



Processing cottage cheese whey components for functional food production

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Abstract:

Introduction. The study offers a new rational approach to processing cottage cheese whey and using it as a highly nutritional functional ingredient in food production. We proposed a scientifically viable method for hydrolyzing cottage cheese whey with enzyme preparations of acid proteases from *Aspergillus oryzae* with an activity of 400 units/g and a pH range of 3.0 to 5.0.

Study objects and methods. Pre-concentrated whey was enzymatically hydrolyzed at 30°C, 40°C, and 50°C for 60 to 180 min (pH 4.6). Non-hydrolyzed whey protein concentrates were used as a control. The amount of enzyme preparation was determined by calculation. All hydrolysate samples showed an increase in active acidity compared to the control samples. Further, we conducted a full-factor experiment with three levels of variation. The input parameters included temperature, duration of hydrolysis, and a substrate-enzyme ratio; the output parameters were the degree of hydrolysis and antioxidant capacity.

Results and discussion. The experiment showed the following optimal parameters for hydrolyzing cottage cheese whey proteins with the enzyme preparation of proteases produced by *Aspergillus oryzae*: temperature – 46.4°C; duration – 180 min; and the amount of enzyme preparation – 9.5% of the protein content. The antioxidant capacity was 7.51 TE mmol/L and the degree of hydrolysis was 17.96%.

Conclusion. Due to its proven antioxidant capacity, the whey protein hydrolysate obtained in the study can be used as a functional food ingredient.

Keywords: Cottage cheese whey, protein, enzymatic hydrolysis, functional ingredient, *Aspergillus oryzae*, concentration factor

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INTRODUCTION

Processing whey, including cottage cheese whey, is highly relevant today due to several urgent problems. Among them are deficiencies in nutrients and raw materials, as well as low social and environmental efficiency. According to analytical data, 59% of the whey produced in Russia is fed to livestock and only 21% is processed for further use. The remaining 20% is discharged into fields or wastewater, exacerbating the existing environmental problems [1]. When discharged

into the environment, whey acts as a biochemical contaminant. It is characterized by high biological oxygen consumption (50–60 g O₂ per one liter annually) and high chemical oxygen consumption (50.5–54 g O₂ per one liter) [2]. Thus, whey entering sewage systems or, in emergency cases, water bodies can cause serious environmental problems. Simple calculations show that the oxidation of organic compounds contained in 25 tons of whey (an output of a medium-sized cheese factory) needs as much oxygen as the oxidation of household wastewater in a city with a population of

40000 people. Wastewater has a high concentration of readily oxidizable organic compounds. Therefore, they can cause a decrease in dissolved oxygen concentration in water bodies. Moreover, the presence of suspended protein particles can lead to the accumulation of bottom sediments and rotting processes [3].

A positive scenario suggests a growth in production from using highly efficient technologies for the deep processing of raw materials, creating “smart” storage and logistics systems, as well as minimizing losses and waste. For this, we need to focus on the “intravital” formation of the composition and properties of raw materials. It is a prerequisite for modern food technologies and “smart” agriculture. Only this approach can lead to potential progress in technologic development and consistently contribute to positive trends in the nutrition of the population [4].

A healthy lifestyle requires new ways of increasing the nutritional value of foods. Intensive food production often leads to raw materials losing essential micronutrients at all stages of processing (refining, pasteurization, etc.) [5].

Another possible cause of nutritional deficiency is excessive consumption of medicines for chronic diseases, including gastrointestinal disorders, acute respiratory viral infections and flue epidemics, etc. These drugs cause “pharmacological” malabsorption, contributing to the deficiency of essential nutrients supplied with food [6, 7]. As a result, they affect adaptive, compensatory, and regulatory capabilities of the body, change its physiological functions, and lead to chronic diseases of not only the digestive system, but also other organs and systems. These include atherosclerosis, hypertension, type 2 diabetes, metabolic immunosuppression, alimentary obesity, autoimmune pathology, etc. Moreover, the lack of proteins, polyunsaturated fatty acids, vitamins, and minerals in the diet leads to impaired immunoreactivity and resistance to natural and anthropogenic environmental factors [8].

