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# ISOLATION AND PURIFICATION OF LYSOZYME FROM THE HEN EGG WHITE

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The aim of the research was the development of the method of lysozyme isolation from hen egg proteins. Lysozyme was isolated by differential heat denaturation of proteins with changing of the medium pH value, followed by neutralization, dialysis and additional purification by gel chromatography on Sephadex G-50. Activity was determined by bacteriolytic method (with *Micrococcus lysodeikticus* 4698 as a substrate). The enzyme purity and molecular mass were determined using SDS-electrophoresis and mass-spectrometry. The method of lysozyme isolation from hen egg proteins with the enzyme yield of 3.2 ± 0.2% and bacteriolytic activity of 22 025 ± 1 500 U/mg is modified. According to electrophoresis data, the isolated enzyme is characterized by high degree of purity (~95–98%) and is comparable with lysozyme of AppliChem company by main physical and chemical characteristics. The obtaining product is stored in a crystalline form at low temperature (–24 °C) for 9 months. The proposed method allows obtaining active and stable lysozyme with high purity from hen egg protein in laboratory conditions for the usage in biotechnology.

***Key words:*** hen egg white, lysozyme.

Lysozyme (EC 3.2.1.17) is an enzyme of hydrolases class, it is also known as muramidase or N-acetylmuramoylhydrolase. Lysozyme is commercially important enzyme, and it is now widely used in biotechnology to extract intracellular bacterial components, in particular in the food industry as an antibacterial additive to increase the shelf life of products, as well as in medicine in the treatment of chronic septic states and purulent processes, burns, frost bytes, conjunctivitis, corneal erosion, stomatitis and other infectious diseases [1–3].

Lysozymes are widely distributed among eukaryotes and prokaryotes and can be classified into three main types: chicken (c-type), goose (g-type) and the type of invertebrates (i-type) [4, 5].

The lysozyme of c-type is most widespread; it occurs in most organisms including viruses, bacteria, plants, insects, reptiles, birds and mammals, and it is contained in a large amount in hen egg proteins.

Lysozyme is one of the first identified proteins (early 1900s). Initially, its isolation was carried out by salting out with ammonium sulfate, but the change in pH and high concentrations of salt affects the activity of the enzyme. The lysozyme isolated using ammonium sulfate was stable in acidic conditions, but only partly soluble in alkaline ones because of crystals formation [6].

In 1984 the ion exchange chromatography with carboxymethyl cellulose as a carrier was used for enzyme isolation; that was not effective in batch processes due to the small particle size and thus due to slow the flow velocity in column [7, 8].

In 2007 a group of Czech scientists offered to use the magnetic macroporous cation-exchange cellulose in isolation procedure [8]. The advantages of this process are one-stage and the purity of the obtained enzyme (more than 96%), and the main drawback is the high cost of the resin used.

Non-chromatographic methods were also used for lysozyme obtaining. Chang et al. suggested β-mercaptoethanol usage along with the other ingredients to lysozyme isolation [9]. However, this method is not widely used because of protein denaturation and β-mercaptoethanol toxicity, which limits

lysozyme usage in the food industry and medicine.

In 2006 Lu et al. suggested ultrafiltration usage to lysozyme isolation. The method allows the enzyme obtaining with purity and yield of 80%; however, it is applicable to lysozyme purification only in laboratory scale [10].

Since numerous methods for lysozyme isolation from hen egg proteins (lysozyme c), in particular chromatography, ultrafiltration, separation with reversed micelles, in two-phase systems, magnetic separation, metal-affinity precipitation, adsorption on plant waste etc., have several disadvantages, so the search, improvement and development of new methods for lysozyme obtaining are urgent tasks of biotechnology.

The aim of the research was to develop the method of lysozyme isolation from hen egg proteins.

## Materials and Methods

Fresh hen eggs, *Micrococcus lysodeikticus* 4698 cells (Sigma-Aldrich, USA) and egg protein lysozyme as a standard sample (EC 3.2.1.17) (Mw 14.4 kDa, 20 000 units / mg, AppliChem, Belgium) were used.

Lysozyme activity was determined by bacteriolytic method [11]. The amount of enzyme which reduces the optical density of *Micrococcus lysodeikticus* 4698 cell suspension by 0.001 per 1 min was taken as the unit of its activity. Protein content was monitored by Hartree-Lowry method [12].

