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Fractionation and identification of Alaska pollock skin collagen-derived mineral chelating peptides

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ABSTRACT

Peptides with the ability to chelate dietary minerals have been reported to have potential as functional food ingredients. A collagen tryptic hydrolysate (CTH), previously shown to chelate iron, was further investigated for the presence of Ca, Fe and Cu chelating peptides. Sequential purification steps, including immobilised metal affinity chromatography (IMAC) and gel permeation chromatography (GPC) were employed for the separation of chelating peptides. GPC analysis showed that the mineral chelating peptides were mainly between 500 and 2000 Da. Subsequent identification was carried out using UPLC–ESI-QTOF MS/MS. Overall, 10 sequences were identified as potential chelating peptides. The Ca, Fe and Cu chelating activity of GPAGPHGPPG was 11.52 ± 2.23 nmol/μmol, 1.71 ± 0.17 nmol/μmol and 0.43 ± 0.02 μmol/μmol, respectively. This study identifies collagen as a good source of peptides with potential applications as functional ingredients in the management of mineral deficiencies.

1. Introduction

Minerals such as calcium, zinc, iron and copper are present in all body tissues and fluids and are necessary for the maintenance of certain physiological processes essential to life. For example, calcium is known as the most abundant inorganic element in the human body, accounting for 1.5–2.2% of total body weight (Daengprok et al., 2003), and is important for intracellular metabolism, bone growth, blood clotting, nerve conduction, muscle contraction and cardiac function (Bass & Chan, 2006). Zinc is a catalytic component of approximately 100 enzymes and has a structural and biological role in many proteins, hormones, transcriptional and growth factors and cytokines (Bozalioğlu, Özkan, Turan, & Şimşek, 2005). Iron is responsible for oxygen transport within haemoglobin and is a vital substrate for haemoglobin production and sufficient iron stores are necessary to achieve and maintain adequate levels of haemoglobin (Tay et al., 2011). Copper is capable of producing reactive oxygen species inducing oxidation of nucleotide bases and DNA strand breaks (Megias et al., 2007). Numerous diseases occur due to the deficiencies of dietary minerals. For instance, without sufficient calcium, a child's bones and teeth do not develop properly, and an adult's bones lose minerals and weaken. Eyesight, taste, smell and memory are connected with zinc and a deficiency in zinc can cause malfunctions of these faculties. Conservative estimates suggest that 25% of the world's population is at risk of zinc deficiency (Maret & Sandstead, 2006). The eventual consequence of iron deficiency is anaemia, where the body's stores of iron have been depleted and the body is unable to maintain the level of haemoglobin in the blood, this happens to be one of the most common of the nutritional deficiencies (Dlouhy & Outten, 2013).

Nutritional scientists encourage consumers to choose foods rich in metal elements in their daily diets (Keller, Lanou, & Barnard, 2002). For health considerations and dietary preferences, food-derived nutritional supplements are more accepted than metal salts and nutrient supplements. Mineral chelating peptides have been identified as potential functional ingredients to improve mineral bioavailability. For example, casein phosphopeptides (CPPs) derived from milk protein are potential mineral absorption enhancing peptides and have previously been shown to chelate calcium (Park, Swaisgood, & Allen, 1998; Tsuchita, Goto, Yonehara, & Kuwata, 1995), zinc (García-Nebot, Barberá, & Alegría, 2013) and iron (Aït-oukhatar et al., 1997; Pérès et al.,

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1999). The mineral chelating ability of CPPs is associated with highly polar acidic sequences of three phosphoserines followed by two glutamic acid residues (García-Nebot et al., 2013).

Marine products and byproducts are good raw material candidates for the production of bioactive peptides due to their low cost and considerable protein content. Peptides derived from marine products have been shown to exhibit good biofunctional properties and many potential functional food applications have been proposed (Chalamaiah, Rao, Rao, & Jyothirmayi, 2010). Numerous mineral chelating peptides derived from marine products and byproducts have been reported in the last decade. These include peptides from oyster (Chen et al., 2013), anchovy (Wu, Liu, Zhao, & Zeng, 2012), hoki (Jung & Kim, 2007) and pollock (Guo, Hou, Li, Zhang, & Zhao, 2013).

Alaska pollock skin collagen tryptic hydrolysate (CTH) has shown potential iron chelating activity in our previous study and an iron chelating peptide, Ser–Cys–His, was purified and characterised (Guo et al., 2013). The relationship between chelating peptide structure and different minerals, such as Ca, Zn, Fe and Cu, is still not clear. Hence, the aim of this study was to firstly fractionate CTH with the view to identifying more mineral chelating peptide sequences. The second objective was to study the interaction between CTH derived peptides and calcium, zinc, iron and copper.

2. Materials and methods

2.1. Materials and chemicals

Alaska pollock skin was obtained from Qingdao Doest Technology Development Co. (China). HPLC-grade water and acetonitrile were from VWR International (Wicklow, Ireland). Leu-Trp-Met-Arg and Asp-Glu were from Bachem Feinchemikailien (Bubendorf, Switzerland). Casein phosphopeptides (CPPs) were prepared as described by Zhu and FitzGerald (2010). All other reagents were supplied by Sigma-Aldrich Chemical Co. (Dublin, Ireland).

