RESEARCH ARTICLE Legume Research- An International Journal, Volume 43 Issue 3: 337-344 (June 2020)

Inhibitory activity of salivary glycoproteins on phytohemagglutins (PHA): Possible molecules to enhance nutritional quality of red kidney beans

Vishwanath B. Chachadi, Tejashwini R. Nayanegali, Bharamappa G. Pujari, Lakshmi V. Umarji, Vasundhara Budyhalamath, Shashikala R. Inamdar, Pi-Wan Cheng[1]

10.18805/LR-4034

ABSTRACT

Food allergy caused by red kidney bean (Phaseolus *vulgaris* L.) is of serious health concern and is mainly due to its phytohemagglutinins (PHA) content. PHA can enter the circulation after oral uptake and cause IgE mediated allergy. However, studies describing enhancement of nutritional quality of red kidney beans by targeting PHA are not reported. This study was carried out to identify, PHA-inhibitory molecules present in saliva secretions. Results describe that PHA can be effectively inhibited by salivary glycoproteins. Fractionation of salivary proteins by ammonium sulphate precipitation revealed that, PHA-inhibitory proteins can be specifically precipitated at 30-60% of ammonium sulphate saturation. Gel filtration chromatography and lectin-blot analysis of 30-60% ammonium sulphate fraction suggest that only high molecular weight glycoproteins can act as potent inhibitors of PHA. In conclusion, human saliva secretions contain inhibitory glycoproteins which can be used to inhibit PHA effectively. If these glycoproteins are purified to homogeneity, can be used as potent food supplements in order to neutralize allergic potential of PHA, thus increasing the nutritional value of red kidney beans.

Key words: Allergy, Glycoproteins, Mucins, Phytohemagglutinins, Red kidney beans, Saliva.

INTRODUCTION

Food allergy, mainly caused by food proteins (antigens) occurs through cross-linking of immunoglobulin isotype E (IgE) on the surface of mast and/or basophil cells (Sutton and Gould, 1993). This type of allergy may be different from food sensitivity that is caused by components of food such as lactose (Heine, 2015). Common food induced allergies include cereal-induced allergies, particularly wheat-induced allergies. It has been well documented that more than 400 allergens cause food allergies (Boyce *et al.,* 2011). The sensitive individuals may experience adverse reactions such as anaphylaxis and in some instances the reaction can be lethal.

Although most of the proteins are degraded into non-immunogenic peptides while travelling through GI tract, but some proteins may escape and enter enteric vasculature and cause allergy (Sampson, 2004). One of the mechanisms which helps them to escape may include aggregation, which occurs after exposing to high temperature (Zamindar *et al.,* 2016). For instance, vicilin becomes resistance to proteolytic digestion after exposing to high temperature (Beyer *et al.,* 2001; Mankotia and Modgil, 2016). On the contrary, high temperature can reduce the allergenic property of egg ovalbumin and ovomucoid (Martos *et al.,* 2011). Proteins of such calibre which have survived during GI digestion, may bind and enter into the mucus-covered epithelial cells or directly absorbed by immune cells and cause allergy (Sicherer and Sampson, 2010).

Most of the food allergies are due to legumes such as red kidney beans (*Phaseolus vulgaris*), peanut, soybeans, red gram, chickpea, black gram, green gram, lentils, fenugreek and green bean (Verma *et al.,* 2013; Kamboj and Nanda, 2018). Red kidney bean is a routinely consumed bean, which is reported to induce allergic reaction in many individuals (Misra *et al.,* 2009; Kumar *et al.,* 2013) and hence the allergenic potential of red kidney bean is a matter of concern. Protein content of red kidney bean is composed of storage proteins such as phaseolin and other proteins including lectins, arcelin and a-amylase inhibitor (Yamaguchi, 1993; Fitches *et al.,* 2001; Yin *et al.,* 2011; Modgil *et al.* (2016). The lectin comprising leucoagglutinating phytohemagglutinin (PHA-L) and erythroagglutinating phytohemagglutinin (PHA-E) (Fitches *et al.,* 2001). Several

Department of Studies in Biochemistry, Karnatak University, Dharwad-580 003, Karnataka, India.
[1]Veterans Affairs Nebraska and Western Iowa Healthcare System, Omaha, NE, USA.

Corresponding Author: Vishwanath B. Chachadi, Department of Studies in Biochemistry, Karnatak University, Dharwad-580 003, Karnataka, India. Email: vish2879@gmail.com; vbchachadi@kud.ac.in

How to cite this article: Chachadi, V.B., Nayanegali, T.R., Pujari, B.G., Umarji, L.V., Budyhalamath, V., Inamdar, S.R. and Cheng, P.W. (2020). Inhibitory activity of salivary glycoproteins on phytohemagglutins (PHA): Possible molecules to enhance nutritional quality of red kidney beans. Legume Research. 43(3): 337-344.

Submitted: 01-05-2018 **Accepted:** 20-08-2018 **Published:** 15-11-2018

studies regarding PHAs have been carried out to reveal blood grouping, erythrocyte polyagglutination activity, mitogenic stimulation of lymphocytes, lymphocyte subpopulation studies, fractionation of cells and other particles, histochemical studies of normal and pathological conditions (Venter and Thiel, 1995; Sengupta *et al.,* 2001). Further, it was also found that phaseolin and PHA can act as strong allergens causing serious anaphylaxis leading to leaky gut by interacting with glycans of intestinal cells (Rouge *et al.,* 2011). This is the main cause for potent autoimmune destruction that can result when the intestinal lining experiences this level of damage. Hence it is very important to address an issue on allergic potential of PHA.

