*Int.J.Adv.Microbiol.Health.Res.*2018; 2(3):37-46

International Journal of Advanced Microbiology and Health Research
ISSN: 2457-077X. Volume 2, Issue 3 (July-September 2018), PP. 37-46
Available online at www.ijamhr.com

Original Research Article

Isolation, Purification and Characterization of Ovalbumin from Hen Egg White and to Develop and isolate antibodies against it

Mehak Munjal[1*] and Farah Khan[2]

PG Student[1], Assistant Professor[2]

[1-2]Department of Biochemistry, School of Chemical and Life Sciences, Jamia Hamdard, Hamdard Nagar, New Delhi – 110062, India.

Article Info

Received 14th August, 2018
Revised 25th August, 2018
Accepted 30st August, 2018
Published online 7th September, 2018

Keywords

- Ovalbumin
- Hen Egg White
- Antibodies
- Mouse
- New Delhi.

ABSTRACT

Ovalbumin (54%) and lysozymes (3.5%) are among the major proteins in egg white that have many functional properties. Ovalbumin is a phosphoglycoprotein with a molecular size of 45 kDa and an iso-electric point of 4.5. Although, the functions of ovalbumin other than nutritional value for humans are not well known, it is widely used in cell culture, and has high potentials to be used as a drug carrier or to produce various functional peptidesThe aim was to isolate ovalbumin from hen egg white and to develop antibodies against it. First, egg white was homogenized and then crude ovalbumin was precipitated by salt fractionation using 36% Na2SO4. The protein was then subjected to dialysis and later filtered through Sephadex G-100 gel. Its molecular weight was estimated to be 35 kDa using SDS-PAGE. This purified ovalbumin was then injected into Wistar rats every 7 days over 4 weeks.Then blood was collected from these rats and centrifuged at 2000 rpm for 5 minutes. Serum was collected and its OD at 280 nm was measured to determine the IgG concentration which came out to be 10.13 mg/mL. Ouchterlony double immuno-diffusion assay between purified ovalbumin and commercially available ovalbumin showed identity pattern.

INTRODUCTION

Albumin belongs to the family of globular proteins and is a member of a class of water-soluble, heat-coagulating proteins. It is widely distributed in plant and animal tissues, e.g. ovalbumin of egg, myogen of muscle, serum albumin of blood, lactalbumin of milk, legumelin of peas, and leucosin of wheat.[1-2] Ovalbumin (also called *Gal d* 2) is a major allergen present in hen egg white. Its molecular weight is approximately 45 kD. Ovalbumin was previously considered to be the most important allergen of egg white. [1] Its importance was over-estimated due to frequent contamination of commercial preparations with ovomucoid (also called *Gal d* 1). Ovalbumin is a protein of unknown function found in large quantities in avian egg-white. Surprisingly, ovalbumin belongs to the serpin family of protease inhibitors but it does not act as a protease inhibitor. [1-4]

The structure of ovalbumin consists of a single polypeptide chain of about 460 residues (about half of which are hydrophobic), a maximum of 2 phosphate residues per molecule, and an oligosaccharide side chain composed of only mannose and glucosamine residues. Ovalbumin is soluble in electrolyte free water and combines with salts, acids and bases. Its denaturation can be induced by heating at 56°C, by vigorous shaking, by electric current and by various chemicals such as acids, ammonium salts, heavy metal salts and alcohols. Such methods produce complete and irreversible denaturation. The isoelectric point (pI) of ovalbumin is 4.63. [2-4]

Chicken egg yolk has been considered an ideal source of immunoglobulin, and IgY is understood to be the predominant antibody in chicken egg yolk [5]. It has many significant advantages over mammalian IgG. First, the production of IgY is

noninvasive, which makes it suitable for large-scale production (~40 g IgY/y/hen). Second, because of the phylogenetic distance between chickens and mammals, IgY does not interact with rheumatoid factors, bacterial fragment crystallizable (Fc) receptors, or activate mammalian complement [6].

Also, IgY can recognize more epitopes of the highly conserved mammalian proteins than other mammalian IgGs are able to, so IgY can induce an efficient immune response [7]. IgY has also attracted considerable attention as a means of preventing and controlling such diseases as bovine mastitis, diarrhea in piglets, campylobacteriosis, and shrimp white spot syndrome virus [8].

In addition, IgY can be used as an immunological supplement in infant formula and other food [9]. However, the practical use of IgY in research and diagnostics is limited because of necessary complex and time-consuming purification steps.

MATERIALS AND METHODS

LOWRY METHOD

Series of test tubes were filled with the desired volume of the BSA (0.1, 0.2, 0.3… 1 ml) .PBS was added to this to make the volume of 1 mL. 5 mL of copper reagent was added to all the tubes. After proper mixing, all the tubes were incubated at room temperature for 15 minutes. 1 mL Folin reagent was added to each and mixed properly with the help of vortex mixer and incubated for 20 minutes. The intensity of the colour was then determined spectrophotometrically at 680 nm. The graph was than plotted between optical density and the amount of BSA. Proteins estimation was done for the detection for the amount of proteins present in the sample solutions by the Lowry method [10].

SDS PAGE

SDS-PAGE was conducted under reduced conditions using a Mini-Protein II cell (Bio-Rad). Ten percent Trisglycine SDS-polyacrylamide gels with 5% stacking gel were prepared and Coomassie Brilliant Blue R-250 (Sigma) staining was used [11].

To check the purity of lysozyme and ovalbumin, gel pictures were taken after destaining. The purity of protein was calculated by converting the density of protein bands in the gel picture.

IMMUNIZATION OF RAT WITH PURIFIED OVALBUMIN

Before the first immunization, the rats were test bleed to get the pre-immune serum for comparison. 1 mg of purified ovalbumin was injected in to the rat at different sites.

