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Chickpea protein hydrolysates: Production, bioactivity, functional profile, and technological properties

Mahmud Gharaviri[1], Denis I. Aleksanochkin[1], Mahbubeh Ahangaran[1], Ivan A. Fomenko[1,*], Leonid I. Kovalev[2], Marina A. Kovaleva[2], Irina M. Chernukha[1,3], Natalia G. Mashentseva[1]

[1] Russian Biotechnological University (ROSBIOTECH), Moscow, Russia
[2] Bach Institute of Biochemistry, Moscow, Russia
[3] V.M. Gorbatov Federal Research Center for Food Systems of RAS, Moscow, Russia

* e-mail: iv.fomenko@mail.ru

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Abstract:
Chickpea plant protein hydrolysates are an innovative product on the Russian food market. However, they meet many urgent needs and may solve some fundamental food safety problems. This article describes some effective enzymatic biodegradation methods that yield hydrolysates and biopeptides with advanced functional and technological properties that possess antioxidant and other bioactive potentials.

The study featured Kabuli chickpea (*Cicer arietinum* L.) protein isolate, as well as a number of enzyme preparations of animal, plant, and microbial origin. Hydrolysis was followed by a set of FRAP, DPPH, and ORAC analyses to determine the functional, technological, and antioxidant properties. A combined approach made it possible to reveal the proteomic profile, e.g., a combination of two-dimensional electrophoresis and subsequent mass spectrometry was used to identify peptides. The bioactivity of peptide fragments was predicted *in silico* using bioinformatic databases.

The efficiency of protein destruction depended on the degree of hydrolysis. At 10%, it improved the functional and technological properties. The best results regarding the time and enzyme concentration belonged to Alcalase 2.4 L FG (2%). The enzymes of animal origin, e.g., pepsin at a 10% hydrolysis degree, also improved the functional and technological profile. The samples treated with pepsin and Protoferm FP showed the highest antioxidant activity (FRAP, ORAC), increasing it by more than 200% relative to the initial chickpea isolate. Computer densitometry revealed that the hydrolysates treated with trypsin and papain could destroy more than 55% of the initial protein. Biologically active peptides of the hydrolysates obtained were determined using bioinformatic forecasting.

In this research, chickpea protein hydrolysates provided new technological processing methods for commercial products. They made it possible to obtain biopeptides with antithrombotic, antitumor, antibacterial, antioxidant, and antiamnetic properties, which indicates excellent prospects in the food industry and pharmacy.

Keywords: Peptides, hydrolysate, biological activity, isolate, plant protein, proteomic methods, proteolysis

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INTRODUCTION

The relationship between nutrition and health is a cluster of highly relevant research topics, as confirmed by regular publications in authoritative scientific journals [1, 2]. In this respect, food proteins and biologically active components are very popular research subjects [3]. Global food security and sustainable development are the most significant challenges in the XXI century. They are triggered by overpopulation, climate change, and depletion of natural resources. These and other environmental issues require innovative approaches to food production and consumption [4, 5].

Advanced food paradigms classify food not only as a source of nutrition but also as a source of beneficial bioactive compounds that improve human health [6]. More and more food scientists consider the production, properties, and efficacy of biologically active ingredients that people may obtain from food products. These ingredients

may have lower physiological effect than pharmaceutical therapeutic agents, yet they cause little or no toxic or side effects.

Bioactive peptides are one of such kind of food ingredients [7]. They are small fragments of food proteins that consist of 2–20 amino acid subunits with a molecular weight of ≤ 3 kDa. Bioactive peptides of animal or plant proteins possess numerous beneficial properties and applications [8]. Many of them are multifunctional: they have several mechanisms of action and therefore exercise several physiological activities at once [9]. Plant raw material in general and legumes in particular are by far the most promising source of food protein and biopeptides.

Legumes (*Leguminosae* family) are the second largest family of seed plants that includes about 13 000 species grouped into 600 genera [10]. The most common legumes are common beans (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), chickpeas (*Cicer arietinum* L.), lentils (*Lens culinaris* L.), soybeans (*Glycine max* L.), lupines (*Lupinus mutabilis*), mung beans (*Vigna radiata* L.), and peanuts (*Arachis hypogaea* L.) [11, 12]. Legumes occupy 81 million hectares worldwide, with 92 million tons of production capacity. India accounts for almost 25% of the global legume production. China, Myanmar, Canada, Australia, Brazil, Argentina, the United States, and Russia are the largest legume producers [13].

Chickpeas are the most cultivated legume crop number three in the world, with 15 million ha of fields and an annual production volume of 16 million tons [14, 15]. According to the Russian Federal State Statistics Service Rosstat, Russia boasted 492 100 ha of chickpea fields in 2023, which was 34% more than in 2022. Even though this annual legume crop prefers regions with mild climate, it flourishes in arid regions as well because it is highly resistant to hot air if the soil contains enough moisture. Southeast Asia provides about 80% of chickpea production, which makes it the dominant cultivation region. India remains the leader in terms of cultivation volume (11.97 million ha). Geographically, chickpeas come in two types: Kabuli and Desi. The Kabuli type is cultivated in the Mediterranean region, as well as in North and South America. The seeds are large (100–750 mg), round, smooth, and beige, with an energy value of 365 kcal/100 g. The Desi type grows in semi-arid areas; the seeds are small (80–350 mg), angular, rough, and dark, with a thick outer layer and an energy value of 327 kcal/100 g [11, 14, 16, 17].

Chickpeas are rich in carbohydrates (41.1–47.4%) and protein (21.7–23.4%), which makes them a valuable component in different food systems [18]. Chickpea proteins possess an excellent amino acid profile with *in-vitro* digestibility as high as 76%. Chickpea proteins can be classified by solubility, like other plant proteins. They are represented by four fractions: albumins (10–14% total protein content), globulins (55–60%), prolamins (2–3%), and glutelins (18%). Globulins make up the largest fraction, which consists of two main groups, 11S globulins (legumes) and vinicillins (7S vicilin and convicillin, *Cupinaceae* superfamily). The major component of the albumin fraction is 2S albumin. It consists of two subunits of 8–10 and 4–5 kDa, which are linked together by two disulfide bonds. This fraction is rich in cysteine but poor in sulfur amino acids [19–21]. Animal proteins are well studied and popular in the food industry, but their adverse prospects stimulate research into other protein sources, e.g., legumes [22].

As chickpeas are rich in protein, food scientists use them to obtain protein isolates that would enhance the nutritional value of various functional products. Chickpea protein isolate powder typically contains ≥ 90% protein [23]. Alkaline extraction with isoelectric precipitation is the most popular method of protein isolate production in the food industry [24, 25]. Protein isolates are rich in bioactive peptides that are released in the gastrointestinal tract by proteolytic enzymes or during technological processing of high-protein raw materials [26].

Protein hydrolysates are derived from enzymatic or chemical hydrolysis with short-chain peptides and amino acids [27]. Enzymatic hydrolysis is a reliable alternative to the chemical method, which makes it especially important for the food industry. Enzymatic hydrolysis requires milder reaction conditions than the chemical one. In addition, it makes it possible to target specific peptide bond cleavage and minimize the formation of undesirable by-products [28, 29]. As a result, the final protein structure may acquire particular functional properties, e.g., solubility, water-holding, fat-holding, emulsifying, and foaming, but their nutritional value remains the same [30]. Protein hydrolysates are grouped depending on the degree of hydrolysis: partially hydrolyzed (< 10%), intensively hydrolyzed (> 10%), and extensively hydrolyzed (> 20%). At 10%, hydrolysis improves the functional and technological properties of food products that involve hydrolysates [31]. However, the impact depends on the choice of enzyme and substrate.

Protein hydrolysates owe their popularity in the food industry to their functional profile, technological properties, bioactivity, and good digestibility. They give functional products extra nutritional value [32, 33]. Their excellent solubility and bioavailability make them a valuable component in functional drinks [34, 35]. They improve the structure and moisture retention of meat products while increasing their shelf life. Hydrolysates are also part of formulations for modern low-fat and high-protein products [36]. As a result, protein hydrolysates remain a promising research area that renders new innovative foods.

In this research, we used enzyme preparations to modify the physicochemical, functional, and technological properties of Kabuli chickpea (*Cicer arietinum* L.) protein isolates, as well as to identify the bioactive potential of peptides derived from these isolates. The method of enzymatic hydrolysis makes it possible to develop new functional foods with advanced technological and biological properties. In addition, the data obtained may be useful for research and development in the field of food ingredients.

