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

Part II. Its Nature of Action on Blood Coagulation

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THE method of purification and general properties of a blood anticoagulant factor present in the latex of *Carica papaya* were described in an earlier publication (Pillai *et al.*, 1955). Since the factor was found to inhibit effectively the process of coagulation of both plasma and whole blood from different species, it was thought desirable to undertake a detailed investigation to elucidate the probable nature of its inhibitory action on blood coagulation. The process of blood coagulation is a complex reaction brought about by the interplay of a variety of coagulation factors present in the blood. The inhibition of this process of blood coagulation may thus include one or more of the following mechanisms, *viz.*, (1) complex formation with prothrombin preventing its further activation; (2) inhibition of thromboplastic action; (3) inactivation of thrombin and (4) combination with fibrinogen making it incapable of being converted to fibrin.

It was, therefore, found necessary to isolate the different proteins active in blood coagulation and study the effect of the anticoagulant factors on the pure individual components. Similar experiments were carried out with coagulation components prepared from different species.

EXPERIMENTAL

Prothrombin.—Prothrombin was prepared from sheep blood according to the method of Seegers (1952), which consisted of isoelectric precipitation of prothrombin from diluted plasma, adsorption on magnesium hydroxide, elution of the prothrombin by decomposing the latter with carbon dioxide under pressure, fractional precipitation by ammonium sulphate, and later, isoelectric fractionation.

Prothrombin from cow and buffalo plasma was also prepared in a similar manner.

Fibrinogen.—Fibrinogen from cow, sheep and buffalo plasma was prepared by the method of Astrup and Darling (1942). Pure bovine fibrinogen

obtained through the kind courtesy of Prof. W. H. Seegers and Messrs. Parke, Davis & Co., Michigan, was also used.

Thrombin.—Thrombin was prepared from pure prothrombin by using Bacto 2-stage reagent and Bacto Ac-globulin. Both these reagents were generous gift samples obtained from Difco Laboratories, Detroit, Mich.

Russell's viper venom.—Lyophilised venom powder obtained from Haffkeine Institute, Bombay, was diluted with 0·025 M calcium chloride to give a 0·01% solution.

Anticoagulant factor.—The anticoagulant factor was purified by fractional precipitation with acetone (Pillai *et al.*, 1955). A 2% aqueous solution of the dry powder was prepared. For the sake of convenience, the purified anticoagulant factor is designated as AF.

The clotting activity was determined according to the 2-stage procedure of Ware and Seegers (1949). In some experiments Russell's viper venom was used as a source of thromboplastin.

Results

Action of the anticoagulant factor on ideal clotting system

To start with, the action of AF on thrombin activity was determined. The method employed was essentially the same as that described by Ware and Seegers (1949) to determine the prothrombin activity of plasma.

The following solutions were prepared:

A. Bacto 2-stage reagent (containing tissue thromboplastin, imidazole buffer, acacia, sodium chloride and calcium ions).

B. Bacto Ac-globulin. The freeze-dried powder was diluted to 2 c.c. with sterile distilled water. 0·4 c.c. of this solution was further diluted to 60 c.c. with normal saline.

C. To 0·1 c.c. of prothrombin (suitably diluted) were added 2·4 c.c. of diluted Ac-globulin (solution B).

D. 0·1 c.c. of solution C was mixed with 3 c.c. of Bacto 2-stage reagent (A) and incubated for 5 minutes when prothrombin was converted to thrombin.

For determination of thrombin activity the following reaction mixture was compounded.

0·2 c.c. of solution D (thrombin from different species) + 0·2 c.c. distilled water + 0·1 c.c. of fibrinogen from the same species.

The time taken to form a visible clot after the addition of fibrinogen to solution D was taken as the clotting time and was noted with a stop watch. Similarly the coagulation time was measured after the addition of 0·2 c.c. of anticoagulant factor (AF). The results are given in Table I.

