GSC Advanced Research and Reviews, 2020, 05(03), 001–009

Available online at GSC Online Press Directory

GSC Advanced Research and Reviews

e-ISSN: 2582-4597, CODEN (USA): GARRC2

Journal homepage: https://www.gsconlinepress.com/journals/gscarr

(RESEARCH ARTICLE)

Purification and Characterisation of Lectin Isolated from Nigeria *Achatina achatina* Snail.

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Publication history**:** Received on 03 November 2020; revised on 16 November 2020; accepted on 20 November 2020

Article DOI: https://doi.org/10.30574/gscarr.2020.5.3.0095

Abstract

Lectins are carbohydrate-binding proteins that are highly specific for sugar moieties of other molecules. They perform recognition on the cellular and molecular level and play numerous roles in biological recognition phenomena involving cells, carbohydrates, and proteins. Blood groups are inherited characters which give rise to antigen-antibody reaction. A total of 120 samples of local (Nigeria) *Achatina achatina* snail specie were collected, authenticated at the Zoology Department of the University of Nigeria, Nsukka and 80mls of pooled crude lectin extract was obtained. Purifications were performed on 20mls of the crude extract in three steps viz, Ammonium sulphate precipitation and Dialysis (Partial purifications), Con A Sepharose 4B affinity chromatography column (Complete purification). The affinity purified lectin was used for all the actual tests conducted in this research. The crude, partially and complete/affinity purified lectin extracts were subjected to Haemagglutination tests, Protein Assay and Specific Sugar determinations. The molecular weight was assessed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The results of the research showed as follows: On complete/affinity purification, 15mls of pure sample containing only the high molecular weight lectin was obtained. The respective haemagglutination tests on the crude, partially and affinity purified lectin showed on standardisation, preferential agglutination with Blood group A type. The Protein contents of the lectin was deduced to be as follows: The crude extract contains 13.5mg/dl, Dialysed precipitate – 5.7mg/dl, Dialysed supernatant – 5.0mg/dl and the Affinity purified Lectin – 0.422mg/dl. Galactose N-acetyl amine (Gal NAc) residue was determined to be its specific sugar. The SDS-PAGE analysis showed the molecular weight of the lectin to be 250 KDaltons. This research has therefore succeeded in the Purification, Characterisation and illustration of the lectinic properties of the local Nigeria snail - *Achatina achatina*.

Keywords: *Achatina achatina* Lectin Purification and Characterisation

1. Introduction

Lectins are complex multi domain proteins, but sugar binding activity can usually be ascribed to a single protein module within the lectin polypeptide. Such a module is designated a carbohydrate recognition domain (CRD). Each molecule of lectins has two or more sites and each of these sites is complementary for a molecule or more, of sugar. These sugars (carbohydrates) exhibit varied interactions with corresponding or specific lectins. The presence of two or more binding sites for each lectin molecule allows the agglutination of many cell types (Krispin, 2006). The specificity of the binding

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sites of lectins suggests that there are endogenous saccharides receptors in the tissue with which the lectin is specialized to interact; the multi valency of the receptor is a prerequisite for recognition (Sharon and Lis, 2003). The carbohydrate specificity of the lectins is usually determined by carrying out sugar inhibition of haemagglutination activity of the lectins. The attachment of lectins to the sugar on cell surface will result in agglutination (Goldstein and Poretz, 2000). There are several forms/types of sugars found on the red cell membrane viz, galactose, fucose, glucose, mannose, N-acetyl galactoseamine (NAGA), N-acetyl neuraminic acid (NANA) (Ukaejiofo, 2005).

Most lectins studied to date are multimeric, consisting of non- covalently associated subunits. A lectin may contain two or more of the same subunit, such as *Concanavalin A*, or different subunits, such as *Phaseolus vulgaris* agglutinin. It is this multimeric structure which gives lectins their ability to agglutinate cells or form precipitates with glyco-conjugates in a manner similar to antigen-antibody interactions (Weis and Drickamer, 1994). Although certain insecurities concerning their chemical characterization exist, lectins are increasingly used in medicinal pure research. They are used for the characterization of certain cell types or fragments (like different membranes), to detect cells in different states of development to distinguish normal from tumour cell, to mark the different states of cell cycle and to separate different cell types by affinity chromatography (Sengbusch, 2004).