The current situation dictates a need for new directions and technologies for producing healthy foods, including dairy products. However, one of the problems here is introducing functional ingredients. The development of functional foods often involves enriching foods with functional ingredients and/or eliminating those substances which cause negative reactions (food hypersensitivity). For this, we need adequate scientific data on healthy nutrients used as ingredients and their effect on the product’s taste and aroma profile [9, 11].

Controlled biocatalysis with specific enzymes can undoubtedly help food formulators develop functional food products [12].

Many researchers suggest using whey protein hydrolysates as functional ingredients. Due to bioactive peptides, they enhance the beneficial effect of

traditional foods on public health [13, 15]. According to many authors, milk proteins modified by enzymatic hydrolysis have both technological properties (moisture binding, emulsifying, and foaming abilities) and functional properties (antioxidant, immunomodulating, hypotensive, etc.) [16–19].

Whey protein hydrolysates are used in the production of specialized products, for example, in sports nutrition [20].

In addition, whey treated with modern methods can increase the biological value of the end-product and improve functional and technological properties of raw materials and meat systems. For example, introducing whey into the meat system can regulate certain bio- and physicochemical processes by activating the biotechnological potential of natural systems in the ingredients [21]. In one study, hydrated protein preparations of Belcon Alev I and Lactobel ED were used to produce high-quality cooked sausages with a high biological value, digestibility, and prebiotic properties [22].

Whey protein is successfully used in the production of sausages as it not only creates a gelatinous mass that replaces fat, but also retains moisture [23]. This means that the yield of end-products can be increased without reducing the content of valuable animal protein or using additives. In addition, the end-products have better functional and technological properties, as well as improved taste characteristics [24].

Another benefit of using concentrated whey proteins in meat production is improved absorption of the end-product by the human body, which, together with a reduced calorie content, contributes to the physiological value of the product. Thus, replacing the fat component of the meat product with a protein fraction of dairy origin can be a fundamentally new solution to the global problem of obesity [25].

Cottage cheese whey is currently the main source of protein hydrolysates. Despite high volumes of whey produced in Russia and its obvious benefits, there are insufficient data on its use as a raw material for functional ingredients [10, 26].

Our study aimed to prove a possibility of using cottage cheese whey proteins subjected to biocatalysis as functional food ingredients.

STUDY OBJECTS AND METHODS

The objects of the study included cottage cheese whey and enzyme preparations – acid proteases from *Aspergillus oryzae* with an activity of 400 units/g and a pH from 3.0 to 5.0.

The initial samples of whey, concentrate, and hydrolysate were analyzed for active acidity (pH) potentiometrically according to State Standard 32892-2014¹ and for a mass fraction of total protein according

¹ State Standard 32892-2014. Milk and dairy products. Method of pH determination. Moscow: Standartinform; 2015. 13 p.

to State Standard 23327-98¹¹. The assays were performed in triplicate.

Whey protein concentrate was obtained on an AL 362 pilot ultrafiltration unit (Altair, Russia) with a concentration factor of 5.0.

Enzymatic hydrolysis was carried out as follows. An enzyme preparation was introduced into cottage cheese whey preheated to the hydrolysis temperature (namely 30°C, 40°C, and 50°C) for 60–180 min. Active acidity was 4.6. Whey protein concentrates were used as a control. The enzyme amount was calculated by the formula:

$$M_E = \frac{P \cdot \alpha}{A} \times 1000 \quad (1)$$

where M_E is the amount of the enzyme preparation per 1 g protein;

P is the protein content in 100 g whey;

α is the required enzyme activity; and

A is the initial enzyme activity.

The hydrolysis was carried out in a thermostatic water bath with constant stirring. At the end, the samples were heated to 85°C and held for 15 min to inactivate the enzyme preparation.

The samples were then cooled and poured into sterile dishes.

