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Original scientific paper

FUNCTIONAL FOOD COMPOSITIONS BASED ON WHEY PROTEIN ISOLATE, FISH OIL AND SOY PHOSPHOLIPIDS

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ABSTRACT
The aim of the research was to study the impact of whey protein isolate on the solubility and oxidative stability of the lipid composition based on soy phosphatidylcholine and fish oil. The relationship between the molecular parameters (density; ζ-potential) of the formed complex particles and their functional properties was found using laser light scattering (*static, dynamic, electrophoretic)* and spectrophotometry. The studied compositions could be used as the basis for the development of multifunctional food additives with an objective of the enrichment of foods and beverages with omega-3 polyunsaturated fatty acids (PUFAs). The main advantages of such composition are a high level of protection of the lipids against oxidation and degradation, a high solubility in an aqueous medium and the clean label.

1. Introduction

According to the data of the World Health Organization and the state policy of the Russian Federation in the sphere of healthy nutrition of the population, one of the most promising areas in the food industry is the development of new generation of functional food additives with improved functionality. The main objectives of such additives are as follows: to reduce the deficiency of biologically active substances, to improve their water solubility, bioavailability and bioaccessibility [1,2]. Such food additives can be classified as physiologically functional ingredients if the amount of their bioactive components in one serving of food or beverage is not less than 15% from the recommended daily intakes [3]. It is supposed that the regular consumption of such functional foods can provide a solution to the acute problem of the micronutrient deficiency and thus significantly reduce the quantity of nutrition-related noncommunicable diseases (cardiovascular, neurodegenerative and oncological diseases, diabetes, etc.).

Omega-3 polyunsaturated fatty acids are among the most important physiological nutrients. Their biochemical functions include the participation in transmembrane transmission of synaptic signals, synthesis of prostaglandins as well as vasodilator, antithrombotic and antiatherogenic actions [4]. These biochemical processes (with active participation of omega-3 PUFAs) underlie the successful functioning of the cardiovascular, central nervous, visual systems and the regulation of lipid metabolism [4]. In contrast, both low intakes of omega-3 PUFAs and the excessive consumption of omega-6 PUFAs are the key factors of the nutrition-related noncommunicable diseases such as cardiovascular, autoimmune ones, diabetes, asthma, ect. [5]. The deficiency of omega-3 PUFAs among children and adults in Russian Federation is rather high and reaches 80% [6], therefore it is extremely important to develop the production of fortified, functional and specialized food products aimed at increasing daily consumption of these PUFAs.

However, it should be noted that the main factors limiting the omega-3 enrichment of low-fat food and drinks are the following: a low solubility of the PUFAs in an aqueous medium (due to their hydrophobic nature) and a high susceptibility to degradation and oxidation (due to the presence of unsaturated carbon-carbon bonds). In this connection, it was assumed that the joint use of soy phospholipids and food biopolymers could be a promising approach for the development of omega-3 delivery systems, which, in turn, could be the basis of the functional food composition aimed at fortification of foods and drinks with these PUFAs.

Phosphatidylcholine is widely used for delivery of drugs and bioactives due to its ability to form micro- and nanoliposomes in an aqueous medium and thus to effectively encapsulate both hydrophobic and hydrophilic substances [7]. In addition, phosphatidylcholine itself exhibits the biological activity (it is the major delivery form of the essential nutrient choline, the major lipid in all cellular membranes and has the hepatoprotective activity) [8].

Whey protein isolate (WPI) in turn is of a great interest when considering food biopolymers for the functional food composition. Firstly, WPI is a valuable source of essential amino acids and bioactive peptides due to its unique protein composition (β-lactoglobulin (52%), α-lactalbumin (23%), immunoglobulins (16%), bovine serum albumin (8%), bovine lactoferrin and other minor proteins (1%)), and also it has the ability to act as an antioxidant, immune enhancing, anti-inflammatory, antitumor, hypotensive, antidiabetic and osteo-protective agents [9]. Secondly, WPI is widely used in the food industry as a structure-forming agent owing to its emulsifying, thickening, gelling, foaming, and water-binding properties [10]. Thirdly, it has recently been shown that WPI nanoparticles are able to encapsulate different bioactive compounds [10]. In addition, it has been noted that the heat treatment over 60 °C is one of the promising ways to improve the functional properties of WPI [11]. Under such temperature conditions, the heat-induced denaturation of globular proteins of WPI occurs (polypeptide chains unfold) leading to the exposure of a large number of hydrophobic functional groups on the surface of protein particles.

