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Mechanically activated hydrolysis of plant-derived proteins in food industry

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Abstract: A poor consumption of important nutrients triggered a public interest in functional foods that contain easy-to-digest proteins. The present research features fractionation, mechanical activation, and enzymatic hydrolysis of pea protein. According to modern chemical methods, the protein content in the original pea biomass was 24.3% and its molecular weight distribution (MWD) was 5–135 kDa. Fractionation, or protein displacement, resulted in four fractions of biopolymers with different chemical composition, i.e. a different content of protein and carbohydrate molecules. The paper introduces some data on the enzymatic transformations of the substrate. A set of experiments made it possible to define the optimal conditions for the mechanical activation of pea biomass with proteolytic enzymes. The enzymes were obtained from Protosubtilin G3x, a complex enzyme preparation. When the substrate and the enzymes were mechanically activated together, it produced mechanocomposite, an intermediate product with increased reactivity. It increased the specific surface area by 3.2 times and doubled the crystallinity of the substrate. As a result, the rate and yield of the subsequent enzymatic hydrolysis increased from 18% to 61%. The study determined the capacity of the substrate in relation to the enzyme preparation. Under optimal conditions, the pea hydrolysis destroyed protein molecules within two hours. After four hours of hydrolysis, no changes were detected. A polyacrylamide gel electrophoresis revealed non-hydrolysed protein molecules with MW ≈ 20 kDa. Presumably, they corresponded with legumin, which is resistant to neutral and alkaline proteases. The resulting hydrolysates were spray-dried to test their potential use as a food component. The product obtained by spray-drying had a monomodal distribution of particle sizes of spherical shape with a diameter of 5–20 μm.

Keywords: Mechanochemistry, mechanochemical activation, mechanocomposite, plant materials, enzymatic hydrolysis, destruction of protein molecules, polypeptides, amino acids, spray-drying

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INTRODUCTION

The development and subsequent quality assessment of functional foods is one of the priorities of healthy nutrition [1]. Functional foods with a programmed chemical composition can be fortified with important nutrients and are suitable for various categories of population, e.g. athletes, lactating and pregnant women, senior citizens, children, etc. [2].

However, priority goes to gastrointestinal and allergic patients and professional athletes. Their nutrition

requires scientific approaches, since their diet should contain a complex of peptides and free amino acids, as well as simple and complex carbohydrates [3].

Foods fortified with proteins, especially those containing essential amino acids, contribute to the rapid and effective recovery of muscle tissue after intense physical exertion. Peptides and polypeptides are known to accelerate metabolic processes, hormone production, and muscle tissue growth [4].

Food intolerance and allergic reactions are another problem of modern society. Some people are allergic to products that contain proteins of animal or plant origin.

Hence, a new generation of food products with easy-to-digest nutrients remains an important objective of food industry. Modern studies confirm that plant raw materials – and legumes in particular – are suitable for isolation and modification of proteins, short peptides, and amino acids [5, 6]. These groups of compounds are widely used as dietary supplements and ingredients for functional products [7–9]. Pea protein has a better nutritional value, amino acid composition, and anti-nutrients than soybeans, beans, and other legumes [10, 11].

There are many methods to isolate protein from plant materials for subsequent hydrolysis [12–14]. However, most of them remain inefficient for enzymatic transformations of heterogeneous substrate. Preliminary mechanical activation means that raw material has to be processed in specially designed energy-stressed activator mills. The procedure makes it possible to control the reactivity of solid substrates. In addition, it increases the speed and yield of water-soluble products for commercial purposes [15, 16].

However, the process of enzymatic reactions after preliminary mechanical activation remains understudied. A series of studies on the hydrolysis of cellulose showed that the increase in specific surface area and the degree of crystallinity of the substrate affected the rate and yield of enzymatic hydrolysis [17]. In addition, it is important to study the elusive transfer of mechanochemical processes from lignocellulose to protein and starch.

The research objective was to study the mechanically activated hydrolysis of pea biomass, as well as to obtain a hydrolysate fortified with free amino acids and peptides to be used in functional foods.

STUDY OBJECTS AND METHODS

The experiment featured dry biomass of split pea seeds harvested in 2017. The peas corresponded with State Standard 6201-68*, Class I, and was produced by OOO ECO-PAK (Novosibirsk region, Russia). Before the experiment, the pea biomass was subjected to rough grinding in a knife mill to the size of ≤ 2 mm. The ground biomass was vacuum-packed, stored at room temperature, and used for further experiments.

