Fractionation and hydrolysis of proteins of plant raw materials obtaining functional nutrition products

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Abstract. Data on the process of fractionation, mechanical activation and subsequent enzymatic hydrolysis of pea seeds proteins was obtained. As a result of fractionation of plant raw materials, fractions of biopolymers with different chemical composition were obtained. The conditions of mechanical activation for the protein fraction with the enzyme preparation, the optimal substrate-enzyme complex ratio and the optimal conditions for mechanically activated enzymatic hydrolysis were determined. It was shown that under certain optimal conditions for hydrolysis of pea biomass, the protein molecules broke down and a mixture of low molecular weight products formed.

1. Introduction

Currently, one of the priority directions in the field of healthy nutrition is the development and the subsequent evaluation of the quality of functional foods [1]. Functional nutrition products with a predetermined chemical composition due to enrichment with important nutrients are suitable for different categories of the population (athletes, pregnant and lactating women, children and others) [2].

The priority direction in this area is the optimization of food rations for people suffering from diseases of the gastrointestinal tract and allergic reactions, as well as nutrition of highly successful athletes. The organization of high-grade food of this category of the population requires certain scientific approaches for creating food products containing a complex of peptides, free amino acids, simple and complex carbohydrates [3]. Food enriched with proteins, especially those containing essential amino acids, promotes rapid and effective recovery of the athlete's muscular tissue after intense physical exertion. It is well known with using peptides and polypeptides, acceleration of metabolic processes, more intensive stimulation of growth hormone and growth of muscle tissue are observed [4].

Another important problem of society is food intolerance, expressed in allergic reactions. The most pronounced properties of allergens are products that contain proteins of animal or plant origin. So the development of a new generation of food products containing nutrients in an easily accessible form is still relevant, despite the large range of products on the market.

There are many methods for isolating proteins from plant raw materials for subsequent hydrolysis [5, 6]. But most of them have low efficiency due to the heterogeneous origin of enzymatic substrate
conversions. Mechanochemical pretreatment allows controlling the reactivity of solid substrates, to increase the rate and yield of subsequent reactions at the industrial level. However, the features of mechanochemically pretreated reactions are not sufficiently studied.

The purpose of this study is to fractionate and hydrolyse proteins of plant raw materials (from pea seeds) to obtain functional nutrition products that are enriched products of protein hydrolysis – free amino acids and peptides.

2. Materials and methods

2.1. Materials
The biomass of pea seeds GOST 6201-68, grade I (Ltd. EKO-PAK, Novosibirsk region) was chosen as a plant raw material. Before the experiments the plant raw material was subjected to coarse grinding by a knife mill to a size of less than 2 mm. The ground biomass was hermetically packed, stored at room temperature and used for further experiments.

Protosubtilin G3H (Sibbiofarm Ltd., Berdsk) was used as an enzyme preparation because of the profile of its catalytic activity and market availability. It is industrially available enzyme preparation consisted of neutral and alkaline proteases and glycosidases (protease - about 11000 units/g, β-glucanase - up to 200 units/g, xylanase - up to 150 units / g and α-amylase - up to 300 units / g) [7]. Protosubtilin belongs to the group of enzyme feed additives and is a product of the activity of bacteria of the Bacillus subtilis strain.

2.2. Characterization of plant raw materials
Measurement of moisture and ash content of plant raw materials and processed products was carried out using gravimetric methods [8] and [9], respectively.

Elemental analysis to determine the quantitative protein content was carried out according to the procedures [10].

The mass fraction of water-soluble substances was determined by the exhaustive extraction method in the Soxhlet extractor for 24 hours.

Determination of the molecular weight of proteins was carried out by the method of vertical electrophoresis in a polyacrylamide gel according to the procedure [11].

The content of free amino acids was determined by MALDI-TOF mass spectrometry on Autoflex Speed (Bruker Daltonics, Germany) [12].

2.3. Fractionation of plant raw materials
Fractionation of plant raw materials was carried out according to the procedure [13, 14]. The round plant raw stock was subjected to extraction in a slightly alkaline aqueous solution. For this a solution of sodium hydroxide (2.5 ml of solution per gram of biomass, pH 9.0) was added to the pre-ground biomass of pea seeds. Suspensions were placed in a thermo-shaker (45 °C, 180 rpm) for 30 minutes. After extraction, the soluble part was separated by centrifugation (20 minutes, 6000 rpm), and the precipitate was used in the next extraction cycle under the same conditions. The extracted components were precipitated with a three-fold volume of ethanol and dried in a laboratory freeze dryer "Iney 4". The insoluble residue after three extraction cycles was washed twice with ethanol and dried under similar conditions. This fraction was representing the carbohydrate fraction or starch.

