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Separation and Purification of lysozyme from Emu *(Dromaius novaehollandiae)* Egg White and its antimicrobial characterization

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Abstract: The current study focuses on the isolation and purification of the proteins such as Lysozyme and ovotransferrin from Emu egg white. Bio separation methods are applied to break up and purify the proteins like reverse micellar extraction and Cascade membrane filtration, gel filtration chromatography. Lysozyme, considered as one of the most expensive and commercially available enzyme is mostly exploited from the chicken egg white. It delivers a broad range of application in pharmaceutical preparations, in packaging as a preservative, and so forth Ovotransferrin is most important egg white protein it can be use as an innate antimicrobial agent. Reverse micellar extraction yielded a considerable amount of the proteins from emu egg white and was noted to be around 60-70%. The purification factor can be further increased by performing Cascade membrane filtration. Molecular weight of lysozyme and ovotransferrin was found to be ~14kDa by SDS-PAGE.
Keywords: Lysozyme, ovotransferrin, SDS-PAGE, FT-IR.

Introduction

Lysozyme is studied biological compounds found in various kinds of organisms includes birds, plants, mammals, bacteria and insects. It has a broad collection of application in the pharmaceutical industry as well as a main ingredient in ophthalmology arrangements. This enzyme is used as a cell distracting agent, food preservative and in the action of infections and ulcers[1].

It's an antimicrobial enzyme to hydrolyze beta-glycosidic relation between N-acetylmuramic sour as well as N-acetyl Glucosamine in the peptidoglycan of bacterial cell walls and besides attaching polymers of N-acetyl Glucosamine. The grown-up chicken lysozyme is calm of 128 AA (amino acids) and the molecular weight be established to be 14.4 kDa[2].

Ovotransferrin is major egg white proteins in an iron-binding glycoprotein, and it is established in avian EW as well as AS (avian serum), belong to the relations of transparent iron required GP (glycoproteins). It can be employed as a natural antimicrobial mediator. Although, the antimicrobial movement of ovotransferrin beside a collection of microorganisms has been discussed, no effort has been fixed to divide ovotransferrin in large scale and apply it as a likely antimicrobial agent in foods. One of the main issues of the sustainable egg commerce is the capable movement of egg and egg stuff through market channels and growing the value of egg[3].

Various separation and purification techniques like ammonium sulfate precipitation, ethanol precipitation, gel filtration, ion exchange chromatography, aqueous 2 phase extractions etc have been employed so far to isolate proteins from chicken egg white. Lysozyme from the egg white of Agapornis species, lovebird has also been exploited, but with small success. Lysozyme and Ovotransferrin from the egg white of chicken have been exploited[4].

The present work concentrates on the extraction and purification of lysozyme from the EW (egg white) of the Emu (Dromaius novaehollandiae). Emu egg, the least studied can be an alternative to chicken eggs. The egg white was manually separated in aseptic condition void of contamination. Extraction and purification techniques including Reverse micellar extraction, Cascade membrane filtration, and Gel filtration chromato graphy were employed. The Lysozyme and ovotransferrin was confirmed by SDS-PAGE. The protein concentration and the enzyme activity were found using the Lowry *et al.* method and the enzymatic assay respectively.

Experimental

Sample Preparation

The Emu eggs were bought from the market (near Erode). Materials are given in Table 1.

Table.1. Materials

Methods	Chemicals
Hydrolysis	0.05 NaCl, Phosphate buffer, Hydrochloric acid, Ethanol, Distilled water.
Reverse micellar extraction	Crude extract, KCL, CTAB, Isooctane Lowry's buffer, Folins reagent, Calorimeter
Cascade membrane filtration	Crude extract, 30 and 100 kDa membranes, Distilled water.
Protein concentration	Lowry's buffer(10g sodium carbonate in 500 ml(Lowry A),1g sodium potassium tartarate in 100 ml (lowry B), 1g copper sulphate in 100ml(lowry C), follin's reagent, calorimeter.
SDS-PAGE	Acrilamide and bis Acrilamide, Tris HCL, SDS, Ammonium persulphate, TEMED, Bromophenol blue, β-Mercaptoethanol, Coomassie G 250.
Pharmaceutical studies	Standard lysozyme, purified emu egg white lysozyme, Nutrient agar, Petridish plate, Laminar, Inoculums loop, Diphenyl picryl hydroxyl, Purified emu egg white lysozyme, Ethanol,Nutrient broth medium.

