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OBTAINING AND CHARACTERISTICS OF CALCIUM ORGANIC FORMS ON THE BASIS OF METABOLITES AND PROCESSING PRODUCTS OF PROBIOTIC BACTERIA

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Abstract. The possibility of obtaining bioavailable mixed ligand chelate complexes of calcium has been considered. As bioligands, it is proposed to use the metabolic products of probiotic bacteria combination and products of enzymatic hydrolysis of peptidoglycans of their cell walls. The culture fluid of probiotic bacteria composition has been investigated for the determination of metabolites in its composition that can participate in the formation of calcium chelate complexes. The qualitative composition and quantitative content of organic acids of a culture fluid have been determined. It has been established that it contains the following acids: oxalic (1.6 mg/dm^3), citric (22.1 mg/dm^3), acetic (575.8 mg/dm^3), lactic (236.3 mg/dm^3), benzoic (1.5 mg/dm^3). In addition, it has been found that in the composition of the culture liquid, free amino acids and soluble protein are also present in the amount of 1.2 mg/cm^3 and 5 mg/cm^3, respectively.

In order to obtain fragments of peptidoglycans of cell walls of probiotic bacteria as potential bioligands for complex formation, their enzymatic hydrolysis with pancreatin has been performed. It has been established that the highest content of biologically active muropeptides is 5.1 mg/cm^3 and it is accumulated during hydrolysis of the substrate for 180 minutes, the ratio of enzyme: substrate 1: 100 and 5.1 mg/cm^3.

By methods of nephelometry and spectrophotometry, it has been established that the obtained mixed ligand systems are effective chelating agents and, depending on the composition, bind calcium in amounts of 9, 14 and 16 mg/cm^3. Identification of the pH stability of the complex has been shown that in the range of pH values 4–7, the chelate system is stable, at pH 2 only 10% of the complex is stored, at pH 9 60% is preserved. By method of differential scanning calorimetry the thermostability of the complex has been investigated. It has been established that the complex is stable in the temperature range of 20–122°C, and therefore can be used in the composition of health foods, the technology of which involves high-temperature processing.

Key words: calcium, chelate complexes, bioligands, probiotic bacteria, metabolites, muropeptides.

ОТРИМАННЯ ТА ХАРАКТЕРИСТИКА ОРГАНІЧНИХ ФОРМ КАЛЬЦІЮ НА ОСНОВІ МЕТАБОЛІТІВ ТА ПРОДУКТІВ ПЕРЕРОБКИ ПРОБІОТИЧНИХ БАКТЕРІЙ

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Анотація. Розглянуто можливість отримання біодоступних змішанолігандних хелатних комплексів кальцію. У якості біолігандів пропонується використовувати продукти метаболізму комбінації пробіотичних бактерій та продукти ферментативного гідролізу пептидогліканів їхніх клітинних стінок. Досліджено культуральну рідину композиції пробіотичних бактерій на предмет наявності метаболітів, які можуть приймати участь в утворенні хелатних комплексів кальцію. Визначено якісний склад та кількісний вміст органічних кислот культуральної рідини. Встановлено, що у її складі присутні наступні кислоти: щавелева (1,6 мг/дм^3), лимонна (22,1 мг/дм^3), оцтова (575,8 мг/дм^3), молочна (236,3 мг/дм^3), бензойна (1,5 мг/дм^3). Окрім того встановлено, що у складі культуральної рідини присутні також вільні амінокислоти та розчинний білок у кількості 1,2 мг/см^3 та 5 мг/см^3 відповідно.

Із метою отримання фрагментів пептидогліканів клітинних стінок пробіотичних бактерій як потенційних біолігандів, здійснено ферментативний гідроліз клітинних стінок пробіотичних бактерій панкреатином. Встановлено, що найбільший вміст біологічно активних муропептидів накопичується при гідролізі субстрату протягом 180 хв, співвідношенні фермент:субстрат 1:100 та складає 5,1 мг/см^3.

Встановлено, що отримані змішанолігандні системи є ефективними хелатоутворювальними агентами та, в залежності від складу, зв'язують кальцій у кількості 9, 14 та 16 мг/см^3. Визначення pH стабільності комплексу показало, що в інтервалі значень pH 4–7, хелатна система є стабільною, при pH 2 зберігається лише 10% комплексу, при pH 9 – 60%. Методом диференційної скануючої калориметрії досліджено термостабільність комплексу. Встановлено, що комплекс є стійким в діапазоні температур 20–122°С, а отже, може бути використаний як фізіологічно функціональний інгредієнт в рецептурі оздоровчих продуктів харчування, технологія яких передбачає високотемпературну обробку.