PREPARATION OF SAMPLE FOR IMMUNIZATION OF RAT

Since the concentration of sample was 0.76 mg per mL, 1.32 mL of protein solution (containing 1 mg ovalbumin) was mixed with 0.5 mL of alum (potassium aluminium sulphate) and 1.0 ml saline. The pH of the sample was then adjusted to 6.8 with the help of 10 % sodium carbonate. The sample was now ready for immunization. 0.5 mL of this sample was administered intradermally and intramuscularly to the rats at three sites for 4 weeks. After 4th week i.e. after giving the third booster dose, the rat was bled by puncturing the orbital vein behind the eye ball with the help of capillary tube or vessel. The blood was collected in siliconised test tubes. The serum was separated from the blood by centrifugation. The serum isolated from the blood was then analyzed for the presence of anti-bodies against protein ovalbumin with the help of double immunodiffusion.

GEL PREPARATION FOR IMMUNODIFFUSION

1gm of agar was taken in a 250 mL conical flask and was mixed with 100 mL of PBS. To dissolve the agar, the flask was kept on a hot plate for few minutes. The flask was taken out and shaken to obtain homogenously hot dense solution. After cooling, the gel was poured over glass slides and allowed to settle. The wells were made on the agar gel and the sample along with the standard were put in different wells and incubated in Petri dish containing wet cotton in bottom for 24 hrs at 0oC.

ISOLATION AND QUANTIFICATION OF IGG ANTIBODIES FROM RAT ANTISERUM

Rat antiserum was diluted in the ratio 1:2 with saline. Then, 2ml of saturated ammonium sulphate solution was added to this mixture. The solution was left to stand for 1 hour at RT. The solution was centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and precipitate was collected as it contains IgG antibodies. The precipitate was washed with 2ml of 40% ammonium sulphate. The precipitate was centrifuged again and supernatant was discarded.

The precipitate was dissolved in minimum volume of PBS and OD was taken at 280nm. (1 OD = 0.72mg/mL).

RESULTS AND DISCUSSION

Ovalbumin (54%) and lysozymes (3.5%) are among the major proteins in egg white [12] that have many functional properties. Ovalbumin is a phosphoglycoprotein with a molecular size of 45 kDa and an iso-electric point of 4.5 [13]. Although, the functions of ovalbumin other than nutritional value for humans are not well known, it is widely used in cell culture, and has high potentials to be used as a drug carrier or to produce various functional peptides [14]. Hydrolyzed peptides derived from ovalbumin showed excellent ACE inhibitory and antihypertensive activities in spontaneously hypertensive rats [15-16]. Also, hydrolysates of ovalbumin reversed abnormalities associated with metabolic syndromes and reduced oxidative stress in spontaneously hypertensive rats [17].

The most common assay for determination of total protein concentration is the Lowry method. This procedure is particularly sensitive because it employs two colour-forming reactions. It uses the Biuret reaction in which Cu2+ (in the presence of base) reacts with the peptide bond to give a deep blue colour. The first reaction is the formation of copper ion complex with amide bonds, forming reduced copper in alkaline solutions. This is called biuret chromophore. The second reaction is the reduction of Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by tyrosine and tryptophan residues. The reduced Folin-Ciocalteu reagent becomes blue and thus detectable with a spectrophotometer in the range of 500-750 nm. The Biuret reaction itself is not all that sensitive. Using the Folin-Ciocalteu reagent to detect reduced copper makes the assay nearly 100 times more sensitive than the Biuret reaction alone [18].

The assay was relatively more sensitive, but took more time than other assays and was susceptible to many interfering compounds like detergents, carbohydrates, glycerol, EDTA, potassium compounds, sulphydryl compounds, disulphide compounds, magnesium and calcium ions. Most of these interfering substances are commonly used in buffers for preparing proteins. The Lowry assay is sensitive to variations in the content of tyrosine and tryptophan residues. The standard curve with regression analysis was linear which can detect the protein in the range of 1 to 100 µg concentration. The absorbance can be read in the region of 500 to 750 nm. Since, it is obvious that the assay conditions and the instrumentation error are always present, the originally obtained points during the preparation of standard curve by Lowry's method were not straight, and it was adjusted by applying the regression method. In this condition, here the y-intercept was excluded assuming that this will not affect the quantification of protein. Since proteins differ markedly in their solubility at high ionic strength, salting out is a very useful procedure to assist in the purification of a given protein.

In practice, ammonium sulphate is the salt commonly used, since it is highly water-soluble, relatively cheap and available at high purity. Furthermore, it has no adverse effects upon enzyme activity. Alternatively sodium sulphate can be used for this purpose. The procedure of salt fractionation was carried out at 0-40˚C, to maximize the protein stability as it is known that higher temperatures lower protein stability. The ovalbumin was precipitated, after first step of salt fractionation by adjusting the pH at its isoelectric point (pI 4.6), due to low solubility of the protein at its pI. At the pI of the protein, the negative and positive charges on the surface of molecule cancel each other, electrostatic repulsion within the individual molecule no longer occurs and electrostatic attraction between different molecules may occur, resulting in the formation of precipitate. The percentage yield of the final working protein was found to be low, which might have been due to the lack of expertise in the work and protein losses with each subsequent purification step. To assure maximum yield and to avoid unnecessary denaturation of the proteins, most of the purification work was carried out at low temperatures, which is the temperature between 0-40°C. Since the purpose of the experiment is to see the yield of the protein using 36% Na2SO4 at normal temperature, the experiment was carried out at room temperature. All the standards and the sample which were run through the column containing Sephadex G-100 were analyzed for the elution volume and then the graph was plotted from which the molecular weight of the hen egg white ovalbumin was determined to be around 38 kDa. Electrophoresis on the native PAGE & SDS-PAGE gave a single major band along with one or two

other bands, which were compared with the standards. This indicated that the ovalbumin was not purified properly and since it is composed of a single unit, other bands must be of some other proteins. For the reference, the Rf value was determined and again the molecular weight was calculated by plotting a graph between Rf & log (MW). The molecular weight was found to be around 35 kDa. The precipitin line of complete identity between the antisera and lab purified ovalbumin, upon double immunodiffusion confirmed the production of the antibodies in rat serum. The double immunodiffusion method was chosen as it is quick and easy to conduct.

