Protein plant-based composites synthesized with transglutaminase

V V Kolpakova¹, I S Gaivoronskaya¹, V A Kovalenok¹, M I Slozhenkina² and A A Mosolov²

¹ All-Russian Research Institute for Starch Products – Branch of V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences, Kraskovo, Moscow region, Russian Federation
² Volga region research institute of manufacture and processing of meat-and-milk production, Volgograd, Russian Federation

E-mail: val-kolpakova@rambler.ru

Abstract. The aim of the work was the optimization of parameters for the synthesis of protein composites with an increased biological value from dry wheat gluten and pea, rice, amaranth, potato, oat concentrates with the enzyme transglutaminase. Using the program developed on the base of Monte Carlo counting method, taking into the amino acid composition of the concentrates, were determined ratios and amino acid score for the protein-protein composites: dry wheat gluten (DWG), concentrates: pea (PEC), potato (POC), oat (OC), rice (RC), amaranth (AMC). Composites of composition DWG / PEC, DWG / POC, DWG / AMC, POC / OC, PEC / RC, PEC / POC was enriched with deficient amino acids. Using the method of formol titration, was determined the influence duration, concentration of enzyme and hydromodule on the amount of amine nitrogen. Were used methods for planning and processing data in the Matematika and table Curve 3D programs, were obtained equations and optimal values were identified at which the amount of amine nitrogen during the synthesis remained minimal. In the DWG / AMC composite, the functional properties were 1.1–2.0 times higher than the properties of the initial protein products, indicating its greater efficiency in food.

1. Introduction
The problem of human nutrition is socio-economic in nature and requires the creation of food products with an expanded range of dietary protein [1]. Taking into account the special role of protein in human nutrition, the presence of its deficiency and high prices for a component of animal origin, it is obvious that it is expedient to search for alternative sources. Protein products with balanced essential amino acids and good functional properties plays an important role in ensuring the health and prolongation of human life. Interest in such products from legumes and grains continues to grow as consumers seek cheaper and healthier products without compromising their quality and safety [2, 3]. Proteins, providing the human body with essential nutritional factors, perform the functions of enzymes, transmembrane transporters, hormones, structural and signaling proteins [4]. However, the main role of dietary protein in the diet is to provide nine essential amino acids that cannot be synthesized in the body, but are necessary for the formation of new proteins in it. Consumption of dietary protein prevents sarcopenia
Proteins provide the necessary structure and rheological properties to food products due to a complex of indicators united by the term "functional and technological properties". In recent years, to ensure functions in food systems, the principles of complementarity of the composition and properties of grain and leguminous raw materials have been used directly in the technology of manufacturing meat, dairy, bakery and other types of products with the enzyme transglutaminase (TG, EC2.3.2.13) [11]. TG belongs to the class of transferases [12,13], which catalyzes the formation of an isopeptide bond between the group of γ-carboxamides of glutamine residues (donor) and first-order ε-amine groups of various compounds, for example, lysine (acceptors of an acyl residue) [14-16]. In addition, TG catalyzes the deamination reaction in the absence of free amine groups. In this case, water acts as an acyl acceptor [17]. The reactions that are catalyzed by this enzyme lead to significant changes in the physicochemical properties of proteins.

The mechanism of crosslinking protein molecules with TG represents by the following reactions:

\[
\text{Gln-CO-NH}_2 + \text{H}_2\text{N-LYS} \rightarrow \text{Gln-CO-NH-LYS} + \text{NH}_3 \\
\text{Gln-CO-NH}_2 + \text{RNH}_2 \rightarrow \text{Gln-CO-NHR} + \text{NH}_3 \\
\text{Gln-CO-NH}_2 + \text{HOH} \rightarrow \text{Gln-COOH} + \text{NH}_3
\]

New covalent bonds can be formed both between proteins of the same origin and between proteins that differ in type: proteins of milk, cottage cheese, whey, cheese [18-23], fish, seafood [24-26], meat [27-31], egg white [32,33], grain proteins, including wheat gluten [34-38], legume proteins [39-43], etc. The aggregated protein structure of food systems can be stable over a wide range of pH and temperature, be mechanically more stable, have increased elasticity, resilience, water-holding, emulsion capacity, changed viscosity, etc. [39, 44-49]. TG is widely used in the meat and dairy industry in the production of restructured products, less enzyme is used in the production of flour products and quite insignificantly in the production of individual protein compositions from quinoa protein, in particular for edible films [50]. TG attracts special attention when creation of gluten-free products [36, 51-52].

