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OBTAINING AND CHARACTERISTIC OF MUROPEPTIDES OF PROBIOTIC CULTURES CELL WALLS

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Abstract. The possibility of muropeptides obtaining of peptidoglycans of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* cell walls by the combination of the use of autolytic processes and enzyme treatment of biomass with the participation of lysozyme and papain has been considered. It has been established that the most significant autolytic changes in biomass occur in the application of high-temperature processing (90°C for 30 minutes) in the final stage of the logarithmic phase of bacterial growth. Thus, after eighth hour of biomass incubation at 37°C, the amino acid content in the culture medium was 1.8 mg/cm^3, and at 90°C it was 5.7 mg/cm^3. In order to further destruction of biomass autolysate and obtaining of low molecular weight peptidoglycan fragments, the process of its enzymatic hydrolysis was studied with lysozyme and papain separately and at their combination. The highest content of low molecular weight peptides in the reaction medium occurred at enzymatic hydrolysis of biomass *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* by the composition of enzymes at a ratio of lysozyme : papain 1:2. At a concentration of enzymes 10 mg/cm^3, the content of low molecular weight peptides was 7.2 mg/cm^3 after eighth hour of incubation of the reaction mixture. The results of studies have been shown that the efficiency of enzymatic hydrolysis of autolysates is much higher. Thus, the amount of low molecular weight peptides in the hydrolysate obtained by processing the autolysate with the composition of lysozyme : papain 1:2 at an enzymes concentration 10 mg/cm^3 and the duration of the process for 8 hours by 36% higher than for similar hydrolysis parameters without the use of the process of autolysis.

The method of gel chromatography was proved that in the hydrolysate there are fractions of protein compounds with a molecular weight in the range of 70–90 kDa, 30–40 kDa and 294–650 Da. The molecular weight of the latter fraction corresponds to the mass of the muramyl dipeptide. The presence of muropeptides was proved by reaction with the Anthron reagent.

Key words: probiotics, biomass, autolysis, peptidoglycan, enzymatic hydrolysis, papain, lysozyme, low molecular weight peptides, muropeptides, immunotropic properties.

ОТРИМАННЯ ТА ХАРАКТЕРИСТИКА МУРОПЕПТИЛІВ КЛІТИННИХ СТІНОК ПРОБІОТИЧНИХ КУЛЬТУР

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Анотація. Розглянуто можливість отримання муропептидів пептидогліканів клітинних стінок *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* шляхом комбінації застосування автолітичних процесів та ферментативної обробки біомаси за участю лізоциму та папаїну. Встановлено, що найбільш значні автолітичні зміни біомаси мають місце при застосуванні високотемпературної обробки (90°С протягом 30 хв) на завершальній стадії логарифмічної фази росту бактерій. Так, на восьмій годині інкубації при 37°С вміст амінокислот у культуральному середовищі складав 1,8 мг/см^3, а при 90°С – 5,7 мг/см^3. Із метою подальшої деструкції автолізату біомаси та отримання низькомолекулярних фрагментів пептидоглікану, досліджували процес його ферментолізу лізоцимом та папаїном окремо та при їхній комбінації. Найвищий вміст низькомолекулярних пептидів у реакційному середовищі мав місце при ферментолізі біомаси *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 композицією ферментів при співвідношенні лізоцим:папаїн 1:2. При концентрації ферментів 10 мг/см^3 вміст НМП складав 7,2 мг/см^3 на 8-му годину інкубації реакційної суміші. Результати досліджень показали, що ефективність ферментолізу автолізату значно вища. Так, кількість НМП у ферментолізаті, який отримали при обробці автолізату композицією лізоцим:папаїн 1:2 при концентрації ферментів 10 мг/см^3 та тривалості процесу протягом 8 годин на 36% більша, ніж за аналогічних параметрів без застосування процесу автолізу.

Методом гель-хроматографії доведено, що у складі ферментолізату присутні фракції білкових сполук з молекулярною масою в межах 70–90 кДа, 30–40 кДа та 294–650 Да. Молекулярна маса останньої фракції відповідає масі мурамілдипептиду. Наявність муропептидів доведено за допомогою реакції з реактивом Антрона.

Ключові слова: пробіотики, біомаса, автоліз, пептидоглікан, ферментоліз, папаїн, лізоцим, низькомолекулярні пептиди, муропептиди, імунотропні властивості.

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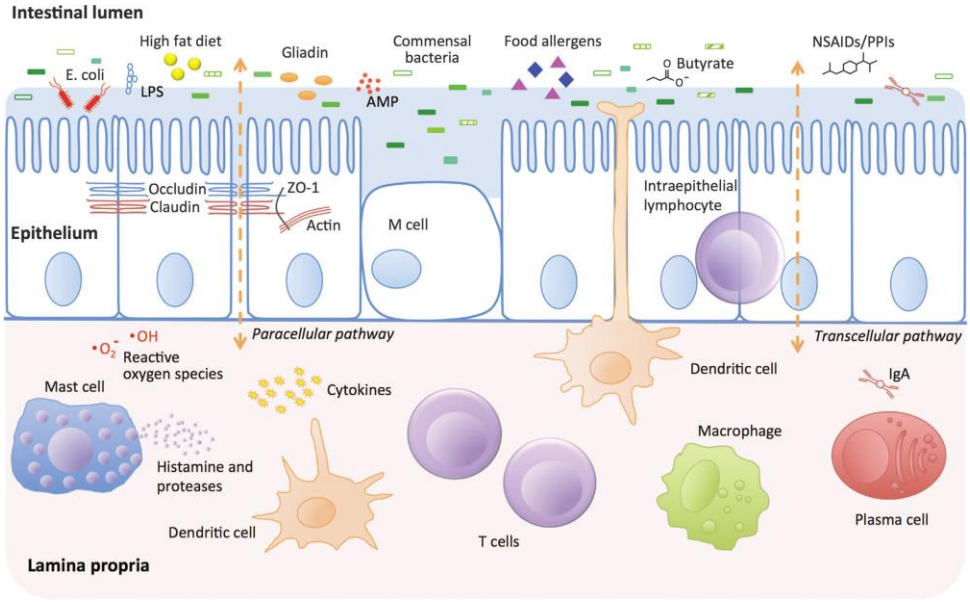
Introduction. Formulation of the problem

