The Scientific and Technological Research Council of Türkiye (TÜBİTAK)

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(BİDEB)

2214-A International Research Fellowship Program for PhD Students

Progress Report

(The report should not exceed 10 pages excluding references)

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Host Institution: Leibniz University Hannover/Germany

Research Title: Determination of Biological Activities of Peptides from Cold Pressed Sunflower Meal, The Stability of Bioactive Peptides and Applications in Food Products

1. **Summary of Research Progress** *(Please provide a summary containing the progress of the research over the last term and overall objectives, all with no more than 1000 words)*

The procurement of hulled sunflower seed samples, the removal of oil from the kernel using cold press equipment, obtaining cold press cake, preparation of protein isolates, and composition analysis of the cake and protein isolate encompass the 1–6-month period of the thesis work. At this stage, a clear definition and understanding of the raw material to be studied significantly influence the remaining process and analyses. In this context, the primary goal is to analyze the nutritional composition of hulled sunflower seeds, cold-pressed cake, and the obtained protein isolate. Additionally, it is crucial within the remaining part of the thesis to determine the suitability of the protein isolation method, decide on and procure the enzymes to be used, establish the working conditions of these enzymes by following similar studies in the literature, and conduct preliminary tests in the laboratory. The all succeeded objectives of 1-6-month period of the project work listed below.

- ✓ Procurement of dehulled sunflower seed samples,
- ✓ Removing oil from sunflower seed and obtaining cold press cake,
- ✓ Preparation of protein isolates,
- ✓ Conducting composition analyses of sunflower seed, meal, and protein isolate,
- ✓ Preparation of enzymatic hydrolysates

In the first stage of the project, two different methods were used for drying the proteins. The methods commonly used in obtaining protein isolates are freeze drying and spray drying. These two methods were also tested for drying sunflower seed protein isolates. *In vitro* antioxidative tests were applied to the obtained isolates. However, due to the low drying efficiency of spray drying specifically for sunflower seed protein isolates and the host institute not having a spray dryer, it was decided to use only the freeze dryer for the remaining stages of the project, as proposed in the project plan, to ensure the continuity of isolate production.

During the protein isolate extraction stage, another noteworthy observation was the green coloration of the waste supernatant, which was quite intriguing. This was explained by the high amount of chlorogenic acid in sunflower seeds, which oxidizes during the quinone alkali extraction stage and interacts with seed proteins to produce a green tint (Wildermuth et al., 2016). Therefore, independently of the project, the *in vitro* antioxidative properties of this green-colored supernatant and the powder obtained by adding maltodextrin were investigated. The potential bioactivity of this by-product has initiated independent studies to be evaluated as a separate research project.

During the protein isolation stage, the pH value of the final protein product obtained after isoelectric precipitation is 4.5. In the literature, studies have also been conducted where the pH value of the obtained protein is adjusted to 7. *In vitro* antioxidant tests have been carried out for protein isolates obtained at different pH values. Due to the higher antioxidant properties of the isolate with a pH value of 7, the pH of the obtained isolates was adjusted to 7 before drying.

2. **Progress Report** *(Please report on the achievement of significant milestones in the research and if milestones have not been met, please explain the reasons why. Work performed from the beginning of the research to the end of the period should be covered by the report.)*

The nutritional composition of samples

The pictures and nutritional compositions of the raw dehulled sunflower kernels used in this study, the sunflower seed cake obtained after cold pressing, and the protein isolate obtained using the alkaline extraction isoelectric precipitation method are shown in Figure 1 and Table 1, respectively. The protein, fat, and ash content of dehulled sunflower kernels were found to be 23.1%, 54.8%, and 3.8%, respectively. These values were found to be consistent with other studies (Srilatha and Krishnakumari, 2003; Nadeem et al., 2010). The cold-pressed sunflower seed cake obtained in this study contained 38.7% protein, 10.8% fat, and 35.6% carbohydrates. The findings are consistent with the literature (Vidal et al., 2022). The protein, fat, ash, and carbohydrate content of sunflower protein isolates obtained using the alkaline extraction and isoelectric precipitation method were determined to be 88.4%, 1.2%, 3.6%, and 2.38%, respectively. In another study, the nutritional composition of the protein isolate obtained using the same method was found to be 78.43% protein, 6.59% ash, and 1.86% carbohydrates (Malik and Saini, 2017). The high protein content of the protein isolate obtained in this study is thought to be due to the use of a different variety of sunflower seeds influenced by environmental factors and differences in the extraction method.

