Chapter

# Strategies to Recover and Purify Lysozyme from Egg White

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## Abstract

The present article analyzes different strategies to purify lysozyme from hen egg white. Since its discovery in 1922, new strategies have arisen to purify lysozyme from hen egg white. However, most of these strategies are based on the use of diluted egg white as the starting material, which has impaired its commercialization path for confectionery. Only a few processes use egg white without any conditioning or pretreatment, which accounts for the difficulty in the egg white processing; however, taking into account the large market for this enzyme and its high concentration in egg white, it is more convenient to obtain lysozyme from its natural source than from recombinant expression.

Here, the different approaches for lysozyme purification are analyzed taking into account the protein-ligand interaction –ionic exchange chromatography, pseudobioaffinity to triazinic dyes and affinity to N-acetyl glucosamine polymers- and considering the chromatographic support material or matrix for an adequate egg white processing.

## Introduction

Lysozyme (E.C.3.2.17) is a ubiquitous enzyme present in secretions, body fluids and animal and human tissues (Lesnieroswski and Kijowski, 2007), which catalyzes the hydrolysis of bacterial cell-wall sugars, specifically the β bonds between muramic acid and N-acetylglucosamine (Chang et al., 2000).

Lysozyme is one of the most studied bacteriolytic enzymes. Because of its relative abundance in nature and its multiple functions and utilities, it is considered a model protein for different research studies.

Several applications have been reported in the fields of food, pharmaceutical and veterinary industries (Cegielska-Radziejewska et al., 2008). Besides its bacteriolytic property, other actions, such as antiviral and anti-inflammatory properties, have been attributed to dimeric forms of the enzyme present in nature (Kicska, 1994; Jolles and Jolles, 1984).

The hen egg white is still the starting material for lysozyme purification: considering its high concentration in such material – 3.5 % on dry basis – the explanation is evident if comparing this amount with that of other starting materials (Lesnieroswski and Kijowski, 2007). Its low cost and high accessibility also contribute in the same way.

On the other hand, from the point of view of the downstream processing, an important challenge of protein purification is to have chromatographic matrices with high adsorption capacity, high affinity, efficient elution of the adsorbed protein and good hydrodynamic behavior. Besides, the performance/cost ratio is critical when the usefulness of the process is assessed (Lienqueo and Asenjo, 2000). With respect to hydrodynamic parameters, not all commercial matrices are useful when the raw starting material is complex. The presence of particulate material, the high viscosity and the presence of fat bring about the need of clarification steps or conditioning of the samples. Besides, for purification processes in batch, in addition to contact time optimization, matrix recovery after the adsorption step becomes difficult when the raw material is not clear and its viscosity is high, as is the case of egg white. In addition, if its purification is intended to be performed with no or a low degree of dilution or conditioning – as it is needed to allow its further utilization in confectionery – there is an added drawback. To overcome this problem, different chromatographic approaches have arisen and analyzing them is the main aim of the present mini-review.

From the point of view of the purification process design, lysozyme is a protein with singular physicochemical properties (Table 1), especially for its high isoelectric point, which leads to believe that cation exchange chromatography is an ideal technique to purify it (Ahvenainen et al., 1979; Palladino et al., 1981: Li-Chan et al., 1986; Weaver and Carta, 1996; Chen et al., 2005; Camperi et al., 1999; Chiu et al., 2007).

On the other hand, taking its biological activity into account (i.e. the fact that N-acetylglucosamine is its target molecule and that this molecule is easy to immobilize on a chromatographic support), numerous works have reported lysozyme purification by affinity chromatography with this monomer as the ligand (Junowicz and Charm, 1975; Weaver et al., 1977; Muzzarelli et al., 1978; Yamasaki and Eto, 1981; Yamada et al., 1985; Chiang et al., 1993; Ruckenstein and Zeng, 1997).

Processes based on membrane filtration have also been used for lysozyme purification from egg white (Chang et al., 1986; Ehsani et al, 1997; Ghosh and Cui, 2000; Wan et al., 2006). However, membrane filtration technology is expensive and the processes for lysozyme purification are rather complex due to egg white high viscosity and molecular associations between the enzyme and other proteins - such as ovomucin - present in egg white.

Despite the numerous strategies to purify lysozyme from egg white reported, very few have found industrial application. A classic production method at industrial level is crystallization from egg white in the presence of 5 % NaCl at pH 9.5.

