

On the potential of lupin protein concentrate made by enzymatic hydrolysis of carbohydrates in dairy-like applications

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Abstract. The aim of this research was to study the parameters of obtaining lupin concentrates by enzymatic hydrolysis of wholegrain lupin flour and application of these concentrates in the technology of high-protein dairy-like products. The following enzymes preparations were used: Celluclast BG, Cellulaza 100, Pentopan Mono BG and α -amylase. The usage of Cellulaza 100 and Pentopan Mono BG showed the highest protein amounts in the lupin concentrates under the test conditions. Three factors were studied to take effect on crude protein content in the product: temperature in the range 50–60°C; cellulase dosage between 0.54–1.62 units g⁻¹; ratio water: flour – 10 : 1, 15 : 1, 20 : 1. Optimum hydrolysis conditions for Cellulaza 100 were temperature of 55°C; ratio water: flour of 15 : 1; cellulase dosage of 1.08 units g⁻¹. Crude protein content in the final product increased on 12% compared with the original flour and on 8–9% compared to the lupin concentrate obtained without enzymes. Hydrolysis by multienzymatic compositions was tried as an alternative way of increasing the efficiency of the process. However hydrolysis by multienzymatic compositions was not yet found so efficient as hydrolysis by pure Cellulaza 100. The lupin protein concentrate was dispersed in water and mixed with skimmed milk to have total product protein content about 5%. The mixture was fermented by yogurt starter culture; consumer properties of final products were investigated. Fermented products supplement the diet with vegetable proteins, fats, carbohydrates and fiber, which have high biological value.

Key words: lupin protein concentrates, lupin whey, multienzymatic compositions, fermented products, analogues of dairy products.

INTRODUCTION

Limitation of resources of food, caused by both environmental and demographic factors, makes the scientists and manufacturers looking for the new ways to meet the needs in essential food nutrients. The main interest in lupin for foods is related to its high content of protein which is considered as a good source of lysine (El-Adawy et al., 2001). Typically, lupin seeds have a crude protein content of 31–42%, which is higher than the content of most other grain legumes (Pollard et al., 2002). Lupin already has many human consumption applications, such as bread making, pasta products, sausage substitutes, egg and milk replacers (Xu & Mohamed, 2003; Xu et al., 2006). Sweet lupin diet helps lowering whey cholesterol level (Chango et al., 1998). Whole lupin flour-enriched foods as well as isolated lupin protein and fiber fractions appear to have a cardioprotective action (Belski, 2012).

Yet the application of lupin products in food is limited, largely due to their ‘green’ and ‘bean-like’ flavor and antinutritional substances. Fermentation of lupin

protein extracts using several lactic acid bacteria was conducted to reduce off-flavour formation in stored samples (Schindler et al., 2011). The main antinutritional substances in lupin are various alkaloids of the quinolizidine group. Hexane-defatted flakes of lupin (*Lupinus rautabilis*) were extracted under various conditions with alcohols to remove alkaloids (Blaicher et al., 1981). Production of protein isolates can overcome this problem too. Alkaloids are water-soluble and would be removed during preparation of the isolates (Lqari et al., 2001). The usage of some organic solvents improves lupin protein isolates quality (Bader et al., 2011).

Protein concentrates are considered to have greater than 50% protein and they are usually native flour (dehulled kernels) from which the carbohydrates (free sugars and oligosaccharides) and other soluble materials have been removed. Australian scientists have proposed the method of obtaining the protein preparation from lupin grains, which includes selection, treatment and fractionation of grains, their fragmentation, extraction of the protein, precipitation and drying (Sipsas, 2003). Muranyi et al. (2013) have studied two important techniques of protein isolation: the alkaline extraction with subsequent isoelectric precipitation and the salt-induced extraction followed by dilutive precipitation.

