

pseudovitamin B₁₂. Comparing this group with the combined results obtained with pseudovitamin B₁₂ and the lactone, statistical analysis demonstrates that the lower uptakes in the presence of anti-vitamin B₁₂ are highly significant ($t = 3.1$; $P = 0.003$). In other words, non-competitive binding is appreciably diminished in the presence of the anti-vitamin; this material is also capable of affecting absorption of cyanocobalamin in the intact animal, whereas no such effect has been observed with either pseudovitamin B₁₂ or the lactone.

It is interesting to note that recent work (Bunge, Schloesser & Schilling, 1956) has demonstrated that normal human gastric juice also sometimes possesses two types of vitamin B₁₂-binding activity. One is highly specific for cyanocobalamin and the other allows for competition with pseudovitamin B₁₂.

Other workers have also studied competition phenomena between vitamin B₁₂ and its analogues (Rosenblum *et al.* 1956; Coates *et al.* 1956). In the chick, high dosage of pseudovitamin B₁₂ by mouth does affect absorption of vitamin B₁₂ (Coates *et al.* 1956).

SUMMARY

1. The measurement of uptake of radioactive vitamin B₁₂ by isolated intestine has been described.

2. The effect of pseudovitamin B₁₂, an anti-vitamin B₁₂ and the lactone of vitamin B₁₂ on uptake of radioactive vitamin B₁₂ has been studied.

3. Two vitamin B₁₂-binding systems have been demonstrated, one competitive and the other non-competitive.

4. The implications of the non-competitive binding system in relation to uptake of vitamin B₁₂ are discussed.

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The Proteolytic Activity of the Venom of *Bothrops jararaca*

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The presence of proteolytic activity in the venom of *Bothrops jararaca* seems to have been the first evidence for the occurrence of enzymes in snake venom (Lacerda, 1884). Since then, knowledge of the proteolytic activity of snake venoms has progressed rather slowly (Zeller, 1951). The hydrolysis of casein by the venom of *B. jararaca* was studied by Taborda & Taborda (1940) under various conditions. Rocha e Silva & Andrade (1945) observed that benzoylarginine amide was also hydrolysed by the venom of *B. jararaca*, a fact which seemed to indicate that the substrate

specificity of the proteolytic enzyme contained in the venom was similar to that of trypsin. More recently, Hamberg & Rocha e Silva (1956, 1957) reported in the venom of *B. jararaca* the presence of both heat-labile and heat-stable proteolytic activity. The latter, after heating, retained the power to hydrolyse benzoylarginine methyl ester, while apparently losing all hydrolytic activity towards casein. Holtz & Raudonat (1956) have separated two fractions from the venom of *B. jararaca*, by precipitation with ammonium sulphate, namely 'protease', which had strong proteo-

lytic activity, and 'koagulin', which is mainly blood-coagulating and weakly proteolytic. Henriques, Lavras & Fichman (1956), also by precipitation with ammonium sulphate, separated two proteolytic fractions from the venom of *B. jararaca*. The first fraction, precipitated at 0.5 saturation with ammonium sulphate, had a high proteolytic activity towards casein and a weak hydrolytic activity towards benzoyl-L-arginine amide, whereas the second, precipitated at 0.7–0.8 saturation with ammonium sulphate, was very active with benzoyl-L-arginine amide and feebly active with casein. The conclusion that more than one proteolytic enzyme was present in the venom was further substantiated by the fact that Ca^{2+} ions did not affect the hydrolytic activity of the second fraction on casein but activated strongly the 'caseinase' activity of the first fraction.

In this paper we present the results of a study of some properties of the proteolytic activity of the crude venom and a method for the isolation of the enzyme hydrolysing benzoyl-L-arginine amide present in the fraction precipitated at 0.7–0.8 saturation with ammonium sulphate.

EXPERIMENTAL

Measurement of enzyme activity. The proteolytic activity was determined with casein, gelatin or benzoyl-L-arginine amide as substrate. As a matter of convenience, in this paper, the hydrolytic power of the venom on casein or gelatin is called caseinase or gelatinase respectively, while the hydrolytic action on benzoyl-L-arginine amide is called benzoylarginine amidase activity.

When casein was used as substrate the method of Kunitz (1946) was applied. Purified casein (Difco Laboratories Inc., Detroit, Mich., U.S.A.) was used as a 1% (w/v) solution in 0.05 M-2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer, pH 8.8. The amount of enzyme was adjusted to give an optical density between 0.080 and 0.315 (corresponding to 50–200 μg . of tyrosine; about 100–400 μg . of dried venom) at 275 $m\mu$ with 1 cm. cells of the Beckman spectrophotometer, DUV model, as at this level of activity the degree of hydrolysis was found to be directly proportional to the amount of enzyme present.