The introduction of purified ovalbumin as an antigen with alum as an adjuvant results in the sequential reaction that leads to the production of antibodies by activating the humoral immunity and mobilization of sensitized T-cell. The first exposure of rat to the ovalbumin was the primary exposure and during this only humoral branch of the immunity got activated. But the later exposure of the same antigen, as the booster dose for 3 weeks, showed profound immunologic effect due to the enhanced immunologic memory, & increased quantity of the antibody formation.

CONCLUSION

The yield of the protein by the use of 36% Na2SO4 was low, that is, about 21%. The molecular weight of ovalbumin determined via column chromatography was 38,905 Da while that determined via SDS PAGE was 35,481 Da. The given dose to the rats produced significant amount of IgG, that is, 10.13 mg/mL.

REFERENCES

1. Aickin R, Hill D, Kemp A (1994) Measles immunisation in children with allergy to eggBMJ 23, 309(6949):223-5
2. Ahmad, F., and Salahuddin, A (1972), The Denatured State Of Ovalbumin, Biochemistry Journal, 128 : 49P.
3. Ansari, A., Ahmad, R., and Salahuddin A (1972), The Native And Denatured States Of Ovalbumin, Biochemistry Journal, 126: 447.
4. Anet J, Back JF, Baker RS, Barnett D, Burley RW & Howden MEH (1985) Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteinsInt Arch Allergy Immunol77:364-371
5. Hamal KR, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens, Poult. Sci., 2006;. 85:1364-1372.
6. Hadge D, Anbrosuius H. Evolution of low molecular weight immunoglobulins-IV: IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA, Mol. Immunol. , 1984; 21:699-707.
7. Edzard S, Ingke B, Kerstin G, Henning S, Simon B, Dion P. Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy, Biologicals. , 2012; 40:313-322.
8. Wang LH, Li XY, Jin LJ, You JS, Zhou Y, Li SY, Xu YP. Characterization of chicken egg yolk immunoglobulins (IgYs) specific for the most prevalent capsular serotypes of mastitis-causing Staphylococcus aureus, Vet. Micro. , 2011; 149:415-421.
9. Facon M, Skura BJ, Nakai S. Potential for immunological supplementation of foods, Food Agric. Immunol. , 1993; 5:85-91.
10. Miguel M, Alonso MJ, Salaices M, Aleixandre A, Fandiño RL. (2007) Antihypertensive, ACE-inhibitory and vasodilator properties of egg white hydrolysates: Effect of a simulated intestinal digestion. Food Chem. 104, 163-168.
11. Price NC and Nairn J. (2009) Exploring proteins: A student's guide to experimental skills and methods. Oxford University Press, pp. 259-278.
12. Stadelman, W. J. and Cotterill, O. J. (2001) Egg science and technology. 4th ed. Avi. Publ. Co., Westport, CT.
13. Huopalahti, R., Fandino, R. L., Anton, M., and Schade, R. (2007) Bioactive egg compounds. Springer, NY. pp. 3-66
14. Datta, D., Bhattacharjee, S., Nath, A., Das, R., Bhattacharjee, C., and Datta, S. (2009). Separation of ovalbumin from chicken egg white using two-stage ultrafiltration technique. Separ. Purif. Technol. 66, 353-361.
15. Miguel, M. and Aleixandre, A. (2006) Antihypertensive peptides derived from egg proteins. J. Nutr. 136, 1457-1460.
16. Miguel, M., Alonso, M. J., Salaices, M., Aleixandre, A., and Fandiño, R. L. (2007) Antihypertensive, ACE-inhibitory and vasodilator properties of egg white hydrolysates: Effect of a simulated intestinal digestion. Food Chem. 104, 163-168.

17. Manso, M. A., Miguel, M., Even, J., Heranández, R., Aleix andre, A., and Fandiño, R. L. (2008) Effect of the long-term intake of egg white hydrolysates on the oxidative status and blood lipid profile of spontaneously hypertensive rats. Food Chem. 109, 361-367.

18. Andrews, A. T. (1986), Electrophoresis: theory, Techniques and Biochemical and Clinical Applications, 2nd edition, Clarendon Press, Oxford University.

