ISOLATION AND CHARACTERIZATION OF AGGLUTININ AND RICIN FROM *RICINUS COMMUNIS*

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CERTIFICATE

This is to certify that the thesis entitled "**Isolation and Characterization of agglutinin and ricin from *Ricinus communis***" which is being submitted by **Miss.Nitu Majhi**, Roll No. **410LS2056**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

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DECLARATION

I do hereby declare that the Project Work entitled "**Isolation and characterization of agglutinin and ricin from *Ricinus communis*"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Sujit Kumar Bhutia, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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Contents

Abstract

Lectins exhibit a promising diagonistic and therapeutic approach for treating cancer . Lectins found, almost in all organisms. On the surface of the cell lectins are attached with their sugar binding site. Lectins are found to be toxic, as it is poisonous, it results in the death of cells both *in vivo* and *in vitro*. Leguminous plant serve as the main source of lectin, most of the lectins are derived from seed, present in cytoplasm of the seed. Ricin is the toxic lectin which is derieved from the castor bean plant *Ricinus communis* belong to the family of Euphorbiaceae. The toxicity of ricin is bound with their two chain A and B chain. The A and B chains of ricin, more toxic to mammalian cells. The study of this project encompasses the purification of ricin through affinity chromatography, characterization of ricin through haemagglutination assay and SDS-PAGE. B chain of ricin bind to the sugar residue on the cell surface of red blood cell .The cytotoxicity on cancer cells is yet to be explored in order to showcase its anti-cancer property.

INTRODUCTION

Lectin is a sugar binding protein, found almost in all organisms mainly in plants; it may be in the solubilised form or membrane-bound widely distributed in nature. The binding specificity of lectins allows them to serve as recognition molecules within a cell, between the cells and between the organisms. The name "lectin" is derived from the Latin word *legere*, meaning, "to select" and derived the term lectin (Kocoureket et al., 1983). Although lectins occur in living organisms, plant lectins were the first proteins that can be studied. Plant lectins are found in root, stem and leaves and it is also present in seed cotyledon especially in cytoplasm. Lectins comprise up to 3% of the weight of a mature seed. Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells and glycoconjugates and are capable of specific recognition and reversible binding to carbohydrate and sugar containing substances, without altering covalent structure of any glycosyl ligands (Goldstein et al., 1980).

Lectin protein binds carbohydrate with high specificity and they have special binding sites for monosaccharide and oligosaccharides. Lectin obtained particularly from the seeds of leguminous plant. Some plant lectins are classified as toxins, and they are the most poisonous proteins on our planet it can result in death of the cells *in vivo* and *in vitro*.

Lectins vary in their composition, molecular weight, subunit structure and the number of sugar binding sites per molecule. They are used as histology and blood transfusion reagents but lectins may be toxic, inflammatory, resistant to digestive enzymes, and are found in much of our foods proteins. Lectins agglutinate animal cells and precipitated in glycoconjugates.

Plant lectins are relatively soluble and it can be easily extracted. The extraction procedures are laborious for the lectins obtain from stems and barks. Lectins can be purified to homogeneity on appropriate immobilized carbohydrate matrices. The lectin activity is usually measured by an agglutination assay that uses red blood cell. Other biological assays are also performed for lectin activity.

Types of lectin: Four types of mature lectins are derived according to their structure, a)Merolectin, b)Hololectins, c) Chimerolectins, d) Superlectins

Based on the sequence of amino acids, plant lectins are sub-divided into 3 different types. These include:

a) Legume lectins,
b) Monocot mannose-binding lectins,
c) Chitin-binding lectins,

Many seeds contain a remarkable amount of lectin. For example, soyabean, castor seed, peanut, abrus agglutinin constitutes 10–15% of the total protein content of the seed. However, not all the seeds contain lectins. For example, "tomato" the tomato lectin occurs in a soluble form in the locular fluid within the tomato fruit and not in tomato seeds. leguminous plant are the main source of lectins isolated and characterized (Van Damme *et al.*, 1998). Leguminaceae the family of leguminous plant, within this family the greater number of the lectins with their three-dimensional structures have been observed (Mourey *et al.*1998). Lectin according to their small carbohydrate binding recognize as two type of binding mannose-binding lectins, galactose-binding lectins or GlcNAc-binding lectins.

