JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Novel Peptide with a Specific Calcium-Binding Capacity from Whey Protein Hydrolysate and the Possible Chelating Mode

Lina Zhao, Qimin Huang, Shunli Huang, Jiaping Lin, Shaoyun Wang,* Yifan Huang, Jing Hong, and Pingfan Rao

College of Bioscience and Biotechnology, Fuzhou University, Fuzhou, Fujian 350002, People's Republic of China

ABSTRACT: A novel peptide with a specific calcium-binding capacity was isolated from whey protein hydrolysates. The isolation procedures included diethylaminoethyl (DEAE) anion-exchange chromatography, Sephadex G-25 gel filtration, and reversed-phase high-performance liquid chromatography (HPLC). A peptide with a molecular mass of 237.99 Da was identified by liquid chromatography−electrospray ionization/mass spectrometry (LC−ESI/MS), and its amino acid sequence was confirmed to be Gly-Tyr. The calcium-binding capacity of Gly-Tyr reached 75.38 μg/mg, increasing by 122% when compared to the hydrolysate complex. The chelating interaction mode between the Gly-Tyr and calcium ion was investigated, indicating that the major binding sites included the oxygen atom of the carbonyl group and nitrogen of the amino or imino group. The folding and structural modification of the peptide arose along with the addition of the calcium ion. The profile of ^{1}H nuclear magnetic resonance (NMR) spectroscopy demonstrated that the electron cloud density around the hydrogen nucleus in the peptide changed was caused by the calcium ion. The results of ζ potential showed that the Gly-Tyr−Ca chelate was a neutral molecule in which the calcium ion was surrounded by the specific binding sites of the peptide. Moreover, thermogravimetry−differential scanning calorimetry (TG−DSC) and calcium-releasing assay revealed that the Gly-Tyr−Ca chelate exerted excellent thermal stability and solubility in both acidic and basic conditions, which were beneficial to calcium absorption in the gastrointestinal tract of the human body and, therefore, improved its bioavailability. These findings further the progress in the research of whey protein, suggesting the potential in making peptide−calcium chelate as a dietary supplement.

KEYWORDS: *whey protein, calcium-binding peptide, purification, characterization, chelating mode*

INTRODUCTION

Whey protein is a mixture of globular proteins isolated from whey as the byproduct in the cheese production process, and it releases a variety of bioactivities through the appropriate protease hydrolysis.[1,2] As one of the good protein sources, whey protein and its hydrolysates can be used as a food ingredient, nutritional supplement, or functional intensifying agent. It is well-reported that whey protein has calcium-binding sites that can bind with calcium ion, such as β-LG, α-LA, and lactoferrin.[3,4] Moreover, α-LA has especially strong calcium-binding sites.[5,6] However, studies on the purification of specific calcium-binding peptide derived from whey protein hydrolysate and the possible chelating mode are scarcely reported.

Calcium is an essential nutrient in the body, and adequate calcium intake is related with the low risk of osteoporosis, hypertension, colon caner, obesity, and kidney stones.[7] The intake of calcium could increase the bone density in children, and it is essential among the middle-aged and aged to prevent osteoporosis.[8,9] With the increase in the population of the aged throughout the world, there is a growing interest in developing calcium supplementary medicine to prevent and treat bone disease.[10] The ionized calcium has served as the main calcium supplements for humans in recent years.[11] However, the disadvantage of ionized calcium is that it is prone to form calcium phosphate deposition in a basic intestine environment.[12] As a result, the bioavailability of dietary calcium is severely lowered. The organic calcium supplement has been becoming one of popular research topics. Both amino acid chelate and small peptide calcium complex can be directly absorbed in the human body. The transportation of metal elements in chelate depends upon ligands. The revolving mechanism of small peptides is much different from that of amino acids. In comparison to amino acids, the absorption of small peptides has many merits, such as consuming little energy, accelerating transport speed, and carriers not being easy to saturate.[13] Hydrolyzed whey peptides, obtained from proteolytic digestion, have shown considerable capacity in incorporating with divalent ions, such as calcium, iron, etc.[14,15] The chelating complex chelated between whey peptides and calcium ion can promote calcium absorption in the human body and, therefore, improve its bioavailability. Peptide-chelated calcium can probably be a suitable candidate as a supplement to improve calcium absorption in the gastrointestinal tract of the human body.

The purpose of this study was to investigate the purification of specific calcium-binding peptide derived from whey protein hydrolysate and the possible chelating mode. To evaluate the chelation mode between the purified calcium-binding peptide and calcium ion, structural characterization methods were applied in detail. The study would be of significance in using the hydrolyzed peptides from whey protein as calcium-binding peptide ingredients in functional foods. The physiological

Received: May 24, 2014
Revised: September 29, 2014
Accepted: September 29, 2014

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

activity of calcium-binding peptides may be used as one kind of food additive that prevents bone disorders.

MATERIALS AND METHODS

Materials. Whey protein (WPC80, containing 80.5% protein) was kindly provided by Hilmar Corporation (Batch 20111107, Hilmar, CA). The commercial proteases, Flavourzyme (EC. 3.4.11.1, 2×10^6 units/g) and Protamex (EC. 3.4.24.28, 1.5×10^6 units/g), were purchased from Novo (Novozymes, Denmark). Toyopearl DEAE-650M and Sephadex G-25 were offered by Amersham Pharmacia (Amersham Pharmacia Co., Uppsala, Sweden). All of the other chemicals and solvents were of analytical grade.

Hydrolysis of Whey Protein. A total of 5% (w/v) whey protein solution was denatured in 80 °C for 20 min, and the pH was adjusted to 7.0. The sample was hydrolyzed using Flavourzyme and Protamex (2:1, w/w) with a substrate/enzyme ratio of 25:1 (w/w) at 49 °C for 7 h. Hydrolysate was heated at boiling water for 10 min to inactive the enzyme and cooled to room temperature. The mixture was subsequently centrifuged at 16000*g* for 20 min, and then the supernatant named whey protein hydrolysate (WPH) was lyophilized and stored at −20 °C for subsequent investigation.

Purification and Identification of Calcium-Binding Peptide. *Ion-Exchange Chromatography on Diethylaminoethyl (DEAE).* The peptide that could bind with calcium and form peptide−calcium chelate was defined as the calcium-binding peptide. The slurry of Toyopearl DEAE-650M was packed in a column (20 × 2.5 cm) and then equilibrated at 5 column volume (CV) of 20 mM Tris-HCl buffer (pH 9.0) as the equilibrating buffer. Afterward, 100 mg of the lyophilized hydrolysates that had been through 0.45 μm filter film was dissolved in 10 mL of the same buffer (pH 9.0) and loaded on the column. Then, after washing with the equilibrating buffer, the collected peak was labeled as the non-absorbed fraction. The bond peptides were eluted by a gradient elution with the same buffer containing 0−0.5 M NaCl. The flow rate was 0.5 mL/min; fraction volume was 5 mL/tube; elution was monitored at 214 nm; all peaks were collected; and calcium-binding capacities of the fractions were determined.

Gel Filtration on Sephadex G-25. The fraction with the highest calcium-binding capacity was pooled and lyophilized; 200 mg of the sample was dissolved in 5 mL of deionized water and loaded onto a Sephadex G-25 column (100 × 2.0 cm), which had previously been equilibrated with deionized water, and then eluted with deionized water at a flow rate of 0.3 mL/min. The eluate was monitored by measuring the absorbance at 214 nm. After calcium-binding capacity was determined, the fraction with the highest activity was pooled and lyophilized.

