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YUN-TAO SUN

COLLEGE OF MEDICINE, NATIONAL TAIWAN UNIVERSITY
TAIWAN, CHINA

A STUDY OF ANTIGENIC ACTIVITY OF SOME PLANT TOXALEMINS

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A STUDY OF ANTIGENIC ACTIVITY OF SOME PLANT TOXALBUMINS

Some plant toxalbumins, such as ricin from Ricinus Communis (Euphorbiacese) and abrin from Abrus precatorius (Leguminosae) are extremely toxic. Ricin, the highly toxic, hemagglutinating protein is of great interest in medicine. For such a toxalbumin when injected in small doses may act as an antigen and produce in the body an antitoxin analogue to that produced against bacteria or venen. The isolation of ricin was first realized by Stillmark[1]. Earlier methods of preparation were carried out before the development of modern physicochemical and immunological methods for characterizing proteins and data are therefore lacking on the purity and homogeneity of the products obtained.

In recent years, the different toxic fractions of ricin have been prepared by Kabat, Heidelberg and Sezer[2] by fractional precipitation with sodium sulfate (1947). Kunits and McDonald[3] have obtained the most toxic fraction by first precipitation with sodium sulfate and then adjusted the isoelectric point of toxalbumin solution at pH 5.2-5.5 (1948). Moullet stated that the highly toxic fraction of ricin was prepared by precipitation with half saturation of ammonium sulfate (1951). The isolated fractions of ricin prepared by previous authors were not pure and homogenous substances.

In the present report a summary is given of a portion of a study on the extraction and purification of ricin. The nature, toxicity and antigenic activity of ricin are also studied.

EXPERIMENTAL

Extraction and Purification of Ricin

1.380 Gm. of castor bean were ground and macerated with 1,400 ml. of ether at room temperature for 12 hours. Pressed out the solvent. Repeated the maceration as the previous time. The castor bean powder was then air dried. The castor meal was extracted twice with 1.5 L. of alcohol at room temperature. Removed the alcohol by pressing and dried the bean powder under reduced pressure. The defatted matter, weighing 410 Gm., was then macerated twice with 2 L. of 10% sodium chloride solution at 3-4°C for 24 hours. By filtration, the filtrate was freed from sodium chloride by dialysis. The non-toxic globulin precipitated was centrifuged off. The proteins of the supernatant liquid were precipitated by saturation with ammonium sulfate, and after 15 hours at about 10°C, centrifuged the mixture. The precipitate was dissolved in water and freed of the insoluble matter by centrifuging. Repeat the precipitation and dissolution.

To the clear liquid measured 90 ml., 45 ml. of saturated ammonium sulfate solution was added (1/3 saturation) and kept the mixture at 3-4°C for 24 hours. The forming precipitate was centrifuged off. To the liquid of a volume of 150 ml., 44 ml. more of saturated ammonium sulfate solution was then added (half saturation) and kept the mixture at about 10°C for 24 hours. The precipitate separated from centrifuging was dissolved in a small amount of water (the supernatant liquid from centrifuging was reserved for further treatment). Repeated the precipitation and dissolution and finally the solution was dialysed. The clear solution was freeze-dried in a Stokes freeze-dryer, model 2003 F2 (equipped with freon as freezing agent, drying under high vacuum at 300 u for 16 hours). The white porous powder, Ricin fraction I (ricin I), 904 mg., was obtained (yield: 0.066%).

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The above reserved supernatant liquid from half saturation of ammonium sulfate was saturated again with ammonium sulfate. Kept the mixture at 10°C for 24 hours. Collect the precipitate after centrifuging. The precipitate was dissolved in a small amount of water and made the solution salt-free by dialysis. The liquid was freeze-dried in a Stokke's freeze-dryer, giving 2.95 g. of a white, porous powder, Ricin fraction II (Ricin II, yield: 0.216%).

Properties of Ricin Fractions

Determination of the sedimentation constant of ricin fractions was carried out by using 1% solution of ricin fractions in 0.2 M sodium phosphate in an ultracentrifuge. The data of determination were as follows:

<table>
<thead>
<tr>
<th>Ricin I</th>
<th>$S_{20} = 4.78 \times 10^{-13}$</th>
<th>75,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricin II</td>
<td>$S_{20} = 5.30 \times 10^{-13}$</td>
<td>85,000</td>
</tr>
</tbody>
</table>

Ricin II was found to have a molecular weight of about 85,000. The sedimentation constant of Ricin I was slightly lower, leading to a molecular weight of 75,000, a value not considered significantly different from that of Ricin II, since the precise temperature control was not possible during the measurement of sedimentation.