The enzyme was isolated according to Mickelson method in our modification [13]: thoroughly washed eggs were wiped with alcohol; protein was separated from the yolk extracting chalazae. The resulting protein was diluted five times in 0.5% solution of sodium chloride, acidified to pH 4.4–4.6 and boiled for 4 min for concomitant proteins coagulation. Then the mixture was neutralized to pH 7.0–7.2 with $NaCO_3$ and the supernatant containing lysozyme was separated by centrifugation (4000 g, 20 min, 4 °C). The supernatant was dialyzed 3 times against 50 volumes of distilled water at 4 °C. At each stage, the protein content and the hydrolytic enzyme activity were controlled. The resulting solution was concentrated by reverse dialysis with dry starch and subjected to gel chromatography on fine-grained Sephadex G-50 eluting with Tris-NaCl buffer (0.05 mol/$dm^3$ of tris (hydroxymethyl) aminomethane, 0.05 mol/$dm^3$ of NaCl, pH 8.2). Protein and hydrolytic activity were determined in eluates. The lysozyme containing fraction was subjected to repeated reverse dialysis with starch yielding transparent colorless plate crystals of the enzyme. The product was stored in a sealed package at −24 °C.

The resulting enzyme were analyzed by MALDI (matrix-assisted laser desorption/ ionization) methods with mass spectrometer Autoflex II Bruker Daltonics Inc. [14, 15]. The results of mass spectrometric analysis were obtained at the Center for collective use at Chuiko Institute of Surface Chemistry of the National Academy of Sciences of Ukraine. SDS-electrophoresis in 10% polyacrylamide gel was carried out according to Laemmli system [16]. We used a set of molecular weight markers — Amersham, High-Range Rainbow Molekular Weight Markers (14 300–220 000), code RPN756. Markers: 1 — myosin (220 kDa); 2 — phosphorylase b (97 kDa); 3 — BSA (66 kDa); 4 — ovalbumin (45 kDa); 5 — carbonic anhydrase (30 kDa); 6 — trypsin inhibitor (20, 1 kDa); 7 — lysozyme (14.3 kDa).

The main physico-chemical and biochemical properties of the isolated enzyme compared with Sigma-Aldrich lysozyme, namely activity, protein content, pH optimum, thermal optimum, thermal stability, storage conditions were determined in accordance with [17].

Experimental data were subjected to statistical analysis in accordance with [18]. The confidence level of differences was evaluated at $n = 3$.

## Results and Discussion

Most of the major pharmaceutical companies (Sigma-Aldrich Pharmaceuticals Inc., Applichem et al.) use hen egg white for lysozyme isolation. This raw material is available and contains about 3.4% of lysozyme which is considered as standard. Besides lysozyme, the main egg white proteins are ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%) and ovomucin (3.5%) [19].

We have proposed a modification of Mickelson method of lysozyme isolation (Fig. 1), lie in:

– carrying out the five times (instead of 30-fold) protein dilution, followed by salting out the nondenaturated proteins with 5% solution of sodium carbonate;

– introduction of three additional stages of dialysis to remove low molecular weight contaminants;

– performing the step of reverse dialysis with dry starch to obtain the enzyme in crystalline form.