Considering that it is convenient to use ready-made protein compositions in food formulations as independent ingredients, technologies of two- or multicomponent composites with specified functional, technological and nutritional properties are relevant. The advantages of composites, including those with biologically active substances [53], are their adjustable composition and properties for use in products for functional or therapeutic and prophylactic purposes. Protein composites can be obtained in various ways: by preparing a fat-protein emulsion with a balanced fatty acid and amino acid composition [54], introducing mineral substances, for example, calcium into proteins, by adsorbing it at the precipitation stage [55], through joint extraction during the isolation of proteins with improved amino acid composition of secondary products of processing of various grain crops for starch [56]. Most cereals are deficient in lysine, threonine, while proteins of legumes, nightshades, pseudo-cereals contain them in sufficient quantities. On the other hand, legumes, despite their widespread use due to their high content of protein, dietary fiber, vitamins, minerals, antioxidants [56] for the prevention of cardiovascular diseases, diabetes, cancer, osteoporosis, etc. [57-60], poor in sulfur-containing amino acids and tryptophan [61]. On the contrary, proteins of cereals, vegetables and other crops are able to supplement legume proteins with amino acids deficient for them, therefore, methods of developing composites with a complementary amino acid composition, based on the principle of crosslinking proteins with a high content of amine groups by the TG enzyme, to increase their reactivity and perform the corresponding technological functions remain relevant. Thus, the range of protein composites obtained with TG to provide the body with dietary protein and improve the quality of food products can be expanded through the development of new or improvement of known technologies, taking into account the addition of the properties of some plant proteins with the properties of their other types.

The aim was to develop a process for the synthesis of two-component composites with a complementary amino acid composition from various plant protein preparations with the TG enzyme for the production of food products.
2. Materials and methods
The main materials used were samples of DWG BM (Kazakhstan), (BioRosva, Kaluga region, Russia), amaranth concentrate, obtained by method explained in work [62], potato concentrate Tubermine GP and pea protein concentrate from Roquette (France), oat protein from Tate & Lyle (Sweden) and rice from Beneo (Belgium). Transglutaminase "classic" TG-EB was obtained from Novozymes (Denmark). The amount of protein in concentrates was determined by the Keldahl method, fat - by the Soxhlet method with hexane, ash content - by ashing with an accelerator with nitric acid, mass fraction of moisture - by drying to constant weight at 105 °C, carbohydrates were calculated by subtracting the sum of the remaining components from 100%. The chemical composition of the concentrates presented in table 1.

Table 1. Chemical composition of protein concentrates, % of the total mass.

| Protein concentrates | Humidity, % | Protein, % | Fat, % | Insoluble fibers, % | Carbohydrates, % |
|----------------------|-------------|------------|--------|---------------------|------------------|
| DWG                  | 3.60±0.2    | 83.7±0.5   | 1.4±0.4| 0.2±0.0             | 11.1±0.4         |
| Pea                  | 10.0±0.3    | 84.0±0.6   | 4.8±0.5| 1.0±0.2             | 0.20±0.1         |
| Potato               | 8.00±0.7    | 78.0±0.7   | 5.0±0.6| 0.5±0.0             | 8.50±0.3         |
| Oat                  | 6.00±0.4    | 56.3±0.6   | 3.0±0.2| 1.5±0.5             | 33.2±0.4         |
| Amaranth             | 6.10±0.3    | 67.5±0.3   | 0.3±0.1| 2.0±0.2             | 24.1±0.3         |
| Rice                 | 12.0±0.8    | 79.0±0.5   | 5.0±0.3| 3.2±0.5             | 6.00±0.4         |

To carry out the synthesis reaction of two-component fermented composites, weighed portions of protein products in certain ratios were mixed on a mixer at a speed of 500 min⁻¹. Samples of EP transglutaminase were placed in a microbiological test tube with a lid, was added 10.5 cm³ of distilled water, in accordance with the specified hydromodule, vigorously stirred, and 1 g of a mixture of protein products was added. The tubes were placed in a thermostat, shaken at 170 min⁻¹ and a temperature of 50 °C, and the reaction of interaction of proteins was carried out at various concentrations and durations. The course of the synthesis reaction with the TG enzyme between proteins of different chemical nature was assessed by the amount of released amine nitrogen, determined by the method of formol titration. When determining nitrogen in each control preparation, 65 cm³ of distilled water was added to 2.9-3.5 g, the mixture was homogenized for 4-5 min and centrifuged at 4000 min⁻¹ for 10 min. Then 5 cm³ of the supernatant was transferred into a cuvette of a pH meter, 0.5 cm³ of a formol mixture with phenolphthalein was added, and titrated with 0.1 N NaOH to pH 9.3.