Protosubtilin G3h was used as enzyme preparation (OOO Sibbiopharm, Berdsk, Russia). The complex was chosen for its catalytic activity and availability for further technological application. This industrially available enzyme preparation contains a complex of enzymes that consists of neutral and alkaline proteases and glycosidases, i.e. ≈ 11000 U/g of protease, ≤ 150 U/g of xylanase, ≤ 200 U/g of β-glucanase, and ≤ 300 U/g of α-amylase [18]. Protosubtilin belongs to the group of enzyme feed additives that are able to break down high-molecular proteins. This enzyme preparation is produced by *Bacillus subtilis*.

Gravimetric methods were used to assess moisture and ash content in the plant materials and processed products, respectively [19, 20].

X-ray diffraction and thermal desorption of gases were employed to measure the degree of crystallinity and specific surface area according to the methods described in [17] and [21], respectively.

The method described by Fadeeva *et al.* was used to perform the elemental analysis that made it possible to determine the quantitative protein content in the peas. After that, the protein content was determined using the nitrogen content with conversion factor of 6.25 according to the Kjeldahl method [22–24].

The mass fraction of soluble substances was determined by the method of exhaustive extraction in a Soxhlet extractor for 24 h. Distilled water was used as extractant. The yield of water-soluble substances was measured according to the reduction of the mass after the extraction.

The content of free amino acids was defined at the Centre of Mass Spectrometry Analysis (Institute of Chemical Biology and Fundamental Medicine). An optimised standard procedure was used as in [25]. A set of isotope-labelled amino acids and acyl carnitines No. 55000 (Chromsystems Instruments & Chemicals, Germany) served as internal standards and solutions. An Agilent-1200 chromatographic system with an Agilent 6410 QQQ mass spectrometer (Agilent Technologies, USA) was employed as an HPLC-MS/MS system. A quantitative analysis was performed in the mode of multiple reactions monitoring; the total analysis time was 2.5 min. The obtained data were processed using MassHunter v.1.3 software.

The molecular weight of protein molecules was measured using the *Laemmli SDS PAGE procedure* [26]. For pre-denaturation, proteins were treated with 1.4-dithiothreitol at a 1:1 ratio. After that, they were placed in a thermoshaker at 95°C (Biosan, Latvia) for 7 min. An Elf-4 power source was used to create electric field (DNA-Technology, Russia). The concentrations of polyacrylamide in the concentrating and separating gel were 4% and 18%, respectively. The pre-phoresis stage lasted 15 min. The current force was 15 mA, while during the phoresis stage it was 35 mA.

To identify the zones of proteins after the electrophoresis, they were stained with Coomassie R-250 pigment according to the procedure described by

* State Standard 6201-68. Polished pea. Specifications. Moscow: Standartinform; 2010. 3 p.

Dyballa *et al.* [27]. Protein markers were represented by Unstained protein MW marker (Thermo Fisher Scientific, USA) with protein molecular weight of 14.5–116 kDa and Unstained protein ladder (Thermo Fisher Scientific, USA) with protein molecular weight of 5–250 kDa. MultiChrom-Planar programme processed the mathematical data [28].

The fractionation of the plant material was conducted according to the method described in [29, 30]. The initial crushed pea biomass was extracted in alkaline water. A 1M solution of sodium hydroxide was added to the pre-ground pea biomass. The solution consisted of 2.5 mL of solution per 1 gram of biomass (pH 9.0). The suspensions were placed in a WSB-30 water bath at 45°C and 180 rpm for 30 min (DAIHAN Scientific, Korea). After the extraction, the soluble portion was separated by centrifugation at 6000 rpm for 20 min. The precipitate was used in the next extraction cycle under the same conditions. The extracted components were precipitated with a threefold volume of cooled ethanol and dried in a laboratory frost dryer Iney 4 (*Institute* for *Biological Instrumentation* of the *Russian Academy* of *Sciences*, Russia).

After three extraction cycles (fraction No. 4), the insoluble residue – a carbohydrate fraction – was washed twice with chilled ethanol and dried under similar conditions.

The mechanical activation of the plant material with enzymes was carried out in an RM-20 roller mill-activator (5.5 kW), which was equipped with a water cooling device (Fig. 1). The pea biomass was mixed with a dry enzyme preparation and processed in an activator at a rotor speed of 1450 rpm. The mixture of raw materials and enzymes was supplied automatically at a rate of 3 kg/h.