Ключові слова: кальцій, хелатні комплекси, біоліганди, пробіотичні бактерії, метаболіти, муропептиди.

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Introduction. Formulation of the problem

Incomplete and unbalanced nutrition provokes the emergence of a number of diseases due to the lack of essential food components. These include minor components – biometals, in particular, calcium [1-2]. Ca^{2+} ions are necessary for the formation of bone tissue, during lactation, for the implementation of cardiac contractions, and they are a factor for coagulation of blood, activation of a number of enzymes, etc. [3]. To overcome calcium deficiencies, dietary adjustments are needed, or the use of effective calcium preparations in an easily digestible form.

Biometals in the composition of inorganic compounds when they enter the body with food have a level of bioavailability of no more than 2–20%. Increasing bioavailability of trace elements is one of the most urgent tasks of modern science. At present, there is a special interest in the prevention and treatment of many hypomicrolementosis through biocoordinative compounds, in which vitally necessary trace elements are contained in the form of a chelating complex with bioligands, that are natural carriers of trace elements.

Analysis of recent research and publications

Amino acids, carboxylic acids, proteins and peptides are usually used as bioligands for chelating metals. There are at least two donor centers in any amino acid, among them are carboxyl and amino groups. In addition, the side chain may contain carboxyl, hydroxyl, thiol and amino groups. This structure of the molecules determines the possibility of chelation at the interaction with metal ions, and in case of participation of the side groups, the ligand's denticity may exceed 2. Peptides are less active in chelation reactions. In the peptide, the carboxyl group and the amino group, which are terminal, are separated by a significant number of atoms, this imposes conformational constraints and increases the role of the side groups for complexation.Although peptides form less stable complexes than the amino acids that comprise them, the stability of the complexes gradually increases with the increase in the number of amino acid residues. Potential donor centers in carbohydrate molecules are carbonyl and hydroxyl groups. But since carbohydrates are mainly in a predominantly cyclic form, the hydroxyl groups play a decisive role in coordination. The effectiveness of the coordination of bioligands strongly depends on the pH of the medium, since protons are able to compete with biomethane for the ligand. Therefore, in acidic medium, when most of the above groups are protonated, complexation proceeds less intensively [4-9].

Most of the processes occurring in biological systems involve the interaction of metal ions with several ligands, so it is of particular interest to obtain and study the properties of mixed ligand complexes of biometals with biologically active ligands [10-14]. The assimilation of the biometal will occur in that case if it is firmly connected with the chelating agent that is a participant in the metabolic processes: amino acids, polybasic acids, vitamins. The study of mixed ligand complexes of biometals has become widespread, the methods of obtaining and characteristics of some of them have been described in the literature .

In [15], complexes of some d-metals ions (Co^{2+}, Cu^{2+}, Zn^{2+}) were obtained in the form of salts and in solution with ligands containing donor nitrogen and oxygen atoms to which the d-metal cations have an affinity. As an oxygen-containing ligand oxycarboxylic acid (malic acid) was used, as a nitrogen-containing ligand imidazole was used.

In work [16], methods for the synthesis of a series of metal-containing (copper, nickel, cobalt, iron) arabinogalactan derivatives have been developed, whose metal content, depending on the reaction conditions and the chemical composition of the initial reagents, can vary from 1% to 5%. As a result of the research, it has been shown that, depending on the properties of the metal, arabinogalactan is able to act either as a ligand or exhibit the properties of a stabilizer of hydrophobic colloidal systems.

In [17] the formation of binary and ternary complexes of metal ions such as Cu(II), Co(II), Pb(II), Zn(II) and Cd(II) with biologically important ligand were investigated. Where nucleic acid Adenine was used as primary ligand and amino acid Histidine was used as secondary ligand.

In [18] the mixed ligand complexes of Cu(II), Ni(II) and Co(II) with uridine and amino acids L-alanine, L-phenylalanine and L-tryptophan were synthesized and characterized by the elemental analysis, conductivity data, infrared spectra, electronic spectra and magnetic susceptibility data.

In paper [19] the synthesis and characterization of mixed ligand complexes of Co(II) and Ni(II) have been described. Malic acid plays the role of primary ligand and Heterocyclic amine bases play the role of secondary ligands in the complexes. The prepared complexes Co(II) and Ni(II) were found to form octahedral structure.

In [20] mixed ligand complexes of Co(II), Ni(II) and Cu(II) with L-glutamine and succinic acid were studied. The increased stability of the ternary complexes compared to their binary complexes was believed to be due to electrostatic interactions of the side chains of the ligands, charge neutralization, chelate effect, stacking interactions and hydrogen bonding

In this study [21], the chelating complex of calcium with soluble collagen peptides was investigated.