To determine the amine nitrogen in composites, 50 cm³ of distilled water was added to 10 g of preparations taken at certain ratios, the mixture was dispersed for 4-5 minutes at 500 min⁻¹. The mixture was centrifuged at 5500 min⁻¹ for 20 minutes. Supernatant poured off, took 5 cm³ and transferred to a glass beaker for measuring pH with 20 cm³ of distilled water. The electrodes of the pH meter were immersed in the suspension. Free carboxyl groups were neutralized with 0.05N NaOH solution. When the pH of the solution reached 7, 0.5 cm³ of a formol mixture with phenolphthalein (50 cm³ of 40% formalin + 2 cm³ of a 1% alcoholic solution of phenolphthalein) was added to it. The mixture was titrated with 0.05N NaOH solution to pH 9.1-9.5, which corresponded to a bright red color of the sample. All reagents were chemically pure. Amine nitrogen (in mg /%) (N) was calculated by the formula: \( N = A \times 0.7 \times 100 / V \), where: A is the amount of cm³ 0.05N NaOH used for titration; V is the amount of titration solution; 0.7 is the amount of nitrogen in g, corresponding to 1 cm³ of 0.05 N NaOH solution.

3. Results and discussion
With the help of the program developed by us on the basis of the Monte Carlo calculation method, were compiled protein compositions with an improved amino acid profile. In time of calculating was used amino acid composition data of protein products for the proposed mixtures, data for the reference protein based on the recommendations of the FAO WHO (2011) [63]. For pea-oat concentrate, the optimum ratio of proteins in the composition was 1: 1, for pea-rice concentrate - 1: 0.6, for pea-DWG 1.5:1, for...
pea-potato- 1:1, potato-DWG 2:1, potato oat- 1:1.5, for amaranth DWG -2:1 (table 2). These ratios provided the optimal amino acid score (table 2).

Table 2. Amino acid score of proteins from compositions from protein concentrates, %.

| Indicators | Protein concentrates (%) | Protein compositions |
|------------|-------------------------|----------------------|
|            | PEC | OC | RC | DWG | POC | AMC | PEC/RCP | DWGPEC/POC | POC/DWG | POC/OC | AMC/DWG |
| Protein mass fraction, % | 84.0 | 56.3 | 79.5 | 83.7 | 78.0 | 71.7 | 81.8 | 84.5 | 80.4 | 80.5 | 67.2 | 73.3 |

| Aminoacid | Score, % | |
|-----------|----------|---|
| Val       | 125      | 157 | 157 | 157 | 157 | 157 | 157 | 157 | 157 | 157 | 157 | 157 |
| Leu       | 134      | 109 | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 |
| Iso       | 156      | 85  | 124 | 124 | 124 | 124 | 124 | 124 | 124 | 124 | 124 | 124 |
| Thr       | 152      | 87  | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 |
| Lys       | 148      | 115 | 141 | 141 | 141 | 141 | 141 | 141 | 141 | 141 | 141 | 141 |
| Try       | 151      | 95  | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 | 129 |
| Met+Cys   | 47       | 85  | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 | 109 |
| Phe+Tyr   | 224      | 115 | 135 | 135 | 135 | 135 | 135 | 135 | 135 | 135 | 135 | 135 |

Note: concentrate: PEC – pea; OC – oat; RC – rice; DWG - gluten; POC – potato; AMC – amaranth.

The data shows, that rice and amaranth concentrates had the highest amino acid score values, next was potato concentrate, the lowest score values for sulfur-containing amino acids were observed in pea concentrate and DWG, lysine, threonine - in DWG and oat concentrate, which does not contradict the literature data. The amino acid composition of composites with pea protein, in comparison with individual samples, was significantly better due to rice, potato and oat concentrates. This increase is especially valuable for all two-component composites for methionine and cysteine. The composition of all composites was practically balanced, with the exception of the PEC / DWG composite due to the low tryptophan score.