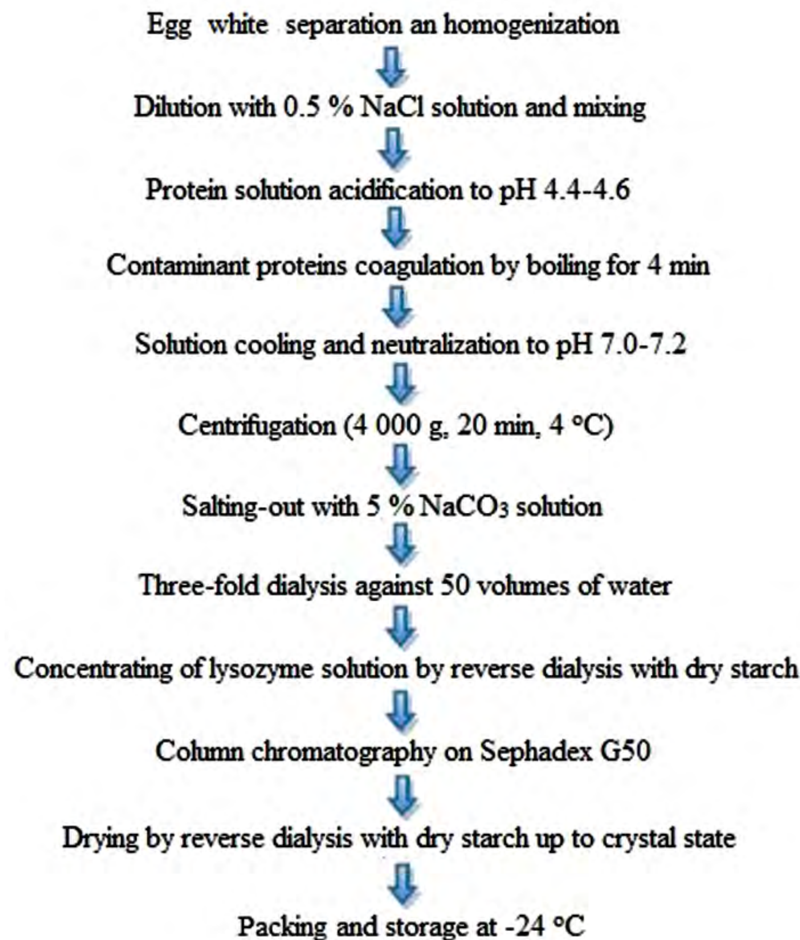


*Fig. 1.* **Diagram of lysozyme isolation from hen egg white**

The main advantages of the proposed method are ease of highly purified enzyme obtaining in the laboratory, economy, getting the product in crystalline form, high activity, and periods of storage comparable to commercial lysozyme of AppliChem firm.

The main results of the enzyme isolation are shown in Table 1. In the process of lysozyme isolation the volume and total protein content in egg, in the supernatant, after dialysis, and also the activity and yield of obtained lysozyme per 1 $cm^3$ of protein solution were determined.

It should be noted that after step of threefold dialysis against distilled water, partially purified enzyme containing, beside lysozyme, contaminants of other proteins is selected. Thus, in the mass spectrum (Fig. 2) peaks with a value of about 14 214 m/z (where m is mass of the particle, and z is its charge), 28 206 m/z, 56 682 m/z and 85 023 m/z are observed, this necessitates additional purification step.

The gel chromatography using Sephadex G-50 (Fig. 3) leads to the separation of partially purified lysozyme proteins to achieve a high degree of enzyme purity, as evidenced by the results of mass spectrometry.

MALDI spectrum of isolated enzyme showed the presence a single peak with m/z value of about 14 181 (Fig. 4) corresponding to the value of commercial lysozyme (Fig. 5). It should be noted that in the aqueous solution of a commercial enzyme, the associates formation is observed because it was lyophilized resulting in the formation of stable aggregates that are not fully degradable in solution.

According to the results of SDS-electrophoresis the most intensive band is in the molecular weight range of 12 to 17 kDa, and the purity of isolated lysozyme is 95–98% (Fig. 6).

As a result of isolating and purifying the colorless transparent plate crystals of the enzyme were obtained (Fig. 7).

The study of physical and chemical properties of commercial and isolated lysozyme

*Table 1.* **Parameters of lysozyme isolation**

| Parameters, measurement units | Indices *, $M \pm m$ |
|---|---|
| The amount of egg protein separated from one egg, $cm^3$ | 35,0 ± 5,5 |
| Total egg protein, mg | 5410,0 ± 380,6 |
| Obtained crystalline lysozyme, mg | 170,2 ± 3,0 |
| Protein content in the isolated enzyme,% | 100,0% |
| Hydrolytic activity of the obtained product, U/mg | 22025 ± 1500 |
| Yield of product,% | 3,15 ± 0,16% |

*Note.* Hereinafter * — $0.02 < P < 0.05$ compared with the control (commercial preparation) at $n = 3$.

