FOOD HYGIENE

DETERMINATION OF PHYSICOCHEMICAL, IMMUNOCHEMICAL AND ANTIOXIDANT PROPERTIES, TOXICOLOGICAL AND HYGIENIC ASSESSMENT OF WHEY PROTEIN COMCENTRATE AND ITS HYDROLYSATE

T. N. Halavach[a,*], V. P. Kurchenko[a], V. G. Zhygankov[b], I. A. Evdokimov[c]

[a] Belarusian State University,
Nezavisimosti Avenue 4, Minsk, 220030 Republic of Belarus

[b] RUE "Scientific Practical Centre of Hygiene",
Academicheskaya Str. 8, Minsk, 220012 Republic of Belarus

[c] North-Caucasus Federal University,
Pushkin Str. 1, Stavropol, 355009 Russian Federation

* e-mail: halavachtn@gmail.com

Received April 24, 2015; Accepted in revised form July 07, 2015; Published October 20, 2015

Abstract: Enzymatic hydrolysis of whey proteins is aimed to obtain products with low allergenic potential and high nutritional value. Whey peptides are protein fraction that possesses a variety of physicochemical, immunochemical and bioactive properties (antioxidant, antibacterial, immunomodulatory effects). Controlled parameters of enzymatic hydrolysates are the degree of hydrolysis of protein substrates, peptide composition, residual antigenicity, antioxidant capacity, etc. The purpose of this work is to characterize peptide profile, antigenicity and free radical scavenging properties of experimental hydrolysate sample, toxicological and hygienic assessment of its impact on a test object (infusoria *Tetrahymena pyriformis*). The research of properties of whey proteins and their enzymatic hydrolysates was conducted using classical and modern methodological approaches: SDS electrophoresis in polyacrylamide gel, HPLC, mass-spectrometry, competitive ELISA, TEAC (Trolox Equivalent Capacity Assay) technique. Peptide composition, antigenic and antioxidant properties of obtained enzymatic hydrolysate were determined, toxicity of raw and digested whey proteins were examined. We established that the investigated hydrolysate sample falls under the category of partial hydrolysates for functional products according to its physicochemical, immunochemical, free radical scavenging and organoleptic properties. According to the results of toxicological and hygienic assessment using *T. pyriformis* model, whey protein concentrate and its hydrolysates are non-toxic and do not possess cumulative properties. Thus, we obtained partial enzymatic hydrolysate of whey proteins from milk, which can be used as physiologically active component in the development of new specialized food.

Keywords: Allergenic milk proteins, protein hydrolysates, peptide composition, residual antigenicity, antioxidant properties, toxicological and hygienic assessment, infusoria *Tetrahymena pyriformis*

DOI 10.12737/13127 *Foods and Raw Materials*, 2015, vol. 3, no. 2, pp. 105–114.

INTRODUCTION

It is well known that the native proteins of cow's milk have diverse physiological properties: immunomodulating, antibacterial, antiviral and antifungal activities [1]. Caseins (α_{S1}-, α_{S2}-, β- and κ- form) are the predominant phosphoprotein fraction in milk of ruminants (80% of total protein). Whey proteins (20% of protein fraction) are presented by β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, lactoferrin, lactoperoxidase and other minor components. During protein hydrolysis the cleavage of peptide bonds with the formation of amino acids and peptides of various lengths occurs. Hydrolyzed milk proteins have low antigenic potential due to destruction of antigenic determinant areas [2].

Caseins and whey proteins act as precursors of biologically active peptides that are produced during digestion or as a result of enzymatic cleavage during technological food processing. Biologically active peptides are particularly interesting for the nutritional science, because they possess opioid-like, immunomodulatory, hypotensive, antimicrobial, antiviral, antioxidant and antitumor effects [3].

The main controlled properties of enzymatic hydrolysates are molecular mass distribution of peptide fractions, degree of hydrolysis of substrates and residual antigenicity (AG) – the amount of undissolved protein that retains the ability to interact with antibodies [1, 4]. Milk proteins are divided into partial and extensive hydrolysates according to the degree of hydrolysis [5]. Partial hydrolysates used in prophylactic mixtures contain peptides of various lengths and a minimum amount of free amino acids. Extensive hydrolysates serving as components of therapeutic products are presented by short-chain peptides and amino acids. In severe cases of food allergy only amino acids without antigenic properties are applied. Significant disadvantage of extensive hydrolysates and mixtures of amino acids is their pronounced bitter taste. Partial hydrolysates with acceptable organoleptic properties are used in the production of baby food, medical, elderly and sports nutrition.

Along with this, a balanced diet requires products with antioxidant capacity or additional components with antiradical properties. Antioxidant activity (AOA) of proteins and peptides is caused by solvent available amino acids (restoring properties of amino acid radicals) [6]. The identified peptides with antiradical properties consist of tryptophan, tyrosine, methionine and histidine. Moreover, antioxidant activity is indicated for amino acid standards (tryptophan > tyrosine, methionine >> cysteine > histidine > phenylalanine) [7, 8]. We applied TEAC (Trolox equivalent antioxidant capacity) technique [9] for determination of the antioxidant properties of natural whey proteins and their corresponding enzymatic hydrolysate.

