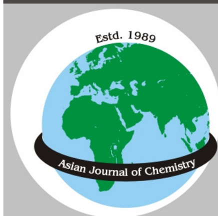
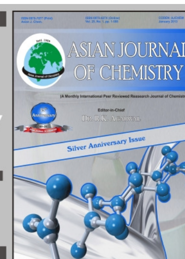


Asian Journal of Chemistry; Vol. 25, No. 5 (2013), 2683-2686

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13656



Simple Two-step Chromatographic Method for Purification of Ovomacroglobulin

FANG GENG, XI HUANG, MEI-HU MA*, ZHI LI and XIAO-WEI ZHANG

National R and D Center for Egg Processing, Huazhong Agricultural University, Wuhan, P.R. China

*Corresponding author: Fax: +86 27 87283177; Tel: +86 27 87283177; E-mail: mameihuhn@yahoo.com.cn; gengfang715@163.com

(*Received*: 27 February 2012; *Accepted*: 19 November 2012) AJC-12428

Ovomacroglobulin, also known as ovostatin, has been demonstrated to possess broad-spectrum inhibitory activity against various types of proteases and some other important biological activities, such as inhibiting sepsis, accelerate wound healing and antiinflammatory. In this study, an improved method was developed to purify ovomacroglobulin. Ovomacroglobulin-rich part obtained from polyethylene glycol precipitation (4-8 %) was separated by using Q Sepharose Fast Flow anion-exchange chromatography and then Sephacryl S-200 was employed to the purification of ovomacroglobulin. The recovery of the whole process was 37.76 %, the obtained protein was identified as ovostatin/ovomacroglobulin (Gallus gallus) by LC-ESI-LTQ. This procedure has an advantage in rapid preparation of ovomacroglobulin with a higher recovery.

Key Words: Ovomacroglobulin, Purification, Anion-exchange chromatography, Gel filtration chromatography.

INTRODUCTION

In the early part of last century, the ability of chicken egg white to inhibit trypsin was recognized and was attributed to ovomucoid[1]. Since then, at last four different proteins of the avian egg white were known to possess proteinase inhibitory activity *i.e.*, ovomucoid, ovoinhibitor, cystatin and ovomacroglobulin[2-4]. Different from ovomucoid and ovoinhibitor, ovomacroglobulin inhibits proteinases of all four mechanistic classes[5,6]. Besides, it has many kinds of biological activities, as antibacterial, inhibiting sepsis, accelerate wound healing and antiinflammatory[7-10]. It completely inhibited the activity of *Serratia* and *Pseudomonas* and reduced corneal destruction in experimental keratitis in rabbits[11]. Ovomacroglobulin can inhibit the generation of bradykinin through preventing bacterial protease activates Hageman factor-kinin system[12]. More noteworthy, ovomacroglobulin are highly homologous with α_2-macroglobulin (α_2M)[13-15], so, it may be it has more important biological activities that like α_2M.

However, the purification of ovomacroglobulin is difficult because of its low content in egg white (only 0.5 % of egg white protein[16]), which limited its research in bioactivity. In the previous research, Nagase *et al.*[6] purified ovomacroglobulin by polyethylene glycol precipitation from egg white following by chromatography on Ultragel AcA 34, DEAE-cellulose and Sephacryl S-300 columns. Ovomacroglobulin could also be prepared by gel filtration of egg white supernatant (obtained by homogenizing egg white in 1 % sodium chloride and 1 M EDTA) on Sephadex G-200 column with further purification on Sepharose CL-4B and DEAE-cellulose columns[3]. But these methods are complex, low recovery, time-consuming and costly due to the application of multiple steps chromatography.

Therefore, it was needed to improve the purification method of ovomacroglobulin by reducing the chromatography steps. Ovomucin will congest the column packing and ovalbumin will weaken the selectivity of anion exchange chromatography due to its high concentration[17,18], so we separated ovomucin and most of the ovalbumin from ovomacroglobulin by using polyethylene glycol precipitation. Then ovomacroglobulin-rich part was separated by anion exchange chromatography and further purified by gel filtration chromatography. Only two steps chromatographies were employed in this method and no desalting or buffer exchange was needed between the two-step chromatography. Benefiting from these improvements, time was saved and a higher recovery was obtained.