It is well documented that all of the lectins can be inhibited by their specific simple/complex sugar derivatives (Sharon, 2007) These specific sugars can be found naturally either in free form or in conjugation with proteins know as glycoproteins. Natural glycoproteins such as mucins which contain 80-85% of carbohydrates are potent molecules to inhibit activity of lectins. Mucins are primary gel-forming components of mucus occurring as major constituents of saliva which nearly accounts 60-70% of dry weight (Nielsen *et al.,* 1997).

Since PHA can be completely inhibited by interacting with its cognitive complex sugar moieties/glycoproteins, it is the best way to inhibit using these sugars/glycoproteins, before it could enter into the digestive system and elicit allergic reactions. Given the fact that lectins are effectively inhibited by glycoproteins and all the salivary mucins are glycoproteins, we exploited these properties to identify whether any salivary fraction from healthy normal could inhibit PHA activity effectively. If so, these salivary fractions can be purified to homogeneity, cloned and produced in bulk and can be supplemented as food additives to minimize allergic implications of PHA.

MATERIALS AND METHODS

Trypsin (type II-S), Bovine serum albumin (BSA, fraction IV, crystallized), Fetuin, Sodium dodecyl sulphate (SDS), acrylamide, N, N'-Methylene-bis-acrylamide, N, N, N', N'-Tetramethylene diamine (TEMED), commassie brilliant blue R-250, N-hydroxysuccinimidobiotin, DMSO and Sephadex G-200 were from Sigma Chemical Co., St. Louis (USA). PHA-E was obtained from EY laboratories, Inc (USA). Standard protein molecular weight marker from Fermentas Glen Burnie, MD (USA). PVDF membrane (Immobilon-P, 0.2 µ) was purchased from Millipore, Bedford, MA. Enhanced chemiluminescent reagent and X-ray films were obtained from Thermo Fisher Scientific, MA, USA. 96 well U bottom plates were from Tarsons (India). All the buffer salts and the solvent chemicals were of analytical reagent grade and the reagents were prepared in distilled water unless otherwise mentioned.

Preparation of PHA

The mixture containing all forms PHA-isolectins was purified using ovomucoid coupled Sepharose 4B affinity column chromatography. Bound protein fractions were eluted with 150 mM sodium tetraborate, pH 8.0 and dialyzed against 50 mM PBS, pH 7.2.

Preparation of trypsinized erythrocytes

Two ml of whole blood from human blood group A, B and O was collected in 1 ml of 4% sodium citrate solution. The erythrocytes of each blood group were separated under centrifugation at 1500 rpm for 15 minutes, washed three times in saline and finally in PBS and adjusted to an OD of 2.5 at 660 nm. Total volume is measured and final concentration of 0.025% trypsin is added and incubated at 37°C for 1 h. Excess trypsin is removed by repeated washing in saline and finally the OD is adjusted to 3.5 at 660 nm and used for hemagglutination assay and inhibition assays.

Collection of saliva samples

The procedure for non-invasive method of collection and processing of saliva was adapted from earlier reports (Navazesh, 1993; Li *et al.,* 2004). Briefly, unstimulated saliva samples (5 ml) were collected from healthy subjects. The individuals selected were without any risk habits like tobacco chewing, smoking or alcohol abuse. Samples were collected and centrifuged at 2000 rpm, at 4°C for 15 min to remove the cells and other debris. Saliva samples collected from individuals with A, B and O blood groups were pooled and used as pool-1, pool-2, pool3 for A, B and O blood groups respectively. To these preparations, 200 mM EDTA was added and stored as whole saliva at 4°C until further use.

Preparation of p-BSA and biotinylated PHA

Periodate-BSA was prepared according to the method of Glass *et al.*, (1981). BSA (fraction V) in 0.1 M sodium acetate buffer, pH 4.5 (4 g/100 ml) was treated with 10 mM periodic acid for 6 h at room temperature. Excess periodate was removed by adding glycerol to a final concentration of 10 mM and then dialyzing against 10 mM PBS and subsequently against distilled water and freeze-dried. Biotinylation of PHA was carried out according to the protocol described by Duk *et al.,* (1994). In brief, the lectin (2 mg/ml in PBS) was mixed with 20-fold molar excess of N-hydroxysuccinimidobiotin prepared in DMSO and left for 1 h at room temperature. The biotinylated lectin was dialyzed for 2-3 h against double distilled water and then dialyzed extensively against tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.3) at 4°C.

Hemagglutination assay (HA)

Hemagglutination assay was performed according to the protocol described by Liener and Hill (1953) using U-bottom 96-well microtitre plates. During assay, each well of the plate received 50 µl of saline followed by 50 µl of assay solution (1mg/ml of lectin) to first well of each row, and a twofold serial dilution was made across the plate until well number 11. From well number 11, 50 µl was discarded. Trypsinized erythrocytes of each blood groups were added (50 µl/well) to each row. For control, well containing only saline and

erythrocytes were included. The plates were incubated at room temperature for 1 h and visualized and photographed. The highest dilution of the sample causing visible agglutination was arbitrarily considered as the "titre".

Fractionation of salivary proteins by ammonium sulphate saturation

Whole saliva was diluted (1:1) with PBS and subjected to 0-30, 30-60 and 60-80% ammonium sulphate precipitation at room temp using 24 ml of diluted saliva sample. After collecting precipitate from each step, the supernatant is measured and ammonium sulphate concentration is raised and proteins were allowed to precipitate. The supernatant is saved as another fraction and precipitates from all the steps were resuspended in PBS and dialyzed extensively against PBS and stored at -20 C until further analysis.

