STUDIES ON THE AGGLUTINATING AND HEMOLYSING FACTORS CONTAINED IN RICINUS COMMUNIS

II. STUDIES ON THE HEMOLYSING FACTOR

By

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Introduction

The fact that organs of various plants, such as seeds of Ricinus communis, contain a certain substance which agglutinate or hemolyze erythrocytes is well known by several authors (1, 2, 3, 4). On the agglutinating substance many investigations were performed and in general it is considered to be ricin itself. In this institute R. Furihata[5] has obtained the results that the agglutinating substance is different from the hemolyzing substance and that the former is different from globulin or slightly from albumin. But there is no chemical experiment on the hemolyzing substance in Ricinus communis and its chemical nature is entirely unknown, so far as I know.[4] Recently K. Kusunoki[6] has experimented on this problem and obtained the results that the hemolyzing substance can be extracted from Ricinus communis in weak alkaline solution and precipitated in acid reaction. However, in detail the other properties have not yet been made clear. In this experiment I attempted to know the chemical properties of the hemolyzing substance and obtained the following results.

Experimental

For the hemolyzing test 0.5 ml. of the preparation of castor bean is diluted with the physiological saline solution to 2, 4, 8, 16, 32, 64 and 128 times, and to each 0.5 ml. of these diluted solutions 0.5 ml. of 3 per cent suspension of sheep erythrocytes is added. After an incubation for one hour and a half at 37°, the degrees of hemolysis are examined. In recording the

results, cases where no hemolysis occurred are designated by (−), and cases of complete hemolysis by (卌). While for intermediate cases the signs (±), (+) and (廾) are used according to the grade of hemolysis.

I. Preparation of the hemolysing substance from Ricinus communis.

Castor beans were freed from shells and pressed with a pressmashine to remove rough oil (signed as A). To the residue (signed as B) was added physiological saline solution five times the volume at pH. 8.0 and the mixture was shaked for several hours. By filtration the supernatant liquid (signed as C) was separated from the water insoluble portion (signed as D). The solution (C) was acidified to pH. 4.4 with acetic acid and the precipitate, there formed, was collected by filtration. The precipitate formed in acid reaction was signed as E and the supernatant liquid as F. And this precipitate (E) was dissolved again in physiological saline solution at pH. 8.0. These procedures above noted were repeated three times. Finally the precipitate was suspended in a small amount of distilled water and was dialysed in a collodium sack in running water to remove acetic acid. The precipitate which developed at the end of dialysation was collected by centrifugation and dried in vacuum at the temperature not exceeding 40°. The white substance (signed as G) was obtained by this procedure. Also the portions such as E and F were dialysed to remove acetic acid.

The hemolyzing power was tested with every portion obtained as above. In the test of rough oil (A), it was emulsified by addition of physiological saline solution 30 times the volume to its alcoholic solution. After 20 minutes at the room temperature the emulsion was submitted to the hemolyzing test.

The results were as follows.

Table I.
Hemolyzing test with each portions of castor bean.

	Number of sheep	Degree of dilutions times 2	4	8	16	32	64	128
(A)	1 2	— —	— —	— —	— —	— —	— —	— —
(B)	1 2	— —	— —	— —	— —	— —	— —	— —
(C)	1 2	+++ +++	+++ +++	+++ +++	+++ +++	— +	— —	— —
(D)	1 2	— —	— —	— —	— —	— —	— —	— —
(E)	1 2	+++ +++	+++ +++	+++ +++	+++ +++	— —	— —	— —
(F)	1 2	— —	— —	— —	— —	— —	— —	— —
(G)	1 2	+++ +++	+++ +++	+++ +++	+++ +++	++ +++	— +	— —

As can be seen from these results, the hemolyzing substance is contained in the residue in an inactive form, which is obtained by removing the rough oil from castor bean. The hemolyzing substance is soluble in weak alkaline solution and precipitated in acid reaction. The intensity of the hemolyzing power of the precipitate in acid reaction increased by repeating the procedure which removes the impurity from C solution by filtration.

II. Some chemical properties of the hemolyzing substance.

(1) Protein reactions of the white substance

The white substance (G) obtained in the preceding experiment was dissolved at the concentration of one per cent in physiological saline solution.

Protein reactions were tested on this solution and the following results were obtained.

Biuret-, xanthoprotein- and Millon's reactions were all positive, but Hopkins-Cole's reaction remained negative. The precipitate was formed from the solution at the heat of 80°, at saturation with ammonium sulfate and by addition of alcohol, sulfosalicylic acid, mercury chloride, picric acid or of nitric acid.