The spray-drying was performed in a Mini Spray Dryer B-290 (Büchi, Switzerland) in the following conditions: nozzle temperature = 110°C, cyclone temperature = 70°C, gas flow rate = 700 L/h, feed rate = 5 mL/min.

The enzymatic hydrolysis lasted 7 h at 50°C. 50 mL of distilled water was added to 15 g of initial or mechanically activated pea biomass with a certain amount of the enzyme preparation. Enzyme loading equalled 0.5%–3%. Suspensions were thoroughly mixed until uniform. For enzymatic hydrolysis, the suspensions were placed in a WSB-30 water bath (DAIHAN Scientific, Korea) at 50°C and 120 rpm. After enzymatic hydrolysis, the supernatant was centrifuged at 6000 rpm for 20 min. No enzyme preparation was added to the control samples.

RESULTS AND DISCUSSION

Suitable protein plant materials were selected for the mechanoenzymatic processing to be used in functional, special, and therapeutic food products. The physical and chemical characteristics are given below. In addition, the selection was based on an analysis of the existing market for high-protein plant materials, state statistics, distribution of croppage, and percentage of various cultures in Russian regions. This approach made it possible to identify raw material with suitable physicochemical parameters, as well as to determine its prospects in subsequent processing and implementation.

Figure 2 shows a distribution diagram of croppage in Russia in 2017. The diagram was based on the data obtained from the Federal State Statistics Service [31]. Cereals and legumes clearly prevail over other cultures. Legumes are richer in protein than grains. An analysis of the distribution of croppage within the group of leguminous crops showed that a large proportion (77%) belongs to peas (Fig. 3).