STUDY OBJECTS AND METHODS

The research featured a protein isolate derived from Kabuli chickpeas (*Cicer arietinum* L.) in line with the protocol for obtaining protein isolate from Desi or Kabuli chickpeas (Patent no. 2803851). The experiment also involved enzyme preparations of plant origin – bromelain and papain (Sigma-Aldrich, USA); animal enzyme preparations – pepsin and trypsin (HIMEDIA, India); and microbial enzyme preparations – Protoferm FP (Alfaprom, China) and Alcalase 2.4 L FG (Novozymes, Denmark).

The enzymatic hydrolysis presupposed a ratio of 0.5, 1, 1.5, and 2% enzyme to the substrate amount. The optimal enzyme operating ranges and hydrolysis conditions were based on the manufacturer's recommendations (Table 1).

Degree of hydrolysis. The degree of hydrolysis was the ratio of amino nitrogen to total nitrogen (Kjeldahl method as in State Standard 13496.4-2019). Amino nitrogen was determined by formal titration (Sorensen method as in General Pharmacopoeia Article 1.2.3.0022.15). The volume of NaOH for titration made it possible to calculate the number of free amino groups.

Chickpea proteins underwent a one-dimensional gel electrophoresis in a 12.5% polyacrylamide gel and a vertical electrophoresis chamber (Helicon, USA) followed by staining with Coomassie Blue R-250 (PanReac, Spain).

Functional and technological profile. The functional and technological properties of the chickpea protein hydrolysates were determined at the 10% hydrolysis, followed by centrifugation at 4000 min^{-1} for 15 min. The sediment was dried in a lyophilic dryer (ProfLyo T50.4, Russia).

Solubility. To measure the solubility, we placed 0.4 g of protein powder in a 100-cm^3 conical flask and added 40 cm^3 of distilled water. The contents were then transferred to a shaker and stirred at 180 min^{-1} for 30 min. After centrifuging the resulting suspension at 4000 min^{-1} for 15 min, the centrate went into a 100-cm^3 flask. The sediment was transferred to another 100-cm^3 flask and extracted twice more with water (25 cm^3). The resulting centrate went into the same flask. The final content of dissolved protein in 100 cm^3 was determined using the Lowry method [37].

Water-holding and fat-holding capacity. To prepare protein solutions, we dissolved 1 g of protein in 10 cm^3 of water or sunflower oil. After 1 min of stirring at 1000 min^{-1} and 15 min of centrifugation at 6000 min^{-1}, the supernatant was decanted, and the tubes were put upside down to remove excess water or oil. After 10 min, we weighed the tubes with the sediment to calculate the water-holding and fat-holding capacity, %, as in Eq. (1):

$$\text{Water-holding and fat-holding capacity} = \frac{M_1 - M_0}{M_n} \times 100 \quad (1)$$

where M_1 is the mass of the test tube with the protein preparation, g; M_0 is the mass of the empty test tube, g; M_n is the mass of the test sample, g [38].

Emulsifying capacity and stability. A 7% suspension of protein solution and water was homogenized at 4000 min^{-1} for 1 min. After adding sunflower oil (1:1), we emulsified the suspension at 8000 min^{-1} for 5 min. The resulting emulsion was divided into four centrifuge tubes and centrifuged at 2000 min^{-1} for 5 min. The emulsifying capacity was calculated as in Eq. (2):

$$\text{Emulsifying capacity} = \frac{V_e}{V_0} \times 100 \quad (2)$$

where V_e is the emulsion volume, cm^3; V_0 is the total system volume, cm^3.

To determine the emulsifying stability, we prepared protein solutions as described above. After heating the emulsion at 80°C for 30 min and cooling it with tap water for 15 min, we poured it into centrifuge tubes to be centrifuged at 2000 min^{-1} for 5 min and used Eq. (3) to calculate its stability, %:

$$\text{Emulsifying stability} = \frac{V_e}{V_0} \times 100 \quad (3)$$

where V_e is the emulsion volume, cm^3; V_0 is the total system volume, cm^3 [38].

Critical gelation concentration. This experiment involved a series of three suspensions (10 cm^3) in distilled water with a concentration step of 2, 12, and 20%, which were homogenized by stirring. The test tubes spent 15 min in a thermostat at 75°C. After heating, the test tubes cooled down in a refrigerator at 5°C for 18 h. After that, lead balls (0.53 g each) were placed on top of suspension to remain there at the same temperature for 2 h. The critical gelation concentration at which the gel was not destroyed under the lead ball loading.

Foaming capacity and stability. Homogeneous 3, 5, and 10% suspensions of a protein preparation was prepared in a graduated cylinder and shaken horizontally for 1 min. After verticalizing the cylinder, we measured the height of the foam column above the liquid level and used Eq. (4) to calculate the foaming capacity, %:

$$\text{Foaming capacity} = \frac{H_1}{H_0} \times 100 \quad (4)$$

where H_1 is the height of the foam after shaking, mm; H_0 is the height of the solution, mm.

To determine the foam stability, we followed the same protocol to prepare the protein and left it for 30 min. After that, we measured the height of the remaining foam and calculated its stability, %, as in Eq. (5):

Table 1 Conditions of enzymatic hydrolysis

Enzyme preparation	Bromelain	Papain	Pepsin	Trypsin	Protoferm F	Alcalase 2.4 L FG
Temperature, °C	45	45	37	37	45	45
pH	4.5	6.2	2.6	7.5	3.5	7.0

$$\text{Foaming stability} = \frac{H_2}{H_1} \times 100 \quad (5)$$

where H_1 is the foam height after shaking, mm; H_2 is the foam height after 30 min of rest, mm [39, 40].

Antioxidant activity. The total antioxidant capacity of the hydrolysates was determined by the method of fluorescence recovery after photobleaching (FRAP) in an SF-2000 spectrophotometer (OKB Spektr, Russia). To determine the total antioxidant capacity, we added 1.45 mL of FRAP and 0.05 mL of hydrolysates pre-diluted with distilled water and incubated the reaction mix at 37°C in the dark for 30 min. After that, we measured the optical density at 594 nm. The FRAP values were calculated using a calibration graph.

The DPPH method involved DPPH (2,2-diphenyl-1-picrylhydrazyl) and an SF-2000 spectrophotometer. To measure the DPPH, we added 1.52 mL DPPH and 0.08 mL of each sample into the test tubes. The reaction mix was incubated in the dark at 22°C for 30 min. The optical density was measured at 517 nm.

The oxygen radical absorbance capacity (ORAC) was determined using a Fluoroskan Ascent FL fluorimeter-luminometer (TermoLabsystems, Finland) and black 96-well plates (Greiner bio-one, Germany).

Proteomic research methods. To identify proteins and provide isoelectric focusing, we used two-dimensional electrophoresis in line with the method first described by O'Farrell [41] which involved isoelectric focusing in an ampholine gradient (IEF-PAGE, equilibrium variant). Proteins were identified on two-dimensional electropherograms by first staining with Coomassie Blue R-250 (CBB R-250) and then with silver nitrate.

Computer densitometry involved wet two-dimensional electropherograms. Complete and/or fragmented digital images were taken with an Epson Expression 1680 scanner at 300 dpi resolution in 48-bit color, with the results saved as TIFF files. The digital images were improved in a graphics editor to calculate proteins using ImageMaster 2D Platinum 7 (GE Healthcare, Switzerland). The procedure included ≥ 3 electropherograms with equal application. The optical density range did not exceed ± 1.5%.

To identify proteins, individual fractions were cut out of two-dimensional electrophoresis: the fragments were crushed and treated with trypsin. The corresponding peptide sets were studied using the methods of matrix assisted laser desorption/ionization (MALDI-TOF MS) and tandem mass spectrometry (MS/MS) on an Ultraflex MALDI – time-of-flight mass spectrometer (Bruker, Germany) with an ultraviolet laser (336 nm) in the positive ion mode (mass range of 500–8000 Da calibrated using trypsin autolysis values). The resulting mass spectra (peptide mass fingerprints) were deciphered using traditional bioinformatics technologies.

Bioinformatics mass spectrometry. The obtained mass spectra of tryptic peptides were analyzed using the peptide mass fingerprint search in the Mascot program (Matrix Science, USA). The 0.01% accuracy of MH^+ mass allowed for the acrylamide modification of cysteines and the oxidation of methionines. The data search involved the databases of the US National Center for Biotechnology Information (NCBI).

The peptide bioinformatics, i.e., determination and prediction of their biological activity, covered the NCBI, BIOPEP, ToxinPred, AntiCP, AntiBP, AHTpin, and Anti-TbPred databases.

