Original article

IRSTI 62.39.54 **https://doi.org/10.70264/jbr.v1.3.2026.3**

PRODUCTION OF HEN EGG-WHITE LYSOZYME: RECOMBINANT EXPRESSION IN *PICHIA PASTORIS* AND ISOLATION OF THE NATIVE ENZYME

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ABSTRACT

Hen egg-white lysozyme (HEWL) is a well-characterized antimicrobial enzyme widely used in the food industry, biotechnology, and biomedical research. The aim of this study was to investigate two approaches to lysozyme production: heterologous expression of its gene in *Pichia pastoris* and isolation of native HEWL from chicken egg white. For recombinant production, the pPICZαA-LysC-His construct was generated and methanol-induced expression was performed in *P. pastoris* GS115. Anti-His-reactive material was detected in induced T3 preparations and selected Ni-NTA fractions; however, selective enrichment of a protein with the expected molecular mass was not achieved, and the available data were therefore not considered definitive evidence for correctly processed recombinant rLysC. For the native approach, fresh chicken egg white was subjected to 30-kDa tangential-flow ultrafiltration followed by cation-exchange chromatography on SP Sepharose Fast Flow. Fractions eluted with 300 mM NaCl were enriched in a low-molecular-weight component with electrophoretic mobility corresponding to the HEWL region, and these preparations exhibited lytic activity in substrate zymography using *Lactobacillus plantarum* cell-wall material. These findings provide preliminary process-oriented observations on obtaining HEWL-enriched, lytically active preparations from fresh egg white. Evaluation of off-grade egg material remains a subject for future study.

Keywords: lysozyme; HEWL; *Pichia pastoris*; recombinant expression; ultrafiltration; cation-exchange chromatography.

Received: September 15, 2026 / *Accepted*: September 24, 2026 / *Published*: September 30, 2026

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Citation: Rakhmetova D., Akishev Zh., Yerzhankyzy Z., Khassenov B. Production of Hen Egg-White Lysozyme: Recombinant Expression in Pichia pastoris and Isolation of the Native Enzyme.Journal of Biological Research, 1(3), 20-29. https://doi.org/10.70264/jbr.v1.3.2026.3.

1. INTRODUCTION

Hen egg-white lysozyme C (HEWL; EC 3.2.1.17; 1,4-β-N-acetylmuramidase) is a classical c-type lysozyme and one of the most extensively studied antimicrobial enzymes. HEWL is a 14.3-kDa protein comprising 129 amino acid residues [1, 2]. The enzyme hydrolyzes β-(1→4)-glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in bacterial cell-wall peptidoglycan [2, 3]. Consequently, lysozyme exhibits lytic activity against Gram-positive bacteria, whereas the outer membrane of Gram-negative bacteria restricts access of the enzyme to peptidoglycan [3, 4]. In addition to its muramidase activity, lysozyme has been reported to exert antibacterial and antibiofilm effects, broadening the range of its potential applications [4, 5].

Lysozyme is widely distributed in animal tissues and biological secretions, and chicken egg white is one of its richest and most technologically accessible sources. HEWL accounts for approximately 3.4–3.5% of total egg-white protein [6], and its concentration may reach several milligrams per milliliter [7]. The high isoelectric point of HEWL (pI approximately 10.7) distinguishes it from most other egg-white proteins and enables efficient use of ion-exchange methods for its isolation and purification [2, 6]. Various approaches have been proposed for obtaining native HEWL, including fractionation, ion-exchange and affinity chromatography, and membrane-based separation methods [6, 8-13].

Owing to its antimicrobial properties, HEWL is of interest for the food industry, biopreservation, the development of antimicrobial coatings and packaging materials, and biomedical and veterinary applications [3-6]. Alongside isolation of the enzyme from natural raw materials, technologies for its recombinant production are being developed. The methylotrophic yeast *Pichia pastoris* is a promising heterologous expression system that enables both intracellular and secretory production of recombinant proteins [14-16]. However, the efficiency of HEWL expression and secretion in this system may be limited by protein processing and folding, requiring experimental assessment not only of protein production

but also of functional activity [14, 15, 17].

Another promising direction is the use of egg raw materials unsuitable for food marketing as a source of biologically active components. Large-scale egg production generates off-grade products that may potentially be considered a secondary resource for obtaining value-added proteins [18, 19]. This approach is also relevant to Kazakhstan, where annual chicken egg production amounts to billions of eggs [20]. Given the relatively high HEWL content of egg white and the possibility of its selective isolation, lysozyme recovery may represent one potential route for the advanced processing of such raw materials.

The aim of this study was to investigate the feasibility of producing hen egg-white lysozyme by heterologous expression of its gene in *Pichia pastoris*, as well as to investigate isolation and enrichment of a native HEWL-region component from egg white and assess its lyticactivity.

