Journal of
Pharmaceutical and BioTech Industry

MDPI

Article

Process for Avidin Recovery from Egg White by Pseudo-Affinity Chromatography

Ezequiel M. Rios [1,2], María S. Peralta [1], Constanza Y. Flores [1,2], Pamela A. Kikot [1,2] and Mariano Grasselli [1,2,*]

[1] Laboratorio de Materiales Biotecnológicos (LaMaBio), Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal B1876, Argentina; msolperalta7@gmail.com (M.S.P.); pamela.kikot@unq.edu.ar (P.A.K.)
[2] Instituto Multidisciplinario de Biología Celular (IMBICE-CONICET), Grupo Vinculado en Biología Estructural y Biotecnología (GBEyB), La Plata B1906, Argentina
* Correspondence: mariano.grasselli@unq.edu.ar

Abstract

Background: Avidin (AV) represents the third most important protein of major commercial interest derived from egg white, alongside ovotransferrin and lysozyme. It constitutes only 0.05% of the total protein content. Despite the widespread natural availability of AV, its purification remains a significant challenge due to its low abundance within a highly concentrated and complex protein matrix. Methods: Developing efficient downstream processing for AV has the potential to significantly enhance profitability within the egg protein industry. This work presents a novel integrated process for AV recovery. It comprises ovomucin removal, AV preconcentration, and final purification using pseudo-affinity chromatography. The latter utilizes a novel resin with 4′-hydroxyazobenzene-2-carboxylic acid (HABA) as the ligand. The HABA–agarose matrix was characterized by an adsorption isotherm and breakthrough curves, indicating an AV adsorption performance higher than that of other pseudo-affinity matrices. Results and Conclusions: The HABA pseudo-affinity chromatographic process was a crucial step to purify AV more than 300-fold with high yield (86%). Despite the low AV recovery, the proposed integrated process aligns with the biorefinery concept, which maximizes the economic value of raw materials by utilizing all components.

Keywords: pseudo-affinity chromatography; avidin; HABA; egg white; dot-blot quantification

1. Introduction

Today, egg whites have become a vital ingredient in the food industry as a high-value ingredient due to their unique technological and nutritional properties. Global production has tripled in the last forty years, reaching a total of 86 million metric tons [1]. Despite the long history of the industry, only about 10% of shell eggs are currently processed into derivatives. This indicates significant potential for growth within the egg processing sector, which continues to evolve by creating innovative, high-value-added products [1].

While egg white is treated as a commodity within the food industry, its commercial value can be significantly enhanced by isolating specific bioactive components for specialized applications. Avidin (AV) is one of the three primary proteins of industrial interest found in egg white, alongside ovotransferrin and lysozyme. Ovalbumin and ovomucoid are also commercially available; however, their applications are not yet fully developed. While ovotransferrin and lysozyme are primarily utilized as functional ingredients in the food industry, AV offers a wider range of high-tech applications. AV is a high-value glycoprotein

Check for updates

Academic Editor: Bozena B. Michniak-Kohn

Received: 8 February 2026
Revised: 22 May 2026
Accepted: 29 May 2026
Published: 22 June 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

characterized by its exceptional affinity for biotin, a property widely exploited in diagnostic assays (i.e., ELISA and Western blot) and immunohistochemical signal amplification [2–5]. Unlike traditional food-grade proteins, AV applications extend to targeted therapies, non-radioactive labeling, and the functionalization of microfluidic surfaces for point-of-care biosensors [6,7]. Its ability to facilitate intelligent drug delivery and enhance vaccine efficacy underscores its growing importance in the pharmaceutical and biotechnology industries [8,9].

Purifying AV from egg white presents significant challenges due to its very low concentration within a high-protein medium. Furthermore, egg white has other physicochemical properties that are incompatible with a chromatographic process.

Reported AV purification processes typically involve the pretreatment of egg whites, using precipitation and solid separation, followed by chromatographic isolation based on ion-exchange or affinity properties. AV is an alkaline protein (MW 68.3 kDa) similar to lysozyme, while other egg white proteins are acidic (Table S1), so its isolation can be performed by ion exchange chromatography. However, the extracted AV is often contaminated with lysozyme, which is present in significantly higher concentrations. Durance et al. optimized ion-exchange chromatography conditions to recover 75% of AV with a purity of up to 40% [10,11]. Rao et al. (2007) proposed a selective elution of AV fixed on an anionic matrix using the azo dye 4-hydroxyazobenzene-2-carboxylic acid (HABA) as an elution agent, resulting in a very high purity AV (98%) [12].

Due to its high biotin affinity, AV is also purified by affinity chromatography with a very high recovery and purity (>95%). Despite the higher costs associated with these selective affinity processes, they remain commercially viable due to the high market value of the protein [13].

Optimizing downstream processing for AV recovery while preserving the food-grade functionality of the remaining proteins presents a significant opportunity to boost profitability within the egg protein industry. By utilizing sequential extraction methods that maintain the structural and functional integrity of the egg white proteins, it can ensure that the residual protein stream remains suitable for high-value food applications.

In addition, the obtention of several egg white proteins through a single, continuous isolation process is of great relevance for realizing their potential applications in the pharmaceutical and food industries. However, most described methods isolate individual proteins while the rest are denatured or discarded [13]. While several researchers have successfully isolated multiple proteins, reports on process scalability remain limited [14]. For instance, Tankrathok et al. (2009) and Vachier et al. (1995) utilized ion-exchange chromatography to separate ovalbumin, lysozyme, and ovotransferrin [15,16]. Using a sequential extraction approach involving gel permeation and anion-exchange chromatography, Awadé et al. (1994) isolated ovomucin, ovalbumin, lysozyme, and ovotransferrin with purities exceeding 80% [17]. Omana et al. (2010) isolated ovomucin using a two-step precipitation technique, followed by ion-exchange chromatography of the supernatants to recover ovalbumin, lysozyme, ovotransferrin, flavoprotein, and ovoinhibitor [18]. Meanwhile, Abeyrathne et al. (2014) proposed a sequential strategy beginning with cation-exchange chromatography to isolate lysozyme, followed by isoelectric precipitation of ovomucin, and finally a salting-out method for ovotransferrin and ovalbumin, resulting in both high yield and purity [19]. Geng et al. (2012) utilized polyethylene glycol precipitation followed by ion-exchange chromatography [20]; similarly, Ji et al. (2020) employed a combination of salt precipitation and ion-exchange chromatography to fractionate the egg white proteins [21]. However, none of these studies included AV among the target proteins.

This work explores a novel biorefinery-based process utilizing food-grade reagents and procedures to recover AV and other proteins. Specifically, the study focuses on AV recovery

through pseudo-affinity chromatography, which was characterized by determining its adsorption isotherms and dynamic adsorption behavior. Finally, an integrated purification process for AV recovery was evaluated using egg white volumes up to 0.7 L.

2. Materials and Methods

Fresh eggs were obtained from the farm of the Agricultural High School N° 1 (Quilmes, Buenos Aires, Argentina) in the morning and used for the experiment on the same day. The average volume of egg whites was 48 ± 6 mL per egg. Standard proteins (bovine serum albumin, BSA, and AV) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate trihydrate (HAuCl4.3H2O, ≥99.9%), sodium citrate (≥98%), and NHS-biotin were purchased from Sigma-Aldrich. All reagents were AR-grade and were used without further purification.

An ionic exchange matrix based on cotton fibers was prepared according to previous reports [22]. The material characterization was performed by Gavara et al. [22]. The dynamic binding capacity is 230 mg of lysozyme/g of fiber at flow rates between 120 and 300 cm/h [22].

The HABA–agarose matrix was synthesized as previously reported [23,24], as schematized in Figure S1. Ligand density was 1.0 μmol/mL [24].

The maximum binding capacity (Qmax) and dissociation constant (Kd) of the adsorbent HABA–agarose matrix were determined by fitting the Langmuir adsorption isotherm. Several tubes contained the HABA matrix and varying amounts of purified AV in a 50 mM citrate buffer, at pH 5.6, plus 425 mM NaCl (n = 2). The tubes were gently shaken for at least 12 h to allow the system to reach equilibrium. The concentration of protein in the supernatant was determined by absorbance at 280 nm (Biochrome Libra S22 spectrophotometer, Cambridge, UK; or Nanodrop 1100, Thermo Scientific, Waltham, MA, USA) using A1%280 nm 15.5 [25]. Kd and Qmax values were determined by non-linear regression of the Langmuir adsorption isotherm fit.

