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Partial purification of Egg white Lysozyme from four different Bird species by Ethanol precipitation method and their Antibacterial Activity Assay: A Comparative study

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Abstract

In the present study, egg white lysozyme (EWL) was partially purified by ethanol precipitation method using varying concentrations of ethanol like 40%, 60% and 80% from the eggs of four different bird species including broiler-bred poultry hen, *Aseel* breed *desi* (country) hen, domesticated duck and pigeon in order to find out the most suitable concentration of ethanol for partial purification of EWL. In addition to this, a comparative anti-bacterial assay was also performed to determine the most effective EWL among the four different egg samples utilizing *Bacillus subtilis* as the test organism. It was found that 80% ethanol was most efficient for partial purification process of EWL when the incubation time is fixed for 6 hrs.after the ethanol treatment and prior to separation of EWL by centrifugation at acidic pH. It was also assessed that the EWL of broiler hen possessed the best lysozomal activity followed by *Aseel* hen, duck and pigeon. The egg white of common Indian domesticated pigeon was also found to exhibit lysozyme activity, although to a lesser extent. The lysozomal activities of the ethanol-precipitated crude EWLs were further confirmed by Lysoplate Assay using the cells of *Micrococcus lysodeikiticus* as the substrate. The study indicates that because of the considerably high lysozyme activity, the egg whites of poultry hen could be used as a potential source of pure lysozyme as the eggs are easily available and much cheaper. The eggs of Aseel breed of hens also contain potential lysozomal activity but they are not economically viable for mass scale production of lysozyme for their higher cost and lower availability.

Lysozyme (E.C.3.2.1.17; N-acetyl-muramic hydrolase), discovered by Alexander Flemming is actually a heterogeneous group of enzymes that has derived its chemical name because of its hydrolyzing activity on the β-(1,4) glycosidic bond found in between the alternating units of N-acetyl-muramic acid and N-acetyl-glucosamine present in the heterpolysaccharide peptidoglycan. This molecule is found as a component of bacterial cell walls, in particular Gram positive bacteria whose cell wall is composed of more than 90% of peptidoglycan. The activity of this enzyme results in rapid lysis of the bacterial cells because of degradation of the cell wall structure and hence, the name lysozyme. Lysozyme is found to be present in all higher eukaryotic organisms including birds and mammals. In vertebrates, including the birds, the major lysozomal type is type-c and it is present in high concentration in the egg white(called as Egg White Lysozyme). In higher mammals (including humans), it is found in the external (secreted) body fluids like tears, saliva, breast milk, urine and is produced by the layers of the epithelial cells in respiratory and intestinal tract[1,4,5,9] and also by the lysosomal granules of neutrophils and macrophages[2]. Lysozyme is considered as one of the major components of the innate immune system of humans (Tenovuo, 1989) as it prevents the colonization and invasion by bacterial pathogens. Lysozyme is a small, stable enzyme, making ideal for research into protein structure and function. It has a long active site cleft that binds to the bacterial carbohydrate chain. Based on computer modeling, it has been proposed that lysozyme distorts the shape of one amino sugar derivative in the peptidoglycan chain in the bacterial cell wall, making it more easy to cleave (although other studies have proposed that different effects, like electrostatics, are more important). Unfortunately, lysozyme is a large molecule that is not particularly useful as a drug. It can be applied topically, but cannot rid the entire body of disease, because it is too large to travel between cells[7]. Egg white lysozyme (EWL) has considerably a wide functional protein exhibiting antibacterial activity mainly against Gram-positive bacteria. The EWL is widely applied in food industry and is considerably safe[10]. Because of its efficient anti-microbial activity, lysozyme is widely used commercially for many purposes like-a) cellulolytic agent for disruption of cells for extraction of intracelleular products[6,] b) food additives in dairy products[8], c) antibacterial agent for the preparation of ophthalmic solutions and wine production process[6], d) drugs for the treatment of ulcers and other gastro-intestinal infections and also for dry mouth, (xerostomia)[8,9], e) drugs for HIV infection and also as an anti-cancer agent and[8] f) germinative agent for bacterial spores etc[8]. Lately, it has also been reported that addition of specific concentrations of lysozyme boosts up antibody production in hybridoma reactors[8]. Lysozyme was the first enzyme whose entire 3D structure was determined by X-ray crystallography technique (Phillips, 1967).