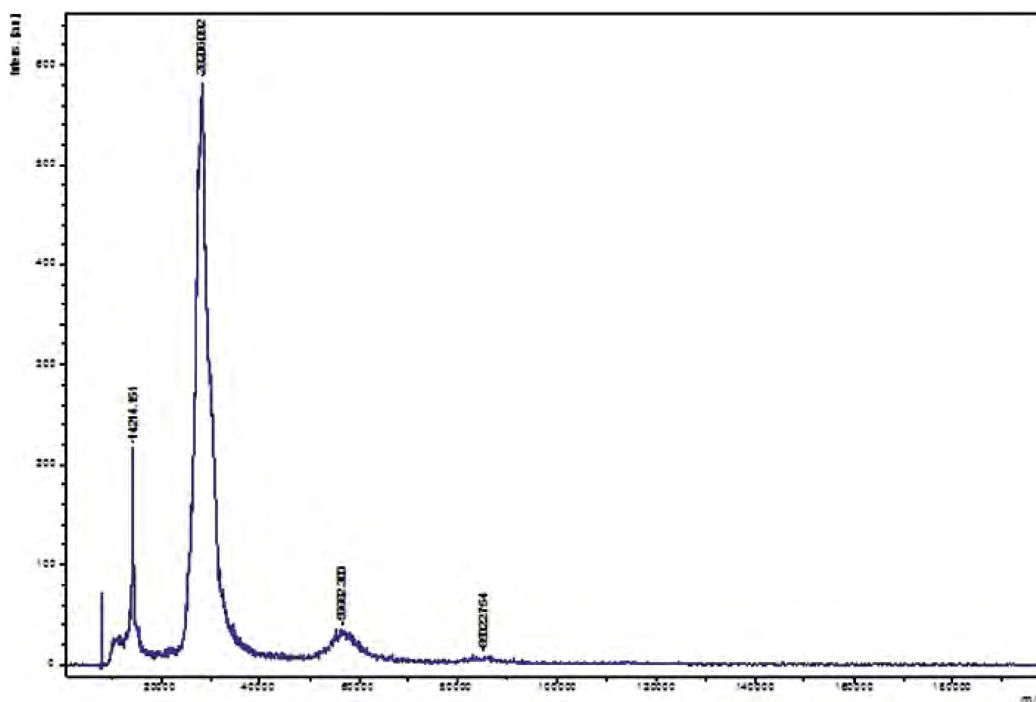

*Fig. 2.* **Mass spectrum of partially purified lysozyme after threefold dialysis step**
Hereinafter — results of a typical experiment

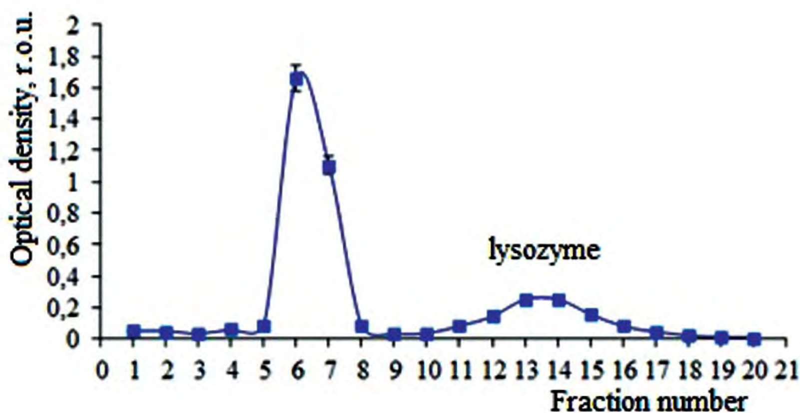


*Fig. 3.* **Chromatography of partially purified lysozyme on a column with Sephadex G-50 (0.05 mol/dm$^3$ of tris (hydroxymethyl) aminomethane, 0.05 mol/dm$^3$ of NaCl, pH 8.2)**