RESULTS AND DISCUSSION

Effect of enzymatic hydrolysis on the native structure of protein isolate. Enzymatic hydrolysis affects the protein isolate by modifying its secondary and tertiary structure, destroying peptide bonds, and improving functional properties [42]. An effective hydrolysis requires a proper concentration of the enzyme preparation and an optimal biodegradation time. The hydrolysis degree is crucial for monitoring the protein hydrolysis [28]. Figure 1 illustrates the effect of enzymatic exposure time on the hydrolysis rate.

For enzymatic hydrolysis, a 10% substrate hydrolysis (relative to the proteolysis time) is the optimal degree. The degree of hydrolysis is the proportion of split peptide bonds in the protein hydrolysate. In this study, the best results relative to time and enzymatic concentration belonged to Alcalase 2.4 L FG. This sample reached the desired 10% as follows: 0.5% – 27 min; 1% – 24 min; 1.5% – 21 min; 2% – 20 min. Alcalase 2.4 L FG possesses a wide catalytic range and is an effective breaker of higher molecular proteins into peptides [43].

The enzymatic hydrolysis induced by plant and animal enzyme preparations (pepsin, trypsin, and papain) proved to be less effective. The average time it took them to reach the 10% protein destruction was as follows: 0.5% – 36 min; 1% – 30 min; 1.5% – 27 min; 2% – 24 min. Bromelain also failed as a hydrolysis intensifier (0.5% – 51 min; 1% – 42 min; 1.5% – 39 min; 2% – 33 min).

Protoferm FP in its initial doses did not reach 10% hydrolysis due to the specificity of the substrate and the activity of the enzyme preparation. We had to bring the dose up to 4% and increase the hydrolysis time up to 120 min.

The degree of hydrolysis increased with time to the stationary phase in 2 h on average. The overall results fell within the predicted range, given the hydrolysis parameters. Further studies involved the following doses: 1.5% for plant and animal enzyme preparations and 2% for microbial preparations.

Subsequent gel electrophoresis (SDS-PAGE) relied on the results obtained for the hydrolysis. The control sample contained a native chickpea protein isolate; the chickpea protein hydrolysates with a 10% hydrolysis degree served as standards. Figure 2 shows the effect of enzyme preparations on the protein and peptide profile of the chickpea protein hydrolysates.

At 0% hydrolysis, the untreated control isolate developed numerous bands in the molecular weight range from ~ 4 to ~ 110 kDa. The bands of ~ 22–24 kDa are known to correspond to legumes (basic subunit); those of ~ 37–41 kDa belong to legumes (acidic subunit); the bands of ~ 50–55 kDa denote vicilin (α, β, γ); the bands

of ~ 70 kDa refer to the convicillin subunit; and the bands of 7 kDa belong to 2S albumin subunit [31]. The molecular weights obtained by SDS-PAGE were below 50 kDa for all the hydrolysates and below 37 kDa for samples 2, 5, and 6, which were treated with trypsin, Protoferm FP, and Alcalase 2.4 L FG. The most effective protein degradation was demonstrated by trypsin, Protoferm FP, and Alcalase 2.4 L FG. At < 15 kDa, each sample had bands with varying staining degrees, which indicated short peptides that developed as a result of protein degradation. The sample treated with trypsin demonstrated a shift in molecular weight and a partial destruction of protein bands represented by vicilin and legumin. The samples treated with papain and bromelain showed

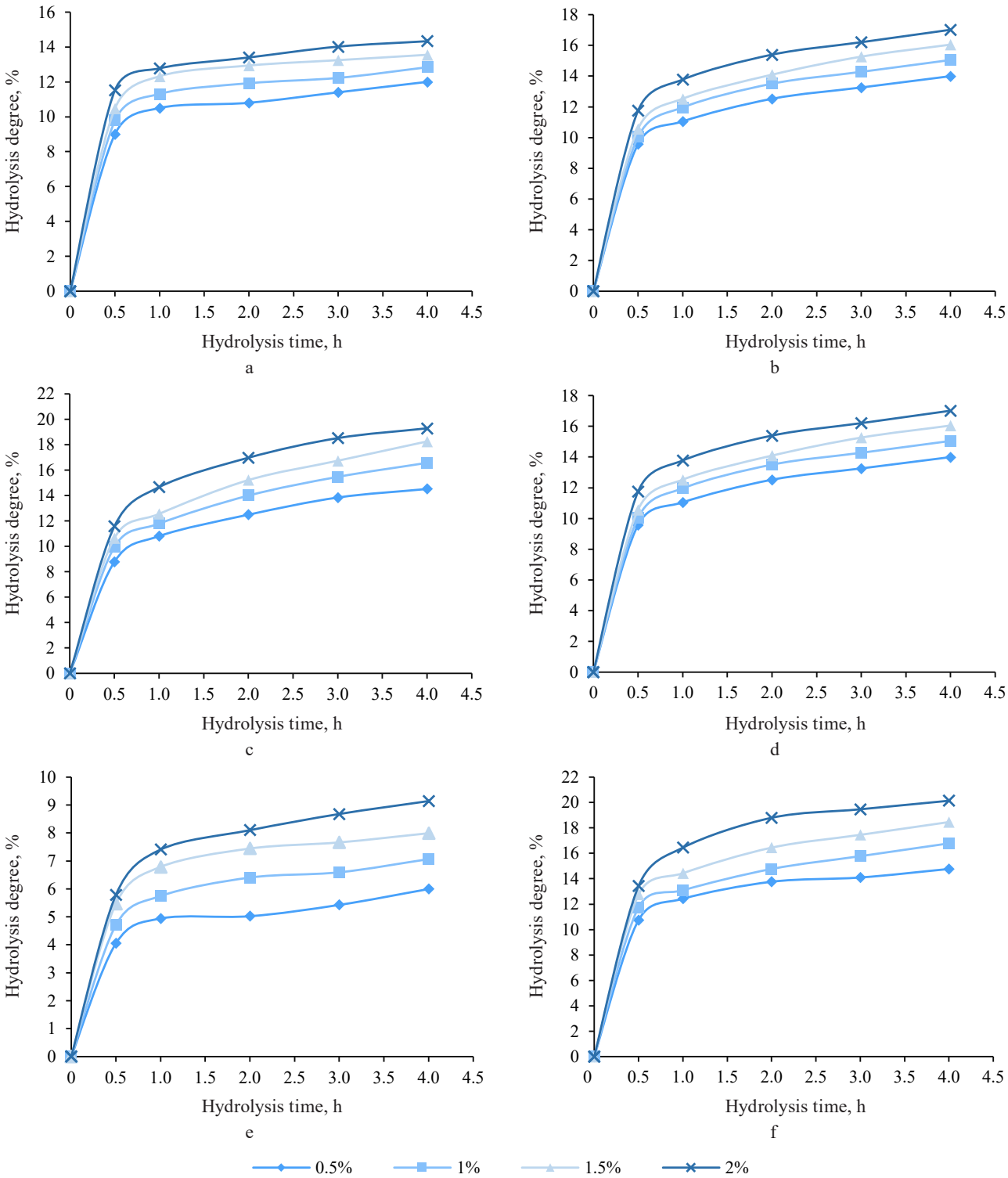


Figure 1 Effect of exposure time to proteolytic enzymes on hydrolysis degree: pepsin (a), trypsin (b), papain(c), bromelain (d), Protoferm FP (e), and Alcalase 2.4 L FG (f)

diffuse tracks of peptides with molecular weights below 25 kDa. These results indicated a stepwise cleavage of amino acid residues from the detected protein fractions. The differences in band profiles between the hydrolysates mean that the associated enzymes were specific for the available catalytic hydrolysis sites.

In addition to nutritional qualities, protein products used in the food industry must possess certain functional and technological properties associated with the operational profile of the final product [44]. The technological characteristics of hydrolysates are known to improve when the protein splits into fragments with a lower molecular weight [45]. Table 2 summarizes the functional and technological properties of the hydrolysates. The control sample contained native chickpea protein isolate, while the experimental samples contained chickpea protein hydrolysates with the 10% hydrolysis.