The aim of the present research was to study the impact of whey protein isolate (original and partially heat-denatured (conditioned at 70 °C for 10 min)) on the solubility and oxidative stability of a lipid composition comprising soy phosphatidylcholine and fish oil.

The amounts of omega-3 and omega-6 PUFAs in this composition were equal (the ratio of omega-6 to omega-3 PUFA was close to 1:1 that is considered the most balanced for the healing effect) [12].

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2. Objects and methods

2.1. Objects

- *Soy phosphatidylcholine* (PC), LIPOID S100 (95.5% purity) was supplied by Lipoid GmbH (Germany). According to the manufacturer's specification, the typical fatty acid composition of this sample (in % to total fatty acids) was as follows: palmitic acid — 12÷17; stearic acid — 2÷5; oleic acid — 11÷15; linoleic acid — 59÷70; linolenic acid — 7÷11.
- The sample of *fish oil* (FO) was «Concentrate of ω-3 Omegadeti», produced by «Ruscaps» (Russia). The fatty acid composition (in % to total fatty acids) of the sample determined by gas-liquid chromatography was as follows: linoleic acid — 1.07%; octadecatrienoic acids — 2.34%; arachidonic acids — 1.67%; docosatetraenoic acid — 3.40%; eicosapentaenoic acid — 27.63%; docosahexaenoic acid — 20.00%.
- The commercial sample of *whey protein isolate* (WPI) (BiPro, Davisco Food International, Inc., USA) contained 91% of whey proteins, 0% of lipids, 0% of carbohydrates, 0% of sucrose and minerals (0.045% — Ca^{2+}; 0.008% — Mg^{2+}; 0.77% — Na^{+}).
- All the other high purity (> 99%) *chemicals* (HCl, NaOH, ethanol, diethyl ether, sodium azide, trichloroacetic acid, 2-thiobarbituric acid, Na_2HPO_4 and NaH_2PO_4 were purchased from «Laverna» (Russia).

All aqueous solutions were prepared using double distilled water. Sodium azide (2×10^{-4} g/ml) was added into each of these solutions as an antimicrobial agent.

2.2. Preparation of aqueous solutions of (PC–FO) liposomes, WPI samples and their supramolecular complexes.

2.2.1. Preparation of PC–FO liposomes. The required amounts of PC and FO were predispersed in the phosphate buffer (pH 7.0, ionic strength (I) = 0.001 M). The concentrations of the lipids ($C_{PC} = 1.56\times10^{-3}$ g/ml; $C_{FO} = 1.46\times10^{-3}$ g/ml) were chosen so that the weight ratio of omega-6 to omega-3 PUFAs in the dispersion was close to 1:1. The preparation of PC–FO liposomes consisted of four successive steps: the stirring at 20000 rpm for 2 min using a mechanical homogenizer (Heidolph, Germany); the sonication (3 times for 5 min each) of the sample (in ice) using a pulse mode of VCX-130 ultrasonic processor (Sonics & Materials, USA); the centrifugation (1800 g for 30 min at 20 °C) to remove the titanium particles released from the ultrasonic probe; and finally the extrusion (19 times) using Avanti Mini Extruder (Avanti Polar Lipids, USA), and a polycarbonate membrane with a pore diameter of 100 nm). The liposome solutions were stored under argon in a refrigerator at 4 °C.

2.2.2. Preparation of WPI solutions (original (WPI) and partially heat-denatured (WPI_{70})). The required amounts of original WPI were dissolved in phosphate buffer (pH 7.0, I = 0.001 M) under magnetic stirring. The centrifugation (1800 g for 40 min at 20 °C) of the protein solutions was performed to remove a small minority of insoluble materials. Thereafter, the solutions were sequentially filtered through filter membranes (Sartorius, Germany) with progressively smaller pore sizes, such as 0.65 μm, 0.45 μm, and 0.22 μm to remove a small fraction (~5%) of large in size and high-molecular-weight aggregates.

The sample of partially heat-denatured protein (WPI_{70}) was prepared by the incubation of original WPI solution at 70 °C for 10 min in GFL 3032 shaking incubator (GFL, Germany).

