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Enzymatic hydrolysis of flaxseed (*Linum usitatissimum* L.) protein and sensory characterization of Maillard reaction products

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ABSTRACT

We aimed to simplify the enzymolysis process for flaxseed protein hydrolysates production as Maillard reaction products (MRPs) to generate different flavor characteristics. More than 50% activity of immobilized enzymes (Alcalase and Flavourzyme) was retained after repeated use. Subsequently, after five weeks, the activities of the immobilized enzymes were also observed to be higher after storage at 4 °C. The optimum conditions for production of flaxseed protein hydrolysates using sequential enzymatic hydrolysis were as follow: 3,000 U/g of Alcalase at 60 °C and pH 8.0 for 2 h and 120 U/g of Flavourzyme at 50 °C and pH 6.5 for 2 h. Partial least squares regression analysis revealed that resulting peptides with the molecular weight (MW) higher than 1,000 Da could improve the mouthfulness and stability in umami soup; whereas, peptides with MW of 128–1,000 Da mainly contributed to the generation of meat-like flavor compounds with a significant effect on umami taste and bitterness.

1. Introduction

Plant-derived proteins are progressively in demand as a better substitute for animal's proteins in human nutrition (Rodriguezpatino et al., 2007). Among plants, legume seeds and oilseeds (soybean, rapeseed, peanut, and sunflower) are the richest sources of proteins (Rusnikova, Straková, & Suchý, 2013). Traditionally, fat extraction from plant seeds yields a large amount of defatted meal as byproducts, which could be used as a food ingredient for animal food (Zawawi et al., 2014). But due to their high protein content, they can be used in human diets with a direct application to human health (Peksa & Miedzianka, 2014).

Flax has been known as one of the most ancient crops with a specific application in cloths and paper industries until the nineteenth century. The flaxseed oil supplies abundance of α-linolenic acid. Lately, flaxseeds have been used in food industry due to the growing consumer's concerns for consumption of functional food with value-added benefits (Shim, Gui, Arnison, Wang, & Reaney, 2014; Wang et al., 2017). Moreover, the occurrence of n-3 fatty acids, soluble fibers, vitamin E, lignans, and other phenolic and peptide composites in flaxseeds was identified as a vital factor for their health-promoting effects as well as their direct use in foods and medicines (Cardoso Carraro, Dantas, Espeschit, Martino & Ribeiro, 2012).

Enzymatic protein hydrolysis is a commonly used method for improving the functional attributes of proteins. Previously, protein hydrolysates displayed elevated solubility at different pH and temperatures as compared to native proteins which had reduced solubility at the isoelectric point with increased temperatures (Zhao et al., 2012). Moreover, the hydrolysis procedure produces smaller fractions of peptides with superior nutritional uniqueness than the original protein (Amza, Balla, Tounkara, Man, & Zhou, 2013). In general, flaxseed constitutes 35–45% proteins which makes the defatted flaxseed meal a remarkable raw material to obtain suitable protein hydrolysates (Rabetafika, Van Remoortel, Danthine, Paquot, & Blecker, 2011).

In addition, the low molecular weight peptides obtained after enzymatic hydrolysis frequently results into a bitter taste due to the formation of hydrophobic amino acids such as isoleucine, tyrosine, phenylalanine, and tryptophan (Polancolugo, Dávilaortiz, Betancurancona, & Chelguerrero, 2014). However, bitterness often decreases with the increased hydrolysis rate which splits the bitter peptides into free amino acids (Hou, Li, Zhao, Zhang, & Li, 2011).

The Maillard reaction plays a considerable function for the resulting flavor in foods during food processing and storage by constituting a multifaceted reaction between different components (Hou et al., 2011). For that reason, MRPs have been used in the form of flavor enhancers to develop the taste of mouthfulness and permanence (Song et al., 2016). Thus, flaxseed protein hydrolysates represent a novel source for these remainders and their modification through the above reaction can be a

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key step in the formation of functional compounds (Gao et al., 2018). So far, a few studies have been conducted to establish the role of flaxseed protein hydrolysates on the generation of flavors through Maillard reaction.

Herein, the aim of the study was to simplify the enzymolysis process by using immobilized enzymes (Alcalase and Flavourzyme) to generate the flaxseed protein hydrolysates with the lowest bitterness taste and improved Maillard peptide content. The latter can be used in MRPs to generate appropriate flavor characteristics.

2. Materials and methods

2.1. Materials and chemicals

Initially, flaxseeds (*Linum usitatissimum* L.) were procured from the local market of Xi'an city, Shanxi, China. The obtained seeds were crushed and passed through a 20-mesh sieve. During this process, moisture content was adjusted to 7–8% and the flaxseed cake was collected through the oil press. The defatted flaxseed meal obtained after the oil extraction was dried at 60 °C until the final moisture content ≤4% The dried meal was grounded to obtain fine powder which was stored at 4 °C. Alcalase and Flavourzyme were purchased from Novozymes (Beijing, China).

2.2. Preparation of Alcalase and Flavourzyme

The immobilized Alcalase and Flavourzyme enzymes were prepared as per the procedure given in previous study (Wang et al., 2016). Briefly, 50 mL, 1.1×10^4 U of Alcalase and 50 mL, 0.13×10^4 U of Flavourzyme were mixed individually with 150 mL of 3.0% of sodium alginate with continuous stirring and flow through a peristaltic pump and subsequent drop from 10 cm height into 400 mL of 3.0% $CaCl_2$ solution to get the gelatinous beads. The beads with uniform particle size and regular shape were washed with deionized water and kept at 4 °C for further use.

