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ARTICLE

Purification of an Iron-Chelating Peptide from Spirulina Protein Hydrolysates

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Abstract Iron-chelating peptide was purified from spirulina protein hydrolysates. Spirulina protein was hydrolyzed using Alcalase and Flavourzyme, and the degree of hydrolysis was determined using a trinitrobenzene sulfonic acid assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis. The spirulina protein hydrolysates were ultra-filtered to isolate the components below 3 kDa, which were then fractionated by Q-Sepharose fast flow and Sephadex G-15 columns. The iron-chelating activity of each fraction was determined, and the peptide with the highest activity was isolated and identified by matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry. Amino acid sequence of the iron-chelating peptide was identified to be Thr-Asp-Pro-Ile(Leu)-Ala-Ala-Cys-Ile(Leu), which has a molecular weight of 802 Da. Moreover, due to its ability to chelate iron, the isolated peptide could be used as an iron supplement.

Keywords amino acid sequence · hydrolysates · iron-chelating peptide · purification · spirulina

Introduction

Iron deficiency is a worldwide health problem. In particular, many children and women requiring a relatively high iron intake are affected (Uchida et al., 2006). Iron deficiency is caused by inadequate iron intake and absorption in the body, which can lead to iron deficiency anemia (Naigamwalla et al., 2012). In addition, it affects the mental and physical health of children and increases the risk of miscarriage in pregnant females (Milman et al., 2011; Smith, 2012). To overcome iron deficiency, iron-fortified products and iron supplements in the form of salts, such as ferrous sulfate, ferrous gluconate, and ferrous fumarate, are widely used (Smith, 2012); however, only 10-35% of iron is absorbed in the body due to limited absorption and bioavailability (Martinez-Navarrete et al., 2002).

Poor iron absorption results from the reactions of iron supplements with other chemicals such as phytic acid, polyphenols, and fibers (Wu et al., 2012). Therefore, studies have been performed on iron-chelating peptides to increase iron absorption and bioavailability (Lee and Song, 2009; Choi et al., 2012). Iron-chelating peptides are generated by the enzymatic hydrolysis of food proteins, and these peptides are combined with iron to form soluble compounds that enhance iron bioavailability by facilitating direct absorption in the small intestine (Wang et al., 2013). Thus, the iron-chelating peptides can replace inorganic salt iron supplements.

Spirulina, a type of cyanobacteria, contains a large amount of protein (Adiba et al., 2011), and its digestibility is relatively high due to the mucopolysaccharide cell wall structure (Kay and Barton, 1991). Various functional properties of spirulina, including its antioxidant and antimicrobial activities, have been studied (Karkos et al., 2011). However, no study has been reported on mineral-chelating peptides from spirulina protein hydrolysates. Considering that spirulina is a good source of food protein, it can be used as a source of iron-chelating peptides. Therefore, this study was performed to isolate and identify an iron-chelating peptide from spirulina protein hydrolysates using anion exchange chromatography, gel permeation chromatography, and matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry.

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Materials and Methods

Materials. The *Spirulina platensis* powder was provided by a local company (ES Biotech, Korea) and the commercial proteases, Alcalase (from *Bacillus licheniformis*, 2.4 AU/g protein) and Flavourzyme (from *Aspergillus oryzae*, 500 LAPU/g protein), were purchased from Novo Nordisk Co. (Denmark). Trinitrobenzene sulfonic acid (TNBS) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate as well as the MALDI matrix, α-cyano-4-hydroxycinnamic acid, were purchased from Sigma-Aldrich (USA). All other reagents for peptide sequencing were purchased from Merck KGaA (Germany).

Extraction of spirulina protein. The spirulina powder (10 g) was dissolved in deionized water (1:20, w/v) for protein extraction, and the solution was homogenized using a homogenizer (Ultra-Turrax® T25 digital, IKA, Germany) at 10,000 rpm for 10 min. The homogenized solution was centrifuged at 10,000×g for 10 min to remove the insoluble materials, and the supernatant was removed. To precipitate the protein from the supernatant, ammonium sulfate (80%, w/w) was added based on a preliminary experiment, and stirred at 4°C for 1 h, then centrifuged at 10,000×g for 10 min. The pellets were collected and dialyzed using a dialysis membrane with a 3.5 kDa molecular weight cut-off (MWCO), Spectrum Laboratories, Inc., USA) for 48 h and freeze-dried using a freeze dryer (ED5505, Ilshin Lab Co., Korea).

