Article

Bean Spread Enriched with Spelt as a Novel Source of Bioactive Peptides with Potential Anti-Metabolic Syndrome Properties

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Abstract: Bean spreads enriched with 10, 20, or 30% of spelt grain were analyzed in the study as a novel product with high nutraceutical potential. The spreads were hydrolyzed in vitro in gastrointestinal conditions. The highest peptide content was noted after the last step of hydrolysis in spread enriched with 10% of spelt grain (1.64 mg/mL). The fraction with molecular mass < 3.0 kDa obtained from this hydrolyzate was also characterized by the highest peptide content (1.50 mg/mL) and the highest antioxidant properties. The highest value of IC_{50} against $ABTS^{\bullet+}$ was 0.078 mg/mL. The highest value of the Fe^{2+} chelating activity was 0.056 mg/mL. Moreover, the trials show inhibitory activity against enzymes involved in the development of metabolic syndrome (with IC_{50} values of 0.072 mg/mL for α-amylase inhibition, 0.028 mg/mL for α-glucosidase, and 0.059 mg/mL ACE—angiotensin-converting enzyme). The fraction with the highest properties was separated using Sephadex G10 and three fractions were obtained. The first and third fractions were characterized by the highest properties. These peptide fractions were identified using the LC–MS/MS technique. The following amino acid sequences were obtained from bean (*Phaseolus vulgaris*) protein: LPIESKWY, FALVAPVGSEPKA, NSILPIESKPWY, RLTDDTEDSMGRA, and KKVELEEEVDDWV, and those isolated from spelt protein had sequences FPQPQPFQ, QPQQPQQPFPQP, WPQQPQQPFPQPQQ, QSQQPQQPFPQPQQ, and QFQPQQPQQPFPQP. The study indicates that the bean spread enriched with 10 % of spelt grain may be used as a new product with special nutritional properties.

Keywords: bioactive peptides; in vitro digestion; anti-metabolic syndrome properties; bean seeds; spelt grain; metabolic syndrome

1. Introduction

Nowadays, there is a growing debate about the health-promoting properties of food products, the bioactive ingredients contained therein, and dietary models that have no negative impact on the environment and do not promote climate change [1,2]. As a result, consumers' health awareness is growing, as they are increasingly inclined to choose food products that are environmentally friendly and rich in nutrients [3]. Eating habits that help to maintain health and take care of environmental aspects primarily include reduction in the consumption of meat products and an increase in the intake of plant products, including legumes. Livestock are thought to be responsible for producing 18% of greenhouse gases, where beef and milk production is estimated to account for 41% and 20% of greenhouse gas emissions, respectively [2]. Consumption of meatless meals based on plants, including legumes, has been shown to have beneficial effects on human health [4–7]. In addition, meatless meals contribute to an over 40% reduction in factors that have a negative impact on the environment (carbon footprint, water consumption, environmental pollution, or resource consumption) [8].

Food is a source of not only energy and macronutrients but also bioactive peptides, which have a wide variety of health-promoting properties [9]. Bioactive peptides are small protein fragments that are inactive in the precursor molecule. To become active and be

able to exert beneficial effects on the human organism, they must be released through enzymatic proteolysis (digestion process) or food processing (fermentation, cooking, ripening) [10]. Many studies show that common beans are rich in bioactive peptides exhibiting antihypertensive, antioxidant, antidiabetic [11], anticancer [12], and anti-obesity effects [13]. However, despite their high protein content, legumes are characterized by an incomplete amino acid composition. Therefore, it seems reasonable to combine legume seeds and cereal grains to obtain a product with the best possible amino acid profile. In the case of legumes, methionine and cysteine, which are abundant in cereal grains, are the limiting amino acids. In turn, lysine, which is the limiting amino acid in grains, is effectively supplemented by legumes [14].

Metabolic syndrome is defined as a syndrome consisting of the co-occurrence of at least three out of five cardio-metabolic abnormalities. These abnormalities include abdominal obesity, hyperglycemia, hypertriglyceridemia, low HDL-cholesterol, and hypertension [15]. Metabolic syndrome is a widespread disease among people living in developed and developing countries and is increasingly affecting children [16]. One of the direct methods of treatment of the component diseases of the metabolic syndrome is long-term pharmacotherapy (including drugs whose action is based on inhibition of certain enzymes [17,18]), whose prolonged use may lead to so-called drug toxicity, causing side effects that have a negative impact on the patient's health [10]. One of the methods supporting the metabolic syndrome pharmacotherapy, which brings positive health effects, is an appropriately matched diet therapy [19]. It involves dietary inclusion of products characterized by high content of inhibitors of the activity of enzymes involved in the pathogenesis of metabolic syndrome diseases. One such product is legumes, including beans [11,13]. The use of a plant-based diet in the dietary management of metabolic syndrome may increase the effectiveness of the diet therapy and contribute to limitation of the amount of applied pharmacological agents owing to the proven health-promoting properties of plants. Plant-based diet therapy has been reported to contribute to a reduction in the risk of the development and/or progression of type 2 diabetes, obesity, or cardiovascular diseases, and a reduction in mortality associated with these diseases [11–13,19].

The aim of this study is to characterize biologically active peptide fractions isolated from bean (*Phaseolus vulgaris*) and spelt grain (cv. Oberkulmer Rotkorn) spreads and determine their impact on the pro-health potential, which can be used in the diet therapy of metabolic syndrome diseases. The spreads made of bean seeds and cereal grains analyzed in this study are a rich source of bioactive compounds and a basis of a plant-based diet, which has documented pro-health effects and is currently one of the most popular diets among consumers.