Table 1. Some physicochemical properties of hen egg white lysozyme

| Property | Value |
|---|---|
| Molecular weight Nº of subunits | 14,400 1 |
| Amino acids | 129 |
| pI Disulfide bonds | 10.7 4 |
| % Carbohydrates | 0 |

This strategy, developed by Alderton and Fevold in 1964, has undergone several modifications (Ahvenainen et al., 1979). A yield of 60-80 % was achieved by using this technology, but the addition of a high concentration of salt and other additives precludes the further commercial utilization of egg white.

A related industrial strategy is a two-step lysozyme isolation by ion exchange chromatography followed by crystallization: the egg white is mixed with an adequate amount of carboxymethyl cellulose and, after washing the matrix with water, lysozyme is eluted with 5 % NaCl and precipitated by pH adjustment to 9.5. The precipitate is then redissolved, desalted and concentrated (Durance and Nakai, 1988).

Recent reports such as those of Chang et al. (2000) and Wang et al. (2009) where lysozyme is purified from egg white by reductants and thermal treatment or using a foam separation method are not subject of this review as they do not maintain the characteristics of the native egg white.

## Lysozyme-Ligand Interaction Mediated by Charge

Lysozyme has an isoelectric point of 10.7, higher than the bulk of egg white proteins. Therefore, the adsorption on matrices of opposite charge becomes one of the main strategies for its purification. In this way, numerous chromatographic systems exploiting this interaction have been developed. As mentioned, only the processes with unmodified or low-diluted egg white as the starting material will be reviewed in this article.

Durance and Nakai (1988) reported a method based on cation exchange chromatography that allows lysozyme and avidin simultaneous purification starting from egg white without any dilution or conditioning. The lysozyme yield was 86 % and the purity was very high as judged by SDS-PAGE. After several cycles of lysozyme purification, the avidin retained by the matrix was eluted, obtaining the protein with 75 % yield and 41 % purity. The matrix used was Duolite C-464, a copolymer of methacrylic acid and divinyl benzene. At pilot scale, 470-ml matrix allowed the processing of 14.2 liters of egg white in eight cycles of load/elution along 6 days.

Another development exploiting the lysozyme adsorption on a matrix by charge interaction was that reported by Bayramoglu et al. in 2007. By the phase inversion technique, these authors synthesized chitosan beads that were further crosslinked with epichlorhydrin and grafted with methacrylic acid using ammonium persulfate as the catalyst. After the grafting, the negative charge density of such matrix – due to carboxyl groups - was 11.24 mmol/g matrix.

The maximum adsorption capacity for lysozyme was 66 mg/g matrix, at pH 6.0, with beads of 800 μm in diameter. A 98 % adsorption of lysozyme from egg white diluted 1:1 with phosphate buffer was achieved, with an 87 % recovery and 94 % purity (by HPLC).

Expanded bed adsorption was developed with the aim to maximize the capture of the target protein from a complex raw material containing particles (Draegger and Chase, 1991; Brobjer, 1999). With this technology and using the commercial matrix Streamline SP, Chang and Chang (2006) developed a method to purify lysozyme from powered egg white redissolved in acetate buffer, pH 4.0.

The yield was 98 % with a purification factor of 11 in a single step, eluting the protein with 2M NaCl. A concentration factor of 4 was achieved. Another interesting strategy based on electrostatic interactions was that reported by Safarik et al. in 2007. These authors used the commercial magnetic beads Iontosorb MG CM 100 and Iontosorb MG SHP 100 to purify lysozyme from unmodified egg

white. These matrices present the advantage of being easy to recover after the target protein capture by subjecting them to a magnetic field.

Maximum adsorption capacities for the protein were 138 mg/ml and 61 mg/ml respectively. The process performed with 5 ml egg white and 0.5 ml matrix allowed obtaining 96 % pure lysozyme, with a specific activity similar to that of the commercial preparations of the enzyme. Here, egg white can be commercialized for confectionery after lysozyme depletion. However, magnetic beads are rather expensive to use at industrial scale.