2. MATERIALS AND METHODS

2.1. Reagents and Culture Media

Unless otherwise specified, chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and AppliChem (Darmstadt, Germany). Luria–Bertani (LB) broth (pH 7.0), containing 0.5% yeast extract (Condalab, Madrid, Spain), 1% tryptone (Sigma-Aldrich), and 0.5% NaCl (Sigma-Aldrich), was used to cultivate *Escherichia coli*. Yeast extract–peptone–dextrose (YEPD) broth (pH 7.0), containing 1% yeast extract (Condalab, Madrid, Spain), 2% peptone (Sigma-Aldrich), and 2% dextrose (Sigma-Aldrich), and buffered glycerol-complex (BMGY) broth (pH 6.0), containing 1% yeast extract (Condalab, Madrid, Spain), 2% peptone (Sigma-Aldrich), 100 mM potassium phosphate buffer prepared from KH_2PO_4 and K_2HPO_4 (Sigma-Aldrich), 1.34% yeast nitrogen base (YNB; AppliChem, Darmstadt, Germany), 4×10^{-5}% biotin (AppliChem, Darmstadt, Germany), and 1% glycerol (TMMEDIA), were used to cultivate *Pichia pastoris.* BMMY contained the same basal components as BMGY, except that glycerol was omitted and methanol was supplied as described in Section 2.4.

2.2. Strains and Vector

Pichia pastoris GS115 and *Escherichia coli* DH5α (Promega, Madison, WI, USA) were used in this study. The pPICZαA vector (Invitrogen, USA) was used for cloning the *lysC* gene and for production of recombinant LysC in *P. pastoris*.

2.3. Lysozyme Gene and Construction of Expression Vectors

The chicken (*Gallus gallus*) lysozyme C gene (*LYZ*; NCBI Gene ID 396218; RefSeq NM_205281.2/NP_990612.2; UniProt P00698) was used as the target sequence [1, 2]. The native precursor contains 147 amino acid residues, whereas mature hen egg-white lysozyme (HEWL), after removal of the N-terminal signal peptide, consists of 129 amino acid residues and has a calculated molecular mass of 14.3 kDa [1, 2]. The expression constructs contained the mature LysC sequence without its native N-terminal signal peptide. A previously generated laboratory construct, pTOP-Blunt-V2-LysC, served as the cloning template.

For secretory production, the 3949-bp pPICZαA-LysC-His plasmid was constructed. Expression was controlled by the methanol-inducible *AOX1* promoter. In pPICZαA-LysC-His, targeting of the recombinant protein to the secretory pathway was mediated by the N-terminal *Saccharomyces cerevisiae* α-factor leader sequence; LysC was cloned without a terminal stop codon, maintaining the reading frame with the vector-encoded C-terminal c-myc/6×His region.

The *LysC* sequence was amplified from pTOP-Blunt-V2-LysC using the primers cLys_EcoRI_alphaA (5′-CCGGAATTCATGAAGGTTTTTGGTAGATGTG-3′) and cLys_NotI_alphaA_his(5′-ATAGTTTAGCGGCCGCATTCTTATTCAATCTACAACCTCTAATCCAAG-3′), containing EcoRI and NotI sites, respectively. PCR amplification was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The 50-µL reaction mixture contained 10 µL of 5× Q5 Reaction Buffer, 1 µL of 10 mM dNTPs, 2.5 µL of each primer (10 µM), 50 ng of template DNA (pTOP-Blunt-V2-LysC), 0.5 µL of Q5 High-Fidelity DNA Polymerase, and nuclease-free water to a final volume of 50 µL. The amplification program consisted of initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 2 min. The amplified gene and vector were digested with EcoRI and NotI in Buffer O and ligated using T4 DNA ligase. Following transformation of *E. coli* DH5α, transformant clones were selected on LB agar containing zeocin (100 µg/mL). Plasmid DNA from the transformants was screened by PCR using the primers AOX1fw (5′-GACTGGTTCCAATTGACAAGC-3′) and AOX1rv (5′-GCAAATGGCATTCTGACATCC-3′). The expected size of the amplification product for clones carrying the pPICZαA-LysC-His expression cassette was 945 bp. The clones were additionally verified by sequencing, resulting in the pPICZαA-LysC-His shuttle vector.

2.4. Generation of Recombinant Strains and Expression of LysC in Pichia pastoris

Pichia pastoris GS115 was selected as the host strain for expression. The pPICZαA-LysC-His plasmid was linearized with the restriction endonuclease PmeI (Thermo Fisher Scientific) in Buffer B. The resulting cassette was purified by phenol/chloroform extraction followed by ethanol precipitation. Competent *P. pastoris* GS115 cells were prepared and transformed according to the EasySelect™ Pichia Expression Kit instructions (Invitrogen). Purified linearized plasmid DNA (3 µg) was added to 80 µL of cells, and electroporation was performed in a cuvette with a 0.2-cm electrode gap at 2 kV (pulse duration, 4.8 ms) using a MicroPulser™ electroporator with the Pic program. Immediately after electroporation, 1 mL of a 1:1 mixture of YEPD and 1 M sorbitol was added, and the cells were allowed to recover for 3 h at 30 °C.*P. pastoris* GS115/pPICZαA-LysC-His transformants were selected on YEPD agar containing an increased concentration of zeocin (200 µg/mL), after which insertion into the yeast genomic DNA was verified by PCR using the AOX1fw and AOX1rv primers. The sequence-verified pPICZαA-LysC-His plasmid clone no. 9 was used for transformation of *P. pastoris* GS115. Among the resulting yeast transformants, T3 was selected as the producer strain.