2.2.3. Preparation of complexes between PC–FO liposomes and WPI (original and partially heat-denatured). The solutions of protein (WPI/ WPI_{70}) and PC–FO liposomes were mixed and shaken (175 rpm) at 40 °C for 1 h in GFL 3032 shaking incubator (GFL, Germany). The weight ratio of protein to lipids in the complex solutions was close to 8:1 and the concentration of the components were as follows: $C_{WPI} = 2.44\times10^{-2}$ g/ml, $C_{PC} = 1.56\times10^{-3}$ g/ml, $C_{FO} = 1.46\times10^{-3}$ g/ml. The prepared complex solutions were stored under argon in a refrigerator at 4 °C. The degree of encapsulation of PC– FO liposomes by protein samples was found to be 93 ± 5 % in the case of WPI and 97 ± 5% for WPI_{70}.

2.3. Characterization of supramolecular complexes of PC–FO liposomes with whey protein isolate (original WPI and partially heat-denatured WPI_{70}).

2.3.1. Determination of the encapsulation efficiency of PC–FO liposomes in WPI/WPI_{70} particles. Aliquots (3 ml) of diethyl ether were added to the glass vials containing 5 ml of PC– FO liposomes or complex particles (PC– FO–WPI or PC– FO–WPI_{70}). All vials were shaken gently, conditioned at room temperature (for 30 min) and then placed in the refrigerator (4 °C) for 24 h. The concentrations of free lipids in the samples were estimated by measuring the optical density of the ether layers at λ = 215 nm (SF-2000 spectrophotometer, OKB SPECTR, Russia). The optical density value for the diethyl ether extract of the (PC — FO) liposomes from their buffered aqueous solutions was considered as 100% in the calculation of the amounts of free (and thus bound) lipids in the solutions of the complexes.

2.3.2. Characterization of the structural parameters of PC–FO liposomes, protein samples (WPI/WPI_{70}) and their supramolecular complexes (PC–FO–WPI and PC– FO–WPI_{70}). The static and dynamic multiangle laser (633 nm, He-Ne laser) light scattering (LS-01 apparatus, Scientific Instruments, St. Petersburg, Russia) were used to determine the following structural parameters of the studied samples: the hydrodynamic radius, R_h; the radius of gyration, R_G; the weight-avarage molar mass, M_w; the particle density, $d = M_w/(N_A V)$, where N_A is Avogadro's number, V is the volume of the particle.

2.3.3. ζ-Potential measurements. The *ζ-potentials* of the studied samples were measured using Zetasizer Nano ZS (Malvern, UK).

2.3.4. Estimation of the level of primary lipid oxidation products (hydroperoxides and ketodienes). The diethyl ether (3 ml) was added to 1 ml aliquots of PC–FO liposomes and complex particles (PC–FO–WPI or PC– FO–WPI_{70}). These two-layer mixtures were shaken gently, conditioned at room temperature (for 30 min) and placed in the refrigerator (4 °C) for 24 h. Thereafter, the organic layers of the samples were separated and investigated spectrophotometrically (SF-2000, OKB SPECTR, Russia). The amounts of hydroperoxides and ketodienes were determined by measuring the optical density of the ether extracts at 232 nm and 270 nm, respectively.

2.3.5. Estimation of the level of secondary lipid oxidation products. The concentrations of the secondary lipid oxidation products (malonic dialdehyde (MDA)) in the studied samples were determined using the thiobarbituric acid (TBA) test (the reaction of MDA with 2-thiobarbituric acid) in the presence of trichloroacetic acid (TCA) as described in [13]. The TBA-TCA reagent was prepared by dissolving 15 g of TBA and 0.67 g of TCA in 100 ml of double distilled water. This reagent (3 ml) was added to the glass vials containing 0.5 ml of the studied samples. The vials were tightly closed (to avoid the loss of MDA by evaporation) and heated in a boiling water bath for 30 min. The optical densities (D_λ) of colored thiobarbituric acid reactive substances (TBARS) were measured using SF-2000 spectrophotometer (OKB Spectr, Russia) at two different wavelengths: λ = 532 nm (the maximum absorbance of TBARS) and λ = 580 nm (the minimum absorbance of TBARS). The MDA concentrations in the samples were calculated using the following equation: $C_{MDA} = (D_{532} - D_{580})\times7\times1000 / 155$, where D_{532} and D_{580} are the optical density values at 532 nm and 580 nm, respectively; C_{MDA} is the concentration of MDA (nmol/ml); 155 is the molar extinction coefficient of MDA at λ = 532 nm and in a cuvette with a path length of 1 cm; 1000 is the conversion factor

from liters to milliliters; 7 is the dilution factor for the samples by TBA-TCA reagent.

2.3.6. Statistical analysis. Each experiment was performed at least twice and no less than three measurements per sample. Results were reported as means ± standard deviations. Data were analyzed by one-way analysis of variance (ANOVA) using Origin 8.0 (OriginLab Co., USA) software at 95% significance level ($p < 0.05$).