In the current study, crude lysozyme was extracted and partially purified by treating with increasing concentrations of ethanol such as 40%, 60% and 80% and the resulting mixture was incubated at room temperature for a period of 6 hrs and was centrifuged. The crude lysozyme was extracted from the egg

whites of four different bird species like hen (*Gallus gallus domesticus*) of two different breeds-the broiler breed and the *desi Aseel* breed, common Indian domesticated duck (*Anas platyrhynchos domesticus*) and the common Indian pigeon (*Columba livia*). A comparative antibacterial assay of the partially purified (ethanol- treated) lysozymes from the said egg samples was performed using *Bacillus subtilis* as the indicator organism in order to determine the effect of ethanol treatment in the purification process of the extracted crude lysozymes. It was assumed the more will be purity of the lysozyme, the greater will be its anti-bacterial activity. The anti-bacterial activity was measured by the Kirby-Bauer Method in terms of the diameter of the corresponding zones of inhibition (appeared as a clear zone surrounding the well containing samples) on the petri plates with *Bacillus subtilis* colonies spread uniformly over the Nutrient Agar (NA) surface. Lysozyme activity in the ethanol-precipitated egg whites was further confirmed by Lysoplate Assay using *Micrococcus lysodeikiticus* cells as the substrate[8].

Materials: Fresh hen and duck eggs were collected from a local poultry farm (located at Galsi, East Burdwan District, West Bengal).The pigeon eggs were collected from the house of a village farmer near Arambagh, Hooghly, West Bengal. The ingredients for nutrient agar/broth medium were purchased form Hi-media®. Other chemicals like the sodium chloride and acetic acid were all purchased from SRL®.

Culture of microorganism used: The pure culture of *Bacillus sublitis* for the current study was obtained from the Deptt. Of Microbiology, The University of Burdwan, Golapbag Campus, West Bengal and was maintained in lab by using NA slants and Nutrient Broth (NB) medium.

The strain *Micrococcus lysodeikiticus* (NCIM No.2170) was collected from National Collection of Industrial Microorganism, National Chemical Laboratory (NCL), Pune, India through The University of Burdwan, West Bengal and its pure culture was maintained in NA slants and NB medium in the lab where the work was performed. This organism was used as the substrate to confirm lysozyme activity of the ethanol-treated extracts obtained from the egg whites of the four birds.

Partial purification of Lysozyme form egg white: The freshly collected hen, duck and pigeon eggs were surface sterilized by dipping them in 70% ethanol. After the surface sterilization, the egg shells were broken carefully using hands and the egg white was separated from the egg yolk with caution by using a Pasteur pipette and was collected in a sterilized and dry petri dish under aseptic condition. The partial purification of the egg white lysozyme was then carried out by the procedure as described by Gemili *et al*.[3] with slight modifications. The egg whites were diluted 3 times using 0.05 M NaCl solution. In order to precipitate the egg white proteins other than lysozyme (as lysozyme is most stable in acidic medium)[6], the pH of the diluted egg white was set to 4.0 by carefully adding 3-5 drops of 1 N acetic acid and the pH was confirmed by testing with pH paper. Following

the acidification of the egg white, it was mixed with equal volume of ethanol of increasing concentrations like 40%, 60% and 80% (v/v) in separate sterile and dry test tubes. The test tubes were marked accordingly with reference to the ethanol concentration and egg sample. All the tubes were then incubated at room temperature for a period of 6 hrs. After the incubation period is over, the mixtures from the respective test tubes were centrifuged at 15,000g for a period of 20 minutes at 4^0C using a Remi® Cooling Centifuge. The precipitates from each of the tubes were discarded and the clear supernatants were tested for their anti-bacterial activity (due to the presence of lysozyme) by Kirby-Bauer Method (also known as 'Agar Cup Assay') using *Bacillus subtilis* as the indicator organism.

Anti-bacterial assay: For the anti-bacterial assay, three wells were created on the surface of the Nutrient Agar (NA) for three samples treated with three different ethanol concentrations for the broiler breed hen egg white and the wells were filled carefully with the respective samples by using a micropipette. The same process was repeated for the other three egg white samples *i.e.*-for *Aseel* breed hen, duck and pigeon eggs. The NA plates were pre-inoculated with pure culture of *Bacillus subtilis* by Spread-Plate Technique under aseptic condition. All the plates were then incubated at 37^0C for 16-20 hours. All the four plates were then examined for the zones of inhibition (the four different plates were as marked as *"Broiler Egg", "Assel Egg", "Duck Egg"* and *"Pigeon Egg")* and the diameter of the corresponding zones of inhibition were recorded accordingly.