Plant proteins isolates and concentrates are used in the production of meat products, analogues of dairy products and combined foods. Lupin seeds and lupin seed protein isolates were used in the manufacture of fermented sausages (Papavergou et al., 1999). Soybeans are used as raw material to obtain soy yoghurt. It is hypoallergenic, so such products can be used in the diet of people suffering from milk protein intolerance (Osman & Razig, 2010; Vij et al., 2011). Soy-coconut yogurts were also studied (Kolapo & Olubamiwa, 2012). The milk-like product from *Lupinus campestris* was obtained by using an alkaline thermal treatment (Jimenez-Martinez et al., 2003).

Usage of enzymes is an alternative removal antinutritional substances method. Microbial hydrolytic enzymes are able to destroy many antinutritional components in plant raw material, which perform linking and protective function, such as phytin, cellulose, hemicellulose and lignin, and others (Ferket & Middleton, 1998). The improvement of carbohydrate extractability due to hydrolysis of polysaccharides allows producing a final product with higher protein content.

The most widespread, commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *Trichoderma reesei* (Olsson & Ahring, 2007). The commercial enzyme preparation 'Celluclast' is a multiactive carbohydrase for degradation of cellulose, cellobiose and higher polymers of glucose that could be used for improving malt quality (Grujic, 1998). Usage of Celluclast 1.5L for pectin extraction increases the pectin yield (Yuliarti et al., 2011). The degradation of biomaterial by cellulase is accompanied by the release of substrates for the action of other enzymes, particularly for xylanase and mannanase. The glykuronoksilan and mannan are rapidly decomposed in a system with cellulase (Viikari et al., 1994). The application of arabinofuranosidase with xylanase leads to complete removal of xylan (Makkonen & Nakas, 2005).

The aim of this research was to study the parameters of obtaining lupin concentrates by enzymatic hydrolysis of wholegrain lupin flour and application of these concentrates in the technology of high-protein dairy-like products.

MATERIALS AND METHODS

Materials

Lupinus angustifolius wholegrain flour was provided by the All-Russian Scientific Research Institute of Lupin, Bryansk, with a crude protein content of 46%, crude fat – 7.1%, fiber – 4.0%.

Selection of enzyme preparations was carried out on the basis of carbohydrate composition of lupin seeds, which includes fiber, small share of starch (about 4%), hemicelluloses and pectin (in total 10%) (Kupcov & Takunov, 2006).

The following enzymes preparations were used:

- Celluclast BG – cellulase preparation made by submerged fermentation of the selected strain of fungus *Trichoderma reesei*, containing 3,500 endoglucanase units gram⁻¹. Preparation was provided by Novozymes, Denmark;
- Cellulaza 100 – cytolytic complex enzyme preparation derived from a mixed culture of fungi *Aspergillus foetidus* and *Trichoderma viride*, containing 540 cellulase units gram⁻¹, Sibbiofarm, Russia;
- Pentopan Mono BG – xylanase preparation from fungi *Aspergillus oryzae*, containing 2,500 fungal xylanase units gram⁻¹, Novozymes, Denmark;
- α -amylase, containing 950 fungal amylase units gram⁻¹. Preparation was provided by State scientific institution All-Russia Scientific Research Institute of Fats of Russian Academy of Agricultural Sciences, Russia.

The yogurt starter culture of *Streptococcus salivarius ssp. thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* were provided by the State scientific institution All-Russia Scientific Research Institute of Fats of Russian Academy of Agricultural Sciences, Russia.

Preparation of the lupin concentrate

The used method is close to the one for obtaining the soy concentrate in the acidic medium (Sair, 1959). The full-fat lupin flour was diluted with water in a ratio of 1 : 10. The resulting solution was adjusted to pH 4.5 by adding 5% HCl to achieve protein isoelectric point. The following process was carried out in a thermostatic vessel with a magnetic stirrer (Khalil et al., 2006). Different variants of enzyme preparations were added after the suspension reached a temperature of 50°C. The mixture was maintained for 40 min.

The mechanical separation of phases was performed by centrifugation at 4000 × g for 30 min. The sediment obtained after centrifugation was the final product (the lupin concentrate). The sediment contained protein and fat fractions as the flour used had a fat content of 7.1%.

The sediment protein content was analyzed after centrifugation. Total content of protein, water-soluble carbohydrates and their component composition in the lupin whey (supernatant) were analyzed.

