Plant Archives Vol. 20, Supplement 2, 2020 pp. 2463-2467 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

BIOCHEMICAL STUDIES ON AGGLUTININ FROM *ABRUS PRECATRIUS*

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Abstract

Agglutinin is a type-2 Ribosome Inactivating protein (RIP) present in the seeds of a common tropical plant Abrusprecatorius. Ribosome inactivating proteins act by depurinatimg a highly conserved α-sarcin loop of 28S rRNA present at A4324 position. The actions of RIPs prevent the ribosome-elongation factor binding, thereby inhibiting protein synthesis resulting in cell death. Agglutinin comprises of two polypeptide subunits: A- Chain, the toxic subunit linked to a galactose binding lectin B-Chain by means of disulphide linkage. The binding of B-Chain to the cell surface initiates endocytosis of the A-B complex facilitating RIP entry and its subsequent inhibition of protein synthesis upon binding to ribosomes.Various biochemical studies conducted in this project are treatment with a reducing agent such as Beta-mercaptoethanol, proteolysis with trypsin and pepsin under varying concentrations and reaction time periods. The effect of denaturation by urea on agglutinin is also observed under varying concentrations and reaction time periods.
Keywords: Ribosome-Inactivating Protein, RIP, Agglutinin, Toxin, Abrus,

Introduction

Toxins are potentially lethal substances which can be produced inside the body itself or could be derived from the other external sources such as plants, bacteria or fungi (Kumar *et al.*, 2019; Singh *et al.*, 2016; 2017). Depending upon their mode and complexity of action their lethality can be determined. Some toxins are not always harmful for the subject. For example: The botulism toxin (derived from a bacterium) is responsible for food poisoning but at the same time, under controlled administration it can be used to treat loose skin and wrinkles (Nigam *et al.*, 2010). Similarly, mycotoxin, a secondary product of fungal metabolism, is a deadly agent for animals (Arjuna, 2014; Bennett *et al.*, 2003; Dagar *et al.*, 2015; Kumar *et al.*, 2018; 2019, Chopra *et al.*, 2019).

Third source of toxins are plants (Ahmad *et al.*, 2015; Barbieri *et al.*, 1993; Chandel and Bagai, 2012; Kaur *et al.*, 2016; Khajuria *et al.*, 2014; Noomansiddique *et al.*, 2018; Prasad, 2014). *Abrus precatorius*'s beads are source of Abrin toxin whereas another plant *Riccinus communis* is a source for Ricin toxin. These two toxins have attracted a great deal of attraction from various international organizations such as UN, US department of defense and many others because of their potential application in biological warfare (Audi *et al.*, 2005; Bhandari *et al.*, 2020; Dua *et al.*, 2018). The source plants can be easily spotted in tropical environments as well as certain Mediterranean regions. Their toxin can be easily extracted at a larger scale for unethical practices.

Abrin and ricin belong to a class of toxins called "Ribosome inhibiting proteins (RIPs)". Ribosome inhibiting proteins are majorly RNA N-glycosidases, involved in depurination of a highly conserved α-sarcin loop of 28S rRNA present at A4324 position (Schrot *et al.*, 2015). The actions of RIPs prevent the ribosome-elongation factor binding, thus effectively shutting down protein synthesis. A potential use of RIPs as immunotoxins to treat cancer by using as a conjugate with monoclonal antibodies has been shown considerable interest (Ghosh *et al.*, 2007). RIPs toxic to humans are found majorly in plants such as *Ricinus communis,* maize, rice etc.

RIPs can be broadly divided into two types:

Type 1 RIPs: Such toxins have a single polypeptide chain approx. 30 KDa size. Example: Trichosanthin (Shi *et al.*, 2018). It is an abortifacient used to induce abortions in animals.

Type 2 RIPs: Such toxins have two polypeptide subunits linked by disulphide linkages. Chain A is the toxic entity which inactivate ribosomes whereas chain B is responsible for binding to the cell surface receptors. Binding of lectin chain – B to the receptor initiates the endocytosis of A-B complex into the cytosol (Schrot *et al.*, 2015; Sidhu *et al.*, 2019). Example: Agglutinin, abrin, ricin etc.