Another alternative process based on electrostatic interaction between lysozyme and a chromatographic matrix is that reported by Ventura et al. (2008), where the ligand – sulfonic group – is immobilized on the porous structure of microfiltration hollow fiber membranes. These systems allow working at high flow rates without a decrease in the resolution or adsorption capacity. In addition, by choosing the adequate inner and outer diameters and the pore size, it is possible to process samples with particles in suspension (Wolman et al., 2010). The maximum adsorption capacity for lysozyme was 140 mg/ml by using polysulfone membranes grafted with glycidyl methacrylate and diethyleneglycol dimethacrylate and then derivatized with sodium sulfite. Egg white was diluted 6-fold with acetate buffer, pH 6.5. The authors proposed purification cycles of 10-15 min, eluting the lysozyme with 1M NaCl. Purity was 95 % and productivity 150 g/l.h. However, egg white cannot be commercialized after lysozyme depletion due to the high degree of dilution of the starting material.

## Lysozyme-Ligand Affinity Interaction

### Triazinic Dyes

Triazinic dyes are interesting molecules for use as affinity ligands (Wolman et al., 2000; Denizli and Piskin, 2001). They have been extensively used to obtain pseudobiospecific ligand affinity chromatography matrices due to their low cost, availability, simple immobilization reaction, biological and chemical degradation resistance as well as acceptable selectivity and capacity. As with many affinity chromatographic ligands, triazine dyes have been immobilized to a wide variety of supports in the search for an ideal system.

By combining the advantages of membranes as the supports with those of the triazinic dyes as the ligands, Grasselli et al. (1999) reported a method involving the use of Red HE-3B immobilized on polyethylene hollow fiber membranes, at a density of 1.7 μmol/ml membrane. Lysozyme was purified from egg white without any conditioning or dilution. Maximum adsorption capacity and dynamic

capacity were 26 and 18 mg/ml membrane respectively. Dynamic capacity did not change after increasing the flow rate three fold. The process productivity was 12 kg lysozyme/$m^3$.h. Elution was achieved with 0.6 M NaCl in phosphate buffer pH 8.0 with a recovery of 92 % and a purity of 88 %. The matrix could be reused for at least 10 times and egg white viscosity was not altered by the treatment.

Arica et al. (2004) reported the use of a matrix composed of plane membranes of poly (2-hydroxy-ethylmethacrylate) with Procion Brown MX-5BR and Procion Green H-4G immobilized. Ligand densities were 0.312 and 0.254 μmol/ml membrane respectively.

By increasing the temperature from 5º C to 35º C, the adsorption capacity of Procion Brown increased by 30 % and that of Procion Green by 27 %. For adsorption, egg white was diluted 1:1 with phosphate buffer, pH 7.0, and elution was performed with 0.5 M NaCl in phosphate buffer, pH 8.0. For membranes derivatized with Procion Brown the lysozyme purity was 76 % and the yield 63 %, whereas for those derivatized with Procion Green, purity was 92 % and yield 77 %. Both membranes recovered their chromatographic performance after regeneration with 0.1 M NaOH and 1 M NaCl.

It should be pointed out that these plane membranes were used in the dead end way, which greatly impaired the egg white processing without dilution. In 2005, the same research group reported the development of a membrane composed of a copolymer poly (2-hydroxy-ethylmethacrylate)/chitosan derivatized with Reactive Green 19 (Yilmaz et al., 2005), at a ligand density of 0.865 μmol/ml membrane. The lysozyme maximum adsorption capacity was 60.8 mg/ml membrane, working with egg white diluted 1:1 with phosphate buffer, pH 7.0. Elution was achieved with 0.5 M NaCl in phosphate buffer, pH 8.0 with a yield of 82 % and a purification factor of 25.4.

The matrix also recovered its chromatographic performance after regeneration. Altintaş and Denizli (2006) immobilized Cibacron Blue F3G-A on beads of poly(glycidyl methacrylate) of 1.6 μm in diameter, at a ligand density of 1.73 mmol/g beads. The lysozyme maximum adsorption capacity was 591.7 mg/g beads. The small size of the beads maximizes the surface area, thus increasing the adsorption capacity of the material. Here, 100 mg matrix were agitated with 10 ml egg white diluted 1:1 with phosphate buffer, pH 7.0 for 2 h. After washing, the matrix was recovered by centrifugation and lysozyme was eluted with 0.5 M NaSCN for 1 h. The purity of the product was 88 % (SDS- PAGE) with a yield of 79 %.