2. Materials and Methods

2.1. Material

Plant material: bean seeds were purchased in PNOS Ożarów Mazowiecki, Poland, and spelt grains were purchased in Orvita, Swarzędz, Poland. The following chemical compounds were used for the measurements: pepsin from porcine gastric mucosa (250 U mg^{-1}) (Sigma Aldrich, St. Louis, MO, USA), α-amylase from hog pancreas (50 U mg^{-1}) Sigma Aldrich (St. Louis, MO, USA), α-Glucosidase from *Saccharomyces cerevisiae* (100 U mg^{-1}) Sigma Aldrich (St. Louis, MO, USA), ACE obtained from pig lungs [20], o-phtaldialdehyde (OPA), 2-Mercaptoethanol, pancreatin from porcine pancreas Sigma Aldrich (St. Louis, MO, USA), bile extract Sigma Aldrich (St. Louis, MO, USA), 2,4,6-trinitrosalicylic acid (TNBS) Sigma Aldrich (St. Louis, MO, USA), 3,5-dinitrosalicylic acid (DNS), starch solution, sucrose solution, hippuryl-L-histidyl-L-leucine (HHL) Sigma Aldrich (St. Louis, MO, USA), 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) Sigma Aldrich (St. Louis, MO, USA), and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) Sigma Aldrich (St. Louis, MO, USA).

2.2. Preparation of the Spread

A total of 100 g of dry bean seeds and 50 g of dry spelt seeds were weighed. The bean seeds were soaked in distilled water for 12 h. Then, the seeds were drained and boiled in water at 100 °C for 90 min. The cooked beans were drained and cooled. The spelt grains were also cooked in water at 100 °C until soft. Next, three types of spread were prepared. They differed in the percentage of grain enrichment: 10, 20, or 30% of spelt grains replaced the beans (10S, 20S, and 30S, respectively). The control sample was prepared using only cooked beans. Then, the spread was thoroughly blended, transferred to containers, and stored at −18 °C until use.

2.3. In Vitro Hydrolysis

In vitro digestion of the spreads in simulated gastrointestinal conditions was carried out according to the method described by Minekus et al. [21]. In this process, 5 g of each type of spread was weighed and transferred into 50 mL centrifuge tubes. After the in vitro digestion, the samples were frozen to inactivate the enzymes. The samples were then unfrozen and centrifuged (45 min, 5500 RMP) and the supernatant fluid was collected. The resulting solution was collected and stored at −18 °C until use.

Preparation of the Fraction with Molecular Mass < 3.0 kDa

The peptide fraction with a molecular weight < 3.0 kDa was obtained from hydrolyzates using a Ultra-15 Centrifugal Filter Units, Merck Millipore (Membrane NMWL, 3 kDa). The hydrolyzates were centrifuged at 5500 RPM at 4 °C for 1 h. The obtained fractions were collected and stored at −18 °C until use.

2.4. Amino Acid Composition Assay

The amino acid profile in the control sample and samples with the highest biological properties was determined, paying particular attention to the content of sulfur-containing amino acids, which are deficient in beans. The analysis was performed by the Central Research Laboratory, Lublin, Poland. Spread samples that had not previously undergone in vitro digestion were submitted to an external accredited laboratory. Acid hydrolysis of proteins for determination of the amino acid composition without oxidation was performed according to the Davies and Thomas method [22]. Hydrolysis of proteins to separate sulfur amino acids was performed according to Schram et al. [23]. For the determination of tryptophan, the samples were subjected to alkaline hydrolysis with barium hydroxide according to the method described by Sławiński and Tyczkowska [24].

2.5. Peptide Content Assay

Peptide content was determined using the method described by Adler-Nissen [25], with L-leucine as a standard. It was determined in the samples during the hydrolysis process and in the fractionated samples with a molecular weight < 3 kDa.

2.6. Nutraceutical Potential of Hydrolyzates and Fractions with Molecular Mass < 3.0 kDa
2.6.1. Inhibition of Enzymes Involved in the Pathogenesis of Metabolic Syndrome
α-Amylase Inhibitory Activity Assay

α-Amylase inhibitory activity (αAI) was determined with the method described by Świeca et al. [26]. All analyses were performed in triplicate. The α-amylase inhibition ability was calculated with the following formula and defined as IC_{50} (IC_{50} is defined as the concentration of the inhibitor that inhibits 50% of the function of the tested compounds):

$$\alpha\text{-Amylase inhibition (\%)} = (1 - ((A_s - A_p)/A_e)) \times 100\%$$

where A_s is the value of absorbance of samples with the tested inhibitor and α-amylase; A_p is the value of absorbance of samples with the tested inhibitor and buffer but without

α-amylase and starch solution; A_e is the value of absorbance of samples with α-amylase but without the tested inhibitor.

ACE Inhibitory Activity Assay

The angiotensin-converting enzyme (ACE) was prepared according to the method described by Jakubczyk and Baraniak [27]. ACE inhibitory activity (ACEI) was determined with the method described by Chang et al. [28] with a slight modification, as in Jakubczyk et al. [29].

All analyses were performed in triplicate. The ability to perform ACE inhibition was calculated using the formula below and IC_{50} value was determined:

$$\text{ACE inhibition (\%)} = (1 - ((A_1 - A_2)/A_3)) \times 100\%$$

where A_1 is the value of absorbance of samples with ACE and the tested inhibitor; A_2 is the value of absorbance of samples with the tested inhibitor but without ACE; A_3 is the value of absorbance of samples with ACE but without the tested inhibitor.

α-Glucosidase Inhibitory Activity Assay

α-Glucosidase inhibitory activity (αGI) was determined with the following method using a sucrose solution as a substrate: 5 μL of α-glucosidase (1 U/mL), 10 μL of sucrose solution (100 mg mL^{-1}), and 5 μL of the sample were added to 10 μL of 100 mM phosphate buffer pH = 6.8. The mixture was incubated for 30 min at 37 $^\circ$C. Next, the reaction was stopped by adding 20 μL of a 1% solution of 3,5-dinitrosalicylic acid (DNS) and heating in boiling water for 10 min. Thereafter, 100 μL of distilled water was added. The final volume of the reaction mixture was 150 μL. Changes in absorbance at 540 nm were measured using a BioTek Microplate Reader (Winooski, VT, USA). The final results were compared with the activity of the same amount of the enzyme without the inhibitor. All analyses were performed in triplicate and IC_{50} value was determined.