EXPERIMENTAL

Fresh hen eggs laid within 24 h from White Leghorns were collected from a local hennery (Jiufeng farm, Wuhan, China) and used within the same day for experiment. Polyethylene glycol (PEG-8000) was made by Merck Chemicals Co. Ltd. (Shanghai China). Q Sepharose Fast Flow was supplied by RuiDaHengHui S & T Co. Ltd. (Beijing, China). Sephacryl S-200 was obtained from GE Healthcare Bio-Sciences AB

(Sweden). The reagents used in SDS-PAGE were purchased from Guge Biotechnology Co. Ltd. (Wuhan, China). Prestained protein molecular weight marker was produced by Fermentas China Co. Ltd. (Shenzhen, China). All the other chemicals used in the experiment were produced by SINOPHARM Chemical Reagent Co. Ltd. (Shanghai, China).

Polyethylene glycol precipitation: All separation procedures in this part were carried out in an ice water bath (2-5 ºC). Egg white (100 mL) was mixed gently with an equal volume of distilled water, stirring the mixture for 2 h to mix the two uniformly[6]. PEG-8000 (4 g, 2 %, w/v) was added while stirring, the dispersion was kept 2 h and the homogenate was centrifuged at 12000 × g (Sigma, Laborzentrifugen, 3-30 K, Germany) at 4 ºC for 10 min. The precipitate (precipitate of 0-2 %, named P-2) was collected and the supernatant was collected and made to 4 % (w/v) by adding PEG-8000; P-4 (the precipitate of 2-4 %) was obtained by the centrifugation at 12,000 × g at 4 ºC for 10 min. In this way, P-6 (the precipitate of 4-6 %), P-8 (the precipitate of 6-8 %), P-10 (the precipitate of 8-10 %) and P-12 (the precipitate of 10-12 %) were obtained. The components of these six samples were identified by SDS-PAGE analysis.

Separation of ovomacroglobulin from precipitate P-6 and P-8: P-6 and P-8 were mixed and suspended in 50 mL *Tris*-HCl buffer (pH 8.0, 20 mM, 80 mM NaCl) for 4 h, then centrifuged at 12000 × g at 4 ºC for 10 min to remove a few amount of insoluble substance. The supernatant was applied (10 mL each time, five times) to a Q Sepharose Fast Flow column (40 × 1.6 cm, automatic low pressure liquid chromatography system, JiaPeng Technology Co. Ltd., Shanghai, China) which had previously equilibrated with Tris-HCl buffer (pH 8.0, 20 mM, contain 80 mM NaCl)[19]. Flow-through fraction was eluted by using equilibrium buffer, followed by gradient elution using 20 mM Tris-HCl buffer (pH 8.0) containing 0.20 and 0.50 M NaCl successively, at a flow of 2.5 mL/min. The peaks was corresponding numbered A, B and C, each peak was pooled respectively. Protein concentrations of each peak were determined according to Bradford's method with bovine serum albumin as standard and components of these peaks were identified by SDS-PAGE analysis.

Purification of ovomacroglobulin by gel filtration chromatography: Peak B was concentrated to 50 mL by employing an ultrafiltration membrane (MWCO = 100 kDa, SINAP membrane science and technology Co. Ltd. Shanghai, China). After centrifuging at 12000 × g at 4 ºC for 10 min, the supernatant (5 mL each time, 10 times) was injected to Sephacryl S-200 column (60 × 1.6 cm), elution was performed by using *Tris*-HCl buffer (20 mM, pH 7.2, 0.15 M NaCl) at a flow rate of 1.0 mL/min. The first peak eluted from the Sephacryl S-200 were pooled and desalted, then identified by SDS-PAGE.