Egg-white protein feedstock used as the AV source was prepared from 740 mL of egg whites from 17 eggs. The ovomucin-free egg-white solution (OMf-EW) was prepared by acidic precipitation. Egg whites were homogenized by gentle magnetic stirring for 30 min, diluted with one volume of water, adjusted to pH 6 with 1 N HCl solution, and incubated overnight at 5 °C. Then, the solution was centrifuged at 3000× g and 4 °C for 15 min to remove particulate material. OM recovery in the precipitate was 88%. The remaining OMf-EW was used to separate and concentrate basic proteins by cation exchange chromatography. A sulfonic matrix based on cotton fibers was packed on a column (with a bed volume of 60 mL) according to Gavara et al. (2012) [22] and connected to a peristaltic pump (Pump P-1, Amersham, Uppsala, Sweden) and a UV detector (UV-2077 Plus, Jasco, Tokyo, Japan) at 280 nm for absorbance online detection (WinPC-Chrom, Buenos Aires, Argentina). The matrix was equilibrated and washed with 20 mM phosphate buffer, pH 8.0, and elution was done with the same buffer containing 0.5 M NaCl. The OMf-EW stock was processed in three chromatographic cycles. Eluted basic proteins were pooled and concentrated by ultrafiltration with a 5 kDa cassette (Omega 5, Pall Co., Fajardo, PR, USA) to different final volumes. Finally, the pH of the sample was adjusted to 5.6 with citric acid. The solution was stored at 5 °C for 48 h and centrifuged prior to use to remove any insoluble material. This basic-protein feedstock (BP-FS) was the material for AV recovery by pseudo-affinity chromatography.

All experiments for dynamic binding capacity at 25% (DBC^{25}) were performed using AKTA prime plus systems, and conducted at a 150 cm/h linear flow rate (3.5 min residence time) with a 9 cm height column (Tricorn, GE, 0.5 cm diameter, GE International, Olivos, Argentina) packed with the HABA–TETA–agarose matrix. The packing efficiency of the

column was analyzed using a 2% acetone pulse. The column was equilibrated with >10 CV of the 50 mM citrate buffer at pH 5.6 plus 425 mM of NaCl (equilibration buffer), followed by overloading of BP-FS at different concentrations (3× and 30× concentrated).

The flow-through was fractionated into equal volume fractions for offline AV determination using the dot-blot assay (see below). The effluent fractions were measured online by absorption at 280 nm. The breakthrough curves were constructed using the quantification of AV in each eluted fraction relative to the amount of AV in the BP-FS (named C/Co), versus the accumulated amount of loaded AV per L of resin (mg/L).

Final AV purification by pseudo-affinity chromatography was performed by loading BP-FS 30× concentrated at 150 cm/h in a 5 mL column (9 cm height). The matrix was equilibrated with >10 CV of equilibration buffer, loaded with 0.7 CV of the sample, and washed with >10 CV until the absorbance was close to zero. Elution was performed using a 5 CV gradient with a 50 mM ammonium carbonate buffer at pH 11, containing 0.5 M NaCl (elution buffer) and 5 mL fractions were collected. Fractions containing pure AV were pooled.

AV quantification based on biological activity was performed using a dot-blot assay with gold nanoparticles (AuNPs). AuNPs were prepared according to the method of Frens (1973) [26] and characterized by UV-vis spectra and dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK) (Figure S2). AuNPs were coated via direct adsorption with biotinylated BSA (b-BSA) prepared by labeling the protein with NHS-biotin [27]. A flocculation test was used to determine the optimum b-BSA concentration on the AuNPs. UV-Vis spectra of the conjugates were obtained using Nanodrop 1000 (Thermo Fisher Scientific, Inc., Madison, WI, USA).

A dot-blot assay was performed on a nitrocellulose membrane mounted in a microfiltration blotting device (Bio-Dot Apparatus, Bio-Rad, Hercules, CA, USA). In total, 100 µL of b-BSA (10 µg/mL in PBS) was loaded in each well and dried at 37 °C for 1 h. After blocking with BSA 0.1 mg/mL in PBS, 100 µL of the samples (or dilutions) containing AV was filtered. Pure AV was used as a quantification standard. Each sample was dotted in triplicate. Wells were washed three times with BSA 0.1 mg/mL in PBS. The strip was incubated in 1 mg/mL BSA at room temperature for 30 min to block non-specific sites. Finally, the membrane was incubated with b-BSA functionalized AuNPs suspension to develop color and was washed with PBS. Photographs of the membranes were taken with a smartphone in a well-lit space, without any external light sources. The images were taken and analyzed using ImageJ software (1.54r, NIH, ML, USA) for each spot. The analysis was performed according to Celikbas et al. (2020) [28]. The color intensity (%) was calculated by subtracting the blank color intensity from the sample intensities, which were then converted to a percentage (the color intensity obtained for the blank was set to 100%). The dynamic range was estimated by fitting the data using non-linear regression analysis in Microsoft Excel®.

AV was quantified by this method at each stage of the purification process, except in raw egg white, where the theoretical content was used [25].

Protein identification by molecular weight (MW) was performed by SDS-PAGE gel electrophoresis. In total, 2 and 10 µg of protein in a final volume of 20 µL were applied per lane for pure proteins and protein mixtures, respectively. After migration, gels were stained with a colloidal Coomassie Blue kit (Novex, Invitrogen, Waltham, MA, USA) and photographed (Gel Doc EZ Imager, Bio-Rad, Hercules, CA, USA). Image analysis was performed to calculate MW using MW markers (ImagLabs software, version 6.1, Bio-Rad, Hercules, CA, USA). AV is a homo-tetrameric protein (MW 68.3 kDa), which is visualized in SDS-PAGE gel as a monomer of 17 kDa.

Total protein concentration was quantified using the Bradford assay with BSA as the standard (Bio-Rad Kit). Inherent underestimation of AV by the Bradford method was addressed by thermal denaturation at 100 °C for 10 min before quantification [29].

For the purification table, purity (%) was defined as the ratio of AV concentration (determined by dot blot assay) to the total protein concentration. The purification factor was estimated as the ratio of purity before and after each step. Finally, yield (%) was determined by the mass of AV recovered, calculated as the product of AV concentration and sample volume, relative to the total AV mass present in the initial or previous step.

3. Results and Discussion

Egg white serves as the primary commercial source of avidin (AV). This complex proteinaceous matrix has a total protein concentration of approximately 11%, with the specific composition and physicochemical characteristics of its major proteins summarized in Table S1. By contrast, AV is a minor constituent, representing less than 0.05% of the total protein profile.

Given the very low proportion of AV in the raw material, an affinity chromatography method is technically required for its efficient recovery. Nevertheless, this method has drawbacks associated with its very low binding capacity, restricted operational lifespan, and high cost.

Pseudo-affinity chromatography with immobilized azo dyes could be a solution for AV recovery from egg white, and is the option explored in this work. In our recent work, a simple protocol for in situ synthesis of 4-hydroxyazobenzene-2-carboxylic acid (HABA) dye onto a chromatographic matrix was described [23]. Furthermore, the ligand spacer and adsorption conditions were optimized to maximize AV recovery using a HABA–TETA–agarose matrix, in which triethylenetetramine (TETA) works as the linker moiety [24]. Figure S1 schematizes the synthesis route of the HABA–TETA–agarose matrix used in this work.

3.1. Adsorption Modeling by Isotherm Analysis

To evaluate the AV recovery efficiency of the pseudo-affinity matrix, the adsorption process was thermodynamically characterized by measuring the adsorption isotherm of pure AV on HABA–TETA–agarose matrix under optimized buffer conditions.

Experimental adsorption data were fitted to the Langmuir single-solute isotherm model (R^2 = 0.98), from which the adsorption parameters were derived (Figure 1). The maximum capacity of the matrix (Qmax) was 20.7 ± 1.2 mg/mL, with a Kd of 2.3 ± 0.4 mg/mL (3.4×10^{-5} M). This relevant Qmax value is consistent with optimized coupling similar to that reported for other proteins adsorbed by dye–agarose matrices [24,30]. As expected, the experimental Kd for immobilized HABA is higher than the value reported for free HABA dye (0.6×10^{-5} M). Notably, the Qmax of the HABA–TETA–agarose support is significantly higher than that of commercial 2-iminobiotin–agarose matrices, which typically range from 1 to 4 mg/mL according to manufacturer specifications.