Probiotic bacteria are an important part of the healthy functioning of the human body. They provide immunomodulatory, antitumor action, contribute to lower cholesterol, to synthesize vitamins. Lactic acid and bifidobacteria (LAB and BB) are responsible for

the resistance of the gastrointestinal tract to pathogenic microorganisms, producing various organic acids, hydrogen peroxide, bacteriocin, short chain fatty acids, diacetyl, and the like. [1–4]. Probiotics promote an increase of immunity at the site of their primary location, that is, in the intestine through the recognition of their surface molecules by immunocompetent Toll-like receptors (TLRs) [5] (Fig. 1).



The intestinal barrier is composed of several layers providing protection against microbial invasion. The intestinal lumen contains anti-microbial peptides, secreted immunoglobulin A, and commensal bacteria, which inhibit the colonization of pathogens by competitive inhibition and by production of, e.g., butyrate, which has barrier-protective properties [6].

Fig. 1. Schematic figure of the intestinal barrier and affecting factors

The intestinal barrier is an obstacle for the penetration of the cells of probiotic cultures into the bloodstream, therefore, in recent years, more and more attention of scientists has attracted the question of the lysis of microbial cells in order to produce low molecular weight biologically active fragments of peptidoglycans of their cell walls, which are called muropeptides [7]. Exactly muropeptides promote the activation of the immune system, because they contain structures that are recognized by immunocompetent TLRs. In [8] it is shown that bacterial breakdown products can penetrate from the gut. Vavricka et al. hypothesized that the apical di/tri-peptide transporter hPepTl in the intestinal epithelium may transport muramyl dipeptides (MDP).

Quite difficult is the issue of the destruction of bacteria, especially grams of positive. Cell walls of microorganisms are extremely resistant to the effects of degrading factors. The role of the main protective barrier is peptidoglycan (PG), the fate of which determines the strength of bacterial cells [9]. Therefore, the development of accessible and simple methods for the destruction of cell walls of probiotic cultures in order to obtain their biologically active fragments – muropeptides are topical today.

Analysis of recent research and publications

Destruction of cell walls of microorganisms is carried out using physical, chemical or combined methods of exposure. As a rule, physical disintegration of microbial cells leads to irreversible violation of their anatomical integrity. In order to produce glycopeptide low molecular weight products of regular structure, chemical and enzymatic methods of degradation are usually used.

The enzymatic methods for the hydrolysis of peptidoglycans of cell walls of bacteria are milder compared with chemical ones. For the destruction of peptidoglycans of bacterial cell walls, it is advisable to use muramidases and proteases that can cleave glycoside and peptide bonds in its structure. In a number of works, combined methods of disintegration of lactobacilli and bifidobacteria are used.

In [10] the heat-killed cells were exhaustively digested with N-acetylmuramidase, followed by treatment with DNase, RNase and trypsin. The digest was then dialyzed against water and lyophilized. The PG fraction was subjected to gel filtration, and the hexosecontaining fractions obtained were designated.

In [11] *Lactobacillus* sp cells were placed in a boiling water bath for 20 min before they were broken by sonication. The suspension was centrifuged at 1 000 r/min for 15 min to sediment unbroken bacteria and the supernatant containing the cell walls was decanted. After the centrifugation at 10000×*g* for 10 min, the pelleted cell walls were incubated in 2% sodium dodecyl sulfate for 30 min at 60°C. After being washed four times with water and twice with dehydrated alcohol, covalently bound proteins were digested with 0.02% trypsin in 0.1 mol/L Tris-HCl buffer (pH 7.5) for 20 h at 37°C. The walls were washed several times

with water and lyophilized, resuspended in 10% trichloroacetic acid and stirred for 20 min at 60°C. PG was concentrated by centrifugation (10 000×*g*, 20 min) and washed extensively with water.

Peptidoglycan isolation method in [12] is based on the bacteria boiling in 1 M NaCl or in 0.25% sodium dodecylsulfate (SDS) solution. The resulting cell walls are washed with water and broken into smaller fragments by sonication. DNAse, RNAse, and trypsin are used to digest residual nucleic acids as well as cell wall bound proteins. The enzymes are inactivated by boiling and the cell walls are washed again with water. Treatment with 1 N HCl releases bound wall teichoic acids or other glycophosphates. Higher concentrations of HCl must be avoided, as they result in clumping of the sample. Washing the pellet until the pH is neutral results in pure PGN that can be digested by cell wall hydrolases. In our case the PGN was digested with mutanolysin for 16 hours at 37°C shaking.

Most of methods of obtaining muropeptides are quite complicated in execution, especially on an industrial scale. They are multistage, using specific and highly reactive reagents. It is worth noting that one of the ways to reduce the number of operations in obtaining biologically active components of the cell walls of bacteria is carry out autolysis. It's well known that the cell wall PG is the target of specific PG hydrolases (also named autolysins), synthesized by the bacteria themselves [13–16].

