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# An Integrated Process for Purification of Lysozyme, Ovalbumin, and Ovomucoid From Hen Egg White

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## Abstract

This article describes an integrated process for simultaneous purification of lysozyme, ovalbumin, and ovomucoid from hen egg white. The crude egg white extract was passed through a cation exchanger Streamline™ SP and the bound lysozyme was eluted with 5% ammonium carbonate, pH 9.0, containing 1 *M* NaCl after elution of avidin. This partially purified lysozyme was further purified 639-fold on dye-linked cellulose beads. Ovalbumin and ovomucoid did not bind to Streamline SP. Ovalbumin could be precipitated from this unbound fraction by 5% trichloroacetic acid, and ovomucoid was removed from the supernatant by precipitation with ethanol. The yields of lysozyme, ovomucoid, and ovalbumin were 77, 94, and 98%, respectively. All the purified proteins showed single bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis. All the steps are easily scalable, and the process described here can be used for large-scale simultaneous purification of these proteins in the pure form.

**Index Entries:** Egg white; lysozyme; ovalbumin; ovomucoid; precipitation; dye-linked chromatography.

## Introduction

Protein purification work is often aimed at obtaining one particular target protein out of many present in a chosen source. However, from the viewpoint of production of enzymes/proteins, it is more useful if many proteins/enzymes present in a single source are simultaneously recovered with adequate purity and yield. Early efforts in this area mostly consisted

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of precipitation and crystallization steps *(1–3)*. The last two decades have seen the development of some efficient bioseparation techniques *(4–6)*. Consequently, the interfacing of these relatively new generation techniques with some classical techniques may be useful in developing efficient integrated processes for simultaneous purification of useful proteins from a single crude extract.

Egg white constitutes one such attractive source, containing many useful proteins. Lysozyme, one of the proteins present in egg white, is extensively used in upstream processing of proteins/enzymes because it hydrolyzes the cell wall of Gram-negative bacteria *(7)*. It is also used to inhibit *Clostridium butyricum* during ripening of cheeses *(8)*, extend shelf life of selected processed foods by inhibiting spore-forming and non-spore-forming spoilage organisms *(9)*, and act synergistically with other antimicrobial compounds *(10)*. A more recent use is in wine preparation as a substitute for sulfites *(11)*.

Ovalbumin has been widely used as a model protein in many biochemical studies *(12,13)*, as a substrate for elastase *(14)*, as a molecular weight marker in gel filtration *(15)* and gel electrophoresis *(16)*, and in pharmaceutical processing *(17)*. Ovomucoid, another egg white protein, continues to find novel uses such as in designing mucoadhesive polymers or microparticulates for drug delivery *(18)*, and for chiral separation of bupropion enantiomers *(19)*. Recently, its interaction with elastase has been the basis for developing biomimetic affinity ligands for the enzyme *(20)*. Thus, it is not surprising that analysis and isolation of egg white proteins continues to be a focus of attention.

Caslavska et al. *(21)* described the purification of ovalbumin and lysozyme by recycling isotachophoresis. Desert et al. *(22)* have recently compared various electrophoretic methods for separation of hen egg white proteins and state that, to their knowledge, their study is "the first proteomic investigation of hen egg white." Ivanov et al. *(23)* have described the partial success of using thermosensitive copolymers as displacers of hen egg white proteins from a Cu(II)-IDA-Sepharose column.

In fact, no efficient integrated process for obtaining hen egg white proteins exists. Therefore, in the present work, the various separation steps were chosen keeping in mind the needs for easy possibility of scale-up, economics of the process, and obtaining high yields and products with adequate purity. We show that all the important proteins present in the hen egg white could be recovered in pure form, in high yields, and in a sequential fashion. Such simultaneous and sequential recoveries of egg white proteins have not been reported previously.

## Materials and Methods

### *Chemicals and Equipment*

Streamline™ SP was purchased from Pharmacia Biotech in Hong Kong.

*Micrococcus lysodekticus* cells, benzoyl-L-arginine-*p*-nitroanilide, trypsin, cellulose beads (cat. no. C7454), Cibacron Blue F3GA, and PD 10 (Sephadex G-25) desalting columns were obtained from Sigma (St. Louis, MO). Fresh hen eggs were obtained from the local market. All others chemicals used were of analytical grade.

