Investigation of the ferric ions complexes with bioligands of probiotic origin

Antonina Kapustian, Natalia Cherno

Odesa National Academy of Food Technologies, Odesa, Ukraine

Abstract

Keywords:

Ferric ion
Chelate
Bioligand
Peptidoglycan
Muropeptide

Article history:

Received 12.06.2019
Received in revised
form 23.12.2019
Accepted 30.03.2020

Corresponding author:

Antonina Kapustian
E-mail:
fst.journal@ukr.net

DOI:

10.24263/2304-
974X-2020-9-1-5

Introduction. The peculiarities of obtaining stable, easily digestible and safe ferric ion complexes with bioligands of the probiotic origin and their characteristics have been investigated.

Materials and research methods. As bioligands, the products of metabolism and processing of biomass *Lactobacillus delbrueckii subsp. Bulgaricus B-3964*, namely, lactic acid, amino acids, low molecular weight peptides and muropeptides, were used. The destruction of biomass peptidoglycans was performed by alternating treatment of sonication and papain. The progress of complexation was monitored using the turbidimetry method.

Results and discussion. The destruction of biomass peptidoglycans, performed by alternating sonication and the papain enzyme preparation, resulted in a mixture of amino acids, low molecular weight peptides and muropeptides, the concentration of which was 10.24 mg/cm³, 6.45 mg/cm³ and 2.25 mg/m³ respectively. Lactic acid was isolated from the culture fluid by crystallization of calcium lactate. Three polydentate systems were used to form Fe³⁺ complexes: peptidoglycan destruction products; lactic acid; a mixture of degradation products and lactic acid. It was found that the bioligand systems under study bind Fe³⁺ ions in the amounts of 32, 40 and 46 mol/dm³·10⁻² respectively. Electrostatic and coordination interactions are involved in the formation of the complex of iron (III) and probiotic bioligands. The behavior of complexes at different pH values and temperatures was studied. Most stable is the complex formed with the participation of the bioligands system containing the products of peptidoglycans degradation and lactic acid. Such system of bioligands provides the formation of ferric ions chelate complexes, stable in the pH range of 1–10 units. Obtained complex is a promising ingredient of dietary supplements and dietetic products whose technology involves high-temperature processing. That was proven that, the complex is stable in the temperature range of 20–122 °C. When the temperature reaches 122–125 °C, the mass loss is 3% for the complex and 14% for the mechanical mixture. In the temperature range of 122–178 °C, an endothermic reaction is observed during the thermal treatment of the complex, and no thermal effects are observed during the mechanical mixture treatment. The weight loss of the complex in this temperature range is 22%, mechanical mixture – 16%. The presence of an endothermic peak on the differential thermal analysis curve of the complex may indicate the presence in its structure of chelate bonds, and during their destruct the enthalpy changes are occurred.

Conclusion. The research results indicate the effectiveness of the use of polydendant mixed ligand systems of probiotic origin for complexation with ferric ions.

Introduction

Iron is one of the most important trace elements that is part of more than 100 enzymes of the human body, participates in respiration, hematopoiesis, immunobiological processes, redox reactions, etc. [1]. Insufficient iron supply to the body can provoke iron deficiency anemia (IDA). It is a disease of the blood system caused by iron deficiency in the body [1, 2]. IDA is accompanied by changes in metabolism parameters, a decrease in hemoglobin concentration, which can lead to disorders of the cardiovascular, nervous system, decrease in women's reproductive function, changes in intelligence and behavioral moods, chronicity of various diseases, etc. [3, 4].

Analysis of recent research and publications

As is known [1], food contains 2 types of iron – heme (red meat) and non-heme, or ionized (vegetables, fruits, cereals). Moreover, non-heme iron covers 70% of the human body's need for iron.

The entry of iron into the body occurs through the intestinal mucosa, where it is adsorbed by food enterocytes (the common name of epithelial cells lining the intestinal mucosa, which are highly specialized cells that coordinate the absorption and transport of iron). The absorption of iron in the intestine occurs in three main ways: with the transporter of divalent cations, as part of the mobilferrin integrated complex, as well as a special way for the absorption of heme iron from food (Figure 1) [2, 3].

Figure 1. Scheme of iron transport in the enterocyte [2]

Inorganic, organic and multivitamin preparations in oral form are usually used to replenish iron deficiency (Figure 2) [4].

