Isolation and identification of an antioxidant collagen peptide from skipjack tuna (Katsuwonus pelamis) bone†

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To date, many researchers have developed active components that are derived from seafood processing for the purposes of healthcare. Here, an antioxidant collagen peptide was obtained from skipjack tuna (Katsuwonus pelamis) bone by using a combination of trypsin and chymotrypsin as the catalyst. The amino acid sequence of the peptide was identified as Ser–Ser–Gly–Pro–Pro–Val–Pro–Gly–Pro–Met–Gly–Pro–Arg (SSGPPVPGPG) by liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) analysis. We found that the as-prepared collagen peptide can efficiently scavenge DPPH radical (IC50 3.149 mM), superoxide anion radical (IC50 3.803 mM) and ABTS radical (IC50 9.489 mM). In addition, it has been found that the methionine (Met) residue in the collagen peptide could provide a precise active site during the scavenging of DPPH radicals by Fourier transform infrared spectroscopy (FTIR) analysis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. These results suggest that the peptide can find wide uses in the food, cosmetic and pharmaceutical industries.

Introduction

Oxidative stress, a vast threat to human health¹–³ results from an imbalance between the generation and scavenging of free radicals.⁴ Excessive free radicals attack membrane lipids, proteins and DNA and cause many health disorders, including diabetes mellitus, neurodegenerative disorders, cancer and inflammatory diseases.⁴–⁷ Currently, many researchers focus on developing functional foods to scavenge free radicals.⁸–¹⁰ As ingredients to such functional foods, antioxidant peptides have received much attention.¹¹–¹⁴ Researchers have tried to find antioxidant peptides from natural sources. Shazly et al. discovered that antioxidant peptides obtained from buffalo casein hydrolysates contain a number of antioxidant amino acids (Ser, Arg, Ala and Leu), and considered these to be key to the high radical scavenging potential of the hydrolysates.¹⁵ Blanca et al. identified an antioxidant peptide in fermented milk and discovered that the Met residue of the peptide is primarily responsible for the antioxidant activities of the peptide.¹⁶

Fish collagen obtained from the byproducts of seafood processing usually contains a large number of antioxidant amino acids residues, such as Val, Met, Trp, Tyr, and Phe.¹⁷ Thus, people have been attempting to obtain antioxidant peptides from fish collagen. Wu et al. isolated an antioxidant peptide from the salmon skin collagen by using extracellular proteases from Vibrio spp. SQS2-3.¹⁸ Alemán et al. hydrolysed a squid collagen with alcalase to isolate a number of antioxidant peptides containing Leu residue which the authors speculated as playing an important role in the antioxidant activities of the peptides.¹⁹ Ngo obtained antioxidant collagen peptides from Nile tilapia and attributed the antioxidant activity of the peptides to the existence of non-aromatic amino acids such as Ala, Pro, and Val.²⁰

Tuna (Thunnus spp.) and tuna-like species have long been significant sources of food for their high contents of bioactive proteins and unsaturated fatty acids.²¹ Currently, tuna generally is processed to produce raw flesh or canned food. Only about one-third of the whole tuna fish is used in the canning process, and the canning industry generates as much as 70% solid wastes from original fish materials. Waste includes viscera, gills, head, bone, and skin. In general, protein-rich by-products from the production of these tuna are processed into products with low market value, such as fishmeal and fertilizer.²² However, these by-products could be used as functional food ingredients. Tuna protein hydrolysates (TPH), which are obtained through hydrolysis of the by-products of tuna processing, can be used as an ingredient in food industries to provide
whipping, gelling, and texturing properties. Many researchers have attempted to derive antioxidant peptides from TPH.23–24

Trypsin (T) and chymotrypsin (C) are enzymes of the digestive system. Each enzyme is responsible for the hydrolysis of different peptide bonds. Trypsin hydrolyses the peptide and ester bonds formed by the carboxyl group of the basic amino acids, such as Arg and Lys.25 Chymotrypsin hydrolyses the peptide bonds formed by the carboxyl group of the aromatic or large hydrophobic amino acids, such as Phe, Tyr, Trp and Met.26 Here, antioxidant collagen hydrolysates from skipjack tuna (Katsuwonus pelamis) bone were prepared by using a combination of trypsin and chymotrypsin as the catalyst. We further purified the antioxidant peptides, determined their sequences and evaluated their antioxidant activities. We also investigated the mechanism of scavenging of free radicals by these peptides via experiments and molecular dynamics (MD) simulations.

