A BLOOD ANTICOAGULANT FACTOR FROM THE LATEX OF *CARICA PAPAYA*

Part I. Purification and General Properties

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In recent years blood anticoagulants have assumed great importance in the prevention and treatment of intravascular thrombosis and embolism, in vascular surgery and in peripheral vascular disease. Well known among the anticoagulants are heparin, dicumarol and a number of complex polysaccharides and certain azo dyes. Of these only heparin and dicumarol are used in routine therapy because of their comparative non-toxicity. The action of dicumarol is slow and is preceded by a lag period and in addition, is difficult to control. In the present-day anticoagulant therapy, intravenous administration of heparin constitutes the method of choice especially because of the complete absence of any untoward effects on the system. The chief drawback of heparin which prevents its extensive use is its prohibitive cost. The urgent need for a cheap substitute for heparin is therefore obvious.

The occurrence of a blood anticoagulant factor in the latex of *Carica papaya* was briefly reported in an earlier publication from this laboratory (Ramakrishnan *et al.*, 1952). The detection for the first time of a blood anticoagulant in papaya latex is of special significance in that it may eventually lead to the isolation of the active factor in a sufficiently pure form for treatment of diseases characterised by the formation of intravascular clots. In the present paper a method is given for the purification of the anticoagulant from the latex of *Carica papaya*. In addition, certain observations are reported on the general properties of the purified preparation.

Experimental

Blood coagulation experiments were carried out according to the method of Rahman and Giri (1945), using fresh citrated plasma (unless otherwise stated) and Russell's viper venom. The venom was obtained in the form of lyophilized powder from the Haffkine Institute, Bombay. 10 mg. of the powder was dissolved in 100 c.c. of 0·025 M calcium chloride solution. The reaction mixture, unless otherwise stated, consisted of 0·2 c.c. venom solution, 0·2 c.c. anticoagulant and 0·2 c.c. plasma.

Fresh latex obtained from plants grown in the Institute Nursery was used as the starting material for the preparation of the anticoagulant.

Preparation of the anticoagulant

A 2% solution of the acetone-dried latex powder was made in distilled water and centrifuged. To the clear supernatant were added three volumes of cold acetone and the precipitate centrifuged. Papain has been reported to coagulate plasma (Ferguson and Ralph, 1943), and hence it was thought desirable to get the anticoagulant without any contamination by papain. Hence the acetone precipitate obtained was repeatedly washed with cold 70% (v/v) alcohol to remove the bulk of papain present. The residue was finally washed twice with cold acetone and dried in a desiccator over fused calcium chloride. This acetone-dried powder was used as starting material for further purification.

Attempts to purify the anticoagulant factor by use of the adsorbents celite, hyflo supercel, tricalcium phosphate, magnesium hydroxide, kaolin, alumina and alumina C_r did not yield a product of satisfactory purity.

Fractional precipitation with organic solvents

As the adsorption techniques were not very promising, fractionation by organic solvents were tried. The results are presented in Table I. Preci-

TABLE I

Fractional precipitation with organic solvents

Precipitating agent	Final concentrations %	Anticoagulant activity of the precipitate
Control ..	..	Clotting at 18 sec.
Methanol ..	50	Clotting at 20 sec.
	60	Precipitate resembling clot.
	75	Active anticoagulant; precipitate formation.
Dioxane ..	40	Thick precipitate resembling clot.
	50	No clotting; precipitate formation.
	75	Clotting at 20 sec.
Ethanol ..	30	Clotting at 22 sec.
	40	Clotting at 21 sec.
	45	No clotting; precipitate formation.
	50	Active anticoagulant; slight precipitate formation.
	60	Formation of precipitate resembling clot.
Acetone ..	40	Heavy precipitate resembling clot.
	45	No clotting; light precipitate formation.
	50	Active anticoagulant.
	60	This fraction accelerates clotting.

pitates obtained at the various fractions were dried in a desiccator over calcium chloride after washing with acetone. The dry powder was dissolved in the same volume of distilled water as the original extracts and their anticoagulant activity tested with sheep plasma and Russell's viper venom.

Exactly similar results were obtained with cow and buffalo plasma. Of the various fractionation procedures tried, acetone fractionation was found to be the best as judged by the anticoagulant activity of the active fractions and also by the fact that preciptate formation in the reaction mixture was minimum with acetone fractions. It can be seen from Table II that the precipitate obtained above 50% up to 60% final concentration of acetone accelerates the clotting of plasma in presence of Russell's viper venom. This pointed to the possibility that this fraction might be a blood coagulant capable of clotting plasma in the absence of venom thromboplastin. This was indeed found to be the case.

Attempts at further purification of the anticoagulant obtained by acetone fractionation were not particularly successful. Careful re-fractionation with acetone, however, showed that a very active preparation could be obtained between 42% and 48% final concentration of acetone. For the sake of convenience, this fraction is designated as Ac 48, and some of its general properties are given below.