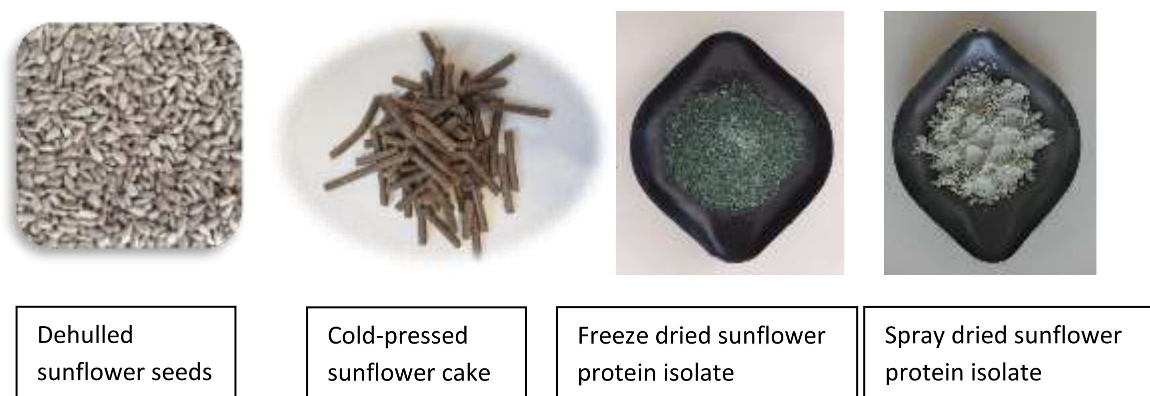

Figure 1. Pictures of sunflower seed samples

Table 1. Nutritional composition of sunflower seed samples*

	Protein (%)	Moisture (%)	Fat (%)	Ash (%)	Carbohydrate** (%)
Dehulled sunflower seed	23.1±0.5	7.5±0.8	54.8±0.15	3.8±0.35	10.8±0.22
Cold press sunflower cake	38.7±0.3	9.6±0.5	10.8±0.7	5.3±0.2	35.6±1.1
Sunflower protein isolate	88.4±0.9	4.4±0.2	1.2±0.3	3.6±0.7	2.38±0.2

*Results are given as the mean ± standard deviation of three measurements.
**Calculated by subtracting the difference from 100.

Antioxidative Properties of Sunflower Seed Meal Samples

Protein isolation from sunflower seed meal, obtained by cold pressing dehulled sunflower seeds, was performed using the alkaline extraction and isoelectric precipitation method with an average yield of 61.6%. The sunflower seed protein isolate was then dried using two different methods: spray drying and freeze drying. For preliminary work, under appropriate conditions, the obtained protein isolates were treated with Alcalase and trypsin enzymes to produce two different sunflower seed protein hydrolysates. Some of the protein isolates were brought to pH 4.5, while the remaining portion was adjusted to pH 7 using a specific amount of pure water and 1 N NaOH before being freeze-dried. Additionally, the supernatant typically discarded at the final stage of the protein isolation process was evaluated and subjected to certain bioactivity tests.
Sunflower seeds are known to be rich in antioxidants and phenolic compounds. Specifically, sunflower seeds contain 4.2 g/100 g total phenolics and approximately 3.0 g/100 g chlorogenic acid in the kernel (Liang and Were, 2018). During the protein isolation stages, a green-blue color was observed at high pH values (pH 9.5) due to alkaline treatment. This green pigment formation is reported to increase due to the oxidation of chlorogenic acid in an alkaline environment (Wildermuth et al., 2016). Therefore, green color development was observed along with proteins intended to dissolve in an alkaline environment. The green pigment-containing supernatant, typically discarded as waste, was mixed with 5% maltodextrin and spray-dried to obtain a powdered form and test antioxidant properties.
The total phenolic content and antioxidant activity values of all the mentioned samples obtained using sunflower seed meal are shown in Table 2. The highest total phenolic content was observed in the alcalase and trypsin protein hydrolysates. The higher phenolic content of hydrolysates compared to protein isolates is thought to be due to peptides released during hydrolysis. Additionally, the freeze-dried sunflower seed protein isolate (SPI) at pH 7 had similar values to the protein isolate obtained by spray drying, while the freeze-dried SPI at pH 4.5 had the lowest values. On the other hand, the supernatant had a higher total phenolic content compared to the spray-dried powder.
To test the antioxidant content, CUPRAC and DPPH analyses were applied to the samples. Similar to the total phenolic content, the lowest antioxidant value was observed in the freeze-dried SPI at pH 4.5. The alcalase protein hydrolysate had the highest antioxidant value in the CUPRAC analysis, with a value of 280.3 TE mg/100 g. Additionally, the antioxidant values of the spray-dried powder containing 5% maltodextrin were higher than those of the supernatant in both methods. This is thought to be because maltodextrin, as a coating agent, preserves the antioxidant properties of the pigment.