In 1900 Dr. Karl Landsteiner discovered the ABO blood group system through his Iso-agglutination experiment in which he mixed red cell from one person with sera from another person that led to discovering A, B, O groups and In 1945, William Boyd discovered the plant, *Phaseolus linensis* as blood group A specific. That is, the plant extract agglutinated the red cells of human blood type A but not those of O and B. In 1950s Walter J.T. Morgan and Winifred M. Watkins found that the agglutination of human type A red cells by lectin was best inhibited by linked N-acetyl-D-galactose and that of type O cells by the lectins of *L. tetragonolobus* was best inhibited by linked L-fucose. They concluded that N-acetyl-D-galactosamine and L-fucose are the sugar determinant conferring A and H (O) blood group specificity respectively (Dutta, 2006).

Proteins are macro molecules which on hydrolysis yield practically nothing except a mixture of amino acids. In the proteins, the amino acids are linked by peptide bonds. The peptide bond is formed by joining the amino group of one amino acid to the carboxyl group of a second. The analytical techniques of gel filtration, chromatography, centrifugation etc are all important in obtaining a pure protein. Here, it is vital to use methods which do not destroy the delicate protein chains in the process of handling them. Molecular weights can also be accurately determined by the process known as gel electrophoresis, if it is carried out in the presence of a detergent, Sodium Dodecyl Sulphate (S.D.S) (Rose, 1982).

Sugars can be written according to the general formula CnH_2nOn; in the simplest case, n = 3. Sugars which contain the aldehyde group - CHO are aldoses, those which contain the ketone group - Co are ketoses. Two possible structures for glyceraldehydes can be drawn, which differ from one another in no other respect but that one is the mirror image of the other (Rose, 1982).

Due to the numerous end uses applications of lectins and the need to produce at cheaper rate indigenous reagents from local sources for routine diagnosis of many disorders including treatment, agglutination and identification studies inform the basis of embarking on this research. Although this research has some limitations including locally unavailable, scarce or very expensive molecular equipment, chemicals, reagents, expertise etc, the objective of the research was achieved. This was made possible by high profile molecular research conducted by us at the Department of Advanced Diagnostics and Biosensors, Cranfield University, Cranfield, Bedfordshire, England, United Kingdom that enabled the Purification and Characterisation of this snail lectin. The specific objectives of this research were to: 1. Isolate/Extract *Achatina achatina* snail lectin (a local snail specie). 2. Purify the crude lectin extract. 3. Determine the Human blood group substances that specifically agglutinate the lectin. 4. Protein Assay of the lectin. 5. To assess the Specific Sugar of the lectin. 6. Deduce the Molecular Weight of the *Achatina achatina* snail lectin.

2. Materials and methods

One Hundred and Twenty (120) samples of the local *Achatina achatina* snail (Ejuna Ojii) were collected for analysis. Their albumin glands were obtained by dissection, weighed and extracted according to the methods of Hammarstrom and kabat, 1969. The crude extracts were mixed with sodium azide preservative and stored frozen at -20oC ready for use.

2.1. Purification of the Crude Extracts

The crude *A. achatina* extracts were purified employing: A. Ammonium Sulphate Precipitation. B. Dialysis and C. Affinity Chromatography purification methods and were carried out based on the following principles:

2.1.1. Ammonium sulphate precipitation method

This is a simple and effective means of fractionating proteins and is based on the fact that at high salt concentrations the natural tendency of proteins not to aggregate is overcome, since the surface charges are neutralized. Charge neutralization means that proteins will tend to bind together, form large complexes and hence are easy to precipitate out by mild centrifugation.

2.1.2. Dialysis method

Dialysis is a classic laboratory technique that relies on selective diffusion of molecules across a semi-permeable membrane to separate molecules based on size.

2.1.3. Affinity chromatography method

The Affinity Chromatography column of choice used in this research for the purification of the *Achatina achatina* lectin is Con A Sepharose 4B (HiTrap Con A 4B). Con A Sepharose 4B is a chromatography medium for separation and purification of glyco-proteins, polysaccharides and glyco-lipids. Con A Sepharose is an affinity medium with Concanavalin A (Con A) coupled to Sepharose 4B by the cyanogens bromide method. HiTrap Con A can be operated with a syringe, a peristaltic pump or a liquid chromatography system such as AKTA design (Ge Healthcare, 2014).

