0.4 g. of butylated hydroxyanisole/kg. account for 61–82 and 11–16 % respectively of the dose, and 5 % of the dose is excreted, unchanged.

3. For single oral doses of 0.002, 0.01, 0.025, 0.05 and 0.1 g. of butylated hydroxyanisole/kg., overall recoveries of the dose from the urine were 81–100 %. Glucuronide excretion accounts for 69–92 % of the dose, and a slight increase in excretion of unchanged butylated hydroxyanisole occurs at the lowest doses.

4. For single oral doses of 0.4 g. of 2-*tert*.-butyl-4-hydroxyanisole 72 % of the dose is excreted as ethereal sulphate in 5 days. With 3-*tert*.-butyl-4-hydroxyanisole 57–71 % of the dose is excreted as glucuronide in 5 days.

5. The administration of several successive daily doses of 0.5 g. of butylated hydroxyanisole/kg., and of 3-*tert*.-butyl-4-hydroxyanisole, decreases the percentage recovery of conjugates and the proportion of ethereal sulphate excreted during dosing; repeated doses of 0.5 g. of 2-*tert*.-butyl-4-hydroxyanisole/kg. cause a considerable increase in glucuronide and a decrease in ethereal sulphate conjugation of the dose.

6. The isolation of the major metabolites, the probable formation of a demethylation product of 2-*tert*.-butyl-4-hydroxyanisole as a minor metabolite, the synthesis of the ethereal sulphates of both isomers and the synthesis of the glucuronamide of 2-*tert*.-butyl-4-hydroxyanisole are described.

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Biochem. J. (1960) **75**, 551

Partial Purification and some Properties of the Blood-Clotting Factor from the Venom of *Bothrops jararaca*

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The blood-clotting activity of the venom of various species of *Bothrops* snakes has been studied by various authors, some of them attributing the clotting effect to the proteolytic activity of the venom (Jánszky, 1950; Zeller, 1951; Slotta, 1953), and others (Taborda, 1940; Deutsch & Diniz, 1955; Rosenfeld, Hampe & Kelen, 1960) giving evidence that separate enzymes are concerned with the two activities. Michl (1954) and Holtz & Raudonat (1956) were able to show that in the venom of

Bothrops jararaca the clotting activity is distinguishable from its proteolytic activity. However, Hamberg & Rocha e Silva (1956) and Henriques, Lavras & Fichman (1956) have shown that in the venom of *B. jararaca* there exist at least two proteolytic enzymes. Therefore one of them could be responsible for the clotting activity. Nevertheless, Henriques, Mandelbaum & Henriques (1959) demonstrated that the clotting activity of the venom of *Bothrops jararaca* is independent of those

two proteolytic enzymes, namely 'caseinase' (Henriques *et al.* 1956) and 'protease' (Holtz & Raudonat, 1956), and *Bothrops* protease A, which hydrolyses benzoyl-L-arginine amide but not casein (Henriques, Lavras, Fichman, Mandelbaum & Henriques, 1958). Habermann (1958), by fractional precipitation with acetone followed by paper electrophoresis, obtained from the venom of *Bothrops jararaca* a fraction that had a 'relative activity' ten times as high as that of the starting material with respect to blood-clotting activity.

In this paper is given a detailed account and extension of observations previously made by Henriques *et al.* (1959). It contains also the study of some properties of the clotting factor and the results of an attempt to isolate it.

MATERIALS AND METHODS

Clotting activity. The clotting activity was measured by adding 0.2 ml. of oxalated horse plasma (0.3 g. of potassium oxalate/100 ml. of plasma) to 0.1 ml. of the sample previously placed on a water bath at 35°; the zero time was noted and the mixture gently agitated until a firm clot was formed. The samples containing the clotting factor were diluted twice or more with 0.2M-sodium cacodylate buffer, pH 6.5. This concentration of buffer was used to obtain pH 7.0 in the final reaction mixture of sample and plasma.

Specific clotting activity. Amounts of venom which clot blood plasma or fibrinogen at between 20 and 50 sec. show a straight-line relationship when the reciprocals of enzyme concentration are plotted against the respective clotting times. Therefore when testing a sample for its blood-clotting activity, three different concentrations of protein were used and, by graphical interpolation, the inverse of the protein concentration which would cause clotting in 30 sec. was inferred from the straight line obtained. This figure, multiplied by 30 for convenience, was taken as the specific clotting activity of the sample analysed, and proved to be accurate enough for the purposes of the present work.

