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Partition and purification of lysozyme from quail egg white using aqueous two phase system

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ABSTRACT

Lysozyme, one of the most expensive and commercially available enzymes is mostly exploited from the chicken egg white. It is commercially used as a cell disrupting agent, food additive and in the treatment of ulcers and infections. It is an antimicrobial enzyme that hydrolyzes the beta-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls and can also bind to polymers of N-acetyl glucosamine. This study focuses on the isolation and purification of the enzyme from Quail egg white. Experiments were carried out using Ammonium sulphate precipitation and Aqueous two phase extraction and the protein content was found to be high in Aqueous two phase extraction when compared to the Ammonium sulphate precipitation. The protein concentration and enzyme activity were estimated using Lowry's method and turbidimetric assay respectively. The purification process was further increased by performing Gel filtration Chromatography and the fractions were confirmed using 12% SDS gel and the molecular weight was found to be ~14kDa which is almost similar to that of the chicken egg. The secondary structure of lysozyme extracted from Quail egg white was performed by FTIR analysis and compared against standard lysozyme sample.
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KEYWORDS

Lysozyme;
Aqueous two phase extraction;
Gel filtration chromatography;
Purification.

INTRODUCTION

Lysozyme [3.2.1.17] is one of the most widely studied biological compounds found in various living beings comprising of flora and fauna, mammals and microbes. The Lysozyme of chicken egg white has been most widely considered[1]. It has an extensive range of applications in the pharmaceutical field and as a main ingredient in opthamologic preparations. Commercial grade of the enzyme is used as a cell disrupting agent, food additive and in the treatment of ulcers and infections[2]. It is an antimicrobial enzyme which affects the beta-glycosidic bonding that exist in N-acetylmuramic acid and N-acetyl glucosamine by hydrolyzation[3]. It is present in the peptidoglycan of bacterial unit wall and it also fastens polymers of N-acetyl glucosamine[4].

Various separation and purification techniques like

Ammonium sulphate precipitation, Reverse micellar extraction, aqueous two phase systems were employed for the isolation of protein from its crude source[5]. The challenging task faced by most of the large scale protein industries is the effective protein extraction. Ammonium precipitation method is one of the traditional methods used for the lysozyme extraction followed by various chromatographic techniques like ion exchange chromatography, covalent or affinity chromatography[6]. Aqueous two phase extraction is showing appreciable potential in the extraction and purification of proteins[7,8]. They are also applied in large scale applications for enzyme separation[9]. Mixing of two polymers, or a polymer/salt combination in aqueous phase forms an aqueous two phase system[10]. This system provides a gentle, simple process, efficient extraction of target proteins[11].

The present study focuses on comparative work of Ammonium sulphate precipitation and Aqueous two phase system for the extraction and purification of the Lysozyme from the egg white of the least studied avian members viz, Quail (*Coturnix ypsilophora*) and Emu (*Dromaius novaehollandiae*)[12]. Quail egg, considered as one of the cheapest source and the Emu egg, the least studied can be an alternative to chicken egg[4,13]. The egg whites were manually separated in aseptic condition void of contamination[14]. Gel filtration chromatography was employed for the purification process. The Lysozyme enzyme was confirmed by SDS-PAGE analysis.

MATERIALS AND METHODS

Materials

The Quail and the Emu eggs were purchased from the market. Phosphate buffer (pH 7), Hexane for de-fatting, ammonium sulphate salt, chemicals for SDS-PAGE, protein estimation were purchased from Hi-media and Fischer chemicals. *Micrococcus luteus* culture was used for the Turbidimetric assay to find out the enzyme activity.

DE-Fatting & crude preparation

The egg white was separated from the egg manually in aseptic conditions. Raw pH of the egg was checked and the sample was suspended in the phosphate buffer. Equal parts of solvent (hexane) was added and stirred for 2-3 hrs. The lipid molecules gets absorbed by the solvent and the de-fatted solution is collected and used as the crude solution. The solution was diluted 3 times to keep the protein concentration in observation.

