Estimation of Toxic and Hemagglutinating Activity of Ricin From Different Varieties of Castor Beans

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Estimation of Toxic and Hemagglutinating Activity from Different Varieties of Castor Beans

by

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The seeds of the castor plant, *Ricinus communis* L., have long been used as a source of castor oil. The press cake obtained after removal of the oil, called castor meal, contains about 50 to 55 percent protein. The highly toxic protein, ricin, and a very powerful allergen present in castor seeds renders the protein-rich castor meal unsuitable for use as an animal feed. The oil contains no toxin or allergen since these components are insoluble in the oil. The utilization of castor meal as a feed depends upon its successful detoxification and deallergenation.

The two substances of extreme physiological potency are in the aqueous extract of the castor bean. Stillmark (13) was the first to isolate the active ricin and Alilaire (1) recognized an allergen sometime later. Considerable interest in both the ricin and the allergen has developed through the years and reviews by Corwin (4), Bolley (2), Das Gupta (5), and Scroggs (11) summarize these contributions. Waller and Negi (15) estimated that commercial castor seeds contained about 1 percent ricin, 0.2 percent dialyzable allergen and 0.1 percent of a mildly toxic alkaloid, ricinine.

This study was undertaken to determine if there was a difference in the quantity or activity of ricin present in different varieties of castor beans. If it were possible to locate a ricin-free variety of castor bean or a variety with a very low ricin content it might then be possible for the plant breeders to produce an entirely new variety of non toxic *Ricinus communis*. The castor meal from such a bean could then be used as an animal feed without going through a detoxification process.

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Knapp (9) tested the hemagglutinating ability of 1760 *Ricinus communis* plants and found that all were active but that differences in the ricin content seemed to exist; however, it is not known if different varietes were included in his study.

Until very recently the toxic and hemagglutinating properties of ricin have been attributed to one protein. The method used by industrial companies to measure the toxicity of castor meal is based only on a hemagglutination test (2). Cooper *et al.* (3) have recently reported that this is not a satisfactory way to assay the toxic activity of commercial castor bean pomace samples. Since a measure of only the hemaggluti-nating activity of ricin is not entirely definitive the method used in this study included a toxicity test also. Both tests were used to measure differences in hemagglutinating activity and toxicity in different varieties of castor beans.

Materials and Methods

A. Photometric method for estimating the hemagglutinating activity of ricin

Selection of a hemagglutination method: A photometric procedure was developed to establish a rapid, accurate and sensitive method of assaying the hemagglutinating activity of ricin. The objective was to evaluate the activity of a crude ricin preparation by determining the amount of protein required for 25 percent hemagglutination of a washed suspension of readily available erythrocytes. The present approach was based on a method proposed by Liener (10) for the detection of the soya bean hemagglutnin and on a ricin hemmagglutination test developed by Gardner *et al.* (6).

Photometric (turbidimetric) measurements were made in an Evelyn Photometric colorimeter. All readings were made at a wave length of 720 mμ which was selected to reduce the absorption of light attributed to hemolysis. Preliminary experiments were conducted to determine the most suitable type of blood; what anticoagulant was most desirable; the incubation time required; and the need of shaking during incubation. Blood obtained from three different sources was evaluated: (a) human blood, "type O," rejected due to aging, obtained from the Stillwater Hospital blood bank; (b) chicken blood; and (c) rabbit blood. Lithium citrate, heparin and Alservers solution (8) modified to include Phenergan[1] were tried as anti coagulants.

[1]Trade name for N-(2'-dimethylamino-2'-methyl) ethylphenothiazine, Wyeth, Inc. Philadelphia, Pa.

Freshly drawn rabbit blood and lithium citrate provided the best combination. Good reproducibility was obtained from an incubation period of two hours at 25° C. After incubation most of the cells had settled out. To make accurate turbidimetric measurements the cells or clumps of cells had to remain in suspension. Consequently, it was necessary to resuspend them without breaking the clumps. Mechanical methods of shaking were unsatisfactory. By gently inverting the stoppered tubes four times by hand through a 180° angle and then letting the tubes stand for five minutes, reproducable percentage transmittance readings were obtained. Care was taken not to re-agitate the settled cells before the readings were taken. Duplicate readings checked within two percent.