The method of Duthie & Lorenz (1949), slightly modified, was used when gelatin was the substrate. Bacteriological Difco standardized gelatin (Difco Laboratories Inc., Detroit, Mich., U.S.A.), 20% (w/v) in 0.9% NaCl, was autoclaved for 30 min. at 110° and stored in the cold. The substrate mixture was prepared freshly each day by adding 40 ml. of 0.5% gum acacia in 0.2 M-tris buffer, pH 8.8, to 60 ml. of warmed gelatin solution. For the determinations, equal volumes of enzyme and substrate solutions were mixed at 35° and 5 ml. of this mixture was transferred to Ostwald viscosimeters kept in a water bath at 35°. The flow time of the mixture was measured 20 min. after mixing. The viscosimeters used had a bulb capacity of about 2 ml., a capillary of 1 mm. diam. and an average flow time of about 20 sec. with water. The flow-time index v

(Swyer & Emmens, 1947) was then calculated from the equation

$$v = 1000 [(f_s - f_e)/(f_s - f_o)],$$

in which f_s is the flow time of the enzyme-substrate mixture after incubation for 20 min.; f_e is the flow time from a similar mixture from which only the enzyme was excluded; f_o is the flow time of another similar mixture, from which gelatin was also excluded. In the estimation, the amount of enzyme was adjusted to give a flow-time index (v) of 170–450, since in this range v was found to be directly proportional to the logarithm of the amount of venom present (50–200 μg . of dried-venom protein). The assay used to measure the potency of the fractions separated from the dried venom included a comparison between the activities of 60 and 120 μg . of protein of the initial venom (standard) with those of the fraction at two levels of concentration (unknown), the higher concentration of unknown being also double the lower one. In most assays four determinations were run with each concentration and the results were submitted to the standard method of biological assay as used by Swyer & Emmens (1947) for the assay of hyaluronidase. As described here, this assay usually estimated the potency of the unknown with 0.95 fiducial limits of 90–110% of the correct value; in the worst case (two replicates/dose) the fiducial limits were 86–116.

The activity on benzoyl-L-arginine amide was assayed by the method of Schwert, Neurath, Kaufman & Snoko (1948) adapted to the diffusion apparatus of Tompkins & Kirk (1942). The sample containing the enzyme (0.1 ml.) was pipetted into the small bulb of the vessel. To this were added 0.2 ml. of 0.05 M-benzoyl-L-arginine amide immediately followed by 0.1 ml. of 0.3 M-tris buffer, pH 8.8, the zero time being noted. The mixture was incubated for 20 hr. at 25°. At the end of this period 1 ml. of saturated K_2CO_3 solution was added to the main chamber of the vessel and 0.5 ml. of 2% (w/v) boric acid was pipetted into the absorption cup, which had a capacity of 1 ml. The system was then closed, the carbonate was mixed with the enzyme-substrate incubated mixture and the diffusion of ammonia took place for 2 hr. at room temperature. The stopper carrying the acid cup was then withdrawn, 1 drop of Tashiro's indicator (described by Davis & Smith, 1955) was added and the ammonia was titrated with 0.01 N-HCl as recommended by Schwert *et al.* (1948). Suitable blanks were run for all enzyme samples. The amount of enzyme in the estimations was adjusted to give an evolution of 15–50 μg . of ammonia (0.25–2 mg. of crude venom), as in this range the amount of ammonia liberated is directly proportional to the weight of venom used. The benzoyl-L-arginine amide used, either prepared by the method of Bergmann, Fruton & Pollok (1939) or obtained from the Mann Research Laboratories, New York, was always recrystallized to give a blank not higher than 0.05 ml. of 0.01 N-HCl when used in the assay.

Protein determination. Protein was estimated colorimetrically by the biuret method of Wolfson, Cohn, Calvary & Ichiba (1948) or in terms of its ultraviolet absorption at 280 $m\mu$ in the 1 cm. cell of a Beckman spectrophotometer. In both cases dry *B. jararaca* venom, which contained 70% (w/w) of protein, was used as the standard.

Experiments with inhibitors and activators. The solutions of inhibitors or activators were always prepared freshly in water and incubated for 1 hr. at 35° with the enzyme solution, before the addition of an equal volume of casein

substrate dissolved in tris buffer, or gelatin dissolved in 0.9% NaCl-tris buffer, for proteinase activity, or of benzoyl-L-arginine amide substrate and tris buffer in the proportions previously described for the determination of amidase activity. Suitable control determinations were made, in which enzyme and inhibitor or substrate and inhibitor were added to the solvents of the reaction mixture. When gelatin was used as substrate the activator or inhibitor was included in the solution used for the determination of f_0 and f_s in the same concentrations as in the final enzyme-substrate-activator (or -inhibitor) mixture.

