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Antioxidant and Structural Properties of Tilapia Protein Hydrolysates-Calcium Complex

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Abstract: Tilapia (*Oreochromisniloticus*) is a fairly low value fish. In this study, the Tilapia Protein Hydrolysates (TPH) were obtained by proteolysis of tilapia muscle protein and then the TPH binded with calcium ions to form TPH-Calcium Complex (TPHCC). The antioxidant and structural properties of TPH and TPHCC were investigated. The results showed that the TPH possessed antioxidant properties and calcium-binding capacities. After TPH chelated with calcium ions to form the TPHCC, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and the reducing power was not significantly ($p<0.05$) decreased but the lipid peroxidation inhibition activity was improved. The scanning electron micrographs and FTIR spectra demonstrated that the micro structure of the TPHCC was obviously different from that of the TPH and the primary binding sites for calcium ions in TPH were the carboxyl oxygen atoms and amino nitrogen atoms.

Key words: Tilapia, hydrolysates, calcium, antioxidant properties, structural properties

INTRODUCTION

Reactive Oxygen Species (ROS) and free radicals can interact with cellular biomolecules such as DNA, proteins and membrane lipids leading to cell and tissue injury or death (Tahara *et al*., 2001; Moskovitz *et al*., 2002; Calabrese *et al*., 2005). Accordingly, it is important to find new antioxidants from natural resources in order to inhibit oxidation and formation of ROS and free radicals occurring in the living organism. In recent years, proteins hydrolysates from many plant and animal sources have been found to possess strong antioxidant activities such as chickpea (Li *et al*., 2008), hey (Peng *et al*., 2009), silver carp (Zhong *et al*., 2011) and croaker (Nazeer *et al*., 2012).

Tilapia (*Oreochromisniloticus*) is an important species in fresh water aquaculture which is the third most widely cultured fish after carp and salmonids (El-Sayed, 2006). China is one of the major farmed tilapia producing countries. The production of tilapia in China is the highest world and the tilapia is usually processed into frozen tilapia fillet. Recently, hydrolysates derived from tilapia protein have been found to possess antioxidant properties (Ngo *et al*., 2010; Zhang *et al*., 2012). Therefore, proteolysis can be considered as a reliable way to produce value-added products of tilapia.

Calcium is an essential element for living body which plays a very important role in intracellular metabolism, bone health, blood clotting, nerve conduction, muscle contraction and cardiac functions (Anderson and Garner, 1995; Bass and Chan, 2006). Previous studies reported that mineral salts used as the mineral supplements had low bio availability and could cause some side effects (Megias *et al*., 2007; Lee and Song, 2009). It is anticipated that Tilapia Hydrolysates (TPH) binded with calcium ions might be a good calcium supplement. However, limited study regarding the effect of calcium-binding on the antioxidant properties of hydrolysates, has been done. The objective of this study was to investigate the antioxidant and structural properties of the TPH-calcium complex.

MATERIALS AND METHODS

Live tilapia (*Oreochromisniloticus*) was purchased from a local market in Guangzhou, China and transported to our laboratory within 20 min. After being sacrificed, the meat (without head, tail, skin, bone and blood) was collected and minced twice through a meat mincer (MM12, Shaoguan Food Machine Co., Shaoguan, China). The minced meat was frozen and stored at 20°C for further use.

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Papain with a nominal activity of 8×10^5 U g^{-1} was obtained from Qiyun biological Technology Co., Ltd. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Linoleic acid was purchased from Feibo Biology Technology Co. (Shanghai, China). All other chemicals and solvents were of analytical grade.