Agglutinin toxin is derived from the beads of a common tropical plant *Abrusprecatorius.* Agglutinin, a type 2 RIP (Bagaria *et al.*, 2006; Rajeshkumar *et al.*, 2019; Singh *et al.*, 2016), is a heterodimeric glycoprotein. It consists of a toxic A-chain of size 30 kDa linked by disulphide bonds to the galactose binding lectin B-chain having size 31kDa (Fig. 1).

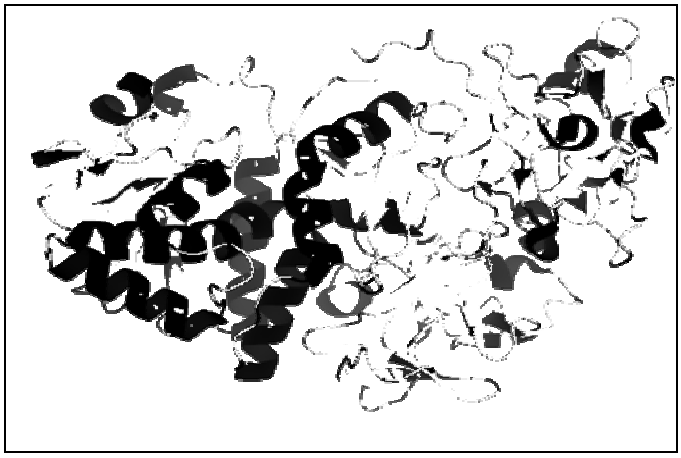

Fig. 1: Crystal structure of Agglutinin (PDB ID: 2Q3N)

The individual chains of Agglutinin have a distinct function. Where A-chain is an N-glycosidase responsible for cleavage of adenine residues from the 28S rRNA backbone, on the other hand, B-chain is responsible for the binding of toxin to the galactose residues on the cell surface. This binding facilitates endocytosis which helps in entry of A-

chain into the cytosol thus, causing an irreversible inactivation of ribosomes and hindered protein synthesis.

Materials and Methods

Protein extraction

The seeds were collected from the plant *Abrus precatorius* grown in the botanical garden of DAV University, Jalandhar. The seeds were soaked overnight in 5% acetic acid. The seeds were crushed to a paste and seed coat was separated. The crude extract was subjected to 30% ammonium sulphate precipitation at 4°Cfor an hour with regular stirring and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and subjected to 90% ammonium sulphate precipitation again at 4°Cfor an hour with regular stirring followed by centrifugation at 10,000 rpm at 4°C for 10 minutes and the supernatant was collected. The supernatant was dialyzed with phosphate buffered saline overnight at 4°C.

Protein purification

After dialysis obtained protein was subjected to anion exchange chromatography to purify the protein of interest. For this, a glass column was packed with sepaharose-Q. The column was washed with distilled water before packing. The beads of Sepharose-Q are stored in 90% ethanol and can settle at the base of the container. Therefore, the beads were shaken well before pouring into the column. The beads were poured 2 ml at a time. The column was packed up to 3-4 cm in length. The ethanol in the bead pouring was allowed to flow through to allow the column packing under the effect of gravity. As soon as the ethanol flow reduced the column was washed with 20 ml Tris-Cl buffer (pH 8). The buffer was pipetted 2 ml at a time along the side of the column drop by drop to prevent any disturbance to the bed. The washing with buffer also helps in the efficient packing of the column. Once the column washed and the entire Tris-Cl buffer flowed through the column, the stored protein extract was passed through the column in the same way. 2 ml of the extract was poured into the column along the wall of the column so as to not disturb the column bed. The flow through content was collected in a tube labelled FT and stored at 0°C. The protein species which had suitable electrostatic potential was bound to the beads of sepharose-Q. Then, the bound proteins were extracted in 26 fractions using 2ml of different elution buffers consisting of 500mM NaCl and Tris-Cl made in varying concentrations. The eluents were collected in clean, well labelled eppendorf tubes and stored at 0°C

Limited proteolysis

Limited proteolysis was performed using trypsin and pepsin (Hi-media). Agglutinin was incubated with varying concentrations of proteases for varying time periods and the results were analyzed using SDS-PAGE.