High-Performance Liquid Chromatography (HPLC) on C18. The lyophilized sample collected from G-25 was dissolved in distilled water approximately equivalent to 30 mg/mL and purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on C18 reversed silica gel chromatography (Gemini 5 μm C18, 250 × 10 mm, Phenomenex, Inc., Torrance, CA). Elution was performed on solution A [0.05% trifluoroacetic acid (TFA) in water] and solution B (0.05% TFA in acetonitrile) with a gradient of 0−30% B at 1.0 mL/min for 50 min. The elution was monitored at 214 nm, and the absorption peaks were fractionated for measuring the calcium-binding activity. The injection volume was generally 200 μL.

Identification of the Purified Calcium-Binding Peptide. The molecular mass and amino acid sequence of the purified calcium-binding peptide were determined using liquid chromatography−electrospray ionization/mass spectrometry (LC−ESI/MS, Delta Prep 4000, Waters Co., Milford, MA) over the m/z range of 300−3000.

Calcium-Binding Capacity Assay. The calcium-binding capacity was defined as the content of calcium (μg) bound with peptide (mg) after the chelating reaction. Lyophilized whey protein hydrolysate was dissolved in deionized water to be 1.0 mg/mL and mixed with 5 mM $CaCl_2$ in 0.2 M sodium phosphate buffer (pH 8.0). The solution was stirred at 37 °C for 2 h, and pH was maintained at 8.0 with a pH meter. The reaction mixture was centrifuged at 10000*g* at room temperature for 10 min to remove insoluble calcium phosphate salts. Sodium phosphate was used as the calcium-binding buffer, which could react with calcium ion and peptide, separately, and show obviously different reactions. Calcium ion could combine with phosphate to form calcium phosphate precipitation, while peptide could take calcium ion from precipitation. As a result, peptide−Ca chelate was a water-soluble substance, and a soluble calcium content in the supernatant represented the calcium content associated with the peptide.[16] The calcium contents in the supernatant were determined using a colorimetric method with *ortho*-cresolphthalein complexone reagent.[17] The absorbance at 570 nm was determined after adding the working solution to the sample. The experiments were performed in triplicate, and values were expressed as the mean ± standard deviation (SD).

Characterization of the Purified Calcium-Binding Peptide. *Formation of the Peptide−Calcium Chelate.* Peptide−calcium chelate was the chelating component of the reaction between dipeptide Gly-Tyr and calcium ion. The calcium-binding chelate was prepared by adding 5 mL of 1% (w/v) $CaCl_2$ into 20 mL of 2.5% (w/v) calcium-binding peptide solution. The reaction was placed in a controlled water bath with constant agitation (100 rpm) at 37 °C for 2 h after the pH of the solution was adjusted to 7.0 by the addition of 0.1 M NaOH. Then, the mixture was added with absolute ethanol (9 times of solution volume) to remove free calcium and centrifuged at 10000*g* for 10 min, and the precipitates was lyophilized for analysis.

Ultraviolet (UV) Absorption Spectroscopy Assay. The UV spectra of the calcium-binding peptide and peptide−calcium chelate were recorded over the wavelength range from 190 to 400 nm by a UV−vis spectrophotometer (UV-2600, UNICO Instrument Co., Ltd., Shanghai, China) as the method described by Chen et al.[18] The calcium-binding peptide of 0.2 mg/mL was prepared. The peptide−calcium chelate was prepared by adding 0, 2, 4, 6, 8, and 10 μM $CaCl_2$ to 0.2 mg/mL calcium-binding peptide solution, separately. The mixed solution reacted at room temperature for 30 min.

Fluorescence Spectra Analysis. Fluorescence spectra were measured to monitor conformational changes in the peptide induced by calcium chelation using the F-4600 fluorescence spectrophotometer (Hitachi Co., Japan). The excitation wavelength was 280 nm, and emission wavelengths between 290 and 360 nm were recorded.

Fourier Transform Infrared Spectroscopy (FTIR) Measurement. A total of 1 mg of calcium-binding peptide powder or peptide−calcium chelate was ground evenly with 100 mg of dry KBr under infrared light to the size below 2.5 μm. Then, the transparent KBr piece was made at 60 MPa. To examine whether the variation in pellet thickness causes significant interference in the measured spectra, three different pellets were prepared from the same sample and their FTIR spectra were compared. The average of these three spectra was then used for analysis. The FTIR spectra were recorded using an infrared spectrophotometer (360 Intelligent, Thermo Nicolet Co., Waltham, MA) over a wavenumber region between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}, and the sample spectra were background-subtracted using a spectrum collected in the absence of a peptide−calcium chelate sample. Therefore, the physical variation in the FTIR spectra caused by the KBr technique had been removed prior to the FTIR spectra analysis. A total of 64 scans were recorded per sample in the FTIR spectra, and the peak signals in the spectra were analyzed using OMNIC 8.2 software (Thermo Nicolet Co., Madison, WI).

^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy Assay. The peptide−calcium chelate and calcium-binding peptide (0.5 mg) were dissolved in 500 μL of deionized water, separately. A total of 50 μL of deuterium oxide (D_2O) was added after the pH of the solution was adjusted to 6.5. The samples were transferred into 5 mm NMR tubes and subjected to NMR analysis with a Bruker Avance III spectrometer (Bruker Biospin, Rheinstetten, Germany).

Differential Scanning Calorimetry (DSC) Analysis. Thermogravimetry (TG)−DSC simultaneous thermal analyzer (STA449C, NETZSCH, Germany) was employed to measure the thermal property. Hermetic pans with lyophilized powder samples (5 mg) were heated from 50 to 600 °C at a programmed heating rate of 10 °C/min in a argon atmosphere. An empty pan was used as a reference.

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

ζ Potential Measurement. A Malvern Nano ZS Zetasizer based on dynamic light scattering was employed to measure the ζ potential. The system works according to the phase analysis light scattering (PALS) principle. The volume of all samples (3 mg) was 15 mL with autotitration by 0.25 M NaOH or HCl. The samples were equilibrated for 30 s before titration.

Calcium-Releasing Percentage Experiment. The calcium-releasing percentage determination of peptide−calcium chelate and $CaCl_2$ was examined according to the method described by Wang et al.[19] Peptide−calcium chelate and $CaCl_2$ were dissolved in deionized water, separately, equivalent to 10 μg/mL. The pH of these solutions was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. After incubation in a shaking water bath for 2 h at 37 °C, the solutions were centrifuged in a refrigerated centrifuge at 10000*g* for 10 min. The calcium amount in the supernatant and the total calcium in the whole solution were determined by the colorimetric method with an *ortho*-cresolphthalein complexone reagent. The calcium releasing percentage was calculated as follows:

$$\text{calcium-releasing percentage (\%)} = \text{Ca amount in supernatant (g)/total Ca in solution (g)} \times 100$$

Statistical Analyses. All data were presented as means (SDs) conducted with three replicates. The statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). Analysis of variance (ANOVA) was performed to determine the significance of the main effects. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Purification and Identification of the Calcium-Binding Peptide. Bioactive peptides are usually purified using a combination of chromatographic techniques.[20] Throughout the purification process, calcium-binding activity of WPH was assessed using the colorimetric method with an *ortho*-cresolphthalein complexone reagent.[17] Three fractions (F1, F2, and F3) of the WPHs were divided on a DEAE anion-exchange chromatography column (Figure 1). The calcium-binding capacity was noticeably higher in F2 and F3 compared to F1 (Figure 1). Moreover, in comparison to WPH (33.92 μg/mg), the calcium-binding capacity of F2 was significantly improved (57.02 μg/mg).