SDS-PAGE analysis: SDS-PAGE employs 8 % polyacrylamide gel. The loaded amount of proteins was about 2-10 µg of samples. Prestained protein markers of molecular weight obtained from Fermentas (Shenzhen, China) were loaded for comparison of molecular weight. The protein bands were stained with 0.1 % Coomassie Brilliant Blue R-250 solution (25 % ethanol and 8 % acetic acid) for 0.5 h at 45 ºC and destainned by diffusion in destaining solution (25 % ethanol and 8 % acetic acid)[20]. The molecular weight and purity of ovomacroglobulin was estimated by Gel-Pro Analyzer 4.0 (Media Cybernetics, America).

Identification of ovomacroglobulin by LC-ESI-LTQ: The sample of ovomacroglobulin was send to Institute of Biochemistry and Cell Biology (CAS Shanghai) and identified by liquid chromatography-electrospray ionization-linear ion trap mass spectrometry (LC-ESI-LTQ).

Yield determination: Yield of ovomacroglobulin was measured by the ratio of the total amount obtained from purification process to the total amount (reference to the theoretical content) in 100 g egg white according to the formula given below.

$$\text{Yield (\%)} = \frac{\text{Volume of OVM} \times \text{Protein content} \times \text{Purity}}{\text{Weight of EW} \times \text{Protein content of EW} \times \text{Theoretical content of OVM}} \times 100\%$$

Protein content in egg white (EW) was accounted to 10.2 %[16,21]. The protein content of finally sample was determined by Bradford's method. The purity of ovomacroglobulin was obtained by SDS-PAGE analysis as described above and theoretical content of ovomacroglobulin was accounted for 0.5 % of egg white protein as reported by Mine[16].

RESULTS AND DISCUSSION

Polyethylene glycol precipitation: Polyethylene glycol is widely used as the precipitant and crystallization agent in proteins separation[22-26]. According to existing theory, mechanism of precipitation of proteins by polyethylene glycol is on the basis of the volume exclusion effects[27]. Protein molecules are sterically excluded from the space of polyethylene glycol molecules and protein precipitates when its concentration exceeds the solubility. Because steric exclusion of polyethylene glycol is prior to the hydration of the protein, preferential exclusion would help to maintain the structure and activity of protein[28,29].

The samples obtained by polyethylene glycol precipitation were analyzed by SDS-PAGE (Fig. 1). The band of ovomacroglobulin on SDS-PAGE gels should be located about 175 kDa, thus, all of P-2, P-4, P-6 and P-8 contained ovomacroglobulin. Considering the congest effect of ovomucin contained in P-2 and P-4 to column packing, P-6 and P-8 was chosen to use in following experiments. In the polyethylene glycol precipitation step, ovomacroglobulin was separated from ovomucin and most of ovalbumin and ovotransferrin and the content of ovomacroglobulin increased from 0.5 % to about 11 %, so this step has a pivotal role for purification.

Polyethylene glycol precipitation was used in Nagase's method, they obtained ovomacroglobulin-rich fraction by precipitating with 5.5-10 % PEG-8000 and got a 70 % recovery. A detailed analysis of the precipitate formed with PEG-8000 was done in this study and according to these results, the PEG -8000 concentration range for the precipitation of ovomacroglobulin was improved to 4-8 % and a 78.15 % recovery of ovomacroglobulin was obtained.

Separation of ovomacroglobulin by anion-exchange chromatography: Anion-exchange chromatography of the mixture of P-6 and P-8 resulted in three peaks by a Q Sepharose fast flow column. Each peak was analyzed by SDS-PAGE (Fig. 2). The molecular weight of proteins in the flow-through

fraction (peak A) is about 78 kDa, consistent with that of ovotransferrin. Peak B (eluted with 0.20 M NaCl solution) contained a band with the molecular weight of around 175 kDa, which was regarded as ovomacroglobulin. Main component of peak C (eluted with 0.50 M NaCl solution) was conjectured as ovoflavoprotein since its molecular weight was about 33 kDa. Peak B was further purified in the gel filtration chromatography.