### *Estimation of Protein and Activities of Various Egg White Proteins*

Lysozyme activity was measured by the rate of lysis of *M. lysodekticus* cells. One unit is equal to a decrease in turbidity of 0.001/min at 450 nm at pH 7.0 and 25°C under the specified conditions *(24)*. The presence of ovomucoid was determined by the antitryptic activity of the protein *(3)*. Trypsin activity was assayed according to the method of Erlanger et al. *(25)*. Protein concentration was determined according to the procedure described by Bradford *(26)*.

### *Polyacrylamide Gel Electrophoresis*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the samples using 12% gel was performed according to Hames *(16)* using a Bangalore Genei electrophoresis unit, and standard molecular weight markers (Bangalore Genei, Bangalore, India) for the purified ovomucoid and ovalbumin, and 15% gel for the purified lysozyme from egg white.

### *Preparation of Crude Egg White Solution*

The whites of four eggs were separated and homogenized. The homogenized solution was lyophilized to obtain about 8 g of solid material. This was dissolved in 75 mL of 0.1 *M* acetate buffer, pH 5.5, and used as the crude source of egg white proteins.

### *Purification of Lysozyme on a Cation Exchanger in Batch Mode*

Streamline SP (5 mL) was equilibrated with 50 mL of 0.1 *M* acetate buffer, pH 5.5. After equilibration, the excess buffer was drained completely, and the dissolved egg white (75 mL) was added to the cation exchanger and allowed to equilibrate for 1 h at 25°C (at 80 rpm on an orbital shaker). The unbound egg white solution was removed, and the matrix was washed twice with 50 mL of 0.1 *M* acetate buffer, pH 5.5. The supernatant and the washings were collected and labeled as S1. Bound avidin was washed off the column. (This step for the purification of avidin is the basis of a patent application that is under preparation and, hence, is not described here.) Twenty-five milliliters of 5% ammonium carbonate, pH 9.0, containing 1 *M* NaCl was added to this, and the protein-bound resin was equilibrated for 1 h at 25°C. The supernatant was then removed and passed through a PD-10 column; preequilibrated and eluted with 0.01 *M* potassium phosphate buffer, pH 7.0. The resultant solution (labeled as S2) was assayed for lysozyme and the amount of protein.

### *Immobilization of Dye on Cellulose Beads*

Cibacron Blue F3GA was coupled to the cellulose beads according to the protocol described by Hermanson et al. *(27)*. Ten milliliters of the beads were washed thoroughly with 200 mL of distilled water. The dye (0.1 g, dissolved in 5 mL of distilled water) was added to the beads and stirred for 30 min at 60°C. NaCl (1.5 g) was added to the reaction mixture, which was stirred for another hour at 60°C. The temperature of the mixture was raised to 80°C. $Na_2CO_3$ (0.15 g) was added and the reaction mixture was stirred for 2 h. The reaction mixture was cooled, filtered (on Whatman No. 1 filter paper), and extensively washed with warm water until the washings were colorless. Finally, the dye-conjugated beads were washed with 200 mL each of 1 *M* NaCl and distilled water and stored in 0.02% sodium azide at 4°C. The amount of dye immobilized on cellulose beads was calculated by subtracting the amount of dye recovered in the supernatant and washings from the amount of dye initially added to the cellulose beads. It was found that 6.8 µmol of dye could be immobilized per milliliter of cellulose beads.

### *Further Purification of Lysozyme on Cibacron Blue-Linked Cellulose Beads*

Five milliliters of dye-linked cellulose beads was equilibrated by washing with 10 mL of 0.05 *M* Tris-HCl, pH 7.0, three times. The supernatant was decanted and 20 mL of S2 was equilibrated with the prewashed beads (with constant shaking in an orbital shaker at 85 rpm) at 25°C for 1 h. After equilibration, the shaking was stopped and the supernatant decanted. The enzyme-linked beads were then washed twice with 10 mL each of equilibration buffer. The supernatant and washings were checked to determine the amount of activity bound to the beads. The activity initially added has been taken as 100%. The bound enzyme was eluted with 0.05 *M* Tris-HCl containing 1 *M* NaCl. The beads were regenerated by washing extensively with 8 *M* urea. The eluate was checked for lysozyme activity after desalting it with a PD 10 column; preequilibrated and eluted with 0.01 *M* potassium phosphate buffer, pH 7.0.