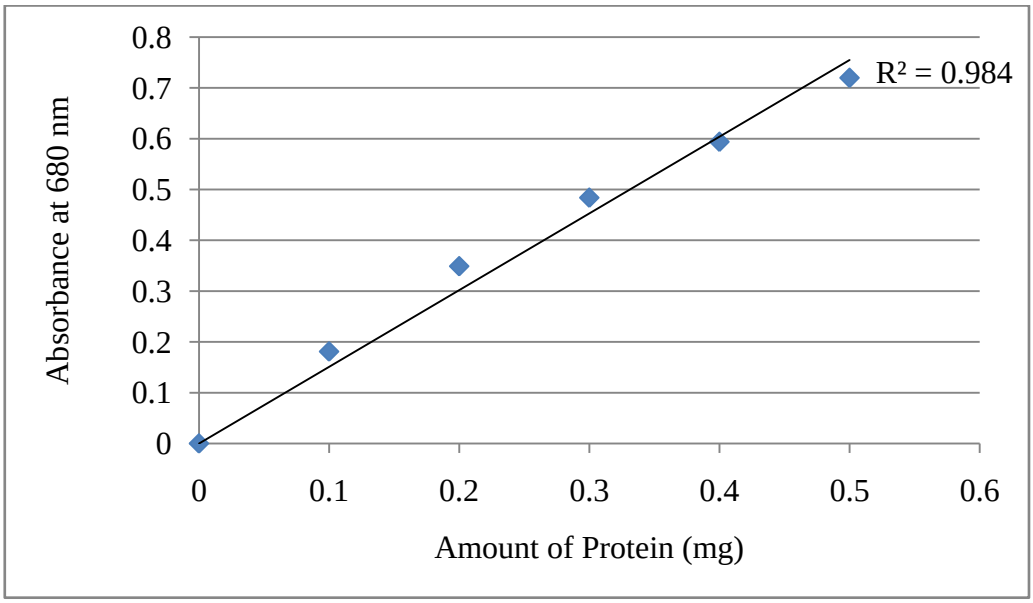


Fig.1. Shows BSA standard curve

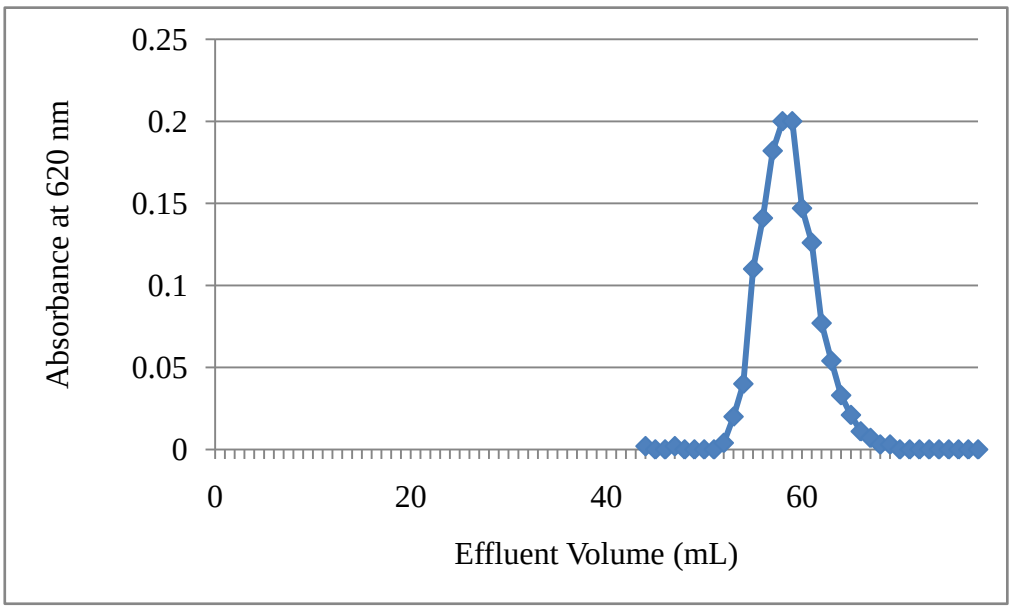


Fig.2. Shows Elution Profile of Blue Dextran (Standard)

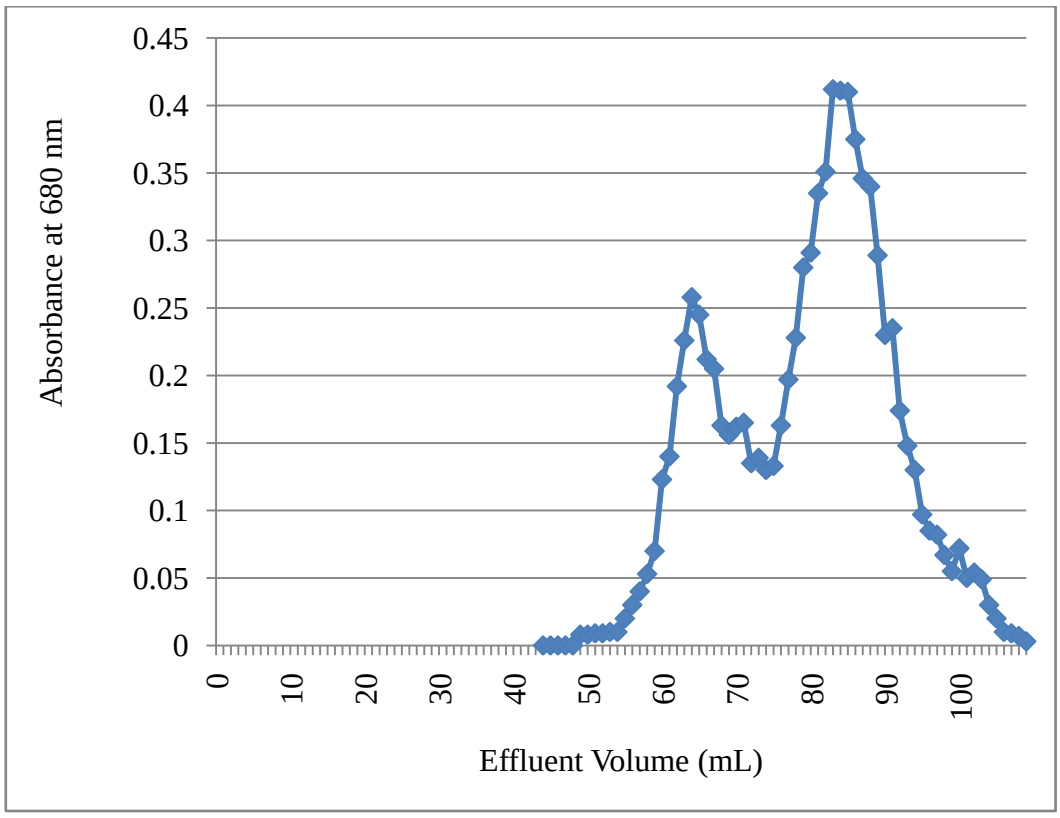


Fig.3. Shows Elution Profile of Ovalbumin (Standard)