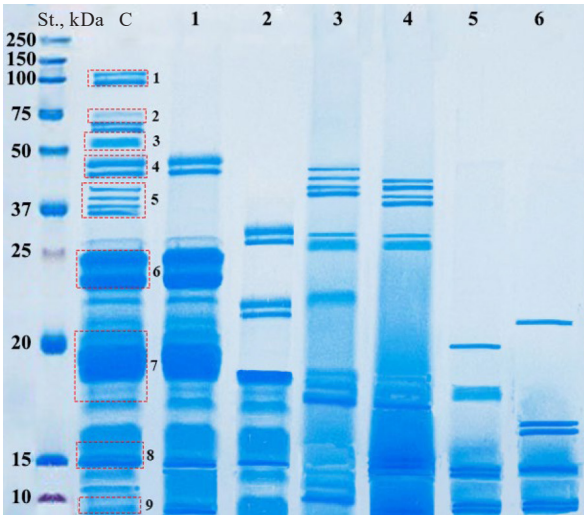


Figure 2 Electrophoresis of hydrolysates: St. – standard, PageRuler™; C – control; 1 – pepsin; 2 – trypsin; 3 – papain; 4 – bromelain; 5 – Protoferm FP; and 6 – Alcalase 2.4 L FG

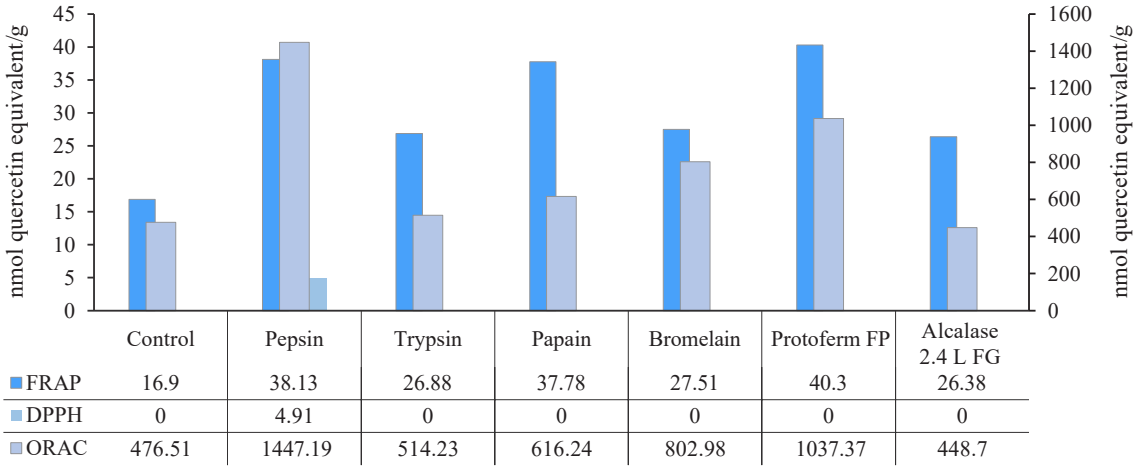
The samples treated with pepsin and Protoferm FP increased the control solubility by 86.4 and 189.8%, respectively. The hydrolysates treated with pepsin and trypsin also increased their water-holding capacity. All the experimental samples demonstrated an advanced fat-holding capacity. In general, hydrolysate biostructures retain water and fat molecules by accumulating free amino acids and peptides during protein hydrolysis, which, in turn, stabilize food systems. None of the hydrolysates demonstrated significant increases in emulsion properties and stability. The critical gelation concentration in the samples treated with pepsin, papain, and Alcalase 2.4 L FG was approximately the same as in the isolate; other samples demonstrated even lower values. The foaming capacity increased in all samples except the one treated with trypsin. The hydrolysate treated with papain demonstrated one of the highest values (160–168%). Due to the high emulsifying capacity, the foaming stability remained low only in the hydrolysate treated with pepsin, where it was as low as in the original isolate. The hydrolysate treated with pepsin increased all its functional and technological indicators except for critical gelation and foaming stability, which was the same as in the native chickpea protein isolate.

The recent years have seen major scientific interest in natural antioxidants, in particular, the antioxidant potential of bioactive peptides derived from protein products [45, 46]. In this research, we identified the total antioxidant capacity of the experimental hydrolysates using the methods of FRAP, DPPH, and ORAC (Fig. 3).

The highest iron-reducing antioxidant capacity (FRAP) belonged to the hydrolysates treated with pepsin, papain, and Protoferm FP. The highest DPPH radical scavenging activity belonged to the sample treated with pepsin. Peptides with smaller molecular size of protein hydrolysate are known to possess stronger DPPH radical scavenging properties than those with higher molecular weight peptides [47–49]. In addition, a high DPPH

Table 2 Functional and technological properties of hydrolysates and chickpea protein isolate

Properties			Control (native chickpea protein isolate)		Pepsin		Trypsin		Papain		Bromelain		Protoferm FP		Alcalase 2.4 L FG	
Solubility, %			17.23		32.11		13.44		20.72		17.48		49.93		21.79	
Water-holding capacity, %			388		442		563		299		299		275		298	
Fat-holding capacity, %			195		435		488		370		280		299		373	
Emulsion	Emulsifying capacity, %		72		75		80		78		77		79		74	
	Emulsifying stability, %y		95		94		96		95		98		92		96	
Critical gelation concentration, %	Concentration, %	2	1		1		1		2		1		1		2	
		12	4		5		5		5		2		1		6	
		20	15		13		9		13		3		1		14	
Foaming capacity, %	Concentration, %	3	70	47	90	39	70	22	160	31	40	10	80	7	90	6
		5	73	49	92	43	73	30	163	34	42	15	89	10	98	8
		10	76	58	95	52	78	35	168	39	50	17	100	12	103	15
Foaming stability, min			0	30	0	30	0	30	0	30	0	30	0	30	0	30



	Control	Pepsin	Trypsin	Papain	Bromelain	Protoferm FP	Alcalase 2.4 L FG
FRAP	16.9	38.13	26.88	37.78	27.51	40.3	26.38
DPPH	0	4.91	0	0	0	0	0
ORAC	476.51	1447.19	514.23	616.24	802.98	1037.37	448.7

Figure 3 Total antioxidant capacity: control vs. experimental samples

activity of protein hydrolysates or peptide fractions was reported to depend on the high content of hydrophobic and positively charged amino acids or biopeptides [50]. In terms of oxygen radical absorption (ORAC), the best results belonged to the hydrolysates treated with pepsin (1447.19 ± 50.60) and Protoferm FP (1037.37 ± 16.80); they increased the initial activity by 303.7 and 217.7%, respectively. The high antioxidant capacity of the hydrolysate treated with Protoferm FP could be explained by the fact that this sample yielded a peptide with the sequence SSSSPDIYIPQAGR, which possesses an antioxidant activity.

Protein composition of chickpea isolates. Proteomic methods proved efficient when applied to proteins of plant origin [51]. They make it possible to assess enzymatic hydrolysis of proteins and determine the biological potential of peptides obtained. Figure 4 shows a two-dimensional electropherogram of proteins in the chickpea isolate: it registers about 100 protein fractions in the mass range of 10–80 kDa and an isoelectric point (pI) of 4.8–10.0.

Only eleven (11) fractions in the chickpea protein isolate corresponded to full-length protein molecules (no. 1–3, 6, 19, 20–22, 24, 26, 27, 29). However, we revealed a certain pattern: some of these proteins changed their physical and chemical properties due to thermochemical effects, and the resulting fractions had different isoelectric points, e.g., no. 21 and 22, 18 and 20, 28 and 30.

Ten (10) fractions proved to be mixes of two or more proteins or their fragments. Some of these proteins retained the native post-translational modifications, e.g., acetylation of the N-terminal part of the molecule (no. 5, 22, 24), lysine methylation (no. 12), and hexoses (no. 10, 11). Twenty fractions demonstrated various protein fragments (no. 4, 5, 7–18, 23, 25, 26, 28, 30).

The two-dimensional electrophoresis revealed the following key proteins: vicilin and its analogues, legumin and its analogues, sucrose-binding proteins, storage proteins, globulins, and some enzymes. Since the chickpea genome remains poorly annotated, it was only the gene locus that was indicated in most cases. As a result, some proteins were identified as resembling (-like), although they might be isoforms of proteins of one and the same gene.

In some cases, we detected tryptic peptides: they were in small quantities, but clearly associated with other proteins (no. 3–5, 9, 21, 22, 24, 29, 30). Table 4 shows their amino acid sequences and positions in the protein chain. Most of these peptides occurred more than once, but in the fractions with different molecular weights.