The complex structure analysis showed that collagen peptide chelated calcium is a five-membered ring structure, calcium is in the center and was combined strongly with both the amino- and carboxyl-group.

A specific peptide Tyr-Leu with calcium-binding capacity was purified from defatted Schizochytrium sp. protein hydrolysates through gel filtration chromatography and RP-HPLC. The results showed that calcium ions could form dative bonds with carboxyl oxygen atoms and amino nitrogen atoms as well as the nitrogen and oxygen atoms of amide bonds. Complex exhibited excellent thermal stability and solubility, which was beneficial for its absorption and transport in the basic intestinal tract of the human body [22].

After analyzing a number of scientific studies in the field of obtaining chelate complexes of biometals, we came to the conclusion that the literature lacks information on the possibility of using metabolites and probiotic bacteria processing products as bioligands. Taking in the account the great experience and volumes of cultivation of probiotic cultures, such an idea is very relevant. In the production of probiotic cultures a large amount of by-products is utilized. Such is the culture fluid that remains after the separation of the bacterial mass. The culture fluid contains a large number of metabolites, in particular organic acids, capable of chelate complexation with biometals. In addition, non-conditioned biomass is often disposed of, which can be sent for recycling to produce degradation products of peptidoglycans from their cell walls – compounds of the muramilpeptide series, which also contain functional groups that can form ionic and coordination bonds with metal ions. In addition, the substances of the muramilpeptide series have their own physiological effect – they are powerful immunotropic compounds [23-26].

The purpose of this work is to obtain the chelate complexes of Ca^{2+} ions with metabolites and low molecular weight degradation products of peptidoglycans of combination of probiotic bacteria cell walls.

Research tasks:

- obtaining and characteristic of bioligands - metabolic products of probiotic bacteria and degradation products of their cell walls;
- obtaining chelate complexes of calcium with bioligands;
- Studying the stability of received complexes depending on the pH medium and temperature.

Research Materials and Methods

The research was conducted on the basis of laboratory of Scientific and Production Enterprise "Ariadna" (Odesa, Ukraine), Scientific-Research Laboratory of the Department of Food Chemistry and Expertise of the Odesa National Academy of Food Technologies (Odesa, Ukraine), the Laboratory of the State Scientific-Research Control Institute for Veterinary Medicines and Feed Additives (Lviv, Ukraine).

Materials. Composition of LAB and BB represents a sum of test cultures: *Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Bifidobacterium bifidum, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Streptococcus thermophilus*, from the collection of Scientific and Production Enterprise "Ariadna" (Odesa) with a concentration $5 \cdot 10^9$ CFU/cm^3. This bacterial composition has a commercial name "Bacterial starter cultures Symbinorm".

Enzymatic degradation of BM cell was performed by pancreatin treatment with a proteolytic activity of 370 UN (Ternopharm, Ternopil). $CaCl_2$ (STAB, the Netherlands) was used as a source of Ca^{2+}.

Obtaining of metabolic products of probiotic bacteria and degradation products of their cell walls and their characteristics. Working cultures were prepared from frozen cultures in 12% (w/v) low-heat RSM before use, and stored at 4°C. Initially, the first generation of each of the bacterial monocultures was cultivated separately on special nutrient medium developed by the "Ariadna" company. Bacterial strains were grown in sterile conditions at 37°C. After reaching the number of bacteria up to $5–9 \cdot 10^9$ and more, the culture fluids were combined, nutrient medium was added and the second generation of bacteria was cultivated under sterile conditions at 37°C. Cultivation was stopped by an emergency heating up to 90°C for 30 minutes after 8 hours incubation, which corresponded to the end of the logarithmic phase of bacterial growth [27].At the same time there was a maximum decrease in pH of the medium to a value of 4.5.

The culture fluid was then cooled to room temperature, centrifuged for 10 min at 8000 min^{-1}, then decantation was performed. In the supernatant, the content of metabolites of probiotic bacteria, namely, organic acids, amino acids, peptides and proteins, which are potential biologic agents for obtaining chelate forms of calcium, was investigated.

The precipitate obtained after centrifugation was resuspended, re-centrifuged, decanted. The washed cells of probiotic bacteria were resuspended with distilled water to the dry matter content in a suspension of 6.5±2%. After this the enzymatic hydrolysis was carried out. The constant parameters of hydrolysis were: temperature –37°C and pH=7.4. The ratio of the enzyme to the substrate (dry matter content of BM) was varied in the range from 1:50 to 1:150 and the duration of the incubation of the reaction mixture was varied in the range 10–300 min. In the obtained hydrolysates, the content of free amino acids, soluble protein, low molecular weight peptides (LMWP) were investigated. Enzymatic hydrolysis was stopped by heating at the temperature 100°C during 15 min, the mixture was cooled, centrifuged for 10 min at 8000 min^{-1}, decanted, further evaporation of supernatant containing low molecular weight soluble biological active substances was carried out up to 6,5±2% dry matter content.