The safety assessment of certain food types, especially food for particular nutritional uses, is usually carried out using laboratory animals (rats, mice, guinea pigs). Alternative biomodels must meet several requirements: they must have similar physiological response as the higher animals, higher speed and efficiency in comparison with traditional methods and specific properties that enable to expand the activity spectrum of investigated factor on the biosystem. Infusoria *Tetrahymena pyriformis* is currently successfully applied for various biological studies including assessment of toxicity and biological value of food products and feed [10, 11]. Toxicity is usually determined by the time of death of 50% of exposed organisms in environment with a certain toxicant concentration (CL_{50}). Advantages of *T. pyriformis* as a test object are following: 1) it is both a cell and an eukaryotic organism – this fact allows evaluating food effects and drawing corresponding analogies on both cellular and organism levels; 2) it is much more similar to the higher organisms by main biochemical indicators and biological needs than other models; 3) many test functions of *T. pyriformis* correspond to the basic vital factors of the higher animals. Thus, *T. pyriformis* is a universal test organism suitable for studying of safety and biological value of food products and for biological evaluation of other natural and artificial objects.

Constantly growing application of enzymatic hydrolysates determines the relevance of toxicological and hygienic assessment of obtained sample of milk whey protein hydrolysate using *T. pyriformis* as a test object.

The purpose of this work is to characterize protein-peptide composition; to assess the antigenic and antioxidant properties of whey protein and its enzymatic hydrolysate; to carry out toxicological and hygienic assessment of sample on the test object (infusoria *T. pyriformis*).

OBJECTS AND METHODS OF STUDY

Obtaing of enzymatic hydrolysates of milk whey proteins

Whey protein concentrate derived using ultrafiltration method (WPC-UF-80, TNLA BY 100377914.550-2008) with protein m.f. 80% and serine protease (alcalase, EC 3.4.21.62, protease from *Bacillus licheniformis*, activity 2.64 u/g; Sigma, USA) were used for enzymatic hydrolysis. To obtain the experimental hydrolysate sample the 8% solution of WPC-UF-80 was made, protein substrate was thermally treated and then cooled to optimal hydrolysis temperature. The enzymatic agent was added in the resulting heat-treated solution; hydrolysis was carried out in thermostatic conditions. After proteolysis completion the agent was inactivated by heating. Finally, the resulting liquid hydrolysate was dried according to method [12].

Analysis of physicochemical and bioactive properties of hydrolysates

The basis for electrophoretic separation of milk proteins and their enzymatic hydrolysates was the methodology used in operational manual [13]. HPLC analysis of hydrolysis products was conducted on Agilent 1100 chromatograph (Agilent, United States) using Zorbax-300SB C8 column (4.6×250 mm, 5 μm; Agilent, United States) according to the technique [14]. Molecular mass distribution of peptides was studied using Bruker Microflex instrument (Bruker, United States). Techniques described in [15, 16] became the basis for competitive ELISA for determination of residual AG of whey proteins and their hydrolysates.

Whey protein hydrolysate was fractionized using filters Amicon Ultra-4 10K (Millipore, United States; permeability 10 kDa). Protein concentration (TN) in hydrolysate and ultrafiltrate was determined according to GOST 30648.2-99. The fraction with molecular weight ≤ 10 kDa (%) was evaluated as the ratio of TN value for ultrafiltrate to the concentration of protein in the original hydrolysate. The content of α-amino

nitrogen (AN) in hydrolysate samples was determined by formol titration according to GOST 13805-76 (p. 3.9). Degree of hydrolysis was evaluated as the ratio AN/TN.

TEAC (Trolox equivalent antioxidant capacity) technique was used to assess the AOA level. ABTS radical scavenging activity measurement required previously obtained cation radical of diammonium salt of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [17]. $ABTS^{\bullet+}$ is a metastable radical, which can exist in solution for a long time. After introduction of various antiradical agents (trolox) into medium, quick reduction of this radical is observed. Reaction was monitored spectrophotometrically at λ_{734}: $ABTS^{\bullet+}$ radical (blue staining of solution) is converted back to its colorless neutral form during reduction. The described system has only one type of radical and antioxidant influence on its formation is impossible, therefore, the mechanism of direct interaction between the antioxidant and cation radical is carried out. AOA measurement was performed on the basis of modified technique described in article [9].

Toxicological and hygienic study of impact of whey protein concentrate and its enzymatic hydrolysate on *T. pyriformis* was based on principles and methods of hygienic regulation adopted in general toxicology [18, 19]. The principle of research on *T. pyriformis* is to analyze the nature of population growth in culture medium containing researched objects.

Primary toxicological assessment of objects on *T. pyriformis*

The study was carried out on the test object *T. pyriformis* in stationary growth phase, supported in standard nutrient medium at 25°C. Depending on toxicity of the researched object acute experiment duration was 0.5–4 h, subacute experiment – 24 h. The toxic effect was estimated by alternative state "life-death" [19]. Suspensions of the WPC and its hydrolysate containing 10–150 mg/ml of protein was prepared, the pH of suspension was adjusted to 7.1-7.2 units. Lethal and malfunctioning concentration was determined and then several intermediate concentrations were prepared. 1 ml of each concentration was put into two 10 ml flasks. Inoculate of infusoria in stationary growth phase (100 000 ± 1 000 organisms) was added to each sample. For acute experiment samples were incubated at 25°C for 30–240 min; for subacute experiment – for 24 h. Intoxication picture in native specimen was observed under the microscope after the incubation stage. The number of dead infusoria prior to fixation and the total number of infusoria after fixation (with 5% iodine solution) was counted in Fuchs-Rosenthal counting chamber. Lethality in % was calculates with consideration of lysed organisms. Probit analysis of direct lethality was carried out using general toxicology methods, in particular the method of V.B. Prozorovsky [20]. According to calculations of the test object lethality (in %) in acute and subacute experiments the basic toxicity parameters were determined (LD_{50}, LD_{16}, LD_{84}). Cumulation coefficient in acute experiment (K_{CUMas}) was calculated as ratio of subacute and acute experiment LD_{50}.