REVIEW OF LITERATURE

Many plants contain 'lectins' or agglutinins in their seed. The first lectin was discoverd by Stillmark in 1888. He found a proteinaceous hemagglutinating factor in castor beans (ricinus communis). As several hundred lectins or hemaagglutinins have been isolated and studied at biochemical or physiochemical level.

Lectins defined as proteins which interact non-covalently with carbohydrate moieties, showing high affinity and specificity for their ligands. Large numbers of lectins have been isolated at present with established biochemical characteristics. Recently by x-ray diffraction number of three-dimensional structures of plant lectins is observed (Bourne *et al*., 1990). Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells and glycoconjugates, capable of specific recognition and reversible binding to carbohydrate and sugar containing substances, without altering covalent structure of any glycosyl ligands (Goldstein et al.1980)

In glycoconjugates research, plant lectins have been use as analytical tool, these applications is extended and refined by understanding structure and specificity functional relationship of different groups.

A lectin researcher, Irwin J. Goldstein introduced affinity chromatography for the isolation of lectin and this was published in the *Biochemical Journal*. He described the purification process of concanavalin A by affinity chromatography. For the isolation of specific lectin sephadex (a polymer of dextran) is used (Moreira et al., 1983).

Mostly all lectins isolated and characterized from *Leguminoseae* ,serve as a main source, (Van Damme *et al.*, 1998). Large number of the lectins with their three-dimensional structures have been described within this family (Mourey *et al.*, 1998). Different monosaccharide binding specificities is exhibited by these proteins, belonging to the same family with their common biochemical features (Debray *et al.*, 1981).

Castor bean (*Ricinus communis)*

Fig 1: Castor plant

Fig 2: Castor seed

Castor comes under the genus *Ricinus* of Euphorbiaceae or spurge family (Atsmon 1989) . It is dicot plant Castor grown in many areas even across the country one of the country is Pakistan .In India it is grown as cash crop .Castor is grown as an ornamental and as an oilseed crop. The height of castor plant is in between 7 to 10 m in the tropics area.

Botanically:

Castor is monoecious, (having both male and female flowers), (Moshkin and Perestova, 1986). Castor perform both crosspollination and self-pollination and do not suffer from reduce fitness (Moshkin, et al.,1986). Under dryland conditions yields seeds of castor around 300 to 400 kg/ha (Duke and Wain, 1981).

Lectin

Lectin is found almost in all organisms with their specific binding sites of sugar i.e carbohydrates.On the surface of the cell lectins are attached with their sugar binding site. This sugar is utilized in differnt types of cell functioning or cell recognization ,signaling inside the cell(Sharon 1989). Lectin is tested by haemagglutination ,and the crosslinked between sugar residue results in the clumping of red blood cell that is haemagglutination.

Classification of plant lectin:

Plant lectins classified into small numbers of families:

Seven lectin families were distinguished:

i. Legume lectin.

ii.Mmonocot mannose-binding lectin.

iii.Chitin-binding protein containing domain.

iv.The type 2 ribosome inactivating protein.

v. Cucurbitaceae phloem lectins.

vi.Jacalin family.

vii.Amaranthaceae lectins.

Toxin from Castor Bean Plant**,** *Ricinus communis* the most natural poisonous substance is ricin they are poisonous to animal, plants, etc. Stillmark in 1888 tested the beans extract on red blood cell and found their agglutination activity, from there it came to be known that agglutination was due to activity of another protein i.e., RCA **(Ricinus communis agglutinin).** Ricin is catagorized under cytotoxin and weak hemagglutinin whereas RCA cartagorised as weak cytotoxin and powerful hemagglutinin.lectins can be isolated (Nicolsan et al .,1974).

Ricin:

Ricin is the protein component of *Ricinus communis* .Castor seeds contain two proteins which are highly toxic (Lord et al., 1994). *R. communis* agglutinin (RCA) and ricin having 102 kD and 65 kDa each. Ricin is a cytotoxic lectin. Toxic to the mammalian cells. In the late nineteenth century H. Stillmark, in 1888 firstly introduced these two proteins in castor seeds. Two polypeptides are found from ricin they are A chain and B chain. After that agglutinin protein has found with their four polypeptides, and they are linked by disulfide bonds (Butterworth and Lord, 1983). Toxicity is measured by agglutinate red blood cell.