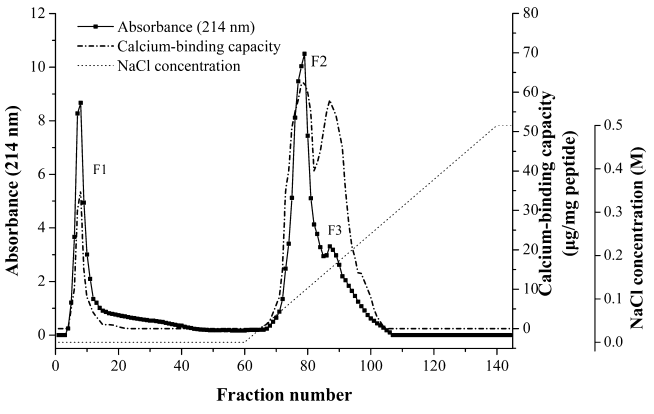

Figure 1. Fractionation on a DEAE anion-exchange chromatography column and the calcium-binding capacity of the three fractions separated from the DEAE column. The peptide was washed with the binding buffer (0.02 M Tris−HCl buffer at pH 9.0) and eluted with a linear gradient of 0−0.5 M NaCl in the same buffer.

Fraction F2 with the highest calcium-binding capacity was further separated using size-dependent Sephadex G-25 gel filtration chromatography resulting in four major fractions, named F21, F22, F23, and F24 (Figure 2). F23 exerted the most excellent calcium-binding capacity (Figure 2). The result

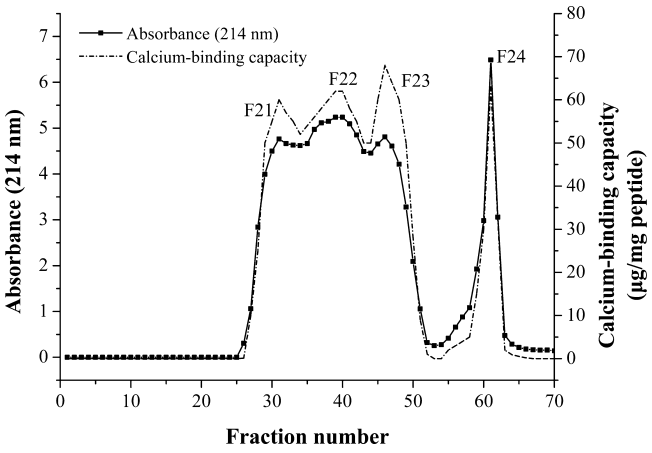

Figure 2. Fractionation of the fraction F2 with the highest calcium-binding activity from DEAE chromatography on a Sephadex G-25 gel filtration chromatography column and the calcium-binding capacity of the fractions F21−F24 separated from a Sephadex G-25 column.

was consistent with some previous reports, which showed that a lower molecular mass peptide exhibited a tendency to combine with calcium ion.[21,23] It is reported that the lowest molecular weight peptide fraction (<1 kDa) had the higher calcium-binding activity through ultrafiltration and small molecular peptide fractions purified on a G-25 column possessed higher calcium-binding capacity.[21,22]

The active peak F23 was pooled, dissolved in distilled water, and loaded onto semi-preparative C18 RP-HPLC. The fraction F23 was separated into 13 major distinct fractions (Figure 3A). Each fraction was pooled, and calcium-binding capacity was determined subsequently (Figure 3B). Among 13 distinct fractions, fraction 7 showed the highest calcium-binding ability, and then it was further fractionated by RP-HPLC on a C18 column. The elution profile showed that fraction 7 was composed of five peaks, and fraction 2 (Figure 4A), one of the eluted peaks, was the purifed peptide. The calcium-binding capacity of fraction 2 reached 75.38 μg/mg (Figure 4B). The chromatographic profile of fraction 2 on analytic C18 RP-HPLC (Figure 5) showed only a single peak called WPH-A, indicating the high purity of the isolated peptide.

The amino acid sequence of WPH-A eluted from RP-HPLC chromatography was identified by LC−ESI/MS (Figure 6). According to the mass/charge ratio (m/z) of 238.99 Da of the molecular ion peak ($[M + H]^+$), the molecular mass of WPH-A was confirmed to be 237.99 Da. The m/z of fragment ion y1 was 181.99 Da, which resulted from the relative molecular mass of tyrosine in addition with one hydrogen atom. On the basis of calculation in ESI ion molecular mass, one residue in WPH-A was Tyr and the other one was Gly. Therefore, the animo acid sequence was determined to be Gly-Tyr. The result was confirmed by comparison to data from the National Center for Biotechnology Information (NCBI) database.

Not only could the differences in the net charge and length of peptides influence the extent of chelate formation with metal ion, but also the different amino acid residues of peptides affected their activities,[14,24] including the amino acid composition and sequence of the peptides.[25] Chaud et al.[14] reported that tyrosine seemed to bind with divalent metal

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

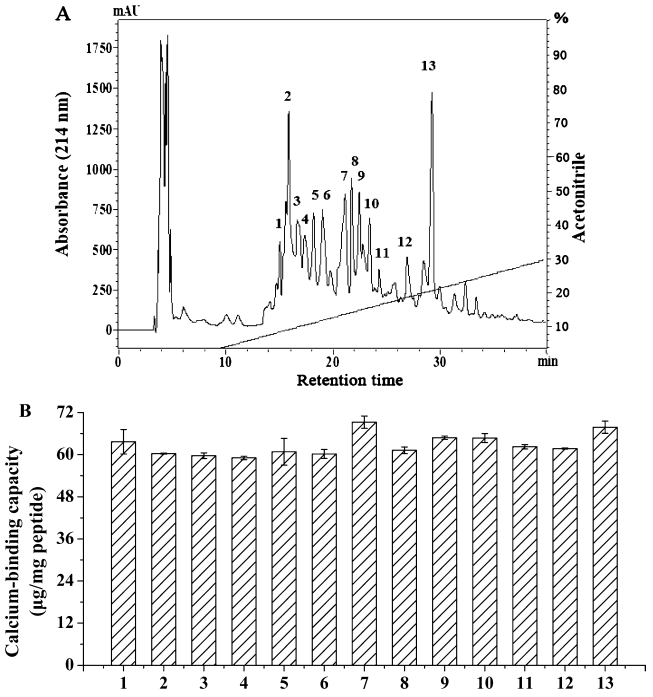

Figure 3. (A) Fraction F23 from a Sephadex G-25 column and then applied to a semi-preparative C18 RP-HPLC column. The column was then washed with a linear gradient of 0−30% acetonitrile containing 0.05% TFA. (B) Calcium-binding capacity for the fractions 1−13 of F23 separated from semi-preparative HPLC.