Removal of impurities from venom solution on calcium phosphate gel. The venom used for this work was part of a sample stocked in this Institute, where snake venoms are always dried *in vacuo* at room temperature. The venom (20 g.) was dissolved in 2 l. of 0.05 M-tris-maleate buffer, pH 6.0, prepared according to Gomori (1955). This solution was centrifuged and the clear supernatant separated. The supernatant was used for comparison of proteinase and benzoylarginine amidase activities with the fractions obtained subsequently. The remaining clear solution was treated with $\text{Ca}_3(\text{PO}_4)_2$ gel (2-6 months' old) prepared according to Keilin & Hartree (1938) in a gel-protein ratio of 2, expressed in terms of dry weight. The gel was slowly added while the solution was mechanically stirred and the stirring was continued for 20 min. The mixture was centrifuged, a sample of the supernatant was kept for control of the next steps, and the remaining solution was used as starting material for the fractionations with $(\text{NH}_4)_2\text{SO}_4$. In a series of preliminary experiments with small amounts of venom it was verified that in itself the treatment with $\text{Ca}_3(\text{PO}_4)_2$ gel does not always increase significantly the proteinase or amidase specific activity of the supernatant, but the gel treatment leads to better fractionation with $(\text{NH}_4)_2\text{SO}_4$. The whole procedure and subsequent fractionation with $(\text{NH}_4)_2\text{SO}_4$ were carried out at room temperature (not above 25°). The proteolytic activity of the venom proved to be rather stable at room temperature.

Fractional precipitation with ammonium sulphate. Sufficient solid $(\text{NH}_4)_2\text{SO}_4$, divided into small portions, was stirred with the supernatant 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). When the fractions were reprecipitated, a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was used. The resulting precipitates were collected by centrifuging, dissolved in a small volume of 0.9% NaCl and dialysed in cellophane tubing at 2-6° against 0.9% NaCl until the diffusate became free of NH_4^+ ions. The diffusate was then centrifuged to separate the precipitate of denaturated proteins.

Heating of venom solution for subsequent fractionation with ammonium sulphate. A 4% solution of *B. jararaca* venom was made up in 0.9% NaCl and the insoluble material centrifuged off. An equal volume of 0.9% NaCl heated to 90° was added to the venom solution and the mixture was heated with stirring in a boiling-water bath for 5 min., the temperature reaching 88° at the end of this period. The mixture was immediately cooled in an ice bath and the denaturated protein centrifuged off. The supernatant was fractionally precipitated with solid $(\text{NH}_4)_2\text{SO}_4$ as described above.

Electrophoresis on starch columns. The glass accessories of the electrophoresis apparatus used were similar to those described by Kunkel (1954), and its electrode compart-

ments, with a capacity of 1 l. each, were similar to those described by Kunkel & Tiselius (1951). Commercial starch, suitable for bacteriological purposes, was thoroughly washed with water and air-dried. For the preparation of a column of 1.2 cm. \times 30 cm., 36 g. of starch was further washed twice with tris buffer, pH 7.2, $I=0.05$, by resuspension and filtering. The washed starch, resuspended in 4 vol. of buffer, was transferred to the electrophoresis tube and a positive pressure of 150 mm. Hg was applied to filter off the excess of buffer. When the level of the buffer was just above the upper starch surface, 1 ml. of the sample (containing up to 10 mg. of protein, and dialysed against 2 l. of tris buffer, pH 7.2, $I=0.05$, for 20 hr. in the cold) was added, and the same pressure was again applied until the level of the sample reached the starch surface. The protein band was displaced downwards by filtering 1 ml. of buffer through the column as described before; the electrophoresis apparatus was assembled, filled up with cold buffer and put in a cold room (about 10°). After 30 min., 330 v was applied in such a way that the upper part of the column became negative in relation to its lower part. The electrophoresis was allowed to proceed for 20 hr., the intensity of current being of about 3.5 ma and practically constant. The apparatus was then dismantled and the protein bands were eluted by filtering about 15 ml. of buffer through the column under a positive pressure of 150 mm. Hg. Fractions were collected at intervals of 3 min., with a volume of about 0.5 ml. each. The separate fractions were appropriately diluted for photometric determination of protein in terms of its ultraviolet absorption and for the determination of amidase and proteinase activities.

The homogeneity of the starch column used was tested by putting on the column 8 mg. of cytochrome *c*, dissolved in 0.067 M-phosphate buffer, pH 7, and displacing the resulting band with the aid of the same buffer. The column appeared homogeneous since throughout its whole length the band of cytochrome *c* travelled downwards about 2 cm./ml. of outcoming effluent buffer; it had a liquid cross-sectional area of 0.46 cm.² and a ratio (starch volume: mobile liquid volume) of 1.5:1.0 (Flodin & Porath, 1954); it gave a good electrophoresis pattern with the proteins of rat serum (in diethylbarbiturate buffer, pH 8.6; $I=0.1$) and did not adsorb cytochrome *c*, rat-serum proteins or the proteins of *Bothrops* venom.