Proteolytic activities. The activities of the samples towards casein and benzoyl-L-arginine amide were determined respectively by the methods of Kunitz (1946) and Schwert, Neurath, Kaufman & Snoko (1948) as previously described (Henriques *et al.* 1958).

Fractionation of the venom. The venom used for this work was collected about 1 month before starting the experiments here reported, the samples being immediately dried *in vacuo* over CaCl_2 at room temperature. The whole procedure of fractionation and subsequent dialysis was done at 5–10°. The venom (2 g.) was dissolved in 100 ml. of 0.05M-sodium cacodylate buffer, pH 6.10, and centrifuged to remove the insoluble residue. The proteins of the venom were fractionally precipitated with $(\text{NH}_4)_2\text{SO}_4$. The solid salt, divided into small portions, was stirred with the venom solution to give the desired percentage saturation; the amount of $(\text{NH}_4)_2\text{SO}_4$ added was calculated from the table of Green & Hughes (1955). The precipitates were centrifuged, dissolved in a small volume of 0.05M-sodium cacodylate buffer, pH 6.10, and dialysed in cellophan tubing against the same buffer until the diffusate was free of NH_4^+ ions.

Protein determination. Protein was estimated photometrically by the biuret method of Wolfson, Cohn, Calvary & Ichiba (1948) or in terms of its ultraviolet absorption at 280 m μ in the 1 cm. cell of a Beckman spectrophotometer.

Electrophoresis on starch columns. The procedure was the same as that described by Henriques *et al.* (1958).

Activators and inhibitors. Solutions of activators or inhibitors were always freshly prepared in water and incubated for 1 hr. at 35° with the enzyme before the addition of the oxalated plasma.

RESULTS

It was observed that the clotting activity varies considerably with the buffer employed. Fig. 1 shows the clotting activity in the presence of cacodylate, phosphate and acetate buffers. In the presence of 0.2M-sodium cacodylate the activity increased with the pH, reaching a plateau at pH 6.5 which was maintained throughout the upper useful range of the buffer. In the presence of 0.2M-phosphate (NaH_2PO_4 – Na_2HPO_4) buffer, pH 6.2, the activity was comparable with that shown with cacodylate buffer. With the rise in pH, however, the activity fell sharply. In the presence of 0.2M-acetate (sodium acetate–acetic acid) buffer the clotting activity was shown to be higher than in the presence of cacodylate below pH 5.6, the situation being reversed at higher pH values. Other buffers, such as citrate, 2-amino-2-hydroxy-methylpropane-1:3-diol (tris)–hydrochloric acid and tris maleate were tried at the pH of maximal activity in cacodylate. In all cases the activity was lower than in the presence of cacodylate. Cacodylate buffer has the further advantage of main-

Fig. 1. Effect of pH and buffers on the blood-clotting activity of the venom of *B. jararaca*. Reaction mixture contained 0.1 ml. of 0.1% (w/v) solution of venom in the corresponding 0.2M-buffer and 0.2 ml. of oxalated horse plasma. ○, Acetate; ●, phosphate; △, cacodylate.

taining unaltered, for at least 2 months, the clotting activities both of the solution of venom and of its fractions, when kept at 1–2° at pH 6.2.

Effect of activators and inhibitors. In these experiments the effect of the ions was tested in a crude venom solution and in a fraction purified ninefold by $(\text{NH}_4)_2\text{SO}_4$ fractionation and electrophoresis on a starch column. It can be seen from Table 1 that the ions tested affected both samples in the same way. In the presence of 2 mM-metal-ion concentration only Cu^{2+} and Hg^{2+} ions had an inhibitory effect (Table 1). Calcium had a slight activating effect, confirming a previous report by Hargreaves (1944). In the presence of 1 mM-iodoacetate, -fluoride, -sulphide, -cyanide and -azide, no effect was observed. Reduced glutathione in 50 mM concentration and ethylenediaminetetra-acetic acid (disodium salt) in 2 mM concentration strongly inhibited the clotting activity (Table 2). The inhibition by ethylenediaminetetra-acetic acid has been previously reported by Rosenfeld & Jánosky (1952).