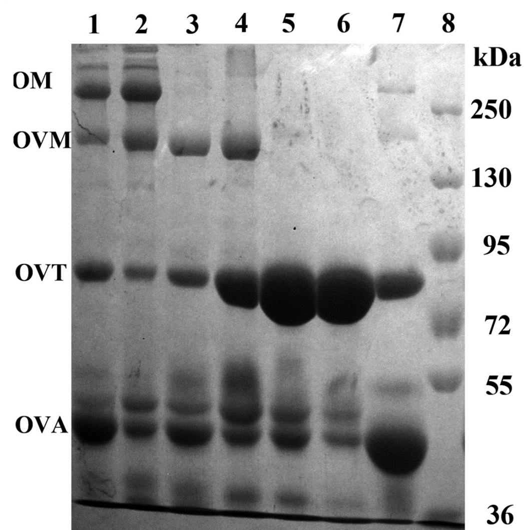


Fig. 1. SDS-PAGE analysis of precipitates obtained by PEG precipitation. Lane 1-6, precipitate of P-2, P-4, P-6, P-8, P-10 and P-12; lane 7, chicken egg white; lane 8, protein marker. OM: ovomucin; OVM: ovomacroglobulin; OVT: ovotransferrin; OVA: ovalbumin

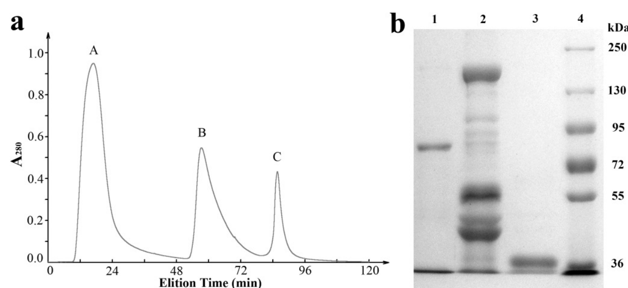


Fig. 2. Separation of ovomacroglobulin by anion-exchange chromatography. a: anion exchange chromatogram of the mixture of P-6 and P-8; b: SDS-PAGE analysis of fractions obtained by anion-exchange chromatography; lane 1, peak A; lane 2, peak B; lane 3, peak C; lane 4, protein marker

In this step, ovomacroglobulin was eluted with 0.20 M NaCl solution, well removed from ovotransferrin and ovoflavoprotein, only some protein contaminants with the molecular weight of about 42-55 kDa were detected on gel electrophoresis. The recovery of ovomacroglobulin after this step was 57.73 %.

Purification of ovomacroglobulin by gel filtration chromatography: The gel filtration chromatography of peak B obtained by Q Sepharose fast flow displayed two peaks. The two peaks were pooled respectively and then analyzed by SDS-PAGE (Fig. 3), the first peak showed a single band on the gel, was regarded as ovomacroglobulin. In this step, ovalbumin and another protein, which has a molecular weight about 55 kDa (may be ovalbumin Y[30-32]), was well removed.

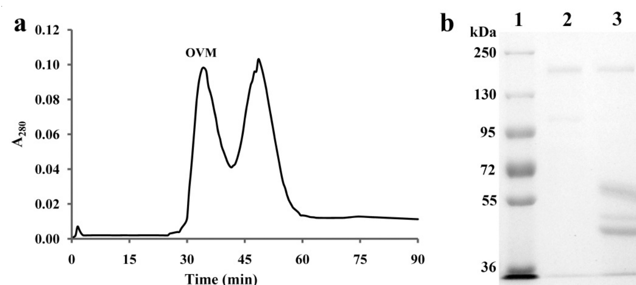


Fig. 3. Purification of ovomacroglobulin by gel filtration chromatography. a: gel filtration chromatogram of peak B obtained by anion-exchange chromatography. b: SDS-PAGE analysis of peaks obtained by gel filtration chromatography; lane 1, protein marker; lane 2, the first peak of gel filtration chromatography; lane 3, the second peak of gel filtration chromatography

Identification of ovomacroglobulin: The sample of purified ovomacroglobulin was identified by liquid chromatography-electrospray ionization-linear ion trap mass spectrometry. One hundred fourteen unique peptides were generated and the accepted amino acids residues was 923, corresponding to the total 1457 amino acids residues of ovomacroglobulin, the sequence coverage rate was 63.34 %[14]. After retrieving and comparing in database, the protein was identified as ovostatin/ ovomacroglobulin (Gallus gallus).