For expression, the selected T3 transformant was first grown in YEPD at 30 °C and 230–250 rpm, then transferred to

200 mL of BMGY and cultured for 12–16 h at 30 °C and 200–250 rpm. Cells were harvested at 3500 × g for 15 min at 4 °C and resuspended in 200 mL of buffered methanol-complex medium (BMMY). Induction was carried out at 25 °C and 200 rpm for 120 h. Methanol was supplied at nominal concentrations of 1% (v/v) during days 1–3 and 2% (v/v) during days 4–5. These values describe the nominal supplementation regimen rather than measured residual methanol concentrations. Samples were collected at 0, 24, 48, 72, 96, and 120 h. After induction, cells were removed by centrifugation, and the clarified culture supernatants were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and attempted affinity purification.

For Ni-NTA purification, 15 mL of T3 culture supernatant was mixed with an equal volume of Buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl) and applied to 1 mL of Ni-NTA resin. After washing with the same buffer, bound proteins were eluted with buffer containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 250 mM imidazole. The resulting fractions were analyzed electrophoretically and immunologically.

2.5. SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE in a 15% polyacrylamide gel according to Laemmli [21] and stained with Coomassie Brilliant Blue R-250. When required, culture supernatants were preconcentrated by precipitation with 10% trichloroacetic acid (TCA). For analysis of native HEWL, 10 µL of each test fraction was loaded; band intensity was used only for qualitative assessment because protein mass loading was not standardized across lanes.

For Western blotting, proteins were transferred after SDS-PAGE to an Immun-Blot PVDF membrane (Bio-Rad) at a constant current of 350 mA for 20 min in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 h. His-tagged proteins were detected using rabbit Anti-His Tag antibodies (Sigma-Aldrich, SAB1306082-40TST; 1:500–1:1000) and laboratory anti-rabbit-HRP secondary antibodies (1:20,000). Primary antibodies were incubated with the membrane overnight at 4 °C, and secondary antibodies for 1 h at room temperature. Chemiluminescent signal was developed using EZ-ECL (Biological Industries) and recorded with a ChemiDoc system.

2.6. Isolation of Native Lysozyme from Chicken Egg White

Fresh chicken eggs were used for isolation of native HEWL. The shell surface was treated with 70% ethanol for surface disinfection, and the egg white was separated from the yolk. Subsequently, 125 mL of egg white was mixed 1:1 with 125 mL of 100 mM sodium acetate, pH 5.2, yielding a nominal total volume of 250 mL and a final nominal sodium acetate concentration of 50 mM. After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant was subjected to tangential-flow ultrafiltration using a Sartorius Vivaflow® 50 cassette equipped with a polyethersulfone membrane with a 30-kDa molecular-weight cut-off (MWCO; Product No. VF05P2), operated with an external VELP Scientifica SP 311/60 peristaltic pump. The sample reservoir was maintained in an ice bath during filtration to minimize sample warming. No inline pressure sensor was used; therefore, pressure was not quantitatively recorded during the experiment. Ultrafiltration was performed in batch concentration mode with collection of the permeate, without diafiltration or buffer replenishment. The exact volumetric flow rate was not recorded during the original experiment. At the end of the run, the permeate and retentate were collected separately and their volumes were measured directly using a graduated measuring cylinder, yielding approximately 200 mL of permeate and 50 mL of retentate. These are measured final volumes; a predefined stopping criterion was not documented in the available records. According to SDS-PAGE, a low-molecular-weight component with electrophoretic mobility in the HEWL region was found predominantly in the 30-kDa permeate, which was used for subsequent cation-exchange chromatography.

Cation-exchange fractionation of the 30-kDa permeate was performed on SP Sepharose Fast Flow (Cytiva) using 2 mL of settled resin. The resin was equilibrated with 50 mM sodium acetate buffer (pH 5.2). After sample application, unbound components were removed with the same buffer. Bound proteins were eluted stepwise with buffers containing 50 mM sodium acetate, pH 5.2, and 50, 150, 300, or 750 mM NaCl; final desorption was performed with 2 M NaCl. At the 300 mM NaCl step, sequential 0.5-mL fractions were collected and combined into a 10-mL pool. Fractions were analyzed by SDS-PAGE and substrate zymography.