After each adsorption cycle, the matrix has to be recovered by centrifugation, which adds a cost to the process. Başar et al. (2007) developed magnetic beads of poly(2-hydroxy-ethylmethacrylate) with immobilized Cibacron Blue F3G-A by suspension-polymerization of 2-hydroxy-ethylmethacrylate in the presence of

$Fe_3O_4$ nano-powder. Particle diameter was 80-120 μm, surface area 56.0 $m^2/g$ beads and ligand density 28.5 μmol/g beads. Maximum adsorption capacity was 342 mg/g beads. Egg white diluted 1:1 with phosphate buffer, pH 7.0, was recirculated at a flow rate of 1 ml/min for 2 h. In order to maintain the beads in suspension, the system was subjected to a 33 G magnetic field around the column. Desorption was performed with 0.5 M NaCl under a magnetic field.

Product purity was 87.4 % (SDS-PAGE) with a yield of 79.6 % and a specific activity similar to that of the commercial enzyme, evidencing that the process does not affect the enzyme activity. Another contribution from this research group was the development of magnetic beads of poly(glycidyl methacrylate) prepared by the dispersion-polymerization method in the presence of $Fe_3O_4$ nano-powder. In this case, the ligand was L-tryptophan (Altintaş et al., 2007).

Lysozyme maximum adsorption capacity was 259.6 mg/g beads. After adsorption from egg white diluted 1:1 in phosphate buffer, pH 7.0, the enzyme was eluted by agitation with 0.1 M ethylene glycol for 1 h at 25º C. The process yield was 76 % and lysozyme purity was 85 % with a purification factor of 71. Specific activity was was similar to that of the commercial enzyme.

**N-Acetylglucosamine**

Being chitin a polymer of N-acetylglucosamine and taking into account the capacity of lysozyme to hydrolyze sugar bonds involving N-acetylglucosamine residues, in the 1960s and early 1970s, this polymer was used for affinity purification of lysozyme, either unmodified (Cherkasov et al., 1967; Jensen and Kleppe, 1972) or chemically modified (Imoto et al., 1968, Cherkasov and Kravchenko, 1969), or as coating of cellulose beads (Imoto and Yagishita, 1973). Moreover, based on the inhibitory properties of N-acetylglucosamine on lysozyme activity, Junowics and Charm (1975) purified the enzyme by using N-acetylglucosamine immobilized on Sepharose 4B. However, these methods do not allow the processing of unmodified egg white due to its high viscosity and the characteristics of the chromatographic matrix, which preclude an efficient fractionation under the conditions of the published works. These are dynamic processes, using Sepharose or other supports that load a pulse of egg white that is diluted with the equilibration buffer (e.g. Junowics and Charm loaded a pulse of 0.5 ml egg white). These methods are not useful for industrial scale.

Safarik and Safarikova (1993) reported lysozyme purification from diluted egg white by using magnetic chitin beads synthesized from chitosan and magnetite as the adsorbent. Chitosan was then acetylated with acetic anhydride to yield chitin. Lysozyme maximum adsorption capacity was only 2.5 mg/ml matrix,

a low value in comparison with other approaches. The adsorbed lysozyme was eluted with diluted HCl and the purification factor was 10.

By combining the advantages of the high flow rates achieved with adsorptive membranes and the affinity of lysozyme for chitin, Ruckenstein and Zeng (1997) developed chitin plane membranes of controlled porosity using silica as the porogen. Maximum adsorption capacity for lysozyme was 50 mg/ml membrane. The process was carried out with egg white diluted 1:10 with PBS, the elution was performed with 0.1 M acetic acid and the purity (HPLC) was 98%.

Wolman et al. (2010) reported a method that allows purifying lysozyme from egg white without any conditioning, based on a chromatographic matrix made with a copolymer of chitin and silicon oxide. Here, 1 g matrix allowed removal of 87 % of the lysozyme present in 10 ml egg white, with a purification factor of 20 and a global yield of 64 %. The enzyme maximum adsorption capacity was 117 mg/g matrix. Due to the high density and the size of the beads (2 mm), this method has the advantage that the matrix can be easily recovered by simple sieving after lysozyme adsorption, which prevents the need of a centrifugation step.

**Tris (Hydroxymethyl) Aminomethane**

Quan et al. (2008) demonstrated an affinity interaction between the buffer Tris and lysozyme. Taking this fact into account, Quan et al. (2009) developed a matrix based on silica particles derivatized with Tris for high performance affinity chromatography (HPAC).

Although the method is not suitable for industrial application, the ligand results original and of low cost. After injection of 100 µl egg white diluted 1:20, the enzyme was eluted with 0.6 M NaCl and had a specific activity similar to the commercial lysozyme. The matrix kept its chromatographic performance after 20 cycles in a 6-month period.