TABLE I

Effect of anticoagulant factor on the activity of thrombin prepared from different species

Reaction mixture	Clotting activity
1. Cow thrombin + cow fibrinogen (control)	Clots in 22 sec.
2. Cow thrombin + cow fibrinogen (in presence of AF)	No clotting even after 24 hours. No precipitate formation.
3. Sheep thrombin + sheep fibrinogen (control)	Clotting in 17 sec.
4. Sheep thrombin + sheep fibrinogen (in presence of AF)	No clotting even after 24 hours. No precipitate formation.
5. Buffalo thrombin + buffalo fibrinogen (control)	Clotting in 14 sec.
6. Buffalo thrombin + buffalo fibrinogen (in presence of AF)	No clotting even after 24 hours. No precipitate formation.

Experiments were also carried out with thrombin prepared by incubating prothrombin with Russell's viper venom in calcium chloride solution.

To 0·1 c.c. of suitably diluted prothrombin 0·2 c.c. of venom was added and incubated for 5 min. to convert prothrombin to thrombin. 0·2 c.c. of a 2% solution of AF was added to this and after incubation for 2 min., 0·1 c.c. of fibrinogen was added. Suitable controls were also run without the addition of AF.

In both these cases it was observed that AF effectively inhibited the action of thrombin on fibrinogen. In other words, it interferes with the second stage of blood coagulation.

Since the anticoagulant factor (AF) was found to inhibit thrombin action, different concentrations of AF were added to thrombin as well as pure prothrombin solutions to see whether there will be any interaction between the two.

At the concentration of prothrombin employed for the above clotting experiments, no visible interaction with AF could be detected. But at very high concentrations, the anticoagulant factor formed varying amounts of a white precipitate with both thrombin and prothrombin depending upon the respective concentrations of the reactants.

After inactivation by heating at 85° C. for 30 min., AF lost its property of precipitate formation when added to concentrated solutions of prothrombin.

In another experiment, pure fibrinogen was incubated with a 2% solution of AF for 5 minutes. When this clear solution was added to prothrombin, precipitate formation was retarded to a certain extent. This obviously indicates that although there is no precipitate formation when fibrinogen and AF are mixed, some soluble complex is formed so that further interaction between AF and prothrombin is retarded. When prothrombin is added to the mixture, possibly dissociation of the fibrinogen-AF complex occurs and the free AF generated forms a precipitate with the added prothrombin.

The ability of AF to form a thick precipitate with high concentrations of prothrombin suggests that it might form a soluble complex with prothrombin in dilute solutions usually employed in the 2-stage procedure of Ware and Seegers (1949), and thereby interfere with the first stage as well. The main difficulty in testing this possibility was to remove the excess of AF after it has reacted with prothrombin so that thrombin formed, if any, from the prothrombin complex by the action of thromboplastin is free to act on fibrinogen. This difficulty was circumvented by adopting the following procedure.

A 2% solution of AF was added dropwise to 0·1 c.c. of a concentrated solution of prothrombin until there was no further formation of precipitate. The granular precipitate formed easily settled down to the bottom of the tube. The mixture was then centrifuged.

(*a*) To an aliquot of the supernatant were added 0·1 c.c. of diluted AC-globulin and 0·2 c.c. venom. After incubating for 5 min. to convert the residual prothrombin, if any, to thrombin, 0·1 c.c. of fibrinogen was added to this mixture and the coagulation time was noted.

(*b*) The precipitate of prothrombin-AF complex was insoluble in water and was therefore washed thrice with distilled water to remove the last traces of free AF.

The washed precipitate was suspended in 0·2 c.c. of distilled water. After adding 0·1 c.c. of dilute Ac-globulin and 0·2 c.c. venom to this suspension, the mixture was incubated for 5 min. After addition of 0·1 c.c. of fibrinogen, the coagulation time was measured.

(*c*) The above experiment was repeated with Bacto 2-stage reagent as a source of thromboplastin.

To 0·1 c.c. of the suspension of the prothrombin-AF complex were added 2·4 c.c. of diluted Ac-globulin. After mixing well, 0·1 c.c. of this suspension was taken and 0·3 c.c. of Bacto 2-stage reagent added. After incubating for 5 min. 0·1 c.c. of fibrinogen was added and the clotting time noted.