In samples, the content of free amino acids was controlled by the method of formolithic titration [28],

soluble protein by Benedict's method [29], low molecular weight peptides (LMWP) by the Benedict method after precipitation of high molecular-weight proteins by 10% solution of trichloroacetic acid.

Qualitative and quantitative content of organic acids was determined by the method of capillary electrophoresis (device Capel 105/105M). Up to 0.5 g of the 50 cm^3 preparation of distilled water heated to 70°C was added. The mixture was stirred on a laboratory shaker for 10 min. After that, 1 cm^3 of filtrate was taken out, centrifuged and the determination of quantitative and qualitative content of organic acids was carried out. Detection was performed at wavelengths of 190 nm [30].

Obtaining of chelate complexes of calcium with bioligands. To obtain chelate complexes of calcium, as a source of bioligands, a mixture of two fluids was used. Liquid I (L1) is a supernatant of a culture fluid obtained after cultivation and centrifugation of the second generation of bacteria. Liquid II (L2) is a concentrated supernatant obtained after enzymatic hydrolysis of a suspension of bacterial cells. The combination of liquids was carried out in bulk proportions L1:L2 2:1, 1:1, 1:2. The complexing ability of calcium ions in relation to a mixture containing products of the metabolism of probiotic bacteria (L1) and degradation products of peptidoglycans of their cell walls (L2) was determined by a nephelometric method in the presence of Na_2CO_3 on a spectrophotometer SF-2000 at a wavelength of 450 nm [26].

To the aliquot of the mixtures containing the bioligands, various volumes of 0.5n $CaCl_2$ were added, stirred and left for 15 minutes to complete chelation. Thereafter, an equimolar amount of Na_2CO_3 was added to the solutions. Ions of Ca^{2+}, which did not participate in complex formation, in interaction with sodium carbonate form insoluble particles of $CaCO_3$ of white color, which provoked turbidity of the system.

Investigation of the stability of the received complexes depending on the pH of the medium. The stability of chelate complexes of calcium, depending on the pH of the medium, was determined by changing the intensity of absorption of light at a wavelength of 270 nm using a spectrophotometer SF-2000. The required pH of the solutions was created by solutions of NaOH and H_2SO_4 "ap". The constancy of the ionic strength (I≈0.1) was maintained by a solution of Na_2SO_4 "ap". The activity of hydrogen ions was measured on an ionometer I-160 using a working electrode ES-10601/7 and a reference electrode ESR-10101. The instrument was calibrated using standard buffer solutions prepared from fixanal. The measurements were carried out at room temperature 20±2°C. Distilled water was used as the reference solution.

Investigation of the stability of obtained complexes depending on temperature. The research was conducted using the method of differential scanning calorimetry (DSC) in dynamic mode. Thermograms of DSC were obtained in the temperature range of 25–250°C at a constant heating rate of 5°C/min on a Derivatorgaph Q1500-D.

In order to determine under what conditions the complete destruction of the samples will occur, the heating was extended to a maximum temperature of 450°C. A weight of 500 mg was placed in a ceramic tigel. The accuracy of the temperature determination was ±1°C, the thermal effect – ±3%.

Results of the research and their discussion

Metabolic products of the combination of probiotic bacteria and the products of enzymatic hydrolysis of peptidoglycans of their cell walls have been proposed to use as ligands for complexation with Ca^{2+} ions.

Obtaining and characteristic of metabolic products of probiotic bacteria. The content of metabolites was investigated in the composition of the culture fluid, which was obtained by probiotic bacteria cultivation. Cultivation was stopped at the end of the logarithmic phase of bacterial growth (after 8 hours of exposure) by heating up to 90°C for 30 minutes. This manipulation was carried out for two reasons: firstly, in order to minimize the content of bacterial nucleic acids in the composition of the culture fluid, as it is known that at the end of this particular phase of growth, their content is minimal; and secondly, to weaken the strength of bacterial cells for more effective enzymatic degradation of the peptidoglycans of their cell walls at subsequent stages of the experiment. It is known that at the end of the logarithmic phase of growth, bacterial cells are most vulnerable to the effects of aggressive factors that can disrupt the integrity of the bacterial cell by destroying their protective shell or can cause partial perforation of the bacterial wall.