Preparation of Tilapia Protein Hydrolysates (TPH): Fifty gram of tilapia meat was mixed with 100 mL distilled water and homogenized at 10,000 rpm for 1 min using a basic homogenizer (T25, Ika, Staufen, Germany). The homogenate was hydrolyzed with 0.3% papain (w/w, enzyme/substrate) at 55°C for 4 h in a water bath shaker. After hydrolysis, the solutions was inactivated in boiling water for 15 min. The hydrolysates were then centrifuged in a refrigerated centrifuge (3K30, Sigma Laboratory, Osterode, Germany) at 6000 g for 20 min and the supernatants were lyophilized (Alpha 1-4 LSC, Marin Christ, Osterode, Germany) and stored in a desiccator for further use.

Preparation of TPH-Calcium Complexes (TPHCC): TPH was dissolved in deionised water and mixed with $CaCl_2$ at various mass ratios. The dispersions were adjusted to pH 7 with NaOH and then the mixtures were incubated at 37°C for 40 min. After binding reaction, the resulting solutions were dialysed with a 100 Da molecular-weight cut-of semipermeable membrane (Spectrum Medical Industries, Houston, TX, USA) to remove the free Ca^{2+}. TPHCC I, TPHCC II and TPHCC III represented the TPH binded with calcium ions at the mass ratios (TPH/$CaCl_2$) of 5:1, 10:1 and 20:1, respectively. The content of the calcium ions was analyzed via flame atomic absorption spectrometry (AA240FS, Varian Inc., USA). Calcium binding rate was calculated asfollows:

$$\text{Calcium binding rate (\%)} = \frac{M_1}{M_2}\times100$$

where, M_1 is the calcium content in complex (g), M_2 is total calcium content in solution before binding (g).

DPPH radical scavenging activity assay: DPPH radical scavenging activity was determined according to the method of Wu *et al.* (2003) with a slight modification. Two milliliters of sample and 2 mL of 0.15 mM DPPH were mixed. The mixture was placed in the dark at room temperature for 30 min. The absorbance of the resultant solution was recorded at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = 1-\frac{A_{\text{DPPH sample}}-A_{\text{Sample control}}}{A_{\text{DPPH blank}}}\times100$$

where, $A_{\text{DPPH sample}}$ is the value for 2 mL of sample solution mixed with 2 mL of DPPH solution, $A_{\text{sample control}}$ is the value for 2 mL of sample solution mixed with 2 mL of 95% ethanol, $A_{\text{DPPH blank}}$ is the value for 2 mL of 95% ethanol mixed with 2 mL of DPPH solution.

Reducing power assay: Reducing power activity was determined according to the method of Ahmadi *et al.* (2007) with a slight modification. One milliliter of each sample was mixed with 2.0 mL phosphate buffer (0.2 M, pH 6.6) and 2.0 mL potassium ferrocyanate (1%, w/v). The mixture was incubated at 50°C for 20 min. Then, 2.0 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 1500 g for 10 min. The supernatant (2.0 mL) was mixed with 2.0 mL distilled water and ferric chloride (0.4 mL, 0.1%) and the absorbance was measured at 700 nm after incubation at 50°C for 10 min. An equivalent volume of distilled water instead of the sample was used as the blank.

Lipid peroxidation inhibition activity assay: Lipid peroxidation inhibition activity was determined in a linoleic acid emulsion system according to the method of You *et al.* (2010). Briefly, 2 mL of 2.5% linoleic acid, 4 mL sodium phosphate buffer (50 mM, pH 7.0) and 2 mL distilled water were successively added to a 2.0 mL sample. The mixture was incubated in a screw-capped round tube at 40°C in the dark. The degree of linoleic acid oxidation was evaluated at specified time intervals by measuring the FeSCN values described below. A 0.1 mL reaction solution incubated in the linoleic acid model system was mixed with 75% ethanol, 30% NH_4SCN and 20 mM $FeCl_2$ solution in 3.5% HCl. After 3 min, the degree of color development, representing the linoleic acid oxidation, was measured at 500 nm. An equivalent volume of distilled water instead of the sample was used as the blank:

$$\text{Lipid peroxidation inhibition activity (\%)} = 1-\frac{A_{S,168h}-A_{S,0h}}{A_{0,168h}-A_{0,0h}}\times100$$

where, $A_{S,\,168h}$ and $A_{S,\,0h}$ are the absorbances of the sample at168 and 0 h, respectively $A_{0,\,168h}$ and $A_{0,\,0h}$ are the absorbances of the blank at 168h and 0h, respectively.