Urea denaturation

Denaturation of the protein was performed by incubating with different concentrations of urea (Hi-media). The results were analyzed using SDS-PAGE.

Results and discussion

The seed extract was subjected to dialysis after the ammonium sulphate precipitation. The product was eluted through an anion exchange column. The absorbance of eluents was measured at 280 nm. The eluents were also analysed by SDS-PAGE. Based on the outcomes, fractions containing agglutinin were selected and subjected to further analysis.

Beta-mercaptoethanol treatment

Beta-mercaptoethanolis a reducing agent that reduces disulphide linkage(s) in a polypeptide. The sample protein was subjected to PAGE by preparing a reaction mixture of 10 µL sample proteins each and 5 µL BMCE containing sample loading buffer and heating the reaction mixture in a boiling water bath. The electrophoresis was allowed to take place at 90 V for an hour (figure 2).

A dimer formation was observed in the Beta-mercaptoethanol treated samples which implies that the protomer-protomer interaction surface is locked by the disulphide bonds and the cleavage of these bonds exposes the interaction surface, facilitating the dimer formation.

Limited Proteolysis with Trypsin

Trypsin is a serine threonine with a catalytic triad of histidine, aspartate and serine.

To analyse the effect of trypsin on agglutinin, the protein was treated with trypsin of concentration 100 µg/ml. The reaction preparations were made with ratios 1:100, 1:50 and 1:20 with 1 being the volume of tryspin and other being the volume of sample protein. The treatment was allowed for 1 hour at 30°C and after that the reaction was stopped using a non-reducing sample loading buffer. The reaction preparations were subjected to SDS-PAGE at 90 V for an hour(figure 3).

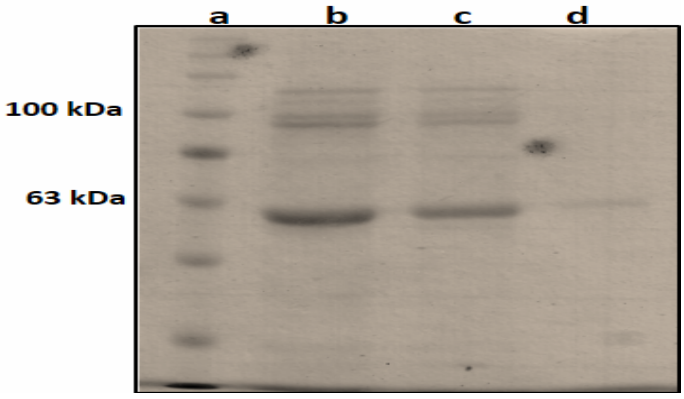

Fig. 2 : SDS-PAGE of different agglutinin samples after betamercaptoethanol treatment (a-prestained protein ladder, b-agglutinin, c-agglutinin, d-agglutinin)

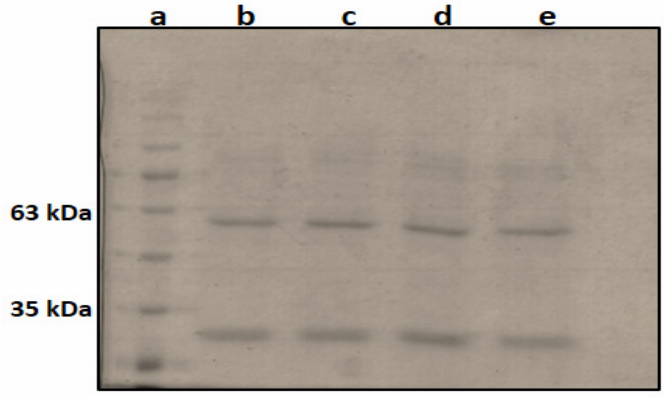

Fig. 3 : SDS-PAGE of agglutinin after different trypsin treatments(a-prestained protein ladder, b-Non tryspsin treated agglutinin (Negative control), c-trypsin:agglutinin (1:100),d-trypsin:agglutinin (1:50), e-trypsin:agglutinin (1:20)