### *Purification of Ovalbumin and Ovomucoid Present in Wash (S1)* (3)

The pH of the wash (previously labeled as S1) was adjusted to 3.5 with 5 *N* sulfuric acid. An equal volume of 10% trichloroacetic acid (TCA) (pH adjusted to 3.0) was added to this. The resultant solution was stirred and kept at room temperature for 15 min. The precipitate formed (ovalbumin) was dissolved in a minimum amount of distilled water and its purity determined by SDS-PAGE. Ethanol was added to the supernatant, and the precipitate (ovomucoid) so obtained was dissolved in a minimum amount of distilled water. The presence and purity of ovomucoid were established by trypsin inhibitory activity and SDS-PAGE, respectively.

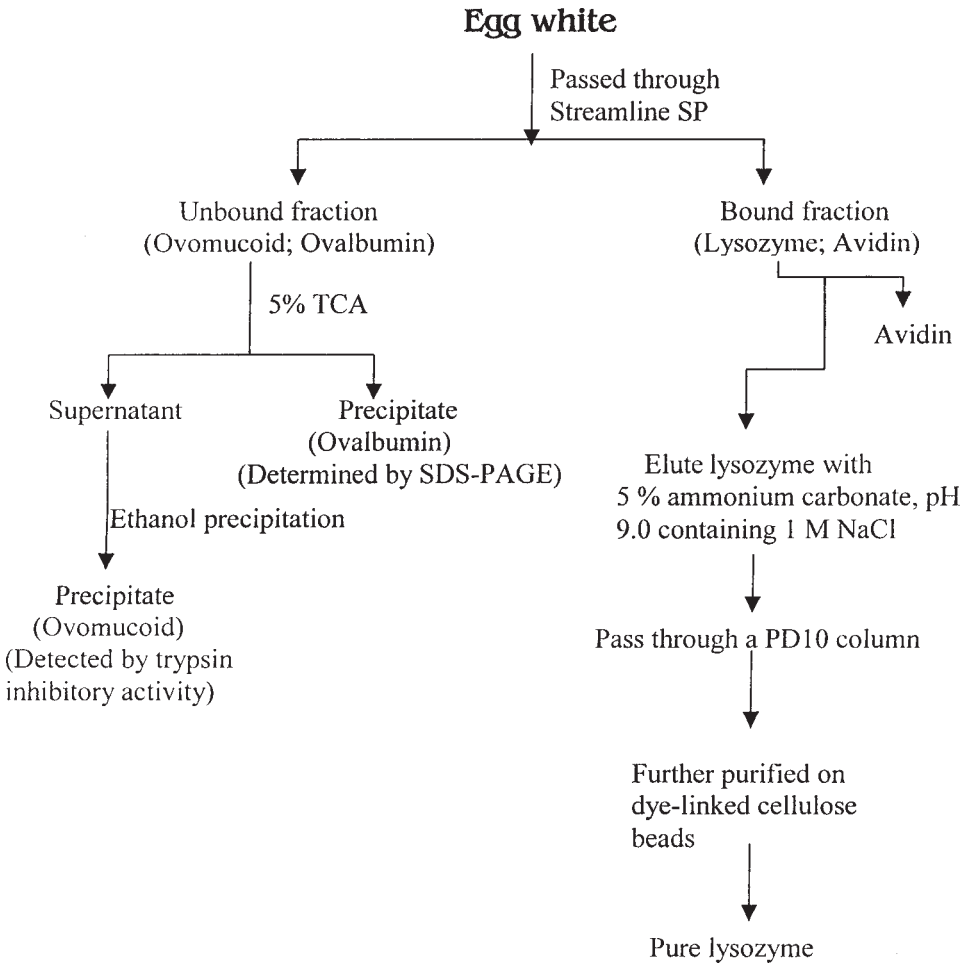


Fig. 1. Flow sheet for integrated protocol for purification of major proteins present in egg white. The individual steps are described in Materials and Methods.

