IMPACT OF FLAXSEED CAKE HYDROLYSIS ON ANTIOXIDANT CAPACITY

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ABSTRACT

Cold pressing of flaxseed produces high-quality oil along with a nutritionally rich by-product—flaxseed cake. While the cold-pressing process is valued for its minimal processing and preservation of oil quality, it is also characterized by low resource utilization efficiency and significant by-product generation. Specifically, the production of 1 kg of flaxseed oil results in approximately 2 kg of residual cake. Given its high protein content (approximately 30–40%), flaxseed cake holds considerable potential as a source of bioactive peptides and other value-added compounds

In this study, flaxseed cake obtained from cold pressing was milled and sieved to a particle size below 0.6 mm. Its chemical composition (protein, oil, fiber) was analyzed. Controlled enzymatic hydrolysis was performed with trypsin for 2 h. An E/S ratio was 5 %, and the temperature used for hydrolysis was 37 °C. The antioxidant capacity of the non-hydrolyzed sample and hydrolysate was determined.

Protein content was 37 %, oil was about 21 %, and fiber was approximately 28 %. The results showed a degree of hydrolysis of about 5 % and a significant increase in antioxidant activity. By determining the concentration of flaxseed powder required to inhibit 50 % of the DPPH free radicals, it was observed that this concentration was significantly ($p < 0.05$) higher in the non-hydrolyzed sample (11.0 mg/mL) compared to the hydrolysate (9.1 mg/mL).

Keywords: hydrolysis, protein, trypsin, bioactivity.

INTRODUCTION

Flaxseed (*Linum usitatissimum L.*) has gained increasing attention in both the food and nutraceutical industries due to its rich nutritional composition and health-promoting properties, including high levels of α-linolenic acid, lignans, and dietary fiber [1]. Cold pressing is a widely used method for extracting flaxseed oil, favoured for its ability to preserve bioactive compounds through minimal thermal degradation [2]. However, this process results in significant amounts of by-product named flaxseed cake.

Despite being commonly used as animal feed, flaxseed cake is rich in valuable nutrients, particularly proteins (>35), residual oil (up to 20%), and dietary fiber with carbohydrate (up to 40%) [3]. These attributes position it as a promising raw material for extracting bioactive compounds, particularly peptides with antioxidant potential. Enzymatic hydrolysis has proven to be an effective strategy for releasing such peptides from proteins [4], [5]. Through controlled proteolytic action, enzymatic treatment can enhance the functional and biological properties of proteins by generating low-molecular-weight peptides with higher bioavailability and antioxidant capacity [4], [6].

In addition to enzymatic hydrolysis, microbial hydrolysis is another method used to break down proteins into bioactive peptides and amino acids. Microbial hydrolysis relies on the natural proteolytic activity of live microorganisms, such as *Lactobacillus* species [7], which are commonly used in traditional food fermentations like yogurt, miso, and tempeh [8]. These microbes secrete a variety of enzymes that degrade proteins during fermentation.

While microbial hydrolysis has benefits, such as lower cost, natural processing, and additional nutritional enrichment (e.g., vitamins, probiotics), it also presents several limitations. The most common limitations are:

- The less controlled process, as microorganisms can produce a mixture of enzymes and metabolites, resulting in variability in peptide profiles.
- The slower process often requires several hours or days.
- The higher risk of contamination, especially in non-sterile environments.
- The process is hard to standardize- the bioactivity and composition of the final product are more difficult to standardize [9], [5].

In contrast, enzymatic hydrolysis uses purified, well-characterized enzymes under strictly controlled conditions. This allows for:

- Precise and reproducible hydrolysis,
- Faster reaction times, typically within 3 hours,
- Improved safety, due to the absence of live microbes,
- Targeted generation of specific bioactive peptides, which is crucial for maximizing health benefits such as antioxidant, antihypertensive, or immunomodulatory effects [10], [5], [4].

Although microbial hydrolysis remains valuable in traditional food systems, enzymatic hydrolysis is generally preferred in industrial and functional food applications due to its higher efficiency, reproducibility, and safety.

This study investigates the valorisation of cold-pressed by-product via enzymatic hydrolysis using trypsin. The focus is on characterizing its chemical composition and evaluating the antioxidant potential of the resulting protein hydrolysate. The work aims to contribute to the development of sustainable strategies for the utilization of flaxseed by-products in functional food and nutraceutical applications.