Microstructure: The samples were deposited on silicon wafers and coated with a conductive material to ensure sufficient electron refraction. Then, the samples were mounted onto an aluminum stub with epoxy and coated with a gold platinum alloy in a sputter-coating device. The samples were observed by a scanning electron microscope (QUANTA 400F Field Emission SEM) at an operating voltage of 20 kV and high vacuum.

Fourier transform infrared (FTIR) spectra: One milligram of sample was mixed with 100 mg of KBr and ground gently with an agate pestle and mortar under an infrared lamp. The powder was pressed into a 13 mm diameter disk by applying 15 tons of pressure for 2 min. FTIR spectra were recorded using an Equinox 55 FTIR spectrophotometer (Bruker, Wissemburg, France) over a wavenumber region between 400-4000 cm^{-1}.

Statistical analysis: All analyses in this study were conducted in triplicate with the values reported as the Mean±SD. The data of the experiments were subjected to one-way analysis of variance (ANOVA) using SPSS version 13.0 for Windows (SPSSInc.).

RESULTS AND DISCUSSION

Calcium-binding rate: The calcium-binding rate of samples is shown in Fig. 1. The calcium -binding rate of TPHCC III was higher than that of TPHCC I and TPHCC II, suggesting that the increase of the mass ratios (TPH/$CaCl_2$) from 5:1 to 20:1 could facilitate the TPH chelated with calcium ions. Similarly, Wang *et al.* (2011) found the ferrous binding capacity was improved when the mass ratio of yak casein hydrolysate to $FeSO_4$ was increased from 1:1 to 15:1.

DPPH radical scavenging activity: It is well known that antioxidants can interact with free radicals and form stable species which terminate the oxidation. DPPH has been used extensively as a free radical to evaluate reducing substances. The DPPH radical scavenging activity of samples is shown in Fig. 2. The DPPH radical scavenging activities of TPHCC I, II and III were not significantly ($p>0.05$) lower than that of TPH. The results indicated that the biological material of TPH-calcium complexes obtained under the selected chelating condition could also act as a good electron donor and react with free radicals to terminate the radical chain reaction.

Reducing power activity of the sample: For the reducing power assay, the presence of antioxidants could result in reducing the Fe^{3+}/ferricyanide complex to the ferrous form and the high absorbance suggests a strong reducing power. As shown in Fig. 3, the reducing power of TPHCC II and III was higher than that of TPHCC I. Besides, the reducing power of all the TPH-calcium complexes was not significantly ($p>0.05$) lower than that of TPH. The result was consistent with the data of the DPPH radical scavenging activity.

Lipid peroxidation inhibition activity of the sample: Figure 4 shows lipid peroxidation inhibition activity of samples. At the end of the 7 day lipid peroxidation, the inhibition rates were 39.7, 61.6, 80.4 and 81.3% for TPH,

