WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 8, Issue 3, 1610-1622. Research Article ISSN 2277– 7105

ISOLATION AND PURIFICATION OF LECTIN FROM PISUM SATIVUM

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Article Received on
19 Jan. 2019,

Revised on 08 Feb. 2019,
Accepted on 01 March 2019

DOI: 10.20959/wjpr20193-14499

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ABSTRACT

Lectins are glycoprotein or carbohydrate binding proteins having agglutinating property.Lectins were first found in plants and were later isolated from animals as well .interaction of lectines with the carbohydrates is very specific. Their specificity to particular sugar is defined by the mono or oligosaccharide that inhibits agglutination plant. Lectins have great potential as tools in identification, purification and stimulation of specific glycoconjugates. Leguminosae or fabaceae is the best characterized family of plant lectin. Other family of plant lectins includes Gramineae, solanaceae, euprobiaceae etc. Their function in plants include enzymatic activity and storage of protein defense mechanism, cell wall extension mutagenic stimulation, transport of carbohydrate and packaging and mobilization of storage materials .Pisum sativum lectin is a leguminous or fabaceae plant. Pea lectin shows specificity towards mannose or glucose. In the following report, it was the aim to isolate and purified the Pisum sativum lectin and its characterization by heamoagglutation assay.

KEYWORDS: Isolation, purification, lectin, Pisum Sativum, SDS- PAGE, Lowry method.

INTRODUCTION

Lectins are naturally occurring glycoprotein's that bind carbohydrate residues selectively and non-covalently (Van Damme, Van Damme, Prumans, Barre, & Rouge, 1998). The name "lectin" is derived from the Latin word legere, meaning, "to select" and derived the term lectin (Kocoureket et al., 1983). Lectins are 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). Although lectins occur in living organisms, plant lectins were the first proteins to be studied. These proteins form reversible complexes with mono-or oligosaccharide structures. Lectins are well distributed in nature found in both plants and animals. Although lectins are present in almost all organisms, plants are the richest source of lectin. Interaction of lectins with carbohydrate is very specific. Lectins with different carbohydrate specificity have been isolated from forty-nine different plant species, primarily from seeds. Lectin-carbohydrate interactions are comparable to enzyme-substrate or antigen-antibody interaction. In many cases the cellular ligands for lectins are the carbohydrate chains of glycoprotein's and glycolipids. Binding of lectins to the cell surface glycoprotein and glycolipids results in the formation of cross-linked complexes which are often associated with the biological responses of cells (Nicolson, 1976).

Classification of Lectin

- On the basis of carbohydrate binding specificities, lectins are classified into-
- Mannose-binding lectins
- Galactose-binding lectins
- GLcNAc –binding lectins

Based on the specific saccharide binding sites, the lectins are classified into-Metal binding sites, hydrophobic sites, Glycosylation sites, Carbohydrate binding sites and some others. According to the structure, lectins are classified into 4 mature types of lectins i.e. merolectins, hololectins, chimerolectins and superlectins.

PLANT LECTIN

Plant lectins are a class of highly diverse non-immune origin and carbohydrate-binding proteins that have at least one non-catalytic domain. This enables them to selectively recognize and reversibly bind to specific free sugars or glycols, present on glycoprotein and glycolipids, without altering structures of carbohydrates. Lectins with a molecular weight of 60,000-1, 00,000 are present in various plants. Many of the plant lectins are toxic in nature. They primarily affect gastrointestinal tract, cell membrane and other organelles. Their function in plants includes-

- Enzymatic activity
- Storage of protein
- Defense mechanism

- Cell wall extension
- Mutagenic stimulation
- Transport of carbohydrate
- Packaging and mobilization of storage materials.

Biological activity of many lectins can be attributed to metal ions which are important components of the native structure of most leguminous lectins. Among the plant lectin groups, leguminosae or fabaceae is the best characterized family of plant lectin. Other families of plant lectins includegramineae, solanaceae and euphorbiaceous etc.

