ISSN 2308-4057. Foods and Raw Materials, 2016, vol. 4, no. 2

EVALUATION OF ANTIMUTAGENIC AND ANTIFUNGAL PROPERTIES, PARAMETERS OF ACUTE TOXICITY AND SENSITIZING ACTIVITY OF ENZYMATIC WHEY PROTEIN HYDROLYSATE

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Received June 01, 2016; Accepted in revised form August 27, 2016; Published December 30, 2016

Abstract: Biologically active peptides with antioxidative, antibacterial, immunomodulating and other properties result from the reaction between the whey proteins and proteolytic enzymes of the gastrointestinal tract or purified proteases. This work aims to determine antimutagenic and antifungal effect of the enzymatic hydrolysate of whey protein obtained, to assess its acute toxicity characteristics and sensitizing power. Antimutagenic action of native whey proteins and hydrolysates (test sample and hydrolyzate analog) was assessed by the Ames test using indicator strains of *Salmonella typhimurium* TA 98 and TA 100. When determining the antifungal activity, opportunistic strains of *Aspergillus niger* and *Candida albicans* were used. The toxicity degree of samples was defined in studies to evaluate the acute intragastric toxicity in white rats as well as in the single abdominal dose study on the white mice. Irritating influence of whey proteins and peptides on was evaluated when applied to the eye mucosa of rabbits. The sensitizing capacity of samples was evaluated using the experimental model to reproduce the delayed hyperresponsiveness in white mice. It is identified that the developed hydrolisate is classified as the safe agent and has low sensitizing ability. The sample obtained has the comparative values of antioxidant and antimutagenic activity level as compared with the analog "Vital Armor H 801 LB" (Armor Protéines, France) used to manufacture functional products. The advantages of the hydrolisate developed include the increase in the content of peptide fraction and more pronounced antifungal activity towards *A. niger*.

Keywords: whey proteins, enzymatic protein hydrolisates, peptide profile, antimutagenic activity, antifungal effect, antioxidant properties, acute toxicity, sensitizing activity

DOI: 10.21179/2308-4057-2016-2-38-47 *Foods and Raw Materials*, 2016, vol. 4, no. 2, pp. 38–47.

INTRODUCTION

Milk proteins (casein and whey fractions) are the precursors of the wide range of biologically active peptides with the immunomodulating, antioxidant, antimutagenic (gene protector), hypotensive, antimicrobial, antiviral, antitumoral effect [1, 2].

Genotoxicity is identified as the general biologic feature of various factors to cause the destructing effect to the genetic structures of the body. Search of natural antimutagens is advantageous when they are able to prevent the genotoxic effect of environmental factors on the human genetic structures [3]. Antimutagenesis is the biological process that suppresses the mutation characterized in the reduction of spontaneous and induced mutation affected by natural and synthetic compounds [4]. Most data on the effect of biologically active compounds on spontaneous and induced mutagenesis are obtained from organism studies. The Ames test is widely applied in the laboratory practice to evaluate the mutagenic features of chemicals, pharmaceutical agents, environmental medium. The test is based on the count of reverse mutations to prototrophy in histidine for *Salmonella typhimurium* strain [5, 6].

It is known that alimentary proteins and peptides are of great significance for anti-infectious defense mechanism that is caused by the stimulated immune system and inhibition of the growth of pathogenic and opportunistic pathogens (antimicrobial and antifungal effects) [7]. In general, antimicrobial peptides are

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positively charged; they have amphiphilic properties and specific secondary structure [8]. The micro-organism sensitivity relates to membranous components that contribute to peptide transport through the outer membrane. However, destruction of the outer membrane structure is not the major factor resulting in the death of microbial cell. Peptides penetrate the outer and cytoplasmic membranes and cause antimicrobial effect resulting from the multifactor mechanism including the impact on several intracellular ion targets [9].

To obtain enzymatic hydrolisates with preset parameters (peptide profile, biological activity), various endopeptidases and exopeptidases, of which the enzymes of microbial (alcalase, neutrase), plant (papin, ficine) and animal (pepsine, trypsin) origin may be isolated [10]. Distinctive features of enzymatic segregation of protein substrates are defined by the best efficient conditions for catalytic activity of the enzyme, its substrate and site specificity, physical and chemical parameters of proteins segregated.

Urgency of studies is associated with the demand in improvement of processes to prepare enzymatic hydrolisates of milk proteins with the required physical and chemical parameters and biologically active parameters for functional food products.

The scientific novelty lies in identification of new data on antifungal and antimutagenic effect of certain hydrolisates of serum proteins, as well as in the use of comprehensive approach to specify the correlation between the physical and chemical parameters and biologically active properties (degree of hydrolysis, antioxidant and antimutagenic activity) of native whey proteins and products of their hydrolysis.

The study was aimed to describe the biologically active properties (antimutagenic and antifungal effects) of the enzymatic hydrolisate of serum proteins obtained, define parameters of its acute toxicity and senstizing power.