The mechanism behind this phenomenon is as follows. The thermochemical action yields short peptides, mainly of the legumin and vicilin series. These interact with proteins of other origin, and together they fractionate by charge and molecular weight. When proteins are treated with trypsin for identification, it destroys the corresponding conservative peptides, they are detected in the mass spectra, and tandem mass spectrometry (MS/MS) reveals their amino acid sequence. Figure 5 is an example of tandem mass spectrometry for a peptide with 1623.8 m/z that corresponds to positions 181–193 in

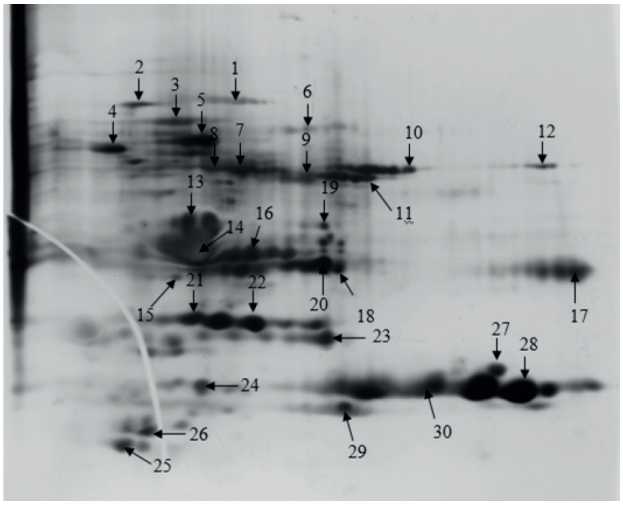


Figure 4 Two-dimensional electropherogram of chickpea isolate proteins (control) stained with silver nitrate. The arrows and numbers mark the fractions identified (see Table 3)

Table 3 Mass spectrometry of protein fractions of chickpea isolate

No. (Fig.3)	Protein (gene symbol)	Protein NCBI	S/M/C*	Mm/pI (experimental)**	Mm/pI (calculated)**
1	Mix of seed biotin-containing protein SBP65 (LOC101504303), legumin (leg3)***(1), and vicilin-like (LOC101515515)	XP_004487170.1 Q9SMJ4.1 XP_004493035.1	180/20/44 135/8/22 80/10/30	71.0/5.40	71.3/5.97 56.2/6.20 51.8/5.73
2	Mix of luminal-binding protein (LOC101513614) ***(1), heat shock 70 kDa protein (LOC101492688), and a fragment of sequence 28-320 of vicilin-like (LOC101510695)***(1)	XP_004503582.1 XP_004507753.1 XP_004496704.1	215/27/53 137/20/45 103/18/30	70.0/4.90	73.5/5.02 71.2/5.21 69.4/5.59
3	Vicilin-like (LOC101510695)	XP_004496704.1	199/41/41	68.0/5.20	69.4/5.59
4	Mix of fragments of protein disulfide-isomerase (LOC101512854) and ruBisCO large subunit-binding protein subunit alpha, chloroplastic (LOC101503231) + Acetyl (Protein N-term)	XP_004502489.2 XP_004506047.1	299/33/64 78/13/32	52.0/4.80	59.7/5.13 62.0/5.12
5	Fragment without N-terminal region of vicilin-like seed storage protein At2g18540 (LOC101506263) + Acetyl (Protein N-term)	XP_004512222.3	155/37/41	54.0/5.30	82.5/5.78
6	Mix of legumin A-like (LOC101489278), vicilin-like (LOC101515515), and provicilin-like (LOC101510367)	XP_004493780.1 XP_004493035.1 XP_004496703.1	67/8/21 65/8/19 49/7/15	56.0/6.50	59.3/5.87 51.8/5.73 64.6/6.61
7	Mix of fragment without N-terminal region of sucrose-binding protein-like (LOC101499776), fragment without N-terminal region of provicilin-like (LOC101510367), and legumin A-like (LOC101489278)***(1)	XP_004495184.1 XP_004496703.1 XP_004493780.1	136/16/38 120/13/29 136/9/18	45.0/5.80	53.8/5.85 64.6/6.61 59.3/5.87
8	Fragment of homologue 8S globulin alpha isoform (ABG02260) [*Vigna radiata* L.]	ABG02260.1	220/27/57	45.0/5.30	52.0/5.81
9	Fragment without N-terminal region of provicilin-like (LOC101510367) + hexose and fragment without N region of sucrose-binding protein-like (LOC101499776)	XP_004496703.1 XP_004495184.1	162/23/51 117/18/45	42.0/6.90	64.6/6.61 53.8/5.85
10	Fragment without N-terminal region of provicilin-like (LOC101510367) + hexose	XP_004496703.1	192/34/61	43.0/9.30	64.6/6.61
11	Fragment without N-terminal region of provicilin-like (LOC101510367) + hexose	XP_004496703.1	226/37/63	41.0/9.00	64.6/6.61
12	Fragment without C region of elongation factor 1-alpha (EF1A) with methylation of K (lysine) at 55K and 87K	NP_001352092.1	87/19/58	42.0/9.60	49.3/9.15
13	Fragment of N-terminal region of legumin A-like (LOC101489278)***(2)	XP_004493780.1	142/24/48	36.0/5.20	59.8/5.87
14	Fragment of N-terminal region of Legumin (LEG3)***(2)	Q9SMJ4.1	168/25/39	32.0/5.20	56.2/5.97
15	Mix of fragments of legumin A-like (LOC101489278)***(1) and vicilin-like (LOC101515515)	XP_004493780.1 XP_004493035.1	168/28/23 60/8/23	32.0/5.00	56.8/5.87 51.9/5.73
16	Fragment without C region of vicilin-like (LOC101515515)***(2)	XP_004493035.1	312/30/50	28.0/5.90	51.8/5.73
17	Central fragment of basic 7S globulin-like (LOC101504144)***(1)	XP_004494958.1	126/16/45	28.0/9.80	48.2/8.37
18	Mix of fragments of vicilin-like (LOC101515515) and NADPH-dependent aldehyde reductase 1, chloroplastic-like (LOC101506833)	XP_004493035.1 XP_004494625.2	107/12/35 80/9/32	28.0/7.80	51.8/5.73 35.5/8.11
19	Mix of fructose-bisphosphate aldolase cytoplasmic isozyme (ALDC) and fragments of legumin A-like (LOC101489278) and vicilin-like (LOC101505411)	NP_001265896.2 XP_004493780.1 XP_004492829.1	195/15/64 74/9/25 71/9/25	36.0/7.60	38.6/6.17 59.8/5.87 51.3/6.10
20	NADPH-dependent aldehyde reductase 1, chloroplastic-like (LOC101505375)	XP_004507706.1	270/16/71	28.0/7.60	32.2/6.40
21	Albumin-2-like (LOC101512722)	NP_001351664.1	184/13/53	25.0/5.10	26.1/5.67
22	Albumin-2-like (LOC101512722) + Acetyl (Protein N-term)	NP_001351664.1	254/24/87	25.0/5.90	26.1/5.67
23	Fragment of N-terminal region of vicilin-like (LOC101505411)	XP_004492829.1	178/32/33	24.0/7.70	51.1/6.10
24	18.5 kDa class I heat shock protein (LOC101514603)***(1) + Acetyl (Protein N-term)	XP_004505083.1	174/25/76	18.5/7.60	18.4/5.57
25	Central fragment of vicilin-like (LOC101505411)	XP_004492829.1	109/13/20	14.0/4.90	51.1/6.10
26	Mix of fragment of C region of vicilin-like (LOC101505411) and fragment of C region of basic 7S globulin-like (LOC101504144)***(1) with admixture of desiccation protectant protein Lea14 homologue (LOC101489566)***(1)	XP_004492829.1 XP_004494958.1 XP_004510736.1	110/19/39 97/13 /20 104/7/46	14.5/5.30	51.1/6.10 48.2/8.37 16.3/5.34
27	P24 oleosin-like (LOC101492171)	XP_004493360.1	64/16/40	21.0/9.30	21.4/9.56
28	Fragment of C region of legumin-like (LEG)***(1)	XP_027188788.1	99/23/30	20.0/9.40	56.2/5.97
29	Mix of 17.5 kDa class I heat shock protein-like (LOC101513658), outer envelope pore protein 16–2, chloroplastic-like (LOC101490648)***(1), and oleosin 16 kDa-like (LOC101488878)***(1)	XP_004490930.1 XP_004487776.1 XP_004501216.1	100/9/59 95/6/46 76/4/26	16.5/7.80	17.4/6.85 18.5/6.90 16.1/8.71
30	Fragment of C region of legumin-A-like (LOC101489278)*** (1)	XP_004493780.1	62/25/28	20.0/9.30	59.3/5.87

* S/M/C – Score, indicator of compliance (significant protein scores are ≥ 68, $p < 0.05$); the number of Matching peptides; Coverage (%) of the complete amino acid sequence of the protein by the peptide; ** Mm/pI (experimental) – estimates based on the electrophoretic mobility on two-dimensional electrophoresis; Mm/pI (calculated) – estimates made from amino acid sequence data based on the removal of the signal peptide but without other postsynthetic modifications (ExPASy Compute pI/Mw tool); *** msms – identification confirmed by tandem mass spectrometry with the number of sequenced tryptic peptides in brackets