2.6.2. Antioxidant Activities

Fe^{2+} Chelating Activity

The Fe^{2+} chelating activity was determined with the method described by Decker and Welch [30]. The absorbance was measured at 562 nm using a BioTek Microplate Reader. All analyses were performed in triplicate. The ability of the analyzed samples to chelate ferrous iron(II) was calculated with the following formula and IC_{50} value was determined:

$$\text{Scavenging (\%)} = (1 - (A_1/A_0)) \times 100\%$$

where A_1 is the value of absorbance of the analyzed sample; A_0 is the value of absorbance of the control.

Antiradical Activity (ABTS$^{\bullet+}$)

Antiradical activity against ABTS$^{\bullet+}$ was determined using the method described by Re et al. [31]. The absorbance was measured at 734 nm at time zero and after 10 min. An ABTS$^{\bullet+}$ solution was used as a blank. All analyses were performed in triplicate. The ability of the analyzed samples to neutralize ABTS$^{\bullet+}$ free radicals was calculated with the following formula and IC_{50} value was determined:

$$\text{Scavenging (\%)} = (1 - (A_s/A_c)) \times 100\%$$

where A_s is the value of absorbance of the analyzed sample; A_c is the value of absorbance of the control.

2.7. Separation and Identification of Peptides

2.7.1. Gel Filtration with the Use of Sephadex G-10

Fractions with the highest bioactive properties (spread with 10% of spelt added) were separated by gel filtration chromatography on Sephadex G10 (column: 1.5×30 cm; eluent: distilled water; flow rate: 0.8 mL/min). The absorbance of one milliliter fractions was monitored at 220 nm and collected to determine their bioactive properties.

2.7.2. Determination of the Structure and Composition of Amino Acids

The peptide fraction was analyzed with the LC–MS-MS/MS technique (liquid chromatography coupled to tandem mass spectrometry) using a Nano-Acquity (Waters) LC system and an Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). The analysis was performed by the Mass Spectrometry Laboratory in Warsaw, Poland. The procedure was presented in the study conducted by Jakubczyk et al. [20]

2.8. Assay of the Physicochemical Characteristics of Peptides

The physicochemical parameters of the peptides, i.e., molecular mass, isoelectric point, instability index, hydrophobicity, and grand average of hydropathicity (GRAVY), were calculated with the method described in a previous study [32]. The Boman index was calculated with the method described in http://www.pep-lab.info/dmpep (accessed on 1 February 2023).

2.9. Statistical Analysis

All tests were conducted in a completely randomized design in independent triplicates to confirm the reproducibility of the results. The report of the data was given as mean $\pm$ SD. Statistical analysis was performed using STATISTICA 13.3 for comparison of means using Tukey's test, which determined significant differences at the 95% confidential level ($p < 0.05$).

3. Results

*3.1. Peptide Content during the Hydrolysis Process*

The spreads were hydrolyzed in simulated gastrointestinal conditions using the method described by [21], and the peptide content was determined in all steps of hydrolysis (Figure 1). The results show that the addition of spelt does not increase the peptide content in the hydrolyzates of the spreads, compared to the control. After hydrolysis, the highest peptide content (1.72 mg mL^{-1}) was determined to be in the hydrolyzate of the control bean spread. The peptide content in the spread supplemented with 10% and 20% of spelt is lower than in the control (1.64 and 1.62 mg mL^{-1}, respectively). The differences between the two samples are not statistically significant. The lowest peptide content (1.57 mg mL^{-1}) after hydrolysis is exhibited by the hydrolyzate of the spread enriched with 30% of spelt. In comparison with the values of the other spreads and controls, this value is statistically significant.

3.2. Antioxidant Activity and Peptide Content in Hydrolyzates and Peptide Fractions with Molecular Mass < 3.0 kDa

After in vitro digestion, the hydrolyzates of all the samples were subjected to separation to peptide fractions < 3.0 kDa, and the antioxidant activity of these fractions and the hydrolyzates was assessed. Moreover, the peptide content was compared between the trials. The results show (Table 1) that all the samples demonstrate antioxidant activity against ABTS$^{\bullet+}$. The hydrolyzates show no statistically significant differences in their anti-radical activity against ABTS$^{\bullet+}$. No statistically significant differences in the values of antioxidant activity against ABTS$^{\bullet+}$ are observed between the peptide fractions < 3.0 kDa of the tested spreads either. Based on these results, it can be concluded that peptide fractions with a molecular weight < 3.0 kDa show higher ABTS$^{\bullet+}$ free radical neutralizing ability, as their IC$_{50}$ values are lower than those of the hydrolyzates. In the case of both the