Figure 2. Classification of iron preparations [4]

The use of inorganic preparations in the ferrous form (Fe^{2+}) can provoke a number of complications. In the gastrointestinal tract, the ferrous ion is oxidized to the ferric-ion, inducing the appearance of a free electron, resulting in the generation of free radicals that disrupt DNA synthesis, affect the activity of several enzymes, cause the peroxidation of polyunsaturated lipids in cell membranes [4]. Moreover, increased concentrations of ferrous iron in the intestine contribute to the its passive transport of into enterocytes, which leads to the entry of toxic ions into the cells of the intestinal mucosa and can cause their death. Direct cytotoxic effect of ferrous ions on cells of different organs, especially brain and liver, was also noted [3]. To counteract these disadvantages, to ferrous drugs additionally include antioxidants (such as ascorbic acid), or fix Fe^{2+} ions on special matrices that cause their prolonged release [4].

Ferric ion salts (Fe^{3+}) have better solubility and higher elemental iron content than ferrous salts, but Fe^{3+} has a higher charge/radius ratio than Fe^{2+} ion, which causes a significant degree of hydrolysis of iron (III) salts [2, 5]. During the hydrolysis of salts of ferric ions, the formation of insoluble $\text{Fe}(\text{OH})_3$ hydroxide occurs. Thus, inorganic agents containing Fe^{3+} are more likely to be converted to ferric hydroxide in an alkaline medium of the intestine, which makes it impossible for them to absorb. Such the ferric properties complicate its use as an oral agent for the correction of IDA [2, 4].

Organic iron compounds are more widely used as a means of correcting iron deficiency [4, 5]. Thus, the use of an organic Fe^{2+} complex with two nicotinamide molecules is known [4]. Ferum (II) salts and carboxylic acids, namely fumaric, lactic, gluconic, have also been used as they have been shown to have protective properties similar to those of ascorbic acid. The problem of introduction of ferric ion into the body was solved in the group of chelating agents. There is an agent based on the chelate complex of ferum protein succinylate, in which the ligands provide the stability of the ferric ion, and the stability constants are not less than in the ferric hydroxide. This provides preservation of Fe^{3+} in a soluble state in the intestinal lumen and increases the likelihood of its absorption [4, 6].

There are agents to combat IDA based on insoluble iron oxide in colloidal solution. In the micelles of such a colloidal solution, insoluble $\text{Fe}(\text{OH})_3$ was used as the nucleus of the micelles, and the dense layer was created by carbohydrate compounds, such as maltose, sucrose, sorbitol, isomaltose [4].

Despite some advances in the development of effective anti anemia agents, the search for new, more perfect forms of iron for oral use is still ongoing [7, 8]. Since iron in the living body is always in the composition of bioorganic complexes with amino acids, proteins and other bioligands [2, 4], the use of mixed ligand systems to obtain safe soluble easily digestible forms of non-toxic ferric ions is worthy of attention [9]. The study of mixed ligand complexes of biometals has become widespread, the methods of obtaining and characteristics of some of them are described in the literature [10–12]. Both natural and synthetic compounds are used as bioligands, but there is no information in the literature on the possibility of using metabolism products and processing of probiotic bacteria to produce mixed ligand systems containing iron [13–15]. Despite the extensive experience and production of probiotic cultures cultivation, this idea is very relevant [16–17]. The culture fluid contains a large number of metabolites, in particular organic acids, capable of chelation with biometals [18, 19]. With the disintegration of probiotic cells, degradation products of the peptidoglycans of their cell walls can be obtained, namely, amino acids, low molecular weight peptides, mucopeptides, which also contain functional groups capable to form ionic and coordination bonds with metal ions. In addition, the substances of the mucopeptide series have their own physiological activity – they are powerful immunotropic compounds [20–21].

The purpose of this work is obtaining and characteristic of the ferric ions complexes with metabolites and low molecular weight degradation products of peptidoglycans of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964*.

Materials and methods

Materials

Biomass (BM) of lactic acid bacteria *Lactobacillus delbrueckii subsp Bulgaricus B-3964* with a concentration of $4.8 \cdot 10^9$ CFU/cm³ from the collection of the Scientific and Production enterprise Ariadna (Odesa), papain with proteolytic activity of 10 Un/mg (Swanson Health Products, USA), FeCl₃·6H₂O h (China) were used for the research.