Table 2. The total phenolic and antioxidant content of sunflower protein isolate, hydrolysate, and supernatant

Assays*	
Total phenolic content (GAE mg/100 g)	
Freeze dried sunflower protein isolate at pH 7.0	53.4±4.9

	Freeze dried sunflower protein isolate at pH 4.5	24.5±0.5	
	Spray dried sunflower protein isolate	56.3±7.4	
	Sunflower trypsin hydrolysate	81.5±4.0	
	Sunflower alcalase hydrolysate	97.6±0.1	
	Supernatant from protein extraction process (pH 9.5)	59.9±1.2	
	Green color pigment with 5% Maltodextrin	49.3±0.7	
	CUPRAC (TE mg/100 g)		
	Freeze dried sunflower protein isolate at pH 7.0	174.6±2.9	
	Freeze dried sunflower protein isolate at pH 4.5	73.1±2.9	
	Spray dried sunflower protein isolate	179.5±14.0	
	Sunflower trypsin hydrolysate	175.7±1.1	
	Sunflower alcalase hydrolysate	280.3±4.1	
	Supernatant from protein extraction process (pH 9.5)	175.7±1.1	
	Green color pigment with 5% Maltodextrin	192.9±22.2	
	DPPH (TE mg/ 100 g)		

Freeze dried sunflower protein isolate at pH 7.0	118.5±2.4
Freeze dried sunflower protein isolate at pH 4.5	92.1±3.4
Spray dried sunflower protein isolate	132.8±4.5
Sunflower trypsin hydrolysate	100.6±12.9
Sunflower alcalase hydrolysate	115.5±0.5
Supernatant from protein extraction process (pH 9.5)	107.8±2.1
Green color pigment with 5% Maltodextrin	128.3±4.4

References

Liang, S., & Were, L. M. (2018). Chlorogenic acid induced colored reactions and their effect on carbonyls, phenolic content, and antioxidant capacity in sunflower butter cookies. LWT, 87, 16-22.

Malik, M. A., & Saini, C. S. (2017). Polyphenol removal from sunflower seed and kernel: Effect on functional and rheological properties of protein isolates. Food Hydrocolloids, 63, 705-715.

Nadeem, M., Anjum, F. M., Arshad, M. U., & Hussain, S. (2010). Chemical characteristics and antioxidant activity of different sunflower hybrids and their utilization in bread. African Journal of Food Science, 4(10), 618-626.

Vidal, N. P., Roman, L., Swaraj, V. S., Ragavan, K. V., Simsek, S., Rahimi, J., ... & Martinez, M. M. (2022). Enhancing the nutritional value of cold-pressed oilseed cakes through extrusion cooking. Innovative Food Science & Emerging Technologies, 77, 102956.

Srilatha, K., & Krishnakumari, K. (2003). Proximate composition and protein quality evaluation of recipes containing sunflower cake. Plant Foods for Human Nutrition, 58(3), 1-11.

Wildermuth, S. R., Young, E. E., & Were, L. M. (2016). Chlorogenic acid oxidation and its reaction with sunflower proteins to form green-colored complexes. Comprehensive Reviews in Food Science and Food Safety, 15(5), 829-843.

The text box can be expanded as needed.

3. Planning for the Following Periods *(Please summarize the next stages of your research here.)*

Fractionation of protein hydrolysates

In the following periods, obtaining fractions from hydrolysates is planned to be the first step. Subsequently, antimicrobial, antioxidant, ACE inhibition, and antidiabetic tests will be conducted on the collected fractions in sufficient quantities. Fractions that stand out due to their high bioactivity will be selected for further purification stages.
After the enzymatic hydrolysis processes, protein hydrolysates will undergo ultrafiltration using appropriate ultrafiltration cassettes (3 kDa, 5 kDa, or 10 kDa MWCO) and will be concentrated to a volume between 0-10 times. Four different fractions will be obtained using three different molecular weight cut-off membranes. The fractions will first be passed through the largest MWCO membrane cartridge (10 kDa). The retentate and filtrate will be collected separately, and the filtrate will be fed into the 5 kDa cassette. The filtrate obtained after the 5 kDa cassette will be fed into the 3 kDa cassette, and the fractions will be collected. The fractions will be named according to whether they are retained by or pass through the membrane (<3 kDa, 3-5 kDa, 5-10 kDa, and >10 kDa) (Segura-Campos et al., 2011). On the other hand, due to the absence of an FPLC system at the host university and the FPLC system being destroyed and unusable at the primary university where the PhD is being conducted, alternative fractionation techniques will be researched, and method development at the host university is aimed.