hydrolyzates and the peptide fractions with a molecular weight < 3.0 kDa, the samples tested have higher IC_{50} values of activity against $ABTS^{\bullet+}$ radicals than the IC_{50} value of Trolox. These differences are statistically significant. The results also indicate Fe^{2+} chelating activity. The lowest IC_{50} value among the hydrolyzates in terms of ferrous iron(II) chelation (0.085 mg mL^{-1}) was determined to be for the spread supplemented with 20% of spelt. This value is statistically significant, compared with the control. Moreover, the spread enriched with 10% of spelt also exhibits high Fe^{2+} chelating activity (0.087 mg mL^{-1}), and this value is also statistically significant, compared with the control. In the peptide fraction < 3.0 kDa, both spreads with the 10% and 20% spelt addition have the lowest IC_{50} value (0.056 and 0.078 mg mL^{-1}, respectively) of Fe^{2+} chelating activity. The differences in the IC_{50} value between these samples and compared with the control are statistically significant. Table 1 also shows the peptide content in each of the samples. In the hydrolyzates, the highest peptide content (1.716 mg mL^{-1}) was determined to be in the control spread. The spread enriched with 30% of spelt has the lowest peptide content. In the peptide fraction < 3.0 kDa, the highest peptide content was determined to be in the spread supplemented with 10 and 20% of spelt (1.501 and 1.483 mg mL^{-1}, respectively). These values are statistically significantly different compared with the control. However, spread 10S is not statistically significantly different from spread 20S but is statistically significantly different compared with spread 30S. The lowest value (1.373 mg mL^{-1}) was determined to be in the control spread.
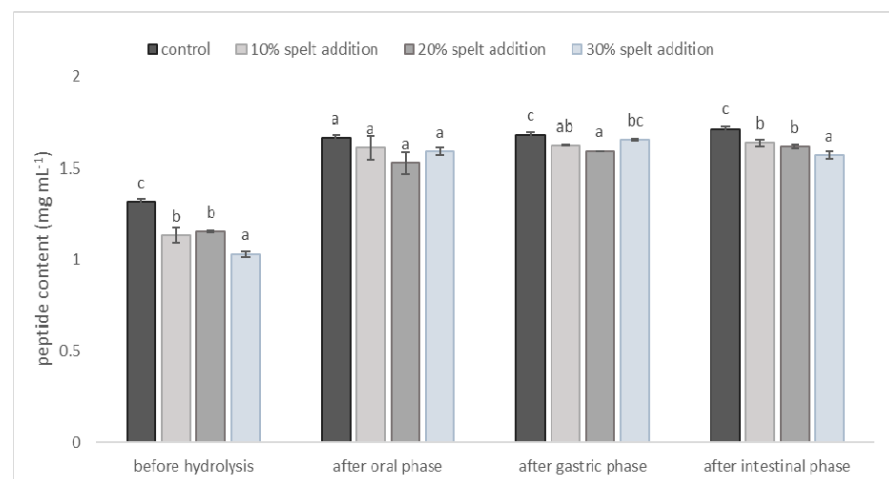
Figure 1. Peptide content in each step of hydrolysis. Different letters in the same step of hydrolysis indicate a significant difference (α = 0.05). Control: sample without spelt grain; 10 S: spread with 10% of spelt grains; 20 S: spread with 20% of spelt grains; 30 S: spread with 30% of spelt grains.

Table 1. Antioxidant activity of samples (IC_{50}) and peptide content (mg mL^{-1}).

Samples	Peptide Content (mg mL^{-1})	$ABTS^{\bullet+}$ (mg mL^{-1})	Fe^{2+} Chelation (mg mL^{-1})
	Hydrolyzates		
Control	1.716 ± 0.012 [a]	0.081 ± 0.003 [a]	0.241 ± 0.027 [a]
10 S	1.637 ± 0.019 [b]	0.084 ± 0.0008 [a]	0.087 ± 0.002 [c]
20 S	1.617 ± 0.010 [b]	0.080 ± 0.003 [a]	0.085 ± 0.003 [c]
30 S	1.568 ± 0.020 [c]	0.081 ± 0.003 [a]	0.162 ± 0.006 [b]
Trolox	-	0.0421 [b]	-
	Fraction with molecular mass < 3.0 kDa		
Control	1.373 ± 0.005 [c]	0.076 ± 0.0004 [a]	0.099 ± 0.004 [a]
10 S	1.501 ± 0.014 [a]	0.078 ± 0.0005 [a]	0.056 ± 0.006 [c]
20 S	1.483 ± 0.004 [ab]	0.080 ± 0.002 [a]	0.078 ± 0.003 [b]
30 S	1.463 ± 0.019 [b]	0.077 ± 0.002 [a]	0.103 ± 0.005 [a]
Trolox	-	0.0421 [b]	-

All values are mean ± standard deviation for triplicate experiments. Different letters at the same sample indicate a significant difference (α = 0.05). Control: sample without spelt grain; 10 S: spread with 10% of spelt grains; 20 S: spread with 20% of spelt grains; 30 S: spread with 30% of spelt grains.

3.3. Amino Acid Composition in the Spreads

Proteins from legumes have a lower digestible indispensable amino acid score (DI-ASS), i.e., a measure developed by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and the Food and Drug Administration (FDA), than milk and meat proteins, as they contain lower amounts of methionine. Cereals, on the other hand, are rich in this amino acid and poor in lysine and tryptophan, which can be found, e.g., in beans. In order to obtain complete protein in a plant-based or semi-vegetarian diet, these two ingredients should be combined with each other. Table 2 shows the amino acid content (mg/g product) of the control bean spread and the spread enriched with 10% of spelt, which has the best properties. The addition of spelt to the bean-based spread has a positive effect on the content of methionine sulfonate, i.e., it increases its content in the final product. The spelt and bean spread is also characterized by higher tryptophan, glutamine, and cysteic acid contents. In turn, the addition of spelt results in a decrease in the content of other amino acids in the final product.

Table 2. Content of individual amino acids (mg/g) in the control sample and the tested sample with the best analyzed properties.

Amino Acid	Control (mg/g)	10 S (mg/g)
Cysteic acid	1.30	1.45
Valine	4.40	3.89
Methionine sulphonate	1.75	2.00
Isoleucine	3.53	3.06
Leucine	7.01	6.20
Tyrosine	2.98	2.65
Phenylalanine	5.22	4.60
Histidine	2.39	2.13
Lysine	6.23	5.44
Arginine	5.02	4.53
Tryptophan	1.32	1.83
Aspartic acid	10.30	9.03
Threonine	4.42	3.75
Serine	5.23	4.74
Glutamine	11.8	11.9
Proline	3.00	2.54
Glycine	3.53	3.25
Alanine	3.87	3.50

Control: sample without spelt grain; 10 S: spread enriched with 10% of spelt grains.