Hydrolysis For Egg White Preparation

The egg white was detached from the egg manually in aseptic conditions. The EW was dilute using 3-crease with 0.05M Sodium chloride (NaCl) solution. The pH was set to 4.0 by carefully adding up some drops of 1N HCL and it was watery with equivalent volume of 40%, 60% ethanol[5]. Afterward that the combination was reserved at room temperature for 3-4 hrs incubation. After the incubation the mixtures were centrifuged at 8000 rpm for 15 minutes at 4°celsious. Then the clear solution was used as crude[6].

Reverse Micellar Extraction

The Reverse micellar removal process concerned in two steps that is FW (forward) and BW (backward) extraction. In forward mining the former, proteins are transport as of mass water phase to the water puddle of RMs in an organic phase; as in the last these proteins are better from the RMs into a new aqueous phase. There are two most important problems with the reverse extraction process, namely, a ease in activity yield due to structural adjust in proteins as a result of the powerful interactions among proteins and micelles; and a sluggish rate of back extraction due to the better interfacial confrontation in the direction of protein discharge at the oil in water boundary during the BE (backward extraction)[7].

The forward extraction action was carried out in combination an 5 ml quantity of crude extract in that add 0.1 M KCL which serves as an aqueous phase of the reverse muscular system. For reverse micellar phase add 50 mM CTAB in 5 ml of isooctane. Mix both phase and vortex for 10 min, then centrifuge the mixture for 10 min at 3000 rpm. In backward extraction, take 2 ml of reverse micellar phase from forward extraction in that add 2 ml of fresh aqueous phase of pH 4 containing 1M of KCL in distilled water. Then vortex it for 10 min after that the mixture was centrifuged for 10 min at 3000 rpm[8].

HIC (Hydrostatic interaction chromatography)

To attain the Lysozyme isocratic preservation information, the butyl sepharose 4 fast flows Column was primarily equilibrated by 5 column volume of the transporter bumper, 25 milli Molar phosphate, pH-7.0, contain a variety of salt concentration range from 0.1- 1.4 Molar of ammonium sulfate as well as 0.2 - 4 Molar of NaCl. Samples were then injected into the column as well as the protein preservation moment was calculated. The column has to be equilibrated through the operational buffer used for 5 column volume after that injection be completed. This method was frequent near get reproduction statistics for every preservation time. Column sewage was monitored at 280 nanometre, FR (flow rate) is 0.5 ml/minutes with column was maintained at 20 °Celsius. The preservation point of an uninhibited tracer was gritty as of the capability feature calculation. The sewage is being monitored at 310 nm for the sodium nitrate experiments[9].

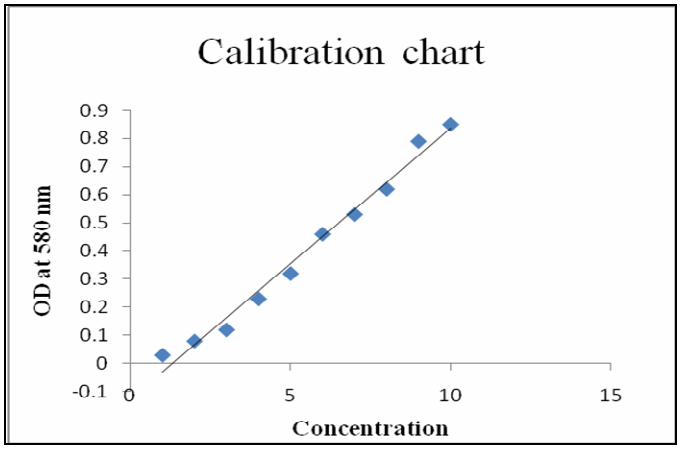
SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis), according to the method of Laemmli, was performed to analyze the proteins present in samples, with a 12 percentage separating gel and 4.5% stacking gel. The criterion lysozyme was used to recognize the lysozyme bands. 20µl samples were burdened in every well and the electrophoresis departure was performed at 100 Voltage for about 45 min. The protein bands were discoloured with Coomassie Brilliant Blue R-250 (0.1g CBB in 40 ml methanol and 10 ml acetic acid and 50 ml distilled water) for 1hr and destained by dispersal in destaining solution (10 ml methanol and 5ml acetic acid and 85 ml of distilled water)[10].

Protein Concentration Estimation

Here 1 ml of sample was added in 2 ml of Lowry's buffer. The Lowry's buffer was prepared by using 49 ml of Lowry A (sodium carbonate) and 0.5 ml of Lowry B (sodium, potassium tartrate) and 0.5 ml of Lowry C (copper sulfate). Then above the mixture was incubated for 10 minutes at room temperature. Then 0.2 ml of diluted follin's-phenol reagent was added to the mixture and it was mixed immediately. Then the mixture was incubated at room temperature for 30 minutes. After incubation, absorbance was calculated at 580 nm using a calorimeter. The standard calibration chart for protein was shown in figure 1[11].