Ricin I showed a lower optical rotation ($-28^\circ$) than did the Ricin II ($-32^\circ$).

Table I Properties of Ricin Fractions

<table>
<thead>
<tr>
<th></th>
<th>Ricin I</th>
<th>Ricin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ degrees</td>
<td>$-28^\circ$</td>
<td>$-32^\circ$</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>75,000</td>
<td>85,000</td>
</tr>
</tbody>
</table>

The Ricin I and II were determined qualitatively by paper chromatography. A descending method, on Whatman paper No. 1 and a solvent of citrate buffer with pH 6.0 (0.75 ml. of 0.02 M sodium citrate, 9.25 ml. of 2 N HCl, and 50 g. of sodium chloride per liter) were employed. The temperature was kept at 20°C. Using ninhydrin as the spraying agent, it showed that Ricin I gave two nearly spots, and Ricin II, three spots.

Toxicity Test of Ricin Fractions

The toxicity test of ricin fractions was determined by Intraperitoneal injection of 0.5 ml. of the ricin solution in serial concentrations. Three mice weighing 20 g. were used as a lot, being injected with each concentration of the ricin solution. Death or survival for four days was used as the end point. A dose of 1 mg. of Ricin I had no significant toxic effect on mice weighing 20 g. when perorally administered but was lethal when administered intraperitoneally.

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Table II: Toxicity Test of Ricin Fractions

<table>
<thead>
<tr>
<th>Quantity of ricin fractions injected (mcg.)</th>
<th>Intraperitoneal toxicity for mice</th>
<th>Ricin I</th>
<th>Ricin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>3/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>3/3&lt;60hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>3/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>3/3&lt;96hr.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzymatic Hydrolysis of Ricin Fractions

The digestion of Ricin I by pepsin at pH 4 or by trypsin at 7.4 was found to take place slowly. After 3 days, the digested ricin gave a slight decline in its toxicity. A 1 mcg. dose of the digested Ricin I by intraperitoneal injection caused death of the mice in 72 - 96 hours. When a prolonged enzymatic digestion was made for 2 weeks, it broke down about 35 - 48% of the low molecular products.

Table III: Two-weeks Enzymatic Digestion of Ricin I

<table>
<thead>
<tr>
<th>Precipitable by Trichloroacetic acid, %</th>
<th>Control Digest</th>
<th>Control Digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichloroacetic acid, %</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>Half saturation with ammonium sulfate, %</td>
<td>100</td>
<td>52</td>
</tr>
</tbody>
</table>

Immuno-chemical Properties of Ricin Fractions

The antitoxic serum to ricin I was prepared by immunization of rabbits with a formalinized toxoid prepared as follows: A solution containing 5 mg. of Ricin I per ml. buffered at 7.4 with 0.02 M sodium phosphate and 0.15 M sodium chloride and with 0.5% formalin was kept at 37°C for 5 days (in some instances 5% formalin was used). This procedure resulted in about a 100 to a 1000 fold reduction in toxicity when 0.5% formalin was used, and about 1000-fold reduction in toxicity with 5% formalin. Marked loss of antigenicity occurred with formalin at pH 8.5 and above.
Because of the decrease of toxicity of the ricin toxoid and the extreme susceptibility of the rabbit to ricin, it was found necessary to give each rabbit subcutaneous injections of 25, 50 and 50 mcg. of toxoid at 5 day intervals to induce some immunity before intravenous injections were started. Each rabbit then received 2-4 intravenous injections weekly for 4 weeks, as follows: two injections of 0.1 mg., two of 0.5 mg., four of 0.5 mg., four of 1.5 mg., and four of 5.0 mg. of ricin toxoid. The animals became so resistant to the toxic effects of ricin that immunization could be continued with equal doses of an alum precipitated undetoxified ricin. Rabbits were bled 5 days after the injection. The effect of antitoxic serum has been shown by neutralising the toxic effect of ricin on intraperitoneal injection into mice.