The **purpose** of the work is the obtaining and characteristic of immunotropic cell wall fragments of autolysate of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 biomass, that is muropeptides.

Research objectives:

1. to investigate the autolytic changes of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964;
2. to study the pattern of the enzymatic hydrolysis of autolysate of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 by hydrolases that catalyze specific bonds in the structure of the bacteria cell walls peptidoglycans;
3. to provide the characterization of the obtained low molecular weight degradation products by means of spectroscopy and gel-chromatography for the purpose of detection of target muropeptides.

Research Materials and Methods

Materials. The strain *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* from the collection of Scientific and production enterprise "Ariadna", Odessa (Ukraine) was maintained in 12% sterile reconstituted skim milk (RSM) supplemented with 2% glucose (Fluka RdH, Buchs, Switzerland) and 1% yeast extract (Fluka RdH, Buchs, Switzerland) and stored at –80°C. Working cultures were prepared from frozen cultures by three successive transfers in 12% (w/v) low-heat RSM before use, and stored at 4°C. Bacterial strain was grown at 37°C without shaking [14].

Enzymatic degradation of BM cells was performed by papain (10 Un/mg) and lysozyme (40,000 Un/mg) treatment.

Establishment of growth phases of Lactobacillus delbrueckii subsp. Bulgaricus B-3964. In order to obtain cell suspensions at different growth stages, a growth curve of the bacterial culture was constructed for 48 hours. For this purpose, the cultures were inoculated with the Koch method at intervals of one hour. The concentration of bacterial cells was determined by seeding tenfold dilutions on the surface of the MCA agar medium and expressed in colony forming units in 1 cm^3 (CFU/cm^3).

BM autolysis was carried out at the end of the logarithmic phase of growth at temperatures of 37–90°C (after eight hours of cultivation, during 30 min) and after stationary growth phase (24 hours of cultivation), maintaining it at temperatures of 37–90°C for 7 days.

The autolysates were cooled to room temperature after exposure, centrifuged for 10 min at 8000 min^{-1}, then decantation was performed. In the supernatant, the content of free amino acids was controlled by the method of formolithic titration [17], soluble protein by Benedict's method [17], low molecular weight peptides (LMWP) by the Benedict method after precipitation of high molecular-weight proteins by 10% solution of trichloroacetic acid.

Destruction of cells of bacterial composition. BM after autolysis processes was heated to a temperature of 90°C and kept for 15 min, then the mixture was cooled and enzymatic hydrolysis was carried out. The constant parameters of hydrolysis were temperature 37 C and pH=7,4. Hydrolysis was performed with lysozyme (Lysate I), papain (Lysate II) separately and with the composition of these hydrolases. The concentration of enzymes was varied from 1 to 20 mg/cm^3, the ratio of lysozyme : papain in the composition was varied from 2:1 to 1:2 (2:1 – Lysate III, 1:1 – Lysate IV, 1:2 – Lysate V). The duration of the incubation of the reaction mixture was varied in the range 0.5–24 hours. As a control, enzymatic hydrolysis was carried out without autolysis. In the obtained hydrolysates, the content of free amino acids, soluble protein, LMWP was monitored by the methods described above. Enzymatic hydrolysis was stopped by heating at the temperature 100°C, the mixture was cooled, centrifuged for 10 min at 8000 min^{-1}, decanted. The supernatant was subjected to ion exchange chromatography with cation exchanger KU-2 (column sizes: H=30 cm, D=1.8 cm) [18]. Received preparation contained amino acids and low molecular weight peptides and deprived of acids, neutral carbohydrates and salts. The presence of muropeptides in the composition LMWP was proved by the Anthron method.

The molecular weight of the soluble protein nature compounds of hydrolysate was determined by gel chromatography on column with sephadex G-15 and sephadex G-150. The column with sephadex G-15 (H=28 cm, D=2,8 cm, V=112 cm^3) was calibrated with markers of known molecular weight, namely: I – GMDP (MW 650 Da), II – aspartame (L-Aspartil-L-

phenylalanine, MW 294 Da), III – glycine (MW 75 Da). The column G-150 (H=38 cm, D=3,8 cm, V=121 cm^3) was calibrated by markers I – phosphorylase (97 kDa), II – bovine serum albumin (65 kDa), III – egg albumin (45 kDa), IV – carbohydrase (30 kDa), V – lactalbumin (14 kDa) [15].

Results of the research and their discussion

The kinetics of CFU accumulation of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 was investigated to determine the phases of their growth. It is known that the autolytic enzyme system exhibits its greatest activity during the exponential growth phase and is not detectable during the stationary phase [14,16]. The growth curve of the bacterial mass is shown in Fig. 2. Based on the data in Fig. 2, the completion of the logarithmic phase of growth is after 8-th hour of incubation, and the end of stationary phase is after 24 hours. From the literature, the LAB and BB are the most labile to degrading factors at the end of the logarithmic phase of growth, but for completeness of the experiment, autolytic changes were observed for seven days and at incubation at temperatures in range 37–90°C.