Preparation of red blood cells: Whole rabbit blood was drawn aseptically by heart puncture. One ml of 11.4 percent lithium citrate was used for every 15 ml of whole blood. Citrated blood was transferred to a graduated centrifuge tube and centrifuged at 1000 to 1200 rpm for three minutes. The supernatant was pipeted off the red blood corpuscles (RBC) and physiological saline solution (0.85 percent NaCl in glass-distilled water) was added in the proportion of one volume of packed blood cells to one volume of saline. The cells were redispersed and centrifuged at 1000 to 1200 rpm for three minutes, and the supernatant removed.

This washing step was repeated four to five times until a clear supernatant was obtained. A trace of hemolysis was noticeable occasionally. Sufficient saline was added to the packed cells in the centrifuge tube to give a final cell concentration of four percent. Red blood cells so prepared could be preserved at 4° C for one day without any noticeable change occurring in the hemagglutination reaction.

Crude ricin solution (CRS) was prepared as follows: Four to five decorticated seeds (about 1 gm) were crushed in a mortar. One gm of the decorticated and crushed seeds was wrapped in filter paper and extracted with ether for 16 hours in a Goldfisch extractor to remove the oil. From 73 to 76 percent of oil was extracted with this treatment. The castor meal was air-dried for 16 hours to remove the ether. Special handling was necessary to prevent the spreading of the allergen from the dry, powdery castor meal into the laboratory atmosphere. It was then extracted with 50 ml. of 0.85 percent saline in a micro Waring blender for three minutes at 4° C. The solution was filtered through Whatman No. 42 paper. This filtrate was stored at 4° C for future use and was labeled crude ricin solution (CRS). The pH of CRS was 6.8 to 7.0. The protein (N x 6.25) content of CRS was determined by a modified Kjeldahl method (8). The varieties of castor beans used, the location and year of growth are shown in Table I.

TABLE I — Comparison of Hemagglutination and Toxicity Assay of Castor Seed Varieties

1	2	3	4	5	6	7
Variety	Place Grown	Year Grown	mg Protein/ml in CRS	γ Prot. Req. for 25% Hem. a	LD γ Prot. / 100 gm Body Wt. b	Ratio a:b
Cimarron	Perkins, Okla.	1958	1.55	55.0	4.4	12.5
Cimarron	Chickasha, Okla.	1952	1.86	48.8	5.1	9.6
US 74	Blackwell Lake, Okla.	1951	1.23	78.9[1]	3.8	20.5
N224A-1-6-4-3	Paradise, Okla.	1955	1.48	49.9	4.0	12.5
Custer	Perkins, Okla.	1958	1.73	52.9	2.2	24.2
USDA 65	Perkins, Okla.	1958	1.54	44.2	2.0	21.7
US 3/384-8	Perkins, Okla.	1958	1.28	90.9[1]	3.6	25.2
Baker 296	Perkins, Okla.	1958	1.37	45.4	2.1	21.2
Dawn	Perkins, Okla.	1958	1.61	35.3		
Interspread Dawn	Perkins, Okla.	1958	2.03	46.6		
OK 8/201-B	Stillwater, Okla.	1958	1.75	50.0		
OK 8/205-B	Stillwater, Okla.	1958	1.31	36.6		
Cimarron Hybrid	Perkins, Okla.	1958	1.67	38.5		
US-51 Hybrid	Perkins, Okla.	1958	1.69	43.7		
Baker Hybrid 45	Perkins, Okla.	1958	1.73	50.4		
Pacific Oilseeds Hybrid 6	Perkins, Okla.	1958	1.84	49.0		

[1]Values obtained by extrapolation of curves similar to those shown in Figure 1.