From the SDS-PAGE it was analyzed that trypsin has no significant effect on the structural integrity of agglutinin. In another test for proteolysis with trypsin, agglutinin was treated with three different concentrations of trypsin 1mg/ml, 5 mg/ml and 10 mg/ml. Each concentration trypsin was used inn two volumes 1 µL and 0.5 µL. The protein quantity was kept constant. The trypsin treatment was allowed to take place for an hour and the reaction was stopped using non reducing sample loading buffer. The samples were ran through SDS-PAGE at 90 V for an hour (figure 4).

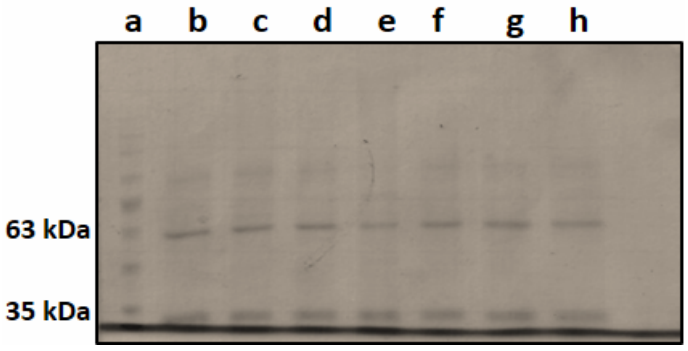

Fig. 4 : SDS-PAGE of agglutinin after different trypsin treatments(a-prestained protein ladder, b-agglutinin without trypsin treatment, c-agglutinin with 1µL 1mg/ml trypsin, d-agglutinin with 0.5µL 1mg/ml trypsin, e-agglutinin with 1µL 5 mg/ml trypsin, f-agglutinin with 0.5µL 5 mg/ml trypsin, g-agglutinin with 1µL 10 mg/ml trypsin, h-agglutinin with 0.5 µL 10 mg/ml trypsin)

From the resulting gel it was analyzed that varying concentration treatment of trypsin has no significant effect on the structural integrity of agglutinin.

Limited Proteolysis with pepsin

To check the stability of agglutinin, implied by trypsin treatment, the protein fraction was subjected to pepsin treatment. For this, two pepsin preparations; 1mg/ml and 5mg/ml were used and the treatment was carried out on different samples for periods of 1 hour, 3 hours, 6 hours and 8 hours. The reactions were stopped by adding non reducing sample loading buffers to the reaction preparations. The samples were run through SDS-PAGE at 90 V for about an hour (figure 5).

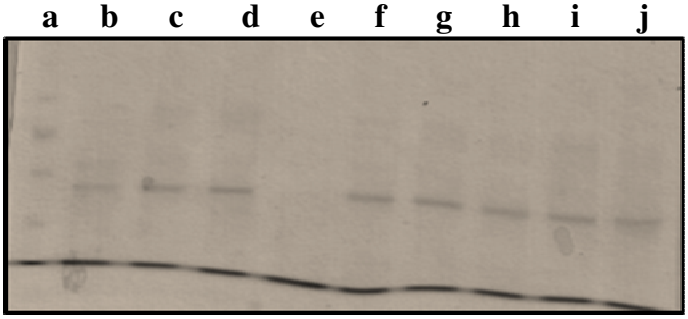

Fig. 5: SDS-PAGE of agglutinin after different pepsin treatments(a-prestained protein ladder, b-agglutinin with no pepsin treatment, c-agglutininwith pepsin (1 mg/ml) for 1 hour, d-agglutinin with pepsin (1 mg/ml) for 3 hours, e-agglutinin with pepsin (1 mg/ml) for 6 hours, f-agglutinin with pepsin (1 mg/ml) for 8 hours, g-agglutinin withpepsin (5 mg/ml) for 1 hour, h-agglutinin with pepsin (5 mg/ml) for 3 hours, i-agglutinin withpepsin (5 mg/ml) for 6 hours, j-agglutinin with pepsin (5 mg/ml) for 8 hours.