One of the simplest and most accessible methods for detecting of the destruction degree of bacterial cells is a definition of the free amino acids content. The dependence of the accumulation of amino acids in the medium of autolysates on the temperature and duration of the process are depicted in fig.3

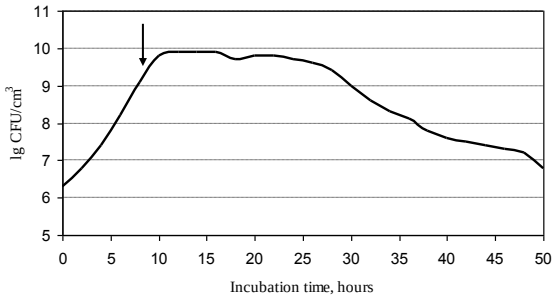


Fig. 2. The growth curve *Lactobacillus delbrueckii subsp. Bulgaricus B-3964*

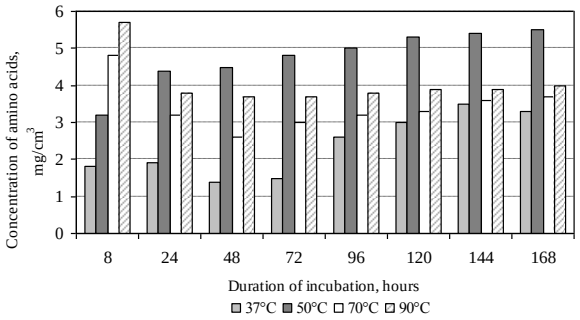


Fig. 3. Comparative dynamics of accumulation of amino acids in autolysates at different exposure temperatures

Results of research in Fig. 3 are demonstrated that the largest accumulation of amino acids occurs in the autolysate, which was obtained at the end of the logarithmic phase of the growth of the bacterial mass at exposures at a temperature of 90°C. In the autolysis of biomass after the stationary phase, the increase of amino acids in the reaction medium is linear in different incubation temperatures. The maximum content of amino acids occurs when exposed to samples at a temperature of 50°C.

Thus, the study of the dynamics of lactobacilli growth has allowed to establish specific time frames for the stages of their development. The most significant influence of high temperatures on the integrity of bacterial cells took place at eight hours of biomass incubation, as evidenced by the intense accumulation of free amino acids in the culture medium.

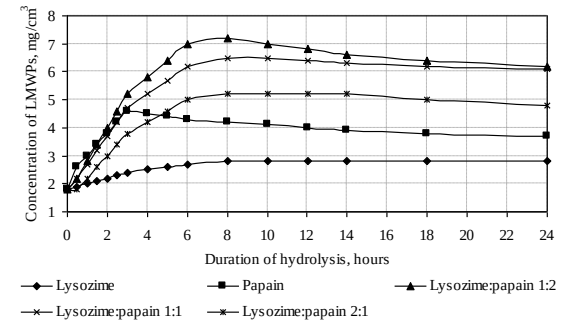
Thus, after eight hour incubation at 37°C, the amino acid content is 1.8 mg/cm^3, and after 90°C – 5.7 mg/cm^3. Based on the data of Fig. 2, eighth hour of biomass *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 incubation corresponds to the final stage of the logarithmic phase of bacterial growth. In this phase bacteria are most labile to aggressive influence factors.

The biomass behavior during incubation at 37°C for a long time in the aspect of the accumulation of free amino acids is rather ambiguous. Thus, in the range of 8–48 hours there is a decrease in the content of amino acids from 1.8 mg/cm^3 to 1.3 mg/cm^3, and after the 3rd day of biomass exposure an increase in the amount of amino acids is observed. This tendency can be explained by the fact that in the first 2-3 days of incubation in the culture medium there is a sufficient amount of viable cells that use nutrients, which are amino acids, for their own nutrition.

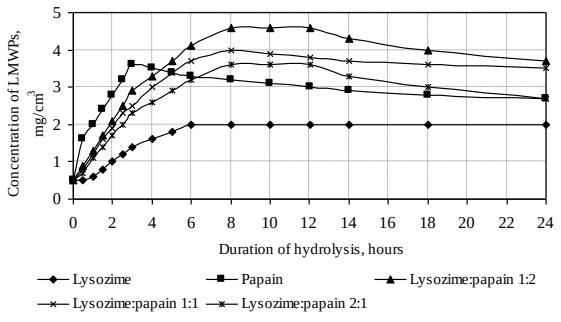
When incubating biomass at 50°C, a more stable picture of the accumulation of amino acids is observed. This is due to the fact that such a temperature is not optimal for maintaining the normal life activity of biomass, and, therefore, the amino acids released upon possible destruction of bacterial cells are not a nutrient medium for viable bacteria. But the literature data indicate that autolysins activates at temperature of 40–50°C that contribute to the degradation of bacteria. The highest content of amino acids in the culture medium at biomass exposures at 50°C was 5.5 mg/cm^3 on the seventh day of the experiment, which was not inferior to the values of autolysis (5.7 mg/cm^3 of amino acids), which was carried out after 8 hours of cultivation, exposing biomass temperature treatment at 90°C for 30 minutes. Therefore, it is more expedient to use the latter version of autolysis as the first stage of destruction of bacterial cells *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964.