Procedure used for measuring the hemagglutinating activity of ricin: Duplicate experiments were carried out for each variety of castor beans. Seven serial dilutions of CRS and eight tubes were used in each set of dilutions. In the first tube 0.2 ml of CRS and 19.8 ml of saline were mixed. From this mixture 5.0 ml were transferred into a second tube, 3.5 ml into a third tube, 5.0 ml into a fourth tube, 2.0 ml into a fifth tube, 1.5 ml into a sixth tube, and 1.0 ml into an eighth tube. The required volume of saline was added in tubes 3, 5, 6, and 8 to bring the volume up to 5.0 ml in each tube. Five ml of saline was added to tubes 4 and 7. Tube 4 was mixed thoroughly, then 5.0 ml of this mixture was withdrawn and pipetted into tube 7. Tube 7 was shaken to mix the diluted CRS and saline, and 5.0 ml of this mixture was withdrawn and discarded. Thus, the final dilutions of CRS in the seven tubes (2-8) were 1:100, 1:143, 1:200, 1:250, 1:333, 1:400, and 1:500 respectively, all tubes having exactly 5.0 ml in volume.

The four percent RBC suspension was diluted with saline to 30 percent transmittance (optical density = 0.52) at 720 mμ. The RBC suspension was stirred continuously with a magnetic stirring bar. To each of the seven tubes containing diluted CRS, 5.0 ml of the RBC suspension (30 percent T) was mixed and incubated for two hours at 25° C. The transmittance of each tube was read after shaking and letting the tubes stand for five minutes. Smooth curves were obtained by plotting percent transmittance from each tube against the reciprocal of the dilution. Figure 1 shows a typical set of such curves.

In the control tube, prepared with 5.0 ml RBC suspension plus 5.0 ml saline, percent T = 52 ± 2 (O. D. = .284) was constant for the two-hour incubation period. Very light settling of the RBC occurred during this period in the control. It is evident that increasing concentration of CRS would cause more clumping of the suspended red blood cells. After the incubation period, therefore, the concentration of cells remaining in suspension should bear a nearly direct relationship to the quantity of ricin present in the diluted CRS. To measure the point at which 25 percent of the red blood cells were agglutinated by ricin, the end point was taken as 61 percent transmittance (optical density = 0.213), and this value was obtained from the graphs drawn for each CRS sample. Quantitative estimation of ricin present at the dilution corresponding to 61 percent T was made. The two main variables governing the extent of hemagglutination were; (a) quantity of ricin present and, (b) activity or potency of the ricin present. The dilution range of 100 to 500 was used because, below a dilution of 100, the hemagglutination was so strong that nearly all the red cells had settled leaving only the clear supernatant in the path of the light beam of the photometer.

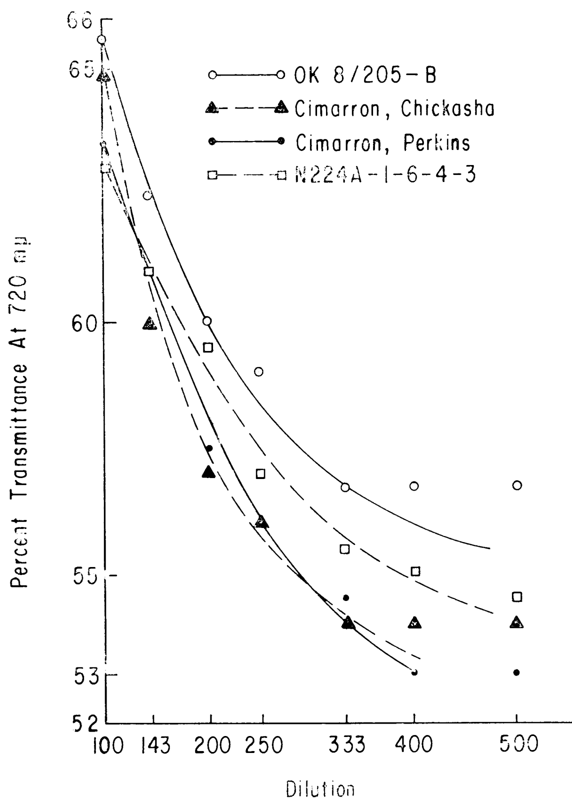

Figure 1. Hemagglutinating activity of selected varieties of castor seeds.