Ricin A Chain and B Chain: four polypeptides chains are similar to the two chains of ricin. A chain is similar with the two chain of agglutinin and other two chains of agglutin are also similar to the B chain of ricin. The A and B chains of ricin, more toxic to mammalian cells, while the *R. Communis* agglutinin shows low cytoxicity to the cell.

Ribosome-inactivating protein is the part of A chain (Lord et al., 1994). Having its molecular weight 32 kDa . protein synthesis is inhibited by alteration of the ribosomal RNA subunits that involved in the translation. 28S ribosomal subunits is binded with the A chain causing in its structural change, without the help of B chain A chain is not able to enter into the cell. A chain and B chain both are heterodimeric toxins. On the cell surface terminating in galactose or N-acetylgalactosamine Ricin B chain completly binds with the glycoprotein and glycolipids (Lord et al., 1994). The galactose/N-acetylgalactosaminebinding activity is initiated by four disulphide bond of B chain. Bilobed structure is arises when bond breaks between N-terminal and C-terminal then two disulfide bonds aligned , this bilobed structure introduced galactose-binding sites halves of the B chain, contain This bilobal structure allows for two galactose binding sites. Endocytosis is initiated by the binding of mannose residue to the mannose receptor (Montfort et al., 1987).

Structue of Ricin:

From the above figure it shows a 3-dimensional structure of ricin (Montfort et al 1987). Obtained from X-ray crystallography data. The right half, the thick red ribbon, is the A chain, and the left half, the thin yellow ribbon, is the B chain.

The A chain (or RTA) contain 267-amino acids globular protein. Comprises of 8 alpha helices and 8 beta sheets. The B chain (or RTB) comprises of 262-amino acid protein. It has a binding site for galactose . These two site allows hydrogen bonding to specific membrane sugars (galactose and N-acetyl galactosamine). A disulfide bridge (-S-S-) joins RTA with RTB . A chain is toxic, ricin A chain cannot enter the cell without B Chain.

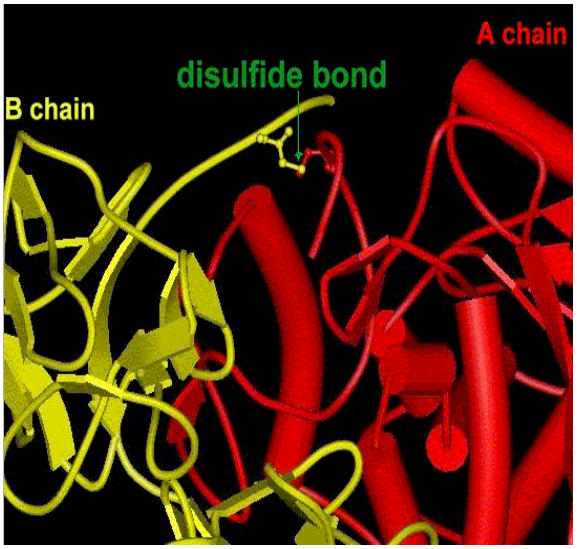

Fig: 3 Structure of Ricin

Mode of Action:

In mammalian cells mode of action is studied (Lord et al., 1994). The B chain of ricin binds to sugar residues on the cell surface. Entry of toxin into the cytoplasm is due to endocytosis in which protein is first enter to the golgi apparatus after that it is transported to the endoplasmic reticulum, in this way cytoplasm got toxicated.

APPLICATION OF LECTIN:

- Lectins use as diagnostic and therapeutic tools for cancer.
- Lectins applied in bacteriology, mycobacteriology,and virology for the identification and differentiation of various microorganisms.
- It also used as epidemiologic as well as taxonomic markers of specific microorganism.
- Use as analytical or preparative tools in glycoconjugates research.
- Lectin is also used in immunological studies.

MATERIALS AND METHODS :

CHEMICALS :

Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3), glycine,Cuppersulphate($CuSO_{4)}$, Potassium sodium tartarate ($KNaC_4H_4O_6$) were purchased from SRL, Sisco Research laboratories Pvt. Ltd., Mumbai. Acrylamide, bisacrylamide, Ammonium persulphate (APS), Sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), Bovine serum albumin(BSA), Tris were purchased from Sigma Aldrich, USA. Folin-Ciocalteau phenol reagent, Potassium Dihydrogen Phosphate (KH_2PO_4), Potassium hydrogen phosphate (K_2HPO_4) were purchased from S.D. fine chem. Ltd., Mumbai. Acetic acid, Bromophenol blue, agarose were purchased from Himedia, Mumbai. Glycerol was purchased from Rankem Pvt Ltd. Ethanol purchased from Trimurty Chemicals, India. Pre stained molecular weight marker was purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from Nice chemicals Pvt.Ltd. India.