Preparation of spirulina protein hydrolysates. The hydrolysis of the spirulina protein was performed according to the method described by Lee and Song (2003) with minor modifications. Alcalase and Flavourzyme were used for enzymatic hydrolysis. Lyophilized spirulina protein (2%, w/v) was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and the solution was partially hydrolyzed using Alcalase (500:1 w/w) at 55°C and pH 8.0. After 1 h, Flavourzyme (50:1, w/w) was added to the solution and further hydrolyzed at 50°C and pH 7.0 for 8 h to complete the hydrolysis of the spirulina protein for production of more low-molecular weight peptides. After the reaction, the mixture was heated to 95°C for 10 min to inactivate the enzyme and then centrifuged at 3,500×g for 20 min.

Determination of the concentration of available amino groups. The degree of hydrolysis of the spirulina protein hydrolysates with different hydrolysis times was determined by measuring the concentration of available amino groups with the TNBS assay (Eklund, 1976). Sodium borate buffer (0.4 mL, 0.1 M, pH 9.2) was added to the hydrolyzed sample solution (0.76 mL). Subsequently, 0.2 mL of 5 mM TNBS reagent was added and kept at room temperature for 30 min. After the reaction, 18 mM sodium sulfite and 2 M monobasic sodium phosphate were added to stop the reaction. The absorbance of the reaction mixture was determined at 420 nm using a spectrophotometer (UV-2450, Shimadzu Co., Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed to analyze the degree of hydrolysis of the spirulina proteins. The spirulina protein hydrolysates were resolved on a 16.5% polyacrylamide gel (Mini-PROTEAN® Tris-Tricine gel, Bio-Rad Laboratories, Inc., USA) and stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). The molecular weight marker proteins were triosephosphate isomerase (26,625 Da), myoglobin (16,950 Da), α-lactalbumin (14,437 Da), aprotinin (6,512 Da), and oxidized insulin B chain (3,496 Da) and bacitracin (1,423 Da).

Purification of iron-chelating peptides. To isolate compounds with molecular weights below 3,000 Da, the spirulina protein hydrolysates were ultra-filtered using an Ultracel PL-3 ultrafiltration disk membrane (Millipore Co., USA), and the membrane-filtered solution was lyophilized. The solution of spirulina protein hydrolysates (0.3%, w/v), prepared by dissolving these hydrolysates in 10 mM Tris-HCl buffer (pH 8.0), was loaded onto a Q-Sepharose fast flow column (2.5×10 cm, GE Healthcare Co., Sweden) equilibrated with a 10 mM Tris-HCl buffer (pH 8.0). A linear gradient using Tris-HCl buffers containing 0 to 0.5 M NaCl was employed at a 1.5 mL/min flow rate. The eluate was monitored by measuring the absorbance at 214 nm, and the appropriate eluate fractions were dialyzed using a dialysis membrane (0.1–0.5 kDa MWCO, Spectrum Laboratories, Inc., USA) for 24 h to remove the NaCl. The iron-chelating activity of each fraction was determined. The fraction with the highest iron-chelating activity was pooled and loaded onto a Sephadex G-15 column (1.5×100 cm, GE Healthcare Co., Sweden) equilibrated with deionized water and eluted with deionized water at 0.8 mL/min. The eluate was collected and monitored by measuring the absorbance at 214 nm. The iron-chelating activity of each fraction from the Sephadex G-15 column was determined and the fraction with the highest iron-chelating activity was used for amino acid sequencing.

Determination of iron-chelating activity. The iron-chelating activity was determined according to the method described by Decker and Welch (1990) with minor modifications. The sample solution (1 mL) was reacted with 0.05 mL of 2 mM ferrous chloride solution at room temperature for 10 min. After the reaction, 1.85 mL of deionized water and 0.1 mL of 5 mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-tri-azine-4',4"-disulfonic acid sodium salt) reagent were added and gently mixed for 10 min, and the absorbance at 562 nm was determined using a spectrophotometer (UV-2450, Shimadzu). Deionized water was used as a control. The iron-chelating activity was calculated by the following equation:

$$\text{Iron-chelating activity (\%)} = [(Ac - As)/Ac] \times 100$$

Ac: absorbance of control
As: absorbance of sample

Peptide sequencing using mass spectrometry. MALDI TOF/TOF mass spectrometry analysis was performed using a Bruker ultrafleXtreme™ system (Bruker Daltonics, USA). The ions were accelerated by 19 kV and detected in positive ion mode. Each mass spectrum was obtained as a sum of 2400 laser shots. On the MALDI plate, 1 μL of purified peptide sample was mixed with

1 μL of matrix, a saturated α-cyano-4-hydroxycinnamic acid solution, and then dried in air to make a homogeneous crystal. Argon gas (1.7 bars) was used to obtain fragment ions in the collision induced dissociation (CID) cell. The raw mass spectrometry data were processed with Flex Analysis Software, version 3.3 (Bruker Daltonics).

