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Effects of rice protein hydrolysates prepared by microbial proteases and ultrafiltration on free radicals and meat lipid oxidation

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ABSTRACT

This study was to evaluate whether rice protein hydrolysates with strong antioxidant activity can be used to prevent lipid oxidation and improve shelf-life of meat products. Rice proteins were hydrolysed by three microbial proteases and further separated by sequential ultrafiltration to 12 hydrolysate fractions. The resulting hydrolysates were first evaluated for free radical scavenging capacity. The oxygen radical absorbance capacity (ORAC) of the fractions varied significantly ranging between 34.2 and 87.3 μmol Trolox equivalents (TE)/g dry weight with two small peptide fractions: Val-F3 produced by Validase from *Aspergillus oryzae* and AP-F3 produced by alkaline protease from *Bacillus licheniformis* possessing the highest values. The hydrolysate fractions at 100 mg/mL scavenged 31.2–49.7% of 1,1-diphenyl-2-picrylhydrazyl radicals ($DPPH^{\bullet}$). The hydrolysates also exerted remarkable cation radical 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+\bullet}$) scavenging activity (10.8 and 28.3 μmol TE/g dry weight). Val-F3 and AP-F3 were incorporated into ground beef to determine their effect on lipid oxidation during a 15-day storage period. Val-F3 treatment at 500 μg/g inhibited lipid oxidation by 19% and 15% at storage day 8 and 15, suggesting that rice protein hydrolysates could be further developed and used to improve shelf-life of meat products.

1. Introduction

Unsaturated lipids deteriorate in food products during processing, handling, and storage since their oxidation can be catalysed by heat, light, and trace metals as well as enzymatically by lipoxygenase. Lipid oxidation therefore is the major cause of the development of rancidity and a number of other byproducts that reduce shelf-life and nutrient value of food products (Frankel, 1996; Shahidi & Zhong, 2008). For this reason, natural and synthetic antioxidants are used in lipid-rich food products to retard lipid oxidation with the former being the most common due to its effectiveness and low cost (Huber, Pike, & Huber, 1995). However, there are increased concerns over the safety of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) (Gharavi, Haggarty, & El-Kadi, 2007; Williams, Iatropoulos, & Whysner, 1999), which have prompted food scientists to identify and develop new natural and cost-effective antioxidants. Food protein hydrolysates could be such a promising source of non-toxic natural antioxidants. A number of studies showed that protein hydrolysates or specific peptides produced from milk, zein, and soybean proteins exerted significant antioxidant properties such as scavenging free radicals, chelating transitional metals, inhibiting lipid peroxidation in food products and *in vitro* lipid-rich models (Cervato, Cazzola, & Cestaro, 1999; Diaz, Dunn, McClements, & Decker, 2003; Kong & Xiong, 2006).

Rice is a principle food source for approximately one-half of the world's population (Pimentel, Wilson, McCullum, & Huang, 1997). As a staple food, rice not only supplies body with energy but also provides essential and unique micronutrients such as vitamins, minerals, and phenolic antioxidants. Rice is rich in a specific group of flavonoids and other unique compounds that have been found to exert significant free radical scavenging activities and inhibit cholesterol oxidation *in vitro* (Qiu, Liu, & Beta, 2009; Xu, Hua, & Godber, 2001). Although rice has a relatively low protein content but rice protein is superior in lysine content which is a limiting essential amino acid in cereal proteins (Schaeffer & Sharpe, 1987). Furthermore, the main rice protein does not contain gliadin, a specific protein found in the cereal grains responsible for allergic gastrointestinal tract coeliac disease (Dieterich et al., 1997). Rice protein consists mainly of alkali-soluble glutelins and water-soluble albumins (Lim, Lee, Shin, & Lim, 1999). These protein components have unique emulsifying and gelling properties. There

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is limited research on the preparation of rice protein hydrolysates and their functional properties. A recent study found that rice protein hydrolysates prepared by alcalase hydrolysis exerted an antihypertensive effect in spontaneously hypertensive rats involving the inhibition of angiotensin I-converting enzyme (Li, Qu, Wan, & You, 2007). The objective of this study is to evaluate whether rice protein can be used to produce specific hydrolysates/peptides with strong antioxidant activity that may be used for improving quality and shelf-life of meat products. In this study, we selected 3 commercial microbial proteases: neutral protease from *Bacillus subtilis*, validase from *Aspergillus oryzae*, and alkaline protease from *Bacillus licheniformis* for the investigation. We choose microbial proteases because proteases from microbial sources are more renewable than the plant and animal proteases and they also possess the characteristics desired for their biotechnological applications (Rao, Tanksale, Ghatge, & Deshpande, 1998). Most commercial neutral proteases are produced by organisms belonging to the genus *Bacillus* (Feder & Schuck, 1970). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity (Kumar & Takagi, 1999). The selected microbial neutral and alkaline proteases are commonly used for food protein digestion but with considerably different substrate specialities and reaction characteristics. After protease digestion, the protein hydrolysates were fractionated through sequential ultrafiltration to obtain different fractions and their antioxidant properties were assessed by various methods including ORAC, DPPH$^{\bullet}$ and ABTS$^{\bullet}$ scavenging activities. Those fractions with strong antioxidant activities were selected for further antioxidant assessment against lipid peroxidation in cooked ground beef during a 15-day storage period.