Hapten inhibition assay

Inhibition assays were carried out by incubating the lectin sample with serially diluted haptens and salivary protein fraction prior to the addition of erythrocytes. The lowest concentration of the sugar/glycoprotein, which inhibited the agglutination, was taken as the inhibitory titre of the hapten. 25 µl of lectin solution containing thrice the minimum hemagglutination dose (titre 8) was added to each well containing 25 µl of serially diluted saliva fraction under test up to 9th well. To the 10th well, saline is added in place of saliva solution, while in 11th well, saline is added in place of lectin. These wells served as both positive and negative controls respectively for inhibition studies. 12th well served as regular control which had received only 50 µl of saline and erythrocyte suspension. Lectin and saliva samples were mixed and incubated for 1 h at room temperature and then 50 µl of erythrocyte suspension of "O" blood group was added and incubated further for 1 h at room temperature. Finally, Inhibition of lectin activity was visualized and photographed. Minimum inhibitory concentration (MIC) which is defined as "The lowest concentration of the sugar/ glycoprotein, inhibiting agglutination" was determined for each fraction.

Sodium-Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins from 30-60% ammonium sulphate precipitated saliva fraction were separated on 7% acrylamide gel. Protein sample was treated with 4x SDS buffer and boiled for 5 mins at 100°C. Cooled and 200 or 400 µg protein was loaded in to the wells and electrophoresed at 100 V for 2.5 hours. After completion of electrophoresis, gels were subjected to commassie brilliant blue R-250 staining. A standard molecular weight protein ladder ranging from 60-250 kDa was also processed and electrophoresed as described above.

Separation of proteins by gel-filtration chromatography

To separate further and to determine the number of protein present in the fraction 30-60% saturation, gel filtration chromatography was performed on a column (75×1.0 cm) of Sephadex G-200 equilibrated with PBS. Total 1.5 mg of protein sample was loaded on to column and fractions of 0.75 ml were collected at a flow rate of 15 ml/h. The elution of the proteins was monitored by measuring the absorbance at 280 nm. Lectin peaks were detected by performing hemagglutination activity of each fractions using human O blood group erythrocytes. Void volume of the column was determined by using blue dextran.

Western blotting

Protein samples precipitated at 30-60% ammonium sulphate saturation were boiled with SDS sample buffer (4x) for 5 min and then run on 4.5% SDS-PAGE (7.5 cm×8.5 cm) under reducing conditions. After electrotransfer, the PVDF membrane was blocked with TBS containing 0.05% Tween 20 and 5% (w/v) p-BSA for 60 min. After washing for 5 times with TBST, blot was incubated with 20 µg/ml of biotinylated-PHA prepared in washing buffer for 1 hour at room temperature. After five washings in same TBST buffer, the membrane was treated for 1 h at room temperature with HRP-conjugated streptavidin. The membrane was then washed five times (5 min each) with TBST and once with milli-Q water. Then, the blot was developed with ECL sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden) and photographed.

RESULTS AND DISCUSSION

PHA shows high affinity for human O blood group erythrocyte glycans

Affinity purified PHA was examined for its hemagglutination activity with human A, B and O blood group erythrocytes. Lectin recognized all the blood groups, but showed highest titre (2048) for O and lowest titre (4) for B blood group erythrocytes (Fig 1). Erythrocytes from blood group A showed moderate binding with titre of 16. Since lectin recognized O erythrocytes with highest titre, further studies were carried out with O erythrocytes. These results indicated that PHA did not recognized cell surface blood group antigens and their non-involvement in PHA binding.

Inhibition of PHA hemagglutinating activity by human saliva

In order to determine the inhibitory property of salivary glycoproteins on PHA activity, unstimulated human saliva was processed as described in materials and methods. Freeze dried saliva samples were reconstituted in PBS (1 mg/ml) and used for the inhibition studies along with standard glycoprotein, fetuin. PHA activity is inhibited by all the saliva samples irrespective of blood groups (Fig 2) using O blood group erythrocytes. Interestingly, pool -3 containing saliva samples from individuals having blood group O, showed lower MIC (1.56 µg) compared to saliva of A and B blood groups (6.25 and 12.5 µg respectively). Positive control, fetuin showed significant inhibition (MIC-3.125 µg), while negative control, p-BSA did not inhibited the activity of PHA.

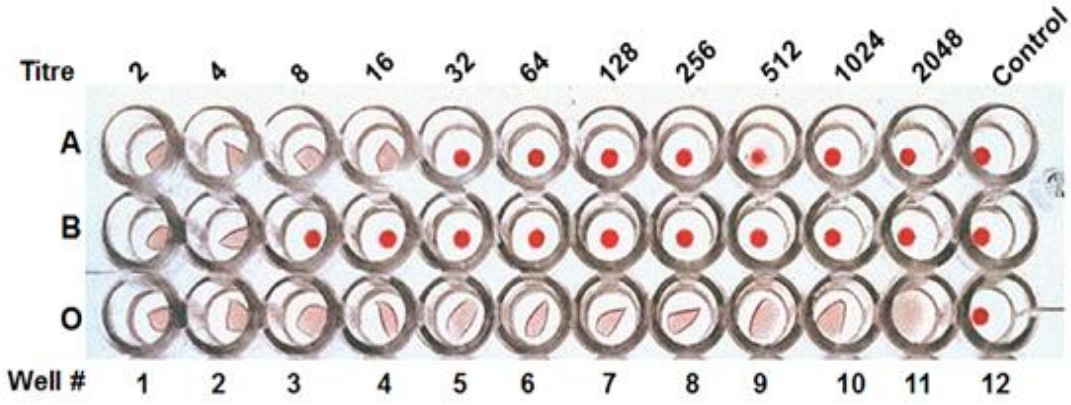

Fig 1: Hemagglutination activity of PHA with human trypsinized A, B and O blood group erythrocytes.