Obtaining degradation products of peptidoglycans *Lactobacillus delbrueckii subsp. Bulgaricus B-3964*

The isolation of cells from the culture fluid was carried out by centrifugation for 15 min at 8000 min⁻¹. The precipitated cells were washed with distilled water, resuspended and carried out the ultrason treatment using ultrasonic baths “PSB-1335-05” with a working frequency of 40 kHz, duration of the treatment was 300 s. The enzymatic destruction of BM cell walls was carried out by papain treatment at 37 °C and pH = 7.4. The enzyme: substrate ratio (BM solids content) was 1:200, the incubation time of the reaction mixture was 300 min. The enzymatic hydrolysis was stopped by emergency heating to a temperature of 100 °C, the mixture was cooled, the liquid phase was separated from the solid by centrifugation for 10 min at 8000 min⁻¹. In the liquid phase, the content of free amino acids was controlled by formol titration method [22]. The content of low molecular weight peptides (LMWP) was determined by the Benedict method [22] after precipitation of high molecular weight proteins by 10% trichloroacetic acid. The content of mucopeptides was determined after purification of the hydrolysate on an ion exchange column with cation exchanger [23] and the subsequent determination of the carbohydrate component in the composition of mucopeptides by the Antron method [24].

Lactic acid isolation

The isolation of lactic acid from the culture fluid was performed by a classical method, which included crystallization of calcium lactate with subsequent processing of crystals with sulfuric acid and removal of insoluble calcium sulfate precipitate [25].

Ferric complexes preparation

The complexes were obtained by combining solutions of bioligands and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ with vigorous stirring for 180 s, the temperature of complexation was 40 °C. Iron-containing complexes were obtained by combining $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solutions with: hydrolysate of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* (Complex I), lactic acid at a concentration of 10 mg/cm³ (Complex II); hydrolysate and lactic acid with concentration 5 mg/cm³ in the mixture (Complex III). The complex formation ability of Fe^{3+} ions with the bioligands was determined by a turbidimetric method in the presence of Na_2CO_3 on a spectrophotometer SF-2000 at 450 nm [26]. Different volumes of 0.5n $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were added to aliquots of mixtures containing bioligands, mixed and left for 180 s for complete chelation. 1N Na_2CO_3 was then added to the solutions to achieve a pH of 7 units. Under these conditions, Fe^{3+} ions, which did not participate in the complexation, interact with sodium carbonate to form insoluble $\text{Fe}(\text{OH})_3$ particles, which provoke turbidity of the system [27].

Complexes pH-stability study

The behavior of the complexes was investigated in the range of pH values of the medium 1–10 units. The concentration of Fe^{3+} was determined by the thiocyanate method [28]. The required pH was achieved using standard NaOH and H_2SO_4 solutions. The stability of the chelate complexes was calculated by the formula (1):

$$C = \frac{a-b}{a} \cdot 100, \% \quad (1)$$

where a is the total amount of Fe^{3+} ions in the complex, mg/cm³; b is the amount of Fe^{3+} ions released into the reaction medium, mg/cm³.

Complexes thermal stability study

The studies were performed using the differential scanning calorimetry (DSC) method. DSC thermograms were obtained in the temperature range of 25–250 °C at a constant heating rate of 5 °C/min on a Derivatograph Q1500-D calorimeter. In order to determine under what conditions the complete decomposition of the samples would take place, the heating was continued to a maximum temperature of 450 °C. A 500 mg portion was placed in a ceramic crucible. The accuracy of determining the temperature was ± 1 °C, the thermal effect – $\pm 3\%$.

IR spectra of samples were recorded in the range of wavelengths from 4000 to 400 cm⁻¹ in a spectrometer with a Fourier transformer FTIR IR Affinity-1, Shimadzu (Japan) [29].