OBJECTS AND METHODS OF STUDY

Production of enzymatic hydrolisate of whey proteins

The whey protein concentrate was used for the enzymatic hydrolysis obtained by ultrafiltration (WPC–UF–80, TU BY 100377914.550–2008) with protein content equal to 80 %, and the serine protease (alcalase, EC 3.4.21.62, protease from *Bacillus licheniformis*, with 2.64 U/g activity; Sigma, USA). To obtain the test hydrolisate sample, 8 % of WPC–UF–80 solution was prepared; the protein substrate was thermally treated and cooled to the temperature best suitable for hydrolysis. The enzymatic agent was added to the heat-treated solution obtained; then, the solution was hydrolised in temperature-controlled conditions. Upon the proteolysis completion, the enzyme was heat-inactivated; the liquid hydrolisate obtained was then allowed to dry (procedure as per [11]). The Table 1 shows characteristics of organoleptic and physical and chemical parameters of the hydrolisate obtained [12].

Table 1. Organoleptic and physical and chemical parameters of whey protein hydrolysate

Parameter	Parameter value
Appearance and consistency	Powder, yellow to creamy
Flavor and odor	Flavor typical to milk Poor bitter milk taste
Solubility	Water-soluble
Active acidity, pH (1 % solution)	6.7
Total protein w/w%	80.0
Peptide profile: Fragments with molecular mass > 10 kDa, %	2*
Native whey proteins	Not detected**
Residual antigenicity, 10^{-3} per unit value.	1.22 ± 0.07
Antioxidative activity, µmol of trolox / mg of protein	0.551 ± 0.035

Note. * − Parameter values are found through determination of general nitrogen in the hydrolisate and ultrafiltrate obtained by using Amicon Ultra–4 10K filters (Millipore, USA) with 10 kDa permeability. ** − As per SDS electrophoresis-data, HPLC and mass spectrometry [12].

Analysis of bioactive properties of the obtained hydrolisate

Method to determine the antimutagenic activity of enzymatic hydrolisates of whey proteins (as per requirements [13]). In the course of short-term analysis to define antimutagenic properties, indicator strains *Salmonella typhimurium* TA 98 and TA 100 (Ames test) were used. These strains are auxotroph mutants for histidine. Antimutagenic effect in the test preparation is identified by the reduction in frequency of reverse mutations from auxotrophy in histidine to prototrophy. In this analysis, positive results indicate that the test compound induces gene mutations of base pair replacement (for TA 100 strain) or reading frame shift (for TA 98 strain). Negative results indicate that the test compound has no mutagenic effect in these conditions on *S. typhimurium* strains used. Antimutagenic activity in native whey proteins (WPC) and the hydrolysate obtained was assessed. The whey protein hydrolisate "Vital Armor H 801 LB" (Armor Protéines, France) was used as the reference sample.

Test samples (WPC and hydrolysates) were assessed for antimutagenic properties when the following was added to the mutagen test-system in the volume of 10 µg/plate: etidium bromide (when exposed to TA 98 strain) and sodium azide (when exposed to

TA 100). The quantity of revertants is obtained when 1.88–30 μg of test compounds was added to the plate; 3 plates were used for each control and test samples.

Culture media components: microbiologic agar-agar (3AO "Pyat' okeanov" CJSC, RF), glucose (as per GOST 6038–79), beef-extract broth (as per GOST 20730–75), beef-extract agar (as per GOST 29112–91), trisodium citrate (as per GOST 31227–2004), $K_2HPO_4 \times 3H_2O$ (as per GOST 2493–75), KH_2PO_4 (as per GOST 4198–75), $(NH_4)_2SO_4$ (as per GOST 9027–82), $MgSO_4 \times 7H_2O$ (as per GOST 20490–75), NaC1 (as per GOST 4233–77), KCl (as per GOST 4234–77); biotin, histidine, L-tryptophan, sodium azide, etidium bromide (Merck, USA).

The standard microbiological laboratory equipment was used for the study purposes as follows: refrigerated heating circulator, electronic laboratory balance, control water bath GFL 1031 (GFL, Germany), pH–meter Hanna pH–211 (Hanna Instruments, Germany), thermal hygrometer IVA–6 (Scientific-Industrial Complex "Mikrofor", RF), Vortex mixer (IKA, Germany). Test conditions: temperature – 21–22ºC, humidity – 63–67%, pressure – 737–742 Hg mm.

Media and solutions used. All media and solutions were made using the distilled water. Upon preparation, they were autoclaved. Beef-extract agar containing 0.6%, 1.5% and 2% agar: 6, 15 and 20 g of agar, accordingly, are added to 1 L of the beef-extract broth. Aqueous agar (2 %): 20 g of agar is dissolved in 1000 ml of water. Evaporate concentrate: 2.0 g of trisodium citrate, 42.0 g of $K_2HPO_4 \times 3H_2O$, 18 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$ are diluted with water to make up 1 L (pH 7.2). Glucose solution (20%): 200 g of D-glucose is dissolved in 800 ml of water. The minimal medium is prepared based on the evaporate concentrate that is 4-fold diluted with water. Seletive agar to assess mutagenesis: 300 ml of aqueous agar, 100 ml of evaporate concentrate, 10 ml of glucose solution and 2 ml of 2% aqueous liquid of $MgSO_4 \times 7H_2O$ are mixed. Semiliquid minimal agar (0.7%): 7 g of agar and 6 g of NaC1 are diluted with water to make up 1 L. Histidine solution (0.5 mmol): 9.6 mg of histidine is dissolved in water to make up a final volume of 100 ml. Biotin solution (0.25 mmol): 6.1 mg of biotin is dissolved in water to make up a final volume of 100 ml. Surface semi-liquid semi-enriched agar: 80 ml of semi-liquid minimal agar (0.7%), 10 ml of histidine solution and 10 ml of biotin solution were mixed well. The mixture is poured in 2 ml tubes. Potash chloride solution (0.15 mol): 11.5 g of KCl is diluted with water to make up 1 L.

