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Isolation of a Calcium-binding Peptide from Chlorella Protein Hydrolysates

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Abstract

To isolate a calcium-binding peptide from chlorella protein hydrolysates, chlorella protein was extracted and hydrolyzed using Flavourzyme, a commercial protease. The degree of hydrolysis and calcium-binding capacity were determined using trinitrobenzenesulfonic acid and orthophenanthroline methods, respectively. The enzymatic hydrolysis of chlorella protein for 6 hr was sufficient for the preparation of chlorella protein hydrolysates. The hydrolysates of chlorella protein were then ultra-filtered under 5 kDa as molecular weight. The membrane-filtered solution was fractionated using ion exchange, reverse phase, normal phase chromatography, and fast protein liquid chromatography to identify a calcium-binding peptide. The purified calcium-binding peptide had a calcium binding activity of 0.166 mM and was determined to be 700.48 Da as molecular weight, and partially identified as a peptide containing Asn-Ser-Gly-Cys based on liquid chromatography/electrospray ionization tandem mass spectrum.

Key words: calcium-binding peptide, chlorella protein, enzymatic hydrolysates, purification

INTRODUCTION

Chlorella, a type of single-celled green algae, is known to have high levels of nutrients and biologically active substances (1,2). Chlorella has various functional properties such as immune-modulation (3,4), anti-cancer (5), and elimination of heavy metal and dioxin (5,6). In particular, the hydrolysis of chlorella protein has been studied regarding ACE-inhibitory peptides (7), inhibition of melanin synthesis (8), and anti-oxidative property (9).

Although the protein content of chlorella is more than 50%, it is usually used as animal feed after production of algae essence (9,10). Therefore, appropriate processing of the chlorella protein is further needed to better obtain of all of its potential benefits (10,11). Chlorella has low protein digestibility due to its strong multiple cell walls (12,13); thus, to efficiently isolate the chlorella protein, the cell wall should be disrupted, which would result in an increase of both the digestion and absorption rates of the chlorella components. Enzymatic hydrolysis of the cell wall is looked on favorably by the food industry, because it results in improved protein digestion and gastrointestinal absorption (14).

Calcium, an essential mineral in the human body, is most commonly obtained through milk or other dairy products (15). However, calcium intake by milk is not sufficient, especially for those who are allergic or lactose intolerant (16). Insufficient calcium intake is related to diseases like osteoporosis, and, consequently, people have growing interest in calcium absorption to prevent bone diseases (17).

Calcium is mostly fortified as calcium salts, which have low bioavailability in the body because of high reactivity with other chemicals such as phosphate and oxalate (18). Therefore, there have been studies on calcium supplements with improved absorption and bioavailability. It has been reported that casein phosphopeptides (CPP) are bound to minerals, and the resulting complex increases the stability and bioavailability of calcium (19). Hence, calcium-binding peptides might be a suitable candidate as calcium supplement.

Therefore, the objectives in this study were to develop the chlorella protein as a food ingredient and calcium supplement and to isolate a calcium binding peptide from its hydrolysates.

MATERIALS AND METHODS

Materials

Chlorella (*Chlorella vulgaris*) powder used in this study was purchased from Backjangsaeng Co. (Seoul, Korea), and the commercial protease Flavourzyme (from *Aspergillus oryzae*, activity 500 LAPU/g protein) was obtained from Novo Nordisk Co. (Bagsvaerd, Denmark). Calcium chloride, 2-amino-2-methyl-1-propanol, *o*-cresolphthalein complexone, and trinitrobenzene sulfornic acid of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade solvents were purchased from J. T. Baker Inc. (Phillipsburg, NJ, USA).

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Sample preparation

Powdered chlorella was dissolved in distilled water for preparation of a 10% (w/v) chlorella solution containing 0.5% (w/v) ethylenediamine tetraacetic acid (EDTA). To disrupt the cell membrane, the chlorella solution was treated by ultrasonication (Model-GE 750, Sonics& Materials, Newtown, CT, USA) and stirred at 4°C for 24 hr, and insoluble materials were removed by centrifugation at 3,500×*g* for 20 min. The supernatant was lyophilized and stored at -20°C for further use.

Preparation of chlorella protein hydrolysates

Hydrolysates of chlorella protein were prepared according to the method of a previous study (20). The lyophilized sample was dissolved in 10 mM phosphate buffer (pH 7.0) for preparation of a 5% (w/v) chlorella protein solution. The chlorella protein was hydrolyzed using Flavourzyme with a 50:1 substrate to enzyme ratio (w/w) at 50°C for 8 hr. The hydrolysis mixtures were then heated at 95°C for 5 min for the inactivation of the enzyme. The reaction mixture was centrifuged at 3,500×*g* for 20 min, and the supernatant was filtered using Pellicon XL (Millipore Co., Billerica, MA, USA) to obtain molecules below molecular weight 5 kDa. The membrane-filtered solution was lyophilized, and the degree of hydrolysis was determined using trinitrobenzene sulfonic acid (TNBS) assay (21). In addition, the TNBS assay, which is a common method to determine the concentration of peptide as well as amino acid, was also used for the determination of peptide concentration in the samples (22).