Total protein concentration in the pooled 300 mM NaCl SP-Sepharose fraction was estimated using the Bradford assay [22]. For each determination, 890 µL of diluent consisting of 10% (v/v) 1× PBS in water supplemented with 1% (v/v) glycerol, 10 µL of sample, and 100 µL of Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, cat. no. 5000006) were combined to a final assay volume of 1.0 mL. For the blank, the 10 µL sample aliquot was replaced with 10 µL of the corresponding diluent. Absorbance at 595 nm was measured using a GeneQuant 100 spectrophotometer. The pooled fraction was analyzed undiluted and after 1:2, 1:5, and 1:10 dilution. Protein mass was calculated using the laboratory BSA calibration equation $y = -49.293x^3 + 76.314x^2 + 10.635x - 0.0257$, where x is A595 and y is the calculated total protein mass (µg) in the analyzed 10-µL aliquot. Each dilution was measured once; no replicate Bradford determinations were performed. The calibration range and the interval between reagent addition and absorbance measurement were not available in the records used for this revision, limiting assessment of the quantitative estimate.

2.7. Zymographic Analysis of Lysozyme Activity

Lytic activity was assessed qualitatively by substrate zymography in a 15% polyacrylamide gel. A *Lactobacillus plantarum* cell-wall preparation was incorporated into the resolving gel to a final nominal concentration of 0.2% (w/v). Before loading, samples were not boiled, reduced with dithiothreitol (DTT) or β-mercaptoethanol, or subjected to TCA precipitation. Electrophoresis was performed at a reduced current of 7–10 mA.

After electrophoresis, SDS was removed by incubating the gel for 1 h at room temperature in 25 mM Tris-HCl, pH 8.0, containing 1% Triton X-100. The gel was then incubated for at least 16 h at 37 °C in the same solution, transferred to 25 mM Tris-HCl, pH 8.0, without Triton X-100, and incubated for an additional 3–4 h at 37 °C. Clearing zones were docu-

mented before protein staining and interpreted as regions of substrate lysis. Commercial HEWL (Fluka Analytical, Sigma-Aldrich, 62971-10G-F, Lot BCBP6286V) was used as a positive control.

2.8. Bioinformatic Analysis and Data Processing

Plasmid maps, restriction-site positions, and sequenced regions were analyzed using SnapGene Viewer. Reference information on the gene and protein was obtained from NCBI Gene/RefSeq and UniProtKB [1, 2]. SDS-PAGE gels, Western blots, and zymograms were evaluated on the basis of the presence and relative mobility of protein bands and clearing zones.

The experimental work was exploratory and included method-development and technical analytical runs rather than standardized independent biological replicates. The complete native 30-kDa ultrafiltration/SP-Sepharose workflow was performed once; a preceding pilot cation-exchange experiment used material from the same egg-white batch and was not considered an independent replicate. Recombinant experiments comprised several method-development series with modified conditions rather than independent repeats of a single standardized protocol. Accordingly, no inferential statistics or estimates of biological reproducibility were calculated.

2.9. Statistical Analysis

The study was exploratory and primarily qualitative. The experimental work included method-development and technical analytical runs; however, these did not constitute independent biological replicates. The complete 30-kDa ultrafiltration/SP-Sepharose workflow was performed once and was not independently replicated using separate egg-white batches. Recombinant-expression experiments comprised several method-development series performed under modified conditions rather than standardized independent biological replicates. SDS-PAGE, Western blotting, and substrate zymography were evaluated qualitatively based on the presence and relative electrophoretic mobility of protein bands and clearing zones. Bradford measurements of the pooled 300 mM NaCl fraction were performed once for each tested dilution and were used only to obtain an approximate estimate of total protein concentration. Because standardized independent biological replication was not performed, mean ± standard deviation, inferential statistical tests, and p values were not calculated.

3. RESULTS

3.1. Assessment of Recombinant LysC Expressionin Pichia pastoris

The pPICZαA-LysC-His construct was designed for extracellular expression of recombinant rLysC (Figure 1). Secretory targeting of the recombinant product is mediated by the *Saccharomyces cerevisiae* α-factor secretion signal sequence (MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAEF), which directs the recombinant product into the secretory pathway during induction in BMMY. During secretion, the *S. cerevisiae* α-factor leader is proteolytically processed by Kex2, followed by removal of the Glu–Ala repeats by Ste13. Because *LysC* was cloned at the EcoRI site downstream of the α-factor processing region, the predicted processed recombinant product contains vector-derived residues at its N terminus and the C-terminal c-myc/6×His region. The calculated molecular mass and predicted pI of the fully processed recombinant product were 18.09 kDa and 8.75, respectively.

Following methanol induction, SDS-PAGE of the culture supernatants did not reveal pronounced accumulation of a protein that could be unequivocally assigned to rLysC on the basis of electrophoretic mobility. Anti-His Western blotting showed the presence of immunoreactive material in induced T3 preparations and in selected Ni-NTA fractions. However, Ni-NTA chromatography did not provide convincing selective enrichment of a protein with the expected molecular mass. Accordingly, the detected anti-His-reactive material was considered consistent with, but not definitive evidence for, accumulation of correctly processed recombinant rLysC.