A separate NA plate with pre-inoculated *Bacillus subtilis* was used in which commercially available pure lysozyme (purchased from Merck®) was added in the well as the positive control. Likewise, a negative control NA plate was used using distilled water in the well.

Cross-validation of the result: The respective ethanol treated crude lysozyme samples that showed maximum zone of inhibition were identified and were again tested individually (single well on a plate containing that particular sample only) to cross-check their anti-bacterial activity and to see if the same result (*i.e.* the diameter of the zone of inhibition) could be obtained since a single well on a single NA plate would reproduce more clear-cut result as compared to 3 wells on a single NA plate.

Determining of the activity of the partially purified lysozyme by Lysoplate Assay[8]*:* As the cell wall of *M. lysodeikiticus* is sensitive to the lysozyme activity, the lysis of the cells of this organism in Lysoplate Assay is directly proportional to the lysozyme concentration in the egg whites. To assess this, Lysoplate Assay was done using the ethanol-treated crude samples that exhibited maximum *Zone of Clearance* in the Agar Cup Assay by Kirby-Bauer Method.

Anti-bacterial assay of crude Lysozyme:

The result of the anti-bacterial assay of the crude lysozyme extracts (in terms of diameter of zone of inhibition on NA plates with *Bacillus subtilis*) obtained from four different egg whites and treated with different concentrations of ethanol are represented in tabular format as below:

Sample-1. Broiler Hen egg white Lysozyme:

Conc. of Ethanol	Incubation Time	Diameter of *Zone of Inhibition*
40%	6 hrs.	0.8 cm.
60%		1.8 cm.
80%		2.2 cm.
Positive Control (pure lysozyme)	__	2.8 cm.
Negative Control (distilled water)	__	No *Zone of Inhibition*

Sample-2. *Aseel* Breed Hen egg white Lysozyme:

Conc. of Ethanol	Incubation Time	Diameter of *Zone of Inhibition*
40%	6 hrs.	0.6 cm.
60%		1.2 cm.
80%		1.9 cm.
Positive control (pure lysozyme)	__	2.8 cm.
Negative Control (distilled water)	__	No *Zone of Inhibition*

Sample-3. Duck egg white Lysozyme:

Conc.of Ethanol	Incubation Time	Diameter of *Zone of Inhibition*
40%	6 hrs.	0.4 cm.
60%		1.4 cm.
80%		1.8 cm.
Positive control (pure lysozyme)	__	2.8 cm.
Negative Control (distilled water)	__	No *Zone of Inhibition*

Sample-4. Pigeon egg white Lysozyme

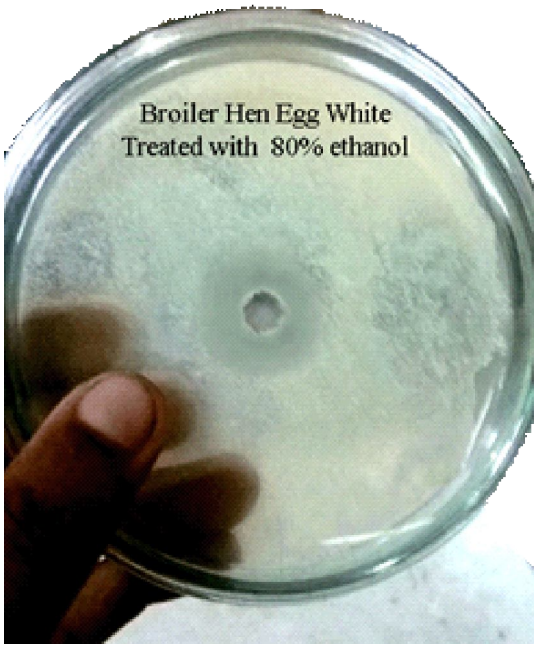
Conc.of Ethanol	Incubation Time	Diameter of *Zone of Inhibition*
40%	6 hrs.	0.2 cm.
60%		0.8 cm.
80%		1.2 cm.
Positive control (pure lysozyme)	__	2.8 cm.
Negative control (distilled water)	__	No *zone of Inhibition*