Preparation of calcium-binding peptide and determination of calcium-binding capacity

Chlorella protein hydrolysates were dissolved in a 20 mM phosphate buffer (pH 7.5). After adding 2.5 mM $CaCl_2$, the solution was reacted by stirring at room temperature for 30 min, and the reaction mixture was centrifuged at 3,500×*g* for 20 min to remove precipitates. Calcium concentration in the supernatant was determined using a colorimetric method (23) with *o*-cresolphthalein complexone reagent. The experiment was performed in triplicate, and values were expressed as means±SD.

Purification of a calcium-binding peptide

Chlorella protein hydrolysates were separated on fast protein liquid chromatography (FPLC) (Amersharm Pharmacia Co., Uppsala, Sweden) with an anion exchange column (Mono Q, Amersham Pharmacia Co.) equilibrated with a 10 mM Tris buffer (pH 8.0) and eluted with a linear gradient of the buffer containing NaCl from 0 to 0.3 M at 0.5 mL/min. The eluate was monitored for peptides by measuring the absorbance at 214 nm. After calcium binding capacity and peptide concentration of each fraction from the Mono Q column were determined, the fraction of the highest calcium/peptide ratio was loaded onto the HPLC (Waters, Milford, MA, USA) with a C_{18} column (4.6 mm×250 mm, SunFire™, Milford, MA, USA). Elution was performed on the condition of solvent A (0.1% trifluroacetic acid in water) and solvent B (0.1% trifluroacetic acid in acetonitrile), having gradient of 0% to 100% at 0.5 mL/min. The fraction of the highest calcium/peptide ratio from the C_{18} column was further purified using normal phase HPLC with an amino column (4.6 mm×250 mm, Luna NH_2, Phenomenet Inc., Torrance, CA, USA) equilibrated with acetonitrile (containing 0.1% trifluroacetic acid), and eluted with a gradient of solvent B (0.1% trifluroacetic acid in water) from 0 to 25%, and 25 to 100% at 0.5 mL/min. After normal phase HPLC, the fraction of the highest calcium binding was finally isolated using FPLC (10 mm×300 mm, Superdex™ peptide 10/300 GL, GE healthcare, Uppsala, Sweden).

Amino acid sequencing of a calcium binding peptide

Molecular mass and sequence of the purified calcium binding peptide were determined using a liquid chromatography/electrospray ionization (LC/ESI) tandem mass spectrometer (QTOF, AB Sciex Instruments, Dublin, CA, USA). The peptide for sequencing was obtained over the *m/z* range 50～2000 and sequenced using the PepSeq *de-novo* sequencing algorithm.

Statistical analysis

Analysis of variance and Duncan's multiple range tests were performed to analyze the results using the SAS program (24).

RESULTS AND DISCUSSION

Chlorella protein was hydrolyzed using commercial protease, Flavourzyme, under optimal condition of pH 7.0 and 50°C. Hydrolysis of proteins using commercial enzyme is the most common method for producing a bioactive peptide (25). To determine the degree of hydrolysis of proteins, available amino group concentration of chlorella protein hydrolysates was determined using the TNBS assay. Available amino group concentration of the chlorella protein hydrolysates increased according to hydrolysis time up to 6 hr (Table 1), and the progress of hydrolysis was confirmed on the SDS-PAGE (Fig. 1), indicating the degradation of protein molecules. The results suggest that the enzymatic hydrolysis of chlorella protein for 6 hr is sufficient for the preparation of chlorella protein hydrolysates.

The enzymatic hydrolysates of chlorella protein were

Table 1. Available amino group concentration of chlorella protein hydrolysates at various time points

Hydrolysis time (hr)	0	2	4	6	8
Available amino group conc. (mM)	2.43 ± 0.17^{d}	19.40 ± 0.71^{c}	23.84 ± 0.63^{b}	25.18 ± 0.07^{a}	25.82 ± 0.48^{a}

[a-d]Any means followed by different letters are significantly ($p<0.05$) different by Duncan's multiple range test.

