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ORIGINAL RESEARCH PAPER

Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate

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Abstract
Objectives To improve the potential value of feather, which is a valuable protein resource, we have separated and identified antioxidant peptide(s) from feather hydrolysate.
Results Feather hydrolysate was prepared by fermentation with *Bacillus subtilis* S1–4. Antioxidative peptides were separated by sequential acid precipitation, cation exchange, and reversed-phase fast performance liquid chromatography. Finally, a peptide with antioxidative activity was identified as Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly by MALDI time-of-flight (TOF)/TOF analysis, and determined to represent a portion of feather keratin near its *N*-terminal. A synthesized peptide with the same sequence was used to characterize its antioxidative properties, including scavenging free radicals, reducing power, and Fe^{2+} chelation. In terms of the peptide's amino acid composition, the antioxidative activity might be mainly attributed to Cys and other amino acid residues.
Conclusion Feather keratin is a good source for the quantitative preparation of antioxidative peptides.

Keywords Antioxidative peptide · *Bacillus subtilis* · Degradation · Feather · Hydrolysate · Keratin

Introduction

Chicken feathers are produced in large amounts as a waste byproduct by the poultry industry (Lasekan et al. 2013). Feather mainly consists of keratin, an insoluble structural protein. Keratin is recognized as a useful protein resource and could be used to prepare animal feed or fertilizers (Brandelli et al. 2015). Prior to utilization of feather keratin, it is necessary to degrade feather keratin into soluble peptides or even amino acids. In consideration of economic costs and environmental constraints, feather biodegradation is the best prospect (Brandelli et al. 2015). Currently, in feather degradation studies, many microorganisms have been isolated or characterized that usually produce keratinases or proteases for catalyzing keratinolytic reactions to transform keratin into soluble peptides and free amino acids (Onifade et al. 1998; Brandelli et al. 2015).

Antioxidants play an important role in human health and food processing. Therefore, growing interest is turning to natural antioxidants, especially those from various protein hydrolysates (López-Barrios

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et al. 1997; Wu et al. 2015). Many antioxidative peptides have been identified and characterized. Feather hydrolysate has antioxidant activities (Fakhfakh et al. 2011; Fontoura et al. 2014) suggesting that it might present an interesting source of bioactive peptides.

In this work, an antioxidative peptide was separated and identified from chicken feather hydrolysate generated by bacterial fermentation followed by a series of separation procedures. The peptide's antioxidative properties were characterized using a synthesized version of the peptide.

Materials and methods

Microorganism and feather

Bacillus subtilis S1–4 was isolated from chicken feathers (Yong et al. 2013) and grown in lysogeny broth or agar plate. Chicken feathers were collected from a local poultry farm and washed with distilled water.

Feather degradation with *B. subtilis* fermentation

Feather fermentation was performed, as described previously (Yong et al. 2013). Briefly, 100 ml salt solution, containing 0.02 g $MgSO_4 \cdot 7H_2O$, 0.03 g K_2HPO_4, 0.04 g KH_2PO_4, 0.02 g $CaCl_2$, and 5 g chopped feather fragments, in 500 ml flasks was autoclaved at 114 °C for 15 min. The flasks were then inoculated with 1 % (v/v) of an overnight culture of *B. subtilis* S1–4 and incubated 37 °C for 72 h with shaking. A clarified supernatant was obtained by filtration through four layers of gauze and centrifugation at 15,500×*g* for 10 min. The supernatant was used for antioxidative activity assay by the ferric reducing ability of plasma (FRAP) method described below.

Purification of antioxidative peptides from feather hydrolysate

The clear supernatant was adjusted to pH 2 with 6 M HCl and allowed to stand at 4 °C overnight. The pellet was then collected by centrifugation at 15,500×*g* for 10 min and lyophilized to dryness to yield feather hydrolysate product.

Feather hydrolysate was redissolved in dimethyl sulfoxide (DMSO)/80 % aqueous methanol (1/1, v/v) and then loaded onto a cation-exchange resin column (2.6 × 35 cm) previously equilibrated with 80 % aqueous methanol. The column was sequentially eluted with 0.1, 0.3, and 0.5 M NaCl in 80 % aqueous methanol at 2 ml/min. Fractions (5 ml each) showing antioxidant activity were pooled and lyophilized to dryness. The resulting samples were dissolved in DMSO and subjected to reversed-phase fast performance liquid chromatography (RP-FPLC) using a Resource 15RPC column (6.4 × 100 mm, GE Healthcare Bio Sciences). The column was eluted with a linear gradient (0–80 %) of acetonitrile containing 0.05 % trifluoroacetic acid (v/v) at 0.5 ml/min. Fractions showing antioxidant activity were collected and lyophilized.