Effect of heating. As shown in Table 3, heating the venom to 63° in 0.05 M-sodium cacodylate buffer, pH 6.2, followed by immediate cooling in an ice bath, reduces the clotting specific activity to 25 % of that of the crude venom, and heating to 87° followed by cooling in the same conditions reduces it to about 12 %. By contrast, the amidase specific activity, confirming previous observations (Hamberg & Rocha e Silva, 1956, 1957; Henriques *et al.* 1958), is more heat resistant, its activity being

reduced to 75 % at 63° and to 60 % at 87°. The caseinase specific activity falls to 50 % at 63° and is completely destroyed when the venom is heated to 83°.

Separation of the clotting fraction from the venom. It can be observed from Table 4 that the highest clotting activity was found in the fraction precipitated with $(\text{NH}_4)_2\text{SO}_4$ between 0.60 and 0.65 saturation. This confirms a previous report (Henriques *et al.* 1959), in which the highest clotting activity was observed at 0.60–0.70 saturation with $(\text{NH}_4)_2\text{SO}_4$. The most active fraction obtained in this experiment had a specific clotting activity 2.4 times as great (602) as that of the crude venom (Table 4, fraction 7). Table 4 shows that some

Table 2. *Effect of certain anions and metal-binding agents on the clotting activity of the venom of Bothrops jararaca*

Added substance	Final concn. (mM)	Clotting time (sec.)
None	—	20
Iodoacetate	1	21
Fluoride	1	20
Sulphide	1	21
Cyanide	1	21
Azide	1	21
Reduced glutathione	50	80
Ethylenediaminetetra-acetic acid (disodium salt)	2	240*

* Partially clotted.

Table 1. *Effect of metal ions on the clotting activity of the venom of Bothrops jararaca*

Venom solution was incubated for 1 hr. at 35° with test substances (final concn. 2 mM) before addition of oxalated plasma. Reaction mixture for the determination of clotting activity: 0.1 ml. of venom or fraction and metal-binding-agent mixture and 0.2 ml. of oxalated horse plasma.

Metal ion	Clotting time (sec.)	
	Crude venom	Purified fraction*
None	20.0	20.0
Mn^{2+}	19.5	19.0
Cu^{2+}	41.0	44.0
Ca^{2+}	16.0	14.5
Fe^{3+}	19.8	18.5
Ag^+	21.0	20.0
Pb^{2+}	19.5	19.0
Cd^{2+}	19.2	19.2
Co^{2+}	22.5	21.5
Zn^{2+}	19.5	17.5
Hg^{2+}	39.5	35.5
Mg^{2+}	19.5	17.8

* Similar to fraction 5, Table 5, obtained from venom collected in 1946; nine times purified with respect to clotting activity.

Table 3. *Effect of heating on the clotting, benzoyl-L-arginine-amidase and caseinase specific activities of the venom*

A 2 % (w/v) solution of *B. jararaca* venom in 0.05 M-sodium cacodylate buffer, pH 6.2, was heated in a boiling-water bath until the desired temperature was reached. Immediately afterwards the tube containing the sample was transferred to an ice-water bath. Specific clotting activity is expressed as 30 (mg. of protein/ml. of the sample that clots the plasma in 30 sec.). Benzoylarginine-amidase specific activity is expressed as $\mu\text{g.}$ of NH_3 liberated from benzoyl-L-arginine amide/mg. of protein in the sample. Caseinase specific activity is expressed as the increase in extinction of the trichloroacetic acid filtrate at 275 $\mu\text{m.}$ /mg. of protein in the sample. All the reactions were performed under standard conditions as described in the text.

Temp. reached	Specific activities		
	Clotting	Amidase	Caseinase
Room temp.	120	37	1.85
63°	30	28	0.96
87°	15	22	Nil

Table 4. *Blood-clotting activity and hydrolytic activity towards casein and benzoyl-L-arginine amide of fractions precipitated with ammonium sulphate*

Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was done in 0.05M-sodium cacodylate buffer, pH 6.10. The clotting, amidase and caseinase specific activities are expressed as in Table 3. All the reactions were performed under standard conditions as described in the text. U indicates 'undetectable'.

