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Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system

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Abstract

The extraction behavior of lysozyme from chicken egg white by PEG/salt aqueous two-phase system (ATPS) was investigated. Considering the excluded volume effect and the hydrophobic character, polyethylene glycol (PEG) with an intermediate molecular weight (6000) was used to form the ATPS. Sulfate was chosen as the phase-forming salt because of its ability of promoting the hydrophobic difference between the phases. Optimal conditions of the process were investigated using the Box–Wilson experimental method. It was found that the ATPS should be composed of 16.1% (w/w) PEG and 12% (w/w) sodium sulfate, with addition of 500 mM of sodium perchlorate. And the egg white should be diluted 7.6-fold before being extracted by the ATPS. When this system was operated at room temperature (25 °C) and pH 10, approximately 70% of lysozyme could be extracted from the diluted chicken egg white. The specific activity of lysozyme resulting from the partitioning was estimated to be 39 500 unit/mg protein.

Keywords: Lysozyme; Chicken egg white; Aqueous two-phase system; Hydrophobicity; Purification

1. Introduction

When two aqueous solutions of certain incompatible substances, such as polyethylene glycol (PEG) and dextran or PEG and salt, are mixed above a critical concentration, a liquid–liquid phase separation occurs [1]. Mixtures of proteins added to this two-phase system tend to partition unequally between the phases thus allowing for the extraction of a particular protein. Separation techniques based on this partitioning have come to be known as aqueous two-phase system (ATPS) [1,2]. The ATPS is advantageous over the other separation techniques because of the high water content of both phases, which means high biocompatibility and low interfacial tension, thus minimizing degradation of biomolecules [1]. The ATPS also provides good resolution, high yield, and a relatively high capacity. In addition, this system is easily scaled-up.

The mechanism governing the partition of biomolecules in ATPS is still not fully understood. It is believed that the differential partition is driven by the van der Waals, hydrophobic, hydrogen bond, and ionic interactions between the biomolecules and the surrounding phase. Therefore, the partition may be influenced by the concentrations and molecular mass of phase-forming polymer, concentration of phase-forming salt, type and concentration of added salts, temperature and pH [3]. The molecular weight, shape, specific bonding sites, and the surface hydrophobicity of the biomolecules also affect the partition behavior [4]. In fact, the surface properties, such as the surface charge of biomolecules are probably the most important factors affecting the separation performance [5].

The added salts affect the partitioning of proteins in the ATPS because of the differential distribution of the salt ions in the two phases. The added salts contain ions with different hydrophobicities, i.e., ions at different positions in the Hofmeister or lyotropic series, which can direct the partitioning behavior of the proteins. The most hydrophobic anions or cations will drive the partitioning of their counterions to the more hydrophobic phase, and the less hydrophobic co-ions will be forced to partition to the more hydrophilic phase [5,6].

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Lysozyme (EC 3.2.1.17) is a commercially valuable enzyme and it may be used as a cell disrupting agent for extraction of bacterial intercellular product, as an antibacterial agent in ophthalmologic preparations, as a food additive, and as a drug for treatment of ulcers and infections [7]. Lysozyme occurs naturally in chicken egg white (CEW) although other natural sources of lysozyme are also known to exist. Commercially available lysozyme is produced from CEW using a combination of conventional processes such as chromatography, adsorption, crystallization, and precipitation [8]. However, the ATPS appears to be another potential method for the extraction of lysozyme from CEW because this system is adequate for continuous large-scale purification of biomolecules and allow the use of traditional liquid–liquid extraction equipment [9,10]. The objectives of this work were to investigate the possibility of using aqueous two-phase system for partitioning of lysozyme from CEW and to optimize the operation conditions for this process.

2. Materials and methods

2.1. Materials

Hen eggs were purchased from a supermarket in Taipei. The polyethylene glycol (PEG; MW 1500, 6000, 8000, and 20 000), lysozyme (EC3.2.1.17, L6876), BSA (A7641), conalbumin (C0755), ovomucoid (T2011), and ovalbumin (O4754), and *Micrococcus lysodeikticus* (M3770) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of reagent grade.