The degree of hydrolysis was determined spectrophotometrically according to the method of Spencer *et al.* [27]. In particular, we took 2 mL of the sample into a 15 mL plastic falcon and added 10 mL of a 1% aqueous solution of sodium dodecyl sulfate. For this, we used an automatic pipette (Eppendorf, Germany) with a measurement range of 500–5000 μ L. The resulting reaction mixture was incubated in a water bath at 75 \pm 1°C for 15 min.

A series of dilutions of L-leucine in a 1% SDS aqueous solution with concentrations of 0.15–3.0 mmol/dm³ were used as standards for determining the degree of hydrolysis according to the Spencer *et al.* method. In particular, the falcons with different standard concentrations were successively filled with 2 mL of 0.2125 M sodium phosphate buffer

(pH 8.20) and 250 μ L of a standard solution, as well as 50 μ L of a hydrolysate sample in the first case, 250 μ L of a UV concentrate sample in the second case, and a blank sample in the third case. In addition, 2 mL of a 0.1% solution of 2,4,6-trinitrobenzenesulfonic acid was added to all the falcons. The falcons were tightly closed and shaken. The samples were then incubated in a water bath at 50°C for one hour. At the end of the incubation, 4.0 mL of a 0.1 M hydrochloric acid solution was added to each falcon to stop the reaction. The falcons were tightly closed, shaken, and kept for 30 min at room temperature for cooling. The optical density of the solutions was determined on a Synergy 2 microplate photometer-fluorometer (BioTek, USA) at a wavelength of 340 nm.

To determine the amount of leucine equivalents, we took 0.75 mL of the hydrolysate with the maximum degree of hydrolysis (100%) and transferred it into a 5.0 mL microreaction vessel. Then, we added 0.75 mL of distilled water and 2.4 L of concentrated hydrochloric acid. The vessel was incubated in an oven at 120 \pm 2°C for 23 h. After incubation, the samples were cooled for one hour at room temperature and filtered under vacuum in a funnel with a glass filter. The contents of the microreaction vessel were quantitatively transferred to the filter and rinsed with distilled water. The pH of the wash water entering the Bunsen flask was monitored using Lach-Ner universal paper. The contents of the Bunsen flask were quantitatively transferred into a 100 mL laboratory glass beaker. The active acidity of the filtrate was adjusted to 7.00 \pm 0.02 pH by adding a 40% aqueous solution of sodium hydroxide. The neutralized filtrate was quantitatively transferred into a 100 mL volumetric flask and the volume was adjusted to the mark with a 1% SDS aqueous solution. The contents of the flask were thoroughly mixed.

After foam collapse, a 0.25 mL sample was taken from the volumetric flask and analyzed. The degree of hydrolysis of the hydrolysate protein was calculated according to the equation:

$$DH = \frac{\left(\frac{(D_{hydrolysate}^{340} - D_{blanksample}^{340}) \cdot 30}{K} - \frac{(D_{UV-concentrate}^{340} - D_{blanksample}^{340}) \cdot 6}{K} \right)}{\left(\frac{(D_{AH}^{340} - D_{blanksample}^{340}) \cdot 13333}{K} - \frac{(D_{UV-concentrate}^{340} - D_{blanksample}^{340}) \cdot 6}{K} \right)} \times 100 \quad (2)$$

where

$D_{hydrolysate}^{340}$ is the optical density in the hydrolysate sample at 340 nm;

$D_{blanksample}^{340}$ is the optical density in the blank sample at 340 nm;

$D_{UV-concentrate}^{340}$ is the optical density in the UV - concentrate sample at 340 nm;

30 is the hydrolysate dilution factor;

6 is a dilution factor for raw materials to obtain a hydrolysate;

K is the slope of the calibration graph showing the dependence of the optical density of the solution at 340 nm on the concentration of the standard in the sample (0.1733 L/mol);

¹¹ State Standard 23327-98. Milk and milk products. Determination of mass fraction of total nitrogen by Kjeldahl method and determination of mass fraction of protein. Moscow: Standartinform; 2009. 11 p.