Ammonium sulphate precipitation

Salt concentration ranging from 30 to 80 % was used to precipitate the protein. 5ml of sample along with the desired concentration of ammonium sulphate was added and vortexes till the salt dissolves. The samples were placed at 4^0C overnight. The pellet and supernatant were separated and the protein and enzymes activities were found.

Aqueous two phase extraction

The ATP systems were formed by the mixing of PEG (4000,6000,8000) at various mass fractions with the $(NH_4)_2SO_4$ salt in the aqueous medium maintained at 25°C. The pH of the system was modified by adding 6M HCl. The crude extract was added to the above mixture and centrifuged at 6,000 rpm for 10 minutes. The two phases formed were isolated carefully and the top phases having appreciable protein in the PEG rich phase were taken for backward extraction. Sodium chloride salt was added to the top phase of the forward extraction and centrifuged at 5,000 rpm for 15 minutes. The salt rich bottom phase having target protein was taken separately to undergo purification process.

Gel filtration chromatography

The precipitated sample and optimized sample from backward extraction of aqueous two phase system were subjected to Gel filtration chromatography. Size exclusion experiments were carried out on a G-50 Sephadex column. At 20°C, the assays were executed at 0.5 mL min^{-1} flow rate. The sodium phosphate buffer maintained at 0.05 $molL^{-1}$ and Nacl of 0.2 $molL^{-1}$ with pH 7 were used for the elution of purified fraction.

SDS-Page

The purified lysozyme was confirmed using 12 % SDS gel. Lysozyme standard and protein marker were used as reference.

Protein concentration estimation & enzyme assay

The protein concentration was estimated using the

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Lowry *et al.* method at each step. The enzyme activity was found out using the Turbidimetric assay based on Weaver *et al*. The rate of lysis of *the bacterial cells* was verified as recommended by Shugar (1952). A diminishing turbidity of 0.001 per minute at 450 nm with pH 7.0 and 25°C following the precised state is equal to one unit. The activity was calculated using the below,

$$\text{Units/mg} = \frac{\text{Abs450/min x 1000}}{\text{mg enzyme in reaction mixture}}$$

RESULTS AND DISCUSSION

Ammonium sulphate precipitation

The effect of different concentrations of the ammonium sulphate salt ranging from 30 to 80% was studied for purification of the enzyme. Precipitation of the quail egg white was considerable in all salt concentrations. However, enzyme activity 11440 (U/mg) was found in case of 45% of ammonium sulphate. The protein concentration increased proportionally with raise in salt concentration and the enzyme activity was found to be decreasing with increase in salt concentration. In the Emu egg white, the protein concentration was high in each case, but the enzyme activity was undetectable in all the salt concentrations of ammonium sulphate.

Aqueous two phase extraction

The aqueous two phase system was applied for the extraction of Lysozyme from Quail egg. It shows an appreciable value of 49411 U/mg specific lysozyme activity and gives 46% yield compared to the conventional ammonium sulphate precipitation method. Various PEG molecular mass like PEG 4000, 6000, 8000 were employed of which the PEG 6000 shows the maximum specific activity and 54.58% yield at 25%w/v of PEG6000 and 18%w/vof ammonium sulphate. The effect of different parameters like pH, temperature and PEG Molecular mass were analysed over the specific activity of the lysozyme.

Effect of pH on lysozyme activity

The effect of pH on the specific activity was tagged in Figure 1. As the pH value raised from 4 to 10 the specific activity and the protein extraction rate starts to increase. Generally, the protein partition towards the top phase is due to the negative charge of the protein particles and the positive charges automatically settle to the bottom phase of the system. As the pH value is raised, protein particles were more negatively charged and give high specific activity values at pH 9 than at low pH values. Hence, the protein and PEG interactions become stronger. Further increase of pH than the optimized value leads to protein denaturation.