## Results and Discussion

An outline of the integrated protocol for the separation of various egg white proteins is shown in Fig. 1.

### *Purification of Lysozyme*

As indicated in Materials and Methods, lysozyme could be eluted from Streamline SP (after elution of avidin) with 5% ammonium carbonate, pH 9.0, containing 1 *M* NaCl (Table 1). Lysozyme obtained at this stage was not pure but could be purified by batch adsorption on dye-linked cellulose beads. Elution with 0.05 *M* Tris-HCl, pH 7.0, containing 1 *M* NaCl gave pure lysozyme (Table 1). SDS-PAGE of the purified preparation is given in Fig. 2.

Table 1
Purification of Lysozyme
Using Streamline SP Followed by Dye-Ligand Chromatography

| Steps | Lysozyme (U) | Protein (mg) | Yield (%) | Specific activity (U/mg) | Fold purification |
|---|---|---|---|---|---|
| Egg white | 750,000 | 3471.5 | 100 | 216 | 1 |
| Wash | 0.0 | 3388.6 | 0.0 | 0.0 | - |
| Eluate (Streamline SP) (after passing through PD10 column) | 750,000 | 34.5 | 100 | 21,739 | 100 |
| Eluate (dye-linked cellulose beads) (after passing through PD10 column) | 580,000 | 4.2 | 77 | 138,095 | 639 |

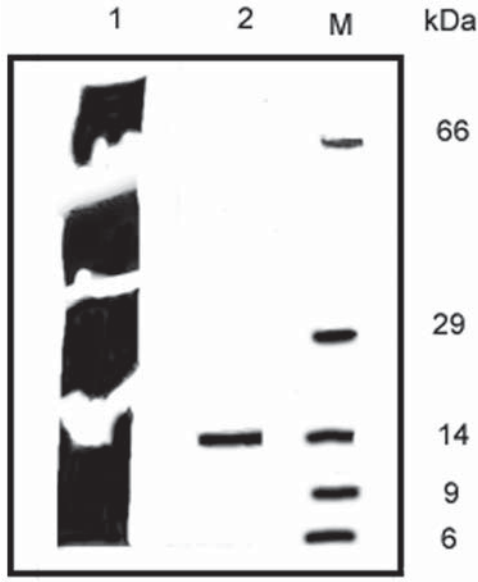


Fig. 2. SDS-PAGE of lysozyme. *Lane 1*, crude egg white extract (15 µg); *lane 2*, pure lysozyme (after purification on dye-linked cellulose beads) (15 µg); *lane M*, molecular weight marker proteins. The gel was stained with Coomassie Brilliant Blue R-250 for 45 min and then destained in 40% methanol and 10% acetic acid.

## *Purification of Ovalbumin and Ovomucoid*

The unbound fraction from the batch adsorption on Streamline ion exchanger (S1) contained both ovalbumin and ovomucoid. Their separa-

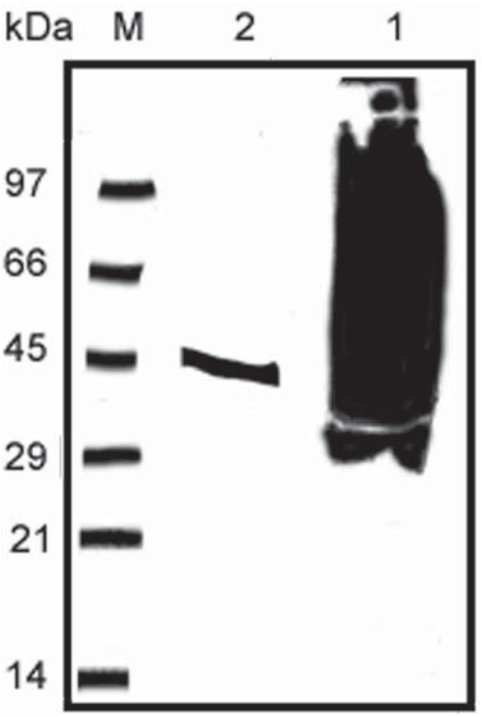


Fig. 3. SDS-PAGE of ovalbumin. *Lane 1*, crude egg white extract (15 µg); *lane 2*, pure ovalbumin (15 µg); *lane M*, molecular weight marker proteins. The gel was stained and destained as in Fig. 2.