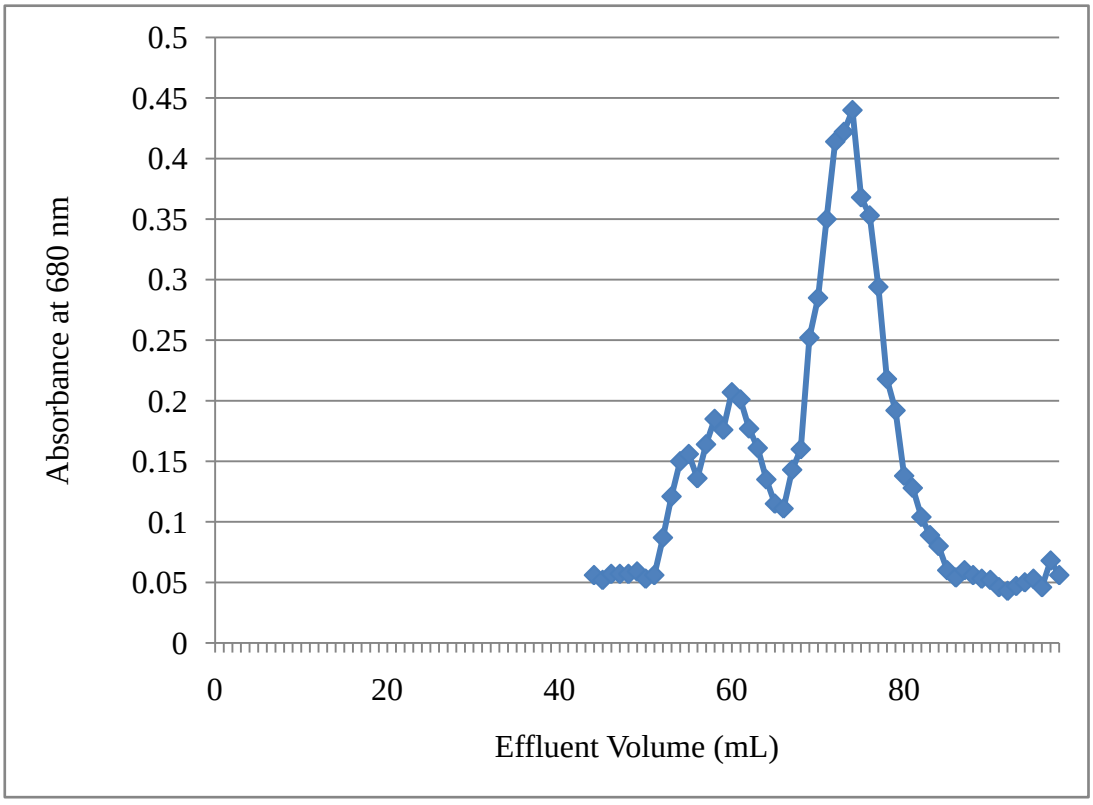


Fig.4. Shows Elution Profile of BSA (Standard)

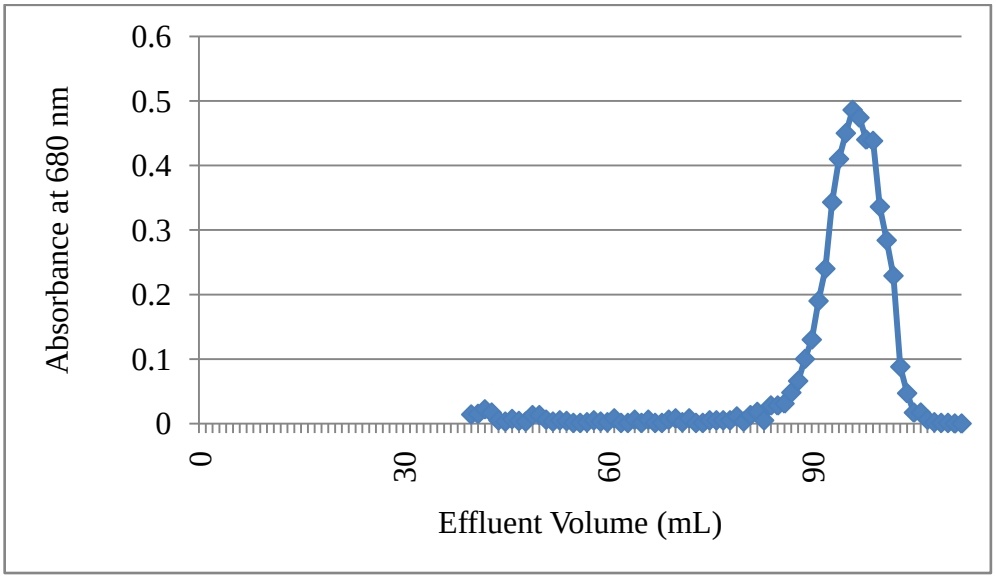


Fig.5. Shows Elution Profile of Chymotrypsin (Standard)

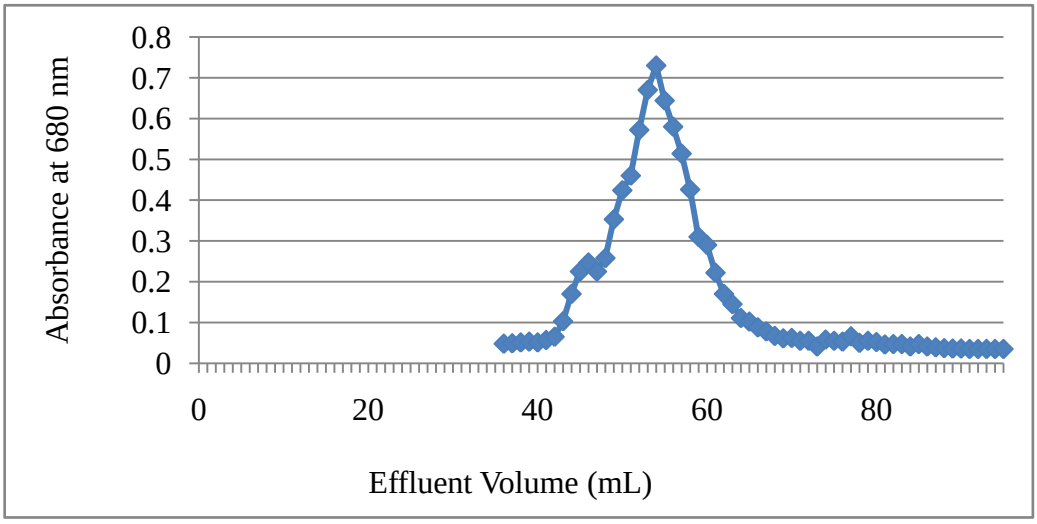
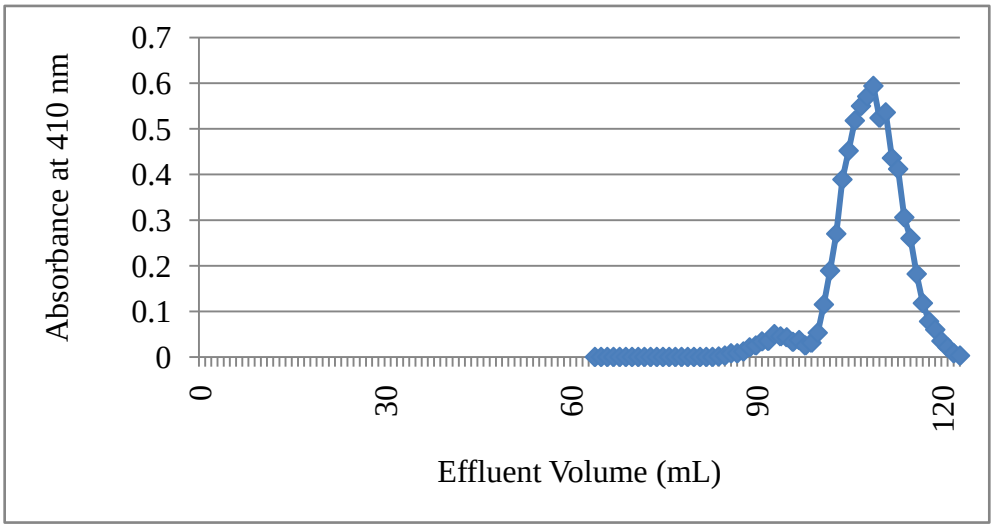


