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EFFECT OF RIBOSOME-INACTIVATING PROTEIN FROM CASTOR BEAN ON TOBACCO MOSAIC TOBAMOVIRUS

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ABSTRACT:

Ribosome-inactivating proteins (RIPs) are homogenous family of plant proteins. It is of special interest due to its unique activity. All RIPs possess a highly specific rRNA N-glycosidase activity and capable of catalytically inactivating ribosome and so inhibit the protein biosynthesis. The ricin, a RIP, was extracted and purified from castor bean (*Ricinus communis*) seeds and its effect on tobacco mosaic virus (TMV) infectivity was studied. The tobacco plant cells inoculated with ricin-TMV, under light microscope, a reduced number of both of crystalline and amorphous inclusions induced by TMV was observed in the cytoplasm of inoculated cells. Electron micrographs of inoculated leaves showed the induction of apoptotic body formation in the ricin treated cells, which is the indication of apoptosis.

INTRODUCTION:

Ribosome inactivating roteins (RIPs) are homogenous family of plant protein, which possess a highly specific rRNA N-glycosidase activity and capable of depurinate, catalytically inactivating ribosomes and stop protein synthesis inside the cells (Stripe *et al*., 1992). Based on the molecular structure of RIPs, they are subdivided into three types. Type 1 consists of a single catalytically active subunit, whereas Type 2 builts up of two structurally and functionally different subunits (Endo *et al*., 1987). In contrast to the Type 1 RIPs, which are not toxic, some but not all Type 2 RIPs are potent toxic agents (Stripe *et al*., 1992). Type III RIPs have a single chain but are distinctly different from both type I and II RIPs in their sequence. Ricin is a Type 2 RIPs, isolated from the seeds of castor bean (*Ricinus communis*) (Endo and Tsurugi, 1988, Barieri *et al*., 1997).

The natural roles of RIPs have been proposed in an attempt to understand the function of RIPs in nature. First, defensive roles against plant viruses by arresting protein synthesis, cell death and prevention of viral replication and spread (Barieri *et al*., 1992). Also, RIPs found to inhibit the frame shifting of the ribosomes and so can inhibit the virus proteins expression (Tumer *et. al*., 1998). According to the unique property of the RIPs, they have different application in both agricultural and medical fields. The applications in agriculture include the screening for their actions on viral infection (Chen *et al.,* 1991), insect

attack and transformation of plant with the RIPs for viral resistance (Lodge *et al*., 1993). The expression of RIPs in bacteria as recombinant proteins is complicated because of their cellular toxicity. Example for successful expression of RIPs in bacteria is the expression of ME1, a ribosome-inactivating protein from *Mirabilis expansa* (Vepachedu *et al*., 2005)

The aim of this study was to focus on the partial purification of ricin from the castor bean and examining its possible use as plant protecting protein against tobacco mosaic tobamovirus (TMV) infection.

MATERIALS AND METHODS:

Ricin extraction from castor bean: The crude extract of ricin was prepared according to the method of (Stripe *et. al*., 1983) modified by Ferreras *et. al*. (1993). Briefly, a weight of 100 g of castor bean seeds were ground into a fine powder in liquid nitrogen. The powder was homogenized in 500 ml of 5 mM sodium phosphate buffer (SPB), pH 7.2. The homogenate was stirred overnight at 4°C, filtrated through double layer of cheese clothes and centrifuged at 13,000 rpm at 4°C for 30 min by using GSA rotor (Beckman Instr., USA). The clear supernatant was centrifuged at 28,000 rpm by using SW28 rotor (Beckman Instr., Palo USA) at 4°C for 30 min. The final supernatant was kept at -80°C until used as a source of the crude extract of ricin. Crude extract was then applied to Q-sepharose column. The column was previously equilibrated with 5mM sodium phosphate buffer (SPB), pH 7.2. The crude extract (30 mg) was applied to the column and unbound proteins washed out by 6 ml of the 5 mM SPB. Ricin was eluted by stepwise elution by applying 0.1, 0.3 and 0.5 M NaCl in 5mM SPB, pH7.2. Ricin activity in different purification steps was detected by using *in vivo* SP6/T7 tran-scription /translation system (Endo *et al.*, 1987). Also, the β-fragment due to rRNA *N*-glycosidase activity was detected after treatment of rabbit ribosome with the purified ricin as a specific activity of all RIPs according to method of Endo and Tsurugi (1988).