RESULTS

Factors influencing the proteolytic activity of the venom

Optimum pH. The pH-activity curves varied with the buffer employed and with the substrate. In tris and ammonium (2-amino-2-methylpropane-1:3-diol) buffers the rate of hydrolysis of casein, low on the acid side, was found to rise throughout their useful pH range, although the activity seemed to have reached a plateau at the alkaline end of the ammonium pH range (Fig. 1). With borate buffer and casein as substrate two maxima were observed, one at pH 8.8 and the other at pH 9.5-9.8. With benzoyl-L-arginine amide as substrate, in tris buffer the activity seemed to have reached a

plateau at pH 9 and in ammediol and borate buffer the pH optima found were respectively 8.5-9 and 9.5-11 (Fig. 2).

Effect of metal-binding agents. The caseinase and gelatinase activities of the venom were found to be strongly inhibited by 50 mM-cysteine (Table 1). Inhibition of the caseinase activity of the venom of *B. atrox* has been previously reported by Rocha e Silva & Andrade (1945). As a high concentration of L-amino acid oxidase is present in the venom, the effect of cysteine on the benzoylarginine amidase activity could not be determined. Glutathione in a final concentration of 50 mM reduced the caseinase activity to 18%, inhibited completely the gela-

tinase activity and reduced the amidase activity to 42%. The addition of 2 mM-ethylenediaminetetra-acetic acid reduced the caseinase activity to 22%, the gelatinase activity to 7% and had no effect on the amidase activity, a fact that has previously been noted by Deutsch & Diniz (1955). 8-Hydroxyquinoline in a concentration of 2 mM inhibited the caseinase activity almost completely, reduced the gelatinase activity to 38% but did not affect the amidase activity. Histamine in a 50 mM-concentration had a moderate inhibitory effect on the three activities tested.

Effect of metal ions. In the presence of 2 mM metal-ion concentration only Cd^{2+} and Hg^{2+} ions had an inhibitory effect greater than 15% on the amidase activity. Caseinase activity was almost

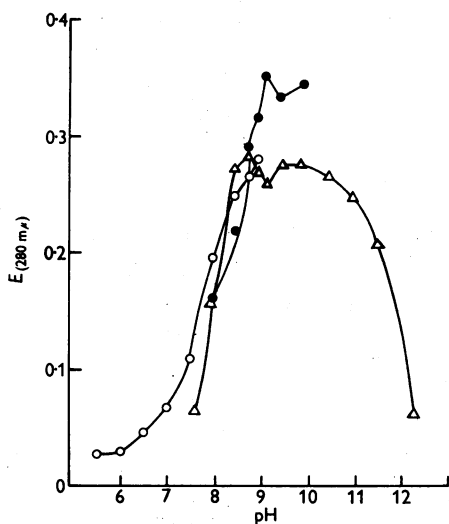


Fig. 1. Effect of pH on the proteolytic activity of the venom of *B. jararaca*. Reaction mixture contained 1 ml. of 1% (w/v) casein dissolved in the corresponding 0.05M buffer, 0.2 ml. of 0.08M- CaCl_2 and 0.8 ml. of venom solution in 0.9% NaCl. O, Tris; ●, ammediol; Δ, borate.

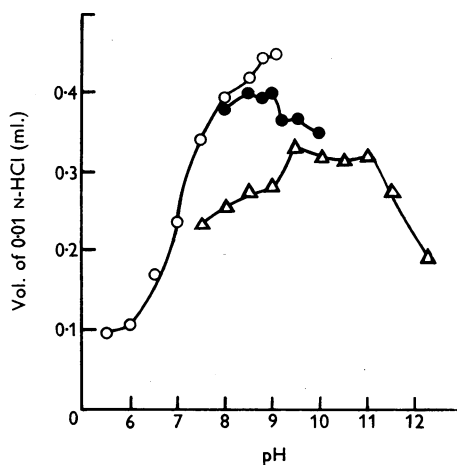


Fig. 2. Effect of pH on the benzoylarginine amidase activity of the venom of *B. jararaca*. Reaction mixture contained 0.2 ml. of 0.05M-benzoyl-L-arginine amide, 0.1 ml. of 0.3M-soln. of corresponding buffer and 0.1 ml. of venom solution in 0.9% NaCl. O, Tris; ●, ammediol; Δ, borate.

Table 1. *Effect of metal-binding agents on proteinase and amidase activity of the venom of Bothrops jararaca*

Test substances were incubated for 1 hr. at 35° with venom before addition of corresponding substrate. Reaction mixture for the determination of caseinase activity: 1 ml. of venom-metal-binding agent mixture and 1 ml. of 1% (w/v) casein in 0.05M-tris buffer, pH 8.8. For gelatinase activity: 2.5 ml. of venom-metal-binding agent mixture and 2.5 ml. of 12% (w/v) gelatin in 0.08M-tris buffer, pH 8.8, containing 0.2% gum acacia. For amidase activity: 0.2 ml. of venom-metal-binding agent mixture, 0.4 ml. of 0.05M-benzoyl-L-arginine amide (BAA) and 0.2 ml. of 0.3M-tris buffer, pH 8.8.