**Experimental**

**Materials**
The frozen skipjack tuna bones were supplied by Xinfu Aquatic Food Co., Ltd. (Rongcheng, China). The frozen tuna bones were packed in zip-lock bags that were contained in a poly styrene box. The samples were transported to the Beijing University of Chemical Technology within 72 h, and stored at −20 °C. Trypsin (2.50 × 10⁴ U g⁻¹), chymotrypsin (1.70 × 10⁵ U g⁻¹), reduced glutathione (GSH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diethylaminoethyl cellulose-52 (DEAE-52) and trifluoroacetic acid (TFA) were purchased from Aladdin Trading Co., Ltd. (Shanghai, China). Acetonitrile (ACN) was purchased by Thermo Fisher Scientific Co., Ltd. (Beijing, China). Synthetic peptides (SSGPPVPQPMPGMQPR, GEOQSGTPQAF, GFPGER, SGGPPVPQPMGMQPR, purity > 99%) were obtained from Botai Co., Ltd. (Shanghai, China). Human hepatocellular carcinoma ( HepG2) cell line was obtained from the BeNa Culture Collection (BNCC, Beijing, China).

**Preparation of skipjack tuna bone collagen hydrolysates (STCHs)**
The bones were first demineralised using 1.0 M HCl solution at ambient temperature (~27 °C) with a bone/solution ratio of 1:2 (g mL⁻¹) for 12 h, and the HCl solution was changed every 1.5 h. The acid treated bones were washed with tap water and soaked in 4% NaCl solution for 12 h to remove non-collagenous proteins. The pretreated bones were washed thoroughly with tap water and then subjected to a final wash with distilled water to remove any residual matter. Then tuna bone collagen was obtained.

We designed three sets of enzymatic hydrolysis experiments. One set involved a simultaneous addition of two enzymes at the trypsin and chymotrypsin (TC), while the other two sets involved the sequential addition of trypsin + chymotrypsin (T+C) or chymotrypsin + trypsin (C+T). The enzymolysis conditions were shown in Table S1.† The enzymes were inactivated by heating at 90–95 °C for approximately 15 min. The degree of hydrolysis (DH) was determined by the pH-state method.27 After filtering and drying, powders containing hydrolysates were obtained and used in subsequent investigations.

**Determination of amino acid composition of skipjack tuna bone collagen**

Amino acid analysis of collagen from skipjack tuna bone was performed using Hitachi L-8900 High Speed Amino Acid Analyzer (Tokyo, Japan) according to Liu and Sun.28

**Purification and identification of antioxidant peptides from STCH-TC**

**Anion-exchange chromatography.** A STCH-TC solution (5 mL, 30.0 mg mL⁻¹) was added into a DEAE-52 cellulose column (1.0 × 60.0 cm) that was pre-equilibrated with distilled water and stepwise eluted with distilled water and NaCl (0.1, 0.5 and 1.0 M) at a flow rate of 1.0 mL min⁻¹. Each eluted fraction (5 mL) was collected and monitored at 280 nm. Six fractions (Frac. 1, Frac. 2, Frac. 3, Frac. 4, Frac. 5 and Frac. 6) were collected and their DPPH radical scavenging activities were measured.