2.2. Two-phase systems

Stock solutions of PEG (50%, w/w), sodium phosphate (30%, w/w), sodium citrate (30%, w/w), and sodium sulfate (30%, w/w) were prepared in deionized water. The polymers, buffer, salt and egg white solutions were weighed in graded 15 ml tubes. Polymers, buffer, salt and deionized water were mixed before adding the egg white to avoid protein precipitation. The total weight of the phase system was 10 g. The pH of the phase systems was adjusted with NaOH or HCl. The phases were dispersed by gentle mixing for 30 min at different temperatures. Complete phase separation was achieved by low-speed batch centrifugation at $1500 \times g$ for 20 min. After dilution the phases were analyzed for lysozyme activity and protein concentration.

2.3. Experimental design

There were six experimental factors that were initially thought to affect the partitioning behavior of lysozyme in aqueous two-phase system. To reduce the number of experimental works, a 2^{6-3} fractional factorial design was used to screen and find the factors which were most important for the partitioning. The coded and actual levels of the experimental variables are shown in the footnote of Table 4. After representing the specific activity and recovery as linear functions of the coded levels of the six experimental variables using multilinear regression techniques, the relative magnitude of the regression coefficients associated with the experimental variables allowed us to determine the importance of these variables.The Box–Wilson experimental design was used for the optimization of partitioning of lysozyme in ATPS. PEG concentration (X_1, w/w), sulfate concentration (X_2, w/w), and egg white concentration (dilution fold) (X_3) were chosen as independent variables in the partitioning experiments. Specific activity and recovery of lysozyme were the dependent output variables. For statistical calculations the variables X_i were coded as x_i according to the equation,

$$x_i = (X_i - X_0)/\Delta X_i$$

where x_i is the coded value of the variable i, X_0 is the value of the variable i at the center point of the investigated area, and ΔX_i is the step size. In the footnote of Table 5 the values of these quantities are displayed.

2.4. Model building and data analysis

A stepwise regression procedure in the SAS package (SAS Institute, Inc., Cary, NC) was used to fit the specific activity and recovery data to second order polynomial equations with interaction terms:

$$Y = B_0 + B_i \sum X_i + B_{ii} \sum X_i^2 + B_{ij} \sum X_{ij} \quad (i \neq j)$$

where Y is the dependent variable (in percentage), B_0, B_i, B_{ii}, and B_{ij} are regression coefficients of the model, and X_i is the magnitude of the selected critical variable.

2.5. Analytical methods

The protein concentration was determined based on reduction of Cu^{2+} to Cu^{+} ions by protein in an alkaline solution [11] using Micro BCA protein assay reagent kit (Pierce Co., Rockford, IL) with bovine serum albumin (BSA) as the standard.

A *M. lysodeikticus* turbidity method suggested by Sigma Chemical Co. was used for determining lysozyme activity, and one unit of lysozyme activity is equal to a decrease in turbidity of 0.001 per minute at 450 nm at pH 7.0 and 25 °C [12]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the proteins of the samples according to the method of Laemmli [13] using a 15% polyacrylamide separating gel and a 4% stacking gel. Samples containing 1–2 mg protein/ml were dissolved in equal volumes of buffer solution (0.1 M Tris–HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 0.05% bromophenol blue, 4% SDS, and 10% β-mercaptoethanol), and heated at 100 °C for 5 min. Gels were stained with

Coomassie Brilliant Blue R250 for detection of protein, and destained by diffusion in a solution containing 10% acetic acid, 20% methanol, and 70% water.

The surface hydrophobicity of proteins was characterized by ammonium sulfate precipitation according to Franco et al. [4]. The proteins were dissolved in 0.05 M sodium phosphate buffer (pH 7.0) at a concentration of 2 mg/ml. To a measured volume of each protein solution, solid ammonium sulfate was added slowly and with continuous stirring to reach certain saturation. After all of the salt had dissolved, the solutions were left to equilibrate for 15 min at 25 °C. After centrifugation (25 000 × *g* for 20 min), more ammonium sulfate was added to the supernatant to make its concentration higher. Again, the solution was left to equilibrate and centrifuged. The above procedure was repeated several times and the supernatants obtained were assayed for protein after dilution. A blank was prepared for each level of saturation and diluted in the same way as the solution containing the protein. The concentration of ammonium sulfate at which the protein started to precipitate was determined by plotting the logarithm of solubility of protein against the concentration of salt. The inverse of the salt concentration at the discontinuity point was used as the indicator of the protein hydrophobicity in solution.