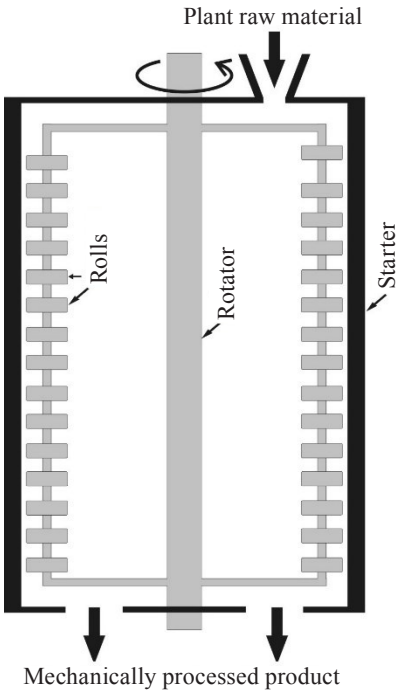

Figure 1 Scheme of the roller type activator mill RM-20

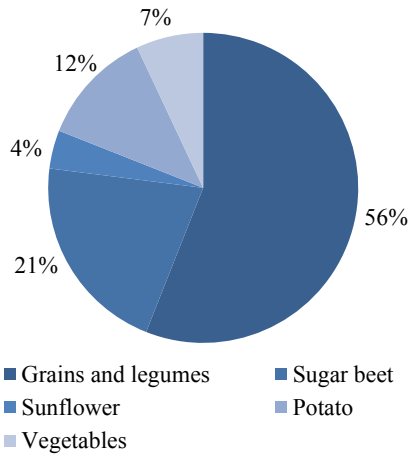

Figure 2 Croppage distribution in Russia

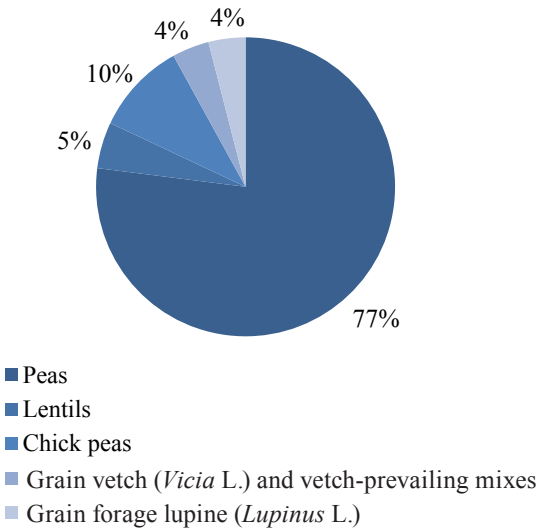

Figure 3 Percentage ratio of the croppage of legumes in Russia

Thus, legumes proved to be the most advantageous source of vegetable protein in Russia, especially peas, which contain about 25% of protein. In spite of the fact that soy contains up to 35% of protein, it was not considered in this study since it is rich in anti-nutritional substances, Moreover, it has a low consumer loyalty, which cannot be ignored in product development [5–7, 10, 32]. The protein content in peas varies greatly according to genotypic characteristics and the cultivation conditions. Leguminous proteins are poor in methionine and cysteine. This is typical of plant proteins. For instance, grain crops are poor in lysine and threonine. However, the biological value of products obtained from them can be fortified by a limiting amino acid or other types of plant materials.

The present research involved a comparative analysis of the protein content and amino acid composition together with its coefficient of imbalance and functionality in high-protein plant raw materials. Peas demonstrated the highest functionality ratio of amino acid composition (FRAAC) – 0.6, while soybeans had 0.4 and beans and lentils had 0.3. This indicated that peas possessed the optimal ratio of amino acids if compared with reference chicken egg protein.

Thus, pea biomass appeared to have a high nutritional value and a balanced amino acid composition, which made it an optimal research subject. Its physical and chemical patterns can subsequently be transferred to other types of biomass. The samples obtained after fractionation (Fig. 4) and freeze drying were analysed for the protein content in the dry product. The results are presented in Table 1. A polyacrylamide gel electrophoresis defined the molecular weight of the proteins in the fractions.

The obtained data are consistent with those already published Mession *et al.*: the pea biomass contained 23–24.4% of protein and 48–60.3% of starch [33].

Figure 4 Frozen fractionation products before freeze drying: 1–4 are numbers of corresponding fractions

Fractions No. 1 and 2 isolated from the biomass were fortified with proteins, while fraction No. 3 was fortified with proteins and carbohydrates, and fraction No. 4 – with carbohydrates.

The electrophoregram (Fig. 5) shows that fractions 1–3 contained proteins with molecular weight = 5–135 kDa, which corresponded to molecules that consisted of 50–1350 amino acid residues. The predominating molecules were those with molecular weight = 24–135 kDa (240–1350 amino acid residues). They were most likely to be sub-units of 11S-globulins [34]. Both the elemental analysis and the gel electrophoresis showed that the content of protein molecules in fraction 4 was at the level of trace amounts.

As proved by cellulose processing, enzyme preparation increases the efficiency of subsequent enzymatic hydrolysis, if added at the stage of mechanic-chemical processing [34]. The enzyme complex used in the present research had a suitable catalytic activity profile and was cheaper than its analogues, such as proteases AP1, Alcalase, Savinase, Esperase, and Neutrase (Shandong Longda Bio-Products and Novozymes).

A set of experiments made it possible to determine the effect of the conditions of mechanical activation on the subsequent enzymatic hydrolysis. The pea biomass was subjected to mechanical activation 1) without enzymes and 2) with an insufficient amount

Table 1 Protein content in the initial raw material and in the fractions

Sample	Protein content, %	Fraction content in the raw material, %
Raw material	24.3	–
Fraction No. 1	97.1	19.0
Fraction No. 2	86.7	6.5
Fraction No. 3	45.7	0.4
Fraction No. 4	Trace	74.1