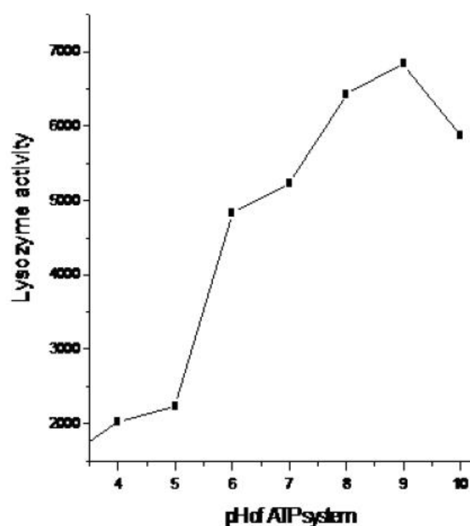

Figure 1 : Effect of pH on the lysozyme activity

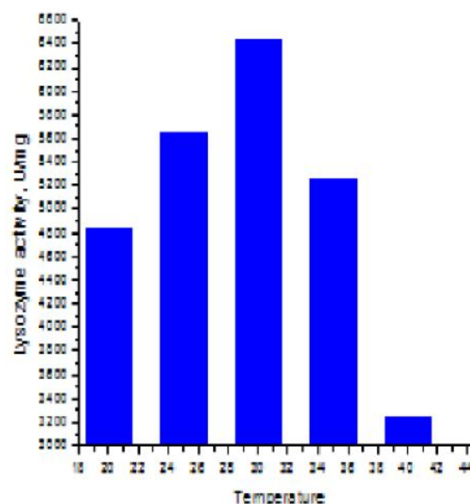

Figure 2 : Effect of Temperature on Lysozyme

Effect of Temperature and PEG molecular weight on lysozyme activity in ATP systemPEG molecular mass is one of the major factors that affects the extraction efficiency of the protein. The protein movement towards the top phase rich in PEG is due to the bonding that exists between the protein and PEG molecules. Figure 3 shows that the PEG molecular mass increases, the tendency of protein to get attracted towards the PEG

increases and larger amount of proteins stay in the top phase itself which leads to maximum extraction. Specific activity was found to be high at PEG 6000. Temperature is also a key factor for the lysozyme extraction. From Figure 2 it was found that increase in temperature also favours the protein extraction and the maximum specific activity observed at 30°C. This explores the endothermic nature of the system.

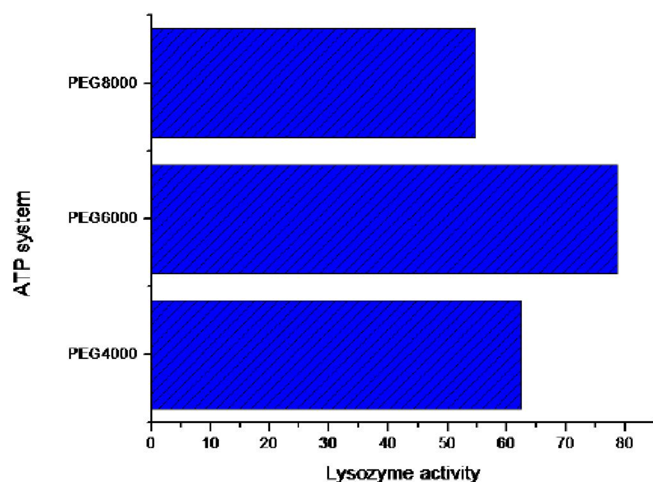

Figure 3 : Effect of molecular weight of ATP system

Gel filtration chromatography

The fraction with the high enzyme activity from quail egg white was subjected to Gel filtration Chromatography. The enzyme activity was found to be increased several folds. A sharp peak was obtained which indicates the high enzyme activity and void of foreign compounds is shown on Figure 5.