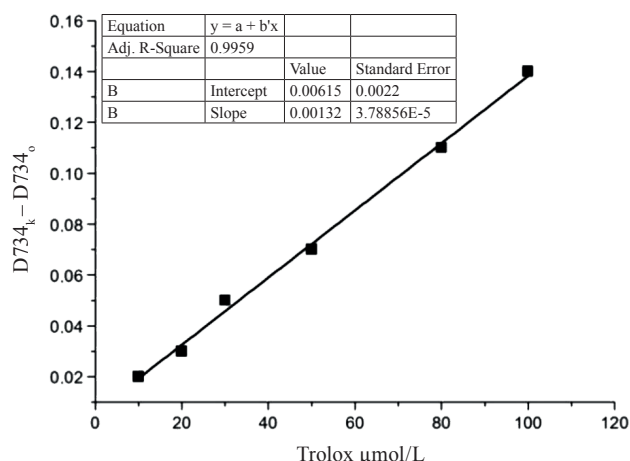


Figure 1 Decrease in optical density of ABTS radical cation solutions vs. Trolox concentrations in the samples

D_{AH}^{340} is the optical density in the acid hydrolysate sample (100% hydrolysis) at 340 nm; and 133.33 is the acid hydrolysate dilution factor.

The *in vitro* antioxidant capacity (TEAC) was measured using the ABTS radical cation.

The ABTS radical cation was obtained according to the Re *et al.* method by incubating a solution of 7 mM ABTS and 2.45 mM potassium peroxodisulfate in the dark at room temperature for 12–18 h [28]. The concentrated solution of ABTS radical cation was diluted with a 50 mM phosphate-buffered saline (with 100 mM sodium chloride), pH 7.4, to $OD_{734} = 0.70 \pm 0.02$. This value corresponded to the final concentration of ABTS radical cation = 47 μM ($\epsilon_{734} = 1.5 \times 10^4 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$).

To determine the antioxidant capacity (AOC), 20 μL of the test samples or a Trolox solution and 180 μL of the ABTS radical cation solution were added to the wells of 96-well non-absorbing polystyrene microplates with a flat bottom. The control was 180 μL of the ABTS radical cation solution and 20 μL of a 50 mM phosphate-buffered saline (with 100 mM sodium chloride), pH 7.4. The reaction was recorded as OD_{734} decreased during 40.5 min with a measurement interval of 60 s at 25°C on a Synergy 2 photometer-fluorimeter (BioTek, USA). The assays were performed in quadruplicate.

The calibration curve of decreased optical density versus Trolox concentrations varying within 1–10 μM can be seen in Fig. 1. Equivalent concentrations of antioxidants in the samples were determined in relation

Table 1 Variation levels of independent parameters in multifactorial experiments to optimize the hydrolysis of cottage cheese whey

Factor	Variable	Level of variation		
		-1	0	+1
Temperature, °C	X_1	30	40	50
Hydrolysis duration, min	X_2	60	120	180
E/S, %	X_3	0.5	4.5	9.5

Table 2 Enzyme amounts per 1 g protein

Enzyme-substrate ratio, %	Enzyme amount per 100 g whey, mg
0.5	4.225
4.5	38.025
9.5	80.275

to the decrease in optical density of the reaction medium in the presence of the studied compounds. The AOC of the samples was expressed in μM TE. When testing the antioxidant activity of hydrolysate samples with respect to the ABTS radical cation, the working range of dilution factors for a 50 mM phosphate-buffered saline (pH 7.4) was 150.

Sensory analysis described by Spellman was used to determine bitterness in enzymatic hydrolysates [29].