Identification of amino acid sequence by MALDI TOF/TOF

After RP-FPLC purification, peptide samples were subjected to matrix-assisted laser desorption/ionization time-of-flight/TOF tandem mass spectroscopy (MS/MS) using an ABI 4800 Plus MALDI TOF/TOF system (Life Technologies) at the Shanghai Boyuan Institute of Biotechnology (Shanghai, China). Data were gathered in centroid mode covering the mass/charge (*m/z*) and used to search the NCBInr database on the Mascot Server (http://www.matrixscience.com) to identify the peptides' amino acid sequences.

Peptide synthesis and antioxidative activity assay

Peptides with an identified amino acid sequence were chemically synthesized at the Top Institute of Biotechnology (Shanghai, China).

FRAP antioxidative activity was measured with a total antioxidant capacity assay kit (Beyotime Institution of Biotechnology, Nanjing, China) using the FRAP method following manufacturer's instruction. Samples (5 µl) were mixed with 180 µl of FRAP working solution, incubated at 37 °C for 5 min, and their A_{593} measured. A standard curve was prepared using $FeSO_4$ from 15 to 60 mM. FRAP activities for samples were evaluated as the equivalent $FeSO_4$ concentrations according to a standard curve.

2,2,-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was assayed as described previously

(Bougatef et al. 2010). Briefly, a sample aliquot (60 µl) was mixed with 60 µl 0.1 mM DPPH in 95 % aqueous ethanol. After 20 min in the dark, the OA_{517} was measured; 95 % (v/v) ethanol served as the blank control. Antioxidant activity was evaluated as %-inhibition of DPPH by the following equation:

$$\text{Scavenging activity } (\%) = (A_0 - A_S)/A_0 \times 100,$$

where A_0 is the blank control absorbance and A_S is the sample absorbance.

An [2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate)], ABTS radical-scavenging assay was conducted as previously described (Re et al. 1999). $ABTS^+$ ions were generated by blending 7.4 mM ABTS stock solution with 2.6 mM $K_2S_2O_8$, which was then diluted in phosphate buffer (pH 7.4) and equilibrated to an OD_{734} of 0.7 ± 0.02. Then, different sample concentrations (25 µl) were mixed with 100 µl of $ABTS^+$ solution and the A_{734} recorded; 95 % (v/v) ethanol served as the blank control. $ABTS^+$-scavenging activity was calculated by the following equation:

$$\text{Scavenging activity } (\%) = (A_0 - A_S)/A_0 \times 100,$$

where A_0 is the blank control absorbance and A_S is the sample absorbance.

Reducing power was quantified based on reducing Fe^{3+} to Fe^{2+} following an established method (Yildirim et al. 2001). Samples (20 µl)were first mixed with 20 µl sodium phosphate buffer (pH 6.6) and 20 µl 10 mg ferricyanide/l. The mixture was incubated at 50 °C for 20 min, followed by addition of 20 µl 10 % (w/v) trichloroacetic acid to terminate the reaction. Then, 70 µl of the resulting solution was mixed with 70 µl ddH_20 and 14 µl 10 mg $FeCl_3$/ml. Finally, the A_{700} was recorded, with distilled water as the blank control.

The Fe^{2+}-chelating activity was measured by the method of Decker and Welch (1990), with a slight modification. Briefly, a 25 µl sample was mixed with 50 µl 2 mM $FeCl_2$ and 50 µl 0.05 mM ferrozine. After standing for 10 min, the A_{562} was recorded, with DMSO and EDTA as the blank and positive controls, respectively. Chelating activity was calculated as:

$$\text{Chelating activity } (\%) = (A_0 - A_1)/A_0 \times 100,$$

where A_0 is the blank control absorbance and A_1 is the sample absorbance.

Results and discussion

Preparation of feather hydrolysate by *B. subtilis* S1–4 fermentation

Degradation of chicken feather was performed by fermentation with *B. subtilis* S1–4. Under these conditions, feather is efficiently degraded to produce free amino acids, soluble peptides, and thiol groups (Yong et al. 2013). The resulting fermentation supernatant was sampled and used for evaluation of antioxidative activity by the FRAP assay. Antioxidative activity occurred across the whole time course of fermentation, being highest at 72 h, which was similar to previous studies even when feather fermentation was performed using other bacterial species (Fakhfakh et al. 2011; Fontoura et al. 2014).