Sample Collection:

The Castor Seeds (*Ricinus communis*) were collected for isolation and purification of Lectins from the Department of Biotechnology , Indian Institute of Technology, Kharagpur and 1 ml of blood was collected from CWS Hospital , Rourkela.

Seed Coat Removal:

Castor seeds were taken and grinded in a mixer for removal of sead coats and 45gms of uncoated seed were taken for the study. The uncoated seeds were deeped in PBS of 100 ml for one day.Then the seeds are grinded with PBS and the pastes were collected in 50 ml Centrifuge tubes and the weights were made equal by measuring the weights by the electronic weight balance. Then the samples were centrifuged by the Eppendorf centifuge with 7500rpm, at 4^0 c for 20 mins. The supernatant were taken after centrifuge and measured by a measuring cylinder. Some supernatant were stored in an eppendorf tube as crude at 4^0 C and the remaining were taken as salting out process.

Salting Out:

Salting out is a process in which separation of proteins takes place as they are less soluble at high salt concentrations. The concentration of salt requires for precipitation of the protein out of the solution is varies greatly in different proteins. It is also used to concentrate dilute solutions of proteins. Ammonium sulphate salt was taken in the salting out process. 40ml of crude were taken for 30% cut off in the salting out process. According to the salt chart 6.56 gm of ammonium sulphate were added to the crude by pinch wise and continues stirring was done by magnetic stirrer.

Fig.4: Salting out with Magnetic Stirrer

Then the sample was stored for overnight at 4^0 C and in the next day the sample was taken for centrifuge, then supernatant and pellet was collected. The amount of supernatant was measured by a measuring cylinder and taken for 60% cut off. 38ml of supernatant was taken and 15.28 gm of ammonium sulphate salt was added in pinch wise and continues stirring was done by magnetic stirrer.

Preparation of Lactamyl Sepharose 4B affinity matrix

4gm of lactamyl Sepharose 4B matrix was washed with 6ml distill water and mixed with 2.6ml of 2N NaOH and 0.66ml epichlorohydrin were added so that the final concentration of the various components were 30% v/v sepharose, 5% epichlorohydrin, 0.4 M NaOH. It was cover with aluminum foil and incubated at 40^0c for 2h with shaking. It was then transferred to a glass filter funnel and the gel was washed with 500 ml of distilled water.

Preparation of Amino Sepharose 4B

Epoxy activated sepharose 4B was suspended in 1.5 volume of concentrated ammonia solution i.e. 6 ml. The suspension was incubated at 40 c for one and half hour. It was then again transferred to a glass filter funnel and the gel was washed with distilled water.

Coupling of Lactose with Amino Sepharose 4B:

4 gms of Suction dried Amino Sepharose 4B was suspended in 3ml of 0.2M K_2HPO_4 buffer, which was contain 51mg $NaCNBH_3$ and 104 mg of Lactose. The Suspension was incubated at room temperature for 10 days with occasionally shaking. The free amino groups which remained in the gel were acetylated by adding 2 ml of acetic anhydride. The suspension was incubated in the room temperature for 1 hour. The Lactamyl sepharose 4B thus obtained was subsequently washed with distilled water, 0.1 M NaOH, distilled water and 10 mM PBS subsequently. It was stored in distilled water with traces of sodium azide at 4C.

Affinity chromatography:

The lactamyl sepharose column washed by PBS solution (pH7.2) and O.D of the washed PBS was measured at 280nm. When the OD value decreases and tend to zero then the protein sample of 60% cut off was passed through lactamyl sepharose beads and the elute sample was collected and its O.D was determined at 280nmLactatamyl sepharose beads were again washed with PBS solution (pH7.2) and the O.D of the washed PBS was measured at 280nm. When the OD value decreases and tend to zero then 20ml lactose solution was loaded on lactose sepharose beads and O.D of the eluent was measured at 280nm. The eluent was collected for dialysis in PBS (pH 7.2) and stored at 4^0c for 1day. Same procedure was followed for the 90% cut off. In 90% cut off 30 ml of Lactose solution was passed through the Lactose sepharose 4B column.