The AV concentration in raw egg white is 0.055 g/L (0.8×10^{-5} M), which is relatively low compared to the dissociation constant (Kd = 3.4×10^{-5} M). From a process engineering perspective, maintaining a concentration ratio of [AV] $\geq 5 \times$ Kd is a common reference parameter to ensure that the adsorption equilibrium favors a high matrix loading. Therefore, increasing the initial AV concentration is essential to achieve a high-performance recovery process.

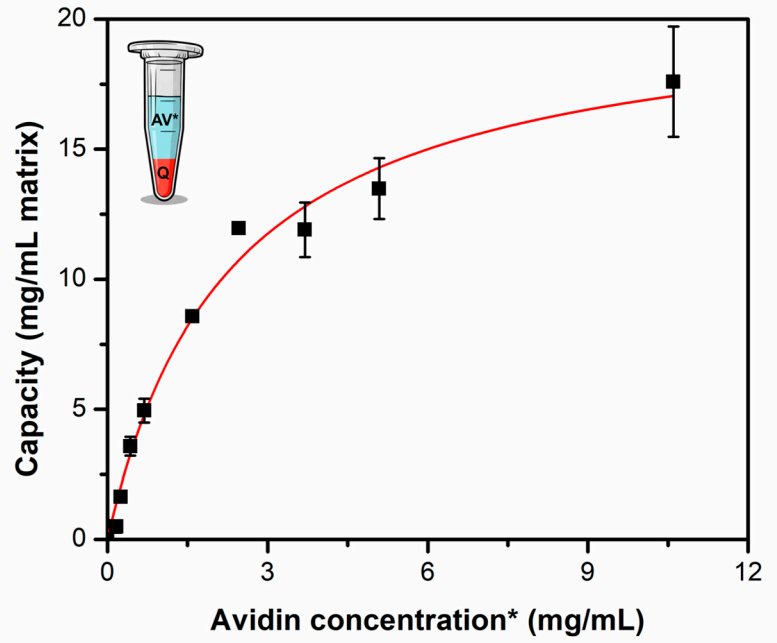


Figure 1. AV adsorption isotherm using HABA–agarose matrix in 50 mM citrate buffer at pH 5.6 + NaCl 425 mM. AV concentration* (mg/mL) is the AV equilibrium concentration in the supernatant, and capacity is the amount of AV adsorbed per matrix volume (mg/mL). Experimental points were fitted to the Langmuir isotherm of the single-solute isotherm model (Qmax = 20.7 ± 1.2 mg/mL; Kd = 2.3 ± 0.4 mg/mL; adjusted R^2 = 0.98). *: In equilibrium.

3.2. Downstream Process

Given that contaminant proteins constitute over 99% of the total protein content, the proposed downstream process was designed to recover AV while preserving the food-grade functionality of the remaining protein fraction. This approach ensures that the residual protein stream remains suitable for high-value food applications. The process design was based on two fundamental premises. First, given the high availability of industrial egg white, significant volume reduction was prioritized over maximizing absolute recovery to improve volumetric yield. Second, all reagents were limited to food-grade specifications to ensure the subsequent valorization of residual protein fractions.

The downstream process is organized into three main stages: OM removal (dilution and precipitation), AV recovery and concentration (ion-exchange fiber adsorption and ultrafiltration), and AV purification (HABA–agarose affinity chromatography). This recovery strategy, detailed in Figure 2, utilizes an initial dilution for pretreatment and sulfonic fibers for AV enrichment. Following these steps, the raw material is concentrated by ultrafiltration before the final dye affinity chromatography, which produces the purified product.

3.3. Avidin Quantification

To monitor purification performance, a reliable quantification method for AV is essential. This technique should ideally be simple, fast, and inexpensive. However, given the low concentration of AV in the raw material, a highly sensitive limit of quantification is the most critical requirement. The low concentration of AV in the initial feedstocks specifically limits the effectiveness of SDS-PAGE for accurate quantification. Among specific detection methods, the HABA assay [25,31] is recognized for its simplicity and reliability; however, its sensitivity is limited, with recommended concentrations typically above 20 µg/mL. While standard curve optimizations have extended this range down to approximately 4 µg/mL [11], it remains insufficient for the initial samples. Alternatively, Shawkey et al. (2008) developed a microplate method using biotin/horseradish peroxidase reagent with

a much lower dynamic range (0.002–5 µg/mL) [32]. Despite its sensitivity, this method involves multiple stages and lengthy incubation periods, ranging from 25 min to overnight. Consequently, an analytical method based on the dot blot assay using AuNPs was developed to monitor AV levels in subsequent studies.

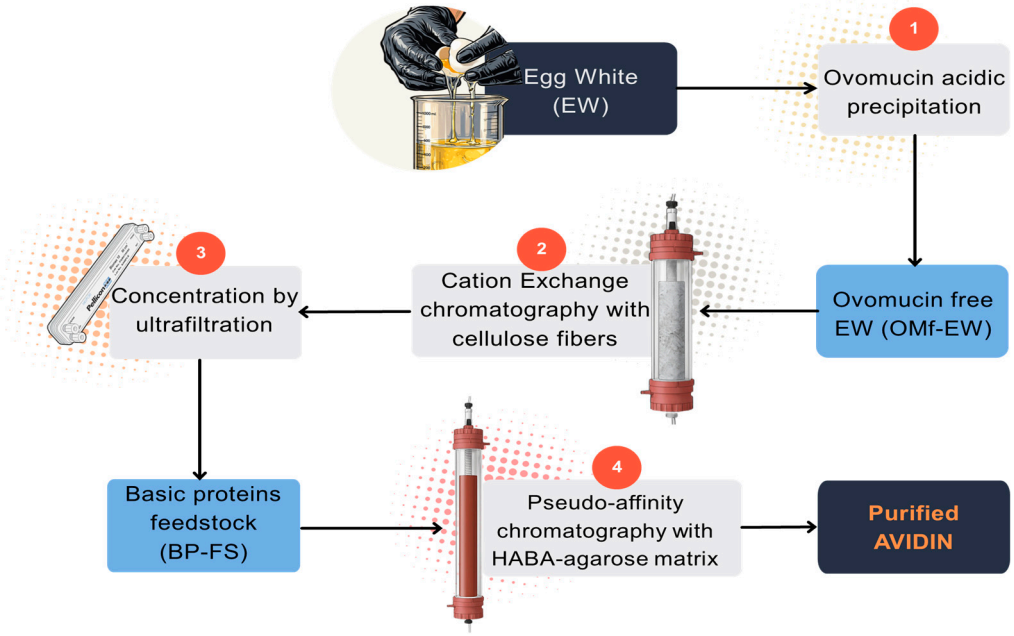


Figure 2. Proposed process scheme: (1) acidic precipitation of ovomucin; (2) recovery of basic proteins via ion-exchange adsorption; (3) concentration by ultrafiltration; and (4) final purification using HABA–agarose pseudo-affinity chromatographic matrix.

The quantification method takes advantage of the unique structural characteristics of the AV molecule. AV is a tetramer capable of binding four biotin molecules; however, these binding sites are distributed in two pairs on opposite faces of the molecule. This spatial arrangement allows AV to act as a molecular bridge between two large entities, such as a solid support (e.g., a polymeric membrane) and an AuNP. Due to steric hindrance, only one binding site on each face remains functional in this configuration. This principle was applied to develop a quantitative dot blot assay, where each AV molecule immobilized on the membrane surface captures exactly one AuNP, ensuring a stoichiometric relationship for accurate quantification.

A schematic representation of the dot blot assay is depicted in Figure 3A. A nitrocellulose membrane, functionalized with biotinylated BSA (b-BSA), serves to capture the AV present in either standards or unknown samples. Once immobilized, the AV is detected using AuNPs that have been previously conjugated with b-BSA, thereby completing the "sandwich" complex for colorimetric visualization. The characterization of AuNPs and the b-BSA coating procedure are detailed in Figure S2. A positive interaction between the b-BSA-AuNP and AV results is denoted in the formation of wine-red dots, whereas negative samples remain colorless. Based on the color intensity of the standards, a linear quantification range of 1 to 10 µg/mL was established (Figure 3B). The validity of the logistic regression model was confirmed using analysis of variance (ANOVA) (Table S2) and residual plot analysis (Figure S3). These results, supported by an adjusted R^2 of 0.96, demonstrate a robust regression model characterized by homoscedasticity (constant variance in residuals).

