sharp peak with 0.2M-Tris–HCl buffer (containing 3%

Table 1. *Proteolytic activity of bovine thrombin, three preparations of Arvin and venom peak 6 on the substrates 1% casein and 2% haemoglobin*

For experimental details see the text.

Enzyme	E_{280}^{1cm}	Caseinolytic activity (units/ml)	Haemoglobinolytic activity (unit/ml)
Bovine thrombin	0.050	0.01	0.00
Arvin	0.075	1.30	0.00
	0.080	1.42	0.01
	0.072	1.05	0.02
Venom peak 6	0.060	1.21	0.85

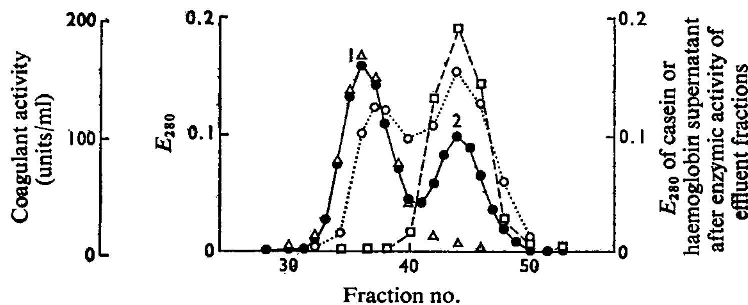

Fig. 1. *Fractionation of peak 6 material on Sephadex G-100 and examination of the fractions for proteolytic activity*

Peak 6 separated into Arvin (peak 1) and the haemorrhagic factor (peak 2), as described by Esnouf & Tunnah (1967). ●, E_{280} of effluent fractions; △, coagulant activity (units/ml); ○, caseinolytic action (E_{280} of supernatant); □, haemoglobinolytic action (E_{280} of supernatant).

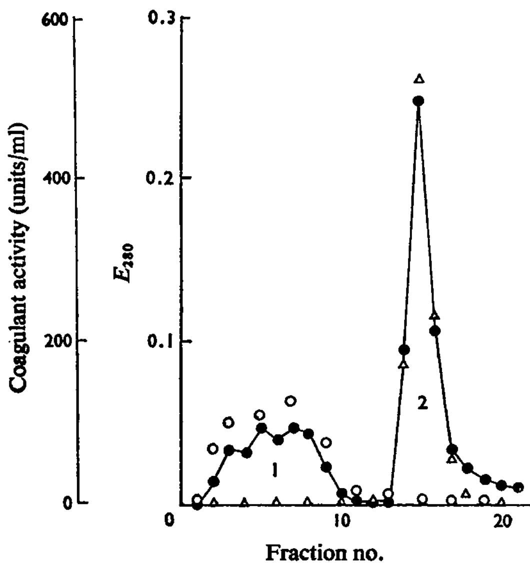

Fig. 2. *Fractionation of Arvin into the caseinolytic fraction (peak* 1) *and the coagulant enzyme (peak* 2) *by chromatography on Amberlite IRC*-50

After elution of the caseinolytic fraction, the eluent was changed to 0.3M-sodium phosphate buffer, pH 8.0, at fraction 12 to elute the coagulant enzyme. ●, E_{280}; △, coagulant activity (units/ml); ○, caseinolytic action (E_{280} of casein supernatant).

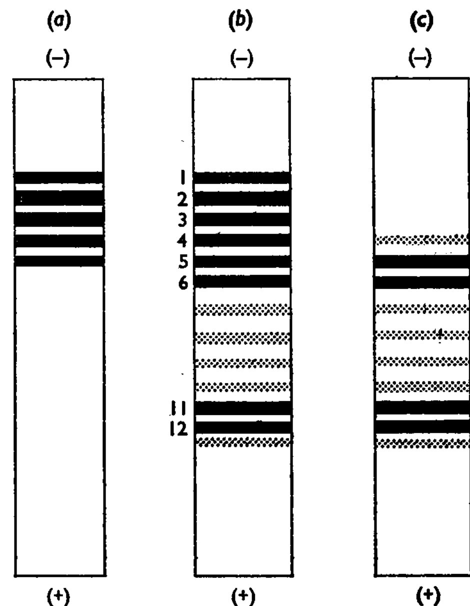

Fig. 3. *Polyacrylamide-gel electrophoresis at pH*8.6 *of the coagulant enzyme* (*a*), *venom peak* 6 (*b*), *and the haemorrhagic and caseinolytic fractions* (*c*)

All gels were loaded at the cathode end and stained with Coomassie Brilliant Blue. For experimental details, see the text.