Results and discussion

Preparation and characterization of mixed-ligand complexes of ferric ions

Probiotic bioligands, namely, peptidoglycan degradation products and metabolism of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964, were used to form Fe^{3+} chelate complexes. Destruction of peptidoglycans of cell walls of BM was performed by the procedure above. As a result, a mixture of amino acids, LMWPs and mucopeptides was obtained, the concentration of which was 10.24 mg/cm^3 , 6.45 mg/cm^3 and 2.25 mg/cm^3 , respectively. An effective chelating agent, namely lactic acid, that is a metabolism product, was also isolated from the BM culture fluid. As a result of the manipulations above, a solution of lactic acid at a concentration of 10% was obtained. A mixture of amino acids, LMWPs, mucopeptides and lactic acid is a mixed ligand system that can cause the formation of stable chelate complexes of biometals, but it is difficult enough to determine the exact denticity of such system, that complicates the calculation of the required amount of metal in inorganic form for its complete chelation at complex formation [9, 27].

As a rule, the synthesis of biometals chelate complexes is carried out with a known composition of ligands of definite denticity. This allows to take on to the complexation reaction required amount of metal, which provides the effect of complete chelation [7, 27]. In our case, it was necessary to determine in what form the metal is in the system of biological agents: organic or inorganic. The classical methods for determining the concentration of a metal ion in a reaction medium are unjustified because the reagents used are sufficiently aggressive. This may cause the destruction of the ionic, coordination bonds of the complexes, which will not provide reliable results and will not allow to determine what form the metal was in the system. Therefore, the determination of free Fe^{3+} ions in the reaction medium was carried out using nephelometry, namely, by determining the turbidity of the system, the presence of which was provided by the reaction products of free ferric ions and sodium carbonate. Three probiotic bioligand systems were used for the studies: low molecular weight hydrolysis products of peptidoglycans (Complex I), lactic acid (Complex II) and their mixture (Complex III). The concentration of additional ligands for complexation was chosen based on theoretical quantum-chemical ideas about possible configurations of Fe^{3+} chelate complexes. The results of the studies are presented in Figure 3.

As can be seen from Figure 3, the turbidity of mixed ligand systems in the presence of Fe^{3+} and Na_2CO_3 ions is minimally stable (0.08 opt. Units) until a certain concentration of metal is reached in the mixture. For the systems under study, this value has a significant difference. Thus, the rapid turbidity growth of Complex I system occurs at concentrations of Fe^{3+} ions in a mixture of $32 \text{ mol/dm}^3 \cdot 10^{-2}$, Complex II – $40 \text{ mol/dm}^3 \cdot 10^{-2}$, Complex III – $40 \text{ mol/dm}^3 \cdot 10^{-2}$. Such behavior of the investigated systems indicates that, before reaching these concentrations, the metal is in bound state in the composition of mixed ligand complexes, that makes impossible its interact with the sodium carbonate present in the system and the appearance of insoluble ferum hydroxide, which causes turbidity. From figure 3 implies that Complex III contains the largest amount of bound metal. This is because the denticity of the mixed ligand system of this complex is the highest, based on the total concentration of all its components [7, 30].

Figure 3. Dependence of the turbidity of mixed ligand system of probiotic origin on the content of Fe³⁺ ions in the presence of Na₂CO₃

IR spectroscopy was used for a deeper study of the chemical structure of the complex. The following samples were studied by IR spectroscopy: 1 – bioligands (a mixture of peptidoglycic hydrolysis products and lactic acid); 2 – a complex of ferric ions with bioligands (Figure 4). Ferric ions and the mechanical mixture of the complex components were not investigated by IR spectroscopy, since the FeCl₃·6H₂O compound is very hygroscopic, that complicates the analysis and may affects on the correct interpretation of the results.

When comparing the IR spectra of bioligands and the resulting complex, there is a difference between the absorption bands. In particular, a significant band broadening in the region of 3000–3500 cm⁻¹ was observed, which corresponds to the valence vibrations of primary amino groups of amino acids, protonated amino groups, free hydroxyl groups [29], metal complexes of amino acids (3200–3400 cm⁻¹) [31]. In addition, a band at frequencies of 2933 cm⁻¹ appears in the spectrum of the complex, which corresponds to the fluctuations of the bound OH- groups [29], which may confirm their participation in the formation of the complex. The spectra lack absorption bands at frequencies of 1700–1760 cm⁻¹, which are characteristic of vibrations of the carboxyl group (-COOH), but there are peaks in the range of 1550–1650 cm⁻¹, which correspond to the vibrations of carboxylate anions (-COO⁻) [29].