The dot-blot quantification technique was utilized at each purification stage to determine recovery and construct a purification table (Table 1). However, this assay could not

be directly applied to raw egg white due to membrane fouling; consequently, the initial AV concentration was estimated based on established values in the literature [25].

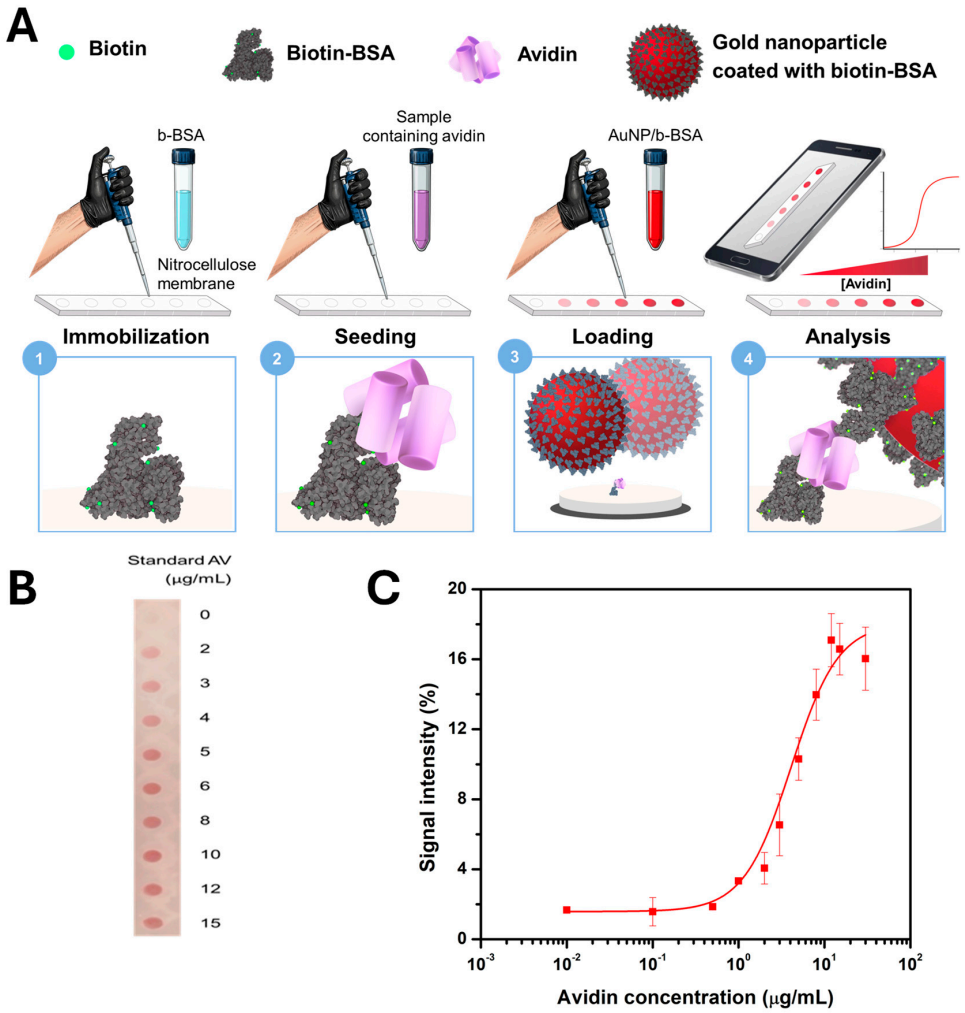


Figure 3. (**A**) Scheme of dot-blot assay for AV quantification. (1) Biotin-BSA (b-BSA) was physiosorbed onto a nitrocellulose membrane. (2) Samples containing AV were filtered in individual spots. (3) Then, gold nanoparticles (AuNP) decorated with b-BSA were used to reveal the presence of AV. (4) Photographs of the membranes were taken using a smartphone, and the color intensity of each dot was analyzed using ImageJ software to calculate the signal intensity (%). (**B**) Dot blot membrane of AV standard curve. (**C**) Quantitative analysis of the signal intensity of the dots (%) against standard AV concentrations. This graph was obtained by analyzing three (n = 3) independent dot blots for each target concentration (adjusted R^2 = 0.96).

Table 1. Purity and yield of AV during the purification process.

Step	AV (mg)	Proteins (g)	Purity (%)	Yield (%)	Purification Factor
Egg white [1]	40.7	81.4	0.05	100	1
Isoelectric precipitation supernatant (OMf-EW)	15.6 ± 0.7	71 ± 2	0.02	38	0.44
Cation exchange chromatography (BP-FS) + ultrafiltration	2.6 ± 0.1	0.79 ± 0.02	0.33	17	15
Pseudo-affinity chromatography	2.2 ± 0.2	0.0021 ± 0.0004	100	84	318

[1] Theoretical protein content is 11 g with 5.5 mg of AV per 100 mL of egg white.

3.4. Ovomucin Removal

During downstream processing, the homogenized egg white is diluted with the minimum volume of water required to induce ovomucin (OM) precipitation. OMs, which constitute 3.5% of the total protein content, are highly glycosylated proteins responsible for the characteristic viscosity and gel-like structure of the egg white matrix. These proteins comprise a carbohydrate-poor group (α1 and α2, of different MWs) and a carbohydrate-rich group (β). Their presence typically compromises chromatographic performance by fouling the matrices, while their inherent high viscosity limits mass transfer [33]. Although their isoelectric point (pI) ranges from 4.5 to 5.0, OMs can be removed via isoelectric precipitation at pH 6.0 through dilution followed by acidification [34]. Notably, OM precipitation can occur at pH levels above the pI when they form complexes with other proteins [34].

In this study, a modified version of the protocol described by Omana and Wu (2009) was employed to precipitate OM, utilizing a 1:1 dilution of egg white with water [35]. While this modification is less efficient for OM removal than the original method, the resulting low ionic strength of the supernatant facilitates direct ion-exchange adsorption in the subsequent unit operation without additional conditioning. Furthermore, the column pass-through remains suitable for reintegration in standard egg white processing streams. However, the lower ionic strength leads to increased AV losses due to its co-precipitation with the OM fraction.

Following precipitation, the sample was centrifuged at 3000× *g* to produce a clarified, slightly viscous supernatant (OM-free egg white solution, OMf-EW). Due to its inherent consistency, this fraction could not be applied to conventional packed-bed chromatographic matrices without further extensive dilution and pre-filtration. According to protein quantification, the OMf-EW retained 87% of the total egg white proteins (see Table 1). SDS-PAGE analysis confirmed the successful removal of OMs, as evidenced by the absence of high-MW bands in the corresponding lane (Figure 4, lane OMf-EW). Consistent with the findings of Omana and Wu (2009) [35], the estimated MWs for α1-OM and α2-OM, and β-OM are 150 kDa, 220 kDa, and 400 kDa, respectively.

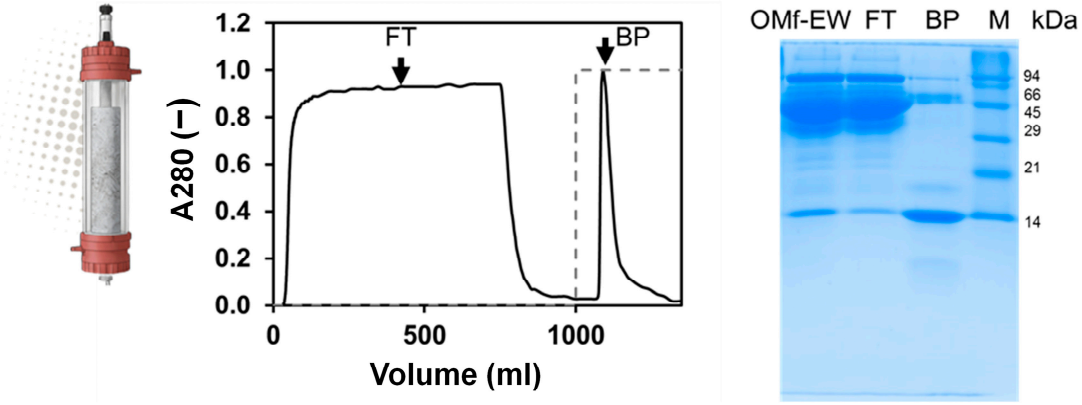


Figure 4. Cation exchange chromatography profile of OMf-EW and 12% SDS-PAGE of chromatographic fractions. The dashed gray line represents the elution step with 0.5 M NaCl. OMf-EW: OM-free egg white solution; FT: flow-through fraction; BP: elution fraction containing basic proteins. For SDS-PAGE, lane M is the MW standard.