Preparation of the fermented products

For making the non-dairy vegetable yoghurt analog lupin protein concentrate was dissolved in water to a dry matter content of 10%. The dispersion was neutralized with alkali to pH 6.8–6.9, homogenized at 60–65°C, pasteurized at 92°C for 3 min and

cooled to a temperature of fermentation. Sucrose was added in an amount of 1% to increase the quantity of nutrients for a starter culture. Fermentation was carried out to achieve the required pH values of 3.8–4.2.

The combined dairy vegetable product was made by adding skim milk instead of sucrose to the pasteurized dispersion in ratio of dairy and lupin proteins 50 : 50. Total protein content of the mixture was about 5%. Fermentation was held to pH 4.4–4.5.

For all products the yogurt starter culture was used in an amount of 5% of the system mass. The temperature of fermentation was $42 \pm 2^\circ\text{C}$.

Measurements

Water content in the lupin protein concentrate was determined by the gravimetric method (AOAC, 1998). The content of crude protein was determined by Kjeldahl method on automated analyzer Kjeltec Auto (Tecator, Sweden) according to standard protocol of manufacturer. Crude protein content was estimated using a conversion factor 6.25 from total nitrogen.

The content of crude fat was determined by the Soxhlet method on automated analyzer SER 148 (VELP Scientifica, Italy) according to standard protocol of manufacturer. The ceramic fiber filter method was used to determine the crude fiber (AOAC, 1980).

Analysis of the total content of water-soluble carbohydrates was conducted by Bertrand method (Bertrand & Thomas, 1910). Changes in pH were measured with Orion 920A pH-meter (Russia).

Component analysis of mono- and disaccharides was conducted by HPLC ‘Stayer’ (Akvilon, Russia) with refractometric detector, the column ‘Luna NH₂ 5μ’, (Phenomenex, USA). The mobile phase consisted of acetonitrile and water in volume ratio of 77 : 23. ‘Stayer’ HPLC system with spectrophotometric detector and column ‘Luna C18’ (Phenomenex, USA) was used for analysis of the organic acids in whey, which was obtained by centrifugation of the fermented products samples at $4,000 \times g$ for 30 min. Solution of 0.1% orthophosphoric acid in distilled water was used as a mobile phase.

Statistical evaluation of the data

All experiments were performed with at least three replicates; data was processed by methods of mathematical statistics at theoretical frequency 0.95. Statistical processing of data was carried out using computer programs Microsoft Office Excel 2010 and Mathcad 15.0.

RESULTS AND DISCUSSION

Effect of the enzyme preparations on the lupin concentrate

Enzymes with different substrate specificities were tried for the hydrolysis of lupin flour polysaccharides. Bioconversion efficiency was evaluated by the content of crude protein in the lupin concentrate and in the lupin whey (Table 1). The data was compared with the results for the negative control sample (the lupin concentrate obtained without enzymes).

Part of the water-soluble protein fraction is transferred to the whey during the lupin concentrate making; despite the system pH value of 4.5 is near the isoelectric

point. On average lupin concentrate loses 19% of protein with the lupin whey according to the mass balance. The usage of Cellulaza 100 and xylanase preparation leads to the highest protein amounts in the lupin concentrates under the test conditions. Cellulaza 100 was used for further optimization of the hydrolysis conditions.

Table 1. Crude protein and water content in the products of the lupin concentrate making, %

The name of the enzyme	Concentrate protein, on a dry basis	Concentrate water	Whey protein, on a dry basis
Control sample	50.4 ± 1.3	75.2 ± 1.1	28.0 ± 1.0
Cellulaza 100	53.3 ± 1.1	77.9 ± 0.5	34.0 ± 1.5
Celluclast BG	50.0 ± 1.1	79.2 ± 0.6	30.4 ± 1.0
Pentopan Mono	53.0 ± 1.2	78.6 ± 0.9	34.1 ± 1.4
α-amylase	48.2 ± 1.1	71.6 ± 0.4	29.4 ± 1.2

Optimization of the hydrolysis conditions

Optimum hydrolysis conditions were necessary for increasing the efficiency of the process. Three factors were studied to take effect on crude protein content in the product (Y, % to dry substance): temperature in the range of 50–60°C (Z₁); cellulase dosage of 0.54–1.62 u g⁻¹ (Z₂); ratio water: flour – 10 : 1, 15 : 1, 20 : 1 (Z₃). The mixture was exposed to the hydrolysis for 40 min.