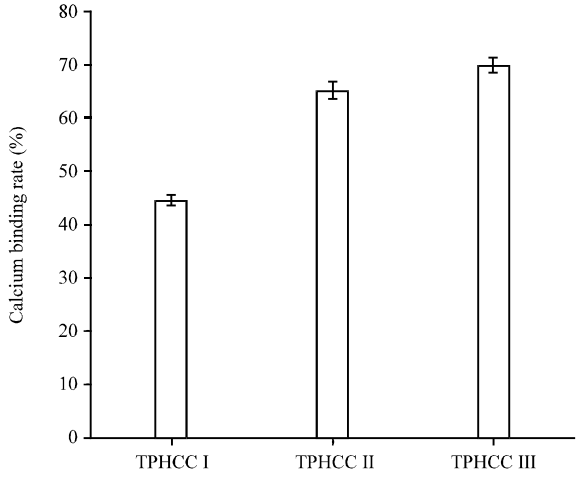

Fig. 1: Calcium-binding rate of TPH-calcium complexes

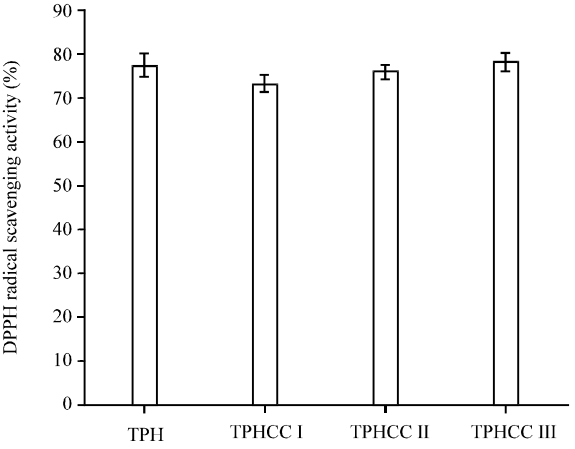

Fig. 2: DPPH radical scavenging activity of TPH and TPH calcium complexes

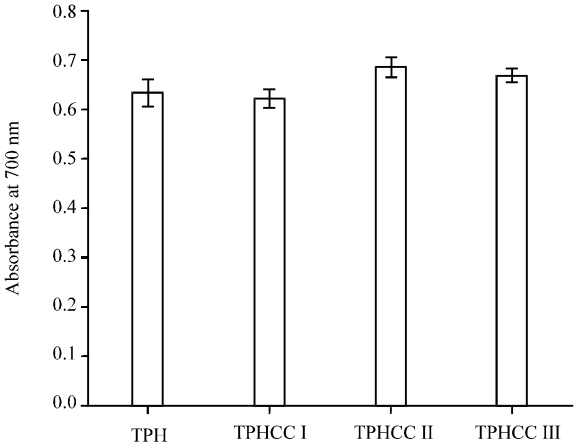

Fig. 3: Reducing power activity of TPH and TPH calcium complexes

TPHCC I, TPHCC II and TPHCC III, respectively. The lipid peroxidation inhibition of TPH calcium complexes was significantly ($p<0.05$) higher than that of TPH. Deng *et al.* (2008) also found that the biological material of protein hydrolysates chelated with ferrous ions could improve the antioxidant activity of the hydrolysates in the linoleic acid system. This might be due to the chelation resulting in the increased hydrophobicity of the hydrolysates.

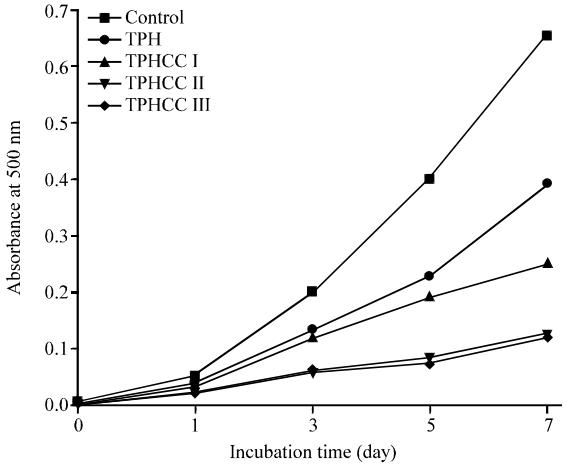

Fig. 4: Lipid peroxidation inhibition activityof TPH and TPH calcium complexes

Microstructure: The microstructures of TPH and TPH calcium complex were investigated by scanning electron microscopy at a magnification factor of 250 and 2000 fold as shown in Fig. 5. TPH exhibited a complex heterogeneous structure consisting of clumps in different sizes and shapes (Fig. 5a). For the TPH-calcium complex, the binding reaction induced the decrease of small clumps and the formation of large clumps (Fig. 5c). Moreover, a number of white spots could be observed in the chelate (Fig. 5d) which might be the chelated calcium ions. The changes in the microstructure were suggested that TPH binded with calcium ions to form the higher molecular weight conjugate.