Chronic toxicity study on *T. pyriformis*

Study of toxicity in chronic experiment was carried out throughout the life cycle of *T. pyriformis* population. The chronic experiment setting was based on primary toxicological assessment results. Suspensions of investigated objects were poured into sterile tubes with gauze plugs. Each concentration was studied in at least three replications. Tubes with suspensions were sterilized at 85°C for 30 min. 20 000 of infusoria in stationary growth phase were added to samples after cooling. Samples were stored in thermostat at 25°C for 96 h. Registration of infusoria state and counting of organisms were carried out after 24 h (lag-phase), 48 hours (logarithmic phase), 72 h (slow growth phase), 96 h (stationary phase). For this purpose 1 ml sample was taken from each tube under sterile conditions. State of organisms in native sample was determined: the presence of dead organisms, nature of morphological and functional changes. After fixation with 1 drop of 5% iodine solution the number of organisms was counted in the Fuchs-Rosenthal counting chamber in 10 large squares. The number of organisms in 1 ml of culture were calculated as mean number of organisms in 1 square multiplied by 5 000 (if selected inoculate was not diluted) or by 20 000 (inoculate was diluted 4 times). The constant of instantaneous population growth rate, number of generations, generation time [20] were calculated using following formulas:

$$r = \frac{\ln \frac{N_t}{2000}}{t}, \quad (1)$$

$$n = \frac{\ln \frac{N_t}{2000}}{\ln 2}, \quad (2)$$

$$g = \frac{t}{n}, \quad (3)$$

where 2 000 is the number of organisms added to 1 ml of cultivation medium; N_t is the number of organisms grown in cultivation medium with the studied agent during time t; r is the constant of instantaneous growth rate; n is the number of generations; g is the generation time.

Chronic toxicity parameters were determined in the concentration range, where inhibition of population growth rate was proportional to the increase in agent concentration. Growth inhibition (ED) for each dose was calculated according to formula:

$$\mathrm{ED}(\%) = 100 - \frac{N_0}{N_C} \times 100, \quad (4)$$

where N_0 is the number of organisms in experiment; N_C is the number of organisms in control; ED_{16}, ED_{50}, ED_{84} were calculated using tables and formulas for determination of LD_{16}, LD_{50}, LD_{84}.

Acute experiment results were estimated with consideration for following parameters: ED_{50} – dose that causes 50% inhibition of generative functions in logarithmic (24–48 h) and stationary (72–96 h) phases of growth; K_{CUMchr} – cumulation coefficient at chronic exposure, calculated as ratio of ED_{50} determined in stationary phase to ED_{50} determined in logarithmic growth phase; cumulation coefficient value more than 1 suggests samples having adaptogenic properties; Z_{chr} – area of chronic action calculated as ratio of mean lethal dose in acute experiment to the dose, which inhibits population growth by 50% in the stationary phase of chronic experiment; MND – maximum non-effective dose determined by limitative indicator; LD_{50}/MND – hazard indicator calculated as ratio of mean lethal dose in acute experiment and maximum non-efficient dose determined in chronic experiment. The estimation results of investigated samples on *T. pyriformis* model allowed classifying them according to toxicity and hazard indexes (Table 1).

The researched object was ranged in a hazard class according to indicator, which corresponds to the highest hazard class.

Graph plotting and mathematical processing of research data were carried out using computer program "Microsoft Office Excel 2003" (Microsoft Corporation, United States).

RESULTS AND DISCUSSION

We performed integrated analysis of organoleptic, physicochemical and immunochemical properties of obtained partial hydrolysate of milk whey proteins (Table 2). According to results of SDS electrophoresis the hydrolysate exhibits almost full proteolysis of β-lactoglobulin, α-lactalbumin and BSA into peptides. HPLC study of sample profiles revealed that the sample does not contain native whey protein.

According to the experimental data, the sample contains 98% of fractions with $m_r \leq 10$ kDa, and the residual AG is reduced by 0.8×10^3 times (Table 2). According to literature, the residual AG of partial hydrolysates used in prophylactic mixtures is $\geq 10^{-3}$ rel. u. (or $\leq 10^3$ times smaller than AG of native proteins) [5]. Mass spectra study confirmed the absence of high molecular fractions in the obtained hydrolysate; peptides with $m_r < 5$ kDa dominate among proteolysis products. Thus, the investigated hydrolysate is proved to meet requirements for the category of partial enzymatic hydrolysates.

The antioxidant capacity of obtained hydrolysate sample was studied. Free radical scavenging efficiency of standard (trolox) upon inactivation of $ABTS^{\bullet+}$ was characterized during experiments. The degree of cation radical reduction within certain time interval (10 min) was determined. This indicator represents the actual decrease in concentration of free radicals in system caused by antioxidant. It was demonstrated that the process is characterized by rapid phase of $ABTS^{\bullet+}$ reduction during the first minute of reaction. Thus, $ABTS^{\bullet+}$ quickly reacts with active antioxidants, which allows to use it as a selective reagent for polycomponent sample analysis [17]. IC_{50} or trolox concentration was calculated (16.45 μM). At this concentration, speed of the process reduces 2 times. EC_{50} of the standard was used to calculate TEAC (free radical scavenging activity indicator, expressed in micromoles of trolox on 1 mg of protein).