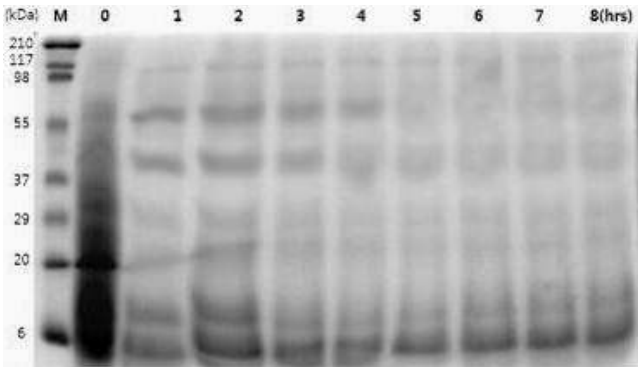

Fig. 1. SDS-PAGE profile of chlorella protein according to hydrolysis time.

filtered to obtain the peptides having molecular weighs below 5 kDa and then lyophilized. Membrane filtration is a suitable technique for the concentration of peptides (26), and small molecular weight peptides can be suitable for intestinal absorption (27).

To purify a calcium binding peptide from chlorella proteins, the chlorella protein hydrolysates were first separated on ion exchange chromatography with a Mono Q column equilibrated with a 10 mM Tris buffer (pH 8.0). As shown in Fig. 2a, there were six major peaks detected. Each fraction was reacted with 5 mM $CaCl_2$, and the calcium and peptide concentrations were determined accordingly. The F4 fraction showed the highest calcium-binding activity than others (Table 2), whereas the F2 fraction showed the lowest-binding capacity. The F4 fraction was then pooled and subjected to reverse phase liquid chromatography with a C_{18} column. There were two major peaks separated during the linear gradient of 0 to 100% of solvent B (Fig. 2b). Between them, the F41 fraction had higher calcium/peptide concentration ratio than the F42 (Table 2). The F41 fraction was pooled and further purified using normal phase high performance liquid chromatography with NH_2 column (Fig. 2c). Two major peaks were separated during gradient elution, and the F411 fraction having the highest calcium-binding capacity (Table 2) was then loaded on the Superdex^{TM} column for further purification, and a single peak (F4111) was finally obtained (Fig. 2d).

Consequently, the isolated fraction was analyzed for peptide sequence using an LC/ESI tandem mass spec-

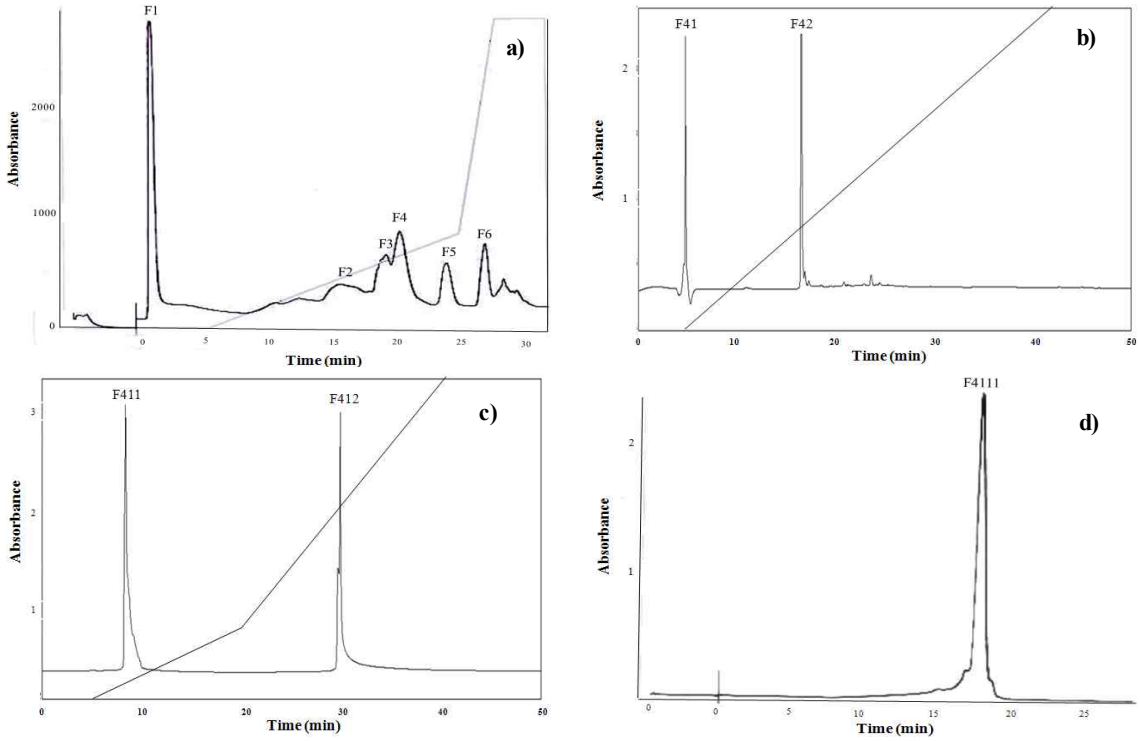

Fig. 2. Elution profiles of chlorella protein hydrolysates from different chromatographic methods. The absorbance was measured at 214 nm. a) ion exchange chromatography, b) reverse phase HPLC, c) normal phase HPLC, d) Superdex™ column.