tion was carried out as described in Materials and Methods. Ovalbumin was purified by precipitation with TCA (Fig. 3), and ovomucoid was precipitated from the supernatant by precipitation with alcohol (Fig. 4). The single band obtained in the SDS-PAGE (Fig. 3) shows that the ovalbumin preparation obtained using this integrated protocol is pure. The minimum molecular mass (43,000 Daltons) obtained agrees well with that reported in the literature *(3)*. The single band obtained for ovomucoid in the SDS-PAGE (Fig. 4) reflects the high purity achieved using this integrated protocol. The minimum molecular mass (28,000 Daltons) obtained agrees well with that reported in the literature *(3)*. Thus, three major proteins present in the egg white were purified to a level where each showed a single band on SDS-PAGE. In the case of lysozyme, 77% of the activity present in egg white could be recovered. The recoveries of ovomucoid and ovalbumin were 94 and 98%, respectively, of the amounts of these proteins reported to be present in egg white *(3)*.

The adequate yields reported here are comparable with those obtained with existing individual multistep procedures for purification of these proteins. For example, starting with 3471.5 mg of egg white protein, 415 mg of ovomucoid and 2374.5 mg of ovalbumin were obtained. These are

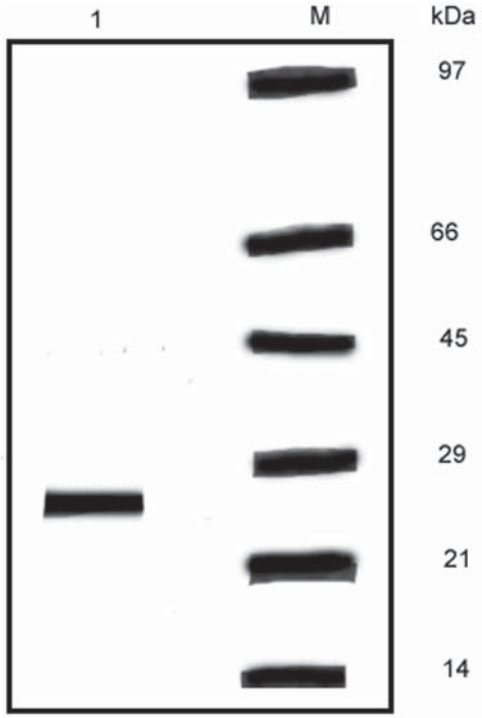


Fig. 4. SDS-PAGE of ovomucoid. *Lane 1*, pure ovomucoid (15 µg); *lane M*, molecular weight marker proteins. The gel was stained and destained as in Fig. 2.

12 and 68% of the starting total protein started. The percentages reported in the literature *(3)* are 12.9 and 69.5%, respectively.

Recently, affinity-based strategies have dominated the individual protocols for obtaining industrial enzymes and pharmaceutically important proteins *(28,29)*. In the past, efforts to obtain various proteins present in a particular crude extract have also relied on affinity-based methods *(30–32)*. It may sometimes be useful to change the strategy because it is not realistic to design and use affinity medium for all the proteins found in a particular cell. In the present case, the separation is based on a combination of ion exchange, precipitation, and an affinity medium. This kind of optimized integration of various methods may turn out to be necessary in quite a few cases.