Pisum Sativum Lectin

Pisum Sativum is a leguminous plant. The protein pea lectin or Pisum sativum lectin (PSL) is a legume lectin isolated from the seeds of garden pea (Pisum sativum). It is a glycoprotein composed of four subunit of about 50,000 dalton that is a dimeric structure of two monomers, each consisting of one alpha- and one beta- subunit. Pisum sativum lectin shows its specificities towards glucose (2mol/mol of protein) and stabilized by 2 atoms of each of Ca2+ and Mn2+ per molecule of protein.

Types of Lectin

Lectins are classified on the basis of carbohydrate binding specificities into –mannose binding lectins, galactose binding lectins and GLcNAc binding lectin, monocot mannose-binding lectins, legume lectins, type II ribosome-inactivating proteins and other lectins.

There are four types of mature lectin according to their structure-

- Merolectin
- Hololectin
- Chimerolectin
- Superlectin

Merolectin – Merolectins are proteins that are built exclusively of a single carbohydrate binding domain. They are small, single polypeptide proteins, which because of their monovalent nature are incapable of precipitating glycoconjugates or agglutinating cells. Hololectin- Hololectin also is built exclusively of carbohydrate binding domains but contain two or more such domains that are either identical or very homologous. This group comprises all lectins that have multiple binding precipitating glycoconjugates. Chimerolectin-

Chimerolectin are fusion proteins possessing a carbohydrate binding domain tenderly arrayed with an unrelated domain, which has a well-defined catalytic activity(or another biological activity) that acts independently of the carbohydrate binding domain.

Lectin Structure

One major property of lectins is their specific saccharide binding sites. Some lectins are composed of subunits with different binding sites. Since subunits have very different specifities for cell surface receptors, each combination is considered to have a different function. The specificity of the binding sites of the lectins suggests that there are endogenous saccharide receptors in the tissues from which they are derived or on other cells or glycoconjugates with which the lectin is specialized to interact.

Metal binding sites

Biological activity of the lectins may be attributed to the metal ions which are the essential part of the native structure of most leguminous lectins. The most studies and fully sequenced lectin is concanavalin A. The metal binding sites of the concanavalin- Are situated in the amino terminal part of the polypeptide chain. In this lectin, each subunit has aspartic 10 and 19, asparagines 14, histamines 24, serine 34, glutamic acid 8, and tyrosine 12 that are involved in the binding to one calcium and one magnesium ion. Lectins of soybean, peas, faba bean, lentils, and sainfoin have amino acids that are involved in metal binding, which are conserved. The exception is of the tyrosine residue at position 12 of concanavalina which is replaced with by phenylalanine in the other legume lectins. Hydrophobic sites-The stability of the native structure of most lectins is thought to be caused by the hydrophobic interactions. Such hydrophobic sites, forming cavities in the lectins structure, may play an important biological role. The hydrophobic binding sites of auxins, or cytokinin and adenine, for instance, by concanavalin A may enhance the functions of lectins on the plant life cycle

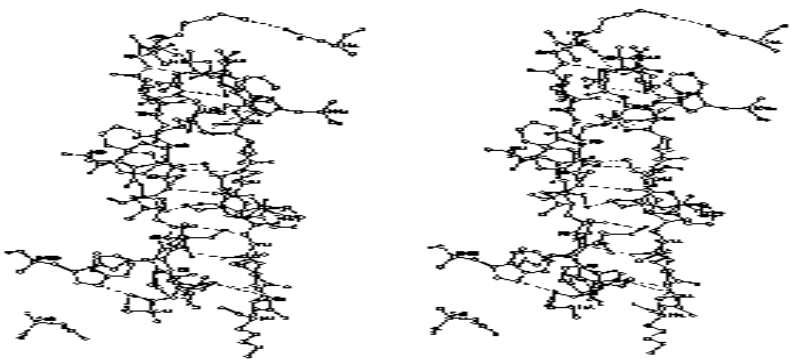


Figure 1: Stereo diagram of monomer.