Purification of bioactive peptides

After fractionation of sunflower seed meal protein hydrolysate, further purification will be performed with the aid of RP-HPLC. The samples with the highest antioxidant and ACE inhibition activities from the fractions obtained by ultrafiltration will be subjected to further purification using the RP-HPLC system. Fractions will be collected based on elution peaks. At this stage, the purified fraction with the highest antioxidant and ACE inhibition activity will be analyzed for amino acid sequence identification (Wang et al., 2017; Tang et al., 2018).

For the obtained fractions, cell line studies will be intensively conducted in the remaining process. *In vitro* protein digestion will be studied in detail. Additionally, product trials will be carried out using the facilities available at the host institute.

References

Segura-Campos, M. R., Chel-Guerrero, L. A., & Betancur-Ancona, D. A. (2011). Purification of angiotensin I-converting enzyme inhibitory peptides from a cowpea (Vigna unguiculata) enzymatic hydrolysate. Process Biochemistry, 46(4), 864-872.

Tang, S. S., Prodhan, Z. H., Biswas, S. K., Le, C. F., & Sekaran, S. D. (2018). Antimicrobial peptides from different plant sources: Isolation, characterisation, and purification. Phytochemistry, 154, 94-105.

Wang, X., Chen, H., Fu, X., Li, S., & Wei, J. (2017). A novel antioxidant and ACE inhibitory peptide from rice bran protein: Biochemical characterization and molecular docking study. Lwt, 75, 93-99.

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3.1 Research Effort per Work Packages *(Please indicate the percentage of the outcome undertaken in each work package).*

Work Package	During reporting period (%)	Total (%)
WP 1 (1-6 months) The procurement of dehulled sunflower seed samples, removal of oil from the kernels using cold press equipment to obtain cold press cake, preparation of protein isolates, composition analysis of meal and protein isolates, and preparation of enzymatic hydrolysates,	-Production of press cake (100%) -Extraction proteins and preparation of dried protein isolates (100%) -Compositional analysis of press cake and protein isolates (100%) -Preparation of different enzymatic protein hydrolysates (100%)	100 %
WP 2 (6-12 months) Conducting antimicrobial, antioxidant, ACE inhibition, and antidiabetic tests for the fractions. Selecting the fractions with high bioactivity and performing further purification steps using RP-HPLC.	-Production of fractions with ultrafiltration (0%) The antioxidant, antimicrobial, ACE inhibition and antidiabetic assays tried to be adopted in Leibniz University Hannover (40%)	20%
WP 3 (12-18 months) Conducting anticancer bioactivity tests, performing cell cycle distribution analyses, and examining the transepithelial permeability of peptides.	In this period, culture analysis like splitting cells, media change and plate splitting were learned before starting bioactivity tests. Adopting and establishing cell culture assays took some time.	10 %
WP 4 (12-24 months) Identifying peptide sequences with prominent antioxidative and anticancer properties using LC-Q-TOF/MS analysis after the	In silico tools like CABSdock, HPEPdock, peptide ranker and BIOPEP were learned and studied. After enlightening the peptide	10 %

purification stage, evaluating their properties through in silico methods	fragments via LC- Q-TOF/MS, in silico analysis will conducted.	

New lines can be added as needed.

4. Barriers to Research *(Please list any issues that you have encountered that have hindered progress of your research activities.)*

During the period from June to January (second half of project), the stage involving the fractionation of sunflower seed protein hydrolysates using ultrafiltration and fast protein liquid chromatography methods and the collection of sufficient amounts of fractions, which constitutes the final part of the study, would be faced with some difficulties. The host university does not have any fractionation units for both ultrafiltration and fast protein liquid chromatography.

Due to problems encountered with the UF and FPLC devices, efficient results could not be obtained, and maintenance was required. The maintenance process has still not been completed due to high costs and the fact that the company is based abroad. Due to such significant issues, the final part of the study would not be completed and has been postponed to the next period.

The fractionation of hydrolysates was planned as the first step for the upcoming period. Following this, antimicrobial, antioxidant, ACE inhibition, and antidiabetic tests will be conducted on the obtained and sufficiently collected fractions. Fractions that stand out for their high bioactivity will be selected and subjected to further purification stages.

The text box can be expanded as needed.

I declare that I comply with the ethical rules and legislation while executing the research and the information I provided above is correct.

Name of the Researcher	Signature	Date
Zeynep Saliha Güneş		30.07.2024
Name of the Host	**Signature**	**Date**
Prof. Dr. Tuba Esatbeyoğlu		30.07.2024