Dialysis:

Dialysis was done against PBS at 4^0C of 30%cut off, 90% cut off and 90% Affinity samples.

Fig.5 : Dialysis of sample with PBS

Determination of concentration of protein:

The concentration of crude, 30% cut, 90% cut, and 90% affinity were measured by Lowry Method.

Lowry's Method:

REAGENT A=Sodium hydroxide(0.5%)

Sodium carbonate(2%) make it upto 1 litre

REAGENT B1=1% Copper sulphate

REAGENT B2=2% Sodium potassium tartarate

REAGENT C=A:B1:B2=100:1:1

BSA STANDARD=1mg/ml

Folinciocalteau's reagent=1N (5 ml solution +5 ml distill water)

Take different concentration of BSA solution from stock solution and add distill water to it and made up to 2ml.Ricin protein taken unknown quantity dissolved in 1ml distill water ,and add reagent C of 5 ml and protein of 0.5ml. Mixed properly and incubate for 10 mins. Then 0.5 ml of Folin reagent was added and incubate for 30min. Take OD at 750nm.

Preparation of Human Erythrocyte:

Healthy human venous blood was collected by a syringes and poured into a 15 ml tube to which the anticoagulant EDTA was previously added. . In my experiment EDTA was added as anti-coagulant.

Haemagglutination Assay:

1ml blood sample was Centrifuged in 2ml microtube at 1000 rpm for 5min at room temperature by Eppendrof mini spin. The the pellet was collected and was added 10ml of PBS. The mixture of blood and PBS was centrifuged at 1000 rpm for 5min at room temperature. After centrifuge the Pellets were collected and c 100µl of pellet was added to10ml of PBS solution (pH 7.2). The Haemaglutination activity of Soyabean lectin was

detected when blood erythrocytes were added to it. The assay was carried out in a 96 well round bottom microtitre plate. The first well of each row was served as positive control to which 100µl of normalized sample and 100µl of blood was added and the last well served as negative control since it contain 100µl of blood and 100µl of PBS solution. Between the positive and negative control each well contains blood, PBS and lectins. First of all 100µl PBS was added to all the wells. Then 10µl of normalized crude was poured to the first well and it was serially diluted till the negative control. Simillar procedure was followed for the ohther samples. Finally 100µl of processed blood sample was poured to each well. After that the placed was placed in a plane surface without disturbing it. After 30mins the haemaglutination assay result was observed.

