Journal of Chemistry and Technologies, 2021, *29*(3), 410-416

Journal of Chemistry and Technologies

pISSN 2663-2934 (Print), ISSN 2663-2942 (Online).

journal homepage: http://chemistry.dnu.dp.ua

UDC 577.15:582.683.2

ISOLATION OF LYSOZYME OF BLACK SEA MUSSEL MYTILUS GALLOPROVINCIALIS

Galyna V. Krusir[1*], Valentina G. Zakharchuk[2], Elena V. Sevastyanova[1], Liudmyla N. Pylypenko[1], Kseniia I. Mazurenko[1]

[1]Odessa National Academy of Food Technologies, St. Kanatna, 112, Odessa, 65039 Ukraine,
[2]Odessa National Economic University, str. Preobrazhenskaya 8, Odessa, Ukraine 65000

Received 17 June 2021; accepted 2 October 2021; available online 27 October 2021

Abstract

The analytical review showed the promising use of lysozyme as a valuable natural antibacterial drug. The eperimental monitoring of natural sources of drugs with antibacterial activity showed the feasibility of using secondary raw materials from water resources of the Black Sea basin, obtained during the mussels processing. The isolation and chromatographic purification of lysozyme was carried out. Only the samples eluted with 0.6 M NaCl solution in 0.02 M acetate buffer, pH 5.0, were found out to exhibit lytic activity. The fraction possessinglytic activity was 54 cm³ and corresponded to the elution with 0.6 M solution of NaCl in 0.02 M acetate buffer, pH 5.0. The lysozyme under study, according to the results of gel electrophoresis in 15% PAH using the calculated calibration curve, is characterized by a molecular weight of 18 kDa, which confirms its belonging to low molecular weight proteins. A preparation of lysozyme with a specific activity of 206 u/mg with a molecular weight of 18 kDa and purification degree of 946.6 was obtained from the juice of Black Sea mussels. The antibacterial activity of the obtained lysozyme preparation against pathogens of food poisoning and food spoilage was studied. The data obtained allow us to predict the prospects of using mussels as a source of highly active lysozyme.

Key words: lysozyme, isolation, Black Sea mussels, secondary raw materials, chromatographic methods, gel electrophoresis, antibacterial activity.

ВИДІЛЕННЯ ЛІЗОЦИМУ ЧОРНОМОРСЬКОЇ МІДІЇ MYTILUS GALLOPROVINCIALIS

Галина В. Крусір[1*], Валентина Г. Захарчук[2], Олена В. Севастьянова[1], Людмила М. Пилипенко[1], Ксенія І. Мазуренко[1]

[1]Одеська національна академія харчових технологій, вул. Канатна, 112, Одеса, 65039 Україна,
[2]Одеський національний економічний університет, вул. Преображенська 8, Одеса, Україна 65000

Анотація

Аналітичний огляд показав перспективність використання лізоциму як цінного природного антибактеріального препарату. Експериментальний моніторинг природних джерел препаратів з антибактеріальною активністю виявив доцільність використання вторинної сировини з водних ресурсів чорноморського басейну, отриманого в процесі переробці мідій. Проведено виділення і хроматографічне очищення ферментного препарату лізоциму. Визначено, що тільки зразки, елюйовані 0.6 М розчином NaCl в 0.02 М ацетатному буфері, pH 5.0, виявляють літичну активність. Фракція з літичною активністю склала 54 см³ при елююванні 0.6 М розчином NaCl в 0.02 М ацетатному буфері, pH 5.0. Лізоцим, досліджений за результатами гель-електрофорезу в 15 % ПААГ з використанням калібрувальної кривої, характеризується молекулярною масою 18 кДа, що підтверджує його приналежність до низькомолекулярних білків. Отримано препарат лізоциму з соку чорноморських мідій з питомою активністю 206 од / мг, молекулярна маса якого становить 18 кДа, ступінь очищення – 946.6. Досліджено антибактеріальну активність отриманого препарату лізоциму до збудників харчових отруєнь і псування харчових продуктів. Отримані результати дозволяють прогнозувати перспективи використання мідій як джерела високоактивного лізоциму.