Added substance	Final concn. (mM)	Relative activity on		
		Casein	Gelatin	BAA
None	—	100	100	100
DL-Cysteine	50	21	17	—*
Reduced glutathione	50	18	1	42
Ethylenediaminetetra-acetic acid	2	22	7	100
8-Hydroxyquinoline	2	13	38	95
Histamine	50	82	67	67

* The effect of histamine could not be determined because of the presence of L-amino acid oxidase in the venom.

unaffected by Fe^{2+} ions, reduced to 89% by Mg^{2+} ions, 83% by Mn^{2+} ions, 46% by Zn^{2+} ions, 17% by Co^{2+} ions and 15% by Cd^{2+} ions (Table 2). Calcium ions increased the caseinase activity by 36% and did not affect the amidase activity. The influence of the concentration of Ca^{2+} ions on the caseinase activity was also studied, the optimum concentration being 2 mM or above (Fig. 3). In a separate experiment it was found that Ca^{2+} ions (final concn. 2 mM) increased the gelatinase activity by 22%.

Effect of enzyme-inhibiting anions. Table 3 shows that sulphide and cyanide (final concn., mM) strongly inhibited the caseinase and gelatinase activities of the venom. This effect on the caseinase activity has been previously reported by Taborda & Taborda (1940). Fluoride had no effect on the caseinase or gelatinase activity. Iodoacetate inhibited strongly the gelatinase activity and did not affect the caseinase activity. None of the anions studied had any effect on the benzoylarginine amidase activity of the venom.

Effect of heating on the caseinase and amidase activities of the venom. As shown in Table 4, heating the venom in a boiling-water bath for 1 min. is sufficient to destroy all caseinase activity which is activated by Ca^{2+} ions, the caseinase specific activity (in the absence of Ca^{2+} ions) falling to about 28% of that of the crude venom. By contrast, the amidase specific activity is virtually unaffected by heating up to 5 min., falling only to 60% of that of the crude venom after heating for 20 min.

Separation of proteolytic fractions from the venom

Comparison of hydrolytic activity on casein, gelatin and benzoyl-L-arginine amide of fractions precipitated with ammonium sulphate after treatment with calcium phosphate gel. It can be seen (Table 5) that for the caseinase activity in the presence of Ca^{2+} ions, the highest specific activity (3.43) was obtained in the fraction precipitated between 0.50 and 0.55 saturation (fraction no. 5)

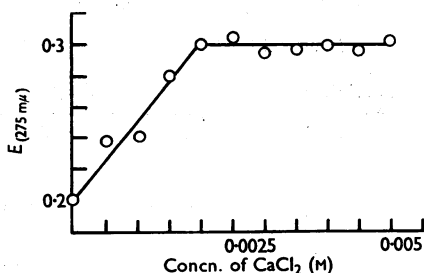


Fig. 3. Effect of Ca^{2+} ion concentration on the caseinase activity of the venom of *B. jararaca*. The venom was incubated for 1 hr. with the CaCl_2 solution at 35° , before addition of the casein.

Table 2. *Effect of metal ions on proteinase and amidase activity of the venom of Bothrops jararaca*

Solutions of metal ions (final concn. 2 mM) were incubated for 1 hr. at 35° with the venom before addition of substrate. Venom-metal-ion mixture was incubated with substrate as in Table 1. BAA: benzoyl-L-arginine amide.

Metal ion	Relative activity	
	Casein as substrate	BAA as substrate
Ca^{2+}	136	100
None	100	100
Fe^{2+}	94	89
Mg^{2+}	89	88
Mn^{2+}	83	100
Zn^{2+}	46	92
Co^{2+}	17	86
Cd^{2+}	15	79
Hg^{2+}	—	81

Table 3. *Effect of certain anions on proteinase and amidase activity of the venom of Bothrops jararaca*

Venom solution was incubated for 1 hr. at 35° with test substances (final concn., mM) before addition of substrate. Venom-test-substance mixture was incubated with substrate as in Table 1. BAA: benzoyl-L-arginine amide.

Substance	Relative activity on		
	Casein	Gelatin	BAA
None	100	100	100
Sulphide	33	34	95
Cyanide	59	35	100
Fluoride	100	93	100
Iodoacetate	100	34	100

Table 4. *Effect of heating on the caseinase and benzoylarginine amidase specific activities of the venom*

A 2% (w/v) solution of *B. jararaca* venom in saline was heated in a boiling-water bath for different periods. At the end of each period the tube containing the sample was immediately transferred to an ice-cold water bath. Venom was incubated with substrate as in Table 1. Caseinase specific activity is expressed as the increase in optical density of the trichloroacetic acid filtrate at 275 m μ /mg. of protein in the sample. 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.

