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Identification and hydrolysis kinetic of a novel antioxidant peptide from pecan meal using Alcalase

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ABSTRACT

In this study, the hydrolysis of a pecan protein isolate (PPI) with Alcalase was carried out to generate antioxidant peptides. We proposed a kinetic model to illustrate the enzymolysis process of PPI, which was found suitable for depiction of the kinetic behavior for PPI hydrolysis by Alcalase. The PPI hydrolysis products were gradually fractionated by ultrafiltration through cut-off membranes with molecular weights of 10, 5 and 3 kDa and their antioxidant activities were evaluated *in vitro*. Further, the strongest antioxidant fraction (< 3 kDa) and novel antioxidative peptide were successfully purified. The amino acid sequence of the purified peptide was identified as LAYLQYTDFETR. The purified fraction exhibited appreciable scavenging activities on ABTS radical (67.67%), DPPH radical (56.25%) and hydroxyl radical (47.42%) at 0.1 mg/mL. The results suggested that this novel peptide may serve as a potential antioxidant and it should be evaluated for development of functional foods and pharmaceuticals products.

1. Introduction

Past years have witnessed a huge concern towards the lipid profile of various nuts because they seem to be an affluent reservoir of bioactive components with significant health effects (Bouali et al., 2014). Many reports have proved that materials such as oils, proteins, phenolics extracted from nuts harbor appreciable antioxidant attributes. Moreover, nuts have occupied various new prospects in food industry due to the growing consumer's demand for functional food with value added benefits (Silva, Hernández-Ledesma, Amigo, Netto, & Miralles, 2017).

Among the nuts, pecan (*Carya cathayensis*) has been recognized as one of the most popular and important species of Carya planted in China with an abundant nutritional value comprising majority of oil content followed by protein and dietary fibers (Jiang & Wang, 2016). Due to the presence of unsaturated fatty acids, phytosterols, tocopherols and other micronutrients, pecan oil was reported to decrease the risk of obesity and oxidative stress (Domínguez-Avila, Alvarez-Parrilla, López-Díaz, Maldonado-Mendoza, & Rosa, 2015; Zhang et al., 2017). As a result, increased focused has been given on pecan oil production in past years. After the oil extraction, the residues are the main members of the food industry waste which are often discarded and ultimately lead to important environmental pollution and various health risks. Interestingly, these waste residues have also been used as animal feed due to low cost (González-García, Puchalska, Marina, & García, 2015). It is reported that the pecan meal contains abundant proteins which range from 30% to 40% and these proteins constitute most of the essential amino acid for human consumption. Therefore, to enhance the economical value in the market, more attention should be drawn for the development of pecan meal products by biotransformation of waste residues. These changes will lead to reduced environmental pollution and also aid in minimizing the wastage of vital protein. Generally, bioactive peptides constitute small 3–20 amino acid residues and possess different physiological functions (Chen, Yang, Sun, Niu, & Liu, 2012). The antioxidant peptides have been purified and identified from various plant sources such as walnut (*Juglans regia* L.), corn gluten meal (Zhuang, Tang, & Yuan, 2013), chickpea (*Cicer arietinum* L.) (Ghribi et al., 2015), Struthio camelus egg white (Asoodeh, Homayouni-Tabrizi, Shabestarian, Emtenani, & Emtenani, 2016), and Pinto beans (Ngoh & Gan, 2016) protein hydrolysates. The obtained peptides have demonstrated potent anti-oxidative, α-amylase inhibitory activities as well as antithrombotic and antimicrobial activities. The above reports have proved the immense nutraceutical value of resulting protein hydrolysis products which can be considered beneficial for improvement of human health.

Oxidation of biomolecules is an essential reaction in all the living organisms which results into release of free radicals. Excessive amounts of these free radicals may induce numerous harmful impacts on food

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and biological systems and give rise to some chronic diseases. These free radicals behave as highly reactive species in human body due to loss and imbalance of electrons. At present, many synthetic antioxidants such as BHT (butylatedhydroxytoluene), BHA (butylatedhydroxyanisole) are being used in food industry to prevent lipid peroxidation. However, these synthetic antioxidants may pose some health risks to a certain extent if consumed in inadequate amount. Many researchers are engaged in identifying and characterizing the potential of non-hazardous natural antioxidants in different oxidative systems (Zhang, Li, Miao, & Jiang, 2011). Various protein sources have been explored to extract the peptide sequences with proven potent antioxidant action (Je, Byun, & Kim, 2007; Kim, Je, & Kim, 2007) Therefore, it is necessary to develop new natural antioxidant substitutes because bioactive peptides derived from plant proteins have many advantages over synthetic ones. The natural peptides can be used as nutritional supplements and natural antioxidants in oxidative stress management. Though, there are few studies revealing novel peptides with anti-oxidant attributes, but there is no existing report for peptides extraction from pecan meal. Therefore, we have isolated the pecan protein from pecan meal and later hydrolyzed them by alkaline protease which resulted into bioactive peptides. The obtained peptide fractions were subjected to ultrafiltration, anion exchange column and Sephadex column chromatography. The fractions were evaluated for antioxidant potential by using various assays and then amino acid sequence of selected antioxidant peptide fraction was determined by mass spectrometry.