In order to further destruction of the biomass autolysate and obtaining low molecular weight fractions of PG, the process of its enzymatic hydrolysis with lysozyme and papain separately and in combination of them was studied. With the disintegration of bacterial cells with a combination of hydrolases, processing by enzymes was carried out not in turns, but at the same time. Because in [19] it is established that in this case there is a synergistic effect of enzymes. In addition, the

total time of disintegration of cells is significantly reduced. To establish the parameters of destruction of cell walls *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964, in which there is a maximum accumulation of immunotropic LMWPs with a molecular weight <1500 Da, a series of experiments was conducted in which the concentration of enzymes in the reaction mixture and the exposure time were varied. It is known that peptides with a molecular weight of up to 1500 Da are not precipitated by trichloroacetic acid solutions and are compounds having high immunological activity [20–24]. The research results are shown in Fig. 4.5



a)

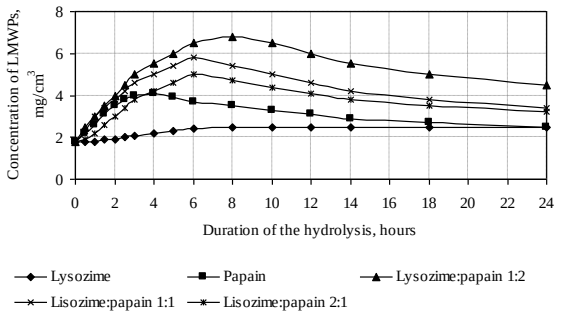


b)

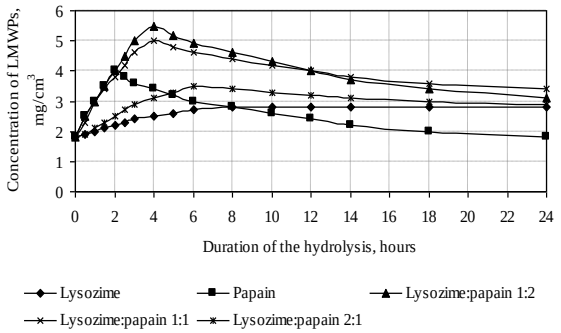
Fig. 4. Dependence of LMWPs accumulation at enzyme's concentration 10 mg/cm^3; a) using autolysis; b) without autolysis

Based on these figures, we can conclude that at the all enzymes concentration variations the same tendency is observed. Namely, the highest content of LMWs in the reaction medium takes place at the enzymatic hydrolysis of biomass

Lactobacillus delbrueckii subsp. Bulgaricus B-3964 by enzymes composition in the ratio lysozyme: papain 1: 2. At a concentration of enzymes 10 mg/cm^3, the content of LMWPs is 7.2 mg/cm^3 at 8 hours of incubation of the reaction mixture (Fig. 4a). When the concentration of enzymes decreases to 5 mg/cm^3, the content of LMWPs in the hydrolisate decreases insignificantly and amounts to 6.5 mg/cm^3 with the same duration of process (Fig. 5a). With an increase in the concentration of enzymes up to 20 mg/cm^3, the highest content of LMWPs in the hydrolisate is 5.5 mg/cm^3 at the 4th hour of the incubation of the reaction mixture (Fig. 5b).



a)



b)

Fig. 5. Dependence of LMWPs accumulation using autolysis at enzyme's concentration a); 5 mg/cm^3; b) 20 mg/cm^3

If combination lysozyme : papain is used at ratio 1:1, hydrolysis indicators are somewhat reduced in all variations of concentrations compared to combination lysozyme : papain 1:2. At the same time, the amount of LMWPs in hydrolysates obtaned by lysozyme : papain treatment at ratio 1:1 is on average less on 5–15%. When using the composition of lysozyme: papain 2:1, the content of LMWPs, as compared to the hydrolysate, obtained at biomass processing by combination lysozyme: papain 1: 2, is less on 30–40%. Reducing of the LMWPs amount in the reaction medium with an increase in the amount of lysozyme in the hydrolases composition can be explained by the fact that in the peptidoglycan structure of bacteria cell walls the amount of specific bonds between the remnants of the muramic acid and N-acetylglucosamine, which are targets for catalysis by lysozyme, are significantly less than peptide bonds [22].

Therefore, to produce low molecular weight muropeptides, it is advisable to use the composition of lysozyme and papain with the advantage of the latter, of course, given the unit of enzyme activity.

When the enzymatic hydrolysis of *Lactobacillus delbrueckii subsp. Bulgaricus* B-3964 biomass was carried out with lysozyme and papain separately, a higher content of LMWPs was in the hydrolysate obtained with the use of papain.

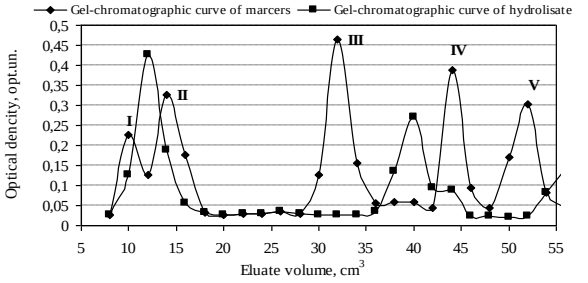
Papain has a wide range of substrate specificity and is capable to hydrolyse the peptide bonds constructed from residues of amino acids inherent in peptidoglycans of bacterial origin. But if we compare the enzymatic hydrolysis of biomass by papain with

the hydrolysis by hydrolases combination at the ratio of lysozyme: papain 1: 2, the content of LMWPs in the hydrolyzate is 35–40% lower.