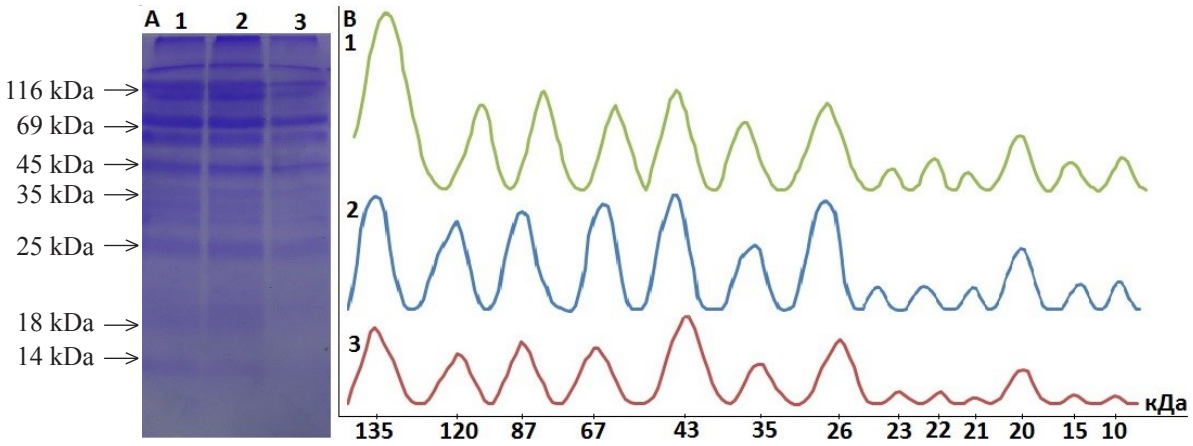

Figure 5 Electrophoregram (A) and MWD profilograms (B) of proteins in the fractions; 1, 2, and 3 – fraction numbers. Fraction No. 4 is not represented as it appeared to have no proteins in its composition

Table 2 Yield of water-soluble substances according to the processing conditions

	Extraction from the initial raw material	Extraction from the product of mechanical activation without enzymes	Product after mechanical activation (without enzymes) and hydrolysis	Product after mechanical activation with 1% of enzyme preparation after hydrolysis
Yield of water-soluble substances, %	18.0	18.5	25.1	60.6

of enzymes (1%) in relation to the substrate. The subsequent hydrolysis and complete extraction (Table 2) showed that the mechanical activation without enzymes barely increased the yield of the subsequent hydrolysis. However, the mechanical activation with enzymes increased the yield during subsequent hydrolysis from 18% to 60%, i.e. by ≥ 3 times.

The results can be explained by the fact that a simultaneous activation of substrate and enzymes produced mechanocomposite. The mechanocomposite was an intermediate solid-phase product with a high reactivity. In such mechanocomposites, enzyme particles are distributed non-diffusively, or mechanically, over the surface of the substrate, which was disordered during the activation process. Similar effects were observed in other cases of activation of food and non-food plant raw materials [35, 36]. When mechanocomposite is formed, it usually increases the rate and yield of the subsequent proteolytic and glycolytic processes. In this case, a preliminary chemical interaction preceded the mixing of the enzymes and the substrate. This interaction resulted from a significant increase in surface area, which enlarged from 0.6 to1.9 m^2/g, and an extra disordering of the substrate structure, whose crystallinity decreased from 25% to 14%.

The conversion of enzymatic hydrolysis was studied under the same conditions, according to the substrate – enzyme ratio. The enzyme preparation was added in 0.5, 1, 2, 2.5, and 3% (Table 3).

Table 3 shows that the amount of water-soluble substances increased, as the amount of enzymes increased from 0.5% to 2%. The water-soluble substances included reducing carbohydrates, which are low molecular weight products of starch hydrolysis. When the load of the enzyme complex increased to 2.5%–3%, the number of reaction products did not increase. This might have been caused by the fact that the sorption sites of the substrate were completely filled with enzymes. The situation was fully consistent with the idea that the heterogeneous stage of enzymatic hydrolysis has a limiting effect.

The polyacrylamide gel electrophoresis was used to study the changes in the molecular weight during the enzymatic hydrolysis. Figure 6 shows the electrophoregram of the proteins contained in the hydrolysate 1–7 h after the hydrolysis. The data prove that the amount of the original protein molecules significantly decreased within 7 h. As a rule, proteins degrade within 2 h. The molecular weight of the degradation products of the original polypeptide proteins revealed no significant changes after 4 h. After

Table 3 Yield of water-soluble substances and reducing carbohydrates according to the amount of enzyme preparation

Enzyme preparation, %	Yield of water-soluble substances, %	Yield of reducing carbohydrates, %
0.0	25.8	1.5
0.5	38.7	4.0
1.0	55.4	7.6
2.0	78.5	16.3
2.5	78.5	16.3
3.0	78.5	16.3