Ключові слова: лізоцим, виділення, чорноморські мідії, вторинна сировина, хроматографічні методи, гель-електрофорез, антибактеріальна активність.

**Corresponding author: e-mail address: krussir.65@gmail.com*

doi: 10.15421/jchemtech.v29i3.223378

Journal of Chemistry and Technologies, 2021, *29*(3), 410-416

ВЫДЕЛЕНИЕ ЛИЗОЦИМА ЧЕРНОМОРСКОЙ МИДИИ MYTILUS GALLOPROVINCIALIS

Галина В. Крусир[1*], Валентина Г. Захарчук[2], Елена В. Севастьянова[1], Людмила Н. Пилипенко[1], Ксения І. Мазуренко[1]

[1]Одесская национальная академия пищевых технологий, ул. Канатная, 112, Одесса, 65039 Украина,
[2]Одесский национальный экономический университет, ул. Преображенская 8, Одесса, Украина 65000

Аннотация

Аналитический обзор показал перспективность использования лизоцима как ценного природного антибактериального препарата. Экспериментальный мониторинг природных источников препаратов с антибактериальной активностью выявил целесообразность использования вторичного сырья из водных ресурсов Черноморского бассейна, полученного при переработке мидий. Проведено выделение и хроматографическая очистка ферментного препарата лизоцима Было определено, что только образцы, элюированные 0.6 М раствором NaCl в 0.02 М ацетатном буфере, pH 5.0, проявляют литическую активность. Фракция с литической активностью составила 54 см^3 и соответствует элюированию 0.6 М раствором NaCl в 0.02 М ацетатном буфере, pH 5.0. Лизоцим, изученный по результатам гель-электрофореза в 15 % -ном ПААГ с использованием калибровочной кривой, характеризуется молекулярной массой 18 кДа, что подтверждает его принадлежность к низкомолекулярным белкам. Получен препарат лизоцима из сока черноморских мидий с удельной активностью 206 ед / мг, молекулярная масса которого составляет 18 кДа, степень очистки – 946.6. Исследована антибактериальная активность полученного препарата лизоцима к возбудителям пищевых отравлений и порчи пищевых продуктов. Полученные данные позволяют прогнозировать перспективы использования мидий как источника высокоактивного лизоцима.

Ключевые слова: лизоцим, выделение, черноморские мидии, вторичное сырье, хроматографические методы, гель-электрофорез, антибактериальная активность.

Introduction

It is known that during the storage of ready-to-eat foods various physical, chemical and microbiological changes occur, affecting their quality indicators and shelf life. Food products are often exposed to surface contamination, leading to a reduction in shelf life [1–3]. The main cause of food spoilage is the microbial contamination of their surface. The antibacterial film acts as a protective barrier that prevents microbial spoilage [4–5]. If an antibacterial agent is included in the film, the shelf life of the food product is increased by creating a microenvironment in the film that delays or prevents the growth of microorganisms on the surface of the product. The antibacterial agents are incorporated into the edible films and are deposited onto the surface of the food product. The protective film serves as a barrier to hydrolytic deterioration of the product by reducing the access of water to the product, as well as oxidative damage due to reduced oxygen exposure. These coatings are effective, inexpensive and protect the product even if the seal of the package is broken. The application of antibacterial preparations is carried out either by introducing into the packaging material, or directly on the surface of the food product [1; 2; 4; 5].

The use of edible packaging leads to the suppression of the growth of pathogenic microflora, the ability to control gas exchange, which helps to prevent oxidative damage to food sensitive components, improve the structural and mechanical properties of food products, preserve the sensory properties of products (color, flavor, etc.) [2; 4; 6; 7].

The use of lysozyme as an antibacterial agent in active (edible) packaging is extremely relevant [1; 5]. Lysozymes (mucopeptide N-acetylmuramyl hydrolase (EC 3.2.1.17)) are antibacterial enzymes that catalyze the breaking of the β-1–4-glycosidic bond between N – acetylmuramic acid and N – acetylglucosamine peptidoglycan, the components that form the bacterial cell walls.