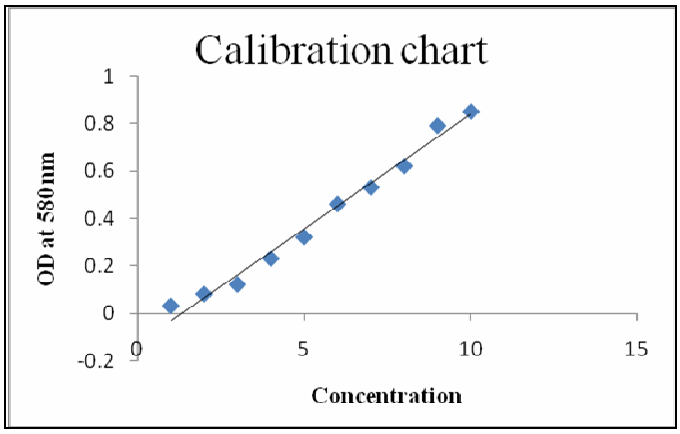
Fig.1. standard chart for BSA



Enzyme Activity

The Lysozyme activity was calculated by lysis of 0.25 mg/ml *Micrococcus Luteus* cells in 100milli Molar sodium phosphate at 25 ◦Celsius. 1 unit of the activity of lysozyme was defining while the reduction of OD450 with 0.001 per min[12]. The standard chart for Lysozyme shown in fig. 2.

Fig.2. Standard chart for Lysozyme



Antimicrobial Activity

The certain antibiotic disk is positioned on the exterior of an agar plate which has previously been inoculated with test bacteria of Bacillus subtilis. Through the incubation time, the antibiotics/chemicals spread superficial from the disks addicted to the agar. This will produce a concentration incline in the agar which depends on the solubility of the chemical and its molecular mass. The deficiency of enlargement of the organism approximately the antibiotic disks indicate that, the appreciated organism is vulnerable to that antibiotic and the existence of enlargement more or less the antibiotic disk indicate the organism is defiant to that exacting antibiotic. This region of no growth about the disk is recognized as a zone of inhibition, which is regularly circular among a confluent lawn of development in the medium[13].

Antioxidant activity: 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

DPPH is a constant free radical through red colour. On scavenge, these free radical revolve to yellow. This frequent theory has been utilised in this assess.

Assay: 0.25 ml of the test sample is added to 1.5 ml of ethanol and assorted with 0.3 ml of 0.5 mM DPPH in ethanol. Then 1.6 ml of ethanol varied with 0.25 ml of standard (nutrient broth) this mixture was consider as a blank and 1.75 ml of ethanol mix with 0.3 ml of DPPH this mixture was measured as a control. These 3 mixtures were reserved in room temperature for 30 min. The absorbance of the resultant solution is calculated at 517 nm[14].

Formula for calculating scavenging activity

$$\% \text{ SA} = 100 - \left\{ \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100 \right\}$$

A - Absorbance of sample
A - Absorbance of the blank
A – Absorbance of the control

Results and Discussion

Reverse Micellar Extraction

From the reverse micellar forward extraction carried out with 50mM SDS and CTAB, the K was determined to be 3.9 in forward extraction using CTAB. By SDS, the K was determined to be 3.2 in forward extraction this shown in table 2 and 3. This shows that most of the proteins have been extracted in the top phase. Compare to CTAB and SDS the maximum yield of partition coefficient was observed in CTAB system. So the CTAB forward extracted sample from the top phase was taken for further optimization studies. The two phase and colour formation of reverse micellar extraction shown in fig 3 and 4.

Table.2 Reverse micellar extraction

	CTAB conc. (mM)	KCL conc. (M)	Injection volume	Top phase	Bottom phase	Partition coefficient
Forward extraction	50	0.1	5	0.71	0.18	3.9
Backward extraction	50	1	5	0.37	0.44	0.84

Table.3. Reverse micellar extraction using SDS

	SDS conc. (mM)	KCL conc. (M)	Injection volume (ml)	Top phase	Bottom phase	Partition Coefficient
Forward extraction	5omM	0.1 M	5	0.49	0.15	3.2
Backward extraction	5omM	0.1 M	5	0.23	0.37	0.62