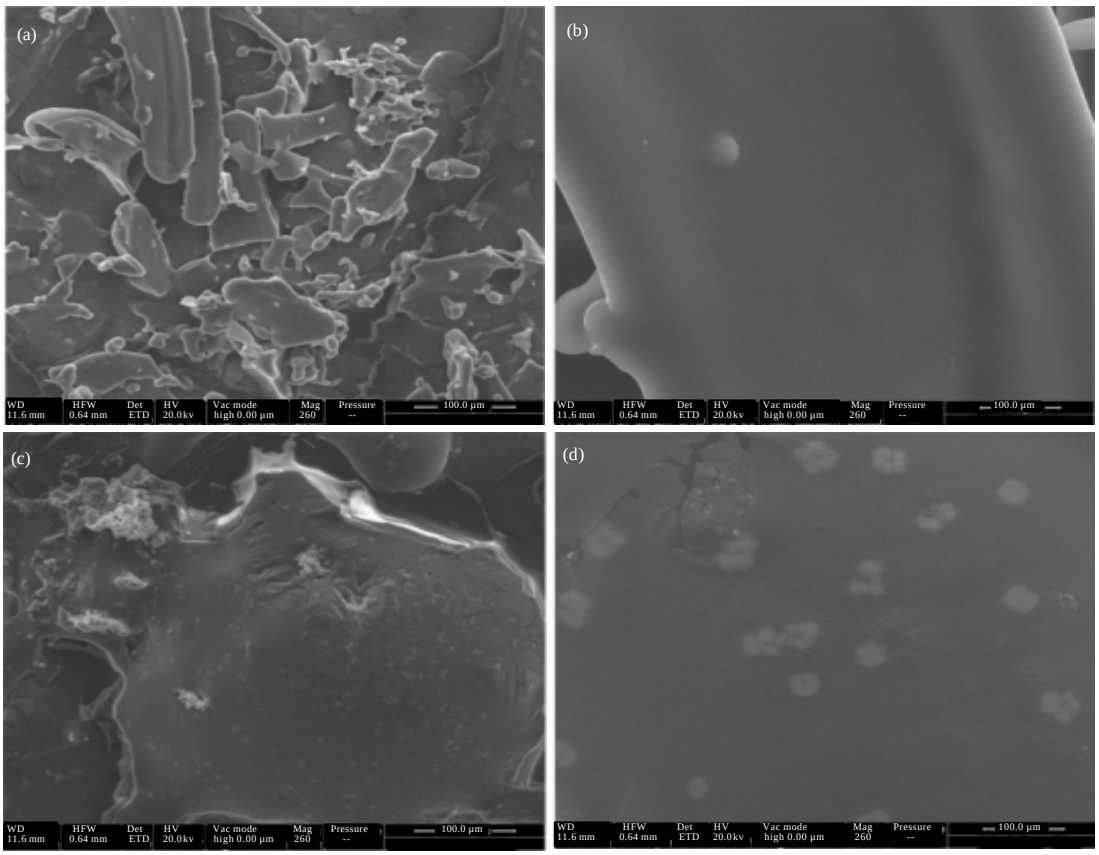

Fig. 5(a-d): Microstructure of TPH and TPH calcium complex determined by scanning electron microscopy, TPH at a magnification of, (a) 250 and (b) 2000-fold, respectively and TPH-calcium complex at a magnification of, (c) 250 and (d) 2000-fold, respectively