Antagonistic activity of ricin on TMV infection: A number of eight concen-trations of ricin i.e., 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μg/ml were prepared using sterile distilled water (dH_2O). To each concentration, an equal volume of crude infectious sap prepared from TMV-infected tobacco leaves (Allam *et al*. 2000) was added. After one hour incubation at room temperature (RT), a volume of 1.0ml of the mixtures were separately rubbed on five half leaves of *Nicotiana glutinosa* as a local lesions indicator host for TMV. In case of negative control, half leaves of the same indicator host were rubbed with pure extraction buffer. Furthermore, un-inoculated leaves were left as a negative control. As a positive control, the crude infectious sap without any additions was used. All inoculated plants as well as controls were kept under a controlled greenhouse at 28±2°C for 25 days for developing the characteristic symptoms of TMV. The experiment was carried out in triplets.

Effect of ricin on systematically TMV-infected tobacco plants: In this experiment, the cytological changes as

well as the presence of inclusion bodies (IB) in TMV-infected tobacco (*N. tabacum* cv. Samson) plant(s) treated with and without ricin were determined by using both of light and electron microscopy. To reach such goal, the crude infectious sap was mixed with 2.0μg/ml ricin and left for one hour at RT, and the leaves of the tobacco growing plants were then inoculated as mentioned before in triplets. Within twenty five days, the presence of IB was detected every five days. According to the method given by Allam *et al.* (2000), in case of amourphous inclusions, the stripes were treated with 0.5% Triton X-100 for 5 min, followed by staining with a mixture of 0.5% methyl green and pyronine-Y (0.5% MGP-Y) for 30 min and then washed by tap water and subjected to light microscopy (Zeiss KL1500) While, the crystal inclusions were directly examined by the same light microscope in water. Moreover, ultrathin sections from the same treated leaves (inoculated with TMV or TMV-ricin mixture) after 7, 14 and 21 days were prepared as described by Soweha *et al.* (1992). Negative and positive controls were similarly used as mentioned above. The grids containing the gold sections were subjected to staining and examination by electron microscope using Joel EM906 transmission electron microscope at Electron Microscope Unit (Ain Shams Specialized Hospital, Cairo, Egypt).

RESULTS:

In this study, ricin was successfully extracted and purified from castor bean and its activity during the purification steps as well as β-fragment were detected (unpublished data)

Effect of ricin on TMV: The degree of inhibiting activity of the purified ricin was determined by detecting the induction of IB (i.e. crystal and amorphous) inclusions in tobacco leaves. Data in Table-1 revealed that the absence of IB of TMV in the cells of ricin-TMV-treated leaves of *N. tabacum* cv. Samson, up to 25 days from mixture inoculation.

In case of TMV-inoculated leaves (as a positive control),IB were found 10 days post virus inoculation in the cytoplasm of virus-infected cells and till the 21 day while, the largest number of these increases was recorded after 15 days from virus inoculation. It was also noted that the number of inclusions began to decrease 21 days post inoculation (Fig.1A). No inclusions were appeared in the three negative controls (healthy, buffer- treated leaves and ricin-treated leaves. Also, no crystalline or amorphous inclusions were found in the plants treated with TMV-ricin (Fig-1B).

Table (1): Effect of ricin on the presence of inclusion bodies induced by TMV in tobacco treated leaves.

Treatments	Crystal inclusions					Amorphous inclusions				
	Days post inoculation									
	5	10	15	21	25	5	10	15	21	25
Healthy Untreated	-	-	-	-	-	-	-	-	-	-
Buffer	-	-	-	-	-	-	-	-	-	-
Ricin	-	-	-	-	-	-	-	-	-	-
TMV	-	+	++	+	-	-	+	++	+	-
TMV-ricin	-	-	-	-	-	-	-	-	-	-

- : No inclusion in the field, + : 5-10 inclusions per microscopic field. ++ : 10-20 inclusions per microscopic field.