Fig.6. Elution Profile of γ-globulin (Standard)



Graph 7: Elution Profile of Cytochrome C (Standard)

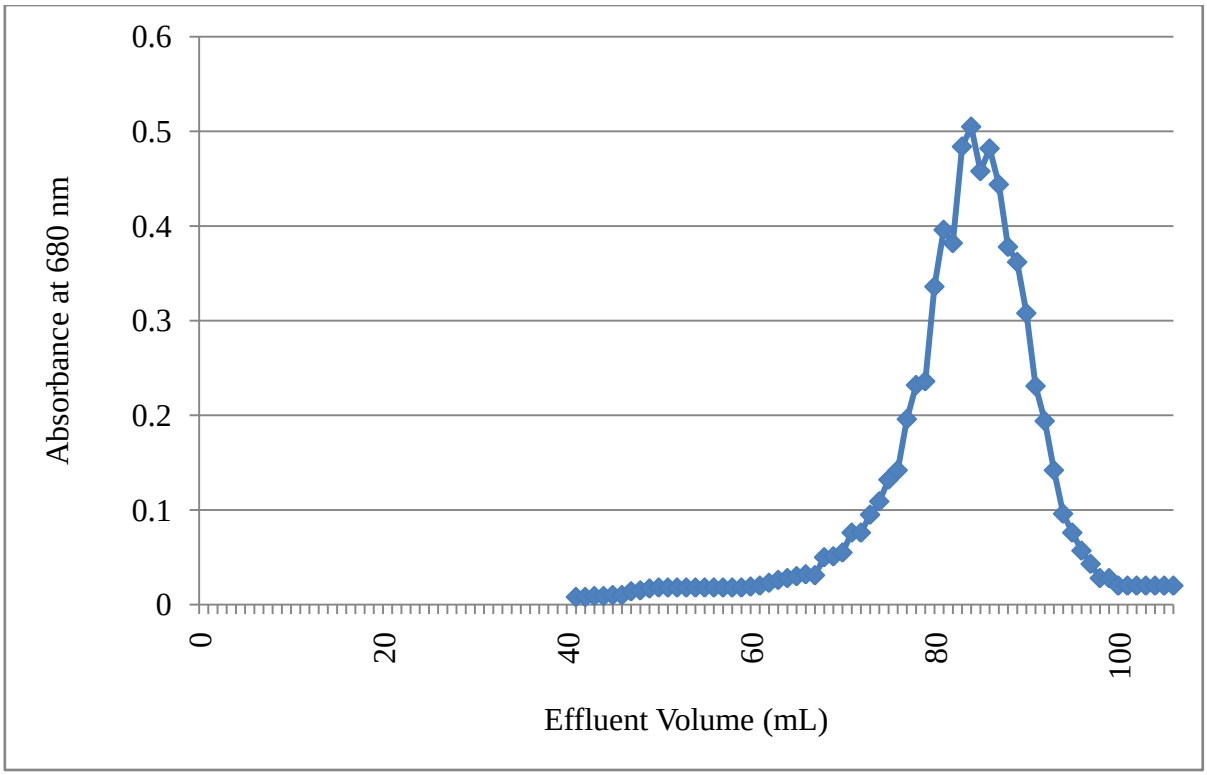


Fig.8. Shows Elution Profile of Ovalbumin from Egg White

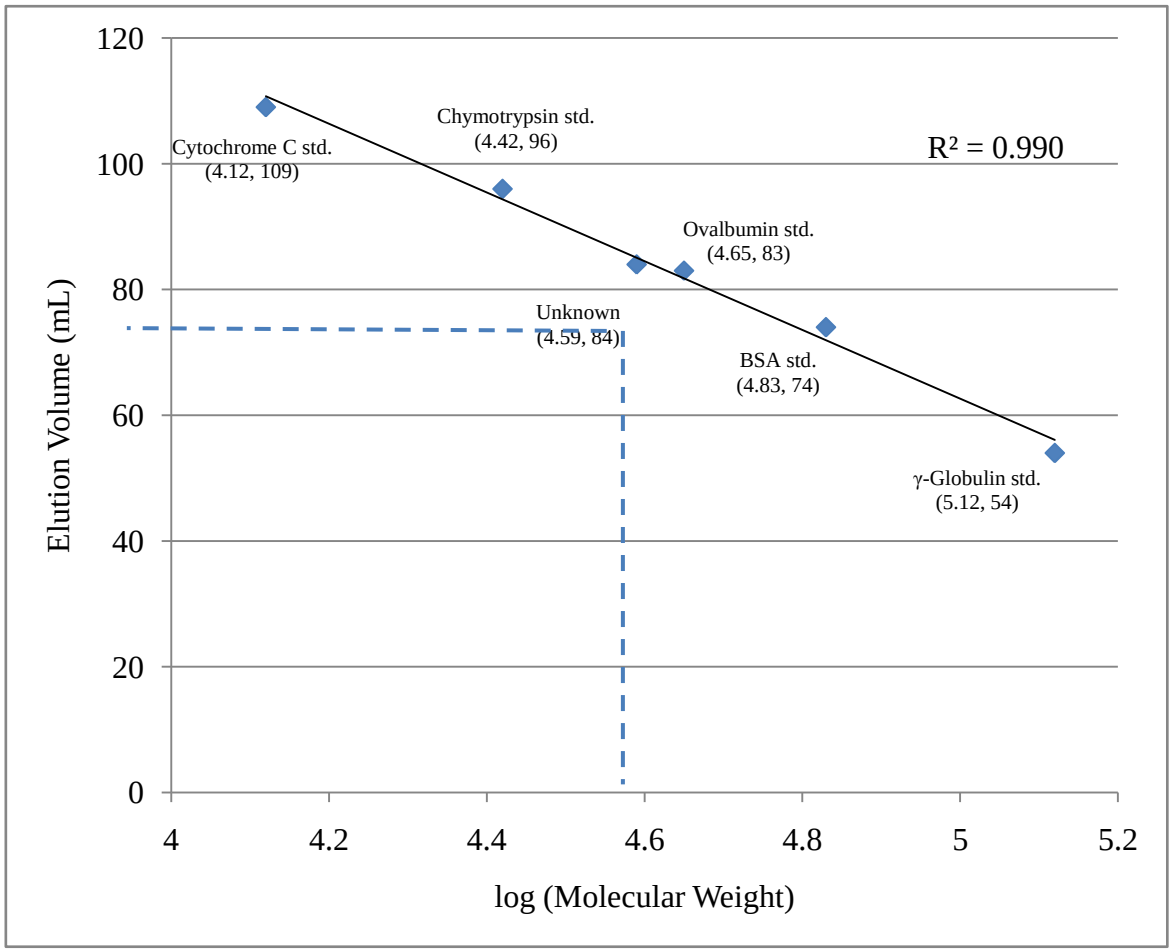


Fig.9. Shows Elution Volume of Standards vs their log (MW)