Figure 4. IR spectra of bioligands (1) and iron complex (2)

The presence of functional groups of bioligands in ionic form proves that electrostatic interactions can take part in the formation of the complex. In the spectrum of the complex, unlike the spectrum of bioligands, there is an absorption band at 1040 cm^{-1} . Its presence may indicate the formation of coordination bonds with amides [31]. In the low-frequency region of the complex spectrum there are bands (345 cm^{-1} , 540 cm^{-1}), corresponding to the fluctuations of the ferrum. Moreover, the absorption at frequencies of 540 cm^{-1} is somewhat offset compared to the inorganic forms of the ferrum, for which oscillations in the region $460\text{--}500\text{ cm}^{-1}$ are characteristic [31]. The IR spectra of the studied materials also show that metal ions interact with the lactic acid OH^- group. For example, in the complex obtained there is a significant increase in the intensities of the peaks in the region of $1100\text{--}1160\text{ cm}^{-1}$, which indicates the coordination interaction of metal ions with the OH^- groups [31].

Thus, since the spectra of the obtained metal complex, as compared to the IR spectrum of the starting material, show an increase in some absorption bands and the formation of new peaks, it can be assumed that the incorporation of ferric ions into the structure of bioligands does not occur mechanically, but as a result of ionic and coordination interactions with their functional groups.

Since chelate complexes of ferric ions with products of metabolism and processing of BM *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 is planned to be used as dietary supplements and biologically active food ingredients, it is advisable to study their behavior at different pH values (Figure 5) and temperatures (Figure 6).

Figure 5. pH-stability of complexes

Based on the data of Figure 5, the complex, formed with the participation of degradation products of peptidoglycans and metabolites of BM, has the greatest stability at different pH values. According to the literature, the mixed ligand polydentate systems exactly can cause a stable “chelating effect”. Complexation of metals with polydentate ligands is more advantageous in terms of thermodynamics than monodentate ligands. The Fe^{3+} complexing agent has d^2sp^3 hybridization of atomic orbitals [30] and may cause the formation of an octahedral complex with bioligands, which also explains the considerable stability of the complexes in environments with different ion activity. The stability of the obtained complexes in the pH range of 8–10 allows us to predict the possibility of the presence of a ferric ion in the dissolved state in the small intestine, where its absorption by enterocytes occurs [2–3]. The stability of chelates at elevated pH values is also due to the competition between metal ions and proton per bioligand anion [30]. Therefore, the resulting chelate structures are stable in the range of pH values inherent in most food systems, which makes the prospect of their use as biologically active food ingredients.

To predict the behavior of the obtained chelate complexes in the composition of food systems that can be subjected to temperature treatment, their analysis was performed by the DSC method (Figure 6a, b). For this study Complex III and mechanical mixture of its components were used.

a

b

Figure 6. Termograms DSC:

a – complex; b – a mechanical mixture of complex components

TG is the thermogravimetric curve that characterizes the mass loss of the sample, depending on the temperature;

DTG is the curve of differential thermogravimetry based on the registration of the rate of change in mass with continuous heating, which is a more accurate interpretation of the TG curve;

DTA is the curve of differential thermal analysis, which serves to fix the presence of certain thermal effects.

In Figure 6a the curves TG, DTG, and DTA obtained from the DSC analysis of the complex are shown, and in Figure. 6b – the curves of the mechanical mixture (MM) of the complex components. When comparing the DSC analysis data, it can be stated that the initial weight loss of the samples begins at a temperature of 49 °C during the thermal treatment of the complex and at 59 °C during the thermal treatment of the MS. These figures show that the first mass loss is not accompanied by thermal effects, indicating that at these temperatures does not destroy the chelate bonds of the complex, which can provoke a change in the enthalpy of the process and the appearance of peaks in the DTA curves. Therefore, the first mass loss is associated with the removal of free moisture in the samples. When the temperature reaches 122–125 °C, the mass loss is 3% for the complex and 14% for the MM. In the temperature range of 122–178 °C, an endothermic reaction is observed during the thermal treatment of the complex, and no thermal effects are observed during the mechanical mixture treatment. The weight loss of the complex in this temperature range is 22%, MM – 16%. The presence of an endothermic peak on the DTA curve of the complex may indicate the presence in its structure of chelate bonds, and during their destruct the enthalpy changes are occurred.