METHODS AND MATERIALS

Materials

In this work, flaxseed cake was obtained from Linum d.o.o.. Commercially available protease used in this work was trypsin (EC 3.4.21.4, Sigma-Aldrich Chemie Gmbh, USA). DPPH and other analytical grade reagents were also purchased from Sigma-Aldrich Chemie Gmbh, USA.

Flaxseed cake pre-treatment

Flaxseed cake, obtained from the cold-pressed oils industry, was used. The composition of the raw material was determined before hydrolysis. Flaxseed cake was ground in a ball mill and sieved through a sieve with a pore size of 0.6 mm. The flaxseed powder obtained in this way was sterilized in an autoclave at 120 °C, with a pressure of 1.5 bar for 30 minutes. After sterilization, the flaxseed powder was diluted in distilled water to a 5% (w/v) concentration.

Flaxseed cake composition

Moisture and dry matter content

The moisture/dry matter content in the flaxseed powders was determined by the

KERN moisture analyzer DBS 60-3 (Mechanikus Gottlieb KERN, Germany).

Oil content

To determine the oil content in flaxseed cake, the Soxhlet extraction method was used, employing a semi-automatic Behr ES 2+2 apparatus (Behr-Labor Technik GmBh, Germany, Figure 1).

Determination of fiber content

To determine the fiber content in flaxseed cake, the Behr EN 4V WBMR was used (Behr-Labor Technik GmBh, Germany, Figure 2).

Determination of protein content

To determine protein content in flaxseed cake, the Kjeldahl method was used. The equipment for this analysis was a Kjeldahl infrared rapid digestion unit from Behr-Labor Technik GmBh, Germany (Figure 3).

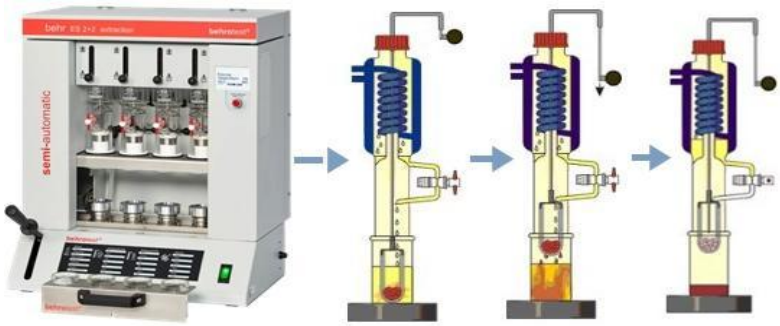

Figure 1. Determination of oil amount in flaxseed cake

Figure 2. Apparatus for the determination of fibre content

Figure 3. Apparatus for determining protein amount by the Kjeldahl method in flaxseed cake

Controlled enzymatic hydrolysis

Flaxseed powder was reconstructed in distilled water to a concentration of 5.0 % (w/v). The mixture was allowed to hydrate for 1 h at room temperature while gently stirring. Hydrolysis was performed as described by Krunic and Rakin (2025) using trypsin (EC 3.4.21.4) under optimal conditions: pH 8.0 / 37 °C. The enzyme/substrate ratio was 5.0% (w/w). During the reaction, pH was kept at a constant value by adding 1M NaOH, using the pH-stat method with automatic dosage of the base. The reaction was stopped after 2 h by heating the mixture at 100°C for 10 min to inactivate the enzyme. The hydrolysate obtained was cooled down to room temperature for future analysis. The degree of hydrolysis (DH) was determined using the pH stat method described by Alder-Nissen (1986). It was calculated by the following equation.

$$DH\ \ (\%) = B \times Nb \times (1/\alpha) \times (1/mp) \times (1/htot) \times 100 \quad (1)$$

Where B is the consumption of the base in mL, Nb is the normality of the base, mp is the mass of protein in g, htot is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition (for flaxseed htot is 7.8 mmol/g protein [11]), α is the degree of dissociation of the α-amino groups (1/α=1.26 at 37 °C and pH 8.0 (Adler-Nissen, 1986)).