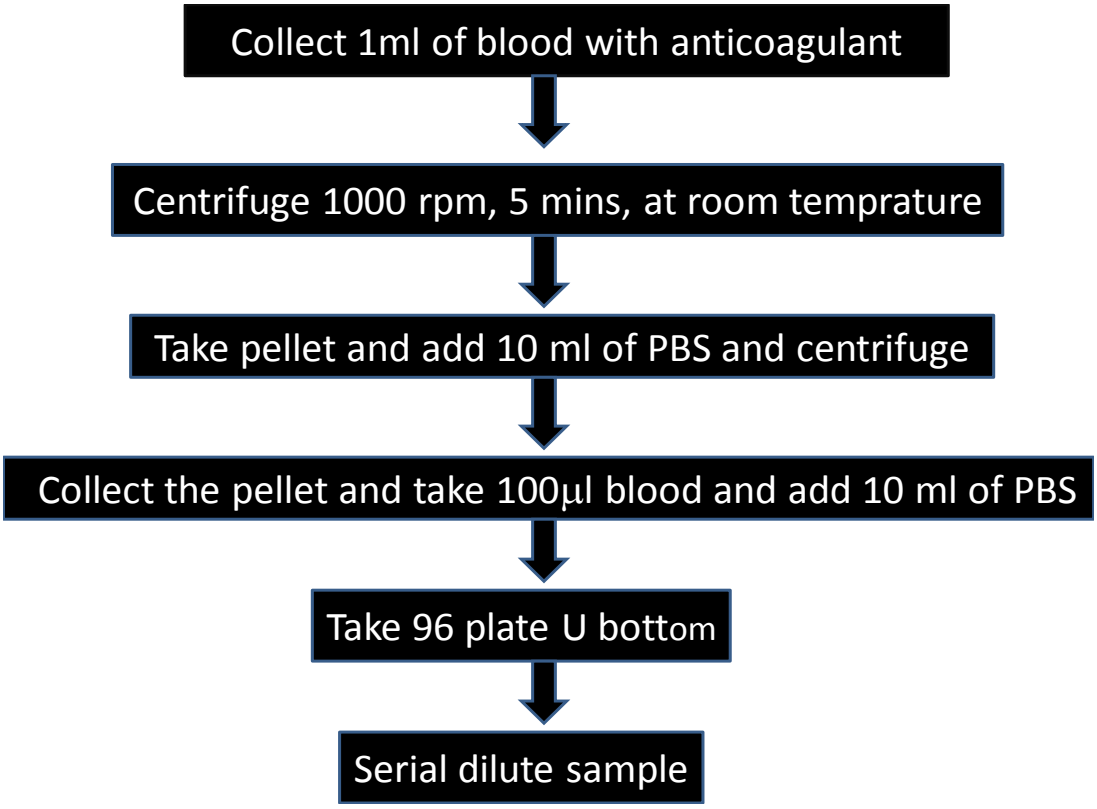

Fig.6 : Protocol for Haemaglutination assay

SDS-PAGE:

The molecular mass of the subunits of the lectins was estimated by SDS-PAGE. The poly acrylamide gel electrophoresis was done according to the protocol given in the Book "Molecular Cloning" by Sambrook & Russell on a 12% gel. For the native 12% polyacrylamide was employed and SDS along with B- mercaptoethanol was not added. The mixture of 10µl of sample, 10µl of Sample loading buffer and 5µl of Coomassie Brilliant Blue were added to the well. In my experiment crude, 60%, 90%, 60% affinity and 90% affinity was added with sample loading buffer and Coomassie Brilliant Blue. The gel was again stained with Silver salts. Silver nitrate was used in the preparation of silver staining.

RESULT

Purification of Ricin:

- The solutions of eluted protein had bound on Lactose sugar of lactamyl beads.
- Lactose was removed from the protein by the method of dialysis in PBS solution (pH-7.2) for 1day.

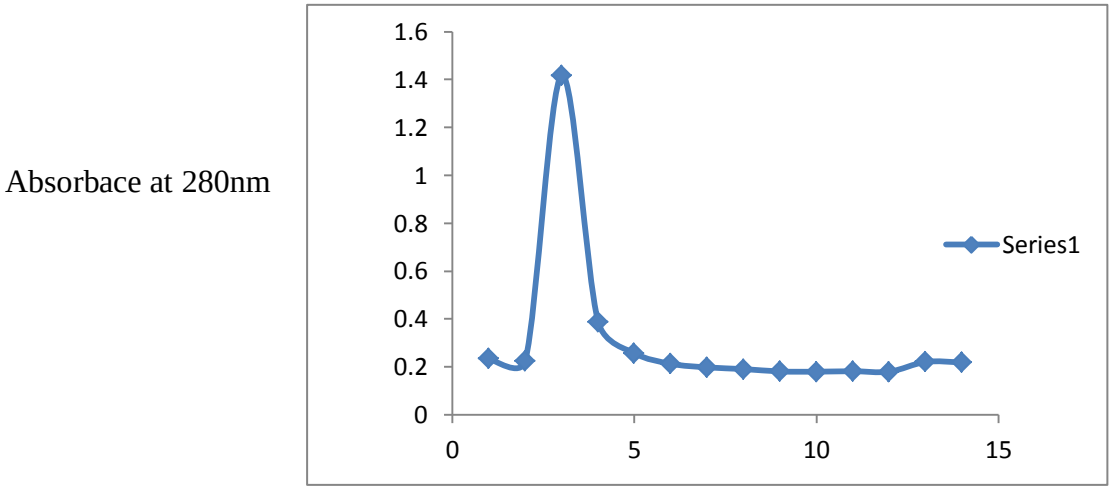

Fig 6: lactose elution graph

Estimation of protein concentration by taking OD at 280 nm:

Sample	Volume	OD(280nm)	Concentration (mg/ml)	Total protein concentration
Crude	40ml	0.770	46.20	1848
30%	38ml	0.640	38.40	1459.20
90%	35ml	0.496	3.174	111.09
Affinity	33ml	0.439	1.756	57.948

Haemagglutination Result:

- Agglutination activity of normalized sample of ricin was tested with human RBC.
- The haemagglutination assay is used to determine titer value of proteins based on their ability to attach to molecules present on the surface of red blood cells.