3. Results and discussion

3.1. Characterization of the structural parameters of the complex particles (PC– FO–WPI and PC– FO–WPI_{70}).

All the formed complexes were nanoscale, according to the data of dynamic light scattering (Table 1). From the data presented in Table 1, it follows that the values of hydrodynamic radii, R_h, and the width of the peaks ($\Delta R = R_h^{max} - R_h^{min}$) of the size distributions of R_h for complex particles are smaller than the corresponding parameters for the proteins. Such result indicates directly to the less polydispersity of the complex particles in their size compared with the same parameter of the protein particles.

Table 1

Hydrodynamic radii and the main characteristics of the size distribution for phosphatidylcholine liposomes enriched by fish oil (PC-FO); whey protein isolate (original (WPI) and partially heat-denatured (WPI_{70})); and their complexes in an aqueous medium (pH = 7.0, I = 0.001 M, 25 °C).

Sample	$R_h^{average}$, nm	R_h^{min}, nm	R_h^{max}, nm	ΔR, nm
PC–FO	74 ± 7	57 ± 6	90 ± 9	33 ± 3
WPI	79 ± 8	43 ± 4	134 ± 13	91 ± 9
PC–FO– WPI	72 ± 7	62 ± 6	85 ± 9	23 ± 2
WPI_{70}	93 ± 9	48 ± 5	232 ± 23	184 ± 18
PC–FO–WPI_{70}	64 ± 6	60 ± 6	80 ± 8	20 ± 2

In addition, the data of static laser light scattering (Table 2), namely M_w, indicated that the interaction of PC–FO liposomes with proteins led to a pronounced association (k_{Mw}) of the latter. Such an association of the proteins in the complexes led to a significant increase in their density (k_d) against the background of minor changes in their sizes (k_{RG}) (Table 2). The literature and previous experimental data suggest that such changes in the structural parameters of proteins can be associated with the formation of multiple inter- and intramolecular interactions (hydrophobic, hydrogen, electrostatic) involving PC–FO liposomes [14,15,16,17].

Table 2

Coefficients characterizing the degree of change in the structural parameters of the complex particles formed between phosphatidylcholine liposomes enriched with fish oil (PC–FO) and whey protein isolates (original (WPI) or heat-denatured (WPI_{70})) compared with those of the corresponding proteins in an aqueous medium (pH=7.0, I=0.001 M, 25 °C).

Complex particles	k_{Mw} [1]	k_{RG} [2]	k_d [3]
PC– FO–WPI	13.3 ± 1.7	1.10 ± 0.07	9.9 ± 1.4
PC–FO–WPI_{70}	2.8 ± 0.4	0.80 ± 0.06	6.2 ± 0.7

[1] $k_{Mw} = M_w^{comlex} / M_w^{protein}$, where k_{Mw} is the degree of the association for the protein particles in the complexes, calculated as the ratio of the weight-averaged molar mass of the complex ($M_w^{complex}$) to that of original WPI or heat-denatured WPI_{70} ($M_w^{protein}$);

[2] $k_{RG} = R_G^{complex} / R_G^{protein}$, where k_{RG} is the degree of an increase in the size of the complexes, calculated as the ratio of the radius of gyration of the complex ($R_G^{complex}$) to that of original WPI or heat-denatured WPI_{70} ($R_G^{protein}$);

[3] $k_d = d^{complex} / d^{protein}$, where k_d is the degree of an increase in the density of the complexes, calculated as the ratio of the density of the complex ($d^{complex}$) to that of original WPI or heat-denatured WPI_{70} ($d^{protein}$) (the calculation of the density, d, was carried out according to the 2.3.2).

3.2. Functional properties of the complex particles (PC–FO–WPI and PC–FO–WPI_{70}).

3.2.1. Solubility of the complex particles in an aqueous medium. The solutions of all the studied complex particles were characterized by optical transparency, which was in a good agreement with their nanoscale sizes (Table 1). In addition, the data of electrophoretic light scattering indicated the high absolute values of ζ-potentials for them –34.2 ± 1.7 mV for the PC–FO–WPI; –36.2 ± 1.9 mV for the PC–FO–WPI_{70}. This led to the high solubility of complex particles in the aqueous medium.