2.2. Method of analysis

Agglutination tests were carried out on the crude, partially purified and affinity chromatography purified extracts using scrupulously cleaned precipitation tubes in the standard tube technique and examined macroscopically and microscopically. The separately pooled and washed human ABO cells were washed four times in saline, and 5% suspension of the cells were made and used for the agglutination tests both in the control test and actual tests as follows:

2.2.1. Control test

Equal volumes of the commercially prepared anti-sera were mixed with separately pooled and washed human ABO cells and examined after five (5) minutes at room temperature.

2.2.2. Actual test

Equal volumes of the crude, ammonium sulphate, dialysed, (partially purified) and Affinity purified (Completely purified) *Achatina achatina* snail extracts were each separately mixed with the separately pooled and washed human ABO cells and their reactions examined macroscopically and microscopically. The crude extract, ammonium sulphate, dialysed and Affinity purified extracts were subjected to protein assay using Pierce BCA Protein Assay Kit method.

2.3. Protein assay of the crude, partially purified and affinity purified *A. achatina* Extracts

2.3.1. Extracts Protein Assay *Using Pierce BCA Protein Assay Kit Method (Thermo Scientific)*

The Thermo Scientific Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of Cu++ to Cu+ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu+) using a unique reagent containing bicinchoninic acid. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion.

2.3.2. Preparation of standards and working reagent

Preparation of diluted albumin (BSA) standards

- The dilution scheme for standard test tube and micro plate procedures, the working range of 20–2000ug/ml was chosen because the isolated lectin is supposed to be a high molecular weight protein. Thus, various concentrations of the BSA standards were prepared or diluted using Phosphate buffered saline diluents in test tubes.
- The various concentrations of the BSA standards were properly mixed using Vortex Genie 2 and transferred into micro plate wells by means of automatic pipettes.
- Thereafter, the Pierce BCA protein assay reagents A and B were mixed together in a separate clean tube in a ratio of 50 volumes of reagent A to 1 volume of reagent B.

- Using automatic pipettes, 200uL of the working BCA reagents mixture were transferred into the micro plate wells containing the various concentrations of the BSA standards (A – I) and the lectin extract sample under assay.
- The samples in the micro plate wells were rocked to mix and incubated at 37 ^{0}C for 30 minutes and protein content viewed and estimated.
- At the end of the incubation period, the micro plates contents were read using Varioskan Flash UV/Visible and Flourescence Spectrophotometer (Micro plate reader).
- The absorbance values for the BSA standards and sample(s) were deduced and plotted, the absorbance on the vertical axis and BSA standard concentrations on the horizontal axis.
- By using the equation: Y= 0.0009X + 0.1205, X (which is the concentration of the unknown) can be calculated.

2.4. Determination of specific sugar

PRINCIPLE: The sugar specificity of the high molecular weight lectin was tested by inhibiting the haemagglutination activity using simple sugars. The results were expressed as the minimal concentration of carbohydrate required to inhibit four haemagglutinating dose units (HDU) of the lectin effectively (Ito et al., 2011).

2.4.1. Procedure

- The sugars used for the haemagglutination inhibition experiments viz, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, D-Fucose, D-glucose, D-Mannose and D-Galactose were diluted to various concentrations.
- The *A. achatina* lectin was diluted to various concentrations (serial double dilutions) in a micro plate wells using Phosphate buffered saline.
- Rabbit erythrocyte (5% suspension) was added to the various dilutions of the lectin and agglutination reactions noted.
- Inhibition of the *A. achatina* lectin haemagglutinating activity against the rabbit erythrocyte was tested with the various concentrations of the variety of saccharides in the micro plate wells.
- The concentration of each saccharide leading to complete inhibition was determined.

2.5. Determination of the Molecular weight of the Affinity purified *Achatina achatina* Lectin

The analytical techniques of Soduim Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS - PAGE), Gel filtration, Ultracentrifugation etc are all important in obtaining or isolating a pure protein including its molecular weight determination. However, SDS – PAGE is usually the first choice as an assay of protein purity due to its reliability and ease. The presence of SDS and the denaturing step causes proteins to be separated approximately based on size, although aberrant migration of some proteins may occur. Also, different proteins may stain differentially. Therefore, the SDS-PAGE technique is the method of choice used in this research for the determination of the molecular weight of the *Achatina achatina* snail lectin.