The feather hydrolysate was prepared by acid precipitation. FRAP assay results indicated that antioxidative activity was only recovered from the pellet and that no activity remained in the supernatant (data not shown), suggesting that acid precipitation was effective for preparation of peptide-containing hydrolysate. Finally, the precipitate was lyophilized to dryness and regarded as feather hydrolysate.

Separation of an antioxidative peptide

An antioxidative peptide was isolated from the pellet by first dissolving the pellet in DMSO/80 % (v/v) aqueous ethanol (1/1, v/v) and then loaded onto a cation-exchange resin column. The eluate was monitored at 215 nm to trace peptide content, and antioxidative activity indicated by the A_{593} was then measured by FRAP assay for each fraction (5 ml). Figure 1a shows a typical elution profile, indicating that the major antioxidative activity (fractions 12–22 in this example) was eluted with 100 mM NaCl. Next, the fractions with high antioxidant activity were pooled and applied to preparative RP-FPLC eluted with a linear gradient of aqueous acetonitrile (0–80 % by volume). Figure 1b shows a representative RP-FPLC elution curve. FRAP assay results revealed four antioxidative peaks, assigned as Fa-1, Fa-2, Fa-3, and Fa-4. Fraction Fa-1 was applied to RF-HPLC again, and the resulting fractions possessing antioxidative activity were pooled and used for amino acid sequence identification.

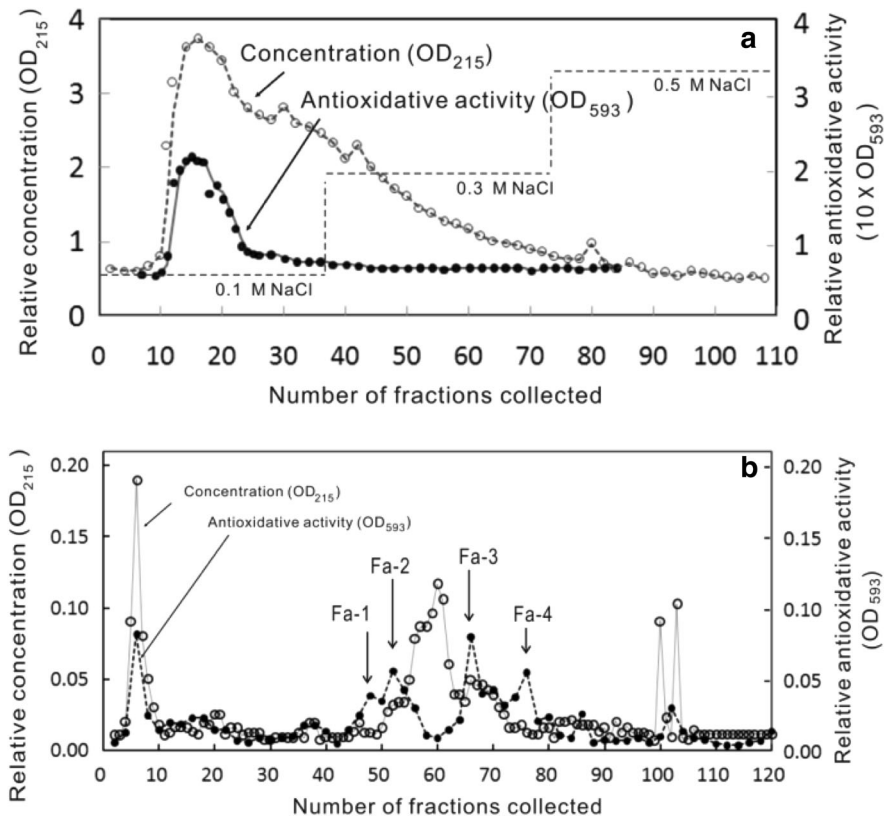

Fig. 1 Separation of peptides from feather hydrolysate. **a** Elution profile of feather hydrolysate by FPLC on cation-exchange resin column, and **b** elution curve of feather hydrolysate by reverse-phase HPLC on commercial Resource 15RPC column performed with a linear gradient (0–80 %) of aqueous acetonitrile containing 0.05 % trifluoroacetic acid (TFA) at 0.5 ml/min

Determination of peptide sequence

Fraction Fa-1 was subjected to tandem MS (MALDI TOF/TOF MS/MS) analysis. The first-order MS of Fa-1 was found to contain several peaks (data not shown), indicating that Fa-1 was a mixture. Further, the second-order mass spectrometric analysis for an individual peak was performed. Figure 2a shows the second-order MS of a peak with an *m*/*z* value of 849.3208. According to mass spectrometric data, the peptide's amino acid sequence was identified as Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly by searching the database NCBInr via the Mascot Server. This peptide consisted of eight amino acids with a mass of 848.8 Da and a predicated p*I* value of 8.23.