2. Materials and methods

2.1. Materials

Defatted pecan seed meals were obtained from Zhan Shi food company at Ning Guo Industry, Anhui Province, China. Alcalase (62,000 U/g) was purchased from Aladdin, USA; whereas, acetonitrile and trifluoroacetic acid (HPLC grade), and other reagents (analytical grade) were purchased from Sigma-Aldrich (Malaysia).

2.2. Preparation of protein from pecan meals

The pecan meals were dried in a tray drier with air circulation at 55 °C, then crushed into powder with a mill and finally grounded by passing through 60 mesh. The resulting pecan meal powder was dissolved into distilled water (DW) at 90 °C for 30 min followed by cooling at room temperature. The pH of the homogenate was adjusted to 10.0 with NaOH (0.1 mol/L) at 55 °C for 3 h. Then after, it was subjected to centrifugation using tubular centrifuge (8,000 r/min) for 20 min. The pH of collected supernatant was adjusted to 4.0 with HCl (0.1 mol/L) after 3 h. The supernatant was further centrifuged at 8,000 r/min for 20 min to obtain the proteins followed by lyophilization and storage at 4 °C until next use.

2.3. Preparation of pecan protein hydrolysate (PPH)

The pecan protein hydrolysis was performed according to previously described method (Evangelho et al., 2017). Briefly, the pecan meal proteins were dissolved into DW at the ratio of 1:25 (W/V) followed by stirring at 100 °C for 10 min. Then after, the resulting solution was cooled to 55 °C and pH was adjusted to 10.0 prior to addition of Alcalase. The enzyme: substrate ratio of 1:20 (w:w) was maintained with a reaction time of 180 min. The hydrolysates were immediately heated under 100 °C for 10 min to stop the enzyme reaction. Subsequently, the hydrolysates were centrifuged at 10,000 r/min for 15 min and the obtained supernatants were lyophilized.

2.4. Degree of hydrolysis (DH)

The pH-stat method was followed for determination of DH (Yang, Li, Lin, Zhang, & Chen, 2017) and following equation was used for its calculation:

$$\mathrm{DH} = \frac{B \times N_b}{\alpha \times M_p \times h_{tot}} \times 100\% \quad (1)$$

Where, B represented the NaOH volume consumption (mL); N_b, the molarity of NaOH solution; parameter "α" was the average degree of dissociation of α-NH_2 group in protein isolate substrate; Mp, the mass of hydrolyzed protein (g); h_{tot}, the total number of peptide bonds in the protein.

2.5. Hydrolysis kinetic

2.5.1. Enzyme hydrolysis

Pecan protein isolate (PPI) was hydrolyzed by using Alcalase (2.0 mg/mL) at substrate concentrations of 20, 40, 60 and 80 mg/mL. The substrate concentration (40 mg/mL) was hydrolyzed to analyze the effect of enzyme concentrations (0.6, 0.8, 1.0 and 1.2 mg/mL) on DH. The remaining hydrolysis conditions were maintained as follow: hydrolysis temperature of 55 °C, pH at 10.0 and hydrolysis time of 0–180 min. The DHs for 30 min interval were calculated to estimate the hydrolysis kinetic for PPI.

2.5.2. Hydrolysis kinetic model

The protein hydrolysis reaction could be indicated as: $E+ S \overset{k_1,k_{-1}}{\longleftrightarrow} ES \overset{k_2}{\rightarrow} E + P$; where, E, S, ES and P represented free enzyme, substrate, enzyme-substrate complex and product, respectively; where, k_{-1} represented the rate constant of reverse reaction and k_2 corresponded to the rate constants of reaction. The reaction rate was calculated by the irreversible process and expressed as Eq. (2).

$$R = S_0 \times \frac{dDH}{dt} = k_2[ES] \quad (2)$$

Where, S_0 was initial substrate concentration and 't' was reaction time. If the mechanism of enzyme passivation reaction was $E+ ES \overset{k_3}{\rightarrow} E_\alpha + E_p + S$. ($E_a$ represented as the active form of enzyme; E_p represented the passivation form of enzyme) and the kinetic equation of this process was as follow:

$$-\frac{de}{dt} = k_3[E][ES] \quad (3)$$

Where, 'e' was the total enzyme. Combination of Eq. (2) and Eq. (3) provided the ratio as follow:

$$-S_0\frac{dDH}{de} = \frac{k_2}{k_3[E]} \quad (4)$$

When enzyme was inhibited, the reactions could be expressed as $S+ ES \overset{K_S}{\leftrightarrow} SES$ and $E+ P \overset{K_p}{\leftrightarrow} EP$, $K_S = k_{-4}/k_4$ and $K_p = k_{-5}/k_5$. At the approximate steady condition, the balance for enzyme-substrate complex resulted into the following equation:

$$[SES] = \frac{[S][ES]}{K_S} = \frac{[S]^2[E]}{K_M K_S} \quad (5)$$

$$[EP] = \frac{[E][P]}{K_P} = \frac{K_M[ES][P]}{K_P[S]} \quad (6)$$

$$[ES] = \frac{[E][S]}{K_M} \quad (7)$$

The total enzyme concentration could be displayed as the following form at a given condition:

$$e = [E] + [ES] + [SES] + [EP] \quad (8)$$