Table 4 Peptides in complexes with other proteins

No. (see Table 1)	Source protein	Positions in amino acid sequence	Sequence confirmed by MS/MS	Mass to charge ratio, m/z
3	Legumin	181–193	FYLAGNHEQEFLR	1623.8
4	Legumin Vicilin-like	181–193 176–190	FYLAGNHEQEFLR ILEASFNSDYEEIER	1623.8 1814.9
5	Legumin Vicilin-like	181–193 176–190	FYLAGNHEQEFLR ILEASFNSDYEEIER	1623.8 1814.9
9	Legumin A-like Vicilin-like и seed storage protein At2g18540	181–200 380–388	FYLAGNQEQEFLQYQQQEGR QGDVFLVPR	2476.2 1030.6
21	Vicilin-like	59–69	SQLFENLQNYR	1411.7
22	Vicilin-like	59–69	SQLFENLQNYR	1411.7
24	Legumin-like Vicilin-like	449–472 59–69	LLGASSLINGMPEEVVAAAFNMER SQLFENLQNYR	2519.2 1411.7
29	Outer envelope pore protein 16-2, chloroplastic-like isoform X2 leosin 16 kDa-like	56–75 136–144	QAYFTAIQGGLPPSDVSATR EYGNYAQGR	2079.0 1057.5
30	Vicilin-like	141–157	VLDLAIPVNRPGQFQSF	1901.0

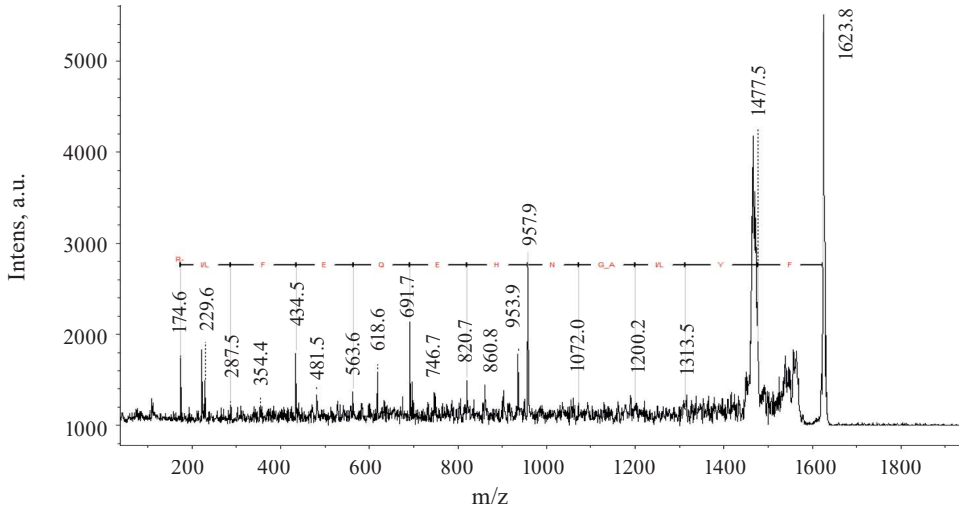


Figure 5 Fragmentation spectrum of ion with 1623.8 m/z as an illustration of amino acid sequence identified: the image is inverted in line with the processing algorithm

the amino acid sequence of chickpea legumin protein. The resulting sequence was FY I/L GA NHEF I/LR, which corresponded to FYLAGNHEQEFLR in Table 2. Isoleucine and leucine are not differentiated by mass during ionization because they are identical in mass (that is why they are often given as a variant, i.e., I/L). Sometimes, it is not one amino acid residue that is cleaved, but two or three. In this case, the program sees them not as single amino acid residues, but as a combination of residues with corresponding masses.

The abovementioned key peptides were generated by thermochemical action during the production of chickpea isolate and could bind to proteins of a different genesis.

Effect of proteases on peptide formation from chickpea protein isolate. Different types of proteases had different effects on the chickpea isolate proteins. Figure 6 shows the two-dimensional electrophoresis of chickpea isolate proteins before and after exposure to proteases.

The protease-induced fractionation of chickpea proteins was visible on the two-dimensional electrophoresis. The proteases demonstrated different efficiency in forming a pool of short peptides. They also differed in the utilization of proteins, especially the genes of legumin and albumin-2-like protein. Computer densitometry made it possible to compare the effect of proteases (Fig. 7) on the amount of total protein in the control.

The computer densitometry results were calculated based on the two-dimensional electrophoresis with Coomassie Blue R-250 staining. This stain binds to proteins more effectively than silver nitrate.

Trypsin and papain proved to be the most effective proteases in terms of functional peptides: they processed ≥ 55% of total protein into short peptides and intermediate forms. Bromelain and Protoferm FP proved to be the least effective proteases (25–35%).

In addition to peptides, the two-dimensional electrophoresis also identified some intermediate, relatively large fragments of chickpea isolate proteins formed by different types of proteases (Fig. 8, Table 5).

The proteomic study revealed that intermediate fragments mostly came from the legumin, vicilin, and albumin-like protein families.

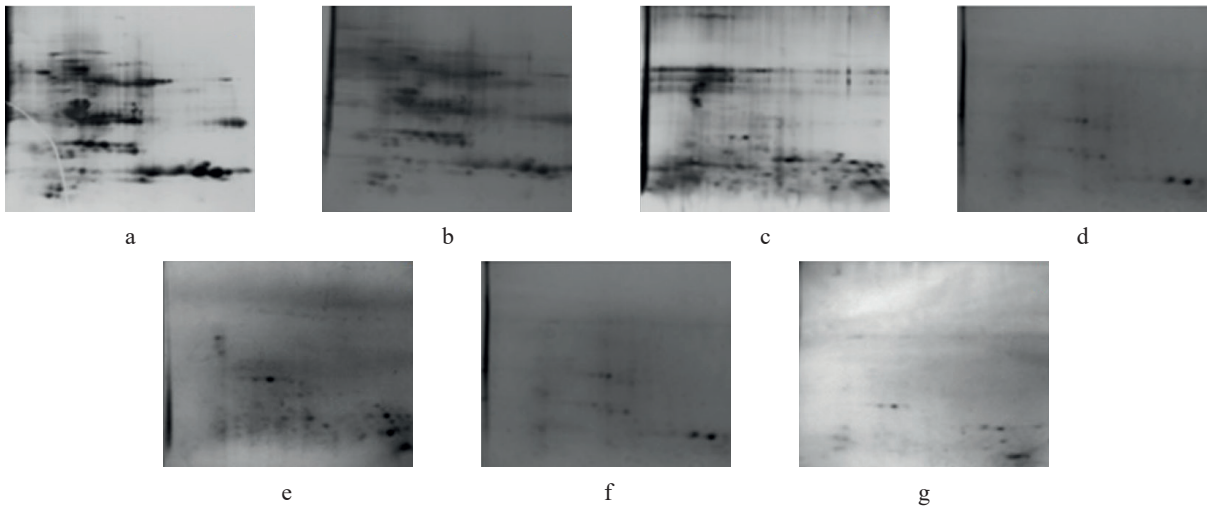


Figure 6 Dimensional electrophoresis of chickpea isolate proteins before and after exposure to proteases: (a) control, (b) trypsin, (c) pepsin, (d) papain, (e) bromelain, (f) Alcalase 2.4 L FG, and (g) Protoferm FP