Fraction no.	Saturation with $(\text{NH}_4)_2\text{SO}_4$	Specific activities			Yield (%)			
		Clotting	Amidase	Caseinase	Protein	Clotting	Amidase	Caseinase
1	None	248	32	2.05	100	100	100	100
2	0.35-0.40	77.5	10.6	2.30	5.5	1.7	1.8	12.8
3	0.40-0.45	162	17.8	2.00	10.3	6.7	5.7	21
4	0.45-0.50	219	27	2.50	19.0	16.8	16.0	47
5	0.50-0.55	318	32	1.60	18.4	23.6	18.6	29
6	0.55-0.60	511	51	0.86	12.2	25.2	19.6	10.0
7	0.60-0.65	602	71	0.31	7.8	19.0	17.4	4.9
8	0.65-0.70	456	98	0.17	2.7	0.50	8.3	0.46
9	0.70-0.80	U	154	0.20	2.3	—	1.1	0.54
10	0.80-1.0	U	22.6	0.18	0.23	—	0.17	0.042

Table 5. *Comparison of the clotting, caseinase and benzoylarginine-amidase specific activities of samples obtained during purification of the clotting activity of a mixture of fractions 6, 7 and 8 of Table 4*

The specific activities are expressed as described in Table 3. All the reactions were performed under standard conditions. U indicates 'undetectable'.

Fraction no.	Step	Specific activities			Yield (%)		
		Clotting	Amidase	Caseinase	Clotting	Amidase	Caseinase
1	Mixture of fractions 6, 7 and 8 (Table 4)	501	61	0.70	100	100	100
2	Reprecipitation of fraction no. 1 at 0.0-0.55 saturation with $(\text{NH}_4)_2\text{SO}_4$	517	47.5	0.93	56	42.6	75.9
3	Reprecipitation of fraction no. 1 at 0.55-0.60 saturation with $(\text{NH}_4)_2\text{SO}_4$	609	65	0.36	24.7	20.8	10.5
4	Reprecipitation of fraction no. 1 at 0.60-0.65 saturation with $(\text{NH}_4)_2\text{SO}_4$	570	94	0.19	6	8.2	1.5
5	Electrophoresis peak of clotting activity (starch column) of fraction no. 3	1020	127	U	2.98	1.4	—

clotting activity was found in all fractions, no sharp separation being obtained. In order to increase the yield, fractions 6, 7 and 8 (Table 4), which had similar specific clotting activities, were mixed and fractionally reprecipitated with $(\text{NH}_4)_2\text{SO}_4$. The highest specific clotting activity was obtained at a slightly lower saturation with $(\text{NH}_4)_2\text{SO}_4$, i.e. 0.55-0.60, and the specific activity reached the value 609 (Table 5), slightly higher than that of the initial mixture (501). A second reprecipitation with $(\text{NH}_4)_2\text{SO}_4$ did not lead to further purification. When this reprecipitated fraction was submitted to electrophoresis on a starch column (cacodylate buffer, pH 6.10, I 0.05) further purification was obtained, the clotting activity reaching the value 1020, i.e. about four times as great as that of the crude venom from which it was obtained. As can be seen from Fig. 2, a

clear separation of the peaks of *Bothrops*-protease A and clotting activities was obtained.

It should be noted here that the degree of purification obtained depends on the age of the venom used. When the purified clotting factor was obtained from old venoms (1946-48) by the technique described in this paper, the overall increase in specific activity was about nine- to ten-fold instead of fourfold as given here. The final specific activity of the purified material obtained either from old or new venoms was, however, about the same.

Attempts were made to purify the clotting factor with gels of aluminum hydroxide (Colowick, 1955), calcium phosphate (Keilin & Hartree, 1938), barium carbonate (Fantl & Nance, 1947) and Amberlite IRC-50 (XE-64), varying buffers and pH. Doery (1958) reported the purification of the blood-

Fig. 2. Protein concentration and amidase and blood-clotting specific activities of fractions obtained by electrophoresis of 220 mg. of protein of fraction no. 3 (Table 5) on a starch column. Cacodylate buffer, pH 6.2, I 0.05; about 11 mA; under these conditions the proteins moved to the anode. Δ , Benzoyl-L-arginine-amidase specific activity; $\bullet$, blood-clotting specific activity; $\circ$, mg. of protein/ml.

clotting factor of the venom of *Notechis scutatus scutatus* by adsorption on to barium carbonate. Treatment of the venom of *Bothrops jararaca* under the conditions specified by Doery (1958) did not lead to adsorption of the clotting enzyme on to barium carbonate. The clotting enzyme was not adsorbed by calcium phosphate but it was adsorbed by alumina- $C\gamma$; however, the adsorption was not selective. On Amberlite IRC-50 (XE-64) the blood-clotting factor was adsorbed and eluted at pH 7.1 in cacodylate buffer when the tube test recommended by Hirs, Moore & Stein (1953) was used. However, when the ion-exchange resin was used in a column no purification was obtained.