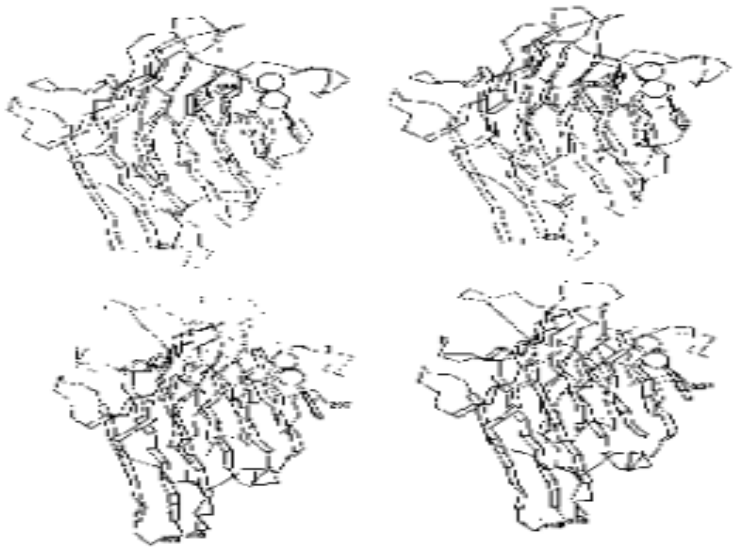


Figure 2: Stereo diagram of pea lectin monomer interface in pea lectin.

Function of Lectin

There is evidence that lectins may be involved in the recognition between cells or cells and various carbohydrate containing molecules. This suggests that they may be involved in the regulating physiological functions. They seem to play an important role in the defense mechanism of plants against the attack of microorganisms, pests and insects. Fungal infection or wounding of the plant seems to increase lectins. In the legumes, the role of lectins in the recognition of nitrogen-fixing bacteria Rhizobium genus, which have sugar containing substances, has received a special attention.

Binding nitrogen – fixing bacteria to legume roots: among the possible functions of plant lectins is their participation in binding nitrogen- fixing bacteria to legume roots.

Other functions of lectins in plants may include –enzymes, storage of protein for the plants, defense mechanism against pathogens, cell wall extension for protection, mutagenic stimulation, packaging and mobilization of storage materials.

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.
- Lectin use as analytical or preparative tools in glycoconjugates research.
- Lectin is also used in immunological studies.

Plant Lectin

Plant lectins are a class of highly diverse non-immune origin and carbohydrate binding proteins that have at least one non-catalytic domain. This enables them to selectively recognize and reversibly bind to specific free sugars or glycans, present on glycoprotein and glycolipids, without altering structure of carbohydrates. The lectin contents in some parts of plants are higher, e.g., 390 and 75 mg of the purified lectin was recovered from 100 g Remusatia viviparous tubers (Bhatt et al. 2010) and Astragalus mongholicus roots (Yan et al. 2005), respectively. Among the plant lectin groups, fabaceae or leguminosae plant is the best characterized plant lectin family. Landsteiner and Raubitschek (1907) presented evidence for nontoxic lectins from legume seeds, in particular Pharsalus vulgarize (bean), Pisum sativum (pea), Lens culinary(lentil), and Vicia sativa(vetch).

Mostly all lectins isolated and characterized from Leguminoseae serve as a main source, (Van Damme et al., 1998). Large numbers 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 (Debary et al., 1981).

Structure

Fig: 3 –Pisum s. seeds.

Fig: 4-Pisum s. plant.

Pisum sativum lectin has a molecular weight of 49,000 consists of two pairs of non-covalently linked polypeptide subunits and contains less than 0.5% by weight of carbohydrate. The small subunit (alpha-) has a molecular weight of 7,000 and the large subunit (beta-) has a molecular weight of 17,000. (Ian S. Trowbridge$., 1974). Pisum sativum lectin show its specifities towards mannose (Sitohy et al., 2007). A plant lectin isolated in its pure state from the Egyptian seeds of *Pisum sativum* (PSL) produced two bands in SDS–

PAGE (5.53 and 19.3 kDa; i.e. a and b chain) but one peak by gel filtration chromatography on Sephardic G-100, corresponding to 50 kDa, i.e., a dimeric structure of two monomers, each consisting of one a and one b subunit. PSL is a glycoprotein bound with glucose (2 mol/mol of protein) and stabilized by 2 atoms of each of Ca2+ and Mn2+ per molecule of protein (Sitohy et al., 2007).