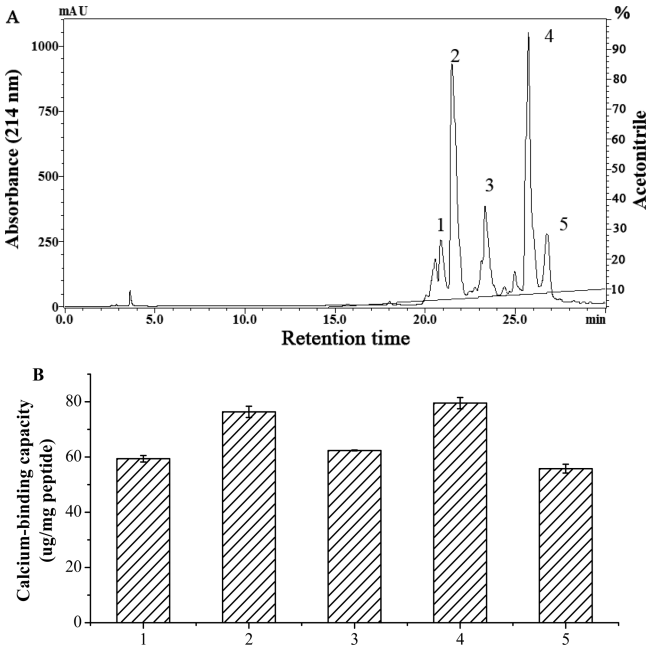

Figure 4. (A) RP-HPLC of fraction 7 derived from preparative HPLC. The column was then washed with a linear gradient of 0−10% acetonitrile containing 0.05% TFA. (B) Calcium-binding capacity of fractions 1−5 from RP-HPLC.

cations through oxygen of the phenolic hydroxyl group. The phosphorylation of tyrosine and serine residues could provided suitable binding sites for positively charged metals, such as calcium, iron, or zinc.[26] Moreover, the animo acid glycine was considered as an organic ligand having the metal ion binding function. Jung et al.[27] purified a calcium-binding peptide from

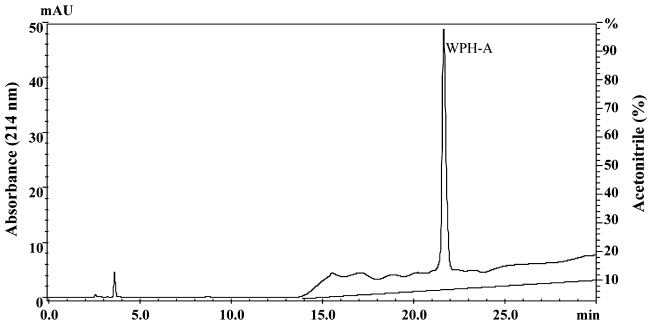

Figure 5. Analytic RP-HPLC of the peak WPH-A for purify identification. The column was washed with a linear gradient of 0−10% acetonitrile containing 0.05% TFA.

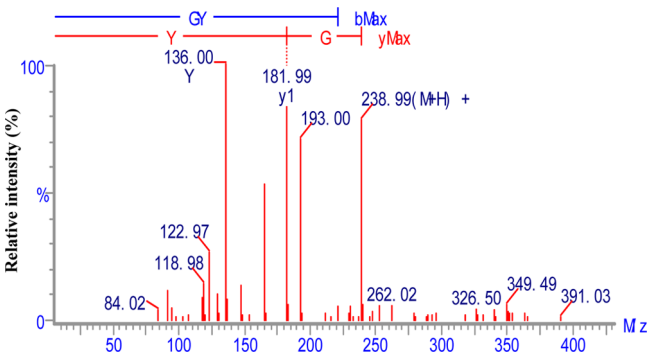

Figure 6. Identification of the amino acid sequence of the calcium-binding peptide using LC−ESI/MS.

Alaska pollack containing Gly, Ser, and Val. Hoki bone hydrolysates had a high affinity to calcium, and the major animo acid composition of the peptide included Gly, Asp, and Glu.[28] An iron-chelating peptide with multi-glycine residues was also purified from anchovy muscle protein.[29] In this study, a calcium-binding peptide was identified to contain Gly and Tyr residues; both of them contributed to combine with divalent minerals.

Characterization of the Purified Calcium-Binding Peptide. *UV Absorption Spectroscopy Assay.* The UV spectra of calcium-binding peptide Gly-Tyr showed an obvious difference from that of the Gly-Tyr−Ca chelate (Figure 7). A strong absorption band was observed at about 200 nm, which could be regarded as the characteristics of the amide bond in peptide. Another absorption peak at around 280 nm appeared in both Gly-Tyr and Gly-Tyr−Ca chelate, resulting from the characteristic UV absorption of Tyr. With the addition of calcium ion to dipeptide Gly-Tyr, the intensity of the absorption band of the amide bond became higher than that of dipeptide alone. In the magnified UV spectra image, the red shifts of the band, such as the red curve in Figure 7, could be clearly observed and a small peak arose in the dipeptide−calcium chelate.

Through observing the shifts and intensity changes of UV bands, the UV absorption situation of the amide bond in peptide and the side group in Tyr could be analyzed. The UV spectra phenomenon was consistent with some other studies, which demonstrated that the spatial structure with the chirality of the chromospheres (C═O and −COOH) and auxochromes (−OH and $-NH_2$) of peptides changed after incorporating with the calcium ion.[30,31] The main reason for the intensity changes of the band indicated that the oxygen atom of the

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

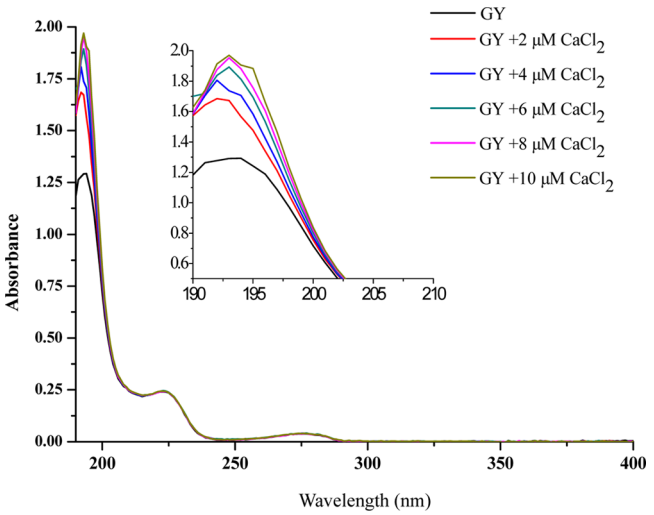

Figure 7. UV spectra of Gly-Tyr and Gly-Tyr−Ca chelate over the wavelength range from 190 to 400 nm.

carbonyl group and nitrogen of the amino group in the peptide bond with calcium.[32] The appearance of a small peak reflected the difference of relevant valence electron transition when more calcium ion reacted with Gly-Tyr. All of these band changes suggest that Gly-Tyr could bind with calcium ion and form the Gly-Tyr−Ca chelate.

Fluorescence Spectra Analysis. The purified calcium-binding dipeptide Gly-Tyr contained aromatic amino acid Tyr, which could generate endogenous fluorescent at a proper excitation wavelength. The fluorescence emission spectra of Gly-Tyr was examined to monitor its structural modification with $CaCl_2$ treatment. Through fluorescence quenching, the calcium binding sites could be speculated. The fluorescence spectra of Gly-Tyr and Gly-Tyr−Ca chelate are shown in Figure 8. When excited at 280 nm, Tyr in Gly-Tyr exhibited an intrinsic fluorescence emission maximum at 310 nm. With the increased calcium ion concentration, the endogenous fluorescence decreased dramatically, which meant that calcium ion had a significant effect on the Gly-Tyr structure. Especially, apparent folding and conformation modification of Gly-Tyr happened at the beginning of the addition with 5 μM $CaCl_2$.