NaCl), pH 8.5. Neither caseinolytic nor haemoglobinolytic activities were detected, and the haemorrhagic factor was either absent or present in only trace amounts. The solution of coagulant enzyme maintained its activity for several weeks when stored at 2°C.

Electrophoretic properties of the coagulant enzyme

After electrophoresis and staining, the coagulant enzyme appeared as five regularly spaced bands (Fig. 3). Two bands (nos. 2 and 3) stained strongly, but the other three were less obvious. Slight variations in the intensity of staining of some of the bands were observed with different enzyme preparations. Carbohydrate staining produced a similar five-band pattern, but no lipid constituent was detected after staining with Toluidine Blue.

The gel patterns obtained for peak 6 material and the caseinolytic fraction are also shown in Fig. 3 in comparison with the coagulant enzyme. In peak 6 material the two fast-running strongly stained bands (nos. 11 and 12) represent the haemorrhagic factor.

*Action of the coagulant enzyme on N-α-benzoyl-*L-*arginine ethyl ester*

(i) *Determination of optimum pH of esterase activity.* From a plot of the rate of hydrolysis of the ester versus pH of reaction mixture, a pH of 8.5 was taken as the point of optimum activity for the enzyme at 25°C and all subsequent esterase experiments were made at this pH value unless otherwise stated.

(ii) *Correlation of esterase and coagulant activities on inhibition.* Work by Esnouf & Tunnah (1967) demonstrated the serine proteinase nature of Arvin from inhibition studies with both di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride. With the latter inhibitor the rates of inhibition of the coagulant and esterase activities were compared for an enzyme preparation. Fig. 4 shows that the inhibition of both activities takes place at a similar rate. It may be concluded from this that the two activities are the responsibility of a single active site on the enzyme.

(iii) *Determination of* K_m, K_i *and* $V_{max.}$. The rates of enzyme activity on different substrate concentrations are shown in Fig. 5 as a Lineweaver–Burk plot. From regression analysis and calculation of the points

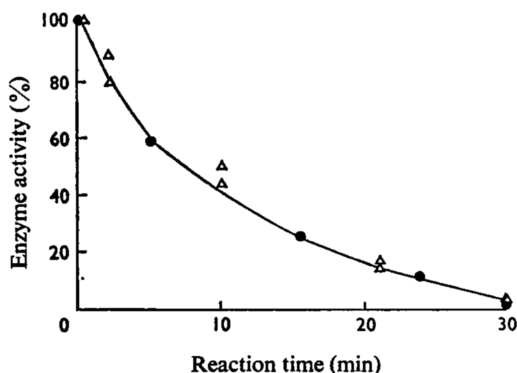

Fig. 4. *Inhibition of coagulant and esterase activities by phenylmethanesulphonyl fluoride at* 37°C

For experimental details, see the text. ●, Arginine esterase activity; △, coagulant activity.

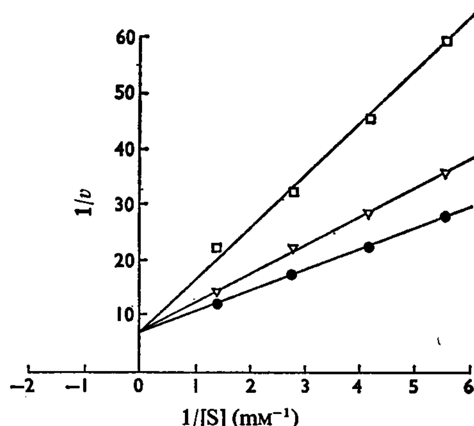

Fig. 5. *Lineweaver–Burk plot showing competitive inhibition of the coagulant enzyme by guanidine and arginine in the presence of N-α-benzoylarginine ethyl ester*

For experimental details, see the text. v is expressed as μmol of benzoylarginine ethyl ester hydrolysed/min. ●, Enzyme (no inhibitor); ▽, enzyme in 32mM-arginine–HCl; □, enzyme in 16mM-guanidine–HCl.

of interception of abscissa and ordinate, K_m = 0.52mM and $V_{max.}$ = 0.026μmol of ester/min per unit of enzyme at 25°C. In the presence of guanidine or arginine, the esterase activity was competitively inhibited. Measurement of the inhibitor constants, K_i, from Fig. 5 gave 9.5mM-guanidine–HCl and 93mM-arginine–HCl. The caseinolytic fraction from Arvin displayed no measurable activity towards the arginine ester.