(2) Effect of heat upon the activity of the hemolyzing substance.

To know whether the activity of the hemolyzing substance is destroyed by heat or not, the one per cent solution of the white substance in physiological saline solution was heated at 100° in the various reactions such as pH 5.6, 7.0 or 8.0 respectively for 30 minutes. After which the each solution was neutralyzed and their hemolyzing powers were tested.

The results were as follows.

Table II.

Hemolyzing test with the white substance heated at 100° in various pH.

	Number of sheep	Degree of dilution times 2	4	8	16	32	64	128
Heated at pH. 5.6	3	+++	+++	+++	+++	+	—	—
	4	+++	+++	+++	+++	++	—	—
Heated at pH. 7.0	3	+++	+++	+++	+++	+++	±	—
	4	+++	+++	+++	+++	+++	—	—
Heated at pH. 3.0	3	+++	+++	+++	+++	+++	—	—
	4	+++	+++	+++	+++	+++	—	—

From the above result we know that the hemolyzing activity of the white substance is stable against the heat especially in neutral and weak alkaline reaction.

(3) Effect of the protease upon the white substance.

From the fact that the hemolyzing substance was stable against the heat, it can be suggested that the hemolyzing substance was other than protein. To confirm that the hemolyzing substance was other than protein, the white substance was digested with proteinase and the effect of it upon the hemolyzing activity was observed.

As proteinase, trypsin and pepsin were used. Trypsin was prepared from pig's pancreas tissue by Willstätter's method and purified until no more glycolytic nor lipolytic action was observed. As pepsin, the preparation of Sankyo was used after purification as in the case of trypsin. The actions of both enzymes against albumin were ascertained before the experiment. One ml. of 2 per cent solution of the white substance was mixed with 1 ml. of 1 per cent solution of trypsin or pepsin at pH. 8.0 or 1.8 respectively and digested at 37° for 3 hours. After which the mixtures were heated at 70° for 30 minutes to inactivate the enzyme and then neutralized. As a control test the enzyme inactivated by heating was used in the same way as above. The hemolyzing power was examined with those digested solutions and the following results were obtained.

Table III.

Hemolyzing test with the white substance treated with proteinase.

	Number of of sheep	Degree of dilution						
		2	times 4	8	16	32	64	128
Digested with trypsin	5 6	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	+ ⧺	— —	— —
Digested with pepsin	5 6	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	— —	— —
Treated with the heated trypsin (as control)	5 6	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	— —	— —
Treated with the heated pepsin (as control)	5 6	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧻	— —	— —

As can be seen from the results, no effect was observed on the hemolyzing power by the treatment with trypsin or pepsin.

(4) Effect of ether upon the activity of the white substance.

I found from the above experiments that the hemolyzing substance was other than protein. In this experiment the white substance was extracted with ether in Soxlet's apparatus completely and it was examined into which fraction, ether soluble or insoluble one, the active substance moved. The ether was evaporated from each fraction at 37°, and from the ether soluble fraction was obtained the sticky matter. With the both fractions the hemolyzing power was tested. The ether insoluble fraction was dissolved in physiological saline solution at the concentration of one per cent. The ether soluble fraction was dissolved in a small amount of alcohol and was emulsified by addition of physiological saline solution 30 times the volume.

The results were indicated in the table IV.

Table IV.

Hemolyzing test with the ether soluble and insoluble fractions of the white substance.

	Number of sheep	Degree of dilution						
		times 2	4	8	16	32	64	128
Ether soluble fraction	7 8	⧻ ⧻	⧻ ⧻	⧻ ⧻	⧻ ⧺	— —	— —	— —
Ether insoluble fraction	7 8	— —	— —	— —	— —	— —	— —	— —

It is made clear from this experiment that the hemolyzing substance is soluble in ether and can be extracted completely in the ether soluble fraction.

(5) Effect of acetone upon the activity of the white substance.

The hemolyzing substance is soluble in ether. Is it also soluble in acetone? In this experiment I treated the white substance with acetone instead of ether. The substance was extracted with acetone completely in Soxlet's apparatus, and with the both fractions, acetone soluble or insoluble one, the hemolyzing power was tested.

The results were follows.

Table V.

Hemolyzing test with the acetone soluble and insoluble fractions of the white substance.