To optimize the conditions for enzymatic hydrolysis of cottage cheese whey, we conducted a full-factor experiment with three variables: temperature (X_1),

Table 3 Results of full-factor experiments to optimize enzymatic hydrolysis of cottage cheese whey proteins

Sample	Bitter taste	Degree of hydrolysis (DH), %	TEAC, TE mmol/L
control samples			
Control 1	–	0	5.30
Control 2	–	0	4.17
Control 3	–	0	2.65
fermented samples			
4	–	7.82	4.17
5	–	10.02	7.58
6	+	12.68	7.20
7	–	8.41	4.55
8	–	11.29	5.68
9	+	13.07	6.74
10	–	8.59	5.19
11	+	11.92	6.36
12	++	13.06	8.71
13	–	7.31	7.58
14	–	11.43	6.06
15	–	13.13	8.71
16	–	8.21	5.30
17	–	11.88	9.09
18	–	15.35	9.81
19	–	8.24	1.14
20	–	12.08	8.71
21	+	15.75	4.55
22	–	8.43	3.41
23	–	11.28	5.30
24	–	15.08	4.55
25	+	9.01	3.03
26	–	13.07	4.92
27	–	18.01	4.55
28	–	9.59	3.79
29	–	14.96	5.68
30	–	19.66	8.71

Table 4 Effects of variable factors on the hydrolysis degree of cottage cheese whey proteins

Factor	Effect	Std. dev.	Student t-test	P	−95, %	+95,%
	11.95086	0.098745	121.0273	0.000000	11.74253	12.15920
Temperature, °C (L)	2.54302	0.241874	10.5138	0.000000	2.03271	3.05333
Temperature, °C (Q)	−0.51056	0.209255	−2.4399	0.025942	−0.95204	−0.06907
Duration, min (L)	1.88078	0.241874	7.7759	0.000001	1.37047	2.39109
Duration, min (Q)	0.30944	0.209255	1.4788	0.157487	−0.13204	0.75093
E/S (L)	6.68667	0.241627	27.6735	0.000000	6.17688	7.19645
E/S (Q)	0.61926	0.209685	2.9533	0.008897	0.17686	1.06166
1L by 2L	1.06167	0.295931	3.5875	0.002269	0.43731	1.68603
1L by 3L	1.97152	0.295324	6.6758	0.000004	1.34844	2.59460
2L by 3L	0.77102	0.295324	2.6108	0.018268	0.14795	1.39410

duration of hydrolysis (X_2), and enzyme-substrate ratio E/S (X_3). Each of the parameters varied at three levels (Table 1). The output parameters were the degree of hydrolysis (DH) and antioxidant capacity (TEAC).

The variation levels of independent parameters in the multifactorial experiments conducted to optimize the hydrolysis of cottage cheese whey are shown in Table 1.

The results of the multifactorial experiments were statistically processed using the DOE block of Statistica 10.0 (StatSoft Inc., USA).

RESULTS AND DISCUSSION

The protein content was 0.56% in the initial whey and 1.35% in the concentrate. The enzyme amounts per 1 g of protein are shown in Table 2.

The results of the full-factor experiments conducted to optimize enzymatic hydrolysis of cottage cheese whey proteins are demonstrated in Table 3.

The experiments showed an increase in the degree of hydrolysis and antioxidant activity of cottage cheese whey proteins with larger amounts of enzyme preparations and longer fermentation at 30°C and 50°C. The maximum degree of hydrolysis (19.66%) was recorded at 50°C, 180 min fermentation, and

9.5% enzyme. However, higher temperatures led to a noticeable, almost two-fold decrease in antioxidant activity in the control samples, which were not hydrolyzed. Thus, temperature had a significant effect on this indicator (Table 4).

According to sensory evaluation, the most bitter taste was registered in the sample that was hydrolyzed at 30°C with the maximum duration and enzyme amount (13.06% degree of hydrolysis). However, sample No. 30, which was obtained at the maximum temperature, duration of hydrolysis, and enzyme amount, did not taste bitter. It means that these conditions make the process more directional, producing hydrolysates that do not contain peptides with bitter amino acids at the end of the chain. At the same time, this sample had the highest degree of hydrolysis.

Table 4 shows the statistical analysis of effects that variable factors have on the degree of hydrolysis of cottage cheese whey proteins. As we can see, all the variable factors, except for the quadratic duration factor, have a significant ($P < 0.05$) effect on the degree of hydrolysis.