3.4. Inhibitors of Enzymes Involved in the Pathogenesis of Metabolic Syndrome Determined in Hydrolyzates and Peptide Fractions < 3 kDa

The hydrolyzates and peptide fractions < 3.0 kDa were also tested to determine their inhibitory properties against enzymes involved in the development of type 2 diabetes. As shown in Table 3, none of the hydrolyzates or the control sample exhibit inhibitory activity against α-amylase. However, the peptide fractions < 3.0 kDa have inhibitory properties against this enzyme. The lowest IC_{50} value (0.072 mg mL^{-1}) is exhibited by the spread with the 10% spelt addition. The inhibitory properties against α-glucosidase were also determined at this stage of the study. Both the hydrolyzates and the peptide fractions < 3.0 kDa exhibit these properties. Among the hydrolyzates, the control spread has the lowest IC_{50} value (0.054 mg mL^{-1}). Among the peptide fractions < 3.0 kDa, the lowest value of α-glucosidase inhibitory activity was determined to be in the control and the spreads supplemented with 10% and 20% of spelt (0.033; 0.028, and 0.03 mg mL^{-1}, respectively). These values are not statistically significantly different compared to the control. Only the spreads supplemented with 10 and 30% of spelt show the ACE inhibitory property. Among the hydrolysates, the ACE inhibition values of these spreads show no statistically significant differences. However, in the case of peptide fractions < 3.0 kDa, the 10S spread has higher activity against ACE (0.059 mg mL^{-1}).
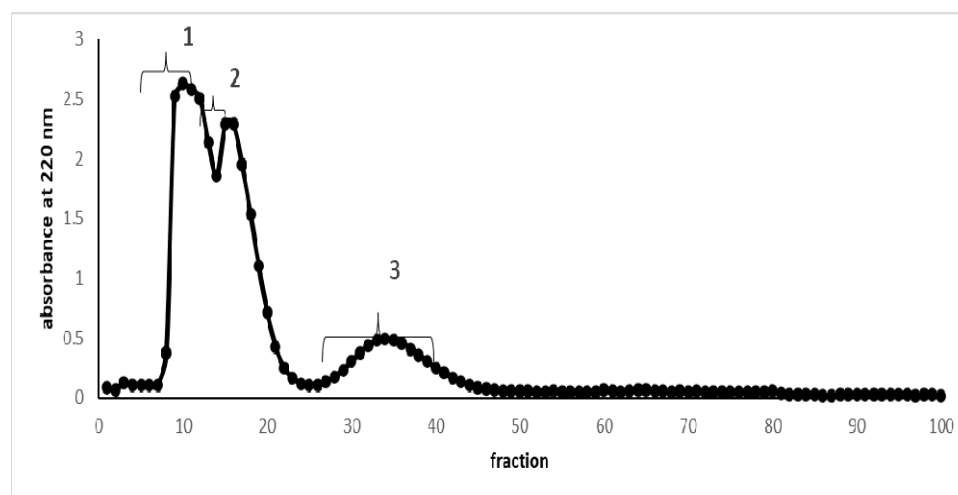
Table 3. Inhibition of enzymes involved in development of diabetes type 2 (IC_{50}) and the angiotensin-converting enzyme.

Samples	α-Amylase (mg mL^{-1})	α-Glucosidase (mg mL^{-1})	ACE (mg mL^{-1})
		Hydrolyzates	
Control	ND	0.054 ± 0.0 [b]	ND
10 S	ND	0.065 ± 0.002 [b]	0.434 ± 0.029 [a]
20 S	ND	0.086 ± 0.009 [a]	ND
30 S	ND	0.079 ± 0.003 [a]	0.397 ± 0.013 [a]
		Fractions < 3.0 kDa	
Control	0.240 ± 0.028 [a]	0.033 ± 0.004 [ab]	ND
10 S	0.072 ± 0.007 [c]	0.028 ± 0.003 [b]	0.059 ± 0.005 [b]
20 S	0.170 ± 0.017 [b]	0.03 ± 0.0007 [ab]	ND
30 S	0.179 ± 0.016 [b]	0.035 ± 0.001 [a]	0.253 ± 0.031 [a]

ND—not detected; all values are mean ± standard deviation for triplicate experiments; different letters indicate significant differences ($\alpha = 0.05$).

3.5. Peptide Profile

Based on the results obtained, one fraction of peptides with molecular mass < 3.0 kDa isolated from the spread with the 10% spelt addition was selected for further separation using Sephadex G-10. This spread shows similar anti-diabetic properties. As shown in Figure 2, three peptide fractions were separated from the < 3.0 kDa fraction for further analysis.

Figure 2. Sephadex G-10 peptide profile (fraction with molecular mass < 3 kDa obtained from spread enriched with 10% of spelt). 1–3—obtained peptide fraction.

3.6. Antioxidant Activity and Inhibitors of Enzymes Involved in the Development of Diabetes Type 2 in the Separated Peptide Fractions

The separation with Sephadex G-10 yielded three peptide fractions. All these fractions were tested for their antioxidant activity and inhibition activity against α-amylase and α-glucosidase. The results show (Table 4) that the lowest IC_{50} value of antioxidant activity against ABTS$^{\bullet+}$ is in fractions 1 and 2 (1.710 and 1.480 mg mL^{-1}, respectively). These values are not statistically significantly different between each other. Fraction 3 has the highest IC_{50} value (14.493 mg mL^{-1}) of antioxidant activity against ABTS$^{\bullet+}$. This value is statistically significantly different compared to the values determined for fraction 1 and 2. All the peptide fractions tested have higher IC_{50} values of activity against ABTS radicals than the IC_{50} value of Trolox. These differences are statistically significant. Table 4 also shows the Fe^{2+} chelating activity, which is exhibited by all fractions. The lowest IC_{50} value (0.326 mg mL^{-1}) is recorded in fraction 1. It is statistically significantly different compared to the other samples. The tested samples demonstrate anti-diabetic properties as well. The

highest inhibitory activity against α-amylase is shown in fractions 2 and 3 (IC_{50} value of 0.103 and 0.094 mg mL^{-1}, respectively). These values are statistically significant compared with fraction 1. Moreover, all fractions have inhibitory activity against α-glucosidase. No statistically significant differences are found between the samples. In addition, there is no inhibitory activity of the tested fractions against ACE.