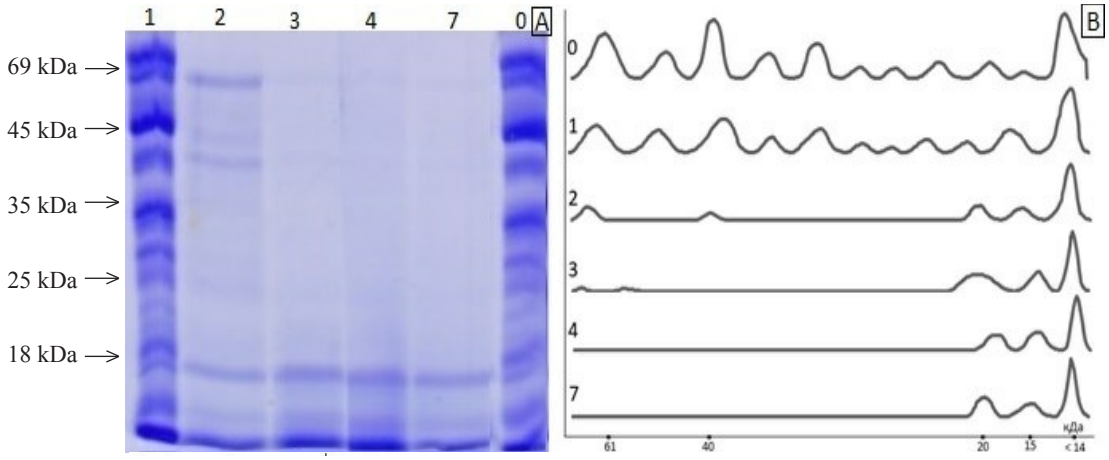

Figure 6 Electrophoregram (A) and profilogram (B) of the molecular weight distribution of proteins in the hydrolysate after 0, 1, 2, 3, 4, and 7 h

7 h, there remained an insignificant amount of stable polypeptides with a molecular weight of ≈ 20 kDa. These polypeptides were associated with the polypeptide chain of legumin, which is resistant to neutral and alkaline proteases [33].

Thus, an enzymatic hydrolysis that exceeded 4 h is ineffective, since the number of low molecular weight polypeptides did not increase much after that time.

Table 4 Content of essential amino acids in the hydrolysate and in the control sample

Amino acid	Amino acid content, μg/g	
	Pea biomass extract	Hydrolysed pea biomass
Ile+Leu	515	10479
Met	110	1320
Phe	514	7681
Val	441	3921