**Reversed-phase high-performance liquid chromatography (RP-HPLC).** Frac. 1 was dissolved in 1 mL TFA (0.1%, v/v) and separated by RP-HPLC (Agilent 1260 HPLC, Agilent Ltd., USA). Frac. 1 solution (20 μL, 1 μg mL⁻¹) was then injected into a Zorbax SB-C18 column (4.6 mm × 250 mm, Agilent, USA). The mobile phase, which was composed of A (0.1% aqueous TFA (v/v)) and B (ACN), was injected into the column according to a gradient program (2% B from 0 to 10 min and 2–50% from 10 to 60 min). The active fraction was isolated at a flow rate of 0.8 mL min⁻¹. The elution solution was monitored at 280 nm and collected, and their DPPH radical scavenging activities were measured.

**Identification of the peptide sequence.** The target peak fractions (27.6 min) exhibiting the highest antioxidant activity were applied to LC-ESI-QTOF-MS analysis for sequence identification. The experiments were performed according to the method described by Piu et al.29

**Determination of antioxidant activity**

**DPPH radical scavenging activity assay.** The DPPH radical scavenging activity was measured using the method described by Kirby and Schmidt.30 One hundred microliters of each sample at different concentrations was added to 75 μL of ethanol and 25 μL of DPPH solution (0.2 mM in ethanol) as a source of radicals. The mixtures were incubated for 1 h in the dark at room temperature. The scavenging capacity was assessed based on measurements at 517 nm using a microplate reader (SpectraMax M2/M2ª, CA, USA). DPPH has an absorption peak at 517 nm; when it is reduced by an antiradical compound, this absorption no longer occurs. Therefore, a lower absorbance for the reaction mixture indicated the presence of greater radical scavenging activity. The DPPH radical scavenging activity (RSA_{DPPH}) was calculated using the following equation:

\[
RSA_{DPPH} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100%
\]
The cell culture and treatment were investigated using a previously described method. In detail, cell cytotoxicity was measured by using a tetrazolium salt WST-8 (Cell Counting Kit 8, CCK-8). HepG2 cells (6 × 10^4 per well) were pretreated with the synthetic peptide (SSGPPVPGMPGMGPFR, GEQGSTGPAGF and GFPGER) at different concentrations (5 and 15 μM) in a 96-well plate for 24 h. After treatment with 500 μM of H₂O₂ for 6 h, 10 μL of WST-8 was added and the mixture was incubated at 37 °C for 2 h. Cell viability was calculated in the light of the absorbance at 450 nm by SpectraMax® M2/M2® (CA, USA).

**Determination of peptide structure**

**Fourier transform infrared spectroscopy-attenuated total reflection (FTIR-ATR) spectrometry.** The interaction of the SSGPPVPGMPGMGPFR peptide with DPPH radicals was observed using a Nicolet 6700 FTIR-ATR (Thermo Nicolet, USA). The scans were performed in a spectral range spanning from 500 cm⁻¹ to 4000 cm⁻¹.

**Secondary structure determination using circular dichroism (CD).** Secondary structure analyses of the samples were performed using a Jasco J-810 circular dichroism spectrope (Shimadzu, Japan) at room temperature. 200 μL of peptide solution at a concentration of 0.1 mg mL⁻¹ was added into a cell with a 0.01 cm optical path. The spectral range was 195–270 nm, the resolution was 0.2 nm and the bandwidth was 1 nm. A scanning speed of 100 nm min⁻¹ with a 2 s response time was used to scan each sample in triplicate.

**Determination of the molecular weights and sequences of the synthetic peptides**

The molecular weight distribution of the samples was analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. For the identification of the sequences, the synthetic peptides were applied to MALDI-TOF/TOF mass spectrometry analysis (Ultraflextreme, Bruker, Germany).