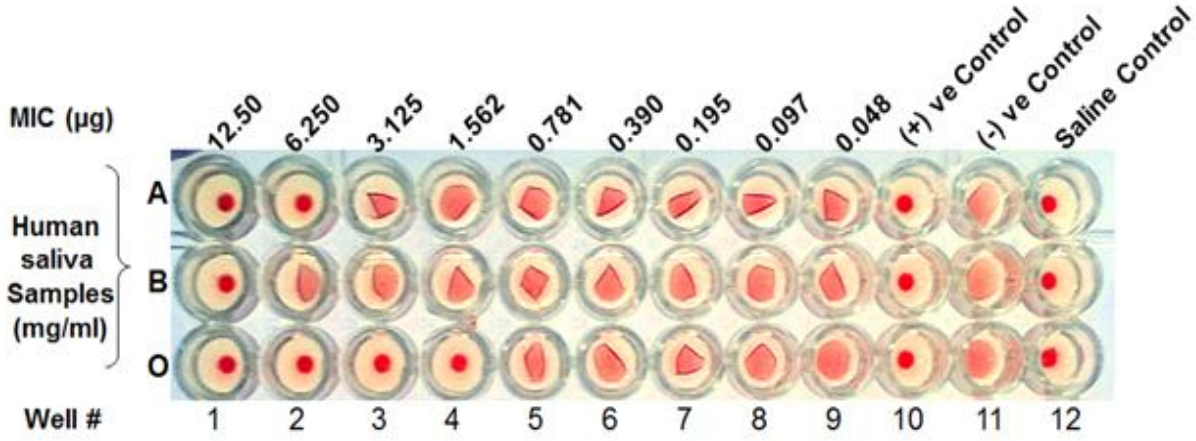

Fig 2: Inhibition of hemagglutinating activity of PHA by human A, B and O blood group saliva.

Ammonium sulphate precipitation of salivary glycoproteins

Next, we fractionated saliva sample from O blood group individuals (pool-3) to purify and characterize glycoproteins having PHA inhibitory activity. Pool-3 samples were reconstituted in distilled water and subjected to 0-30, 30-60 and 60-80% ammonium sulphate saturation at room temperature. Among different fractions of ammonium sulphate precipitated saliva samples, proteins at 30-60% saturation exhibited strong inhibition with 0.039 µg of MIC (Fig 3A). Proteins precipitated at 60-80% of saturation, did not show any inhibition on PHA activity. However, 0-30% fraction showed slight inhibitory activity (MIC-1.25 µg). SDS-PAGE profiling of 30-60% fraction on 7.5% acrylamide gel-electrophoresis revealed that, there are 10 different major protein bands corresponding to approximate molecular weight of 80, 110, 150, 200, 260, 300 and other proteins are of high molecular weight with more than 300 kDa (Fig 3B).

High molecular weight proteins exhibited strong inhibitory property on PHA activity

To further purify proteins exhibiting inhibitory property, 30-60% ammonium precipitated proteins were subjected to gel-filtration chromatography. As indicated in Fig 4, gel filtration chromatography yielded 5 major peaks (peak 1-5). Hapten inhibition studies with these protein peaks revealed that only peak 1 corresponding to high molecular weight, inhibited the hemagglutination activity of PHA. Since majority of proteins in saliva secretions contain glycoproteins, we pooled fractions corresponding to peak 1 (27-31), dialyzed against buffer and then against water and analyzed by lectin blot assay.

Lectin blot analysis of salivary glycoproteins by biotinylated PHA

Since only high molecular weight proteins have exhibited PHA inhibitory activity, we wanted to confirm these results by lectin blot analysis using biotinylated PHA. Proteins from 30-60% fraction were separated on 7.5% acrylamide gel electrophoresis and then blotted on to PVDF membrane and probed with biotinylated PHA lectin. Binding of biotinylated PHA to glycoproteins was detected with streptavidin-HRP reaction. Fig 5 clearly demonstrate that, PHA detected only high molecular weight proteins well above 300 kDa. Although, there are 5 proteins bands (Band# 6-10) below