Table 1. Hygienic classification of objects according to results of the study of their toxicity on *T. pyriformis*

Indicator	Classes (decreasing toxicity and hazard level)				
	1 (extra hazardous)	2 (highly hazardous)	3 (moderately hazardous)	4 (low hazardous)	5 (non-toxic)
LD_{50}, mg/ml	less than 0.1	0.1–1.0	1.1–20	21–50	more than 50
K_{CUMac}, K_{CUMchr}	less than 0.1	0.10–0.30	0.31–0.49	0.50–1.0	more than 1.0
Z_{chr}	more than 10	10.0–5.0	4.9–2.5	less than 2.5	–
MND, mg/ml	less than 10–6	10^{-6}–10^{-4}	10^{-4}–10^{-1}	more than 10^{-1}	–
LD_{50}/MND	more than 106	10^6–10^5	10^5–10^4	less than 10^4	–

Table 2. Organoleptic, physicochemical and immunochemical properties of milk whey protein hydrolysate

Parameter name	Parameter value
Appearance and consistency	Yellow-cream powder
Flavor and smell	Typical milk flavor. Weak bitter milk taste
Solubility	Soluble in water
Active acidity, u. pH (1% solution)	6.7
Weight fraction of total protein, %	80
Peptide profile: fragments with molecular weight >10% kDa, %	2*
Native whey proteins	Not found **
Decreasing of residual AG (compared to native WPC)	0.8×10^3

Note. * indicator values are set after determination of total nitrogen in hydrolysate and ultrafiltrate obtained using filters Amicon Ultra-4 10 K with permeability 10 kDa. ** According to SDS electrophoresis, HPLC and mass spectrometry.

The data on radical scavenging activity of initial substrate for production of hydrolysates (native whey protein concentrate WPC) were obtained. Study of the reaction kinetics of WPC cation radical reduction revealed the stage of rapid decrease in $ABTS^{\bullet+}$ amount during the first minute of reaction and relatively slow increase in degree of reduction within time interval 1-30 min. Rapid absorption inhibition stage (duration 1 min) is associated with the presence of highly active antioxidants. We estimated the overall free radical scavenging activity within 30 min of reaction to determine the total content of antioxidants with different $ABTS^{\bullet+}$ reduction efficiency. IC_{50} value for the WPC sample had been achieved at concentration 95.1 ± 2.8 μg/ml of protein component; the radical scavenging activity in u. TEAC was 0.173 ± 0.005 μmol/mg.

A study of cation radical reduction kinetics after introduction of enzymatic hydrolysate experimental sample into the test system was performed. As in case of native WPC, rapid $ABTS^{\bullet+}$ decrease stage during the first minute of reaction was observed. One should also note process slowdown at the 4th min and gradual fading of radical reduction at the 30th min of reaction. To determine the total content of antioxidants (compounds with various efficiency of interaction with $ABTS^{\bullet+}$) key AOA indicators for 30 min of reaction time were calculated. The increase in free radical scavenging activity caused by enzymatic cleavage of the protein component in comparison with the original WPC was obvious. So, IC_{50} value for the experimental hydrolysate sample had been achieved at concentration 29.02 ± 1.92 μg/ml of protein component as shown in Fig. 1; AOA was 0.551 ± 0.035 μmol of trolox/mg of protein. $ABTS^{\bullet+}$ reduction ability after introduction of hydrolyzed substrate increased 2.98–3.39 times in comparison with native WPC.

According to literature, a significant increase in DPPH radical reducing activity of whey appeared after Flavourzyme 500L® hydrolysis [8]. Another study [21] mentions increased free radical scavenging potential of WPC hydrolysate ultrafiltrates (5 kDa) in comparison with the original substrate (ORAC-method). These ultrafiltrates were obtained using alcalase, neutrase, flavourzyme and Corolase PP. Increasing of hydrolysate AOA compared to native proteins is associated with cleavage of protein macromolecules into peptides, accompanied by additional exposure of amino acid radicals. Expressed antioxidant effect is associated with proton donor properties of indole and phenol groups of tryptophan and tyrosine respectively, as well as with the formation of methionine sulfoxide and oxidation of cysteine sulfhydryl group (−SH) [7].

Next, we studied antioxidant and antigenic properties, peptide profile of whey protein partial enzymatic hydrolysates of foreign manufacture: PRODIET GF 006 (Ingredia, France), Hilmar 8350 (Hilmar, United States). Table 3 presents the comparison between the obtained hydrolysate sample and foreign analogues.

We obtained dependences between $ABTS^{\bullet+}$ degree of reduction by enzymatic hydrolysates and reaction time (30 min). The process is characterized by rapid decrease of $ABTS^{\bullet+}$ amount during the first minute of reaction followed by less intensive increase of reduced radical fraction at 4th−6th minute. This is followed by a slow reaction stage (up to 30th min). The purpose of experiment was to assess antiradical properties of hydrolysate as a set of different antioxidants, that is why AOA indicators were calculated for 30 min of reaction time.

Study on hydrolysates of foreign manufacture and experimental sample allowed to obtain correlation between depth of whey protein cleavage and the level of AOA. Thus, when the degree of hydrolysis is equal to 12.5% (Hilmar 8350) and 15.5 ± 0.6% (experimental sample), free radical scavenging activity of the studied protein components reaches 0.559 ± 0.022 and 0.551 ± 0.035 μmol of trolox/mg of protein, respectively. Along with this, degree of hydrolysis of protein component PRODIET GF 006 is 20–25%; radical scavenging activity raises to 0.982 ± 0.014 μmol of trolox/mg of protein. It is obvious that increase of low molecular peptide fraction yield causes increase of antioxidant capacity.