Figure 1. Induction of amorphous inclusions (AI) stained with MGP-Y in TMV-infected tobacco leaves (A). Detection of AI in ricin-TMV treated tobacco leaves. Note: no inclusions were appeared (B).

Electron microscopy of the plant leaves gave some features about the interaction between the ricin and the TMV inside the cells. Healthy plants as a control (Fig. 2) showed normal shape of the chloroplast, mitochondria and nucleus. Results in Figure 3 and 4 showed the infected cells with TMV after inoculation times of 7 and 14 days. Results showed that viral particles were increased inside the infected cells by time. Parallel to that severe damage in some organelles of the cells was found. Chloroplast tended to be spherical in shape, breakdown of bounding membrane and granulation of stroma. Nucleus always lay close to chloroplast and apart from the cell membrane. Mitochondria showed disrupting in their ultrastructure. Their cristae and bounding membrane were disintegrated. Crystal containing micro-bodies seems to be increased in number. However, when ricin mixed with the TMV, less numbers of viral particles were found in cytoplasm of the cells. The general damages in the cells were less and delayed for longer times when compared to the damages resulted from the rubbing with TMV alone, at corresponding time interval, as shown in Figure 5.

Twenty one days post ricin-TMV treatment, the cells started to show the

characteristic features of apoptosis or programmed cell death. This feature was induced by the presence of ricin. Results in Figure 6 showed the bubbling in the membranes and starting of formation of engulfing vacuoles.

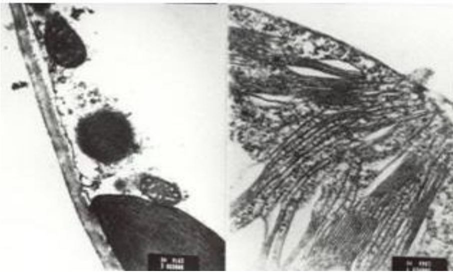


Figure 2. Ultastructure of healthy normal tobacco cells. All organelles appear in normal shapes and sizes**.**

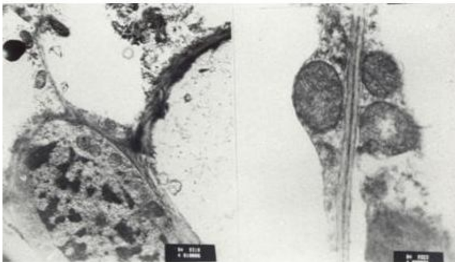
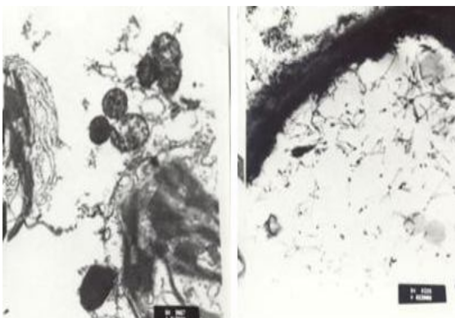


Figure 3. Ultastructure of TMV-infected tobacco cells, 7 days post treatment. All organelles appear to be affected by the viral infection. Note: degenerated nucleus (Left) and mitochondria (Right).



tobacco cells, 14 days post treatment. All organelles appear to be affected by the viral infection. Virus particles appeared inside the cytoplasm and chloroplast. The chloroplast started to be round in shape with torn membranes and burst of vacuoles.

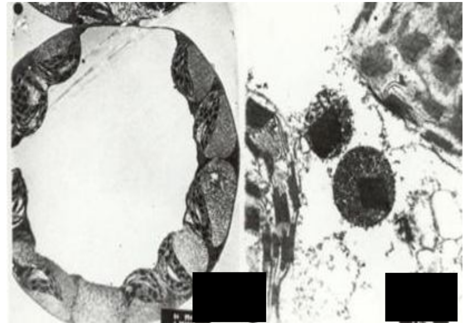


Figure 5. Ultastructure of ricin-TMV treated tobacco cells (14 days post treatment). All organelles appear to be affected by the viral infection but to less degree in comparison to that treated by TMV alone. Virus particles appeared inside the chloroplasts. However, all organelles, in general, still keep its shapes. A: after 7 days, B: after 14 days of inoculation