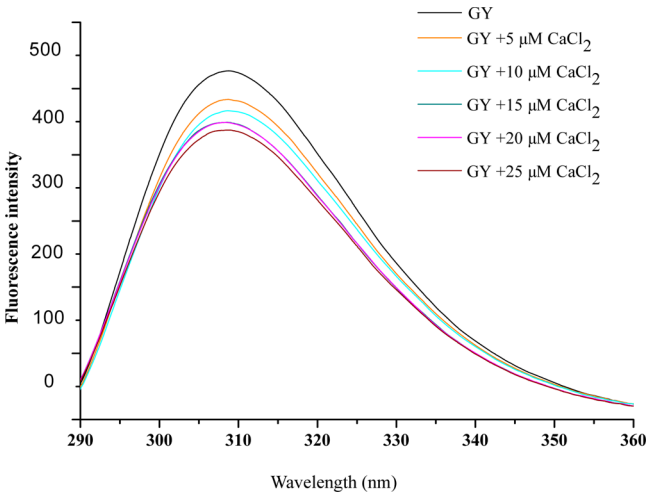

Figure 8. Fluorescence emission spectra of the peptide Gly-Tyr at various concentrations of Ca^{2+}.

This result is consistent with that of the iron-chelating peptides from anchovy (*Engraulis japonicus*) muscle protein.[29] Reddi et al.[33] demonstrated that the Zn^{II} binding energy was used to fold the peptide in the zinc-binding reaction of a zinc-finger peptide. As the concentration of $CaCl_2$ increased, fluorescence quenching became weaker than before. It meant that there were not any spared binding sites in Gly-Tyr for the calcium ion. This experimental evidence suggested that the structure of functional binding sites in Gly-Tyr changed, resulting in the formation of the Gly-Tyr−Ca chelate when $CaCl_2$ reacted with Gly-Tyr. The addition of metal ion induced the intrinsic fluorescence quenching of protein or peptide, especially for the oligo-peptide without thermodynamic structure stability.[34] Generally, the calcium ion could cause fluorescence quenching of the calcium-binding peptide, which likely contributed to the decrease in the fluorescence intensity.[19]

FTIR Measurement. Changes in the characteristic FTIR absorption peaks of some functional binding sites in peptide could reflect the interaction of metal ion with organic ligand groups in the peptide. To analyze calcium-ion-induced changes in the structure of the calcium-binding peptide Gly-Tyr, the FTIR technique was employed.

Figure 9 depicted the infrared spectrum of Gly-Tyr in the absence and presence of calcium. There were significant

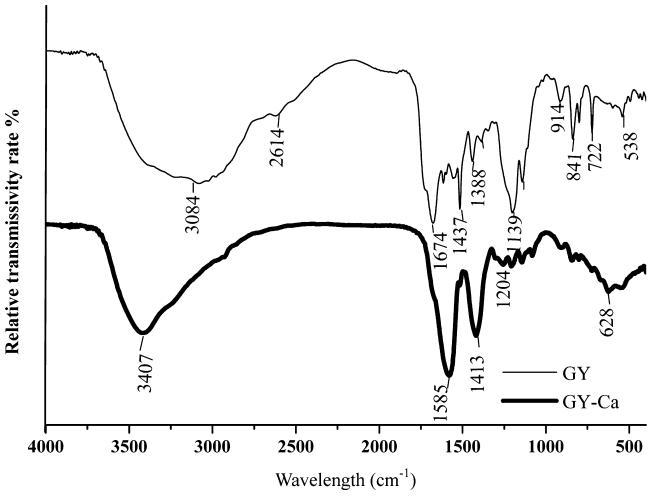

Figure 9. FTIR spectra of Gly-Tyr and Gly-Tyr−Ca chelate in the regions from 4000 to 400 cm^{-1}.

differences between Gly-Tyr and Gly-Tyr−Ca chelate. The results implied that the wavenumbers (1674 and 1437 cm^{-1}) of the amide I band were shifted to lower frequencies (1585 and 1413 cm^{-1}) after the addition of calcium, which stood for infrared absorption of the carbanyl group caused by the antisymmetric stretching vibration υ_{as} (COO^-) and the symmetric stretching vibration υ_s (COO^-) of carboxylic acid ions. Upon coordination with the Gly-Tyr−Ca chelate, the C bands corresponding to the carboxyl groups at 1204 and 1139 cm^{-1} exhibited two weaker peaks in the fingerprint region. It is reported that ferric chloride could bind with casein enzymatic hydrolysates to form the Fe^{3+}−peptide complex, and the chief iron-binding site relates to the amide and carboxylate groups.[14] When metal ions combined with ligand atoms, such as O, N, and S, of organic compounds to form the chelate, the absorption peaks because of vibration of the coordinate bonds were typically located in the far-infrared region. The

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

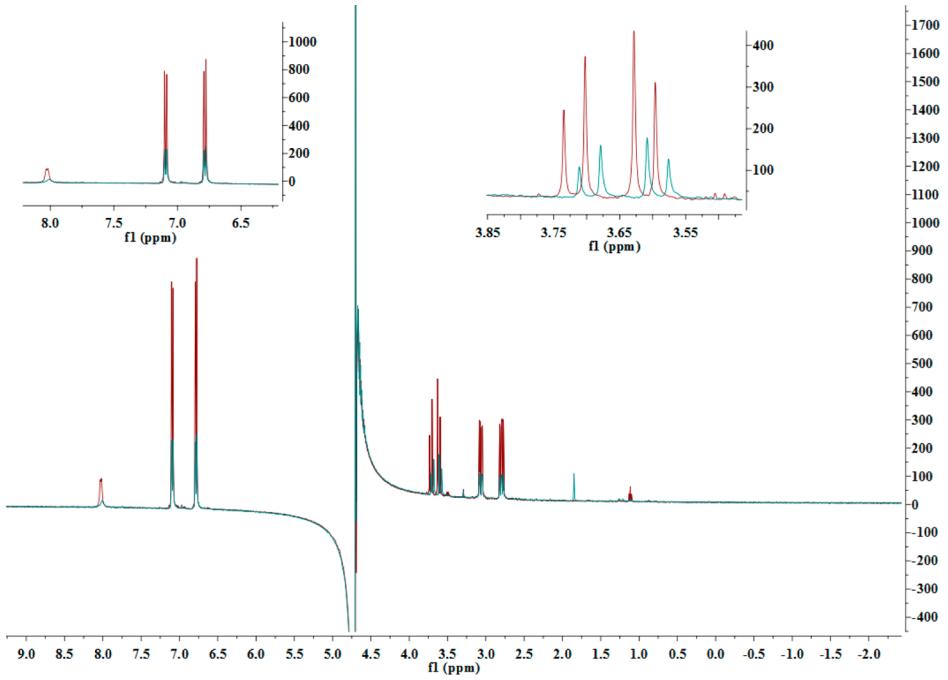

Figure 10. ^{1}H NMR spectral region from −0.30 to 10.00 ppm of Gly-Tyr and Gly-Tyr−Ca chelate (the red line means Gly-Tyr, and the green line means Gly-Tyr−Ca).

Ca−O vibration band was located between 500 and 800 cm^{-1}, while the interaction between the peptide and calcium ion resulted in a broadening and decreased intensity of the peaks; even some band absorptions disappeared, which was in agreement with the report by Chen et al.[18] Absorption of a high frequency at 3084 cm^{-1} referred to the antisymmetric stretching vibration υ_{as} (N−H) and the symmetric stretching vibration υ_{s} (N−H) of the N−H bond in the Gly-Tyr spectrum. Absorption of a high frequency at 3200−3600 cm^{-1} referred to the possible Ph−O-H strech and H bonds of hydration in the Gly-Tyr spectrum. With the coordination of the calcium−peptide complex, the waveform and the absorbance values had greatly changed. After the addition of calcium, the chelation reaction resulted in a shift to a higher wavenumber (3407 cm^{-1}), which indicated that the electron cloud density of N−H in Gly-Tyr became stronger because of the inductive effect or dipole field effect.