TABLE II

Effect of anticoagulant factor on prothrombin activation

Reaction mixture	Clotting time
(*a*) 0·1 c.c. of supernatant obtained after complete precipitation of prothrombin-AF complex + 0·1 c.c. Bacto Ac-globulin (150 times dilution) + 0·2 c.c. venom + 0·1 c.c. fibrinogen	No clotting.
(*b*) 0·2 c.c. of suspension of prothrombin-AF complex + 0·1 c.c. Ac-globulin + 0·2 c.c. venom + 0·1 c.c. fibrinogen	Thick precipitate formation after 1 min. indicating partial inactivation.
(*c*) 0·1 c.c. of suspension of prothrombin-AF complex containing Ac-globulin + 0·3 c.c. Bacto 2-stage reagent + 0·1 c.c. fibrinogen	Clotting in 100 sec.
(*d*) Control experiment using same concentration of prothrombin and other reagents but without AF	Clotting in 7 sec.

The data presented in Table II show that AF does inhibit the first stage of blood clotting, *i.e.*, it interferes with prothrombin activation. It can also be seen that the supernatant obtained after precipitation of prothrombin-AF complex was devoid of prothrombin, because it was incapable of clotting fibrinogen in the presence of thromboplastin, Ac-globulin and calcium ions. This suggests that combination between prothrombin and AF is possibly quantitative.

Iodacetate inactivation

In our earlier publication (Pillai *et al.*, 1955) it was reported that iodacetate treatment caused only a partial inactivation of the anticoagulant factor when plasma was used to test the anticoagulant activity. It was thought that the use of pure coagulation components would throw more light on the mechanism of iodacetate inactivation.

Results obtained with iodacetate-treated AF are summarised in Table III.

TABLE III

Effect of iodacetate-treated anticoagulant factor on the blood coagulation systems

	Reaction mixture	Clotting time
1.	0·4 c.c. thrombin + 0·2 c.c. water + 0·1 c.c. cow fibrinogen	10 sec.
2.	0·4 c.c. thrombin + 0·2 c.c. iodacetate-treated AF + 0·1 c.c. cow fibrinogen	11 sec.
3.	(*a*) 0·2 c.c. citrated sheep plasma + 0·2 c.c. dilute iodacetate solution (0·07%)+0·4 c.c. thrombin	20 sec.
	(*b*) 0·2 c.c. citrated sheep plasma + 0·2 c.c. iodacetate-treated AF + 0·4 c.c. thrombin	No clotting, increase in viscosity of the solution
4.	(*a*) 0·1 c.c. of 0·6% fibrinogen (Parke, Davis) + 0·2 c.c. dilute iodacetate solution (0·07%) + 0·4 c.c. thrombin	35 sec.
	(*b*) 0·1 c.c. of 0·6% fibrinogen (Parke, Davis) + 0·2 c.c. iodacetate-treated AF + 0·4 c.c. thrombin	40 sec.

Details of the reaction mixtures are given below:

A mixture of 2 c.c. of a 2% solution of AF and 0·8 c.c. of neutralised iodacetate solution (0·25%) was incubated at room temperature for two hours.

1. To 0·1 c.c. of suitably diluted prothrombin containing Ac-globulin was added 0·3 c.c. of Bacto 2-stage reagent and the mixture incubated for 5 minutes at 37° C. to complete the conversion of prothrombin to thrombin.

To this were added 0·2 c.c. of water and 0·1 c.c. of cow fibrinogen and the clotting time was determined.

2. To the thrombin solution prepared as described above, 0·2 c.c. of iodacetate-treated AF and 0·1 c.c. of cow fibrinogen were added and the clotting time measured.

3. In this experiment, citrated sheep plasma was used as a source of fibrinogen.

A mixture of 0·2 c.c. citrated sheep plasma and 0·2 c.c. of iodacetate-treated AF was incubated for 2 min. at 37° C. To this was added 0·4 c.c. of thrombin prepared as indicated above and the clotting time noted.