**DISCUSSION**

The defatted castor meal firstly extracted with 10% sodium chloride solution, followed by fractional precipitation with ammonium sulfate (1/3, 1/2 and full saturation), the highly toxic ricin fractions I and II were obtained. The qualitative determination of ricin fractions by paper chromatographic method showed that Ricin I gave two nearby spots, and Ricin II, three. It seems that Ricin I and II are still not pure, homogeneous substances, same as those ricin fractions prepared by Kabat, Heidelberg and Boser, Knits and McDonald as well as Houle's etc. An attempt was made for the purification of Ricin I & II by using ion-exchange resin column chromatography. No favorable results obtained.

Ricin I & II are different by their molecular weight of 75,000-85,000. Ricin I showed a lower rotation (-28') than did the Ricin II (-32'). As to the toxicity of ricin, 1 mg. dose of Ricin I killed a mouse (20 Gm.) by intraperitoneal injection after an interval of 4 days, while in the case of Ricin II, a dose of 5 mg. gave the same lethal effect. It is interest to notice that a dose of 1 mg. of Ricin I had no significant toxic effect on mice weighing 20 Gm. when perorally administered but was lethal when administered intraperitoneally. It seems that the ricin undergo hydrolysis and lose part of its toxicity in the gastro-intestinal tract of mouse.

In the enzymatic hydrolysis of ricin fractions, the digestion of Ricin I by pepsin at pH 4 or by trypsin at 7.4 was found to take place slowly. Action for 3 days, the digested Ricin I gave a slight decline in its toxicity. It has been shown that ricin was somewhat resistant to the proteolytic enzymes. It is somewhat contradictory to the phenomenon that the ricin I had no significant toxic effect in mouse when administered perorally. A prolonged enzymatic digestion for 2 weeks, it broke down about 39 - 43% of the low molecular products.

**ACKNOWLEDGMENT**

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REFERENCES


DISTRIBUTION LIST

Director
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Director
Armed Forces Institute of Pathology
Walter Reed Army Medical Center
Washington 12, D. C.

Commanding Officer
US Army Tropical Research Medical Laboratory
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Dr. Mary H. Aldridge
The American University
Massachusetts & Nebraska Avenues, N. W.
Washington, D. C.

Dr. Alf S. Alving
University of Chicago
Department of Medicine
950 E. 59th Street
Chicago 37, Illinois

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Silliman University
Mission Hospital
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University of Maryland
College Park, Maryland

Dr. Paul E. Carson
University of Chicago
Department of Medicine
940 E. 59th Street
Chicago 37, Illinois

Dr. D. H. Connor
Makere College
The University College of East Africa
P. O. Box 262
Kampala, Uganda

Professor J. D. Duguid
Institute for Medical Research
Kuala Lumpur, Malaya

Dr. Lorenzo Calindo
University of Puerto Rico
San Juan, Puerto Rico

Dr. Camlong Harinasuta
The Bangkok School of Tropical Medicine
Rajvithi Road, Phayathai
Bangkok, Thailand

Dr. H. F. Hsu
State University of Iowa
Iowa City, Iowa

Dr. Hongkol Kruatrachue
The Bangkok School of Tropical Medicine
Rajvithi Road, Phayathai
Bangkok, Thailand

Dr. Mary Joyce Marples
Department of Microbiology
Medical School
University of Otago
P. O. Box 913
Dunedin, New Zealand

Dr. Edward P. Munford
Palo Alto Medical Research Foundation
380 Bryant Street
Palo Alto, California

Dr. William P. Murphy, Jr.
Cordis Corporation
241 N. E. 36th Street
Miami 37, Florida

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University of Puerto Rico
San Juan, Puerto Rico
Dr. Leo Rane
University of Miami
Coral Gables, Florida

Dr. Swaja Vajraothira
The Bangkok School of Tropical Medicine
Rajavithi Road, Phayathai
Bangkok, Thailand

Dr. Robert Charles Wood
Department of Microbiology
The George Washington University
Washington 5, D. C.

Dr. Wen-Hsiun Huang
College of Medicine
National Taiwan University
Taipei, Taiwan

Professor O. K. Khaw
National Defense Medical Center
Taipei, Taiwan

Dr. Tongchai Papasaranthron
Faculty of Public Health
University of Medical Sciences
Bangkok, Thailand