3. Results and discussion

3.1. Effect of phase-forming salt on protein separation

Three PEG1500/salt systems were tested to investigate the effects of phase-forming salt on the separation of lysozyme. PEG/phosphate system has been demonstrated to be able to extract macromolecules from fermentation broth [14–16]. Citrate was used due to its low environmental polluting properties. It also has similar properties to phosphate in PEG/phosphate system [4]. Sulfate salt was used because of its ability to promote hydrophobic interactions between proteins [17]. Sulfate salts are also preferentially excluded from the surface of proteins, and it could be used to lead to optimum selectivity during purification of proteins by hydrophobic interaction chromatography [18,19]. Table 1 shows the activities of lysozyme in the upper phases of the three systems after extraction. The PEG/sulfate system extracted the highest amount of lysozyme to the upper phase, suggesting that the extraction of lysozyme in ATPS was influenced by hydrophobicity. Increasing the difference in hydrophobicity between the phases would increase the strength of hydrophobic interaction between the protein and PEG molecules, thus improving extraction. Given the better partition behavior of lysozyme in the PEG/sulfate system, this was chosen as the system for further investigation.

Table 1
The activity of lysozyme in the upper phase (PEG phase) after extraction by aqueous two-phase system using different phase-forming salts

Component 1 (10%, w/w)	Component 2 (12%, w/w)	Activity of lysozyme (unit/ml)
PEG 1500	Phosphate	2300[c]
PEG 1500	Citrate	3700[b]
PEG 1500	Sulfate	6350[a]

a, b, c: means superscripted with different letters are significantly different ($p < 0.05$).

3.2. Effect of PEG molecular weight on protein separation

The 10% PEG–12% sulfate systems were used for the separation of lysozyme from egg white to investigate the effect of molecular mass of PEG on the partitioning, and the results are shown in Table 2. It appeared that the higher the molecular mass of PEG, the lower the extraction efficiency of the system. Due to an excluded volume effect, the general tendency expected would be an increase of the partition behavior in the upper phase as the PEG molecular mass decreases [20]. Therefore, in general, the partition coefficients decrease with increasing PEG (upper phase) molecular weight [21]. However, in PEG/salt system the protein partition behavior may also be driven by the salting-out forces and most proteins strongly favor the lower salt-rich phase [22,23]. Probably under the influence of both excluded volume effect and the salting out forces, there was no significant difference in the partitioning behavior of lysozyme between the phase-forming polymers of PEG 1500 and PEG 6000. On the other hand, with a further increase in the chain length of PEG, its hydrophobic character increases [24]. Because lysozyme has the highest total surface hydrophobicity among the chicken egg white proteins as found by ammonium sulfate precipitation studies (the surface hydrophobicities of lysozyme, BSA, conalbumin, and ovalbumin were determined to be 0.41, 0.31, 0.35, and 0.31, respectively), increase in the PEG molecular mass should increase the lysozyme affinity to the upper, PEG-rich, phase. This fact explains why the PEG 20 000 phase did not show dramatic decrease in lysozyme extraction. Moreover, further reduction of the size of PEG would not be a reasonable choice because too low of PEG molecular weight would virtually attract all proteins to the PEG phase, which

Table 2
The activity of lysozyme in the upper phase (PEG phase) after extraction by PEG/sulfate aqueous two-phase system using PEG of different molecular weight

Component 1 (10%, w/w)	Component 2 (12%, w/w)	Activity of lysozyme (unit/ml)
PEG 1500	Sulfate	7200[a]
PEG 6000	Sulfate	8800[a]
PEG 8000	Sulfate	2900[b]
PEG 20 000	Sulfate	3800[b]

a, b, c: means superscripted with different letters are significantly different ($p < 0.05$).

Table 3
The specific activity of lysozyme in the upper phase (PEG phase) after extraction by PEG/sulfate aqueous two-phase system with addition of various salts

Added salt	Specific activity of lysozyme (unit/mg)
Sodium perchlorate	13250[a]
Sodium acetate	6753[b]
Sodium carbonate	3869[c]

a, b, c: means superscripted with different letters are significantly different ($p < 0.05$).

would lead to very poor separation and purification from contaminating proteins [25]. Therefore, an intermediate value of PEG molecular weight, PEG 6000, was chosen for the subsequent studies.