It is known that Lactic acid bacteria (LAB) and bifidobacteria (BB) produce a number of organic acids that play a decisive role in maintaining colonization resistance and antagonistic activity against pathogenic microflora. In the aspect of this work, the functional groups of organic acids synthesized by polyspecific bacterial leaven are potential donors for the formation of ionic and coordination bonds in chelate structures of Calcium. That is why it was expedient to determine the qualitative and quantitative content of organic acids in the composition of the culture fluid. Definition of these indicators was carried out by capillary electrophoresis. The study showed that there is a number of organic acids in the composition of the culture fluid of the combination of the LAB and the BB. The following organic acids were identified and their quantitative content was determined: oxalic acid – 1.6 mg/dm^3, lemon – 22.1 mg/dm^3, acetic acid – 575.8 mg/dm3, dairy – 236.3 mg/dm^3, benzoic acid – 1, 5 mg/dm^3.

It has also been found that except organic acids in the culture fluid, there are free amino acids in the amount of 1.2 mg/cm^3 and a soluble protein in the amount of 5 mg/cm^3.

Obtaining of degradation products of probiotic bacteria cell walls and their characteristic. The degradation of the cell walls peptidoglycans of the probiotic bacteria composition was carried out using enzymatic hydrolysis with pancreatin. The pancreatin contains a number of exo- and endoproteases that are capable of

breaking down the specific peptide bonds of high-molecular peptidoglycan cell walls of bacteria, forming products of its degradation: amino acids, low molecular weight muropeptides, which have a powerful immunotropic effect.

The effectiveness of enzymatic hydrolysis was evaluated on the basis of dynamics of LMWP and amino acids accumulation in the hydrolysate. A series of experiments was conducted in which the concentration of pancreatin in the reaction mixture and the time of hydrolysis were varied. The research results are shown in Fig. 1.

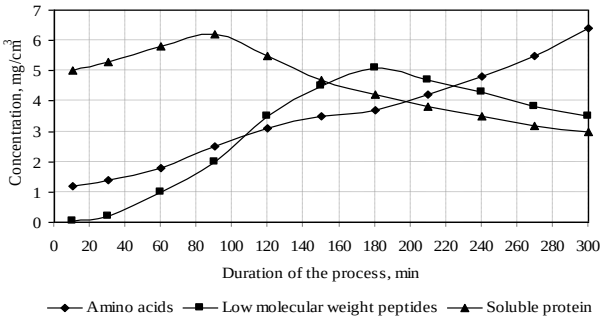


Fig. 1. Dependence of the degradation products accumulation on the duration of the process of enzymatic hydrolysis of the bacterial composition (with a ratio of enzyme : substrate 1 : 100)

When analyzing research results presented in the graphic dependence of Figure 1, it can be stated that when the ratio of the enzyme: substrate is 1:100, the accumulation of amino acids in enzymatic hydrolysis for 10–300 minutes is almost linear. Accumulation of soluble protein in the hydrolysate during the first 90 minutes increases from 5 to 6.2 mg/cm^3, after which its amount gradually decreases. Some increase in the amount of soluble protein can be explained by the beginning of cell walls destruction of the LAB and BB composition and the release of intracellular proteinaceous compounds. The graphic representation of the LMWPs accumulation in the hydrolysate is parabolic with a maximum corresponding to a LMWPs concentration of 5.1 mg/cm^3. In the course of the process, the amount of LMWPs decreases in the hydrolysate, which is apparently due to the destruction of peptide bonds in their structure under the influence of pancreatin. Since LMWPs of peptidoglycans possess immunotropic activity, it is expedient that the time of enzymatic hydrolysis of the bacterial mass, in which there is a maximum accumulation, that is, 180 minutes. Since LMWPs peptidoglycans possess immunotropic activity, it is expedient that the duration of hydrolysis must correspond to their maximum accumulation, that is, 180 min.

With an increase in the concentration of the enzyme in the reaction mixture (the ratio of the enzyme:substrate is 1:50), the regularities of kinetic of the hydrolysis products accumulation are the same as at the hydrolysis with the ratio of the enzyme: substrate being 1:100. But when splitting peptidoglycans of bacterial cells with the participation of a higher concentration of the enzyme, the content of amino acids after 180 minutes of hydrolysis is 18% smaller, and the content of LMWPs is less than 22%. This tendency can be explained by a certain inhibition of peptidoglycan digestion products of further enzymatic degradation, since, at elevated concentration of the enzyme, the hydrolysis products accumulate rather actively in the first stages, and a sharp increase in the concentration of low molecular weight compounds in the reaction mixture may inhibit the process of enzymatic hydrolysis. When studying the regularities of enzymatic hydrolysis, with the decrease of the concentration of the enzyme in the reaction mixture (the ratio of enzyme: substrate is 1: 150), it was established that the values of the indexes of amino acids and LMWPs are also inferior to those in hydrolysis with the ratio of the enzyme: substrate being 1:100.