According to the results of competitive ELISA, residual AG of “PRODIET GF 006” sample is $(0.84 \pm 0.04) \times 10^{-3}$ rel. u. and the free radical scavenging activity level reaches 0.982 ± 0.014 μmol of trolox /mg of protein, whereas the same indicators for "Hilmar 8350" are equal to $(52.9 \pm 2.0) \times 10^{-3}$ rel. u. and 0.559 ± 0.022 μmol of trolox/mg of protein, respectively. The obtained data confirm that increasing degree of substrate cleavage is accompanied by increase in free radical scavenging activity and decrease of hydrolysate allergenic potential.

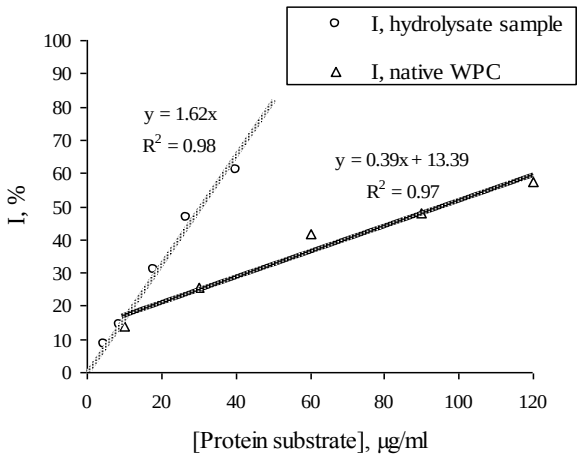

Fig. 1. Dependence of absorption inhibition (I, %) from concentration of WPC and hydrolysate sample.

Table 3. Characteristic of partial whey protein hydrolysates of foreign manufacture

Name of hydrolysate	Peptide profile	Ratio between α-amino and general nitrogen *, AN/TN, %	IC50, μg/ml	TEAC, μmol of trolox / mg of protein	TEAC (h-t) / TEAC (WPC)	Residual AG, 10^{-3} rel. u.
Experimental hydrolysate sample	≤ 10 kDa 98%	15.5 ± 0.6	29.88 ± 1.92	0.551 ± 0.035	3.19 ± 0.20	1.22 ± 0.07
Hilmar 8350 (Hilmar, United States)	< 20 kDa 83.0% *	12.5 *	29.46 ± 1.18	0.559 ± 0.022	3.23 ± 0.13	52.9 ± 2.0
PRODIET GF 006 (Ingredia, France)	< 5 kDa 97.2% *	22.5 ± 2.5 *	16.75 ± 0.24	0.982 ± 0.014	5.68 ± 0.08	0.84 ± 0.04

Note. * indicators are presented according to the data from manufacturers.

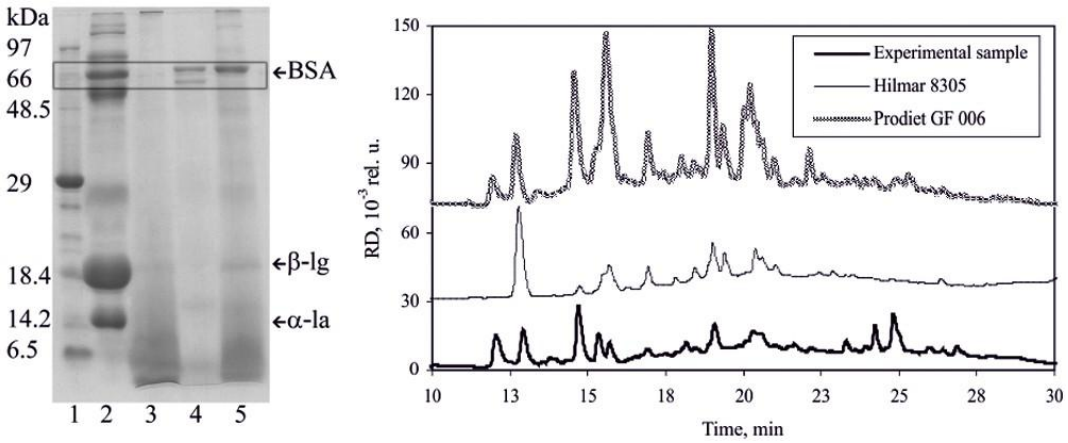

Fig. 2. SDS electrophoregram (a) and HPLC profiles (b) of whey protein hydrolysates: 1 – marker; 2 – WPC control; 3 – experimental hydrolysate sample; 4 – "PRODIET GF 006"; 5 – "Hilmar 8350"; β-lg – β-lactoglobulin, α-la – α-lactalbumin.

Comparable degree of hydrolysis and AOA level were indicated for experimental hydrolysate sample and "Hilmar 8350" whereas decrease of antigenic potential of hydrolyzed WPC to 1.22 ± 0.07 rel. units (similar to "PRODIET GF 006") are probably associated with prior thermal treatment of WPC [22]. Heating of whey protein solution in optimal conditions and subsequent hydrolysis by alcalase resulted in cleavage of all substrates into intermediate peptides. According to SDS electrophoretic analysis (Fig. 2a, framed) PRODIET GF 006 and Hilmar 8350 hydrolysates have native bovine serum albumin (BSA), which possesses allergenic potential. Thus, advantage of the experimental sample is the total cleavage of all allergenic whey proteins. Moreover, comparison between peptide profiles of experimental sample and analogues (Fig. 2b) indicates the use of proteolytic enzymes with different catalytic activity mechanisms and substrate specificity that ensures obtaining of hydrolysates with specific composition.

According to experimental data, the enzymatic hydrolysis of whey proteins (WPC) resulted in increase of free radical scavenging activity of obtained peptide fraction by 2.98–3.39 times. It was established that increasing degree of substrate cleavage is accompanied by increasing radical scavenging activity and decreasing allergenic potential of hydrolysates.