**Modeling platform and simulations of peptide–DPPH radical complexes**

Molecular dynamics (MD) simulation was performed using Material Studio 6.0 software with a canonical (NVT) ensemble. A cubic box with a size of 47.43 × 47.43 × 47.43 Å (x, y, and z dimensions) containing one peptide molecule and one DPPH radical molecule was built. Periodic boundary conditions were employed for all three dimensions of the box, and a PVFF force field was chosen to model the interactions among all the particles. A velocity scale thermostat was used to fix the temperature at 298 K with a 0.1 ps decay constant. Energy minimization with integration (5000 steps) was first used to relax the molecules prior to the beginning of the simulation. The system was then equilibrated using a 5 ns MD simulation with a time step of 1 fs.
Results and discussion

Antioxidant peptides obtained from skipjack tuna bone

For skipjack tuna bone collagen, the relative content of each amino acid residue in the bone collagen was determined (Table 1). We found that the antioxidant amino acids, such as Arg, Lys, Phe and Met, were present. By comparison, skipjack tuna bone collagen contains a higher ratio of Met residues than tilapia skin collagen.  

Researchers have previously demonstrated that the presence of Trp, Tyr, and Phe residues within the C-terminus of peptides appeared to be essential for radical scavenging, and those antioxidant peptides which contain Lys or Arg residues in the C-terminus have higher antioxidant activity. Therefore, based on the structure and amino acid composition of skipjack tuna bone collagen, we used a combination of trypsin and chymotrypsin as the catalyst to prepare collagen hydrolysate (STCH-TC) with high antioxidant activity. Actually, chymotrypsin specically cleaves peptide bonds on the carboxyl side of Phe, Tyr, and Try residues, and trypsin specifically cleaves peptides on the carboxyl side of Arg and Lys residues. Here, we studied the optimal conditions for the preparation of STCH-TC by implementing Box–Behnken Design (BBD) in combination with response surface methodology (RSM). At the same time, we prepared STCH-T + C and STCH-C + T as control samples to investigate the interaction between trypsin and chymotrypsin.

Table 1  Amino acid content in skipjack tuna bone collagen

| Amino acid | Number of residues/1000 residues |
|------------|----------------------------------|
| Asp        | 46                               |
| Thr        | 30                               |
| Ser        | 37                               |
| Glu        | 77                               |
| Gly        | 337                              |
| Ala        | 125                              |
| Val        | 25                               |
| Met        | 15                               |
| Ile        | 13                               |
| Leu        | 27                               |
| Tyr        | 7                                |
| Phe        | 18                               |
| Lys        | 23                               |
| His        | 13                               |
| Arg        | 43                               |
| Hyp        | 70                               |
| Pro        | 94                               |

The results are shown in Table S1.† STCH-TC exhibited much higher antioxidant activity than the control samples, indicating that there are synergistic effect between trypsin and chymotrypsin for the hydrolysis of skipjack tuna bone collagen. We believe that peptides with high antioxidant activity could be isolated from the STCH-TC.

As shown in Fig. 1A, six fractions (Frac. 1 to Frac. 6) were separated from STCH-TC using a DEAE-52 cellulose column. Frac. 1 exhibited the highest DPPH radical scavenging ratio (23.41%) at 1 mg mL⁻¹ (Fig. 1B). Thus, it was subsequently purified using RP-HPLC (Fig. 1C). According to the elution profile obtained using RP-HPLC, one major fraction with the highest DPPH activity (22.09%) was identified (27.6 min) (Fig. 1D).

The peptides with the highest antioxidant activity were determined to reside in the main peak (27.6 min) obtained from Frac. 1 and were evaluated using LC-ESI-QTOF-MS analysis to identify antioxidant peptides. As shown in Table 2, the peptides with antioxidant activity were identified. We did not find any information on these peptides in the BIOPEP database. Previous studies have shown that there is at least one antioxidant amino acid residue (Val, Try, Tyr, Phe, Met, or His) within antioxidant peptide chains. Therefore, we believe the peptide (SSGPPVPGMGMGPR) which contains a Gly–Pro–Met (GPM) fragment from STCH-TC could be an antioxidant peptide. In addition, the Arg residue in its C-terminus may contribute to the antioxidant activity of the peptide.