The use of microbial, plant material, insects, and animal tissues is known as sources of lysozyme [5; 8–12]. Native g-, c-, and i-type lysozymes were identified in the different tissues of worm-like-marine animal-providing amphioxus [10]. The classification features of lysozymes are the sources of their production, amino acid sequence and the resulting three-dimensional structures of protein molecules. The main types of lysozymes are presented in table 1.

Table 1

Types of lysozyme

A type	Source	Literary sources
c-type (chicken)	Chicken Egg Protein	[8; 11]
g-type (goose)	Birds	[11; 13]
h-type (hevea type)	Plants	[14]
b-type (barley type)	Plants	[14]
i-type (type of invertebrate animals)	Mollusks, insects	[12; 15]
g-type, c-type, and i-type (type of invertebrate animals)	Amphioxus Branchiostoma japonicum	[10]

In recent years, significant interest has been formed and is expanding towards a new type of lysozyme belonging to the invertebrate type (i-type) [12; 16]. One of the first studies of invertebrate lysozyme, which proved the existence of highly active lysozyme in the oyster lymph of Crassostrea virginica, is the work of McDade and Trip [16]. Later, Jols and Jols [15; 17] isolated, purified, and partially determined the lysozyme structure from the mollusk Asterias rubens.

The biochemical properties of bivalve mollusks purified lysozymes are diverse and differ from those of chicken egg white lysozyme [18]. The enzymatic properties of lysozyme are confirmed if the enzyme has the ability to:

1) reduce the density of isolated structures of the cell walls;

2) catalyze the hydrolysis of intact bacterial cells;

3) release complex groups and the acetylaminosaccharide complex of glucosamine and hexosamine and muramic acids [19; 20].

It is known that the lytic activity of bivalve mollusks lysozyme is comparable to that of the most studied chicken egg lysozyme, and, according to some literature, exceeds the activity of the latter. Therefore, it was of interest to develop a method for isolating lysozyme from mussel juice, a waste product of Black Sea mussels processing, a liquid that remains after the separation of mussel carcasses from the cusps and is a mixture of liquid and plasma, in order to use it later as an antibacterial agent of active coatings. The aim of the study was the isolation and purification of the lysozyme of Black Sea mussels Mytilus galloprovincialis and estimation of the antibacterial activity of the obtained lysozyme preparation against causative agents of food poisoning and food spoilage.

Experimental

The mussel juice was obtained by manual cleaning the shells of mussels. The initial volume of mussel juice, comprising 100 cm^3, was centrifuged at 4000 rpm for 30 minutes in order to remove fragments of shells, tissue parts and mussel meat. Lysozyme activity was determined spectrophotometrically [21; 22], the Lowry protein was modified by Hartree [23]. The supernatant was chromatographed on a KM – Sepharose column (2.6 x 35 cm) at a rate of 6 cm^3 / min, previously equilibrated with 0.02 M acetate buffer, pH 5.0. The column was washed sequentially with water, 0.1, 0.3 and 0.6 M NaCl solution in 0.02 M acetate buffer, pH 5.0 at a rate of 6 cm^3 / min. The elution was controlled by determining the optical density at 280 nm.

The main stages of the lysozyme isolation from the mussel juice are given in table 2, from which it follows that the juice with a protein concentration of 0.66 mg / cm^3 and a specific activity of 206 u / mg was purified to a protein concentration of 0.17 mg / cm^3 with a specific concentration of 195.000 u / mg.

Table 2

Isolation of lysozyme from mussel juice (100 cm^3 of juice (0.5 kg of mussels))

Stage selection	Volume, cm^3	LA, u / cm^3	Protein, mg / cm^3	Total protein, mg	Total LA, units	Specific LA, units / mg	Degree of purification	Output, %
Juice	100	136	0.66	66	13596	206	1.0	100
Ion-exchange chromatography on KM – Sepharose I	90	2666	0.18	16	240000	15000	72.8	1765.2
Ion-exchange chromatography on KM – Sepharose II	54	34306	0.17	9.5	1852500	195000	946.6	13625

The results of determining the optical density of the collected eluate samples at 280 nm during chromatography using KM – Sepharose as a carrier are shown in Fig. 1.