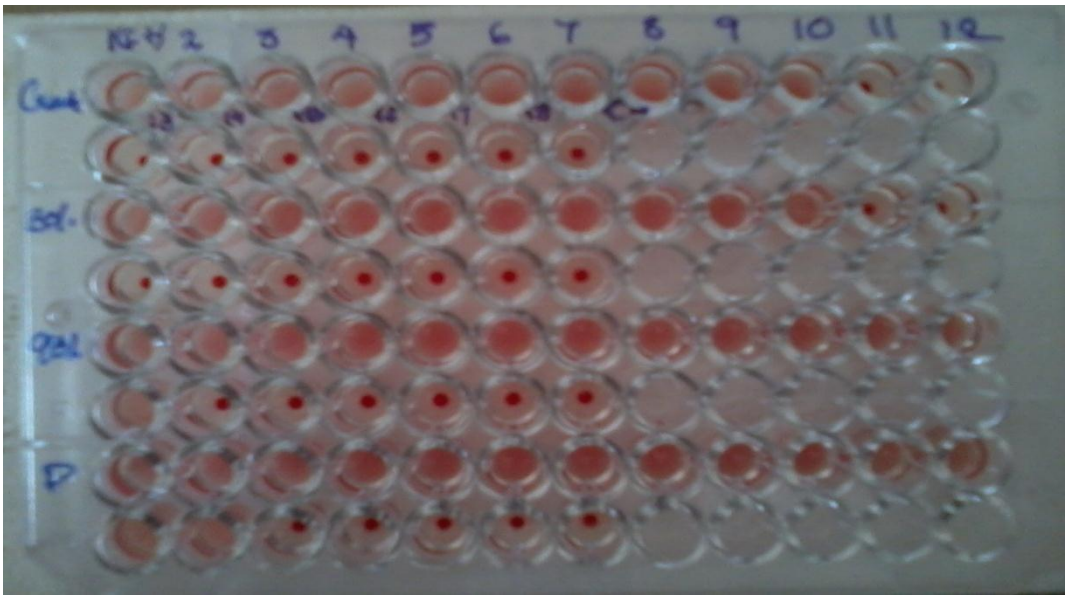

Fig 7: Haemagglutination Assay

Table 1: Haemagglutination result

SAMPLE	HA VALUE
Crude	$1:2^{10}$
30%	$1:2^{10}$
90%	$1:2^{13}$
Affinity	$1:2^{14}$

1) Crude HA=$1:2^{10}$,means that the protein was titered by Hemagglutination Assay (HA), and the endpoint for the assay was a dilution of $1:2^{10}$, that is 1: 1024.
2) 30% HA=$1:2^{10}$ means that the protein was tittered by Hemagglutination Assay (HA), and the endpoint for the assay was a dilution of $1:2^{10}$, that is 1: 1024.
3) 90% HA=1: 2^{13} means that the protein was tittered by Hemagglutination Assay (HA), and the endpoint for the assay was a dilution of $1:2^{10}$, that is 1:8190
4) Affinity HA =1: 2^{14} means that the protein was tittered by Hemagglutination Assay (HA), and the endpoint for the assay was a dilution of $1:2^{10}$, that is 1:16,384

So, from the haemagglutination result it is found that affinity value is higher which indicate the presence of high concentration of protein.