Determination of the inhibition of DPPH free radicals

The flaxseed powder, un-hydrolysed and hydrolysed, was taken in two different dilutions (0.5 and 1%) and mixed with 0.1 mM–thanolic DPPH free radical, in a ratio 1 : 18. Mixtures were homogenized and left in the dark for 30 min. The absorbance was measured at 517 nm using a UV-visible spectrophotometer (Ultrospec 3300 pro, Amerischam Bioscienc). For making control, ethanol was used instead of the sample. The antioxidant activity was expressed as a percentage of DPPH activity calculated as:

$$DPPH\ capacity\ (\%) = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank} \times 100 \quad (2)$$

The concentration of flaxseed powder required to inhibit 5% of the DPPH free radicals (IC_{50}) was calculated.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as means with standard deviations. The Tukey test was used to determine significant differences between samples ($p < 0.05$). Data analysis was conducted using Microsoft Excel (Microsoft Office 2013 Edition, USA) and OriginPro 8 (Origin Lab Co., Northampton, USA).

RESULTS AND DISCUSSION

This study investigates the potential use of flaxseed cake, a by-product generated in large quantities during the production of cold-pressed flaxseed oil, as a component of functional food. As can be seen in Table 1, the protein component is 37.1 ± 2.5% which makes flaxseed cake a good raw material for controlled enzymatic hydrolysis to obtain bioactive peptides.

Many food proteins contain biologically active peptides that can be released through gastrointestinal digestion or controlled enzymatic hydrolysis. Research has demonstrated that controlled enzymatic hydrolysis of food protein can enhance the functional and nutritional properties, as well as

biological activities of food [5], [4], [6]. Hydrolysis of food protein with digestive enzymes (such as trypsin, chymotrypsin, and pepsin) is a very common practice, and many results are achieved. In this study, the digestive enzyme trypsin was used, and the degree of hydrolysis obtained after 2h is shown in Figure 4.

Table 1. Flaxseed cake composition

	Protein, %	Oil, %	Fiber, %	Moister, %
Flaxseed cake	37.1 ± 2.5	20.7 ± 1.2	28.0 ± 3.5	8.1 ± 0.9

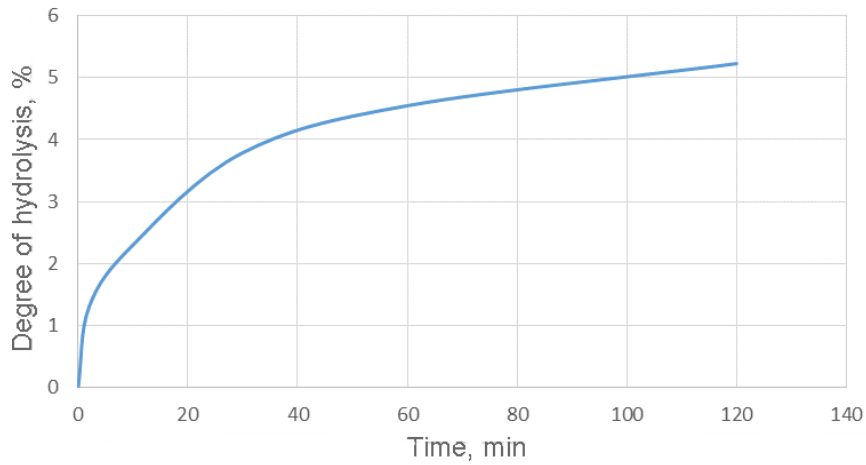

Figure 4. The degree of hydrolysis after 120 min of trypsin hydrolysis of flaxseed powder (Enzyme/Protein ratio is 5:100)

A degree of hydrolysis of 5% is significantly lower compared to the degree of hydrolysis obtained under identical conditions, but in half the time for whey protein, where the hydrolysis was twice as high [12], [4], [6]. This is consistent with literature reports that describe the resistance of cereal proteins to trypsin. Research has confirmed that cereal proteins, such as those from wheat and barley, contain α-amylase/trypsin inhibitors that are resistant to enzymatic digestion. These inhibitors form part of a multigene family and are structurally adapted to resist degradation by trypsin, contributing to the inherent resistance of cereal proteins to proteolysis [13]. Flaxseed contains trypsin inhibitors and compounds that inhibit α-amylase, but these are likely separate mechanisms rather than a classical bifunctional inhibitor protein commonly found in cereal grains [14], [15]. Flaxseed contains measurable levels of trypsin inhibitor activity. Laboratory-prepared flaxseed meal samples showed trypsin inhibitor levels ranging from 42–51 units, while commercial samples had 14–37 units, which is significantly lower than soybean meal (1650 units), but still present and relevant [14].

