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Purification and evaluation of a novel antioxidant peptide from corn protein hydrolysate

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ABSTRACT

In the present study, corn protein hydrolysate (CPH) with antioxidant activity was obtained by enzymatic hydrolysis. Corn gluten meal (CGM) was hydrolyzed using two proteases (Alcalase and Protamex) to produce the antioxidant peptide. Extrusion and starch removal of corn protein were used as pretreatment procedures before proteolysis. Hydrolysis by Alcalase has more remarkable digesting efficiency on corn protein than that by Protamex. Therefore, the hydrolysate catalyzed by Alcalase was fractionated by ultrafiltration, and peptide with the highest antioxidant activity was purified from <6 kDa molecular weight fraction. The amino acid sequence of the novel peptide was Gln-Gln-Pro-Gln-Pro-Trp as identified by a quadrupole time-of-flight mass spectrometer (Q-TOF2), with molecular weight of 782.34 Da, which was matched to γ-zein f (50–55). The new peptide was further synthesized by Fmoc solid-phase method. It showed scavenging activity against DPPH, ABTS, and hydroxyl free radicals in dose dependent manner with EC_{50} values of 0.95, 0.0112 and 4.43 mg/mL, respectively. It also exhibited notable reducing power of 0.54 at 2.0 mg/mL, but showed weaker Fe^{2+}-chelating capacity with EC_{50} value of 6.27 mg/mL. These results suggest that the hexapeptide is a potential natural antioxidant that can be used as drug or functional food ingredient.

1. Introduction

According to International Grist Council (IGC) statistics, the total output of corn in the world has reached 863 million tons in 2012/2013, and about 23.8% of this total output was produced in P.R. China. In industry, corn is used to make corn starch and fuel alcohol. Corn gluten meal (CGM) is a major by-product of corn wet-milling, contains a minimum of 60% total protein [1]. Corn protein has low solubility in aqueous systems and lacks essential amino acids such as lysine and tryptophan, which greatly limits its application in food industry, and is mostly utilized as animal feed. If the protein from CGM is modified and utilized in food industry, its value and presence in market place can be increased.

Oxidation is thought to have a significant role in the occurrence of diseases, such as cancer, aging, arteriosclerosis, cardiovascular diseases and Alzheimer's disease [2,3]. These adverse health conditions have been attributed to oxidative damage to cell components, including membranes, lipoproteins, enzymes, and nucleic acids [4,5]. In foods, oxidation also directly affects food quality, and commonly associated with changes of food flavor and texture. Oxidation is the result of excessive production of free radicals and/or depletion of endogenous antioxidant compounds. Therefore, it is important to inhibit oxidation and the formation of free radicals occurring in the living body and food-stuffs. Appropriate intake of antioxidant compounds will significantly reduce the level of free radicals in the body, and play a major role in maintaining health and preventing diseases. Similarly, incorporation of antioxidant compounds into food can improve quality and nutrition of food. According to their origin, antioxidants can be divided into synthetic (butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ)) and natural (ascorbic acid, tocopherols, catechin, phenolic compounds from plants and peptides). Natural antioxidants have been the focus of growing interest because of their heightened safety, high activity, easy absorption, little or no negative side effects, and wide distribution properties. Recently, antioxidant peptides have been isolated from several food proteins [6–12].

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Table 1
Conditions for Alcalase and Protamex and their hydrolysis results for CGM pretreated by extrusion and starch removal.

Condition	Alcalase	Protamex
Temperature (°C)	60	50
pH value	8.5	7.0
Hydrolysis time (h)	2	2
Enzyme unit (U)	9.42×10^4	9.42×10^4
Degree of hydrolysis (%)	40.34 ± 0.67^a	30.87 ± 0.84^b
Soluble protein content (mg/mL)	24.85 ± 1.35^a	15.05 ± 0.68^b

Each value was carried out in triplicate ($n = 3$). Values with the same letters denote no significant difference ($P < 0.05$).

Amino acids, such as Trp, Tyr, His, Leu, Pro and Cys, have been reported to exhibit radical scavenging activities and metal chelating capacity [13,14]. Corn protein contains high proportions of hydrophobic amino acids, such as Leu (19.3–21.1%), Ala (8.3–10.5%), Ile (5.0–6.2%), and Pro (9.0–10.5%). Therefore, corn protein can be a good source for preparation of antioxidant peptides.