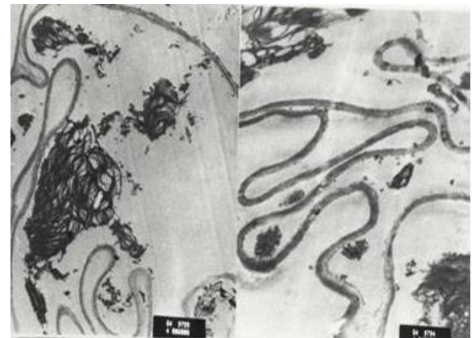


Figure 6. Ultastructure of ricin-TMV treated tobacco cells (21 days post treatment). Note: induction of apoptosis by ricin as indicated by bubbling in the membranes and starting of formation of engulfing vacuoles.

DISCUSSION:

Ricin is a potent RIPs and considered as one of the very toxic protein that produced in *Ricinus communis* (Stripe *et al.*, 1992). Due to its high toxicity the partial purified ricin was used here to study its effect on the viral infection in tobacco plant. Partial purification of ricin was carried out by anion exchange column Q-Sepharose. TMV and tobacco were to achive such purpose. In a previous work the leaves of *Nictoniana tabacum* cv. Xanthi were rubbed with a mixture of the virus supernatant and the PAP (RIP from *Phytolacca americana*) in an equal volume (Chen *et al.*, 1991). The infection with TMV was retained if the protein separated from the virus supernatant. After different times course they determined the numbers of local lesions that indicate the degree of infection (Chen *et al.*, 1991). Their results indicated that the antiviral activity of PAP depends on the concentration of PAP and not on virus. They also showed that the RIP in general could be effectively used as antiviral agent because this activity of RIP does not depend on the type of the virus.

In this study, similar strategy for studying the effect of ricin on TMV was used. Here, we extended the study to cover the details of the inhibition of viral infection and effect of ricin itself on the plant cells. The experimental results showed the absence of crystal and amorphous inclusions in the cells treated with TMV-ricin mixture in comparison with that of the infected cells treated with TMV only. For more details, EM was used to examine leaf-cells of treated tobacco. It was reported before that there were some ultra-structure changes of the plant cells due to TMV infection (Soweha *et al.*, 1992).

Healthy untreated plant showed normal cell components i.e. chloroplast, cell wall, mitochondria and nucleus. On other hand, cytological changes were observed in the infected cells with TMV in the chloroplast and mitochondria. Viral particles were also increased inside the chloroplast as well as the cytoplasm of the virus-infected cells with time. Soweha *et al.* (1992) have reported that the infection with TMV caused most organells to be affected.

However, when ricin mixed with TMV, viral particles were found with less number after the same periods of incubation when compared to that in the treated leaves with TMV only. It was also observed that the mentioned changes are delayed markedly when leaves treated with both TMV and ricin. According to these results, it was suggested that the ricin prevent virus replication by inhibition of protein synthesis of the virus particles and degradation of its genome. Results also showed that the formation of the apoptotic bodies induced by ricin. This finding consistent with that data reported for induction of apoptosis by ricin (Hughes *et al.*, 1996, Waring, 1990). Apoptosis features vary according to the way of the induction of apoptosis, time after induction and type of cells. It has been reported that the apoptosis processes affect the mitochondria, membrane of the cells and the chromatin structure (Hughes *et al.*, 1996). Hughes *et al.*, (1996) and Waring (1990) have reported that that

the plant cell and all multicelluar organisms have an apoptosis machine, which is active in normal and disease conditions. Bubbling in the cell membranes could be explained as an initial step for fragmentation of the apoptotic cells form apoptotic bodies by vacuolar autophage. This result is in agreement with the results reported by Golstein (1998).The present results showed some importance of using RIPs in general and ricin specifically in plant protection against the viral infections. As a conclusion, ricin inhibited the viral replication thought inhibition of protein synthesis, inhibition of ribosomal frame shift, induction of viral nucleic acid hydrolysis and induction of apoptosis in the infected cells. These criteria are involved in the inhibition of the viral infection and decrease the viral propagation in plant. Further more, according to the results, as a plant protecting protein against viral infection, more attention must be taken due to the high toxicity of ricin as a plant protecting protein against viral infected.

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