Conclusions

1. The results of studies indicate the effectiveness of the use of polydendant mixed ligand systems of probiotic origin for complexation with ferric ions.
2. The study of the complex of iron (III) and bioligands of probiotic origin by IR spectroscopy showed that electrostatic and coordination interactions are involved in its formation.
3. According to studies, a system of bioligands containing the peptidoglycan degradation products and metabolites of lactobacilli, provides the formation of chelate complexes of ferric ions, stable in a wide range of pH environments (1-10 units).
4. The DSC method shows that the obtained complex is a promising ingredient of dietary supplements and wellness products whose technology involves high-temperature processing, since the complex is stable in the temperature range of 20–122 °C.
5. The presence in the composition of these complexes of low molecular weight muropeptides with high immunotropic activity, allows to classify these compounds to the category of polyfunctional.

The prospect of further research is to study the physiological activity of the resulting complex in animal experiment.

References

1. Vatutin M.T. ta in. (2012), Rol zaliza v orhanizmi liudyny, *Visnyk KhNU imeni V.N. Karazina*, 1024, pp. 74–80.
2. Shafran L.M., Pyihteeva E.G., Shitko E.S. (2012), Sistema transporta zheleza v kletkah: fiziologiya i toksikologiya pogloscheniya iz pischi enterotsitami kishechnika, *Suchasni problemi toksikologii*, 2, pp. 5–16.
3. Jesse P. Goff (2018), Invited review: Mineral absorption mechanisms, mineral interactions that affect acid-base and antioxidant status, and diet considerations to improve mineral status, *Journal of Dairy Science*, 101(4), pp. 2763–2813, DOI: 10.3168/jds.2017-13112.

4. Kruglov D.S. (2017), Lekarstvennyie sredstva primenyaemye dlya profilaktiki i lecheniya zhelezodefitsitnyih sostoyaniy, *Nauchnoe obozrenie. Meditsinskie nauki*, 4, pp. 26–41.
5. Tong Liu et al. (2019), Preparation and Characterization of a Novel Polysaccharide-Iron(III) Complex in Auricularia, *BioMed Research International*, 2019, DOI: 10.1155/2019/6416941.
6. Yanan Li, Han Jiang, Guangrong Huang (2017), Protein Hydrolysates as Promoters of Non-Haem Iron Absorption, *Nutrients*, 9, pp. 609. DOI: 10.3390/nu9060609.
7. Kapustian A., Chernov N. (2017), Chelate forms of biometals. Theoretical aspects of obtaining and characteristics, *Food science and technology*, 11(1), pp. 37–49, DOI: 10.15673/fst.v11i1.297.
8. Belayet Hossain M.D., Saidul Islam M., Rafiqul Islam M., Abdus M.D. et al. (2012), Synthesis and characterization of mixed ligand complexes of Co(II) and Fe(III) ions with maleic acid and heterocyclic amines, *Journal of Bangladesh Chemical Society*, 25(2), pp. 139–145, DOI: 10.3329/jbcs.v25i2.15066.
9. Zolotareva N.V., Semenov V.V., Petrov B.I. (2013), Preparation of Water-Soluble Iron and Manganese Chelates with Oxyethylidenediphosphonic Acid, *Russian Journal of General Chemistry*, 83(11), pp. 1985–1989, DOI: 10.1134/S1070363213110030.
10. Scheglova N.V., Pechnikova A.S., Shevchenko A.I. (2014), Smeshannoligandnyie kompleksy kobalta (III) s etilendiaminom i etilendiamintetrausnoy kislotoy v vodnyih rastvorah, *Vestnik Kazanskogo tehnologicheskogo universiteta*, 17(17), pp. 56–59.
11. Nikolaeva L.S., Semenov A.N., Burova L.I. (2011), Smeshannoligandnoe kompleksobrazovanie ionov kaltsiya i magniya s geparinom i gliitsinom, *Zhurn. neorg. Himii*, 56(4), pp. 689–696, DOI: 10.1134/S003602361104022X.
12. Zolotuhina N.A. (2015), Kompleksy perehodnyih metallov s organicheskimi ligandami, *Polzunovskiy vestn IK*, 4(2), pp. 58–60.
13. Verma Sh. (2015), Equilibrium study and Stability constants of mixed Ligand complexes of Biomolecules and Amino acids with Metal ions by Potentiometric method, *Research Journal of Chemical Sciences*, 5(3), pp. 42–48.
14. Rabindra P., Mohan A. (2000), Synthesis and characterization of mixed ligand complexes of bio-metals with pyrimidine nucleoside (uridine) and amino acids, *Proc. Indian Acad. Sci. (Chem. Sci.)*, 112(6), pp. 593–600, DOI: 10.1007/BF02704366.
15. Gandham Hima Bindu, Gollapalli Nageswara Rao (2012), Mixed ligand complexes of essential metal ions with L-glutamine and succinic acid in sodium dodecyl sulfate–water mixtures, *J. Serb. Chem. Soc.*, 77(4), pp. 453–63, DOI: 10.2298/JSC110201177B.
16. Kapustian A.I., Chernov N.K. (2015), Perspektivy ispolzovaniya biologicheskii aktivnyih bakterialnyih gidrolizatorov dlya nutritivnoy podderzhki naseleniya s rastroystvami immunnogo sistema, *Pischevaya nauka i tehnologiya*, 2(31), pp. 18–25, DOI: 10.15673/2073-8684.31/2015.44263.
17. Chernov N., Kapustian A. (2016), Immunological properties of the bacterial origin compounds, *Food science and technology*, 10(3), pp. 19–28, DOI: 10.15673/fst.v10i3.175.
18. Traub S., Von S. Aulock, Hartung T., Hermann C. (2006), MDP and other muropeptides – direct and synergistic effects on the immune system, *J Endotoxin Res*, 12, pp. 69–85, DOI: 10.1179/096805106X89044.
19. Qingshan Lv. et al. (2012), MDP Up-Regulates the Gene Expression of Type I Interferons in Human Aortic Endothelial Cells, *Molecules*, 17, pp. 3599–3608, DOI: 10.3390/molecules17043599.