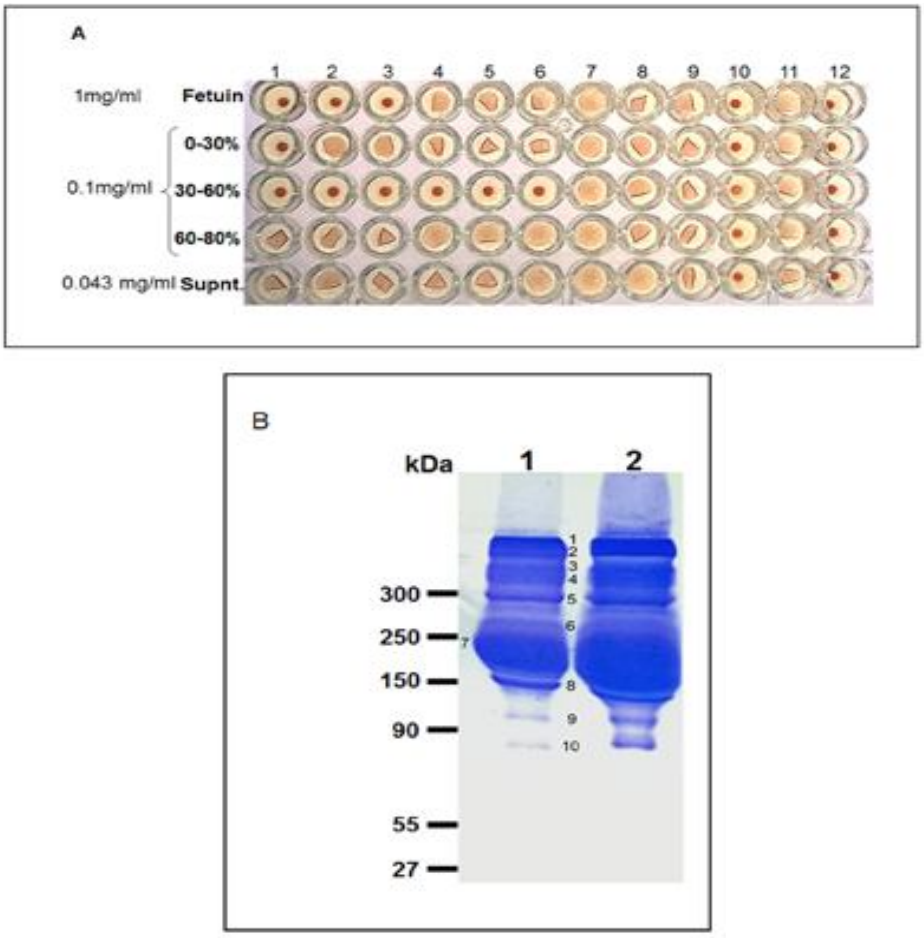

Fig 3: Fractionation of saliva proteins by ammonium sulphate precipitation that inhibited PHA activity.

300 kDa size, with very high intensity band around 200 kDa (Fig 3B), but lectin did not detect any of these bands. Lectin has also detected fetuin which was used as positive control suggesting the true recognition of N-linked glycans in saliva-glycoproteins.

Although there are many reports on detailed composition and allergic property of red kidney beans in humans (Misra *et al.,* 2009; Kumar *et al.,* 2014), information regarding lowering the allergic potential, especially from the perspective of hemagglutinins are scanty. Since major portion of protein content in red kidney bean is PHA and it is known to play vital role in eliciting allergic reaction, it would greatly enhance nutritional value of kidney beans if they are devoid of PHA content. Alternatively, activity of PHA can be neutralized by inhibiting with its potent hapten/s, before it could enter into the cells and elicit allergic stimuli. In this connection, we have made an attempt to determine whether glycoproteins of normal human saliva can exhibit any inhibitory property of PHA. If so, these glycoprotein/s from saliva are identified, purified and characterized completely to use as food additives. This way, inhibited PHA may not get absorbed by intestinal cells, making red kidney beans safe to consume in allergic individuals.

Hemagglutination study using human blood group A, B and O erythrocytes has validated earlier findings that blood group non-specific nature of this lectin and requirement of complex sugars as its haptens. Although there are reports on human A, B, O blood group non-specific nature of PHA, there are no reports describing the preference among these blood groups (Wiener *et al.,* 1969; Manage *et al.,* 1972). However, current study has shown that PHA has more affinity towards O but not A and B blood groups. This result suggested that O erythrocytes expressed high number of PHA-inhibitory glycans on their cell surfaces. In line with this, saliva from blood group O individuals showed very strong inhibition for PHA activity when compared to A and B, suggesting the fact that expression of high concentration of PHA-inhibitory glycans in the saliva secretions of O blood group individuals.

It is well known fact that many types of modifications are observed on salivary proteins including glycosylation, phosphorylation, sulfation, etc., (Pol *et al.,* 2007; Helmerhorst and Oppenheim 2007). Modification of salivary proteins by glycosylation has important functions in the oral cavity such as lubrication and protection of oral cavity and

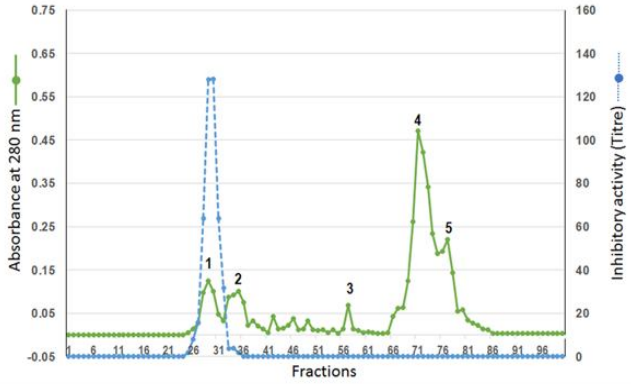

Fig 4: Gel filtration chromatography of 30-60% Ammonium Sulphate precipitate of saliva that inhibited PHA.