The OMf-EW fraction recovered 38% of the initial AV (15.6 mg of the estimated 40.7 mg in raw egg white). This significant reduction in yield is attributed to the strong electrostatic association between basic AV and the acidic subunits of OM, leading to co-precipitation and mechanical occlusion within the protein clot, a phenomenon well-documented in salt-free systems [18,34,36]. Consequently, the purification factor fell below unity (PF < 1),

indicating that the rate of AV loss during this negative precipitation stage exceeded the overall protein removal rate (Table 1).

3.5. AV Preconcentration

The OMf-EW was loaded onto a fiber-based cation-exchange column to concentrate basic proteins [22]. These fibrous materials have emerged as promising chromatographic media due to their high throughput and high capacity. Their structure provides superior mechanical strength compared to traditional hydrogel beads, facilitating higher flow rates and enhanced mass transfer kinetics relative to columns packed with agarose particles [22]. Furthermore, feedstock can be loaded without complete clarification, thereby mitigating clogging issues common in packed-bed systems. Their cost-effectiveness and the widespread availability of raw materials are defining attributes that facilitate large-scale biomolecule purification, making these fibers highly attractive for industrial applications in the food sector.

The adsorption process onto a fiber-based column was conducted at pH 8.0, a condition designed to promote electrostatic interactions between the sulfonic groups of the matrix and proteins with an alkaline isoelectric point (pI). Most egg white proteins are acidic and negatively charged at this pH, except lysozyme and AV (Table S1). Sample loading onto the cotton-based matrix proceeded without clogging, obstruction, or overpressure, which are typically associated with this type of raw material. Elution was subsequently triggered by increasing the ionic strength with NaCl.

As shown in the chromatogram and corresponding SDS-PAGE (Figure 4), the initial acidic precipitation and fiber adsorption flow-through (FT) fractions did not exhibit a defined band for AV (lanes OMf-EW and FT), likely due to its low relative concentration. However, the more sensitive dot-blot method successfully detected AV in the crude sample, while also revealing its presence in the FT fraction. This indicates a partial loss of the target protein during the loading stage (Figure S4). In the eluate, AV was clearly resolved as a 17 kDa monomeric band, alongside the major basic protein lysozyme (14 kDa) and other minor contaminants. Quantitative dot-blot analysis (Figure S4) confirmed an AV recovery performance of 17% for this step, further corroborating the losses observed in the FT. The loss of AV during this operation may be attributed to its low initial concentration, which reduces binding efficiency in ion-exchange chromatography. However, this step eliminated a significant amount of contaminating proteins, resulting in a 15-fold increase in purity. Furthermore, the FT solution remains a viable byproduct that can be utilized for subsequent protein fractionation or repurposed within the food industry following concentration and/or drying. This material primarily consists of diluted egg white that has been pH-adjusted using a minimal concentration of food-grade additives.

The pooled eluate (volume 540 mL and A280 4.5 AU, equivalent to 2430 AU·mL^{-1}) contained AV at a concentration of 5 µg/mL. To enhance the efficiency of the subsequent pseudo-affinity chromatography step and minimize processing time, the sample was concentrated by ultrafiltration in two stages: first to 3× (200 mL and 13 AU, equivalent to 2600 AU·mL^{-1}) and then to 30× (20 mL and 115 AU, equivalent to 2300 AU·mL^{-1}). In accordance with the A280 measurements, no protein loss was detected during the concentration process. These prepared feedstocks, designated BP-FS 3× and BP-FS 30×, were then subjected to the final AV purification stage.

After sample storage at 5 °C, partial lysozyme precipitation occurred due to the high protein concentration and NaCl content. This behavior was described decades ago [37]. The sample was centrifuged to remove solid material prior to use in pseudo-affinity chromatography.

3.6. Pseudo-Affinity Chromatography: Dynamic Binding Capacity

The adsorption and desorption parameters for AV using HABA–agarose were established in our previous study [24]. Optimal adsorption was achieved using a 50 mM citrate buffer at pH 5.6, supplemented with 425 mM NaCl; this condition maximized binding capacity while simultaneously minimizing non-specific protein interactions. To enhance purity, a 5 CV gradient elution was employed using a carbonate buffer, pH 11.0, containing 0.5 M NaCl, which successfully increased AV purity to 82%.

To optimize the affinity purification process, column performance was evaluated through dynamic binding capacity (DBC) experiments. The DBC represents the maximum mass of target protein that can be loaded onto a column before a significant breakthrough occurs in the flow-through, under defined operational parameters (flow rate and feedstock composition). To determine the DBC experimentally, BP-FS was prepared according to the previous sections at 3× and 30× concentrations. While the sample required pH adjustment to 5.6 for adsorption, the NaCl concentration (425 mM) was maintained. This was because the eluate from the preceding ion-exchange step already contained sufficient salt, resulting in a conductivity equivalent to that of the equilibration buffer.

Chromatographic runs were conducted using a 9 cm packed bed of the HABA–TETA–agarose matrix (1.8 mL CV). Flow-through fractions were collected and analyzed for total protein content via A280 and for avidin (AV) concentration using a dot-blot assay. The breakthrough curves were generated by plotting the relative AV concentration (C/Co) against the mass of AV loaded per L of affinity matrix (mg/L).

The DBC at 25% breakthrough (DBC^{25}) was determined by overloading the column with BP-FS at various concentrations. We evaluated linear flow rates of 60, 150, and 300 cm/h, corresponding to residence times of 8.5, 3.4, and 1.7 min, respectively. Since the DBC^{25} values for the FS 3× remained nearly identical across these flow rates, 150 cm/h was selected as the optimal operating velocity to balance throughput and capacity. The observed independence from flow rate suggests that the interaction between AV and the HABA ligand is governed by rapid adsorption kinetics, rather than diffusion-limited mass transfer. This behavior implies that the system can maintain binding efficiency even with reduced residence times, allowing it to operate at high linear velocities, which are crucial in industrial applications.

Increasing the feedstock concentration tenfold (from 3× to 30×) significantly enhanced the capacity from 28 to 98 mg of AV per L of matrix (Figure 5). This improvement is attributed to the increased ratio between the AV concentration in the BP-FS and the Kd. As expected in a complex feedstock under dynamic conditions, these DBC^{25} values are lower than the static Qmax.

In the design of an effective downstream process, maximizing protein capture efficiency during the chromatographic step is essential. To ensure optimal utilization of the matrix capacity, the ratio of the AV concentration to the Kd should be maintained at a value greater than five. Consequently, to achieve high-efficiency binding, the egg-white-based feedstock should be concentrated to a minimum AV concentration of 12 mg/mL based on the Kd determined for this system. Furthermore, the feedstock must be OM-free; removing this macro-component is vital to prevent matrix occlusion and fouling, which would otherwise lead to increased backpressure and reduced resin longevity.

Pseudo-affinity chromatography utilizing the HABA–TETA–agarose matrix exhibits a Kd in the millimolar range. This Kd value reflects a relatively low binding affinity, which limits the efficiency of AV adsorption when using the current BP-FS concentration. To overcome this limitation and maximize matrix utilization, it is essential to increase the AV concentration in the feedstock. Increasing the AV concentration in the feedstock shifts the equilibrium toward the bound state, effectively compensating for the high Kd, and

maximizing the utilization of Qmax. Additionally, reducing the total BP-FS volume through preconcentration provides a secondary benefit by significantly decreasing the loading time, thereby improving overall process throughput and productivity.