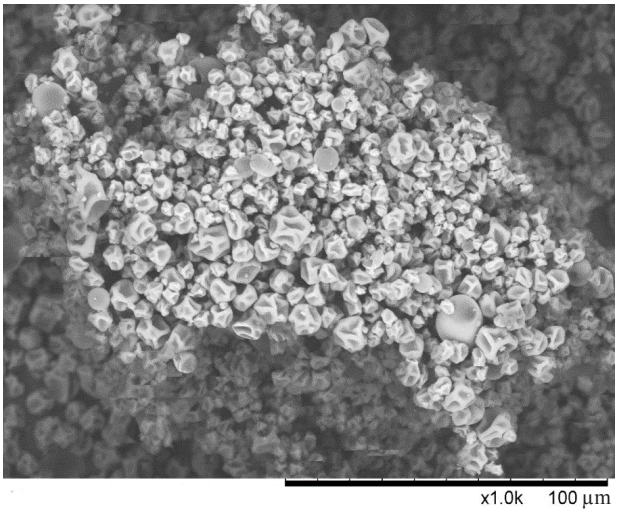

Figure 7 Scanning electron microscopy of the hydrolysate after spray-drying

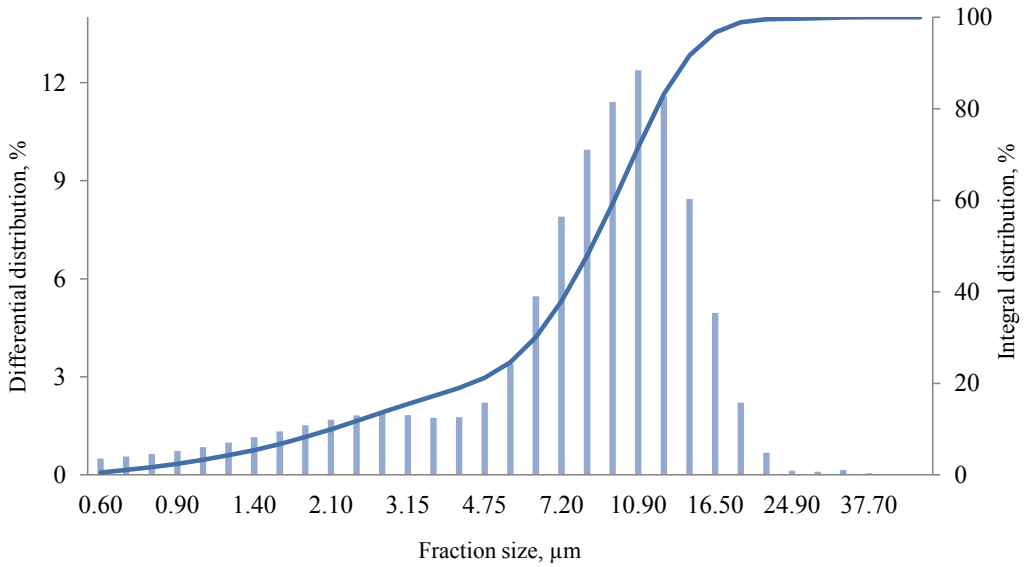

Figure 8 Granulometric composition of the hydrolysate after spray-drying

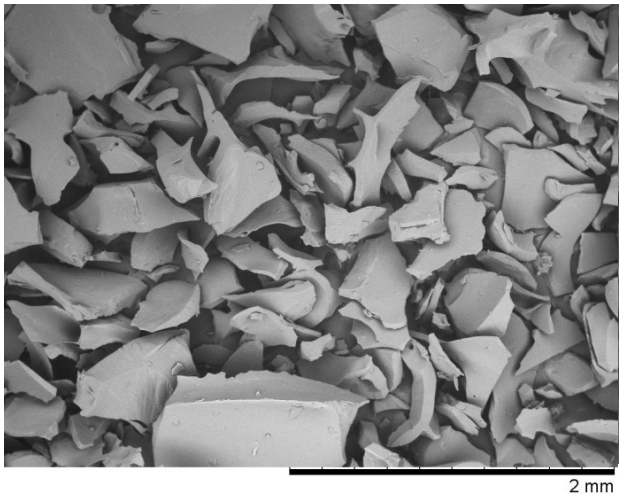

Figure 9 Scanning electron microscopy of the material after vacuum drying

A mass-spectrometric analysis of amino acids was performed to study the low molecular weight products of the enzymatic hydrolysis. Table 4 shows that the hydrolysis resulted in a significant increase in the number of essential amino acids in comparison with the control sample obtained without enzymes.

For the hydrolysates to be widely implemented, there have to be new ready-made food products with prolonged shelf life. Thus, a set of experiments on spray-drying had to be performed [37]. The spray-drying process can be easily scaled and is widely used in food industry to produce dry enzymes, foodstuffs, and unstable compounds [38–40].

The product obtained by spray drying (Figs. 7 and 8) had a monomodal particle size distribution. The main share belonged to spherical particles with a diameter of 5–20μм. The size was associated with the characteristics of the equipment: the nozzle opening was 25μм in diameter.

Most of the particles were concave, which made it possible to describe the mechanism of drying. Initially, a powerful inward-directed deformation removed the solvent from the surface of the drop. As a result, there formed a layer of the product. The solvent diffused the layer of the dry product, after which the particle deformed and collapsed.

In the control experiment, vacuum drying without splashing the hydrolysate resulted in the formation of a layer that was not dispersed into individual particles. An electron scanning microscopy of the ground product (Fig. 9) showed that it had a dense structure without pores. This confirms the spray-drying mechanism: the drying occurs on the surface, while the dry layer captures the solvent, and a high mechanical tension deforms the particle, giving it a concave shape.

CONCLUSION

Thus, the paper featured the process of mechanical activation and subsequent enzymatic hydrolysis of pea proteins. The original pea biomass was described using modern chemical methods. The protein content was 24.3%, and MWD was 5–135 kDa.

The fractionation produced four fractions of biopolymers with various contents of protein and carbohydrate molecules. The experiment made it possible to define the optimal conditions for the mechanical activation performed together with proteolytic enzymes. The enzymes were obtained from the complex enzyme preparation Protosubtilin G3x. When both the substrate and the enzymes were mechanically activated, it produced mechanocomposite. As a result, the specific surface area increased by 3.2 times, while the crystallinity decreased by 2 times, which raised the yield of the subsequent enzymatic hydrolysis from 18% to 61%.

During hydrolysis, protein broke down within 2 h, and there was almost no change after 4 h. The experiment detected non-hydrolysed protein molecules with a molecular weight of ≈ 20 kDa. They presumably corresponded with legumin, which is resistant to neutral and alkaline proteases.

The research involved an experiment on spray-drying of the obtained hydrolysates for their potential use as food components. The resulting product had a monomodal particle size distribution. The particles had a spherical shape with a diameter of 5–20 μ.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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