The relation between the degree of whey protein hydrolysis and variable parameters is graphically

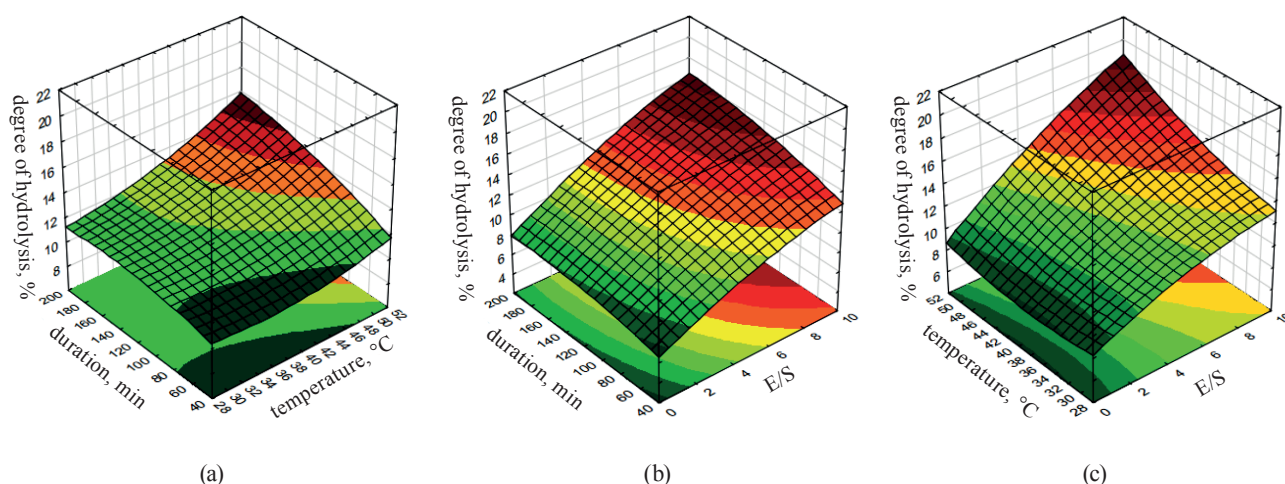


Figure 2 Degree of hydrolysis in UV concentrate hydrolysates of cottage cheese whey versus variable parameters with an average third factor: (a) Degree of hydrolysis versus duration and temperature; (b) Degree of hydrolysis versus duration and enzyme-substrate ratio; (c) Degree of hydrolysis versus temperature and enzyme-substrate ratio

Table 5 Effects of variable factors on antioxidant activity of cottage cheese whey proteins

Factor	Effect	Std. dev.	Student t-test	P	–95, %	+95,%
	6.01776	0.350932	17.14794	0.000000	5.27736	6.758159
Temperature, °C (L)	–1.36738	0.859600	–1.59071	0.130097	–3.18098	0.446221
Temperature, °C (Q)	1.21000	0.743674	1.62706	0.122116	–0.35902	2.779015
Duration, min (L)	–0.15247	0.859600	–0.17738	0.861309	–1.96607	1.661126
Duration, min (Q)	–0.00333	0.743674	–0.00448	0.996476	–1.57235	1.565682
E/S (L)	2.81889	0.858721	3.28266	0.004391	1.00715	4.630632
E/S (Q)	1.10494	0.745203	1.48273	0.156446	–0.46730	2.677179
1L by 2L	0.60167	1.051714	0.57208	0.574754	–1.61726	2.820590
1L by 3L	–0.19918	1.049557	–0.18978	0.851732	–2.41355	2.015191
2L by 3L	1.04324	1.049557	0.99398	0.334171	–1.17113	3.257609