Through a database search (http://blast.ncbi.nlm.nih.gov/) using this peptide's amino acid sequence, several proteins annotated as feather keratin-1 were retrieved from the database (Fig. 2b). This indicated that the peptide was derived from feather keratin and encoded in feather keratin near its *N*-terminal, matching the 3rd–10th amino acid residues.

Characterization of the antioxidative peptide

The observed antioxidative activity derived from the identified peptide was confirmed by chemically synthesizing a peptide with the sequence Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly and then characterizing its antioxidative properties. The peptide was found to scavenge radicals of DPPH and ABTS with IC_{50} values of ~0.39 and ~0.35 mg/ml, respectively (Fig. 3a), which were almost equivalent to those of antioxidative peptides identified from enzymatic hydrolysate of crocein croaker (*Pseudosciaena crocea*) (Wang et al. 2013).

The reducing power and antioxidative activity of this peptide were also evaluated. The peptide's antioxidative activity, as determined by FRAP assay, was linear in relation to peptide concentration

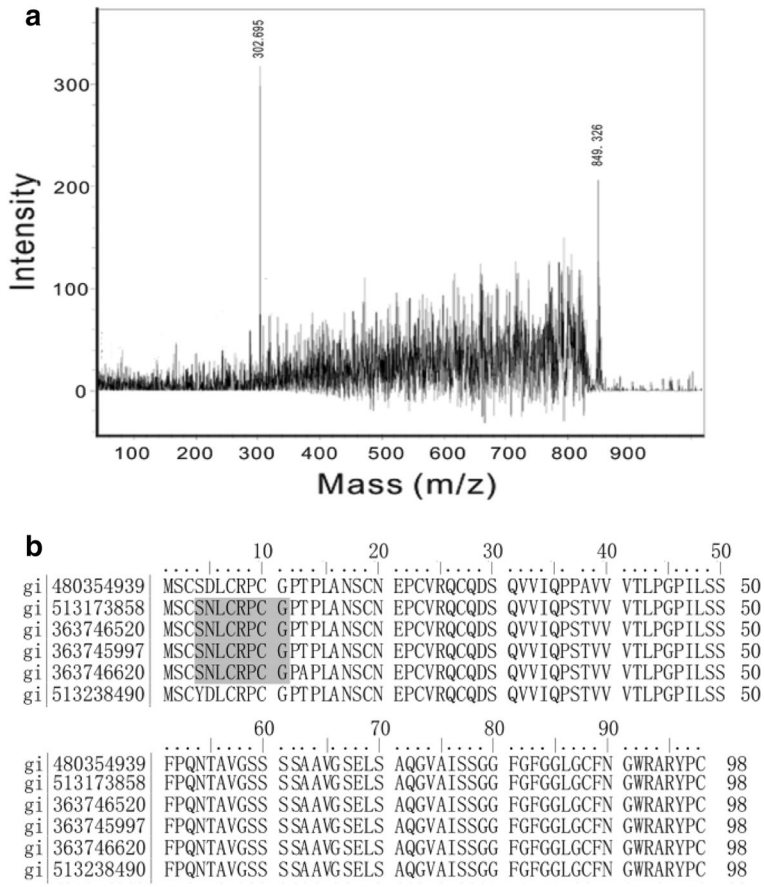

Fig. 2 Identification of the amino acid sequence of the antioxidative peptide. **a** Profile of second-order mass spectrometry, and **b** alignment of multiple amino acid sequences of feather keratin-1 retrieved from GenBank containing the identified antioxidative peptide (Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly)

(Fig. 3b), and the peptide was able to reduce Fe^{3+} to Fe^{2+} in a dosage-dependent manner (Fig. 3c). Finally, the peptide is also able to chelate Fe^{2+} with an IC_{50} value of $\sim$1.85 mg/ml (Fig. 3d).