Table 4. Antioxidant activity (IC_{50}) and inhibition of enzymes involved in development of diabetes type 2 (IC_{50}).

Samples	ABTS$^{\bullet+}$ (mg mL^{-1})	Fe^{2+} Chelation (mg mL^{-1})	α-Amylase (mg mL^{-1})	α-Glucosidase (mg mL^{-1})	ACE (mg mL^{-1})
Fraction 1	1.710 ± 0.119 [b]	0.326 ± 0.024 [c]	0.644 ± 0.108 [a]	0.051 ± 0.001 [a]	ND
Fraction 2	1.480 ± 0.038 [b]	3.278 ± 0.375 [a]	0.103 ± 0.004 [b]	0.056 ± 0.005 [a]	ND
Fraction 3	14.493 ± 1.386 [a]	1.342 ± 0.332 [b]	0.094 ± 0.005 [b]	0.055 ± 0.002 [a]	ND
Trolox	0.0421 [c]	-	-	-	-

ND—not detected; all values are mean ± standard deviation for triplicate experiments; different letters indicate significant differences (α = 0.05).

3.7. Identification of Peptide Sequences and Physicochemical Properties

Peptides from fractions 1 and 3 were analyzed using the LC–MS-MS/MS technique. Five peptides originating from *Phaseolus vulgaris* protein and five from *Triticum urartu* were identified (Table 5). The molecular mass of the peptides is in the range from 988.1 to 1733.88. Only two peptides have a net charge other than zero. The theoretical pI value for all identified peptides is higher than 3 or 6. An instability index less than 40 indicates that the peptide is stable under in vitro conditions. Only one peptide (KKVELEEEVDDWV) has an instability index indicating its stability in in vitro conditions. The value of this factor is 34.76. The peptides identified from bean protein are characterized by a high aliphatic index, which indicates their potential thermostability. According to the GRAVY index, almost all peptides except FALVAPVGSEPKA have a hydrophilic character. Moreover, the Boman index indicates that the peptides with the sequences RLTDDTEDSMGRA, KKVELEEEVDDWV, QPQQPQQPFPQP, and QSQQPQQPFPQPQQ have high binding potential, as their factor is higher than 2.48. Only three of the peptides have good water solubility (LPIESKWY, RLTDDTEDSMGRA, and KKVELEEEVDDWV).

Table 5. Characterization of the peptides.

Peptide Sequence	Abbreviation	Molecular Mass (g/mol)	Net Charge	Theoretical pI	Instability Index	Aliphatic Index	GRAVY Index	Boman Index	Water Solubility
α-amylase inhibitor-like protein from *Phaseolus vulgaris*
LPIESKWY	LY-8	1035.19	0	6.79	111.83	97.50	−0.463	0.4663	Good
FALVAPVGSEPKA	FA-13	1285.49	0	6.61	61.96	97.69	0.662	−0.5069	Poor
NSILPIESKPWY	NY-12	1446.64	0	6.43	85.25	97.50	−0.425	0.7375	Poor
RLTDDTEDSMGRA	RA-13	1466.53	−2	3.82	42.12	37.69	−1.392	4.7177	Good
KKVELEEEVDDWV	KV-13	1617.75	−4	3.69	34.76	96.92	−1.023	2.8008	Good
Gamma-hordein-1 from *Triticum urartu*
FPQPQPFQ	FQ-8	988.1	0	3.45	153.20	0	−1.212	1.3325	Poor
QPQQPQQPFPQP	QP-12	1419.54	0	3.52	185.72	0	−2.183	2.5217	Poor
WPQQPQQPFPQPQQ	WQ-14	1733.88	0	3.58	174.37	0	−2.186	2.3907	Poor
QSQQPQQPFPQPQQ	QQ-14	1665.76	0	3.41	205.76	0	−2.314	3.1957	Poor
QFQPQQPQQPFPQP	QP-14	1694.84	0	3.52	155.23	0	−1.921	2.3443	Poor

4. Discussion

One of the consequences of the increasingly fast-paced lifestyle, physical inactivity, and poor eating habits in the populations of developing and highly developed countries is metabolic syndrome. Consequently, many researchers are looking for nutritional products rich in active ingredients (e.g., bioactive peptides) to help prevent the onset of these diseases or to support the effects of pharmacotherapy. One of the aims of food product research is to find ingredients with the best possible antioxidant properties and the ability to inhibit enzymes involved in metabolic syndrome development but with no side effects.

The main focus of the study was to determine the antioxidant properties and inhibitory activity against α-amylase, α-glucosidase, and ACE of the obtained hydrolyzates, peptide fractions with a molecular weight < 3 kDa, and peptide fractions isolated from spreads exhibiting the best properties.

All the hydrolyzates and peptide fractions with a molecular weight < 3.0 kDa show $ABTS^{\bullet+}$ free radical scavenging properties. In the group of hydrolyzates and peptide fractions, the differences between the results are not statistically significant, suggesting that the addition of spelt does not increase the analyzed properties. It can be noted that the peptide fractions are characterized by slightly lower IC_{50} values. In their study conducted on fermented faba bean seeds, Jakubczyk and co-authors obtained higher IC_{50} values of $ABTS^{\bullet+}$ scavenging activity of both hydrolyzates and peptide fractions with molecular masses < 3.0 kDa than in the spelt-supplemented spreads and the control spread [33]. This means that common beans show higher $ABTS^{\bullet+}$ free radical scavenging properties than faba beans. In turn, Marathe and co-authors, who studied the most widely consumed legumes in India, prove that red, beige, black, and brown common beans have high $ABTS^{\bullet+}$ free radical scavenging capacity (>12.0 µmol TEAC/g legume). Red beans are shown to have the highest values (23.856 $\pm$ 1.323 µmol TEAC/g legume), while plain white beans exhibit moderate $ABTS^{\bullet+}$ free radical scavenging activity (06.039 $\pm$ 0.019 µmol TEAC/g legume) [34].