Toxicological and hygienic assessment of milk whey protein concentrate and its enzymatic hydrolysate on *T. pyriformis* model

For toxicity study in the acute experiment 100 000 infusoria in stationary growth phase were added to suspensions containing 30, 90, 120 and 150 mg/ml of WPC and hydrolysate (24, 72, 96, and 120 mg/ml of protein respectively). After 30, 60 and 120 min of exposure there was no dead infusoria in studied samples, so the exposure time for acute toxicity determination was increased up to 240 min. One could observe decrease in number of organisms by 10% compared to control in samples containing 90, 120 and 150 mg/ml of enzymatic hydrolysates of milk whey proteins. Single dead infusoria were found in sample with concentration 90 mg/ml. Along with this, a sample of milk whey protein concentrate had no visible impact on the organisms and did not cause their death.

For toxicity study in subacute experiment 100 000 infusoria in stationary growth phase were added to suspensions containing 30, 90, 120 and 150 mg/ml of WPC and hydrolysate (24, 72, 96, and 120 mg/ml of protein respectively). Exposure time was 24 hours. The number of infusoria in samples with concentration 30, 60 and 90 mg/ml increased compared to the control by 152, 171 and 145%,

respectively. Population size increased by 34 and 24% at concentrations 120 and 150 mg/ml. However, these samples contained dead organisms: lethality 10 and 19%, respectively. Infusoria in all samples were large, they acquired rounded shape and became darker in comparison with the control. But WPC sample had no visible impact on the organisms. Population size increased 3 times compared to the control in samples with concentration 90, 120, 150 mg/ml and 2.7 times – in samples with concentration 30, 60 mg/ml.

Noncarbohydrate medium with 4.0 mg/ml of peptone, 1.0 mg/ml of NaCl, 1.0 mg/ml of yeast extract was prepared for chronic toxicity experiment. For chronic toxicity study of whey protein hydrolysates, *T. pyriformis* population was cultivated throughout life cycle in medium with 10, 30, 60, 90, 120 and 150 mg/ml of experimental sample (Table 4). The chronic toxicity of whey protein concentrate was studied at concentrations 10, 30 and 60 mg/ml, because the protein in samples with concentration 90, 120 and 150 mg/ml has folded on sample preparation stage (during tyndalization).

Cultivation medium of *T. pyriformis* with previously added enzymatic hydrolysate of milk whey protein at concentration 10 mg/ml stimulated infusoria growth during 48–96 h of population life cycle in comparison with the control level ($p > 0.05$). Increasing of sample content up to 30 mg/ml lead to decrease in population size in lag-phase and logarithmic growth phase by 30% and to subsequent stimulation in slow growth and stationary phases by 11 and 131%, respectively, relative to the control level ($p > 0.05$). At concentrations 60 and 90 mg/ml population growth was inhibited during 24–48–72 h of life cycle. In addition, with increasing cultivation time the population was gradually approaching the control level and reached it after 96 h, or even exceeded it by 51% ($r > 0.05$) at sample concentration 60 mg/ml. Increase of hydrolysate content in medium up to 120 and 150 mg/ml caused infusoria growth inhibition to increase and to reach 77 and 88% in logarithmic growth phase, respectively, relative to the control level ($p > 0.05$) (Table 4).

Table 4. Changes in size of *T. pyriformis* population cultivated in medium with enzymatic hydrolysate of milk whey proteins and whey protein concentrate

Content of hydrolysate/ WPC, mg/ml	Exposure time, h			
	24	48	72	96
Population				
0 (control)	12 500 ± 928	100 500 ± 577	251 000 ± 6 360	289 000 ± 2 404
Population to control,%				
0 (control)	100 ± 7.4	100 ± 0.6	100 ± 2.5	100 ± 0.8
Milk whey protein hydrolysate				
10.0	11 000 ± 0	121 500 ± 167 *	394 500 ± 19 641 *	559 000 ± 577 *
30.0	10 000 ± 167	70 000 ± 2 309 *	278 000 ± 4 619 *	668 000 ± 8 083 *
60.0	6 000 ± 0 *	56 000 ± 1 443 *	243 000 ± 6 351	436 000 ± 1 155 *
90.0	6 500 ± 441 *	37 000 ± 2 179 *	128 000 ± 2 646 *	298 000 ± 8 083
120.0	3 500 ± 167 *	23 000 ± 1 014 *	60 500 ± 2 309 *	182 000 ± 1 155 *
150.0	4 500 ± 0 *	12 000 ± 167 *	47 000 ± 289 *	131 000 ± 1 732 *
Population to control,%				
10.0	88 ± 0	121 ± 0.2 *	157 ± 7.8 *	193 ± 0.2 *
30.0	80 ± 1.3	70 ± 2.3 *	111 ± 1.8 *	231 ± 2.8 *
60.0	48 ± 0 *	56 ± 1.4 *	97 ± 2.5	151 ± 0.4 *
90.0	52 ± 3.5 *	37 ± 2.2 *	51 ± 1.1 *	103 ± 2.8
120.0	28 ± 1.3 *	23 ± 1.0 *	24 ± 0.9 *	63 ± 0.4 *
150.0	36 ± 0 *	12 ± 0.2 *	19 ± 0.1 *	45 ± 0.4 *
Milk whey protein concentrate				
10.0	11 000 ± 601	69 500 ± 1 302 *	322 500 ± 26 238	569 000 ± 4 041 *
30.0	12 000 ± 289	56 000 ± 2 309 *	191 000 ± 8 743 *	418 000 ± 8 083 *
60.0	9 000 ± 577 *	77 000 ± 0 *	307 000 ± 68 537	439 000 ± 2 887 *
Population to control,%				
10.0	88 ± 4.8	69 ± 1.3 *	128 ± 10.5	197 ± 1.4 *
30.0	96 ± 2.3	56 ± 2.3 *	76 ± 3.5 *	145 ± 2.8 *
60.0	72 ± 4.6 *	77 ± 0 *	122 ± 27.3	152 ± 1.0 *

Note. * statistically significant differences in relation to control level ($p < 0.05$).