Fig.3. Phase formation of reverse Micellar extraction

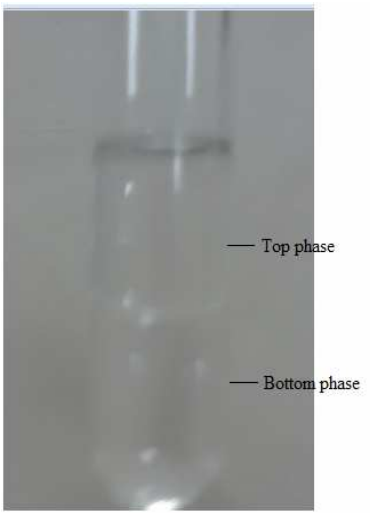


Fig.4. Colour formation of RME

Hydrostatic Interaction Chromatography

The experimental part of the HIC, the preservation times for Lysozyme is calculated in HIC experiments using NH_2SO_4 concentrations ranges like 0.1 to 1.5 Molar, and Sodium chloride concentrations range as of 0.2 – 4 Molar. Column ability factor be intended as of the resultant preservation statistics. Fig.5 shows in the agent semi-log plot of the ability thing verses salt conc. for some of lysozyme. The situation examines the capability feature augmented by rising salt conc. Representative these experiments be passed away in the salting away command. The majority of the data, obtains with NaCl, the curves are moderately ‘‘flat” at lesser salt conc., which might be owing to compensatory property among electrostatic as well as hydrophobic connections below of these conditions[15].

SDS-PAGE

Purification of lysozyme is done by gel filtration chromatography and the results are analyzed and conformed using SDS PAGE analysis. Standard lysozyme marker of molecular weight 14.3KDa is used to compare the isolated and purified Emu egg white lysozyme. We observe heavy and light chains on lane 1and 2 indicate the presence of lysozyme. Light bands were observed due to low concentration of lysozyme in increasing the concentration of lysozyme we observe clear bands, that indicates and confirms the presence of lysozyme[16]. By comparing the standard marker of lysozyme and the isolated lysozyme we conclude that the molecular weight of the isolated and purified Emu egg white lysozyme was found to be 14.3 KDa (Fig.6).