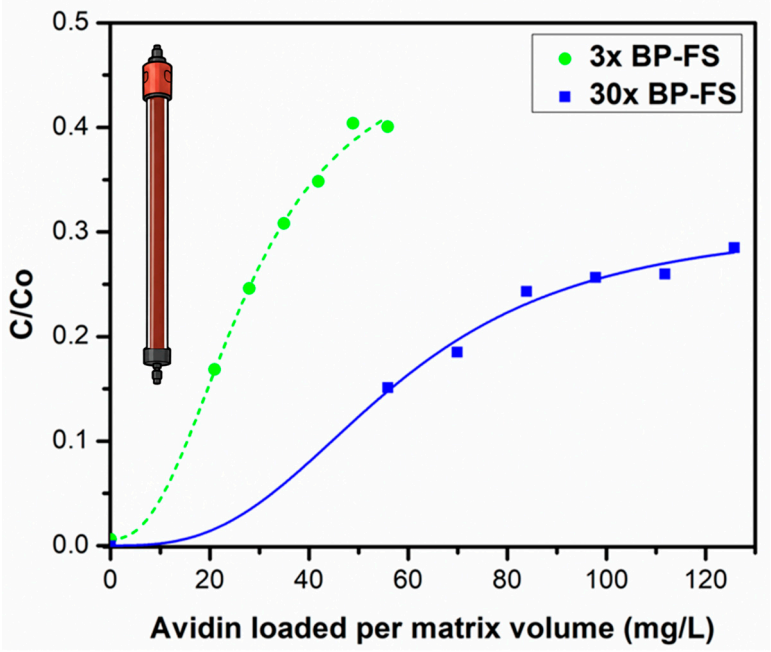


Figure 5. The breakthrough curves of the pseudo-affinity matrix HABA–agarose were constructed by the overload of BP-FS at a linear flow rate of 150 cm/h. Dots: 3× concentrated BP-FS; Squares: 30× concentrated BP-FS. The lines are only included to guide the view.

3.7. AV Purification

Final AV purification was performed using a 5 mL column in six cycles. Each loading step consists of 0.7 CV of a 30× concentrated feedstock (around 3.5 mL). This loading volume was determined based on the previously established DBC[25] and the specific AV concentration of the BP-FS 30× sample. The chromatographic process operated with minimal back pressure throughout the runs. Figure 6 illustrates a representative chromatographic cycle, and the SDS-PAGE and dot-blot analysis of the resulting fractions. Elution was conducted using a 5 CV linear gradient with 5 mL fractions, which resolved the adsorbed proteins into two distinct peaks: a major peak (Figure 6, E1) consisting of contaminating proteins, and a minor peak (Figure 6, E2) containing mainly AV. The absence of AV in the flow-through and its presence in E2 were confirmed by the appearance of a 17 kDa band on SDS-PAGE (Figure 6) and further validated by dot-blot assay (Figure S5). The E2 fractions from all chromatographic cycles (20 mL each) were pooled (total 120 mL) for final AV quantification.

In this chromatographic step, 99.7% of the total protein content was successfully removed. AV recovery reached 84%, corresponding to a 318-fold purification (Table 1). Furthermore, gradient elution yielded high-purity AV, as confirmed by SDS-PAGE and the quantitative assay. This performance significantly exceeds that of other pseudo-affinity methods utilizing immobilized azo-dyes, which typically report yields of 40–80% and purification factors ranging from 5- to 100-fold, depending on the specific enzyme and ligand [38].

Evaluation of the complete process described in this work, comprising three purification steps (precipitation, ion exchange, and pseudo-affinity chromatography) and one concentration step (ultrafiltration), reveals an overall AV recovery of 5% with an overall purification factor greater than 2000. This relatively low yield is primarily attributed to AV

losses during ovomucin (OM) separation and the subsequent ion-exchange step on cotton fibers. While co-precipitation with OM could be mitigated by increasing ionic strength (to shield protein charges), salt addition was avoided to ensure compatibility with the subsequent ion-exchange step and to preserve the remaining egg white for food-grade applications or the co-extraction of other proteins. Furthermore, the efficiency of the ion-exchange stage could be enhanced by increasing the cotton matrix volume to compensate for the low adsorption capacity inherent when processing dilute AV from the raw material.

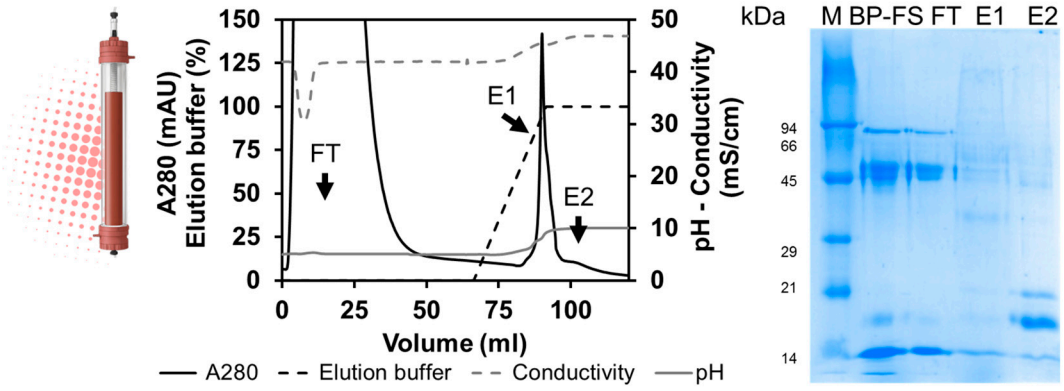


Figure 6. Chromatogram of HABA–agarose column loaded with BP-FS 30× and 12% SDS-PAGE of chromatographic fractions. Line reference: A280 nm (black solid line); elution % (black dashed line); pH (gray solid line); conductivity (gray dashed line). BP-FS: basic-protein feedstock 30×; FT: flow-through fraction; E1: major elution peak containing contaminant proteins; E2: minor elution peak containing AV. For SDS-PAGE, lane M is the MW standard.

Table 2 compares our process with previously described AV isolation methods, focusing on purity, yield, and food compatibility. Generally, higher purity is achieved using affinity chromatography, with the sole exception of the crystallization-based method by Green and Toms [39]; on the other hand, ion-exchange processes tend to improve overall yield. Other techniques, such as preparative electrophoresis, remain constrained by technical complexities that make it difficult to scale effectively for industrial protein recovery. Although biotin [40] and biocytin [41] ligands provide superior selectivity, their extreme binding affinity for avidin requires harsh elution conditions that compromise protein stability. In contrast, the 2-iminobiotin approach reported by Orr et al. (1986) achieved over 90% recovery [42], a yield competitive with the 84% observed in our affinity step. Notably, conventional pre-chromatographic steps like ammonium sulfate precipitation remain labor-intensive and require extensive washing to prevent significant non-specific protein adsorption [43].

Table 2. Reported processes for the purification of AV from egg white.

Process	Purity (%)	Global Yield (%)	Food-Grade Compatibility	Reference
Isoelectric precipitation + Ion exchange adsorption + Ultrafiltration + Pseudo-affinity chromatography (HABA ligand)	100	5	Yes	This work
Ammonium sulfate precipitation + Affinity chromatography (Iminobiotin ligand)	>95 [1]	38–50 [2]	No	[42]
Ammonium sulfate precipitation + Pseudo-affinity chromatography (HABA ligand)	N.R. [3]	36 [2]	No	[43]

Table 2. *Cont.*

Process	Purity (%)	Global Yield (%)	Food-Grade Compatibility	Reference
Ion-exchange chromatography (HABA eluent)	98	N.R. [3]	No	[12]
Ion exchange chromatography	41	74–80	Yes	[10]
Dilution + two Ion-exchange chromatography + Crystallization	100	62	No	[39]
Dilution + Preparative electrophoresis	97	65	No	[44]
PEG precipitation + Aqueous two-phase system + Mix-mode chromatography	97 [4]	N.R. [3]	No	[45]

[1] Considering the specific activity of 14.4 ug/[^{14}C] biotin/mg protein reported by the authors and the maximum specific activity of 15.1 ug/[^{14}C] biotin/mg [39] [2] Considering 1.65 mg AV per egg (where the average volume of egg white is 30 mL [46] and the theoretical content of AV is 0.05%). [3] N.R.: Not reported. [4] Starting from a pre-purified AV solution (initial AV 11%).

In all the processes described in Table 2, the raw material was subjected to rigorous conditions that compromised the functional integrity or use of the non-target proteins in the food industry, except those that directly adsorbed on ion exchange resins. In contrast, despite having good performance in HABA-based chromatography, the overall recovery was low (2.2 mg of AV from 17 eggs) in favor of strategic prioritization of an integrated process.

This approach allows for the recovery of the remaining egg white fractions in their native state. When a single protein is the sole focus, isolation methods are dictated by its specific physicochemical tolerances, often at the expense of the broader protein matrix. For instance, chemical precipitants can induce irreversible denaturation and increase environmental footprints. Conversely, multi-protein co-purification strategies must balance high purity and yield for each target with the preservation of biological activity across all fractions. Such integrated methods are essential for optimizing egg utilization and enhancing economic efficiency [47].