MATERIALS AND METHODS

Chemicals

Sodium Phosphate monobasic dehydrate (NaH2PO4.2H2O), Sodium Phosphate dibasic dehydrate (Na2HPO4.2H2O) were purchased from SRL, Sysco Research Laboratories Pvt. Ltd. Mumbai. Sodium chloride (NaCl) was purchased from Fischer scientific. Bovine serum albumin (BSA) was purchased from Himedia, Mumbai, India. Sodium hydroxides (NaOH), Sodium carbonate (Na2CO3), Sodium potassium tartarate (KNaC4H4O6), Copper sulphate (CuSO4) were bought from Himedia, Mumbai, India. Folin-Ciocalteau phenol reagent purchased from Himedia, Mumbai.0.05 M TrisHCl(Ph-8.0), 1Mm CaCl2, 1Mm MgCl2, 0.02% NaN3 , 0.25 M Glucose and acetic acid.

Sample collection

The Pisum sativum seeds (pea) were collected for isolation and purification of lectin from local market, Rourkela. The blood sample was collected from CWS Hospital, Rourkela.

Isolation and purification of lectin

Pea (*Pisum sativum*) seeds were taken and grinded in a mixer for removal of seed coat. 50 gms of uncoated seeds were taken for the study. Uncoated seeds were soaked in milli-Q water overnight at 4^0c.Then the seeds are grinded or homogenized with 500 ml of 0.05 M TrisHcl pH-8.0 containing 1mM $CaCl_2$, 1mM $MgCl_2$, 1 mM $MnCl_2$ for 2 hours at 4^0c. Then filter the suspension by using nylon membrane tissue. The supernatant were collected in 50 ml centrifuge tubes and the weights were made equal by measuring the weights by electronic weight balance. Then the samples were centrifuged by the eppendrof centrifuge with 23,500 g for 20 minutes. Storage proteins are precipitated by slowly added 1 M acetic acid to the stirred solution until pH-5.0 is reached. After an additional hour of stirred at 4^0c, the suspension was centrifuged again at 23,500 g for 20 minutes. And the supernatant readjusted to pH -8.0 by addition of 1 M NaOH. About 2 ml of supernatant was taken for estimation of proteins and haemagglutination and remaining supernatant used for the process of dialysis.

Dialysis

Dialysis helps in the separation of the dissolved molecules via semi-permeable membrane. The semi-permeable membrane contains pores that allow smaller molecules, such as salts and solvents to diffuse across the membrane but the diffusion of the larger molecules is restricted. It is a method in which an aqueous solution containing both small and large molecules is placed in a dialysis bag made out of the cellulose acetate membrane. Cellophane, also known as cellulose acetate is commonly used semi-permeable membrane for dialysis. Other substances such as collodion or nitrocellulose can also be used. The dialysis bag is placed in a large vial containing either distilled water or same buffer used earlier. In this way, the smaller molecules and salts will easily diffuse out of the membrane. After sometime the concentration of the smaller molecules present inside the bag will be same to that present outside the bag whereas the macromolecules still remain inside the dialysis bag. During dialysis the fluid containing dialysis bags must be changed at the regular interval of time. The pellet obtained after precipitation with acetic acid was dissolved in TrisHCl buffer (pH-8.0) and taken for dialysis. it was kept in dialysis for 3 days. One important thing to be taken care during dialysis is that the dialysis bag containing the sample should immerse completely in water or buffer being used. Upon completion of dialysis the sample was centrifuged at 7500 rpm, 4°C for 20 minutes. The supernatant was collected and filtered using Whattman filter paper followed by filtration using a 0.22µsyringe filter. The filtered supernatant was stored in -20°C to be used for affinity chromatography.

Sds-Page

The molecular mass of the subunits of the lectins was estimated by SDS-PAGE. The poly acryl amide gel electrophoresis was done according to the protocol given in the Book "Molecular Cloning" by Sam brook & 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, dialysis, affinity, and dialysis 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.

Estimation Of Protein Concentration By Lowry's Method

Protocol

The concentration of crude, after dialysis sample and after affinity chromatography was estimated by Lowry's method. Bovine serum albumin (BSA) was used as the standard protein. The reagents required:

1. BSA stock solution (1 mg/ml)
2. Analytical Reagents

Reagent A: Sodium hydroxide (0.5%)

Sodium carbonates (2%)

Reagent B1: 1% copper sulphate

Reagent B2: 2% sodium potassium tartarate

The analytical reagents were prepared by mixing 1ml of both reagents B1 and B2 and 100 ml of reagent A.