Protein yield: SDS-PAGE of samples from each stage of ovomacroglobulin purification was shown in Fig. 4 and the yields of ovomacroglobulin in purification process were calculated and summarized in Table-1. About 19 mg of ovomacroglobulin was obtained from 100 mL of egg white and the overall yield of the protein was 37.76 %. This yield has greatly improved, compared with Nagase's method (17 %)[6].

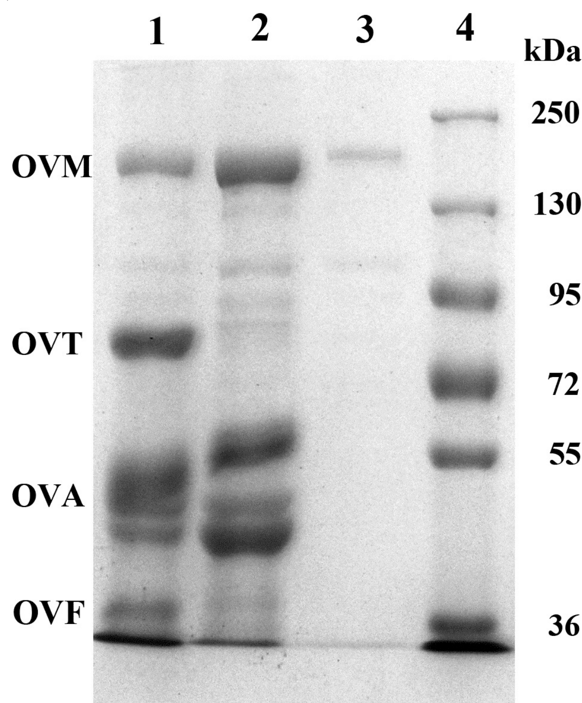


Fig. 4. SDS-PAGE pattern of samples from each stage of ovomacroglobulin purification. Lane 1, the mixture of P-6 and P-8; lane 2, sample after Q Sepharose FF; lane 3, purified ovomacroglobulin; lane 4, protein marker. OVM: ovomacroglobulin; OVT: ovotransferrin; OVA: ovalbumin; OVF: ovoflavoprotein

TABLE-1
PURIFICATION OF OVOMACROGLOBULIN FROM CHICKEN EGG WHITE

Steps	Sample (mL)	Protein Content (mg/mL)	Purity (%)	OVM (mg)	Recovery (%)
Egg white	100.0	102.00 [1]	0.50 [2]	51.00	100.00
PEG precipitation	50.1	6.90	11.53	39.78	78.15
Q Sepharose FF	245.3	0.52	23.08	29.44	57.73
Sephacryl S-200	128.4	0.15	100.00	19.26	37.76

1,2: The protein content in egg white and theoretical concentration of OVM was according to references [16, 21]

Conclusion

Ovomacroglobulin was purified from egg white by polyethylene glycol precipitation, Q Sepharose fast flow column and Sephacryl S-200. Ovomucin and most of the ovalbumin were firstly eliminated in the polyethylene glycol precipitation step, then ovotransferrin and ovoflavoprotein were well removed by anion-exchange chromatography, finally, the remaining ovalbumin was completely eliminated through gel filtration chromatography. Attribute to the improvement of polyethylene glycol precipitation and simplification of chromatography steps, the overall yield was higher than the present methods. This protocol has an advantage of being rapid and applicable at the laboratory scale.

ACKNOWLEDGEMENTS

This work was supported by the Earmarked Fund for Modern Agro-industry Technology Research System (CARS-41-K23) and Huazhong Agricultural University Scientific and Technological Self-innovation Foundation (2010SC09).