Optimal process parameters were obtained by means of rotatable plan of the second order and regression equation coefficients were found. The resulting response surfaces have the form of an elliptic paraboloid (Fig. 1).

The significance of the regression equation coefficients was determined by the Student's criterion. The adequacy of the regression equation was estimated by the Fisher test.

Optimum hydrolysis conditions for Cellulaza 100 were temperature of 55°C; ratio water: flour of 15 : 1; cellulase dosage of 1.08 units g⁻¹. Crude protein content in the final product under these conditions was 59.3 ± 1.1% on a dry basis. Crude protein content increased on 12–13% compared with the initial flour and on 8–9% compared to the lupin concentrate obtained without enzymes.

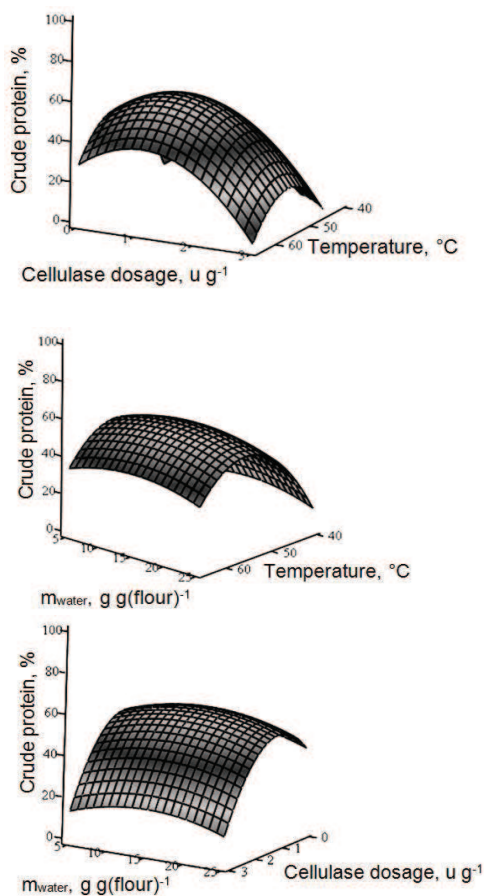


Figure 1. Dependence of crude protein content in the lupin concentrate on temperature, ratio water: flour and cellulase dosage.

Hydrolysis by multienzymatic compositions

Hydrolysis by multienzymatic compositions was tried as an alternative way of increasing the efficiency of the process. The degradation of non-starchy polysaccharides was conducted in the presence of Cellulaza 100 or Celluclast BG. An attempt was made to improve Cellulaza 100 hydrolysis efficiency with help of α -amylase. The synergetic effect is known between xylanase and cellulase. These enzymes act on cellulose, xylan and other hemicelluloses of lupin flour (Jeffries, 1996). So there was an attempt to raise the protein yield of the concentrates made with Celluclast BG with help of the xylanase preparation Pentopan Mono.

The compositions of the ferments included cellulases in the optimal dosage of 1.08 u g^{-1} and other enzymes in recommended or higher dosages. The ratio of enzymes in the compositions was calculated according to their declared activity (Table 2). Hydrolysis was carried out in the previously found optimal conditions for Cellulaza 100.