The antioxidative activities of a given peptide have been recognized to relate to its amino acid composition. For example, Dávalos et al. (2004) have reported that Cys residues have antioxidative activity, as a Cys –SH group in a peptide acts as an effective hydrogen donor to free radicals (Wu et al. 2015). In addition, Pro and Gly residues are commonly found in antioxidative peptides from various sources (López-Barrios et al. 2014; Wu et al. 2015). Furthermore, a high correlation commonly occurs between reducing power and antioxidative activity in some antioxidative peptides (Zhang et al. 2009). Taken together, the antioxidative properties of this particular peptide might be attributed to specific amino acid residues (e.g., Cys, Gly, Pro, and Arg) in this peptide.

Feather keratin consists of insoluble protein rich in Cys and hydrophobic amino acid residues (Fig. 2b), which are usually related to antioxidative activity (Wu et al. 2015). In addition to antioxidative activity, other bioactivities, such as inhibitory activities toward angiotensin I-converting enzyme and dipeptidyl peptidase-IV, have been demonstrated in feather hydrolysate (Fontoura et al. 2014). In fact, RP-FPLC analysis in the present study resolved more than one peak with antioxidative activity (Fig. 1b). Considering the considerable amounts of waste feather produced each year by the poultry industry, feather keratin is an attractive and potentially useful source of bioactive peptides.

Conclusions

To our knowledge, this is the first report identifying a novel antioxidant peptide from chicken feather hydrolysate. The peptide's amino acid sequence was Ser-Asn-Leu-Cys-Arg-Pro-Cys-Gly, representing a portion of feather keratin-1. This peptide exhibited

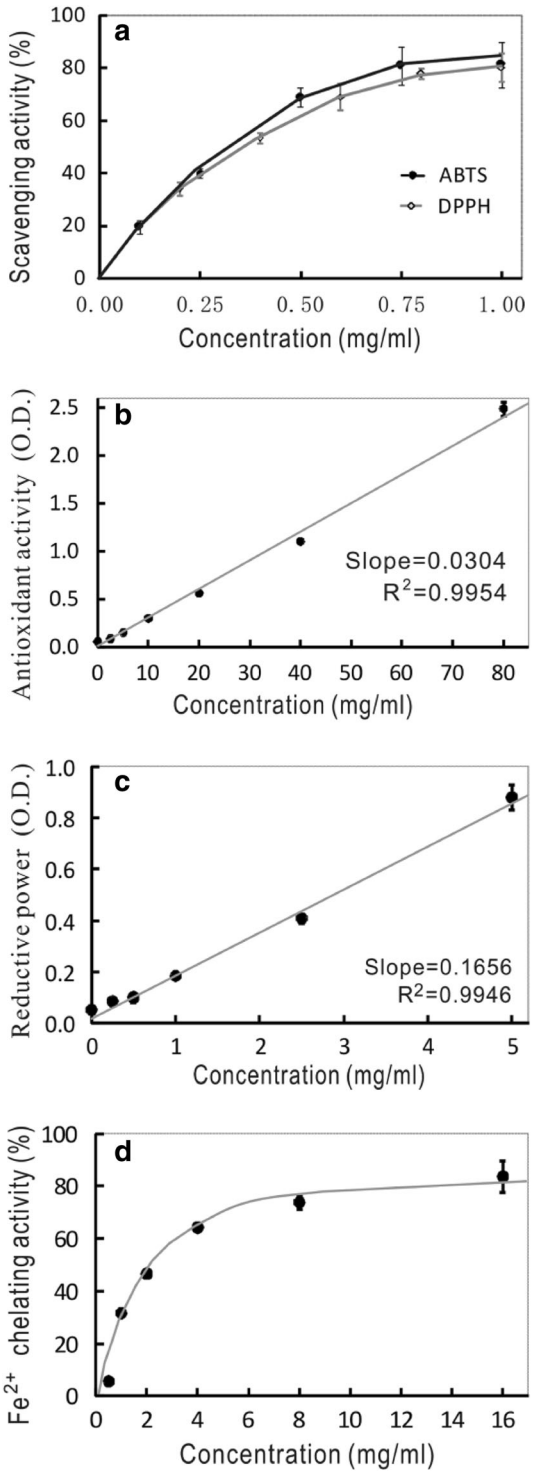

Fig. 3 Antioxidative properties of synthesized peptide (SNLCRPCG). **a** ABTS and DPPH radical scavenging activity, **b** FRAP antioxidative activity, **c** reducing power, and **d** Fe^{2+} chelating activity

good antioxidative properties, such as free radical scavenging and reducing and effectively chelating Fe^{2+}. These results suggested that feather keratin might be a good source for the quantitative preparation of antioxidative peptides.

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