From the results of chromatography on the KM-Sepharose column shown in Fig. 1, we can see that the elution curve is accompanied by four peaks: the first peak corresponds to the elution with water, the second with 0.1 M NaCl solution in 0.02 M acetate buffer, pH 5.0, the third with 0.3 M NaCl solution in 0.02 M acetate buffer, pH 5.0 and the fourth with 0.6 M NaCl solution in 0.02 M acetate buffer, pH 5.0. It was determined that only the samples eluted with a 0.6 M NaCl solution in 0.02 M acetate buffer, pH 5.0, showed lytic activity.

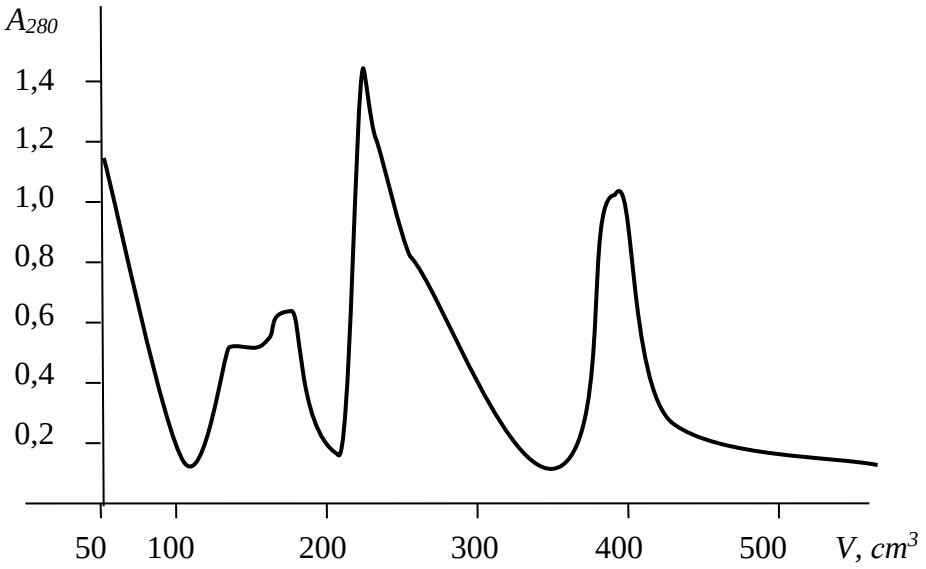

Fig. 1. The outgoing chromatographic curve of the lysozyme from mussel juice on KM-sepharose

These eluted samples were collected (total fraction volume was 90 cm^3) and subjected to secondary chromatography on a KM – Sepharose column under the identical chromatographic conditions. The results of the fraction IV secondary chromatography are presented in Fig. 2.

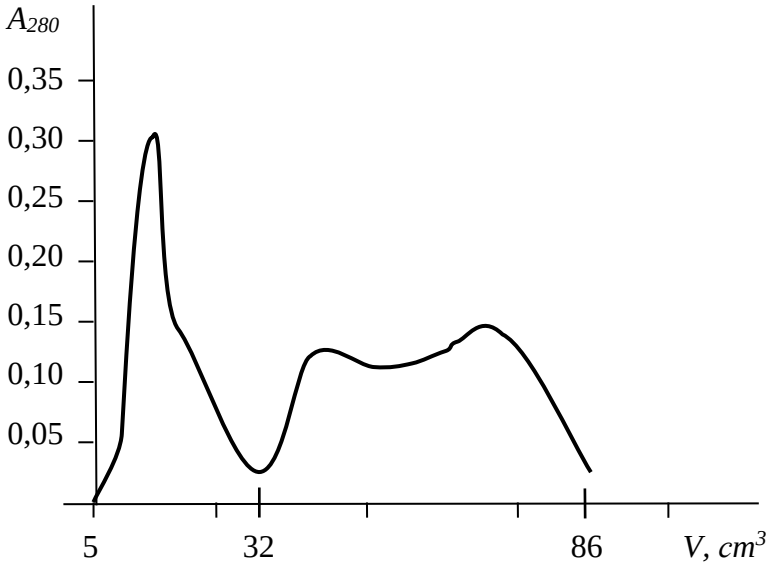

Fig. 2. The yield curve of the lysozyme fraction IV on KM-sepharose

From the results shown in Fig. 2, we can conclude that the fraction with lytic activity was 54 cm^3 and corresponds to the elution with a 0.6 M solution of NaCl in 0.02 M acetate buffer, pH 5.0 (peak II)

The lysozyme under study, in accordance with the results of gel electrophoresis in 15% PAGE using the calculated calibration curve (Fig. 3, 4) is characterized by a molecular weight of 18 kDa, which confirms its belonging to low molecular weight proteins.