3.3. Effect of added salt on protein separation

The difference in affinity of the ions for different polymers, and consequently for different phases, affects the partitioning of any charged molecule in the ATPS. The most hydrophobic anions or cations will drive the partitioning of their counterions to the more hydrophobic phase. Co-ions which are less hydrophobic will in the same way be forced to partition to the hydrophilic phase [6,26]. Salts containing ions with different hydrophobicities, i.e., ions at different positions in the Hofmeister or lyotropic series, including sodium acetate, sodium carbonate, and sodium perchlorate were added to the PEG 6000/sulfate ATPS in order to direct the partitioning of the proteins. As can be seen in Table 3, lysozyme preferred to partition to the upper phase of the system with addition of sodium perchlorate since its counterion (ClO_4^-) is most hydrophobic among the three salts investigated. The interactions between the properties of the surface of the proteins and the properties of the systems determine the partitioning behavior [27]. Lysozyme has the highest total surface hydrophobicity of the proteins of chicken egg white. Although the salt had a strong effect on the partitioning of lysozyme, it was affected even stronger by the hydrophobic interaction with the polymer [28]. When these effects are in the same direction in the two-phase system the total effect is strong. Therefore, lysozyme was strongly partitioned to the upper phase with the hydrophobic polymer PEG and the hydrophobic counterion (ClO_4^-) as compared with the other chicken egg white proteins which were relatively more hydrophilic and mostly remained in the bottom phase. A similar result was obtained by Berggren et al. [28] with lysozyme in a model solution.

3.4. Processing optimization

There were six independent variables which might affect the separation of lysozyme in the aqueous two-phase system, including concentrations of PEG, sodium sulfate, sodium perchlorate, egg white (i.e. the dilution fold of egg white), pH of the system, and the temperature during extraction. The aim of this part of study was to identify the processing variables which had decisive effect on the extraction performance. Six variables were investigated using the fractional factorial design, and the result is given in Table 4. Based on the experimental data, two linear regression models for the specific activity (Y_1) and recovery (Y_2) of lysozyme were obtained:

$$Y_1 = 22\,431.6 + 5915.8X_1 + 174.8X_2 + 9755.4X_3 - 9103.5X_4 + 18\,113.3X_5 + 9193.5X_6 \quad (1)$$

$$Y_2 = 38.4 + 5.9X_1 - 7.9X_2 + 18.3X_3 + 10.7X_4 + 8.0X_5 - 6.0X_6 \quad (2)$$

Table 4
Effects of processing variables on the specific activity and the recovery of lysozyme separated by ATPS based on a 2^{6-3} fractional factorial design

No.	X_1	X_2	X_3	X_4	X_5	X_6	Specific activity (unit/mg)	Recovery (%)
1	−1	−1	−1	−1	−1	−1	3427 ± 57	9 ± 2
2	−1	−1	−1	+1	−1	+1	1317 ± 0	30 ± 3
3	−1	−1	+1	−1	+1	−1	25023 ± 677	70 ± 5
4	−1	−1	+1	+1	+1	−1	22845 ± 973	80 ± 3
5	−1	+1	−1	−1	+1	−1	19490 ± 922	18 ± 3
6	−1	+1	−1	+1	+1	−1	11649 ± 972	45 ± 5
7	−1	+1	+1	−1	−1	+1	0	0
8	−1	+1	+1	+1	−1	−1	16100 ± 1720	44 ± 5
9	+1	−1	−1	−1	+1	+1	10677 ± 526	33 ± 2
10	+1	−1	−1	+1	+1	−1	7577 ± 546	33 ± 2
11	+1	−1	+1	−1	−1	−1	8915 ± 108	57 ± 2
12	+1	−1	+1	+1	−1	+1	18145 ± 425	85 ± 3
13	+1	+1	−1	−1	−1	+1	1577 ± 149	2 ± 0.5
14	+1	+1	−1	+1	−1	+1	960 ± 6	16 ± 2
15	+1	+1	+1	−1	+1	+1	19602 ± 2543	44 ± 5
16	+1	+1	+1	+1	+1	+1	12946 ± 54	65 ± 5

The actual values corresponding to the coded levels of each variable—X_1: PEG concentration (w/w) (−1: 10%, 1: 20%); X_2: sodium sulfate concentration (w/w) (−1: 10%, 1: 20%); X_3: $NaClO_4$ concentration (−1: 100 mM, 1: 500 mM); X_4: egg white dilution fold (−1: 10-fold, +1: 4-fold), X_5: pH (−1: 6, 1: 10); X_6: temperature (−1: 25 °C, 1: 40 °C).

In general, the magnitude of the coefficients of the linear regression models could be used to judge the extent of contribution of the corresponding independent variables to the dependent variables. Considering both the specific activity and recovery of lysozyme, we could not ignore any of the six independent variables.