Results and Discussion

Preparation of spirulina protein hydrolysates. The spirulina protein was extracted by cell disruption and ammonium sulfate precipitation, and the spirulina protein hydrolysates were prepared using a combination of Alcalase and Flavourzyme to facilitate the degree of hydrolysis. In general, peptides obtained from the enzymatic hydrolysis of proteins have high digestibility and bioavailability, compared to proteins (Korhonen, 2009). To determine the degree of hydrolysis at different hydrolysis times, the concentration of available amino groups, which indicates the degree of hydrolysis, was determined (Fig. 1). The available amino group concentration increased with increasing hydrolysis time. The concentration of available amino groups after 1 h of addition of Alcalase and Flavourzyme were 6.3 and 11.0 mM, respectively, whereas the initial concentration was 2.6 mM. After 8 h of hydrolysis, the concentration of available amino groups was 15.3 mM, which appeared to be sufficient for the preparation of hydrolysates, based on the SDS-PAGE data (Fig. 2). The 15-kDa major band on the SDS-PAGE profile gradually disappeared as the enzymatic hydrolysis of the spirulina protein proceeded, resulting in low molecular weight bands only. The resulting spirulina protein hydrolysates were used for the isolation of the iron-chelating peptides.

Purification of iron-chelating peptides. Huang et al. (2012) and Lee and Song (2009) reported that the molecular weights of the peptides with iron-chelating activity were 699 and 1,055 Da, respectively. The iron-binding peptides from cottonseed meal protein hydrolysates had a molecular weight less than 3 kDa (Choi et al., 2012). Therefore, to isolate the iron-chelating peptides derived from the enzymatic hydrolysis of spirulina protein, the hydrolysates were ultra-filtered to isolate compounds below 3 kDa. The ultra-filtered hydrolysates were then fractionated using anion exchange chromatography and gel permeation chromatography. For anion exchange chromatography, the hydrolysates were first fractionated from a Q-Sepharose fast flow column. Amino acid side chains with negatively charged carboxylate groups have the capacity to chelate positively charged metal ions such as iron (Dudev and Lim, 2007). Vegarud et al. (2000) also reported that peptides should have negatively charged side chains to bind divalent cations. From the elution profile of the Q-Sepharose column, two major fractions were obtained (Fig. 3).

The iron-chelating activity of each fraction from anion exchange chromatography was determined, and the F2 fraction had higher iron-chelating activity than the F1 fraction (Fig. 4).

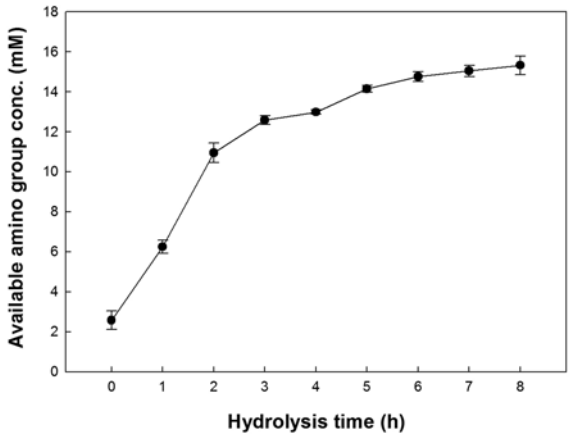

Fig. 1 Effects of hydrolysis time on the available amino group concentration of spirulina protein hydrolysates. Alcalase and Flavourzyme were used for enzymatic hydrolysis of lyophilized spirulina protein (2%, w/v) dissolved in 10 mM sodium phosphate buffer (pH 7.0). The concentration of available amino groups during hydrolysis was determined with the TNBS assay.