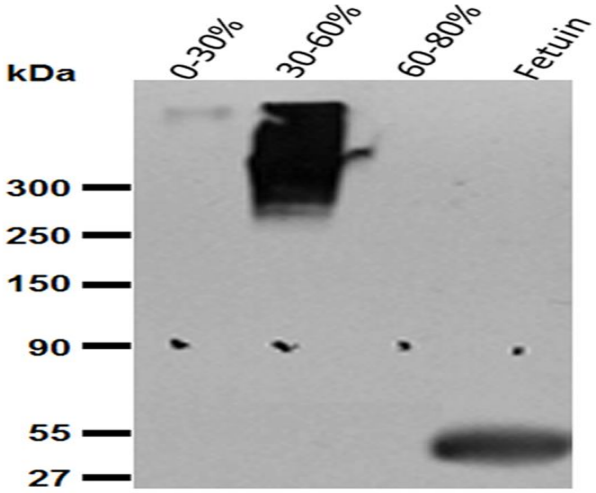

Fig 5: Lectin-blot analysis of saliva proteins.

teeth (Hatton *et al.,* 1985). Glycans make up to 50% of the saliva's weight and some of the salivary glycoproteins involved in lubrication include proline-rich glycoproteins and mucins (Ramachandran *et al.,* 2008). Considering the fact that PHA recognizes trisaccharide Galβ1-4GlcNAcb1-2Man present in the biantennary galactosylated N-glycans (Nagae *et al.,* 2014), and these N-linked glycans are also part of mucins (Guile *et al.,* 1998; Ramachandran *et al.,* 2008; Hall *et al.,* 2017), it is quite possible that inhibitory property of saliva may due these N-linked glycans either in free form or in association with proteins. Results of ammonium sulphate fractionation studies indicated that proteins precipitated at 30-60% of ammonium sulphate have inhibited the PHA activity, suggesting the role of high molecular weight proteins in inhibiting the activity of PHA. Also, supernatant from these fractions did not show any inhibition indicating the absence of free glycans in saliva secretions. In addition, gel filtration chromatography and lectin blot analysis have also suggested that only high molecular weight proteins (< 250 kDa) exhibited inhibitory activity. Since mucins are high molecular weight (>400 kDa) glycoproteins and major constituents of saliva secretions (Zalewska *et al.,* 2000; Pol *et al.,* 2007), we presumed that inhibitory activity of saliva on PHA is mainly due to these mucins.

There are at least 20 identified mucins throughout the human body that cover wet epithelial surfaces such as the gastrointestinal tract, respiratory tract, and eyes. Out of these 20 mucins, MUC1, MUC4, MUC5B, MUC7 and MUC19 were found predominantly in saliva of oral cavity (Linden *et al.,* 2008). Although MUC19 is gel forming mucin in saliva, MUC5B is the primary and predominant gel-forming mucin in the mouth that is secreted by mucous cells in the submandibular, sublingual, palatine, and labial salivary glands (Nielsen *et al.,* 1997; Chen *et al.,* 2004; Rousseau *et al.,* 2008; Culp *et al.,* 2015). Since MUC1 and MUC4 are membrane-associated mucins (Sengupta *et al.,* 2001; Liu *et al.,* 2002) where as MUC 5B is the secretory protein, we predicted that MUC5B is the glycoprotein which may contributing inhibitory property to saliva in neutralizing the activity of PHA. However, this prediction needs further analysis by other specific glycan-lectin interaction studies such as surface plasmon resonance (Lonardi *et al.,* 2013) and isothermal calorimetry (Bouckaert *et al.,* 2013) using purified MUC5B mucin or its glycans.

In conclusion, the findings from our study suggest that PHA can be effectively inhibited by human salivary glycoproteins. If these glycoproteins (mucins) are purified to homogeneity and characterized completely, one can easily target allergenic property of red kidney beans by neutralizing the activity of PHA through these mucins. Additionally, if these glycoproteins are produced in bulk, which can be used as food additives to minimize allergic potential and to increase the nutritional value of red kidney beans.

ACKNOWLEDGEMENT

We thank University Scientific Instrument Center (USIC), Karnatak University, Dharwad for technical assistance.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Beyer, K., Morrow, E., Li, X.M., Bardina, L., Bannon, G.A., Burks, A.W., Sampson, H.A. (2001). Effects of cooking methods on peanut allergenicity. The Journal of Allergy and Clinical Immunology, 107:1077-1081. https://doi.org/10.1067/mai.2001.115480

Bouckaert, J., Li, Z., Xavier, C., Almant, M., Caveliers V, Lahoutte, T., Weeks, S.D., Kovensky, J., Gouin, S. G. (2013). Heptyl alpha-D-mannosides grafted on a beta-cyclodextrin core to interfere with Escherichia coli adhesion: an *in vivo* multivalent effect. Chemistry (Weinheim an Der Bergstrasse, Germany), 19:7847-7855. https://doi.org/10.1002/chem.201204015.

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., Schwaninger, J. M. (2011). Guidelines

for the diagnosis and management of food allergy in the United States: summary of the NIAID-sponsored expert panel report. Nutrition Research (New York, N.Y.), 31: 61-75. https://doi.org/10.1016/j.nutres.2011.01.001.

Chen, Y., Zhao, Y.H., Kalaslavadi, T.B., Hamati, E., Nehrke, K., Le, A.D., Ann, D.K., Wu, R. (2004). Genome-wide search and identification of a novel gel-forming mucin MUC19/ Muc19 in glandular tissues. American Journal of Respiratory Cell and Molecular Biology, 30:155–165. https://doi.org/ 10.1165/rcmb.2003-0103OC.

Culp, D.J., Robinson, B., Cash, M.N., Bhattacharyya, I., Stewart, C., Cuadra-Saenz, G. (2015). Salivary mucin 19 glycoproteins: innate immune functions in Streptococcus mutans-induced caries in mice and evidence for expression in human saliva. The Journal of Biological Chemistry, 290: 2993-3008. https://doi.org/10.1074/jbc.M114.597906.