According to the above two equations, pH (X_5) was an important factor for both of the specific activity and recovery. Increase of the system's pH could increase both the specific activity and recovery of lysozyme. The p*I* of lysozyme is 10.5–11.0, and the p*I*s of other proteins in egg white are below 6.5. Forciniti and Hall [29] reported that hydrophobic interactions are stronger at a pH closer to the p*I* of the protein. Therefore, there was a general increase of the affinity behavior to the PEG-rich phase with increasing the system pH. Accordingly, it was decided to fix the pH of the system at 10 for the subsequent studies.

The regression equations also indicated that the specific activity and recovery of lysozyme increased with increasing sodium perchlorate concentration (X_3). Increasing the concentration of sodium perchlorate not only increased the hydrophobic interactions [30] but also changed the ionic strength so as to increase the electrical potential difference between the phases. However, a too high salt concentration might cause loss of lysozyme activity. Therefore, the concentration of sodium perchlorate was fixed at 500 mM for the subsequent experiments.

According to the regression coefficients of equations, the extraction temperature (X_6) had conflicting effect on the process performance. Increasing the extraction temperature increased the specific activity of lysozyme in the upper phase, but decreased the rate of lysozyme recovery. For the convenience of the operation, it was decided to carry out the aqueous two-phase extraction at room temperature (25 °C).

Table 5 shows the effects of the independent variables, namely PEG concentration (X_1), sodium sulfate concentration (X_2) and egg white concentration (dilution fold, X_3), on the specific activity and recovery of lysozyme based on a Box–Wilson design. Two models based on stepwise regression analysis were then developed showing the specific activity (Y_1) and recovery (Y_2) as the function of the processing variables:

$$Y_1 = 37\,002 - 3827.3X_1 - 833.1X_2 - 1229.0X_3 - 10\,847.0X_1^2 - 7635.2X_2^2 - 5813.4X_3^2 - 3181.4X_1X_2 + 4914.9X_1X_3 \quad (3)$$

$$Y_2 = 0.798 - 0.079X_1 - 0.01X_2 + 0.247X_3 - 0.127X_1^2 - 0.143X_2^2 - 0.092X_1X_2 \quad (4)$$

The high value of the R^2 ($R^2 = 0.88$ for Y_1; $R^2 = 0.85$ for Y_2) indicated that the data fitted the models well. The highest possible specific activity of lysozyme resulting from PEG/sulfate two-phase system was estimated to be 39 549 unit/mg protein, which was obtained by operating the partition at PEG concentration 16.1% (w/w), sodium sulfate concentration 12.0% (w/w), and 7.6-fold of egg white dilution. The estimated lysozyme recovery rate at this operating condition was 70%. Fig. 1 shows the response surface of the specific activity of lysozyme when one of the three variables was fixed. The maximum values of the response appeared near the centers of the graph, indicating that the center points chosen for this experiment were appropriate.