These phenomena indicated that the interaction between Gly-Tyr and calcium occurred via the carboxyl oxygen atoms and amino group nitrogen atoms in the peptide, which were similar to the calcium-binding peptides derived from soybean protein hydrolysates.[35]

^{1}H NMR Spectroscopy Assay. The low-field region, from 6.75 to 8.00 ppm, of the Gly-Tyr and Gly-Tyr−Ca NMR spectrum (Figure 10) contained signals from aromatic group tyrosine (6.75 and 7.10 ppm) and N−H of the amide bond (8.00 ppm). The symmetric doublet in the region of 3.55−3.75 ppm corresponded to H spin coupling cracking in α-H of Gly and Tyr. The signals at 3.00 and 2.75 ppm arose from two β-H atoms of tyrosine. With the addition of the calcium ion, the symmetric doublet signals of α-H in Gly-Tyr shifted to lower parts per million (ppm) values, whose chemical shift was approximately 0.025 ppm. The change in hydrogen atom spin coupling cracking resonance signal peaks was caused by the combination of Gly-Tyr and calcium, thereby the electron density around the hydrogen nucleus in Gly-Tyr was affected.

The chemical shift information on ^{1}H NMR could explain the distribution of the electron cloud density around the hydrogen nucleus among different substances; consequently, the reaction situation between the purified calcium-binding dipeptide Gly-Tyr and calcium ion could be revealed. A declined electron cloud density along with a decreased shielding effect and intensive resonant frequency led to the movement of resonance signal peaks to a lower field region.

TG−DSC Analysis. The thermostability of samples was investigated through DSC and TG thermal analysis. There were remarkable differences of peak temperatures between dipeptide Gly-Tyr and Gly-Tyr−Ca chelate (Figure 11). The TG−DSC curve of Figure 11A showed that the thermal decomposition reaction of Gly-Tyr was not single in the whole process of weight loss. The thermal transitions at 179.40, 269.15, 359.99, and 530.51 °C were accompanied by weight loss of 25.74, 14.23, 15.15, and 14.32%, respectively. These endothermic peaks were caused by the C−N bond in Gly-Tyr. Whereas, obviously high shift endothermic peaks were observed at 428.77, 462.05, 504.07, and 550.93 °C after calcium ion treatment (Figure 11B). Meanwhile, TG of Gly-Tyr−Ca showed a flat and decline curve with a mass loss of 80%, which demonstrated that calcium-ion-processed Gly-Tyr resulted in a great increase in the DSC peak temperature accompanied by less and reduced weight loss, indicating that the Gly-Tyr−Ca chelate exhibited distinct thermal stability. This result suggested that Ca-processed dipeptide Gly-Tyr was less sensitive to heat denaturation with a higher peak temperature because of the formation of the Gly-Tyr−Ca chelate compound and the thermal stability of Gly-Tyr−Ca chelate was higher than that of Gly-Tyr alone.

ζ Potential. The principal of ζ potential was polarization of the water that occurred in the interface of two phases and then inducing that potential difference that existed in the phases. Figure 12 presented the ζ potential values of the Gly-Tyr−Ca chelate compared to Gly-Tyr without the calcium ion. The

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

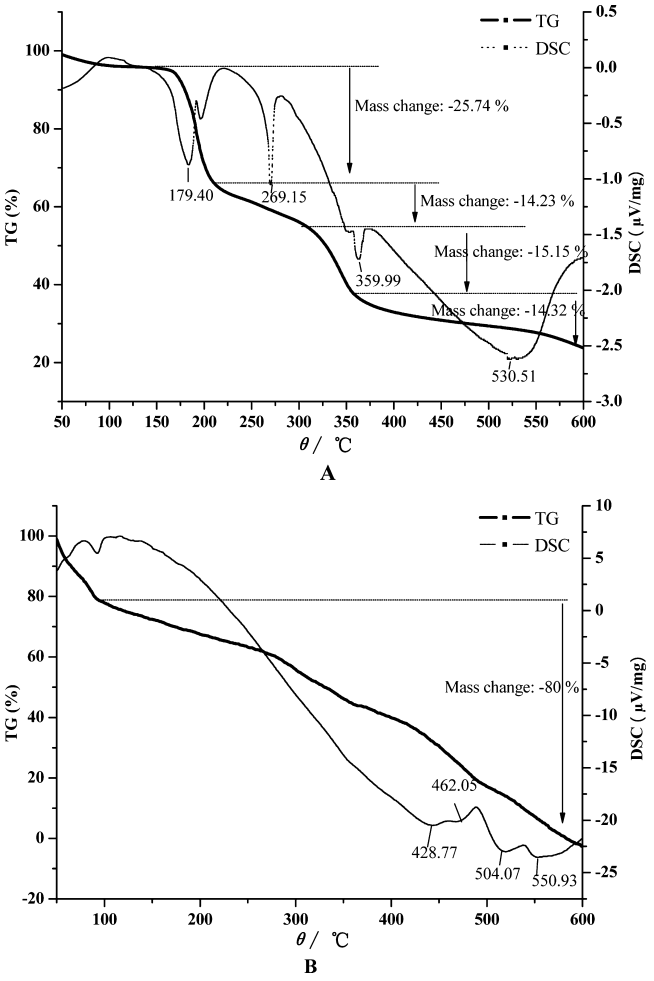

Figure 11. Typical DSC thermograms of (A) Gly-Tyr and (B) Gly-Tyr−Ca chelate.

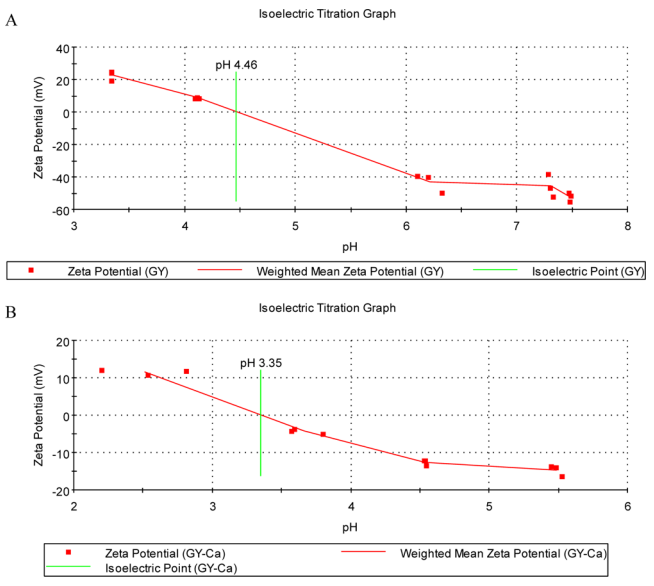

Figure 12. ζ potential of (A) Gly-Tyr and (B) Gly-Tyr−Ca chelate.

initial pH of Gly-Tyr in aqueous solution was measured to be 3.34 (Figure 12A). It meant that dipeptide Gly-Tyr in aqueous solution exhibited acid property. While in the Gly-Tyr−Ca chelate, the initial pH 5.45 approached neutral pH 7.0, which indicated that the addition of calcium improved the pH value of Gly-Tyr, inducing a weaker charge, and made it prone to be an neutral molecule (Figure 12B). Besides, the ζ potential ranged from positive charge (19.3 mV) to negative charge (−13.8 mV) when the calcium ion was added to dipeptide Gly-Tyr. Figure 12A also showed that the pI of Gly-Tyr was 4.46, while that of Gly-Tyr−Ca was 3.35. The pI decreased with the calcium ion treated on Gly-Tyr. In addition, the pH 3.34 of Gly-Tyr in aqueous solution was closer to its pI 4.46 than that of Gly-Tyr−Ca, which suggested that Gly-Tyr−Ca was more stable than Gly-Tyr alone. In the same pH value range, the changes of the ζ potential absolute value of Gly-Tyr−Ca were less than those of Gly-Tyr and Gly-Tyr−Ca possessed a lower ζ potential absolute value. The result indicated that Gly-Tyr−Ca existed as a molecular structure in aqueous solution without two different phases or double electrode layers. Through this experimental evidence, it could be speculated that the calcium ion was surrounded by the functional binding sites, such as the carboxyl and amino groups, of Gly-Tyr by a coordinate bond exhibiting an neutral molecule without being a form of inorganic calcium, to enhance its stability in the gastrointestinal tract.