One could observe following processes in *T. pyriformis* cultivation medium with 10 and 60 mg/ml of WPC: decrease of *T. pyriformis* population size in lag-phase and logarithmic growth phase up to 31%; subsequent stimulation by 97 and 52% in slow growth and stationary phases, respectively ($p > 0.05$). Decrease in population size by 14% in lag-phase, by 44% in logarithmic growth phase and by 24% in slow growth phase was observed at WPC concentration 30 mg/ml. The 45% increase relative to the control level was reached after 96 h.

Probit analysis was used to calculate parameters of acute, subacute and chronic toxicity of milk whey protein concentrate and its enzymatic hydrolysate in acute, subacute (infusoria lethality) and chronic (inhibition of generative function under the influence of investigated samples) experiments.

According to experimental data of toxicological and hygienic assessment on *T. pyriformis* model presented in Tables 5 and 6, whey protein concentrate and its enzymatic hydrolysate belong to hazard class 4 with absence of cumulative properties.

Thus, the presented samples of milk and whey protein concentrate and partial enzymatic hydrolysate of milk whey proteins are non-toxic and do not possess any cumulative properties.

Table 5. Toxicity parameters of whey protein enzymatic hydrolysate according to assessment results obtained on *T. pyriformis* model

Toxicity index	Toxicity value	Hazard class
Acute toxicity		
LD_{16}, mg/ml	–	–
LD_{50}, mg/ml	more than 100	5
LD_{84}, mg/ml	–	–
Subacute toxicity		
LD_{16}, mg/ml	–	–
LD_{50}, mg/ml	more than 150	–
LD_{84}, mg/ml	more than 200	–
K_{CUMac}	more than 1	5
Chronic toxicity in logarithmic phase (48 h)		
ED_{16}, mg/ml	8.13	–
ED_{50}, mg/ml	70.0 ± 0.12	–
ED_{84}, mg/ml	131.88	–
Chronic toxicity in stationary growth phase (96 h)		
ED_{16}, mg/ml	105.18	–
ED_{50}, mg/ml	140.96 ± 0.09	–
ED_{84}, mg/ml	176.75	–
K_{CUMchr}	2.01	5
Z_{chr}	less than 2.5	4

Table 6. Toxicity parameters of milk whey proteins according to the results obtained on *T. pyriformis* model

Toxicity index	Toxicity value	Hazard class
Acute toxicity		
LD_{16}, mg/ml	–	–
LD_{50}, mg/ml	more than 100	5
LD_{84}, mg/ml	–	–
Subacute toxicity		
LD_{16}, mg/ml	–	–
LD_{50}, mg/ml	more than 150	–
LD_{84}, mg/ml	more than 200	–
K_{CUMac}	more than 1	5
Chronic toxicity		
ED_{50}, mg/ml in logarithmic phase (48 h)	more than 50	–
ED_{50}, mg/ml in stationary growth phase (96 h)	more than 50	–
K_{CUMchr}	more than 1	5
Z_{chr}	less than 2.5	4

CONCLUSION

We performed integrated analysis of physico-chemical and bioactive properties (antigenic and free radical scavenging activity) of obtained enzymatic hydrolysates of milk whey proteins. It has been established that the experimental hydrolysate sample possesses acceptable organoleptic properties and low antigenicity due to absence of high molecular fraction.

Antioxidant properties of whey protein concentrate from cow's milk and its enzymatic hydrolysate were studied using TEAC (Trolox equivalent antioxidant capacity) technique. It has been established that enzymatic hydrolysis of whey proteins leads to the increase in free radical scavenging activity of obtained peptide fraction by 2.98–3.39 times. According to the comparative characteristics of experimental hydrolysate sample and foreign analogues, increasing degree of protein substrate cleavage is accompanied by rising of radical scavenging activity and decreasing of allergenic potential of hydrolysates.

Higher antiradical properties of hydrolysates (compared to native whey protein) are associated with splitting of protein macromolecules into peptides. This process is accompanied by exhibiting of additional amino acid radicals with proton donor properties of indole and phenol groups of tryptophan and tyrosine, respectively, and the formation of methionine sulfoxide and oxidation of cysteine sulfhydryl groups (−SH). Enzymatic hydrolysis of substrates also leads to splitting of antigenic determinant areas, and hence provides the protein component with low antigenic potential.

Enzymatic hydrolysate experimental sample obtained using bacterial endopeptidase (alcalase) is comparable by physicochemical parameters, antigenic and antioxidant activity with foreign analogues: PRODIET GF 006 (Ingredia, France) and Hilmar 8350 (Hilmar, United States), which are used in the manufacture of functional products. Advantage of the experimental hydrolysate sample is the total cleavage of all whey protein allergens.

According to the results of toxicological and hygienic assessment in acute and subacute experiments on *T. pyriformis*, whey protein concentrate and its enzymatic hydrolysate belong to hazard class 5 with absence of cumulative properties and to hazard class 4 according to zone of chronic action. Investigated agents do not possess toxic effects that lead to vital activity disruption of individual organs, systems or the whole organism. This indicates the absence of cumulative properties of these agents.

The obtained partial whey proteins hydrolysate can serve as physiologically active component in the development of new specialized food including functional products.