Duk, M., Lisowska, E., Wu, J.H., Wu, A.M. (1994). The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Analytical Biochemistry, 221: 266-272. https://doi.org/10.1006/abio. 1994.1410.

Fitches, E., Ilett, C., Gatehouse, A.M.R., Gatehouse, L.N., Greene, R., Edwards, J.P., Gatehouse, J.A. (2001). The effects of *Phaseolus vulgaris* erythro- and leucoagglutinating isolectins (PHA-E and PHA-L) delivered via artificial diet and transgenic plants on the growth and development of tomato moth (Lacanobia oleracea) larvae; lectin binding to gut glycoprotein. Journal of Insect Physiology. 47: 1389-1398.

Glass, W.F. 2nd, Briggs, R.C., Hnilica, L.S. (1981). Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Analytical Biochemistry. 115: 219-224.

Guile, G. R., Harvey, D. J., O'Donnell, N., Powell, A. K., Hunter, A. P., Zamze, S., *et al*. (1998). Identification of highly fucosylated N-linked oligosaccharides from the human parotid gland. European Journal of Biochemistry. 258: 623-656.

Hall, S.C., Hassis, M.E., Williams, K.E., Albertolle, M.E., Prakobphol, A., Dykstra, A. B., Laurance, M., Ona, K., Niles, *et al*. (2017). Alterations in the Salivary Proteome and N-Glycome of Sjogren's Syndrome Patients. Journal of Proteome Research, 16: 1693-1705. https://doi.org/ 10.1021/acs.jproteome.6b01051.

Hatton, M. N., Loomis, R. E., Levine, M. J., Tabak, L. A. (1985). Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex. The Biochemical Journal. 230: 817-820.

Heine, R. G. (2015). Food intolerance and allergy. World Review of Nutrition and Dietetics. 113: 195-202. https://doi.org/ 10.1159/000360340.

Helmerhorst, E. J. and Oppenheim, F. G. (2007). Saliva: a dynamic proteome. Journal of Dental Research. 86: 680-693. https://doi.org/10.1177/154405910708600802.

Kamboj, R. and Nanda, V. (2018), Proximate composition, nutritional profile and health benefits of legumes – A review. Legume Research-An international Journal. 41: 325-332, DOI: 10.18805/LR-3748.

Kumar, D., Kumar, S., Verma, A. K., Sharma, A., Tripathi, A., Chaudhari, B.P., Kant, S., Das, M., Jain, S.K. and Dwivedi, P.D. (2014). Hypersensitivity linked to exposure of broad bean protein(s) in allergic patients and BALB/c mice. Nutrition (Burbank, Los Angeles County, Calif.). 30: 903-914. https://doi.org/10.1016/j.nut.2013.11.024.

Kumar, S., Verma, A. K., Sharma, A., Kumar, D., Tripathi, A., Chaudhari, B. P., Das, M., Jain, S. K. and Dwivedi, P. D. (2013). Phytohemagglutinins augment red kidney bean (*Phaseolus vulgaris* L.) induced allergic manifestations. Journal of Proteomics. 93: 50-64. https://doi.org/10.1016/ j.jprot.2013.02.003.

Li, Y., St John, M. A. R., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C. K., Eisele, D., *et al.* (2004). Salivary transcriptome diagnostics for oral cancer detection. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research. 10: 8442-8450. https://doi.org/10.1158/1078-0432.CCR-04-1167.

Liener, I. E. and Hill, E.G. (1953). The effect of heat treatment on the nutritive value and hemagglutinating activity of soybean oil meal. The Journal of Nutrition. 49: 609-620. https:// doi.org/10.1093/jn/49.4.609

Linden, S.K., Sutton, P., Karlsson, N.G., Korolik, V., McGuckin, M.A. (2008). Mucins in the mucosal barrier to infection. Mucosal Immunology. 1: 183-197. https://doi.org/10. 1038/mi.2008.5.

Liu, B., Lague, J.R., Nunes, D.P., Toselli, P., Oppenheim, F.G., Soares, R.V, Troxler, R.F., Offner, G.D. (2002). Expression of membrane-associated mucins MUC1 and MUC4 in major human salivary glands. The Journal of Histochemistry and Cytochemistry/ : Official Journal of the Histochemistry Society, 50: 811-820. https://doi.org/10.1177/00221554 0205000607.

Lonardi, E., Moonens, K., Buts, L., de Boer, A.R., Olsson, J.D.M., Weiss, M.S., *et al.* (2013). Structural sampling of glycan. interaction profiles reveals mucosal receptors for fimbrial adhesins of enterotoxigenic escherichia coli. Biology. 2: 894-917. https://doi.org/10.3390/biology2030894.

Manage, L., Joshi, A., Sohonie, K. (1972). Toxicity to rats and mice of purified phytohaemagglutinins from four Indian legumes. Toxicon/ : Official Journal of the International Society on Toxinology. 10: 89-91.

Mankotia, K., Modgil, R., (2004). Nutritional and physico-chemical quality changes in soaked, sprouted and cooked kidney beans (*Phaseolus vulgaris*). Legume Research-An International Journal. 27: ARCC3822.

Martos, G., Lopez-Exposito, I., Bencharitiwong, R., Berin, M. C., Nowak-Wegrzyn, A. (2011). Mechanisms underlying differential food allergy response to heated egg. The Journal of Allergy and Clinical Immunology. 127: 990-992. https://doi.org/10.1016/j.jaci.2011.01.057.