Currently, the preparation methods, antioxidant activity, amino acid composition and fractionation by ultrafiltration of antioxidant hydrolysates from corn protein have been studied [15–17]. But sequences information of peptides from the hydrolysate of corn protein and the evaluation of antioxidant activity were limited. In our earlier work, an octapeptide Phe-Pro-Leu-Glu-Met-Met-Pro-Phe possessing antioxidant activity was reported from the hydrolysate of CGM [15], and Zhuang et al. reported other three peptides Leu-Pro-Phe, Leu-Leu-Pro-Phe and Phe-Leu-Pro-Phe from hydrolysates of CGM, which exhibited good free radical scavenging activity and lipid peroxidation inhibitory effect [18]. More information on physiological active peptides amino acids sequences of protein hydrolysate will be beneficial for choosing the specificity of protease and hydrolysis conditions to obtain the hydrolysate with higher activity. Therefore, the primary objective of this study was to purify an antioxidant active peptide, and identify its primary structure. The secondary objective was to evaluate the antioxidant activities of the corn peptide by different in vitro methods.

2. Material and methods

2.1. Materials and chemicals

CGM was purchased from Longfeng Corn Development Co., Ltd. (Heilongjiang, China), with a total protein content of 61.25%. Protease Alcalase (6.28×10^5 U/mL) and Protamex (3.7×10^5 U/mL) were gifts from Novo Nordisk (Bagsvaerd, Denmark), and α-amylase was obtained from Wuxi Syder Bio-Products (Wuxi, China). Resource RPC (3 mL) and Source 5RPC ST 4.6/150 chromatography columns were purchased from GE healthcare (Uppsala, Sweden). C18 column was purchased from Waters Corporation (Milford, Massachusetts, USA). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox, trifluoroacetic acid, reduced L-glutathione, bacitracin and 2-deoxy-D-ribose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.2. Extrusion of CGM and starch removal of the extrudate

Moisturized CGM was introduced into a double-screw-rod extruder (DS32-II, China) and extruded under 160–180 °C and 1–1.5 MPa. The extrudate was dried at room temperature, ground, and sieved through an 80-mesh sieve. Suspension of extruded CGM of 10% (w/v) was adjusted to pH 6.5 with 1 M NaOH and incubated with α-amylase (30 U/g) at 70 °C for 2 h in a shaking apparatus. The reaction mixture was centrifuged to remove hydrolysis products of starch and washed three times with an equal volume of distilled water. Finally, the mixture was centrifuged at $3000 \times g$ for 10 min, and the precipitate was dried at 60 °C and stored in a desiccator.

2.3. Preparation of corn protein hydrolysate by enzymatic hydrolysis

Enzymatic hydrolysis of CGM was performed in a water bath with a constant agitation. Pretreated CGM was hydrolyzed with two enzymes (Alcalase and Protamex) according to their optimum hydrolysis conditions, respectively (Table 1). Briefly, CGM was suspended with the distilled water to a substrate concentration of 5% (w/v), then the enzyme was added and the mixture was hydrolyzed at its optimum temperature and pH. The degree of hydrolysis (DH) and soluble protein content as well as antioxidant activity of the hydrolysate were determined. DH was measured by pH-stat method [19], and soluble protein content was estimated according to Lowry method [20]. Antioxidant activity was determined at 5, 10, 15, 20, 30, 60, and 120 min of hydrolysis according to the pyrogallol autoxidation method [21]. After hydrolysis, the reaction was terminated in a boiling water bath for 10 min. Finally, the hydrolysate was centrifuged at $3000 \times g$ for 20 min to remove insoluble substrate fragments, and the supernatant was stored at −20 °C.