Test description (without metabolic activation). 0.1 ml of test agents (WPC solutions and hydrolysates with the protein concentration 18.8–300 μg/ml) is put in tubes with agar. Then, 0.1 ml of bacteria suspension, positive control (mutagen solution) are added, quickly mixed in the mixer and poured out over the layer of the bottom minimal agar in Petri dish. An even and thin layer of the semi-liquid agar covers the surface of the bottom agar. The Petri dish is kept at the room temperature for 30−40 min and thermostated, upon full jelling, at 37°C. The results are read in 48 h upon incubation. The relevant volume of the solvent (physiological solution) is added into the layer of the top semi-liquid agar with the bacteria suspension for the control sample. The test is proved with positive control. 3 replications are made for each control and test samples.

Decrease of mutation level (I_m, %) was assessed by the formula:

$$I_m = 100 - \frac{N_1}{N_2} \times 100,$$

where N_1 is the number of revertants in an experiment, N_2 is the number of revertants in positive control.

Statistic result analysis. To validate test results, calculations were made by the method of Dunnett's multiple comparisons [14]. The significance of revertants reduced in number is assessed in test versus control samples. The efficiency and sustainability of the method of Dunnett's multiple comparisons versus pair-wise comparison method for test and control samples are proved by the reduction in the rate of false-positive and false-negative results recorded, since the type I error probability (usually 5%) may be specified for the entire test by this method but not only for selected comparison. This approach does not consider using experiments with zero value of revertant number when testing. This is feasible in practice when handling substances with bactericidal effect, or in case of procedure error.

Method to determine antimicrobial (antifungal) activity of whey proteins and peptides. Opportunistic strains *Aspergillus niger* and *Candida albicans*, obtained from the bank of the Educational Institution “Belorusian State Medical University” as the test subject.

To prepare culture media, pancreatic casein hydrolysate (as per TU (Standard Specifications) 9385–002–00479327–94), peptone for bacteriological culture media (manufactured by the “Pharmacotherapy Research and Development Center” CJSC, Russia), glucose (as per GOST 6038–79), dry nutrient broth for microorganism cultivation (GRM-broth based on pancreatic hydrolysate of fish meal, as per TU 9398–021–78095326–2006) and microbiological agar-agar (manufactured by "Pyat' okeanov" CJSC, Russia).

The test sample of the milk whey protein hydrolysate was assessed for antimicrobial properties. Whey protein concentrate (WPC–UF–80, TU BY 100377914.550–2008; primary substrate to obtain hydrolysates) was used as the reference sample. Whey protein hydrolysate "Vital Armor H 801 LB" (Armor Protéines, France) was used as the standard.

Media and solutions used. Opportunistic fungal pathogenes (*A. niger* and *C. albicans*) were cultured on the agarized native culture media (Saburo and FMH-medium) in the course of the study to assess he antifungal effect. To obtain Saburo medium, 0.5 g of pancreatic casein hydrolysate, 0.5 g of peptone, 2 g of glucose and 1.5 g of agar-agar were dissolved in 100 ml of distilled water. To prepare the medium based on pancreatic hydrolysate of fish meal, 3.8 g of dry

nutrient broth to cultivate microorganisms and 1.7 g of agar-agar were dissolved in 100 ml of distilled water. The active acidity of solutions was made up to 7.0 pH and then, the solutions were autoclaved at 0.5 atm for 30 minutes (FMH-medium) and 60 minutes (Saburo).

WPC solutions and whey protein hydrolysates were used in the physiological solution with the protein original concentration of 50 mg/ml. To eliminate microbiological contamination, the solutions obtained were twice filtered using polypropilene filters (Rolitabo®-syringe filters, Ø 25 mm, 0.45 µm; Carl Roth, Germany).

Test description. Mycelium fragment *A. niger* or *C. albicans* was cultured in Petri dishes with the culture medium (Saburo or fish hydrolysate-based medium), containing 5.0 mg/ml of WPC and whey protein hydrolysates. Then, they were cultivated in the thermostat at 37 ºC for 4 days. Petri dishes with the medium without the tested protein component (WPC and whey protein hydrolysates) were used as the reference. Three Petri dishes were used for each control and test samples. The degree of inhibition (*DI*, %) of the mycelium growth was assessed by the formula:

$$DI = 100 - \frac{d}{d_k} \times 100,$$

where d is the arithmetic mean value of the fungal mycelium diameter of the test sample (with WPC and whey protein hydrolysates added), $n = 3$; d_k is the arithmetic mean value of the fungal mycelium diameter of the control sample, $n = 3$.