Action of the coagulant enzyme on lysine esters

Enzymic action on lysine methyl ester by the coagulant enzyme was not detected. Non-enzymic solvolysis of the substrate in aqueous solution was a problem even at pH6.7, but nevertheless no significant difference in the rates of hydrolysis of test and control reactions was observed.

The action of the coagulant enzyme on a lysine ester with a blocked α-amino group was also studied. *N*-α-Benzyloxycarbonyl-lysine *p*-nitrophenyl ester was chosen for this purpose. Again solvolysis by the aqueous buffer at pH6.5 interfered with the measurement of the true enzymic activity, but the enzyme showed esterolytic action toward the substrate. Apparent values for K_m, $V_{max.}$ and K_i were determined as 8.5mM in the lysine ester, 0.05μmol/min per unit of enzyme activity and 16.5mM-guanidine–HCl respectively.

Action of Arvin fractions on N-benzyloxycarbonyl-phenylalanine p-nitrophenyl ester

Esnouf & Tunnah (1967) showed that Arvin had esterase action on *N*-benzyloxycarbonylphenylalanine *p*-nitrophenyl ester. In view of the caseinolytic (non-coagulant) impurity detected in Arvin, it was decided to study the action of both the coagulant and the caseinolytic fractions on this substrate. The coagulant enzyme was wholly inactive whereas the caseinolytic fraction showed good esterolytic properties. For Arvin preparations the esterolytic action was determined as approx. 1.40nmol of *p*-nitrophenol released/min per unit of enzyme activity. By this method it was possible to determine the extent of contamination of the caseinolytic fraction in Arvin. Values of 17–25% have been calculated for this fraction in several Arvin batches, and the result closely resembles the yield of the caseinolytic fraction obtained by Amberlite IRC-50 fractionation.

Analysis of the coagulant enzyme

The *N*-terminal amino acid labelled with dansyl chloride in the presence of 8M-urea was released from the protein by acid hydrolysis and identified as valine by polyamide plate chromatography. A sample of the enzyme was also sent to Dr. B. Blomback (Karolinska Institute, Stockholm, Sweden), who kindly offered to determine the *N*-terminal residue by the phenyl[^{35}S]isothiocyanate procedure (Irion & Blomback, 1970). Valine was the only labelled amino acid detected.

Carboxypeptidase A cleaved several amino acids from the *C*-terminal end of the protein over 24h.

Table 2. *Chemical analysis of the coagulant enzyme*

For experimental details, see the text. An amount of enzyme with $E_{280}^{1cm} = 1.00$ was used.

	Content	
Amino acid analysis	(μg)	(mol/10^5 g of protein)
Aspartic acid	77.8	88.8
Threonine	19.0	24.8
Serine	28.9	42.9
Glutamic acid	37.2	37.7
Proline	31.1	41.6
Glycine	23.1	52.6
Alanine	17.9	32.5
Half-cystine	10.8	13.7
Valine	30.9	40.2
Methionine	15.7	15.6
Isoleucine	40.2	46.8
Leucine	37.1	42.9
Tyrosine	19.4	15.6
Phenylalanine	26.4	23.4
Lysine	30.4	31.2
Histidine	24.2	23.4
Arginine	68.1	57.1
Tryptophan	13.8	9.4
	546.0 μg	
Carbohydrate analysis		
Non-nitrogenous sugars	107.8	86.2
Hexosamines	74.4	60.0
Sialic acid	40.0	17.9
	222.2 μg	

Total weight: 768.2 μg/ml of coagulant enzyme

After incubation for 1 h isoleucine was identified as the most strongly represented spot, any other amino acids being present in trace quantities only. It is therefore concluded that isoleucine is the *C*-terminal amino acid.

The amino acid analysis of the coagulant enzyme is given in Table 2, together with the quantities of carbohydrate residues. The non-nitrogenous sugars identified by paper chromatography were galactose and mannose. Neither fucose nor glucose was detected. Analysis of the hexosamines revealed only glucosamine. A trace of galactosamine was, however, reported from the amino acid analysis. From the sialic acid preparation, only *N*-acetylneuraminic acid was found.