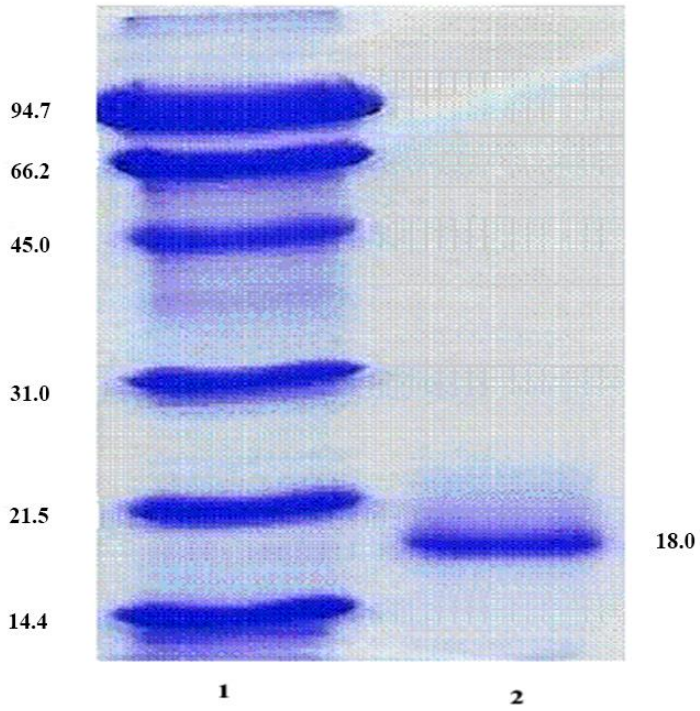

Fig. 3. The electrophoregram of the Black Sea mussel lysozyme (determination of molecular weight): 1 – markers; 2 – sample of lysozyme

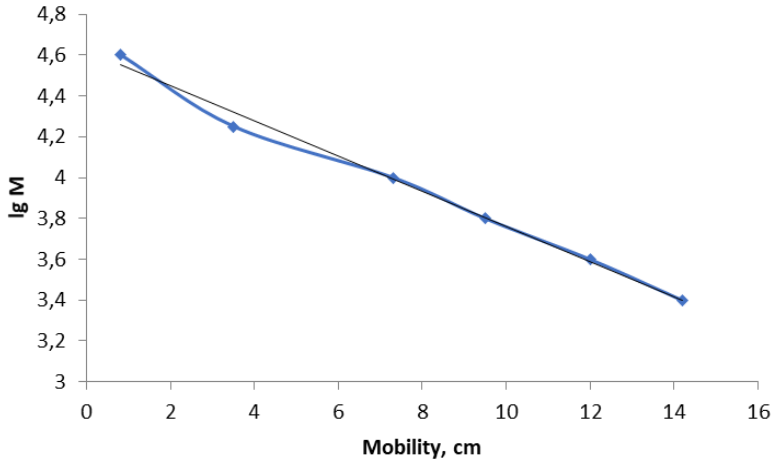

Fig. 4. The calibration curve for calculating the molecular weight of the Black Sea mussel lysozyme.

The results of the study of the antimicrobial activity of the lysozyme preparation obtained from the juice of the Black Sea mussels are shown in Table 3 (s – microorganisms sensitive; i – microorganisms with intermediate sensitivity; r – microorganisms stable).