The results of quantitative test data processing are shown as the arithmetic mean value of inhibition (*I*, %), calculated for three independent experiments.

The toxicological-hygienic assessment of WPC and enzymatic whey proteins hydrolysate (EWPH). The degree of toxicity effect of native whey proteins and products of their enzymatic hydrolysis was determined in tests on the white rats and mice. The irritating effect of WPC and EWPH on eye mucosa of rabbits was evaluated. The sensitizing effect of samples was analysed using the experimental model to reproduce the delayed hyperresponsiveness in white mice. Experiments were made to comply with requirements of technological regulations and using new practices and customization of existing ones [15–17]. Toxicological and hygienic study of submitted samples was performed on laboratory animals as follow: white mice, non-linear male and female white rats (baseline body weight is 180–220 g) and albino rabbits (2500–3000 g) supplied from the vivarium of the Republican unitary enterprise "Applied Research Centre of Hygiene". The study design, scope and methods are shown in the Table 2.

Exposure groups were randomly formed in view of the body weight as the key factor (the difference in the body weight was ≤ 10%). During experiments, the animal health state was assessed on a daily basis, as well as the rate of food and water consumption. Toxicological and hygienic studies were performed as per requirements of the Instruction 1.1.11-12-35-2004 [15].

Graph plotting and mathematical treatment of study results were made via the Microsoft Office Excel 2003 (Microsoft Corporation, USA) software. The statistic data was processed using the Student t-test [14] and *X*-Van der Waerden Criteria [18].

RESULTS AND DISCUSSION

Evaluation of antimutagenic activity of enzymatic hydrolysates of whey proteins. Studies were performed to evaluate antimutagenic properties of the test sample of whey protein hydrolisates (WPH) and the analogue "Vital Armor H 801 LB". The required condition to process results of this study was the mutagenic effect available in positive control samples for all test strains (*S. typhimurium* TA 98 and TA 100). As per the results of study data processing by the method of Dunnett's multiple comparisons, the specified values in the number of revertants in the control and test samples were statistically valid.

Statistically significant reduction in the induced mutation for all study variations was reported in the test sample of the WPC hydrolysate and "Vital Armor H 801 LB". Differences in the number of revertant in the control and test samples revealed were statistically valid ($p < 0.05$) when 1.88–30 µg of hydrolysate samples were added to the test system which is shown in Tables 3–6.

Table 2. Design and scope of toxicological-hygienic studies

Name of the test and its design	Animal species	Test methods (observation period)
Determination of toxicity and hazard parameters of the medicinal product at a single intragastric dose administered by animal subjects.	Rats ($n = 18$)	Records of clinical signs of toxication and test animal survival (14 days)
Study of the drug toxicity parameters at a single dose administered by animals intraperitoneally	Mice ($n = 24$)	Records of clinical signs of toxication and test animal survival (14 days)
Single-dose evaluation of the local irritation of the drug for 4-hours at 20 mg/cm^2 (application area is 16 cm^2) on the animal back skin	Rats ($n = 12$)	Control of clinical signs of intoxication and cutaneous skin condition (4 h, 24 h, 10 days). Evaluation of the skin fitness state by the erythema intensity and edema size
The study of the drug action on the mucous membranes and the visual organ; 20% single dose administration of the drug to the lower conjunctival fornix of the tested eye of the animal	Rabbits ($n = 6$)	Visual monitoring of the eye mucosa and conjunctiva (14 days)

Table 3. Statistical evaluation of antimutagenic activity of the hydrolysate test sample by the Ames test performed on the strain *S. typhimurium* TA 98

Sample volume, µg per plate	Quantity of revertants, $x_{ave} \pm \sigma$	Decrease of mutation level, %
30	97 ± 6	49.2
15	105 ± 12	45.0
7.5	116 ± 5	39.3
3.75	147 ± 12	23.0
1.88	161 ± 33	15.7
0	21 ± 2	–
Positive control	191 ± 12	–

Note. Mutagen – ethidium bromide, 10 µg per plate. The strain response to mutagens was within normal limits.

Table 4. Statistical evaluation of antimutagenic activity of the hydrolysate test sample by the Ames test on the strain *S. typhimurium* TA 100

Sample volume, µg per plate	Quantity of revertants, $x_{ave} \pm \sigma$	Decrease of mutation level, %
30	346 ± 11	52.1
15	368 ± 25	49.0
7.5	454 ± 61	37.1
3.75	541 ± 52	25.1
1.88	586 ± 53	18.8
0	98 ± 2	–
Positive control	722 ± 75	–

Note. Mutagen– sodium azide, 10 µg per plate. The strain response to mutagens was within normal limits.

Table 5. Statistical evaluation of antimutagenic activity of the hydrolysate “Vital Armor H 801 LB” sample by the Ames test on the strain *S. typhimurium* TA 98

Sample volume, µg per plate	Quantity of revertants, $x_{ave} \pm \sigma$	Decrease of mutation level, %
30	105 ± 9	45.0
15	114 ± 12	40.3
7.5	122 ± 17	36.1
3.75	143 ± 21	25.1
1.88	155 ± 15	18.8
0	27 ± 7	–
Positive control	191 ± 12	–

Note. Mutagen – ethidium bromide, 10 µg per plate. The strain response to mutagens was within normal limits.