Principles of SDS – PAGE: An electric field is applied across the Gel, causing the negatively charged proteins to migrate across the Gel towards the positive (+) electrode (anode). Depending on their size, each protein will move differently through the Gel matrix: Short proteins will be more easily fit through the pores in the Gel; while larger ones will have more difficulty (they encounter more resistance). After a set amount of time (usually a few hours – though this depends on the voltage applied across the Gel; higher voltages run faster but tend to produce somewhat poorer resolution), the proteins will have differentially migrated based on their size; smaller proteins will have travelled further down the Gel, while larger ones will have remained closer to the point of origin. Proteins may therefore be separated roughly according to size (and thus molecular weight); however, certain glyco-proteins behave anomalously on SDS Gels (Wikipedia, 2011).

3. Results

The results of the analysis obtained in this research are as illustrated below in Figures 1, 2, 3, 4, 5, 6 and 7. Figures 1 and 2 show the Haemagglutination patterns of the *A. achatina* lectin in saline control and with Blood group A Cells respectively. Figure 3 is the Bicinchoninic acid (BCA) Protein Calibration curve performed with Bovine Serum Albumin (BSA) Standards. Figure 4 illustrates the photograph of the micro plate containing the BCA protein content Quantification analysis of the standards, the crude, dialysed/partially purified *A. achatina* lectin samples (neat and 10 times diluted). Figure 5 shows the photograph of Micro plate of BCA Protein Quantification of the affinity purified *A. achatina* lectin. Figure 6 demonstrates the Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) migration bands patterns of the crude, partially purified and the completely /affinity purified *A. achatina* lectin as well as the molecular weight markers/standards. Figure 7 is the annotated SDS-PAGE migration bands patterns of the crude,

partially purified and the affinity/completely purified *A. achatina* lectin compared with the known molecular weight markers/standards. The results of the analysis are as shown below:

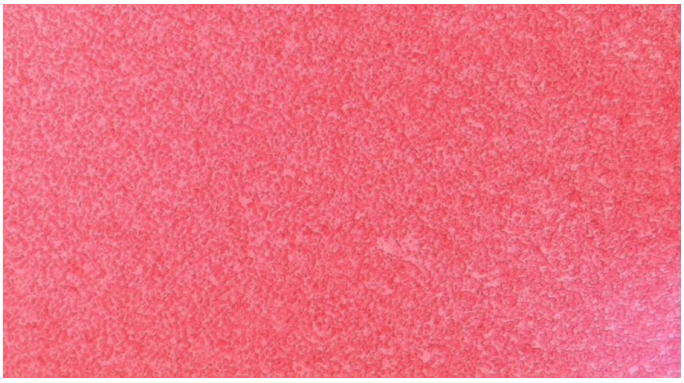


Figure 1: 10 x Photomicrograph of phosphate buffered saline haemagglutination negative control.

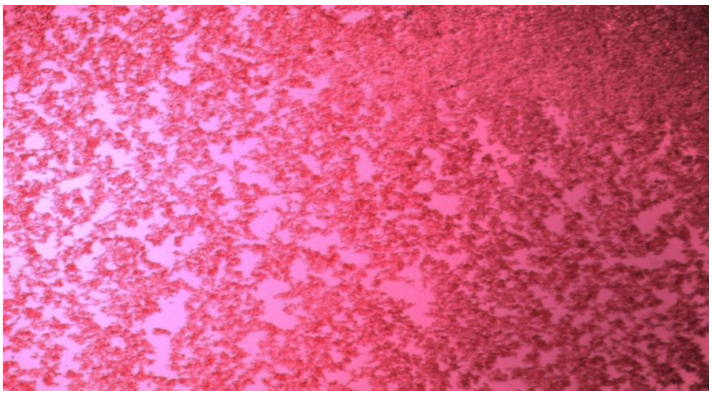


Figure 2: 10 x Photomicrograph haemagglutination pattern of the *Achatina achatina* lectin with human blood group A cells.