3. Folinciocalteau reagent: 1N of this reagent was prepared by mixing equal volume of the reagent and water i.e., 5 ml of the reagent was mixed with 5 ml of distilled water. Different dilutions of BSA stock solutions were prepared.ml of protein sample was taken to which 2ml of Lowry's reagent was added, mixed and then kept for 15 minutes incubation. Then 200µl of Folin's reagent was added to it and incubated for 30minutes.O.D was taken at 595 nm. The concentration of the unknown protein was plotted in a graph taking absorbance in the Y-axis and concentration in the X-axis.

Haemagglutination Assay

Protocol

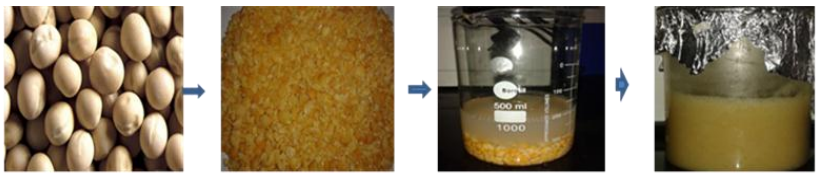
1. About 2 ml of human blood from different blood groups was collected from Common Welfare Society Hospital, Rourkela in a 15 ml falcon tube and EDTA was added to it.
2. About 1 ml of blood was taken and centrifuged at 1000 rpm for 5mins at room temperature. The serum was discarded and pellet was collected. About 10 ml of PBS was added to the pellet and centrifuged again for 5 minute at 1000 rpm in room temperature.
3. The supernatant was discarded and the pellet was collected. About 200 µl of the pellet was taken and mixed with 10 ml of PBS. This was the final blood sample for the assay.

The haemagglutination assay was carried out in 96 well round bottom microtitre plate. The first well of each row served as positive control and the last well as negative control. The positive control contains sample and RBC's whereas the negative control contained PBS and

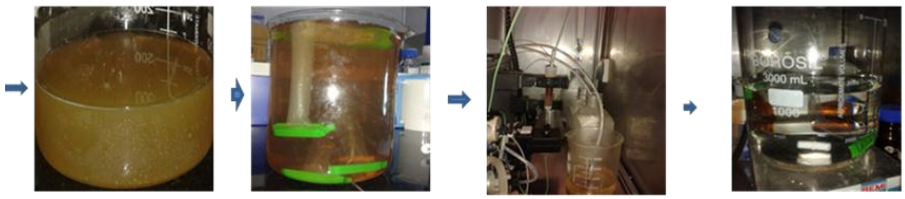
RBC's. Individual wells were provided with 100µl of PBS and then the protein sample was added and serially diluted till the negative control. About 100µl of prepared blood was added to each of the well. Care should be taken so that the final volume of each well should be 200µl.

RESULT

1. Isolation of Pisum sativum lectin:



Seeds of pea → Decoated seeds → Seeds soaked in TrisHCl → Seeds grinded into paste