Table 6. Statistical evaluation of antimutagenic activity of the “Vital Armor H 801 LB” hydrolysate by the Ames test using the strain *S. typhimurium* TA 100

Sample volume, µg per plate	Quantity of revertants, $x_{ave} \pm \sigma$	Decrease of mutation level, %
30	375 ± 29	48.1
15	425 ± 41	41.1
7.5	455 ± 56	37.0
3.75	505 ± 32	30.1
1.88	570 ± 89	21.1
0	98 ± 2	–
Positive control	722 ± 75	–

Note. Mutagen – sodium azide, 10 µg per plate. The strain response to mutagens was within normal limits.

The effect of mutation level reduction is more evident in studies of the WPC hydrolysate test sample which made 15.7–49.2% for the strain *S. typhimurium* TA 98 and 18.8–52.1% for the strain TA 100. When using the “Vital Armor H 801 LB” hydrolysate, the effect of induced mutation reduction reached 18.8–45.0% when the strain *S. typhimurium* TA 98 was tested and 21.1–48.1% – when the strain TA 100 was tested.

As per experimental data, the antimutagenic activity of WPC was not revealed within the tested concentrations via the model test using strains *S. typhimurium* TA 98 and TA 100. The results obtained likely relate to physical and chemical

properties of proteic macromolecules, and in particular, to inaccessibility to penetrate the cellular membrane of auxotroph strains.

The studies were further conducted to evaluate **antifungal properties of peptides of the cow milk whey proteins** when opportunistic fungi strains *Aspergillus niger* and *Candida albicans* are exposed.

A. niger is the species of mold fungi of the genus *Aspergillus* (or black mould). The long-term exposure to antigens *A. niger* induces the allergic reaction resulting in allergic rinities, allergic-bronchopulmonary aspergillosis or bronchial asthma. The pathogenicity of *Aspergillus* spp. is related to heterotrophy and synthesis of amylolytic, proteolytic, lipolytic and other enzymes. *C. albicans* is the yeast-like fungi that produce pseudomycelium. *Candida* species are the part of the normal microflora in most healthy humans (80%). The disease is induced through intense propagation and/or intrusion of more pathogenic fungal strains. Pathogenic factors in fungi of genus *Candida* include the secretion of proteolytic enzyme and haemolysins, dermato-necrotic activity and adhesiveness [19].

The effect of whey proteins and peptides on the growth of opportunistic microorganism *A. niger* is studied when cultured on the agar culture media (Saburo and FMH-medium) containing 5.0 mg/ml of WPC and hydrolysates. Since the *C. albicans* pathogen is more demanding to the culture media components, it was cultured on the Saburo agar medium. The concentration of tested compounds (WPC and hydrolysates of whey proteins) was determined to comply with that the Saburo medium contains 5.0 mg/ml of the enzymatic casein hydrolysate and 5.0 mg/ml of the peptone as the source of nitrogen compound. FMH-medium includes the pancreatic fish meal hydrolysate as the protein component. So, the culture media used contain the protein substrate of various origin (milk protein hydrolysates, in particular, casein and animal meat, as well as the fish meal hydrolysate). Test samples added resulted in supplementary enrichment of culture media with milk whey proteins (WPC) and their hydrolysates.

Fig. 1 and 2 show results of the test to culture *A. niger* on the agarized culture media (Saburo and FMH-medium) containing WPC and whey protein hydrolysates. Suppression of the *A. niger* mycelium is reported when the experimental sample of the hydrolysate and "Vital Armor H 801 LB" is added when both culture media are used. At the same time, the *A. niger* mycelium more intensely grows when the fungi is cultivated on culture media with WPC added.

The experimental data obtained show that specific peptides with antagonist properties are available in the tested hydrolysates of whey proteins. The quantitative degree of inhibition (*DI*, %) of the *A. niger* mycelium was assessed by cultivation in Petri dishes with FMH-medium. This medium was chosen since it does not have the milk protein hydrolysates (casein) as a component and ensures lower rate of mycelium growth where the results are better reproduced. The degree of the *A. niger* mycelium inhibition, when it is added to the FMH-medium of the experimental sample of whey protein hydrolysates dosed at 5.0 mg/ml was about 25% and 11% when the “Vital Armor H 801 LB” sample is added.