In vitro evaluation of antioxidant peptides

The three peptides (SSGPPVPGMGMGPR, GEQGSTGPAGF and GFPGER) identified in the highly-antioxidant fractions from STCH-TC were synthesized (Fig. S1†). These peptides were selected to meet certain requirements, such as the presence of Phe residues at the C-terminus or the presence of Val, Met and Phe residues within the peptide chain. To evaluate the antioxidant activities of these three peptides, DPPH, superoxide anion and ABTS radical scavenging assays were performed, and their activities were compared with those of the GSH positive controls (0–1.0 mM). The IC₅₀ values of GSH (as standards) during the inhibition of DPPH, superoxide anion and ABTS are 0.329 mM, 0.335 mM and 0.105 mM, respectively (Fig. S2†).

One peptide (SSGPPVPGMGMGPR) exhibited higher DPPH radical scavenging activity (IC₅₀ 3.149 mM) than the other two peptides (no more than 25%; Fig. 2A). The IC₅₀ of the SSGPPVPGMGMGPR peptide was also lower than that of GPP (IC₅₀ 7.16 mM) and WDR (IC₅₀ 7.63 mM) derived from hydrolysates from bluefin leatherjacket and S. lewini proteins, respectively, 38,39 DPPH is a radical that contains an unpaired electron, which could capture a proton when it encounters antioxidants. Recently, it has been reported that the radical scavenging mechanism utilized by antioxidant peptides containing Met residues is single electron transfer (SET), 40 which indicates that peptides containing Met residues may have high DPPH radical scavenging activity. In addition, the position of the antioxidant amino acid residues within peptide sequences also affects the antioxidant activity of the peptide, and
antioxidant peptides containing Lys and Arg residues at the C-terminus have higher antioxidant activity.

From the Fig. 2B and C, we observed that the SSGPPVPGPMGPMGPR peptide also exhibited high scavenging activity in the presence of superoxide anion radicals (IC_{50} 3.803 mM) and ABTS radicals (IC_{50} 9.489 mM). Thus, we inferred that the presence of Val, Arg and Met residues within a peptide sequence may be beneficial to its antioxidant activity.

The above results indicate that the SSGPPVPGPMGPMGPR peptide has high antioxidant activity. Using ESR measurements, the DPPH radical scavenging activity of the SSGPPVPGPMGPMGPR peptide was obtained. The changes in the ESR signal intensity of the SSGPPVPGPMGPMGPR at different concentrations are shown in Fig. 2D. The ESR signal intensities can be determined based on the peak height, which is the sum of the up-peak value and the down-peak value. For the SSGPPVPGPMGPMGPR peptide, the

Fig. 1  (A) Elution profile of STCH obtained using DEAE-52 cellulose chromatography;  (B) inhibition of DPPH radicals in the fractions was determined (1 mg mL^{-1});  (C) elution profile of Frac.1 obtained using RP-HPLC;  (D) DPPH radical scavenging activity of the active fraction based on the RP-HPLC chromatogram (1 mg mL^{-1}).
comparison between the signal heights of the blank and the samples in each ESR plot indicated that the decrease in the ESR signal intensity is 43.10% at a 5 mM concentration and 79.7% at the 10 mM concentration. In addition, upon searching the BLAST protein database, the sequence of this antioxidant peptide was only found in a few species of fish, almost all of which are uncommon.

In summary, the SSGPPVPGPMGPMGPR peptide not only has high bioactivity in terms of scavenging DPPH radicals, but could also be used to distinguish tuna collagen from other common collagens on the market. Some peptides with 3–9 amino acid residues have been found to have strong antioxidant activities. In fact, many factors affect the antioxidant activity of peptides, including the amino acid composition, amino acid sequence and spatial conformation.43 Nowadays, some antioxidant peptides with high molecular weight, such as NTDGSTDYGILQINSR and TVTSLDLPLVRW, have also been reported.44,45 The radical scavenging mechanism of the peptide SSGPPVPGPMGPMGPR is worth further exploring.