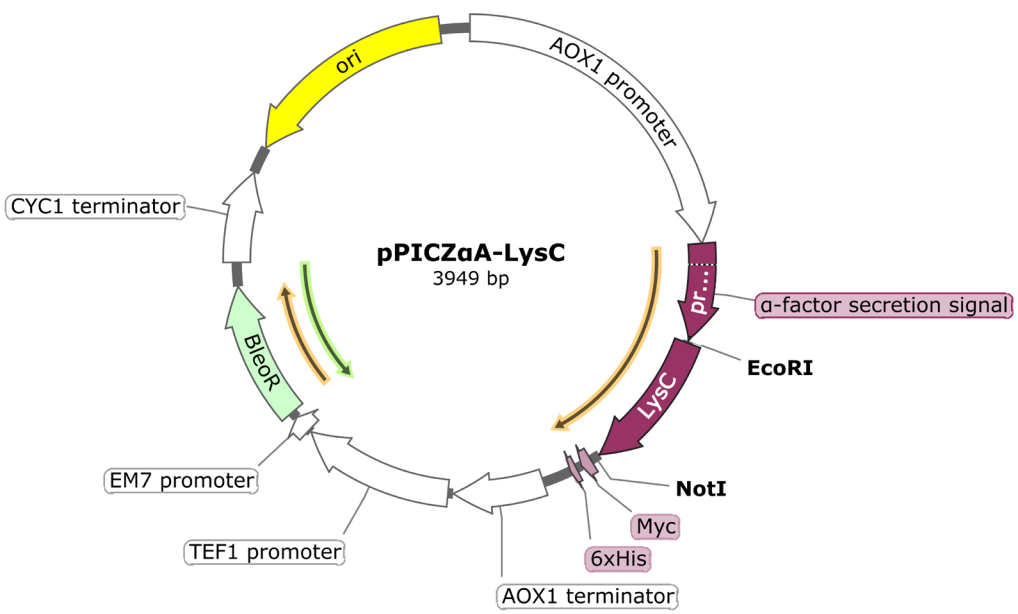

Figure 1 – Plasmid map of the pPICZαA-LysC-His vector.

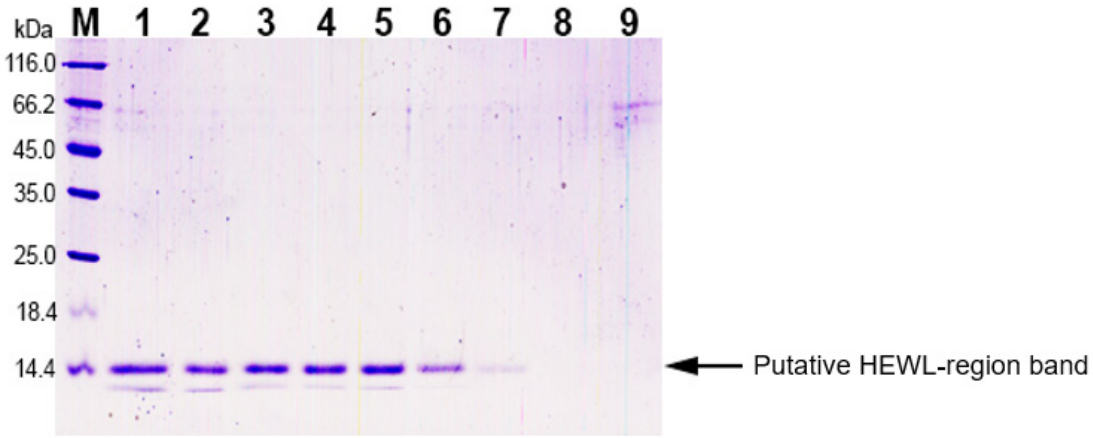

Figure 2 – SDS-PAGE analysis of fractions obtained after SP Sepharose Fast Flow chromatography of the 30-kDa egg-white permeate and an additional recombinant-expression preparation. Lane M, protein molecular-weight marker (Thermo Fisher Scientific, cat. no. 26610); lanes 1–6, fractions 300.11, 300.13, 300.15, 300.17, 300.19, and 300.20, respectively, obtained following purification of the 30-kDa egg-white permeate on an SP Sepharose Fast Flow column and eluted with 300 mM NaCl; lane 7, fraction eluted with 750 mM NaCl; lane 8, fraction eluted with 2 M NaCl; lane 9, 10-kDa ultrafiltration permeate obtained from a recombinant-expression preparation, included as an additional comparison and excluded from interpretation of the native HEWL purification. Test samples were loaded at 10 µL per lane. The low-molecular-weight band is labelled as a putative HEWL-region band.Lanes 1–8 derive from the single complete 30-kDa ultrafiltration/SP-Sepharose workflow, which was performed once and was not independently replicated. The image documents an individual analytical run.