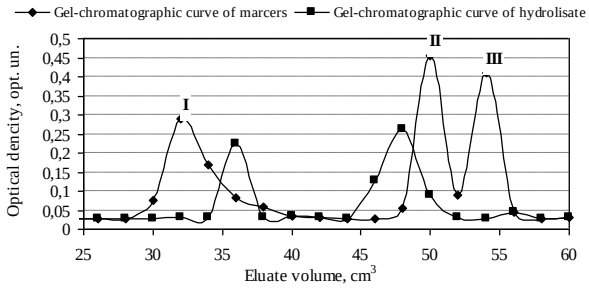
In control experiments, enzymatic hydrolysis of biomass was carried out, which was not subjected to pretreatment after 8 hours of cultivation at 90°C for 30 minutes. The results of studies have shown (Figs. 4a, b) that the efficiency of enzymatic hydrolysis of biomass autolysates is much higher than that of biomass, which was not subjected to autolysis. Thus, the amount of LMWPs in the hydrolyzate obtained by treatment of autolysate with the combination lysozyme : papain 1: 2 at an enzymes concentration of 10 mg/cm^3 and the duration of the process for 8 hours is on 36% higher than under similar conditions without the use of autolysis.

Consequently, autolytic changes in biomass significantly affect the effectiveness of enzymatic degradation of bacterial cells. As a result of autolysis, the surface protective layer of lactobacilli is destroyed, which is a barrier for external biological degrading factors, to which enzymes belong. Such changes lead to the fact that the specific bonds of bacterial peptidoglycan that forms their cell wall and the content of which can reach up to 70% of the total mass of gram-positive bacteria become more accessible to proteases and muramidases that catalyse these bonds.

At the next stage, the molecular-weight distribution of the proteinaceous compounds of the hydrolysate was determined to detect the structural components of bacterial cell walls corresponding to the molecular weight of the compounds of the muropeptide series (up to 1500 Da). The liquid phase of the hydrolysate was previously subjected to ion exchange chromatography in order to deprive from the by-products of degradation, metabolites or nutrients of the culture medium. The results of investigations of the hydrolysate liquid phase on the gel-chromatography column with the Sephadex G-150 are shown (Fig. 6a) the presence of three main protein fractions in it, as evidenced by the presence of three distinct peaks on the gel-chromatographic curve corresponding to fractions of molecular weight in within the limits of 70–90 kDa, 30–40 kDa and a fraction whose molecular weight is less than 14 kDa. For a more detailed study of the latter fraction, at the next stage, its molecular-mass distribution was investigated (Fig. 6b). There are two pronounced peaks on the gel chromatographic curve of the low molecular weight fraction of hydrolysate on the 36th, 50th cm^3 of elute, which are between the peaks of markers with molecular weights of 294–650 Da. The results of the studies allow us to state that in the obtained hydrolysate there are peptides with molecular weight in the range corresponding to the molecular weight of the muropeptides, namely MDP. Exactly MDP initiates a signal of cascade reactions that leads to the synthesis of proinflammatory cytokines by immunocompetent cells and activation of immunological defense mechanisms of the organism [23–28].



a)



b)

Fig. 6. Molecular weight distribution of low molecular compounds of protein nature
a) column with Sephadex G-150,
b) column with sSphadex G-15

Since the gel chromatography method was proved that the low molecular weight compounds of peptide nature are presented in the resulting hydrolysate, it is advisable to carry out studies on identification of the muropeptides in their composition. The presence of the compounds of the muropeptide series was determined by reaction with the Anthron reagent, which is based on the fact that the carbohydrate part of the muropeptides (muramic acid and N-acetylglucosamine), by the action of concentrated sulfuric acid, is converted to furfural, or its derivatives, which, by condensation with the reagent Anthon, form compounds of green color. These manipulations were performed with samples that were previously subjected to ion exchange chromatography in order to get rid of neutral carbohydrates that could interfere with the purity of the experiment. The results of the studies are presented in Table 1, where lysates I–V were obtained by enzymatic hydrolysis of biomass autolysate with the ratio of enzymes described in the section "Materials and Methods of Research". The concentration of enzymes was 10 mg/cm^3, the duration of hydrolysis was 8 hours.

Table 1 – Characteristics of lysates of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* (n=3, p≥0,95)

Samples	Characteristics of hydrolyzates	
	LMWPs, mg/cm^3	LMWPs of muropeptide serias,% from total LMWPs
Autolysate	1,83	5.48
Lysate I	2.85	13.52
Lysate II	4.28	18.41
Lysate III	7.21	43.46
Lysate IV	6.52	32.12
Lysate V	5.22	22.46

So, based on the data of the table. 1, it can be stated that among all samples there are low molecular weight muropeptides. The most higher content of muropeptides (43.46% of total LMWPs) occurs during hydrolysis of biomass Lactobacillus delbrueckii subsp. Bulgaricus B-3964 with a ratio of lysozyme: papain 1: 2. It should be noted that with such parameters of enzymatic hydrolysis by the combination of hydrolases, the maximum amount of total LMWPs is accumulated. Obviously, such hydrolysis regimes provide the highest degree of enzymatic degradation of the peptidoglycans of the cell walls of the biomass under study.

Conclusions

1. Autolysis processes of biomass *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* at the end of the logarithmic and after the stationary phase of growth are significantly differentiated. The autolysis is most effectively at the end of the logarithmic phase of biomass growth at a temperature of 90°C. This is, obviously, due to the fact that during this period, biomass is most vulnerable to the effects of external stress. After the stationary phase of growth, autolysis of biomass also takes place, but the maximum accumulation of free amino acids occurs at incubation at a temperature of 50–70°C, which may be due to the activation of the action of bacterial autolysins and the partial denaturation of the protein component.