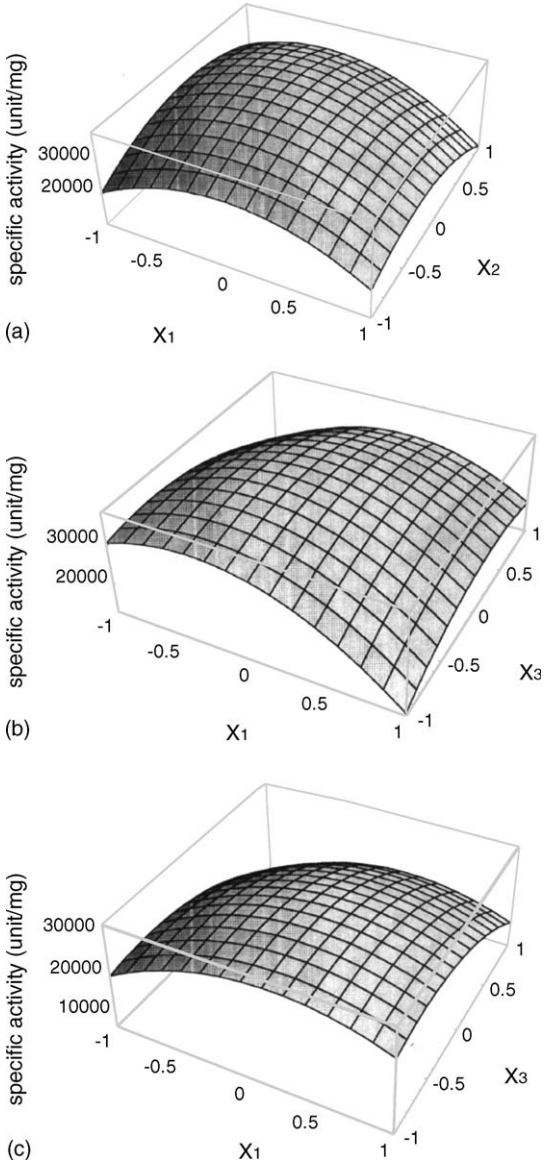

Fig. 1. Response surface of the specific activity of lysozyme of the upper phase of aqueous two-phase system. The actual levels corresponding to the coded levels of each variable—X_1: PEG concentration (w/w) (−1: 10%, 1 20%); X_2: sodium sulfate concentration (w/w) (−1: 8%, 1: 16%); X_3: egg white dilution fold (−1: 10-fold, 1: 4-fold). (a) the egg white dilution fold was 7.6, (b) the sodium sulfate concentration was 12%, (c) the PEG concentration was 16.1%.

Electrophoretic patterns of the chicken egg white, the upper and the bottom solutions resulting from PEG–salt aqueous two-phase extraction operated at the optimal conditions were compared (Fig. 2). The highly purified lysozyme product was found in the upper PEG-rich phase.

Table 5
Effects of processing variables on the specific activity and the recovery of lysozyme separating by ATPS based on a Box–Wilson design

No.	[PEG] (X_1)	[Sodium sulfate] (X_2)	Protein dilution fold (X_3)	Specific activity (unit/mg)	Recovery (%)
1	−1	−1	0	21561	63
2	−1	0	−1	28711	46
3	−1	0	1	21067	85
4	−1	1	0	14011	80
5	−1	1	0	19615	56
6	0	−1	−1	26883	30
7	0	−1	−1	26923	40
8	0	−1	1	21342	85
9	0	0	0	39204	80
10	0	0	0	39883	82
11	0	0	0	36638	83
12	0	1	−1	28052	50
13	0	1	1	21101	80
14	1	−1	0	19623	60
15	1	0	−1	15769	40
16	1	0	−1	8571.4	14
17	1	0	1	23784	80
18	1	1	0	7238	25

The actual values corresponding to the coded levels of each variable—X_1: PEG concentration (w/w) (−1: 10%, 0: 15%, 1: 20%); X_2: sodium sulfate concentration (w/w) (−1: 8%, 0: 12%, 1: 16%); X_3: egg white dilution fold (−1: 10-fold, 0: 7-fold, 1: 4-fold).

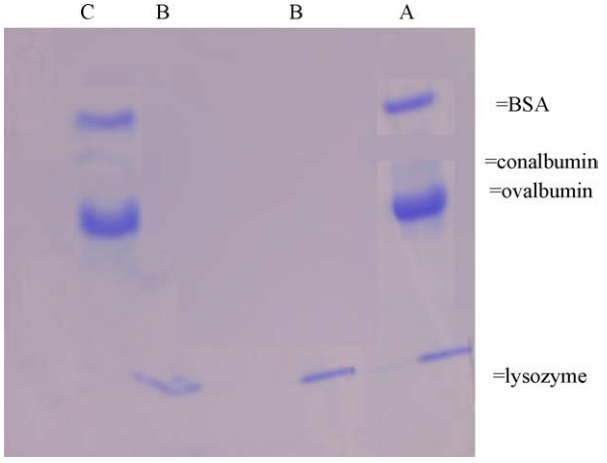

Fig. 2. SDS-PAGE profile of lysozyme separated from egg white by aqueous two-phase systems. Lane A: chicken egg white; lane B: the upper phase product after extraction by aqueous two-phase systems (lane B is presented in duplicate); lane C: the bottom phase product after extraction by aqueous two-phase systems.

4. Conclusion

Efficient and inexpensive lysozyme purification from chicken egg white can be achieved by using PEG–salt ATPS, if the correct salt is selected. The salts have a strong contribution to lysozyme partition behavior. The partitioning of lysozyme can be directed to upper-phase in an aqueous two-phase system by the addition of salt with hydrophobic counter ion, such as sodium perchlorate. When the PEG–sulfate ATPS was operated at pH 10 and room temperature (25 °C) with addition of 500 mM of sodium perchlorate, approximately 70% of lysozyme can be extracted from the 7.6-fold diluted chicken egg white. The specific activity of lysozyme in the extracted product was ca. 39 500 unit/mg protein.

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