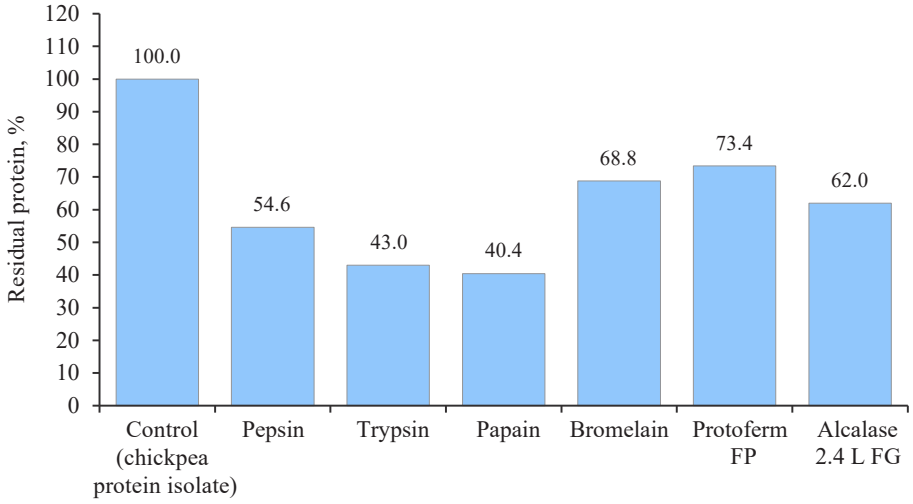


Figure 7 Effect of different proteases on total proteins in chickpea isolate

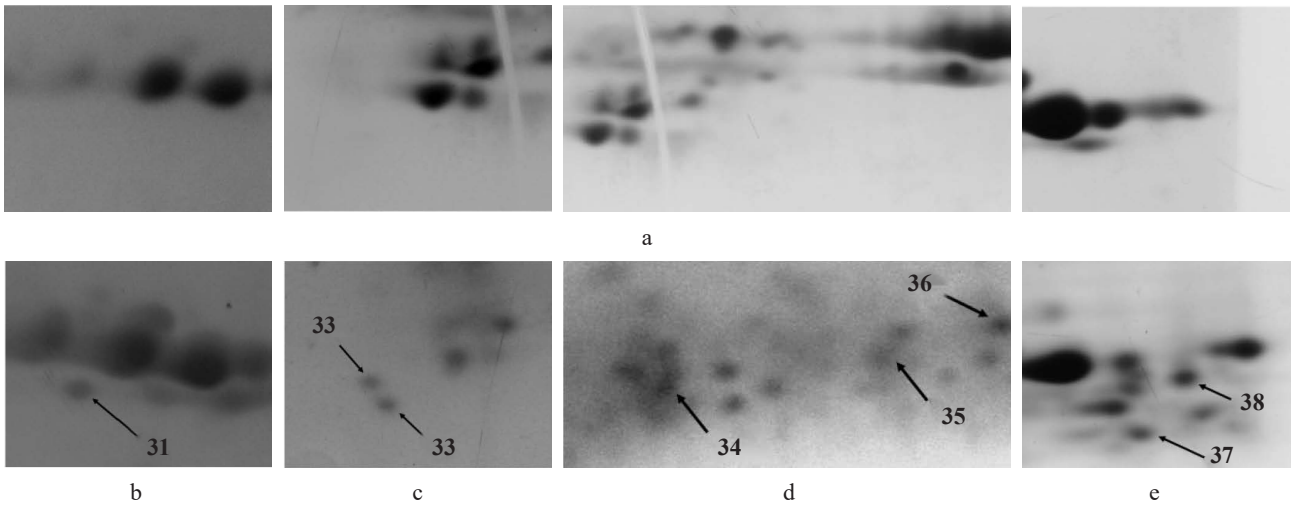


Figure 8 Intermediate peptides on fragments of two-dimensional electrophoresis created by different proteases (Table 5): (a) control, (b) trypsin, (c) bromelain, (d) Alcalase 2.4 L FG, and (e) Protoferm FP

Table 5 Intermediate fragments of protein fractions of chickpea isolate exposed to different proteases: mass spectrometry

No.	Protein (gene symbol)	Protein NCBI	S/M/C*	Mm/pI (experimental)**	Mm/pI (calculated)**
31	Fragment of C-terminal region of positions 344–431, legumin A-like (LOC101489278) + (1)	XP_004493780.1	77/16/25	18.0/9.35	59.3/5.87
32	Fragment of positions 252–448 vicilin-like (LOC101515194) + (2)	NP_001296635.1	114/12/18	14.0/5.00	51.5/6.04
33	Fragment of positions 252–441 vicilin-like (LOC101515194) + (1)	NP_001296635.1	85/12/18	13.5/5.20	51.5/6.04
34	Mix of fragment of positions 24–279 vicilin-like (LOC101515515)***(3), fragment of positions 347–414 legumin-like (LEG)***(1), and positions 154–183 provicilin-like (LOC101510367)***(1)	XP_004493035.1 XP_027188788.1 XP_004496703.1	286/19/33 91/2/4 89/3/4	14.5/5.30	51.9/5.73 56.7/5.97 65.0/6.61
35	Mix of fragment of positions 25–190 vicilin-like (LOC101515515), fragment of positions 105–182 albumin-2-like (LOC101512722)***(1), fragment of positions 252–427 legumin-like (LEG)***(1), and fragment of positions 163–309 provicilin-like (LOC101510367)***(1)	XP_004493035.1 NP_001351664.1 XP_027188788.1 XP_004496703.1	71/7/17 125/3/20 125/5/14 85/2/4	16.0/6.60	51.9/5.73 26.3/5.67 26.3/5.67 65.0/6.61
36	Mix of fragment of positions 219–427 legumin-like (LEG)***(1), fragment of positions 59-251 vicilin-like (LOC101505411), fragment of positions 14–71 17.1 kDa class II heat shock protein-like (LOC101504383)***(1), and fragment of positions 30–154 P24 oleosin (LOC101509783)***(1)	XP_027188788.1 XP_004492829.1 XP_004501442.1 XP_004489219.1	78/6/15 72/6/ 15 118/2/23 80/2/12	8.0/5.90	56.7/6.61 51.2/6.10 21.4/5.46 20.7/7.85
37	Fragment of positions 334–510 legumin A-like (LOC101489278)***(1)	XP_004493780.1	107/10/24	15.0/9.60	59.8/5.87
38	Mix of fragments of positions 325–491 legumin-like (LEG)***(1) and positions of 365–507 legumin J-like (LOC101501269)***(1)	XP_027188788.1 XP_004495100.1	78/18/22 67/18/18	16.0/9.80	56.7/5.97 61.0/5.50

* S/M/C – Score, indicator of compliance (significant protein scores are ≥ 68, $p < 0.05$); the number of Matching peptides; Coverage (%) of the complete amino acid sequence of the protein by the peptides; ** Mm/pI (experimental) – estimates based on the electrophoretic mobility on two-dimensional electrophoresis; Mm/pI (calculated) – estimates made from amino acid sequence data based on the removal of the signal peptide but without other postsynthetic modifications (ExPASy Compute pI/Mw tool); *** msms – identification confirmed by tandem mass spectrometry with the number of sequenced tryptic peptides in brackets.

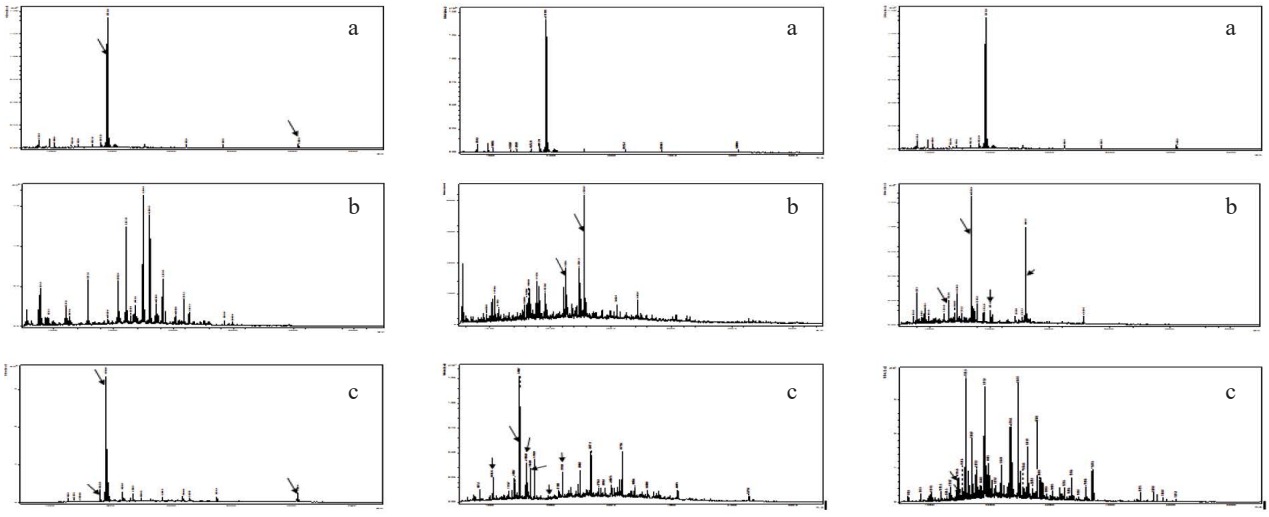


Figure 9 Mass spectra of short peptides after protease treatment. Peptides are marked with arrows

Protease effect on generation of short peptides (m/z ≤ 6000 Da) from chickpea isolate. When dealing with short peptides generated by different types of proteases, we encountered with a technical problem that prevented the identification of mass peaks by tandem mass spectrometry. The isolate samples contained high concentrations of residual chemicals that had been involved in the process of isolate formation, e.g., salts, acids, and alkalis. These substances hindered ionization and identification of the amino acid sequence of particular peptides. Moreover, some of the mass peaks were not of protein nature (high salt concentration). Figure 9 illustrates the mass spectra of short peptides in protease-treated hydrolysates and the control.