Table 3

Sensitivity of microorganisms to lysozyme of various origins

Type of microorganism	Lysozyme isolated from mussel juice	Lysozyme isolated from chicken egg protein
Bacillus cereus ATCC 11778	s	s
Bacillus cereus ATCC 14579	s	-
Paenibacillus macerans B-5803^T	s	s
Paenibacillus polymyxa B-5760^T	s	i
Bacillus thuringiensis IMV 7173	i	i
Bacillus cereus P90-9 (from eggplant)	s	s
Bacillus cereus P90-1 (from carrots)	i	r
E.coli ONU 90 (UCM B-906)	s	s
Staphylococcus aureus ONU 443 (ATCC 6538)	s	s
Pseudomonas aeruginosa ONU 441	i	r
E.coli ONU 447 (ATCC 25922)	s	s

The isolated and identified types of microorganisms that make up the epiphytic microflora of food raw materials, as well as collection strains of microorganisms that are causative agents of food spoilage and food poisoning in humans were used as test cultures [3; 24; 25]. The lysozyme isolated from the juice of mussels exhibits a pronounced antimicrobial effect against both bacillary microorganisms and non-spore-bearing microorganisms.

Conclusions

Thus, the lysozyme preparation was obtained from the Black Sea mussel juice with a specific activity of 206 u / mg, the molecular weight of which is 18 kDa, and the purification degree is 946.6. A higher antimicrobial activity of the mussel juice lysozyme was found compared to the lysozyme isolated from chicken egg protein against *Pseudomonas aeruginosa*, *Bacillus cereus* P90-1 isolated from carrots, *Paenibacillus polymyxa* B-5760^T. The data obtained make it possible to predict the prospects of using mussels as a source of highly active lysozyme.

References

[1] Cha, D.S., Shinnan, M.S. (2004). Biopolymer-based packaging: a review. *Crit. Rev. Food Sci. Nutr.*, *44*, 223–237.

[2] Barbiroli, A., Bonomi, F., Capretti, G., Iametti, S., Manzoni, M., Piergiovanni, L., Rollini, M. (2012). Antimicrobial activity of lysozyme and lactoferrin incorporated in cellulose-based food packaging. *Food Control*, *26*(2), 387-392. https://doi.org/10.1016/j.foodcont.2012.01.046

[3] Danylova, O., Serdyuk, M., Pylypenko, L., Pelykh, V., Lopotan, I., Yegorova, A. (2019). Screening of Agricultural Raw Materials and Long-term Storage Products to Identify Bacillary Contaminants. *Modern Development Paths of Agricultural Production*, 641-654. https://doi.org/10.1007/978-3-030-14918-5_63

[4] Chawla, R., Sivakumar, S., Kaur, H. (2021). Antimicrobial edible films in food packaging: Current scenario and recent nanotechnological advancements: a review. *Carbohydrate polymer technologies and applications*, *2*, 100024. https://doi.org/10.1016/j.carpta.2020.100024

[5] Dekina, S.S. (2020). Biotekhnologhija mukoadghezyvnykh lizocym-polimernykh system medychnogho pryznachennja: dys. d-ra biol.nauk. 265. https://ela.kpi.ua/handle/123456789/35577

[6] Masschalck, B., Michiels, C.W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit. Rev. Microbiol.*, *29*, 191-214. https://doi.org/10.1080/713610448

[7] Proctor, V.A., Cunningham, F.E., Fung, D. Y. C. (1988). The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Crit. Rev. Food Sci. Nutr.*, *26*(4), 359-395. https://doi.org/10.1080/10408398809527473

[8] Gálvez-Iriqui, A.C., Plascencia-Jatomea, M., Bautista-Baños, S. (2020). Lysozymes: characteristics, mechanism of action and technological applications on the control of pathogenic microorganisms. *Rev. mex. fitopatol.*, *38*(3), 1-24. https://doi.org/10.18781/r.mex.fit.2005-6

[9] Benkerroum, N. (2008). Antimicrobial activity of lysozyme with special relevance to milk. *African Journal of Biotechnology*, *7*(25), 4856-4867. http://www.academicjournals.org/AJB.

[10] Xu, N., Pan, J., Liu, S., Xue, Q., Zhang, S. (2014). Three in one: Identification, expression and enzymatic activity of lysozymes in amphioxus. *Dev. Comp. Immunol.*, *46*(2), 508-517. https://doi.org/10.1016/j.dci.2014.06.007

[11] Prager, E.M., Jollès, P. (1996). Animal lysozymes c and g: an overview. *EXS.*, 75, 9-31. https://doi.org/10.1007/978-3-0348-9225-4_2.