Determination of nitrogen showed the enzyme to contain 127.5 μg of N/ml of solution of $E_{280} = 1.00$. However, calculation of the N content from Table 2 accounts for only 103.5 μg. It is possible that amide nitrogen may be responsible for part of this difference.

Discussion

The choice of resin most likely to effect separation of the coagulant enzyme from Arvin was largely due to the earlier studies of Rasmussen (1955) and Magnusson (1965) on bovine thrombin and the obvious enzymic similarities between Arvin and thrombin (Esnouf & Tunnah, 1967; Ewart *et al.*, 1969, 1970). In addition the affinity of the coagulant enzyme for other cation exchangers has been reported elsewhere (Hatton, 1972). It is noteworthy that Henriques *et al.* (1960) also used Amberlite IRC-50 resin to adsorb the coagulant activity from *Bothrops jararaca* venom when using a batch procedure, although attempts to isolate the enzyme by column chromatography with this resin were unsuccessful.

Having purified the coagulant activity, it was decided to investigate the homogeneity of the preparation. The coagulant and esterase specific activities of the enzyme are constant across an eluted peak from

an Amberlite IRC-50 column. However, non-homogeneity was revealed in the form of five protein bands after polyacrylamide-gel electrophoresis of the enzyme. The five-band pattern was similar in appearance when stained for either protein or carbohydrate. A polymorphic appearance is not unusual for certain glycoproteins and is reported to be due to an unequal distribution of sialic acid residues on the molecules within the enzyme preparation (Schmid, 1968). With α_1-acid glycoprotein, removal of the sialyl groups by neuraminidase produced a residual protein with a much simplified electrophoretic pattern (Schmid & Binette, 1961).

The coagulant enzyme has also been subjected to the action of neuraminidase and the modified coagulant enzyme produced only a single protein band on subsequent electrophoresis (M. W. C. Hatton, unpublished work). Thus the microheterogeneity shown by the native coagulant enzyme on electrophoresis is concluded to be a property of the enzyme and not due to the contaminants.

An interesting parallel has been reported for bovine thrombin by Rosenberg & Waugh (1970); these authors obtained a six-band pattern for the enzyme after polyacrylamide-gel electrophoresis at pH 8.9. Further, a partial separation of the components was achieved by column chromatography on cellulose phosphate.

Chemical analysis has revealed a high proportion of carbohydrate (29%) in the enzyme. Esnouf & Tunnah (1967) accounted for approx. 70% of the weight of the protein from the amino acid analysis of Arvin. These authors proposed that the remainder was due to the carbohydrate moiety. It is noticeable that the amino acid analysis reported for Arvin contained a disproportionately large half-cystine component, which might be explained by the glucosamine and half-cystine peaks merging as one peak. The presence of glucosamine was not reported in the Esnouf & Tunnah (1967) analysis and yet a substantial peak was obtained in the amino acid analysis of the coagulant enzyme in the present work.

The serine proteinase nature of the coagulant enzyme originally shown for Arvin by Esnouf & Tunnah (1967) is further demonstrated by the phenylmethanesulphonyl fluoride inhibition curve in Fig. 5. The rates of esterase and coagulant activities were inhibited to the same degree, from which it can be concluded that the two activities are the responsibility of the same active site. Thompson (1970) has reported a similar result by using bovine thrombin in the presence of Sarin, an inhibitor of the organophosphate type.

The trypsin-like activity of the coagulant enzyme was further studied on lysine esters. No detectable activity was shown with lysine methyl ester, which resembles the very low or undetectable activity of thrombin on the same ester (Sherry & Troll, 1954; Sherry *et al.*, 1965; Exner & Koppel, 1972). However, with *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester, where the α-amino group is blocked, substantial esterase activity was detected. Again, a parallel with thrombin can be found. Kezdy *et al.* (1965) observed that human thrombin possessed strong lytic activity for this ester at pH 5.02.

I thank Professor F. A. Robinson and Dr. T. L. V. Ulbricht for their encouragement and Mr. G. W. Tunnah for much helpful advice, Dr. B. Blomback for help and advice witht he *N*-terminal amino acid determination and Mrs. V. Winckless for the nitrogen analysis.

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