The mass spectra of short peptides had clearly different sets. The samples treated with pepsin, papain, bromelain, and Protoferm FP demonstrated a large number of different mass peaks. The hydrolysate treated with

Alcalase 2.4 L FG had fewer peaks and was less effective in biodegrading proteins into biopeptides. Trypsin mainly converted the processed proteins into single amino acid residues.

The mass spectra made it possible to identify eighteen biopeptides; their potential was predicted based on bioinformatic databases (Table 6). The *in-silico* proteolysis was able to connect protein sequence databases and bioactive peptide databases to accelerate the identification of bioactive peptides and their efficiency assessment [52].

The proteolytic enzyme preparations made it possible to determine the amino acid sequence of eighteen (18) peptides generated by different types of proteases. However, pepsin proved useless in this respect. In the trypsin sample, one peptide, which was initially present in the native protein structure, was identical to the control

Table 6 Identified biologically active peptides of chickpea proteins

No.	Source protein	Positions in amino acid sequence	Sequence confirmed by MS/MS	Mass to charge ratio, m/z	Bioactivity
			Control		
1	XP_004487601.1	24–65	SKDEKEEIPESCHKQLKSLNL KHCEKFLMKRMQKDEDEDDDN	5089.1	ACE inhibitor, DPP-IV inhibitor, regulating, AntiCP, non-toxin
			Trypsin		
2	Unknown	–	SSNPFTFLVPPRESEN	1820.9	AntiCP, AHTpin, non-toxin
3	XP_004487601.1	24–65	SKDEKEEIPESCHKQLKSLNL KHCEKFLMKRMQKDEDEDDDN	5089.1	ACE inhibitor, DPP-IV inhibitor, regulating, AntiCP, non-toxin
			Papain		
4	legumin J-like XP_004495100.1	159–178	ITLLDTSNFANQLDSTPRVF	2252.1	ACE inhibitor, DPP-IV inhibitor, AntiCP, non-toxin
5	provicilin-like XP_004496703.1	485–507	FGINAQNNQRNFLAGEDDNVISQ	2564.2	ACE inhibitor, DPP-IV inhibitor, AntiCP, non-toxin
			Bromelain		
6	vicilin-like XP_004492829.1	485–497	FGINAQNNQRNFL	1535.7	ACE inhibitor, DPP-IV inhibitor, AntiCP, AntiBP, non-toxin
7		485–498	FGINAQNNQRNFLA	1606.8	ACE inhibitor, DPP-IV inhibitor, AntiCP, non-toxin
8		485–499	FGINAQNNQRNFLAG	1663.8	ACE inhibitor, DPP-IV inhibitor, AntiCP, non-toxin
9	legumin-like XP_027188788.1	484–492	FSFLVPPRS	1049.6	ACE inhibitor, DPP-IV inhibitor, Renin inhibitor, AntiCP, AHTpin, non-toxin
10	vicilin-like XP_004496704.1	547–563	FPGSAQEVNRLIKNQRQ	1985.0	Regulating, Antithrombotic, Antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, AntiCP, non-toxin
11		545–563	LTFPGSAQEVNRLIKNQRQ	2199.2	Regulating, Antithrombotic, Antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, AntiCP, non-toxin
			Alcalase 2.4 L FG		
12	vicilin-like XP_004492829.1	143–154	DLAIPVNRPGQF	1326.7	Regulating, antithrombotic, antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, AntiCP, AntiBP, AHTpin, non-toxin
13		143–157	DLAIPVNRPGQFQSF	1688.8	Regulating, antithrombotic, antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, renin inhibitor, AntiCP, non-toxin
14	legumin-like XP_027188788.1	169–184	SFQNQLDQMPRRFYLA	2013.9	ACE inhibitor, DPP-IV inhibitor, renin inhibitor, AntiCP, non-toxin
15		214–235	SGFKRDFLEDALNVNRRIVNKL	2604.3	ACE inhibitor, DPP-IV inhibitor, AntiCP, non-toxin
16	legumin A-like XP_004493780.1	350–364	SSSSPDIYIPQAGR	1477.7	ACE inhibitor, antioxidative, DPP-IV inhibitor, AntiCP, AHTpin, non-toxin
17	vicilin-like XP_004492829.1	143–155	DLAIPVNRPGQFQ	1454.7	Regulating, antithrombotic, antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, AntiCP, AntiBP, non-toxin
18		143–157	DLAIPVNRPGQFQSF	1688.8	Regulating, antithrombotic, antiamnestic, ACE inhibitor, DPP-IV inhibitor, PAM inhibitor, renin inhibitor, AntiCP, non-toxin
19	legumin-like XP_027188788.1	250–269	VKGGLSIITPPEKEPRQKRG	2190.2	ACE inhibitor, DPP-IV inhibitor, DPP-III inhibitor, AntiCP, AntiBP, AHTpin, non-toxin, Anti-Tb

AntiCP – anticancer, AntiBP – antibacterial, AHTpin – antihypertensive inhibitor, AntiFP – antifungal, AntiTb – anti-tuberculosis, ACE inhibitor – angiotensin-converting enzyme inhibitor

sample with the amino acid sequence SKDEKEEIPES-CHKQLKSLNLKHCEKFLMKRMQKDEDEDDDN. Another peptide was of unknown origin: although its amino acid sequence was identified, its source remained unknown. The plant proteases generated short peptides from legumin and vicilin-like proteins. The bacterial proteases also formed peptides from the same series of proteins, but with a different amino acid sequence. The bromelain sample demonstrated a specific pattern: it yielded complexes of almost identical peptides that differed only in one amino acid residue, which fortified the solution with specific amino acids. A similar effect was observed in the samples with Alcalase 2.4 L FG and Protoferm FP; however, the cleavage yielded 2–3 amino acid residues, forming di- and tripeptides. The treatment with Alcalase 2.4 L FG and Protoferm FP resulted in an absolutely identical peptide with the amino acid sequence DLAIPVNRPGQFQSF. It demonstrated antithrombotic, antiamnestic, and antitumor activities, as well as inhibited the angiotensin-converting enzyme, DPP-4, proton pump, and renin.

According to the bioinformatics forecasting, all the peptides had antitumor activity, promoting the destruction of cancer cells by a direct or indirect mechanism [53]. All hydrolysates, except for the one treated with trypsin, could inhibit the angiotensin-converting enzyme. Some peptides possessed antithrombotic, antibacterial, antioxidant, and antiamnestic activities or could inhibit DPP-4, DPP-3, adenylate cyclase, and renin, as well as control blood glucose.

The remaining mass peaks rendered it impossible to read the amino acid sequence because of the high concentration of salts. Apparently, a different technological base is required for a more complete identification of the peptides generated by different types of proteases, e.g., HPLC combined with tandem mass spectrometry.

CONCLUSION

In this study, the enzymatic processing of plant protein yielded biologically active peptides of low molecular weight. In addition to bioactivity, these biopeptides exhibited good functional, technological, and antioxidant properties. These results may open up new prospects for the application of plant proteins. Hydrolysates treated with proteolytic enzymes of animal origin improved their functional and technological profile. Commercial enzymes of microbial origin proved to be more effective producers of biologically active peptides. The plant proteases demonstrated the greatest balance between the advanced functional and technological properties and the biopeptide yield.

Chickpea protein hydrolysates and biopeptides are a new potential natural source of bioactive substances to be used in the food industry and pharmacy. Advanced hydrolysis technologies may resolve a lot of current issues in the spheres of nutrition, ecology, and economy, contributing to the development of more sustainable and affordable food systems.

CONTRIBUTION

All authors contributed equally to the study and are equally responsible for the information published in this article.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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ORCID IDs
Mahmud Gharaviri https://orcid.org/0000-0002-4318-7077
Denis I. Aleksanochkin https://orcid.org/0009-0000-7677-6583
Mahbubeh Ahangaran https://orcid.org/0000-0003-4796-2066
Ivan A. Fomenko https://orcid.org/0000-0003-2478-1705
Leonid I. Kovalev https://orcid.org/0000-0001-6519-8247
Marina A. Kovaleva https://orcid.org/0000-0002-3486-2122
Irina M. Chernukha https://orcid.org/0000-0003-4298-0927
Natalia G. Mashentseva https://orcid.org/0000-0002-9287-0585