Flaxseed possesses a high antioxidant capacity, primarily attributed to its lignan content, which is up to 30 times higher than that of any other plant. The antioxidant potential of flaxseed can be further enhanced through microbial or enzymatic protein hydrolysis. Figure 5 presents the antioxidant capacity of flaxseed powder before and after hydrolysis at concentrations of 0.5 % and 1 %. A significant increase in antioxidant capacity is evident following controlled hydrolysis using trypsin. By determining the

concentration of flaxseed powder required to inhibit 50 % of the DPPH free radicals (IC_{50}), it was observed that this concentration was significantly higher in the non-hydrolyzed sample (11.0 mg/mL) compared to the hydrolyzed one (9.1 mg/mL). This is in accordance with the literature [16], [17].

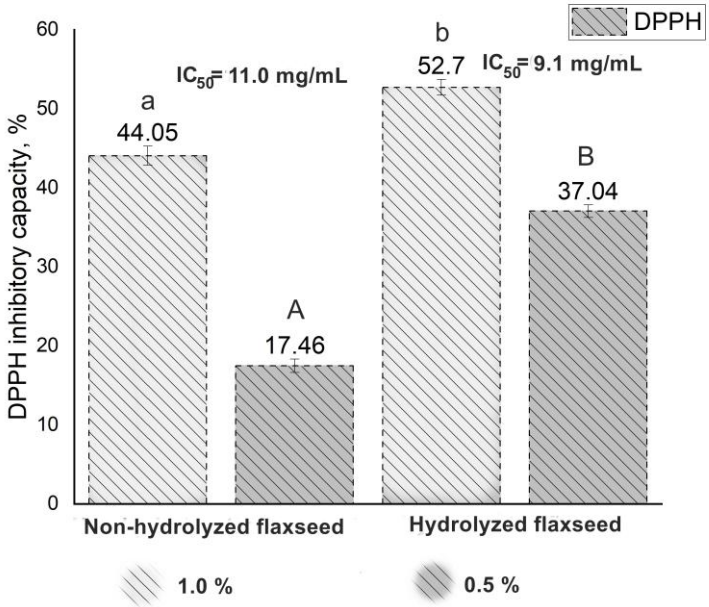

Figure 5. Antioxidant capacity of flaxseed powder water after 120 min of hydrolysis by trypsin with an Enzyme/Protein ratio 5:100 (flaxseed powder was diluted to 1 and 0.5 % in distilled water). Values followed by different letters are significantly different from each other ($p < 0.05$)

Trypsin hydrolysis significantly increased the antioxidant capacity of flaxseed protein compared to its native form. This included improvements in DPPH radical scavenging, reducing power, and metal ion chelating activity [16]. Hydrolysates from trypsin-treated flaxseed protein exhibit improved antioxidant effects due to the release of bioactive peptides with lower molecular weight, which are more effective in scavenging radicals and chelating metals [18]. Also, pre-treating flaxseed protein with high hydrostatic pressure before trypsin hydrolysis resulted in peptides with significantly higher antioxidant activity, suggesting pressure can enhance enzyme accessibility and peptide bioactivity [19], [17]. The antioxidant capacity of the obtained peptides depends more on the type of enzyme used than on the duration of hydrolysis, since enzymes have specific binding and cleavage sites on proteins, which determine the bioactivity of the resulting peptides [5], [4].

CONCLUSION

This study investigates the potential use of flaxseed cake, a by-product generated in large quantities during the production of cold-pressed flaxseed oil, as a component of functional food. The protein component of 37.1 ± 2.5% makes flaxseed cake a good raw material for controlled enzymatic hydrolysis to obtain bioactive peptides. This study demonstrates that controlled enzymatic hydrolysis using trypsin can enhance the antioxidant capacity of flaxseed powder by releasing bioactive peptides. Although flaxseed proteins exhibit partial resistance to enzymatic digestion, likely due to the presence of trypsin inhibitors, the hydrolysis achieved

was sufficient to significantly improve functional properties. The antioxidant activity, as measured by DPPH radical scavenging capacity, increased notably after hydrolysis, with the IC_{50} value of the hydrolyzed sample being significantly lower (9.1 mg/mL) compared to the non-hydrolyzed one (11.0 mg/mL). These findings highlight the potential of enzymatic treatment to improve the bioactivity of flaxseed-derived ingredients, despite inherent inhibitory factors.

Overall, hydrolyzed milled flaxseed cake can be incorporated into functional food formulations such as protein bars, yogurts, or bakery products, where it enhances nutritional value, improves fiber content, and provides bioactive compounds beneficial for gut health.

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DECLARATIONS OF INTEREST STATEMENT

The authors affirm that there are no conflicts of interest to declare in relation to the research presented in this paper.

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