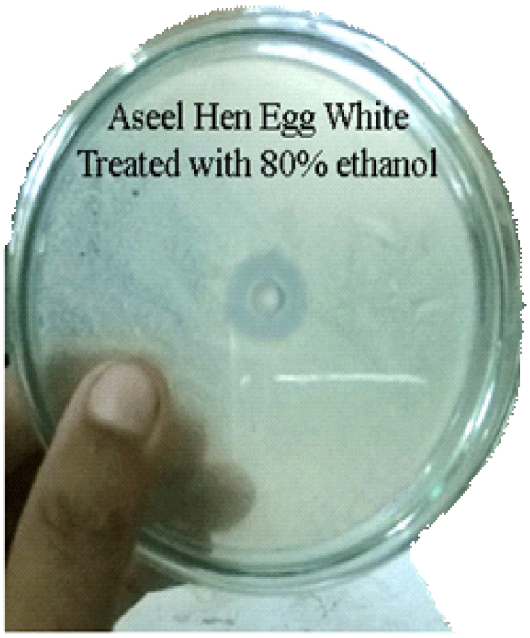
The results clearly show that the lysozyme extracted with 80% ethanol treatment exhibited maximum anti-bacterial effect on the growth of *Bacillus subtilis* indicating that 80% ethanol was most efficient for crude lysozyme precipitation than the other concentrations of ethanol used i.e.40% and 60%. The incubation time used for lysozyme extraction (*i.e.* 6 hrs.)

was also proved to be ideal for the partial purification of lysozyme as manifested by the anti-bacterial activity. It could as well be concluded that the broiler breed hen egg white lysozyme showed maximum anti-bacterial activity on *Bacillius sublitis* when treated and precipitated with 80% ethanol as compared with the egg whites of *Aseel* breed hen, domesticated duck and pigeon treated with similar concentrations of ethanol.

Cross-validation of the result: In order to ensure that the crude egg white samples extracted and precipitated with 80% ethanol showed maximum lysozomal (anti-bacterial) activity, each of the 80% ethanol-treated lysozyme extracts from four different egg whites were again tested for their anti-bacterial activities by putting each one of them in four separate wells in four separate NA plates with pre-inoculated *Bacillus subtilis*. The results are tabulated as follows:

Sample	**% of ethanol treated with**	**Incubation time**	**Diameter of *Zone of Inhibition***
Broiler Hen egg white	80%	6 Hrs.	2.1 cm.
Aseel Breed Hen egg white			1.7 cm.
Duck egg white			1.8 cm.
Pigeon egg white			1.2 cm.





This repeat test shows that 80% ethanol-treated egg white samples reproduced almost same results in terms of their lysozomal activity (i.e.the size of the *Zone of Inhibition*) against the organism *Bacillus subtilis* as were obtained in the initial test. This confirms the fact that the broiler breed hen egg white contains maximum lysozomal activity and 80% ethanol is the most suitable concentration for the partial purification process of egg white lysozyme when the incubation period after ethanol treatment is fixed at 6 hrs.

Determining of the activity of the partially purified lysozyme by Lysoplate Assay: It was observed that all the ethanol-treated (of 80% concentration) egg-white samples resulted in the lysis of the cells of *M. lysodeikiticus* among which the egg white of broiler-bred poultry hen exhibited the maximum and that of the pigeon exhibited minimum lysis.

The present study indicated that the egg whites of the four types of birds viz. broiler-bred poultry hen, *Asee*l breed of hen, duck and pigeon contains lysozomal activity against the test organism. As far as the partial purification and precipitation of egg white lysozyme is considered, using 80% ethanol resulted in maximum purification of the crude egg white lysozyme following 6 hrs. of incubation at room temperature. The egg white of common broiler bred hen that is mainly bred for the production of meat in the poultry farms has considerably high lysozyme activity and hence, could be exploited as a good source of commercially available pure lysozyme. As the eggs of such poultry hens are easily available at a low cost, they could be used as industrial source of lysozyme which will lead to a significant reduction in the production cost of the enzyme. Eggs of *Aseel* bred hens also can be used for industrial production of lysozyme but the eggs are much costlier and so, are not as economically viable as the eggs of broiler bred hens. The study also revealed that the eggs of the common domesticated pigeon also have lysozyme activity although to a much

lesser extent.

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