4. Conclusions

A novel process to recover AV and other proteins based on the concept of bio-refinery is studied using different procedures and reagents compatible with the food industry. In particular, the AV recovery is achieved by a pseudo-affinity chromatography step.

The novel HABA pseudo-affinity chromatographic process allows an AV purification factor higher than 300 and a yield of 86% in this step. Starting from 0.7 L of egg white, 2 mg of pure AV was recovered, representing a global yield of 5%. Although this recovery is relatively low, the process remains feasible; yields could be enhanced by optimizing concentration steps without significantly altering the proposed workflow.

Additionally, a novel dot-blot assay is reported to enable the sensitive detection and quantification of AV concentrations within complex matrices. The versatility of this assay allows it to be adapted for various target proteins. By substituting b-BSA with specific antibodies, the gold nanoparticle-based detection system can be tailored to identify different proteins in raw materials, serving as a cost-effective alternative to traditional immunoassays.

The proposed integrated recovery process for AV aligns with the biorefinery concept, which is a strategic pillar of the modern bioeconomy that maximizes the economic value of raw materials by utilizing all components. By implementing a cascading utilization approach, the process prioritizes the extraction of high-value, low-abundance bioactive molecules such as AV.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jpbi3020014/s1, Figure S1. Synthesis of HABA-agarose matrix. Sepharose 4B CL (purchased from Cytiva) was activated with allyl glycidyl ether (AGE) (Sigma), and the allylic group was brominated with N-bromosuccinimide (NBS) (Sigma-Aldrich, St. Louis, USA). Then, a substitution reaction was done using triethylenetetramine (TETA) (technical grade, Merck) as a spacer arm for the dye ligand. HABA synthesis was carried out in situ by the formation of an amide bond between the amine of the spacer arm and the carboxyl of salicylic acid (Biopack, Buenos Aires, Argentina), by N′-dicyclohexylcarbodiimide (DCC) (Fluka, actual Sigma-Aldrich) reaction. Finally, anthranilic acid (BDH, Merck) was incorporated by diazotation as diazonium salt. All other salts and solvents were obtained from Anedra (Buenos Aires, Argentina); Figure S2. AuNp characterization. A. DLS of naked 24 nm AuNP; B. Photo of the resulting gold aggregation test. AuNP were coated with b-BSA at different concentrations, and KCl was added to test AuNP-bBSA stability. Nanoparticle aggregation was determined by color turning from red to purple or blue. Even by the naked eye, the color gradient at different b-BSA concentrations is clear. C. SPR of AuNP decorated with different concentrations of b-BSA in the gold aggregation test. D. The graph obtained with the absorbance at 520 nm with respect to 620 nm, allows to estimate the minimum protein concentration to give stable conjugates (65 ug/mL). Figure S3. Plots of residuals demonstrated random distributions of residuals without any trends of logistic regression used to calibrate dot-blot assay for AV quantification. Figure S4. Photography of dot-blot membrane used to measure AV in OMf-EW solution, flow-through (FT) and basic proteins (BP) obtained by cation exchange chromatography. (d) indicates samples diluted in phosphate saline buffer (PBS). Negative control was obtained by PBS filtration. Quantification standards of pure AV were applied at different concentrations. Figure S5. Photography of dot-blot membrane used to measure AV in flow-through (FT) and eluted proteins (E1 and E2, for both peaks) obtained by pseudo-affinity chromatography of BP-FT 30x. (d) indicates samples diluted in phosphate saline buffer (PBS). Negative control was obtained by PBS filtration. Quantification standards of pure AV were applied at different concentrations. Table S1. Content and characteristics (isoelectric point, molecular weight) of major proteins found in egg white [48]. Table S2. ANOVA analysis of logistic regression used to calibrate dot-blot assay for AV quantification. ANOVA results made the models valid for the actual system ($\alpha = 0.05$).

Author Contributions: Conceptualization, P.A.K. and M.G.; methodology, E.M.R., M.S.P., C.Y.F. and P.A.K.; software, E.M.R. and P.A.K.; validation, E.M.R., M.S.P. and C.Y.F.; formal analysis, P.A.K.; investigation, E.M.R., M.S.P., C.Y.F. and P.A.K.; resources, P.A.K.; data curation, C.Y.F. and P.A.K.; writing—original draft preparation, P.A.K. and M.G.; writing—review and editing, P.A.K. and M.G.; visualization, P.A.K.; supervision, M.G.; project administration, M.G.; funding acquisition, P.A.K. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: E.M.R. thanks CONICET for the fellowship. P.A.K., C.Y.F. and M.G. are researchers from Universidad Nacional de Quilmes and members of CONICET. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET) PIP 2021/23-1565 and PIBBA 2022/23-0006 and Universidad Nacional de Quilmes PUNQ EBIODEM 1600/25.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AV	Avidin
BP-EW	Basic-protein feedstock
HABA	4′-hydroxyazobenzene-2-carboxylic acid
Kd	Dissociation constant

Qmax	Maximum binding capacity
OM	Ovomucin
OMf-EW	Ovomucin-free egg white solution
TETA	Triethylenetetramine

References

1. Guyonnet, V. Chapter 2 World Egg Production and Marketing Trends. In *Handbook of Egg Science and Technology*, 1st ed.; CRC Press: Boca Raton, FL, USA, 2025. [CrossRef]
2. Jain, A.; Cheng, K. The principles and applications of avidin-based nanoparticles in drug delivery and diagnosis. *J. Control. Release* **2017**, *245*, 27–40. [CrossRef] [PubMed]
3. Balzer, A.H.; Whitehurst, C.B. An analysis of the biotin–(strept) avidin system in immunoassays: Interference and mitigation strategies. *Curr. Issues Mol. Biol.* **2023**, *45*, 8733–8754. [CrossRef]
4. Mishra, M.; Tiwari, S.; Gunaseelan, A.; Li, D.; Hammock, B.D.; Gomes, A.V. Improving the sensitivity of traditional Western blotting via Streptavidin containing Poly-horseradish peroxidase (PolyHRP). *Electrophoresis* **2019**, *40*, 1731–1739. [CrossRef]
5. Janardhan, K.S.; Jensen, H.; Clayton, N.P.; Herbert, R.A. Immunohistochemistry in investigative and toxicologic pathology. *Toxicol. Pathol.* **2018**, *46*, 488–510. [CrossRef] [PubMed]
6. Liao, T.Y.A.; Lau, A.; Joseph, S.; Hytönen, V.; Hmama, Z. Improving the immunogenicity of the Mycobacterium bovis BCG vaccine by non-genetic bacterial surface decoration using the avidin-biotin system. *PLoS ONE* **2015**, *10*, e0145833. [CrossRef] [PubMed]
7. Wang, S.; Hossain, M.Z.; Han, T.; Shinozuka, K.; Suzuki, T.; Kuwana, A.; Kobayashi, H. Avidin–biotin technology in gold nanoparticle-decorated graphene field effect transistors for detection of biotinylated macromolecules with ultrahigh sensitivity and specificity. *ACS Omega* **2020**, *5*, 30037–30046. [CrossRef]
8. Bajpayee, A.G.; Quadir, M.A.; Hammond, P.T.; Grodzinsky, A.J. Charge based intra-cartilage delivery of single dose dexamethasone using Avidin nano-carriers suppresses cytokine-induced catabolism long term. *Osteoarthr. Cartil.* **2016**, *24*, 71–81. [CrossRef]
9. He, T.; Zhang, C.; Vedadghavami, A.; Mehta, S.; Clark, H.A.; Porter, R.M.; Bajpayee, A.G. Multi-arm Avidin nano-construct for intra-cartilage delivery of small molecule drugs. *J. Control. Release* **2020**, *318*, 109–123. [CrossRef]
10. Durance, T.D.; Nakai, S. Simultaneous isolation of avidin and lysozyme from egg albumen. *J. Food Sci.* **1988**, *53*, 1096–1101. [CrossRef]
11. Durance, T.; Li-Chan, E.; Nakai, S. Process for the Isolation and Separation of Lysozyme and Avidin from Egg White. US Patent 4,966,851, 30 October 1990.
12. Rao, M.; Gupta, M.; Roy, I. Process for the Isolation and Purification of a Glycoprotein Avidin. US Patent 7,205,393 B2, 17 April 2007.
13. Gésan-Guiziou, G. 12—Separation technologies in dairy and egg processing. In *Separation, Extraction and Concentration Processes in the Food, Beverage and Nutraceutical Industries*; Rizvi, S.S.H., Ed.; Woodhead Publishing Series in Food Science, Technology and Nutrition; Woodhead Publishing: Sawston, UK, 2013; pp. 341–380, ISBN 9781845696450. [CrossRef]
14. Abeyrathne, E.N.S.; Lee, H.Y.; Ahn, D.U. Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents—A review. *Poult. Sci.* **2013**, *92*, 3292–3299. [CrossRef]
15. Tankrathok, A.; Daduang, S.; Patramanon, R.; Araki, T.; Thammasirirak, S. Purification process for the preparation and characterizations of hen egg white ovalbumin, lysozyme, ovotransferrin, and ovomucoid. *Prep. Biochem. Biotechnol.* **2009**, *39*, 380–399. [CrossRef] [PubMed]
16. Vachier, M.C.; Piot, M.; Awade, A.C. Isolation of hen egg white lysozyme, ovotransferrin and ovalbumin, using a quaternary ammonium bound to a highly crosslinked agarose matrix. *J. Chromatogr. B Biomed. Sci. Appl.* **1995**, *664*, 201–210. [CrossRef]
17. Awade, A.C.; Moreau, S.; Molle, D.; Brulé, G.; Maubois, J.L. Two-step chromatographic procedure for the purification of hen egg white ovomucin, lysozyme, ovotransferrin and ovalbumin and characterization of purified proteins. *J. Chromatogr. A* **1994**, *677*, 279–288. [CrossRef] [PubMed]
18. Omana, D.A.; Wang, J.; Wu, J. Co-extraction of egg white proteins using ion-exchange chromatography from ovomucin-removed egg whites. *J. Chromatogr. B* **2010**, *878*, 1771–1776. [CrossRef] [PubMed]
19. Abeyrathne, E.D.N.S.; Lee, H.Y.; Ahn, D.U. Sequential separation of lysozyme, ovomucin, ovotransferrin, and ovalbumin from egg white. *Poult. Sci.* **2014**, *93*, 1001–1009. [CrossRef]
20. Geng, F.; Huang, Q.; Wu, X.; Ren, G.; Shan, Y.; Jin, G.; Ma, M. Co-purification of chicken egg white proteins using polyethylene glycol precipitation and anion-exchange chromatography. *Sep. Purif. Technol.* **2012**, *96*, 75–80. [CrossRef]
21. Ji, S.; Ahn, D.U.; Zhao, Y.; Li, K.; Li, S.; Huang, X. An easy and rapid separation method for five major proteins from egg white: Successive extraction and MALDI-TOF-MS identification. *Food Chem.* **2020**, *315*, 126207. [CrossRef]