3.2.2. Evaluation of the stability of the lipids encapsulated into complex particles against oxidation. The lipid peroxidation is a multistage reaction proceeding with the formation of a wide range of products of their degradation (hydroperoxides, conjugated dienes, aldehydes, ketones) [18]. Thus, it is advisable to simultaneously register both primary and secondary lipid peroxidation (LPO) products using a set of methods (Table 3, 4).

First of all, it is necessary to note the high optical density at 232 nm (D_{232}) for the solution of the nonencapsulated PC–FO liposomes. This result indicates the formation of primary lipid peroxidation products — conjugated dienes and hydroperoxides (the absorption peaks are in the region from 220 nm to 240 nm) [18]. In addition, the high optical density at 270 nm (D270) reveals the active formation of other primary products of lipid peroxidation, which are ketodienes (absorption peaks range from 270 nm to 285 nm) [18].

Table 3

The formation of the primary products of lipid peroxidation (hydroperoxides at λ = 232 nm and ketodienes at λ = 270 nm) in the aqueous solutions (pH = 7.0, I = 0.001 M) of phosphatidylcholine liposomes enriched with fish oil (PC–FO) and their complexes with whey protein isolate (original (WPI) and partially heat-denatured (WPI_{70})) during their storage at a room temperature in the light

	3 days		14 days	
	D_{232}	D_{270}	D_{232}	D_{270}
PC–FO	1.53 ± 0.02	0.41 ± 0.03	1.42 ± 0.02	0.8 ± 0.01
PC–FO–WPI	0.01 ± 0.02	0.01 ± 0.02	0.37 ± 0.02	1.12 ± 0.01
PC–FO–WPI_{70}	0.01 ± 0.01	0.01 ± 0.02	0.37 ± 0.02	1.12 ± 0.01

In the case of the solutions of the complex particles, the found values of D_{232} and D_{270} were many times lower than those measured for the solution of the pure PC–FO liposomes. This result indicates a significant stabilization of the lipids encapsulated by WPI in relation to oxidation. The same level of the protection of PC–FO liposomes was observed both in a combination with WPI and WPI_{70}.

The concentrations of the secondary lipid peroxidation product (malonic dialdehyde (MDA)) are presented in Table 4 for the studied solutions on the 3rd, 7th and 14th day of storage. The maximum concentration of MDA was found on the 7th day of storage (108 nmol/ ml) in the solution of noncapsulated PC–FO liposomes. This result, apparently, may indicate the most active LPO process in this solution during the storage period. At the same time, the concentration of MDA in the solutions of the complex particles was ten times lower (Table 4).

Table 4

The concentration of the secondary product of lipid peroxidation (malonic dialdehyde (MDA)) in the aqueous solutions (pH=7.0, I=0.001 M) of phosphatidylcholine liposomes enriched with fish oil (PC–FO) and their complexes with whey protein isolate (original (WPI) and partially heat-denatured (WPI_{70})) during their storage at a room temperature in the light

Sample	MDA, nmol/ml (3 days)	MDA, nmol/ml (7 days)	MDA, nmol/ml (14 days)
PC — FO	20 ± 1.2	108 ± 4.5	55 ± 2.6
PC — FO — WPI	2 ± 0.1	2.5 ± 0.2	3 ± 0.1
PC — FO — WPI_{70}	2 ± 0.2	2 ± 0.1	2 ± 0.1

Relying on the previous studies [13,17], the high density of complex particles (⩾ 2 mg / ml) is one of the key factors underlying the protective effect of biopolymers relative to the lipids. Indeed, the studied complex particles have a density close to this value (d = 2.97 mg/ml for the PC–FO–WPI and d = 1.8 mg/ml for PC–FO–WPI_{70}). Thus, the observed protective effect is most likely associated with an obstacle in the diffusion of air oxygen to the hydrocarbon chains of lipids encapsulated in the protein. In addition, according to published data, the WPI amino acid residues of tyrosine and methionine exhibit antioxidant properties, which can also contribute to the protective properties of the complexes [19,20,21].

4. Conclusions

1. Whey protein isolate (original and partially heat-denatured) forms complex particles with phosphatidylcholine liposomes enriched by fish oil. Moreover, the degree of lipid encapsulation with proteins at a given ratio is more than 90%.

2. The studied complexes are nanoscale and have the high solubility in an aqueous medium, which was confirmed visually, and was also due to the absolute value of the ζ potentials >|30| mV.

3. The whey protein isolate (original and partially heat-denatured) exhibits high protective properties with respect to the oxidation of the studied lipids. The basis of this is a significant increase in the density of the complex particles during their formation, as well as the antioxidant activity of whey proteins.

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