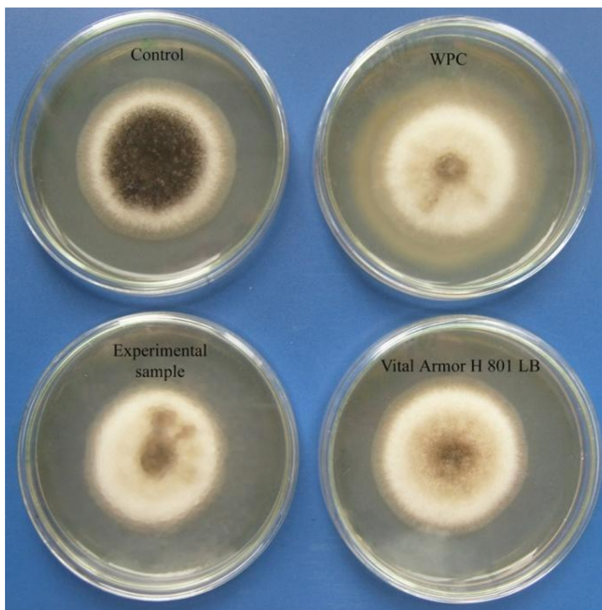

Fig. 1. Antifungal activity evaluation of whey proteins (WPC) and hydrolysates (experimental sample and “Vital Armor H 801 LB”) in the volume of 5.0 mg/ml against the opportunistic fungi *A. niger* when cultivated on the agarized Saburo medium.

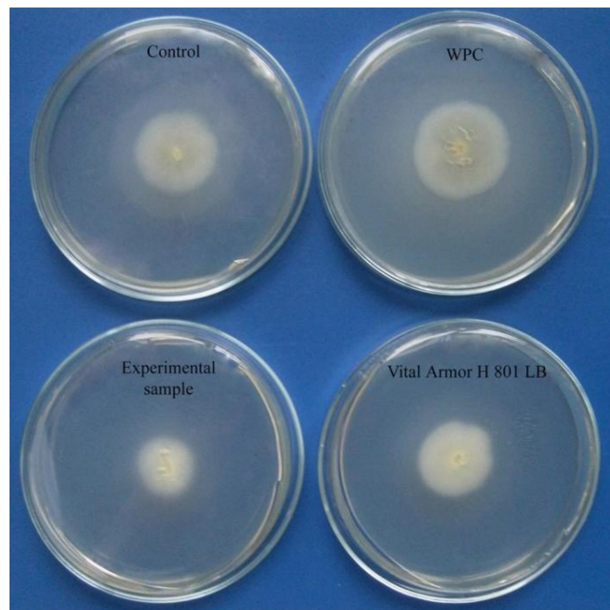

Fig. 2. Antifungal activity evaluation of whey proteins (WPC) and hydrolysates (experimental sample and “Vital Armor H 801 LB”) of 5.0 mg/ml against the opportunistic fungi *A. niger* when cultivated on the agarized FMH-medium.

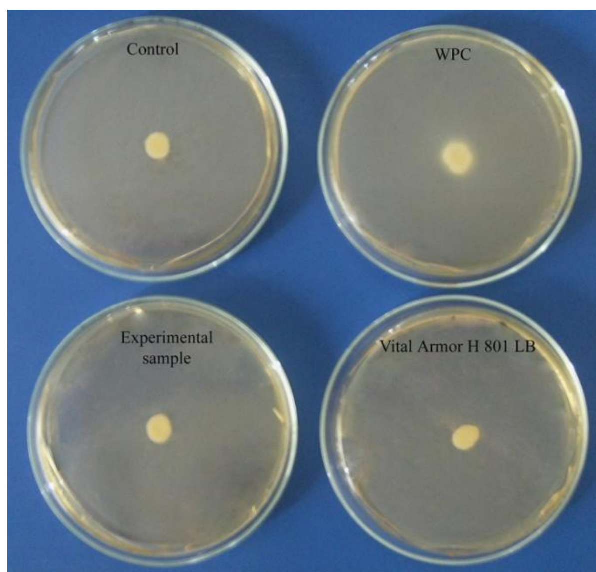

Fig. 3. Antifungal activity evaluation of whey proteins (WPC) and hydrolysates (experimental sample and "Vital Armor H 801 LB") at the level of 5.0 mg/ml against the opportunistic fungi *C. albicans* when cultivated on the agarized Saburo medium.

The Fig. 3 shows the test results of *C. albicans* cultivation on the agarized FMH-medium containing WPC and whey protein hydrolysates. Significant differences of the *C. albicans* mycelium diameter in control and test samples (when added to the test system of hydrolysate samples) are not determined. At the same time, the mycelium growth stimulation was induced by adding the native whey proteins (WPC) to the culture medium. Thus, the whey protein peptides available in the culture media did not cause any antagonistic effect on the growth of the opportunistic *C. albicans*.

The works by N. Nandhini et al. (2015) [20] with the use of whey proteins also show the varied level of antibacterial and antifungal activity when a range of opportunistic microorganisms are used. Particularly, as per the tested inhibition zone, the larger inhibition zone was reported in studies where *A. niger* was used rather than *C. albicans.*

Comparative characteristics of physical, chemical and bioactive properties of the obtained whey protein hydrolysate and "Vital Armor H 801 LB" sample.

The physical, chemical and bioactive properties of the hydrolysate test sample and its analog are given in the Table 7. Reference samples are consistent by the degree of substrate cleavage (AN/TN). In addition, the quantity of low-molecular proteic fraction (mr < 10 kDa) in the experimental hydrolysate sample is 11% greater than in the "Vital Armor H 801 LB" sample.