3.2. Isolation and Enrichment of a HEWL-Region Component from Egg White

Before the final ultrafiltration-based workflow, a pilot SP-Sepharose cation-exchange experiment was performed using 20 mL of egg white from the same original batch, mixed with 20 mL of 100 mM sodium acetate, pH 5.2, to give a total loading volume of 40 mL. Chromatographic monitoring with the ÄKTA/UNICORN system and SDS-PAGE indicated that a low-molecular-weight component appeared in salt eluates, including the 300 mM NaCl step, whereas the starting material and early fractions retained a more complex protein profile. This pilot experiment was analyzed separately and was used to select the subsequent workflow in which 125 mL of egg white from the same batch was first subjected to 30-kDa ultrafiltration followed by SP-Sepharose chromatography.

After dilution and clarification, the preparation derived

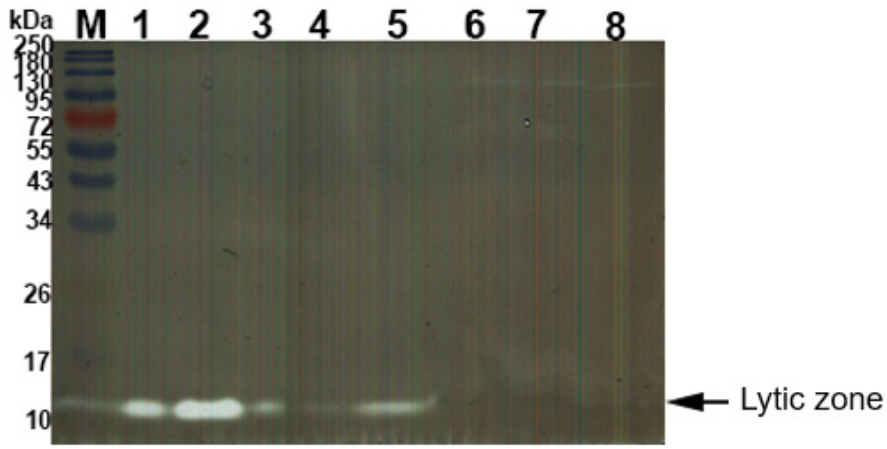

Figure 3 – Substrate zymography of egg-white-derived preparations and preparations from the recombinant-expression series. Lane M, protein molecular-weight marker (New England Biolabs, cat. no. P7719S); lane 1, commercial HEWLpositive control (Fluka Analytical, Sigma-Aldrich, cat. no. 62971); lanes 2 and 5, egg-white-derived SP-Sepharose preparations, lane 2, a pilot batch, and lane 5, 30-kDa permeate subjected to fractionation on SP Sepharose Fast Flow; lane 3, 30-kDa retentate; lane 4, 30-kDa permeate; lanes 6–8, preparations from the recombinant-expression series. Lanes 6–8 were not used to establish recombinant LysC activity. The annotation identifies a lytic zone without assigning an exact molecular mass. Clearing zones in egg-white-derived samples migrated approximately at the position of the commercial HEWL control under these assay conditions; this correspondence does not establish protein identity, purity, or quantitative enzymatic activity. Lane 2 derives from the separate pilot SP-Sepharose experiment, whereas lanes 3–5 derive from the single complete 30-kDa ultrafiltration/SP-Sepharose workflow. The pilot and complete workflow were not independent purification replicates. The image documents an individual analytical run. Additional analytical runs were technical or method-development analyses and did not constitute independent purification replicates.

from 125 mL of egg white was subjected to tangential-flow ultrafiltration through a membrane with a 30-kDa MWCO. Approximately 50 mL of retentate and 200 mL of permeate were obtainedby direct volumetric measurement. SDS-PAGE revealed differences in the protein composition of these fractions; the low-molecular-weight component with electrophoretic mobility in the HEWL region was found predominantly in the 30-kDa permeate. Therefore, this fraction was used for subsequent fractionation on SP Sepharose Fast Flow.

During stepwise column elution, a low-molecular-weight band was observed in fractions obtained at 300 mM NaCl (Figure 2). Its electrophoretic mobility corresponded to the molecular-mass region of native HEWL (~14.4 kDa). In some lanes, an additional low-molecular-weight component was present below the main band; its identity was not determined in this study.

The pooled 300 mM NaCl fraction had a final volume of 10 mL. Bradford analysis of the undiluted sample gave a provisional total-protein estimate of approximately 0.140 mg/mL, corresponding to approximately 1.40 mg of total protein in the pooled fraction. Because the assay measured total protein rather than HEWL specifically and replicate quantitative determinations were not performed, this value was treated only as an approximate estimate and was not used to calculate HEWL-specific recovery, purity, or purification fold.

3.3. Lytic Activity of HEWL-Enriched Egg-White Preparations

Lytic activity of the obtained preparations was assessed qualitatively by substrate zymography using *Lactobacillus plantarum* cell-wall material as the substrate. Commercial HEWL used as a positive control produced a clearing zone. Clearing zones were also observed in egg-white preparations obtained after 30-kDa ultrafiltration and SP-Sepharose chromatography (Figure 3). These observations demonstrate lytic activity in the tested preparations; however, zymography alone does not establish that the activity originates exclusively from the low-molecular-weight band assigned to the HEWL region, nor does it provide a quantitative measure of enzyme activity or protein purity. The clearing zones in the egg-white-derived samples migrated approximately at the position of the commercial HEWL control under the zymography conditions used. No exact molecular mass was assigned to these zones, and their correspondence with the control was not considered definitive evidence of protein identity. The incompletely documented recombinant-series lanes were not used to establish recombinant LysC activity.