Zhang et al. (2011) reported a method where Tris was immobilized on silica-coated magnetic microspheres of 350-430 nm in diameter. Maximum adsorption capacity for lysozyme was 108.6 mg/g matrix. After egg white dilution 1:1 with phosphate buffer, pH 7.0, 0.1 M NaCl, 30 mg matrix was agitated with 5 ml egg white for 20 min. The matrix was recovered with a magneto and, after washing, elution was accomplished with 0.6 M NaCl. Specific activity of the eluted lysozyme was 8140 U/mg.

## Conclusion

Without the slightest doubt, lysozyme purification from egg white continues to be a subject of interest and represents a hard downstream processing challenge, mainly due to its high viscosity and the presence of mucoproteins. Finding methods that allow the purification of the enzyme in a simple way, maintaining its activity, with adequate yields, in short time and low cost, is still the aim of most studies. In the case egg white is used as the starting material for lysozyme purification, it is economically convenient that egg white can follow its commercialization route after the partial or total depletion of the enzyme. Any methodology that alters the properties of egg white will impact negatively on the profitability of the process. Since the first research on lysozyme purification from egg white, few strategies have satisfied all these conditions and, therefore, can exceed the step of the academic research to reach the industry.

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## References

Ahvenainen, R.; Heikonen, M.; Kreula, M. and Linko, P. (1979). Separation of lysozyme from egg white. *Food Process Eng.*, 2, 301-310.

Alderton, G. and Fevold, H. L. (1946). Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme**.** *J. Biol. Chem*., 164, 1-5.

Altintas, E. B; Tüzmen, N.; Candan, N. and Denizli, A. (2007). Use of magnetic poly(glydicyl methacrylate) monisize beads for the purification of lysozyme in batch system. *J. Chrom. B.*, 853, 105-113.

Altintas, E. B. and Denizli, A. (2006). Monosize poly(glycidyl methacrylate) beads for dye-affinity purification of lysozyme. *Int. J. Bio.l Macromol.*, 38, 99-106.

Basar, N.; Uzun, L.; Güner, A. and Denizli, A. (2007). Lysozyme purification with dye-affinity beads under magnetic field. *Int. J. Bio.l Macromol.*, 41, 234-242.

Cherkasov, I. A.; Kravchenko, N. A. and Kaverzneva, E. D. (1967). Isolation and purification of lysozyme by fractionation on a chitin column. *Mol. Biol.*, 1, 34-38.

Arica, M. Y.; Yilmaz, M.; Yalcin, E. and Bayramoglu, G. (2004). Affinity membrane chromatography: relationship of dye-ligand type to surface polarity and their affect on lysozyme separation and purification. *J. Chromatogr. B.,* 805: 315-323.

Bayramoglu, G.; Ekici, G.; Besirli, N. and Arica, Y. (2007) Preparation of ion-exchange beads based on poly(methacrylic acid) brush grafted chitosan beads: Isolation of lysozyme from egg white in batch system *Colloids and surfaces A: Physicochem. Eng. Aspects,* 310, 68-77.

Brobjer, M. (1989) Development and scale up of a capture step (expanded bed chromatography) for a fusion protein expressed intracellularly in *E. Coli. Bioseparation*, 8, 218-228.

Camperi, S. A.; Navarro del Cañizo, A. A.; Wolman, F. J.; Smolko, E. E.; Cascone O. and Grasselli, M. (1999) Protein adsorption onto tentacle cation-exchange hollow fiber membranes. *Biotechnol. Prog.,* 15, 500-505.

Cegielska-Radziejewska, R.; Leśnierowski, G. and Kijowski, J. (2008). Properties and application of egg white lysozyme and its modified preparations- A review. *Pol. J. Food. Nutr. Sci.*, 58, 5-10.

Chang, H. M.; Yang, C. C. and Chang, Y. C. (2000). Rapid separation of lysozyme from chicken egg white by reductants and thermal treatment. *J. Agric. Food Chem.*, 48, 161-164.

Chang, Y. K. and Chang, I. P. (2006). Method development for direct recovery of lysozyme from highly crude chicken egg white by stirred fluidized bed technique. *Biochem. Bioeng. J.*, 30, 63-75.