Properties of Ac 48

The anticoagulant activity of the dry powder was well preserved on storage. The dried material dissolves readily in water to yield clear colourless solutions. The factor was found to be active in preventing the coagulation of plasma in presence of active thromboplastin and calcium (Russell's viper venom solution) at a final concentration of 0·25% (w/v). The use of a higher concentration (*i.e.*, 0·5% w/v) resulted in the total absence of precipitate formation in the reaction mixture. The clotting of whole blood (human) was completely prevented by the addition of Ac 48 at a final concentration of 0·05%.

It has been reported by Grob (1943) that serum antitrypsin and pancreatic trypsin inhibitor strongly inhibit the coagulation of plasma *in vitro*. Horwitt (1940) has observed that many basic dyes which are potent anticoagulants are also antiproteolytic.

It was, therefore, thought to be of interest to see whether the anticoagulant factor Ac 48 exerts any inhibiting action on trypsin.

The reaction mixture consisted of:

10 c.c. of phosphate buffer (pH 8·4)
10 c.c. of 2% gelatin solution
5 c.c. of trypsin solution
5 c.c. of Ac 48 (2% solution)

30 c.c. .. Total volume

A control experiment was also carried out with 5 c.c. of distilled water instead of the anticoagulant solution. The reaction mixture was kept in an incubator at 37° C. At various intervals of time 5 c.c. aliquots of the enzyme digest was taken out and the proteolytic activity determined by Sörensen's formol titration method as modified by Northrop (1926).

TABLE II

Action of Ac 48 *on trypsin*

Time of incubation in hours	No. of c.c. of 0·04 N NaOH		
	Trypsin	Ac 48	Trypsin+Ac 48
3	0·34	0·45	0·64
12	1·53	1·30	1·35
24	1·83	1·61	2·00

It can be seen from the results presented in Table II, that Ac 48 fraction contains an antitryptic factor. All the same, the Ac 48 itself was found to exert some proteolytic activity at pH 8·4, which could not be removed by repeated washings with cold 70% alcohol. The proteolytic activity of the various acetone fractions at pH 5·0 was then determined by the formol titration method.

From the date given in Table III, it is clear that there is no correlation between blood coagulant or anticoagulant activities and the proteolytic activity.

Experiments were therefore performed to find out the relative heat stability of the anticoagulant factor and the proteolytic component in Ac 48. It was found that the anticoagulant factor was resistant to thermal inactivation when heated at 70° C. for 30 min. 5 c.c. of a 2% solution of Ac 48 was heated at 85° C. for 30 min. After cooling, the anticoagulant and the proteolytic activities of the solution were determined. The data given in Table IV (*a*) and (*b*) show that while the anticoagulant activity is completely destroyed

TABLE III

Proteolytic activity of the various acetone fractions

Final concentration of acetone %	Proteolytic activity of the precipitates obtained at the specified final concentrations of acetone at various intervals of time (in c.c. of 0·04 N NaOH)		
	3 hours	12 hours	24 hours
42	1·16	1·55	1·70
48	0·97	1·28	1·82
60	1·13	1·38	1·83

TABLE IV (*a*)

Effect of heating at 85° C. for 30 min. on the anticoagulant activity of Ac 48

Reaction mixture	Clotting time with oxalated plasma	Clotting time with citrated plasma
(0·2 c.c. venom + 0·2 c.c. distilled water) incubated at 38° C. for 2 min. + 0·2 c.c. plasma ..	20 sec.	25 sec.
(0·2 c.c. venom + 0·2 c.c. of heated Ac 48) incubated at 38° C. for 2 min. + 0·2 c.c. plasma ..	23 sec.	28 sec.

TABLE IV (*b*)

The effect of heating at 85° C. for 30 min. on the proteolytic activity of Ac 48

Time of incubation in hours	Proteolytic activity in c.c. of 0·04 N NaOH	
	Ac 48 unheated	Ac 48 heated
3	0·97	0·52
12	1·28	0·67
24	1·82	0·67

by heating at 85° C. for 30 min., some proteolytic activity still persists after this heat treatment.

Inactivation and Reactivation experiments

It is well known that papain, the proteolytic enzyme present in papaya latex, is inactivated by dilute hydrogen peroxide and iodacetate, while hydrogen sulphide and hydrogen cyanide exert an activating action. The effect of these compounds on the anticoagulant activity of Ac 48 was tested with a view to find out the difference, if any, between the anticoagulant and proteolytic activities of Ac 48 as regards their susceptibility. It was found that the anticoagulant activity is destroyed by iodacetate and hydrogen peroxide. H_2O_2-inactivated Ac 48 could be regenerated only partially by passing H_2S. Suitable controls were also run to ascertain that the blood coagulation is in no way affected in presence of iodacetate, H_2O_2, HCN or H_2S alone. The results are presented in Table V.