Calcium-Releasing Percentage Assay. The calcium-releasing percentage of the Gly-Tyr−Ca chelate and $CaCl_2$ at different pH values is shown in Figure 13. The solubility

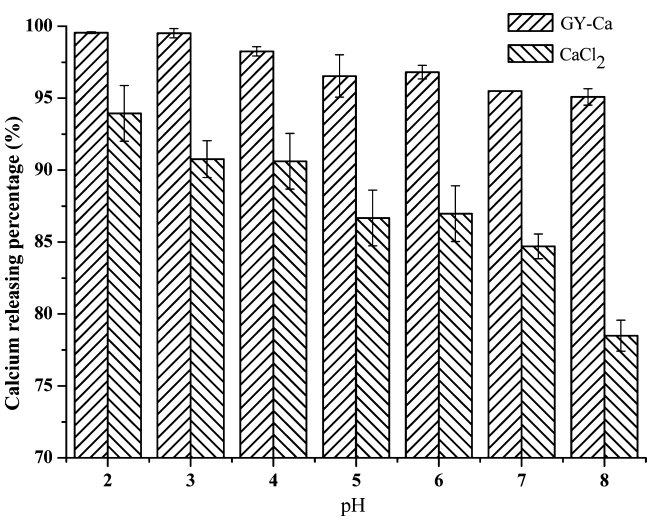

Figure 13. Calcium-releasing percentage of the Gly-Tyr−Ca chelate and $CaCl_2$ at pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, separately.

between Gly-Tyr−Ca and $CaCl_2$ was apparently different. For both of them, the calcium-releasing percentage reduced with the increase of pH. While at any pH value, the calcium-releasing percentage of Gly-Tyr−Ca was obviously higher than that of $CaCl_2$. The calcium-releasing amount in the Gly-Tyr−Ca chelate was relatively stable in the pH that ranged from 2.0 to 8.0, maintaining above 95%, whereas that of $CaCl_2$ decreased significantly from 93.94% at pH 2.0 to 78.48% at pH 8.0. The result implied that calcium in Gly-Tyr−Ca had good solubility whether at pH 2.0 or 8.0, which was similar to the pH values of the human stomach or gastric environment; consequently, precipitation could not form.[36]

It is essential to discuss the dissolved status of the peptide−calcium chelate in the human gastrointestinal tract because the calcium ion probably reacted with phytic or oxalic acid to become insoluble in the stomach and form $Ca(OH)_2$ in the

dx.doi.org/10.1021/jf502412f | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

intestinal tract. The finding of our study suggested that the purified dipeptide Gly-Tyr contributed to improve the solubility of Ca under gastrointestinal tract pH values and was effectively absorbed by intestinal epithelial cells. The result suggests that it is feasible to produce natural calcium-chelating peptide as functional food additives.

Hypothetical Molecular Structural Formula of the Gly-Tyr−Ca Chelate. According to the results of frontal structural characterizations, including UV absorption spectroscopy, FTIR, fluorescence spectra, and ^{1}H NMR spectroscopy, the functional binding sites of Gly-Tyr contained an oxygen atom of the carbonyl group and nitrogen of the amino or imino group in the peptide bond. Futhermore, the ζ potential experiment indicated that the calcium ion was surrounded by these functional binding bonds in Gly-Tyr through a coordinate bond. Therefore, the molecular structural formula of the Gly-Tyr−Ca chelate was speculated in Figure 14.

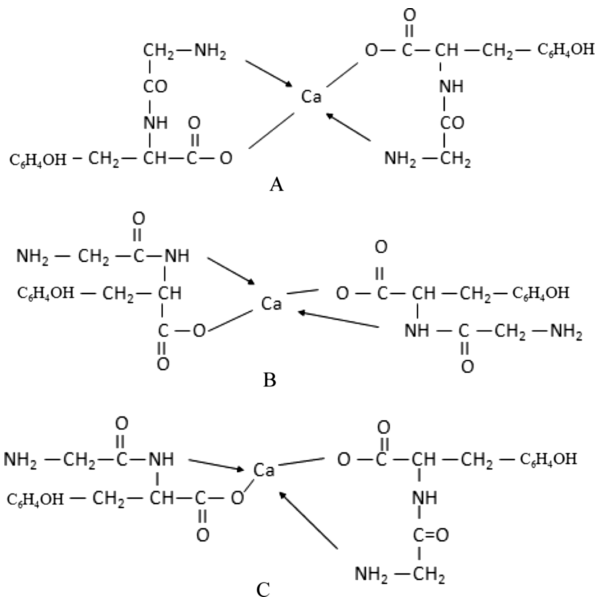

Figure 14. Hypothetical molecular structural formula of the Gly-Tyr−Ca chelate.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +86-591-22866375. Fax: +86-591-22866278. E-mail: shywang@fzu.edu.cn.

Funding

This work was supported by the National Marine Public Welfare Projects (201305022), the Fujian Natural Science Foundation of China (2013J01132), the open funding of Fujian Provincial Key Laboratory of Photocatalysis−State Key Laboratory Breeding Base of China (038018), Natural Science Foundation of China (31471623).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) O'Keeffe, M. B.; FitzGerald, R. J. Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells. *Int. Dairy J.* **2014**, *36*, 128−135.

(2) O'Loughlin, I. B.; Murray, B. A.; Brodkorb, A.; FitzGerald, R. J.; Kelly, P. M. Production of whey protein isolate hydrolysate fractions with enriched ACE-inhibitory activity. *Int. Dairy J.* **2014**, *38*, 101−103.

(3) Zhao, L. N.; Huang, S. L.; Cai, X. X.; Hong, J.; Wang, S. Y. A specific peptide with calcium chelating capacity isolated from whey protein hydrolysate. *J. Funct. Foods* **2014**, *10*, 46−53.

(4) Xu, R. Calcium binding of peptides derived from enzymatic hydrolysates of whey protein concentrate. *Int. J. Dairy Technol.* **2009**, *62*, 170−173.

(5) Bennett, T.; Desmond, A.; Harrington, M.; McDonagh, D.; FitzGerald, R.; Flynn, A.; Cashman, K. D. The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *Br. J. Nutr.* **2000**, *83*, 673−680.

(6) Kim, S.; Shin, H.; Lim, J. Separation of calcium-binding protein derived from enzymatic hydrolysates of cheese whey protein. *Asian-Australas. J. Anim. Sci.* **2004**, *17*, 712−718.

(7) Singh, G.; Muthukumarappan, K. Influence of calcium fortification on sensory, physical and rheological characteristics of fruit yogurt. *LWT—Food Sci. Technol.* **2008**, *41*, 1145−1152.

(8) Guéguen, L.; Pointillart, A. The bioavailability of dietary calcium. *J. Am. Coll. Nutr.* **2000**, *19*, 119S−136S.