2.4. Mechanochemical activation of plant raw materials
Mechanical activation of plant raw materials with enzymes was carried out on a roller mill RM-20 (5.5 kW) equipped with water cooling. For this the biomass of pea seeds subjected to coarse grinding on a knife mill was mixed with 1% by weight of dry enzyme preparation Protosubtilin. The supply of plant raw materials along with the enzymes was automated. The rotation speed was 1450 rpm. The productivity was 3 kg / h.
2.5. Enzymatic hydrolysis of plant raw materials

Enzymatic hydrolysis was carried out for 7 hours at 50 °C. For this, an enzyme preparation (1 wt%) and distilled water (50 ml per 15 g biomass) were added to the previously mechanochemically activated biomass of pea seeds. The enzyme preparation was not added to the control samples. Suspensions were placed in a thermo-shaker (120 rpm) to perform enzymatic hydrolysis. Every 30 minutes for a better mixing, the slurries were shaken by hand. After enzymatic hydrolysis, the supernatant was separated by centrifugation (20 minutes, 6000 rpm).

3. Results and discussion

The samples obtained after fractionation and freeze drying were analyzed for the protein content in the dry product. Also the molecular weight of the proteins contained in the fractions was determined by electrophoresis in a polyacrylamide gel. The results are presented in Table 1.

**Table 1.** The protein content in the plant raw material (pea seeds) and in the isolated fractions.

| The sample                          | Protein content, wt % | The protein content in a dry product per 100 grams of raw material, g / 100 g |
|-------------------------------------|-----------------------|--------------------------------------------------------------------------------|
| biomass before fractionation        | 100.0                 | 26.0                                                                              |
| fraction 1                          | 97.1                  | 18.5                                                                              |
| fraction 2                          | 86.7                  | 5.6                                                                               |
| fraction 3                          | 45.7                  | 0.2                                                                               |
| fraction 4                          | 0.0                   | 0.0                                                                               |

The data obtained are consistent with those already published [15], according to which the biomass of pea seeds contains 23-24.4% protein and 48-60.3% starch.

The fractions 1 and 2, isolated from biomass, are enriched in proteins. The fraction 3 is protein-carbohydrate. The fraction 4 is carbohydrate. Determination of the molecular masses of protein molecules in the obtained fractions was carried out by electrophoresis. The prophylogram for the scanned gels was done according to the program “Planar” [16].

Figure 1 shows the electrophoregram (Figure 1). Fractions 1-3 contain a set of proteins with molecular weights from 5 to 135 kDa, which accords to molecules consisting of 50-1350 amino acid residues, respectively. Molecules with molecular masses from 24 to 135 kDa (240-1350 amino acids) are quantitatively predominant. Probably these molecules are subunits of 11S-globulins [17].

![Figure 1. The electrophoregram (A) and the profilogram (B) of the molecular weight distribution of proteins in the isolated fractions.](image_url)
Early in the experiments it was established that addition of the enzyme preparation in the mechanochemical treatment step increases the efficiency of subsequent enzymatic hydrolysis [18]. It was shown the addition of the Enzyme preparation Protosubtilin G3H in the mechanochemical treatment step increases the efficiency of subsequent enzymatic hydrolysis. So at processing of cellulosic raw materials on the example of wheat straw Enzyme complex Protosubtilin G3H was chosen for experiments, because it has a suitable profile of catalytic activity. For our purposes the enzymatic complex Protosubtilin G3H has a suitable catalytic activity profile. In addition, it is economically more affordable than analogues (for example, proteases AR1, Alcalase, Savinase, Esperase and Neutrase from biotechnology and newzymes of Shandong Longda).