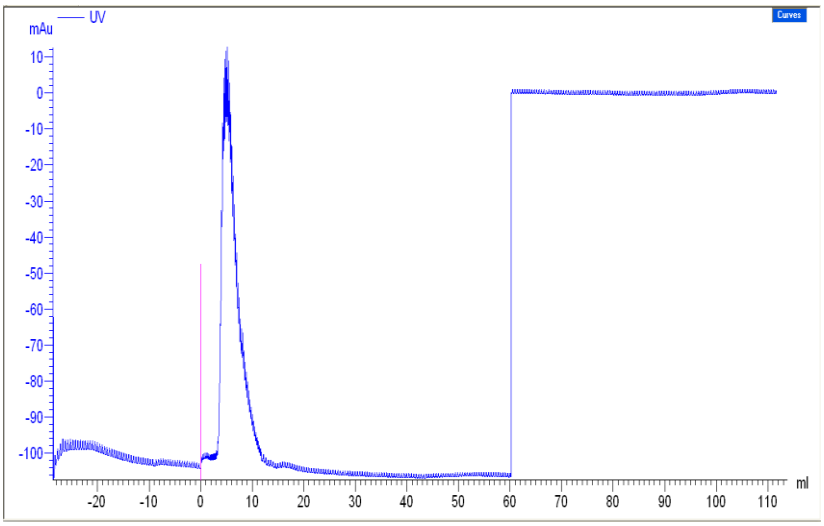


Precipitate with Acetic acid → dialysis → Affinity Chromatography → Dialysis

⇨ Characterization by Haemagglutination assay.

2. Purification of Pea Lectin By Affinity Chromatography

Abs. at 280 nm



Fraction number (ml)

Figure 5: Affinity chromatography peak using lactamylsephadex G-100 column.

Inference

The isolated protein is purified by affinity chromatography using lactamylSephadex G100 column and the graph of the eluted protein is prepared. This peak shows that the protein elution.

Haemagglutination assay

+ve $1/2^1$ $1/2^2$ $1/2^3$ $1/2^4$ $1/2^5$ $1/2^6$ $1/2^7$ $1/2^8$ $1/2^9$ $1/2^{10}$ $1/2^{11}$

Crude

Dialysis

Affinity Chromatography

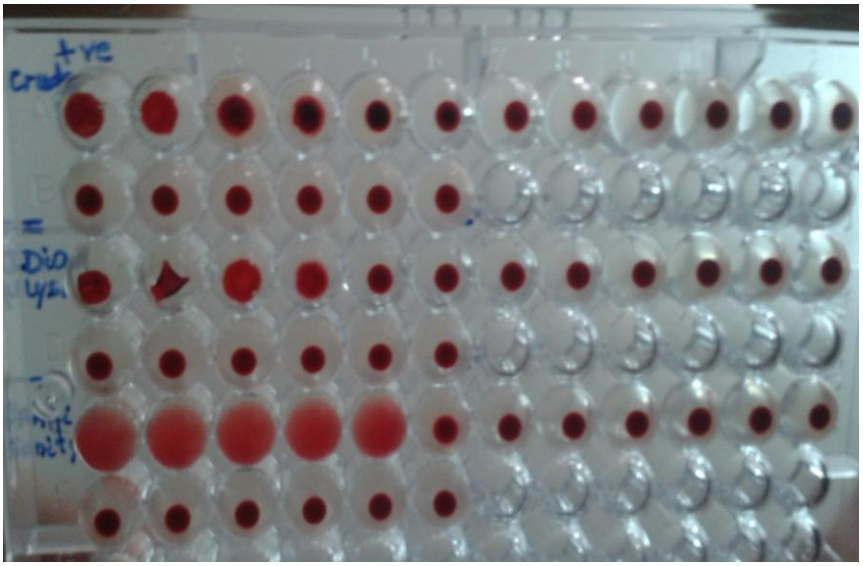


Figure 6: Haemagglutination assay.

Table 1: Haemagglutination result.

Sample	Ha value
Crude	1:22
Dialysis	1:24
Affinity chromatography	1:25

1) Crude HA = 1:22, means that the protein was tittered by hem agglutination assay (HA), and the endpoint for the assay was a dilution of 1:22, that is 1:4.

2) Dialysis HA=1:24, means that the protein was tittered by hemagglutinationassay (HA), and the assay was a dilution of 1:24, that is 1:16.

3) Affinity HA=1:25, means that the protein was tittered by hem agglutination assay (HA), and the assay was a dilution of 1:25, that is 1:32.

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

Table 2: Estimation of protein concentration by lowry's method.

Sample	o.d at 595nm	Concentration(mg/ml)
Crude	1.354	2.46
Dialysis	1.336	1.75
Affinity chromatography	1.325	1.43

Inference

The eluted protein concentration check was done by Lowry's method and was found to 10 mg when calculated with total volume and this was collected by lyophilisation and is further.

CONCLUSION

Pea lectin is a weak agglutinin. As it contains lectin, it is assumed to shows anti-cancer property. Further researches are required for its application in different cancer cell lines to see whether it has inhibitory action on proliferation.

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