Chang, C. T.; Chen, L. H.; Sung, H. Y. and Kao, M. D. (1986). Studies on the purification of lysozyme from egg white by ultrafiltration. *J. Chin. Agr. Chem. Soc.,* 24, 86-93.

Chen, X.; Liu, J. H.; Feng, Z. C. and Shao, Z. Z. (2005) Macroporous chitosan/carboxymethyl cellulose blend membranes and their application for lysozyme adsorption. *J. Appl. Polym. Sci.,* 96, 1267–1274.

Cherkasov, I. A. and Kravchenko, N. A. (1969) Improved method for isolating lysozymes by enzyme-substrate chromatography. *Biokhimya*, 34, 885-886.

Chiang, B. H.; Su, C. K.; Tsai, G. J. and Tsao, G. T. (1993) Egg white lysozyme purification by ultrafiltration and affinity chromatography. *J. Food Sci.*, 58, 303-306.

Chiu, H. C.; Lin, C. W. and Suen, S. Y. (2007) Isolation of lysozyme from hen egg albumen using glass fiber-based cation exchange membranes. *J. Membrane Sci.,* 290, 259-266.

Denizli, A. and Piskin, E. (2001) Dye-ligand affinity systems. *J. Biochem. Biophys. Methods*, 49, 391-416.

Draegger, M. M. and Chase, H. A. (1991) Liquid fluidized bed adsorption of protein in the presence of cells. *Bioseparation,* 2, 67– 80.

Durance, T. D. and Nakai, S. (1988) Simultaneous isolation of avidin and lysozyme from egg albumen. *J. Food Sci.,* 53, 1096-1102.

Ehsani, N.; Parkkinen, S. and Nyström M. (1997) Fractionation of natural and model egg-white protein solutions with modified and unmodified polysulfone UF membranes. *J. Membr. Sci.*, 123, 105-119.

Ghosh, R. and Cui, Z. F. (2000) Purification of lysozyme using ultrafiltration. *Biotechnol. Bioeng*., 68, 191-203.

Grasselli, M.; Camperi, S. A.; Navarro del Cañizo, A. A. and Cascone, O. (1999) Direct lysozyme separation from egg white by dye membrane affinity chromatography. *J. Sci. Food Agric.,* 79, 333-339.

Hung-Min, C.; Ching-Chuan, Y. and Yung-Chung, Y. (2000) Rapid Separation of lysozyme from chicken egg white by reductants and thermal treatment. *J. Agric. Food. Chem.*, 48, 161-164.

Imoto, T.; Hayashl, K. and Funatsu, M. (1968) Characterization of enzyme-substrate complex of lysozyme I. Two types of complex. *J. Biochem.*, 64, 387-392.

Imoto, T. and Yagishita, K. (1973) Chitin coated cellulose as an adsorbent of lysozyme-like enzymes. Preparation and properties. *Agric. Biol. Chem*., 37**,** 465-470.

Jensen, H. B. and Kleppe, K. (1972) Studies on T4 Lysozyme. Affinity for chitin and the use of chitin in the purification of the enzyme. *Eur. J. Biochem*., 26, 305-312.

Jolles, P. and Jolles, J. (1984) What's new in lysozyme research? *Mol. Cell Biochem.*, 63, 165–189.

Junowicz, E. and Charm, S. E. (1975) Purification of lysozyme by affinity chromatography. *FEBS Lett.,* 57, 219-221.

Kiczka, W. (1994) From monomer to dimer of lysozyme. Życie Wet., 4A, 131-136 (in Polish; English abstract).

Lesnieroswski, G. and Kijowski, J. (2007) Lysozyme. In R. Huopalahti, R. López-Fandiño, M. Anton and R. Schade (Eds.), Bioactive Egg Compounds (chapter 6, pp. 33-42). Berlin Heidelberg: Springer-Verlag.

Li-Chan, E.; Nakai, S.; Sim, J.; Bragg, D. B. and Lo, K. V. (1986) Lysozyme separation from egg white by cation exchange column chromatography. *J. Food Sci.,* 51, 1032–1039.

Li, Q.; Cao, Q.; Li, Z.; Li, N.; Li, K. and Liu F. (2009) Highly efficient and low-cost purification of lysozyme: A novel tris(hydroxymethyl)aminomethane immobilized affinity column. *J. Chromatogr. B*, 877**,** 594–598.

Lienqueo, M. E. and Asenjo, J. A. (2000) Use of expert systems for the synthesis of downstream protein process. *Computers Chem. Eng.,* 24, 2339-2350.