Among the hydrolyzates obtained, the spreads with the 10 and 20% spelt addition exhibit the best Fe^{2+} ion chelating properties. These results are statistically significant with respect to the control spread, which supports the thesis assuming that the addition of spelt grain can increase the health-promoting properties of bean spreads. Karaś and co-authors investigated the antioxidant properties of protein hydrolyzates extracted from heat-treated yellow string beans. The IC_{50} value of Fe^{2+} ion chelation was 0.19 $\pm$0.01 mg/mL [35]. This value is higher than in the aforementioned variants supplemented with 10 and 20% spelt addition (IC_{50} = 0.087 $\pm$ 0.002 and IC_{50} = 0.085 $\pm$ 0.003, respectively) but lower than in the control bean spread (IC_{50} = 0.241 $\pm$ 0.027). In addition, as shown by Gujral and co-authors, cooked beans have a lower capacity to chelate Fe^{2+} ions than soybean, moth, or green mung beans [36].

The bean-based spread with spelt is an innovative product; therefore, there are no similar studies analyzing the health-promoting properties discussed here. In the case of the peptide fraction with a molecular weight < 3 kDa, the spread with the 10% spelt addition shows the best Fe^{2+} ion chelating properties (IC_{50} = 0.056 $\pm$ 0.006). A similar IC_{50} value in a low molecular weight peptide fraction was obtained by Jakubczyk and co-authors in analyses of packaged string beans cooked sous vide at 100 °C (IC_{50} = 0.054 $\pm$ 0.003) and unpackaged beans cooked in identical conditions (IC_{50} = 0.055 $\pm$ 0.004) [29]. Despite the similar values, it is worth noting that the spelt-enriched bean spread is a better source of protein than string beans. Moreover, samples analyzed by Mundi and Rotimi exhibit various degrees of iron-chelating potency, with 3–5 and 5–10 kDa peptide fractions showing significantly higher chelating activity than the hydrolyzate of kidney beans and the 1–3 kDa fraction [37]. This proves that the activity of peptides depends on the size of the molecule.

No ability to inhibit α-amylase is observed in the hydrolyzates analyzed in the present study. However, this ability is exhibited by peptide fractions with a molecular weight < 3.0 kDa. The spread with the 10% spelt addition has the lowest IC_{50} values, and the differences between this result and the other samples, including the control spread, are statistically significant. This may indicate that the components present in the hydrolyzates with a molecular weight >3.0 kDa exert an anti-diabetic effect. Jakubczyk and co-authors observed α-amylase inhibitory properties in both hydrolyzate and peptide fractions < 3.5 kDa in cooked, packaged, and non-packaged string beans [29]. Interestingly, in their study conducted in a mouse model, Micheli and co-authors assessed the effect of standardized white kidney bean extract (standardized dry extract containing an alpha-amylase inhibitor and phytohaemagglutinin, where *P. vulgaris* dry extract was prepared by means of aqueous extraction and alcoholic precipitation from the common kidney bean (*P. vulgaris*))

on induction of metabolic syndrome in the tested animals. They found that the efficacy of repeated per os treatment with white kidney bean extract (500 mg/kg) was comparable to that of metformin (100 mg/kg) and atorvastatin (10 mg/kg). Furthermore, the tested extract reduced body weight over time, effectively lowered glycemia, triglycerides, and cholesterol, reduced hepatic steatosis and hepatic lipid peroxidation, and protected the heart from oxidative changes [38]. The results of our study correspond well with the findings reported by Oseguera-Toledo, M. E and co-authors (2015), who studied the effect of bioactive peptide fractions from de-hulled hard-to-cook (HTC) beans on enzyme targets of type 2 diabetes and oxidative stress. The authors obtained similar results, where the best α-amylase and α-glucosidase inhibitory properties were exhibited by fractions with very low molecular weights, with the best properties in fractions with a molecular weight was less than 1.0 kDa [39]. As reported by Bosi and co-authors, all extracts from the legume varieties tested in their study inactivated α-amylase to varying degrees. However, four varieties of common bean ('Great Northern', 'Kidney China', 'Verdone', 'Roviotto') show the highest values, i.e., over 30% inhibition. Such values are considered the threshold value for formulating dietary supplements that effectively reduce α-amylase absorption [40]. Combining beans with oatmeal may also prove interesting, as Fuentes L.R. and co-authors (2021) demonstrated the presence of bioactive peptides with a molecular weight of 1–5 kDa exhibiting inhibitory activity against enzymes associated with glucose digestion, absorption, and metabolism, including α-amylase [41]. Another test was carried out in the present study to determine the α-glucosidase inhibitory ability. The results show that both hydrolyzates and peptide fractions with a molecular weight < 3.0 kDa exhibit this property. In the case of the hydrolyzates, the control spread and the spread supplemented with 10% of spelt show the best α-glucosidase inhibitory properties. The differences between the results are not statistically significant. In turn, the IC_{50} values of the peptide fractions are much lower than those of the hydrolyzates, indicating a higher inhibitory effect, but the highest properties are shown by the control spread and the spread with the 10 and 20% spelt addition. The differences between these results are not statistically significant. Interestingly, eleven genotypes of legumes studied by Bosi et al. showed high levels of α-glucosidase inhibition of >80%. All the eleven plants were characterized by a dark color (red, brown, or creamy) of the outer seed coat [40]. Seed germination and its duration may also affect the a-glucosidase inhibitory activity. As reported by Stefano et al., the α-glucosidase inhibitory activity was significantly different between pulses after germination. In the case of chickpeas, sprouting for 3 days resulted in a significant approx. 36% increase in bioactivity, which then slightly decreased on the 5th day of further germination. The situation was similar in the case of bean and yellow pea germination, which led to a moderate increase in α-glucosidase inhibition [42].