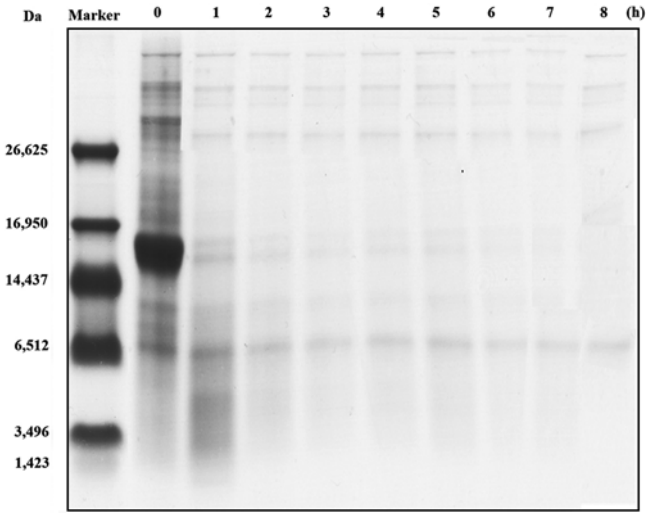

Fig. 2 SDS-PAGE profile of spirulina protein hydrolysates. Spirulina protein hydrolysates were resolved on a 16.5% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. The molecular weight marker proteins were triosephosphate isomerase (26,625 Da), myoglobin (16,950 Da), α-lactalbumin (14,437 Da), aprotinin (6,512 Da), oxidized insulin B chain (3,496 Da), and bacitracin (1,423 Da).

Using the F2 fraction, gel permeation chromatography was used for further purification. As a result, three major fractions were obtained (Fig. 5), and the F21 fraction had the highest iron-chelating activity among the isolated fractions based on the iron-chelating activity data (data not shown). Consequently, the F21 fraction was analyzed for its amino acid sequence using MALDI-TOF/TOF mass spectrometry.

The MALDI mass spectrum of the peptide (F21 fraction) isolated from the spirulina protein hydrolysates is shown in Fig. 6.

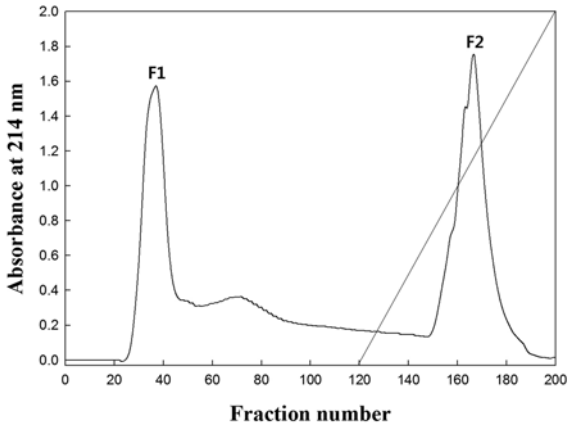

Fig. 3 Elution profile of spirulina protein hydrolysates from ion exchange chromatography. The absorbances of the eluted fractions were measured at 214 nm.

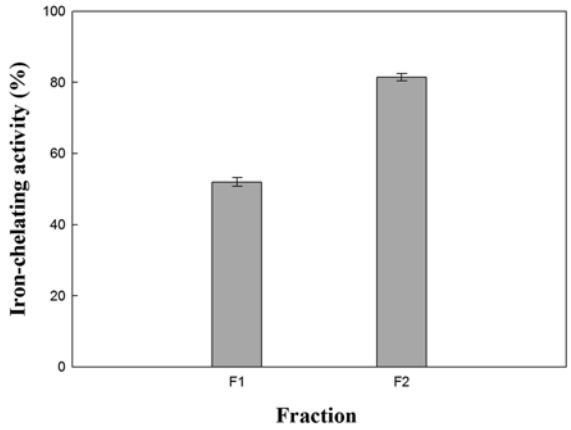

Fig. 4 Iron-chelating activity for each fraction (F1 and F2) from ion exchange chromatography (Fig. 3).

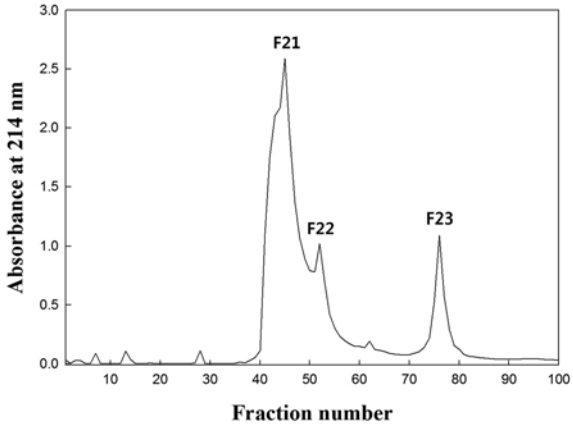

Fig. 5 Elution profile of the F2 Fraction (from Fig. 3) from gel permeation chromatography. Absorbances of the eluted fractions were measured at 214 nm.