4. DISCUSSION

The efficiency of secretory production of heterologous proteins in *P. pastoris* depends on processing of the secretion leader, folding, disulfide-bond formation, and proteolytic stability of the product [14, 15]. For rLysC, the initial production level in *P. pastoris* has also been reported to be low and to require optimization of the expression system [17]. Thus, the present observations support the need for further optimization of the recombinant system and specific identification of the target product, for example by mass spectrometry. Owing to the ambiguity of recombinant production, the subsequent part of the study focused on fractionation of native egg-white preparations.

The contribution of the present work is process-oriented rather than the introduction of a fundamentally new lysozyme-production principle. Specifically, the study documents the behavior and limitations of a sequential 30-kDa ultrafiltration/SP-Sepharose workflow under the tested laboratory conditions and places these observations alongside the limitations encountered during recombinant rLysC production.

The observed chromatographic behavior is consistent with the physicochemical properties of HEWL. Owing to its high isoelectric point (pI approximately 10.7), HEWL carries a net positive charge at pH 5.2 and can interact efficiently with a cation-exchange resin [2, 6]. Efficient isolation of HEWL by cation-exchange and membrane-based methods has also been demonstrated in other studies [8, 11-13]. Nevertheless, in the present study the identity of the low-molecular-weight component was inferred from electrophoretic mobility and preparation-level lytic activity rather than confirmed by a HEWL-specific analytical method.

The relatively low amount of total protein measured in the pooled 300 mM NaCl fraction may reflect cumulative losses during membrane fractionation, chromatographic binding, washing and elution, and sample handling. However, because HEWL was not quantified specifically in the starting material, ultrafiltration fractions, chromatographic flow-through, wash fractions, or individual eluates, stage-specific HEWL recovery and losses could not be determined.

Recovery of biologically active components from egg-production by-products is considered one potential route for their high-value valorization [18, 19]. The relevance of this direction is supported by the large production volumes: according to FAO data, global egg production in 2024 was approximately 100 million tonnes [23], while approximately 2.82 billion chicken eggs were produced in Kazakhstan in January–July 2026 [20]. The present experiments, however, were performed using fresh chicken eggs and therefore do not demonstrate recovery from off-grade egg material.

HEWL is present in egg white at concentrations of several milligrams per milliliter [7], and its high pI provides favorable conditions for cation-exchange fractionation. Off-grade egg material not intended for food marketing may therefore be evaluated as a potentialsource of lysozymein future studies, but this application remains prospective in the present work.

Study limitations. The present study was exploratory and primarily qualitative. The complete native HEWL fractionation workflow was performed once, and its reproducibility across independent egg-white batches was therefore not assessed. Protein identity was inferred from electrophoretic mobility in the HEWL molecular-mass region together with preparation-level lytic activity, but was not independently confirmed by HEWL-specific immunoblotting or mass spectrometry. Enzymatic activity was evaluated qualitatively by substrate zymography, and HEWL-specific recovery, purity, purification fold, and specific activity were not determined. Accordingly, the results should be interpreted as preliminary process-oriented observations rather than as quantitative validation of purification efficiency. Further studies using independent biological replicates, HEWL-specific identification, and quantitative activity measurements will be required to validate and optimize the workflow.

Future studies should include independent biological replicates, HEWL-specific protein identification, and quantitative measurements of enzymatic activity and process recovery.

5. CONCLUSION

This study investigated two approaches to obtaining hen egg-white lysozyme: recombinant production in *Pichia pastoris* and fractionation of native HEWL from fresh egg white. In the recombinant series, anti-His-reactive material was detected in induced T3 preparations and selected Ni-NTA fractions, but the available data did not provide definitive evidence for accumulation of correctly processed rLysC. In the native series, sequential 30-kDa ultrafiltration and SP-Sepharose chromatography yielded fractions enriched in a low-molecular-weight component with electrophoretic mobility corresponding to the HEWL region, and these preparations exhibited lytic activity by substrate zymography. Because protein identity was not confirmed by a target-specific method, activity was assessed qualitatively, and the complete workflow was not independently replicated, the findings should be regarded as preliminary process-oriented observations. Off-grade egg material was not tested and remains a prospective subject for future investigation.

AUTHORS' CONTRIBUTIONS

D.R.: investigation, data curation, writing – original draft. Zh.A.: conceptualization, methodology, investigation, supervision. Z.Y.: investigation, data curation. B.K.: supervision, project administration, writing – review and editing. All authors reviewed and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors acknowledge the National Center for Biotechnology for providing the laboratory facilities and infrastructure used in this study.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AI DECLARATION

During the preparation of this manuscript, the authors used OpenAI ChatGPT for English-language translation and for improving the readability and language of the manuscript. The authors subsequently reviewed and edited the content and take full responsibility for the final manuscript.