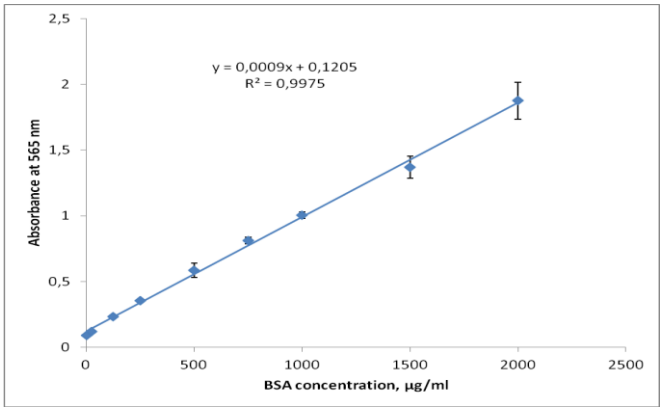


Figure 3: BCA protein calibration curve performed with BSA standards.

Samples were analysed neat and diluted 10 times. The neat samples were too concentrated and could not be used to calculate the protein content. The samples were diluted ten times and after fitting the absorbance values to the calibration curve (and multiplication by 10) the following concentrations were found:

Crude = 13.5 mg/ml
Dialysed Precipitate = 5.7 mg/ml
Dialysed Supernatant = 5.0 mg/ml
Affinity Purified = 0.422mg/ml

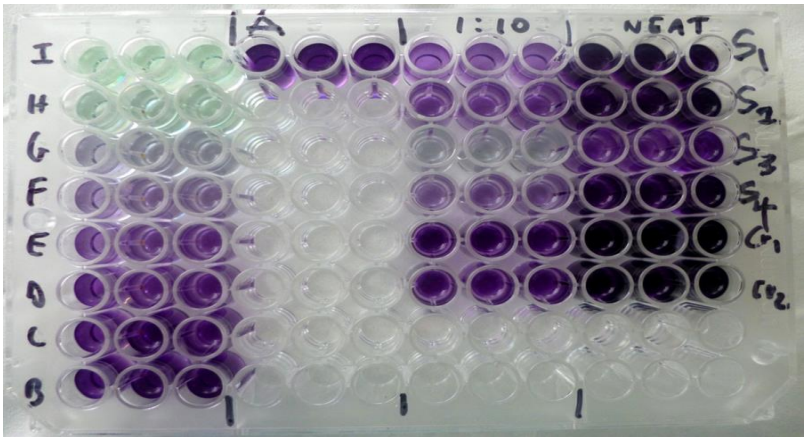


Figure 4: The photograph of the micro plate containing the BCA protein content quantification analysis of the standards, the crude and dialysed/partially purified *Achatina achatina* lectin samples (neat and diluted 10 times). Standards and samples were analysed in triplicates.

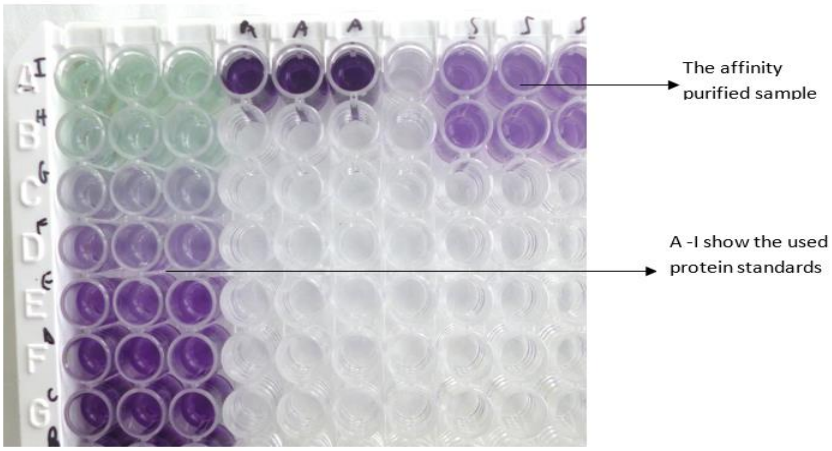


Figure 5: Photograph of Micro plate of BCA protein quantification of the affinity purified *A. achatina* lectin**.**