2.3. Determination of enzyme activity

The Alcalase and Flavourzyme were targeted for their proteolytic activities as per the previous method (Wang et al., 2016). For immobilization of two enzymes, mixtures of 0.25 mL of casein solution (2%, w/v of casein in 0.2 mol/L buffer) and 0.25 mL of each enzyme solution such as Alcalase and Flavourzyme were incubated at 60 °C and 50 °C for 10 min, respectively. The reaction was ended by addition of 0.5 mL of 0.4 mol/L of $C_2HCl_3O_2$. The resulting suspension was then centrifuged at 10,000 r/min for 15 min at 4 °C. Then after, 0.25 mL of collected supernatants for each enzyme was mixed with 1.25 mL of 0.4 mol/L sodium bicarbonate and 0.25 mL of 0.4 mol/L Folin-Ciocalteu phenol Reagent and incubated for 30 min at 60 °C and 50 °C, respectively. The resulting mixture was used for absorbance measurement at 660 nm. In our study, one unit of protease (U) represented amount of enzyme required for the color equal to 1.0 μmol/L of tyrosine in 1.0 mL of reaction solution per minute at 60 °C and 50 °C, respectively. Enzyme activity (U/g) was calculated as substrate concentration.

2.4. Preparation of flaxseed protein hydrolysates

Briefly, 20 g of defatted flaxseed meals were suspended into distilled water (DW) to obtain diverse proportions. The samples were pre-treated at 85 °C for 30 min. The pH of suspension was accustomed with 1.0 mol/L of sodium hydroxide after cooling to desired temperature prior to enzymatic hydrolysis. The immobilized enzymes were recovered individually after enzymatic hydrolysis was completed. The precipitates were separated through centrifugation at 10,000 r/min for 20 min at 4 °C. The resulting supernatants were collected separately for each enzyme and then freeze-dried to get flaxseed protein hydrolysates.

2.5. Preparation of MRPs

For this, 2.0 g of flaxseed protein hydrolysates, 1.0 g of D-xylose, and an appropriate amount of L-cysteine (0.55 g) were mixed with DW with an aim to obtain the final concentration of 10% (w/v). The resulting suspensions were poured into three neck flasks (100 mL) followed by pH adjustment to 7.4 and heating in a thermostatic oil bath with magnetic stirring at 120 °C for 2 h and then immediate cooling in iced water. The final samples were stored at -20 °C until further use.

2.6. Physico-chemical analysis methods

Contents of moisture, fat, protein, and crude fiber were determined according to AOAC Method (Thiex, 2009).

2.7. Determination of degree of hydrolysis (DH) and protein recovery (PR)

DH relates to cleavage of free amino groups from proteins and it can be determined from the ratio of α-amino nitrogen to total nitrogen. For calculation of this ratio, formaldehyde titration method was used; whereas, Kjeldahl method (the conversion factor is 5.30 for protein) was followed for determination of total nitrogen content. PR was evaluated in the form of the ratio of protein content (Nitrogen percentage × 5.30) available in the hydrolysates compared to the amount in the reaction mixtures (Pagán, Ibarz, Falguera, & Benítez, 2013).

2.8. Sensory characterization

For sensory evaluation, scores given by well-trained team of 14 personnel (8 females and 6 males) between the age group of 23–28 were applied under suitable laboratory environment for evaluation of the flavor characteristics generated in various MRPs. Before the final descriptive sensory study, aroma and taste characteristics of samples through three preliminary sessions were meticulously discussed. In total, eight descriptions, including meat-like; umami; salty; caramel-like; bitterness and "Kokumi" flavor consisting of mouthfulness; continuity (long-lasting taste development) and total acceptance were used for the descriptive analysis. The control samples such as 10.0 g of bouillon cube for "mouthfulness and continuity" attributes, defatted beef brisket for "meat-like" attribute, 2.5 g of burning white sugar for caramel-like aroma, 4.0 mmol/L of monosodium glutamate (MSG) for umami taste, 2.0 mmol/L of NaCl for salty taste and 1.5 mmol/L of Caffeine for bitter taste were used for sensory evaluation. The MRPs sample solutions (0.5%, w/w) were suspended into umami solution (1.0% (w/v) MSG and 0.5% (w/v) NaCl) as per the previous descriptions (Masashi Ogasawara, Katsumata, & Egi, 2006; M gasawara, Yamada, & Egi, 2006). For sensory analysis, 30 mL of samples were served in opaque disposable plastic cups simultaneously to minimize the temperature differences which may influence the evaluation. The samples were randomly coded with three-digit numbers for presentation to each panel to obtain more clarity in judgment.

2.9. MW distribution

High-performance gel-filtration chromatography was used for estimation of MW distribution profiles of the samples (Eric et al., 2013). Waters e2695 Alliance HPLC system (Waters, Milford, MA, USA) equipped with 2487 UV detector and Empower workstation was used with a column specification such as TSK gel 2000 SWXL 7.8 i.d. × 300 mm (Tosoh Co., Tokyo, Japan) at 30 °C. The mobile phase consisted of acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v) which was delivered at a flow rate of 0.5 mL/min with a sample injection volume of 10.0 μL. After preparation of calibration curve using different standards, the obtained data was analyzed using gel permeation chromatography software (Eric et al., 2013).