At the hydrolysis degree of 15.5 ± 0.6% (test sample) and 16 % ("Vital Armor H 801 LB"), the antiradical activity of proteic components tested reaches 0.551 ± 0.035 and 0.618 ± 0.001 µmol/mg, accordingly. So, the enzymatic hydrolysis of the whey proteins was responsible for 3.0−3.6 times increase in the anti-radical activity of enzymatic hydrolysates (Table 7).

At the same time, the reduction in the mutation level in experiments with the test sample of WPC hydrolysate was 15.7–49.2% for the *S. typhimurium* TA 98 strain and 18.8–52.1% for the strain TA 100. For the Vital Armor H 801 LB hydrolysate, the effect of the induced mutation decrease was less to some extent: 18.8–45.0% when tested on the *S. typhimurium* TA 98 and 21.1–48.1% – on the strain TA 100.

Both hydrolysates showed antagonistic activity against the opportunistic fungi *A. niger*. The experimental hydrolysate sample (25%) has more evident antifugal effect.

Overall, the comparable values of protein substrat hydrolysis degree, the level of antioxydant and antimutagenic activity are reported for the test sample of the partial whey protein hysrolysate and "Vital Armor H 801 LB" that is currently used for functional food production. The test hydrolysate sample has the advantage to increase the content of the peptide fraction and more evident antifungal activity against *A. niger*.

Table 7. Characteristics of partial hydrolysates of whey proteins

Name of hydrolysate	Peptide profile	Ratio of α-amino nitrogen and total nitrogen, AN/TN, %	TEAC, µmol trolox/mg of protein**	Antimutagenic activity (TA 98/ TA 100 strains), %	Antifungal activity, %
Experimental sample of hydrolysate	≤10 kDa, 98%	15.5 ± 0.6	0.551 ± 0.035	15.7–49.2 / 18.8–52.1	25
"Vital Armor H 801 LB"	<10 kDa, 87% *	16 *	0.618 ± 0.001	18.8–45.0 / 21.1–48.1	11

Note. * – parameters are shown as per manufacturer's data, ** – as per the study results as described in the article [12].

Parameters of acute toxicity and sensitizing activity of obtained hydrolysate are determined at the following study stage.

Acute toxicity parameters (median lethal dose, LD_{50}) of WPC and EWPH are determined on the white rats. Each group is of 6 animals. To evaluate the acute intragastric toxicity, the white rats received drug samples under fasting condition in 20%-aqueous solutions (by protein)- injected in the stomach with the needle probe in the maximum permissible volume (3.0 ml per 200 g of body weight). Control animals received the water in equivalent volume. The test

animals were monitored for 14 days since the date of experiment. High doses of drugs did not result in any ataxia, adynamia, clinical and tonic contractions, and paralysis in animals. The maximum dose of drugs did not result in the animal death. It is established WPC and EWPH refer to low-hazard chemical compounds (Hazard class 4 – as per GOST 12.1.007–76) by the parameters of acute intragastric toxicity.

The irritative effect of WPC and EWPH on the eye mucosa of test animals was evaluated. Two drops of 20 %-solution of WPC (by protein) and its hydrolysate instilled in the lower conjunctival fornix of the right "test" eye of animals (the distilled water was instilled in the left control eye of animals in the equivalent volume) were followed by the intense winking and lacrimation for 10–15 min, and weak mucosa irritation. The irritation effect in all animals disappeared in 1 hour upon instillation. Thus, the single exposure of tested drugs as 20% solutions to the eye mucosa - hardly resulted in the irritation.

Parameters of acute toxicity of WPC and EWPH were analyzed at a single abdominal dose administered by the white mice of both genders. Thee groups (8 animals per group) were formed for the study purpose. The control mice administered the drug in the highest possible dose of LD_{50} (3784 ± 17.0 mg (protein)/kg for EWPH, 3265 ± 34.3 mg (protein)/kg for WPC), control animals received the physiological solution. Toxication signs disappeared by the end of the first study day; the general health state of animals, their behavioral pattern, need in food and appearance did not differ from those in control animals during the 2-week observation period. Thus, as per the median lethal doses, the test drugs may be classified as relatively safe (> 3000 mg/kg at intraperitoneal administration).

The irritative effect of WPC and EWPH at a single exposure on the intact cutaneous skin of test animals was studied. A single dose of 20%- drug solutions (by protein) applied to the chipped skin of the white rack back (6 animals per group) at 20 mg/cm^2 did not result in any visible signs of intoxication of animals within 4 hours and their death within 10 days. Cutaneous skin irritations and infection were not reported in application sites.

A comparative study of the WPC and EWPH sensitizing property was performed (as per [15, 16]) on the experimental model of delayed hypersensitivity reproduction in white mice. WPC solution (1-st test group) and EWPH solution (2-nd test group) were intradermally injected to the white mice at the tail base at a single dose of 300 μg (in terms of protein) in the 1 : 1 mixture with the complete Freund's adjunct at 0.06 cm^3 per animal. Control group animals received the mixture of the physiological solution and complete Freund's adjunct (CFA). Sensibilization by the delayed hypersensitivity reproduction was defined on the 6-th day of the test by using the provocative test – paw swelling intracutaneous test. The test was performed by injection of the sample dosed at 400 μg (at the volume of 0.04 cm^3) in the pad of the hind feet (under the aponeuroses) of test and control animals. The results of paw swelling intracutaneous test were evaluated by the difference in thickness of the test paw of animals in both test and control groups using the micrometer prior to and in 24 hours upon injection on the provocative test site with the rate of accuracy up to 0.01 mm. The paw swelling intracutaneous test absolute index per each animal was expressed in 10^{-2} mm. To evaluate the sensibilization intensity, the paw swelling intracutaneous test results was scored [17].