The HepG2 cell line is considered a good model with which to research in vitro toxicity of the liver, since it retains many of the specific functions that are characteristic of normal human hepatocytes.46 Prior to the evaluating the effect of the three peptides on scavenging free radicals, the cytotoxicity of the synthetic peptides in HepG2 cells was also assessed (Fig. 3). H2O2 at 500 μM significantly decreased cell viability by 50.0%. We found that pre-treatment of SSGPPVPGPMGPMGPR at concentrations of 5 and 15 μM significantly enhanced cell viability by 64.92 ± 5.73% and 74.93 ± 2.55%, respectively. In contrast, the other two peptides cannot reduce cell viability induced by H2O2. In addition, we found that the three peptides have no toxic side effects on HepG2 cells (Fig. S4†). These results demonstrate that the peptide SSGPPVPGPMGPMGPR possess protective effect on H2O2-induced oxidative stress in HepG2 cell.

### DPPH radical scavenging mechanism utilized by antioxidant peptides

The measurement of DPPH scavenging is a quick, convenient, and efficient method to determine antioxidant activity. Here, we

| Theo. MH⁺ [Da] | Sequence                   | Ions score mascot | Location |
|----------------|----------------------------|-------------------|----------|
| 1520.7348      | SSGPPVPGPMGPMGPR           | 42                | f 159–175 |
| 702.3570       | APDPFR                     | 35                | f 1195–1200|
| 1327.6641      | GFPGLPGQSGEPGK             | 33                | f 959–972 |
| 976.4371       | GETGPSGPAGF                | 32                | f 791–801 |
| 830.4268       | GHRGFSGL                  | 29                | f 253–261 |
| 662.3257       | GFPGER                     | 27                | f 668–673 |
| 1007.4429      | GEOQGSTGPAGF               | 18                | f 620–630 |

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Fig. 2  Antioxidant activities of the peptides in terms of: (A) DPPH radical scavenging activity; (B) superoxide anion radical scavenging activity; (C) ABTS radical scavenging activity; (D) ESR spectra in the presence of DPPH radicals.
are interested to understand the mechanism utilized by the SSGPPVGPMGPMGPR peptide during scavenging of DPPH radicals. Due to the complexity of the peptides and the lack of effective experimental methods, there are some challenges for exploring their radical scavenging mechanism. The secondary structure of peptides affects its biological activity, and it could be investigated by CD. The CD spectra (Fig. 4A) of the SSGPPVGPMGPMGPR peptide and the SSGPPVGPMGPMGPR peptide in the presence of DPPH radicals exhibited one negative minimum at approximately 200 nm, which is due to a random coil conformation. Although there are only slight differences between the curves generated for the SSGPPVGPMGPMGPR and the SSGPPVGPMGPMGPR peptides in the presence of DPPH radicals, the conformation of the SSGPPVGPMGPMGPR peptide was altered after the reaction. After scavenging of DPPH radicals, the proportion of β-turns increases from 34.3% to 35.1%, and the proportion of random coil increases from 46.5% to 47.6%, while the proportion of β-sheets decreases from 15.1% to 13.9%. After reacting with the DPPH radicals, the secondary structures of the peptides assumed a more relaxed conformation. Their FTIR spectra displayed similar curves between 4000 and 500 cm\(^{-1}\) (Fig. 4B). The CH\(_3\) stretching vibrations were blue-shifted from 2972.78 cm\(^{-1}\) to 2925.04 cm\(^{-1}\). In addition, the emergence of an S-C bond at 1056.14 cm\(^{-1}\) demonstrated that the structure of the Met residue was changed. These results indicate that there was a reaction between the Met residue and DPPH.