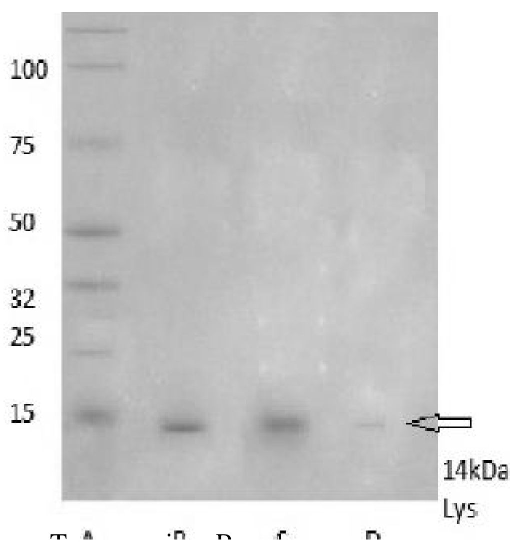

Figure 4 : SDS Page analysis

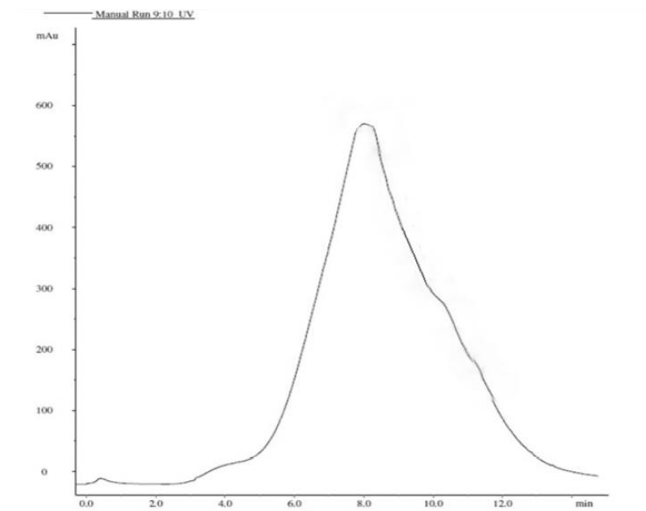

Figure 5 : Gel filtration chromatography of lysozyme fraction from ATP system.

SDS-Page

The samples were run on a 12% SDS-PAGE. Standard lysozyme and protein marker were used for reference. The molecular weight of lysozyme was found to be 14kDa and is shown in Figure 4.

TABLE 1 : Lysozyme extraction using Ammonium sulphate Precipitation

Stage	Specific Activity (U/mg)	% Yield	Purity Factor
Crude	1580	100	1
45% $(NH_4)_2SO_4$ Precipitation	3640	58	2.3
GFC	11440	32	7.24

TABLE 2 : Lysozyme extraction using Aqueous two phase extraction system

Stages	Specific Activity (U/mg)	% Yield	Purity Factor
Crude	1580	100	1
PEG 4000+ $(NH_4)_2SO_4$	19615	62.4	12.4
PEG 6000+ $(NH_4)_2SO_4$	25645	78.65	16.23
PEG 8000+ $(NH_4)_2SO_4$	34946	54.58	22.11
GFC_PEG8000+$(NH_4)_2SO_4$	49411	46	31.29

CONCLUSIONS

The enz*BTAIJ, 4(1) March 2010*BioTechnologyAn Indian

JournalBioTechnology yme activity in Quail egg is almost similar to that of the chicken egg. The lysozyme can be a major component of opthamologic solutions, which can serve as an anti bacterial agent especially against gram positive organisms and in certain gram negative microbes. Using conventional ammonium sulphate precipitation and aqueous two phase system, the extraction efficiency of both the systems were compared. It leads to maximum extraction with 46% yield and 49,411 U/mg specific activity using (PEG6000+$(NH_4)_2SO_4$) system. The results expose that the lysozyme can be extracted in large scale by applying the aqueous two phase extraction method.

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