REFERENCES

1. Hurley W.L. *Milk protein*. Vol. 1. Rijeka, Croatia: InTech, 2012. 352 p.
2. Wal J.M. Bovine milk allergenicity. *Ann. Allergy Asthma Immunol*., 2004, vol. 93, pp. 2–11.
3. Raikos V. and Dassios T. Health-promoting properties of bioactive peptides derived from milk proteins in infant food: a review. *Dairy Sci. & Technol*., 2014, vol. 94, pp. 91–101.
4. Mahmoud M.I. The physicochemical and functional properties of protein hydrolysates in nutritional products. *Food Technol*., 1994, vol. 48, no. 10, pp. 89–95.
5. Maldonado J., Gil A., Narbona E. and Molina J.A. Special formulas in infant nutrition: a review. *Early Hum. Dev*., 1998, vol. 53, no. 1, pp. 23–32.
6. Zulueta A., Maurizi A., Frígola A., Esteve M.J., Coli, R. and Burini G. Antioxidant capacity of cow milk, whey and milk deproteinized. *Int. Dairy J*., 2009, vol. 19, no. 6–7, pp. 380–385.
7. Hernández-Ledesma B., Dávalos A., Bartolomé B. and Amigo L. Preparation of antioxidant enzymatic hydrolysates from alpha- lactalbumin and beta-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem*., 2005, vol. 53, no. 3, pp. 588–593.
8. De Castro R.J.S. and Sato H.H. Comparison and synergistic effects of intact proteins and their hydrolysates on the functional properties and antioxidant activities in a simultaneous process of enzymatic hydrolysis. *Food and Bioproducts Proc*., 2014, vol. 92, no. 1, pp. 80–88.
9. Hernández-Ledesma B., Quirós A., Amigo L. and Recio I. Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *Int. Dairy J*., 2007, vol. 17, no. 1, pp. 42–49.
10. Sinks G. and Schultz T. Correlation of Tetrahymena periformis and Pimephales toxicity: evaluation of 100 additional compounds. *Environ. Toxicol. Chem*., 2001, vol. 20, no. 4, pp. 917–921.
11. Zhu H., Tropsha A., Fourches D., Varnek A., Papa E., Gramatica P., Oberg T., Dao P., Cherkasov A. and Tetko I.V. Combinatorial QSAR modeling of chemical toxicants tested against Tetrahymena periformis. *J. Chem. Inf. Model*., 2008, vol. 48, no. 4, pp. 766–784.
12. Kurchenko V.P., Kapustin M.A., Butkevich T.V., Rizevskiy S.V., Halavach T.N. and Strogiy V.N. Integrated technology of whey processing. *Molochnaya Industriya*, 2013, no. 2, pp. 38–41. (In Russian).
13. Osterman L.A. *Research methods of proteins and nucleic acids: Electrophoresis and ultracentrifugation (practice note)*. Moscow: Nauka Publ., 1981. 288 p. (In Russian).
14. Kim S.B., Seo I.S., Khan A., Ki K.S., Lee W.S. and Lee H.J. Enzymatic hydrolysis of whey is heated: iron-binding ability of peptides and antigenic protein fractions. *J. Dairy Sci*., 2007, vol. 90, pp. 4033–4042.
15. Kim S.B., Ki K.S., Khan A., Lee W.S., Lee H.J. and Ahn B.S. Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *J. Dairy Sci*., 2007, vol. 90, pp. 4043–4050.

16. Kruglik V.I., Zorin S.N., Gmoshinskiy I.V., Ponomarev D.V., Nikitina N.E., Abramova A.A., Volkova I.N. and Revyakina N.V. *Sposob polucheniya fermentativnogo gidrolizata syvorotochnykh belkov so sredney stepen'yu gidroliza* [Obtaining method of whey protein enzymatic hydrolysate with moderate degree of hydrolysis]. Patent RF, no. 2375910, 2009.
17. Kiselev P.A., Oreshko N.A., Bovdey N.A., Le Min Kha, Fam Kuok Long, Baranovskiy A.V., Khripach N.B., Khlebnikova T.S. and Lakhvich F.A. *Obtaining and characterization of antioxidant activity of Iris Domestica extracts and their individual components. Proceedings of the Belarusian State University*, 2012, vol. 7, pp. 228–237. (In Russian).
18. Kutsenko S.A. *Toxicology basics: method. pub*. St. Petersburg: Foliant Publ., 2004. 720 p. (In Russian).
19. Podunova L.G., Menshikova, T.A. and Dvoskin Ya.G. *Alternative research methods (express methods) for toxicological-hygienic assessment of materials, products and environmental objects: method. pub*. Moscow, 1999. 108 p. (In Russian).
20. Prozorovsky V.B. Least square method for probit analysis of mortality curves. *Farmakologiya i toxikologiya*, 1962, no. 1, pp. 41–63. (In Russian).
21. O'Keeffe M.B. and FitzGerald, J.R. Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells. *Int. Dairy J*., 2014, vol. 36, no. 2, pp. 128–135.
22. Halavach T.M., Kurchenko V.P. and Albulov A.I. Enzymatic hydrolysis of milk proteins as a basis of specialized food products biotechnology. *Ural Scientific Bulletin*, 2014, vol. 104, no. 25, pp. 69–79.

Please cite this article in press as: Halavach T.N., Kurchenko V.P., Zhygankov V.G. and Evdokimov I.A. Determination of physicochemical, immunochemical and antioxidant properties, toxicological and hygienic assessment of whey protein concentrate and its hydrolysate. *Foods and Raw Materials*, 2015, vol. 3, no. 1, pp. 105–114. doi: 10.12737/13127.