So, proceeding from the above, it has been found out that organic acids, amino acids, soluble protein are contained in the composition of the culture liquid of the LAB and BB combination, and amino acids and LMWPs are contained also in the hydrolysate of the bacterial cell wall peptidoglycans that can serve as biologically active ligands for the formation of chelate mixed ligand complexes of Calcium.

Obtaining of Calcium chelate complexes with bioligands. The preparation of Calcium chelate structures was carried out according to the scheme given in the section "Methods of investigation". In fig. 2 the results of nephelometric titration are shown, where the arrow indicates the point of equivalence of the maximum binding of Ca^{2+} ions with a mixed ligand system. The use of the classical method for Calcium determining by means of complexometric titration was not possible, since the chelating agent of this reaction (EDTA) competes for the binding of calcium with bioligands of the investigated system. The use of this method in determining the amount of calcium that participates in complex formation would not be correct.

Analyzing the data of Figure 2 it can be stated that the highest ability to bind Calcium ions has a mixed ligand system, formed by the combination of L1 and L2 in the ratio 2:1. In this case, the maximum binding of Calcium by this system is 16 mg/cm^3. With the subsequent addition of $CaCl_2$ to the system of bioligands, Calcium ions remain in the free state, which, when interacting with Na_2CO_3, form insoluble particles of $CaCO_3$ of white color, which cause a sharp turbidity of the system. The maximum binding of Calcium ions by the system formed by the combination of L1 and L2 in the ratio 1:1 is 14 mg/cm^3, and at a ratio of 1:2 is 9 mg/cm^3. Such a difference in the amount of bound Calcium ions by different systems of bioligands due to the fact that when comparing mixtures L1 and L2 in ratios 2:1, 1:1 and 1:2, in the first system the content of organic acids, which are donors of anions for ionotropic binding of Calcium ions is the highest. In the second system, the amount of binding of calcium is 2 mg/cm^3 less, compared with the first, obviously, the complex formation is provided by the system of ionic bonds of acids and coordination bonds, which are formed between amino groups of free amino acids and peptide bonds of LMWPs with metal. In the prediction of the behavior of these complexes in acidic medium, it

can be assumed that the complex formed by the system L1 and L2 in the ratio 2:1 will be less stable, since dissociation of acidic groups is suppressed in the acidic medium, which can provoke the destruction of ion bonds with calcium, and, respectively, chelate systems. That is why, for further research, the chelate system created by combination of L1 and L2 in the ratio of 1:1 was used. In this mixture apart from a significant amount of carboxyl groups, more amount of products of enzymatic hydrolysis of bacterial cell walls peptidoglycans is contained, which, due to their amphoteric properties, can improve stability of Calcium chelate complexes.

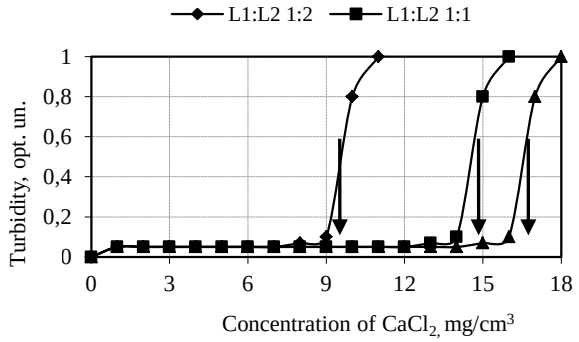


Fig.2 Maximum binding of Ca^{2+} ions by mixed ligand systems (λ=450 nm)

Complex formation was also proved by changing of the spectrum of the mixed ligand system formed by the combination of L1 and L2 in the ratio of 1:1 in the ultraviolet region, depending on the content of Calcium ions in it (Fig. 3).