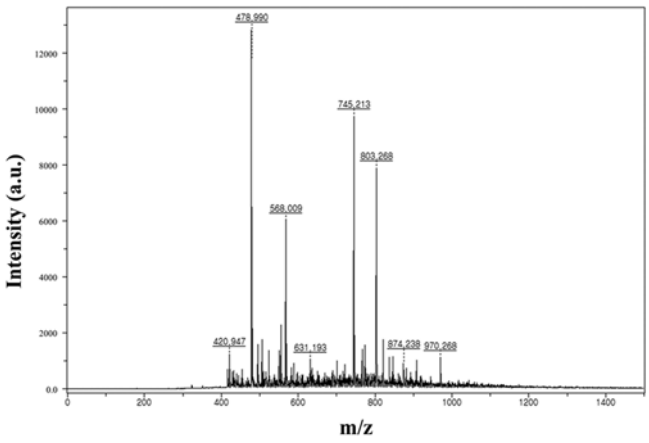

Fig. 6 The representative MALDI mass spectrum of the peptide (F21 fraction) isolated from spirulina protein hydrolysates.

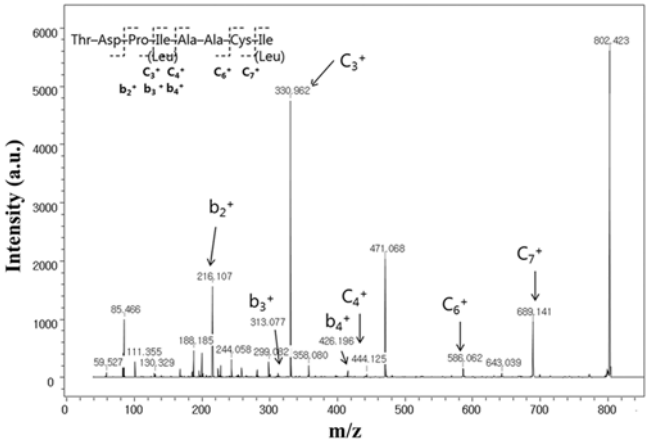

Fig. 7 Identification of amino acid sequence of the iron-chelating peptide (F21 fraction) using MALDI TOF/TOF mass spectrometry.

All ions have a protonated molecular ion form ($[M+H]^+$). There were four major and several minor peaks, and all major peaks were further fragmented by high energy CID. The ion at m/z 802.423 was identified as a unique peptide, and de novo peptide sequencing for this unique peptide was performed using tandem mass spectrometry. The peptide sequence for the peak at m/z 802.423 corresponded to Thr-Asp-Pro-Ile(Leu)-Ala-Ala-Cys-Ile(Leu) (Fig. 7). However, Ile and Leu are not differentiable by mass spectrometry, because they have identical masses.

In general, bioactive peptides, such as metal-chelating peptides, are composed of 3-20 amino acids. Kim et al. (2013) and Choi et al. (2013) reported that the peptides with iron-binding activity had a molecular weight below 3 kDa. An iron-binding peptide with a molecular weight of 1,055 Da from porcine blood plasma protein hydrolysates had 9 amino acids (Lee and Song, 2009), and the 699 Da peptide with iron-binding activity from shrimp processing by-

product hydrolysates had 7 amino acids (Huang et al., 2012). Similarly, the iron-chelating peptide isolated from the spirulina protein hydrolysates in the present study had 8 amino acids.

Thr, Asp, and Pro are frequently reported in the amino acid composition of metal-binding peptides (Storchksdieck et al., 2007; Huang et al., 2012), and an iron-chelating peptide from spirulina protein hydrolysates also contains Thr, Asp, and Pro. There have been studies on iron-chelating peptides containing Asp, in particular, which is one of key amino acids for iron binding (Gaucheron, 2000; Jiang and Mine, 2000). Cys, another amino acid in the iron-chelating peptide isolated from spirulina protein hydrolysates, has also been known to play a role in the formation of a chelate with iron (Taylor et al., 1986). Guo et al. (2013) also reported that the iron-chelating peptide isolated from Alaska pollock skin hydrolysates contained Cys. Therefore, it appears that Thr, Asp, Pro, and Cys are the major amino acids of the peptide with iron-chelating activity isolated from spirulina protein hydrolysates. In addition, because of its iron-chelating ability, it should be noted that the isolated peptide could be utilized as an iron supplement, if scale-up processing is developed for commercial application in the future.

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