[12] Xue, Q., Hellberg, M.E., Schey, K.L., Itoh, N., Eytan, R.I., Cooper, R.K., La Peyre J.F.(2010). A new lysozyme from the eastern oyster, Crassostrea virginica, and a possible evolutionary pathway for i-type lysozymes in bivalves from host defense to digestion. *BMC evolutionary biology*, *10*, 213. https://doi.org/10.1186/1471-2148-10-213

[13] Debeaufort, F., Quezada–Gallo, J.A. (1998). Voilley Edible films and coatings: tomorrow's packagings: a review. *Crit. Rev. Food Sci. Nutr.*, *38*(4), 299–313. https://doi.org/10.1080/10408699891274219

[14] Beintema, J.J., Terwisscha van Scheltinga, A.C. (1996). Plant lysozymes. *EXS.*, *75*, 75–86.

[15] Bachali, S., Bailly, X., Jollès, J., Jollès, P., Deutsch, J. S. (2004). The lysozyme of the starfish Asterias rubens. A paradygmatic type i lysozyme. *Eur J Biochem.*, *271*(2), 237-242. https://doi.org/10.1046/j.1432-1033.2003.03915.x

[16] Itoh, N., Okada, Y., Takahashi, K.G., Osada, M. (2010). Presence and characterization of multiple mantle lysozymes in the Pacific oyster, Crassostrea gigas. *Fish Shellfish Immunol.*, *29*(1), 126-135. https://doi.org/10.1016/j.fsi.2010.02.027

[17] Jolles, P. (1996). From the discovery of lysozyme to the characterization of several lysozyme families. *EXS., 75*, 3–5.

[18] McHenery, J.G., Birkbeck, T.H. (1982). Characterization of the lysozyme of Mytilus edulis (L). *Comp. Biochem. Physiol., 71*, 583–589. https://doi.org/10.1016/0305-0491(82)90466-7

[19] Bukharin, O.V., Valyshev, A.V. (2006). Microbial inhibitors of lysozyme. *Zhurnal mikrobiologii, èpidemiologii i immunobiologii. 4*, 8-13.

[20] Goncharova, A.I., Okulich, V.K., Zemko, V.Yu., Senkovich, S.A. (2019). Antimikrobnaya aktivnost lizotsima kak faktor nespetsificheskoy rezistentnosti. *Vestnik VGMU*, *18*(4), 40-45. https://doi.org/10.22263/2312-4156.2019.4.40.

[21] Solovykh, G. N. Sposob opredeleniya lizotsimnoy aktivnosti biologicheskikh obektov: pat. 2294373. Rossiya: MPK C12Q 1/02 / – № 2005103265/13; zayavl. 20.07.06; opubl. 27.02.07, Byul. № 6.

[22] Mine, Y., Ma, F., Laurian, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *J. Agric. Food Chem., 52*, 1088–1094. https://doi.org/10.1021/jf0345752

[23] Komsa-Penkova, R., Spirova, R., Bechev, B. (1996). Modification of Lowry's method for collagen concentration measurement. *Journal of Biochemical and Biophysical Methods, 32*(1), 33-43. https://doi.org/10.1016/0165-022X(95)00046-T

[24] Pylypenko, L.M., Pylypenko, I.V., Danylova, O.I., Ilieva, O.S., Ivanytsia, V.O., Yamborko, G.V. Sposib vyznachennja bacyljarnykh zbudnykiv kharchovykh otrujenj ta psuvannja kharchovykh produktiv: pat. UA 117644. MPK G01N 33/02 (2006.01) C12Q 1/68 (2018.01) C12N 15/31 (2006.01) C12R 1/00 (2006.01); zayavl. 12.16.17; opubl. 28.08.18, Byul. № 16.

[25] Pylypenko, L.M., Pylypenko, I.V., Yamborko, G.V., Marinova, I.V. (2017). Toxin production ability of *Bacillus cereus* strains from food product of Ukraine. *Food Science and Technology*, *11*(3), 93-98. https://doi.org/10.15673/fst.v11i3.612