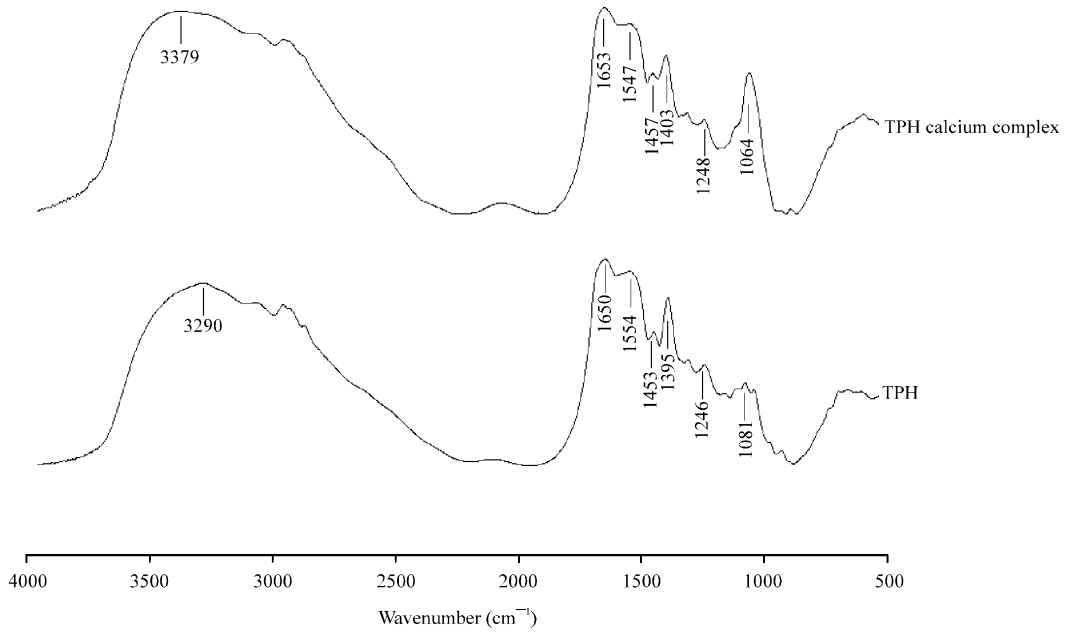

Fig. 6: FTIR spectra of TPH and TPH calcium complex

Analysis of FTIR spectra: The FTIR spectra of TPH and TPH calcium complex are shown in Fig. 6. For the spectrum of TPH, the bands located at 1650 and 1554 cm^{-1} can be mainly attributed to the vibration of amide I (C = O) and amide II (N-H and C-N). After TPH chelated with calcium ions, the band corresponding to the amide I group shifted from 1650 to 1653 cm^{-1}. This might be induced by the amide carbonyl (C = O) stretching vibration of peptide groups. It was also observed that the band of 3290 cm^{-1} in the spectrum of TPH shifted to 3379 cm^{-1}, which could be caused by the N-H stretch and the hydrogen bonds were replaced by Ca-N bonds after the chelation. Besides, the band of 1395 cm^{-1} corresponding to the carboxyl group (COO^-) shifted to 1403 cm^{-1}. The results were further confirmed that the TPH had binded with calcium ions to form the complex and the calcium binding sites might be the amino nitrogen atoms and the oxygen atoms belonging to the carboxylate groups. Similar changes in the FTIR spectra were found in β-lactoglobulin hydrolysate binded with iron (Zhou *et al*., 2012).

CONCLUSION

TPH chelated with calcium ions at the selected condition exhibited good DPPH radical scavenging activity, reducing power and lipid peroxidation inhibition activity. After TPH binded with calcium ions, the conjugate of TPH calcium was formed and the structure was changed as proved by scanning electron micrographs and FTIR spectra. The antioxidant activities of the TPH-calcium complex were not lower even higher than that of TPH. The results indicated that the TPH calcium complex might be used as the biological materials related to antioxidant agent and calcium-fortified supplements.

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