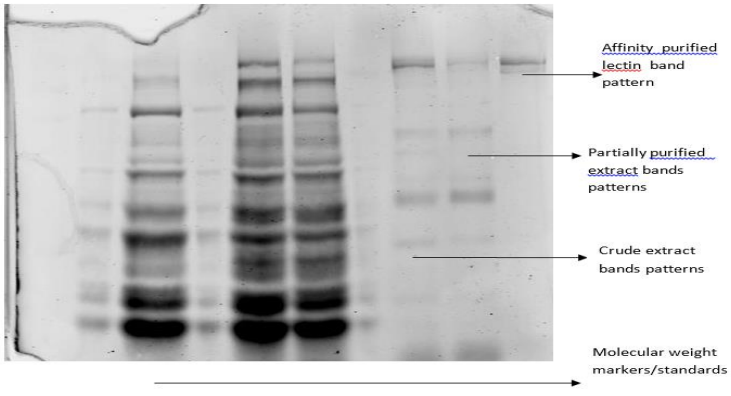


Figure 6: Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) migration bands patterns of the crude, partially purified and completely/ affinity purified *Achatina achatina* lectin (on the right) as well as molecular weight markers/standards (on the left).

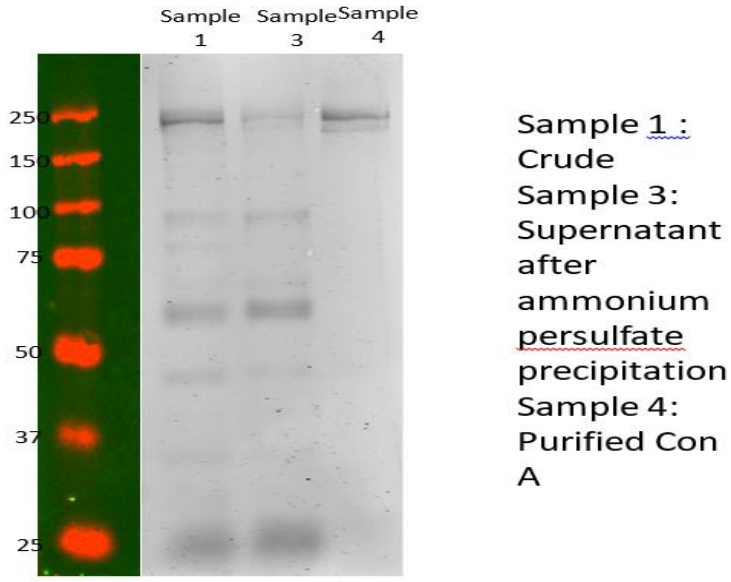


Figure 7: The annotated SDS-PAGE migration bands patterns of the crude, partially purified and affinity/completely purified *A. achatina* lectin compared with the known molecular weight markers/standards.

4. Discussion

In this research, the *Achatina achatina* snail lectin was characterised as much as possible and with particular attention to its haemagglutination properties, the lectins SDS-PAGE electrophoretic mobility band pattern aimed at determining its molecular weight, as well as its activities in presence of various monosaccharides geared towards deducing its specific sugar and the colorimetric detection and quantification of the total protein content of the lectin. The Ammonium sulphate and Dialysed partially purified active combined snail extracts and which demonstrated lectinic activities were next completely purified or isolated by means of Con A Sepharose 4B Affinity chromatography column method (Ito et al., 2011; Shapiro et al., 1967; Weber and Osborn, 1969; Caprette, 2009).