2. The highest content of low molecular weight peptides in the reaction medium occurred at enzymatic hydrolysis of biomass *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* by the composition of enzymes at a ratio of lysozyme : papain 1:2. At a concentration of enzymes 10 mg/cm^3, the content of low molecular weight peptides was 7.2 mg/cm^3 after eighth hour of incubation of the reaction mixture. The results of studies have been shown that the efficiency of enzymatic hydrolysis of autolysates is much higher. Thus, the amount of low molecular weight peptides in the hydrolysate obtained by processing the autolysate with the composition of lysozyme : papain 1:2 at an enzymes concentration 10 mg/cm^3 and the duration of the process for 8 hours by 36% higher than for similar hydrolysis parameters without the use of the process of autolysis.

3. The method of gel chromatography was proved that in the hydrolysate there are fractions of protein compounds with a molecular weight in the range of 70–90 kDa, 30–40 kDa and 294–650 Da. The molecular weight of the latter fraction corresponds to the mass of the muramyl dipeptide. The presence of muropeptides was proved by reaction with the Anthron reagent.

List of references

1. Erickson K.L., Hubbard N.E. Probiotic immunomodulation in health and disease // J Nutri. 2000. V. 130. P. 403-409.
2. Глушанова Н.А. Биологические свойства лактобацилл // Бюллетень сибирской медицины. 2003. №4. С. 50-58
3. Стоянова Л.Г., Устюгова Е.А., Нетрусов А.И. Антимикробные метаболиты молочнокислых бактерий: разнообразие и свойства (обзор) // Прикладная биохимия и микробиология. 2012. Том 48. №3. С. 259-275
4. Молохова Е.И., Сорокина Ю.В. Разработки отечественных метаболитных пробиотиков и их стандартизация // Сибирский медицинский журнал. 2011. Т. 26. № 15. С. 29-33.
5. Recognition of commensal microflora by Toll-Like receptors is required for intestinal homeostasis / Rakoff-Nahoum S. // Cell. 2004. V. 118. P. 229-241.
6. Human Intestinal Barrier Function in Health and Disease / Julia Konig et al. // Clinical and Translational Gastroenterology. 2016. V 7(196). DOI:10.1038/ctg.2016.54
7. Traub S. MDP and other muropeptides – direct and synergistic effects on the immune system. J. Endotoxin Res. 2006. V. 12(2).P. 69-85. DOI:10.1179/096805106X89044
8. hPepTl transports muramyl dipeptide, activating NF -kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells / Vavricka S.R. et al. // Gastroenterology. 2004. V. 127. P. 1401-1409.
9. Chapot-Chartier M.-P., Kulakauskas S. Cell wall structure and function in lactic acid bacteria // Microbial Cell Factories. 2014. 13(Suppl 1):S9. P.1-23. DOI: 10.1186/1475-2859-13-S1-S9.
10. A component of polysaccharide peptidoglycan complex on Lactobacillus induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer / Matsumoto S. et al. // Immunology. 2009. DOI: 10.1111/j.1365-2567.2008.02942.x
11. Ku¨hner, D., Stahl, M., Demircioglu, D.D., Bertsche, U. From cells to muropeptide structures in 24 h: Peptidoglycan mapping by UPLC-MS. // Sci. Rep. 2014. V. 4. P. 74-94. DOI:10.1038/srep07494
12. Analysis of the Peptidoglycan Hydrolase Complement of Lactobacillus casei and Characterization of the Major c-D-Glutamyl-L-Lysyl-Endopeptidase / Regulski K. // PLoS ONE. 2012. V. 7(2). e32301. DOI:10.1371/journal.pone.0032301
13. Requirement of Autolytic Activity for Bacteriocin-Induced Lysis / Martinez-Cuesta M.-C. // Applied and environmental microbiology. 2000. Aug., P. 3174-3179 DOI: 10.1128/AEM.66.8.3174-3179.2000
14. Simova E., Beshkova D. Effect of growth phase and growth medium on peptidase activities of starter lactic acid bacteria // Le Lait, INRA Editions. 2007. V. 87 (6). P. 555-573. DOI: 10.1051/lait:2007036
15. Kapustian A., Cherno,N. Obtaining and characteristic of the autolysate of lactic acid bacteria // EUREKA: Life Sciences. 2018. V.1. P. 24-31. DOI: 10.21303/2504-5695.2018.00558
16. Wood B.J., Warner P. J. Genetics of Lactic Acid Bacteria. 2012.
17. Биохимия белков: практикум для студентов биол. Фак. спец. 1-31 01 01 «Биология» / авт.И. В. Семак, Т. Н. Зырянова, О. И. Губич. Минск: БГУ, 2007. 49 с.
18. Патент на корисну модель № 100637 Україна МПК (2011.01) C12N 11/00, Спосіб одержання глікопептидного продукту із клітинних стінок бактерій заявл. 29.09.2014; опубл. 10.08.2015 Бюл. № 15.
19. Гаврилин М.В., Сеньчукова Г.В., Сенченко С.П. Выбор оптимальных условий получения гидролизатов молочнокислых бактерий термокислотным способом // Хим.-фарм. журн. 2007. Т. 41, № 2. С. 54-56.
20. Fournier B., Philpott D.J. Recognition of Staphylococcus aureus by the Innate Immune System. Clinical Microbiology Reviews. 2005. V. 18(3). P. 521-540. DOI:10.1128/CMR.18.3.521-540.2005
21. Kawai T., Akira S. The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat. Immunol. 2010. V. 11. P. 373-384. DOI:10.1038/ni.1863
22. How the Immune System Recognizes Self and Nonself. Immunoreceptors and Their Signaling / Harris G. et al. // Dordresht, the Netherlands: Springer. 2008. 251. DOI:10.1007/978-4-431-73884-8