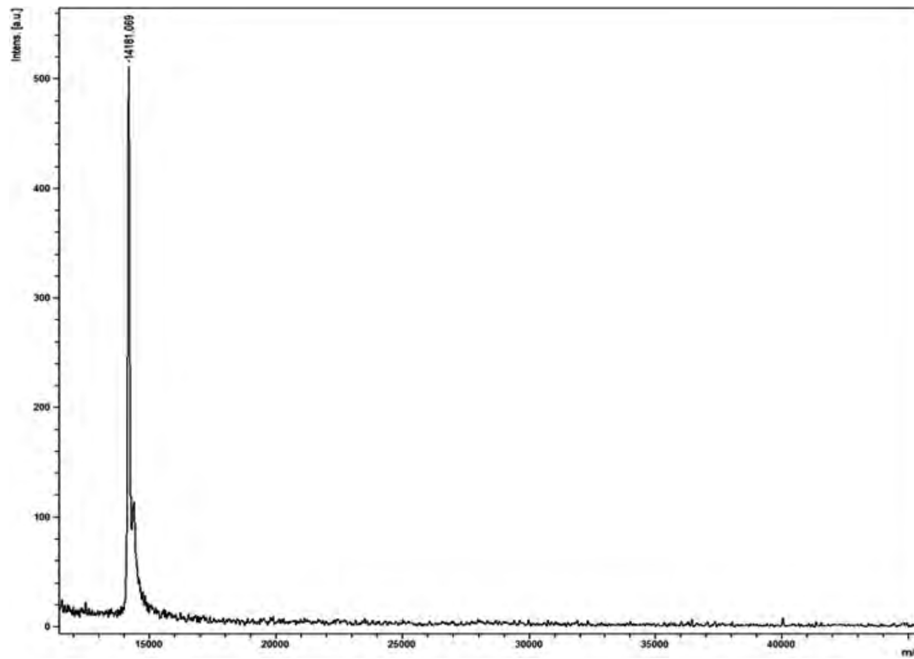


*Fig. 4.* **Mass spectrum of purified lysozyme**

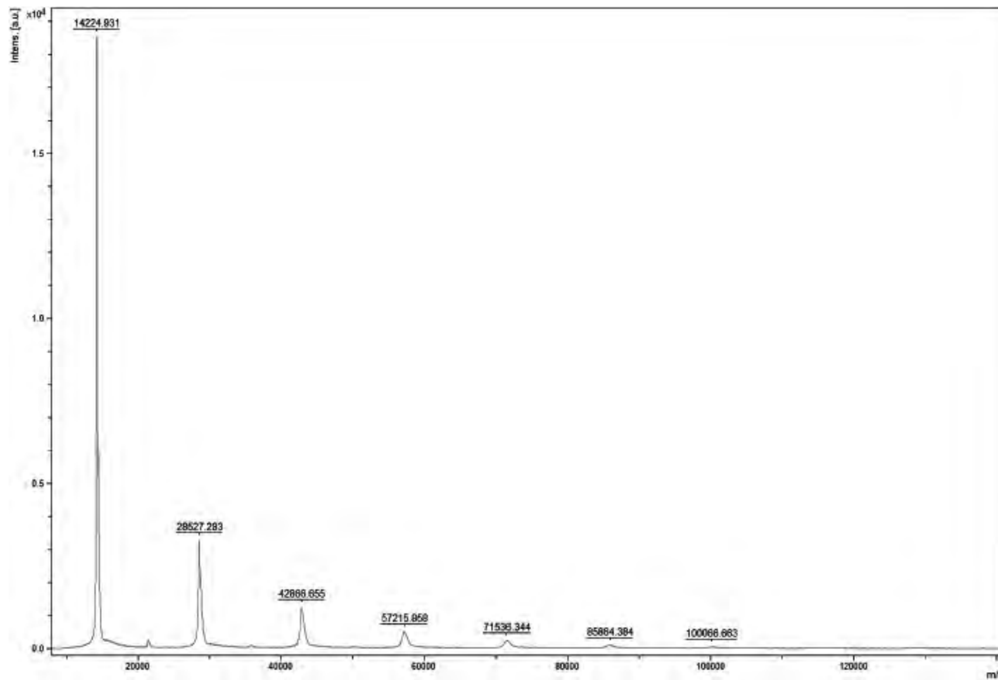


*Fig. 5.* **Mass spectrum of Sigma-Aldrich lysozyme**

shows the coincidence of bacteriolytic activity pH- and thermal optimum, similar storage periods and specific activity of the isolated enzyme (Table 2).

The resulting product retains the bacteriolytic activity for 9 months at a temperature of −24 °C. Isolated lysozyme shelf life increasing is possible using freeze-drying of the product.

Thus as a result of Michelson method modification [13], the lysozyme from hen egg protein in a yield of 3.2% and activity of 22 025 ± 1 500 U/mg is obtained. The purity of isolated enzyme (~ 95–98%) is confirmed by mass spectrometry and SDS-electrophoresis in a 10% PAAG.

The proposed method is economical and is available for implementation in the laboratory conditions; it allows obtaining a stable crystalline form of the active enzyme from hen egg proteins with a high degree of purity; and it is promising for the usage in biotechnology.

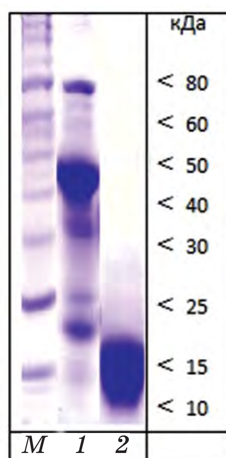


*Fig. 6*. **SDS-electrophoresis of lysozyme in 10% polyacrylamide gel:**
*M* — markers (15 µl); *1* — whole egg protein (5 µl in a slot); *2* — purified lysozyme (15 µl in a slot)