In reactions with the participation of TG, the transfer of −NH₂ groups between protein molecules with the formation of new covalent bonds took place, the amount of amine nitrogen decreased, and the degree of the synthesis process was judged from the number of unreacted groups. To develop the process parameters for the AMC: DWG composite, we first studied the effect of temperature, pH, reaction duration, water: DWG hydromodule on the amine nitrogen content in a system of one DWG. It was shown that the minimum amount of amine nitrogen in the reaction with TG in the DWG was reached at 50°C, pH 5.8 (figure 1), the hydromodule DWG water 1: 3.5 and the concentration of EP 0.0015 U / g (figure 2).

![Figure 1](image_url) 

Figure 1. Influence of temperature and pH on the amount of amine nitrogen in DWG.
Figure 2. Influence of the reaction time and the hydromodule of water: DWG on the amount of amine nitrogen in DWG.

When studying the number of components for creating a composite from DWG and AMC (mass fraction of moisture 70%), it was found that with a ratio of 1:2 amine nitrogen after the reaction with the previously identified parameters, it remained 1.8 times less than with other values (table 3), therefore, it was chosen to obtain lyophilized preparations with a moisture content of 5-6%.

Table 3. Influence of the ratio of DWG and AMC on the amount of amine nitrogen.

| Ratio DWG:AMC | Volume of 0.1N NaOH, sm³ | Amine nitrogen, mg/% |
|---------------|--------------------------|----------------------|
| Control, without TG | 0.090±0.005 | 2290±128 |
| 1:1           | 0.160±0.008 | 4072±203 |
| 1:2           | 0.050±0.007 | 1272±177 |
| 1:3           | 0.150±0.010 | 3818±254 |

The biological value of the composite, in comparison with DWG, due to AMC increased: score of Val., Leu, Iso, Thr, Lys increased by 25, 16, 28, 8 and 47%, respectively (table 2). At the same time, the functional properties of the composition improved as compared with the original preparations (table 4). In the composite, the WBC increased by 1.2–2 times, FBA by 1.1–1.4 times, FEA by 1.15–2 times, FA - by 1.1–1.5 times, which indirectly indicated that were formed aggregates with a new protein structure, possibly with a higher molecular weight.

Table 4. Functional properties of protein products.

| Protein products | WBC, g/g | FBA, g/g | FEA, % | SE, % | FA, % | FS, % |
|------------------|----------|----------|--------|-------|-------|-------|
| DWG              | 2.27±0.21| 1.24±0.23| 50±2.0 | 70±2.0| 182±2.5| 59±0.0 |
| SPC              | 3.75±0.11| 1.64±0.15| 87±3.5 | 84±1.0| 200±5.0| 65±0.5 |
| Protein composition | 4.51±0.42| 1.80±0.34| 100±4.0| 95±3.0| 280±3.0| 70±1.0 |

Note: WBC - water binding capacity; FBA - fat-binding ability; FEA - fat-emulsifying ability; SE-stability of the emulsion; FA – foaming ability, FS - foam stability.

To determine the parameters of the biosynthesis of composites from DWG with other protein concentrates, the effect of the concentration of EP and the exposure time on the amount of amine nitrogen remaining during the reaction was studied. Preliminarily, the amount of amine nitrogen in the initial protein preparations was determined (figure 3).
The smallest amount of it was contained in DWG, the largest - in POC, more than 3 times compared to DWG. The other two concentrates occupied an intermediate position in this indicator, and a greater reactivity could be expected from potato and pea concentrates. The studies were carried out at TG concentrations: 0.0015 and 0.0024 g / g protein and reaction times of 5, 10, 15, 30, 90, and 120 min. For the DWG / PEC composite, the minimum amount of amine nitrogen was observed at both TG concentrations over an exposure time of about 10 minutes (figure 4a). Over 10 minutes of the reaction, and especially at 30 minutes of its course, was observed a sharp jump in the number of free groups. This is probably due to the competitive inhibition of the transfer of amino groups by

**Figure 3.** Amount of amine nitrogen in the initial protein preparations.

From the plan of the experiment by the method of Latin polygons. The results were processed with the table Curve 2D program and are shown in figure 5.
Figure 5. Partial empirical dependences of the amount of amine nitrogen $az\%$ of the TG concentration $sv$, U / g (a) and the holding time $t$, h (b).