DISCUSSION

The results presented in this paper give evidence that the blood-clotting factor differs from the two other proteolytic enzymes known to be present in the venom of *Bothrops jararaca*, namely *Bothrops* protease A and 'caseinase'. These proteolytic enzymes can already be distinguished from the blood-clotting enzyme by some of its properties. The caseinase is almost completely inhibited by Cd^{2+} ions (Henriques *et al.* 1958) whereas the clotting enzyme is not affected by the same concentration of Cd^{2+} ions. Heating the venom to 87° in cacodylate buffer (Table 3) decreased the benzoyl-arginine-amidase specific activity to about two-thirds, completely destroyed the caseinase activity

and decreased the specific clotting activity to about one-tenth. The pH optimum found for the clotting enzyme was about 7.0 and the enzyme activity was found to be activated by cacodylate buffer and inhibited by tris-hydrochloric acid. The latter was found to be the best medium for caseinase and *Bothrops*-protease A activities and the pH optimum for both of these enzymes was found to be approx. 8.8 (Henriques *et al.* 1958). The three enzymes can be partially separated by ammonium sulphate precipitation (Table 4). The fractions precipitated at 0.50–0.55, 0.60–0.65 and 0.70–0.80 saturation were found respectively to contain the highest specific activity of caseinase, clotting enzyme and *Bothrops* protease A. By fractional reprecipitation of the most strongly clotting fractions a preparation was obtained which still contained a high specific activity as *Bothrops* protease A, but when it was submitted to electrophoresis on a starch column two distinguishable peaks of activity were obtained (Fig. 2), one for the blood-clotting enzyme and the other for *Bothrops* protease A. The partial separation of caseinase and the clotting enzyme by ammonium sulphate precipitation confirms previous work by Holtz & Raudonat (1956), who reported a fraction precipitated at 40% saturation with ammonium sulphate, strongly proteolytic and with slight clotting activity and a fraction precipitated at 60% which had strong clotting activity and was weakly proteolytic.

The clotting activity caused by *Bothrops* venom may be due to a third proteolytic enzyme. In fact evidence has been presented that Reptilase, a pharmaceutical preparation of clotting enzyme obtained from *Bothrops* venom, can split from fibrinogen a peptide (Blombäck & Vestermarck, 1958) which in Dowex columns shows the same behaviour as peptide A, one of the two peptides which are liberated by thrombin from fibrinogen (Bettelheim & Bailey, 1952).

Data presented in this paper do not exclude the hypothesis proposed by Habermann (1959) that there exist in the venom three enzymes which hydrolyse N^{α} -toluene- p -sulphonyl-L-arginine methyl ester and, therefore, benzoylarginine amide. His hypothesis seems to be based on an electrophoresis diagram of purified blood-clotting enzyme, in which he plotted the yield of the N^{α} -toluene- p -sulphonyl-L-arginine methyl esterase activity against the effluent volume (Habermann, 1959; fig. 1a). As judged from the diagram published by Habermann the fraction he analysed was about as polydisperse as the one included in this paper (fig. 2). Under these circumstances it is questionable whether the appearance of three peaks of yield should be accepted as evidence for the existence of three distinct N^{α} -toluene- p -sulphonyl-

L-arginine methyl esterases in the venom; but a final conclusion must await further purification of the blood-clotting enzyme.

SUMMARY

1. The pH optimum for the blood-clotting activity of the venom of *Bothrops jararaca* was found to be about 7.0; at this pH the enzyme was more active in cacodylate buffer, being inhibited by phosphate, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-hydrochloric acid, citrate and tris maleate. The clotting enzyme is thermolabile, its activity being reduced to about one-tenth when it is rapidly heated to 87°.

2. The venom fraction precipitated between 0.60 and 0.65 saturation with ammonium sulphate had a clotting activity about twice as high as that of the crude venom; its benzoylarginine-amidase specific activity was also about twice that of the crude venom; its caseinase specific activity was about one-sixth of that of the crude venom.

3. Fractional reprecipitation of the venom fraction precipitated between 0.60 and 0.65 saturation with ammonium sulphate followed by electrophoresis on a starch column produced a clotting fraction about four times as active as the crude venom.

4. The clotting enzyme was found to be different from the two other proteolytic enzymes found in the venom, namely 'caseinase' and *Bothrops* protease A.

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