	Number of sheep	Degree of dilution						
		times 2	4	8	16	32	64	128
Acetone soluble fraction	9	⧻	⧻	⧻	⧻	+	—	—
	10	⧻	⧻	⧻	⧻	—	—	—
Acetone insoluble fraction	9	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—

The hemolyzing substance is also soluble in acetone and therefore it is considered that the substance belongs to the lipid other than the phospholipid. I found also by the similar experiment with chloroform instead of acetone that the hemolyzing substance was extracted completely in the chloroform soluble fraction.

(6) Effect of the lipase upon the hemolyzing substance.

It has become clear that the hemolyzing substance is other than protein and probably of lipid nature. So in this experiment I attempted to know whether the activity of the white substance is destroyed by the lipase action or not.

Lipase was prepared from pig's pancreas tissue by Willstätter's procedure and purified carefully. The activity of the lipase was confirmed by the method with Traube's Stalagmometer using Tributylen before this experiment. One ml. of 2 per cent solution of the white substance was mixed with 1 ml. of 1 per cent solution of lipase and digested at 37° for 2 hours at pH 8.0. After which the mixture was heated at 100° for 30 minutes to inactivate the lipase. As a control test the enzyme solution, which was inactivated previously by heating, was used as above. The hemolyzing test was performed on the white substance digested with the lipase.

The result was as follows.

Table VI.

Hemolyzing test with the white substance digested with lipase.

	Number of sheep	Degree of dilution times 2	4	8	16	32	64	128
the white substance digested with lipase	11	⧻	⧻	⧻	⧻	±	—	—
	12	⧻	⧻	⧻	⧻	—	—	—
control test	11	⧻	⧻	⧻	⧻	+	—	—
	12	⧻	⧻	⧻	⧻	±	—	—

As indicated in the table VI, no reduction of the hemolyzing activity can be seen by the digestion with the lipase. It is suggested from this experiment that the hemolyzing substance does not exist in the form of an ester of fatty acid and alcohol.

(7) Nitrogen content and iodine number in the hemolyzing substance.

The light yellow substance was obtained by drying the ether soluble fraction of the white substance. The iodine number and the total nitrogen content of this yellow substance were determined by Hanus's method and micro Kjeldahl's method respectively. It's iodine number was 82.0 and it contained, on dry weight basis, 0.106 per cent nitrogen. Then it was examined what was the effect of hydrogenation on the hemolyzing substance.

The ethereal extract of the white substance was evaporated and the residue was dissolved in hot alcohol at the concentration of 2 per cent. The hydrogen gas, which was purified by bubbling through 2 per cent mercury chloride solution, 2 per cent potassium permanganate solution and 5 per cent sodium sulfate solution, was passed for 2 hours through the alcoholic solution, warmed in the water bath at 40°, of the hemolyzing substance, which was hydrogenated under the existence of the platinum black as a catalyzer. After this procedure, the alcoholic solution was filtered and the filtrate was evaporated to dryness. Thus, the iodine number of the ethereal extract of the white substance decreased to 2.35. With this reduced substance the hemolyzing test was carried out.

Table VII.
Hemolyzing test with the hydrogenated substance.

	Number of sheep	Degree of dilution times 2	4	8	16	32	64	128
hydrogenated substance	13	+	−	−	−	−	−	−
	14	+	−	−	−	−	−	−
control (non hydrogenated substance)	13	+++	+++	+++	+++	+	−	−
	14	+++	+++	+++	+++	−	−	−

We know from this results that the hemolyzing substance loses its activity by hydrogenation almost completely.

Conclusion

The hemolyzing substance contained in Ricinus communis was isolated and tested about its chemical properties.

1) The hemolyzing substance is contained in an inactive form in the residue of castor beans where the rough oil was removed by pressing. The active substance can be extracted from the residue with the physiological saline solution at pH. 8.5 and precipitated from its alkaline solution by acidification.
2) The hemolyzing substance is stable against the heat of 100°, especially in neutral or weak alkaline reaction.
3) The activity of the hemolyzing substance is not reduced by digestion with proteinase.
4) The hemolyzing substance is soluble in ether, acetone or chloroform.
5) The activity of the hemolyzing substance is not reduced by digestion with lipase.
6) The iodine number of the hemolyzing substance obtained by me is 82.0 and by hydrogenation loses its activity. It contains 0.106 per cent of nitrogen.

From these properties I can consider that the hemolyzing substance contained in castor beans is a free unsaturated fatty acid.

I wish to express my sincerest thanks to Prof. S. Fujimura for his kind advice and encouragement in carrying out this experiment.

References

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