22. Gavara, P.R.; Cabrera, R.; Vennapusa, R.R.; Grasselli, M.; Fernandez-Lahore, M. Preparation, characterization, and process performance of composite fibrous adsorbents as cation exchangers for high throughput and high capacity bioseparations. *J. Chromatogr. B* **2012**, *903*, 14–22. [CrossRef]
23. Kikot, P.A.; Vazquez, D.S.; Carreras, J.G.; Laurella, S.L.; Grasselli, M. Synthesis of an HABA-based affinity matrix for Avidin purification from egg white proteins. *Braz. J. Chem. Eng.* **2025**, 1–11. [CrossRef]
24. Kikot, P.A.; Rios, E.M.; Vazquez, D.S.; Grasselli, M. Optimization of affinity chromatography using HABA ligand for avidin purification. *J. Chem. Technol. Biotechnol.* **2026**. [CrossRef]
25. Green, N.M. Avidin. *Adv. Protein Chem.* **1975**, *29*, 85–133. [CrossRef]
26. Frens, G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nat. Phys. Sci.* **1973**, *241*, 20–22. [CrossRef]
27. Hermanson, G.T. *Bioconjugate Techniques*, 3rd ed.; Editorial Academic Press: Cambridge, MA, USA, 2013; p. 485, ISBN 978-0-12-382239-0.
28. Celikbas, E.; Ceylan, A.E.; Timur, S. Paper-based colorimetric spot test utilizing smartphone sensing for detection of biomarkers. *Talanta* **2020**, *208*, 120446. [CrossRef] [PubMed]
29. Sharma, H.K.; Tihon, C. Determination of avidin and streptavidin by a modified Bradford method. *Anal. Biochem.* **1988**, *170*, 135–139. [CrossRef] [PubMed]
30. Wolman, F.J.; Maglio, D.G.; Grasselli, M.; Cascone, O. One-step lactoferrin purification from bovine whey and colostrum by affinity membrane chromatography. *J. Membr. Sci.* **2007**, *288*, 132–138. [CrossRef]
31. Green, N.M. [74] Spectrophotometric determination of avidin and biotin. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 1970; Volume 18, Part A, pp. 418–424, ISBN 9780121818791, ISSN 0076-6879. [CrossRef]
32. Shawkey, M.D.; Kosciuch, K.L.; Liu, M.; Rohwer, F.C.; Loos, E.R.; Wang, J.M.; Beissinger, S.R. Do birds differentially distribute antimicrobial proteins within clutches of eggs? *Behav. Ecol.* **2008**, *19*, 920–927. [CrossRef]
33. Guérin-Dubiard, C.; Pasco, M.; Hietanen, A.; Del Bosque, A.Q.; Nau, F.; Croguennec, T. Hen egg white fractionation by ion-exchange chromatography. *J. Chromatogr. A* **2005**, *1090*, 58–67. [CrossRef]
34. Kato, A.; Yoshida, K.; Matsudomi, N.; Kobayashi, K. The interaction between ovomucin and egg white proteins. *Agric. Biol. Chem.* **1976**, *40*, 2361–2366.
35. Omana, D.A.; Wu, J. A new method of separating ovomucin from egg white. *J. Agric. Food Chem.* **2009**, *57*, 3596–3603. [CrossRef]
36. Wang, J.; Wu, J. Effect of operating conditions on the extraction of ovomucin. *Process Biochem.* **2012**, *47*, 94–98. [CrossRef]
37. Alderton, G.; Fevold, H.L. Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. *J. Biol. Chem.* **1946**, *164*, 1–5. [CrossRef]
38. Clonis, Y.D.; Labrou, N.E.; Kotsira, V.P.; Mazitsos, C.; Melissis, S.; Gogolas, G. Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A* **2000**, *891*, 33–44. [CrossRef]
39. Green, N.M.; Toms, E.J. Purification and crystallization of avidin. *Biochem. J.* **1970**, *118*, 67–70. [CrossRef]
40. McCormick, D.B. Specific purification of avidin by column chromatography on biotin-cellulose. *Anal. Biochem.* **1965**, *13*, 194–198. [CrossRef]
41. Cuatrecasas, P.; Wilchek, M. Single-step purification of avidin from egg white by affinity chromatography on biocytin-Sepharose columns. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 235–239. [CrossRef]
42. Orr, G.A.; Heney, G.C.; Zeheb, R. [14] Purification of avidin and its derivatives on 2-iminobiotin-6-aminohexyl-sepharose 4B. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 1986; Volume 122, pp. 83–87.
43. Vetter, S.; Miron, T.; Wilchek, M. Affinity purification of the avidin protein family, based on crystal structures of avidin-HABA complexes. *J. Chromatogr. B* **2018**, *1093*, 113–118. [CrossRef] [PubMed]
44. Rothemund, D.L.; Thomas, T.M.; Rylatt, D.B. Purification of the basic protein avidin using Gradiflow technology. *Protein Expr. Purif.* **2002**, *26*, 149–152. [CrossRef] [PubMed]
45. Diederich, P.; Hoffmann, M.; Hubbuch, J. High-throughput process development of purification alternatives for the protein avidin. *Biotechnol. Prog.* **2015**, *31*, 957–973. [CrossRef]
46. Réhault-Godbert, S.; Guyot, N.; Nys, Y. The golden egg: Nutritional value, bioactivities, and emerging benefits for human health. *Nutrients* **2019**, *11*, 684. [CrossRef]
47. Li, Z.; Huang, X.; Tang, Q.; Ma, M.; Jin, Y.; Sheng, L. Functional properties and extraction techniques of chicken egg white proteins. *Foods* **2022**, *11*, 2434. [CrossRef]
48. Li-Chan, E.C.Y.; Kim, H.O. Structure and chemical composition of eggs. In *Egg Bioscience and Biotechnology*; Wiley Interscience: Hoboken, NJ, USA, 2008; pp. 1–95.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.