2.2. Sample preparation

Collagen tryptic hydrolysate (CTH) was generated as previously described (Guo et al., 2013). Briefly, collagen was extracted from Alaska pollock skin and was then hydrolysed with trypsin (Pangbo Biotech Co., Ltd., China) at 50 °C (pH 8.0) for 4 h with 0.6% (w/w) enzyme to substrate.

2.3. Measurement of metal chelating activity

2.3.1. Ca chelating activity

The measurement of Ca chelating activity was carried out by the method described in previous studies (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2013; Jung & Kim, 2007). Peptide sample (0.5 mg/ml) was mixed with 5 mmol/l $CaCl_2$ in 20 mmol/l sodium phosphate buffer (pH 7.5) and incubated at 37 °C for 30 min. The precipitate was then removed by centrifuge at 4000 rpm for 20 min. The soluble calcium content in the supernatant was determined by flame atomic absorption spectrometry (FAAS). Sample without peptides was used as a blank. The amount of bound Ca was calculated as follows:

$$\text{Bound Ca} = \text{soluble Ca in the supernatant with added peptide} - \text{soluble Ca in the supernatant of the blank.}$$

The Ca chelating activity (nmol/μmol) was defined as the amount of Ca (nmol) bound by the peptide (μmol). The experiment was performed in triplicate, and values were expressed as mean ± SD.

2.3.2. Fe chelating activity

Ferrozine forms a coloured complex, Fe^{2+}-ferrozine, with a ferrous ion which can be measured at 562 nm (Carter, 1971). Samples (250 μl, 1 mg/ml) were dissolved in assay buffer (0.05 mol/l sodium acetate, pH 5.0) and were then pipetted into a 96-well plate and pre-incubated for 5 min at 37 °C using a BioTek Synergy HT plate reader (BioTek Instruments Inc., VT, USA). The reaction was initiated with the addition of 20 μl of 0.25 mmol/l $FeSO_4$. After 30 min, the reaction was terminated with the addition of 15 μl of 2.5 mmol/l ferrozine. The absorbance (Abs) of the solution at 562 nm was measured after 10 min. Deionised water was used as a control. The Fe chelating activity (nmol/μmol) was defined as the amount of Fe (nmol) bound by the peptide (μmol). CPPs were used as the positive control. The experiment was performed in triplicate and values were expressed as mean ± SD.

2.3.3. Cu chelating activity

Pyrocatechol violet (PV) forms a coloured complex with Cu^{2+} which can be measured at 632 nm. The assay used for copper chelating activity was as described by Saiga, Tanabe, and Nishimura (2003). Samples (200 μl, 100 μg/ml) were dissolved in assay buffer (0.05 M sodium acetate, pH 5.0) and were then pipetted into a 96-well plate and preincubated for 5 min at 37 °C. The reaction was initiated with the addition of 20 μl of 1 mg/ml $CuSO_4$. After 30 min, the reaction was terminated with 10 μl of 2 mmol/l PV. The absorbance of the solution at 632 nm was then measured. Deionised water was used as a control. The Cu chelating activity (μmol/μmol) was defined as the amount of Cu (μmol) bound by the peptide (μmol). CPPs were used as the positive control. The experiment was performed in triplicate and values were expressed as mean ± SD.

2.4. Enrichment of chelating peptides using immobilised metal affinity chromatography (IMAC)

The IMAC column was prepared and packed as previously described with some modifications (Guo et al., 2013). Briefly, Sepharose 6B matrix was initially activated by epichlorohydrin at 40 °C for 3.5 h and was then fully washed with deionised water. Iminodiacetic acid (IDA) solution was prepared by dissolving 6 g IDA in 40 ml 2 mol/l Na_2CO_3 and the pH was adjusted to 13.0 with 6 mol/l NaOH. The epichlorohydrin activated Sepharose 6B matrix was incubated with the IDA solution and gently stirred at 120 rpm at 60 °C for 8 h prior to being packed into a glass column (200 mm × 10 mm).

The IMAC column was coupled to a fast protein liquid chromatography (FPLC) Äkta-Purifier 100 with a Frac-920 fraction collector (GE Healthcare, Piscataway, NJ, USA). Ca^{2+}-, Zn^{2+}-, Fe^{2+}- and Cu^{2+}-IMAC columns were prepared by charging the column with 5 column volumes of 0.2 mol/L $CaCl_2$, $ZnSO_4$, $FeSO_4$ and $CuSO_4$, respectively. CTH (2 ml, 15 mg/ml) dissolved in HPLC grade water was loaded onto the IMAC column with H_2O at pH 7.6 and the absorbance at 214 nm was monitored. The unbound peptides were washed off until the absorbance at 214 nm returned to baseline. The bound peptides were then eluted using H_2O at pH 3.0 at a flow rate of 1 ml/min. One ml fractions were collected. The peptide fractions eluted during this step were pooled, freeze-dried and named $F_{Ca\text{-}IMAC}$, $F_{Zn\text{-}IMAC}$, $F_{Fe\text{-}IMAC}$ and $F_{Cu\text{-}IMAC}$.

2.5. Separation of chelating peptides using gel permeation chromatography (GPC-FPLC)

A Hiload 16/60 Superdex 30 column (GE Healthcare, USA) was coupled with the FPLC Äkta-Purifier 100 and equilibrated with HPLC grade H_2O. The column was calibrated using thyroglobulin (MW = 660 kDa, Vo = 40.0 ml) and l-proline (MW = 115 Da,