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## References


1. Edsall, J. T. (1947), in *Advances in Protein Chemistry*, vol. 3, Anson, M. L. and Edsall, J. T., eds., Academic, New York, pp. 383–479.
2. Cohn, E. J., Gurd, F. R. N., and Surgenor, D. M. (1950), *J. Am. Chem. Soc.* **72,** 465–474.
3. Fevold, H. L. (1951), in *Advances in Protein Chemistry*, vol. 6, Anson, M. L., Edsall, J. T., and Bailey, K., eds., Academic, New York, pp. 187–252.
4. Scopes, R. (1996), *Biotechnol. Appl. Biochem.* **23,** 197–204.
5. Hjorth, R. (1997), *Trends Biotechnol.* **15,** 230–235.
6. Gupta, M. N. (2002), *Methods for Affinity-Based Separation of Proteins/Enzymes*, Birkhauser Verlag, Basel, Switzerland.
7. Middelberg, A. P. J. (1998), in *Bioseparation and Bioprocessing*, vol. 2, Subramanian, G., ed., Wiley-VCH, Berlin, pp. 131–164.
8. Maullu, C., Lampis, G., Basile, T., Ingianni, A., Rossolini, G. M., and Pompei, R. (1999), *J. Appl. Microbiol.* **86,** 182–186.
9. Peck, M. W. and Fernandez, P. S. (1995), *Lett. Appl. Microbiol.* **21,** 50–54.
10. Leitch, E. C. and Willcox, M. D. (1998), *J. Med. Microbiol.* **47,** 837–842.
11. Marchal, R., Chaboche, D., Douillard, R., and Jeandet, P. (2002), *J. Agric. Food Chem.* **50,** 1420–1428.
12. Nichol, L. W., Owen, E. A., and Winzor, D. J. (1985), *Arch. Biochem. Biophys.* **236,** 338–341.
13. Boschetti, E. and Coffman, J. L. (1998), in *Bioseparation and Bioprocessing*, vol. 1, Subramanian, G., ed., Wiley-VCH, Berlin, pp. 157–198.
14. Wright, H. T. (1984), *J. Biol. Chem.* **259,** 14,335, 14,336.
15. Mann, K. G. and Fish, W. W. (1968), in *Advances in Protein Chemistry*, vol. 23, Anfinsen, C. B., Edsall, J. T., and Richards, F. M., eds., Academic, New York, pp. 28–42.
16. Hames, B. D. (1986), in *Gel Electrophoresis of Proteins: A Practical Approach*, Hames, B. D. and Rickwood, D., eds., IRL, Oxford, pp. 1–86.
17. Nierkens, S., van Helden, P., Bol, M., Bleumink, R., van Kooten, P., Ramdien-Murli, S., Boon, L., and Pieters, R. (2002), *J. Immunol.* **168,** 3747–3754.
18. Plate, N. A., Valuev, I. L., Sytov, G. A., and Valuev, L. I. (2002), *Biomaterials* **23,** 1673–1677.
19. Munro, J. S. and Walker, T. A. (2001), *J. Chromatogr. A* **913,** 275–282.
20. Filippusson, H., Erlendsson, L. S., and Lowe, C. R. (2000), *J. Mol. Recognit.* **13,** 370–381.
21. Caslavska, J., Gebauer, P., and Thormann, W. (1991), *J. Chromatog.* **585,** 145–152.
22. Desert, C., Guerin-Dubiard, C., Nau, F., Jan, G., Val, F., and Mallard, J. (2001), *J. Agric. Food Chem.* **49,** 4553–4561
23. Ivanov, A. E., Galaev, I. Y., Kazakov, S. V., and Mattiasson, B. (2001), *J. Chromatogr. A* **907,** 115–130.
24. Shugar, D. (1952), *Biochim. Biophys. Acta* **8,** 302–308.
25. Erlanger, B. E., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys.* **95,** 271–278.
26. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
27. Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992), *Immobilized Affinity Ligand Techniques*, Academic, California.
28. Hage, D. S. (1999), *Clin. Chem.* **45,** 593–615.
29. Graslund, S., Falk, R., Brundell, E., Hoog, C., and Stahl, S. (2002), *Biotechnol. Appl. Biochem.* **35,** 75–82.
30. Reyes, L. B. and Scopes, R. K. (1991), *Bioseparation* **2,** 137–146.
31. Labrou, N. and Clonis, Y. D. (1994), *J. Biotechnol.* **36,** 95–119.
32. Narayanan, S. R. (1994), *J. Chromatogr.* **658,** 237–258.