Fig.5. Hydrophobic interaction chromatography showing the purified lysozyme

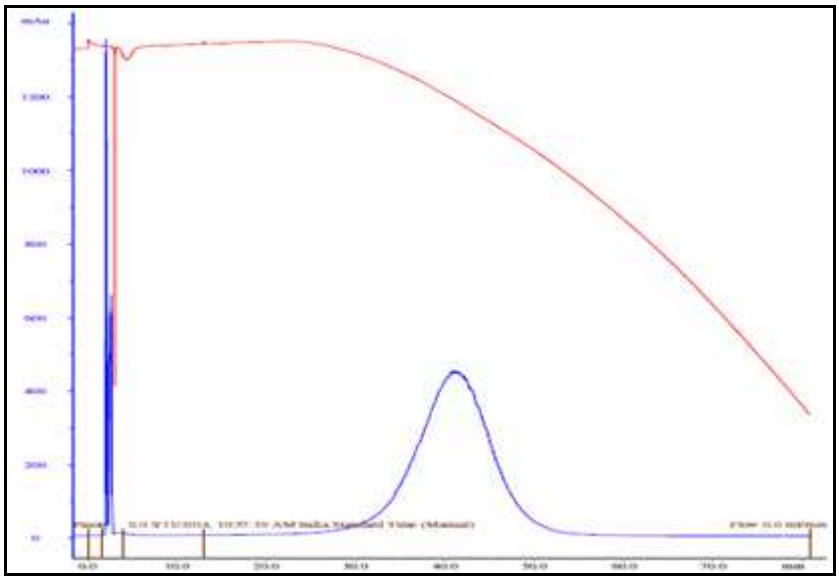


Fig.6. SDS-PAGE

Antimicrobial Activity

Standard Lysozyme

Zone of inhibition = 18 mm

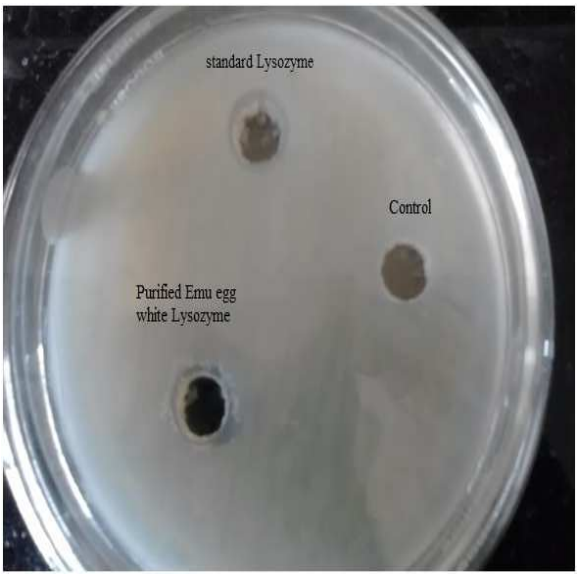
Purified Emu Egg White Lysozyme

Zone of inhibition = 14 mm

Antimicrobial activity of isolated and purified Emu egg white lysozyme was tested against *Bacillus subtilis*. We observe zone of inhibition after 24 hrs of incubation. Zone formation was measured to be 14mm for the isolated and purified Emu egg white lysozyme while we observe the zone formation for standard lysozyme was 18mm **(fig.7).** From this we conclude that lysozyme has potent antibacterial activities that can be used as

antibiotics in Pharmaceutical industries. When compared with standard marker of lysozyme we observe more or less equal activities of isolated and purified Emu egg white lysozyme that indicates the purity of isolated lysozyme[17]. Lysozyme can also be used in food industries for inhibiting the growth of bacteria since lysozyme is well known for its cell lysis.

Fig.7. Antimicrobial activity of purified emu egg white lysozyme



Antioxidant Activity

Fig.8. Antioxidant activity of purified emu egg white lysozyme

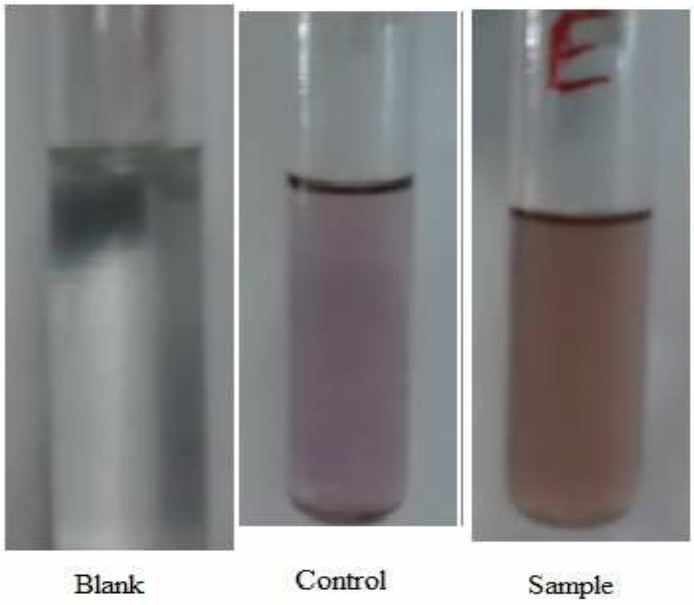
$$\% \text{ SA} = 100 - \left\{ \frac{A_{\text{sample}} - A_{\text{blank}} \times 100}{A_{\text{control}}} \right\}$$

$$\% \text{ SA} = 100 - \left\{ \frac{0.31 \times 100}{0.57} \right\}$$

% SA = 45.62

Antioxidant activity of isolated and purified Emu egg white Lysozyme was analyzed for oxidation capacity of free radicals produced. Free radicals produced by our body needs to be stabilized by the supply of antioxidants, which otherwise leads to cell damage. In order to analyze the isolated and purified lysozyme produces any antioxidants we go for analysis using the DPPH assay, which produces free radicals by undergoing oxidation reaction in the presence of light. When the free radicals are produced it should be controlled by the isolated lysozyme then it is proven that lysozyme has antioxidant capacity. By analyzing this we found that the scavenging activity of isolated and purified Emu egg white lysozyme is 45.62%, (fig. 8) which is considered as a good antioxidant. Hence we conclude that lysozyme can also be used in the treatment of various diseases produced by free radicals[18].

Fig.8. **Antioxidant activity of purified emu egg white lysozyme**



Conclusions

Major egg white proteins like lysozyme and ovotransferrin are isolated and purified from the crude extract using the bio separation method. Here total proteins are extracted using reverse micellar extraction and the concentrations are determined by using the Lowry's method. In RME, the best results are obtained by forward extraction so in that optimization was performed by using response surface methodology. Here the maximum partition coefficient was obtained in 125mM CTAB cons. (35.3). The crude egg white proteins are purified by using cascade membrane filtration. Here lysozyme and ovotransferrin are purified under the condition of pH- 4 and pressure is 2 bars and stirring speed is 200 rpm. FT_IR analysis was done by confirming the Lysozyme presence. Finally the SDS-PAGE was performed for the molecular weight determination of lysozyme is 14.3kDa. This enzyme is used as a cell disrupting agent, food additive and in the treatment of infections and ulcers. Ovotransferrin can be used as a natural antimicrobial agent.

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