Time (min.)	Specific activity		
	Caseinase		Benzoyl-arginine amidase
	Ca^{2+} ions absent	Ca^{2+} ions present	
0	1.54	2.06	45.2
0.5	0.74	1.11	41.8
1	0.43	0.44	54.2
2	0.44	0.41	55.4
3	0.47	0.47	54.3
5	0.44	0.46	53.1
10	0.36	—	39.3
20	0.39	—	27.1

Table 5. Comparison of hydrolytic activity on casein, gelatin and benzoyl-L-arginine amide of the various fractions precipitated with ammonium sulphate

Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was from the supernatant of the calcium phosphate gel. The caseinase and benzoylarginine amidase specific activities are calculated as described in Table 4. The gelatinase specific activity is expressed as v (flow-time index)/ μg . of protein in the sample. All the reactions were performed under standard conditions (see text).

Fraction no.	Saturation with $(\text{NH}_4)_2\text{SO}_4$	Specific activities				Yield (%) as			
		Caseinase		Gelatinase	Amidase	Protein	Caseinase	Gelatinase	Amidase
		Ca ²⁺ ions absent	Ca ²⁺ ions present						
1	None	1.53	2.03	3.15	35.6	100	100	100	100
2	0 -0.40	2.09	2.92	2.24	11.3	4.7	6.4	3.3	1.5
3	0.40-0.45	1.33	2.52	2.76	19.7	5.0	4.3	4.4	2.7
4	0.45-0.50	1.60	2.86	6.28	27.7	11.6	12.1	23.1	8.9
5	0.50-0.55	1.59	3.43	7.15	33.1	13.2	13.8	30.0	12.3
6	0.55-0.70	0.61	0.88	1.61	69.0	14.8	6.0	7.5	28.8
7	0.70-0.80	0.66	0.66	0.94	164.0	1.4	0.61	0.42	6.4
8	0.80-1.00	0.34	0.24	0.46	45.0	0.24	0.05	0.04	0.29
Total recovery from fraction no. 1						50.9	43.3	68.8	60.9

Table 6. Benzoylarginine amidase activity of fractions obtained from heated venom by precipitation with ammonium sulphate

Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was from the supernatant of the heated venom. The venom was heated in a boiling-water bath for 5 min., the temperature reaching 88° (see text). Specific activities were calculated as described in Table 4.

Fraction no.	Saturation with $(\text{NH}_4)_2\text{SO}_4$	Amidase specific activity	Yield (%)	
			Protein	Amidase
1	Nil (crude venom)	40.7	100	100
2	Nil (heated venom)	32.8	36.5	29.3
3*	0 -0.45	—	—	3.0
4	0.45-0.55	43.6	1.7	1.8
5	0.55-0.60	52.0	1.2	1.6
6	0.60-0.70	162	2.0	7.8
7	0.70-0.80	570	0.46	6.4
8	0.80-1.00	43.6	0.28	0.3
Total recovery from fraction no. 1			5.64	20.9

* Most of this fraction was insoluble in saline and the solution was very turbid even after centrifuging at high speed.

Table 7. Comparison of the gelatinase and benzoylarginine amidase specific activities of samples obtained in the course of purification of the amidase activity of a fraction similar to fraction no. 7 of Table 6

Gelatinase and benzoylarginine amidase specific activities are expressed as described in Table 5. All the reactions were performed under standard conditions.

Fraction no.	Step	Specific activities		Yield (%)	
		Gelatinase	Amidase	Gelatinase	Amidase
1*	Precipitation at 0.7-0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$	1.89	279	100	100
2	Reprecipitation of fraction no. 1 at 0.7-0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$	5.86	783	39.2	35.5
3	Reprecipitation of fraction no. 2 at 0.7-0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$	6.84	1161	26.3	30.3
4	Electrophoretic peak (starch column) of fraction no. 3	7.88	1548	8.9	11.8

* The amidase specific activity of the crude venom from which this fraction was obtained was 29.5.

with ammonium sulphate, and that this fraction was also the one most activated by Ca^{2+} ions (100%). The peak of gelatinase specific activity (7.15) was also found in this fraction, but its amidase specific activity was the same as that of the crude venom. By reprecipitation with ammonium sulphate further purification of this fraction was obtained in its caseinase specific activity (4.39) and gelatinase specific activity (9.48), whereas its amidase activity remained about the same (results are not included in the tables).

Fraction no. 7, which had a caseinase or gelatinase specific activity about one-third of that of the initial venom, was found to have an amidase specific activity approximately five times as great as that of the crude venom, and its caseinase activity was not affected by Ca^{2+} ions. The proteins

present in this fraction migrated towards the anode at pH 7.2 (tris buffer, $I=0.05$), when submitted to electrophoresis on a starch column; the electrophoresis diagram obtained was very broad and the recorded maximum benzoylarginine amidase specific activity was found ahead of the protein peak (Fig. 4). Considering the electrophoretic fraction with the highest specific activity, an overall purification of 45 times was observed for the amidase activity, giving, however, a very low yield.