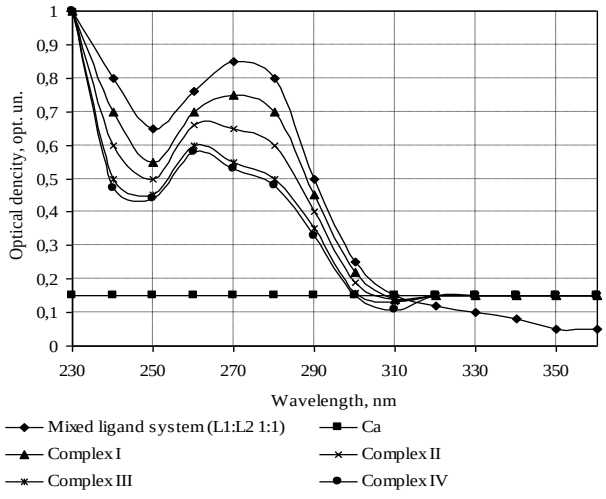


Fig. 3. Absorption pectrum of a mixed ligand systems with different Calcium content:
Complex I – 4 mg/cm^3 $CaCl_2$, Complex II – 9 mg/cm^3 $CaCl_2$, Complex III – 14 mg/cm^3 $CaCl_2$, Complex IV – 19 mg/cm^3 $CaCl_2$

As can be seen from Fig. 3, the absorption spectrum of a solution of calcium chloride has no peaks in the ultraviolet region, in contrast to the spectrum of the studied mixed-ligand system, which has a clear peak in the region of 260–280 nm. Namely in this area of the spectrum organic acids, amino acids and proteins are absorbed. When adding calcium chloride in the amount of 4–19 mg/cm^3 to the bioligand system, the intensity of spectral absorption gradually decreases with increasing calcium ion concentration. Such changes in the spectrum indicate that the number of free functional groups responsible for oscillations in the spectrum is reduced due to their participation in the formation of calcium chelate complexes. Moreover, with the addition of calcium chloride in the amount of 4–14 mg/cm^3 to the system, the absorption intensity decreases linearly with a step of ≈12%, with further increase of calcium chloride in the system (19 mg/cm^3), the absorption intensity decreases by only ≈2%, this confirms that the saturation of bioligands with calcium take place at a concentration of calcium chloride equal to 14 mg/cm^3.

Since chelate complexes of Calcium with metabolites and processing products of LAB and BB composition are planned to be used as dietary supplements and biologically active food ingredients, it is expedient to study their behavior at different pH values of the medium and temperatures.

Investigation of the stability of the obtained complexes depending on the pH of the medium

The research methodology of the Calcium chelate complex pH stability is described in detail in the section "Research Methods". The intensity of absorption of the complex was determined in the range of pH values 2–10 at a wavelength of 270 nm (Fig. 4), in which the maximum absorption of chelate mixed ligand systems (Fig. 3) was defined.

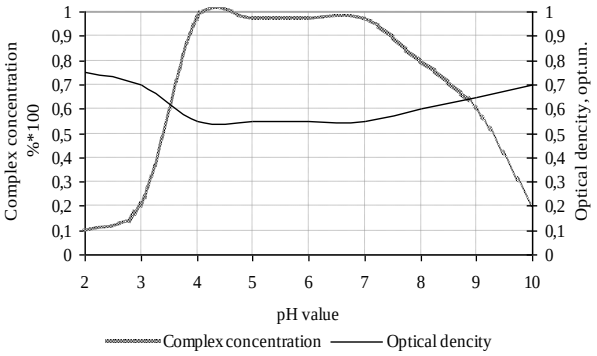
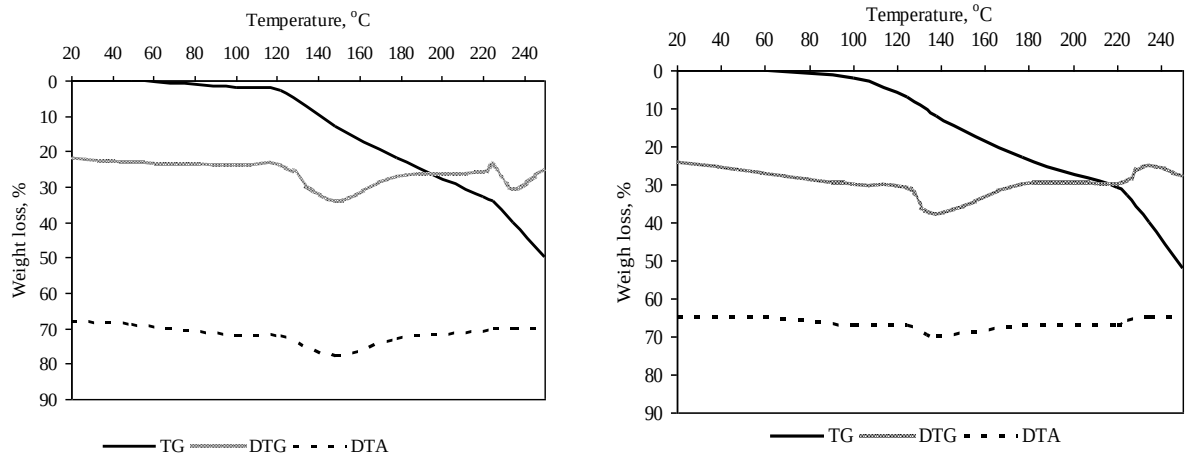