Table 2. Effect of multienzymatic compositions on the content of crude protein, fat and fiber in the lupin concentrates

No	Multienzymatic composition	Content in % on a dry basis		
		crude protein	crude fat	crude fiber
1	$1.08 \pm 0.02 \text{ u g}^{-1}$ 'Cellulaza 100' & $0.7 \pm 0.2 \text{ u g}^{-1}$ α -amylase	54.9 ± 1.4	10 ± 1	5 ± 1
2	$1.08 \pm 0.02 \text{ u g}^{-1}$ 'Celluclast BG' & $5 \pm 1 \text{ u g}^{-1}$ 'Pentopan Mono'	56.0 ± 1.1	10 ± 1	5 ± 1
3	$1.08 \pm 0.02 \text{ u g}^{-1}$ 'Celluclast BG' & $25 \pm 1 \text{ u g}^{-1}$ 'Pentopan Mono'	51.9 ± 1.3	9 ± 1	5 ± 1

About 20% of cellulose was subjected to bioconversion to soluble carbohydrates according to the material balance. Multienzymatic composition based on cellulase $1.08 \pm 0.02 \text{ u g}^{-1}$ and xylanase $5 \pm 1 \text{ u g}^{-1}$ showed highest protein yeild results under the used conditions. However hydrolysis by multienzymatic compositions was not yet found so efficient as hydrolysis by pure Cellulaza 100. Moreover, increased content of Pentopan Mono resulted in reduced crude protein content in the lupin concentrate of the third sample. This may be due to the inhibition of the action of one enzyme preparation by hydrolysis products of other one. This issue requires further study.

Transition of carbohydrate and protein fractions in the lupin whey

The lupin whey of samples after hydrolysis with $1.08 \pm 0.02 \text{ u g}^{-1}$ 'Celluclast BG' & $5 \pm 1 \text{ u g}^{-1}$ 'Pentopan Mono' was analyzed as they showed highest protein yeild results under the used conditions for multienzimatic compositions. The resulting lupin whey contains some extractive substances from lupin seeds (organic acids, soluble carbohydrates and vitamins, other biologically active substances), molecular nitrogen compounds (amino acids, peptides, albumin fraction of proteins) and lipids, which were released from the initial substrate in the process of hydrolytic destruction of cellular structures (Table 3). The initial lupin flour and the resulting lupin whey had a dry substance content of 90% and 2%, respectively.

Table 3. Crude protein, mono- and disaccharides percentage of the initial lupin flour and resulting lupin whey

	% to dry substance	
	flour	whey
Crude protein	46.3 ± 1.1	30.0 ± 1.4
Mono- and disaccharides	3.4 ± 0.5	25.0 ± 0.3

The balance of material is shown in equation 1:

$$M_{flour} \cdot S_{flour} \cdot C_{flour} = M_{whey} \cdot S_{whey} \cdot C_{whey}, \text{ or } 100 \cdot 0,90 \cdot 0,034 \rightarrow 1280 \cdot 0,02 \cdot 0,25 \quad (1)$$

where: *M* – mass of the product (g); *S* – solids (dry substance), share units; *C* – carbohydrates, share units.

Total content of mono- and disaccharides in the lupin whey according to the material balance has increased in 2 times in comparison with their content in the initial flour.

Mass fraction of total sugar (to invert sugar) in the whey was 0.5% (25% to dry substance). Non-starchy polysaccharides hydrolysis increases the concentration of soluble disaccharides (in particular, sucrose) and monosaccharides in the whey up to 17% and 3% to dry substance, respectively. High performance liquid chromatography of the lupin whey showed that the ratio sucrose: glucose: fructose is 10 : 1 : 1.

Transition of protein fraction to the whey was 18% of the amount contained in the flour. That corresponds to the data obtained for the samples hydrolysed with Cellulaza 100.

Thus, 20% of the hydrolyzed cellulose and some quantity of the hydrolyzed xylan increase yields of mono- and disaccharides in the whey twice but accompanied by the protein loss of 18%.

Application of the lupin concentrates in dairy-like products

Three fermented products were studied: the non-dairy vegetable yoghurt analog, the combined dairy vegetable product and the control product based on a skim cow milk. All received clots had homogeneous consistency with minor release of whey, sour-sweet taste and fruity smell. The usage of vegetable raw material in the combined product leads to dynamic reduction of pH in the first 6 h of fermentation comparing to the control product (Fig. 2). This gives the possibility to reduce the fermentation time.

The initial pH value of the lupin dispersion was 1.2 units lower than in the control sample. Buffer capacity of this dispersion was lower compared to the skim milk because of its plant origin.