20. Matsui K., Ikeda R. (2014), Peptidoglycan in combination with muramyl dipeptide synergistically induces an interleukin-10-dependent T helper 2-dominant immune response, *Microbiol Immunol*, 58, pp. 260–265, DOI: 10.1111/1348-0421.12139.
21. Matsumoto S. et al. (2009), A component of polysaccharide peptidoglycan complex on *Lactobacillus* induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer, *Immunology*, 128, pp. 170–80, DOI: 10.1111/j.1365-2567.2008.02942.x.
22. Semak I.V., Zyryanova T. N., Gubich O. I. (2007), *Biohimiya belkov*, Minsk.
23. Kapustian A.I., Chernon N.K., Pukas A.S. (2019), Rozroblennia metodu vyznachennia funktsionalnykh kharchovykh inhredientiv muropeptydnoho pokhodzhennia u bakterialnykh dezintehratakh, *Naukovi pratsi NUKhT*, 25(62), pp. 157–164.
24. Morris D.L. (1948). Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent, *Science*, 107(2775), pp. 254–255, DOI: 10.1126/science.107.2775.254.
25. Starr J.N., Westhoff G. Lactic Acid (2014), *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley, DOI: 10.1002/14356007.a15_097.pub3.
26. Kapustian A.I., Chernon N.K. (2019), Vyznachennia kompleksoutvoriuvanoi zdatnosti zmishanolihandnykh orhanichnykh system po vidnoshenniu do yoniv metaliv, *Naukovyi visnyk Lvivskoho natsionalnoho universytetu veterinarynoi medytsyny ta biotekhnolohii im. S.Z. Hzhyskoho*, 21(91), pp. 130–135.
27. Klenin V.I., Schegolev S.Yu., Lavrushin V.I. (1977), *Harakteristicheskie funktsii svetorasseyaniya dispersnykh sistem*, Saratov.
28. Mophan N., Vinitnantharat S., Somsook E. (2010), Enhancing iron (III) solubility using cassava and arrowroot starch, *Science Asia*, 36, 172–173, DOI: 10.2306/scienceasia1513-1874.2010.36.172.
29. Tarasevich B.N. (2012), *IK-spektryi osnovnykh klassov organicheskikh soedineniy*, Moscow.
30. Neudachina L.K., Lakiza N.V. (2014), *Fiziko-himicheskie osnovy primeneniya koordinatsionnykh soedineniy*, Ekaterinburg.
31. Nakamoto K. (1966), *Infrakrasnyie spektryi neorganicheskikh i koordinatsionnykh soedineniy*, Moscow.