A control experiment was carried out with the addition of 0·2 c.c. dilute iodacetate instead of the iodacetate-treated AF.

4. To test whether iodacetate-treated AF can combine with fibrinogen and may thus inhibit fibrin formation the following experiment was carried out.

0·1 c.c. of diluted fibrinogen (0·6% solution of a sample of fibrinogen obtained from Messrs. Parke, Davis & Co.) and 0·2 c.c. of iodacetate-treated AF were mixed well and incubated at 37° C. for 2 min. To this solution 0·4 c.c. of thrombin was added and the clotting time measured.

A control was also run with 0·2 c.c. of dilute iodacetate (approx. 0·07%) instead of iodacetate-treated AF.

It was observed that iodacetate treatment completely destroys the anti-thrombin activity of the anticoagulant factor. Therefore, if thrombin is added to plasma which had been previously incubated with iodacetate-treated AF one would expect that thrombin would be free to act on fibrinogen of the plasma and convert it to fibrin. It was, however, observed that there was no clot formation in presence of iodacetate-treated AF and active thrombin, when citrated plasma was used as a source of fibrinogen. The reason for this anomaly is not clear.

DISCUSSION

As has been shown in the foregoing paragraphs, the anticoagulant factor present in the latex of *Carica papaya* acts as a potent antithrombin inhibiting the second stage of blood coagulation. The factor was equally effective when blood coagulation components prepared from the blood of different species were used (Table I).

That the anticoagulant factor has a definite inhibiting action on the first stage of blood clotting, *viz.*, the activation of prothrombin to give

thrombin, was established by removing the excess anticoagulant from the reaction mixture after incubating it with a concentrated solution of prothrombin (Table II). This was easily accomplished by simple centrifugation since the anticoagulant factor formed an insoluble granular precipitate with prothrombin at high concentrations.

It is probable that the anticoagulant factor has an inhibiting action on thromboplastin, thereby rendering the activation of prothrombin impossible. This could not, however, be ascertained because of the property of AF to form a complex with prothrombin itself.

It was also found that AF forms a precipitate when added to concentrated solutions of thrombin. It can therefore be suggested that this factor forms a soluble complex with thrombin as well as prothrombin at the very low concentrations usually employed in the 2-stage procedure of Ware and Seegers.

Another interesting observation is the retardation of precipitate formation between AF and prothrombin (at high concentrations) when AF is initially incubated with fibrinogen. How exactly fibrinogen alters the property of AF to form a precipitate with prothrombin is not known. The use of conventional physico-chemical procedures like electrophoresis and light-scattering will no doubt help in elucidating the mechanism of this complicated process.

The results obtained with iodacetate-treated AF are as intriguing as they are interesting. It was observed that on treatment with iodacetate the antithrombin activity of AF was completely destroyed. However, when fresh citrated plasma used as a source of fibrinogen was incubated with iodacetate-treated AF, further addition of active thrombin did not result in the formation of fibrin clot (Table III). This suggests that iodacetate-treated AF may form a complex with fibrinogen thus rendering it refractory to the action of thrombin. This could not be confirmed when a dilute solution of purified fibrinogen (Parke, Davis) was incubated with iodacetate-treated AF (Table III).

The only conclusion that could be drawn from these experiments is that iodacetate-treated AF inhibits the formation of a perfect fibrin clot from plasma, probably by forming a complex with fibrinogen in the plasma and that plasma contains a co-factor which promotes this complex formation.

Summary

A detailed investigation has been carried out on the mode of action of the anticoagulant factor present in the latex of *Carica papaya*.

The dual function of the anticoagulant factor, *viz.*, the inhibition of the conversion of prothrombin to thrombin, and the destruction of thrombin activity has been established.

It has been found that the iodacetate treatment destroys the antithrombin activity of the anticoagulant factor. However, the iodacetate-treated anticoagulant factor inhibits the formation of a perfect fibrin clot from plasma, even in the presence of active thrombin, probably through complex formation with the fibrinogen of the plasma. The possibility of the presence of a co-factor in plasma which promotes this complex formation is also envisaged.

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