Muzzarelli, R. A. A.; Barontini, G. and Rochetti, R. (1978) Isolation of lysozyme on chitosan. *Biotechnol. Bioeng.,* 20, 87-94.

Palladino, D. K.; Ball, H. R. and Swaisgood, H. E. (1981) Separation of native and acylated egg white proteins with gel chromatography and DEAE-cellulose ion exchange. J. *Food Sci.,* 46, 778-780.

Quan, L.; Wei, D. G.; Jiang, X. L.; Liu, Y.; Li, Z. Y.; Li, N.; Li, K. A.; Liu, F. and Lai, L. H. (2008) Resurveying the Tris buffer solution: the specific interaction between tris(hydroxymethyl)aminomethane and lysozyme. *Anal. Biochem*., 378, 144–150.

Ruckenstein, E. and Zeng, X. (1997) Macroporous chitin affinity membranes for lysozyme separation. *Biotechnol. Bioeng.,* 56, 610-617.

Safarik, I. and Safarikova, M. (1993) Batch isolation of hen egg white lysozyme with magnetic chitin. *J. Biochem. Biophys. Methods,* 27, 327-330.

Safarik, I.; Sabatkova, Z.; Tokar, O. and Safarikova, M. (2007) Magnetic cation exchange isolation of lysozyme from native hen egg white. *Food Technol. Biotechnol.*, 45, 355-359.

Ventura, A. M.; Fernández Lahore, H. M.; Smolko, E. E. and Grasselli, M. (2008) High-speed protein purification by adsorptive cation-exchange hollow-fiber cartridges. *J. Membr. Sci.,* 321, 350-355.

Wan, Y.; Lu, J. and Cui, Z. (2006) Separation of lysozyme from chicken egg white using ultrafiltration. *Sep. Purif. Technol*., 48, 133-142.

Wang, W.; Yue, H. and Yuan, Q. (2009) A primary study on partial purification of lysozyme from chicken egg white using foam separation method *Biotechnol. and Biotechnol. EQ.,* 23, 1237-1241.

Weaver, G. L. and Carta, G. (1996) Protein adsorption on cation exchangers: comparison of macroporous and gel-composite media. *Biotechnol. Progress*, 12, 342-345.

Weaver, G.L.; Kroge, M. and Katz, F. (1977) Deaminated chitin affinity chromatography: a method for isolation, purification and concentration of lysozyme. *J. Food Sci.,* 42, 1084-1087.

Wolman, F. J.; Grasselli, M. and Cascone, O. (2010) Application of dye affinity membrane chromatography to protein purification from raw materials. Comparison with soft-gel matrices. In S. V. Gorley (Ed.), Membranes: Properties, Performance and Applications (Chapt. 11, pp. 367-385). Hauppauge, New York: Nova Science Publishers.

Wolman, F. J.; Grasselli, M.; Smolko, E. E. and Cascone, O. (2000) Preparation and characterisation of Cibacron Blue F3G-A poly(ethylene) hollow-fibre affinity membranes. *Biotechnol. Lett.*, 22, 1407-1411.

Wolman, F. J.; Copello, G. J.; Mebert, A. M.; Targovnik, A. M.; Miranda, M. V.; Navarro del Cañizo, A. A.; Díaz, L. E. and Cascone, O. (2010) Egg white lysozyme purification with a chitin-silica based affinity chromatographic matrix. *Eur. Food Res. Technol.*, 231, 181-188.

Yamada, H.; Fukumura, T.; Ito, Y. and Imoto, T. (1985) Chitin-coated celite as an affinity adsorbent for high-performance liquid chromatography of lysozyme. *Anal. Biochem.*, 146, 71-75.

Yamasaki, N. and Eto, T. (1981) A novel adsorbent for affinity chromatography of lysozyme. *Agric. Biol. Chem.,* 45, 2939-2941.

Yilmaz, M.; Bayramoglu, G. and Arica, M. Y. (2005) Separation and purification of lysozyme by reactive green 19 immobilised membrane affinity chromatography. *Food Chem*., 89, 11–18.

Zhang, G.; Cao, Q.; Li, N.; Li, K. and Liu, F. (2011) Tris(hydroxymethyl)aminomethane-modified magnetic microspheres for rapid affinity purification of lysozyme. *Talanta,* 83, 1515–1520.

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