Fig. 4. pH-stability of chelate complexes of Calcium (λ=270 nm)

Fig. 4 indicates that in the range of pH values of 4–7, the intensity of absorption of optical density by the complex is 0.55 opt. un., this corresponds to the value of the maximum absorption peak of optical density by a complex with a 14 mg/cm^3 concentration of calcium in it (Fig. 3). This means that in this range of pH values of the medium, the complex retains its chelate structure and its concentration in the mixture is maximal – 100%. If the pH value is deviated to a more acidic side, the stability of the complex decreases sharply. Thus, at pH 3, the concentration of the complex in the mixture is 20%, at pH 2–10%. With the deviation of the pH to the alkaline side, the stability of the complex is also somewhat lost, at pH 8 ≈ up to

20% and at pH 9 ≈ up to 40%. Consequently, the obtained Calcium chelate structures are stable in the range of pH values of the medium inherent for most food systems, which determines the promising use of them as biologically active food ingredients.

Investigation of stability of the obtained complexes depending on temperature. In order to predict the behavior of the calcium chelate complexes in the composition of food systems that can be subjected to temperature processing, they were analyzed by the DSC method (Figs. 5a, b).



a) b)

Fig. 5. Termograms DSC: a) Chelate complex of calcium; 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 fig. 5a the curves of TG, DTG and DTA are shown, which were obtained as a result of research of Calcium chelate complex by DSC method, and in Fig. 5b the curves obtained as a result of studies of the mechanical mixture (MM) of the components of the complex are depicted. When comparing the data of the DSC analysis, we can state that the initial mass loss of the complex begins at the temperature of 49°C, and MS at 59°C. From these figures it can be seen that the first mass loss is not accompanied by thermal effects, which indicates that at these temperatures there is no destruction of the chelate bonds of the complex, which can provoke a change in the enthalpy of the process and the appearance of peaks on the DTA curves. Consequently, the first loss of mass is associated with the removal of free moisture in the sample. When the temperature reaches 122–125°C, the mass loss is 3% for the complex and 7% for the MM. In the temperature range of 122–178°C, an endothermic reaction is observed at the heat treatment of the complex, while thermal effects at the treatment of MM is not observed. The mass loss of the complex in this range of temperatures is 18%, MM – 16%. The presence of an endothermic peak on the DTA curve of the complex may indicate the presence of chelate bonds in its structure, with the destruction of which there are changes in the enthalpy of the process.

Consequently, the results of the analysis of the DSC have shown that the obtained complex is stable in the temperature range of 25–122°C, and therefore can be used in the composition of health foods, the technology of which involves high-temperature processing.

Conclusions

1. The culture fluid of probiotic bacteria composition has been investigated for the presence of metabolites that can participate in the formation of calcium chelate complexes. The qualitative composition and quantitative content of organic acids of a culture fluid are determined. It has been established that it contains the following acids: oxalic (1.6 mg/dm^3), citric (22.1 mg/dm^3), acetic (575.8 mg/dm^3), lactic (236.3 mg/dm^3) benzoic (1.5 mg/dm^3). In addition, it has been found that in the composition of the culture liquid, free amino acids and soluble protein are also present in the amount of 1.2 mg/cm^3 and 5 mg/cm^3, respectively.

2. In order to obtain fragments of cell walls peptidoglycans of probiotic bacteria as potential bioligands for complex formation, their enzymatic hydrolysis with pancreatin was performed. It has been found that the highest content of biologically active muropeptides (5.1 mg/cm^3) is accumulated during hydrolysis for 180 minutes and the ratio of enzyme: substrate 1:100.

3. By methods of nephelometry and spectrophotometry in the ultraviolet region, it has been found that obtained mixed ligand systems are effective chelating agents and, depending on the composition, bind calcium in amounts of 9, 14 and 16 mg/cm^3.

4. Identification of the pH stability of the complex showed that in the range of pH values 4–7 chelate system is stable, at pH 2, only 10% of the complex is stored, at pH 9–60%.

5. According to the results of the DSC analysis, it has been proven that the obtained complex is formed with the participation of chelate bonds of bioligands with calcium, at the destruction of which an endothermic effect is observed. The complex is stable in the range of temperatures of 20–122°C, and, consequently, can be used in the formulation of healthy foods, the technology of which involves high-temperature processing.

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