REFERENCES

1. H. Lineweaver and C.W. Murray, *J. Biol. Chem.*, **171**, 565 (1947).
2. I. Matsushima, *Science*, **127**, 1178 (1958).
3. T. Kitamoto, M. Nakashima and A. Ikai, *J. Biochem.*, **92**, 1679 (1982).
4. I. Saxena and S. Tayyab, *Cellul, Mol, Life Sci,*, **53**, 13 (1997).
5. H. Nagase and E.D. Harris Jr., *J. Biol. Chem.*, **258**, 7490 (1983).
6. H. Nagase, E.D. Harris Jr., J.F. Woessner Jr. and K. Brew, *J. Biol. Chem.*, **258**, 7481 (1983).
7. S. Miyagawa, R. Kamata, K. Matsumoto, R. Okamura and H. Maeda, *Graefe's Archive for Clinical and Experimental Ophthalmology*, **232**, 488 (1994).
8. K. Maruo, T. Akaike, T. Ono and H. Maeda, *Infect. Immun.*, **66**, 866 (1998).
9. A. Ikai, M. Nakashima and Y. Aoki, *Biochem. Biophys. Res. Commun.*, **158**, 831 (1989).
10. Y. Ofuji, T. Suzuki, H. Yoshie, K. Hara and M. Adachi, Periodontal Clinical Investigations: Official Publication of the Northeastern Society of Periodontists, **14**, 13 (1992).
11. S. Miyagawa, R. Kamata, K. Matsumoto, R. Okamura and H. Maeda, *Graefe's Archive for Clinical and Experimental Ophthalmology*, **229**, 281 (1991).
12. A. Molla, T. Yamamoto, T. Akaike, S. Miyoshi and H. Maeda, *J. Biol. Chem.*, **264**, 10589 (1989).
13. J.J. Enghild, G. Salvesen, K. Brew and H. Nagase, *J. Biol. Chem.*, **264**, 8779 (1989).
14. K.L. Nielsen, L. Sottrup-Jensen, H. Nagase, H.C. Thogersen and M. Etzerodt, *DNA Sequence: The Journal of DNA Sequencing and Mapping*, **5**, 111 (1994).
15. N.A. Zorin, V.N. Zorina and R.M. Zorina, *J. Evolut. Biochem. Physiol.*, **42**, 112 (2006).
16. Y. Mine, Egg Bioscience and Biotechnology, Wiley Online Library, (2008).
17. D. Pinto, E.A. Arriaga, R.M. Schoenherr, S.S.H. Chou and N.J. Dovichi, *J. Chromatogr. B*, **793**, 107 (2003).
18. D.A. Omana, J. Wang and J. Wu, *J. Chromatogr. B*, **878**, 1771 (2010).
19. A. Tankrathok, S. Daduang, R. Patramanon, T. Araki and S. Thammasirirak, *Prep. Biochem. Biotechnol.*, **39**, 380 (2009).
20. Y. Guo, Protein Electrophoresis Technology, Science Press, Beijing, (1999).
21. R. Huopalahti, Bioactive Egg Compounds, Springer Verlag (2007).
22. X. Zhang, N. Qiu, F. Geng and M. Ma, *J. Sep. Sci.*, **34**, 3295 (2011).
23. R. Bhat and S.N. Timasheff, *Protein Sci.*, **1**, 1133 (1992).
24. K.C. Ingham, *Methods Enzymol.*, **182**, 301 (1990).
25. G. Bassani, B. Farruggia, B. Nerli, D. Rornanini and G. Pico, *J. Chromatogr. B*, **859**, 222 (2007).
26. Y. Shibusawa, N. Takeuchi, K. Sugawara, A. Yanagida, H. Shindo and Y. Ito, *J. Chromatogr. B*, **844**, 217 (2006).
27. V. Kumar, V.K. Sharma and D.S. Kalonia, *Int. J. Pharmaceut.*, **366**, 38 (2009).
28. D.H. Atha and K.C. Ingham, *J. Biol. Chem.*, **256**, 12108 (1981).
29. W. Honig and M.R. Kula, *Anal. Biochem.*, **72**, 502 (1976).
30. J. Hirose, Y. Doi, N. Kitabatake and H. Narita, *Biosci. Biotechnol. Biochem.*, **70**, 144 (2006).
31. C. Guerin-Dubiard, M. Pasco, A. Hietanen, A.Q. del Bosque, F. Nau and T. Croguennec, *J. Chromatogr. A*, **1090**, 58 (2005).
32. F. Nau, M. Pasco, C. Desert, D. Molle, T. Croguennec and C. Guérin-Dubiard, *J. Agric. Food Chem.*, **53**, 2158 (2005).