Misra, A., Prasad, R., Das, M., Dwivedi, P. D. (2009). Probing novel allergenic proteins of commonly consumed legumes. Immunopharmacology and Immunotoxicology. 31: 186-194. https://doi.org/10.1080/08923970802578782.

Modgil, R., Mankotia, K., Verma, R., Sandal, A. (2016). Biological protein quality and phytic acid content of domestically processed kidney beans (*Phaseolus vulgaris*). Legume Research-An International Journal. 35: 315-318. DOI: 10.18805/ajdfr.v35i4.6631

Nagae, M., Soga, K., Morita-Matsumoto, K., Hanashima, S., Ikeda,

A., Yamamoto, K., Yamaguchi, Y. (2014). Phytohemagglutinin from Phaseolus vulgaris (PHA-E) displays a novel glycan recognition mode using a common legume lectin fold. Glycobiology. 24: 368-378. https://doi.org/10.1093/glycob/cwu004.

Navazesh, M. (1993). Methods for collecting saliva. Annals of the New York Academy of Sciences. 694: 72-77.

Nielsen, P.A., Bennett, E.P., Wandall, H.H., Therkildsen, M.H., Hannibal, J., Clausen, H. (1997). Identification of a major human high molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B. Glycobiology. 7: 413-419.

Pol, J., Buczkowska-Radlinska, J., Binczak-Kuleta, A., Trusewicz, M. (2007). [Human salivary mucins-their role and meaning]. Annales Academiae Medicae Stetinensis. 53: 87-91.

Ramachandran, P., Boontheung, P., Pang, E., Yan, W., Wong, D.T., Loo, J.A. (2008). Comparison of N-linked Glycoproteins in Human Whole Saliva, Parotid, Submandibular and Sublingual Glandular Secretions Identified using Hydrazide Chemistry and Mass Spectrometry. Clinical Proteomics. 4: 80-104. https://doi.org/10.1007/s12014-008-9005-0.

Rouge, P., Culerrier, R., Thibau, F., Didier, A., Barre, A. (2011). A case of severe anaphylaxis to kidney bean: phaseolin (vicilin) and PHA (lectin) identified as putative allergens. Allergy. 66: 301-302. https://doi.org/10.1111/j.1398-9995.2010.02466.x.

Rousseau, K., Kirkham, S., Johnson, L., Fitzpatrick, B., Howard, M., Adams, E.J., *et al.* (2008). Proteomic analysis of polymeric salivary mucins: no evidence for MUC19 in human saliva. The Biochemical Journal. 413: 545-552. https://doi.org/10.1042/BJ20080260.

Sampson, H.A. (2004). Update on food allergy. The Journal of Allergy and Clinical Immunology. 113: 805-19; quiz 820. https://doi.org/10.1016/j.jaci.2004.03.014.

Sengupta, A., Valdramidou, D., Huntley, S., Hicks, S.J., Carrington, S.D., Corfield, A.P. (2001). Distribution of MUC1 in the normal human oral cavity is localized to the ducts of minor salivary glands. Archives of Oral Biology. 46: 529-538.

Sharon, N. (2007). Lectins: carbohydrate-specific reagents and biological recognition molecules. The Journal of Biological Chemistry. 282: 2753-2764. https://doi.org/10.1074/jbc.X600004200.

Sicherer, S.H., Sampson, H.A. (2010). Food allergy. The Journal of Allergy and Clinical Immunology, 125:116-25. https://doi.org/10.1016/j.jaci.2009.08.028.

Sutton, B.J., Gould, H.J. (1993). The human IgE network. Nature. 366: 421–428. https://doi.org/10.1038/366421a0.

Venter, F.S., Thiel, P.G. (1995). Red kidney beans-to eat or not to eat? South African Medical Journal Suid-Afrikaanse Tydskrif Vir Geneeskunde. 85: 250-252.

Verma, A.K., Kumar, S., Das, M., Dwivedi, P.D. (2013). A comprehen--sive review of legume allergy. Clinical Reviews in Allergy & Immunology. 45: 30-46. https://doi.org/10.1007/s12016-012-8310-6.

Wiener, A.S., Moor-Jankowski, J., Gordon, E.B. (1969). The specificity of hemagglutinating bean and seed extracts (lectins). Implications for the nature of A-B-H agglutinins. International Archives of Allergy and Applied Immunology. 36: 582-591.

Yamaguchi, H. (1993). Isolation and characterization of the subunits of a heat-labile alpha-amylase inhibitor from *Phaseolus vulgaris* white kidney bean. Bioscience, Biotechnology, and Biochemistry. 57: 297-302.

Yin, S.W., Tang, C.H., Wen, Q.B., Yang, X.Q. (2011). Conforma--tional and thermal properties of phaseolin, the major storage protein of red kidney bean (*Phaseolus vulgaris* L.). Journal of the Science of Food and Agriculture. 91: 94-99. https://doi.org/10.1002/jsfa.4155.

Zalewska, A., Zwierz, K., Zolkowski, K., Gindzienski, A. (2000). Structure and biosynthesis of human salivary mucins. Acta Biochimica Polonica. 47: 1067-1079.

Zamindar, N., Mosaffa, L., Bashash, M., Amoheidari, M., Golabadi, M. (2016). The effect of diverse treatments on biophysical characteristics of red kidney beans. Legume Research-An International Journal. 39: 550-557. DOI: 10.18805/lr.v39i4.11258.