2. Material and methods

2.1. Materials

The rice protein isolate (*Oryza sativa L.*) was obtained from EnerGenetics International, Inc. (Keokuk, IA). Protease Validase® FP concentrate and Alkaline Protease concentrate was kindly provided by Valley Research Corporate (South Bend, IN). Neutral Protease (NP) was provided by Bio-CAT Inc (Troy, VA). Val is a fungal peptidase complex produced by submerged fermentation of a selected strain of *A. oryzae* and contains both endoprotease and exopeptidase activities. The AP is a serine alkaline protease prepared from *B. licheniformis* which possesses endopeptidase activity. The NP is an extracellular endopeptidase produced from *B. subtilis*. These enzymes are active in the neutral pH range. These proteases were selected because they have been commonly used in industrial processes with high-quality thermostability. Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), and DPPH were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 2,2′-azobis(2-amino-propane) dihydrochloride (AAPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was purchased from Wako Chemicals (Richmond, VA). All other chemicals and solvents were of analytical or HPLC-grade. The ultrafiltration system (Model No. 8050) and cellulose membranes for the preparation of protein hydrolysates were purchased from Millipore Co. (Billerica, MA).

2.2. Preparation of rice protein hydrolysates

Three microbial proteases, Val, AP, and NP, were used to hydrolyse the rice protein isolate according to the peptide guidelines from the manufacturer with slight modification. The protein was first suspended and homogenized in water at 50 g/500 g and then hydrolysed by the individual proteases at a concentration of 1 g/50 g (enzyme/substrate). The enzymatic hydrolysis was conducted for 6 h in a water bath at 55 °C under shaking. For Val and NP, the reaction mixture was adjusted to pH 7.0 using 0.5 mol equi/L NaOH or 0.6 mol equi/L HCl during hydrolysis. For AP, the solution was adjusted to the optimum pH 10. The enzymatic reactions were terminated by boiling the mixtures for 5 min. Each mixture was centrifuged at 15,000× *g* and the soluble fraction was then filtered using filtration paper (Whatman 4) for further fractionation.

2.3. Determination of the degree of hydrolysis (DH)

The DH of rice protein by the three proteases was determined via the trinitrobenzenesulfonic acid (TNBS) reaction according to a previously established protocol (Zhang, Li, & Zhou, 2010).

2.4. Fractionation of protein hydrolysates by ultrafiltration

The collected protein hydrolysates were ultra-filtered sequentially using a Millipore 8050 ultrafiltration unit through cellulose membranes with different molecular weight (MW) limits. The hydrolysates were diluted with water and ultra-filtered through a membrane with 10 k Dalton (kDa) molecular weight-cut-off (MWCO) under 276 kPa nitrogen gas to afford two fractions: retentate (fraction 1, F1, represented hydrolysates >10 kDa) and permeate (MW < 10 kDa). The permeate was further ultra-filtered through a 3 kDa MWCO membrane to obtain the second retentate (fraction 2, F2, represented hydrolysates between 3 and 10 kDa) and permeate. The permeate was further ultra-filtered through a 1 kDa MWCO membrane to yield the third retentate (fraction 3, F3, represented hydrolysates between 1 and 3 kDa) and permeate (fraction 4, F4, represented hydrolysates <1 kDa). All retentates and permeates were lyophilized and stored at −20 °C until further analysis.

2.5. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity of peptides with Trolox as the antioxidant standard (Hogan, Zhang, Li, Wang, & Zhou, 2009). Fluorescein was used as the fluorescent probe and the peroxyl radicals were generated from AAPH in 75 mmol/L phosphate buffer (pH 7.4). The ORAC value was expressed in micromoles of Trolox™ equivalents per gram dry weight hydrolysates (μmoles TE/g).

2.6. Measurement of DPPH$^{\bullet}$ scavenging activity

The assay was conducted according to the previously reported procedure using the stable DPPH$^{\bullet}$ (Zhou, Yin, & Yu, 2005). A reaction mixture containing 100 μL of each sample and 100 μL of 0.2 mmol/L DPPH$^{\bullet}$ solution were prepared. The absorbance at 517 nm was measured against a blank of deionized water at 40 min and 50 mmol/L ascorbic acid and BHT were used for comparison.

2.7. Measurement of ABTS$^{\bullet+}$ scavenging activity

The assay was performed according to a previously reported protocol (Zhou, Laux, & Yu, 2004). ABTS$^{\bullet+}$ was prepared by oxidizing a 5 mmol/L aqueous solution of ABTS with manganese dioxide at ambient temperature for 30 min. The reaction mixture contained 1.0 mL of ABTS$^{\bullet+}$ with an initial absorbance around 0.7 at 734 nm and 80 μL of the protein hydrolysates or Trolox as an antioxidant standard. The absorbance at 734 nm was measured following 1 min of the reaction, and the Trolox equivalent was calculated using a standard curve prepared with Trolox.