B. Evaluation of toxicity of CRS

Male rats of the Sprague-Dawley strain, about 100-150 gms, were injected subcutaneously in the abdominal region with graded doses of CRS. The lethal dose (LD) within 48 hours was found. These values given as the LD should not be confused with LD_{50} values, i.e. the dose which would kill 50 percent of the animals tested.

Results and Discussion

The figures in Table I, column 6, indicate the micrograms (γ) of protein per 100 gm body weight for the LD. In column 5 the γ of protein required for 25 percent hemagglutination is shown. These values were obtained from curves such as those shown in Figure 1. In the last column,

the ration of γ of protein causing 25 percent hemagglutination to the γ of protein required for LD is shown. Of the 16 varieties tested for hemagglutinating potency, variety Dawn required 35 γ protein, the least quantity for 25 percent hemagglutination, hence it was considered to be the most active. Variety United States Department of Agriculture No. 65 was similarly the most potent among the eight varieties tested for toxicity and hemagglutinating potency. This variety was also the most toxic since 2 γ, the least amount of protein, was required to kill a 100 gm rat in 48 hours. The ratios in column 7 came under at least two groups; (1) a:b = approximately 10, and (2) a:b = above 20.

All of the 16 varieties tested possessed hemagglutinating activity and all eight varieties tested were toxic. The absence of a consistent relation between hemagglutination and toxicity made it impossible to evaluate toxicty based on hemagglutinating activity. The ratios of the amount of protein required for hemagglutinating activity and toxicity of 10:1 and 20:1 might be interpreted to indicate that the active groups in ricin causing these two effects were not produced by castor plants in the same ratio. Perhaps two proteins produced in different quantities by castor plants are responsible for these two unrelated effects. Recent studies by Takahashi *et al.* (14) and Ishiguro *et al.* (7) have shown that crystalline ricin from *Ricinus communis* L. and *Ricinus sanguineus* L. can be separated into proteins possessing only toxic or hemagglutinating activity.

Pathological examinations on rats which had been injected with CRS were kindly performed by Dr. J. B. Corcoran of the Pathology Department, College of Veterinary Medicine, Oklahoma State University. Marked degenerative changes in the mesenteric lymph nodes, spleen, mucosa of the small intestine and liver were found. These findings agreed with those previously reported (12). The pathological studies are to be described more fully in a separate publication (16) which also describes some studies on the biochemical action of ricin.

The method for measuring ricin activity can be readily adapted to fit in with the present pollination techniques. According to Zimmerman (17) up to 10,000 genotypes might need to be examined to determine if significant variation in ricin content really exists in castor. For rapid screening the hemagglutinating assay would be the quickest way to screen a large number of samples at the lowest cost. Random toxicity assays would be adequate except for those samples showing low hemagglutinating activity and then it would be advisable to determine the toxicity of each one.

Summary

Three to five castor beans were extracted with physiological saline solution. The total protein in the solution was determined and appropriate dilutions made for toxicity and hemagglutinating activity measurements. Toxicity was evaluated by determining the minimum lethal dosage required to kill a rat within 48 hours. Hemagglutinating activity was estimated by determining the amount of protein required to agglutinate a 4 percent rabbit red blood corpuscles suspension.

A study of 16 varieties of castor seeds showed that differences between varieties existed but all samples showed both toxic and hemagglutinating activity. The ratio of toxicity to hemagglutinating activity was not constant which suggested that these two properties cannot be attributed to a single protein and that at least two proteins, produced in different quantities by castor plants, are required to account for such variation. The tests for evaluating toxicity and hemagglutinating activity are simple, rapid and inexpensive.

"Precautionary Note"

The mechanism by which ricin produces its toxic action is not known. There is no effective antidote for the action of ricin. Consequently, the best way of protection from ricin poisoning is to maintain a supply of antiserum in the laboratory at all times. This can be prepared by injecting a goat with CRS at a concentration which is about 1/4 the amount required to kill a rat, withdrawing some blood 96 hours later and removing the red cells by centrifugation. The serum should then be sterilized by filtering through a sterile filter. The serum can then be stored at 4° C. It is advisable to have a medical doctor available as a consultant who could administer the antiserum.

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