A series of experiments was performed to determine the effect of mechanical activation conditions on subsequent enzymatic hydrolysis. For this, the biomass of the pea was subjected to mechanical activation in an individual form (without enzymes) and with the addition of 1% of the enzyme preparation. Conducting the subsequent hydrolysis within 7 hours and exhaustive extraction (Figure 2) showed that mechanical activation of plant raw materials without enzymes does not lead to a significant increase in the yield of subsequent hydrolysis. At the same time, the mechanical activation of biomass together with the enzymes allows to increase the yield by subsequent hydrolysis more than 3 times (from 18 to 60%). These results can be explained by the formation of a mechanocomposite. It is formed by preliminary mechanochemical activation of dried pea biomass with the addition of the enzymes. The mechanocomposite, in our case, is an intermediate solid phase product with increased reactivity. It particles of the enzyme preparation are distributed indiffusively (mechanically) over the surface. The surface was disordered during the activation of the plant raw material (biomass of pea seeds) on a roller mill PM-20. Similar effects were published earlier in the case of food and non-food raw materials [19]. It is shown that the formation of mechanocomposites in similar experiments leads to an increase in the reactivity of proteolytic and glycolytic enzymes. Otherwise, simple mixing of the components occurs. This reaction provides a significant increase in surface area and an additional disorder structural organization.

![Figure 2](image_url)

**Figure 2.** Dependence of the yield of water-soluble products of hydrolysis on the conditions of the experiment on mechanical activation and subsequent enzymatic hydrolysis.

*MA – mechanical activation.

Under the same conditions the depth of the enzymatic hydrolysis was investigated depending on the ratio of the amount of substrate and enzyme preparation. The amount of degradation products of the mechanically activated biomass of pea seeds was measured by the amount of water-soluble
substances formed. Loads of the preparation Protosubtilin in the reaction were taken respectively 0.5 wt %, 1 wt %, 2 wt % and 3 wt %. The results of the experiments are shown in Figure 3.

![Figure 3](image)

**Figure 3.** The yield of water-soluble products of hydrolysis depending on the amount of enzymes.

Figure 3 shows that the amount of water-soluble substances, including reducing carbohydrates (low-molecular products of starch hydrolysis), naturally increases with an increase in the quantity of enzymes from 0.5 to 2 %. The amount of reaction products no longer increases with an increase in the loading of the enzyme complex to 3 %.

The flow of enzymatic hydrolysis by 1 % of Protosubtilin G3H over time was studied by electrophoresis in a polyacrylamide gel. Figure 4 shows the electrophoregram of proteins contained in the hydrolysate after 1-7 hours of hydrolysis. It was shown that within 7 hours the quantity of full-length original protein molecules significantly decreased. In general, protein degradation occurs within two hours. After four hours there is practically no significant change in the molecular masses of degradation products of the original protein. As a result, a small amount of polypeptides with a molecular weight of 20 kDa remains.

![Figure 4](image)

**Figure 4.** The electrophoregram (A) and the profilogram (B) of the molecular weight distribution of in the samples during 0, 1, 2, 3, 4 and 7 hours by 1 % of Protosubtilin G3H.
Thus, the enzymatic hydrolysis of more than 4 hours is ineffective. The quantity of low molecular weight polypeptides increases slightly after this time.

The chromatography-mass spectrometric analysis for amino acids was performed to characterize the hydrolysis process. Two samples were prepared for this. Hydrolysis was carried out for 4 hours at 50°C. In the first case, the enzyme preparation Protosubtilin (enzyme loading 1 %) and 50 ml of distilled water was added to 15 g of biomass of pea seeds. In the second case, the enzyme preparation was not added. The results are shown in Table 2.

**Table 2.** The content of essential amino acids in the hydrolisate and extract of pea biomass.

| Content of essential amino acids, mg/g | The samples | Hydrolysate of pea biomass | Extract of pea biomass |
|---------------------------------------|-------------|----------------------------|------------------------|
| Ile + Leu                             | 10478.72    | 514.73                     |
| Met                                   | 1320.47     | 109.57                     |
| Phe                                   | 7681.42     | 513.89                     |
| Val                                   | 3921.19     | 441.42                     |

It is shown that during the course of enzymatic hydrolysis a significant increase in essential amino acids occurs. In the case of extraction of pea biomass under the same conditions, this effect is less noticeable.

4. Conclusions

The data of proteins’ enzymatic hydrolysis of pea seeds with the enzyme preparation Protosubtilin was obtained. The proteins’ mixture of pea seeds was obtained by grinding biomass of pea seeds and by fractionating into an aqueous solution. Three isolated fractions containing predominantly protein substances were dried. Then the protein fraction was mixed with the enzyme complex Protosubtilin and subjected to mechanical activation on a roller mill PM-20 (5.5 kW). A mixture of low molecular weight products that contained only low molecular weight peptides and free amino acids was obtained with the subsequent enzymatic hydrolysis with Protosubtilin of the formed mechanocomposite. Also essential amino acids were obtained in the hydrolysate. Degradation of pea proteins was much deeper and more effective if the mechanocomposite was with the enzymes than without it.

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