23. Qingshan Lv. MDP Up-Regulates the Gene Expression of Type I Interferons in Human Aortic Endothelial // Cells. Molecules. 2012. V. 17. P. 3599-3608. DOI:10.3390/molecules17043599
24. Matsui K., Ikeda R. Peptidoglycan in combination with muramyldipeptide synergistically induces an interleukin-10-dependent T helper 2-dominant immune response // Microbiol Immunol. 2014. V. 58. P. 260-265. DOI:10.1111/1348-0421.12139

References

1. Erickson KL, Hubbard NE. Probiotic immunomodulation in health and disease. J Nutri 2000; 130: 403S-409S].
2. Glushanova NA. Biologicheskie svoystva laktobatsill. Byulleten sibirskoy meditsinyi. 2003;4: 50-58
3. Stoyanova LG, Ustyugova EA, Netrusov AI. Antimikrobnyie metabolityi molochnokislyih bakteriy: raznoobrazie i svoystva (obzor). Prikladnaya biohimiya i mikrobiologiya. 2012; 48(3): 259-275
4. Molohova EI, Sorokina YuV. Razrabotki otechestvennyih metabolitnyih probiotikov i ih standa-rtizatsiya. Sibirskiy meditsinskiy zhurnal. 2011; 26(1): 29-33.
5. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by Toll-Like receptors is required for intestinal homeostasis. Cell 2004;118: 229-241].
6. Julia Konig et al. Human Intestinal Barrier Function in Health and Disease Clinical and Translational Gastroenterology. 2016; 7: e196; DOI:10.1038/ctg.2016.54
7. Traub S. MDP and other muropeptides – direct and synergistic effects on the immune system. J. Endotoxin Res. 2006;12(2): 69-85. DOI:10.1179/096805106X89044
8. Vavricka SR, Musch MW, Chang JE et al. hPepTl transports muramyl dipeptide, activating NF -kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. Gastroenterology. 2004; 127: 1401-1409.
9. Chapot-Chartier M.-P, Kulakauskas S. Cell wall structure and function in lactic acid bacteria // Microbial Cell Factories. 2014; 13 (Suppl 1):.1-23. DOI: 10.1186/1475-2859-13-S1-S9.
10. Matsumoto S et al. A component of polysaccharide peptidoglycan complex on Lactobacillus induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer. Immunology 2009. DOI: 10.1111/j.1365-2567.2008.02942.x
11. Ku¨hner D, Stahl M, Demircioglu DD, Bertsche U. From cells to muropeptide structures in 24 h: Peptidoglycan mapping by UPLC-MS. Sci. Rep. 2014;.4: 74-94. DOI:10.1038/srep07494
12. Regulski K, Courtin P, Meyrand M, Claes IJJ, Lebeer S, et al. Analysis of the Peptidoglycan Hydrolase Complement of Lactobacillus casei and Characterization of the Major c-D-Glutamyl-L-Lysyl-Endopeptidase. PLoS ONE. 2012; 7(2): e32301. DOI:10.1371/journal.pone.0032301
13. Martinez-Cuesta M-C, Kok J, Herranz E, Pelaez C, Requena T, Buist G. Requirement of Autolytic Activity for Bacteriocin-Induced Lysis. Applied and environmental microbiology. 2000;Aug: 3174–3179. DOI: 10.1128/AEM.66.8.3174-3179.2000
14. Simova E, Beshkova D. Effect of growth phase and growth medium on peptidase activities of starter lactic acid bacteria. Le Lait, INRA Editions. 2007; 87(6):555-573. DOI: 10.1051/lait:2007036
15. Kapustian A, Cherno N. Obtaining and characteristic of the autolysate of lactic acid bacteria. EUREKA: Life Sciences. (2018); 1: 24-31. doi: 10.21303/2504-5695.2018.00558
16. Wood, B.J., Warner, P. J. Genetics of Lactic Acid Bacteria; 2012.
17. Semak IV, Zyryanova TN, Gubich OI. Biohimiya belkov: praktikum dlya studentov biol. Fak. spec. 1-31 01 01 «Biologiya». Minsk: BSU; 2007.
18. Патент на корисну модель № 100637 Україна МПК (2011.01) C12N 11/00, Спосіб одержання глікопептидного продукту із клітинних стінок бактерій заявл. 29.09.2014; опубл. 10.08.2015 Бюл. № 15.
19. Gavrilin MV, Sen'chukova GV, Senchenko SP. Vybor optimal'nyh uslovii polucheniya gidrolizatov molochnokislyh bakterii termokislotnym sposobom. Him.-farm. Zhurn. 2007; 41(2): 54-56.
20. Fournier B, Philpott DJ. Recognition of Staphylococcus aureus by the Innate Immune System. Clinical Microbiology Reviews. 2005; 18(3): 521-540. DOI:10.1128/CMR.18.3.521-540.2005
21. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat. Immunol. 2010; 11: 373-384. DOI:10.1038/ni.1863
22. Harris G et al. How the Immune System Recognizes Self and Nonself. Immunoreceptors and Their Signaling. Dordresht, the Netherlands: Springer; 2008. DOI:10.1007/978-4-431-73884-8
23. Qingshan Lv. MDP Up-Regulates the Gene Expression of Type I Interferons in Human Aortic Endothelial Cells. Molecules. 2012; 17: 3599-3608. DOI:10.3390/molecules17043599
24. Matsui K, Ikeda R. Peptidoglycan in combination with muramyldipeptide synergistically induces an interleukin-10-dependent T helper 2-dominant immune response. Microbiol Immunol. 2014; 58: 260-265. DOI:10.1111/1348-0421.12139

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