2.4. Fractionation of corn protein hydrolysate by ultrafiltration

The collected corn protein hydrolysate (CPH) catalyzed by Alcalase was ultrafiltered using hollow-fiber membranes with molecular weight cut-off 20–50 kDa, 6–10 kDa and 6 kDa. Ultrafiltration was performed sequentially: first, the hydrolysate was passed through a 20–50 kDa membrane; and permeate was pumped through 6–10 kDa; permeate from 6 to 10 kDa was further ultrafiltered through 6 kDa membrane to obtain retentate and permeate. Four fractions were obtained: fraction A with molecular weight >20 kDa (retentate of a 20–50 kDa membrane), fraction B with molecular weight 20–10 kDa (retentate of a 6–10 kDa membrane), fraction C with molecular weight 10–6 kDa (retentate of a 6 kDa membrane) and fraction D with molecular weight <6 kDa (permeate of a 6 kDa membrane). Soluble protein content and antioxidant activity of each fraction were evaluated, and their specific activities were calculated. Fraction D was lyophilized using a freeze drier (Flexi-Dry™ MP, FTS SYSTEMS Inc., USA) and stored at −20 °C for further purification.

2.5. Purification of corn peptides with antioxidant activity

2.5.1. Anion exchange chromatography

Fraction D was dissolved in buffer A (20 mM Tris–HCl buffer, pH 8.0) at a concentration of 12 mg/mL, and 10 mL was loaded onto a Q-Sepharose Fast Flow column (1.6 cm × 20 cm), which was previously equilibrated with buffer A and washed with another 200 mL of buffer A to remove any unbound peptides. The bound peptides were eluted with an increasing linear gradient of buffer B (1 M NaCl in 20 mM Tris–HCl buffer, pH 8.0) from 0% to 100% at a flow rate of 2 mL/min. The elution was detected at 214 nm, and the fractions eluted under same elution peak were pooled and the antioxidant activity was determined according to the pyrogallol autoxidation method. The fractions exhibiting highest activity were lyophilized, and subjected to next step of separation.

2.5.2. Size exclusion chromatography

The lyophilized active fraction II from the Q-Sepharose column was dissolved in distilled water at a concentration of 6 mg/mL and applied on a Sephadex G-25 column (2.6 cm × 80 cm), which was previously equilibrated and eluted with distilled water at a flow rate of 2 mL/min. Fractions were collected at 6 mL per tube, absorbance was monitored at 214 nm, and antioxidant activity of each tube was determined according to pyrogallol autoxidation method. The column was calibrated with bacitracin (1400 Da). The highest antioxidant fractions of molecular weight <1400 Da were pooled and lyophilized.

2.5.3. Reversed phase high performance liquid chromatography

The lyophilized fraction from the Sephadex G-25 column was used for further purification on a Resource™ RPC 3 mL column (6.4 mm × 100 mm, 15 μm). After sample application, the column was initially washed with solvent A (2% (v/v) acetonitrile containing 0.065% (v/v) trifluoroacetic acid (TFA)) for 40 min at a flow rate of 1 mL/min, and the bound peptides were eluted with a increasing linear gradient (0–100%) of solvent B (80% (v/v) acetonitrile containing 0.05% (v/v) TFA) for another 45 min. Fractions were collected at 1 mL per tube and determined the antioxidant activity according to the pyrogallol autoxidation method. The most active peaks were further purified by a Source 5RPC ST 4.6/150 (5 μm) column. In this purification step also, same solvents A and B with similar conditions were used except for the elution gradient of 0–100% B in 50 mL. The lyophilized active peak was injected onto a Sunfire™ C18 (4.6 mm × 150 mm, 5 μm) column and bound peptides eluted using a linear gradient of acetonitrile containing 0.065% TFA (10–55% in 45 mL) at a flow rate of 1 mL/min. All the peaks were collected by monitoring the absorbance at 214 nm, and fractions showing antioxidant activity were lyophilized.

2.6. Amino acid sequence identification of the purified antioxidant peptide

The amino acid sequence of the purified antioxidant peptide was determined by a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass, Manchester, UK) coupled to an electrospray ionization (ESI) source, at National Center of Biomedical Analysis (Beijing, China).

2.7. Synthesis of corn antioxidant peptide

Corn antioxidant peptide isolated from CPH was synthesized by the solid-phase method using standard Fmoc-chemistry at Bootech Bioscience & Technology Co., Ltd. (Shanghai, China).