Preparation of a purified proteolytic fraction. The fraction precipitated between 0.7 and 0.8 saturation with ammonium sulphate (Table 6, fraction no. 7) had the highest benzoylarginine amidase specific activity, and this was 14 times as great as that of the crude venom. By reprecipitation of a similar fraction, obtained in another preparation (specific activity 279), the amidase specific activity reached the value of 1161 (Table 7). A further

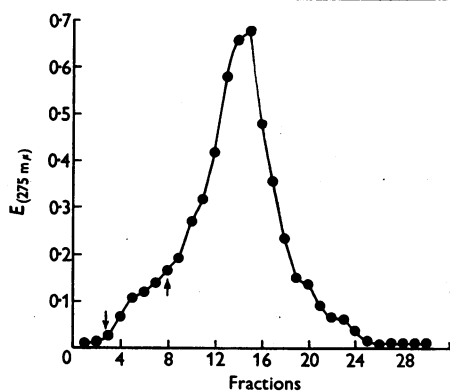


Fig. 4. Optical densities of fractions obtained by electrophoresis of 10 mg. of protein of fraction no. 7 (Table 5), on a starch column. Tris buffer, pH 7.2, $I=0.05$; about 3 mA; under these conditions the proteins moved to the anode. The arrows indicate the highest levels of benzoylarginine amidase activity.

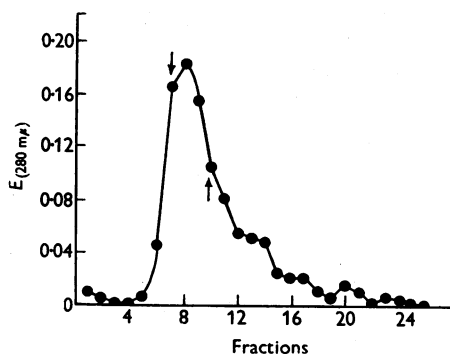


Fig. 5. Optical densities of fractions obtained by electrophoresis of 1.67 mg. of protein of fraction no. 4 (Table 7), on a starch column. Same conditions as described for Fig. 4. The part of the curve between the arrows contained the highest benzoylarginine amidase activity.

Table 8. Comparison of benzoylarginine amidase and gelatinase specific activities during purification of *Bothrops* protease A

Reaction mixtures and calculation of benzoylarginine amidase and viscosimetric specific activities were as indicated in the text. Incubation mixture for gelatinase activity, as measured from non-protein nitrogen release: 1.5 ml. of 1% gelatin + 1 ml. of 0.2M-borate buffer, pH 8.8, + enzyme sample, and the volume made up to 5 ml. with 0.9% NaCl. The control for each sample consisted of the same mixture, the enzyme sample being added after the deproteinizing agent. Non-protein nitrogen determinations were made by standard micro-Kjeldahl technique in Folin-Wu supernatants after high-speed centrifuging.

Phase of preparation	Specific activities		
	Amidase	Gelatinase	
		Viscosity method	Non-protein N*
After heating and precipitation with $(\text{NH}_4)_2\text{SO}_4$ (0.55-0.80 saturation)	123	1.20	0.21
Precipitation with $(\text{NH}_4)_2\text{SO}_4$ (0.70-0.80 saturation)	475	4.40	0.65
Electrophoretic peak (starch column)	887	7.20	0.93

* Released nitrogen (mg./mg. of enzyme protein).

purification was obtained when this reprecipitated fraction was submitted to electrophoresis on a starch column (tris buffer, pH 7.2, $I=0.05$). The benzoylarginine amidase specific activity of the fraction which corresponded to the electrophoresis protein peak (Fig. 5) was found to be 52 times (1548/29.5) greater than that of the crude venom from which it was prepared. It can be seen (Table 8) that the increase in gelatinase specific activity of the purified fractions follows approximately the increase in the amidase specific activity.

DISCUSSION

The results presented here show similar pH optima for benzoylarginine amidase and caseinase activities of the venom of *Bothrops jararaca* (Figs. 1, 2). The pertinent results also show that, in the crude venom, the amidase activity differs markedly from either the caseinase or the gelatinase activity in sensitivity to metal-binding agents, metal ions and some anions. Thus in the presence of ethylenediaminetetra-acetic acid and 8-hydroxyquinoline the caseinase and gelatinase activities were very much decreased, whereas the amidase activity was unaltered; Ca^{2+} ions increased the caseinase and gelatinase activities but did not affect the amidase activity; sulphide and cyanide strongly inhibited the caseinase and gelatinase activities, but did not affect the amidase activity. Therefore the effects of activators and inhibitors on the caseinase and on the gelatinase activity of the crude venom were roughly parallel, an exception being the action of iodoacetate, which strongly inhibited the gelatinase activity without affecting either the caseinase or the amidase activity. Heating the venom in a boiling-water bath further distinguished the Ca^{2+} ion-sensitive caseinase activity from the amidase activity. Thus heating for 1 min., without changing the amidase specific activity, caused a total destruction of the calcium-activated caseinase activity and a sharp decrease of the caseinase specific activity; on the other hand, the remaining caseinase, as well as the amidase specific activity, was not affected by a more prolonged heating in a boiling-water bath, at least up to 5 min. These observations confirm the finding of Hamberg & Rocha e Silva (1956, 1957) who reported a heat-resistant benzoylarginine methyl esterase in the venom of *B. jararaca*. The difference between the benzoylarginine amidase activity and the calcium-activated caseinase and gelatinase activities is also apparent from experiments on the fractionation of the venom with ammonium sulphate after treatment with calcium phosphate gel, since the fraction obtained at 0.50–0.55 saturation, with about the same specific amidase activity as the initial venom, had the highest calcium-activated caseinase (about