The mechanism of haemagglutination by lectins relies upon the bridging of red cells with the extract molecules coupled with the specific lattices of the erythrocytes. Since several thousand sites for each antigen are present on each single erythrocyte, there is ample opportunity for the lattice formation needed to create easily visible clumps of erythrocytes. One other factor that influences the cross reactions between lectins and human blood groups, border on their combination with several sugar components on the red cell membrane. Thus the Haemagglutination activity assay performed in this research depicts the *A. achatina* extract as a glyco-protein with lectinic properties which is quite in agreement with the works of Hammerstrom and Kabat, 1969; Tsutsui et al., 2003; Ukaejiofo and Odiegwu, 2010; Ito et al., 2011 etc. The colorimetric detection and quantification of total protein content of the lectin was carried out using Thermo Scientific Pierce BCA Protein Assay which is a detergent-compatible formulation based on bicinchoninic acid (BCA). The sugar specificity of the high molecular weight lectin was tested by inhibiting the haem-agglutination activity using simple sugars. The results were expressed as the minimal concentration of carbohydrate required to inhibit four haemagglutinating dose units (HDU) of the lectin effectively (Ito et al., 2011). The results obtained from this research showed as follows: The Crude, Ammonium sulphate precipitate, Dialysed and Affinity Chromatography purified *A. achatina* snail lectin extracts, all cross reacted in different agglutinating strengths with human ABO blood cells and at dilution of 256, reacted only with group A type and thus possess lectinic properties. This is in support of the works of Hammerstrom and Kabat, 1969; Ito et al., 2011.The Phosphate buffered saline negative control and the Haemagglutination pattern of the *A. achatina* lectin with human blood group A cells respectively are shown in Figures 1 and 2. The molecular weight of the affinity purified lectin as illustrated in Figures 6 and 7 was found to be 250kDa. The concentration of each saccharide leading to complete inhibition was determined and it was deduced that N-acetyl-D-galactosamine (D-GalNAc) inhibited the agglutinating activity at high concentrations. These findings have some relationships with the works of Ito et al., 2011; Tsutsui et al., 2003. The results of the protein assay as presented in Figures 3, 4 and 5 show that the *A. achatina* snail extracts are high molecular weight proteins and since they agglutinated ABO cells means that they are glyco-proteins with lectinic properties. The protein contents were deduced to be as follows: Crude = 13.5 mg/ml, Dialysed Precipitate = 5.7 mg/ml, Dialysed Supernatant = 5.0 mg/ml and Affinity Purified = 0.422mg/ml.

5. Conclusion

The research findings show that the *Achatina achatina* snail extract demonstrated lectinic properties as it cross reacted with human ABO erythrocytes. The reaction with human ABO cells was at different agglutinating strengths and at titre of 256 it reacted specifically with human blood group A type. The affinity purified snail lectin was found to be a high molecular weight protein with protein content of 0.422mg/ml and molecular weight of 250kDa, that show specificity with Gal-NAc residues of glyco-proteins. This research has therefore succeeded in the Purification and Characterisation of the Isolated Nigeria *Achatina achatina* snail lectin.

RECOMMENDATIONS The importance end uses applications of lectins, *A. achatina* lectin inclusive in Molecular Medicine/Biology, Biomedical and Life Sciences cannot be over emphasised. Therefore, it is hereby recommended that further research activities on this and other local animals and plants lectins be intensified with a view to commercially producing local or indigenous lectins products that could serve as an adjunct or potent agents for treatments and routine diagnosis of many health disorders.

Compliance with ethical standards

Acknowledgments

We are ever grateful and hereby acknowledge the Tertiary Education Trust Fund (TETFund) of Nigeria that gave Research grant to the corresponding Author which made possible this high profile molecular research to be conducted by us at the Department of Advanced Diagnostics and Biosensors, Cranfield University, Cranfield, Bedfordshire, England, United Kingdom and hence enabled the Purification and Characterisation of this snail lectin. This is because, this research has some limitations including locally unavailable, scarce or very expensive molecular equipment, chemicals, reagents, expertise etc, but through the TETFund Research grant intervention, the objective of this research was achieved. Thus, we appreciate the corresponding author Re: Dr. C.N.C. Odiegwu for the release of funds to Cranfield University, the expertise, technology, equipment etc. provided by Prof. I.E. Tothill and Dr. I. Chianella of the Department of Advanced Diagnostics and Biosensors, Cranfield University, Cranfield, Bedfordshire, England, United Kingdom which led to the comprehensive analysis of the *Achatina achatina* snail lectin.

Disclosure of conflict of interest

There is no conflict of interest amongst the authors as all the authors contributed in one way or the other in conducting the research and in writing the manuscript which was eventually articulated and sent for publication by the corresponding author.

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2020-12-14
GSC Online Press
https://doi.org/10.30574/gscarr.2020.5.3.0095
Odiegwu CNC, Ukaejiofo EO, Tothill IE, et al., (2020) Purification and characterisation of lectin isolated from Nigeria achatina achatina snail. GSC Advanced Research and Reviews, Volume 5, Issue 3, November 2020, pp.1-9

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