On analyzing the gel it was inferred that time bound and varying concentration pepsin treatment has no significant effect on agglutinin's structural integrity.

Urea denaturation

The effect of urea treatment on a protein structure is because of its various effects. It not only changes the dynamics of the water surrounding the protein but also affects the charge balance in the biomolecule. To study the effects of urea on agglutinin, the sample protein was treated with urea concentrations of 0M and 3M in a time bound manner. The time period ranging from 1 hour and 3 hours was used as the reaction benchmark. To stop the reaction after allotted time period, non-reducing and reducing sample buffers were used consecutively. The reaction preparations were then subjected to SDS-PAGE at 90 V for about an hour (Figure 6).

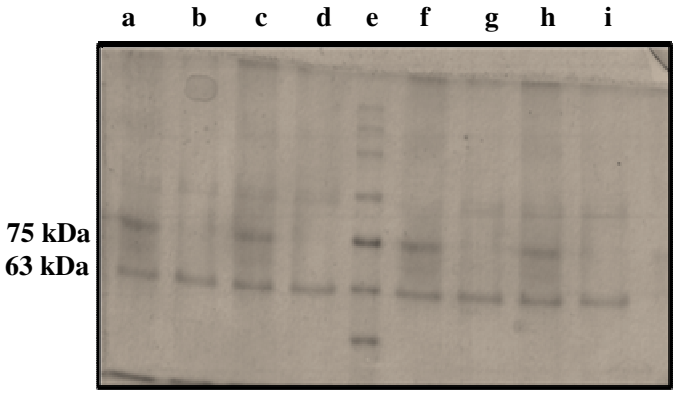

Fig. 6: SDS-PAGE of agglutinin samples after urea treatment (a - agglutinin- urea (0M) with BMCE/ 1 hour, b- agglutinin- urea (0M) without BMCE/ 1 hour, c - agglutinin- urea (3M) with BMCE/ 1 hour, d - agglutinin- urea (3M) without BMCE/ 1 hour, e – prestained protein ladder, f - agglutinin- urea (0M) with BMCE/ 3 hours, g - agglutinin- urea (0M) without BMCE/ 3 hours, h - agglutinin- urea (3M) with BMCE/ 3 hours,i - agglutinin- urea (3M) without BMCE/ 3 hours)

From the resulting gel it was analysed that there was a dimer formation in reaction preparations which contained BMCE. Urea had no effect on dimer formation. This implies that the protomer-protomer interaction interface is locked by the disulphide bonds. Denaturation does not expose the interface but only reducing conditions does.

Conclusion

The beads of *Abrus precatorius* were crushed and homogenized. The sample was subjected to dialysis and anion exchange chromatography to get protein in smaller sample fractions before further analysis. Measurement of optical density and successive SDS-PAGE showed that last three fractions 23, 24 and 25 were fit for further analysis. Under reducing conditions agglutinin formed a dimer. However, no such formation took place under denaturing conditions. This showed that the protomer-protomer interaction interface is locked under the surface disulphide linkages and any amount of denaturation is unable to expose this interaction surface. Agglutinin resisted any effect of proteases trypsin and pepsin under varying conditions. Pepsin treatment of upto 5mg/ml and 3 hour reaction time had no effect on the structure of agglutinin. Similarly, the protein resisted any change in its structure because of trypsin treatment of upto 10mg/ml. Both of these observations show that agglutinin is a quite stable protein. We also concluded that the dimer formed under the reducing conditions is highly stable as it remained intact under denaturing conditions of urea.

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