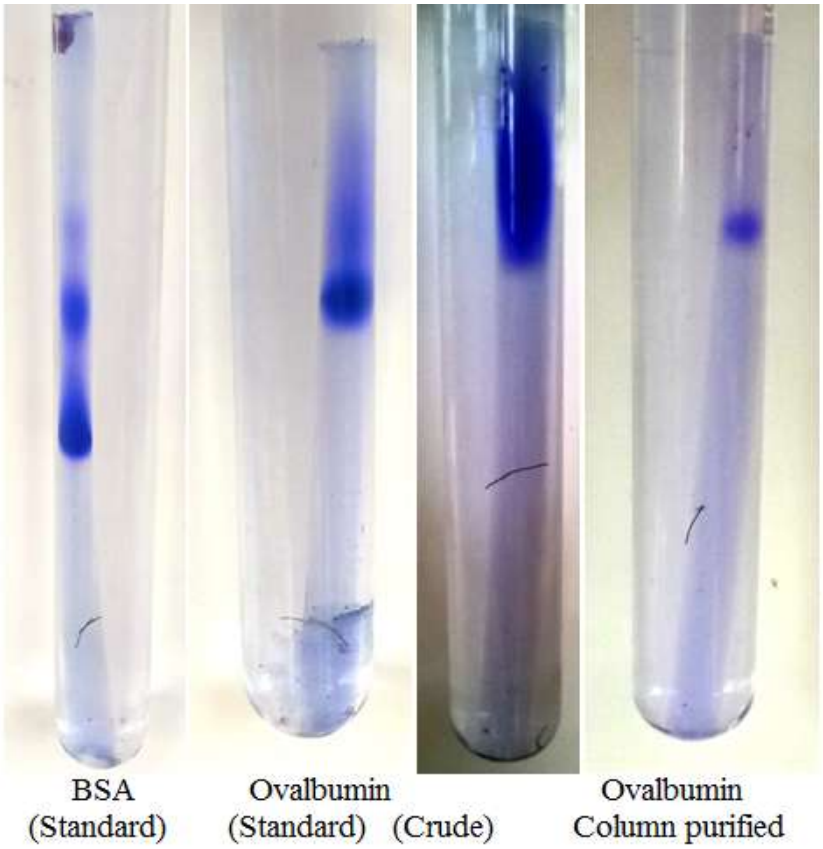


Fig.10. Bands obtained in Native PAGE

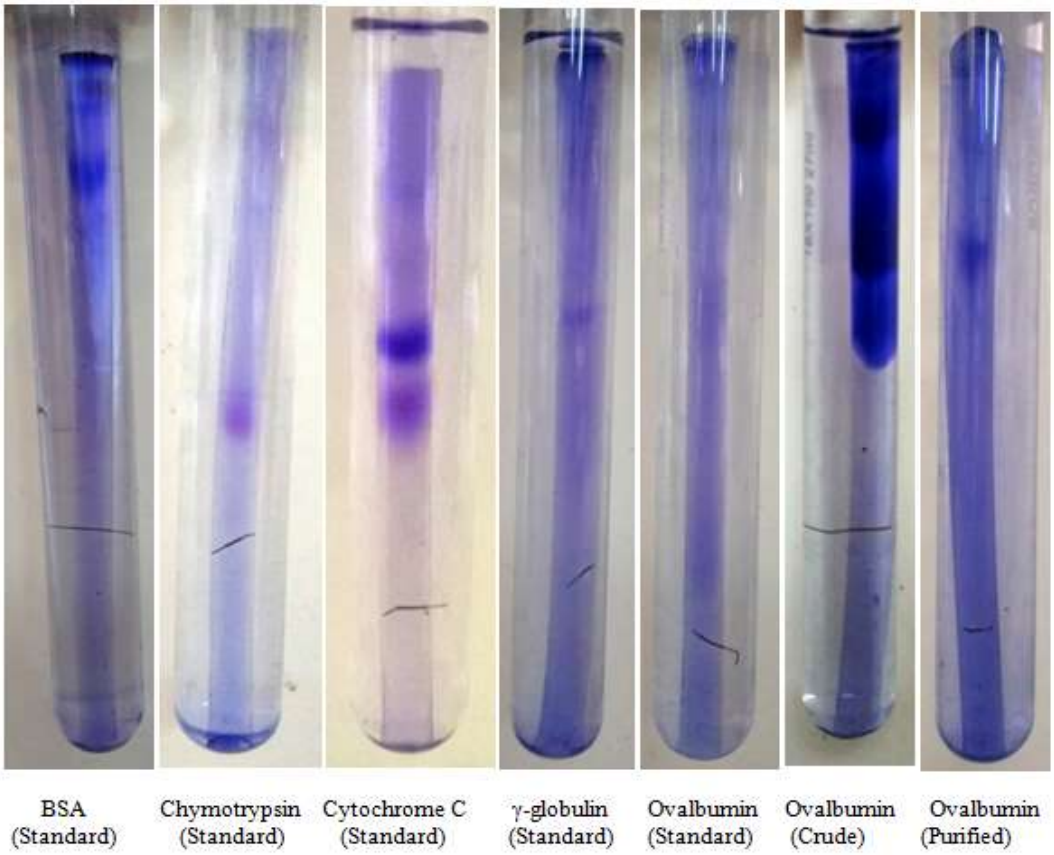


Fig.11. Bands obtained in SDS PAGE

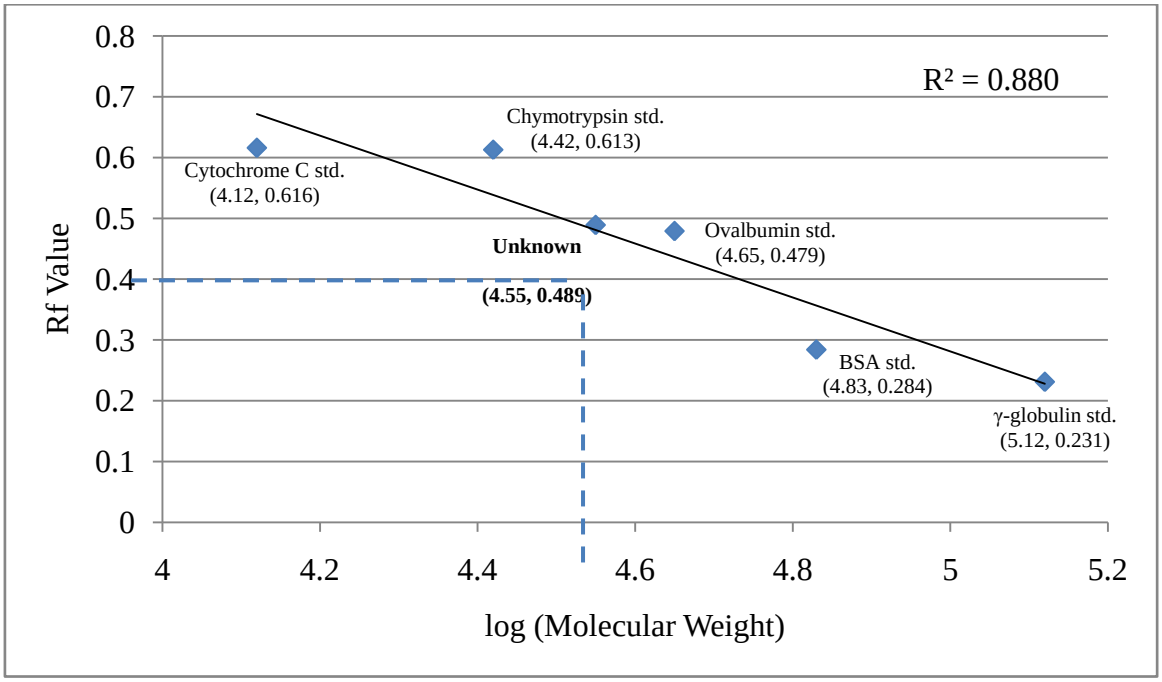


Fig.12. Shows Rf Value of Standards vs their log (MW)

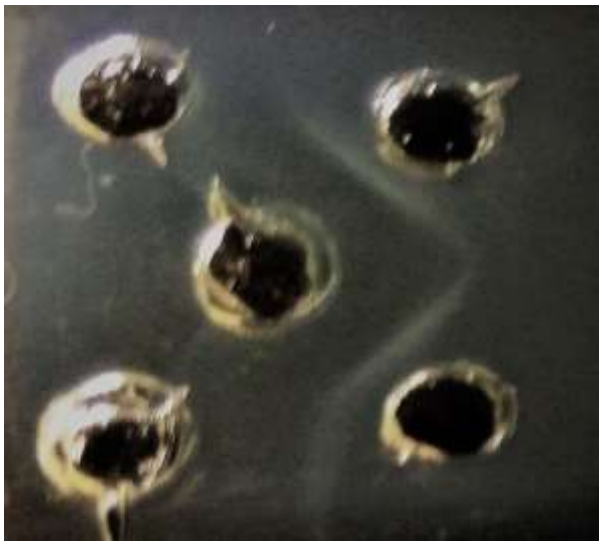


Fig.13. Shows formation of precipitin line between antisera & ovalbumin

Corresponding Author: Ms. Mehak Munjal
House No. 20, Ground Floor, Pocket B 8, Sector -19 Rohini,
New Delhi -110085
E-mail: mehakmunjal8130@gmail.com

How to cite this article:
Munjal M, Khan F. Isolation, Purification and Characterization of Ovalbumin from Hen Egg White and to Develop and isolate antibodies against it. Int. J. Adv.Microbiol.Health.Res., 2018; 2(3):37-46.

Source of Financial Support: Nil, **Conflict of interest:** Nil.