1.7 higher than the crude venom) or gelatinase specific activity (about 2.2 higher than the crude venom); on the other hand, the fraction precipitated at 0.7–0.8 saturation, with a caseinase or gelatinase specific activity of about one-third as high as the starting material, had the highest amidase specific activity (about 4.6 times higher than the crude venom).

Heating of the venom before precipitation of the benzoylarginine amidase at 0.7–0.8 saturation with ammonium sulphate proved to be advantageous. This procedure, followed by two reprecipitations and zone electrophoresis, led to a fraction 52 times more active than the crude venom. The concomitant increase in gelatinase specific activity observed during the purification of the amidase (Table 7) shows that, as expected, the amidase of the venom of *B. jararaca* is a real endopeptidase, and accordingly we propose to call it *Bothrops* protease *A*. That the decrease in viscosity of gelatin is due to proteolysis was shown by parallel determinations of viscosity and non-protein nitrogen run with samples obtained in different phases of a separate preparation of *Bothrops* protease *A* (see Table 8). It can be seen that the proportional increases in specific activities as calculated from hydrolysis of benzoyl-L-arginine amide, viscosity and non-protein nitrogen were about the same in the two last phases of purification after heating. It is noteworthy, however, that the purified preparations of *Bothrops* protease *A* had a doubtful casein-hydrolysing activity when used in amounts ten times greater than for benzoylarginine amidase activity. Although *Bothrops* protease *A* proved to behave like trypsin in its action upon benzoyl-L-arginine amide, it differs from the latter with gelatin and casein, the first of which is very slowly hydrolysed and the second of which seems not to be hydrolysed by this protease. It must also be mentioned that soya-bean trypsin inhibitor or ovomucoid, which inhibits the hydrolysis of benzoylarginine ethyl ester, does not affect the action of *Bothrops* venom on this synthetic substrate (Deutsch & Diniz, 1955).

SUMMARY

1. The hydrolysis of casein and gelatin by the venom of *Bothrops jararaca* is enhanced by Ca^{2+} ions and inhibited by sulphide, cyanide, cysteine, reduced glutathione, ethylenediaminetetra-acetic acid, 8-hydroxyquinoline and histamine, whereas the hydrolysis of benzoylarginine amide is moderately inhibited only by reduced glutathione and histamine.

2. The venom fraction precipitated between 0.50 and 0.55 saturation with ammonium sulphate proved to be activated by Ca^{2+} ions when hydro-

lysing casein and had a specific activity about twice as high as that of the crude venom when hydrolysing casein or gelatin; its benzoylarginine amidase specific activity was about the same as that of the crude venom.

3. The venom fraction precipitated between 0.7 and 0.8 saturation with ammonium sulphate had a specific activity about four times higher than the crude venom when hydrolysing benzoylarginine amide; it was insensitive to Ca^{2+} ions when hydrolysing casein and its caseinase and gelatinase activities were lower than those of the crude venom.

4. An isolation technique has been described which leads to the preparation from the venom of a heat-resistant proteolytic enzyme which is precipitated between 0.7 and 0.8 saturation with ammonium sulphate and has a benzoylarginine amidase specific activity 52 times higher than the crude venom.

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A Microdetermination of Cellulose in Studies with Cellulase

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Cellulose preparations may contain other polysaccharides as impurities (cf. Huffman, Rabers, Spriesterbach & Smith, 1955). When such preparations are used as substrates for cellulase care must be taken to ensure that the degradation observed is of the cellulose and not of the impurities (Halliwell, 1957*a, b*). This will be so if a substantial degradation of the cellulose is allowed to take place, and a measure of such degradation is the rate of solubilization of the polysaccharide, a process which does not necessarily involve complete degradation to sugars. This rate can be determined by measuring the loss in weight of the

cellulose. Until recently microprocedures have not been available for the determination of cellulose. Schramm & Hestrin (1954) describe an acetolytic micromethod which is time-consuming and involves a critical alkali neutralization. More recently, Dearing (1957) has reported a chromogenic reaction with concentrated sulphuric acid which can be used in the quantitative estimation of cellulose. Both these methods, however, involve preliminary and lengthy centrifuging of the cellulose and it has been found that the cellulose often fails to precipitate even in the presence of talcum powder. This failure of the cellulose to