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ПОЛУЧЕНИЕ ЛИЗОЦИМА КУРИНОГО ЯЙЦА: РЕКОМБИНАНТНАЯ ЭКСПРЕССИЯ В *PICHIA PASTORIS* И ВЫДЕЛЕНИЕ НАТИВНОГО ФЕРМЕНТА

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АННОТАЦИЯ

Лизоцим белка куриного яйца (HEWL) – хорошо изученный антимикробный фермент, широко применяемый в пищевой промышленности, биотехнологии и биомедицинских исследованиях. Целью работы было изучение двух подходов к получению лизоцима: гетерологичной экспрессии его гена в *Pichiapastoris* и выделения нативного HEWL из белка куриного яйца. Для получения рекомбинантного белка была создана конструкция pPICZαA-LysC-His и проведена индуцируемая метанолом экспрессия в *P. pastoris* GS115. В индуцированных препаратах T3 и отдельных Ni-NTA-фракциях выявлялся материал, иммунореактивный к антителам против His-tag; однако селективного обогащения белка ожидаемой молекулярной массы достигнуто не было, поэтому полученные данные не рассматривались как однозначное подтверждение накопления корректно процессированного рекомбинантного rLysC. Для нативного подхода све-

жий белок куриного яйца подвергали тангенциальной ультрафильтрации через мембрану с порогом отсечения 30 кДа с последующей катионообменной хроматографией на SP Sepharose Fast Flow. Фракции, элюированные 300 мМ NaCl, были обогащены низкомолекулярным компонентом с электрофоретической подвижностью, соответствующей области HEWL, и проявляли литическую активность при субстратной зимографии с использованием материала клеточной стенки *Lactobacillus plantarum*. Полученные результаты представляют предварительные процесс-ориентированные наблюдения по получению из свежего яичного белка препаратов, обогащенных компонентом в области HEWL и обладающих литической активностью. Использование некондиционного яичного сырья остается предметом будущих исследований.

Ключевые слова: лизоцим; HEWL; *Pichia pastoris*; рекомбинантная экспрессия; ультрафильтрация; катионообменная хроматография.

ТАУЫҚ ЖҰМЫРТҚАСЫНЫҢ АҚУЫЗЫНЫҢ ЛИЗОЦИМІН АЛУ: *PICHIA PASTORIS*-ТАҒЫ РЕКОМБИНАНТТЫ ЭКСПРЕССИЯ ЖӘНЕ НАТИВТІ ФЕРМЕНТТІ БӨЛІП АЛУ

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АҢДАТПА

Тауық жұмыртқасының ақуызының лизоцимі (HEWL) – тамақ өнеркәсібінде, биотехнологияда және биомедициналық зерттеулерде кеңінен қолданылатын, қасиеттері жақсы зерттелген микробтарға қарсы фермент. Бұл жұмыстың мақсаты лизоцим алудың екі тәсілін зерттеу болды: оның генін *Pichia pastoris* ашытқысында гетерологиялық экспрессиялау және тауық жұмыртқасының ақуызынан нативті HEWL бөліп алу. Рекомбинантты өнім алу үшін pPICZαA-LysC-His конструкциясы жасалып, *P. pastoris* GS115 штамында метанолмен индукцияланатын экспрессия жүргізілді. Индукцияланған T3 препараттарында және кейбір Ni-NTA фракцияларында His-tag-ке қарсы антиденелермен реакция беретін материал анықталды; алайда күтілетін молекулалық массадағы ақуыздың селективті байытылуы алынбады, сондықтан бұл деректер дұрыс процессингтен өткен рекомбинантты rLysC жиналуының нақты дәлелі ретінде қарастырылмады. Нативті тәсілде жаңа тауық жұмыртқасының ақуызы 30 кДа молекулалық масса бойынша бөлу шегі бар мембрана арқылы тангенциалды ультрафильтрациядан өткізіліп, кейін SP Sepharose Fast Flow сорбентінде катионалмасу хроматографиясы жүргізілді. 300 мМ NaCl кезінде элюцияланған фракциялар HEWL аймағына сәйкес электрофоретикалық қозғалғыштығы бар төмен молекулалық компонентпен байытылды және *Lactobacillus plantarum* жасуша қабырғасының материалын пайдаланған субстратты зимографияда литикалық белсенділік көрсетті. Нәтижелер жаңа жұмыртқа ақуызынан HEWL аймағындағы компонентпен байытылған және литикалық белсенділігі бар препараттарды алуға қатысты алдын ала процесс-бағытталған бақылауларды көрсетеді. Кондицияға сәйкес келмейтін жұмыртқа шикізатын бағалау болашақ зерттеулердің тақырыбы болып қалады.

Түйін сөздер: лизоцим; HEWL; *Pichia pastoris*; рекомбинантты экспрессия; ультрафильтрация; катионалмасу хроматографиясы.