The WPC sample showed the strong sensitizing property (antigenic activity class 1) since it caused induction in the delayed hypersensitivity reproduction in more than 75% of test animals with significant differences of relative paw swelling intracutaneous test indexes in the test and control animals a s per *X*-criterion ($p < 0.01$) (Table 8).

Table 8. Delayed hypersensitivity reproduction indexes in the white mice upon administration of the sample of native whey proteins (WPC) and its enzymatic hydrolysate (EWPH)

Parameter	Unit of measurement	Control group (n = 12)	Test group (n = 12)
Paw swelling intracutaneous test results upon intracutaneous WPC testing (test group 1):			
absolute values	10^{-2} mm	6.33 ± 1.41	18.8 ± 1.96 *
	t-criterium	–	5.15
relative values	H	1/12	12/12
	Score	0.08 ± 0.08	1.58 ± 0.19 $^{*+}$
	t-criterium	–	7.14
	X-criterium	–	8.43
Paw swelling intracutaneous test results upon intracutaneous EWPH testing (test group 2):			
absolute values	10^{-2} mm	5.75 ± 1.39	7.67 ± 1.60
	t- criterium	–	0.91
relative values	H	1/12	5/12
	Score	0.08 ± 0.08	0.42 ± 0.15 ^{0}
	t-criterium	–	1.96
	X-criterium	–	3.06

Note. Significant differences as compared with control values by t-criterion (* – p < 0.001; ^{0} – p < 0.1) and X-criterion ($^{+}$ – p < 0.05). Data on the number of species with positive results and total number of animals used in the study are given in slash.

A weaker induction in the delayed hypersensitivity reproduction (≤1 point) is reported in 5 of 12 animals sensitized with enzymatic WPH. The value of the provocative test integral indicator was 5.2 times higher than the control results though the difference in between statistically tended to increase in terms of *t*-criterion ($p < 0.1$) based on which the hydrolysate sample was classified as the agent with the weak sensitizing property (Class 4).

CONCLUSION

Bioactive properties (antimutagenic, antioxidant and antifungal effect) of the enzymatic whey protein hydrolysate are described. The comparable values of protein substrate hydrolysis intensity, the level of antioxidant and antimutagenic activity are given for the test hydrolysate sample and “Vital Armor H 801 LB” (Armor Protéines, France), used for functional food production. With 15–16% hydrolysis, the antiradical activity of these protein components is comparable and reaches 0.551–0.618 TEAC units. Overall, the enzymatic hydrolysis of the milk whey proteins caused an increase in antiradical properties of peptide fraction which is 3.0−3.6 times higher. When tested, a decrease in the mutation level of the test hydrolysate sample was 15.7–49.2% in the test system for *S. typhimurium* TA 98 and 18.8–52.1% for the TA 100 strain. When the foreign analog "Vital Armor H 801 LB" was tested, the reduction effect of the induced mutation reached 18.8–45.0% when the *S. typhimurium* TA 98 strain was tested and 21.1–48.1% – when the TA 100 strain was tested which is lower than values typical for the test hydrolysate sample. Antifungal property study showed that when the obtained enzymatic hydrolysate of whey proteins and the sample of “Vital Armor H 801 LB” dosed at 5.0 mg/ml were added to the culture medium, they suppressed the growth of opportunistic fungi *A. niger* mycelium for 25 and 11%, accordingly. The effect of tested hydrolysate samples on the suppression of *C. albicans* mycelium growth is not determined. At the same time, when native whey proteins (WPC) were cultured on the culture medium, they stimulated the growth of these microorganisms. The test hydrolysate sample benefits to increase the content of peptide fractions and have more evident antifungal effect against the opportunistic pathogen *A. niger*.

It is established in studies to evaluate the acute toxicity and sensitizing activity parameters on animal models that the obtained enzymatic hydrolysate of whey proteins is classified as the safe agent and has low sensitizing property. The developed partial hydrolysate is proposed to be used as the proteic component with the specified peptide structure and bioactive properties to manufacture specialized food.

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Please cite this article in press as: Golovach T.N., Dudchik N.V., Veremeenko E.G., Tsygankov V.G., Bondarchuk A.M., Filonyuk V.A., Shevlyakov V.V., Ushkov A.A., Sobol' Yu.A., Erm G.I., and Kurchenko V.P. Evaluation of antimutagenic and antifungal properties, parameters of acute toxicity and sensitizing activity of enzymatic whey protein hydrolysate. *Foods and Raw Materials*, 2016, vol. 4, no. 2, pp. 38–47. DOI: 10.21179/2308-4057-2016-2-38-47.