Table 2. Calcium binding and peptide concentration for each fraction

	Fraction	Ca binding (mM)	Peptide (mM)	Ca/peptide
Mono Q	F1	0.432±0.026[a]	2.523±0.014[a]	0.165
	F2	0.410±0.013[a]	2.451±0.003[a]	0.162
	F3	0.376±0.037[ab]	1.82±0.043[b]	0.219
	F4	0.420±0.017[b]	0.97±0.216[c]	0.447
	F5	0.223±0.003[c]	0.78±0.015[d]	0.281
	F6	0.223±0.003[c]	0.78±0.051[d]	0.283
C_{18}	F41	0.058±0.000[a]	0.050±0.000[a]	1.155
	F42	0.030±0.000[b]	0.032±0.001[b]	0.938
NH_2	F411	0.166±0.000[a]	0.045±0.000[a]	3.662
	F412	0.015±0.000[b]	0.034±0.000[b]	0.446

[a-d]Any means followed by different letters in the same column are significantly ($p<0.05$) different by Duncan's multiple range test.

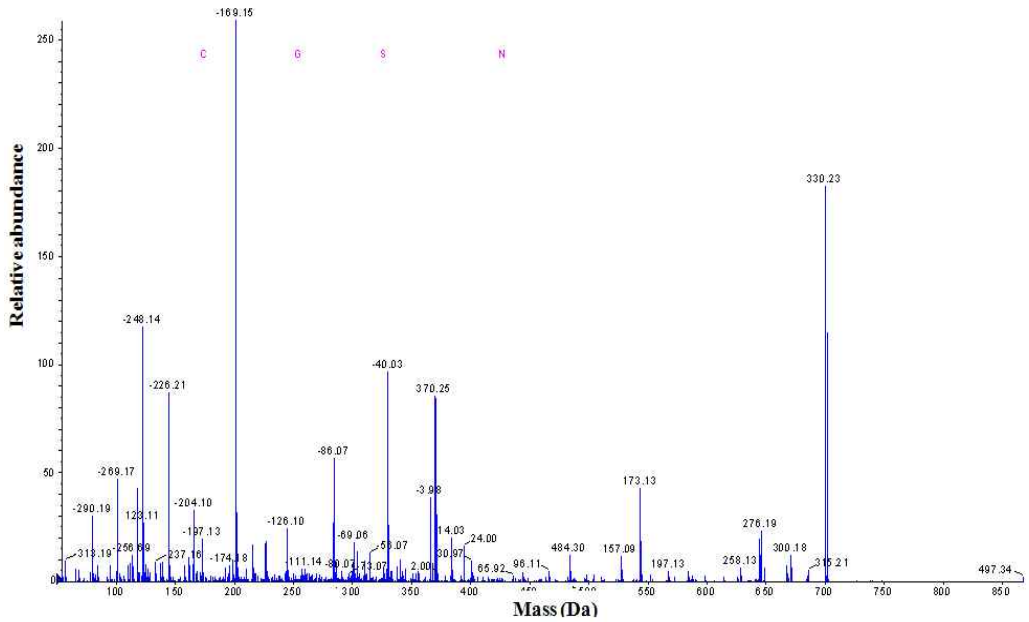

Fig. 3. Amino acid sequence of a calcium-binding peptide using LC/ESI tandem mass spectrometer.

trometer. The calcium-binding peptide from the chlorella protein hydrolysates was partially identified to be the peptide containing Asn-Ser-Gly-Cys, whose molecular weight was 700.48 Da (Fig. 3).

Food-derived peptides such as casein phosphopeptide can enhance calcium absorption by increasing calcium solubility in the duodenum (28,29), resulting in prevention of osteoporosis and dental caries. Miquel et al. (30) reported that α_{S1}-, α_{S2}-, and β-casein contained Ser, Glu, and phosphoserine residues, which could form a complex with calcium, thus enhancing their general bioavailability. Alaska Pollack (*Theragra chalcogramma*) backbone hydrolysates had 14 amino acids, including Ser and Gly for calcium binding (31), reflecting a similarity to the peptide sequence isolated in our study. Furthermore, the calcium-binding peptide from porcine blood plasma protein (PBPP) hydrolysates had also Ser, Asn, and Gly residues, similar to our study (18). Maliarik et al. (32) also reported that the Cys residue seemed to be responsible for calcium binding. Therefore, in accordance with these previous reports, our results strongly suggest that Ser, Gly, and Cys residues are the major amino acids that are responsible for calcium binding.

This is the first report dealing with the isolation of a calcium-binding peptide from chlorella protein hydrolysates. This peptide might prove to be applicable as a food ingredient and dietary supplement for calcium deficiency.

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