Based on the Mathematica 12 program, an equation was derived for the dependence of the amount of amine nitrogen $az\%$ of influencing factors:

$$az = \frac{(1.126 - 0.0892e^{-0.01093(-14.21 + t)^2})(16.08 - 8984.3 + 1250002sv)}{gm^{0.0508}}$$

Figure 6. Dependence of the amount of amine nitrogen $az\%$, on the concentration of TG $sv$, units / g and duration $t$, min.

The correlation coefficient of the equation is $R = 0.87$, which confirmed the adequacy of the description of the experimental data. Figure 6 shows the dependence of the amount of amine nitrogen $az\%$ on the concentration of TG $sv$, units / g and duration $t$, h in 3D.

The values of the influencing factors that provide the minimum amount of amine nitrogen $az$ in the synthesis reaction of the composite are as follows:

- the concentration of the enzyme preparation $sv = 0.0024$ U / g,
- the duration of the process $t = 14.2$ min.,
- hydromodule $gm = 8$.

To obtain results on the influence of various factors on the amine nitrogen content for the PEC / POC and PEC / RC compositions, methods of mathematical design of the experiment were also used at pH 6.8–7.0 and a reaction temperature of 50 °C. The exposure time ($X3$) was varied in the range of 5–20 min, the hydromodule ($X1$) - in the range 1: to 1: 8, the concentration of TG ($X2$) - from 0.0015 to 0.003.
U / g protein. The matrices of the experiment were compiled from 16 experiments and the response surfaces for amine nitrogen were constructed using the table Curve 3D software (figure 2). Data processing in programs Matematika and table Curve 3D (figure 7a, figure 7b).

\[
Y = 7.4865 + 149X_1 + 0.0335X_2 - 0.1145X_3
\]

\[
Y = 7.5775 + 567X_1 + 0.0245X_2 - 0.2555X_3
\]

**Figure 7.** The dependence of the amount of amine nitrogen on the reaction parameters for the composites of the PEC/PO (a) and the PRC/RC (b), where x – concentration of EP, g/g of protein; y – time of exposition, min; z – back hydromodule.

For the composition of pea concentrate-oat concentrate, the patterns of change in the amount of amine nitrogen in the course of the reaction were similar to the patterns characteristic of the composition of pea concentrate-rice concentrate. The minimum amount of amino nitrogen after reaction with TG in the composition of pea concentrate-oat concentrate was observed in the reaction medium at a concentration of 0.0015 g / g of protein, exposure time of 15 minutes and a water ratio of 1: 7. The minimum amount of amino nitrogen after reaction with TG in the composition of pea concentrate-rice concentrate was observed in the reaction medium at a concentration of 0.0015 g / g of protein, exposure time of 20 minutes and a water ratio of 1: 8.

4. Conclusions
Methods have been developed for the synthesis of protein composites of increased biological value from plant concentrates with limiting essential amino acids using the TG enzyme. Previously, based on the Monte Carlo counting method, were developed programs and compositions of composites with a complementary amino acid composition. Investigating the influence of technological factors on the amine nitrogen content in the DWG / AMC protein concentrate system, rational values were determined: temperature - 50 ° C, reaction time - 15 minutes, pH - 5.8, TG concentration - 0.08 units / g, hydromodule DWG: water - 1: 3.5, the ratio DWG: AMC is 1: 2. The composition did not contain deficient essential amino acids (lysine, threonine), in contrast to DWG, and the functional properties exceeded those of the original protein products by 1.1–2.0 times.

Using mathematical planning and data processing methods in the Matematika and table Curve 3D programs, were obtained the dependences of the amount of amine nitrogen (function) on the reaction parameters, were solved the equations, were found the optimal parameters at which the amount of amine nitrogen during the synthesis remained minimal. For composites of composition DWG / PEC and DWG / POC, the smallest amount of amine nitrogen after reaction in a medium with TG were achieved at a concentration of 0.0024 g / g protein and a reaction time of 10 minutes, for a composite POC / OC - at a TG concentration of 0.0015 g / g protein and time exposure 15 minutes. These data indicated a high intensity of the reaction of synthesis of new forms of composite proteins. Composites with potato and pea proteins, prepared in conjunction with other proteins, did not contain deficient essential amino acids, quickly approached the reference protein as much as possible, or was higher than that of it. Further
experiments will show what functional properties the new protein composites will have and in which food products they can be used.

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