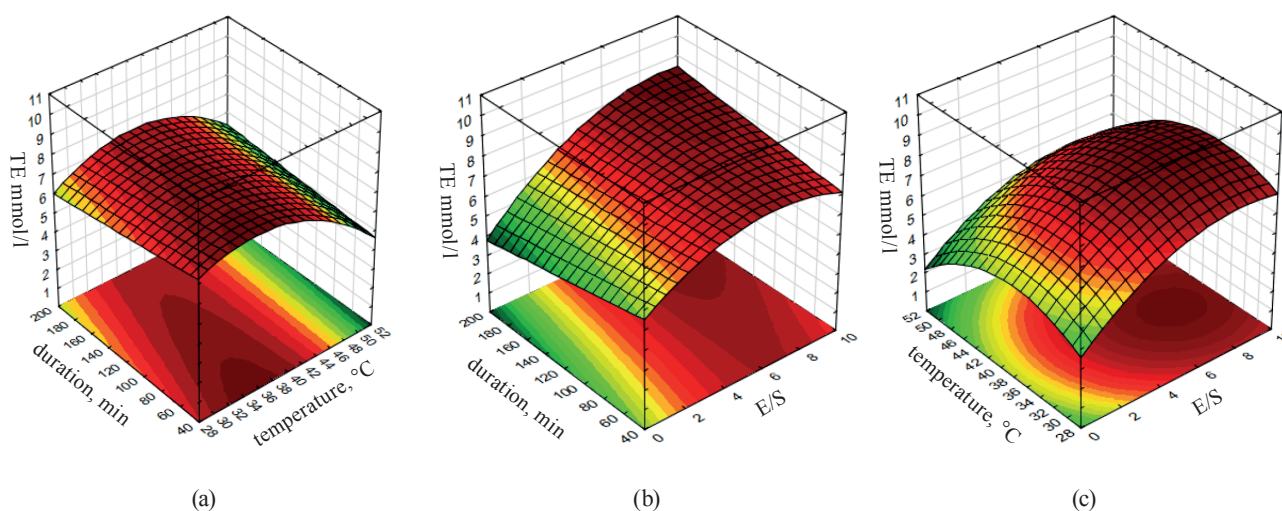


Figure 3 Antioxidant capacity of UV-concentrate hydrolysates versus variable parameters with an average third factor: (a) Antioxidant capacity versus duration and temperature; (b) Antioxidant activity versus duration and enzyme-substrate ratio; (c) Antioxidant activity versus temperature and enzyme-substrate ratio

illustrated in Fig. 2. The graphs show no local maxima or minima, suggesting that the degree of hydrolysis rises with an increase in each of the parameters.

Table 5 shows the statistical analysis of effects that variable factors have on the antioxidant activity of cottage cheese whey proteins. As we can see, only the linear factor of enzyme amount has a significant ($P > 0.05$) effect on the antioxidant capacity.

The surfaces of equal response of hydrolysates TEAC versus variable parameters of whey concentrate hydrolysis are presented in Fig. 3. We can clearly see the presence of a local maximum of antioxidant activity in Figs. 3a and 3b.

Finally, we correlated the key factors in the multifactorial experiments in order to select optimal conditions for the enzymatic hydrolysis of the UV-concentrate of cottage cheese whey using the enzyme preparation from *Aspergillus oryzae*.

CONCLUSION

Based on the statistical analysis, we selected the following optimal conditions for the hydrolysis of cottage cheese whey proteins: temperature – 46.4°C;

duration – 180 min; and the enzyme amount – 9.5% of the protein content. These conditions provided the antioxidant capacity of 7.5 TE mmol/L with a 17.96% degree of hydrolysis.

The given data open up new prospects for processing acid cottage cheese whey and using whey proteins as potential functional components with increased antioxidant activity. We showed that targeted biocatalytic conversion can make whey proteins more functional. The obtained hydrolysate of cottage cheese whey proteins can be used to develop new functional foods, including meat and dairy products.

CONTRIBUTION

E.Yu. Agarkova led the research. A.G. Kruchinin statistically processed the data. N.A. Zolotarev developed and analyzed the test samples. N.S. Pryanichnikova checked the data reliability. Z.Yu. Belyakova systematized the data. T.V. Fedorova summarized the data.

CONFLICT OF INTEREST







The authors state that there is no conflict of interest.

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