SDS-PAGE Result

(std .protein marker) affinity 90% 30% crude

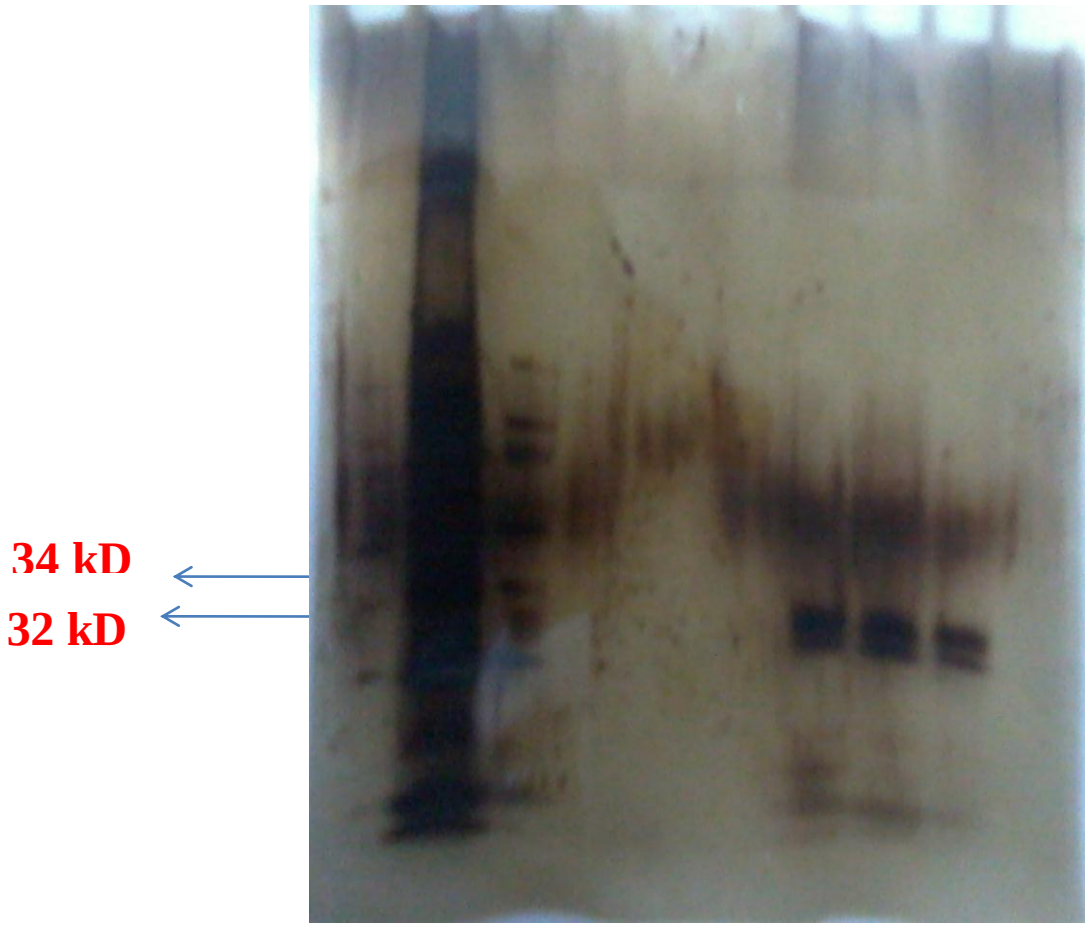

Fig 8 : SDS- PAGE

Two chain of ricin is identified A chain with its molecular weight 32 KD and B chain with its molecular weight 34 KD.

DISCUSSION

The hemagglutination assay is used to determine titre value of proteins based on their ability to attach to molecules present on the surface of red blood cells. The red blood cells are agglutinated by protein suspension which ensures there is no settling of RBCs out of suspension. Serial dilution of a protein is performed in a 96-well plate and with consistent addition of red blood cells, an estimation of the amount of protein in crude, 30 %, 90 % and purified protein present is estimated.

Positive control are seen with a uniform film with indistinctive shape covering the bottom of the tube. Negative control is seen perfectly outlined with round "button" of cells settled at the bottom of the tube. Irregular clumps of cells are specified for the intermediately positive results and are seen at the bottom of the tube.

The highest dilution of protein suspension that produces a positive result is termed as the end point . HA (hemagglutination assay) is where the protein is mixed with diluted red blood cells, the protein forms a network (lattice formation) with the red blood cells. The red blood cells spread out as these "lattice" formations settle to the bottom of the tubes. If the protein is absent, then the red blood cells are unable to form the lattice, and they settle down at the bottom of the tube as a condensed button; interpreting the immunological property of ricin, which is a weak agglutinin.

SDS PAGE : The purpose of SDS-PAGE is to separate proteins according to their size. The molecular weight of desired protein after affinity was determined with respect to the corresponding molecular weight of standard protein marker. We found out that Ricin comprises of two chains A chain : 32 KD and B chain : 34 KD.

CONCLUSION:

SDS-PAGE enhanced our measurement of protein of interest, RICIN. Significantly, which are of 32kD and 34 KD, characterised as A-Chain and B- chain.

Ricin is a weak agglutinin. As it is toxic, its cytotoxicity on normal cells and cancer cells should be further explored to quote its anti-cancer property. Further research are required for its application in different cancer cell lines to see whether it has inhibitory action on proliferation.

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