The following solutions were prepared:

1. 0·5 c.c. water + 0·2 c.c. iodacetate (0·125% solution, neutralised with NaOH).
2. 0·5 c.c. Ac 48 (2%) + 0·2 c.c. iodacetate.
3. 0·5 c.c. water + 0·2 c.c. 3% solution of NaCN (neutralised).
4. 0·5 c.c. Ac 48 + 0·2 c.c. NaCN.
5. 0·5 c.c. water + 0·1 c.c. saturated H_2S solution.
6. 0·5 c.c. Ac 48 + 0·1 c.c. saturated H_2S solution.
7. 0·5 c.c. water + 0·2 c.c. H_2O_2 (N/100).
8. 0·5 c.c. Ac 48 + 0·2 c.c. H_2O_2.

The solutions were incubated for 1 hour at room temperature.

TABLE V

Effect of oxidising and reducing agents on the anticoagulant activity of Ac 48

Reaction mixture	Clotting time
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. water	21 sec.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 1	24 sec.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 2	No clotting, increase in viscocity and precipitate formation.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. solution 3	41 sec.

TABLE V—(*Contd.*)

Reaction mixture	Clotting time
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 4	No clotting
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 5	22 sec.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 6	No clotting, formation of turbidity
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 7	18 sec.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 8	40 sec.
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 2 through which H_2S was passed	No clotting, but increase in viscosity
0·2 c.c. plasma + 0·2 c.c. venom + 0·2 c.c. of solution 8 saturated with H_2S	No clotting; but thick precipitate formation after 25 sec. indicating partial regeneration of activity.

DISCUSSION

In the present investigation, conclusive evidence has been obtained for the presence of two factors in the latex of *Carica papaya*, one inhibiting and the other accelerating the process of blood coagulation.

Purification of the anticoagulant could not be effected by the usual adsorption procedures. Although the active factor could be adsorbed on Alumina C_r and Kaolin, the adsorption was only partial and difficulty was experienced in eluting the anticoagulant from the adsorbent.

One of the disturbing effects of the anticoagulant was the formation of a very light precipitate in the presence of plasma and Russell's viper venom. Attempts to remove any impurities which might be responsible for precipitate formation, by the use of adsorbents, were not very fruitful.

Fractional precipitation with organic solvents proved to be an effective method for purification of the anticoagulant factor. Fractional precipitation with cold acetone yielded the most active material and was far superior to salting out with ammonium sulphate described in a previous note ((Ramakrishnan *et al.*, 1952). By acetone fractionation it was possible to separate the blood coagulant and anticoagulant factors. While the anticoagulant factor was precipitated at a final acetone concentration of 50%, the precipitate obtained between 50% and 60% final concentration of acetone was very

active in clotting blood plasma in the absence of venom thromboplastin. This finding may explain the observations of earlier workers (Eagle and Harris, 1937; Dyckerhoff and Gigante, 1940; Ferguson and Ralph, 1943) on the blood coagulating property of papain preparations.

It is generally believed that the process of blood coagulation is essentially a proteolytic reaction. A natural corollary of this would be to conceive blood anticoagulants as inhibitors of proteolytic activity. It was found that the Ac 48 fraction exerts an antitryptic activity although it still contained a residual proteolytic factor (Table II). Experiments designed to establish a correlation between anticoagulant and proteolytic activities were, however, inconclusive (Table III). As judged by experiments with plasma, it was observed that iodacetate which is known to inactivate papain could only partially inactivate the anticoagulant property of Ac 48. There was no strict parallelism between the heat stability of the anticoagulant activity and the proteolytic activity present in Ac 48 [Table IV (*a*) and (*b*)]. When a solution of Ac 48 was heated at 85° C. for 30 min., there was still some residual proteolytic activity even though the anticoagulant property was completely destroyed.

On the other hand, indication of the identity of the anticoagulant factor with papain was obtained from experiments with H_2O_2. It was found that the anticoagulant property of Ac 48 was destroyed by treatment with dilute H_2O_2, but could be partially regenerated by subsequent saturation of the solution with hydrogen sulphide (Table V).

As was stated earlier, when a mixture of Ac 48, Russell's viper venom and oxalated sheep plasma were kept at room temperature for more than 24 hours a very fine precipitate was found to settle down at the bottom of the coagulation tube. When citrated plasma was used, the formation of precipitate was considerably reduced. This difference can possibly be explained by the fact that Ac 48 does form a fine granular precipitate with dilute solutions of oxalate.

Summary

A factor capable of effectively inhibiting the process of coagulation of plasma as well as whole blood has been isolated from the latex of *Carica papaya*. Attempts were made at purification of the factor by adsorption and fractional precipitation with organic solvents. A comparatively pure product was obtained by cold acetone fractionation at a final concentration of 42 to 48%. It was found that there was no correlation between the residual proteolytic activity and the anticoagulant property of the factor. Some of the general properties of the anticoagulant factor have also been studied.

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