(9) Cilla, A.; Lagarda, M. J.; Alegría, A.; de Ancos, B.; Cano, M. P.; Sánchez-Moreno, C.; Plaza, L.; Barberá, R. Effect of processing and food matrix on calcium and phosphorous bioavailability from milk-based fruit beverages in Caco-2 cells. *Food Res. Int.* **2011**, *44*, 3030−3038.

(10) Kim, S.; Lim, J. Calcium-binding peptides derived from tryptic hydrolysates of cheese whey protein. *Asian-Australas. J. Anim. Sci.* **2004**, *17*, 1459−1464.

(11) Lee, S. H.; Song, K. B. Isolation of a calcium-binding peptide from enzymatic hydrolysates of porcine blood plasma protein. *J. Korean Soc. Appl. Biol. Chem.* **2009**, *52*, 290−294.

(12) Bronner, F.; Pansu, D. Nutritional aspects of calcium absorption. *J. Nutr.* **1999**, *129*, 9−12.

(13) Rerat, A.; Nunes, C. S.; Mendy, F.; Roger, L. Amino acid absorption and production of pancreatic hormones in non-anaesthetized pigs after duodenal infusions of a milk enzymic hydrolysate or free amino acids. *Br. J. Nutr.* **1988**, *60*, 121−136.

(14) Chaud, M. V.; Izumi, C.; Nahaal, Z.; Shuhama, T.; Bianchi, M. D. L. P.; Freitas, O. D. Iron derivatives from casein hydrolysates as a potential source in the treatment of iron deficiency. *J. Agric. Food Chem.* **2002**, *50*, 871−877.

(15) Kim, S.; Seo, I.; Khan, M.; Ki, K.; Lee, W.; Lee, H.; Shin, H.; Kim, H. Enzymatic hydrolysis of heated whey: Iron-binding ability of peptides and antigenic protein fractions. *J. Dairy Sci.* **2007**, *90*, 4033−4042.

(16) Sato, R.; Shindo, M.; Gunshin, H.; Noguchi, T.; Naito, H. Characterization of phosphopeptide derived from bovine β-casein: An inhibitor to intraintestinal precipitation of calcium phosphate. *Biochim. Biophys. Acta* **1991**, *1077*, 413−415.

(17) Gitelman, H. J. An improved automated procedure for the determination of calcium in biological specimens. *Anal. Biochem.* **1967**, *18*, 521−531.

(18) Chen, D.; Liu, Z.; Huang, W.; Zhao, Y.; Dong, S.; Zeng, M. Purification and characterisation of a zinc-binding peptide from oyster protein hydrolysate. *J. Funct. Foods* **2013**, *5*, 689−697.

(19) Zhou, J.; Wang, X.; Ai, T.; Cheng, X.; Guo, H.; Teng, G.; Mao, X. Preparation and characterization of β-lactoglobulin hydrolysate−iron complexes. *J. Dairy Sci.* **2012**, *95*, 4230−4236.

(20) Megías, C.; Pedroche, J.; Yust, M. M.; Girón-Calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Affinity purification of copper-chelating peptides from sunflower protein hydrolysates. *J. Agric. Food Chem.* **2007**, *55*, 6509−6514.

(21) Huang, G. R.; Ren, Z. Y.; Jiang, J. X.; Chen, W. W. Purification of a hepta-peptide with iron binding activity from shrimp processing by-products hydrolysates. *Adv. J. Food Sci. Technol.* **2012**, *4*, 207−212.

(22) Huang, G.; Ren, L.; Jiang, J. Purification of a histidine-containing peptide with calcium binding activity from shrimp

processing byproducts hydrolysate. *Eur. Food Res. Technol.* **2011**, *232*, 281−287.
(23) Huang, G.; Ren, Z.; Jiang, J. Separation of iron-binding peptides from shrimp processing by-products hydrolysates. *Food Bioprocess Technol.* **2011**, *4*, 1527−1532.
(24) Reddy, I. M.; Mahoney, A. W. Solution visible difference spectral properties of Fe^{3+}−L-amino acid complexes at pH 6.60. *J. Agric. Food Chem.* **1995**, *43*, 1436−1443.
(25) Lv, Y.; Liu, Q.; Bao, X.; Tang, W.; Yang, B.; Guo, S. Identification and characteristics of iron-chelating peptides from soybean protein hydrolysates using IMAC-Fe^{3+}. *J. Agric. Food Chem.* **2009**, *57*, 4593−4597.
(26) Miquel, E.; Farré, R. Effects and future trends of casein phosphopeptides on zinc bioavailability. *Trends Food Sci. Technol.* **2007**, *18*, 139−143.
(27) Jung, W. K.; Karawita, R.; Heo, S. J.; Lee, B. J.; Kim, S. K.; Jeon, Y. J. Recovery of a novel Ca-binding peptide from Alaska Pollack (*Theragra chalcogramma*) backbone by pepsinolytic hydrolysis. *Process Biochem.* **2006**, *41*, 2097−2100.
(28) Jung, W. K.; Park, P. J.; Byun, H. G.; Moon, S. H.; Kim, S. K. Preparation of hoki (*Johnius belengerii*) bone oligophosphopeptide with a high affinity to calcium by carnivorous intestine crude proteinase. *Food Chem.* **2005**, *91*, 333−340.
(29) Wu, H. H.; Liu, Z. Y.; Zhao, Y. H.; Zeng, M. Y. Enzymatic preparation and characterization of iron-chelating peptides from anchovy (*Engraulis japonicus*) muscle protein. *Food Res. Int.* **2012**, *48*, 435−441.
(30) Houser, R. P.; Fitzsimons, M. P.; Barton, J. K. Metal-dependent intramolecular chiral induction: The Zn^{2+} complex of an ethidium−peptide conjugate. *Inorg. Chem.* **1999**, *38*, 1368−1370.
(31) Armas, A.; Sonois, V.; Mothes, E.; Mazarguil, H.; Faller, P. Zinc(II) binds to the neuroprotective peptide humanin. *J. Inorg. Biochem.* **2006**, *100*, 1672−1678.
(32) Blindauer, C. A.; Harvey, I.; Bunyan, K. E.; Stewart, A. J.; Sleep, D.; Harrison, D. J.; Berezenko, S.; Sadler, P. J. Structure, properties, and engineering of the major zinc binding site on human albumin. *J. Biol. Chem.* **2009**, *284*, 23116−23124.
(33) Reddi, A. R.; Guzman, T. R.; Breece, R. M.; Tierney, D. L.; Gibney, B. R. Deducing the energetic cost of protein folding in zinc finger proteins using designed metallopeptides. *J. Am. Chem. Soc.* **2007**, *129*, 12815−12827.
(34) Beyer, R. L.; Hoang, H. N.; Appleton, T. G.; Fairlie, D. P. Metal clips induce folding of a short unstructured peptide into an α-helix via turn conformations in water. Kinetic versus thermodynamic products. *J. Am. Chem. Soc.* **2004**, *126*, 15096−15105.
(35) Bao, X. L.; Lv, Y.; Yang, B. C.; Ren, C. G.; Guo, S. T. A study of the soluble complexes formed during calcium binding by soybean protein hydrolysates. *J. Food Sci.* **2008**, *73*, C117−C121.
(36) Wang, X.; Zhou, J.; Tong, P.; Mao, X. Zinc-binding capacity of yak casein hydrolysate and the zinc-releasing characteristics of casein hydrolysate−zinc complexes. *J. Dairy Sci.* **2011**, *94*, 2731−2740.