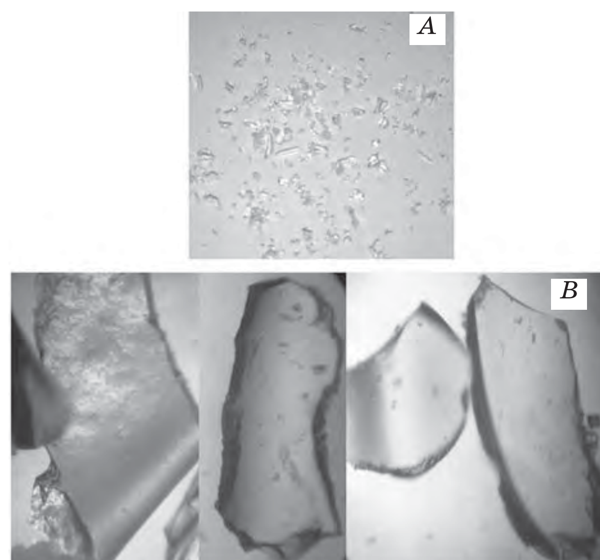


*Fig. 7*. **Microphotographs of isolated lysozyme:**
*A* ×8, *B* ×120

*Table 2*. **Characteristics of the isolated and commercial lysozyme main properties**

| Properties | Lysozyme | |
|---|---|---|
| | AppliChem | Isolated |
| Specific activity, unit/mg, $M \pm m$ | 20 000 ± 5 000 | 22 025 ± 1 500* |
| pH-optimum | 6.0 | 6.0 |
| Thermal optimum, °C | 55 °C | 55 °C |
| The constants of thermal inactivation at 80 °C, $min^{-1}$ | $5.2 \cdot 10^{-3}$ | $4.7 \cdot 10^{-3}$ |
| The shelf life of the enzyme at −24 °C, months | 12 | 9* |

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## ВИДІЛЕННЯ Й ОЧИЩЕННЯ ЛІЗОЦИМУ З ПРОТЕЇНІВ КУРЯЧОГО ЯЙЦЯ

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Метою роботи було розроблення методу виділення лізоциму з протеїну курячого яйця. Лізоцим виділяли методом диференційної денатурації протеїнів шляхом нагрівання зі зміною рН середовища, наступною нейтралізацією, діалізом і доочищенням з використанням гель-хроматографії на Sephadex G-50. Активність визначали бактеріолітичним методом (субстрат — *Micrococcus lysodeikticus* 4698), чистоту ензиму і молекулярну масу — SDS-електрофорезом і мас-спектрометрією. Модифіковано метод виділення лізоциму із протеїну курячого яйця з виходом ензиму 3,2 ± 0,2% і бактеріолітичною активністю 22 025 ± 1 500 од/мг. Згідно з даними електрофорезу виділений ензим характеризується високим ступенем чистоти (~95–98%) і за основними фізико-хімічними характеристиками подібний до лізоциму фірми AppliChem. Одержаний продукт зберігається в кристалічному стані в умовах низьких температур (–24 °C) упродовж 9 міс. Запропонований спосіб виділення дає змогу одержувати в лабораторних умовах стабільний активний лізоцим із протеїну курячого яйця з високим ступенем чистоти для використання у біотехнології.

***Ключові слова:*** протеїн курячих яєць, лізоцим.

## ВЫДЕЛЕНИЕ И ОЧИСТКА ЛИЗОЦИМА ИЗ ПРОТЕИНОВ КУРИНОГО ЯЙЦА

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Целью работы была разработка метода выделения лизоцима из протеина куриного яйца. Лизоцим выделяли путем дифференциальной денатурации протеинов нагреванием с изменением рН среды, последующей нейтрализацией, диализом и доочисткой с использованием гель-хроматографии на Sephadex G-50. Активность определяли бактериолитическим методом (субстрат — *Micrococcus lysodeikticus* 4698), чистоту энзима и молекулярную массу — SDS-электрофорезом и масс-спектрометрически. Модифицирован метод выделения лизоцима из протеина куриного яйца с выходом энзима 3,2 ± 0,2% и бактериолитической активностью 22 025 ± 1 500 ед/мг. Согласно данным электрофореза выделенный энзим характеризуется высокой степенью чистоты (~95–98%) и по основным физико-химическим характеристикам сравним с лизоцимом фирмы AppliChem. Полученный продукт хранится в кристаллическом состоянии в условиях низких температур (–24 °C) на протяжении 9 мес. Предложенный способ выделения позволяет получать в лабораторных условиях стабильный активный лизоцим из протеина куриного яйца с высокой степенью чистоты для использования в биотехнологии.

***Ключевые слова:*** протеин куриных яиц, лизоцим.