Identification of peptide sequences is crucial in studying their activity and the relationship between food components or enzymes that regulate the functions of the organism. The amino acid residues that build peptides also play an important role in peptides that inhibit enzymes involved in the pathogenesis of diabetes, e.g., α-amylase, α-glucosidase, and dipeptidyl peptidase-IV (DPP-IV). The types and sequences of amino acids determine the interaction and bonds formed between the peptide and the enzyme as well as the strength and location of the interaction [43]. Molecular docking studies indicate that peptide α-amylase inhibitors interact with two or three amino acid residues with the catalytic center of the enzyme. Moreover, the interactions of peptide inhibitors with enzymes are hydrophobic, polar, and formed by hydrogen bonds [39]. The peptides obtained in our work also contain hydrophobic residues of two or three amino acids in their sequences, such as A, L, I, F, P, G, or V, which may confirm their α-amylase inhibitory properties.

The presence of basic and hydrophobic amino acids, such as R, P, and F, in the structure of peptides increases their inhibitory activity against α-glucosidase [44]. Among the peptides obtained from the bean spread enriched with spelt grain, only peptide KV-13 does not contain the listed amino acid residues in its composition, which may indicate its lower activity against α-glucosidase. In addition, some studies indicate that the inhibitory activity

against α-glucosidase is influenced by polar and hydrophobic interactions, as in the case of peptides KTYGL, KKSSG, CPGNK, and GGGLHK obtained from common bean [44] or AKSPLF, QTPF, FEELN, and LSKSVL obtained from black bean [45]. There are no data confirming that the isoelectric point affects the inhibitory activity against α-glucosidase, while peptides that inhibit the activity of this enzyme have net charges in the range of 0 to +1 [43]. In our work, the identified peptides show a net charge in the range from −4 to 0, which may confirm that this property has no effect on the inhibition of the enzyme in question.

The amino acid composition and sequences also affect the antioxidant properties of peptides. It was found that peptides containing acidic, basic, aromatic, and hydrophobic amino acids show higher antioxidant activity [46]. Moreover, an amino acid residue in the C-terminus in the peptide structure with a polar charge was also found to increase antioxidant potential [47]. Some of the identified peptides have polar amino acids, such as Q or Y at the C-terminus, which indicates their antioxidant properties. Also, the presence of aromatic amino acid residues (Y, W, and F) can affect electron capture or ion chelation, which increases their antioxidant potential [48].

Based on the amino acid sequence of a protein or peptide, the Boman index, which estimates the potential of a peptide to bind to cell membranes, other proteins, or receptors, can be determined. An index above 2.48 indicates high binding potential [49]. Four peptides identified from the spread enriched with 10% of spelt grain are characterized by a Boman index indicating their high binding potential. These include RA-13, KV-13, QP-12, and QQ-14, but only the first two have good solubility in water.

Despite the many promising research results relating to bioactive peptides found in foods, these studies have their limitations. For many people already struggling with such civilization diseases as type 2 diabetes, hypertension, or obesity, foods, even those showing high health-promoting potential, will not replace preparations used in pharmacotherapy in 100% of situations. These products can only support the treatment process and help to possibly reduce the doses of drugs taken by patients.

5. Conclusions

The incorporation of legume seeds into a diet brings many health benefits, especially in the context of prevention and support of the treatment of such lifestyle diseases as type 2 diabetes, obesity, and hypertension. The occurrence of all these conditions, called metabolic syndrome, is a heavy burden on the body. Bioactive peptides contained in legumes and grains can inhibit enzymes responsible for the development of these diseases (α-amylase and α-glucosidase) and have high antioxidant properties (antioxidant activity against ABTS$^{•+}$ and against ferrous iron (II)). The presence of peptides that inhibit the enzymes studied supports the conclusion that legume- and grain-based products can counteract the development and/or support the treatment of diseases included in the metabolic syndrome (especially diabetes and obesity). The product tested do not exhibit inhibitory properties against ACE; therefore, its effectiveness in the prevention of hypertension is negligible. In addition, the combination of beans with the 10% supplementation of spelt grains yields a spread with a higher content of amino acids, such as methionine, tryptophan, cysteine, and glutamic acid. Due to the improvement in the amino acid composition of the tested product compared to the control spread, a conclusion can be formulated that supports the statement regarding the validity of combining pulses with cereal grains to provide complete protein. The ten peptides are found to contain between 8 and 14 amino acid residues, which indicates their biological activity. In addition, four of the peptides (RA-13, KV-13, QP-12, and QQ-14) show high potential to bind to cell membranes, other proteins, and receptors, which has a positive effect on their health-promoting potential and their effects on the human organism. The high antioxidant properties of the tested peptide fractions are confirmed by the structure of the amino acid sequences, which are characterized by polarity and have Q or Y at the C-terminus and aromatic amino acid residues. The α-amylase inhibitory properties are confirmed by the hydrophobic nature of

two or three amino acids in the peptide sequences (A, L, I, F, P, G, or V). In contrast, high inhibitory activity against α-glucosidase is confirmed in nine of the ten peptides, as they contain basic and hydrophobic amino acids (R, P, and F). The results indicate that bean spread with 10% spelt grains may be used as a dietary and functional food product as an addition to sandwiches, salads, or pasta sauces. Further studies on the characterization of IC_{50} value of single peptides and in vivo studies about their influence on organisms are needed.

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