The products were measured using MALDI-TOF mass spectrometry (Fig. 5A). According to the mass spectra, the molecular weights of products were determined to be 1520.486 and 1472.466 Da, which indicated that a single novel product was present. To determine the changes in the amino acids residues that resulted in the decrease of the molecular weight, further MALDI-TOF/TOF mass spectrometry analysis was conducted (Fig. 5B and C). The results showed that the molecular weight of the Met residue was reduced by 48 Da, which is consistent with the decrease in the total molecular weight of the peptide. This means one of the Met residues in the SSGPPVGPMGPMGPR peptide could play a key role in the scavenging of DPPH radicals. As the peptide contains two Met residues, only one serves as an active site during scavenging of DPPH radicals. It may be a result of a spatial configuration structure for the peptide.

We designed a peptide that could be used to verify the above results. Gly was selected to replace the reactive Met residues, resulting in a synthetic peptide with the sequence of SSGPPVGPGPMGPR peptide as a control sample. The results of the scavenging DPPH radical assay (Fig. 6A) showed that the DPPH radical scavenging activity of the SSGPPVGPGPMGPR peptide was lower than that of the SSGPPVGPMGPMGPR peptide. The result further demonstrates that the Met residue plays an essential role during scavenging of DPPH free radicals.

The ESR results (Fig. 6B) showed that the inhibition potency of the SSGPPVGPGPMGPR peptide scavenging DPPH radical is 43.10%, whereas that of the SSGPPVGPGPMGPR peptide scavenging DPPH radical is 29.78%, which is consistent with the above results. We deduced that the sulfur in the Met residue reacts with DPPH radicals; the structure of the SSGPPVGPMGPMGPR peptide is shown in Fig. 6C.

In addition to the sequences of the peptides, the configuration of the peptides also strongly affects their bioactivity. This may be the key reason why only one Met residue in the SSGPPVGPMGPMGPR peptide contributes to the scavenging of DPPH free radicals. However, because of the limitations of
the experiment, the conformational structure of the peptide could not be observed directly. Therefore, a molecular dynamics (MD) simulation was used to explore the scavenging behaviour of the peptide. The conformational structure of the SSGPPVPGPGPMGPR peptide in the presence of DPPH radicals was investigated. We placed one unfolded the SSGPPVPGPGPMGPR peptide and one DPPH radical molecule in a box for the purposes of the simulation. The time variation of the potential energy of this system is shown in Fig. S5.† The initial potential energy was at a relatively high value and fluctuated within a large range. As the simulation progressed, the peptide folded and coalesced, and the corresponding potential energy decreased to a much lower value and was maintained at that level. This demonstrated that the curled peptide exhibited a higher stability than the unfolded peptide. In the curled peptide, the S–CH₃ bond in the Met residue, which is on top of a peptide clump and has been experimentally observed to be effective, is exposed to the environment (shown inside the red circle in Fig. S5†), while another Met residue, which is inside the peptide clump, would hardly contact with the dissociated radical. This phenomenon provides further insight regarding the important role of the Met residue in the SSGPPVPGPGPMGPR peptide during the scavenging of DPPH radicals.

Fig. 5  (A) MALDI-TOF mass spectrometry of the SSGPPVPGPGPMGPR peptide after the reaction with DPPH radicals; (B) MALDI-TOF/TOF mass spectrometry of the SSGPPVPGPGPMGPR peptide; (C) MALDI-TOF/TOF mass spectrometry of the SSGPPVPGPGPMGPR peptide after the reaction with DPPH radicals.
Conclusions
Skipjack tuna bone has been used to obtain collagen peptides with high antioxidant activity. We found that a combination of trypsin and chymotrypsin can effectively hydrolyse the collagen of skipjack tuna bone to produce antioxidant hydrolysates. Among the antioxidant peptides isolated, the SSGPPVPGPMGPMGPR peptide exhibits particularly high antioxidant activity. In addition, a Met residue within this peptide chain was demonstrated to provide an active site for the scavenging of DPPH radicals. We believe that these findings will provide guidance for the development of peptides with high antioxidant activity from natural sources for functional foods.

Conflicts of interest
There are no conflicts to declare.

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