Composition of organic acids in the whey obtained by centrifugation of the products samples characterizes the biochemical process of fermentation (Table 4).

Increase in the acidity of the combined product in comparison with the control product mainly depends on the malic acid production. This is due to the formation of by-products of homofermentative lactic acid fermentation, in particular malic acid. Organic acids are produced in varying degrees in the process of fermentation and storage of yoghurts (Fernandez-Garcia & McGregor, 1994). The course of lactic acid

fermentation can be different depending on environmental conditions. Malic, propionic and some other organic acids are formed on the Embden-Meyerhof-Parnas pathway.

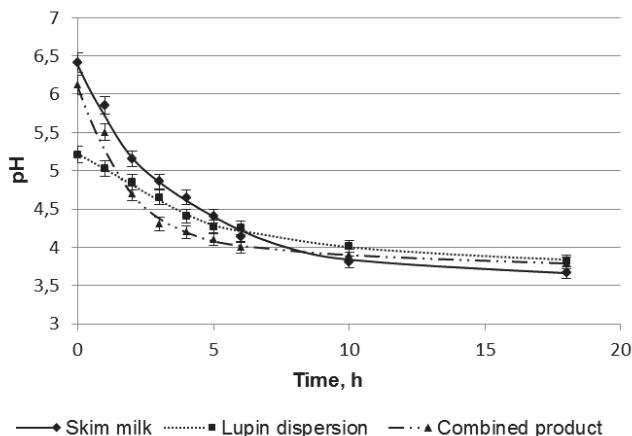


Figure 2. Dependence of active acidity on the fermentation time and the product base.

Table 4. Composition of organic acids in the whey samples

Whey sample	Content of lactic acid, g dm ⁻³	Content of malic acid, g dm ⁻³	The amount of lactic and malic acids, g dm ⁻³
Vegetable analog	3.93 ± 0.19	0.17 ± 0.03	4.1
Combined product	6.7 ± 1.3	1.9 ± 0.4	8.6
Control product	6.9 ± 1.2	–	6.9

Table 5. Macronutrients and energy value of the fermented products

Parameter	Vegetable yoghurt analog	Combined product	Control product
Protein, %	4.8 ± 0.2	4.8 ± 0.2	5.0
Fat, %	0.8 ± 0.1	0.6 ± 0.2	1.5
Fiber, %	0.4 ± 0.1	0.2 ± 0.1	–
Energy value, kcal (per 100 g)	61.3	67.4	75.1

The fermented products supplement the diet with vegetable proteins, fats, carbohydrates and fiber, which are necessary for the proper functioning of the gastrointestinal tract and have high biological value (Table 5).

The vegetable yoghurt analog and the combined product can be classified as low-calorie dietary foods. Fiber in the composition of these products would have a positive effect on intestinal motility. Sensory analysis of the products with hydrolyzed lupine flour revealed no ‘green’ and ‘bean-like’ flavors.

CONCLUSIONS

Enzymatic treatment of the lupin flour is an effective method for increasing the protein content in the concentrate production. Hydrolysis of the lupin flour with Cellulaza 100 increased crude protein content in the lupin concentrate on 12–13% compared with the initial flour and on 8–9% compared to the lupin concentrate obtained without enzymes. This led to crude protein content in the concentrate of $59.3 \pm 1.1\%$ on a dry basis. Average protein loss from moving in the whey amounted 18–19%. Improving efficiency of hydrolysis using a combination of enzyme preparations still not yielded the expected results, and requires further study.

The formation of malic acid during lactic acid fermentation of products containing lupin concentrates gives the possibility to reduce the fermentation time. The received products have homogeneous consistency, sour-sweet taste and fruity smell without negative flavor. They can be classified as low-calorie dietary foods with energy value less than 70 kcal per 100 g.

The proposed ways of making fermented products allow reducing the cost of vegetable yoghurt analogue in 1.7 times, the combined product in 1.3 times compared with the traditional product in Russia. Implementation of these products can help diversifying production due to the necessity of compensation of technological risks and of using new food sources.

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